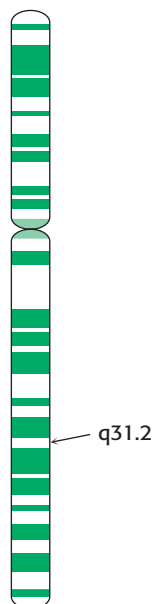
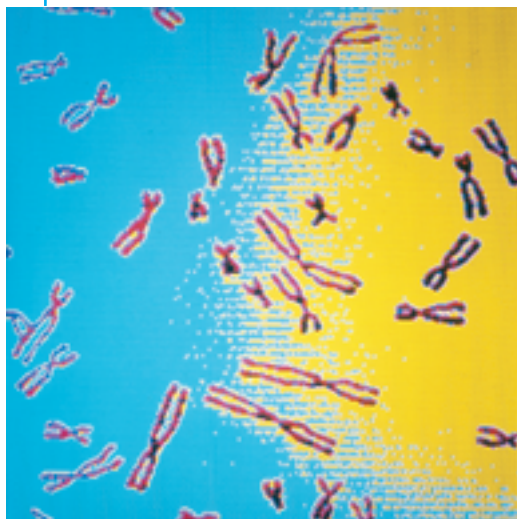


Prelude: Biochemistry and the Genomic Revolution



Disease and the genome. Studies of the human genome are revealing disease origins and other biochemical mysteries. Human chromosomes, left, contain the DNA molecules that constitute the human genome. The staining pattern serves to identify specific regions of a chromosome. On the right is a diagram of human chromosome 7, with band q31.2 indicated by an arrow. A gene in this region encodes a protein that, when malfunctioning, causes cystic fibrosis. [(Left) Alfred Pasiaka/Peter Arnold.]

GACTTCACTTCTAATGATGATTATGGGAGA AACTGGAGCCT
TCAGAGGGTAAAAATTAAGCACAGTGGGAAGAATTTTCATTC
TGTTCTCAGTTTTTCCTGGATTATGCCTGGCACCATTAAAG
AAAATATCTTTGGTGTTTCCTATGATGAATATAGATACAG
AAGCGTCATCAAAGCATGCCAACTAGAAGAG... This string of letters A, C, G, and T is a part of a DNA sequence. Since the biochemical techniques for DNA sequencing were first developed more than three decades ago, the genomes of dozens of organisms have been sequenced, and many more such sequences will be forthcoming. The information contained in these DNA sequences promises to shed light on many fascinating and important questions. What genes in *Vibrio cholera*, the bacterium that causes cholera, for example, distinguish it from its more benign relatives? How is the development of complex organisms controlled? What are the evolutionary relationships between organisms?

Sequencing studies have led us to a tremendous landmark in the history of biology and, indeed, humanity. A nearly complete sequence of the entire human genome has been determined. The string of As, Cs, Gs, and Ts with which we began this book is a tiny part of the human genome sequence, which is more than 3 billion letters long. If we included the entire sequence, our opening sentence would fill more than 500,000 pages.

The implications of this knowledge cannot be overestimated. By using this blueprint for much of what it means to be human, scientists can begin the identification and

OUTLINE

- 1.1 DNA Illustrates the Relation Between Form and Function
- 1.2 Biochemical Unity Underlies Biological Diversity
- 1.3 Chemical Bonds in Biochemistry
- 1.4 Biochemistry and Human Biology

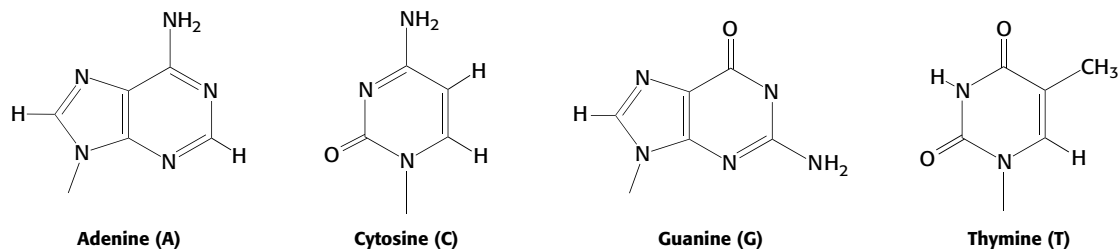
characterization of sequences that foretell the appearance of specific diseases and particular physical attributes. One consequence will be the development of better means of diagnosing and treating diseases. Ultimately, physicians will be able to devise plans for preventing or managing heart disease or cancer that take account of individual variations. Although the sequencing of the human genome is an enormous step toward a complete understanding of living systems, much work needs to be done. Where are the functional genes within the sequence, and how do they interact with one another? How is the information in genes converted into the functional characteristics of an organism? Some of our goals in the study of biochemistry are to learn the concepts, tools, and facts that will allow us to address these questions. It is indeed an exciting time, the beginning of a new era in biochemistry.

1.1 DNA ILLUSTRATES THE RELATION BETWEEN FORM AND FUNCTION

The structure of DNA, an abbreviation for *deoxyribonucleic acid*, illustrates a basic principle common to all biomolecules: the intimate relation between structure and function. The remarkable properties of this chemical substance allow it to function as a very efficient and robust vehicle for storing information. We begin with an examination of the covalent structure of DNA and its extension into three dimensions.

1.1.1 DNA Is Constructed from Four Building Blocks

DNA is a *linear polymer* made up of four different monomers. It has a fixed backbone from which protrude variable substituents (Figure 1.1). The backbone is built of repeating sugar-phosphate units. The sugars are molecules of *deoxyribose* from which DNA receives its name. Joined to each deoxyribose is one of four possible bases: adenine (A), cytosine (C), guanine (G), and thymine (T).



All four bases are planar but differ significantly in other respects. Thus, the monomers of DNA consist of a sugar-phosphate unit, with one of four bases attached to the sugar. *These bases can be arranged in any order along a strand of DNA.* The order of these bases is what is displayed in the sequence that begins this chapter. For example, the first base in the sequence shown is G

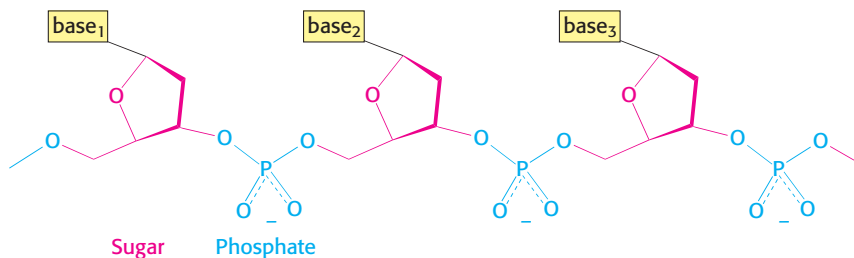


FIGURE 1.1 Covalent structure of DNA. Each unit of the polymeric structure is composed of a sugar (deoxyribose), a phosphate, and a variable base that protrudes from the sugar-phosphate backbone.

(guanine), the second is A (adenine), and so on. *The sequence of bases along a DNA strand constitutes the genetic information*—the instructions for assembling proteins, which themselves orchestrate the synthesis of a host of other biomolecules that form cells and ultimately organisms.

1.1.2 Two Single Strands of DNA Combine to Form a Double Helix

Most DNA molecules consist of not one but two strands (Figure 1.2). How are these strands positioned with respect to one another? In 1953, James Watson and Francis Crick deduced the arrangement of these strands and proposed a three-dimensional structure for DNA molecules. This structure is a *double helix* composed of two intertwined strands arranged such that the sugar-phosphate backbone lies on the outside and the bases on the inside. The key to this structure is that the bases form *specific base pairs* (bp) held together by *hydrogen bonds* (Section 1.3.1): adenine pairs with thymine (A–T) and guanine pairs with cytosine (G–C), as shown in Figure 1.3. Hydrogen bonds are much weaker than covalent bonds such as the carbon–carbon or carbon–nitrogen bonds that define the structures of the bases themselves. Such weak bonds are crucial to biochemical systems; they are weak enough to be reversibly broken in biochemical processes, yet they are strong enough, when many form simultaneously, to help stabilize specific structures such as the double helix.

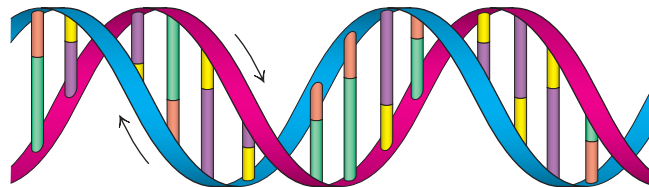


FIGURE 1.2 The double helix. The double-helical structure of DNA proposed by Watson and Crick. The sugar-phosphate backbones of the two chains are shown in red and blue and the bases are shown in green, purple, orange, and yellow.

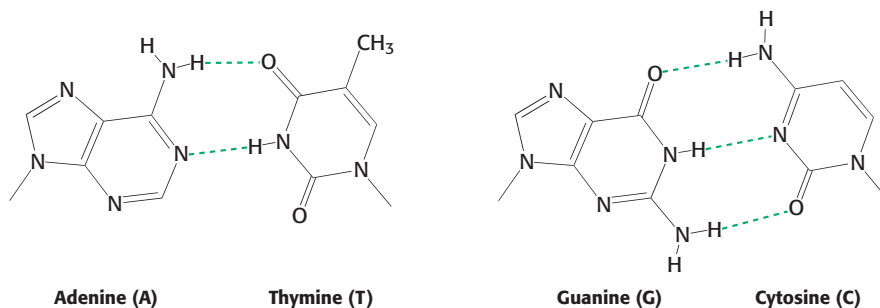


FIGURE 1.3 Watson-Crick base pairs. Adenine pairs with thymine (A–T), and guanine with cytosine (G–C). The dashed lines represent hydrogen bonds.

The structure proposed by Watson and Crick has two properties of central importance to the role of DNA as the hereditary material. First, the structure is compatible with *any sequence of bases*. The base pairs have essentially the same shape (Figure 1.4) and thus fit equally well into the center of the double-helical structure. Second, because of base-pairing, *the sequence of bases along one strand completely determines the sequence along the other strand*. As Watson and Crick so coyly wrote: “It has not escaped our notice that the specific pairing we have postulated immediately suggests a possible copying mechanism for the genetic material.” Thus, if the DNA double helix is separated into two single strands, each strand can act as a template for the generation of its partner strand through specific base-pair formation (Figure 1.5). *The three-dimensional structure of DNA beautifully illustrates the close connection between molecular form and function.*

FIGURE 1.4 Base-pairing in DNA. The base-pairs A–T (blue) and C–G (red) are shown overlaid. The Watson-Crick base-pairs have the same overall size and shape, allowing them to fit neatly within the double helix.

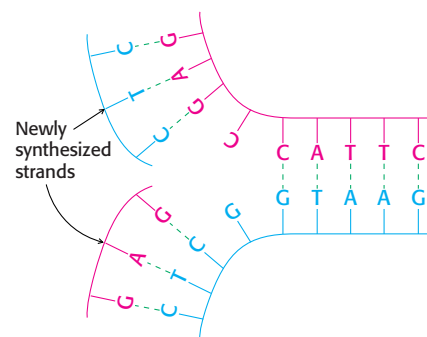
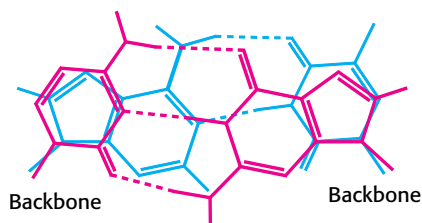
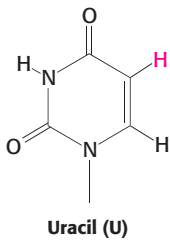
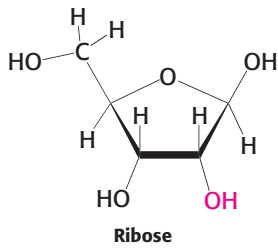


FIGURE 1.5 DNA replication. If a DNA molecule is separated into two strands, each strand can act as the template for the generation of its partner strand.



1.1.3 RNA Is an Intermediate in the Flow of Genetic Information

An important nucleic acid in addition to DNA is *ribonucleic acid* (RNA). Some viruses use RNA as the genetic material, and even those organisms that employ DNA must first convert the genetic information into RNA for the information to be accessible or functional. Structurally, RNA is quite similar to DNA. It is a linear polymer made up of a limited number of repeating monomers, each composed of a sugar, a phosphate, and a base. The sugar is ribose instead of deoxyribose (hence, RNA) and one of the bases is uracil (U) instead of thymine (T). Unlike DNA, an RNA molecule usually exists as a single strand, although significant segments within an RNA molecule may be double stranded, with G pairing primarily with C and A pairing with U. This intrastrand base-pairing generates RNA molecules with complex structures and activities, including catalysis.

RNA has three basic roles in the cell. First, it serves as the intermediate in the flow of information from DNA to protein, the primary functional molecules of the cell. The DNA is copied, or *transcribed*, into messenger RNA (mRNA), and the mRNA is *translated* into protein. Second, RNA molecules serve as adaptors that translate the information in the nucleic acid sequence of mRNA into information designating the sequence of constituents that make up a protein. Finally, RNA molecules are important functional components of the molecular machinery, called ribosomes, that carries out the translation process. As will be discussed in Chapter 2, the unique position of RNA between the storage of genetic information in DNA and the functional expression of this information as protein as well as its potential to combine genetic and catalytic capabilities are indications that RNA played an important role in the evolution of life.

1.1.4 Proteins, Encoded by Nucleic Acids, Perform Most Cell Functions

A major role for many sequences of DNA is to encode the sequences of *proteins*, the workhorses within cells, participating in essentially all processes. Some proteins are key structural components, whereas others are specific catalysts (termed *enzymes*) that promote chemical reactions. Like DNA and RNA, proteins are linear polymers. However, proteins are more complicated in that they are formed from a selection of 20 building blocks, called *amino acids*, rather than 4.

The functional properties of proteins, like those of other biomolecules, are determined by their three-dimensional structures. Proteins possess an extremely important property: a protein spontaneously folds into a well-defined and elaborate three-dimensional structure that is dictated entirely by the sequence of amino acids along its chain (Figure 1.6). *The self-folding nature of proteins constitutes the transition from the one-dimensional world of sequence information to the three-dimensional world of biological function.* This marvelous ability of proteins to self assemble into complex structures is responsible for their dominant role in biochemistry.

How is the sequence of bases along DNA translated into a sequence of amino acids along a protein chain? We will consider the details of this process in later chapters, but the important finding is that *three bases along a DNA chain encode a single amino acid.* The specific correspondence between a set of three bases and 1 of the 20 amino acids is called the *genetic code*. Like the use of DNA as the genetic material, the genetic code is essentially universal; the same sequences of three bases encode the same amino acids in all life forms from simple microorganisms to complex, multicellular organisms such as human beings.

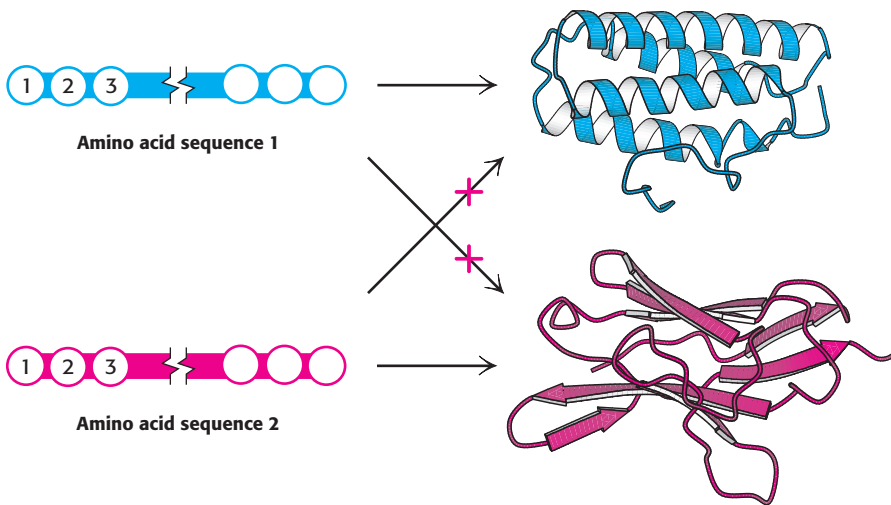


FIGURE 1.6 Folding of a protein. The three-dimensional structure of a protein, a linear polymer of amino acids, is dictated by its amino acid sequence.

Knowledge of the functional and structural properties of proteins is absolutely essential to understanding the significance of the human genome sequence. For example, the sequence at the beginning of this chapter corresponds to a region of the genome that differs in people who have the genetic disorder *cystic fibrosis*. The most common mutation causing cystic fibrosis, the loss of three consecutive T's from the gene sequence, leads to the loss of a single amino acid within a protein chain of 1480 amino acids. This seemingly slight difference—a loss of 1 amino acid of nearly 1500—creates a life-threatening condition. What is the normal function of the protein encoded by this gene? What properties of the encoded protein are compromised by this subtle defect? Can this knowledge be used to develop new treatments? These questions fall in the realm of biochemistry. Knowledge of the human genome sequence will greatly accelerate the pace at which connections are made between DNA sequences and disease as well as other human characteristics. However, these connections will be nearly meaningless without the knowledge of biochemistry necessary to interpret and exploit them.

Cystic fibrosis—

A disease that results from a decrease in fluid and salt secretion by a transport protein referred to as the cystic fibrosis transmembrane conductance regulator (CFTR). As a result of this defect, secretion from the pancreas is blocked, and heavy, dehydrated mucus accumulates in the lungs, leading to chronic lung infections.

1.2 BIOCHEMICAL UNITY UNDERLIES BIOLOGICAL DIVERSITY

The stunning variety of living systems (Figure 1.7) belies a striking similarity. The common use of DNA and the genetic code by all organisms underlies one of the most powerful discoveries of the past century—namely, that *organisms are remarkably uniform at the molecular level*. All organisms are built from similar molecular components distinguishable by relatively minor variations. *This uniformity reveals that all organisms on Earth have*

FIGURE 1.7 The diversity of living systems. The distinct morphologies of the three organisms shown—a plant (the false hellebora, or Indian poke) and two animals (sea urchins and a common house cat)—might suggest that they have little in common. Yet biochemically they display a remarkable commonality that attests to a common ancestry. [(Left and right) John Dudak/Phototake. (Middle) Jeffrey L. Rotman/Peter Arnold.]



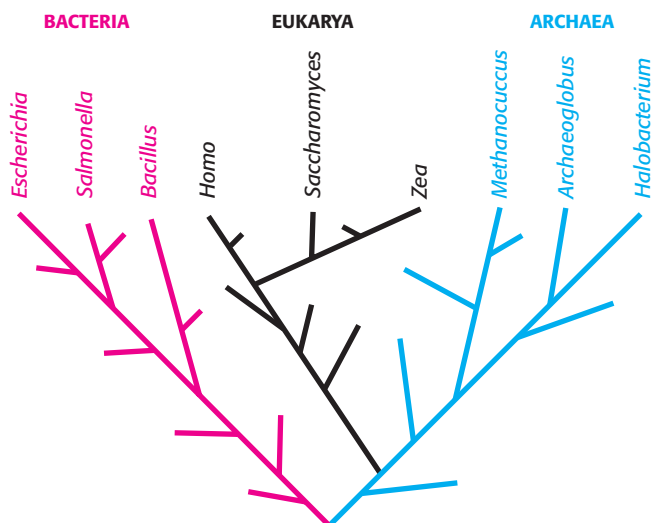


FIGURE 1.8 The tree of life. A possible evolutionary path from a common ancestral cell to the diverse species present in the modern world can be deduced from DNA sequence analysis.

arisen from a common ancestor. A core of essential biochemical processes, common to all organisms, appeared early in the evolution of life. The diversity of life in the modern world has been generated by evolutionary processes acting on these core processes through millions or even billions of years. As we will see repeatedly, the generation of diversity has very often resulted from the adaptation of existing biochemical components to new roles rather than the development of fundamentally new biochemical technology. The striking uniformity of life at the molecular level affords the student of biochemistry a particularly clear view into the essence of biological processes that applies to all organisms from human beings to the simplest microorganisms.

On the basis of their biochemical characteristics, the diverse organisms of the modern world can be divided into three fundamental groups called *domains*: *Eukarya* (eukaryotes), *Bacteria* (formerly Eubacteria), and *Archaea* (formerly Archaeobacteria). Eukarya comprise all macroscopic organisms, including human beings as well as many microscopic, unicellular organisms such as yeast. The defining characteristic of *eukaryotes* is the presence of a well-defined nucleus within each cell. Unicellular organisms such as bacteria, which lack a nucleus, are referred to as *prokaryotes*. The prokaryotes were reclassified as two separate domains in response to Carl Woese's discovery in 1977 that certain bacteria-like organisms are biochemically quite distinct from better-characterized bacterial species. These organisms, now recognized as having diverged from bacteria early in evolution, are archaea. Evolutionary paths from a common ancestor to modern organisms can be developed and analyzed on the basis of biochemical information. One such path is shown in Figure 1.8.

By examining biochemistry in the context of the tree of life, we can often understand how particular molecules or processes helped organisms adapt to specific environments or life styles. We can ask not only *what* biochemical processes take place, but also *why* particular strategies appeared in the course of evolution. In addition to being sources of historical insights, *the answers to such questions are often highly instructive with regard to the biochemistry of contemporary organisms.*

1.3 CHEMICAL BONDS IN BIOCHEMISTRY

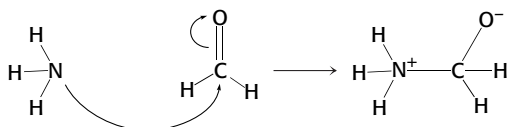
The essence of biological processes—the basis of the uniformity of living systems—is in its most fundamental sense molecular interactions; in other words, the chemistry that takes place between molecules. Biochemistry is the *chemistry* that takes place within living systems. To truly understand biochemistry, we need to understand chemical bonding. We review here the types of chemical bonds that are important for biochemicals and their transformations.

The strongest bonds that are present in biochemicals are *covalent bonds*, such as the bonds that hold the atoms together within the individual bases shown in Figure 1.3. A covalent bond is formed by the sharing of a pair of electrons between adjacent atoms. A typical carbon–carbon (C–C) covalent bond has a bond length of 1.54 Å and bond energy of 85 kcal mol⁻¹ (356 kJ mol⁻¹). Because this energy is relatively high, considerable energy must be expended to break covalent bonds. More than one electron pair can

be shared between two atoms to form a multiple covalent bond. For example, three of the bases in Figure 1.4 include carbon–oxygen (C=O) double bonds. These bonds are even stronger than C–C single bonds, with energies near $175 \text{ kcal mol}^{-1}$ (732 kJ mol^{-1}).

For some molecules, more than one pattern of covalent bonding can be written. For example, benzene can be written in two equivalent ways called *resonance structures*. Benzene's true structure is a composite of its two resonance structures. A molecule that can be written as several resonance structures of approximately equal energies has greater stability than does a molecule without multiple resonance structures. Thus, because of its resonance structures, benzene is unusually stable.

Chemical reactions entail the breaking and forming of covalent bonds. The flow of electrons in the course of a reaction can be depicted by curved arrows, a method of representation called “arrow pushing.” Each arrow represents an electron pair.



1.3.1 Reversible Interactions of Biomolecules Are Mediated by Three Kinds of Noncovalent Bonds

Readily reversible, noncovalent molecular interactions are key steps in the dance of life. Such weak, noncovalent forces play essential roles in the faithful replication of DNA, the folding of proteins into intricate three-dimensional forms, the specific recognition of substrates by enzymes, and the detection of molecular signals. Indeed, all biological structures and processes depend on the interplay of noncovalent interactions as well as covalent ones. The three fundamental noncovalent bonds are *electrostatic interactions*, *hydrogen bonds*, and *van der Waals interactions*. They differ in geometry, strength, and specificity. Furthermore, these bonds are greatly affected in different ways by the presence of water. Let us consider the characteristics of each:

1. *Electrostatic interactions.* An electrostatic interaction depends on the electric charges on atoms. The energy of an electrostatic interaction is given by *Coulomb's law*:

$$E = kq_1q_2/Dr$$

where E is the energy, q_1 and q_2 are the charges on the two atoms (in units of the electronic charge), r is the distance between the two atoms (in angstroms), D is the dielectric constant (which accounts for the effects of the intervening medium), and k is a proportionality constant ($k = 332$, to give energies in units of kilocalories per mole, or 1389 , for energies in kilojoules per mole). Thus, the electrostatic interaction between two atoms bearing single opposite charges separated by 3 \AA in water (which has a dielectric constant of 80) has an energy of $1.4 \text{ kcal mol}^{-1}$ (5.9 kJ mol^{-1}).

2. *Hydrogen bonds.* Hydrogen bonds are relatively weak interactions, which nonetheless are crucial for biological macromolecules such as DNA and proteins. These interactions are also responsible for many of the properties of water that make it such a special solvent. The hydrogen atom in a hydrogen bond is partly shared between two relatively electronegative atoms such as nitrogen or oxygen. The *hydrogen-bond donor* is the group that includes both the atom to which the hydrogen is more tightly linked and the hydrogen atom itself, whereas the *hydrogen-bond acceptor* is the atom less tightly linked to the hydrogen atom (Figure 1.9). Hydrogen bonds are fundamentally

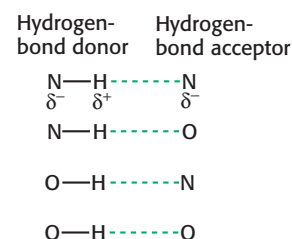
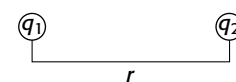
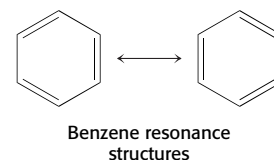


FIGURE 1.9 Hydrogen bonds that include nitrogen and oxygen atoms. The positions of the partial charges (δ^+ and δ^-) are shown.

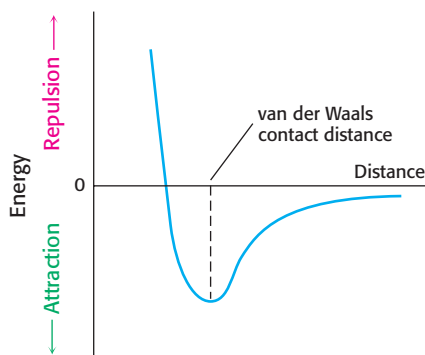
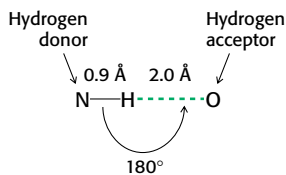


FIGURE 1.10 Energy of a van der Waals interaction as two atoms approach one another. The energy is most favorable at the van der Waals contact distance. The energy rises rapidly owing to electron–electron repulsion as the atoms move closer together than this distance.

electrostatic interactions. The relatively electronegative atom to which the hydrogen atom is covalently bonded pulls electron density away from the hydrogen atom so that it develops a partial positive charge (δ^+). Thus, it can interact with an atom having a partial negative charge (δ^-) through an electrostatic interaction.

Hydrogen bonds are much weaker than covalent bonds. They have energies of 1–3 kcal mol⁻¹ (4–13 kJ mol⁻¹) compared with approximately 100 kcal mol⁻¹ (418 kJ mol⁻¹) for a carbon–hydrogen covalent bond. Hydrogen bonds are also somewhat longer than are covalent bonds; their bond distances (measured from the hydrogen atom) range from 1.5 to 2.6 Å; hence, distances ranging from 2.4 to 3.5 Å separate the two nonhydrogen atoms in a hydrogen bond. The strongest hydrogen bonds have a tendency to be approximately straight, such that the hydrogen-bond donor, the hydrogen atom, and the hydrogen-bond acceptor lie along a straight line.

3. *van der Waals interactions.* The basis of a van der Waals interaction is that the distribution of electronic charge around an atom changes with time. At any instant, the charge distribution is not perfectly symmetric. This transient asymmetry in the electronic charge around an atom acts through electrostatic interactions to induce a complementary asymmetry in the electron distribution around its neighboring atoms. The resulting attraction between two atoms increases as they come closer to each other, until they are separated by the van der Waals contact distance (Figure 1.10). At a shorter distance, very strong repulsive forces become dominant because the outer electron clouds overlap.

Energies associated with van der Waals interactions are quite small; typical interactions contribute from 0.5 to 1.0 kcal mol⁻¹ (from 2 to 4 kJ mol⁻¹) per atom pair. When the surfaces of two large molecules come together, however, a large number of atoms are in van der Waals contact, and the net effect, summed over many atom pairs, can be substantial.

1.3.2 The Properties of Water Affect the Bonding Abilities of Biomolecules

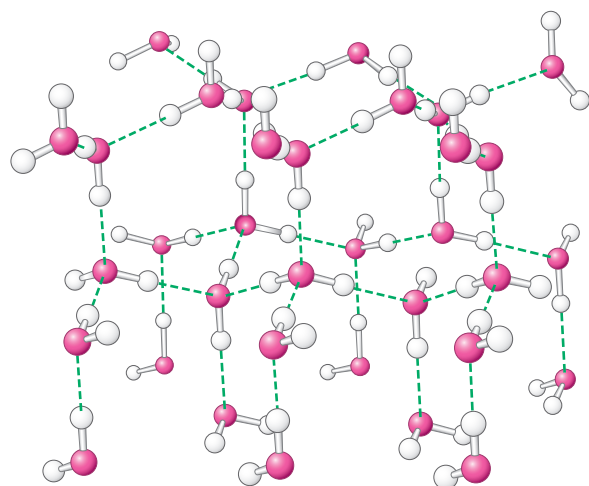
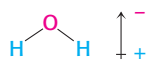


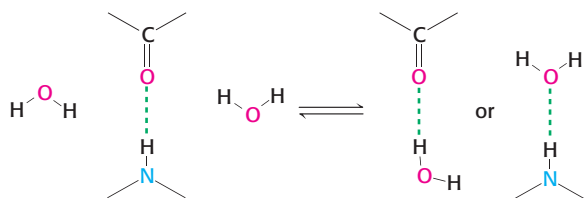
FIGURE 1.11 Structure of ice. Hydrogen bonds (shown as dashed lines) are formed between water molecules.

Weak interactions are the key means by which molecules interact with one another—enzymes with their substrates, hormones with their receptors, antibodies with their antigens. The strength and specificity of weak interactions are highly dependent on the medium in which they take place, and the majority of biological interactions take place in water. Two properties of water are especially important biologically:

1. *Water is a polar molecule.* The water molecule is bent, not linear, and so the distribution of charge is asymmetric. The oxygen nucleus draws electrons away from the hydrogen nuclei, which leaves the region around the hydrogen nuclei with a net positive charge. The water molecule is thus an electrically polar structure.

2. *Water is highly cohesive.* Water molecules interact strongly with one another through hydrogen bonds. These interactions are apparent in the structure of ice (Figure 1.11). Networks of hydrogen bonds hold the structure together; similar interactions link molecules in liquid water and account for the cohesion of liquid water, although, in the liquid state, some of the hydrogen bonds are broken. The highly cohesive nature of water dramatically affects the interactions between molecules in aqueous solution.

What is the effect of the properties of water on the weak interactions discussed in Section 1.3.1? The polarity and hydrogen-bonding capability of water make it a highly interacting molecule. Water is an excellent solvent for polar molecules. The reason is that water greatly weakens electrostatic forces and hydrogen bonding between polar molecules by competing for their attractions. For example, consider the effect of water on hydrogen bonding between a carbonyl group and the NH group of an amide.



A hydrogen atom of water can replace the amide hydrogen atom as a hydrogen-bond donor, whereas the oxygen atom of water can replace the carbonyl oxygen atom as a hydrogen-bond acceptor. Hence, a strong hydrogen bond between a CO group and an NH group forms only if water is excluded.

The dielectric constant of water is 80, so water diminishes the strength of electrostatic attractions by a factor of 80 compared with the strength of those same interactions in a vacuum. The dielectric constant of water is unusually high because of its polarity and capacity to form oriented solvent shells around ions. These oriented solvent shells produce electric fields of their own, which oppose the fields produced by the ions. Consequently, the presence of water markedly weakens electrostatic interactions between ions.

The existence of life on Earth depends critically on the capacity of water to dissolve a remarkable array of polar molecules that serve as fuels, building blocks, catalysts, and information carriers. High concentrations of these polar molecules can coexist in water, where they are free to diffuse and interact with one another. However, the excellence of water as a solvent poses a problem, because it also weakens interactions between polar molecules. *The presence of water-free microenvironments within biological systems largely circumvents this problem.* We will see many examples of these specially constructed niches in protein molecules. Moreover, the presence of water with its polar nature permits another kind of weak interaction to take place, one that drives the folding of proteins (Section 1.3.4) and the formation of cell boundaries (Section 12.3).

The essence of these interactions, like that of all interactions in biochemistry, is energy. To understand much of biochemistry—bond formation, molecular structure, enzyme catalysis—we need to understand energy. Thermodynamics provides a valuable tool for approaching this topic. We will revisit this topic in more detail when we consider enzymes (Chapter 8) and the basic concepts of metabolism (Chapter 14).

1.3.3 Entropy and the Laws of Thermodynamics

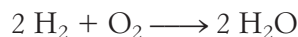
The highly structured, organized nature of living organisms is apparent and astonishing. This organization extends from the organismal through the cellular to the molecular level. Indeed, biological processes can seem magical in that the well-ordered structures and patterns emerge from the chaotic and disordered world of inanimate objects. However, the organization visible in a cell or a molecule arises from biological events that are subject to the same physical laws that govern all processes—in particular, the *laws of thermodynamics*.

How can we understand the creation of order out of chaos? We begin by noting that the laws of thermodynamics make a distinction between a system and its surroundings. A *system* is defined as the matter within a defined region of space. The matter in the rest of the universe is called the *surroundings*. *The First Law of Thermodynamics states that the total energy of a system and its surroundings is constant.* In other words, the energy content of the universe is constant; energy can be neither created nor destroyed. Energy can take different forms, however. Heat, for example, is one form of energy. Heat is a manifestation of the *kinetic energy* associated with the random motion of molecules. Alternatively, energy can be present as *potential energy*, referring to the ability of energy to be released on the occurrence of some process. Consider, for example, a ball held at the top of a tower. The ball has considerable potential energy because, when it is released, the ball will develop kinetic energy associated with its motion as it falls. Within chemical systems, potential energy is related to the likelihood that atoms can react with one another. For instance, a mixture of gasoline and oxygen has much potential energy because these molecules may react to form carbon dioxide and release energy as heat. The First Law requires that any energy released in the formation of chemical bonds be used to break other bonds, be released as heat, or be stored in some other form.

Another important thermodynamic concept is that of *entropy*. Entropy is a measure of the level of randomness or disorder in a system. *The Second Law of Thermodynamics states that the total entropy of a system and its surroundings always increases for a spontaneous process.* At first glance, this law appears to contradict much common experience, particularly about biological systems. Many biological processes, such as the generation of a well-defined structure such as a leaf from carbon dioxide gas and other nutrients, clearly increase the level of order and hence decrease entropy. Entropy may be decreased locally in the formation of such ordered structures only if the entropy of other parts of the universe is increased by an equal or greater amount.

An example may help clarify the application of the laws of thermodynamics to a chemical system. Consider a container with 2 moles of hydrogen gas on one side of a divider and 1 mole of oxygen gas on the other (Figure 1.12). If the divider is removed, the gases will intermingle spontaneously to form a uniform mixture. The process of mixing increases entropy as an ordered arrangement is replaced by a randomly distributed mixture.

Other processes within this system can decrease the entropy locally while increasing the entropy of the universe. A spark applied to the mixture initiates a chemical reaction in which hydrogen and oxygen combine to form water:



If the temperature of the system is held constant, the entropy of the system decreases because 3 moles of two differing reactants have been combined to form 2 moles of a single product. The gas now consists of a uniform set of indistinguishable molecules. However, the reaction releases a significant amount of heat into the surroundings, and this heat will increase the entropy of the surrounding molecules by increasing their random movement.

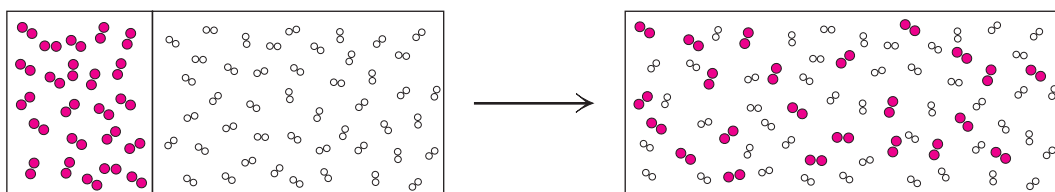


FIGURE 1.12 From order to disorder. The spontaneous mixing of gases is driven by an increase in entropy.

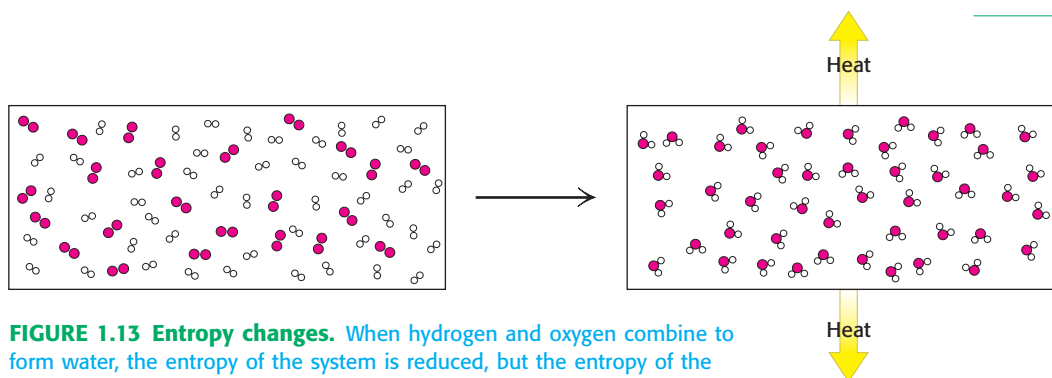


FIGURE 1.13 Entropy changes. When hydrogen and oxygen combine to form water, the entropy of the system is reduced, but the entropy of the universe is increased owing to the release of heat to the surroundings.

The entropy increase in the surroundings is enough to allow water to form spontaneously from hydrogen and oxygen (Figure 1.13).

The change in the entropy of the surroundings will be proportional to the amount of heat transferred from the system and inversely proportional to the temperature of the surroundings, because an input of heat leads to a greater increase in entropy at lower temperatures than at higher temperatures. In biological systems, T [in kelvin (K), absolute temperature] is assumed to be constant. If we define the heat content of a system as *enthalpy* (H), then we can express the relation linking the entropy (S) of the surroundings to the transferred heat and temperature as a simple equation:

$$\Delta S_{\text{surroundings}} = -\Delta H_{\text{system}}/T \quad (1)$$

The total entropy change is given by the expression

$$\Delta S_{\text{total}} = \Delta S_{\text{system}} + \Delta S_{\text{surroundings}} \quad (2)$$

Substituting equation 1 into equation 2 yields

$$\Delta S_{\text{total}} = \Delta S_{\text{system}} - \Delta H_{\text{system}}/T \quad (3)$$

Multiplying by $-T$ gives

$$-T\Delta S_{\text{total}} = \Delta H_{\text{system}} - T\Delta S_{\text{system}} \quad (4)$$

The function $-T\Delta S$ has units of energy and is referred to as *free energy* or *Gibbs free energy*, after Josiah Willard Gibbs, who developed this function in 1878:

$$\Delta G = \Delta H_{\text{system}} - T\Delta S_{\text{system}} \quad (5)$$

The free-energy change, ΔG , will be used throughout this book to describe the energetics of biochemical reactions.

Recall that the Second Law of Thermodynamics states that, for a reaction to be spontaneous, the entropy of the universe must increase. Examination of equation 3 shows that the total entropy will increase if and only if

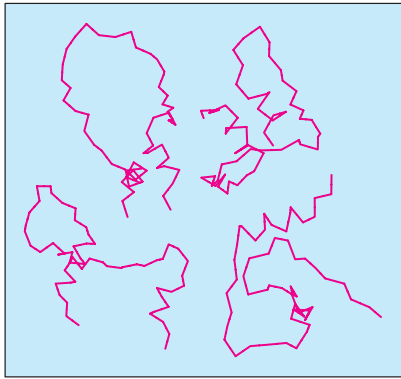
$$\Delta S_{\text{system}} > \Delta H_{\text{system}}/T \quad (6)$$

Rearranging gives $T\Delta S_{\text{system}} > \Delta H$, or entropy will increase if and only if

$$\Delta G = \Delta H_{\text{system}} - T\Delta S_{\text{system}} < 0 \quad (7)$$

In other words, *the free-energy change must be negative for a reaction to be spontaneous*. A negative free-energy change occurs with an increase in the overall entropy of the universe. Thus, we need to consider only one term, the free energy of the system, to decide whether a reaction can occur spontaneously; any effects of the changes within the system on the rest of the universe are automatically taken into account.

Unfolded ensemble



Folded ensemble

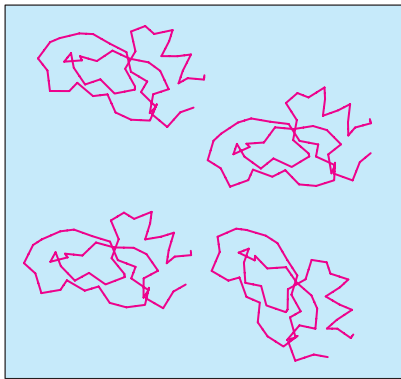


FIGURE 1.14 Protein folding. Protein folding entails the transition from a disordered mixture of unfolded molecules to a relatively uniform solution of folded protein molecules.

1.3.4 Protein Folding Can Be Understood in Terms of Free-Energy Changes

The problem of protein folding illustrates the utility of the concept of free energy. Consider a system consisting of a solution of unfolded protein molecules in aqueous solution (Figure 1.14). Each unfolded protein molecule can adopt a unique conformation, so the system is quite disordered and the entropy of the collection of molecules is relatively high. Yet, protein folding proceeds spontaneously under appropriate conditions. Thus, entropy must be increasing elsewhere in the system or in the surroundings. How can we reconcile the apparent contradiction that proteins spontaneously assume an ordered structure, and yet entropy increases? The entropy decrease in the system on folding is not as large as it appears to be, because of the properties of water. Molecules in aqueous solution interact with water molecules through the formation of hydrogen and ionic interactions. However, some molecules (termed *nonpolar molecules*) cannot participate in hydrogen or ionic interactions. The interactions of nonpolar molecules with water are not as favorable as are interactions between the water molecules themselves. The water molecules in contact with these nonpolar surfaces form “cages” around the nonpolar molecule, becoming more well ordered (and, hence, lower in entropy) than water molecules free in solution. As two such nonpolar molecules come together, some of the water molecules are released, and so they can interact freely with bulk water (Figure 1.15). Hence, nonpolar molecules have a tendency to aggregate in water because the entropy of the water is increased through the release of water molecules. This phenomenon, termed the *hydrophobic effect*, helps promote many biochemical processes.

How does the hydrophobic effect favor protein folding? Some of the amino acids that make up proteins have nonpolar groups. These nonpolar amino acids have a strong tendency to associate with one another inside the interior of the folded protein. The increased entropy of water resulting from the interaction of these hydrophobic amino acids helps to compensate for the entropy losses inherent in the folding process.

Hydrophobic interactions are not the only means of stabilizing protein structure. Many weak bonds, including hydrogen bonds and van der Waals interactions, are formed in the protein-folding process, and heat is released into the surroundings as a consequence. Although these interactions replace interactions with water that take place in the unfolded protein, the net result is the release of heat to the surroundings and thus a negative (favorable) change in enthalpy for the system.

The folding process can occur when the combination of the entropy associated with the hydrophobic effect and the enthalpy change associated with hydrogen bonds and van der Waals interactions makes the overall free energy negative.

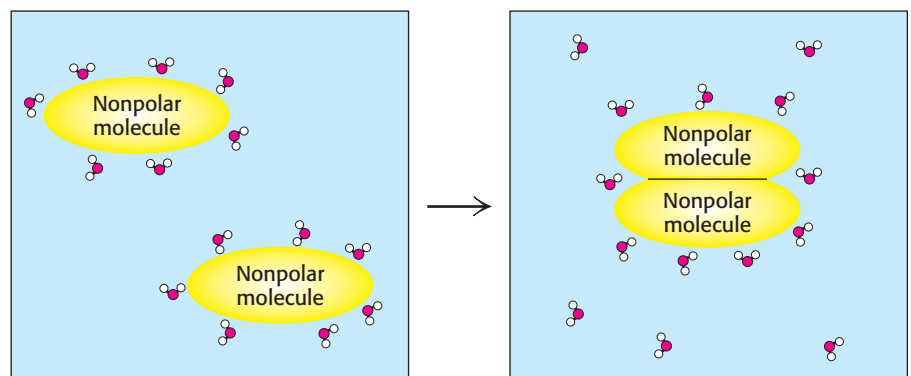


FIGURE 1.15 The hydrophobic effect. The aggregation of nonpolar groups in water leads to an increase in entropy owing to the release of water molecules into bulk water.

Our understanding of biochemistry has had and will continue to have extensive effects on many aspects of human endeavor. *First, biochemistry is an intrinsically beautiful and fascinating body of knowledge.* We now know the essence and many of the details of the most fundamental processes in biochemistry, such as how a single molecule of DNA replicates to generate two identical copies of itself and how the sequence of bases in a DNA molecule determines the sequence of amino acids in an encoded protein. Our ability to describe these processes in detailed, mechanistic terms places a firm chemical foundation under other biological sciences. Moreover, the realization that we can understand essential life processes, such as the transmission of hereditary information, as chemical structures and their reactions has significant philosophical implications. What does it mean, biochemically, to be human? What are the biochemical differences between a human being, a chimpanzee, a mouse, and a fruit fly? Are we more similar than we are different?

Second, biochemistry is greatly influencing medicine and other fields. The molecular lesions causing sickle-cell anemia, cystic fibrosis, hemophilia, and many other genetic diseases have been elucidated at the biochemical level. Some of the molecular events that contribute to cancer development have been identified. An understanding of the underlying defects opens the door to the discovery of effective therapies. Biochemistry makes possible the rational design of new drugs, including specific inhibitors of enzymes required for the replication of viruses such as human immunodeficiency virus (HIV). Genetically engineered bacteria or other organisms can be used as “factories” to produce valuable proteins such as insulin and stimulators of blood-cell development. Biochemistry is also contributing richly to clinical diagnostics. For example, elevated levels of telltale enzymes in the blood reveal whether a patient has recently had a myocardial infarction (heart attack). DNA probes are coming into play in the precise diagnosis of inherited disorders, infectious diseases, and cancers. Agriculture, too, is benefiting from advances in biochemistry with the development of more effective, environmentally safer herbicides and pesticides and the creation of genetically engineered plants that are, for example, more resistant to insects. All of these endeavors are being accelerated by the advances in genomic sequencing.

Third, advances in biochemistry are enabling researchers to tackle some of the most exciting questions in biology and medicine. How does a fertilized egg give rise to cells as different as those in muscle, brain, and liver? How do the senses work? What are the molecular bases for mental disorders such as Alzheimer disease and schizophrenia? How does the immune system distinguish between self and nonself? What are the molecular mechanisms of short-term and long-term memory? The answers to such questions, which once seemed remote, have been partly uncovered and are likely to be more thoroughly revealed in the near future.

Because all living organisms on Earth are linked by a common origin, evolution provides a powerful organizing theme for biochemistry. This book is organized to emphasize the unifying principles revealed by evolutionary considerations. We begin in the next chapter with a brief tour along a plausible evolutionary path from the formation of some of the chemicals that we now associate with living organisms through the evolution of the processes essential for the development of complex, multicellular organisms. The remainder of Part I of the book more fully introduces the most important classes of biochemicals as well as catalysis and regulation. Part II, Transducing and Storing Energy, describes how energy from chemicals or from sunlight is converted into usable forms and how this conversion is regulated. As we will see, a small set of molecules such as adenosine triphosphate

(ATP) act as energy currencies that allow energy, however captured, to be utilized in a variety of biochemical processes. This part of the text examines the important pathways for the conversion of environmental energy into molecules such as ATP and uncovers many unifying principles. Part III, *Synthesizing the Molecules of Life*, illustrates the use of the molecules discussed in Part II to synthesize key molecular building blocks, such as the bases of DNA and amino acids, and then shows how these precursors are assembled into DNA, RNA, and proteins. In Parts II and III, we will highlight the relation between the reactions within each pathway and between those in different pathways so as to suggest how these individual reactions may have combined early in evolutionary history to produce the necessary molecules. From the student's perspective, the existence of features common to several pathways enables material mastered in one context to be readily applied to new contexts. Part IV, *Responding to Environmental Changes*, explores some of the mechanisms that cells and multicellular organisms have evolved to detect and respond to changes in the environment. The topics range from general mechanisms, common to all organisms, for regulating the expression of genes to the sensory systems used by human beings and other complex organisms. In many cases, we can now see how these elaborate systems evolved from pathways that existed earlier in evolutionary history. Many of the sections in Part IV link biochemistry with other fields such as cell biology, immunology, and neuroscience. We are now ready to begin our journey into biochemistry with events that took place more than 3 billion years ago.

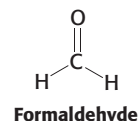
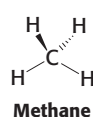
APPENDIX: DEPICTING MOLECULAR STRUCTURES

The authors of a biochemistry text face the problem of trying to present three-dimensional molecules in the two dimensions available on the printed page. The interplay between the three-dimensional structures of biomolecules and their biological functions will be discussed extensively throughout this book. Toward this end, we will frequently use representations that, although of necessity are rendered in two dimensions, emphasize the three-dimensional structures of molecules.

Stereochemical Renderings

Most of the chemical formulas in this text are drawn to depict the geometric arrangement of atoms, crucial to chemical bonding and reactivity, as accurately as possible. For example, the carbon atom of methane is sp^3 hybridized and tetrahedral, with

H–C–H angles of 109.5 degrees while the carbon atom in formaldehyde is sp^2 hybridized with bond angles of 120 degrees.



To illustrate the correct *stereochemistry* about carbon atoms, wedges will be used to depict the direction of a bond into or out of the plane of the page. A solid wedge with the broad end away from the carbon denotes a bond coming toward the viewer out of the plane. A dashed wedge, with the broad end of the bond at the carbon represents a bond going away from the viewer into the plane of the page. The remaining two bonds are depicted as straight lines.

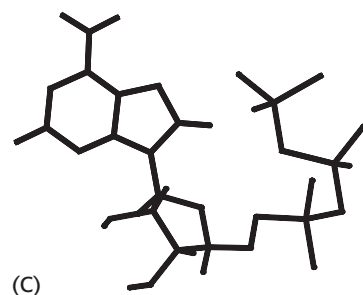
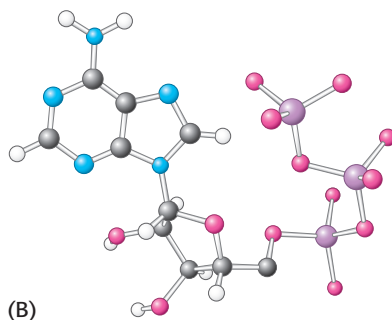
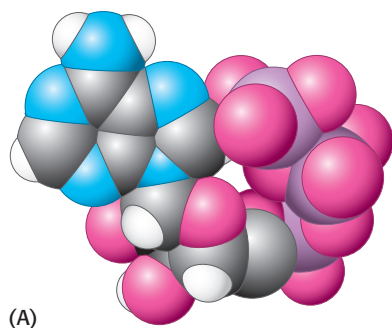
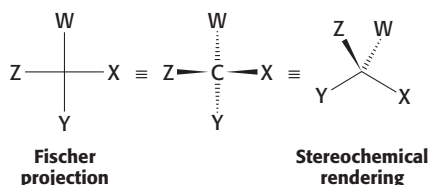


FIGURE 1.16 Molecular representations. Comparison of (A) space-filling, (B) ball-and-stick, and (C) skeletal models of ATP.

Fischer Projections

Although more representative of the actual structure of a compound, stereochemical structures are often difficult to draw quickly. An alternative method of depicting structures with tetrahedral carbon centers relies on the use of *Fischer projections*.



In a Fischer projection, the bonds to the central carbon are represented by horizontal and vertical lines from the substituent atoms to the carbon atom, which is assumed to be at the center of the cross. By convention, the horizontal bonds are assumed to project out of the page toward the viewer, whereas the vertical bonds are assumed to project into the page away from the viewer. Appendix xx at the back of the book is a structural glossary of the key molecules in biochemistry, presented both as stereochemically accurate structures and as Fischer projections.

For depicting molecular architecture in more detail, five types of models will be used: space filling, ball and stick, skeletal, ribbon, and surface representations (Figure 1.16). The first three types show structures at the atomic level.

1. *Space-filling models*. The space-filling models are the most realistic. The size and position of an atom in a space-filling model are determined by its bonding properties and van der Waals radius, or contact distance (Section 1.3.1). A van der Waals radius describes how closely two atoms can approach each other when they are not linked by a covalent bond. The colors of the model are set by convention.

Carbon, black	Hydrogen, white	Nitrogen, blue
Oxygen, red	Sulfur, yellow	Phosphorus, purple

Space-filling models of several simple molecules are shown in Figure 1.17.

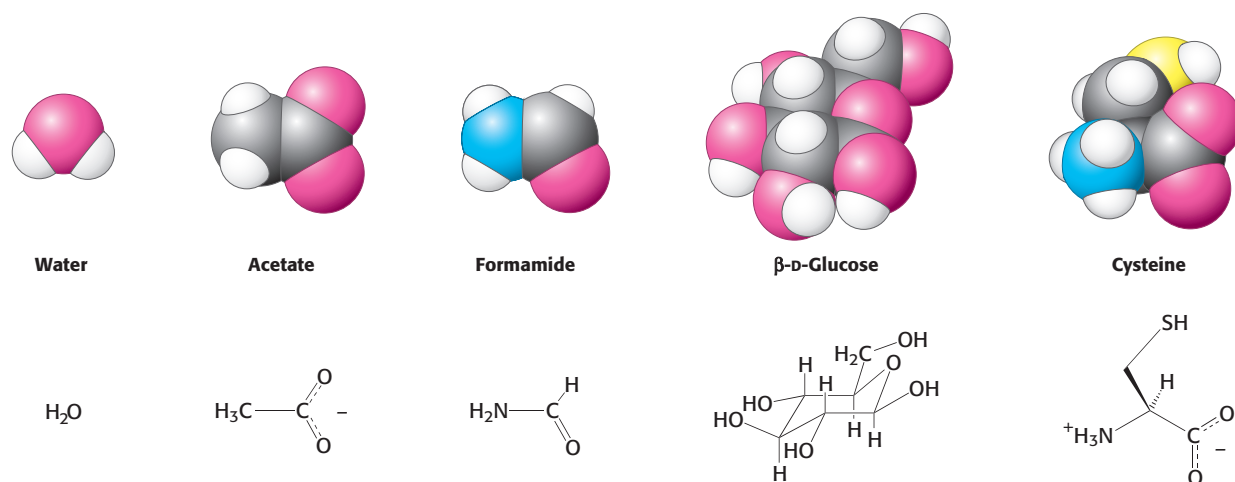


FIGURE 1.17 Space-filling models. Structural formulas and space-filling representations of selected molecules are shown.

2. *Ball-and-stick models*. Ball-and-stick models are not as realistic as space-filling models, because the atoms are depicted as spheres of radii smaller than their van der Waals radii. However, the bonding arrangement is easier to see because the bonds are explicitly represented as sticks. In an illustration, the taper of a stick, representing parallax, tells which of a pair of bonded atoms is closer to the reader. A ball-and-stick model reveals a complex structure more clearly than a space-filling model does.

3. *Skeletal models*. An even simpler image is achieved with a skeletal model, which shows only the molecular framework. In skeletal models, atoms are not shown explicitly. Rather, their positions are implied by the junctions and ends of bonds. Skeletal models are frequently used to depict larger, more complex structures.

As biochemistry has advanced, more attention has been focused on the structures of biological macromolecules and their complexes. These structures comprise thousands or even tens of thousands of atoms. Although these structures can be depicted at the atomic level, it is difficult to discern the relevant structural features because of the large number of atoms. Thus, more schematic representations—ribbon diagrams and surface representations—have been developed for the depiction of macromolecular structures in which atoms are not shown explicitly (Figure 1.18).

4. *Ribbon diagrams*. These diagrams are highly schematic and most commonly used to accent a few dramatic aspects of protein structure, such as the α helix (a coiled ribbon), the β strand (a broad arrow), and loops (simple lines), so as to provide simple and clear views of the folding patterns of proteins.

5. *Surface representations*. Often, the interactions between macromolecules take place exclusively at their surfaces. Surface representations have been developed to better visualize macromolecular surfaces. These representations display the overall shapes of macromolecules and can be shaded or colored to indicate particular features such as surface topography or the distribution of electric charges.

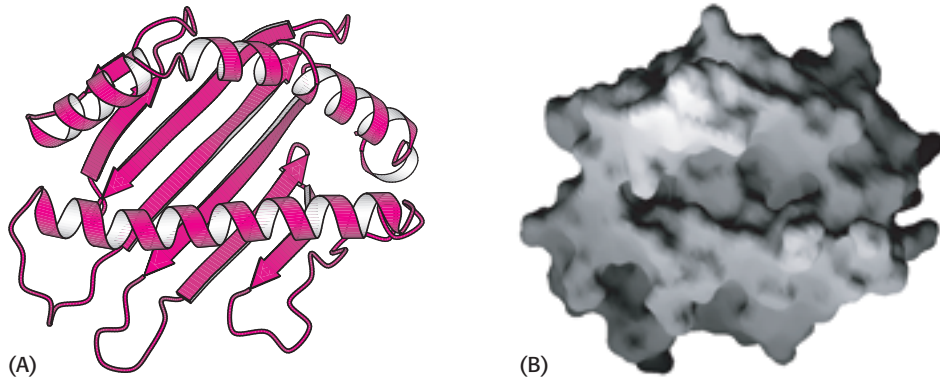


FIGURE 1.18 Alternative representations of protein structure. A ribbon diagram (A) and a surface representation (B) of a key protein from the immune system emphasize different aspects of structure.

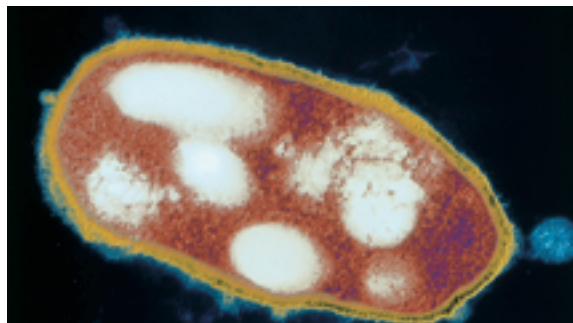
KEY TERMS

deoxyribonucleic acid (DNA) (p. 4)
 double helix (p. 5)
 ribonucleic acid (RNA) (p. 6)
 protein (p. 6)
 amino acid (p. 6)
 genetic code (p. 6)
 Eukarya (p. 8)
 Bacteria (p. 8)
 Archaea (p. 8)

eukaryote (p. 8)
 prokaryote (p. 8)
 covalent bond (p. 8)
 resonance structure (p. 9)
 electrostatic interaction (p. 9)
 hydrogen bond (p. 9)
 van der Waals interaction (p. 9)
 entropy (p. 12)
 enthalpy (p. 13)

free energy (p. 13)
 hydrophobic effect (p. 14)
 stereochemistry (p. 16)
 Fischer projection (p. 17)
 space-filling model (p. 17)
 ball-and-stick-model (p. 17)
 skeletal model (p. 17)
 ribbon diagram (p. 17)
 surface presentation (p. 17)

Biochemical Evolution



Natural selection, one of the key forces powering evolution, opens an array of improbable ecological niches to species that can adapt biochemically. (Left) Salt pools, where the salt concentration can be greater than 1.5 M, would seem to be highly inhospitable environments for life. Yet certain halophilic archaea, such as *Haloferax mediterranei* (right), possess biochemical adaptations that enable them to thrive under these harsh conditions. [(Left) Kaj R. Svensson/Science Photo Library/Photo Researchers; (right) Wanner/Eye of Science/Photo Researchers.]

Earth is approximately 4.5 billion years old. Remarkably, there is convincing fossil evidence that organisms morphologically (and very probably biochemically) resembling certain modern bacteria were in existence 3.5 billion years ago. With the use of the results of directed studies and accidental discoveries, it is now possible to construct a hypothetical yet plausible evolutionary path from the prebiotic world to the present. A number of uncertainties remain, particularly with regard to the earliest events. Nonetheless, a consideration of the steps along this path and the biochemical problems that had to be solved provides a useful perspective from which to regard the processes found in modern organisms. *These evolutionary connections make many aspects of biochemistry easier to understand.*

We can think of the path leading to modern living species as consisting of stages, although it is important to keep in mind that these stages were almost certainly not as distinct as presented here. The first stage was the initial generation of some of the key molecules of life—nucleic acids, proteins, carbohydrates, and lipids—by nonbiological processes. The second stage was fundamental—the transition from prebiotic chemistry to replicating systems. With the passage of time, these systems became increasingly sophisticated, enabling the formation of living cells. In the third stage, mechanisms evolved for interconverting energy from chemical sources and sunlight into forms that can be utilized to drive biochemical reactions. Intertwined with these energy-conversion processes are pathways for synthesizing the components of nucleic acids, proteins, and

OUTLINE

- 2.1 Key Organic Molecules Are Used by Living Systems
- 2.2 Evolution Requires Reproduction, Variation, and Selective Pressure
- 2.3 Energy Transformations Are Necessary to Sustain Living Systems
- 2.4 Cells Can Respond to Changes in Their Environments

other key substances from simpler molecules. With the development of energy-conversion processes and biosynthetic pathways, a wide variety of unicellular organisms evolved. The fourth stage was the evolution of mechanisms that allowed cells to adjust their biochemistry to different, and often changing, environments. Organisms with these capabilities could form colonies comprising groups of interacting cells, and some eventually evolved into complex multicellular organisms.

This chapter introduces key challenges posed in the evolution of life, whose solutions are elaborated in later chapters. Exploring a possible evolutionary origin for these fundamental processes makes their use, in contrast with that of potential alternatives, more understandable.

2.1 KEY ORGANIC MOLECULES ARE USED BY LIVING SYSTEMS

Approximately 1 billion years after Earth's formation, life appeared, as already mentioned. Before life could exist, though, another major process needed to have taken place—the synthesis of the organic molecules required for living systems from simpler molecules found in the environment. The components of nucleic acids and proteins are relatively complex organic molecules, and one might expect that only sophisticated synthetic routes could produce them. However, this requirement appears not to have been the case. How did the building blocks of life come to be?

2.1.1 Many Components of Biochemical Macromolecules Can Be Produced in Simple, Prebiotic Reactions

Among several competing theories about the conditions of the *prebiotic world*, none is completely satisfactory or problem-free. One theory holds that Earth's early atmosphere was highly reduced, rich in methane (CH_4), ammonia (NH_3), water (H_2O), and hydrogen (H_2), and that this atmosphere was subjected to large amounts of solar radiation and lightning. For the sake of argument, we will assume that these conditions were indeed those of prebiotic Earth. Can complex organic molecules be synthesized under these conditions? In the 1950s, Stanley Miller and Harold Urey set out to answer this question. An electric discharge, simulating lightning, was passed through a mixture of methane, ammonia, water, and hydrogen (Figure 2.1). Remarkably, these experiments yielded a highly nonrandom mixture of organic compounds, including amino acids and other substances fundamental to biochemistry. The procedure produces the amino acids glycine and alanine in approximately 2% yield, depending on the amount of carbon supplied as methane. More complex amino acids such as glutamic acid and leucine are produced in smaller amounts (Figure 2.2). Hydrogen cyanide (HCN), another likely component of the early atmosphere, will con-

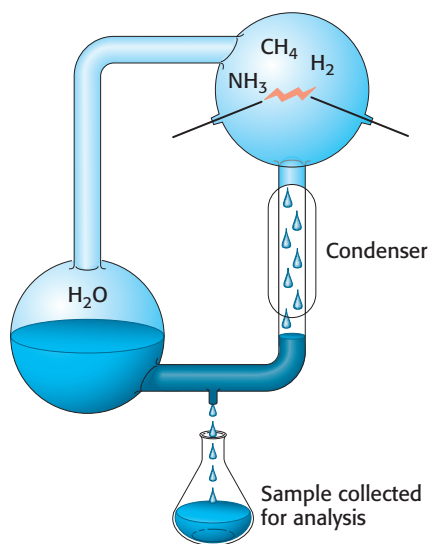


FIGURE 2.1 The Urey-Miller experiment. An electric discharge (simulating lightning) passed through an atmosphere of CH_4 , NH_3 , H_2O , and H_2 leads to the generation of key organic compounds such as amino acids.

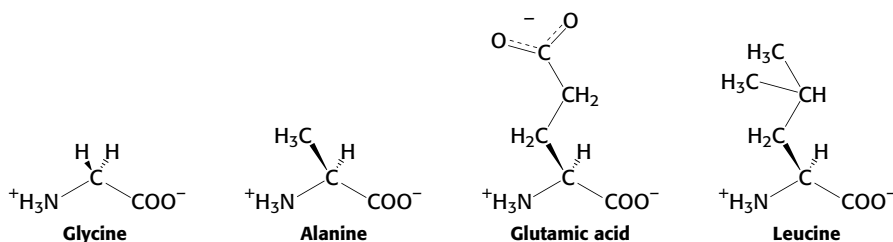


FIGURE 2.2 Products of prebiotic synthesis. Amino acids produced in the Urey-Miller experiment.

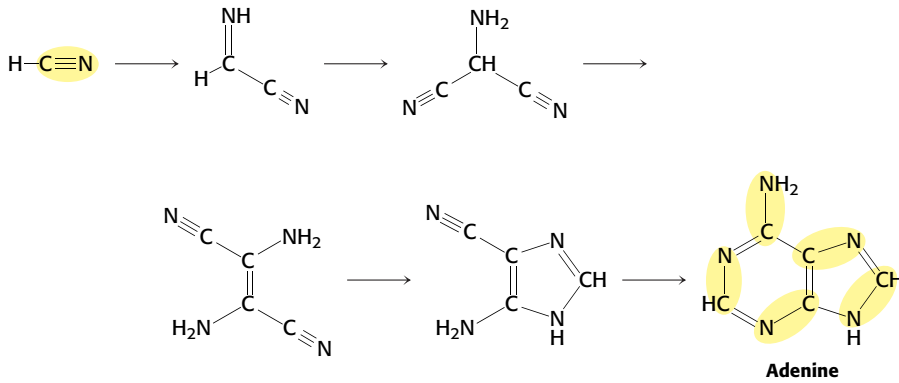


FIGURE 2.3 Prebiotic synthesis of a nucleic acid component. Adenine can be generated by the condensation of HCN.

dense on exposure to heat or light to produce adenine, one of the four nucleic acid bases (Figure 2.3). Other simple molecules combine to form the remaining bases. A wide array of sugars, including ribose, can be formed from formaldehyde under prebiotic conditions.

2.1.2 Uncertainties Obscure the Origins of Some Key Biomolecules

The preceding observations suggest that many of the building blocks found in biology are unusually easy to synthesize and that significant amounts could have accumulated through the action of nonbiological processes. However, it is important to keep in mind that there are many uncertainties. For instance, ribose is just one of many sugars formed under prebiotic conditions. In addition, ribose is rather unstable under possible prebiotic conditions. Furthermore, ribose occurs in two mirror-image forms, only one of which occurs in modern RNA. To circumvent those problems, the first nucleic acid-like molecules have been suggested to have been bases attached to a different backbone and only later in evolutionary time was ribose incorporated to form nucleic acids as we know them today. Despite these uncertainties, an assortment of prebiotic molecules did arise in some fashion, and from this assortment *those with properties favorable for the processes that we now associate with life began to interact and to form more complicated compounds*. The processes through which modern organisms synthesize molecular building blocks will be discussed in Chapters 24, 25, and 26.

2.2 EVOLUTION REQUIRES REPRODUCTION, VARIATION, AND SELECTIVE PRESSURE

Once the necessary building blocks were available, how did a living system arise and evolve? Before the appearance of life, simple molecular systems must have existed that subsequently evolved into the complex chemical systems that are characteristic of organisms. To address how this evolution occurred, we need to consider the *process* of evolution. There are several basic principles common to evolving systems, whether they are simple collections of molecules or competing populations of organisms. First, the most fundamental property of evolving systems is their ability to *replicate* or *reproduce*. Without this ability of *reproduction*, each “species” of molecule that might appear is doomed to extinction as soon as all its individual molecules degrade. For example, individual molecules of biological polymers such as ribonucleic acid are degraded by hydrolysis reactions and other processes. However, *molecules that can replicate will continue to be represented in the population even if the lifetime of each individual molecule remains short*.

A second principle fundamental to evolution is *variation*. The replicating systems must undergo changes. After all, if a system always replicates perfectly, the replicated molecule will always be the same as the parent molecule. Evolution cannot occur. The nature of these variations in living systems are considered in Section 2.2.5.

A third basic principle of evolution is *competition*. Replicating molecules compete with one another for available resources such as chemical precursors, and the competition allows the process of *evolution by natural selection* to occur. Variation will produce differing populations of molecules. Some variant offspring may, by chance, be better suited for survival and replication under the prevailing conditions than are their parent molecules. The prevailing conditions exert a *selective pressure* that gives an advantage to one of the variants. Those molecules that are best able to survive and to replicate themselves will increase in relative concentration. Thus, new molecules arise that are better able to replicate under the conditions of their environment. The same principles hold true for modern organisms. Organisms reproduce, show variation among individual organisms, and compete for resources; those variants with a selective advantage will reproduce more successfully. The changes leading to variation still take place at the molecular level, but the selective advantage is manifest at the organismal level.

2.2.1 The Principles of Evolution Can Be Demonstrated in Vitro

Is there any evidence that evolution can take place at the molecular level? In 1967, Sol Spiegelman showed that replicating molecules could evolve new forms in an experiment that allowed him to observe molecular evolution in the test tube. He used as his evolving molecules RNA molecules derived from a bacterial virus called bacteriophage Q β . The genome of bacteriophage Q β , a single RNA strand of approximately 3300 bases, depends for its replication on the activity of a protein complex termed Q β replicase. Spiegelman mixed the replicase with a starting population of Q β RNA molecules. Under conditions in which there are ample amounts of precursors, no time constraints, and no other selective pressures, the composition of the population does not change from that of the parent molecules on replication. When selective pressures are applied, however, the composition of the population of molecules can change dramatically. For example, decreasing the time available for replication from 20 minutes to 5 minutes yielded, incrementally over 75 generations, a population of molecules dominated by a single species comprising only 550 bases. This species is replicated 15 times as rapidly as the parental Q β RNA (Figure 2.4). Spiegelman applied other selective pressures by, for example, limiting the concentrations of precursors or adding compounds that inhibit the replication process. In each case, new species appeared that replicated more effectively under the conditions imposed.

The process of evolution demonstrated in these studies depended on the existence of machinery for the replication of RNA fragments in the form of the Q β replicase.

As noted in Chapter 1, one of the most elegant characteristics of nucleic acids is that the mechanism for their replication follows naturally from their molecular structure. This observation suggests that nucleic acids, perhaps RNA, could have become *self-replicating*. Indeed, the results of studies have revealed that single-stranded nucleic acids can serve as templates for the synthesis of their complementary strands and that this synthesis can occur spontaneously—that is, without biologically de-

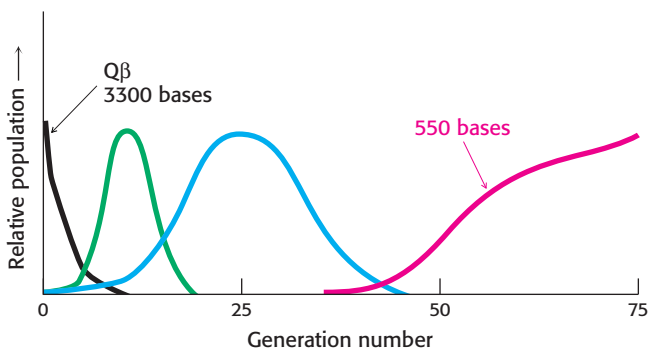


FIGURE 2.4 Evolution in a test tube. Rapidly replicating species of RNA molecules were generated from Q β RNA by exerting selective pressure. The green and blue curves correspond to species of intermediate size that accumulated and then became extinct in the course of the experiment.

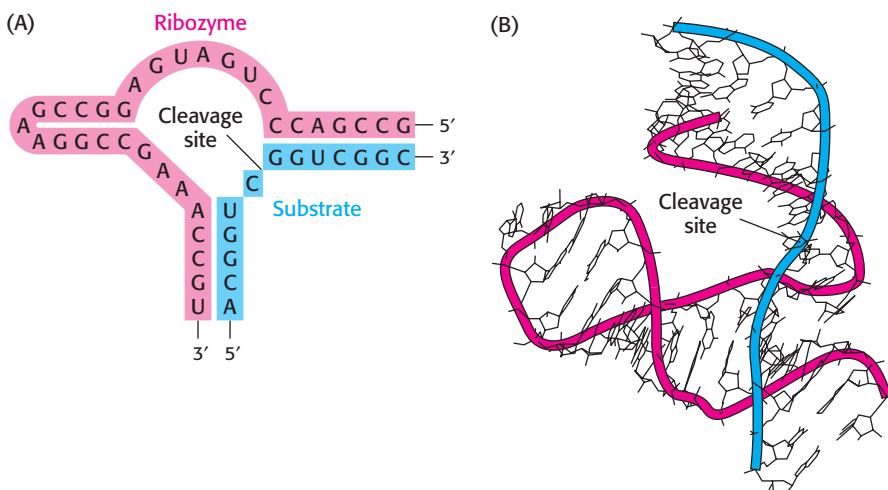
rived replication machinery. However, investigators have not yet found conditions in which an RNA molecule is fully capable of independent self-replication from simple starting materials.

2.2.2 RNA Molecules Can Act As Catalysts

The development of capabilities beyond simple replication required the generation of specific catalysts. A *catalyst* is a molecule that accelerates a particular chemical reaction without itself being chemically altered in the process. The properties of catalysts will be discussed in detail in Chapters 8 and 9. Some catalysts are highly specific; they promote certain reactions without substantially affecting closely related processes. Such catalysts allow the reactions of specific pathways to take place in preference to those of potential alternative pathways. Until the 1980s, all biological catalysts, termed *enzymes*, were believed to be proteins. Then, Tom Cech and Sidney Altman independently discovered that certain RNA molecules can be effective catalysts. These RNA catalysts have come to be known as *ribozymes*. The discovery of ribozymes suggested the possibility that catalytic RNA molecules could have played fundamental roles early in the evolution of life.

The catalytic ability of RNA molecules is related to their ability to adopt specific yet complex structures. This principle is illustrated by a “hammerhead” ribozyme, an RNA structure first identified in plant viruses (Figure 2.5). This RNA molecule promotes the cleavage of specific RNA molecules at specific sites; this cleavage is necessary for certain aspects of the viral life cycle. The ribozyme, which requires Mg^{2+} ion or other ions for the cleavage step to take place, forms a complex with its substrate RNA molecule that can adopt a reactive conformation.

The existence of RNA molecules that possess specific binding and catalytic properties makes plausible the idea of an early “RNA world” inhabited by life forms dependent on RNA molecules to play all major roles, including those important in heredity, the storage of information, and the promotion of specific reactions—that is, biosynthesis and energy metabolism.



This icon, appearing throughout the book, indicates an opportunity to explore further resources available on the *Biochemistry* Web site; www.whfreeman.com/biochem5. This icon in a figure caption indicates a **Living Figure** that allows you to interact with three-dimensional representations of the illustration. Go to the Website and select the chapter and figure number.



FIGURE 2.5 Catalytic RNA.

(A) The base-pairing pattern of a “hammerhead” ribozyme and its substrate. (B) The folded conformation of the complex. The ribozyme cleaves the bond at the cleavage site. The paths of the nucleic acid backbones are highlighted in red and blue.

2.2.3 Amino Acids and Their Polymers Can Play Biosynthetic and Catalytic Roles

In the early RNA world, the increasing populations of replicating RNA molecules would have consumed the building blocks of RNA that had been generated over long periods of time by prebiotic reactions. A shortage of these compounds would have favored the evolution of alternative mechanisms

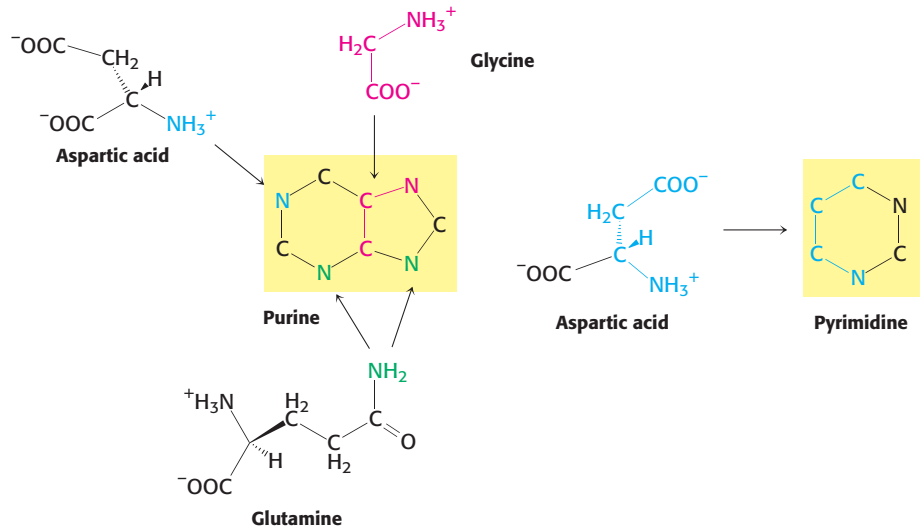


FIGURE 2.6 Biosynthesis of RNA bases. Amino acids are building blocks for the biosynthesis of purines and pyrimidines.

for their synthesis. A large number of pathways are possible. Examining the biosynthetic routes utilized by modern organisms can be a source of insight into which pathways survived. A striking observation is that simple amino acids are used as building blocks for the RNA bases (Figure 2.6). For both purines (adenine and guanine) and pyrimidines (uracil and cytosine), an amino acid serves as a core onto which the remainder of the base is elaborated. In addition, nitrogen atoms are donated by the amino group of the amino acid aspartic acid and by the amide group of the glutamine side chain.

Amino acids are chemically more versatile than nucleic acids because their side chains carry a wider range of chemical functionality. Thus, amino acids or short polymers of amino acids linked by *peptide bonds*, called *polypeptides* (Figure 2.7), may have functioned as components of ribozymes to provide a specific reactivity. Furthermore, longer polypeptides are capable of spontaneously folding to form well-defined three-dimensional structures, dictated by the sequence of amino acids along their polypeptide chains. The ability of polypeptides to fold spontaneously into elaborate structures, which permit highly specific chemical interactions with other molecules, may have favored the expansion of their roles in the course of evolution and is crucial to their dominant position in modern organisms. Today, most biological catalysts (enzymes) are not nucleic acids but are instead large polypeptides called *proteins*.

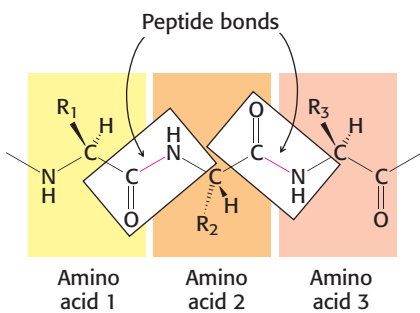


FIGURE 2.7 An alternative functional polymer. Proteins are built of amino acids linked by peptide bonds.

2.2.4 RNA Template-Directed Polypeptide Synthesis Links the RNA and Protein Worlds

Polypeptides would have played only a limited role early in the evolution of life because their structures are not suited to self-replication in the way that nucleic acid structures are. However, polypeptides could have been included in evolutionary processes indirectly. For example, if the properties of a particular polypeptide favored the survival and replication of a class of RNA molecules, then these RNA molecules could have evolved ribozyme activities that promoted the synthesis of that polypeptide. This method of producing polypeptides with specific amino acid sequences has several limitations. First, it seems likely that only relatively short specific polypeptides could have been produced in this manner. Second, it would have been difficult to accurately link the particular amino acids in the polypeptide in a reproducible manner. Finally, a different ribozyme would have been required for each polypeptide. A critical point in evolution was reached when

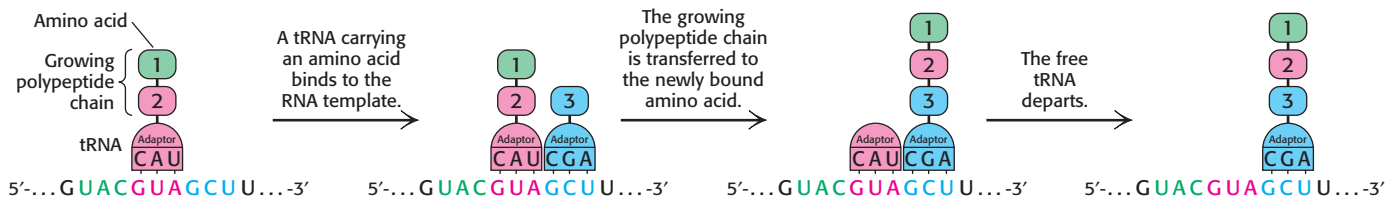


FIGURE 2.8 Linking the RNA and protein worlds. Polypeptide synthesis is directed by an RNA template. Adaptor RNA molecules, with amino acids attached, sequentially bind to the template RNA to facilitate the formation of a peptide bond between two amino acids. The growing polypeptide chain remains attached to an adaptor RNA until the completion of synthesis.

an apparatus for polypeptide synthesis developed that allowed *the sequence of bases in an RNA molecule to directly dictate the sequence of amino acids in a polypeptide*. A code evolved that established a relation between a specific sequence of three bases in RNA and an amino acid. We now call this set of three-base combinations, each encoding an amino acid, the *genetic code*. A decoding, or *translation*, system exists today as the *ribosome* and associated factors that are responsible for essentially all polypeptide synthesis from RNA templates in modern organisms. The essence of this mode of polypeptide synthesis is illustrated in Figure 2.8.

An RNA molecule (*messenger RNA*, or *mRNA*), containing in its base sequence the information that specifies a particular protein, acts as a template to direct the synthesis of the polypeptide. Each amino acid is brought to the template attached to an adapter molecule specific to that amino acid. These adapters are specialized RNA molecules (called *transfer RNAs* or *tRNAs*). After initiation of the polypeptide chain, a tRNA molecule with its associated amino acid binds to the template through specific Watson-Crick base-pairing interactions. Two such molecules bind to the ribosome and peptide-bond formation is catalyzed by an RNA component (called *ribosomal RNA* or *rRNA*) of the ribosome. The first RNA departs (with neither the polypeptide chain nor an amino acid attached) and another tRNA with its associated amino acid bonds to the ribosome. The growing polypeptide chain is transferred to this newly bound amino acid with the formation of a new peptide bond. This cycle then repeats itself. This scheme allows the sequence of the RNA template to encode the sequence of the polypeptide and thereby makes possible the production of long polypeptides with specified sequences. The mechanism of protein synthesis will be discussed in Chapter 29. Importantly, the ribosome is composed largely of RNA and is a highly sophisticated ribozyme, suggesting that it might be a surviving relic of the RNA world.

2.2.5 The Genetic Code Elucidates the Mechanisms of Evolution

The sequence of bases that encodes a functional protein molecule is called a *gene*. The genetic code—that is, the relation between the base sequence of a gene and the amino acid sequence of the polypeptide whose synthesis the gene directs—applies to all modern organisms with only very minor exceptions. This universality reveals that the genetic code was fixed early in the course of evolution and has been maintained to the present day.

We can now examine the mechanisms of evolution. Earlier, we considered how variation is required for evolution. We can now see that such variations in living systems are changes that alter the meaning of the genetic message. These variations are called *mutations*. A mutation can be as simple as a change in a single nucleotide (called a *point mutation*), such that a

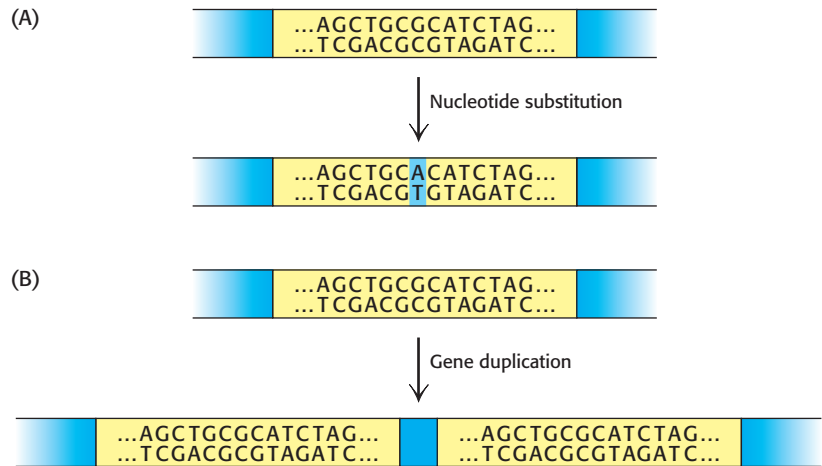


FIGURE 2.9 Mechanisms of evolution. A change in a gene can be (A) as simple as a single base change or (B) as dramatic as partial or complete gene duplication.

sequence of bases that encoded a particular amino acid may now encode another (Figure 2.9A). A mutation can also be the insertion or deletion of several nucleotides.

Other types of alteration permit the more rapid evolution of new biochemical activities. For instance, entire sections of the coding material can be duplicated, a process called *gene duplication* (Figure 2.9B). One of the duplication products may accumulate mutations and eventually evolve into a gene with a different, but related, function. Furthermore, parts of a gene may be duplicated and added to parts of another to give rise to a completely new gene, which encodes a protein with properties associated with each parent gene. Higher organisms contain many large families of enzymes and other macromolecules that are clearly related to one another in the same manner. Thus, gene duplication followed by specialization has been a crucial process in evolution. It allows the generation of macromolecules having particular functions without the need to start from scratch. The accumulation of genes with subtle and large differences allows for the generation of more complex biochemical processes and pathways and thus more complex organisms.

2.2.6 Transfer RNAs Illustrate Evolution by Gene Duplication

Transfer RNA molecules are the adaptors that associate an amino acid with its correct base sequence. Transfer RNA molecules are structurally similar to one another: each adopts a three-dimensional cloverleaf pattern of base-paired groups (Figure 2.10). Subtle differences in structure enable the protein-synthesis machinery to distinguish transfer RNA molecules with different amino acid specificities.

This family of related RNA molecules likely was generated by gene duplication followed by specialization. A nucleic acid sequence encoding one member of the family was duplicated, and the two copies evolved independently to generate molecules with specificities for different amino acids. This process was repeated, starting from one primordial transfer RNA gene until the 20 (or more) distinct members of the transfer RNA family present in modern organisms arose.

2.2.7 DNA Is a Stable Storage Form for Genetic Information

It is plausible that RNA was utilized to store genetic information early in the history of life. However, in modern organisms (with the exception of some viruses), the RNA derivative DNA (*deoxyribonucleic acid*) performs this function (Sections 1.1.1 and 1.1.3). The 2'-hydroxyl group in the

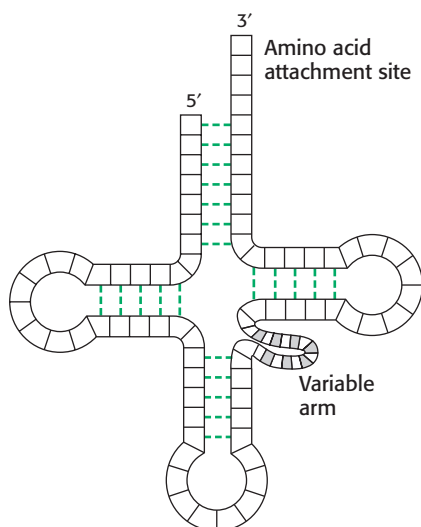


FIGURE 2.10 Cloverleaf pattern of tRNA. The pattern of base-pairing interactions observed for all transfer RNA molecules reveals that these molecules had a common evolutionary origin.

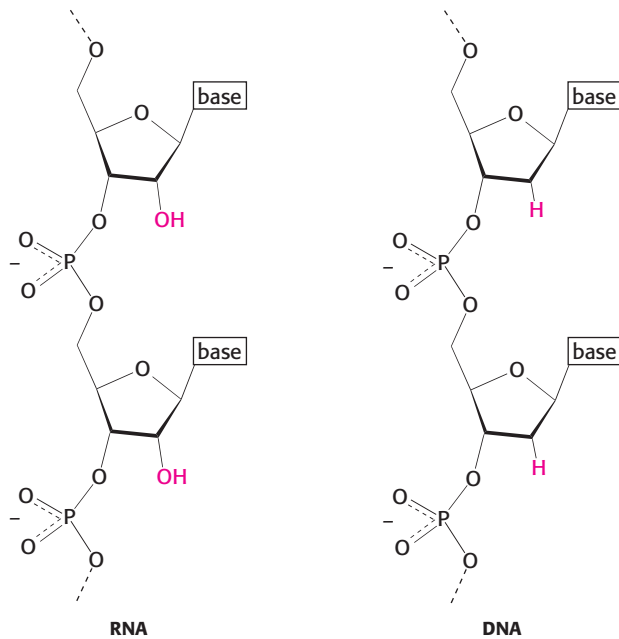
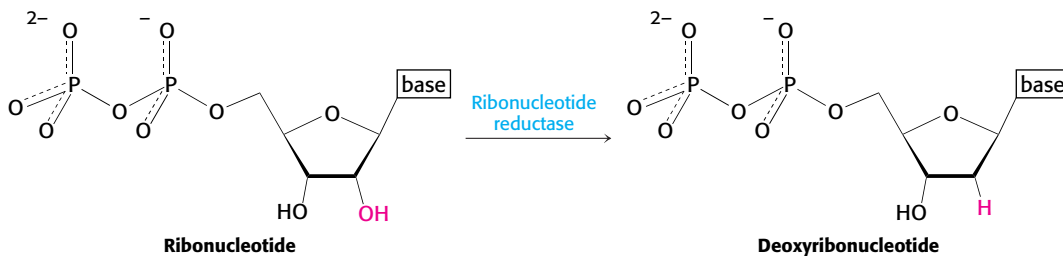


FIGURE 2.11 RNA and DNA compared. Removal of the 2'-hydroxyl group from RNA to form DNA results in a backbone that is less susceptible to cleavage by hydrolysis and thus enables more-stable storage of genetic information.

ribose unit of the RNA backbone is replaced by a hydrogen atom in DNA (Figure 2.11).

What is the selective advantage of DNA over RNA as the genetic material? The genetic material must be extremely stable so that sequence information can be passed on from generation to generation without degradation. RNA itself is a remarkably stable molecule; negative charges in the sugar-phosphate backbone protect it from attack by hydroxide ions that would lead to hydrolytic cleavage. However, the 2'-hydroxyl group makes the RNA susceptible to base-catalyzed hydrolysis. The removal of the 2'-hydroxyl group from the ribose decreases the rate of hydrolysis by approximately 100-fold under neutral conditions and perhaps even more under extreme conditions. Thus, the conversion of the genetic material from RNA into DNA would have substantially increased its chemical stability.

The evolutionary transition from RNA to DNA is recapitulated in the biosynthesis of DNA in modern organisms. In all cases, the building blocks used in the synthesis of DNA are synthesized from the corresponding building blocks of RNA by the action of enzymes termed *ribonucleotide reductases*. These enzymes convert ribonucleotides (a base and phosphate groups linked to a *ribose* sugar) into deoxyribonucleotides (a base and phosphates linked to *deoxyribose* sugar).

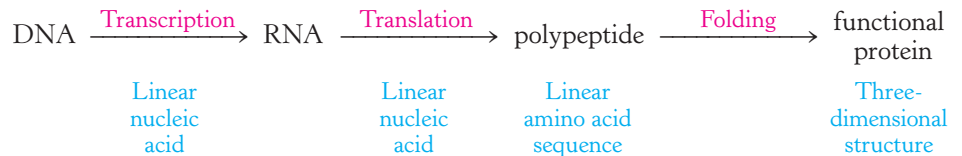


The properties of the ribonucleotide reductases vary substantially from species to species, but evidence suggests that they have a common mechanism of action and appear to have evolved from a common primordial enzyme.

The covalent structures of RNA and DNA differ in one other way. Whereas RNA contains *uracil*, DNA contains a methylated uracil derivative termed

thymine. This modification also serves to protect the integrity of the genetic sequence, although it does so in a less direct manner. As we will see in Chapter 27, the methyl group present in thymine facilitates the repair of damaged DNA, providing an additional selective advantage.

Although DNA replaced RNA in the role of storing the genetic information, RNA maintained many of its other functions. RNA still provides the template that directs polypeptide synthesis, the adaptor molecules, the catalytic activity of the ribosomes, and other functions. Thus, the genetic message is *transcribed* from DNA into RNA and then *translated* into protein.



This flow of sequence information from DNA to RNA to protein (to be considered in detail in Chapters 5, 28, and 29) applies to all modern organisms (with minor exceptions for certain viruses).

2.3 ENERGY TRANSFORMATIONS ARE NECESSARY TO SUSTAIN LIVING SYSTEMS

Most of the reactions that lead to the biosynthesis of nucleic acids and other biomolecules are not thermodynamically favorable under most conditions; they require an input of energy to proceed. Thus, they can proceed only if they are coupled to processes that release energy. How can energy-requiring and energy-releasing reactions be linked? How is energy from the environment transformed into a form that living systems can use? Answering these questions fundamental to biochemistry is the objective of much of this book.

2.3.1 ATP, a Common Currency for Biochemical Energy, Can Be Generated Through the Breakdown of Organic Molecules

Just as most economies simplify trade by using currency rather than bartering, biochemical systems have evolved common currencies for the exchange of energy. The most important of these currencies are molecules related to *adenosine triphosphate* (ATP) that contain an array of three linked phosphates (Figure 2.12). The bonds linking the phosphates persist in solution under a variety of conditions, but, when they are broken, an unusually large amount of energy is released that can be used to promote other processes. The roles of ATP and its use in driving other processes will be presented in detail in Chapter 14 and within many other chapters throughout this book.

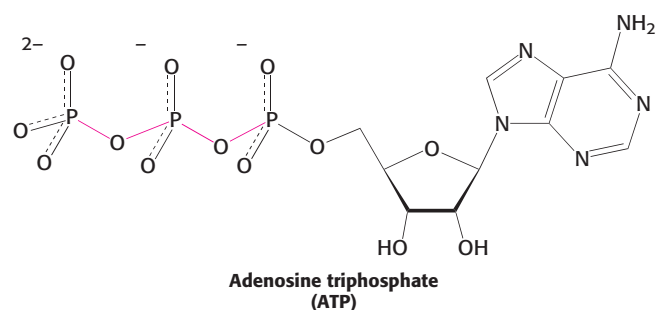


FIGURE 2.12 ATP, the energy currency of living systems. The phosphodiester bonds (red) release considerable energy when cleaved by hydrolysis or other processes.

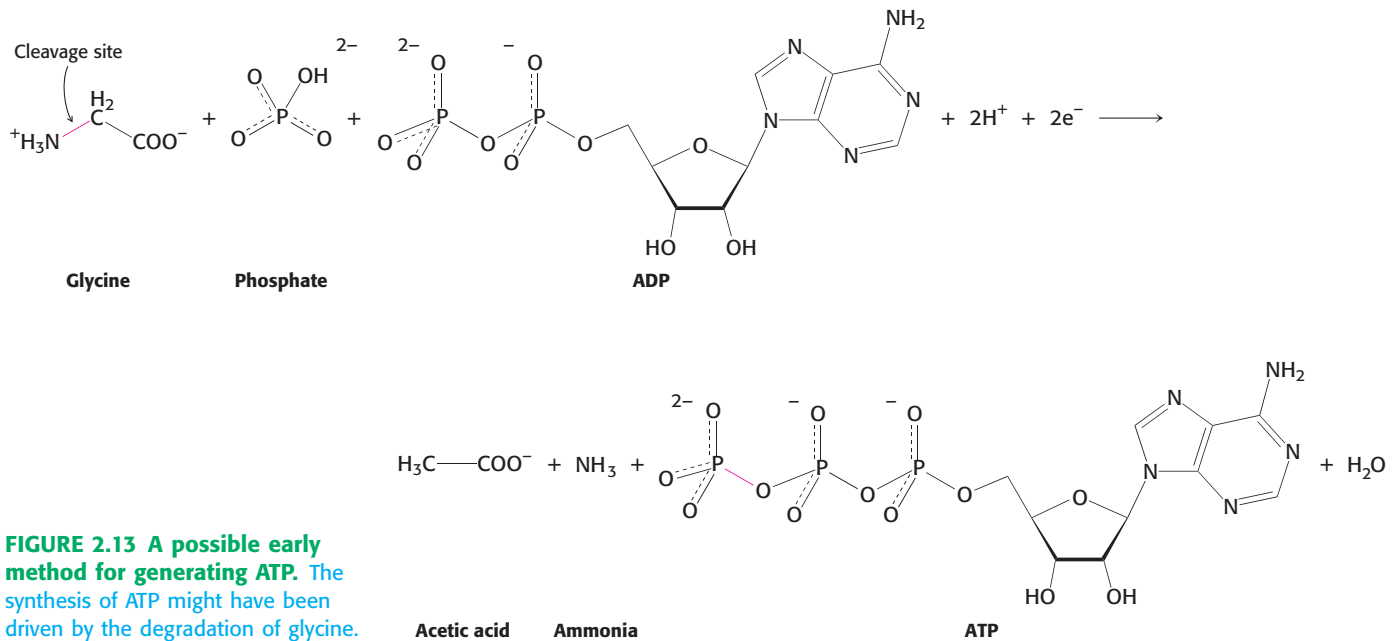
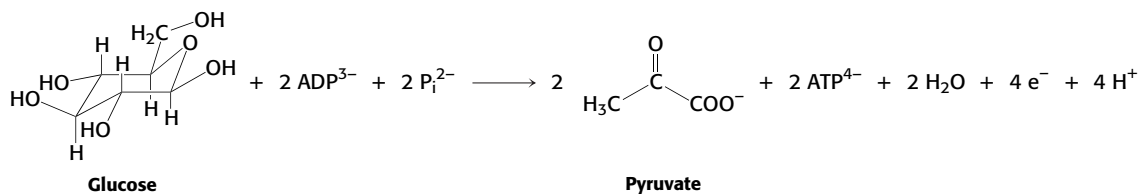


FIGURE 2.13 A possible early method for generating ATP. The synthesis of ATP might have been driven by the degradation of glycine.

ATP must be generated in appropriate quantities to be available for such reactions. The energy necessary for the synthesis of ATP can be obtained by the breakdown of other chemicals. Specific enzymes have evolved to couple these degradative processes to the phosphorylation of adenosine diphosphate (ADP) to yield ATP. Amino acids such as glycine, which were probably present in relatively large quantities in the prebiotic world and early in evolution, were likely sources of energy for ATP generation. The degradation of glycine to acetic acid may be an ATP-generation system that functioned early in evolution (Figure 2.13). In this reaction, the carbon–nitrogen bond in glycine is cleaved by reduction (the addition of electrons), and the energy released from the cleavage of this bond drives the coupling of ADP and orthophosphate (P_i) to produce ATP.

Amino acids are still broken down to produce ATP in modern organisms. However, sugars such as glucose are a more commonly utilized energy source because they are more readily metabolized and can be stored. The most important process for the direct synthesis of ATP in modern organisms is *glycolysis*, a complex process that derives energy from glucose.



Glycolysis presumably evolved as a process for ATP generation after carbohydrates such as glucose were being produced in significant quantities by other pathways. Glycolysis will be discussed in detail in Chapter 16.

2.3.2 Cells Were Formed by the Inclusion of Nucleic Acids Within Membranes

Modern organisms are made up of *cells*. A cell is composed of nucleic acids, proteins, and other biochemicals surrounded by a *membrane* built from lipids. These membranes completely enclose their contents, and so cells have a defined inside and outside. A typical membrane-forming lipid is phosphatidyl choline.

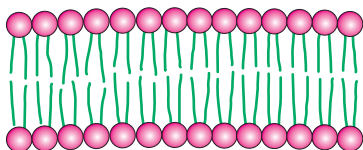
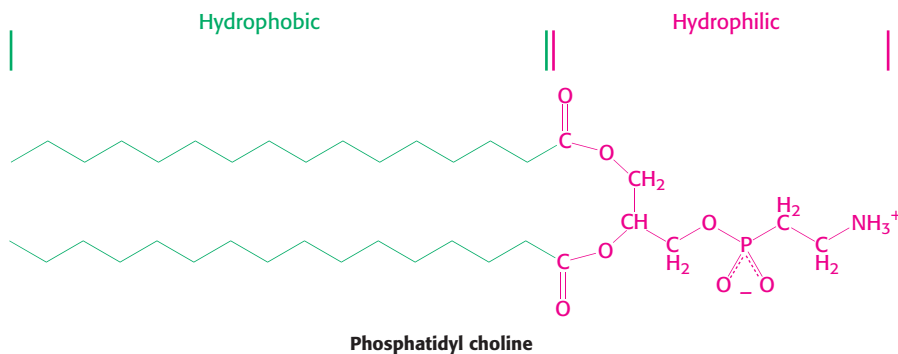


FIGURE 2.14 Schematic view of a lipid bilayer. These structures define the boundaries of cells.



The most important feature of membrane-forming molecules such as phosphatidyl choline is that they are *amphipathic*—that is, they contain both *hydrophilic* (water-loving) and *hydrophobic* (water-avoiding) components. Membrane-forming molecules consist of fatty acids, whose long alkyl groups are hydrophobic, connected to shorter hydrophilic “head groups.” When such lipids are in contact with water, they spontaneously aggregate to form specific structures such that the hydrophobic parts of the molecules are packed together away from water, whereas the hydrophilic parts are exposed to the aqueous solution. The structure that is important for membrane formation is the *lipid bilayer* (Figure 2.14). A bilayer is formed from two layers of lipids arranged such that the fatty acid tails of each layer interact with each other to form a hydrophobic interior while the hydrophilic head groups interact with the aqueous solution on each side. Such bilayer structures can fold onto themselves to form hollow spheres having interior compartments filled with water. The hydrophobic interior of the bilayer serves as a barrier between two aqueous phases. If such structures are formed in the presence of other molecules such as nucleic acids and proteins, these molecules can become trapped inside, thus forming cell-like structures. The structures of lipids and lipid bilayers will be considered in detail in Chapter 12.

At some stage in evolution, sufficient quantities of appropriate amphipathic molecules must have accumulated from biosynthetic or other processes to allow some nucleic acids to become entrapped and cell-like organisms to form. Such compartmentalization has many advantages. When the components of a cell are enclosed in a membrane, the products of enzymatic reactions do not simply diffuse away into the environment but instead are contained where they can be used by the cell that produced them. The containment is aided by the fact that nearly all biosynthetic intermediates and other biochemicals include one or more charged groups such as phosphates or carboxylates. Unlike more nonpolar or neutral molecules, charged molecules do not readily pass through lipid membranes.

2.3.3 Compartmentalization Required the Development of Ion Pumps

Despite its many advantages, the enclosure of nucleic acids and proteins within membranes introduced several complications. Perhaps the most significant were the effects of *osmosis*. Membranes are somewhat permeable to water and small nonpolar molecules, whereas they are impermeable to macromolecules such as nucleic acids. When macromolecules are concentrated inside a compartment surrounded by such a semipermeable membrane, osmotic forces drive water through the membrane into the compartment. Without counterbalancing effects, the flow of water will burst the cell (Figure 2.15).

Osmosis—

The movement of a solvent across a membrane in the direction that tends to equalize concentrations of solute on the two sides of the membrane.

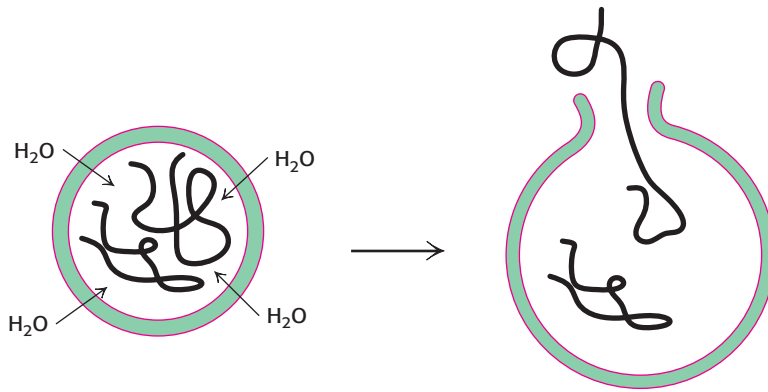


FIGURE 2.15 The “osmotic crisis.”

A cell consisting of macromolecules surrounded by a semipermeable membrane will take up water from outside the cell and burst.

Modern cells have two distinct mechanisms for resisting these osmotic forces. One mechanism is to toughen the cell membrane by the introduction of an additional structure such as a cell wall. However, such a chemically elaborate structure may not have evolved quickly, especially because it must completely surround a cell to be effective. The other mechanism is the use of *energy-dependent ion pumps*. These pumps can lower the concentration of ions inside a cell relative to the outside, favoring the flow of water molecules from inside to outside. The resulting unequal distribution of ions across an inherently impermeable membrane is called an *ion gradient*. Appropriate ion gradients can balance the osmotic forces and maintain a cell at a constant volume. Membrane proteins such as ion pumps will be considered in Chapter 13.

Ion gradients can prevent osmotic crises, but they require energy to be produced. Most likely, an ATP-driven proton pump was the first existing component of the machinery for generating an ion gradient (Figure 2.16). Such pumps, which are found in essentially all modern cells, hydrolyze ATP to ADP and inorganic phosphate and utilize the energy released to transport protons from the inside to the outside of a cell. The pump thus establishes a proton gradient that, in turn, can be coupled to other membrane-transport processes such as the removal of sodium ions from the cell. The proton gradient and other ion gradients generated from it act together to counteract osmotic effects and prevent the cell from swelling and bursting.

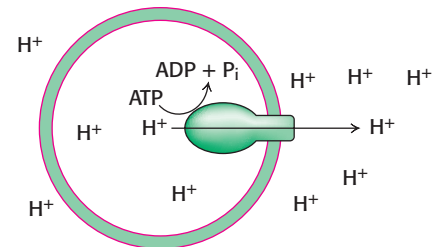


FIGURE 2.16 Generating an ion gradient. ATP hydrolysis can be used to drive the pumping of protons (or other ions) across a membrane.

2.3.4 Proton Gradients Can Be Used to Drive the Synthesis of ATP

Enzymes act to accelerate reactions, but they cannot alter the position of chemical equilibria. An enzyme that accelerates a reaction in the forward direction must also accelerate the reaction to the same extent in the reverse direction. Thus, the existence of an enzyme that utilized the hydrolysis of ATP to generate a proton gradient presented a tremendous opportunity for the evolution of alternative systems for generating ATP. Such an enzyme could synthesize ATP by reversing the process that produces the gradient. Enzymes, now called *ATP synthases*, do in fact use proton gradients to drive the bonding of ADP and P_i to form ATP (Figure 2.17). These proteins will be considered in detail in Chapter 18.

Organisms have evolved a number of elaborate mechanisms for the generation of proton gradients across membranes. An example is *photosynthesis*, a process first used by bacteria and now also used by plants to harness the light energy from the sun. The essence of photosynthesis is the light-driven transfer of an electron across a membrane. The fundamental processes are illustrated in Figure 2.18.

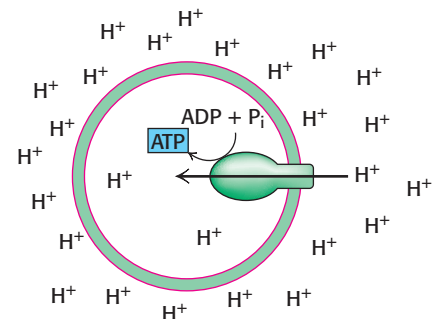


FIGURE 2.17 Use of proton gradients to synthesize ATP. ATP can be synthesized by the action of an ATP-driven proton pump running in reverse.

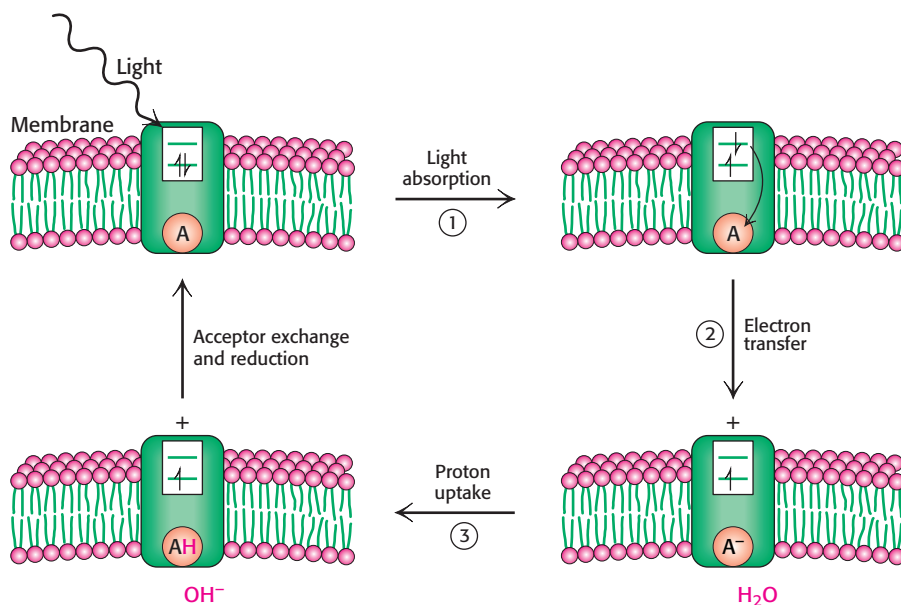


FIGURE 2.18 Photosynthesis.

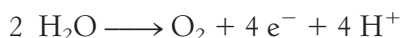
Absorption of light (1) leads to electron transfer across a membrane (2). For each electron transfer, one excess hydroxide ion is generated inside the cell (3). The process produces a proton gradient across the membrane that can drive ATP synthesis.

The photosynthetic apparatus, which is embedded in a membrane, contains pigments that efficiently absorb light from the sun. The absorbed light provides the energy to promote an electron in the pigment molecule to an excited state. The high-energy electron can then jump to an appropriate acceptor molecule located in the part of the membrane facing the inside of the cell. The acceptor molecule, now reduced, binds a proton from a water molecule, generating an hydroxide ion inside the cell. The electronic “hole” left in the pigment on the outside of the membrane can then be filled by the donation of an electron from a suitable reductant on the outside of the membrane. Because the generation of an hydroxide ion inside the cell is equivalent to the generation of a proton outside the cell, a proton gradient develops across the membrane. Protons flow down this gradient through ATP synthases to generate ATP.

Photosynthesis is but one of a range of processes in different organisms that lead to ATP synthesis through the action of proteins evolutionarily related to the primordial ATP-driven pumps. In animals, the degradation of carbohydrates and other organic compounds is the source of the electron flow across membranes that can be used to develop proton gradients. The formation of ATP-generating proton gradients by fuel metabolism will be considered in Chapter 18 and by light absorption in Chapter 19.

2.3.5 Molecular Oxygen, a Toxic By-Product of Some Photosynthetic Processes, Can Be Utilized for Metabolic Purposes

As stated earlier, photosynthesis generates electronic “holes” in the photosynthetic apparatus on the outside of the membrane. These holes are powerful oxidizing agents; that is, they have very high affinities for electrons and can pull electrons from many types of molecules. They can even oxidize water. Thus, for many photosynthetic organisms, the electron donor that completes the photosynthetic cycle is water. The product of water oxidation is oxygen gas—that is, molecular oxygen (O_2).



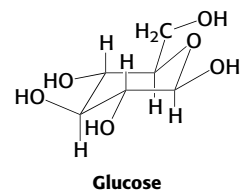
The use of water as the electron donor significantly increases the efficiency of photosynthetic ATP synthesis because the generation of one molecule of oxygen is accompanied not only by the release of four electrons (e^-), but

also by the release of four protons on one side of the membrane. Thus, an additional proton is released for each proton equivalent produced by the initial electron-transfer process, so twice as many protons are available to drive ATP synthesis. Oxygen generation will be considered in Chapter 19.

Oxygen was present in only small amounts in the atmosphere before organisms evolved that could oxidize water. The “pollution” of the air with oxygen produced by photosynthetic organisms greatly affected the course of evolution. Oxygen is quite reactive and thus extremely toxic to many organisms. Many biochemical processes have evolved to protect cells from the deleterious effects of oxygen and other reactive species that can be generated from this molecule. Subsequently, organisms evolved mechanisms for taking advantage of the high reactivity of oxygen to promote favorable processes. Most important among these mechanisms are those for the oxidation of organic compounds such as glucose. Through the action of oxygen, a glucose molecule can be completely converted into carbon dioxide and water, releasing enough energy to synthesize approximately 30 molecules of ATP.



This number represents a 15-fold increase in ATP yield compared with the yield from the breakdown of glucose in the absence of oxygen in the process of glycolysis. This increased efficiency is apparent in everyday life; our muscles exhaust their fuel supply and tire quickly if they do not receive enough oxygen and are forced to use glycolysis as the sole ATP source. The role of oxygen in the extraction of energy from organic molecules will be considered in Chapter 18.



2.4 CELLS CAN RESPOND TO CHANGES IN THEIR ENVIRONMENTS

The environments in which cells grow often change rapidly. For example, cells may consume all of a particular food source and must utilize others. To survive in a changing world, cells evolved mechanisms for adjusting their biochemistry in response to signals indicating environmental change. The adjustments can take many forms, including changes in the activities of pre-existing enzyme molecules, changes in the rates of synthesis of new enzyme molecules, and changes in membrane-transport processes.

Initially, the detection of environmental signals occurred inside cells. Chemicals that could pass into cells, either by diffusion through the cell membrane or by the action of transport proteins, and could bind directly to proteins inside the cell and modulate their activities. An example is the use of the sugar arabinose by the bacterium *Escherichia coli* (Figure 2.19). *E. coli* cells are normally unable to use arabinose efficiently as a source of

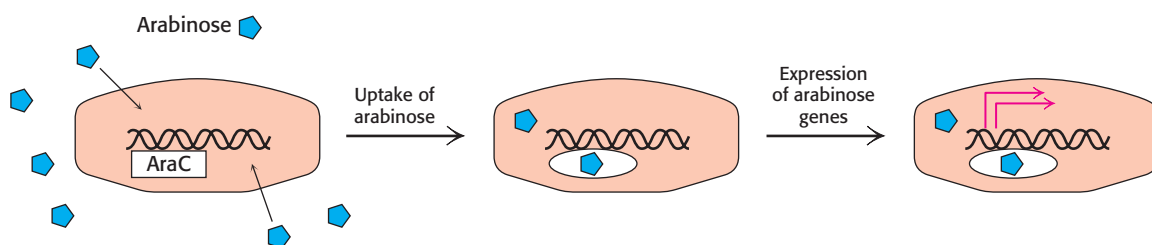


FIGURE 2.19 Responding to environmental conditions. In *E. coli* cells, the uptake of arabinose from the environment triggers the production of enzymes necessary for its utilization.

energy. However, if arabinose is their only source of carbon, *E. coli* cells synthesize enzymes that catalyze the conversion of this sugar into useful forms. This response is mediated by arabinose itself. If present in sufficient quantity outside the cell, arabinose can enter the cell through transport proteins. Once inside the cell, arabinose binds to a protein called AraC. This binding alters the structure of AraC so that it can now bind to specific sites in the bacterial DNA and increase RNA transcription from genes encoding enzymes that metabolize arabinose. The mechanisms of gene regulation will be considered in Chapter 31.

Subsequently, mechanisms appeared for detecting signals at the cell surface. Cells could thus respond to signaling molecules even if those molecules did not pass into the cell. Receptor proteins evolved that, embedded in the membrane, could bind chemicals present in the cellular environment. Binding produced changes in the protein structure that could be detected at the inside surface of the cell membrane. By this means, chemicals outside the cell could influence events inside the cell. Many of these *signal-transduction pathways* make use of substances such as cyclic adenosine monophosphate (cAMP) and calcium ion as “second messengers” that can diffuse throughout the cell, spreading the word of environmental change.

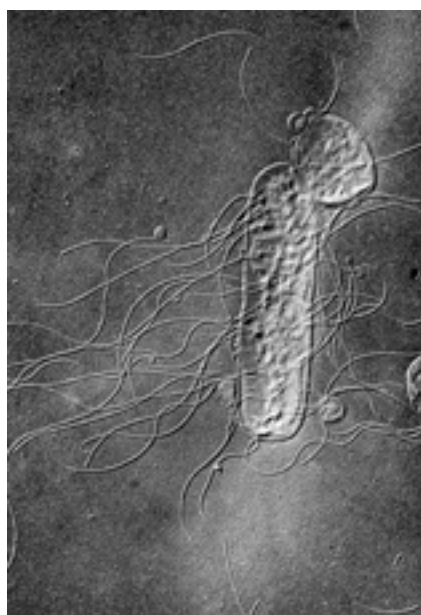
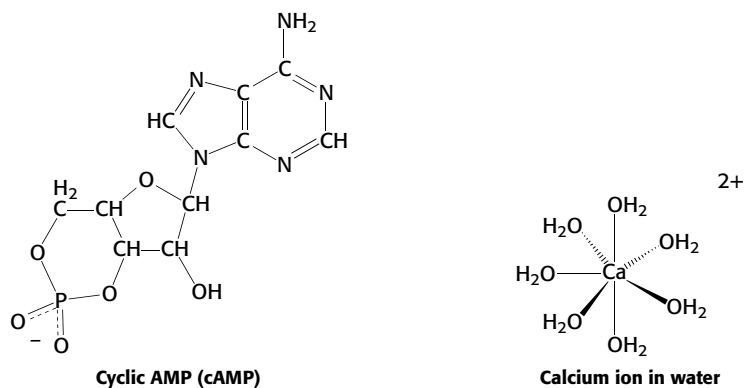


FIGURE 2.20 Bacteria with flagella. A bacterium (*Proteus mirabilis*) swims through the rotation of filamentous structures called flagella. [Fred E. Hossler/Visuals Unlimited.]

The second messengers may bind to specific sensor proteins inside the cell and trigger responses such as the activation of enzymes. Signal-transduction mechanisms will be considered in detail in Chapter 15 and in many other chapters throughout this book.

2.4.1 Filamentous Structures and Molecular Motors Enable Intracellular and Cellular Movement

The development of the ability to move was another important stage in the evolution of cells capable of adapting to a changing environment. Without this ability, nonphotosynthetic cells might have starved after consuming the nutrients available in their immediate vicinity.

Bacteria swim through the use of filamentous structures termed *flagella* that extend from their cell membranes (Figure 2.20). Each bacterial cell has several flagella, which, under appropriate conditions, form rotating bundles that efficiently propel the cell through the water. These flagella are long polymers consisting primarily of thousands of identical protein subunits. At the base of each flagellum are assemblies of proteins that act as motors to drive its rotation. The rotation of the flagellar motor is driven by the flow of protons from outside to inside the cell. Thus, energy stored in the form of a proton gradient is transduced into another form, rotatory motion.

Other mechanisms for motion, also depending on filamentous structures, evolved in other cells. The most important of these structures are *microfilaments* and *microtubules*. Microfilaments are polymers of the protein

actin, and microtubules are polymers of two closely related proteins termed α - and β -*tubulin*. Unlike a bacterial flagellum, these filamentous structures are highly dynamic: they can rapidly increase or decrease in length through the addition or subtraction of component protein molecules. Microfilaments and microtubules also serve as tracks on which other proteins move, driven by the hydrolysis of ATP. Cells can change shape through the motion of *molecular motor proteins* along such filamentous structures that are changing in shape as a result of dynamic polymerization (Figure 2.21). Coordinated shape changes can be a means of moving a cell across a surface and are crucial to cell division. The motor proteins are also responsible for the transport of organelles and other structures within eukaryotic cells. Molecular motors will be considered in Chapter 34.

2.4.2 Some Cells Can Interact to Form Colonies with Specialized Functions

Early organisms lived exclusively as single cells. Such organisms interacted with one another only indirectly by competing for resources in their environments. Certain of these organisms, however, developed the ability to form colonies comprising many interacting cells. In such groups, the environment of a cell is dominated by the presence of surrounding cells, which may be in direct contact with one another. These cells communicate with one another by a variety of signaling mechanisms and may respond to signals by altering enzyme activity or levels of gene expression. One result may be *cell differentiation*; differentiated cells are genetically identical but have different properties because their genes are expressed differently.

Several modern organisms are able to switch back and forth from existence as independent single cells to existence as multicellular colonies of differentiated cells. One of the most well characterized is the slime mold *Dictyostelium*. In favorable environments, this organism lives as individual cells; under conditions of starvation, however, the cells come together to form a cell aggregate. This aggregate, sometimes called a *slug*, can move as a unit to a potentially more favorable environment where it then forms a multicellular structure, termed a *fruiting body*, that rises substantially above the surface on which the cells are growing. Wind may carry cells released from the top of the fruiting body to sites where the food supply is more plentiful. On arriving in a well-stocked location, the cells grow, reproduce, and live as individual cells until the food supply is again exhausted (Figure 2.22).

The transition from unicellular to multicellular growth is triggered by cell–cell communication and reveals much about signaling processes between and within cells. Under starvation conditions, *Dictyostelium* cells release the signal molecule cyclic AMP. This molecule signals surrounding cells by binding to a membrane-bound protein receptor on the cell surface.

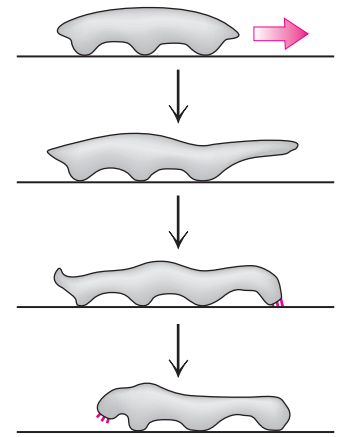


FIGURE 2.21 Alternative movement. Cell mobility can be achieved by changes in cell shape.

FIGURE 2.22 Unicellular to multicellular transition in *Dictyostelium*. This scanning electron micrograph shows the transformation undergone by the slime mold *Dictyostelium*. Hundreds of thousands of single cells aggregate to form a migrating slug, seen in the lower left. Once the slug comes to a stop, it gradually elongates to form the fruiting body. [Courtesy of M. J. Grimsom and R. L. Blanton, Texas Tech University.]

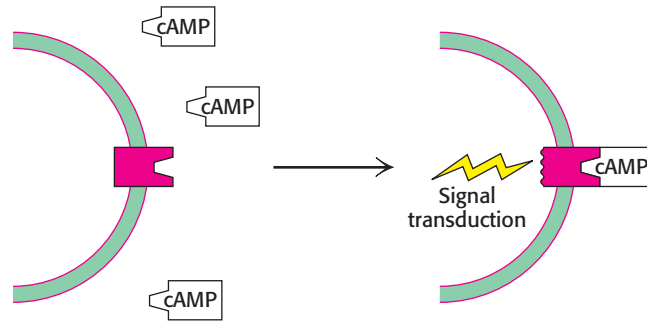


FIGURE 2.23 Intracellular signaling. Cyclic AMP, detected by cell-surface receptors, initiates the formation of aggregates in *Dictyostelium*.

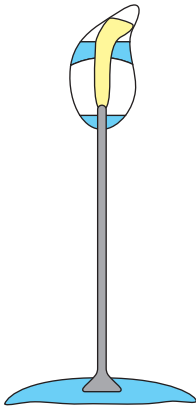


FIGURE 2.24 Cell differentiation in *Dictyostelium*. The colors represent the distribution of cell types expressing similar sets of genes in the *Dictyostelium* fruiting body.

The binding of cAMP molecules to these receptors triggers several responses, including movement in the direction of higher cAMP concentration, as well as the generation and release of additional cAMP molecules (Figure 2.23).

The cells aggregate by following cAMP gradients. Once in contact, they exchange additional signals and then differentiate into distinct *cell types*, each of which expresses the set of genes appropriate for its eventual role in forming the fruiting body (Figure 2.24). The life cycles of organisms such as *Dictyostelium* foreshadow the evolution of organisms that are multicellular throughout their lifetimes. It is also interesting to note the cAMP signals starvation in many organisms, including human beings.

2.4.3 The Development of Multicellular Organisms Requires the Orchestrated Differentiation of Cells

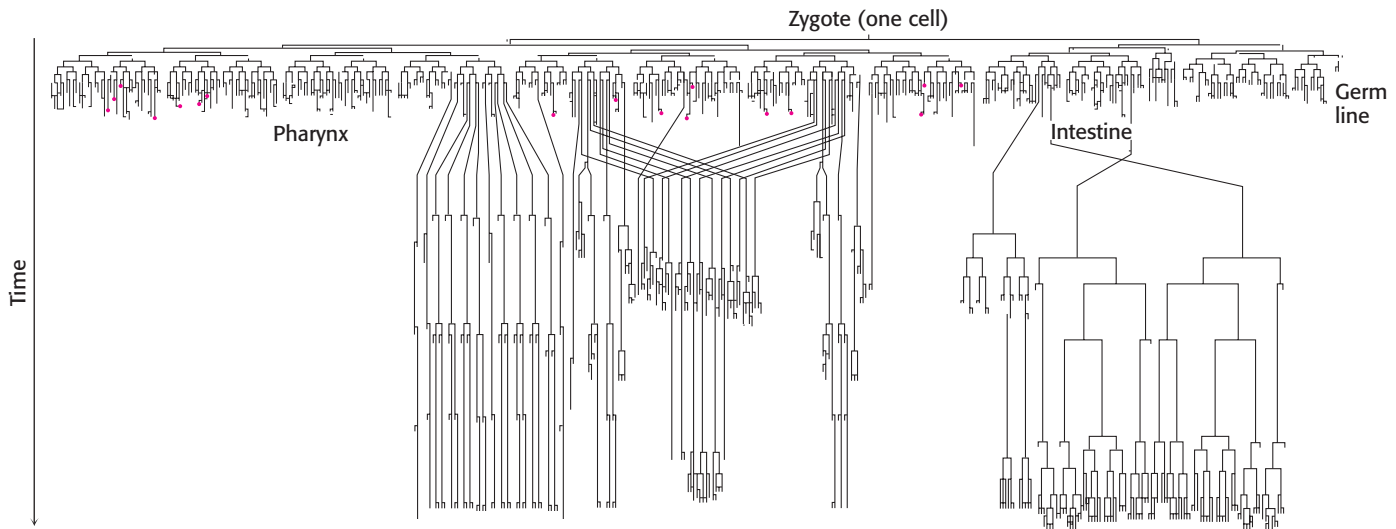
The fossil record indicates that macroscopic, multicellular organisms appeared approximately 600 million years ago. Most of the organisms familiar to us consist of many cells. For example, an adult human being contains approximately 100,000,000,000,000 cells. The cells that make up different organs are distinct and, even within one organ, many different cell types are present. Nonetheless, the DNA sequence in each cell is identical. The differences between cell types are the result of differences in how these genes are expressed.

Each multicellular organism begins as a single cell. For this cell to develop into a complex organism, the embryonic cells must follow an intricate program of regulated gene expression, cell division, and cell movement. The developmental program relies substantially on the responses of cells to the environment created by neighboring cells. Cells in specific positions within the developing embryo divide to form particular tissues, such as muscle. Developmental pathways have been extensively studied in a number of organisms, including the nematode *Caenorhabditis elegans* (Figure 2.25), a 1-mm-long worm containing 959 cells. A detailed map describing the fate of each cell in *C. elegans* from the fertilized egg to the adult is shown in Figure 2.26. Interestingly, proper development requires not only cell division but also the death of specific cells at particular points in time through a process called programmed cell death or *apoptosis*.

Investigations of genes and proteins that control development in a wide range of organisms have revealed a great many common features. Many of the molecules that control human development are evolutionarily related to those in relatively simple organisms such as *C. elegans*. Thus, solutions to the problem of controlling development in multicellular organisms arose early in evolution and have been adapted many times in the course of evolution, generating the great diversity of complex organisms.



FIGURE 2.25 The nematode *Caenorhabditis elegans*. This organism serves as a useful model for development. [Sinclair Stammers Science Photo Library/Photo Researchers.]



2.4.4 The Unity of Biochemistry Allows Human Biology to Be Effectively Probed Through Studies of Other Organisms

All organisms on Earth have a common origin (Figure 2.27). How could complex organisms such as human beings have evolved from the simple organisms that existed at life's start? The path outlined in this chapter reveals that most of the fundamental processes of biochemistry were largely fixed early in the history of life. The complexity of organisms such as human beings is manifest, at a biochemical level, in the interactions between overlapping and competing pathways, which lead to the generation of intricately connected groups of specialized cells. The evolution of biochemical and physiological complexity is made possible by the effects of gene duplication followed by specialization. Paradoxically, the reliance on gene duplication also makes this complexity easier to comprehend. Consider, for example, the protein kinases—enzymes that transfer phosphoryl groups from ATP to specific amino acids in proteins. These enzymes play essential roles in many signal-transduction pathways and in the control of cell growth and differentiation. The human genome encodes approximately 500 proteins of this class; even a relatively simple, unicellular organism such as brewer's yeast has more than 100 protein kinases. Yet each of these enzymes is the evolutionary descendant of a common ancestral enzyme. Thus, *we can learn much about the essential behavior of this large collection of proteins through studies of a single family member*. After the essential behavior is understood, we can evaluate the specific adaptations that allow each family member to perform its particular biological functions.

Most central processes in biology have been characterized first in relatively simple organisms, often through a combination of genetic, physiological, and biochemical studies. Many of the processes controlling early

FIGURE 2.26 Developmental pathways of *C. elegans*. The nematode develops from a single cell, called a zygote, into a complex organism. The fate of each individual cell in *C. elegans* is known and can be followed by referring to the cell-lineage diagram. The labels indicate cells that form specific organs. Cells that undergo programmed cell death are shown in red.

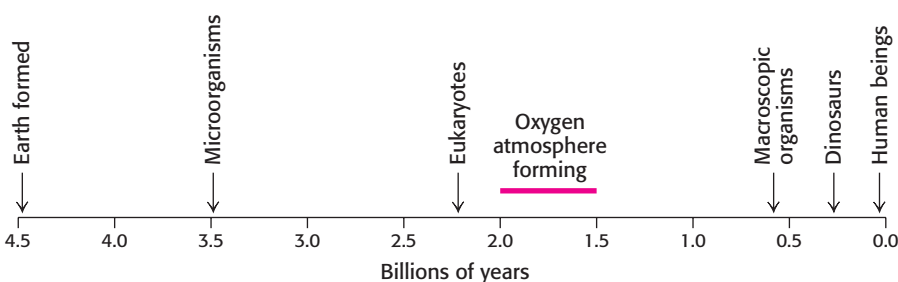


FIGURE 2.27 A possible time line for biochemical evolution. Key events are indicated.

embryonic development were elucidated by the results of studies of the fruit fly. The events controlling DNA replication and the cell cycle were first deciphered in yeast. Investigators can now test the functions of particular proteins in mammals by disrupting the genes that encode these proteins in mice and examining the effects. The investigations of organisms linked to us by common evolutionary pathways are powerful tools for exploring all of biology and for developing new understanding of normal human function and disease.

SUMMARY

• Key Organic Molecules Are Used by Living Systems

The evolution of life required a series of transitions, beginning with the generation of organic molecules that could serve as the building blocks for complex biomolecules. How these molecules arose is a matter of conjecture, but experiments have established that they could have formed under hypothesized prebiotic conditions.

• Evolution Requires Reproduction, Variation, and Selective Pressure

The next major transition in the evolution of life was the formation of replicating molecules. Replication, coupled with variation and selective pressure, marked the beginning of evolution. Variation was introduced by a number of means, from simple base substitutions to the duplication of entire genes. RNA appears to have been an early replicating molecule. Furthermore, some RNA molecules possess catalytic activity. However, the range of reactions that RNA is capable of catalyzing is limited. With time, the catalytic activity was transferred to proteins—linear polymers of the chemically versatile amino acids. RNA directed the synthesis of these proteins and still does in modern organisms through the development of a genetic code, which relates base sequence to amino acid sequence. Eventually, RNA lost its role as the gene to the chemically similar but more stable nucleic acid DNA. In modern organisms, RNA still serves as the link between DNA and protein.

• Energy Transformations Are Necessary to Sustain Living Systems

Another major transition in evolution was the ability to transform environmental energy into forms capable of being used by living systems. ATP serves as the cellular energy currency that links energy-yielding reactions with energy-requiring reactions. ATP itself is a product of the oxidation of fuel molecules, such as amino acids and sugars. With the evolution of membranes—hydrophobic barriers that delineate the borders of cells—ion gradients were required to prevent osmotic crises. These gradients were formed at the expense of ATP hydrolysis. Later, ion gradients generated by light or the oxidation of fuel molecules were used to synthesize ATP.

• Cells Can Respond to Changes in Their Environments

The final transition was the evolution of sensing and signaling mechanisms that enabled a cell to respond to changes in its environment. These signaling mechanisms eventually led to cell–cell communication, which allowed the development of more-complex organisms. The record of much of what has occurred since the formation of primitive organisms is written in the genomes of extant organisms. Knowledge of these genomes and the mechanisms of evolution will enhance our understanding of the history of life on Earth as well as our understanding of existing organisms.

KEY TERMS

prebiotic world (p. 20)	RNA world (p. 23)	membrane (p. 29)
reproduction (p. 21)	proteins (p. 24)	ion pump (p. 31)
variation (p. 22)	genetic code (p. 25)	ion gradient (p. 31)
competition (p. 22)	translation (p. 25)	photosynthesis (p. 31)
selective pressure (p. 22)	gene (p. 25)	signal transduction pathway (p. 34)
catalyst (p. 23)	mutation (p. 25)	molecular motor protein (p. 35)
enzyme (p. 23)	gene duplication (p. 26)	cell differentiation (p. 35)
ribozyme (p. 23)	ATP (adenosine triphosphate) (p. 28)	unity of biochemistry (p. 37)

SELECTED READINGS

Where to start

- Pace, N. R., 2000. The universal nature of biochemistry. *Proc. Natl. Acad. Sci. U. S. A.* 98:805–808.
- Orgel, L. E., 1987. Evolution of the genetic apparatus: A review. *Cold Spring Harbor Symp. Quant. Biol.* 52:9–16.
- Lazcano, A., and Miller, S. L., 1996. The origin and early evolution of life: Prebiotic chemistry, the pre-RNA world, and time. *Cell* 85:793–798.
- Orgel, L. E. 1998. The origin of life: A review of facts and speculations. *Trends Biochem. Sci.* 23:491–495.

Books

- Darwin, C., 1975. *On the Origin of Species, a Facsimile of the First Edition.* Harvard University Press.
- Gesteland, R. F., Cech, T., and Atkins, J. F., 1999. *The RNA World.* Cold Spring Harbor Laboratory Press.
- Dawkins, R., 1996. *The Blind Watchmaker.* Norton.
- Smith, J. M., and Szathmáry, E., 1995. *The Major Transitions in Evolution.* W. H. Freeman and Company.

Prebiotic chemistry

- Miller, S. L., 1987. Which organic compounds could have occurred on the prebiotic earth? *Cold Spring Harbor Symp. Quant. Biol.* 52:17–27.
- Westheimer, F. H., 1987. Why nature chose phosphates. *Science* 235:1173–1178.
- Levy, M., and Miller, S. L., 1998. The stability of the RNA bases: Implications for the origin of life. *Proc. Natl. Acad. Sci. U. S. A.* 95:7933–7938.
- Sanchez, R., Ferris, J., and Orgel, L. E., 1966. Conditions for purine synthesis: Did prebiotic synthesis occur at low temperatures? *Science* 153:72–73.

In vitro evolution

- Mills, D. R., Peterson, R. L., and Spiegelman, S., 1967. An extracellular Darwinian experiment with a self-duplicating nucleic acid molecule. *Proc. Natl. Acad. Sci. U. S. A.* 58:217–224.
- Levisohn, R., and Spiegelman, S., 1969. Further extracellular Darwinian experiments with replicating RNA molecules: Diverse variants

- isolated under different selective conditions. *Proc. Natl. Acad. Sci. U. S. A.* 63:805–811.
- Wilson, D. S., and Szostak, J. W., 1999. In vitro selection of functional nucleic acids. *Annu. Rev. Biochem.* 68:611–647.

Replication and catalytic RNA

- Cech, T. R., 1993. The efficiency and versatility of catalytic RNA: Implications for an RNA world. *Gene* 135:33–36.
- Orgel, L. E., 1992. Molecular replication. *Nature* 358:203–209.
- Zielinski, W. S., and Orgel, L. E., 1987. Autocatalytic synthesis of a tetranucleotide analogue. *Nature* 327:346–347.
- Nelson, K. E., Levy, M., and Miller, S. L., 2000. Peptide nucleic acids rather than RNA may have been the first genetic molecule. *Proc. Natl. Acad. Sci. U. S. A.* 97:3868–3871.

Transition from RNA to DNA

- Reichard, P., 1997. The evolution of ribonucleotide reduction. *Trends Biochem. Sci.* 22:81–85.
- Jordan, A., and Reichard, P., 1998. Ribonucleotide reductases. *Annu. Rev. Biochem.* 67:71–98.

Membranes

- Wilson, T. H., and Maloney, P. C., 1976. Speculations on the evolution of ion transport mechanisms. *Fed. Proc.* 35:2174–2179.
- Wilson, T. H., and Lin, E. C., 1980. Evolution of membrane bioenergetics. *J. Supramol. Struct.* 13:421–446.

Multicellular organisms and development

- Mangiarotti, G., Bozzaro, S., Landfear, S., and Lodish, H. F., 1983. Cell–cell contact, cyclic AMP, and gene expression during development of *Dictyostelium discoideum*. *Curr. Top. Dev. Biol.* 18:117–154.
- Kenyon, C., 1988. The nematode *Caenorhabditis elegans*. *Science* 240:1448–1453.
- Hodgkin, J., Plasterk, R. H., and Waterston, R. H., 1995. The nematode *Caenorhabditis elegans* and its genome. *Science* 270:410–414.

PROBLEMS

1. *Finding the fragments.* Identify the likely source (CH_4 , NH_3 , H_2O , or H_2) of each atom in alanine generated in the Miller-Urey experiment.
2. *Following the populations.* In an experiment analogous to the Spiegelman experiment, suppose that a population of RNA

molecules consists of 99 identical molecules, each of which replicates once in 15 minutes, and 1 molecule that replicates once in 5 minutes. Estimate the composition of the population after 1, 10, and 25 “generations” if a generation is defined as 15 minutes of replication. Assume that all necessary components are readily available.

3. *Selective advantage.* Suppose that a replicating RNA molecule has a mutation (genotypic change) and the phenotypic result is that it binds nucleotide monomers more tightly than do other RNA molecules in its population. What might the selective advantage of this mutation be? Under what conditions would you expect this selective advantage to be most important?

4. *Opposite of randomness.* Ion gradients prevent osmotic crises, but they require energy to be produced. Why does the formation of a gradient require an energy input?

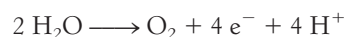
5. *Coupled gradients.* How could a proton gradient with a higher concentration of protons inside a cell be used to pump ions out of a cell?

6. *Proton counting.* Consider the reactions that take place across a photosynthetic membrane. On one side of the membrane, the following reaction takes place:



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whereas, on the other side of the membrane, the reaction is:

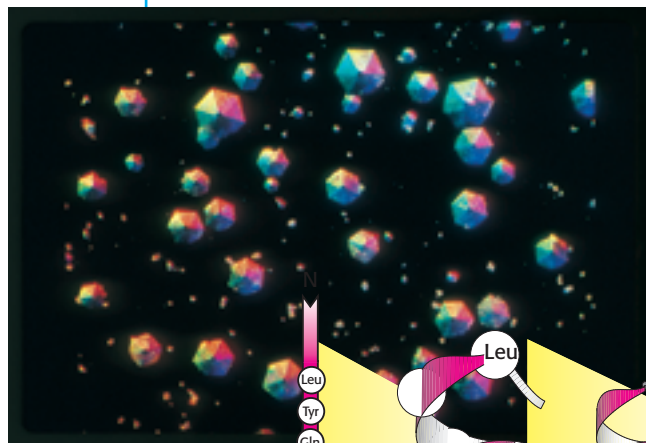


How many protons are made available to drive ATP synthesis for each reaction cycle?

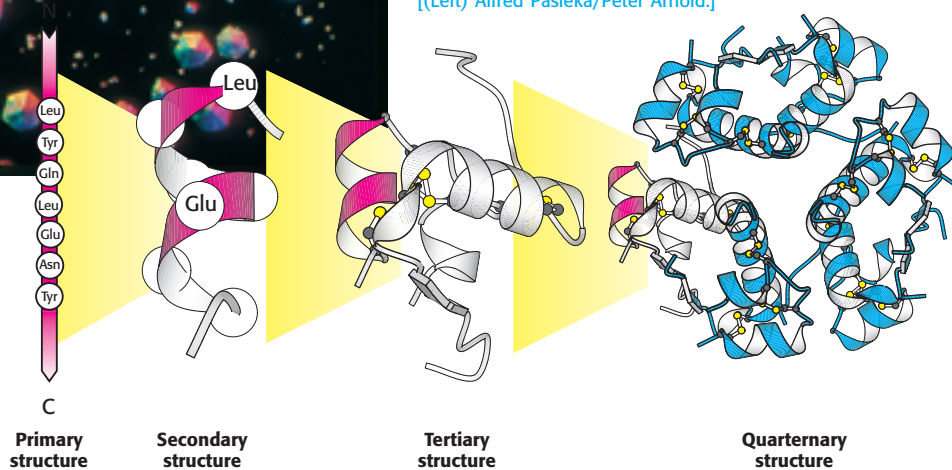
7. *An alternative pathway.* To respond to the availability of sugars such as arabinose, a cell must have at least two types of proteins: a transport protein to allow the arabinose to enter the cell and a gene-control protein, which binds the arabinose and modifies gene expression. To respond to the availability of some very hydrophobic molecules, a cell requires only one protein. Which one and why?

8. *How many divisions?* In the development pathway of *C. elegans*, cell division is initially synchronous—that is, all cells divide at the same rate. Later in development, some cells divide more frequently than do others. How many times does each cell divide in the synchronous period? Refer to Figure 2.26.

Protein Structure and Function



Crystals of human insulin. Insulin is a protein hormone, crucial for maintaining blood sugar at appropriate levels. (Below) Chains of amino acids in a specific sequence (the primary structure) define a protein like insulin. These chains fold into well-defined structures (the tertiary structure)—in this case a single insulin molecule. Such structures assemble with other chains to form arrays such as the complex of six insulin molecules shown at the far right (the quaternary structure). These arrays can often be induced to form well-defined crystals (photo at left), which allows determination of these structures in detail. [(Left) Alfred Pasieka/Peter Arnold.]



Proteins are the most versatile macromolecules in living systems and serve crucial functions in essentially all biological processes. They function as catalysts, they transport and store other molecules such as oxygen, they provide mechanical support and immune protection, they generate movement, they transmit nerve impulses, and they control growth and differentiation. Indeed, much of this text will focus on understanding what proteins do and how they perform these functions.

Several key properties enable proteins to participate in such a wide range of functions.

1. *Proteins are linear polymers built of monomer units called amino acids.* The construction of a vast array of macromolecules from a limited number of monomer building blocks is a recurring theme in biochemistry. Does protein function depend on the linear sequence of amino acids? The function of a protein is directly dependent on its three-dimensional structure (Figure 3.1). Remarkably, proteins spontaneously fold up into three-dimensional structures that are determined by the sequence of amino acids in the protein polymer. Thus, *proteins are the embodiment of the transition from the one-dimensional world of sequences to the three-dimensional world of molecules capable of diverse activities.*

2. *Proteins contain a wide range of functional groups.* These functional groups include alcohols, thiols, thioethers, carboxylic

OUTLINE

- **3.1 Proteins Are Built from a Repertoire of 20 Amino Acids**
- **3.2 Primary Structure: Amino Acids Are Linked by Peptide Bonds to Form Polypeptide Chains**
- **3.3 Secondary Structure: Polypeptide Chains Can Fold into Regular Structures Such as the Alpha Helix, the Beta Sheet, and Turns and Loops**
- **3.4 Tertiary Structure: Water-Soluble Proteins Fold into Compact Structures with Nonpolar Cores**
- **3.5 Quaternary Structure: Polypeptide Chains Can Assemble into Multisubunit Structures**
- **3.6 The Amino Acid Sequence of a Protein Determines Its Three-Dimensional Structure**

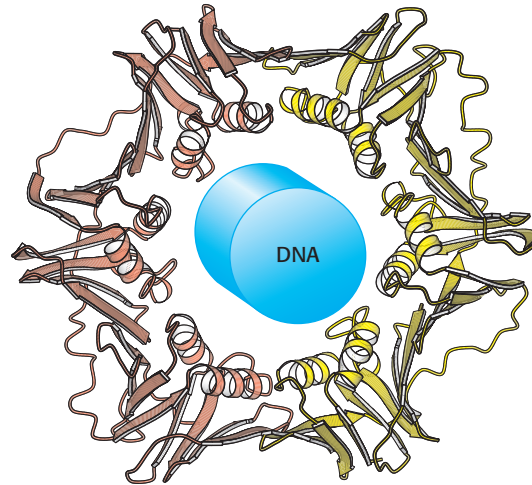


FIGURE 3.1 Structure dictates function. A protein component of the DNA replication machinery surrounds a section of DNA double helix. The structure of the protein allows large segments of DNA to be copied without the replication machinery dissociating from the DNA.

acids, carboxamides, and a variety of basic groups. When combined in various sequences, this array of functional groups accounts for the broad spectrum of protein function. For instance, the chemical reactivity associated with these groups is essential to the function of *enzymes*, the proteins that catalyze specific chemical reactions in biological systems (see Chapters 8–10).

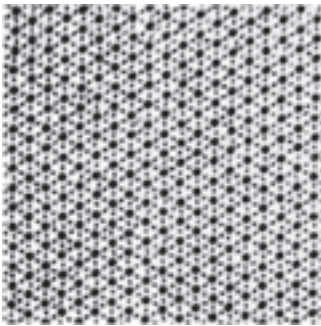


FIGURE 3.2 A complex protein assembly. An electron micrograph of insect flight tissue in cross section shows a hexagonal array of two kinds of protein filaments. [Courtesy of Dr. Michael Reedy.]

3. *Proteins can interact with one another and with other biological macromolecules to form complex assemblies.* The proteins within these assemblies can act synergistically to generate capabilities not afforded by the individual component proteins (Figure 3.2). These assemblies include macromolecular machines that carry out the accurate replication of DNA, the transmission of signals within cells, and many other essential processes.

4. *Some proteins are quite rigid, whereas others display limited flexibility.* Rigid units can function as structural elements in the cytoskeleton (the internal scaffolding within cells) or in connective tissue. Parts of proteins with limited flexibility may act as hinges, springs, and levers that are crucial to protein function, to the assembly of proteins with one another and with other molecules into complex units, and to the transmission of information within and between cells (Figure 3.3).

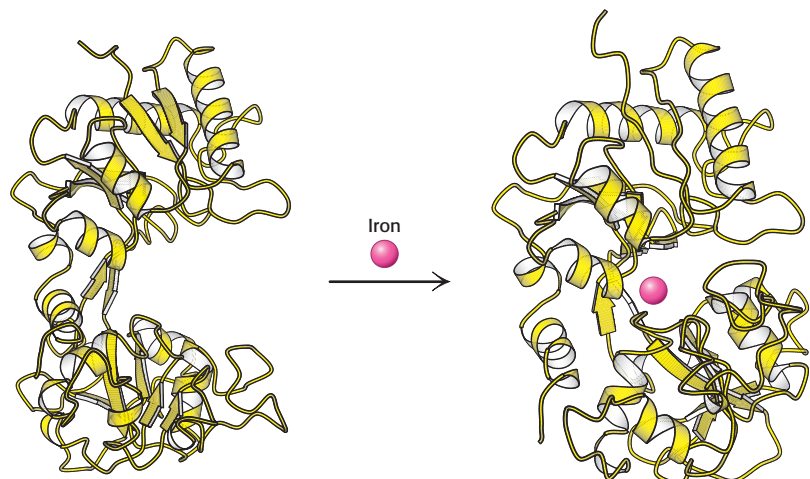


FIGURE 3.3 Flexibility and function. Upon binding iron, the protein lactoferrin undergoes conformational changes that allow other molecules to distinguish between the iron-free and the iron-bound forms.

3.1 PROTEINS ARE BUILT FROM A REPERTOIRE OF 20 AMINO ACIDS

Amino acids are the building blocks of proteins. An α -amino acid consists of a central carbon atom, called the α carbon, linked to an amino group, a carboxylic acid group, a hydrogen atom, and a distinctive R group. The R group is often referred to as the *side chain*. With four different groups connected to the tetrahedral α -carbon atom, α -amino acids are *chiral*; the two mirror-image forms are called the L isomer and the D isomer (Figure 3.4).

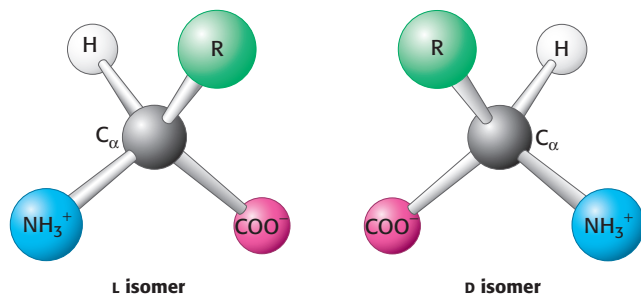


FIGURE 3.4 The L and D isomers of amino acids. R refers to the side chain. The L and D isomers are mirror images of each other.

Only L amino acids are constituents of proteins. For almost all amino acids, the L isomer has S (rather than R) absolute configuration (Figure 3.5). Although considerable effort has gone into understanding why amino acids in proteins have this absolute configuration, no satisfactory explanation has been arrived at. It seems plausible that the selection of L over D was arbitrary but, once made, was fixed early in evolutionary history.

Amino acids in solution at neutral pH exist predominantly as *dipolar ions* (also called *zwitterions*). In the dipolar form, the amino group is protonated ($-\text{NH}_3^+$) and the carboxyl group is deprotonated ($-\text{COO}^-$). The ionization state of an amino acid varies with pH (Figure 3.6). In acid solution (e.g., pH 1), the amino group is protonated ($-\text{NH}_3^+$) and the carboxyl group is not dissociated ($-\text{COOH}$). As the pH is raised, the carboxylic acid is the first group to give up a proton, inasmuch as its pK_a is near 2. The dipolar form persists until the pH approaches 9, when the protonated amino group

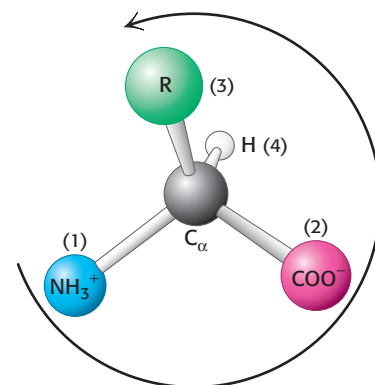


FIGURE 3.5 Only L amino acids are found in proteins. Almost all L amino acids have an S absolute configuration (from the Latin *sinister* meaning “left”). The counterclockwise direction of the arrow from highest- to lowest-priority substituents indicates that the chiral center is of the S configuration.

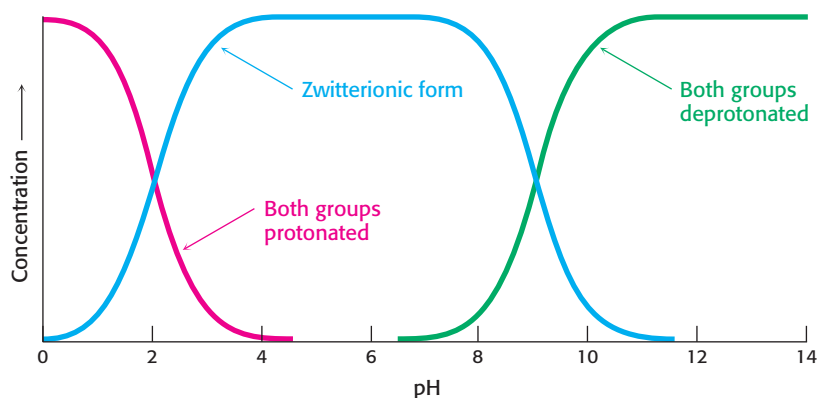
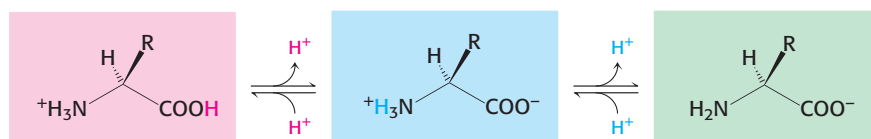


FIGURE 3.6 Ionization state as a function of pH. The ionization state of amino acids is altered by a change in pH. The zwitterionic form predominates near physiological pH.

Notation for distinguishing stereoisomers—

The four different substituents of an asymmetric carbon atom are assigned a priority according to atomic number. The lowest-priority substituent, often hydrogen, is pointed away from the viewer. The configuration about the carbon is called *S*, from the Latin *sinister* for “left,” if the progression from the highest to the lowest priority is counterclockwise. The configuration is called *R*, from the Latin *rectus* for “right,” if the progression is clockwise.

FIGURE 3.7 Structures of glycine and alanine. (Top) Ball-and-stick models show the arrangement of atoms and bonds in space. (Middle) Stereochemically realistic formulas show the geometrical arrangement of bonds around atoms (see Chapter 1 Appendix). (Bottom) Fischer projections show all bonds as being perpendicular for a simplified representation (see Chapter 1 Appendix).

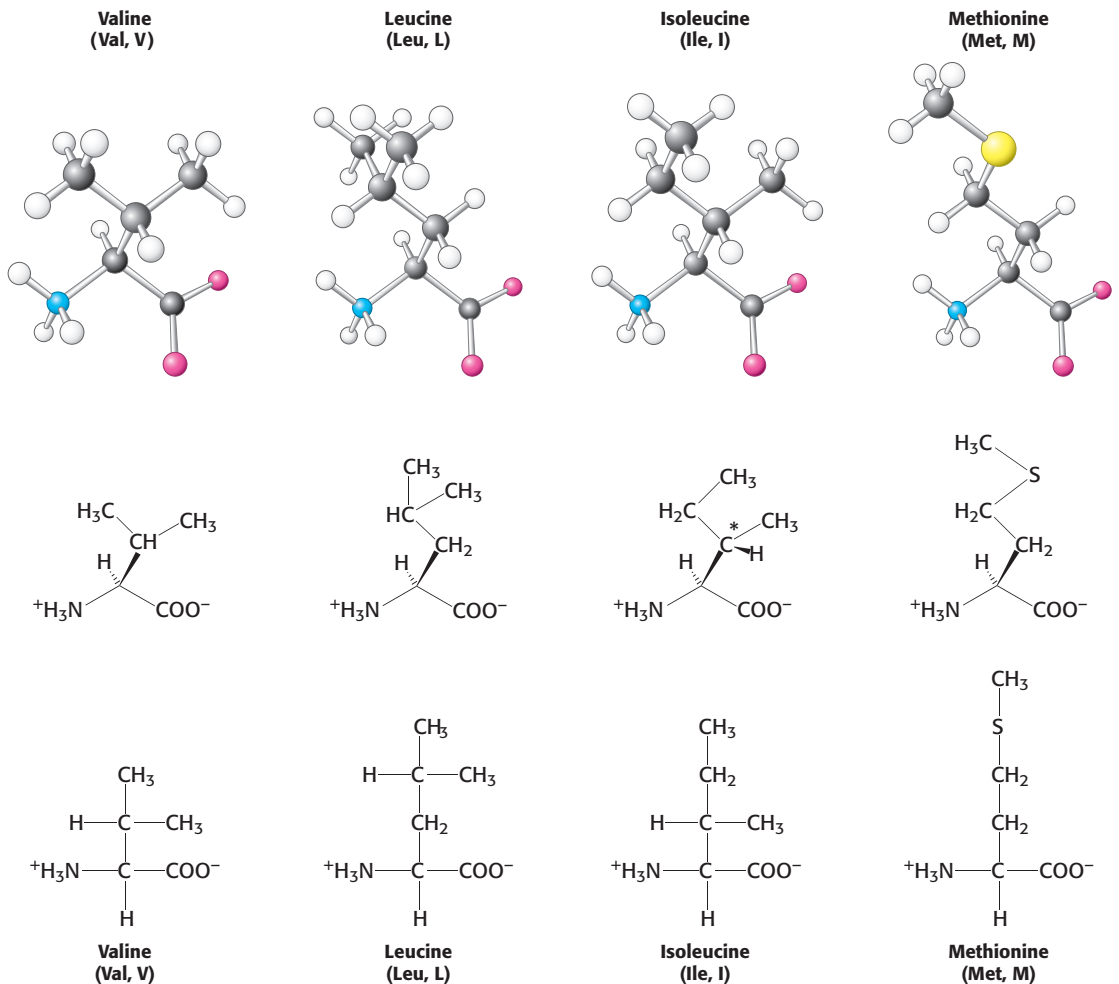
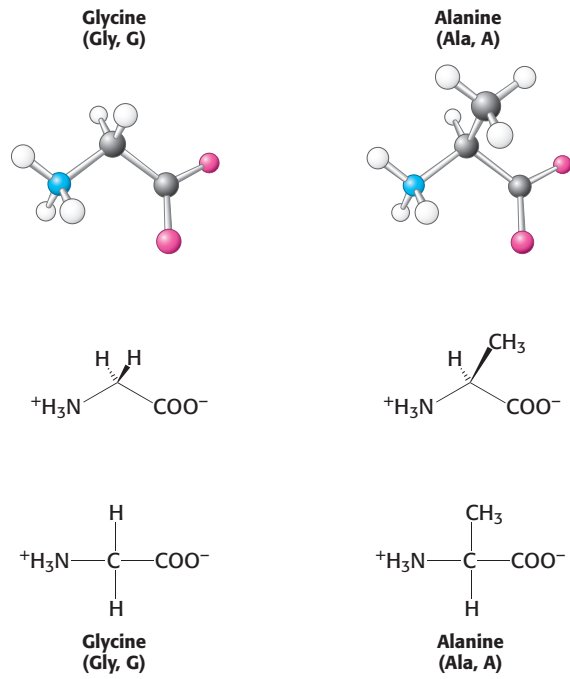


FIGURE 3.8 Amino acids with aliphatic side chains. The additional chiral center of isoleucine is indicated by an asterisk.

loses a proton. For a review of acid–base concepts and pH, see the appendix to this chapter.

Twenty kinds of side chains varying in *size*, *shape*, *charge*, *hydrogen-bonding capacity*, *hydrophobic character*, and *chemical reactivity* are commonly found in proteins. Indeed, all proteins in all species—bacterial, archaeal, and eukaryotic—are constructed from the same set of 20 amino acids. This fundamental alphabet of proteins is several billion years old. The remarkable range of functions mediated by proteins results from the diversity and versatility of these 20 building blocks. Understanding how this alphabet is used to create the intricate three-dimensional structures that enable proteins to carry out so many biological processes is an exciting area of biochemistry and one that we will return to in Section 3.6.

Let us look at this set of amino acids. The simplest one is *glycine*, which has just a hydrogen atom as its side chain. With two hydrogen atoms bonded to the α -carbon atom, glycine is unique in being *achiral*. *Alanine*, the next simplest amino acid, has a methyl group ($-\text{CH}_3$) as its side chain (Figure 3.7).

Larger hydrocarbon side chains are found in *valine*, *leucine*, and *isoleucine* (Figure 3.8). *Methionine* contains a largely *aliphatic* side chain that includes a *thioether* ($-\text{S}-$) group. The side chain of isoleucine includes an additional chiral center; only the isomer shown in Figure 3.8 is found in proteins. The larger aliphatic side chains are *hydrophobic*—that is, they tend to cluster together rather than contact water. The three-dimensional structures of water-soluble proteins are stabilized by this tendency of hydrophobic groups to come together, called *the hydrophobic effect* (see Section 1.3.3). The different sizes and shapes of these hydrocarbon side chains enable them to pack together to form compact structures with few holes. *Proline* also has an aliphatic side chain, but it differs from other members of the set of 20 in that its side chain is bonded to both the nitrogen and the α -carbon atoms (Figure 3.9). Proline markedly influences protein architecture because its ring structure makes it more conformationally restricted than the other amino acids.

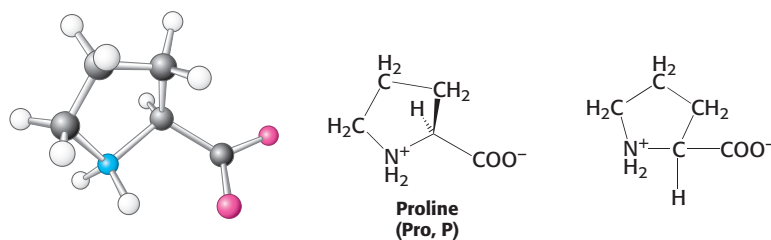


FIGURE 3.9 Cyclic structure of proline.
The side chain is joined to both the α carbon and the amino group.

Three amino acids with relatively simple *aromatic side chains* are part of the fundamental repertoire (Figure 3.10). *Phenylalanine*, as its name indicates, contains a phenyl ring attached in place of one of the hydrogens of alanine. The aromatic ring of *tyrosine* contains a hydroxyl group. This hydroxyl group is reactive, in contrast with the rather inert side chains of the other amino acids discussed thus far. *Tryptophan* has an indole ring joined to a methylene ($-\text{CH}_2-$) group; the indole group comprises two fused rings and an NH group. Phenylalanine is purely hydrophobic, whereas tyrosine and tryptophan are less so because of their hydroxyl and NH groups. The aromatic rings of tryptophan and tyrosine contain delocalized π electrons that strongly absorb ultraviolet light (Figure 3.11).

A compound's *extinction coefficient* indicates its ability to absorb light. Beer's law gives the absorbance (A) of light at a given wavelength:

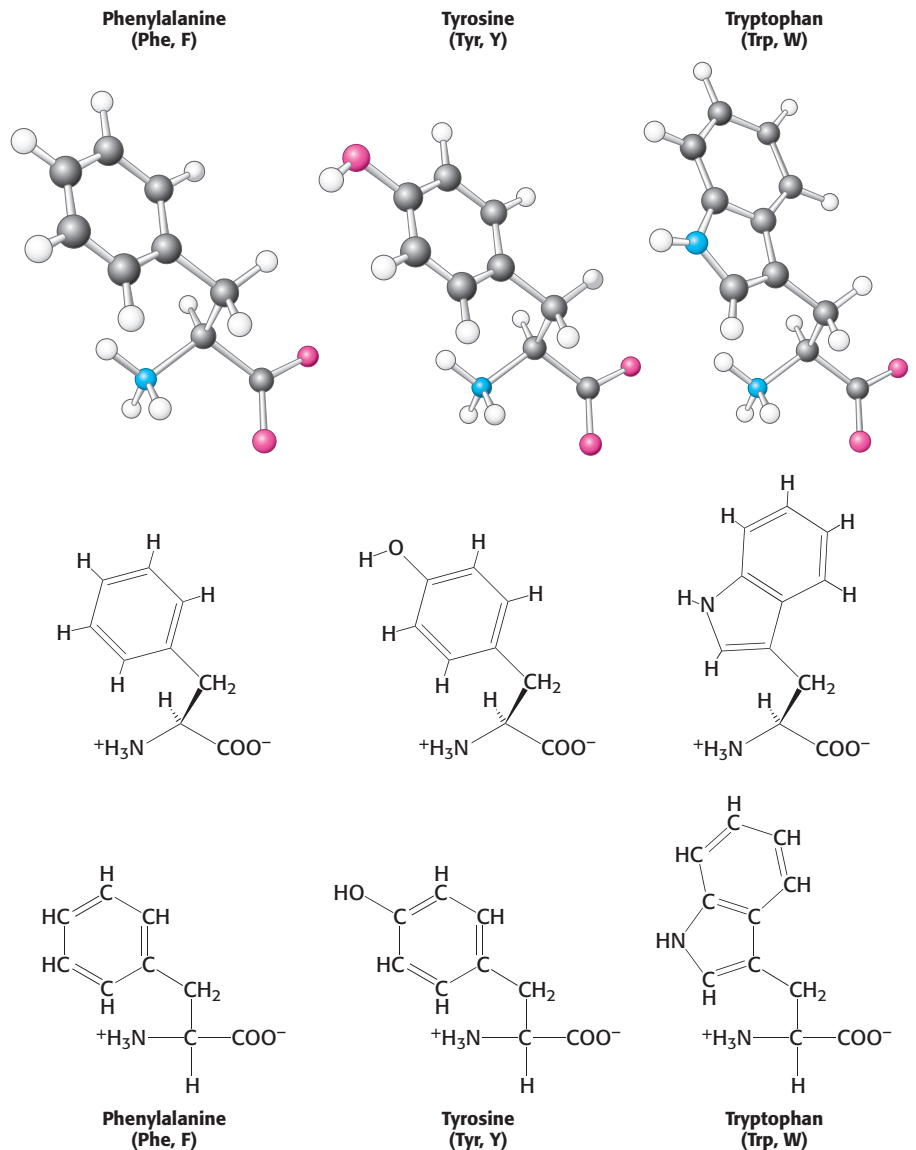
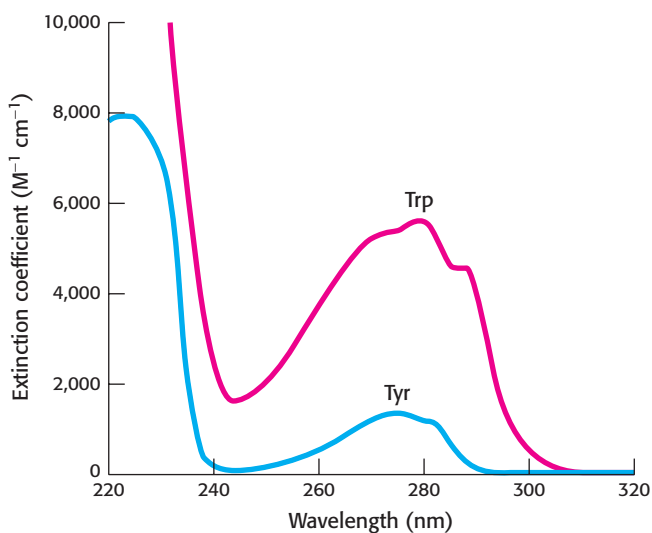


FIGURE 3.10 Amino acids with aromatic side chains. Phenylalanine, tyrosine, and tryptophan have hydrophobic character. Tyrosine and tryptophan also have hydrophilic properties because of their $-OH$ and $-NH-$ groups, respectively.



$A = \epsilon cl$ Beer's law

where ϵ is the extinction coefficient [in units that are the reciprocals of molarity and distance in centimeters ($M^{-1} cm^{-1}$)], c is the concentration of the absorbing species (in units of molarity, M), and l is the length through which the light passes (in units of centimeters). For tryptophan, absorption is maximum at 280 nm and the extinction coefficient is $3400 M^{-1} cm^{-1}$ whereas, for tyrosine, absorption is maximum at 276 nm and the extinction coefficient is a less-intense $1400 M^{-1} cm^{-1}$. Phenylalanine absorbs light less strongly and at shorter wavelengths. The absorption of light at 280 nm can be used to estimate

FIGURE 3.11 Absorption spectra of the aromatic amino acids tryptophan (red) and tyrosine (blue). Only these amino acids absorb strongly near 280 nm. [Courtesy of Greg Gatto].

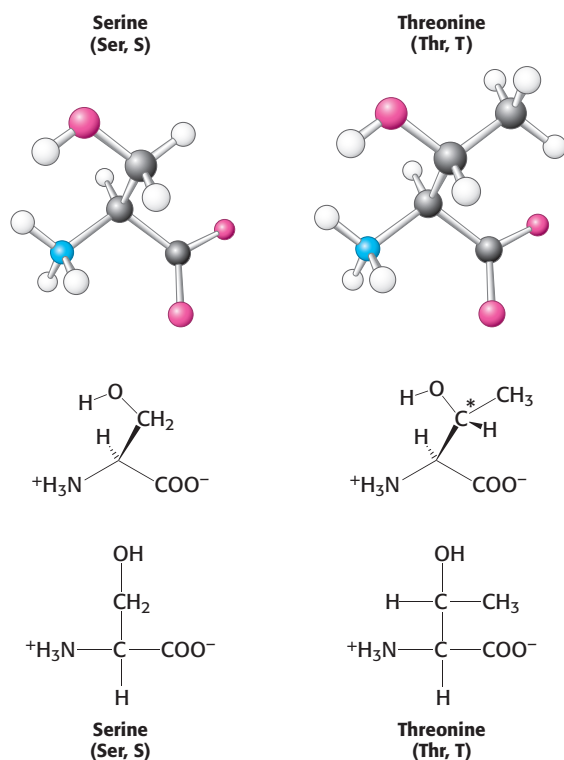


FIGURE 3.12 Amino acids containing aliphatic hydroxyl groups. Serine and threonine contain hydroxyl groups that render them hydrophilic. The additional chiral center in threonine is indicated by an asterisk.

the concentration of a protein in solution if the number of tryptophan and tyrosine residues in the protein is known.

Two amino acids, *serine* and *threonine*, contain aliphatic *hydroxyl groups* (Figure 3.12). Serine can be thought of as a hydroxylated version of alanine, whereas threonine resembles valine with a hydroxyl group in place of one of the valine methyl groups. The hydroxyl groups on serine and threonine make them much more *hydrophilic* (water loving) and *reactive* than alanine and valine. Threonine, like isoleucine, contains an additional asymmetric center; again only one isomer is present in proteins.

Cysteine is structurally similar to serine but contains a *sulfhydryl*, or *thiol* ($-\text{SH}$), group in place of the hydroxyl ($-\text{OH}$) group (Figure 3.13). The sulfhydryl group is much more reactive. Pairs of sulfhydryl groups may come together to form disulfide bonds, which are particularly important in stabilizing some proteins, as will be discussed shortly.

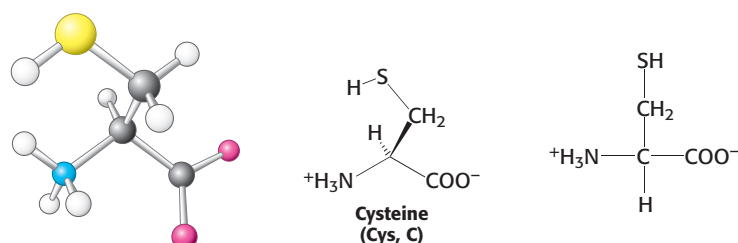


FIGURE 3.13 Structure of cysteine.

We turn now to amino acids with very polar side chains that render them highly hydrophilic. *Lysine* and *arginine* have relatively long side chains that terminate with groups that are *positively charged* at neutral pH. Lysine is capped by a primary amino group and arginine by a guanidinium group. *Histidine* contains an imidazole group, an aromatic ring that also can be positively charged (Figure 3.14).

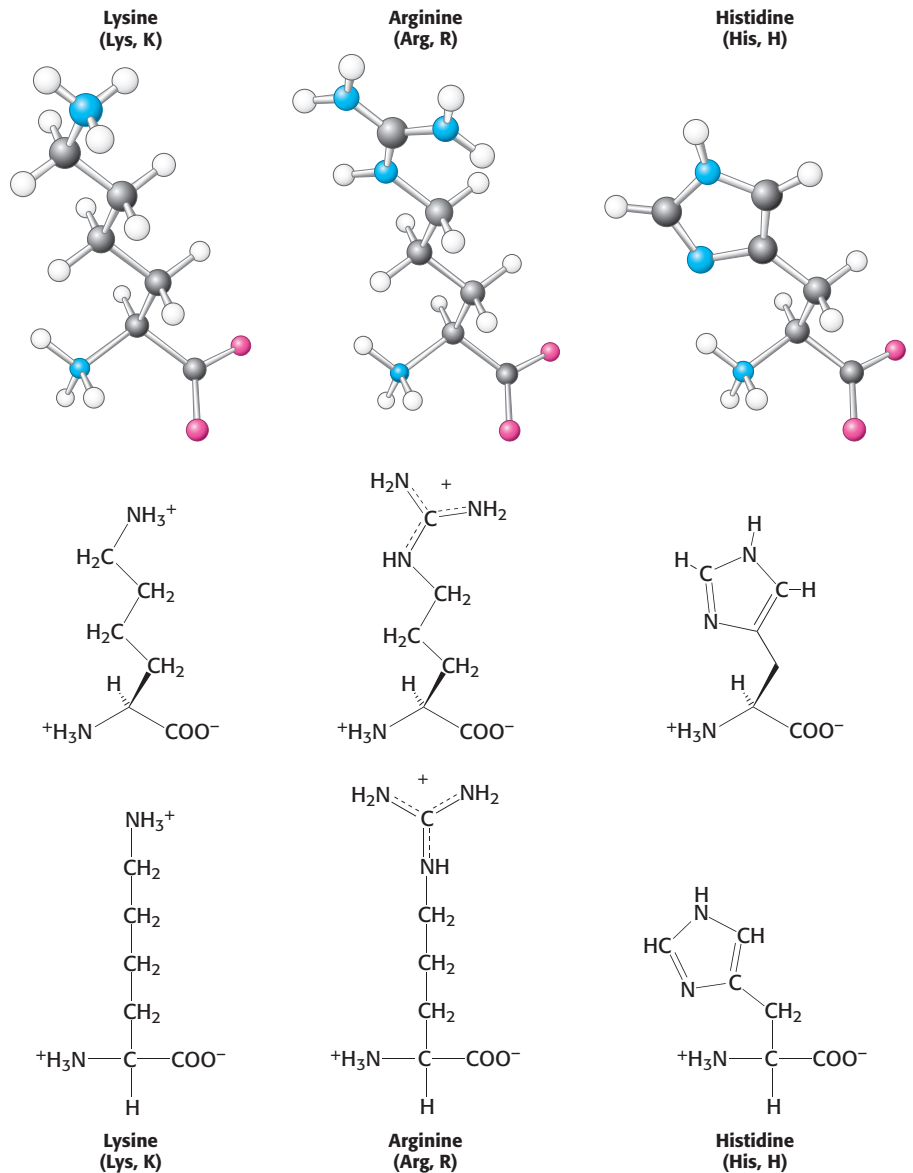


FIGURE 3.14 The basic amino acids lysine, arginine, and histidine.

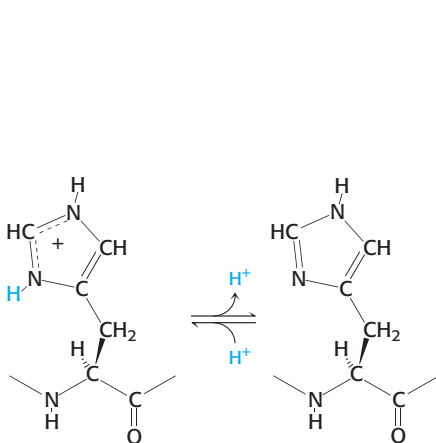
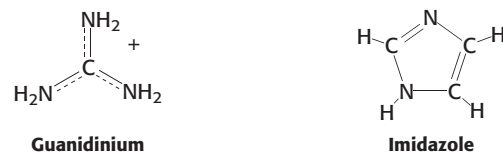


FIGURE 3.15 Histidine ionization. Histidine can bind or release protons near physiological pH.



With a pK_a value near 6, the imidazole group can be uncharged or positively charged near neutral pH, depending on its local environment (Figure 3.15). Indeed, histidine is often found in the active sites of enzymes, where the imidazole ring can bind and release protons in the course of enzymatic reactions.

The set of amino acids also contains two with *acidic side chains*: *aspartic acid* and *glutamic acid* (Figure 3.16). These amino acids are often called *aspartate* and *glutamate* to emphasize that their side chains are usually negatively charged at physiological pH. Nonetheless, in some proteins these side chains do accept protons, and this ability is often functionally important. In addition, the set includes uncharged derivatives of aspartate and

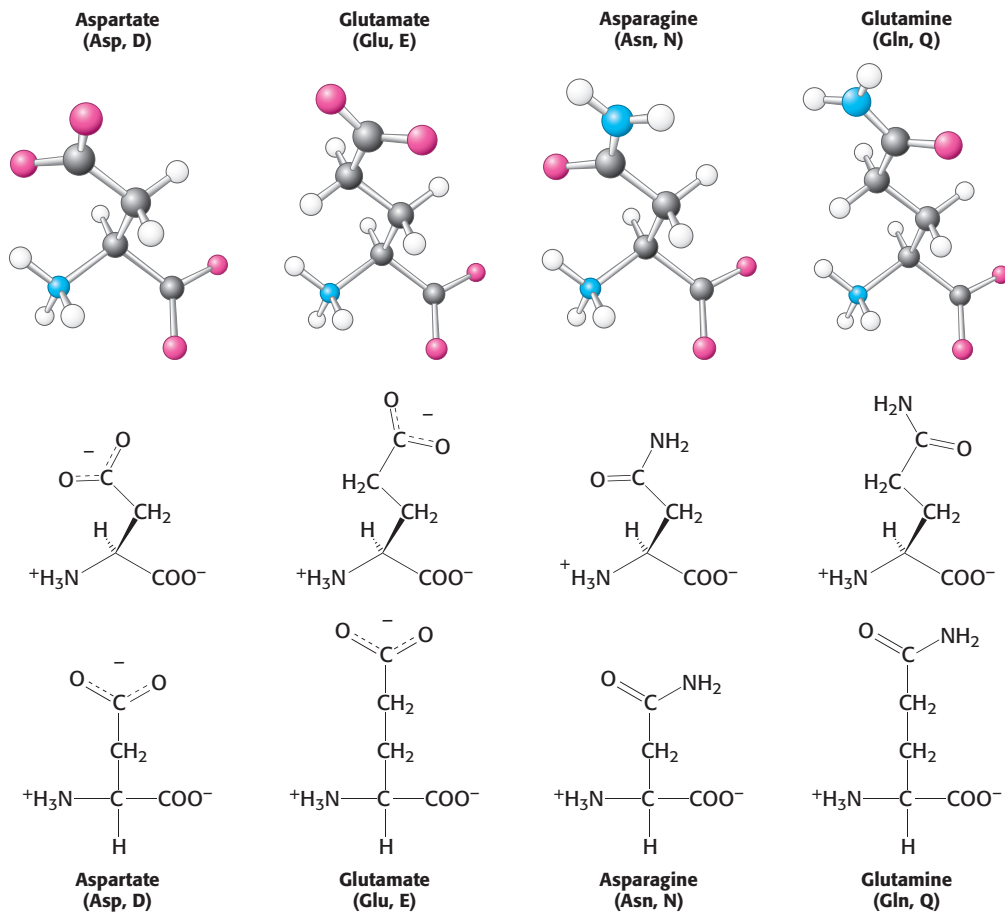


FIGURE 3.16 Amino acids with side-chain carboxylates and carboxamides.

glutamate—*asparagine* and *glutamine*—each of which contains a terminal *carboxamide* in place of a carboxylic acid (Figure 3.16).

Seven of the 20 amino acids have readily ionizable side chains. These 7 amino acids are able to donate or accept protons to facilitate reactions as well as to form ionic bonds. Table 3.1 gives equilibria and typical pK_a values for ionization of the side chains of tyrosine, cysteine, arginine, lysine, histidine, and aspartic and glutamic acids in proteins. Two other groups in proteins—the terminal α -amino group and the terminal α -carboxyl group—can be ionized, and typical pK_a values are also included in Table 3.1.

Amino acids are often designated by either a three-letter abbreviation or a one-letter symbol (Table 3.2). The abbreviations for amino acids are the first three letters of their names, except for asparagine (Asn), glutamine (Gln), isoleucine (Ile), and tryptophan (Trp). The symbols for many amino acids are the first letters of their names (e.g., G for glycine and L for leucine); the other symbols have been agreed on by convention. These abbreviations and symbols are an integral part of the vocabulary of biochemists.


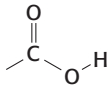
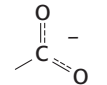
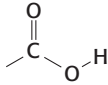
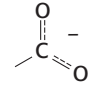
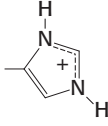
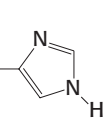
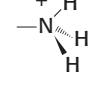
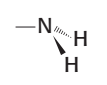
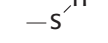
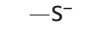
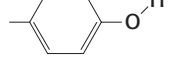
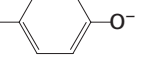
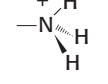
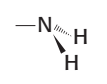
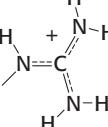
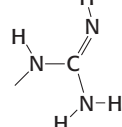
 How did this particular set of amino acids become the building blocks of proteins? First, as a set, they are diverse; their structural and chemical properties span a wide range, endowing proteins with the versatility to assume many functional roles. Second, as noted in Section 2.1.1, many of these amino acids were probably available from prebiotic reactions. Finally, excessive intrinsic reactivity may have eliminated other possible amino

TABLE 3.1 Typical pK_a values of ionizable groups in proteins

Group	Acid	\rightleftharpoons	Base	Typical pK_a^*
Terminal α -carboxyl group		\rightleftharpoons		3.1
Aspartic acid Glutamic acid		\rightleftharpoons		4.1
Histidine		\rightleftharpoons		6.0
Terminal α -amino group		\rightleftharpoons		8.0
Cysteine		\rightleftharpoons		8.3
Tyrosine		\rightleftharpoons		10.9
Lysine		\rightleftharpoons		10.8
Arginine		\rightleftharpoons		12.5

* pK_a values depend on temperature, ionic strength, and the microenvironment of the ionizable group.

TABLE 3.2 Abbreviations for amino acids

Amino acid	Three-letter abbreviation	One-letter abbreviation	Amino acid	Three-letter abbreviation	One-letter abbreviation
Alanine	Ala	A	Methionine	Met	M
Arginine	Arg	R	Phenylalanine	Phe	F
Asparagine	Asn	N	Proline	Pro	P
Aspartic Acid	Asp	D	Serine	Ser	S
Cysteine	Cys	C	Threonine	Thr	T
Glutamine	Gln	Q	Tryptophan	Trp	W
Glutamic Acid	Glu	E	Tyrosine	Tyr	Y
Glycine	Gly	G	Valine	Val	V
Histidine	His	H	Asparagine or aspartic acid	Asx	B
Isoleucine	Ile	I	Glutamine or glutamic acid	Glx	Z
Leucine	Leu	L			
Lysine	Lys	K			

acids. For example, amino acids such as homoserine and homocysteine tend to form five-membered cyclic forms that limit their use in proteins; the alternative amino acids that are found in proteins—serine and cysteine—do not readily cyclize, because the rings in their cyclic forms are too small (Figure 3.17).

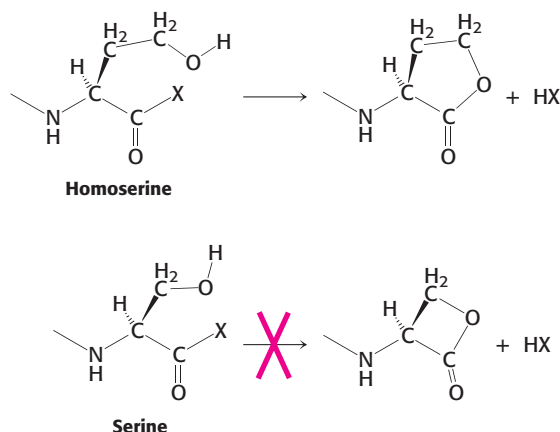


FIGURE 3.17 Undesirable reactivity in amino acids. Some amino acids are unsuitable for proteins because of undesirable cyclization. Homoserine can cyclize to form a stable, five-membered ring, potentially resulting in peptide-bond cleavage. Cyclization of serine would form a strained, four-membered ring and thus is unfavored. X can be an amino group from a neighboring amino acid or another potential leaving group.

3.2 PRIMARY STRUCTURE: AMINO ACIDS ARE LINKED BY PEPTIDE BONDS TO FORM POLYPEPTIDE CHAINS

Proteins are *linear polymers* formed by linking the α -carboxyl group of one amino acid to the α -amino group of another amino acid with a *peptide bond* (also called an *amide bond*). The formation of a dipeptide from two amino acids is accompanied by the loss of a water molecule (Figure 3.18). The equilibrium of this reaction lies on the side of hydrolysis rather than synthesis. Hence, the biosynthesis of peptide bonds requires an input of free energy. Nonetheless, peptide bonds are quite *stable kinetically*; the lifetime of a peptide bond in aqueous solution in the absence of a catalyst approaches 1000 years.

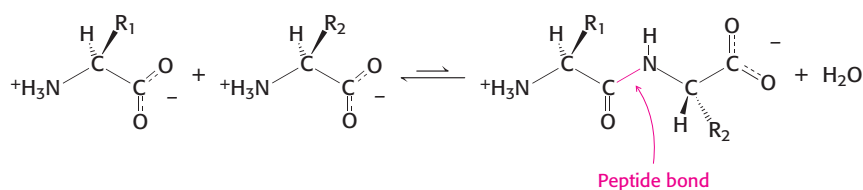


FIGURE 3.18 Peptide-bond formation. The linking of two amino acids is accompanied by the loss of a molecule of water.

A series of amino acids joined by peptide bonds form a *polypeptide chain*, and each amino acid unit in a polypeptide is called a *residue*. A *polypeptide chain has polarity* because its ends are different, with an α -amino group at one end and an α -carboxyl group at the other. By convention, *the amino end is taken to be the beginning of a polypeptide chain*, and so the sequence of amino acids in a polypeptide chain is written starting with the amino-terminal residue. Thus, in the pentapeptide Tyr-Gly-Gly-Phe-Leu (YGGFL), phenylalanine is the amino-terminal (N-terminal) residue and leucine is the carboxyl-terminal (C-terminal) residue (Figure 3.19). Leu-Phe-Gly-Gly-Tyr (LFGGY) is a different pentapeptide, with different chemical properties.

A polypeptide chain consists of a regularly repeating part, called the *main chain* or *backbone*, and a variable part, comprising the distinctive *side chains* (Figure 3.20). The polypeptide backbone is rich in hydrogen-bonding potential. Each residue contains a carbonyl group, which is a good hydrogen-bond acceptor and, with the exception of proline, an NH group, which is a

FIGURE 3.19 Amino acid sequences have direction. This illustration of the pentapeptide Try-Gly-Gly-Phe-Leu (YGGFL) shows the sequence from the amino terminus to the carboxyl terminus. This pentapeptide, Leu-enkephalin, is an opioid peptide that modulates the perception of pain. The reverse pentapeptide, Leu-Phe-Gly-Gly-Tyr (LFGGY), is a different molecule and shows no such effects.

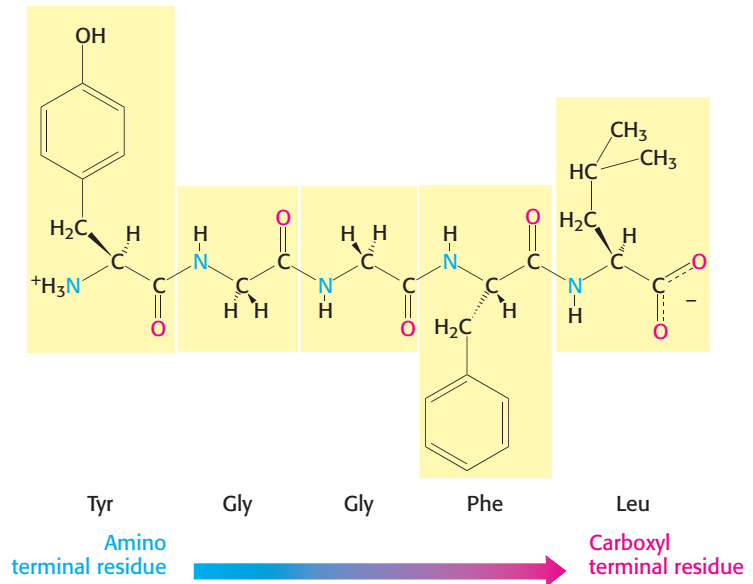
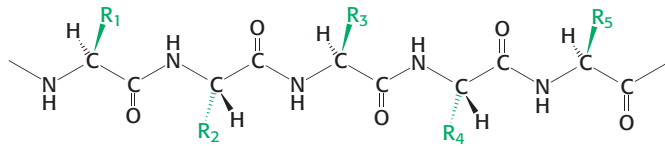


FIGURE 3.20 Components of a polypeptide chain. A polypeptide chain consists of a constant backbone (shown in black) and variable side chains (shown in green).



good hydrogen-bond donor. These groups interact with each other and with functional groups from side chains to stabilize particular structures, as will be discussed in detail.

Most natural polypeptide chains contain between 50 and 2000 amino acid residues and are commonly referred to as *proteins*. Peptides made of small numbers of amino acids are called *oligopeptides* or simply *peptides*. The mean molecular weight of an amino acid residue is about 110, and so the molecular weights of most proteins are between 5500 and 220,000. We can also refer to the mass of a protein, which is expressed in units of daltons; one *dalton* is equal to one atomic mass unit. A protein with a molecular weight of 50,000 has a mass of 50,000 daltons, or 50 kd (kilodaltons).

In some proteins, the linear polypeptide chain is cross-linked. The most common cross-links are *disulfide bonds*, formed by the oxidation of a pair of cysteine residues (Figure 3.21). The resulting unit of linked cysteines is

Dalton—

A unit of mass very nearly equal to that of a hydrogen atom. Named after John Dalton (1766–1844), who developed the atomic theory of matter.

Kilodalton (kd)—

A unit of mass equal to 1000 daltons.

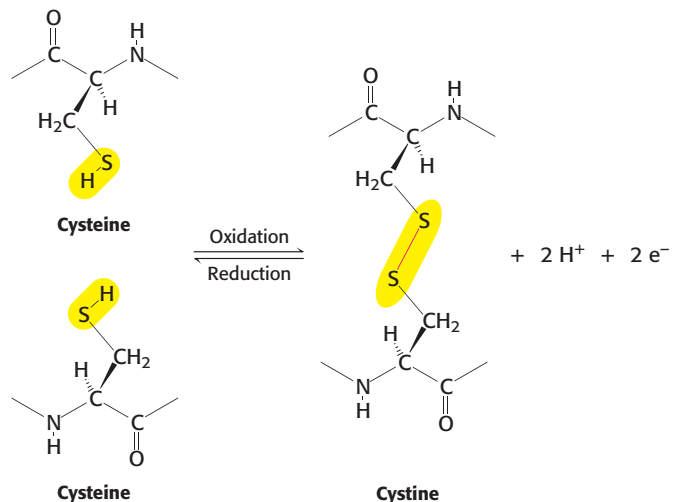


FIGURE 3.21 Cross-links. The formation of a disulfide bond from two cysteine residues is an oxidation reaction.

called *cystine*. Extracellular proteins often have several disulfide bonds, whereas intracellular proteins usually lack them. Rarely, nondisulfide cross-links derived from other side chains are present in some proteins. For example, collagen fibers in connective tissue are strengthened in this way, as are fibrin blood clots.

3.2.1 Proteins Have Unique Amino Acid Sequences That Are Specified by Genes

In 1953, Frederick Sanger determined the amino acid sequence of insulin, a protein hormone (Figure 3.22). *This work is a landmark in biochemistry because it showed for the first time that a protein has a precisely defined amino acid sequence.* Moreover, it demonstrated that insulin consists only of L amino acids linked by peptide bonds between α -amino and α -carboxyl groups. This accomplishment stimulated other scientists to carry out sequence studies of a wide variety of proteins. Indeed, the complete amino acid sequences of more than 100,000 proteins are now known. *The striking fact is that each protein has a unique, precisely defined amino acid sequence.* The amino acid sequence of a protein is often referred to as its *primary structure*.

A series of incisive studies in the late 1950s and early 1960s revealed that the amino acid sequences of proteins are genetically determined. The sequence of nucleotides in DNA, the molecule of heredity, specifies a complementary sequence of nucleotides in RNA, which in turn specifies the amino acid sequence of a protein. In particular, each of the 20 amino acids of the repertoire is encoded by one or more specific sequences of three nucleotides (Section 5.5).

Knowing amino acid sequences is important for several reasons. First, knowledge of the sequence of a protein is usually essential to elucidating its mechanism of action (e.g., the catalytic mechanism of an enzyme). Moreover, proteins with novel properties can be generated by varying the sequence of known proteins. Second, amino acid sequences determine the three-dimensional structures of proteins. Amino acid sequence is the link between the genetic message in DNA and the three-dimensional structure that performs a protein's biological function. Analyses of relations between amino acid sequences and three-dimensional structures of proteins are uncovering the rules that govern the folding of polypeptide chains. Third, sequence determination is a component of molecular pathology, a rapidly growing area of medicine. Alterations in amino acid sequence can produce abnormal function and disease. Severe and sometimes fatal diseases, such as sickle-cell anemia and cystic fibrosis, can result from a change in a single amino acid within a protein. Fourth, the sequence of a protein reveals much about its evolutionary history (see Chapter 7). Proteins resemble one another in amino acid sequence only if they have a common ancestor. Consequently, molecular events in evolution can be traced from amino acid sequences; molecular paleontology is a flourishing area of research.

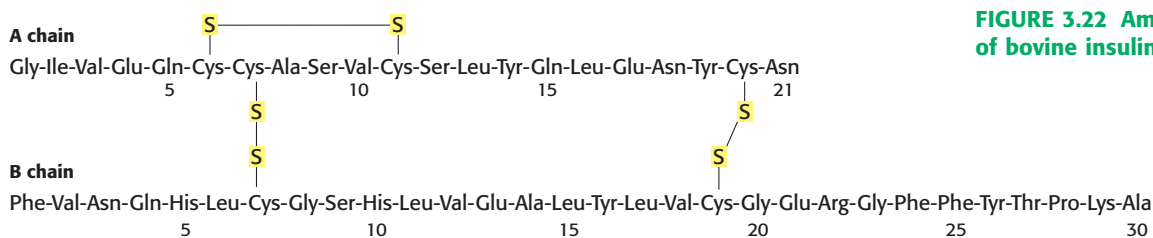


FIGURE 3.22 Amino acid sequence of bovine insulin.

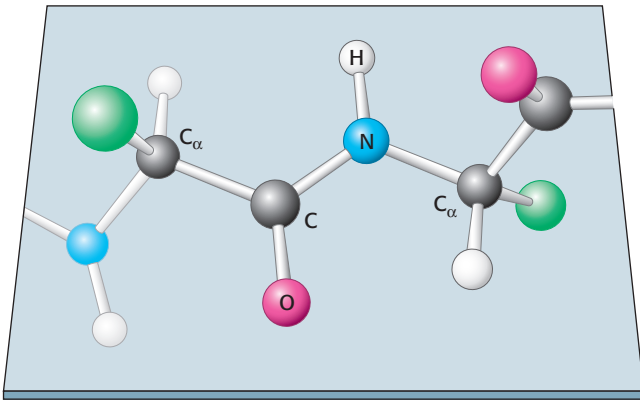


FIGURE 3.23 Peptide bonds are planar. In a pair of linked amino acids, six atoms (C_{α} , C, O, N, H, and C_{α}) lie in a plane. Side chains are shown as green balls.

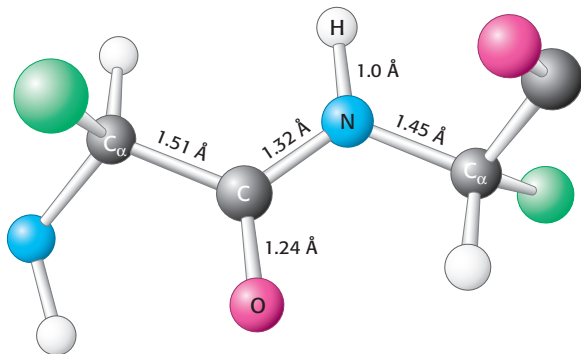
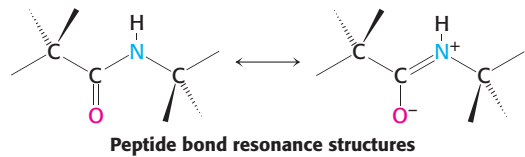


FIGURE 3.24 Typical bond lengths within a peptide unit. The peptide unit is shown in the trans configuration.

3.2.2 Polypeptide Chains Are Flexible Yet Conformationally Restricted

Examination of the geometry of the protein backbone reveals several important features. First, *the peptide bond is essentially planar* (Figure 3.23). Thus, for a pair of amino acids linked by a peptide bond, six atoms lie in the same plane: the α -carbon atom and CO group from the first amino acid and the NH group and α -carbon atom from the second amino acid. The nature of the chemical bonding within a peptide explains this geometric preference. The peptide bond has considerable *double-bond character*, which prevents rotation about this bond.

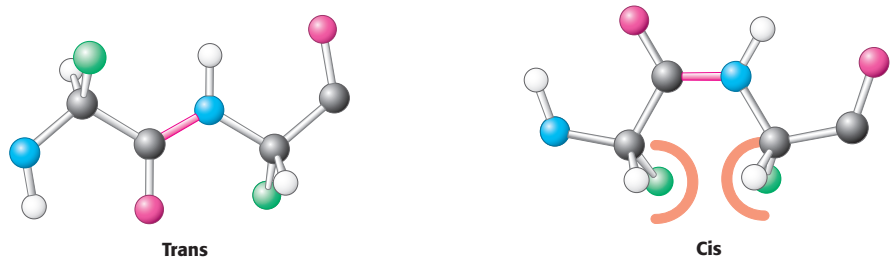


The inability of the bond to rotate constrains the conformation of the peptide backbone and accounts for the bond's planarity. This double-bond character is also expressed in the length of the bond between the CO and NH groups. The C–N distance in a peptide bond is typically 1.32 Å, which is between the values expected for a C–N single bond (1.49 Å) and a C=N double bond (1.27 Å), as shown in Figure 3.24. Finally, the peptide bond is uncharged, allowing polymers of amino acids linked by peptide bonds to form tightly packed globular structures.

Two configurations are possible for a planar peptide bond. In the trans configuration, the two α -carbon atoms are on opposite sides of the peptide bond. In the cis configuration, these groups are on the same side of the peptide bond. *Almost all peptide bonds in proteins are trans.* This preference for trans over cis can be explained by the fact that steric clashes between groups attached to the α -carbon atoms hinder formation of the cis form but do not occur in the trans configuration (Figure 3.25). By far the most common cis peptide bonds are X–Pro linkages. Such bonds show less preference for the trans configuration because the nitrogen of proline is bonded to two tetrahedral carbon atoms, limiting the steric differences between the trans and cis forms (Figure 3.26).

In contrast with the peptide bond, the bonds between the amino group and the α -carbon atom and between the α -carbon atom and the carbonyl group are pure single bonds. The two adjacent rigid peptide units may rotate about these bonds, taking on various orientations. *This freedom of rotation*

FIGURE 3.25 Trans and cis peptide bonds. The trans form is strongly favored because of steric clashes that occur in the cis form.



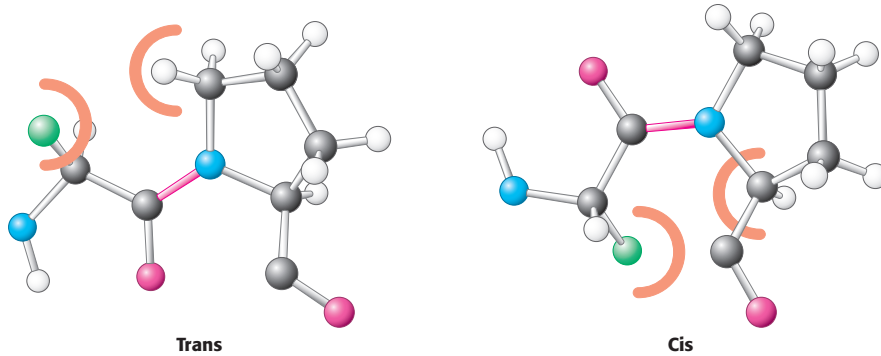


FIGURE 3.26 Trans and cis X-Pro bonds. The energies of these forms are relatively balanced because steric clashes occur in both forms.

about two bonds of each amino acid allows proteins to fold in many different ways. The rotations about these bonds can be specified by dihedral angles (Figure 3.27). The angle of rotation about the bond between the nitrogen and the α -carbon atoms is called *phi* (ϕ). The angle of rotation about the bond between the α -carbon and the carbonyl carbon atoms is called *psi* (ψ). A clockwise rotation about either bond as viewed from the front of the back group corresponds to a positive value. The ϕ and ψ angles determine the path of the polypeptide chain.

Are all combinations of ϕ and ψ possible? G. N. Ramachandran recognized that many combinations are forbidden because of steric collisions between atoms. The allowed values can be visualized on a two-dimensional plot called a *Ramachandran diagram* (Figure 3.28). Three-quarters of the possible (ϕ , ψ) combinations are excluded simply by local steric clashes. *Steric exclusion, the fact that two atoms cannot be in the same place at the same time, can be a powerful organizing principle.*

The ability of biological polymers such as proteins to fold into well-defined structures is remarkable thermodynamically. Consider the equilibrium between an unfolded polymer that exists as a random coil—that is, as a mixture of many possible conformations—and the folded form that adopts a unique conformation. The favorable entropy associated with the large number of conformations in the unfolded form opposes folding and must be overcome by interactions favoring the folded form. Thus, highly flexible polymers with a large number of possible conformations do not fold into unique structures. *The rigidity of the peptide unit and the restricted set of allowed ϕ and ψ angles limits the number of structures accessible to the unfolded form sufficiently to allow protein folding to occur.*

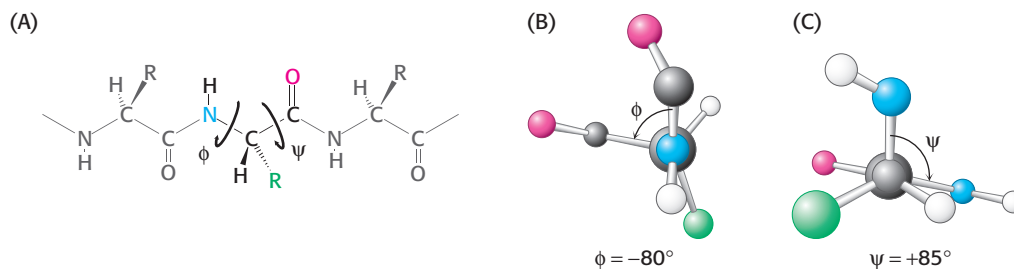
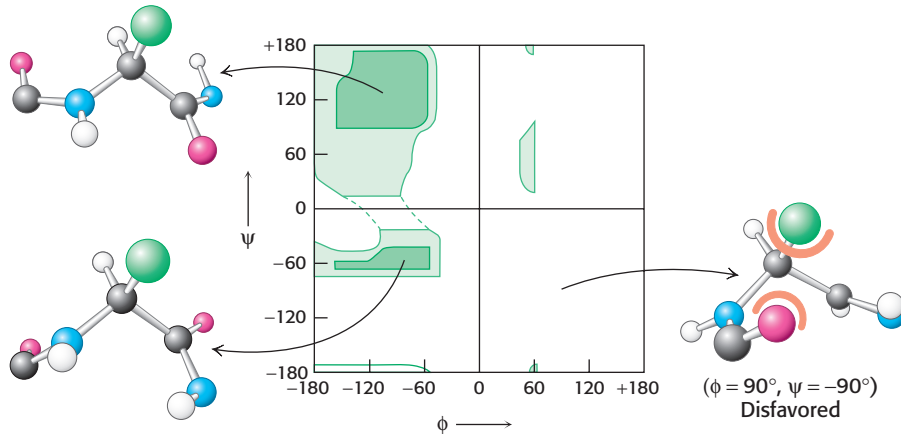


FIGURE 3.27 Rotation about bonds in a polypeptide. The structure of each amino acid in a polypeptide can be adjusted by rotation about two single bonds. (A) Phi (ϕ) is the angle of rotation about the bond between the nitrogen and the α -carbon atoms, whereas psi (ψ) is the angle of rotation about the bond between the α -carbon and the carbonyl carbon atoms. (B) A view down the bond between the nitrogen and the α -carbon atoms, showing how ϕ is measured. (C) A view down the bond between the α -carbon and the carbonyl carbon atoms, showing how ψ is measured.

Dihedral angle—

A measure of the rotation about a bond, usually taken to lie between -180° and $+180^\circ$. Dihedral angles are sometimes called torsion angles.

FIGURE 3.28 A Ramachandran diagram showing the values of ϕ and ψ . Not all ϕ and ψ values are possible without collisions between atoms. The most favorable regions are shown in dark green; borderline regions are shown in light green. The structure on the right is disfavored because of steric clashes.



3.3 SECONDARY STRUCTURE: POLYPEPTIDE CHAINS CAN FOLD INTO REGULAR STRUCTURES SUCH AS THE ALPHA HELIX, THE BETA SHEET, AND TURNS AND LOOPS

Can a polypeptide chain fold into a regularly repeating structure? In 1951, Linus Pauling and Robert Corey proposed two periodic structures called the α helix (alpha helix) and the β pleated sheet (beta pleated sheet). Subsequently, other structures such as the β turn and omega (Ω) loop were identified. Although not periodic, these common turn or loop structures are well defined and contribute with α helices and β sheets to form the final protein structure.



STRUCTURAL INSIGHTS, appearing throughout the book, are molecular modeling-based tutorials that enable you to review structure and learn what the latest research tells us about the workings of the molecule. To access, go to the Web site: www.whfreeman.com/biochem5, and select the chapter, Structural Insights, and the title.



STRUCTURAL INSIGHTS, Elements of Protein Structure provides interactive representations of some of the important elements of protein architecture described in this chapter, including a summary of secondary structure motifs.

3.3.1 The Alpha Helix Is a Coiled Structure Stabilized by Intrachain Hydrogen Bonds

In evaluating potential structures, Pauling and Corey considered which conformations of peptides were sterically allowed and which most fully exploited the hydrogen-bonding capacity of the backbone NH and CO groups. The first of their proposed structures, the α helix, is a rodlike structure (Figure 3.29). A tightly coiled backbone forms the inner part of the rod and the side chains extend outward in a helical array. The α helix is stabilized by hydrogen bonds between the NH and CO groups of the main chain. In particular, the CO group of each amino acid forms a hydrogen bond with the NH group of the amino acid that is situated four residues ahead in the sequence (Figure 3.30). Thus, except for amino acids near the ends of an α helix, all the main-chain CO and NH groups are hydrogen bonded. Each residue is related to the next one by a rise of 1.5 Å along the helix axis and a rotation of 100 degrees, which gives 3.6 amino acid residues per turn of helix. Thus, amino acids spaced three and four apart in the sequence are spatially quite close to one another in an α helix. In contrast, amino acids two apart in the sequence are situated on opposite sides of the helix and so are unlikely to make contact. The pitch of the α helix, which is equal to the product of the translation (1.5 Å) and the number of residues per turn (3.6), is 5.4 Å. The screw sense of a helix can be right-handed (clockwise) or left-handed (counterclockwise). The Ramachandran diagram reveals that both

Screw sense—

Describes the direction in which a helical structure rotates with respect to its axis. If, viewed down the axis of a helix, the chain turns in a clockwise direction, it has a right-handed screw sense. If the turning is counterclockwise, the screw sense is left-handed.

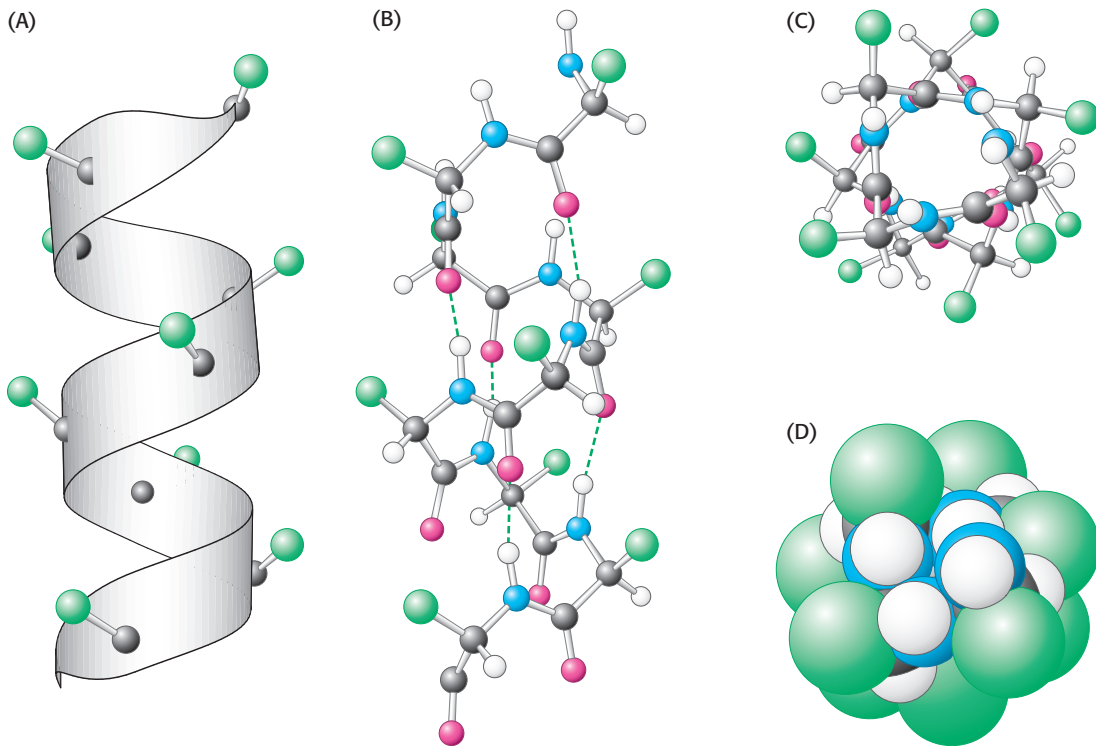


FIGURE 3.29 Structure of the α helix. (A) A ribbon depiction with the α -carbon atoms and side chains (green) shown. (B) A side view of a ball-and-stick version depicts the hydrogen bonds (dashed lines) between NH and CO groups. (C) An end view shows the coiled backbone as the inside of the helix and the side chains (green) projecting outward. (D) A space-filling view of part C shows the tightly packed interior core of the helix.

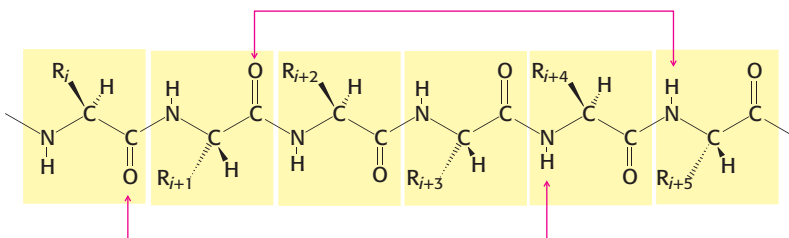


FIGURE 3.30 Hydrogen-bonding scheme for an α helix. In the α helix, the CO group of residue n forms a hydrogen bond with the NH group of residue $n + 4$.

the right-handed and the left-handed helices are among allowed conformations (Figure 3.31). However, right-handed helices are energetically more favorable because there is less steric clash between the side chains and the backbone. *Essentially all α helices found in proteins are right-handed.* In schematic diagrams of proteins, α helices are depicted as twisted ribbons or rods (Figure 3.32).

Pauling and Corey predicted the structure of the α helix 6 years before it was actually seen in the x-ray reconstruction of the structure of myoglobin. *The elucidation of the structure of the α helix is a landmark in biochemistry because it demonstrated that the conformation of a polypeptide chain can be predicted if the properties of its components are rigorously and precisely known.*

The α -helical content of proteins ranges widely, from nearly none to almost 100%. For example, about 75% of the residues in ferritin, a protein that helps store iron, are in α helices (Figure 3.33). Single α helices are usually less than 45 Å long. However, two or more α helices can entwine to form a very stable structure, which can have a length of 1000 Å (100 nm, or 0.1 μ m)

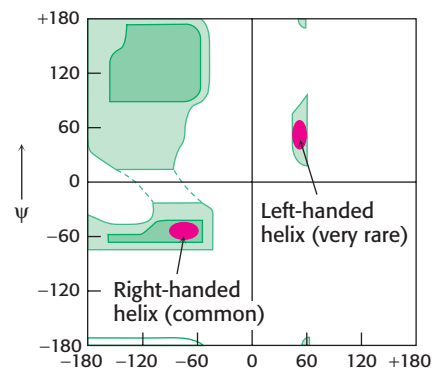


FIGURE 3.31 Ramachandran diagram for helices. Both right- and left-handed helices lie in regions of allowed conformations in the Ramachandran diagram. However, essentially all α helices in proteins are right-handed.

FIGURE 3.32 Schematic views of α helices. (A) A ball-and-stick model. (B) A ribbon depiction. (C) A cylindrical depiction.

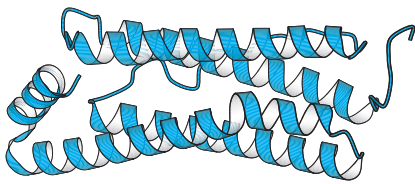
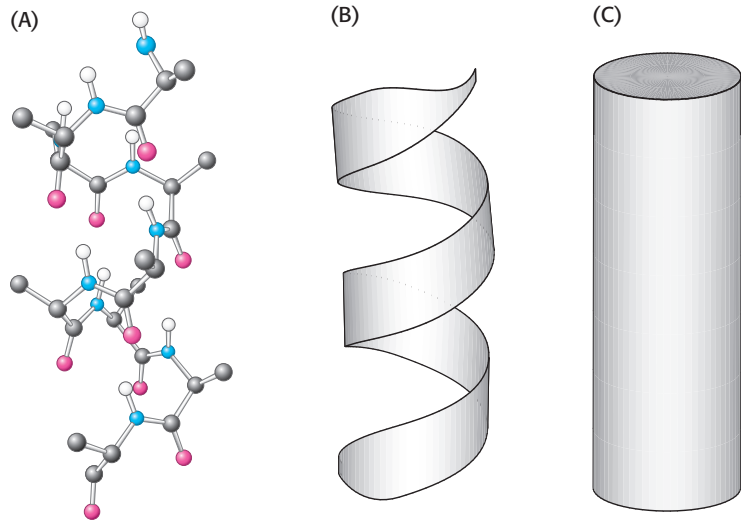


FIGURE 3.33 A largely α helical protein. Ferritin, an iron-storage protein, is built from a bundle of α helices.

or more (Figure 3.34). Such α -helical coiled coils are found in myosin and tropomyosin in muscle, in fibrin in blood clots, and in keratin in hair. The helical cables in these proteins serve a mechanical role in forming stiff bundles of fibers, as in porcupine quills. The cytoskeleton (internal scaffolding) of cells is rich in so-called intermediate filaments, which also are two-stranded α -helical coiled coils. Many proteins that span biological membranes also contain α helices.

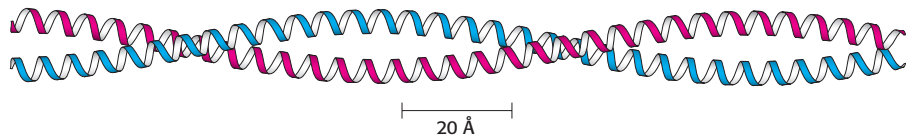


FIGURE 3.34 An α -helical coiled coil. The two helices wind around one another to form a superhelix. Such structures are found in many proteins including keratin in hair, quills, claws, and horns.

3.3.2 Beta Sheets Are Stabilized by Hydrogen Bonding Between Polypeptide Strands

Pauling and Corey discovered another periodic structural motif, which they named the β pleated sheet (β because it was the second structure that they elucidated, the α helix having been the first). The β pleated sheet (or, more simply, the β sheet) differs markedly from the rodlike α helix. A polypeptide chain, called a β strand, in a β sheet is almost fully extended rather than being tightly coiled as in the α helix. A range of extended structures are sterically allowed (Figure 3.35).

The distance between adjacent amino acids along a β strand is approximately 3.5 \AA , in contrast with a distance of 1.5 \AA along an α helix. The side chains of adjacent amino acids point in opposite directions (Figure 3.36). A β sheet is formed by linking two or more β strands by hydrogen bonds. Adjacent chains in a β sheet can run in opposite directions (antiparallel β sheet) or in the same direction (parallel β sheet). In the antiparallel arrangement, the NH group and the CO group of each amino acid are respectively hydrogen bonded to the CO group and the NH group of a partner on the adjacent chain (Figure 3.37). In the parallel arrangement, the hydrogen-bonding scheme is slightly more complicated. For each amino acid, the NH group is hydrogen

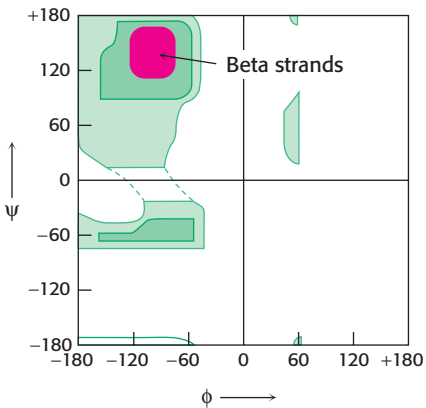


FIGURE 3.35 Ramachandran diagram for β strands. The red area shows the sterically allowed conformations of extended, β -strand-like structures.

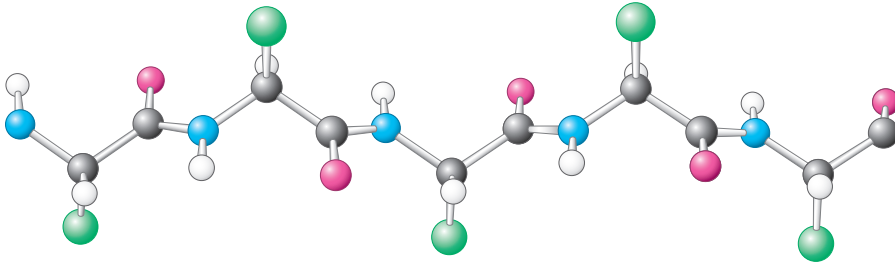


FIGURE 3.36 Structure of a β strand.
The side chains (green) are alternately above and below the plane of the strand.

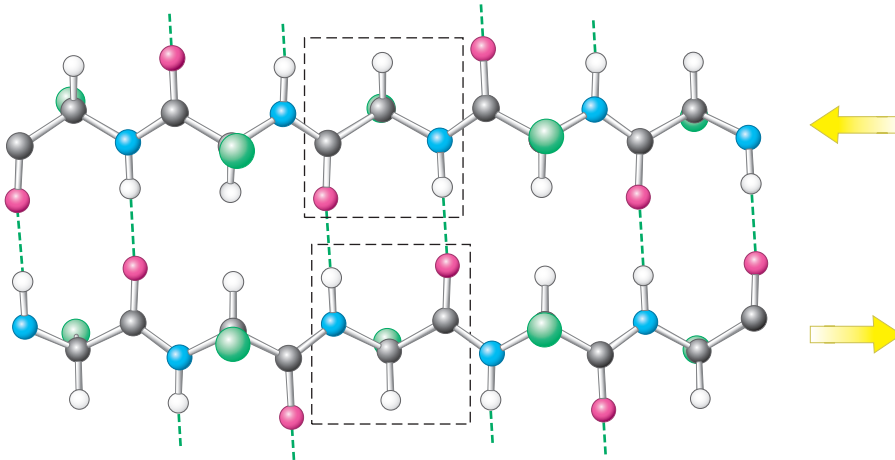


FIGURE 3.37 An antiparallel β sheet.
Adjacent β strands run in opposite directions. Hydrogen bonds between NH and CO groups connect each amino acid to a single amino acid on an adjacent strand, stabilizing the structure.

bonded to the CO group of one amino acid on the adjacent strand, whereas the CO group is hydrogen bonded to the NH group on the amino acid two residues farther along the chain (Figure 3.38). Many strands, typically 4 or 5 but as many as 10 or more, can come together in β sheets. Such β sheets can be purely antiparallel, purely parallel, or mixed (Figure 3.39).

In schematic diagrams, β strands are usually depicted by broad arrows pointing in the direction of the carboxyl-terminal end to indicate the type of β sheet formed—parallel or antiparallel. More structurally diverse than α helices, β sheets can be relatively flat but most adopt a somewhat twisted shape (Figure 3.40). The β sheet is an important structural element in many proteins. For example, fatty acid-binding proteins, important for lipid metabolism, are built almost entirely from β sheets (Figure 3.41).

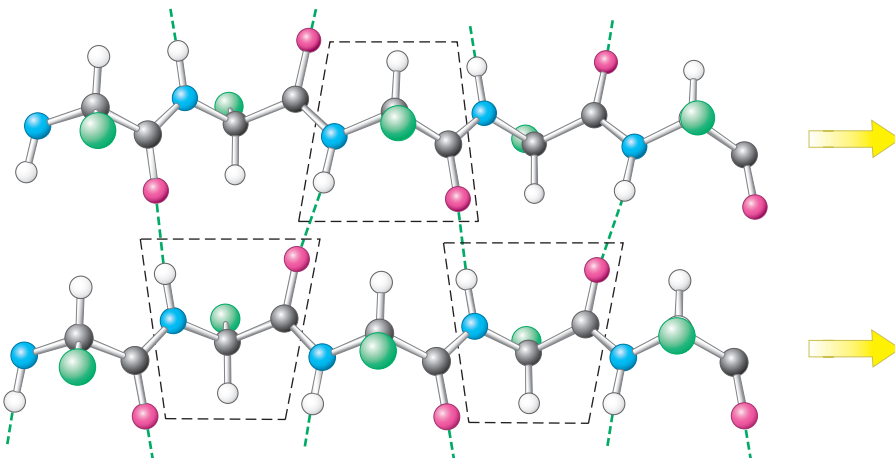


FIGURE 3.38 A parallel β sheet.
Adjacent β strands run in the same direction. Hydrogen bonds connect each amino acid on one strand with two different amino acids on the adjacent strand.

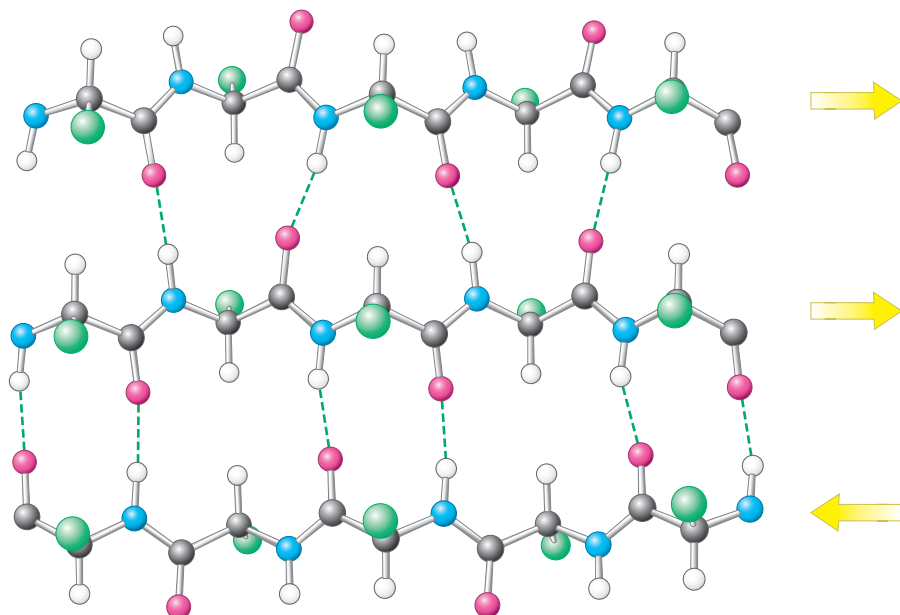


FIGURE 3.39 Structure of a mixed β sheet.

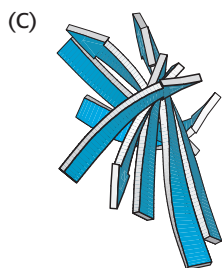
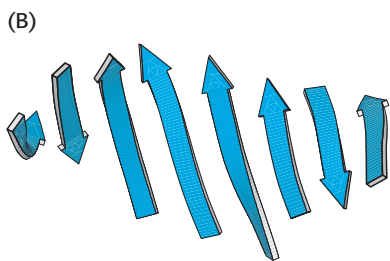
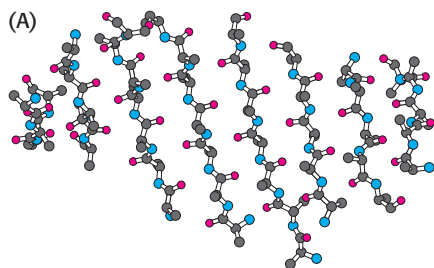


FIGURE 3.40 A twisted β sheet. (A) A ball-and-stick model. (B) A schematic model. (C) The schematic view rotated by 90 degrees to illustrate the twist more clearly.

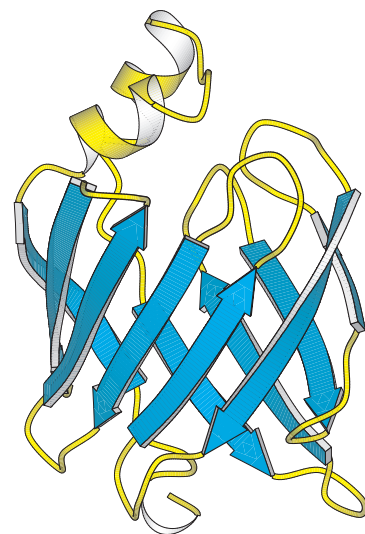


FIGURE 3.41 A protein rich in β sheets. The structure of a fatty acid-binding protein.

3.3.3 Polypeptide Chains Can Change Direction by Making Reverse Turns and Loops

Most proteins have compact, globular shapes, requiring reversals in the direction of their polypeptide chains. Many of these reversals are accomplished by a common structural element called the *reverse turn* (also known as the β turn or *hairpin bend*), illustrated in Figure 3.42. In many reverse turns, the CO group of residue i of a polypeptide is hydrogen bonded to the NH group of residue $i + 3$. This interaction stabilizes abrupt changes in direction of the polypeptide chain. In other cases, more elaborate structures are responsible for chain reversals. These structures are called *loops* or sometimes Ω loops (omega loops) to suggest their overall shape. Unlike α helices and β strands, loops do not have regular, periodic structures. Nonetheless, loop structures are often rigid and well defined (Figure 3.43). Turns and loops invariably lie on the surfaces of proteins and thus often participate in

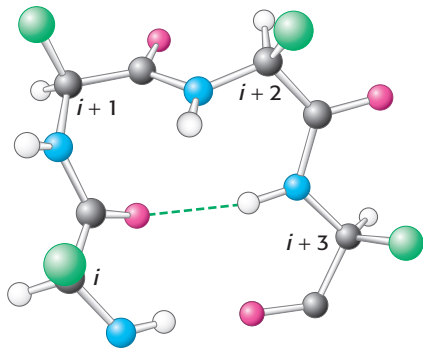


FIGURE 3.42 Structure of a reverse turn. The CO group of residue i of the polypeptide chain is hydrogen bonded to the NH group of residue $i + 3$ to stabilize the turn.

interactions between proteins and other molecules. The distribution of α helices, β strands, and turns along a protein chain is often referred to as its *secondary structure*.

3.4 TERTIARY STRUCTURE: WATER-SOLUBLE PROTEINS FOLD INTO COMPACT STRUCTURES WITH NONPOLAR CORES

Let us now examine how amino acids are grouped together in a complete protein. X-ray crystallographic and nuclear magnetic resonance studies (Section 4.5) have revealed the detailed three-dimensional structures of thousands of proteins. We begin here with a preview of *myoglobin*, the first protein to be seen in atomic detail.

Myoglobin, the oxygen carrier in muscle, is a single polypeptide chain of 153 amino acids (see also Chapters 7 and 10). The capacity of myoglobin to bind oxygen depends on the presence of *heme*, a nonpolypeptide *prosthetic (helper) group* consisting of protoporphyrin IX and a central iron atom. *Myoglobin is an extremely compact molecule*. Its overall dimensions are $45 \times 35 \times 25 \text{ \AA}$, an order of magnitude less than if it were fully stretched out (Figure 3.44).

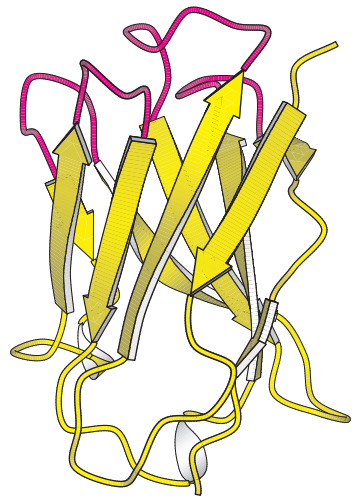


FIGURE 3.43 Loops on a protein surface. A part of an antibody molecule has surface loops (shown in red) that mediate interactions with other molecules.

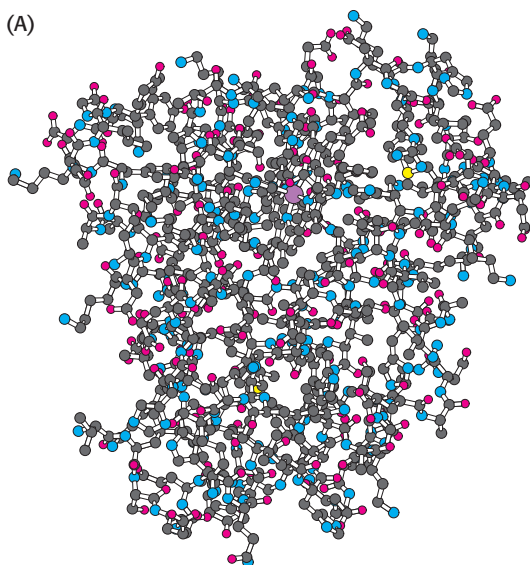
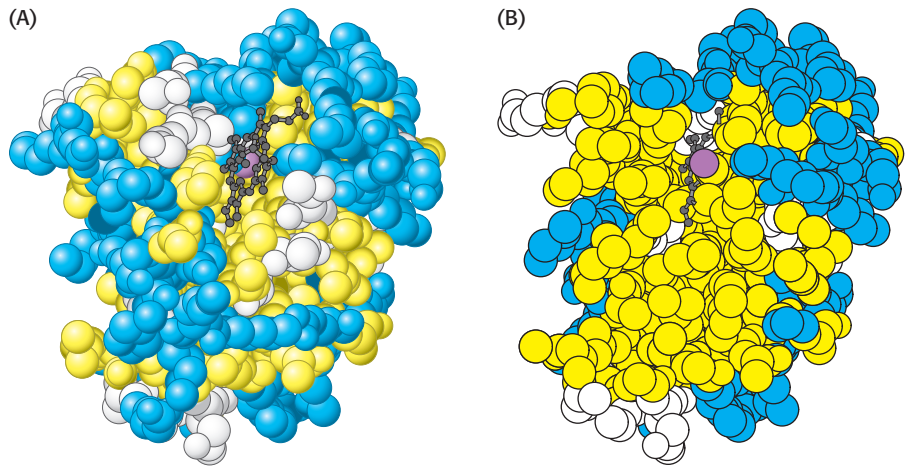


FIGURE 3.44 Three-dimensional structure of myoglobin. (A) This ball-and-stick model shows all nonhydrogen atoms and reveals many interactions between the amino acids. (B) A schematic view shows that the protein consists largely of α helices. The heme group is shown in black and the iron atom is shown as a purple sphere.



FIGURE 3.45 Distribution of amino acids in myoglobin. (A) A space-filling model of myoglobin with hydrophobic amino acids shown in yellow, charged amino acids shown in blue, and others shown in white. The surface of the molecule has many charged amino acids, as well as some hydrophobic amino acids. (B) A cross-sectional view shows that mostly hydrophobic amino acids are found on the inside of the structure, whereas the charged amino acids are found on the protein surface.



About 70% of the main chain is folded into eight α helices, and much of the rest of the chain forms turns and loops between helices.

The folding of the main chain of myoglobin, like that of most other proteins, is complex and devoid of symmetry. The overall course of the polypeptide chain of a protein is referred to as its *tertiary structure*. A unifying principle emerges from the distribution of side chains. The striking fact is that *the interior consists almost entirely of nonpolar residues* such as leucine, valine, methionine, and phenylalanine (Figure 3.45). Charged residues such as aspartate, glutamate, lysine, and arginine are absent from the inside of myoglobin. The only polar residues inside are two histidine residues, which play critical roles in binding iron and oxygen. The outside of myoglobin, on the other hand, consists of both polar and nonpolar residues. The space-filling model shows that there is very little empty space inside.

This contrasting distribution of polar and nonpolar residues reveals a key facet of protein architecture. In an aqueous environment, protein folding is driven by the strong tendency of hydrophobic residues to be excluded from water (see Section 1.3.3). Recall that a system is more thermodynamically stable when hydrophobic groups are clustered rather than extended into the aqueous surroundings. *The polypeptide chain therefore folds so that its hydrophobic side chains are buried and its polar, charged chains are on the surface.* Many α helices and β strands are amphipathic; that is, the α helix or β strand has a hydrophobic face, which points into the protein interior, and a more polar face, which points into solution. The fate of the main chain accompanying the hydrophobic side chains is important, too. An unpaired peptide NH or CO group markedly prefers water to a nonpolar milieu. The secret of burying a segment of main chain in a hydrophobic environment is pairing all the NH and CO groups by hydrogen bonding. This pairing is neatly accomplished in an α helix or β sheet. Van der Waals interactions between tightly packed hydrocarbon side chains also contribute to the stability of proteins. We can now understand why the set of 20 amino acids contains several that differ subtly in size and shape. They provide a palette from which to choose to fill the interior of a protein neatly and thereby maximize van der Waals interactions, which require intimate contact.

Some proteins that span biological membranes are “the exceptions that prove the rule” regarding the distribution of hydrophobic and hydrophilic amino acids throughout three-dimensional structures. For example, consider porins, proteins found in the outer membranes of many bacteria (Figure 3.46). The permeability barriers of membranes are built largely of alkane chains that are quite hydrophobic (Section 12.2.1). Thus, porins are

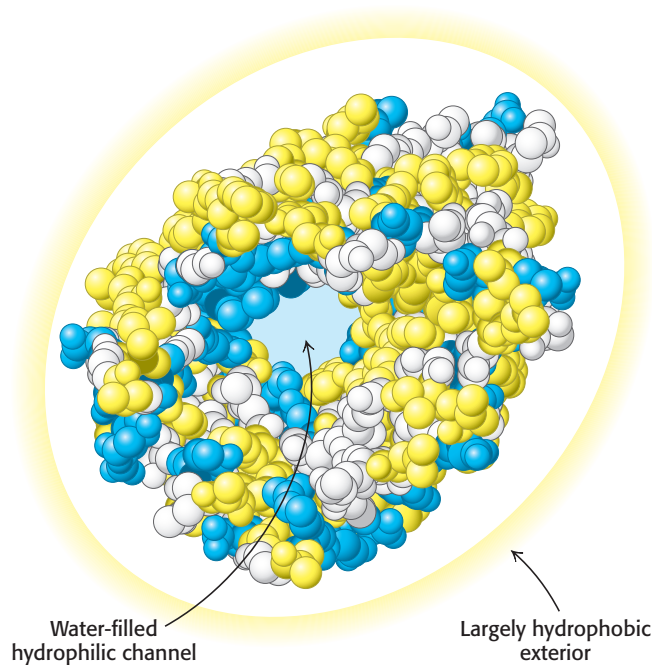


FIGURE 3.46 “Inside out” amino acid distribution in porin. The outside of porin (which contacts hydrophobic groups in membranes) is covered largely with hydrophobic residues, whereas the center includes a water-filled channel lined with charged and polar amino acids.

covered on the outside largely with hydrophobic residues that interact with the neighboring alkane chains. In contrast, the center of the protein contains many charged and polar amino acids that surround a water-filled channel running through the middle of the protein. Thus, because porins function in hydrophobic environments, they are “inside out” relative to proteins that function in aqueous solution.

Some polypeptide chains fold into two or more compact regions that may be connected by a flexible segment of polypeptide chain, rather like pearls on a string. These compact globular units, called *domains*, range in size from about 30 to 400 amino acid residues. For example, the extracellular part of CD4, the cell-surface protein on certain cells of the immune system to which the human immunodeficiency virus (HIV) attaches itself, comprises four similar domains of approximately 100 amino acids each (Figure 3.47). Often, proteins are found to have domains in common even if their overall tertiary structures are different.

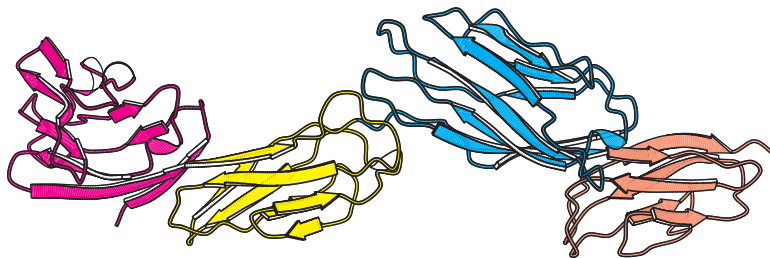


FIGURE 3.47 Protein domains. The cell-surface protein CD4 consists of four similar domains.

3.5 QUATERNARY STRUCTURE: POLYPEPTIDE CHAINS CAN ASSEMBLE INTO MULTISUBUNIT STRUCTURES

Four levels of structure are frequently cited in discussions of protein architecture. So far, we have considered three of them. *Primary structure* is the amino acid sequence. *Secondary structure* refers to the spatial arrangement of amino acid residues that are nearby in the sequence. Some of these arrangements are of a regular kind, giving rise to a periodic structure. The α helix and β strand are elements of secondary structure. *Tertiary structure*

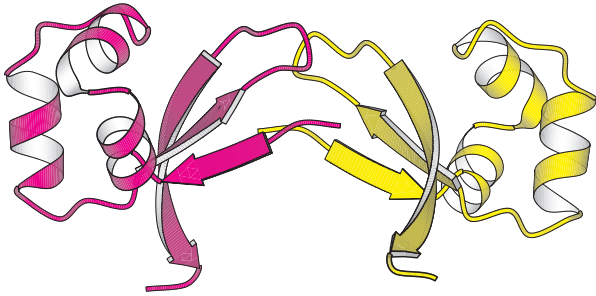


FIGURE 3.48 Quaternary structure. The Cro protein of bacteriophage λ is a dimer of identical subunits.

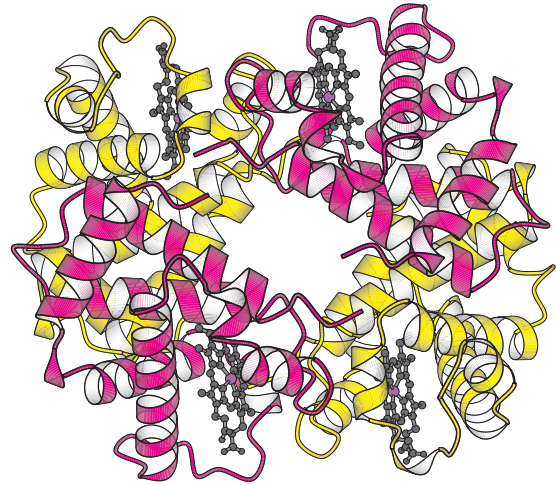


FIGURE 3.49 The $\alpha_2\beta_2$ tetramer of human hemoglobin. The structure of the two identical α subunits (red) is similar to but not identical with that of the two identical β subunits (yellow). The molecule contains four heme groups (black with the iron atom shown in purple).

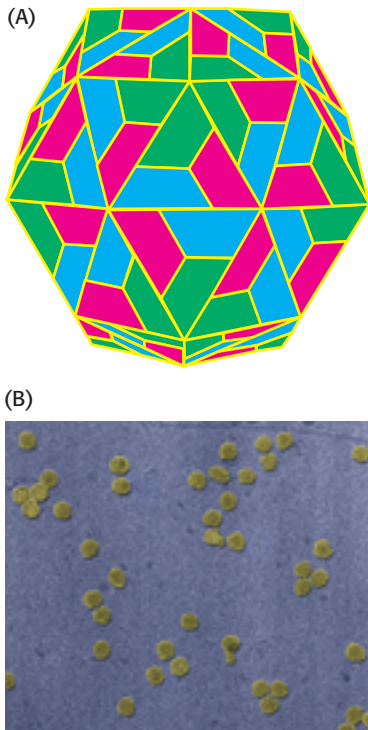


FIGURE 3.50 Complex quaternary structure. The coat of rhinovirus comprises 60 copies of each of four subunits. (A) A schematic view depicting the three types of subunits (shown in red, blue, and green) visible from outside the virus. (B) An electron micrograph showing rhinovirus particles. [Courtesy of Norm Olson, Dept. of Biological Sciences, Purdue University.]

refers to the spatial arrangement of amino acid residues that are far apart in the sequence and to the pattern of disulfide bonds. We now turn to proteins containing more than one polypeptide chain. Such proteins exhibit a fourth level of structural organization. Each polypeptide chain in such a protein is called a *subunit*. *Quaternary structure* refers to the spatial arrangement of subunits and the nature of their interactions. The simplest sort of quaternary structure is a *dimer*, consisting of two identical subunits. This organization is present in the DNA-binding protein Cro found in a bacterial virus called λ (Figure 3.48). More complicated quaternary structures also are common. More than one type of subunit can be present, often in variable numbers. For example, human hemoglobin, the oxygen-carrying protein in blood, consists of two subunits of one type (designated α) and two subunits of another type (designated β), as illustrated in Figure 3.49. Thus, the hemoglobin molecule exists as an $\alpha_2\beta_2$ tetramer. Subtle changes in the arrangement of subunits within the hemoglobin molecule allow it to carry oxygen from the lungs to tissues with great efficiency (Section 10.4.2).

Viruses make the most of a limited amount of genetic information by forming coats that use the same kind of subunit repetitively in a symmetric array. The coat of rhinovirus, the virus that causes the common cold, includes 60 copies each of four subunits (Figure 3.50). The subunits come together to form a nearly spherical shell that encloses the viral genome.

3.6 THE AMINO ACID SEQUENCE OF A PROTEIN DETERMINES ITS THREE-DIMENSIONAL STRUCTURE

How is the elaborate three-dimensional structure of proteins attained, and how is the three-dimensional structure related to the one-dimensional amino acid sequence information? The classic work of Christian Anfinsen in the 1950s on the enzyme ribonuclease revealed the relation between the amino acid sequence of a protein and its conformation. Ribonuclease is a single polypeptide chain consisting of 124 amino acid residues cross-linked by four disulfide bonds (Figure 3.51). Anfinsen's plan was to destroy the

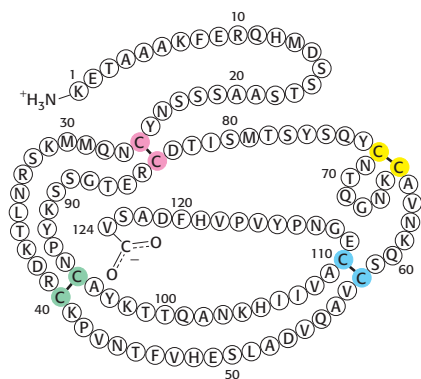


FIGURE 3.51 Amino acid sequence of bovine ribonuclease. The four disulfide bonds are shown in color. [After C. H. W. Hirs, S. Moore, and W. H. Stein, *J. Biol. Chem.* 235 (1960):633.]

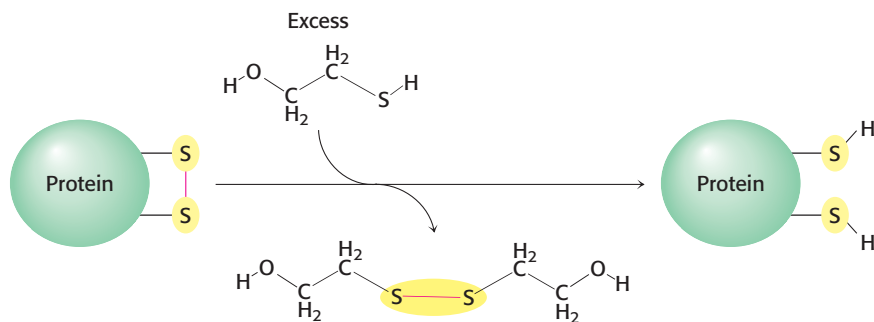


FIGURE 3.52 Role of β -mercaptoethanol in reducing disulfide bonds. Note that, as the disulfides are reduced, the β -mercaptoethanol is oxidized and forms dimers.

three-dimensional structure of the enzyme and to then determine what conditions were required to restore the structure.

Agents such as urea or guanidinium chloride effectively disrupt the non-covalent bonds, although the mechanism of action of these agents is not fully understood. The disulfide bonds can be cleaved reversibly by reducing them with a reagent such as β -mercaptoethanol (Figure 3.52). In the presence of a large excess of β -mercaptoethanol, a protein is produced in which the disulfides (cystines) are fully converted into sulfhydryls (cysteines).

Most polypeptide chains devoid of cross-links assume a *random-coil conformation* in 8 M urea or 6 M guanidinium chloride, as evidenced by physical properties such as viscosity and optical activity. When ribonuclease was treated with β -mercaptoethanol in 8 M urea, the product was a fully reduced, randomly coiled polypeptide chain *devoid of enzymatic activity*. In other words, ribonuclease was *denatured* by this treatment (Figure 3.53).

Anfinsen then made the critical observation that the denatured ribonuclease, freed of urea and β -mercaptoethanol by dialysis, slowly regained enzymatic activity. He immediately perceived the significance of this chance finding: the sulfhydryl groups of the denatured enzyme became oxidized by air, and the enzyme spontaneously refolded into a catalytically active form. Detailed studies then showed that nearly all the original enzymatic activity was regained if the sulfhydryl groups were oxidized under suitable conditions. All the measured physical and chemical properties of the refolded enzyme were virtually identical with those of the native enzyme. These experiments showed that *the information needed to specify the catalytically active structure of ribonuclease is contained in its amino acid sequence*. Subsequent studies have established the generality of this central principle of biochemistry: *sequence specifies conformation*. The dependence of conformation on sequence is especially significant because of the intimate connection between conformation and function.

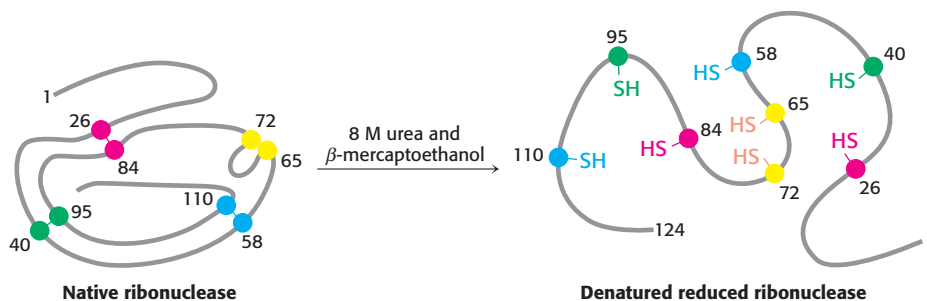
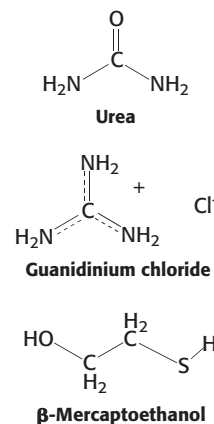


FIGURE 3.53 Reduction and denaturation of ribonuclease.

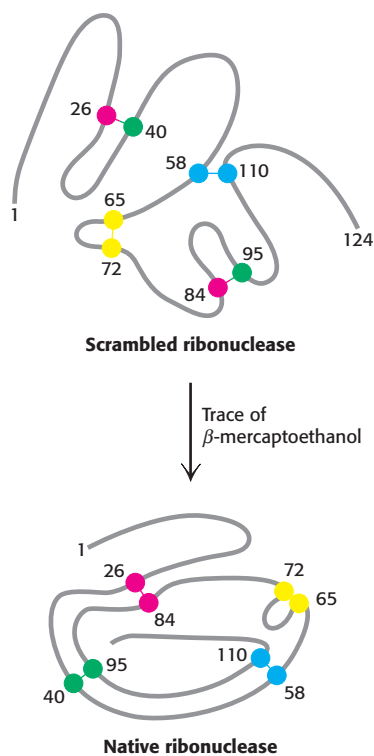


FIGURE 3.54 Reestablishing correct disulfide pairing. Native ribonuclease can be reformed from scrambled ribonuclease in the presence of a trace of β -mercaptoethanol.

A quite different result was obtained when reduced ribonuclease was reoxidized while it was still in 8 M urea and the preparation was then dialyzed to remove the urea. Ribonuclease reoxidized in this way had only 1% of the enzymatic activity of the native protein. Why were the outcomes so different when reduced ribonuclease was reoxidized in the presence and absence of urea? The reason is that the wrong disulfides formed pairs in urea. There are 105 different ways of pairing eight cysteine molecules to form four disulfides; only one of these combinations is enzymatically active. The 104 wrong pairings have been picturesquely termed “scrambled” ribonuclease. Anfinsen found that scrambled ribonuclease spontaneously converted into fully active, native ribonuclease when trace amounts of β -mercaptoethanol were added to an aqueous solution of the protein (Figure 3.54). The added β -mercaptoethanol catalyzed the rearrangement of disulfide pairings until the native structure was regained in about 10 hours. *This process was driven by the decrease in free energy as the scrambled conformations were converted into the stable, native conformation of the enzyme.* The native disulfide pairings of ribonuclease thus contribute to the stabilization of the thermodynamically preferred structure.

Similar refolding experiments have been performed on many other proteins. In many cases, the native structure can be generated under suitable conditions. For other proteins, however, refolding does not proceed efficiently. In these cases, the unfolded protein molecules usually become tangled up with one another to form aggregates. Inside cells, proteins called *chaperones* block such illicit interactions (Sections 11.36 and 29.x.x).

3.6.1 Amino Acids Have Different Propensities for Forming Alpha Helices, Beta Sheets, and Beta Turns

How does the amino acid sequence of a protein specify its three-dimensional structure? How does an unfolded polypeptide chain acquire the form of the native protein? These fundamental questions in biochemistry can be approached by first asking a simpler one: What determines whether a particular sequence in a protein forms an α helix, a β strand, or a turn? Examining the frequency of occurrence of particular amino acid residues in these secondary structures (Table 3.3) can be a source of insight into this determination. Residues such as alanine, glutamate, and leucine tend to be present in α helices, whereas valine and isoleucine tend to be present in β strands. Glycine, asparagine, and proline have a propensity for being in turns.

The results of studies of proteins and synthetic peptides have revealed some reasons for these preferences. The α helix can be regarded as the default conformation. Branching at the β -carbon atom, as in valine, threonine, and isoleucine, tends to destabilize α helices because of steric clashes. These residues are readily accommodated in β strands, in which their side chains project out of the plane containing the main chain. Serine, aspartate, and asparagine tend to disrupt α helices because their side chains contain hydrogen-bond donors or acceptors in close proximity to the main chain, where they compete for main-chain NH and CO groups. Proline tends to disrupt both α helices and β strands because it lacks an NH group and because its ring structure restricts its ϕ value to near -60 degrees. Glycine readily fits into all structures and for that reason does not favor helix formation in particular.

Can one predict the secondary structure of proteins by using this knowledge of the conformational preferences of amino acid residues? Predictions of secondary structure adopted by a stretch of six or fewer residues have proved to be about 60 to 70% accurate. What stands in the way of more accurate prediction? Note that the conformational preferences of amino acid residues are not tipped all the way to one structure (see Table 3.3). For


TABLE 3.3 Relative frequencies of amino acid residues in secondary structures

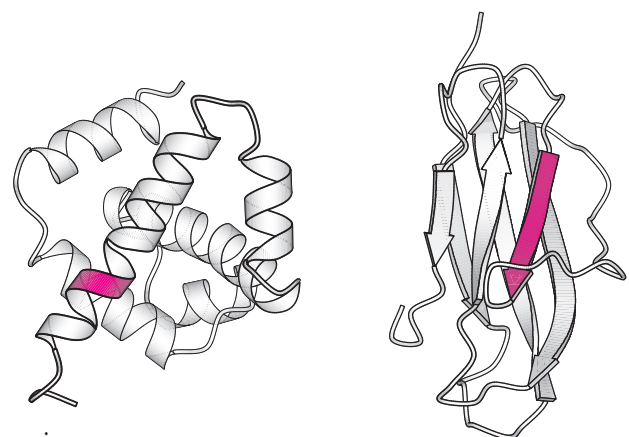
Amino acid	α helix	β sheet	Turn
Ala	1.29	0.90	0.78
Cys	1.11	0.74	0.80
Leu	1.30	1.02	0.59
Met	1.47	0.97	0.39
Glu	1.44	0.75	1.00
Gln	1.27	0.80	0.97
His	1.22	1.08	0.69
Lys	1.23	0.77	0.96
Val	0.91	1.49	0.47
Ile	0.97	1.45	0.51
Phe	1.07	1.32	0.58
Tyr	0.72	1.25	1.05
Trp	0.99	1.14	0.75
Thr	0.82	1.21	1.03
Gly	0.56	0.92	1.64
Ser	0.82	0.95	1.33
Asp	1.04	0.72	1.41
Asn	0.90	0.76	1.28
Pro	0.52	0.64	1.91
Arg	0.96	0.99	0.88


Note: The amino acids are grouped according to their preference for α helices (top group), β sheets (second group), or turns (third group). Arginine shows no significant preference for any of the structures.

After T. E. Creighton, *Proteins: Structures and Molecular Properties*, 2d ed. (W. H. Freeman and Company, 1992), p. 256.

example, glutamate, one of the strongest helix formers, prefers α helix to β strand by only a factor of two. The preference ratios of most other residues are smaller. Indeed, some penta- and hexapeptide sequences have been found to adopt one structure in one protein and an entirely different structure in another (Figure 3.55). Hence, some amino acid sequences do not uniquely determine secondary structure. Tertiary interactions—interactions between residues that are far apart in the sequence—may be decisive in specifying the secondary structure of some segments. *The context is often crucial in determining the conformational outcome.* The conformation of a protein evolved to work in a particular environment or context.

 Pathological conditions can result if a protein assumes an inappropriate conformation for the context. Striking examples are *prion diseases*, such as Creutzfeldt-Jacob disease, kuru, and mad cow disease. These conditions result when a brain protein called a prion converts from its normal conformation (designated PrP^C) to an altered one (PrP^{SC}). This conversion is self-propagating, leading to large aggregates of PrP^{SC}. The role of these aggregates in the generation of the pathological conditions is not yet understood.



 **FIGURE 3.55** Alternative conformations of a peptide sequence. Many sequences can adopt alternative conformations in different proteins. Here the sequence VDLLKN shown in red assumes an α helix in one protein context (left) and a β strand in another (right).

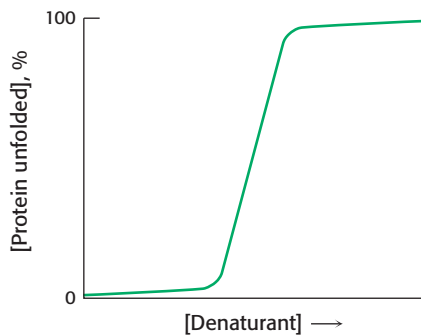


FIGURE 3.56 Transition from folded to unfolded state. Most proteins show a sharp transition from the folded to unfolded form on treatment with increasing concentrations of denaturants.

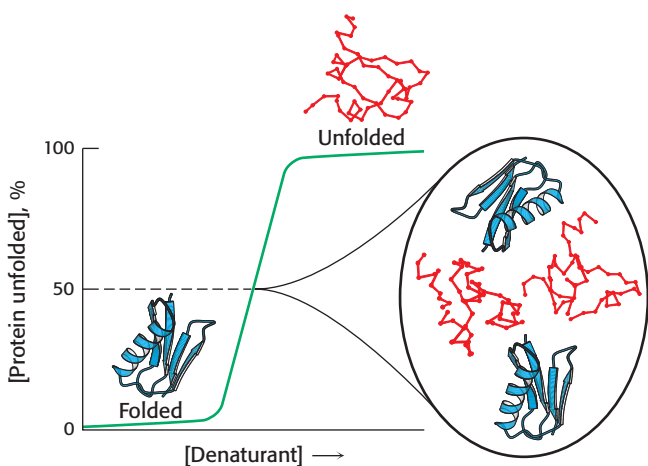


FIGURE 3.57 Components of a partially denatured protein solution. In a half-unfolded protein solution, half the molecules are fully folded and half are fully unfolded.

3.6.2 Protein Folding Is a Highly Cooperative Process

As stated earlier, proteins can be denatured by heat or by chemical denaturants such as urea or guanidium chloride. For many proteins, a comparison of the degree of unfolding as the concentration of denaturant increases has revealed a relatively sharp transition from the folded, or native, form to the unfolded, or denatured, form, suggesting that only these two conformational states are present to any significant extent (Figure 3.56). A similar sharp transition is observed if one starts with unfolded proteins and removes the denaturants, allowing the proteins to fold.

Protein folding and unfolding is thus largely an “*all or none*” process that results from a *cooperative transition*. For example, suppose that a protein is placed in conditions under which some part of the protein structure is thermodynamically unstable. As this part of the folded structure is disrupted, the interactions between it and the remainder of the protein will be lost. The loss of these interactions, in turn, will destabilize the remainder of the structure. Thus, conditions that lead to the disruption of any part of a protein structure are likely to unravel the protein completely. The structural properties of proteins provide a clear rationale for the cooperative transition.

The consequences of cooperative folding can be illustrated by considering the contents of a protein solution under conditions corresponding to the middle of the transition between the folded and unfolded forms. Under these conditions, the protein is “half folded.” Yet the solution will contain no half-folded molecules but, instead, will be a 50/50 mixture of fully folded and fully unfolded molecules (Figure 3.57). Structures that are partly intact and partly disrupted are not thermodynamically stable and exist only transiently. Cooperative folding ensures that partly folded structures that might interfere with processes within cells do not accumulate.

3.6.3 Proteins Fold by Progressive Stabilization of Intermediates Rather Than by Random Search

The cooperative folding of proteins is a thermodynamic property; its occurrence reveals nothing about the kinetics and mechanism of protein folding. How does a protein make the transition from a diverse ensemble of unfolded structures into a unique conformation in the native form? One possibility a priori would be that all possible conformations are tried out to find the energetically most favorable one. How long would such a random search take? Consider a small protein with 100 residues. Cyrus Levinthal calculated that, if each residue can assume three different conformations, the total number of structures would be 3^{100} , which is equal to 5×10^{47} . If it takes 10^{-13} s to convert one structure into another, the total search time would be $5 \times 10^{47} \times 10^{-13}$ s, which is equal to 5×10^{34} s, or 1.6×10^{27} years. Clearly, it would take much too long for even a small protein to fold properly by randomly trying out all possible conformations. The enormous difference between calculated and actual folding times is called *Levinthal’s paradox*.

The way out of this dilemma is to recognize the power of *cumulative selection*. Richard Dawkins, in *The Blind Watchmaker*, asked how long it would take a monkey poking randomly at a typewriter to reproduce Hamlet’s remark to Polonius, “Methinks it is like a weasel” (Figure 3.58). An astronomically large number of keystrokes, of the order of 10^{40} , would be required. However, suppose that we preserved each correct character and

allowed the monkey to retype only the wrong ones. In this case, only a few thousand keystrokes, on average, would be needed. The crucial difference between these cases is that the first employs a completely random search, whereas, in the second, *partly correct intermediates are retained*.

The essence of protein folding is the retention of partly correct intermediates. However, the protein-folding problem is much more difficult than the one presented to our simian Shakespeare. First, the criterion of correctness is not a residue-by-residue scrutiny of conformation by an omniscient observer but rather the total free energy of the transient species. Second, proteins are only marginally stable. The free-energy difference between the folded and the unfolded states of a typical 100-residue protein is 10 kcal mol⁻¹ (42 kJ mol⁻¹), and thus each residue contributes on average only 0.1 kcal mol⁻¹ (0.42 kJ mol⁻¹) of energy to maintain the folded state. This amount is less than that of thermal energy, which is 0.6 kcal mol⁻¹ (2.5 kJ mol⁻¹) at room temperature. This meager stabilization energy means that correct intermediates, especially those formed early in folding, can be lost. The analogy is that the monkey would be somewhat free to undo its correct keystrokes. Nonetheless, the interactions that lead to cooperative folding can stabilize intermediates as structure builds up. Thus, local regions, which have significant structural preference, though not necessarily stable on their own, will tend to adopt their favored structures and, as they form, can interact with one other, leading to increasing stabilization.

3.6.4 Prediction of Three-Dimensional Structure from Sequence Remains a Great Challenge

The amino acid sequence completely determines the three-dimensional structure of a protein. However, the prediction of three-dimensional structure from sequence has proved to be extremely difficult. As we have seen, the local sequence appears to determine only between 60% and 70% of the secondary structure; long-range interactions are required to fix the full secondary structure and the tertiary structure.

Investigators are exploring two fundamentally different approaches to predicting three-dimensional structure from amino acid sequence. The first is *ab initio prediction*, which attempts to predict the folding of an amino acid sequence without any direct reference to other known protein structures. Computer-based calculations are employed that attempt to minimize the free energy of a structure with a given amino acid sequence or to simulate the folding process. The utility of these methods is limited by the vast number of possible conformations, the marginal stability of proteins, and the subtle energetics of weak interactions in aqueous solution. The second approach takes advantage of our growing knowledge of the three-dimensional structures of many proteins. In these *knowledge-based methods*, an amino acid sequence of unknown structure is examined for compatibility with any known protein structures. If a significant match is detected, the known structure can be used as an initial model. Knowledge-based methods have been a source of many insights into the three-dimensional conformation of proteins of known sequence but unknown structure.

3.6.5 Protein Modification and Cleavage Confer New Capabilities



Proteins are able to perform numerous functions relying solely on the versatility of their 20 amino acids. However, many proteins are covalently modified, through the attachment of groups other than amino acids, to augment their functions (Figure 3.59). For example, *acetyl groups* are attached to the amino termini of many proteins, a modification that

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400 oDr`Jh7s DFR:W4l'u+^v6zpJseOi
600 e2ih'8zs n527x8l8d_ih=Hldseb.
800 S#dh>)/s ]tZqC%lP&DK<!|^aseZ.
1000 V0th>nLs ut/isjl kwojjwMasef.
1200 juth+nvs it is[lukh?SCw=ase5.
1400 Iithdn4s it is0l/Ks/IxwLase~.
1600 M?thinr's it is lXK?T"woasel.
1800 Msthinws it is lwKN7DKw(asel.
2000 Mhthin`s it is likv,aww_asel.
2200 Mmthinns it is lik+5avwlasel.
2400 MethinXs it is likydaqw)asel.
2600 Methin4s it is lik2dasweasel.
2800 MethinHs it is like□aTweasel.
2883 Methinks it is like a weasel.

200 )z~hg)W4{ {cu!k0{d6jS!NlEyUx)p
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1000 OFthinksP)@fZO li8v] /+ElN26B
1200 6ithinksMvt -v likm+g1#K~)BFk
1400 vxthinksaEt □w like.SlGeutks.
1600 :Othinks<it MC likesN2[eaVe4.
1800 uxthinksqit Or likeQh)weaoeW.
2000 Y/thinks it id like7alwea)e&.
2200 Methinks it iw like a(weaWel.
2400 Methinks it is like a;weasel.
2431 Methinks it is like a weasel.

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FIGURE 3.58 Typing monkey analogy. A monkey randomly poking a typewriter could write a line from Shakespeare's *Hamlet*, provided that correct keystrokes were retained. In the two computer simulations shown, the cumulative number of keystrokes is given at the left of each line.

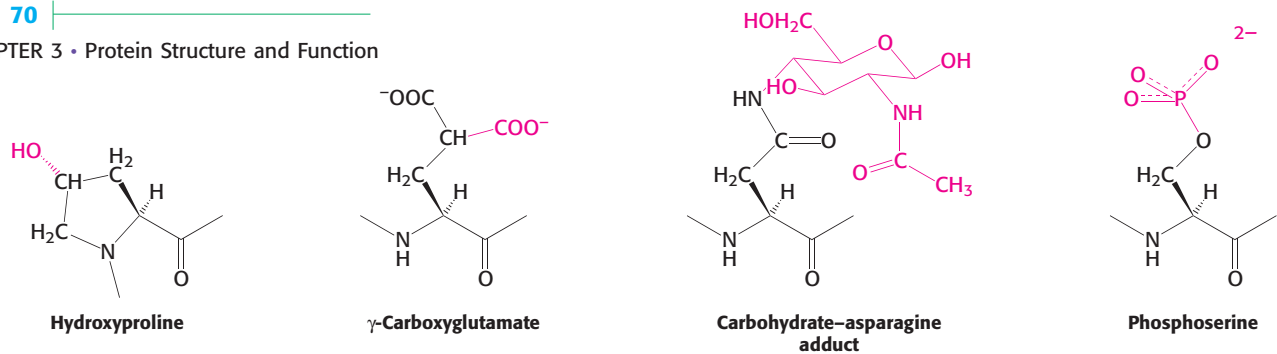


FIGURE 3.59 Finishing touches. Some common and important covalent modifications of amino acid side chains are shown.

makes these proteins more resistant to degradation. The addition of *hydroxyl groups* to many proline residues stabilizes fibers of newly synthesized collagen, a fibrous protein found in connective tissue and bone. The biological significance of this modification is evident in the disease scurvy: a deficiency of vitamin C results in insufficient hydroxylation of collagen and the abnormal collagen fibers that result are unable to maintain normal tissue strength. Another specialized amino acid produced by a finishing touch is *γ -carboxyglutamate*. In vitamin K deficiency, insufficient carboxylation of glutamate in prothrombin, a clotting protein, can lead to hemorrhage. Many proteins, especially those that are present on the surfaces of cells or are secreted, acquire *carbohydrate units* on specific asparagine residues. The addition of sugars makes the proteins more hydrophilic and able to participate in interactions with other proteins. Conversely, the addition of a *fatty acid* to an α -amino group or a cysteine sulfhydryl group produces a more hydrophobic protein.

Many hormones, such as epinephrine (adrenaline), alter the activities of enzymes by stimulating the phosphorylation of the hydroxyl amino acids serine and threonine; *phosphoserine* and *phosphothreonine* are the most ubiquitous modified amino acids in proteins. Growth factors such as insulin act by triggering the phosphorylation of the hydroxyl group of tyrosine residues to form *phosphotyrosine*. The phosphoryl groups on these three modified amino acids are readily removed; thus they are able to act as reversible switches in regulating cellular processes. The roles of phosphorylation in signal transduction will be discussed extensively in Chapter 15.

The preceding modifications consist of the addition of special groups to amino acids. Other special groups are generated by chemical rearrangements of side chains and, sometimes, the peptide backbone. For example, certain jellyfish produce a fluorescent green protein (Figure 3.60). The source of the fluorescence is a group formed by the spontaneous rearrangement and oxidation of the sequence Ser-Tyr-Gly within the center of the protein. This protein is of great utility to researchers as a marker within cells (Section 4.3.5).

Finally, many proteins are cleaved and trimmed after synthesis. For example, digestive enzymes are synthesized as inactive precursors that can be stored safely in the pancreas. After release into the intestine, these precursors become activated by peptide-bond cleavage. In blood clotting, peptide-bond cleavage converts soluble fibrinogen into insoluble fibrin. A number of polypeptide hormones, such as adrenocorticotrophic hormone, arise from the splitting of a single large precursor protein. Likewise, many virus proteins are produced by the cleavage of large polyprotein precursors. We shall encounter many more examples of modification and cleavage as essential features of protein formation and function. Indeed, these finishing touches account for much of the versatility, precision, and elegance of protein action and regulation.

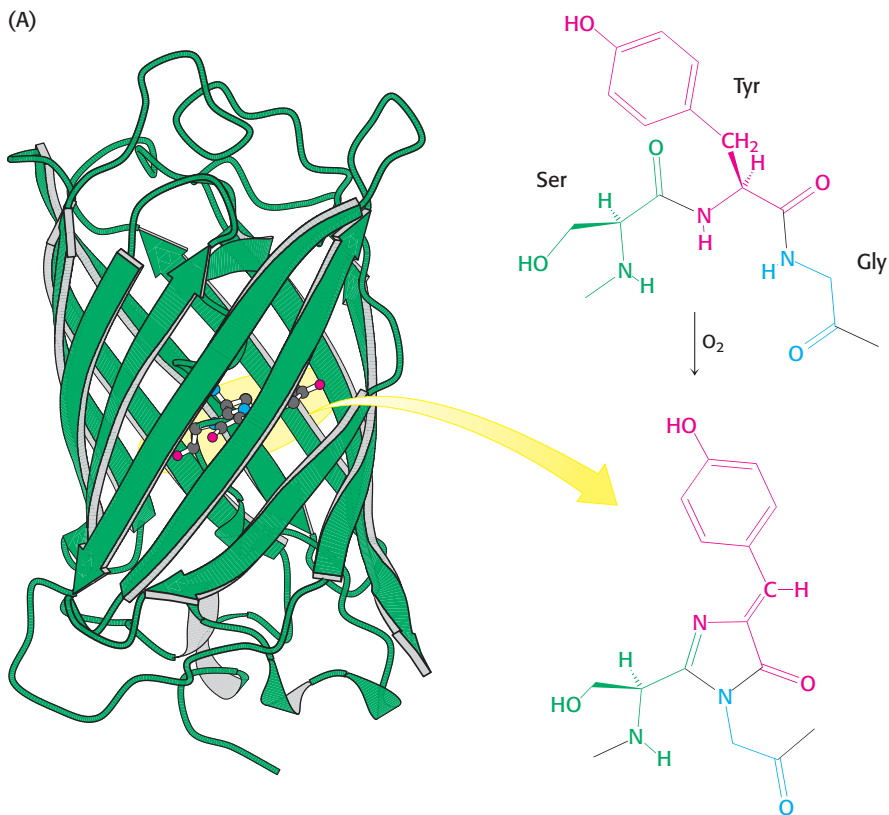


FIGURE 3.60 Chemical rearrangement in GFP. (A) The structure of green fluorescent protein (GFP). The rearrangement and oxidation of the sequence Ser-Tyr-Gly is the source of fluorescence. (B) Fluorescence micrograph of a four-cell embryo (cells are outlined) from the roundworm *C. elegans* containing a protein, PIE-1, labeled with GFP. The protein is expressed only in the cell (top) that will give rise to the germline. [(B) Courtesy of Geraldine Seydoux.]

SUMMARY

- Proteins are the workhorses of biochemistry, participating in essentially all cellular processes. Protein structure can be described at four levels. The primary structure refers to the amino acid sequence. The secondary structure refers to the conformation adopted by local regions of the polypeptide chain. Tertiary structure describes the overall folding of the polypeptide chain. Finally, quaternary structure refers to the specific association of multiple polypeptide chains to form multisubunit complexes.
- Proteins Are Built from a Repertoire of 20 Amino Acids**
Proteins are linear polymers of amino acids. Each amino acid consists of a central tetrahedral carbon atom linked to an amino group, a carboxylic acid group, a distinctive side chain, and a hydrogen. These tetrahedral centers, with the exception of that of glycine, are chiral; only the L isomer exists in natural proteins. All natural proteins are constructed from the same set of 20 amino acids. The side chains of these 20 building blocks vary tremendously in size, shape, and the presence of functional groups. They can be grouped as follows: (1) aliphatic side chains—glycine, alanine, valine, leucine, isoleucine, methionine, and proline; (2) aromatic side chains—phenylalanine, tyrosine, and tryptophan; (3) hydroxyl-containing aliphatic side chains—serine and threonine; (4) sulfhydryl-containing cysteine; (5) basic side chains—lysine, arginine, and histidine; (6) acidic side chains—aspartic acid and glutamic acid; and (7) carboxamide-containing side chains—asparagine and glutamine. These groupings are somewhat arbitrary and many other sensible groupings are possible.

- **Primary Structure: Amino Acids Are Linked by Peptide Bonds to Form Polypeptide Chains**

The amino acids in a polypeptide are linked by amide bonds formed between the carboxyl group of one amino acid and the amino group of the next. This linkage, called a peptide bond, has several important properties. First, it is resistant to hydrolysis so that proteins are remarkably stable kinetically. Second, the peptide group is planar because the C–N bond has considerable double-bond character. Third, each peptide bond has both a hydrogen-bond donor (the NH group) and a hydrogen-bond acceptor (the CO group). Hydrogen bonding between these backbone groups is a distinctive feature of protein structure. Finally, the peptide bond is uncharged, which allows proteins to form tightly packed globular structures having significant amounts of the backbone buried within the protein interior. Because they are linear polymers, proteins can be described as sequences of amino acids. Such sequences are written from the amino to the carboxyl terminus.

- **Secondary Structure: Polypeptide Chains Can Fold into Regular Structures Such as the Alpha Helix, the Beta Sheet, and Turns and Loops**

Two major elements of secondary structure are the α helix and the β strand. In the α helix, the polypeptide chain twists into a tightly packed rod. Within the helix, the CO group of each amino acid is hydrogen bonded to the NH group of the amino acid four residues along the polypeptide chain. In the β strand, the polypeptide chain is nearly fully extended. Two or more β strands connected by NH-to-CO hydrogen bonds come together to form β sheets.

- **Tertiary Structure: Water-Soluble Proteins Fold into Compact Structures with Nonpolar Cores**

The compact, asymmetric structure that individual polypeptides attain is called tertiary structure. The tertiary structures of water-soluble proteins have features in common: (1) an interior formed of amino acids with hydrophobic side chains and (2) a surface formed largely of hydrophilic amino acids that interact with the aqueous environment. The driving force for the formation of the tertiary structure of water-soluble proteins is the hydrophobic interactions between the interior residues. Some proteins that exist in a hydrophobic environment, in membranes, display the inverse distribution of hydrophobic and hydrophilic amino acids. In these proteins, the hydrophobic amino acids are on the surface to interact with the environment, whereas the hydrophilic groups are shielded from the environment in the interior of the protein.

- **Quaternary Structure: Polypeptide Chains Can Assemble into Multisubunit Structures**

Proteins consisting of more than one polypeptide chain display quaternary structure, and each individual polypeptide chain is called a subunit. Quaternary structure can be as simple as two identical subunits or as complex as dozens of different subunits. In most cases, the subunits are held together by noncovalent bonds.

- **The Amino Acid Sequence of a Protein Determines Its Three-Dimensional Structure**

The amino acid sequence completely determines the three-dimensional structure and, hence, all other properties of a protein. Some proteins can be unfolded completely yet refold efficiently when placed under conditions in which the folded form of the protein is stable. The amino acid sequence of a protein is determined by the sequences of bases in a DNA molecule. This one-dimensional sequence information is extended into

the three-dimensional world by the ability of proteins to fold spontaneously. Protein folding is a highly cooperative process; structural intermediates between the unfolded and folded forms do not accumulate.

The versatility of proteins is further enhanced by covalent modifications. Such modifications can incorporate functional groups not present in the 20 amino acids. Other modifications are important to the regulation of protein activity. Through their structural stability, diversity, and chemical reactivity, proteins make possible most of the key processes associated with life.

APPENDIX: ACID-BASE CONCEPTS

Ionization of Water

Water dissociates into hydronium (H_3O^+) and hydroxyl (OH^-) ions. For simplicity, we refer to the hydronium ion as a hydrogen ion (H^+) and write the equilibrium as



The equilibrium constant K_{eq} of this dissociation is given by

$$K_{\text{eq}} = [\text{H}^+][\text{OH}^-]/[\text{H}_2\text{O}] \quad (1)$$

in which the terms in brackets denote molar concentrations. Because the concentration of water (55.5 M) is changed little by ionization, expression 1 can be simplified to give

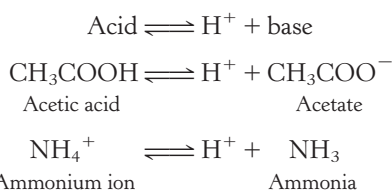
$$K_{\text{w}} = [\text{H}^+][\text{OH}^-] \quad (2)$$

in which K_{w} is the ion product of water. At 25°C, K_{w} is 1.0×10^{-14} .

Note that the concentrations of H^+ and OH^- are reciprocally related. If the concentration of H^+ is high, then the concentration of OH^- must be low, and vice versa. For example, if $[\text{H}^+] = 10^{-2}$ M, then $[\text{OH}^-] = 10^{-12}$ M.

Definition of Acid and Base

An acid is a proton donor. A base is a proton acceptor.



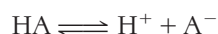
The species formed by the ionization of an acid is its conjugate base. Conversely, protonation of a base yields its conjugate acid. Acetic acid and acetate ion are a conjugate acid–base pair.

Definition of pH and pK

The pH of a solution is a measure of its concentration of H^+ . The pH is defined as

$$\text{pH} = \log_{10}(1/[\text{H}^+]) = -\log_{10}[\text{H}^+] \quad (3)$$

The ionization equilibrium of a weak acid is given by



The apparent equilibrium constant K_{a} for this ionization is

$$K_{\text{a}} = [\text{H}^+][\text{A}^-]/[\text{HA}] \quad (4)$$

The $\text{p}K_{\text{a}}$ of an acid is defined as

$$\text{p}K_{\text{a}} = -\log K_{\text{a}} = \log(1/K_{\text{a}}) \quad (5)$$

Inspection of equation 4 shows that the $\text{p}K_{\text{a}}$ of an acid is the pH at which it is half dissociated, when $[\text{A}^-] = [\text{HA}]$.

Henderson-Hasselbalch Equation

What is the relation between pH and the ratio of acid to base? A useful expression can be derived from equation 4. Rearrangement of that equation gives

$$1/[\text{H}^+] = 1/K_{\text{a}}[\text{A}^-]/[\text{HA}] \quad (6)$$

Taking the logarithm of both sides of equation 6 gives

$$\log(1/[\text{H}^+]) = \log(1/K_{\text{a}}) + \log([\text{A}^-]/[\text{HA}]) \quad (7)$$

Substituting pH for $\log 1/[\text{H}^+]$ and $\text{p}K_{\text{a}}$ for $\log 1/K_{\text{a}}$ in equation 7 yields

$$\text{pH} = \text{p}K_{\text{a}} + \log([\text{A}^-]/[\text{HA}]) \quad (8)$$

which is commonly known as the Henderson-Hasselbalch equation.

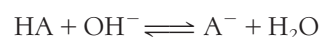
The pH of a solution can be calculated from equation 8 if the molar proportion of A^- to HA and the $\text{p}K_{\text{a}}$ of HA are known. Consider a solution of 0.1 M acetic acid and 0.2 M acetate ion. The $\text{p}K_{\text{a}}$ of acetic acid is 4.8. Hence, the pH of the solution is given by

$$\text{pH} = 4.8 + \log(0.2/0.1) = 4.8 + \log 2.0 = 4.8 + 0.3 = 5.1$$

Conversely, the $\text{p}K_{\text{a}}$ of an acid can be calculated if the molar proportion of A^- to HA and the pH of the solution are known.

Buffers

An acid–base conjugate pair (such as acetic acid and acetate ion) has an important property: it resists changes in the pH of a solution. In other words, it acts as a *buffer*. Consider the addition of OH^- to a solution of acetic acid (HA):



A plot of the dependence of the pH of this solution on the amount of OH^- added is called a *titration curve* (Figure 3.61). Note that there is an inflection point in the curve at pH 4.8, which is the $\text{p}K_{\text{a}}$ of acetic acid. In the vicinity of this pH, a relatively large amount of OH^- produces little change in pH. In other words, the buffer maintains the value of pH near a given value, despite the addition of other either protons or hydroxide

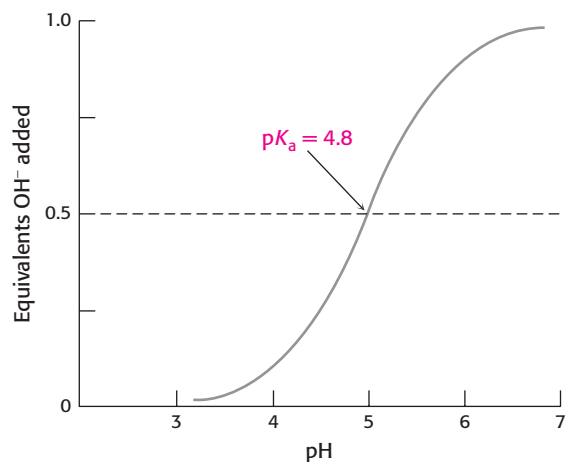


FIGURE 3.61 Titration curve of acetic acid.

ions. In general, a weak acid is most effective in buffering against pH changes in the vicinity of its pK_a value.

pK_a Values of Amino Acids

An amino acid such as glycine contains two ionizable groups: an α -carboxyl group and a protonated α -amino group. As base is added, these two groups are titrated (Figure 3.62). The pK_a of the α -COOH group is 2.4, whereas that of the α -NH₃⁺ group is 9.8. The pK_a values of these groups in other amino

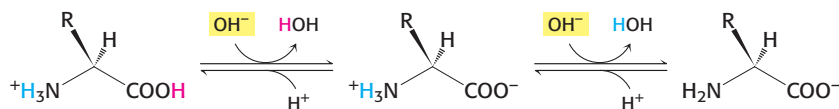


FIGURE 3.62 Titration of the α -carboxyl and α -amino groups of an amino acid.

TABLE 3.4 pK_a values of some amino acids

Amino acid	pK_a values (25°C)		
	α -COOH group	α -NH ₃ ⁺ group	Side chain
Alanine	2.3	9.9	
Glycine	2.4	9.8	
Phenylalanine	1.8	9.1	
Serine	2.1	9.2	
Valine	2.3	9.6	
Aspartic acid	2.0	10.0	3.9
Glutamic acid	2.2	9.7	4.3
Histidine	1.8	9.2	6.0
Cysteine	1.8	10.8	8.3
Tyrosine	2.2	9.1	10.9
Lysine	2.2	9.2	10.8
Arginine	1.8	9.0	12.5

After J. T. Edsall and J. Wyman, *Biophysical Chemistry* (Academic Press, 1958), Chapter 8.

acids are similar (Table 3.4). Some amino acids, such as aspartic acid, also contain an ionizable side chain. The pK_a values of ionizable side chains in amino acids range from 3.9 (aspartic acid) to 12.5 (arginine).

KEY TERMS

side chain (R group) (p. 43)

L amino acid (p. 43)

dipolar ion (zwitterion) (p. 43)

peptide bond (amide bond) (p. 51)

disulfide bond (p. 52)

primary structure (p. 53)

phi (ϕ) angle (p. 55)

psi (ψ) angle (p. 55)

Ramachandran diagram (p. 55)

α helix (p. 56)

β pleated sheet (p. 58)

β strand (p. 58)

reverse turn (β turn; hairpin turn) (p. 60)

secondary structure (p. 61)

tertiary structure (p. 62)

domain (p. 63)

subunit (p. 64)

quaternary structure (p. 64)

cooperative transition (p. 68)

SELECTED READINGS

Where to start

Richardson, J. S., 1981. The anatomy and taxonomy of protein structure. *Adv. Protein Chem.* 34:167–339.

Doolittle, R. F., 1985. Proteins. *Sci. Am.* 253(4):88–99.

Richards, F. M., 1991. The protein folding problem. *Sci. Am.* 264(1): 54–57.

Weber, A. L., and Miller, S. L., 1981. Reasons for the occurrence of the twenty coded protein amino acids. *J. Mol. Evol.* 17:273–284.

Books

Branden, C., Tooze, J., 1999. *Introduction to Protein Structure* (2d ed.). Garland.

Perutz, M. F., 1992. *Protein Structure: New Approaches to Disease and Therapy*. W. H. Freeman and Company.

Creighton, T. E., 1992. *Proteins: Structures and Molecular Principles* (2d ed.). W. H. Freeman and Company.

Schultz, G. E., and Schirmer, R. H., 1979. *Principles of Protein Structure*. Springer-Verlag.

Conformation of proteins

Richardson, J. S., Richardson, D. C., Tweedy, N. B., Gernert, K. M., Quinn, T. P., Hecht, M. H., Erickson, B. W., Yan, Y., McClain, R. D., Donlan, M. E., and Suries, M. C., 1992. Looking at proteins: Representations, folding, packing, and design. *Biophys. J.* 63: 1186–1220.

Chothia, C., and Finkelstein, A. V., 1990. The classification and origin of protein folding patterns. *Annu. Rev. Biochem.* 59:1007–1039.

Alpha helices, beta sheets, and loops

O'Neil, K. T., and DeGrado, W. F., 1990. A thermodynamic scale for the helix-forming tendencies of the commonly occurring amino acids. *Science* 250:646–651.

Zhang, C., and Kim, S. H., 2000. The anatomy of protein beta-sheet topology. *J. Mol. Biol.* 299:1075–1089.

Regan, L., 1994. Protein structure: Born to be beta. *Curr. Biol.* 4:656–658.

Leszczynski, J. F., and Rose, G. D., 1986. Loops in globular proteins: A novel category of secondary structure. *Science* 234:849–855.

Srinivasan, R., and Rose, G. D., 1999. A physical basis for protein secondary structure. *Proc. Natl. Acad. Sci. U. S. A.* 96:14258–14263.

Domains

Bennett, M. J., Choe, S., and Eisenberg, D., 1994. Domain swapping: Entangling alliances between proteins. *Proc. Natl. Acad. Sci. U. S. A.* 91:3127–3131.

Bergdoll, M., Eltis, L. D., Cameron, A. D., Dumas, P., and Bolin, J. T., 1998. All in the family: Structural and evolutionary relationships among three modular proteins with diverse functions and variable assembly. *Protein Sci.* 7:1661–1670.

Hopfner, K. P., Kopetzki, E., Kresse, G. B., Bode, W., Huber, R., and Engh, R. A., 1998. New enzyme lineages by subdomain shuffling. *Proc. Natl. Acad. Sci. U. S. A.* 95:9813–9818.

Ponting, C. P., Schultz, J., Copley, R. R., Andrade, M. A., and Bork, P., 2000. Evolution of domain families. *Adv. Protein Chem.* 54:185–244.

Protein folding

Anfinsen, C. B., 1973. Principles that govern the folding of protein chains. *Science* 181:223–230.

Baldwin, R. L., and Rose, G. D., 1999. Is protein folding hierarchic? I. Local structure and peptide folding. *Trends Biochem. Sci.* 24:26–33.

Baldwin, R. L., and Rose, G. D., 1999. Is protein folding hierarchic? II. Folding intermediates and transition states. *Trends Biochem. Sci.* 24:77–83.

Staley, J. P., and Kim, P. S., 1990. Role of a subdomain in the folding of bovine pancreatic trypsin inhibitor. *Nature* 344:685–688.

Neira, J. L., and Fersht, A. R., 1999. Exploring the folding funnel of a polypeptide chain by biophysical studies on protein fragments. *J. Mol. Biol.* 285:1309–1333.

Covalent modification of proteins

Krishna, R. G., and Wold, F., 1993. Post-translational modification of proteins. *Adv. Enzymol. Relat. Areas. Mol. Biol.* 67:265–298.

Aletta, J. M., Cimato, T. R., and Ettinger, M. J., 1998. Protein methylation: A signal event in post-translational modification. *Trends Biochem. Sci.* 23:89–91.

Glazer, A. N., DeLange, R. J., and Sigman, D. S., 1975. *Chemical Modification of Proteins*. North-Holland.

Tsien, R. Y., 1998. The green fluorescent protein. *Annu. Rev. Biochem.* 67:509–544.

Molecular graphics

Kraulis, P., 1991. MOLSCRIPT: A program to produce both detailed and schematic plots of protein structures. *J. Appl. Cryst.* 24:946–950.

Ferrin, T., Huang, C., Jarvis, L., and Langridge, R., 1988. The MIDAS display system. *J. Mol. Graphics* 6:13–27.

Richardson, D. C., and Richardson, J. S., 1994. Kinemages: Simple macromolecular graphics for interactive teaching and publication. *Trends Biochem. Sci.* 19:135–138.

PROBLEMS

1. *Shape and dimension.* (a) Tropomyosin, a 70-kd muscle protein, is a two-stranded α -helical coiled coil. Estimate the length of the molecule? (b) Suppose that a 40-residue segment of a protein folds into a two-stranded antiparallel β structure with a 4-residue hairpin turn. What is the longest dimension of this motif?

2. *Contrasting isomers.* Poly-L-leucine in an organic solvent such as dioxane is α helical, whereas poly-L-isoleucine is not. Why do these amino acids with the same number and kinds of atoms have different helix-forming tendencies?

3. *Active again.* A mutation that changes an alanine residue in the interior of a protein to valine is found to lead to a loss of activity. However, activity is regained when a second mutation at a different position changes an isoleucine residue to glycine. How might this second mutation lead to a restoration of activity?

4. *Shuffle test.* An enzyme that catalyzes disulfide–sulfhydryl exchange reactions, called protein disulfide isomerase (PDI), has been isolated. PDI rapidly converts inactive scrambled ribonuclease into enzymatically active ribonuclease. In contrast, insulin is rapidly inactivated by PDI. What does this important observation imply about the relation between the amino acid sequence of insulin and its three-dimensional structure?

5. *Stretching a target.* A protease is an enzyme that catalyzes the hydrolysis of the peptide bonds of target proteins. How might a protease bind a target protein so that its main chain be-

comes fully extended in the vicinity of the vulnerable peptide bond?

6. *Often irreplaceable.* Glycine is a highly conserved amino acid residue in the evolution of proteins. Why?

7. *Potential partners.* Identify the groups in a protein that can form hydrogen bonds or electrostatic bonds with an arginine side chain at pH 7.

8. *Permanent waves.* The shape of hair is determined in part by the pattern of disulfide bonds in keratin, its major protein. How can curls be induced?

9. *Location is everything.* Proteins that span biological membranes often contain α helices. Given that the insides of membranes are highly hydrophobic (Section 12.2.1), predict what type of amino acids would be in such a helix. Why is an α helix particularly suited to exist in the hydrophobic environment of the interior of a membrane?

10. *Issues of stability.* Proteins are quite stable. The lifetime of a peptide bond in aqueous solution is nearly 1000 years. However, the ΔG° of hydrolysis of proteins is negative and quite large. How can you account for the stability of the peptide bond in light of the fact that hydrolysis releases much energy?

11. *Minor species.* For an amino acid such as alanine, the major species in solution at pH 7 is the zwitterionic form. Assume a pK_a value of 8 for the amino group and a pK_a value of 3 for the

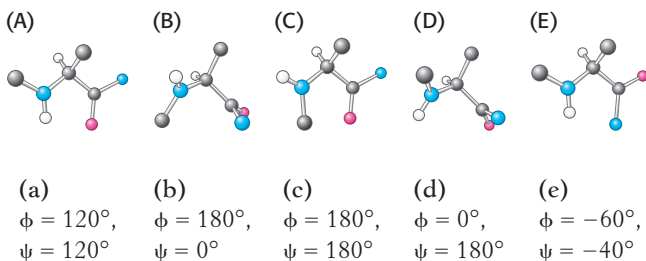
carboxylic acid and estimate the ratio of the concentration of neutral amino acid species (with the carboxylic acid protonated and the amino group neutral) to that of the zwitterionic species at pH 7.

12. *A matter of convention.* All L amino acids have an S absolute configuration except L-cysteine, which has the R configuration. Explain why L-cysteine is designated as the R absolute configuration.

13. *Hidden message.* Translate the following amino acid sequence into one-letter code: Ser-Ala-Val-Glu-Met-Glu-Ile-Met-Thr-Arg-Ala-Pro-Pro-Glu-Asp-Ile-Asn-Ala-Gly-Glu-Asn-Glu. Note the message.

14. *Who goes first?* Would you expect Pro-X peptide bonds to tend to have cis conformations like those of X-Pro bonds? Why or why not?

15. *Matching.* For each of the amino acid derivatives shown below (A-E), find the matching set of ϕ and ψ values (a-e).



16. *Concentrate of the concentration.* A solution of a protein whose sequence includes three tryptophan residues, no tyrosine residues, and no phenylalanine residues has an absorbance of 0.1 at 280 nm in a cell with a path length of 1 cm. Estimate the concentration of the protein in units of molarity. If the protein has a molecular mass of 100 kd, estimate the concentration in units of milligrams of protein per milliliter of solution.

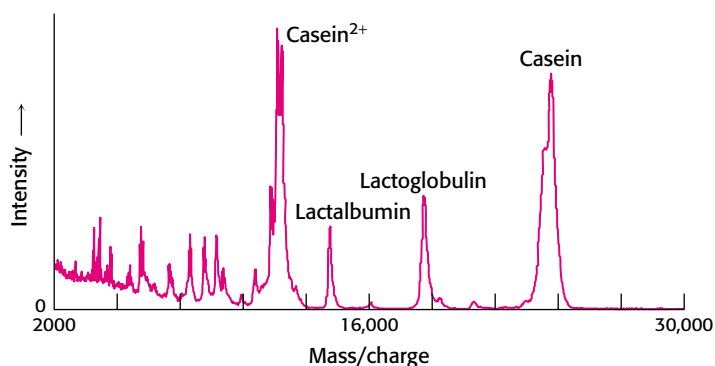


Media Problem

You can use the **Structural Insights** and **Conceptual Insights** as visual aids to help you answer Media Problems. Go to the Website: www.whfreeman.com/biochem5, and select the applicable module.

17. *Inside-out, back-to-front.* In the Media Problem section of the **Structural Insights** module on protein structure, you can examine molecular models of four putative protein structures. One of the four structures has been determined by x-ray crystallography. The other three have been made-up, and in fact are very unlikely to occur. Which are the structures that are unlikely to occur and why?

Exploring Proteins



Milk, a source of nourishment for all mammals, is composed, in part, of a variety of proteins. The protein components of milk are revealed by the technique of MALDI-TOF mass spectrometry, which separates molecules on the basis of their mass to charge ratio. [(Left) Jean Paul Iris/FPG (Right) courtesy of Brian Chait.]

In the preceding chapter, we saw that proteins play crucial roles in nearly all biological processes—in catalysis, signal transmission, and structural support. This remarkable range of functions arises from the existence of thousands of proteins, each folded into a distinctive three-dimensional structure that enables it to interact with one or more of a highly diverse array of molecules. A major goal of biochemistry is to determine how amino acid sequences specify the conformations of proteins. Other goals are to learn how individual proteins bind specific substrates and other molecules, mediate catalysis, and transduce energy and information.

The purification of the protein of interest is the indispensable first step in a series of studies aimed at exploring protein function. Proteins can be separated from one another on the basis of solubility, size, charge, and binding ability. When a protein has been purified, the amino acid sequence can be determined. The strategy is to divide and conquer, to obtain specific fragments that can be readily sequenced. Automated peptide sequencing and the application of recombinant DNA methods are providing a wealth of amino acid sequence data that are opening new vistas. To understand the physiological context of a protein, antibodies are choice probes for locating proteins *in vivo* and measuring their quantities. Monoclonal antibodies able to probe for specific proteins can be obtained in large amounts. The synthesis of peptides is possible, which makes feasible the

OUTLINE

- **4.1 The Purification of Proteins Is an Essential First Step in Understanding Their Function**
- **4.2 Amino Acid Sequences Can Be Determined by Automated Edman Degradation**
- **4.3 Immunology Provides Important Techniques with Which to Investigate Proteins**
- **4.4 Peptides Can Be Synthesized by Automated Solid-Phase Methods**
- **4.5 Three-Dimensional Protein Structure Can Be Determined by NMR Spectroscopy and X-Ray Crystallography**

synthesis of new drugs, functional protein fragments, and antigens for inducing the formation of specific antibodies. Nuclear magnetic resonance (NMR) spectroscopy and x-ray crystallography are the principal techniques for elucidating three-dimensional structure, the key determinant of function.

The exploration of proteins by this array of physical and chemical techniques has greatly enriched our understanding of the molecular basis of life and makes it possible to tackle some of the most challenging questions of biology in molecular terms.

4.0.1 The Proteome Is the Functional Representation of the Genome

Many organisms are yielding their DNA base sequences to gene sequencers, including several metazoans. The roundworm *Caenorhabditis elegans* has a genome of 97 million bases and about 19,000 protein-encoding genes, whereas that of the fruit fly *Drosophila melanogaster* contains 180 million bases and about 14,000 genes. The incredible progress being made in gene sequencing has already culminated in the elucidation of the complete sequence of the human genome, all 3 billion bases with an estimated 40,000 genes. But this genomic knowledge is analogous to a list of parts for a car—it does not explain how the parts work together. A new word has been coined, the *proteome*, to signify a more complex level of information content, the level of *functional information*, which encompasses the type, functions, and interactions of proteins that yield a functional unit.

The term proteome is derived from *proteins* expressed by the *genome*. Whereas the genome tells us what is possible, the proteome tells us what is functionally present—for example, which proteins interact to form a signal-transduction pathway or an ion channel in a membrane. The proteome is not a fixed characteristic of the cell. Rather, because it represents the functional expression of information, it varies with cell type, developmental stage, and environmental conditions, such as the presence of hormones. The proteome is much larger than the genome because of such factors as alternatively spliced RNA, the posttranslational modification of proteins, the temporal regulation of protein synthesis, and varying protein–protein interactions. Unlike the genome, the proteome is not static.

An understanding of the proteome is acquired by investigating, characterizing, and cataloging proteins. An investigator often begins the process by separating a particular protein from all other biomolecules in the cell.

4.1 THE PURIFICATION OF PROTEINS IS AN ESSENTIAL FIRST STEP IN UNDERSTANDING THEIR FUNCTION

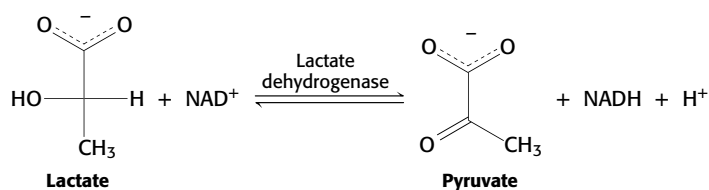
An adage of biochemistry is, Never waste pure thoughts on an impure protein. Starting from pure proteins, we can determine amino acid sequences and evolutionary relationships between proteins in diverse organisms and we can investigate a protein's biochemical function. Moreover, crystals of the protein may be grown from pure protein, and from such crystals we can obtain x-ray data that will provide us with a picture of the protein's tertiary structure—the actual *functional* unit.

4.1.1 The Assay: How Do We Recognize the Protein That We Are Looking For?

Purification should yield a sample of protein containing only one type of molecule, the protein in which the biochemist is interested. This protein

sample may be only a fraction of 1% of the starting material, whether that starting material consists of cells in culture or a particular organ from a plant or animal. How is the biochemist able to isolate a particular protein from a complex mixture of proteins?

The biochemist needs a test, called an *assay*, for some unique identifying property of the protein so that he or she can tell when the protein is present. Determining an effective assay is often difficult; but the more specific the assay, the more effective the purification. For enzymes, which are protein catalysts (Chapter 8), the assay is usually based on the reaction that the enzyme catalyzes in the cell. Consider the enzyme lactate dehydrogenase, an important player in the anaerobic generation of energy from glucose as well as in the synthesis of glucose from lactate. Lactate dehydrogenase carries out the following reaction:



Nicotinamide adenine dinucleotide [reduced (NADH); Section 14.3.1] is distinguishable from the other components of the reaction by its ability to absorb light at 340 nm. Consequently, we can follow the progress of the reaction by examining how much light the reaction mixture absorbs at 340 nm in unit time—for instance, within 1 minute after the addition of the enzyme. Our assay for enzyme activity during the purification of lactate dehydrogenase is thus the increase in absorbance of light at 340 nm observed in 1 minute.

To be certain that our purification scheme is working, we need one additional piece of information—the amount of protein present in the mixture being assayed. There are various rapid and accurate means of determining protein concentration. With these two experimentally determined numbers—enzyme activity and protein concentration—we then calculate the *specific activity*, the ratio of enzyme activity to the amount of protein in the enzyme assay. The specific activity will rise as the purification proceeds and the protein mixture being assayed consists to a greater and greater extent of lactate dehydrogenase. In essence, the point of the purification is to maximize the specific activity.

4.1.2 Proteins Must Be Released from the Cell to Be Purified

Having found an assay and chosen a source of protein, we must now fractionate the cell into components and determine which component is enriched in the protein of interest. Such fractionation schemes are developed by trial and error, on the basis of previous experience. In the first step, a *homogenate* is formed by disrupting the cell membrane, and the mixture is fractionated by centrifugation, yielding a dense pellet of heavy material at the bottom of the centrifuge tube and a lighter supernatant above (Figure 4.1). The supernatant is again centrifuged at a greater force to yield yet another pellet and supernatant. The procedure, called *differential centrifugation*, yields several fractions of decreasing density, each still containing hundreds of different proteins, which are subsequently assayed for the activity being purified. Usually, one fraction will be enriched for such activity, and it then serves as the source of material to which more discriminating purification techniques are applied.

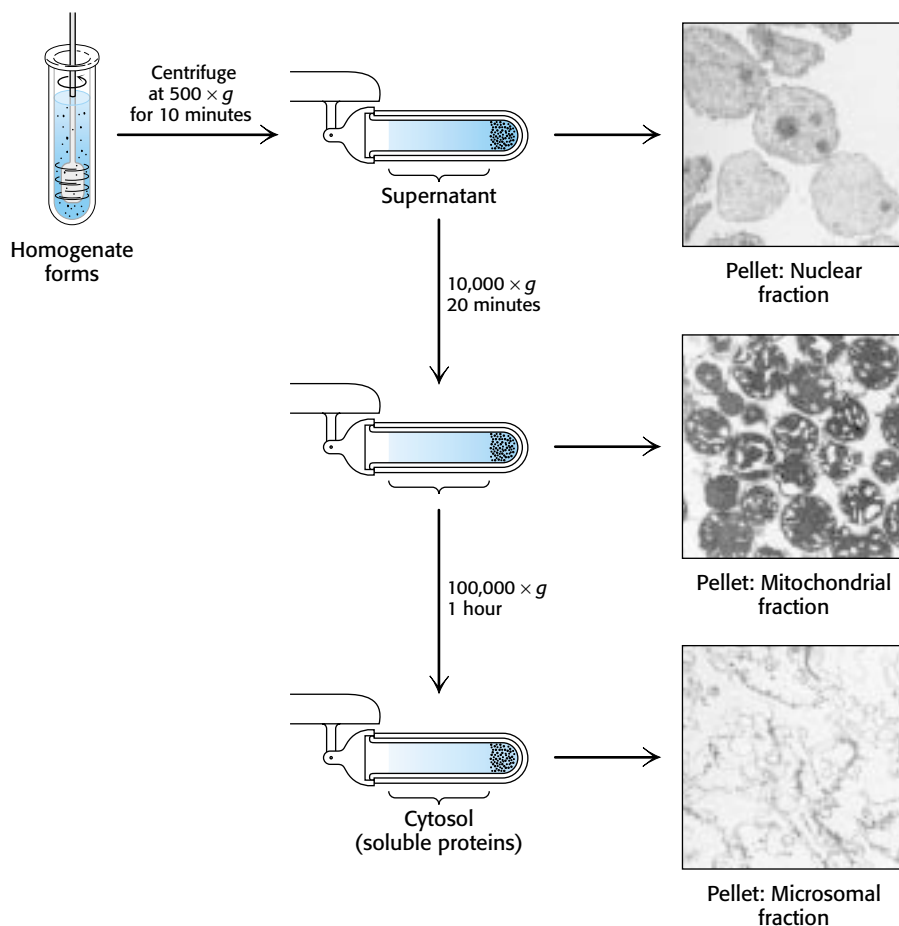


FIGURE 4.1 Differential centrifugation.

Cells are disrupted in a homogenizer and the resulting mixture, called the homogenate, is centrifuged in a step-by-step fashion of increasing centrifugal force. The denser material will form a pellet at lower centrifugal force than will the less-dense material. The isolated fractions can be used for further purification. [Photographs courtesy of S. Fleischer and B. Fleischer.]

4.1.3 Proteins Can Be Purified According to Solubility, Size, Charge, and Binding Affinity

Several thousand proteins have been purified in active form on the basis of such characteristics as *solubility*, *size*, *charge*, and *specific binding affinity*. Usually, protein mixtures are subjected to a series of separations, each based on a different property to yield a pure protein. At each step in the purification, the preparation is assayed and the protein concentration is determined. Substantial quantities of purified proteins, of the order of many milligrams, are needed to fully elucidate their three-dimensional structures and their mechanisms of action. Thus, the overall yield is an important feature of a purification scheme. A variety of purification techniques are available.

Salting Out. Most proteins are less soluble at high salt concentrations, an effect called *salting out*. The salt concentration at which a protein precipitates differs from one protein to another. Hence, salting out can be used to fractionate proteins. For example, 0.8 M ammonium sulfate precipitates fibrinogen, a blood-clotting protein, whereas a concentration of 2.4 M is needed to precipitate serum albumin. Salting out is also useful for concentrating dilute solutions of proteins, including active fractions obtained from other purification steps. Dialysis can be used to remove the salt if necessary.

Dialysis. Proteins can be separated from small molecules by *dialysis* through a semipermeable membrane, such as a cellulose membrane with

pores (Figure 4.2). Molecules having dimensions significantly greater than the pore diameter are retained inside the dialysis bag, whereas smaller molecules and ions traverse the pores of such a membrane and emerge in the dialysate outside the bag. This technique is useful for removing a salt or other small molecule, but it will not distinguish between proteins effectively.

Gel-Filtration Chromatography. More discriminating separations on the basis of size can be achieved by the technique of *gel-filtration chromatography* (Figure 4.3). The sample is applied to the top of a column consisting of porous beads made of an insoluble but highly hydrated polymer such as dextran or agarose (which are carbohydrates) or polyacrylamide. Sephadex, Sepharose, and Bio-gel are commonly used commercial preparations of these beads, which are typically 100 μm (0.1 mm) in diameter. Small molecules can enter these beads, but large ones cannot. The result is that small molecules are distributed in the aqueous solution both inside the beads and between them, whereas large molecules are located only in the solution between the beads. *Large molecules flow more rapidly through this column and emerge first because a smaller volume is accessible to them.* Molecules that are of a size to occasionally enter a bead will flow from the column at an intermediate position, and small molecules, which take a longer, tortuous path, will exit last.

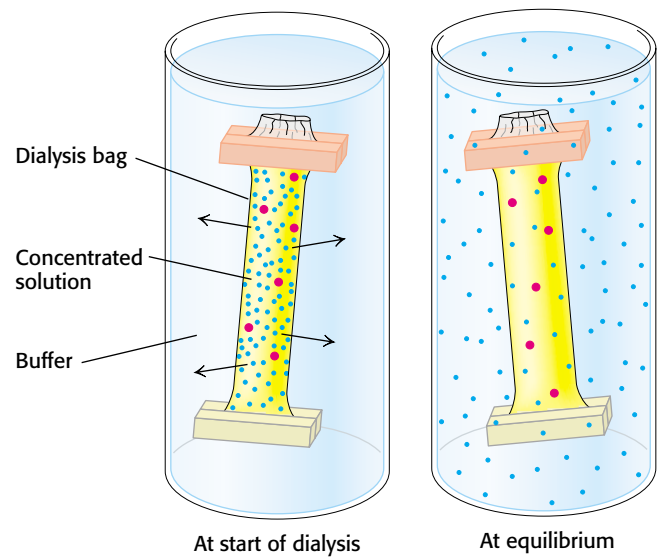


FIGURE 4.2 Dialysis. Protein molecules (red) are retained within the dialysis bag, whereas small molecules (blue) diffuse into the surrounding medium.

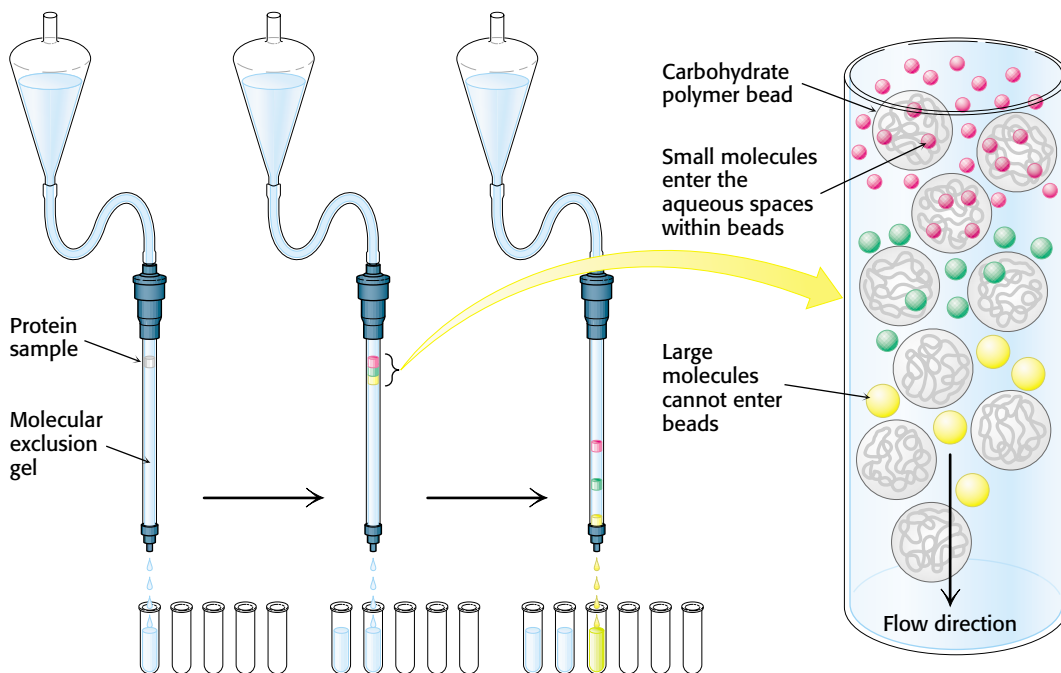


FIGURE 4.3 Gel filtration chromatography. A mixture of proteins in a small volume is applied to a column filled with porous beads. Because large proteins cannot enter the internal volume of the beads, they emerge sooner than do small ones.

Ion-Exchange Chromatography. Proteins can be separated on the basis of their net charge by *ion-exchange chromatography*. If a protein has a net positive charge at pH 7, it will usually bind to a column of beads containing carboxylate groups, whereas a negatively charged protein will not

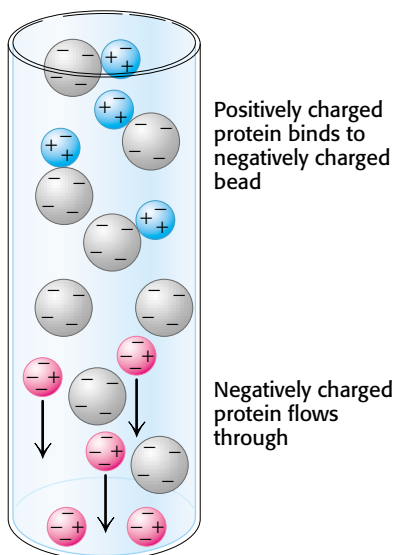
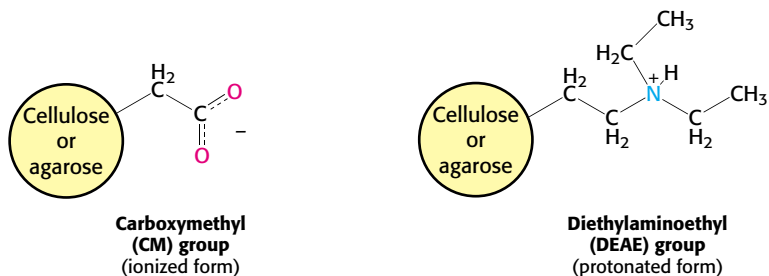


FIGURE 4.4 Ion-exchange chromatography. This technique separates proteins mainly according to their net charge.



Affinity Chromatography. *Affinity chromatography* is another powerful and generally applicable means of purifying proteins. This technique takes advantage of the high affinity of many proteins for specific chemical groups. For example, the plant protein concanavalin A can be purified by passing a crude extract through a column of beads containing covalently attached glucose residues. Concanavalin A binds to such a column because it has affinity for glucose, whereas most other proteins do not. The bound concanavalin A can then be released from the column by adding a concentrated solution of glucose. The glucose in solution displaces the column-attached glucose residues from binding sites on concanavalin A (Figure 4.5). Affinity chromatography is a powerful means of isolating transcription factors, proteins that regulate gene expression by binding to specific DNA sequences. A protein mixture is percolated through a column containing specific DNA sequences attached to a matrix; proteins with a high affinity for the sequence will bind and be retained. In this instance, the transcription factor is released by washing with a solution containing a high concentration of salt. In general, affinity chromatography can be effectively used to isolate a protein that recognizes group X by (1) covalently attaching X or a derivative of it to a column, (2) adding a mixture of proteins to this column, which is then washed with buffer to remove unbound proteins, and (3) eluting the desired protein by adding a high concentration of a soluble form of X or altering the conditions to decrease binding affinity. Affinity chromatography is most effective when the interaction of the protein and the molecule that is used as the bait is highly specific.

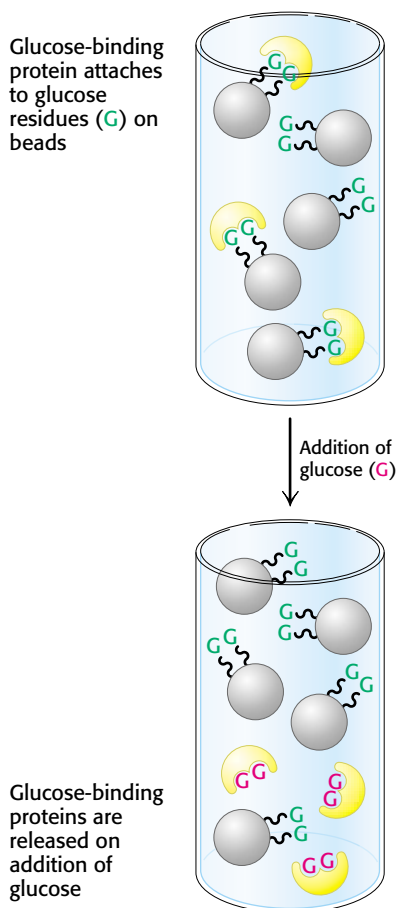


FIGURE 4.5 Affinity chromatography. Affinity chromatography of concanavalin A (shown in yellow) on a solid support containing covalently attached glucose residues (G).

High-Pressure Liquid Chromatography. The resolving power of all of the column techniques can be improved substantially through the use of a technique called *high-pressure liquid chromatography (HPLC)*, which is an enhanced version of the column techniques already discussed. The column materials themselves are much more finely divided and, as a consequence, there are more interaction sites and thus greater resolving power. Because the column is made of finer material, pressure must be applied to the column to obtain adequate flow rates. The net result is high resolution as well as rapid separation (Figure 4.6).

4.1.4 Proteins Can Be Separated by Gel Electrophoresis and Displayed

How can we tell whether a purification scheme is effective? One way is to ascertain that the specific activity rises with each purification step. Another is to visualize the effectiveness by displaying the proteins present at each step. The technique of electrophoresis makes the latter method possible.

Gel Electrophoresis. A molecule with a net charge will move in an electric field. This phenomenon, termed *electrophoresis*, offers a powerful means of separating proteins and other macromolecules, such as DNA and RNA. The velocity of migration (v) of a protein (or any molecule) in an electric field depends on the electric field strength (E), the net charge on the protein (z), and the frictional coefficient (f).

$$v = \frac{Ez}{f} \quad (1)$$

The electric force Ez driving the charged molecule toward the oppositely charged electrode is opposed by the viscous drag fv arising from friction between the moving molecule and the medium. The frictional coefficient f depends on both the mass and shape of the migrating molecule and the viscosity (η) of the medium. For a sphere of radius r ,

$$f = 6\pi\eta r \quad (2)$$

Electrophoretic separations are nearly always carried out in gels (or on solid supports such as paper) because the gel serves as a molecular sieve that enhances separation (Figure 4.7). Molecules that are small compared with the pores in the gel readily move through the gel, whereas molecules much larger than the pores are almost immobile. Intermediate-size molecules move through the gel with various degrees of facility. Electrophoresis is performed

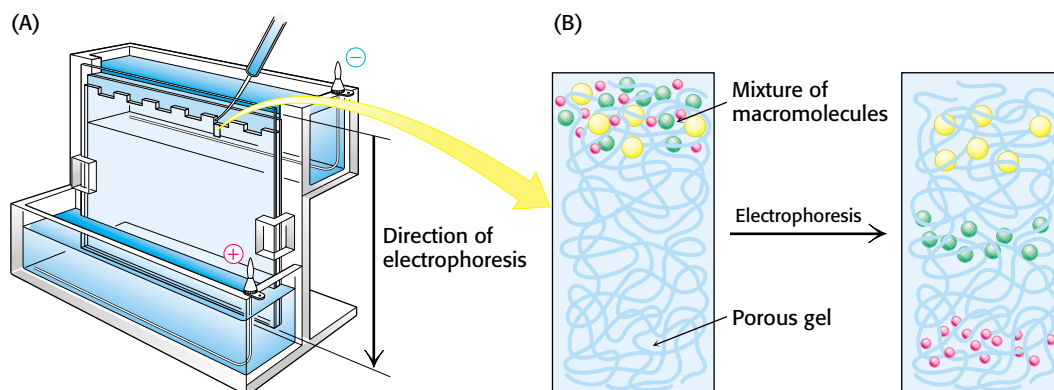


FIGURE 4.7 Polyacrylamide gel electrophoresis. (A) Gel electrophoresis apparatus. Typically, several samples undergo electrophoresis on one flat polyacrylamide gel. A microliter pipette is used to place solutions of proteins in the wells of the slab. A cover is then placed over the gel chamber and voltage is applied. The negatively charged SDS (sodium dodecyl sulfate)–protein complexes migrate in the direction of the anode, at the bottom of the gel. (B) The sieving action of a porous polyacrylamide gel separates proteins according to size, with the smallest moving most rapidly.

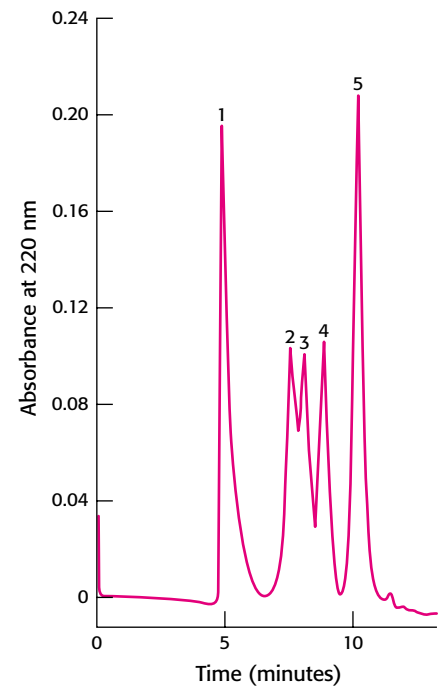
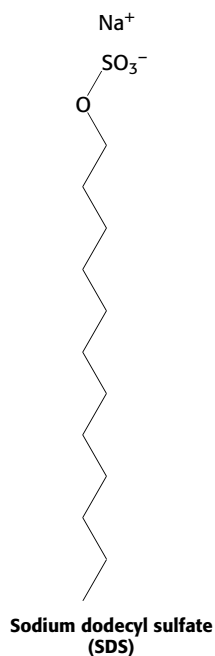
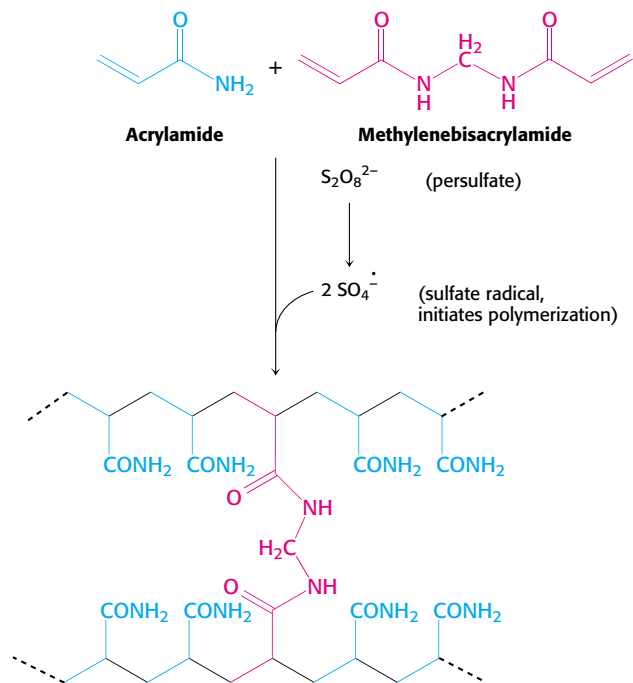


FIGURE 4.6 High-pressure liquid chromatography (HPLC). Gel filtration by HPLC clearly defines the individual proteins because of its greater resolving power: (1) thyroglobulin (669 kd), (2) catalase (232 kd), (3) bovine serum albumin (67 kd), (4) ovalbumin (43 kd), and (5) ribonuclease (13.4 kd). [After K. J. Wilson and T. D. Schlabach. In *Current Protocols in Molecular Biology*, vol. 2, suppl. 41, F. M. Ausbel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl, Eds. (Wiley, 1998), p. 10.14.1.]

FIGURE 4.8 Formation of a polyacrylamide gel. A three-dimensional mesh is formed by co-polymerizing activated monomer (blue) and cross-linker (red).



in a thin, vertical slab of polyacrylamide. The direction of flow is from top to bottom. Polyacrylamide gels, formed by the polymerization of acrylamide and cross-linked by methylenebisacrylamide, are choice supporting media for electrophoresis because they are chemically inert and are readily formed (Figure 4.8). Electrophoresis is the opposite of gel filtration in that all of the molecules, regardless of size, are forced to move through the same matrix. The gel behaves as one bead of a gel-filtration column.

Proteins can be separated largely on the basis of mass by electrophoresis in a polyacrylamide gel under denaturing conditions. The mixture of proteins is first dissolved in a solution of sodium dodecyl sulfate (SDS), an anionic detergent that disrupts nearly all noncovalent interactions in native proteins. Mercaptoethanol (2-thioethanol) or dithiothreitol also is added to reduce disulfide bonds. Anions of SDS bind to main chains at a ratio of about one SDS anion for every two amino acid residues. This complex of SDS with a denatured protein has a large net negative charge that is roughly proportional to the mass of the protein. The negative charge acquired on binding SDS is usually much greater than the charge on the native protein; this native charge is thus rendered insignificant. The SDS–protein complexes are then subjected to electrophoresis. When the electrophoresis is complete, the proteins in the gel can be visualized by staining them with silver or a dye such as Coomassie blue, which reveals a series of bands (Figure 4.9). Radioactive

FIGURE 4.9 Staining of proteins after electrophoresis. Proteins subjected to electrophoresis on an SDS–polyacrylamide gel can be visualized by staining with Coomassie blue. [Courtesy of Kodak Scientific Imaging Systems.]

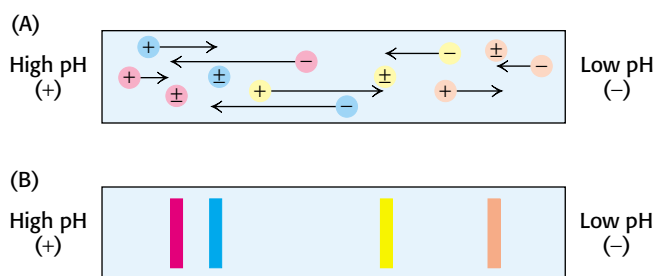


labels can be detected by placing a sheet of x-ray film over the gel, a procedure called *autoradiography*.

Small proteins move rapidly through the gel, whereas large proteins stay at the top, near the point of application of the mixture. The mobility of most polypeptide chains under these conditions is linearly proportional to the logarithm of their mass (Figure 4.10). Some carbohydrate-rich proteins and membrane proteins do not obey this empirical relation, however. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) is rapid, sensitive, and capable of a high degree of resolution. As little as $0.1 \mu\text{g}$ ($\sim 2 \text{ pmol}$) of a protein gives a distinct band when stained with Coomassie blue, and even less ($\sim 0.02 \mu\text{g}$) can be detected with a silver stain. Proteins that differ in mass by about 2% (e.g., 40 and 41 kd, arising from a difference of about 10 residues) can usually be distinguished.

We can examine the efficacy of our purification scheme by analyzing a part of each fraction by SDS-PAGE. The initial fractions will display dozens to hundreds of proteins. As the purification progresses, the number of bands will diminish, and the prominence of one of the bands should increase. This band will correspond to the protein of interest.

Isoelectric Focusing. Proteins can also be separated electrophoretically on the basis of their relative contents of acidic and basic residues. The *isoelectric point* (pI) of a protein is the pH at which its net charge is zero. At this pH, its electrophoretic mobility is zero because z in equation 1 is equal to zero. For example, the pI of cytochrome *c*, a highly basic electron-transport protein, is 10.6, whereas that of serum albumin, an acidic protein in blood, is 4.8. Suppose that a mixture of proteins undergoes electrophoresis in a pH gradient in a gel in the absence of SDS. Each protein will move until it reaches a position in the gel at which the pH is equal to the pI of the protein. This method of separating proteins according to their isoelectric point is called *isoelectric focusing*. The pH gradient in the gel is formed first by subjecting a mixture of *polyampholytes* (small multicharged polymers) having many pI values to electrophoresis. Isoelectric focusing can readily resolve proteins that differ in pI by as little as 0.01, which means that proteins differing by one net charge can be separated (Figure 4.11).



Two-Dimensional Electrophoresis. Isoelectric focusing can be combined with SDS-PAGE to obtain very high resolution separations. A single sample is first subjected to isoelectric focusing. This single-lane gel is then placed horizontally on top of an SDS-polyacrylamide slab. The proteins are thus spread across the top of the polyacrylamide gel according to how far they migrated during isoelectric focusing. They then undergo electrophoresis again in a perpendicular direction (vertically) to yield a two-dimensional pattern of spots. In such a gel, proteins have been separated in the horizontal direction on the basis of isoelectric point and in the vertical direction on the basis of mass. It is remarkable that more than a thousand different proteins in the bacterium *Escherichia coli* can be resolved in a single experiment by two-dimensional electrophoresis (Figure 4.12).

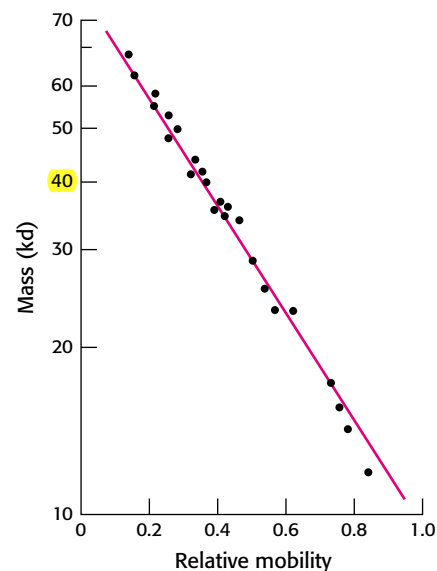


FIGURE 4.10 Electrophoresis can determine mass. The electrophoretic mobility of many proteins in SDS-polyacrylamide gels is inversely proportional to the logarithm of their mass. [After K. Weber and M. Osborn, *The Proteins*, vol. 1, 3d ed. (Academic Press, 1975), p. 179.]

FIGURE 4.11 The principle of isoelectric focusing. A pH gradient is established in a gel before loading the sample. (A) The sample is loaded and voltage is applied. The proteins will migrate to their isoelectric pH, the location at which they have no net charge. (B) The proteins form bands that can be excised and used for further experimentation.

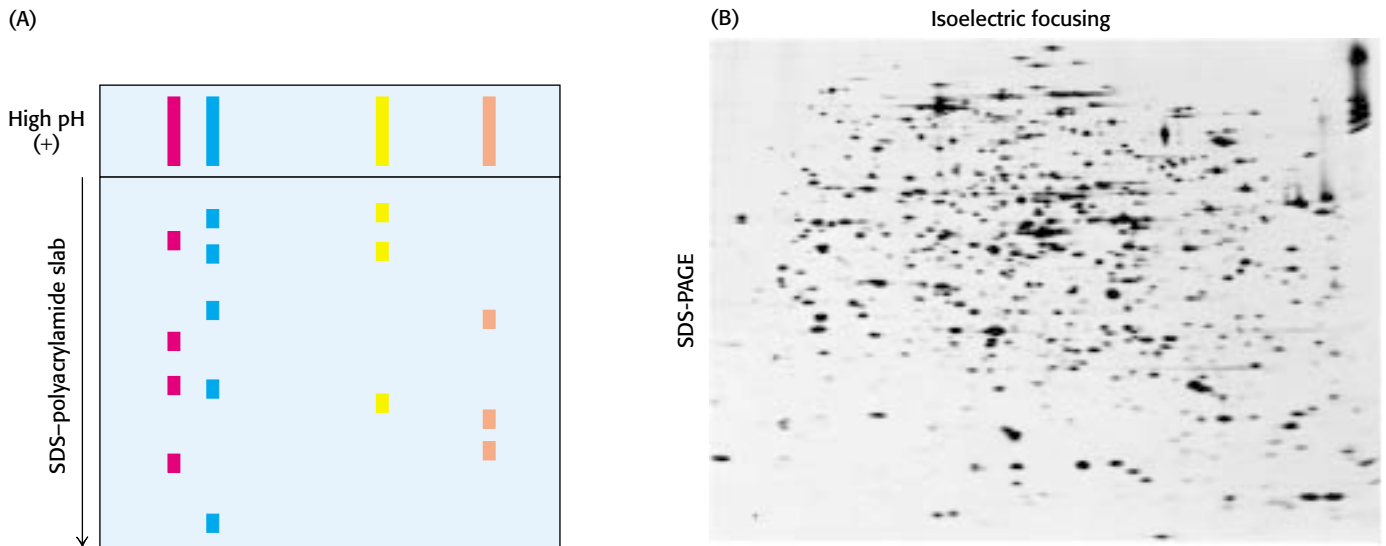


FIGURE 4.12 Two-dimensional gel electrophoresis. (A) A protein sample is initially fractionated in one dimension by isoelectric focusing as described in Figure 4.11. The isoelectric focusing gel is then attached to an SDS-polyacrylamide gel, and electrophoresis is performed in the second dimension, perpendicular to the original separation. Proteins with the same pI are now separated on the basis of mass. (B) Proteins from *E. coli* were separated by two-dimensional gel electrophoresis, resolving more than a thousand different proteins. The proteins were first separated according to their isoelectric pH in the horizontal direction and then by their apparent mass in the vertical direction. [(B) Courtesy of Dr. Patrick H. O'Farrell.]

Proteins isolated from cells under different physiological conditions can be subjected to two-dimensional electrophoresis, followed by an examination of the intensity of the signals. In this way, particular proteins can be seen to increase or decrease in concentration in response to the physiological state. How can we tell what protein is being regulated? A former drawback to the power of the two-dimensional gel is that, although many proteins are displayed, they are not identified. It is now possible to identify proteins by coupling two-dimensional gel electrophoresis with mass spectrometric techniques. We will consider these techniques when we examine how the mass of a protein is determined (Section 4.1.6).

4.1.5 A Protein Purification Scheme Can Be Quantitatively Evaluated

To determine the success of a protein purification scheme, we monitor the procedure at each step by determining specific activity and by performing an SDS-PAGE analysis. Consider the results for the purification of a fictitious protein, summarized in Table 4.1 and Figure 4.13. At each step, the following parameters are measured:

Total protein. The quantity of protein present in a fraction is obtained by determining the protein concentration of a part of each fraction and multiplying by the fraction's total volume.

Total activity. The enzyme activity for the fraction is obtained by measuring the enzyme activity in the volume of fraction used in the assay and multiplying by the fraction's total volume.

TABLE 4.1 Quantification of a purification protocol for a fictitious protein

Step	Total protein (mg)	Total activity (units)	Specific activity, (units mg ⁻¹)	Yield (%)	Purification level
Homogenization	15,000	150,000	10	100	1
Salt fractionation	4,600	138,000	30	92	3
Ion-exchange chromatography	1,278	115,500	90	77	9
Molecular exclusion chromatography	68.8	75,000	1,100	50	110
Affinity chromatography	1.75	52,500	30,000	35	3,000

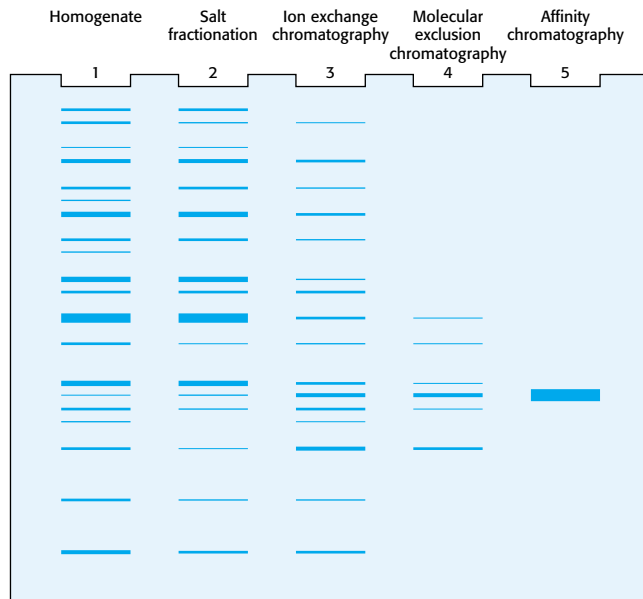


FIGURE 4.13 Electrophoretic analysis of a protein purification. The purification scheme in Table 4.1 was analyzed by SDS-PAGE. Each lane contained 50 μg of sample. The effectiveness of the purification can be seen as the band for the protein of interest becomes more prominent relative to other bands.

Specific activity. This parameter is obtained by dividing total activity by total protein.

Yield. This parameter is a measure of the activity retained after each purification step as a percentage of the activity in the crude extract. The amount of activity in the initial extract is taken to be 100%.

Purification level. This parameter is a measure of the increase in purity and is obtained by dividing the specific activity, calculated after each purification step, by the specific activity of the initial extract.

As we see in Table 4.1, the first purification step, salt fractionation, leads to an increase in purity of only 3-fold, but we recover nearly all the target protein in the original extract, given that the yield is 92%. After dialysis to lower the high concentration of salt remaining from the salt fractionation, the fraction is passed through an ion-exchange column. The purification now increases to 9-fold compared with the original extract, whereas the yield falls to 77%. Molecular exclusion chromatography brings the level of purification to 100-fold, but the yield is now at 50%. The final step is affinity chromatography with the use of a ligand specific for the target enzyme. This step, the most powerful of these purification procedures, results in a purification level of 3000-fold, while lowering the yield to 35%. The SDS-PAGE in Figure 4.13 shows that, if we load a constant amount of protein onto each lane after each step, the number of bands decreases in proportion to the level of purification, and the amount of protein of interest increases as a proportion of the total protein present.

A good purification scheme takes into account both purification levels and yield. A high degree of purification and a poor yield leave little protein with which to experiment. A high yield with low purification leaves many contaminants (proteins other than the one of interest) in the fraction and complicates the interpretation of experiments.

4.1.6 Ultracentrifugation Is Valuable for Separating Biomolecules and Determining Their Masses

We have already seen that centrifugation is a powerful and generally applicable method for separating a crude mixture of cell components, but it is also useful for separating and analyzing biomolecules themselves. With this

TABLE 4.2 S values and molecular weights of sample proteins

Protein	S value (Svedberg units)	Molecular weight
Pancreatic trypsin inhibitor	1	6,520
Cytochrome <i>c</i>	1.83	12,310
Ribonuclease A	1.78	13,690
Myoglobin	1.97	17,800
Trypsin	2.5	23,200
Carbonic anhydrase	3.23	28,800
Concanavlin A	3.8	51,260
Malate dehydrogenase	5.76	74,900
Lactate dehydrogenase	7.54	146,200

From T. Creighton, *Proteins*, 2nd Edition (W. H. Freeman and Company, 1993), Table 7.1.

technique, we can determine such parameters as mass and density, learn something about the shape of a molecule, and investigate the interactions between molecules. To deduce these properties from the centrifugation data, we need a mathematical description of how a particle behaves in a centrifugal force.

A particle will move through a liquid medium when subjected to a centrifugal force. A convenient means of quantifying the rate of movement is to calculate the sedimentation coefficient, s , of a particle by using the following equation:

$$s = m(1 - \bar{v}\rho)/f$$

where m is the mass of the particle, \bar{v} is the partial specific volume (the reciprocal of the particle density), ρ is the density of the medium and f is the frictional coefficient (a measure of the shape of the particle). The $(1 - \bar{v}\rho)$ term is the buoyant force exerted by liquid medium.

Sedimentation coefficients are usually expressed in **Svedberg units (S)**, equal to 10^{-13} s. The smaller the S value, the slower a molecule moves in a centrifugal field. The S values for a number of biomolecules and cellular components are listed in Table 4.2 and Figure 4.14.

Several important conclusions can be drawn from the preceding equation:

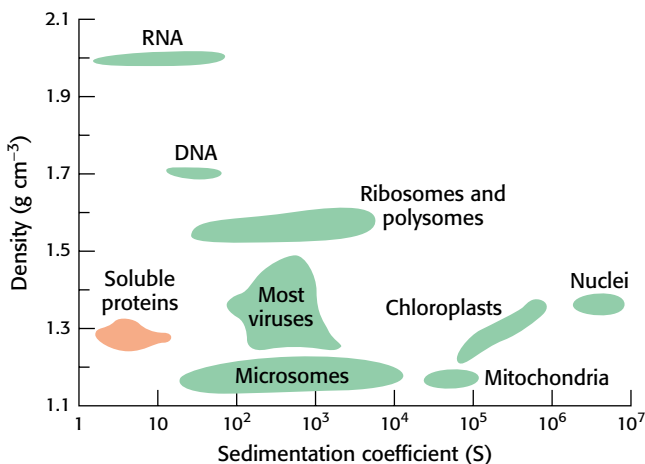


FIGURE 4.14 Density and sedimentation coefficients of cellular components. [After L. J. Kleinsmith and V. M. Kish, *Principles of Cell and Molecular Biology*, 2d ed. (Harper Collins, 1995), p. 138.]

1. The sedimentation velocity of a particle depends in part on its mass. A more massive particle sediments more rapidly than does a less massive particle of the same shape and density.

2. Shape, too, influences the sedimentation velocity because it affects the viscous drag. The frictional coefficient f of a compact particle is smaller than that of an extended particle of the same mass. Hence, elongated particles sediment more slowly than do spherical ones of the same mass.

3. A dense particle moves more rapidly than does a less dense one because the opposing buoyant force $(1 - \bar{v}\rho)$ is smaller for the denser particle.

4. The sedimentation velocity also depends on the density of the solution. (ρ). Particles sink when $\bar{v}\rho < 1$, float when $\bar{v}\rho > 1$, and do not move when $\bar{v}\rho = 1$.

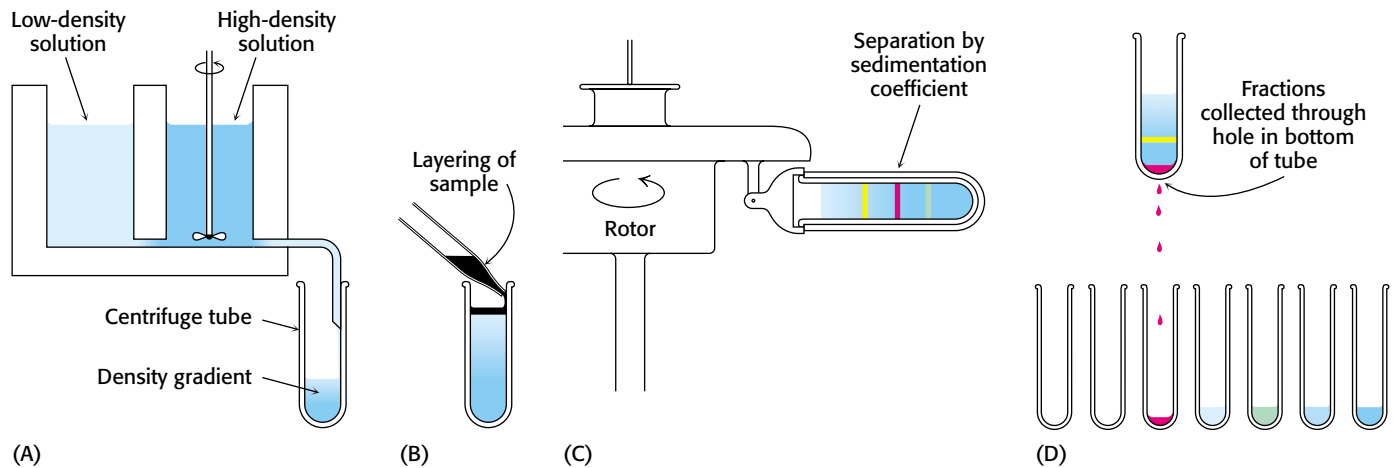


FIGURE 4.15 Zonal centrifugation. The steps are as follows: (A) form a density gradient, (B) layer the sample on top of the gradient, (C) place the tube in a swinging-bucket rotor and centrifuge it, and (D) collect the samples. [After D. Freifelder, *Physical Biochemistry*, 2d ed. (W. H. Freeman and Company, 1982), p. 397.]

A technique called *zonal*, *band*, or most commonly *gradient* centrifugation can be used to separate proteins with different sedimentation coefficients. The first step is to form a density gradient in a centrifuge tube. Differing proportions of a low-density solution (such as 5% sucrose) and a high-density solution (such as 20% sucrose) are mixed to create a linear gradient of sucrose concentration ranging from 20% at the bottom of the tube to 5% at the top (Figure 4.15). The role of the gradient is to prevent convective flow. A small volume of a solution containing the mixture of proteins to be separated is placed on top of the density gradient. When the rotor is spun, proteins move through the gradient and separate according to their sedimentation coefficients. The time and speed of the centrifugation is determined empirically. The separated bands, or zones, of protein can be harvested by making a hole in the bottom of the tube and collecting drops. The drops can be measured for protein content and catalytic activity or another functional property. This sedimentation-velocity technique readily separates proteins differing in sedimentation coefficient by a factor of two or more.

The mass of a protein can be directly determined by *sedimentation equilibrium*, in which a sample is centrifuged at relatively low speed so that sedimentation is counterbalanced by diffusion. The *sedimentation-equilibrium technique for determining mass is very accurate and can be applied under non-denaturing conditions in which the native quaternary structure of multimeric proteins is preserved*. In contrast, SDS–polyacrylamide gel electrophoresis (Section 4.1.4) provides an *estimate* of the mass of *dissociated polypeptide chains under denaturing conditions*. Note that, if we know the mass of the dissociated components of a multimeric protein as determined by SDS–polyacrylamide analysis and the mass of the intact multimeric protein as determined by sedimentation equilibrium analysis, we can determine how many copies of each polypeptide chain is present in the multimeric protein.

4.1.7 The Mass of a Protein Can Be Precisely Determined by Mass Spectrometry

Mass spectrometry has been an established analytical technique in organic chemistry for many years. Until recently, however, the very low volatility of proteins made mass spectrometry useless for the investigation of these molecules. This difficulty has been circumvented by the introduction of techniques for effectively dispersing proteins and other macromolecules into the gas phase. These methods are called *matrix-assisted laser desorption–ionization (MALDI)* and *electrospray spectrometry*. We will focus on

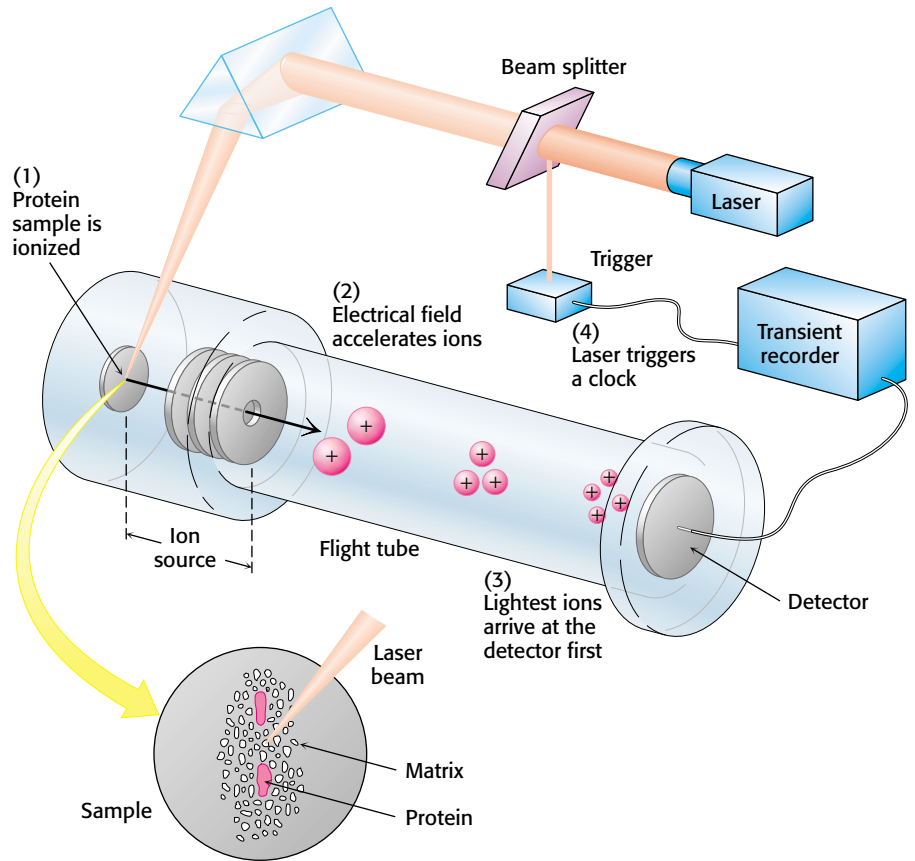
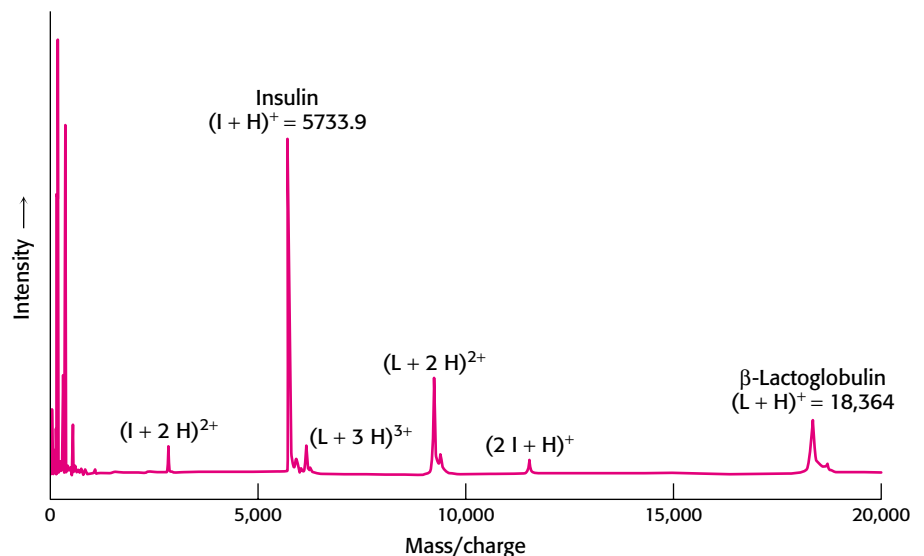


FIGURE 4.16 MALDI-TOF mass spectrometry. (1) The protein sample, embedded in an appropriate matrix, is ionized by the application of a laser beam. (2) An electrical field accelerates the ions formed through the flight tube toward the detector. (3) The lightest ions arrive first. (4) The ionizing laser pulse also triggers a clock that measures the time of flight (TOF) for the ions. [After J. T. Watson, *Introduction to Mass Spectrometry*, 3d ed. (Lippincott-Raven, 1997), p. 279.]

MALDI spectrometry. In this technique, protein ions are generated and then accelerated through an electrical field (Figure 4.16). They travel through the flight tube, with the smallest traveling fastest and arriving at the detector first. Thus, the *time of flight* (TOF) in the electrical field is a measure of the mass (or, more precisely, the mass/charge ratio). Tiny amounts of biomolecules, as small as a few picomoles (pmol) to femtomoles (fmol), can be analyzed in this manner. A MALDI-TOF mass spectrum for a mixture of the proteins insulin and β -lactoglobulin is shown in Figure 4.17. The masses determined by MALDI-TOF are 5733.9 and 18,364, respectively, compared with calculated values of 5733.5 and 18,388. MALDI-TOF is indeed an accurate means of determining protein mass.

FIGURE 4.17 MALDI-TOF mass spectrum of insulin and β -lactoglobulin. A mixture of 5 pmol each of insulin (I) and β -lactoglobulin (L) was ionized by MALDI, which produces predominately singly charged molecular ions from peptides and proteins ($I + H^+$ for insulin and $L + H^+$ for lactoglobulin). However, molecules with multiple charges as well as small quantities of a singly charged dimer of insulin, $(2I + H)^+$, also are produced. [After J. T. Watson, *Introduction to Mass Spectrometry*, 3d ed. (Lippincott-Raven, 1997), p. 282.]



Mass spectrometry has permitted the development of *peptide mass fingerprinting*. This technique for identifying peptides has greatly enhanced the utility of two-dimensional gels. Two-dimensional electrophoresis is performed as described in Section 4.1.4. The sample of interest is extracted and cleaved *specifically* by chemical or enzymatic means. The masses of the protein fragments are then determined with the use of mass spectrometry. Finally, the peptide masses, or *fingerprint*, are matched against the fingerprint found in databases of proteins that have been “electronically cleaved” by a computer simulating the same fragmentation technique used for the experimental sample. This technique has provided some outstanding results. For example, of 150 yeast proteins analyzed with the use of two-dimensional gels, peptide mass fingerprinting unambiguously identified 80%. Mass spectrometry has provided name tags for many of the proteins in two-dimensional gels.

4.2 AMINO ACID SEQUENCES CAN BE DETERMINED BY AUTOMATED EDMAN DEGRADATION

The protein of interest having been purified and its mass determined, the next analysis usually performed is to determine the protein’s amino acid sequence, or primary structure. As stated previously (Section 3.2.1), a wealth of information about a protein’s function and evolutionary history can often be obtained from the primary structure. Let us examine first how we can sequence a simple peptide, such as



The first step is to determine the *amino acid composition* of the peptide. The peptide is hydrolyzed into its constituent amino acids by heating it in 6 N HCl at 110°C for 24 hours. Amino acids in hydrolysates can be separated by ion-exchange chromatography on columns of sulfonated polystyrene. The identity of the amino acid is revealed by its elution volume, which is the volume of buffer used to remove the amino acid from the column (Figure 4.18), and quantified by reaction with *ninhydrin*. Amino acids treated with *ninhydrin* give an intense blue color, except for *proline*, which gives a yellow color because it contains a secondary amino group. The concentration of an amino acid in a solution, after heating with *ninhydrin*, is proportional to the optical absorbance of the solution. This technique can detect a microgram (10 nmol) of an amino acid, which is about the amount

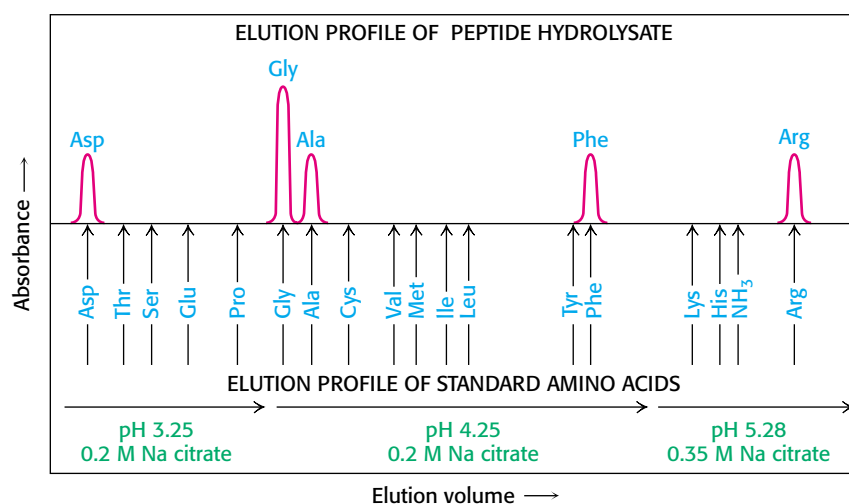
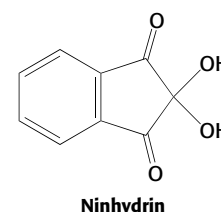
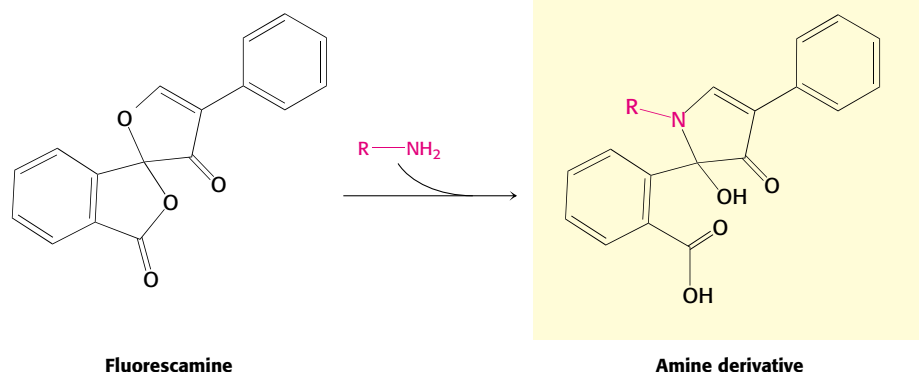


FIGURE 4.18 Determination of amino acid composition. Different amino acids in a peptide hydrolysate can be separated by ion-exchange chromatography on a sulfonated polystyrene resin (such as Dowex-50). Buffers (in this case, sodium citrate) of increasing pH are used to elute the amino acids from the column. The amount of each amino acid present is determined from the absorbance. Aspartate, which has an acidic side chain, is first to emerge, whereas arginine, which has a basic side chain, is the last. The original peptide is revealed to be composed of one aspartate, one alanine, one phenylalanine, one arginine, and two glycine residues.

FIGURE 4.19 Fluorescent derivatives of amino acids. Fluorescamine reacts with the α -amino group of an amino acid to form a fluorescent derivative.

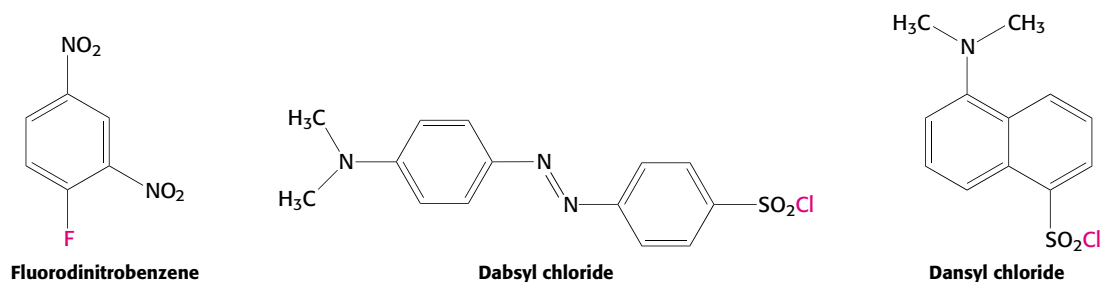


present in a thumbprint. As little as a nanogram (10 pmol) of an amino acid can be detected by replacing ninhydrin with **fluorescamine**, which reacts with the α -amino group to form a highly fluorescent product (Figure 4.19). A comparison of the chromatographic patterns of our sample hydrolysate with that of a standard mixture of amino acids would show that the amino acid composition of the peptide is

(Ala, Arg, Asp, Gly₂, Phe)

The parentheses denote that this is the amino acid composition of the peptide, not its sequence.

The next step is often to identify the N-terminal amino acid by labeling it with a compound that forms a stable covalent bond. *Fluorodinitrobenzene* (FDNB) was first used for this purpose by Frederick Sanger. *Dabsyl chloride* is now commonly used because it forms fluorescent derivatives that can be detected with high sensitivity. It reacts with an uncharged α -NH₂ group to form a sulfonamide derivative that is stable under conditions that hydrolyze peptide bonds (Figure 4.20). Hydrolysis of our sample dabsyl-peptide in 6 N HCl would yield a dabsyl-amino acid, which could be identified as dabsyl-alanine by its chromatographic properties. *Dansyl chloride*, too, is a valuable labeling reagent because it forms fluorescent sulfonamides.



Although the dabsyl method for determining the amino-terminal residue is sensitive and powerful, it cannot be used repeatedly on the same peptide, because the peptide is totally degraded in the acid-hydrolysis step and thus all sequence information is lost. Pehr Edman devised a method for labeling the amino-terminal residue and cleaving it from the peptide without disrupting the peptide bonds between the other amino acid residues. The *Edman degradation* sequentially removes one residue at a time from the amino end of a peptide (Figure 4.21). *Phenyl isothiocyanate* reacts with the uncharged terminal amino group of the peptide to form a phenylthiocarbamoyl derivative. Then, under mildly acidic conditions, a cyclic derivative of the terminal amino acid is liberated, which leaves an intact peptide shortened by one amino acid. The cyclic compound is a phenylthiohydantoin

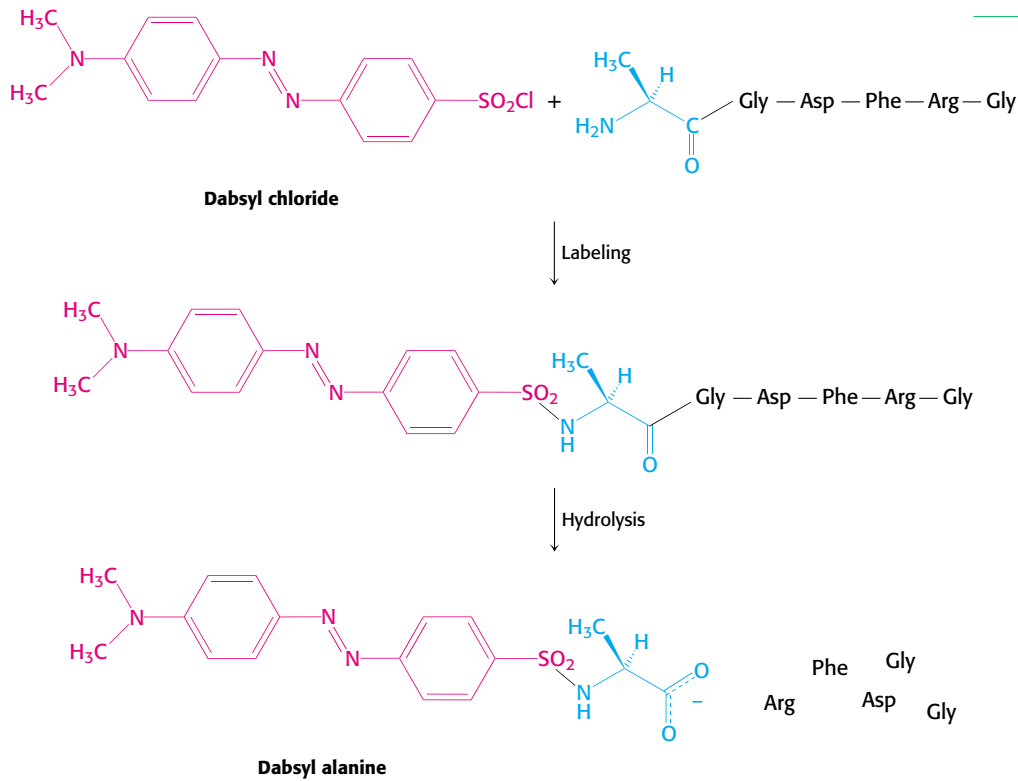


FIGURE 4.20 Determination of the amino-terminal residue of a peptide. Dabsyl chloride labels the peptide, which is then hydrolyzed with the use of hydrochloric acid. The dabsyl-amino acid (dabsyl-alanine in this example) is identified by its chromatographic characteristics.

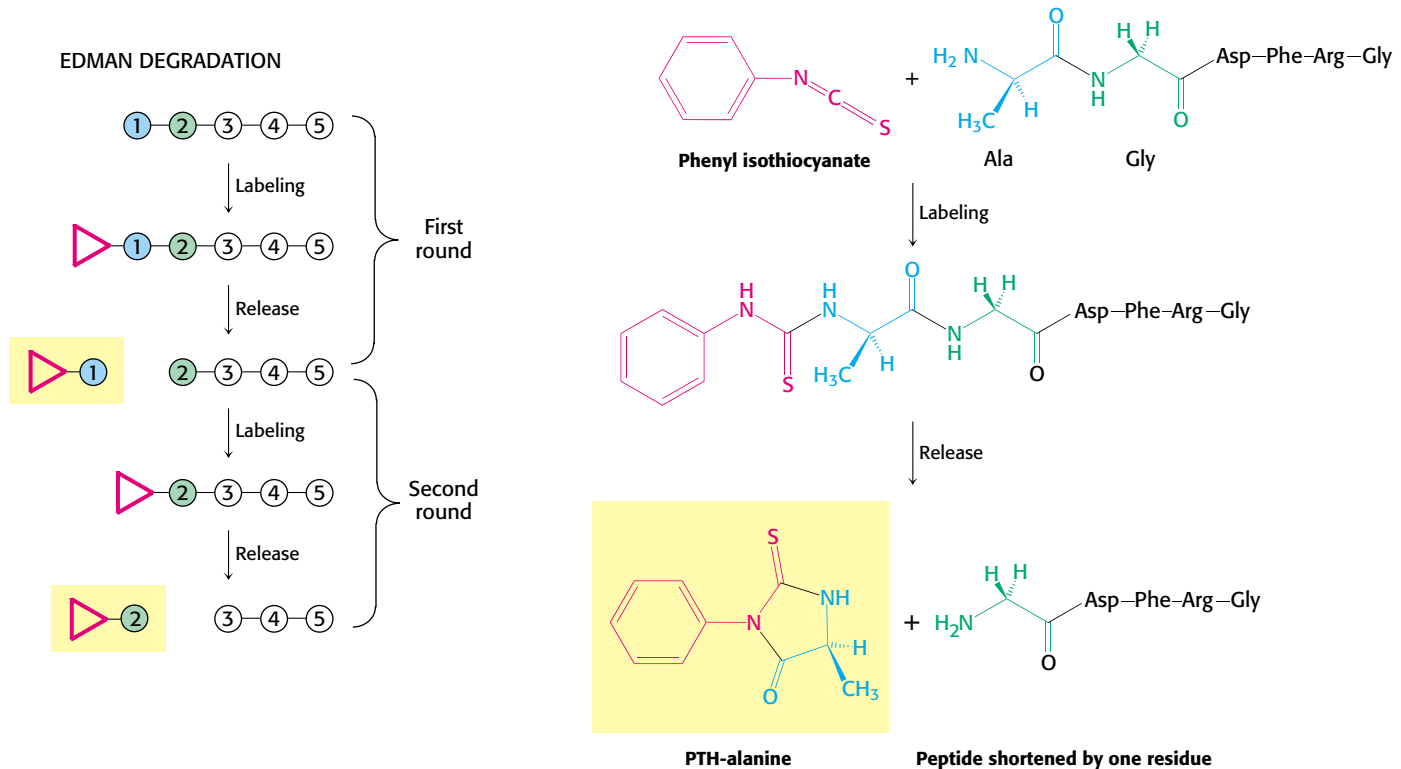


FIGURE 4.21 The Edman degradation. The labeled amino-terminal residue (PTH-alanine in the first round) can be released without hydrolyzing the rest of the peptide. Hence, the amino-terminal residue of the shortened peptide (Gly-Asp-Phe-Arg-Gly) can be determined in the second round. Three more rounds of the Edman degradation reveal the complete sequence of the original peptide.

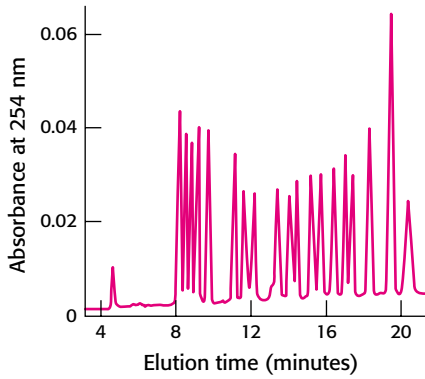


FIGURE 4.22 Separation of PTH-amino acids. PTH-amino acids can be rapidly separated by high-pressure liquid chromatography (HPLC). In this HPLC profile, a mixture of PTH-amino acids is clearly resolved into its components. An unknown amino acid can be identified by its elution position relative to the known ones.

(PTH)-amino acid, which can be identified by chromatographic procedures. The Edman procedure can then be repeated on the shortened peptide, yielding another PTH-amino acid, which can again be identified by chromatography. Three more rounds of the Edman degradation will reveal the complete sequence of the original peptide pentapeptide.

The development of automated sequencers has markedly decreased the time required to determine protein sequences. One cycle of the Edman degradation—the cleavage of an amino acid from a peptide and its identification—is carried out in less than 1 hour. By repeated degradations, the amino acid sequence of some **50 residues** in a protein can be determined. High-pressure liquid chromatography provides a sensitive means of distinguishing the various amino acids (Figure 4.22). Gas-phase sequencers can analyze picomole quantities of peptides and proteins. This high sensitivity makes it feasible to analyze the sequence of a protein sample eluted from a single band of an SDS-polyacrylamide gel.

4.2.1 Proteins Can Be Specifically Cleaved into Small Peptides to Facilitate Analysis

In principle, it should be possible to sequence an entire protein by using the Edman method. In practice, the peptides cannot be much longer than about 50 residues. This is so because the reactions of the Edman method, especially the release step, are not 100% efficient, and so not all peptides in the reaction mixture release the amino acid derivative at each step. For instance, if the efficiency of release for each round were 98%, the proportion of “correct” amino acid released after 60 rounds would be (0.98^{60}) , or 0.3—a hopelessly impure mix. This obstacle can be circumvented by cleaving the original protein at specific amino acids into smaller peptides that can be sequenced. In essence, the strategy is to *divide and conquer*.

Specific cleavage can be achieved by chemical or enzymatic methods. For example, *cyanogen bromide* (CNBr) splits polypeptide chains only on the carboxyl side of methionine residues (Figure 4.23).

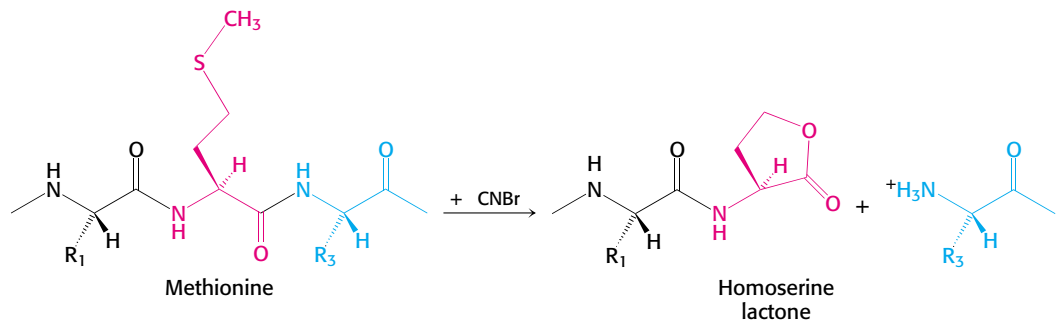


FIGURE 4.23 Cleavage by cyanogen bromide. Cyanogen bromide cleaves polypeptides on the carboxyl side of methionine residues.

A protein that has 10 methionine residues will usually yield 11 peptides on cleavage with CNBr. Highly specific cleavage is also obtained with *trypsin*, a proteolytic enzyme from pancreatic juice. Trypsin cleaves polypeptide chains on the carboxyl side of arginine and lysine residues (Figure 4.24 and Section 9.1.4). A protein that contains 9 lysine and 7 arginine residues will usually yield 17 peptides on digestion with trypsin. Each of these tryptic

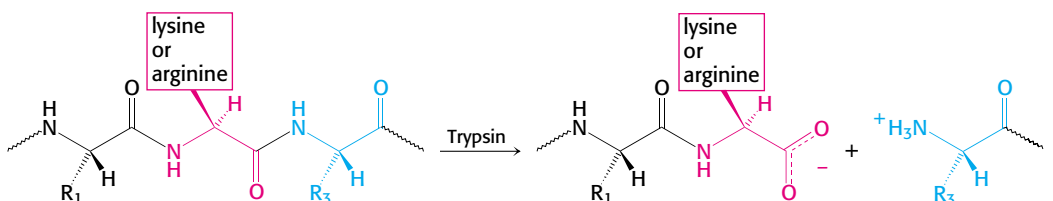


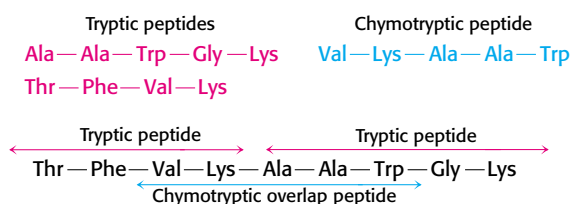
FIGURE 4.24 Cleavage by trypsin. Trypsin hydrolyzes polypeptides on the carboxyl side of arginine and lysine residues.

TABLE 4.3 Specific cleavage of polypeptides

Reagent	Cleavage site
Chemical cleavage	
Cyanogen bromide	Carboxyl side of methionine residues
O-Iodosobenzoate	Carboxyl side of tryptophan residues
Hydroxylamine	Asparagine–glycine bonds
2-Nitro-5-thiocyanobenzoate	Amino side of cysteine residues
Enzymatic cleavage	
Trypsin	Carboxyl side of lysine and arginine residues
Clostripain	Carboxyl side of arginine residues
Staphylococcal protease	Carboxyl side of aspartate and glutamate residues (glutamate only under certain conditions)
Thrombin	Carboxyl side of arginine
Chymotrypsin	Carboxyl side of tyrosine, tryptophan, phenylalanine, leucine, and methionine
Carboxypeptidase A	Amino side of C-terminal amino acid (not arginine, lysine, or proline)

peptides, except for the carboxyl-terminal peptide of the protein, will end with either arginine or lysine. Table 4.3 gives several other ways of specifically cleaving polypeptide chains.

The peptides obtained by specific chemical or enzymatic cleavage are separated by some type of chromatography. The sequence of each purified peptide is then determined by the Edman method. At this point, the amino acid sequences of segments of the protein are known, but the order of these segments is not yet defined. How can we order the peptides to obtain the primary structure of the original protein? The necessary additional information is obtained from *overlap peptides* (Figure 4.25). A second enzyme is used to split the polypeptide chain at different linkages. For example, chymotrypsin cleaves preferentially on the carboxyl side of aromatic and some other bulky nonpolar residues (Section 9.1.3). Because these chymotryptic peptides overlap two or more tryptic peptides, they can be used to establish the order of the peptides. The entire amino acid sequence of the polypeptide chain is then known.

**FIGURE 4.25** Overlap peptides.

The peptide obtained by chymotryptic digestion overlaps two tryptic peptides, establishing their order.

Additional steps are necessary if the initial protein sample is actually several polypeptide chains. SDS–gel electrophoresis under reducing conditions should display the number of chains. Alternatively, the number of distinct N-terminal amino acids could be determined. For a protein made up of two or more polypeptide chains held together by noncovalent bonds, denaturing agents, such as urea or guanidine hydrochloride, are used to dissociate

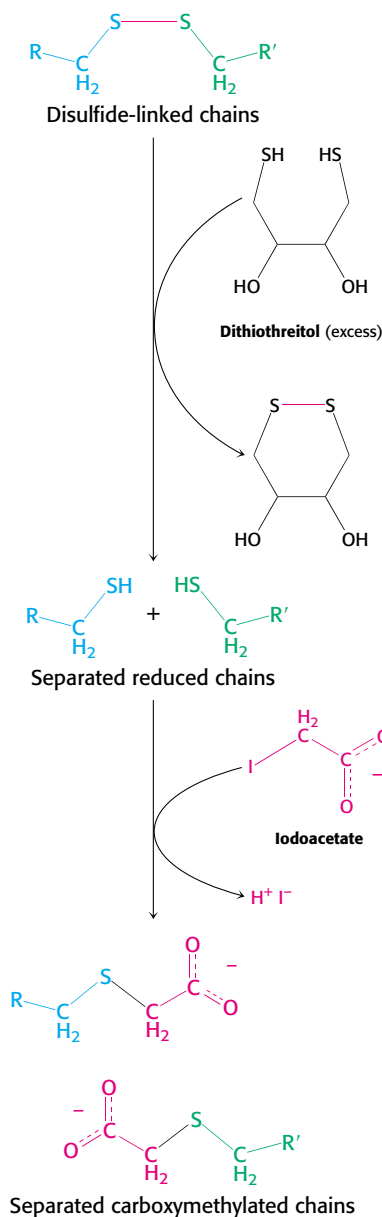
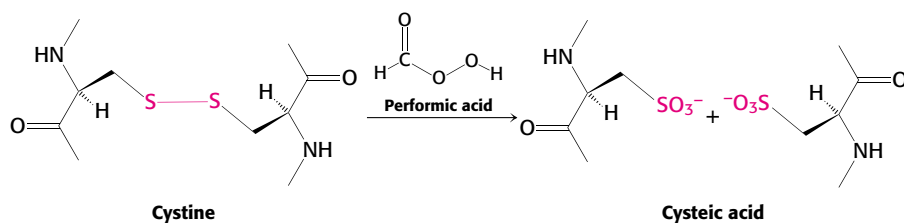


FIGURE 4.26 Disulfide-bond reduction. Polypeptides linked by disulfide bonds can be separated by reduction with dithiothreitol followed by alkylation to prevent reformation.

the chains from one another. The dissociated chains must be separated from one another before sequence determination of the individual chains can begin. Polypeptide chains linked by disulfide bonds are separated by reduction with thiols such as β -mercaptoethanol or dithiothreitol. To prevent the cysteine residues from recombining, they are then alkylated with iodoacetate to form stable *S*-carboxymethyl derivatives (Figure 4.26). Sequencing can then be performed as heretofore described.

To complete our understanding of the protein's structure, we need to determine the positions of the original disulfide bonds. This information can be obtained by using a *diagonal electrophoresis* technique to isolate the peptide sequences containing such bonds (Figure 4.27). First, the protein is specifically cleaved into peptides under conditions in which the disulfides remain intact. The mixture of peptides is applied to a corner of a sheet of paper and subjected to electrophoresis in a single lane along one side. The resulting sheet is exposed to vapors of performic acid, which cleaves disulfides and converts them into cysteic acid residues. Peptides originally linked by disulfides are now independent and more acidic because of the formation of an SO_3^- group.



This mixture is subjected to electrophoresis in the perpendicular direction under the same conditions as those of the first electrophoresis. Peptides that were devoid of disulfides will have the same mobility as before, and consequently all will be located on a single diagonal line. In contrast, the newly formed peptides containing cysteic acid will usually migrate differently from their parent disulfide-linked peptides and hence will lie off the diagonal. These peptides can then be isolated and sequenced, and the location of the disulfide bond can be established.

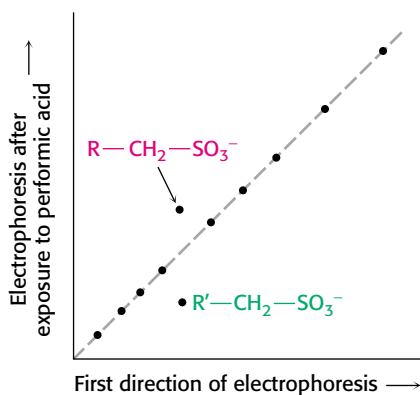


FIGURE 4.27 Diagonal electrophoresis. Peptides joined together by disulfide bonds can be detected by diagonal electrophoresis. The mixture of peptides is subjected to electrophoresis in a single lane in one direction (horizontal) and then treated with performic acid, which cleaves and oxidizes the disulfide bonds. The sample is then subjected to electrophoresis in the perpendicular direction (vertical).

4.2.2 Amino Acid Sequences Are Sources of Many Kinds of Insight

A protein's amino acid sequence, once determined, is a valuable source of insight into the protein's function, structure, and history.

1. *The sequence of a protein of interest can be compared with all other known sequences to ascertain whether significant similarities exist. Does this protein belong to one of the established families? A search for kinship between a newly sequenced protein and the thousands of previously sequenced ones takes*

only a few seconds on a personal computer (Section 7.2). If the newly isolated protein is a member of one of the established classes of protein, we can begin to infer information about the protein's function. For instance, chymotrypsin and trypsin are members of the serine protease family, a clan of proteolytic enzymes that have a common catalytic mechanism based on a reactive serine residue (Section 9.1.4). If the sequence of the newly isolated protein shows sequence similarity with trypsin or chymotrypsin, the result suggests that it may be a serine protease.

2. *Comparison of sequences of the same protein in different species yields a wealth of information about evolutionary pathways.* Genealogical relations between species can be inferred from sequence differences between their proteins. We can even estimate the time at which two evolutionary lines diverged, thanks to the clocklike nature of random mutations. For example, a comparison of serum albumins found in primates indicates that human beings and African apes diverged 5 million years ago, not 30 million years ago as was once thought. Sequence analyses have opened a new perspective on the fossil record and the pathway of human evolution.

3. *Amino acid sequences can be searched for the presence of internal repeats.* Such internal repeats can reveal information about the history of an individual protein itself. Many proteins apparently have arisen by duplication of a primordial gene followed by its diversification. For example, calmodulin, a ubiquitous calcium sensor in eukaryotes, contains four similar calcium-binding modules that arose by gene duplication (Figure 4.28).

4. *Many proteins contain amino acid sequences that serve as signals designating their destinations or controlling their processing.* A protein destined for export from a cell or for location in a membrane, for example, contains a signal sequence, a stretch of about 20 hydrophobic residues near the amino terminus that directs the protein to the appropriate membrane. Another protein may contain a stretch of amino acids that functions as a nuclear localization signal, directing the protein to the nucleus.

5. *Sequence data provide a basis for preparing antibodies specific for a protein of interest.* Careful examination of the amino acid sequence of a protein can reveal which sequences will be most likely to elicit an antibody when injected into a mouse or rabbit. Peptides with these sequences can be synthesized and used to generate antibodies to the protein. These specific antibodies can be very useful in determining the amount of a protein present in solution or in the blood, ascertaining its distribution within a cell, or cloning its gene (Section 4.3.3).

6. *Amino acid sequences are valuable for making DNA probes that are specific for the genes encoding the corresponding proteins* (Section 6.1.4). Knowledge of a protein's primary structure permits the use of reverse genetics. DNA probes that correspond to a part of the amino acid sequence can be constructed on the basis of the genetic code. These probes can be used to isolate the gene of the protein so that the entire sequence of the protein can be determined. The gene in turn can provide valuable information about the physiological regulation of the protein. Protein sequencing is an integral part of molecular genetics, just as DNA cloning is central to the analysis of protein structure and function.

4.2.3 Recombinant DNA Technology Has Revolutionized Protein Sequencing

Hundreds of proteins have been sequenced by Edman degradation of peptides derived from specific cleavages. Nevertheless, heroic effort is required to elucidate the sequence of large proteins, those with more than 1000

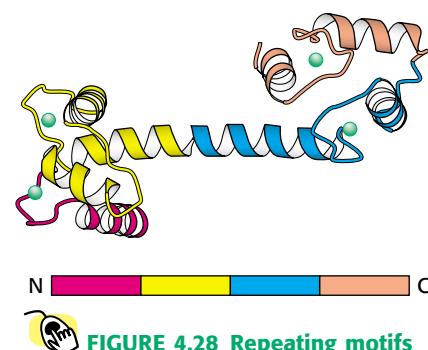


FIGURE 4.28 Repeating motifs in a protein chain. Calmodulin, a calcium sensor, contains four similar units in a single polypeptide chain shown in red, yellow, blue, and orange. Each unit binds a calcium ion (shown in green).

residues. For sequencing such proteins, a complementary experimental approach based on recombinant DNA technology is often more efficient. As will be discussed in Chapter 6, long stretches of DNA can be cloned and sequenced, and the nucleotide sequence directly reveals the amino acid sequence of the protein encoded by the gene (Figure 4.29). Recombinant DNA technology is producing a wealth of amino acid sequence information at a remarkable rate.

DNA sequence	GGG	TTC	TTG	GGA	GCA	GCA	GGA	AGC	ACT	ATG	GGC	GCA
Amino acid sequence	Gly	Phe	Leu	Gly	Ala	Ala	Gly	Ser	Thr	Met	Gly	Ala

FIGURE 4.29 DNA sequence yields the amino acid sequence. The complete nucleotide sequence of HIV-1 (human immunodeficiency virus), the cause of AIDS (acquired immune deficiency syndrome), was determined within a year after the isolation of the virus. A part of the DNA sequence specified by the RNA genome of the virus is shown here with the corresponding amino acid sequence (deduced from a knowledge of the genetic code).

Even with the use of the DNA base sequence to determine primary structure, there is still a need to work with isolated proteins. The amino acid sequence deduced by reading the DNA sequence is that of the *nascent* protein, the direct product of the translational machinery. Many proteins are modified after synthesis. Some have their ends trimmed, and others arise by cleavage of a larger initial polypeptide chain. Cysteine residues in some proteins are oxidized to form disulfide links, connecting either parts within a chain or separate polypeptide chains. Specific side chains of some proteins are altered. Amino acid sequences derived from DNA sequences are rich in information, but they do not disclose such posttranslational modifications. Chemical analyses of proteins in their final form are needed to delineate the nature of these changes, which are critical for the biological activities of most proteins. *Thus, genomic and proteomic analyses are complementary approaches to elucidating the structural basis of protein function.*

4.3 IMMUNOLOGY PROVIDES IMPORTANT TECHNIQUES WITH WHICH TO INVESTIGATE PROTEINS

Immunological methods have become important tools used to **purify** a protein, **locate** it in the cell, or **quantify** how much of the protein is present. These methods are predicated on the exquisite specificity of antibodies for their target proteins. Labeled antibodies provide a means to tag a specific protein so that it can be isolated, quantified, or **visualized**.

4.3.1 Antibodies to Specific Proteins Can Be Generated

Immunological techniques begin with the generation of antibodies to a particular protein. An *antibody* (also called an *immunoglobulin*, Ig) is a protein synthesized by an animal in response to the presence of a foreign substance, called an *antigen*, and normally functions to protect the animal from infection (Chapter 33). Antibodies have specific and high affinity for the antigens that elicited their synthesis. **Proteins, polysaccharides, and nucleic acids can be effective antigens.** An antibody recognizes a specific group or cluster of amino acids on a large molecule called an *antigenic determinant*, or **epitope** (Figures 4.30 and 4.31). Small foreign molecules, such as synthetic peptides, also can elicit antibodies, provided that the **small molecule contains a recognized epitope** and is attached to a macromolecular carrier. The small foreign molecule itself is called a **hapten**. Animals have a very

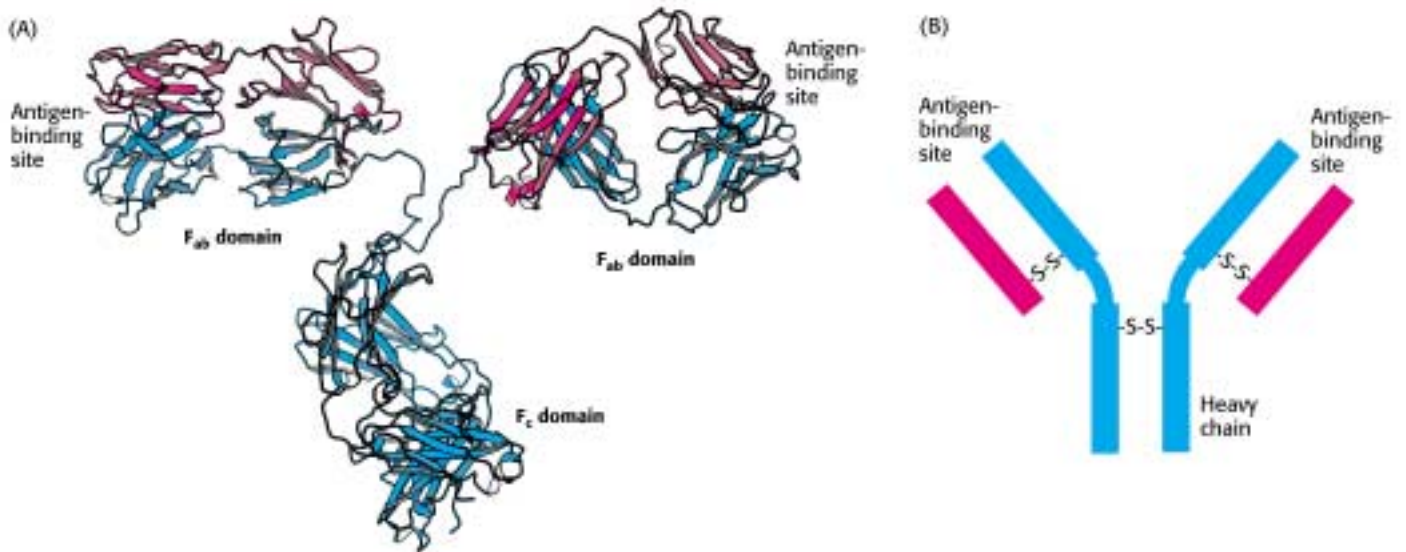


FIGURE 4.30 Antibody structure.

(A) IgG antibodies consist of four chains, two heavy chains (blue) and two light chains (red), linked by disulfide bonds. The heavy and light chains come together to form F_{ab} domains, which have the antigen-binding sites at the ends. The two heavy chains form the F_c domain. The F_{ab} domains are linked to the F_c domain by flexible linkers. (B) A more schematic representation of an IgG molecule.

large repertoire of antibody-producing cells, each producing an antibody of a single specificity. An antigen acts by stimulating the proliferation of the small number of cells that were already forming an antibody capable of recognizing the antigen (Chapter 33).

Immunological techniques depend on our being able to generate antibodies to a specific antigen. To obtain antibodies that recognize a particular protein, a biochemist injects the protein into a rabbit twice, 3 weeks apart.

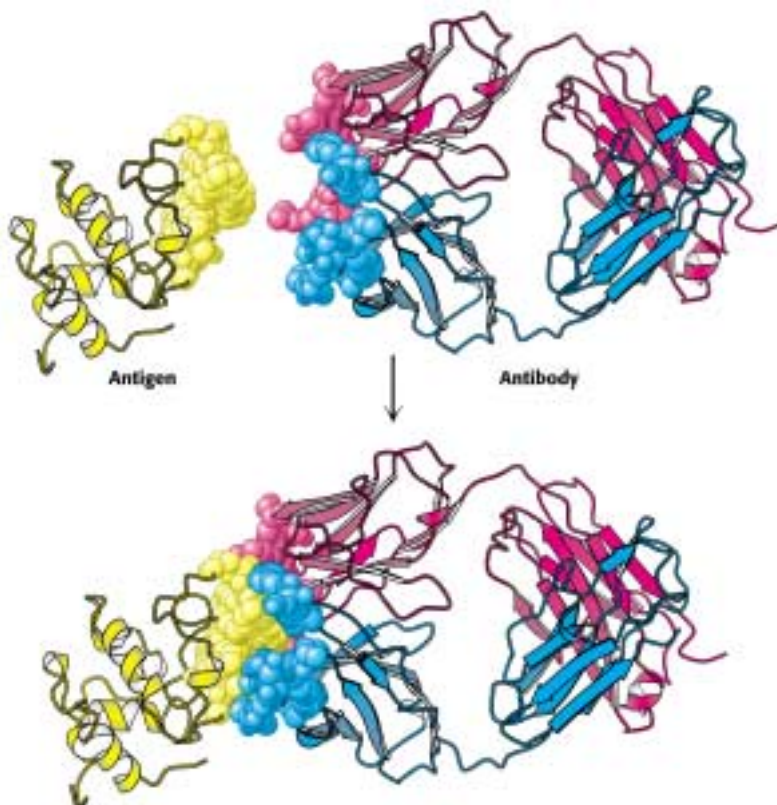


FIGURE 4.31 Antigen-antibody interactions. A protein antigen, in this case lysozyme, binds to the end of an F_{ab} domain from an antibody. The end of the antibody and the antigen have complementary shapes, allowing a large amount of surface to be buried on binding.

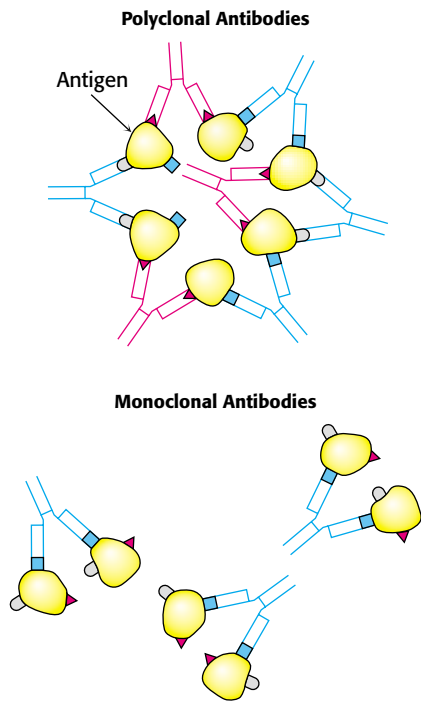


FIGURE 4.32 Polyclonal and monoclonal antibodies. Most antigens have several epitopes. Polyclonal antibodies are heterogeneous mixtures of antibodies, each specific for one of the various epitopes on an antigen. Monoclonal antibodies are all identical, produced by clones of a single antibody-producing cell. They recognize one specific epitope. [After R. A. Goldsby, T. J. Kindt, B. A. Osborne, *Kuby Immunology*, 4th ed. (W. H. Freeman and Company, 2000), p. 154.]

The injected protein stimulates the reproduction of cells producing antibodies that recognize the foreign substance. Blood is drawn from the immunized rabbit several weeks later and centrifuged to separate blood cells from the supernatant, or serum. The serum, called an *antisera*, contains antibodies to all antigens to which the rabbit has been exposed. Only some of them will be antibodies to the injected protein. Moreover, antibodies of a given specificity are not a single molecular species. For instance, 2,4-dinitrophenol (DNP) has been used as a hapten to generate antibodies to DNP. Analyses of anti-DNP antibodies revealed a wide range of binding affinities—the dissociation constants ranged from about 0.1 nM to 1 μ M. Correspondingly, a large number of bands were evident when anti-DNP antibody was subjected to isoelectric focusing. These results indicate that cells are producing many different antibodies, each recognizing a different surface feature of the same antigen. The antibodies are heterogeneous, or *polyclonal* (Figure 4.32). This heterogeneity is a barrier, which can complicate the use of these antibodies.

4.3.2 Monoclonal Antibodies with Virtually Any Desired Specificity Can Be Readily Prepared

The discovery of a means of producing *monoclonal antibodies* of virtually any desired specificity was a major breakthrough that intensified the power of immunological approaches. Just as working with impure proteins makes it difficult to interpret data and understand function, so too does working with an impure mixture of antibodies. The ideal would be to isolate a clone of cells that produce only a single antibody. The problem is that antibody-producing cells isolated from an organism die in a short time.

Immortal cell lines that produce monoclonal antibodies do exist. These cell lines are derived from a type of cancer, *multiple myeloma*, a malignant disorder of antibody-producing cells. In this cancer, a single transformed plasma cell divides uncontrollably, generating a very large number of *cells of a single kind*. They are a *clone* because they are descended from the same cell and have identical properties. The identical cells of the myeloma secrete large amounts of normal *immunoglobulin of a single kind* generation after generation. A myeloma can be transplanted from one mouse to another, where it continues to proliferate. These antibodies were useful for elucidating antibody structure, but nothing is known about their specificity and so they are useless for the immunological methods described in the next pages.

Cesar Milstein and Georges Köhler discovered that *large amounts of homogeneous antibody of nearly any desired specificity could be obtained by fusing a short-lived antibody-producing cell with an immortal myeloma cell*. An antigen is injected into a mouse, and its spleen is removed several weeks later (Figure 4.33). *A mixture of plasma cells from this spleen is fused in vitro with myeloma cells. Each of the resulting hybrid cells, called hybridoma cells,* indefinitely produces homogeneous antibody specified by the parent cell from the spleen. Hybridoma cells can then be screened, by using some sort of assay for the antigen–antibody interaction, to determine which ones produce antibody having the desired specificity. Collections of cells shown to produce the desired antibody are subdivided and reassayed. This process is repeated until a pure cell line, a clone producing a single antibody, is isolated. These positive cells can be grown in culture medium or injected into mice to induce myelomas. Alternatively, the cells can be frozen and stored for long periods.

The hybridoma method of producing monoclonal antibodies has opened new vistas in biology and medicine. *Large amounts of homogeneous antibod-*

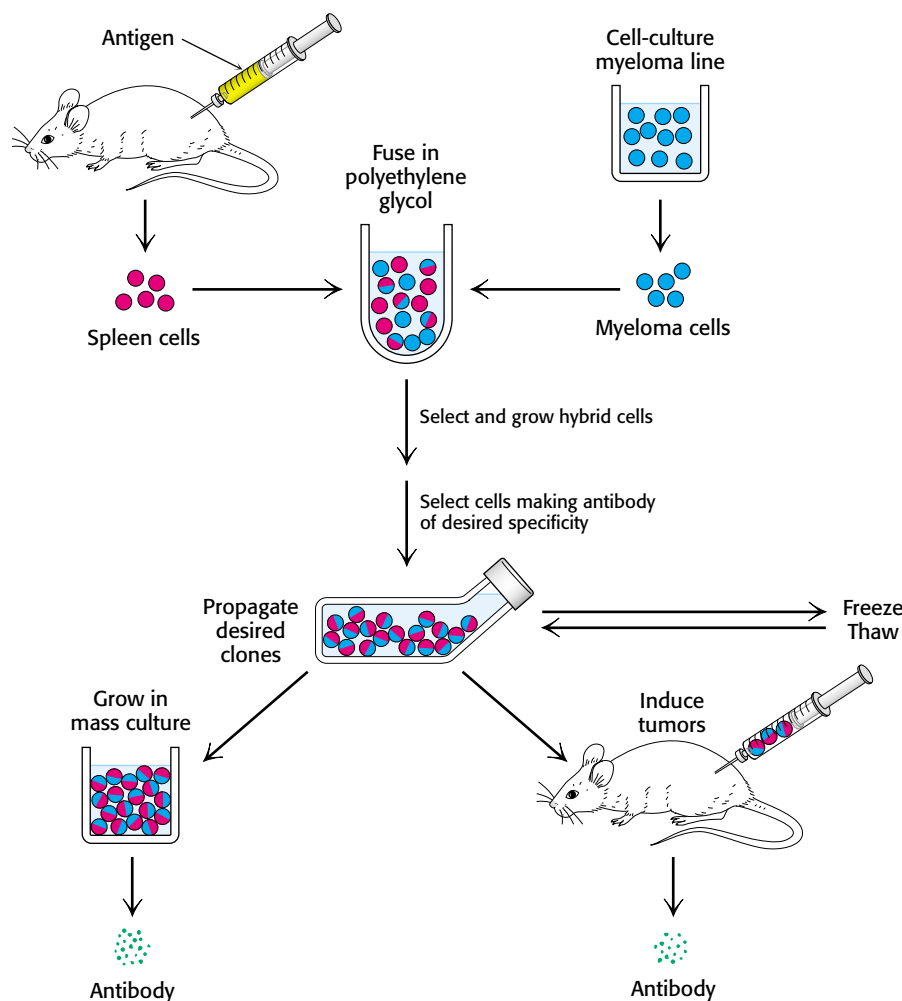


FIGURE 4.33 Preparation of monoclonal antibodies. Hybridoma cells are formed by fusion of antibody-producing cells and myeloma cells. The hybrid cells are allowed to proliferate by growing them in selective medium. They are then screened to determine which ones produce antibody of the desired specificity. [After C. Milstein. *Monoclonal Antibodies*. Copyright © 1980 by Scientific American, Inc. All rights reserved.]

ies with tailor-made specificities can be readily prepared. They are sources of insight into relations between antibody structure and specificity. Moreover, monoclonal antibodies can serve as precise analytical and preparative reagents. For example, a pure antibody can be obtained against an antigen that has not yet been isolated (Section 4.4). Proteins that guide development have been identified with the use of monoclonal antibodies as tags (Figure 4.34). Monoclonal antibodies attached to solid supports can be used as affinity columns to purify scarce proteins. This method has been used to purify interferon (an antiviral protein) 5000-fold from a crude mixture. Clinical laboratories are using monoclonal antibodies in many assays. For example, the detection in blood of isozymes that are normally localized in the heart points to a myocardial infarction (heart attack). Blood transfusions have been made safer by antibody screening of donor blood for viruses that cause AIDS (acquired immune deficiency syndrome), hepatitis, and other infectious diseases. Monoclonal antibodies are also being evaluated for use as therapeutic agents, as in the treatment of cancer. Furthermore, the vast repertoire of antibody specificity can be tapped to generate catalytic antibodies having novel features not found in naturally occurring enzymes.

4.3.3 Proteins Can Be Detected and Quantitated by Using an Enzyme-Linked Immunosorbent Assay

Antibodies can be used as exquisitely specific analytic reagents to quantify the amount of a protein or other antigen. The technique is the *enzyme-linked*



FIGURE 4.34 Fluorescence micrograph of a developing *Drosophila* embryo. The embryo was stained with a fluorescently labeled monoclonal antibody for the DNA-binding protein encoded by *engrailed*, an essential gene in specifying the body plan. [Courtesy of Dr. Nipam Patel and Dr. Corey Goodman.]

immunosorbent assay (ELISA). In this method, an enzyme, which reacts with a colorless substrate to produce a colored product, is covalently linked to a specific antibody that recognizes a target antigen. If the antigen is present, the antibody–enzyme complex will bind to it, and the enzyme component of the antibody–enzyme complex will catalyze the reaction generating the colored product. Thus, the presence of the colored product indicates the presence of the antigen. Such an enzyme-linked immunosorbent assay, which is rapid and convenient, can detect less than a nanogram (10^{-9} g) of a protein. ELISA can be performed with either polyclonal or monoclonal antibodies, but the use of monoclonal antibodies yields more reliable results.



We will consider two among the several types of ELISA. *The indirect ELISA is used to detect the presence of antibody* and is the basis of the test for HIV infection. In that test, viral core proteins (the antigen) are absorbed to the bottom of a well. Antibodies from a patient are then added to the coated well and allowed to bind to the antigen. Finally, enzyme-linked antibodies to human antibodies (for instance, goat antibodies that recognize human antibodies) are allowed to react in the well and unbound antibodies are removed by washing. Substrate is then applied. An enzyme reaction suggests that the enzyme-linked antibodies were bound to human antibodies, which in turn implies that the patient had antibodies to the viral antigen (Figure 4.35).

The sandwich ELISA allows both the detection and the quantitation of antigen. Antibody to a particular antigen is first absorbed to the bottom of a well. Next, the antigen (or blood or urine containing the antigen) is added to the well and binds to the antibody. Finally, a second, different antibody to the antigen is added. This antibody is enzyme linked and is processed as described for indirect ELISA. In this case, the extent of reaction is directly proportional to the amount of antigen present. Consequently, it permits the measurement of small quantities of antigen (see Figure 4.35).

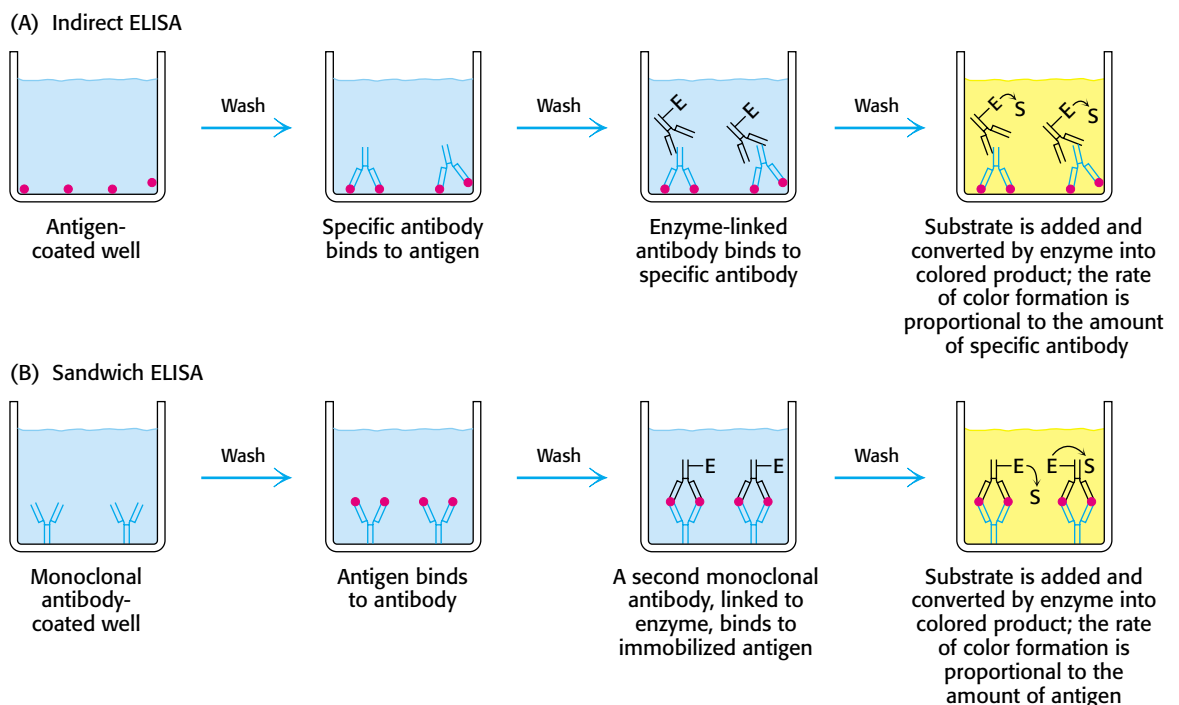


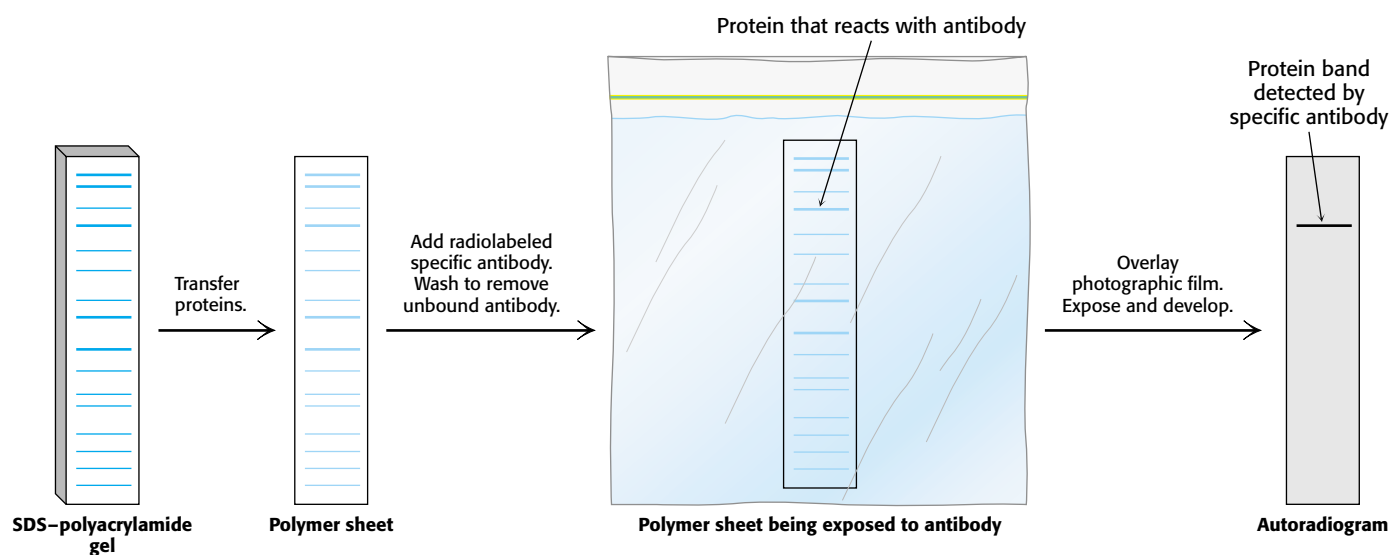
FIGURE 4.35 Indirect ELISA and sandwich ELISA (A) In indirect ELISA, the production of color indicates the amount of an antibody to a specific antigen. (B) In sandwich ELISA, the production of color indicates the quantity of antigen. [After R. A. Goldsby, T. J. Kindt, B. A. Osborne, *Kuby Immunology*, 4th ed. (W. H. Freeman and Company, 2000), p. 162.]

4.3.4 Western Blotting Permits the Detection of Proteins Separated by Gel Electrophoresis

Often it is necessary to detect small quantities of a particular protein in the presence of many other proteins, such as a viral protein in the blood. Very small quantities of a protein of interest in a cell or in body fluid can be detected by an immunoassay technique called *Western blotting* (Figure 4.36). A sample is subjected to electrophoresis on an SDS–polyacrylamide gel. Blotting (or more typically electroblotting) transfers the resolved proteins on the gel to the surface of a polymer sheet to make them more accessible for reaction. An antibody that is specific for the protein of interest is added to the sheet and reacts with the antigen. The antibody–antigen complex on the sheet then can be detected by rinsing the sheet with a second antibody specific for the first (e.g., goat antibody that recognizes mouse antibody). A radioactive label on the second antibody produces a dark band on x-ray film (an autoradiogram). Alternatively, an enzyme on the second antibody generates a colored product, as in the ELISA method. Western blotting makes it possible to find a protein in a complex mixture, the proverbial needle in a haystack. It is the basis for the test for infection by hepatitis C, where it is used to detect a core protein of the virus. This technique is also very useful in the cloning of genes.

FIGURE 4.36 Western blotting.

Proteins on an SDS–polyacrylamide gel are transferred to a polymer sheet and stained with radioactive antibody. A band corresponding to the protein to which the antibody binds appears in the autoradiogram.



4.3.5 Fluorescent Markers Make Possible the Visualization of Proteins in the Cell

Biochemistry is often performed in test tubes or polyacrylamide gels. However, most proteins function in the context of a cell. Fluorescent markers provide a powerful means of examining proteins in their biological context. For instance, cells can be stained with fluorescence-labeled antibodies or other fluorescent proteins and examined by *fluorescence microscopy* to reveal the location of a protein of interest. Arrays of parallel bundles are evident in cells stained with antibody specific for actin, a protein that polymerizes into filaments (Figure 4.37). Actin filaments are constituents of the cytoskeleton, the internal scaffolding of cells that controls their shape and movement. By tracking protein location, fluorescent markers also provide clues to protein function. For instance, the glucocorticoid receptor protein is a transcription factor that controls gene expression in response to the steroid hormone cortisol. The receptor was linked to *green fluorescent*

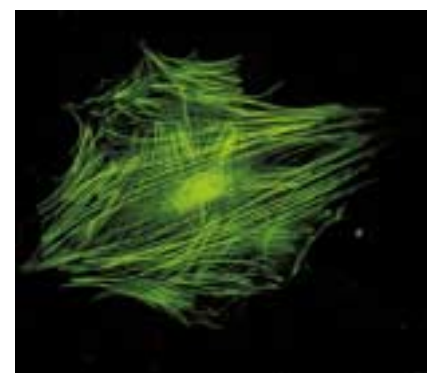


FIGURE 4.37 Actin filaments.

Fluorescence micrograph of actin filaments in a cell stained with an antibody specific to actin. [Courtesy of Dr. Elias Lazarides.]

FIGURE 4.38 Nuclear localization of a steroid receptor. (A) The receptor, made visible by attachment of the green fluorescent protein, is located predominantly in the cytoplasm of the cultured cell. (B) Subsequent to the addition of corticosterone (a glucocorticoid steroid), the receptor moves into the nucleus. [Courtesy of Professor William B. Pratt/Department of Pharmacology, University of Michigan.]

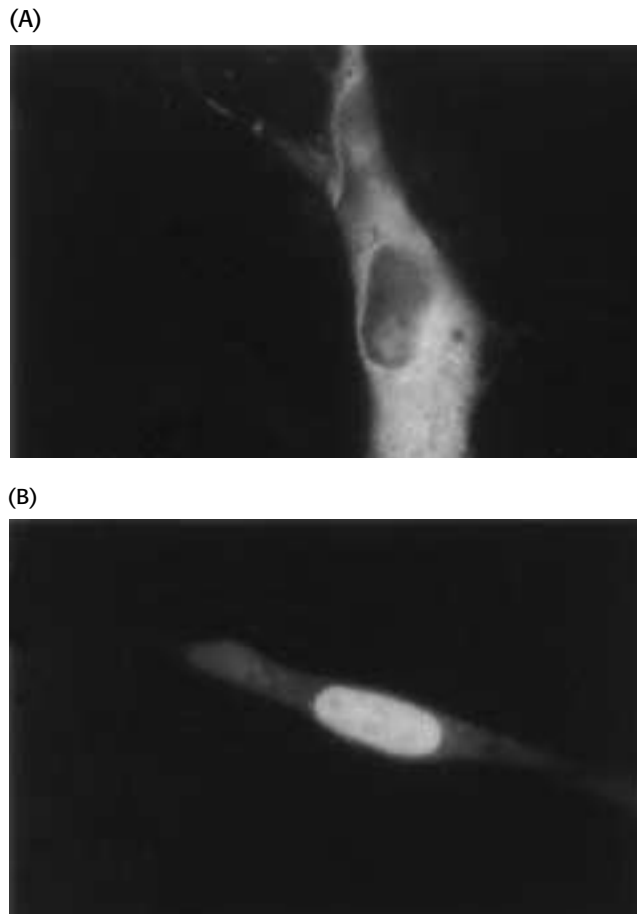


FIGURE 4.39 Immunoelectron microscopy. The opaque particles (150-Å, or 15-nm, diameter) in this electron micrograph are clusters of gold atoms bound to antibody molecules. These membrane vesicles from the synapses of neurons contain a channel protein that is recognized by the specific antibody. [Courtesy of Dr. Peter Sargent.]

protein (GFP), a naturally fluorescent protein isolated from the jellyfish *Aequorea victoria* (Section 3.6.5). Fluorescence microscopy revealed that, in the absence of the hormone, the receptor is located in the cytoplasm (Figure 4.38A). On addition of the steroid, the receptor is translocated to the nucleus, where it binds to DNA (Figure 4.38B).

The highest resolution of fluorescence microscopy is about $0.2 \mu\text{m}$ (200 nm, or 2000 Å), the wavelength of visible light. Finer spatial resolution can be achieved by electron microscopy by using antibodies tagged with electron-dense markers. For example, ferritin conjugated to an antibody can be readily visualized by electron microscopy because it contains an electron-dense core rich in iron. Clusters of gold also can be conjugated to antibodies to make them highly visible under the electron microscope. *Immunoelectron microscopy* can define the position of antigens to a resolution of 10 nm (100 Å) or finer (Figure 4.39).


4.4 PEPTIDES CAN BE SYNTHESIZED BY AUTOMATED SOLID-PHASE METHODS

The ability to synthesize peptides of defined sequence is a powerful technique for extending biochemical analysis for several reasons.

1. *Synthetic peptides can serve as antigens to stimulate the formation of specific antibodies.* For instance, as discussed earlier, it is often more efficient to obtain a protein sequence from a nucleic acid sequence than by sequencing the protein itself (see also Chapter 6). Peptides can be synthesized on the basis of the nucleic acid sequence, and antibodies can be raised that

target these peptides. These antibodies can then be used to isolate the intact protein from the cell.

2. *Synthetic peptides can be used to isolate receptors for many hormones and other signal molecules.* For example, white blood cells are attracted to bacteria by formylmethionyl (fMet) peptides released in the breakdown of bacterial proteins. Synthetic formylmethionyl peptides have been useful in identifying the cell-surface receptor for this class of peptide. Moreover, synthetic peptides can be attached to agarose beads to prepare affinity chromatography columns for the purification of receptor proteins that specifically recognize the peptides.

 3. *Synthetic peptides can serve as drugs.* Vasopressin is a peptide hormone that stimulates the reabsorption of water in the distal tubules of the kidney, leading to the formation of more concentrated urine. Patients with diabetes insipidus are deficient in *vasopressin* (also called *antidiuretic hormone*), and so they excrete large volumes of urine (more than 5 liters per day) and are continually thirsty. This defect can be treated by administering 1-desamino-8-D-arginine vasopressin, a synthetic analog of the missing hormone (Figure 4.40). This synthetic peptide is degraded *in vivo* much more slowly than vasopressin and, additionally, does not increase the blood pressure.

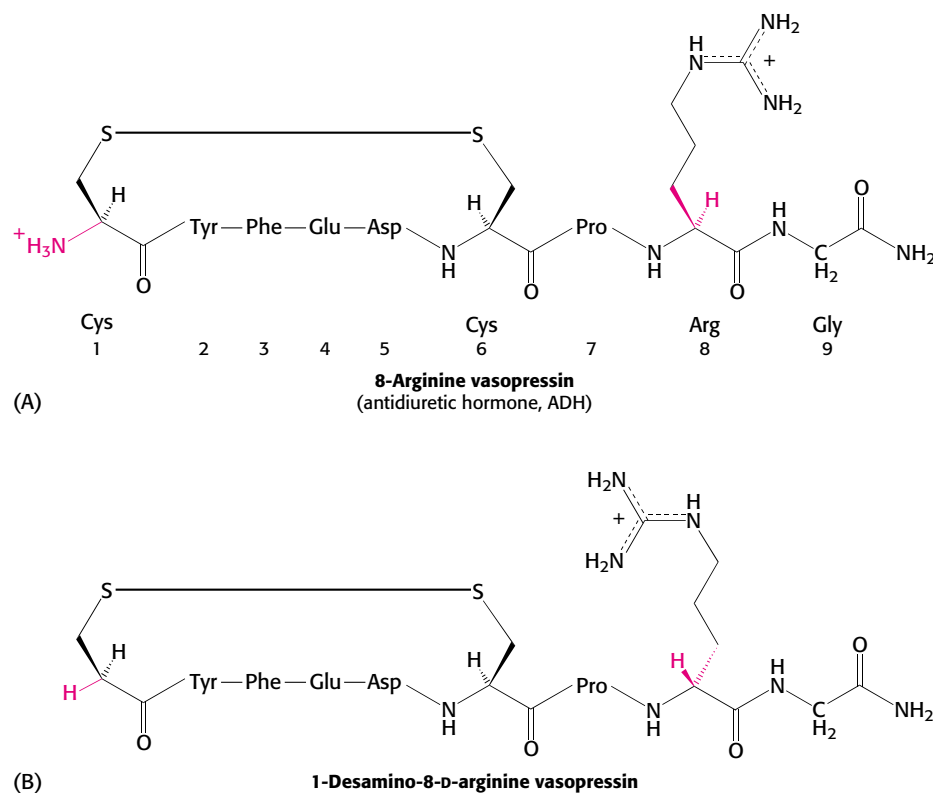
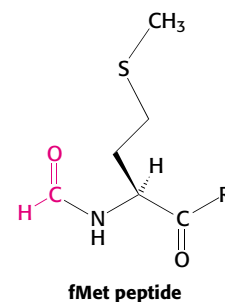


FIGURE 4.40 Vasopressin and synthetic vasopressin. Structural formulas of (A) vasopressin, a peptide hormone that stimulates water resorption, and (B) 1-desamino-8-D-arginine vasopressin, a more stable synthetic analog of this antidiuretic hormone.

4. Finally, *studying synthetic peptides can help define the rules governing the three-dimensional structure of proteins.* We can ask whether a particular sequence by itself folds into an α helix, β strand, or hairpin turn or behaves as a random coil.

How are these peptides constructed? The amino group of one amino acid is linked to the carboxyl group of another. However, a unique product is formed only if a single amino group and a single carboxyl group are available for reaction. Therefore, it is necessary to block some groups and to

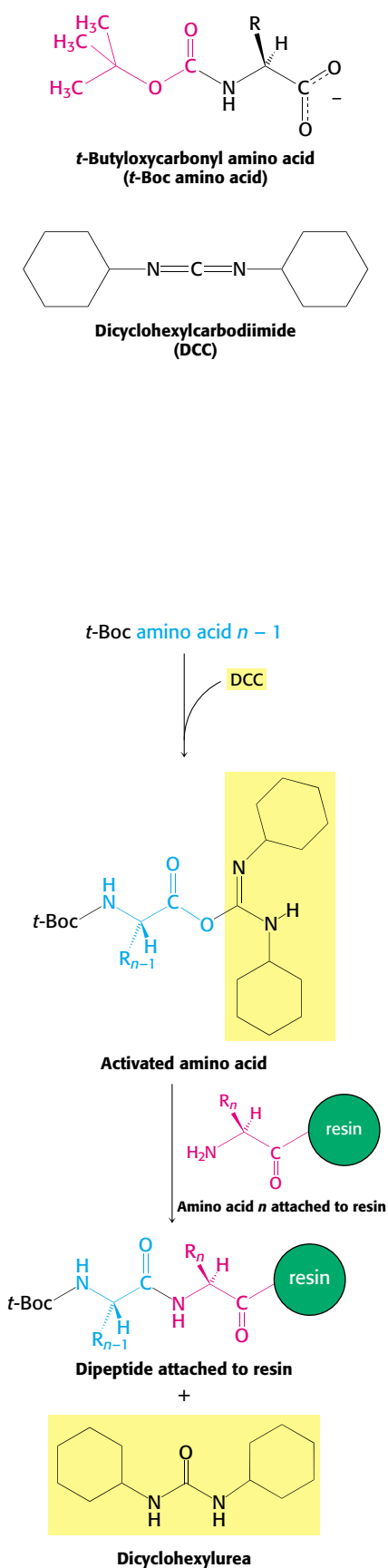


FIGURE 4.41 Amino acid activation. Dicyclohexylcarbodiimide is used to activate carboxyl groups for the formation of peptide bonds.

activate others to prevent unwanted reactions. The α -amino group of the first amino acid of the desired peptide is blocked with a *tert*-butyloxycarbonyl (*t*-Boc) group, yielding a *t*-Boc amino acid. The carboxyl group of this same amino acid is activated by reacting it with a reagent such as *dicyclohexylcarbodiimide* (DCC), as illustrated in Figure 4.41. The free amino group of the next amino acid to be linked attacks the activated carboxyl, leading to the formation of a peptide bond and the release of dicyclohexylurea. The carboxyl group of the resulting dipeptide is activated with DCC and reacted with the free amino group of the amino acid that will be the third residue

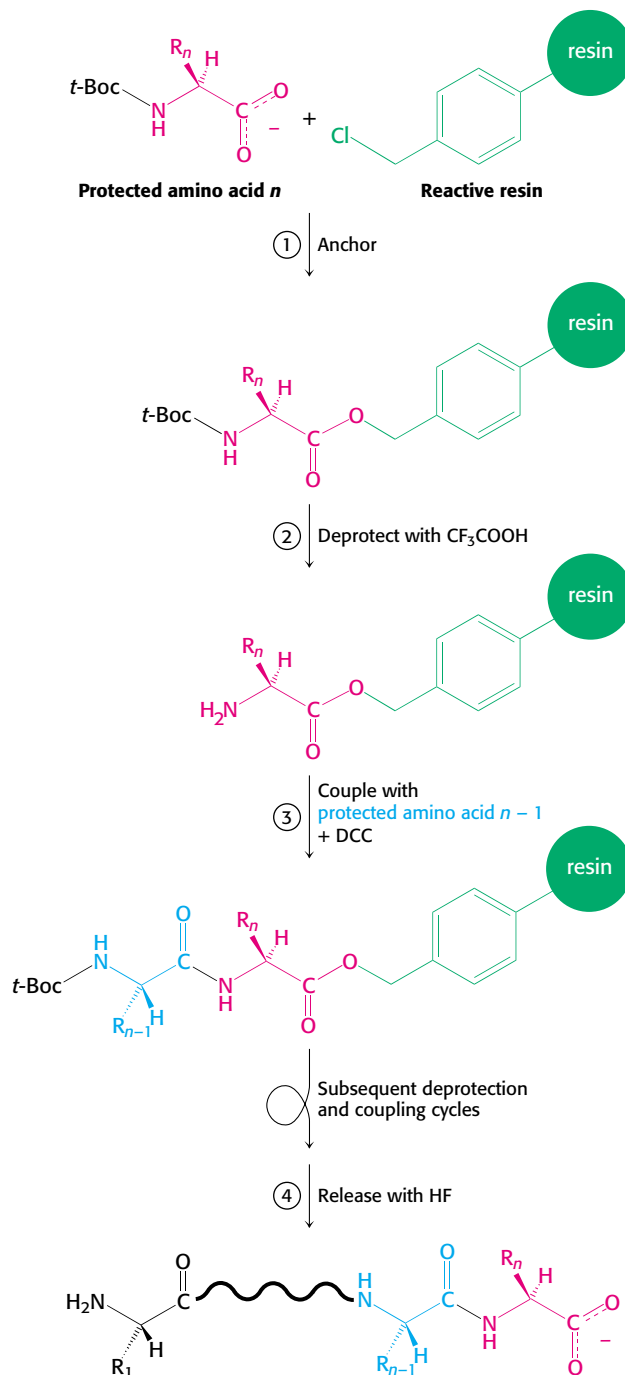


FIGURE 4.42 Solid-phase peptide synthesis. The sequence of steps in solid-phase synthesis is: (1) anchoring of the C-terminal amino acid, (2) deprotection of the amino terminus, and (3) coupling of the next residue. Steps 2 and 3 are repeated for each added amino acid. Finally, in step 4, the completed peptide is released from the resin.

in the peptide. This process is repeated until the desired peptide is synthesized. Exposing the peptide to dilute acid removes the *t*-Boc protecting group from the first amino acid while leaving peptide bonds intact.

Peptides containing more than 100 amino acids can be synthesized by sequential repetition of the preceding reactions. Linking the growing peptide chain to an insoluble matrix, such as polystyrene beads, further enhances efficiency. A major advantage of this *solid-phase method* is that the desired product at each stage is bound to beads that can be rapidly filtered and washed, and so there is no need to purify intermediates. All reactions are carried out in a single vessel, eliminating losses caused by repeated transfers of products. The carboxyl-terminal amino acid of the desired peptide sequence is first anchored to the polystyrene beads (Figure 4.42). The *t*-Boc protecting group of this amino acid is then removed. The next amino acid (in the protected *t*-Boc form) and dicyclohexylcarbodiimide, the coupling agent, are added together. After the peptide bond forms, excess reagents and dicyclohexylurea are washed away, leaving the desired dipeptide product attached to the beads. Additional amino acids are linked by the same sequence of reactions. At the end of the synthesis, the peptide is released from the beads by adding hydrofluoric acid (HF), which cleaves the carboxyl ester anchor without disrupting peptide bonds. Protecting groups on potentially reactive side chains, such as that of lysine, also are removed at this time. This cycle of reactions can be readily automated, which makes it feasible to routinely synthesize peptides containing about 50 residues in good yield and purity. In fact, the solid-phase method has been used to synthesize interferons (155 residues) that have antiviral activity and ribonuclease (124 residues) that is catalytically active.

4.5 THREE-DIMENSIONAL PROTEIN STRUCTURE CAN BE DETERMINED BY NMR SPECTROSCOPY AND X-RAY CRYSTALLOGRAPHY

A crucial question is, What does the three-dimensional structure of a specific protein look like? Protein structure determines function, given that the specificity of active sites and binding sites depends on the precise three-dimensional conformation. Nuclear magnetic resonance spectroscopy and x-ray crystallography are two of the most important techniques for elucidating the conformation of proteins.

4.5.1 Nuclear Magnetic Resonance Spectroscopy Can Reveal the Structures of Proteins in Solution

Nuclear magnetic resonance (NMR) spectroscopy is unique in being able to reveal the *atomic structure* of macromolecules *in solution*, provided that highly concentrated solutions (~ 1 mM, or 15 mg ml^{-1} for a 15-kd protein) can be obtained. This technique depends on the fact that certain atomic nuclei are intrinsically magnetic. Only a limited number of isotopes display this property, called *spin*, and the ones most important to biochemistry are listed in Table 4.4. The simplest example is the hydrogen nucleus (^1H), which is a proton. The spinning of a proton generates a magnetic moment. This moment can take either of two orientations, or spin states (called α and β), when an external magnetic field is applied (Figure 4.43). The energy difference between these states is proportional to the strength of the imposed magnetic field. The α state has a slightly lower energy and hence is slightly more populated (by a factor of the order of 1.00001 in a typical experiment) because it is aligned with the field. A spinning proton in an α state can be

TABLE 4.4 Biologically important nuclei giving NMR signals

Nucleus	Natural abundance (% by weight of the element)
^1H	99.984
^2H	0.016
^{13}C	1.108
^{14}N	99.635
^{15}N	0.365
^{17}O	0.037
^{23}Na	100.0
^{25}Mg	10.05
^{31}P	100.0
^{35}Cl	75.4
^{39}K	93.1

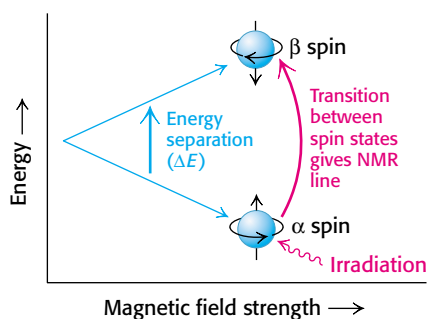
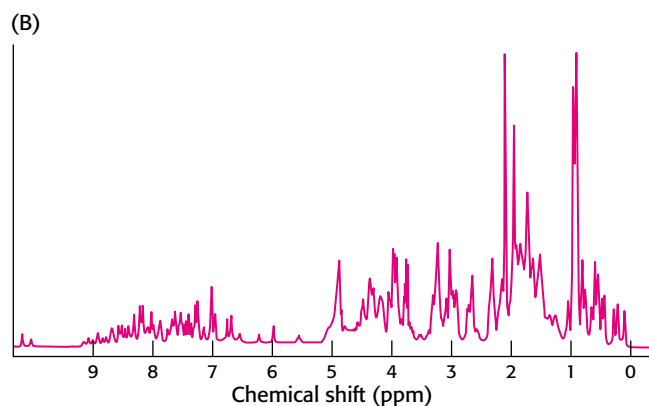
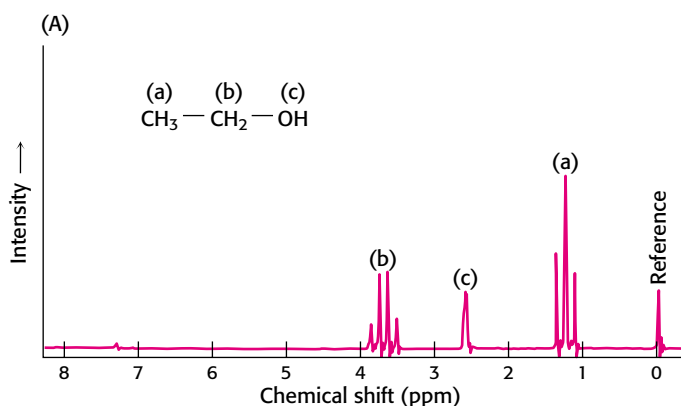


FIGURE 4.43 Basis of NMR spectroscopy. The energies of the two orientations of a nucleus of spin $\frac{1}{2}$ (such as ^{31}P and ^1H) depend on the strength of the applied magnetic field. Absorption of electromagnetic radiation of appropriate frequency induces a transition from the lower to the upper level.

FIGURE 4.44 One-dimensional NMR spectra. (A) ^1H -NMR spectrum of ethanol ($\text{CH}_3\text{CH}_2\text{OH}$) shows that the chemical shifts for the hydrogen are clearly resolved. (B) ^1H -NMR spectrum from a 55 amino acid fragment of a protein with a role in RNA splicing shows a greater degree of complexity. A large number of peaks are present and many overlap. [(A) After C. Branden and J. Tooze, *Introduction to Protein Structure* (Garland, 1991), p. 280; (B) courtesy of Barbara Amann and Wesley McDermott.]



raised to an excited state (β state) by applying a pulse of electromagnetic radiation (a radio-frequency, or RF, pulse), provided the frequency corresponds to the energy difference between the α and the β states. In these circumstances, the spin will change from α to β ; in other words, *resonance* will be obtained. A resonance spectrum for a molecule can be obtained by varying the magnetic field at a constant frequency of electromagnetic radiation or by keeping the magnetic field constant and varying electromagnetic radiation.

These properties can be used to examine the chemical surroundings of the hydrogen nucleus. The flow of electrons around a magnetic nucleus generates a small local magnetic field that opposes the applied field. The degree of such shielding depends on the surrounding electron density. Consequently, nuclei in different environments will change states, or resonate, at slightly different field strengths or radiation frequencies. The nuclei of the perturbed sample absorb electromagnetic radiation at a frequency that can be measured. The different frequencies, termed *chemical shifts*, are expressed in fractional units δ (parts per million, or ppm) relative to the shifts of a standard compound, such as a water-soluble derivative of tetramethylsilane, that is added with the sample. For example, a $-\text{CH}_3$ proton typically exhibits a chemical shift (δ) of 1 ppm, compared with a chemical shift of 7 ppm for an aromatic proton. The chemical shifts of most protons in protein molecules fall between 0 and 9 ppm (Figure 4.44). It is possible to resolve most protons in many proteins by using this technique of *one-dimensional NMR*. With this information, we can then deduce changes to a particular chemical group under different conditions, such as the conformational change of a protein from a disordered structure to an α helix in response to a change in pH.

We can garner even more information by examining how the spins on different protons affect their neighbors. By inducing a transient magnetization in a sample through the application a radio-frequency pulse, it is possible to alter the spin on one nucleus and examine the effect on the spin of a neighboring nucleus. Especially revealing is a *two-dimensional spectrum* obtained by *nuclear Overhauser enhancement spectroscopy* (NOESY), which graphically displays pairs of protons that are in close proximity, even if they are not close together in the primary structure. The basis for this technique is the *nuclear Overhauser effect* (NOE), an interaction between nuclei that is proportional to the inverse sixth power of the distance between them. Magnetization is transferred from an excited nucleus to an unexcited one if they are less than about 5 \AA apart (Figure 4.45A). In other words, the effect provides a means of detecting the location of atoms relative to one another in the three-dimensional structure of the protein. The diagonal of a NOESY spectrum corresponds to a one-dimensional spectrum. The off-diagonal peaks provide crucial new information: *they identify pairs of protons*

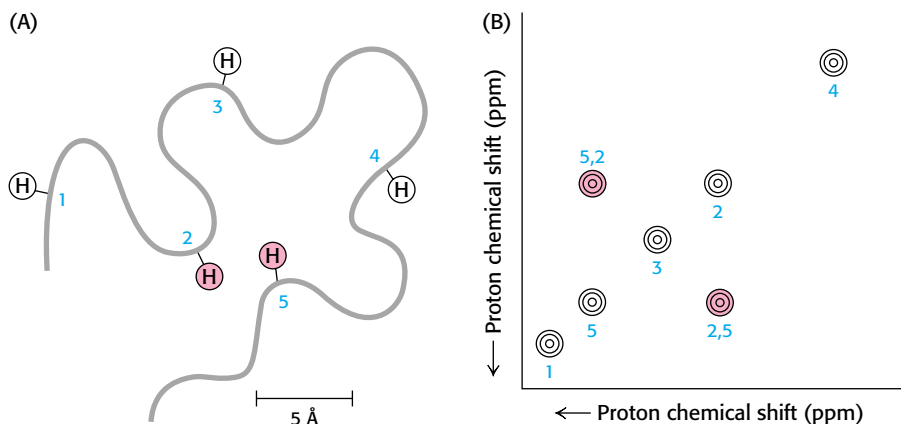


FIGURE 4.45 The nuclear Overhauser effect. The nuclear Overhauser effect (NOE) identifies pairs of protons that are in close proximity. (A) Schematic representation of a polypeptide chain highlighting five particular protons. Protons 2 and 5 are in close proximity (~ 4 Å apart), whereas other pairs are farther apart. (B) A highly simplified NOESY spectrum. The diagonal shows five peaks corresponding to the five protons in part A. The peaks above the diagonal and the symmetrically related one below reveal that proton 2 is close to proton 5.

that are less than 5 Å apart (Figure 4.45B). A two-dimensional NOESY spectrum for a protein comprising 55 amino acids is shown in Figure 4.46. The large number of off-diagonal peaks reveals short proton–proton distances. The three-dimensional structure of a protein can be reconstructed with the use of such proximity relations. Structures are calculated such that protons that must be separated by less than 5 Å on the basis of NOESY spectra are close to one another in the three-dimensional structure (Figure 4.47). If a sufficient number of distance constraints are applied, the three-dimensional structure can be determined nearly uniquely. A family of related structures is generated for three reasons (Figure 4.48). First, not enough constraints may be experimentally accessible to fully specify the structure. Second, the distances obtained from analysis of the NOESY spectrum are only approximate. Finally, the experimental observations are made not on single molecules but on a large number of molecules in solution that may have slightly different structures at any given moment. Thus, the family of structures generated from NMR structure analysis indicates the range of conformations for the protein in solution. At present, NMR spectroscopy can determine the structures of only relatively small proteins (< 40 kd), but its revolving power is certain to increase. The power of

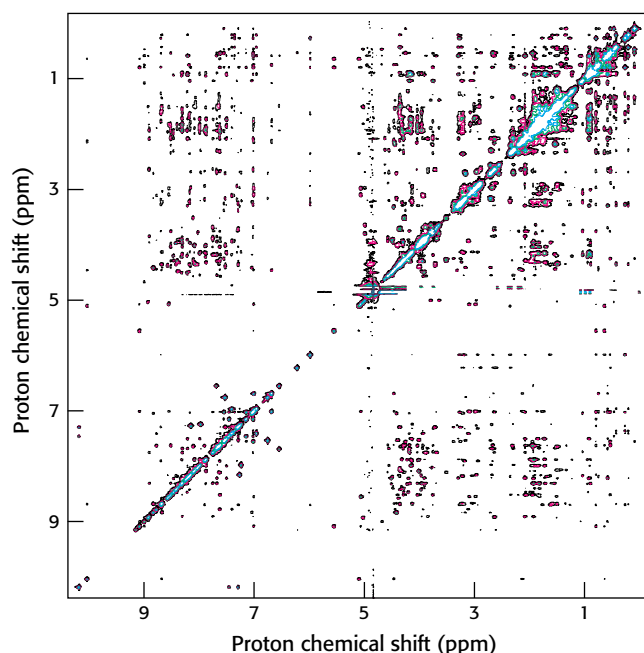


FIGURE 4.46 Detecting short proton–proton distances. A NOESY spectrum for a 55 amino acid domain from a protein having a role in RNA splicing. Each off-diagonal peak corresponds to a short proton–proton separation. This spectrum reveals hundreds of such short proton–proton distances, which can be used to determine the three-dimensional structure of this domain. [Courtesy of Barbara Amann and Wesley McDermott.]

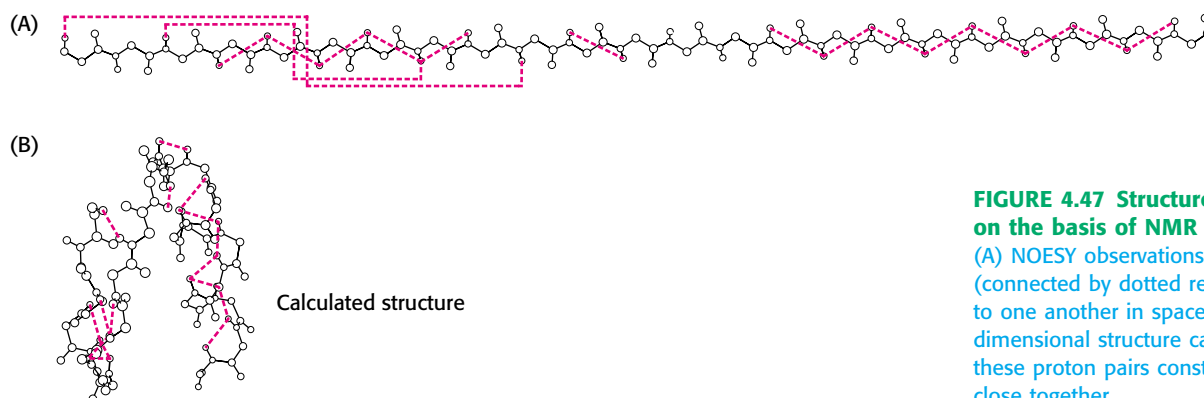


FIGURE 4.47 Structures calculated on the basis of NMR constraints. (A) NOESY observations show that protons (connected by dotted red lines) are close to one another in space. (B) A three-dimensional structure calculated with these proton pairs constrained to be close together.



FIGURE 4.48 A family of structures. A set of 25 structures for a 28 amino acid domain from a zinc-finger-DNA-binding protein. The red line traces the average course of the protein backbone. Each of these structures is consistent with hundreds of constraints derived from NMR experiments. The differences between the individual structures are due to a combination of imperfections in the experimental data and the dynamic nature of proteins in solution. [Courtesy of Barbara Amann.]

NMR has been greatly enhanced by the ability to produce proteins labeled uniformly or at specific sites with ^{13}C , ^{15}N , and ^2H with the use of recombinant DNA technology (Chapter 6).

4.5.2 X-Ray Crystallography Reveals Three-Dimensional Structure in Atomic Detail

X-ray crystallography provides the finest visualization of protein structure currently available. This technique can reveal the precise three-dimensional positions of most atoms in a protein molecule. The use of x-rays provides the best resolution because the wavelength of x-rays is about the same length as that of a covalent bond. The three components in an x-ray crystallographic analysis are a *protein crystal*, a *source of x-rays*, and a *detector* (Figure 4.49).

The technique requires that all molecules be precisely oriented, so the first step is to obtain crystals of the protein of interest. Slowly adding ammonium sulfate or another salt to a concentrated solution of protein to reduce its solubility favors the formation of highly ordered crystals. This is the process of salting out discussed in Section 4.1.3. For example, myoglobin crystallizes in 3 M ammonium sulfate (Figure 4.50). Some proteins crystallize readily, whereas others do so only after much effort has been expended in identifying the right conditions. Crystallization is an art; the best practitioners have great perseverance and patience. Increasingly large and complex proteins are being crystallized. For example, poliovirus, an 8500-kd assembly of 240 protein subunits surrounding an RNA core, has been crystallized and its structure solved by x-ray methods. Crucially, protein crystals frequently display their biological activity, indicating that the proteins have crystallized in their biologically active configuration. For instance, enzyme crystals may display catalytic activity if the crystals are suffused with substrate.

Next, a source of x-rays is required. A beam of x-rays of wavelength 1.54 \AA is produced by accelerating electrons against a copper target. A narrow beam of x-rays strikes the protein crystal. Part of the beam goes straight through the crystal; the rest is *scattered* in various directions. Finally, these scattered, or *diffracted*, x-rays are detected by x-ray film, the blackening of the emulsion being proportional to the intensity of the scattered x-ray beam, or by a solid-state electronic detector. The scattering pattern provides abundant information about protein structure. The basic physical principles underlying the technique are:

1. *Electrons scatter x-rays.* The amplitude of the wave scattered by an atom is proportional to its number of electrons. Thus, a carbon atom scatters six times as strongly as a hydrogen atom does.
2. *The scattered waves recombine.* Each atom contributes to each scattered beam. The scattered waves reinforce one another at the film or detector if they are in phase (in step) there, and they cancel one another if they are out of phase.
3. *The way in which the scattered waves recombine depends only on the atomic arrangement.*

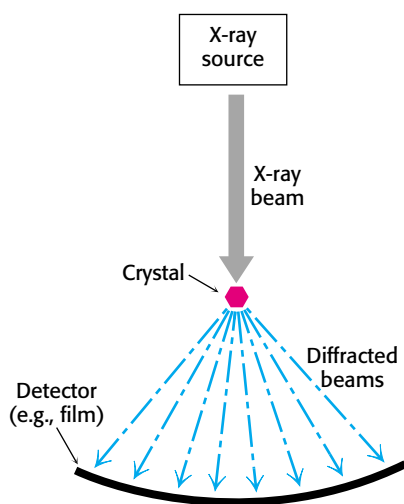


FIGURE 4.49 Essence of an x-ray crystallographic experiment: an x-ray beam, a crystal, and a detector.

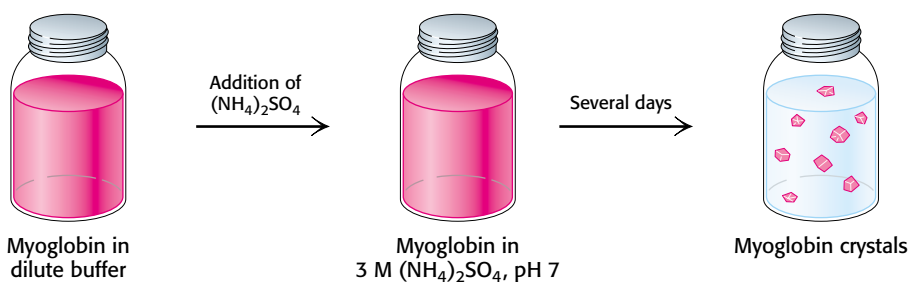


FIGURE 4.50 Crystallization of myoglobin.

The protein crystal is mounted and positioned in a precise orientation with respect to the x-ray beam and the film. The crystal is rotated so that the beam can strike the crystal from many directions. This rotational motion results in an x-ray photograph consisting of a regular array of spots called *reflections*. The x-ray photograph shown in Figure 4.51 is a two-dimensional section through a three-dimensional array of 25,000 spots. The intensity of each spot is measured. These *intensities and their positions* are the basic experimental data of an x-ray crystallographic analysis. The next step is to reconstruct an image of the protein from the observed intensities. In light microscopy or electron microscopy, the diffracted beams are focused by lenses to directly form an image. However, appropriate lenses for focusing x-rays do not exist. Instead, the image is formed by applying a mathematical relation called a Fourier transform. For each spot, this operation yields a wave of electron density whose amplitude is proportional to the square root of the observed intensity of the spot. Each wave also has a *phase*—that is, the timing of its crests and troughs relative to those of other waves. The phase of each wave determines whether the wave reinforces or cancels the waves contributed by the other spots. These phases can be deduced from the well-understood diffraction patterns produced by electron-dense heavy-atom reference markers such as uranium or mercury at specific sites in the protein.

The stage is then set for the calculation of an electron-density map, which gives the density of electrons at a large number of regularly spaced points in the crystal. This three-dimensional electron-density distribution is represented by a series of parallel sections stacked on top of one another. Each section is a transparent plastic sheet (or, more recently, a layer in a computer image) on which the electron-density distribution is represented by contour lines (Figure 4.52), like the contour lines used in geological survey maps to depict altitude (Figure 4.53). The next step is to interpret the electron-density map. A critical factor is the *resolution* of the x-ray analysis, which is determined by the number of scattered intensities used in the Fourier synthesis. The fidelity of the image depends on the resolution of the

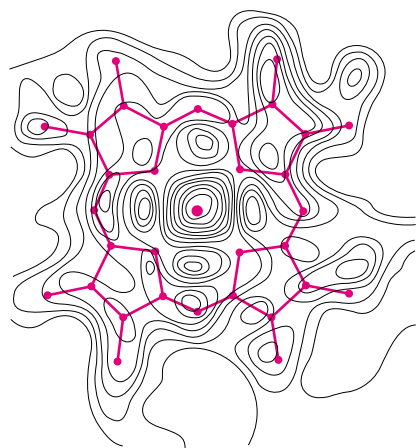


FIGURE 4.52 Section of the electron-density map of myoglobin. This section of the electron-density map shows the heme group. The peak of the center of this section corresponds to the position of the iron atom. [From J. C. Kendrew. The three-dimensional structure of a protein molecule. Copyright © 1961 by Scientific American, Inc. All rights reserved.]

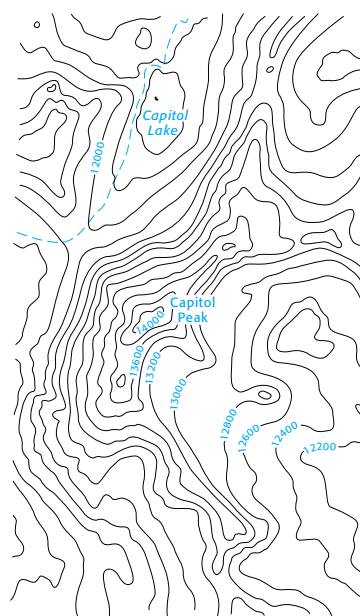


FIGURE 4.53 Section of a U.S. Geological Survey map. Capitol Peak Quadrangle, Colorado.

(A)



(B)

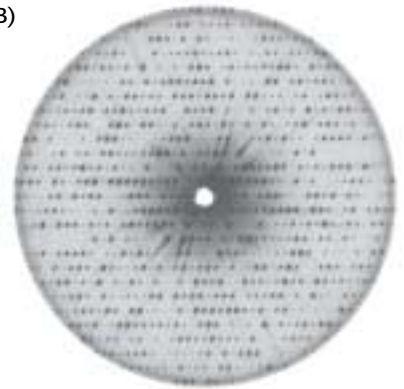
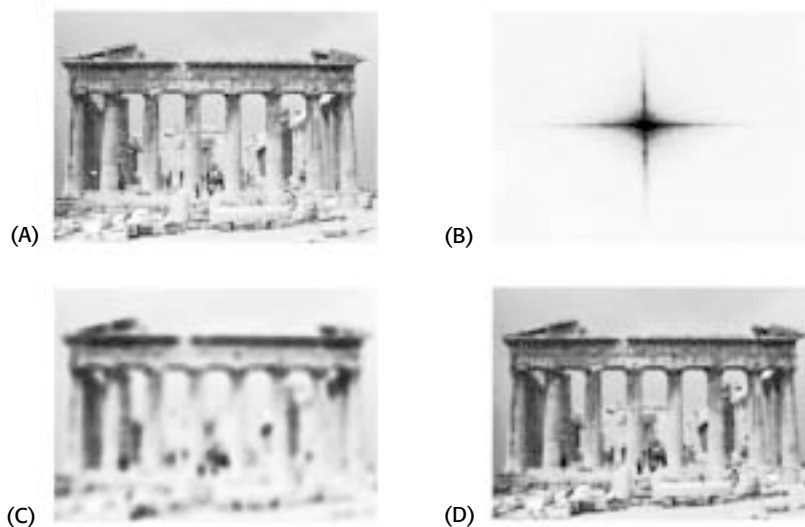


FIGURE 4.51 Myoglobin crystal and x-ray. (A) Crystal of myoglobin. (B) X-ray precession photograph of a myoglobin crystal. [(A) Mel Pollinger/Fran Heyl Associates.]

FIGURE 4.54 Resolution affects the quality of an image. The effect of resolution on the quality of a reconstructed image is shown by an optical analog of x-ray diffraction: (A) a photograph of the Parthenon; (B) an optical diffraction pattern of the Parthenon; (C and D) images reconstructed from the pattern in part B. More data were used to obtain image D than image C, which accounts for the higher quality of image D. [(A) Courtesy of Dr. Thomas Steitz. (B) Courtesy of Dr. David DeRosier.]



Fourier synthesis, as shown by the optical analogy in Figure 4.54. A resolution of 6 Å reveals the course of the polypeptide chain but few other structural details. The reason is that polypeptide chains pack together so that their centers are between 5 Å and 10 Å apart. Maps at higher resolution are needed to delineate groups of atoms, which lie between 2.8 Å and 4.0 Å apart, and individual atoms, which are between 1.0 Å and 1.5 Å apart. The ultimate resolution of an x-ray analysis is determined by the degree of perfection of the crystal. For proteins, this limiting resolution is usually about 2 Å.

The structures of more than 10,000 proteins had been elucidated by NMR and x-ray crystallography by mid-2000, and several new structures are now determined each day. The coordinates are collected at the Protein Data Bank (<http://www.rcsb.org/pdb>) and the structures can be accessed for visualization and analysis. Knowledge of the detailed molecular architecture of proteins has been a source of insight into how proteins recognize and bind other molecules, how they function as enzymes, how they fold, and how they evolved. This extraordinarily rich harvest is continuing at a rapid pace and is greatly influencing the entire field of biochemistry.

SUMMARY

- The rapid progress in gene sequencing has advanced another goal of biochemistry—elucidation of the proteome. The proteome is the complete set of proteins expressed and includes information about how they are modified, how they function, and how they interact with other molecules.

- **The Purification of Proteins Is an Essential Step in Understanding Their Function**

Proteins can be separated from one another and from other molecules on the basis of such characteristics as solubility, size, charge, and binding affinity. SDS–polyacrylamide gel electrophoresis separates the polypeptide chains of proteins under denaturing conditions largely according to mass. Proteins can also be separated electrophoretically on the basis of net charge by isoelectric focusing in a pH gradient. Ultracentrifugation and gel-filtration chromatography resolve proteins according to size, whereas ion-exchange chromatography separates them mainly on the basis of net charge. The high affinity of many proteins for specific chemical groups is exploited in affinity chromatography, in

which proteins bind to columns containing beads bearing covalently linked substrates, inhibitors, or other specifically recognized groups. The mass of a protein can be precisely determined by sedimentation equilibrium measurements or by mass spectrometry.

- **Amino Acid Sequences Can Be Determined by Automated Edman Degradation**

The amino acid composition of a protein can be ascertained by hydrolyzing it into its constituent amino acids in 6 N HCl at 110°C. The amino acids can be separated by ion-exchange chromatography and quantitated by reacting them with ninhydrin or fluorescamine. Amino acid sequences can be determined by Edman degradation, which removes one amino acid at a time from the amino end of a peptide. Phenyl isothiocyanate reacts with the terminal amino group to form a phenylthiocarbamoyl derivative, which cyclizes under mildly acidic conditions to give a phenylthiohydantoin–amino acid and a peptide shortened by one residue. Automated repeated Edman degradations by a sequenator can analyze sequences of about 50 residues. Longer polypeptide chains are broken into shorter ones for analysis by specifically cleaving them with a reagent such as cyanogen bromide, which splits peptide bonds on the carboxyl side of methionine residues. Enzymes such as trypsin, which cleaves on the carboxyl side of lysine and arginine residues, also are very useful in splitting proteins. Amino acid sequences are rich in information concerning the kinship of proteins, their evolutionary relations, and diseases produced by mutations. Knowledge of a sequence provides valuable clues to conformation and function.

- **Immunology Provides Important Techniques with Which to Investigate Proteins**

Proteins can be detected and quantitated by highly specific antibodies; monoclonal antibodies are especially useful because they are homogeneous. Enzyme-linked immunosorbent assays and Western blots of SDS–polyacrylamide gels are used extensively. Proteins can also be localized within cells by immunofluorescence microscopy and immunoelectron microscopy.

- **Peptides Can Be Synthesized by Automated Solid-Phase Methods**

Polypeptide chains can be synthesized by automated solid-phase methods in which the carboxyl end of the growing chain is linked to an insoluble support. The α -carboxyl group of the incoming amino acid is activated by dicyclohexylcarbodiimide and joined to the α -amino group of the growing chain. Synthetic peptides can serve as drugs and as antigens to stimulate the formation of specific antibodies. They can also be sources of insight into relations between amino acid sequence and conformation.

- **Three-Dimensional Protein Structure Can Be Determined by NMR Spectroscopy and X-Ray Crystallography**

Nuclear magnetic resonance spectroscopy and x-ray crystallography have greatly enriched our understanding of how proteins fold, recognize other molecules, and catalyze chemical reactions. Nuclear magnetic resonance spectroscopy reveals the structure and dynamics of proteins in solution. The chemical shift of nuclei depends on their local environment. Furthermore, the spins of neighboring nuclei interact with each other in ways that provide definitive structural information.

X-ray crystallography is possible because electrons scatter x-rays; the way in which the scattered waves recombine depends only on the atomic arrangement. The three-dimensional structures of thousands of proteins are now known in atomic detail.

KEY TERMS

- proteome (p. 78)
 assay (p. 79)
 homogenate (p. 79)
 salting out (p. 80)
 dialysis (p. 80)
 gel-filtration chromatography (p. 81)
 ion-exchange chromatography (p. 81)
 affinity chromatography (p. 82)
 high-pressure liquid chromatography (HPLC) (p. 82)
 gel electrophoresis (p. 83)
 isoelectric point (p. 85)
 isoelectric focusing (p. 85)
- two-dimensional electrophoresis (p. 85)
 sedimentation coefficient (Svedberg units, S) (p. 88)
 matrix-assisted laser desorption–ionization–time of flight spectrometry (MALDI-TOF) (p. 89)
 dansyl chloride (p. 92)
 dansyl chloride (p. 92)
 Edman degradation (p. 92)
 phenyl isothiocyanate (p. 92)
 cyanogen bromide (CNBr) (p. 94)
 overlap peptides (p. 95)
 diagonal electrophoresis (p. 96)
- antibody (p. 98)
 antigen (p. 98)
 antigenic determinant (epitope) (p. 98)
 monoclonal antibodies (p. 100)
 enzyme-linked immunosorbent assay (ELISA) (p. 101)
 Western blotting (p. 103)
 fluorescence microscopy (p. 103)
 green fluorescent protein (GFP) (p. 103)
 solid-phase method (p. 107)
 nuclear magnetic resonance (NMR) spectroscopy (p. 107)
 x-ray crystallography (p. 110)

SELECTED READINGS

Where to start

- Hunkapiller, M. W., and Hood, L. E., 1983. Protein sequence analysis: Automated microsequencing. *Science* 219:650–659.
 Merrifield, B., 1986. Solid phase synthesis. *Science* 232:341–347.
 Sanger, F., 1988. Sequences, sequences, sequences. *Annu. Rev. Biochem.* 57:1–28.
 Milstein, C., 1980. Monoclonal antibodies. *Sci. Am.* 243(4):66–74.
 Moore, S., and Stein, W. H., 1973. Chemical structures of pancreatic ribonuclease and deoxyribonuclease. *Science* 180:458–464.

Books

- Creighton, T. E., 1993. *Proteins: Structure and Molecular Properties* (2d ed.). W. H. Freeman and Company.
 Kyte, J., 1994. *Structure in Protein Chemistry*. Garland.
 Van Holde, K. E., Johnson, W. C., and Ho, P.-S., 1998. *Principles of Physical Biochemistry*. Prentice Hall.
Methods in Enzymology. Academic Press. [The more than 200 volumes of this series are a treasure house of experimental procedures.]
 Cantor, C. R., and Schimmel, P. R., 1980. *Biophysical Chemistry*. W. H. Freeman and Company.
 Freifelder, D., 1982. *Physical Biochemistry: Applications to Biochemistry and Molecular Biology*. W. H. Freeman and Company.
 Johnstone, R. A. W., 1996. *Mass Spectroscopy for Chemists and Biochemists* (2d ed.). Cambridge University Press.
 Wilkins, M. R., Williams, K. L., Appel, R. D., and Hochstrasser, D. F., 1997. *Proteome Research: New Frontiers in Functional Genomics (Principles and Practice)*. Springer Verlag

Protein purification and analysis

- Deutscher, M. (Ed.), 1997. *Guide to Protein Purification*. Academic Press.
 Scopes, R. K., and Cantor, C., 1994. *Protein Purification: Principles and Practice* (3d ed.). Springer Verlag.
 Dunn, M. J., 1997. Quantitative two-dimensional gel electrophoresis: From proteins to proteomes. *Biochem. Soc. Trans.* 25:248–254.
 Aebersold, R., Pipes, G. D., Wettenhall, R. E., Nika, H., and Hood, L. E., 1990. Covalent attachment of peptides for high sensitivity solid-phase sequence analysis. *Anal. Biochem.* 187:56–65.
 Blackstock, W. P., and Weir, M. P., 1999. Proteomics: Quantitative and physical mapping of cellular proteins. *Trends Biotechnol.* 17:121–127.
 Dutt, M. J., and Lee, K. H., 2000. Proteomic analysis. *Curr. Opin. Biotechnol.* 11:176–179.
 Pandey, A., and Mann, M., 2000. Proteomics to study genes and genomes. *Nature* 405:837–846.

Ultracentrifugation and mass spectrometry

- Schuster, T. M., and Laue, T. M., 1994. *Modern Analytical Ultracentrifugation*. Springer Verlag.
 Arnott, D., Shabanowitz, J., and Hunt, D. F., 1993. Mass spectrometry of proteins and peptides: Sensitive and accurate mass measurement and sequence analysis. *Clin. Chem.* 39:2005–2010.
 Chait, B. T., and Kent, S. B. H., 1992. Weighing naked proteins: Practical, high-accuracy mass measurement of peptides and proteins. *Science* 257:1885–1894.
 Jardine, I., 1990. Molecular weight analysis of proteins. *Methods Enzymol.* 193:441–455.
 Edmonds, C. G., Loo, J. A., Loo, R. R., Udseth, H. R., Barinaga, C. J., and Smith, R. D., 1991. Application of electrospray ionization mass spectrometry and tandem mass spectrometry in combination with capillary electrophoresis for biochemical investigations. *Biochem. Soc. Trans.* 19:943–947.
 Li, L., Garden, R. W., and Sweedler, J. V., 2000. Single-cell MALDI: A new tool for direct peptide profiling. *Trends Biotechnol.* 18:51–60.
 Pappin, D. J., 1997. Peptide mass fingerprinting using MALDI-TOF mass spectrometry. *Methods Mol. Biol.* 64:165–173.
 Yates, J. R., 3rd, 1998. Mass spectrometry and the age of the proteome. *J. Mass Spectrom.* 33:1–19.

X-ray crystallography and spectroscopy

- Glusker, J. P., 1994. X-ray crystallography of proteins. *Methods Biochem. Anal.* 37:1–72.
 Wery, J. P., and Schevitz, R. W., 1997. New trends in macromolecular x-ray crystallography. *Curr. Opin. Chem. Biol.* 1:365–369.
 Brunger, A. T., 1997. X-ray crystallography and NMR reveal complementary views of structure and dynamics. *Nat. Struct. Biol.* 4 (suppl.):862–865.
 Wüthrich, K., 1989. Protein structure determination in solution by nuclear magnetic resonance spectroscopy. *Science* 243:45–50.
 Clore, G. M., and Gronenborn, A. M., 1991. Structures of larger proteins in solution: Three- and four-dimensional heteronuclear NMR spectroscopy. *Science* 252:1390–1399.
 Wüthrich, K., 1986. *NMR of Proteins and Nucleic Acids*. Wiley-Interscience.

Monoclonal antibodies and fluorescent molecules

- Köhler, G., and Milstein, C., 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* 256:495–497.

- Goding, J. W., 1996. *Monoclonal Antibodies: Principles and Practice*. Academic Press.
- Immunology Today*, 2000. Volume 21, issue 8.
- Tsien, R. Y., 1998. The green fluorescent protein. *Annu. Rev. Biochem.* 67:509–544.
- Kendall, J. M., and Badminton, M. N., 1998. *Aequorea victoria* bioluminescence moves into an exciting era. *Trends Biotechnol.* 16:216–234.

Chemical synthesis of proteins

- Mayo, K. H., 2000. Recent advances in the design and construction of synthetic peptides: For the love of basics or just for the technology of it. *Trends Biotechnol.* 18:212–217.
- Borgia, J. A., and Fields, G. B., 2000. Chemical synthesis of proteins. *Trends Biotechnol.* 18:243–251.

PROBLEMS

1. *Valuable reagents.* The following reagents are often used in protein chemistry:

CNBr	Dabsyl chloride
Urea	6 N HCl
Mercaptoethanol	Ninhydrin
Trypsin	Phenyl isothiocyanate
Performic acid	Chymotrypsin

Which one is the best suited for accomplishing each of the following tasks?

- Determination of the amino acid sequence of a small peptide.
 - Identification of the amino-terminal residue of a peptide (of which you have less than 0.1 μg).
 - Reversible denaturation of a protein devoid of disulfide bonds. Which additional reagent would you need if disulfide bonds were present?
 - Hydrolysis of peptide bonds on the carboxyl side of aromatic residues.
 - Cleavage of peptide bonds on the carboxyl side of methionines.
 - Hydrolysis of peptide bonds on the carboxyl side of lysine and arginine residues.
2. *Finding an end.* Anhydrous hydrazine ($\text{H}_2\text{N}-\text{NH}_2$) has been used to cleave peptide bonds in proteins. What are the reaction products? How might this technique be used to identify the carboxyl-terminal amino acid?
3. *Crafting a new breakpoint.* Ethyleneimine reacts with cysteine side chains in proteins to form *S*-aminoethyl derivatives. The peptide bonds on the carboxyl side of these modified cysteine residues are susceptible to hydrolysis by trypsin. Why?
4. *Spectrometry.* The absorbance A of a solution is defined as
- $$A = \log_{10} (I_0/I)$$
- in which I_0 is the incident light intensity and I is the transmitted light intensity. The absorbance is related to the molar absorption coefficient (extinction coefficient) ϵ (in $\text{M}^{-1} \text{cm}^{-1}$), concentration c (in M), and path length l (in cm) by
- $$A = \epsilon c l$$
- The absorption coefficient of myoglobin at 580 nm is $15,000 \text{ M}^{-1} \text{cm}^{-1}$. What is the absorbance of a 1 mg ml^{-1} solution across a 1-cm path? What percentage of the incident light is transmitted by this solution?
5. *A slow mover.* Tropomyosin, a 93-kd muscle protein, sediments more slowly than does hemoglobin (65 kd). Their sedimentation coefficients are 2.6S and 4.31S, respectively. Which structural feature of tropomyosin accounts for its slow sedimentation?
6. *Sedimenting spheres.* What is the dependence of the sedimentation coefficient S of a spherical protein on its mass? How much more rapidly does an 80-kd protein sediment than does a 40-kd protein?
7. *Size estimate.* The relative electrophoretic mobilities of a 30-kd protein and a 92-kd protein used as standards on an SDS-polyacrylamide gel are 0.80 and 0.41, respectively. What is the apparent mass of a protein having a mobility of 0.62 on this gel?
8. *A new partnership?* The gene encoding a protein with a single disulfide bond undergoes a mutation that changes a serine residue into a cysteine residue. You want to find out whether the disulfide pairing in this mutant is the same as in the original protein. Propose an experiment to directly answer this question.
9. *Sorting cells.* Fluorescence-activated cell sorting (FACS) is a powerful technique for separating cells according to their content of particular molecules. For example, a fluorescence-labeled antibody specific for a cell-surface protein can be used to detect cells containing such a molecule. Suppose that you want to isolate cells that possess a receptor enabling them to detect bacterial degradation products. However, you do not yet have an antibody directed against this receptor. Which fluorescence-labeled molecule would you prepare to identify such cells?
10. *Column choice.* (a) The octapeptide AVGW RVKS was digested with the enzyme trypsin. Would ion exchange or molecular exclusion be most appropriate for separating the products? Explain. (b) Suppose that the peptide was digested with chymotrypsin. What would be the optimal separation technique? Explain.
11. *Making more enzyme?* In the course of purifying an enzyme, a researcher performs a purification step that results in an increase in the total activity to a value greater than that present in the original crude extract. Explain how the amount of total activity might increase.
12. *Protein purification problem.* Complete the table at the top of the next page.

Purification procedure	Total protein (mg)	Total activity (units)	Specific activity (units mg ⁻¹)	Purification level	Yield (%)
Crude extract	20,000	4,000,000		1	100
(NH) ₄ SO ₄ precipitation	5,000	3,000,000			
DEAE-cellulose chromatography	1,500	1,000,000			
Size-exclusion chromatography	500	750,000			
Affinity chromatography	45	675,000			

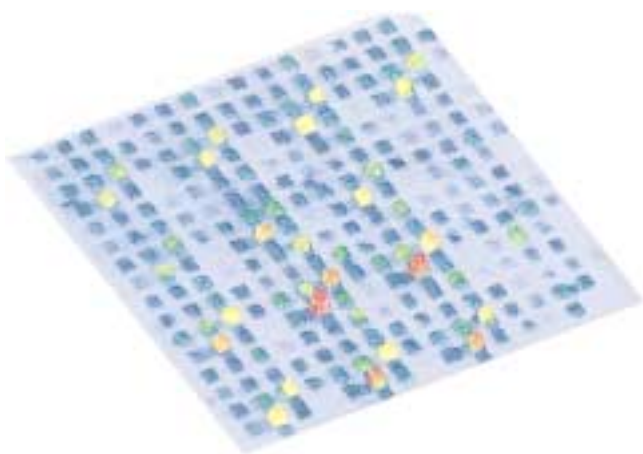
Chapter Integration Problems

13. *Quaternary structure.* A protein was purified to homogeneity. Determination of the molecular weight by molecular exclusion chromatography yields 60 kd. Chromatography in the presence of 6 M urea yields a 30-kd species. When the chromatography is repeated in the presence of 6 M urea and 10 mM β -mercaptoethanol, a single molecular species of 15 kd results. Describe the structure of the molecule.

14. *Helix-coil transitions.*

(a) NMR measurements have shown that poly-L-lysine is a random coil at pH 7 but becomes α helical as the pH is raised above 10. Account for this pH-dependent conformational transition.
 (b) Predict the pH dependence of the helix-coil transition of poly-L-glutamate.

15. *Peptides on a chip.* Large numbers of different peptides can be synthesized in a small area on a solid support. This high-density array can then be probed with a fluorescence-labeled protein to find out which peptides are recognized. The binding of an antibody to an array of 1024 different peptides occupying a total area the size of a thumbnail is shown in the figure below. How would you synthesize such a peptide array? [Hint: Use light instead of acid to deprotect the terminal amino group in each round of synthesis.]



Fluorescence scan of an array of 1024 peptides in a 1.6-cm² area. Each synthesis site is a 400- μ m square. A fluorescently labeled monoclonal antibody was added to the array to identify peptides that are recognized. The height and color of each square denote the fluorescence intensity. [After S. P. A. Fodor, J. O. Read, M. C. Pirrung, L. Stryer, A. T. Lu, and D. Solas. *Science* 251(1991):767.]

Data Interpretation Problems

16. *Protein sequencing I.* Determine the sequence of hexapeptide based on the following data. Note: When the sequence is not known, a comma separates the amino acids. (See Table 4.3)

Amino acid composition: (2R,A,S,V,Y)

N-terminal analysis of the hexapeptide: A

Trypsin digestion: (R,A,V) and (R,S,Y)

Carboxypeptidase digestion: No digestion.

Chymotrypsin digestion: (A,R,V,Y) and (R,S)

17. *Protein sequencing II.* Determine the sequence of a peptide consisting of 14 amino acids on the basis of the following data.

Amino acid composition: (4S,2L,F,G,I,M,T,W,Y)

N-terminal analysis: S

Carboxypeptidase digestion: L

Trypsin digestion: (3S,2L,F,I,M,T,W) (G,K,S,Y)

Chymotrypsin digestion:

(F,I,S) (G,K,L) (L,S) (M,T) (S,W) (S,Y)

N-terminal analysis of (F,I,S) peptide: S

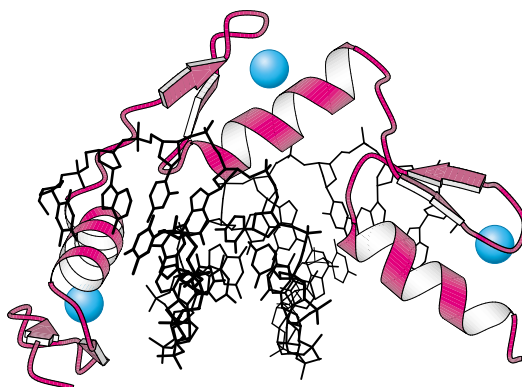
Cyanogen bromide treatment:

(2S,F,G,I,K,L,M*,T,Y) (2S,L,W)

M*, methionine detected as homoserine

18. *Edman degradation.* Alanine amide was treated with phenyl isothiocyanate to form PTH-alanine. Write a mechanism for this reaction.

DNA, RNA, and the Flow of Genetic Information



Having genes in common accounts for the resemblance of a mother and her daughters. Genes must be expressed to exert an effect, and proteins regulate such expression. One such regulatory protein, a zinc-finger protein (zinc ion is blue, protein is red), is shown bound to a control or promoter region of DNA (black). [Left] Barnaby Hall/Photonica.]

DNA and RNA are long linear polymers, called nucleic acids, that carry information in a form that can be passed from one generation to the next. These macromolecules consist of a large number of linked nucleotides, each composed of a sugar, a phosphate, and a base. Sugars linked by phosphates form a common backbone, whereas the bases vary among four kinds. *Genetic information is stored in the sequence of bases along a nucleic acid chain.* The bases have an additional special property: they form specific pairs with one another that are stabilized by hydrogen bonds. The base pairing results in the formation of a double helix, a helical structure consisting of two strands. *These base pairs provide a mechanism for copying the genetic information in an existing nucleic acid chain to form a new chain.* Although RNA probably functioned as the genetic material very early in evolutionary history, the genes of all modern cells and many viruses are made of DNA. DNA is replicated by the action of DNA polymerase enzymes. These exquisitely specific enzymes copy sequences from nucleic acid templates with an error rate of less than 1 in 100 million nucleotides.

Genes specify the kinds of proteins that are made by cells, but DNA is not the direct template for protein synthesis. Rather, the templates for protein synthesis are RNA (ribonucleic acid) molecules. In particular, a class of RNA molecules called *messenger RNA* (mRNA) are the information-carrying intermediates in protein synthesis. Other RNA molecules, such as *transfer RNA* (tRNA) and

OUTLINE

- 5.1 A Nucleic Acid Consists of Four Kinds of Bases Linked to a Sugar-Phosphate Backbone
- 5.2 A Pair of Nucleic Acid Chains with Complementary Sequences Can Form a Double-Helical Structure
- 5.3 DNA Is Replicated by Polymerases That Take Instructions from Templates
- 5.4 Gene Expression Is the Transformation of DNA Information into Functional Molecules
- 5.5 Amino Acids Are Encoded by Groups of Three Bases Starting from a Fixed Point
- 5.6 Most Eukaryotic Genes Are Mosaics of Introns and Exons

ribosomal RNA (rRNA), are part of the protein-synthesizing machinery. All forms of cellular RNA are synthesized by RNA polymerases that take instructions from DNA templates. This process of *transcription* is followed by *translation*, the synthesis of proteins according to instructions given by mRNA templates. Thus, the flow of genetic information, or *gene expression*, in normal cells is:



This flow of information is dependent on the genetic code, which defines the relation between the sequence of bases in DNA (or its mRNA transcript) and the sequence of amino acids in a protein. The code is nearly the same in all organisms: a sequence of three bases, called a *codon*, specifies an amino acid. Codons in mRNA are read sequentially by tRNA molecules, which serve as adaptors in protein synthesis. Protein synthesis takes place on ribosomes, which are complex assemblies of rRNAs and more than 50 kinds of proteins.

The last theme to be considered is the interrupted character of most eukaryotic genes, which are mosaics of nucleic acid sequences called *introns* and *exons*. Both are transcribed, but introns are cut out of newly synthesized RNA molecules, leaving mature RNA molecules with continuous exons. The existence of introns and exons has crucial implications for the evolution of proteins.

5.1 A NUCLEIC ACID CONSISTS OF FOUR KINDS OF BASES LINKED TO A SUGAR-PHOSPHATE BACKBONE

The nucleic acids DNA and RNA are well suited to function as the carriers of genetic information by virtue of their covalent structures. These macromolecules are *linear polymers* built up from similar units connected end to end (Figure 5.1). Each monomer unit within the polymer consists of three components: a sugar, a phosphate, and a base. The sequence of bases uniquely characterizes a nucleic acid and represents a form of linear information.

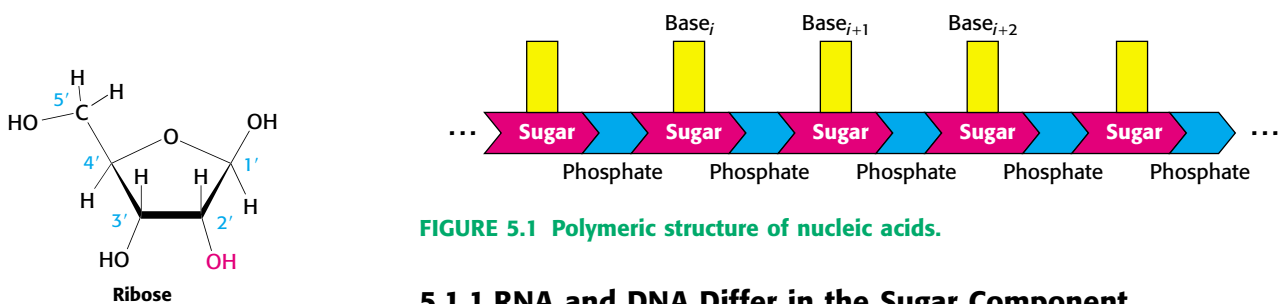


FIGURE 5.1 Polymeric structure of nucleic acids.

5.1.1 RNA and DNA Differ in the Sugar Component and One of the Bases

The sugar in *deoxyribonucleic acid* (DNA) is *deoxyribose*. The deoxy prefix indicates that the 2' carbon atom of the sugar lacks the oxygen atom that is linked to the 2' carbon atom of *ribose* (the sugar in *ribonucleic acid*, or RNA), as shown in Figure 5.2. The sugars in nucleic acids are linked to one another by phosphodiester bridges. Specifically, the 3'-hydroxyl (3'-OH) group of the sugar moiety of one nucleotide is esterified to a phosphate group, which is, in turn, joined to the 5'-hydroxyl group of the adjacent sugar. The chain of sugars linked by phosphodiester bridges is referred to as the *backbone* of the nucleic acid (Figure 5.3). Whereas the backbone is constant in DNA and RNA, the bases vary from one monomer to the next. Two of the bases

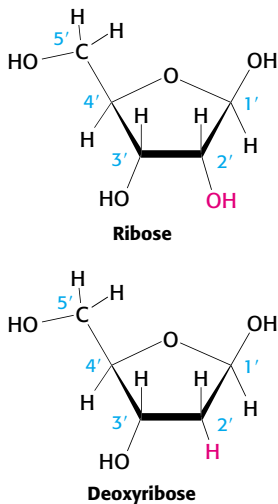


FIGURE 5.2 Ribose and deoxyribose. Atoms are numbered with primes to distinguish them from atoms in bases (see Figure 5.4).

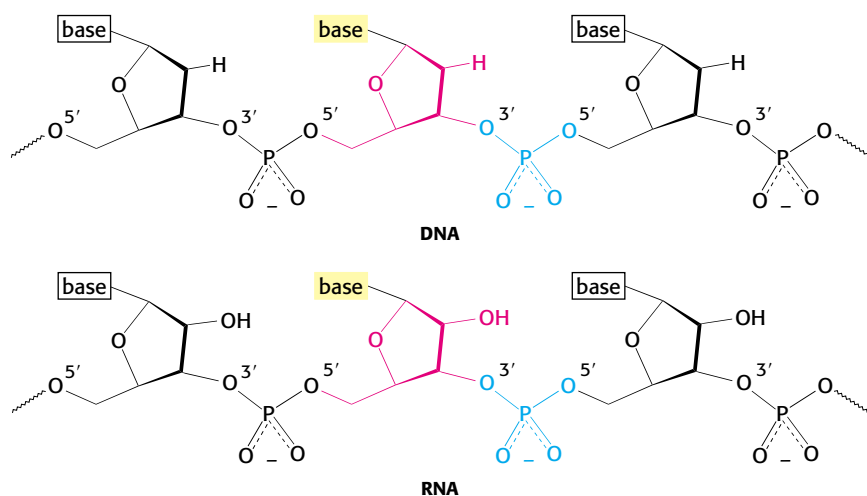


FIGURE 5.3 Backbones of DNA and RNA. The backbones of these nucleic acids are formed by 3'-to-5' phosphodiester linkages. A sugar unit is highlighted in red and a phosphate group in blue.

are derivatives of *purine*—adenine (A) and guanine (G)—and two of *pyrimidine*—cytosine (C) and thymine (T, DNA only) or uracil (U, RNA only), as shown in Figure 5.4.

RNA, like DNA, is a long unbranched polymer consisting of nucleotides joined by 3' → 5' phosphodiester bonds (see Figure 5.3). The covalent structure of RNA differs from that of DNA in two respects. As stated earlier and as indicated by its name, the sugar units in RNA are riboses rather than deoxyriboses. Ribose contains a 2'-hydroxyl group not present in deoxyribose. As a consequence, in addition to the standard 3' → 5' linkage, a 2' → 5' linkage is possible for RNA. This later linkage is important in the removal of introns and the joining of exons for the formation of mature RNA (Section 28.x.x). The other difference, as already mentioned, is that one of the four major bases in RNA is uracil (U) instead of thymine (T).

Note that each phosphodiester bridge has a negative charge. This negative charge repels nucleophilic species such as hydroxide ion; consequently, phosphodiester linkages are much less susceptible to hydrolytic attack than are other esters such as carboxylic acid esters. This resistance is crucial for maintaining the integrity of information stored in nucleic acids. The absence of the 2'-hydroxyl group in DNA further increases its resistance to hydrolysis. The greater stability of DNA probably accounts for its use rather than RNA as the hereditary material in all modern cells and in many viruses.

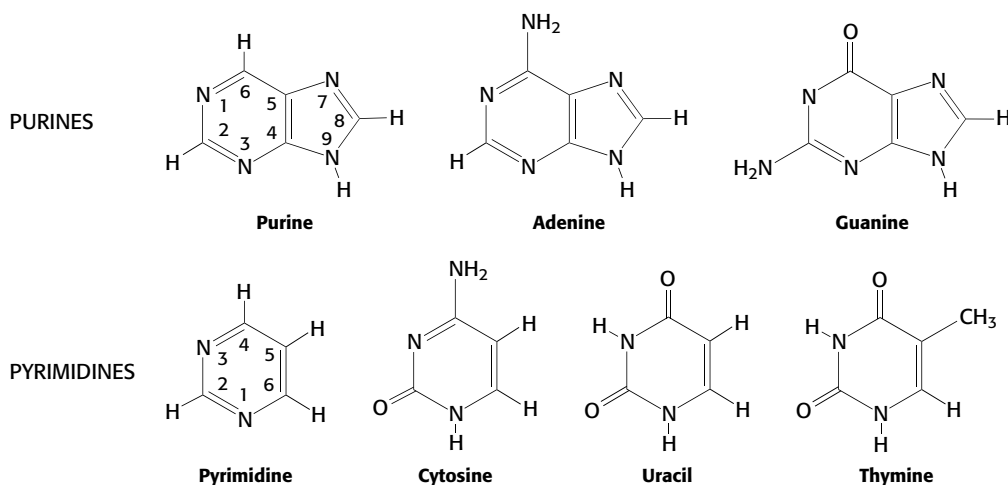


FIGURE 5.4 Purines and pyrimidines. Atoms within bases are numbered without primes. Uracil instead of thymine is used in RNA.

5.1.2 Nucleotides Are the Monomeric Units of Nucleic Acids

STRUCTURAL INSIGHTS, Nucleic Acids offers a three-dimensional perspective on nucleotide structure, base pairing, and other aspects of DNA and RNA structure.

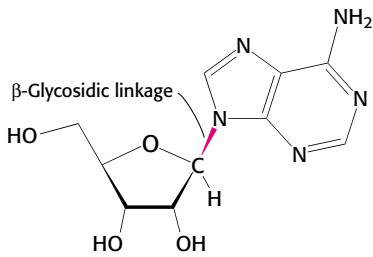


FIGURE 5.5 β -Glycosidic linkage in a nucleoside.

A unit consisting of a base bonded to a sugar is referred to as a *nucleoside*. The four nucleoside units in RNA are called *adenosine*, *guanosine*, *cytidine*, and *uridine*, whereas those in DNA are called *deoxyadenosine*, *deoxyguanosine*, *deoxycytidine*, and *thymidine*. In each case, N-9 of a purine or N-1 of a pyrimidine is attached to C-1' of the sugar (Figure 5.5). The base lies above the plane of sugar when the structure is written in the standard orientation; that is, the configuration of the *N*-glycosidic linkage is β . A *nucleotide* is a nucleoside joined to one or more phosphate groups by an ester linkage. The most common site of esterification in naturally occurring nucleotides is the hydroxyl group attached to C-5' of the sugar. A compound formed by the attachment of a phosphate group to the C-5' of a nucleoside sugar is called a *nucleoside 5'-phosphate* or a *5'-nucleotide*. For example, ATP is *adenosine 5'-triphosphate*. Another nucleotide is deoxyguanosine 3'-monophosphate (3'-dGMP; Figure 5.6). This nucleotide differs from ATP in that it contains guanine rather than adenine, contains deoxyribose rather than ribose (indicated by the prefix “d”), contains one rather than three phosphates, and has the phosphate esterified to the hydroxyl group in the 3' rather than the 5' position. Nucleotides are the monomers that are linked to form RNA and DNA. The four nucleotide units in DNA are called *deoxyadenylate*, *deoxyguanylate*, *deoxycytidylate*, and *thymidylate*. Note that thymidylate contains deoxyribose; by convention, the prefix deoxy is not added because thymine-containing nucleotides are only rarely found in RNA.

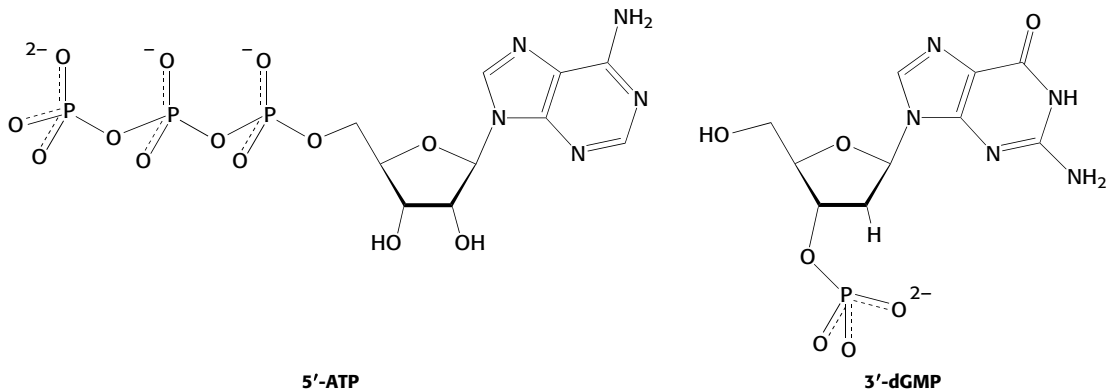


FIGURE 5.6 Nucleotides adenosine 5'-triphosphate (5'-ATP) and deoxyguanosine 3'-monophosphate (3'-dGMP).

The abbreviated notations pApCpG or pACG denote a trinucleotide of DNA consisting of the building blocks deoxyadenylate monophosphate, deoxycytidylate monophosphate, and deoxyguanylate monophosphate linked by a phosphodiester bridge, where “p” denotes a phosphate group (Figure 5.7). The 5' end will often have a phosphate attached to the 5'-OH group. Note that, like a polypeptide (see Section 3.2), a *DNA chain has polarity*. One end of the chain has a free 5'-OH group (or a 5'-OH group attached to a phosphate), whereas the other end has a 3'-OH group, neither of which is linked to another nucleotide. By convention, *the base sequence is written in the 5'-to-3' direction*. Thus, the symbol ACG indicates that the unlinked 5'-OH group is on deoxyadenylate, whereas the unlinked 3'-OH group is on deoxyguanylate. Because of this polarity, ACG and GCA correspond to different compounds.

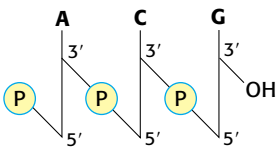


FIGURE 5.7 Structure of a DNA chain. The chain has a 5' end, which is usually attached to a phosphate, and a 3' end, which is usually a free hydroxyl group.

A striking characteristic of naturally occurring DNA molecules is their length. A DNA molecule must comprise many nucleotides to carry the genetic information necessary for even the simplest organisms. For example, the DNA of a virus such as polyoma, which can cause cancer in certain organisms, is 5100 nucleotides in length. We can quantify the information carrying capacity of nucleic acids in the following way. Each position can be one of four bases, corresponding to two bits of information ($2^2 = 4$). Thus, a chain of 5100 nucleotides corresponds to $2 \times 5100 = 10,200$ bits, or 1275 bytes (1 byte = 8 bits). The *E. coli* genome is a single DNA molecule consisting of two chains of 4.6 million nucleotides, corresponding to 9.2 million bits, or 1.15 megabytes, of information (Figure 5.8).

DNA molecules from higher organisms can be much larger. The human genome comprises approximately 3 billion nucleotides, divided among 24 distinct DNA molecules (22 autosomes, x and y sex chromosomes) of different sizes. One of the largest known DNA molecules is found in the Indian muntjak, an Asiatic deer; its genome is nearly as large as the human genome but is distributed on only 3 chromosomes (Figure 5.9). The largest of these chromosomes has chains of more than 1 billion nucleotides. If such a DNA molecule could be fully extended, it would stretch more than 1 foot in length. Some plants contain even larger DNA molecules.

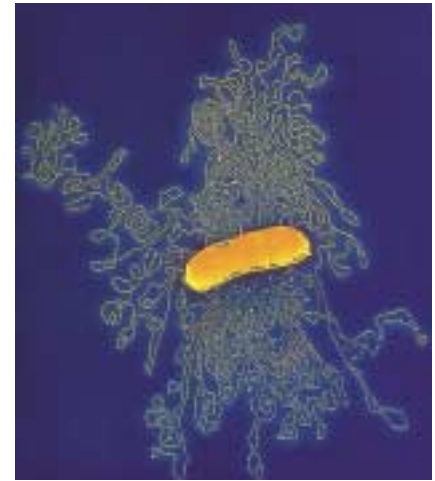
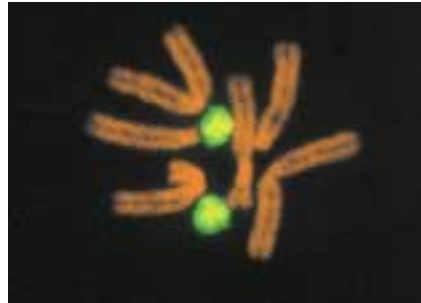


FIGURE 5.8 Electron micrograph of part of the *E. coli* genome. [Dr. Gopal Murti/Science Photo Library/Photo Researchers.]

FIGURE 5.9 The Indian muntjak and its chromosomes. Cells from a female Indian muntjak (right) contain three pairs of very large chromosomes (stained orange). The cell shown is a hybrid containing a pair of human chromosomes (stained green) for comparison. [(Left) M. Birkhead, OSF/Animals Animals. (Right) J-Y Lee, M. Koi, E. J. Stanbridge, M. Oshimura, A. T. Kumamoto, and A. P. Feinberg. *Nature Genetics* 7(1994):30.]

5.2 A PAIR OF NUCLEIC ACID CHAINS WITH COMPLEMENTARY SEQUENCES CAN FORM A DOUBLE-HELICAL STRUCTURE

The covalent structure of nucleic acids accounts for their ability to carry information in the form of a sequence of bases along a nucleic acid chain. Other features of nucleic acid structure facilitate the process of *replication*—that is, the generation of two copies of a nucleic acid from one. These features depend on the ability of the bases found in nucleic acids to form *specific base pairs* in such a way that a helical structure consisting of two strands is formed. The double-helical structure of DNA facilitates the replication of the genetic material (Section 5.2.2).

5.2.1 The Double Helix Is Stabilized by Hydrogen Bonds and Hydrophobic Interactions

The existence of specific base-pairing interactions was discovered in the course of studies directed at determining the three-dimensional structure of DNA. Maurice Wilkins and Rosalind Franklin obtained x-ray diffraction photographs of fibers of DNA (Figure 5.10). The characteristics of these diffraction patterns indicated that DNA was formed of two chains that wound in a regular helical structure. From these and other data, James Watson and Francis Crick inferred a structural model for DNA that accounted

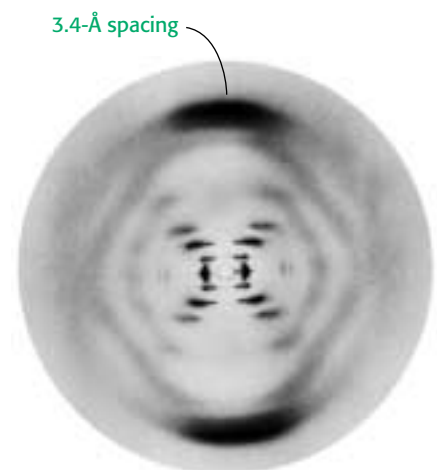


FIGURE 5.10 X-ray diffraction photograph of a hydrated DNA fiber. The central cross is diagnostic of a helical structure. The strong arcs on the meridian arise from the stack of nucleotide bases, which are 3.4 Å apart. [Courtesy of Dr. Maurice Wilkins.]

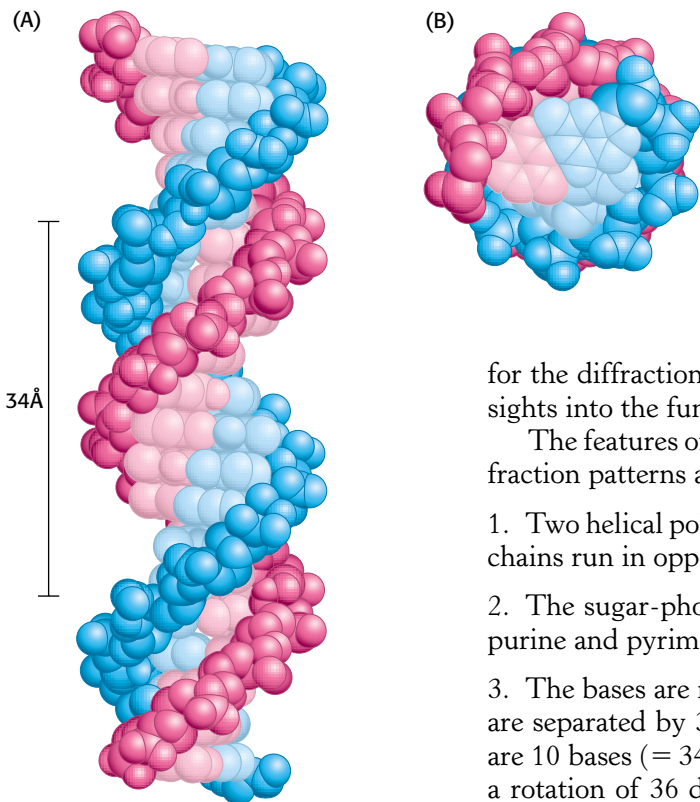


FIGURE 5.11 Watson-Crick model of double-helical DNA.

One polynucleotide chain is shown in blue and the other in red. The purine and pyrimidine bases are shown in lighter colors than the sugar-phosphate backbone. (A) Axial view. The structure repeats along the helical axis (vertical) at intervals of 34 Å, which corresponds to 10 nucleotides on each chain. (B) Radial view, looking down the helix axis.

for the diffraction pattern and was also the source of some remarkable insights into the functional properties of nucleic acids (Figure 5.11).

The features of the Watson-Crick model of DNA deduced from the diffraction patterns are:

1. Two helical polynucleotide chains are coiled around a common axis. The chains run in opposite directions.
2. The sugar-phosphate backbones are on the outside and, therefore, the purine and pyrimidine bases lie on the inside of the helix.
3. The bases are nearly perpendicular to the helix axis, and adjacent bases are separated by 3.4 Å. The helical structure repeats every 34 Å, so there are 10 bases (= 34 Å per repeat/3.4 Å per base) per turn of helix. There is a rotation of 36 degrees per base (360 degrees per full turn/10 bases per turn).
4. The diameter of the helix is 20 Å.

How is such a regular structure able to accommodate an arbitrary sequence of bases, given the different sizes and shapes of the purines and pyrimidines? In attempting to answer this question, Watson and Crick discovered that guanine can be paired with cytosine and adenine with thymine to form base pairs that have essentially the same shape (Figure 5.12). These base pairs are held together by specific hydrogen bonds. This base-pairing scheme was supported by earlier studies of the base composition of DNA from different species. In 1950, Erwin Chargaff reported that the ratios of adenine to thymine and of guanine to cytosine were nearly the same in all species studied. Note in Table 5.1 that all the adenine:thymine and guanine:cytosine ratios are close to 1, whereas the adenine-to-guanine ratio varies considerably. The meaning of these equivalences was not evident until the Watson-Crick model was proposed, when it became clear that they represent an essential facet of DNA structure.

The spacing of approximately 3.4 Å between nearly parallel base pairs is readily apparent in the DNA diffraction pattern (see Figure 5.10). The

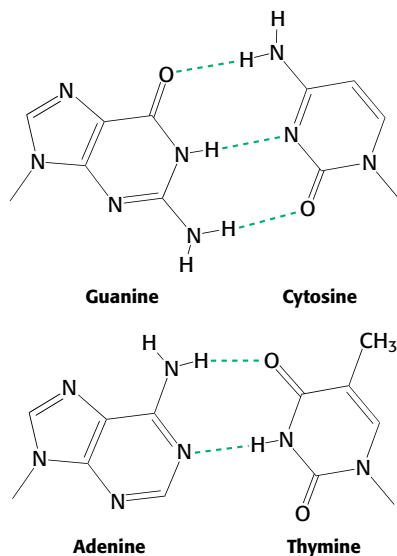


FIGURE 5.12 Structures of the base pairs proposed by Watson and Crick.

TABLE 5.1 Base compositions experimentally determined for a variety of organisms

Species	A:T	G:C	A:G
Human being	1.00	1.00	1.56
Salmon	1.02	1.02	1.43
Wheat	1.00	0.97	1.22
Yeast	1.03	1.02	1.67
<i>Escherichia coli</i>	1.09	0.99	1.05
<i>Serratia marcescens</i>	0.95	0.86	0.70

stacking of bases one on top of another contributes to the stability of the double helix in two ways (Figure 5.13). First, adjacent base pairs attract one another through van der Waals forces (Section 1.3.1). Energies associated with van der Waals interactions are quite small, such that typical interactions contribute from 0.5 to 1.0 kcal mol⁻¹ per atom pair. In the double helix, however, a large number of atoms are in van der Waals contact, and the net effect, summed over these atom pairs, is substantial. In addition, the double helix is stabilized by the hydrophobic effect (Section 1.3.4): base stacking, or hydrophobic interactions between the bases, results in the exposure of the more polar surfaces to the surrounding water. This arrangement is reminiscent of protein folding, where hydrophobic amino acids are interior in the protein and hydrophilic are exterior (Section 3.4). Base stacking in DNA is also favored by the conformations of the relatively rigid five-membered rings of the backbone sugars. The sugar rigidity affects both the single-stranded and the double-helical forms.

5.2.2 The Double Helix Facilitates the Accurate Transmission of Hereditary Information

The double-helical model of DNA and the presence of specific base pairs immediately suggested how the genetic material might replicate. The sequence of bases of one strand of the double helix precisely determines the sequence of the other strand; a guanine base on one strand is always paired with a cytosine base on the other strand, and so on. Thus, separation of a double helix into its two component chains would yield two single-stranded templates onto which new double helices could be constructed, each of which would have the same sequence of bases as the parent double helix. Consequently, as DNA is replicated, one of the chains of each daughter DNA molecule would be newly synthesized, whereas the other would be passed unchanged from the parent DNA molecule. This distribution of parental atoms is achieved by *semiconservative replication*.

Matthew Meselson and Franklin Stahl carried out a critical test of this hypothesis in 1958. They labeled the parent DNA with ¹⁵N, a heavy isotope of nitrogen, to make it denser than ordinary DNA. The labeled DNA was generated by growing *E. coli* for many generations in a medium that contained ¹⁵NH₄Cl as the sole nitrogen source. After the incorporation of heavy nitrogen was complete, the bacteria were abruptly transferred to a medium that contained ¹⁴N, the ordinary isotope of nitrogen. The question asked was: What is the distribution of ¹⁴N and ¹⁵N in the DNA molecules after successive rounds of replication?

The distribution of ¹⁴N and ¹⁵N was revealed by the technique of *density-gradient equilibrium sedimentation*. A small amount of DNA was dissolved in a concentrated solution of cesium chloride having a density close to that of the DNA (1.7 g cm⁻³). This solution was centrifuged until it was nearly at equilibrium. The opposing processes of sedimentation and diffusion created a gradient in the concentration of cesium chloride across the centrifuge cell. The result was a stable density gradient, ranging from 1.66 to 1.76 g cm⁻³. The DNA molecules in this density gradient were driven by centrifugal force into the region where the solution's density was equal to their own. The genomic DNA yielded a narrow band that was detected by its absorption of ultraviolet light. A mixture of ¹⁴N DNA and ¹⁵N DNA molecules gave clearly separate bands because they differ in density by about 1% (Figure 5.14).

DNA was extracted from the bacteria at various times after they were transferred from a ¹⁵N to a ¹⁴N medium and centrifuged. Analysis of these samples showed that there was a single band of DNA after one generation. The density of this band was precisely halfway between the densities of the

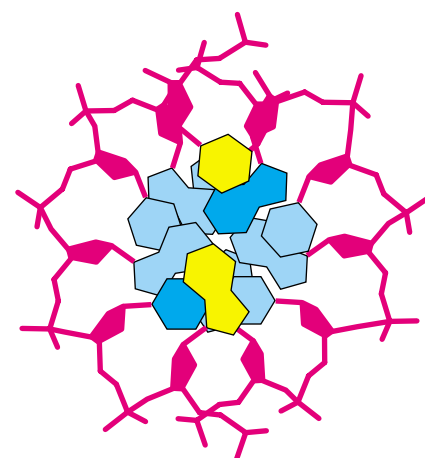


FIGURE 5.13 Axial view of DNA. Base pairs are stacked nearly one on top of another in the double helix.

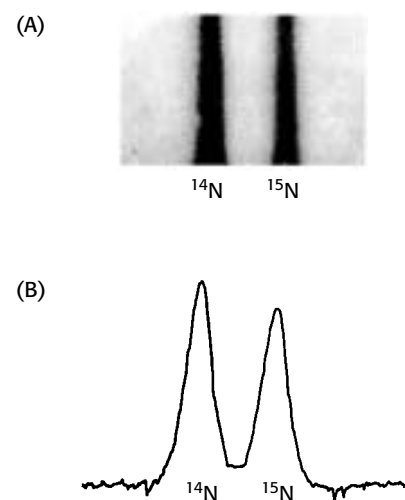


FIGURE 5.14 Resolution of ¹⁴N DNA and ¹⁵N DNA by density-gradient centrifugation. (A) Ultraviolet absorption photograph of a centrifuge cell showing the two distinct bands of DNA. (B) Densitometric tracing of the absorption photograph. [From M. Meselson and F. W. Stahl. *Proc. Natl. Acad. Sci. U.S.A.* 44(1958):671.]

FIGURE 5.15 Detection of semiconservative replication of *E. coli* DNA by density-gradient centrifugation. The position of a band of DNA depends on its content of ^{14}N and ^{15}N . After 1.0 generation, all of the DNA molecules were hybrids containing equal amounts of ^{14}N and ^{15}N . [From M. Meselson and F. W. Stahl. *Proc. Natl. Acad. Sci. U.S.A.* 44(1958):671.]

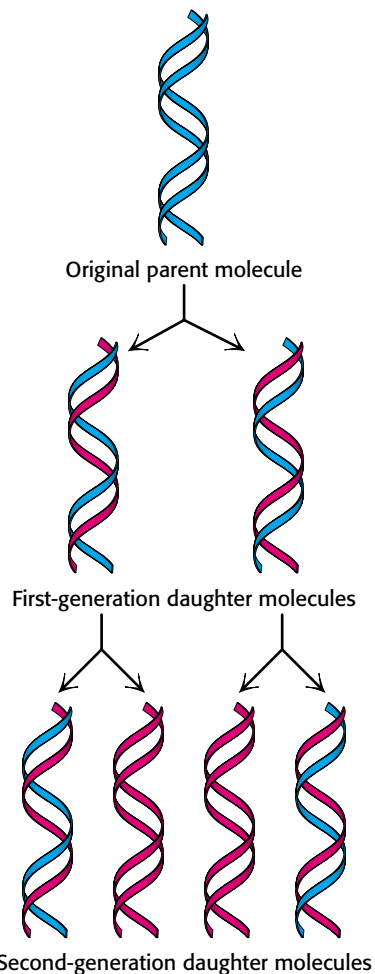
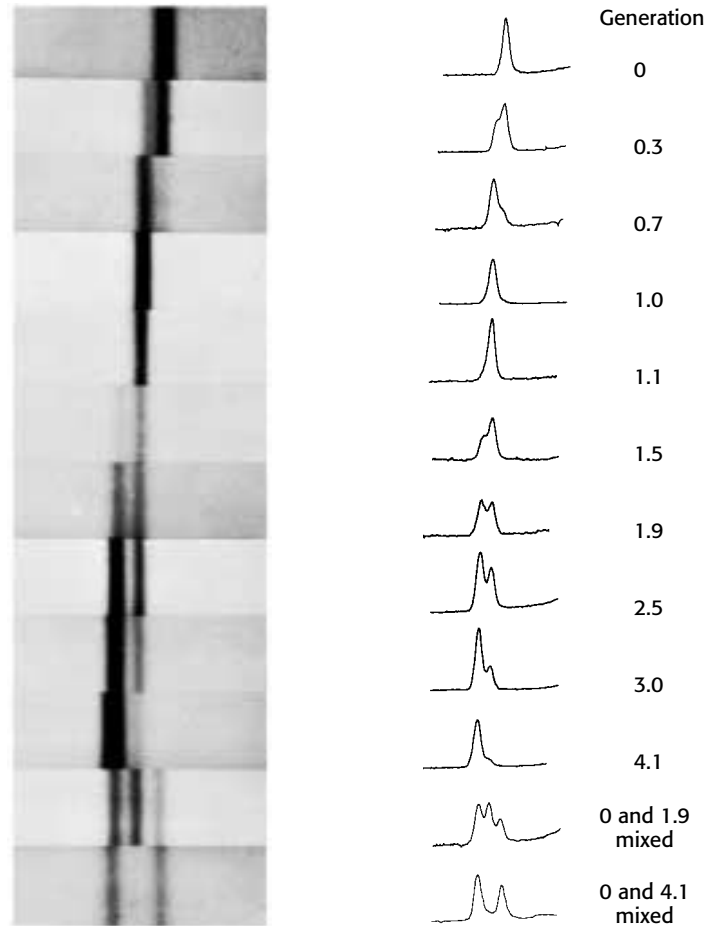


FIGURE 5.16 Diagram of semiconservative replication. Parental DNA is shown in blue and newly synthesized DNA in red. [After M. Meselson and F. W. Stahl. *Proc. Natl. Acad. Sci. U.S.A.* 44(1958):671.]

^{14}N DNA and ^{15}N DNA bands (Figure 5.15). The absence of ^{15}N DNA indicated that parental DNA was not preserved as an intact unit after replication. The absence of ^{14}N DNA indicated that all the daughter DNA derived some of their atoms from the parent DNA. This proportion had to be half because the density of the hybrid DNA band was halfway between the densities of the ^{14}N DNA and ^{15}N DNA bands.

After two generations, there were equal amounts of two bands of DNA. One was hybrid DNA, and the other was ^{14}N DNA. Meselson and Stahl concluded from these incisive experiments “that the nitrogen in a DNA molecule is divided equally between two physically continuous subunits; that following duplication, each daughter molecule receives one of these; and that the subunits are conserved through many duplications.” Their results agreed perfectly with the Watson-Crick model for DNA replication (Figure 5.16).

5.2.3 The Double Helix Can Be Reversibly Melted

During DNA replication and other processes, the two strands of the double helix must be separated from one another, at least in a local region. In the laboratory, the double helix can be disrupted by heating a solution of DNA. The heating disrupts the hydrogen bonds between base pairs and thereby causes the strands to separate. The dissociation of the double helix is often called *melting* because it occurs relatively abruptly at a certain temperature. The *melting temperature* (T_m) is defined as the temperature at which half the helical structure is lost. Strands may also be separated by adding acid or alkali to ionize the nucleotide bases and disrupt base pairing.

Stacked bases in nucleic acids absorb less ultraviolet light than do unstacked bases, an effect called *hypochromism*. Thus, the melting of nucleic

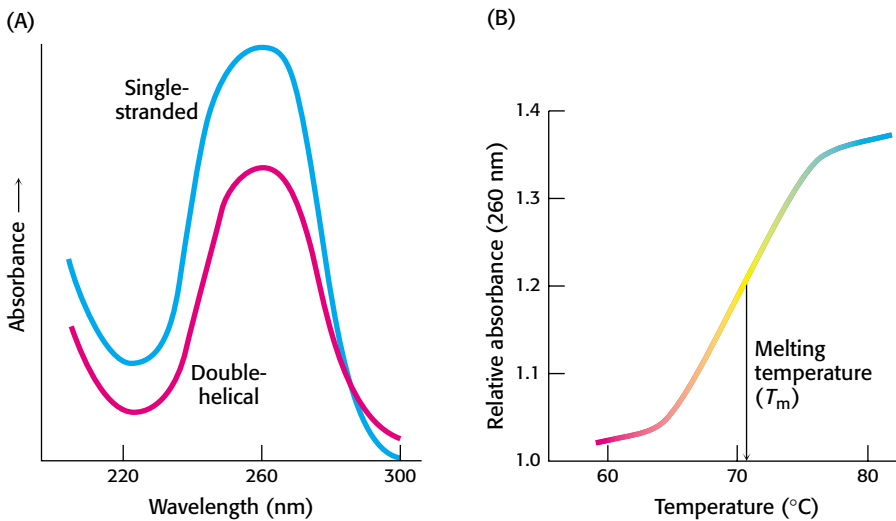


FIGURE 5.17 Hypochromism.

(A) Single-stranded DNA absorbs light more effectively than does double-helical DNA. (B) The absorbance of a DNA solution at a wavelength of 260 nm increases when the double helix is melted into single strands.

acids is easily followed by monitoring their absorption of light, which peaks at a wavelength of 260 nm (Figure 5.17).

Separated complementary strands of nucleic acids spontaneously reassociate to form a double helix when the temperature is lowered below T_m . This renaturation process is sometimes called *annealing*. The facility with which double helices can be melted and then reassociated is crucial for the biological functions of nucleic acids. Of course, inside cells, the double helix is not melted by the addition of heat. Instead, proteins called *helicases* use chemical energy (from ATP) to disrupt the structure of double-stranded nucleic acid molecules.

The ability to melt and reanneal DNA reversibly in the laboratory provides a powerful tool for investigating sequence similarity as well as gene structure and expression. For instance, DNA molecules from two different organisms can be melted and allowed to reanneal or *hybridize* in the presence of each other. If the sequences are similar, hybrid DNA duplexes, with DNA from each organism contributing a strand of the double helix, can form. Indeed, the degree of hybridization is an indication of the relatedness of the genomes and hence the organisms. Similar hybridization experiments with RNA and DNA can locate genes in a cell's DNA that correspond to a particular RNA. We will return to this important technique in Chapter 6.

5.2.4 Some DNA Molecules Are Circular and Supercoiled

The DNA molecules in human chromosomes are linear. However, electron microscopic and other studies have shown that intact DNA molecules from some other organisms are circular (Figure 5.18A). The term *circular* refers to the continuity of the DNA chains, not to their geometrical form. DNA molecules inside cells necessarily have a very compact shape. Note that the *E. coli* chromosome, fully extended, would be about 1000 times as long as the greatest diameter of the bacterium.

A new property appears in the conversion of a linear DNA molecule into a closed circular molecule. The axis of the double helix can itself be twisted into a *superhelix* (Figure 5.18B). A circular DNA molecule without any superhelical turns is known as a *relaxed molecule*. Supercoiling is biologically important for two reasons. First, a *supercoiled DNA molecule has a more compact shape than does its relaxed counterpart*. Second, *supercoiling may hinder or favor the capacity of the double helix to unwind and thereby affects the interactions between DNA and other molecules*. These topological features of DNA will be considered further in Section 27.x.x.

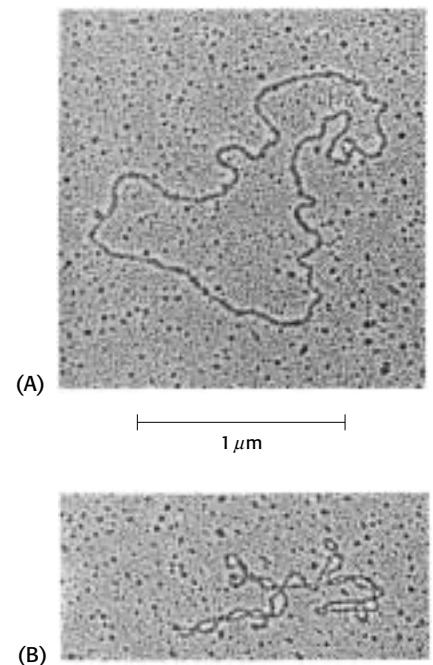


FIGURE 5.18 Electron micrographs of circular DNA from mitochondria. (A) Relaxed form. (B) Supercoiled form. [Courtesy of Dr. David Clayton.]

5.2.5 Single-Stranded Nucleic Acids Can Adopt Elaborate Structures

Single-stranded nucleic acids often fold back on themselves to form well-defined structures. Early in evolutionary history, nucleic acids, particularly RNA, may have adopted complex and diverse structures both to store genetic information and to catalyze its transmission (Section 2.2.2). Such structures are also important in all modern organisms in entities such as the ribosome, a large complex of RNAs and proteins on which proteins are synthesized.

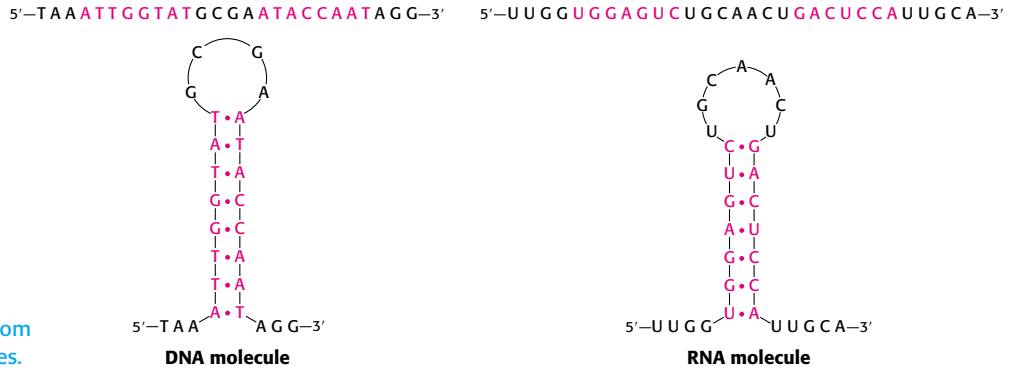


FIGURE 5.19 Stem-loop structures. Stem-loop structures may be formed from single-stranded DNA and RNA molecules.

The simplest and most common structural motif formed is a *stem-loop*, created when two complementary sequences within a single strand come together to form double-helical structures (Figure 5.19). In many cases, these double helices are made up entirely of Watson-Crick base pairs. In other cases, however, the structures include mismatched or unmatched (bulged) bases. Such mismatches destabilize the local structure but introduce deviations from the standard double-helical structure that can be important for higher-order folding and for function (Figure 5.20).

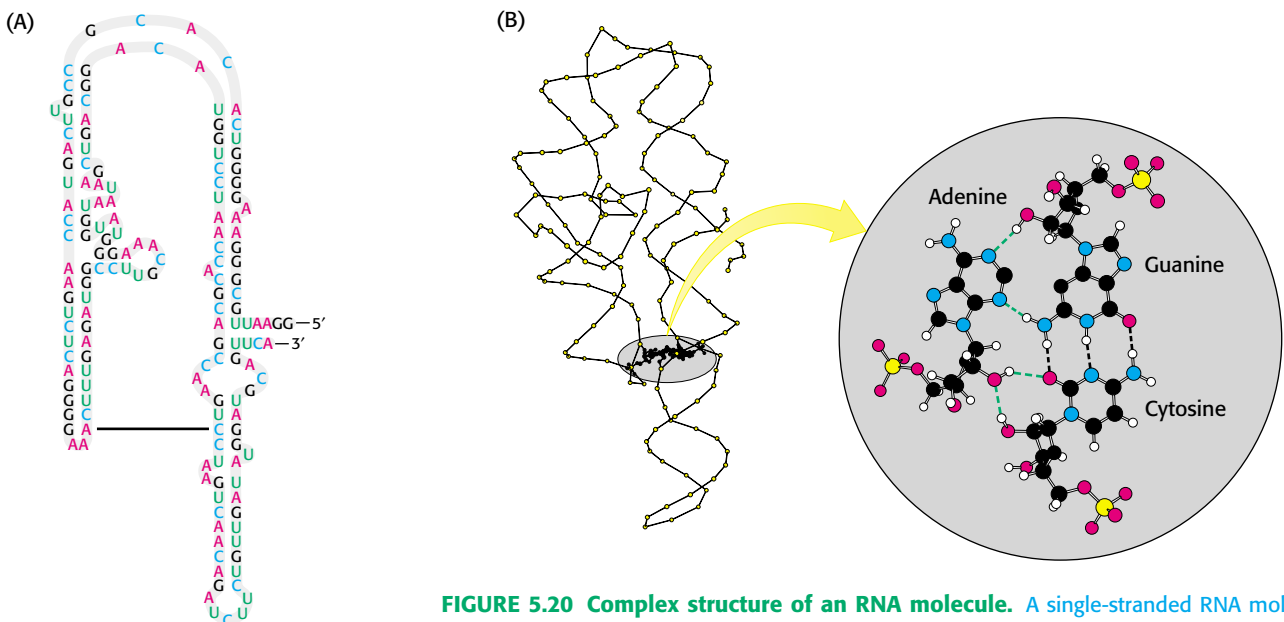


FIGURE 5.20 Complex structure of an RNA molecule. A single-stranded RNA molecule may fold back on itself to form a complex structure. (A) The nucleotide sequence showing Watson-Crick base pairs and other nonstandard base pairings in stem-loop structures. (B) The three-dimensional structure and one important long-range interaction between three bases. Hydrogen bonds within the Watson-Crick base pair are shown as dashed black lines; additional hydrogen bonds are shown as dashed green lines.

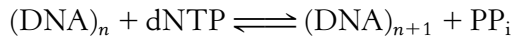
Single-stranded nucleic acids can adopt structures more complex than simple stem-loops through the interaction of more widely separated bases. Often, three or more bases may interact to stabilize these structures. In such cases, hydrogen-bond donors and acceptors that do not participate in Watson-Crick base pairs may participate in hydrogen bonds of nonstandard pairings. Metal ions such as magnesium ion (Mg^{2+}) often assist in the stabilization of these more elaborate structures.

5.3 DNA IS REPLICATED BY POLYMERASES THAT TAKE INSTRUCTIONS FROM TEMPLATES

We now turn to the molecular mechanism of DNA replication. The full replication machinery in cells comprises more than 20 proteins engaged in intricate and coordinated interplay. In 1958, Arthur Kornberg and his colleagues isolated the first known of the enzymes, called DNA polymerases, that promote the formation of the bonds joining units of the DNA backbone.

5.3.1 DNA Polymerase Catalyzes Phosphodiester-Bond Formation

DNA polymerases catalyze the step-by-step addition of deoxyribonucleotide units to a DNA chain (Figure 5.21). Importantly, *the new DNA chain is assembled directly on a preexisting DNA template*. The reaction catalyzed, in its simplest form, is:



where dNTP stands for any deoxyribonucleotide and PP_i is a pyrophosphate molecule. The template can be a single strand of DNA or a double strand with one of the chains broken at one or more sites. If single stranded, the template DNA must be bound to a *primer* strand having a free 3'-hydroxyl group. The reaction also requires all four activated precursors—that is, the deoxynucleoside 5'-triphosphates dATP, dGTP, TTP, and dCTP—as well as Mg^{2+} ion.

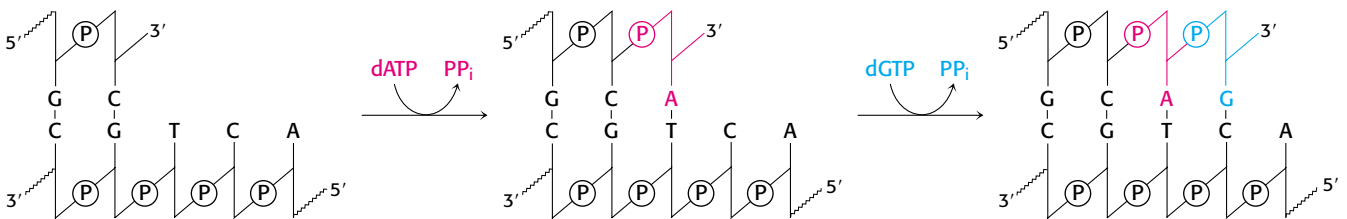


FIGURE 5.21 Polymerization reaction catalyzed by DNA polymerases.

The chain-elongation reaction catalyzed by DNA polymerases is a nucleophilic attack by the 3'-hydroxyl group of the primer on the innermost phosphorus atom of the deoxynucleoside triphosphate (Figure 5.22). A phosphodiester bridge forms with the concomitant release of pyrophosphate. The subsequent hydrolysis of pyrophosphate by pyrophosphatase, a ubiquitous enzyme, helps drive the polymerization forward. Elongation of the DNA chain proceeds in the 5'-to-3' direction.

DNA polymerases catalyze the formation of a phosphodiester bond efficiently only if the base on the incoming nucleoside triphosphate is complementary to the base on the template strand. Thus, DNA polymerase is

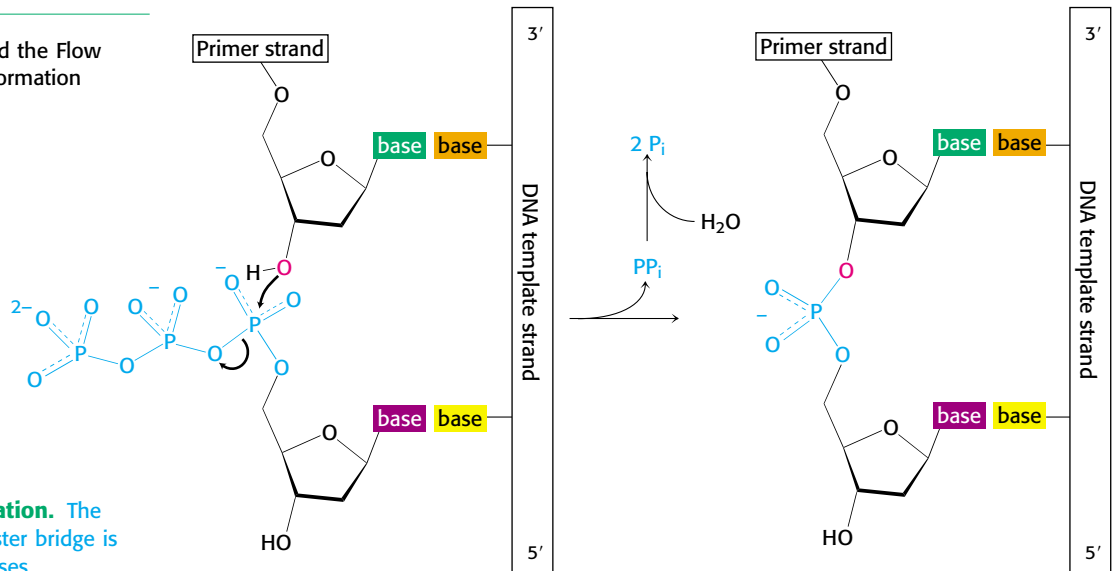


FIGURE 5.22 DNA replication. The formation of a phosphodiester bridge is catalyzed by DNA polymerases.

a *template-directed enzyme* that synthesizes a product with a base sequence complementary to that of the template. Many DNA polymerases also have a separate nuclease activity that allows them to correct mistakes in DNA by using a different reaction to remove mismatched nucleotides. These properties of DNA polymerases contribute to the remarkably high fidelity of DNA replication, which has an error rate of less than 10^{-8} per base pair.

5.3.2 The Genes of Some Viruses Are Made of RNA

Genes in all cellular organisms are made of DNA. The same is true for some viruses, but for others the genetic material is RNA. Viruses are genetic elements enclosed in protein coats that can move from one cell to another but are not capable of independent growth. One well-studied example of an RNA virus is the tobacco mosaic virus, which infects the leaves of tobacco plants. This virus consists of a single strand of RNA (6930 nucleotides) surrounded by a protein coat of 2130 identical subunits. An RNA-directed RNA polymerase catalyzes the replication of this viral RNA.

Another important class of RNA virus comprises the *retroviruses*, so called because the genetic information flows from RNA to DNA rather than from DNA to RNA. This class includes human immunodeficiency virus 1 (HIV-1), the cause of AIDS, as well as a number of RNA viruses that produce tumors in susceptible animals. Retrovirus particles contain two copies of a single-stranded RNA molecule. On entering the cell, the RNA is copied into DNA through the action of a viral enzyme called *reverse transcriptase* (Figure 5.23). The resulting double-helical DNA version of the viral

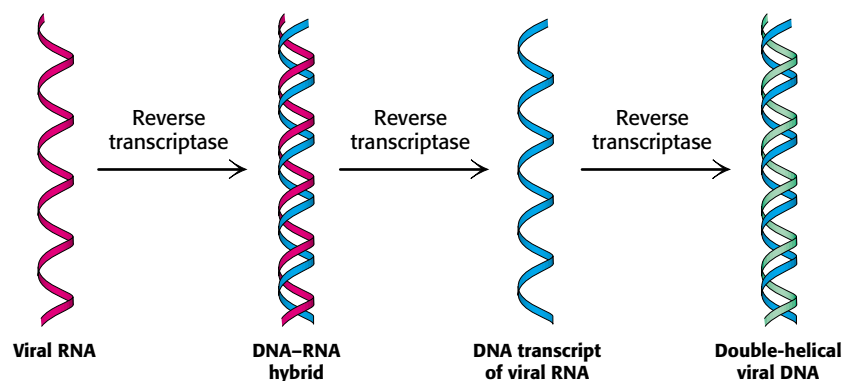


FIGURE 5.23 Flow of information from RNA to DNA in retroviruses. The RNA genome of a retrovirus is converted into DNA by reverse transcriptase, an enzyme brought into the cell by the infecting virus particle. Reverse transcriptase catalyzes the synthesis of a complementary DNA strand, the digestion of the RNA, and the subsequent synthesis of the DNA strand.

genome can become incorporated into the chromosomal DNA of the host and is replicated along with the normal cellular DNA. At a later time, the integrated viral genome is expressed to form viral RNA and viral proteins, which assemble into new virus particles.

Note that RNA viruses are not vestiges of the RNA world. Instead, fragments of RNA in these viruses have evolved to encode their protein coats and other structures needed for transferring from cell to cell and replicating.

5.4 GENE EXPRESSION IS THE TRANSFORMATION OF DNA INFORMATION INTO FUNCTIONAL MOLECULES

The information stored as DNA becomes useful when it is expressed in the production of RNA and proteins. This rich and complex topic is the subject of several chapters later in this book, but here we introduce the basics of gene expression. DNA can be thought of as archival information, stored and manipulated judiciously to minimize damage (mutations). It is expressed in two steps. First, an RNA copy is made. An RNA molecule that encodes proteins can be thought of as a photocopy of the original information—it can be made in multiple copies, used, and then disposed of. Second, an RNA molecule can be further thought of as encoding directions for protein synthesis that must be translated to be of use. The information in messenger RNA is translated into a functional protein. Other types of RNA molecules exist to facilitate this translation. We now examine the transcription of DNA information into RNA, the translation of RNA information into protein, and the genetic code that links nucleotide sequence with amino acid sequence.

5.4.1 Several Kinds of RNA Play Key Roles in Gene Expression

Cells contain several kinds of RNA (Table 5.2).

1. *Messenger RNA* is the template for protein synthesis or *translation*. An mRNA molecule may be produced for each gene or group of genes that is to be expressed in *E. coli*, whereas a distinct mRNA is produced for each gene in eukaryotes. Consequently, mRNA is a heterogeneous class of molecules. In *E. coli*, the average length of an mRNA molecule is about 1.2 kilobases (kb).
2. *Transfer RNA* carries amino acids in an activated form to the ribosome for peptide-bond formation, in a sequence dictated by the mRNA template. There is at least one kind of tRNA for each of the 20 amino acids. Transfer RNA consists of about 75 nucleotides (having a mass of about 25 kd), which makes it one of the smallest of the RNA molecules.

Kilobase (kb)–

A unit of length equal to 1000 base pairs of a double-stranded nucleic acid molecule (or 1000 bases of a single-stranded molecule).

One kilobase of double-stranded DNA has a contour length of 0.34 μm and a mass of about 660 kd.

TABLE 5.2 RNA molecules in *E. coli*

Type	Relative amount (%)	Sedimentation coefficient (S)	Mass (kd)	Number of nucleotides
Ribosomal RNA (rRNA)	80	23	1.2×10^3	3700
		16	0.55×10^3	1700
		5	3.6×10^1	120
Transfer RNA (tRNA)	15	4	2.5×10^1	75
Messenger RNA (mRNA)	5		Heterogeneous	

3. *Ribosomal RNA (rRNA)*, the major component of ribosomes, plays both a catalytic and a structural role in protein synthesis (Section 29.x.x). In *E. coli*, there are three kinds of rRNA, called 23S, 16S, and 5S RNA because of their sedimentation behavior. One molecule of each of these species of rRNA is present in each ribosome.

Ribosomal RNA is the most abundant of the three types of RNA. Transfer RNA comes next, followed by messenger RNA, which constitutes only 5% of the total RNA. Eukaryotic cells contain additional small RNA molecules. *Small nuclear RNA (snRNA)* molecules, for example, participate in the splicing of RNA exons. A small RNA molecule in the cytosol plays a role in the targeting of newly synthesized proteins to intracellular compartments and extracellular destinations.

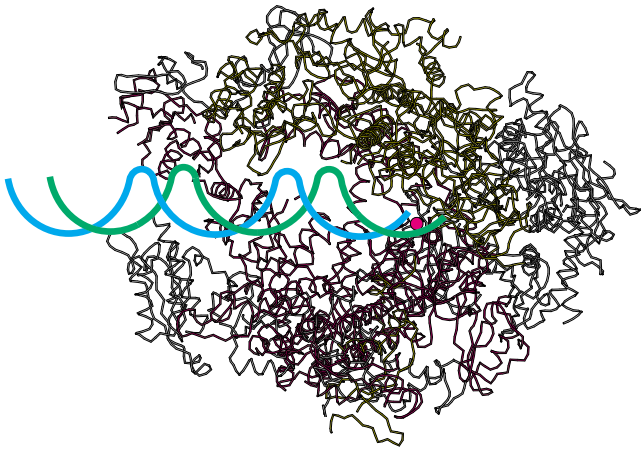


FIGURE 5.24 RNA polymerase. A large enzyme comprising many subunits including β (red) and β' (yellow), which form a “claw” that holds the DNA to be transcribed. The active site includes a Mg^{2+} ion (red ball) at the center of the structure.

5.4.2 All Cellular RNA Is Synthesized by RNA Polymerases

The synthesis of RNA from a DNA template is called *transcription* and is catalyzed by the enzyme *RNA polymerase* (Figure 5.24). RNA polymerase requires the following components:

1. *A template.* The preferred template is *double-stranded DNA*. Single-stranded DNA also can serve as a template. RNA, whether single or double stranded, is not an effective template; nor are RNA–DNA hybrids.
2. *Activated precursors.* All four *ribonucleoside triphosphates*—ATP, GTP, UTP, and CTP—are required.
3. *A divalent metal ion.* Mg^{2+} or Mn^{2+} are effective.

RNA polymerase catalyzes the initiation and elongation of RNA chains. The reaction catalyzed by this enzyme is:



The synthesis of RNA is like that of DNA in several respects (Figure 5.25). First, the direction of synthesis is $5' \rightarrow 3'$. Second, the mechanism of elongation is similar: the $3'$ -OH group at the terminus of the growing chain

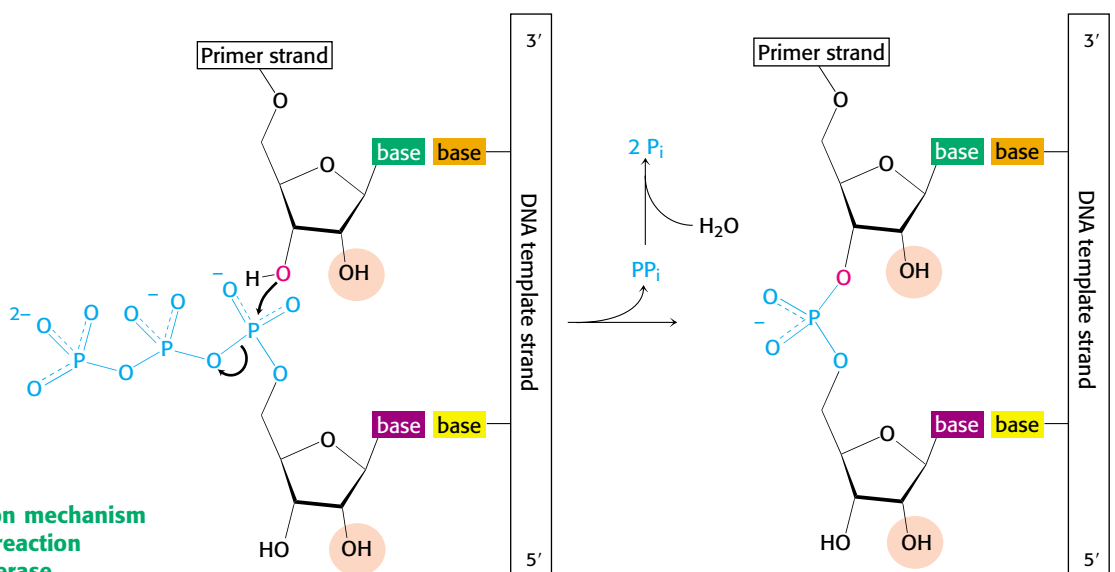


FIGURE 5.25 Transcription mechanism of the chain-elongation reaction catalyzed by RNA polymerase.

makes a nucleophilic attack on the innermost phosphate of the incoming nucleoside triphosphate. Third, the synthesis is driven forward by the hydrolysis of pyrophosphate. In contrast with DNA polymerase, however, RNA polymerase does not require a primer. In addition, RNA polymerase lacks the nuclease capability used by DNA polymerase to excise mismatched nucleotides.

All three types of cellular RNA—mRNA, tRNA, and rRNA—are synthesized in *E. coli* by the same RNA polymerase according to instructions given by a DNA template. In mammalian cells, there is a division of labor among several different kinds of RNA polymerases. We shall return to these RNA polymerases in Chapter 28.

5.4.3 RNA Polymerases Take Instructions from DNA Templates

RNA polymerase, like the DNA polymerases described earlier, takes instructions from a DNA template. The earliest evidence was the finding that the *base composition* of newly synthesized RNA is the complement of that of the DNA template strand, as exemplified by the RNA synthesized from a template of single-stranded ϕ X174 DNA (Table 5.3). *Hybridization experiments* also revealed that RNA synthesized by RNA polymerase is complementary to its DNA template. In these experiments, DNA is melted and allowed to reassociate in the presence of mRNA. RNA–DNA hybrids will form if the RNA and DNA have complementary sequences. The strongest evidence for the fidelity of transcription came from base-sequence studies showing that the RNA sequence is the precise complement of the DNA template sequence (Figure 5.26).

TABLE 5.3 Base composition (percentage) of RNA synthesized from a viral DNA template

DNA template (plus strand of ϕ X174)		RNA product	
A	25	25	U
T	33	32	A
G	24	23	C
C	18	20	G

5'—CGGGCAGCGCGCAGUUAUCCACAGCCGCGCAGUUCGCGUGGCGGCAUUUU—3' mRNA
 3'—CGCCGCTGCGCGTCAATTAGGGTGTGCGCGGTCAAGGCGACCGCCGTAAAA—5' Template strand of DNA
 5'—CGGGCAGCGCGCAGTTAATCCACAGCCGCGCAGTTCGCTGGCGGCATTTT—3' Coding strand of DNA

FIGURE 5.26 Complementarity between mRNA and DNA. The base sequence of mRNA (red) is the complement of that of the DNA template strand (blue). The sequence shown here is from the tryptophan operon, a segment of DNA containing the genes for five enzymes that catalyze the synthesis of tryptophan. The other strand of DNA (black) is called the coding strand because it has the same sequence as the RNA transcript except for thymine (T) in place of uracil (U).

5.4.4 Transcription Begins near Promoter Sites and Ends at Terminator Sites

RNA polymerase must detect and transcribe discrete genes from within large stretches of DNA. What marks the beginning of a transcriptional unit? DNA templates contain regions called *promoter sites* that specifically bind RNA polymerase and determine where transcription begins. In bacteria, two sequences on the 5' (upstream) side of the first nucleotide to be transcribed function as promoter sites (Figure 5.27A). One of them, called the *Pribnow box*, has the consensus sequence TATAAT and is centered at -10 (10 nucleotides on the 5' side of the first nucleotide transcribed, which is denoted by $+1$). The other, called the -35 region, has the consensus sequence TTGACA. The first nucleotide transcribed is usually a purine.

Eukaryotic genes encoding proteins have promoter sites with a TATAAA consensus sequence, called a *TATA box* or a *Hogness box*, centered at about -25 (Figure 5.27B). Many eukaryotic promoters also have a

Consensus sequence—

The base sequences of promoter sites are not all identical. However, they do possess common features, which can be represented by an idealized consensus sequence. Each base in the consensus sequence TATAAT is found in a majority of prokaryotic promoters. Nearly all promoter sequences differ from this consensus sequence at only one or two bases.

FIGURE 5.27 Promoter sites for transcription. Promoter sites are required for the initiation of transcription in both (A) prokaryotes and (B) eukaryotes. Consensus sequences are shown. The first nucleotide to be transcribed is numbered +1. The adjacent nucleotide on the 5' side is numbered -1. The sequences shown are those of the coding strand of DNA.

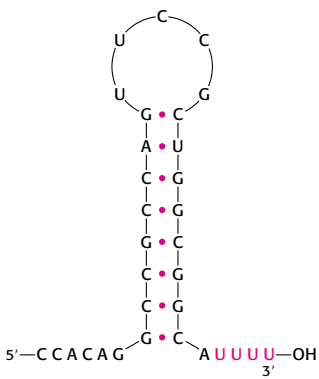
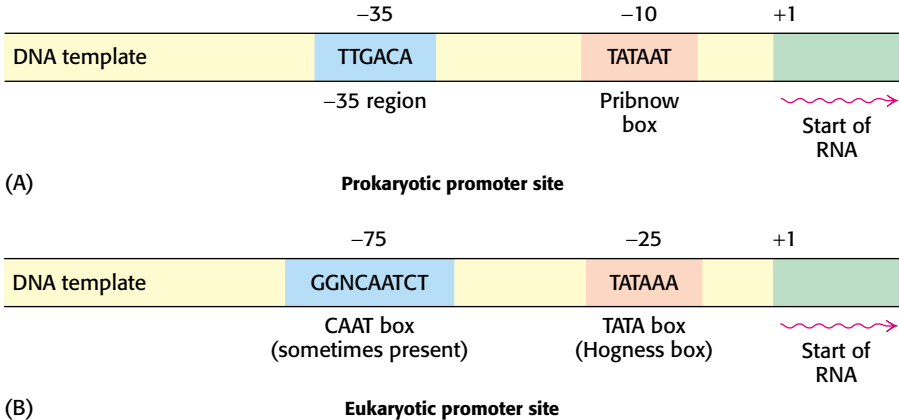


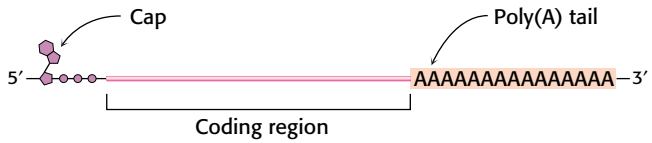
FIGURE 5.28 Base sequence of the 3' end of an mRNA transcript in *E. coli*. A stable hairpin structure is followed by a sequence of uridine (U) residues.

CAAT box with a GGNCAATCT consensus sequence centered at about -75. Transcription of eukaryotic genes is further stimulated by *enhancer sequences*, which can be quite distant (as many as several kilobases) from the start site, on either its 5' or its 3' side.

RNA polymerase proceeds along the DNA template, transcribing one of its strands until it reaches a terminator sequence. This sequence encodes a termination signal, which in *E. coli* is a *base-paired hairpin* on the newly synthesized RNA molecule (Figure 5.28). This hairpin is formed by base pairing of self-complementary sequences that are rich in G and C. Nascent RNA spontaneously dissociates from RNA polymerase when this hairpin is followed by a string of U residues. Alternatively, RNA synthesis can be terminated by the action of *rho*, a protein. Less is known about the termination of transcription in eukaryotes. A more detailed discussion of the initiation and termination of transcription will be given in Chapter 28. The important point now is that *discrete start and stop signals for transcription are encoded in the DNA template*.

In eukaryotes, the mRNA is modified after transcription (Figure 5.29). A "cap" structure is attached to the 5' end, and a sequence of adenylates, the poly(A) tail, is added to the 3' end. These modifications will be presented in detail in Section 28.x.x.

FIGURE 5.29 Modification of mRNA. Messenger RNA in eukaryotes is modified after transcription. A nucleotide "cap" structure is added to the 5' end, and a poly(A) tail is added at the 3' end.



5.4.5 Transfer RNA Is the Adaptor Molecule in Protein Synthesis

We have seen that mRNA is the template for protein synthesis. How then does it direct amino acids to become joined in the correct sequence to form a protein? In 1958, Francis Crick wrote:

RNA presents mainly a sequence of sites where hydrogen bonding could occur. One would expect, therefore, that whatever went onto the template in a *specific* way did so by forming hydrogen bonds. It is therefore a natural hypothesis that the amino acid is carried to the template by an adaptor molecule, and that the adaptor is the part that actually fits onto the RNA. In its simplest form, one would require twenty adaptors, one for each amino acid.

This highly innovative hypothesis soon became established as fact. *The adaptor in protein synthesis is transfer RNA*. The structure and reactions of

these remarkable molecules will be considered in detail in Chapter 29. For the moment, it suffices to note that tRNA contains an *amino acid-attachment site* and a *template-recognition site*. A tRNA molecule carries a specific amino acid in an activated form to the site of protein synthesis. The carboxyl group of this amino acid is esterified to the 3'- or 2'-hydroxyl group of the ribose unit at the 3' end of the tRNA chain (Figure 5.30). The joining of an amino acid to a tRNA molecule to form an *aminoacyl-tRNA* is catalyzed by a specific enzyme called an *aminoacyl-tRNA synthetase*. This esterification reaction is driven by ATP cleavage. There is at least one specific synthetase for each of the 20 amino acids. The template-recognition site on tRNA is a sequence of three bases called an *anticodon* (Figure 5.31). The anticodon on tRNA recognizes a complementary sequence of three bases, called a *codon*, on mRNA.

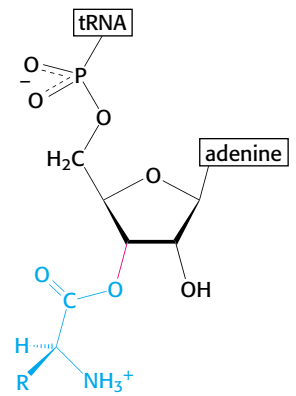
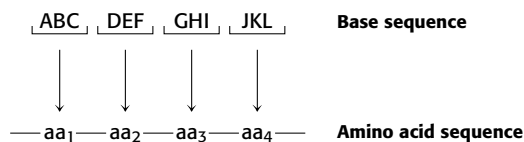


FIGURE 5.30 Attachment of an amino acid to a tRNA molecule. The amino acid (shown in blue) is esterified to the 3'-hydroxyl group of the terminal adenosine of tRNA.

5.5 AMINO ACIDS ARE ENCODED BY GROUPS OF THREE BASES STARTING FROM A FIXED POINT

The *genetic code* is the relation between the sequence of bases in DNA (or its RNA transcripts) and the sequence of amino acids in proteins. Experiments by Francis Crick, Sydney Brenner, and others established the following features of the genetic code by 1961:

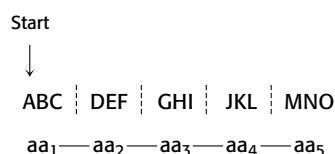
1. *Three nucleotides encode an amino acid.* Proteins are built from a basic set of 20 amino acids, but there are only four bases. Simple calculations show that a minimum of three bases is required to encode at least 20 amino acids. Genetic experiments showed that *an amino acid is in fact encoded by a group of three bases, or codon.*
2. *The code is nonoverlapping.* Consider a base sequence ABCDEF. In an overlapping code, ABC specifies the first amino acid, BCD the next, CDE the next, and so on. In a nonoverlapping code, ABC designates the first amino acid, EFG the second, and so forth. Genetics experiments again established the code to be nonoverlapping.



3. *The code has no punctuation.* In principle, one base (denoted as Q) might serve as a “comma” between groups of three bases.

... QABCQDEFQGHIQJKLQ ...

This is not the case. Rather, *the sequence of bases is read sequentially from a fixed starting point*, without punctuation.



4. *The genetic code is degenerate.* Some amino acids are encoded by more than one codon, inasmuch as there are 64 possible base triplets and only 20 amino acids. In fact, 61 of the 64 possible triplets specify particular amino acids and 3 triplets (called stop codons) designate the termination of translation. Thus, *for most amino acids, there is more than one code word.*

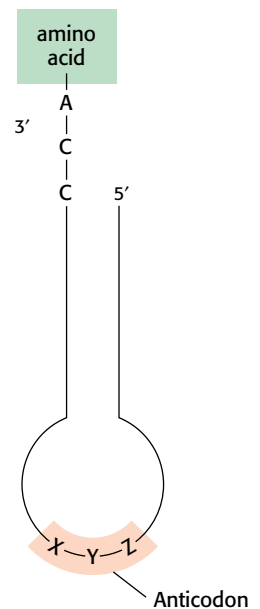


FIGURE 5.31 Symbolic diagram of an aminoacyl-tRNA. The amino acid is attached at the 3' end of the RNA. The anticodon is the template-recognition site.

5.5.1 Major Features of the Genetic Code

All 64 codons have been deciphered (Table 5.4). Because the code is highly degenerate, only tryptophan and methionine are encoded by just one triplet each. The other 18 amino acids are each encoded by two or more. Indeed, leucine, arginine, and serine are specified by six codons each. The number of codons for a particular amino acid correlates with its frequency of occurrence in proteins.

Codons that specify the same amino acid are called *synonyms*. For example, CAU and CAC are synonyms for histidine. Note that synonyms are not distributed haphazardly throughout the genetic code (depicted in Table 5.4). An amino acid specified by two or more synonyms occupies a single box (unless it is specified by more than four synonyms). The amino acids in a box are specified by codons that have the same first two bases but differ in the third base, as exemplified by GUU, GUC, GUA, and GUG. Thus, *most synonyms differ only in the last base of the triplet*. Inspection of the code shows that XYC and XYU always encode the same amino acid, whereas XYG and XYA usually encode the same amino acid. The structural basis for these equivalences of codons will become evident when we consider the nature of the anticodons of tRNA molecules (Section 29.x.x).

What is the biological significance of the extensive degeneracy of the genetic code? If the code were not degenerate, 20 codons would designate amino acids and 44 would lead to chain termination. The probability of mutating to chain termination would therefore be much higher with a nondegenerate code. Chain-termination mutations usually lead to inactive proteins, whereas substitutions of one amino acid for another are usually rather

TABLE 5.4 The genetic code

First position (5' end)	Second position				Third position (3' end)
	U	C	A	G	
U	Phe	Ser	Tyr	Cys	U
	Phe	Ser	Tyr	Cys	C
	Leu	Ser	Stop	Stop	A
	Leu	Ser	Stop	Trp	G
C	Leu	Pro	His	Arg	U
	Leu	Pro	His	Arg	C
	Leu	Pro	Gln	Arg	A
	Leu	Pro	Gln	Arg	G
A	Ile	Thr	Asn	Ser	U
	Ile	Thr	Asn	Ser	C
	Ile	Thr	Lys	Arg	A
	Met	Thr	Lys	Arg	G
G	Val	Ala	Asp	Gly	U
	Val	Ala	Asp	Gly	C
	Val	Ala	Glu	Gly	A
	Val	Ala	Glu	Gly	G

Note: This table identifies the amino acid encoded by each triplet. For example, the codon 5' AUG 3' on mRNA specifies methionine, whereas CAU specifies histidine. UAA, UAG, and UGA are termination signals. AUG is part of the initiation signal, in addition to coding for internal methionine residues.

harmless. Thus, *degeneracy minimizes the deleterious effects of mutations*. Degeneracy of the code may also be significant in permitting DNA base composition to vary over a wide range without altering the amino acid sequence of the proteins encoded by the DNA. The G + C content of bacterial DNA ranges from less than 30% to more than 70%. DNA molecules with quite different G + C contents could encode the same proteins if different synonyms of the genetic code were consistently used.

5.5.2 Messenger RNA Contains Start and Stop Signals for Protein Synthesis

Messenger RNA is translated into proteins on *ribosomes*, large molecular complexes assembled from proteins and ribosomal RNA. How is mRNA interpreted by the translation apparatus? As already mentioned, *UAA*, *UAG*, and *UGA* designate chain termination. These codons are read not by tRNA molecules but rather by specific proteins called *release factors* (Section 29.x.x). Binding of the release factors to the ribosomes releases the newly synthesized protein. The start signal for protein synthesis is more complex. Polypeptide chains in bacteria start with a modified amino acid—namely, formylmethionine (fMet). A specific tRNA, the initiator tRNA, carries fMet. This fMet-tRNA recognizes the codon AUG or, less frequently, GUG. However, AUG is also the codon for an internal methionine residue, and GUG is the codon for an internal valine residue. Hence, the signal for the first amino acid in a prokaryotic polypeptide chain must be more complex than that for all subsequent ones. *AUG (or GUG) is only part of the initiation signal* (Figure 5.32). In bacteria, the initiating AUG (or GUG) codon is preceded several nucleotides away by a purine-rich sequence that base-pairs with a complementary sequence in a ribosomal RNA molecule (Section 29.x.x). In eukaryotes, the AUG closest to the 5' end of an mRNA molecule is usually the start signal for protein synthesis. This particular AUG is read by an initiator tRNA conjugated to methionine. Once the initiator AUG is located, the *reading frame* is established—groups of three nonoverlapping nucleotides are defined, beginning with the initiator AUG codon.

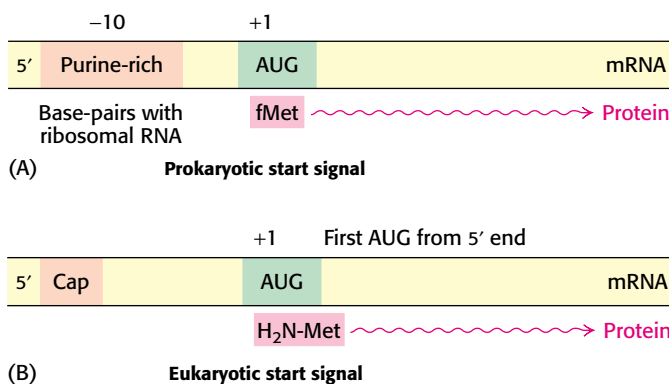
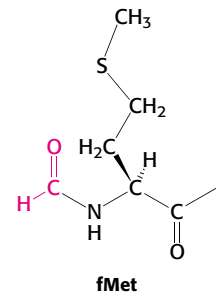


FIGURE 5.32 Initiation of protein synthesis. Start signals are required for the initiation of protein synthesis in (A) prokaryotes and (B) eukaryotes.

5.5.3 The Genetic Code Is Nearly Universal


 Is the genetic code the same in all organisms? The base sequences of many wild-type and mutant genes are known, as are the amino acid sequences of their encoded proteins. In each case, the nucleotide change in the gene and the amino acid change in the protein are as predicted by the genetic code. Furthermore, mRNAs can be correctly translated by the protein-synthesizing machinery of very different species. For example, human hemoglobin mRNA is correctly translated by a wheat germ extract, and bacteria efficiently express recombinant DNA molecules encoding human

TABLE 5.5 Distinctive codons of human mitochondria

Codon	Standard code	Mitochondrial code
UGA	Stop	Trp
UGG	Trp	Trp
AUA	Ile	Met
AUG	Met	Met
AGA	Arg	Stop
AGG	Arg	Stop

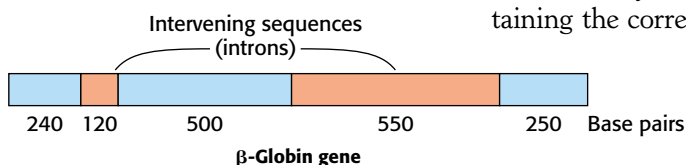
proteins such as insulin. These experimental findings strongly suggested that the genetic code is universal.

A surprise was encountered when the sequence of human mitochondrial DNA became known. Human mitochondria read UGA as a codon for tryptophan rather than as a stop signal (Table 5.5). Furthermore, AGA and AGG are read as stop signals rather than as codons for arginine, and AUA is read as a codon for methionine instead of isoleucine. Mitochondria of other species, such as those of yeast, also have genetic codes that differ slightly from the standard one. The genetic code of mitochondria can differ from that of the rest of the cell because mitochondrial DNA encodes a distinct set of tRNAs. Do any cellular protein-synthesizing systems deviate from the standard genetic code? Ciliated protozoa differ from most organisms in reading UAA and UAG as codons for amino acids rather than as stop signals; UGA is their sole termination signal. Thus, *the genetic code is nearly but not absolutely universal*. Variations clearly exist in mitochondria and in species, such as ciliates, that branched off very early in eukaryotic evolution. It is interesting to note that two of the codon reassignments in human mitochondria diminish the information content of the third base of the triplet (e.g., both AUA and AUG specify methionine). Most variations from the standard genetic code are in the direction of a simpler code.

Why has the code remained nearly invariant through billions of years of evolution, from bacteria to human beings? A mutation that altered the reading of mRNA would change the amino acid sequence of most, if not all, proteins synthesized by that particular organism. Many of these changes would undoubtedly be deleterious, and so there would be strong selection against a mutation with such pervasive consequences.

5.6 MOST EUKARYOTIC GENES ARE MOSAICS OF INTRONS AND EXONS

In bacteria, polypeptide chains are encoded by a continuous array of triplet codons in DNA. For many years, genes in higher organisms also were assumed to be continuous. This view was unexpectedly shattered in 1977, when investigators in several laboratories discovered that several genes are *discontinuous*. The mosaic nature of eukaryotic genes was revealed by electron microscopic studies of hybrids formed between mRNA and a segment of DNA containing the corresponding gene (Figure 5.33). For example, the gene for the β chain of hemoglobin is interrupted within its amino acid-coding sequence by a long *intervening sequence* of 550 base pairs and a short one of 120 base pairs. Thus, the β -globin gene is split into three coding sequences.



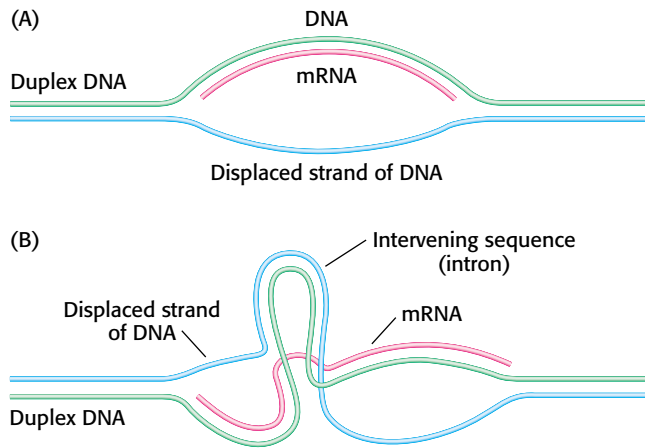


FIGURE 5.33 Detection of intervening sequences by electron microscopy. An mRNA molecule (shown in red) is hybridized to genomic DNA containing the corresponding gene. (A) A single loop of single-stranded DNA (shown in blue) is seen if the gene is continuous. (B) Two loops of single-stranded DNA (blue) and a loop of double-stranded DNA (blue and green) are seen if the gene contains an intervening sequence. Additional loops are evident if more than one intervening sequence is present.

5.6.1 RNA Processing Generates Mature RNA

At what stage in gene expression are intervening sequences removed? Newly synthesized RNA chains (pre-mRNA) isolated from nuclei are much larger than the mRNA molecules derived from them; in the case of β -globin RNA, the former sediment at 15S in zonal centrifugation experiments (Section 4.1.6) and the latter at 9S. In fact, the primary transcript of the β -globin gene contains two regions that are not present in the mRNA. *These intervening sequences in the 15S primary transcript are excised, and the coding sequences are simultaneously linked by a precise splicing enzyme to form the mature 9S mRNA* (Figure 5.34). Regions that are removed from the primary transcript are called *introns* (for *intervening sequences*), whereas those that are retained in the mature RNA are called *exons* (for *expressed regions*). A common feature in the expression of split genes is that their exons are ordered in the same sequence in mRNA as in DNA. Thus, split genes, like continuous genes, are colinear with their polypeptide products.

Splicing is a facile complex operation that is carried out by *spliceosomes*, which are assemblies of proteins and small RNA molecules (Section 28.x.x). This enzymatic machinery recognizes signals in the nascent RNA that specify the splice sites. *Introns nearly always begin with GU and end with an AG that is preceded by a pyrimidine-rich tract* (Figure 5.35). *This consensus sequence is part of the signal for splicing.*

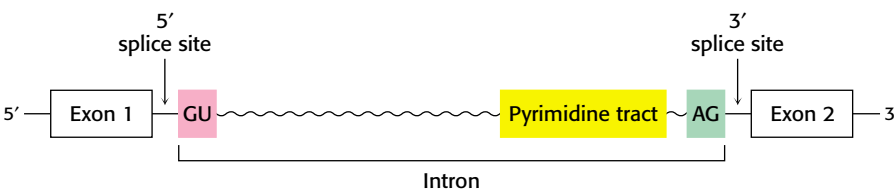



FIGURE 5.35 Consensus sequence for the splicing of mRNA precursors.

5.6.2 Many Exons Encode Protein Domains

 Most genes of higher eukaryotes, such as birds and mammals, are split. Lower eukaryotes, such as yeast, have a much higher proportion of continuous genes. In prokaryotes, split genes are extremely rare. Have introns been inserted into genes in the evolution of higher organisms? Or have introns been removed from genes to form the streamlined genomes of prokaryotes and simple eukaryotes? Comparisons of the DNA sequences of genes encoding proteins that are highly conserved in evolution suggest that *introns were present in ancestral genes and were lost in the evolution of*

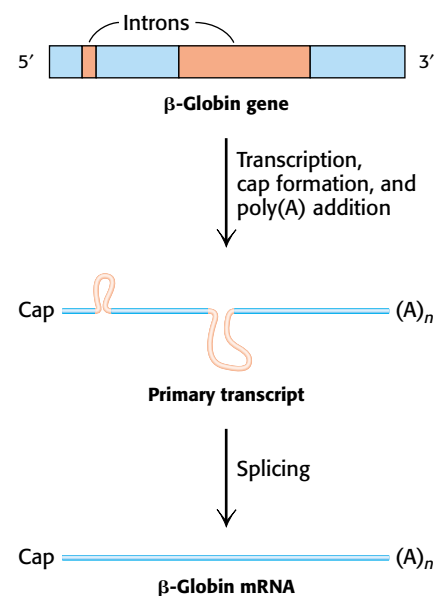


FIGURE 5.34 Transcription and processing of the β -globin gene. The gene is transcribed to yield the primary transcript, which is modified by cap and poly(A) addition. The intervening sequences in the primary RNA transcript are removed to form the mRNA.

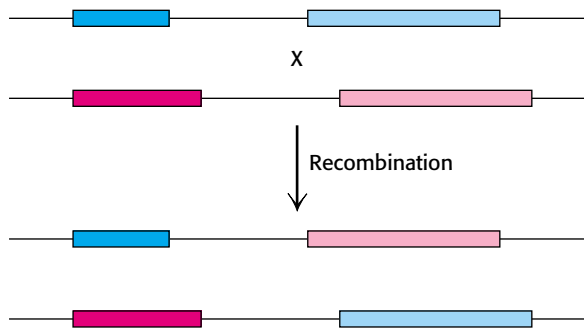


FIGURE 5.36 Exon shuffling. Exons can be readily shuffled by recombination of DNA to expand the genetic repertoire.

organisms that have become optimized for very rapid growth, such as prokaryotes. The positions of introns in some genes are at least 1 billion years old. Furthermore, a common mechanism of splicing developed before the divergence of fungi, plants, and vertebrates, as shown by the finding that mammalian cell extracts can splice yeast RNA. Many exons encode discrete structural and functional units of proteins. An attractive hypothesis is that new proteins arose in evolution by the rearrangement of exons encoding discrete structural elements, binding sites, and catalytic sites, a process called exon shuffling. Because it preserves functional units but allows them to interact in new ways, exon shuffling is a rapid and efficient means of generating novel genes (Figure 5.36). Introns are extensive regions in which DNA can break and recombine with

no deleterious effect on encoded proteins. In contrast, the exchange of sequences between different exons usually leads to loss of function.

Another advantage conferred by split genes is the potentiality for generating a series of related proteins by splicing a nascent RNA transcript in different ways. For example, a precursor of an antibody-producing cell forms an antibody that is anchored in the cell's plasma membrane (Figure 5.37). Stimulation of such a cell by a specific foreign antigen that is recognized by the attached antibody leads to cell differentiation and proliferation. The activated antibody-producing cells then splice their nascent RNA transcript in an alternative manner to form soluble antibody molecules that are secreted rather than retained on the cell surface. We see here a clear-cut example of a benefit conferred by the complex arrangement of introns and exons in higher organisms. Alternative splicing is a facile means of forming a set of proteins that are variations of a basic motif according to a developmental program without requiring a gene for each protein.

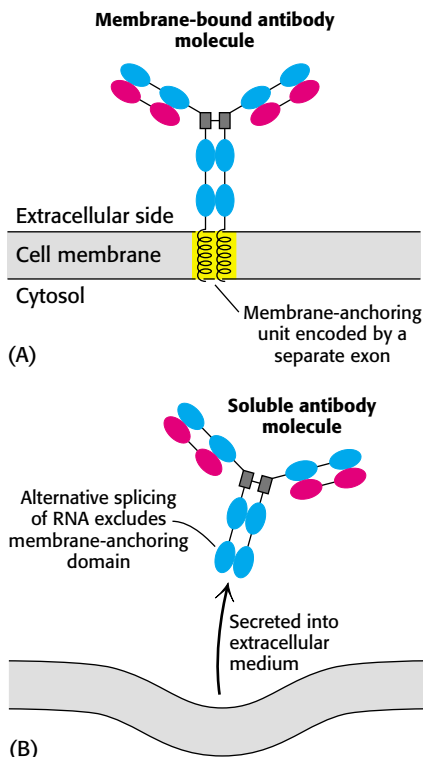


FIGURE 5.37 Alternative splicing. Alternative splicing generates mRNAs that are templates for different forms of a protein: (A) a membrane-bound antibody on the surface of a lymphocyte, and (B) its soluble counterpart, exported from the cell. The membrane-bound antibody is anchored to the plasma membrane by a helical segment (highlighted in yellow) that is encoded by its own exon.

SUMMARY

- **A Nucleic Acid Consists of Four Kinds of Bases Linked to a Sugar-Phosphate Backbone**

DNA and RNA are linear polymers of a limited number of monomers. In DNA, the repeating units are nucleotides with the sugar being a deoxyribose and the bases being adenine (A), thymine (T), guanine (G), and cytosine (C). In RNA, the sugar is a ribose and the base uracil (U) is used in place of thymine. DNA is the molecule of heredity in all prokaryotic and eukaryotic organisms. In viruses, the genetic material is either DNA or RNA.

- **A Pair of Nucleic Acid Chains with Complementary Sequences Can Form a Double-Helical Structure**

All cellular DNA consists of two very long, helical polynucleotide chains coiled around a common axis. The sugar-phosphate backbone of each strand is on the outside of the double helix, whereas the purine and pyrimidine bases are on the inside. The two chains are held together by hydrogen bonds between pairs of bases: adenine is always paired with thymine, and guanine is always paired with cytosine. Hence, one strand of a double helix is the complement of the other. The two strands of the double helix run in opposite directions. Genetic information is encoded in the precise sequence of bases along a strand. Most RNA molecules are single stranded, but many contain extensive double-helical regions that arise from the folding of the chain into hairpins.

- **DNA Is Replicated by Polymerases That Take Instructions from Templates**

In the replication of DNA, the two strands of a double helix unwind and separate as new chains are synthesized. Each parent strand acts as a template for the formation of a new complementary strand. Thus, the replication of DNA is semiconservative—each daughter molecule receives one strand from the parent DNA molecule. The replication of DNA is a complex process carried out by many proteins, including several DNA polymerases. The activated precursors in the synthesis of DNA are the four deoxyribonucleoside 5'-triphosphates. The new strand is synthesized in the 5' → 3' direction by a nucleophilic attack by the 3'-hydroxyl terminus of the primer strand on the innermost phosphorus atom of the incoming deoxyribonucleoside triphosphate. Most important, DNA polymerases catalyze the formation of a phosphodiester bond only if the base on the incoming nucleotide is complementary to the base on the template strand. In other words, DNA polymerases are template-directed enzymes. The genes of some viruses, such as tobacco mosaic virus, are made of single-stranded RNA. An RNA-directed RNA polymerase mediates the replication of this viral RNA. Retroviruses, exemplified by HIV-1, have a single-stranded RNA genome that is transcribed into double-stranded DNA by reverse transcriptase, an RNA-directed DNA polymerase.

- **Gene Expression Is the Transformation of DNA Information into Functional Molecules**

The flow of genetic information in normal cells is from DNA to RNA to protein. The synthesis of RNA from a DNA template is called transcription, whereas the synthesis of a protein from an RNA template is termed translation. Cells contain several kinds of RNA: messenger RNA (mRNA), transfer RNA (tRNA), and ribosomal RNA (rRNA), which vary in size from 75 to more than 5000 nucleotides. All cellular RNA is synthesized by RNA polymerases according to instructions given by DNA templates. The activated intermediates are ribonucleoside triphosphates and the direction of synthesis, like that of DNA, is 5' → 3'. RNA polymerase differs from DNA polymerase in not requiring a primer.

- **Amino Acids Are Encoded by Groups of Three Bases Starting from a Fixed Point**

The genetic code is the relation between the sequence of bases in DNA (or its RNA transcript) and the sequence of amino acids in proteins. Amino acids are encoded by groups of three bases (called codons) starting from a fixed point. Sixty-one of the 64 codons specify particular amino acids, whereas the other 3 codons (UAA, UAG, and UGA) are signals for chain termination. Thus, for most amino acids, there is more than one code word. In other words, the code is degenerate. The genetic code is nearly the same in all organisms. Natural mRNAs contain start and stop signals for translation, just as genes do for directing where transcription begins and ends.

- **Most Eukaryotic Genes Are Mosaics of Introns and Exons**

Most genes in higher eukaryotes are discontinuous. Coding sequences (exons) in these split genes are separated by intervening sequences (introns), which are removed in the conversion of the primary transcript into mRNA and other functional mature RNA molecules. Split genes, like continuous genes, are colinear with their polypeptide products. A striking feature of many exons is that they encode functional domains in proteins. New proteins probably arose in the course of evolution by the shuffling of exons. Introns may have been present in primordial genes but were lost in the evolution of such fast-growing organisms as bacteria and yeast.

KEY TERMS

deoxyribonucleic acid (DNA) (p. 118)	DNA polymerase (p. 127)	promoter site (p. 131)
deoxyribose (p. 118)	template (p. 127)	codon (p. 133)
ribose (p. 118)	primer (p. 127)	genetic code (p. 133)
ribonucleic acid (RNA) (p. 118)	reverse transcriptase (p. 128)	ribosome (p. 135)
purine (p. 119)	messenger RNA (mRNA) (p. 129)	intron (p. 137)
pyrimidine (p. 119)	translation (p. 129)	exon (p. 137)
nucleoside (p. 120)	transfer RNA (tRNA) (p. 129)	splicing (p. 137)
nucleotide (p. 120)	ribosomal RNA (rRNA) (p. 130)	spliceosomes (p. 137)
replication (p. 121)	small nuclear RNA (snRNA) (p. 130)	exon shuffling (p. 138)
double helix (p. 121)	transcription (p. 130)	alternative splicing (p. 138)
semiconservative replication (p. 123)	RNA polymerase (p. 130)	

SELECTED READINGS

Where to start

- Felsenfeld, G., 1985. DNA. *Sci. Am.* 253(4):58–67.
- Darnell, J. E., Jr., 1985. RNA. *Sci. Am.* 253(4):68–78.
- Dickerson, R. E., 1983. The DNA helix and how it is read. *Sci. Am.* 249(6):94–111.
- Crick, F. H. C., 1954. The structure of the hereditary material. *Sci. Am.* 191(4): 54–61.
- Chambon, P., 1981. Split genes. *Sci. Am.* 244(5):60–71.
- Watson, J. D., and Crick, F. H. C., 1953. Molecular structure of nucleic acids. A structure for deoxyribose nucleic acid. *Nature* 171:737–738.
- Watson, J. D., and Crick, F. H. C., 1953. Genetic implications of the structure of deoxyribonucleic acid. *Nature* 171:964–967.
- Meselson, M., and Stahl, F. W., 1958. The replication of DNA in *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.* 44:671–682.

Books

- Bloomfield, V. A., Crothers, D. M., Tinoco, I., and Hearst, J., 2000. *Nucleic Acids: Structures, Properties, and Functions*. University Science Books.
- Singer, M., Berg, P., 1991. *Genes and Genomes: A Changing Perspective*. University Science Books.
- Lodish, H., Berk, A., Zipursky, L., and Matsudaira, P., 1999. *Molecular Cell Biology* (4th ed.). W. H. Freeman and Company.
- Lewin, B., 2000. *Genes VII*. Oxford University Press.
- Watson, J. D., Hopkins, N. H., Roberts, J. W., Steitz, J. A., and Weiner, A. M., 2000. *Molecular Biology of the Gene* (5th ed.). Benjamin Cummings.

DNA structure

- Saenger, W., 1984. *Principles of Nucleic Acid Structure*. Springer Verlag.
- Dickerson, R. E., Drew, H. R., Conner, B. N., Wing, R. M., Fratini, A. V., and Kopka, M. L., 1982. The anatomy of A-, B-, and Z-DNA. *Science* 216:475–485.
- Sinden, R. R., 1994. *DNA structure and function*. Academic Press.

DNA replication

- Kornberg, A., and Baker, T. A., 1992. *DNA Replication* (2d ed.). W. H. Freeman and Company.
- Hübscher, U., Nasheuer, H.-P., and Syväoja, J. E. 2000. Eukaryotic DNA polymerases: A growing family. *Trends Biochem. Sci.* 25:143–147.
- Brautigam, C. A., and Steitz, T. A., 1998. Structural and functional insights provided by crystal structures of DNA polymerases and their substrate complexes. *Curr. Opin. Struct. Biol.* 8:54–63.

Discovery of messenger RNA

- Jacob, F., and Monod, J., 1961. Genetic regulatory mechanisms in the synthesis of proteins. *J. Mol. Biol.* 3:318–356.

- Brenner, S., Jacob, F., and Meselson, M., 1961. An unstable intermediate carrying information from genes to ribosomes for protein synthesis. *Nature* 190:576–581.
- Hall, B. D., and Spiegelman, S., 1961. Sequence complementarity of T2-DNA and T2-specific RNA. *Proc. Natl. Acad. Sci. U.S.A.* 47:137–146.

Genetic code

- Crick, F. H. C., Barnett, L., Brenner, S., and Watts-Tobin, R. J., 1961. General nature of the genetic code for proteins. *Nature* 192:1227–1232.
- Nirenberg, M., 1968. The genetic code. In *Nobel Lectures: Physiology or Medicine* (1963–1970), pp. 372–395. American Elsevier (1973).
- Crick, F. H. C., 1958. On protein synthesis. *Symp. Soc. Exp. Biol.* 12:138–163.
- Woese, C. R., 1967. *The Genetic Code*. Harper & Row.
- Knight, R. D., Freeland, S. J., and Landweber L. F., 1999. Selection, history and chemistry: The three faces of the genetic code. *Trends Biochem. Sci.* 24(6):241–247

Introns, exons, and split genes

- Sharp, P. A., 1988. RNA splicing and genes. *J. Am. Med. Assoc.* 260:3035–3041.
- Dorit, R. L., Schoenbach, L., and Gilbert, W., 1990. How big is the universe of exons? *Science* 250:1377–1382.
- Cochet, M., Gannon, F., Hen, R., Maroteaux, L., Perrin, F., and Chambon, P., 1979. Organization and sequence studies of the 17-piece chicken conalbumin gene. *Nature* 282:567–574.
- Tilghman, S. M., Tiemeier, D. C., Seidman, J. G., Peterlin, B. M., Sullivan, M., Maizel, J. V., and Leder, P., 1978. Intervening sequence of DNA identified in the structural portion of a mouse β -globin gene. *Proc. Natl. Acad. Sci. U.S.A.* 75:725–729.

Reminiscences and historical accounts

- Watson, J. D., 1968. *The Double Helix*. Atheneum.
- McCarty, M., 1985. *The Transforming Principle: Discovering That Genes Are Made of DNA*. Norton.
- Cairns, J., Stent, G. S., and Watson, J. D., 2000. *Phage and the Origins of Molecular Biology*. Cold Spring Harbor Laboratory.
- Olby, R., 1974. *The Path to the Double Helix*. University of Washington Press.
- Portugal, F. H., and Cohen, J. S., 1977. *A Century of DNA: A History of the Discovery of the Structure and Function of the Genetic Substance*. MIT Press.
- Judson, H., 1996. *The Eighth Day of Creation*. Cold Spring Harbor Laboratory.
- Sayre, A. 2000. *Rosalind Franklin and DNA*. Norton.

PROBLEMS

1. *Complements.* Write the complementary sequence (in the standard 5' → 3' notation) for (a) GATCAA, (b) TCGAAC, (c) ACGCGT, and (d) TACCAT.

2. *Compositional constraint.* The composition (in mole-fraction units) of one of the strands of a double-helical DNA molecule is [A] = 0.30 and [G] = 0.24. (a) What can you say about [T] and [C] for the same strand? (b) What can you say about [A], [G], [T], and [C] of the complementary strand?

3. *Lost DNA.* The DNA of a deletion mutant of λ bacteriophage has a length of 15 μm instead of 17 μm . How many base pairs are missing from this mutant?

4. *An unseen pattern.* What result would Meselson and Stahl have obtained if the replication of DNA were conservative (i.e., the parental double helix stayed together)? Give the expected distribution of DNA molecules after 1.0 and 2.0 generations for conservative replication.

5. *Tagging DNA.* (a) Suppose that you want to radioactively label DNA but not RNA in dividing and growing bacterial cells. Which radioactive molecule would you add to the culture medium? (b) Suppose that you want to prepare DNA in which the backbone phosphorus atoms are uniformly labeled with ^{32}P . Which precursors should be added to a solution containing DNA polymerase I and primed template DNA? Specify the position of radioactive atoms in these precursors.

6. *Finding a template.* A solution contains DNA polymerase I and the Mg^{2+} salts of dATP, dGTP, dCTP, and TTP. The following DNA molecules are added to aliquots of this solution. Which of them would lead to DNA synthesis? (a) A single-stranded closed circle containing 1000 nucleotide units. (b) A double-stranded closed circle containing 1000 nucleotide pairs. (c) A single-stranded closed circle of 1000 nucleotides base-paired to a linear strand of 500 nucleotides with a free 3'-OH terminus. (d) A double-stranded linear molecule of 1000 nucleotide pairs with a free 3'-OH group at each end.

7. *The right start.* Suppose that you want to assay reverse transcriptase activity. If polyriboadenylate is the template in the assay, what should you use as the primer? Which radioactive nucleotide should you use to follow chain elongation?

8. *Essential degradation.* Reverse transcriptase has ribonuclease activity as well as polymerase activity. What is the role of its ribonuclease activity?

9. *Virus hunting.* You have purified a virus that infects turnip leaves. Treatment of a sample with phenol removes viral proteins. Application of the residual material to scraped leaves results in the formation of progeny virus particles. You infer that the infectious substance is a nucleic acid. Propose a simple and highly sensitive means of determining whether the infectious nucleic acid is DNA or RNA.

10. *Mutagenic consequences.* Spontaneous deamination of cytosine bases in DNA occurs at low but measurable frequency. Cytosine is converted into uracil by loss of its amino group. After this conversion, which base pair occupies this position in each of the daughter strands resulting from one round of replication? Two rounds of replication?

11. *Information content.* (a) How many different 8-mer sequences of DNA are there? (Hint: There are 16 possible dinucleotides and 64 possible trinucleotides.) (b) How many bits of information are stored in an 8-mer DNA sequence? In the *E. coli* genome? In the human genome? (c) Compare each of these values with the amount of information that can be stored on a personal computer diskette. A byte is equal to 8 bits.

12. *Key polymerases.* Compare DNA polymerase I and RNA polymerase from *E. coli* in regard to each of the following features: (a) activated precursors, (b) direction of chain elongation, (c) conservation of the template, and (d) need for a primer.

13. *Encoded sequences.* (a) Write the sequence of the mRNA molecule synthesized from a DNA template strand having the sequence



(b) What amino acid sequence is encoded by the following base sequence of an mRNA molecule? Assume that the reading frame starts at the 5' end.



(c) What is the sequence of the polypeptide formed on addition of poly(UUAC) to a cell-free protein-synthesizing system?

14. *A tougher chain.* RNA is readily hydrolyzed by alkali, whereas DNA is not. Why?

15. *A potent blocker.* How does cordycepin (3'-deoxyadenosine) block the synthesis of RNA?

16. *Silent RNA.* The code word GGG cannot be deciphered in the same way as can UUU, CCC, and AAA, because poly(G) does not act as a template. Poly(G) forms a triple-stranded helical structure. Why is it an ineffective template?

17. *Two from one.* Synthetic RNA molecules of defined sequence were instrumental in deciphering the genetic code. Their synthesis first required the synthesis of DNA molecules to serve as a template. H. Gobind Khorana synthesized, by organic-chemical methods, two complementary deoxyribonucleotides, each with nine residues: d(TAC)₃ and d(GTA)₃. Partly overlapping duplexes that formed on mixing these oligonucleotides then served as templates for the synthesis by DNA polymerase of long, repeating double-helical DNA chains. The next step was to obtain long polyribonucleotide chains with a sequence complementary to only one of the two DNA strands. How did he obtain only poly(UAC)? Only poly(GUA)?

18. *Overlapping or not.* In a nonoverlapping triplet code, each group of three bases in a sequence ABCDEF ... specifies only one amino acid—ABC specifies the first, DEF the second, and so forth—whereas, in a completely overlapping triplet code, ABC specifies the first amino acid, BCD the second, CDE the third, and so forth. Assume that you can mutate an individual nucleotide of a codon and detect the mutation in the amino acid sequence. Design an experiment that would establish whether the genetic code is overlapping or nonoverlapping.

19. *Triple entendre.* The RNA transcript of a region of T4 phage DNA contains the sequence 5'-AAAUGAGGA-3'. This sequence encodes three different polypeptides. What are they?

20. *Valuable synonyms.* Proteins generally have low contents of Met and Trp, intermediate ones of His and Cys, and high ones of Leu and Ser. What is the relation between the number of codons of an amino acid and its frequency of occurrence in proteins? What might be the selective advantage of this relation?

21. *A new translation.* A transfer RNA with a UGU anticodon is enzymatically conjugated to ^{14}C -labeled cysteine. The cysteine unit is then chemically modified to alanine (with the use of Raney nickel, which removes the sulfur atom of cysteine). The altered aminoacyl-tRNA is added to a protein-synthesizing system containing normal components except for this tRNA. The mRNA added to this mixture contains the following sequence:

5'-UUUUGCCAUGUUUGUGCU-3'

What is the sequence of the corresponding radiolabeled peptide?

Chapter Integration Problems

22. *Eons ago.* The atmosphere of the primitive Earth before the emergence of life contained N_2 , NH_3 , H_2 , HCN , CO , and H_2O . Which of these compounds is a likely precursor of most of the atoms in adenine? Why?

23. *Back to the bench.* A protein chemist told a molecular geneticist that he had found a new mutant hemoglobin in which

aspartate replaced lysine. The molecular geneticist expressed surprise and sent his friend scurrying back to the laboratory. (a) Why did the molecular geneticist doubt the reported amino acid substitution? (b) Which amino acid substitutions would have been more palatable to the molecular geneticist?

24. *Eons apart.* The amino acid sequences of a yeast protein and a human protein carrying out the same function are found to be 60% identical. However, the corresponding DNA sequences are only 45% identical. Account for this differing degree of identity.



Media Problem

25. *More than one way to pair a base.* Genetic mutations can arise due to nonstandard base pairing during DNA replication. Such mispairing can be made more likely by the chemical modification of bases (which is how mutagens work). One example is oxidation of guanine to 8-oxoguanine. An effect of this modification is to introduce some steric strain into the *anti* configuration of the glycosylic bond, making the *syn* configuration more favorable than usual. Look at the Media Problem section of the **Structural Insights** module on nucleic acids and explain why 8-oxoguanine often mispairs with adenine.

Exploring Genes



Processes such as development from a caterpillar into a butterfly involve dramatic changes in patterns of gene expression. The expression levels of thousands of genes can be monitored through the use of DNA arrays. At right, a GeneChip[®] reveals the expression levels of more than 12,000 human genes; the brightness of each spot indicates the expression level of the corresponding gene. [Left) Roger Hart/Rainbow. (Right) GeneChip courtesy of Affymetrix.]

Recombinant DNA technology has revolutionized biochemistry since it came into being in the 1970s. The genetic endowment of organisms can now be precisely changed in designed ways. Recombinant DNA technology is a fruit of several decades of basic research on DNA, RNA, and viruses. It depends, first, on having enzymes that can cut, join, and replicate DNA and reverse transcribe RNA. Restriction enzymes cut very long DNA molecules into specific fragments that can be manipulated; DNA ligases join the fragments together. The availability of many kinds of restriction enzymes and DNA ligases makes it feasible to treat DNA sequences as modules that can be moved at will from one DNA molecule to another. Thus, recombinant DNA technology is based on nucleic acid enzymology.

A second foundation is the base-pairing language that allows complementary sequences to recognize and bind to each other. Hybridization with complementary DNA or RNA probes is a sensitive and powerful means of detecting specific nucleotide sequences. In recombinant DNA technology, base-pairing is used to construct new combinations of DNA as well as to detect and amplify particular sequences. This revolutionary technology is also critically dependent on our understanding of viruses, the ultimate parasites. Viruses efficiently deliver their own DNA (or RNA) into hosts, subverting them either to replicate the viral genome and produce viral proteins or to incorporate viral DNA into the host genome. Likewise, plasmids, which are accessory chromosomes found in bacteria, have been indispensable in recombinant DNA technology.

OUTLINE

- 6.1 The Basic Tools of Gene Exploration
- 6.2 Recombinant DNA Technology Has Revolutionized All Aspects of Biology
- 6.3 Manipulating the Genes of Eukaryotes
- 6.4 Novel Proteins Can Be Engineered by Site-Specific Mutagenesis

These new methods have wide-ranging benefits. Entire genomes, including the human genome, are being deciphered. New insights are emerging, for example, into the regulation of gene expression in cancer and development and the evolutionary history of proteins as well as organisms. New proteins can be created by altering genes in specific ways to provide detailed views into protein function. Clinically useful proteins, such as hormones, are now synthesized by recombinant DNA techniques. Crops are being generated to resist pests and harsh conditions. The new opportunities opened by recombinant DNA technology promise to have broad effects.

6.1 THE BASIC TOOLS OF GENE EXPLORATION

The rapid progress in biotechnology—indeed its very existence—is a result of a relatively few techniques.

1. *Restriction-enzyme analysis.* Restriction enzymes are precise, molecular scalpels that allow the investigator to manipulate DNA segments.
2. *Blotting techniques.* The Southern and Northern blots are used to separate and characterize DNA and RNA, respectively. The Western blot, which uses antibodies to characterize proteins, was described in Section 4.3.4.
3. *DNA sequencing.* The precise nucleotide sequence of a molecule of DNA can be determined. Sequencing has yielded a wealth of information concerning gene architecture, the control of gene expression, and protein structure.
4. *Solid-phase synthesis of nucleic acids.* Precise sequences of nucleic acids can be synthesized de novo and used to identify or amplify other nucleic acids.
5. *The polymerase chain reaction (PCR).* The polymerase chain reaction leads to a billionfold amplification of a segment of DNA. One molecule of DNA can be amplified to quantities that permit characterization and manipulation. This powerful technique is being used to detect pathogens and genetic diseases, to determine the source of a hair left at the scene of a crime, and to resurrect genes from fossils.

A final tool, the use of which will be highlighted in the next chapter, is the computer. Without the computer, it would be impossible to catalog, access, and characterize the abundant information, especially DNA sequence information, that the techniques just outlined are rapidly generating.

6.1.1 Restriction Enzymes Split DNA into Specific Fragments

Restriction enzymes, also called *restriction endonucleases*, recognize specific base sequences in double-helical DNA and cleave, at specific places, both strands of a duplex containing the recognized sequences. To biochemists, these exquisitely precise scalpels are marvelous gifts of nature. They are indispensable for analyzing chromosome structure, sequencing very long DNA molecules, isolating genes, and creating new DNA molecules that can be cloned. Werner Arber and Hamilton Smith discovered restriction enzymes, and Daniel Nathans pioneered their use in the late 1960s.

Restriction enzymes are found in a wide variety of prokaryotes. Their biological role is to cleave foreign DNA molecules. The cell's own DNA is not degraded, because the sites recognized by its own restriction enzymes are methylated. Many restriction enzymes recognize specific sequences of four to eight base pairs and hydrolyze a phosphodiester bond in each strand in this region. A striking characteristic of these cleavage sites is that they almost always possess *twofold rotational symmetry*. In other words, the recognized sequence is *palindromic*, or an inverted repeat, and the cleavage sites

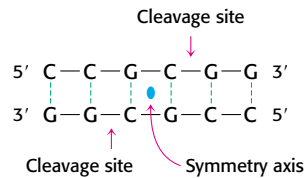
Palindrome—

A word, sentence, or verse that reads the same from right to left as it does from left to right.

Radar
Madam, I'm Adam
Able was I ere I saw Elba
Roma tibi subito motibus ibit amor

Derived from the Greek *palindromos*, "running back again."

are symmetrically positioned. For example, the sequence recognized by a restriction enzyme from *Streptomyces achromogenes* is:



In each strand, the enzyme cleaves the C–G phosphodiester bond on the 3' side of the symmetry axis. As we shall see in Chapter 9, this symmetry reflects that of structures of the restriction enzymes themselves.

More than 100 restriction enzymes have been purified and characterized. Their names consist of a three-letter abbreviation for the host organism (e.g., *Eco* for *Escherichia coli*, *Hin* for *Haemophilus influenzae*, *Hae* for *Haemophilus aegyptius*) followed by a strain designation (if needed) and a roman numeral (if more than one restriction enzyme from the same strain has been identified). The specificities of several of these enzymes are shown in Figure 6.1. Note that the cuts may be staggered or even.

Restriction enzymes are used to cleave DNA molecules into specific fragments that are more readily analyzed and manipulated than the entire parent molecule. For example, the 5.1-kb circular duplex DNA of the tumor-producing SV40 virus is cleaved at 1 site by *EcoRI*, 4 sites by *HpaI*, and 11 sites by *HindIII*. A piece of DNA produced by the action of one restriction enzyme can be specifically cleaved into smaller fragments by another restriction enzyme. The pattern of such fragments can serve as a *fingerprint* of a DNA molecule, as will be discussed shortly. Indeed, complex chromosomes containing hundreds of millions of base pairs can be mapped by using a series of restriction enzymes.

6.1.2 Restriction Fragments Can Be Separated by Gel Electrophoresis and Visualized

Small differences between related DNA molecules can be readily detected because their restriction fragments can be separated and displayed by gel electrophoresis. In many types of gels, the electrophoretic mobility of a DNA fragment is inversely proportional to the logarithm of the number of base pairs, up to a certain limit. Polyacrylamide gels are used to separate fragments containing about as many as 1000 base pairs, whereas more porous agarose gels are used to resolve mixtures of larger fragments (about as many as 20 kb). An important feature of these gels is their high resolving power. In certain kinds of gels, *fragments differing in length by just one nucleotide of several hundred can be distinguished*. Moreover, entire chromosomes containing millions of nucleotides can be separated on agarose gels by applying pulsed electric fields (pulsed-field gel electrophoresis, PFGE) in different directions. This technique depends on the differential stretching and relaxing of large DNA molecules as an electric field is turned off and on at short intervals. Bands or spots of radioactive DNA in gels can be visualized by autoradiography (Section 4.1.4). Alternatively, a gel can be stained with ethidium bromide, which fluoresces an intense orange when bound to double-helical DNA molecule (Figure 6.2). A band containing only 50 ng of DNA can be readily seen.

FIGURE 6.2 Gel electrophoresis pattern of a restriction digest. This gel shows the fragments produced by cleaving SV40 DNA with each of three restriction enzymes. These fragments were made fluorescent by staining the gel with ethidium bromide. [Courtesy of Dr. Jeffrey Sklar.]

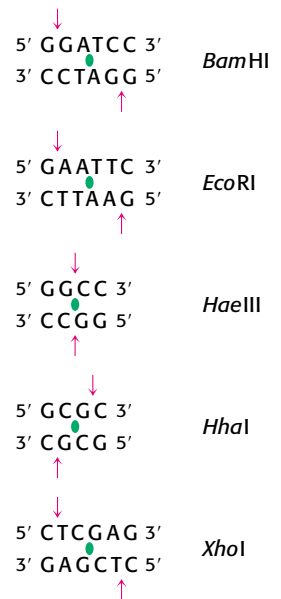


FIGURE 6.1 Specificities of some restriction endonucleases. The base-pair sequences that are recognized by these enzymes contain a twofold axis of symmetry. The two strands in these regions are related by a 180-degree rotation about the axis marked by the green symbol. The cleavage sites are denoted by red arrows. The abbreviated name of each restriction enzyme is given at the right of the sequence that it recognizes.

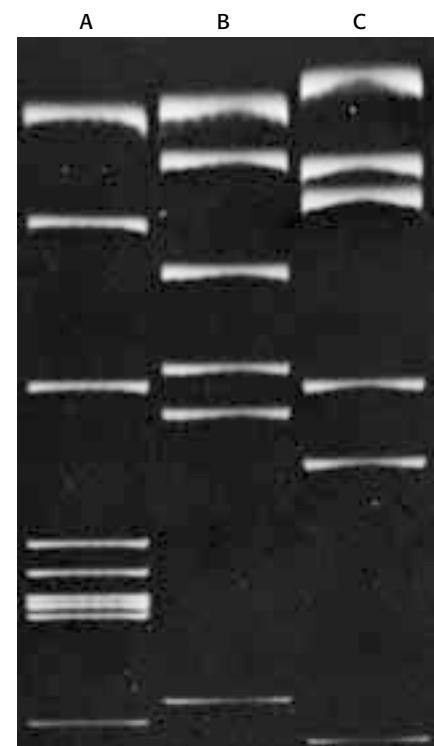
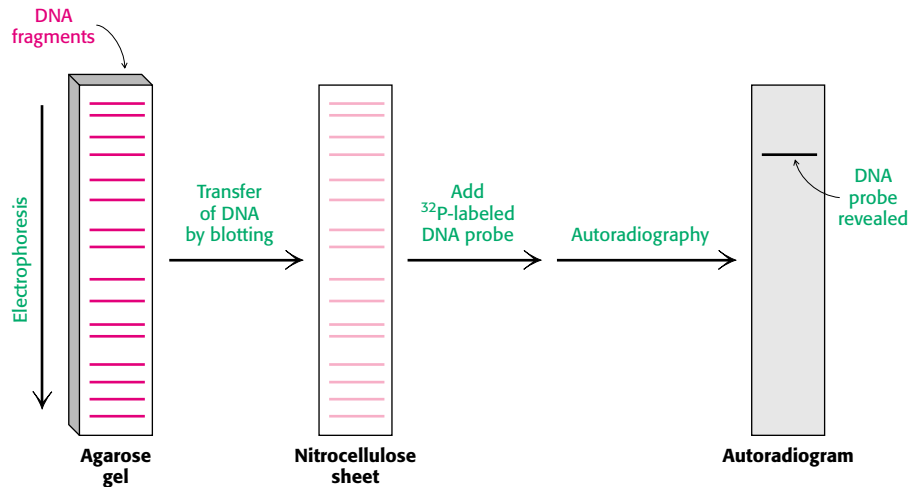


FIGURE 6.3 Southern blotting. A DNA fragment containing a specific sequence can be identified by separating a mixture of fragments by electrophoresis, transferring them to nitrocellulose, and hybridizing with a ^{32}P -labeled probe complementary to the sequence. The fragment containing the sequence is then visualized by autoradiography.



A restriction fragment containing a specific base sequence can be identified by hybridizing it with a labeled complementary DNA strand (Figure 6.3). A mixture of restriction fragments is separated by electrophoresis through an agarose gel, denatured to form single-stranded DNA, and transferred to a nitrocellulose sheet. The positions of the DNA fragments in the gel are preserved on the nitrocellulose sheet, where they are exposed to a ^{32}P -labeled single-stranded DNA probe. The probe hybridizes with a restriction fragment having a complementary sequence, and autoradiography then reveals the position of the restriction-fragment-probe duplex. A particular fragment in the midst of a million others can be readily identified in this way, like finding a needle in a haystack. This powerful technique is known as *Southern blotting* because it was devised by Edwin Southern.

Similarly, RNA molecules can be separated by gel electrophoresis, and specific sequences can be identified by hybridization subsequent to their transfer to nitrocellulose. This analogous technique for the analysis of RNA has been whimsically termed *Northern blotting*. A further play on words accounts for the term *Western blotting*, which refers to a technique for detecting a particular protein by staining with specific antibody (Section 4.3.4). Southern, Northern, and Western blots are also known respectively as *DNA*, *RNA*, and *protein blots*.

Restriction-fragment-length polymorphism (RFLP)—

Southern blotting can be used to follow the inheritance of selected genes. Mutations within restriction sites change the sizes of restriction fragments and hence the positions of bands in Southern-blot analyses. The existence of genetic diversity in a population is termed polymorphism. The detected mutation may itself cause disease or it may be closely linked to one that does. Genetic diseases such as sickle-cell anemia, cystic fibrosis, and Huntington chorea can be detected by RFLP analyses.

6.1.3 DNA Is Usually Sequenced by Controlled Termination of Replication (Sanger Dideoxy Method)

The analysis of DNA structure and its role in gene expression also have been markedly facilitated by the development of powerful techniques for the *sequencing* of DNA molecules. The key to DNA sequencing is the generation of DNA fragments whose length depends on the last base in the sequence. Collections of such fragments can be generated through the *controlled interruption of enzymatic replication*, a method developed by Frederick Sanger and coworkers. This technique has superseded alternative methods because of its simplicity. The same procedure is performed on four reaction mixtures at the same time. In all these mixtures, a DNA polymerase is used to make the complement of a particular sequence within a single-stranded DNA molecule. The synthesis is primed by a fragment, usually obtained by chemical synthetic methods described in Section 6.1.4, that is complementary to a part of the sequence known from other studies. In addition to the four deoxyribonucleoside triphosphates (radioactively labeled), each reaction mixture contains a small amount of the *2',3'-dideoxy analog* of one of the nucleotides, a different nucleotide for each reaction mixture.

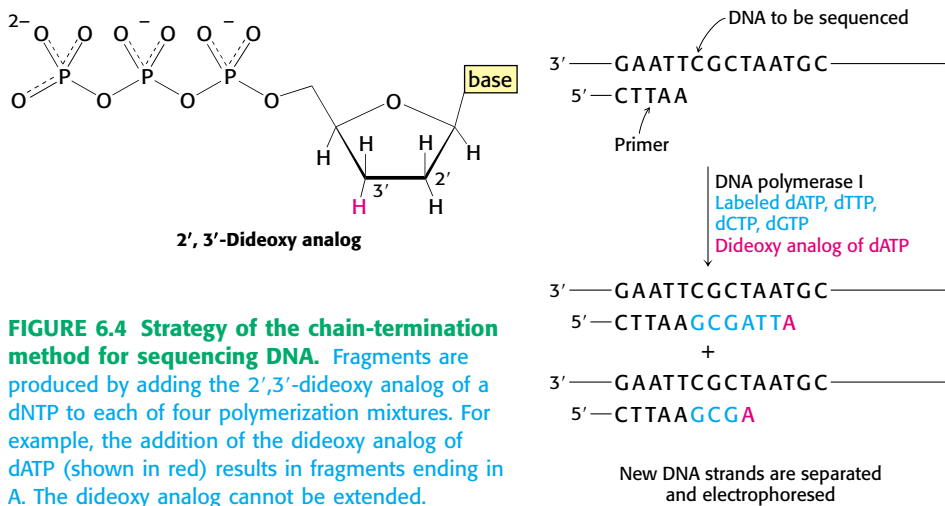


FIGURE 6.4 Strategy of the chain-termination method for sequencing DNA. Fragments are produced by adding the 2',3'-dideoxy analog of a dNTP to each of four polymerization mixtures. For example, the addition of the dideoxy analog of dATP (shown in red) results in fragments ending in A. The dideoxy analog cannot be extended.

The incorporation of this analog blocks further growth of the new chain because it lacks the 3'-hydroxyl terminus needed to form the next phosphodiester bond. The concentration of the dideoxy analog is low enough that chain termination will take place only occasionally. The polymerase will sometimes insert the correct nucleotide and other times the dideoxy analog, stopping the reaction. For instance, if the dideoxy analog of dATP is present, fragments of various lengths are produced, but all will be terminated by the dideoxy analog (Figure 6.4). Importantly, this dideoxy analog of dATP will be inserted only where a T was located in the DNA being sequenced. Thus, the fragments of different length will correspond to the positions of T. Four such sets of *chain-terminated fragments* (one for each dideoxy analog) then undergo electrophoresis, and the base sequence of the new DNA is read from the autoradiogram of the four lanes.

Fluorescence detection is a highly effective alternative to autoradiography. A fluorescent tag is attached to an oligonucleotide priming fragment—a differently colored one in each of the four chain-terminating reaction mixtures (e.g., a blue emitter for termination at A and a red one for termination at C). The reaction mixtures are combined and subjected to electrophoresis together. The separated bands of DNA are then detected by their fluorescence as they emerge from the gel; the sequence of their colors directly gives the base sequence (Figure 6.5). Sequences of as many as 500 bases can be determined in this way. Alternatively, the dideoxy analogs can be labeled, each with a specific fluorescent label. When this method is used, all four terminators can be placed in a single tube, and only one reaction is necessary. Fluorescence detection is attractive because it eliminates the use of radioactive reagents and can be readily automated.

Sanger and coworkers determined the complete sequence of the 5386 bases in the DNA of the ϕ X174 DNA virus in 1977, just a quarter century after Sanger's pioneering elucidation of the amino acid sequence of a protein. This accomplishment is a landmark in molecular biology because it revealed the total information content of a DNA genome. This tour de force was followed several years later by the determination of the sequence of human mitochondrial DNA, a double-stranded circular DNA molecule containing 16,569 base pairs. It encodes 2 ribosomal RNAs, 22 transfer RNAs, and 13 proteins. In

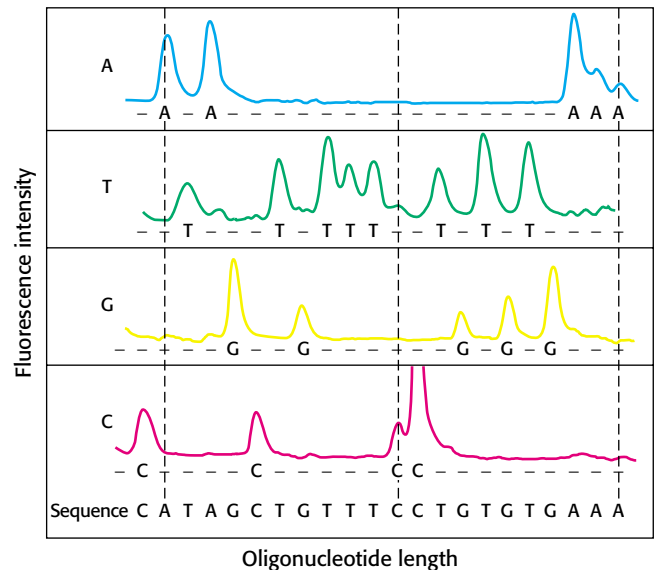


FIGURE 6.5 Fluorescence detection of oligonucleotide fragments produced by the dideoxy method. Each of the four chain-terminating mixtures is primed with a tag that fluoresces at a different wavelength (e.g., blue for A). The sequence determined by fluorescence measurements at four wavelengths is shown at the bottom. [From L. M. Smith, J. Z. Sanders, R. J. Kaiser, P. Hughes, C. Dodd, C. R. Connell, C. Heiner, S. B. H. Kent, and L. E. Hood. *Nature* 321(1986):674.]

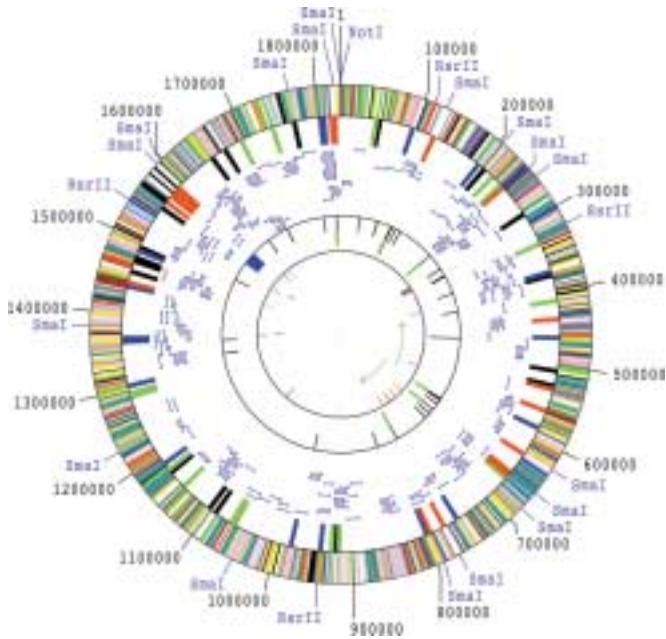


FIGURE 6.6 A complete genome. The diagram depicts the genome of *Haemophilus influenzae*, the first complete genome of a free-living organism to be sequenced. The genome encodes more than 1700 proteins and 70 RNA molecules. The likely function of approximately one-half of the proteins was determined by comparisons with sequences from proteins previously characterized in other species. [From R. D. Fleischmann et al., *Science* 269(1995):496; scan courtesy of TIGR.]

recent years, the complete genomes of free-living organisms have been sequenced. The first such sequence to be completed was that of the bacterium *Haemophilus influenzae*. Its genome comprises 1,830,137 base pairs and encodes approximately 1740 proteins (Figure 6.6).

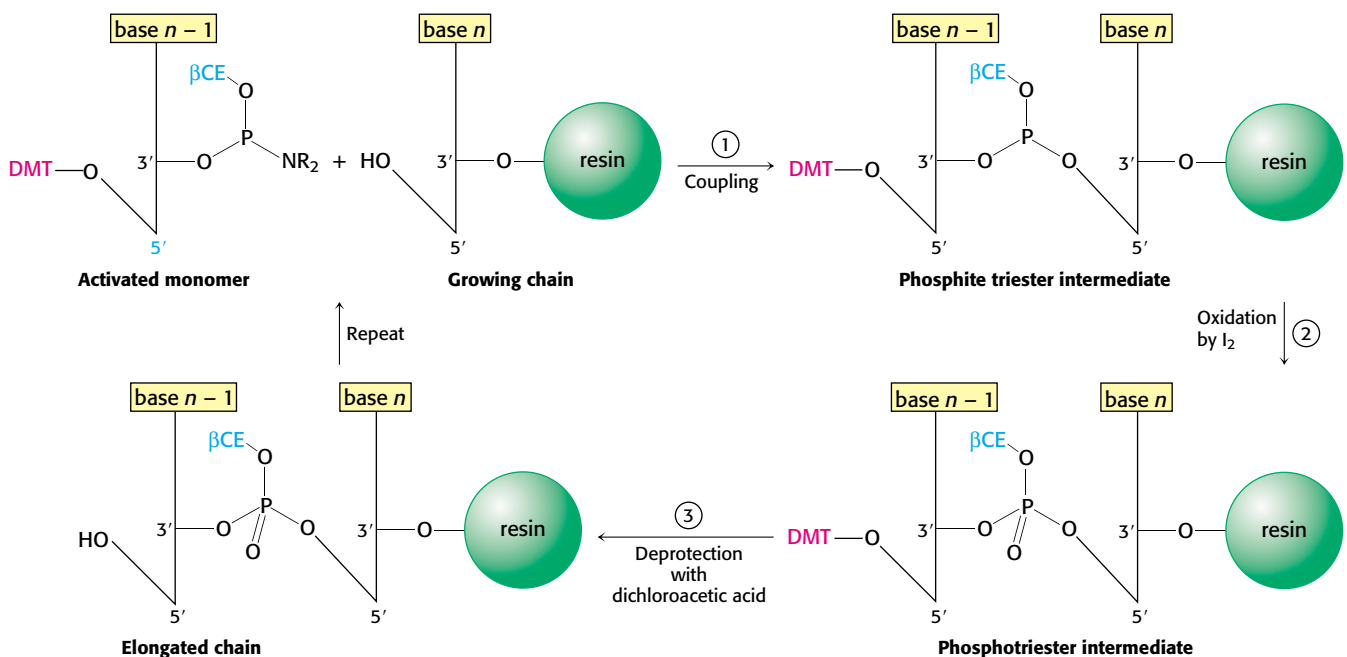
Many other bacterial and archaeal genomes have since been sequenced. The first eukaryotic genome to be completely sequenced was that of baker's yeast, *Saccharomyces cerevisiae*, which comprises approximately 12 million base pairs, distributed on 16 chromosomes, and encodes more than 6000 proteins. This achievement was followed by the first complete sequencing of the genome

of a multicellular organism, the nematode *Caenorhabditis elegans*, which contains nearly 100 million base pairs. The human genome is considerably larger at more than 3 billion base pairs, but it has been essentially completely sequenced. *The ability to determine complete genome sequences has revolutionized biochemistry and biology.*

FIGURE 6.7 Solid-phase synthesis of a DNA chain by the phosphite triester method. The activated monomer added to the growing chain is a deoxyribonucleoside 3'-phosphoramidite containing a DMT protecting group on its 5' oxygen atom, a β -cyanoethyl (β CE) protecting group on its 3' phosphoryl oxygen, and a protecting group on the base.

6.1.4 DNA Probes and Genes Can Be Synthesized by Automated Solid-Phase Methods

DNA strands, like polypeptides (Section 4.4), can be synthesized by the sequential addition of activated monomers to a growing chain that is linked to an insoluble support. The activated monomers are protonated *deoxyribonucleoside 3'-phosphoramidites*. In step 1, the 3' phosphorus atom of this incoming unit becomes joined to the 5' oxygen atom of the growing chain to form a *phosphite triester* (Figure 6.7). The 5'-OH group of the activated



monomer is unreactive because it is blocked by a dimethoxytrityl (DMT) protecting group, and the 3'-phosphoryl group is rendered unreactive by attachment of the β -cyanoethyl (β CE) group. Likewise, amino groups on the purine and pyrimidine bases are blocked.

Coupling is carried out under anhydrous conditions because water reacts with phosphoramidites. In step 2, the phosphite triester (in which P is trivalent) is oxidized by iodine to form a *phosphotriester* (in which P is pentavalent). In step 3, the DMT protecting group on the 5'-OH of the growing chain is removed by the addition of dichloroacetic acid, which leaves other protecting groups intact. The DNA chain is now elongated by one unit and ready for another cycle of addition. Each cycle takes only about 10 minutes and elongates more than 98% of the chains.

This solid-phase approach is ideal for the synthesis of DNA, as it is for polypeptides, because the desired product stays on the insoluble support until the final release step. All the reactions take place in a single vessel, and excess soluble reagents can be added to drive reactions to completion. At the end of each step, soluble reagents and by-products are washed away from the glass beads that bear the growing chains. At the end of the synthesis, NH_3 is added to remove all protecting groups and release the oligonucleotide from the solid support. Because elongation is never 100% complete, the new DNA chains are of diverse lengths—the desired chain is the longest one. The sample can be purified by high-pressure liquid chromatography or by electrophoresis on polyacrylamide gels. DNA chains of as many as 100 nucleotides can be readily synthesized by this automated method.

The ability to rapidly synthesize DNA chains of any selected sequence opens many experimental avenues. For example, synthesized oligonucleotide labeled at one end with ^{32}P or a fluorescent tag can be used to search for a complementary sequence in a very long DNA molecule or even in a genome consisting of many chromosomes. The use of labeled oligonucleotides as DNA probes is powerful and general. For example, a DNA probe that can base-pair to a known complementary sequence in a chromosome can serve as the starting point of an exploration of adjacent uncharted DNA. Such a probe can be used as a *primer* to initiate the replication of neighboring DNA by DNA polymerase. One of the most exciting applications of the solid-phase approach is the *synthesis of new tailor-made genes*. New proteins with novel properties can now be produced in abundance by expressing synthetic genes. *Protein engineering* has become a reality.

6.1.5 Selected DNA Sequences Can Be Greatly Amplified by the Polymerase Chain Reaction

In 1984, Kary Mullis devised an ingenious method called the *polymerase chain reaction (PCR)* for amplifying specific DNA sequences. Consider a DNA duplex consisting of a target sequence surrounded by nontarget DNA. Millions of the target sequences can be readily obtained by PCR if the flanking sequences of the target are known. PCR is carried out by adding the following components to a solution containing the target sequence: (1) a pair of primers that hybridize with the flanking sequences of the target, (2) all four deoxyribonucleoside triphosphates (dNTPs), and (3) a heat-stable DNA polymerase. A PCR cycle consists of three steps (Figure 6.8).

1. *Strand separation.* The two strands of the parent DNA molecule are separated by heating the solution to 95°C for 15 s.

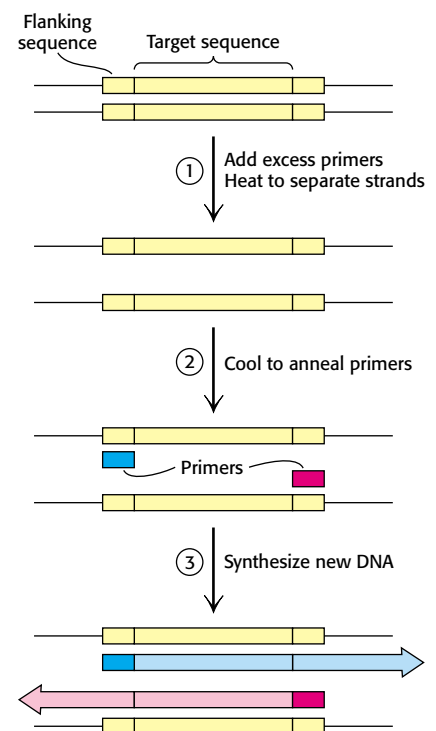
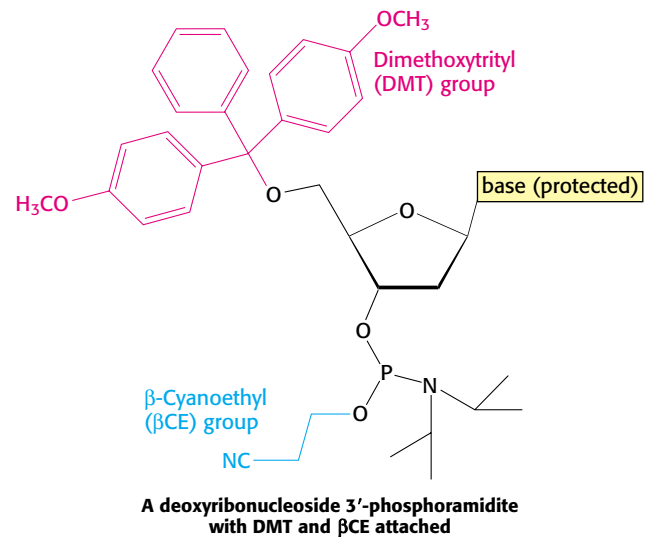


FIGURE 6.8 The first cycle in the polymerase chain reaction (PCR). A cycle consists of three steps: strand separation, hybridization of primers, and extension of primers by DNA synthesis.

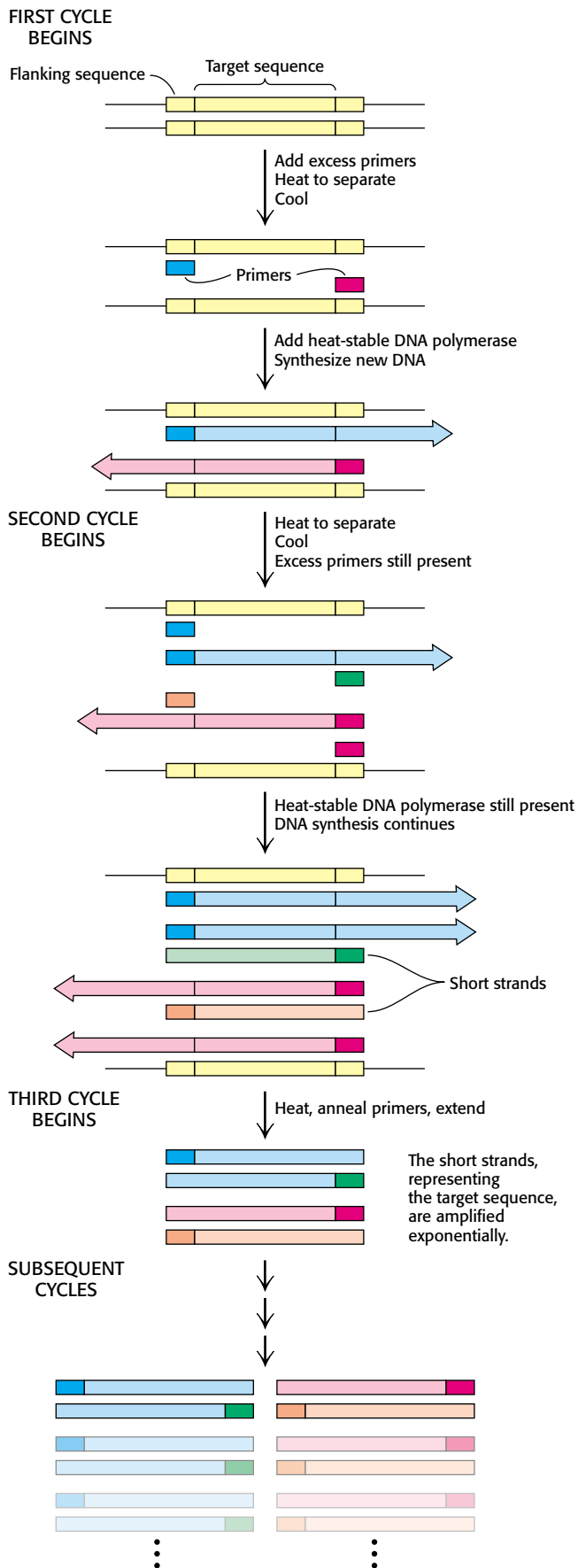


FIGURE 6.9 Multiple cycles of the polymerase chain reaction.

The two short strands produced at the end of the third cycle (along with longer strands not shown) represent the target sequence. Subsequent cycles will amplify the target sequence exponentially and the parent sequence arithmetically.

2. *Hybridization of primers.* The solution is then abruptly cooled to 54°C to allow each primer to hybridize to a DNA strand. One primer hybridizes to the 3'-end of the target on one strand, and the other primer hybridizes to the 3' end on the complementary target strand. Parent DNA duplexes do not form, because the primers are present in large excess. Primers are typically from 20 to 30 nucleotides long.

3. *DNA synthesis.* The solution is then heated to 72°C, the optimal temperature for *Taq* DNA polymerase. This heat-stable polymerase comes from *Thermus aquaticus*, a thermophilic bacterium that lives in hot springs. The polymerase elongates both primers in the direction of the target sequence because DNA synthesis is in the 5'-to-3' direction. DNA synthesis takes place on both strands but extends beyond the target sequence.

These three steps—strand separation, hybridization of primers, and DNA synthesis—constitute one cycle of the PCR amplification and can be carried out repetitively just by changing the temperature of the reaction mixture. The thermostability of the polymerase makes it feasible to carry out PCR in a closed container; no reagents are added after the first cycle. The duplexes are heated to begin the second cycle, which produces four duplexes, and then the third cycle is initiated (Figure 6.9). At the end of the third cycle, two short strands appear that constitute only the target sequence—the sequence including and bounded by the primers. Subsequent cycles will amplify the target sequence exponentially. The larger strands increase in number arithmetically and serve as a source for the synthesis of more short strands. Ideally, after n cycles, this sequence is amplified 2^n -fold. The amplification is a millionfold after 20 cycles and a billionfold after 30 cycles, which can be carried out in less than an hour.

Several features of this remarkable method for amplifying DNA are noteworthy. First, the sequence of the target need not be known. All that is required is knowledge of the flanking sequences. Second, the target can be much larger than the primers. Targets larger than 10 kb have been amplified by PCR. Third, primers do not have to be perfectly matched to flanking sequences to amplify targets. With the use of primers derived from a gene of known sequence, it is possible to search for variations on the theme. In this way, families of genes are being discovered by PCR. Fourth, PCR is highly specific because of the stringency of hybridization at high temperature (54°C). Stringency is the required closeness of the match

between primer and target, which can be controlled by temperature and salt. At high temperatures, the only DNA that is amplified is that situated be-

tween primers that have hybridized. A gene constituting less than a millionth of the total DNA of a higher organism is accessible by PCR. Fifth, PCR is exquisitely sensitive. A single DNA molecule can be amplified and detected.

6.1.6 PCR Is a Powerful Technique in Medical Diagnostics, Forensics, and Molecular Evolution

PCR can provide valuable diagnostic information in medicine. Bacteria and viruses can be readily detected with the use of specific primers. For example, PCR can reveal the presence of human immunodeficiency virus in people who have not mounted an immune response to this pathogen and would therefore be missed with an antibody assay. Finding *Mycobacterium tuberculosis* bacilli in tissue specimens is slow and laborious. With PCR, as few as 10 tubercle bacilli per million human cells can be readily detected. PCR is a promising method for the early detection of certain cancers. This technique can identify mutations of certain growth-control genes, such as the *ras* genes (Section 15.x.x). The capacity to greatly amplify selected regions of DNA can also be highly informative in monitoring cancer chemotherapy. Tests using PCR can detect when cancerous cells have been eliminated and treatment can be stopped; they can also detect a relapse and the need to immediately resume treatment. PCR is ideal for detecting leukemias caused by chromosomal rearrangements.

PCR is also having an effect in forensics and legal medicine. An individual DNA profile is highly distinctive because many genetic loci are highly variable within a population. For example, variations at a specific one of these locations determines a person's HLA type (human leukocyte antigen type); organ transplants are rejected when the HLA types of the donor and recipient are not sufficiently matched. PCR amplification of multiple genes is being used to establish biological parentage in disputed paternity and immigration cases. Analyses of blood stains and semen samples by PCR have implicated guilt or innocence in numerous assault and rape cases. The root of a single shed hair found at a crime scene contains enough DNA for typing by PCR (Figure 6.10).

DNA is a remarkably stable molecule, particularly when relatively shielded from air, light, and water. Under such circumstances, large fragments of DNA can remain intact for thousands of years or longer. PCR provides an ideal method for amplifying such ancient DNA molecules so that they can be detected and characterized (Section 7.5.1). PCR can also be used to amplify DNA from microorganisms that have not yet been isolated and cultured. As will be discussed in the next chapter, sequences from these PCR products can be sources of considerable insight into evolutionary relationships between organisms.

6.2 RECOMBINANT DNA TECHNOLOGY HAS REVOLUTIONIZED ALL ASPECTS OF BIOLOGY

The pioneering work of Paul Berg, Herbert Boyer, and Stanley Cohen in the early 1970s led to the development of recombinant DNA technology, which has permitted biology to move from an exclusively analytical science to a synthetic one. New combinations of unrelated genes can be constructed in the laboratory by applying recombinant DNA techniques. These novel combinations can be cloned—amplified manifold—by introducing them into suitable cells, where they are replicated by the DNA-synthesizing machinery of the host. The inserted genes are often transcribed and translated in their new setting. What is most striking is that the genetic endowment of the host can be permanently altered in a designed way.



FIGURE 6.10 DNA and forensics. DNA analysis can be used to establish guilt in criminal cases. Here, DNA was isolated from bloodstains on the pants and shirt of a defendant and amplified by PCR. The DNA was then compared to the DNA from the victim as well as the defendant using gel electrophoresis and autoradiography. DNA from the bloodstains on the defendant's clothing matched the pattern of the victim, but not that of the defendant. The frequency of a coincidental match of the DNA pattern on the clothing and the victim is approximately 1 in 33 billion. Lanes λ , 1kb, and TS = Control DNA samples; lane D = DNA from the defendant; jeans = DNA isolated from bloodstains on defendant's pants; shirt = DNA isolated from bloodstains of the defendant's shirt (two different amounts analyzed); V = DNA sample from victim's blood. [Courtesy of Cellmark Diagnostics, Germantown MD.]

6.2.1 Restriction Enzymes and DNA Ligase Are Key Tools in Forming Recombinant DNA Molecules

Let us begin by seeing how novel DNA molecules can be constructed in the laboratory. A DNA fragment of interest is covalently joined to a DNA *vector*. The essential feature of a vector is that it can replicate autonomously in an appropriate host. *Plasmids* (naturally occurring circles of DNA that act as accessory chromosomes in bacteria) and bacteriophage λ , a virus, are choice vectors for cloning in *E. coli*. The vector can be prepared for accepting a new DNA fragment by cleaving it at a single specific site with a restriction enzyme. For example, the plasmid pSC101, a 9.9-kb double-helical circular DNA molecule, is split at a unique site by the *EcoRI* restriction enzyme. The staggered cuts made by this enzyme produce *complementary single-stranded ends*, which have specific affinity for each other and hence are known as *cohesive* or *sticky ends*. Any DNA fragment can be inserted into this plasmid if it has the same cohesive ends. Such a fragment can be prepared from a larger piece of DNA by using the same restriction enzyme as was used to open the plasmid DNA (Figure 6.11).

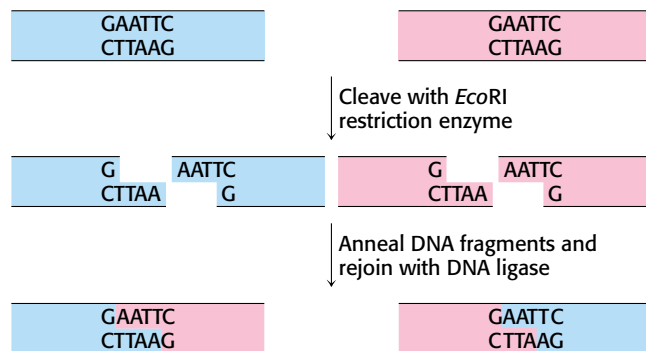


FIGURE 6.11 Joining of DNA molecules by the cohesive-end method. Two DNA molecules, cleaved with a common restriction enzyme such as *EcoRI*, can be ligated to form recombinant molecules.

The single-stranded ends of the fragment are then complementary to those of the cut plasmid. The DNA fragment and the cut plasmid can be annealed and then joined by *DNA ligase*, which catalyzes the formation of a phosphodiester bond at a break in a DNA chain. DNA ligase requires a free 3'-hydroxyl group and a 5'-phosphoryl group. Furthermore, the chains joined by ligase must be in a double helix. An energy source such as ATP or NAD^+ is required for the joining reaction, as will be discussed in Chapter 27.

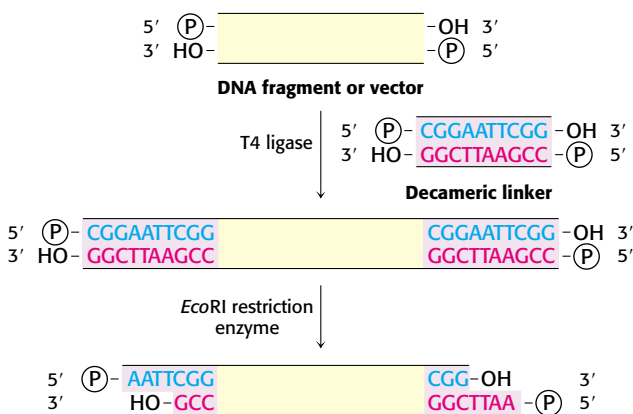


FIGURE 6.12 Formation of cohesive ends. Cohesive ends are formed by the addition and cleavage of a chemically synthesized linker.

This cohesive-end method for joining DNA molecules can be made general by using a *short, chemically synthesized DNA linker* that can be cleaved by restriction enzymes. First, the linker is covalently joined to the ends of a DNA fragment or vector. For example, the 5' ends of a decameric linker and a DNA molecule are phosphorylated by polynucleotide kinase and then joined by the ligase from T4 phage (Figure 6.12). This ligase can form a covalent bond between blunt-ended (flush-ended) double-helical DNA molecules. Cohesive ends are produced when these terminal extensions are cut by an appropriate restriction enzyme. Thus, *cohesive ends corresponding to a particular restriction enzyme can be added to virtually any DNA molecule*. We see here the fruits of combining enzymatic and synthetic chemical approaches in crafting new DNA molecules.

6.2.2 Plasmids and Lambda Phage Are Choice Vectors for DNA Cloning in Bacteria

Many plasmids and bacteriophages have been ingeniously modified to enhance the delivery of recombinant DNA molecules into bacteria and to facilitate the selection of bacteria harboring these vectors. Plasmids are circular duplex DNA molecules occurring naturally in some bacteria and ranging in size from 2 to several hundred kilobases. They carry genes for the inactivation of antibiotics, the production of toxins, and the breakdown of natural products. These *accessory chromosomes* can replicate independently of the host chromosome. In contrast with the host genome, they are dispensable under certain conditions. A bacterial cell may have no plasmids at all or it may house as many as 20 copies of a plasmid.

pBR322 Plasmid. One of the most useful plasmids for cloning is *pBR322*, which contains genes for resistance to tetracycline and ampicillin (an antibiotic like penicillin). Different endonucleases can cleave this plasmid at a variety of unique sites, at which DNA fragments can be inserted. Insertion of DNA at the *EcoRI* restriction site does not alter either of the genes for antibiotic resistance (Figure 6.13). However, insertion at the *HindIII*, *Sall*, or *BamHI* restriction site inactivates the gene for tetracycline resistance, an effect called *insertional inactivation*. Cells containing *pBR322* with a DNA insert at one of these restriction sites are resistant to ampicillin but sensitive to tetracycline, and so they can be readily *selected*. Cells that failed to take up the vector are sensitive to both antibiotics, whereas cells containing *pBR322* without a DNA insert are resistant to both.

Lambda (λ) Phage. Another widely used vector, λ phage, enjoys a choice of life styles: this bacteriophage can destroy its host or it can become part of its host (Figure 6.14). In the *lytic pathway*, viral functions are fully expressed: viral DNA and proteins are quickly produced and packaged into virus particles, leading to the lysis (destruction) of the host cell and the sudden appearance of about 100 progeny virus particles, or *virions*. In the *lysogenic pathway*, the phage DNA becomes inserted into the host-cell genome and can be replicated together with host-cell DNA for many generations, remaining inactive. Certain environmental changes can trigger the expression of this dormant viral DNA, which leads to the formation of progeny virus and lysis of the host. Large segments of the 48-kb DNA of λ phage are not essential for productive infection and can be replaced by foreign DNA, thus making λ phage an ideal vector.

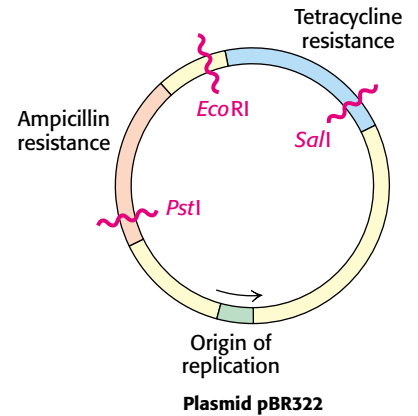
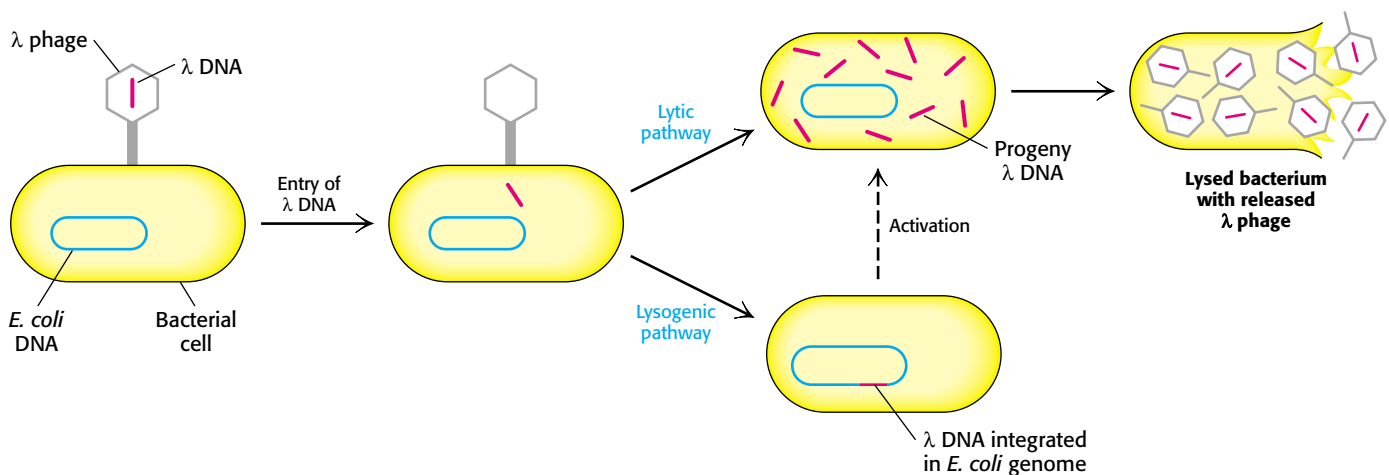


FIGURE 6.13 Genetic map of the plasmid pBR322. This plasmid carries two genes for antibiotic resistance. Like all other plasmids, it is a circular duplex DNA.

FIGURE 6.14 Alternative infection modes for λ phage. Lambda phage can multiply within a host and lyse it (lytic pathway), or its DNA can become integrated into the host genome (lysogenic pathway), where it is dormant until activated.



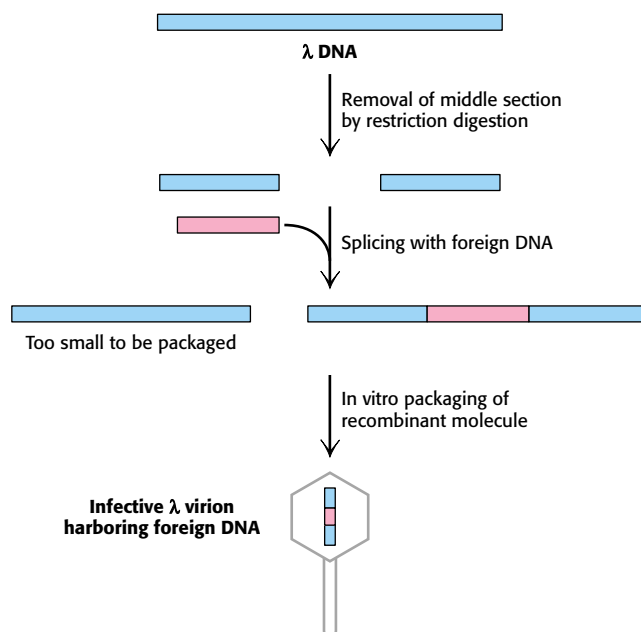


FIGURE 6.15 Mutant λ phage as a cloning vector. The packaging process selects DNA molecules that contain an insert.

Mutant λ phages designed for cloning have been constructed. An especially useful one called λ gt- $\lambda\beta$ contains only two *EcoRI* cleavage sites instead of the five normally present (Figure 6.15). After cleavage, the middle segment of this λ DNA molecule can be removed. The two remaining pieces of DNA (called arms) have a combined length equal to 72% of a normal genome length. This amount of DNA is too little to be packaged into a λ particle, because only DNA measuring from 75% to 105% of a normal genome in length can be readily packaged. However, a suitably long DNA insert (such as 10 kb) between the two ends of λ DNA enables such a recombinant DNA molecule (93% of normal length) to be packaged. Nearly all infective λ particles formed in this way will contain an inserted piece of foreign DNA. Another advantage of using these modified viruses as vectors is that they enter bacteria much more easily than do plasmids. Among the variety of λ mutants that have been constructed for use as cloning vectors, one of them, called a *cosmid*, is essentially a hybrid of λ phage and a plasmid that can serve as a vector for large DNA inserts (as large as 45 kb).

M13 Phage. Another very useful vector for cloning DNA, *M13 phage* is especially useful for sequencing the inserted DNA. This filamentous virus is 900 nm long and only 9 nm wide (Figure 6.16). Its 6.4-kb single-stranded circle of DNA is protected by a coat of 2710 identical protein subunits. M13 enters *E. coli* through the bacterial sex pilus, a protein appendage that permits the transfer of DNA between bacteria. The single-stranded DNA in the virus particle [called the (+) strand] is replicated through an intermediate circular double-stranded replicative form (RF) containing (+) and (-) strands. Only the (+) strand is packaged into new virus particles. About a thousand progeny M13 are produced per generation. A striking feature of M13 is that it does not kill its bacterial host. Consequently, large quantities of M13 can be grown and easily harvested (1 gram from 10 liters of culture fluid).

An M13 vector is prepared for cloning by cutting its circular RF DNA at a single site with a restriction enzyme. The cut is made in a *polylinker* region that contains a series of closely spaced recognition sites for restriction enzymes; only one of each such sites is present in the vector. A double-stranded foreign DNA fragment produced by cleavage with the same restriction enzyme is then ligated to the cut vector (Figure 6.17). The foreign DNA can be inserted in two different orientations because the ends of both

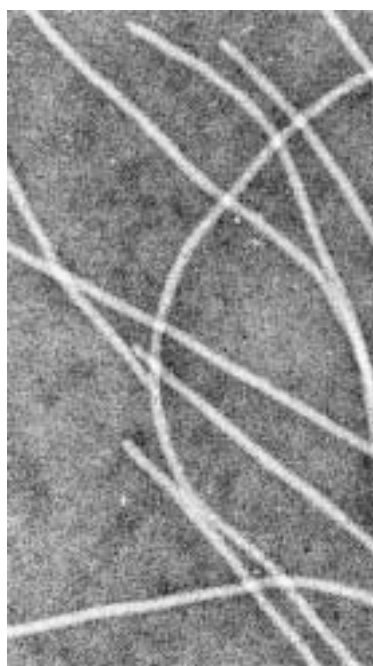


FIGURE 6.16 Electron micrograph of M13 filamentous phage. [Courtesy of Dr. Robley Williams.]

DNA molecules are the same. Hence, half the new (+) strands packaged into virus particles will contain one of the strands of the foreign DNA, and half will contain the other strand. Infection of *E. coli* by a single virus particle will yield a large amount of single-stranded M13 DNA containing the same strand of the foreign DNA. DNA cloned into M13 can be easily sequenced. An oligonucleotide that hybridizes adjacent to the polylinker region is used as a primer for sequencing the insert. This oligomer is called a *universal sequencing primer* because it can be used to sequence *any* insert. M13 is ideal for sequencing but not for long-term propagation of recombinant DNA, because inserts longer than about 1 kb are not stably maintained.

6.2.3 Specific Genes Can Be Cloned from Digests of Genomic DNA

Ingenious cloning and selection methods have made feasible the isolation of a specific DNA segment several kilobases long out of a genome containing more than 3×10^6 kb. Let us see how a gene that is present just once in a human genome can be cloned. A sample containing many molecules of total genomic DNA is first mechanically sheared or partly digested by restriction enzymes into large fragments (Figure 6.18). This nearly random population of overlapping DNA fragments is then separated by gel electrophoresis to isolate a set about 15 kb long. Synthetic linkers are attached to the ends of these fragments, cohesive ends are formed, and the fragments are then inserted into a vector, such as λ phage DNA, prepared with the same cohesive ends. *E. coli* bacteria are then infected with these recombinant phages. The resulting lysate contains fragments of human DNA housed in a sufficiently large number of virus particles to ensure that nearly the entire genome is represented. These phages constitute a *genomic library*. Phages can be propagated indefinitely, and so the library can be used repeatedly over long periods.

This genomic library is then screened to find the very small portion of phages harboring the gene of interest. For the human genome, a calculation shows that a 99% probability of success requires screening about 500,000 clones; hence, a very rapid and efficient screening process is essential. Rapid screening can be accomplished by DNA hybridization.

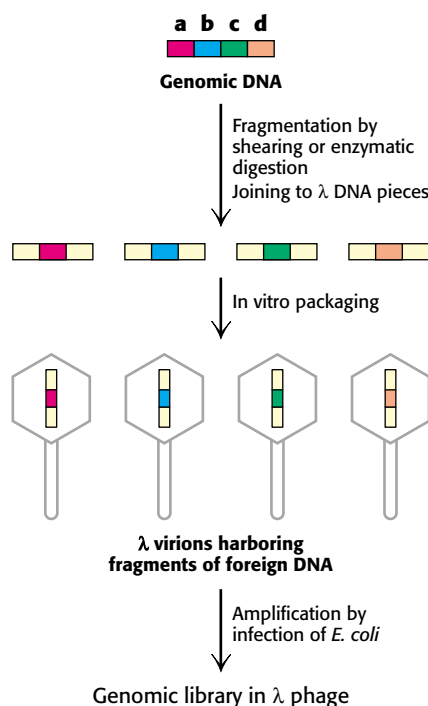


FIGURE 6.18 Creation of a genomic library. A genomic library can be created from a digest of a whole eukaryotic genome.

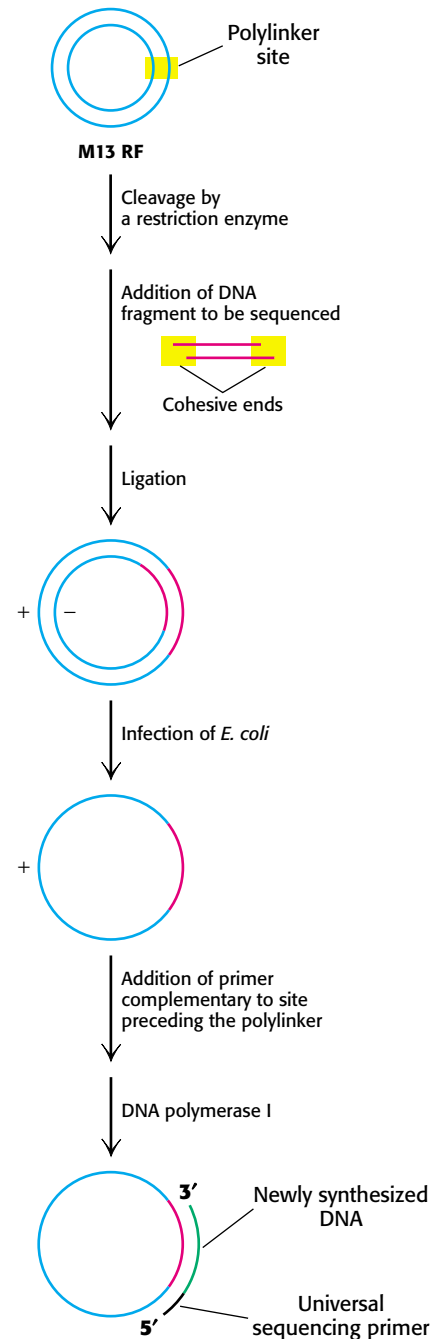


FIGURE 6.17 M13 phage DNA, a cloning and sequencing vector. M13 phage DNA is very useful in sequencing DNA fragments by the dideoxy method. A double-stranded DNA fragment is inserted into M13 RF DNA. Synthesis of new strand is primed by an oligonucleotide that is complementary to a sequence near the inserted DNA.

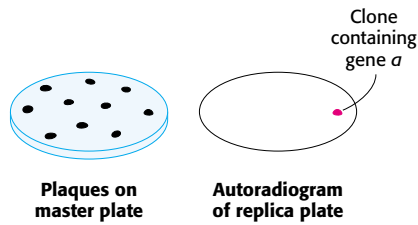


FIGURE 6.19 Screening a genomic library for a specific gene. Here, a plate is tested from plaques containing gene α of Figure 6.18.

A dilute suspension of the recombinant phages is first plated on a lawn of bacteria (Figure 6.19). Where each phage particle has landed and infected a bacterium, a *plaque* containing identical phages develops on the plate. A replica of this master plate is then made by applying a sheet of nitrocellulose. Infected bacteria and phage DNA released from lysed cells adhere to the sheet in a pattern of spots corresponding to the plaques. Intact bacteria on this sheet are lysed with NaOH, which also serves to denature the DNA so that it becomes accessible for hybridization with a ^{32}P -labeled probe. The presence of a specific DNA sequence in a single spot on the replica can be detected by using a radioactive complementary DNA or RNA molecule as a probe. Autoradiography then reveals the positions of spots harboring recombinant DNA. The corresponding plaques are picked out of the intact master plate and grown. A single investigator can readily screen a million clones in a day.

This method makes it possible to isolate virtually any gene, *provided that a probe is available*. How does one obtain a specific probe? One approach is to start with the corresponding mRNA from cells in which it is abundant. For example, precursors of red blood cells contain large amounts of mRNA for hemoglobin, and plasma cells are rich in mRNAs for antibody molecules. The mRNAs from these cells can be fractionated by size to enrich for the one of interest. As will be described shortly, a DNA complementary to this mRNA can be synthesized in vitro and cloned to produce a highly specific probe.

Alternatively, a probe for a gene can be prepared if part of the amino acid sequence of the protein encoded by the gene is known. A problem arises because a given peptide sequence can be encoded by a number of oligonucleotides (Figure 6.20). Thus, for this purpose, peptide sequences containing tryptophan and methionine are preferred, because these amino acids are specified by a single codon, whereas other amino acid residues have between two and six codons (Section 5.5.1).

Amino acid sequence	...	Cys	Pro	Asn	Lys	Trp	Thr	His	...
Potential oligonucleotide sequences		TG ^C _T	CG ^C _G	AA ^C _T	AA ^A _G	TGG	AC ^C _G	CA ^C _T	

FIGURE 6.20 Probes generated from a protein sequence. A probe can be generated by synthesizing all possible oligonucleotides encoding a particular sequence of amino acids. Because of the degeneracy of the genetic code, 256 distinct oligonucleotides must be synthesized to ensure that the probe matching the sequence of seven amino acids is present.

All the DNA sequences (or their complements) that encode the selected peptide sequence are synthesized by the solid-phase method and made radioactive by phosphorylating their 5' ends with ^{32}P from [^{32}P]-ATP. The replica plate is exposed to a mixture containing all these probes and autoradiographed to identify clones with a complementary DNA sequence. Positive clones are then sequenced to determine which ones have a sequence matching that of the protein of interest. Some of them may contain the desired gene or a significant segment of it.

6.2.4 Long Stretches of DNA Can Be Efficiently Analyzed by Chromosome Walking

A typical genomic DNA library housed in λ phage vectors consists of DNA fragments about 15 kb long. However, many eukaryotic genes are much longer—for example, the *dystrophin* gene, which is mutated in Duchenne muscular dystrophy, is 2000 kb long. How can such long stretches of DNA be analyzed? The development of cosmids helped because these chimeras

of plasmids and λ phages can house 45-kb inserts. Much larger pieces of DNA can now be propagated in *bacterial artificial chromosomes (BACs)* or *yeast artificial chromosomes (YACs)*. YACs contain a centromere, an *autonomous replication sequence (ARS)*, where replication begins), a pair of telomeres (normal ends of eukaryotic chromosomes), selectable marker genes, and a cloning site (Figure 6.21). Genomic DNA is partly digested by a restriction endonuclease that cuts, on the average, at distant sites. The fragments are then separated by pulsed-field gel electrophoresis, and the large ones (~ 450 kb) are eluted and ligated into YACs. Artificial chromosomes bearing inserts ranging from 100 to 1000 kb are efficiently replicated in yeast cells.

Equally important in analyzing large genes is the capacity to scan long regions of DNA. The principle technique for this purpose makes use of overlaps in the library fragments. The fragments in a cosmid or YAC library are produced by random cleavage of many DNA molecules, and so some of the fragments overlap one another. Suppose that a fragment containing region A selected by hybridization with a complementary probe A' also contains region B (Figure 6.22). A new probe B' can be prepared by cleaving this fragment between regions A and B and subcloning region B. If the library is screened again with probe B', new fragments containing region B will be found. Some will contain a previously unknown region C. Hence, we now have information about a segment of DNA encompassing regions A, B, and C. This process of subcloning and rescreening is called *chromosome walking*. Long stretches of DNA can be analyzed in this way, provided that each of the new probes is complementary to a unique region.

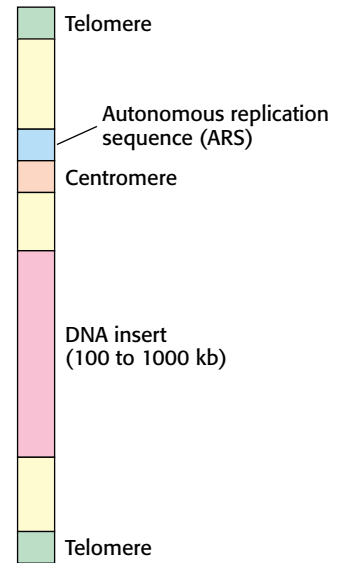
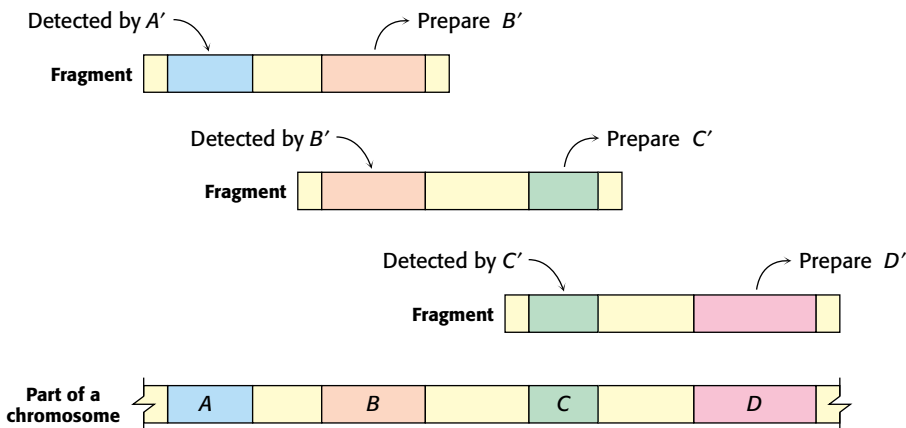


FIGURE 6.21 Diagram of a yeast artificial chromosome (YAC). DNA inserts as large as 1000 kb can be propagated in this vector.

FIGURE 6.22 Chromosome walking. Long regions of unknown DNA can be explored, starting with a known base sequence, by subcloning and rescreening. New probes are designed on the basis of the DNA sequences that have been determined.

6.3 MANIPULATING THE GENES OF EUKARYOTES

Eukaryotic genes, in a simplified form, can be introduced into bacteria, and the bacteria can be used as factories to produce a desired protein product. It is also possible to introduce DNA into higher organisms. In regard to animals, this ability provides a valuable tool for examining gene action, and it will be the basis of gene therapy. In regard to plants, introduced genes may make a plant resistant to pests or capable of growing in harsh conditions or able to carry greater quantities of essential nutrients. The manipulation of eukaryotic genes holds much promise for medical and agricultural benefits, but it is also the source of controversy.

6.3.1 Complementary DNA Prepared from mRNA Can Be Expressed in Host Cells

How can mammalian DNA be cloned and expressed by *E. coli*? Recall that most mammalian genes are mosaics of introns and exons (Section 5.6). These

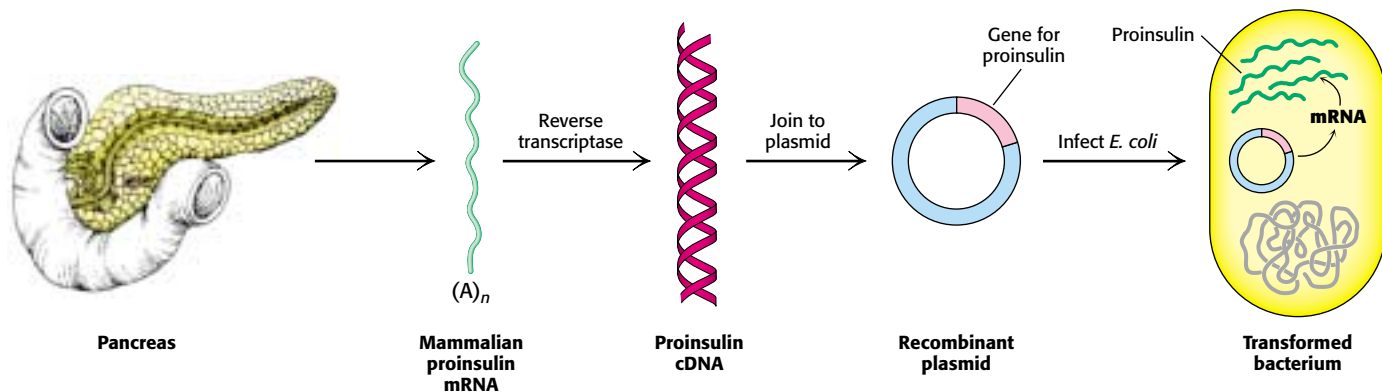


FIGURE 6.23 Synthesis of proinsulin by bacteria. Proinsulin, a precursor of insulin, can be synthesized by transformed (genetically altered) clones of *E. coli*. The clones contain the mammalian proinsulin gene.

interrupted genes cannot be expressed by bacteria, which lack the machinery to splice introns out of the primary transcript. However, this difficulty can be circumvented by causing bacteria to take up recombinant DNA that is complementary to mRNA. For example, proinsulin, a precursor of insulin, is synthesized by bacteria harboring plasmids that contain DNA complementary to mRNA for proinsulin (Figure 6.23). Indeed, bacteria produce much of the insulin used today by millions of diabetics.

The key to forming *complementary DNA (cDNA)* is the enzyme *reverse transcriptase*. As discussed in Section 5.3.1, a retrovirus uses this enzyme to form a DNA–RNA hybrid in replicating its genomic RNA. Reverse transcriptase synthesizes a DNA strand complementary to an RNA template if it is provided with a DNA primer that is base-paired to the RNA and contains a free 3'-OH group. We can use a simple sequence of linked thymidine [oligo(T)] residues as the primer. This oligo(T) sequence pairs with the poly(A) sequence at the 3' end of most eukaryotic mRNA molecules (Section 5.4.4), as shown in Figure 6.24. The reverse transcriptase then synthesizes the rest of the cDNA strand in the presence of the four deoxyribonucleoside triphosphates. The RNA strand of this RNA–DNA hybrid is subsequently hydrolyzed by raising the pH. Unlike RNA, DNA is resistant to alkaline hydrolysis. The single-stranded DNA is converted into double-stranded DNA by creating another primer site. The enzyme *terminal transferase* adds nucleotides—for instance, several residues of dG—to the 3' end of DNA. Oligo(dC) can bind to dG residues and prime the synthesis of the second DNA strand. Synthetic linkers can be added to this double-helical DNA for ligation to a suitable vector. Complementary DNA for all mRNA that a cell contains can be made, inserted into vectors, and then inserted into bacteria. Such a collection is called a *cDNA library*.

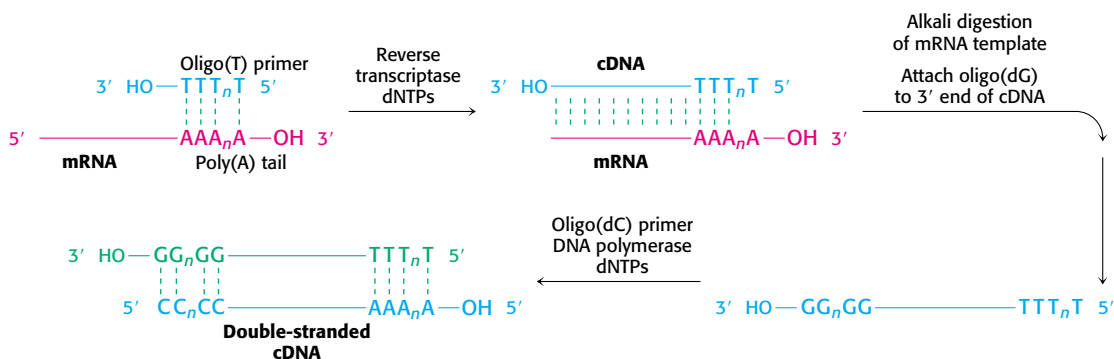


FIGURE 6.24 Formation of a cDNA duplex. A cDNA duplex is created from mRNA by using reverse transcriptase to synthesize a cDNA strand, first along the mRNA template and then, after digestion of the mRNA, along that same newly synthesized cDNA strand.

Complementary DNA molecules can be inserted into vectors that favor their efficient expression in hosts such as *E. coli*. Such plasmids or phages are called *expression vectors*. To maximize transcription, the cDNA is inserted into the vector in the correct reading frame near a strong bacterial promoter site. In addition, these vectors ensure efficient translation by encoding a ribosome-binding site on the mRNA near the initiation codon. Clones of cDNA can be screened on the basis of their capacity to direct the synthesis of a foreign protein in bacteria. A radioactive antibody specific for the protein of interest can be used to identify colonies of bacteria that harbor the corresponding cDNA vector (Figure 6.25). As described in Section 6.2.3, spots of bacteria on a replica plate are lysed to release proteins, which bind to an applied nitrocellulose filter. A ^{125}I -labeled antibody specific for the protein of interest is added, and autoradiography reveals the location of the desired colonies on the master plate. This immunochemical screening approach can be used whenever a protein is expressed and corresponding antibody is available.

6.3.2 Gene-Expression Levels Can Be Comprehensively Examined

Most genes are present in the same quantity in every cell—namely, one copy per haploid cell or two copies per diploid cell. However, the level at which a gene is expressed, as indicated by mRNA quantities, can vary widely, ranging from no expression to hundreds of mRNA copies per cell. Gene-expression patterns vary from cell type to cell type, distinguishing, for example, a muscle cell from a nerve cell. Even within the same cell, gene-expression levels may vary as the cell responds to changes in physiological circumstances.

Using our knowledge of complete genome sequences, it is now possible to analyze the pattern and level of expression of all genes in a particular cell or tissue. One of the most powerful methods developed to date for this purpose is based on hybridization. High-density arrays of oligonucleotides, called *DNA microarrays* or *gene chips*, can be constructed either through light-directed chemical synthesis carried out with photolithographic microfabrication techniques used in the semiconductor industry or by placing very small dots of oligonucleotides or cDNAs on a solid support such as a microscope slide. Fluorescently labeled cDNA is hybridized to the chip to reveal the expression level for each gene, identifiable by its known location on the chip. (Figure 6.26).

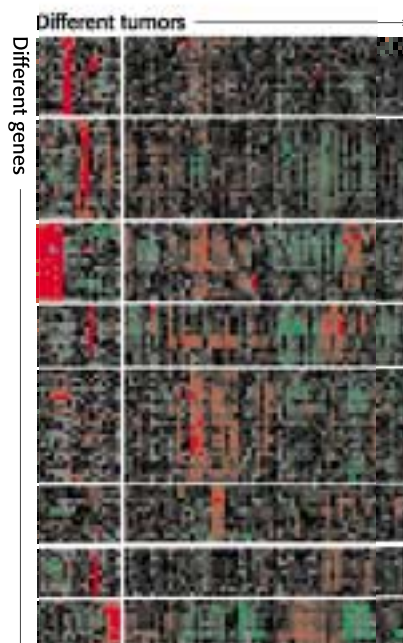


FIGURE 6.26 Gene expression analysis using microarrays. The expression levels of thousands of genes can be simultaneously analyzed using DNA microarrays (gene chips). Here, analysis of 1733 genes in 84 breast tumor samples reveals that the tumors can be divided into distinct classes based on their gene expression patterns. Red corresponds to gene induction and green corresponds to gene repression. [Adapted from C. M. Perou et al., *Nature* 406(2000):747.]

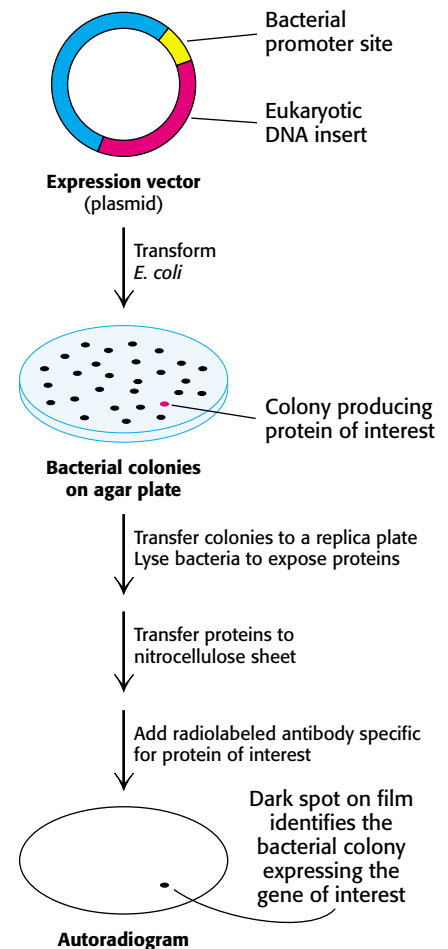


FIGURE 6.25 Screening of cDNA clones. A method of screening for cDNA clones is to identify expressed products by staining with specific antibody.

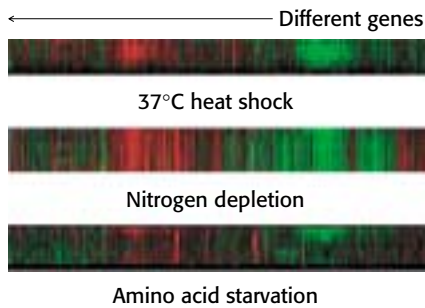


FIGURE 6.27 Monitoring changes in yeast gene expression. This microarray analysis shows levels of gene expression for yeast genes under different conditions. [Adapted from Iyer et al., *Nature* 409(2000):533.]

The intensity of the fluorescent spot on the chip reveals the extent of transcription of a particular gene. DNA chips have been prepared that contain oligonucleotides complementary to all known open reading frames, 6200 in number, within the yeast genome (Figure 6.27). An analysis of mRNA pools with the use of these chips revealed, for example, that approximately 50% of all yeast genes are expressed at steady-state levels of between 0.1 and 1.0 mRNA copy per cell. This method readily detected variations in expression levels displayed by specific genes under different growth conditions. These tools will continue to grow in power as genome sequencing efforts continue.

6.3.3 New Genes Inserted into Eukaryotic Cells Can Be Efficiently Expressed

Bacteria are ideal hosts for the amplification of DNA molecules. They can also serve as factories for the production of a wide range of prokaryotic and eukaryotic proteins. However, bacteria lack the necessary enzymes to carry out posttranslational modifications such as the specific cleavage of polypeptides and the attachment of carbohydrate units. Thus, many eukaryotic genes can be correctly expressed only in eukaryotic host cells. The introduction of recombinant DNA molecules into cells of higher organisms can also be a source of insight into how their genes are organized and expressed. How are genes turned on and off in embryological development? How does a fertilized egg give rise to an organism with highly differentiated cells that are organized in space and time? These central questions of biology can now be fruitfully approached by expressing foreign genes in mammalian cells.

Recombinant DNA molecules can be introduced into animal cells in several ways. In one method, foreign DNA molecules precipitated by calcium phosphate are taken up by animal cells. A small fraction of the imported DNA becomes stably integrated into the chromosomal DNA. The efficiency of incorporation is low, but the method is useful because it is easy to apply. In another method, DNA is *microinjected* into cells. A fine-tipped (0.1- μm -diameter) glass micropipet containing a solution of foreign DNA is inserted into a nucleus (Figure 6.28). A skilled investigator can inject hundreds of cells per hour. About 2% of injected mouse cells are viable and contain the new gene. In a third method, *viruses* are used to bring new genes into animal cells. The most effective vectors are *retroviruses*. As discussed in Section 5.3.1, retroviruses replicate through DNA intermediates, the reverse of the normal flow of information. A striking feature of the life cycle of a retrovirus is that the double-helical DNA form of its genome, produced by the action of reverse transcriptase, becomes randomly incorporated into host chromosomal DNA. This DNA version of the viral genome, called *proviral DNA*, can be efficiently expressed by the host cell and replicated along with normal cellular DNA. Retroviruses do not usually kill their hosts. Foreign genes have been efficiently introduced into mammalian cells by infecting them with vectors derived from *Moloney murine leukemia virus*, which can accept inserts as long as 6 kb. Some genes introduced by this retroviral vector into the genome of a transformed host cell are efficiently expressed.



FIGURE 6.28 Microinjection of DNA. Cloned plasmid DNA is being microinjected into the male pronucleus of a fertilized mouse egg.

Two other viral vectors are extensively used. *Vaccinia virus*, a large DNA-containing virus, replicates in the cytoplasm of mammalian cells, where it shuts down host-cell protein synthesis. *Baculovirus* infects insect cells, which can be conveniently cultured. Insect larvae infected with this virus

can serve as efficient protein factories. Vectors based on these large-genome viruses have been engineered to express DNA inserts efficiently.

6.3.4 Transgenic Animals Harbor and Express Genes That Were Introduced into Their Germ Lines

Genetically engineered giant mice illustrate the expression of foreign genes in mammalian cells (Figure 6.29). Giant mice were produced by introducing the gene for rat growth hormone into a fertilized mouse egg. *Growth hormone (somatotropin)*, a 21-kd protein, is normally synthesized by the pituitary gland. A deficiency of this hormone produces dwarfism, and an excess leads to gigantism. The gene for rat growth hormone was placed on a plasmid next to the mouse metallothionein *promoter* (Figure 6.30). This promoter site is normally located on a chromosome, where it controls the transcription of *metallothionein*, a cysteine-rich protein that has high affinity for heavy metals. Metallothionein binds to and sequesters heavy metals, many of which are toxic for metabolic processes (Section 17.3.2). The synthesis of this protective protein by the liver is induced by heavy-metal ions such as cadmium. Hence, if mice contain the new gene, its expression can be initiated by the addition of cadmium to the drinking water.



FIGURE 6.29 Transgenic mice. Injection of the gene for growth hormone into a fertilized mouse egg gave rise to a giant mouse (left), about twice the weight of his sibling (right). [Courtesy of Dr. Ralph Brinster.]

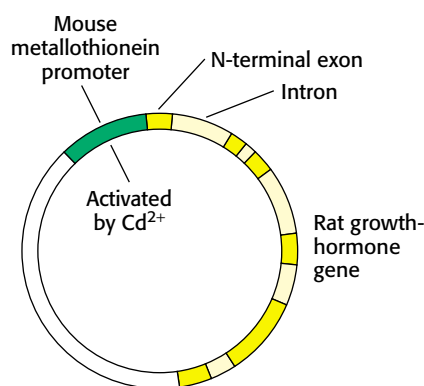
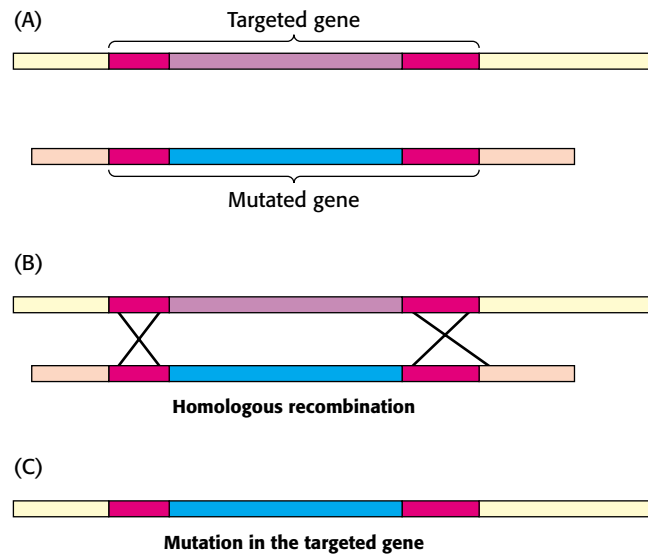


FIGURE 6.30 Rat growth hormone–metallothionein gene construct. The gene for rat growth hormone (shown in yellow) was inserted into a plasmid next to the metallothionein promoter, which is activated by the addition of heavy metals, such as cadmium ion.

Several hundred copies of the plasmid containing the promoter and growth-hormone gene were microinjected into the male pronucleus of a fertilized mouse egg, which was then inserted into the uterus of a foster mother mouse. A number of mice that developed from such microinjected eggs contained the gene for rat growth hormone, as shown by Southern blots of their DNA. These *transgenic mice*, containing multiple copies (~ 30 per cell) of the rat growth-hormone gene, grew much more rapidly than did control mice. In the presence of cadmium, the level of growth hormone in these mice was 500 times as high as in normal mice, and their body weight at maturity was twice normal. The foreign DNA had been transcribed and its five introns correctly spliced out to form functional mRNA. *These experiments strikingly demonstrate that a foreign gene under the control of a new promoter site can be integrated and efficiently expressed in mammalian cells.*

FIGURE 6.31 Gene disruption by homologous recombination. (A) A mutated version of the gene to be disrupted is constructed, maintaining some regions of homology with the normal gene (red). When the foreign mutated gene is introduced into an embryonic stem cell, (B) recombination takes place at regions of homology and (C) the normal (targeted) gene is replaced, or “knocked out,” by the foreign gene. The cell is inserted into embryos, and mice lacking the gene (knockout mice) are produced.



6.3.5 Gene Disruption Provides Clues to Gene Function

A gene's function can also be probed by inactivating the gene and looking for resulting abnormalities. Powerful methods have been developed for accomplishing *gene disruption* (also called *gene knockout*) in organisms such as yeast and mice. These methods rely on the process of *homologous recombination*. Through this process, regions of strong sequence similarity exchange segments of DNA. Foreign DNA inserted into a cell thus can disrupt any gene that is at least in part homologous by exchanging segments (Figure 6.31). Specific genes can be targeted if their nucleotide sequences are known.

For example, the gene knockout approach has been applied to the genes encoding gene regulatory proteins (also called transcription factors) that control the differentiation of muscle cells. When both copies of the gene for the regulatory protein *myogenin* are disrupted, an animal dies at birth because it lacks functional skeletal muscle. Microscopic inspection reveals that the tissues from which muscle normally forms contain precursor cells that have failed to differentiate fully (Figure 6.32). Heterozygous mice containing one normal *myogenin* gene and one disrupted gene appear normal, indicating that the level of gene expression is not essential for its function. Analogous studies have probed the function of many other genes to generate animal models for known human genetic diseases.

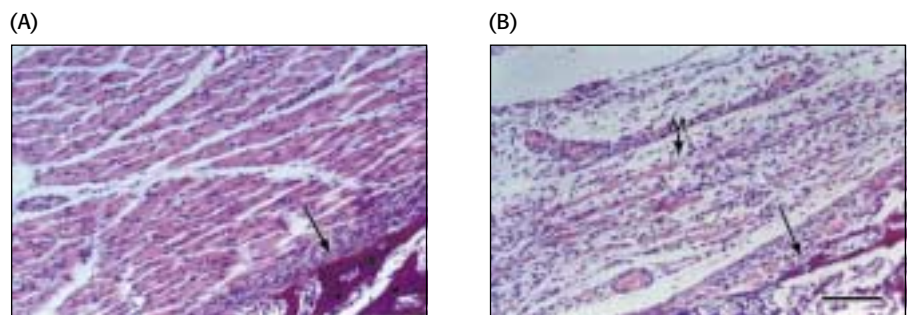


FIGURE 6.32 Consequences of gene disruption. Sections of muscle from normal (A) and gene-disrupted (B) mice, as viewed under the light microscope. Muscles do not develop properly in mice having both *myogenin* genes disrupted. [From P. Hasty, A. Bradley, J. H. Morris, D. G. Edmondson, J. M. Venuti, E. N. Olson, and W. H. Klein, *Nature* 364(1993):501.]

6.3.6 Tumor-Inducing Plasmids Can Be Used to Introduce New Genes into Plant Cells

The common soil bacterium *Agrobacterium tumefaciens* infects plants and introduces foreign genes into plants cells (Figure 6.33). A lump of tumor tissue called a *crown gall* grows at the site of infection. Crown galls synthesize opines, a group of amino acid derivatives that are metabolized by the infecting bacteria. In essence, the metabolism of the plant cell is diverted to satisfy the highly distinctive appetite of the intruder. *Tumor-inducing plasmids* (*Ti plasmids*) that are carried by *Agrobacterium* carry instructions for the switch to the tumor state and the synthesis of opines. A small part of the *Ti* plasmid becomes integrated into the genome of infected plant cells; this 20-kb segment is called *T-DNA* (transferred DNA; Figure 6.34).

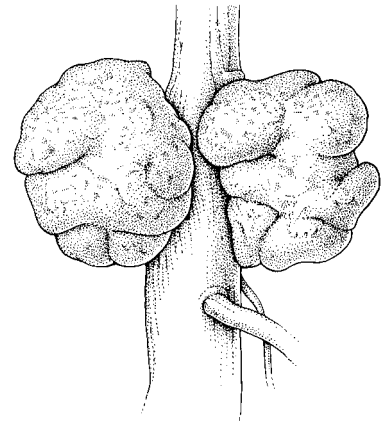


FIGURE 6.33 Tumors in plants. Crown gall, a plant tumor, is caused by a bacterium (*Agrobacterium tumefaciens*) that carries a tumor-inducing plasmid (*Ti* plasmid).

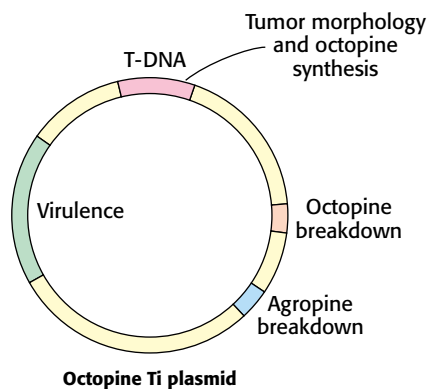


FIGURE 6.34 *Ti* plasmids. *Agrobacteria* containing *Ti* plasmids can deliver foreign genes into some plant cells. [After M. Chilton. A vector for introducing new genes into plants. Copyright ©1983 by Scientific American, Inc. All rights reserved.]

Ti plasmid derivatives can be used as vectors to deliver foreign genes into plant cells. First, a segment of foreign DNA is inserted into the *T-DNA* region of a small plasmid through the use of restriction enzymes and ligases. This synthetic plasmid is added to *Agrobacterium* colonies harboring naturally occurring *Ti* plasmids. By recombination, *Ti* plasmids containing the foreign gene are formed. These *Ti* vectors hold great promise as tools for exploring the genomes of plant cells and modifying plants to improve their agricultural value and crop yield. However, they are not suitable for transforming all types of plants. *Ti*-plasmid transfer is effective with dicots (broad-leaved plants such as grapes) and a few kinds of monocots but not with economically important cereal monocots.

Foreign DNA can be introduced into cereal monocots as well as dicots by applying intense electric fields, a technique called *electroporation* (Figure 6.35). First, the cellulose wall surrounding plant cells is removed by adding cellulase; this treatment produces *protoplasts*, plant cells with exposed plasma membranes. Electric pulses are then applied to a suspension of protoplasts and plasmid DNA. Because high electric fields make membranes transiently permeable to large molecules, plasmid DNA molecules enter the cells. The cell wall is then allowed to reform, and the plant cells are again viable. Maize cells and carrot cells have been stably transformed in this way with the use of plasmid DNA that includes genes for resistance to antibiotics. Moreover, the transformed cells efficiently express the plasmid DNA. Electroporation is also an effective means of delivering foreign DNA into animal cells.

The most effective means of transforming plant cells is through the use of “gene guns,” or bombardment-mediated transformation. DNA is coated onto 1- μm -diameter tungsten pellets, and these microprojectiles are fired

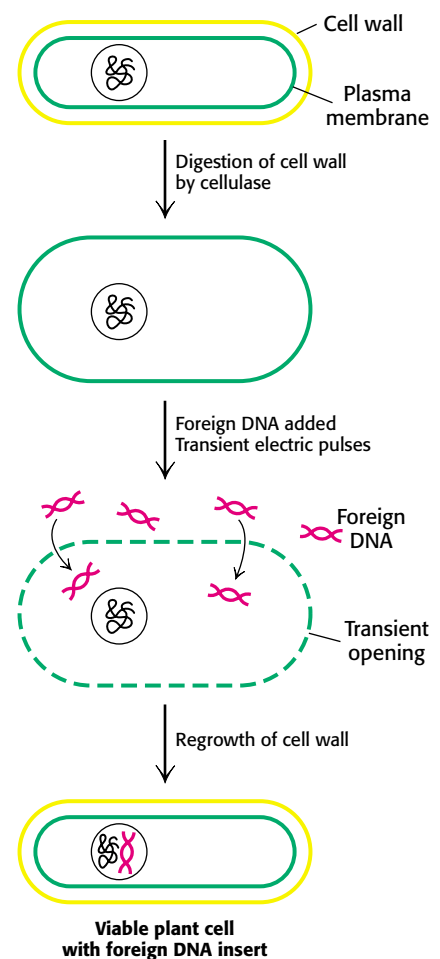


FIGURE 6.35 Electroporation. Foreign DNA can be introduced into plant cells by electroporation, the application of intense electric fields to make their plasma membranes transiently permeable.

at the target cells with a velocity greater than 400 m s^{-1} . Despite its apparent crudeness, this technique is proving to be the most effective way of transforming plants, especially important crop species such as soybean, corn, wheat, and rice. The gene-gun technique affords an opportunity to develop genetically modified organisms (GMOs) with beneficial characteristics. Such characteristics could include the ability to grow in poor soils, resistance to natural climatic variation, resistance to pests, and nutritional fortification. These crops might be most useful in developing countries. The use of genetically modified organisms is highly controversial at this point because of fears of unexpected side effects.

The first GMO to come to market was a tomato characterized by delayed ripening, rendering it ideal for shipment. Pectin is a polysaccharide that gives tomatoes their firmness and is naturally destroyed by the enzyme *polygalacturonase*. As pectin is destroyed, the tomatoes soften, making shipment difficult. DNA was introduced that disrupts the polygalacturonase gene. Less of the enzyme was produced, and the tomatoes stayed fresh longer. However, the tomato's poor taste hindered its commercial success.

6.4 NOVEL PROTEINS CAN BE ENGINEERED BY SITE-SPECIFIC MUTAGENESIS

Much has been learned about genes and proteins by analyzing mutated genes selected from the repertoire offered by nature. In the classic genetic approach, mutations are generated randomly throughout the genome, and those exhibiting a particular phenotype are selected. Analysis of these mutants then reveals which genes are altered, and DNA sequencing identifies the precise nature of the changes. *Recombinant DNA technology now makes it feasible to create specific mutations in vitro.*

6.4.1 Proteins with New Functions Can Be Created Through Directed Changes in DNA

We can construct new genes with designed properties by making three kinds of directed changes: *deletions*, *insertions*, and *substitutions*.

Deletions. A specific deletion can be produced by cleaving a plasmid at two sites with a restriction enzyme and ligating to form a smaller circle. This simple approach usually removes a large block of DNA. A smaller deletion can be made by cutting a plasmid at a single site. The ends of the linear DNA are then digested with an exonuclease that removes nucleotides from both strands. The shortened piece of DNA is then ligated to form a circle that is missing a short length of DNA about the restriction site.

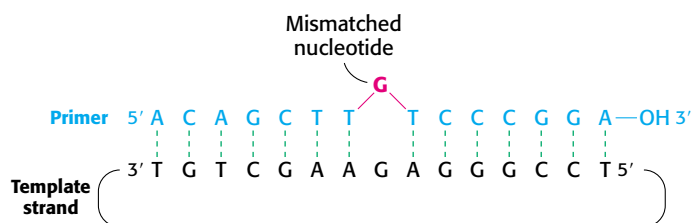


FIGURE 6.36 Oligonucleotide-directed mutagenesis. A primer containing a mismatched nucleotide is used to produce a desired change in the DNA sequence.

Substitutions: Oligonucleotide-Directed Mutagenesis. Mutant proteins with single amino acid substitutions can be readily produced by *oligonucleotide-directed mutagenesis* (Figure 6.36). Suppose that we want to replace a particular serine residue with cysteine. This mutation can be made if (1) we have a plasmid containing the gene or cDNA for the protein and (2) we know the base sequence around the site to be altered. If the serine of interest is encoded by TCT, we need to change the C to a G to get cysteine, which is encoded by TGT. This type of mutation is called a point mutation because only one base is altered. The key to this mutation is to prepare an oligonucleotide primer that is complementary to this region of the gene except that it contains TGT instead of TCT. The two strands of the plasmid are separated, and the primer is then annealed to the complementary strand. After annealing to the complementary strand, the primer is elongated by DNA polymerase, and the double-stranded circle is closed by adding DNA ligase. Subsequent replication of this duplex yields two kinds of progeny plasmid, half with the original TCT sequence and half with the mutant TGT sequence. Expression of the plasmid containing the new TGT sequence will produce a protein with the desired substitution of serine for cysteine at a unique site. We will encounter many examples of the use of oligonucleotide-directed mutagenesis to precisely alter regulatory regions of genes and to produce proteins with tailor-made features.

Insertions: Cassette Mutagenesis. In another valuable approach, *cassette mutagenesis*, plasmid DNA is cut with a pair of restriction enzymes to remove a short segment (Figure 6.37). A synthetic double-stranded oligonucleotide (the *cassette*) with cohesive ends that are complementary to the ends of the cut plasmid is then added and ligated. Each plasmid now contains the desired mutation. It is convenient to introduce into the plasmid unique restriction sites spaced about 40 nucleotides apart so that mutations can be readily made anywhere in the sequence.

Designer Genes. Novel proteins can also be created by splicing together gene segments that encode domains that are not associated in nature. For example, a gene for an antibody can be joined to a gene for a toxin to produce a chimeric protein that kills cells that are recognized by the antibody. These *immunotoxins* are being evaluated as anticancer agents. Entirely new genes can be synthesized *de novo* by the solid-phase method. Furthermore, noninfectious coat proteins of viruses can be produced in large amounts by recombinant DNA methods. They can serve as *synthetic vaccines* that are safer than conventional vaccines prepared by inactivating pathogenic viruses. A subunit of the hepatitis B virus produced in yeast is proving to be an effective vaccine against this debilitating viral disease.

6.4.2 Recombinant DNA Technology Has Opened New Vistas

Recombinant DNA technology has revolutionized the analysis of the molecular basis of life. Complex chromosomes are being rapidly mapped and dissected into units that can be manipulated and deciphered. The amplification of genes by cloning has provided abundant quantities of DNA for sequencing. Genes are now open books that can be read. New insights are emerging, as exemplified by the discovery of introns in eukaryotic genes. Central questions of biology, such as the molecular basis of development, are now being fruitfully explored. DNA and RNA sequences provide a wealth of information about evolution. Biochemists now move back and forth between gene and protein and feel at home in both areas of inquiry.

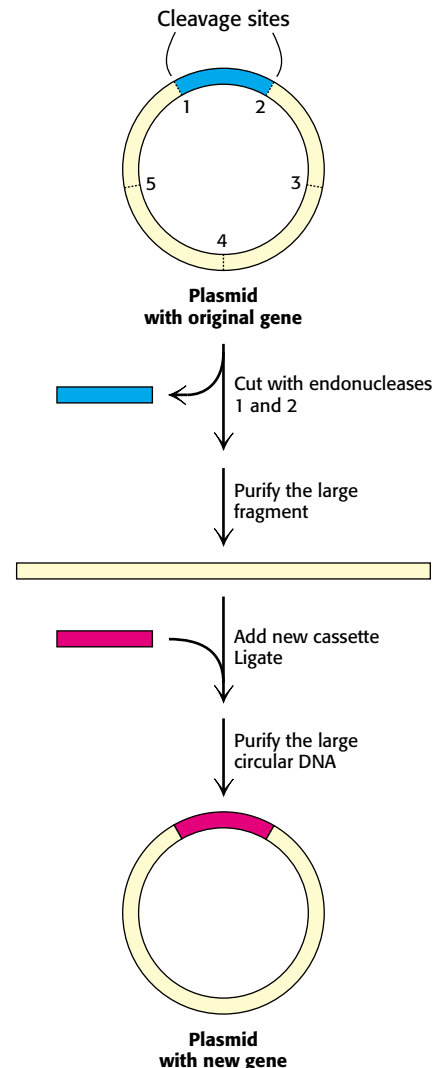


FIGURE 6.37 Cassette mutagenesis. DNA is cleaved at a pair of unique restriction sites by two different restriction endonuclease. A synthetic oligonucleotide with ends that are complementary to these sites (the *cassette*) is then ligated to the cleaved DNA. The method is highly versatile because the inserted DNA can have any desired sequence.

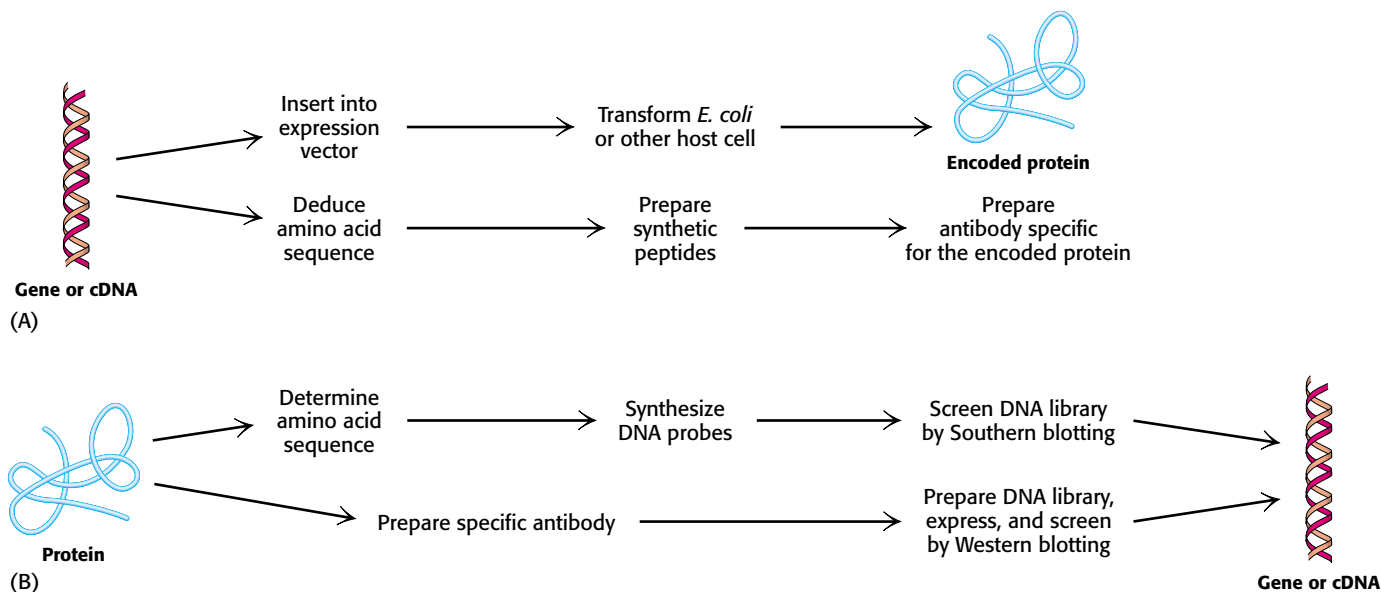


FIGURE 6.38 The techniques of protein chemistry and nucleic acid chemistry are mutually reinforcing. (A) From DNA (or RNA) to protein and (B) from protein to DNA.

Analyses of genes and cDNA can reveal the existence of previously unknown proteins, which can be isolated and purified (Figure 6.38A). Conversely, purification of a protein can be the starting point for the isolation and cloning of its gene or cDNA (Figure 6.39B). Very small amounts of protein or nucleic acid suffice because of the sensitivity of recently developed microchemical techniques and the amplification afforded by gene cloning and the polymerase chain reaction. The powerful techniques of protein chemistry, nucleic acid chemistry, immunology, and molecular genetics are highly synergistic.

New kinds of proteins can be created by altering genes in specific ways. Site-specific mutagenesis opens the door to understanding how proteins fold, recognize other molecules, catalyze reactions, and process information. Large amounts of protein can be obtained by expressing cloned genes or cDNAs in bacteria or eukaryotic cells. Hormones, such as insulin, and antiviral agents, such as interferon, are being produced by bacteria. Tissue plasminogen activator, which is administered to a patient after a heart attack, is made in large quantities in mammalian cells. A new pharmacology, using proteins produced by recombinant DNA technology as drugs, is beginning to significantly alter the practice of medicine. Recombinant DNA technology is also providing highly specific diagnostic reagents, such as DNA probes for the detection of genetic diseases, infections, and cancers. Human gene therapy has been successfully initiated. White blood cells deficient in adenosine deaminase, an essential enzyme, are taken from patients and returned after being transformed *in vitro* to correct the genetic error. Agriculture, too, is benefiting from genetic engineering. Transgenic crops with increased resistance to insects, herbicides, and drought have been produced.

SUMMARY

- **The Basic Tools of Gene Exploration**

The recombinant DNA revolution in biology is rooted in the repertoire of enzymes that act on nucleic acids. Restriction enzymes are a key group among them. These endonucleases recognize specific base sequences in double-helical DNA and cleave both strands of the duplex, forming specific fragments of DNA. These restriction fragments can be separated

and displayed by gel electrophoresis. The pattern that they form on the gel is a fingerprint of a DNA molecule. A DNA fragment containing a particular sequence can be identified by hybridizing it with a labeled single-stranded DNA probe (Southern blotting).

Rapid sequencing techniques have been developed to further the analysis of DNA molecules. DNA can be sequenced by controlled interruption of replication (Sanger dideoxy method). The fragments produced are separated by gel electrophoresis and visualized by autoradiography of a ^{32}P label at the 5' end or by fluorescent tags. The recent sequencing of many complete genomes demonstrates the power of these techniques.

DNA probes for hybridization reactions, as well as new genes, can be synthesized by the automated solid-phase method. The technique is to add deoxyribonucleoside 3'-phosphoramidites to one another to form a growing chain that is linked to an insoluble support. DNA chains a hundred nucleotides long can be readily synthesized by this automated solid-phase method. The polymerase chain reaction makes it possible to greatly amplify specific segments of DNA in vitro. The region amplified is determined by the placement of a pair of primers that are added to the target DNA along with a thermostable DNA polymerase and deoxyribonucleoside triphosphates. The exquisite sensitivity of PCR makes it a choice technique in detecting pathogens and cancer markers, in genotyping, and in reading DNA from fossils that are many thousands of years old.

- **Recombinant DNA Technology Has Revolutionized All Aspects of Biology**

New genes can be constructed in the laboratory, introduced into host cells, and expressed. Novel DNA molecules are made by joining fragments that have complementary cohesive ends produced by the action of a restriction enzyme. DNA ligase seals breaks in DNA chains. Vectors for propagating the DNA include plasmids, λ phage, and yeast artificial chromosomes. Specific genes can be cloned from a genomic library using a DNA or RNA probe. Foreign DNA can be expressed after insertion into prokaryotic and eukaryotic cells by the appropriate vector.

- **Manipulating the Genes of Eukaryotes**

The production of giant mice by injecting the gene for rat growth hormone into fertilized mouse eggs vividly shows that mammalian cells can be genetically altered in a designed way. New DNA can be brought into plant cells by the soil bacterium *Agrobacterium tumefaciens*, which harbors Ti (tumor-inducing) plasmids. DNA can also be introduced into plant cells by applying intense electric fields, which render them transiently permeable to very large molecules, or by bombarding them with DNA-coated microparticles. Gene-expression levels can be examined through the hybridization of cellular mRNA to arrays of oligonucleotides synthesized on solid supports (gene chips). The functions of particular genes can also be investigated by disruption.

- **Novel Proteins Can Be Engineered by Site-Specific Mutagenesis**

Specific mutations can be generated in vitro to engineer novel proteins. A mutant protein with a single amino acid substitution can be produced by priming DNA replication with an oligonucleotide encoding the new amino acid. Plasmids can be engineered to permit the facile insertion of a DNA cassette containing any desired mutation. The techniques of protein and nucleic acid chemistry are highly synergistic. Investigators now move back and forth between gene and protein with great facility. Recombinant DNA technology is beginning to significantly alter the practice of medicine by providing new diagnostic and therapeutic agents and revealing molecular mechanisms of disease.

KEY TERMS

- restriction enzyme (p. 144)
 palindrome (p. 144)
 DNA probe (p. 146)
 Southern blotting (p. 146)
 Northern blotting (p. 146)
 controlled termination of replication (Sanger dideoxy method) (p. 146)
 polymerase chain reaction (PCR) (p. 149)
 vector (p. 152)
 plasmid (p. 152)
- sticky ends (p. 152)
 DNA ligase (p. 152)
 lambda (λ) phage (p. 153)
 genomic library (p. 155)
 bacterial artificial chromosome (BAC) (p. 157)
 yeast artificial chromosome (YAC) (p. 157)
 chromosome walking (p. 157)
 complementary DNA (cDNA) (p. 158)
 reverse transcriptase (p. 158)
- cDNA library (p. 158)
 expression vector (p. 159)
 DNA microarray (gene chip) (p. 159)
 transgenic mice (p. 161)
 gene disruption (gene knockout) (p. 162)
 tumor-inducing plasmid (Ti plasmid) (p. 163)
 oligonucleotide-directed mutagenesis (p. 165)
 cassette mutagenesis (p. 165)

SELECTED READINGS

Where to start

- Berg, P., 1981. Dissections and reconstructions of genes and chromosomes. *Science* 213:296–303.
 Gilbert, W., 1981. DNA sequencing and gene structure. *Science* 214:1305–1312.
 Sanger, F., 1981. Determination of nucleotide sequences in DNA. *Science* 214:1205–1210.
 Mullis, K.B., 1990. The unusual origin of the polymerase chain reaction. *Sci. Am.* 262(4):56–65.

Books on recombinant DNA technology

- Watson, J. D., Gilman, M., Witkowski, J., and Zoller, M., 1992. *Recombinant DNA* (2d ed.). Scientific American Books.
 Grierson, D. (Ed.), 1991. *Plant Genetic Engineering*. Chapman and Hall.
 Mullis, K. B., Ferré, F., and Gibbs, R. A. (Eds.), 1994. *The Polymerase Chain Reaction*. Birkhäuser.
 Russel, D., Sambrook, J., and Russel, D., 2000. *Molecular Cloning: A Laboratory Manual* (3d ed.). Cold Spring Harbor Laboratory Press.
 Ausubel, F. M., Brent, R., Kingston, R. E., and Moore, D. D., (Eds.) 1999. *Short Protocols in Molecular Biology: A Compendium of Methods from Current Protocols in Molecular Biology*. Wiley.
 Birren, B., Green, E. D., Klapholz, S., Myers, R. M., Roskams, J., Riethamn, H., and Hieter, P. (Eds.), 1999. *Genome Analysis* (vols. 1–4). Cold Spring Harbor Laboratory Press.
Methods in Enzymology. Academic Press. [Many volumes in this series deal with recombinant DNA technology.]

DNA sequencing and synthesis

- Hunkapiller, T., Kaiser, R. J., Koop, B. F., and Hood, L., 1991. Large-scale and automated DNA sequence determination. *Science* 254:59–67.
 Sanger, F., Nicklen, S., and Coulson, A. R., 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. U.S.A.* 74:5463–5467.
 Maxam, A. M., and Gilbert, W., 1977. A new method for sequencing DNA. *Proc. Natl. Acad. Sci. U.S.A.* 74:560–564.
 Smith, L. M., Sanders, J. Z., Kaiser, R. J., Hughes, P., Dodd, C., Connell, C. R., Heiner, C., Kent, S. B. H., and Hood, L. E., 1986. Fluorescence detection in automated DNA sequence analysis. *Nature* 321:674–679.
 Pease, A. C., Solas, D., Sullivan, E. J., Cronin, M. T., Holmes, C. P., and Fodor, S. P. A., 1994. Light-generated oligonucleotide arrays for rapid DNA sequence analysis. *Proc. Natl. Acad. Sci. U.S.A.* 91:5022–5026.
 Venter, J. C., Adams, M. D., Sutton, G. G., Kerlavage, A.R., Smith, H.O., and Hunkapiller, M., 1998. Shotgun sequencing of the human genome. *Science* 280:1540–1542.

Polymerase chain reaction (PCR)

- Arnheim, N., and Erlich, H., 1992. Polymerase chain reaction strategy. *Annu. Rev. Biochem.* 61:131–156.
 Kirby, L. T. (Ed.), 1997. *DNA Fingerprinting: An Introduction*. Stockton Press.
 Eisenstein, B. I., 1990. The polymerase chain reaction: A new method for using molecular genetics for medical diagnosis. *N. Engl. J. Med.* 322:178–183.
 Foley, K. P., Leonard, M. W., and Engel, J. D., 1993. Quantitation of RNA using the polymerase chain reaction. *Trends Genet.* 9:380–386.
 Pääbo, S., 1993. Ancient DNA. *Sci. Am.* 269(5):86–92.
 Hagelberg, E., Gray, I. C., and Jeffreys, A. J., 1991. Identification of the skeletal remains of a murder victim by DNA analysis. *Nature* 352:427–429.
 Lawlor, D. A., Dickel, C. D., Hauswirth, W. W., and Parham, P., 1991. Ancient HLA genes from 7500-year-old archaeological remains. *Nature* 349:785–788.
 Krings, M., Geisert, H., Schmitz, R. W., Krainitzki, H., and Pääbo, S., 1999. DNA sequence of the mitochondrial hypervariable region II for the Neanderthal type specimen. *Proc. Natl. Acad. Sci. U.S.A.* 96:5581–5585.
 Ovchinnikov, I. V., Götherström, A., Romanova, G. P., Kharitonov, V. M., Lidén, K., and Goodwin, W., 2000. Molecular analysis of Neanderthal DNA from the northern Caucasus. *Nature* 404:490–493.

DNA arrays

- Duggan, D. J., Bittner, J. M., Chen, Y., Meltzer, P., and Trent, J. M., 1999. Expression profiling using cDNA microarrays. *Nat. Genet.* 21:10–14.
 Golub, T. R., Slonim, D. K., Tamayo, P., Huard, C., Gaasenbeek, M., Mesirov, J. P., Coller, H., Loh, M. L., Downing, J. R., Caligiuri, M. A., Bloomfield, C. D., and Lander, E. S., 1999. Molecular classification of cancer: Class discovery and class prediction by gene expression monitoring. *Science* 286:531–537.
 Perou, C. M., Sørlie, T., Eisen, M. B., van de Rijn, M., Jeffery, S. S., Rees, C. A., Pollack, J. R., Ross, D. T., Johnsen, H., Akslen, L. A., Fluge, Ø., Pergamenschikov, A., Williams, C., Zhu, S. X., Lønning, P. E., Børresen-Dale, A.-L., Brown, P. O., and Botstein, D., 2000. Molecular portraits of human breast tumours. *Nature* 406:747–752.

Introduction of genes into animal cells

- Anderson, W. F., 1992. Human gene therapy. *Science* 256:808–813.
 Friedmann, T., 1997. Overcoming the obstacles to gene therapy. *Sci. Am.* 277(6):96–101.

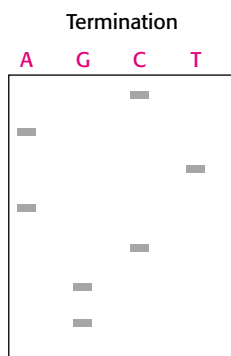
- Blaese, R. M., 1997. Gene therapy for cancer. *Sci. Am.* 277 (6):111–115.
- Brinster, R. L., and Palmiter, R. D., 1986. Introduction of genes into the germ lines of animals. *Harvey Lect.* 80:1–38.
- Capecchi, M. R., 1989. Altering the genome by homologous recombination. *Science* 244:1288–1292.
- Hasty, P., Bradley, A., Morris, J. H., Edmondson, D. G., Venuti, J. M., Olson, E. N., and Klein, W. H., 1993. Muscle deficiency and neonatal death in mice with a targeted mutation in the myogenin gene. *Nature* 364:501–506
- Parkmann, R., Weinberg, K., Crooks, G., Nolta, J., Kapoor, N., and Kohn, D., 2000. Gene therapy for adenosine deaminase deficiency. *Annu. Rev. Med.* 51:33–47.

Genetic engineering of plants

- Gasser, C. S., and Fraley, R. T., 1992. Transgenic crops. *Sci. Am.* 266(6):62–69.

PROBLEMS

1. *Reading sequences.* An autoradiogram of a sequencing gel containing four lanes of DNA fragments is shown in the adjoining illustration. (a) What is the sequence of the DNA fragment? (b) Suppose that the Sanger dideoxy method shows that the template strand sequence is 5'-TGCAATGGC-3'. Sketch the gel pattern that would lead to this conclusion.



2. *The right template.* Ovalbumin is the major protein of egg white. The chicken ovalbumin gene contains eight exons separated by seven introns. Should one use ovalbumin cDNA or ovalbumin genomic DNA to form the protein in *E. coli*? Why?
3. *Cleavage frequency.* The restriction enzyme *AluI* cleaves at the sequence 5'-AGCT-3', and *NotI* cleaves at 5'-GCGGCCGC-3'. What would be the average distance between cleavage sites for each enzyme on digestion of double-stranded DNA?
4. *The right cuts.* Suppose that a human genomic library is prepared by exhaustive digestion of human DNA with the *EcoRI* restriction enzyme. Fragments averaging about 4 kb in length would be generated.
- (a) Is this procedure suitable for cloning large genes? Why?
- (b) Is this procedure suitable for mapping extensive stretches of the genome by chromosome walking? Why?
5. *A revealing cleavage.* Sickle-cell anemia arises from a mutation in the gene for the β chain of human hemoglobin. The change from GAG to GTG in the mutant eliminates a cleavage site for the restriction enzyme *MstII*, which recognizes the target sequence CCTGAGG. These findings form the basis of a diagnostic test for the sickle-cell gene. Propose a rapid procedure for distinguishing between the normal and the mutant gene. Would a positive result prove that the mutant contains GTG in place of GAG?
6. *Many melodies from one cassette.* Suppose that you have isolated an enzyme that digests paper pulp and have obtained its cDNA. The goal is to produce a mutant that is effective at high temperature. You have engineered a pair of unique restriction sites

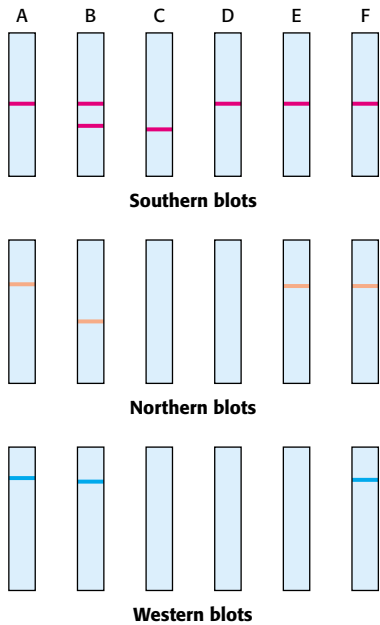
- Gasser, C. S., and Fraley, R. T., 1989. Genetically engineering plants for crop improvement. *Science* 244:1293–1299.
- Shimamoto, K., Terada, R., Izawa, T., and Fujimoto, H., 1989. Fertile transgenic rice plants regenerated from transformed protoplasts. *Nature* 338:274–276.
- Chilton, M.-D., 1983. A vector for introducing new genes into plants. *Sci. Am.* 248(6):50.
- Hansen, G., Wright, M. S., 1999. Recent advances in the transformation of plants. *Trends Plant Sci.* 4:226–231.
- Hammond, J., 1999. Overview: The many uses of transgenic plants. *Curr. Top. Microbiol. Immunol.* 240:1–20.
- Finer, J. J., Finer, K. R., and Ponappa, T., 1999. Particle bombardment mediated transformation. *Curr. Top. Microbiol. Immunol.* 240:60–80.

in the cDNA that flank a 30-bp coding region. Propose a rapid technique for generating many different mutations in this region.

7. *A blessing and a curse.* The power of PCR can also create problems. Suppose someone claims to have isolated dinosaur DNA by using PCR. What questions might you ask to determine if it is indeed dinosaur DNA?
8. *Questions of accuracy.* The stringency (Section 6.1.5) of PCR amplification can be controlled by altering the temperature at which the hybridization of the primers to the target DNA occurs. How would altering the temperature of hybridization effect the amplification? Suppose that you have a particular yeast gene A, and you wish to see if it has a counterpart in humans. How would controlling the stringency of the hybridization help you?
9. *Terra incognita.* PCR is typically used to amplify DNA that lies between two known sequences. Suppose that you want to explore DNA on both sides of a single known sequence. Devise a variation of the usual PCR protocol that would enable you to amplify entirely new genomic terrain.
10. *A puzzling ladder.* A gel pattern displaying PCR products shows four strong bands. The four pieces of DNA have lengths that are approximately in the ratio of 1:2:3:4. The largest band is cut out of the gel, and PCR is repeated with the same primers. Again, a ladder of four bands is evident in the gel. What does this result reveal about the structure of the encoded protein?
11. *Landmarks in the genome.* Many laboratories throughout the world are mapping the human genome. It is essential that the results be merged at an early stage to provide a working physical map of each chromosome. In particular, it is necessary to know whether a YAC studied in one laboratory overlaps a YAC studied in another when only a small proportion of each (less than 5%) has been sequenced. Propose a simple test for overlap based on the *transfer of information but not of materials* between the two laboratories.
- Chapter Integration Problem**
12. *Designing primers.* A successful PCR experiment often depends on designing the correct primers. In particular, the T_m for each primer should be approximately the same. What is the basis of this requirement?

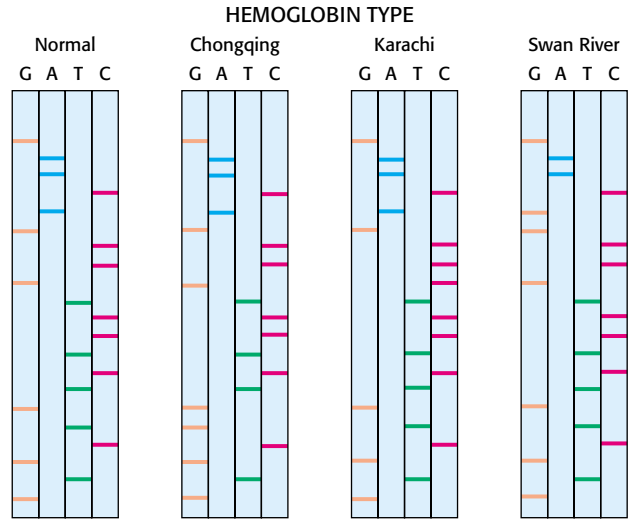
Chapter Integration and Data Analysis Problem

13. *Any direction but east.* A series of people are found to have difficulty eliminating certain types of drugs from their bloodstreams. The problem has been linked to a gene *X*, which encodes an enzyme *Y*. Six people were tested with the use of various techniques of molecular biology. Person A is a normal control, person B is asymptomatic but some of his children have the metabolic problem, and persons C through F display the trait. Tissue samples from each person were obtained. Southern analysis was performed on the DNA after digestion with the restriction enzyme *Hind*III. Northern analysis of mRNA also was done. In both types of analysis, the gels were probed with labeled *X* cDNA. Finally, a Western blot with an enzyme-linked monoclonal antibody was used to test for the presence of protein *Y*. The results are shown here. Why is person B without symptoms? Suggest possible defects in the other people.



Data Interpretation Problem

14. *DNA diagnostics.* Representations of sequencing gels for variants of the α chain of human hemoglobin are shown here. What is the nature of the amino acid change in each of the variants? The first triplet encodes valine.



Exploring Evolution



Evolutionary relationships are manifest in protein sequences. The close kinship between human beings and chimpanzees, hinted at by the mutual interest shown by Jane Goodall and a chimpanzee in the photograph, is revealed in the amino acid sequences of myoglobin. The human sequence (red) differs from the chimpanzee sequence (blue) in only one amino acid in a protein chain of 153 residues. [(Left) Kennan Ward/Corbis.]

GLSDGEWQLVLNVWGKVEAD I PGHGQEVLI R L F K G H P E T L E K F D K F K H L K S E D E M K A S E D L K K H G A T V L T A L G G I L -
GLSDGEWQLVLNVWGKVEAD I PGHGQEVLI R L F K G H P E T L E K F D K F K H L K S E D E M K A S E D L K K H G A T V L T A L G G I L -

KKKGHHEAEIKPLAQSHATKHK I PVKYLEFI SEC I I QV L H S K H P G D F G A D A Q G A M N K A L E L F R K D M A S N Y K E L G F Q G
KKKGHHEAEIKPLAQSHATKHK I PVKYLEFI SEC I I QV L C S K H P G D F G A D A Q G A M N K A L E L F R K D M A S N Y K E L G F Q G

Like members of a human family, members of molecular families often have features in common. Such family resemblance is most easily detected by comparing three-dimensional structure, the aspect of a molecule most closely linked to function. Consider, for example, ribonuclease from cows, which was introduced in our consideration of protein folding (Section 3.6). Comparing structures reveals that the three-dimensional structure of this protein and that of a human ribonuclease are quite similar (Figure 7.1). Although this similarity is not unexpected, given the similarity in biological function, similarities revealed by comparisons are sometimes surprising. For example, angiogenin, a protein identified on the basis of its ability to stimulate the growth of new blood vessels, also turns out to be structurally similar to ribonuclease—so similar that it is clear that both angiogenin and ribonuclease are members of the same protein family (Figure 7.2). Angiogenin and ribonuclease must have had a common ancestor at some earlier stage of evolution.

Unfortunately, three-dimensional structures have been determined for only a relatively small number of proteins. In contrast, gene sequences and the corresponding amino acid sequences are available for a great number of proteins, largely owing to the tremendous power of DNA cloning and sequencing techniques. Evolutionary relationships also are manifest in amino acid sequences. For example, comparison of the amino acid sequences of bovine ribonuclease and angiogenin reveals that 35% of amino acids in corresponding

OUTLINE

- 7.1 Homologs Are Descended from a Common Ancestor
- 7.2 Statistical Analysis of Sequence Alignments Can Detect Homology
- 7.3 Examination of Three-Dimensional Structure Enhances Our Understanding of Evolutionary Relationships
- 7.4 Evolutionary Trees Can Be Constructed on the Basis of Sequence Information
- 7.5 Modern Techniques Make the Experimental Exploration of Evolution Possible

FIGURE 7.1 Structures of ribonucleases from cows and human beings. Structural similarity often follows functional similarity.

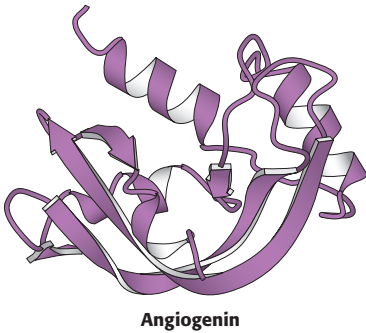
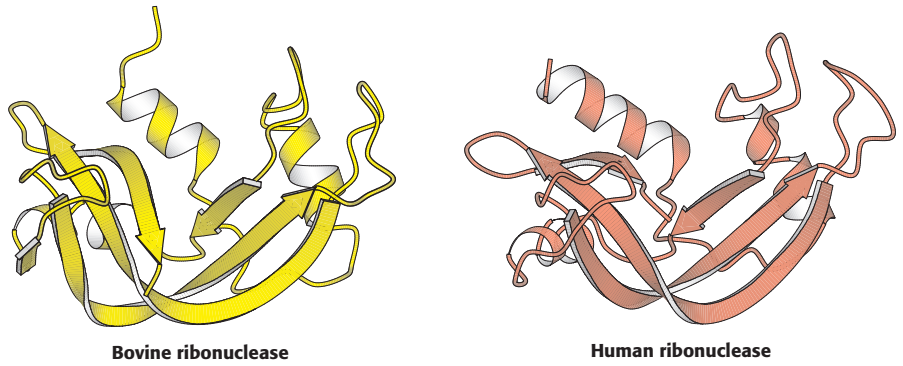


FIGURE 7.2 Structure of angiogenin. The protein angiogenin, identified on the basis of its ability to stimulate blood-vessel growth, is highly similar to ribonuclease.

positions are identical. Is this level sufficiently high to ensure an evolutionary relationship? If not, what level is required? In this chapter, we shall examine the methods that are used to compare amino acid sequences and to deduce such evolutionary relationships.

Sequence-comparison methods have become a powerful tool in modern biochemistry. Sequence databases can be probed for matches to a newly elucidated sequence in order to identify related molecules. This information can often be a source of considerable insight into the function and mechanism of the newly sequenced molecule. When three-dimensional structures are available, they may be compared to confirm relationships suggested by sequence comparisons and to reveal others that are not readily detected at the level of sequence alone.

By examining the footprints present in modern protein sequences, the biochemist can become a molecular archeologist able to learn about events in the evolutionary past. Sequences comparisons can often reveal both pathways of evolutionary descent and estimated dates of specific evolutionary landmarks. This information can be used to construct evolutionary trees that trace the evolution of a particular protein or nucleic acid in many cases from Archaea and Bacteria through Eukarya, including human beings. Molecular evolution can also be studied experimentally. In some cases, DNA from fossils can be amplified by PCR methods (Section 6.1.5) and sequenced, giving a direct view into the past. In addition, investigators can observe molecular evolution taking place in the laboratory, through experiments based on nucleic acid replication. The results of such studies are revealing more about how evolution proceeds.

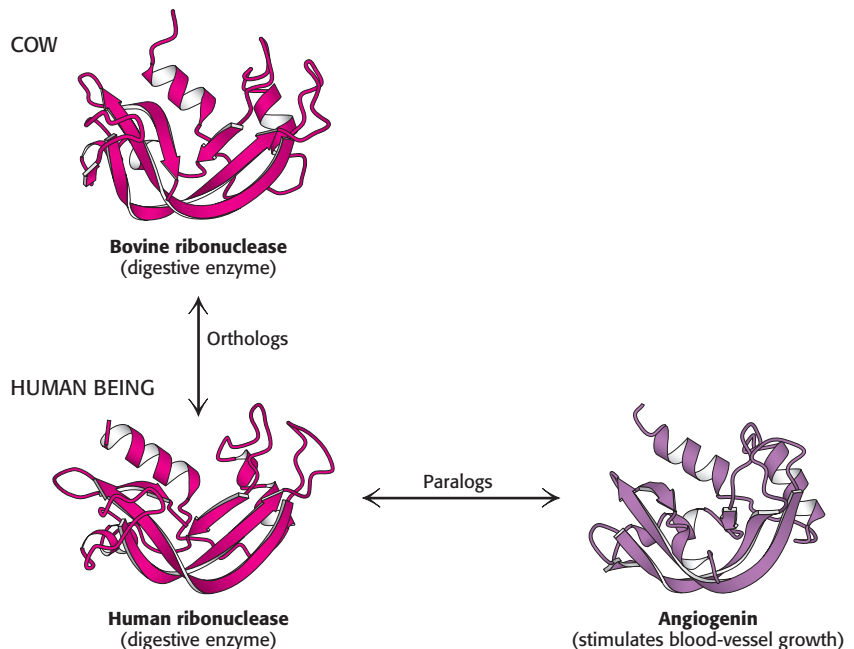


FIGURE 7.3 Two classes of homologs. Homologs that perform identical or very similar functions in different organisms are called orthologs, whereas homologs that perform different functions within one organism are called paralogs.

7.1 HOMOLOGS ARE DESCENDED FROM A COMMON ANCESTOR

The exploration of biochemical evolution consists largely of an attempt to determine how proteins, other molecules, and biochemical pathways have been transformed through time. The most fundamental relationship between two entities is *homology*; two molecules are said to be *homologous* if they have been derived from a common ancestor. Homologous molecules, or *homologs*, can be divided into two classes (Figure 7.3). *Paralogs* are homologs that are present within one species. Paralogs often differ in their detailed biochemical functions. *Orthologs* are homologs that are present within different species and have very similar or identical functions. Understanding the homology between molecules can reveal the evolutionary history of the molecules as well as information about their function; if a newly sequenced protein is homologous to an already characterized protein, we have a strong indication of the new protein's biochemical function.

How can we tell whether two human proteins are paralogs or whether a yeast protein is the ortholog of a human protein? As will be discussed in Section 7.2, *homology is often manifested by significant similarity in nucleotide or amino acid sequence and almost always manifested in three-dimensional structure.*

7.2 STATISTICAL ANALYSIS OF SEQUENCE ALIGNMENTS CAN DETECT HOMOLOGY



CONCEPTUAL INSIGHTS, Sequence Analysis, provides opportunities to interactively explore issues involved in sequence alignment.

A significant sequence similarity between two molecules implies that they are likely to have the same evolutionary origin and, therefore, the same three-dimensional structure, function, and mechanism. Although both nucleic acid and protein sequences can be compared to detect homology, a comparison of protein sequences is much more effective for several reasons, most notably that proteins are built from 20 different building blocks, whereas RNA and DNA are synthesized from only 4 building blocks.

To illustrate sequence-comparison methods, let us consider a class of proteins called the *globins*. Myoglobin is a protein that binds oxygen in muscle, whereas hemoglobin is the oxygen-carrying protein in blood (Section 10.2). Both proteins cradle a heme group, an iron-containing organic molecule that binds the oxygen. Each human hemoglobin molecule is composed of four heme-containing polypeptide chains, two identical α chains and two identical β chains. Here, we consider only the α chain. We wish to examine the similarity between the amino acid sequence of the human α chain and that of human myoglobin (Figure 7.4). To detect such similarity, methods have been developed for *sequence alignment*.

Human hemoglobin (α chain)

VLSPADKTNVKAAWGKVGAGHAGEYGAEALERMFLSFPTTKTYFPHFDLSHG
SAQVKGHGKKVADALTNAVAHVDDMPNALSALSDLHAHKLRVDPVNFKLLS
HCLLVTLAAHLPAEFTPAVHASLDKFLASVSTVLTISKYR

Human myoglobin

GLSDGEWQLVLNWKVVEADIPGHGQEVLIIRLFGKHPETLEKFDKFKHLKLS
EDEMKASEDLKKHGATVLTALGGILKKGHHEAEIKPLAQSHATKHKIPVK
YLEFISECIQVQLQSKHPGDFGADAQGAMNKALELFRKDMASNYKELGFQG



CONCEPTUAL INSIGHTS, appearing throughout the book, are interactive animations that help you build your understanding of key biochemical principles and concepts. To access, go to the Web site: www.whfreeman.com/biochem5, and select the chapter, Conceptual Insights, and the title.

FIGURE 7.4 Amino acid sequences of human hemoglobin (α chain) and human myoglobin. Hemoglobin α is composed of 141 amino acids; myoglobin consists of 153 amino acids. (One-letter abbreviations designating amino acids are used; see Table 3.2.)

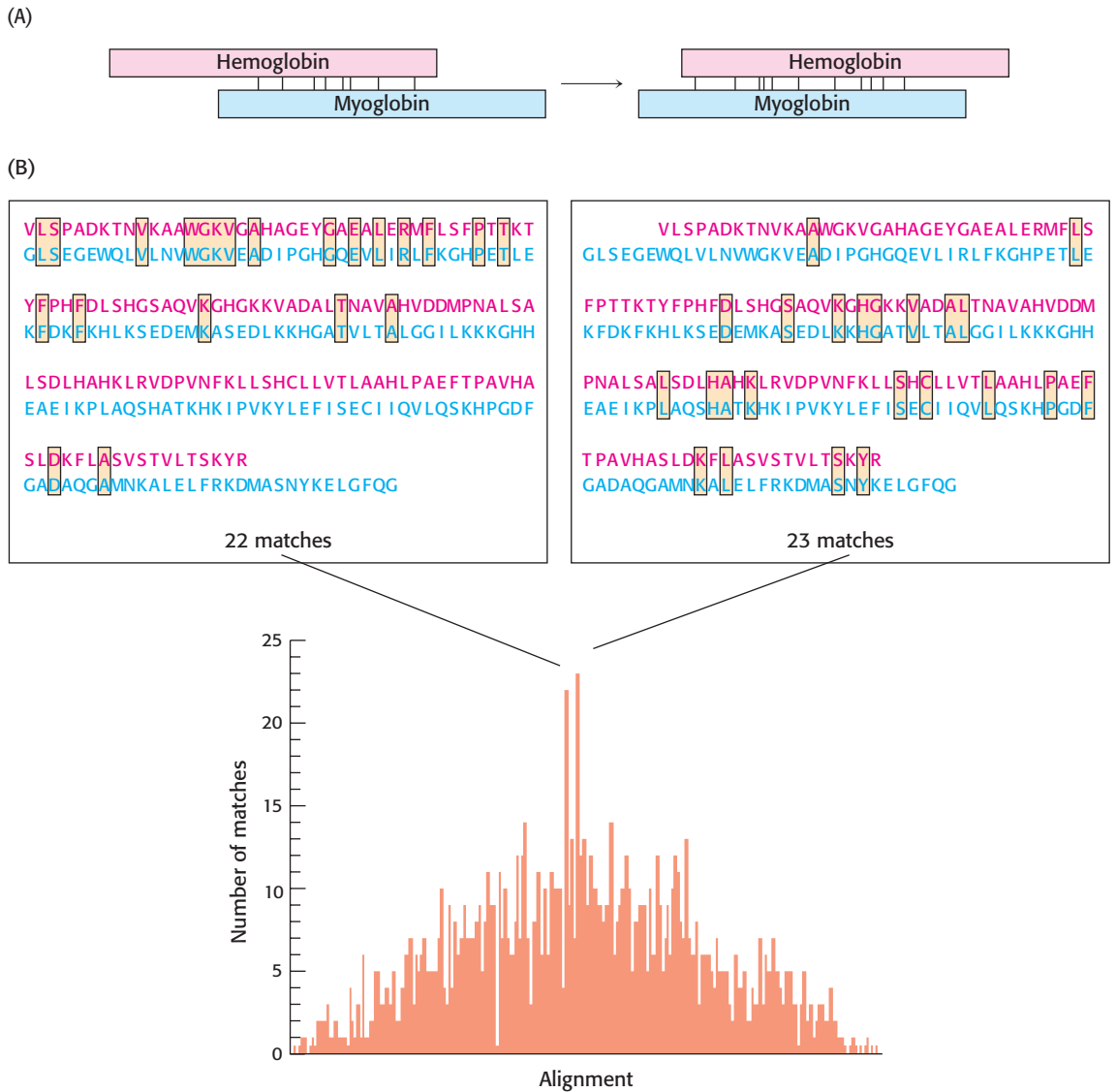


FIGURE 7.5 Comparing the amino acid sequences of hemoglobin α and myoglobin. (A) A comparison is made by sliding the sequences of the two proteins past one another, one amino acid at a time, and counting the number of amino acid identities between the proteins. (B) The two alignments with the largest number of matches are shown above the graph, which plots the matches as a function of alignment.

How can we tell where to align the two sequences? The simplest approach is to compare all possible juxtapositions of one protein sequence with another, in each case recording the number of identical residues that are aligned with one another. This comparison can be accomplished by simply sliding one sequence past the other, one amino acid at a time, and counting the number of matched residues (Figure 7.5).

For hemoglobin α and myoglobin, the best alignment reveals 23 sequence identities, spread throughout the central parts of the sequences. However, a nearby alignment showing 22 identities is nearly as good. In this alignment, the identities are concentrated toward the amino-terminal end of the sequences. The sequences can be aligned to capture most of the identities in *both* alignments by introducing a *gap* into one of the sequences (Figure 7.6). Such gaps must often be inserted to compensate for the insertions or deletions of nucleotides that may have taken place in the gene for one molecule but not the other in the course of evolution.

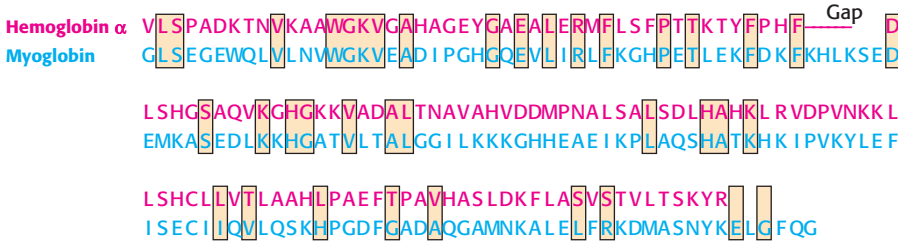


FIGURE 7.6 Alignment with gap insertion. The alignment of hemoglobin α and myoglobin after a gap has been inserted into the hemoglobin α sequence.

The use of gaps substantially increases the complexity of sequence alignment because, in principle, the insertion of gaps of arbitrary sizes must be considered throughout each sequence. However, methods have been developed for the insertion of gaps in the automatic alignment of sequences. These methods use scoring systems to compare different alignments, and they include penalties for gaps to prevent the insertion of an unreasonable number of them. Here is an example of such a scoring system: each identity between aligned sequences results in +10 points, whereas each gap introduced, regardless of size, results in -25 points. For the alignment shown in Figure 7.6, there are 38 identities and 1 gap, producing a score of $(38 \times 10 - 1 \times 25 = 355)$. Overall, there are 38 matched amino acids in an average length of 147 residues; so the sequences are 25.9% identical. The next step is to ask, Is this percentage of identity significant?

7.2.1 The Statistical Significance of Alignments Can Be Estimated by Shuffling

The similarities in sequence in Figure 7.5 appear striking, yet there remains the possibility that a grouping of sequence identities has occurred by chance alone. How can we estimate the probability that a specific series of identities is a chance occurrence? To make such an estimate, the amino acid sequence in one of the proteins is “shuffled”—that is, randomly rearranged—and the alignment procedure is repeated (Figure 7.7). This process is repeated to build up a distribution showing, for each possible score, the number of shuffled sequences that received that score.

When this procedure is applied to the sequences of myoglobin and hemoglobin α , the authentic alignment clearly stands out (Figure 7.8). Its score is far above the mean for the alignment scores based on shuffled sequences. The odds of such a deviation occurring owing due to chance alone are

THISISTHEAUTHENTICSEQUENCE

↓ Shuffling

SNUCSNSEATEEITUHQIHHTTCEI

FIGURE 7.7 The generation of a shuffled sequence.

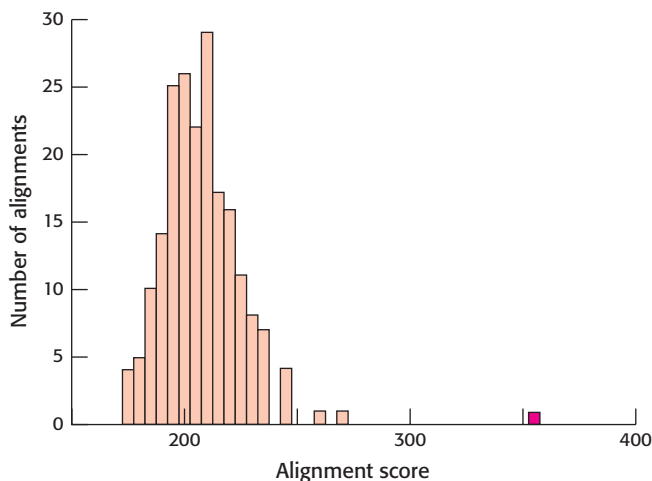


FIGURE 7.8 Statistical comparison of alignment scores. Alignment scores are calculated for many shuffled sequences, and the number of sequences generating a particular score is plotted against the score. The resulting plot is a distribution of alignment scores occurring by chance. The alignment score for hemoglobin α and myoglobin (shown in red) is substantially greater than any of these scores, strongly suggesting that the sequence similarity is significant.

approximately 1 in 10^{20} . Thus, we can comfortably conclude that the two sequences are genuinely similar; the simplest explanation for this similarity is that these sequences are homologous—that is, that the two molecules have descended by divergence from a common ancestor.

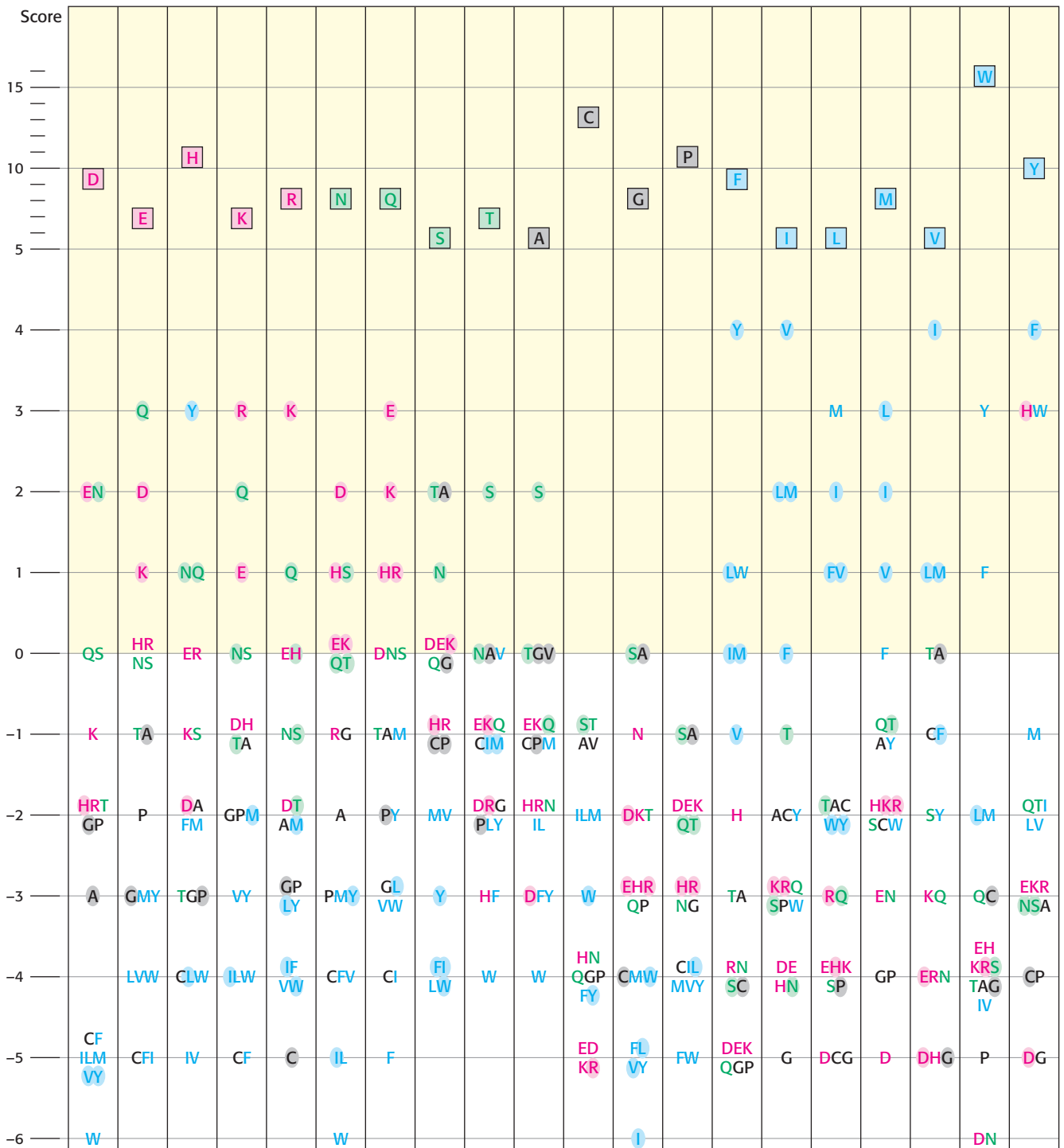


FIGURE 7.9 A graphic view of the Blosum-62 substitution matrix. This scoring scheme was derived by examining substitutions that occur within aligned sequence blocks in related proteins. Amino acids are classified into four groups (charged, red; polar, green; large and hydrophobic, blue; other, black). Substitutions that require the change of only a single nucleotide are shaded. To find the score for a substitution of, for instance, a Y for an H, you find the Y in the column having H (boxed) at the top and check the number at the left. In this case, the resulting score is 3.

7.2.2 Distant Evolutionary Relationships Can Be Detected Through the Use of Substitution Matrices

The scoring scheme in Section 7.2.1 assigns points only to positions occupied by identical amino acids in the two sequences being compared. No credit is given for any pairing that is not an identity. However, not all substitutions are equivalent. Some are structurally *conservative substitutions*, replacing one amino acid with another that is similar in size and chemical properties. Such conservative amino acid substitutions may have relatively minor effects on protein structure and can thus be tolerated without compromising function. In other substitutions, an amino acid replaces one that is dissimilar. Furthermore, some amino acid substitutions result from the replacement of only a single nucleotide in the gene sequence; whereas others require two or three replacements. Conservative and single-nucleotide substitutions are likely to be more common than are substitutions with more radical effects. How can we account for the type of substitution when comparing sequences? We can approach this problem by first examining the substitutions that have actually taken place in evolutionarily related proteins.

From the examination of appropriately aligned sequences, *substitution matrices* can be deduced. In these matrices, a large positive score corresponds to a substitution that occurs relatively frequently, whereas a large negative score corresponds to a substitution that occurs only rarely. The Blossum-62 substitution matrix illustrated in Figure 7.9 is an example. The highest scores in this substitution matrix indicate that amino acids such as cysteine (C) and tryptophan (W) tend to be conserved more than those such as serine (S) and alanine (A). Furthermore, structurally conservative substitutions such as lysine (K) for arginine (R) and isoleucine (I) for valine (V) have relatively high scores. When two sequences are compared, each substitution is assigned a score based on the matrix. In addition, a gap penalty is often assigned according to the size of the gap. For example, the introduction of a gap lowers the alignment score by 12 points and the extension of an existing gap costs 2 points per residue. Using this scoring system, the alignment shown in Figure 7.6 receives a score of 115. In many regions, most substitutions are conservative (defined as those substitutions with scores greater than 0) and relatively few are strongly disfavored types (Figure 7.10).

This scoring system detects homology between less obviously related sequences with greater sensitivity than would a comparison of identities only. Consider, for example, the protein leghemoglobin, an oxygen-binding protein found in the roots of some plants. The amino acid sequence of leghemoglobin from the herb lupine can be aligned with that of human myoglobin and scored by using either the simple scoring scheme based on identities only or the Blossum-62 scoring matrix (see Figure 7.9). Repeated

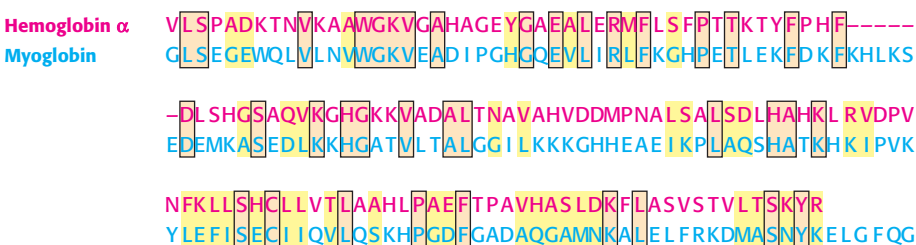
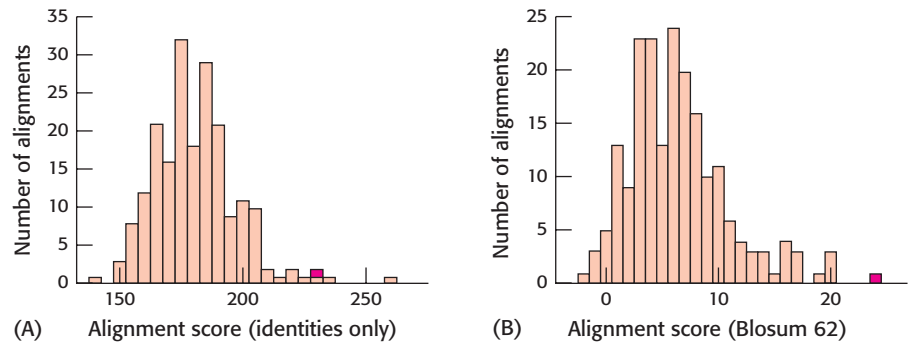


FIGURE 7.10 Alignment with conservative substitutions noted. The alignment of hemoglobin α and myoglobin with conservative substitutions indicated by yellow shading and identities by orange.

FIGURE 7.11 Alignment of identities only versus the Blossum 62 matrix. Repeated shuffling and scoring reveal the significance of sequence alignment for human myoglobin versus lupine leghemoglobin with the use of either (A) the simple, identity-based scoring system or (B) the Blossum-62 matrix. The scores for the alignment of the authentic sequences are shown in red. The Blossum matrix provides greater statistical power.



shuffling and scoring provides a distribution of alignment scores (Figure 7.11). Scoring based on identities only indicates that the odds of the alignment between myoglobin and leghemoglobin occurring by chance alone are 1 in 20. Thus, although the level of similarity suggests a relationship, there is a 5% chance that the similarity is accidental on the basis of this analysis. In contrast, users of the substitution matrix are able to incorporate the effects of conservative substitutions. From such an analysis, the odds of the alignment occurring by chance are calculated to be approximately 1 in 300. Thus, an analysis performed by using the substitution matrix reaches a much firmer conclusion about the evolutionary relationship between these proteins (Figure 7.12).

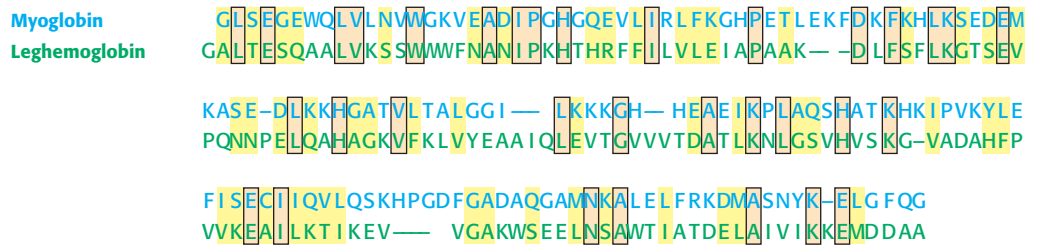


FIGURE 7.12 Alignment of human myoglobin and lupine leghemoglobin. The use of the Blossum-62 substitution matrix yields the alignment shown between human myoglobin and lupine leghemoglobin, illustrating identities (orange) and conservative substitutions (yellow). These sequences are 23% identical.

Experience with sequence analysis has led to the development of simpler rules of thumb. For sequences longer than 100 amino acids, sequence identities greater than 25% are almost certainly not the result of chance alone; such sequences are probably homologous. In contrast, if two sequences are less than 15% identical, pairwise comparison alone is unlikely to indicate statistically significant similarity. For sequences that are between 15% and 25% identical, further analysis is necessary to determine the statistical significance of the alignment. It must be emphasized that *the lack of a statistically significant degree of sequence similarity does not rule out homology*. The sequences of many proteins that have descended from common ancestors have diverged to such an extent that the relationship between the proteins can no longer be detected from their sequences alone. As we will see, such homologous proteins can often be detected by examining three-dimensional structures.

7.2.3 Databases Can Be Searched to Identify Homologous Sequences

When the sequence of a protein is first determined, comparing it with all previously characterized sequences can be a source of tremendous insight into its evolutionary relatives and, hence, its structure and function. *Indeed,*

an extensive sequence comparison is almost always the first analysis performed on a newly elucidated sequence. The sequence alignment methods heretofore described are used to compare an individual sequence with all members of a database of known sequences.


In 1995, investigators reported the first complete sequence of the genome of a free-living organism, the bacterium *Haemophilus influenzae*. Of 1743 identified open reading frames (Section 6.3.2), 1007 (58%) could be linked by sequence-comparison methods to some protein of known function that had been previously characterized in another organism. An additional 347 open reading frames could be linked to sequences in the database for which no function had yet been assigned (“hypothetical proteins”). The remaining 389 sequences did not match any sequence present in the database at the time at which the *Haemophilus influenzae* sequence was completed. Thus, investigators were able to identify likely functions for more than half the proteins within this organism solely through the use of sequence-comparison methods.

7.3 EXAMINATION OF THREE-DIMENSIONAL STRUCTURE ENHANCES OUR UNDERSTANDING OF EVOLUTIONARY RELATIONSHIPS

Sequence comparison is a powerful tool for extending our knowledge of protein function and kinship. However, biomolecules generally function as intricate three-dimensional structures rather than as linear polymers. Mutations occur at the level of sequence, but the effects of the mutations are at the level of function, and function is directly related to tertiary structure. Consequently, to gain a deeper understanding of evolutionary relationships between proteins, we must examine three-dimensional structures, especially in conjunction with sequence information. The techniques of structural determination are presented in Chapter 4.

7.3.1 Tertiary Structure Is More Conserved Than Primary Structure

Because three-dimensional structure is much more closely associated with function than is sequence, tertiary structure is more evolutionarily conserved than is primary structure. This conservation is apparent in the tertiary structures of the globins (Figure 7.13), which are extremely similar even though the similarity between human myoglobin and lupine leghemoglobin is just barely detectable at the sequence level and that between human hemoglobin (α chain) and lupine leghemoglobin is not statistically

 **FIGURE 7.13 Conservation of three-dimensional structure.** The tertiary structures of human hemoglobin (α chain), human myoglobin, and lupine leghemoglobin are conserved. Each heme group contains an iron atom to which oxygen binds.

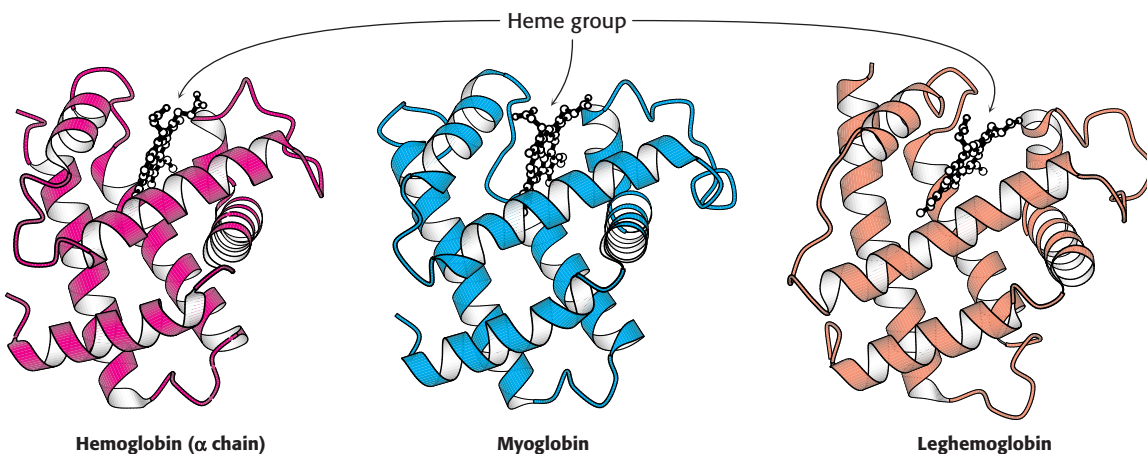
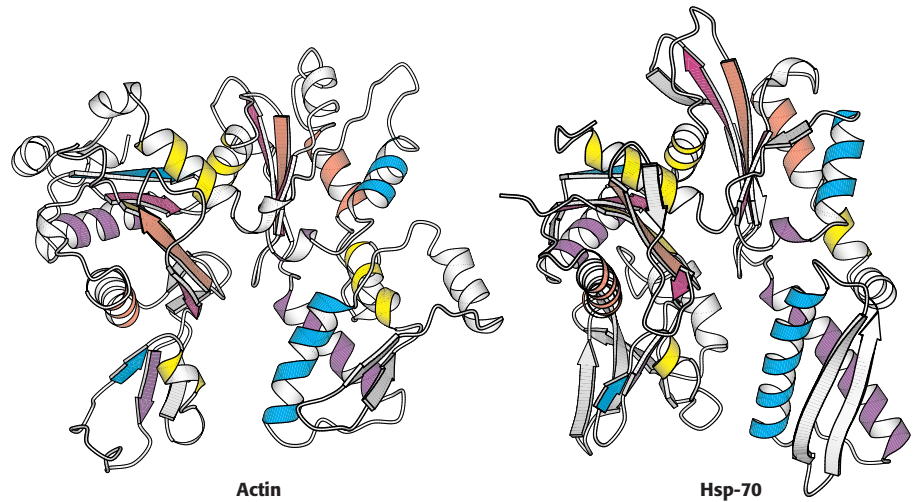




FIGURE 7.14 Structures of actin and the large fragment of heat shock protein 70 (Hsp-70). A comparison of the identically colored elements of secondary structure reveals the overall similarity in structure despite the difference in biochemical activities.



significant (15.6% identity). This structural similarity firmly establishes that the framework that binds the heme group and facilitates the reversible binding of oxygen has been conserved over a long evolutionary period.

Anyone aware of the similar biochemical functions of hemoglobin, myoglobin, and leghemoglobin could expect the structural similarities. In a growing number of other cases, however, a comparison of three-dimensional structures has revealed striking similarities between proteins that were *not* expected to be related. A case in point is the protein actin, a major component of the cytoskeleton, and heat shock protein 70 (Hsp-70), which assists protein folding inside cells. These two proteins were found to be noticeably similar in structure despite only 15.6% sequence identity (Figure 7.14). On the basis of their three-dimensional structures, actin and Hsp-70 are paralogs. The level of structural similarity strongly suggests that, despite their different biological roles in modern organisms, these proteins descended from a common ancestor. As the three-dimensional structures of more proteins are determined, such unexpected kinships are being discovered with increasing frequency. The search for such kinships relies ever more frequently on computer-based search procedures that allow the three-dimensional structure of any protein to be compared with all other known structures.

7.3.2 Knowledge of Three-Dimensional Structures Can Aid in the Evaluation of Sequence Alignments

The sequence-comparison methods described thus far treat all positions within a sequence equally. However, examination of families of homologous proteins for which at least one three-dimensional structure is known has revealed that regions and residues critical to protein function are more strongly conserved than are other residues. For example, each type of globin contains a bound heme group with an iron atom at its center. A histidine residue that interacts directly with this iron (residue 64 in human myoglobin) is conserved in all globins. After we have identified key residues or highly conserved sequences within a family of proteins, we can sometimes identify other family members even when the overall level of sequence similarity is below statistical significance. Thus, the generation of *sequence templates*—conserved residues that are structurally and functionally important and are characteristic of particular families of proteins—can be useful for recognizing new family members that might be undetectable by other means. A variety of other methods for sequence classification that take advantage of known three-dimensional structures also are being developed. Still other

methods are able to identify relatively conserved residues within a family of homologous proteins, even without a known three-dimensional structure. These methods are proving to be powerful in identifying distant evolutionary relationships.

7.3.3 Repeated Motifs Can Be Detected by Aligning Sequences with Themselves

More than 10% of all proteins contain sets of two or more domains that are similar to one another. The afore-described sequence search methods can often detect internally repeated sequences that have been characterized in other proteins. Where repeated units do not correspond to previously identified domains, their presence can be detected by attempting to align a given sequence with itself. This alignment is most easily visualized with the use of a *self-diagonal plot*. Here, the protein sequence is displayed on both the vertical and the horizontal axes, running from amino to carboxyl terminus; a dot is placed at each point in the space defined by the axes at which the amino acid directly below along the horizontal axis is the same as that directly across along the vertical axis. The central diagonal represents the sequence aligned with itself. Internal repeats are manifested as lines of dots parallel to the central diagonal, illustrated by the plot in Figure 7.15 prepared for the TATA-box-binding protein, a key protein in the initiation of gene transcription (Section 28.x.x).

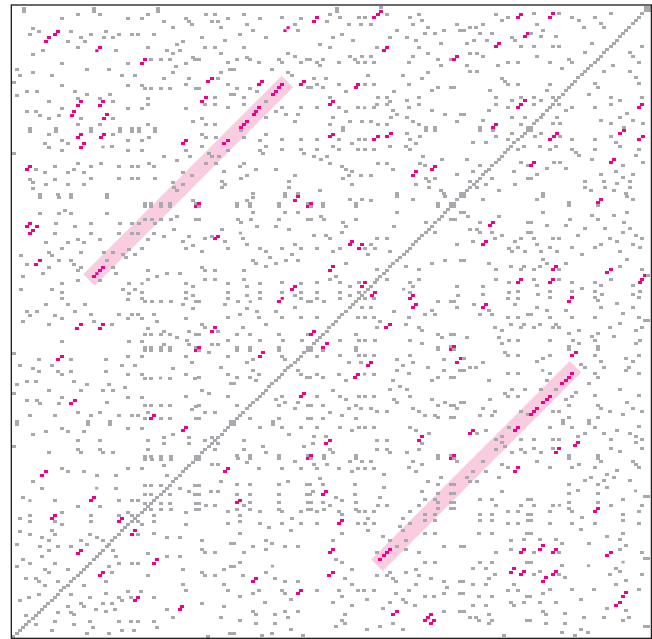


FIGURE 7.15 A self-diagonal plot for the TATA-box-binding protein from the plant *Arabidopsis*. Self-diagonal plots are used to search for amino acid sequence repeats within a protein. The central diagonal is the sequence aligned with itself. Red dots indicating a correspondence of amino acids appear where two or more amino acids in a row match. Lines of dots, highlighted in pink, parallel to the central diagonal suggest an internal repeat.

The statistical significance of such repeats can be tested by aligning the regions in question as if these regions were sequences from separate proteins. For the TATA-box-binding protein, the alignment is highly significant: 30% of the amino acids are identical over 90 residues (Figure 7.16A). The estimated probability of such an alignment occurring by chance is 1 in 10^{13} . The determination of the three-dimensional structure of the TATA-box-binding protein confirmed the presence of repeated structures; the protein is formed of two nearly identical domains (Figure 7.16B). The evidence is convincing that the gene encoding this protein evolved by duplication of a gene encoding a single domain.

(A)

1	MTDQGLEGNPVDLSKHPS
20	GIVPTLQNIIVSTVNLDCCKLDLKAIALQ-ARNAEYNPKRFAAVIMRI R
110	FKDFKIQNIIVGSCDVKFPRLLEGLAYSHAAFFSSYEPELFPGLIYRMK
66	EPKTTALIFASGKMWCTGAKSEDFSKMAARKYARIVQKLGFPK
157	VPKIVLLIFVSGKIVITGAKMRDETYKAFENIYPLSEFRKIQQ

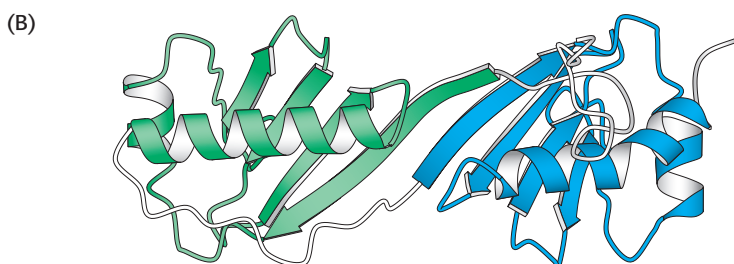


FIGURE 7.16 Sequence alignment of internal repeats. (A) An alignment of the sequences of the two repeats of the TATA-box-binding protein. The amino-terminal repeat is shown in green and the carboxyl-terminal repeat in blue. (B) Structure of the TATA-box-binding protein. The amino-terminal domain is shown in green and the carboxyl-terminal domain in blue.

7.3.4 Convergent Evolution: Common Solutions to Biochemical Challenges

Thus far, we have been exploring proteins derived from common ancestors—that is, through *divergent evolution*. In other cases, clear examples have been found of proteins that are structurally similar in important ways but are not descended from a common ancestor. How might two unrelated proteins come to resemble each other structurally? Two proteins evolving independently may have converged on a similar structure in order to perform a similar biochemical activity. Perhaps that structure was an especially effective solution to a biochemical problem that organisms face. The process by which very different evolutionary pathways lead to the same solution is called *convergent evolution*.

One example of convergent evolution is found among the serine proteases. These enzymes, to be discussed in more detail in Chapter 9, cleave peptide bonds by hydrolysis. Figure 7.17 shows for two such enzymes the structure of the active sites—that is, the sites on the proteins at which the hydrolysis reaction takes place. These active-site structures are remarkably similar. In each case, a serine residue, a histidine residue, and an aspartic acid residue are positioned in space in nearly identical arrangements. As we will see, this is the case because chymotrypsin and subtilisin use the same mechanistic solution to the problem of peptide hydrolysis. At first glance, this similarity might suggest that these proteins are homologous. However, striking differences in the overall structures of these proteins make an evolutionary relationship extremely unlikely (Figure 7.18). Whereas chymotrypsin consists almost entirely of β sheets, subtilisin contains extensive α -helical structure. Moreover, the key serine, histidine, and aspartic acid residues do not occupy similar positions or even appear in the same order within the two sequences. It is extremely unlikely that two proteins evolving from a common ancestor could have retained similar active-site structures while other aspects of the structure changed so dramatically.

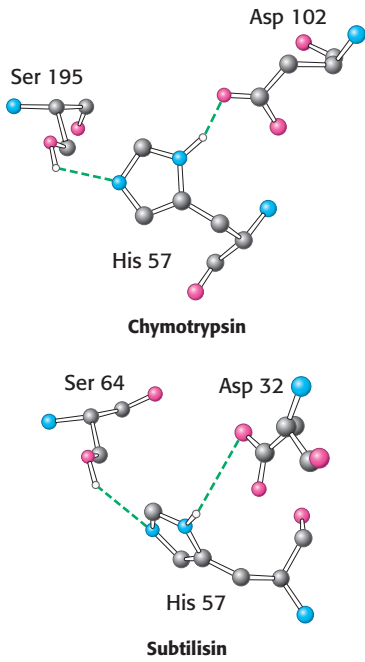


FIGURE 7.17 Convergent evolution of protease active sites. The relative positions of the three key residues shown are nearly identical in the active sites of the serine proteases chymotrypsin and subtilisin.

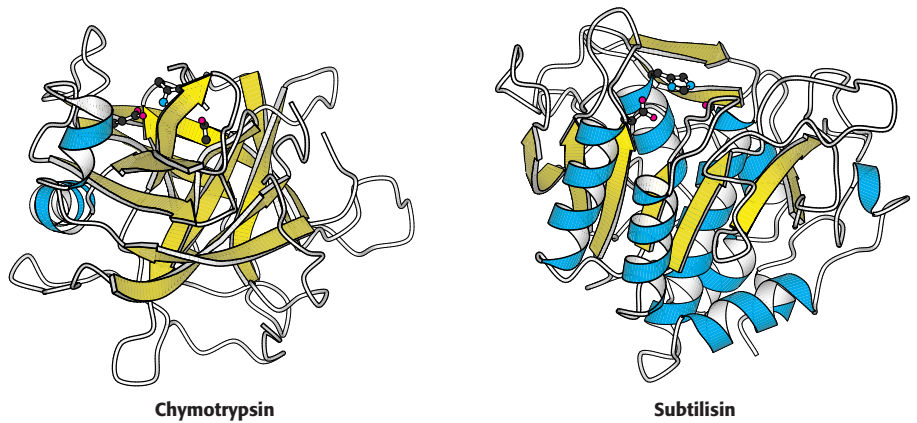


FIGURE 7.18 Structures of chymotrypsin and subtilisin. The β strands are shown in yellow and α helices in blue. The overall structures are quite dissimilar, in stark contrast with the active sites, shown at the top of each structure.

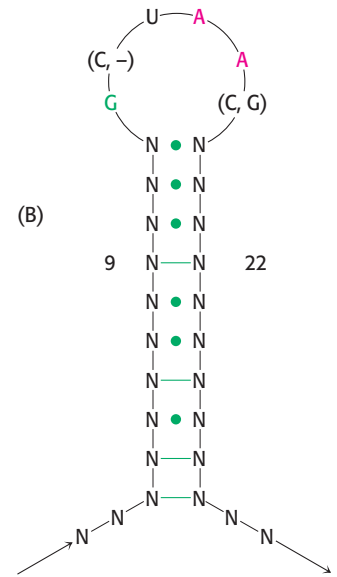
7.3.5 Comparison of RNA Sequences Can Be a Source of Insight into Secondary Structures

A comparison of homologous RNA sequences can be a source of important insights into evolutionary relationships in a manner similar to that already described. In addition, such comparisons provide clues to the three-dimensional structure of the RNA itself. As noted in Chapter 5, single-stranded nucleic acid molecules fold back on themselves to form elaborate structures held together by Watson-Crick base-pairing and other interactions. In a family of sequences that form such base-paired structures, base sequences may vary, but base-pairing ability is conserved. Consider, for example, a region from a large RNA molecule present in the ribosomes of

all organisms (Figures 7.19). In the region shown, the *E. coli* sequence has a guanine (G) residue in position 9 and a cytosine (C) residue in position 22, whereas the human sequence has uracil (U) in position 9 and adenine (A) in position 22. Examination of the six sequences shown in Figure 7.20 (and many others) reveals that the bases in positions 9 and 22 retain the ability to form a Watson-Crick base pair even though the identities of the bases in these positions vary. Base-pairing ability is also conserved in neighboring positions; we can deduce that two segments with such compensating mutations are likely to form a double helix. Where sequences are known for several homologous RNA molecules, this type of sequence analysis can often suggest complete secondary structures as well as some additional interactions.

		↓ 9	↓ 22
BACTERIA	<i>Escherichia coli</i>	C A C A C G G C G G G U G C U A A C G U C C G U C G U G A A	
	<i>Pseudomonas aeruginosa</i>	A C C A C G G C G G G U G C U A A C G U C C G U C G U G A A	
ARCHAEA	<i>Halobacterium halobium</i>	C C G G U G U G C G G G G - U A A G C C U G U G C A C C G U	
	<i>Methanococcus vannielii</i>	G A G G G C A U A C G G G - U A A G C U G U A U G U C C G A	
EUKARYA	<i>Homo sapiens</i>	G G G C C A C U U U U G G - U A A G C A G A A C U G G C G C	
	<i>Saccharomyces cerevisiae</i>	G G G C C A U U U U U G G - U A A G C A G A A C U G G C G A	

FIGURE 7.19 Comparison of RNA sequences. (A) A comparison of sequences in a part of ribosomal RNA taken from a variety of species. (B) The implied secondary structure. Bars indicate positions at which Watson-Crick base-pairing is completely conserved in the sequences shown, whereas dots indicate positions at which Watson-Crick base-pairing is conserved in most cases.



7.4 EVOLUTIONARY TREES CAN BE CONSTRUCTED ON THE BASIS OF SEQUENCE INFORMATION



CONCEPTUAL INSIGHTS, Sequence Analysis, offers insights into evolutionary trees through interactive analysis of simulated evolutionary histories.

The observation that homology is often manifested as sequence similarity suggests that the evolutionary pathway relating the members of a family of proteins may be deduced by examination of sequence similarity. This approach is based on the notion that sequences that are more similar to one another have had less evolutionary time to diverge from one another than have sequences that are less similar. This method can be illustrated by using the three globin sequences in Figures 7.10 and 7.12, as well as the sequence for the human hemoglobin β chain. These sequences can be aligned with the additional constraint that gaps, if present, should be at the same positions in all of the proteins. These aligned sequences can be used to construct an *evolutionary tree* in which the length of the branch connecting each pair of proteins is proportional to the number of amino acid differences between the sequences (Figure 7.20).

Such comparisons reveal only the relative divergence times—for example, that myoglobin diverged from hemoglobin twice as long ago as the α chain diverged from the β chain. How can we estimate the approximate dates of gene duplications and other evolutionary events? Evolutionary

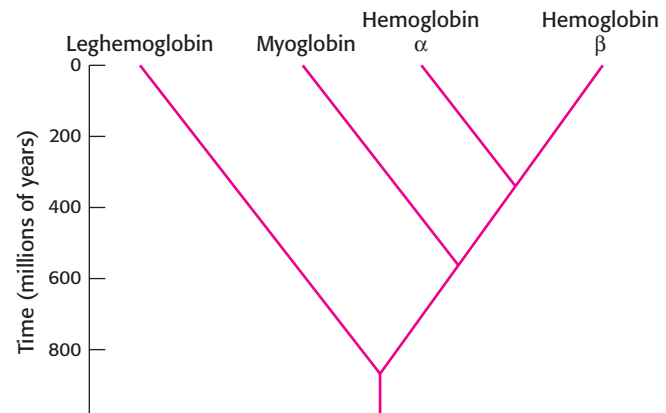


FIGURE 7.20 An evolutionary tree for globins. The branching structure was deduced by sequence comparison, whereas the results of fossil studies provided the overall time scale showing when divergence occurred.



FIGURE 7.21 The lamprey. A jawless fish whose ancestors diverged from bony fish approximately 400 million years ago, the lamprey contains hemoglobin molecules that contain only a single type of polypeptide chain. [Brent P. Kent.]

trees can be calibrated by comparing the deduced branch points with divergence times determined from the fossil record. For example, the duplication leading to the two chains of hemoglobin appears to have occurred 350 million years ago. This estimate is supported by the observation that jawless fish such as the lamprey, which diverged from bony fish approximately 400 million years ago, contain hemoglobins built from a single type of subunit (Figure 7.21).

These methods can be applied to both relatively modern and very ancient molecules, such as the ribosomal RNAs that are found in all organisms. Indeed, it was such an RNA sequence analysis that led to the suggestion that Archaea are a distinct group of organisms that diverged from Bacteria very early in evolutionary history.

7.5 MODERN TECHNIQUES MAKE THE EXPERIMENTAL EXPLORATION OF EVOLUTION POSSIBLE

Two techniques of biochemistry have made it possible to examine the course of evolution more directly and not simply by inference. The polymerase chain reaction (Section 6.1.5) allows the direct examination of ancient DNA sequences, releasing us, at least in some cases, from the constraints of being able to examine existing genomes from living organisms only. Molecular evolution may be investigated through the use of *combinatorial chemistry*, the process of producing large populations of molecules en masse and selecting for a biochemical property. This exciting process provides a glimpse into the types of molecules that may have existed in the RNA world.

7.5.1 Ancient DNA Can Sometimes Be Amplified and Sequenced

The tremendous chemical stability of DNA (Section 2.2.7) makes the molecule well suited to its role as the storage site of genetic information. So stable is the molecule that samples of DNA have survived for many thousands of years under appropriate conditions. With the development of PCR methods, such ancient DNA can sometimes be amplified and sequenced. This approach has been applied to mitochondrial DNA from a Neanderthal fossil estimated at between 30,000 and 100,000 years of age found near Düsseldorf, Germany, in 1956. Investigators managed to identify a total of 379 bases of sequence. Comparison with a number of the corresponding sequences from *Homo sapiens* revealed between 22 and 36 substitutions, considerably fewer than the average of 55 differences between human beings and chimpanzees over the common bases in this region. Further analysis suggested that the common ancestor of modern human beings and Neanderthals lived approximately 600 million years ago. An evolutionary tree constructed by using these and other data revealed that the Neanderthal was not an intermediate between chimpanzees and human beings but, instead, was an evolutionary “dead end” that became extinct (Figure 7.22).

Note that earlier studies describing the sequencing of much more ancient DNA such as that found in insects trapped in amber appear to have been flawed; contaminating modern DNA was responsible for the sequences determined. Successful sequencing of ancient DNA requires sufficient DNA for reliable amplification and the rigorous exclusion of all sources of contamination.

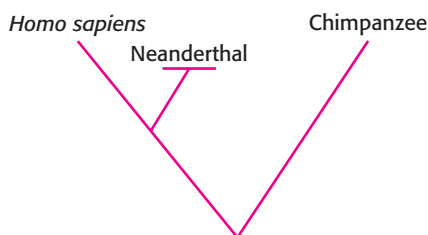


FIGURE 7.22 Placing Neanderthal on an evolutionary tree. Comparison of DNA sequences revealed that Neanderthal is not on the line of direct descent leading to *Homo sapiens* but, instead, branched off earlier and then became extinct.

7.5.2 Molecular Evolution Can Be Examined Experimentally

Evolution requires three processes: (1) the generation of a diverse population, (2) the selection of members based on some criterion of fitness, and (3) reproduction to enrich the population in more fit members (Section 2.2).

Nucleic acid molecules are capable of undergoing all three processes *in vitro* under appropriate conditions. The results of such studies enable us to glimpse how evolutionary processes might have generated catalytic activities and specific binding abilities—important biochemical functions in all living systems.

A diverse population of nucleic acid molecules can be synthesized in the laboratory by the process of combinatorial chemistry, which rapidly produces large populations of a particular type of molecule such as a nucleic acid. A population of molecules of a given size can be generated randomly so that many or all possible sequences are present in the mixture. When an initial population has been generated, it is subjected to a selection process that isolates specific molecules with desired binding or reactivity properties. Finally, molecules that have survived the selection process are allowed to reproduce through the use of PCR; primers are directed toward specific sequences included at the ends of each member of the population.

As an example of this approach, consider an experiment that set a goal of creating an RNA molecule capable of binding adenosine triphosphate and related nucleotides. Such ATP-binding molecules are of interest because they might have been present in the RNA world. An initial population of RNA molecules 169 nucleotides long was created; 120 of the positions differed randomly, with equimolar mixtures of adenine, cytosine, guanine, and uracil. The initial synthetic pool that was used contained approximately 10^{14} RNA molecules. Note that this number is a very small fraction of the total possible pool of random 120-base sequences. From this pool, those molecules that bound to ATP, which had been immobilized on a column, were selected (Figure 7.23).

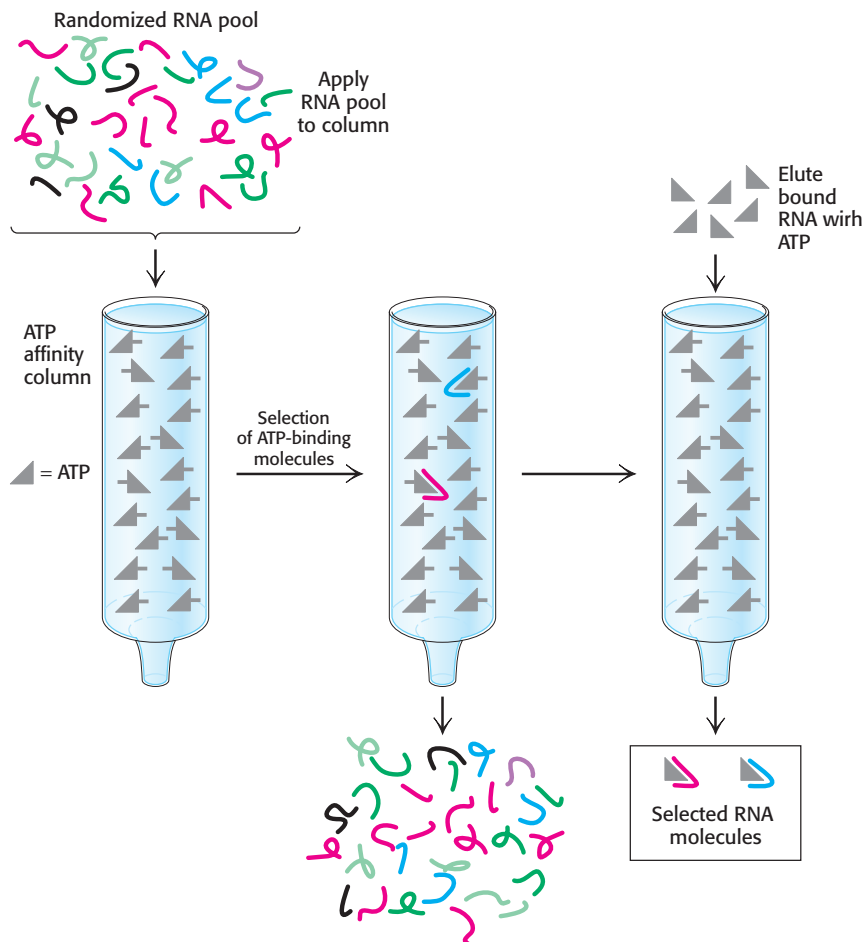


FIGURE 7.23 Evolution in the laboratory.

A collection of RNA molecules of random sequences is synthesized by combinatorial chemistry. This collection is selected for the ability to bind ATP by passing the RNA through an ATP affinity column (Section 4.1.3). The ATP-binding RNA molecules are released from the column by washing with excess ATP, and replicated. The process of selection and replication is then repeated several times. The final RNA products with significant ATP-binding ability are isolated and characterized.

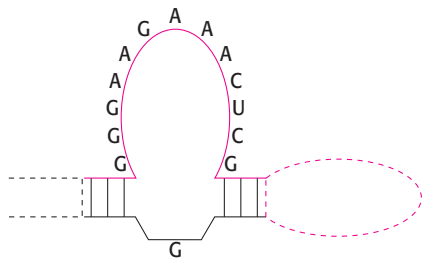


FIGURE 7.24 A conserved secondary structure. The secondary structure shown is common to RNA molecules selected for ATP binding.

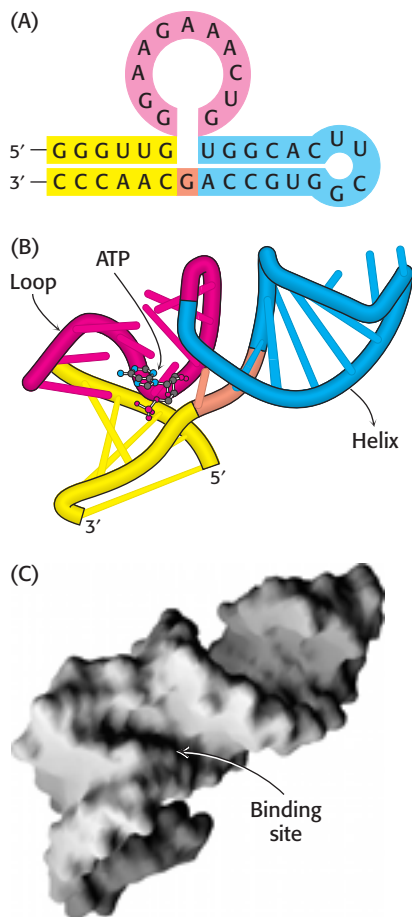


FIGURE 7.25 RNA molecule binds ATP. (A) The Watson-Crick base-pairing pattern, (B) the folding pattern, and (C) a surface representation of an RNA molecule selected to bind adenosine nucleotides. The bound ATP is shown in part B, and the binding site is revealed as a deep pocket in part C.

The collection of molecules that were bound well by the ATP affinity column was allowed to reproduce by reverse transcription into DNA, amplification by PCR, and transcription back into RNA. This new population was subjected to additional rounds of selection for ATP-binding activity. After eight generations, members of the selected population were characterized by sequencing. Seventeen different sequences were obtained, 16 of which could form the structure shown in Figure 7.24. Each of these molecules bound ATP with high affinity, as indicated by dissociation constants less than $50 \mu\text{M}$.

The folded structure of the ATP-binding region from one of these RNAs was determined by nuclear magnetic resonance (Section 4.5.1) methods (Figure 7.25). As expected, this 40-nucleotide molecule is composed of two Watson-Crick base-paired helical regions separated by an 11-nucleotide loop. This loop folds back on itself in an intricate way to form a deep pocket into which the adenine ring can fit. Thus, a structure was generated, or evolved, that was capable of a specific interaction.

SUMMARY

• Homologs Are Descended from a Common Ancestor

Exploring evolution biochemically often means searching for homology, or relatedness, between molecules, because homologous molecules, or homologs, evolved from a common ancestor. Paralogs are homologous molecules that are found in one species and have acquired different functions through evolutionary time. Orthologs are homologous molecules that are found in different species and have similar or identical functions.

• Statistical Analysis of Sequence Alignments Can Detect Homology

Protein and nucleic acid sequences are two of the primary languages of biochemistry. Sequence-alignment methods are the most powerful tools of the evolutionary detective. Sequences can be aligned to maximize their similarity, and the significance of these alignments can be judged by statistical tests. The detection of a statistically significant alignment between two sequences strongly suggests that two sequences are related by divergent evolution from a common ancestor. The use of substitution matrices makes the detection of more distant evolutionary relationships possible. Any sequence can be used to probe sequence databases to identify related sequences present in the same organism or in other organisms.

• Examination of Three-Dimensional Structure Enhances Our Understanding of Evolutionary Relationships

The evolutionary kinship between proteins may be even more profoundly evident in the conserved three-dimensional structures. The analysis of three-dimensional structure in combination with analysis of especially conserved sequences has made it possible to determine evolutionary relationships that are not possible to detect by other means. Sequence-comparison methods can also be used to detect imperfectly repeated sequences within a protein, indicative of linked similar domains.

• Evolutionary Trees Can Be Constructed on the Basis of Sequence Information

Construction of an evolutionary tree based on sequence comparisons revealed approximate times for the gene duplication events separating myoglobin and hemoglobin as well as the α and β subunits of hemoglobin. Evolutionary trees based on sequences can be compared to those based on fossil records.

The exploration of evolution can also be a laboratory science. In favorable cases, PCR amplification of well-preserved samples allows the determination of the nucleotide sequences from extinct organisms. Sequences so determined can help authenticate aspects of an evolutionary tree constructed by other means. Molecular evolutionary experiments performed in the test tube can examine how molecules such as ligand-binding RNA molecules might have been generated.

KEY TERMS

homolog (p. 173)

paralog (p. 173)

ortholog (p. 173)

sequence alignment (p. 173)

conservative substitution (p. 177)

substitution matrix (p. 177)

sequence template (p. 180)

self-diagonal plot (p. 181)

divergent evolution (p. 182)

convergent evolution (p. 182)

evolutionary tree (p. 183)

combinatorial chemistry (p. 184)

SELECTED READINGS

Book

Doolittle, R. F., 1987. *Of UFS and ORFS*. University Science Books.

Sequence alignment

Henikoff, S., and Henikoff, J. G., 1992. Amino acid substitution matrices from protein blocks. *Proc. Natl. Acad. Sci. U.S.A.* 89: 10915–10919.

Johnson, M. S., and Overington, J. P., 1993. A structural basis for sequence comparisons: An evaluation of scoring methodologies. *J. Mol. Biol.* 233:716–738.

Aravind, L., and Koonin, E. V., 1999. Gleaning non-trivial structural, functional and evolutionary information about proteins by iterative database searches. *J. Mol. Biol.* 287:1023–1040.

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J., 1997. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Res.* 25:3389–3402.

Structure comparison

Bashford, D., Chothia, C., and Lesk, A. M., 1987. Determinants of a protein fold: Unique features of the globin amino acid sequences. *J. Mol. Biol.* 196:199–216.

Harutyunyan, E. H., Safonova, T. N., Kuranova, I. P., Popov, A. N., Teplyakov, A. V., Obmolova, G. V., Rusakov, A. A., Vainshtein, B. K., Dodson, G. G., Wilson, J. C., et al., 1995. The structure of deoxy- and oxy-leghaemoglobin from lupin. *J. Mol. Biol.* 251:104–115.

Flaherty, K. M., McKay, D. B., Kabsch, W., and Holmes, K. C., 1991. Similarity of the three-dimensional structures of actin and the ATPase fragment of a 70-kDa heat shock cognate protein. *Proc. Natl. Acad. Sci. U. S. A.* 88:5041–5045.

Murzin, A. G., Brenner, S. E., Hubbard, T., and Chothia, C., 1995. SCOP: A structural classification of proteins database for the investigation of sequences and structures. *J. Mol. Biol.* 247:536–540.

Hadley, C., and Jones, D. T., 1999. A systematic comparison of protein structure classification: SCOP, CATH and FSSP. *Structure Fold. Des.* 7:1099–1112.

Domain detection

Ploegman, J. H., Drent, G., Kalk, K. H., and Hol, W. G., 1978. Structure of bovine liver rhodanese I: Structure determination at 2.5

Å resolution and a comparison of the conformation and sequence of its two domains. *J. Mol. Biol.* 123:557–594.

Nikolov, D. B., Hu, S. H., Lin, J., Gasch, A., Hoffmann, A., Horikoshi, M., Chua, N. H., Roeder, R. G., and Burley, S. K., 1992. Crystal structure of TFIID TATA-box binding protein. *Nature* 360:40–46.

Doolittle, R. F., 1995. The multiplicity of domains in proteins. *Annu. Rev. Biochem.* 64:287–314.

Heger, A., and Holm, L., 2000. Rapid automatic detection and alignment of repeats in protein sequences. *Proteins* 41:224–237.

Evolutionary trees

Doolittle, R. F., 1992. Stein and Moore Award address. Reconstructing history with amino acid sequences. *Protein Sci.* 1:191–200.

Zukerkandl, E., and Pauling, L., 1965. Molecules as documents of evolutionary history. *J. Theor. Biol.* 8:357–366.

Ancient DNA

Krings, M., Stone, A., Schmitz, R. W., Krainitzki, H., Stoneking, M., and Pääbo, S., 1997. Neandertal DNA sequences and the origin of modern humans [see comments]. *Cell* 90:19–30.

Krings, M., Geisert, H., Schmitz, R. W., Krainitzki, H., and Pääbo, S., 1999. DNA sequence of the mitochondrial hypervariable region II from the Neandertal type specimen. *Proc. Natl. Acad. Sci. U. S. A.* 96:5581–5585.

Evolution in the laboratory

Gold, L., Polisky, B., Uhlenbeck, O., and Yarus, M., 1995. Diversity of oligonucleotide functions. *Annu. Rev. Biochem.* 64:763–797.

Wilson, D. S., and Szostak, J. W., 1999. In vitro selection of functional nucleic acids. *Annu. Rev. Biochem.* 68:611–647.

Hermann, T., and Patel, D. J., 2000. Adaptive recognition by nucleic acid aptamers. *Science* 287:820–825.

Web sites

The Protein Databank (PDB) site is the repository for three-dimensional macromolecular structures. It currently contains nearly 14,000 structures. (<http://www.rcsb.org/pdb/>)

National Center for Biotechnology Information (NCBI) contains molecular biological databases and software for analysis. (<http://www.ncbi.nlm.nih.gov/>)

PROBLEMS

1. *What's the score?* Using the identity-based scoring system (Section 7.2), calculate the score for the following alignment. Do you think the score is statistically significant?

- (1) WYLKQITRMDAEEVLLKKPTVRDGHFLVTQCESSPGEF-
 (2) WYFGKITRRESERLLLNPENPRGTFLVRESEETTKGAY-

SISVRFQDSVQ-----HFKVLRLDQNGKYYLWAVK-FN-
 CLSVSDFDNAKGLNVKHYKIRKLDSGGFYITSRTQFS-

SLNELVAYHRTASVSRTHHTILLSDMNV
 SSLQQLVAYYSKHADGLCHRLTNV

2. *Sequence and structure.* A comparison of the aligned amino acid sequences of two proteins each consisting of 150 amino acids reveals them to be only 8% identical. However, their three-dimensional structures are very similar. Are these two proteins related evolutionarily? Explain.

3. *It depends on how you count.* Consider the following two sequence alignments:

- (a) A-SNFLDIRLIG (b) ASNFLDIRLI-G
 GSNDFYEVKIMD GSNDFYEVKIMD

Which alignment has a higher score if the identity-based scoring system (Section 7.2) is used? Which alignment has a higher score if the Blosom-62 substitution matrix (Figure 7.9) is used?

4. *Discovering a new base pair.* Examine the ribosomal RNA sequences in Figure 7.19. In sequences that do not contain Watson-Crick base pairs, what base tends to be paired with G? Propose a structure for your new base pair.

5. *Overwhelmed by numbers.* Suppose that you wish to synthesize a pool of RNA molecules that contain all four bases at each of 40 positions. How much RNA must you have in grams if the pool is to have at least a single molecule of each sequence? The average molecular weight of a nucleotide is 330 g mol^{-1} .

6. *Form follows function.* The three-dimensional structure of biomolecules is more conserved evolutionarily than is sequence. Why is this the case?

7. *Shuffling.* Using the identity-based scoring system (Section 7.2), calculate the alignment score for the alignment of the following two short sequences:

- (1) ASNFLDKAGK (2) ATDYLEKAGK

Generate a shuffled version of sequence 2 by randomly reordering these 10 amino acids. Align your shuffled sequence with sequence 1 without allowing gaps, and calculate the alignment score between sequence 1 and your shuffled sequence.

8. *Interpreting the score.* Suppose that the sequences of two proteins each with 200 amino acids are aligned and that the percentage of identical residues has been calculated. How would you interpret each of the following results in regard to the possible divergence of the two proteins from a common ancestor?

- (a) 80%, (b) 50%, (c) 20%, (d) 10%

9. *A set of three.* The sequences of three proteins (A, B, and C) are compared with one another, yielding the following levels of identity:

	A	B	C
A	100%	65%	15%
B	65%	100%	55%
C	15%	55%	100%

Assume that the sequence matches are distributed relatively uniformly along each aligned sequence pair. Would you expect protein A and protein C to have similar three-dimensional structures? Explain.

10. *RNA alignment.* Sequences of an RNA fragment from five species have been determined and aligned. Propose a likely secondary structure for these fragments.

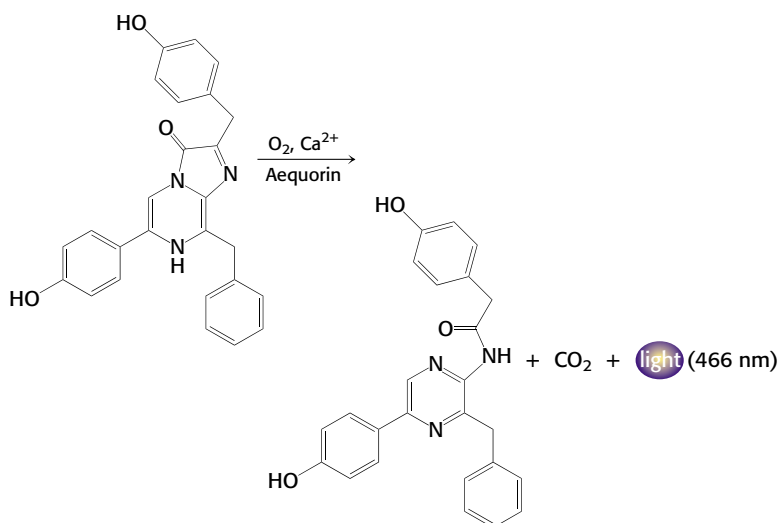
- (1) UUGGAGAUUCGGUAGAAUCUCCC
 (2) GCCGGGAAUCGACAGAUUCCCCG
 (3) CCCAAGUCCCGGCAGGGACUUAC
 (4) CUCACUGCCGAUAGGCAGGUCA
 (5) AAUACCACCCGGUAGGGUGGUUC



Media Problem

11. *Evolutionary time machine.* It has been suggested that ancestral protein sequences might be inferred from evolutionary trees of sequences that exist today. The **Conceptual Insights** module on sequence analysis allows you to try your hand at inferring ancestral sequences from model evolutionary trees. Based on this experience, explain why you would *not* expect to be able to successfully infer dinosaur sequences.

Enzymes: Basic Concepts and Kinetics



The activity of an enzyme is responsible for the glow of the luminescent jellyfish at left. The enzyme aequorin catalyzes the oxidation of a compound by oxygen in the presence of calcium to release CO_2 and light. [(Left) Fred Bavendam/Peter Arnold.]

Enzymes, the catalysts of biological systems, are remarkable molecular devices that determine the patterns of chemical transformations. They also mediate the transformation of one form of energy into another. The most striking characteristics of enzymes are their *catalytic power and specificity*. Catalysis takes place at a particular site on the enzyme called the *active site*. *Nearly all known enzymes are proteins*. However, proteins do not have an absolute monopoly on catalysis; the discovery of catalytically active RNA molecules provides compelling evidence that RNA was an early biocatalyst (Section 2.2.2).

Proteins as a class of macromolecules are highly effective catalysts for an enormous diversity of chemical reactions because of their capacity to *specifically bind a very wide range of molecules*. By utilizing the full repertoire of intermolecular forces, enzymes bring substrates together in an optimal orientation, the prelude to making and breaking chemical bonds. They catalyze reactions *by stabilizing transition states*, the highest-energy species in reaction pathways. By selectively stabilizing a transition state, an enzyme determines which one of several potential chemical reactions actually takes place.

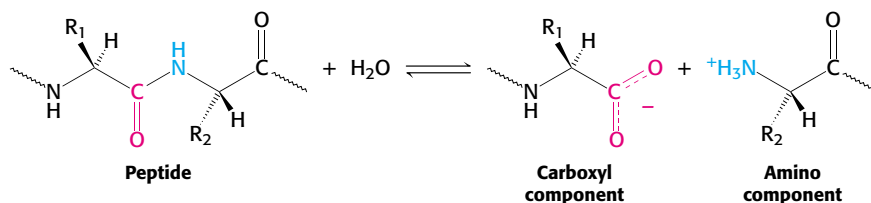
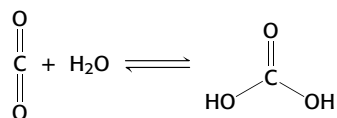
OUTLINE

- 8.1 Enzymes Are Powerful and Highly Specific Catalysts
- 8.2 Free Energy Is a Useful Thermodynamic Function for Understanding Enzymes
- 8.3 Enzymes Accelerate Reactions by Facilitating the Formation of the Transition State
- 8.4 The Michaelis-Menten Model Accounts for the Kinetic Properties of Many Enzymes
- 8.5 Enzymes Can Be Inhibited by Specific Molecules
- 8.6 Vitamins Are Often Precursors to Coenzymes

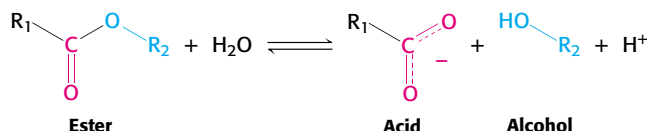
8.1 ENZYMES ARE POWERFUL AND HIGHLY SPECIFIC CATALYSTS

Enzymes accelerate reactions by factors of as much as a million or more (Table 8.1). Indeed, most reactions in biological systems do not take place at perceptible rates in the absence of enzymes. Even a reaction as simple as the hydration of carbon dioxide is catalyzed by an enzyme—namely, carbonic anhydrase (Section 9.2). The transfer of CO₂ from the tissues into the blood and then to the alveolar air would be less complete in the absence of this enzyme. In fact, carbonic anhydrase is one of the fastest enzymes known. Each enzyme molecule can hydrate 10⁶ molecules of CO₂ *per second*. This catalyzed reaction is 10⁷ times as fast as the uncatalyzed one. We will consider the mechanism of carbonic anhydrase catalysis in Chapter 9. Enzymes are highly specific both in the reactions that they catalyze and in their choice of reactants, which are called *substrates*. An enzyme usually catalyzes a single chemical reaction or a set of closely related reactions. Side reactions leading to the wasteful formation of by-products are rare in enzyme-catalyzed reactions, in contrast with uncatalyzed ones.

Let us consider *proteolytic enzymes* as an example. *In vivo*, these enzymes catalyze *proteolysis*, the hydrolysis of a peptide bond.



Most proteolytic enzymes also catalyze a different but related reaction *in vitro*—namely, the hydrolysis of an ester bond. Such reactions are more easily monitored than is proteolysis and are useful in experimental investigations of these enzymes (Section 9.1.2).



Proteolytic enzymes differ markedly in their degree of substrate specificity. Subtilisin, which is found in certain bacteria, is quite indiscriminat-

TABLE 8.1 Rate enhancement by selected enzymes

Enzyme	Nonenzymatic half-life	Uncatalyzed rate ($k_{\text{un}} \text{ s}^{-1}$)	Catalyzed rate ($k_{\text{cat}} \text{ s}^{-1}$)	Rate enhancement ($k_{\text{cat}}/k_{\text{un}}$)
OMP decarboxylase	78,000,000 years	2.8×10^{-16}	39	1.4×10^{17}
Staphylococcal nuclease	130,000 years	1.7×10^{-13}	95	5.6×10^{14}
AMP nucleosidase	69,000 years	1.0×10^{-11}	60	6.0×10^{12}
Carboxypeptidase A	7.3 years	3.0×10^{-9}	578	1.9×10^{11}
Ketosteroid isomerase	7 weeks	1.7×10^{-7}	66,000	3.9×10^{11}
Triose phosphate isomerase	1.9 days	4.3×10^{-6}	4,300	1.0×10^9
Chorismate mutase	7.4 hours	2.6×10^{-5}	50	1.9×10^6
Carbonic anhydrase	5 seconds	1.3×10^{-1}	1×10^6	7.7×10^6

Abbreviations: OMP, orotidine monophosphate; AMP, adenosine monophosphate.

Source: After A. Radzicka and R. Wofenden. *Science* 267 (1995):90–93.

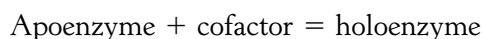
ing: it will cleave any peptide bond with little regard to the identity of the adjacent side chains. Trypsin, a digestive enzyme, is quite specific and catalyzes the splitting of peptide bonds only on the carboxyl side of lysine and arginine residues (Figure 8.1A). Thrombin, an enzyme that participates in blood clotting, is even more specific than trypsin. It catalyzes the hydrolysis of Arg–Gly bonds in particular peptide sequences only (Figure 8.1B).

DNA polymerase I, a template-directed enzyme (Section 27.x.x), is another highly specific catalyst. It adds nucleotides to a DNA strand that is being synthesized, in a sequence determined by the sequence of nucleotides in another DNA strand that serves as a template. DNA polymerase I is remarkably precise in carrying out the instructions given by the template. It inserts the wrong nucleotide into a new DNA strand less than one in a million times.

The specificity of an enzyme is due to the precise interaction of the substrate with the enzyme. This precision is a result of the intricate three-dimensional structure of the enzyme protein.

8.1.1 Many Enzymes Require Cofactors for Activity

The catalytic activity of many enzymes depends on the presence of small molecules termed *cofactors*, although the precise role varies with the cofactor and the enzyme. Such an enzyme without its cofactor is referred to as an *apoenzyme*; the complete, catalytically active enzyme is called a *holoenzyme*.



Cofactors can be subdivided into two groups: metals and small organic molecules (Table 8.2). The enzyme carbonic anhydrase, for example, requires Zn^{2+} for its activity (Section 9.2.1). Glycogen phosphorylase (Section 21.1.5), which mobilizes glycogen for energy, requires the small organic molecule pyridoxal phosphate (PLP).

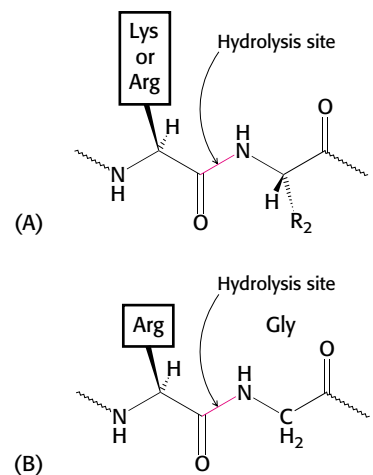


FIGURE 8.1 Enzyme specificity. (A) Trypsin cleaves on the carboxyl side of arginine and lysine residues, whereas (B) thrombin cleaves Arg–Gly bonds in particular sequences specifically.

TABLE 8.2 Enzyme cofactors

Cofactor	Enzyme
Coenzyme	
Thiamine pyrophosphate	Pyruvate dehydrogenase
Flavin adenine nucleotide	Monoamine oxidase
Nicotinamide adenine dinucleotide	Lactate dehydrogenase
Pyridoxal phosphate	Glycogen phosphorylase
Coenzyme A (CoA)	Acetyl CoA carboxylase
Biotin	Pyruvate carboxylase
5'-Deoxyadenosyl cobalamin	Methylmalonyl mutase
Tetrahydrofolate	Thymidylate synthase
Metal	
Zn^{2+}	Carbonic anhydrase
Zn^{2+}	Carboxypeptidase
Mg^{2+}	<i>EcoRV</i>
Mg^{2+}	Hexokinase
Ni^{2+}	Urease
Mo	Nitrate reductase
Se	Glutathione peroxidase
Mn^{2+}	Superoxide dismutase
K^+	Propionyl CoA carboxylase

Cofactors that are small organic molecules are called *coenzymes*. Often derived from vitamins, coenzymes can be either tightly or loosely bound to the enzyme. If tightly bound, they are called *prosthetic groups*. Loosely associated coenzymes are more like cosubstrates because they bind to and are released from the enzyme just as substrates and products are. The use of the same coenzyme by a variety of enzymes and their source in vitamins sets coenzymes apart from normal substrates, however. Enzymes that use the same coenzyme are usually mechanistically similar. In Chapter 9, we will examine the mechanistic importance of cofactors to enzyme activity. A more detailed discussion of coenzyme vitamins can be found in Section 8.6.

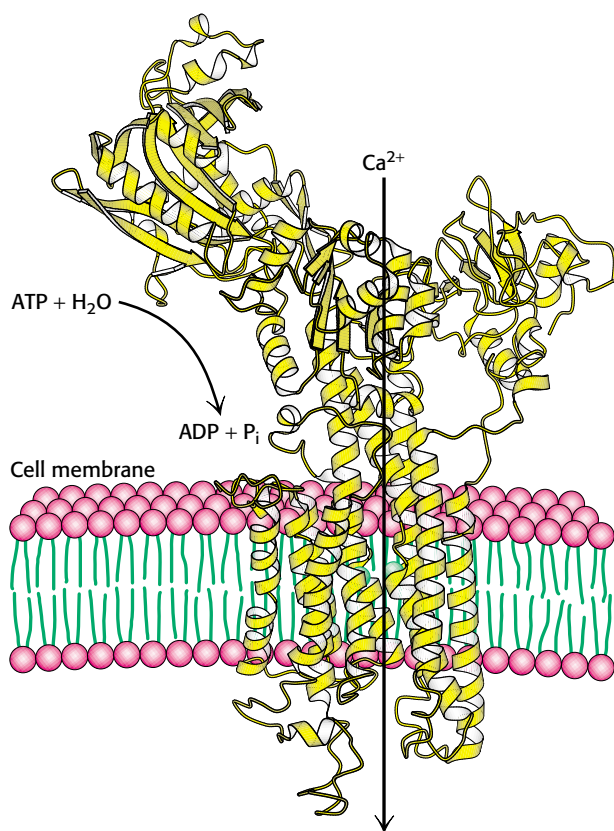


FIGURE 8.2 An energy-transforming enzyme. Ca^{2+} ATPase uses the energy of ATP hydrolysis to transport Ca^{2+} across the membrane, generating a Ca^{2+} gradient.

8.1.2 Enzymes May Transform Energy from One Form into Another

In many biochemical reactions, *the energy of the reactants is converted with high efficiency into a different form*. For example, in photosynthesis, light energy is converted into chemical-bond energy through an ion gradient. In mitochondria, the free energy contained in small molecules derived from food is converted first into the free energy of an ion gradient and then into a different currency, the free energy of adenosine triphosphate. Enzymes may then use the chemical-bond energy of ATP in many ways. The enzyme myosin converts the energy of ATP into the mechanical energy of contracting muscles. Pumps in the membranes of cells and organelles, which can be thought of as enzymes that move substrates rather than chemically altering them, create chemical and electrical gradients by using the energy of ATP to transport molecules and ions (Figure 8.2). The molecular mechanisms of these energy-transducing enzymes are being unraveled. We will see in subsequent chapters how unidirectional cycles of discrete steps—binding, chemical transformation, and release—lead to the conversion of one form of energy into another.

8.1.3 Enzymes Are Classified on the Basis of the Types of Reactions That They Catalyze

Many enzymes have common names that provide little information about the reactions that they catalyze. For example, a proteolytic enzyme secreted by the pancreas is called trypsin. Most other enzymes are named for their substrates and for the reactions that they catalyze, with the suffix “ase” added. Thus, an ATPase is an enzyme that breaks down ATP, whereas ATP synthase is an enzyme that synthesizes ATP.

To bring some consistency to the classification of enzymes, in 1964 the International Union of Biochemistry established an Enzyme Commission to develop a nomenclature for enzymes. Reactions were divided into six major groups numbered 1 through 6 (Table 8.3). These groups were subdivided and further subdivided, so that a four-digit number preceded by the letters *EC* for Enzyme Commission could precisely identify all enzymes.

Consider as an example nucleoside monophosphate (NMP) kinase, an enzyme that we will examine in detail in the next chapter (Section 9.4). It catalyzes the following reaction:

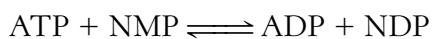


TABLE 8.3 Six major classes of enzymes

Class	Type of reaction	Example	Chapter
1. Oxidoreductases	Oxidation-reduction	Lactate dehydrogenase	16
2. Transferases	Group transfer	Nucleoside monophosphate kinase (NMP kinase)	9
3. Hydrolases	Hydrolysis reactions (transfer of functional groups to water)	Chymotrypsin	9
4. Lyases	Addition or removal of groups to form double bonds	Fumarase	18
5. Isomerases	Isomerization (intramolecular group transfer)	Triose phosphate isomerase	16
6. Ligases	Ligation of two substrates at the expense of ATP hydrolysis	Aminoacyl-tRNA synthetase	29

NMP kinase transfers a phosphoryl group from ATP to NMP to form a nucleoside diphosphate (NDP) and ADP. Consequently, it is a transferase, or member of group 2. Many groups in addition to phosphoryl groups, such as sugars and carbon units, can be transferred. Transferases that shift a phosphoryl group are designated 2.7. Various functional groups can accept the phosphoryl group. If a phosphate is the acceptor, the transferase is designated 2.7.4. The final number designates the acceptor more precisely. In regard to NMP kinase, a nucleoside monophosphate is the acceptor, and the enzyme's designation is EC 2.7.4.4. Although the common names are used routinely, the classification number is used when the precise identity of the enzyme might be ambiguous.

8.2 FREE ENERGY IS A USEFUL THERMODYNAMIC FUNCTION FOR UNDERSTANDING ENZYMES

Some of the principles of thermodynamics were introduced in Chapter 1—notably the idea of *free energy* (G). To fully understand how enzymes operate, we need to consider two thermodynamic properties of the reaction: (1) the free-energy difference (ΔG) between the products and reactants and (2) the energy required to initiate the conversion of reactants to products. The former determines whether the reaction will be spontaneous, whereas the latter determines the rate of the reaction. Enzymes affect only the latter. First, we will consider the thermodynamics of reactions and then, in Section 8.3, the rates of reactions.

8.2.1 The Free-Energy Change Provides Information About the Spontaneity but Not the Rate of a Reaction

As stated in Section 1.3.3, the free-energy change of a reaction (ΔG) tells us if the reaction can occur spontaneously:

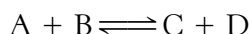
1. A reaction can occur spontaneously only if ΔG is negative. Such reactions are said to be exergonic.
2. A system is at equilibrium and no net change can take place if ΔG is zero.
3. A reaction cannot occur spontaneously if ΔG is positive. An input of free energy is required to drive such a reaction. These reactions are termed endergonic.

Two additional points need to be emphasized. The ΔG of a reaction depends only on the free energy of the products (the final state) minus the free energy of the reactants (the initial state). *The ΔG of a reaction is independent of the path (or molecular mechanism) of the transformation.* The mechanism of a reaction has no effect on ΔG . For example, the ΔG for the oxidation of glucose to CO_2 and H_2O is the same whether it occurs by combustion in vitro or by a series of enzyme-catalyzed steps in a cell. *The ΔG provides no information about the rate of a reaction.* A negative ΔG indicates that a reaction *can* occur spontaneously, but it does not signify whether it will proceed at a perceptible rate. As will be discussed shortly (Section 8.3), the rate of a reaction depends on the *free energy of activation* (ΔG^\ddagger), which is largely unrelated to the ΔG of the reaction.

8.2.2 The Standard Free-Energy Change of a Reaction Is Related to the Equilibrium Constant

As for any reaction, we need to be able to determine ΔG for an enzyme-catalyzed reaction in order to know whether the reaction is spontaneous or an input of energy is required. To determine this important thermodynamic parameter, we need to take into account the nature of both the reactants and the products as well as their concentrations.

Consider the reaction



The ΔG of this reaction is given by

$$\Delta G = \Delta G^\circ + RT \ln \frac{[\text{C}][\text{D}]}{[\text{A}][\text{B}]} \quad (1)$$

in which ΔG° is the *standard free-energy change*, R is the gas constant, T is the absolute temperature, and $[\text{A}]$, $[\text{B}]$, $[\text{C}]$, and $[\text{D}]$ are the molar concentrations (more precisely, the activities) of the reactants. ΔG° is the free-energy change for this reaction under standard conditions—that is, when each of the reactants A, B, C, and D is present at a concentration of 1.0 M (for a gas, the standard state is usually chosen to be 1 atmosphere). Thus, the ΔG of a reaction depends on the *nature* of the reactants (expressed in the ΔG° term of equation 1) and on their *concentrations* (expressed in the logarithmic term of equation 1).

A convention has been adopted to simplify free-energy calculations for biochemical reactions. The standard state is defined as having a pH of 7. Consequently, when H^+ is a reactant, its activity has the value 1 (corresponding to a pH of 7) in equations 1 and 4 (below). The activity of water also is taken to be 1 in these equations. The *standard free-energy change at pH 7*, denoted by the symbol $\Delta G^{\circ'}$ will be used throughout this book. The *kilocalorie* (abbreviated *kcal*) and the *kilojoule* (*kJ*) will be used as the units of energy. One kilocalorie is equivalent to 4.184 kilojoules.

The relation between the standard free energy and the equilibrium constant of a reaction can be readily derived. This equation is important because it displays the energetic relation between products and reactants in terms of their concentrations. At equilibrium, $\Delta G = 0$. Equation 1 then becomes

$$0 = \Delta G^{\circ'} + RT \ln \frac{[\text{C}][\text{D}]}{[\text{A}][\text{B}]} \quad (2)$$

and so

$$\Delta G^{\circ'} = -RT \ln \frac{[\text{C}][\text{D}]}{[\text{A}][\text{B}]} \quad (3)$$

Units of energy—

A *calorie* (cal) is equivalent to the amount of heat required to raise the temperature of 1 gram of water from 14.5°C to 15.5°C.

A *kilocalorie* (kcal) is equal to 1000 cal.

A *joule* (J) is the amount of energy needed to apply a 1-newton force over a distance of 1 meter.

A *kilojoule* (kJ) is equal to 1000 J.

$$1 \text{ kcal} = 4.184 \text{ kJ}$$

The equilibrium constant under standard conditions, K'_{eq} , is defined as

$$K'_{\text{eq}} = \frac{[C][D]}{[A][B]} \quad (4)$$

Substituting equation 4 into equation 3 gives

$$\Delta G^{\circ'} = -RT \ln K'_{\text{eq}} \quad (5)$$

$$\Delta G^{\circ'} = -2.303RT \log_{10} K'_{\text{eq}} \quad (6)$$

which can be rearranged to give

$$K'_{\text{eq}} = 10^{-\Delta G^{\circ'}/(2.303RT)} \quad (7)$$

Substituting $R = 1.987 \times 10^{-3} \text{ kcal mol}^{-1} \text{ deg}^{-1}$ and $T = 298 \text{ K}$ (corresponding to 25°C) gives

$$K'_{\text{eq}} = 10^{-\Delta G^{\circ'}/1.36} \quad (8)$$

where $\Delta G^{\circ'}$ is here expressed in kilocalories per mole because of the choice of the units for R in equation 7. Thus, the standard free energy and the equilibrium constant of a reaction are related by a simple expression. For example, an equilibrium constant of 10 gives a standard free-energy change of $-1.36 \text{ kcal mol}^{-1}$ ($-5.69 \text{ kJ mol}^{-1}$) at 25°C (Table 8.4). Note that, for each 10-fold change in the equilibrium constant, the $\Delta G^{\circ'}$ changes by $1.36 \text{ kcal mol}^{-1}$ (5.69 kJ mol^{-1}).

As an example, let us calculate $\Delta G^{\circ'}$ and ΔG for the isomerization of dihydroxyacetone phosphate (DHAP) to glyceraldehyde 3-phosphate (GAP). This reaction takes place in glycolysis (Section 16.1.4). At equilibrium, the ratio of GAP to DHAP is 0.0475 at 25°C (298 K) and pH 7. Hence, $K'_{\text{eq}} = 0.0475$. The standard free-energy change for this reaction is then calculated from equation 6:

$$\begin{aligned} \Delta G^{\circ'} &= -2.303RT \log_{10} K'_{\text{eq}} \\ &= -2.303 \times 1.987 \times 10^{-3} \times 298 \times \log_{10}(0.0475) \\ &= +1.80 \text{ kcal mol}^{-1} (7.53 \text{ kJ mol}^{-1}) \end{aligned}$$

Under these conditions, the reaction is endergonic. DHAP will not spontaneously convert to GAP.

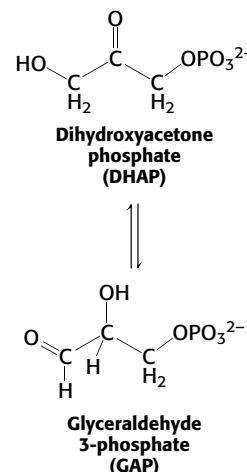
Now let us calculate ΔG for this reaction when the initial concentration of DHAP is $2 \times 10^{-4} \text{ M}$ and the initial concentration of GAP is $3 \times 10^{-6} \text{ M}$. Substituting these values into equation 1 gives

$$\begin{aligned} \Delta G &= 1.80 \text{ kcal mol}^{-1} + 2.303RT \log_{10} \frac{3 \times 10^{-6} \text{ M}}{2 \times 10^{-4} \text{ M}} \\ &= 1.80 \text{ kcal mol}^{-1} - 2.49 \text{ kcal mol}^{-1} \\ &= -0.69 \text{ kcal mol}^{-1} (-2.89 \text{ kJ mol}^{-1}) \end{aligned}$$

This negative value for the ΔG indicates that the isomerization of DHAP to GAP is exergonic and can occur spontaneously when these species are present at the aforesaid concentrations. Note that ΔG for this reaction is negative, although $\Delta G^{\circ'}$ is positive. *It is important to stress that whether the ΔG for a reaction is larger, smaller, or the same as $\Delta G^{\circ'}$ depends on the concentrations of the reactants and products.* The criterion of spontaneity for a reaction is ΔG , not $\Delta G^{\circ'}$. This point is important because reactions that are not spontaneous based on $\Delta G^{\circ'}$ can be made spontaneous by adjusting the concentrations of reactants and products. This principle is the basis of the coupling of reactions to form metabolic pathways (Chapter 14).

TABLE 8.4 Relation between $\Delta G^{\circ'}$ and K'_{eq} (at 25°C)

K'_{eq}	$\Delta G^{\circ'}$	
	kcal mol ⁻¹	kJ/mol ⁻¹
10^{-5}	6.82	28.53
10^{-4}	5.46	22.84
10^{-3}	4.09	17.11
10^{-2}	2.73	11.42
10^{-1}	1.36	5.69
1	0	0
10	-1.36	-5.69
10^2	-2.73	-11.42
10^3	-4.09	-17.11
10^4	-5.46	-22.84
10^5	-6.82	-28.53



8.2.3 Enzymes Alter Only the Reaction Rate and Not the Reaction Equilibrium

Because enzymes are such superb catalysts, it is tempting to ascribe to them powers that they do not have. An enzyme cannot alter the laws of thermodynamics and *consequently cannot alter the equilibrium of a chemical reaction*. This inability means that an enzyme accelerates the forward and reverse reactions by precisely the same factor. Consider the interconversion of A and B. Suppose that, in the absence of enzyme, the forward rate constant (k_F) is 10^{-4} s^{-1} and the reverse rate constant (k_R) is 10^{-6} s^{-1} . The equilibrium constant K is given by the ratio of these rate constants:

$$A \xrightleftharpoons[10^{-6} \text{ s}^{-1}]{10^{-4} \text{ s}^{-1}} B$$

$$K = \frac{[B]}{[A]} = \frac{k_F}{k_R} = \frac{10^{-4}}{10^{-6}} = 100$$

The equilibrium concentration of B is 100 times that of A, whether or not enzyme is present. However, it might take considerable time to approach this equilibrium without enzyme, whereas equilibrium would be attained rapidly in the presence of a suitable enzyme. *Enzymes accelerate the attainment of equilibria but do not shift their positions. The equilibrium position is a function only of the free-energy difference between reactants and products.*

8.3 ENZYMES ACCELERATE REACTIONS BY FACILITATING THE FORMATION OF THE TRANSITION STATE

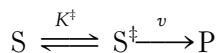
The free-energy difference between reactants and products accounts for the equilibrium of the reaction, but enzymes accelerate how quickly this equilibrium is attained. How can we explain the rate enhancement in terms of thermodynamics? To do so, we have to consider not the end points of the reaction but the chemical pathway between the end points.

A chemical reaction of substrate S to form product P goes through a *transition state* S^\ddagger that has a higher free energy than does either S or P. The double dagger denotes a thermodynamic property of the transition state. The transition state is the most seldom occupied species along the reaction pathway because it is the one with the highest free energy. The difference in free energy between the transition state and the substrate is called the *Gibbs free energy of activation* or simply the *activation energy*, symbolized by ΔG^\ddagger , as mentioned in Section 8.2.1 (Figure 8.3).

$$\Delta G^\ddagger = G_{S^\ddagger} - G_S$$

Note that the energy of activation, or ΔG^\ddagger , does not enter into the final ΔG calculation for the reaction, because the energy input required to reach the transition state is returned when the transition state forms the product. The activation-energy barrier immediately suggests how enzymes enhance reaction rate without altering ΔG of the reaction: enzymes function to lower the activation energy, or, in other words, *enzymes facilitate the formation of the transition state*.

One approach to understanding how enzymes achieve this facilitation is to assume that the transition state (S^\ddagger) and the substrate (S) are in equilibrium.



in which K^\ddagger is the equilibrium constant for the formation of S^\ddagger , and v is the rate of formation of product from S^\ddagger .

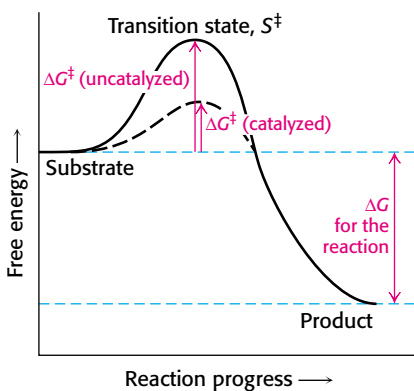


FIGURE 8.3 Enzymes decrease the activation energy. Enzymes accelerate reactions by decreasing ΔG^\ddagger , the free energy of activation.

The rate of the reaction is proportional to the concentration of S^\ddagger :

$$\text{Rate} \propto [S^\ddagger]$$

because only S^\ddagger can be converted into product. The concentration of S^\ddagger is in turn related to the free energy difference between S^\ddagger and S , because these two chemical species are assumed to be in equilibrium; the greater the difference between these two states, the smaller the amount of S^\ddagger .

Because the reaction rate is proportional to the concentration of S^\ddagger , and the concentration of S^\ddagger depends on ΔG^\ddagger , the rate of reaction V depends on ΔG^\ddagger . Specifically,

$$V = v[S^\ddagger] = \frac{kT}{h}[S]e^{-\Delta G^\ddagger/RT}$$

In this equation, k is Boltzmann's constant, and h is Planck's constant. The value of kT/h at 25°C is $6.2 \times 10^{12} \text{ s}^{-1}$. Suppose that the free energy of activation is $6.82 \text{ kcal mol}^{-1}$ ($28.53 \text{ kJ mol}^{-1}$). The ratio $[S^\ddagger]/[S]$ is then 10^{-5} (see Table 8.4). If we assume for simplicity's sake that $[S] = 1\text{M}$, then the reaction rate V is $6.2 \times 10^7 \text{ s}^{-1}$. If ΔG^\ddagger were lowered by $1.36 \text{ kcal mol}^{-1}$ (5.69 kJ mol^{-1}), the ratio $[S^\ddagger]/[S]$ is then 10^{-4} , and the reaction rate would be $6.2 \times 10^8 \text{ s}^{-1}$. As Table 8.4 shows, a decrease of $1.36 \text{ kcal mol}^{-1}$ in ΔG^\ddagger yields a tenfold larger V . A relatively small decrease in ΔG^\ddagger (20% in this particular reaction) results in a much greater increase in V .

Thus, we see the key to how enzymes operate: *Enzymes accelerate reactions by decreasing ΔG^\ddagger , the activation energy.* The combination of substrate and enzyme creates a new reaction pathway whose transition-state energy is lower than that of the reaction in the absence of enzyme (see Figure 8.3). The lower activation energy means that more molecules have the required energy to reach the transition state. Decreasing the activation barrier is analogous to lowering the height of a high-jump bar; more athletes will be able to clear the bar. *The essence of catalysis is specific binding of the transition state.*

8.3.1 The Formation of an Enzyme–Substrate Complex Is the First Step in Enzymatic Catalysis

Much of the catalytic power of enzymes comes from their bringing substrates together in favorable orientations to promote the formation of the transition states in *enzyme–substrate* (ES) complexes. The substrates are bound to a specific region of the enzyme called the *active site*. Most enzymes are highly selective in the substrates that they bind. Indeed, the catalytic specificity of enzymes depends in part on the specificity of binding.

What is the evidence for the existence of an enzyme–substrate complex?

1. The first clue was the observation that, at a constant concentration of enzyme, the reaction rate increases with increasing substrate concentration until a maximal velocity is reached (Figure 8.4). In contrast, uncatalyzed reactions do not show this saturation effect. *The fact that an enzyme-catalyzed reaction has a maximal velocity suggests the formation of a discrete ES complex.* At a sufficiently high substrate concentration, all the catalytic sites are filled and so the reaction rate cannot increase. Although indirect, this is the most general evidence for the existence of ES complexes.

2. *X-ray crystallography* has provided high-resolution images of substrates and substrate analogs bound to the active sites of many enzymes (Figure 8.5). In Chapter 9, we will take a close look at several of these complexes. X-ray studies carried out at low temperatures (to slow reactions down) are providing revealing views of enzyme–substrate complexes and their subsequent

"I think that enzymes are molecules that are complementary in structure to the activated complexes of the reactions that they catalyze, that is, to the molecular configuration that is intermediate between the reacting substances and the products of reaction for these catalyzed processes. The attraction of the enzyme molecule for the activated complex would thus lead to a decrease in its energy and hence to a decrease in the energy of activation of the reaction and to an increase in the rate of reaction."

—LINUS PAULING
Nature 161(1948):707

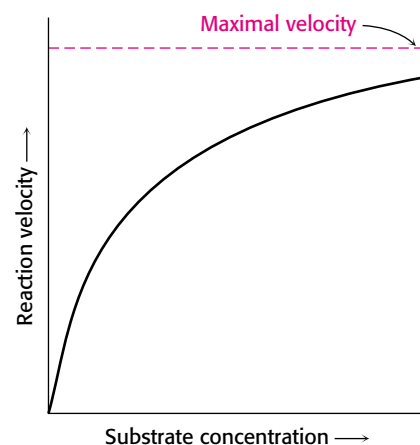


FIGURE 8.4 Reaction velocity versus substrate concentration in an enzyme-catalyzed reaction. An enzyme-catalyzed reaction reaches a maximal velocity.

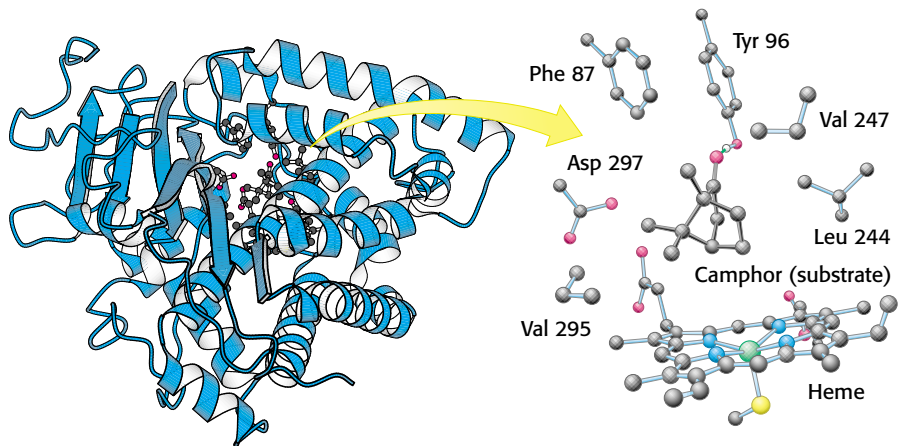


FIGURE 8.5 Structure of an enzyme-substrate complex. (Left) The enzyme cytochrome P-450 is illustrated bound to its substrate camphor. (Right) In the active site, the substrate is surrounded by residues from the enzyme. Note also the presence of a heme cofactor.

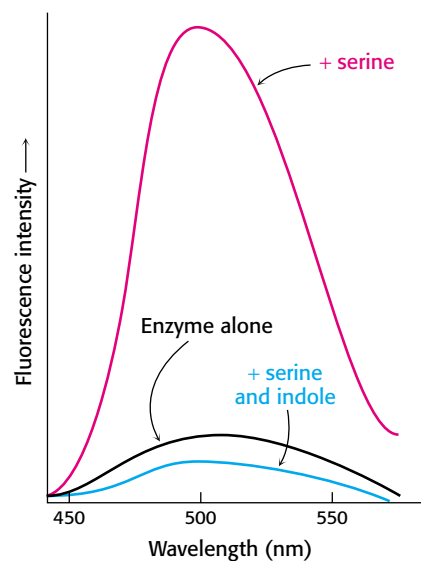


FIGURE 8.6 Change in spectroscopic characteristics with the formation of an enzyme-substrate complex. Fluorescence intensity of the pyridoxal phosphate group at the active site of tryptophan synthetase changes on addition of serine and indole, the substrates.

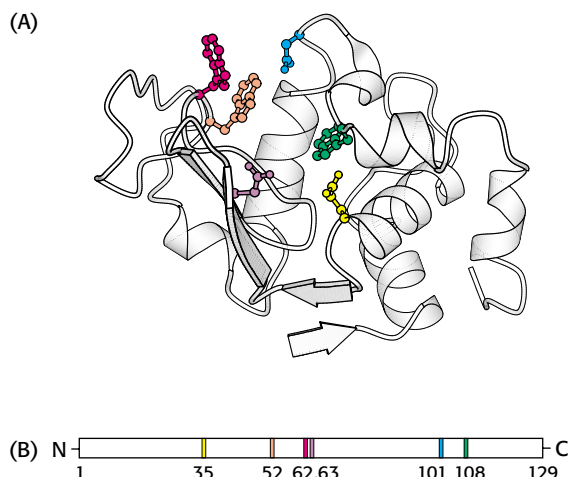
reactions. A new technique, *time-resolved crystallography*, depends on co-crystallizing a photolabile substrate analog with the enzyme. The substrate analog can be converted to substrate light, and images of the enzyme–substrate complex are obtained in a fraction of a second by scanning the crystal with intense, polychromatic x-rays from a synchrotron.

3. The *spectroscopic characteristics* of many enzymes and substrates change on formation of an ES complex. These changes are particularly striking if the enzyme contains a colored prosthetic group. Tryptophan synthetase, a bacterial enzyme that contains a pyridoxal phosphate (PLP) prosthetic group, provides a nice illustration. This enzyme catalyzes the synthesis of L-tryptophan from L-serine and indole-derivative. The addition of L-serine to the enzyme produces a marked increase in the fluorescence of the PLP group (Figure 8.6). The subsequent addition of indole, the second substrate, quenches this fluorescence to a level even lower than that of the enzyme alone. Thus, fluorescence spectroscopy reveals the existence of an enzyme–serine complex and of an enzyme–serine–indole complex. Other spectroscopic techniques, such as nuclear magnetic resonance and electron spin resonance, also are highly informative about ES interactions.

8.3.2 The Active Sites of Enzymes Have Some Common Features

The active site of an enzyme is the region that binds the substrates (and the cofactor, if any). It also contains the residues that directly participate in the making and breaking of bonds. These residues are called the *catalytic groups*. In essence, *the interaction of the enzyme and substrate at the active site promotes the formation of the transition state*. The active site is the region of the enzyme that most directly lowers the ΔG^\ddagger of the reaction, which results in the rate enhancement characteristic of enzyme action. Although enzymes differ widely in structure, specificity, and mode of catalysis, a number of generalizations concerning their active sites can be stated:

1. *The active site is a three-dimensional cleft* formed by groups that come from different parts of the amino acid sequence—indeed, residues far apart in the sequence may interact more strongly than adjacent residues in the amino acid sequence. In lysozyme, an enzyme that degrades the cell walls of some bacteria, the important groups in the active site are contributed by




residues numbered 35, 52, 62, 63, and 101 in the sequence of the 129 amino acids (Figure 8.7).

2. *The active site takes up a relatively small part of the total volume of an enzyme.* Most of the amino acid residues in an enzyme are not in contact with the substrate, which raises the intriguing question of why enzymes are so big. Nearly all enzymes are made up of more than 100 amino acid residues, which gives them a mass greater than 10 kd and a diameter of more than 25 Å. The “extra” amino acids serve as a scaffold to create the three-dimensional active site from amino acids that are far apart in the primary structure. Amino acids near to one another in the primary structure are often sterically constrained from adopting the structural relations necessary to form the active site. In many proteins, the remaining amino acids also constitute regulatory sites, sites of interaction with other proteins, or channels to bring the substrates to the active sites.

3. *Active sites are clefts or crevices.* In all enzymes of known structure, substrate molecules are bound to a cleft or crevice. Water is usually excluded unless it is a reactant. The nonpolar character of much of the cleft enhances the binding of substrate as well as catalysis. Nevertheless, the cleft may also contain polar residues. In the nonpolar microenvironment of the active site, certain of these polar residues acquire special properties essential for substrate binding or catalysis. The internal positions of these polar residues are biologically crucial exceptions to the general rule that polar residues are exposed to water.

4. *Substrates are bound to enzymes by multiple weak attractions.* ES complexes usually have equilibrium constants that range from 10^{-2} to 10^{-8} M, corresponding to free energies of interaction ranging from about -3 to -12 kcal mol $^{-1}$ (from -13 to -50 kJ mol $^{-1}$). The noncovalent interactions in ES complexes are much weaker than covalent bonds, which have energies between -50 and -110 kcal mol $^{-1}$ (between -210 and -460 kJ mol $^{-1}$). As discussed in Chapter 1 (Section 1.3.1), electrostatic interactions, hydrogen bonds, van der Waals forces, and hydrophobic interactions mediate reversible interactions of biomolecules. Van der Waals forces become significant in binding only when numerous substrate atoms simultaneously come close to many enzyme atoms. Hence, the enzyme and substrate should have complementary enzyme shapes. The directional character of hydrogen bonds between enzyme and substrate often enforces a high degree of specificity, as seen in the RNA-degrading enzyme ribonuclease (Figure 8.8).

 **FIGURE 8.7 Active sites may include distant residues.** (A) Ribbon diagram of the enzyme lysozyme with several components of the active site shown in color. (B) A schematic representation of the primary structure of lysozyme shows that the active site is composed of residues that come from different parts of the polypeptide chain.

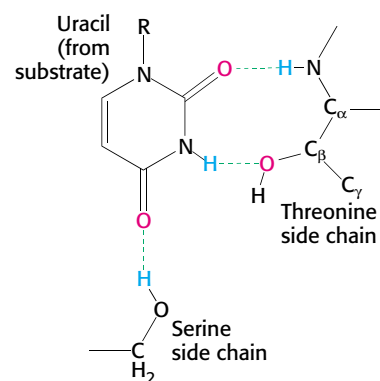
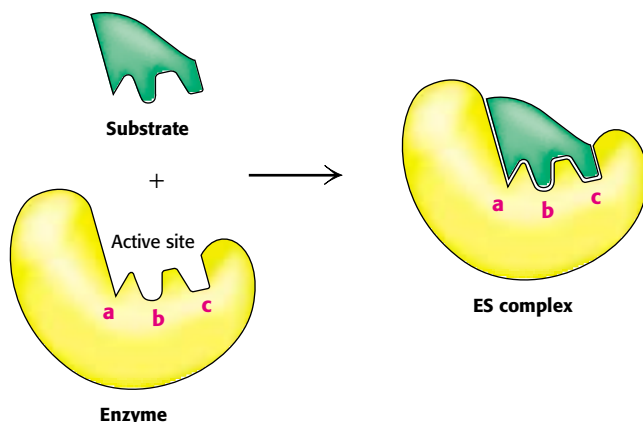


FIGURE 8.8 Hydrogen bonds between an enzyme and substrate. The enzyme ribonuclease forms hydrogen bonds with the uridine component of the substrate. [After F. M. Richards, H. W. Wyckoff, and N. Allewel. In *The Neurosciences: Second Study Program*, F. O. Schmidt, Ed. (Rockefeller University Press, 1970), p. 970.]

FIGURE 8.9 Lock-and-key model of enzyme–substrate binding. In this model, the active site of the unbound enzyme is complementary in shape to the substrate.



5. The specificity of binding depends on the precisely defined arrangement of atoms in an active site. Because the enzyme and the substrate interact by means of short-range forces that require close contact, a substrate must have a matching shape to fit into the site. Emil Fischer's analogy of the lock and key (Figure 8.9), expressed in 1890, has proved to be highly stimulating and fruitful. However, we now know that enzymes are flexible and that the shapes of the active sites can be markedly modified by the binding of substrate, as was postulated by Daniel E. Koshland, Jr., in 1958. The active site of some enzymes assume a shape that is complementary to that of the transition state only *after* the substrate is bound. This process of dynamic recognition is called *induced fit* (Figure 8.10).

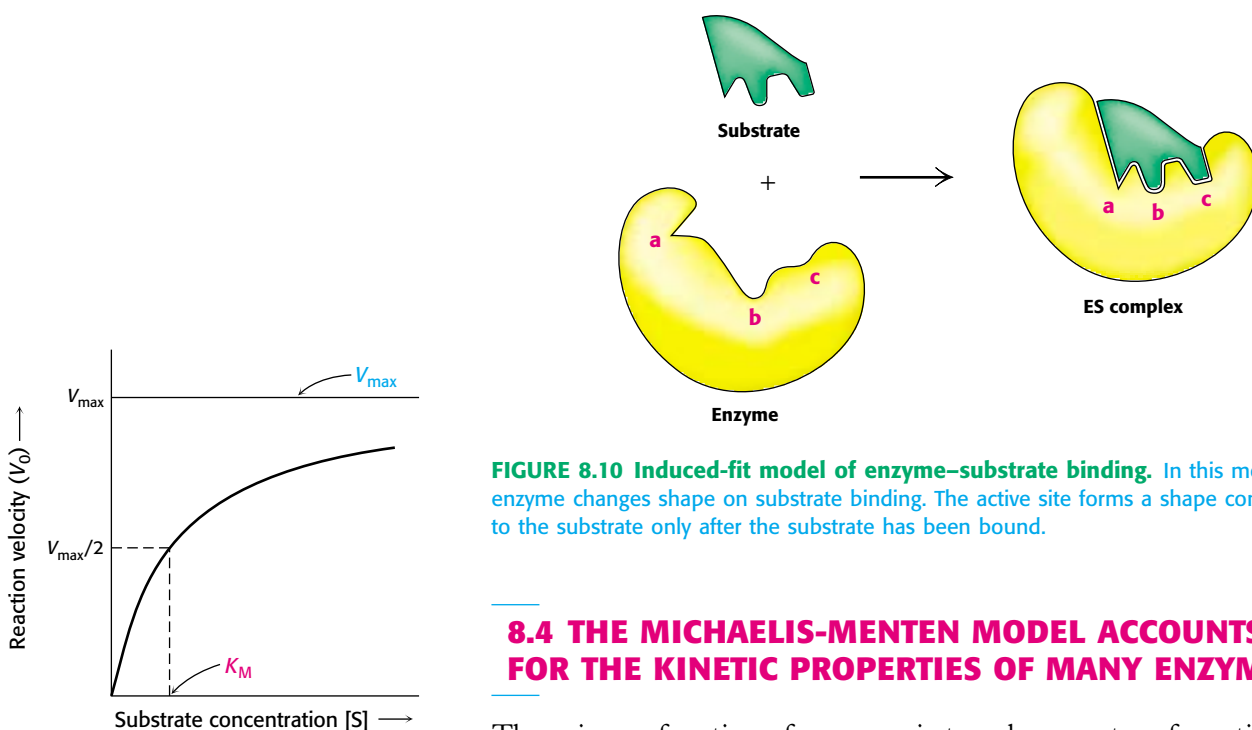


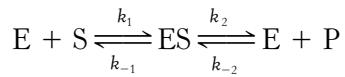
FIGURE 8.10 Induced-fit model of enzyme–substrate binding. In this model, the enzyme changes shape on substrate binding. The active site forms a shape complementary to the substrate only after the substrate has been bound.

8.4 THE MICHAELIS-MENTEN MODEL ACCOUNTS FOR THE KINETIC PROPERTIES OF MANY ENZYMES

The primary function of enzymes is to enhance rates of reactions so that they are compatible with the needs of the organism. To understand how enzymes function, we need a kinetic description of their activity. For many enzymes, the rate of catalysis V_0 , which is defined as the number of moles of product formed per second, varies with the substrate concentration $[S]$ in a manner shown in Figure 8.11. The rate of catalysis rises linearly as substrate concentration increases and then begins to level off and approach a maximum at higher substrate concentrations. Before we can accurately interpret

FIGURE 8.11 Michaelis-Menten kinetics. A plot of the reaction velocity (V_0) as a function of the substrate concentration $[S]$ for an enzyme that obeys Michaelis-Menten kinetics shows that the maximal velocity (V_{\max}) is approached asymptotically. The Michaelis constant (K_M) is the substrate concentration yielding a velocity of $V_{\max}/2$.

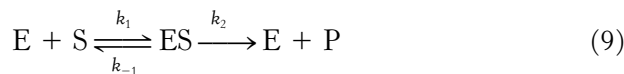
this graph, we need to understand how it is generated. Consider an enzyme that catalyzes the S to P by the following pathway:



The extent of product formation is determined as a function of time for a series of substrate concentrations (Figure 8.12). As expected, in each case, the amount of product formed increases with time, although eventually a time is reached when there is *no net change* in the concentration of S or P. The enzyme is still actively converting substrate into product and *visa versa*, but the reaction equilibrium has been attained. Figure 8.13A illustrates the changes in concentration observed in all of the reaction participants with time until equilibrium has been reached.

Enzyme kinetics are more easily approached if we can ignore the back reaction. We define V_0 as the rate of increase in product with time when [P] is low; that is, at times close to zero (hence, V_0) (Figure 8.13B). Thus, for the graph in Figure 8.11, V_0 is determined for each substrate concentration by measuring the rate of product formation at early times before P accumulates (see Figure 8.12).

We begin our kinetic examination of enzyme activity with the graph shown in Figure 8.11. At a fixed concentration of enzyme, V_0 is almost linearly proportional to [S] when [S] is small but is nearly independent of [S] when [S] is large. In 1913, Leonor Michaelis and Maud Menten proposed a simple model to account for these kinetic characteristics. The critical feature in their treatment is that a specific ES complex is a necessary intermediate in catalysis. The model proposed, which is the simplest one that accounts for the kinetic properties of many enzymes, is



An enzyme E combines with substrate S to form an ES complex, with a rate constant k_1 . The ES complex has two possible fates. It can dissociate to E and S, with a rate constant k_{-1} , or it can proceed to form product P, with a rate constant k_2 . Again, we assume that almost none of the product reverts to the initial substrate, a condition that holds in the initial stage of a reaction before the concentration of product is appreciable.

We want an expression that relates the rate of catalysis to the concentrations of substrate and enzyme and the rates of the individual steps. Our

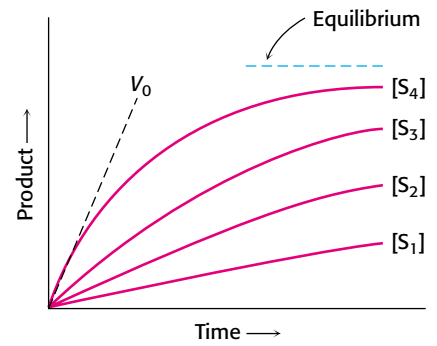


FIGURE 8.12 Determining initial velocity. The amount of product formed at different substrate concentrations is plotted as a function of time. The initial velocity (V_0) for each substrate concentration is determined from the slope of the curve at the beginning of a reaction, when the reverse reaction is insignificant.

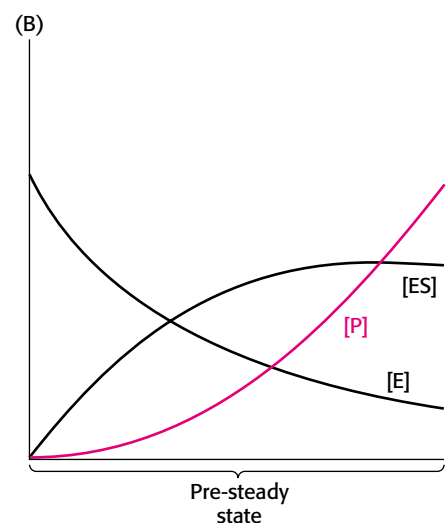
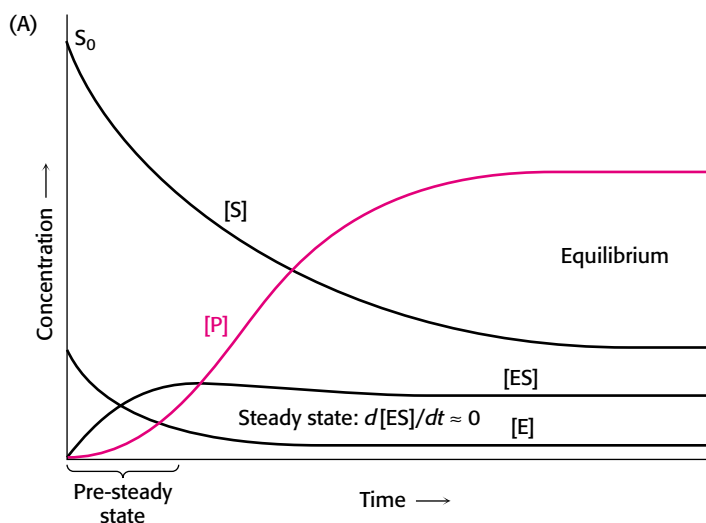


FIGURE 8.13 Changes in the concentration of reaction participants of an enzyme-catalyzed reaction with time. Concentration changes under (A) steady-state conditions, and (B) the pre-steady-state conditions.

starting point is that the catalytic rate is equal to the product of the concentration of the ES complex and k_2 .

$$V_0 = k_2[\text{ES}] \quad (10)$$

Now we need to express $[\text{ES}]$ in terms of known quantities. The rates of formation and breakdown of ES are given by:

$$\text{Rate of formation of ES} = k_1[\text{E}][\text{S}] \quad (11)$$

$$\text{Rate of breakdown of ES} = (k_{-1} + k_2)[\text{ES}] \quad (12)$$

To simplify matters, we will work under the *steady-state assumption*. In a steady state, the concentrations of intermediates, in this case $[\text{ES}]$, stay the same even if the concentrations of starting materials and products are changing. This occurs when the rates of formation and breakdown of the ES complex are equal. Setting the right-hand sides of equations 11 and 12 equal gives

$$k_1[\text{E}][\text{S}] = (k_{-1} + k_2)[\text{ES}] \quad (13)$$

By rearranging equation 13, we obtain

$$[\text{E}][\text{S}]/[\text{ES}] = (k_{-1} + k_2)/k_1 \quad (14)$$

Equation 14 can be simplified by defining a new constant, K_M , called the *Michaelis constant*:

$$K_M = \frac{k_{-1} + k_2}{k_1} \quad (15)$$

Note that K_M has the units of concentration. K_M is an important characteristic of enzyme–substrate interactions and is independent of enzyme and substrate concentrations.

Inserting equation 15 into equation 14 and solving for $[\text{ES}]$ yields

$$[\text{ES}] = \frac{[\text{E}][\text{S}]}{K_M} \quad (16)$$

Now let us examine the numerator of equation 16. The concentration of uncombined substrate $[\text{S}]$ is very nearly equal to the total substrate concentration, provided that the concentration of enzyme is much lower than that of substrate. The concentration of uncombined enzyme $[\text{E}]$ is equal to the total enzyme concentration $[\text{E}]_T$ minus the concentration of the ES complex.

$$[\text{E}] = [\text{E}]_T - [\text{ES}] \quad (17)$$

Substituting this expression for $[\text{E}]$ in equation 16 gives

$$[\text{ES}] = \frac{([\text{E}]_T - [\text{ES}])[\text{S}]}{K_M} \quad (18)$$

Solving equation 18 for $[\text{ES}]$ gives

$$[\text{ES}] = \frac{[\text{E}]_T/K_M}{1 + [\text{S}]/K_M} \quad (19)$$

or

$$[\text{ES}] = [\text{E}]_T \frac{[\text{S}]}{[\text{S}] + K_M} \quad (20)$$

By substituting this expression for $[ES]$ into equation 10, we obtain

$$V_0 = k_2 [E]_T \frac{[S]}{[S] + K_M} \quad (21)$$

The maximal rate, V_{\max} , is attained when the catalytic sites on the enzyme are saturated with substrate—that is, when $[ES] = [E]_T$. Thus,

$$V_{\max} = k_2 [E]_T \quad (22)$$

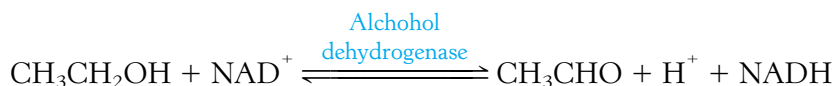
Substituting equation 22 into equation 21 yields the *Michaelis-Menten equation*:

$$V_0 = V_{\max} \frac{[S]}{[S] + K_M} \quad (23)$$

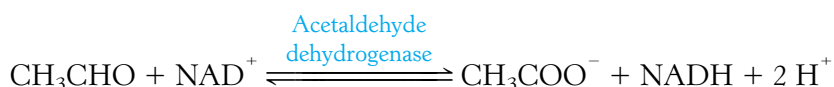
This equation accounts for the kinetic data given in Figure 8.11. At very low substrate concentration, when $[S]$ is much less than K_M , $V_0 = (V_{\max}/K_M)[S]$; that is, the rate is directly proportional to the substrate concentration. At high substrate concentration, when $[S]$ is much greater than K_M , $V_0 = V_{\max}$; that is, the rate is maximal, independent of substrate concentration.

The meaning of K_M is evident from equation 23. When $[S] = K_M$, then $V_0 = V_{\max}/2$. Thus, K_M is equal to the substrate concentration at which the reaction rate is half its maximal value. K_M is an important characteristic of an enzyme-catalyzed reaction and is significant for its biological function.

The physiological consequence of K_M is illustrated by the sensitivity of some individuals to ethanol. Such persons exhibit facial flushing and rapid heart rate (tachycardia) after ingesting even small amounts of alcohol. In the liver, alcohol dehydrogenase converts ethanol into acetaldehyde.



Normally, the acetaldehyde, which is the cause of the symptoms when present at high concentrations, is processed to acetate by acetaldehyde dehydrogenase.



Most people have two forms of the acetaldehyde dehydrogenase, a low K_M mitochondrial form and a high K_M cytosolic form. In susceptible persons, the mitochondrial enzyme is less active due to the substitution of a single amino acid, and acetaldehyde is processed only by the cytosolic enzyme. Because this enzyme has a high K_M , less acetaldehyde is converted into acetate; excess acetaldehyde escapes into the blood and accounts for the physiological effects.

8.4.1 The Significance of K_M and V_{\max} Values



CONCEPTUAL INSIGHTS, Steady-State Enzyme Kinetics. Learn how the kinetic parameters K_M and V_{\max} can be determined experimentally using the enzyme kinetics lab simulation in this media module.

The Michaelis constant, K_M , and the maximal rate, V_{\max} , can be readily derived from rates of catalysis measured at a variety of substrate concentrations if an enzyme operates according to the simple scheme given in equation 23. The derivation of K_M and V_{\max} is most commonly achieved

TABLE 8.5 K_M values of some enzymes

Enzyme	Substrate	$K_M(\mu\text{M})$
Chymotrypsin	Acetyl-L-tryptophanamide	5000
Lysozyme	Hexa-N-acetylglucosamine	6
β -Galactosidase	Lactose	4000
Threonine deaminase	Threonine	5000
Carbonic anhydrase	CO_2	8000
Penicillinase	Benzylpenicillin	50
Pyruvate carboxylase	Pyruvate	400
	HCO_3^-	1000
	ATP	60
Arginine-tRNA synthetase	Arginine	3
	tRNA	0.4
	ATP	300

with the use of curve-fitting programs on a computer (see the appendix to this chapter for alternative means of determining K_M and V_{\max}). The K_M values of enzymes range widely (Table 8.5). For most enzymes, K_M lies between 10^{-1} and 10^{-7} M. The K_M value for an enzyme depends on the particular substrate and on environmental conditions such as pH, temperature, and ionic strength. The Michaelis constant, K_M , has two meanings. First, K_M is the concentration of substrate at which half the active sites are filled. Thus, K_M provides a measure of the substrate concentration required for significant catalysis to occur. In fact, for many enzymes, experimental evidence suggests that K_M provides an approximation of substrate concentration in vivo. When the K_M is known, the fraction of sites filled, f_{ES} , at any substrate concentration can be calculated from

$$f_{ES} = \frac{V}{V_{\max}} = \frac{[S]}{[S] + K_M} \quad (24)$$

Second, K_M is related to the rate constants of the individual steps in the catalytic scheme given in equation 9. In equation 15, K_M is defined as $(k_{-1} + k_2)/k_1$. Consider a limiting case in which k_{-1} is much greater than k_2 . Under such circumstances, the ES complex dissociates to E and S much more rapidly than product is formed. Under these conditions ($k_{-1} \gg k_2$),

$$K_M = \frac{k_{-1}}{k_1} \quad (25)$$

The dissociation constant of the ES complex is given by

$$K_{ES} = \frac{[E][S]}{[ES]} = \frac{k_{-1}}{k_1} \quad (26)$$

In other words, K_M is equal to the dissociation constant of the ES complex if k_2 is much smaller than k_{-1} . When this condition is met, K_M is a measure of the strength of the ES complex: a high K_M indicates weak binding; a low K_M indicates strong binding. It must be stressed that K_M indicates the affinity of the ES complex only when k_{-1} is much greater than k_2 .

The maximal rate, V_{\max} , reveals the *turnover number* of an enzyme, which is the number of substrate molecules converted into product by an enzyme molecule in a unit time when the enzyme is fully saturated with substrate. It is equal to the kinetic constant k_2 , which is also called k_{cat} . The maximal rate, V_{\max} , reveals the turnover number of an enzyme if the concentration of active sites

$[E]_T$ is known, because

$$V_{\max} = k_2[E]_T$$

and thus

$$k_2 = V_{\max}/[E]_T \quad (27)$$

For example, a 10^{-6} M solution of carbonic anhydrase catalyzes the formation of 0.6 M H_2CO_3 per second when it is fully saturated with substrate. Hence, k_2 is $6 \times 10^5 \text{ s}^{-1}$. This turnover number is one of the largest known. Each catalyzed reaction takes place in a time equal to $1/k_2$, which is 1.7 μs for carbonic anhydrase. The turnover numbers of most enzymes with their physiological substrates fall in the range from 1 to 10^4 per second (Table 8.6).

8.4.2 Kinetic Perfection in Enzymatic Catalysis: The k_{cat}/K_M Criterion

When the substrate concentration is much greater than K_M , the rate of catalysis is equal to k_{cat} , the turnover number, as described in Section 8.4.1. However, most enzymes are not normally saturated with substrate. Under physiological conditions, the $[S]/K_M$ ratio is typically between 0.01 and 1.0. When $[S] \ll K_M$, the enzymatic rate is much less than k_{cat} because most of the active sites are unoccupied. Is there a number that characterizes the kinetics of an enzyme under these more typical cellular conditions? Indeed there is, as can be shown by combining equations 10 and 16 to give

$$V_0 = \frac{k_{\text{cat}}}{K_M} [E][S] \quad (28)$$

When $[S] \ll K_M$, the concentration of free enzyme, $[E]$, is nearly equal to the total concentration of enzyme $[E]_T$, so


$$V_0 = \frac{k_{\text{cat}}}{K_M} [S][E]_T \quad (29)$$

Thus, when $[S] \ll K_M$, the enzymatic velocity depends on the values of k_{cat}/K_M , $[S]$, and $[E]_T$. Under these conditions, k_{cat}/K_M is the rate constant for the interaction of S and E and can be used as a measure of catalytic efficiency. For instance, by using k_{cat}/K_M values, one can compare an enzyme's preference for different substrates. Table 8.7 shows the k_{cat}/K_M

TABLE 8.6 Maximum turnover numbers of some enzymes

Enzyme	Turnover number (per second)
Carbonic anhydrase	600,000
3-Ketosteroid isomerase	280,000
Acetylcholinesterase	25,000
Penicillinase	2,000
Lactate dehydrogenase	1,000
Chymotrypsin	100
DNA polymerase I	15
Tryptophan synthetase	2
Lysozyme	0.5

TABLE 8.7 Substrate preferences of chymotrypsin

Amino acid in ester	Amino acid side chain	k_{cat}/K_M ($\text{s}^{-1} \text{ M}^{-1}$)
Glycine	—H	1.3×10^{-1}
Valine	$\begin{array}{c} \text{CH}_3 \\ \\ \text{—CH} \\ \\ \text{CH}_3 \end{array}$	2.0
Norvaline	— $\text{CH}_2\text{CH}_2\text{CH}_3$	3.6×10^2
Norleucine	— $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_3$	3.0×10^3
Phenylalanine	— CH_2 — 	1.0×10^5

Source: After A. Fersht, *Structure and Mechanism in Protein Science: A Guide to Enzyme Catalysis and Protein Folding* (W. H. Freeman and Company, 1999), Table 7.3.

values for several different substrates of chymotrypsin (Section 9.1.1). Chymotrypsin clearly has a preference for cleaving next to bulky, hydrophobic side chains.

How efficient can an enzyme be? We can approach this question by determining whether there are any physical limits on the value of $k_{\text{cat}}/K_{\text{M}}$. Note that this ratio depends on k_1 , k_{-1} , and k_2 , as can be shown by substituting for K_{M} .

$$\frac{k_{\text{cat}}}{K_{\text{M}}} = \frac{k_{\text{cat}}}{k_{-1} + k_{\text{cat}}/k_1} = \frac{k_{\text{cat}}}{k_{\text{cat}} + k_{-1}} k_1 < k_1 \quad (30)$$

Suppose that the rate of formation of product (k_{cat}) is much faster than the rate of dissociation of the ES complex (k_{-1}). The value of $k_{\text{cat}}/K_{\text{M}}$ then approaches k_1 . Thus, the ultimate limit on the value of $k_{\text{cat}}/K_{\text{M}}$ is set by k_1 , the rate of formation of the ES complex. *This rate cannot be faster than the diffusion-controlled encounter of an enzyme and its substrate.* Diffusion limits the value of k_1 so that it cannot be higher than between 10^8 and $10^9 \text{ s}^{-1} \text{ M}^{-1}$. Hence, the upper limit on $k_{\text{cat}}/K_{\text{M}}$ is between 10^8 and $10^9 \text{ s}^{-1} \text{ M}^{-1}$.

The $k_{\text{cat}}/K_{\text{M}}$ ratios of the enzymes superoxide dismutase, acetylcholinesterase, and triosephosphate isomerase are between 10^8 and $10^9 \text{ s}^{-1} \text{ M}^{-1}$. Enzymes such as these that have $k_{\text{cat}}/K_{\text{M}}$ ratios at the upper limits have attained *kinetic perfection*. *Their catalytic velocity is restricted only by the rate at which they encounter substrate in the solution* (Table 8.8). Any further gain in catalytic rate can come only by decreasing the time for diffusion. Remember that the active site is only a small part of the total enzyme structure. Yet, for catalytically perfect enzymes, every encounter between enzyme and substrate is productive. In these cases, there may be attractive electrostatic forces on the enzyme that entice the substrate to the active site. These forces are sometimes referred to poetically as *Circe effects*.

The limit imposed by the rate of diffusion in solution can also be partly overcome by confining substrates and products in the limited volume of a multienzyme complex. Indeed, some series of enzymes are associated into organized assemblies (Section 17.1.9) so that the product of one enzyme is very rapidly found by the next enzyme. In effect, products are channeled from one enzyme to the next, much as in an assembly line.

Circe effect—

The utilization of attractive forces to lure a substrate into a site in which it undergoes a transformation of structure, as defined by William P. Jencks, an enzymologist, who coined the term.

A goddess of Greek mythology, Circe lured Odysseus's men to her house and then transformed them into pigs.

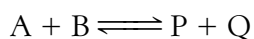
TABLE 8.8 Enzymes for which $k_{\text{cat}}/K_{\text{M}}$ is close to the diffusion-controlled rate of encounter

Enzyme	$k_{\text{cat}}/K_{\text{M}}$ ($\text{s}^{-1}\text{M}^{-1}$)
Acetylcholinesterase	1.6×10^8
Carbonic anhydrase	8.3×10^7
Catalase	4×10^7
Crotonase	2.8×10^8
Fumarase	1.6×10^8
Triose phosphate isomerase	2.4×10^8
β -Lactamase	1×10^8
Superoxide dismutase	7×10^9

Source: After A. Fersht, *Structure and Mechanism in Protein Science: A Guide to Enzyme Catalysis and Protein Folding* (W. H. Freeman and Company, 1999), Table 4.5.

8.4.3 Most Biochemical Reactions Include Multiple Substrates

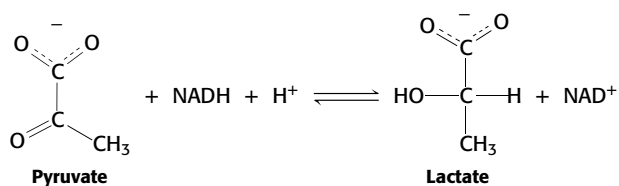
Most reactions in biological systems usually include two substrates and two products and can be represented by the bisubstrate reaction:



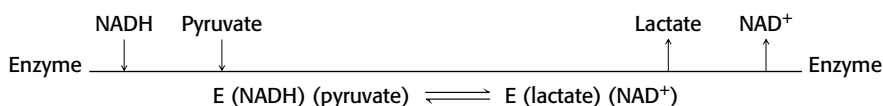
The majority of such reactions entail the transfer of a functional group, such as a phosphoryl or an ammonium group, from one substrate to the other. In oxidation–reduction reactions, electrons are transferred between substrates. Multiple substrate reactions can be divided into two classes: *sequential displacement* and *double displacement*.

Sequential Displacement. In the sequential mechanism, all substrates must bind to the enzyme before any product is released. Consequently, in a bisubstrate reaction, a *ternary complex* of the enzyme and both substrates forms. Sequential mechanisms are of two types: ordered, in which the substrates bind the enzyme in a defined sequence, and random.

Many enzymes that have NAD^+ or NADH as a substrate exhibit the sequential ordered mechanism. Consider lactate dehydrogenase, an important enzyme in glucose metabolism (Section 16.1.9). This enzyme reduces pyruvate to lactate while oxidizing NADH to NAD^+ .

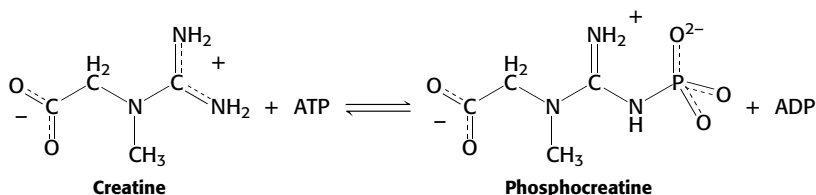


In the ordered sequential mechanism, the coenzyme always binds first and the lactate is always released first. This sequence can be represented as follows in a notation developed by W. Wallace Cleland:

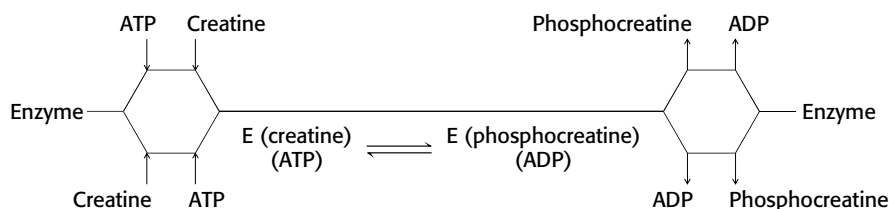


The enzyme exists as a ternary complex: first, consisting of the enzyme and substrates and, after catalysis, the enzyme and products.

In the random sequential mechanism, the order of addition of substrates and release of products is random. Sequential random reactions are illustrated by the formation of phosphocreatine and ADP from ATP and creatine, a reaction catalyzed by creatine kinase (Section 30.x.x).

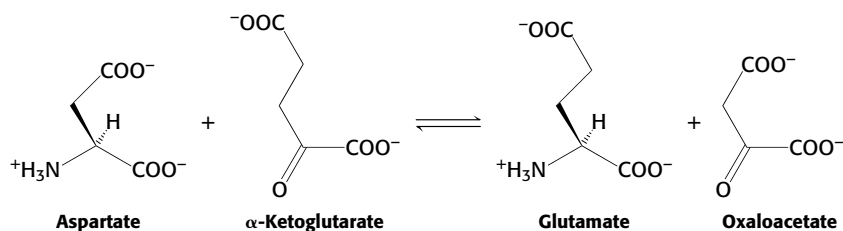


Phosphocreatine is an important energy source in muscle. Sequential random reactions can also be depicted in the Cleland notation.

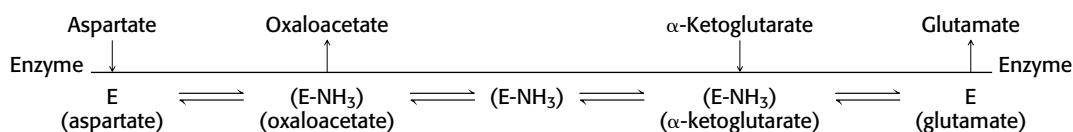


Although the order of certain events is random, the reaction still passes through the ternary complexes including, first, substrates and, then, products.

Double-Displacement (Ping-Pong) Reactions. In double-displacement, or Ping-Pong, reactions, one or more products are released before all substrates bind the enzyme. The defining feature of double-displacement reactions is the existence of a *substituted enzyme intermediate*, in which the enzyme is temporarily modified. Reactions that shuttle amino groups between amino acids and α -keto acids are classic examples of double-displacement mechanisms. The enzyme aspartate aminotransferase (Section 23.x.x) catalyzes the transfer of an amino group from aspartate to α -ketoglutarate.



The sequence of events can be portrayed as the following diagram.



After aspartate binds to the enzyme, the enzyme removes aspartate's amino group to form the substituted enzyme intermediate. The first product, oxaloacetate, subsequently departs. The second substrate, α -ketoglutarate, binds to the enzyme, accepts the amino group from the modified enzyme, and is then released as the final product, glutamate. In the Cleland notation, the substrates appear to bounce on and off the enzyme analogously to a Ping-Pong ball bouncing on a table.

8.4.4 Allosteric Enzymes Do Not Obey Michaelis-Menten Kinetics

The Michaelis-Menten model has greatly assisted the development of enzyme chemistry. Its virtues are simplicity and broad applicability. However, the Michaelis-Menten model cannot account for the kinetic properties of many enzymes. An important group of enzymes that do not obey Michaelis-Menten kinetics comprises the *allosteric enzymes*. These enzymes consist of multiple subunits and multiple active sites.

Allosteric enzymes often display sigmoidal plots (Figure 8.14) of the reaction velocity V_0 versus substrate concentration $[S]$, rather than the hyperbolic plots predicted by the Michaelis-Menten equation (equation 23). In allosteric enzymes, the binding of substrate to one active site can affect the properties of other active sites in the same enzyme molecule. A possible outcome of this interaction between subunits is that the binding of substrate becomes *cooperative*; that is, the binding of substrate to one active site of the enzyme facilitates substrate binding to the other active sites. As will be considered in Chapter 10, such cooperativity results in a sigmoidal plot of V_0 versus $[S]$. In addition, the activity of an allosteric enzyme may be altered by regulatory molecules that are reversibly bound to specific sites other than the catalytic sites. The catalytic properties of allosteric enzymes can thus be adjusted to meet the immediate needs of a cell (Chapter 10). For this reason, allosteric enzymes are key regulators of metabolic pathways in the cell.

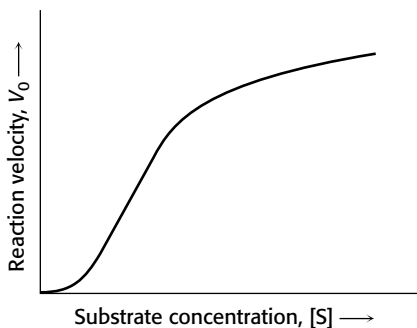


FIGURE 8.14 Kinetics for an allosteric enzyme. Allosteric enzymes display a sigmoidal dependence of reaction velocity on substrate concentration.

8.5 ENZYMES CAN BE INHIBITED BY SPECIFIC MOLECULES

The activity of many enzymes can be inhibited by the binding of specific small molecules and ions. This means of inhibiting enzyme activity serves as a major control mechanism in biological systems. The regulation of allosteric enzymes typifies this type of control. In addition, many drugs and toxic agents act by inhibiting enzymes. Inhibition by particular chemicals can be a source of insight into the mechanism of enzyme action: specific inhibitors can often be used to identify residues critical for catalysis. The value of transition-state analogs as potent inhibitors will be discussed shortly.

Enzyme inhibition can be either reversible or irreversible. An *irreversible inhibitor* dissociates very slowly from its target enzyme because it has become tightly bound to the enzyme, either covalently or noncovalently. Some irreversible inhibitors are important drugs. Penicillin acts by covalently modifying the enzyme transpeptidase, thereby preventing the synthesis of bacterial cell walls and thus killing the bacteria (Section 8.5.5). Aspirin acts by covalently modifying the enzyme cyclooxygenase, reducing the synthesis of inflammatory signals.

Reversible inhibition, in contrast with irreversible inhibition, is characterized by a rapid dissociation of the enzyme–inhibitor complex. In *competitive inhibition*, an enzyme can bind substrate (forming an ES complex) or inhibitor (EI) but not both (ESI). The competitive inhibitor resembles the substrate and binds to the active site of the enzyme (Figure 8.15). The substrate is thereby prevented from binding to the same active site. A *competitive inhibitor diminishes the rate of catalysis by reducing the proportion of enzyme molecules bound to a substrate*. At any given inhibitor concentration, competitive inhibition can be relieved by increasing the substrate concentration. Under these conditions, the substrate “outcompetes” the inhibitor for the active site. Methotrexate is a structural analog of tetrahydrofolate, a cofactor for the enzyme dihydrofolate reductase, which plays a role in the biosynthesis of purines and pyrimidines (Figure 8.16). It binds to dihydrofolate reductase 1000-fold more tightly than the natural substrate and inhibits nucleotide base synthesis. It is used to treat cancer.

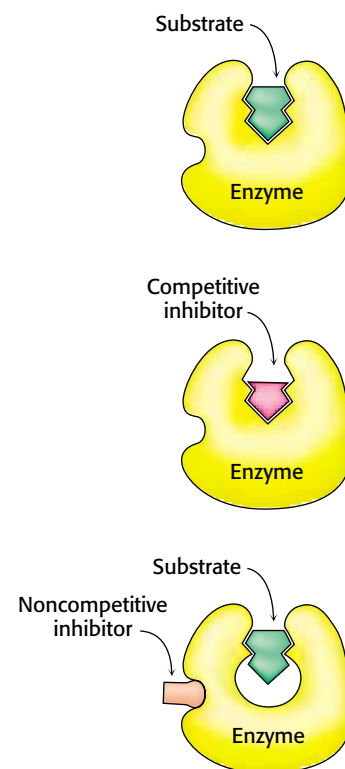
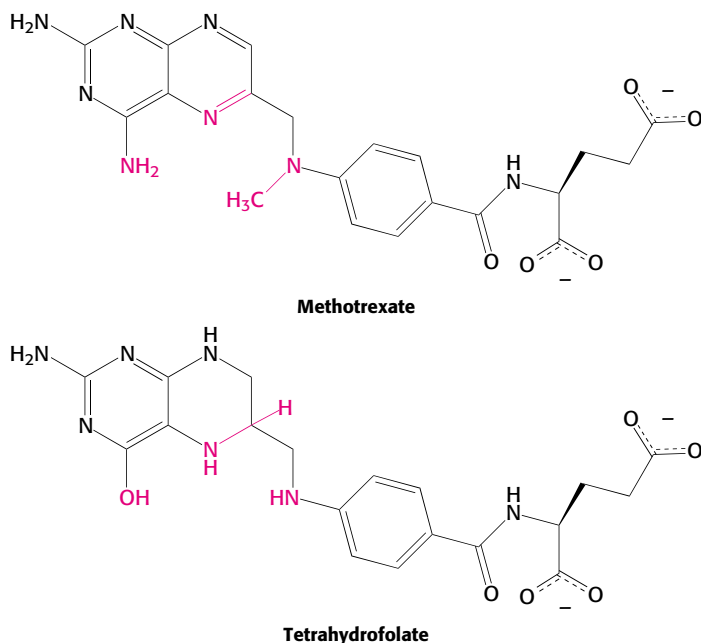


FIGURE 8.15 Distinction between a competitive and a noncompetitive inhibitor. (Top) enzyme–substrate complex; (middle) a competitive inhibitor binds at the active site and thus prevents the substrate from binding; (bottom) a noncompetitive inhibitor does not prevent the substrate from binding.

FIGURE 8.16 Enzyme inhibitors. The cofactor tetrahydrofolate and its structural analog methotrexate. Regions with structural differences are shown in red.

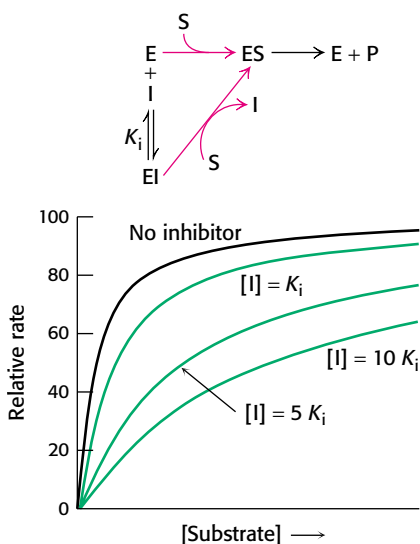


FIGURE 8.17 Kinetics of a competitive inhibitor. As the concentration of a competitive inhibitor increases, higher concentrations of substrate are required to attain a particular reaction velocity. The reaction pathway suggests how sufficiently high concentrations of substrate can completely relieve competitive inhibition.

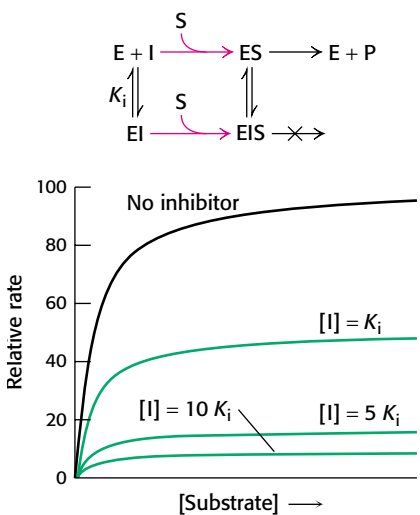


FIGURE 8.18 Kinetics of a noncompetitive inhibitor. The reaction pathway shows that the inhibitor binds both to free enzyme and to enzyme complex. Consequently, V_{\max} cannot be attained, even at high substrate concentrations.

In *noncompetitive inhibition*, which also is reversible, the inhibitor and substrate can bind simultaneously to an enzyme molecule at different binding sites (see Figure 8.16). A noncompetitive inhibitor acts by decreasing the turnover number rather than by diminishing the proportion of enzyme molecules that are bound to substrate. Noncompetitive inhibition, in contrast with competitive inhibition, cannot be overcome by increasing the substrate concentration. A more complex pattern, called *mixed inhibition*, is produced when a single inhibitor both hinders the binding of substrate and decreases the turnover number of the enzyme.

8.5.1 Competitive and Noncompetitive Inhibition Are Kinetically Distinguishable

How can we determine whether a reversible inhibitor acts by competitive or noncompetitive inhibition? Let us consider only enzymes that exhibit Michaelis-Menten kinetics. Measurements of the rates of catalysis at different concentrations of substrate and inhibitor serve to distinguish the two types of inhibition. In *competitive inhibition*, the inhibitor competes with the substrate for the active site. The dissociation constant for the inhibitor is given by

$$K_i = \frac{[E][I]}{[EI]}$$

Because increasing the amount of substrate can overcome the inhibition, V_{\max} can be attained in the presence of a competitive inhibitor (Figure 8.17). *The hallmark of competitive inhibition is that it can be overcome by a sufficiently high concentration of substrate.* However, the apparent value of K_M is altered; the effect of a competitive inhibitor is to increase the apparent value of K_M . This new value of K_M , called K_M^{app} , is numerically equal to

$$K_M^{\text{app}} = K_M(1 + [I]/K_i)$$

where $[I]$ is the concentration of inhibitor and K_i is the dissociation constant for the enzyme–inhibitor complex. As the value of $[I]$ increases, the value of K_M^{app} increases (see Figure 8.17). In the presence of a competitive inhibitor, an enzyme will have the same V_{\max} as in the absence of an inhibitor.

In *noncompetitive inhibition* (Figure 8.18), substrate can still bind to the enzyme–inhibitor complex. However, the enzyme–inhibitor–substrate complex *does not* proceed to form product. The value of V_{\max} is decreased to a new value called V_{\max}^{app} while the value of K_M is unchanged. Why is V_{\max} lowered while K_M remains unchanged? In essence, the inhibitor simply lowers the concentration of functional enzyme. The remaining enzyme behaves like a more dilute solution of enzyme; V_{\max} is lower, but K_M is the same. *Noncompetitive inhibition cannot be overcome by increasing the substrate concentration.*

8.5.2 Irreversible Inhibitors Can Be Used to Map the Active Site

In Chapter 9, we will examine the chemical details of how enzymes function. The first step in obtaining the chemical mechanism of an enzyme is to determine what functional groups are required for enzyme activity. How can we ascertain these functional groups? X-ray crystallography (Section 4.5.2) of the enzyme bound to its substrate provides one approach. Irreversible inhibitors that covalently bond to the enzyme provide an alternative and often complementary means for elucidating functional groups at the enzyme active site because they modify the functional groups, which can then be identified. Irreversible inhibitors can be divided into three categories: group-specific reagents, substrate analogs, and suicide inhibitors.

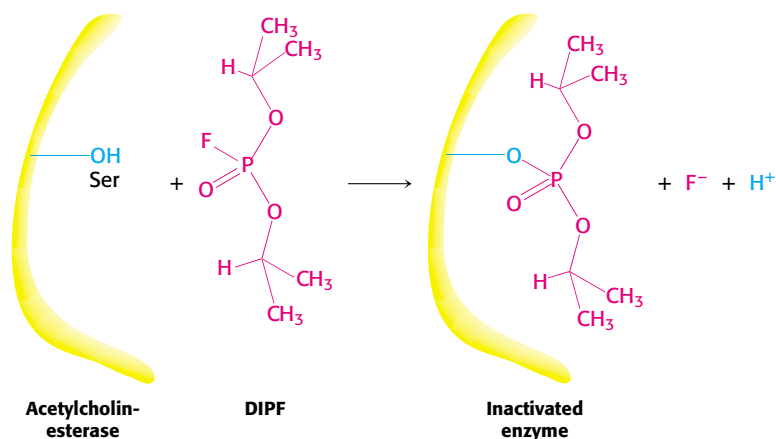


FIGURE 8.19 Enzyme inhibition by diisopropylphosphofluoridate (DIPF), a group-specific reagent. DIPF can inhibit an enzyme by covalently modifying a crucial serine residue (Section 9.1.1).

Group-specific reagents react with specific R groups of amino acids. Two examples of group-specific reagents are diisopropylphosphofluoridate (DIPF; Figure 8.19) and iodoacetamide (Figure 8.20). DIPF modifies only 1 of the 27 serine residues in the proteolytic enzyme chymotrypsin, implying that this serine residue is especially reactive. As we will see in Chapter 9, it is indeed the case that this serine residue is at the active site. DIPF also revealed a reactive serine residue in acetylcholinesterase, an enzyme important in the transmission of nerve impulses (see Figure 8.19). Thus, DIPF and similar compounds that bind and inactivate acetylcholinesterase are potent nerve gases.

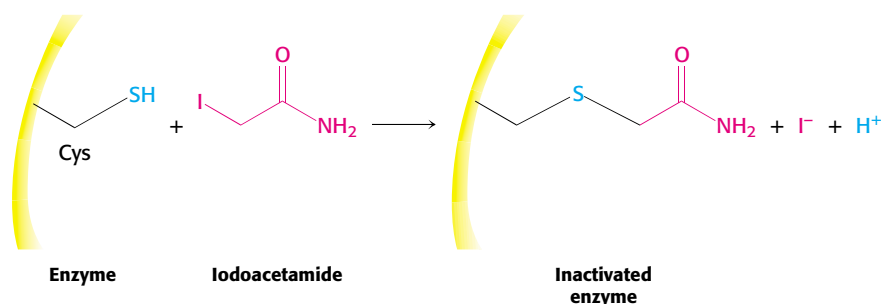
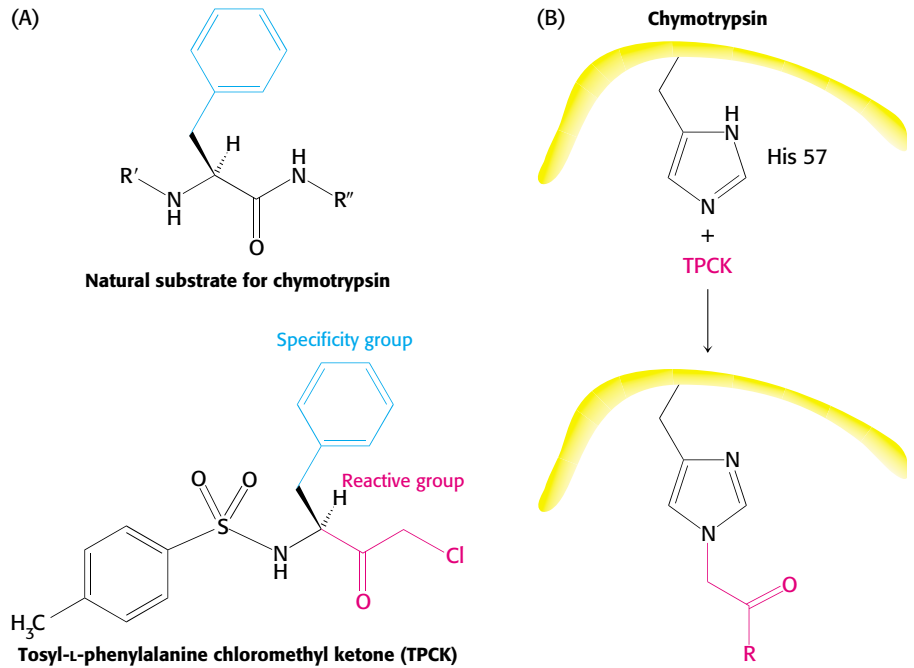


FIGURE 8.20 Enzyme inactivation by iodoacetamide, a group-specific reagent. Iodoacetamide can inactivate an enzyme by reacting with a critical cysteine residue.

Affinity labels are molecules that are structurally similar to the substrate for the enzyme that covalently modify active site residues. They are thus more specific for the enzyme active site than are group-specific reagents. Tosyl-L-phenylalanine chloromethyl ketone (TPCK) is a substrate analog for chymotrypsin (Figure 8.21). TPCK binds at the active site; and then reacts irreversibly with a histidine residue at that site, inhibiting the enzyme. The compound 3-bromoacetol is an affinity label for the enzyme triose phosphate isomerase (TIM). It mimics the normal substrate, dihydroxyacetone phosphate, by binding at the active site; then it covalently modifies the enzyme such that the enzyme is irreversibly inhibited (Figure 8.22).

Suicide inhibitors, or *mechanism-based inhibitors* are modified substrates that provide the most specific means to modify an enzyme active site. The inhibitor binds to the enzyme as a substrate and is initially processed by the normal catalytic mechanism. The mechanism of catalysis then generates a chemically reactive intermediate that inactivates the enzyme through covalent modification. The fact that the enzyme participates in its own irreversible inhibition strongly suggests that the covalently modified group on the enzyme is catalytically vital. One example of such an inhibitor is *N,N*-dimethylpropargylamine. A flavin prosthetic group of monoamine oxidase

**FIGURE 8.21 Affinity labeling.**

(A) Tosyl-L-phenylalanine chloromethyl ketone (TPCK) is a reactive analog of the normal substrate for the enzyme chymotrypsin. (B) TPCK binds at the active site of chymotrypsin and modifies an essential histidine residue.

FIGURE 8.22 Bromoacetyl phosphate, an affinity label for triose phosphate isomerase (TIM). Bromoacetyl phosphate, an analog of dihydroxyacetone phosphate, binds at the active site of the enzyme and covalently modifies a glutamic acid residue required for enzyme activity.

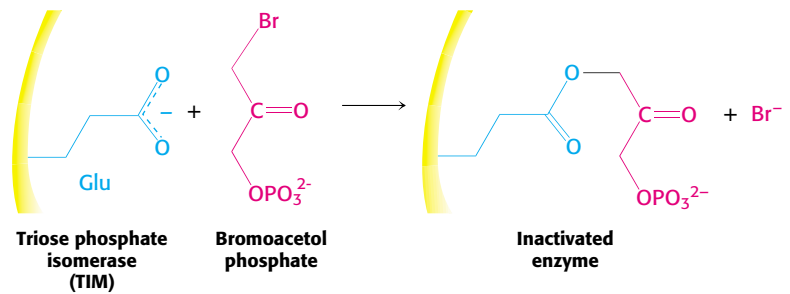
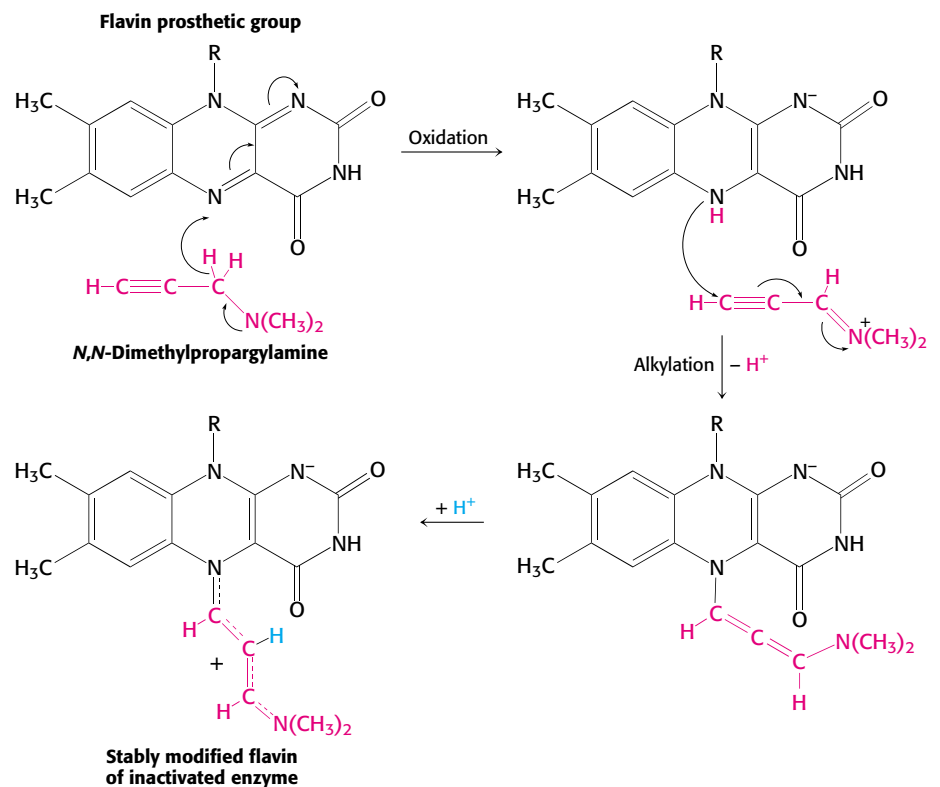


FIGURE 8.23 Mechanism-based (suicide) inhibition. Monoamine oxidase, an enzyme important for neurotransmitter synthesis, requires the cofactor FAD (flavin adenine dinucleotide). *N,N*-Dimethylpropargylamine inhibits monoamine oxidase by covalently modifying the flavin prosthetic group only after the inhibitor is first oxidized. The N-5 flavin adduct is stabilized by the addition of a proton.



(MAO) oxidizes the *N,N*-dimethylpropargylamine, which in turn inactivates the enzyme by covalently modifying the flavin prosthetic group by alkylating N-5 (Figure 8.23). Monoamine oxidase deaminates neurotransmitters such as dopamine and serotonin, lowering their levels in the brain. Parkinson disease is associated with low levels of dopamine, and depression is associated with low levels of serotonin. The drug (–)deprenyl, which is used to treat Parkinson disease and depression, is a suicide inhibitor of monoamine oxidase.

8.5.3 Transition-State Analogs Are Potent Inhibitors of Enzymes

We turn now to compounds that provide the most intimate views of the catalytic process itself. Linus Pauling proposed in 1948 that compounds resembling the transition state of a catalyzed reaction should be very effective inhibitors of enzymes. These mimics are called *transition-state analogs*. The inhibition of proline racemase is an instructive example. *The racemization of proline proceeds through a transition state in which the tetrahedral α -carbon atom has become trigonal by loss of a proton* (Figure 8.24). In the trigonal form, all three bonds are in the same plane; C_{α} also carries a net negative charge. This symmetric carbanion can be reprotonated on one side to give the L isomer or on the other side to give the D isomer. This picture is supported by the finding that the inhibitor pyrrole 2-carboxylate binds to the racemase 160 times as tightly as does proline. *The α -carbon atom of this inhibitor, like that of the transition state, is trigonal*. An analog that also carries a negative charge on C_{α} would be expected to bind even more tightly. In general, synthesizing compounds that more closely resemble the transition state than the substrate itself can produce highly potent and specific inhibitors of enzymes. The inhibitory power of transition-state analogs underscores the essence of catalysis: *selective binding of the transition state*.

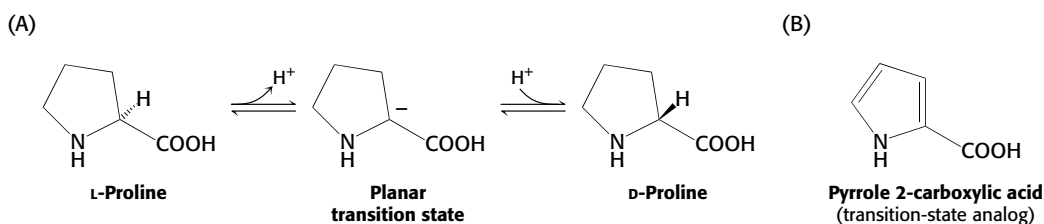
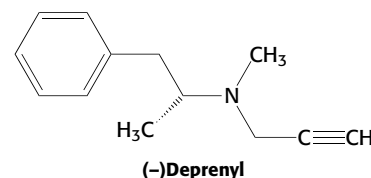


FIGURE 8.24 Inhibition by transition state analogs. (A) The isomerization of L-proline to D-proline by proline racemase, a bacterial enzyme, proceeds through a planar transition state in which the α carbon is trigonal rather than tetrahedral. (B) Pyrrole 2-carboxylate, a transition state analog because of its trigonal geometry, is a potent inhibitor of proline racemase.

8.5.4 Catalytic Antibodies Demonstrate the Importance of Selective Binding of the Transition State to Enzymatic Activity

Antibodies that recognize transition states should function as catalysts, if our understanding of the importance of the transition state to catalysis is correct. The preparation of an antibody that catalyzes the insertion of a metal ion into a porphyrin nicely illustrates the validity of this approach. Ferrochelatase, the final enzyme in the biosynthetic pathway for the production of heme, catalyzes the insertion of Fe^{2+} into protoporphyrin IX. The nearly planar porphyrin must be bent for iron to enter. The recently determined crystal structure of the ferrochelatase bound to a substrate analog confirms that the enzyme does indeed bend one of the pyrole rings, distorting it 36 degrees to insert the iron.

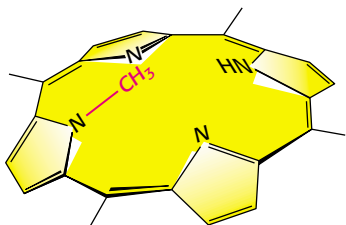


FIGURE 8.25 Use of transition-state analogs to generate catalytic antibodies. The insertion of a metal ion into a porphyrin by ferrochelatase proceeds through a transition state in which the porphyrin is bent. *N*-Methylmesoporphyrin, a bent porphyrin that resembles the transition state of the ferrochelatase-catalyzed reaction, was used to generate an antibody that also catalyzes the insertion of a metal ion into a porphyrin ring.

The problem was to find a transition-state analog for this metallation reaction that could be used as an antigen (immunogen) to generate an antibody. The solution came from the results of studies showing that an alkylated porphyrin, *N*-methylprotoporphyrin, is a potent inhibitor of ferrochelatase. This compound resembles the transition state because *N*-alkylation forces the porphyrin to be bent. Moreover, it was known that *N*-alkylporphyrins chelate metal ions 10^4 times as fast as their unalkylated counterparts do. Bending increases the exposure of the pyrrole nitrogen lone pairs of electrons to solvent, which facilitates metal ion binding.

An antibody catalyst was produced with the use of an *N*-alkylporphyrin as the immunogen. The resulting antibody presumably distorts a planar porphyrin (Figure 8.25) to facilitate the entry of a metal. On average, an antibody molecule metallated 80 porphyrin molecules per hour, a rate only 10-fold less than that of ferrochelatase and 2500-fold faster than the uncatalyzed reaction. *Catalytic antibodies (abzymes) can indeed be produced by using transition-state analogs as antigens.* Antibodies catalyzing many other kinds of chemical reactions—exemplified by ester and amide hydrolysis, amide-bond formation, transesterification, photoinduced cleavage, photoinduced dimerization, decarboxylation, and oxidation—have been produced with the use of similar strategies. The results of studies with transition-state analogs provide strong evidence that enzymes can function complementary in structure to the transition state. *The power of transition-state analogs is now evident: (1) they are sources of insight into catalytic mechanisms, (2) they can serve as potent and specific inhibitors of enzymes, and (3) they can be used as immunogens to generate a wide range of novel catalysts.*

8.5.5 Penicillin Irreversibly Inactivates a Key Enzyme in Bacterial Cell-Wall Synthesis



Penicillin, the first antibiotic discovered, consists of a thiazolidine ring fused to a β -lactam ring, to which a variable R group is attached by a peptide bond (Figure 8.26A). In benzyl penicillin, for example, R is a benzyl group (Figure 8.26B). This structure can undergo a variety of rearrangements, and, in particular, the β -lactam ring is very labile. Indeed, this instability is closely tied to the antibiotic action of penicillin, as will be evident shortly.

How does penicillin inhibit bacterial growth? In 1957, Joshua Lederberg showed that bacteria ordinarily susceptible to penicillin could be grown in its presence if a hypertonic medium were used. The organisms obtained in this way, called *protoplasts*, are devoid of a cell wall and consequently lyse when transferred to a normal medium. Hence, penicillin was inferred to interfere with the synthesis of the bacterial cell wall. The cell-wall macromolecule, called a *peptidoglycan*, consists of linear polysaccharide chains that

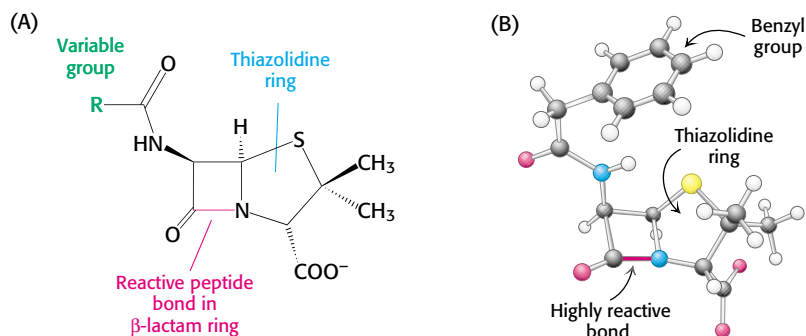


FIGURE 8.26 Structure of penicillin. The reactive site of penicillin is the peptide bond of its β -lactam ring. (A) Structural formula of penicillin. (B) Representation of benzyl penicillin.

are cross-linked by short peptides (Figure 8.27). The enormous bag-shaped peptidoglycan confers mechanical support and prevents bacteria from bursting in response to their high internal osmotic pressure.

In 1965, James Park and Jack Strominger independently deduced that penicillin blocks the last step in cell-wall synthesis—namely, the cross-linking of different peptidoglycan strands. In the formation of the cell wall of *Staphylococcus aureus*, the amino group at one end of a pentaglycine chain attacks the peptide bond between two D-alanine residues in another peptide unit (Figure 8.28). A peptide bond is formed between glycine and one of the D-alanine residues; the other D-alanine residue is released. This cross-linking reaction is catalyzed by *glycopeptide transpeptidase*. Bacterial cell walls are unique in containing D amino acids, which form cross-links by a mechanism different from that used to synthesize proteins.

Penicillin inhibits the cross-linking transpeptidase by the Trojan horse stratagem. The transpeptidase normally forms an *acyl intermediate* with the penultimate D-alanine residue of the D-Ala-D-Ala peptide (Figure 8.29). This covalent acyl-enzyme intermediate then reacts with the amino group of the terminal glycine in another peptide to form the cross-link. Penicillin is welcomed into the active site of the transpeptidase because it mimics the D-Ala-D-Ala moiety of the normal substrate (Figure 8.30). Bound penicillin then forms a covalent bond with a serine residue at the active site of the enzyme. *This penicilloyl-enzyme does not react further. Hence, the transpeptidase is irreversibly inhibited and cell-wall synthesis cannot take place.*

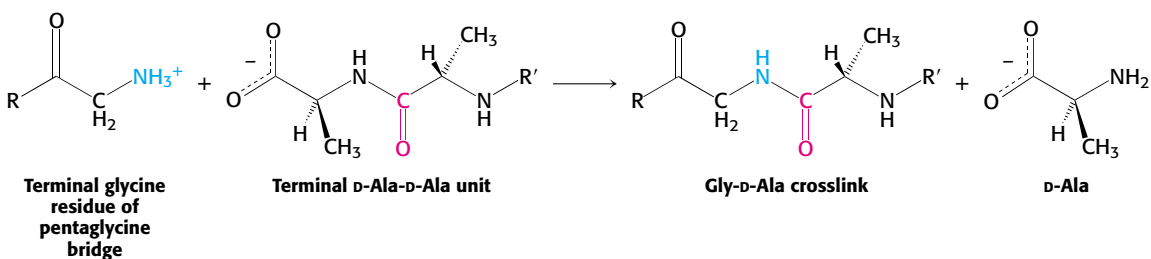


FIGURE 8.28 Formation of cross-links in *S. aureus* peptidoglycan. The terminal amino group of the pentaglycine bridge in the cell wall attacks the peptide bond between two D-alanine residues to form a cross-link.

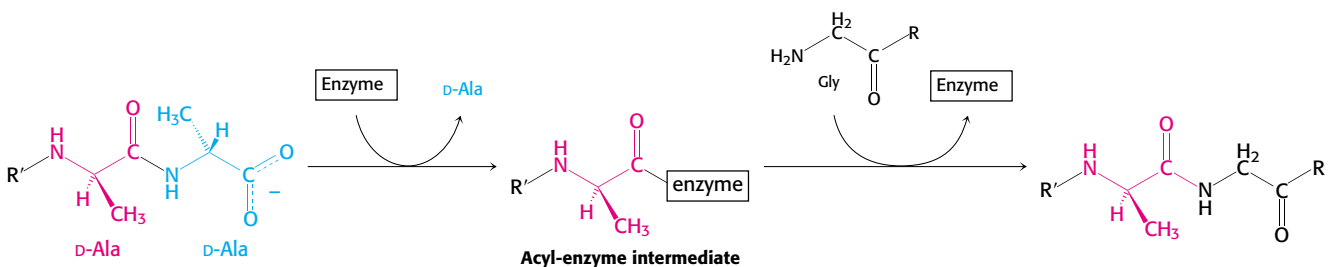


FIGURE 8.29 Transpeptidation reaction. An acyl-enzyme intermediate is formed in the transpeptidation reaction leading to cross-link formation.

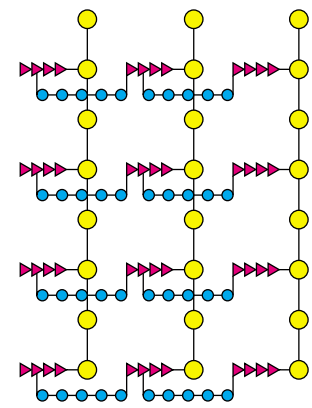
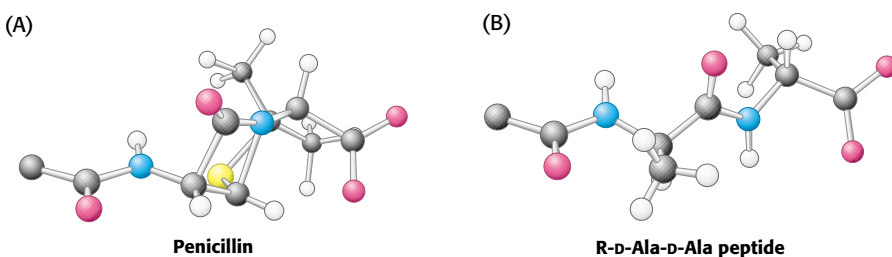
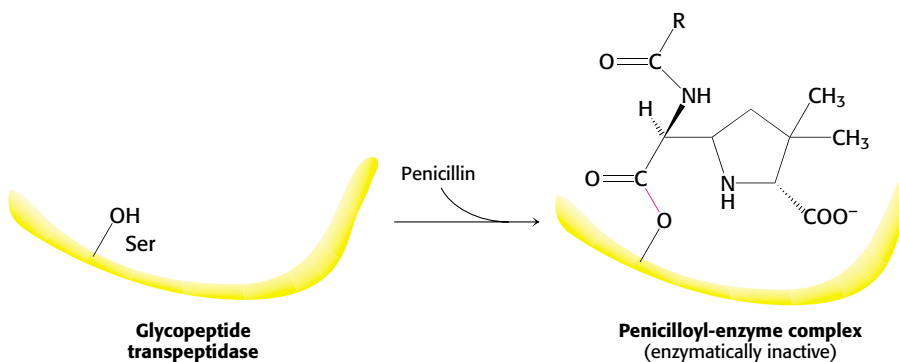


FIGURE 8.27 Schematic representation of the peptidoglycan in *Staphylococcus aureus*. The sugars are shown in yellow, the tetrapeptides in red, and the pentaglycine bridges in blue. The cell wall is a single, enormous, bag-shaped macromolecule because of extensive cross-linking.

FIGURE 8.30 Conformations of penicillin and a normal substrate. The conformation of penicillin in the vicinity of its reactive peptide bond (A) resembles the postulated conformation of the transition state of R-D-Ala-D-Ala (B) in the transpeptidation reaction. [After B. Lee. *J. Mol. Biol.* 61(1971):464.]

FIGURE 8.31 Formation of a penicilloyl-enzyme complex. Penicillin reacts with the transpeptidase to form an inactive complex, which is indefinitely stable.



Why is penicillin such an effective inhibitor of the transpeptidase? The highly strained, four-membered β -lactam ring of penicillin makes it especially reactive. On binding to the transpeptidase, the serine residue at the active site attacks the carbonyl carbon atom of the lactam ring to form the penicilloyl-serine derivative (Figure 8.31). Because the peptidase participates in its own inactivation, penicillin acts as a suicide inhibitor.

8.6 VITAMINS ARE OFTEN PRECURSORS TO COENZYMES



Earlier (Section 8.1.1), we considered the fact that many enzymes require cofactors to be catalytically active. One class of these cofactors, termed coenzymes, consists of small organic molecules, many of which are derived from *vitamins*. Vitamins themselves are organic molecules that are needed in small amounts in the diets of some higher animals. These mole-

TABLE 8.9 Water-Soluble Vitamins

Vitamin	Coenzyme	Typical reaction type	Consequences of deficiency
Thiamine (B_1)	Thiamine pyrophosphate	Aldehyde transfer	Beriberi (weight loss, heart problems, neurological dysfunction)
Riboflavin (B_2)	Flavin adenine dinucleotide (FAD)	Oxidation–reduction	Cheliosis and angular stomatitis (lesions of the mouth), dermatitis
Pyridoxine (B_6)	Pyridoxal phosphate	Group transfer to or from amino acids	Depression, confusion, convulsions
Nicotinic acid (niacin)	Nicotinamide adenine dinucleotide (NAD^+)	Oxidation–reduction	Pellagra (dermatitis, depression, diarrhea)
Pantothenic acid	Coenzyme A	Acyl–group transfer	Hypertension
Biotin	Biotin–lysine complexes (biocytin)	ATP-dependent carboxylation and carboxyl–group transfer	Rash about the eyebrows, muscle pain, fatigue (rare)
Folic acid	Tetrahydrofolate	Transfer of one-carbon components; thymine synthesis	Anemia, neural-tube defects in development
B_{12}	5'-Deoxyadenosyl cobalamin	Transfer of methyl groups; intramolecular rearrangements	Anemia, pernicious anemia, methylmalonic acidosis
C (ascorbic acid)		Antioxidant	Scurvy (swollen and bleeding gums, subdermal hemorrhages)

TABLE 8.10 Fat-soluble vitamins

Vitamin	Function	Deficiency
A	Antioxidant	Inhibition of sperm production; lesions in muscles and nerves (rare)
D	Regulation of calcium and phosphate metabolism	Rickets (children): skeletal deformities, impaired growth Osteomalacia (adults): soft, bending bones
E	Roles in vision, growth, reproduction	Night blindness, cornea damage, damage to respiratory and gastrointestinal tract
K	Blood coagulation	Subdermal hemorrhaging

cules serve the same roles in nearly all forms of life, but higher animals lost the capacity to synthesize them in the course of evolution. For instance, whereas *E. coli* can thrive on glucose and organic salts, human beings require at least 12 vitamins in the diet. The biosynthetic pathways for vitamins can be complex; thus, it is biologically more efficient to ingest vitamins than to synthesize the enzymes required to construct them from simple molecules. This efficiency comes at the cost of dependence on other organisms for chemicals essential for life. Indeed, vitamin deficiency can generate diseases in all organisms requiring these molecules (Tables 8.9 and 8.10). Vitamins can be grouped according to whether they are soluble in water or in nonpolar solvents.

8.6.1 Water-Soluble Vitamins Function As Coenzymes



Table 8.9 lists the *water-soluble vitamins*—ascorbic acid (vitamin C) and a series known as the vitamin B complex (Figure 8.32). Ascorbate, the ionized form of ascorbic acid, serves as a reducing agent (an antioxidant), as will be discussed shortly. The vitamin B series comprises components of coenzymes. Note that, in all cases except vitamin C, the vitamin must be modified before it can serve its function.

Vitamin deficiencies are capable of causing a variety of pathological conditions (see Table 8.9). However, many of the same symptoms can result from conditions other than lack of a vitamin. For this reason and because vitamins are required in relatively small amounts, pathological conditions resulting from vitamin deficiencies are often difficult to diagnose.

The requirement for vitamin C proved relatively straightforward to demonstrate. This water-soluble vitamin is not used as a coenzyme but is

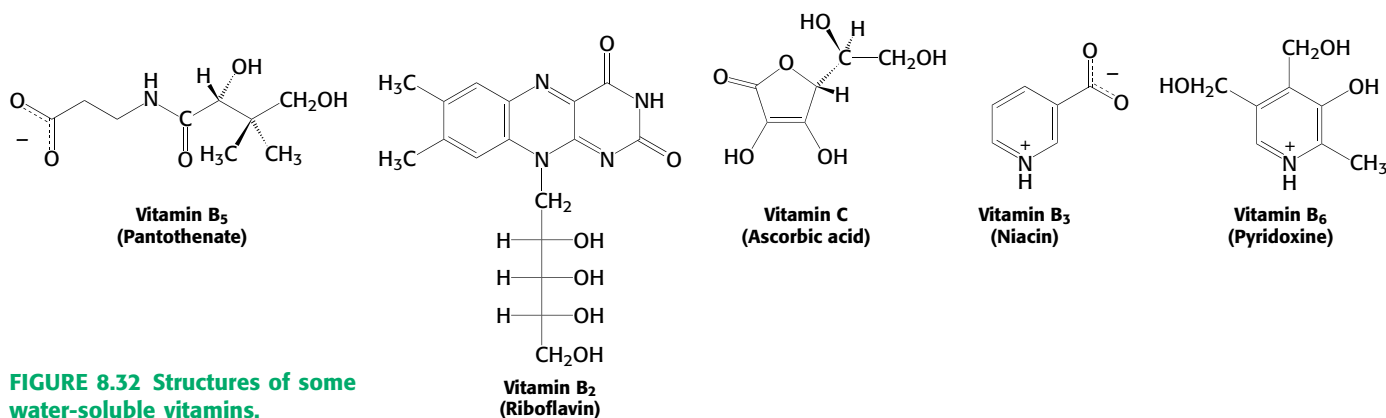


FIGURE 8.32 Structures of some water-soluble vitamins.

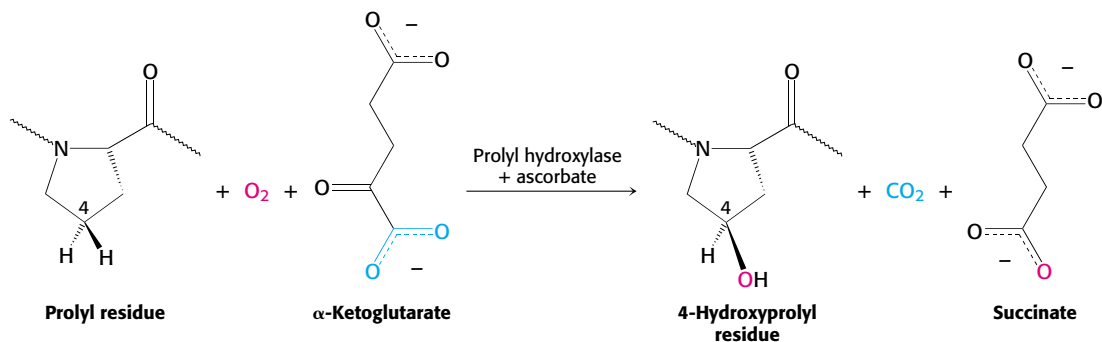


FIGURE 8.33 Formation of 4-hydroxyproline. Proline is hydroxylated at C-4 by the action of prolyl hydroxylase, an enzyme that activates molecular oxygen.

still required for the continued activity of prolyl hydroxylase. This enzyme synthesizes 4-hydroxyproline, an amino acid that is required in collagen, the major connective tissue in vertebrates, but is rarely found anywhere else. How is this unusual amino acid formed and what is its role? The results of radioactive-labeling studies showed that proline residues on the amino side of glycine residues in nascent collagen chains become hydroxylated. The oxygen atom that becomes attached to C-4 of proline comes from molecular oxygen, O_2 . The other oxygen atom of O_2 is taken up by α -ketoglutarate, which is converted into succinate (Figure 8.33). This complex reaction is catalyzed by *prolyl hydroxylase*, a *dioxygenase*. It is assisted by an Fe^{2+} ion, which is tightly bound to it and needed to activate O_2 . The enzyme also converts α -ketoglutarate into succinate without hydroxylating proline. In this partial reaction, an oxidized iron complex is formed, which inactivates the enzyme. How is the active enzyme regenerated? *Ascorbate* (vitamin C) comes to the rescue by reducing the ferric ion of the inactivated enzyme. In the recovery process, ascorbate is oxidized to dehydroascorbic acid (Figure 8.34). Thus, ascorbate serves here as a specific *antioxidant*.

Primates are unable to synthesize ascorbic acid and hence must acquire it from their diets. The importance of ascorbate becomes strikingly evident in *scurvy*. Jacques Cartier in 1536 gave a vivid description of this dietary deficiency disease, which afflicted his men as they were exploring the Saint Lawrence River:

Some did lose all their strength, and could not stand on their feet. ... Others also had all their skins spotted with spots of blood of a purple colour: then did it ascend up to their ankles, knees, thighs, shoulders, arms, and necks. Their mouths became stinking, their gums so rotten, that all the flesh did fall off, even to the roots of the teeth, which did also almost all fall out.

James Lind, a Scottish physician, illuminated the means of preventing scurvy in an article titled "A Treatise of the Scurvy" published in 1747. Lind described a controlled study establishing that scurvy could be prevented by including citrus fruits in the diet. The Royal Navy eventually began issuing lime rations to sailors, from which custom British sailors acquired the nickname "limeys." Lind's research was inspired by the plight of an expedition commanded by Commodore George Anson. Anson left England in 1740 with a fleet of six ships and more than 1000 men and returned with an enormous amount of treasure, but of his crew only 145 survived to reach home. The remainder had died of scurvy.

Why does impaired hydroxylation have such devastating consequences? *Collagen synthesized in the absence of ascorbate is less stable than the normal protein.* Studies of the thermal stability of synthetic polypeptides have been

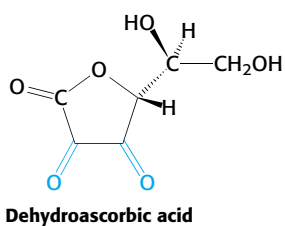
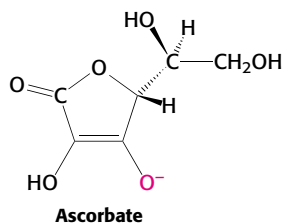
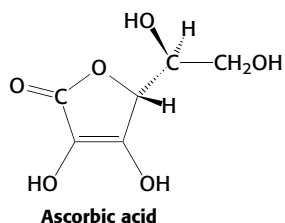


FIGURE 8.34 Forms of ascorbic acid (vitamin C). Ascorbate is the ionized form of vitamin C, and dehydroascorbic acid is the oxidized form of ascorbate.

especially informative. Hydroxyproline stabilizes the collagen triple helix by forming interstrand hydrogen bonds. The abnormal fibers formed by insufficiently hydroxylated collagen contribute to the skin lesions and blood-vessel fragility seen in scurvy.

8.6.2 Fat-Soluble Vitamins Participate in Diverse Processes Such as Blood Clotting and Vision

Not all vitamins function as coenzymes. The *fat-soluble vitamins*, which are designated by the letters A, D, E, and K (Figure 8.35, Table 8.10), have a diverse array of functions. Vitamin K, which is required for normal blood clotting (*K* from the German *koagulation*), participates in the carboxylation of glutamate residues to γ -carboxyglutamate, which makes modified glutamic acid a much stronger chelator of Ca^{2+} (Section 10.7.7). Vitamin A (retinol) is the precursor of retinal, the light-sensitive group in rhodopsin and other visual pigments (Section 32.3.1). A deficiency of this vitamin leads to night blindness. In addition, young animals require vitamin A for growth. Retinoic acid, which contains a terminal carboxylate in place of the alcohol terminus of retinol, serves as a signal molecule and activates the transcription of specific genes that mediate growth and development (Section 31.x.x). A metabolite of vitamin D is a hormone that regulates the metabolism of calcium and phosphorus. A deficiency in vitamin D impairs bone formation in growing animals. Infertility in rats is a consequence of vitamin E (α -tocopherol) deficiency. This vitamin reacts with and neutralizes reactive oxygen species such as hydroxyl radicals before they can oxidize unsaturated membrane lipids, damaging cell structures.

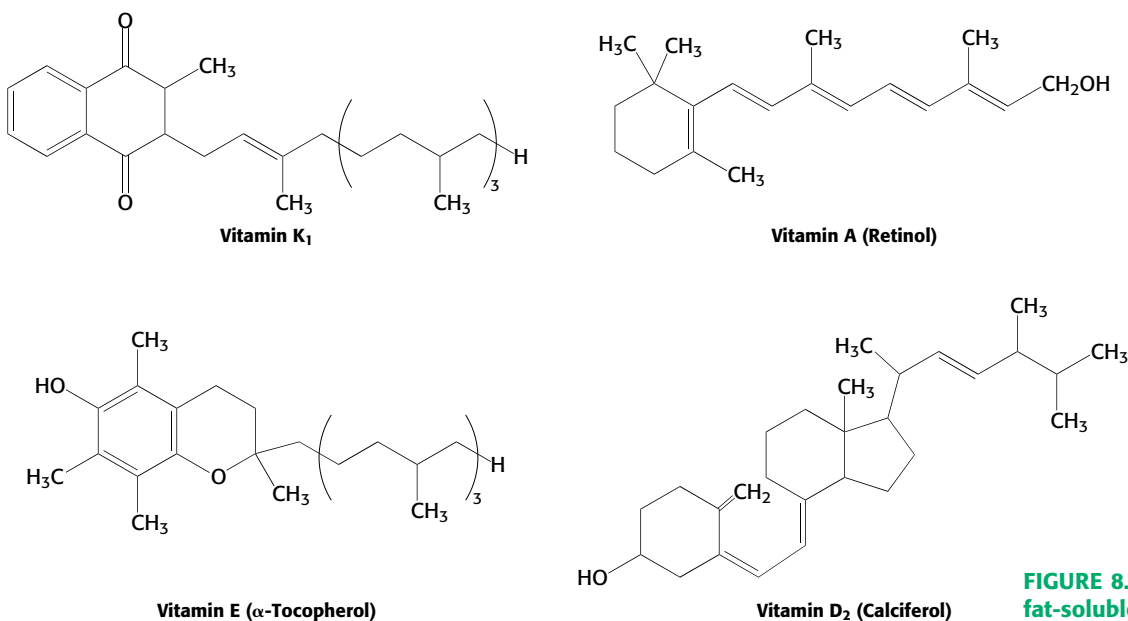


FIGURE 8.35 Structures of some fat-soluble vitamins.

SUMMARY

Enzymes are Powerful and Highly Specific Catalysts

The catalysts in biological systems are enzymes, and nearly all enzymes are proteins. Enzymes are highly specific and have great catalytic power. They can enhance reaction rates by factors of 10^6 or more. Many enzymes require cofactors for activity. Such cofactors can be metal ions or small, vitamin-derived organic molecules called coenzymes.

• Free Energy Is a Useful Thermodynamic Function for Understanding Enzymes

Free energy (G) is the most valuable thermodynamic function for determining whether a reaction can take place and for understanding the energetics of catalysis. A reaction can occur spontaneously only if the change in free energy (ΔG) is negative. The free-energy change of a reaction that takes place when reactants and products are at unit activity is called the standard free-energy change (ΔG°). Biochemists usually use $\Delta G^\circ'$, the standard free-energy change at pH 7. Enzymes do not alter reaction equilibria; rather, they increase reaction rates.

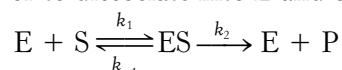
• Enzymes Accelerate Reactions by Facilitating the Formation of the Transition State

Enzymes serve as catalysts by decreasing the free energy of activation of chemical reactions. Enzymes accelerate reactions by providing a reaction pathway in which the transition state (the highest-energy species) has a lower free energy and hence is more rapidly formed than in the uncatalyzed reaction.

The first step in catalysis is the formation of an enzyme–substrate complex. Substrates are bound to enzymes at active-site clefts from which water is largely excluded when the substrate is bound. The specificity of enzyme–substrate interactions arises mainly from hydrogen bonding, which is directional, and the shape of the active site, which rejects molecules that do not have a sufficiently complementary shape. The recognition of substrates by enzymes is accompanied by conformational changes at active sites, and such changes facilitate the formation of the transition state.

• The Michaelis-Menten Model Accounts for the Kinetic Properties of Many Enzymes

The Michaelis-Menten model accounts for the kinetic properties of some enzymes. In this model, an enzyme (E) combines with a substrate (S) to form an enzyme–substrate (ES) complex, which can proceed to form a product (P) or to dissociate into E and S.



The rate V_0 of formation of product is given by the Michaelis-Menten equation:

$$V_0 = V_{\max} \frac{[S]}{[S] + K_M}$$

in which V_{\max} is the reaction rate when the enzyme is fully saturated with substrate and K_M , the Michaelis constant, is the substrate concentration at which the reaction rate is half maximal. The maximal rate, V_{\max} , is equal to the product of k_2 or k_{cat} and the total concentration of enzyme. The kinetic constant k_{cat} , called the turnover number, is the number of substrate molecules converted into product per unit time at a single catalytic site when the enzyme is fully saturated with substrate. Turnover numbers for most enzymes are between 1 and 10^4 per second. The ratio of k_{cat}/K_M provides a penetrating probe into enzyme efficiency.

Allosteric enzymes constitute an important class of enzymes whose catalytic activity can be regulated. These enzymes, which do not conform to Michaelis-Menten kinetics, have multiple active sites. These active sites display cooperativity, as evidenced by a sigmoidal dependence of reaction velocity on substrate concentration.

Enzymes Can Be Inhibited by Specific Molecules

Specific small molecules or ions can inhibit even nonallosteric enzymes. In irreversible inhibition, the inhibitor is covalently linked to the enzyme or bound so tightly that its dissociation from the enzyme is very slow. Covalent inhibitors provide a means of mapping the enzyme's active site. In contrast, reversible inhibition is characterized by a rapid equilibrium between enzyme and inhibitor. A competitive inhibitor prevents the substrate from binding to the active site. It reduces the reaction velocity by diminishing the proportion of enzyme molecules that are bound to substrate. In noncompetitive inhibition, the inhibitor decreases the turnover number. Competitive inhibition can be distinguished from noncompetitive inhibition by determining whether the inhibition can be overcome by raising the substrate concentration.

The essence of catalysis is selective stabilization of the transition state. Hence, an enzyme binds the transition state more tightly than the substrate. Transition-state analogs are stable compounds that mimic key features of this highest-energy species. They are potent and specific inhibitors of enzymes. Proof that transition-state stabilization is a key aspect of enzyme activity comes from the generation of catalytic antibodies. Transition-state analogs are used as antigens, or immunogens, in generating catalytic antibodies.

Vitamins Are Often Precursors to Coenzymes

Vitamins are small biomolecules that are needed in small amounts in the diets of higher animals. The water-soluble vitamins are vitamin C (ascorbate, an antioxidant) and the vitamin B complex (components of coenzymes). Ascorbate is required for the hydroxylation of proline residues in collagen, a key protein of connective tissue. The fat-soluble vitamins are vitamin A (a precursor of retinal), D (a regulator of calcium and phosphorus metabolism), E (an antioxidant in membranes), and K (a participant in the carboxylation of glutamate).

APPENDIX: V_{\max} AND K_M CAN BE DETERMINED BY DOUBLE-RECIPROCAL PLOTS

Before the availability of computers, the determination of K_M and V_{\max} values required algebraic manipulation of the basic Michaelis-Menten equation. Because V_{\max} is approached asymptotically (see Figure 8.11), it is impossible to obtain a definitive value from a typical Michaelis-Menten plot. Because K_M is the concentration of substrate at $V_{\max}/2$, it is likewise impossible to determine an accurate value of K_M . However, V_{\max} can be accurately determined if the Michaelis-Menten equation is transformed into one that gives a straight-line plot. Taking the reciprocal of both sides of equation 23 gives

$$\frac{1}{V_0} = \frac{1}{V_{\max}} + \frac{K_M}{V_{\max}} \cdot \frac{1}{[S]} \quad (31)$$

A plot of $1/V_0$ versus $1/[S]$, called a *Lineweaver-Burk* or *double-reciprocal plot*, yields a straight line with an intercept of $1/V_{\max}$ and a slope of K_M/V_{\max} (Figure 8.36). The intercept on the y-axis is $1/V_{\max}$.

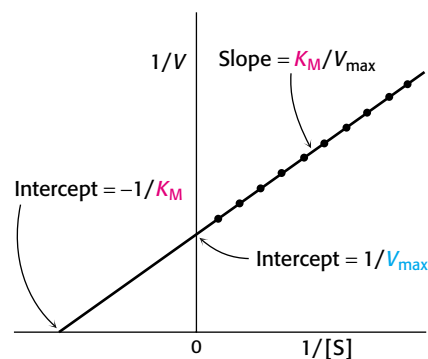


FIGURE 8.36 A double-reciprocal or Lineweaver-Burk plot. A double-reciprocal plot of enzyme kinetics is generated by plotting $1/V_0$ as a function $1/[S]$. The slope is the K_M/V_{\max} , the intercept on the vertical axis is $1/V_{\max}$, and the intercept on the horizontal axis is $-1/K_M$.

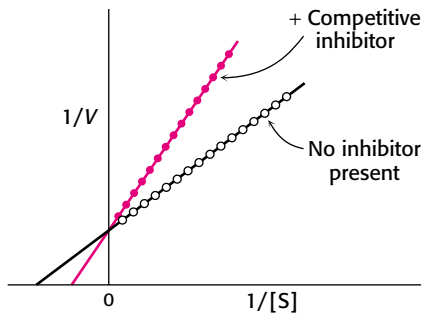


FIGURE 8.37 Competitive inhibition illustrated on a double-reciprocal plot. A double-reciprocal plot of enzyme kinetics in the presence (—▲—▲—▲) and absence (—○—○—○) of a competitive inhibitor illustrates that the inhibitor has no effect on V_{\max} but increases K_M .

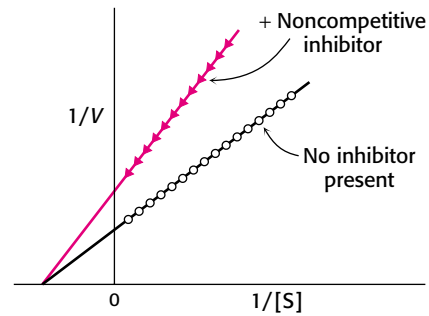
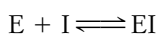


FIGURE 8.38 Noncompetitive inhibition illustrated on a double-reciprocal plot. A double-reciprocal plot of enzyme kinetics in the presence (—▲—▲—▲) and absence (—○—○—○) of a noncompetitive inhibitor shows that K_M is unaltered and V_{\max} is decreased.

Double-reciprocal plots are especially useful for distinguishing between competitive and noncompetitive inhibitors. In competitive inhibition, the intercept on the y-axis of the plot of $1/V_0$ versus $1/[S]$ is the same in the presence and in the absence of inhibitor, although the slope is increased (Figure 8.37). That the intercept is unchanged is because a competitive inhibitor does not alter V_{\max} . At a sufficiently high concentration, virtually all the active sites are filled by substrate, and the enzyme is fully operative. The increase in the slope of the $1/V_0$ versus $1/[S]$ plot indicates the strength of binding of competitive inhibitor. In the presence of a competitive inhibitor, equation 31 is replaced by

$$\frac{1}{V_0} = \frac{1}{V_{\max}} + \frac{K_M}{V_{\max}} \left(1 + \frac{[I]}{K_i} \right) \left(\frac{1}{[S]} \right)$$

in which $[I]$ is the concentration of inhibitor and K_i is the dissociation constant of the enzyme–inhibitor complex.



$$K_i = \frac{[E][I]}{[EI]}$$

In other words, the slope of the plot is increased by the factor $(1 + [I]/K_i)$ in the presence of a competitive inhibitor. Consider an enzyme with a K_M of 10^{-4} M. In the absence of inhibitor, $V_0 = V_{\max}/2$ when $[S] = 10^{-4}$ M. In the presence of 2×10^{-3} M competitive inhibitor that is bound to the enzyme with a K_i of 10^{-3} M, the apparent K_M (K_M^{app}) will be equal to $K_M \times (1 + [I]/K_i)$, or 3×10^{-4} M. Substitution of these values into equation 23 gives $V_0 = V_{\max}/4$, when $[S] = 10^{-4}$ M. The presence of this competitive inhibitor thus cuts the reaction rate in half at this substrate concentration.

In noncompetitive inhibition (Figure 8.38), the inhibitor can combine with either the enzyme or the enzyme–substrate complex. In pure noncompetitive inhibition, the values of the dissociation constants of the inhibitor and enzyme and of the inhibitor and enzyme–substrate complex are equal (Section 8.5.1). The value of V_{\max} is decreased to a new value called V_{\max}^{app} , and so the intercept on the vertical axis is increased. The new slope, which is equal to $K_M/V_{\max}^{\text{app}}$, is larger by the same factor. In contrast with V_{\max} , K_M is not affected by pure noncompetitive inhibition. The maximal velocity in the presence of a pure noncompetitive inhibitor, V_{\max}^{app} , is given by

$$V_{\max}^{\text{app}} = \frac{V_{\max}}{1 + [I]/K_i}$$

KEY TERMS

enzyme (p. 189)
 substrate (p. 190)
 cofactor (p. 191)
 apoenzyme (p. 191)
 holoenzyme (p. 191)
 coenzyme (p. 192)
 prosthetic group (p. 192)
 free energy (p. 193)
 transition state (p. 196)
 free energy of activation (p. 196)
 active site (p. 197)

induced fit (p. 200)
 K_M (the Michaelis constant) (p. 202)
 V_{\max} (p. 203)
 Michaelis-Menten equation (p. 203)
 turnover number (p. 204)
 k_{cat}/K_M (p. 205)
 sequential displacement reaction (p. 207)
 double-displacement (Ping-Pong) reaction (p. 207)

allosteric enzyme (p. 208)
 competitive inhibition (p. 209)
 noncompetitive inhibition (p. 210)
 group-specific reagent (p. 211)
 affinity label (p. 211)
 mechanism-based (suicide) inhibition (p. 211)
 transition-state analog (p. 213)
 catalytic antibody (abzyme) (p. 214)
 vitamin (p. 216)

SELECTED READINGS

Where to start

- Koshland, D. E., Jr., 1987. Evolution of catalytic function. *Cold Spring Harbor Symp. Quant. Biol.* 52:1–7.
- Jencks, W. P., 1987. Economics of enzyme catalysis. *Cold Spring Harbor Symp. Quant. Biol.* 52:65–73.
- Lerner, R. A., and Tramontano, A., 1988. Catalytic antibodies. *Sci. Am.* 258(3):58–70.

Books

- Fersht, A., 1999. *Structure and Mechanism in Protein Science: A Guide to Enzyme Catalysis and Protein Folding*. W. H. Freeman and Company.
- Walsh, C., 1979. *Enzymatic Reaction Mechanisms*. W. H. Freeman and Company.
- Page, M. I., and Williams, A. (Eds.), 1987. *Enzyme Mechanisms*. Royal Society of Chemistry.
- Bender, M. L., Bergeron, R. J., and Komiya, M., 1984. *The Bioorganic Chemistry of Enzymatic Catalysis*. Wiley-Interscience.
- Abelson, J. N., and Simon, M. I. (Eds.), 1992. *Methods in Enzymology*. Academic Press.
- Boyer, P. D. (Ed.), 1970. *The Enzymes* (3d ed.). Academic Press.
- Friedmann, H. (ed.), 1981. *Benchmark Papers in Biochemistry*. Vol. 1, *Enzymes*. Hutchinson Ross.

Transition-state stabilization, analogs, and other enzyme inhibitors

- Schramm, V. L., 1998. Enzymatic transition states and transition state analog design. *Annu. Rev. Biochem.* 67:693–720.
- Pauling, L., 1948. Nature of forces between large molecules of biological interest. *Nature* 161:707–709.
- Leinhard, G. E., 1973. Enzymatic catalysis and transition-state theory. *Science* 180:149–154.
- Kraut, J., 1988. How do enzymes work? *Science* 242:533–540.
- Waxman, D. J., and Strominger, J. L., 1983. Penicillin-binding proteins and the mechanism of action of β -lactam antibiotics. *Annu. Rev. Biochem.* 52:825–869.
- Abraham, E. P., 1981. The β -lactam antibiotics. *Sci. Am.* 244:76–86.
- Walsh, C. T., 1984. Suicide substrates, mechanism-based enzyme inactivators: Recent developments. *Annu. Rev. Biochem.* 53:493–535.

Catalytic antibodies

- Hilvert, D., 2000. Critical analysis of antibody catalysis. *Annu. Rev. Biochem.* 69:751–794.
- Wade, H., and Scanlan, T. S., 1997. The structural and functional basis of antibody catalysis. *Annu. Rev. Biophys. Biomol. Struct.* 26:461–493.
- Lerner, R. A., Benkovic, S. J., and Schultz, P. G., 1991. At the crossroads of chemistry and immunology: Catalytic antibodies. *Science* 252:659–667.
- Cochran, A. G., and Schultz, P. G., 1990. Antibody-catalyzed porphyrin metallation. *Science* 249:781–783.

Enzyme kinetics and mechanisms

- Xie, X. S., and Lu, H. P., 1999. Single-molecule enzymology. *J. Biol. Chem.* 274:15967–15970.
- Miles, E. W., Rhee, S., and Davies, D. R., 1999. The molecular basis of substrate channeling. *J. Biol. Chem.* 274:12193–12196.
- Warshel, A., 1998. Electrostatic origin of the catalytic power of enzymes and the role of preorganized active sites. *J. Biol. Chem.* 273:27035–27038.
- Cannon, W. R., and Benkovic, S. J., 1999. Solvation, reorganization energy, and biological catalysis. *J. Biol. Chem.* 273:26257–26260.
- Cleland, W. W., Frey, P. A., and Gerlt, J. A., 1998. The low barrier hydrogen bond in enzymatic catalysis. *J. Biol. Chem.* 273:25529–25532.
- Romesberg, F. E., Santarsiero, B. D., Spiller, B., Yin, J., Barnes, D., Schultz, P. G., and Stevens, R. C., 1998. Structural and kinetic evidence for strain in biological catalysis. *Biochemistry* 37:14404–14409.
- Lu, H. P., Xun, L., and Xie, X. S., 1998. Single-molecule enzymatic dynamics. *Science* 282:1877–1882.
- Fersht, A. R., Leatherbarrow, R. J., and Wells, T. N. C., 1986. Binding energy and catalysis: A lesson from protein engineering of the tyrosyl-tRNA synthetase. *Trends Biochem. Sci.* 11:321–325.
- Jencks, W. P., 1975. Binding energy, specificity, and enzymic catalysis: The Circe effect. *Adv. Enzymol.* 43:219–410.
- Knowles, J. R., and Alberty, W. J., 1976. Evolution of enzyme function and the development of catalytic efficiency. *Biochemistry* 15:5631–5640.

PROBLEMS

1. *Hydrolytic driving force*. The hydrolysis of pyrophosphate to orthophosphate is important in driving forward biosynthetic reactions such as the synthesis of DNA. This hydrolytic reaction is catalyzed in *Escherichia coli* by a pyrophosphatase that has a mass of 120 kd and consists of six identical subunits. For this enzyme, a unit of activity is defined as the amount of enzyme that hydrolyzes 10 μmol of pyrophosphate in 15 minutes at 37°C under standard assay conditions. The purified enzyme has a V_{max} of 2800 units per milligram of enzyme.

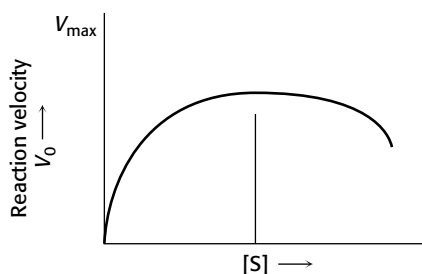
- (a) How many moles of substrate are hydrolyzed per second per milligram of enzyme when the substrate concentration is much greater than K_M ?
- (b) How many moles of active site are there in 1 mg of enzyme? Assume that each subunit has one active site.
- (c) What is the turnover number of the enzyme? Compare this value with others mentioned in this chapter.

2. *Destroying the Trojan horse*. Penicillin is hydrolyzed and thereby rendered inactive by penicillinase (also known as β -lac

tamase), an enzyme present in some resistant bacteria. The mass of this enzyme in *Staphylococcus aureus* is 29.6 kd. The amount of penicillin hydrolyzed in 1 minute in a 10-ml solution containing 10^{-9} g of purified penicillinase was measured as a function of the concentration of penicillin. Assume that the concentration of penicillin does not change appreciably during the assay.

[Penicillin] (μM)	Amount hydrolyzed (nanomoles)
1	0.11
3	0.25
5	0.34
10	0.45
30	0.58
50	0.61

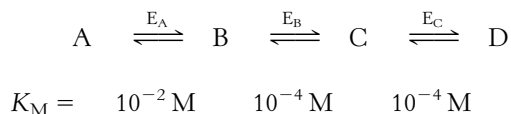
13. *Too much of a good thing.* A simple Michaelis-Menten enzyme, in the absence of any inhibitor, displayed the following kinetic behavior. The expected value of V_{\max} is shown on the y-axis.



(a) Draw a double-reciprocal plot that corresponds to the velocity-versus-substrate curve.

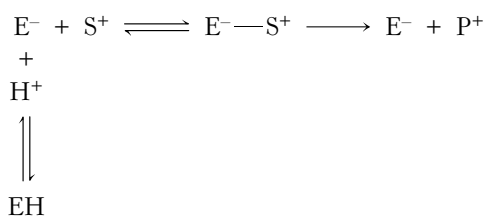
(b) Provide an explanation for the kinetic results.

14. *Rate-limiting step.* In the conversion of A into D in the following biochemical pathway, enzymes E_A , E_B , and E_C have the K_M values indicated under each enzyme. If all of the substrates and products are present at a concentration of 10^{-4} M, which step will be rate limiting and why?



Chapter Integration Problems

15. *Titration experiment.* The effect of pH on the activity of an enzyme was examined. At its active site, the enzyme has an ionizable group that must be negatively charged for substrate binding and catalysis to take place. The ionizable group has a pK_a of 6.0. The substrate is positively charged throughout the pH range of the experiment.

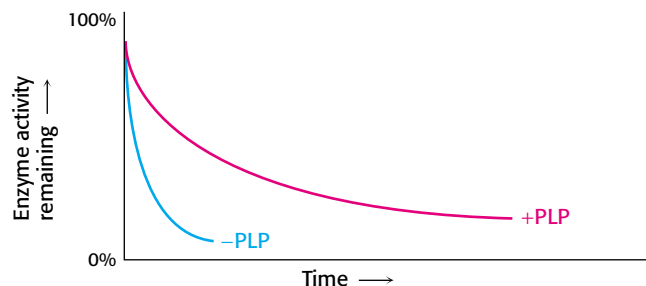


(a) Draw the V_0 -versus-pH curve when the substrate concentration is much greater than the enzyme K_M .

(b) Draw the V_0 -versus-pH curve when the substrate concentration is much less than the enzyme K_M .

(c) At which pH will the velocity equal one-half of the maximal velocity attainable under these conditions?

16. *A question of stability.* Pyridoxal phosphate (PLP) is a coenzyme for the enzyme ornithine aminotransferase. The enzyme was purified from cells grown in PLP-deficient media as well as from cells grown in media that contained pyridoxal phosphate. The stability of the enzyme was then measured by incubating the enzyme at 37°C and assaying for the amount of enzyme activity remaining. The following results were obtained.



(a) Why does the amount of active enzyme decrease with the time of incubation?

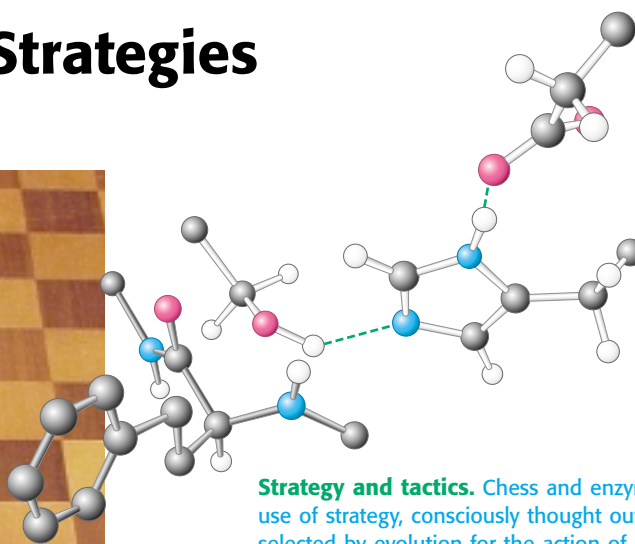
(b) Why does the amount of enzyme from the PLP-deficient cells decline more rapidly?



Media Problem

17. *Not all data points are created equal.* Your lab partner, who is both systematic and frugal, decides to perform a series of enzyme assays at substrate concentrations of 1, 2, 4, and $8 \mu\text{M}$. You argue for doing the experiments at $[S] = 1, 4, 16,$ and $100 \mu\text{M}$. Try both sets of experiments using the simulated enzyme kinetics lab in the Steady-State Enzyme Kinetics **Conceptual Insights** module. Who had the better idea, and why?

Catalytic Strategies



Strategy and tactics. Chess and enzymes have in common the use of strategy, consciously thought out in the game of chess and selected by evolution for the action of an enzyme. The three amino acid residues at the right, denoted by the white bonds, constitute a catalytic triad found in the active site of a class of enzymes that cleave peptide bonds. The substrate, represented by the molecule with black bonds, is as hopelessly trapped as the king in the photograph of a chess match at the left and is sure to be cleaved. [Photograph courtesy of Wendie Berg.]

What are the sources of the catalytic power and specificity of enzymes? This chapter presents the catalytic strategies used by four classes of enzymes: the serine proteases, carbonic anhydrases, restriction endonucleases, and nucleoside monophosphate (NMP) kinases. The first three classes of enzymes catalyze reactions that require the addition of water to a substrate. For the serine proteases, exemplified by chymotrypsin, the challenge is to promote a reaction that is almost immeasurably slow at neutral pH in the absence of a catalyst. For carbonic anhydrases, the challenge is to achieve a high absolute rate of reaction, suitable for integration with other rapid physiological processes. For restriction endonucleases such as *EcoRV*, the challenge is to attain a very high level of specificity. Finally, for NMP kinases, the challenge is to transfer a phosphoryl group from ATP to a nucleotide and not to water. The actions of these enzymes illustrate many important principles of catalysis. The mechanisms of these enzymes have been revealed through the use of incisive experimental probes, including the techniques of protein structure determination (Chapter 4) and site-directed mutagenesis (Chapter 6). These mechanisms include the use of binding energy and induced fit as well as several specific catalytic strategies. Properties common to an enzyme family reveal how their enzyme active sites have evolved and been refined. Structural and mechanistic comparisons of enzyme action are thus sources of insight into the evolutionary history of enzymes. These comparisons also reveal particularly effective solutions to biochemical problems that are used repeatedly in biological

OUTLINE

- **9.1 Proteases: Facilitating a Difficult Reaction**
- **9.2 Carbonic Anhydrases: Making a Fast Reaction Faster**
- **9.3 Restriction Enzymes: Performing Highly Specific DNA Cleavage Reactions**
- **9.4 Nucleoside Monophosphate Kinases: Catalyzing Phosphoryl Group Exchange Without Promoting Hydrolysis**

systems. In addition, our knowledge of catalytic strategies has been used to develop practical applications, including drugs that are potent and specific enzyme inhibitors. Finally, although we shall not consider catalytic RNA molecules (Section 28.x.x) explicitly in this chapter, the principles apply to these catalysts in addition to protein catalysts.

9.0.1 A Few Basic Catalytic Principles Are Used by Many Enzymes

In Chapter 8, we learned that enzymatic catalysis begins with substrate binding. The *binding energy* is the free energy released in the formation of a large number of weak interactions between the enzyme and the substrate. We can envision this binding energy as serving two purposes: it establishes substrate specificity and increases catalytic efficiency. Only the correct substrate can participate in most or all of the interactions with the enzyme and thus maximize binding energy, accounting for the exquisite substrate specificity exhibited by many enzymes. Furthermore, the full complement of such interactions is formed only when the substrate is in the transition state. Thus, interactions between the enzyme and the substrate not only favor substrate binding but stabilize the transition state, thereby lowering the activation energy. The binding energy can also promote structural changes in both the enzyme and the substrate that facilitate catalysis, a process referred to as *induced fit*.

Enzymes commonly employ one or more of the following strategies to catalyze specific reactions:

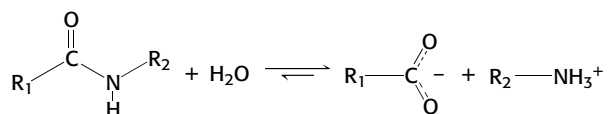
1. *Covalent catalysis*. In covalent catalysis, the active site contains a reactive group, usually a powerful nucleophile that becomes temporarily covalently modified in the course of catalysis. The proteolytic enzyme chymotrypsin provides an excellent example of this mechanism (Section 9.1.2).
2. *General acid–base catalysis*. In general acid–base catalysis, a molecule other than water plays the role of a proton donor or acceptor. Chymotrypsin uses a histidine residue as a base catalyst to enhance the nucleophilic power of serine (Section 9.1.3).
3. *Metal ion catalysis*. Metal ions can function catalytically in several ways. For instance, a metal ion may serve as an electrophilic catalyst, stabilizing a negative charge on a reaction intermediate. Alternatively, the metal ion may generate a nucleophile by increasing the acidity of a nearby molecule, such as water in the hydration of CO_2 by carbonic anhydrase (Section 9.2.2). Finally, the metal ion may bind to substrate, increasing the number of interactions with the enzyme and thus the binding energy. This strategy is used by NMP kinases (Section 9.4.2).
4. *Catalysis by approximation*. Many reactions include two distinct substrates. In such cases, the reaction rate may be considerably enhanced by bringing the two substrates together along a single binding surface on an enzyme. NMP kinases bring two nucleotides together to facilitate the transfer of a phosphoryl group from one nucleotide to the other (Section 9.4.3).

9.1 PROTEASES: FACILITATING A DIFFICULT REACTION

Protein turnover is an important process in living systems (Chapter 23). Proteins that have served their purpose must be degraded so that their constituent amino acids can be recycled for the synthesis of new proteins. Proteins ingested in the diet must be broken down into small peptides and

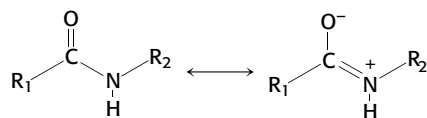
amino acids for absorption in the gut. Furthermore, as described in detail in Chapter 10, proteolytic reactions are important in regulating the activity of certain enzymes and other proteins.

Proteases cleave proteins by a hydrolysis reaction—the addition of a molecule of water to a peptide bond:



Although the hydrolysis of peptide bonds is thermodynamically favored, such hydrolysis reactions are extremely slow. In the absence of a catalyst, the half-life for the hydrolysis of a typical peptide at neutral pH is estimated to be between 10 and 1000 years. Yet, peptide bonds must be hydrolyzed within milliseconds in some biochemical processes.

The chemical bonding in peptide bonds is responsible for their kinetic stability. Specifically, the resonance structure that accounts for the planarity of a peptide bond (Section 3.2.2) also makes such bonds resistant to hydrolysis. This resonance structure endows the peptide bond with partial double-bond character:



The carbon–nitrogen bond is strengthened by its double-bond character, and the carbonyl carbon atom is less electrophilic and less susceptible to nucleophilic attack than are the carbonyl carbon atoms in compounds such as carboxylate esters. Consequently, to promote peptide-bond cleavage, an enzyme must facilitate nucleophilic attack at a normally unreactive carbonyl group.

9.1.1 Chymotrypsin Possesses a Highly Reactive Serine Residue

A number of proteolytic enzymes participate in the breakdown of proteins in the digestive systems of mammals and other organisms. One such enzyme, chymotrypsin, cleaves peptide bonds selectively on the carboxyl-terminal side of the large hydrophobic amino acids such as tryptophan, tyrosine, phenylalanine, and methionine (Figure 9.1). Chymotrypsin is a good example of the use of *covalent modification* as a catalytic strategy. The enzyme employs a powerful nucleophile to attack the unreactive carbonyl group of the substrate. This nucleophile becomes covalently attached to the substrate briefly in the course of catalysis.

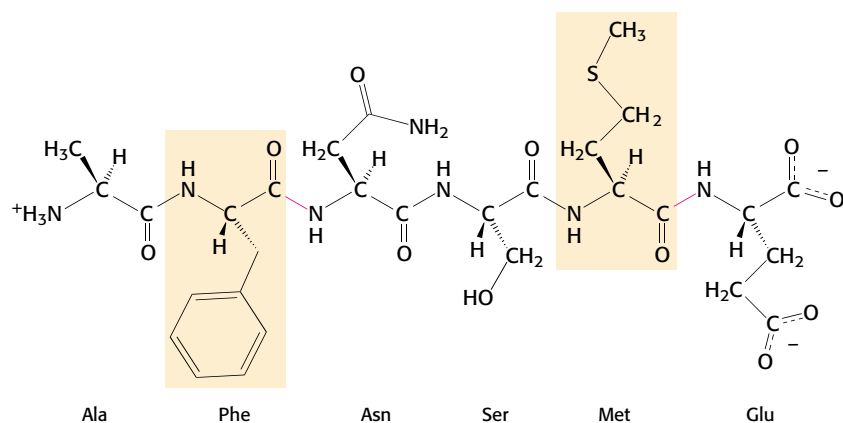
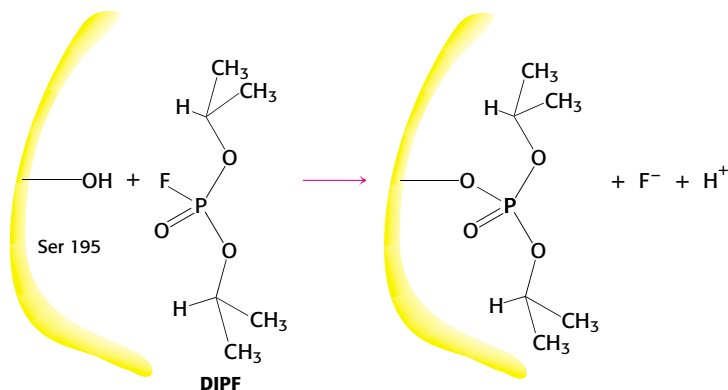


FIGURE 9.1 Specificity of chymotrypsin. Chymotrypsin cleaves proteins on the carboxyl side of aromatic or large hydrophobic amino acids (shaded yellow). The likely bonds cleaved by chymotrypsin are indicated in red.

FIGURE 9.2 An unusually reactive serine in chymotrypsin. Chymotrypsin is inactivated by treatment with diisopropylphosphofluoridate (DIPF), which reacts only with serine 195 among 28 possible serine residues.



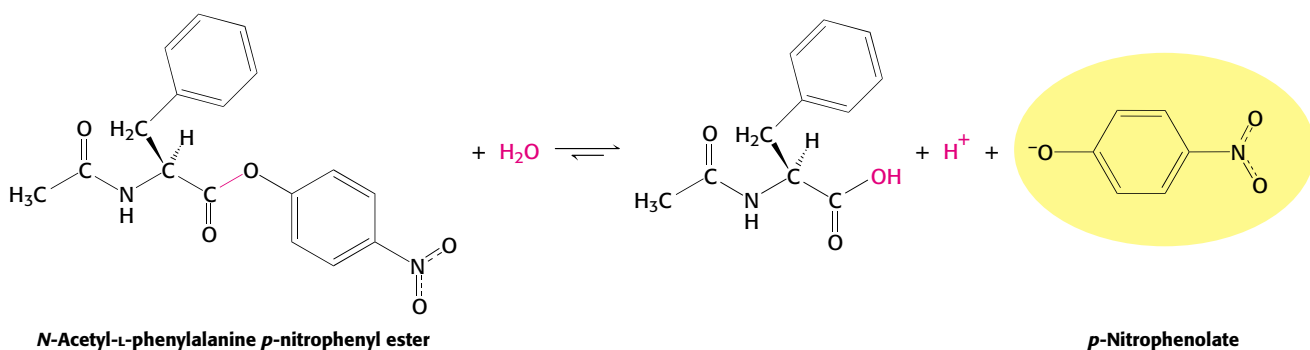
What is the nucleophile that chymotrypsin employs to attack the substrate carbonyl group? A clue came from the fact that chymotrypsin contains an extraordinarily reactive serine residue. Treatment with organofluorophosphates such as diisopropylphosphofluoridate (DIPF); (Section 8.4.1) was found to inactivate the enzyme irreversibly (Figure 9.2). Despite the fact that the enzyme possesses 28 serine residues, only one, serine 195, was modified, resulting in a total loss of enzyme activity. This *chemical modification reaction* suggested that this unusually reactive serine residue plays a central role in the catalytic mechanism of chymotrypsin.

9.1.2 Chymotrypsin Action Proceeds in Two Steps Linked by a Covalently Bound Intermediate

How can we elucidate the role of serine 195 in chymotrypsin action? A study of the enzyme's kinetics provided a second clue to chymotrypsin's catalytic mechanism and the role of serine 195. The kinetics of enzyme action are often easily monitored by having the enzyme act on a substrate analog that forms a colored product. For chymotrypsin, such a *chromogenic substrate* is *N*-acetyl-L-phenylalanine *p*-nitrophenyl ester. This substrate is an ester rather than an amide, but many proteases will also hydrolyze esters. One of the products formed by chymotrypsin's cleavage of this substrate is *p*-nitrophenolate, which has a yellow color (Figure 9.3). Measurements of the absorbance of light revealed the amount of *p*-nitrophenolate being produced.

Under steady-state conditions, the cleavage of this substrate obeys Michaelis-Menten kinetics with a K_M of 20 μM and a k_{cat} of 77 s^{-1} . The initial phase of the reaction was examined by using the stopped-flow method. This technique permits the rapid mixing of enzyme and substrate and allows almost instantaneous monitoring of the reaction. At the beginning of the reaction, this method revealed a "burst" phase during which the

FIGURE 9.3 Chromogenic substrate. *N*-Acetyl-L-phenylalanine *p*-nitrophenyl ester yields a yellow product, *p*-nitrophenolate, on cleavage by chymotrypsin. *p*-Nitrophenolate forms by deprotonation of *p*-nitrophenol at pH 7.



colored product was produced rapidly (Figure 9.4). Product was then produced more slowly as the reaction reached the steady state. These results suggest that hydrolysis proceeds in two steps. The burst is observed because, for this substrate, the first step is more rapid than the second step.

The two steps are explained by the reaction of the serine nucleophile with the substrate to form the covalently bound enzyme–substrate intermediate (Figure 9.5). First, the highly reactive serine 195 hydroxyl group attacks the carbonyl group of the substrate to form the acyl-enzyme intermediate, releasing the alcohol *p*-nitrophenol (or an amine if the substrate is an amide rather than an ester). Second, the acyl-enzyme intermediate is hydrolyzed to release the carboxylic acid component of the substrate and regenerate the free enzyme. Thus, *p*-nitrophenolate is produced rapidly on the addition of the substrate as the acyl-enzyme intermediate is formed, but it takes longer for the enzyme to be “reset” by the hydrolysis of the acyl-enzyme intermediate.

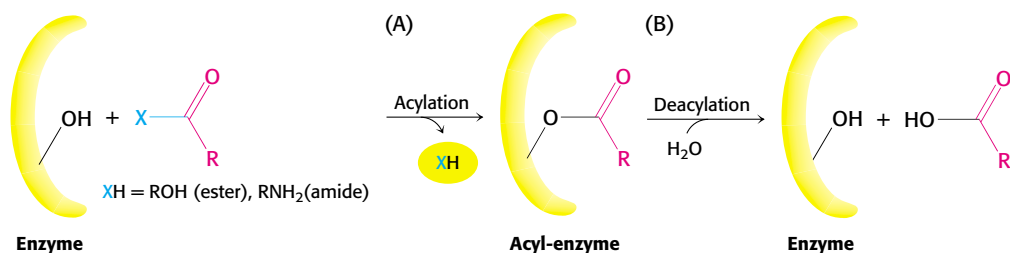


FIGURE 9.5 Covalent catalysis. Hydrolysis by chymotrypsin takes place in two stages: (A) acylation to form the acyl-enzyme intermediate followed by (B) deacylation to regenerate the free enzyme.

9.1.3 Serine is Part of a Catalytic Triad That Also Includes Histidine and Aspartic Acid

The determination of the three-dimensional structure of chymotrypsin by David Blow in 1967 was a source of further insight into its mechanism of action. Overall, chymotrypsin is roughly spherical and comprises three polypeptide chains, linked by disulfide bonds. It is synthesized as a single polypeptide, termed chymotrypsinogen, which is activated by the proteolytic cleavage of the polypeptide to yield the three chains. The active site of chymotrypsin, marked by serine 195, lies in a cleft on the surface of the enzyme (Figure 9.6). The structural analysis revealed the chemical basis of the special reactivity of serine 195 (Figure 9.7). The side chain of serine 195 is hydrogen bonded to the imidazole ring of histidine 57. The –NH group of this imidazole ring is, in turn, hydrogen bonded to the carboxylate group of aspartate 102. This constellation of residues is referred to as the *catalytic triad*. How does this arrangement of residues lead to the high reactivity of serine 195? The histidine residue serves to position the serine side chain and to polarize its hydroxyl group. In doing so, the residue acts as a general

FIGURE 9.6 Three-dimensional structure of chymotrypsin. The three chains are shown in ribbon form in orange, blue, and green. The side chains of the catalytic triad residues, including serine 195, are shown as ball-and-stick representations, as are two intrastrand and interstrand disulfide bonds.

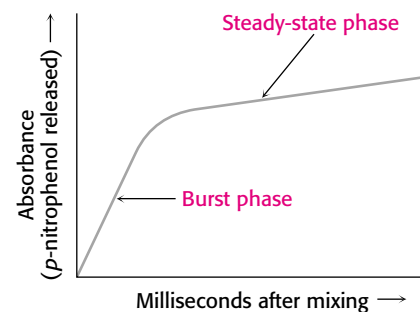
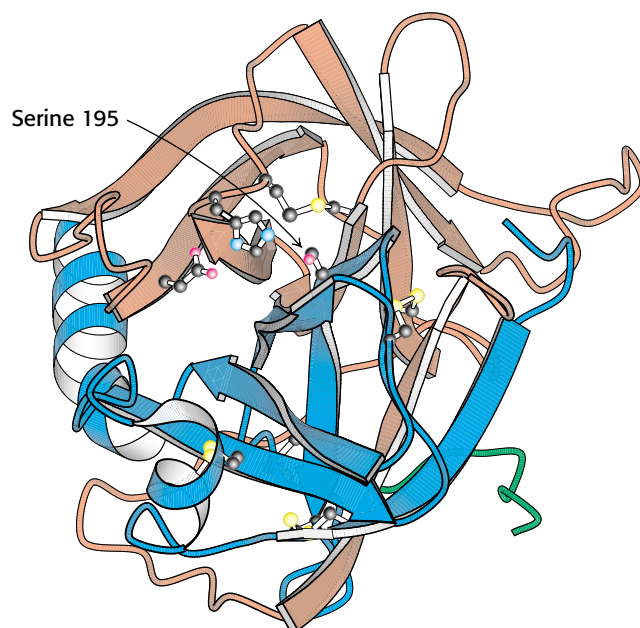


FIGURE 9.4 Kinetics of chymotrypsin catalysis. Two stages are evident in the cleaving of *N*-acetyl-L-phenylalanine *p*-nitrophenyl ester by chymotrypsin: a rapid burst phase (pre-steady state) and a steady-state phase.



CONCEPTUAL INSIGHTS, Enzyme Kinetics. See the section entitled “Pre-Steady-State Kinetics” in *Conceptual Insights* module to better understand why a “burst” phase at short reaction times implies the existence of an enzyme-substrate intermediate.

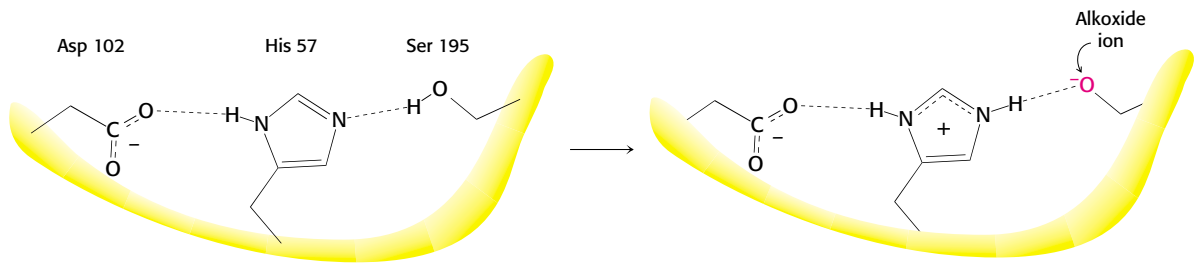
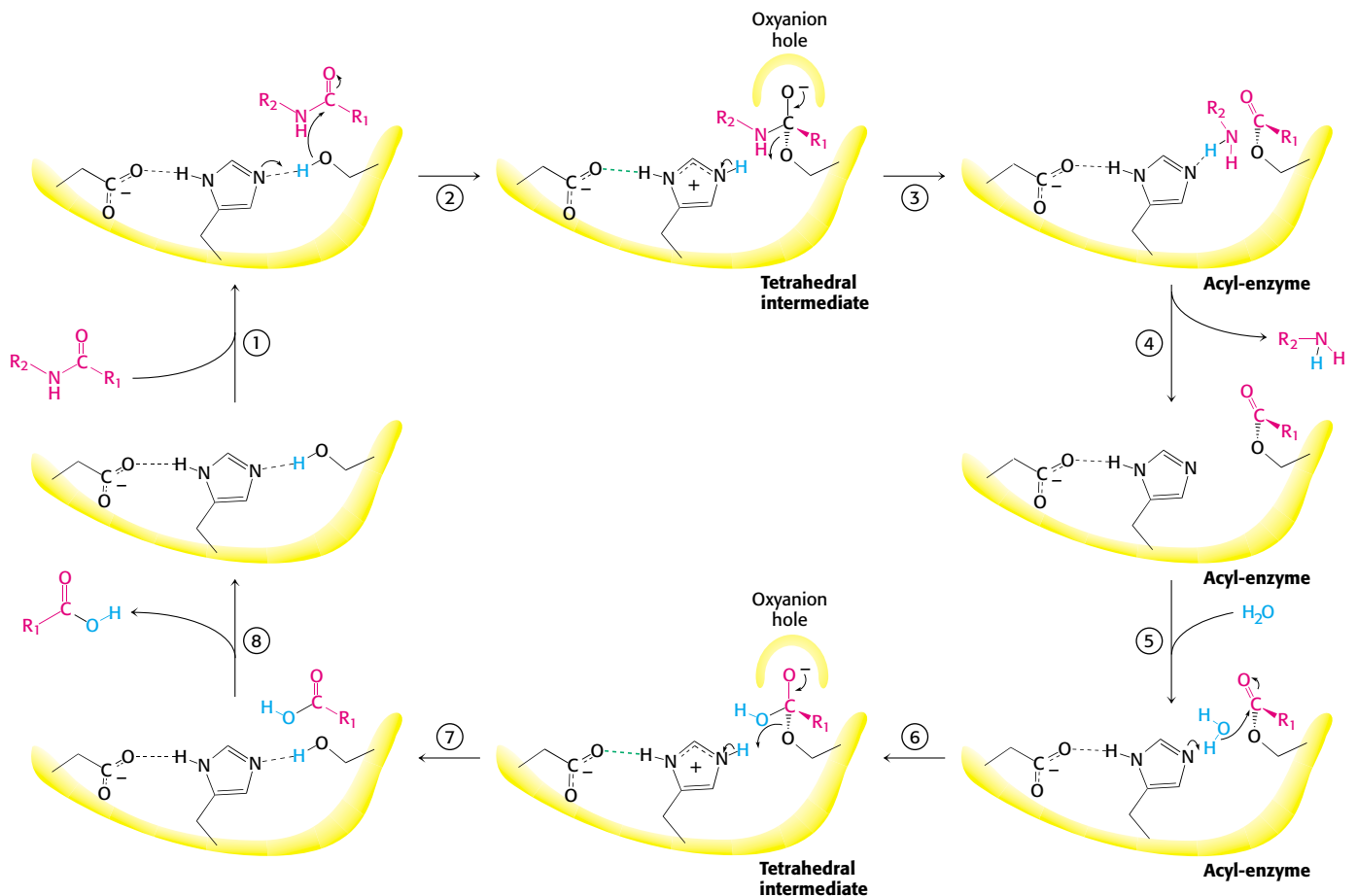


FIGURE 9.7 The catalytic triad. The catalytic triad, shown on the left, converts serine 195 into a potent nucleophile, as illustrated on the right.

base catalyst, a hydrogen ion acceptor, because the polarized hydroxyl group of the serine residue is poised for deprotonation. The withdrawal of the proton from the hydroxyl group generates an alkoxide ion, which is a much more powerful nucleophile than an alcohol is. The aspartate residue helps orient the histidine residue and make it a better proton acceptor through electrostatic effects.

FIGURE 9.8 Peptide hydrolysis by chymotrypsin. The mechanism of peptide hydrolysis illustrates the principles of covalent and acid–base catalysis. The dashed green lines indicate favorable interactions between the negatively charged aspartate residue and the positively charged histidine residue, which make the histidine residue a more powerful base.

These observations suggest a mechanism for peptide hydrolysis (Figure 9.8). After substrate binding (step 1), the reaction begins with the hydroxyl group of serine 195 making a nucleophilic attack on the carbonyl carbon atom of the substrate (step 2). The nucleophilic attack changes the geometry around this carbon atom from trigonal planar to tetrahedral. The inherently unstable *tetrahedral intermediate* formed bears a formal negative charge on the oxygen atom derived from the carbonyl group. This charge is stabilized by interactions with NH groups from the protein in a site



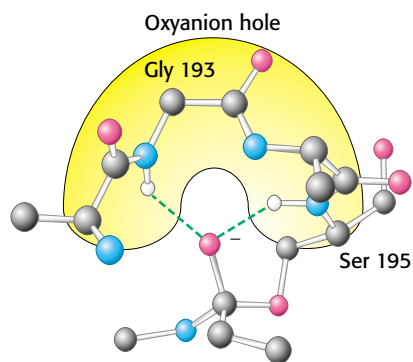


FIGURE 9.9 The oxyanion hole. The structure stabilizes the tetrahedral intermediate of the chymotrypsin reaction. Hydrogen bonds (shown in green) link peptide NH groups and the negatively charged oxygen.

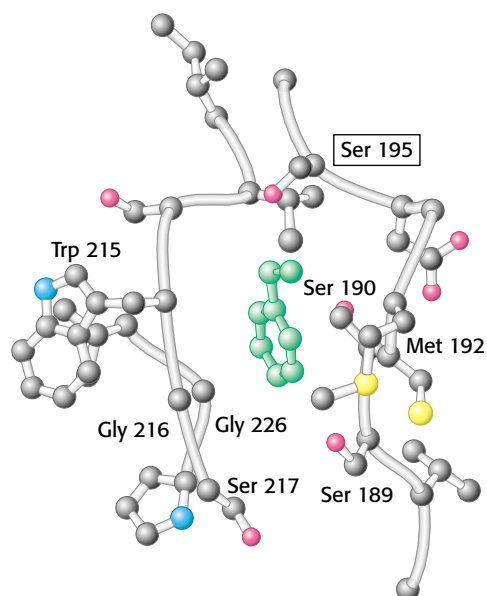


FIGURE 9.10 The hydrophobic pocket of chymotrypsin. The hydrophobic pocket of chymotrypsin is responsible for its substrate specificity. The key amino acids that constitute the binding site are labeled, including the active-site serine residue (boxed). The position of an aromatic ring bound in the pocket is shown in green.

termed the *oxyanion hole* (Figure 9.9). These interactions also help stabilize the transition state that precedes the formation of the tetrahedral intermediate. This tetrahedral intermediate then collapses to generate the acyl-enzyme (step 3). This step is facilitated by the transfer of a proton from the positively charged histidine residue to the amino group formed by cleavage of the peptide bond. The amine component is now free to depart from the enzyme (step 4) and is replaced by a water molecule (step 5). The ester group of the acyl-enzyme is now hydrolyzed by a process that is essentially a repeat of steps 2 through 4. The water molecule attacks the carbonyl group while a proton is concomitantly removed by the histidine residue, which now acts as a general acid catalyst, forming a tetrahedral intermediate (step 6). This structure breaks down to form the carboxylic acid product (step 7). Finally, the release of the carboxylic acid product (step 8) readies the enzyme for another round of catalysis.

This mechanism accounts for all characteristics of chymotrypsin action except the observed preference for cleaving the peptide bonds just past residues with large, hydrophobic side chains. Examination of the three-dimensional structure of chymotrypsin with substrate analogs and enzyme inhibitors revealed the presence of a deep, relatively hydrophobic pocket, called the S_1 pocket, into which the long, uncharged side chains of residues such as phenylalanine and tryptophan can fit. *The binding of an appropriate side chain into this pocket positions the adjacent peptide bond into the active site for cleavage* (Figure 9.10). The specificity of chymotrypsin depends almost entirely on which amino acid is directly on the amino-terminal side of the peptide bond to be cleaved. Other proteases have more-complex specificity patterns, as illustrated in Figure 9.11. Such enzymes have additional pockets on their surfaces for the recognition of other residues in the substrate. Residues on the amino-terminal side of the scissile bond (the bond

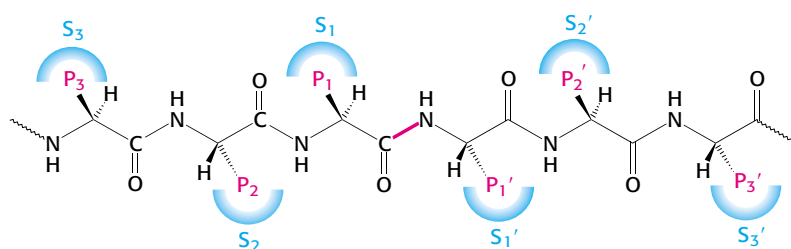


FIGURE 9.11 Specificity nomenclature for protease-substrate interactions. The potential sites of interaction of the substrate with the enzyme are designated P (shown in red), and corresponding binding sites on the enzyme are designated S. The scissile bond (also shown in red) is the reference point.

to be cleaved) are labeled P_1 , P_2 , P_3 , and so forth, indicating their positions in relation to the scissile bond. Likewise, residues on the carboxyl side of the scissile bond are labeled P_1' , P_2' , P_3' , and so forth. The corresponding sites on the enzyme are referred to as S_1 , S_2 or S_1' , S_2' , and so forth.



STRUCTURAL INSIGHTS, Chymotrypsin: A Serine Protease. Work with interactive molecular models to learn more about the structural bases of active site specificity and reactivity, and some of the ways in which active site residues can be identified.

9.1.4 Catalytic Triads Are Found in Other Hydrolytic Enzymes



Many other proteins have subsequently been found to contain catalytic triads similar to that discovered in chymotrypsin. Some, such as trypsin and elastase, are obvious homologs of chymotrypsin. The sequences of these proteins are approximately 40% identical with that of chymotrypsin, and their overall structures are nearly the same (Figure 9.12). These proteins operate by mechanisms identical with that of chymotrypsin. However, they have very different substrate specificities. Trypsin cleaves at the peptide bond after residues with long, positively charged side chains—namely, arginine and lysine—whereas elastase cleaves at the peptide bond after amino acids with small side chains—such as alanine and serine. Comparison of the S_1 pockets of these enzymes reveals the basis of the specificity. In trypsin, an aspartate residue (Asp 189) is present at the bottom of the S_1 pocket in place of a serine residue in chymotrypsin. The aspartate residue attracts and stabilizes a positively charged arginine or lysine residue in the substrate. In elastase, two residues at the top of the pocket in chymotrypsin and trypsin are replaced with valine (Val 190 and Val 216). These residues close off the mouth of the pocket so that only small side chains may enter (Figure 9.13).

Other members of the chymotrypsin family include a collection of proteins that take part in blood clotting, to be discussed in Chapter 10. In addition, a wide range of proteases found in bacteria and viruses also belong to this clan. Furthermore, other enzymes that are not homologs of chymotrypsin have been found to contain very similar active sites. As noted in Chapter 7, the presence of very similar active sites in these different protein families is a consequence of convergent evolution. Subtilisin, a protease in bacteria such as *Bacillus amyloliquefaciens*, is a particularly well characterized example. The active site of this enzyme includes both the catalytic triad and the oxyanion hole. However, one of the NH groups that forms the oxyanion hole comes from the side chain of an asparagine residue rather than from the peptide backbone (Figure 9.14). Subtilisin is the founding member of another large family of proteases that includes representatives from Archaea, Eubacteria, and Eukarya.

Yet another example of the catalytic triad has been found in carboxypeptidase II from wheat. The structure of this enzyme is not significantly similar to either chymotrypsin or subtilisin (Figure 9.15). This protein is a member of an intriguing family of homologous proteins that includes esterases such as acetylcholine esterase and certain lipases. These enzymes all make use of histidine-activated nucleophiles, but the nucleophiles may be cysteine rather than serine. Finally, other proteases have been discovered that contain an active-site serine or threonine residue that is activated not by a histidine–aspartate pair but by a primary amino group from the side chain of lysine or by the N-terminal amino group of the polypeptide chain.

Thus, the catalytic triad in proteases has emerged at least three times in the course of evolution. We can conclude that this catalytic strategy must be an especially effective approach to the hydrolysis of peptides and related bonds.



FIGURE 9.12 Structural similarity of trypsin and chymotrypsin. An overlay of the structure of chymotrypsin (red) on that of trypsin (blue) shows the high degree of similarity. Only α -carbon atom positions are shown. The mean deviation in position between corresponding α -carbon atoms is 1.7 Å.

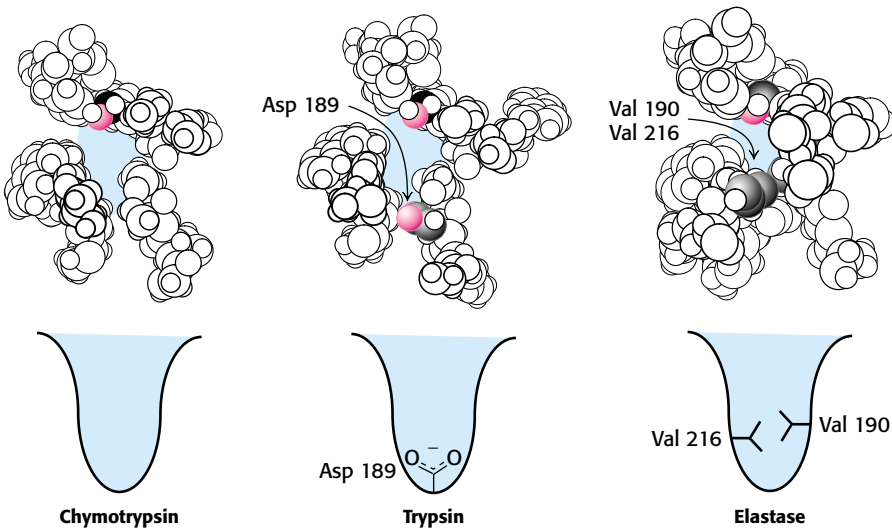


FIGURE 9.13 The S₁ pockets of chymotrypsin, trypsin, and elastase. Certain residues play key roles in determining the specificity of these enzymes. The side chains of these residues, as well as those of the active-site serine residues, are shown in color.

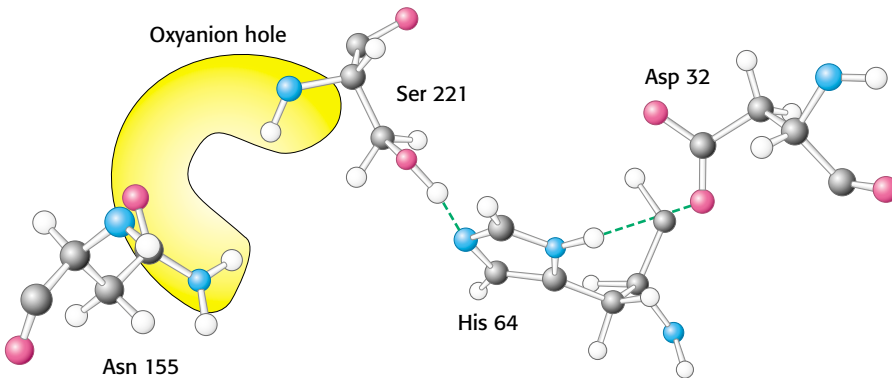


FIGURE 9.14 The catalytic triad and oxyanion hole of subtilisin. The peptide bond attacked by nucleophilic serine 221 of the catalytic triad will develop a negative charge, which is stabilized by enzyme NH groups (both in the backbone and in the side chain of Asn 155) located in the oxyanion hole.

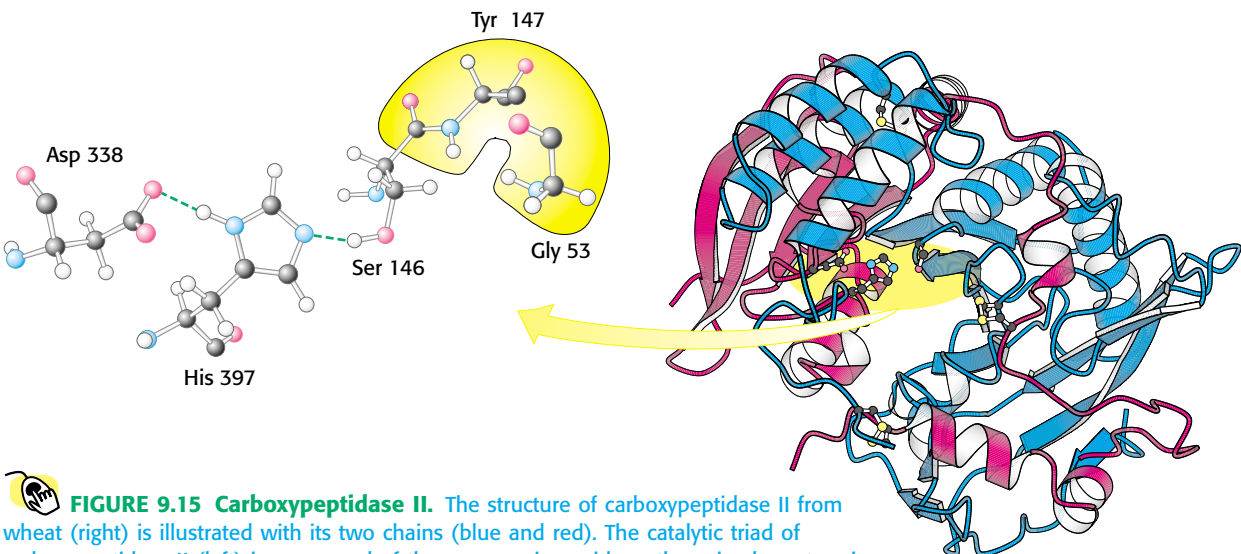


FIGURE 9.15 Carboxypeptidase II. The structure of carboxypeptidase II from wheat (right) is illustrated with two chains (blue and red). The catalytic triad of carboxypeptidase II (left) is composed of the same amino acids as those in chymotrypsin, despite the fact that the enzymes display no structural similarity. The residues that form the oxyanion hole are highlighted in yellow.

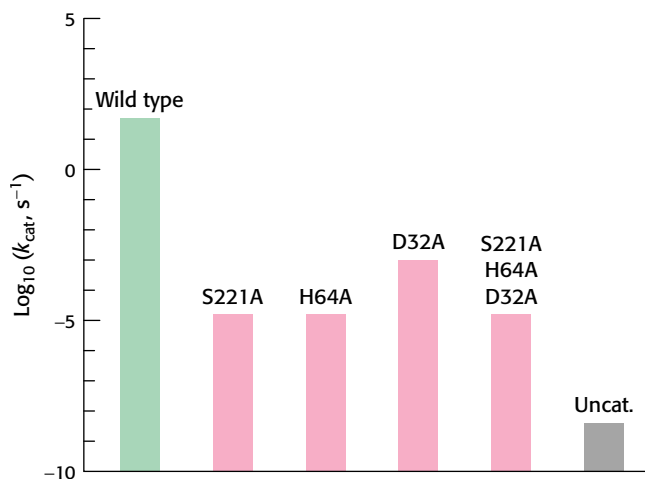


FIGURE 9.16 Site-directed mutagenesis of subtilisin. Residues of the catalytic triad were mutated to alanine, and the activity of the mutated enzyme was measured. Mutations in any component of the catalytic triad cause a dramatic loss of enzyme activity. Note that the activity is displayed on a logarithmic scale. The mutations are identified as follows: the first letter is the one-letter abbreviation for the amino acid being altered; the number identifies the position of the residue in the primary structure; and the second letter is the one-letter abbreviation for the amino acid replacing the original one.

9.1.5 The Catalytic Triad Has Been Dissected by Site-Directed Mutagenesis

The techniques of molecular biology discussed in Chapter 6 have permitted detailed examination of the catalytic triad. In particular, site-directed mutagenesis has been used to test the contribution of individual amino acid residues to the catalytic power of an enzyme. Subtilisin has been extensively studied by this method. Each of the residues within the catalytic triad, consisting of aspartic acid 32, histidine 64, and serine 221, has been individually converted into alanine, and the ability of each mutant enzyme to cleave a model substrate has been examined (Figure 9.16). As expected, the conversion of active-site serine 221 into alanine dramatically reduced catalytic power; the value of k_{cat} fell to less than *one-millionth* of its value for the wild-type enzyme.

The value of K_M was essentially unchanged: its increase by no more than a factor of two indicated that substrate binding is not significantly affected. The mutation of histidine 64 to alanine had very similar effects. These observations support the notion that the serine–histidine pair act together to generate a nucleophile of sufficient power to attack the carbonyl group of a peptide bond. The conversion of aspartate 32 into alanine had a smaller effect, although the value of k_{cat} still fell to less than 0.005% of its wild-type value. The simultaneously conversion of all three catalytic triad residues into alanine was no more deleterious than the conversion of serine or histidine alone. Despite the reduction in their catalytic power, the mutated enzymes still hydrolyze peptides a thousand times as rapidly as does buffer at pH 8.6.

Because the oxyanion hole of subtilisin includes a side-chain NH group in addition to backbone NH groups, it is possible to probe the importance of the oxyanion hole for catalysis by site-directed mutagenesis. The mutation of asparagine 155 to glycine reduced the value of k_{cat} to 0.2% of its wild-type value but increased the value of K_M by only a factor of two. These observations demonstrate that the NH group of the asparagine residue plays a significant role in stabilizing the tetrahedral intermediate and the transition state leading to it.

9.1.6 Cysteine, Aspartyl, and Metalloproteases Are Other Major Classes of Peptide-Cleaving Enzymes



Not all proteases utilize strategies based on activated serine residues. Classes of proteins have been discovered that employ three alternative approaches to peptide-bond hydrolysis (Figure 9.17). These classes are the (1) cysteine proteases, (2) aspartyl proteases, and (3) metalloproteases. In each case, the strategy generates a nucleophile that attacks the peptide carbonyl group (Figure 9.18).

The strategy used by the *cysteine proteases* is most similar to that used by the chymotrypsin family. In these enzymes, a cysteine residue, activated by a histidine residue, plays the role of the nucleophile that attacks the peptide bond (see Figure 9.18), in a manner quite analogous to that of the serine residue in serine proteases. An ideal example of these proteins is papain, an enzyme purified from the fruit of the papaya. Mammalian proteases homologous to papain have been discovered, most notably the cathepsins, proteins having a role in the immune and other systems. The cysteine-based active site arose independently at least twice in the course of evolution; the caspases, enzymes that play a major role in apoptosis (Section 2.4.3), have active sites similar to that of papain, but their overall structures are unrelated.

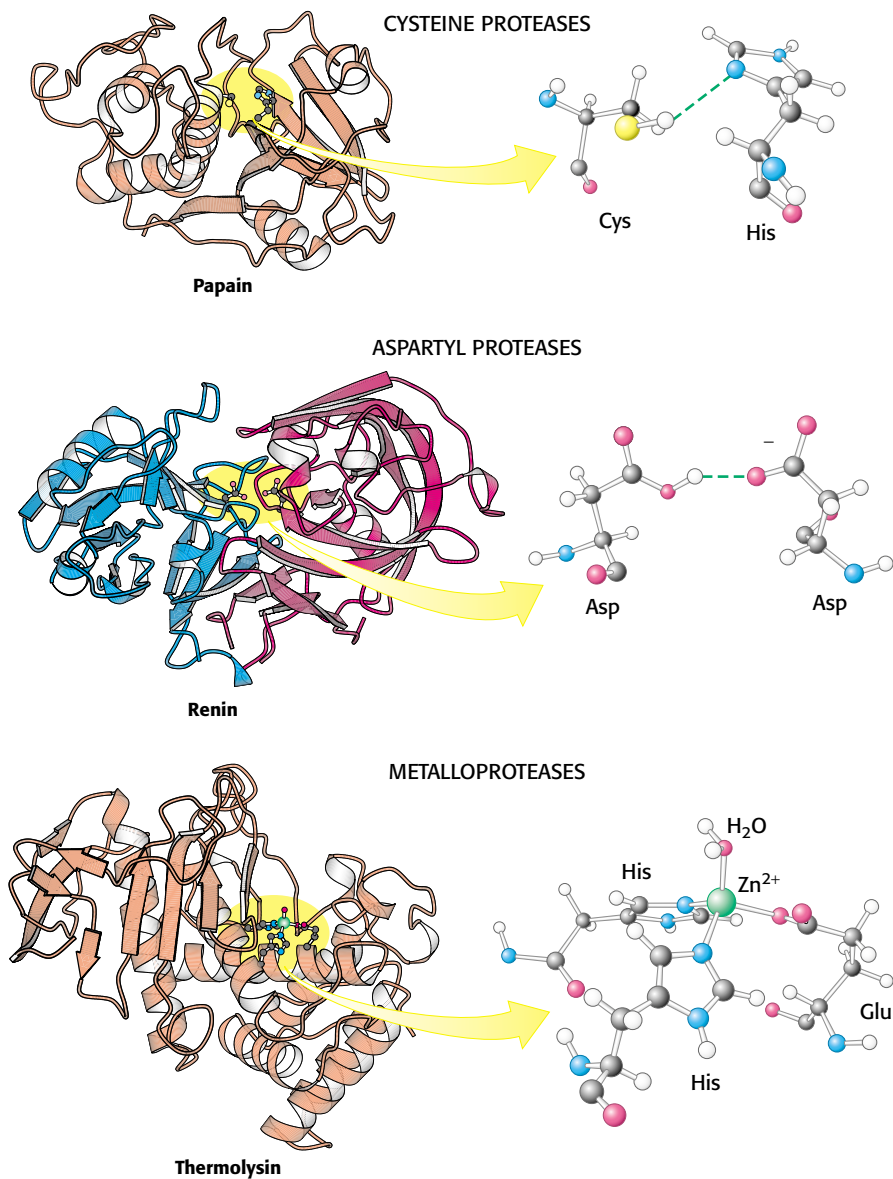
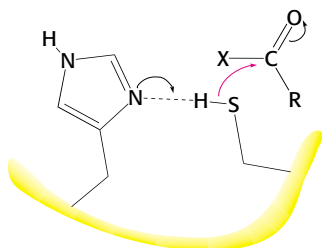
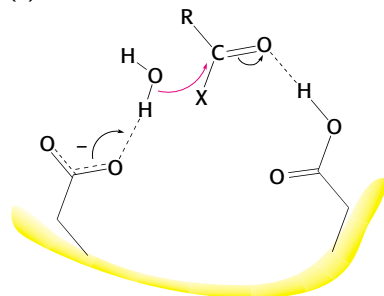


FIGURE 9.17 Three classes of proteases and their active sites. These examples of a cysteine protease, an aspartyl protease, and a metalloprotease use a histidine-activated cysteine residue, an aspartate-activated water molecule, and a metal-activated water molecule, respectively, as the nucleophile. The two halves of renin are in blue and red to highlight the approximate twofold symmetry of aspartyl proteases.

(A) CYSTEINE PROTEASES



(B) ASPARTYL PROTEASES



(C) METALLOPROTEASES

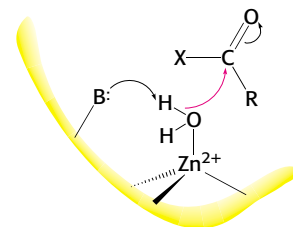


FIGURE 9.18 The activation strategies for three classes of proteases. The peptide carbonyl group is attacked by (A) a histidine-activated cysteine, in the cysteine proteases; (B) an aspartate-activated water molecule, in the aspartyl proteases; and (C) a metal-activated water molecule, in the metalloproteases. For the metalloproteases, the letter B represents a base (often a glutamate) that helps deprotonate the metal-bound water.

The second class comprises the *aspartyl proteases*. The central feature of the active sites is a pair of aspartic acid residues that act together to allow a water molecule to attack the peptide bond. One aspartic acid residue (in its deprotonated form) activates the attacking water molecule by poisoning it for deprotonation, whereas the other aspartic acid residue (in its protonated form) polarizes the peptide carbonyl, increasing its susceptibility to attack (see Figure 9.18). Members of this class include renin, an enzyme having a role in the regulation of blood pressure, and the digestive enzyme pepsin. These proteins possess approximate twofold symmetry, suggesting that the two halves are evolutionarily related. A likely scenario is that two copies of a gene for the ancestral enzyme fused to form a single gene that encoded a single-chain enzyme. Each copy of the gene would have contributed an aspartate residue to the active site. The human immunodeficiency virus (HIV) and other retroviruses contain an unfused dimeric aspartyl protease that is similar to the fused protein, but the individual chains are not joined to make a single chain (Figure 9-19). This observation is consistent with the idea that the enzyme may have originally existed as separate subunits.

The *metalloproteases* constitute the final major class of peptide-cleaving enzymes. The active site of such a protein contains a bound metal ion, almost always zinc, that activates a water molecule to act as a nucleophile to attack the peptide carbonyl group. The bacterial enzyme thermolysin and the digestive enzyme carboxypeptidase A are classic examples of the zinc proteases. Thermolysin, but not carboxypeptidase A, is a member of a large and diverse family of homologous zinc proteases that includes the matrix metalloproteases, enzymes that catalyze the reactions in tissue remodeling and degradation.

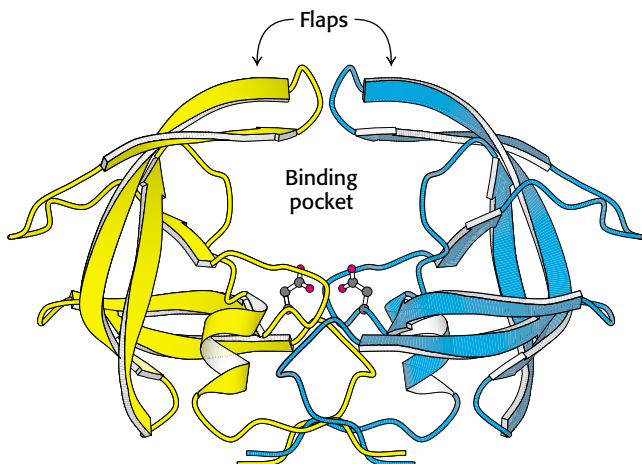
In each of these three classes of enzymes, *the active site includes features that allow for the activation of water or another nucleophile as well as for the polarization of the peptide carbonyl group and subsequent stabilization of a tetrahedral intermediate* (see Figure 9.18).

9.1.7 Protease Inhibitors Are Important Drugs

Compounds that block or modulate the activities of proteases can have dramatic biological effects. Most natural *protease inhibitors* are similar in structure to the peptide substrates of the enzyme that each inhibits (Section 10.7.4). *Several important drugs are protease inhibitors*. For example, captopril, an inhibitor of the metalloprotease angiotensin-converting enzyme (ACE), has been used to regulate blood pressure. Crixivan, an inhibitor of the HIV protease, is used in the treatment of AIDS. This protease cleaves multidomain viral proteins into their active forms; blocking this process completely prevents the virus from being infectious (see Figure 9.19). To prevent unwanted side effects, protease inhibitors used as drugs must be specific for one enzyme without inhibiting other proteins within the body.

Let us examine the interaction of Crixivan with HIV protease in more detail. Crixivan is constructed around an alcohol that mimics the tetrahedral intermediate; other groups are present to bind into the S_2 , S_1 , S_1' , and S_2' recognition sites on the enzyme (Figure 9.20). The results of x-ray crystallographic studies revealed the structure of the enzyme–Crixivan complex, showing that Crixivan adopts a conformation that approximates the twofold symmetry of the enzyme (Figure 9.21). The active site of HIV protease is covered by two apparently flexible flaps that fold down on top of the bound in-

FIGURE 9.19 The structure of HIV protease and its binding pocket. The protease is a dimer of identical subunits, shown in blue and yellow, consisting of 99 amino acids each. The active-site aspartic acid residues, one from each chain, are shown as ball-and-stick structures. The flaps will close down on the binding pocket after substrate has been bound.



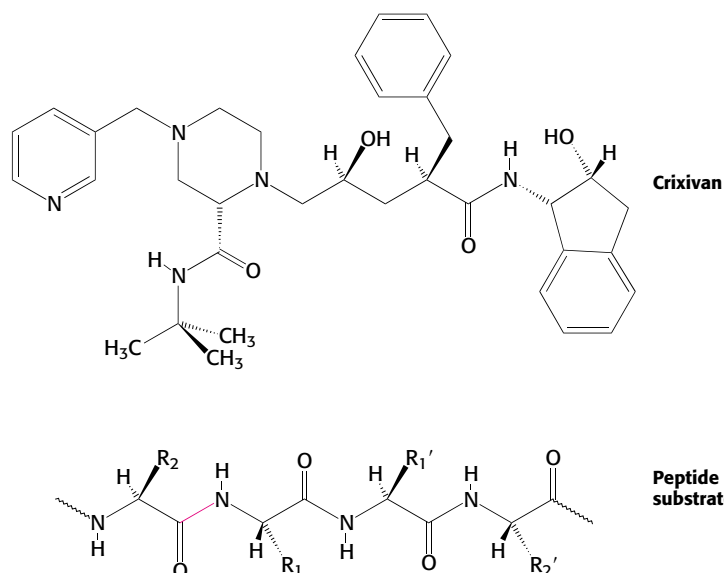


FIGURE 9.20 Crixivan, an HIV protease inhibitor. The structure of Crixivan is shown in comparison with that of a peptide substrate of HIV protease. The scissile bond in the substrate is highlighted in red.

hibitor. The hydroxyl group of the central alcohol interacts with two aspartate residues of the active site, one in each subunit. In addition, two carbonyl groups of the inhibitor are hydrogen bonded to a water molecule (not shown), which, in turn, is hydrogen bonded to a peptide NH group in each of the flaps. This interaction of the inhibitor with water and the enzyme is not possible with cellular aspartyl proteases such as renin and thus may contribute to the specificity of Crixivan and other inhibitors for HIV protease.

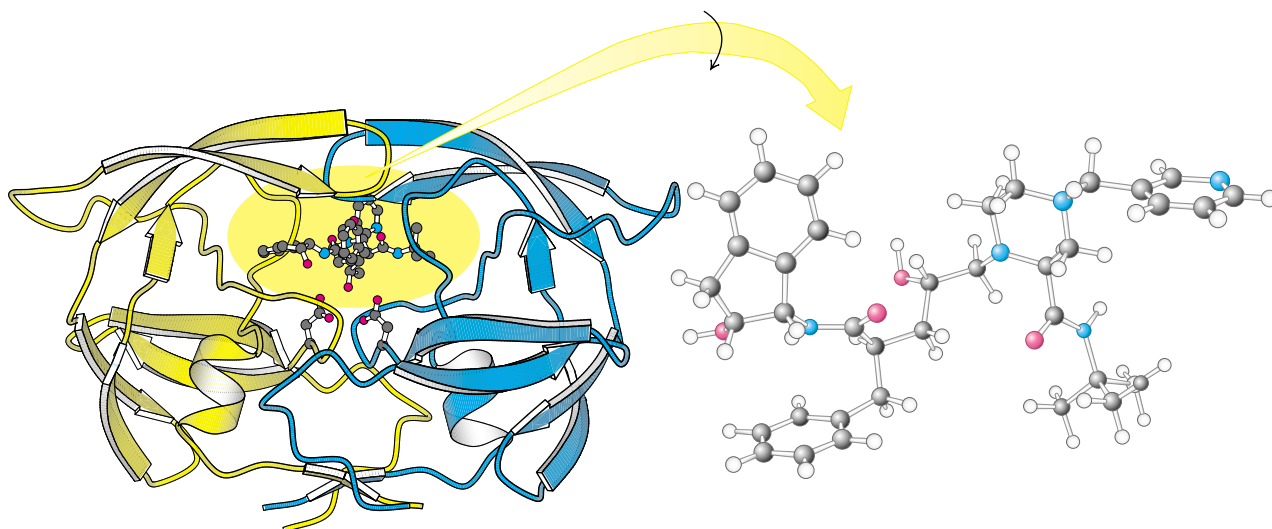
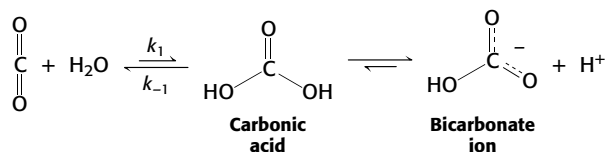


FIGURE 9.21 HIV protease–Crixivan complex. (Left) The HIV protease is shown with the inhibitor crixivan bound at the active site. (Right) The drug has been rotated to reveal its approximately twofold symmetric conformation.

9.2 MAKING A FAST REACTION FASTER: CARBONIC ANHYDRASES

Carbon dioxide is a major end product of aerobic metabolism. In complex organisms, this carbon dioxide is released into the blood and transported to the lungs for exhalation. While in the blood, carbon dioxide reacts with

water. The product of this reaction is a moderately strong acid, carbonic acid ($\text{p}K_{\text{a}} = 3.5$), which becomes bicarbonate ion on the loss of a proton.



Even in the absence of a catalyst, this hydration reaction proceeds at a moderate pace. At 37°C near neutral pH, the second-order rate constant k_1 is $0.0027 \text{ M}^{-1} \text{ s}^{-1}$. This corresponds to an effective first-order rate constant of 0.15 s^{-1} in water ($[\text{H}_2\text{O}] = 55.5 \text{ M}$). Similarly, the reverse reaction, the dehydration of bicarbonate, is relatively rapid, with a rate constant of $k_{-1} = 50 \text{ s}^{-1}$. These rate constants correspond to an equilibrium constant of $K_1 = 5.4 \times 10^{-5}$ and a ratio of $[\text{CO}_2]$ to $[\text{H}_2\text{CO}_3]$ of 340:1.



Despite the fact that CO_2 hydration and HCO_3^- dehydration occur spontaneously at reasonable rates in the absence of catalysts, almost all organisms contain enzymes, referred to as *carbonic anhydrases*, that catalyze these processes. Such enzymes are required because CO_2 hydration and HCO_3^- dehydration are often coupled to rapid processes, particularly transport processes. For example, HCO_3^- in the blood must be dehydrated to form CO_2 for exhalation as the blood passes through the lungs. Conversely, CO_2 must be converted into HCO_3^- for the generation of the aqueous humor of the eye and other secretions. Furthermore, both CO_2 and HCO_3^- are substrates and products for a variety of enzymes, and the rapid interconversion of these species may be necessary to ensure appropriate substrate levels. So important are these enzymes in human beings that mutations in some carbonic anhydrases have been found to cause osteopetrosis (excessive formation of dense bones accompanied by anemia) and mental retardation.

Carbonic anhydrases accelerate CO_2 hydration dramatically. The most active enzymes, typified by human carbonic anhydrase II, hydrate CO_2 at rates as high as $k_{\text{cat}} = 10^6 \text{ s}^{-1}$, or a million times a second. Fundamental physical processes such as diffusion and proton transfer ordinarily limit the rate of hydration, and so special strategies are required to attain such prodigious rates.

9.2.1 Carbonic Anhydrase Contains a Bound Zinc Ion Essential for Catalytic Activity

Less than 10 years after the discovery of carbonic anhydrase in 1932, this enzyme was found to contain bound zinc, associated with catalytic activity. This discovery, remarkable at the time, made carbonic anhydrase the first known zinc-containing enzyme. At present, hundreds of enzymes are known to contain zinc. In fact, more than one-third of all enzymes either contain bound metal ions or require the addition of such ions for activity. The chemical reactivity of metal ions—associated with their positive charges, with their ability to form relatively strong yet kinetically labile bonds, and, in some cases, with their capacity to be stable in more than one oxidation state—explains why catalytic strategies that employ metal ions have been adopted throughout evolution.

The results of x-ray crystallographic studies have supplied the most detailed and direct information about the zinc site in carbonic anhydrase. At least seven carbonic anhydrases, each with its own gene, are present in human beings. They are all clearly homologous, as revealed by substantial levels of sequence identity. Carbonic anhydrase II, present in relatively

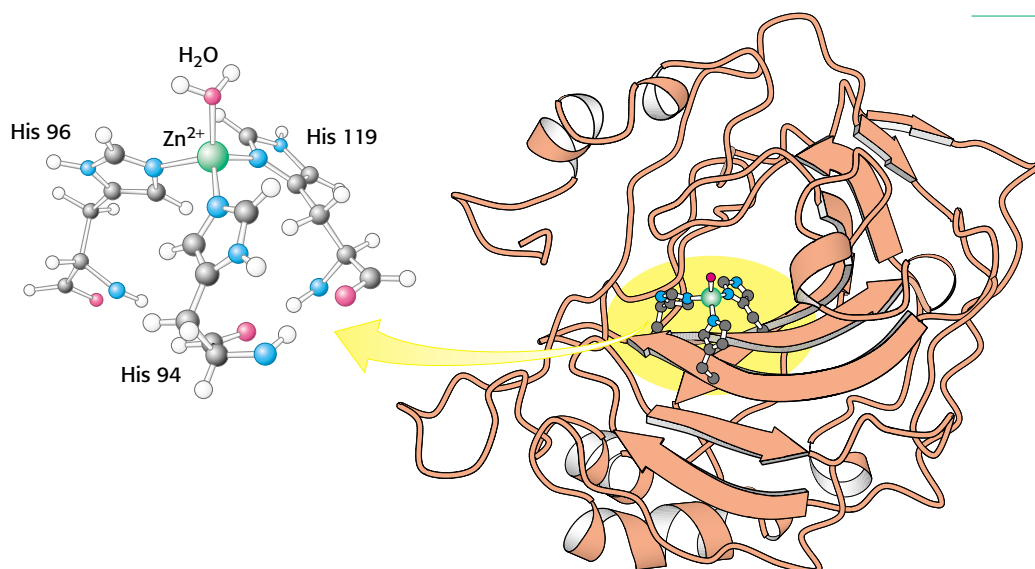


FIGURE 9.22 The structure of human carbonic anhydrase II and its zinc site. (Left) The zinc is bound to the imidazole rings of three histidine residues as well as to a water molecule. (Right) The location of the zinc site in the enzyme.

high concentrations in red blood cells, has been the most extensively studied (Figure 9.22).

Zinc is found only in the +2 state in biological systems; so we need consider only this oxidation level as we examine the mechanism of carbonic anhydrase. A zinc atom is essentially always bound to four or more ligands; in carbonic anhydrase, three coordination sites are occupied by the imidazole rings of three histidine residues and an additional coordination site is occupied by a water molecule (or hydroxide ion, depending on pH). Because all of the molecules occupying the coordination sites are neutral, the overall charge on the $\text{Zn}(\text{His})_3$ unit remains +2.

9.2.2 Catalysis Entails Zinc Activation of Water

How does this zinc complex facilitate carbon dioxide hydration? A major clue comes from the pH profile of enzymatically catalyzed carbon dioxide hydration (Figure 9.23). At pH 8, the reaction proceeds near its maximal rate. As the pH decreases, the rate of the reaction drops. The midpoint of this transition is near pH 7, suggesting that a group with $\text{p}K_a = 7$ plays an important role in the activity of carbonic anhydrase and that the deprotonated (high pH) form of this group participates more effectively in catalysis. Although some amino acids, notably histidine, have $\text{p}K_a$ values near 7, a variety of evidence suggests that the group responsible for this transition is not an amino acid but is the zinc-bound water molecule. Thus, the binding of a water molecule to the positively charged zinc center reduces the $\text{p}K_a$ of the water molecule from 15.7 to 7 (Figure 9.24). With the lowered $\text{p}K_a$, a

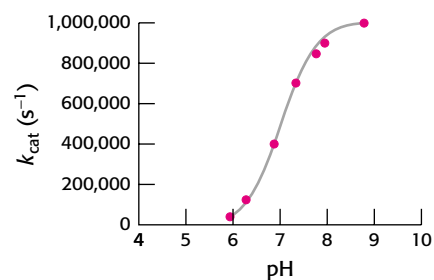


FIGURE 9.23 Effect of pH on carbonic anhydrase activity. Changes in pH alter the rate of carbon dioxide hydration catalyzed by carbonic anhydrase II. The enzyme is maximally active at high pH.

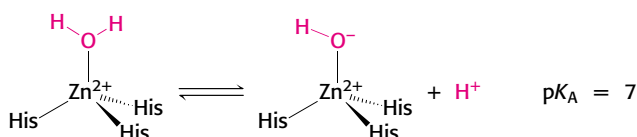


FIGURE 9.24 The $\text{p}K_a$ of water-bound zinc. Binding to zinc lowers the $\text{p}K_a$ of water from 15.7 to 7.

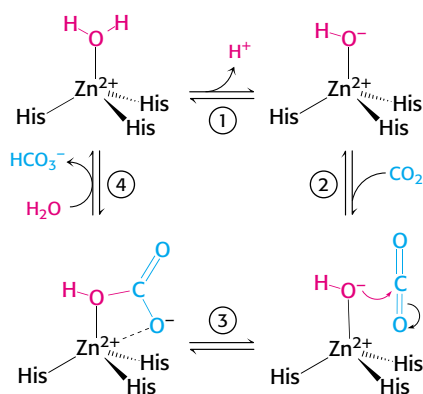


FIGURE 9.25 Mechanism of carbonic anhydrase. The zinc-bound hydroxide mechanism for the hydration of carbon dioxide catalyzed by carbonic anhydrase.

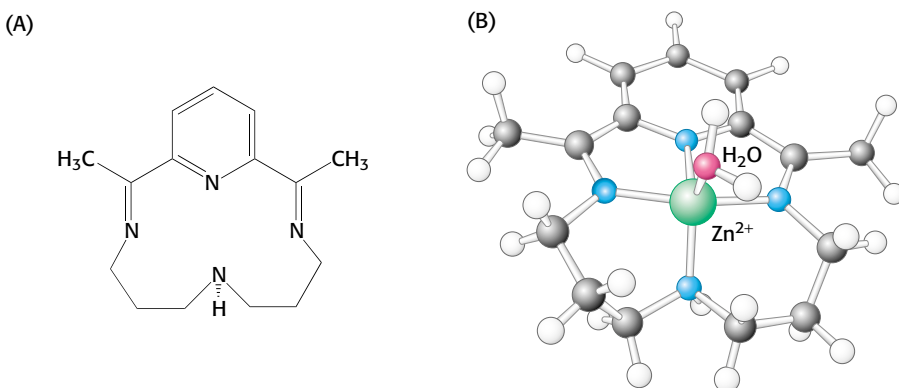
substantial concentration of hydroxide ion (bound to zinc) is generated at neutral pH. A zinc-bound hydroxide ion is sufficiently nucleophilic to attack carbon dioxide much more readily than water does. The importance of the zinc-bound hydroxide ion suggests a simple mechanism for carbon dioxide hydration (Figure 9.25).

1. Zinc facilitates the release of a proton from a water molecule, which generates a hydroxide ion.
2. The carbon dioxide substrate binds to the enzyme's active site and is positioned to react with the hydroxide ion.
3. The hydroxide ion attacks the carbon dioxide, converting it into bicarbonate ion.
4. The catalytic site is regenerated with the release of the bicarbonate ion and the binding of another molecule of water.

Thus, the binding of water to zinc favors the formation of the transition state, leading to bicarbonate formation by facilitating proton release and by bringing the two reactants into close proximity. A range of studies supports this mechanism. In particular, studies of a *synthetic analog model system* provide evidence for its plausibility. A simple synthetic ligand binds zinc through four nitrogen atoms (compared with three histidine nitrogen atoms in the enzyme), as shown in Figure 9.26. One water molecule remains bound to the zinc ion in the complex. Direct measurements reveal that this water molecule has a pK_a value of 8.7, not as low as the value for the water molecule in carbonic anhydrase but substantially lower than the value for free water. At pH 9.2, this complex accelerates the hydration of carbon dioxide more than 100-fold. Although catalysis by this synthetic system is much less efficient than catalysis by carbonic anhydrase, the model system strongly suggests that the zinc-bound hydroxide mechanism is likely to be correct. Carbonic anhydrases have evolved to utilize the reactivity intrinsic to a zinc-bound hydroxide ion as a potent catalyst.

FIGURE 9.26 A synthetic analog model system for carbonic anhydrase.

(A) An organic compound, capable of binding zinc, was synthesized as a model for carbonic anhydrase. The zinc complex of this ligand accelerates the hydration of carbon dioxide more than 100-fold under appropriate conditions. (B) The structure of the presumed active complex showing zinc bound to the ligand and to one water molecule.



9.2.3 A Proton Shuttle Facilitates Rapid Regeneration of the Active Form of the Enzyme

As noted earlier, some carbonic anhydrases can hydrate carbon dioxide at rates as high as a million times a second (10^6 s^{-1}). The magnitude of this rate can be understood from the following observations. At the conclusion of a carbon dioxide hydration reaction, the zinc-bound water molecule must lose a proton to regenerate the active form of the enzyme (Figure 9.27). The rate of the reverse reaction, the protonation of the zinc-bound hydroxide ion, is limited by the rate of proton diffusion. Protons diffuse very rapidly with second-

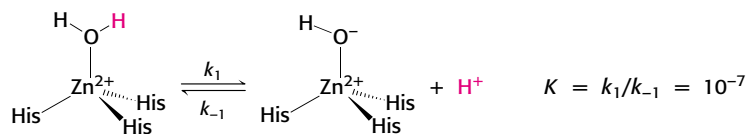


FIGURE 9.27 Kinetics of water deprotonation. The kinetics of deprotonation and protonation of the zinc-bound water molecule in carbonic anhydrase.

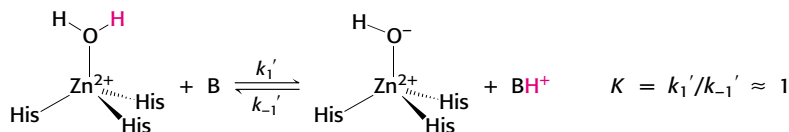


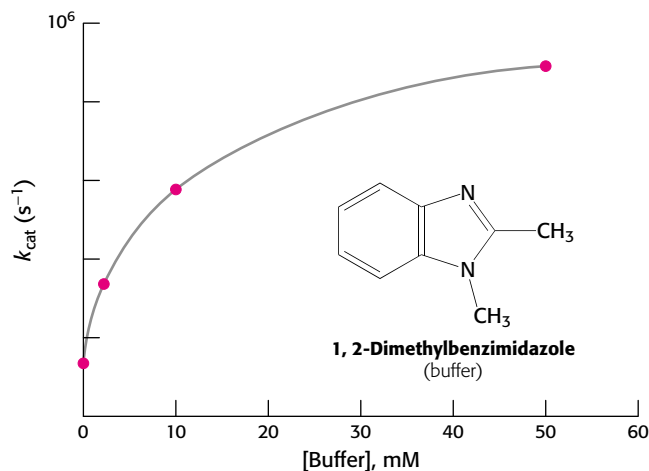
FIGURE 9.28 The effect of buffer on deprotonation. The deprotonation of the zinc-bound water molecule in carbonic anhydrase is aided by buffer component B.

order rate constants near $10^{-11} \text{ M}^{-1} \text{ s}^{-1}$. Thus, the backward rate constant k_{-1} must be less than $10^{11} \text{ M}^{-1} \text{ s}^{-1}$. Because the equilibrium constant K is equal to k_1/k_{-1} , the forward rate constant is given by $k_1 = K \cdot k_{-1}$. Thus, if $k_{-1} \leq 10^{11} \text{ M}^{-1} \text{ s}^{-1}$ and $K = 10^{-7} \text{ M}$ (because $\text{p}K_a = 7$), then k_1 must be less than or equal to 10^4 s^{-1} . In other words, the rate of proton diffusion limits the rate of proton release to less than 10^4 s^{-1} for a group with $\text{p}K_a = 7$. However, if carbon dioxide is hydrated at a rate of 10^6 s^{-1} , then every step in the mechanism (see Figure 9.25) must take place at least this fast. How can this apparent paradox be resolved?

The answer became clear with the realization that *the highest rates of carbon dioxide hydration require the presence of buffer, suggesting that the buffer components participate in the reaction*. The buffer can bind or release protons. The advantage is that, whereas the concentrations of protons and hydroxide ions are limited to 10^{-7} M at neutral pH, the concentration of buffer components can be much higher, on the order of several millimolar. If the buffer component BH^+ has a $\text{p}K_a$ of 7 (matching that for the zinc-bound water), then the equilibrium constant for the reaction in Figure 9.28 is 1. The rate of proton abstraction is given by $k_1' \cdot [\text{B}]$. The second-order rate constants k_1' and k_{-1}' will be limited by buffer diffusion to values less than approximately $10^9 \text{ M}^{-1} \text{ s}^{-1}$. Thus, buffer concentrations greater than $[\text{B}] = 10^{-3} \text{ M}$ (1 mM) may be high enough to support carbon dioxide hydration rates of $10^6 \text{ M}^{-1} \text{ s}^{-1}$ because $k_1' \cdot [\text{B}] = (10^9 \text{ M}^{-1} \text{ s}^{-1}) \cdot (10^{-3} \text{ M}) = 10^6 \text{ s}^{-1}$. This prediction is confirmed experimentally (Figure 9.29).

The molecular components of many buffers are too large to reach the active site of carbonic anhydrase. Carbonic anhydrase II has evolved a *proton shuttle* to allow buffer components to participate in the reaction from solution. The primary component of this shuttle is histidine 64. This residue transfers protons from the zinc-bound water molecule to the protein surface and then to the buffer (Figure 9.30). Thus, *catalytic function has been enhanced through the evolution of an apparatus for controlling proton transfer from and to the active site*. Because protons participate in many biochemical reactions, the manipulation of the proton inventory within active sites is crucial to the function of many enzymes and explains the prominence of acid–base catalysis.

FIGURE 9.29 The effect of buffer concentration on the rate of carbon dioxide hydration. The rate of carbon dioxide hydration increases with the concentration of the buffer 1,2-dimethylbenzimidazole. The buffer enables the enzyme to achieve its high catalytic rates.



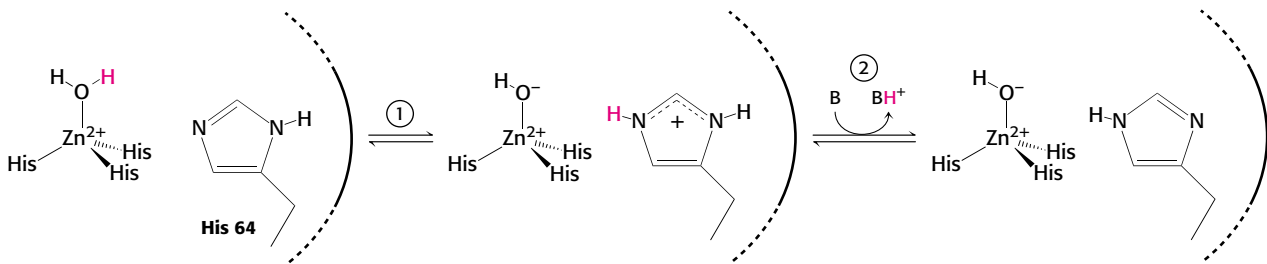


FIGURE 9.30 Histidine proton shuttle. (1) Histidine 64 abstracts a proton from the zinc bound water molecule, generating a nucleophilic hydroxide ion and a protonated histidine. (2) The buffer (B) removes a proton from the histidine, regenerating the unprotonated form.

9.2.4 Convergent Evolution Has Generated Zinc-Based Active Sites in Different Carbonic Anhydrases



Carbonic anhydrases homologous to the human enzymes, referred to as α -carbonic anhydrases, are common in animals and in some bacteria and algae. In addition, two other families of carbonic anhydrases have been discovered. The β -carbonic anhydrases are found in higher plants and in many bacterial species, including *E. coli*. These proteins contain the zinc required for catalytic activity but are not significantly similar in sequence to the α -carbonic anhydrases. Furthermore, the β -carbonic anhydrases have only one conserved histidine residue, whereas the α -carbonic anhydrases have three. No three-dimensional structure is yet available, but spectroscopic studies suggest that the zinc is bound by one histidine residue, two cysteine residues (conserved among β -carbonic anhydrases), and a water molecule. A third family, the γ -carbonic anhydrases, also has been identified, initially in the archaeon *Methanosarcina thermophila*. The crystal structure of this enzyme reveals three zinc sites extremely similar to those in the α -carbonic anhydrases. In this case, however, the three zinc sites lie at the interfaces between the three subunits of a trimeric enzyme (Figure 9.31). The very striking left-handed β -helix (a β strand twisted into a left-handed helix) structure present in this enzyme has also been found in enzymes that catalyze reactions unrelated to those of carbonic anhydrase. Thus, convergent evolution has generated carbonic anhydrases that rely on coordinated zinc ions at least three times. In each

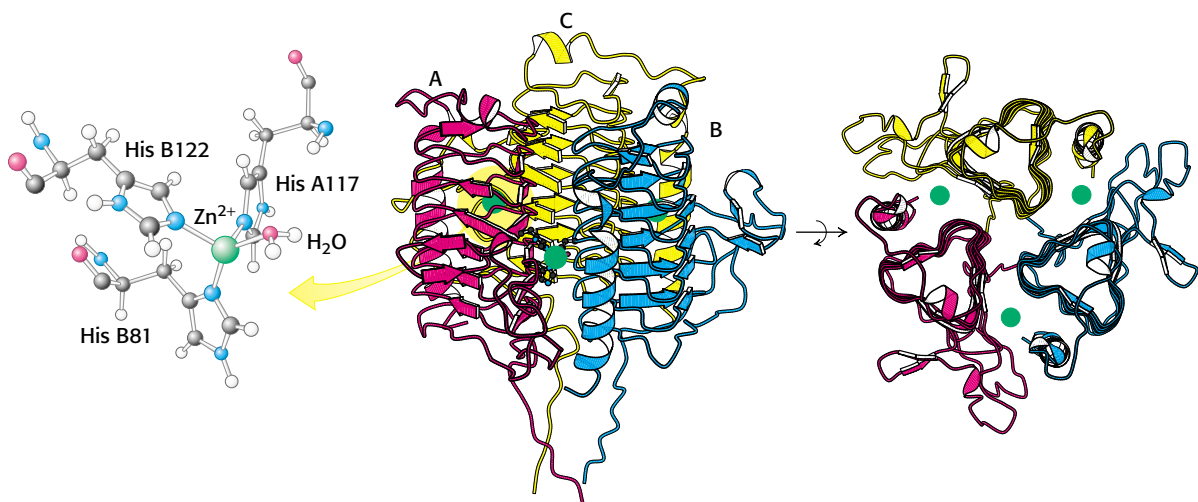


FIGURE 9.31 γ -Carbonic anhydrase. (Left) The zinc site of γ -carbonic anhydrase. (Middle) The trimeric structure of the protein (individual chains are labeled A, B, and C). (Right) The protein is rotated to show a top-down view that highlights its threefold symmetry and the position of the zinc sites (green) at the interfaces between subunits.

case, the catalytic activity appears to be associated with zinc-bound water molecules.

9.3 RESTRICTION ENZYMES: PERFORMING HIGHLY SPECIFIC DNA-CLEAVAGE REACTIONS

Let us next consider a hydrolytic reaction that results in the cleavage of DNA. Bacteria and archaea have evolved mechanisms to protect themselves from viral infections. Many viruses inject their DNA genomes into cells; once inside, the viral DNA hijacks the cell's machinery to drive the production of viral proteins and, eventually, of progeny virus. Often, a viral infection results in the death of the host. A major protective strategy for the host is to use *restriction endonucleases* (restriction enzymes) to degrade the viral DNA on its introduction into a cell. These enzymes recognize particular base sequences, called *recognition sequences* or *recognition sites*, in their target DNA and cleave that DNA at defined positions. The most well studied class of restriction enzymes comprises the so-called type II restriction enzymes, which cleave DNA *within* their recognition sequences. Other types of restriction enzymes cleave DNA at positions somewhat distant from their recognition sites.

Restriction endonucleases must show tremendous specificity at two levels. First, they must cleave only DNA molecules that contain recognition sites (hereafter referred to as *cognate DNA*) without cleaving DNA molecules that lack these sites. Suppose that a recognition sequence is six base pairs long. Because there are 4^6 , or 4096, sequences having six base pairs, the concentration of sites that must not be cleaved will be approximately 5000-fold as high as the concentration of sites that should be cleaved. Thus, to keep from damaging host-cell DNA, endonucleases must cleave cognate DNA molecules much more than 5000 times as efficiently as they cleave nonspecific sites. Second, restriction enzymes must not degrade the host DNA. How do these enzymes manage to degrade viral DNA while sparing their own?

The restriction endonuclease *EcoRV* (from *E. coli*) cleaves double-stranded viral DNA molecules that contain the sequence 5'-GATATC-3' but leaves intact host DNA containing hundreds of such sequences. The host DNA is protected by other enzymes called methylases, which methylate adenine bases within host recognition sequences (Figure 9.32). For each restriction endonuclease, the host cell produces a corresponding methylase that marks the host DNA and prevents its degradation. These pairs of enzymes are referred to as *restriction-modification systems*. We shall return to the mechanism used to achieve the necessary levels of specificity after considering the chemistry of the cleavage process.

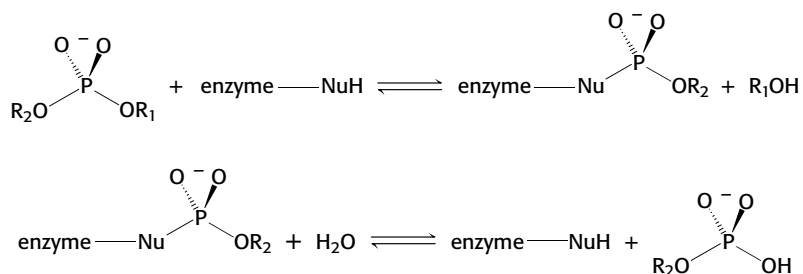


FIGURE 9.32 Protection by methylation. The recognition sequence for *EcoRV* endonuclease (left) and the sites of methylation (right) in DNA protected from the catalytic action of the enzyme.

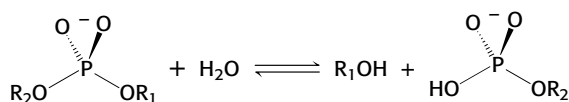
9.3.1 Cleavage Is by In-Line Displacement of 3' Oxygen from Phosphorus by Magnesium-Activated Water

The fundamental reaction catalyzed by restriction endonucleases is the hydrolysis of the phosphodiester backbone of DNA. Specifically, the bond between the 3' oxygen atom and the phosphorus atom is broken. The products of this reaction are DNA strands with a free 3'-hydroxyl group and a 5'-phosphoryl group (Figure 9.33). This reaction proceeds by nucleophilic attack at the phosphorus atom. We will consider two types of mechanism, as suggested by analogy with the proteases. The restriction endonuclease might cleave DNA in mechanism 1 through a covalent intermediate, employing a potent nucleophile (Nu), or in mechanism 2 by direct hydrolysis:

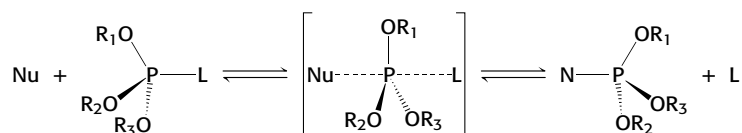
Mechanism Type 1 (covalent intermediate)



Mechanism Type 2 (direct hydrolysis)



Each postulates a different nucleophile to carry out the attack on the phosphorus. In either case, each reaction takes place by an *in-line displacement* path:



The incoming nucleophile attacks the phosphorus atom, and a pentacoordinate transition state is formed. This species has a trigonal bipyramidal geometry centered at the phosphorus atom, with the incoming nucleophile at one

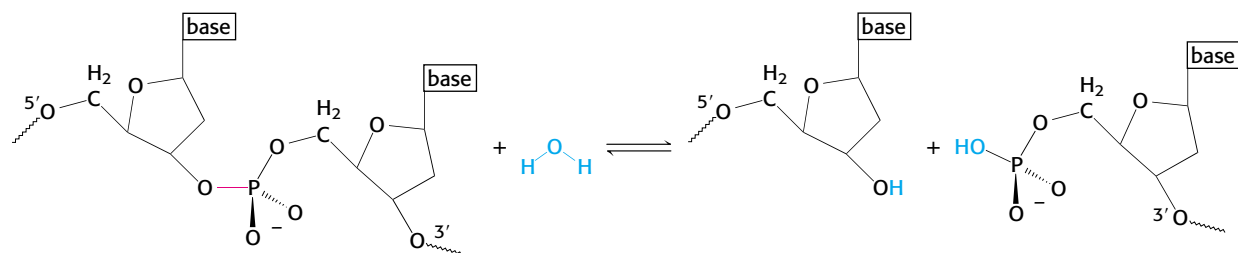


FIGURE 9.33 Hydrolysis of a phosphodiester bond. All restriction enzymes catalyze the hydrolysis of DNA phosphodiester bonds, leaving a phosphoryl group attached to the 5' end. The bond that is cleaved is shown in red.

apex of the two pyramids and the group that is displaced (the leaving group, L) at the other apex. The two mechanisms differ in the number of times the displacement occurs in the course of the reaction.

In the first type of mechanism, a nucleophile in the enzyme (analogous to serine 195 in chymotrypsin) attacks the phosphoryl group to form a covalent intermediate. In a second step, this intermediate is hydrolyzed to produce the final products. Because two displacement reactions take place at the phosphorus atom in the first mechanism, the stereochemical configuration at the phosphorus atom would be inverted and then inverted again, and the overall configuration would be retained. In the second type of mechanism, analogous to that used by the aspartyl and metalloproteases, an activated water molecule attacks the phosphorus atom directly. In this mechanism, a single displacement reaction takes place at the phosphorus atom. Hence, the stereochemical configuration of the tetrahedral phosphorus atom is inverted each time a displacement reaction takes place. Monitoring the stereochemical changes of the phosphorus could be one approach to determining the mechanism of restriction endonuclease action.

A difficulty is that the phosphorus centers in DNA are not chiral, because two of the groups bound to the phosphorus atom are simple oxygen atoms, identical with each other. This difficulty can be circumvented by preparing DNA molecules that contain chiral phosphoryl groups, made by replacing one oxygen atom with sulfur (called a phosphorothioate). Let us consider *EcoRV* endonuclease. This enzyme cleaves the phosphodiester bond between the T and the A at the center of the recognition sequence 5'-GATATC-3'. The first step in monitoring the activity of the enzyme is to synthesize an appropriate substrate for *EcoRV* containing phosphorothioates at the sites of cleavage (Figure 9.34). The reaction is then performed in water that has been greatly enriched in ^{18}O to allow the incoming oxygen atom to be marked. The location of the ^{18}O label with respect to the sulfur atom indicates whether the reaction proceeds with inversion or retention of stereochemistry. *The analysis revealed that the stereochemical configuration at the phosphorus atom was inverted only once with cleavage.* This result is consistent with a direct attack of water at phosphorus and rules out the formation of any covalently bound intermediate (Figure 9.35).

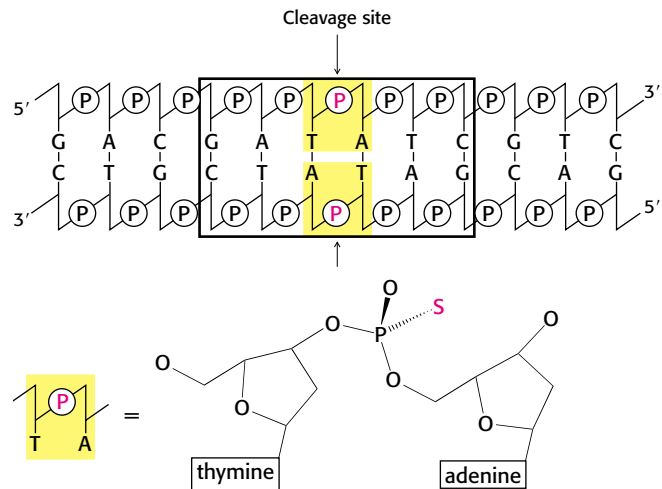


FIGURE 9.34 Labeling with phosphorothioates. Phosphorothioates, groups in which one of the nonbridging oxygen atoms is replaced with a sulfur atom, can be used to label specific sites in the DNA backbone to determine the overall stereochemical course of a displacement reaction. Here, a phosphorothioate is placed at sites that can be cleaved by *EcoRV* endonuclease.

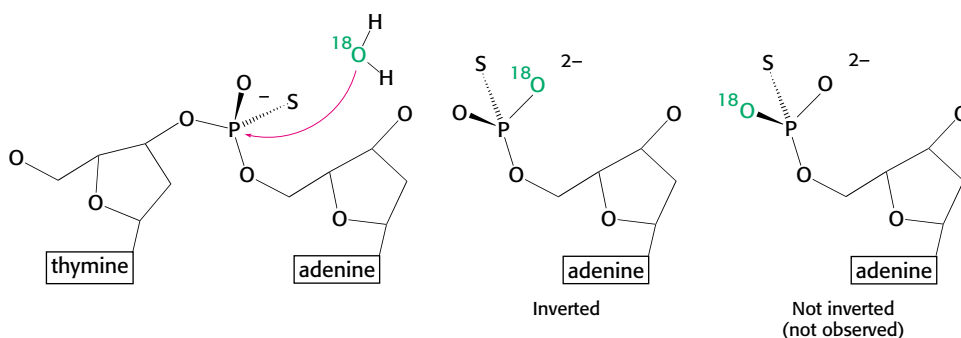


FIGURE 9.35 Stereochemistry of cleaved DNA. Cleavage of DNA by *EcoRV* endonuclease results in overall inversion of the stereochemical configuration at the phosphorus atom, as indicated by the stereochemistry of the phosphorus atom bound to one bridging oxygen atom, one ^{16}O , one ^{18}O , and one sulfur atom. This configuration strongly suggests that the hydrolysis takes place by the direct attack of water on the phosphorus atom.

9.3.2 Restriction Enzymes Require Magnesium for Catalytic Activity

Restriction endonucleases as well as many other enzymes that act on phosphate-containing substrates require Mg^{2+} or some other similar divalent cation for activity. What is the function of this metal?

It has been possible to examine the interactions of the magnesium ion when it is bound to the enzyme. Crystals have been produced of *EcoRV* endonuclease bound to oligonucleotides that contain the appropriate recognition sequences. These crystals are grown in the absence of magnesium to prevent cleavage; then, when produced, the crystals are soaked in solutions containing the metal. No cleavage takes place, allowing the location of the magnesium ion binding sites to be determined (Figure 9.36). The magnesium ion was found to be bound to six ligands: three are water molecules, two are carboxylates of the enzyme's aspartate residues, and one is an oxygen atom of the phosphoryl group at the site of cleavage. The magnesium ion holds a water molecule in a position from which the water molecule can attack the phosphoryl group and, in conjunction with the aspartate residues, helps polarize the water molecule toward deprotonation. Because cleavage does not take place within these crystals, the observed structure cannot be the true catalytic conformation. Additional studies have revealed that a second magnesium ion must be present in an adjacent site for *EcoRV* endonuclease to cleave its substrate.

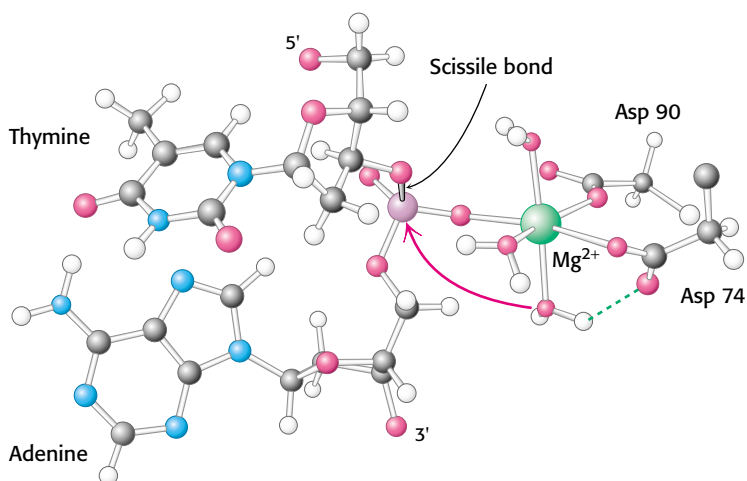


FIGURE 9.36 Magnesium ion binding site in *EcoRV* endonuclease. The magnesium ion helps to activate a water molecule and positions it so that it can attack the phosphate.

9.3.3 The Complete Catalytic Apparatus Is Assembled Only Within Complexes of Cognate DNA Molecules, Ensuring Specificity

We now return to the question of specificity, the defining feature of restriction enzymes. The recognition sequences for most restriction endonucleases are *inverted repeats*. This arrangement gives the three-dimensional structure of the recognition site a *twofold rotational symmetry* (Figure 9.37). The restriction enzymes display a corresponding symmetry to facilitate recognition: they are dimers whose two subunits are related by twofold rotational symmetry. The matching symmetry of the recognition sequence and the enzyme has been confirmed by the determination of the structure of the complex between *EcoRV* endonuclease and DNA fragments containing its recognition sequence (Figure 9.38). The enzyme surrounds the DNA in a tight embrace. Examination of this structure reveals features that are highly significant in determining specificity.

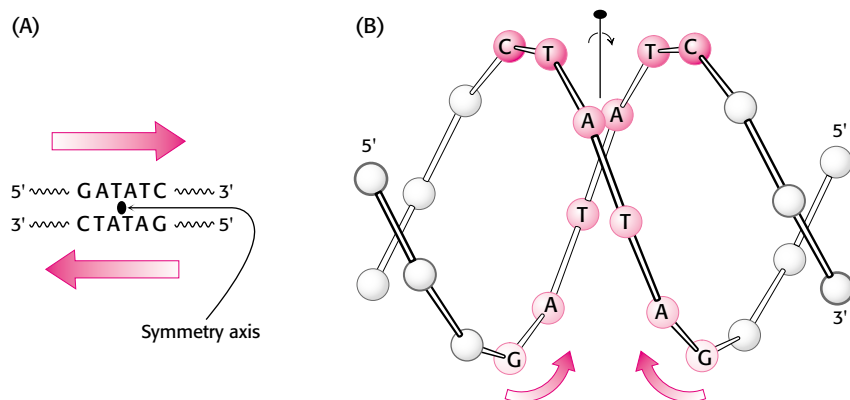


FIGURE 9.37 Structure of the recognition site of *EcoRV* endonuclease. (A) The sequence of the recognition site, which is symmetric around the axis of rotation designated in green. (B) The inverted repeat within the recognition sequence of *EcoRV* (and most other restriction endonucleases) endows the DNA site with twofold rotational symmetry.

A unique set of interactions occurs between the enzyme and a cognate DNA sequence. Within the 5'-GATATC-3' sequence, the G and A bases at the 5' end of each strand and their Watson-Crick partners directly contact the enzyme by hydrogen bonding with residues that are located in two loops, one projecting from the surface of each enzyme subunit (Figure 9.39). The most striking feature of this complex is the *distortion of the DNA*, which is substantially kinked in the center (Figure 9.40). The central two TA base pairs in the recognition sequence play a key role in producing the kink. They do not make contact with the enzyme but appear to be required because of their ease of distortion. 5'-TA-3' sequences are known to be among the most easily deformed base pairs. The distortion of the DNA at this site has severe effects on the specificity of enzyme action.

Specificity is often determined by an enzyme's binding affinity for substrates. In regard to *EcoRV* endonuclease, however, binding studies performed in the absence of magnesium have demonstrated that the enzyme binds to all sequences, both cognate and noncognate, with approximately equal affinity. However, the structures of complexes formed with noncognate DNA fragments are strikingly different from those formed with cognate DNA: the noncognate DNA conformation is not substantially distorted (Figure 9.41). *This lack of distortion has important consequences with regard to catalysis. No phosphate is positioned sufficiently close to the active-site*

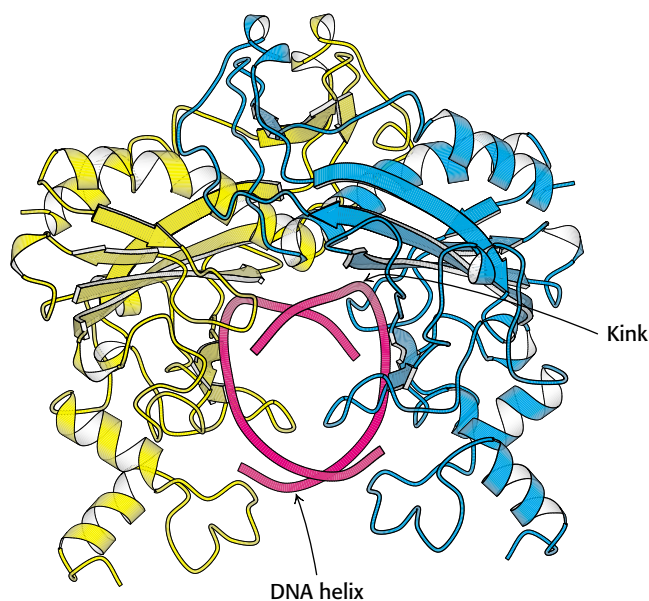


FIGURE 9.38 Structure of the *EcoRV*-cognate DNA complex. This view of the structure of *EcoRV* endonuclease bound to a cognate DNA fragment is down the helical axis of the DNA. The two protein subunits are in yellow and blue, and the DNA backbone is in red. The twofold axes of the enzyme dimer and the DNA are aligned.

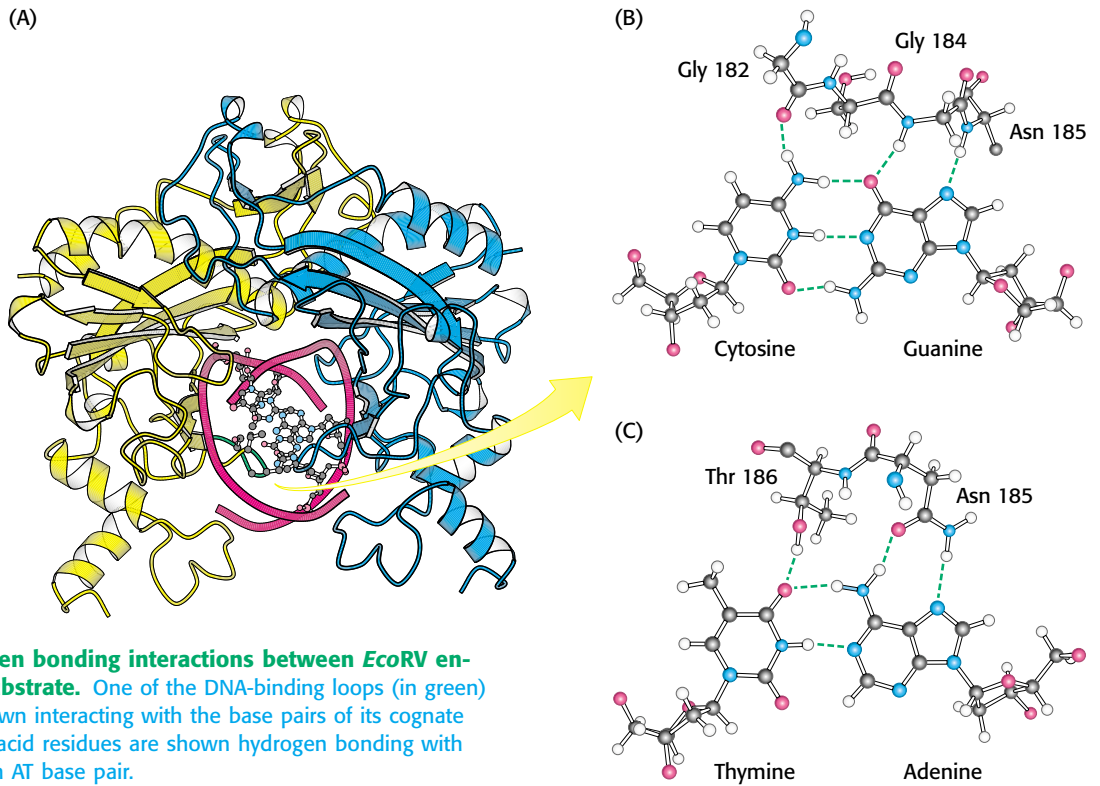


FIGURE 9.39 Hydrogen bonding interactions between *EcoRV* endonuclease and its DNA substrate. One of the DNA-binding loops (in green) of *EcoRV* endonuclease is shown interacting with the base pairs of its cognate DNA binding site. Key amino acid residues are shown hydrogen bonding with (B) a CG base pair and (C) an AT base pair.

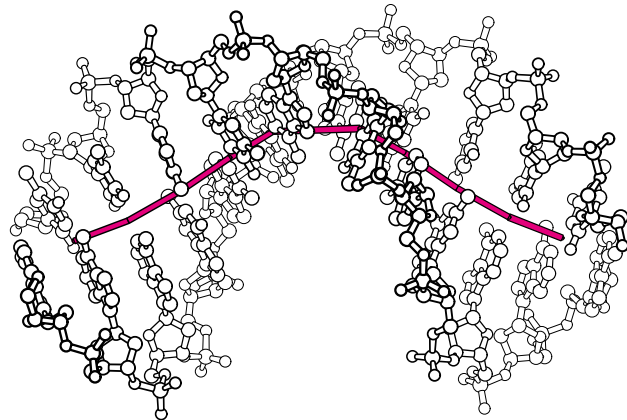


FIGURE 9.40 Distortion of the recognition site. The DNA is represented as a ball-and-stick model. The path of the DNA helical axis, shown in red, is substantially distorted on binding to the enzyme. For the B form of DNA, the axis is straight (not shown).

aspartate residues to complete a magnesium ion binding site (see Figure 9.36). Hence, the nonspecific complexes do not bind the magnesium ion and the complete catalytic apparatus is never assembled. The distortion of the substrate and the subsequent binding of the magnesium ion account for the catalytic specificity of more than 1,000,000-fold that is observed for *EcoRV* endonuclease despite very little preference at the level of substrate binding.

We can now see the role of binding energy in this strategy for attaining catalytic specificity. In binding to the enzyme, the DNA is distorted in such a way that additional contacts are made between the enzyme and the substrate, increasing the binding energy. However, this increase is canceled by the energetic cost of distorting the DNA from its relaxed conformation (Figure 9.42). Thus, for *EcoRV* endonuclease, there is little difference in binding affinity for cognate and nonspecific DNA fragments. However, the distortion in the cognate complex dramatically affects catalysis by



FIGURE 9.41 Nonspecific and cognate DNA within *EcoRV* endonuclease. A comparison of the positions of the nonspecific (orange) and the cognate DNA (red) within *EcoRV* reveals that, in the nonspecific complex, the DNA backbone is too far from the enzyme to complete the magnesium ion binding sites.

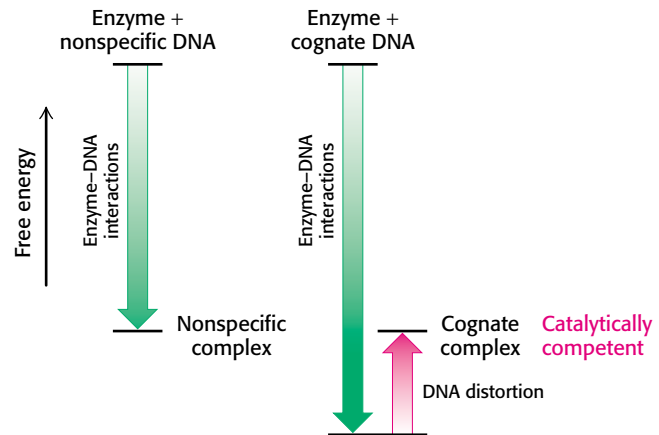


FIGURE 9.42 Greater binding energy of *EcoRV* endonuclease bound to cognate versus noncognate DNA. The additional interactions between *EcoRV* endonuclease and cognate DNA increase the binding energy, which can be used to drive DNA distortions necessary for forming a catalytically competent complex.

completing the magnesium ion binding site. This example illustrates how enzymes can utilize available binding energy to deform substrates and poise them for chemical transformation. Interactions that take place within the distorted substrate complex stabilize the transition state leading to DNA hydrolysis.

The distortion in the DNA explains how methylation blocks catalysis and protects host-cell DNA. When a methyl group is added to the amino group of the adenine nucleotide at the 5' end of the recognition sequence, the methyl group's presence precludes the formation of a hydrogen bond between the amino group and the side-chain carbonyl group of asparagine 185 (Figure 9.43). This asparagine residue is closely linked to the other amino acids that form specific contacts with the DNA. The absence of the hydrogen bond disrupts other interactions between the enzyme and the DNA substrate, and the distortion necessary for cleavage will not take place.

9.3.4 Type II Restriction Enzymes Have a Catalytic Core in Common and Are Probably Related by Horizontal Gene Transfer

Type II restriction enzymes are prevalent in Archaea and Eubacteria. What can we tell of the evolutionary history of these enzymes? Comparison of the amino acid sequences of a variety of type II restriction endonucleases did not reveal significant sequence similarity between most pairs of enzymes. However, a careful examination of three-dimensional structures, taking into account the location of the active sites, revealed the presence of a core structure conserved in the different enzymes. This structure includes β strands that contain the aspartate (or, in some cases, glutamate) residues forming the magnesium ion binding sites (Figure 9.44).

These observations indicate that many type II restriction enzymes are indeed evolutionary related. Analyses of the sequences in greater detail suggest that bacteria may have obtained genes encoding these enzymes from

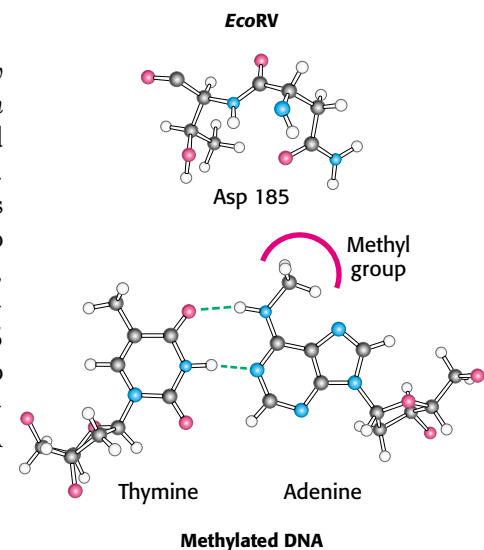


FIGURE 9.43 Methylation of adenine. The methylation of adenine blocks the formation of hydrogen bonds between *EcoRV* endonuclease and cognate DNA molecules and prevents their hydrolysis.

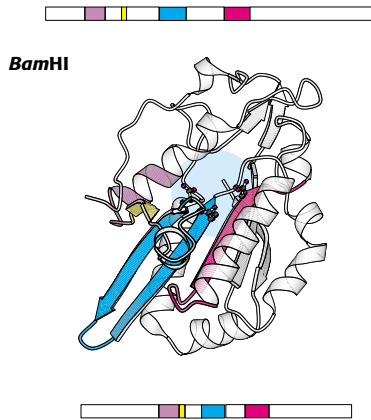
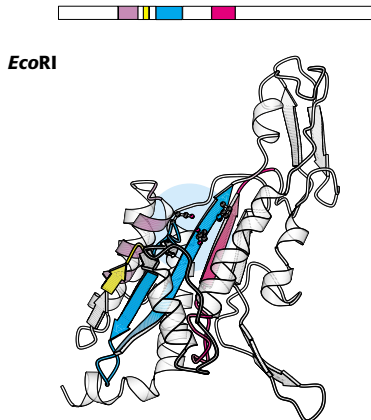
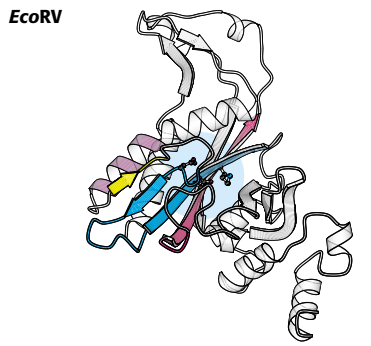


FIGURE 9.44 A conserved structural core in type II restriction enzymes. Four conserved structural elements, including the active-site region (in blue), are highlighted in color in these models of a single monomer from each dimeric enzyme. The positions of the amino acid sequences that form these elements within each overall sequence are represented schematically below each structure.

other species by *horizontal gene transfer*, the passing between species of pieces of DNA (such as plasmids) that provide a selective advantage in a particular environment. For example, *EcoRI* (from *E. coli*) and *RsrI* (from *Rhodobacter sphaeroides*) are 50% identical in sequence over 266 amino acids, clearly indicative of a close evolutionary relationship. However, these species of bacteria are not closely related, as is known from sequence comparisons of other genes and other evidence. Thus, *it appears that these species obtained the gene for this restriction endonuclease from a common source more recently than the time of their evolutionary divergence*. Moreover, the gene encoding *EcoRI* endonuclease uses particular codons to specify given amino acids that are strikingly different from the codons used by most *E. coli* genes, which suggests that the gene did not originate in *E. coli*. Horizontal gene transfer may be a relatively common event. For example, genes that inactivate antibiotics are often transferred, leading to the transmission of antibiotic resistance from one species to another. For restriction-modification systems, protection against viral infections may have favored horizontal gene transfer.

9.4 NUCLEOSIDE MONOPHOSPHATE KINASES: CATALYZING PHOSPHORYL GROUP EXCHANGE BETWEEN NUCLEOTIDES WITHOUT PROMOTING HYDROLYSIS

The final enzymes that we shall consider are the nucleoside monophosphate kinases (NMP kinases), typified by adenylate kinase. These enzymes catalyze the transfer of the terminal phosphoryl group from a nucleoside triphosphate (NTP), usually ATP, to the phosphoryl group on a nucleoside monophosphate (Figure 9.45). The challenge for NMP kinases is to promote the transfer of the phosphoryl group from NTP to NMP without promoting the competing reaction—the transfer of a phosphoryl group from NTP to water; that is, NTP hydrolysis. We shall see how the use of induced fit by these enzymes is used to solve this problem. Moreover, these enzymes employ metal ion catalysis; but, in this case, the metal forms a complex with the substrate to enhance enzyme–substrate interaction.

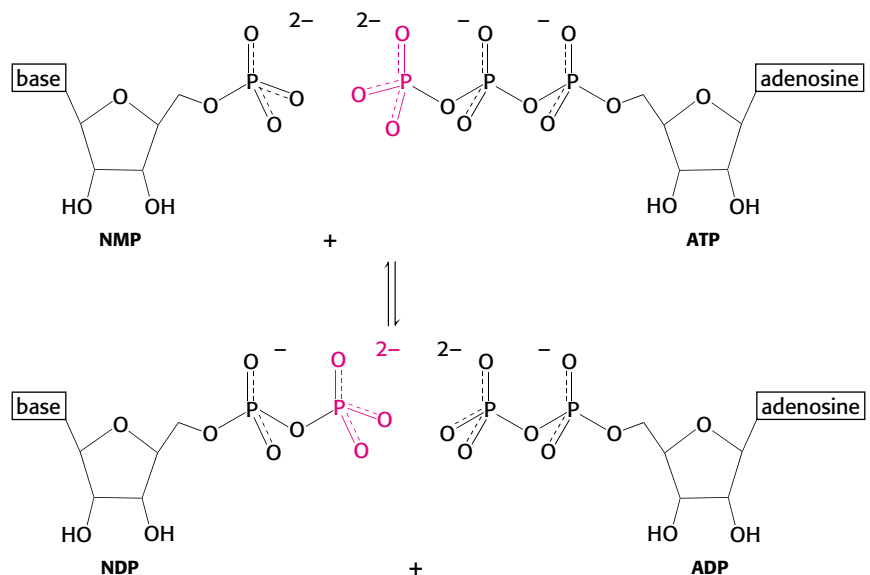


FIGURE 9.45 Phosphoryl group transfer by nucleoside monophosphate kinases. These enzymes catalyze the interconversion of nucleoside triphosphate (here, ATP) and a nucleoside monophosphate (NMP) into two nucleoside diphosphates by the transfer of a phosphoryl group (shown in red).

9.4.1 NMP Kinases Are a Family of Enzymes Containing P-Loop Structures

X-ray crystallographic methods have yielded the three-dimensional structures of a number of different NMP kinases, both free and bound to substrates or substrate analogs. Comparison of these structures reveals that these enzymes form a family of homologous proteins (Figure 9.46). In particular, such comparisons reveal the presence of a conserved NTP-binding domain. This domain consists of a central β sheet, surrounded on both sides by α helices (Figure 9.47). A characteristic feature of this domain is a loop between the first β strand and the first helix. This loop, which typically has an amino acid sequence of the form Gly-X-X-X-Gly-Lys, is often referred to as the *P-loop* because it interacts with phosphoryl groups on the bound nucleotide (Figure 9.48). As described in Section 9.4.4, similar domains containing P-loops are present in a wide variety of important nucleotide-binding proteins.

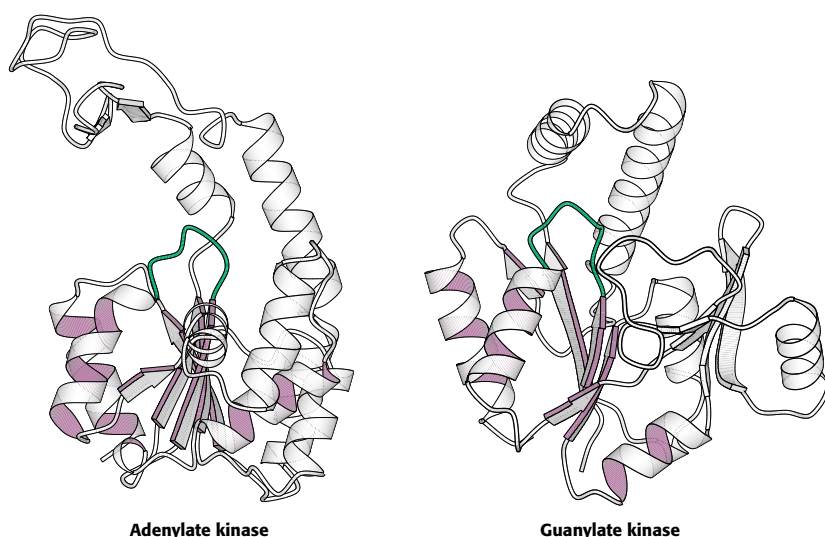


FIGURE 9.46 Structures of adenylate kinase and guanylate kinase. The nucleoside triphosphate-binding domain is a common feature in these and other homologous nucleotide kinases. The domain consists of a central β -pleated sheet surrounded on both sides by α helices (highlighted in purple) as well as a key loop (shown in green).

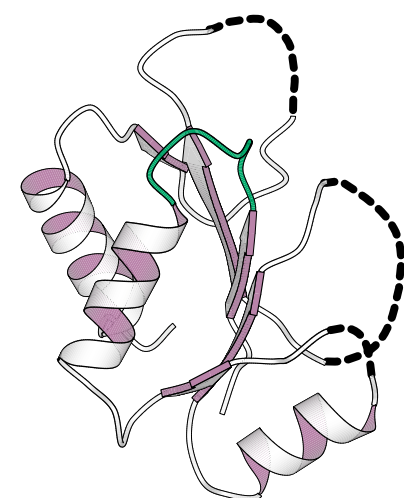


FIGURE 9.47 The core domain of NMP kinases. The P-loop is shown in green. The dashed lines represent the remainder of the protein structure.

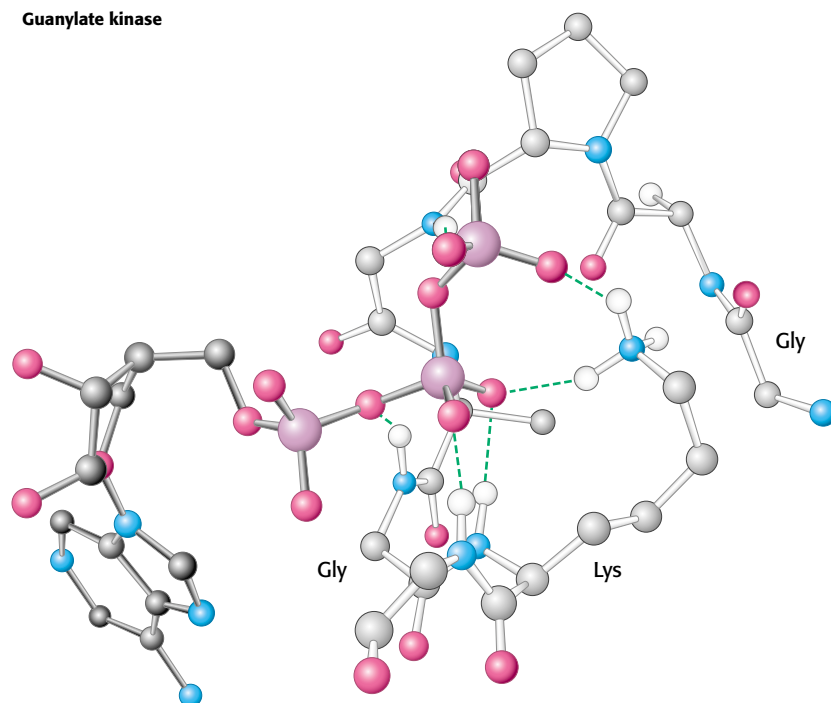


FIGURE 9.48 P-loop interaction with ATP. The P-loop of adenylate kinase interacts with the phosphoryl groups of ATP (shown with dark bonds). Hydrogen bonds (green) link ATP to peptide NH groups as well as a lysine residue conserved among NMP kinases.

9.4.2 Magnesium (or Manganese) Complexes of Nucleoside Triphosphates Are the True Substrates for Essentially All NTP-Dependent Enzymes

Kinetic studies of NMP kinases, as well as many other enzymes having ATP or other nucleoside triphosphates as a substrate, reveal that these enzymes are essentially inactive in the absence of divalent metal ions such as magnesium (Mg^{2+}) or manganese (Mn^{2+}), but acquire activity on the addition of these ions. In contrast with the enzymes discussed so far, the metal is not a component of the active site. Rather, nucleotides such as ATP bind these ions, and *it is the metal ion–nucleotide complex that is the true substrate for the enzymes*. The dissociation constant for the ATP–Mg^{2+} complex is approximately 0.1 mM, and thus, given that intracellular Mg^{2+} concentrations are typically in the millimolar range, essentially all nucleoside triphosphates are present as NTP–Mg^{2+} complexes.

How does the binding of the magnesium ion to the nucleotide affect catalysis? There are a number of related consequences, but all serve to enhance the specificity of the enzyme–substrate interactions by enhancing binding energy. First, the magnesium ion neutralizes some of the negative charges present on the polyphosphate chain, reducing nonspecific ionic interactions between the enzyme and the polyphosphate group of the nucleotide. Second, the interactions between the magnesium ion and the oxygen atoms in the phosphoryl group hold the nucleotide in well-defined conformations that can be specifically bound by the enzyme (Figure 9.49). Magnesium ions are typically coordinated to six groups in an octahedral arrangement. Typically, two oxygen atoms are directly coordinated to a magnesium ion, with the remaining coordination positions often occupied

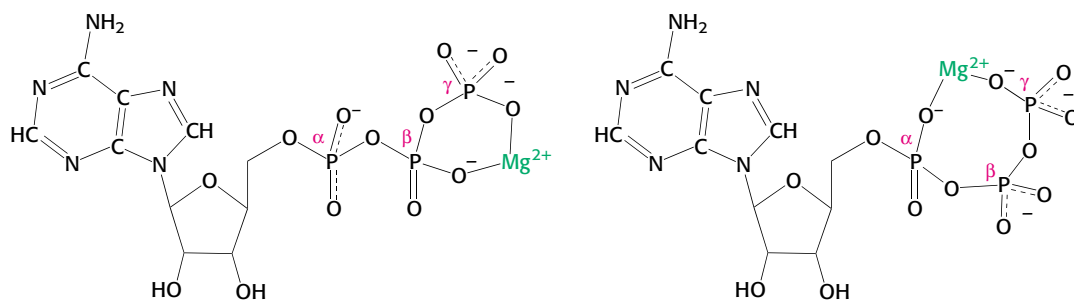
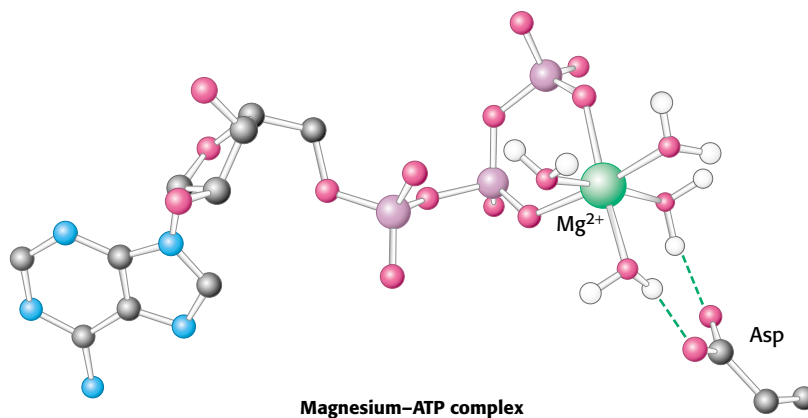


FIGURE 9.49 The structures of two isomeric forms of the ATP–Mg^{2+} complex. Other groups coordinated to the magnesium ion have been omitted for clarity.

FIGURE 9.50 ATP–Mg^{2+} complex bound to adenylate kinase. The magnesium ion is bound to the β and γ phosphoryl groups, and the four water molecules bound to the remaining coordination positions interact with groups on the enzyme, including a conserved aspartate residue. Other interactions have been omitted for clarity.




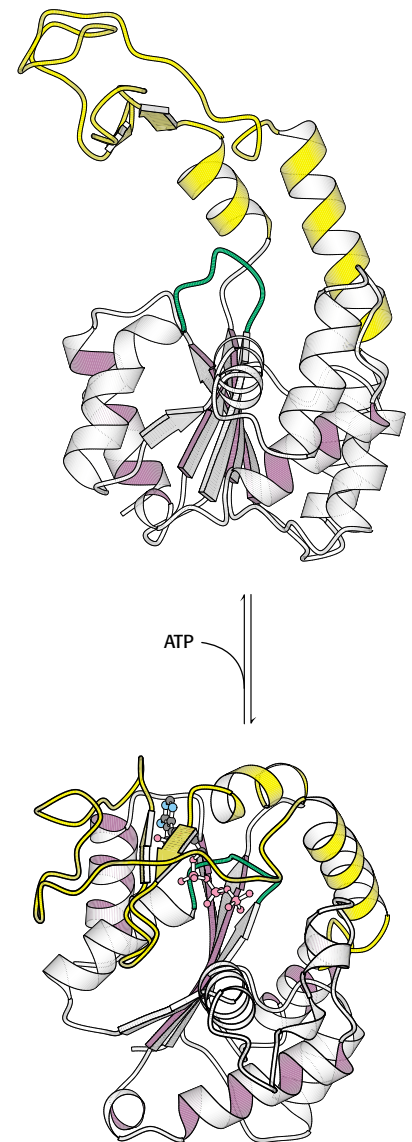
by water molecules. Oxygen atoms of the α and β , β and γ , or α and γ phosphoryl groups may contribute, depending on the particular enzyme. In addition, different stereoisomers are produced, depending on exactly which oxygen atoms bind to the metal ion. Third, the magnesium ion provides additional points of interaction between the ATP-Mg²⁺ complex and the enzyme, thus increasing the binding energy. In some cases, such as the DNA polymerases (Section 27.x.x), side chains (often aspartate and glutamate residues) of the enzyme can bind directly to the magnesium ion. In other cases, the enzyme interacts indirectly with the magnesium ion through hydrogen bonds to the coordinated water molecules (Figure 9.50). Such interactions have been observed in adenylate kinases bound to ATP analogs.


9.4.3 ATP Binding Induces Large Conformational Changes

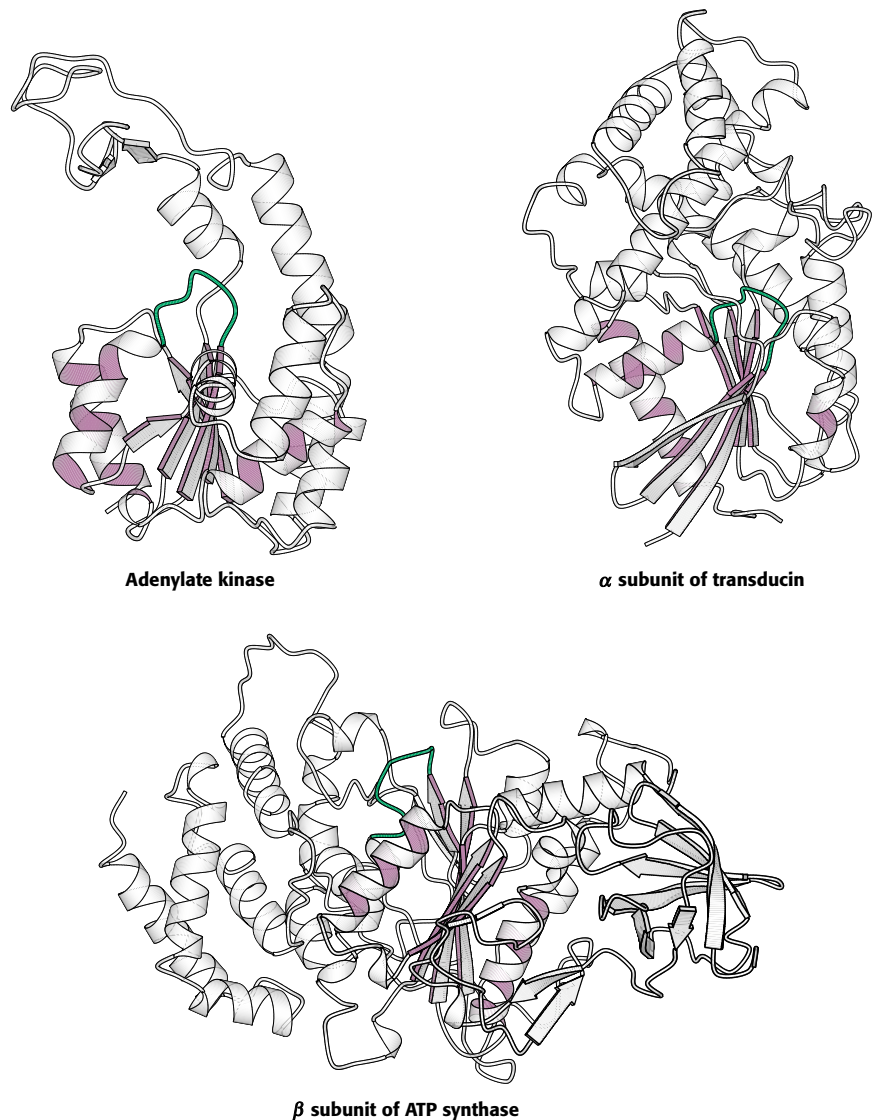
Comparison of the structure of adenylate kinase in the presence and absence of an ATP analog reveals that substrate binding induces large structural changes in the kinase, providing a classic example of the use of induced fit (Figure 9.51). The P-loop closes down on top of the polyphosphate chain, interacting most extensively with the β phosphoryl group. The movement of the P-loop permits the top domain of the enzyme to move down to form a lid over the bound nucleotide. This motion is favored by interactions between basic residues (conserved among the NMP kinases), the peptide backbone NH groups, and the nucleotide. With the ATP nucleotide held in this position, its γ phosphoryl group is positioned next to the binding site for the second substrate, NMP. In sum, the direct interactions with the nucleotide substrate lead to local structural rearrangements (movement of the P-loop) within the enzyme, which in turn allow more extensive changes (the closing down of the top domain) to take place. The binding of the second substrate, NMP, induces additional conformational changes. Both sets of changes ensure that a catalytically competent conformation is formed only when *both* the donor and the acceptor are bound, preventing wasteful transfer of the phosphoryl group to water. The enzyme holds its two substrates close together and appropriately oriented to stabilize the transition state that leads to the transfer of a phosphoryl group from the ATP to the NMP. This is an example of *catalysis by approximation*. We will see such examples of a catalytically competent active site being generated only on substrate binding many times in our study of biochemistry.


9.4.4 P-Loop NTPase Domains Are Present in a Range of Important Proteins

 Domains similar (and almost certainly homologous) to those found in NMP kinases are present in a remarkably wide array of proteins, many of which participate in essential biochemical processes. Examples include ATP synthase, the key enzyme responsible for ATP generation; molecular motor proteins such as myosin; signal-transduction proteins such as transducin; proteins essential for translating mRNA into proteins, such as elongation factor Tu; and DNA and RNA unwinding helicases. The wide utility of P-loop NTPase domains is perhaps best explained by their ability to undergo substantial conformational changes on nucleoside triphosphate binding and hydrolysis. We shall encounter these domains (hereafter referred to as P-loop NTPases) throughout the book and shall observe how they function as springs, motors, and clocks. To allow easy recognition of these domains, they, like the binding domains of the NMP kinases, will be depicted with the inner surfaces of the ribbons in a ribbon diagram shown in purple and the P-loop shown in green (Figure 9.52).



 **FIGURE 9.51** Conformational changes in adenylate kinase. Large conformational changes are associated with the binding of ATP by adenylate kinase. The P-loop is shown in green in each structure. The lid domain is highlighted in yellow.



 **FIGURE 9.52 Three proteins containing P-loop NTPase domains.** For the conserved domain, the inner surfaces of the ribbons are purple and the P-loops are green.

SUMMARY

Enzymes adopt conformations that are structurally and chemically complementary to the transition states of the reactions that they catalyze. Sets of interacting amino acid residues make up sites with the special structural and chemical properties necessary to stabilize the transition state. Enzymes use five basic strategies to form and stabilize the transition state: (1) the use of binding energy, (2) covalent catalysis, (3) general acid–base catalysis, (4) metal ion catalysis, and (5) catalysis by approximation. Of the enzymes examined in this chapter, three groups of enzymes catalyze the addition of water to their substrates but have different requirements for catalytic speed and specificity, and a fourth group of enzymes must prevent reaction with water.

- **Proteases: Facilitating a Difficult Reaction**

The cleavage of peptide bonds by chymotrypsin is initiated by the attack of a serine residue on the peptide carbonyl group. The attacking hydroxyl group is activated by interaction with the imidazole group of a histidine residue, which is, in turn, linked to an aspartate residue. This Ser-His-Asp catalytic triad generates a powerful nucleophile. The product of this

initial reaction is a covalent intermediate formed by the enzyme and an acyl group derived from the bound substrate. The hydrolysis of this acyl-enzyme intermediate completes the cleavage process. The tetrahedral intermediates for these reactions have a negative charge on the peptide carbonyl oxygen atom. This negative charge is stabilized by interactions with peptide NH groups in a region on the enzyme termed the oxyanion hole.

Other proteases employ the same catalytic strategy. Some of these proteases, such as trypsin and elastase, are homologs of chymotrypsin. In other proteases, such as subtilisin, a very similar catalytic triad has arisen by convergent evolution. Active-site structures that differ from the catalytic triad are present in a number of other classes of proteases. These classes employ a range of catalytic strategies but, in each case, a nucleophile is generated that is sufficiently powerful to attack the peptide carbonyl group. In some enzymes, the nucleophile is derived from a side chain; whereas, in others, an activated water molecule attacks the peptide carbonyl directly.

- **Carbonic Anhydrases: Making a Fast Reaction Faster**

Carbonic anhydrases catalyze the reaction of water with carbon dioxide to generate carbonic acid. The catalysis can be extremely fast: molecules of some carbonic anhydrases hydrate carbon dioxide at rates as high as 1 million times per second. A tightly bound zinc ion is a crucial component of the active sites of these enzymes. Each zinc ion binds a water molecule and promotes its deprotonation to generate a hydroxide ion at neutral pH. This hydroxide attacks carbon dioxide to form bicarbonate ion, HCO_3^- . Because of the physiological roles of carbon dioxide and bicarbonate ions, speed is of the essence for this enzyme. To overcome limitations imposed by the rate of proton transfer from the zinc-bound water molecule, the most active carbonic anhydrases have evolved a proton shuttle to transfer protons to a buffer.

- **Restriction Enzymes: Performing Highly Specific DNA Cleavage Reactions**

A high level of substrate specificity is often the key to biological function. Restriction endonucleases that cleave DNA at specific recognition sequences discriminate between molecules that contain these recognition sequences and those that do not. Within the enzyme–substrate complex, the DNA substrate is distorted in a manner that generates a magnesium ion binding site between the enzyme and DNA. The magnesium ion binds and activates a water molecule, which attacks the phosphodiester backbone.

Some enzymes discriminate between potential substrates by binding them with different affinities. Others may bind many potential substrates but promote chemical reactions efficiently only on specific molecules. Restriction endonucleases such as *EcoRV* endonuclease employ the latter mechanism to achieve levels of discrimination as high as million-fold. Structural studies reveal that these enzymes may bind nonspecific DNA molecules, but such molecules are not distorted in a manner that allows magnesium ion binding and, hence, catalysis. Restriction enzymes are prevented from acting on the DNA of a host cell by the methylation of key sites within their recognition sequences. The added methyl groups block specific interactions between the enzymes and the DNA such that the distortion necessary for cleavage does not take place.

- **Nucleoside Monophosphate Kinases: Catalyzing Phosphoryl Group Exchange Without Promoting Hydrolysis**

Finally, NMP kinases illustrate that induced fit—the alteration of enzyme structure on substrate binding—facilitates phosphoryl transfer between nucleotides rather than to a molecule of water. This class of

enzyme displays a structural motif called the P-loop NTPase domain that is present in a wide array of nucleotide-binding proteins. The closing of the P-loop over a bound nucleoside triphosphate substrate permits the top domain of the enzyme to form a lid over the bound nucleotide, positioning the triphosphate near the monophosphate with which it will react, in an example of catalysis by approximation. These enzymes are dependent on metal ions, but the ions bind to substrate instead of directly to the enzyme. The binding of the metal ion to the nucleoside triphosphate enhances the specificity of the enzyme–substrate interactions by holding the nucleotide in a well-defined conformation and providing additional points of interaction, thus increasing binding energy.

KEY TERMS

binding energy (p. 9-2)	chemical modification reaction (p. 9-4)	restriction-modification system (p. 9-19)
induced fit (p. 9-2)	catalytic triad (p. 9-5)	in-line displacement (p. 9-20)
covalent catalysis (p. 9-2)	oxyanion hole (p. 9-7)	horizontal gene transfer (p. 9-26)
general acid–base catalysis (p. 9-2)	protease inhibitor (p. 9-12)	P-loop (p. 9-27)
metal ion catalysis (p. 9-2)	proton shuttle (p. 9-17)	
catalysis by approximation (p. 9-2)	recognition sequence (p. 9-19)	

SELECTED READINGS

Where to start

- Stroud, R. M., 1974. A family of protein-cutting proteins. *Sci. Am.* 231(1):74–88.
- Kraut, J., 1977. Serine proteases: structure and mechanism of catalysis. *Annu. Rev. Biochem.* 46:331–358.
- Lindskog, S., 1997. Structure and mechanism of carbonic anhydrase. *Pharmacol. Ther.* 74:1–20.
- Jeltsch, A., Alves, J., Maass, G., Pingoud, A., 1992. On the catalytic mechanism of EcoRI and EcoRV: A detailed proposal based on biochemical results, structural data and molecular modelling. *FEBS Lett.* 304:4–8.
- Yan, H., and Tsai, M.-D., 1999. Nucleoside monophosphate kinases: Structure, mechanism, and substrate specificity. *Adv. Enzymol. Relat. Areas Mol. Biol.* 73:103–134.
- Lolis, E., and Petsko, G. A., 1990. Transition-state analogues in protein crystallography: Probes of the structural source of enzyme catalysis. *Annu. Rev. Biochem.* 59:597–630.

Books

- Fersht, A., 1999. *Structure and Mechanism in Protein Science: A Guide to Enzyme Catalysis and Protein Folding*. W. H. Freeman and Company.
- Silverman, R. B., 2000. *The Organic Chemistry of Enzyme-Catalyzed Reactions*. Academic Press.
- Page, M., and Williams, A., 1997. *Organic and Bio-organic Mechanisms*. Addison Wesley Longman.

Chymotrypsin and other serine proteases

- Fastrez, J., and Fersht, A. R., 1973. Demonstration of the acyl-enzyme mechanism for the hydrolysis of peptides and anilides by chymotrypsin. *Biochemistry* 12:2025–2034.
- Sigler, P. B., Blow, D. M., Matthews, B. W., and Henderson, R., 1968. Structure of crystalline-chymotrypsin II: A preliminary report including a hypothesis for the activation mechanism. *J. Mol. Biol.* 35:143–164.
- Kossiakoff, A. A., and Spencer, S. A., 1981. Direct determination of the protonation states of aspartic acid-102 and histidine-57 in the tetrahedral intermediate of the serine proteases: Neutron structure of trypsin. *Biochemistry* 20:6462–6474.

- Carter, P., and Wells, J. A., 1988. Dissecting the catalytic triad of a serine protease. *Nature* 332:564–568.
- Carter, P., and Wells, J. A., 1990. Functional interaction among catalytic residues in subtilisin BPN'. *Proteins* 7:335–342.
- Koepke, J., Ermler, U., Warkentin, E., Wenzl, G., and Flecker, P., 2000. Crystal structure of cancer chemopreventive Bowman-Birk inhibitor in ternary complex with bovine trypsin at 2.3 Å resolution: Structural basis of Janus-faced serine protease inhibitor specificity. *J. Mol. Biol.* 298:477–491.
- Gaboriaud, C., Rossi, V., Bally, I., Arlaud, G. J., and Fontecilla-Camps, J. C., 2000. Crystal structure of the catalytic domain of human complement C1s: A serine protease with a handle. *EMBO J.* 19:1755–1765.

Other proteases

- Kamphuis, I. G., Kalk, K. H., Swarte, M. B., and Drenth, J., 1984. Structure of papain refined at 1.65 Å resolution. *J. Mol. Biol.* 179:233–256.
- Kamphuis, I. G., Drenth, J., and Baker, E. N., 1985. Thiol proteases: Comparative studies based on the high-resolution structures of papain and actinidin, and on amino acid sequence information for cathepsins B and H, and stem bromelain. *J. Mol. Biol.* 182:317–329.
- Sivaraman, J., Nagler, D. K., Zhang, R., Menard, R., and Cygler, M., 2000. Crystal structure of human procathepsin X: A cysteine protease with the proregion covalently linked to the active site cysteine. *J. Mol. Biol.* 295:939–951.
- Davies, D. R., 1990. The structure and function of the aspartic proteinases. *Annu. Rev. Biophys. Chem.* 19:189–215.
- Dorsey, B. D., Levin, R. B., McDaniel, S. L., Vacca, J. P., Guare, J. P., Darke, P. L., Zugay, J. A., Emini, E. A., Schleif, W. A., Quintero, J. C., et al., 1994. L-735,524: The design of a potent and orally bioavailable HIV protease inhibitor. *J. Med. Chem.* 37:3443–3451.
- Chen, Z., Li, Y., Chen, E., Hall, D. L., Darke, P. L., Culbertson, C., Shafer, J. A., and Kuo, L. C., 1994. Crystal structure at 1.9-Å resolution of human immunodeficiency virus (HIV) II protease complexed with L-735,524, an orally bioavailable inhibitor of the HIV proteases. *J. Biol. Chem.* 269:26344–26348.
- Ollis, D. L., Cheah, E., Cygler, M., Dijkstra, B., Frolov, F., Franken, S. M., Harel, M., Remington, S. J., Silman, I., Schrag, J., et al., 1992. The α/β hydrolase fold. *Protein Eng.* 5:197–211.

Carbonic anhydrase

- Lindskog, S., and Coleman, J. E., 1973. The catalytic mechanism of carbonic anhydrase. *Proc. Natl. Acad. Sci. USA* 70:2505–2508.
- Kannan, K. K., Notstrand, B., Fridborg, K., Lovgren, S., Ohlsson, A., and Petef, M., 1975. Crystal structure of human erythrocyte carbonic anhydrase B: Three-dimensional structure at a nominal 2.2-Å resolution. *Proc. Natl. Acad. Sci. U.S.A.* 72:51–55.
- Boriack-Sjodin, P. A., Zeitlin, S., Chen, H. H., Crenshaw, L., Gross, S., Dantanarayana, A., Delgado, P., May, J. A., Dean, T., and Christianson, D. W., 1998. Structural analysis of inhibitor binding to human carbonic anhydrase II. *Protein Sci.* 7:2483–2489.
- Woolley, P., 1975. Models for metal ion function in carbonic anhydrase. *Nature* 258:677–682.
- Jonsson, B. H., Steiner, H., and Lindskog, S., 1976. Participation of buffer in the catalytic mechanism of carbonic anhydrase. *FEBS Lett.* 64:310–314.
- Sly, W. S., and Hu, P. Y., 1995. Human carbonic anhydrases and carbonic anhydrase deficiencies. *Annu. Rev. Biochem.* 64:375–401.
- Maren, T. H., 1988. The kinetics of HCO_3^- synthesis related to fluid secretion, pH control, and CO_2 elimination. *Annu. Rev. Physiol.* 50:695–717.
- Kisker, C., Schindelin, H., Alber, B. E., Ferry, J. G., and Rees, D. C., 1996. A left-hand beta-helix revealed by the crystal structure of a carbonic anhydrase from the archaeon *Methanosarcina thermophila*. *EMBO J.* 15:2323–2330.

Restriction enzymes

- Winkler, F. K., Banner, D. W., Oefner, C., Tsernoglou, D., Brown, R. S., Heathman, S. P., Bryan, R. K., Martin, P. D., Petratos, K., and Wilson, K. S., 1993. The crystal structure of EcoRV endonuclease and of its complexes with cognate and non-cognate DNA fragments. *EMBO J.* 12:1781–1795.

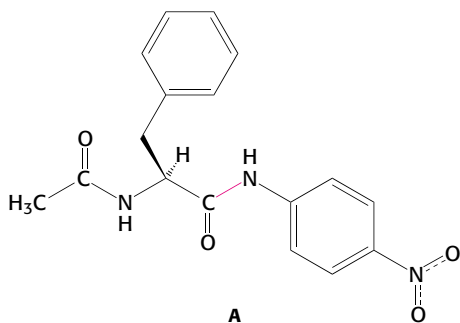
- Kostrewa, D., and Winkler, F. K., 1995. Mg^{2+} binding to the active site of EcoRV endonuclease: A crystallographic study of complexes with substrate and product DNA at 2 Å resolution. *Biochemistry* 34:683–696.
- Athanasiadis, A., Vlasi, M., Kotsifaki, D., Tucker, P. A., Wilson, K. S., and Kokkinidis, M., 1994. Crystal structure of PvuII endonuclease reveals extensive structural homologies to EcoRV. *Nat. Struct. Biol.* 1:469–475.
- Sam, M. D., and Perona, J. J., 1999. Catalytic roles of divalent metal ions in phosphoryl transfer by EcoRV endonuclease. *Biochemistry* 38:6576–6586.
- Jeltsch, A., and Pingoud, A., 1996. Horizontal gene transfer contributes to the wide distribution and evolution of type II restriction-modification systems. *J. Mol. Evol.* 42:91–96.

NMP kinases

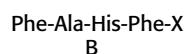
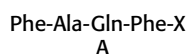
- Byeon, L., Shi, Z., and Tsai, M. D., 1995. Mechanism of adenylate kinase: The “essential lysine” helps to orient the phosphates and the active site residues to proper conformations. *Biochemistry* 34:3172–3182.
- Dreusicke, D., and Schulz, G. E., 1986. The glycine-rich loop of adenylate kinase forms a giant anion hole. *FEBS Lett.* 208:301–304.
- Pai, E. F., Sachsenheimer, W., Schirmer, R. H., and Schulz, G. E., 1977. Substrate positions and induced-fit in crystalline adenylate kinase. *J. Mol. Biol.* 114:37–45.
- Schlauderer, G. J., Proba, K., and Schulz, G. E., 1996. Structure of a mutant adenylate kinase ligated with an ATP-analogue showing domain closure over ATP. *J. Mol. Biol.* 256:223–227.
- Vonrhein, C., Schlauderer, G. J., and Schulz, G. E., 1995. Movie of the structural changes during a catalytic cycle of nucleoside monophosphate kinases. *Structure* 3:483–490.
- Muller-Dieckmann, H. J., and Schulz, G. E., 1994. The structure of uridylylase kinase with its substrates, showing the transition state geometry. *J. Mol. Biol.* 236:361–367.

PROBLEMS

1. *No burst.* Examination of the cleavage of the chromogenic amide substrate, A, by chymotrypsin with the use of stopped-flow kinetic methods reveals no burst. Why?



2. *Contributing to your own demise.* Consider the subtilisin substrates A and B.



These substrates are cleaved (between Phe and X) by native subtilisin at essentially the same rate. However, the His 64-to-Ala mutant of subtilisin cleaves substrate B more than 1000-fold as rapidly as it cleaves substrate A. Propose an explanation.

3. *1 + 1 ≠ 2.* Consider the following argument. In subtilisin, mutation of Ser 221 to Ala results in a 10^6 -fold decrease in activity. Mutation of His 64 to Ala results in a similar 10^6 -fold de-

crease. Therefore, simultaneous mutation of Ser 221 to Ala and His 64 to Ala should result in a $10^6 \times 10^6 = 10^{12}$ -fold reduction in activity. Is this correct? Why or why not?

4. *Adding a charge.* In chymotrypsin, a mutant was constructed with Ser 189, which is in the bottom of the substrate specificity pocket, changed to Asp. What effect would you predict for this Ser 189 → Asp 189 mutation?

5. *Conditional results.* In carbonic anhydrase II, mutation of the proton-shuttle residue His 64 to Ala was expected to result in a decrease in the maximal catalytic rate. However, in buffers such as imidazole with relatively small molecular components, no rate reduction was observed. In buffers with larger molecular components, significant rate reductions were observed. Propose an explanation.

6. *How many sites?* A researcher has isolated a restriction endonuclease that cleaves at only one particular 10-base-pair site. Would this enzyme be useful in protecting cells from viral infections, given that a typical viral genome is 50,000 base pairs long? Explain.

7. *Is faster better?* Restriction endonucleases are, in general, quite slow enzymes with typical turnover numbers of 1 s^{-1} . Suppose that endonucleases were faster with turnover numbers similar to those for carbonic anhydrase (10^6 s^{-1}). Would this increased rate be beneficial to host cells, assuming that the fast enzymes have similar levels of specificity?

8. *Adopting a new gene.* Suppose that one species of bacteria obtained one gene encoding a restriction endonuclease by horizontal gene transfer. Would you expect this acquisition to be beneficial?

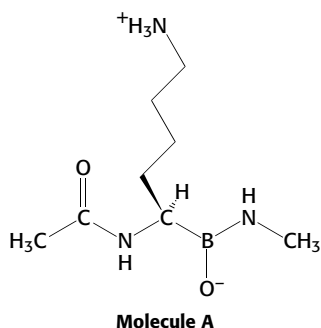
9. *Predict the product.* Adenylate kinase is treated with adenosine diphosphate (ADP).

(a) What products will be generated?

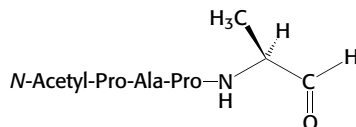
(b) If the initial concentration of ADP is 1 mM, estimate the concentrations of ADP and the products from part *a* after incubation with adenylate kinase for a long time.

10. *Chelation therapy.* Treatment of carbonic anhydrase with high concentrations of the metal chelator EDTA (ethylenediaminetetraacetic acid) results in the loss of enzyme activity. Propose an explanation.

11. *Identify the enzyme.* Consider the structure of molecule A. Which enzyme discussed in this chapter do you think molecule A will most effectively inhibit?



12. *An aldehyde inhibitor.* Elastase is specifically inhibited by an aldehyde derivative of one of its substrates:



(a) Which residue in the active site of elastase is most likely to form a covalent bond with this aldehyde?

(b) What type of covalent link would be formed?

Mechanism Problem

13. *Complete the mechanism.* On the basis of the information provided in Figure 9.18, complete the mechanisms for peptide-bond cleavage by (a) a cysteine protease, (b) an aspartyl protease, and (c) a metalloprotease.

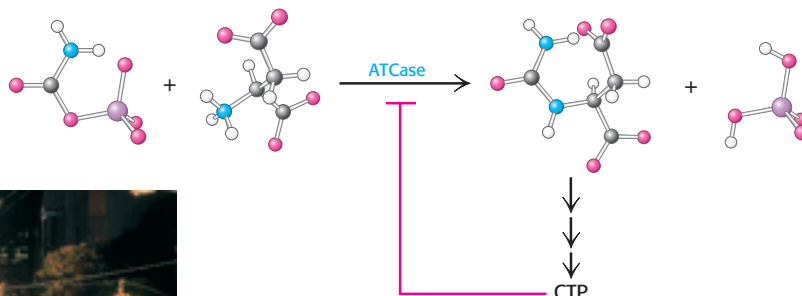


Media Problems

14. *Now you see it, now you don't.* Pre-steady-state experiments using chymotrypsin and a chromogenic substrate (*N*-acetyl-L-phenylalanine *p*-nitrophenyl ester) show a “burst” of product at very short times (Figure 9.4). The **Conceptual Insights** module on enzyme kinetics explains this result. What results would you see if the product detected by the assay was the free N-terminal component of the substrate instead of the C-terminal component? (*Hint:* Use the pre-steady-state reaction simulation to simulate the experiment. Select different times following mixing and observe the amount of each product.)

15. *Seeing is disbelieving.* DIPF reacts specifically with serine 195 of chymotrypsin. One hypothesis as to why this is so might be that serine 195 is unusually exposed on the surface of the protein compared to other serines. After looking at the **Structural Insights** module on chymotrypsin, what do you think of this hypothesis?

Regulatory Strategies: Enzymes and Hemoglobin



Like motor traffic, metabolic pathways flow more efficiently when regulated by signals. CTP, the final product of a multistep pathway, controls flux through the pathway by inhibiting the committed step catalyzed by aspartate transcarbamoylase (ATCase).
 [(Left) Richard Berenholtz/The Stock Market.]

The activity of proteins, including enzymes, often must be regulated so that they function at the proper time and place. The biological activity of proteins is regulated in four principal ways:

1. **Allosteric control.** Allosteric proteins contain distinct regulatory sites and multiple functional sites. Regulation by small signal molecules is a significant means of controlling the activity of many proteins. The binding of these regulatory molecules at sites distinct from the active site triggers conformational changes that are transmitted to the active site. Moreover, allosteric proteins show the property of *cooperativity*: activity at one functional site affects the activity at others. As a consequence, a slight change in substrate concentration can produce substantial changes in activity. Proteins displaying allosteric control are thus information transducers: their activity can be modified in response to signal molecules or to information shared among active sites. This chapter examines two of the best-understood allosteric proteins: the enzyme *aspartate transcarbamoylase* (ATCase) and the oxygen-carrying protein *hemoglobin*. Catalysis by aspartate transcarbamoylase of the first step in pyrimidine biosynthesis is inhibited by cytidine triphosphate, the final product of that biosynthesis, in an example of *feedback inhibition*. The binding of O_2 by hemoglobin is cooperative and is regulated by H^+ , CO_2 and 2,3-bisphosphoglycerate (2,3-BPG).

OUTLINE

- 10.1 Aspartate Transcarbamoylase Is Allosterically Inhibited by the End Product of Its Pathway
- 10.2 Hemoglobin Transports Oxygen Efficiently by Binding Oxygen Cooperatively
- 10.3 Isozymes Provide a Means of Regulation Specific to Distinct Tissues and Developmental Stages
- 10.4 Covalent Modification Is a Means of Regulating Enzyme Activity
- 10.5 Many Enzymes Are Activated by Specific Proteolytic Cleavage

2. *Multiple forms of enzymes.* Isozymes, or isoenzymes, provide an avenue for varying regulation of the same reaction at distinct locations or times. Isozymes are homologous enzymes within a single organism that catalyze the same reaction but differ slightly in structure and more obviously in K_M and V_{max} values, as well as regulatory properties. Often, isozymes are expressed in a distinct tissue or organelle or at a distinct stage of development.

3. *Reversible covalent modification.* The catalytic properties of many enzymes are markedly altered by the covalent attachment of a modifying group, most commonly a phosphoryl group. ATP serves as the phosphoryl donor in these reactions, which are catalyzed by *protein kinases*. The removal of phosphoryl groups by hydrolysis is catalyzed by *protein phosphatases*. This chapter considers the structure, specificity, and control of *protein kinase A* (PKA), a ubiquitous eukaryotic enzyme that regulates diverse target proteins.

4. *Proteolytic activation.* The enzymes controlled by some of these mechanisms cycle between active and inactive states. A different regulatory motif is used to *irreversibly* convert an inactive enzyme into an active one. Many enzymes are activated by the hydrolysis of a few or even one peptide bond in inactive precursors called *zymogens* or *proenzymes*. This regulatory mechanism generates digestive enzymes such as chymotrypsin, trypsin, and pepsin. Caspases, which are proteolytic enzymes that are the executioners in *programmed cell death*, or *apoptosis* (Section 2.4.3), are proteolytically activated from the procaspase form. Blood clotting is due to a remarkable cascade of zymogen activations. Active digestive and clotting enzymes are switched off by the irreversible binding of specific inhibitory proteins that are irresistible lures to their molecular prey.

To begin, we will consider the principles of allostery by examining two proteins: the enzyme aspartate transcarbamoylase and the oxygen-transporting protein hemoglobin.

10.1 ASPARTATE TRANSCARBAMOYLASE IS ALLOSTERICALLY INHIBITED BY THE END PRODUCT OF ITS PATHWAY

Aspartate transcarbamoylase catalyzes the first step in the biosynthesis of pyrimidines, bases that are components of nucleic acids. The reaction catalyzed by this enzyme is the condensation of aspartate and carbamoyl phosphate to form *N*-carbamoylaspartate and orthophosphate (Figure 10.1).

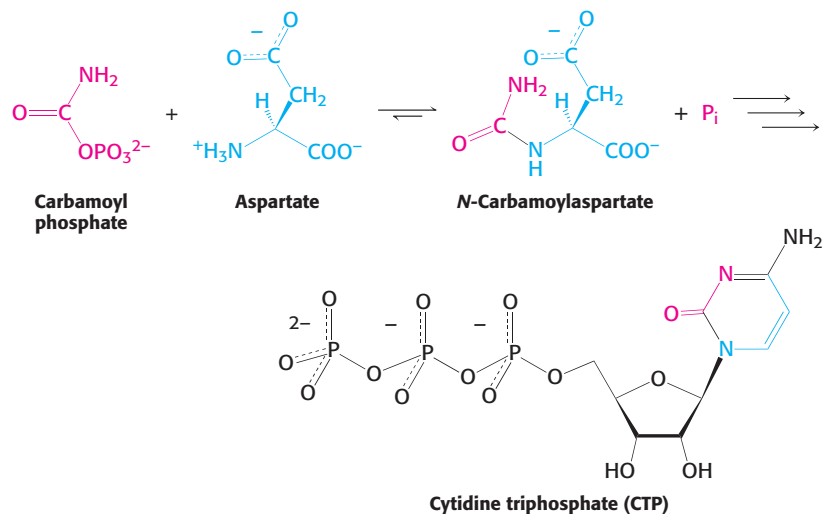


FIGURE 10.1 ATCase reaction. Aspartate transcarbamoylase catalyzes the committed step, the condensation of aspartate and carbamoyl phosphate to form *N*-carbamoylaspartate, in pyrimidine synthesis.

ATCase catalyzes the committed step in the pathway that will ultimately yield pyrimidine nucleotides such as cytidine triphosphate (CTP). How is this enzyme regulated to generate precisely the amount of CTP needed by the cell?

John Gerhart and Arthur Pardee found that ATCase is inhibited by CTP, the final product of the ATCase-controlled pathway. The rate of the reaction catalyzed by ATCase is fast in the absence of high concentrations of CTP but decreases as the CTP concentration increases (Figure 10.2). Thus, more molecules are sent along the pathway to make new pyrimidines until sufficient quantities of CTP have accumulated. The effect of CTP on the enzyme exemplifies the *feedback*, or *end-product, inhibition* mentioned earlier. Despite the fact that end-product regulation makes considerable physiological sense, the observation that ATCase is inhibited by CTP is remarkable because *CTP is structurally quite different from the substrates of the reaction* (see Figure 10.1). Owing to this structural dissimilarity, CTP must bind to a site distinct from the active site where substrate binds. Such sites are called *allosteric* (from the Greek *allos*, “other,” and *stereos*, “structure”) or *regulatory sites*. CTP is an example of an *allosteric inhibitor*. In ATCase (but not all allosterically regulated enzymes), the catalytic sites and the regulatory sites are on separate polypeptide chains.

10.1.1 ATCase Consists of Separable Catalytic and Regulatory Subunits

What is the evidence that ATCase has distinct regulatory and catalytic sites? ATCase can be literally separated into regulatory and catalytic subunits by treatment with a mercurial compound such as *p*-hydroxymercuribenzoate, which reacts with sulfhydryl groups (Figure 10.3). The results of ultracentrifugation studies carried out by Gerhart and Howard Schachman showed that *p*-hydroxymercuribenzoate dissociates ATCase into two kinds of subunits (Figure 10.4). The sedimentation coefficient of the native enzyme is 11.6S, whereas those of the dissociated subunits are 2.8S and 5.8S, indicating subunits of different size. The subunits can be readily separated by ion-exchange chromatography because they differ markedly in charge (Section 4.1.3) or by centrifugation in a sucrose density gradient because they differ in size (Section 4.1.6). Furthermore, the attached *p*-mercuribenzoate groups can be removed from the separated subunits by adding an excess of mercaptoethanol. The isolated subunits provide materials that can be used to investigate and characterize the individual subunits and their interactions with one another.

The larger subunit is called the *catalytic* (or *c*) *subunit*. This subunit displays catalytic activity, but it is not affected by CTP. The isolated smaller subunit can bind CTP, but has no catalytic activity. Hence, that subunit is called the *regulatory* (or *r*) *subunit*. The catalytic subunit, which consists of

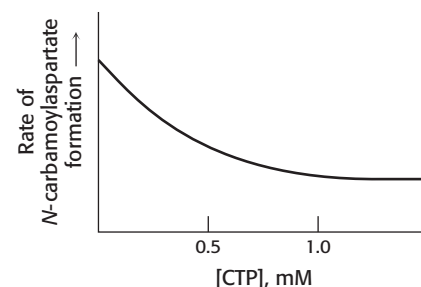


FIGURE 10.2 CTP inhibits ATCase. Cytidine triphosphate, an end product of the pyrimidine synthesis pathway, inhibits aspartate transcarbamoylase despite having little structural similarity to reactants or products.

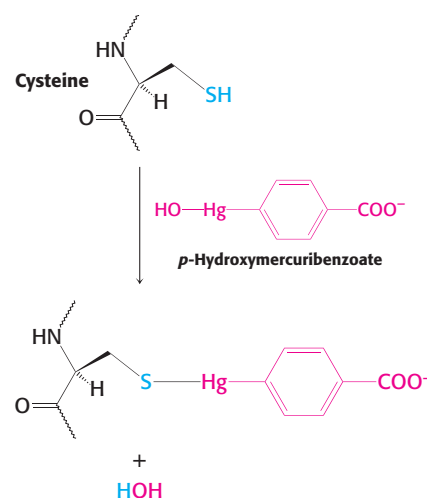


FIGURE 10.3 Modification of cysteine residues. *p*-Hydroxymercuribenzoate reacts with crucial cysteine residues in aspartate transcarbamoylase.

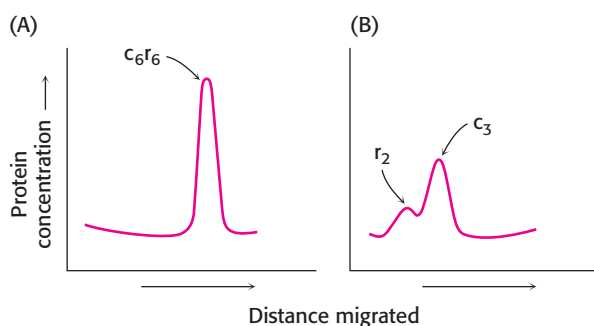
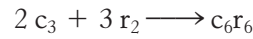


FIGURE 10.4 Ultracentrifugation studies of ATCase. Sedimentation velocity patterns of (A) native ATCase and (B) the enzyme after treatment with *p*-hydroxymercuribenzoate show that the enzyme can be dissociated into regulatory and catalytic subunits. [After J. C. Gerhart and H. K. Schachman. *Biochemistry* 4(1965):1054.]

three chains (34 kd each), is referred to as c_3 . The regulatory subunit, which consists of two chains (17 kd each), is referred to as r_2 . The catalytic and regulatory subunits combine rapidly when they are mixed. The resulting complex has the same structure, c_6r_6 , as the native enzyme: two catalytic trimers and three regulatory dimers.



Furthermore, the reconstituted enzyme has the same allosteric properties as the native enzyme. Thus, ATCase is composed of discrete catalytic and regulatory subunits, which interact in the native enzyme to produce its allosteric behavior.

10.1.2 Allosteric Interactions in ATCase Are Mediated by Large Changes in Quaternary Structure

How can the binding of CTP to a regulatory subunit influence reactions at the active site of a catalytic subunit? Significant clues have been provided by the determination of the three-dimensional structure of ATCase in various forms by x-ray crystallography in the laboratory of William Lipscomb. The structure of the enzyme without any ligands bound to it confirms the overall structure of the enzyme. Two catalytic trimers are stacked one on top of the other, linked by three dimers of the regulatory chains (Figure 10.5). There are significant contacts between the two catalytic trimers: each r chain within a regulatory dimer interacts with a c chain within a catalytic trimer through a structural domain stabilized by a zinc ion bound to four cysteine residues.

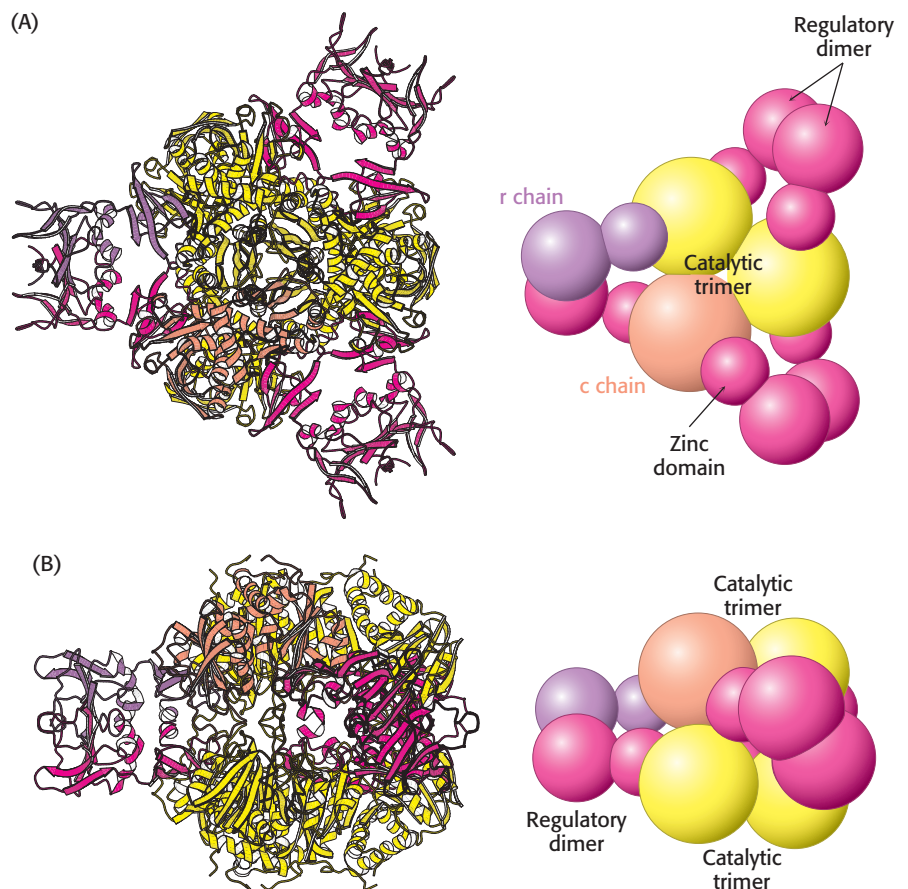


FIGURE 10.5 Structure of ATCase. (A) The quaternary structure of aspartate transcarbamoylase as viewed from the top. The schematic drawing at the right is a simplified representation of the relationships between subunits. A single trimer [catalytic (c) chains, shown in orange and yellow] is visible; in this view, the second trimer is hidden behind the one visible. (B) A side view of the complex.

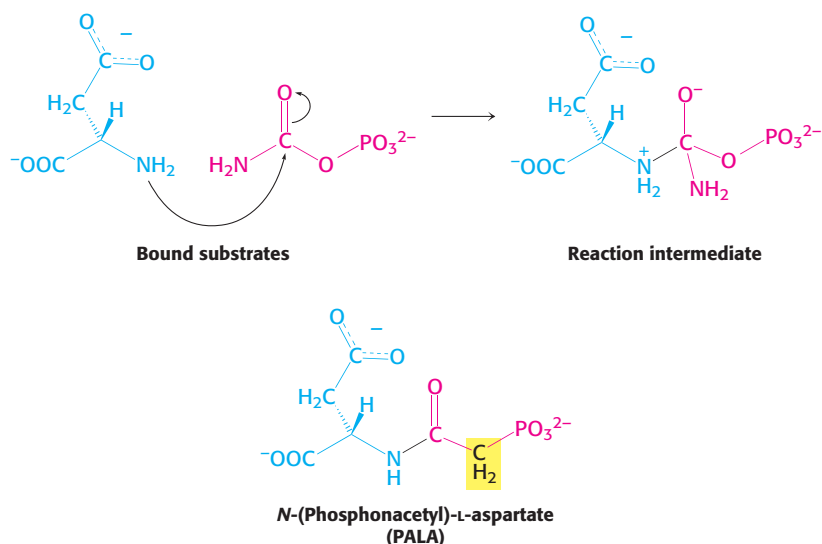


FIGURE 10.6 PALA, a bisubstrate analog. (Top) Nucleophilic attack by the amino group of aspartate on the carbonyl carbon atom of carbamoyl phosphate generates an intermediate on the pathway to the formation of *N*-carbamoylaspartate. (Bottom) *N*-(Phosphonacetyl)-L-aspartate (PALA) is an analog of the reaction intermediate and a potent competitive inhibitor of aspartate transcarbamoylase.

The ability of *p*-hydroxymercuribenzoate to dissociate the catalytic and regulatory subunits is related to the ability of mercury to bind strongly to the cysteine residues, displacing the zinc and destabilizing this domain.

To understand the mechanism of allosteric regulation, it is crucial to locate each active site and each regulatory site in the three-dimensional structure. To locate the active sites, the enzyme was crystallized in the presence of *N*-(phosphonacetyl)-L-aspartate (PALA), a bisubstrate analog (an analog of the two substrates) that resembles an intermediate along the pathway of catalysis (Figure 10.6). PALA is a potent competitive inhibitor of ATCase; it binds to and blocks the active sites. The structure of the ATCase–PALA complex reveals that PALA binds at sites lying at the boundaries between pairs of *c* chains within a catalytic trimer (Figure 10.7). Note that, though most of the residues belong to one subunit, several key residues belong to a neighboring subunit. Thus, because the active sites are at the subunit interface, each catalytic trimer contributes three active sites to the complete enzyme. Suitable amino acid residues are available in the active sites for recognizing all features of the bisubstrate analog, including the phosphate and both carboxylate groups.

FIGURE 10.7 The active site of ATCase. Some of the crucial active-site residues are shown binding to the inhibitor PALA. The active site is composed mainly of residues from one subunit, but an adjacent subunit also contributes important residues (boxed in green).

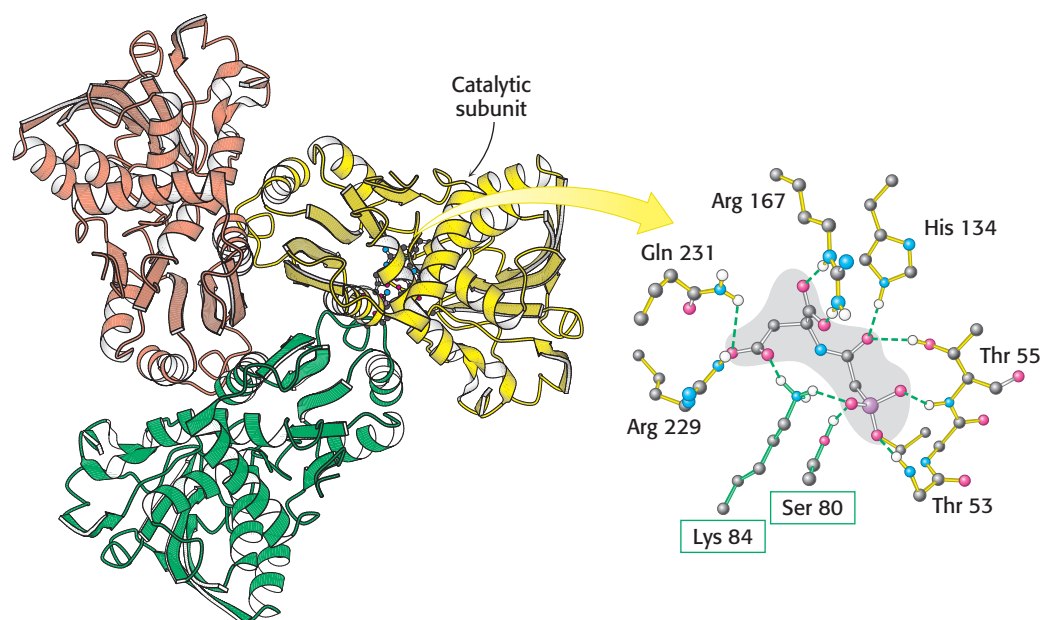
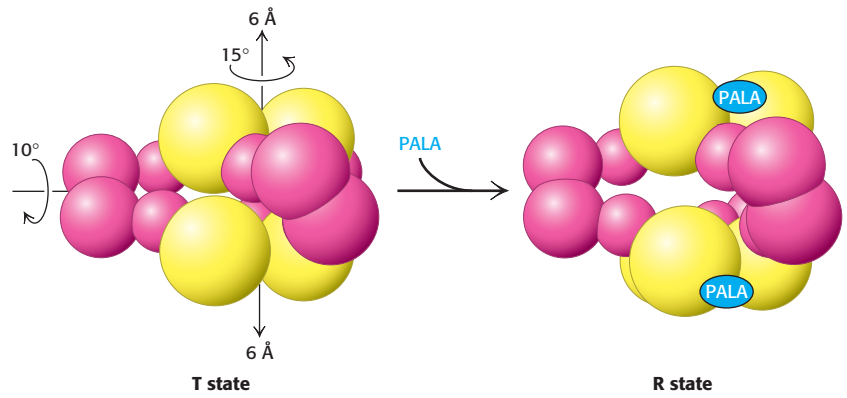


FIGURE 10.8 The T-to-R state transition in ATCase. Aspartate transcarbamoylase exists in two conformations: a compact, relatively inactive form called the tense (T) state and an expanded form called the relaxed (R) state. PALA binding stabilizes the R state.



Further examination of the ATCase–PALA complex reveals a remarkable change in quaternary structure on binding of PALA. The two catalytic trimers move 12 Å farther apart and rotate approximately 10 degrees about their common threefold axis of symmetry. Moreover, the regulatory dimers rotate approximately 15 degrees to accommodate this motion (Figure 10.8). The enzyme literally expands on PALA binding. In essence, ATCase has two distinct quaternary forms: one that predominates in the absence of substrate or substrate analogs and another that predominates when substrates or analogs are bound. These forms will be referred to as the T (for tense) state and the R (for relaxed) state, respectively. The T state has lower affinity for substrates and, hence, lower catalytic activity than does the R state. In the presence of any fixed concentration of aspartate and carbamoyl phosphate, the enzyme exists in equilibrium between the T and the R forms. *The position of the equilibrium depends on the number of active sites that are occupied by substrate.*

Having located the active sites and seen that PALA binding results in substantial structural changes in the entire ATCase molecule, we now turn our attention to the effects of CTP. Where on the regulatory subunit does CTP bind? Determination of the structure of ATCase in the presence of CTP reveals a binding site for this nucleotide in each regulatory chain in a domain that does not interact with the catalytic subunit (Figure 10.9). The question naturally arises as to how CTP can inhibit the catalytic activity of the enzyme when it does not interact with the catalytic chain. Each active site is more than 50 Å from the nearest CTP binding site. The CTP-bound form is in the T quaternary state in the absence of bound substrate.

The quaternary structural changes observed on substrate-analog binding suggest a mechanism for the allosteric regulation of ATCase by CTP (Figure 10.10). The binding of the inhibitor CTP shifts the equilibrium toward the T state, decreasing the net enzyme activity and reducing the rate of *N*-carbamoylaspartate generation. This mechanism for allosteric regulation is referred to as the *concerted mechanism* because the change in the en-

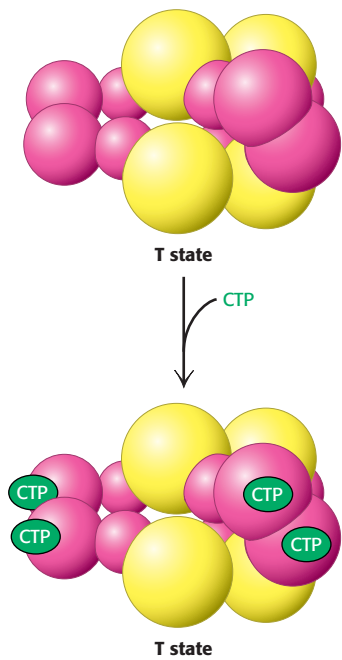
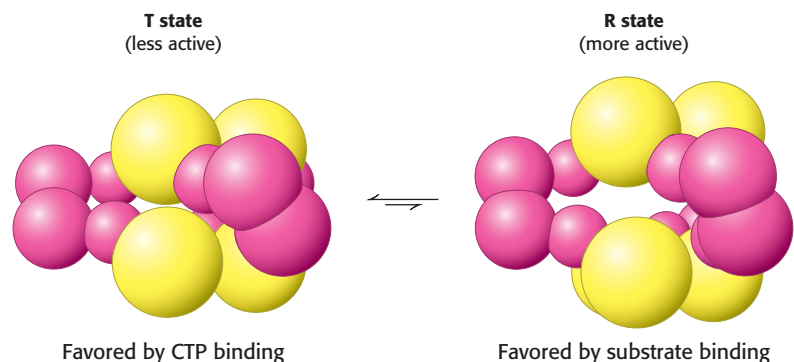


FIGURE 10.9 CTP stabilizes the T state. The binding of CTP to the regulatory subunit of aspartate transcarbamoylase stabilizes the T state.

FIGURE 10.10 The R state and the T state are in equilibrium. Even in the absence of any substrate or regulators, aspartate transcarbamoylase exists in an equilibrium between the R and the T states. Under these conditions, the T state is favored by a factor of approximately 200.



zyme is “all or none”; the entire enzyme is converted from T into R, affecting all of the catalytic sites equally. The concerted mechanism stands in contrast with the sequential mechanism, which will be discussed shortly.

10.1.3 Allosterically Regulated Enzymes Do Not Follow Michaelis-Menten Kinetics

Allosteric enzymes are distinguished by their response to substrate concentration in addition to their susceptibility to regulation by other molecules. Examining the rate of product formation as a function of substrate concentration can be a source of further insights into the mechanism of regulation of ATCase (Figure 10.11). The curve differs from that expected for an enzyme that follows Michaelis-Menten kinetics. The observed curve is referred to as sigmoid because it resembles an “S.” How can we explain this kinetic behavior in light of the structural observations? In the absence of substrate, the enzyme exists almost entirely in the T state. However, the binding of substrate molecules to the enzyme shifts the enzyme toward the R state. A transition from T to R favored by substrate binding to one site will increase the enzymatic activity of the remaining five sites, leading to an overall increase in enzyme activity. This important property is called *cooperativity* because the subunits cooperate with one another. If one subunit switches conformation, they all do. The sigmoid curve can be pictured as a composite of two Michaelis-Menten curves, one corresponding to the T state and the other to the R state. An increase in substrate concentration favors a transition from the T-state curve to the R-state curve (Figure 10.12).

The importance of the changes in quaternary structure in determining the sigmoidal curve is illustrated nicely by studies of the isolated catalytic trimer, freed by *p*-hydroxymercuribenzoate treatment. The catalytic subunit shows Michaelis-Menten kinetics with kinetic parameters that are indistinguishable from those deduced for the R state. Thus, the term *tense* is apt: in the T state, the regulatory dimers hold the two catalytic trimers sufficiently close to one other that key loops on their surfaces collide and interfere with conformational adjustments necessary for high-affinity substrate binding and catalysis.

10.1.4 Allosteric Regulators Modulate the T-to-R Equilibrium

What is the effect of CTP on the kinetic profile of ATCase? CTP increases the initial phase of the sigmoidal curve (Figure 10.13). As noted earlier, CTP inhibits the activity of ATCase. In the presence of CTP, the enzyme becomes less responsive to the cooperative effects facilitated by substrate binding; more substrate is required to attain a given reaction rate. Interestingly, ATP, too, is an allosteric effector of ATCase. However, the effect of ATP is to *increase* the reaction rate at a given aspartate concentration (Figure 10.14). At high concentrations of ATP, the kinetic profile shows a less-pronounced sigmoidal behavior. Note that such sigmoidal behavior has an

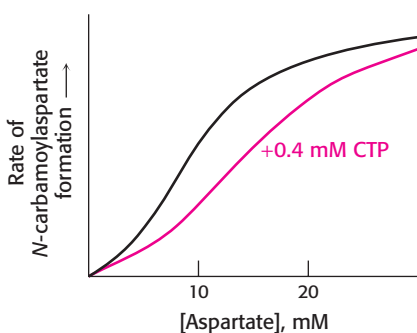


FIGURE 10.13 Effect of CTP on ATCase kinetics. Cytidine triphosphate (CTP) stabilizes the T state of aspartate transcarbamoylase, making it more difficult for substrate binding to convert the enzyme into the R state. As a result, the curve is shifted to the right, as shown in red.

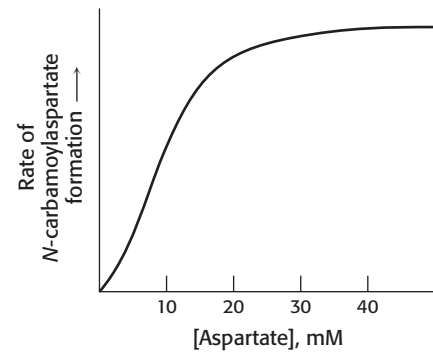


FIGURE 10.11 ATCase displays sigmoidal kinetics. A plot of product formation as a function of substrate concentration produces a sigmoidal curve because the binding of substrate to one active site favors the conversion of the entire enzyme into the R state, increasing the activity at the other active sites. Thus, the active sites show cooperativity.

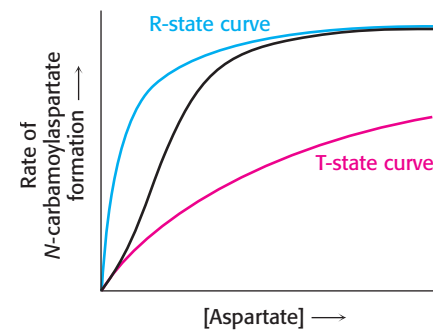


FIGURE 10.12 Basis for the sigmoidal curve. The generation of the sigmoidal curve by the property of cooperativity can be understood by imagining an allosteric enzyme as a mixture of two Michaelis-Menten enzymes, one with a high value of K_m that corresponds to the T state and another with a low value of K_m that corresponds to the R state. As the concentration of substrate is increased, the equilibrium shifts from the T state to the R state, which results in a steep rise in activity with respect to substrate concentration.

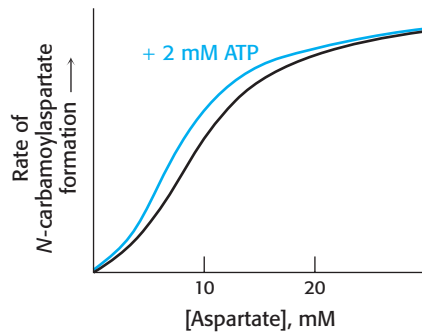


FIGURE 10.14 Effect of ATP on ATCase kinetics. ATP is an allosteric activator of aspartate transcarbamoylase because it stabilizes the R state, making it easier for substrate to bind. As a result, the curve is shifted to the left, as shown in blue.

additional consequence: in the concentration range where the T-to-R transition is taking place, the curve depends quite steeply on the substrate concentration. The effects of substrates on allosteric enzymes are referred to as *homotropic effects* (from the Greek *homós*, “same”). In contrast, the effects of nonsubstrate molecules on allosteric enzymes (such as those of CTP and ATP on ATCase) are referred to as *heterotropic effects* (from the Greek *héteros*, “different”).

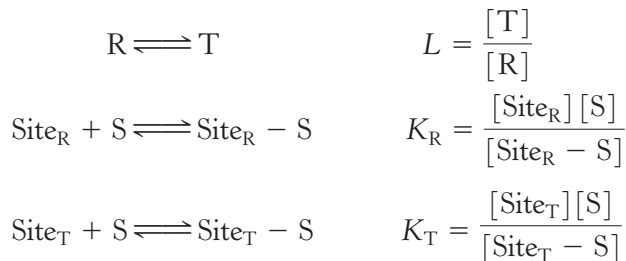
The increase in ATCase activity in response to increased ATP concentration has two potential physiological explanations. First, high ATP concentration signals a high concentration of purine nucleotides in the cell; the increase in ATCase activity will tend to balance the purine and pyrimidine pools. Second, a high concentration of ATP indicates that there is significant energy stored in the cell to promote mRNA synthesis and DNA replication.

10.1.5 The Concerted Model Can Be Formulated in Quantitative Terms



CONCEPTUAL INSIGHTS, Cooperative Binding and Kinetics. Interactive graphing activities allow you to experiment with changes in the parameters and conditions of the MWC model in order to increase your understanding of the model and its implications for cooperative binding and kinetics.

The concerted model was first proposed by Jacques Monod, Jeffries Wyman, and Jean-Pierre Changeux; hence, it is often referred to as the MWC model. This model can be formulated in quantitative terms. Consider an enzyme with n identical active sites. Suppose that the enzyme exists in equilibrium between a T form with a low affinity for its substrate and an R form with a high affinity for the substrate. We can define L as the equilibrium constant between the R and the T forms; c as the ratio of the affinities of the two forms for the substrate, S, measured as dissociation constants; and α as the ratio of substrate concentration to the dissociation constant K_R .



Define

$$c = \frac{K_R}{K_T} \quad \text{and} \quad \alpha = \frac{[S]}{K_R}$$

The fraction of active sites bound to substrate (fractional saturation, Y_S) is given by

$$Y_S = \frac{\alpha(1 - \alpha)^{n-1} + Lc\alpha(1 + c\alpha)^{n-1}}{(1 + \alpha)^n + L(1 + c\alpha)^n}$$

where n is the number of sites in the enzyme.

This quantitative model can be used to examine the data from ATCase, for which $n = 6$. Excellent agreement with experimental data is obtained

with $L \approx 200$ and $c \approx 0.1$. Thus, in the absence of bound substrate, the equilibrium favors the T form by a factor of 200 (i.e., only 1 in 200 molecules is in the R form), and the affinity of the R form for substrate is approximately 10 times as high as that of the T form. As substrate binds to each active site, the equilibrium shifts toward the R form. For example, with these parameters, when half the active sites (three of six) are occupied by substrate, the equilibrium has shifted so that the ratio of T to R is now 1 to 5; that is, nearly all the molecules are in the R form.

The effects of CTP and ATP can be modeled simply by changing the value of L . For the CTP-saturated form, the value of L increases to 1250. Thus, it takes more substrate to shift the equilibrium appreciably to the R form. For the ATP saturated form, the value of L decreases to 70 (Figure 10.15).

10.1.6 Sequential Models Also Can Account for Allosteric Effects

In the concerted model, an allosteric enzyme can exist in one of only two states, T and R; no intermediate states are allowed. An alternative, first proposed by Daniel Koshland, posits that *sequential* changes in structure take place within an oligomeric enzyme as active sites are occupied. The binding of substrate to one site influences the substrate affinity of neighboring active sites *without necessarily inducing a transition encompassing the entire enzyme* (Figure 10.16). An important feature of sequential in contrast with concerted models is that the former can account for *negative cooperativity*, in which the binding of substrate to one active site *decreases* the affinity of other sites for substrate. The results of studies of a number of allosteric proteins suggest that most behave according to some combination of the sequential and cooperative models.

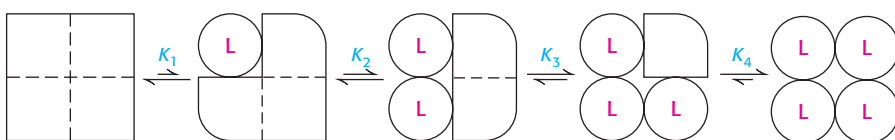


FIGURE 10.16 Simple sequential model for a tetrameric allosteric enzyme. The binding of a ligand (L) to a subunit changes the conformation of that particular subunit from the T (square) to the R (circle) form. This transition affects the affinity of the other subunits for the ligand.

10.2 HEMOGLOBIN TRANSPORTS OXYGEN EFFICIENTLY BY BINDING OXYGEN COOPERATIVELY

Allostery is a property not limited to enzymes. The basic principles of allostery are also well illustrated by the oxygen-transport protein hemoglobin (Section 7.2). The binding of oxygen to hemoglobin isolated from red blood cells displays marked sigmoidal behavior (similar to that observed for the activity of ATCase, as a function of substrate concentration), which is indicative of cooperation between subunits (Figure 10.17). What is the physiological significance of the cooperative binding of oxygen by hemoglobin? Oxygen must be transported in the blood from the lungs, where the partial pressure of oxygen (pO_2) is relatively high (approximately 100 torr), to the tissues, where the partial pressure of oxygen is much lower (typically 20 torr). Let us consider how the cooperative behavior represented by the sigmoidal

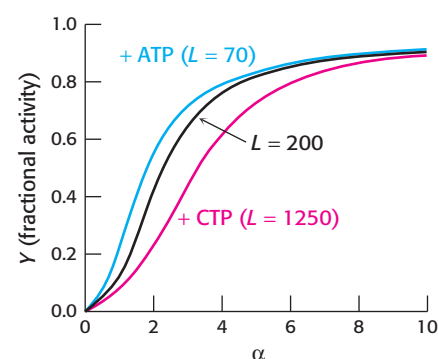


FIGURE 10.15 Quantitative description of the MWC model. Fractional activity, Y , is the fraction of active sites bound to substrate and is directly proportional to reaction velocity; α is the ratio of $[S]$ to the dissociation constant of S with the enzyme in the R state; L is the ratio of the concentration of enzyme in the T state to that in the R state. The binding of the regulators ATP and CTP to ATCase changes the value of L and thus the response to substrate concentration.

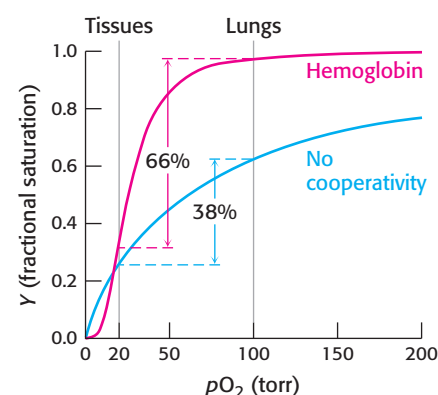


FIGURE 10.17 Cooperativity enhances oxygen delivery by hemoglobin. Because of cooperativity between O_2 -binding sites, hemoglobin delivers more O_2 to tissues than would a noncooperative protein (pO_2 , partial pressure of oxygen.)

Torr—

A unit of pressure equal to that exerted by a column of mercury 1 mm high at 0°C and standard gravity (1 mm Hg). Named after Evangelista Torricelli (1608–1647), inventor of the mercury barometer.

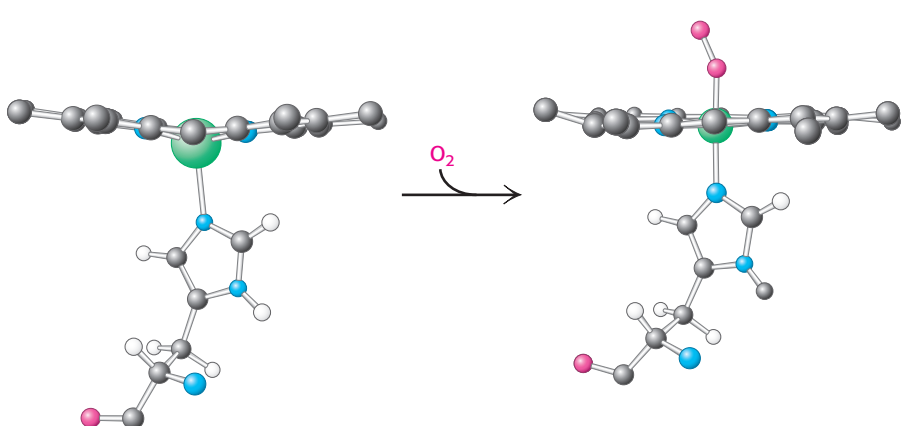
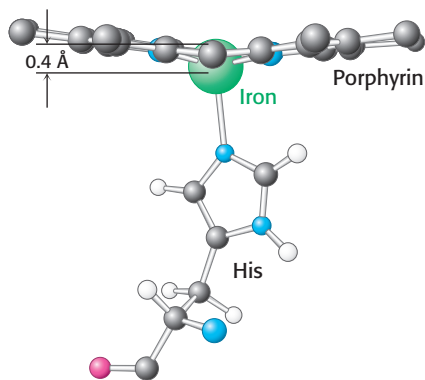
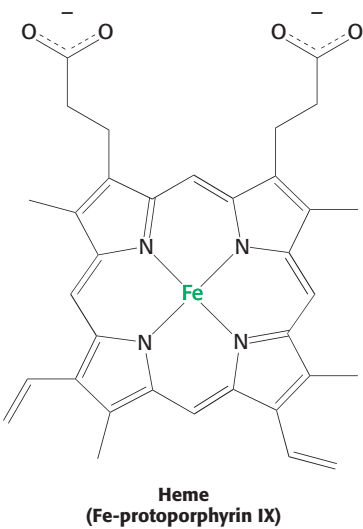
curve leads to efficient oxygen transport. In the lungs, hemoglobin becomes nearly saturated with oxygen such that 98% of the oxygen-binding sites are occupied. When hemoglobin moves to the tissues, the saturation level drops to 32%. Thus, a total of $98 - 32 = 66\%$ of the potential oxygen-binding sites contribute to oxygen transport. In comparison, for a hypothetical noncooperative transport protein, the most oxygen that can be transported from a region in which pO_2 is 100 torr to one in which it is 20 torr is $63 - 25 = 38\%$ (see Figure 10.17). Thus, *the cooperative binding of oxygen by hemoglobin enables it to deliver 1.7 times as much oxygen as it would if the sites were independent*. The homotropic regulation of hemoglobin by its ligand oxygen dramatically increases its physiological oxygen-carrying capacity.

10.2.1 Oxygen Binding Induces Substantial Structural Changes at the Iron Sites in Hemoglobin

The results of structural studies pioneered by Max Perutz revealed the structure of hemoglobin in various forms. Human hemoglobin A, present in adults, consists of four subunits: two α subunits and two β subunits. The α and β subunits are homologous and have similar three-dimensional structures (Section 7.4). The capacity of hemoglobin to bind oxygen depends on the presence of a bound prosthetic group called *heme*. The heme group consists of an organic component and a central iron atom. The organic component, called *protoporphyrin*, is made up of four pyrrole rings linked by methene bridges to form a tetrapyrrole ring. Four methyl groups, two vinyl groups, and two propionate side chains are attached.

The iron atom lies in the center of the protoporphyrin, bonded to the four pyrrole nitrogen atoms. Under normal conditions, the iron is in the ferrous (Fe^{2+}) oxidation state. The iron ion can form two additional bonds, one on each side of the heme plane. These binding sites are called the fifth and sixth coordination sites. In hemoglobin, the fifth coordination site is occupied by the imidazole ring of a histidine residue from the protein. In deoxyhemoglobin, the sixth coordination site remains unoccupied. The iron ion lies approximately 0.4 Å outside the porphyrin plane because iron, in this form, is slightly too large to fit into the well-defined hole within the porphyrin ring (Figure 10.18).

The binding of the oxygen molecule at the sixth coordination site of the iron ion substantially rearranges the electrons within the iron so that the ion becomes effectively smaller, allowing it to move into the plane of the porphyrin (Figure 10.19). This change in electronic structure is paralleled by



changes in the magnetic properties of hemoglobin, which are the basis for functional magnetic resonance imaging (fMRI; Section 32.1.3). Indeed, the structural changes that take place on oxygen binding were anticipated by Linus Pauling, based on magnetic measurements in 1936, nearly 25 years before the three-dimensional structure of hemoglobin was elucidated.

10.2.2 Oxygen Binding Markedly Changes the Quaternary Structure of Hemoglobin

The three-dimensional structure of hemoglobin is best described as a pair of identical $\alpha\beta$ dimers ($\alpha_1\beta_1$ and $\alpha_2\beta_2$) that associate to form the hemoglobin tetramer (Figure 10.20). In deoxyhemoglobin, these $\alpha\beta$ dimers are linked by an extensive interface, which includes, among other regions, the carboxyl terminus of each chain. The heme groups are well separated in the tetramer with iron–iron distances ranging from 24 to 40 Å. The deoxy form corresponds to the T state in the context of either the concerted or the sequential model for hemoglobin cooperativity. On oxygen binding, there are substantial changes in quaternary structure that correspond to the T-to-R state transition (Figure 10.21).

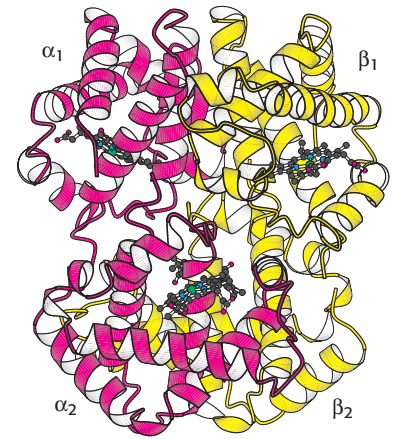


FIGURE 10.20 Quaternary structure of hemoglobin. Hemoglobin, which is composed of two α chains and two β chains, functions as a pair of $\alpha\beta$ dimers.

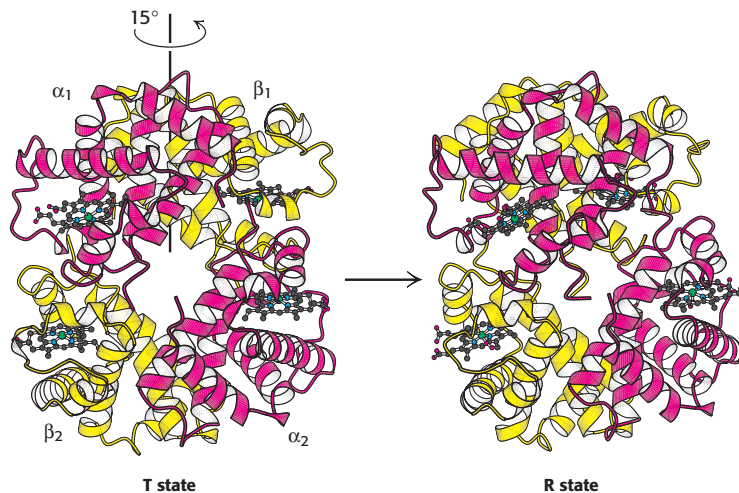


FIGURE 10.21 Transition from T to R state in hemoglobin. On oxygenation, one pair of $\alpha\beta$ subunits shifts with respect to the other by a rotation of 15 degrees.

The $\alpha_1\beta_1$ and $\alpha_2\beta_2$ dimers rotate approximately 15 degrees with respect to one another. The dimers themselves are relatively unchanged, although localized conformational shifts do occur. Thus, the interface between the $\alpha_1\beta_1$ and $\alpha_2\beta_2$ dimers is most effected by this structural transition.

How does oxygen binding lead to the structural transition from the T state to the R state? When the iron ion moves into the plane of the porphyrin, the histidine residue bound in the fifth coordination site moves with it. This histidine residue is part of an α helix, which also moves (Figure 10.22). The carboxyl terminal end of this α helix lies in the interface between the two $\alpha\beta$ dimers. Consequently, the structural transition at the iron ion is directly transmitted to the other subunits. The rearrangement of the dimer interface provides a pathway for communication between subunits, enabling the cooperative binding of oxygen.

Is the cooperative binding of oxygen by hemoglobin best described by the concerted or the sequential model? Neither model in its pure form fully accounts for the behavior of hemoglobin. Instead, a combined model

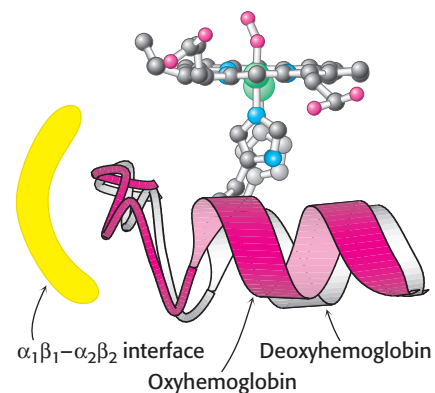


FIGURE 10.22 Conformational changes in hemoglobin. The movement of the iron ion on oxygenation brings the iron-associated histidine residue toward the porphyrin ring. The corresponding movement of the histidine-containing α helix alters the interface between the $\alpha\beta$ pairs, instigating other structural changes. For comparison, the deoxyhemoglobin structure is shown in gray behind the oxyhemoglobin structure in color.

is required. Hemoglobin behavior is concerted in that hemoglobin with three sites occupied by oxygen is in the quaternary structure associated with the R state. The remaining open binding site has an affinity for oxygen more than 20-fold as great as that of fully deoxygenated hemoglobin binding its first oxygen. However, the behavior is not fully concerted, because hemoglobin with oxygen bound to only one of four sites remains in the T-state quaternary structure. Yet, this molecule binds oxygen 3 times as strongly as does fully deoxygenated hemoglobin, an observation consistent only with a sequential model. These results highlight the fact that the concerted and sequential models represent idealized limiting cases, which real systems may approach but rarely attain.

10.2.3 Tuning the Oxygen Affinity of Hemoglobin: The Effect of 2,3-Bisphosphoglycerate

Examination of the oxygen binding of hemoglobin fully purified from red blood cells revealed that the oxygen affinity of purified hemoglobin is much greater than that for hemoglobin within red blood cells. This dramatic difference is due to the presence within these cells of 2,3-bisphosphoglycerate (2,3-BPG) (also known as 2,3-diphosphoglycerate or 2,3-DPG). This highly anionic compound is present in red blood cells at approximately the same concentration as that of hemoglobin (~ 2 mM). Without 2,3-BPG, hemoglobin would be an extremely inefficient oxygen transporter, releasing only 8% of its cargo in the tissues.

How does 2,3-BPG affect oxygen affinity so significantly? Examination of the crystal structure of deoxyhemoglobin in the presence of 2,3-BPG reveals that a single molecule of 2,3-BPG binds in a pocket, present only in the T form, in the center of the hemoglobin tetramer (Figure 10.23). On T-to-R transition, this pocket collapses. Thus, 2,3-BPG binds preferentially to deoxyhemoglobin and stabilizes it, effectively reducing the oxygen affinity. In order for the structural transition from T to R to take place, the bonds between hemoglobin and 2,3-BPG must be broken and 2,3-BPG must be expelled.

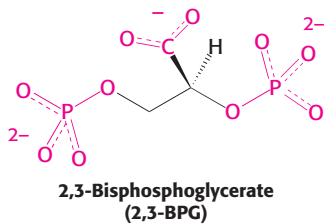
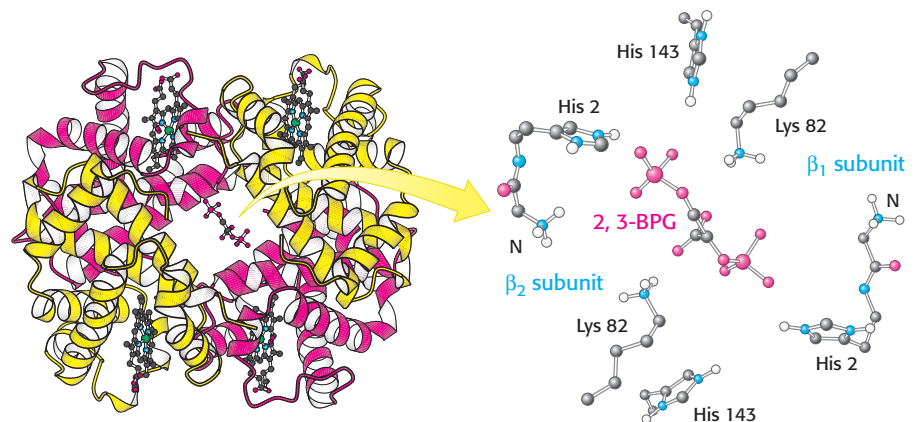


FIGURE 10.23 Mode of binding of 2,3-BPG to human deoxyhemoglobin. 2,3-BPG binds in the central cavity of deoxyhemoglobin. There, it interacts with three positively charged groups on each β chain.



2,3-BPG binding to hemoglobin has other crucial physiological consequences. The globin gene expressed by fetuses differs from that expressed by human adults; fetal hemoglobin tetramers include two α chains and two γ chains. The γ chain, a result of another gene duplication, is 72% identical in amino acid sequence with the β chain. One noteworthy change is the substitution of a serine residue for His 143 in the β chain of the 2,3-BPG-binding site. This change removes two positive charges from the 2,3-BPG-binding site (one from each chain) and reduces the affinity of 2,3-BPG for fetal hemoglobin, thereby increasing the oxygen-

binding affinity of fetal hemoglobin relative to that of maternal (adult) hemoglobin (Figure 10.24). This difference in oxygen affinity allows oxygen to be effectively transferred from maternal to fetal red cells. We see again an example of where gene duplication and specialization produced a ready solution to a biological challenge—in this case, the transport of oxygen from mother to fetus.

10.2.4 The Bohr Effect: Hydrogen Ions and Carbon Dioxide Promote the Release of Oxygen

Rapidly metabolizing tissues, such as contracting muscle, have a high need for oxygen and generate large amounts of hydrogen ions and carbon dioxide as well (Sections 16.1.9 and 17.1). Both of these species are heterotropic effectors of hemoglobin that enhance oxygen release. The oxygen affinity of hemoglobin decreases as pH decreases from the value of 7.4 found in the lungs (Figure 10.25). Thus, as hemoglobin moves into a region of low pH, its tendency to release oxygen increases. For example, transport from the lungs, with pH 7.4 and an oxygen partial pressure of 100 torr, to active muscle, with a pH of 7.2 and an oxygen partial pressure of 20 torr, results in a release of oxygen amounting to 77% of total carrying capacity. Recall that only 66% of the oxygen would be released in the absence of any change in pH. In addition, hemoglobin responds to carbon dioxide with a decrease in oxygen affinity, thus facilitating the release of oxygen in tissues with a high carbon dioxide concentration. In the presence of carbon dioxide at a partial pressure of 40 torr, the amount of oxygen released approaches 90% of the maximum carrying capacity. Thus, *the heterotropic regulation of hemoglobin by hydrogen ions and carbon dioxide further increases the oxygen-transporting efficiency of this magnificent allosteric protein.*

The regulation of oxygen binding by hydrogen ions and carbon dioxide is called the *Bohr effect* after Christian Bohr, who described this phenomenon in 1904. The results of structural and chemical studies have revealed much about the chemical basis of the Bohr effect. At least two sets of chemical groups are responsible for the effect of protons: the amino termini and the side chains of histidines β_{146} and α_{122} , which have pK_a values near pH 7. Consider histidine β_{146} . In deoxyhemoglobin, the terminal carboxylate group of β_{146} forms a salt bridge with a lysine residue in the α subunit of the other $\alpha\beta$ dimer. This interaction locks the side chain of histidine β_{146} in a position where it can participate in a salt bridge with negatively charged aspartate 94 in the same chain, provided that the imidazole group of the histidine residue is protonated (Figure 10.26). At high pH, the side chain of histidine β_{146} is not protonated and the salt bridge does not form. As the pH drops, however, the side chain of histidine β_{146} becomes protonated, the

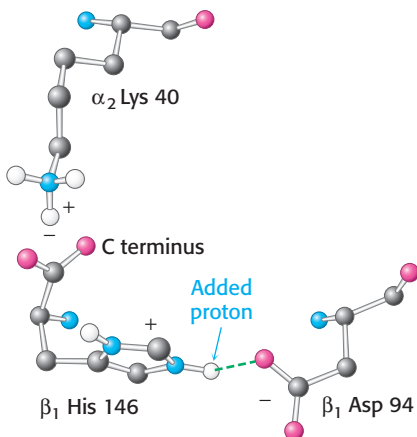


FIGURE 10.26 Chemical basis of the Bohr effect. In deoxyhemoglobin, shown here, three amino acid residues form two salt bridges that stabilize the T quaternary structure. The formation of one of the salt bridges depends on the presence of an added proton on histidine β_{146} . The proximity of the negative charge on aspartate 94 favors protonation of histidine β_{146} in deoxyhemoglobin.

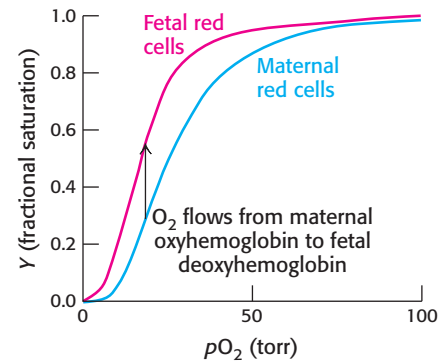


FIGURE 10.24 Oxygen affinity of fetal red blood cells. Fetal red blood cells have a higher oxygen affinity than that of maternal red blood cells because fetal hemoglobin does not bind 2,3-BPG as well as maternal hemoglobin does.

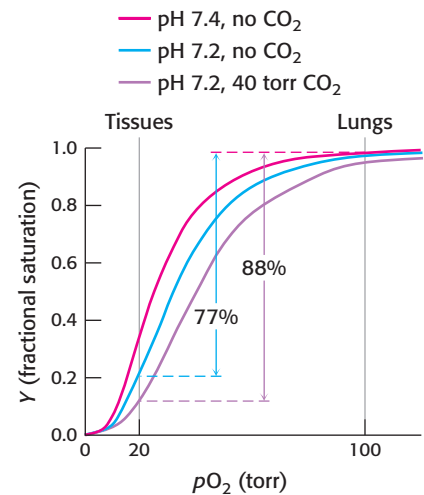
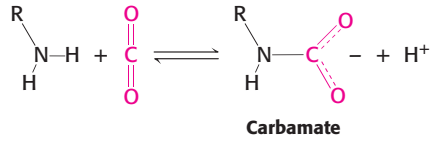


FIGURE 10.25 Effect of pH and CO_2 concentration on the oxygen affinity of hemoglobin. Lowering the pH from 7.4 (red curve) to 7.2 (blue curve) results in the release of O_2 from oxyhemoglobin. Raising the CO_2 partial pressure from 0 to 40 torr (purple curve) also favors the release of O_2 from oxyhemoglobin.



salt bridge with aspartate $\beta 94$ forms, and the quaternary structure characteristic of deoxyhemoglobin is stabilized, leading to a greater tendency for oxygen to be released at actively metabolizing tissues. No significant change takes place in oxyhemoglobin over the same pH range.

Carbon dioxide also stabilizes deoxyhemoglobin by reacting with the terminal amino groups to form carbamate groups, which are negatively charged, in contrast with the neutral or positive charges on the free amino groups. The amino termini lie at the interface between the $\alpha\beta$ dimers, and these negatively charged carbamate groups participate in salt-bridge interactions, characteristic of the T-state structure, which stabilize deoxyhemoglobin's structure and favor the release of oxygen.

Hemoglobin with bound carbon dioxide and hydrogen ions is carried in the blood back to the lungs, where it releases the hydrogen ions and carbon dioxide and rebinds oxygen. Thus, hemoglobin helps to transport hydrogen ions and carbon dioxide in addition to transporting oxygen. However, transport by hemoglobin accounts for only about 14% of the total transport of these species; both hydrogen ions and carbon dioxide are also transported in the blood as bicarbonate (HCO_3^-) formed spontaneously or through the action of carbonic anhydrase (Section 9.2), an abundant enzyme in red blood cells.

10.3 ISOZYMES PROVIDE A MEANS OF REGULATION SPECIFIC TO DISTINCT TISSUES AND DEVELOPMENTAL STAGES

Isozymes or *isoenzymes*, are enzymes that differ in amino acid sequence yet catalyze the same reaction. Usually, these enzymes display different kinetic parameters, such as K_M , or different regulatory properties. They are encoded by different genetic loci, which usually arise through gene duplication and divergence (Section 2.2.5). Isozymes differ from allozymes, which are enzymes that arise from allelic variation at one gene locus. Isozymes can often be distinguished from one another by biochemical properties such as electrophoretic mobility.

The existence of isozymes permits the fine-tuning of metabolism to meet the particular needs of a given tissue or developmental stage. Consider the example of lactate dehydrogenase (LDH), an enzyme that functions in anaerobic glucose metabolism and glucose synthesis. Human beings have two isozymic polypeptide chains for this enzyme: the H isozyme highly expressed in heart and the M isozyme found in skeletal muscle. The amino acid sequences are 75% identical. The functional enzyme is tetrameric, and many different combinations of the two subunits are possible. The H_4 isozyme, found in the heart, has a higher affinity for substrates than does the M_4 isozyme. The two isozymes also differ in that high levels of pyruvate allosterically inhibit the H_4 but not the M_4 isozyme. The other combinations, such as H_3M , have intermediate properties depending on the ratio of the two kinds of chains. We will consider these isozymes in their biological context in Chapter 16.



The M_4 isozyme functions optimally in an anaerobic environment, whereas the H_4 isozyme does so in an aerobic environment. Indeed, the proportions of these isozymes are altered in the course of development of the rat heart as the tissue switches from an anaerobic environment to an aerobic one (Figure 10.27A). Figure 10.27B shows the tissue-specific forms of lactate dehydrogenase in adult rat tissues. The appearance of some

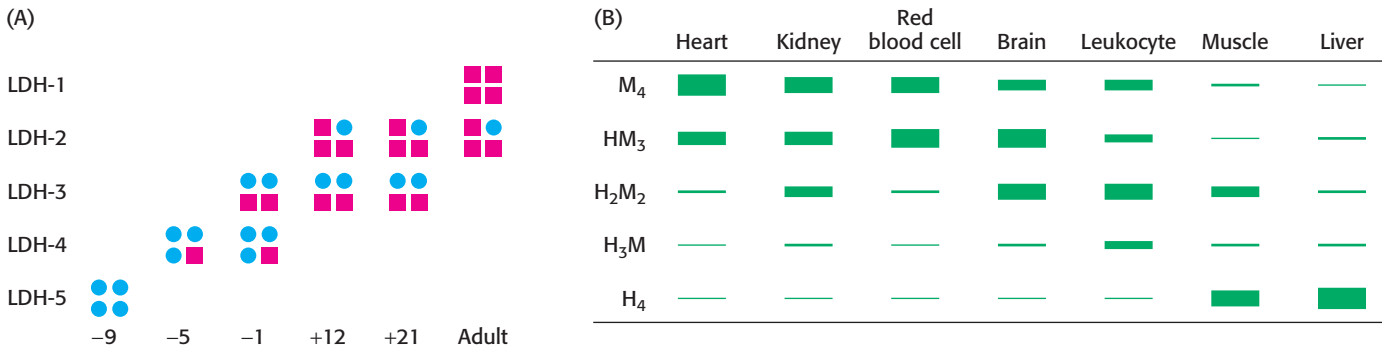


FIGURE 10.27 Isozymes of lactate dehydrogenase. (A) The rat heart LDH isozyme profile changes in the course of development. The H isozyme is represented by squares and the M isozyme by circles. The negative and positive numbers denote the days before and after birth, respectively. (B) LDH isozyme content varies by tissue. [(A) After W.-H. Li, *Molecular Evolution* (Sinauer, 1997), p. 283; (B) After K. Urich, *Comparative Animal Biochemistry* (Springer Verlag, 1990), p. 542.]

isozymes in the blood is indicative of tissue damage and can be used for clinical diagnosis. For instance, an increase in serum levels of H₄ relative to H₃M is an indication that a myocardial infarction, or heart attack, has occurred.

10.4 COVALENT MODIFICATION IS A MEANS OF REGULATING ENZYME ACTIVITY

The covalent attachment of another molecule can modify the activity of enzymes and many other proteins. In these instances, a donor molecule provides a functional moiety that modifies the properties of the enzyme. Most modifications are reversible. Phosphorylation and dephosphorylation are the most common but not the only means of covalent modification. Histones—proteins that assist in the packaging of DNA into chromosomes as well as in gene regulation—are rapidly acetylated and deacetylated in vivo (Section 31.X.X). More heavily acetylated histones are associated with genes that are being actively transcribed. The acetyltransferase and deacetylase enzymes are themselves regulated by phosphorylation, showing that the covalent modification of histones may be controlled by the covalent modification of the modifying enzymes.

Modification is not readily reversible in some cases. Some proteins in signal-transduction pathways, such as Ras and Src (a protein tyrosine kinase), are localized to the cytoplasmic face of the plasma membrane by the irreversible attachment of a lipid group (Section 12.5.3). Fixed in this location, the proteins are better able to receive and transmit information that is being passed along their signaling pathways (Chapter 15). The attachment of ubiquitin, a protein comprising 72 amino acids, is a signal that a protein is to be destroyed, the ultimate means of regulation (Chapter 23). Cyclin, an important protein in cell-cycle regulation, must be ubiquitinated and destroyed before a cell can enter anaphase and proceed through the cell cycle (Table 10.1).

Virtually all the metabolic processes that we will examine are regulated in part by covalent modification. Indeed, the allosteric properties of many enzymes are modified by covalent modification. Table 10.1 lists some of the common covalent modifications.

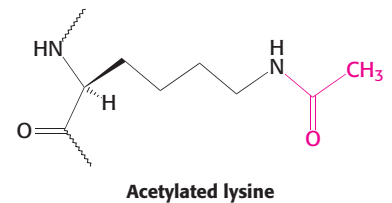


TABLE 10.1 Common covalent modifications of protein activity

Modification	Donor molecule	Example of modified protein	Protein function
Phosphorylation	ATP	Glycogen phosphorylase	Glucose homeostasis; energy transduction
Acetylation	Acetyl CoA	Histones	DNA packing; transcription
Myristoylation	Myristoyl CoA	Src	Signal transduction
ADP-ribosylation	NAD	RNA polymerase	Transcription
Farnesylation	Farnesyl pyrophosphate	Ras	Signal transduction
γ -Carboxylation	HCO_3^-	Thrombin	Blood clotting
Sulfation	3'-Phosphoadenosine-5'-phosphosulfate	Fibrinogen	Blood-clot formation
Ubiquitination	Ubiquitin	Cyclin	Control of cell cycle

10.4.1 Phosphorylation Is a Highly Effective Means of Regulating the Activities of Target Proteins

The activities of many enzymes, membrane channels, and other target proteins are regulated by phosphorylation, the most prevalent reversible covalent modification. Indeed, we will see this regulatory mechanism in virtually every metabolic process in eukaryotic cells. The enzymes catalyzing phosphorylation reactions are called *protein kinases*, which constitute one of the largest protein families known, with more than 100 homologous enzymes in yeast and more than 550 in human beings. This multiplicity of enzymes allows regulation to be fine-tuned according to a specific tissue, time, or substrate.

The terminal (γ) phosphoryl group of ATP is transferred to specific *serine* and *threonine* residues by one class of protein kinases and to specific *tyrosine* residues by another.

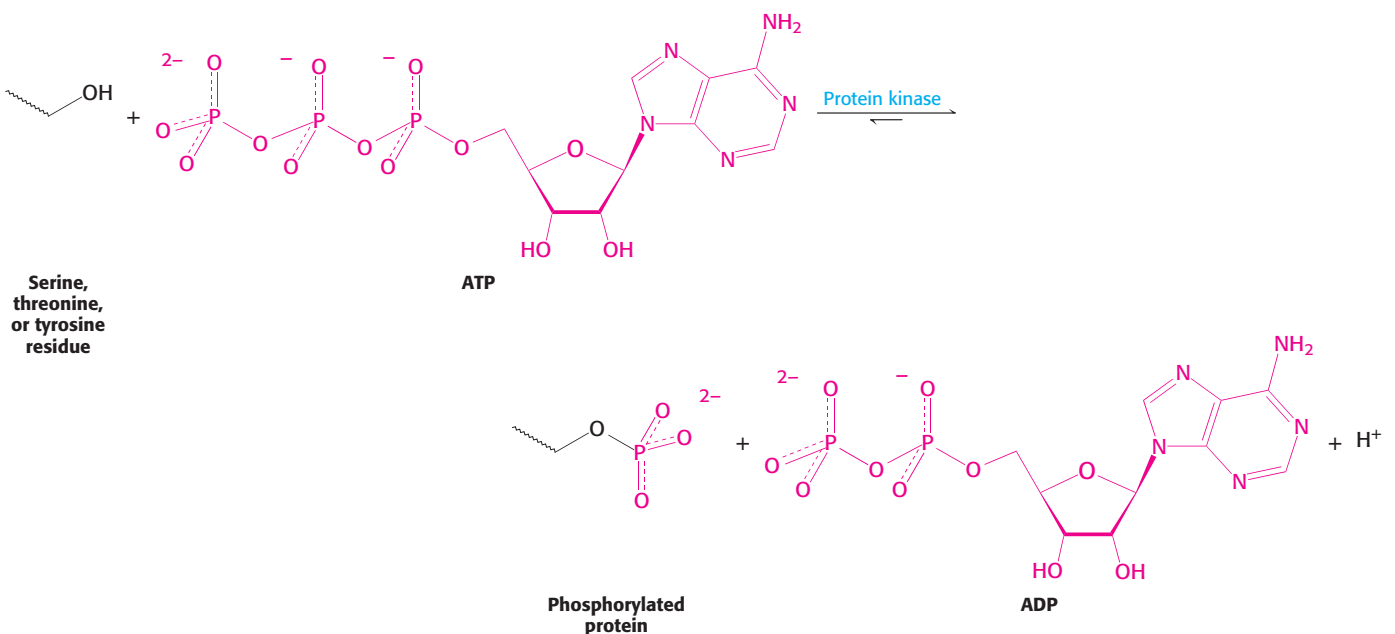
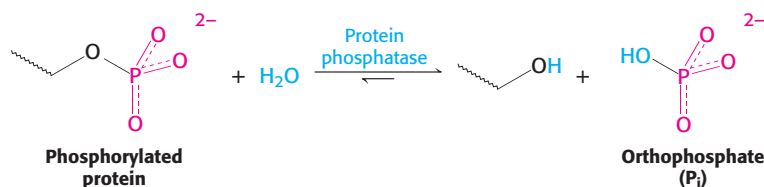


TABLE 10.2 Examples of serine and threonine kinases and their activating signals

Signal	Enzyme
Cyclic nucleotides	Cyclic AMP-dependent protein kinase Cyclic GMP-dependent protein kinase
Ca ²⁺ and calmodulin	Ca ²⁺ -calmodulin protein kinase Phosphorylase kinase or glycogen synthase kinase 2
AMP	AMP-activated kinase
Diacylglycerol	Protein kinase C
Metabolic intermediates and other “local” effectors	Many target specific enzymes, such as pyruvate dehydrogenase kinase and branched-chain ketoacid dehydrogenase kinase

Source: After D. Fell, *Understanding the Control of Metabolism* (Portland Press, 1997), Table 7.2.

The acceptors in protein phosphorylation reactions are located inside cells, where the phosphoryl-group donor ATP is abundant. Proteins that are entirely extracellular are not regulated by reversible phosphorylation. Table 10.2 lists a few of the known protein kinases. *Protein phosphatases* reverse the effects of kinases by catalyzing the hydrolytic removal of phosphoryl groups attached to proteins.

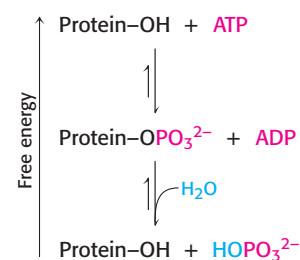


The unmodified hydroxyl-containing side chain is regenerated and orthophosphate (P_i) is produced.

It is important to note that phosphorylation and dephosphorylation are not the reverse of one another; each is essentially irreversible under physiological conditions. Furthermore, both reactions take place at negligible rates in the absence of enzymes. Thus, phosphorylation of a protein substrate will take place only through the action of a specific protein kinase and at the expense of ATP cleavage, and dephosphorylation will result only through the action of a phosphatase. The rate of cycling between the phosphorylated and the dephosphorylated states depends on the relative activities of kinases and phosphatases. Note that the net outcome of the two reactions is the hydrolysis of ATP to ADP and P_i, which has a ΔG of $-12 \text{ kcal mol}^{-1}$ (-50 kJ mol^{-1}) under cellular conditions (Section 14.1.2). This highly favorable free-energy change ensures that target proteins cycle unidirectionally between unphosphorylated and phosphorylated forms.

Phosphorylation is a highly effective means of controlling the activity of proteins for structural, thermodynamic, kinetic, and regulatory reasons:

1. A phosphoryl group adds two negative charges to a modified protein. Electrostatic interactions in the unmodified protein can be disrupted and new electrostatic interactions can be formed. Such structural changes can markedly alter substrate binding and catalytic activity.

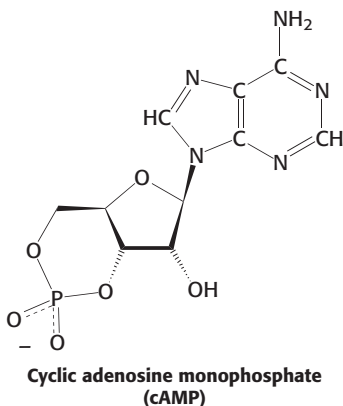


2. A phosphate group can form three or more hydrogen bonds. The tetrahedral geometry of the phosphoryl group makes these hydrogen bonds highly directional, allowing for specific interactions with hydrogen-bond donors.
3. The free energy of phosphorylation is large. Of the $-12 \text{ kcal mol}^{-1}$ (-50 kJ mol^{-1}) provided by ATP, about half is consumed in making phosphorylation irreversible; the other half is conserved in the phosphorylated protein. Recall that a free-energy change of $1.36 \text{ kcal mol}^{-1}$ (5.69 kJ mol^{-1}) corresponds to a factor of 10 in an equilibrium constant (Section 14.1.3). Hence, phosphorylation can change the conformational equilibrium between different functional states by a large factor, of the order of 10^4 .
4. Phosphorylation and dephosphorylation can take place in less than a second or over a span of hours. The kinetics can be adjusted to meet the timing needs of a physiological process.
5. Phosphorylation often evokes *highly amplified* effects. A single activated kinase can phosphorylate hundreds of target proteins in a short interval. Further amplification can take place because the target proteins may be enzymes, each of which can then transform a large number of substrate molecules.
6. ATP is the cellular energy currency (Chapter 14). The use of this compound as a phosphoryl group donor links the energy status of the cell to the regulation of metabolism.

Protein kinases vary in their degree of specificity. *Dedicated protein kinases* phosphorylate a single protein or several closely related ones. *Multifunctional protein kinases* modify many different targets; they have a wide reach and can coordinate diverse processes. Comparisons of amino acid sequences of many phosphorylation sites show that a multifunctional kinase recognizes related sequences. For example, the *consensus sequence* recognized by protein kinase A is Arg-Arg-X-Ser-Z or Arg-Arg-X-Thr-Z, in which X is a small residue, Z is a large hydrophobic one, and Ser or Thr is the site of phosphorylation. It should be noted that this sequence is not absolutely required. Lysine, for example, can substitute for one of the arginine residues but with some loss of affinity. Short synthetic peptides containing a consensus motif are nearly always phosphorylated by serine-threonine protein kinases. Thus, *the primary determinant of specificity is the amino acid sequence surrounding the serine or threonine phosphorylation site*. However, distant residues can contribute to specificity. For instance, changes in protein conformation may alter the accessibility of a possible phosphorylation site.

10.4.2 Cyclic AMP Activates Protein Kinase A by Altering the Quaternary Structure

Protein kinases modulate the activity of many proteins, but what leads to the activation of a kinase? Activation is often a multistep process initiated by hormones (Chapter 15). In some cases, the hormones trigger the formation of cyclic AMP, a molecule formed by cyclization of ATP. Cyclic AMP serves as an intracellular messenger in mediating the physiological actions of hormones, as will be discussed in Chapter 15. The striking finding is that *most effects of cAMP in eukaryotic cells are achieved through the activation by cAMP of a single protein kinase*. This key enzyme is called *protein kinase A* or *PKA*. The kinase alters the activities of target proteins by phosphorylating specific serine or threonine residues. As we shall see, PKA provides a clear example of the integration of allosteric regulation and phosphorylation.



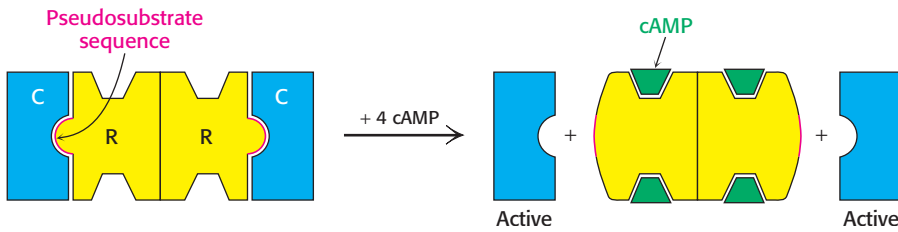



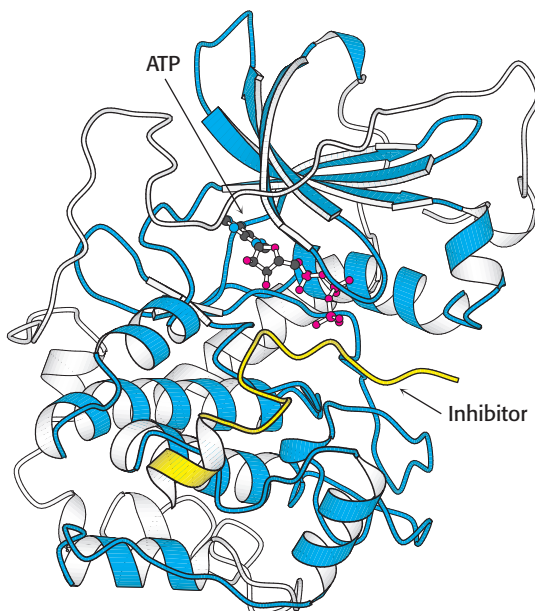
FIGURE 10.28 Regulation of protein kinase A. The binding of four molecules of cAMP activates protein kinase A by dissociating the inhibited holoenzyme (R_2C_2) into a regulatory subunit (R_2) and two catalytically active subunits (C).


PKA is activated by cAMP concentrations of the order of 10 nM. The activation mechanism is reminiscent of that of aspartate transcarbamoylase. Like that enzyme, PKA in muscle consists of two kinds of subunits: a 49-kd regulatory (R) subunit, which has high affinity for cAMP, and a 38-kd catalytic (C) subunit. In the absence of cAMP, the regulatory and catalytic subunits form an R_2C_2 complex that is enzymatically inactive (Figure 10.28). The binding of two molecules of cAMP to each of the regulatory subunits leads to the dissociation of R_2C_2 into an R_2 subunit and two C subunits. These free catalytic subunits are then enzymatically active. Thus, *the binding of cAMP to the regulatory subunit relieves its inhibition of the catalytic subunit*. PKA and most other kinases exist in isozymic forms for fine-tuning regulation to meet the needs of a specific cell or developmental stage.

How does the binding of cAMP activate the kinase? Each R chain contains the sequence Arg-Arg-Gly-Ala-Ile, which matches the consensus sequence for phosphorylation except for the presence of alanine in place of serine. In the R_2C_2 complex, this *pseudosubstrate sequence* of R occupies the catalytic site of C, thereby preventing the entry of protein substrates (see Figure 10.28). The binding of cAMP to the R chains allosterically moves the pseudosubstrate sequences out of the catalytic sites. The released C chains are then free to bind and phosphorylate substrate proteins.

10.4.3 ATP and the Target Protein Bind to a Deep Cleft in the Catalytic Subunit of Protein Kinase A

 The three-dimensional structure of the catalytic subunit of PKA containing a bound 20-residue peptide inhibitor was determined by x-ray crystallography. The 350-residue catalytic subunit has two lobes (Figure 10.29). ATP and part of the inhibitor fill a deep cleft between



 **FIGURE 10.29 Protein kinase A bound to an inhibitor.** Three-dimensional structure of a complex of the catalytic subunit of protein kinase A and an inhibitor bearing a pseudosubstrate sequence. The inhibitor (yellow) binds in a cleft between the domains of the enzyme. The bound ATP is adjacent to the active site.

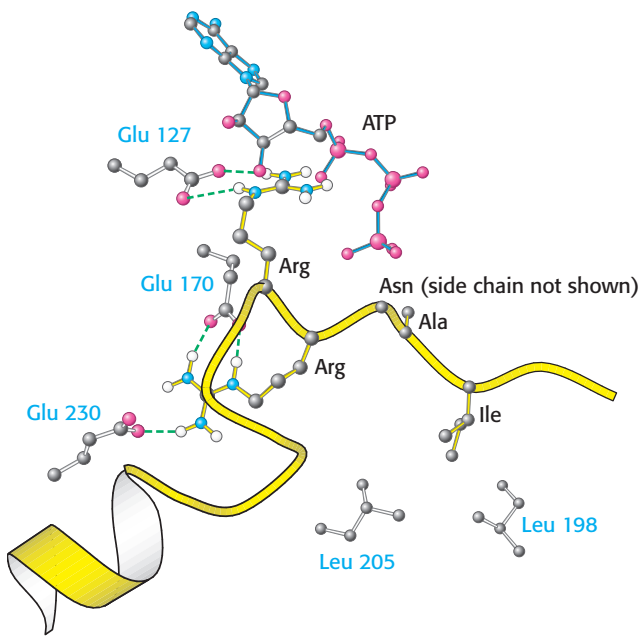


FIGURE 10.30 Binding of pseudosubstrate to protein kinase A. The two arginine side chains of the pseudosubstrate form salt bridges with three glutamate carboxylates. Hydrophobic interactions are also important in the recognition of substrate. The isoleucine residue of the pseudosubstrate is in contact with a pair of leucine residues of the enzyme.

the lobes. The smaller lobe makes many contacts with ATP-Mg^{2+} , whereas the larger lobe binds peptide and contributes the key catalytic residues. Like other kinases (Section 16.1.1), the two lobes move closer to one another on substrate binding; mechanisms for restricting this domain closure provides a means for regulating protein kinase activity. *The PKA structure has broad significance because residues 40 to 280 constitute a conserved catalytic core that is common to essentially all known protein kinases.* We see here an example of a successful biochemical solution to the problem of protein phosphorylation being employed many times in the course of evolution.

The bound peptide in this crystal occupies the active site because it contains the pseudosubstrate sequence Arg-Arg-Asn-Ala-Ile (Figure 10.30). The structure of the complex reveals the basis for the consensus sequence. The guanidinium group of the first arginine residue forms an ion pair with the carboxylate side chain of a glutamate residue (Glu 127) of the enzyme. The second arginine likewise interacts with two other carboxylates. The nonpolar side chain of isoleucine, which matches Z in the consensus sequence, fits snugly in a hydrophobic groove formed by two leucine residues of the enzyme.

10.5 MANY ENZYMES ARE ACTIVATED BY SPECIFIC PROTEOLYTIC CLEAVAGE

We turn now to a different mechanism of enzyme regulation. Many enzymes acquire full enzymatic activity as they spontaneously fold into their characteristic three-dimensional forms. In contrast, other enzymes are synthesized as inactive precursors that are subsequently activated by cleavage of one or a few specific peptide bonds. The inactive precursor is called a *zymogen* (or a *proenzyme*). A energy source (ATP) is not needed for cleavage. Therefore, in contrast with reversible regulation by phosphorylation, even proteins located outside cells can be activated by this means. Another noteworthy difference is that proteolytic activation, in contrast with allosteric control and reversible covalent modification, occurs just once in the life of an enzyme molecule.

Specific proteolysis is a common means of activating enzymes and other proteins in biological systems. For example:

1. The *digestive enzymes* that hydrolyze proteins are synthesized as zymogens in the stomach and pancreas (Table 10.3).

TABLE 10.3 Gastric and pancreatic zymogens

Site of synthesis	Zymogen	Active enzyme
Stomach	Pepsinogen	Pepsin
Pancreas	Chymotrypsinogen	Chymotrypsin
Pancreas	Trypsinogen	Trypsin
Pancreas	Procarboxypeptidase	Carboxypeptidase
Pancreas	Proelastase	Elastase

- Blood clotting* is mediated by a cascade of proteolytic activations that ensures a rapid and amplified response to trauma.
- Some protein hormones are synthesized as inactive precursors. For example, *insulin* is derived from *proinsulin* by proteolytic removal of a peptide.
- The fibrous protein *collagen*, the major constituent of skin and bone, is derived from *procollagen*, a soluble precursor.
- Many *developmental processes* are controlled by the activation of zymogens. For example, in the metamorphosis of a tadpole into a frog, large amounts of collagen are resorbed from the tail in the course of a few days. Likewise, much collagen is broken down in a mammalian uterus after delivery. The conversion of *procollagenase* into *collagenase*, the active protease, is precisely timed in these remodeling processes.
- Programmed cell death*, or *apoptosis*, is mediated by proteolytic enzymes called *caspases*, which are synthesized in precursor form as *procaspases*. When activated by various signals, caspases function to cause cell death in most organisms, ranging from *C. elegans* (Section 2.4.3) to human beings. Apoptosis provides a means sculpting the shapes of body parts in the course of development and a means of eliminating cells producing anti-self antibodies or infected with pathogens as well as cells containing large amounts of damaged DNA.

We next examine the activation and control of zymogens, using as examples several digestive enzymes as well as blood-clot formation.

10.5.1 Chymotrypsinogen Is Activated by Specific Cleavage of a Single Peptide Bond

Chymotrypsin is a digestive enzyme that hydrolyzes proteins in the small intestine. Its mechanism of action was discussed in detail in Chapter 9. Its inactive precursor, *chymotrypsinogen*, is synthesized in the pancreas, as are several other zymogens and digestive enzymes. Indeed, the pancreas is one of the most active organs in synthesizing and secreting proteins. The enzymes and zymogens are synthesized in the acinar cells of the pancreas and stored inside membrane-bounded granules (Figure 10.31). The zymogen granules accumulate at the apex of the acinar cell; when the cell is stimulated by a hormonal signal or a nerve impulse, the contents of the granules are released into a duct leading into the duodenum.

Chymotrypsinogen, a single polypeptide chain consisting of 245 amino acid residues, is virtually devoid of enzymatic activity. It is converted into a fully active enzyme when the peptide bond joining arginine 15 and isoleucine 16 is cleaved by trypsin (Figure 10.32). The resulting active enzyme, called π -chymotrypsin, then acts on other π -chymotrypsin molecules. Two dipeptides are removed to yield α -chymotrypsin, the stable form of the enzyme. The three resulting chains in α -chymotrypsin remain linked to one another by two interchain disulfide bonds. The striking feature of this activation process is that *cleavage of a single specific peptide bond transforms the protein from a catalytically inactive form into one that is fully active*.

10.5.2 Proteolytic Activation of Chymotrypsinogen Leads to the Formation of a Substrate-Binding Site

How does cleavage of a single peptide bond activate the zymogen? Key conformational changes, which were revealed by the elucidation of the three-dimensional structure of chymotrypsinogen, result from the cleavage of the peptide bond between amino acids 15 and 16.

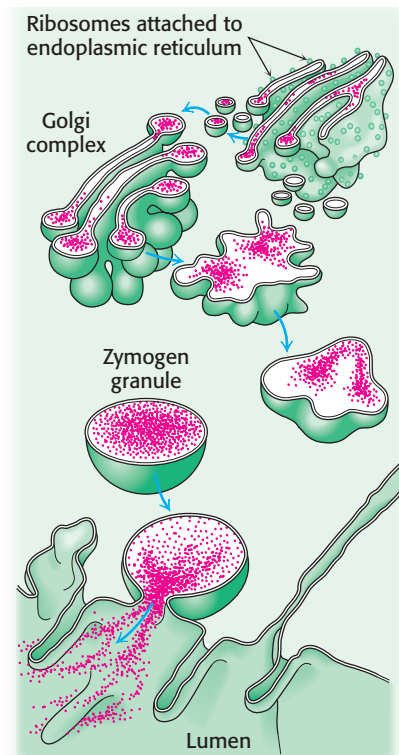


FIGURE 10.31 Secretion of zymogens by an acinar cell of the pancreas.

Chymotrypsinogen (inactive)

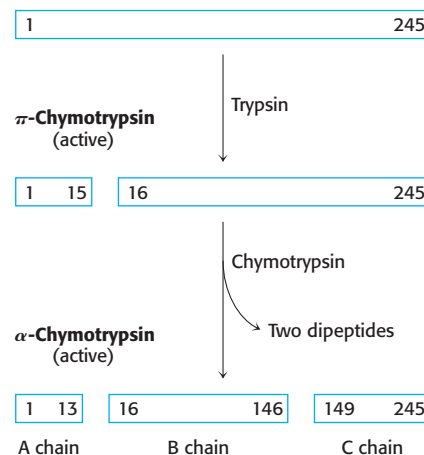


FIGURE 10.32 Proteolytic activation of chymotrypsinogen. The three chains of α -chymotrypsin are linked by two interchain disulfide bonds (A to B, and B to C).

1. The newly formed *amino-terminal group of isoleucine 16 turns inward and forms an ionic bond with aspartate 194 in the interior of the chymotrypsin molecule* (Figure 10.33). Protonation of this amino group stabilizes the active form of chymotrypsin.

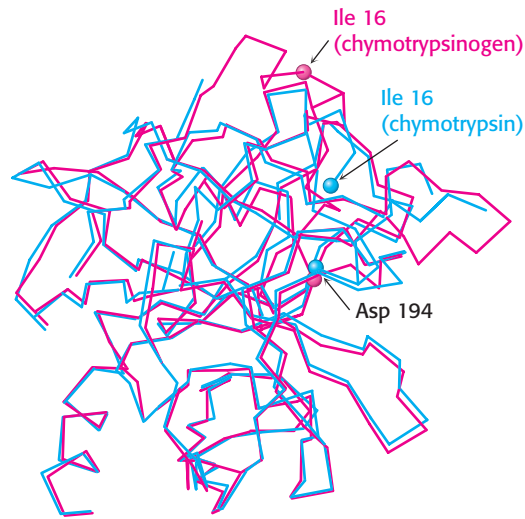


FIGURE 10.33 Conformations of chymotrypsinogen (red) and chymotrypsin (blue). The electrostatic interaction between the carboxylate of aspartate 194 and the α -amino group of isoleucine 16, essential for the structure of active chymotrypsin, is possible only in chymotrypsin.

2. This electrostatic interaction triggers a number of conformational changes. Methionine 192 moves from a deeply buried position in the zymogen to the surface of the active enzyme, and residues 187 and 193 become more extended. These changes result in the formation of the *substrate-specificity site* for aromatic and bulky nonpolar groups. One side of this site is made up of residues 189 through 192. *This cavity for binding part of the substrate is not fully formed in the zymogen.*

3. The tetrahedral transition state in catalysis by chymotrypsin is stabilized by hydrogen bonds between the negatively charged carbonyl oxygen atom of the substrate and two NH groups of the main chain of the enzyme (Section 9.1.3). One of these NH groups is not appropriately located in chymotrypsinogen, and so *the oxyanion hole is incomplete in the zymogen.*

4. The conformational changes elsewhere in the molecule are very small. Thus, *the switching on of enzymatic activity in a protein can be accomplished by discrete, highly localized conformational changes that are triggered by the hydrolysis of a single peptide bond.*

10.5.3 The Generation of Trypsin from Trypsinogen Leads to the Activation of Other Zymogens

The structural changes accompanying the activation of trypsinogen, the precursor of the proteolytic enzyme trypsin, are somewhat different from those in the activation of chymotrypsinogen. X-ray analyses have shown that the conformation of four stretches of polypeptide, constituting about 15% of the molecule, changes markedly on activation. *These regions, called the activation domain, are very flexible in the zymogen, whereas they have a well-defined conformation in trypsin.* Furthermore, the oxyanion hole (Section 9.1.3) in trypsinogen is too far from histidine 57 to promote the formation of the tetrahedral transition state.

The digestion of proteins in the duodenum requires the concurrent action of several proteolytic enzymes, because each is specific for a limited number of side chains. Thus, the zymogens must be switched on at the same

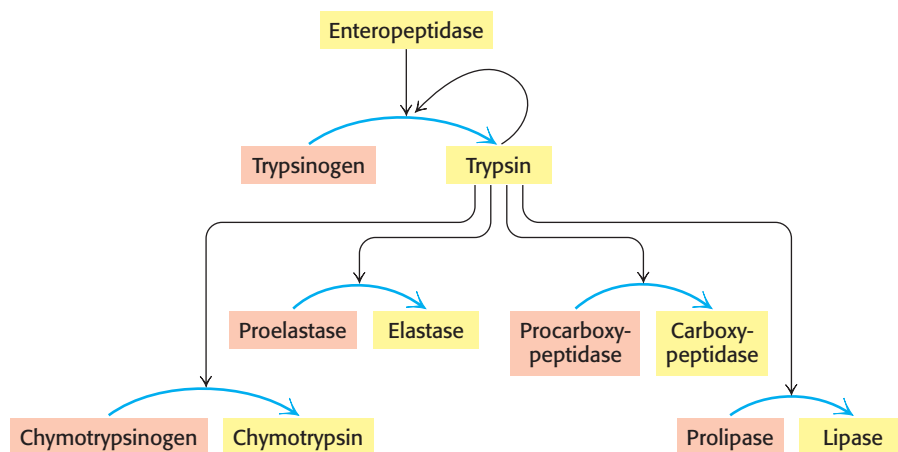


FIGURE 10.34 Zymogen activation by proteolytic cleavage. Enteropeptidase initiates the activation of the pancreatic zymogens by activating trypsin, which then activates other zymogens. Active enzymes are shown in yellow; zymogens are shown in orange.

time. Coordinated control is achieved by the action of *trypsin* as the common activator of all the pancreatic zymogens—trypsinogen, chymotrypsinogen, proelastase, procarboxypeptidase, and prolipase, a lipid degrading enzyme. To produce active trypsin, the cells that line the duodenum secrete an enzyme, *enteropeptidase*, that hydrolyzes a unique lysine–isoleucine peptide bond in trypsinogen as the zymogen enters the duodenum from the pancreas. The small amount of trypsin produced in this way activates more trypsinogen and the other zymogens (Figure 10.34). Thus, the formation of trypsin by enteropeptidase is the master activation step.

10.5.4 Some Proteolytic Enzymes Have Specific Inhibitors

The conversion of a zymogen into a protease by cleavage of a single peptide bond is a precise means of switching on enzymatic activity. However, this activation step is irreversible, and so a different mechanism is needed to stop proteolysis. Specific protease inhibitors accomplish this task. For example, *pancreatic trypsin inhibitor*, a 6-kd protein, inhibits trypsin by binding very tightly to its active site. The dissociation constant of the complex is 0.1 pM, which corresponds to a standard free energy of binding of about $-18 \text{ kcal mol}^{-1}$ (-75 kJ mol^{-1}). In contrast with nearly all known protein assemblies, this complex is not dissociated into its constituent chains by treatment with denaturing agents such as 8 M urea or 6 M guanidine hydrochloride.

The reason for the exceptional stability of the complex is that pancreatic trypsin inhibitor is a very effective substrate analog. X-ray analyses showed that the inhibitor lies in the active site of the enzyme, positioned such that the side chain of lysine 15 of this inhibitor interacts with the aspartate side chain in the specificity pocket of trypsin. In addition, there are many hydrogen bonds between the main chain of trypsin and that of its inhibitor. Furthermore, the carbonyl group of lysine 15 and the surrounding atoms of the inhibitor fit snugly in the active site of the enzyme. Comparison of the structure of the inhibitor bound to the enzyme with that of the free inhibitor reveals that the structure is essentially unchanged on binding to the enzyme (Figure 10.35). Thus, the inhibitor is preorganized into a structure that is highly complementary to the enzyme's active site. Indeed, the

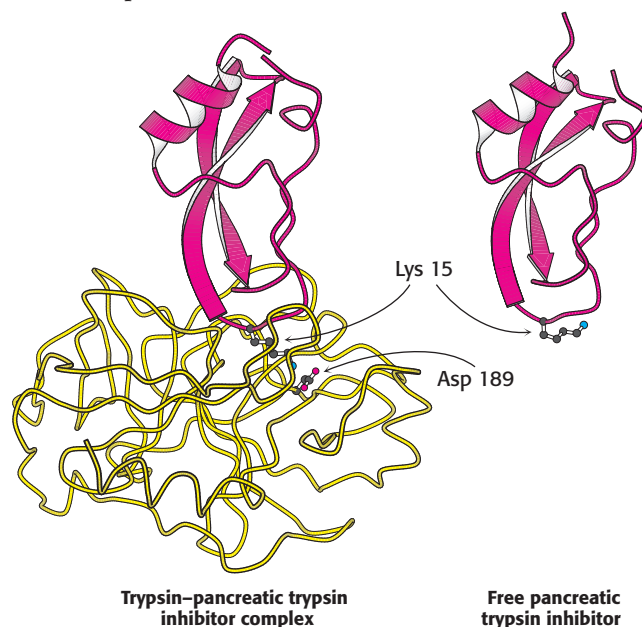


FIGURE 10.35 Interaction of trypsin with its inhibitor. Structure of a complex of trypsin (yellow) and pancreatic trypsin inhibitor (red). Lysine 15 of the inhibitor penetrates into the active site of the enzyme and forms a salt bridge with aspartate 189 in the active site. The bound inhibitor and the free inhibitor are almost identical in structure.

peptide bond between lysine 15 and alanine 16 in pancreatic trypsin inhibitor is cleaved but at a very slow rate: the half-life of the trypsin–inhibitor complex is several months. In essence, the inhibitor is a substrate, but its intrinsic structure is so nicely complementary to the enzyme’s active site that it binds very tightly and is turned over slowly.



Why does trypsin inhibitor exist? Indeed, the amount of trypsin is much greater than that of the inhibitor. Under what circumstances is it beneficial to inhibit trypsin? Recall that trypsin activates other zymogens. Consequently, it is vital that even small amounts of trypsin be prevented from initiating the cascade prematurely. Trypsin molecules activated in the pancreas or pancreatic ducts could severely damage those tissues, leading to acute pancreatitis. Tissue necrosis may result from the activation of proteolytic enzymes (as well as prolipases) by trypsin, and hemorrhaging may result from its activation of elastase. We see here the physiological need for the tight binding of the inhibitor to trypsin.

Pancreatic trypsin inhibitor is not the only important protease inhibitor. α_1 -Antitrypsin (also called α_1 -antiproteinase), a 53-kd plasma protein, protects tissues from digestion by elastase, a secretory product of neutrophils (white blood cells that engulf bacteria). *Antielastase* would be a more accurate name for this inhibitor, because it blocks elastase much more effectively than it blocks trypsin. Like pancreatic trypsin inhibitor, α_1 -antitrypsin blocks the action of target enzymes by binding nearly irreversibly to their active sites. Genetic disorders leading to a deficiency of α_1 -antitrypsin show that this inhibitor is physiologically important. For example, the substitution of lysine for glutamate at residue 53 in the type Z mutant slows the secretion of this inhibitor from liver cells. Serum levels of the inhibitor are about 15% of normal in people homozygous for this defect. The consequence is that excess elastase destroys alveolar walls in the lungs by digesting elastic fibers and other connective-tissue proteins.

The resulting clinical condition is called *emphysema* (also known as *destructive lung disease*). People with emphysema must breathe much harder than normal people to exchange the same volume of air, because their alveoli are much less resilient than normal. Cigarette smoking markedly increases the likelihood that even a type Z heterozygote will develop emphysema. The reason is that smoke oxidizes methionine 358 of the inhibitor (Figure 10.36), a residue essential for binding elastase. Indeed, this methionine side chain is the bait that selectively traps elastase. The *methionine sulfoxide* oxidation product, in contrast, does not lure elastase, a striking consequence of the insertion of just one oxygen atom into a protein. We will consider another protease inhibitor, antithrombin III, when we examine the control of blood clotting.

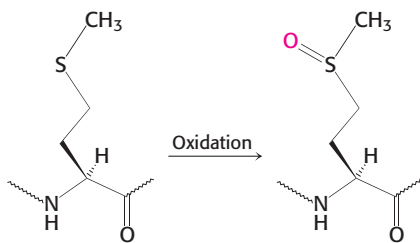


FIGURE 10.36 Oxidation of methionine to the sulfoxide.

10.5.5 Blood Clotting Is Accomplished by a Cascade of Zymogen Activations

Enzymatic cascades are often employed in biochemical systems to achieve a rapid response. In a cascade, an initial signal institutes a series of steps, each of which is catalyzed by an enzyme. At each step, the signal is amplified. For instance, if a signal molecule activates an enzyme that in turn activates 10 enzymes and each of the 10 enzymes in turn activates 10 additional enzymes, after four steps the original signal will have been amplified 10,000-fold. Blood clots are formed by a *cascade of zymogen activations*: the activated form of one clotting factor catalyzes the activation of the next (Figure 10.37). Thus, very small amounts of the initial factors suffice to trigger the cascade, ensuring a rapid response to trauma.

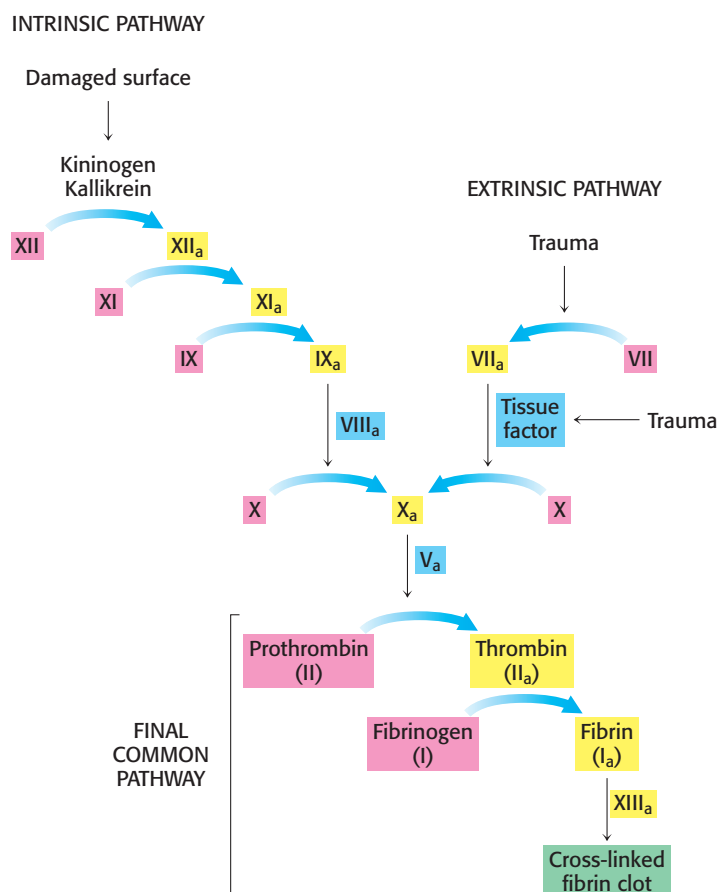


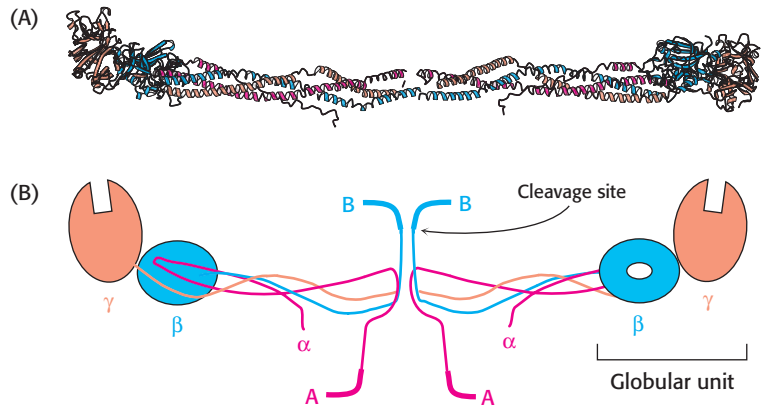
FIGURE 10.37 Blood-clotting cascade. A fibrin clot is formed by the interplay of the intrinsic, extrinsic, and final common pathways. The intrinsic pathway begins with the activation of factor XII (Hageman factor) by contact with abnormal surfaces produced by injury. The extrinsic pathway is triggered by trauma, which activates factor VII and releases a lipoprotein, called tissue factor, from blood vessels. Inactive forms of clotting factors are shown in red; their activated counterparts (indicated by the subscript "a") are in yellow. Stimulatory proteins that are not themselves enzymes are shown in blue. A striking feature of this process is that the activated form of one clotting factor catalyzes the activation of the next factor.

There are two means of initiating clotting: the intrinsic and extrinsic pathways. The *intrinsic clotting pathway* is activated by exposure of anionic surfaces on rupture of the endothelial lining of the blood vessels. These surfaces serve as binding sites for factors in the clotting cascade. Substances that are released from tissues as a consequence of trauma to them trigger the *extrinsic clotting pathway*. The extrinsic and intrinsic pathways converge on a common sequence of final steps to form a clot composed of the protein fibrin. The two pathways interact with each other *in vivo*. Indeed, both are needed for proper clotting, as evidenced by clotting disorders caused by a deficiency of a single protein in one of the pathways. Note that the active forms of the clotting factors are designated with a subscript "a."

10.5.6 Fibrinogen Is Converted by Thrombin into a Fibrin Clot

The best-characterized part of the clotting process is the final step in the cascade: the conversion of fibrinogen into fibrin by thrombin, a proteolytic enzyme. Fibrinogen is made up of three globular units connected by two

FIGURE 10.38 Structure of a fibrinogen molecule. (A) A ribbon diagram. The two rod regions are α -helical coiled coils, connected to a globular region at each end. (B) A schematic representation showing the positions of the fibrinopeptides A and B.



rods (Figure 10.38). This 340-kd protein consists of six chains: two each of $A\alpha$, $B\beta$, and γ . The rod regions are triple-stranded α -helical coiled coils, a recurring motif in proteins. Thrombin cleaves four *arginine–glycine peptide bonds* in the central globular region of fibrinogen. On cleavage, an A peptide of 18 residues from each of the two $A\alpha$ chains and a B peptide of 20 residues from each of the two $B\beta$ chains are released from the globular region. These A and B peptides are called *fibrinopeptides*. A fibrinogen molecule devoid of these fibrinopeptides is called a *fibrin monomer* and has the subunit structure $(\alpha\beta\gamma)_2$.

Fibrin monomers spontaneously assemble into ordered fibrous arrays called *fibrin*. Electron micrographs and low-angle x-ray patterns show that fibrin has a periodic structure that repeats every 23 nm (Figure 10.39).

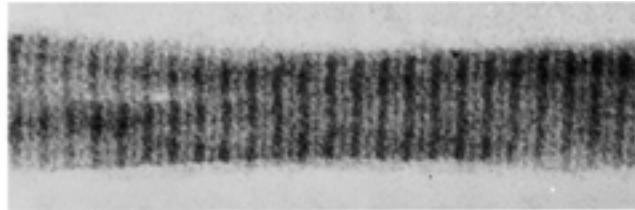


FIGURE 10.39 Electron micrograph of fibrin. The 23-nm period along the fiber axis is half the length of a fibrinogen molecule. [Courtesy of Dr. Henry Slayter.]

Higher-resolution images reveal the detailed structure of the fibrin monomer, how the fibrin monomers come together, and how this assembly is facilitated by removal of the fibrinopeptides. The homologous β and γ chains have globular domains at the carboxyl-terminal ends (Figure 10.40). These domains have binding “holes” that interact with peptides. The β domain is specific for sequences of the form H_3N^+ -Gly-His-Arg-, whereas the γ domain binds H_3N^+ -Gly-Pro-Arg-. Exactly these sequences (some-

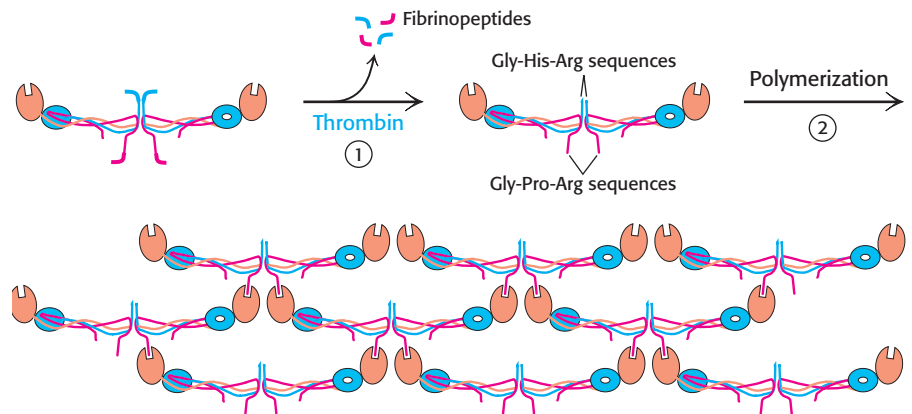
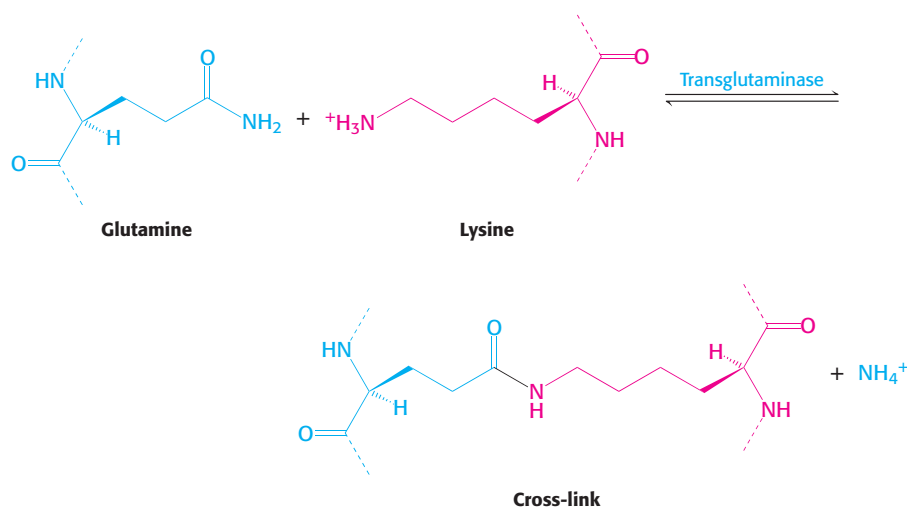


FIGURE 10.40 Formation of a fibrin clot. (1) Thrombin cleaves fibrinopeptides A and B from the central globule of fibrinogen. (2) Globular domains at the carboxyl-terminal ends of the β and γ chains interact with “knobs” exposed at the amino-terminal ends of the β and α chains to form clots.

times called “knobs”) are exposed at the amino-terminal ends of the β and α chains, respectively, on thrombin cleavage. The knobs of the α subunits fit into the holes on the γ subunits of another monomer to form a protofibril. This protofibril is extended when the knobs of the β subunits fit into holes of β subunits of other protofibrils. Thus, analogous to the activation of chymotrypsinogen, peptide-bond cleavage exposes new amino termini that can participate in specific interactions. The newly formed clot is stabilized by the formation of amide bonds between the side chains of lysine and glutamine residues in different monomers.



This cross-linking reaction is catalyzed by *transglutaminase* (factor XIII_a), which itself is activated from the protransglutaminase form by thrombin.

12.5.7 Prothrombin Is Readied for Activation by a Vitamin K-Dependent Modification

Thrombin is synthesized as a zymogen called *prothrombin*, which comprises four major domains, with the serine protease domain at its carboxyl terminus. The first domain is called a *gla domain*, whereas domains 2 and 3 are called *kringle domains* (Figure 10.41). These domains work in concert to keep prothrombin in an inactive form and to target it to appropriate sites for its activation by factor X_a (a serine protease) and factor V_a (a stimulatory protein). Activation is accomplished by proteolytic cleavage of the bond between arginine 274 and threonine 275 to release a fragment containing the first three domains and by cleavage of the bond between arginine 323 and isoleucine 324 (analogous to the key bond in chymotrypsinogen) to yield active thrombin.

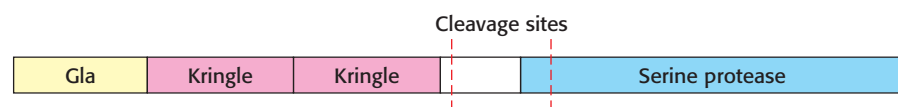



FIGURE 10.41 Modular structure of prothrombin. Cleavage of two peptide bonds yields thrombin. All the γ -carboxyglutamate residues are in the gla domain.

 Vitamin K (Section 8.6.2 and Figure 10.42) has been known for many years to be essential for the synthesis of prothrombin and several other clotting factors. The results of studies of the abnormal prothrombin synthesized in the absence of vitamin K or in the presence of vitamin K antagonists, such as dicoumarol, revealed the mode of action of this vitamin. *Dicoumarol* is found in spoiled sweet clover and causes a fatal

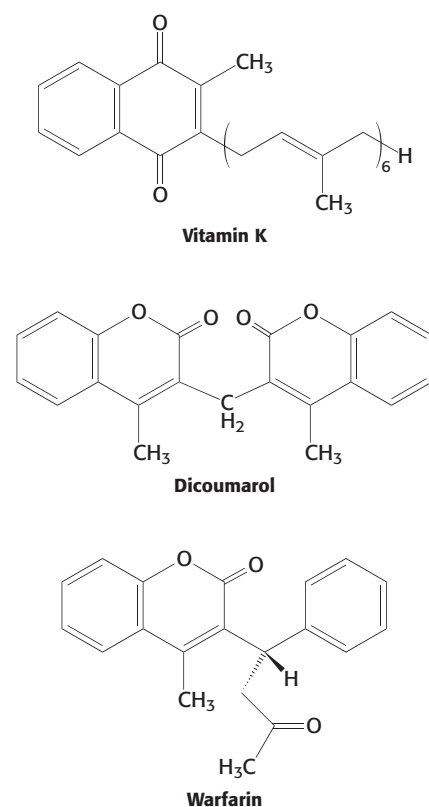


FIGURE 10.42 Structures of vitamin K and two antagonists, dicoumarol and warfarin.

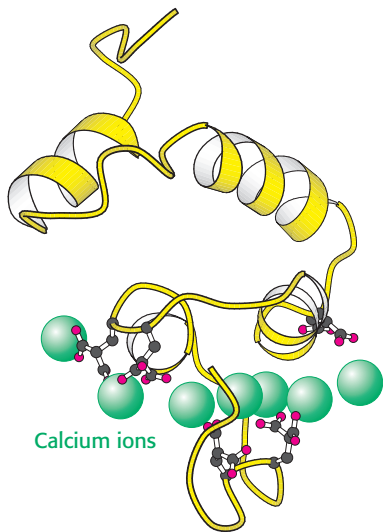
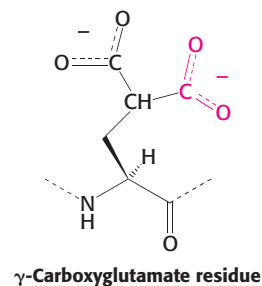


FIGURE 10.43 The calcium-binding region of prothrombin. Prothrombin binds calcium ions with the modified amino acid γ -carboxyglutamate (red).

hemorrhagic disease in cattle fed on this hay. This coumarin derivative is used clinically as an *anticoagulant* to prevent thromboses in patients prone to clot formation. Dicoumarol and such related vitamin K antagonists as *warfarin* also serve as effective rat poisons. Cows fed dicoumarol synthesize an abnormal prothrombin that does not bind Ca^{2+} , in contrast with normal prothrombin. This difference was puzzling for some time because abnormal prothrombin has the same number of amino acid residues and gives the same amino acid analysis after acid hydrolysis as does normal prothrombin.

Nuclear magnetic resonance studies revealed that normal prothrombin contains γ -carboxyglutamate, a previously unknown residue that evaded detection because its second carboxyl group is lost on acid hydrolysis. The abnormal prothrombin formed subsequent to the administration of anticoagulants lacks this modified amino acid. In fact, the first 10 glutamate residues in the amino-terminal region of prothrombin are carboxylated to γ -carboxyglutamate by a vitamin K-dependent enzyme system (Figure 10.43). *The vitamin K-dependent carboxylation reaction converts glutamate, a weak chelator of Ca^{2+} , into γ -carboxyglutamate, a much stronger chelator.* Prothrombin is thus able to bind Ca^{2+} , but what is the effect of this binding? The binding of Ca^{2+} by prothrombin anchors the zymogen to phospholipid membranes derived from blood platelets after injury. The binding of prothrombin to phospholipid surfaces is crucial because it brings prothrombin into close proximity to two clotting proteins that catalyze its conversion into thrombin. The proteolytic activation of prothrombin removes the calcium-binding domain and frees thrombin from the membrane so that it can cleave fibrinogen and other targets.



10.5.8 Hemophilia Revealed an Early Step in Clotting

Some important breakthroughs in the elucidation of clotting pathways have come from studies of patients with bleeding disorders. *Classic hemophilia*, or *hemophilia A*, the best-known clotting defect, is genetically transmitted as a sex-linked recessive characteristic. *In classic hemophilia, factor VIII (antihemophilic factor) of the intrinsic pathway is missing or has markedly reduced activity.* Although factor VIII is not itself a protease, it markedly stimulates the activation of factor X, the final protease of the intrinsic pathway, by factor IX_a , a serine protease (Figure 10.44). Thus, activation of the intrinsic pathway is severely impaired in hemophilia.

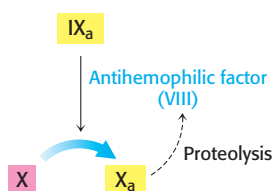


FIGURE 10.44 Action of antihemophilic factor. Antihemophilic factor (VIII) stimulates the activation of factor X by factor IX_a . It is interesting to note that the activity of factor VIII is markedly increased by limited proteolysis by thrombin and factor X_a . This positive feedback amplifies the clotting signal and accelerates clot formation after a threshold has been reached.


In the past, hemophiliacs were treated with transfusions of a concentrated plasma fraction containing factor VIII. This therapy carried the risk of infection. Indeed, many hemophiliacs contracted hepatitis and AIDS. A safer preparation of factor VIII was urgently needed. With the use of biochemical purification and recombinant DNA techniques, the gene for factor VIII was isolated and expressed in cells grown in culture. Recombinant factor VIII purified from these cells has largely replaced plasma concentrates in treating hemophilia.

An account of a hemorrhagic disposition existing in certain families—

“About seventy or eighty years ago, a woman by the name of Smith settled in the vicinity of Plymouth, New Hampshire, and transmitted the following idiosyncrasy to her descendants. It is one, she observed, to which her family is unfortunately subject and has been the source not only of great solicitude, but frequently the cause of death. If the least scratch is made on the skin of some of them, as mortal a hemorrhage will eventually ensue as if the largest wound is inflicted.... It is a surprising circumstance that the males only are subject to this strange affection, and that all of them are not liable to it.... Although the females are exempt, they are still capable of transmitting it to their male children.”

JOHN OTTO (1803)

10.5.9 The Clotting Process Must Be Precisely Regulated

 There is a fine line between hemorrhage and thrombosis. Clots must form rapidly yet remain confined to the area of injury. What are the mechanisms that normally limit clot formation to the site of injury? The lability of clotting factors contributes significantly to the control of clotting. Activated factors are short-lived because they are diluted by blood flow, removed by the liver, and degraded by proteases. For example, the stimulatory proteins factors V_a and $VIII_a$ are digested by protein C, a protease that is switched on by the action of thrombin. Thus, thrombin has a dual function: it catalyzes the formation of fibrin and it initiates the deactivation of the clotting cascade.

Specific inhibitors of clotting factors are also critical in the termination of clotting. The most important one is *antithrombin III*, a plasma protein that inactivates thrombin by forming an irreversible complex with it. Antithrombin III resembles α_1 -antitrypsin except that it inhibits thrombin much more strongly than it inhibits elastase. Antithrombin III also blocks other serine proteases in the clotting cascade—namely, factors XII_a , XI_a , IX_a , and X_a . The inhibitory action of antithrombin III is enhanced by *heparin*, a negatively charged polysaccharide found in mast cells near the walls of blood vessels and on the surfaces of endothelial cells (Figure 10.45). Heparin acts as an *anticoagulant* by increasing the rate of formation of irreversible complexes between antithrombin III and the serine protease clotting factors. Antitrypsin and antithrombin are *serpins*, a family of *serine protease inhibitors*.

The importance of the ratio of thrombin to antithrombin is illustrated in the case in which a 14-year-old boy died of a bleeding disorder because of a mutation in his α_1 -antitrypsin, which normally inhibits elastase (Section 10.5.4). Methionine 358 in α_1 -antitrypsin's binding pocket for elastase was replaced by arginine, resulting in a change in specificity from an elastase inhibitor to a thrombin inhibitor. α_1 -Antitrypsin activity normally increases markedly after injury to counteract excess elastase arising from stimulated neutrophils. The mutant α_1 -antitrypsin caused the patient's thrombin activity to drop to such a low level that hemorrhage ensued. We see here a striking example of how a change of a single residue in a protein can dramatically alter specificity and an example of the critical importance of having the right amount of a protease inhibitor.

Antithrombin limits the extent of clot formation, but what happens to the clots themselves? Clots are not permanent structures but are designed to be dissolved when the structural integrity of damaged areas is restored. Fibrin is split by *plasmin*, a serine protease that hydrolyzes peptide bonds in the coiled-coil regions. Plasmin molecules can diffuse through aqueous channels in the porous fibrin clot to cut the accessible connector rods. Plasmin is formed by proteolytic activation of *plasminogen*, an inactive precursor that has a high affinity for the fibrin clots. This conversion is carried out by *tissue-type plasminogen activator* (TPA), a 72-kd protein that has a domain structure closely related to that of prothrombin (Figure 10.46).

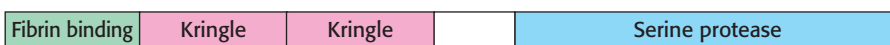


FIGURE 10.46 Modular structure of tissue-type plasminogen activator (TPA).

However, a domain that targets TPA to fibrin clots replaces the membrane-targeting gla domain of prothrombin. The TPA bound to fibrin clots swiftly activates adhering plasminogen. In contrast, TPA activates free plasminogen very slowly. The gene for TPA has been cloned and expressed in cultured mammalian cells. The results of clinical studies have shown that TPA administered intravenously within an hour of the formation of a blood clot in a coronary artery markedly increases the likelihood of surviving a heart attack (Figure 10.47).

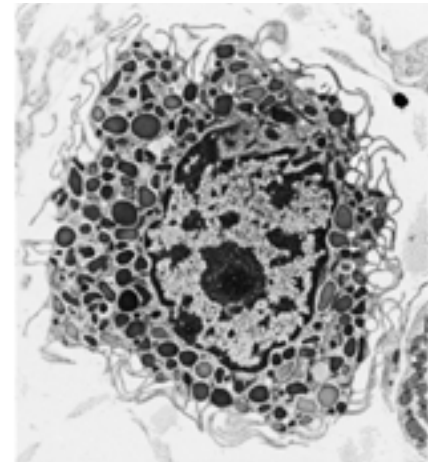


FIGURE 10.45 Electron micrograph of a mast cell. Heparin and other molecules in the dense granules are released into the extracellular space when the cell is triggered to secrete. [Courtesy of Lynne Mercer.]

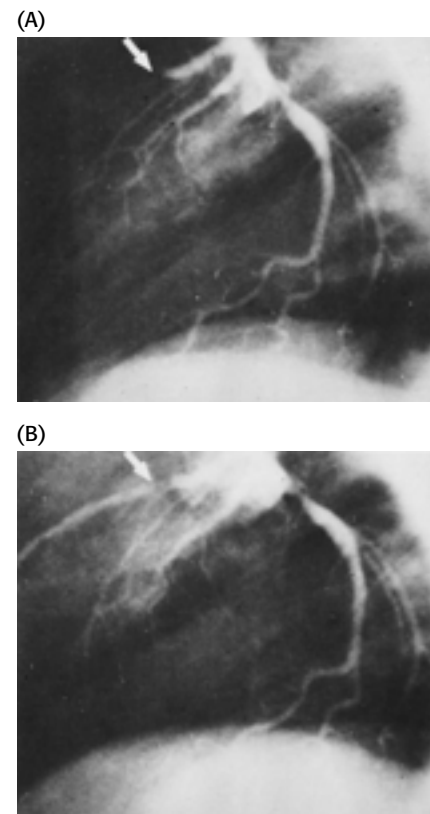


FIGURE 10.47 The effect of tissue-type plasminogen factor. TPA leads to the dissolution of blood clots, as shown by x-ray images of blood vessels in the heart (A) before and (B) 3 hours after the administration of TPA. The position of the clot is marked by the arrow in part A. [After F. Van de Werf, P. A. Ludbrook, S. R. Bergmann, A. J. Tiefenbrunn, K. A. A. Fox, H. de Geest, M. Verstraete, D. Collen, and B. E. Sobel. *New Engl. J. Med.* 310(1984):609.]

SUMMARY

- **Aspartate Transcarbamoylase Is Allosterically Inhibited by the End Product of Its Pathway**

Allosteric proteins constitute an important class of proteins whose biological activity can be regulated. Specific regulatory molecules can modulate the activity of allosteric proteins by binding to distinct regulatory sites, separate from the functional site. These proteins have multiple functional sites, which display cooperation as evidenced by a sigmoidal dependence of function on substrate concentration. Aspartate transcarbamoylase (ATCase), one of the best-understood allosteric enzymes, catalyzes the synthesis of *N*-carbamoylaspartate, the first intermediate in the synthesis of pyrimidines. ATCase is feedback inhibited by cytidine triphosphate (CTP), the final product of the pathway. ATP reverses this inhibition. ATCase consists of separable catalytic (c_3) subunits (which bind the substrates) and regulatory (r_2) subunits (which bind CTP and ATP). The inhibitory effect of CTP, the stimulatory action of ATP, and the cooperative binding of substrates are mediated by large changes in quaternary structure. On binding substrates, the c_3 subunits of the c_6r_6 enzyme move apart and reorient themselves. This allosteric transition is highly concerted, as postulated by the Monod-Wyman-Changeux (MWC) model. All subunits of an ATCase molecule simultaneously interconvert from the T (low-affinity) to the R (high-affinity) state.

- **Hemoglobin Transports Oxygen Efficiently by Binding Oxygen Cooperatively**

Hemoglobin, the oxygen carrier in the blood, is an allosteric protein. Hemoglobin consists of four polypeptide chains, each with a heme group—a substituted porphyrin with a central iron. Hemoglobin A, the predominant hemoglobin in adults, has the subunit structure $\alpha_2\beta_2$. Hemoglobin transports H^+ and CO_2 in addition to O_2 . Hemoglobin exhibits three kinds of allosteric effects. First, the oxygen-binding curve of hemoglobin is sigmoidal, which indicates that the binding of oxygen is cooperative. The binding of oxygen to one heme group facilitates the binding of oxygen to the other heme groups in the same molecule. Second, the binding of H^+ and CO_2 promotes the release of O_2 from hemoglobin, an effect that is physiologically important in enhancing the release of O_2 in metabolically active tissues such as muscle. These allosteric linkages between the binding of H^+ , CO_2 , and O_2 are known as the Bohr effect. Third, the affinity of hemoglobin for O_2 is further regulated by 2,3-bisphosphoglycerate (2,3-BPG), a small molecule with a very high density of negative charge. 2,3-Bisphosphoglycerate binds tightly to deoxyhemoglobin but not to oxyhemoglobin. Hence, 2,3-BPG lowers the oxygen affinity of hemoglobin. Fetal hemoglobin ($\alpha_2\gamma_2$) has a higher oxygen affinity than human adult hemoglobin because fetal hemoglobin binds 2,3-BPG less tightly. Neither the sequential nor the concerted model completely describes the allosteric behavior of hemoglobin. Rather, the behavior of hemoglobin is best described by a combined model that employs features of both models.

- **Isozymes Provide a Means of Regulation Specific to Distinct Tissues and Developmental Stages**

Isozymes differ in structural characteristics but catalyze the same reaction. They provide a means of fine-tuning metabolism to meet the needs of a given tissue or developmental stage. The results of gene-duplication events provide the means for subtle regulation of enzyme function.

● Covalent Modification Is a Means of Regulating Enzyme Activity

Covalent modification of proteins is a potent means of controlling the activity of enzymes and other proteins. Phosphorylation is the most common type of reversible covalent modification. Signals can be highly amplified by phosphorylation because a single kinase can act on many target molecules. The regulatory actions of protein kinases are reversed by protein phosphatases, which catalyze the hydrolysis of attached phosphoryl groups.

Cyclic AMP serves as an intracellular messenger in the transduction of many hormonal and sensory stimuli. Cyclic AMP switches on protein kinase A (PKA), a major multifunctional kinase, by binding to the regulatory subunit of the enzyme, thereby releasing the active catalytic subunits of PKA. In the absence of cAMP, the catalytic sites of PKA are occupied by pseudosubstrate sequences of the regulatory subunit.

● Many Enzymes Are Activated by Specific Proteolytic Cleavage

The activation of an enzyme by proteolytic cleavage of one or a few peptide bonds is a recurring control mechanism seen in processes as diverse as the activation of digestive enzymes and blood clotting. The inactive precursor is called a zymogen (or a proenzyme). Trypsinogen is activated by enteropeptidase or trypsin, and trypsin then activates a host of other zymogens, leading to the digestion of foodstuffs. For instance, trypsin converts chymotrypsinogen, a zymogen, into active chymotrypsin by hydrolyzing a single peptide bond.

A striking feature of the clotting process is that it is accomplished by a cascade of zymogen conversions, in which the activated form of one clotting factor catalyzes the activation of the next precursor. Many of the activated clotting factors are serine proteases. In the final step of clot formation, fibrinogen, a highly soluble molecule in the plasma, is converted by thrombin into fibrin by the hydrolysis of four arginine–glycine bonds. The resulting fibrin monomer spontaneously forms long, insoluble fibers called fibrin. Zymogen activation is also essential in the lysis of clots. Plasminogen is converted into plasmin, a serine protease that cleaves fibrin, by tissue-type plasminogen activator (TPA). Although zymogen activation is irreversible, specific inhibitors of some proteases exert control. The irreversible protein inhibitor antithrombin III holds blood clotting in check in the clotting cascade.

KEY TERMS

feedback (end-product) inhibition (p. 263)
 regulatory site (p. 263)
 concerted mechanism (p. 266)
 cooperativity (p. 267)
 homotropic effect (p. 268)
 heterotropic effect (p. 268)
 sequential model (p. 269)

heme (p. 270)
 protoporphyrin (p. 270)
 Bohr effect (p. 273)
 isozyme (isoenzyme) (p. 274)
 covalent modification (p. 275)
 protein kinase (p. 276)
 protein phosphatase (p. 277)

consensus sequence (p. 278)
 protein kinase A (PKA) (p. 278)
 pseudosubstrate sequence (p. 279)
 zymogen (proenzyme) (p. 280)
 enzymatic cascade (p. 284)
 intrinsic clotting pathway (p. 285)
 extrinsic clotting pathway (p. 285)

SELECTED READINGS

Where to start

Kantrowitz, E. R., and Lipscomb, W. N., 1990. *Escherichia coli* aspartate transcarbamoylase: The molecular basis for a concerted allosteric transition. *Trends Biochem. Sci.* 15:53–59.

Schachman, H. K., 1988. Can a simple model account for the allosteric transition of aspartate transcarbamoylase? *J. Biol. Chem.* 263: 18583–18586.
 Dickerson, R. E., and Geis, I., 1983. *Hemoglobin: Structure, Function, Evolution and Pathology*. Benjamin Cummings.

- Neurath, H., 1989. Proteolytic processing and physiological regulation. *Trends Biochem. Sci.* 14:268–271.
- Bode, W., and Huber, R., 1992. Natural protein proteinase inhibitors and their interaction with proteinases. *Eur. J. Biochem.* 204: 433–451.
- Aspartate transcarbamoylase and allosteric interactions**
- Endrizzi, J. A., Beernink, P. T., Alber, T., and Schachman, H. K., 2000. Binding of bisubstrate analog promotes large structural changes in the unregulated catalytic trimer of aspartate transcarbamoylase: Implications for allosteric regulation. *Proc. Natl. Acad. Sci. U. S. A.* 97:5077–5082.
- Beernink, P. T., Endrizzi, J. A., Alber, T., and Schachman, H. K., 1999. Assessment of the allosteric mechanism of aspartate transcarbamoylase based on the crystalline structure of the unregulated catalytic subunit. *Proc. Natl. Acad. Sci. U. S. A.* 96:5388–5393.
- Wales, M. E., Madison, L. L., Glaser, S. S., and Wild, J. R., 1999. Divergent allosteric patterns verify the regulatory paradigm for aspartate transcarbamoylase. *J. Mol. Biol.* 294:1387–1400.
- LiCata, V. J., Burz, D. S., Moerke, N. J., and Allewell, N. M., 1998. The magnitude of the allosteric conformational transition of aspartate transcarbamoylase is altered by mutations. *Biochemistry* 37: 17381–17385.
- Eisenstein, E., Markby, D. W., and Schachman, H. K., 1990. Heterotropic effectors promote a global conformational change in aspartate transcarbamoylase. *Biochemistry* 29:3724–3731.
- Werner, W. E., and Schachman, H. K., 1989. Analysis of the ligand-promoted global conformational change in aspartate transcarbamoylase: Evidence for a two-state transition from boundary spreading in sedimentation velocity experiments. *J. Mol. Biol.* 206:221–230.
- Newell, J. O., Markby, D. W., and Schachman, H. K., 1989. Cooperative binding of the bisubstrate analog *N*-(phosphonacetyl)-*L*-aspartate to aspartate transcarbamoylase and the heterotropic effects of ATP and CTP. *J. Biol. Chem.* 264:2476–2481.
- Stevens, R. C., Reinisch, K. M., and Lipscomb, W. N., 1991. Molecular structure of *Bacillus subtilis* aspartate transcarbamoylase at 3.0 Å resolution. *Proc. Natl. Acad. Sci. U. S. A.* 88:6087–6091.
- Stevens, R. C., Gouaux, J. E., and Lipscomb, W. N., 1990. Structural consequences of effector binding to the T state of aspartate carbamoyltransferase: Crystal structures of the unligated and ATP- and CTP-complexed enzymes at 2.6-Å resolution. *Biochemistry* 29:7691–7701.
- Gouaux, J. E., and Lipscomb, W. N., 1990. Crystal structures of phosphonoacetamide ligated T and phosphonoacetamide and malonate ligated R states of aspartate carbamoyltransferase at 2.8-Å resolution and neutral pH. *Biochemistry* 29:389–402.
- Labadan, B., Boyen, A., Baetens, M., Charlier, D., Chen, P., Cunin, R., Durbeco, V., Glansdorff, N., Herve, G., Legrain, C., Liang, Z., Purcarea, C., Roovers, M., Sanchez, R., Toong, T. L., Van de Castele, M., van Vliet, F., Xu, Y., and Zhang, Y. F., 1999. The evolutionary history of carbamoyltransferases: A complex set of paralogous genes was already present in the last universal common ancestor. *J. Mol. Evol.* 49:461–473.
- Hemoglobin**
- Perutz, M. F., Wilkinson, A. J., Paoli, M., and Dodson, G. G., 1998. The stereochemical mechanism of the cooperative effects in hemoglobin revisited. *Annu. Rev. Biophys. Biomol. Struct.* 27:1–34.
- Ackers, G. K., 1998. Deciphering the molecular code of hemoglobin allostery. *Adv. Protein Chem.* 51:185–253.
- Ackers, G. K., Doyle, M. L., Myers, D., and Daugherty, M. A., 1992. Molecular code for cooperativity in hemoglobin. *Science* 255:54–63.
- Fermi, G., Perutz, M. F., Shaanan, B., and Fourme, R., 1984. The crystal structure of human deoxyhaemoglobin at 1.74 Å resolution. *J. Mol. Biol.* 175:159–174.
- Kilmartin, J. V. and Rossi-Bernardi, L., 1973. Interaction of hemoglobin with hydrogen ions, carbon dioxide, and organic phosphates. *Physiol. Rev.* 53:836–890.
- Covalent modification**
- Johnson, L. N., and Barford, D., 1993. The effects of phosphorylation on the structure and function of proteins. *Annu. Rev. Biophys. Biomol. Struct.* 22:199–232.
- Ziegler, M., 2000. New functions of a long-known molecule: Emerging roles of NAD in cellular signaling. *Eur. J. Biochem.* 267:1550–1564.
- Ng, H. H. and Bird, A., 2000. Histone deacetylases: Silencers for hire. *Trends Biochem. Sci.* 25:121–126.
- Raju, R. V., Kakkar, R., Radhi, J. M., and Sharma, R. K., 1997. Biological significance of phosphorylation and myristoylation in the regulation of cardiac muscle proteins. *Mol. Cell. Biochem.* 176:135–143.
- Jacobson, M. K., and Jacobson, E. L., 1999. Discovering new ADP-ribose polymer cycles: Protecting the genome and more. *Trends Biochem. Sci.* 24:415–417.
- Barford, D., Das, A. K., and Egloff, M. P., 1998. The structure and mechanism of protein phosphatases: Insights into catalysis and regulation. *Annu. Rev. Biophys. Biomol. Struct.* 27:133–164.
- Protein kinase A**
- Taylor, S. S., Knighton, D. R., Zheng, J., Sowadski, J. M., Gibbs, C. S., and Zoller, M. J., 1993. A template for the protein kinase family. *Trends Biochem. Sci.* 18:84–89.
- Gibbs, C. S., Knighton, D. R., Sowadski, J. M., Taylor, S. S., and Zoller, M. J., 1992. Systematic mutational analysis of cAMP-dependent protein kinase identifies unregulated catalytic subunits and defines regions important for the recognition of the regulatory subunit. *J. Biol. Chem.* 267:4806–4814.
- Knighton, D. R., Zheng, J. H., TenEyck, L., Ashford, V. A., Xuong, N. H., Taylor, S. S., and Sowadski, J. M., 1991. Crystal structure of the catalytic subunit of cyclic adenosine monophosphate-dependent protein kinase. *Science* 253:407–414.
- Knighton, D. R., Zheng, J. H., TenEyck, L., Xuong, N. H., Taylor, S. S., and Sowadski, J. M., 1991. Structure of a peptide inhibitor bound to the catalytic subunit of cyclic adenosine monophosphate-dependent protein kinase. *Science* 253:414–420.
- Adams, S. R., Harootunian, A. T., Buechler, Y. J., Taylor, S. S., and Tsien, R. Y., 1991. Fluorescence ratio imaging of cyclic AMP in single cells. *Nature* 349:694–697.
- Zymogen activation**
- Neurath, H., 1986. The versatility of proteolytic enzymes. *J. Cell. Biochem.* 32:35–49.
- Bode, W., and Huber, R., 1986. Crystal structure of pancreatic serine endopeptidases. In *Molecular and Cellular Basis of Digestion* (pp. 213–234), edited by P. Desnuelle, H. Sjöstrom, and O. Noren. Elsevier.
- Huber, R., and Bode, W., 1978. Structural basis of the activation and action of trypsin. *Acc. Chem. Res.* 11:114–122.
- Stroud, R. M., Kossiakoff, A. A., and Chambers, J. L., 1977. Mechanism of zymogen activation. *Annu. Rev. Biophys. Bioeng.* 6:177–193.
- Sielecki, A. R., Fujinaga, M., Read, R. J., and James, M. N., 1991. Refined structure of porcine pepsinogen at 1.8 Å resolution. *J. Mol. Biol.* 219:671–692.
- Protease inhibitors**
- Carrell, R., and Travis, J., 1985. α_1 -Antitrypsin and the serpins: Variation and countervariation. *Trends Biochem. Sci.* 10:20–24.
- Carp, H., Miller, F., Hoidal, J. R., and Janoff, A., 1982. Potential mechanism of emphysema: α_1 -Proteinase inhibitor recovered from lungs of cigarette smokers contains oxidized methionine and has decreased elastase inhibitory capacity. *Proc. Natl. Acad. Sci. U. S. A.* 79:2041–2045.
- Owen, M. C., Brennan, S. O., Lewis, J. H., and Carrell, R. W., 1983. Mutation of antitrypsin to antithrombin. *New Engl. J. Med.* 309:694–698.
- Travis, J., and Salvesen, G. S., 1983. Human plasma proteinase inhibitors. *Annu. Rev. Biochem.* 52:655–709.
- Clotting cascade**
- Fuentes-Prior, P., Iwanaga, Y., Huber, R., Pagila, R., Rumennik, G., Seto, M., Morser, J., Light, D. R., and Bode, W., 2000. Structural basis for the anticoagulant activity of the thrombin-thrombomodulin complex. *Nature*. 404:518–525.
- Herzog, R. W., and High, K. A., 1998. Problems and prospects in gene therapy for hemophilia. *Curr. Opin. Hematol.* 5:321–326.

Doolittle, R. F., 1981. Fibrinogen and fibrin. *Sci. Am.* 245(12):126–135.
 Lawn, R. M., and Vehar, G. A., 1986. The molecular genetics of hemophilia. *Sci. Am.* 254(3):48–65.
 Brown, J. H., Volkmann, N., Jun, G., Henschen-Edman, A. H. and Cohen, C., 2000. The crystal structure of modified bovine fibrinogen. *Proc. Natl. Acad. Sci. U. S. A.* 97:85–90.

Stubbs, M. T., Oschkinat, H., Mayr, I., Huber, R., Anglikler, H., Stone, S. R., and Bode, W., 1992. The interaction of thrombin with fibrinogen: A structural basis for its specificity. *Eur. J. Biochem.* 206:187–195.
 Rydel, T. J., Tulinsky, A., Bode, W., and Huber, R., 1991. Refined structure of the hirudin-thrombin complex. *J. Mol. Biol.* 221: 583–601.

PROBLEMS

1. *Activity profile.* A histidine residue in the active site of aspartate transcarbamoylase is thought to be important in stabilizing the transition state of the bound substrates. Predict the pH dependence of the catalytic rate, assuming that this interaction is essential and dominates the pH-activity profile of the enzyme.

2. *Allosteric switching.* A substrate binds 100 times as tightly to the R state of an allosteric enzyme as to its T state. Assume that the concerted (MWC) model applies to this enzyme.

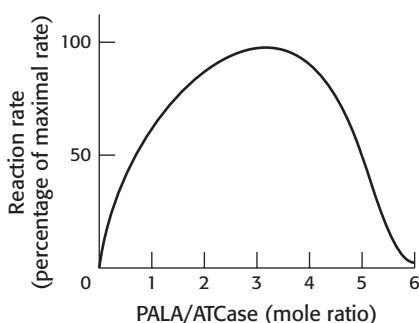
(a) By what factor does the binding of one substrate molecule per enzyme molecule alter the ratio of the concentrations of enzyme molecules in the R and T states?

(b) Suppose that L , the ratio of [T] to [R] in the absence of substrate, is 10^7 and that the enzyme contains four binding sites for substrate. What is the ratio of enzyme molecules in the R state to that in the T state in the presence of saturating amounts of substrate, assuming that the concerted model is obeyed?

3. *Allosteric transition.* Consider an allosteric protein that obeys the concerted model. Suppose that the ratio of T to R formed in the absence of ligand is 10^5 , $K_T = 2$ mM, and $K_R = 5$ μ M. The protein contains four binding sites for ligand. What is the fraction of molecules in the R form when 0, 1, 2, 3, and 4 ligands are bound?

4. *Negative cooperativity.* You have isolated a dimeric enzyme that contains two identical active sites. The binding of substrate to one active site decreases the substrate affinity of the other active site. Which allosteric model best accounts for this negative cooperativity?

5. *Paradoxical at first glance.* Recall that phosphonacetyl-L-aspartate (PALA) is a potent inhibitor of ATCase because it mimics the two physiological substrates. However, low concentrations of this unreactive bisubstrate analog *increase* the reaction velocity. On the addition of PALA, the reaction rate increases until an average of three molecules of PALA are bound per molecule of enzyme. This maximal velocity is 17-fold as great as it is in the absence of PALA. The reaction rate then decreases to nearly zero on the addition of three more molecules of PALA per molecule of enzyme. Why do low concentrations of PALA activate ATCase?



Effect of PALA on ATCase rate.

6. *R versus T.* An allosteric enzyme that follows the concerted mechanism has a T/R ratio of 300 in the absence of substrate. Suppose that a mutation reversed the ratio. How would this mutation affect the relation between the rate of the reaction and substrate concentration?

7. *Tuning oxygen affinity.* What is the effect of each of the following treatments on the oxygen affinity of hemoglobin A in vitro?

(a) Increase in pH from 7.2 to 7.4.

(b) Increase in $p\text{CO}_2$ from 10 to 40 torr.

(c) Increase in [2,3-BPG] from 2×10^{-4} to 8×10^{-4} M.

(d) Dissociation of $\alpha_2\beta_2$ into monomeric subunits.

8. *Avian and reptilian counterparts.* The erythrocytes of birds and turtles contain a regulatory molecule different from 2,3-BPG. This substance is also effective in reducing the oxygen affinity of human hemoglobin stripped of 2,3-BPG. Which of the following substances would you predict to be most effective in this regard?

(a) Glucose 6-phosphate

(d) Malonate

(b) Inositol hexaphosphate

(e) Arginine

(c) HPO_4^{2-}

(f) Lactate

9. *Tuning proton affinity.* The pK of an acid depends partly on its environment. Predict the effect of the following environmental changes on the pK of a glutamic acid side chain.

(a) A lysine side chain is brought into close proximity.

(b) The terminal carboxyl group of the protein is brought into close proximity.

(c) The glutamic acid side chain is shifted from the outside of the protein to a nonpolar site inside.

10. *Zymogen activation.* When very low concentrations of pepsinogen are added to acidic media, how does the half-time for activation depend on zymogen concentration?

11. *A revealing assay.* Suppose that you have just examined a young boy with a bleeding disorder highly suggestive of classic hemophilia (factor VIII deficiency). Because of the late hour, the laboratory that carries out specialized coagulation assays is closed. However, you happen to have a sample of blood from a classic hemophiliac whom you admitted to the hospital an hour earlier. What is the simplest and most rapid test that you can perform to determine whether your present patient also is deficient in factor VIII activity?

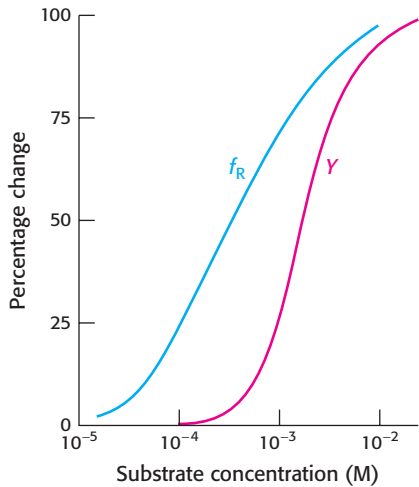
12. *Counterpoint.* The synthesis of factor X, like that of prothrombin, requires vitamin K. Factor X also contains γ -carboxyglutamate residues in its amino-terminal region. However, activated factor X, in contrast with thrombin, retains this region of the molecule. What is a likely functional consequence of this difference between the two activated species?

13. *A discerning inhibitor.* Antithrombin III forms an irreversible complex with thrombin but not with prothrombin. What is the most likely reason for this difference in reactivity?

14. *Repeating heptads.* Each of the three types of fibrin chains contains repeating heptapeptide units (*abcdefg*) in which residues *a* and *d* are hydrophobic. Propose a reason for this regularity.
15. *Drug design.* A drug company has decided to use recombinant DNA methods to prepare a modified α_1 -antitrypsin that will be more resistant to oxidation than is the naturally occurring inhibitor. Which single amino acid substitution would you recommend?

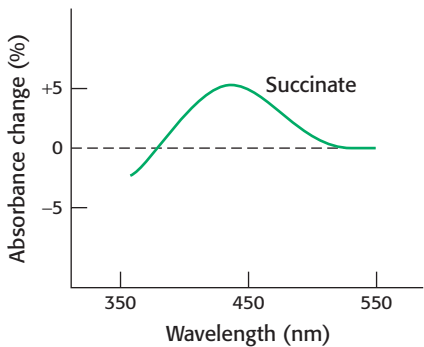
Data Interpretation Problems

16. *Distinguishing between models.* The graph below shows the fraction of an allosteric enzyme in the R state (f_R) and the fraction of active sites bound to substrate (Y) as a function of substrate concentration. Which model, the concerted or sequential, best explains these results?



[From M. W. Kirschner and H. K. Schachman, *Biochemistry* 12 (1966):2997.]

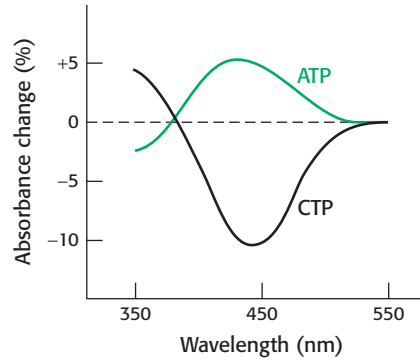
17. *Reporting live from ATCase.* ATCase was reacted with nitromethane to form a colored nitrotyrosine group ($\lambda_{\max} = 430 \text{ nm}$) in each of its catalytic chains. The absorption by this reporter group depends on its immediate environment. An essential lysine residue at each catalytic site also was modified to block the binding of substrate. Catalytic trimers from this doubly modified enzyme were then combined with native trimers to form a hybrid enzyme. The absorption by the nitrotyrosine group was measured on addition of the substrate analog succinate. What is the significance of the alteration in the absorbance at 430 nm?



[After H. K. Schachman, *J. Biol. Chem.* 263 (1988):18583]

The binding of succinate, an unreactive analog of aspartate, to one trimer changed the visible absorption spectrum of nitrotyrosine residues attached to the other trimer.

18. *Reporting live from ATCase 2.* A different ATCase hybrid was constructed to test the effects of allosteric activators and inhibitors. Normal regulatory subunits were combined with nitrotyrosine-containing catalytic subunits. The addition of ATP in the absence of substrate increased the absorbance at 430 nm, the same change elicited by the addition of succinate (see the graph in Problem 17). Conversely, CTP in the absence of substrate decreased the absorbance at 430 nm. What is the significance of the changes in absorption of the reporter groups?



[After H. K. Schachman, *J. Biol. Chem.* 263(1988):18583.]

Chapter Integration Problem

19. *Sticky patches.* The substitution of valine for glutamate at position 6 of the β chains of hemoglobin places a nonpolar residue on the outside of hemoglobin S, the version of hemoglobin responsible for sickle-cell anemia. The oxygen affinity and allosteric properties of hemoglobin are virtually unaffected by this change. However, the valine side chain of hemoglobin S interacts with a complementary sticky patch (formed by phenylalanine $\beta 85$ and leucine $\beta 88$) on another hemoglobin molecule—a patch that is exposed in deoxygenated but not in oxygenated hemoglobin. What is the chemical basis for the interaction between the hemoglobin molecules? What would be the effect of this interaction?

Mechanism Problems

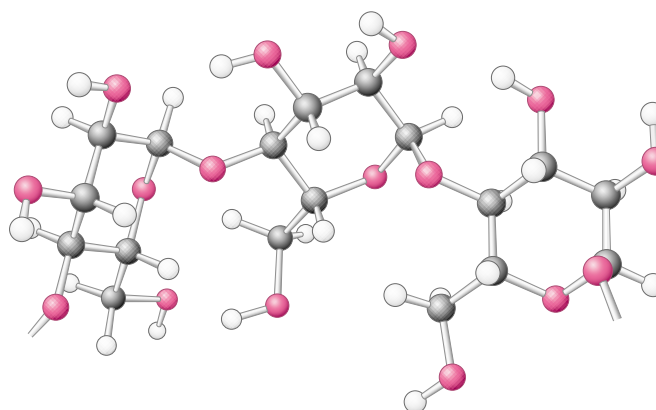
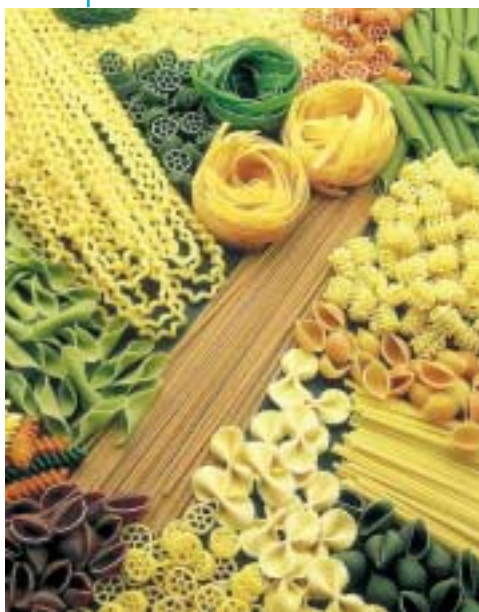
20. *Aspartate transcarbamoylase.* Write the mechanism (in detail) for the conversion of aspartate and carbamoyl phosphate into *N*-carbamoylaspartate. Include a role for the residue histidine present in the active site.
21. *Protein kinases.* Write a mechanism (in detail) for the phosphorylation of a serine residue by ATP catalyzed by a protein kinase. What groups might you expect to find in the enzyme active site?



Media Problem

22. *Cooperative, more or less.* (a) Suppose you purify the same enzyme from two different species and find that one is a tetramer ($n = 4$) and the other a hexamer ($n = 6$). All other things being equal, which would you expect would show the greater cooperativity? (b) To your surprise, both enzymes show similar cooperativity. Explain how differences in the MWC parameter c could explain this. (*Hint:* Make sure you do the exercise in Section 4 of the **Conceptual Insights** module on cooperative binding and kinetics.)

Carbohydrates



Carbohydrates in food are important sources of energy. Starch, found in plant-derived food such as pasta, consists of chains of linked glucose molecules. These chains are broken down into individual glucose molecules for eventual use in generation of ATP and building blocks for other molecules. [(Left) Superstock.]

Let us take an overview of carbohydrates, one of the four major classes of biomolecules along with proteins, nucleic acids, and lipids. Carbohydrates are aldehyde or ketone compounds with multiple hydroxyl groups. They make up most of the organic matter on Earth because of their extensive roles in all forms of life. First, carbohydrates serve as *energy stores, fuels, and metabolic intermediates*. Second, ribose and deoxyribose sugars form part of the *structural framework of RNA and DNA*. Third, polysaccharides are *structural elements in the cell walls of bacteria and plants*. In fact, cellulose, the main constituent of plant cell walls, is one of the most abundant organic compounds in the biosphere. Fourth, carbohydrates are *linked to many proteins and lipids*, where they play key roles in mediating interactions among cells and interactions between cells and other elements in the cellular environment.

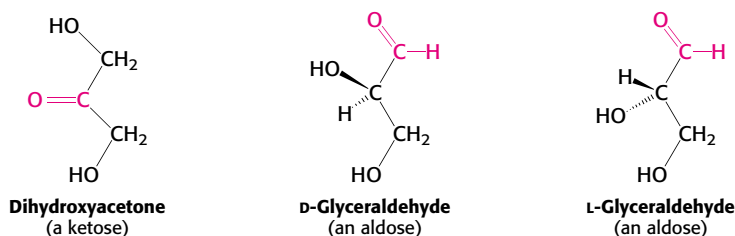
A key related property of carbohydrates in their role as mediators of cellular interactions is the tremendous *structural diversity* possible within this class of molecules. Carbohydrates are built from monosaccharides, small molecules that typically contain from three to nine carbon atoms and vary in size and in the stereochemical configuration at one or more carbon centers. These monosaccharides may be linked together to form a large variety of oligosaccharide structures. The unraveling of these oligosaccharide structures, the discovery of their placement at specific sites within proteins, and the determination of their function are tremendous challenges in the field of proteomics.

OUTLINE

- **11.1 Monosaccharides Are Aldehydes or Ketones with Multiple Hydroxyl Groups**
- **11.2 Complex Carbohydrates Are Formed by Linkage of Monosaccharides**
- **11.3 Carbohydrates Can be Attached to Proteins to Form Glycoproteins**
- **11.4 Lectins Are Specific Carbohydrate-Binding Proteins**

11.1 MONOSACCHARIDES ARE ALDEHYDES OR KETONES WITH MULTIPLE HYDROXYL GROUPS

Monosaccharides, the simplest carbohydrates, are aldehydes or ketones that have two or more hydroxyl groups; the empirical formula of many is $(C-H_2O)_n$, literally a “carbon hydrate.” Monosaccharides are important fuel molecules as well as building blocks for nucleic acids. The smallest monosaccharides, for which $n = 3$, are dihydroxyacetone and D- and L-glyceraldehyde.



They are referred to as *trioses* (tri- for 3). Dihydroxyacetone is called a *ketose* because it contains a keto group, whereas glyceraldehyde is called an *aldose* because it contains an aldehyde group. Glyceraldehyde has a single asymmetric carbon and, thus, there are two stereoisomers of this sugar. D-Glyceraldehyde and L-glyceraldehyde are *enantiomers*, or mirror images of each other. As mentioned in Chapter 3, the prefixes D and L designate the absolute configuration. Monosaccharides and other sugars will often be represented in this book by *Fischer projections* (Figure 11.1). Recall that, in a Fischer projection of a molecule, atoms joined to an asymmetric carbon atom by horizontal bonds are in front of the plane of the page, and those joined by vertical bonds are behind (see the Appendix in Chapter 1). Fischer projections are useful for depicting carbohydrate structures because they provide clear and simple views of the stereochemistry at each carbon center.

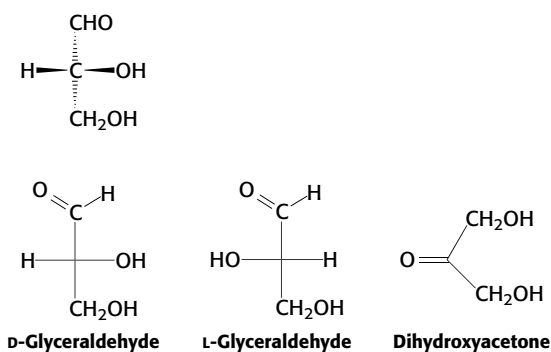


FIGURE 11.1 Fischer projections of trioses. The top structure reveals the stereochemical relations assumed for Fischer projections.

Simple monosaccharides with four, five, six, and seven carbon atoms are called *tetroses*, *pentoses*, *hexoses*, and *heptoses*, respectively. Because these molecules have multiple asymmetric carbons, they exist as *diastereoisomers*, isomers that are not mirror images of each other, as well as enantiomers. In regard to these monosaccharides, the symbols D and L designate the ab-

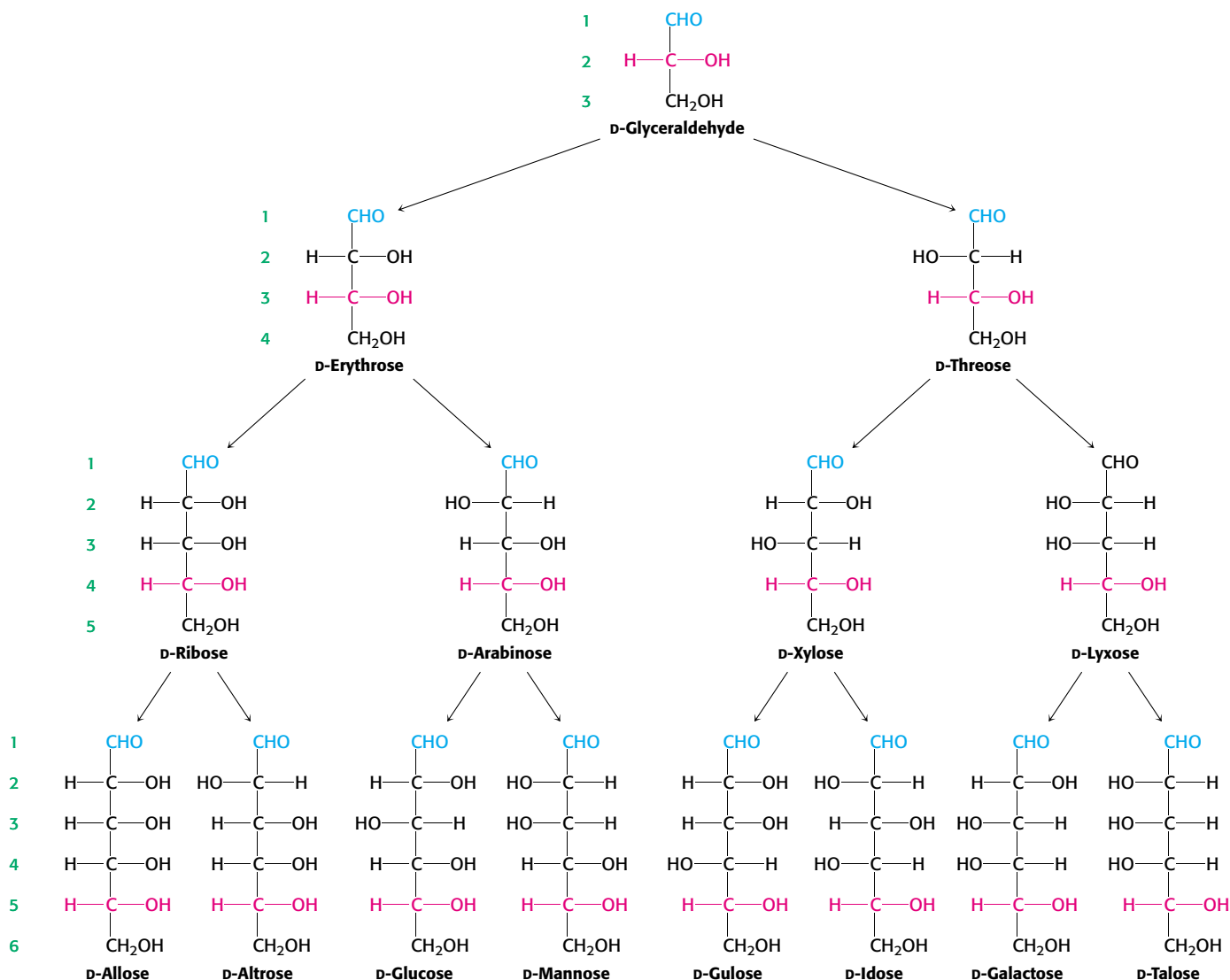


FIGURE 11.2 D-Aldoses containing three, four, five, and six carbon atoms. D-Aldoses contain an aldehyde group (shown in blue) and have the absolute configuration of D-glyceraldehyde at the asymmetric center (shown in red) farthest from the aldehyde group. The numbers indicate the standard designations for each carbon atom.

solite configuration of the asymmetric carbon farthest from the aldehyde or keto group. Figure 11.2 shows the common D-aldose sugars. D-Ribose, the carbohydrate component of RNA, is a five-carbon aldose. D-Glucose, D-mannose, and D-galactose are abundant six-carbon aldoses. Note that D-glucose and D-mannose differ in configuration only at C-2. Sugars differing in configuration at a single asymmetric center are called *epimers*. Thus, D-glucose and D-mannose are epimeric at C-2; D-glucose and D-galactose are epimeric at C-4.

Dihydroxyacetone is the simplest ketose. The stereochemical relation between D-ketoses containing as many as six carbon atoms are shown in Figure 11.3. Note that ketoses have one fewer asymmetric center than do aldoses with the same number of carbons. D-Fructose is the most abundant ketohexose.

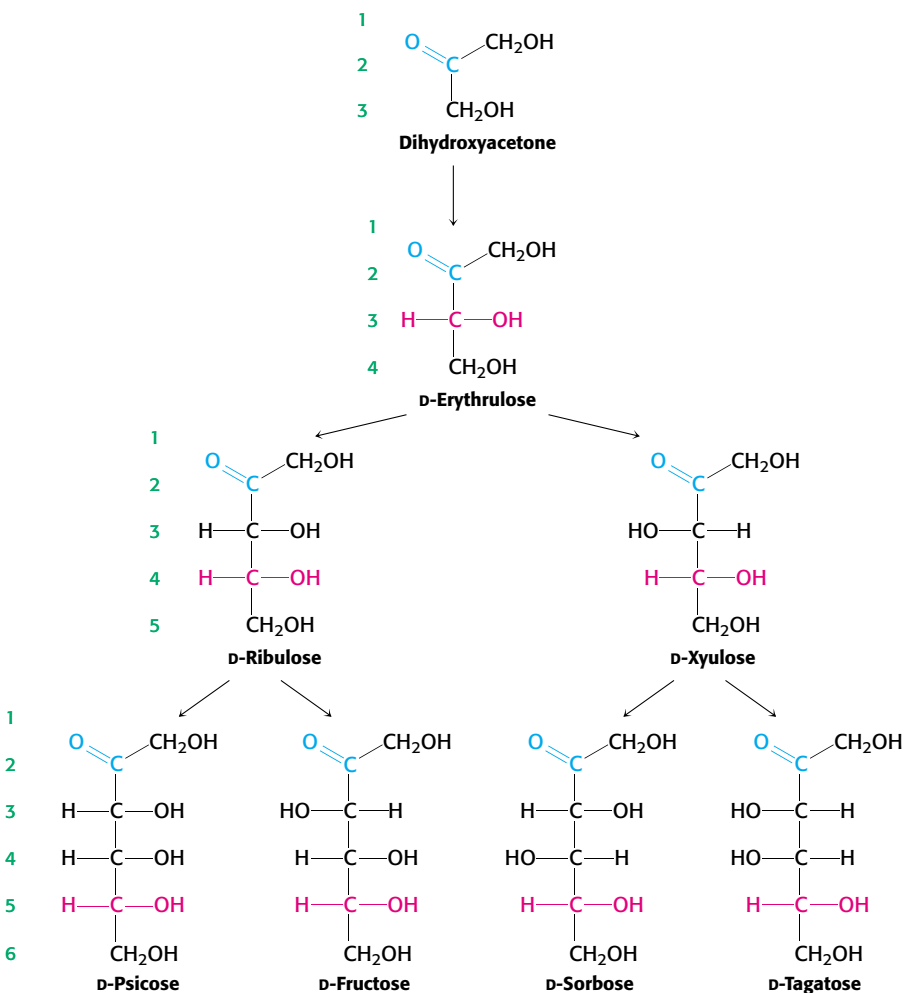
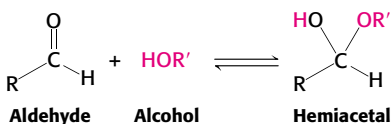


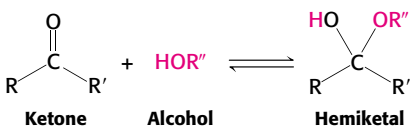
FIGURE 11.3 D-Ketoses containing three- four, five, and six carbon atoms. The keto group is shown in blue. The asymmetric center farthest from the keto group, which determines the D designation, is shown in red.

11.1.1 Pentoses and Hexoses Cyclize to Form Furanose and Pyranose Rings

The predominant forms of ribose, glucose, fructose, and many other sugars in solution are not open chains. Rather, the open-chain forms of these sugars cyclize into rings. In general, an aldehyde can react with an alcohol to form a *hemiacetal*.



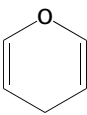
For an aldohexose such as glucose, the C-1 aldehyde in the open-chain form of glucose reacts with the C-5 hydroxyl group to form an *intramolecular hemiacetal*. The resulting cyclic hemiacetal, a six-membered ring, is called *pyranose* because of its similarity to *pyran* (Figure 11.4). Similarly, a ketone can react with an alcohol to form a *hemiketal*.



The C-2 keto group in the open-chain form of a ketohexose, such as fructose, can form an *intramolecular hemiketal* by reacting with either the



Furan



Pyran

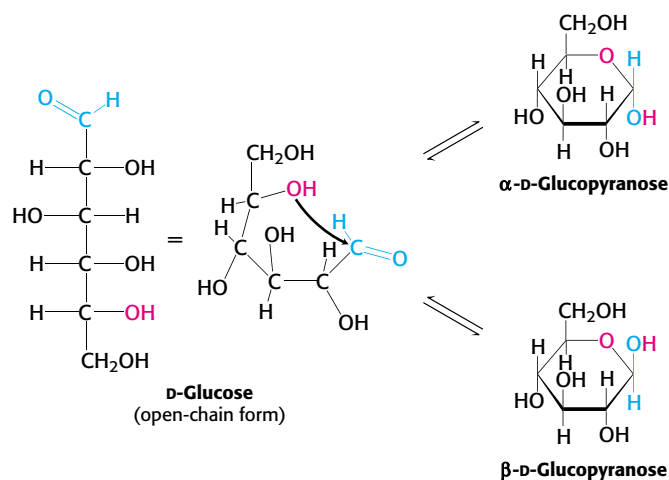


FIGURE 11.4 Pyranose formation. The open-chain form of glucose cyclizes when the C-5 hydroxyl group attacks the oxygen atom of the C-1 aldehyde group to form an intramolecular hemiacetal. Two anomeric forms, designated α and β , can result.

C-6 hydroxyl group to form a six-membered cyclic hemiketal or the C-5 hydroxyl group to form a five-membered cyclic hemiketal (Figure 11.5). The five-membered ring is called a *furanose* because of its similarity to *furan*.

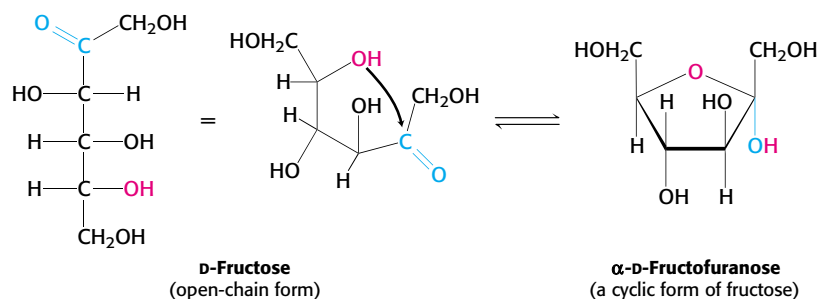


FIGURE 11.5 Furanose formation. The open-chain form of fructose cyclizes to a five-membered ring when the C-5 hydroxyl group attacks the C-2 ketone to form an intramolecular hemiketal. Two anomers are possible, but only the α anomer is shown.

The depictions of glucopyranose and fructofuranose shown in Figures 11.4 and 11.5 are *Haworth projections*. In such projections, the carbon atoms in the ring are not explicitly shown. The approximate plane of the ring is perpendicular to the plane of the paper, with the heavy line on the ring projecting toward the reader. Like Fischer projections, Haworth projections allow easy depiction of the stereochemistry of sugars. We will return to a more structurally realistic view of the conformations of cyclic monosaccharides shortly.

An additional asymmetric center is created when a cyclic hemiacetal is formed. In glucose, C-1, the carbonyl carbon atom in the open-chain form, becomes an asymmetric center. Thus, two ring structures can be formed: α -D-glucopyranose and β -D-glucopyranose (see Figure 11.4). For D sugars drawn as Haworth projections, the designation α means that the hydroxyl group attached to C-1 is below the plane of the ring; β means that it is above the plane of the ring. The C-1 carbon atom is called the *anomeric carbon atom*, and the α and β forms are called *anomers*. An equilibrium mixture of glucose contains approximately one-third α anomer, two-thirds β anomer, and <1% of the open-chain form.

The same nomenclature applies to the furanose ring form of fructose, except that α and β refer to the hydroxyl groups attached to C-2, the anomeric carbon atom (see Figure 11.5). Fructose forms both pyranose and furanose rings. The pyranose form predominates in fructose free in solution, and the furanose form predominates in many fructose derivatives (Figure 11.6). Pentoses such as D-ribose and 2-deoxy-D-ribose form furanose rings, as we have seen in the structure of these units in RNA and DNA.

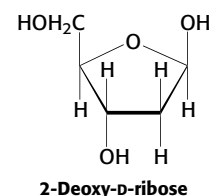
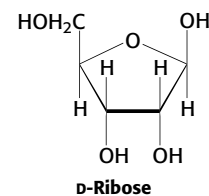
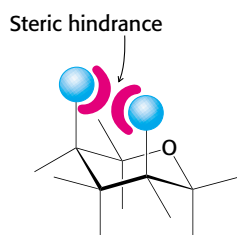
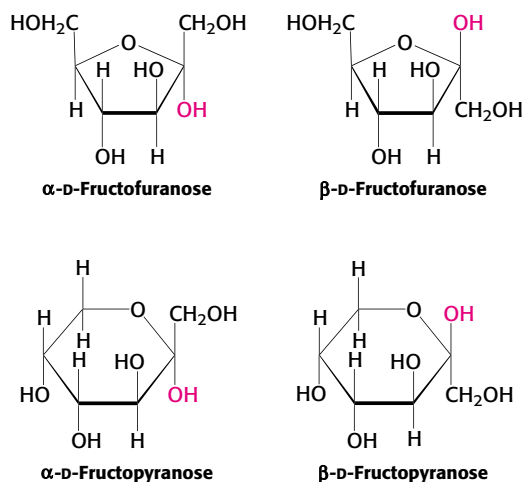


FIGURE 11.6 Ring structures of fructose. Fructose can form both five-membered furanose and six-membered pyranose rings. In each case, both α and β anomers are possible.



11.1.2 Conformation of Pyranose and Furanose Rings

The six-membered pyranose ring is not planar, because of the tetrahedral geometry of its saturated carbon atoms. Instead, pyranose rings adopt two classes of conformations, termed *chair* and *boat* because of the resemblance to these objects (Figure 11.7). In the chair form, the substituents on the ring carbon atoms have two orientations: *axial* and *equatorial*. Axial bonds are nearly perpendicular to the average plane of the ring, whereas equatorial bonds are nearly parallel to this plane. Axial substituents sterically hinder each other if they emerge on the same side of the ring (e.g., 1,3-diaxial groups). In contrast, equatorial substituents are less crowded. The chair form of β -D-glucopyranose predominates because all axial positions are occupied by hydrogen atoms. The bulkier -OH and $\text{-CH}_2\text{OH}$ groups emerge at the less-hindered periphery. The boat form of glucose is disfavored because it is quite sterically hindered.

FIGURE 11.7 Chair and boat forms of β -D-glucopyranose. The chair form is more stable because of less steric hindrance as the axial positions are occupied by hydrogen atoms.

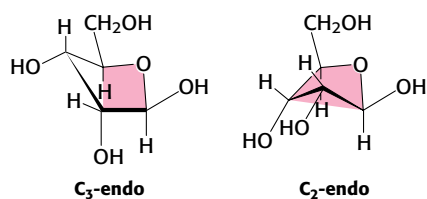
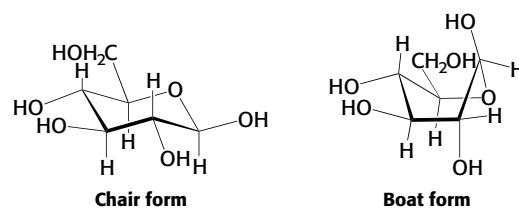


FIGURE 11.8 Envelope conformations of β -D-ribose. The C_2 -endo and C_3 -endo forms of β -D-ribose are shown. The color indicates the four atoms that lie approximately in a plane.

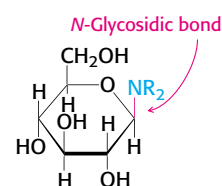
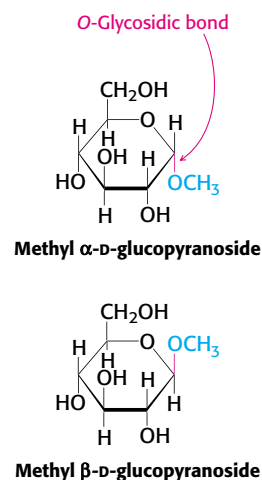
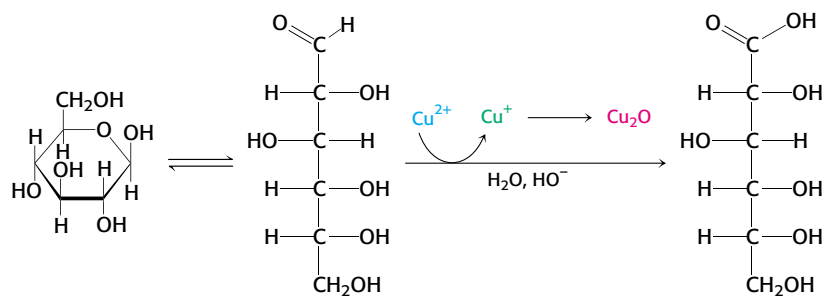
Furanose rings, like pyranose rings, are not planar. They can be puckered so that four atoms are nearly coplanar and the fifth is about 0.5 Å away from this plane (Figure 11.8). This conformation is called an *envelope form* because the structure resembles an opened envelope with the back flap raised. In the ribose moiety of most biomolecules, either C-2 or C-3 is out of the plane on the same side as C-5. These conformations are called C_2 -endo and C_3 -endo, respectively.

11.1.3 Monosaccharides Are Joined to Alcohols and Amines Through Glycosidic Bonds

Monosaccharides can be modified by reaction with alcohols and amines to form *adducts*. For example, D-glucose will react with methanol in an acid-catalyzed process: the anomeric carbon atom reacts with the hydroxyl group

of methanol to form two products, methyl α -D-glucopyranoside and methyl β -D-glucopyranoside. These two glucopyranosides differ in the configuration at the anomeric carbon atom. The new bond formed between the anomeric carbon atom of glucose and the hydroxyl oxygen atom of methanol is called a *glycosidic bond*—specifically, an *O-glycosidic bond*. The anomeric carbon atom of a sugar can be linked to the nitrogen atom of an amine to form an *N-glycosidic bond*.

Indeed, we have previously encountered such reaction products; nucleosides are adducts between sugars such as ribose and amines such as adenine (Section 5.1.1). Some other important modified sugars are shown in Figure 11.9. Compounds such as methyl glucopyranoside show differences in reactivity from that of the parent monosaccharide. For example, unmodified glucose reacts with oxidizing agents such as cupric ion (Cu^{2+}) because the open-chain form has a free aldehyde group that is readily oxidized.



Glycosides such as methyl glucopyranoside do not react, because they are not readily interconverted with a form that includes a free aldehyde group. Solutions of cupric ion (known as Fehling's solution) provide a simple test for sugars such as glucose. Sugars that react are called *reducing sugars*; those that do not are called *nonreducing sugars*.

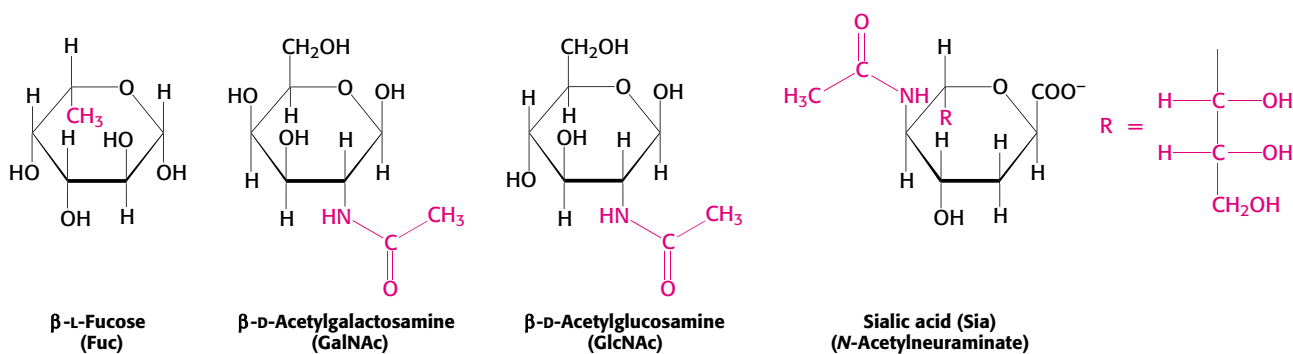


FIGURE 11.9 Modified monosaccharides. Carbohydrates can be modified by the addition of substituents (shown in red) other than hydroxyl groups. Such modified carbohydrates are often expressed on cell surfaces.

11.2 COMPLEX CARBOHYDRATES ARE FORMED BY LINKAGE OF MONOSACCHARIDES

Because sugars contain many hydroxyl groups, glycosidic bonds can join one monosaccharide to another. *Oligosaccharides* are built by the linkage of two or more monosaccharides by *O-glycosidic bonds* (Figure 11.10). In maltose, for example, two D-glucose residues are joined by a glycosidic linkage between the α -anomeric form of C-1 on one sugar and the hydroxyl oxygen atom on C-4 of the adjacent sugar. Such a linkage is called an α -1,4-glycosidic bond. The fact that monosaccharides have multiple hydroxyl groups means that

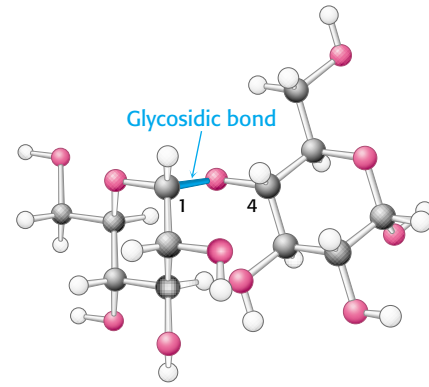


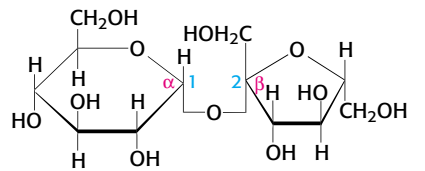
FIGURE 11.10 Maltose, a disaccharide. Two molecules of glucose are linked by an α -1,4-glycosidic bond to form the disaccharide maltose.

various glycosidic linkages are possible. Indeed, the wide array of these linkages in concert with the wide variety of monosaccharides and their many isomeric forms makes complex carbohydrates information-rich molecules.

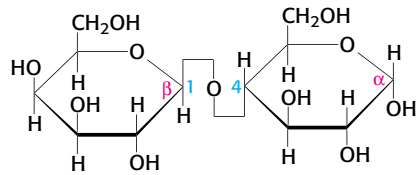
11.2.1 Sucrose, Lactose, and Maltose Are the Common Disaccharides

A *disaccharide* consists of two sugars joined by an *O*-glycosidic bond. Three abundant disaccharides are sucrose, lactose, and maltose (Figure 11.11). *Sucrose* (common table sugar) is obtained commercially from cane or beet. The anomeric carbon atoms of a glucose unit and a fructose unit are joined in this disaccharide; the configuration of this glycosidic linkage is α for glucose and β for fructose. Sucrose can be cleaved into its component monosaccharides by the enzyme *sucrase*.

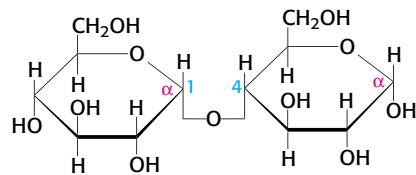
Lactose, the disaccharide of milk, consists of galactose joined to glucose by a β -1,4-glycosidic linkage. Lactose is hydrolyzed to these monosaccharides by *lactase* in human beings (Section 16.1.12) and by β -*galactosidase* in bacteria. In *maltose*, two glucose units are joined by an α -1,4 glycosidic linkage, as stated earlier. Maltose comes from the hydrolysis of starch and is in turn hydrolyzed to glucose by *maltase*. Sucrase, lactase, and maltase are located on the outer surfaces of epithelial cells lining the small intestine (Figure 11.12).



Sucrose
 $(\alpha$ -D-Glucopyranosyl-(1 \rightarrow 2)- β -D-fructofuranose)



Lactose
 $(\beta$ -D-Galactopyranosyl-(1 \rightarrow 4)- α -D-glucopyranose)



Maltose
 $(\alpha$ -D-Glucopyranosyl-(1 \rightarrow 4)- α -D-glucopyranose)

FIGURE 11.11 Common disaccharides. Sucrose, lactose, and maltose are common dietary components.

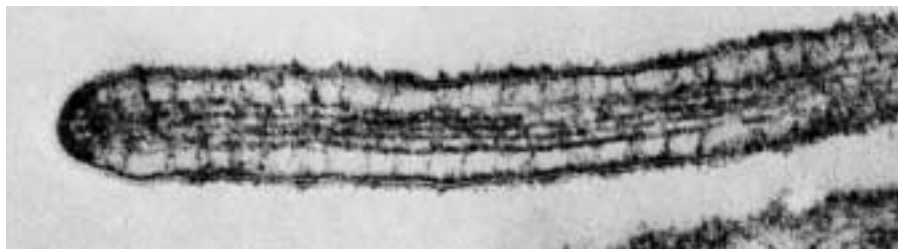


FIGURE 11.12 Electron micrograph of a microvillus. Lactase and other enzymes that hydrolyze carbohydrates are present on microvilli that project from the outer face of the plasma membrane of intestinal epithelial cells. [From M. S. Mooseker and L. G. Tilney, *J. Cell. Biol.* 67(1975):725.]

11.2.2 Glycogen and Starch Are Mobilizable Stores of Glucose

Large polymeric oligosaccharides, formed by the linkage of multiple monosaccharides, are called *polysaccharides*. Polysaccharides play vital roles in energy storage and in maintaining the structural integrity of an organism. If all of the monosaccharides are the same, these polymers are called *homopolymers*. The most common homopolymer in animal cells is *glycogen*, the storage form

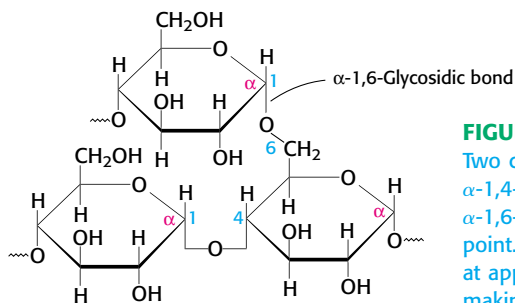
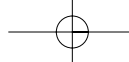


FIGURE 11.13 Branch point in glycogen. Two chains of glucose molecules joined by α -1,4-glycosidic bonds are linked by an α -1,6-glycosidic bond to create a branch point. Such an α -1,6-glycosidic bond forms at approximately every 10 glucose units, making glycogen a highly branched molecule.

of glucose. As will be considered in detail in Chapter 21, glycogen is a very large, branched polymer of glucose residues. Most of the glucose units in glycogen are linked by α -1,4-glycosidic bonds. The branches are formed by α -1,6-glycosidic bonds, present about once in 10 units (Figure 11.13).

The nutritional reservoir in plants is *starch*, of which there are two forms. *Amylose*, the unbranched type of starch, consists of glucose residues in α -1,4 linkage. *Amylopectin*, the branched form, has about 1 α -1,6 linkage per 30 α -1,4 linkages, in similar fashion to glycogen except for its lower degree of branching. More than half the carbohydrate ingested by human beings is starch. Both amylopectin and amylose are rapidly hydrolyzed by α -amylase, an enzyme secreted by the salivary glands and the pancreas.

11.2.3 Cellulose, the Major Structural Polymer of Plants, Consists of Linear Chains of Glucose Units

Cellulose, the other major polysaccharide of glucose found in plants, serves a structural rather than a nutritional role. *Cellulose is one of the most abundant organic compounds in the biosphere.* Some 10^{15} kg of cellulose is synthesized and degraded on Earth each year. It is an unbranched polymer of glucose residues joined by β -1,4 linkages. The β configuration allows cellulose to form very long, straight chains. Fibrils are formed by parallel chains that interact with one another through hydrogen bonds. The α -1,4 linkages in glycogen and starch produce a very different molecular architecture from that of cellulose. A hollow helix is formed instead of a straight chain (Figure 11.14). These differing consequences of the α and β linkages are biologically important. The straight chain formed by β linkages is optimal for the construction of fibers having a high tensile strength. In contrast, the open helix formed by α linkages is well suited to forming an accessible store of sugar. Mammals lack cellulases and therefore cannot digest wood and vegetable fibers.

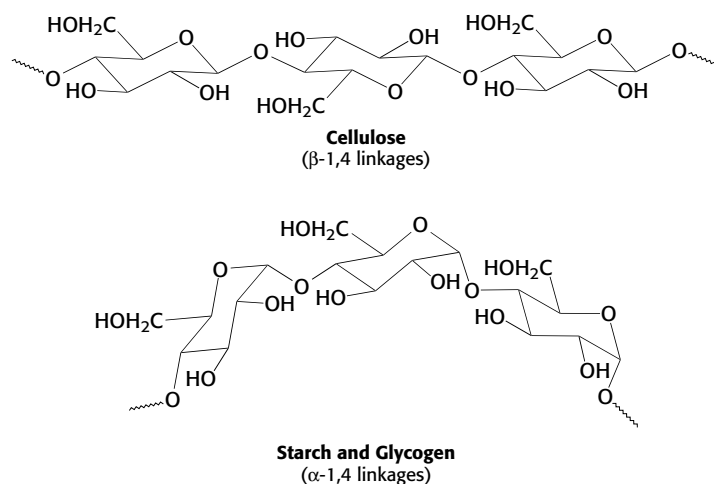
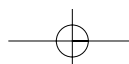


FIGURE 11.14 Glycosidic bonds determine polysaccharide structure. The β -1,4 linkages favor straight chains, which are optimal for structural purposes. The α -1,4 linkages favor bent structures, which are more suitable for storage.



11.2.4 Glycosaminoglycans Are Anionic Polysaccharide Chains Made of Repeating Disaccharide Units

A different kind of repeating polysaccharide is present on the animal cell surface and in the extracellular matrix. Many *glycosaminoglycans* are made of *disaccharide repeating units* containing a derivative of an *amino sugar*, either glucosamine or galactosamine (Figure 11.15). At least one of the sugars in the repeating unit has a *negatively charged carboxylate or sulfate group*. Chondroitin sulfate, keratan sulfate, heparin, heparan sulfate, dermatan sulfate, and hyaluronate are the major glycosaminoglycans.

Glycosaminoglycans are usually attached to proteins to form *proteoglycans*. Heparin is synthesized in a nonsulfated form, which is then deacetylated and sulfated. Incomplete modification leads to a mixture of variously sulfated sequences. Some of them act as anticoagulants by binding specifically to antithrombin, which accelerates its sequestration of thrombin (Section 10.7.8). Heparan sulfate is like heparin except that it has fewer *N*- and *O*-sulfate groups and more acetyl groups.

Proteoglycans resemble polysaccharides more than proteins in as much as the carbohydrate makes up as much as 95% of the biomolecule by weight. Proteoglycans function as lubricants and structural components in connective tissue, mediate adhesion of cells to the extracellular matrix, and bind factors that stimulate cell proliferation.

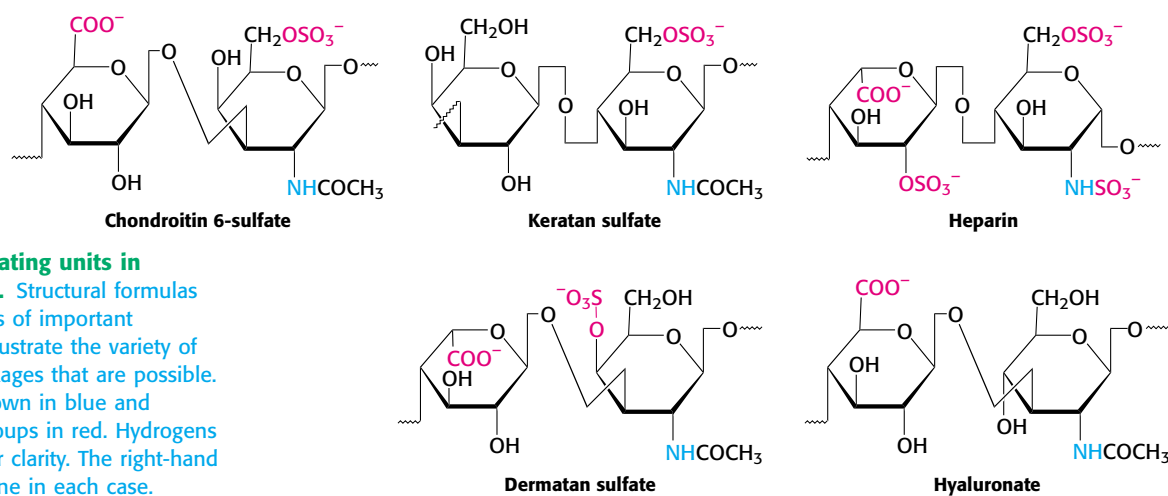


FIGURE 11.15 Repeating units in glycosaminoglycans. Structural formulas for five repeating units of important glycosaminoglycans illustrate the variety of modifications and linkages that are possible. Amino groups are shown in blue and negatively charged groups in red. Hydrogens have been omitted for clarity. The right-hand structure is glucosamine in each case.

11.2.5 Specific Enzymes Are Responsible for Oligosaccharide Assembly

Oligosaccharides are synthesized through the action of specific enzymes, *glycosyltransferases*, which catalyze the formation of glycosidic bonds. Each enzyme must be specific, to a greater or lesser extent, to the sugars being linked. Given the diversity of known glycosidic linkages, many different enzymes are required. Note that this mode of assembly stands in contrast with those used for the other biological polymers heretofore discussed—that is, polypeptides and oligonucleotides. As these polymers are assembled, information about monomer sequence is transferred from a template, and a single catalytic apparatus is responsible for all bond formation.

The general form of the reaction catalyzed by a glycosyltransferase is shown in Figure 11.16. The sugar to be added comes in the form of an activated sugar nucleotide. *Sugar nucleotides are important intermediates in many processes*, and we will encounter these intermediates again in Chapters 16 and 21. Note that such reactions can proceed with either retention or

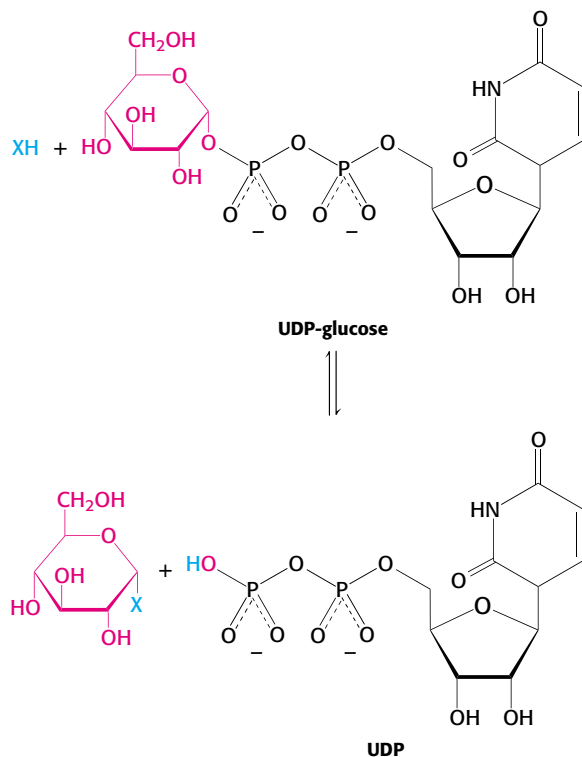




FIGURE 11.16 General form of a glycosyltransferase reaction. The sugar to be added comes from a sugar nucleotide—in this case, UDP-glucose.

inversion of configuration at the glycosidic carbon atom at which the new bond is formed; a given enzyme proceeds by one stereochemical path or the other.

 The human ABO blood groups illustrate the effects of glycosyltransferases. Carbohydrates are attached to glycoproteins and glycolipids on the surfaces of red blood cells. For one type of blood group, one of the three different structures, termed A, B, and O, may be present (Figure 11.17). These structures have in common an oligosaccharide foundation called the O (or sometimes H) antigen. The A and B antigens differ from the O antigen by the addition of one extra monosaccharide, either *N*-acetylgalactosamine (for A) or galactose (for B) through an α -1,3 linkage to a galactose moiety of the O antigen.

Specific glycosyltransferases add the extra monosaccharide to the O antigen. Each person inherits the gene for one glycosyltransferase of this type from each parent. The type A transferase specifically adds *N*-acetylgalactosamine, whereas the type B transferase adds galactose. These enzymes are identical in all but 4 of 354 positions. The O phenotype is the result of a mutation that leads to premature termination of translation and, hence, to the production of no active glycosyltransferase.

These structures have important implications for blood transfusions and other transplantation procedures. If an antigen not normally present in a person is introduced, the person's immune system recognizes it as foreign. Adverse reactions can ensue, initiated by the intravascular destruction of the incompatible red blood cells.

 Why are different blood types present in the human population? Suppose that a pathogenic organism such as a parasite expresses on its cell surface a carbohydrate antigen similar to one of the blood-group

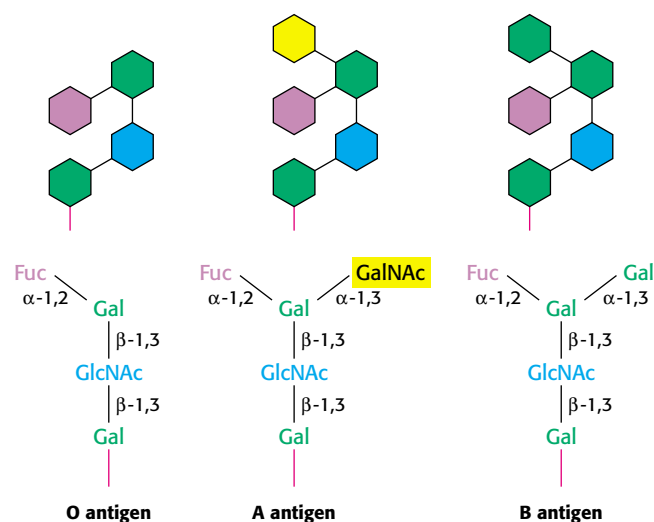


FIGURE 11.17 Structures of A, B, and O oligosaccharide antigens. Abbreviations: Fuc, fucose; Gal, galactose; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine.

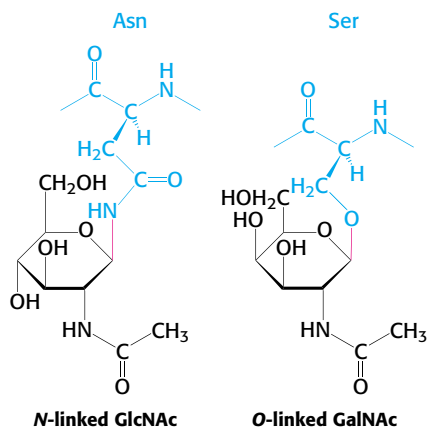


FIGURE 11.18 Glycosidic bonds between proteins and carbohydrates. A glycosidic bond links a carbohydrate to the side chain of asparagine (*N*-linked) or to the side chain of serine or threonine (*O*-linked). The glycosidic bonds are shown in red.

antigens. This antigen may not be readily detected as foreign in a person with the blood type that matches the parasite antigen, and the parasite will flourish. However, other people with different blood types will be protected. Hence, there will be selective pressure on human beings to vary blood type to prevent parasitic mimicry and a corresponding selective pressure on parasites to enhance mimicry. The constant “arms race” between pathogenic microorganisms and human beings drives the evolution of diversity of surface antigens within the human population.

11.3 CARBOHYDRATES CAN BE ATTACHED TO PROTEINS TO FORM GLYCOPROTEINS

Carbohydrate groups are covalently attached to many different proteins to form *glycoproteins*. Carbohydrates are a much smaller percentage of the weight of glycoproteins than of proteoglycans. Many glycoproteins are components of cell membranes, where they play a variety of roles in processes such as cell adhesion and the binding of sperm to eggs.

11.3.1 Carbohydrates May Be Linked to Proteins Through Asparagine (*N*-Linked) or Through Serine or Threonine (*O*-Linked) Residues

In glycoproteins, sugars are attached either to the amide nitrogen atom in the side chain of asparagine (termed an *N-linkage*) or to the oxygen atom in the side chain of serine or threonine (termed an *O-linkage*), as shown in Figure 11.18. An asparagine residue can accept an oligosaccharide only if the residue is part of an Asn-X-Ser or Asn-X-Thr sequence, in which X can be any residue. Thus, *potential glycosylation sites can be detected within amino acid sequences*. However, which of these potential sites is actually glycosylated depends on other aspects of the protein structure and on the cell type in which the protein is expressed. All *N*-linked oligosaccharides have in common a pentasaccharide core consisting of three mannose and two *N*-acetylglucosamine residues. Additional sugars are attached to this core to form the great variety of oligosaccharide patterns found in glycoproteins (Figure 11.19).

Abbreviations for sugars—

Fuc	Fucose
Gal	Galactose
GalNAc	<i>N</i> -Acetylgalactosamine
Glc	Glucose
GlcNAc	<i>N</i> -Acetylglucosamine
Man	Mannose
Sia	Sialic acid
NeuNAc	<i>N</i> -Acetylneuraminate (sialic acid)

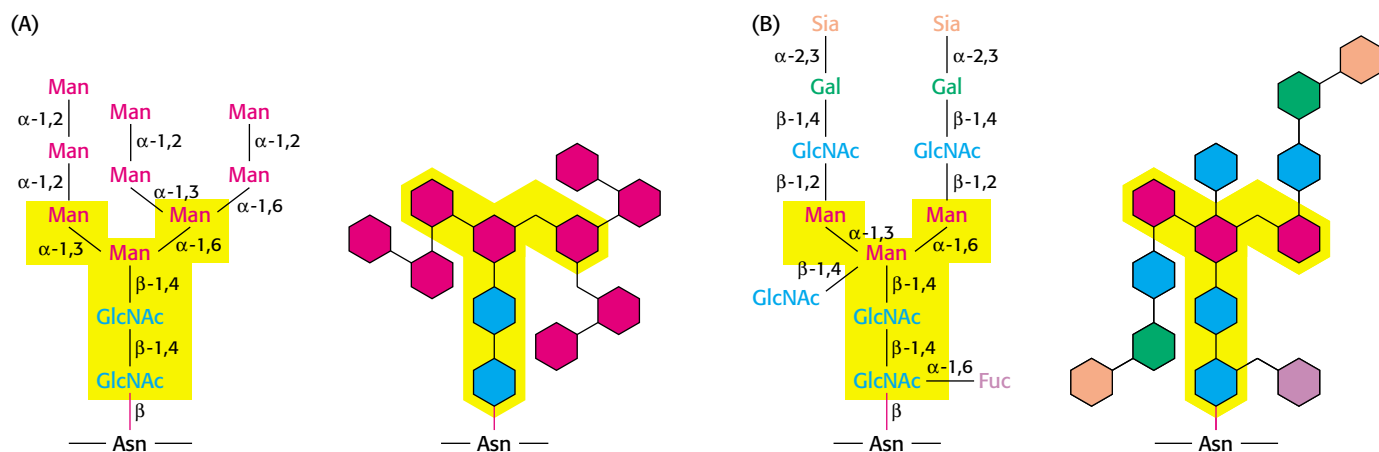


FIGURE 11.19 *N*-linked oligosaccharides. A pentasaccharide core (shaded yellow) is common to all *N*-linked oligosaccharides and serves as the foundation for a wide variety of *N*-linked oligosaccharides, two of which are illustrated: (A) high-mannose type; (B) complex type. Detailed chemical formulas and schematic structures are shown for each type.

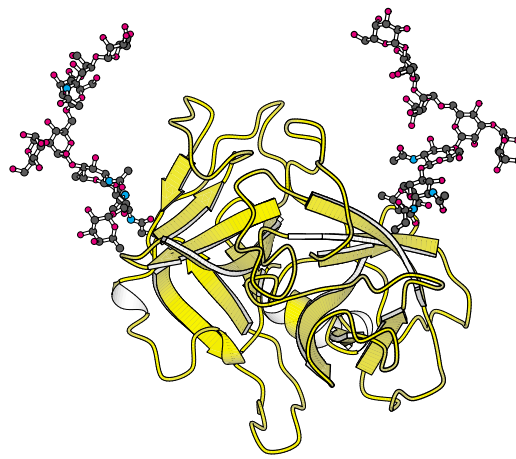


FIGURE 11.20 Elastase, a secreted glycoprotein, showing linked carbohydrates on its surface. Elastase is a protease found in serum. Note that the oligosaccharide chains have substantial size even for this protein, which has a relatively low level of glycosylation.

Carbohydrates are linked to some soluble proteins as well as membrane proteins. In particular, many of the proteins secreted from cells are glycosylated. Most proteins present in the serum component of blood are glycoproteins (Figure 11.20). Furthermore, *N*-acetylglucosamine residues are *O*-linked to some intracellular proteins. The role of these carbohydrates, which are dynamically added and removed, is under active investigation.

11.3.2 Protein Glycosylation Takes Place in the Lumen of the Endoplasmic Reticulum and in the Golgi Complex

Protein glycosylation takes place inside the lumen of the *endoplasmic reticulum* (ER) and the *Golgi complex*, organelles that play central roles in protein trafficking (Figure 11.21). One such glycoprotein (depicted in Figure 11.20) is the proteolytic enzyme elastase (Section 9.1.4), which is secreted by the pancreas as a zymogen (Section 10.7). This protein is synthesized by ribosomes attached to the cytoplasmic face of the ER membrane, and the peptide chain is inserted into the lumen of the ER as it grows, guided by a signal sequence of 29 amino acids at the amino terminus. This signal sequence, which directs the protein through a channel in the ER membrane, is cleaved from the protein in the transport process into the ER (Figure 11.22). After the protein has entered the ER, the glycosylation process begins. The *N*-linked glycosylation begins in the ER and continues in the Golgi complex, whereas the *O*-linked glycosylation takes place exclusively in the Golgi complex.

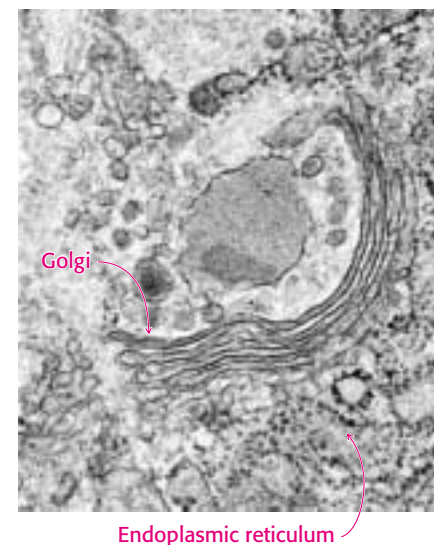


FIGURE 11.21 Golgi complex and endoplasmic reticulum. The electron micrograph shows the Golgi complex and adjacent endoplasmic reticulum. The black dots on the cytoplasmic surface of the ER membrane are ribosomes. [Micrograph courtesy of Lynne Mercer.]

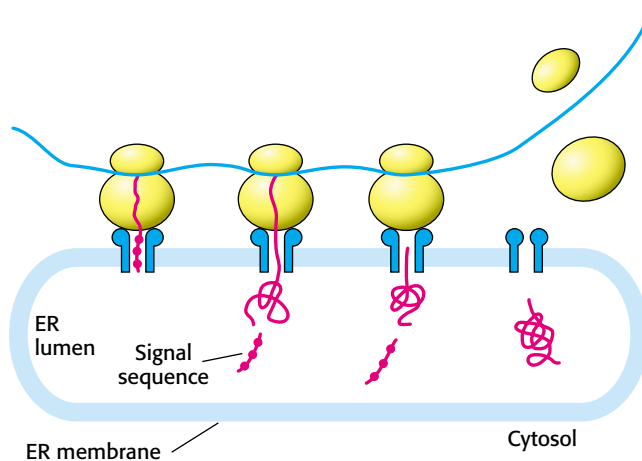
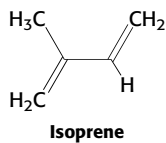
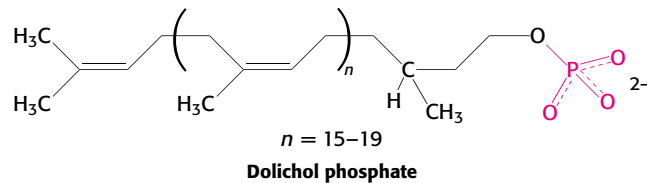


FIGURE 11.22 Transport into the endoplasmic reticulum. As translation takes place, a signal sequence on membrane and secretory proteins directs the nascent protein through channels in the ER membrane and into the lumen. In most cases, the signal sequence is subsequently cleaved and degraded.



11.3.3 N-Linked Glycoproteins Acquire Their Initial Sugars from Dolichol Donors in the Endoplasmic Reticulum

A large oligosaccharide destined for attachment to the asparagine residue of a protein is assembled attached to *dolichol phosphate*, a specialized lipid molecule containing as many as 20 isoprene (C_5) units (Section 26.x.x).



The terminal phosphate group is the site of attachment of the activated oligosaccharide, which is subsequently transferred to the protein acceptor. Dolichol phosphate resides in the ER membrane with its phosphate terminus on the cytoplasmic face.

The assembly process proceeds in three stages. First, 2 *N*-acetylglucosamine residues and 5 mannose residues are added to the dolichol phosphate through the action of a number of cytoplasmic enzymes that catalyze monosaccharide transfer from sugar nucleotides. Then, in a remarkable (and, as yet, not well understood) process, this large structure is “flipped” through the ER membrane into the lumen of the ER. Finally, additional sugars are added by enzymes in the ER lumen, this time with the use of monosaccharides activated by attachment to dolichol phosphate. This process ends with the formation of a 14-residue oligosaccharide attached to dolichol phosphate (Figure 11.23).

The 14-sugar-residue precursor attached to this dolichol phosphate intermediate is then transferred en bloc to a specific asparagine residue of the growing polypeptide chain. In regard to elastase, oligosaccharides are linked

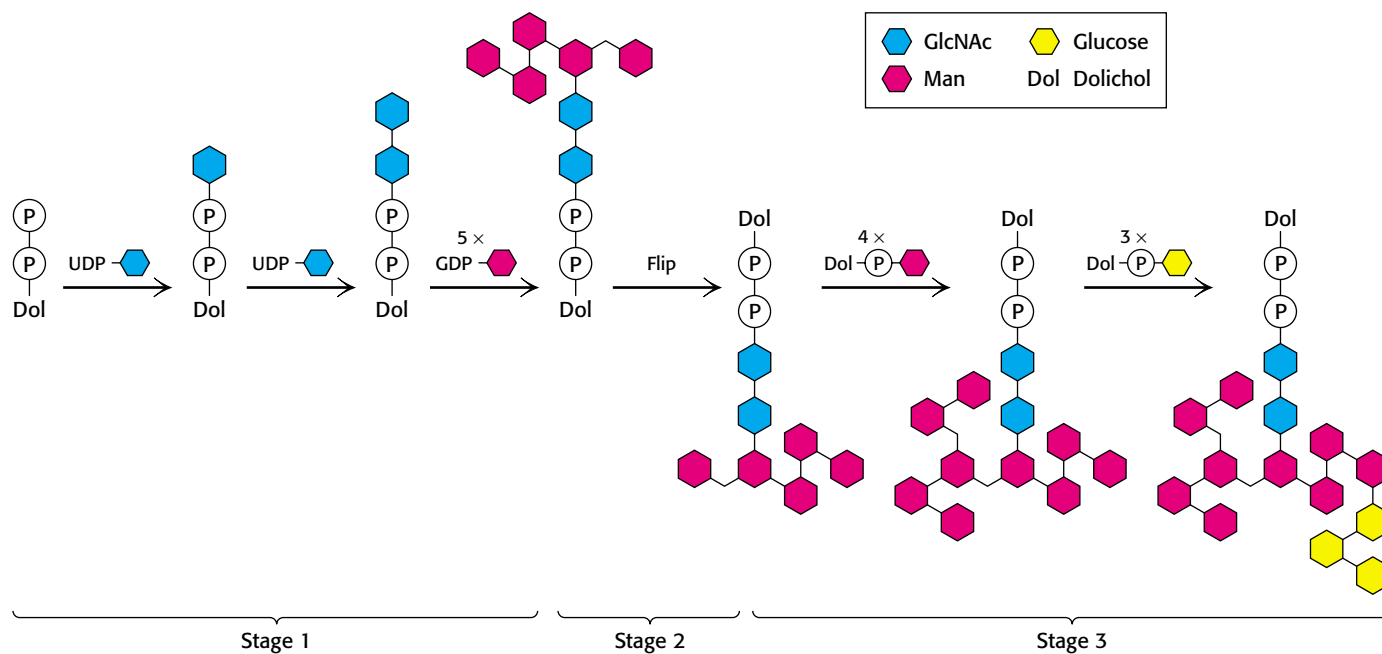



FIGURE 11.23 Assembly of an N-linked oligosaccharide precursor on dolichol phosphate. The first stage of oligosaccharide synthesis takes place in the cytoplasm on the exposed phosphate of a membrane-embedded dolichol molecule. Synthesis of the precursor is completed in the lumen of the ER after flipping of the dolichol phosphate and attached oligosaccharide.

to the asparagine residues in the recognition sequences Asn 109-Gly-Ser and Asn 159-Val-Thr. Both the activated sugars and the complex enzyme that is responsible for transferring the oligosaccharide to the protein are located on the luminal side of the ER, accounting for the fact that proteins in the cytosol are not glycosylated by this pathway. Before the glycoprotein leaves the lumen of the ER, 3 glucose molecules are removed from the 14-residue oligosaccharide. As we will see in Section 11.3.6, the sequential removal of these glucose molecules is a quality-control step that ensures that only properly folded glycoproteins are further processed.

 Dolichol pyrophosphate released in the transfer of the oligosaccharide to the protein is recycled to dolichol phosphate by the action of a phosphatase. This hydrolysis is blocked by *bacitracin*, an antibiotic. Another interesting antibiotic inhibitor of *N*-glycosylation is *tunicamycin*, a hydrophobic analog of the sugar nucleotide uridine diphosphate-*N*-acetylglucosamine (UDP-GlcNAc), the activated form of *N*-acetylglucosamine used as a substrate for the enzymes that synthesize the oligosaccharide unit on dolichol phosphate. Tunicamycin blocks the addition of *N*-acetylglucosamine to dolichol phosphate, the first step in the formation of the core oligosaccharide.

11.3.4 Transport Vesicles Carry Proteins from the Endoplasmic Reticulum to the Golgi Complex for Further Glycosylation and Sorting

Proteins in the lumen of the ER and in the ER membrane are transported to the Golgi complex, which is a stack of flattened membranous sacs. The Golgi complex has two principal roles. First, *carbohydrate units of glycoproteins are altered and elaborated in the Golgi complex*. The *O*-linked sugar units are fashioned there, and the *N*-linked sugars, arriving from the ER as a component of a glycoprotein, are modified in many different ways. Second, *the Golgi complex is the major sorting center of the cell*. Proteins proceed from the Golgi complex to lysosomes, secretory granules (as is the case for the elastase zymogen), or the plasma membrane, according to signals encoded within their amino acid sequences and three-dimensional structures (Figure 11.24).

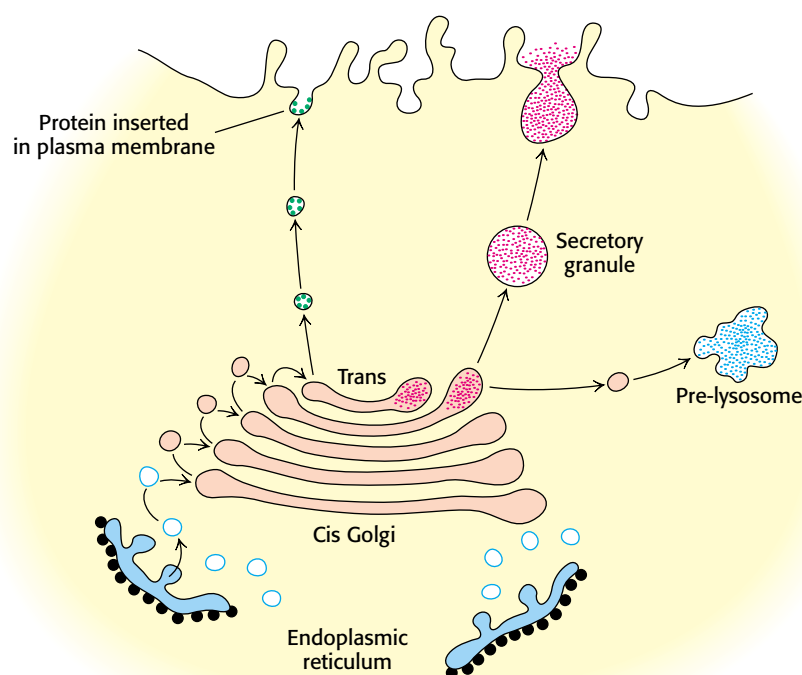


FIGURE 11.24 Golgi complex as sorting center. The Golgi complex is the sorting center in the targeting of proteins to lysosomes, secretory vesicles, and the plasma membrane. The cis face of the Golgi complex receives vesicles from the ER, and the trans face sends a different set of vesicles to target sites. Vesicles also transfer proteins from one compartment of the Golgi complex to another. [Courtesy of Dr. Marilyn Farquhar.]

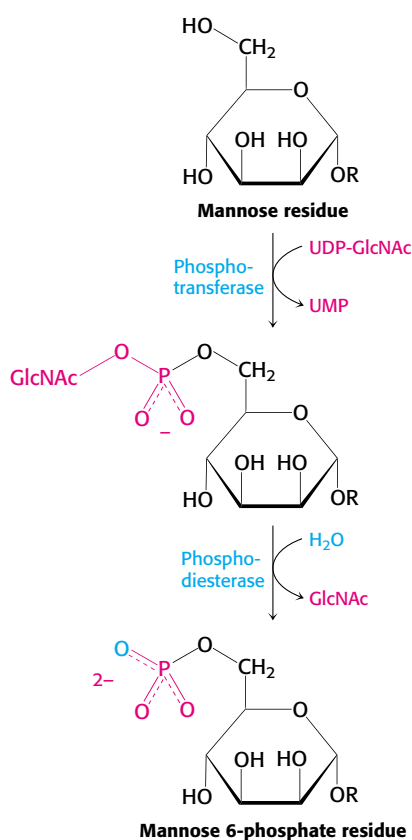



FIGURE 11.25 Formation of a mannose 6-phosphate marker. A glycoprotein destined for delivery to lysosomes acquires a phosphate marker in the *cis* Golgi compartment in a two-step process. First, a phosphotransferase adds a phospho-*N*-acetylglucosamine unit to the 6-OH group of a mannose, and then a phosphodiesterase removes the added sugar to generate a mannose 6-phosphate residue in the core oligosaccharide.

The Golgi complex of a typical mammalian cell has 3 or 4 membranous sacs (cisternae), and those of many plant cells have about 20. The Golgi complex is differentiated into (1) a *cis* compartment, the receiving end, which is closest to the ER; (2) *medial* compartments; and (3) a *trans* compartment, which exports proteins to a variety of destinations. These compartments contain different enzymes and mediate distinctive functions.

The *N*-linked carbohydrate units of glycoproteins are further modified in each of the compartments of the Golgi complex. In the *cis* Golgi compartment, three mannose residues are removed from the oligosaccharide chains of proteins destined for secretion or for insertion in the plasma membrane. The carbohydrate units of glycoproteins targeted to the lumen of lysosomes are further modified. In the *medial* Golgi compartments of some cells, two more mannose residues are removed, and two *N*-acetylglucosamines residues and a fucose residue are added. Finally, in the *trans* Golgi, another *N*-acetylglucosamine residue can be added, followed by galactose and sialic acid, to form a complex oligosaccharide unit. The sequence of *N*-linked oligosaccharide units of a glycoprotein is determined both by (1) the sequence and conformation of the protein undergoing glycosylation and by (2) the glycosyltransferases present in the Golgi compartment in which they are processed. Note that, despite all of this processing, *N*-glycosylated proteins have in common a pentasaccharide core (see Figure 11.19). Carbohydrate processing in the Golgi complex is called *terminal glycosylation* to distinguish it from *core glycosylation*, which takes place in the ER. Tremendous structural diversification can occur as a result of the terminal glycosylation process.

11.3.5 Mannose 6-phosphate Targets Lysosomal Enzymes to Their Destinations

 A carbohydrate marker directs certain proteins from the Golgi complex to lysosomes. A clue to the identity of this marker came from analyses of *I-cell disease* (also called *muco lipidosis II*), a lysosomal storage disease. *Lysosomes* are organelles that degrade and recycle damaged cellular components or material brought into the cell by endocytosis. Patients with *I-cell disease* suffer severe psychomotor retardation and skeletal deformities. Their lysosomes contain large *inclusions* of undigested glycosaminoglycans (Section 11.2.4) and glycolipids (Section 12.2.3)—hence the “I” in the name of the disease. These inclusions are present because at least eight acid hydrolases required for their degradation are missing from affected lysosomes. In contrast, very high levels of the enzymes are present in the blood and urine. Thus, active enzymes are synthesized, but they are exported instead of being sequestered in lysosomes. In other words, *a whole series of enzymes is mislocated in I-cell disease*. Normally, these enzymes contain a mannose 6-phosphate residue, but, in *I-cell disease*, the attached mannose is unmodified (Figure 11.25). *Mannose 6-phosphate is in fact the marker that normally directs many hydrolytic enzymes from the Golgi complex to lysosomes. I-cell patients are deficient in the phosphotransferase catalyzing the first step in the addition of the phosphoryl group; the consequence is the mistargeting of eight essential enzymes.*

11.3.6 Glucose Residues Are Added and Trimmed to Aid in Protein Folding

The oligosaccharide precursors added to proteins may play a role in protein **folding** as well as in protein targeting. As we have seen, before a glycoprotein leaves the ER, two glucosidases cleave the three glucose residues of the oligosaccharide in a step-by-step fashion. If the protein is properly folded, it moves to the Golgi complex for further processing (Section 11.3.3). However,

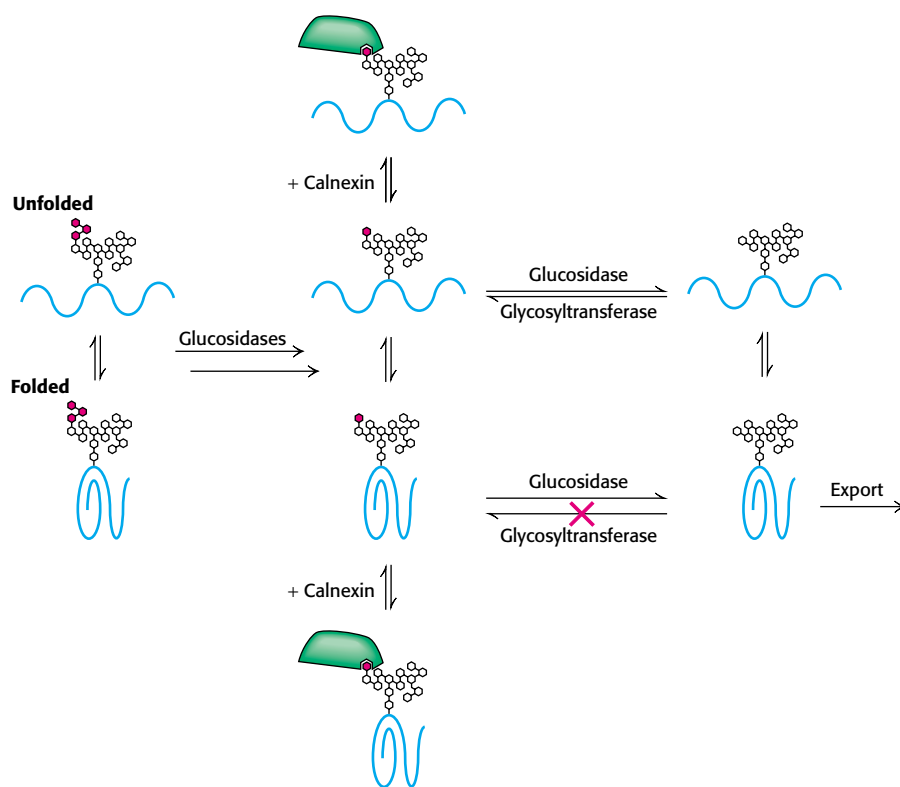
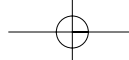


FIGURE 11.26 Quality-control system for protein folding in the ER. A properly folded glycoprotein will move to the Golgi complex after the removal of glucose moieties (shown in red). An unfolded or misfolded protein will receive a glucose residue, through the action of a glucosyltransferase. Such glucosylated glycoproteins bind to calnexin (or the related protein calreticulin), which serves as a chaperone to allow multiple attempts to attain correct folding. Properly folded proteins are not reglucosylated.

if the protein is sufficiently unfolded that the oligosaccharide can act as a substrate for glucosyltransferase, another enzyme residing in the lumen of the ER, a glucose residue will be reattached (Figure 11.26). This residue, in turn, is bound by one of two chaperone proteins called *calnexin* and *calreticulin*. Calnexin, the more fully understood of the two proteins, is membrane bound, whereas calreticulin is a soluble component of the ER lumen. Unfolded proteins held by these carbohydrate-binding proteins (lectins, Section 11.4) cannot leave the ER, giving the unfolded proteins time to fold properly. When a chaperone releases the bound protein, the glucose residue will be cleaved by a glucosidase. If the folding is correct, the protein moves to the Golgi complex. Otherwise, the protein will repeat another cycle of glucose addition and binding until the glucose-free (and, hence, properly folded) protein can be translocated to the Golgi complex. This quality-control system reveals an important principle: **carbohydrates carry information**. Here, the availability of carbohydrates to specific glucosyltransferases conveys information about the folding state of the protein. Moreover, we see the reiteration of a theme in the control of protein folding: other chaperone proteins rely on the same essential mechanism of allowing misfolded proteins multiple attempts to reach a folded state (Section 3.6), even though carbohydrate modification is not a part of their reaction cycles.

11.3.7 Oligosaccharides Can Be “Sequenced”

Given the large diversity of oligosaccharide structures and the many possible points of attachment to most proteins, how is it possible to determine the structure of a glycoprotein? Most approaches are based on the use of enzymes that cleave oligosaccharides at specific types of linkages. For example, *N*-linked oligosaccharides can be released from proteins by an enzyme such as Peptide *N*-glycosidase F, which cleaves the *N*-glycosidic bonds linking the oligosaccharide to the protein. The oligosaccharides can then be isolated and analyzed. Through the use of MALDI-TOF or other mass spectrometric

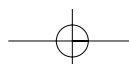
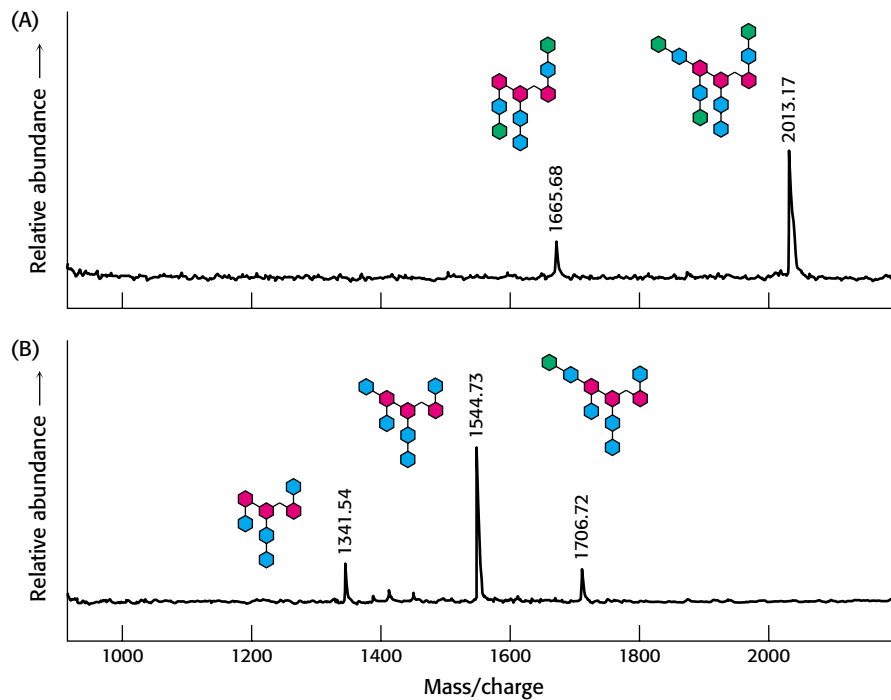


FIGURE 11.27 Mass spectrometric “sequencing” of oligosaccharides.

Carbohydrate-cleaving enzymes were used to release and specifically cleave the oligosaccharide component of the glycoprotein fetuin from bovine serum. Parts A and B show the masses obtained with MALDI-TOF spectrometry as well as the corresponding structures of the oligosaccharide digestion products (using the same scheme as that in Figure 11.19): (A) digestion with Peptide *N*-glycosidase F (to release the oligosaccharide from the protein) and neuraminidase; (B) digestion with Peptide *N*-glycosidase F, neuraminidase, and β -1,4-galactosidase. Knowledge of the enzyme specificities and the masses of the products permits the characterization of the oligosaccharide. [After A. Varki, R. Cummings, J. Esko, H. Freeze, G. Hart, and J. Marth (Eds.), *Essentials of Glycobiology* (Cold Spring Harbor Laboratory Press, 1999), p. 596.]



techniques (Section 4.1.7), the mass of an oligosaccharide fragment can be determined. However, given the large number of potential monosaccharide combinations, many possible oligosaccharide structures are consistent with a given mass. More complete information can be obtained by cleaving the oligosaccharide with enzymes of varying specificities. For example, β -1,4-galactosidase cleaves β -glycosidic bonds exclusively at galactose residues. The products can again be analyzed by mass spectrometry (Figure 11.27). The repetition of this process with the use of an array of enzymes of different specificity will eventually reveal the structure of the oligosaccharide.

The points of oligosaccharide attachment can be determined through the use of proteases. Cleavage of a protein by applying specific proteases yields a characteristic pattern of peptide fragments that can be analyzed chromatographically (Section 4.2.1). The chromatographic properties of peptides attached to oligosaccharides will change on glycosidase treatment. Mass spectrometric analysis or direct peptide sequencing can reveal the identity of the peptide in question and, with additional effort, the exact site of oligosaccharide attachment.

Posttranslational modifications such as glycosylation greatly increase the complexity of the proteome. A given protein with several potential glycosylation sites can have many different glycosylated forms (sometimes called *glycoforms*), each of which may be generated only in a specific cell type or developmental stage. Now that the sequencing of the human genome is essentially complete, the characterization of the much more complex proteome, including the biological roles of specifically modified proteins, can begin in earnest.

11.4 LECTINS ARE SPECIFIC CARBOHYDRATE-BINDING PROTEINS

The diverse carbohydrate structures displayed on cell surfaces are well suited to serve as interaction sites between cells and their environments. Proteins termed *lectins* (from the Latin *legere*, “to select”) are the partners

that bind specific carbohydrate structures. Lectins are ubiquitous, being found in animals, plants, and microorganisms. We have already seen that some lectins, such as calnexin, function as chaperones in protein folding (Section 11.3.6).

11.4.1 Lectins Promote Interactions Between Cells

The chief function of lectins in animals is to facilitate cell–cell contact. A lectin usually contains two or more binding sites for carbohydrate units; some lectins form oligomeric structures with multiple binding sites. The binding sites of lectins on the surface of one cell interact with arrays of carbohydrates displayed on the surface of another cell. Lectins and carbohydrates are linked by a number of relatively weak interactions that ensure specificity yet permit unlinking as needed. The interactions between one cell surface with carbohydrates and another with lectins resemble the action of Velcro; each interaction is relatively weak but the composite is strong.

The exact role of lectins in plants is unclear, although they can serve as potent insecticides. Castor beans contain so much lectin that they are toxic to most organisms. The binding specificities of lectins from plants have been well characterized (Figure 11.28). Bacteria, too, contain lectins. *Escherichia coli* bacteria are able to adhere to epithelial cells of the gastrointestinal tract because lectins on the *E. coli* surface recognize oligosaccharide units on the surfaces of target cells. These lectins are located on slender hairlike appendages called *fimbriae* (*pili*).

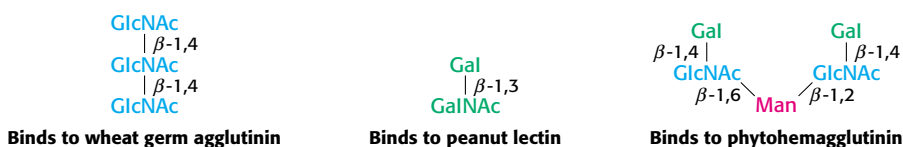


FIGURE 11.28 Binding selectivities of plant lectins. The plant lectins wheat germ agglutinin, peanut lectin, and phytohemagglutinin recognize different oligosaccharides.

Lectins can be divided into classes on the basis of their amino acid sequences and biochemical properties. One large class is the C type (for calcium-requiring) found in animals. These proteins have in common a domain of 120 amino acids that is responsible for carbohydrate binding. The structure of one such domain bound to a carbohydrate target is shown in Figure 11.29.

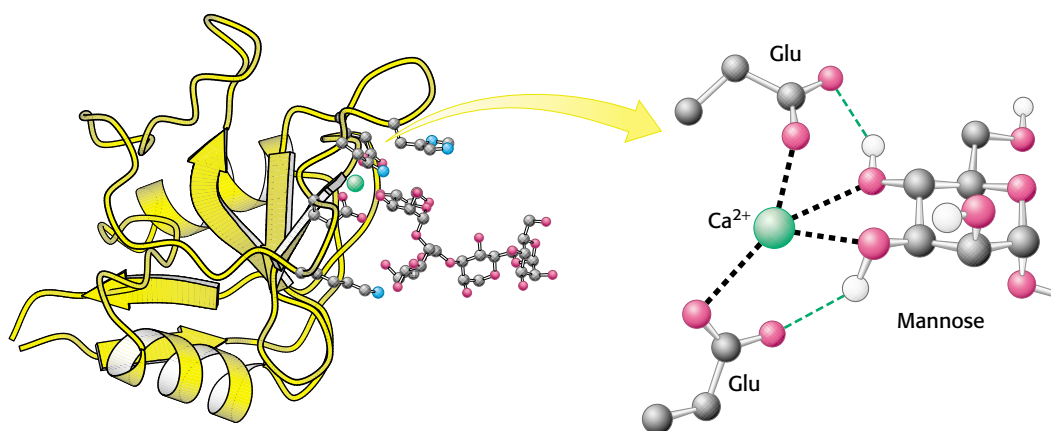


FIGURE 11.29 Structure of a C-type carbohydrate-binding domain from an animal lectin. A calcium ion links a mannose residue to the lectin. Selected interactions are shown, with some hydrogen atoms omitted for clarity.

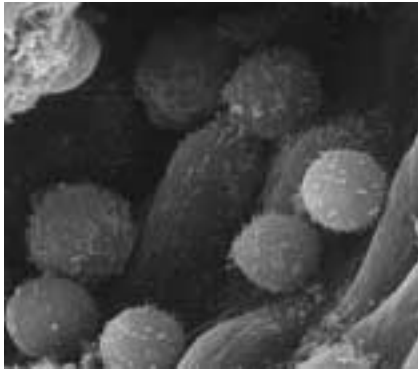


FIGURE 11.30 Selectins mediate cell-cell interactions. The scanning electron micrograph shows lymphocytes adhering to the endothelial lining of a lymph node. The L selectins on the lymphocyte surface bind specifically to carbohydrates on the lining of the lymph-node vessels. [Courtesy of Dr. Eugene Butcher.]

A calcium ion acts as a bridge between the protein and the sugar through direct interactions with sugar hydroxyl groups. In addition, two glutamate residues in the protein bind to both the calcium ion and the sugar, while other protein side chains form hydrogen bonds with other hydroxyl groups on the carbohydrate. Changes in the amino acid residues that interact with the carbohydrate alter the carbohydrate-binding specificity of the lectin.

Proteins termed *selectins* are members of the C-type family. Selectins bind immune-system cells to the sites of injury in the inflammatory response (Figure 11.30). The L, E, and P forms of selectins bind specifically to carbohydrates on lymph-node vessels, endothelium, or activated blood platelets, respectively. New therapeutic agents that control inflammation may emerge from a deeper understanding of how selectins bind and distinguish different carbohydrates.

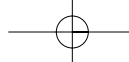
11.4.2 Influenza Virus Binds to Sialic Acid Residues

The ability of viruses to infect specific cell types is dictated in part by the ability of these viruses to bind to particular structures or receptors on the surfaces of cells. In some cases, these receptors are carbohydrates. For example, influenza virus recognizes sialic acid residues present on cell-surface glycoproteins. The viral protein that binds to these sugars is called *hemagglutinin* (Figure 11.31).

After these surface interactions have taken place and the virus has been taken into the cell, another viral protein, *neuramidase*, cleaves the glycosidic bonds to the sialic acid residues, freeing the virus to infect the cell. Inhibitors of this enzyme are showing some promise as anti-influenza agents.



FIGURE 11.31 Structure of a part of influenza hemagglutinin. This viral protein has multiple binding sites for linking to sialic acid residues on the target-cell surface.

**SUMMARY**

- **Monosaccharides Are Aldehydes or Ketones with Multiple Hydroxyl Groups**

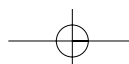
An aldose is a carbohydrate with an aldehyde group (as in glyceraldehyde and glucose), whereas a ketose contains a keto group (as in dihydroxyacetone and fructose). A sugar belongs to the D series if the absolute configuration of its asymmetric carbon farthest from the aldehyde or keto group is the same as that of D-glyceraldehyde. Most naturally occurring sugars belong to the D series. The C-1 aldehyde in the open-chain form of glucose reacts with the C-5 hydroxyl group to form a six-membered pyranose ring. The C-2 keto group in the open-chain form of fructose reacts with the C-5 hydroxyl group to form a five-membered furanose ring. Pentoses such as ribose and deoxyribose also form furanose rings. An additional asymmetric center is formed at the anomeric carbon atom (C-1 in aldoses and C-2 in ketoses) in these cyclizations. The hydroxyl group attached to the anomeric carbon atom is below the plane of the ring (viewed in the standard orientation) in the α anomer, whereas it is above the ring in the β anomer. Not all the atoms in the rings lie in the same plane. Rather, pyranose rings usually adopt the chair conformation, and furanose rings usually adopt the envelope conformation. Sugars are joined to alcohols and amines by glycosidic bonds from the anomeric carbon atom. For example, N-glycosidic bonds link sugars to purines and pyrimidines in nucleotides, RNA, and DNA.

- **Complex Carbohydrates Are Formed by Linkage of Monosaccharides**

Sugars are linked to one another in disaccharides and polysaccharides by O-glycosidic bonds. Sucrose, lactose, and maltose are the common disaccharides. Sucrose (common table sugar), obtained from cane or beet, consists of α -glucose and β -fructose joined by a glycosidic linkage between their anomeric carbon atoms. Lactose (in milk) consists of galactose joined to glucose by a β -1,4 linkage. Maltose (from starch) consists of two glucoses joined by an α -1,4 linkage. Starch is a polymeric form of glucose in plants, and glycogen serves a similar role in animals. Most of the glucose units in starch and glycogen are in α -1,4 linkage. Glycogen has more branch points formed by α -1,6 linkages than does starch, which makes glycogen more soluble. Cellulose, the major structural polymer of plant cell walls, consists of glucose units joined by β -1,4 linkages. These β linkages give rise to long straight chains that form fibrils with high tensile strength. In contrast, the α linkages in starch and glycogen lead to open helices, in keeping with their roles as mobilizable energy stores. Cell surfaces and extracellular matrices of animals contain polymers of repeating disaccharides called glycosaminoglycans. One of the units in each repeat is a derivative of glucosamine or galactosamine. These highly anionic carbohydrates have a high density of carboxylate or sulfate groups. Proteins bearing covalently linked glycosaminoglycans are termed proteoglycans.

- **Carbohydrates Can Attach to Proteins to Form Glycoproteins**

Specific enzymes link the oligosaccharide units on proteins either to the side-chain oxygen atom of a serine or threonine residue or to the side-chain amide nitrogen atom of an asparagine residue. Protein glycosylation takes place in the lumen of the endoplasmic reticulum. The N-linked oligosaccharides are synthesized on dolichol phosphate and subsequently transferred to the protein acceptor. Additional sugars are attached in the Golgi complex to form diverse patterns.



• Lectins Are Specific Carbohydrate-Binding Proteins

Carbohydrates are recognized by proteins called lectins, which are found in animals, plants, and microorganisms. In animals, the interplay of lectins and their sugar targets guides cell–cell contact. The viral protein hemagglutinin on the surface of the influenza virus recognizes sialic acid residues on the surfaces of the cells invaded by the virus. A small number of carbohydrate residues can be joined in many different ways to form highly diverse patterns that can be distinguished by the lectin domains of protein receptors.

KEY TERMS

monosaccharide (p. 296)	hemiketal (p. 298)	proteoglycan (p. 304)
triose (p. 296)	furanose (p. 299)	glycosyltransferase (p. 304)
ketose (p. 296)	anomer (p. 299)	glycoprotein (p. 306)
aldose (p. 296)	glycosidic bond (p. 301)	endoplasmic reticulum (p. 307)
enantiomer (p. 296)	reducing sugar (p. 301)	Golgi complex (p. 307)
tetrose (p. 296)	nonreducing sugar (p. 301)	dolichol phosphate (p. 308)
pentose (p. 296)	oligosaccharide (p. 301)	lectin (p. 312)
hexose (p. 296)	disaccharide (p. 302)	selectin (p. 314)
heptose (p. 296)	polysaccharide (p. 302)	
diastereoisomer (p. 296)	glycogen (p. 302)	
epimer (p. 297)	starch (p. 303)	
hemiacetal (p. 298)	cellulose (p. 303)	
pyranose (p. 298)	glycosaminoglycan (p. 304)	

SELECTED READINGS

Where to start

- Sharon, N., and Lis, H., 1993. Carbohydrates in cell recognition. *Sci. Am.* 268(1):82–89.
- Lasky, L. A., 1992. Selectins: Interpreters of cell-specific carbohydrate information during inflammation. *Science* 258:964–969.
- Weiss, P., and Ashwell, G., 1989. The asialoglycoprotein receptor: Properties and modulation by ligand. *Prog. Clin. Biol. Res.* 300:169–184.
- Sharon, N., 1980. Carbohydrates. *Sci. Am.* 245(5):90–116.
- Paulson, J. C., 1989. Glycoproteins: What are the sugar side chains for? *Trends Biochem. Sci.* 14:272–276.
- Woods, R. J., 1995. Three-dimensional structures of oligosaccharides. *Curr. Opin. Struct. Biol.* 5:591–598.

Books

- Varki, A., Cummings, R., Esko, J., Freeze, H., Hart, G., and Marth, J., 1999. *Essentials of Glycobiology*. Cold Spring Harbor Laboratory Press.
- Fukuda, M., and Hindsgaul, O., 2000. *Molecular Glycobiology*. IRL Press at Oxford University Press.
- El Khadem, H. S., 1988. *Carbohydrate Chemistry*. Academic Press.
- Ginsburg, V., and Robbins, P. W. (Eds.), 1981. *Biology of Carbohydrates* (vols. 1–3). Wiley.
- Fukuda, M. (Ed.), 1992. *Cell Surface Carbohydrates and Cell Development*. CRC Press.
- Preiss, J. (Ed.), 1988. *The Biochemistry of Plants: A Comprehensive Treatise: Carbohydrates*. Academic Press.

Structure of carbohydrate-binding proteins

- Ünlügil, U., and Rini, J. M., 2000. Glycosyltransferase structure and mechanism. *Curr. Opin. Struct. Biol.* 10:510–517.

- Bouckaert, J., Hamelryck, T., Wyns, L., and Loris, R., 1999. Novel structures of plant lectins and their complexes with carbohydrates. *Curr. Opin. Struct. Biol.* 9:572–577.
- Weis, W. I., and Drickamer, K., 1996. Structural basis of lectin-carbohydrate recognition. *Annu. Rev. Biochem.* 65:441–473.
- Vyas, N. K., 1991. Atomic features of protein-carbohydrate interactions. *Curr. Opin. Struct. Biol.* 1:732–740.
- Weis, W. I., Drickamer, K., and Hendrickson, W. A., 1992. Structure of a C-type mannose-binding protein complexed with an oligosaccharide. *Nature* 360:127–134.
- Wright, C. S., 1992. Crystal structure of a wheat germ agglutinin/glycophorin-sialoglycopeptide receptor complex: Structural basis for cooperative lectin-cell binding. *J. Biol. Chem.* 267:14345–14352.
- Shaanan, B., Lis, H., and Sharon, N., 1991. Structure of a legume lectin with an ordered N-linked carbohydrate in complex with lactose. *Science* 254:862–866.

Glycoproteins

- Spiro, R. G., 2000. Glucose residues as key determinants in the biosynthesis and quality control of glycoproteins with N-linked oligosaccharides. *J. Biol. Chem.* 275:35657–35660.
- Bernfield, M., Götte, M., Park, P. W., Reizes, O., Fitzgerald, M. L., Lincecum, J., and Zako, M., 1999. Functions of cell surface heparan sulfate proteoglycans. *Annu. Rev. Biochem.* 68:729–777.
- Iozzo, R. V., 1998. Matrix proteoglycans: From molecular design to cellular function. *Annu. Rev. Biochem.* 67:609–652.
- Trombetta, E. S., and Helenius, A., 1998. Lectins as chaperones in glycoprotein folding. *Curr. Opin. Struct. Biol.* 8:587–592.
- Yanagishita, M., and Hascall, V. C., 1992. Cell surface heparan sulfate proteoglycans. *J. Biol. Chem.* 267:9451–9454.
- Iozzo, R. V., 1999. The biology of small leucine-rich proteoglycans: Functional network of interactive proteins. *J. Biol. Chem.* 274:18843–18846.

Carbohydrates in recognition processes

- Weis, W. I., 1997. Cell-surface carbohydrate recognition by animal and viral lectins. *Curr. Opin. Struct. Biol.* 7:624–630.
- Sharon, N., and Lis, H., 1989. Lectins as cell recognition molecules. *Science* 246:227–234.
- Turner, M. L., 1992. Cell adhesion molecules: A unifying approach to topographic biology. *Biol. Rev. Camb. Philos. Soc.* 67:359–377.
- Feizi, T., 1992. Blood group-related oligosaccharides are ligands in cell-adhesion events. *Biochem. Soc. Trans.* 20:274–278.
- Jessell, T. M., Hynes, M. A., and Dodd, J., 1990. Carbohydrates and carbohydrate-binding proteins in the nervous system. *Annu. Rev. Neurosci.* 13:227–255.

Clothia, C., and Jones, E. V., 1997. The molecular structure of cell adhesion molecules. *Annu. Rev. Biochem.* 66:823–862.

Carbohydrate sequencing

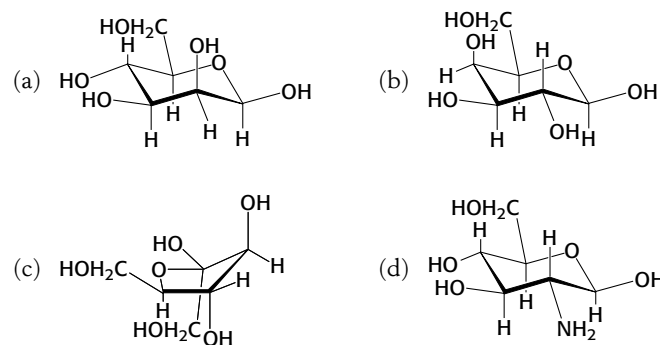
- Venkataraman, G., Shriver, Z., Raman, R., and Sasisekharan, R., 1999. Sequencing complex polysaccharides. *Science* 286:537–542.
- Zhao, Y., Kent, S. B. H., and Chait, B. T., 1997. Rapid, sensitive structure analysis of oligosaccharides. *Proc. Natl. Acad. Sci. U.S.A.* 94:1629–1633.
- Rudd, P. M., Guile, G. R., Küster, B., Harvey, D. J., Opdenakker, G., and Dwek, R. A. 1997. Oligosaccharide sequencing technology. *Nature* 388:205–207.

PROBLEMS

- Word origin.** Account for the origin of the term *carbohydrate*.
- Diversity.** How many different oligosaccharides can be made by linking one glucose, one mannose, and one galactose? Assume that each sugar is in its pyranose form. Compare this number with the number of tripeptides that can be made from three different amino acids.
- Couples.** Indicate whether each of the following pairs of sugars consists of anomers, epimers, or an aldose-ketose pair:
 - D-glyceraldehyde and dihydroxyacetone
 - D-glucose and D-mannose
 - D-glucose and D-fructose
 - α -D-glucose and β -D-glucose
 - D-ribose and D-ribulose
 - D-galactose and D-glucose
- Tollen's test.** Glucose and other aldoses are oxidized by an aqueous solution of a silver-ammonia complex. What are the reaction products?
- Mutarotation.** The specific rotations of the α and β anomers of D-glucose are +112 degrees and +18.7 degrees, respectively. Specific rotation, $[\alpha]_D$, is defined as the observed rotation of light of wavelength 589 nm (the D line of a sodium lamp) passing through 10 cm of a 1 g ml⁻¹ solution of a sample. When a crystalline sample of α -D-glucopyranose is dissolved in water, the specific rotation decreases from 112 degrees to an equilibrium value of 52.7 degrees. On the basis of this result, what are the proportions of the α and β anomers at equilibrium? Assume that the concentration of the open-chain form is negligible.
- Telltale adduct.** Glucose reacts slowly with hemoglobin and other proteins to form covalent compounds. Why is glucose reactive? What is the nature of the adduct formed?
- Periodate cleavage.** Compounds containing hydroxyl groups on adjacent carbon atoms undergo carbon-carbon bond cleavage when treated with periodate ion (IO₄⁻). How can this reaction be used to distinguish between pyranosides and furanosides?

8. **Oxygen source.** Does the oxygen atom attached to C-1 in methyl α -D-glucopyranoside come from glucose or methanol?

9. **Sugar lineup.** Identify the following four sugars.

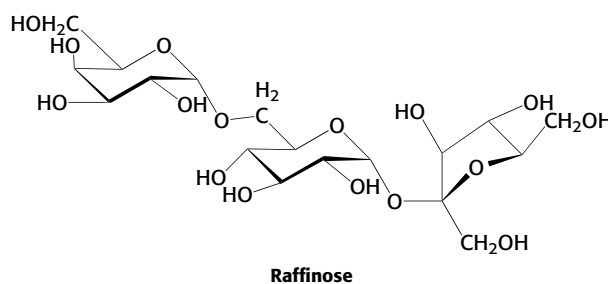


10. **Cellular glue.** A trisaccharide unit of a cell-surface glycoprotein is postulated to play a critical role in mediating cell-cell adhesion in a particular tissue. Design a simple experiment to test this hypothesis.

11. **Mapping the molecule.** Each of the hydroxyl groups of glucose can be methylated with reagents such as dimethylsulfate under basic conditions. Explain how exhaustive methylation followed by complete digestion of a known amount of glycogen would enable you to determine the number of branch points and reducing ends.

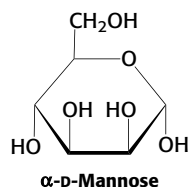
12. **Component parts.** Raffinose is a trisaccharide and a minor constituent in sugar beets.

- Is raffinose a reducing sugar? Explain.
- What are the monosaccharides that compose raffinose?
- β -Galactosidase is an enzyme that will remove galactose residues from an oligosaccharide. What are the products of β -galactosidase treatment of raffinose?



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13. *Anomeric differences.* α -D-Mannose is a sweet-tasting sugar. β -D-Mannose, on the other hand, tastes bitter. A pure solution of α -D-mannose loses its sweet taste with time as it is converted into the β anomer. Draw the β anomer and explain how it is formed from the α anomer.



14. *A taste of honey.* Fructose in its β -D-pyranose form accounts for the powerful sweetness of honey. The β -D-furanose form, although sweet, is not as sweet as the pyranose form. The furanose form is the more stable form. Draw the two forms and explain why it may not always be wise to cook with honey.

15. *Making ends meet.* (a) Compare the number of reducing ends to nonreducing ends in a molecule of glycogen. (b) As we will

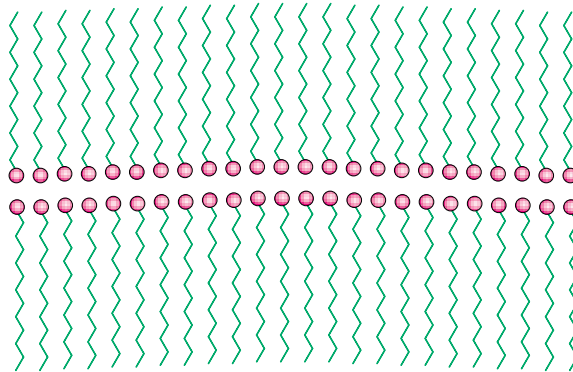
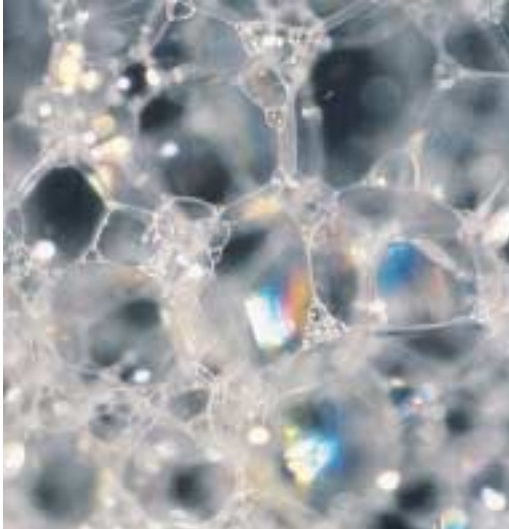
see in Chapter 21, glycogen is an important fuel storage form that is rapidly mobilized. At which end—the reducing or nonreducing—would you expect most metabolism to take place?

16. *Carbohydrates and proteomics.* Suppose that a protein contains six potential *N*-linked glycosylation sites. How many possible proteins can be generated, depending on which of these sites is actually glycosylated? Do not include the effects of diversity within the carbohydrate added.

Chapter Integration Problem

17. *Stereospecificity.* Sucrose, a major product of photosynthesis in green leaves, is synthesized by a battery of enzymes. The substrates for sucrose synthesis, D-glucose and D-fructose, are a mixture of α and β anomers as well as acyclic compounds in solution. Nonetheless, sucrose consists of α -D-glucose linked by its carbon-1 atom to the carbon-2 atom of β -D-fructose. How can the specificity of sucrose be explained in light of the potential substrates?

Lipids and Cell Membranes



The surface of a soap bubble is a bilayer formed by detergent molecules. The polar heads (red) pack together leaving the hydrophobic groups (green) in contact with air on the inside and outside of the bubble. Other bilayer structures define the boundary of a cell. [(Left) Photonica.]

The boundaries of cells are formed by *biological membranes*, the barriers that *define the inside and the outside of a cell* (Figure 12.1). These barriers prevent molecules generated inside the cell from leaking out and unwanted molecules from diffusing in; yet they also contain transport systems that allow specific molecules to be taken up and unwanted compounds to be removed from the cell. Such transport systems confer on membranes the important property of *selective permeability*.

Membranes are dynamic structures in which proteins float in a sea of lipids. The lipid components of the membrane form the permeability barrier, and protein components act as a transport system of pumps and channels that endow the membrane with selective permeability.

In addition to an external cell membrane (called the plasma membrane), eukaryotic cells also contain internal membranes that form the boundaries of organelles such as mitochondria, chloroplasts, peroxisomes, and lysosomes. Functional specialization in the course of evolution has been closely linked to the formation of such compartments. Specific systems have evolved to allow targeting of selected proteins into or through particular internal membranes and, hence, into specific organelles. External and internal membranes have essential features in common, and these essential features are the subject of this chapter.

OUTLINE

- 12.1 Many Common Features Underlie the Diversity of Biological Membranes
- 12.2 Fatty Acids Are Key Constituents of Lipids
- 12.3 There Are Three Common Types of Membrane Lipids
- 12.4 Phospholipids and Glycolipids Readily Form Bimolecular Sheets in Aqueous Media
- 12.5 Proteins Carry Out Most Membrane Processes
- 12.6 Lipids and Many Membrane Proteins Diffuse Rapidly in the Plane of the Membrane
- 12.7 Eukaryotic Cells Contain Compartments Bounded by Internal Membranes

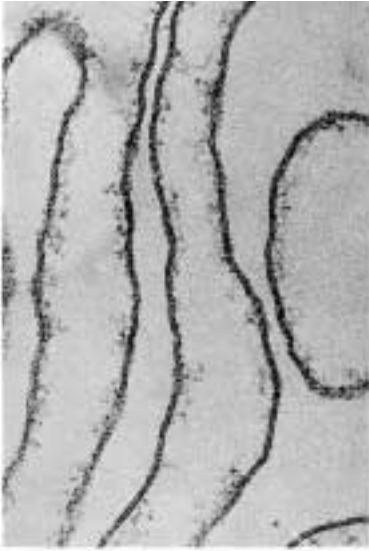


FIGURE 12.1 Red-blood-cell plasma membrane. An electron micrograph of a preparation of plasma membranes from red blood cells showing the membranes as seen “on edge,” in cross section. [Courtesy of Dr. Vincent Marchesi.]

Biological membranes serve several additional important functions indispensable for life, such as energy storage and information transduction, that are dictated by the proteins associated with them. In this chapter, we will examine the general properties of membrane proteins—how they can exist in the hydrophobic environment of the membrane while connecting two hydrophilic environments—and delay a discussion of the functions of these proteins to the next and later chapters.

12.1 MANY COMMON FEATURES UNDERLIE THE DIVERSITY OF BIOLOGICAL MEMBRANES

Membranes are as diverse in structure as they are in function. However, they do have in common a number of important attributes:

1. Membranes are *sheetlike structures*, only two molecules thick, that form *closed boundaries* between different compartments. The thickness of most membranes is between 60 \AA (6 nm) and 100 \AA (10 nm).
2. Membranes consist mainly of *lipids* and *proteins*. Their mass ratio ranges from 1:4 to 4:1. Membranes also contain *carbohydrates* that are linked to lipids and proteins.
3. Membrane lipids are relatively small molecules that have both *hydrophilic* and *hydrophobic* moieties. These lipids spontaneously form *closed bimolecular sheets* in aqueous media. These *lipid bilayers* are barriers to the flow of polar molecules.
4. *Specific proteins mediate distinctive functions of membranes*. Proteins serve as pumps, channels, receptors, energy transducers, and enzymes. Membrane proteins are embedded in lipid bilayers, which create suitable environments for their action.
5. Membranes are *noncovalent assemblies*. The constituent protein and lipid molecules are held together by many noncovalent interactions, which are cooperative.
6. Membranes are *asymmetric*. The two faces of biological membranes always differ from each other.
7. Membranes are *fluid structures*. Lipid molecules diffuse rapidly in the plane of the membrane, as do proteins, unless they are anchored by specific interactions. In contrast, lipid molecules and proteins do not readily rotate across the membrane. Membranes can be regarded as *two-dimensional solutions of oriented proteins and lipids*.
8. Most cell membranes are *electrically polarized*, such that the inside is negative [typically -60 millivolts (mV)]. Membrane potential plays a key role in transport, energy conversion, and excitability (Chapter 13).

12.2 FATTY ACIDS ARE KEY CONSTITUENTS OF LIPIDS

Among the most biologically significant properties of lipids are their hydrophobic properties. These properties are mainly due to a particular component of lipids: fatty acids, or simply fats. Fatty acids also play important roles in signal-transduction pathways (Sections 15.x. x and 22.x.x).

12.2.1 The Naming of Fatty Acids

Fatty acids are hydrocarbon chains of various lengths and degrees of unsaturation that terminate with carboxylic acid groups. The systematic name

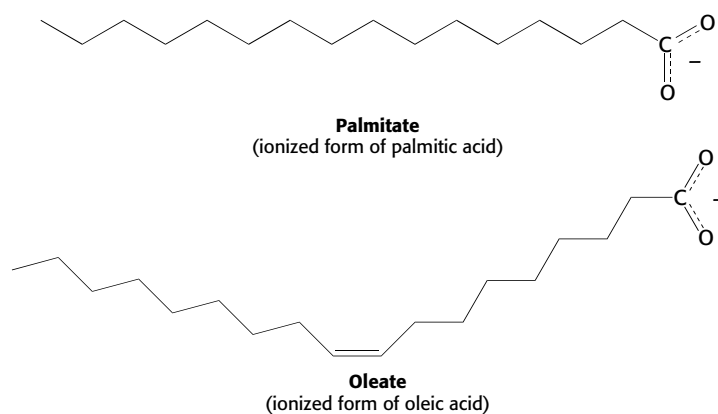
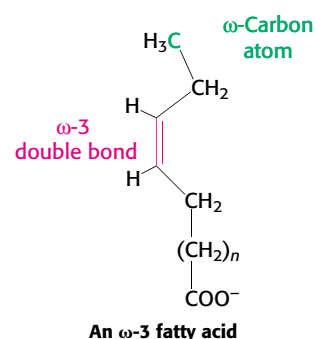
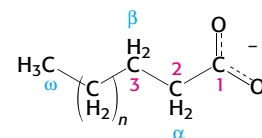


FIGURE 12.2 Structures of two fatty acids. Palmitate is a 16-carbon, saturated fatty acid, and oleate is an 18-carbon fatty acid with a single cis double bond.

for a fatty acid is derived from the name of its parent hydrocarbon by the substitution of *oic* for the final *e*. For example, the C_{18} saturated fatty acid is called *octadecanoic acid* because the parent hydrocarbon is octadecane. A C_{18} fatty acid with one double bond is called *octadecenoic acid*; with two double bonds, *octadecadienoic acid*; and with three double bonds, *octadecatrienoic acid*. The notation 18:0 denotes a C_{18} fatty acid with no double bonds, whereas 18:2 signifies that there are two double bonds. The structures of the ionized forms of two common fatty acids—palmitic acid (C_{16} , saturated) and oleic acid (C_{18} , monounsaturated)—are shown in Figure 12.2.

Fatty acid carbon atoms are numbered starting at the carboxyl terminus, as shown in the margin. Carbon atoms 2 and 3 are often referred to as α and β , respectively. The methyl carbon atom at the distal end of the chain is called the ω -carbon atom. The position of a double bond is represented by the symbol Δ followed by a superscript number. For example, *cis*- Δ^9 means that there is a cis double bond between carbon atoms 9 and 10; *trans*- Δ^2 means that there is a trans double bond between carbon atoms 2 and 3. Alternatively, the position of a double bond can be denoted by counting from the distal end, with the ω -carbon atom (the methyl carbon) as number 1. An ω -3 fatty acid, for example, has the structure shown in the margin. Fatty acids are ionized at physiological pH, and so it is appropriate to refer to them according to their carboxylate form: for example, palmitate or hexadecanoate.



12.2.2 Fatty Acids Vary in Chain Length and Degree of Unsaturation

Fatty acids in biological systems usually contain an even number of carbon atoms, typically between 14 and 24 (Table 12.1). The 16- and 18-carbon fatty acids are most common. The hydrocarbon chain is almost invariably unbranched in animal fatty acids. The alkyl chain may be saturated or it may contain one or more double bonds. The configuration of the double bonds in most unsaturated fatty acids is *cis*. The double bonds in polyunsaturated fatty acids are separated by at least one methylene group.

The properties of fatty acids and of lipids derived from them are markedly dependent on chain length and degree of saturation. Unsaturated fatty acids have lower melting points than saturated fatty acids of the same length. For example, the melting point of stearic acid is 69.6°C , whereas that of oleic acid (which contains one *cis* double bond) is 13.4°C . The melting points of polyunsaturated fatty acids of the C_{18} series are even lower. Chain length also affects the melting point, as illustrated by the fact that the melting temperature of palmitic acid (C_{16}) is 6.5 degrees lower than that of stearic acid (C_{18}). Thus, *short chain length and unsaturation enhance the fluidity of fatty acids and of their derivatives.*

TABLE 12.1 Some naturally occurring fatty acids in animals

Number of carbons	Number of double bonds	Common name	Systematic name	Formula
12	0	Laurate	<i>n</i> -Dodecanoate	CH ₃ (CH ₂) ₁₀ COO ⁻
14	0	Myristate	<i>n</i> -Tetradecanoate	CH ₃ (CH ₂) ₁₂ COO ⁻
16	0	Palmitate	<i>n</i> -Hexadecanoate	CH ₃ (CH ₂) ₁₄ COO ⁻
18	0	Stearate	<i>n</i> -Octadecanoate	CH ₃ (CH ₂) ₁₆ COO ⁻
20	0	Arachidate	<i>n</i> -Eicosanoate	CH ₃ (CH ₂) ₁₈ COO ⁻
22	0	Behenate	<i>n</i> -Docosanoate	CH ₃ (CH ₂) ₂₀ COO ⁻
24	0	Lignocerate	<i>n</i> -Tetracosanoate	CH ₃ (CH ₂) ₂₂ COO ⁻
16	1	Palmitoleate	<i>cis</i> -Δ ⁹ -Hexadecenoate	CH ₃ (CH ₂) ₅ CH=CH(CH ₂) ₇ COO ⁻
18	1	Oleate	<i>cis</i> -Δ ⁹ -Octadecenoate	CH ₃ (CH ₂) ₇ CH=CH(CH ₂) ₇ COO ⁻
18	2	Linoleate	<i>cis,cis</i> -Δ ⁹ ,Δ ¹² - Octadecadienoate	CH ₃ (CH ₂) ₄ (CH=CHCH ₂) ₂ (CH ₂) ₆ COO ⁻
18	3	Linolenate	<i>all-cis</i> -Δ ⁹ ,Δ ¹² ,Δ ¹⁵ - Octadecatrienoate	CH ₃ CH ₂ (CH=CHCH ₂) ₃ (CH ₂) ₆ COO ⁻
20	4	Arachidonate	<i>all-cis</i> -Δ ⁵ ,Δ ⁸ ,Δ ¹¹ ,Δ ¹⁴ - Eicosatetraenoate	CH ₃ (CH ₂) ₄ (CH=CHCH ₂) ₄ (CH ₂) ₂ COO ⁻

12.3 THERE ARE THREE COMMON TYPES OF MEMBRANE LIPIDS

Lipids differ markedly from the other groups of biomolecules considered thus far. By definition, *lipids are water-insoluble biomolecules that are highly soluble in organic solvents such as chloroform*. Lipids have a variety of biological roles: they serve as fuel molecules, highly concentrated energy stores, signal molecules, and components of membranes. The first three roles of lipids will be discussed in later chapters. Here, our focus is on lipids as membrane constituents. The three major kinds of membrane lipids are *phospholipids*, *glycolipids*, and *cholesterol*. We begin with lipids found in eukaryotes and bacteria. The lipids in archaea are distinct, although they have many features related to their membrane-forming function in common with lipids of other organisms.

12.3.1 Phospholipids Are the Major Class of Membrane Lipids

Phospholipids are abundant in all biological membranes. A phospholipid molecule is constructed from four components: fatty acids, a platform to which the fatty acids are attached, a phosphate, and an alcohol attached to the phosphate (Figure 12.3). The fatty acid components provide a hydrophobic barrier, whereas the remainder of the molecule has hydrophilic properties to enable interaction with the environment.

The platform on which phospholipids are built may be *glycerol*, a 3-carbon alcohol, or *sphingosine*, a more complex alcohol. Phospholipids derived from glycerol are called *phosphoglycerides*. A phosphoglyceride consists of a glycerol backbone to which two fatty acid chains (whose characteristics were described in Section 12.2.2) and a phosphorylated alcohol are attached.

In phosphoglycerides, the hydroxyl groups at C-1 and C-2 of glycerol are esterified to the carboxyl groups of the two fatty acid chains. The C-3 hydroxyl group of the glycerol backbone is esterified to phosphoric acid. When no further additions are made, the resulting compound is *phosphatidate* (*diacylglycerol 3-phosphate*), the simplest phosphoglyceride. Only small

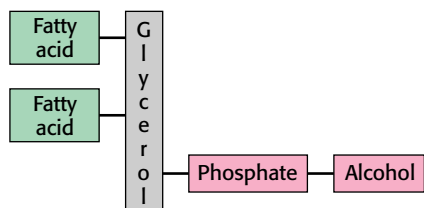


FIGURE 12.3 Schematic structure of a phospholipid.

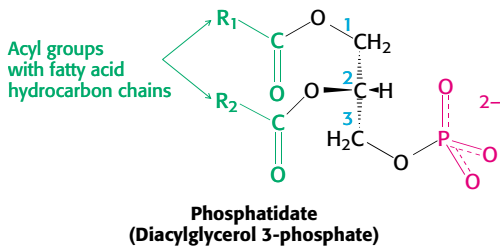
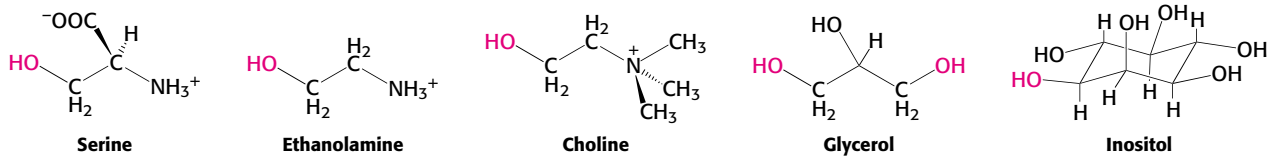


FIGURE 12.4 Structure of phosphatidate (diacylglycerol 3-phosphate). The absolute configuration of the center carbon (C-2) is shown.

amounts of phosphatidate are present in membranes. However, the molecule is a key intermediate in the biosynthesis of the other phosphoglycerides (Section 26.x.x). The absolute configuration of the glycerol 3-phosphate moiety of membrane lipids is shown in Figure 12.4.

The major phosphoglycerides are derived from phosphatidate by the formation of an ester bond between the phosphate group of phosphatidate and the hydroxyl group of one of several alcohols. The common alcohol moieties of phosphoglycerides are the amino acid serine, ethanolamine, choline, glycerol, and the inositol.



The structural formulas of phosphatidyl choline and the other principal phosphoglycerides—namely, phosphatidyl ethanolamine, phosphatidyl serine, phosphatidyl inositol, and diphosphatidyl glycerol—are given in Figure 12.5.

Sphingomyelin is a phospholipid found in membranes that is not derived from glycerol. Instead, the backbone in sphingomyelin is *sphingosine*, an

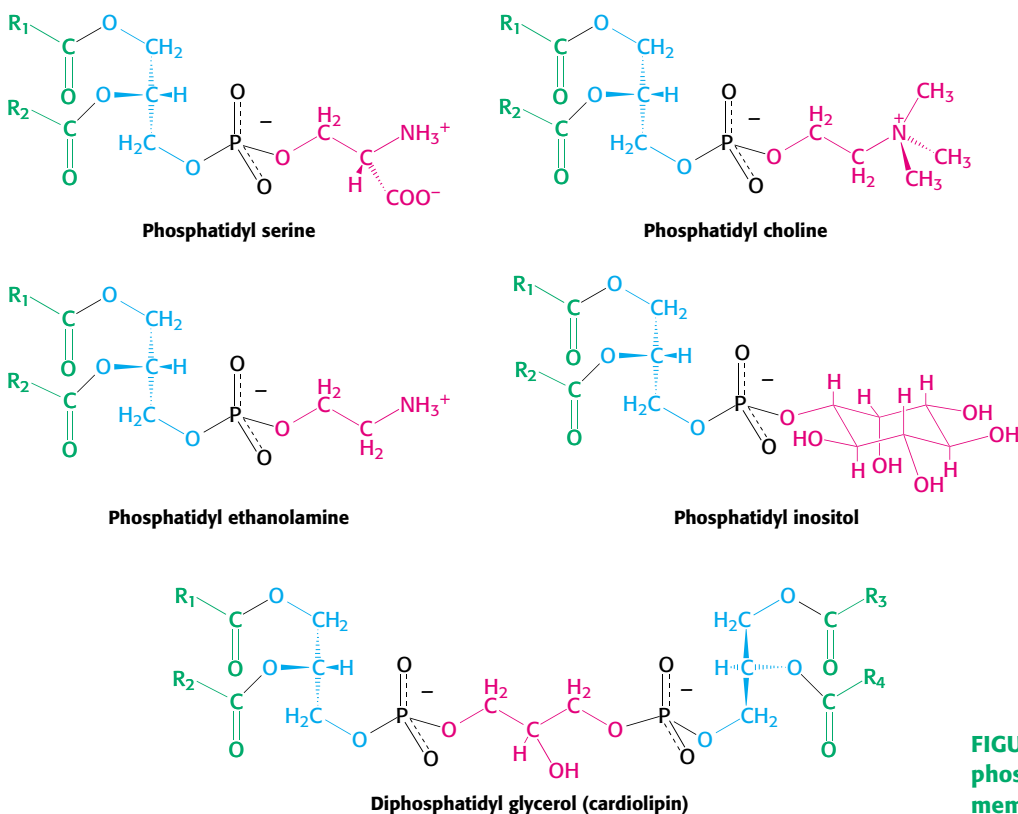


FIGURE 12.5 Some common phosphoglycerides found in membranes.

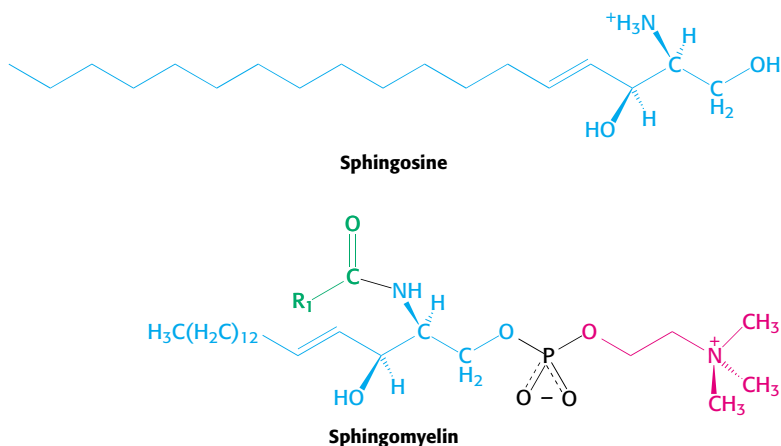


FIGURE 12.6 Structures of sphingosine and sphingomyelin. The sphingosine moiety of sphingomyelin is highlighted in blue.

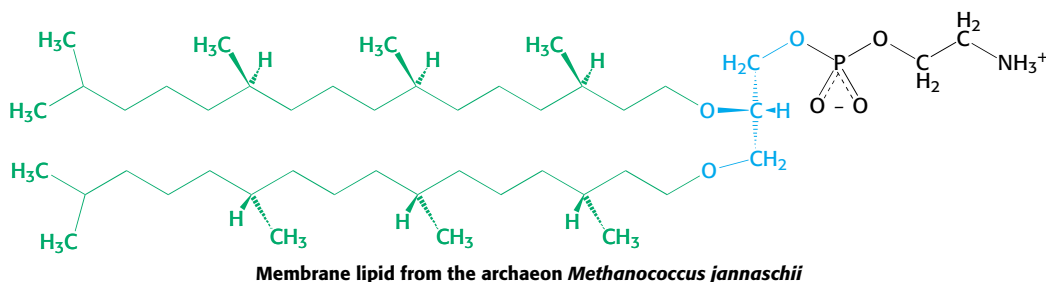


FIGURE 12.7 An archaeon and its environment. Archaea can thrive in habitats as harsh as a volcanic vent. Here, the archaea form an orange mat surrounded by yellow sulfurous deposits. [Krafft-Explorer/Photo Researchers.]

amino alcohol that contains a long, unsaturated hydrocarbon chain (Figure 12.6). In sphingomyelin, the amino group of the sphingosine backbone is linked to a fatty acid by an amide bond. In addition, the primary hydroxyl group of sphingosine is esterified to phosphoryl choline.

12.3.2 Archaeal Membranes Are Built from Ether Lipids with Branched Chains

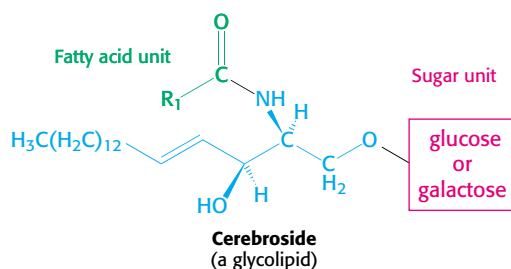
The membranes of archaea differ in composition from those of eukaryotes or bacteria in three important ways. Two of these differences clearly relate to the hostile living conditions of many archaea (Figure 12.7). First, the non-polar chains are joined to a glycerol backbone by *ether* rather than ester linkages. The ether linkage is more resistant to hydrolysis. Second, the alkyl chains are *branched* rather than linear. They are built up from repeats of a fully saturated five-carbon fragment. These branched, saturated hydrocarbons are more resistant to oxidation. The ability of archaeal lipids to resist hydrolysis and oxidation may help these organisms to withstand the extreme conditions, such as high temperature, low pH, or high salt concentration, under which some of these archaea grow. Finally, the stereochemistry of the central glycerol is inverted compared with that shown in Figure 12.4.



12.3.3 Membrane Lipids Can Also Include Carbohydrate Moieties

Glycolipids, as their name implies, are *sugar-containing lipids*. Like sphingomyelin, the glycolipids in animal cells are derived from sphingosine. The amino group of the sphingosine backbone is acylated by a fatty acid, as in sphingomyelin. Glycolipids differ from sphingomyelin in the identity of the unit that is linked to the primary hydroxyl group of the sphingosine backbone. In glycolipids, one or more sugars (rather than phosphoryl choline)

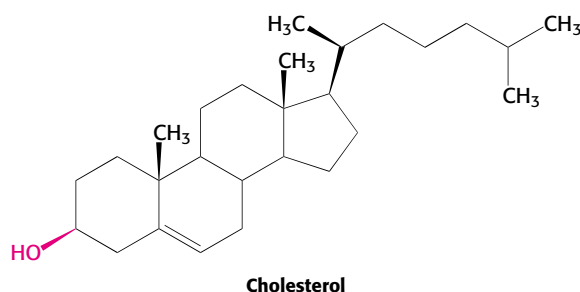
are attached to this group. The simplest glycolipid, called a *cerebroside*, contains a single sugar residue, either glucose or galactose.



More complex glycolipids, such as *gangliosides*, contain a branched chain of as many as seven sugar residues. Glycolipids are oriented in a completely asymmetric fashion with the *sugar residues always on the extracellular side of the membrane*.

12.3.4 Cholesterol Is a Lipid Based on a Steroid Nucleus

Cholesterol is a lipid with a structure quite different from that of phospholipids. It is a steroid, built from four linked hydrocarbon rings.



A hydrocarbon tail is linked to the steroid at one end, and a hydroxyl group is attached at the other end. In membranes, the molecule is oriented parallel to the fatty acid chains of the phospholipids, and the hydroxyl group interacts with the nearby phospholipid head groups. Cholesterol is absent from prokaryotes but is found to varying degrees in virtually all animal membranes. It constitutes almost 25% of the membrane lipids in certain nerve cells but is essentially absent from some intracellular membranes.

12.3.5 A Membrane Lipid Is an Amphipathic Molecule Containing a Hydrophilic and a Hydrophobic Moiety

The repertoire of membrane lipids is extensive, perhaps even bewildering, at first sight. However, they possess a critical common structural theme: *membrane lipids are amphipathic molecules* (amphiphilic molecules). A membrane lipid contains both a *hydrophilic* and a *hydrophobic* moiety.

Let us look at a model of a phosphoglyceride, such as phosphatidyl choline. Its overall shape is roughly rectangular (Figure 12.8A). The two hydrophobic fatty acid chains are approximately parallel to each other, whereas the hydrophilic phosphoryl choline moiety points in the opposite direction. Sphingomyelin has a similar conformation, as does the archaeal lipid depicted. Therefore, the following shorthand has been adopted to represent these membrane lipids: the hydrophilic unit, also called the *polar head group*, is represented by a circle, whereas the hydrocarbon tails are depicted by straight or wavy lines (Figure 12.8B).

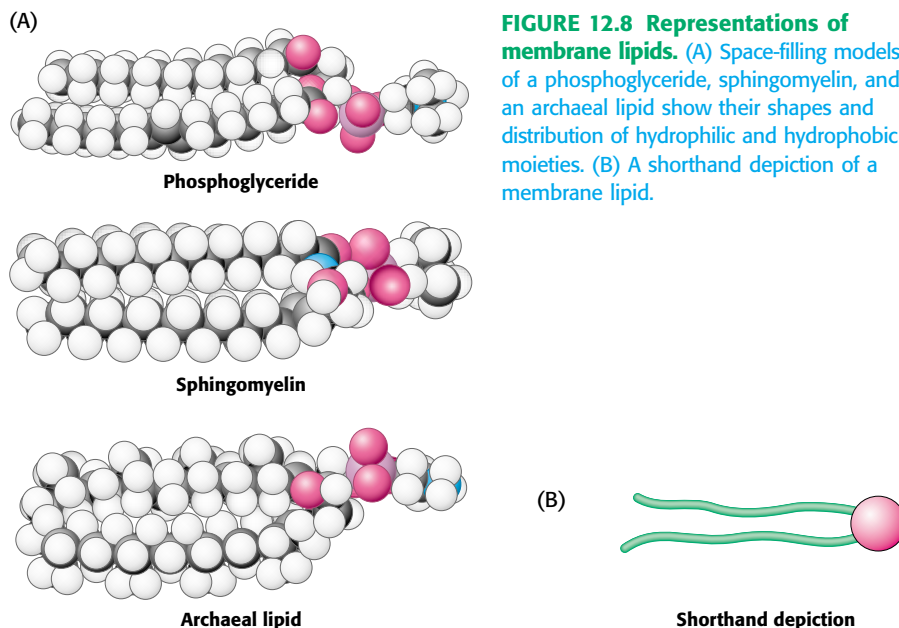


FIGURE 12.8 Representations of membrane lipids. (A) Space-filling models of a phosphoglyceride, sphingomyelin, and an archaeal lipid show their shapes and distribution of hydrophilic and hydrophobic moieties. (B) A shorthand depiction of a membrane lipid.

12.4 PHOSPHOLIPIDS AND GLYCOLIPIDS READILY FORM BIMOLECULAR SHEETS IN AQUEOUS MEDIA

What properties enable phospholipids to form membranes? *Membrane formation is a consequence of the amphipathic nature of the molecules.* Their polar head groups favor contact with water, whereas their hydrocarbon tails interact with one another, in preference to water. How can molecules with these preferences arrange themselves in aqueous solutions? One way is to form a *micelle*, a globular structure in which polar head groups are surrounded by water and hydrocarbon tails are sequestered inside, interacting with one another (Figure 12.9).

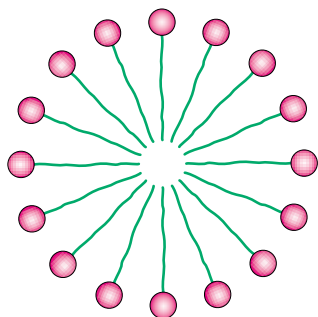


FIGURE 12.9 Diagram of a section of a micelle. Ionized fatty acids readily form such structures, but most phospholipids do not.

Alternatively, the strongly opposed preferences of the hydrophilic and hydrophobic moieties of membrane lipids can be satisfied by forming a *lipid bilayer*, composed of two lipid sheets (Figure 12.10). A lipid bilayer is also called a *bimolecular sheet*. The hydrophobic tails of each individual sheet interact with one another, forming a hydrophobic interior that acts as a permeability barrier. The hydrophilic head groups interact with the aqueous medium on each side of the bilayer. The two opposing sheets are called leaflets.

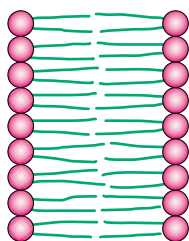


FIGURE 12.10 Diagram of a section of a bilayer membrane.

The favored structure for most phospholipids and glycolipids in aqueous media is a bimolecular sheet rather than a micelle. The reason is that the two fatty acyl chains of a phospholipid or a glycolipid are too bulky to fit into the interior of a micelle. In contrast, salts of fatty acids (such as sodium palmitate, a constituent of soap), which contain only one chain, readily form micelles. *The formation of bilayers instead of micelles by phospholipids is of critical biological importance.* A micelle is a limited structure, usually less than 20 nm (200 Å) in diameter. In contrast, a bimolecular sheet can have macroscopic dimensions, such as a millimeter (10^6 nm, or 10^7 Å). Phospholipids and related molecules are important membrane constituents because they readily form extensive bimolecular sheets (Figure 12.11).

The formation of lipid bilayers is a *self-assembly process*. In other words, the structure of a bimolecular sheet is inherent in the structure of the constituent lipid molecules. The growth of lipid bilayers from phospholipids is a rapid and spontaneous process in water. *Hydrophobic interactions are the*

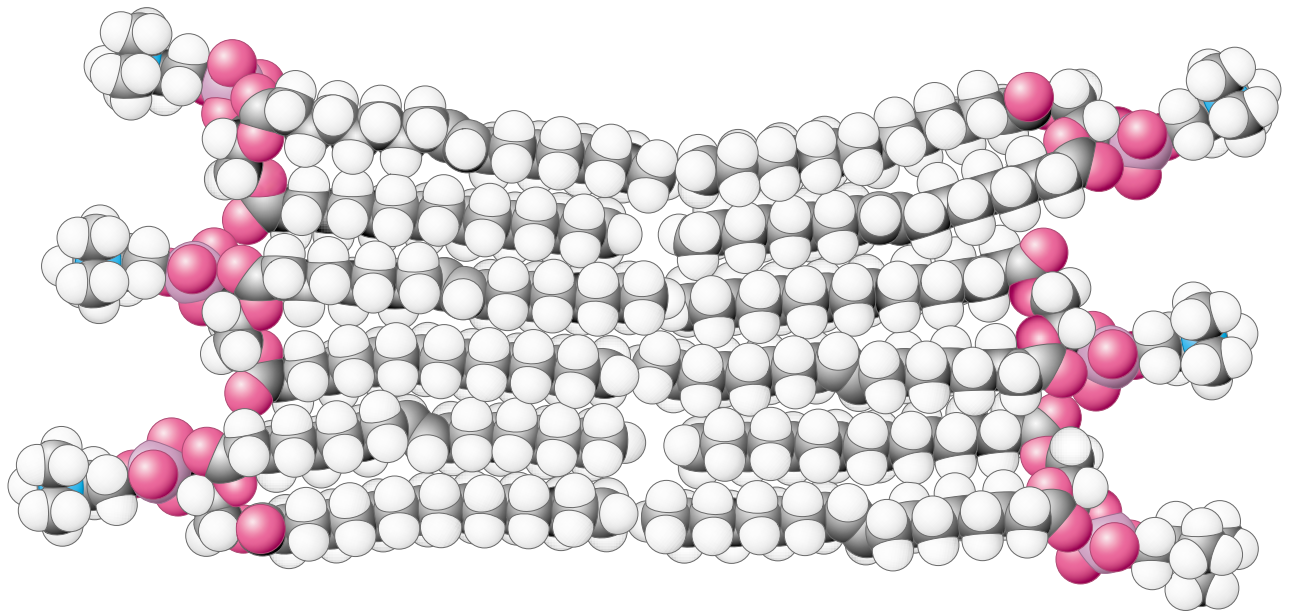


FIGURE 12.11 Space-filling model of a section of phospholipid bilayer membrane.

major driving force for the formation of lipid bilayers. Recall that hydrophobic interactions also play a dominant role in the folding of proteins (Sections 1.3.4 and 3.4) and in the stacking of bases in nucleic acids (Section 5.2.1). Water molecules are released from the hydrocarbon tails of membrane lipids as these tails become sequestered in the nonpolar interior of the bilayer. Furthermore, *van der Waals attractive forces between the hydrocarbon tails favor close packing of the tails*. Finally, there are *electrostatic and hydrogen-bonding attractions between the polar head groups and water molecules*. Thus, lipid bilayers are stabilized by the full array of forces that mediate molecular interactions in biological systems.

Because lipid bilayers are held together by many *reinforcing, noncovalent interactions (predominantly hydrophobic)*, they are *cooperative structures*. These hydrophobic interactions have three significant biological consequences: (1) lipid bilayers have an inherent tendency to be *extensive*; (2) lipid bilayers will tend to *close on themselves* so that there are no edges with exposed hydrocarbon chains, and so they form compartments; and (3) lipid bilayers are *self-sealing* because a hole in a bilayer is energetically unfavorable.

12.4.1 Lipid Vesicles Can Be Formed from Phospholipids

The propensity of phospholipids to form membranes has been used to create an important experimental and clinical tool. *Lipid vesicles*, or *liposomes*, aqueous compartments enclosed by a lipid bilayer (Figure 12.12), can be used to study membrane permeability or to deliver chemicals to cells. Liposomes are formed by suspending a suitable lipid, such as phosphatidylcholine, in an aqueous medium, and then *sonicating* (i.e., agitating by high-frequency sound waves) to give a dispersion of closed vesicles that are quite uniform in size. Vesicles formed by these methods are nearly spherical in shape and have a diameter of about 50 nm (500 Å). Larger vesicles (of the order of 1 μm, or 10⁴ Å, in diameter) can be prepared by slowly evaporating the organic solvent from a suspension of phospholipid in a mixed solvent system.

Ions or molecules can be trapped in the aqueous compartments of lipid vesicles by forming the vesicles in the presence of these substances (Figure 12.13). For example, 50-nm-diameter vesicles formed in a 0.1 M

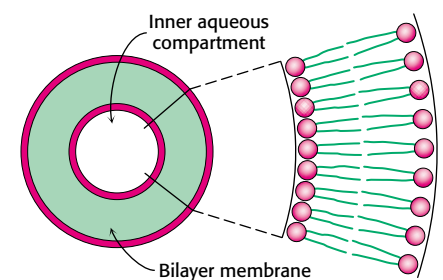


FIGURE 12.12 Liposome. A liposome, or lipid vesicle, is a small aqueous compartment surrounded by a lipid bilayer.

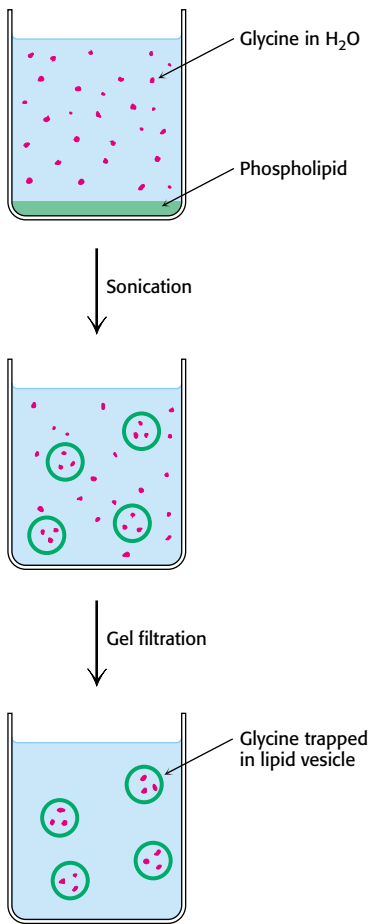


FIGURE 12.13 Preparation of glycine-containing liposomes. Liposomes containing glycine are formed by sonication of phospholipids in the presence of glycine. Free glycine is removed by gel filtration.

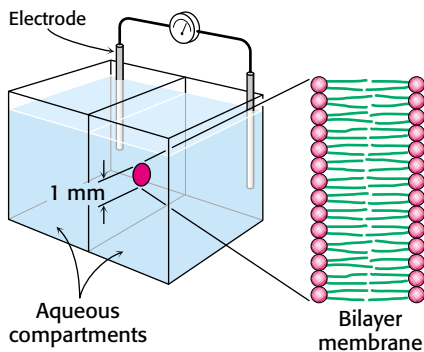


FIGURE 12.14 Experimental arrangement for the study of planar bilayer membrane. A bilayer membrane is formed across a 1-mm hole in a septum that separates two aqueous compartments. This arrangement permits measurements of the permeability and electrical conductance of lipid bilayers.

glycine solution will trap about 2000 molecules of glycine in each inner aqueous compartment. These glycine-containing vesicles can be separated from the surrounding solution of glycine by dialysis or by gel-filtration chromatography (Section 4.1.3). The permeability of the bilayer membrane to glycine can then be determined by measuring the rate of efflux of glycine from the inner compartment of the vesicle to the ambient solution. Specific membrane proteins can be solubilized in the presence of detergents and then added to the phospholipids from which liposomes will be formed. Protein-liposome complexes provide valuable experimental tools for examining a range of membrane-protein functions.

Experiments are underway to develop clinical uses for liposomes. For example, liposomes containing drugs or DNA for gene-therapy experiments can be injected into patients. These liposomes fuse with the plasma membrane of many kinds of cells, introducing into the cells the molecules that they contain. Drug delivery with liposomes also alters the distribution of a drug within the body and often lessens its toxicity. For instance, less of the drug is distributed to normal tissues because long-circulating liposomes have been shown to concentrate in regions of increased blood circulation, such as solid tumors and sites of inflammation. Moreover, the selective fusion of lipid vesicles with particular kinds of cells is a promising means of controlling the delivery of drugs to target cells.

Another well-defined synthetic membrane is a *planar bilayer membrane*. This structure can be formed across a 1-mm hole in a partition between two aqueous compartments by dipping a fine paintbrush into a membrane-forming solution, such as phosphatidyl choline in decane. The tip of the brush is then stroked across a hole (1 mm in diameter) in a partition between two aqueous media. The lipid film across the hole thins spontaneously into a lipid bilayer. The electrical conduction properties of this macroscopic bilayer membrane are readily studied by inserting electrodes into each aqueous compartment (Figure 12.14). For example, its permeability to ions is determined by measuring the current across the membrane as a function of the applied voltage.

12.4.2 Lipid Bilayers Are Highly Impermeable to Ions and Most Polar Molecules

The results of permeability studies of lipid vesicles and electrical-conductance measurements of planar bilayers have shown that *lipid bilayer membranes have a very low permeability for ions and most polar molecules*. Water is a conspicuous exception to this generalization; it readily traverses such membranes because of its small size, high concentration, and lack of a complete charge. The range of measured permeability coefficients is very wide (Figure 12.15). For example, Na^+ and K^+ traverse these membranes 10^9

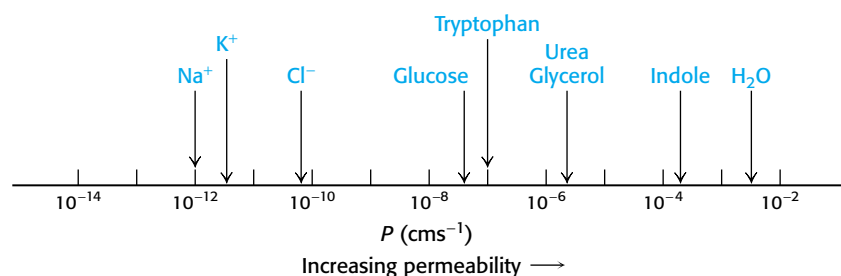


FIGURE 12.15 Permeability coefficients (P) of ions and molecules in a lipid bilayer. The ability of molecules to cross a lipid bilayer spans a wide range of values.

times as slowly as does H_2O . Tryptophan, a zwitterion at pH 7, crosses the membrane 10^3 times as slowly as does indole, a structurally related molecule that lacks ionic groups. In fact, *the permeability coefficients of small molecules are correlated with their solubility in a nonpolar solvent relative to their solubility in water*. This relation suggests that a small molecule might traverse a lipid bilayer membrane in the following way: first, it sheds its solvation shell of water; then, it becomes dissolved in the hydrocarbon core of the membrane; finally, it diffuses through this core to the other side of the membrane, where it becomes resolvated by water. An ion such as Na^+ traverses membranes very slowly because the replacement of its coordination shell of polar water molecules by nonpolar interactions with the membrane interior is highly unfavorable energetically.

12.5 PROTEINS CARRY OUT MOST MEMBRANE PROCESSES

We now turn to membrane proteins, which are responsible for most of the dynamic processes carried out by membranes. Membrane lipids form a permeability barrier and thereby establish compartments, whereas *specific proteins mediate nearly all other membrane functions*. In particular, proteins transport chemicals and information across a membrane. Membrane lipids create the appropriate environment for the action of such proteins.

Membranes differ in their protein content. Myelin, a membrane that serves as an insulator around certain nerve fibers, has a low content of protein (18%). Relatively pure lipids are well suited for insulation. In contrast, the plasma membranes or exterior membranes of most other cells are much more active. They contain many pumps, channels, receptors, and enzymes. The protein content of these plasma membranes is typically 50%. Energy-transduction membranes, such as the internal membranes of mitochondria and chloroplasts, have the highest content of protein, typically 75%.

The protein components of a membrane can be readily visualized by SDS–polyacrylamide gel electrophoresis. As discussed earlier (Section 4.1.4), the electrophoretic mobility of many proteins in SDS-containing gels depends on the mass rather than on the net charge of the protein. The gel-electrophoresis patterns of three membranes—the plasma membrane of erythrocytes, the photoreceptor membrane of retinal rod cells, and the sarcoplasmic reticulum membrane of muscle—are shown in Figure 12.16. It is evident that each of these three membranes contains many proteins but has a distinct protein composition. In general, *membranes performing different functions contain different repertoires of proteins*.

12.5.1 Proteins Associate with the Lipid Bilayer in a Variety of Ways

The ease with which a protein can be dissociated from a membrane indicates how intimately it is associated with the membrane. Some membrane proteins can be solubilized by relatively mild means, such as extraction by a solution of high ionic strength (e.g., 1 M NaCl). Other membrane proteins are bound much more tenaciously; they can be solubilized only by using a detergent or an organic solvent. Membrane proteins can be classified as being either *peripheral* or *integral* on the basis of this difference in dissociability (Figure 12.17). *Integral membrane proteins* interact extensively with the hydrocarbon chains of membrane lipids, and so only agents that compete for these nonpolar interactions can release them. In fact, most integral membrane proteins span the lipid bilayer. In contrast, *peripheral membrane proteins* are bound to

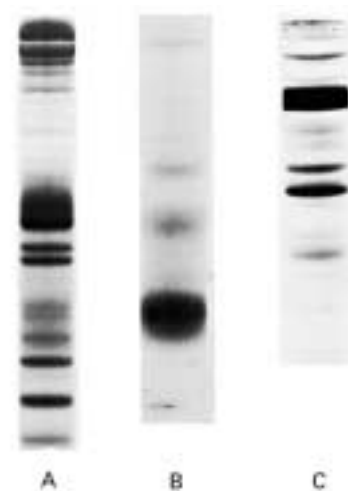
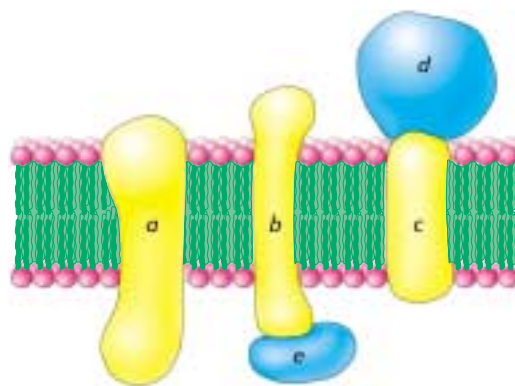


FIGURE 12.16 SDS–acrylamide gel patterns of membrane proteins. (A) The plasma membrane of erythrocytes. (B) The photoreceptor membranes of retinal rod cells. (C) The sarcoplasmic reticulum membrane of muscle cells. [Courtesy of Dr. Theodore Steck (Part A) and Dr. David MacLennan (Part C).]

FIGURE 12.17 Integral and peripheral membrane proteins. Integral membrane proteins (*a*, *b*, and *c*) interact extensively with the hydrocarbon region of the bilayer. Nearly all known integral membrane proteins traverse the lipid bilayer. Peripheral membrane proteins (*d* and *e*) bind to the surfaces of integral proteins. Some peripheral membrane proteins interact with the polar head groups of the lipids (not shown).



membranes primarily by electrostatic and hydrogen-bond interactions with the head groups of lipids. These polar interactions can be disrupted by adding salts or by changing the pH. Many peripheral membrane proteins are bound to the surfaces of integral proteins, on either the cytosolic or the extracellular side of the membrane. Others are anchored to the lipid bilayer by a covalently attached hydrophobic chain, such as a fatty acid.

12.5.2 Proteins Interact with Membranes in a Variety of Ways

Although membrane proteins are more difficult to purify and crystallize than are water-soluble proteins, researchers using x-ray crystallographic or electron microscopic methods have determined the three-dimensional structures of more than 20 such proteins at sufficiently high resolution to discern the molecular details. As noted in Chapter 3, the structures of membrane proteins differ from those of soluble proteins with regard to the distribution of hydrophobic and hydrophilic groups. We will consider the structures of three membrane proteins in some detail.

Proteins Can Span the Membrane with Alpha Helices. The first membrane protein that we consider is the archaeal protein *bacteriorhodopsin*, shown in Figure 12.18. This protein acts in energy transduction by using light energy to transport protons from inside the cell to outside. The proton gradient generated in this way is used to form ATP. Bacteriorhodopsin is built almost entirely of α helices; seven closely packed α helices, arranged almost perpendicularly to the plane of the membrane, span its 45-Å width. Examination of the primary structure of bacteriorhodopsin reveals that most of the amino acids in these membrane-spanning α helices are nonpolar and only a very few are charged (Figure 12.19). This distribution of nonpolar amino acids is sensible because these residues are either in contact with the

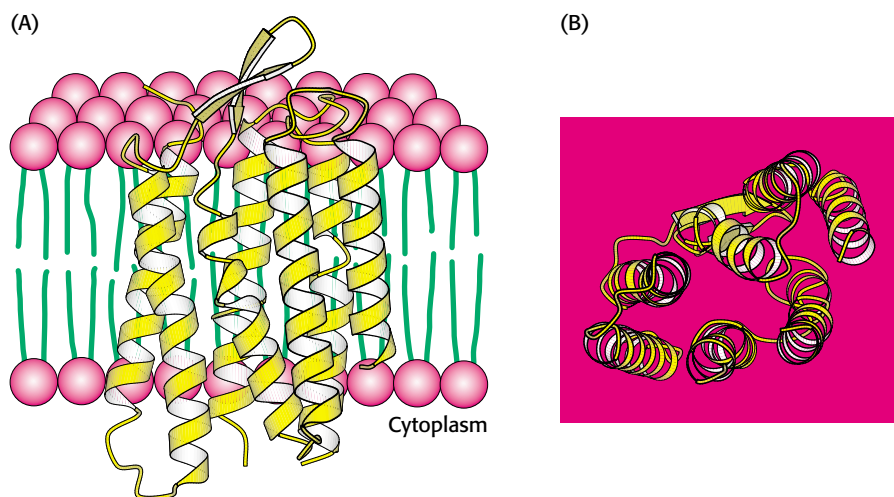


FIGURE 12.18 Structure of bacteriorhodopsin. Bacteriorhodopsin consists largely of membrane-spanning α helices. (A) View through the membrane bilayer. The interior of the membrane is green and the head groups are red. (B) A view from the cytoplasmic side of the membrane.

hydrocarbon core of the membrane or with one another. *Membrane-spanning α helices are the most common structural motif in membrane proteins.* As will be discussed in Section 12.5.4, such regions can often be detected from amino acid sequence information alone.

AQITGRPEWIWLAGLTALMGLGTLYFLVKGMGVSDPDAKKFYAITTLVPA
IAFTMYLSML LGYGLTMVPFGGEQNP IYWAR YADWLF TTP LLLD L L L V
DADQGTILALVGADGIMIGTGLVGALTKVYSYRFVWWAISTAAML Y ILYV
LFFGFTSKAESMRPEVASTFKVLRNVTVVLSAYVVVWLIIGSEGAGIVPL
NIETLLFMVLDVSAKVGFGLLLRSAIFGEAEAPEPSADIGAAATS

FIGURE 12.19 Amino acid sequence of bacteriorhodopsin. The seven helical regions are highlighted in yellow and the charged residues in red.

A Channel Protein Can Be Formed from Beta Strands. Porin, a protein from the outer membranes of bacteria such as *E. coli* and *Rhodobacter capsulatus*, represents a class of membrane proteins with a completely different type of structure. Structures of this type are built from β strands and contain essentially no α helices (Figure 12.20).

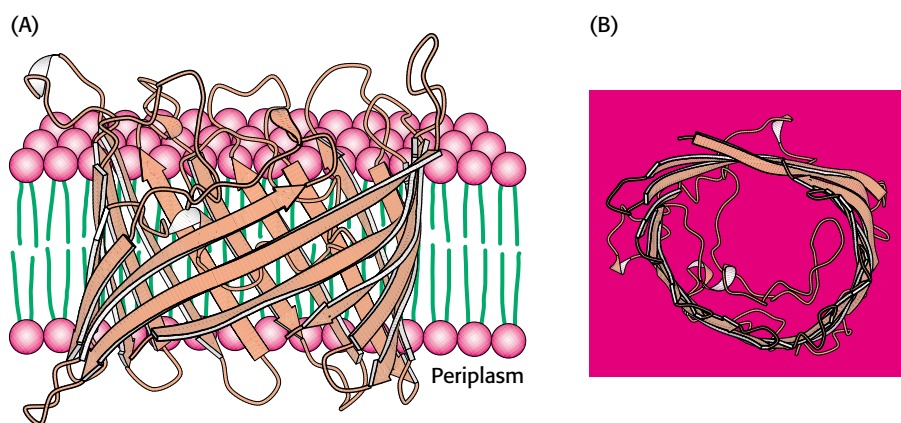


FIGURE 12.20 Structure of bacterial porin (from *Rhodopseudomonas blastica*). Porin is a membrane protein built entirely of beta strands. (A) Side view. (B) View from the periplasmic space. Only one monomer of the trimeric protein is shown.

The arrangement of β strands is quite simple: each strand is hydrogen bonded to its neighbor in an antiparallel arrangement, forming a single β sheet. The β sheet curls up to form a hollow cylinder that functions as the active unit. As its name suggests, porin forms pores, or channels, in the membranes. A pore runs through the center of each cylinder-like protein. The outside surface of porin is appropriately nonpolar, given that it interacts with the hydrocarbon core of the membrane. In contrast, the inside of the channel is quite hydrophilic and is filled with water. This arrangement of nonpolar and polar surfaces is accomplished by the alternation of hydrophobic and hydrophilic amino acids along each β strand (Figure 12.21).

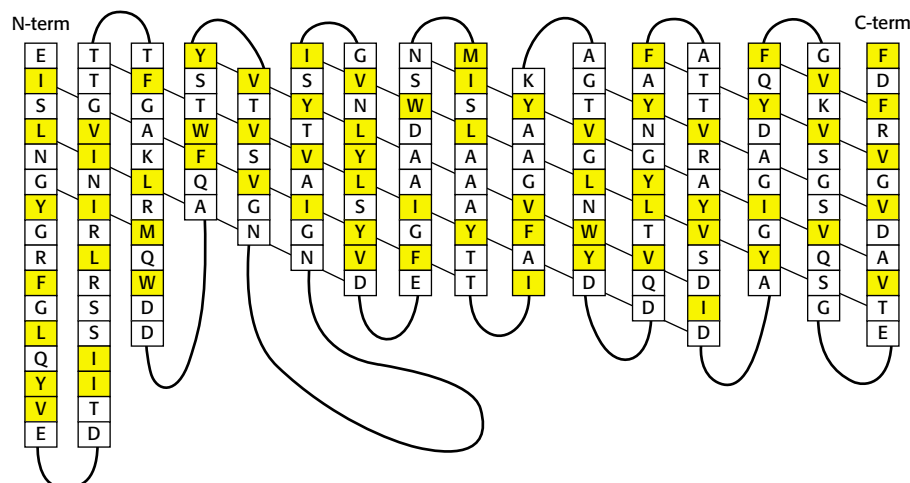


FIGURE 12.21 Amino acid sequence of a porin. Some membrane proteins such as porins are built from β strands that tend to have hydrophobic and hydrophilic amino acids in adjacent positions. The secondary structure of *Rhodopseudomonas blastica* is shown, with the diagonal lines indicating the direction of hydrogen bonding along the β sheet. Hydrophobic residues (F, I, L, M, V, W, and Y) are shown in yellow. These residues tend to lie on the outside of the structure, in contact with the hydrophobic core of the membrane.

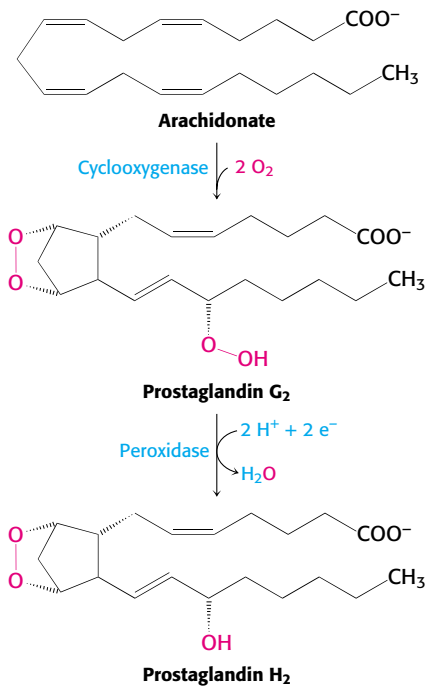


FIGURE 12.22 Formation of prostaglandin H_2 . Prostaglandin H_2 synthase-1 catalyzes the formation of prostaglandin H_2 from arachidonic acid in two steps.

Embedding Part of a Protein in a Membrane Can Link It to the Membrane Surface. The structure of the membrane-bound enzyme prostaglandin H_2 synthase-1 reveals a rather different role for α helices in protein-membrane associations. This enzyme catalyzes the conversion of arachidonic acid into prostaglandin H_2 in two steps: a cyclooxygenase reaction and a peroxidase reaction (Figure 12.22). Prostaglandin H_2 promotes inflammation and modulates gastric acid secretion. The enzyme that produces prostaglandin H_2 is a homodimer with a rather complicated structure consisting primarily of α helices. Unlike bacteriorhodopsin, this protein is not largely embedded in the membrane but, instead, lies along the outer surface of the membrane firmly bound by a set of α helices with hydrophobic surfaces that extend from the bottom of the protein into the membrane (Figure 12.23). This linkage is sufficiently strong that only the action of detergents can release the protein from the membrane. Thus, this enzyme is classified as an integral membrane protein, although it is not a membrane-spanning protein.

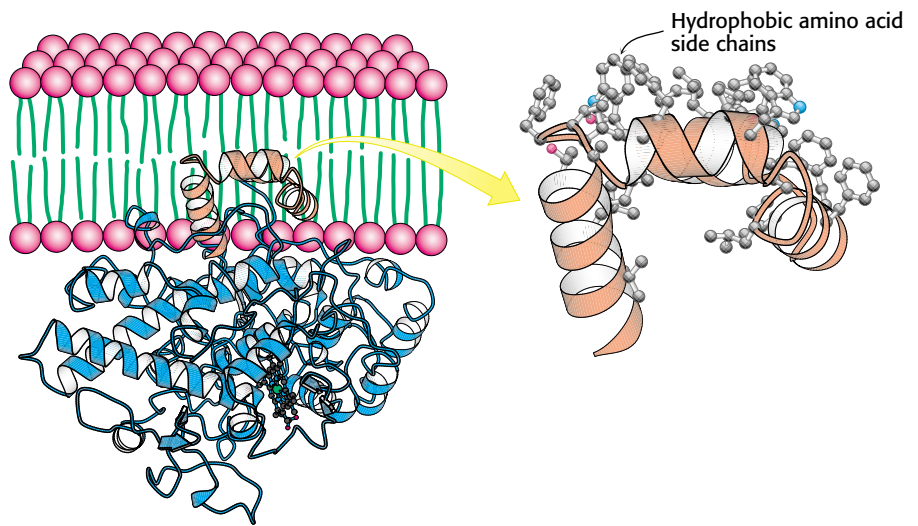


FIGURE 12.23 Attachment of prostaglandin H_2 synthase-1 to the membrane. Prostaglandin H_2 synthase-1 is held in the membrane by a set of α helices coated with hydrophobic side chains. One monomer of the dimeric enzyme is shown.

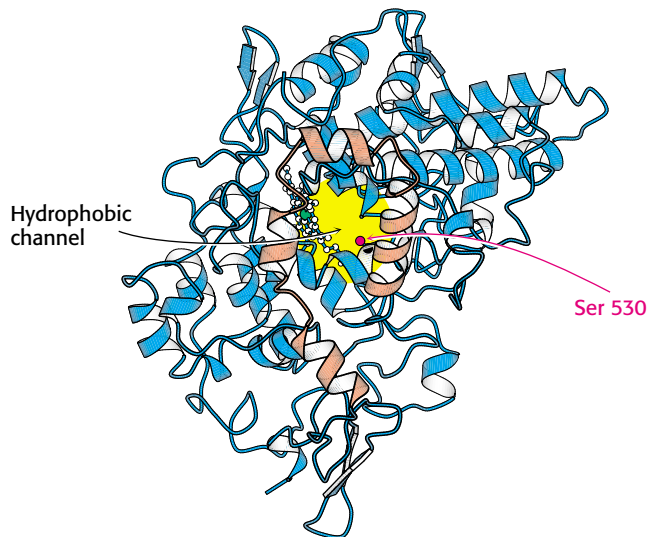


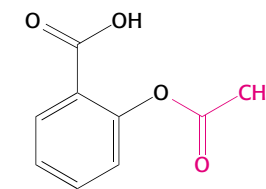
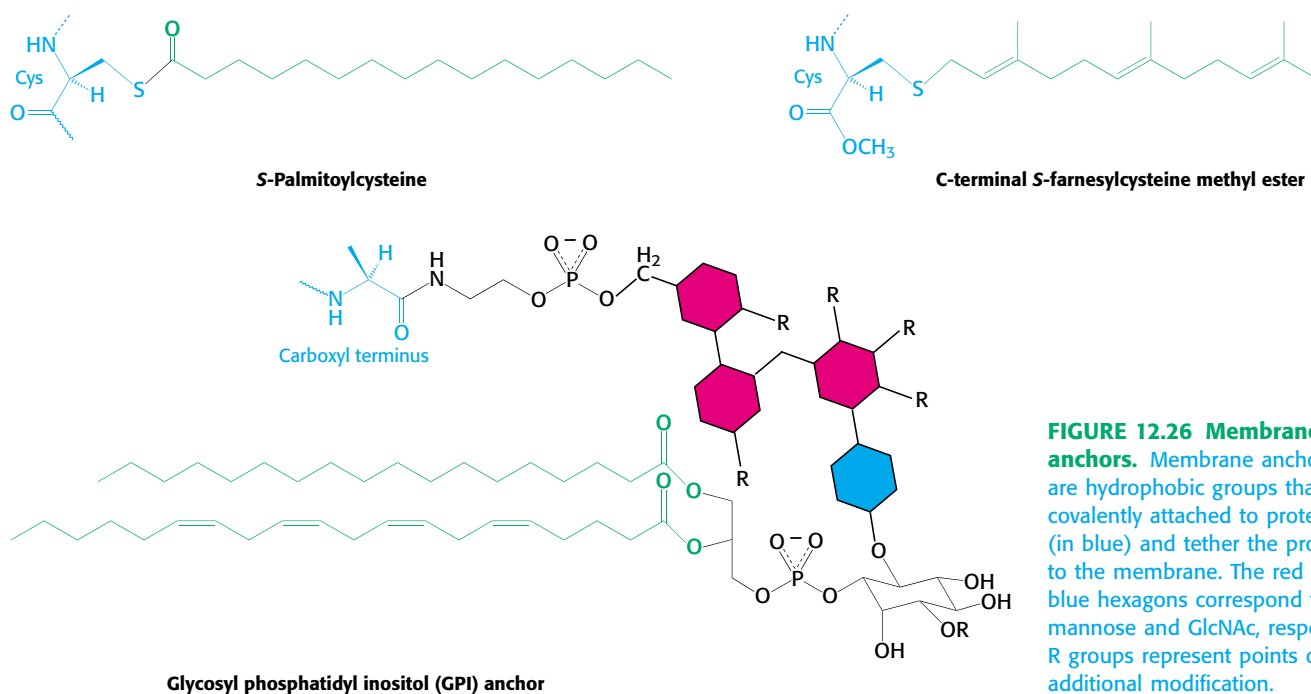
FIGURE 12.24 Hydrophobic channel of prostaglandin H_2 synthase. A view of prostaglandin H_2 synthase from the membrane, showing the hydrophobic channel that leads to the active site. The membrane-anchoring helices are shown in orange.

The localization of prostaglandin H_2 synthase-1 in the membrane is crucial to its function. The substrate for this enzyme, arachidonic acid, is a hydrophobic molecule generated by the hydrolysis of membrane lipids. Arachidonic acid reaches the active site of the enzyme from the membrane without entering an aqueous environment by traveling through a hydrophobic channel in the protein (Figure 12.24). Indeed, nearly all of us have experienced the importance of this channel: drugs such as aspirin and ibuprofen block the channel and prevent prostaglandin synthesis by inhibiting the cyclooxygenase activity of the synthase. In the case of aspirin, the drug acts through the transfer of an acetyl group from the aspirin to a serine residue (Ser 530) that lies along the path to the active site (Figure 12.25).

Two important features emerge from our examination of these three examples of membrane protein structure. First, the parts of the protein that interact with the hydrophobic parts of the membrane are coated with non-polar amino acid side chains, whereas those parts that interact with the aqueous environment are much more hydrophilic. Second, the structures positioned within the membrane are quite regular and, in particular, all backbone hydrogen-bond donors and acceptors participate in hydrogen bonds. *Breaking a hydrogen bond within a membrane is quite unfavorable, because little or no water is present to compete for the polar groups.*

12.5.3 Some Proteins Associate with Membranes Through Covalently Attached Hydrophobic Groups

The membrane proteins considered thus far associate with the membrane through surfaces generated by hydrophobic amino acid side chains. However, even otherwise soluble proteins can associate with membranes if the association is mediated by hydrophobic groups attached to the proteins. Three such groups are shown in Figure 12.26: (1) a palmitoyl group attached to a specific cysteine residue by a thioester bond, (2) a farnesyl group attached to a cysteine residue at the carboxyl terminus, and (3) a glycolipid structure termed a glycosyl phosphatidyl inositol (GPI) anchor attached to the carboxyl terminus. These modifications are attached by enzyme systems that recognize specific signal sequences near the site of attachment.



Aspirin
(Acetylsalicylic acid)

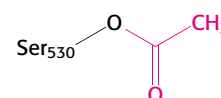


FIGURE 12.25 Aspirin's effects on prostaglandin H_2 synthase-1. Aspirin acts by transferring an acetyl group to a serine residue in prostaglandin H_2 synthase-1.

FIGURE 12.26 Membrane anchors. Membrane anchors are hydrophobic groups that are covalently attached to proteins (in blue) and tether the proteins to the membrane. The red and blue hexagons correspond to mannose and GlcNAc, respectively. R groups represent points of additional modification.

TABLE 12.2 Polarity scale for identifying transmembrane helices

Amino acid residue	Transfer free energy kcal mol ⁻¹ (kJ mol ⁻¹)
Phe	3.7 (15.5)
Met	3.4 (14.3)
Ile	3.1 (13.0)
Leu	2.8 (11.8)
Val	2.6 (10.9)
Cys	2.0 (8.4)
Trp	1.9 (8.0)
Ala	1.6 (6.7)
Thr	1.2 (5.0)
Gly	1.0 (4.2)
Ser	0.6 (2.5)
Pro	-0.2 (-0.8)
Tyr	-0.7 (-2.9)
His	-3.0 (-12.6)
Gln	-4.1 (-17.2)
Asn	-4.8 (-20.2)
Glu	-8.2 (-34.4)
Lys	-8.8 (-37.0)
Asp	-9.2 (-38.6)
Arg	-12.3 (-51.7)

Source: After D. M. Engelman, T. A. Steitz, and A. Goldman. *Annu. Rev. Biophys. Biophys. Chem.* 15(1986):330. Note: The free energies are for the transfer of an amino acid residue in an α helix from the membrane interior (assumed to have a dielectric constant of 2) to water.

12.5.4 Transmembrane Helices Can Be Accurately Predicted from Amino Acid Sequences

Many membrane proteins, like bacteriorhodopsin, employ α helices to span the hydrophobic part of a membrane. As noted earlier, typically most of the residues in these α helices are nonpolar and almost none of them are charged. Can we use this information to identify putative membrane-spanning regions from sequence data alone? One approach to identifying transmembrane helices is to ask whether a postulated helical segment is likely to be most stable in a hydrocarbon milieu or in water. Specifically, we want to estimate the free-energy change when a helical segment is transferred from the interior of a membrane to water. Free-energy changes for the transfer of individual amino acid residues from a hydrophobic to an aqueous environment are given in Table 12.2. For example, the transfer of a poly-L-arginine helix, a homopolymer of a positively charged amino acid, from the interior of a membrane to water would be highly favorable [-12.3 kcal mol⁻¹ (-51.5 kJ mol⁻¹) per arginine residue in the helix], whereas the transfer of

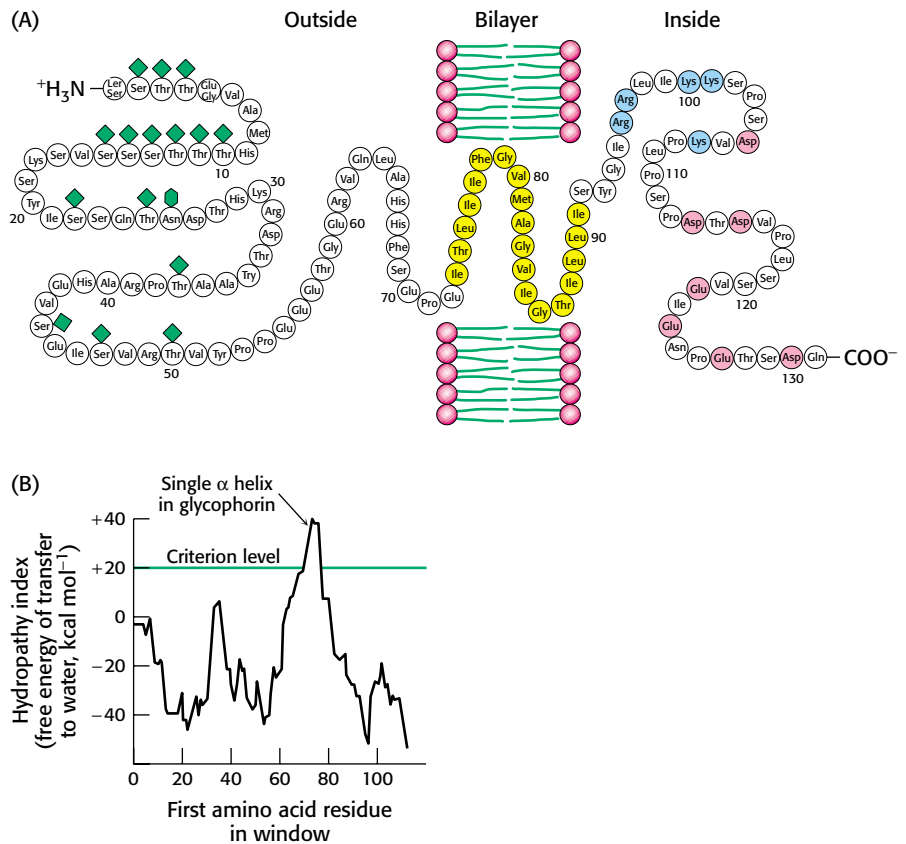


FIGURE 12.27 Locating the membrane-spanning helix of glycoporphin. (A) Amino acid sequence and transmembrane disposition of glycoporphin A from the red-cell membrane. Fifteen O-linked carbohydrate units are shown as diamond shapes, and an N-linked unit is shown as a lozenge shape. The hydrophobic residues (yellow) buried in the bilayer form a transmembrane α helix. The carboxyl-terminal part of the molecule, located on the cytosolic side of the membrane, is rich in negatively charged (red) and positively charged (blue) residues. (B) Hydropathy plot for glycoporphin. The free energy for transferring a helix of 20 residues from the membrane to water is plotted as a function of the position of the first residue of the helix in the sequence of the protein. Peaks of greater than $+20$ kcal mol⁻¹ in hydropathy plots are indicative of potential transmembrane helices. [(A) Courtesy of Dr. Vincent Marchesi; (B) after D. M. Engelman, T. A. Steitz, and A. Goldman. Identifying nonpolar transbilayer helices in amino acid sequences of membrane proteins. *Annu. Rev. Biophys. Biophys. Chem.* 15(1986):343. Copyright © 1986 by Annual Reviews, Inc. All rights reserved.]

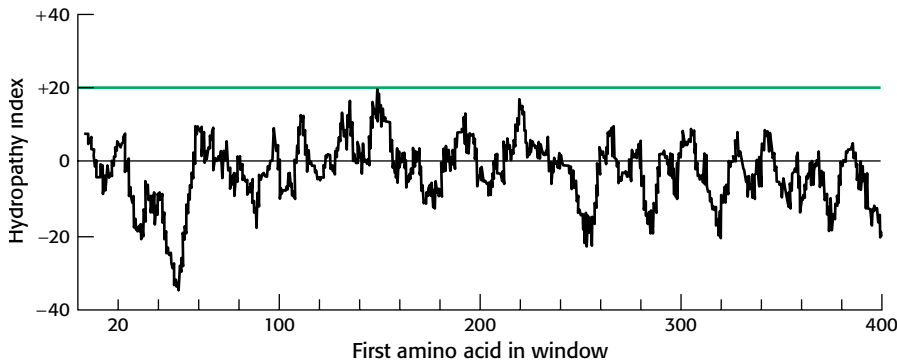


FIGURE 12.28 Hydropathy plot for porin. No strong peaks are observed for this intrinsic membrane protein because it is constructed from membrane-spanning β strands rather than α helices.

a poly-L-phenylalanine helix, a homopolymer of a hydrophobic amino acid, would be unfavorable [$+3.7 \text{ kcal mol}^{-1}$ ($+15.5 \text{ kJ mol}^{-1}$) per phenylalanine residue in the helix].

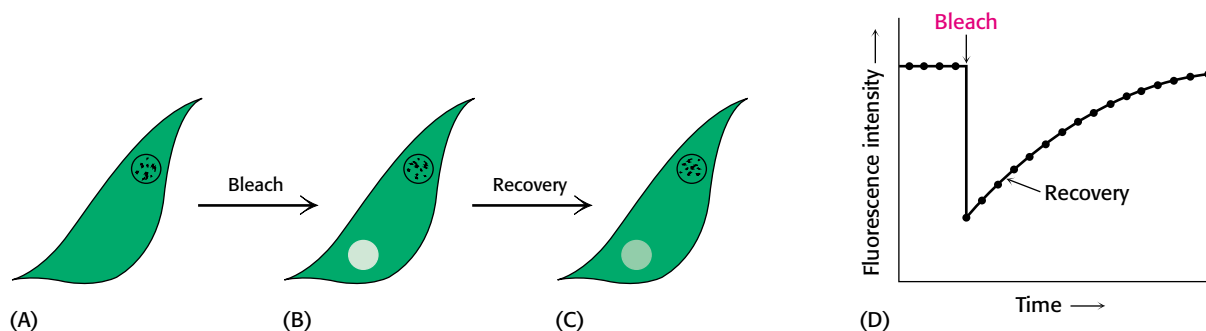
The hydrocarbon core of a membrane is typically 30 \AA wide, a length that can be traversed by an α helix consisting of 20 residues. We can take the amino acid sequence of a protein and estimate the free-energy change that takes place when a hypothetical α helix formed of residues 1 through 20 is transferred from the membrane interior to water. The same calculation can be made for residues 2 through 21, 3 through 22, and so forth, until we reach the end of the sequence. The span of 20 residues chosen for this calculation is called a *window*. The free-energy change for each window is plotted against the first amino acid at the window to create a *hydropathy plot*. Empirically, a peak of $+20 \text{ kcal mol}^{-1}$ ($+84 \text{ kJ mol}^{-1}$) or more in a hydropathy plot based on a window of 20 residues indicates that a polypeptide segment could be a membrane-spanning α helix. For example, glycoporin, a protein found in the membranes of red blood cells, is predicted by this criterion to have one membrane-spanning helix, in agreement with experimental findings (Figure 12.27). It should be noted, however, that a peak in the hydropathy plot does not prove that a segment is a transmembrane helix. Even soluble proteins may have highly nonpolar regions. Conversely, some membrane proteins contain transmembrane structural features (such as membrane-spanning β strands) that escape detection by these plots (Figure 12.28).

12.6 LIPIDS AND MANY MEMBRANE PROTEINS DIFFUSE RAPIDLY IN THE PLANE OF THE MEMBRANE

Biological membranes are not rigid, static structures. On the contrary, lipids and many membrane proteins are constantly in lateral motion, a process called *lateral diffusion*. The rapid lateral movement of membrane proteins has been visualized by means of fluorescence microscopy through the use of the technique of *fluorescence recovery after photobleaching* (FRAP; Figure 12.29).

FIGURE 12.29 Fluorescence recovery after photobleaching (FRAP) technique.

(A) The cell-surface fluoresces because of a labeled surface component. (B) The fluorescent molecules of a small part of the surface are bleached by an intense light pulse. (C) The fluorescence intensity recovers as bleached molecules diffuse out of the region and unbleached molecules diffuse into it. (D) The rate of recovery depends on the diffusion coefficient.



First, a cell-surface component is specifically labeled with a fluorescent chromophore. A small region of the cell surface ($\sim 3 \mu\text{m}^2$) is viewed through a fluorescence microscope. The fluorescent molecules in this region are then destroyed (bleached) by a very intense light pulse from a laser. The fluorescence of this region is subsequently monitored as a function of time by using a light level sufficiently low to prevent further bleaching. If the labeled component is mobile, bleached molecules leave and unbleached molecules enter the illuminated region, which results in an increase in the fluorescence intensity. The rate of recovery of fluorescence depends on the lateral mobility of the fluorescence-labeled component, which can be expressed in terms of a diffusion coefficient, D . The average distance s traversed in time t depends on D according to the expression

$$s = (4Dt)^{1/2}$$

The diffusion coefficient of lipids in a variety of membranes is about $1 \mu\text{m}^2 \text{s}^{-1}$. Thus, a phospholipid molecule diffuses an average distance of $2 \mu\text{m}$ in 1 s. This rate means that *a lipid molecule can travel from one end of a bacterium to the other in a second*. The magnitude of the observed diffusion coefficient indicates that the viscosity of the membrane is about 100 times that of water, rather like that of olive oil.

In contrast, proteins vary markedly in their lateral mobility. *Some proteins are nearly as mobile as lipids, whereas others are virtually immobile*. For example, the photoreceptor protein rhodopsin (Section 32.3.1), a very mobile protein, has a diffusion coefficient of $0.4 \mu\text{m}^2 \text{s}^{-1}$. The rapid movement of rhodopsin is essential for fast signaling. At the other extreme is fibronectin, a peripheral glycoprotein that interacts with the extracellular matrix. For fibronectin, D is less than $10^{-4} \mu\text{m}^2 \text{s}^{-1}$. Fibronectin has a very low mobility because it is anchored to actin filaments on the inside of the plasma membrane through *integrin*, a transmembrane protein that links the extracellular matrix to the cytoskeleton.

12.6.1 The Fluid Mosaic Model Allows Lateral Movement but Not Rotation Through the Membrane

On the basis of the dynamic properties of proteins in membranes, S. Jonathan Singer and Garth Nicolson proposed the concept of a *fluid mosaic model* for the overall organization of biological membranes in 1972 (Figure 12.30). The essence of their model is that *membranes are two-dimensional solutions of oriented lipids and globular proteins*. The lipid bilayer has a dual role: it is both a *solvent* for integral membrane proteins and a *permeability*

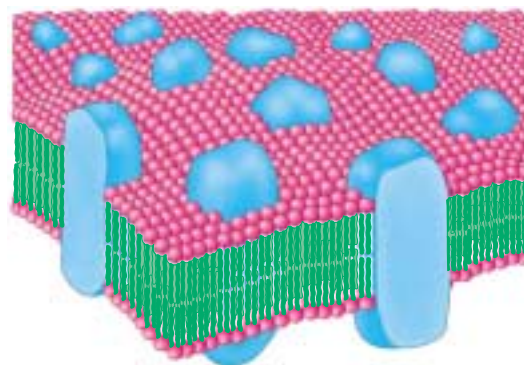


FIGURE 12.30 Fluid mosaic model. [After S. J. Singer and G. L. Nicolson. *Science* 175(1972):723.]

barrier. Membrane proteins are free to diffuse laterally in the lipid matrix unless restricted by special interactions.

Although the lateral diffusion of membrane components can be rapid, the spontaneous rotation of lipids from one face of a membrane to the other is a very slow process. The transition of a molecule from one membrane surface to the other is called *transverse diffusion* or *flip-flop* (Figure 12.31) The flip-flop of phospholipid molecules in phosphatidyl choline vesicles has been directly measured by electron spin resonance techniques, which show that a phospholipid molecule flip-flops once in several hours. Thus, a phospholipid molecule takes about 10^9 times as long to flip-flop across a membrane as it takes to diffuse a distance of 50 Å in the lateral direction. The free-energy barriers to flip-flopping are even larger for protein molecules than for lipids because proteins have more extensive polar regions. In fact, the flip-flop of a protein molecule has not been observed. Hence, *membrane asymmetry can be preserved for long periods.*

12.6.2 Membrane Fluidity Is Controlled by Fatty Acid Composition and Cholesterol Content

Many membrane processes, such as transport or signal transduction, depend on the fluidity of the membrane lipids, which in turn depends on the properties of fatty acid chains, which can exist in an ordered, rigid state or in a relatively disordered, fluid state. The transition from the rigid to the fluid state occurs rather abruptly as the temperature is raised above T_m , the melting temperature (Figure 12.32). *This transition temperature depends on the length of the fatty acyl chains and on their degree of unsaturation* (Table 12.3). The presence of saturated fatty acyl residues favors the rigid state because their straight hydrocarbon chains interact very favorably with each other. On the other hand, a *cis double bond produces a bend in the hydrocarbon chain. This bend interferes with a highly ordered packing of fatty acyl chains, and so T_m is lowered* (Figure 12.33). The length of the fatty acyl chain also affects the transition temperature. Long hydrocarbon chains interact more strongly than do short ones. Specifically, each additional $-\text{CH}_2-$ group makes a favorable contribution of about $-0.5 \text{ kcal mol}^{-1}$ (-2.1 kJ mol^{-1}) to the free energy of interaction of two adjacent hydrocarbon chains.

Bacteria regulate the fluidity of their membranes by varying the number of double bonds and the length of their fatty acyl chains. For example, the ratio of saturated to unsaturated fatty acyl chains in the *E. coli* membrane decreases from 1.6 to 1.0 as the growth temperature is lowered from 42°C to 27°C. This decrease in the proportion of saturated residues prevents the membrane from becoming too rigid at the lower temperature.

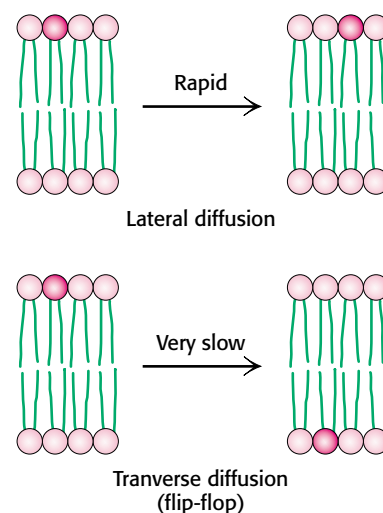


FIGURE 12.31 Lipid movement in membranes. Lateral diffusion of lipids is much more rapid than transverse diffusion (flip-flop).

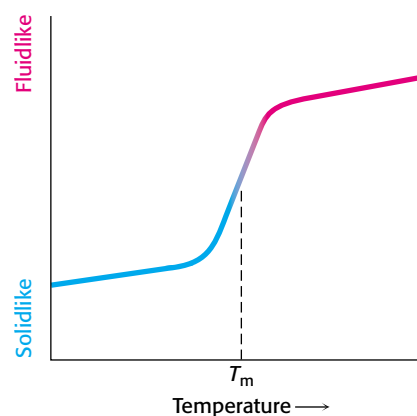
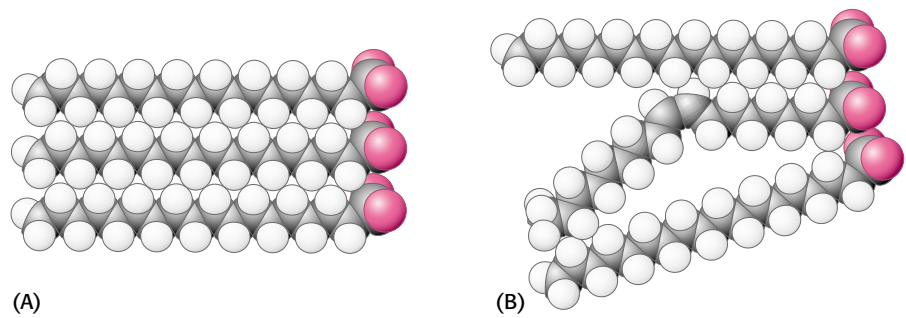


FIGURE 12.32 The phase-transition, or melting, temperature (T_m) for a phospholipid membrane. As the temperature is raised, the phospholipid membrane changes from a packed, ordered state to a more random one.

TABLE 12.3 The melting temperature of phosphatidyl choline containing different pairs of identical fatty acid chains

Number of carbons	Number of double bonds	Fatty acid		T_m (°C)
		Common name	Systematic name	
22	0	Behenate	<i>n</i> -Docosanoate	75
18	0	Stearate	<i>n</i> -Octadecanoate	58
16	0	Palmitate	<i>n</i> -Hexadecanoate	41
14	0	Myristate	<i>n</i> -Tetradecanoate	24
18	1	Oleate	<i>cis</i> - Δ^9 -Octadecenoate	-22

FIGURE 12.33 Packing of fatty acid chains in a membrane. The highly ordered packing of fatty acid chains is disrupted by the presence of cis double bonds. The space-filling models show the packing of (A) three molecules of stearate (C_{18} , saturated) and (B) a molecule of oleate (C_{18} , unsaturated) between two molecules of stearate.



In animals, cholesterol is the key regulator of membrane fluidity. Cholesterol contains a bulky steroid nucleus with a hydroxyl group at one end and a flexible hydrocarbon tail at the other end. Cholesterol inserts into bilayers with its long axis perpendicular to the plane of the membrane. The hydroxyl group of cholesterol forms a hydrogen bond with a carbonyl oxygen atom of a phospholipid head group, whereas the hydrocarbon tail of cholesterol is located in the nonpolar core of the bilayer. The different shape of cholesterol compared with phospholipids disrupts the regular interactions between fatty acyl chains. In addition, cholesterol appears to form specific complexes with some phospholipids. Such complexes may concentrate in specific regions within membranes. One result of these interactions is the *moderation of membrane fluidity*, making membranes less fluid but at the same time less subject to phase transitions.

12.6.3 All Biological Membranes Are Asymmetric

Membranes are structurally and functionally asymmetric. The outer and inner surfaces of *all known biological membranes have different components and different enzymatic activities*. A clear-cut example is the pump that regulates the concentration of Na^+ and K^+ ions in cells (Figure 12.34). This transport protein is located in the plasma membrane of nearly all cells in higher organisms. The Na^+ - K^+ pump is oriented so that it pumps Na^+ out of the cell and K^+ into it. Furthermore, ATP must be on the inside of the cell to drive the pump. Ouabain, a specific inhibitor of the pump, is effective only if it is located outside.

Membrane proteins have a unique orientation because they are synthesized and inserted into the membrane in an asymmetric manner. This absolute asymmetry is preserved because membrane proteins do not rotate from one side of the membrane to the other and because *membranes are always synthesized by the growth of preexisting membranes*. Lipids, too, are asymmetrically distributed as a consequence of their mode of biosynthesis, but this asymmetry is usually not absolute, except for glycolipids. In the red-blood-cell membrane, sphingomyelin and phosphatidyl choline are preferentially located in the outer leaflet of the bilayer, whereas phosphatidyl ethanolamine and phosphatidyl serine are located mainly in the inner leaflet. Large amounts of cholesterol are present in both leaflets.

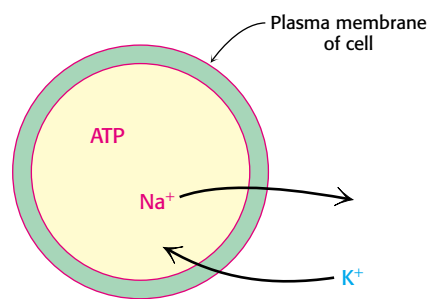


FIGURE 12.34 Asymmetry of the Na^+ - K^+ transport system in plasma membranes. The Na^+ - K^+ transport system pumps Na^+ out of the cell and K^+ into the cell.

12.7 EUKARYOTIC CELLS CONTAIN COMPARTMENTS BOUNDED BY INTERNAL MEMBRANES

Thus far we have considered only the plasma membrane of cells. Many bacteria such as *E. coli* have two membranes separated by a cell wall (made of proteins, peptides, and carbohydrates) lying in between (Figure 12.35). The inner membrane acts as the permeability barrier, and the outer membrane and

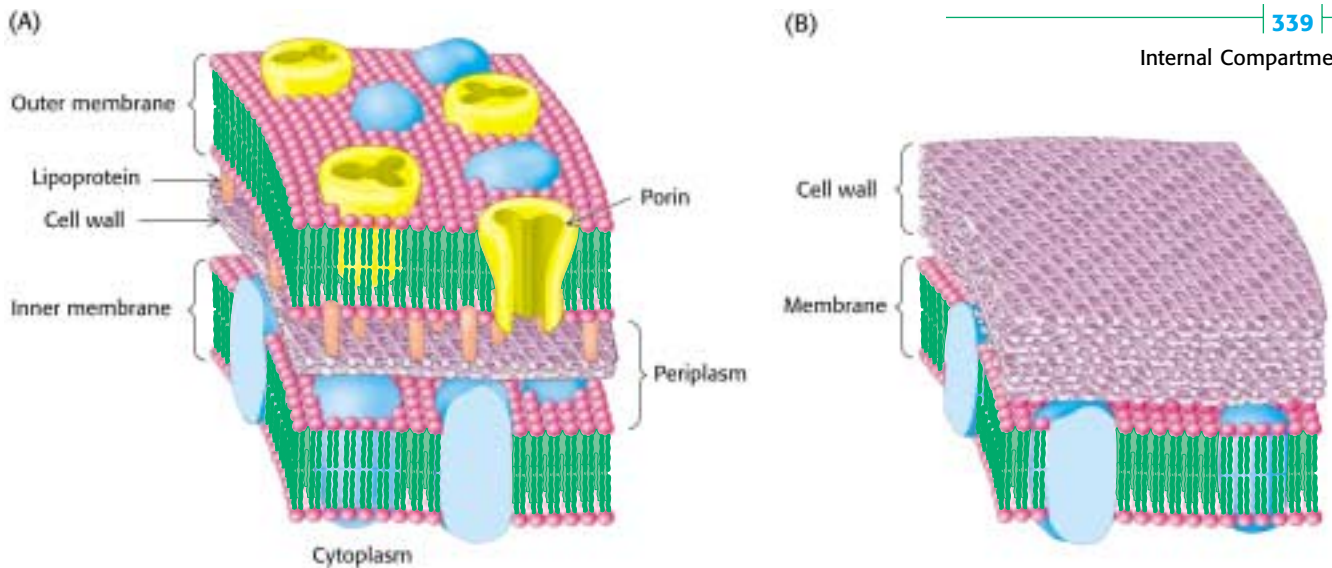


FIGURE 12.35 Cell membranes of prokaryotes. A schematic view of the membrane in bacterial cells surrounded by (A) two membranes or (B) one membrane.

the cell wall provide additional protection. The outer membrane is quite permeable to small molecules owing to the presence of porins. The region between the two membranes containing the cell wall is called the *periplasm*. Other bacteria and archaea have only a single membrane surrounded by a cell wall.

Eukaryotic cells, with the exception of plant cells, do not have cell walls, and their cell membranes consist of a single lipid bilayer. In plant cells, the cell wall is on the outside of the plasma membrane. Eukaryotic cells are distinguished by the use of membranes inside the cell to form internal compartments (Figure 12.36). For example, peroxisomes, organelles that play a major role in the oxidation of fatty acids for energy conversion, are defined by a single membrane. Mitochondria, the organelles in which ATP is synthesized, are surrounded by two membranes. Much like the case for a bacterium, the outer membrane is quite permeable to small molecules, whereas the inner membrane is not. Indeed, considerable evidence now indicates that mitochondria evolved from bacteria by *endosymbiosis* (Section 18.12). A double membrane also surrounds the nucleus. However, the *nuclear envelope* is not continuous but, instead, consists of a set of closed membranes that come together at structures called *nuclear pores*. These pores regulate transport into and out of the nucleus. The nuclear membranes are linked to another membrane-defined structure, the *endoplasmic reticulum*, which plays a host of cellular roles, including drug detoxification and the modification of proteins for secretion (Section 11.4.1). Thus, a eukaryotic cell comprises interacting compartments, and transport into and out of these compartments is essential to many biochemical processes.

12.7.1 Proteins Are Targeted to Specific Compartments by Signal Sequences

The compartmentalization of eukaryotic cells makes possible many processes that must be separated from the remainder of the cellular environment to function properly. Specific proteins are found in peroxisomes, others in mitochondria, and still others in the nucleus. How do proteins end up in the proper compartment? Even for bacteria, some targeting of proteins is required: some proteins are secreted from the cell, whereas others remain in the cytosol.

Proteins include specific sequences that serve as address labels to direct the molecules to the proper location. For example, most peroxisomal proteins end with a sequence, Ser-Lys-Leu-COO⁻, that acts as an autonomous

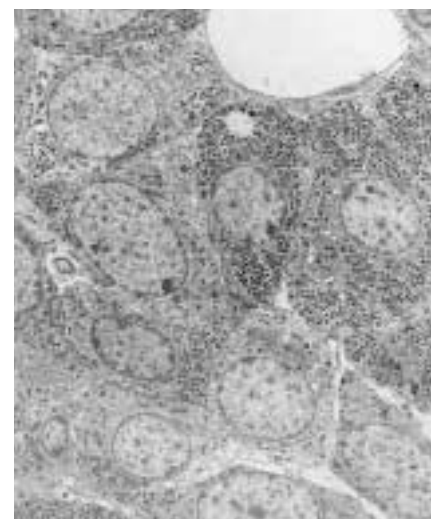


FIGURE 12.36 Internal membranes of eukaryotes. Electron micrograph of a thin section of a hormone-secreting cell for the rat pituitary, showing the presence of internal structures bounded by membranes. [Biophoto Associates/Photo Researchers.]



FIGURE 12.37 A mitochondrial targeting sequence. This sequence is recognized by receptors on the external face of the outer mitochondrial membrane. A protein bearing the sequence will be imported into the mitochondrion. Hydrophobic residues are shown in yellow, basic ones in blue, and serine and threonine in red.

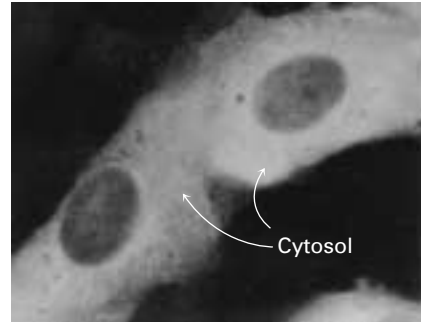
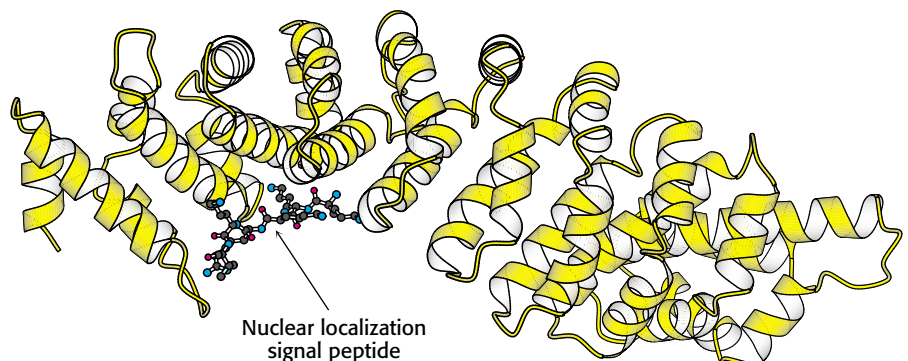
targeting signal. The removal of this sequence from a protein that normally resides in peroxisomes blocks its import into that organelle, whereas the addition of this sequence to a protein that normally resides in the cytosol can direct that protein to peroxisomes. A protein destined to pass through both mitochondrial membranes usually has a targeting sequence at its amino terminus (Figure 12.37). Unlike the peroxisomal targeting sequence, these amino-terminal sequences are highly variable; no clear consensus exists. They are typically from 15 to 35 residues long and rich in positively charged residues and in serines and threonines. Proteins destined for the nucleus have internal targeting sequences. A typical nuclear localization signal contains five consecutive positively charged residues such as Lys-Lys-Lys-Arg-Lys. The addition of such a sequence to a protein not found in the nucleus can direct it to the nucleus (Figure 12.38). Other sequences can direct proteins out of the nucleus. The known targeting sequences are given in Table 12.4.

TABLE 12.4 Targeting sequences

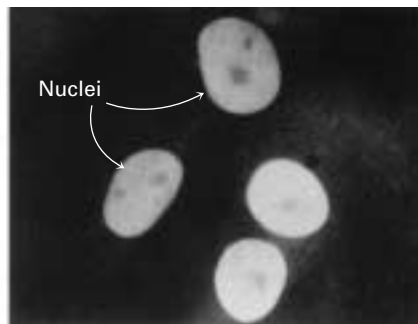
Target	Signal
Nucleus	–KKXK or –(K/R) ₂ –X _{10–12} –(K/R)*
Peroxisome	–SKL–COO [–]
Mitochondrion	N-terminal amphipathic helix
Endoplasmic reticulum	–KDEL–COO [–] (ER retention)

*The “/” means that either K or R is required.

Targeting sequences act by binding to specific proteins associated with each organelle. The determination of the structure of a protein, α -karyopherin, that binds to the nuclear localization signal reveals how the protein recognizes such a targeting sequence (Figure 12.39). A peptide containing the appropriate sequence binds to a specific site on the protein. The target peptide is held in an extended conformation through interactions between the target peptide backbone and asparagine side chains of the α -karyopherin while each of the basic residues lies in a deep pocket near the bottom, lined with negatively charged residues. Proteins that bind to the other targeting signal sequences presumably also have structures that allow recognition of



(A)



(B)

FIGURE 12.38 Movement of a protein into the nucleus. Localization of (A) unmodified pyruvate kinase, and (B) pyruvate kinase containing a nuclear localization signal sequence attached to its amino terminus. The protein was visualized by fluorescence microscopy after staining with a specific antibody. [From W. D. Richardson, B. L. Roberts, and A. E. Smith. *Cell* 44(1986):79.]

FIGURE 12.39 Protein targeting signal recognition. The structure of the nuclear localization signal-binding protein α -karyopherin (also known as α -importin) with a nuclear localization signal peptide bound to its major recognition site.

the required features. Note that we have considered only how proteins are marked for different compartments. Later, we will consider the mechanisms by which proteins actually cross membranes (Section 29.x.x).

12.7.2 Membrane Budding and Fusion Underlie Several Important Biological Processes

Membranes must be able to separate or join together to take up, transport, and release molecules. Many take up molecules through the process of *receptor-mediated endocytosis* (Figure 12.40). Here, a protein or larger complex initially binds to a receptor on the cell surface. After the protein is bound, specialized proteins act to cause the membrane in the vicinity of the bound protein to invaginate. The invaginated membrane eventually breaks off and fuses to form a *vesicle*.

Receptor-mediated endocytosis plays a key role in cholesterol metabolism (Section 26.x.x). Some cholesterol in the blood is in the form of a lipid–protein complex called *low-density lipoprotein* (LDL). Low density lipoprotein binds to an LDL receptor, an integral membrane protein. The segment of the plasma membrane containing the LDL–LDL receptor complex then invaginates and buds off from the membrane. The LDL separates from the receptor, which is recycled back to the membrane in a separate vesicle. The vesicle containing the LDL fuses with a *lysosome*, an organelle containing an array of digestive enzymes. The cholesterol is released into the cell for storage or use in membrane biosynthesis, and the remaining protein components are degraded. Various hormones, transport proteins, and antibodies employ receptor-mediated endocytosis to gain entry into a cell. A less advantageous consequence is that this pathway is available to viruses and toxins as a means of entry into cells. The reverse process—the fusion of a vesicle to a membrane—is a key step in the release of neurotransmitters from a neuron into the synaptic cleft (Figure 12.41). Although the processes of budding and fusion appear deceptively simple, the structures of the intermediates in the budding and fusing processes and the detailed mechanisms remain active areas of investigation.

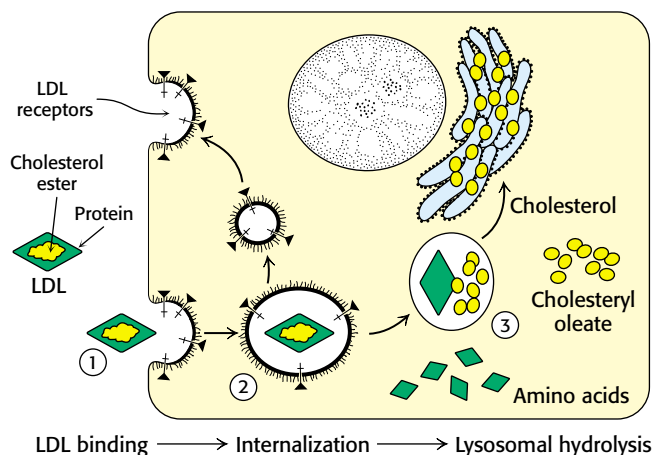


FIGURE 12.40 Receptor-mediated endocytosis. The process of receptor-mediated endocytosis is illustrated for the cholesterol-carrying complex, low-density lipoprotein (LDL): (1) LDL binds to a specific receptor, the LDL receptor; (2) this complex invaginates to form an internal vesicle; (3) after separation from its receptor, the LDL-containing vesicle fuses with a lysosome, leading to degradation of the LDL and release of the cholesterol.

SUMMARY

- Many Common Features Underlie the Diversity of Biological Membranes**
Biological membranes are sheetlike structures, typically from 60 to 100 Å thick, that are composed of protein and lipid molecules held together by noncovalent interactions. Membranes are highly selective permeability barriers. They create closed compartments, which may be entire cells or organelles within a cell. Proteins in membranes regulate the molecular and ionic compositions of these compartments. Membranes also control the flow of information between cells.
- Fatty Acids Are Key Constituents of Lipids**
Fatty acids are hydrocarbon chains of various lengths and degrees of unsaturation that terminate with a carboxylic acid group. The fatty acid chains in membranes usually contain between 14 and 24 carbon atoms; they may be saturated or unsaturated. Short chain length and unsaturation enhance the fluidity of fatty acids and their derivatives by lowering the melting temperature.



FIGURE 12.41 Neurotransmitter release. Neurotransmitter-containing synaptic vesicles are arrayed near the plasma membrane of a nerve cell. Synaptic vesicles fuse with the plasma membrane, releasing the neurotransmitter into the synaptic cleft. [T. Reese/Don Fawcett/Photo Researchers.]

- **There Are Three Common Types of Membrane Lipids**

The major classes of membrane lipids are phospholipids, glycolipids, and cholesterol. Phosphoglycerides, a type of phospholipid, consist of a glycerol backbone, two fatty acid chains, and a phosphorylated alcohol. Phosphatidyl choline, phosphatidyl serine, and phosphatidyl ethanolamine are major phosphoglycerides. Sphingomyelin, a different type of phospholipid, contains a sphingosine backbone instead of glycerol. Glycolipids are sugar-containing lipids derived from sphingosine. Cholesterol, which modulates membrane fluidity, is constructed from a steroid nucleus. A common feature of these membrane lipids is that they are amphipathic molecules, having hydrophobic and hydrophilic ends.

- **Phospholipids and Glycolipids Readily Form Bimolecular Sheets in Aqueous Media**

Membrane lipids spontaneously form extensive bimolecular sheets in aqueous solutions. The driving force for membrane formation is the hydrophobic interactions among the fatty acid tails of membrane lipids. The hydrophilic head groups interact with the aqueous medium. Lipid bilayers are cooperative structures, held together by many weak bonds. These lipid bilayers are highly impermeable to ions and most polar molecules, yet they are quite fluid, which enables them to act as a solvent for membrane proteins.

- **Proteins Carry Out Most Membrane Processes**

Specific proteins mediate distinctive membrane functions such as transport, communication, and energy transduction. Many integral membrane proteins span the lipid bilayer, whereas others are partly embedded in the membrane. Peripheral membrane proteins are bound to membrane surfaces by electrostatic and hydrogen-bond interactions. Membrane-spanning proteins have regular structures, including β strands, although the α helix is the most common membrane-spanning domain. Indeed, sequences of 20 consecutive nonpolar amino acids can be diagnostic of a membrane-spanning α -helical region of a protein.

- **Lipids and Many Membrane Proteins Diffuse Rapidly in the Plane of the Membrane**

Membranes are structurally and functionally asymmetric, as exemplified by the restriction of sugar residues to the external surface of mammalian plasma membranes. Membranes are dynamic structures in which proteins and lipids diffuse rapidly in the plane of the membrane (lateral diffusion), unless restricted by special interactions. In contrast, the rotation of lipids from one face of a membrane to the other (transverse diffusion, or flip-flop) is usually very slow. Proteins do not rotate across bilayers; hence, membrane asymmetry can be preserved. The degree of fluidity of a membrane partly depends on the chain length of its lipids and the extent to which their constituent fatty acids are unsaturated. In animals, cholesterol content also regulates membrane fluidity.

- **Eukaryotic Cells Contain Compartments Bounded by Internal Membranes**

An extensive array of internal membranes in eukaryotes creates compartments within a cell for distinct biochemical functions. For instance, a double membrane surrounds the nucleus, the location of most of the cell's genetic material, and the mitochondria, the location of most ATP synthesis. A single membrane defines the other internal compartments, such as the endoplasmic reticulum. Some compartments can exchange material by the process of membrane budding and fusion. As with all membranes, the proteins associated with these membranes determine the specific biochemical function. Specific amino acid sequences in the proteins direct these molecules to the appropriate compartment.

KEY TERMS

- fatty acid (p. 320)
 phospholipid (p. 322)
 sphingosine (p. 322)
 phosphoglyceride (p. 322)
 sphingomyelin (p. 323)
 glycolipid (p. 324)
 cerebroside (p. 325)
- ganglioside (p. 325)
 cholesterol (p. 325)
 amphipathic molecule (p. 325)
 lipid bilayer (p. 326)
 liposome (p. 327)
 integral membrane protein (p. 329)
 peripheral membrane protein (p. 329)
- hydropathy plot (p. 335)
 lateral diffusion (p. 335)
 fluid mosaic model (p. 336)
 targeting sequence (p. 340)
 receptor-mediated endocytosis (p. 341)

SELECTED READINGS

Where to start

- De Weer, P., 2000. A century of thinking about cell membranes. *Annu. Rev. Physiol.* 62:919–926.
- Bretscher, M. S., 1985. The molecules of the cell membrane. *Sci. Am.* 253(4):100–108.
- Unwin, N., and Henderson, R., 1984. The structure of proteins in biological membranes. *Sci. Am.* 250(2):78–94.
- Deisenhofer, J., and Michel, H., 1989. The photosynthetic reaction centre from the purple bacterium *Rhodospseudomonas viridis*. *EMBO J.* 8:2149–2170.
- Singer, S. J., and Nicolson, G. L., 1972. The fluid mosaic model of the structure of cell membranes. *Science* 175:720–731.
- Jacobson, K., Sheets, E. D., and Simson, R., 1995. Revisiting the fluid mosaic model of membranes. *Science* 268:1441–1442.

Books

- Gennis, R. B., 1989. *Biomembranes: Molecular Structure and Function*. Springer Verlag.
- Vance, D. E., and Vance, J. E. (Eds.), 1996. *Biochemistry of Lipids, Lipoproteins, and Membranes*. Elsevier.
- Lipowsky, R., and Sackmann, E., 1995. *The structure and dynamics of membranes*. Elsevier.
- Racker, E., 1985. *Reconstitutions of Transporters, Receptors, and Pathological States*. Academic Press.
- Tanford, C., 1980. *The Hydrophobic Effect: Formation of Micelles and Biological Membranes* (2d ed.). Wiley-Interscience.

Membrane lipids and dynamics

- Saxton, M. J., and Jacobson, K., 1997. Single-particle tracking: Applications to membrane dynamics. *Annu. Rev. Biophys. Biomol. Struct.* 26:373–399.
- Bloom, M., Evans, E., Mouritsen, O. G., 1991. Physical properties of the fluid lipid-bilayer component of cell membranes: A perspective. *Q. Rev. Biophys.* 24:293–397.
- Elson, E. L., 1986. Membrane dynamics studied by fluorescence correlation spectroscopy and photobleaching recovery. *Soc. Gen. Physiol. Ser.* 40:367–383.
- Zachowski, A., and Devaux, P. F., 1990. Transmembrane movements of lipids. *Experientia* 46:644–656.
- Devaux, P. F., 1992. Protein involvement in transmembrane lipid asymmetry. *Annu. Rev. Biophys. Biomol. Struct.* 21:417–439.
- Silvius, J. R., 1992. Solubilization and functional reconstitution of biomembrane components. *Annu. Rev. Biophys. Biomol. Struct.* 21:323–348.
- Yeagle, P. L., Albert, A. D., Boesze-Battaglia, K., Young, J., and Frye, J., 1990. Cholesterol dynamics in membranes. *Biophys. J.* 57:413–424.
- Nagle, J. F., and Tristram-Nagle, S., 2000. Lipid bilayer structure. *Curr. Opin. Struct. Biol.* 10: 474–480.

- Dowhan, W., 1997. Molecular basis for membrane phospholipid diversity: Why are there so many lipids? *Annu. Rev. Biochem.* 66: 199–232.
- Huijbregts, R. P. H., de Kroon, A. I. P. M., and de Kruijff, B., 1998. Rapid transmembrane movement of newly synthesized phosphatidylethanolamine across the inner membrane of *Escherichia coli*. *J. Biol. Chem.* 273:18936–18942.

Structure of membrane proteins

- Popot, J.-L., and Engleman, D. M., 2000. Helical membrane protein folding, stability and evolution. *Annu. Rev. Biochem.* 69:881–922.
- White, S. H., and Wimley, W. C., 1999. Membrane protein folding and stability: Physical principles. *Annu. Rev. Biophys. Biomol. Struct.* 28:319–365.
- Marassi, F. M., and Opella, S. J., 1998. NMR structural studies of membrane proteins. *Curr. Opin. Struct. Biol.* 8:640–648.
- Lipowsky, R., 1991. The conformation of membranes. *Nature* 349: 475–481.
- Altenbach, C., Marti, T., Khorana, H. G., and Hubbell, W. L., 1990. Transmembrane protein structure: Spin labeling of bacteriorhodopsin mutants. *Science* 248:1088–1092.
- Fasman, G. D., and Gilbert, W. A., 1990. The prediction of transmembrane protein sequences and their conformation: An evaluation. *Trends Biochem. Sci.* 15:89–92.
- Jennings, M. L., 1989. Topography of membrane proteins. *Annu. Rev. Biochem.* 58:999–1027.
- Engelman, D. M., Steitz, T. A., and Goldman, A., 1986. Identifying non-polar transbilayer helices in amino acid sequences of membrane proteins. *Annu. Rev. Biophys. Biomol. Struct.* 15:321–353.
- Udenfriend, S., and Kodukola, K., 1995. How glycosyl-phosphatidylinositol-anchored membrane proteins are made. *Annu. Rev. Biochem.* 64:563–591.

Intracellular membranes

- Shekel, J. J., and Wiley, D. C., 2000. Receptor binding and membrane fusion in virus entry: The influenza hemagglutinin. *Annu. Rev. Biochem.* 69:531–569.
- Roth, M. G., 1999. Lipid regulators of membrane traffic through the Golgi complex. *Trends Cell Biol.* 9:174–179.
- Jahn, R., and Sudhof, T. C., 1999. Membrane fusion and exocytosis. *Annu. Rev. Biochem.* 68:863–911.
- Stroud, R. M., and Walter, P., 1999. Signal sequence recognition and protein targeting. *Curr. Opin. Struct. Biol.* 9:754–759.
- Teter, S. A., and Klionsky, D. J., 1999. How to get a folded protein across a membrane. *Trends Cell Biol.* 9:428–31.
- Hettema, E. H., Distel, B., and Tabak, H. F., 1999. Import of proteins into peroxisomes. *Biochim. Biophys. Acta* 1451:17–34.

PROBLEMS

1. *Population density.* How many phospholipid molecules are there in a $1\text{-}\mu\text{m}^2$ region of a phospholipid bilayer membrane? Assume that a phospholipid molecule occupies 70 \AA^2 of the surface area.

2. *Lipid diffusion.* What is the average distance traversed by a membrane lipid in $1\text{ }\mu\text{s}$, 1 ms , and 1 s ? Assume a diffusion coefficient of $10^{-8}\text{ cm}^2\text{ s}^{-1}$.

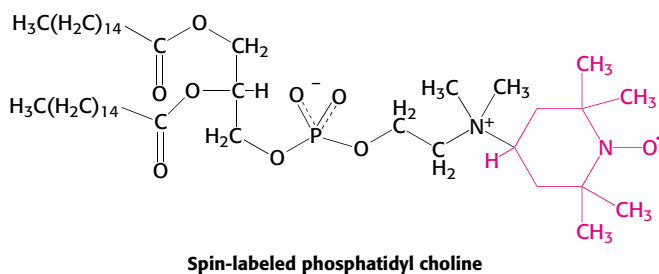
3. *Protein diffusion.* The diffusion coefficient, D , of a rigid spherical molecule is given by

$$D = kT/6\pi\eta r$$

in which η is the viscosity of the solvent, r is the radius of the sphere, k is the Boltzman constant (1.38×10^{-16} erg/degree), and T is the absolute temperature. What is the diffusion coefficient at 37°C of a 100-kd protein in a membrane that has an effective viscosity of 1 poise (1 poise = $1 \text{ erg s}^{-1}/\text{cm}^{-3}$)? What is the average distance traversed by this protein in 1 μs , 1 ms, and 1 s? Assume that this protein is an unhydrated, rigid sphere of density 1.35 g cm^{-3} .

4. *Cold sensitivity.* Some antibiotics act as carriers that bind an ion on one side of a membrane, diffuse through the membrane, and release the ion on the other side. The conductance of a lipid-bilayer membrane containing a carrier antibiotic decreased abruptly when the temperature was lowered from 40°C to 36°C. In contrast, there was little change in conductance of the same bilayer membrane when it contained a channel-forming antibiotic. Why?

5. *Flip-flop.* The transverse diffusion of phospholipids in a bilayer membrane was investigated by using a paramagnetic analog of phosphatidyl choline, called *spin-labeled phosphatidyl choline*.



The nitroxide (NO) group in spin-labeled phosphatidyl choline gives a distinctive paramagnetic resonance spectrum. This spectrum disappears when nitroxides are converted into amines by reducing agents such as ascorbate.

Lipid vesicles containing phosphatidyl choline (95%) and the spin-labeled analog (5%) were prepared by sonication and purified by gel-filtration chromatography. The outside diameter of these liposomes was about 25 nm. The amplitude of the paramagnetic resonance spectrum decreased to 35% of its initial value within a few minutes of the addition of ascorbate. There was no detectable change in the spectrum within a few minutes after the addition of a second aliquot of ascorbate. However, the amplitude of the residual spectrum decayed exponentially with a half-time of 6.5 hours. How would you interpret these changes in the amplitude of the paramagnetic spectrum?

6. *Flip-flop 2.* Although proteins rarely if ever flip-flop across a membrane, distribution of membrane lipids between the membrane leaflets is not absolute except in the case of glycolipids. Why are glycosylated lipids less likely to flip-flop?

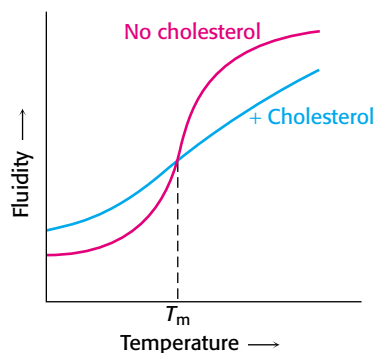
7. *Cis versus trans.* Why are most unsaturated fatty acids found in phospholipids in the *cis* rather than the *trans* conformation? Draw the structure of a 16-carbon fatty acid as saturated, *trans* monounsaturated, and *cis* monounsaturated.

8. *A question of competition.* Would a homopolymer of alanine be more likely to form an α helix in water or in a hydrophobic medium? Explain.

9. *Maintaining fluidity.* A culture of bacteria growing at 37°C was shifted to 25°C. How would you expect this shift to alter the fatty acid composition of the membrane phospholipids? Explain.

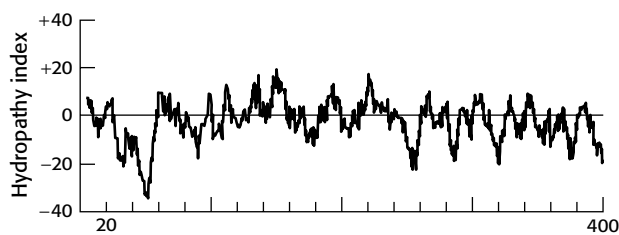
Data Interpretation Problems

10. *Cholesterol effects.* The red line on the following graph shows the fluidity of the fatty acids of a phospholipid bilayer as a function of temperature. The blue line shows the fluidity in the presence of cholesterol.

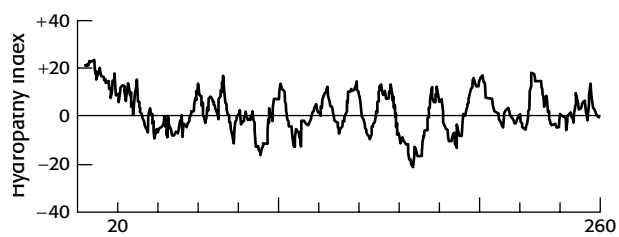


- What is the effect of cholesterol?
- Why might this effect be biologically important?

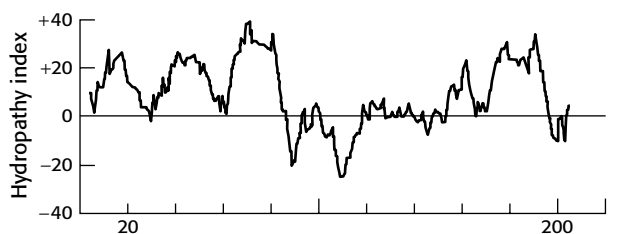
11. *Hydropathy plots.* On the basis of the following hydropathy plots for three proteins, predict which would be membrane proteins. What are the ambiguities with respect to using such plots to determine if a protein is a membrane protein?



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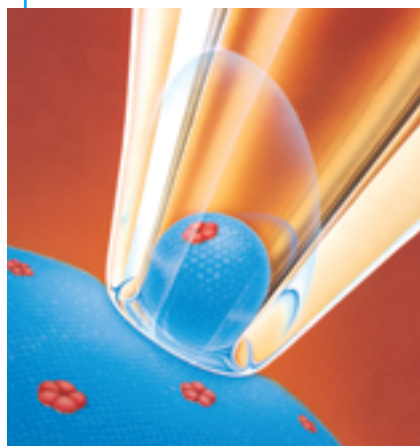


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Chapter Integration Problem

12. *The proper environment.* An understanding of the structure and function of membrane proteins has lagged behind that of other proteins. The primary reason is that membrane proteins are more difficult to purify and crystallize. Why might this be the case?

Membrane Channels and Pumps



The flow of ions through a single membrane channel (channels are shown in red in the illustration at the left) can be detected by the patch clamp technique, which records current changes as the channel transits between the open and closed states. [(Left) After E. Neher and B. Sakmann. *The patch clamp technique*. Copyright © 1992 by Scientific American, Inc. All rights reserved. (Right) Courtesy of Dr. Mauricio Montal.]

The lipid bilayer of biological membranes, as discussed in Chapter 12, is intrinsically impermeable to ions and polar molecules. Permeability is conferred by two classes of membrane proteins, *pumps* and *channels*. Pumps use a source of free energy such as ATP or light to drive the thermodynamically uphill transport of ions or molecules. Pump action is an example of *active transport*. Channels, in contrast, enable ions to flow rapidly through membranes in a downhill direction. Channel action illustrates *passive transport*, or *facilitated diffusion*.

Pumps are energy transducers in that they convert one form of free energy into another. Two types of ATP-driven pumps, P-type ATPases and the ATP-binding cassette pumps, undergo conformational changes on ATP binding and hydrolysis that cause a bound ion to be transported across the membrane. Phosphorylation and dephosphorylation of both the Ca^{2+} -ATPase and the Na^{+} - K^{+} -ATPase pumps, which are representative of P-type ATPase, are coupled to changes in orientation and affinity of their ion-binding sites.

A different mechanism of active transport, one that utilizes the gradient of one ion to drive the active transport of another, will be illustrated by the sodium–calcium exchanger. This pump plays an important role in extruding Ca^{2+} from cells.

We begin our examination of channels with the *acetylcholine receptor*, a channel that mediates the transmission of nerve signals across synapses, the functional junctions between neurons. The acetylcholine receptor is a *ligand-*

OUTLINE

- 13.1 The Transport of Molecules Across a Membrane May Be Active or Passive
- 13.2 A Family of Membrane Proteins Uses ATP Hydrolysis to Pump Ions Across Membranes
- 13.3 Multidrug Resistance and Cystic Fibrosis Highlight a Family of Membrane Proteins with ATP-Binding Cassette Domains
- 13.4 Secondary Transporters Use One Concentration Gradient to Power the Formation of Another
- 13.5 Specific Channels Can Rapidly Transport Ions Across Membranes
- 13.6 Gap Junctions Allow Ions and Small Molecules to Flow Between Communicating Cells

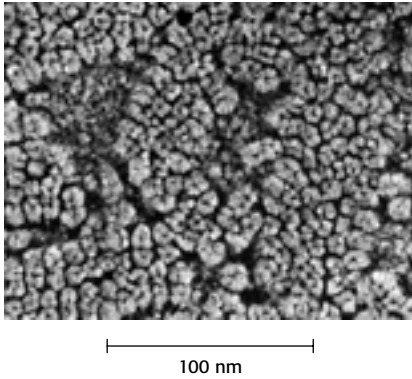


FIGURE 13.1 Acetylcholine receptors. An electron micrograph shows the densely packed acetylcholine receptors embedded in a postsynaptic membrane. [Courtesy of Dr. John Heuser and Dr. Shelly Salpeter.]

gated channel in that the channel opens in response to the binding of acetylcholine (Figure 13.1). In contrast, the sodium and potassium channels, which mediate action potentials in neuron axon membranes, are opened by membrane depolarization rather than by the binding of an allosteric effector. These channels are *voltage-gated*. These channels are also of interest because they swiftly and deftly distinguish between quite similar ions (e.g., Na^+ and K^+). The flow of ions through a single channel in a membrane can readily be detected by using the *patch-clamp technique*.

The chapter concludes with a view of a different kind of channel—the cell-to-cell channel, or *gap junction*. These channels allow the transport of ions and metabolites between cells.

13.1 THE TRANSPORT OF MOLECULES ACROSS A MEMBRANE MAY BE ACTIVE OR PASSIVE

Before we consider the specifics of membrane-protein function, we will consider some general principles of membrane transport. Two factors determine whether a molecule will cross a membrane: (1) the permeability of the molecule in a lipid bilayer and (2) the availability of an energy source.

13.1.1 Many Molecules Require Protein Transporters to Cross Membranes

As discussed in Chapter 12, some molecules can pass through cell membranes because they dissolve in the lipid bilayer. Such molecules are called *lipophilic molecules*. The steroid hormones provide a physiological example. These cholesterol relatives can pass through a membrane in their path of movement, but what determines the direction in which they will move? Such molecules will pass through a membrane located down their concentration gradient in a process called *simple diffusion*. In accord with the Second Law of Thermodynamics, molecules spontaneously move from a region of higher concentration to one of lower concentration. Thus, in this case, an entropy increase powers transport across the membrane.

Matters become more complicated when the molecule is highly polar. For example, sodium ions are present at 143 mM outside the cell and 14 mM inside the cell, yet sodium does not freely enter the cell because the positively charged ion cannot pass through the hydrophobic membrane interior. In some circumstances, as during a nerve impulse (Section 13.5.3), sodium ions must enter the cell. How are they able to do so? Sodium ions pass through specific channels in the hydrophobic barrier formed by membrane proteins. This means of crossing the membrane is called *facilitated diffusion*, because the diffusion across the membrane is facilitated by the channel. It is also called *passive transport*, because the energy driving the ion movement originates from the ion gradient itself, without any contribution by the transport system. Channels, like enzymes, display substrate specificity.

How is the sodium gradient established in the first place? In this case, sodium must move, or be pumped, *against* a concentration gradient. Because moving the ion from a low concentration to a higher concentration results in a decrease in entropy, it requires an input of free energy. Protein transporters embedded in the membrane are capable of using an energy source to move the molecule up a concentration gradient. Because an input of energy from another source is required, this means of crossing the membrane is called *active transport*.

13.1.2 Free Energy Stored in Concentration Gradients Can Be Quantified

An unequal distribution of molecules is an energy-rich condition because free energy is minimized when all concentrations are equal. Consequently, to attain such an unequal distribution of molecules, called a *concentration gradient*, requires an input of free energy. In fact, the creation of a concentration gradient is the result of active transport. Can we quantify the amount of energy required to generate a concentration gradient (Figure 13.2)? Consider an uncharged solute molecule. The free-energy change in transporting this species from side 1, where it is present at a concentration of c_1 , to side 2, where it is present at concentration c_2 , is

$$\Delta G = RT \ln(c_2/c_1) = 2.303RT \log_{10}(c_2/c_1)$$

For a charged species, the unequal distribution across the membrane generates an electrical potential that also must be considered because the ions will be repelled by the like charges. The sum of the concentration and electrical terms is called the *electrochemical potential*. The free-energy change is then given by

$$\Delta G = RT \ln(c_2/c_1) + ZF\Delta V = 2.303RT \log_{10}(c_2/c_1) + ZF\Delta V$$

in which Z is the electrical charge of the transported species, ΔV is the potential in volts across the membrane, and F is the faraday [23.1 kcal V^{-1} mol $^{-1}$ (96.5 kJ V^{-1} mol $^{-1}$)].

A transport process must be active when ΔG is positive, whereas it can be passive when ΔG is negative. For example, consider the transport of an uncharged molecule from $c_1 = 10^{-3}$ M to $c_2 = 10^{-1}$ M.

$$\begin{aligned} \Delta G &= 2.303RT \log_{10}(10^{-1}/10^{-3}) \\ &= 2.303 \times 1.99 \times 298 \times 2 \\ &= +2.7 \text{ kcal mol}^{-1} (+11.3 \text{ kJ mol}^{-1}) \end{aligned}$$

At 25°C (298 K), ΔG is + 2.7 kcal mol $^{-1}$ (+11.3 kJ mol $^{-1}$), indicating that this transport process requires an input of free energy. It could be driven, for example, by the hydrolysis of ATP, which yields -12 kcal mol $^{-1}$ (-50.2 kJ mol $^{-1}$) under typical cellular conditions. If ΔG is negative, the transport process can occur spontaneously without free-energy input.

Ion gradients are important energy storage forms in all biological systems. For instance, bacteriorhodopsin (Section 12.4.2) generates a proton gradient at the expense of light energy, an example of active transport. The energy of the proton gradient in turn can be converted into chemical energy in the form of ATP. This example illustrates the use of membranes and membrane proteins to transform energy: from light energy into an ion gradient into chemical energy.

13.2 A FAMILY OF MEMBRANE PROTEINS USES ATP HYDROLYSIS TO PUMP IONS ACROSS MEMBRANES

The extracellular fluid of animal cells has a salt concentration similar to that of sea water. However, cells must control their intracellular salt concentrations to prevent unfavorable interactions with high concentrations of ions such as calcium and to facilitate specific processes. For instance, most animal cells contain a high concentration of K^+ and a low concentration of Na^+ relative to the external medium. These ionic gradients are generated by a specific transport system, an enzyme that is called the *Na⁺-K⁺ pump* or the

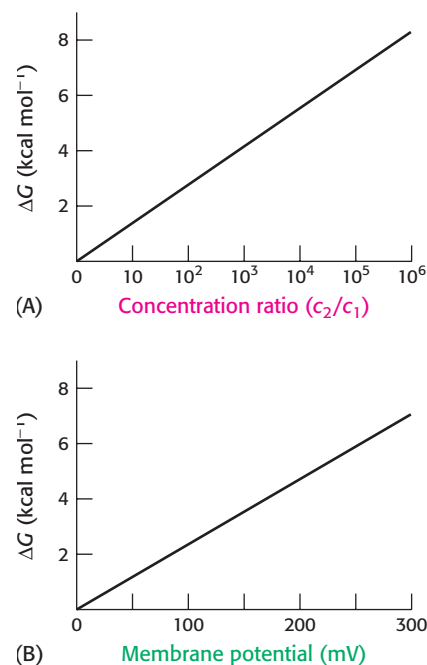


FIGURE 13.2 Free energy and transport. The free-energy change in transporting (A) an uncharged solute from a compartment at concentration c_1 to one at c_2 and (B) a singly charged species across a membrane to the side having the same charge as that of the transported ion. Note that the free-energy change imposed by a membrane potential of 59 mV is equivalent to that imposed by a concentration ratio of 10 for a singly charged ion at 25°C.

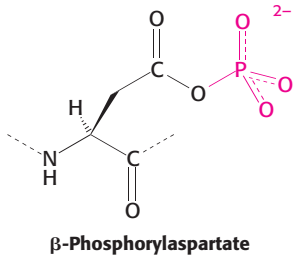


FIGURE 13.3 Phosphoaspartate. Phosphoaspartate (also referred to as β -aspartyl phosphate) is a key intermediate in the reaction cycles of P-type ATPases.

$\text{Na}^+ - \text{K}^+$ ATPase. The hydrolysis of ATP by the pump provides the energy needed for the active transport of Na^+ out of the cell and K^+ into the cell, generating the gradient. The pump is called the $\text{Na}^+ - \text{K}^+$ ATPase because the hydrolysis of ATP occurs only when Na^+ and K^+ are bound to the pump. Moreover, this ATPase, like all such enzymes, requires Mg^{2+} (Section 9.4.2). The active transport of Na^+ and K^+ is of great physiological significance. Indeed, more than a third of the ATP consumed by a resting animal is used to pump these ions. The $\text{Na}^+ - \text{K}^+$ gradient in animal cells controls cell volume, renders neurons and muscle cells electrically excitable, and drives the active transport of sugars and amino acids.

The subsequent purification of other ion pumps has revealed a large family of evolutionarily related ion pumps including proteins from bacteria, archaea, and all eukaryotes. These pumps are specific for an array of ions. Of particular interest are the Ca^{2+} ATPase, the enzyme that transports Ca^{2+} out of the cytoplasm and into the sarcoplasmic reticulum of muscle cells, and the gastric $\text{H}^+ - \text{K}^+$ ATPase, the enzyme responsible for pumping sufficient protons into the stomach to lower the pH below 1.0. These enzymes and the hundreds of known homologs, including the $\text{Na}^+ - \text{K}^+$ ATPase, are referred to as P-type ATPases because they form a key phosphorylated intermediate. In the formation of this intermediate, a phosphoryl group obtained from the hydrolysis of ATP is linked to the side chain of a specific conserved aspartate residue in the ATPase (Figure 13.3).

13.2.1 The Sarcoplasmic Reticulum Ca^{2+} ATPase Is an Integral Membrane Protein

We will consider the structural and mechanistic features of these enzymes by examining the Ca^{2+} ATPase found in the sarcoplasmic reticulum (SR Ca^{2+} ATPase) of muscle cells. This enzyme, which constitutes 80% of the sarcoplasmic reticulum membrane protein, plays an important role in muscle contraction, which is triggered by an abrupt rise in the cytosolic calcium level. Muscle relaxation depends on the rapid removal of Ca^{2+} from the cytosol into the sarcoplasmic reticulum, a specialized compartment for calcium storage, by the SR Ca^{2+} ATPase. This pump maintains a Ca^{2+} concentration of approximately $0.1 \mu\text{M}$ in the cytosol compared with 1.5 mM in the sarcoplasmic reticulum.

The SR Ca^{2+} ATPase is a single 110-kd polypeptide with a transmembrane domain consisting of 10 α helices. A large cytoplasmic head piece constitutes nearly half the molecular weight of the protein and consists of three distinct domains (Figure 13.4). The three cytoplasmic domains of the SR Ca^{2+} ATPase have distinct functions. One domain (N) binds the ATP nucleotide, another (P) accepts the phosphoryl group on its conserved aspartate residue, and the third (A) may serve as an actuator for the N domain. The relation between these three domains changes significantly on ATP hydrolysis. The crystal structure in the absence of ATP shows the likely nucleotide-binding site separated by more than 25 \AA from the phosphorylation site, suggesting that the N and P domains move toward one another during the catalytic cycle. This closure is facilitated by ATP binding and by the binding of Ca^{2+} to the membrane-spanning helices.

The results of mechanistic studies of the SR Ca^{2+} ATPase and other P-type ATPases have revealed two common features. First, as we have seen, each protein can be phosphorylated on a specific aspartate residue. For the SR Ca^{2+} ATPase, this reaction takes place at Asp 351 only in the presence of relatively high cytosolic concentrations of Ca^{2+} . Second, each pump can interconvert between at least two different conformations, denoted by E_1 and E_2 . Thus, at least four conformational states— E_1 , $E_1 - \text{P}$, $E_2 - \text{P}$, and E_2 —participate

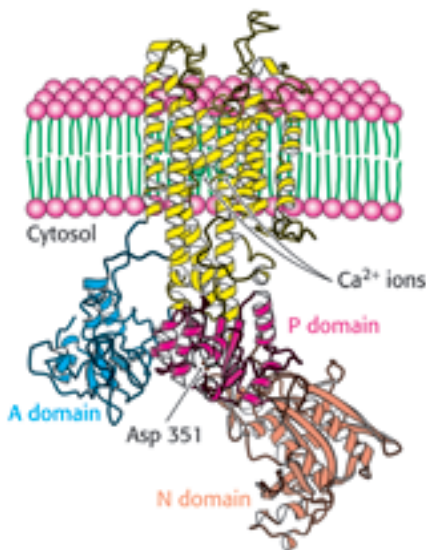


FIGURE 13.4 Structure of SR Ca^{2+} ATPase. This enzyme, the calcium pump of the sarcoplasmic reticulum, comprises a membrane-spanning domain of 10 α helices and a cytoplasmic headpiece consisting of three domains (N, P, and A). Two calcium ions (green) bind within the membrane-spanning region. The aspartate residue characteristic of this protein family is indicated.

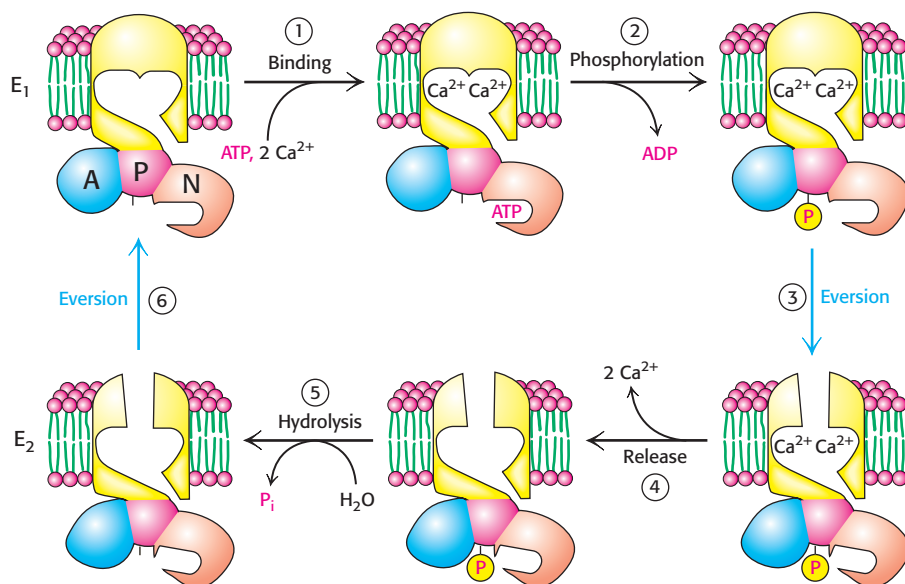


FIGURE 13.5 Mechanism of P-type ATPase action. The binding of Ca^{2+} and the phosphorylation of the ATPase (steps 1 and 2), illustrated here for the Ca^{2+} ATPase, lead to the eversion of the binding sites (step 3) and the release of Ca^{2+} to the luminal side of the membrane (step 4). Hydrolysis of phosphoaspartate (step 5) and eversion (step 6) reset the enzyme to its initial state.

in the transport process. From these four states, it is possible to construct a plausible mechanism of action for these enzymes, although further studies are necessary to confirm the mechanism and provide more details (Figure 13.5):

1. The postulated reaction cycle begins with the binding of ATP and two Ca^{2+} ions to the E_1 state.
2. The enzyme cleaves ATP, transferring the γ -phosphoryl group to the key aspartate residue. Calcium must be bound to the enzyme for the phosphorylation to take place. Phosphorylation shifts the conformational equilibrium of the ATPase toward E_2 .
3. The transition from the E_1 to the E_2 state causes the ion-binding sites to “evert” so that the ions can dissociate only to the luminal side of the membrane.
4. In the E_2 -P state, the enzyme has low affinity for the Ca^{2+} ions, so they are released.
5. With the release of Ca^{2+} , the phosphoaspartate residue is hydrolyzed, and the phosphate group is released.
6. The enzyme, devoid of a covalently attached phosphoryl group, is not stable in the E_2 form. It everts back to the E_1 form, completing the cycle.

Essentially the same mechanism is employed by the Na^+ - K^+ ATPase. The E_1 state binds three Na^+ ions and transports them across the membrane and out of the cell as a result of the protein’s phosphorylation and transition to the E_2 state. The three Na^+ ions are released into the extracellular medium. The E_2 state of this enzyme also binds ions—namely, two K^+ ions. These K^+ ions are carried across the membrane in the opposite direction by eversion driven by the hydrolysis of the phosphoaspartate residue and are released into the cytosol.

The change in free energy accompanying the transport of Na^+ and K^+ can be calculated (Section 13.1.1). Suppose that the concentration of Na^+ outside and inside the cell is 143 and 14 mM, respectively, and that of K^+ is 4 and 157 mM. At a membrane potential of -50 mV and a temperature of 37°C , the free-energy change in transporting 3 moles of Na^+ out of and 2 moles of K^+ into the cell is $+10.0$ kcal ($+41.8$ kJ mol^{-1}). The hydrolysis of a single ATP per transport cycle provides sufficient free energy, about -12 kcal mol^{-1} (-50 kJ mol^{-1}) under cellular conditions, to drive the uphill transport of these ions.

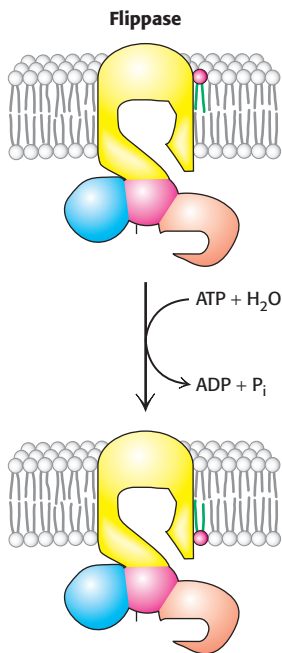



FIGURE 13.6 P-type ATPases can transport lipids. Flippases are enzymes that maintain membrane asymmetry by “flipping” phospholipids (displayed with a red head group) from the outer to the inner layer of the membrane.

13.2.2 P-Type ATPases Are Evolutionarily Conserved and Play a Wide Range of Roles

 Analysis of the complete yeast genome revealed the presence of 16 proteins that clearly belong to the P-type ATPase family. More detailed sequence analysis suggests that 2 of these proteins transport H^+ ions, 2 transport Ca^{2+} , 3 transport Na^+ , and 2 transport metals such as Cu^{2+} . In addition, 5 members of this family appear to participate in the transport of phospholipids with amino acid head groups. These latter proteins assist in the maintenance of membrane asymmetry by transporting lipids such as phosphatidyl serine from the outer to the inner leaflet of the bilayer membrane (Figure 13.6). Such enzymes have been termed “flippases.”

All members of this protein family employ the same fundamental mechanism. The free energy of ATP hydrolysis drives membrane transport by effecting conformational changes associated with the addition and removal of a phosphoryl group at an analogous aspartate site in each protein.

13.2.3 Digitalis Specifically Inhibits the $Na^+ - K^+$ Pump by Blocking Its Dephosphorylation

Certain steroids derived from plants are potent inhibitors ($K_i \approx 10$ nM) of the $Na^+ - K^+$ pump. Digitoxigenin and ouabain are members of this class of inhibitors, which are known as *cardiotonic steroids* because of their strong effects on the heart (Figure 13.7). These compounds inhibit the dephosphorylation of the $E_2 - P$ form of the ATPase when applied on the *extracellular* face of the membrane.

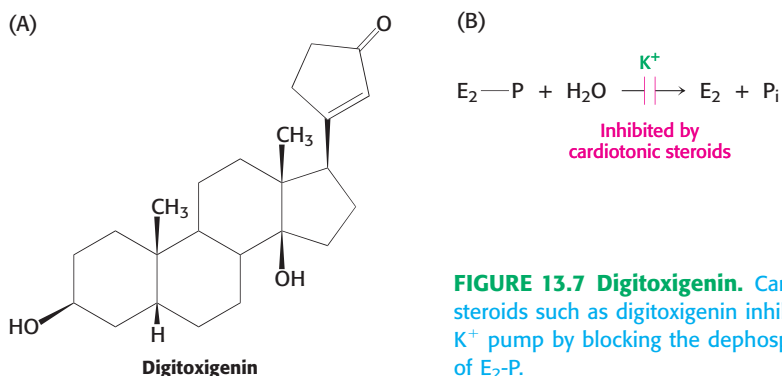




FIGURE 13.7 Digitoxigenin. Cardiotonic steroids such as digitoxigenin inhibit the $Na^+ - K^+$ pump by blocking the dephosphorylation of $E_2 - P$.




Foxglove (*Digitalis purpurea*) is the source of digitalis, one of the most widely used drugs. [Inga Spence/Visuals Unlimited.]

 *Digitalis*, a mixture of cardiotonic steroids derived from the dried leaf of the foxglove plant (*Digitalis purpurea*), is of great clinical significance. Digitalis increases the force of contraction of heart muscle, which makes it a choice drug in the treatment of congestive heart failure. Inhibition of the $Na^+ - K^+$ pump by digitalis leads to a higher level of Na^+ inside the cell. The diminished Na^+ gradient results in slower extrusion of Ca^{2+} by the sodium–calcium exchanger (Section 13.4). The subsequent increase in the intracellular level of Ca^{2+} enhances the contractility of cardiac muscle. It is interesting to note that digitalis was effectively used long before the discovery of the $Na^+ - K^+$ ATPase. In 1785, William Withering, a British physician, heard tales of an elderly woman, known as “the old woman of Shropshire,” who cured people of “dropsy” (which today would be recognized as congestive heart failure) with an extract of foxglove. Withering conducted the first scientific study of the effects of foxglove on congestive heart failure and documented its effectiveness.

13.3 MULTIDRUG RESISTANCE AND CYSTIC FIBROSIS HIGHLIGHT A FAMILY OF MEMBRANE PROTEINS WITH ATP-BINDING CASSETTE DOMAINS

 Tumor cells in culture often become resistant to drugs that were initially quite toxic to the cells. Remarkably, the development of resistance to one drug also makes the cells less sensitive to a range of other compounds. This phenomenon is known as *multidrug resistance*. In a significant discovery, the onset of multidrug resistance was found to correlate with the expression and activity of a membrane protein with an apparent molecular mass of 170 kd. This protein acts as an ATP-dependent pump that extrudes a wide range of small molecules from cells that express it. The protein is called the *multidrug resistance protein (MDR)* or *P-glycoprotein* (“glyco” because it includes a carbohydrate moiety). Thus, when cells are exposed to a drug, the MDR pumps the drug out of the cell before the drug can exert its effects. A related protein was discovered through genetic studies of the hereditary disease *cystic fibrosis* (Section 1.1.4). In one of the first studies leading to the identification of a specific genetic change causing human disease, investigators performed a comparative genetic analysis of many people having this disease and family members who did not have the disease. The gene found to be mutated in the affected persons encodes a protein, now called *cystic fibrosis transmembrane conductance regulator (CFTR)*. CFTR acts as an ATP-regulated chloride channel in the plasma membranes of epithelial cells. As mentioned in Chapter 1, cystic fibrosis results from a decrease in fluid and salt secretion by CFTR. As a consequence of this defect, secretion from the pancreas is blocked and heavy, dehydrated mucus accumulates in the lungs, leading to chronic lung infections.

 Analysis of the amino acid sequences of MDR, CFTR, and homologous proteins revealed a common architecture (Figure 13.8). Each protein comprises four domains: two membrane-binding domains of unknown structure and two ATP-binding domains. The ATP-binding domains of these proteins are called *ATP-binding cassettes* (or ABCs) and are homologous to domains in a large family of transport proteins of bacteria and archaea. Indeed, with 79 members, the ABC proteins are the largest single family identified in the *E. coli* genome. The ABC proteins are members of the P-loop NTPase superfamily (Section 9.4.4). Some ABC proteins, particularly those of prokaryotes, are multisubunit proteins constructed such that the membrane-spanning domains and the ABC domains are present on separate polypeptide chains. The consolidation of enzymatic activities of several polypeptide chains in prokaryotes to a single chain in eukaryotes is a theme that we will see again (Section 22.4.4). For example, the histidine permease of *Salmonella typhimurium*, which transports the amino acid histidine into the bacterium, consists of (1) two different protein subunits with membrane-spanning domains (HisQ and HisM) and (2) a homodimeric protein (HisP) with ABC domains (Figure 13.9). A soluble, histidine-binding protein (HisJ) binds histidine after the amino acid enters the cell.

Like other members of the P-loop NTPase superfamily, proteins with ABC domains undergo conformational changes on ATP binding and hydrolysis. These structural changes are coupled within each dimeric transporter unit in a manner that allows these membrane proteins to drive the uptake or efflux of specific compounds or to act as gates for open membrane channels.

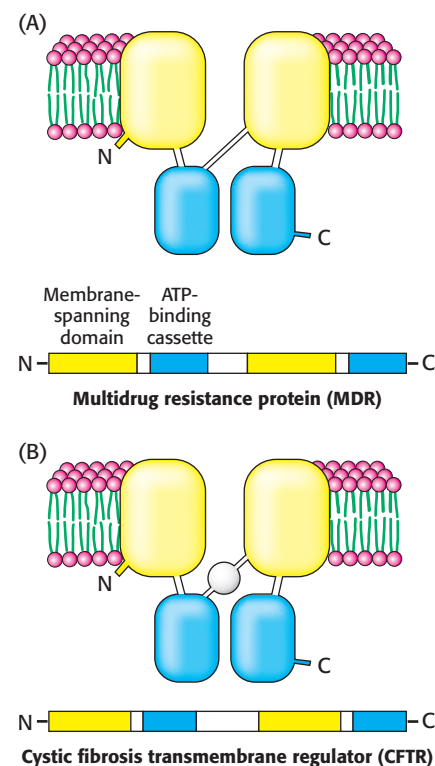


FIGURE 13.8 ABC transporters. The multidrug resistance protein (MDR) and the cystic fibrosis transmembrane regulator (CFTR) are homologous proteins composed of two transmembrane domains and two ATP-binding domains, called ATP-binding cassettes (ABCs).

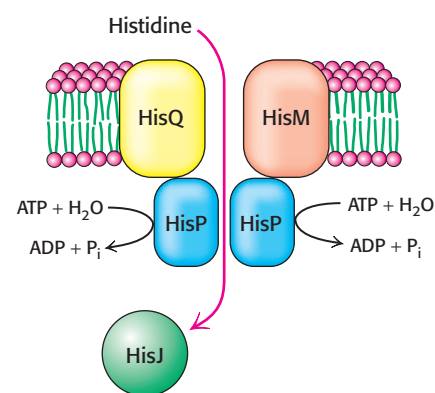


FIGURE 13.9 Histidine permease. In the histidine permease of *S. typhimurium*, the membrane-spanning regions (yellow and orange) and ABC regions (blue) are on separate polypeptide chains (compare with Figure 13.8). ATP hydrolysis drives the transport of histidine into the cell.

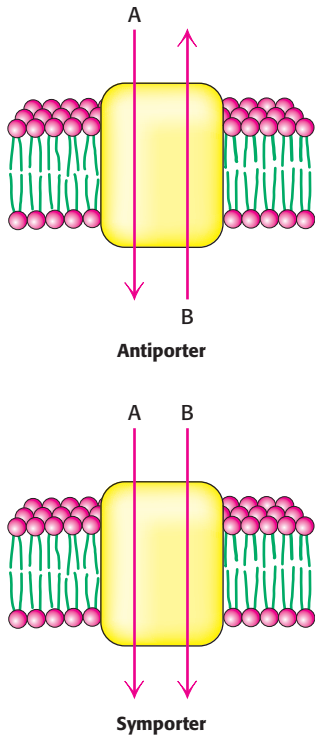


FIGURE 13.10 Secondary transporters. These transporters employ the downhill flow of one gradient to power the formation of another gradient. In antiporters, the chemical species move in opposite directions. In symporters, the two species move in the same direction.

13.4 SECONDARY TRANSPORTERS USE ONE CONCENTRATION GRADIENT TO POWER THE FORMATION OF ANOTHER

Many active-transport processes are not directly driven by the hydrolysis of ATP. Instead, the thermodynamically uphill flow of one species of ion or molecule is coupled to the downhill flow of a different species. Membrane proteins that pump ions or molecules uphill by this means are termed *secondary transporters* or *cotransporters*. These proteins can be classified as either *antiporters* or *symporters*. Antiporters couple the downhill flow of one species to the uphill flow of another in the *opposite direction* across the membrane; symporters use the flow of one species to drive the flow of a different species in the *same direction* across the membrane (Figure 13.10).

The *sodium–calcium exchanger* in the plasma membrane of an animal cell is an antiporter that uses the electrochemical gradient of Na^+ to pump Ca^{2+} out of the cell. Three Na^+ ions enter the cell for each Ca^{2+} ion that is extruded. The cost of transport by this exchanger is paid by the Na^+ - K^+ -ATPase pump, which generates the requisite sodium gradient. Because Ca^{2+} is a vital messenger inside the cell, its concentration must be tightly controlled. *The exchanger has lower affinity for Ca^{2+} than does the Ca^{2+} ATPase (Section 13.2.1), but its capacity to extrude Ca^{2+} is greater.* The exchanger can lower the cytosolic Ca^{2+} level to several micromolar; submicromolar Ca^{2+} levels are attained by the subsequent action of the Ca^{2+} ATPase. The exchanger can extrude about 2000 Ca^{2+} ions per second, compared with only 30 ions per second for the Ca^{2+} -ATPase pump.

Glucose is pumped into some animal cells by a symporter powered by the simultaneous entry of Na^+ . The entry of Na^+ provides a free-energy input of $2.2 \text{ kcal mol}^{-1}$ (9.2 kJ mol^{-1}) under typical cellular conditions (external $[\text{Na}^+] = 143 \text{ mM}$, internal $[\text{Na}^+] = 14 \text{ mM}$, and membrane potential = -50 mV). This free-energy input is sufficient to generate a 66-fold concentration gradient of an uncharged molecule such as glucose.


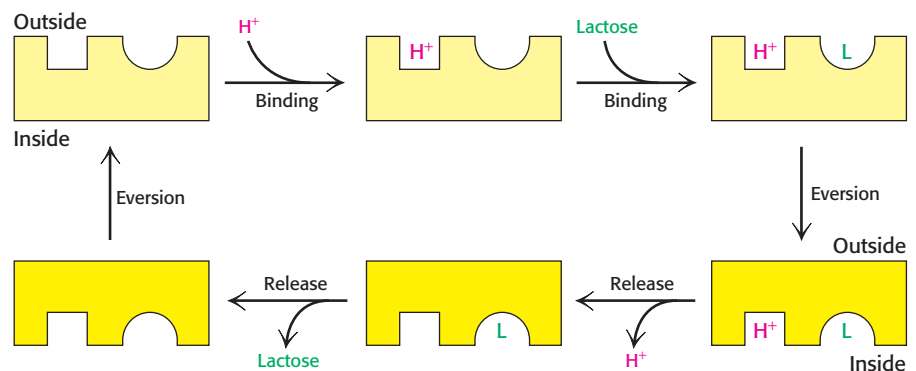
 Secondary transporters are ancient molecular machines, common today in bacteria and archaea as well as in eukaryotes. For example, approximately 160 (of approximately 4000) proteins encoded by the *E. coli* genome appear to be secondary transporters. Sequence comparison and hydrophathy analysis suggest that members of the largest family have 12 transmembrane helices that appear to have arisen by duplication and fusion of a membrane protein with 6 transmembrane helices. Included in this family is the lactose permease of *E. coli*. This symporter uses the H^+ gradient across the *E. coli* membrane generated by the oxidation of fuel molecules to drive the uptake of lactose and other sugars against a concentration gradient. The permease has a proton-binding site and a lactose-binding site (Figure 13.11).

FIGURE 13.11 Action of lactose permease. Lactose permease pumps lactose into bacterial cells by drawing on the proton-motive force. The binding sites evert when a lactose molecule (L) and a proton (H^+) are bound to external sites. After these species are released inside the cell, the binding sites again evert to complete the transport cycle. Lactose permease is an example of a symporter.



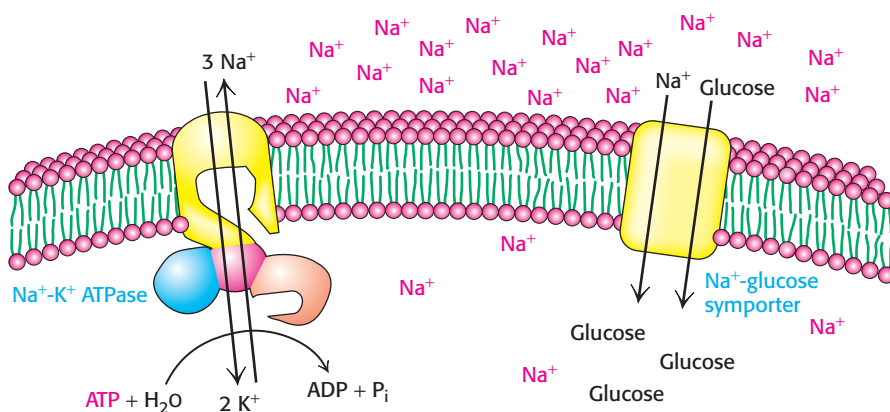


FIGURE 13.12 Energy transduction by membrane proteins. The Na⁺-K⁺ ATPase converts the free energy of phosphoryl transfer into the free energy of a Na⁺ ion gradient. The ion gradient can then be used to pump materials into the cell, through the action of a secondary transporter such as the Na⁺-glucose symporter.

A proton and a lactose molecule bind to sites facing the outside of the cell. The permease, with both binding sites full, everts, releasing first the proton and then the lactose inside the bacterium. Another eversion places the empty sites on the outside. Thus, the energetically uphill transfer of one lactose molecule is coupled to the downhill transport of one proton. Further analysis of the three-dimensional structures is underway and should provide more information about their mechanisms of action as well as the evolutionary relationships within this large group of ancient proteins.

These observations reveal how different energy currencies are interconverted. A single energy currency, ATP, is used by P-type ATPases to generate gradients of a small number of types of ions, particularly Na⁺ and H⁺, across membranes. These gradients then serve as an energy source for the large number of secondary transporters, which allow many different molecules to be taken up or transported out of cells (Figure 13.12).

13.5 SPECIFIC CHANNELS CAN RAPIDLY TRANSPORT IONS ACROSS MEMBRANES

Pumps and secondary transporters can transport ions at rates approaching several thousand ions per second. Other membrane proteins, *ion channels*, which are passive transport systems, are capable of ion-transport rates that are more than 1000 times as high. These rates of transport through ion channels are close to rates expected for ions diffusing freely through aqueous solution. Yet, ion channels are not simply tubes that span membranes through which ions can rapidly flow. Instead, they are highly sophisticated molecular machines that respond to chemical and physical changes in their environments and undergo precisely timed conformational changes that facilitate their roles as essential components of the nervous and other systems.

Several key properties characterize ion channels:

1. *Ion channels can be highly selective for particular ions.* For example, some channels allow the flow of K⁺ very effectively but do not allow appreciable levels of Na⁺ to cross the membrane. Other channels transport positively charged ions (cations), but block the flow of negatively charged ions (anions). The selectivities of some ion-channel proteins are shown in Table 13.1.
2. *Ion channels exist in open and closed states.* These channels undergo transitions from the closed state, incapable of supporting ion transport, to the open state, through which ions can flow.
3. *Transitions between the open and the closed states are regulated.* Ion channels are divided into two classes: *ligand-gated channels* and *voltage-gated*

TABLE 13.1 Relative permeabilities for selected ion channels

	Na ⁺ channel	K ⁺ channel	Acetylcholine receptor	Chloride channel
Li ⁺	0.93	< 0.01	0.87	< 0.01
Na ⁺	1.00	< 0.01	1.00	< 0.01
K ⁺	0.09	1.00	1.11	< 0.01
Rb ⁺	< 0.01	0.91		
Cs ⁺	< 0.01	< 0.08	1.42	
NH ₄ ⁺	0.16	0.13	1.79	
H ₃ NOH ⁺	0.94	< 0.03	1.92	
H ₂ NNH ₃ ⁺	0.59	< 0.03		
H ₃ CNH ₃ ⁺	< 0.01	< 0.02		
Cl ⁻	< 0.01	< 0.01	< 0.01	1.00

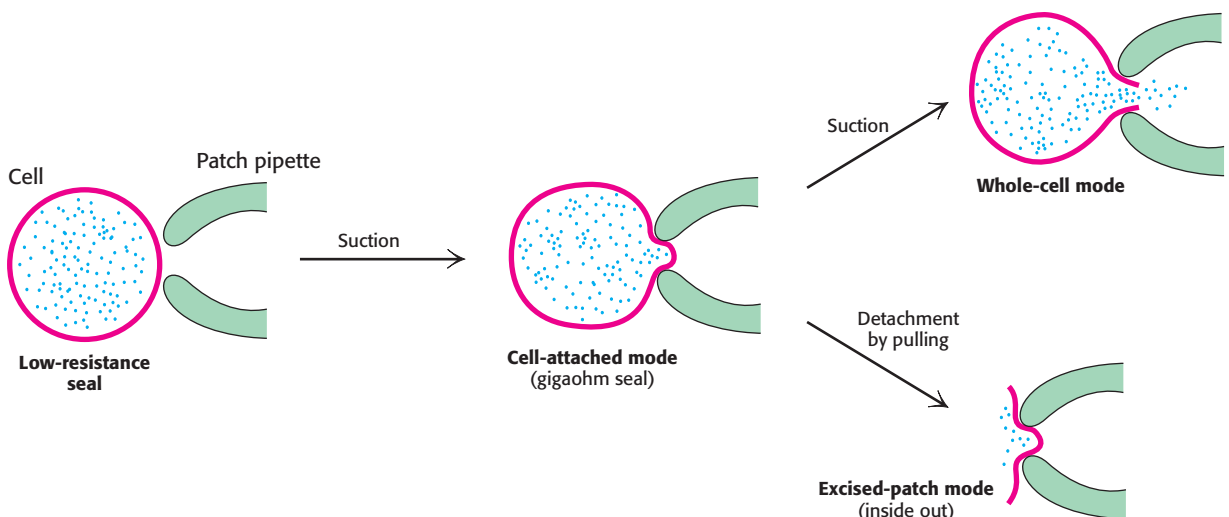
channels. Ligand-gated channels open and close in response to the binding of specific chemicals, whereas voltage-gated channels open and close in response to the electrical potential across the membrane in which they are found.

4. *Open states of channels often spontaneously convert into inactivated states.* Most ion channels do not remain in an open state indefinitely but, instead, spontaneously transform into inactivated states that do not conduct ions. The spontaneous transitions of ion channels from open to inactivated states act as built-in timers that determine the duration of ion flow.

FIGURE 13.13 Patch-clamp modes. The patch-clamp technique for monitoring channel activity is highly versatile. A high-resistance seal (gigaseal) is formed between the pipette and a small patch of plasma membrane. This configuration is called *cell attached*. The breaking of the membrane patch by increased suction produces a low-resistance pathway between the pipette and interior of the cell. The activity of the channels in the entire plasma membrane can be monitored in this *whole-cell mode*. To prepare a membrane in the *excised-patch mode*, the pipette is pulled away from the cell. A piece of plasma membrane with its cytosolic side now facing the medium is monitored by the patch pipette.

13.5.1 Patch-Clamp Conductance Measurements Reveal the Activities of Single Channels

The study of ion channels has been revolutionized by the *patch-clamp technique*, which was introduced by Erwin Neher and Bert Sakmann in 1976 (Figure 13.13). This powerful technique enables the measurement of the activity of a single channel to be measured. A clean glass pipette with a tip diameter of about 1 μm is pressed against an intact cell to form a seal. Slight suction leads to the formation of a very tight seal so that the resistance between the inside of the pipette and the bathing solution is many gigaohms (1 gigaohm is equal to 10^9 ohms). Thus, a gigaohm seal (called a *gigaseal*)



ensures that an electric current flowing through the pipette is identical with the current flowing through the membrane covered by the pipette. The gigaseal makes possible high-resolution current measurements while a known voltage is applied across the membrane. In fact, patch clamping increased the precision of such measurements 100-fold. *The flow of ions through a single channel and transitions between the open and closed states of a channel can be monitored with a time resolution of microseconds.* Furthermore, the activity of a channel in its native membrane environment, even in an intact cell, can be directly observed. Patch-clamp methods provided one of the first views of single biomolecules in action. Subsequently, other methods for observing single molecules were invented, opening new vistas on biochemistry at its most fundamental level.

13.5.2 Ion-Channel Proteins Are Built of Similar Units

How do ion channels, vital to a wide array of biological functions, operate at a molecular level? We will examine three channels important in the propagation of nerve impulses: the ligand-gated channel; the acetylcholine receptor channel, which communicates the nerve impulse between certain neurons; and the voltage-gated Na^+ and K^+ channels, which conduct the nerve impulse down the axon of a neuron.

Nerve impulses are communicated across most synapses by small, diffusible molecules called *neurotransmitters*, of which one is acetylcholine, referred to as a cholinergic neurotransmitter because it is derived from choline (Section 12.2.1). The presynaptic membrane of a synapse is separated from the postsynaptic membrane by a gap of about 50 nm, called the *synaptic cleft*. The end of the presynaptic axon is filled with *synaptic vesicles*, each containing about 10^4 acetylcholine molecules (Figure 13.14). The arrival of a nerve impulse leads to the synchronous export of the contents of some 300 vesicles, which raises the acetylcholine concentration in the cleft from 10 nM to 500 μM in less than a millisecond. The binding of acetylcholine to the postsynaptic membrane markedly changes its ionic permeabilities (Figure 13.15). *The conductance of both Na^+ and K^+ increases greatly within 0.1 ms, leading to a large inward current of Na^+ and a smaller outward current of K^+ .* The inward Na^+ current depolarizes the postsynaptic membrane and triggers an action potential (Section 13.5.3). *Acetylcholine opens a single kind of cation channel, which is almost equally permeable to Na^+ and K^+ .* This change in ion permeability is mediated by the *acetylcholine receptor*.

The acetylcholine receptor is the best-understood ligand-gated channel. The activity of a single such channel is graphically displayed in patch-clamp

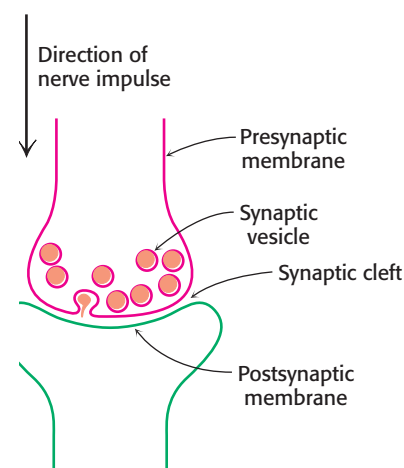


FIGURE 13.14 Schematic representation of a synapse.

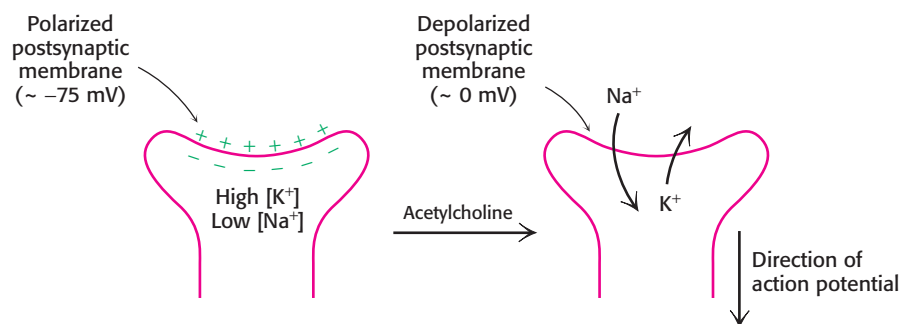
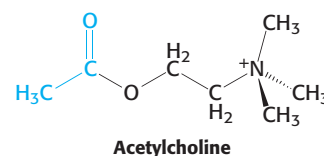


FIGURE 13.15 Membrane depolarization. Acetylcholine depolarizes the postsynaptic membrane by increasing the conductance of Na^+ and K^+ .

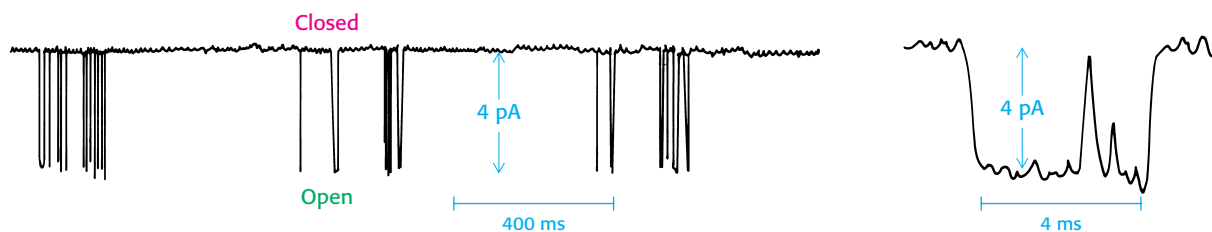



FIGURE 13.16 Patch-clamp recordings of the acetylcholine receptor channel. Patch-clamp recordings illustrate changes in the conductance of an acetylcholine receptor channel in the presence of acetylcholine. The channel undergoes frequent transitions between open and closed states. [Courtesy of Dr. D. Colquhoun and Dr. B. Sakmann.]



The torpedo (*Torpedo marmorata*, also known as the electric ray) has an electric organ, rich in acetylcholine receptors, that can deliver a shock of as much as 200 V for approximately 1 s. [Yves Gladu/Jacana/Photo Researchers.]

recordings of postsynaptic membranes of skeletal muscle (Figure 13.16). The addition of acetylcholine is followed by transient openings of the channel. The current, i , flowing through an open channel is 4 pA (picoamperes) when the membrane potential, V , is -100 mV. An ampere is the flow of 6.24×10^{18} charges per second. Hence, 2.5×10^7 ions per second flow through an open channel.

The electric organ of *Torpedo marmorata*, an electric fish, is a choice source of acetylcholine receptors for study because its electroplaxs (voltage-generating cells) are very rich in cholinergic postsynaptic membranes. The receptor is very densely packed in these membranes ($\sim 20,000/\mu\text{m}^2$). An exotic biological material has been invaluable in the isolation of acetylcholine receptors. Snake neurotoxins such as α -bungarotoxin (from the venom of a Formosan snake) and *cobratoxin* block the transmission of impulses between nerve and muscle. These small (7-kd) basic proteins bind specifically and very tightly to acetylcholine receptors and hence can be used as tags.

 The acetylcholine receptor of the electric organ has been solubilized by adding a nonionic detergent to a postsynaptic membrane preparation and purified by affinity chromatography on a column bearing covalently attached *cobratoxin*. With the use of techniques presented in Chapter 4, the 268-kd receptor was identified as a pentamer of four kinds of membrane-spanning subunits— α_2 , β , γ , and δ —arranged in the form of a ring that creates a pore through the membrane (Figure 13.17). The cloning and sequencing of the cDNAs for the four kinds of subunits (50–58 kd) showed that they have clearly similar sequences; the genes for the α , β , γ , and δ subunits arose by duplication and divergence of a common ancestral gene. Each subunit has a large extracellular domain, followed at the carboxyl end by four predominantly hydrophobic segments that span the bilayer membrane. Acetylcholine binds at the α - γ and α - δ interfaces. Electron microscopic studies of purified acetylcholine receptors demonstrated that the structure has approximately fivefold symmetry, in harmony with the similarity of its five constituent subunits.

What is the basis of channel opening? A comparison of the structures of the closed and open forms of the channel would be highly revealing, but such comparisons have been difficult to obtain. Cryoelectron micrographs indicate that the binding of acetylcholine to the extracellular domain causes a structural alteration, which initiates rotations of the α -helical rods lining the membrane-spanning pore. The amino acid sequences of these helices point to the presence of alternating ridges of small polar or neutral residues (serine, threonine, glycine) and large nonpolar ones (isoleucine, leucine, phenylalanine). In the closed state, the large residues may occlude the channel by forming a tight hydrophobic ring (Figure 13.18). Indeed, each subunit has a bulky

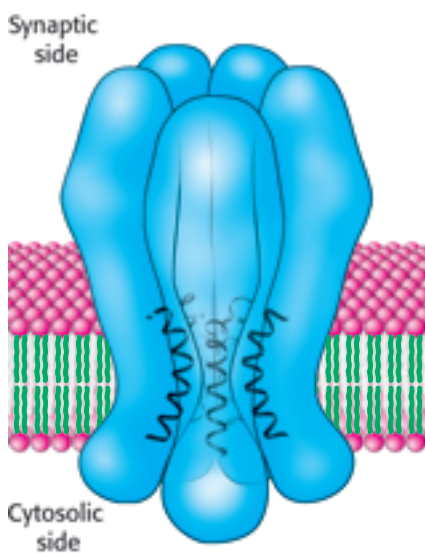


FIGURE 13.17 Schematic representation of the closed form of the acetylcholine receptor channel. In the closed state, the narrowest part of the pore is occluded by side chains coming from five helices. [Courtesy of Dr. Nigel Unwin.]

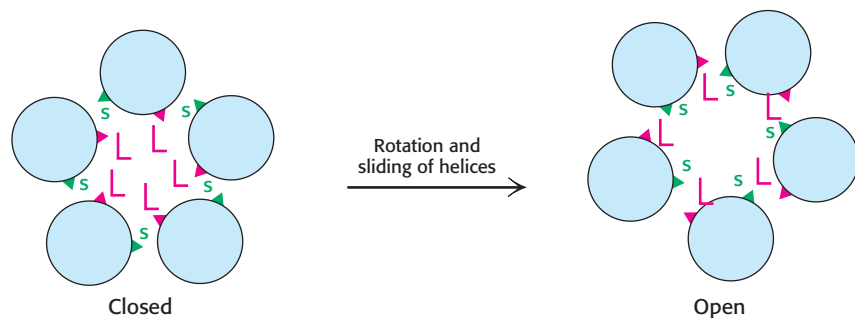


FIGURE 13.18 Opening of the acetylcholine channel pore. Large hydrophobic side chains (L) occlude the pore of the closed form of the acetylcholine receptor channel. Channel opening is probably mediated by the tilting of helices that line the pore. Large residues move away from the pore and small ones (S) take their place. [After N. Unwin. *Neuron* 3(1989):665.]

leucine residue at a critical position. The binding of acetylcholine could allosterically rotate the membrane-spanning helices so that the pore would be lined by small polar residues rather than by large hydrophobic ones. The wider, more polar pore would then be open to the passage of Na^+ and K^+ ions.

13.5.3 Action Potentials Are Mediated by Transient Changes in Na^+ and K^+ Permeability

We turn now from ligand-gated channels to voltage-gated channels, which are responsible for the propagation of nerve impulses. A *nerve impulse* is an electrical signal produced by the flow of ions across the plasma membrane of a neuron and is the fundamental means of communication in the nervous system. The interior of a neuron, like that of most other cells, has a high concentration of K^+ and a low concentration of Na^+ . These ionic gradients are generated by an ATP-driven pump (Section 13.2.1). In the resting state, the membrane potential is -60 mV. A nerve impulse, or *action potential*, is generated when the membrane potential is depolarized beyond a critical threshold value (i.e., from -60 to -40 mV). The membrane potential becomes positive within about a millisecond and attains a value of about $+30$ mV before turning negative again. This amplified depolarization is propagated along the nerve terminal (Figure 13.19)

Ingenuous experiments carried out by Alan Hodgkin and Andrew Huxley revealed that *action potentials arise from large, transient changes in the permeability of the axon membrane to Na^+ and K^+ ions* (see Figure 13.19A). Two kinds of voltage-sensitive channels, one selectively permeable to Na^+ and the other to K^+ , were defined. The conductance of the membrane to Na^+ changes first. Depolarization of the membrane beyond the threshold level leads to an opening of Na^+ channels. Sodium ions begin to flow into

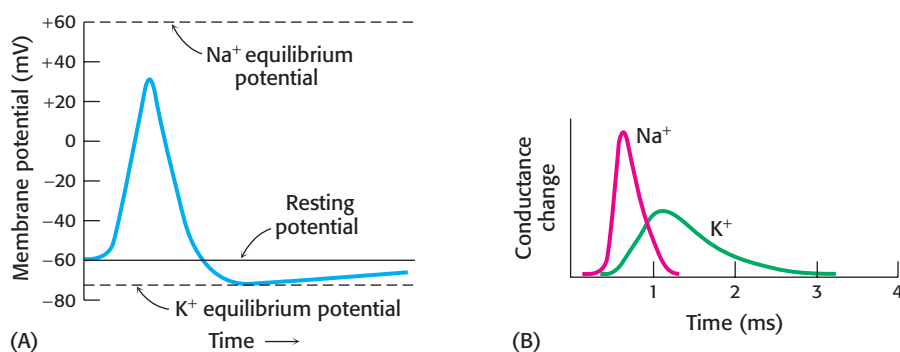
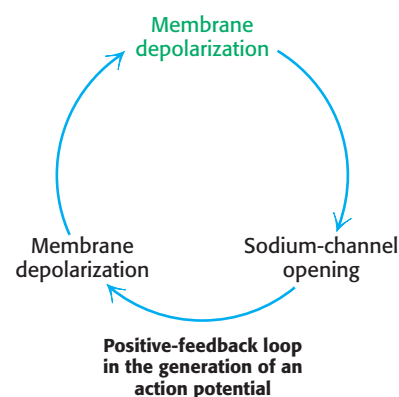


FIGURE 13.19 Membrane potential. Depolarization of an axon membrane results in an action potential. Time course of (A) the change in membrane potential and (B) the change in Na^+ and K^+ conductances.

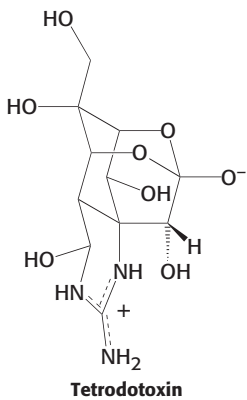
the cell because of the large electrochemical gradient across the plasma membrane. The entry of Na^+ further depolarizes the membrane, and so more gates for Na^+ are opened. This positive feedback between depolarization and Na^+ entry leads to a very rapid and large change in membrane potential, from about -60 mV to $+30$ mV in a millisecond.

Sodium channels spontaneously close and potassium channels begin to open at about this time (see Figure 13.19B). Consequently, potassium ions flow outward, and so the membrane potential returns to a negative value. The resting level of -60 mV is restored in a few milliseconds as the K^+ conductance decreases to the value characteristic of the unstimulated state. Only a very small proportion of the sodium and potassium ions in a nerve cell, of the order of one in a million, flows across the plasma membrane during the action potential. Clearly, the action potential is a very efficient means of signaling over large distances.

13.5.4 The Sodium Channel Is an Example of a Voltage-Gated Channel

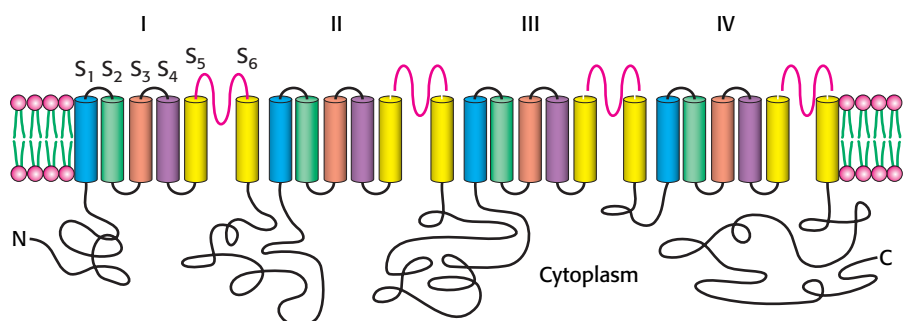
Like the acetylcholine receptor channel, the sodium channel also was purified on the basis of its ability to bind a specific neurotoxin. Tetrodotoxin, an organic compound isolated from the puffer fish, binds to sodium channels with great avidity ($K_i \approx 1$ nM). The lethal dose of this poison for an adult human being is about 10 ng. The sodium channel was first purified from the electric organ of electric eel, which is a rich source of the protein forming this channel. The isolated protein is a single chain of 260 kd.

The availability of purified protein enabled Shosaku Numa and coworkers to clone and sequence the cDNA for the sodium channel from the electroplax cells of the eel electric organ and then from the rat. Subsequently, a large number of sodium channel cDNAs have been cloned from other sources, and sequence comparisons have been made. The eel and rat cDNA sequences are approximately 61% identical, which indicates that the amino acid sequence of the sodium channel has been conserved over a long evolutionary period. Most interesting, the channel contains four internal repeats, or homology units, having similar amino acid sequences, suggesting that gene duplication and divergence have produced the gene for this channel. Hydrophobicity profiles indicate that each homology unit contains five hydrophobic segments (S1, S2, S3, S5, and S6). Each repeat also contains a highly positively charged S4 segment; arginine or lysine residues are present at nearly every third residue. Numa proposed that segments S1 through S6 are membrane-spanning α helices (Figure 13.20). The positively charged residues in S4 segments act as the voltage sensors of the channel. The purification of calcium channels and the subsequent cloning and sequencing of their cDNAs revealed that these proteins are homologous to the sodium channels and have quite similar architectures; each protein comprises four imperfectly repeated units, each of which has regions corresponding to segments S1 through S6.




A puffer fish is regarded as a culinary delicacy in Japan. [Fred Bavendam/Peter Arnold.]

FIGURE 13.20 The sodium channel. The Na^+ channel is a single polypeptide chain with four repeating units (I–IV). Each repeat probably folds into six transmembrane helices. The loops (shown in red) between helices 5 and 6 of each domain form the pore of the channel.



We can thus note similarities between ligand-gated and voltage-gated channels. Like the acetylcholine receptor, the sodium channel is constructed of similar units. The acetylcholine receptor has five units, whereas the sodium channel has four units that have been fused into a single polypeptide chain. The acetylcholine receptor is composed of similar but noncovalently attached subunits.

13.5.5 Potassium Channels Are Homologous to the Sodium Channel

 The purification of potassium channels proved to be much more difficult because of their low abundance and the lack of known high-affinity ligands comparable to tetrodotoxin. The breakthrough came in studies of mutant fruit flies that shake violently when anesthetized with ether. The mapping and cloning of the gene, termed *shaker*, responsible for this defect revealed the amino acid sequence encoded by a potassium-channel gene. The availability of this gene sequence has led to the cloning of potassium-channel cDNAs from many other organisms. *Shaker* cDNA encodes a 70-kd protein that has regions that correspond to one of the homology units of the sodium channel containing the membrane-spanning segments S1 through S6. Thus, a potassium-channel subunit is homologous to one of the repeated homology units of the sodium and calcium channels. Consistent with this hypothesis, four potassium-channel subunits come together to form a functional channel. Subsequently, other potassium channels were discovered, including some from bacteria, which contain only the two membrane-spanning regions corresponding to segments S5 and S6. This and other information pointed to the region between S5 and S6 as a key component of the ion-channel pore in the potassium channel and in the sodium and calcium channels as well. The sequence relationships between these ion channels are summarized in Figure 13.21.

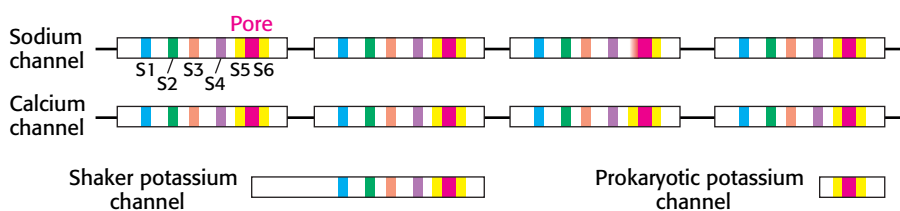


FIGURE 13.21 Sequence relationships of ion channels. Like colors indicate structurally similar regions of the sodium, calcium, and potassium channels. These channels exhibit approximate fourfold symmetry.

13.5.6 The Structure of a Potassium Channel Reveals the Basis of Rapid Ion Flow with Specificity



STRUCTURAL INSIGHTS, The Potassium Channel, examines the structural basis of the potassium channel's ion specificity and high conductivity in further detail.

Scientists were slowly discovering the likely structures of ion channels through a combination of patch-clamp methods, site-directed mutagenesis, and other methods. However, progress was limited by the lack of a high-resolution three-dimensional structure. The need was met by the determination of the structure of a bacterial potassium channel by x-ray crystallography in 1998. The resulting structural framework is a source of insight into many aspects of ion-channel function, including specificity and rapidity of ion flow.

As expected, the potassium channel is a tetramer of identical subunits, each of which includes two membrane-spanning α helices. The four subunits come together to form a pore in the shape of a cone that runs through the center of the structure (Figure 13.22). Beginning from the inside of the cell, the pore starts with a diameter of approximately 10 Å and then constricts

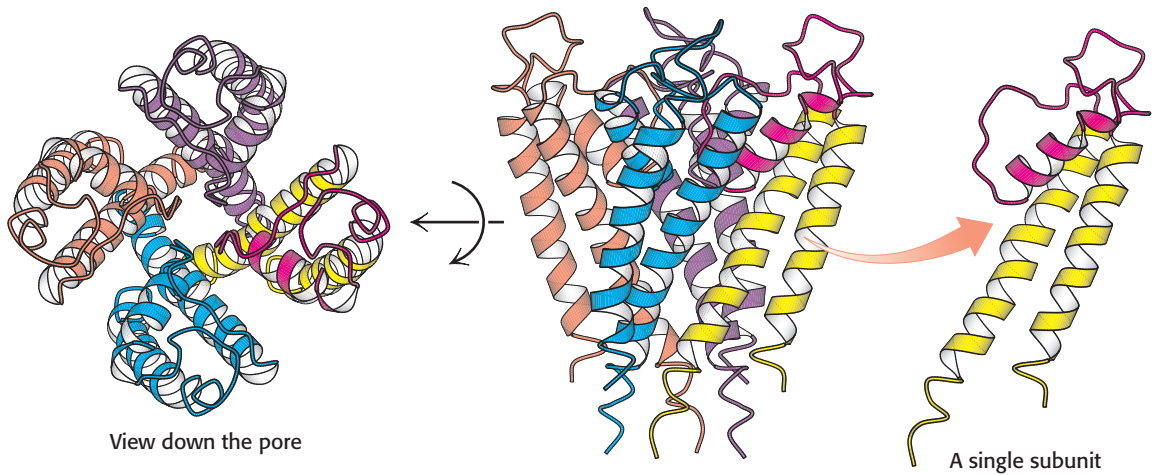


FIGURE 13.22 Structure of the potassium channel. The potassium channel, composed of four identical subunits, is cone shaped, with the larger opening facing the inside of the cell (center). A view down the pore, looking toward the outside of the cell, shows the relations of the individual subunits (left). One of the four identical subunits of the pore is illustrated at the right, with the pore-forming region highlighted in red.

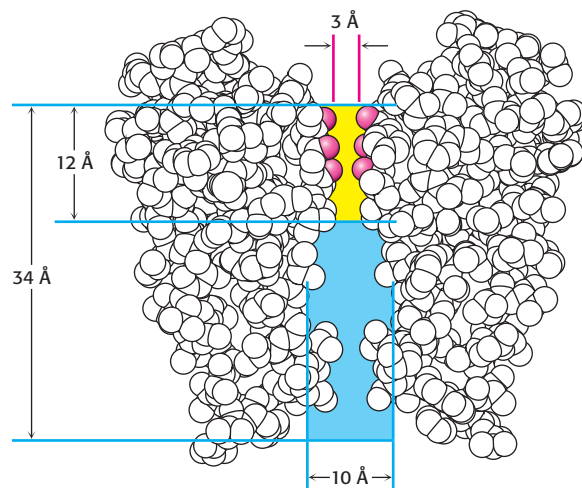


FIGURE 13.23 Path through a channel. A potassium ion entering the potassium channel can pass a distance of 22 Å into the membrane while remaining solvated with water (blue). At this point, the pore diameter narrows to 3 Å (yellow), and potassium must shed its water and interact with carbonyl groups (red) of the pore amino acids.

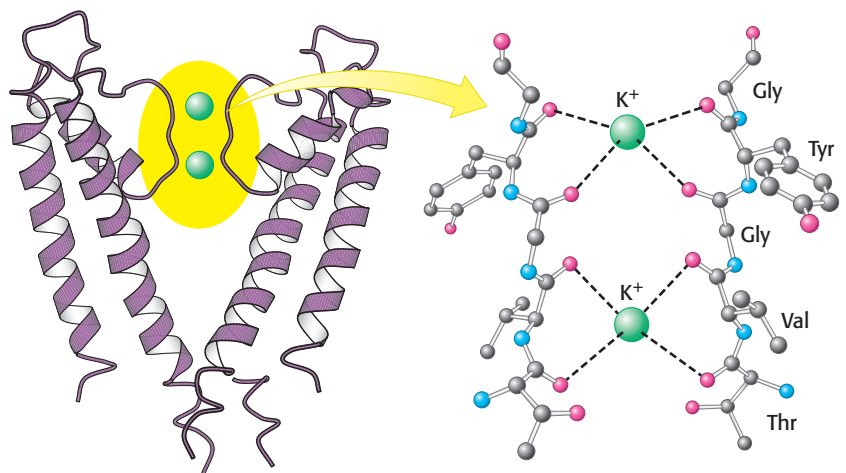


FIGURE 13.24 Selectivity filter of the potassium channel. Potassium ions interact with the carbonyl groups of the TVGYG sequence of the selectivity filter, located at the 3-Å-diameter pore of the potassium channel.

to a smaller cavity with a diameter of 8 Å. Both the opening to the outside and the central cavity of the pore are filled with water, and a K^+ ion can fit in the pore without losing its shell of bound water molecules. Approximately two-thirds of the way through the membrane, the pore becomes more constricted (3-Å diameter). At that point, any K^+ ions must give up their water molecules and interact directly with groups from the protein. The channel structure effectively reduces the thickness of the membrane from 34 Å to 12 Å by allowing the solvated ions to penetrate into the membrane before the ions must directly interact with the channel (Figure 13.23).

For potassium ions to relinquish their water molecules, other polar interactions must replace those with water. The restricted part of the pore is built from residues between the two transmembrane helices (which correspond to segments S5 and S6 in the sodium channel). In particular, a five-amino-acid stretch within this region functions as the *selectivity filter* that determines the preference for K^+ over other ions (Figure 13.24). The stretch has the sequence Thr-Val-Gly-Tyr-Gly, which is nearly completely conserved in all K^+ channels and had already been identified as a signature sequence useful for identifying potential K^+ channels. This region lies in a relatively extended conformation and is oriented such that the peptide carbonyl groups are directed into the channel, facilitating interaction with the potassium ions.

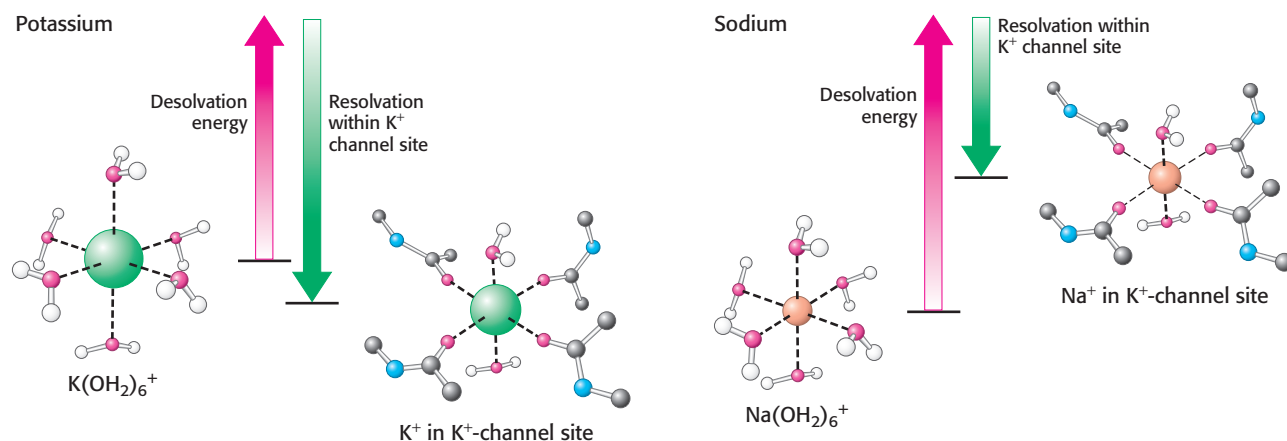
Potassium channels are 100-fold as permeable to K^+ as to Na^+ . How is this high degree of selectivity achieved? The narrow diameter (3 Å) of the selectivity filter of the potassium channel enables the filter to reject ions having a radius larger than 1.5 Å. However, a bare Na^+ is small enough (Table 13.2) to pass through the pore. Indeed, the ionic radius of Na^+ is substantially smaller than that of K^+ . How then is Na^+ rejected?

We need to consider the free-energy cost of dehydrating the Na^+ and K^+ ions, given that they cannot pass through this part of the channel bearing a retinue of water molecules. The key point is that the free-energy costs of dehydrating these ions are considerable [Na^+ , 72 kcal mol⁻¹ (301 kJ mol⁻¹), and K^+ , 55 kcal mol⁻¹ (203 kJ mol⁻¹)]. *The channel pays the cost of dehydrating K^+ by providing compensating interactions with the carbonyl oxygen atoms lining the selectivity filter.* However, these oxygen atoms are positioned such that they do not interact very favorably with Na^+ , because it is too small (Figure 13.25). The higher cost of dehydrating Na^+ would be unrecovered, and so Na^+ would be rejected. The ionic radii of oxygen, potassium, and sodium are 1.4, 1.33, and 0.95 Å, respectively. Hence a ring of oxygen atoms positioned so that the K^+-O distance is 2.73 Å (1.4 + 1.33 Å) would be optimal for interaction with K^+ compared with the shorter Na^+-O bonds (0.95 + 1.4 = 2.35 Å) optimal for interaction with Na^+ . Thus, the potassium channel avoids closely embracing Na^+ ions, which must stay hydrated and hence are impermeant.

TABLE 13.2 Properties of alkali cations

Ion	Ionic radius (Å)	Hydration free energy in kcal mol ⁻¹ (kJ mol ⁻¹)
Li ⁺	0.60	-98 (-410)
Na ⁺	0.95	-72 (-301)
K ⁺	1.33	-55 (-230)
Rb ⁺	1.48	-51 (-213)
Cs ⁺	1.69	-47 (-197)

FIGURE 13.25 Energetic basis of ion selectivity. The energy cost of dehydrating a potassium ion is compensated by favorable interactions with the selectivity filter. Because sodium is too small to interact favorably with the selectivity filter, the free energy of desolvation cannot be compensated and the sodium does not pass through the channel.



13.5.7 The Structure of the Potassium Channel Explains Its Rapid Rates of Transport

In addition to selectivity, ion channels display rapid rates of ion transport. A structural analysis provides an appealing explanation for this proficiency. The results of such studies revealed the presence of two potassium-binding sites in the constricted regions of the potassium channel that are crucial for rapid ion flow. Consider the process of ion conductance. One K^+ ion proceeds into the channel and through the relatively unrestricted part of the channel. It then gives up most or all of its coordinated water molecules and binds to the first site in the selectivity filter region, a favorable binding site. It can then jump to the second site, which appears to have comparable binding energy. However, the binding energy of the second site presents a free-energy barrier, or trap, preventing the ion from completing its journey; there is no energetic reason to leave the second ion-binding site. However, if a second ion moves through the channel into the first site, the electrostatic repulsion between the two ions will destabilize the initially bound ion and help push it into solution (Figure 13.26). This mechanism provides a solution to the apparent paradox of high ion selectivity (requiring tight binding sites) and rapid flow.

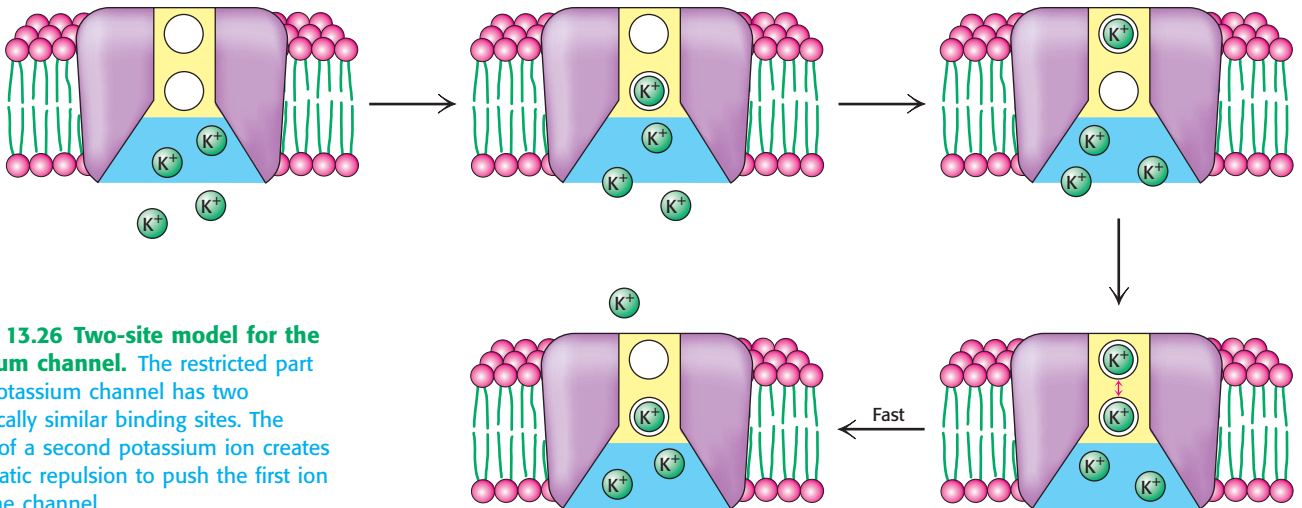
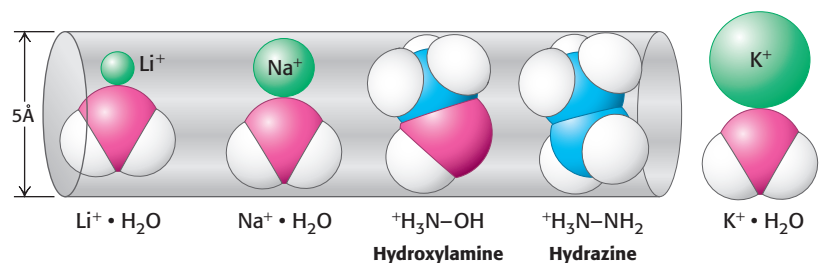


FIGURE 13.26 Two-site model for the potassium channel. The restricted part of the potassium channel has two energetically similar binding sites. The binding of a second potassium ion creates electrostatic repulsion to push the first ion out of the channel.

The structure determined for K^+ channels is a good start for considering the amino acid sequence similarities, as well as the structural and functional relations, for Na^+ and Ca^{2+} channels because of their homology to K^+ channels. Sequence comparisons and the results of mutagenesis experiments have also implicated the region between segments S5 and S6 in ion selectivity in the Ca^{2+} channels. In Ca^{2+} channels, one glutamate residue of this region in each of the four units plays a major role in determining ion selectivity. The Na^+ channel's selection of Na^+ over K^+ depends on ionic radius; the diameter of the pore is sufficiently restricted that small ions such as Na^+ and Li^+ can pass through the channel, but larger ions such as K^+ are significantly hindered (Figure 13.27).

FIGURE 13.27 Selectivity of the sodium channel. The ionic selectivity of the sodium channel partly depends on steric factors. Sodium and lithium ions, together with a water molecule, fit in the channel, as do hydroxylamine and hydrazine. In contrast, K^+ with a water molecule is too large. [After R. D. Keynes. *Ion channels in the nerve-cell membrane*. Copyright © 1979 by Scientific American, Inc. All rights reserved.]



Residues in the positions corresponding to the glutamate residues in Ca^{2+} channels are major components of the selectivity filter of the Na^+ channel. These residues are aspartate, glutamate, lysine, and alanine in units 1, 2, 3, and 4, respectively (the DEKA locus). Thus, the potential fourfold symmetry of the channel is clearly broken in this region, providing one explanation of why Na^+ channels comprise single large polypeptide chains rather than a noncovalent assembly of four identical subunits.

13.5.8 A Channel Can Be Inactivated by Occlusion of the Pore: The Ball-and-Chain Model

The potassium channel and the sodium channel undergo inactivation within milliseconds of channel opening (Figure 13.28). A first clue to the mechanism of inactivation came from exposing the cytoplasmic side of either channel to trypsin; cleavage by trypsin produced a trimmed channel that stayed persistently open after depolarization. A second clue was the finding that alternatively spliced variants of the potassium channel have markedly different inactivation kinetics; these variants differed from one another only near the amino terminus, which is on the cytoplasmic side of the channel. A mutant Shaker channel lacking 42 amino acids near the amino terminus opened in response to depolarization but did not inactivate (see Figure 13.28). Most revealing, inactivation was restored by adding a synthetic peptide corresponding to the first 20 residues of the native channel.

These experiments strongly support the *ball-and-chain model* for channel inactivation that had been proposed years earlier (Figure 13.29). According to this model, the first 20 residues of the potassium channel form a cytoplasmic unit (the *ball*) that is attached to a flexible segment of the polypeptide (the *chain*). When the channel is closed, the ball rotates freely in the aqueous solution. When the channel opens, the ball quickly finds a complementary site in the pore and occludes it. Hence, the channel opens for only a brief interval before it undergoes inactivation by occlusion. Shortening the chain speeds inactivation because the ball finds its target more quickly. Conversely, lengthening the chain slows inactivation. Thus, the duration of the open state can be controlled by the length and flexibility of the tether.

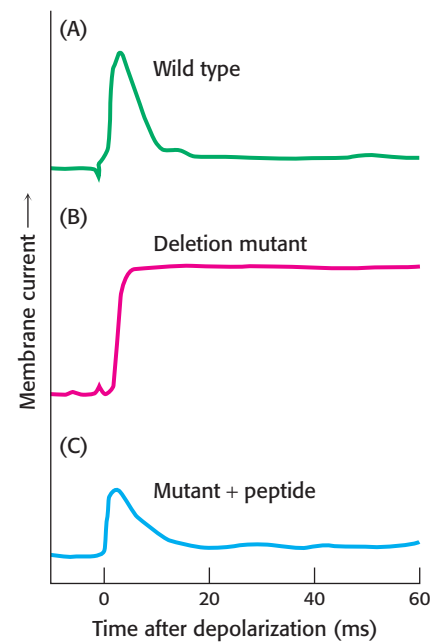
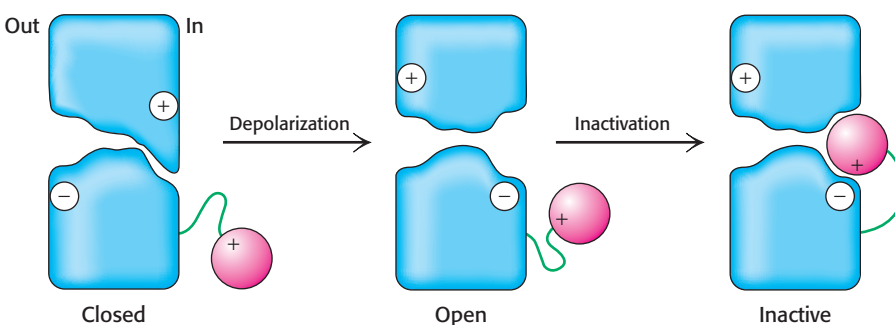


FIGURE 13.28 Inactivation of the potassium channel. The amino-terminal region of the potassium chain is critical for inactivation. (A) The wild-type Shaker potassium channel displays rapid inactivation after opening. (B) A mutant channel lacking residues 6 through 46 does not inactivate. (C) Inactivation can be restored by adding a peptide consisting of residues 1 through 20 at a concentration of 100 μM . [After W. N. Zagotta, T. Hoshi, and R. W. Aldrich. *Science* 250(1990):568.]

FIGURE 13.29 Ball-and-chain model for channel inactivation. The inactivation domain, or “ball” (red), is tethered to the channel by a flexible “chain” (green). In the closed state, the ball is located in the cytosol. Depolarization opens the channel and creates a negatively charged binding site for the positively charged ball near the mouth of the pore. Movement of the ball into this site inactivates the channel by occluding it. [After C. M. Armstrong and F. Bezanilla. *J. Gen. Physiol.* 70(1977):567.]

13.6 GAP JUNCTIONS ALLOW IONS AND SMALL MOLECULES TO FLOW BETWEEN COMMUNICATING CELLS

The ion channels that we have considered thus far have narrow pores and are moderately to highly selective in regard to which ions are permeant. They are closed in the resting state and have short lifetimes in the open state, typically a millisecond, that enable them to transmit highly frequent neural signals. We turn now to a channel with a very different role. *Gap junctions*, also known as *cell-to-cell channels*, serve as passageways between the

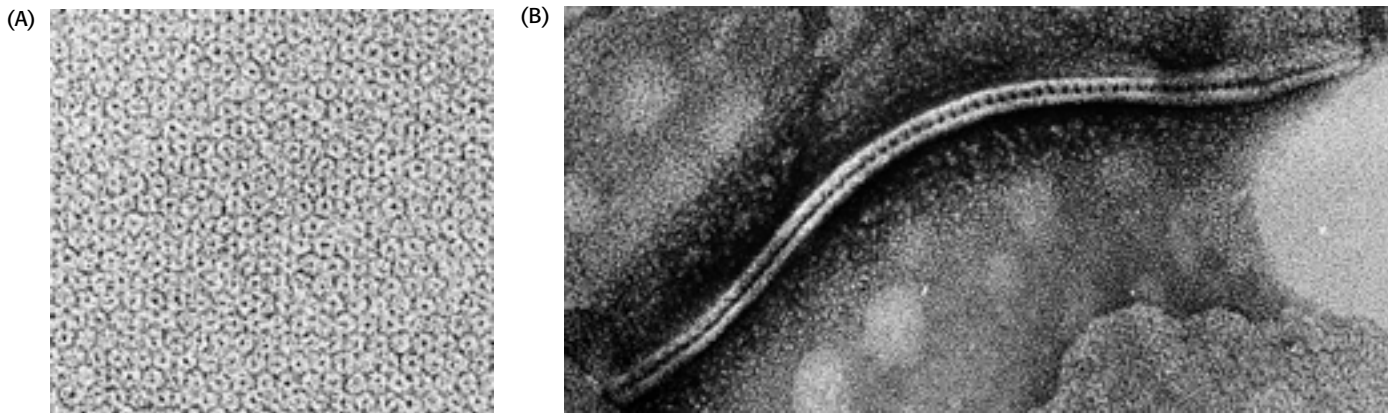


FIGURE 13.30 Gap junctions. (A) This electron micrograph shows a sheet of isolated gap junctions. The cylindrical connexons form a hexagonal lattice having a unit-cell length of 85 Å. The densely stained central hole has a diameter of about 20 Å. (B) Electron micrograph of a tangential view of apposed cell membranes that are joined by gap junctions. [(A) Courtesy of Dr. Nigel Unwin and Dr. Guido Zampighi; (B) from E. L. Hertzberg and N. B. Gilula. *J. Biol. Chem.* 254(1979):2143.]

interiors of contiguous cells. Gap junctions are clustered in discrete regions of the plasma membranes of apposed cells. Electron micrographs of sheets of gap junctions show them tightly packed in a regular hexagonal array (Figure 13.30A). A 20-Å central hole, the lumen of the channel, is prominent in each gap junction. A tangential view (Figure 13.30B) shows that these channels span the intervening space, or gap, between apposed cells (hence, the name *gap junction*). The width of the gap between the cytosols of the two cells is about 35 Å.

Small hydrophilic molecules as well as ions can pass through gap junctions. The pore size of the junctions was determined by microinjecting a series of fluorescent molecules into cells and observing their passage into adjoining cells. All polar molecules with a mass of less than about 1 kd can readily pass through these cell-to-cell channels. Thus, *inorganic ions and most metabolites (e.g., sugars, amino acids, and nucleotides) can flow between the interiors of cells joined by gap junctions.* In contrast, proteins, nucleic acids, and polysaccharides are too large to traverse these channels. *Gap junctions are important for intercellular communication.* Cells in some excitable tissues, such as heart muscle, are coupled by the rapid flow of ions through these junctions, which ensure a rapid and synchronous response to stimuli. Gap junctions are also essential for the nourishment of cells that are distant from blood vessels, as in lens and bone. Moreover, communicating channels are important in development and differentiation. For example, a pregnant uterus is transformed from a quiescent protector of the fetus to a forceful ejector at the onset of labor; the formation of functional gap junctions at that time creates a syncytium of muscle cells that contract in synchrony.

A cell-to-cell channel is made of 12 molecules of *connexin*, one of a family of transmembrane proteins with molecular masses ranging from 30 to 42 kd. Each connexin molecule appears to have four membrane-spanning helices. Six connexin molecules are hexagonally arrayed to form a half channel, called a *connexon* or *hemichannel* (Figure 13.31). Two connexons join end to end in the intercellular space to form a functional channel between the communicating cells. Cell-to-cell channels differ from other membrane channels in three respects: (1) they traverse *two* membranes rather than one; (2) they connect cytosol to cytosol, rather than to the extracellular space or the lumen of an organelle; and (3) the connexons forming a channel are synthesized by different cells. Gap junctions form readily when cells are brought together. A cell-to-cell channel, once formed, tends to stay open for seconds to minutes. They are closed by high concentrations of calcium ion and by low pH. *The closing of gap junctions by Ca^{2+} and H^+ serves to seal normal cells from traumatized or dying neighbors.* Gap junctions are also controlled by membrane potential and by hormone-induced phosphorylation.

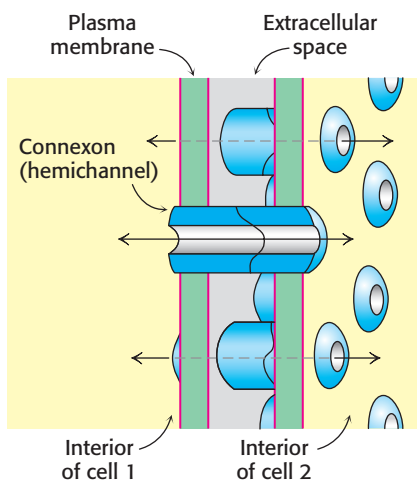


FIGURE 13.31 Schematic representation of a gap junction. [Courtesy of Dr. Werner Loewenstein.]

- **The Transport of Molecules Across a Membrane May Be Active or Passive**

For a net movement of molecules across a membrane, two features are required: (1) the molecule must be able to cross a hydrophobic barrier and (2) an energy source must power the movement. Lipophilic molecules can pass through a membrane's hydrophobic interior by simple diffusion. These molecules will move down their concentration gradients. Polar or charged molecules require proteins to form passages through the hydrophobic barrier. Passive transport or facilitated diffusion occurs when an ion or polar molecule moves down its concentration gradient. If a molecule moves against a concentration gradient, an external energy source is required; this movement is referred to as active transport and results in the generation of concentration gradients. Concentration gradients are a commonly used form of energy in all organisms.

- **A Family of Membrane Proteins Uses ATP Hydrolysis to Pump Ions Across Membranes**

Active transport is often carried out at the expense of ATP hydrolysis. P-type ATPases pump ions against a concentration gradient and become transiently phosphorylated on an aspartic acid residue in the process of transport. P-type ATPases, which include the sarcoplasmic reticulum Ca^{2+} ATPase and the Na^{+} - K^{+} ATPase, are integral membrane proteins with conserved structures and catalytic mechanisms.

- **Multidrug Resistance and Cystic Fibrosis Highlight a Family of Membrane Proteins with ATP-Binding Cassette Domains**

The membrane proteins with ATP-binding cassette (ABC) domains are complex ATP-dependent pumps. Each pump includes four major domains: two domains span the membrane and two others contain ABC P-loop ATPase structures. The multidrug resistance proteins confer resistance on cancer cells by pumping chemotherapeutic drugs out of a cancer cell before the drugs can exert their effects. Another ABC domain protein is the cystic fibrosis transmembrane conductance regulator (CFTR), an ATP-gated chloride channel. Mutations in CFTR can result in cystic fibrosis.

- **Secondary Transporters Use One Concentration Gradient to Power the Formation of Another**

Many active-transport systems couple the uphill flow of one ion or molecule to the downhill flow of another. These membrane proteins, called secondary transporters or cotransporters, can be classified as antiporters or symporters. Antiporters couple the downhill flow of one type of ion in one direction to the uphill flow of another in the opposite direction. Symporters move both ions in the same direction.

- **Specific Channels Can Rapidly Transport Ions Across Membranes**

Ion channels allow the rapid movement of ions across the hydrophobic barrier of the membrane. Such channels allow ions to flow down their concentration gradients. The channels have several features in common: (1) ion specificity, (2) the existence of open and closed states, (3) regulation by ligands or voltage. Ion channels are exemplified by the Na^{+} and K^{+} channels responsible for nerve impulses.

- **Gap Junctions Allow Ions and Small Molecules to Flow Between Communicating Cells**

In contrast with many channels, which connect the cell interior with the environment, gap junctions, or cell-to-cell channels, serve to connect the interiors of contiguous groups of cells. A cell-to-cell channel is composed of 12 molecules of connexin, which associate to form two 6-membered connexons.

KEY TERMS

facilitated diffusion (passive transport) (p. 346)	ATP-binding cassette (ABC) domain (p. 351)	voltage-gated channel (p. 353)
active transport (p. 346)	secondary transporter (cotransporter) (p. 352)	patch-clamp technique (p. 354)
Na ⁺ -K ⁺ pump (Na ⁺ -K ⁺ -ATPase) (p. 347)	antiporter (p. 352)	gigaseal (p. 354)
P-type ATPase (p. 348)	symporter (p. 352)	neurotransmitter (p. 355)
digitalis (p. 350)	ion channel (p. 353)	action potential (p. 357)
multidrug resistance (p. 351)	ligand-gated channel (p. 353)	selectivity filter (p. 361)
		gap junction (p. 363)

SELECTED READINGS

Where to start

- Welsh, M. J., and Smith, A. E., 1995. Cystic fibrosis. *Sci. Am.* 273(6):52–59.
- Unwin, N., 1993. Neurotransmitter action: Opening of ligand-gated ion channels. *Cell* 72:31–41.
- Lienhard, G. E., Slot, J. W., James, D. E., and Mueckler, M. M., 1992. How cells absorb glucose. *Sci. Am.* 266(1):86–91.
- Neher, E., and Sakmann, B., 1992. The patch clamp technique. *Sci. Am.* 266(3):28–35.
- Sakmann, B., 1992. Elementary steps in synaptic transmission revealed by currents through single ion channels. *Science* 256:503–512.

Books

- Ashcroft, F. M., 2000. *Ion Channels and Disease*. Academic Press.
- Conn, P. M. (Ed.), 1998. *Ion Channels*, vol. 293, *Methods in Enzymology*. Academic Press.
- Aidley, D. J., and Stanfield, P. R., 1996. *Ion Channels: Molecules in Action*. Cambridge University Press.
- Hille, B., 1992. *Ionic Channels of Excitable Membranes* (2d ed.). Sinauer.
- Läuger, P., 1991. *Electrogenic Ion Pumps*. Sinauer.
- Stein, W. D., 1990. *Channels, Carriers, and Pumps: An Introduction to Membrane Transport*. Academic Press.
- Hodgkin, A., 1992. *Chance and Design: Reminiscences of Science in Peace and War*. Cambridge University Press.

Voltage-gated ion channels

- Bezannilla, F., 2000. The voltage sensor in voltage-dependent ion channels. *Physiol. Rev.* 80:555–592.
- Biggin, P. C., Roosild, T., and Choe, S., 2000. Potassium channel structure: Domain by domain. *Curr. Opin. Struct. Biol.* 10:456–461.
- Sansom, M. S. P., 2000. Potassium channels: Watching a voltage-sensor tilt and twist. *Curr. Biol.* 10:R206–R209.
- Shieh, C.-C., Coghlan, M., Sullivan, J. P., and Gopalakrishnan, M., 2000. Potassium channels: Molecular defects, diseases, and therapeutic opportunities. *Pharmacol. Rev.* 52:557–594.
- Horn, R., 2000. Conversation between voltage sensors and gates of ion channels. *Biochemistry* 39:15653–15658.
- Perozo, E., Cortes, D. M., and Cuello, L. G., 1999. Structural rearrangements underlying K⁺-channel activation gating. *Science* 285:73–78.
- Doyle, D. A., Morais Cabral, J., Pfuetzner, R. A., Kuo, A., Gulbis, J. M., Cohen, S. L., Chait, B. T., and MacKinnon R., 1998. The structure of the potassium channel: Molecular basis of K⁺ conduction and selectivity. *Science* 280:69–77.
- Marban, E., Yamagishi, T., and Tomaselli, G. F., 1998. Structure and function of the voltage-gated Na⁺ channel. *J. Physiol.* 508:647–657.
- Gonzalez, C., Rosenman, E., Bezannilla, F., Alvarez, O., and Latorre, R., 2000. Modulation of the Shaker K⁺ channel gating kinetics by the S3-S4 linker. *J. Gen. Physiol.* 114:193–297.
- Miller, R. J., 1992. Voltage-sensitive Ca²⁺ channels. *J. Biol. Chem.* 267:1403–1406.

- Catterall, W. A., 1991. Excitation-contraction coupling in vertebrate skeletal muscle: A tale of two calcium channels. *Cell* 64:871–874.

Ligand-gated ion channels

- Miyazawa, A., Fujiyoshi, Y., Stowell, M., and Unwin, N., 1999. Nicotinic acetylcholine receptor at 4.6 Å resolution: Transverse tunnels in the channel wall. *J. Mol. Biol.* 288:765–786.
- Barrantes, F. J., Antollini, S. S., Blanton, M. P., and Prieto, M., 2000. Topography of the nicotinic acetylcholine receptor membrane-embedded domains. *J. Biol. Chem.* 275:37333–37339.
- Cordero-Erausquin, M., Marubio, L. M., Klink, R., and Changeux, J. P., 2000. Nicotinic receptor function: New perspectives from knockout mice. *Trends Pharmacol. Sci.* 21:211–217.
- Le Novère, N., and Changeux, J. P., 1995. Molecular evolution of the nicotinic acetylcholine receptor: An example of multigene family in excitable cells. *J. Mol. Evol.* 40:155–172.
- Kunishima, N., Shimada, Y., Tsuji, Y., Sato, T., Yamamoto, M., Kumasaka, T., Nakanishi, S., Jingami, H., and Morikawa, K., 2000. Structural basis of glutamate recognition by dimeric metabotropic glutamate receptor. *Nature* 407:971–978.
- Betz, H., Kuhse, J., Schmieden, V., Laube, B., Kirsch, J., and Harvey, R. J., 1999. Structure and functions of inhibitory and excitatory glycine receptors. *Ann. N. Y. Acad. Sci.* 868:667–676.
- Unwin, N., 1995. Acetylcholine receptor channel imaged in the open state. *Nature* 373:37–43.
- Colquhoun, D., and Sakmann, B., 1981. Fluctuations in the microsecond time range of the current through single acetylcholine receptor ion channels. *Nature* 294:464–466.
- Seeburg, P. H., Wisden, W., Verdoorn, T. A., Pritchett, D. B., Werner, P., Herb, A., Luddens, H., Sprengel, R., and Sakmann, B., 1990. The GABA_A receptor family: Molecular and functional diversity. *Cold Spring Harbor Symp. Quant. Biol.* 55:29–40.

ATP-driven ion pumps

- Toyoshima, C., Nakasako, M., Nomura, H., and Ogawa, H., 2000. Crystal structure of the calcium pump of sarcoplasmic reticulum at 2.6 Å resolution. *Nature* 405:647–655.
- Auer, M., Scarborough, G. A., and Kuhlbrandt, W., 1998. Three-dimensional map of the plasma membrane H⁺-ATPase in the open conformation. *Nature* 392:840–843.
- Axelsen, K. B., and Palmgren, M. G., 1998. Evolution of substrate specificities in the P-type ATPase superfamily. *J. Mol. Evol.* 46:84–101.
- Pedersen, P. A., Jorgensen, J. R., and Jorgensen, P. L., 2000. Importance of conserved alpha-subunit ⁷⁰⁹GDDGVD for Mg²⁺ binding, phosphorylation, energy transduction in Na, K-ATPase. *J. Biol. Chem.* 275:37588–37595.
- Blanco, G., and Mercer, R. W., 1998. Isozymes of the Na-K-ATPase: Heterogeneity in structure, diversity in function. *Am. J. Physiol.* 275:F633–F650.
- MacLennan, D. H., 1990. Molecular tools to elucidate problems in excitation-contraction coupling. *Biophys. J.* 58:1355–1365.

Estes, J. W., and White, P. D., 1965. William Withering and the purple foxglove. *Sci. Am.* 212(6):110–117.

ATP-binding cassette (ABC) proteins

Akabas, M. H., 2000. Cystic fibrosis transmembrane conductance regulator: Structure and function of an epithelial chloride channel. *J. Biol. Chem.* 275:3729–3732.

Chen, J., Sharma, S., Quioco, F. A., and Davidson, A. L., 2001. Trapping the transition state of an ATP-binding cassette transporter: Evidence for a concerted mechanism of maltose transport. *Proc. Natl. Acad. Sci. USA* 98:1525–1530.

Zhang, Z. R., McDonough, S. I., and McCarty, N. A., 2000. Interaction between permeation and gating in the putative pore domain mutant in the cystic fibrosis transmembrane conductance regulator. *Biophys. J.* 79:298–313.

Sheppard, D. N., and Welsh, M. J., 1999. Structure and function of the CFTR chloride channel. *Physiol. Rev.* 79:S23–S45.

Riordan, J. R., Rommens, J. M., Kerem, B. S., Alon, N., Rozmahel, R., Grzelczak, Z., Zielenski, J., Lok, S., Plavsic, N., Chou, J. L., Drumm, M. T., Iannuzzi, M. C., Collins, F. S., and Tsui, L. C., 1989. Identification of the cystic fibrosis gene: Cloning and characterization of complementary DNA. *Science* 245:1066–1073.

Jones, P. M., and George, A. M., 2000. Symmetry and structure in P-glycoprotein and ABC transporters: What goes around comes around. *Eur. J. Biochem.* 287:5298–5305.

Chen, Y., and Simon, S. M., 2000. In situ biochemical demonstration that P-glycoprotein is a drug efflux pump with broad specificity. *J. Cell Biol.* 148:863–870.

Saier, M. H., Jr., Paulsen, I. T., Sliwinski, M. K., Pao, S. S., Skurray, R. A., and Nikaido, H., 1998. Evolutionary origins of multidrug and drug-specific efflux pumps in bacteria. *FASEB J.* 12:265–74.

Symporters and antiporters

Philipson, K. D., and Nicoll, D. A., 2000. Sodium-calcium exchange: A molecular perspective. *Annu. Rev. Physiol.* 62:111–133.

Green, A. L., Anderson, E. J., and Brooker, R. J., 2000. A revised model for the structure and function of the lactose permease: Evidence that a face on transmembrane segment 2 is important for conformational changes. *J. Biol. Chem.* 275:23240–23246.

Pao, S. S., Paulsen, I. T., and Saier, M. H., Jr., 1998. Major facilitator superfamily. *Microbiol. Mol. Biol. Rev.* 62:1–34.

Wright, E. M., Hirsch, J. R., Loo, D. D., and Zampighi, G. A., 1997. Regulation of Na⁺/glucose cotransporters. *J. Exp. Biol.* 200:287–293.

Wright, E. M., Loo, D. D., Turk, E., and Hirayama, B. A., 1996. Sodium cotransporters. *Curr. Opin. Cell Biol.* 8:468–473.

Kaback, H. R., Bibi, E., and Roepe, P. D., 1990. β-Galactoside transport in *E. coli*: A functional dissection of lac permease. *Trends Biochem. Sci.* 8:309–314.

Hilgemann, D. W., Nicoll, D. A., and Philipson, K. D., 1991. Charge movement during Na⁺ translocation by native and cloned cardiac Na⁺/Ca²⁺ exchanger. *Nature* 352:715–718.

Hediger, M. A., Turk, E., and Wright, E. M., 1989. Homology of the human intestinal Na⁺/glucose and *Escherichia coli* Na⁺/proline cotransporters. *Proc. Natl. Acad. Sci. USA* 86:5748–5752.

Gap junctions

Revilla, A., Bennett, M. V. L., and Barrio, L. C., 2000. Molecular determinants of membrane potential dependence in vertebrate gap junction channels. *Proc. Natl. Acad. Sci. USA* 97:14760–14765.

Unger, V. M., Kumar, N. M., Gilula, N. B., and Yeager, M., 1999. Three-dimensional structure of a recombinant gap junction membrane channel. *Science* 283:1176–1180.

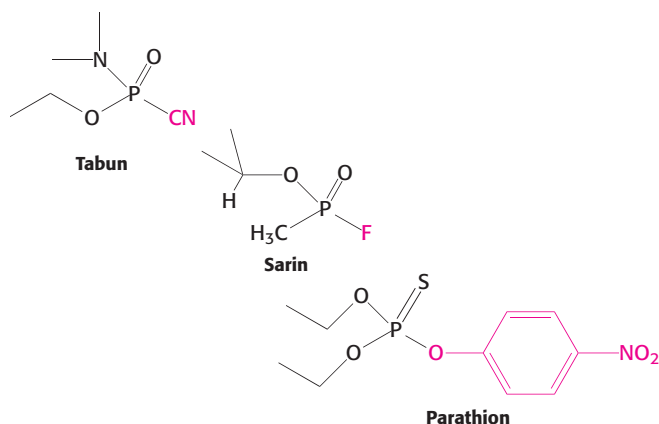
Simon, A. M., 1999. Gap junctions: More roles and new structural data. *Trends Cell Biol.* 9:169–170.

White, T. W., and Paul, D. L., 1999. Genetic diseases and gene knockouts reveal diverse connexin functions. *Annu. Rev. Physiol.* 61:283–310.

PROBLEMS

1. *Concerted opening.* Suppose that a channel obeys the concerted allosteric model (MWC model, Section 10.3.4). The binding of ligand to the R state (the open form) is 20 times as tight as to the T state (the closed form). In the absence of ligand, the ratio of closed to open channels is 10^5 . If the channel is a tetramer, what is the fraction of open channels when 1, 2, 3, and 4 ligands are bound?

2. *Respiratory paralysis.* Tabun and sarin have been used as chemical-warfare agents, and parathion has been employed as an insecticide. What is the molecular basis of their lethal actions?



3. *Ligand-induced channel opening.* The ratio of open to closed forms of the acetylcholine receptor channel containing zero, one, and two bound acetylcholine molecules is 5×10^{-6} , 1.2×10^{-3} , and 14, respectively.

(a) By what factor is the open/closed ratio increased by the binding of the first acetylcholine molecule? The second acetylcholine molecule?

(b) What are the corresponding free-energy contributions to channel opening at 25°C?

(c) Can the allosteric transition be accounted for by the MWC concerted model?

4. *Voltage-induced channel opening.* The fraction of open channels at 5 mV increments beginning at -45 mV and ending at $+5$ mV at 20°C is 0.02, 0.04, 0.09, 0.19, 0.37, 0.59, 0.78, 0.89, 0.95, 0.98, and 0.99.

(a) At what voltage are half the channels open?

(b) What is the value of the gating charge?

(c) How much free energy is contributed by the movement of the gating charge in the transition from -45 mV to $+5$ mV?

5. *Different directions.* The potassium channel and the sodium channel have similar structures and are arranged in the same orientation in the cell membrane. Yet, the sodium channel allows sodium ions to flow into the cell and the potassium channel allows potassium ions to flow out of the cell. Explain.

6. *Structure–activity relations.* On the basis of the structure of tetrodotoxin, propose a mechanism by which the toxin inhibits sodium flow through the sodium channel.

7. *A dangerous snail.* Cone snails are carnivores that inject a powerful set of toxins into their prey, leading to rapid paralysis. Many of these toxins are found to bind to specific ion-channel proteins. Why are such molecules so toxic? How might such toxins be useful for biochemical studies?

8. *Only a few.* Why do only a small number of sodium ions need to flow through the sodium channel to significantly change the membrane potential?

9. *Frog poison.* Batrachotoxin (BTX) is a steroidal alkaloid from the skin of *Phyllobates terribilis*, a poisonous Colombian frog (source of the poison used on blowgun darts). In the presence of BTX, sodium channels in an excised patch stay persistently open when the membrane is depolarized. They close when the membrane is repolarized. Which transition is blocked by BTX?



Phyllobates terribilis. [Tom McHugh, Photo Researchers.]

10. *Valium target.* γ -Aminobutyric acid (GABA) opens channels that are specific for chloride ions. The GABA_A receptor channel is pharmacologically important because it is the target of Valium, which is used to diminish anxiety.

(a) The extracellular concentration of Cl^- is 123 mM and the intracellular concentration is 4 mM. In which direction does Cl^- flow through an open channel when the membrane potential is in the -60 mV to $+30$ mV range?

(b) What is the effect of chloride-channel opening on the excitability of a neuron?

(c) The hydrophathy profile of the GABA_A receptor resembles that of the acetylcholine receptor. Predict the number of subunits in this chloride channel.

11. *The price of extrusion.* What is the free-energy cost of pumping Ca^{2+} out of a cell when the cytosolic concentration is 0.4 μM , the extracellular concentration is 1.5 mM, and the membrane potential is -60 mV?

12. *Rapid transit.* A channel exhibits current increments of 5 pA at a membrane potential of -50 mV. The mean open time is 1 ms.

(a) What is the conductance of this channel?

(b) How many univalent ions flow through the channel during its mean open time?

(c) How long does it take an ion to pass through the channel?

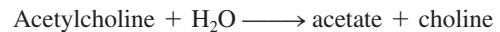
13. *Pumping protons.* Design an experiment to show that lactose permease can be reversed in vitro to pump protons.

Chapter Integration Problem

14. *Speed and efficiency matter.* Acetylcholine is rapidly destroyed by the enzyme acetylcholinesterase. This enzyme, which has a turnover number of 25,000 per second, has attained catalytic perfection with a k_{cat}/K_M of $2 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$. Why is it physiologically crucial that this enzyme be so efficient?

Mechanism Problem

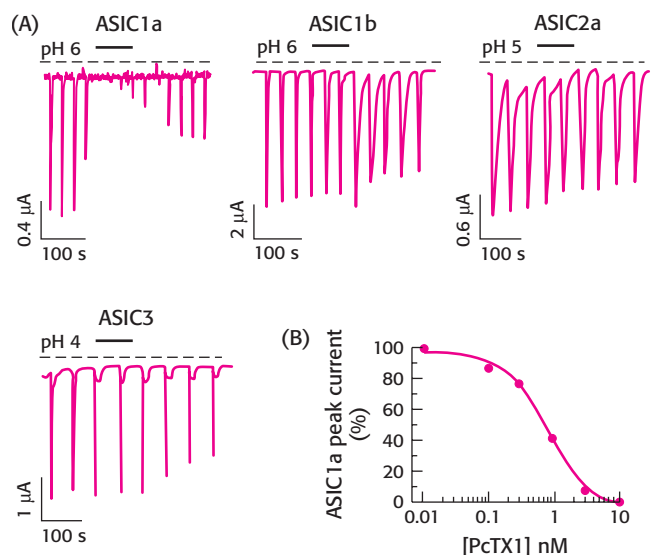
15. *Remembrance of mechanisms past.* Acetylcholinesterase converts acetylcholine into acetate and choline. Show the reaction as chemical structures:



Like serine proteases, acetylcholinesterase is inhibited by DIPF. Propose a catalytic mechanism for acetylcholine digestion by acetylcholinesterase.

Data Interpretation Problem

16. *Tarantula toxin.* Acid sensing is associated with pain, tasting, and other biological activities (Chapter 32). Acid sensing is carried out by a ligand-gated channel that permits sodium influx in response to H^+ . This family of acid-sensitive ion channels (ASICs) comprises a number of members. Psalmotoxin 1 (PcTX1), a venom from the tarantula, inhibits some members of this family. Below are electrophysiological recordings of cells containing one of several members of the ASIC family made in the presence of the toxin at a concentration of 10 nM. The channels were opened by changing the pH from 7.4 to the indicated values. The PcTX1 was present for a short time (indicated by the black bar above the recordings) after which time it was rapidly washed from the system.



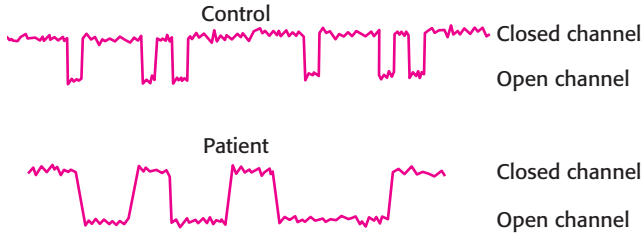
(A) Electrophysiological recordings of cells exposed to tarantula toxin. (B) Plot of peak current of a cell containing the ASIC1a protein versus the toxin concentration. [From P. Escoubas, et al., 2000, *J. Biol. Chem.* 275:25116–25121.]

(a) Which of the ASIC family members—ASIC1a, ASIC1b, ASIC2a, or ASIC3—is most sensitive to the toxin?

(b) Is the effect of the toxin reversible? Explain.

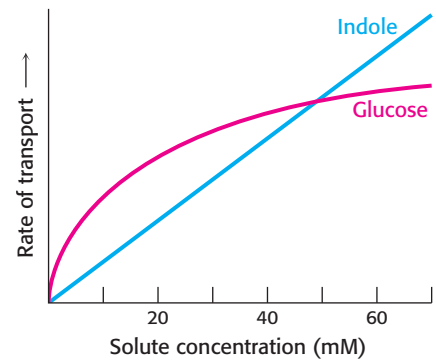
(c) What concentration of PcTX1 yields 50% inhibition of the sensitive channel?

17. *Channel problems 1.* A number of pathological conditions result from mutations in the acetylcholine receptor channel. One such mutation in the β subunit, $\beta V266M$, causes muscle weakness and rapid fatigue. An investigation of the acetylcholine-generated currents through the acetylcholine receptor channel for both a control and a patient yielded the following results. What is the effect of the mutation on channel function? Suggest some possible biochemical explanations for the effect.



18. *Channel problems 2.* The acetylcholine receptor channel can also undergo mutation leading to fast channel syndrome (FCS), with clinical manifestations similar to those of slow channel syndrome (SCS). What would the recordings of ion movement look like in this syndrome? Again, suggest a biochemical explanation.

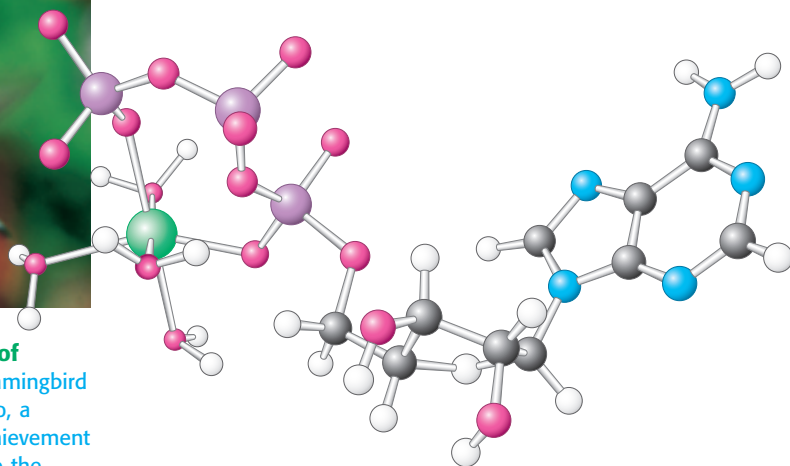
19. *Transport differences.* The rate of transport of two molecules, indole and glucose, across a cell membrane is shown in the right column. What are the differences between the transport mechanisms of the two molecules? Suppose that ouabain inhibited the transport of glucose. What would this inhibition suggest about the mechanism of transport?



Media Problem

20. *The merits of inflexibility.* The selectivity filter of potassium channels restricts passage of sodium ions even though sodium ions are smaller than potassium ions. Mutation of the tyrosine that is part of this filter to leucine has been shown to reduce selectivity against sodium in a channel homologous to the one whose structure has been determined [Chapman, M. L., Krovetz, H. S., and VanDongen, A. M. J., 2001. GYGD pore motifs in neighboring potassium channel subunits interact to determine ion selectivity. *J. Physiol.* 530:21–33]. Look at the three-dimensional structure in the Potassium Channel **Structural Insights** module and propose, in general terms, an explanation for the role of this tyrosine in the wild-type protein and the effect of its mutation to leucine.

Metabolism: Basic Concepts and Design



Hummingbirds are capable of prodigious feats of endurance. For instance, the tiny ruby-throated hummingbird can store enough fuel to fly across the Gulf of Mexico, a distance of some 500 miles, without resting. This achievement is possible because of the ability to convert fuels into the cellular energy currency, ATP, represented by the model at the right. [(Left) K. D. McGraw/Rainbow.]

The concepts of conformation and dynamics developed in Part I—especially those dealing with the specificity and catalytic power of enzymes, the regulation of their catalytic activity, and the transport of molecules and ions across membranes—enable us to now ask questions fundamental to biochemistry:

1. How does a cell extract energy and reducing power from its environment?
2. How does a cell synthesize the building blocks of its macromolecules and then the macromolecules themselves?

These processes are carried out by a highly integrated network of chemical reactions that are collectively known as *metabolism*.

More than a thousand chemical reactions take place in even as simple an organism as *Escherichia coli*. The array of reactions may seem overwhelming at first glance. However, closer scrutiny reveals that metabolism has a *coherent design containing many common motifs*. These motifs include the use of an energy currency and the repeated appearance of a limited number of activated intermediates. In fact, a group of about 100 molecules play central roles in all forms of life. Furthermore, although the number of reactions in metabolism is large, the number of *kinds* of reactions is small and the mechanisms of these reactions are usually quite simple. Metabolic pathways are also regulated in common ways. The purpose of this chapter is to introduce some general principles and motifs of metabolism to provide a foundation for the more detailed studies to follow.

OUTLINE

- 14.1 Metabolism Is Composed of Many Coupled, Interconnecting Reactions
- 14.2 The Oxidation of Carbon Fuels Is an Important Source of Cellular Energy
- 14.3 Metabolic Pathways Contain Many Recurring Motifs

14.0.1 Cells Transform Different Types of Energy

Living organisms require a continual input of free energy for three major purposes: (1) the performance of mechanical work in muscle contraction and other cellular movements, (2) the active transport of molecules and ions, and (3) the synthesis of macromolecules and other biomolecules from simple precursors. The free energy used in these processes, which maintain an organism in a state that is far from equilibrium, is derived from the environment.

The First Law of Thermodynamics states that energy can be neither created nor destroyed. The amount of energy in the universe is constant. Nevertheless, energy can be converted from one form into another.

Photosynthetic organisms, or *phototrophs*, use the energy of sunlight to convert simple energy-poor molecules into more-complex energy-rich molecules that serve as fuels. In other words, photosynthetic organisms transform light energy into chemical energy. Indeed, this transformation is ultimately the primary source of chemical energy for the vast majority of organisms, human beings included. *Chemotrophs*, which include animals, obtain chemical energy through the oxidation of foodstuffs generated by phototrophs.

Chemical energy obtained from the oxidation of carbon compounds may be transformed into the unequal distribution of ions across a membrane, resulting in an ion gradient. This gradient, in turn, is an energy source that can be used to move molecules across membranes, that can be converted into yet other types of chemical energy, or that can convey information in the form of nerve impulses. In addition, chemical energy can be transduced into mechanical energy. We convert the chemical energy of a fuel into structural alterations of contractile proteins that result in muscle contraction and movement. Finally, chemical energy powers the reactions that result in the synthesis of biomolecules.

At any given instant in a cell, thousands of energy transformations are taking place. Energy is being extracted from fuels and used to power biosynthetic processes. These transformations are referred to as *metabolism* or *intermediary metabolism*.

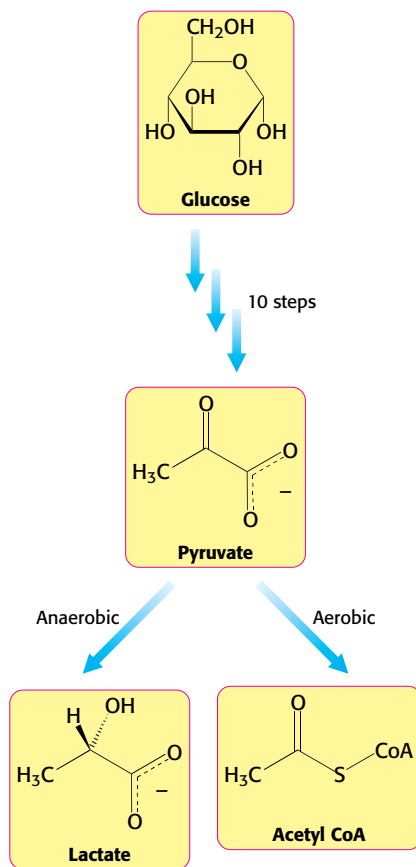
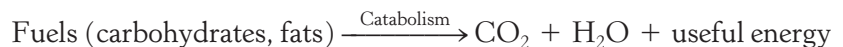


FIGURE 14.1 Glucose metabolism. Glucose is metabolized to pyruvate in 10 linked reactions. Under anaerobic conditions, pyruvate is metabolized to lactate and, under aerobic conditions, to acetyl CoA. The glucose-derived carbons are subsequently oxidized to CO_2 .

14.1 METABOLISM IS COMPOSED OF MANY COUPLED, INTERCONNECTING REACTIONS

Metabolism is essentially a linked series of chemical reactions that begins with a particular molecule and converts it into some other molecule or molecules in a carefully defined fashion (Figure 14.1). There are many such defined pathways in the cell (Figure 14.2), and we will examine a few of them in some detail later. These pathways are interdependent, and their activity is coordinated by exquisitely sensitive means of communication in which allosteric enzymes are predominant (Section 10.1). We will consider the principles of this communication in Chapter 15.

We can divide metabolic pathways into two broad classes: (1) those that convert energy into biologically useful forms and (2) those that require inputs of energy to proceed. Although this division is often imprecise, it is nonetheless a useful distinction in an examination of metabolism. Those reactions that transform fuels into cellular energy are called *catabolic reactions* or, more generally, *catabolism*.



Those reactions that require energy—such as the synthesis of glucose, fats, or DNA—are called *anabolic reactions* or *anabolism*. The useful forms of energy that are produced in catabolism are employed in anabolism to gen-

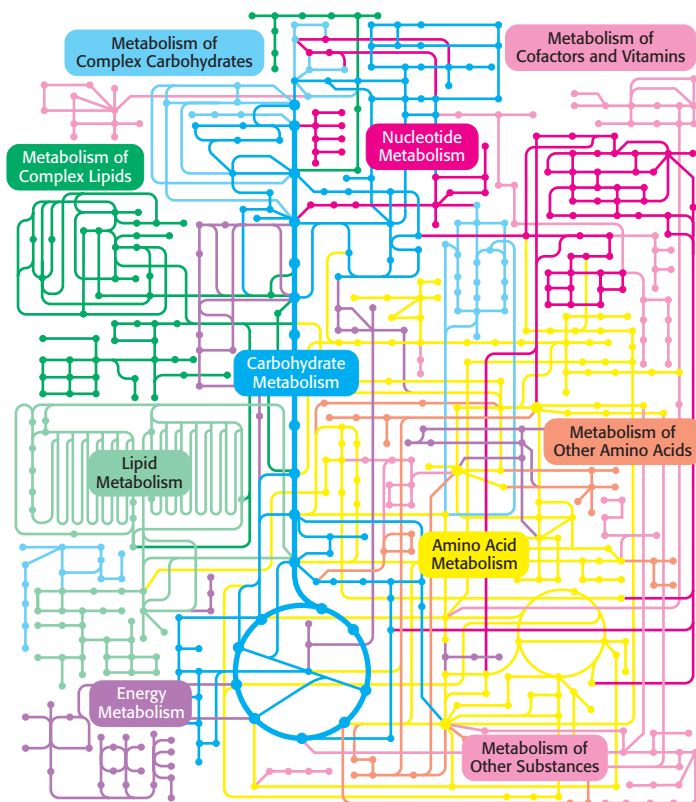


FIGURE 14.2 Metabolic pathways.
[From the Kyoto Encyclopedia of Genes and Genomes (www.genome.ad.jp/kegg).]

erate complex structures from simple ones, or energy-rich states from energy-poor ones.



Some pathways can be either anabolic or catabolic, depending on the energy conditions in the cell. They are referred to as *amphibolic pathways*.

14.1.1 A Thermodynamically Unfavorable Reaction Can Be Driven by a Favorable Reaction



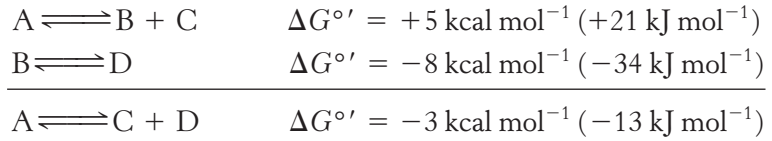
CONCEPTUAL INSIGHTS, Energetic Coupling, offers a graphical presentation of how enzymatic coupling enables a favorable reaction to drive an unfavorable reaction.

How are specific pathways constructed from individual reactions? A pathway must satisfy minimally two criteria: (1) the individual reactions must be *specific* and (2) the entire set of reactions that constitute the pathway must be *thermodynamically favored*. A reaction that is specific will yield only one particular product or set of products from its reactants. As discussed in Chapter 8, a function of enzymes is to provide this specificity. The thermodynamics of metabolism is most readily approached in terms of free energy, which was discussed in Sections 1.3.3, 8.2.1, and 8.2.2. A reaction can occur spontaneously only if ΔG , the change in free energy, is negative. Recall that ΔG for the formation of products C and D from substrates A and B is given by

$$\Delta G = \Delta G^{\circ'} + RT \ln \frac{[C][D]}{[A][B]}$$

Thus, the ΔG of a reaction depends on the *nature* of the reactant products (expressed by the $\Delta G^{\circ'}$ term, the standard free-energy change) and on their *concentrations* (expressed by the second term).

An important thermodynamic fact is that *the overall free-energy change for a chemically coupled series of reactions is equal to the sum of the free-energy changes of the individual steps*. Consider the following reactions:

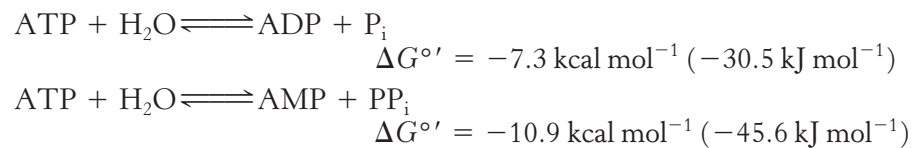


Under standard conditions, A cannot be spontaneously converted into B and C, because ΔG is positive. However, the conversion of B into D under standard conditions is thermodynamically feasible. Because free-energy changes are additive, the conversion of A into C and D has a $\Delta G^{\circ'}$ of -3 kcal mol^{-1} (-13 kJ mol^{-1}), which means that it can occur spontaneously under standard conditions. Thus, *a thermodynamically unfavorable reaction can be driven by a thermodynamically favorable reaction to which it is coupled*. In this example, the chemical intermediate B, common to both reactions, couples the reactions. Thus, metabolic pathways are formed by the coupling of enzyme-catalyzed reactions such that the overall free energy of the pathway is negative.

14.1.2 ATP Is the Universal Currency of Free Energy in Biological Systems

Just as commerce is facilitated by the use of a common currency, the commerce of the cell—metabolism—is facilitated by the use of a common energy currency, *adenosine triphosphate* (ATP). Part of the free energy derived from the oxidation of foodstuffs and from light is transformed into this highly accessible molecule, which acts as the free-energy donor in most energy-requiring processes such as motion, active transport, or biosynthesis.

ATP is a nucleotide consisting of an adenine, a ribose, and a triphosphate unit (Figure 14.3). The active form of ATP is usually a complex of ATP with Mg^{2+} or Mn^{2+} (Section 9.4.2). In considering the role of ATP as an energy carrier, we can focus on its triphosphate moiety. *ATP is an energy-rich molecule because its triphosphate unit contains two phosphoanhydride bonds*. A large amount of free energy is liberated when ATP is hydrolyzed to adenosine diphosphate (ADP) and orthophosphate (P_i) or when ATP is hydrolyzed to adenosine monophosphate (AMP) and pyrophosphate (PP_i).



The precise $\Delta G^{\circ'}$ for these reactions depends on the ionic strength of the medium and on the concentrations of Mg^{2+} and other metal ions. Under typical cellular concentrations, the actual ΔG for these hydrolyses is approximately $-12 \text{ kcal mol}^{-1}$ (-50 kJ mol^{-1}).

The free energy liberated in the hydrolysis of ATP is harnessed to drive reactions that require an input of free energy, such as muscle contraction. In turn, ATP is formed from ADP and P_i when fuel molecules are oxidized in chemotrophs or when light is trapped by phototrophs. *This ATP–ADP cycle is the fundamental mode of energy exchange in biological systems*.

Some biosynthetic reactions are driven by hydrolysis of nucleoside triphosphates that are analogous to ATP—namely, guanosine triphosphate (GTP), uridine triphosphate (UTP), and cytidine triphosphate (CTP). The diphosphate forms of these nucleotides are denoted by GDP, UDP, and CDP, and the monophosphate forms by GMP, UMP, and CMP. Enzymes

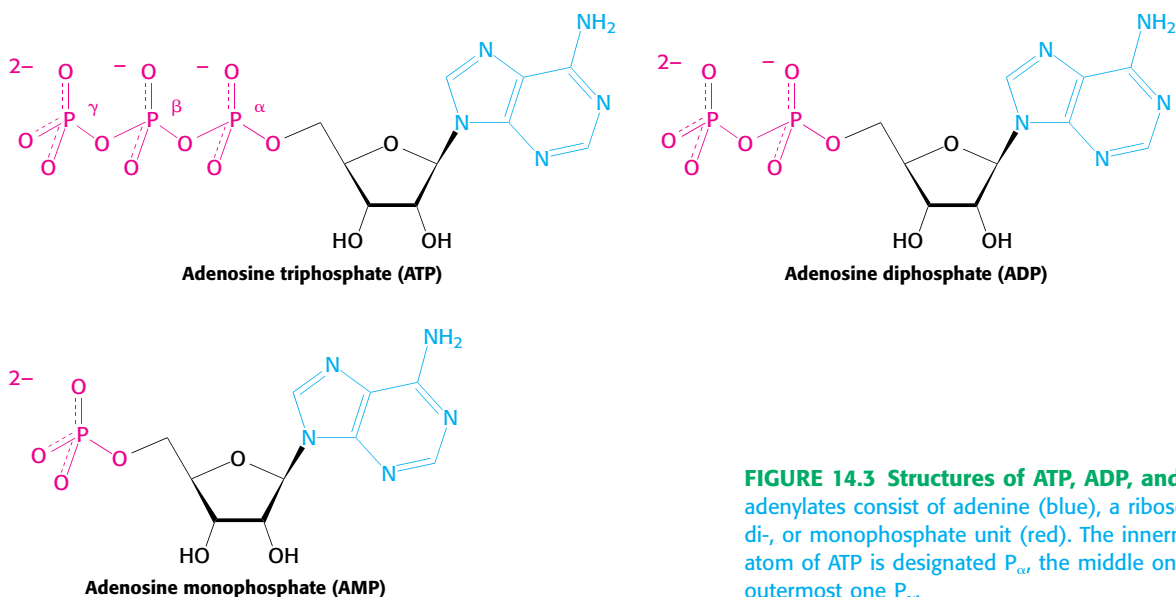
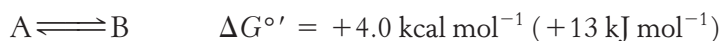


FIGURE 14.3 Structures of ATP, ADP, and AMP. These adenylates consist of adenine (blue), a ribose (black), and a tri-, di-, or monophosphate unit (red). The innermost phosphorus atom of ATP is designated P_{α} , the middle one P_{β} , and the outermost one P_{γ} .

can catalyze the transfer of the terminal phosphoryl group from one nucleotide to another. The phosphorylation of nucleoside monophosphates is catalyzed by a family of nucleoside monophosphate kinases, as discussed in Section 9.4.1. The phosphorylation of nucleoside diphosphates is catalyzed by nucleoside diphosphate kinase, an enzyme with broad specificity. It is intriguing to note that, although all of the nucleotide triphosphates are energetically equivalent, ATP is nonetheless the primary cellular energy carrier. In addition, two important electron carriers, NAD^+ and FAD, are derivatives of ATP. *The role of ATP in energy metabolism is paramount.*

14.1.3 ATP Hydrolysis Drives Metabolism by Shifting the Equilibrium of Coupled Reactions

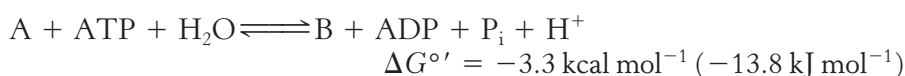
How does coupling to ATP hydrolysis make possible an otherwise unfavorable reaction? Consider a chemical reaction that is thermodynamically unfavorable without an input of free energy, a situation common to many biosynthetic reactions. Suppose that the standard free energy of the conversion of compound A into compound B is $+4.0 \text{ kcal mol}^{-1}$ ($+13 \text{ kJ mol}^{-1}$):



The equilibrium constant K'_{eq} of this reaction at 25°C is related to $\Delta G^{\circ'}$ (in units of kilocalories per mole) by

$$K'_{\text{eq}} = [B]_{\text{eq}}/[A]_{\text{eq}} = 10^{-\Delta G^{\circ'}/1.36} = 1.15 \times 10^{-3}$$

Thus, net conversion of A into B cannot occur when the molar ratio of B to A is equal to or greater than 1.15×10^{-3} . However, A can be converted into B under these conditions if the reaction is coupled to the hydrolysis of ATP. The new overall reaction is



Its standard free-energy change of $-3.3 \text{ kcal mol}^{-1}$ ($-13.8 \text{ kJ mol}^{-1}$) is the sum of the value of $\Delta G^{\circ'}$ for the conversion of A into B [$+4.0 \text{ kcal mol}^{-1}$ ($+12.6 \text{ kJ mol}^{-1}$)] and the value of $\Delta G^{\circ'}$ for the hydrolysis of ATP [$-7.3 \text{ kcal mol}^{-1}$ ($-30.5 \text{ kJ mol}^{-1}$)]. At pH 7, the equilibrium constant of this coupled reaction is

$$K'_{\text{eq}} = \frac{[\text{B}]_{\text{eq}}}{[\text{A}]_{\text{eq}}} \times \frac{[\text{ADP}]_{\text{eq}}[\text{P}_i]_{\text{eq}}}{[\text{ATP}]_{\text{eq}}} = 10^{3.3/1.36} = 2.67 \times 10^2$$

At equilibrium, the ratio of [B] to [A] is given by

$$\frac{[\text{B}]_{\text{eq}}}{[\text{A}]_{\text{eq}}} = K'_{\text{eq}} \frac{[\text{ATP}]_{\text{eq}}}{[\text{ADP}]_{\text{eq}}[\text{P}_i]_{\text{eq}}}$$

The ATP-generating system of cells maintains the [ATP]/[ADP][P_i] ratio at a high level, typically of the order of 500 M⁻¹. For this ratio,

$$\frac{[\text{B}]_{\text{eq}}}{[\text{A}]_{\text{eq}}} = 2.67 \times 10^2 \times 500 = 1.34 \times 10^5$$

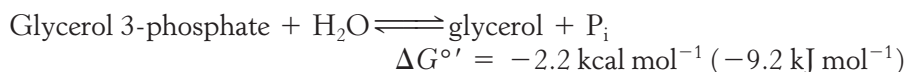
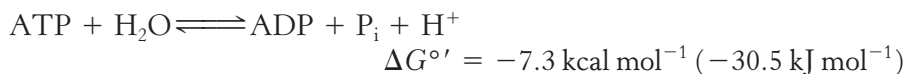
which means that the hydrolysis of ATP enables A to be converted into B until the [B]/[A] ratio reaches a value of 1.34×10^5 . This equilibrium ratio is strikingly different from the value of 1.15×10^{-3} for the reaction $A \rightarrow B$ in the absence of ATP hydrolysis. In other words, coupling the hydrolysis of ATP with the conversion of A into B has changed the equilibrium ratio of B to A by a factor of about 10^8 .

We see here the thermodynamic essence of ATP's action as an *energy-coupling agent*. Cells maintain a high level of ATP by using oxidizable substrates or light as sources of free energy. The hydrolysis of an ATP molecule in a coupled reaction then changes the equilibrium ratio of products to reactants by a very large factor, of the order of 10^8 . More generally, the hydrolysis of n ATP molecules changes the equilibrium ratio of a coupled reaction (or sequence of reactions) by a factor of 10^{8n} . For example, the hydrolysis of three ATP molecules in a coupled reaction changes the equilibrium ratio by a factor of 10^{24} . Thus, *a thermodynamically unfavorable reaction sequence can be converted into a favorable one by coupling it to the hydrolysis of a sufficient number of ATP molecules in a new reaction*. It should also be emphasized that A and B in the preceding coupled reaction may be interpreted very generally, not only as different chemical species. For example, A and B may represent activated and unactivated conformations of a protein; in this case, phosphorylation with ATP may be a means of conversion into an activated conformation. Such a conformation can store free energy, which can then be used to drive a thermodynamically unfavorable reaction. Through such changes in conformation, molecular motors such as myosin, kinesin, and dynein convert the chemical energy of ATP into mechanical energy (Chapter 34). Indeed, this conversion is the basis of muscle contraction.

Alternatively, A and B may refer to the concentrations of an ion or molecule on the outside and inside of a cell, as in the active transport of a nutrient. The active transport of Na⁺ and K⁺ across membranes is driven by the phosphorylation of the sodium–potassium pump by ATP and its subsequent dephosphorylation (Section 13.2.1).

14.1.4 Structural Basis of the High Phosphoryl Transfer Potential of ATP

As illustrated by molecular motors (Chapter 34) and ion pumps (Section 13.2), phosphoryl transfer is a common means of energy coupling. Furthermore, as we shall see in Chapter 15, phosphoryl transfer is also widely used in the intracellular transmission of information. What makes ATP a particularly efficient phosphoryl-group donor? Let us compare the standard free energy of hydrolysis of ATP with that of a phosphate ester, such as glycerol 3-phosphate:



The magnitude of $\Delta G^{\circ'}$ for the hydrolysis of glycerol 3-phosphate is much smaller than that of ATP, which means that ATP has a stronger tendency to transfer its terminal phosphoryl group to water than does glycerol 3-phosphate. In other words, ATP has a higher *phosphoryl transfer potential* (*phosphoryl-group transfer potential*) than does glycerol 3-phosphate.

What is the structural basis of the high phosphoryl transfer potential of ATP? Because $\Delta G^{\circ'}$ depends on the *difference* in free energies of the products and reactants, the structures of both ATP and its hydrolysis products, ADP and P_i , must be examined to answer this question. Three factors are important: *resonance stabilization*, *electrostatic repulsion*, and *stabilization due to hydration*. ADP and, particularly, P_i , have greater resonance stabilization than does ATP. Orthophosphate has a number of resonance forms of similar energy (Figure 14.4), whereas the γ -phosphoryl group of ATP has a smaller number.

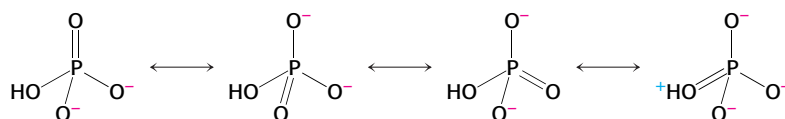


FIGURE 14.4 Resonance structures of orthophosphate.

Forms like that shown in Figure 14.5 are unfavorable because a positively charged oxygen atom is adjacent to a positively charged phosphorus atom, an electrostatically unfavorable juxtaposition. Furthermore, at pH 7, the triphosphate unit of ATP carries about four negative charges. These charges repel one another because they are in close proximity. The repulsion between them is reduced when ATP is hydrolyzed. Finally, water can bind more effectively to ADP and P_i than it can to the phosphoanhydride part of ATP, stabilizing the ADP and P_i by hydration.

ATP is often called a high-energy phosphate compound, and its phosphoanhydride bonds are referred to as high-energy bonds. Indeed, a “squiggle” (\sim P) is often used to indicate such a bond. Nonetheless, there is nothing special about the bonds themselves. *They are high-energy bonds in the sense that much free energy is released when they are hydrolyzed*, for the aforementioned reasons.

14.1.5 Phosphoryl Transfer Potential Is an Important Form of Cellular Energy Transformation

The standard free energies of hydrolysis provide a convenient means of comparing the phosphoryl transfer potential of phosphorylated compounds. Such comparisons reveal that ATP is not the only compound with a high phosphoryl transfer potential. In fact, some compounds in biological systems have a higher phosphoryl transfer potential than that of ATP. These compounds include phosphoenolpyruvate (PEP), 1,3-bisphosphoglycerate (1,3-BPG), and creatine phosphate (Figure 14.6). Thus, PEP can transfer its phosphoryl group to ADP to form ATP. Indeed, this is one of the ways in which ATP is generated in the breakdown of sugars (Sections 14.2.1, 16.1.6, and 16.1.7). It is significant that ATP has a phosphoryl transfer potential that is intermediate among the biologically important phosphorylated molecules (Table 14.1). *This intermediate position enables ATP to function efficiently as a carrier of phosphoryl groups.*

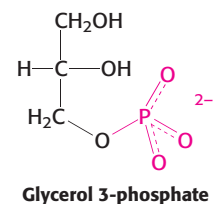
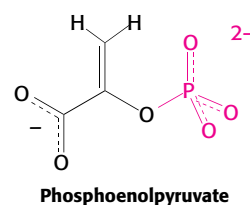
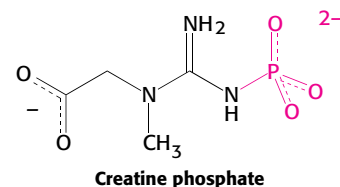


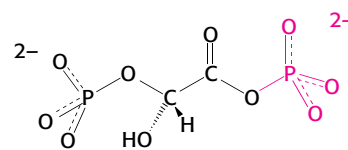
FIGURE 14.5 Improbable resonance structure. The structure contributes little to the terminal part of ATP, because two positive charges are placed adjacent to each other.



Phosphoenolpyruvate



Creatine phosphate



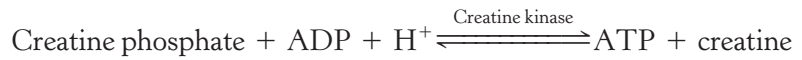
1,3-Bisphosphoglycerate

FIGURE 14.6 High phosphoryl transfer potential compounds. These compounds have a higher phosphoryl transfer potential than that of ATP and can be used to phosphorylate ADP to form ATP.

TABLE 14.1 Standard free energies of hydrolysis of some phosphorylated compounds

Compound	kcal mol ⁻¹	kJ mol ⁻¹
Phosphoenolpyruvate	-14.8	-61.9
1,3-Bisphosphoglycerate	-11.8	-49.4
Creatine phosphate	-10.3	-43.1
ATP (to ADP)	- 7.3	-30.5
Glucose 1-phosphate	- 5.0	-20.9
Pyrophosphate	- 4.6	-19.3
Glucose 6-phosphate	- 3.3	-13.8
Glycerol 3-phosphate	- 2.2	- 9.2

Creatine phosphate in vertebrate muscle serves as a reservoir of high-potential phosphoryl groups that can be readily transferred to ATP. Indeed, we use creatine phosphate to regenerate ATP from ADP every time we exercise strenuously. This reaction is catalyzed by *creatine kinase*.



At pH 7, the standard free energy of hydrolysis of creatine phosphate is $-10.3 \text{ kcal mol}^{-1}$ ($-43.1 \text{ kJ mol}^{-1}$), compared with $-7.3 \text{ kcal mol}^{-1}$ ($-30.5 \text{ kJ mol}^{-1}$) for ATP. Hence, the standard free-energy change in forming ATP from creatine phosphate is $-3.0 \text{ kcal mol}^{-1}$ ($-12.6 \text{ kJ mol}^{-1}$), which corresponds to an equilibrium constant of 162.

$$K_{\text{eq}} = \frac{[\text{ATP}][\text{creatine}]}{[\text{ADP}][\text{creatine phosphate}]} = 10^{-\Delta G^\circ / (2.303RT)} = 10^{3/1.36} = 162$$

In resting muscle, typical concentrations of these metabolites are $[\text{ATP}] = 4 \text{ mM}$, $[\text{ADP}] = 0.013 \text{ mM}$, $[\text{creatine phosphate}] = 25 \text{ mM}$, and $[\text{creatine}] = 13 \text{ mM}$. The amount of ATP in muscle suffices to sustain contractile activity for less than a second. The abundance of creatine phosphate and its high phosphoryl transfer potential relative to that of ATP make it a highly effective phosphoryl buffer. Indeed, creatine phosphate is the major source of phosphoryl groups for ATP regeneration for a runner during the first 4 seconds of a 100-meter sprint. After that, ATP must be generated through metabolism (Figure 14.7).

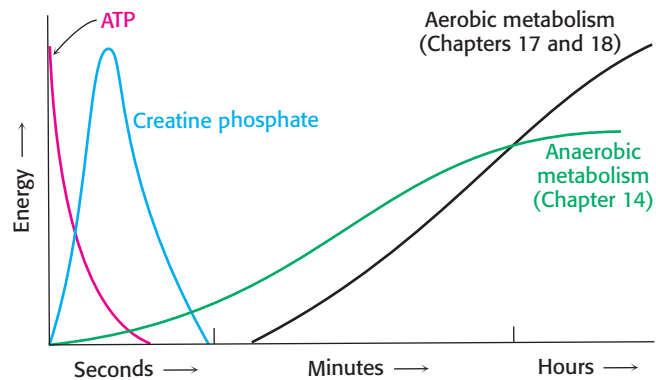


FIGURE 14.7 Sources of ATP during exercise. In the initial seconds, exercise is powered by existing high phosphoryl transfer compounds (ATP and creatine phosphate). Subsequently, the ATP must be regenerated by metabolic pathways.

14.2 THE OXIDATION OF CARBON FUELS IS AN IMPORTANT SOURCE OF CELLULAR ENERGY

ATP serves as the principal *immediate donor of free energy* in biological systems rather than as a long-term storage form of free energy. In a typical cell,

an ATP molecule is consumed within a minute of its formation. Although the total quantity of ATP in the body is limited to approximately 100 g, *the turnover of this small quantity of ATP is very high*. For example, a resting human being consumes about 40 kg of ATP in 24 hours. During strenuous exertion, the rate of utilization of ATP may be as high as 0.5 kg/minute. For a 2-hour run, 60 kg (132 pounds) of ATP is utilized. Clearly, it is vital to have mechanisms for regenerating ATP. Motion, active transport, signal amplification, and biosynthesis can occur only if ATP is continually regenerated from ADP (Figure 14.8). The generation of ATP is one of the primary roles of catabolism. The carbon in fuel molecules—such as glucose and fats—is oxidized to CO₂, and the energy released is used to regenerate ATP from ADP and P_i.

In aerobic organisms, the ultimate electron acceptor in the oxidation of carbon is O₂ and the oxidation product is CO₂. Consequently, the more reduced a carbon is to begin with, the more exergonic its oxidation will be. Figure 14.9 shows the ΔG° of oxidation for one-carbon compounds.

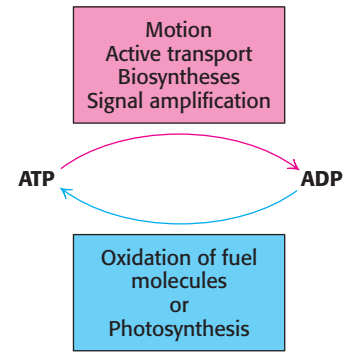


FIGURE 14.8 ATP–ADP cycle. This cycle is the fundamental mode of energy exchange in biological systems.

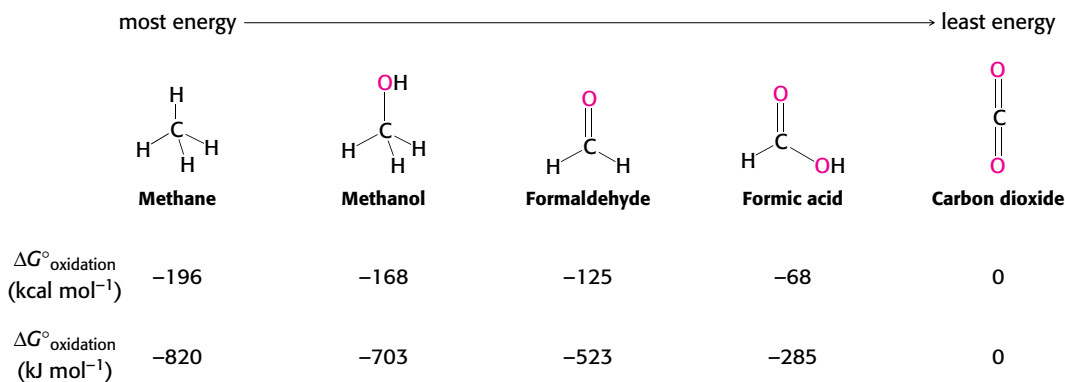


FIGURE 14.9 Free energy of oxidation of single-carbon compounds.

Although fuel molecules are more complex (Figure 14.10) than the single-carbon compounds depicted in Figure 14.9, when a fuel is oxidized, the oxidation takes place one carbon at a time. The carbon oxidation energy is used in some cases to create a compound with high phosphoryl transfer potential and in other cases to create an ion gradient. In either case, the end point is the formation of ATP.

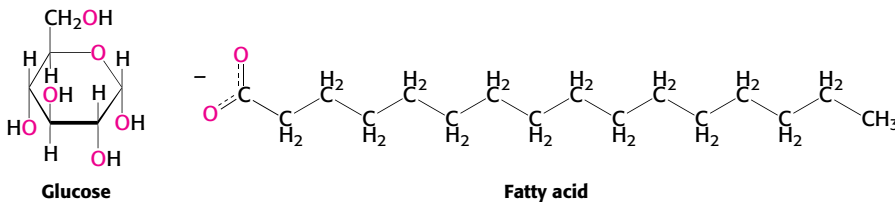
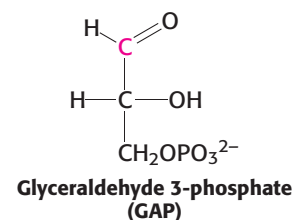
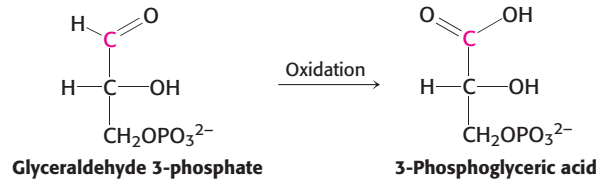


FIGURE 14.10 Prominent fuels. Fats are a more efficient fuel source than carbohydrates such as glucose because the carbon in fats is more reduced.

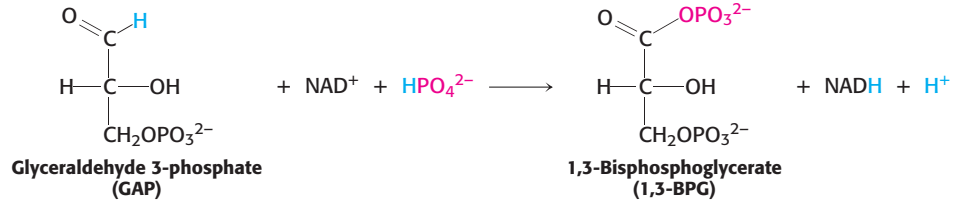
14.2.1 High Phosphoryl Transfer Potential Compounds Can Couple Carbon Oxidation to ATP Synthesis

How is the energy released in the oxidation of a carbon compound converted into ATP? As an example, consider glyceraldehyde 3-phosphate (shown in the margin), which is a metabolite of glucose formed in the oxidation of that sugar. The C-1 carbon (shown in red) is a component of an aldehyde and is not in its most oxidized state. Oxidation of the aldehyde to an acid will release energy.

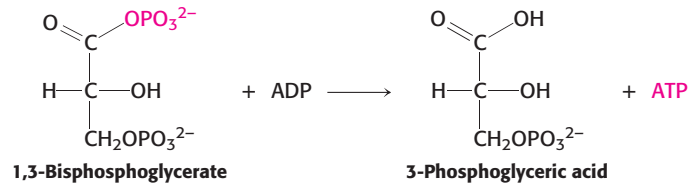




However, the oxidation does not take place directly. Instead, the carbon oxidation generates an acyl phosphate, 1,3-bisphosphoglycerate. The electrons released are captured by NAD^+ , which we will consider shortly.



For reasons similar to those discussed for ATP (Section 14.1.4), 1,3-bisphosphoglycerate has a high phosphoryl transfer potential. Thus, the cleavage of 1,3-BPG can be coupled to the synthesis of ATP.



The energy of oxidation is initially trapped as a high-energy phosphate compound and then used to form ATP. The oxidation energy of a carbon atom is transformed into phosphoryl transfer potential, first as 1,3-bisphosphoglycerate and ultimately as ATP. We will consider these reactions in mechanistic detail in Section 16.1.5.

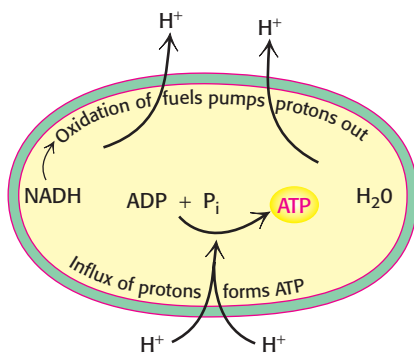


FIGURE 14.11 Proton gradients. The oxidation of fuels can power the formation of proton gradients. These proton gradients can in turn drive the synthesis of ATP.

14.2.2 Ion Gradients Across Membranes Provide an Important Form of Cellular Energy That Can Be Coupled to ATP Synthesis

The electrochemical potential of *ion gradients across membranes*, produced by the oxidation of fuel molecules or by photosynthesis, ultimately powers the synthesis of most of the ATP in cells. In general, ion gradients are versatile means of coupling thermodynamically unfavorable reactions to favorable ones. Indeed, in animals, *proton gradients* generated by the oxidation of carbon fuels account for more than 90% of ATP generation (Figure 14.11). This process is called *oxidative phosphorylation* (Chapter 18). ATP hydrolysis can then be used to form ion gradients of different types and functions. The electrochemical potential of a Na^+ gradient, for example, can be tapped to pump Ca^{2+} out of cells (Section 13.4) or to transport nutrients such as sugars and amino acids into cells.

14.2.3 Stages in the Extraction of Energy from Foodstuffs

Let us take an overall view of the processes of energy conversion in higher organisms before considering them in detail in subsequent chapters. Hans Krebs described three stages in the generation of energy from the oxidation of foodstuffs (Figure 14.12).

In the first stage, large molecules in food are broken down into smaller units. Proteins are hydrolyzed to their 20 kinds of constituent amino acids, poly-

saccharides are hydrolyzed to simple sugars such as glucose, and fats are hydrolyzed to glycerol and fatty acids. This stage is strictly a preparation stage; no useful energy is captured in this phase.

In the second stage, these numerous small molecules are degraded to a few simple units that play a central role in metabolism. In fact, most of them—sugars, fatty acids, glycerol, and several amino acids—are converted into the acetyl unit of acetyl CoA (Section 14.3.1). Some ATP is generated in this stage, but the amount is small compared with that obtained in the third stage.

In the third stage, ATP is produced from the complete oxidation of the acetyl unit of acetyl CoA. The third stage consists of the citric acid cycle and oxidative phosphorylation, which are the final common pathways in the oxidation of fuel molecules. Acetyl CoA brings acetyl units into the citric acid cycle [also called the tricarboxylic acid (TCA) cycle or Krebs cycle], where they are completely oxidized to CO_2 . Four pairs of electrons are transferred (three to NAD^+ and one to FAD) for each acetyl group that is oxidized. Then, a proton gradient is generated as electrons flow from the reduced forms of these carriers to O_2 , and this gradient is used to synthesize ATP.

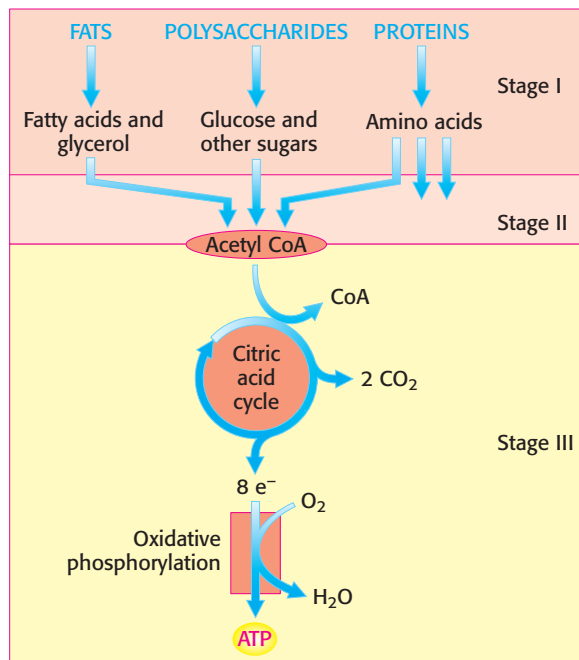


FIGURE 14.12 Stages of catabolism. The extraction of energy from fuels can be divided into three stages.

14.3 METABOLIC PATHWAYS CONTAIN MANY RECURRING MOTIFS

At first glance, metabolism appears intimidating because of the sheer number of reactants and reactions. Nevertheless, there are unifying themes that make the comprehension of this complexity more manageable. These unifying themes include common metabolites, reactions, and regulatory schemes that stem from a common evolutionary heritage.

14.3.1 Activated Carriers Exemplify the Modular Design and Economy of Metabolism

We have seen that phosphoryl transfer can be used to drive otherwise endergonic reactions, alter the energy of conformation of a protein, or serve as a signal to alter the activity of a protein. The phosphoryl-group donor in all of these reactions is ATP. In other words, ATP is an activated carrier of phosphoryl groups because phosphoryl transfer from ATP is an exergonic process. The use of activated carriers is a recurring motif in biochemistry, and we will consider several such carriers here.

1. *Activated carriers of electrons for fuel oxidation.* In aerobic organisms, the ultimate electron acceptor in the oxidation of fuel molecules is O_2 . However, electrons are not transferred directly to O_2 . Instead, fuel molecules transfer electrons to special carriers, which are either *pyridine nucleotides* or *flavins*. The reduced forms of these carriers then transfer their high-potential electrons to O_2 .

Nicotinamide adenine dinucleotide is a major electron carrier in the oxidation of fuel molecules (Figure 14.13). The reactive part of NAD^+ is its nicotinamide ring, a pyridine derivative synthesized from the vitamin niacin. In the oxidation of a substrate, the nicotinamide ring of NAD^+ accepts a hydrogen ion and two electrons, which are equivalent to a hydride ion.

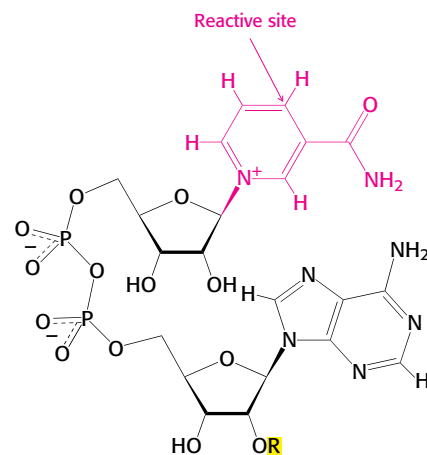


FIGURE 14.13 Structures of the oxidized forms of nicotinamide-derived electron carriers. Nicotinamide adenine dinucleotide (NAD^+) and nicotinamide adenine dinucleotide phosphate (NADP^+) are prominent carriers of high-energy electrons. In NAD^+ , $\text{R} = \text{H}$; in NADP^+ , $\text{R} = \text{PO}_3^{2-}$.

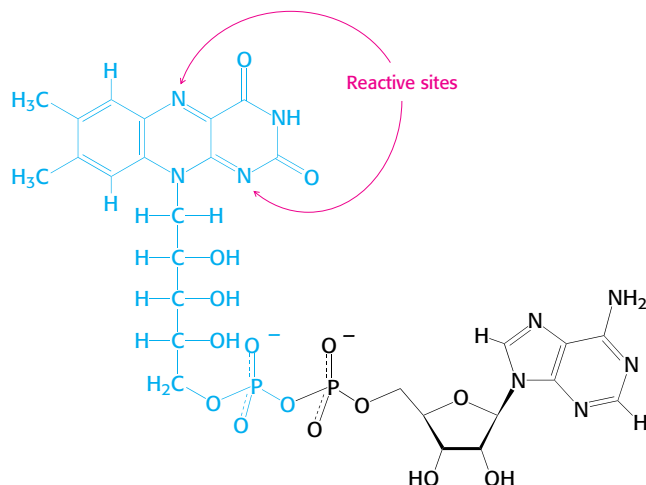
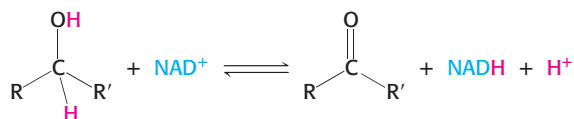


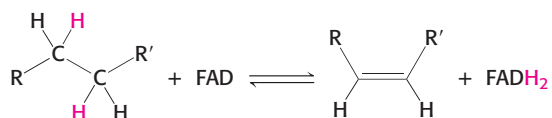
FIGURE 14.14 Structure of the oxidized form of flavin adenine dinucleotide (FAD). This electron carrier consists of a flavin mononucleotide (FMN) unit (shown in blue) and an AMP unit (shown in black).

The reduced form of this carrier is called *NADH*. In the oxidized form, the nitrogen atom carries a positive charge, as indicated by NAD^+ . NAD^+ is the electron acceptor in many reactions of the type



In this dehydrogenation, one hydrogen atom of the substrate is directly transferred to NAD^+ , whereas the other appears in the solvent as a proton. Both electrons lost by the substrate are transferred to the nicotinamide ring.

The other major electron carrier in the oxidation of fuel molecules is the coenzyme *flavin adenine dinucleotide* (Figure 14.14). The abbreviations for the oxidized and reduced forms of this carrier are *FAD* and *FADH₂*, respectively. *FAD* is the electron acceptor in reactions of the type



The reactive part of *FAD* is its isoalloxazine ring, a derivative of the vitamin riboflavin (Figure 14.15). *FAD*, like NAD^+ , can accept two electrons. In doing so, *FAD*, unlike NAD^+ , takes up two protons. These carriers of high-potential electrons as well as flavin mononucleotide (FMN), an electron carrier related to *FAD*, will be considered further in Chapter 18.

2. *An activated carrier of electrons for reductive biosynthesis.* High-potential electrons are required in most biosyntheses because the precursors are more oxidized than the products. Hence, reducing power is needed in addition to

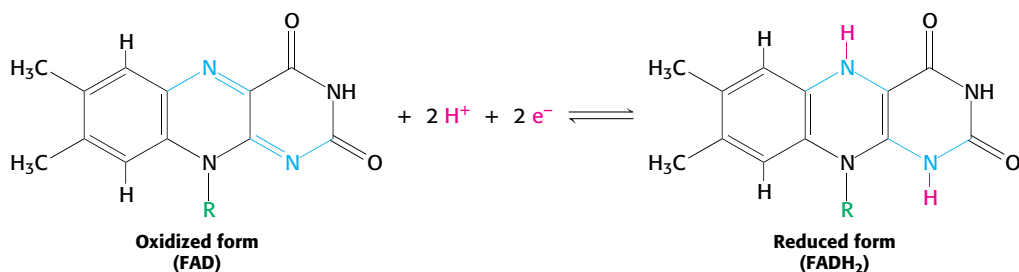
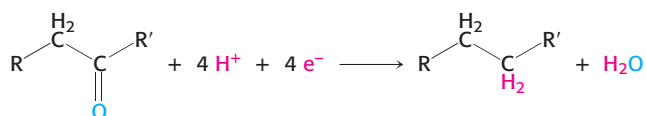


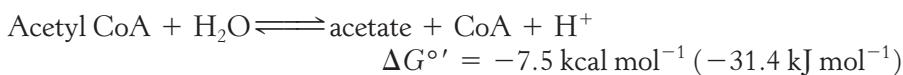
FIGURE 14.15 Structures of the reactive parts of *FAD* and *FADH₂*. The electrons and protons are carried by the isoalloxazine ring component of *FAD* and *FADH₂*.

ATP. For example, in the biosynthesis of fatty acids, the keto group of an added two-carbon unit is reduced to a methylene group in several steps. This sequence of reactions requires an input of four electrons.



The electron donor in most reductive biosyntheses is NADPH, the reduced form of nicotinamide adenine dinucleotide phosphate (NADP⁺; see Figure 14.13). NADPH differs from NADH in that the 2'-hydroxyl group of its adenosine moiety is esterified with phosphate. NADPH carries electrons in the same way as NADH. However, *NADPH is used almost exclusively for reductive biosyntheses, whereas NADH is used primarily for the generation of ATP.* The extra phosphoryl group on NADPH is a tag that enables enzymes to distinguish between high-potential electrons to be used in anabolism and those to be used in catabolism.

3. *An activated carrier of two-carbon fragments.* Coenzyme A, another central molecule in metabolism, is a carrier of acyl groups (Figure 14.16). Acyl groups are important constituents both in catabolism, as in the oxidation of fatty acids, and in anabolism, as in the synthesis of membrane lipids. The terminal sulfhydryl group in CoA is the reactive site. Acyl groups are linked to CoA by thioester bonds. The resulting derivative is called an *acyl CoA*. An acyl group often linked to CoA is the acetyl unit; this derivative is called *acetyl CoA*. The $\Delta G^{\circ'}$ for the hydrolysis of acetyl CoA has a large negative value:



The hydrolysis of a thioester is thermodynamically more favorable than that of an oxygen ester because the electrons of the C=O bond cannot form resonance structures with the C-S bond that are as stable as those that they can form with the C-O bond. Consequently, *acetyl CoA has a high acetyl potential (acetyl group-transfer potential) because transfer of the acetyl group is exergonic.* Acetyl CoA carries an activated acetyl group, just as ATP carries an activated phosphoryl group.

The use of activated carriers illustrates two key aspects of metabolism. First, NADH, NADPH, and FADH₂ react slowly with O₂ in the absence of a catalyst. Likewise, ATP and acetyl CoA are hydrolyzed slowly (in times of many hours or even days) in the absence of a catalyst. These molecules are kinetically quite stable in the face of a large thermodynamic driving force for reaction with O₂ (in regard to the electron carriers) and H₂O (in regard to ATP and acetyl CoA). *The kinetic stability of these molecules in the absence of specific catalysts is essential for their biological function because it enables enzymes to control the flow of free energy and reducing power.*

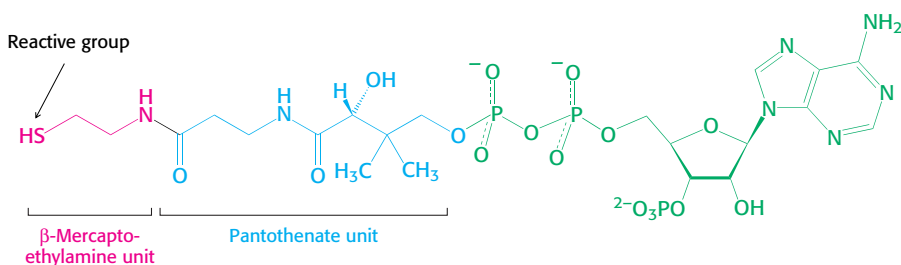
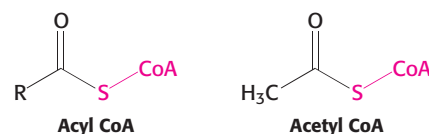


FIGURE 14.16 Structure of coenzyme A (CoA).

TABLE 14.2 Some activated carriers in metabolism

Carrier molecule in activated form	Group carried	Vitamin precursor
ATP	Phosphoryl	
NADH and NADPH	Electrons	Nicotinate (niacin)
FADH ₂	Electrons	Riboflavin (vitamin B ₂)
FMNH ₂	Electrons	Riboflavin (vitamin B ₂)
Coenzyme A	Acyl	Pantothenate
Lipoamide	Acyl	
Thiamine pyrophosphate	Aldehyde	Thiamine (vitamin B ₁)
Biotin	CO ₂	Biotin
Tetrahydrofolate	One-carbon units	Folate
S-Adenosylmethionine	Methyl	
Uridine diphosphate glucose	Glucose	
Cytidine diphosphate diacylglycerol	Phosphatidate	
Nucleoside triphosphates	Nucleotides	

Note: Many of the activated carriers are coenzymes that are derived from water-soluble vitamins (Section 8.6.1).

Second, *most interchanges of activated groups in metabolism are accomplished by a rather small set of carriers* (Table 14.2). The existence of a recurring set of activated carriers in all organisms is one of the unifying motifs of biochemistry. Furthermore, it illustrates the modular design of metabolism. A small set of molecules carries out a very wide range of tasks. Metabolism is readily comprehended because of the economy and elegance of its underlying design.

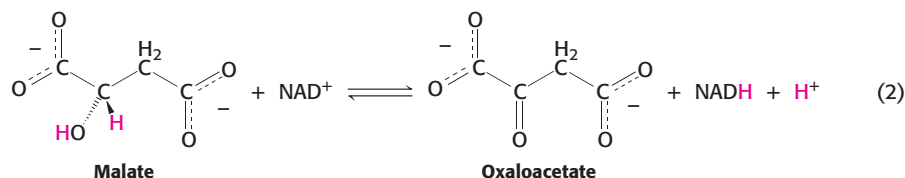
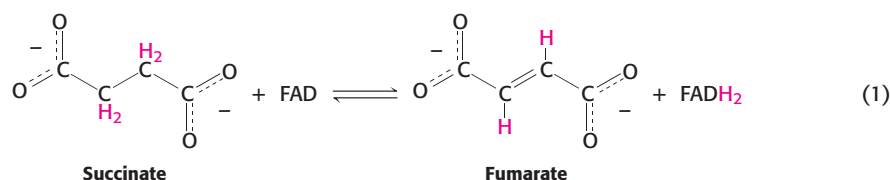
14.3.2 Key Reactions Are Reiterated Throughout Metabolism

Just as there is an economy of design in the use of activated carriers, so is there an economy of design in biochemical reactions. The thousands of metabolic reactions, bewildering at first in their variety, can be subdivided into just six types (Table 14.3). Specific reactions of each type appear repeatedly, further reducing the number of reactions necessary for the student to learn.

TABLE 14.3 Types of chemical reactions in metabolism

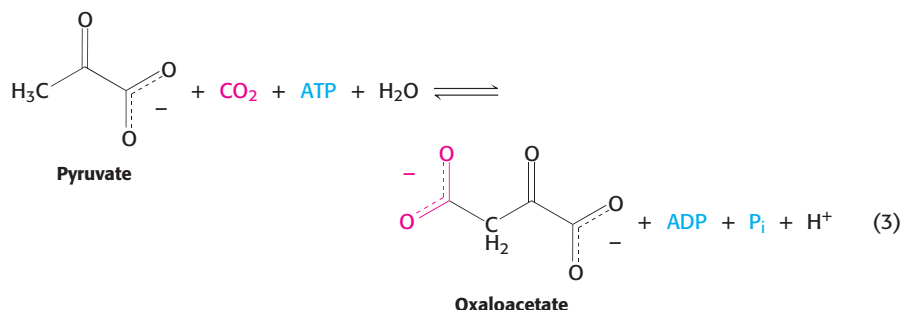
Type of reaction	Description
Oxidation–reduction	Electron transfer
Ligation requiring ATP cleavage	Formation of covalent bonds (i.e., carbon–carbon bonds)
Isomerization	Rearrangement of atoms to form isomers
Group transfer	Transfer of a functional group from one molecule to another
Hydrolytic	Cleavage of bonds by the addition of water
Addition or removal of functional groups	Addition of functional groups to double bonds or their removal to form double bonds

1. *Oxidation–reduction reactions* are essential components of many pathways. Useful energy is often derived from the oxidation of carbon compounds. Consider the following two reactions:



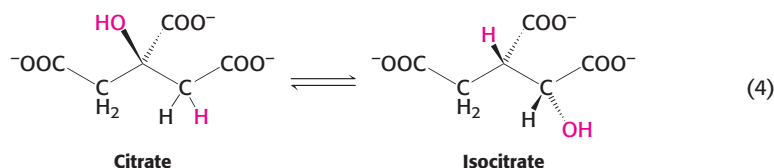
These two oxidation–reduction reactions are components of the citric acid cycle (Chapter 17), which completely oxidizes the activated two-carbon fragment of acetyl CoA to two molecules of CO_2 . In reaction 1, FADH_2 carries the electrons, whereas, in reaction 2, electrons are carried by NADH . In biosynthetic oxidation–reduction reactions, NADPH is the reductant.

2. *Ligation reactions* form bonds by using free energy from ATP cleavage. Reaction 3 illustrates the ATP-dependent formation of a carbon–carbon bond, necessary to combine smaller molecules to form larger ones. Oxaloacetate is formed from pyruvate and CO_2 .



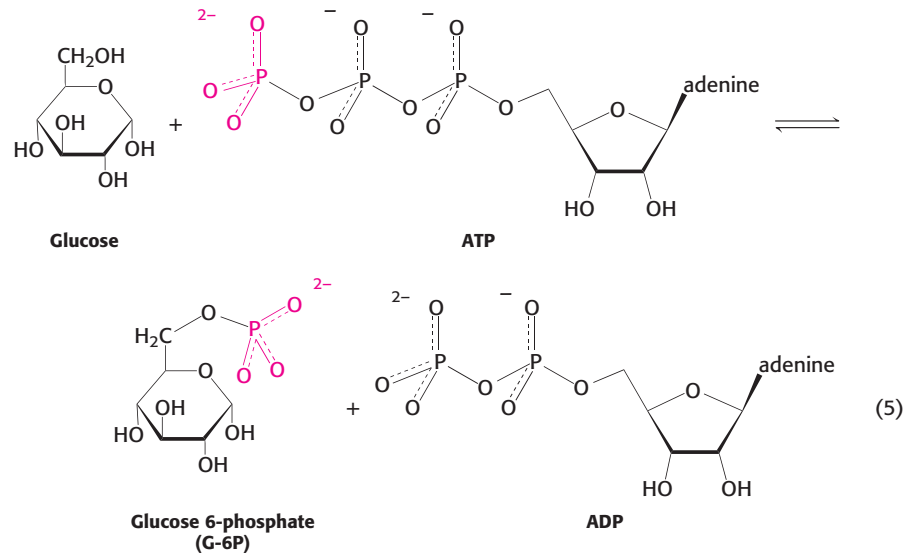
The oxaloacetate can be used in the citric acid cycle or converted into amino acids such as aspartic acid.

3. *Isomerization reactions* rearrange particular atoms within the molecule. Their role is often to prepare a molecule for subsequent reactions such as the oxidation–reduction reactions described in point 1.



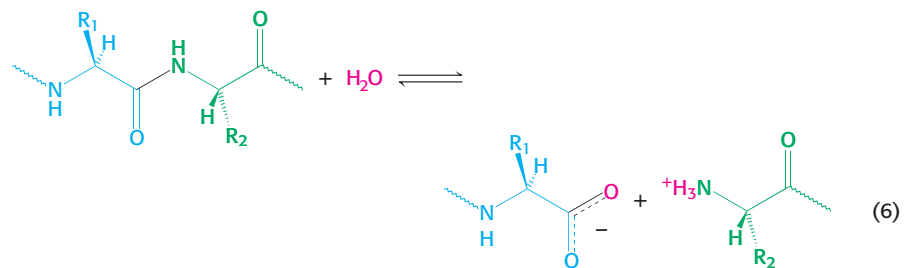
Reaction 4 is, again, a component of the citric acid cycle. This isomerization prepares the molecule for subsequent oxidation and decarboxylation by moving the hydroxyl group of citrate from a tertiary to a secondary position.

4. *Group-transfer reactions* play a variety of roles. Reaction 5 is representative of such a reaction. A phosphoryl group is transferred from the activated phosphoryl-group carrier, ATP, to glucose. This reaction traps glucose in the cell so that further catabolism can take place.

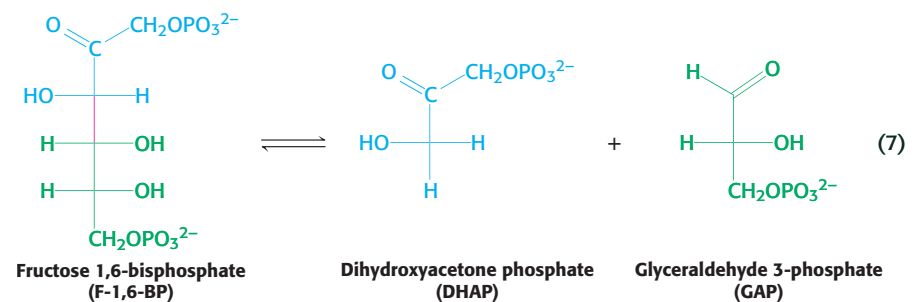


We saw earlier that group-transfer reactions are used to synthesize ATP (14.2.1). We will also see examples of their use in signaling pathways (Chapter 15).

5. *Hydrolytic reactions* cleave bonds by the addition of water. Hydrolysis is a common means employed to break down large molecules, either to facilitate further metabolism or to reutilize some of the components for biosynthetic purposes. Proteins are digested by hydrolytic cleavage (Chapters 9 and 10). Reaction 6 illustrates the hydrolysis of a peptide to yield two smaller peptides.

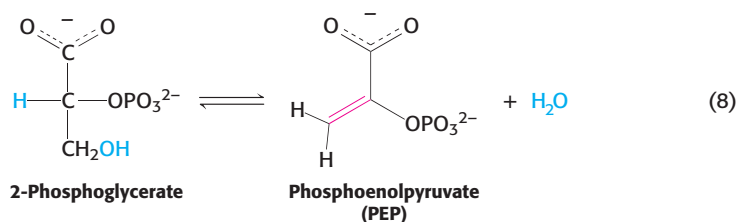


6. *The addition of functional groups to double bonds or the removal of groups to form double bonds* constitutes the final class of reactions. The enzymes that catalyze these types of reaction are classified as lyases (Section 8.1.3). An important example, illustrated in reaction 7, is the conversion of the six-carbon molecule fructose 1,6-bisphosphate (F-1,6-BP) into 2 three-carbon fragments: dihydroxyacetone phosphate and glyceraldehyde 3-phosphate.



This reaction is a key step in glycolysis, a key pathway for extracting energy from glucose (Section 16.1.5). Dehydrations to form double bonds,

such as the formation of phosphoenolpyruvate (Table 14.1) from 2-phosphoglycerate (reaction 8), are important reactions of this type.



The dehydration sets up the next step in the pathway, a group-transfer reaction that uses the high phosphoryl transfer potential of the product PEP to form ATP from ADP.

These six fundamental reaction types are the basis of metabolism. Remember that all six types can proceed in either direction, depending on the standard free energy for the specific reaction and the concentration of the reactants and products inside the cell. As an example of how simple themes are reiterated, consider the reactions shown in Figure 14.17. The same sequence of reactions is employed in the citric acid cycle, fatty acid degradation, the degradation of the amino acid lysine, and (in reverse) the biosynthesis of fatty acids. An effective way to learn is to look for commonalities in the diverse metabolic pathways that we will be studying. There is a chemical logic that, when exposed, renders the complexity of the chemistry of living systems more manageable and reveals its elegance.

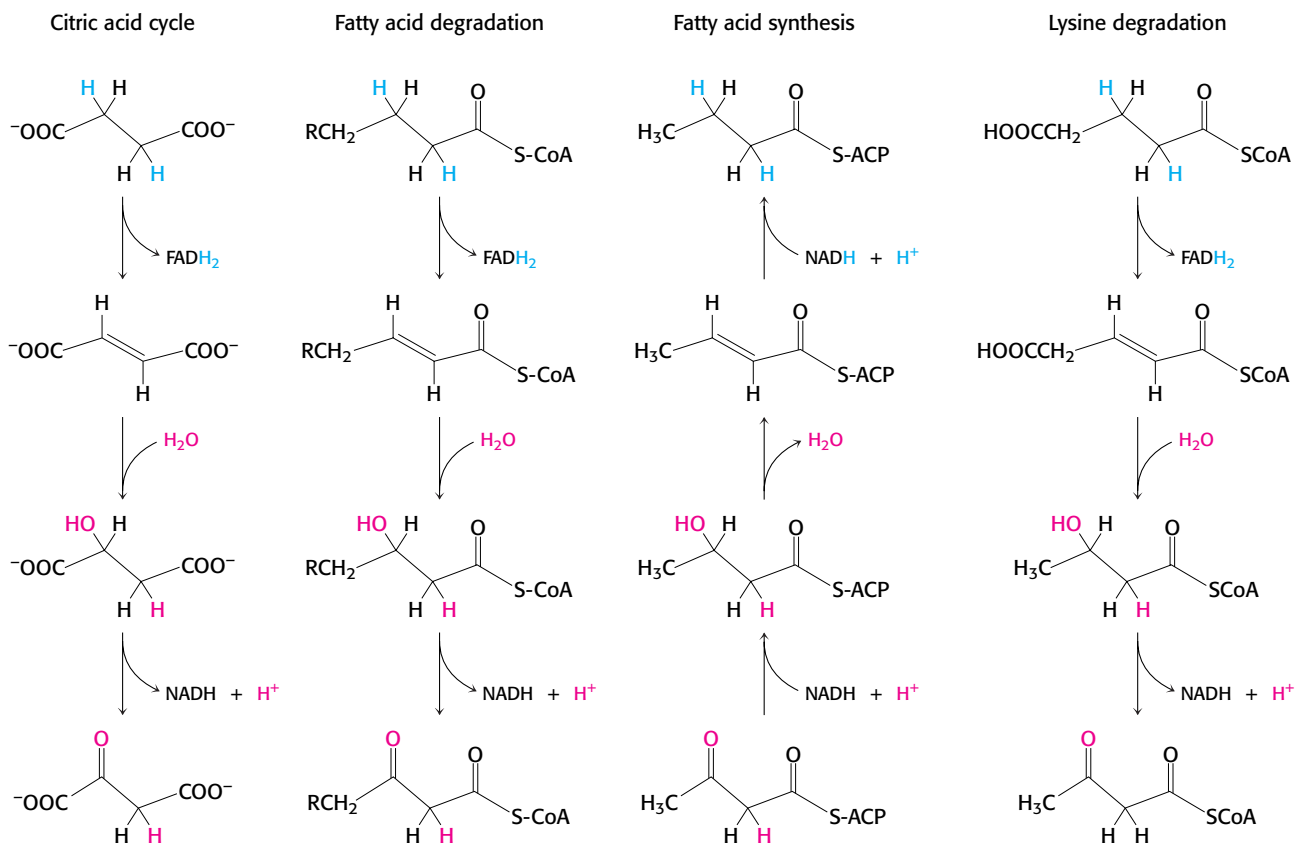


FIGURE 14.17 Metabolic motifs. Some metabolic pathways have similar sequences of reactions in common—in this case, an oxidation, the addition of a functional group (from a water molecule) to a double bond, and another oxidation. ACP designates acyl carrier protein.

14.3.3 Metabolic Processes Are Regulated in Three Principal Ways

It is evident that the complex network of reactions constituting intermediary metabolism must be rigorously regulated. At the same time, metabolic control must be flexible, because the external environments of cells are not constant. Metabolism is regulated by controlling (1) *the amounts of enzymes*, (2) *their catalytic activities*, and (3) *the accessibility of substrates*. The amount of a particular enzyme depends on both its rate of synthesis and its rate of degradation. The level of most enzymes is adjusted primarily by changing the *rate of transcription* of the genes encoding them. In *E. coli*, the presence of lactose, for example, induces within minutes a more than 50-fold increase in the rate of synthesis of β -galactosidase, an enzyme required for the breakdown of this disaccharide.

The catalytic activity of enzymes is controlled in several ways. *Reversible allosteric control* is especially important. For example, the first reaction in many biosynthetic pathways is allosterically inhibited by the ultimate product of the pathway. The inhibition of aspartate transcarbamoylase by cytidine triphosphate (Section 10.1) is a well-understood example of *feedback inhibition*. This type of control can be almost instantaneous. Another recurring mechanism is *reversible covalent modification*. For example, glycogen phosphorylase, the enzyme catalyzing the breakdown of glycogen, a storage form of sugar, is activated by phosphorylation of a particular serine residue when glucose is scarce (Section 21.2.1).

Hormones coordinate metabolic relations between different tissues, often by regulating the reversible modification of key enzymes. Hormones such as epinephrine trigger signal transduction cascades that lead to highly amplified changes in metabolic patterns in target tissues such as the muscle (Section 15.0.1). The hormone insulin promotes the entry of glucose into many kinds of cells. As will be discussed again in Chapter 15, many hormones act through intracellular messengers, such as cyclic AMP and calcium ion, that coordinate the activities of many target proteins.

Controlling the *flux of substrates* also regulates metabolism. The transfer of substrates from one compartment of a cell to another (e.g., from the cytosol to mitochondria) can serve as a control point.

An important general principle of metabolism is that *biosynthetic and degradative pathways are almost always distinct*. This separation is necessary for energetic reasons, as will be evident in subsequent chapters. It also facilitates the control of metabolism. In eukaryotes, metabolic regulation and flexibility also are enhanced by compartmentalization. For example, fatty acid oxidation takes place in mitochondria, whereas fatty acid synthesis takes place in the cytosol. *Compartmentalization segregates opposed reactions*.

Many reactions in metabolism are controlled by the *energy status* of the cell. One index of the energy status is the *energy charge*, which is proportional to the mole fraction of ATP plus half the mole fraction of ADP, given that ATP contains two anhydride bonds, whereas ADP contains one. Hence, the energy charge is defined as

$$\text{Energy charge} = \frac{[\text{ATP}] + \frac{1}{2}[\text{ADP}]}{[\text{ATP}] + [\text{ADP}] + [\text{AMP}]}$$

The energy charge can have a value ranging from 0 (all AMP) to 1 (all ATP). Daniel Atkinson showed that *ATP-generating (catabolic) pathways are inhibited by an energy charge, whereas ATP-utilizing (anabolic) pathways are stimulated by a high-energy charge*. In plots of the reaction rates of such pathways versus the energy charge, the curves are steep near an energy charge of 0.9, where they usually intersect (Figure 14.18). It is evident that the control

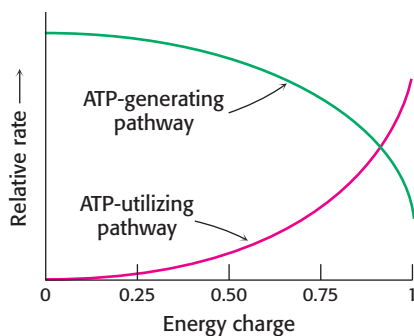


FIGURE 14.18 Energy charge regulates metabolism. High concentrations of ATP inhibit the relative rates of a typical ATP-generating (catabolic) pathway and stimulate the typical ATP-utilizing (anabolic) pathway.

of these pathways has evolved to maintain the energy charge within rather narrow limits. In other words, *the energy charge, like the pH of a cell, is buffered*. The energy charge of most cells ranges from 0.80 to 0.95. An alternative index of the energy status is the *phosphorylation potential*, which is defined as

$$\text{Phosphorylation potential} = \frac{[\text{ATP}]}{[\text{ADP}][\text{P}_i]}$$

The phosphorylation potential, in contrast with the energy charge, depends on the concentration of P_i and is directly related to the free energy-storage available from ATP.

14.3.4 Evolution of Metabolic Pathways



How did the complex pathways that constitute metabolism evolve? This question, a difficult one to address, was approached in Chapter 2. The current thinking is that RNA was an early biomolecule and that, in an early RNA world, RNA served as catalysts and information-storage molecules (Section 2.2.2).

Why do activated carriers such as ATP, NADH, FADH_2 , and coenzyme A contain adenosine diphosphate units (Figure 14.19)? A possible explanation is that these molecules evolved from the early RNA catalysts. Non-RNA units such as the isoalloxazine ring may have been recruited to serve as efficient carriers of activated electrons and chemical units, a function not readily performed by RNA itself. We can picture the adenine ring of FADH_2 binding to a uracil unit in a niche of an RNA enzyme (ribozyme) by base-pairing, whereas the isoalloxazine ring protrudes and functions as an electron carrier. When the more versatile proteins replaced RNA as the major catalysts, the ribonucleotide coenzymes stayed essentially unchanged because they were already well suited to their metabolic roles. The nicotinamide unit of NADH, for example, can readily transfer electrons irrespective of whether the adenine unit interacts with a base in an RNA enzyme or with amino acid residues in a protein enzyme. With the advent of protein enzymes, these important cofactors evolved as free molecules without losing the adenosine diphosphate vestige of their RNA-world ancestry. That molecules and motifs of metabolism are common to all forms of life testifies to their common origin and to the retention of functioning modules through billions of years of evolution. Our understanding of metabolism, like that of other biological processes, is enriched by inquiry into how these beautifully integrated patterns of reactions came into being.

SUMMARY

All cells transform energy. They extract energy from their environment and use this energy to convert simple molecules into cellular components.

- **Metabolism Is Composed of Many Coupled, Interconnecting Reactions**

The process of energy transduction takes place through a highly integrated network of chemical reactions called metabolism. Metabolism can be subdivided into catabolism (reactions employed to extract energy from fuels) and anabolism (reactions that use this energy for biosynthesis). The most valuable thermodynamic concept for understanding bioenergetics is free energy. A reaction can occur spontaneously only if the change in free energy (ΔG) is negative. A thermodynamically unfavorable reaction can be driven by a thermodynamically favorable one, which in many cases is the hydrolysis of ATP. The hydrolysis of ATP shifts the equilibrium of a coupled reaction by a factor of about 10^8 . ATP,

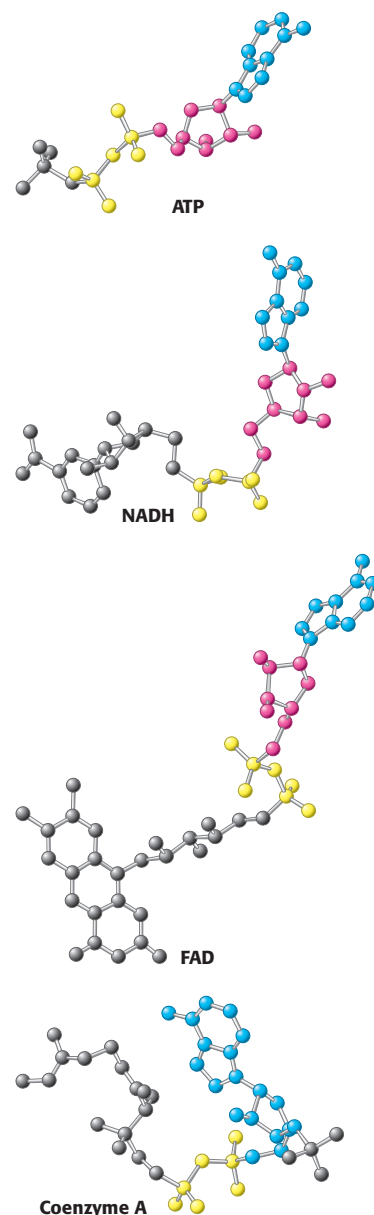


FIGURE 14.19 Adenosine diphosphate (ADP) is an ancient module in metabolism. This fundamental building block is present in key molecules such as ATP, NADH, FAD, and coenzyme A. The adenine unit is shown in blue, the ribose unit in red, and the diphosphate unit in yellow.

the universal currency of energy in biological systems, is an energy-rich molecule because it contains two phosphoanhydride bonds.

• **The Oxidation of Carbon Fuels Is an Important Source of Cellular Energy**

ATP formation is coupled to the oxidation of carbon fuels, either directly or through the formation of ion gradients. Photosynthetic organisms can use light to generate such gradients. ATP is consumed in muscle contraction and other motions of cells, in active transport, in signal transduction processes, and in biosyntheses. There are three stages in the extraction of energy from foodstuffs by aerobic organisms. In the first stage, large molecules are broken down into smaller ones, such as amino acids, sugars, and fatty acids. In the second stage, these small molecules are degraded to a few simple units that have a pervasive role in metabolism. One of them is the acetyl unit of acetyl CoA, a carrier of activated acyl groups. The third stage of metabolism is the citric acid cycle and oxidative phosphorylation, in which ATP is generated as electrons flow to O_2 , the ultimate electron acceptor, and fuels are completely oxidized to CO_2 .

• **Metabolic Pathways Contain Many Recurring Motifs**

Metabolism is characterized by common motifs. A small number of activated carriers, such as ATP, NADH, and acetyl CoA, are used in many metabolic pathways. NADPH, which carries two electrons at a high potential, provides reducing power in the biosynthesis of cell components from more-oxidized precursors. ATP and NADPH are continually generated and consumed. Most transfers of activated groups in metabolism are mediated by a recurring set of carriers. Moreover, key reaction types are used repeatedly in metabolic pathways.

Metabolism is regulated in a variety of ways. The amounts of some critical enzymes are controlled by regulation of the rate of protein synthesis and degradation. In addition, the catalytic activities of many enzymes are regulated by allosteric interactions (as in feedback inhibition) and by covalent modification. The movement of many substrates into cells and subcellular compartments also is controlled. Distinct pathways for biosynthesis and degradation contribute to metabolic regulation. The energy charge, which depends on the relative amounts of ATP, ADP, and AMP, plays a role in metabolic regulation. A high-energy charge inhibits ATP-generating (catabolic) pathways, whereas it stimulates ATP-utilizing (anabolic) pathways.

KEY TERMS

phototroph (p. 374)

chemotroph (p. 374)

metabolism or intermediary metabolism (p. 374)

catabolism (p. 374)

anabolism (p. 374)

amphibolic pathway (p. 375)

coupled reaction (p. 377)

phosphoryl transfer potential (p. 379)

oxidative phosphorylation (p. 382)

activated carrier (p. 383)

oxidation–reduction reaction (p. 387)

ligation reaction (p. 387)

isomerization reaction (p. 387)

group-transfer reaction (p. 387)

hydrolytic reaction (p. 388)

addition to or formation of double-bond reaction (p. 388)

energy charge (p. 390)

phosphorylation potential (p. 391)

SELECTED READINGS

Where to start

McGrane, M. M., Yun, J. S., Patel, Y. M., and Hanson, R. W., 1992. Metabolic control of gene expression: In vivo studies with transgenic mice. *Trends Biochem. Sci.* 17:40–44.

Books

Harold, F. M., 1986. *The Vital Force: A Study of Bioenergetics*. W. H. Freeman and Company.

Krebs, H. A., and Kornberg, H. L., 1957. *Energy Transformations in Living Matter*. Springer Verlag.

- Linder, M. C. (Ed.), 1991. *Nutritional Biochemistry and Metabolism* (2d ed.). Elsevier.
- Gottschalk, G., 1986. *Bacterial Metabolism* (2d ed.). Springer Verlag.
- Nicholls, D. G., and Ferguson, S. J., 1997. *Bioenergetics 2* (2d ed.). Academic Press.
- Martin, B. R., 1987. *Metabolic Regulation: A Molecular Approach*. Blackwell Scientific.
- Frayn, K. N., 1996. *Metabolic Regulation: A Human Perspective*. Portland Press.
- Fell, D., 1997. *Understanding the Control of Metabolism*. Portland Press.
- Harris, D. A., 1995. *Bioenergetics at a Glance*. Blackwell Scientific.

Thermodynamics

- Von Baeyer, H. C., 1999. *Warmth Disperses and Time Passes: A History of Heat*. Modern Library.
- Edsall, J. T., and Gutfreund, H., 1983. *Biothermodynamics: The Study of Biochemical Processes at Equilibrium*. Wiley.
- Klotz, I. M., 1967. *Energy Changes in Biochemical Reactions*. Academic Press.
- Hill, T. L., 1977. *Free Energy Transduction in Biology*. Academic Press.
- Alberty, R. A., 1993. Levels of thermodynamic treatment of biochemical reaction systems. *Biophys. J.* 65:1243–1254.
- Alberty, R. A., and Goldberg, R. N., 1992. Standard thermodynamic formation properties for the adenosine 5'-triphosphate series. *Biochemistry* 31:10610–10615.
- Alberty, R. A., 1968. Effect of pH and metal ion concentration on the equilibrium hydrolysis of adenosine triphosphate to adenosine diphosphate. *J. Biol. Chem.* 243:1337–1343.
- Goldberg, R. N., 1984. *Compiled Thermodynamic Data Sources for Aqueous and Biochemical Systems: An Annotated Bibliography (1930–1983)*. National Bureau of Standards Special Publication 685, U.S. Government Printing Office.
- Frey, P. A., and Arabshahi, A., 1995. Standard free energy change for the hydrolysis of the α , β -phosphoanhydride bridge in ATP. *Biochemistry* 34:11307–11310.

- Westheimer, F. H., 1987. Why nature chose phosphates. *Science* 235:1173–1178.

Bioenergetics and metabolism

- Schilling, C. H., Letscher, D., and Palsson, B. O., 2000. Theory for the systemic definition of metabolic pathways and their use in interpreting metabolic function from a pathway-oriented perspective. *J. Theor. Biol.* 203:229–248.
- DeCoursey, T. E., and Cherny, V. V., 2000. Common themes and problems of bioenergetics and voltage-gated proton channels. *Biochim. Biophys. Acta.* 1458:104–119.
- Giersch, C., 2000. Mathematical modelling of metabolism. *Curr. Opin. Plant Biol.* 3:249–253.
- Rees, D. C., and Howard, J. B., 1999. Structural bioenergetics and energy transduction mechanisms. *J. Mol. Biol.* 293:343–350.

Regulation of metabolism

- Kemp, G. J., 2000. Studying metabolic regulation in human muscle. *Biochem. Soc. Trans.* 28:100–103.
- Towle, H. C., Kaytor, E. N., and Shih, H. M., 1996. Metabolic regulation of hepatic gene expression. *Biochem. Soc. Trans.* 24:364–368.
- Hofmeyr, J. H., 1995. Metabolic regulation: A control analytic perspective. *J. Bioenerg. Biomembr.* 27:479–490.
- Atkinson, D. E., 1977. *Cellular Energy Metabolism and Its Regulation*. Academic Press.
- Erecińska, M., and Wilson, D. F., 1978. Homeostatic regulation of cellular energy metabolism. *Trends Biochem. Sci.* 3:219–223.

Historical aspects

- Kalckar, H. M., 1991. 50 years of biological research: From oxidative phosphorylation to energy requiring transport regulation. *Annu. Rev. Biochem.* 60:1–37.
- Kalckar, H. M. (Ed.), 1969. *Biological Phosphorylations*. Prentice Hall.
- Fruton, J. S., 1972. *Molecules and Life*. Wiley-Interscience.
- Lipmann, F., 1971. *Wanderings of a Biochemist*. Wiley-Interscience.

PROBLEMS

1. *Energy flow*. What is the direction of each of the following reactions when the reactants are initially present in equimolar amounts? Use the data given in Table 14.1.

- (a) $\text{ATP} + \text{creatine} \rightleftharpoons \text{creatine phosphate} + \text{ADP}$
 (b) $\text{ATP} + \text{glycerol} \rightleftharpoons \text{glycerol 3-phosphate} + \text{ADP}$
 (c) $\text{ATP} + \text{pyruvate} \rightleftharpoons \text{phosphoenolpyruvate} + \text{ADP}$
 (d) $\text{ATP} + \text{glucose} \rightleftharpoons \text{glucose 6-phosphate} + \text{ADP}$

2. *A proper inference*. What information do the ΔG° data given in Table 14.1 provide about the relative rates of hydrolysis of pyrophosphate and acetyl phosphate?

3. *A potent donor*. Consider the following reaction:



- (a) Calculate ΔG° and K'_{eq} at 25°C for this reaction, by using the data given in Table 14.1.
 (b) What is the equilibrium ratio of pyruvate to phosphoenolpyruvate if the ratio of ATP to ADP is 10?

4. *Isomeric equilibrium*. Calculate ΔG° for the isomerization of glucose 6-phosphate to glucose 1-phosphate. What is the equilibrium ratio of glucose 6-phosphate to glucose 1-phosphate at 25°C?

5. *Activated acetate*. The formation of acetyl CoA from acetate is an ATP-driven reaction:



(a) Calculate ΔG° for this reaction by using data given in this chapter.

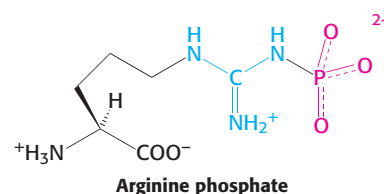
(b) The PP_i formed in the preceding reaction is rapidly hydrolyzed in vivo because of the ubiquity of inorganic pyrophosphatase. The ΔG° for the hydrolysis of PP_i is $-4.6 \text{ kcal mol}^{-1}$. Calculate the ΔG° for the overall reaction. What effect does the hydrolysis of PP_i have on the formation of acetyl CoA?

6. *Acid strength*. The pK of an acid is a measure of its proton-group-transfer potential.

(a) Derive a relation between ΔG° and pK .

(b) What is the ΔG° for the ionization of acetic acid, which has a pK of 4.8?

7. *Raison d'être*. The muscles of some invertebrates are rich in *arginine phosphate* (phosphoarginine). Propose a function for this amino acid derivative. How would you test your hypothesis?

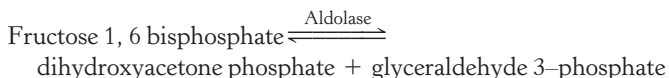


8. *Recurring motif.* What is the structural feature common to ATP, FAD, NAD⁺, and CoA?

9. *Ergogenic help or hindrance?* Creatine is a popular, but untested, dietary supplement.

- (a) What is the biochemical rationale for the use of creatine?
 (b) What type of exercise would most benefit from creatine supplementation?

10. *Standard conditions versus real life.* The enzyme aldolase catalyzes the following reaction in the glycolytic pathway:



The $\Delta G^{\circ'}$ for the reaction is $+5.7 \text{ kcal mol}^{-1}$, whereas the ΔG in the cell is $-0.3 \text{ kcal mol}^{-1}$. Calculate the ratio of reactants to products under equilibrium and intracellular conditions. Using your results, explain how the reaction can be endergonic under standard conditions and exergonic under intracellular conditions.

11. *Not all alike.* The concentrations of ATP, ADP, and P_i differ with cell type. Consequently, the release of free energy with the hydrolysis of ATP will vary with cell type. Using the following table, calculate the ΔG for the hydrolysis of ATP in muscle, liver, and brain cells. In which cell type is the free energy of ATP hydrolysis greatest?

	ATP (mM)	ADP (mM)	P _i (mM)
Liver	3.5	1.8	5.0
Muscle	8.0	0.9	8.0
Brain	2.6	0.7	2.7

12. *Running downhill.* Glycolysis is a series of 10 linked reactions that convert one molecule of glucose into two molecules of pyruvate with the concomitant synthesis of two molecules of ATP (Chapter 16). The $\Delta G^{\circ'}$ for this set of reactions is -8.5

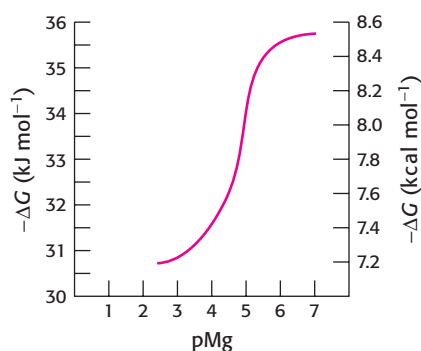
kcal mol^{-1} ($-35.6 \text{ kJ mol}^{-1}$), whereas the ΔG is $-18.3 \text{ kcal mol}^{-1}$ ($-76.6 \text{ kJ mol}^{-1}$). Explain why the free-energy release is so much greater under intracellular conditions than under standard conditions.

Chapter Integration Problem

13. *Activated sulfate.* Fibrinogen contains tyrosine-*O*-sulfate. Propose an activated form of sulfate that could react in vivo with the aromatic hydroxyl group of a tyrosine residue in a protein to form tyrosine-*O*-sulfate.

Data Interpretation

14. *Opposites attract.* The following graph shows how the ΔG for the hydrolysis of ATP varies as a function of the Mg²⁺ concentration ($\text{pMg} = \log 1/[\text{Mg}^{2+}]$).



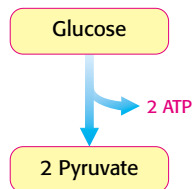
- (a) How does decreasing $[\text{Mg}^{2+}]$ affect the ΔG of hydrolysis for ATP?
 (b) How can you explain this effect?



Media Problem

15. *Coupled reactions.* The most obvious role of enzymes is to accelerate reactions, but a second critical role is to couple reactions that would ordinarily be unrelated. From what you have learned from the text and the **Conceptual Insights** module on energetic coupling, explain why the coupling of chemical reactions in a single enzyme is critical for life.

Glycolysis and Gluconeogenesis



Glycolysis produces energy. Michael Johnson sprints to another victory in the 200-meter semifinals of the 1996 Atlantic Olympics. Johnson, like anyone who sprints, requires a source of energy that can be rapidly accessed. The anaerobic metabolism of glucose—the process of glycolysis—provides such a source of energy for short, intense bouts of exercise. [Simon Bruty/Allsport.]

The first metabolic pathway that we encounter is *glycolysis*, an ancient pathway employed by a host of organisms. *Glycolysis is the sequence of reactions that metabolizes one molecule of glucose to two molecules of pyruvate with the concomitant net production of two molecules of ATP.* This process is anaerobic (i.e., it does not require O_2) inasmuch as it evolved before the accumulation of substantial amounts of oxygen in the atmosphere. Pyruvate can be further processed anaerobically (fermented) to lactate (*lactic acid fermentation*) or ethanol (*alcoholic fermentation*). Under aerobic conditions, pyruvate can be completely oxidized to CO_2 , generating much more ATP, as will be discussed in Chapters 18 and 19.

Glucose can be synthesized from noncarbohydrate precursors, such as pyruvate and lactic acid, in the process of *gluconeogenesis*. Although glycolysis and gluconeogenesis have some of the same enzymes in common, the two pathways are not simply the reverse of each other. In particular, the highly exergonic, irreversible steps of glycolysis are bypassed in gluconeogenesis. Both pathways are stringently controlled by intercellular and intracellular signals, and they are reciprocally regulated so that glycolysis and gluconeogenesis do not take place simultaneously in the same cell to a significant extent.

Our understanding of glucose metabolism, especially glycolysis, has a rich history. Indeed, the development of biochemistry and the delineation of glycolysis went hand in hand. A key discovery was made by Hans Buchner and Eduard Buchner in 1897, quite by accident. The Buchners

OUTLINE

- 16.1 Glycolysis Is an Energy-Conversion Pathway in Many Organisms
- 16.2 The Glycolytic Pathway Is Tightly Controlled
- 16.3 Glucose Can Be Synthesized from Noncarbohydrate Precursors
- 16.4 Gluconeogenesis and Glycolysis Are Reciprocally Regulated

Glycolysis—

Derived from the Greek stem *glyk-*, “sweet,” and the word *lysis*, “dissolution.”

were interested in manufacturing cell-free extracts of yeast for possible therapeutic use. These extracts had to be preserved without the use of antiseptics such as phenol, and so they decided to try sucrose, a commonly used preservative in kitchen chemistry. They obtained a startling result: sucrose was rapidly fermented into alcohol by the yeast juice. The significance of this finding was immense. *The Buchners demonstrated for the first time that fermentation could take place outside living cells.* The accepted view of their day, asserted by Louis Pasteur in 1860, was that fermentation is inextricably tied to living cells. The chance discovery of the Buchners refuted this vitalistic dogma and opened the door to modern biochemistry. *Metabolism became chemistry.*

Studies of muscle extracts then showed that many of the reactions of lactic acid fermentation were the same as those of alcoholic fermentation. *This exciting discovery revealed an underlying unity in biochemistry.* The complete glycolytic pathway was elucidated by 1940, largely through the pioneering contributions of Gustav Embden, Otto Meyerhof, Carl Neuberg, Jacob Parnas, Otto Warburg, Gerty Cori, and Carl Cori. Glycolysis is also known as the *Embden-Meyerhof pathway.*

In our consideration of the glycolytic and gluconeogenic pathways, we shall examine the mechanisms of selected enzymes in some detail. Of particular interest will be the enzymes that play the most central roles in converting one type of chemical energy into another.

16.0.1 Glucose Is an Important Fuel for Most Organisms



Glucose is an important and common fuel. In mammals, glucose is the only fuel that the brain uses under nonstarvation conditions and the only fuel that red blood cells can use at all. Indeed, almost all organisms use glucose, and most that do process it in a similar fashion. Recall from Chapter 11 that there are many carbohydrates. Why is glucose instead of some other monosaccharide such a prominent fuel? We can speculate on the reasons. First, glucose is one of the monosaccharides formed from formaldehyde under prebiotic conditions, so it may have been available as a fuel source for primitive biochemical systems. Second, glucose has a low tendency, relative to other monosaccharides, to nonenzymatically glycosylate proteins. In their open-chain (carbonyl) forms, monosaccharides can react with the amino groups of proteins to form Schiff bases, which rearrange to form a more stable amino ketone linkage. Such nonspecifically modified proteins often do not function effectively. Glucose has a strong tendency to exist in the ring formation and, consequently, relatively little tendency to modify proteins. Recall that all the hydroxyl groups in the ring conformation of β -glucose are equatorial, contributing to this high relative stability (Section 11.x.x).

16.0.2 Fermentations Provide Usable Energy in the Absence of Oxygen

Although glycolysis is a nearly universal process, the fate of its end product, pyruvate, may vary in different organisms or even in different tissues. In the presence of oxygen, the most common situation in multicellular organisms and many unicellular ones, pyruvate is metabolized to carbon dioxide and water through the citric acid cycle and the electron-transport chain. In the absence of oxygen, fermentation generates a lesser amount of energy; pyruvate is converted, or fermented, into lactic acid in lactic acid fermentation or into ethanol in alcoholic fermentation (Figure 16.1). Lactic acid production takes place in skeletal muscle when energy needs outpace the ability to transport oxygen. Although we will consider only these two fer-

Enzyme—

A term coined by Friedrich Wilhelm Kühne in 1878 to designate catalytically active substances that had previously been called ferments. Derived from the Greek words *en*, “in,” and *zyme*, “leaven.”

Fermentation—

An ATP-generating process in which organic compounds act as both donors and acceptors of electrons. Fermentation can take place in the absence of O_2 . Discovered by Louis Pasteur, who described fermentation as “la vie sans l’air” (“life without air”).

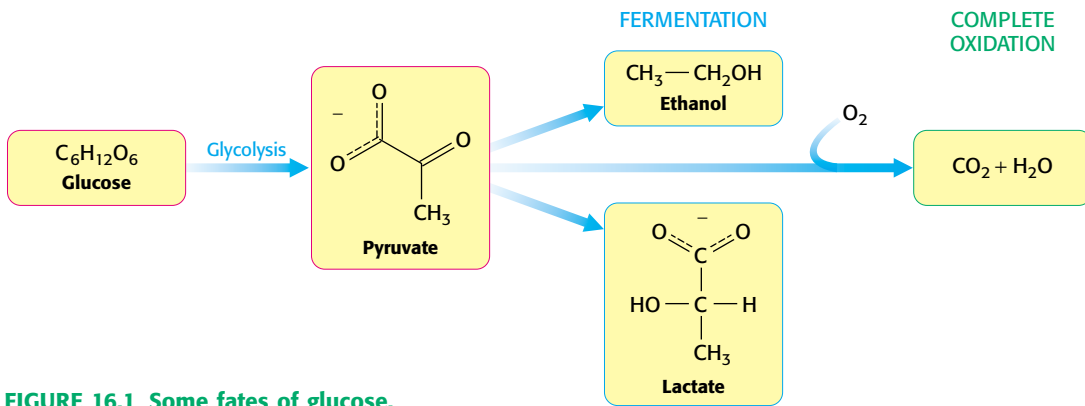


FIGURE 16.1 Some fates of glucose.

mentations, microorganisms are capable of generating a wide array of molecules as end points to fermentation (Table 16.1). Indeed, many food products are the result of fermentations. These foods include sour cream, yogurt, various cheeses, beer, wine, and sauerkraut.

Fermentations yield only a fraction of the energy available from the complete combustion of glucose. Why is a relatively inefficient metabolic pathway so extensively used? The fundamental reason is that oxygen is not required. The ability to survive without oxygen affords a host of living accommodations such as soils, deep water, and skin pores. Some organisms, called *obligate anaerobes*, cannot survive in the presence of O₂, a highly reactive compound. The bacterium *Clostridium perfringens*, the cause of gangrene, is an example of an obligate anaerobe. Other pathogenic obligate anaerobes are listed in Table 16.2.

Facultative anaerobes can function in the presence or absence of oxygen. For instance, organisms that live in the intertidal zone, such as the bivalve *Mytilus* (Figure 16.2), can function aerobically, using gills when they are under water and anaerobically when exposed to the air. Such organisms display habitat-dependent anaerobic functioning, or *habitat-dependent anaerobiosis*. Muscles in most animals display *activity-dependent anaerobiosis*, meaning that they can function anaerobically for short periods. For example, when animals perform bursts of intense exercise, their ATP needs rise faster than the ability of the body to provide oxygen to the muscle.

TABLE 16.1 Starting and ending points of various fermentations

Glucose	—————>	lactate
Lactate	—————>	acetate
Glucose	—————>	ethanol
Ethanol	—————>	acetate
Arginine	—————>	carbon dioxide
Pyrimidines	—————>	carbon dioxide
Purines	—————>	formate
Ethylene glycol	—————>	acetate
Threonine	—————>	propionate
Leucine	—————>	2-alkylacetate
Phenylalanine	—————>	propionate

Note: The products of some fermentations are the substrates for others.

TABLE 16.2 Examples of pathogenic obligate anaerobes

Bacterium	Results of infection
<i>Clostridium tetani</i>	Tetanus (lockjaw)
<i>Clostridium botulinum</i>	Botulism (an especially severe type of food poisoning)
<i>Clostridium perfringens</i>	Gas gangrene (gas is produced as an end point of the fermentation, distorting and destroying the tissue)
<i>Bartonella hensela</i>	Cat scratch fever (flulike symptoms)
<i>Bacteroides fragilis</i>	Abdominal, pelvic, pulmonary, and blood infections

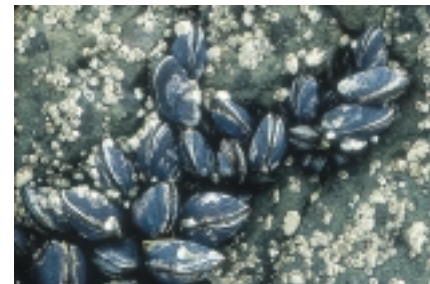
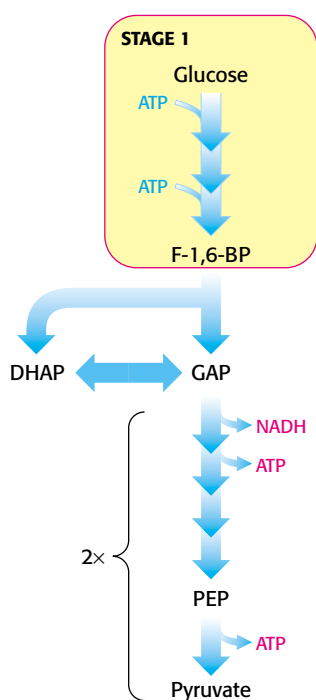


FIGURE 16.2 The bivalve *Mytilus*. These mussels, inhabitants of the intertidal zone, display habitat-dependent anaerobiosis. [Ed Reschke/Peter Arnold.]

The muscle functions anaerobically until the lactic acid builds up to the point at which the fall in pH inhibits the anaerobic pathway (Section 16.2.1).

16.1 GLYCOLYSIS IS AN ENERGY-CONVERSION PATHWAY IN MANY ORGANISMS

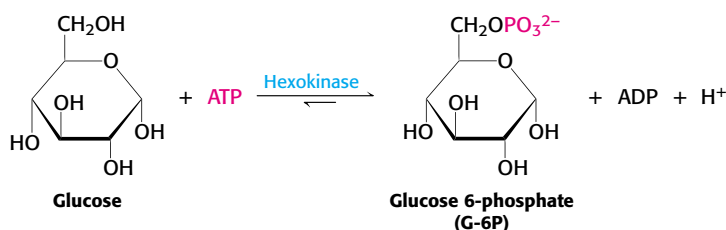
We now start our consideration of the glycolytic pathway. This pathway is common to virtually all cells, both prokaryotic and eukaryotic. In eukaryotic cells, glycolysis takes place in the cytosol. This pathway can be thought of as comprising three stages (Figure 16.3). Stage 1, which is the conversion of glucose into fructose 1,6-bisphosphate, consists of three steps: a phosphorylation, an isomerization, and a second phosphorylation reaction. *The strategy of these initial steps in glycolysis is to trap the glucose in the cell and form a compound that can be readily cleaved into phosphorylated three-carbon units.* Stage 2 is the cleavage of the fructose 1,6-bisphosphate into two three-carbon fragments. These resulting three-carbon units are readily interconvertible. In stage 3, ATP is harvested when the three-carbon fragments are oxidized to pyruvate.



Stage 1 of glycolysis. The three steps of stage 1 begin with the phosphorylation of glucose by hexokinase.

16.1.1 Hexokinase Traps Glucose in the Cell and Begins Glycolysis

Glucose enters cells through specific transport proteins (Section 16.2.4) and has one principal fate: *it is phosphorylated by ATP to form glucose 6-phosphate.* This step is notable for two reasons: (1) glucose 6-phosphate cannot diffuse through the membrane, because of its negative charges, and (2) the addition of the phosphoryl group begins to destabilize glucose, thus facilitating its further metabolism. The transfer of the phosphoryl group from ATP to the hydroxyl group on carbon 6 of glucose is catalyzed by *hexokinase*.



Phosphoryl transfer is a fundamental reaction in biochemistry and is one that was discussed in mechanistic and structural detail earlier (Section 9.4.1). *Kinases* are enzymes that catalyze the transfer of a phosphoryl group from ATP to an acceptor. Hexokinase, then, catalyzes the transfer of a phosphoryl group from ATP to a variety of six-carbon sugars (*hexoses*), such as glucose and mannose. *Hexokinase, like adenylate kinase (Section 9.4.2) and all other kinases, requires Mg^{2+} (or another divalent metal ion such as Mn^{2+}) for activity.* The divalent metal ion forms a complex with ATP.

The results of x-ray crystallographic studies of yeast hexokinase revealed that the binding of glucose induces a large conformational change in the enzyme, analogous to the conformational changes undergone by NMP kinases on substrate binding (Section 9.4.3). Hexokinase consists of two lobes, which move toward each other when glucose is bound (Figure 16.4). On glucose binding, one lobe rotates 12 degrees with respect to the other, resulting in movements of the polypeptide backbone of as much as 8 Å. The cleft between the lobes closes, and the bound glucose becomes surrounded by protein, except for the hydroxyl group of carbon 6, which will accept the phosphoryl group from ATP. The closing of the cleft in hexokinase is a striking example of the role of *induced fit* in enzyme action (Section 8.2.2).

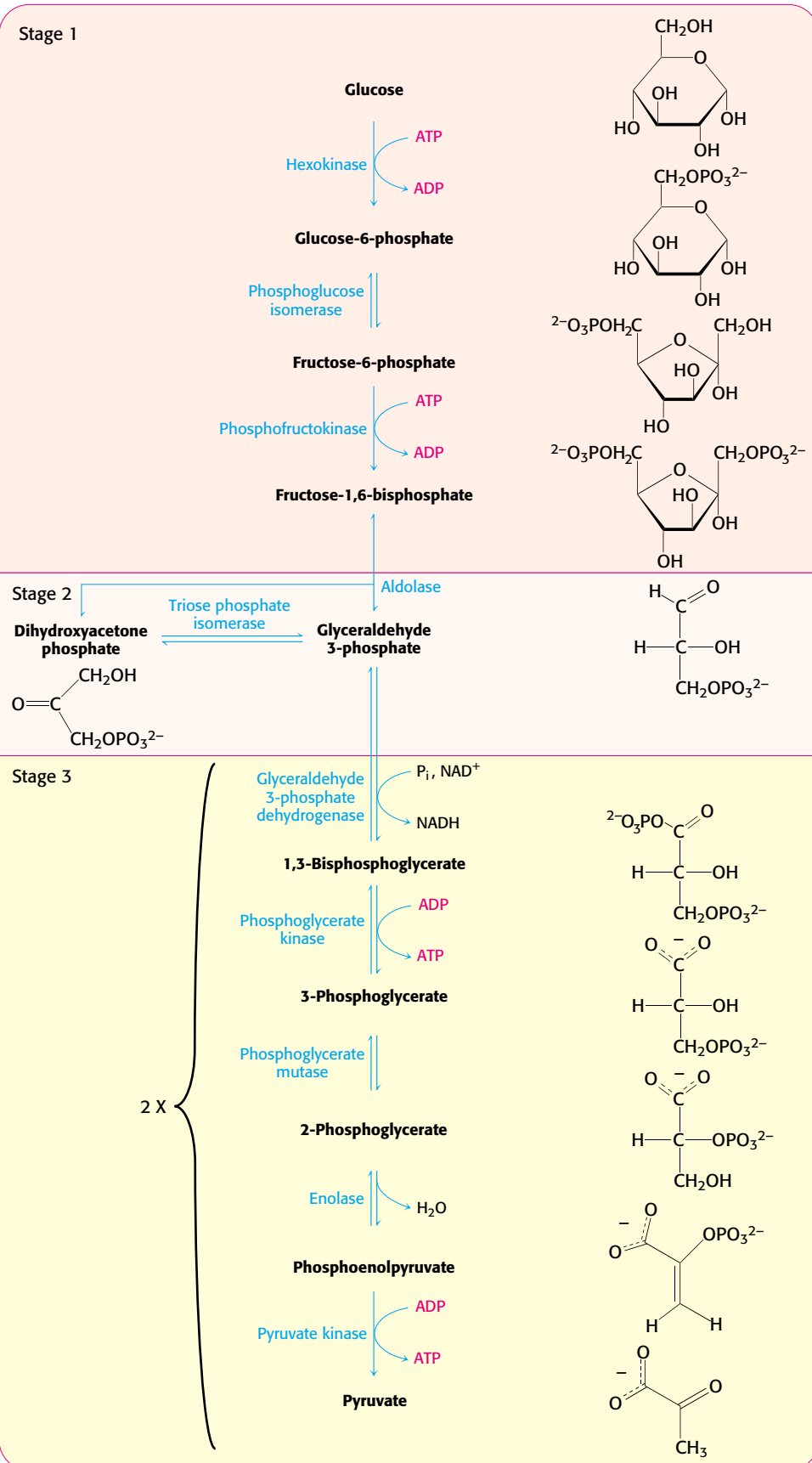


FIGURE 16.3 Stages of glycolysis. The glycolytic pathway can be divided into three stages: (1) glucose is trapped and destabilized; (2) two interchangeable three-carbon molecules are generated by cleavage of six-carbon fructose; and (3) ATP is generated.

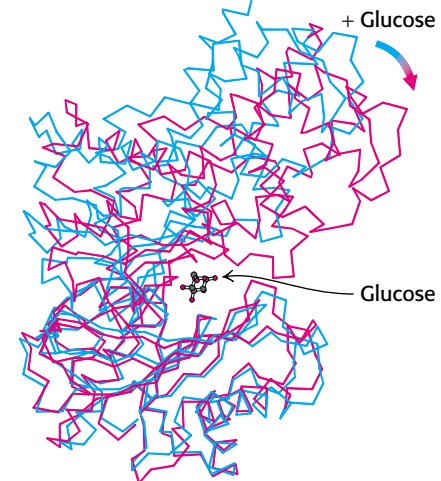
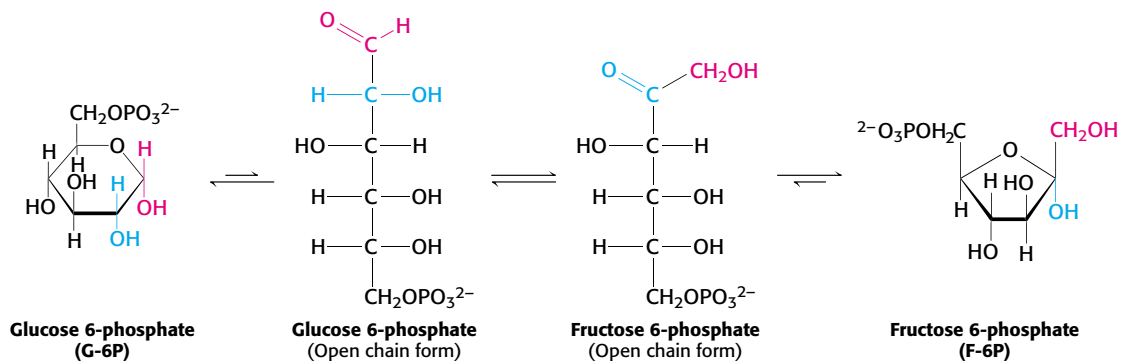


FIGURE 16.4 Induced fit in hexokinase. As shown in blue, the two lobes of hexokinase are separated in the absence of glucose. The conformation of hexokinase changes markedly on binding glucose, as shown in red. The two lobes of the enzyme come together and surround the substrate.

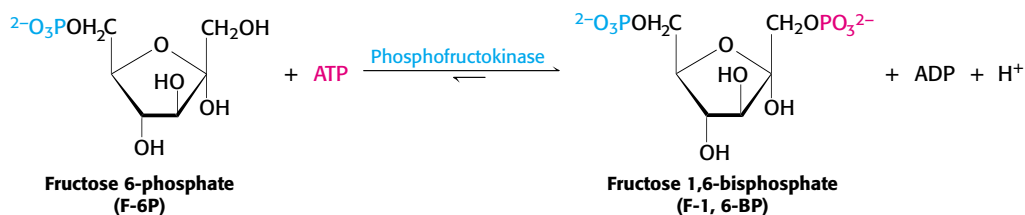
The glucose-induced structural changes are significant in two respects. First, the environment around the glucose becomes much more nonpolar, which favors the donation of the terminal phosphoryl group of ATP. Second, as noted in Section 9.4.3, the substrate-induced conformational changes within the kinase enables it to discriminate against H_2O as a substrate. If hexokinase were rigid, a molecule of H_2O occupying the binding site for the $-\text{CH}_2\text{OH}$ of glucose would attack the γ phosphoryl group of ATP, forming ADP and P_i . In other words, a rigid kinase would necessarily also be an ATPase. It is interesting to note that other kinases taking part in glycolysis—pyruvate kinase, phosphoglycerate kinase, and phosphofructokinase—also contain clefts between lobes that close when substrate is bound, although the structures of these enzymes are different in other regards. *Substrate-induced cleft closing is a general feature of kinases.*

16.1.2 The Formation of Fructose 1,6-bisphosphate from Glucose 6-phosphate

The next step in glycolysis is the *isomerization of glucose 6-phosphate to fructose 6-phosphate*. Recall that the open-chain form of glucose has an aldehyde group at carbon 1, whereas the open-chain form of fructose has a keto group at carbon 2. Thus, the isomerization of glucose 6-phosphate to fructose 6-phosphate is a *conversion of an aldose into a ketose*. The reaction catalyzed by phosphoglucose isomerase includes additional steps because both glucose 6-phosphate and fructose 6-phosphate are present primarily in the cyclic forms. The enzyme must first open the six-membered ring of glucose 6-phosphate, catalyze the isomerization, and then promote the formation of the five-membered ring of fructose 6-phosphate.



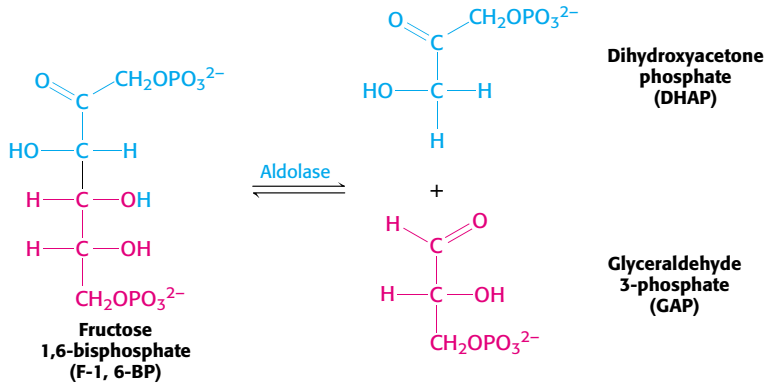
A second phosphorylation reaction follows the isomerization step. *Fructose 6-phosphate is phosphorylated by ATP to fructose 1,6-bisphosphate (F-1,6-BP)*. The prefix *bis-* in bisphosphate means that two separate monophosphate groups are present, whereas the prefix *di-* in diphosphate (as in adenosine diphosphate) means that two phosphate groups are present and are connected by an anhydride bond.



This reaction is catalyzed by *phosphofructokinase* (PFK), an allosteric enzyme that sets the pace of glycolysis (Section 16.2.1). As we will learn, this enzyme plays a central role in the integration of much of metabolism.

16.1.3 The Six-Carbon Sugar Is Cleaved into Two Three-Carbon Fragments by Aldolase

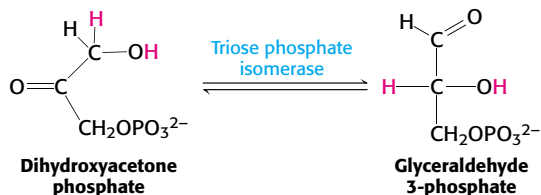
The second stage of glycolysis begins with the splitting of fructose 1,6-bisphosphate into *glyceraldehyde 3-phosphate* (GAP) and *dihydroxyacetone phosphate* (DHAP). The products of the remaining steps in glycolysis consist of three-carbon units rather than six-carbon units.



This reaction is catalyzed by *aldolase*. This enzyme derives its name from the nature of the reverse reaction, an aldol condensation. The reaction catalyzed by aldolase is readily reversible under intracellular conditions.

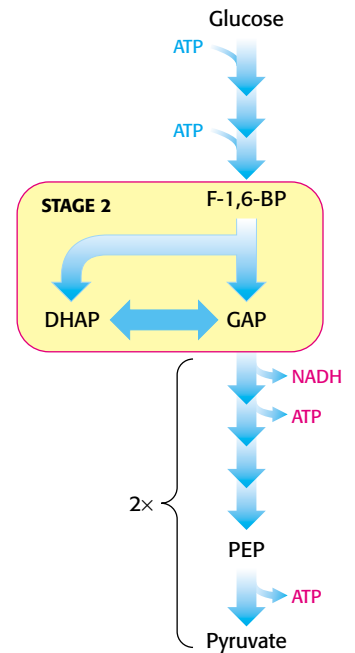
16.1.4 Triose phosphate isomerase Salvages a Three-Carbon Fragment

Glyceraldehyde 3-phosphate is on the direct pathway of glycolysis, whereas dihydroxyacetone phosphate is not. Unless a means exists to convert dihydroxyacetone phosphate into glyceraldehyde 3-phosphate, a three-carbon fragment useful for generating ATP will be lost. These compounds are isomers that can be readily interconverted: dihydroxyacetone phosphate is a ketose, whereas glyceraldehyde 3-phosphate is an aldose. The isomerization of these three-carbon phosphorylated sugars is catalyzed by *triose phosphate isomerase* (TIM; Figure 16.5). This reaction is rapid and reversible. At equilibrium, 96% of the triose phosphate is dihydroxyacetone phosphate. However, the reaction proceeds readily from dihydroxyacetone phosphate to glyceraldehyde 3-phosphate because the subsequent reactions of glycolysis remove this product.



Much is known about the catalytic mechanism of triose phosphate isomerase. TIM catalyzes the transfer of a hydrogen atom from carbon 1 to carbon 2 in converting dihydroxyacetone phosphate into glyceraldehyde 3-phosphate, an intramolecular oxidation–reduction. This isomerization of a ketose into an aldose proceeds through an *enediol intermediate* (Figure 16.6).

X-ray crystallographic and other studies showed that glutamate 165 (see Figure 16.5) plays the role of a general acid–base catalyst. However, this



Stage 2 of glycolysis. Two three-carbon fragments are produced from one six-carbon sugar.

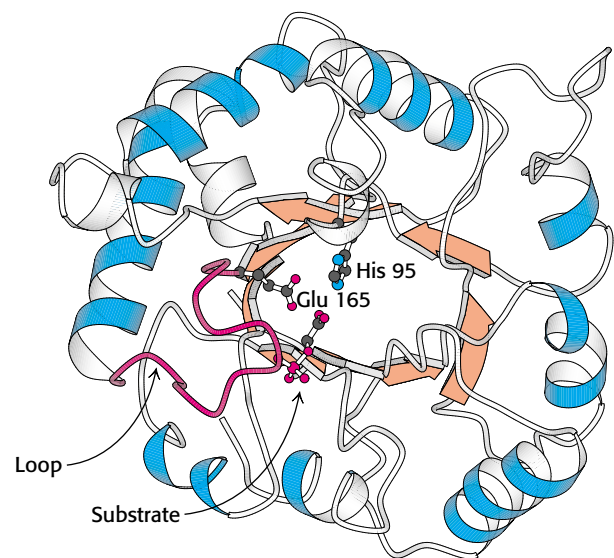


FIGURE 16.5 Structure of triose phosphate isomerase. This enzyme consists of a central core of eight parallel β strands (orange) surrounded by eight α helices (blue). This structural motif, called an $\alpha\beta$ barrel, is also found in the glycolytic enzymes aldolase, enolase, and pyruvate kinase. Histidine 95 and glutamate 165, essential components of the active site of triose phosphate isomerase, are located in the barrel. A loop (red) closes off the active site on substrate binding.

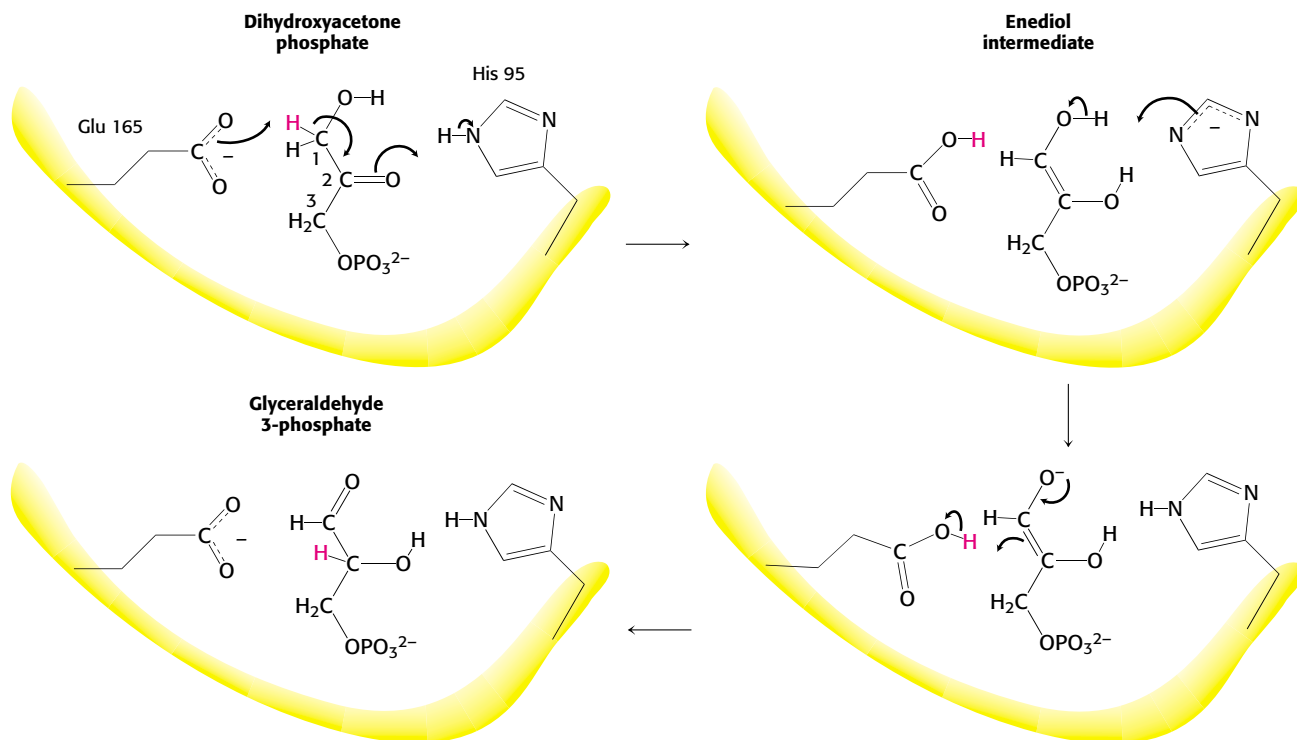
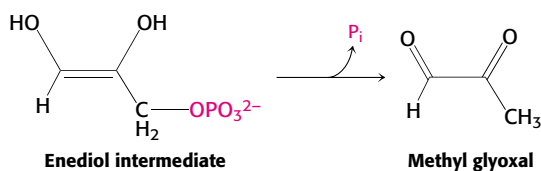


FIGURE 16.6 Catalytic mechanism of triose phosphate isomerase. Glutamate 165 transfers a proton between carbons with the assistance of histidine 95, which shuttles between the neutral and relatively rare negatively charged form. The latter is stabilized by interactions with other parts of the enzyme.

carboxylate group by itself is not basic enough to pull a proton away from a carbon atom adjacent to a carbonyl group. Histidine 95 assists catalysis by donating a proton to stabilize the negative charge that develops on the C-2 carbonyl group.

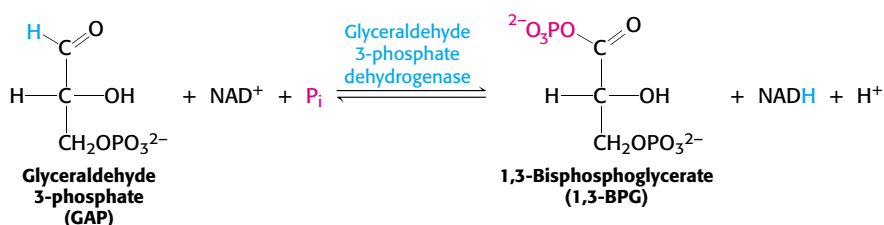
Two features of this enzyme are noteworthy. First, TIM displays great catalytic prowess. It accelerates isomerization by a factor of 10^{10} compared with the rate obtained with a simple base catalyst such as acetate ion. Indeed, the k_{cat}/K_M ratio for isomerization of glyceraldehyde 3-phosphate is $2 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$, which is close to the diffusion-controlled limit. In other words, the rate-limiting step in catalysis is the diffusion-controlled encounter of substrate and enzyme. TIM is an example of a *kinetically perfect enzyme* (Section 8.2.5). Second, TIM suppresses an undesired side reaction, the decomposition of the enediol intermediate into methyl glyoxal and inorganic phosphate.



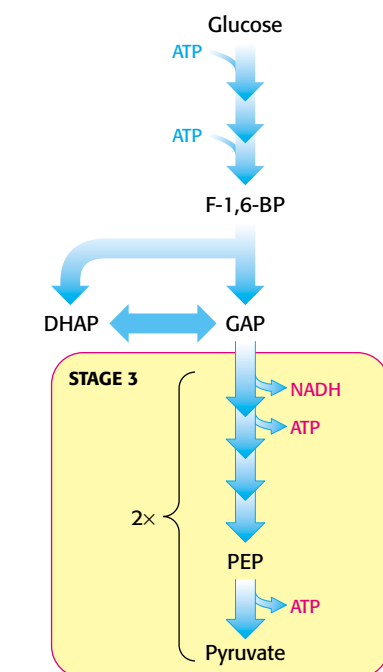
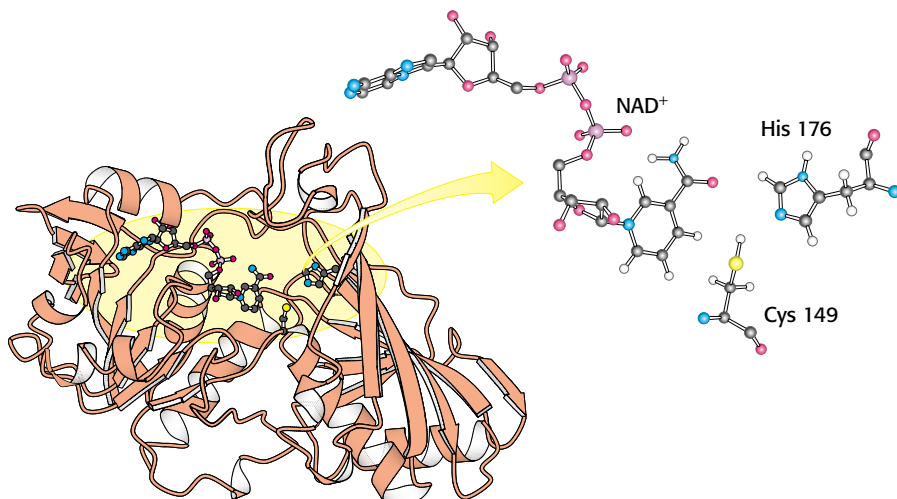
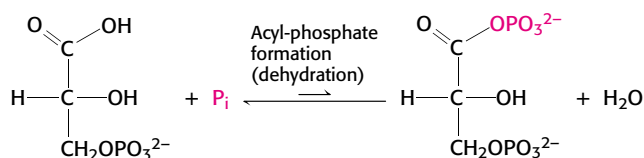
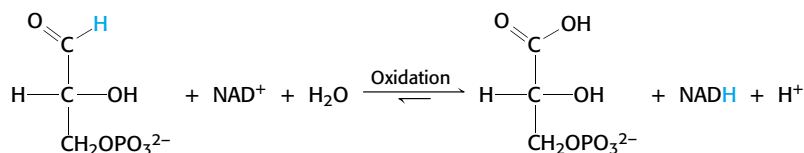
In solution, this physiologically useless reaction is 100 times as fast as isomerization. Hence, TIM must prevent the enediol from leaving the enzyme. This labile intermediate is trapped in the active site by the movement of a loop of 10 residues (see Figure 16.5). This loop serves as a lid on the active site, shutting it when the enediol is present and reopening it when isomerization is completed. *We see here a striking example not only of catalytic perfection, but also of the acceleration of a desirable reaction so that it takes place much faster than an undesirable alternative reaction.* Thus, two molecules of glyceraldehyde 3-phosphate are formed from one molecule of fructose 1,6-bisphosphate by the sequential action of aldolase and triose phosphate isomerase. The economy of metabolism is evident in this reaction sequence. The isomerase funnels dihydroxyacetone phosphate into the main glycolytic pathway—a separate set of reactions is not needed.

16.1.5 Energy Transformation: Phosphorylation Is Coupled to the Oxidation of Glyceraldehyde 3-phosphate by a Thioester Intermediate

The preceding steps in glycolysis have transformed one molecule of glucose into two molecules of glyceraldehyde 3-phosphate, but no energy has yet been extracted. On the contrary, thus far two molecules of ATP have been invested. We come now to a series of steps that harvest some of the energy contained in glyceraldehyde 3-phosphate. The initial reaction in this sequence is the *conversion of glyceraldehyde 3-phosphate into 1,3-bisphosphoglycerate* (1,3-BPG), a reaction catalyzed by *glyceraldehyde 3-phosphate dehydrogenase* (Figure 16.7).



1,3-Bisphosphoglycerate is an acyl phosphate. Such compounds have a high phosphoryl-transfer potential; one of its phosphoryl groups is transferred to ADP in the next step in glycolysis. The reaction catalyzed by glyceraldehyde 3-phosphate dehydrogenase is really the sum of two processes: the *oxidation* of the aldehyde to a carboxylic acid by NAD^+ and the *joining* of the carboxylic acid and orthophosphate to form the acyl-phosphate product.



Stage 3 of glycolysis. The oxidation of three-carbon fragments yields ATP.

FIGURE 16.7 Structure of glyceraldehyde 3-phosphate dehydrogenase. The active site includes a cysteine residue and a histidine residue adjacent to a bound NAD^+ .

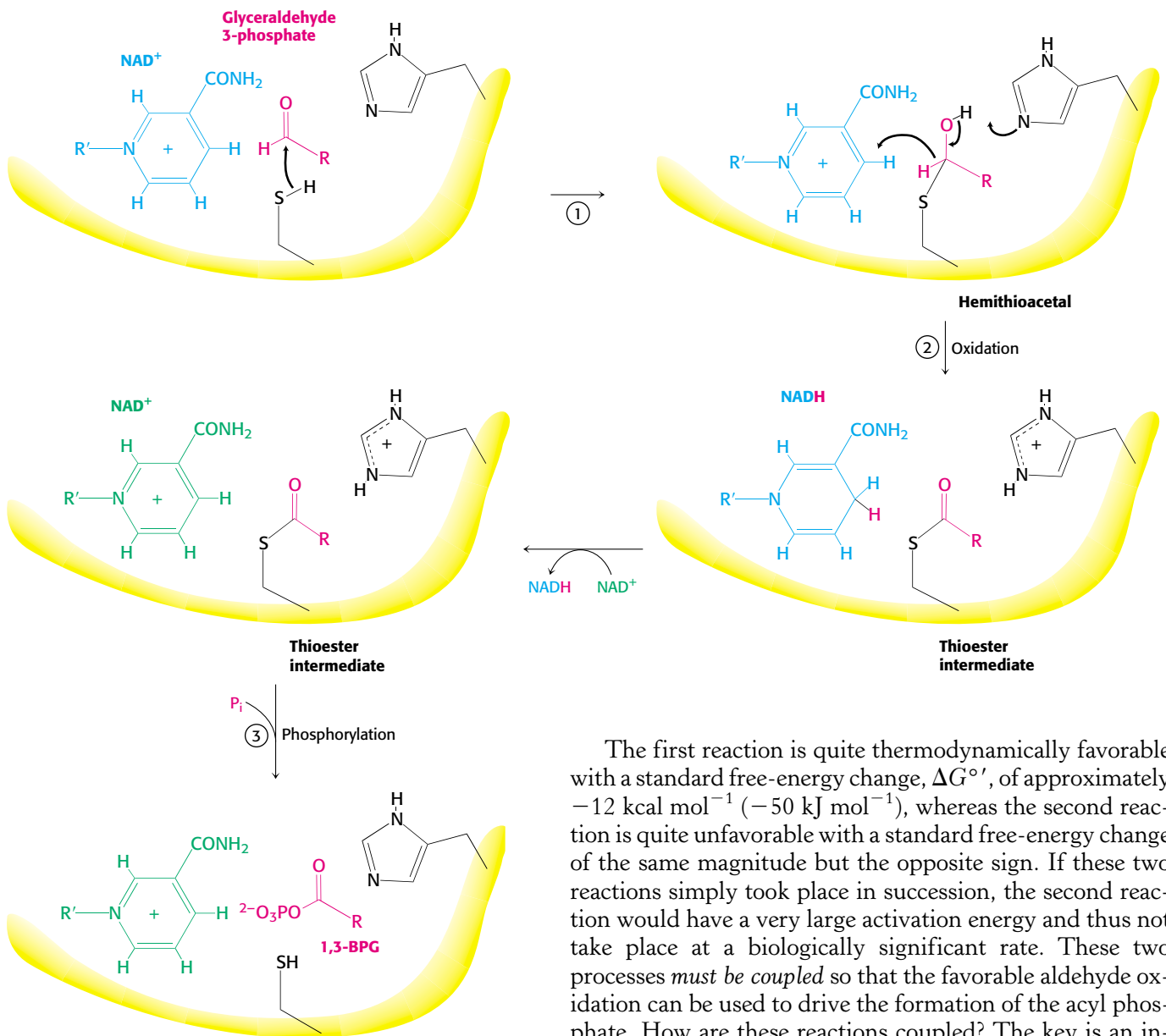


FIGURE 16.8 Catalytic mechanism of glyceraldehyde 3-phosphate dehydrogenase. The reaction proceeds through a thioester intermediate, which allows the oxidation of glyceraldehyde to be coupled to the phosphorylation of 3-phosphoglycerate.

The first reaction is quite thermodynamically favorable with a standard free-energy change, ΔG° , of approximately $-12 \text{ kcal mol}^{-1}$ (-50 kJ mol^{-1}), whereas the second reaction is quite unfavorable with a standard free-energy change of the same magnitude but the opposite sign. If these two reactions simply took place in succession, the second reaction would have a very large activation energy and thus not take place at a biologically significant rate. These two processes *must be coupled* so that the favorable aldehyde oxidation can be used to drive the formation of the acyl phosphate. How are these reactions coupled? The key is an intermediate, formed as a result of the aldehyde oxidation, that is higher in free energy than the free carboxylic acid is. This intermediate reacts with orthophosphate to form the acyl-phosphate product.

Let us consider the mechanism of glyceraldehyde 3-phosphate dehydrogenase in detail (Figure 16.8). In step 1, the aldehyde substrate reacts with the sulfhydryl group of cysteine 149 on the enzyme to form a hemithioacetal. Step 2 is the *transfer of a hydride ion to a molecule of NAD⁺ that is tightly bound to the enzyme and is adjacent to the cysteine residue*. This reaction is favored by the deprotonation of the hemithioacetal by histidine 176. The products of this reaction are the reduced coenzyme NADH and a thioester intermediate. *This thioester intermediate has a free energy close to that of the reactants*. In step 3, orthophosphate attacks the thioester to form 1,3-BPG and free the cysteine residue. This displacement occurs only after the NADH formed from the aldehyde oxidation has left the enzyme and been replaced by a second NAD⁺. The positive charge on the NAD⁺ may help polarize the thioester intermediate to facilitate the attack by orthophosphate.

This example illustrates the essence of energy transformations and of metabolism itself: energy released by carbon oxidation is converted into high phosphoryl-transfer potential. The favorable oxidation and unfavorable

phosphorylation reactions are *coupled by the thioester intermediate*, which preserves much of the free energy released in the oxidation reaction. We see here the *use of a covalent enzyme-bound intermediate as a mechanism of energy coupling*. A free-energy profile of the glyceraldehyde 3-phosphate dehydrogenase reaction, compared with a hypothetical process in which the reaction proceeds without this intermediate, reveals how this intermediate allows a favorable process to drive an unfavorable one (Figure 16.9).

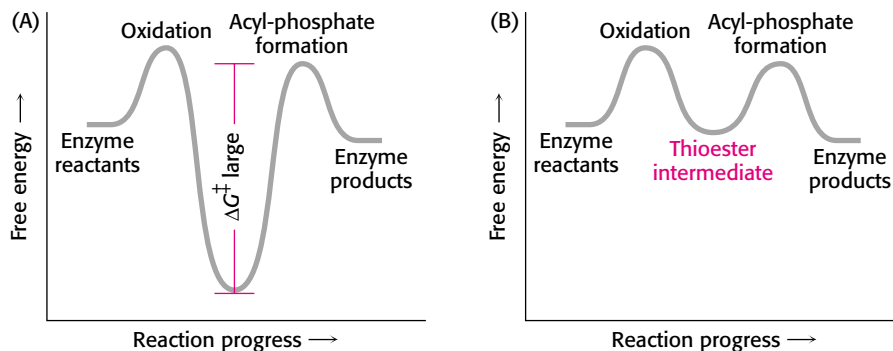
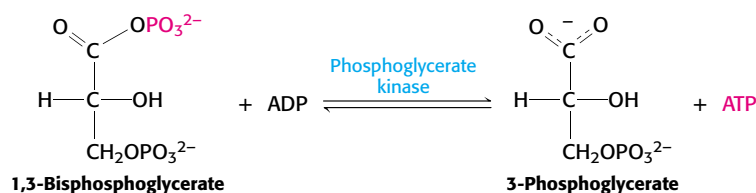


FIGURE 16.9 Free-energy profiles for glyceraldehyde oxidation followed by acyl-phosphate formation. (A) A hypothetical case with no coupling between the two processes. The second step must have a large activation barrier, making the reaction very slow. (B) The actual case with the two reactions coupled through a thioester intermediate.

16.1.6 The Formation of ATP from 1,3-Bisphosphoglycerate

The final stage in glycolysis is the generation of ATP from the phosphorylated three-carbon metabolites of glucose. *Phosphoglycerate kinase* catalyzes the transfer of the phosphoryl group from the acyl phosphate of 1,3-bisphosphoglycerate to ADP. ATP and 3-phosphoglycerate are the products.



The formation of ATP in this manner is referred to as *substrate-level phosphorylation* because the phosphate donor, 1,3-BPG, is a substrate with high phosphoryl-transfer potential. We will contrast this manner of ATP formation with that in which ATP is formed from ionic gradients in Chapters 19 and 20.

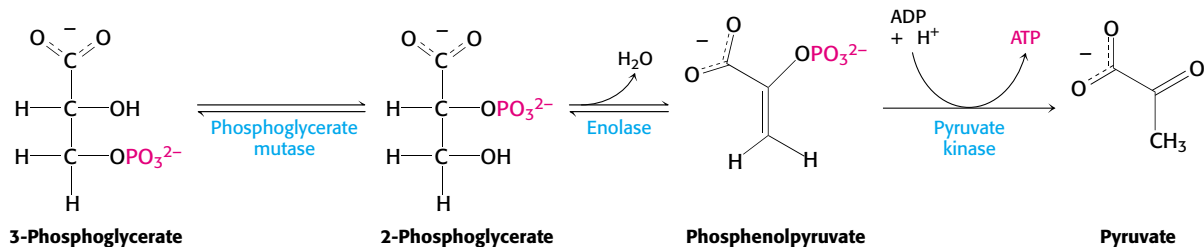
Thus, the outcomes of the reactions catalyzed by glyceraldehyde 3-phosphate dehydrogenase and phosphoglycerate kinase are:

1. Glyceraldehyde 3-phosphate, an aldehyde, is oxidized to 3-phosphoglycerate, a carboxylic acid.
2. NAD^+ is concomitantly reduced to NADH.
3. ATP is formed from P_i and ADP at the expense of carbon oxidation energy.

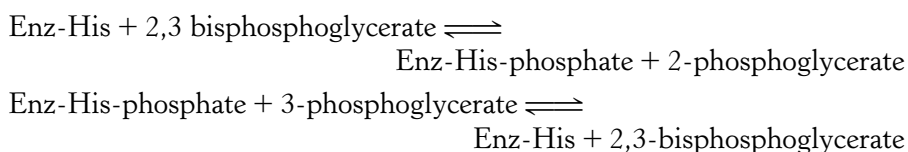
Keep in mind that, because of the actions of aldolase and triose phosphate isomerase, two molecules of glyceraldehyde 3-phosphate were formed and hence two molecules of ATP were generated. These ATP molecules make up for the two molecules of ATP consumed in the first stage of glycolysis.

16.1.7 The Generation of Additional ATP and the Formation of Pyruvate

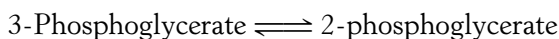
In the remaining steps of glycolysis, 3-phosphoglycerate is converted into pyruvate with the concomitant conversion of ADP into ATP.



The first reaction is a rearrangement. The position of the phosphoryl group shifts in the conversion of *3-phosphoglycerate* into *2-phosphoglycerate*, a reaction catalyzed by *phosphoglycerate mutase*. In general, a *mutase* is an enzyme that catalyzes the intramolecular shift of a chemical group, such as a phosphoryl group. The phosphoglycerate mutase reaction has an interesting mechanism: the phosphoryl group is not simply moved from one carbon to another. This enzyme requires catalytic amounts of 2,3-bisphosphoglycerate to maintain an active-site histidine residue in a phosphorylated form.



The sum of these reactions yields the mutase reaction:



Examination of the first partial reaction reveals that the mutase functions as a phosphatase—it converts 2,3-bisphosphoglycerate into 2-phosphoglycerate. However, the phosphoryl group remains linked to the enzyme. This phosphoryl group is then transferred to 3-phosphoglycerate to reform 2,3-bisphosphoglycerate.

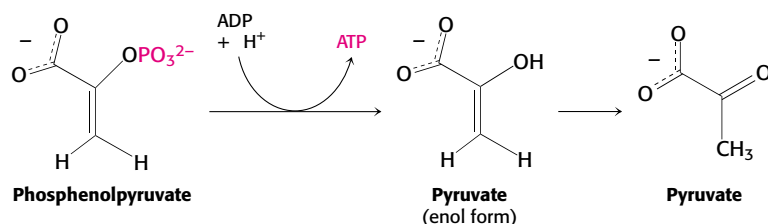
In the next reaction, an *enol* is formed by the dehydration of 2-phosphoglycerate. *Enolase* catalyzes the formation of *phosphoenolpyruvate* (PEP). This dehydration markedly elevates the transfer potential of the phosphoryl group. An *enol phosphate* has a high phosphoryl-transfer potential,

TABLE 16.3 Reactions of glycolysis

Step	Reaction	Enzyme
1	Glucose + ATP \rightarrow glucose 6-phosphate + ADP + H ⁺	Hexokinase
2	Glucose 6-phosphate \rightleftharpoons fructose 6-phosphate	Phosphoglucose isomerase
3	Fructose 6-phosphate + ATP \rightarrow fructose 1,6-bisphosphate + ADP + H ⁺	Phosphofruktokinase
4	Fructose 1,6-bisphosphate \rightleftharpoons dihydroxyacetonephosphate + glyceraldehyde 3-phosphate	Aldolase
5	Dihydroxyacetone phosphate \rightleftharpoons glyceraldehyde 3-phosphate	Triose phosphate isomerase
6	Glyceraldehyde 3-phosphate + P _i + NAD ⁺ \rightleftharpoons 1,3-bisphosphoglycerate + NADH + H ⁺	Glyceraldehyde 3-phosphate dehydrogenase
7	1,3-Bisphosphoglycerate + ADP \rightleftharpoons 3-phosphoglycerate + ATP	Phosphoglycerate kinase
8	3-Phosphoglycerate \rightleftharpoons 2-phosphoglycerate	Phosphoglycerate mutase
9	2-Phosphoglycerate \rightleftharpoons phosphoenolpyruvate + H ₂ O	Enolase
10	Phosphoenolpyruvate + ADP + H ⁺ \rightarrow pyruvate + ATP	Pyruvate kinase

Note: ΔG , the actual free-energy change, has been calculated from ΔG° and known concentrations of reactants under typical physiologic conditions. Glycolysis can proceed only if the ΔG values of all reactions are negative. The small positive ΔG values of three of the

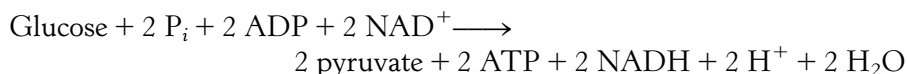
whereas the phosphate ester, such as 2-phosphoglycerate, of an ordinary alcohol has a low one. The $\Delta G^{\circ'}$ of the hydrolysis of a phosphate ester of an ordinary alcohol is -3 kcal mol^{-1} (-13 kJ mol^{-1}), whereas that of phosphoenolpyruvate is $-14.8 \text{ kcal mol}^{-1}$ (-62 kJ mol^{-1}). Why does phosphoenolpyruvate have such a high phosphoryl-transfer potential? The phosphoryl group traps the molecule in its unstable enol form. When the phosphoryl group has been donated to ATP, the enol undergoes a conversion into the more stable ketone—namely, pyruvate.



Thus, the high phosphoryl-transfer potential of phosphoenolpyruvate arises primarily from the large driving force of the subsequent enol–ketone conversion. Hence, pyruvate is formed, and ATP is generated concomitantly. The virtually irreversible transfer of a phosphoryl group from phosphoenolpyruvate to ADP is catalyzed by *pyruvate kinase*. Because the molecules of ATP used in forming fructose 1,6-bisphosphate have already been regenerated, the two molecules of ATP generated from phosphoenolpyruvate are “profit.”

16.1.8 Energy Yield in the Conversion of Glucose into Pyruvate

The net reaction in the transformation of glucose into pyruvate is:



Thus, two molecules of ATP are generated in the conversion of glucose into two molecules of pyruvate. The reactions of glycolysis are summarized in Table 16.3.

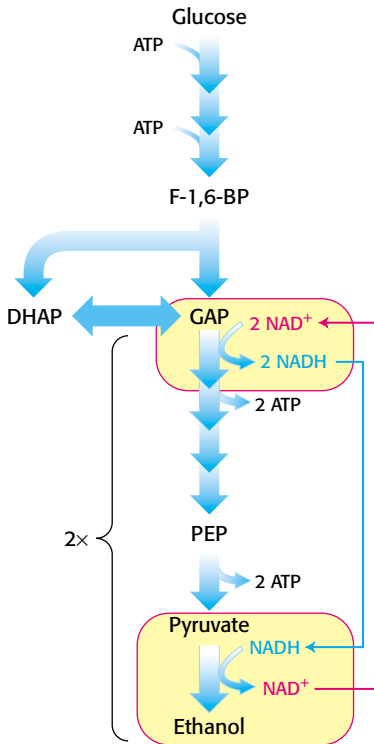
Reaction type	$\Delta G^{\circ'}$ in kcal mol ⁻¹ (kJ mol ⁻¹)	ΔG in kcal mol ⁻¹ (kJ mol ⁻¹)
Phosphoryl transfer	-4.0 (-16.7)	-8.0 (-33.5)
Isomerization	+0.4 (+1.7)	-0.6 (-2.5)
Phosphoryl transfer	-3.4 (-14.2)	-5.3 (-22.2)
Aldol cleavage	+5.7 (+23.8)	-0.3 (-1.3)
Isomerization	+1.8 (+7.5)	+0.6 (+2.5)
Phosphorylation coupled to oxidation	+1.5 (+6.3)	+0.6 (+2.5)
Phosphoryl transfer	-4.5 (-18.8)	+0.3 (+1.3)
Phosphoryl shift	+1.1 (+4.6)	+0.2 (+0.8)
Dehydration	+0.4 (+1.7)	-0.8 (-3.3)
Phosphoryl transfer	-7.5 (-31.4)	-4.0 (-16.7)

above reactions indicate that the concentrations of metabolites in vivo in cells undergoing glycolysis are not precisely known.

Note that the energy released in the anaerobic conversion of glucose into two molecules of pyruvate is $-47 \text{ kcal mol}^{-1}$ (-197 kJ mol^{-1}). We shall see in Chapters 18 and 19 how much more energy can be released from glucose in the presence of oxygen.



CONCEPTUAL INSIGHTS, Energetics of Glucose Metabolism. See the section on the energetics of glycolysis in the Conceptual Insights module for a graphical representation of free energy differences among glycolytic metabolites, and how these differences are used to drive ATP and NADH synthesis in coupled reactions.



Location of redox balance steps. The generation and consumption of NADH, located within the glycolytic pathway.

16.1.9 Maintaining Redox Balance: The Diverse Fates of Pyruvate

The conversion of glucose into two molecules of pyruvate has resulted in the net synthesis of ATP. However, an energy-converting pathway that stopped at pyruvate would not proceed for long, because redox balance has not been maintained. As we have seen, the activity of glyceraldehyde 3-phosphate dehydrogenase, in addition to generating a compound with high phosphoryl-transfer potential, of necessity leads to the reduction of NAD^+ to NADH. There are limited amounts of NAD^+ in the cell, which is derived from the vitamin *niacin*, a dietary requirement in human beings. Consequently, NAD^+ must be regenerated for glycolysis to proceed. Thus, the final process in the pathway is the regeneration of NAD^+ through the metabolism of pyruvate. The sequence of reactions from glucose to pyruvate is similar in most organisms and most types of cells. In contrast, the fate of pyruvate is variable. Three reactions of pyruvate are of prime importance: conversion into ethanol, lactic acid, or carbon dioxide (Figure 16.10).

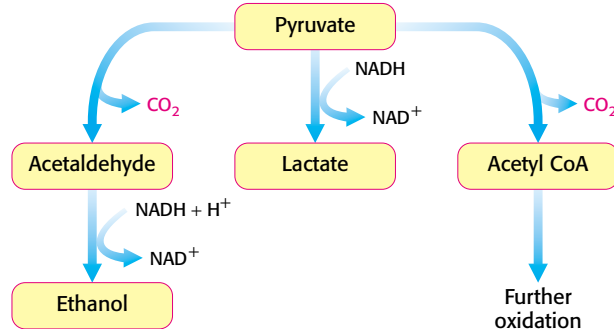
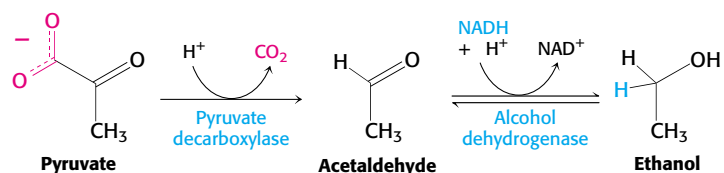


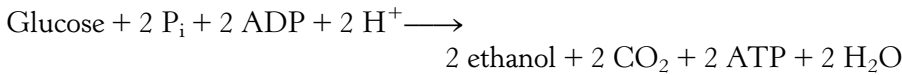
FIGURE 16.10 Diverse fates of pyruvate. Ethanol and lactate can be formed by reactions involving NADH. Alternatively, a two-carbon unit from pyruvate can be coupled to coenzyme A (see Section 18.x.x) to form acetyl CoA.

1. *Ethanol* is formed from pyruvate in yeast and several other microorganisms. The first step is the decarboxylation of pyruvate. This reaction is catalyzed by *pyruvate decarboxylase*, which requires the coenzyme thiamine pyrophosphate. This coenzyme, derived from the vitamin thiamine (B_1), also participates in reactions catalyzed by other enzymes (Section 17.x.x). The second step is the reduction of acetaldehyde to ethanol by NADH, in a reaction catalyzed by *alcohol dehydrogenase*. This process regenerates NAD^+ .



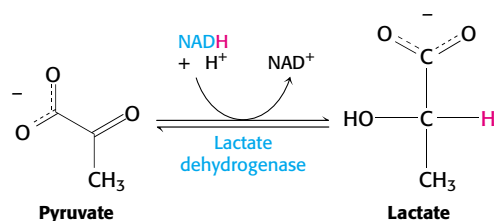
The active site of alcohol dehydrogenase contains a zinc ion that is coordinated to the sulfur atoms of two cysteine residues and a nitrogen atom of histidine (Figure 16.11). This zinc ion polarizes the carbonyl group of the substrate to favor the transfer of a hydride from NADH.

The conversion of glucose into ethanol is an example of *alcoholic fermentation*. The net result of this anaerobic process is:

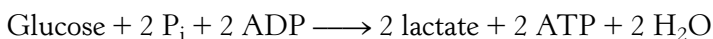


Note that NAD^+ and NADH do not appear in this equation, even though they are crucial for the overall process. NADH generated by the oxidation of glyceraldehyde 3-phosphate is consumed in the reduction of acetaldehyde to ethanol. Thus, *there is no net oxidation–reduction in the conversion of glucose into ethanol* (Figure 16.12). The ethanol formed in alcoholic fermentation provides a key ingredient for brewing and winemaking.

2. *Lactate* is formed from pyruvate in a variety of microorganisms in a process called lactic acid fermentation. The reaction also takes place in the cells of higher organisms when the amount of oxygen is limiting, as in muscle during intense activity. The reduction of pyruvate by NADH to form lactate is catalyzed by *lactate dehydrogenase*.



The overall reaction in the conversion of glucose into lactate is:



As in alcoholic fermentation, there is no net oxidation–reduction. The NADH formed in the oxidation of glyceraldehyde 3-phosphate is consumed in the reduction of pyruvate. *The regeneration of NAD^+ in the reduction of pyruvate to lactate or ethanol sustains the continued operation of glycolysis under anaerobic conditions.*

3. Only a fraction of the energy of glucose is released in its anaerobic conversion into ethanol or lactate. Much more energy can be extracted aerobically by means of the citric acid cycle and the electron-transport chain. The entry point to this oxidative pathway is *acetyl coenzyme A* (acetyl CoA), which is formed inside mitochondria by the oxidative decarboxylation of pyruvate.

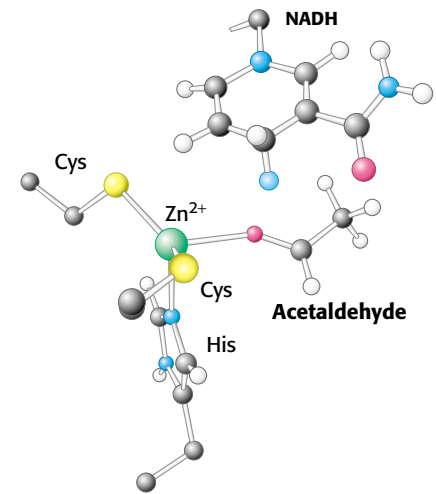
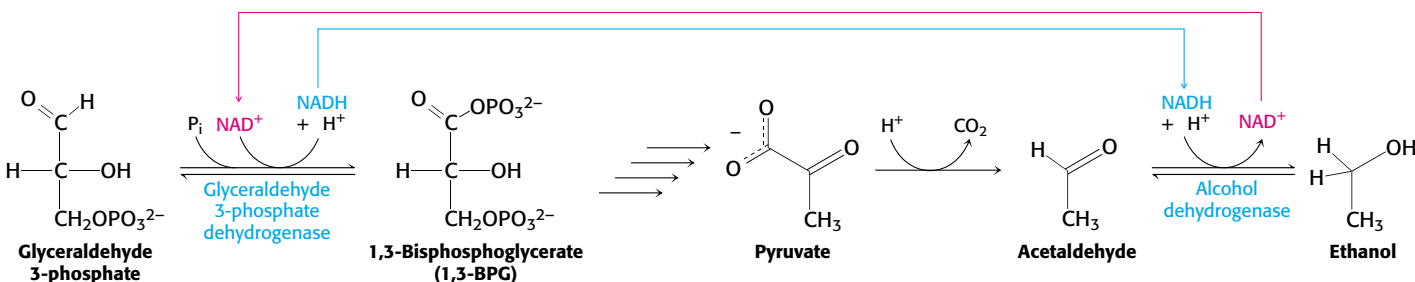
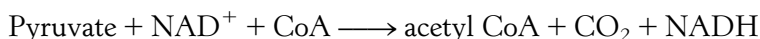


FIGURE 16.11 Active site of alcohol dehydrogenase. The active site contains a zinc ion bound to two cysteine residues and one histidine residue. The zinc ion binds the acetaldehyde substrate through its oxygen atom, polarizing it so that it more easily accepts a hydride (light blue) from NADH . Only the nicotinamide ring of NADH is shown.

FIGURE 16.12 Maintaining redox balance. The NADH produced by the glyceraldehyde 3-phosphate dehydrogenase reaction must be reoxidized to NAD^+ for the glycolytic pathway to continue. In alcoholic fermentation, alcohol dehydrogenase oxidizes NADH and generates ethanol. In lactic acid fermentation (not shown), lactate dehydrogenase oxidizes NADH while generating lactic acid.

This reaction, which is catalyzed by the pyruvate dehydrogenase complex, will be discussed in detail in Chapter 18. The NAD^+ required for this reaction and for the oxidation of glyceraldehyde 3-phosphate is regenerated when NADH ultimately transfers its electrons to O_2 through the electron-transport chain in mitochondria.

16.1.10 The Binding Site for NAD^+ Is Similar in Many Dehydrogenases



Although the enzymes taking part in glycolysis and the subsequent conversion of pyruvate are structurally diverse, the three dehydrogenases—glyceraldehyde 3-phosphate dehydrogenase, alcohol dehydrogenase, and lactate dehydrogenase—have in common a domain for NAD^+ binding (Figure 16.13). This nucleotide-binding region is made up of four α helices and a sheet of six parallel β strands. Moreover, in all cases, the bound NAD^+ displays nearly the same conformation. This common structural domain, one of the first recurring structural domains to be discovered, is often called a *Rossmann fold* after Michael Rossmann, who first recognized it. This fold likely represents a primordial dinucleotide-binding domain that recurs in the dehydrogenases of glycolysis and other enzymes because of their descent from a common ancestor.

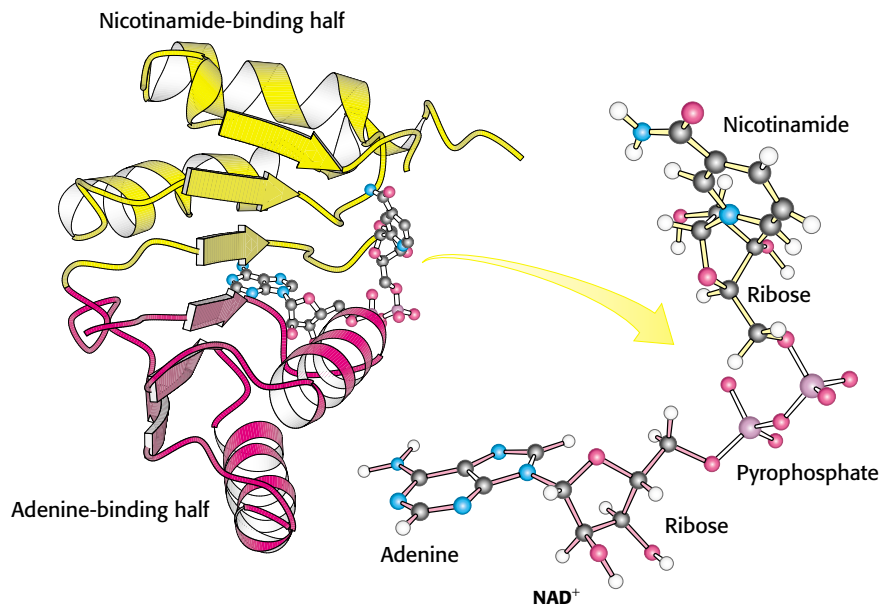


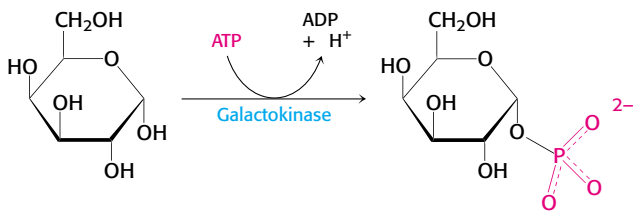
FIGURE 16.13 NAD^+ -binding region in dehydrogenases. The nicotinamide-binding half (yellow) is structurally similar to the adenine-binding half (red). The two halves together form a structural motif called a Rossmann fold. The NAD^+ molecule binds in an extended conformation.

16.1.11 The Entry of Fructose and Galactose into Glycolysis

Although glucose is the most widely used monosaccharide, others also are important fuels. Let us consider how two abundant sugars—fructose and galactose—can be funneled into the glycolytic pathway (Figure 16.14). Much of the ingested fructose is metabolized by the liver, using the *fructose 1-phosphate pathway* (Figure 16.15). The first step is the phosphorylation of fructose to fructose 1-phosphate by *fructokinase*. Fructose 1-phosphate is then split into *glyceraldehyde* and *dihydroxyacetone phosphate*, an intermediate in glycolysis. This aldol cleavage is catalyzed by a specific *fructose 1-phosphate aldolase*. Glyceraldehyde is then phosphorylated to *glyceraldehyde 3-phosphate*, a glycolytic intermediate, by *triose kinase*. Alternatively, fructose can be phosphorylated to fructose 6-phosphate by *hexokinase*. However, the affinity of hexokinase for glucose is 20 times as great as it is for fructose. Little fructose 6-phosphate is formed in the liver because glucose

is so much more abundant in this organ. Moreover, glucose, as the preferred fuel, is also trapped in the muscle by the hexokinase reaction. Because liver and muscle phosphorylate glucose rather than fructose, adipose tissue is exposed to more fructose than glucose. Hence, the formation of fructose 6-phosphate is not competitively inhibited to a biologically significant extent, and most of the fructose in adipose tissue is metabolized through fructose 6-phosphate.

There are no catabolic pathways to metabolize *galactose*, so the strategy is to convert galactose into a metabolite of glucose. *Galactose* is converted into *glucose 6-phosphate* in four steps. The first reaction in the *galactose-glucose interconversion pathway* is the phosphorylation of galactose to galactose 1-phosphate by *galactokinase*.



Galactose 1-phosphate then acquires a uridyl group from uridine diphosphate glucose (UDP-glucose), an intermediate in the synthesis of glycosidic linkages (Section 21.x.x).

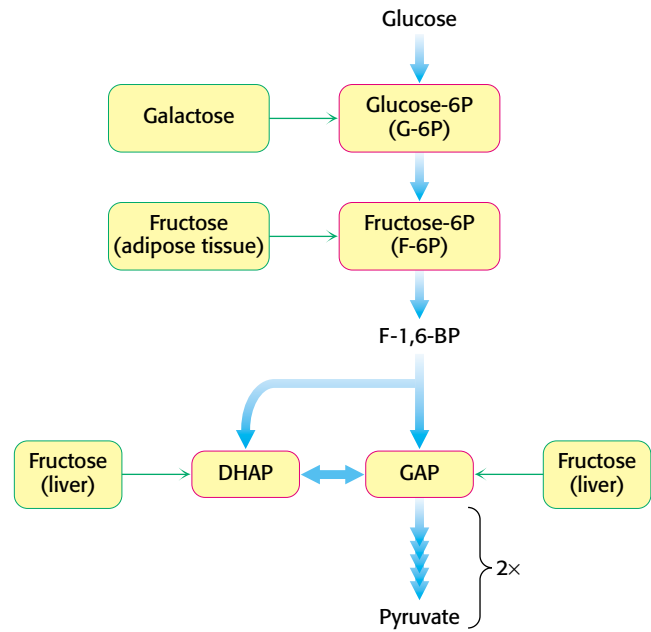
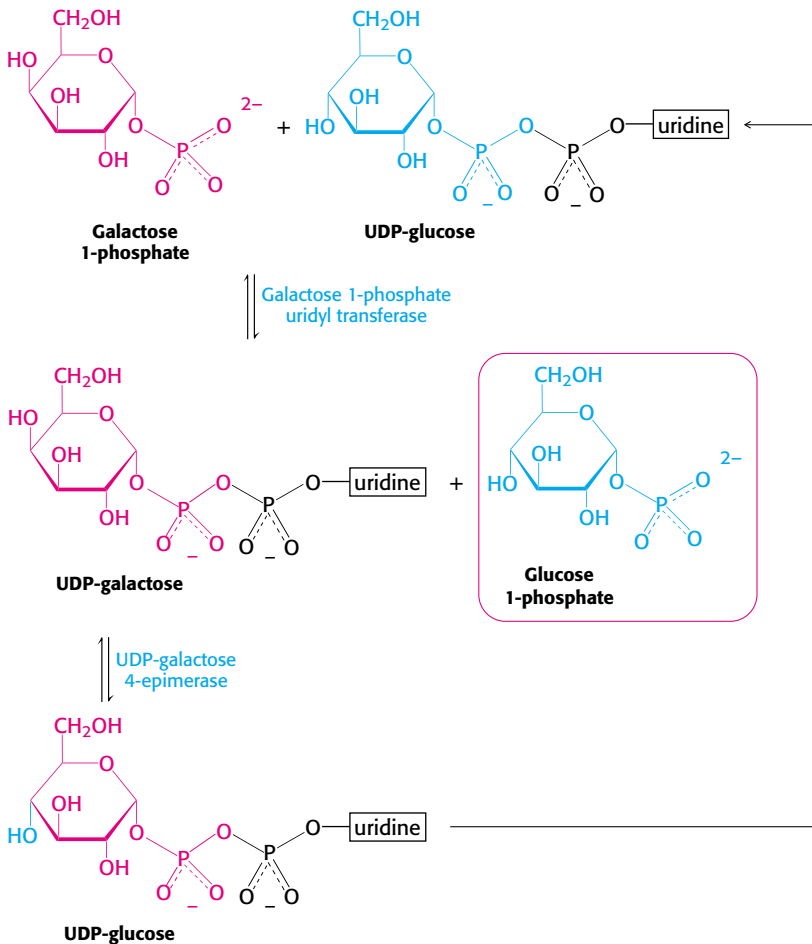


FIGURE 16.14 Entry points in glycolysis for galactose and fructose.

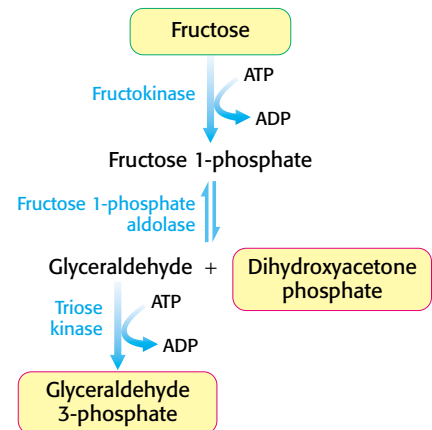
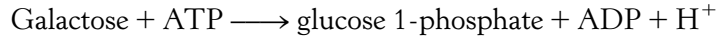


FIGURE 16.15 Fructose metabolism. Fructose enters the glycolytic pathway in the liver through the fructose 1-phosphate pathway.

The products of this reaction, which is catalyzed by *galactose 1-phosphate uridyl transferase*, are UDP-galactose and glucose 1-phosphate. The galactose moiety of UDP-galactose is then epimerized to glucose. The configuration of the hydroxyl group at carbon 4 is inverted by *UDP-galactose 4-epimerase*.

The sum of the reactions catalyzed by galactokinase, the transferase, and the epimerase is:



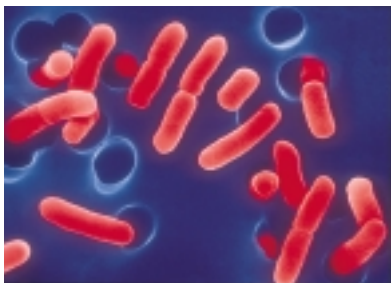
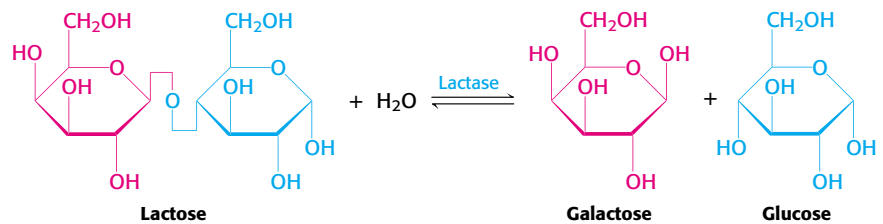
Note that UDP-glucose is not consumed in the conversion of galactose into glucose, because it is regenerated from UDP-galactose by the epimerase. This reaction is reversible, and the product of the reverse direction also is important. *The conversion of UDP-glucose into UDP-galactose is essential for the synthesis of galactosyl residues in complex polysaccharides and glycoproteins if the amount of galactose in the diet is inadequate to meet these needs.*

Finally, glucose 1-phosphate, formed from galactose, is isomerized to glucose 6-phosphate by *phosphoglucomutase*. We shall return to this reaction when we consider the synthesis and degradation of glycogen, which proceeds through glucose 1-phosphate, in Chapter 21.

16.1.12 Many Adults Are Intolerant of Milk Because They Are Deficient in Lactase



Many adults are unable to metabolize the milk sugar lactose and experience gastrointestinal disturbances if they drink milk. *Lactose intolerance*, or hypolactasia, is most commonly caused by a deficiency of the enzyme lactase, which cleaves lactose into glucose and galactose.



Scanning electron micrograph of *Lactobacillus*. The anaerobic bacteria *Lactobacillus* is shown here (artificially colored) at a magnification of 22,245 \times . As suggested by its name, this genus of bacteria ferments glucose into lactic acid, and is widely used in the food industry. *Lactobacillus* is also a component of the normal human bacterial flora of the urogenital tract where, because of its ability to generate an acidic environment, it prevents growth of harmful bacteria. [Dr. Dennis Kunkel/PhotoTake.]

“Deficiency” is not quite the appropriate term, because a decrease in lactase is normal during development in all mammals. As children are weaned and milk becomes less prominent in their diets, lactase activity normally declines to about 5 to 10% of the level at birth. This decrease is not as pronounced with some groups of people, most notably Northern Europeans, and people from these groups can continue to ingest milk without gastrointestinal difficulties. With the appearance of milk-producing domesticated animals, a human being with a genetic alteration endowing high levels of lactase activity in adulthood would hypothetically have a selective advantage in being able to consume calories from the readily available milk.

What happens to the lactose in the intestine of a lactase-deficient person? The lactose is a good energy source for microorganisms in the colon, and they ferment it to lactic acid while also generating methane (CH_4) and hydrogen gas (H_2). The gas produced creates the uncomfortable feeling of gut distention and the annoying problem of flatulence. The lactic acid produced by the microorganisms is osmotically active and draws water into the intestine, as does any undigested lactose, resulting in diarrhea. If severe enough, the gas and diarrhea hinder the absorption of other nutrients such as fats and proteins. The simplest treatment is to avoid the consumption of products containing much lactose. Alternatively, the enzyme lactase can be ingested with milk products.

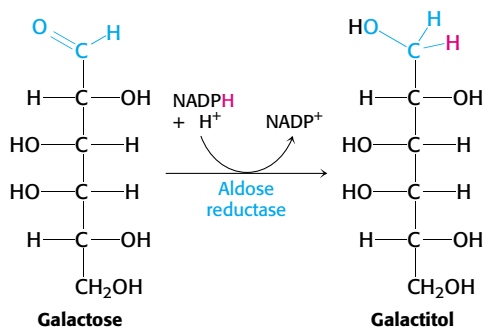
16.1.13 Galactose Is Highly Toxic If the Transferase Is Missing



Less common than lactose intolerance are disorders that interfere with the metabolism of galactose. The disruption of galactose metabolism is referred to as *galactosemia*. The most common form, called classic galactosemia, is an inherited deficiency in galactose 1-phosphate uridyl transferase activity. Afflicted infants fail to thrive. They vomit or have diarrhea after consuming milk, and enlargement of the liver and jaundice are common, sometimes progressing to cirrhosis. Cataracts will form, and lethargy and retarded mental development also are common. The blood-galactose level is markedly elevated, and galactose is found in the urine. The absence of the transferase in red blood cells is a definitive diagnostic criterion.

The most common treatment is to remove galactose (and lactose) from the diet. The enigma of galactosemia is that, although elimination of galactose from the diet prevents liver disease and cataract development, the majority of patients still suffer from central nervous system malfunction, most commonly a delayed acquisition of language skills. Females will also display ovarian failure.

Cataract formation is better understood. A cataract is the clouding of the normally clear lens of the eye. If the transferase is not active in the lens of the eye, the presence of aldose reductase causes the accumulating galactose to be reduced to galactitol.



Galactitol is osmotically active, and water will diffuse into the lens, instigating the formation of cataracts. In fact, there is a high incidence of cataract formation with age in populations that consume substantial amounts of milk into adulthood.

16.2 THE GLYCOLYTIC PATHWAY IS TIGHTLY CONTROLLED

The flux through the glycolytic pathway must be adjusted in response to conditions both inside and outside the cell. The rate of conversion of glucose into pyruvate is regulated to meet two major cellular needs: (1) the production of ATP, generated by the degradation of glucose, and (2) the provision of building blocks for synthetic reactions, such as the formation of fatty acids. *In metabolic pathways, enzymes catalyzing essentially irreversible reactions are potential sites of control.* In glycolysis, the reactions catalyzed by hexokinase, phosphofruktokinase, and pyruvate kinase are virtually irreversible; hence, these enzymes would be expected to have regulatory as well as catalytic roles. In fact, each of them serves as a control site. Their activities are regulated by the reversible binding of allosteric effectors or by covalent modification. In addition, the amounts of these important enzymes are varied by the regulation of transcription to meet changing metabolic

needs. The time required for reversible allosteric control, regulation by phosphorylation, and transcriptional control is typically in milliseconds, seconds, and hours, respectively.

16.2.1 Phosphofructokinase Is the Key Enzyme in the Control of Glycolysis

Phosphofructokinase is the most important control element in the mammalian glycolytic pathway (Figure 16.16). High levels of ATP allosterically inhibit the enzyme in the liver (a 340-kd tetramer), thus lowering its affinity for fructose 6-phosphate. A high concentration of ATP converts the hyperbolic binding curve of fructose 6-phosphate into a sigmoidal one (Figure 16.17). ATP elicits this effect by binding to a specific regulatory site that is distinct from the catalytic site. AMP reverses the inhibitory action of ATP, and so the activity of the enzyme increases when the ATP/AMP ratio is lowered. In other words, glycolysis is stimulated as the energy charge falls. A fall in pH also inhibits phosphofructokinase activity. The inhibition of phosphofructokinase by H^+ prevents excessive formation of lactic acid (Section 16.1.9) and a precipitous drop in blood pH (acidosis).

FIGURE 16.16 Structure of phosphofructokinase. Phosphofructokinase in the liver is a tetramer of four identical subunits. The positions of the catalytic and allosteric sites are indicated.

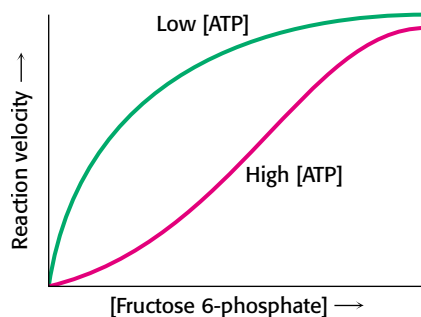
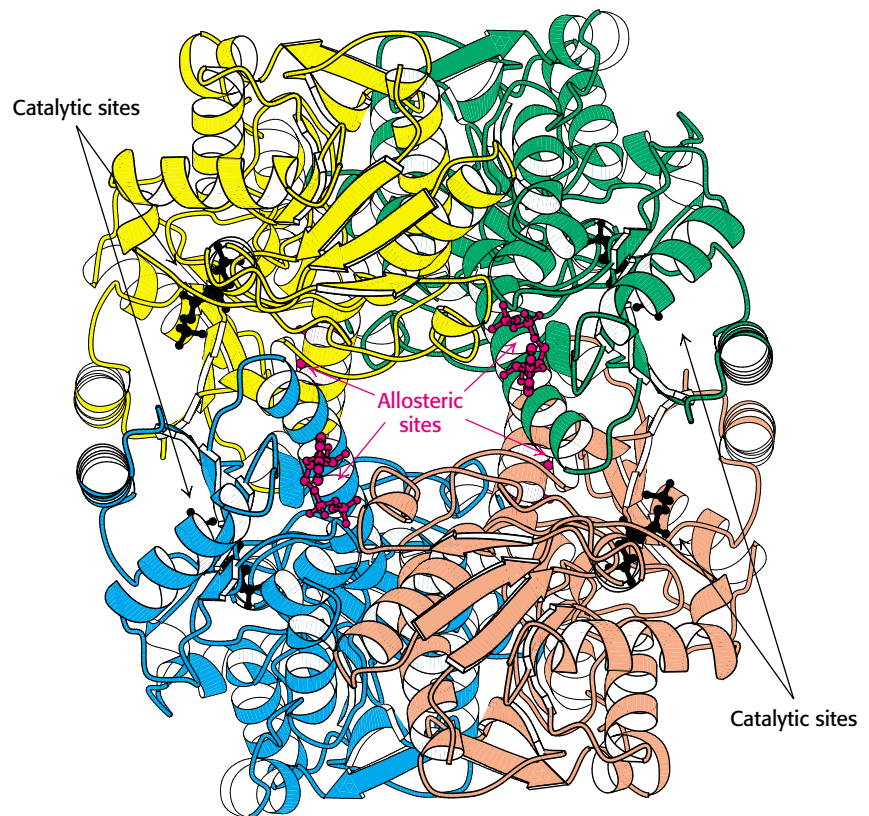


FIGURE 16.17 Allosteric regulation of phosphofructokinase. A high level of ATP inhibits the enzyme by decreasing its affinity for fructose 6-phosphate. AMP diminishes and citrate enhances the inhibitory effect of ATP.

Why is AMP and not ADP the positive regulator of phosphofructokinase? When ATP is being utilized rapidly, the enzyme *adenylate kinase* (Section 9.4) can form ATP from ADP by the following reaction:



Thus, some ATP is salvaged from ADP, and AMP becomes the signal for the low-energy state. Moreover, the use of AMP as an allosteric regulator

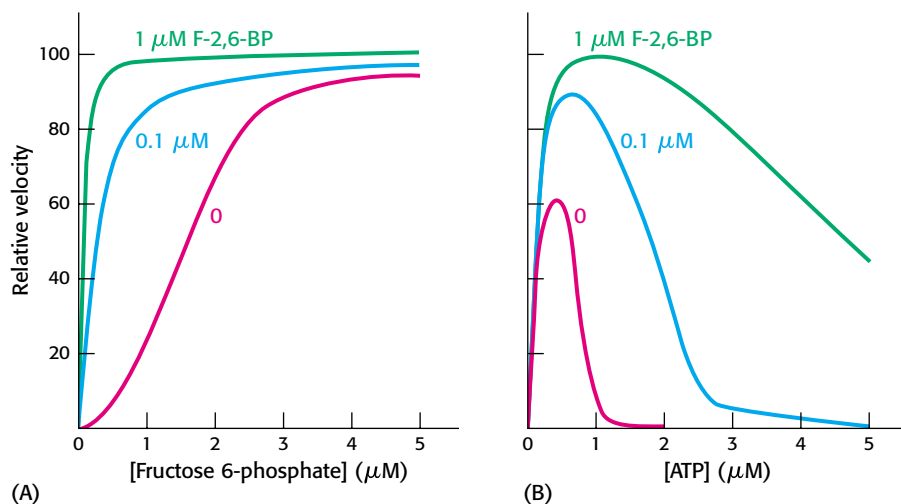
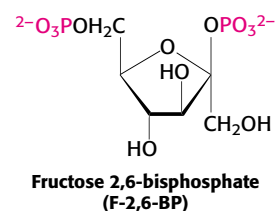


FIGURE 16.18 Activation of phosphofructokinase by fructose 2,6-bisphosphate. (A) The sigmoidal dependence of velocity on substrate concentration becomes hyperbolic in the presence of 1 μM fructose 2,6-bisphosphate. (B) ATP, acting as a substrate, initially stimulates the reaction. As the concentration of ATP increases, it acts as an allosteric inhibitor. The inhibitory effect of ATP is reversed by fructose 2,6-bisphosphate. [After H.-G. Hers and E. Van Schaftingen. *Proc. Natl. Acad. Sci.* 78(1981):2862.]

provides an especially sensitive control. We can understand why by considering, first, that the total adenylate pool ($[\text{ATP}]$, $[\text{ADP}]$, $[\text{AMP}]$) in a cell is constant over the short term and, second, that the concentration of ATP is greater than that of ADP and the concentration of ADP is, in turn, greater than that of AMP. Consequently, small-percentage changes in $[\text{ATP}]$ result in larger-percentage changes in the concentrations of the other adenylate nucleotides. This magnification of small changes in $[\text{ATP}]$ to larger changes in $[\text{AMP}]$ leads to tighter control by increasing the range of sensitivity of phosphofructokinase.

Glycolysis also furnishes carbon skeletons for biosyntheses, and so a signal indicating whether building blocks are abundant or scarce should also regulate phosphofructokinase. Indeed, *phosphofructokinase is inhibited by citrate*, an early intermediate in the citric acid cycle (Section 18.x.x). A high level of citrate means that biosynthetic precursors are abundant and additional glucose should not be degraded for this purpose. Citrate inhibits phosphofructokinase by enhancing the inhibitory effect of ATP.

In 1980, *fructose 2,6-bisphosphate* (F-2,6-BP) was identified as a potent activator of phosphofructokinase. Fructose 2,6-bisphosphate activates phosphofructokinase by increasing its affinity for fructose 6-phosphate and diminishing the inhibitory effect of ATP (Figure 16.18). In essence, *Fructose 2,6-bisphosphate is an allosteric activator that shifts the conformational equilibrium of this tetrameric enzyme from the T state to the R state.*



16.2.2 A Regulated Bifunctional Enzyme Synthesizes and Degrades Fructose 2,6-bisphosphate

How is the concentration of fructose 2,6-bisphosphate appropriately controlled? Two enzymes regulate the concentration of this important regulator of glycolysis by phosphorylating fructose 6-phosphate and dephosphorylating fructose 2,6-bisphosphate. Fructose 2,6-bisphosphate is formed in a reaction catalyzed by *phosphofructokinase 2* (PFK2), a different enzyme from phosphofructokinase. Fructose 2,6-bisphosphate is hydrolyzed to fructose 6-phosphate by a specific phosphatase, *fructose bisphosphatase 2* (FBPase2). The striking finding is that *both PFK2 and FBPase2 are present in a single 55-kd polypeptide chain* (Figure 16.19). This *bifunctional enzyme* contains an N-terminal *regulatory domain*, followed by a *kinase domain* and a *phosphatase domain*. PFK2 resembles adenylate kinase in having a P-loop NTPase domain (Sections 9.4.1 and 9.4.3), whereas FBPase2 resembles phosphoglycerate mutase (Section 16.1.7). Recall that the mutase

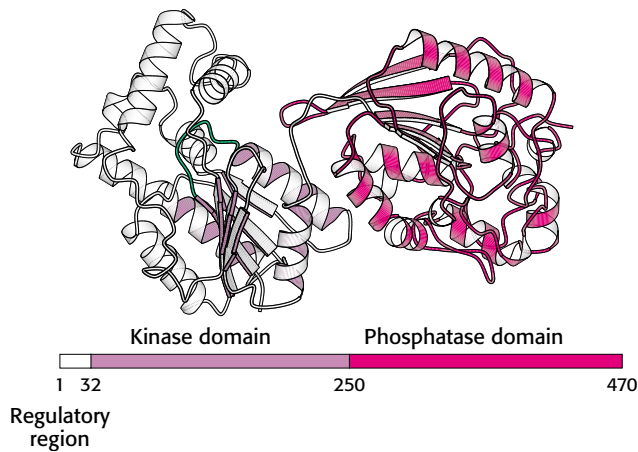
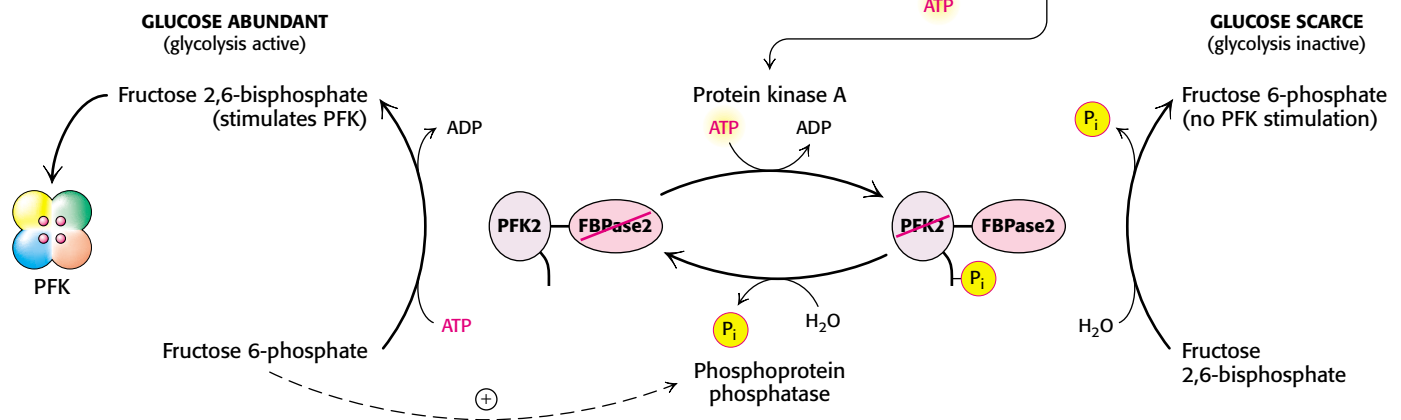


FIGURE 16.19 Domain structure of the bifunctional enzyme phosphofruktokinase 2. The kinase domain (purple) is fused to the phosphatase domain (red). The kinase domain is a P-loop NTP hydrolase domain, as indicated by the purple shading (Section 9.4.4). The bar represents the amino acid sequence of the enzyme.

FIGURE 16.20 Control of the synthesis and degradation of fructose 2,6-bisphosphate. A low blood-glucose level as signaled by glucagon leads to the phosphorylation of the bifunctional enzyme and hence to a lower level of fructose 2,6-bisphosphate, slowing glycolysis. High levels of fructose 6-phosphate accelerate the formation of fructose 2,6-bisphosphate by facilitating the dephosphorylation of the bifunctional enzyme.



is essentially a phosphatase. In the bifunctional enzyme, the phosphatase activity evolved to become specific for F-2,6-BP. The bifunctional enzyme itself probably arose by the fusion of genes encoding the kinase and phosphatase domains.

The bifunctional enzyme exists in five isozymic forms (isoforms) that differ in size and kinetics as well as immunological and regulatory properties. Recall that isoenzymes, or isozymes, have essentially the same architectural plan and catalytic properties but differ in how they are regulated. The L isoform, which predominates in the liver, and the M isoform, found in muscle are generated by alternative splicing (Section 28.x.x) of the transcription product of a single gene. The L isoform helps to maintain blood-glucose homeostasis. In the liver, the

concentration of fructose 6-phosphate rises when blood-glucose concentration is high, and the abundance of fructose 6-phosphate accelerates the synthesis of F-2,6-BP. Hence, an abundance of fructose 6-phosphate leads to a higher concentration of F-2,6-BP, which in turn stimulates phosphofruktokinase. Such a process is called *feedforward stimulation*. What controls whether PFK2 or FBPase2 dominates the bifunctional enzyme's activities in the liver? The activities of PFK2 and FBPase2 are reciprocally controlled by phosphorylation of a single serine residue. When glucose is scarce, a rise in the blood level of the hormone glucagon triggers a cyclic AMP cascade, through its 7TM receptor and $G_s\alpha$ (Section 15.x.x.), leading to the phosphorylation of this bifunctional enzyme by protein kinase A (Figure 16.20). This covalent modification activates FBPase2 and inhibits PFK2, lowering the level of F-2,6-BP. Thus, glucose metabolism by the liver is curtailed. Conversely, when glucose is abundant, the enzyme loses its attached phosphate group. This covalent modification activates PFK2 and inhibits FBPase2, raising the level of F-2,6-BP and accelerating glycolysis. This coordinated control is facilitated by the location of the kinase and phosphatase domains on the same polypeptide chain as the regulatory domain. We shall return to this elegant switch when we consider the integration of carbohydrate metabolism (Section 16.4).

16.2.3 Hexokinase and Pyruvate kinase

Also Set the Pace of Glycolysis

Phosphofructokinase is the most prominent regulatory enzyme in glycolysis, but it is not the only one. Hexokinase, the enzyme catalyzing the first step of glycolysis, is inhibited by its product, glucose 6-phosphate. High concentrations of this molecule signal that the cell no longer requires glucose for energy, for storage in the form of glycogen, or as a source of biosynthetic precursors, and the glucose will be left in the blood. For example, when phosphofructokinase is inactive, the concentration of fructose 6-phosphate rises. In turn, the level of glucose 6-phosphate rises because it is in equilibrium with fructose 6-phosphate. Hence, *the inhibition of phosphofructokinase leads to the inhibition of hexokinase*. However, the liver, in keeping with its role as monitor of blood-glucose levels, possesses a specialized isozyme of hexokinase called *glucokinase* that is not inhibited by glucose 6-phosphate. Glucokinase phosphorylates glucose only when it is abundant because it has about a 50-fold affinity for glucose than does hexokinase (5 mM, compared with 0.1 mM). The role of glucokinase is to provide glucose 6-phosphate for the synthesis of glycogen, a storage form of glucose (Section 21.x.x), and for the formation of fatty acids (Section 22.x.x). The low glucose affinity of glucokinase in the liver gives the brain and muscles first call on glucose when its supply is limited, whereas it ensures that glucose will not be wasted when it is abundant.

Why is phosphofructokinase rather than hexokinase the pacemaker of glycolysis? The reason becomes evident on noting that glucose 6-phosphate is not solely a glycolytic intermediate. Glucose 6-phosphate can also be converted into glycogen or it can be oxidized by the pentose phosphate pathway (Section 17.x.x) to form NADPH. The first irreversible reaction unique to the glycolytic pathway, the *committed step*, (Section 10.2), is the phosphorylation of fructose 6-phosphate to fructose 1,6-bisphosphate. Thus, it is highly appropriate for phosphofructokinase to be the primary control site in glycolysis. In general, *the enzyme catalyzing the committed step in a metabolic sequence is the most important control element in the pathway*.

Pyruvate kinase, the enzyme catalyzing the third irreversible step in glycolysis, controls the outflow from this pathway. This final step yields ATP and pyruvate, a central metabolic intermediate that can be oxidized further or used as a building block. Several isozymic forms of pyruvate kinase (a tetramer of 57-kd subunits) encoded by different genes are present in mammals: the L type predominates in liver, and the M type in muscle and brain. The L and M forms of pyruvate kinase have many properties in common. Both bind phosphoenolpyruvate cooperatively. Fructose 1,6-bisphosphate, the product of the preceding irreversible step in glycolysis, activates both isozymes to enable them to keep pace with the oncoming high flux of intermediates. ATP allosterically inhibits both the L and the M forms of pyruvate kinase to slow glycolysis when the energy charge is high. Finally, alanine (synthesized in one step from pyruvate, Section 24.x.x) also allosterically inhibits the pyruvate kinases—in this case, to signal that building blocks are abundant.

The isozymic forms differ in their susceptibility to covalent modification. The catalytic properties of the L form—but not of the M form—are also controlled by reversible phosphorylation (Figure 16.21). When the blood-glucose level is low, the glucagon-triggered cyclic AMP cascade (Section 15.x.x) leads to the phosphorylation of pyruvate kinase, which diminishes its activity. *These hormone-triggered phosphorylations, like that of the bifunctional enzyme controlling the levels of fructose 2,6-bisphosphate, prevent the liver from consuming glucose when it is more urgently needed by brain*

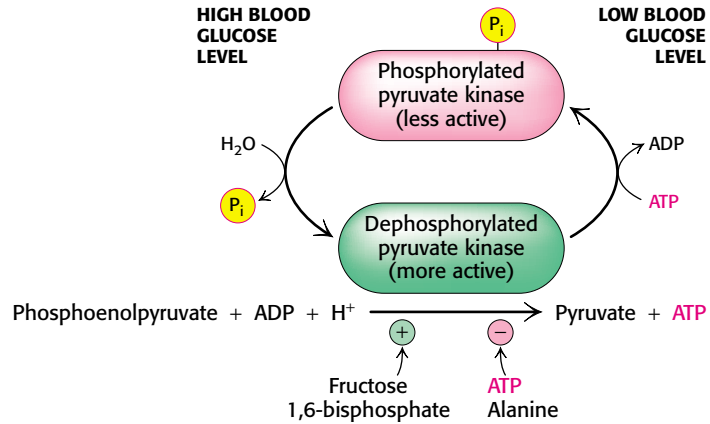


FIGURE 16.21 Control of the catalytic activity of pyruvate kinase. Pyruvate kinase is regulated by allosteric effectors and covalent modification.

and muscle (Section 30.x.x). We see here a clear-cut example of how isoenzymes contribute to the metabolic diversity of different organs. We will return to the control of glycolysis after considering gluconeogenesis.

16.2.4 A Family of Transporters Enables Glucose to Enter and Leave Animal Cells

Several glucose transporters mediate the thermodynamically downhill movement of glucose across the plasma membranes of animal cells. Each member of this protein family, named GLUT1 to GLUT5, consists of a single polypeptide chain about 500 residues long (Table 16.4). The common structural theme is the presence of 12 transmembrane segments (Figure 16.22).

The members of this family have distinctive roles:

1. GLUT1 and GLUT3, present in nearly all mammalian cells, are responsible for basal glucose uptake. Their K_M value for glucose is about 1 mM, significantly less than the normal serum-glucose level, which typically ranges from 4 mM to 8 mM. Hence, GLUT1 and GLUT3 continually transport glucose into cells at an essentially constant rate.
2. GLUT2, present in liver and pancreatic β cells, is distinctive in having a very high K_M value for glucose (15–20 mM). Hence, glucose enters these tissues at a biologically significant rate only when there is much glucose in the blood. The pancreas can thereby sense the glucose level and accordingly adjust the rate of insulin secretion. Insulin signals the need to remove glucose from the blood for storage as glycogen or conversion into fat (Section 30.x.x). The high K_M value of GLUT2 also ensures that glucose rapidly enters liver cells only in times of plenty.

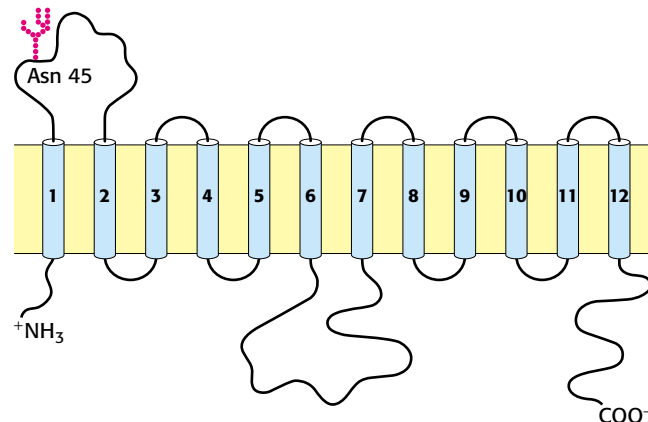


FIGURE 16.22 Model of a mammalian glucose transporter. The hydrophobicity profile of the protein indicates 12 transmembrane α helices. [From M. Muekler, C. Caruso, S. A. Baldwin, M. Panico, M. Blench, H. R. Morris, W. J. Allard, G. E. Lienhard, and H. F. Lodish. *Science* 229(1985):941.]

TABLE 16.4 Family of glucose transporters

Name	Tissue location	K_m	Comments
GLUT1	All mammalian tissues	1 mM	Basal glucose uptake
GLUT2	Liver and pancreatic β cells	15–20 mM	In the pancreas, plays a role in regulation of insulin In the liver, removes excess glucose from the blood
GLUT3	All mammalian tissues	1 mM	Basal glucose uptake
GLUT4	Muscle and fat cells	5 mM	Amount in muscle plasma membrane increases with endurance training
GLUT5	Small intestine	—	Primarily a fructose transporter

3. GLUT4, which has a K_M value of 5 mM, transports glucose into muscle and fat cells. The presence of insulin, which signals the fed state, leads to a rapid increase in the number of GLUT4 transporters in the plasma membrane. Hence, insulin promotes the uptake of glucose by muscle and fat. The amount of this transporter present in muscle membranes increases in response to endurance exercise training.

4. GLUT5, present in the small intestine, functions primarily as a fructose transporter.



This family of transporters vividly illustrates how isoforms of a single protein can significantly shape the metabolic character of cells and contribute to their diversity and functional specialization. The transporters are members of a superfamily of transporters called the major facilitator (MF) superfamily. Members of this family transport sugars in organisms as diverse as *E. coli*, *Trypanosoma brucei* (a parasitic protozoan that causes sleeping sickness), and human beings. An elegant solution to the problem of fuel transport evolved early and has been tailored to meet the needs of different organisms and even different tissues.

16.2.5 Cancer and Glycolysis



It has been known for decades that tumors display enhanced rates of glucose uptake and glycolysis. We now know that these enhanced rates of glucose processing are not fundamental to the development of cancer, but we can ask what selective advantage they might confer on cancer cells.

Cancer cells grow more rapidly than the blood vessels to nourish them; thus, as solid tumors grow, they are unable to obtain oxygen efficiently. In other words, they begin to experience *hypoxia*. Under these conditions, glycolysis leading to lactic acid fermentation becomes the primary source of ATP. Glycolysis is made more efficient in hypoxic tumors by the action of a transcription factor, *hypoxia-inducible transcription factor* (HIF-1). In the absence of oxygen, HIF-1 increases the expression of most glycolytic enzymes and the glucose transporters GLUT1 and GLUT3 (Table 16.5). In

TABLE 16.5 Proteins in glucose metabolism encoded by genes regulated by hypoxia-inducible factor

GLUT1
GLUT3
Hexokinase
Phosphofructokinase
Aldolase
Glyceraldehyde 3-phosphate dehydrogenase
Phosphoglycerate kinase
Enolase
Pyruvate kinase
Lactate dehydrogenase

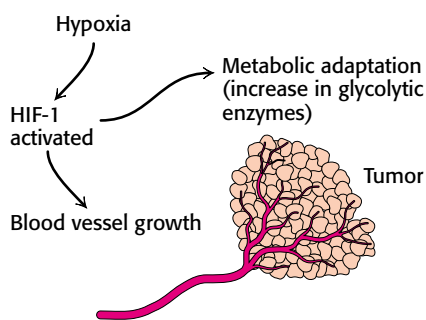


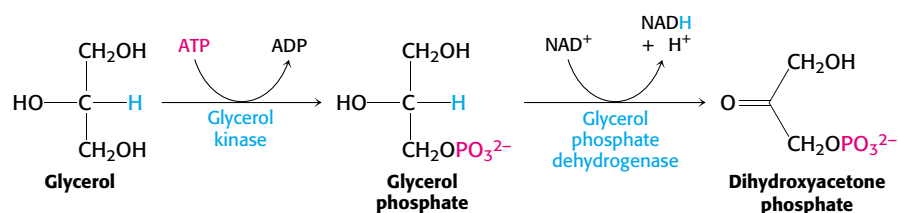
FIGURE 16.23 Alteration of gene expression in tumors due to hypoxia. The hypoxic conditions inside a tumor mass lead to the activation of the hypoxia-inducible transcription factor (HIF-1), which induces metabolic adaptation (increase in glycolytic enzymes) and activates angiogenic factors that stimulate the growth of new blood vessels. [Adapted from C. V. Dang and G. L. Semenza. *Trends Biochem. Sci.* 24(1999):68–72.]

fact, glucose uptake correlates with tumor aggressiveness and a poor prognosis. These adaptations by the cancer cells enable the tumor to survive until vascularization can occur. HIF-1 also stimulates the growth of new tumors by increasing the expression of signal molecules, such as vascular endothelial growth factor (VEGF), that facilitate the growth of blood vessels (Figure 16.23). Without such vascularization, the tumor would cease to grow and either die or remain harmlessly small. Efforts are underway to develop drugs that inhibit vascularization of tumors.

16.3 GLUCOSE CAN BE SYNTHESIZED FROM NONCARBOHYDRATE PRECURSORS

We now turn to the *synthesis of glucose from noncarbohydrate precursors*, a process called *gluconeogenesis*. This metabolic pathway is important because the brain depends on glucose as its primary fuel and red blood cells use only glucose as a fuel. The daily glucose requirement of the brain in a typical adult human being is about 120 g, which accounts for most of the 160 g of glucose needed daily by the whole body. The amount of glucose present in body fluids is about 20 g, and that readily available from glycogen, a storage form of glucose (Section 21.x.x), is approximately 190 g. Thus, the direct glucose reserves are sufficient to meet glucose needs for about a day. During a longer period of starvation, glucose must be formed from noncarbohydrate sources (Section 30.x.x).

The *gluconeogenic pathway converts pyruvate into glucose*. Noncarbohydrate precursors of glucose are first converted into pyruvate or enter the pathway at later intermediates such as oxaloacetate and dihydroxyacetone phosphate (Figure 16.24). The major noncarbohydrate precursors are *lactate*, *amino acids*, and *glycerol*. Lactate is formed by active skeletal muscle when the rate of glycolysis exceeds the rate of oxidative metabolism. Lactate is readily converted into pyruvate by the action of lactate dehydrogenase (Section 16.1.9). Amino acids are derived from proteins in the diet and, during starvation, from the breakdown of proteins in skeletal muscle (Section 30.x.x). The hydrolysis of triacylglycerols (Section 22.x.x) in fat cells yields glycerol and fatty acids. Glycerol is a precursor of glucose, but animals cannot convert fatty acids into glucose, for reasons that will be discussed later (Section 18.x.x). Glycerol may enter either the gluconeogenic or the glycolytic pathway at dihydroxyacetone phosphate.



The major site of gluconeogenesis is the *liver*, with a small amount also taking place in the *kidney*. Little gluconeogenesis takes place in the brain, skeletal muscle, or heart muscle. Rather, *gluconeogenesis in the liver and kidney helps to maintain the glucose level in the blood so that brain and muscle can extract sufficient glucose from it to meet their metabolic demands*.

16.3.1 Gluconeogenesis Is Not a Reversal of Glycolysis

In glycolysis, glucose is converted into pyruvate; in gluconeogenesis, pyruvate is converted into glucose. However, *gluconeogenesis is not a reversal of*

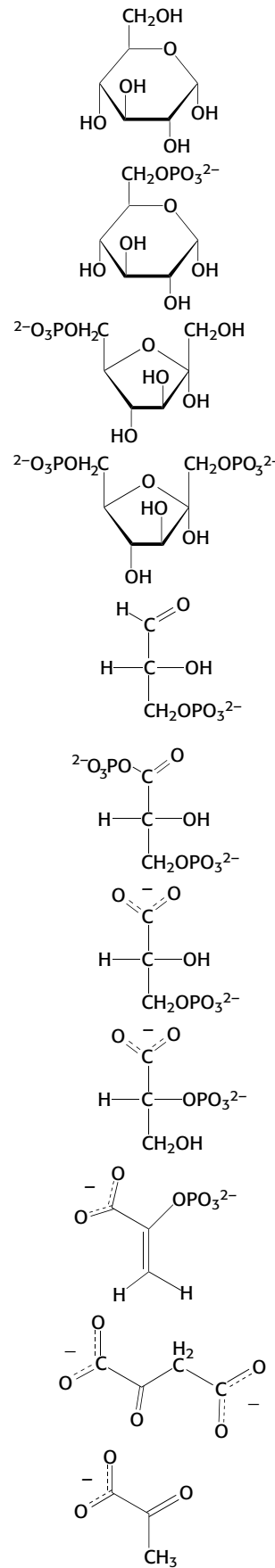
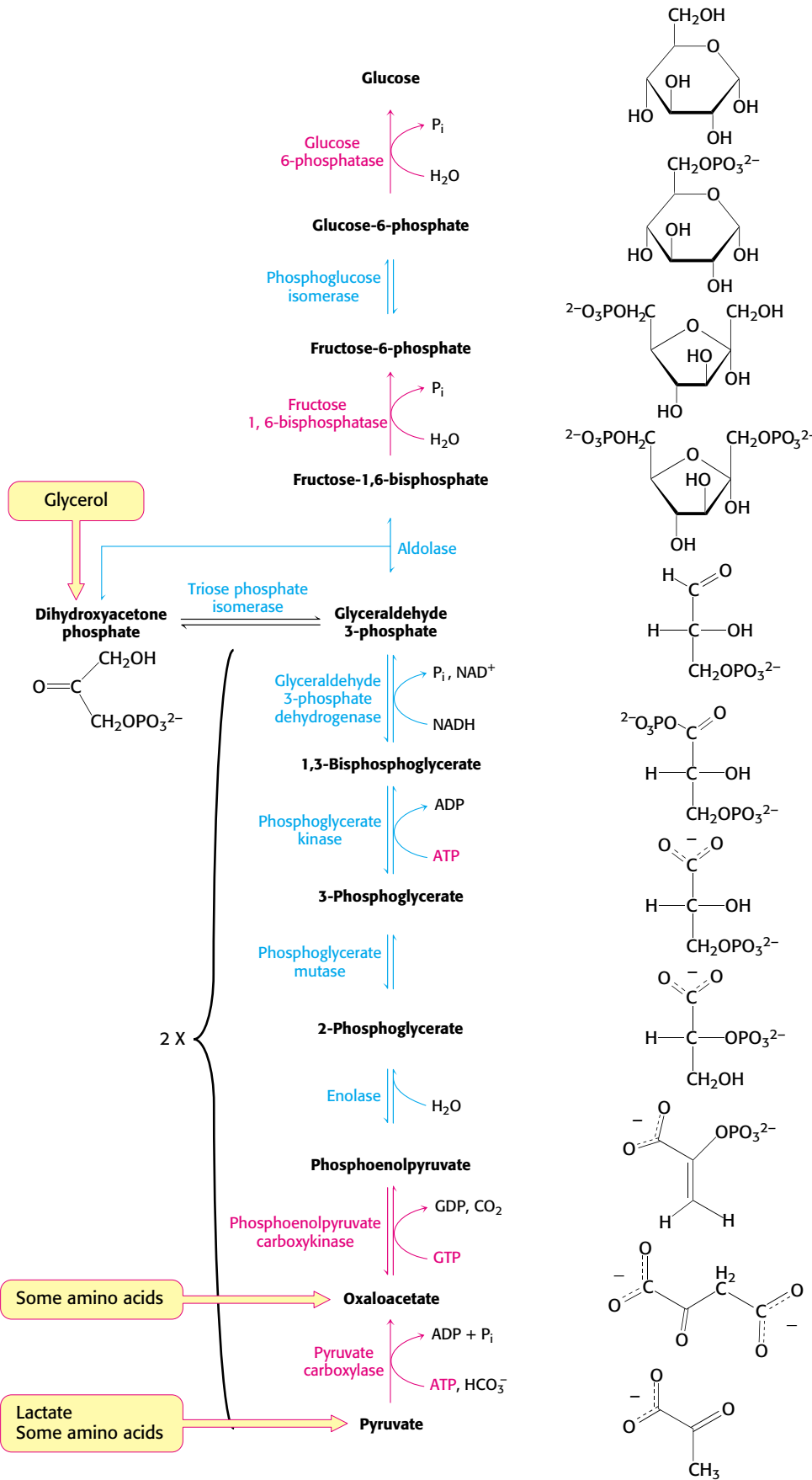
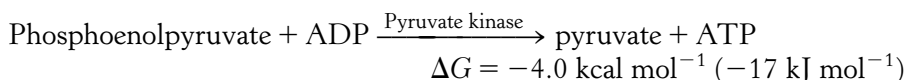
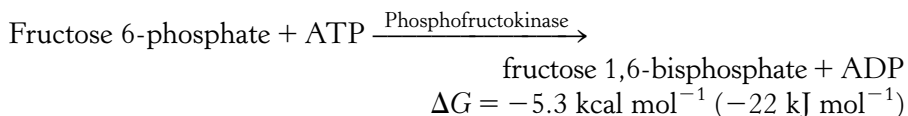
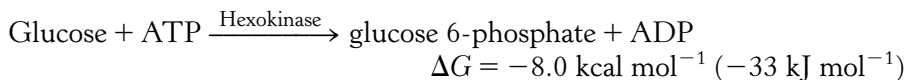


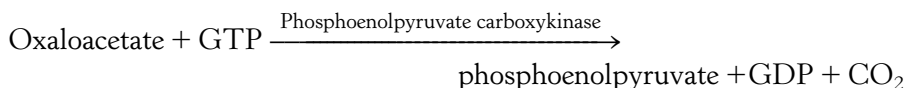
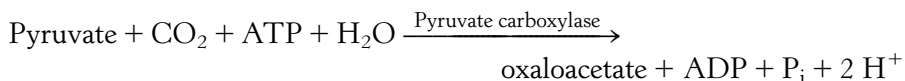
FIGURE 16.24 Pathway of gluconeogenesis. The distinctive reactions and enzymes of this pathway are shown in red. The other reactions are common to glycolysis. The enzymes for gluconeogenesis are located in the cytosol, except for pyruvate carboxylase (in the mitochondria) and glucose 6-phosphatase (membrane bound in the endoplasmic reticulum). The entry points for lactate, glycerol, and amino acids are shown.

glycolysis. Several reactions must differ because the equilibrium of glycolysis lies far on the side of pyruvate formation. The actual ΔG for the formation of pyruvate from glucose is about $-20 \text{ kcal mol}^{-1}$ (-84 kJ mol^{-1}) under typical cellular conditions. Most of the decrease in free energy in glycolysis takes place in the three essentially irreversible steps catalyzed by hexokinase, phosphofructokinase, and pyruvate kinase.



In gluconeogenesis, the following new steps bypass these virtually irreversible reactions of glycolysis:

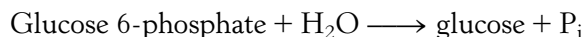
1. *Phosphoenolpyruvate is formed from pyruvate by way of oxaloacetate* through the action of pyruvate carboxylase and phosphoenolpyruvate carboxykinase.



2. *Fructose 6-phosphate is formed from fructose 1,6-bisphosphate by hydrolysis of the phosphate ester at carbon 1.* Fructose 1,6-bisphosphatase catalyzes this exergonic hydrolysis.



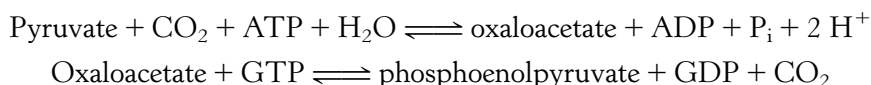
3. *Glucose is formed by hydrolysis of glucose 6-phosphate* in a reaction catalyzed by glucose 6-phosphatase.



We will examine each of these steps in turn.

16.3.2 The Conversion of Pyruvate into Phosphoenolpyruvate Begins with the Formation of Oxaloacetate

The first step in gluconeogenesis is the carboxylation of pyruvate to form oxaloacetate at the expense of a molecule of ATP. Then, oxaloacetate is decarboxylated and phosphorylated to yield phosphoenolpyruvate, at the expense of the high phosphoryl-transfer potential of GTP. Both of these reactions take place inside the mitochondria.



The first reaction is catalyzed by *pyruvate carboxylase* and the second by *phosphoenolpyruvate carboxykinase*. The sum of these reactions is:

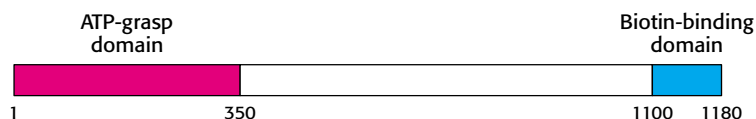
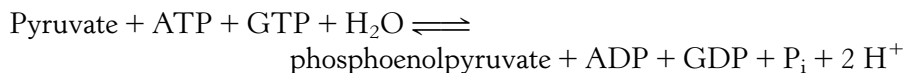
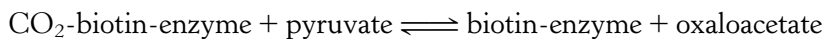
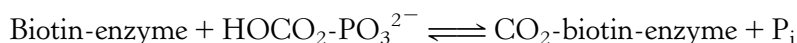


FIGURE 16.25 Domain structure of pyruvate carboxylase. The ATP-grasp domain activates HCO_3^- and transfers CO_2 to the biotin-binding domain. From there, the CO_2 is transferred to pyruvate generated in the central domain.

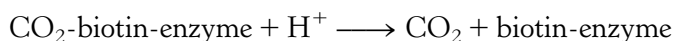


Pyruvate carboxylase is of special interest because of its structural, catalytic, and allosteric properties. The N-terminal 300 to 350 amino acids form an *ATP-grasp domain* (Figure 16.25), which is a widely used ATP-activating domain to be discussed in more detail when we investigate nucleotide biosynthesis (Section 25.x.x). The C-terminal 80 amino acids constitute a biotin-binding domain (Figure 16.26) that we will see again in fatty acid synthesis (Section 22.x.x). *Biotin* is a covalently attached prosthetic group, which serves as a *carrier of activated CO_2* . The carboxylate group of biotin is linked to the ϵ -amino group of a specific lysine residue by an amide bond (Figure 16.27). Note that biotin is attached to pyruvate carboxylase by a long, flexible chain.

The carboxylation of pyruvate takes place in three stages:



Recall that, in aqueous solutions, CO_2 exists as HCO_3^- with the aid of carbonic anhydrase (Section 9.2). The HCO_3^- is activated to carboxyphosphate. This activated CO_2 is subsequently bonded to the N-1 atom of the biotin ring to form the carboxybiotin-enzyme intermediate (see Figure 16.27). The CO_2 attached to the biotin is quite activated. The $\Delta G^{\circ'}$ for its cleavage



is $-4.7 \text{ kcal mol}^{-1}$ (-20 kJ mol^{-1}). This negative $\Delta G^{\circ'}$ indicates that carboxybiotin is able to transfer CO_2 to acceptors without the input of additional free energy.

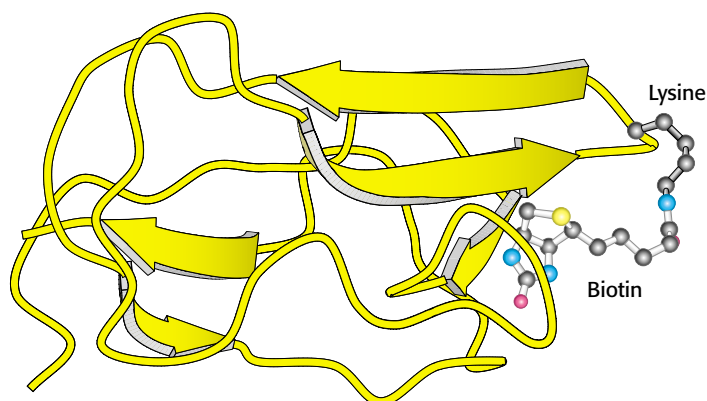


FIGURE 16.26 Biotin-binding domain of pyruvate carboxylase. This likely structure is based on the structure of the homologous domain from the enzyme acetyl CoA carboxylase (Section 22.x.x). The biotin is on a flexible tether, allowing it to move between the ATP-bicarbonate site and the pyruvate site.

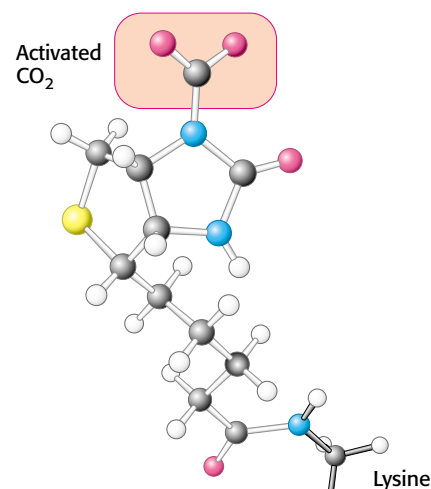


FIGURE 16.27 Structure of carboxybiotin.

The activated carboxyl group is then transferred from carboxybiotin to pyruvate to form oxaloacetate. The long, flexible link between biotin and the enzyme enables this prosthetic group to rotate from one active site of the enzyme (the ATP-bicarbonate site) to the other (the pyruvate site).

The first partial reaction of pyruvate carboxylase, the formation of carboxybiotin, depends on the presence of acetyl CoA. *Biotin is not carboxylated unless acetyl CoA is bound to the enzyme.* Acetyl CoA has no effect on the second partial reaction. The allosteric activation of pyruvate carboxylase by acetyl CoA is an important physiological control mechanism that will be discussed in Section 18.x.x.

16.3.3 Oxaloacetate Is Shuttled into the Cytosol and Converted into Phosphoenolpyruvate

Pyruvate carboxylase is a mitochondrial enzyme, whereas the other enzymes of gluconeogenesis are cytoplasmic. Oxaloacetate, the product of the pyruvate carboxylase reaction, is reduced to malate inside the mitochondrion for transport to the cytosol. The reduction is accomplished by an NADH-linked malate dehydrogenase. When malate has been transported across the mitochondrial membrane, it is reoxidized to oxaloacetate by an NAD⁺-linked malate dehydrogenase in the cytosol (Figure 16.28).

Finally, oxaloacetate is simultaneously *decarboxylated* and *phosphorylated* by phosphoenolpyruvate carboxykinase in the cytosol. The CO₂ that was added to pyruvate by pyruvate carboxylase comes off in this step. Recall that, in glycolysis, the presence of a phosphoryl group traps the unstable enol isomer of pyruvate as phosphoenolpyruvate (Section 16.1.7). In gluconeogenesis, the formation of the unstable enol is driven by decarboxylation—the oxidation of the carboxylic acid to CO₂—and trapped by the addition of a phosphate to carbon 2 from GTP. The two-step pathway for the formation of phosphoenolpyruvate from pyruvate has a ΔG° of +0.2 kcal mol⁻¹ (+0.13 kJ mol⁻¹) in contrast with +7.5 kcal mol⁻¹ (+31 kJ mol⁻¹) for the reaction catalyzed by pyruvate kinase. The much more favorable ΔG° for the two-step pathway results from the use of a molecule of ATP to add a molecule of CO₂ in the carboxylation step that can be removed to power the formation of phosphoenolpyruvate in the decarboxylation step. *Decarboxylations often drive reactions otherwise highly endergonic.* This metabolic motif is used in the citric acid cycle (Section 18.x.x), the pentose phosphate pathway (Section 17.x.x), and fatty acid synthesis (Section 22.x.x).

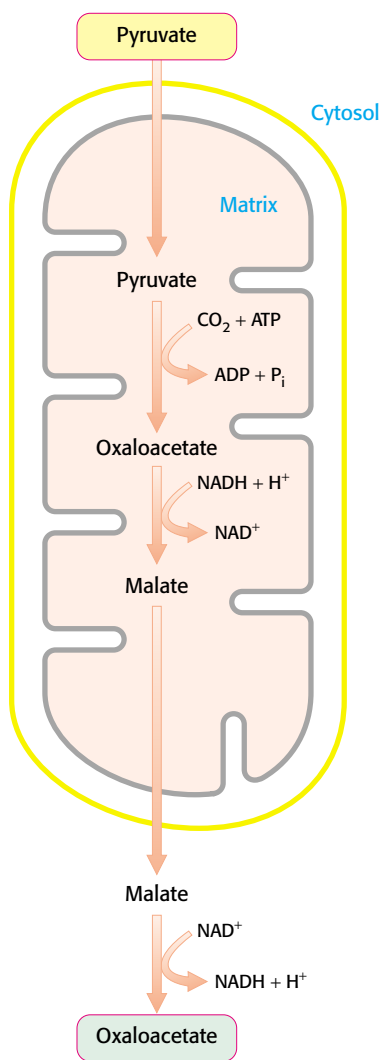
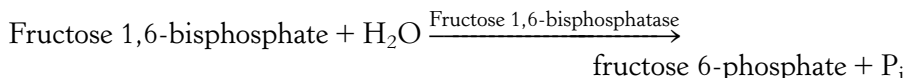


FIGURE 16.28 Compartmental cooperation. Oxaloacetate utilized in the cytosol for gluconeogenesis is formed in the mitochondrial matrix by carboxylation of pyruvate. Oxaloacetate leaves the mitochondrion by a specific transport system (not shown) in the form of malate, which is reoxidized to oxaloacetate in the cytosol.

16.3.4 The Conversion of Fructose 1,6-bisphosphate into Fructose 6-phosphate and P_i Is an Irreversible Step

On formation, phosphoenolpyruvate is metabolized by the enzymes of glycolysis but in the reverse direction. These reactions are near equilibrium under intracellular conditions; so, when conditions favor gluconeogenesis, the reverse reactions will take place until the next irreversible step is reached. This step is the hydrolysis of fructose 1,6-bisphosphate to fructose 6-phosphate and P_i.



The enzyme responsible for this step is fructose 1,6-bisphosphatase. Like its glycolytic counterpart, it is an allosteric enzyme that participates in the regulation of gluconeogenesis. We will return to its regulatory properties later in the chapter.

16.3.5 The Generation of Free Glucose Is an Important Control Point

The fructose 6-phosphate generated by fructose 1,6-bisphosphatase is readily converted into glucose 6-phosphate. In most tissues, gluconeogenesis ends here. Free glucose is not generated; rather, the glucose 6-phosphate is processed in some other fashion, notably to form glycogen. One advantage to ending gluconeogenesis at glucose 6-phosphate is that, unlike free glucose, the molecule cannot diffuse out of the cell. To keep glucose inside the cell, the generation of free glucose is controlled in two ways. First, the enzyme responsible for the conversion of glucose 6-phosphate into glucose, *glucose 6-phosphatase*, is regulated. Second, the enzyme is present only in tissues whose metabolic duty is to maintain blood-glucose homeostasis—tissues that release glucose into the blood. These tissues are the liver and to a lesser extent the kidney.

This final step in the generation of glucose does not take place in the cytosol. Rather, glucose 6-phosphate is transported into the lumen of the endoplasmic reticulum, where it is hydrolyzed to glucose by glucose 6-phosphatase, which is bound to the membrane (Figure 16.29). An associated Ca^{2+} -binding stabilizing protein is essential for phosphatase activity. Glucose and P_i are then shuttled back to the cytosol by a pair of transporters. The glucose transporter in the endoplasmic reticulum membrane is like those found in the plasma membrane (Section 16.2.4). It is striking that five proteins are needed to transform cytosolic glucose 6-phosphate into glucose.

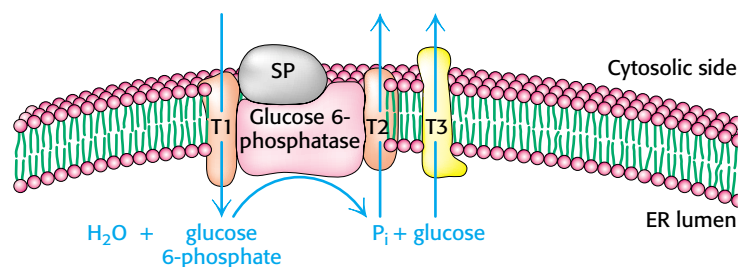
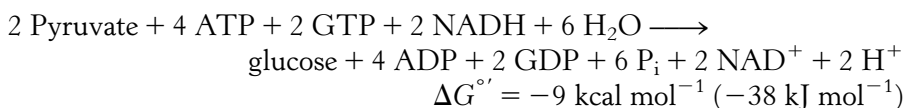


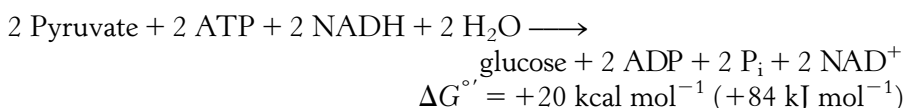
FIGURE 16.29 Generation of glucose from glucose 6-phosphate. Several endoplasmic reticulum (ER) proteins play a role in the generation of glucose from glucose 6-phosphate. T1 transports glucose 6-phosphate into the lumen of the ER, whereas T2 and T3 transport P_i and glucose, respectively, back into the cytosol. Glucose 6-phosphatase is stabilized by a Ca^{2+} -binding protein (SP). [After A. Buchell and I. D. Waddell. *Biochem. Biophys. Acta* 1092(1991):129.]

16.3.6 Six High-Energy Phosphate Bonds Are Spent in Synthesizing Glucose from Pyruvate

The stoichiometry of gluconeogenesis is:



In contrast, the stoichiometry for the reversal of glycolysis is:



Note that six nucleotide triphosphate molecules are hydrolyzed to synthesize glucose from pyruvate in gluconeogenesis, whereas only two molecules of ATP are generated in glycolysis in the conversion of glucose into pyruvate. Thus, the extra cost of gluconeogenesis is four high phosphoryl-transfer potential molecules per molecule of glucose synthesized from pyruvate. The four additional high phosphoryl-transfer potential molecules are needed to turn an energetically unfavorable process (the reversal of glycolysis, $\Delta G^{\circ'} = +20 \text{ kcal mol}^{-1}$ [$+84 \text{ kJ mol}^{-1}$]) into a favorable one

(gluconeogenesis, $\Delta G^{\circ\prime} = -9 \text{ kcal mol}^{-1} [-38 \text{ kJ mol}^{-1}]$). This is a clear example of the coupling of reactions: ATP hydrolysis is used to power an energetically unfavorable reaction.



CONCEPTUAL INSIGHTS, Energetics of Glucose Metabolism. See the section on gluconeogenesis in the Conceptual Insights module to review why and how gluconeogenesis must differ from the reversal of glycolysis.

16.4 GLUCONEOGENESIS AND GLYCOLYSIS ARE RECIPROCALLY REGULATED

Gluconeogenesis and glycolysis are coordinated so that within a cell one pathway is relatively inactive while the other is highly active. If both sets of reactions were highly active at the same time, the net result would be the hydrolysis of four nucleotide triphosphates (two ATP plus two GTP) per reaction cycle. Both glycolysis and gluconeogenesis are highly exergonic under cellular conditions, and so there is no thermodynamic barrier to such simultaneous activity. However, the *amounts* and *activities* of the distinctive enzymes of each pathway are controlled so that both pathways are not highly active at the same time. The rate of glycolysis is also determined by the concentration of glucose, and the rate of gluconeogenesis by the concentrations of lactate and other precursors of glucose.

The interconversion of fructose 6-phosphate and fructose 1,6-bisphosphate is stringently controlled (Figure 16.30). As discussed in Section 16.2.1, AMP stimulates phosphofructokinase, whereas ATP and citrate inhibit it. Fructose 1,6-bisphosphatase, on the other hand, is inhibited by AMP and activated by citrate. A high level of AMP indicates that the energy charge is low and signals the need for ATP generation. Conversely,

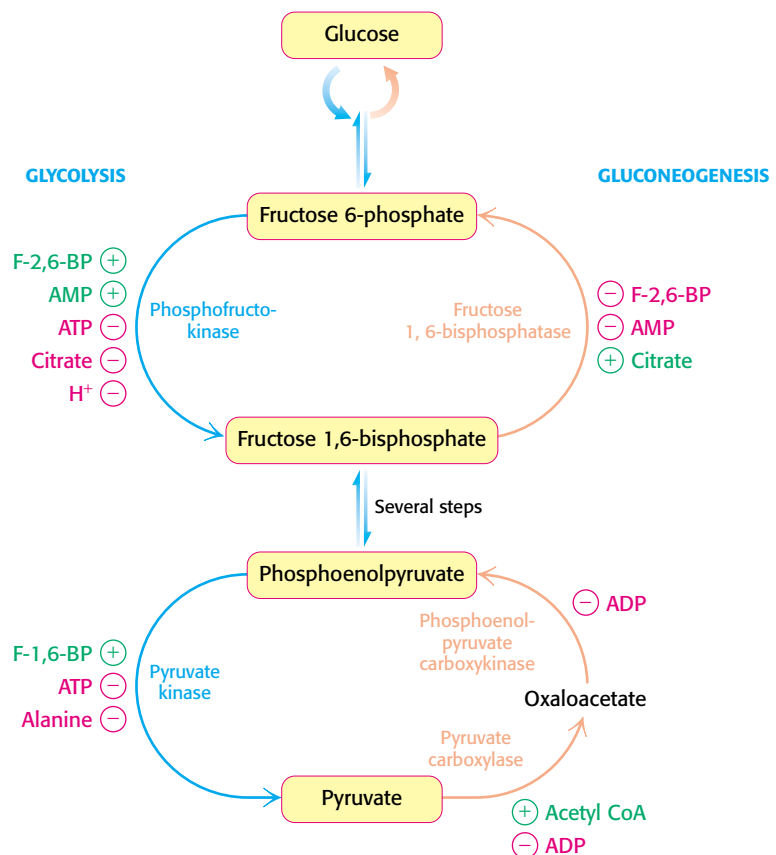


FIGURE 16.30 Reciprocal regulation of gluconeogenesis and glycolysis in the liver. The level of fructose 2,6-bisphosphate is high in the fed state and low in starvation. Another important control is the inhibition of pyruvate kinase by phosphorylation during starvation.

high levels of ATP and citrate indicate that the energy charge is high and that biosynthetic intermediates are abundant. Under these conditions, glycolysis is nearly switched off and gluconeogenesis is promoted.

Phosphofructokinase and fructose 1,6-bisphosphatase are also reciprocally controlled by *fructose 2,6-bisphosphate in the liver* (Section 16.2.2). The level of F-2,6-BP is low during starvation and high in the fed state, because of the antagonistic effects of glucagon and insulin on the production and degradation of this signal molecule. *Fructose 2,6-bisphosphate strongly stimulates phosphofructokinase and inhibits fructose 1,6-bisphosphatase*. Hence, glycolysis is accelerated and gluconeogenesis is diminished in the fed state. During starvation, gluconeogenesis predominates because the level of F-2,6-BP is very low. Glucose formed by the liver under these conditions is essential for the viability of brain and muscle.

The interconversion of phosphoenolpyruvate and pyruvate also is precisely regulated. Recall that pyruvate kinase is controlled by allosteric effectors and by phosphorylation (Section 16.2.3). High levels of ATP and alanine, which signal that the energy charge is high and that building blocks are abundant, inhibit the enzyme in liver. Conversely, pyruvate carboxylase, which catalyzes the first step in gluconeogenesis from pyruvate, is activated by acetyl CoA and inhibited by ADP. Likewise, ADP inhibits phosphoenolpyruvate carboxykinase. Hence, gluconeogenesis is favored when the cell is rich in biosynthetic precursors and ATP.

The amounts and the activities of these essential enzymes also are regulated. The regulators in this case are hormones. Hormones affect gene expression primarily by changing the rate of transcription, as well as by regulating the degradation of mRNA. Insulin, which rises subsequent to eating, stimulates the expression of phosphofructokinase, pyruvate kinase, and the bifunctional enzyme that makes and degrades F-2,6-BP. Glucagon, which rises during starvation, inhibits the expression of these enzymes and stimulates instead the production of two key gluconeogenic enzymes, phosphoenolpyruvate carboxykinase and fructose 1,6-bisphosphatase. Transcriptional control in eukaryotes is much slower than allosteric control; it takes hours or days in contrast with seconds to minutes. The richness and complexity of hormonal control are graphically displayed by the promoter of the phosphoenolpyruvate carboxykinase gene, which contains regulatory sequences that respond to insulin, glucagon, glucocorticoids, and thyroid hormone (Figure 16.31).

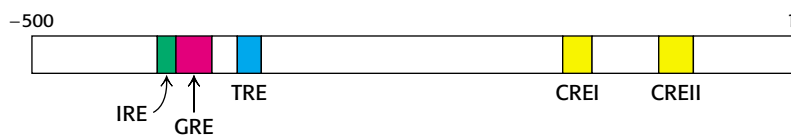


FIGURE 16.31 The promoter of the phosphoenolpyruvate carboxykinase gene. This promoter is approximately 500 bp in length and contains regulatory sequences (response elements) that mediate the action of several hormones. IRE, insulin response element; GRE, glucocorticoid response element; TRE, thyroid hormone response element; CREI and CREII, cAMP response elements. [After M. M. McGrane, J. S. Jun, Y. M. Patel, and R. W. Hanson. *Trends Biochem. Sci.* 17(1992):40.]

16.4.1 Substrate Cycles Amplify Metabolic Signals and Produce Heat

A pair of reactions such as the phosphorylation of fructose 6-phosphate to fructose 1,6-bisphosphate and its hydrolysis back to fructose 6-phosphate is called a *substrate cycle*. As already mentioned, both reactions are not simultaneously fully active in most cells, because of reciprocal allosteric

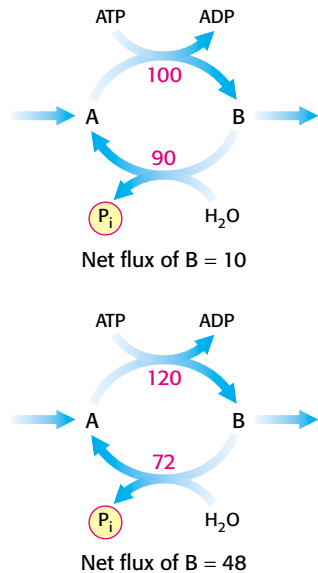


FIGURE 16.32 Substrate cycle. This ATP-driven cycle operates at two different rates. A small change in the rates of the two opposing reactions results in a large change in the *net flux* of product B.

controls. However, the results of isotope-labeling studies have shown that some fructose 6-phosphate is phosphorylated to fructose 1,6-bisphosphate in gluconeogenesis. There also is a limited degree of cycling in other pairs of opposed irreversible reactions. This cycling was regarded as an imperfection in metabolic control, and so substrate cycles have sometimes been called *futile cycles*. Indeed, there are pathological conditions, such as malignant hyperthermia, in which control is lost and both pathways proceed rapidly with the concomitant generation of heat by the rapid, uncontrolled hydrolysis of ATP.

Despite such extraordinary circumstances, it now seems likely that substrate cycles are biologically important. One possibility is that *substrate cycles amplify metabolic signals*. Suppose that the rate of conversion of A into B is 100 and of B into A is 90, giving an initial net flux of 10. Assume that an allosteric effector increases the A → B rate by 20% to 120 and reciprocally decreases the B → A rate by 20% to 72. The new net flux is 48, and so a 20% change in the rates of the opposing reactions has led to a 380% increase in the net flux. In the example shown in Figure 16.32, this amplification is made possible by the rapid hydrolysis of ATP. It has been suggested that the flux down the glycolytic pathway may increase 1000-fold at the initiation of intense exercise. Because it seems unlikely that allosteric activation of enzymes alone could explain this increased flux, the existence of substrate cycles may partly account for the rapid rise in the rate of glycolysis.

The other potential biological role of substrate cycles is the *generation of heat produced by the hydrolysis of ATP*. A striking example is provided by bumblebees, which must maintain a thoracic temperature of about 30°C to fly. A bumblebee is able to maintain this high thoracic temperature and forage for food even when the ambient temperature is only 10°C because phosphofructokinase and fructose 1,6-bisphosphatase in its flight muscle are simultaneously highly active; the continuous hydrolysis of ATP generates heat. This bisphosphatase is not inhibited by AMP, which suggests that the enzyme is specially designed for the generation of heat. In contrast, the honeybee has almost no fructose 1,6-bisphosphatase activity in its flight muscle and consequently cannot fly when the ambient temperature is low.

16.4.2 Lactate and Alanine Formed by Contracting Muscle Are Converted into Glucose by the Liver

The major raw materials of gluconeogenesis are lactate and alanine produced by active skeletal muscle and erythrocytes. Erythrocytes lack mitochondria and can never oxidize glucose completely. In contracting skeletal muscle during vigorous exercise, the rate at which glycolysis produces pyruvate exceeds the rate at which the citric acid cycle oxidizes it. Under these conditions, moreover, the rate of formation of NADH by glycolysis is greater than the rate of its oxidation by aerobic metabolism. Continued glycolysis depends on the availability of NAD⁺ for the oxidation of glyceraldehyde 3-phosphate. The accumulation of both NADH and pyruvate is reversed by lactate dehydrogenase, which oxidizes NADH to NAD⁺ as it reduces pyruvate to lactate (Section 16.1.7). However, lactate is a dead end in metabolism. It must be converted back into pyruvate before it can be metabolized. The only purpose of the reduction of pyruvate to lactate is to regenerate NAD⁺ so that glycolysis can proceed in active skeletal muscle and erythrocytes. *The formation of lactate buys time and shifts part of the metabolic burden from muscle to liver.*

The plasma membrane of most cells contains carriers that render them highly permeable to lactate and pyruvate. Both substances diffuse out of active skeletal muscle into the blood and are carried to the liver. Much more

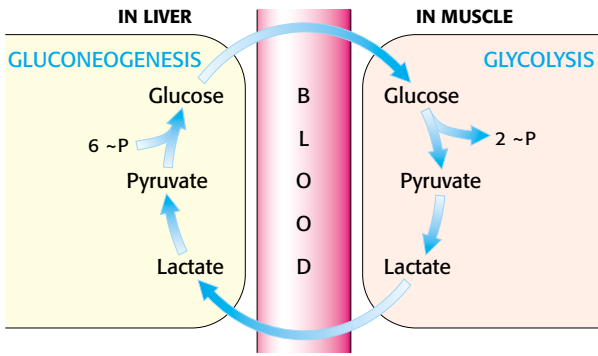


FIGURE 16.33 The Cori cycle. Lactate formed by active muscle is converted into glucose by the liver. This cycle shifts part of the metabolic burden of active muscle to the liver.

lactate than pyruvate is transported out because the high NADH/NAD^+ ratio in contracting skeletal muscle favors the conversion of pyruvate into lactate. The lactate that enters the liver is oxidized to pyruvate, a reaction favored by the low NADH/NAD^+ ratio in the cytosol of liver cells. Pyruvate in the liver is converted into glucose by the gluconeogenic pathway. Glucose then enters the blood and is taken up by skeletal muscle. Thus, *the liver furnishes glucose to contracting skeletal muscle, which derives ATP from the glycolytic conversion of glucose into lactate. Contracting skeletal muscle supplies lactate to the liver, which uses it to synthesize glucose. These reactions constitute the Cori cycle* (Figure 16.33). Studies have shown that alanine, like lactate, is a major precursor of glucose. In muscle, alanine is formed from pyruvate by transamination (Section 24.x.x); the reverse reaction takes place in the liver. The interplay between glycolysis and gluconeogenesis is summarized in Figure 16.34, which shows how these two pathways help to meet the energy needs of different cell types.



Isozymic forms of lactate dehydrogenase in different tissues catalyze the interconversions of pyruvate and lactate. Lactate dehydrogenase is a tetramer of two kinds of 35-kd subunits encoded by similar genes: the H type predominates in the heart, and the homologous M type in skeletal muscle and the liver. These subunits associate to form five types of tetramers: H_4 , H_3M_1 , H_2M_2 , H_1M_3 , and M_4 . The H_4 isozyme (type 1) has higher

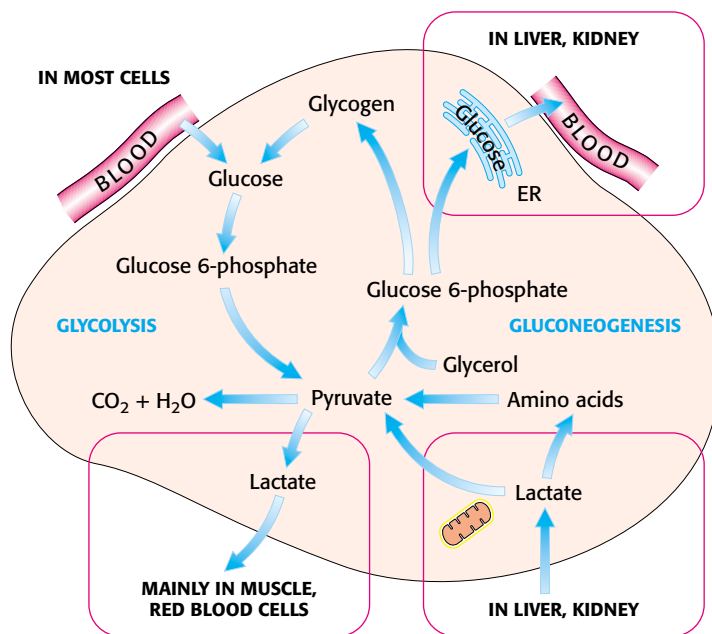


FIGURE 16.34 Cooperation between glycolysis and gluconeogenesis. Glycolysis and gluconeogenesis are coordinated, in a tissue-specific fashion, to ensure that the glucose-dependent energy needs of all cells are met.

affinity for substrates than does the M_4 isozyme (type 5) and, unlike M_4 , is allosterically inhibited by high levels of pyruvate. The other isozymes have intermediate properties, depending on the ratio of the two kinds of chains. H_4 is designed to oxidize lactate to pyruvate, which is then utilized as a fuel by the heart through aerobic metabolism. Indeed, heart muscle never functions anaerobically. In contrast, M_4 is optimized to operate in the reverse direction, to convert pyruvate into lactate to allow glycolysis to proceed under anaerobic conditions. We see here an example of how gene duplication and divergence generate a series of homologous enzymes that foster metabolic cooperation between organs.

16.4.3 Glycolysis and Gluconeogenesis Are Evolutionarily Intertwined



The metabolism of glucose has ancient origins. Organisms living in the early biosphere depended on the anaerobic generation of energy until significant amounts of oxygen began to accumulate 2 billion years ago. The fact that glycolytic enzymes with similar properties do not have similar amino acid sequences also provides a clue to how the pathway originated. Although there are four kinases and two isomerases in the pathway, both sequence and structural comparisons do not suggest that these sets of enzymes are related to one another by divergent evolution. The absence of such similarities implies that glycolytic enzymes were derived independently rather than by gene duplication. The common dinucleotide-binding domain found in the dehydrogenases (Section 16.1.10) and the $\alpha\beta$ barrels are the only major recurring elements.

We can speculate on the relationship between glycolysis and gluconeogenesis if we think of glycolysis as consisting of two segments: the metabolism of hexoses (the upper segment) and the metabolism of trioses (the lower segment). The enzymes from the upper segment are different in some species and are missing entirely in some archaea, whereas enzymes from the lower segment are quite conserved. In fact, four enzymes of the lower segment are present in all species. *This lower part of the pathway is common to glycolysis and gluconeogenesis.* This common part of the two pathways may be the oldest part, constituting the core to which the other steps were added. The upper part would vary according to the sugars that were available to evolving organisms in particular niches. Interestingly, this core part of carbohydrate metabolism can generate triose precursors for ribose sugars, a component of RNA and a critical requirement for the RNA world. Thus, we are left with the unanswered question, Was the original core pathway used for energy conversion or biosynthesis?

SUMMARY

- **Glycolysis Is an Energy-Conversion Pathway in Many Organisms**

Glycolysis is the set of reactions that converts glucose into pyruvate. The 10 reactions of glycolysis take place in the cytosol. In the first stage, glucose is converted into fructose 1,6-bisphosphate by a phosphorylation, an isomerization, and a second phosphorylation reaction. Two molecules of ATP are consumed per molecule of glucose in these reactions, which are the prelude to the net synthesis of ATP. In the second stage, fructose 1,6-bisphosphate is cleaved by aldolase into dihydroxyacetone phosphate and glyceraldehyde 3-phosphate, which are readily interconvertible. In the third stage, ATP is generated. Glyceraldehyde 3-phosphate is oxidized and phosphorylated to form 1,3-bisphospho-

glycerate, an acyl phosphate with a high phosphoryl-transfer potential. This molecule transfers a phosphoryl group to ADP to form ATP and 3-phosphoglycerate. A phosphoryl shift and a dehydration form phosphoenolpyruvate, a second intermediate with a high phosphoryl-transfer potential. Another molecule of ATP is generated as phosphoenolpyruvate is converted into pyruvate. There is a net gain of two molecules of ATP in the formation of two molecules of pyruvate from one molecule of glucose.

The electron acceptor in the oxidation of glyceraldehyde 3-phosphate is NAD^+ , which must be regenerated for glycolysis to continue. In aerobic organisms, the NADH formed in glycolysis transfers its electrons to O_2 through the electron-transport chain, which thereby regenerates NAD^+ . Under anaerobic conditions and in some microorganisms, NAD^+ is regenerated by the reduction of pyruvate to lactate. In other microorganisms, NAD^+ is regenerated by the reduction of pyruvate to ethanol. These two processes are examples of fermentations.

- **The Glycolytic Pathway Is Tightly Controlled**

The glycolytic pathway has a dual role: it degrades glucose to generate ATP, and it provides building blocks for the synthesis of cellular components. The rate of conversion of glucose into pyruvate is regulated to meet these two major cellular needs. Under physiologic conditions, the reactions of glycolysis are readily reversible except for the ones catalyzed by hexokinase, phosphofructokinase, and pyruvate kinase. Phosphofructokinase, the most important control element in glycolysis, is inhibited by high levels of ATP and citrate, and it is activated by AMP and fructose 2,6-bisphosphate. In the liver, this bisphosphate signals that glucose is abundant. Hence, phosphofructokinase is active when either energy or building blocks are needed. Hexokinase is inhibited by glucose 6-phosphate, which accumulates when phosphofructokinase is inactive. ATP and alanine allosterically inhibit pyruvate kinase, the other control site, and fructose 1,6-bisphosphate activates the enzyme. Consequently, pyruvate kinase is maximally active when the energy charge is low and glycolytic intermediates accumulate.

- **Glucose Can Be Synthesized from Noncarbohydrate Precursors**

Gluconeogenesis is the synthesis of glucose from noncarbohydrate sources, such as lactate, amino acids, and glycerol. Several of the reactions that convert pyruvate into glucose are common to glycolysis. Gluconeogenesis, however, requires four new reactions to bypass the essential irreversibility of three reactions in glycolysis. In two of the new reactions, pyruvate is carboxylated in mitochondria to oxaloacetate, which in turn is decarboxylated and phosphorylated in the cytosol to phosphoenolpyruvate. Two high-energy phosphate bonds are consumed in these reactions, which are catalyzed by pyruvate carboxylase and phosphoenolpyruvate carboxykinase. Pyruvate carboxylase contains a biotin prosthetic group. The other distinctive reactions of gluconeogenesis are the hydrolyses of fructose 1,6-bisphosphate and glucose 6-phosphate, which are catalyzed by specific phosphatases. The major raw materials for gluconeogenesis by the liver are lactate and alanine produced from pyruvate by active skeletal muscle. The formation of lactate during intense muscular activity buys time and shifts part of the metabolic burden from muscle to the liver.

- **Gluconeogenesis and Glycolysis Are Reciprocally Regulated**

Gluconeogenesis and glycolysis are reciprocally regulated so that one pathway is relatively inactive while the other is highly active. Phosphofructokinase and fructose 1,6-bisphosphatase are key control points.

Fructose 2,6-bisphosphate, an intracellular signal molecule present at higher levels when glucose is abundant, activates glycolysis and inhibits gluconeogenesis by regulating these enzymes. Pyruvate kinase and pyruvate carboxylase are regulated by other effectors so that both are not maximally active at the same time. Allosteric regulation and reversible phosphorylation, which are rapid, are complemented by transcriptional control, which takes place in hours or days.

KEY TERMS

glycolysis (p. 16-1)	phosphofructokinase (p. 16-6)	feedforward stimulation (p. 16-22)
lactic acid fermentation (p. 16-1)	thioester intermediate (p. 16-10)	pyruvate carboxylase (p. 16-28)
alcoholic fermentation (p. 16-1)	substrate-level phosphorylation (p. 16-11)	biotin (p. 16-29)
gluconeogenesis (p. 16-1)	mutase (p. 16-12)	glucose 6-phosphatase (p. 16-31)
obligate anaerobe (p. 16-3)	enol phosphate (p. 16-12)	substrate cycle (p. 16-33)
facultative anaerobe (p. 16-3)	pyruvate kinase (p. 16-13)	Cori cycle (p. 16-35)
hexokinase (p. 16-4)	committed step (p. 000)	
kinase (p. 16-4)	bifunctional enzyme (p. 16-21)	

SELECTED READINGS

Where to start

- Knowles, J. R., 1991. Enzyme catalysis: Not different, just better. *Nature* 350:121–124.
- Granner, D., and Pilkis, S., 1990. The genes of hepatic glucose metabolism. *J. Biol. Chem.* 265:10173–10176.
- McGrane, M. M., Yun, J. S., Patel, Y. M., and Hanson, R. W., 1992. Metabolic control of gene expression: In vivo studies with transgenic mice. *Trends Biochem. Sci.* 17:40–44.
- Pilkis, S. J., and Granner, D. K., 1992. Molecular physiology of the regulation of hepatic gluconeogenesis and glycolysis. *Annu. Rev. Physiol.* 54:885–909.

Books

- Fell, D., 1997. *Understanding the Control of Metabolism*. Portland.
- Fersht, A., 1999. *Structure and Mechanism in Protein Science: A Guide to Enzyme Catalysis and Protein Folding*. W. H. Freeman and Company.
- Frayn, K. N., 1996. *Metabolic Regulation: A Human Perspective*. Portland.
- Hargreaves, M., and Thompson, M. (Eds.) 1999. *Biochemistry of Exercise X. Human Kinetics*.

Structure of glycolytic and gluconeogenic enzymes

- Aleshin, A. E., Kirby, C., Liu, X., Bourenkov, G. P., Bartunik, H. D., Fromm, H. J., and Honzatko, R. B., 2000. Crystal structures of mutant monomeric hexokinase I reveal multiple ADP binding sites and conformational changes relevant to allosteric regulation. *J. Mol. Biol.* 296:1001–1015.
- Jeffery, C. J., Bahnsen, B. J., Chien, W., Ringe, D., and Petsko, G. A., 2000. Crystal structure of rabbit phosphoglucose isomerase, a glycolytic enzyme that moonlights as neuroleukin, autocrine motility factor, and differentiation mediator. *Biochemistry* 39:955–964.
- Schirmer, T., and Evans, P. R., 1990. Structural basis of the allosteric behaviour of phosphofructokinase. *Nature* 343:140–145.
- Cooper, S. J., Leonard, G. A., McSweeney, S. M., Thompson, A. W., Naismith, J. H., Qamar, S., Plater, A., Berry, A., and Hunter, W. N., 1996. The crystal structure of a class II fructose-1,6-bisphosphate aldolase shows a novel binuclear metal-binding active site embedded in a familiar fold. *Structure* 4:1303–1315.

- Davenport, R. C., Bash, P. A., Seaton, B. A., Karplus, M., Petsko, G. A., and Ringe, D., 1991. Structure of the triosephosphate isomerase–phosphoglycolhydroxamate complex: An analogue of the intermediate on the reaction pathway. *Biochemistry* 30:5821–5826.
- Skarzynski, T., Moody, P. C., and Wonacott, A. J., 1987. Structure of holo-glyceraldehyde-3-phosphate dehydrogenase from *Bacillus stearothermophilus* at 1.8 Å resolution. *J. Mol. Biol.* 193:171–187.
- Bernstein, B. E., and Hol, W. G., 1998. Crystal structures of substrates and products bound to the phosphoglycerate kinase active site reveal the catalytic mechanism. *Biochemistry* 37:4429–4436.
- Rigden, D. J., Alexeev, D., Phillips, S. E. V., and Fothergill-Gilmore, L. A., 1998. The 2.3 Å X-ray crystal structure of *S. cerevisiae* phosphoglycerate mutase. *J. Mol. Biol.* 276:449–459.
- Zhang, E., Brewer, J. M., Minor, W., Carreira, L. A., and Lebioda, L., 1997. Mechanism of enolase: The crystal structure of asymmetric dimer enolase-2-phospho-D-glycerate/enolase-phosphoenolpyruvate at 2.0 Å resolution. *Biochemistry* 36:12526–12534.
- Mattevi, A., Valentini, G., Rizzi, M., Speranza, M. L., Bolognesi, M., and Coda, A., 1995. Crystal structure of *Escherichia coli* pyruvate kinase type I: Molecular basis of the allosteric transition. *Structure* 3:729–741.
- Hasemann, C. A., Istvan E. S., Uyeda, K., and Deisenhofer, J., 1996. The crystal structure of the bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase reveals distinct domain homologies. *Structure* 4:1017–1029.
- Tari, L. W., Matte, A., Pugazhenth, U., Goldie, H., and Delbaere, L. T. J., 1996. Snapshot of an enzyme reaction intermediate in the structure of the ATP-Mg²⁺-oxalate ternary complex of *Escherichia coli* PEP carboxykinase. *Nat. Struct. Biol.* 3:355–363.

Catalytic mechanisms

- Soukri, A., Mougin, A., Corbier, C., Wonacott, A., Branlant, C., and Branlant, G., 1989. Role of the histidine 176 residue in glyceraldehyde-3-phosphate dehydrogenase as probed by site-directed mutagenesis. *Biochemistry* 28:2586–2592.
- Bash, P. A., Field, M. J., Davenport, R. C., Petsko, G. A., Ringe, D., and Karplus, M., 1991. Computer simulation and analysis of the reaction pathway of triosephosphate isomerase. *Biochemistry* 30:5826–5832.

Knowles, J. R., and Albery, W. J., 1977. Perfection in enzyme catalysis: The energetics of triosephosphate isomerase. *Acc. Chem. Res.* 10:105–111.

Rose, I. A., 1981. Chemistry of proton abstraction by glycolytic enzymes (aldolase, isomerases, and pyruvate kinase). *Philos. Trans. R. Soc. Lond.: Series B, Biol. Sci.* 293:131–144.

Regulation

Dang, C. V., and Semenza, G. L., 1999. Oncogenic alterations of metabolism. *Trends Biochem. Sci.* 24:68–72.

Depre, C., Rider, M. H., and Hue, L., 1998. Mechanisms of control of heart glycolysis. *Eur. J. Biochem.* 258:277–290.

Gleeson, T. T., 1996. Post-exercise lactate metabolism: A comparative review of sites, pathways, and regulation. *Annu. Rev. Physiol.* 58:556–581.

Hers, H. -G., and Van Schaftingen, E., 1982. Fructose 2,6-bisphosphate two years after its discovery. *Biochem. J.* 206:1–12.

Middleton, R. J., 1990. Hexokinases and glucokinases. *Biochem. Soc. Trans.* 18:180–183.

Nordlie, R. C., Foster, J. D., and Lange, A. J., 1999. Regulation of glucose production by the liver. *Annu. Rev. Nutr.* 19:379–406.

Jitrapakdee, S., and Wallace, J. C., 1999. Structure, function and regulation of pyruvate carboxylase. *Biochem. J.* 340:1–16.

Pilkis, S. J., and Claus, T. H., 1991. Hepatic gluconeogenesis/glycolysis: Regulation and structure/function relationships of substrate cycle enzymes. *Annu. Rev. Nutr.* 11:465–515.

Plaxton, W. C., 1996. The organization and regulation of plant glycolysis. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 47:185–214.

van de Werve, G., Lange, A., Newgard, C., Mechin, M. C., Li, Y., and Berteloot, A., 2000. New lessons in the regulation of glucose metabolism taught by the glucose 6-phosphatase system. *Eur. J. Biochem.* 267:1533–1549.

Sugar transporters

Czech, M. P., and Corvera, S., 1999. Signaling mechanisms that regulate glucose transport. *J. Biol. Chem.* 274:1865–1868.

Silverman, M., 1991. Structure and function of hexose transporters. *Annu. Rev. Biochem.* 60:757–794.

Thorens, B., Charron, M. J., and Lodish, H. F., 1990. Molecular physiology of glucose transporters. *Diabetes Care* 13:209–218.

Genetic diseases

Scriver, C. R., (Ed.), 2000. *The Metabolic and Molecular Basis of Inherited Disease* (8th ed.). McGraw-Hill.

Evolution

Dandekar, T., Schuster, S., Snel, B., Huynen, M., and Bork, P., 1999. Pathway alignment: Application to the comparative analysis of glycolytic enzymes. *Biochem. J.* 343:115–124.

Heinrich, R., Melendez-Hevia, E., Montero, F., Nuno, J. C., Stephani, A., and Waddell, T. G., 1999. The structural design of glycolysis: An evolutionary approach. *Biochem. Soc. Trans.* 27:294–298.

Walmsley, A. R., Barrett, M. P., Bringaud, F., and Gould, G. W., 1998. Sugar transporters from bacteria, parasites and mammals: Structure-activity relationships. *Trends Biochem. Sci.* 23:476–480.

Maes, D., Zeelen, J. P., Thanki, N., Beaucamp, N., Alvarez, M., Thi, M. H., Backmann, J., Martial, J. A., Wyns, L., Jaenicke, R., and Wierenga, R. K., 1999. The crystal structure of triosephosphate isomerase (TIM) from *Thermotoga maritima*: A comparative thermostability structural analysis of ten different TIM structures. *Proteins* 37:441–453.

Historical aspects

Fruton, J. S., 1972. *Molecules and Life: Historical Essays on the Interplay of Chemistry and Biology*. Wiley Interscience.

Kalckar, H. M. (Ed.), 1969. *Biological Phosphorylations: Development of Concepts*. Prentice Hall.

PROBLEMS

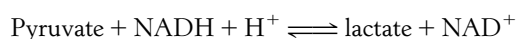
1. *Kitchen chemistry*. Sucrose is commonly used to preserve fruits. Why is glucose not suitable for preserving foods?

2. *Tracing carbon atoms I*. Glucose labeled with ^{14}C at C-1 is incubated with the glycolytic enzymes and necessary cofactors. (a) What is the distribution of ^{14}C in the pyruvate that is formed? (Assume that the interconversion of glyceraldehyde 3-phosphate and dihydroxyacetone phosphate is very rapid compared with the subsequent step.)

(b) If the specific activity of the glucose substrate is 10 mCi mM^{-1} , what is the specific activity of the pyruvate that is formed?

3. *Lactic acid fermentation*. (a) Write a balanced equation for the conversion of glucose into lactate.

(b) Calculate the standard free-energy change of this reaction by using the data given in Table 16-3 and the fact that ΔG° is -6 kcal for the following reaction:



What is the free-energy change (ΔG , not ΔG°) of this reaction when the concentrations of reactants are: glucose, 5 mM; lactate, 0.05 mM; ATP, 2 mM; ADP, 0.2 mM; and P_i , 1 mM?

4. *High potential*. What is the equilibrium ratio of phosphoenolpyruvate to pyruvate under standard conditions when $[\text{ATP}]/[\text{ADP}] = 10$?

5. *Hexose-triose equilibrium*. What are the equilibrium concentrations of fructose 1,6-bisphosphate, dihydroxyacetone phosphate, and glyceraldehyde 3-phosphate when 1 mM fructose 1,6-bisphosphate is incubated with aldolase under standard conditions?

6. *Double labeling*. 3-Phosphoglycerate labeled uniformly with ^{14}C is incubated with 1,3-BPG labeled with ^{32}P at C-1. What is the radioisotope distribution of the 2,3-BPG that is formed on addition of BPG mutase?

7. *An informative analog*. Xylose has the same structure as that of glucose except that it has a hydrogen atom at C-6 in place of a hydroxymethyl group. The rate of ATP hydrolysis by hexokinase is markedly enhanced by the addition of xylose. Why?

8. *Distinctive sugars*. The intravenous infusion of fructose into healthy volunteers leads to a two- to fivefold increase in the level of lactate in the blood, a far greater increase than that observed after the infusion of the same amount of glucose.

(a) Why is glycolysis more rapid after the infusion of fructose?
(a) Fructose has been used in place of glucose for intravenous feeding. Why is this use of fructose unwise?

9. *Metabolic mutants*. Predict the effect of each of the following mutations on the pace of glycolysis in liver cells:

(a) Loss of the allosteric site for ATP in phosphofructokinase.
(b) Loss of the binding site for citrate in phosphofructokinase.

- (c) Loss of the phosphatase domain of the bifunctional enzyme that controls the level of fructose 2,6-bisphosphate.
 (d) Loss of the binding site for fructose 1,6-bisphosphate in pyruvate kinase.

10. *Metabolic mutant.* What are the likely consequences of a genetic disorder rendering fructose 1,6-bisphosphatase in liver less sensitive to regulation by fructose 2,6-bisphosphate?

11. *Biotin snatcher.* Avidin, a 70-kd protein in egg white, has very high affinity for biotin. In fact, it is a highly specific inhibitor of biotin enzymes. Which of the following conversions would be blocked by the addition of avidin to a cell homogenate?

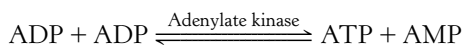
- (a) Glucose \longrightarrow pyruvate
 (b) Pyruvate \longrightarrow glucose
 (c) Oxaloacetate \longrightarrow glucose
 (d) Malate \longrightarrow oxaloacetate
 (e) Pyruvate \longrightarrow oxaloacetate
 (f) Glyceraldehyde 3-phosphate \longrightarrow fructose 1,6-bisphosphate

12. *Tracing carbon atoms II.* If cells synthesizing glucose from lactate are exposed to CO_2 labeled with ^{14}C , what will be the distribution of label in the newly synthesized glucose?

13. *Arsenate poisoning.* Arsenate (AsO_4^{3-}) closely resembles P_i in structure and reactivity. In the reaction catalyzed by glyceraldehyde 3-phosphate dehydrogenase, arsenate can replace phosphate in attacking the energy-rich thioester intermediate. The product of this reaction, 1-arseno-3-phosphoglycerate, is unstable. It and other acyl arsenates are rapidly and spontaneously hydrolyzed. What is the effect of arsenate on energy generation in a cell?

14. *Reduce, reuse, recycle.* In the conversion of glucose into two molecules of lactate, the NADH generated earlier in the pathway is oxidized to NAD^+ . Why is it not to the cells advantage to simply make more NAD^+ so that the regeneration would not be necessary? After all, the cell would save much energy because it would no longer need to synthesize lactic acid dehydrogenase.

15. *Adenylate kinase again.* Adenylate kinase, an enzyme discussed in great detail in Chapter 9, is responsible for interconverting the adenylate nucleotide pool:



The equilibrium constant for this reaction is close to 1, inasmuch as the number of phosphoanhydride bonds is the same on each side of the equation. Using the equation for the equilibrium constant for this reaction, show why changes in $[\text{AMP}]$ are a more effective indicator of the adenylate pool than $[\text{ATP}]$.

16. *Working at cross-purposes?* Gluconeogenesis takes place during intense exercise, which seems counterintuitive. Why would an organism synthesize glucose and at the same time use glucose to generate energy?

17. *Powering pathways.* Compare the stoichiometries of glycolysis and gluconeogenesis (Section 16.1.8). Recall that the input of one ATP equivalent changes the equilibrium constant of a reaction by a factor of about 10^8 (Section 16.3.6). By what factor do the additional high phosphoryl-transfer potential compounds alter the equilibrium constant of gluconeogenesis?

Mechanism Problem

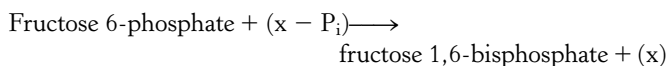
18. *Argument by analogy.* Propose a mechanism for the conversion of glucose 6-phosphate into fructose 6-phosphate by phosphoglucose isomerase based on the mechanism of triose phosphate isomerase.

Chapter Integration Problem

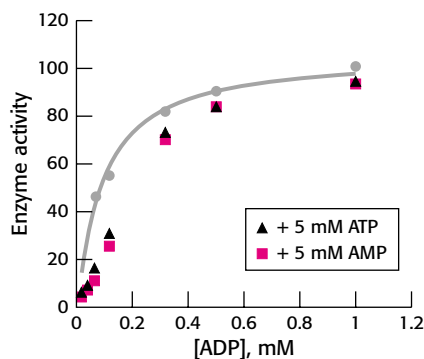
19. *Not just for energy.* People with galactosemia display central nervous system abnormalities even if galactose is eliminated from the diet. The precise reason for it is not known. Suggest a plausible explanation.

Data Interpretation Problem

20. *Now, that's unusual.* Phosphofructokinase has recently been isolated from the hyperthermophilic archaeon *Pyrococcus furiosus*. It was subjected to standard biochemical analysis to determine basic catalytic parameters. The processes under study were of the form:



The assay measured the increase in fructose 1,6-bisphosphate. Selected results are shown in the adjoining graph.



- (a) How does the *P. furiosus* phosphofructokinase differ from the phosphofructokinase discussed in this chapter?
 (b) What effects do AMP and ATP have on the reaction with ADP?



Media Problems

21. *No free lunch.* Suppose a microorganism is discovered to be lacking pyruvate kinase. Nevertheless, the organism does metabolize glucose to pyruvate. Surprisingly, it does so with a net gain of four molecules of ATP (or ATP equivalents) per molecule of metabolized glucose. Looking at the **Conceptual Insights** module on the energetics of glucose metabolism, suggest a biochemical pathway for metabolism of glucose in this organism. Why might evolution have favored the normal glycolytic pathway rather than this alternative?

22. *Oxidation in the absence of oxygen.* The energetics of glycolysis section of the **Conceptual Insights** module shows that there is a large free energy drop upon oxidation of glyceraldehyde 3-phosphate to 1,3-bisphosphoglycerate (reaction 6). In the presence of oxygen, some of this energy is ultimately converted into ATP production. However, no such conversion happens under anaerobic conditions. Explain why.

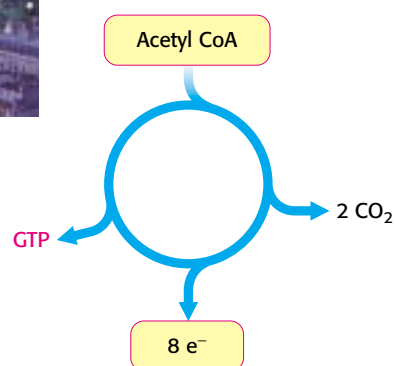
The Citric Acid Cycle



Roundabouts, or traffic circles, function as hubs to facilitate traffic flow.

The citric acid cycle is the biochemical hub of the cell, oxidizing carbon fuels, usually in the form of acetyl CoA, as well as serving as a source of precursors for biosynthesis.

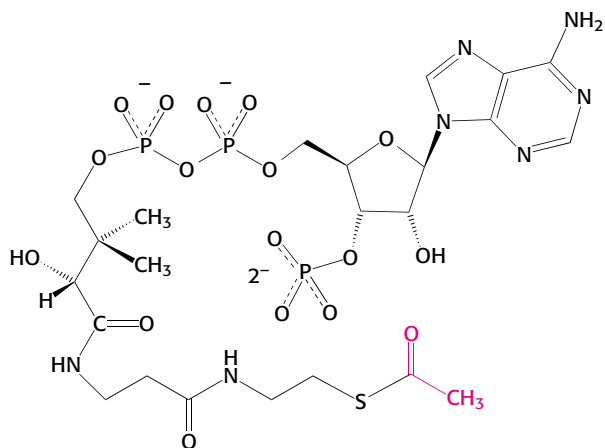
[(Above) Chris Warren/International Stock.]



From Chapter 16, we know that glucose can be metabolized to pyruvate anaerobically to synthesize ATP through the glycolytic pathway. Glycolysis, however, harvests but a fraction of the ATP available from glucose. We now begin an exploration of the aerobic processing of glucose, which is the source of most of the ATP generated in metabolism. The aerobic processing of glucose starts with the complete oxidation of glucose derivatives to carbon dioxide. This oxidation takes place in the *citric acid cycle*, a series of reactions also known as the *tricarboxylic acid (TCA) cycle* or the *Krebs cycle*. The citric acid cycle is the *final common pathway for the oxidation of fuel molecules*—amino acids, fatty acids, and carbohydrates. Most fuel molecules enter the cycle as acetyl coenzyme A.

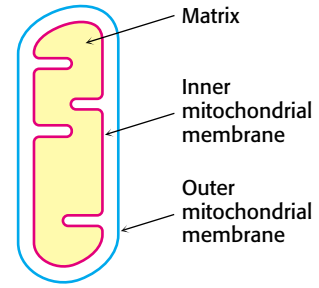
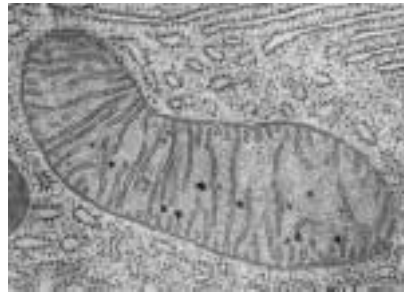
OUTLINE

- 17.1 The Citric Acid Cycle Oxidizes Two-Carbon Units
- 17.2 Entry to the Citric Acid Cycle and Metabolism Through It Are Controlled
- 17.3 The Citric Acid Cycle Is a Source of Biosynthetic Precursors
- 17.4 The Glyoxylate Cycle Enables Plants and Bacteria to Grow on Acetate



Acetyl coenzyme A (Acetyl CoA)

FIGURE 17.1 Mitochondrion. The double membrane of the mitochondrion is evident in this electron micrograph. The numerous invaginations of the inner mitochondrial membrane are called cristae. The oxidative decarboxylation of pyruvate and the sequence of reactions in the citric acid cycle take place within the matrix. [(Left) Omikron/Photo Researchers.]



Under aerobic conditions, the pyruvate generated from glucose is oxidatively decarboxylated to form acetyl CoA. In eukaryotes, the reactions of the citric acid cycle take place inside mitochondria, in contrast with those of glycolysis, which take place in the cytosol (Figure 17.1).

17.0.1 An Overview of the Citric Acid Cycle

The citric acid cycle is the central metabolic hub of the cell. It is the gateway to the aerobic metabolism of any molecule that can be transformed into an acetyl group or dicarboxylic acid. The cycle is also an important source of precursors, not only for the storage forms of fuels, but also for the building blocks of many other molecules such as amino acids, nucleotide bases, cholesterol, and porphyrin (the organic component of heme).

What is the function of the citric acid cycle in transforming fuel molecules into ATP? Recall that fuel molecules are carbon compounds that are capable of being oxidized—of losing electrons (Chapter 14). The citric acid cycle includes a series of oxidation–reduction reactions that result in the oxidation of an acetyl group to two molecules of carbon dioxide.

The overall pattern of the citric acid cycle is shown in Figure 17.2. A four-carbon compound (oxaloacetate) condenses with a two-carbon acetyl unit to yield a six-carbon tricarboxylic acid (citrate). An isomer of citrate is then oxidatively decarboxylated. The resulting five-carbon compound (α -ketoglutarate) also is oxidatively decarboxylated to yield a four-carbon compound (succinate). Oxaloacetate is then regenerated from succinate. Two carbon atoms enter the cycle as an acetyl unit and two carbon atoms leave the cycle in the form of two molecules of carbon dioxide. Three hydride ions (hence, six electrons) are transferred to three molecules of nicotinamide adenine dinucleotide (NAD^+), whereas one pair of hydrogen atoms (hence, two electrons) is transferred to one molecule of flavin adenine dinucleotide (FAD). The function of the citric acid cycle is the harvesting of high-energy electrons from carbon fuels. Note that the citric acid cycle itself nei-

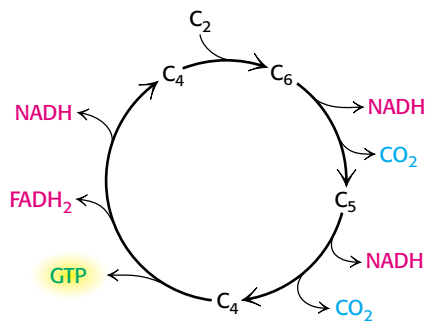
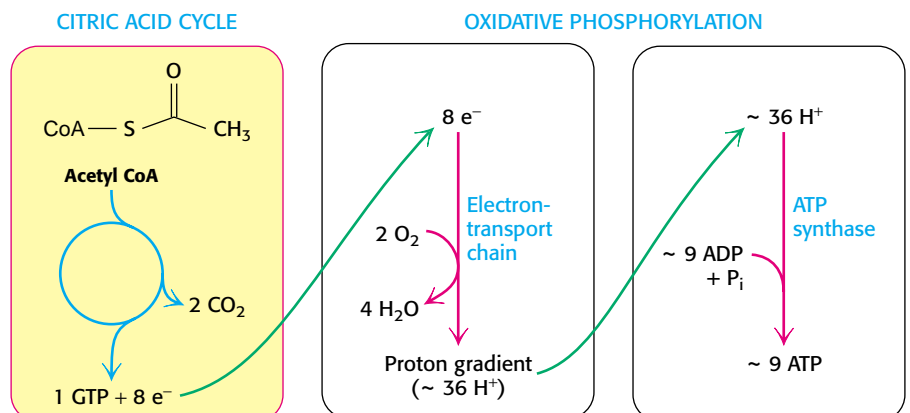


FIGURE 17.2 Overview of the citric acid cycle. The citric acid cycle oxidizes two-carbon units, producing two molecules of CO_2 , one molecule of GTP, and high-energy electrons in the form of NADH and FADH_2 .

FIGURE 17.3 Cellular respiration. The citric acid cycle constitutes the first stage in cellular respiration, the removal of high-energy electrons from carbon fuels (left). These electrons reduce O_2 to generate a proton gradient (middle), which is used to synthesize ATP (right). The reduction of O_2 and the synthesis of ATP constitute oxidative phosphorylation.



ther generates a large amount of ATP nor includes oxygen as a reactant (Figure 17.3). Instead, the citric acid cycle removes electrons from acetyl CoA and uses these electrons to form NADH and FADH₂. In *oxidative phosphorylation* (Chapter 18), electrons released in the reoxidation of NADH and FADH₂ flow through a series of membrane proteins (referred to as the *electron-transport chain*) to generate a proton gradient across the membrane. These protons then flow through ATP synthase to generate ATP from ADP and inorganic phosphate. Oxygen is required for the citric acid cycle indirectly inasmuch as it is the electron acceptor at the end of the electron-transport chain, necessary to regenerate NAD⁺ and FAD.

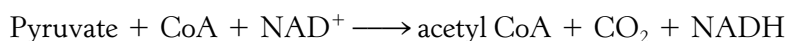
The citric acid cycle, in conjunction with oxidative phosphorylation, provides the vast majority of energy used by aerobic cells—in human beings, greater than 95%. It is highly efficient because a limited number of molecules can generate large amounts of NADH and FADH₂. Note in Figure 17.2 that the four-carbon molecule, oxaloacetate, that initiates the first step in the citric acid cycle is regenerated at the end of one passage through the cycle. The oxaloacetate acts catalytically: it participates in the oxidation of the acetyl group but is itself regenerated. Thus, one molecule of oxaloacetate is capable of participating in the oxidation of many acetyl molecules.

17.1 THE CITRIC ACID CYCLE OXIDIZES TWO-CARBON UNITS

Acetyl CoA is the fuel for the citric acid cycle. This important molecule is formed from the breakdown of glycogen (the storage form of glucose), fats, and many amino acids. Indeed, as we will see in Chapter 22, fats contain strings of reduced two-carbon units that are first oxidized to acetyl CoA and then completely oxidized to CO₂ by the citric acid cycle.

17.1.1 The Formation of Acetyl Coenzyme A from Pyruvate

The formation of acetyl CoA from carbohydrates is less direct than from fat. Recall that carbohydrates, most notably glucose, are processed by glycolysis into pyruvate (Chapter 16). Under anaerobic conditions, the pyruvate is converted into lactic acid or ethanol, depending on the organism. Under aerobic conditions, the pyruvate is transported into mitochondria in exchange for OH⁻ by the pyruvate carrier, an antiporter (Section 13.4). In the mitochondrial matrix, pyruvate is oxidatively decarboxylated by the *pyruvate dehydrogenase complex* to form acetyl CoA.



This irreversible reaction is the link between glycolysis and the citric acid cycle. (Figure 17.4) Note that, in the preparation of the glucose derivative pyruvate for the citric acid cycle, an oxidative decarboxylation takes place and high-transfer-potential electrons in the form of NADH are captured. Thus, the pyruvate dehydrogenase reaction has many of the key features of the reactions of the citric acid cycle itself.

The pyruvate dehydrogenase complex is a large, highly integrated complex of three kinds of enzymes (Table 17.1). Pyruvate dehydrogenase is a member of a family of homologous complexes that includes the citric acid cycle enzyme α -ketoglutarate dehydrogenase (Section 17.1.6), a branched-chain α -ketoacid dehydrogenase, and acetoin dehydrogenase, found in certain prokaryotes. These complexes are giant, with molecular masses ranging from 4 to 10 million daltons (Figure 17.5). As we will see, their elaborate structures allow groups to travel from one active site to another, connected

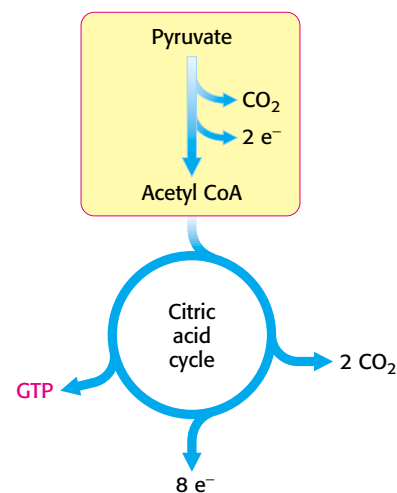


FIGURE 17.4 The link between glycolysis and the citric acid cycle. Pyruvate produced by glycolysis is converted into acetyl CoA, the fuel of the citric acid cycle.

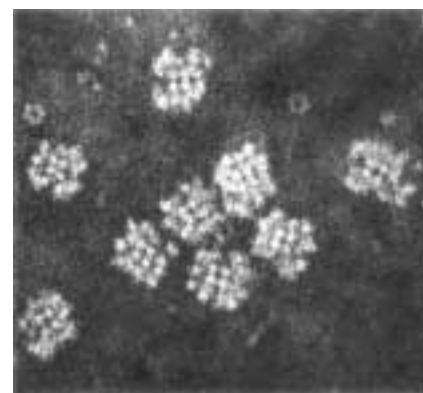
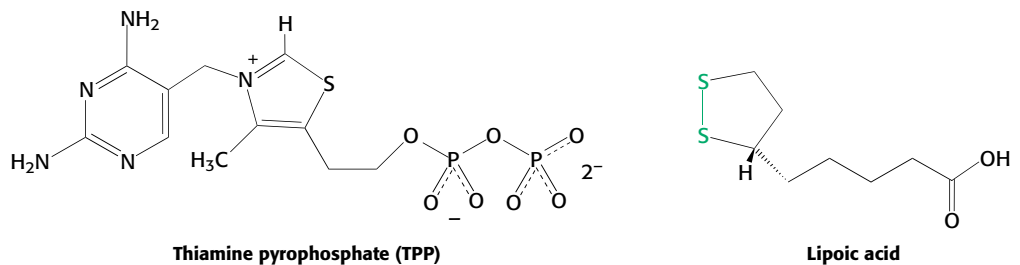


FIGURE 17.5 Electron micrograph of the pyruvate dehydrogenase complex from *E. coli*. [Courtesy of Dr. Lester Reed.]

TABLE 17.1 Pyruvate dehydrogenase complex of *E. coli*

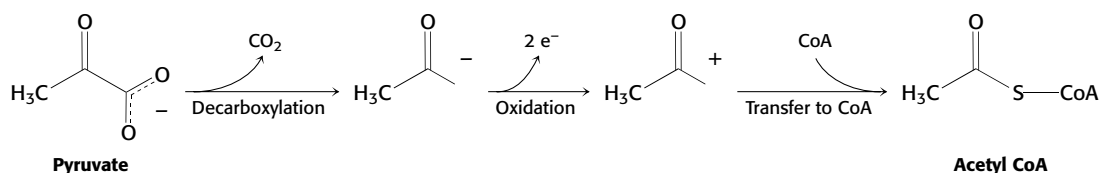
Enzyme	Abbreviation	Number of chains	Prosthetic group	Reaction catalyzed
Pyruvate dehydrogenase component	E ₁	24	TPP	Oxidative decarboxylation of pyruvate
Dihydrolipoyl transacetylase	E ₂	24	Lipoamide	Transfer of the acetyl group to CoA
Dihydrolipoyl dehydrogenase	E ₃	12	FAD	Regeneration of the oxidized form of lipoamide

by tethers to the core of the structure. The mechanism of the pyruvate dehydrogenase reaction is wonderfully complex, more so than is suggested by its relatively simple stoichiometry. The reaction requires the participation of the three enzymes of the pyruvate dehydrogenase complex, each composed of several polypeptide chains, and five coenzymes: *thiamine pyrophosphate (TPP)*, *lipoic acid*, and *FAD* serve as catalytic cofactors, and CoA and NAD⁺ are stoichiometric cofactors.

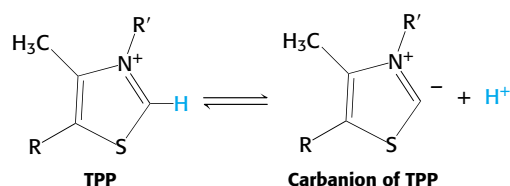


At least two additional enzymes regulate the activity of the complex.

The conversion of pyruvate into acetyl CoA consists of three steps: decarboxylation, oxidation, and transfer of the resultant acetyl group to CoA.



These steps must be coupled to preserve the free energy derived from the decarboxylation step to drive the formation of NADH and acetyl CoA. First, pyruvate combines with TPP and is then decarboxylated (Figure 17.6). This reaction is catalyzed by the *pyruvate dehydrogenase component* (E₁) of the multienzyme complex. A key feature of TPP, the prosthetic group of the pyruvate dehydrogenase component, is that the carbon atom between the nitrogen and sulfur atoms in the thiazole ring is much more acidic than most =CH– groups, with a pK_a value near 10. This center ionizes to form a *carbanion*, which readily adds to the carbonyl group of pyruvate.



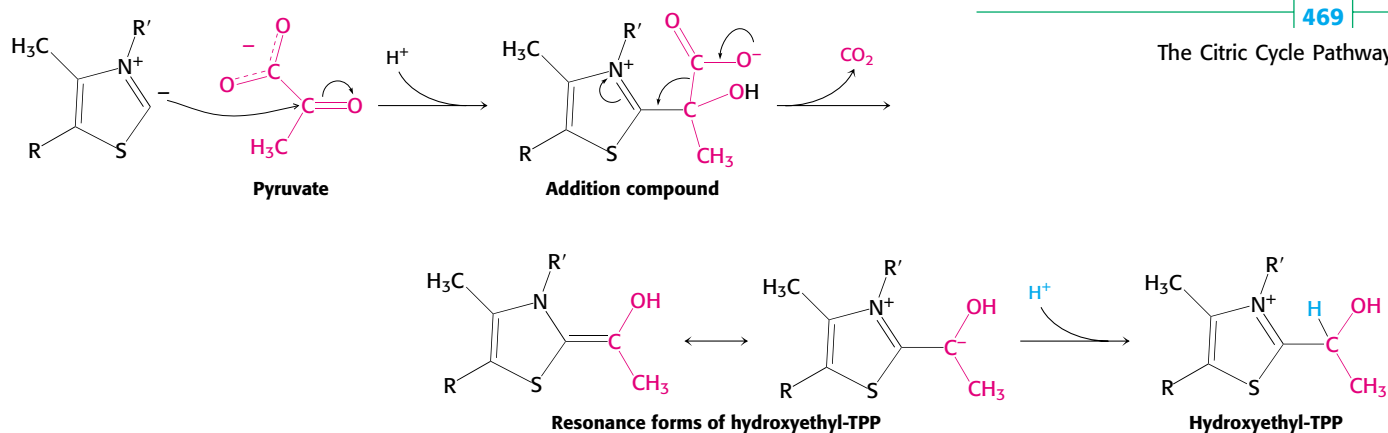
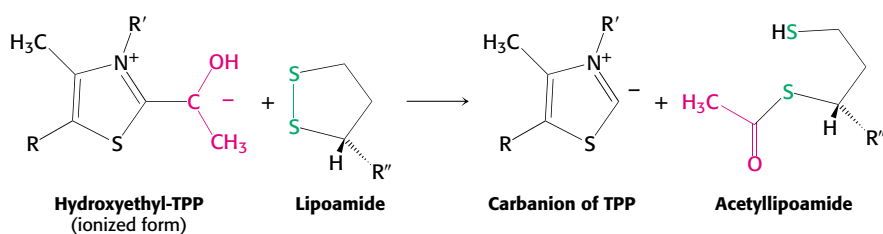


FIGURE 17.6 Mechanism of the decarboxylation reaction of E₁, the pyruvate dehydrogenase component of the pyruvate dehydrogenase complex.

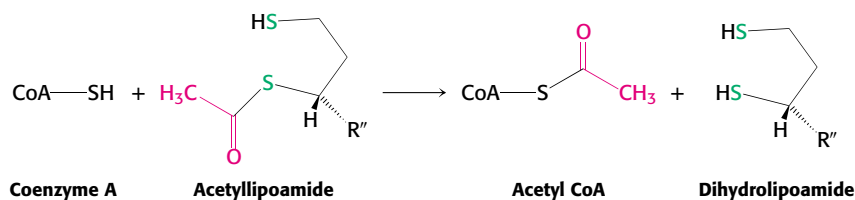
This addition is followed by the decarboxylation of pyruvate. The positively charged ring of TPP acts as an electron sink that stabilizes the negative charge that is transferred to the ring as part of the decarboxylation. Protonation yields hydroxyethyl-TPP.

Second, the hydroxyethyl group attached to TPP is *oxidized* to form an acetyl group and concomitantly *transferred* to lipoamide, a derivative of lipoic acid that is linked to the side chain of a lysine residue by an amide linkage.



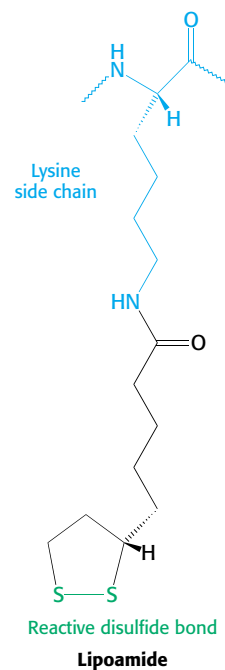
The oxidant in this reaction is the disulfide group of lipoamide, which is reduced to its disulfhydryl form. This reaction, also catalyzed by the pyruvate dehydrogenase component E₁, yields *acetyl-lipoamide*.

Third, the *acetyl group* is transferred from acetyl-lipoamide to CoA to form *acetyl CoA*.

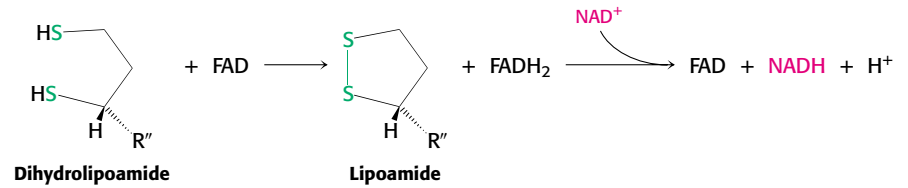


Dihydrolipoyl transacetylase (E₂) catalyzes this reaction. The energy-rich thioester bond is preserved as the acetyl group is transferred to CoA. Recall that CoA serves as a carrier of many activated acyl groups, of which acetyl is the simplest (Section 14.3.1). Acetyl CoA, the fuel for the citric acid cycle, has now been generated from pyruvate.

The pyruvate dehydrogenase complex cannot complete another catalytic cycle until the dihydrolipoamide is oxidized to lipoamide. In a fourth step, the *oxidized form of lipoamide* is regenerated by *dihydrolipoyl dehydrogenase*



(E₃). Two electrons are transferred to an FAD prosthetic group of the enzyme and then to NAD⁺.



This electron transfer to FAD is unusual, because the common role for FAD is to receive electrons from NADH. The electron transfer potential of FAD is altered by its association with the enzyme and enables it to transfer electrons to NAD⁺. Proteins tightly associated with FAD or flavin mononucleotide (FMN) are called *flavoproteins*.

17.1.2 Flexible Linkages Allow Lipoamide to Move Between Different Active Sites

Although the structure of an intact member of the pyruvate dehydrogenase complex family has not yet been determined in atomic detail, the structures of all of the component enzymes are now known, albeit from different complexes and species. Thus, it is now possible to construct an atomic model of the complex to understand its activity (Figure 17.7).

The core of the complex is formed by E₂. Acetyltransferase consists of eight catalytic trimers assembled to form a hollow cube. Each of the three subunits forming a trimer has three major domains (Figure 17.8). At the amino terminus is a small domain that contains a bound lipoamide cofactor attached to a lysine residue. This domain is homologous to biotin-binding domains such as that of pyruvate carboxylase (see Figure 16.26). The lipoamide domain is followed by a small domain that interacts with E₃ within the complex. A larger transacetylase domain completes an E₂ subunit. E₁ is an α₂ β₂ tetramer, and E₃ is an αβ dimer. Twenty-four copies of

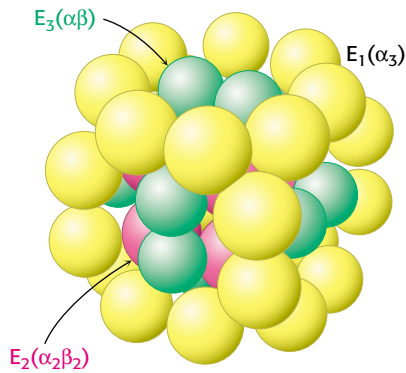


FIGURE 17.7 Schematic representation of the pyruvate dehydrogenase complex. The transacetylase core (E₂) is shown in red, the pyruvate dehydrogenase component (E₁) in yellow, and the dihydrolipoyl dehydrogenase (E₃) in green.

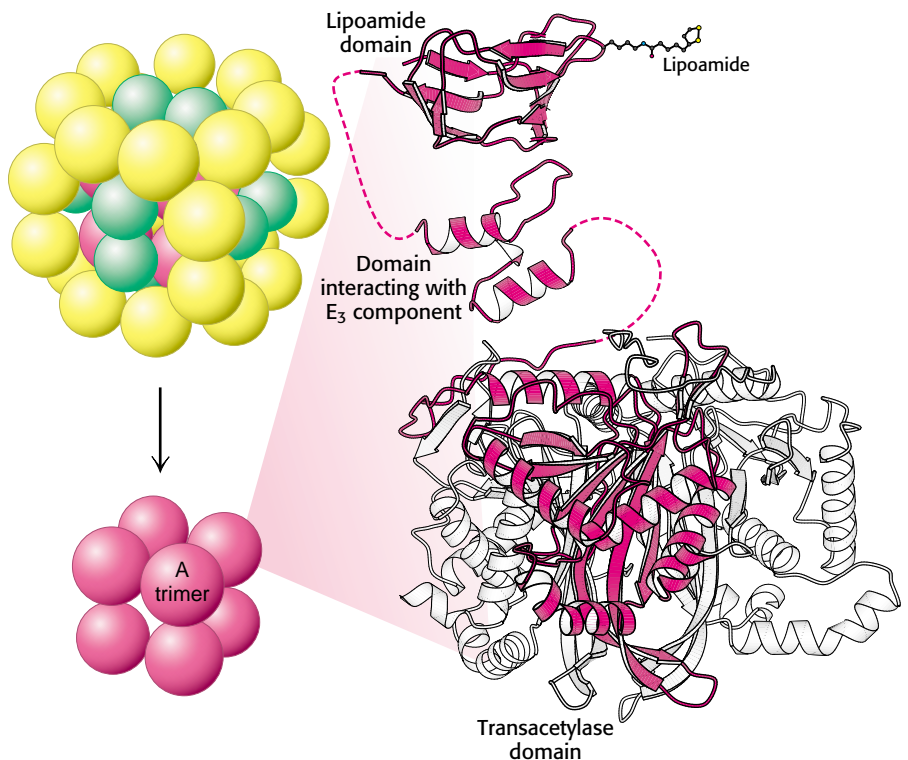


FIGURE 17.8 Structure of the transacetylase (E₂) core. Each red ball represents a trimer of three E₂ subunits. Each subunit consists of three domains: a lipoamide-binding domain, a small domain for interaction with E₃, and a large transacetylase catalytic domain. All three subunits of the transacetylase domain are shown in the ribbon representation, with one depicted in red.

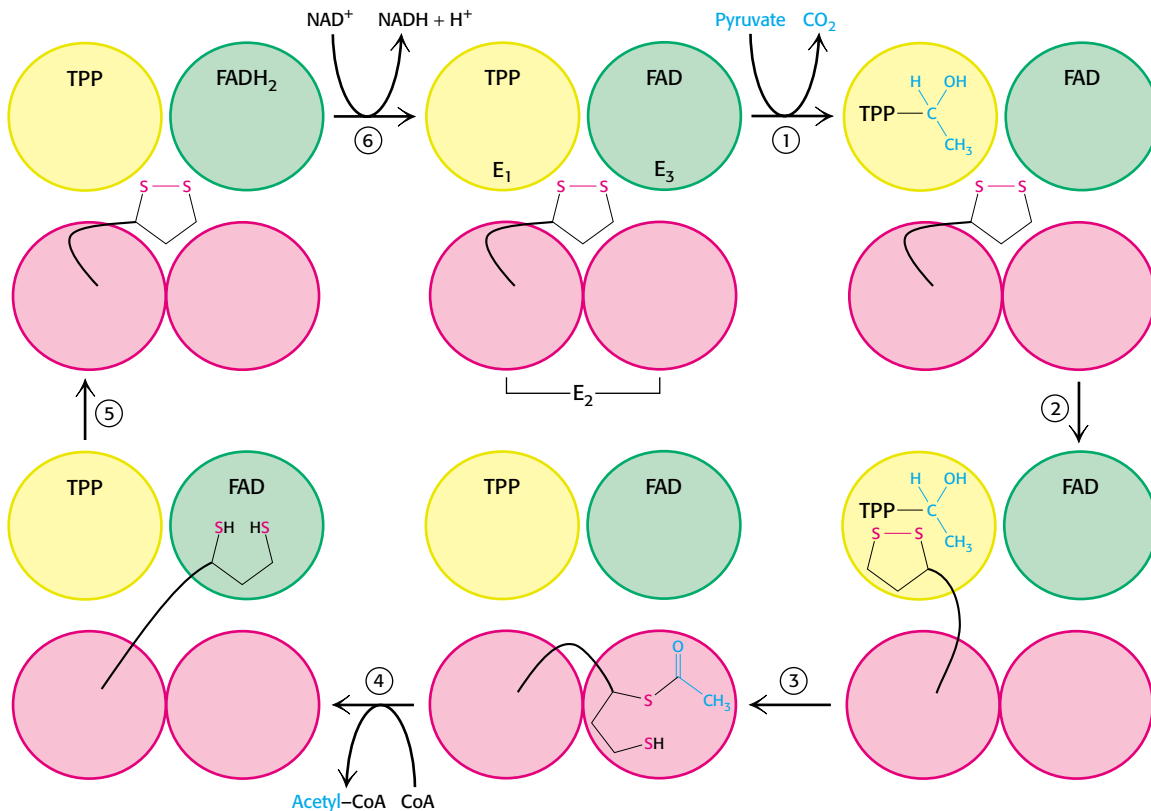


FIGURE 17.9 Reactions of the pyruvate dehydrogenase complex. At the top (center), the enzyme (represented by a yellow, a blue, and two red spheres) is unmodified and ready for a catalytic cycle. (1) Pyruvate is decarboxylated to form the hydroxyethyl TPP. (2) The dihydrolipoyl arm of E_2 moves into the active site of E_1 . (3) E_1 catalyzes the transfer of the two-carbon group to the dihydrolipoyl group to form the acetyl-lipoyl complex. (4) E_2 catalyzes the transfer of the acetyl moiety to CoA to form the product acetyl CoA. The disulfhydryl lipoyl arm then swings to the active site of E_3 . E_3 catalyzes (5) the reduction of the lipoyl acid and (6) the transfer of the protons and electrons to NAD^+ to complete the reaction cycle.

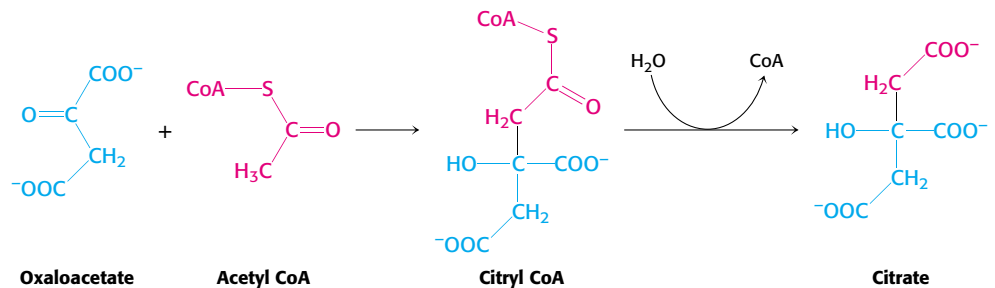
E_1 and 12 copies of E_3 surround the E_2 core. How do the three distinct active sites work in concert (Figure 17.9)?

1. Pyruvate is decarboxylated at the active site of E_1 , forming the substituted TPP intermediate, and CO_2 leaves as the first product. This active site lies within the E_1 complex, connected to the enzyme surface by a 20-Å-long hydrophobic channel.
2. E_2 inserts the lipoyl-lysine arm of the lipoamide domain into the channel in E_1 .
3. E_1 catalyzes the transfer of the acetyl group to the lipoamide. The acetylated lipoyl-lysine arm then leaves E_1 and enters the E_2 cube through 30 Å windows on the sides of the cube to visit the active site of E_2 , located deep in the cube at the subunit interface.
4. The acetyl moiety is then transferred to CoA, and the second product, acetyl CoA, leaves the cube. The reduced lipoyl-lysine arm then swings to the active site of the E_3 flavoprotein.
5. At the E_3 active site, the lipoamide acid is oxidized by coenzyme FAD.
6. The final product, NADH, is produced with the reoxidation of $FADH_2$, and the reactivated lipoamide is ready to begin another reaction cycle.

The structural integration of three kinds of enzymes makes the coordinated catalysis of a complex reaction possible. The proximity of one enzyme to another increases the overall reaction rate and minimizes side reactions. All the intermediates in the oxidative decarboxylation of pyruvate are tightly bound to the complex and are readily transferred because of the ability of the lipoyl-lysine arm of E_2 to call on each active site in turn.

17.1.3 Citrate Synthase Forms Citrate from Oxaloacetate and Acetyl Coenzyme A

The citric acid cycle begins with the condensation of a four-carbon unit, oxaloacetate, and a two-carbon unit, the acetyl group of acetyl CoA. Oxaloacetate reacts with acetyl CoA and H_2O to yield citrate and CoA.



Synthase—

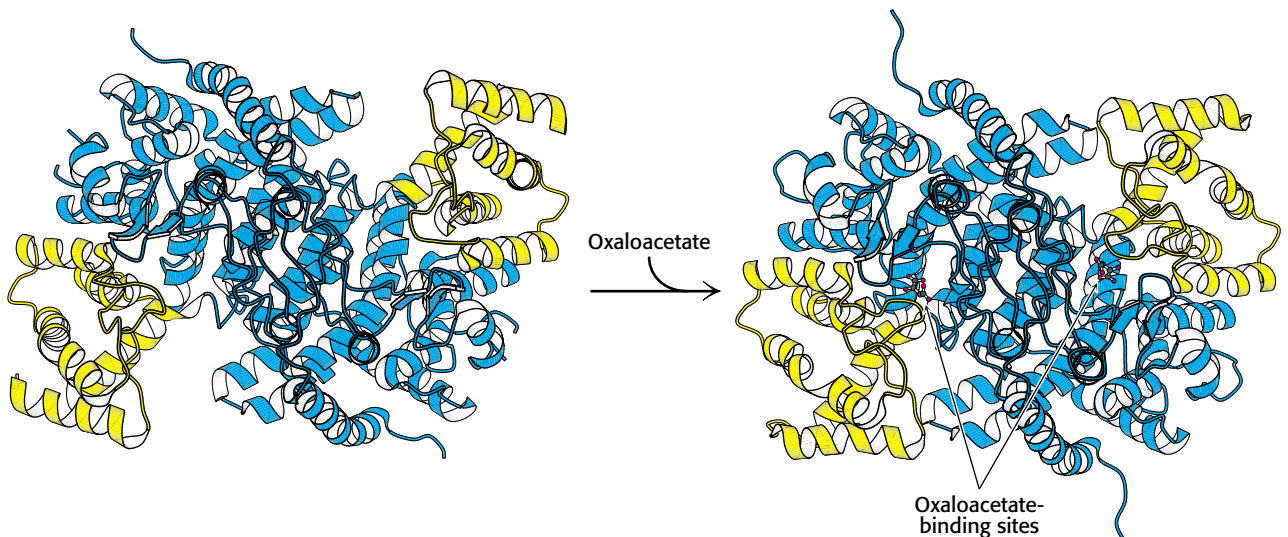
An enzyme catalyzing a synthetic reaction in which two units are joined without the direct participation of ATP (or another nucleoside triphosphate).

This reaction, which is an aldol condensation followed by a hydrolysis, is catalyzed by *citrate synthase*. Oxaloacetate first condenses with acetyl CoA to form *citryl CoA*, which is then hydrolyzed to citrate and CoA. The hydrolysis of citryl CoA, a high-energy thioester intermediate, drives the overall reaction far in the direction of the synthesis of citrate. In essence, the hydrolysis of the thioester powers the synthesis of a new molecule from two precursors. Because this reaction initiates the cycle, it is very important that side reactions be minimized. Let us briefly consider the how citrate synthase prevents wasteful processes such as the hydrolysis of acetyl CoA.

Mammalian citrate synthase is a dimer of identical 49-kd subunits. Each active site is located in a cleft between the large and small domains of a subunit, adjacent to the subunit interface. The results of x-ray crystallographic studies of citrate synthase and its complexes with several substrates and inhibitors revealed that the enzyme undergoes large conformational changes in the course of catalysis. Citrate synthase exhibits sequential, ordered



FIGURE 17.10 Conformational changes in citrate synthase on binding oxaloacetate. The small domain of each subunit of the homodimer is shown in yellow; the large domain is shown in blue. (Left) Open form of enzyme alone. (Right) Closed form of the liganded enzyme.



kinetics: oxaloacetate binds first, followed by acetyl CoA. The reason for the ordered binding is that *oxaloacetate induces a major structural rearrangement leading to the creation of a binding site for acetyl CoA*. The open form of the enzyme observed in the absence of ligands is converted into a closed form by the binding of oxaloacetate (Figure 17.10). In each subunit, the small domain rotates 19 degrees relative to the large domain. *Movements as large as 15 Å are produced by the rotation of α helices elicited by quite small shifts of side chains around bound oxaloacetate*. This conformational transition is reminiscent of the cleft closure in hexokinase induced by the binding of glucose (Section 16.1.1).

Citrate synthase catalyzes the condensation reaction by bringing the substrates into close proximity, orienting them, and polarizing certain bonds. Two histidine residues and an aspartate residue are important players (Figure 17.11). One of the histidine residues (His 274) donates a proton to the carbonyl oxygen of acetyl CoA to promote the removal of a methyl proton by Asp 375. Oxaloacetate is activated by the transfer of a proton from His 320 to its carbonyl carbon atom. The concomitant attack of the enol of acetyl CoA on the carbonyl carbon of oxaloacetate results in the formation of a carbon–carbon bond. The newly formed citryl CoA induces additional structural changes in the enzyme. The active site becomes completely enclosed. His 274 participates again as a proton donor to hydrolyze the thioester. Coenzyme A leaves the enzyme, followed by citrate, and the enzyme returns to the initial open conformation.

We can now understand how the wasteful hydrolysis of acetyl CoA is prevented. Citrate synthase is well suited to hydrolyze *citryl* CoA but not *acetyl* CoA. How is this discrimination accomplished? First, acetyl CoA does not bind to the enzyme until oxaloacetate is bound and ready for condensation. Second, the catalytic residues crucial for hydrolysis of the thioester linkage are not appropriately positioned *until citryl CoA is formed*. As with hexokinase (Section 16.1.1) and triose phosphate isomerase (Section 16.1.4), *induced fit prevents an undesirable side reaction*.

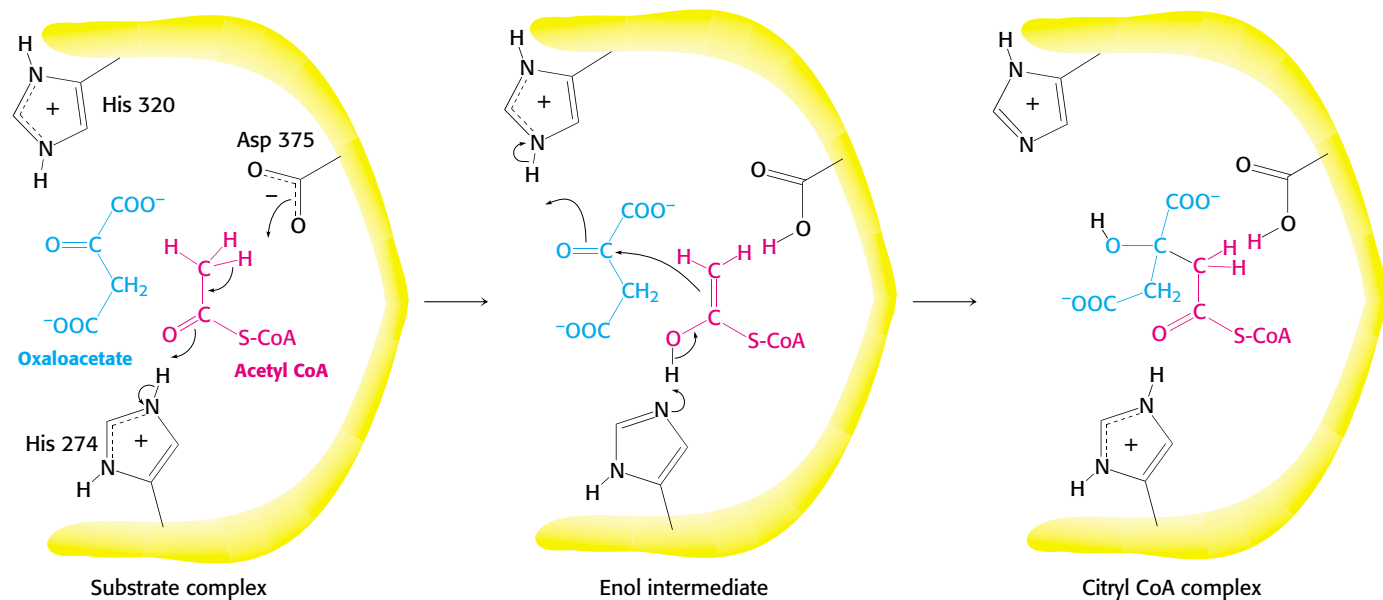
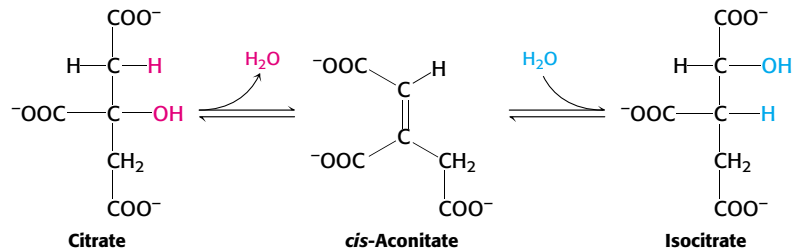


FIGURE 17.11 Mechanism of synthesis of citryl CoA by citrate synthase. The condensation of oxaloacetate and acetyl CoA proceeds through an enol intermediate. The subsequent hydrolysis of citryl CoA yields citrate and CoA.

17.1.4 Citrate Is Isomerized into Isocitrate

The tertiary hydroxyl group is not properly located in the citrate molecule for the oxidative decarboxylations that follow. Thus, citrate is isomerized into isocitrate to enable the six-carbon unit to undergo oxidative decarboxylation.

The isomerization of citrate is accomplished by a *dehydration* step followed by a *hydration* step. The result is an interchange of a hydrogen atom and a hydroxyl group. The enzyme catalyzing both steps is called *aconitase* because *cis-aconitate* is an intermediate.



Aconitase is an *iron-sulfur protein*, or *nonheme iron protein*. It contains four iron atoms that are not incorporated as part of a heme group. The four iron atoms are complexed to four inorganic sulfides and three cysteine sulfur atoms, leaving one iron atom available to bind citrate and then isocitrate through their carboxylate and hydroxyl groups (Figure 17.12). This iron center, in conjunction with other groups on the enzyme, facilitates the dehydration and rehydration reactions. We will consider the role of these iron-sulfur clusters in the electron-transfer reactions of oxidative phosphorylation subsequently (Section 18.3.1).

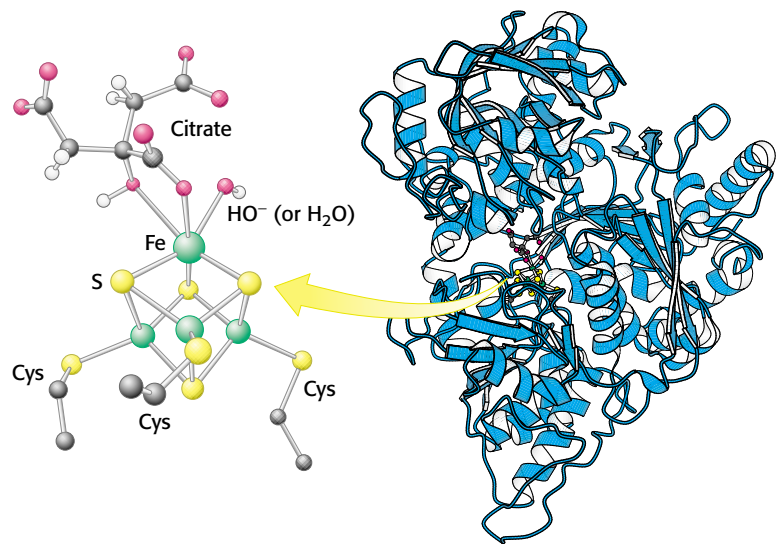


FIGURE 17.12 Binding of citrate to the iron-sulfur complex of aconitase. A 4Fe-4S iron-sulfur cluster is a component of the active site of aconitase. One of the iron atoms of the cluster binds to the carboxylate and hydroxyl groups of citrate.

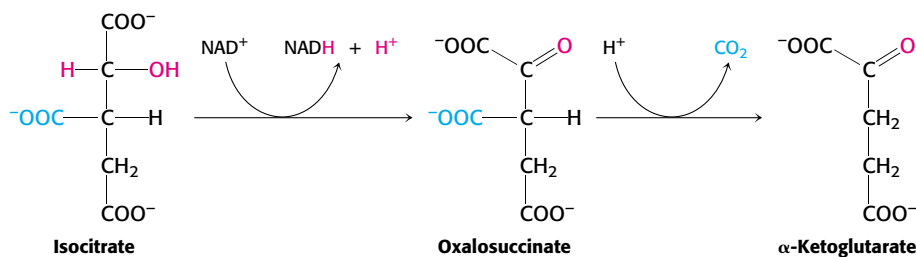
The iron-sulfur cluster in aconitase is somewhat unstable, so one or more iron atoms dissociate under conditions of low iron availability in the cell. Remarkably, this sensitivity to iron level was exploited in the evolution of a mechanism for regulating gene expression in response to iron levels, as will be discussed in Chapter 31.

17.1.5 Isocitrate Is Oxidized and Decarboxylated to α -Ketoglutarate

We come now to the first of four oxidation-reduction reactions in the citric acid cycle. The oxidative decarboxylation of isocitrate is catalyzed by *isocitrate dehydrogenase*.



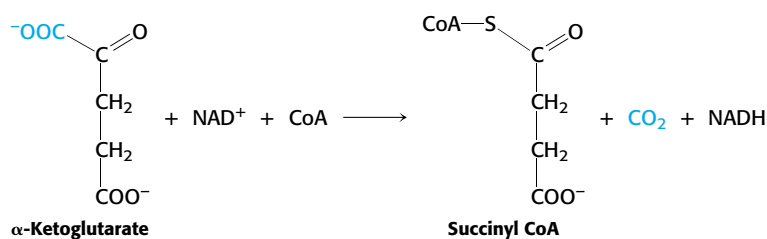
The intermediate in this reaction is oxalosuccinate, an unstable β -ketoacid. While bound to the enzyme, it loses CO_2 to form α -ketoglutarate.



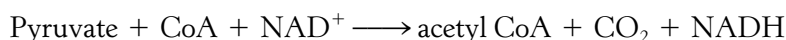
The rate of formation of α -ketoglutarate is important in determining the overall rate of the cycle, as will be discussed in Section 17.2.2. This oxidation generates the first high-transfer-potential electron carrier NADH in the cycle.

17.1.6 Succinyl Coenzyme A Is Formed by the Oxidative Decarboxylation of α -Ketoglutarate

The conversion of isocitrate into α -ketoglutarate is followed by a second oxidative decarboxylation reaction, the formation of succinyl CoA from α -ketoglutarate.



The oxidative decarboxylation of α -ketoglutarate closely resembles that of pyruvate, also an α -ketoacid.

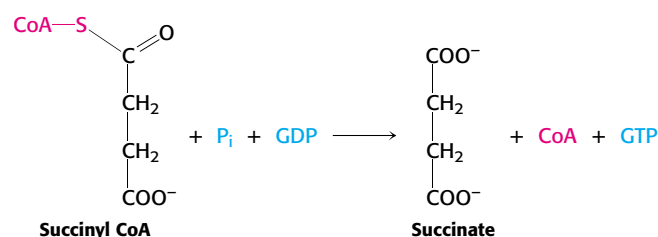


Both reactions include the decarboxylation of an α -ketoacid and the subsequent formation of a high-transfer-potential thioester linkage with CoA. The complex that catalyzes the oxidative decarboxylation of α -ketoglutarate is homologous to the pyruvate dehydrogenase complex, and the reaction mechanism is entirely analogous. The α -ketoglutarate dehydrogenase component (E_2) and transsuccinylase (E_1) are different from but homologous to the corresponding enzymes in the pyruvate dehydrogenase complex, whereas the dihydrolipoyl dehydrogenase components (E_3) of the two complexes are identical.

17.1.7 A High Phosphoryl-Transfer Potential Compound Is Generated from Succinyl Coenzyme A

Succinyl CoA is an energy-rich thioester compound. The $\Delta G^{\circ'}$ for the hydrolysis of succinyl CoA is about -8 kcal mol^{-1} ($-33.5 \text{ kJ mol}^{-1}$), which is comparable to that of ATP ($-7.3 \text{ kcal mol}^{-1}$, or $-30.5 \text{ kJ mol}^{-1}$). In the citrate synthase reaction, the cleavage of the thioester bond powers the synthesis of the six-carbon citrate from the four-carbon oxaloacetate and the two-carbon fragment.

The cleavage of the thioester bond of succinyl CoA is coupled to the phosphorylation of a purine nucleoside diphosphate, usually GDP. This reaction is catalyzed by succinyl CoA synthetase (succinate thiokinase). This enzyme is an $\alpha_2\beta_2$ heterodimer; the functional unit is one $\alpha\beta$ pair. The mechanism is a clear example of energy transformations: energy inherent in



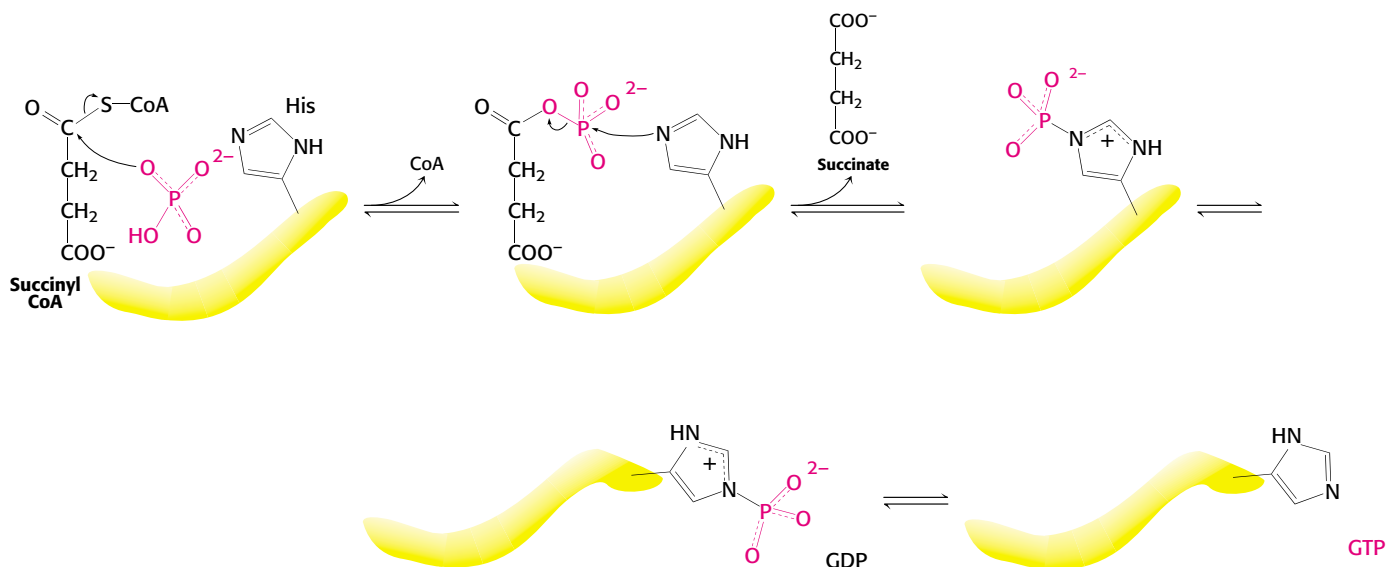
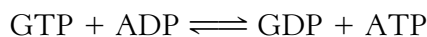



FIGURE 17.13 Reaction mechanism of succinyl CoA synthetase. The formation of GTP at the expense of succinyl CoA is an example of substrate-level phosphorylation. The reaction proceeds through a phosphorylated enzyme intermediate.

the thioester molecule is transformed into phosphoryl-group transfer potential (Figure 17.13). The first step is the displacement of coenzyme A by orthophosphate, which generates another energy-rich compound, succinyl phosphate. A histidine residue of the α subunit removes the phosphoryl group with the concomitant generation of succinate and phosphohistidine. The phosphohistidine residue then swings over to a bound nucleoside diphosphate and the phosphoryl group is transferred to form the nucleoside triphosphate. The participation of high-energy compounds in all the steps is attested to by the fact that the reaction is readily reversible: $\Delta G^{\circ} = -0.8 \text{ kcal mol}^{-1}$ (-3.4 kJ mol^{-1}). This is the only step in the citric acid cycle that directly yields a compound with high phosphoryl transfer potential through a substrate-level phosphorylation. Some mammalian succinyl CoA synthetases are specific for GDP and others for ADP. The *E. coli* enzyme uses either GDP or ADP as the phosphoryl-group acceptor. We have already seen that GTP is an important component of signal-transduction systems (Chapter 15). Alternatively, its γ -phosphoryl group can be readily transferred to ADP to form ATP, in a reaction catalyzed by *nucleoside diphosphokinase*.



 The mechanism of succinyl CoA synthetase reveals that a phosphoryl group is transferred first to succinyl CoA bound in the α subunit and then to a nucleoside diphosphate bound in the β subunit. Examination of the three-dimensional structure of succinyl CoA synthetase shows that each subunit comprises two domains (Figure 17.14). The carboxyl-terminal domains of the two subunits are similar to one another, whereas the amino-terminal domains have different structures, each characteristic of its role in the mechanism. The amino-terminal domain of the α subunit forms a Rossmann fold (Section 16.1.10), which binds the ADP component of succinyl CoA, whereas the amino-terminal domain of the β subunit is an ATP-grasp domain, a nucleotide-activating domain found in many enzymes, especially those catalyzing purine biosynthesis (Section 16.3.2 and Chapter 25). Succinyl CoA synthetase has evolved by adopting these domains and harnessing them to allow the capture of the energy associated with succinyl CoA cleavage to drive the generation of a nucleoside triphosphate.

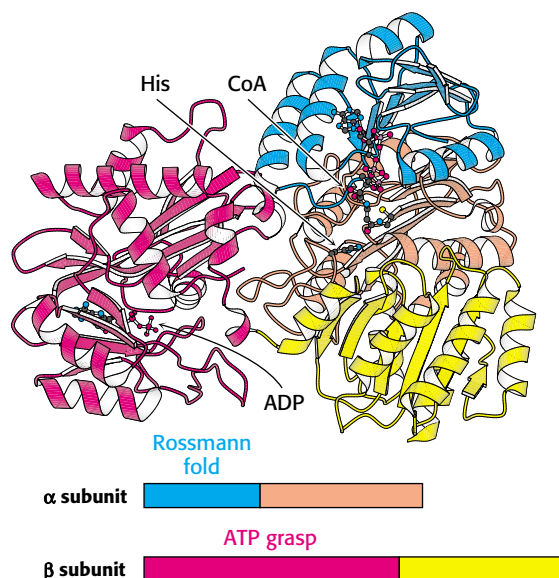
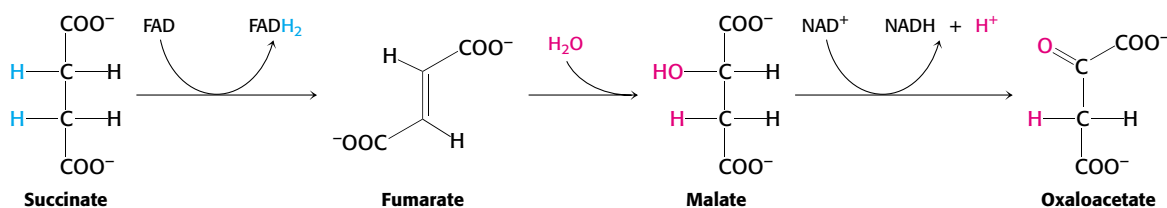


FIGURE 17.14 Structure of succinyl CoA synthetase. The enzyme is composed of two subunits. The α subunit contains a Rossmann fold that binds the ADP component of CoA, and the β subunit contains a nucleotide-activating region called the ATP-grasp domain. The ATP-grasp domain is shown here binding a molecule of ADP. The histidine residue picks up the phosphoryl group from near the CoA and swings over to transfer it to the nucleotide bound in the ATP-grasp domain.

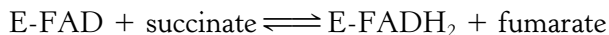
17.1.8 Oxaloacetate Is Regenerated by the Oxidation of Succinate

Reactions of four-carbon compounds constitute the final stage of the citric acid cycle: the regeneration of oxaloacetate.



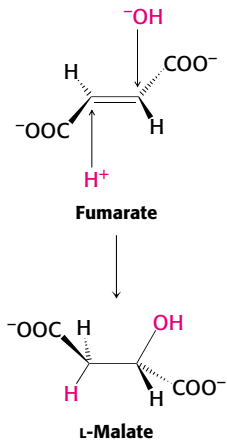
The reactions constitute a metabolic motif that we will see again in fatty acid synthesis and degradation as well as in the degradation of some amino acids (see Figure 14.17). A methylene group (CH₂) is converted into a carbonyl group (C=O) in three steps: an oxidation, a hydration, and a second oxidation reaction. Not only is oxaloacetate thereby regenerated for another round of the cycle, but also more energy is extracted in the form of FADH₂ and NADH.

Succinate is oxidized to fumarate by *succinate dehydrogenase*. The hydrogen acceptor is FAD rather than NAD⁺, which is used in the other three oxidation reactions in the cycle. In succinate dehydrogenase, the isoalloxazine ring of FAD is covalently attached to a histidine side chain of the enzyme (denoted E-FAD).



FAD is the hydrogen acceptor in this reaction because the free-energy change is insufficient to reduce NAD⁺. FAD is nearly always the electron acceptor in oxidations that remove two hydrogen atoms from a substrate.

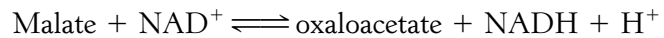
Succinate dehydrogenase, like aconitase, is an iron-sulfur protein. Indeed, succinate dehydrogenase contains three different kinds of iron-sulfur clusters, 2Fe-2S (two iron atoms bonded to two inorganic sulfides), 3Fe-4S, and 4Fe-4S. Succinate dehydrogenase—which consists of two subunits, one 70 kd and the other 27 kd—differs from other enzymes in the citric acid cycle in being embedded in the inner mitochondrial membrane. In fact, *succinate dehydrogenase is directly associated with the electron-transport chain, the link between the citric acid cycle and ATP formation*. FADH₂ produced by the



oxidation of succinate does not dissociate from the enzyme, in contrast with NADH produced in other oxidation–reduction reactions. Rather, two electrons are transferred from FADH₂ directly to iron–sulfur clusters of the enzyme. The ultimate acceptor of these electrons is molecular oxygen, as we shall see in Chapter 18.

The next step is the hydration of fumarate to form L-malate. *Fumarase* catalyzes a stereospecific trans addition of a hydrogen atom and a hydroxyl group. The hydroxyl group adds to only one side of the double bond of fumarate; hence, only the L isomer of malate is formed.

Finally, malate is oxidized to form oxaloacetate. This reaction is catalyzed by *malate dehydrogenase*, and NAD⁺ is again the hydrogen acceptor.



Note that the standard free energy for this reaction, unlike that for the other steps in the citric acid cycle, is significantly positive. The oxidation of malate is driven by the utilization of the products—oxaloacetate by citrate synthase and NADH by the electron-transport chain.

17.1.9 Stoichiometry of the Citric Acid Cycle

The net reaction of the citric acid cycle is:



Let us recapitulate the reactions that give this stoichiometry (Figure 17.15 and Table 17.2):

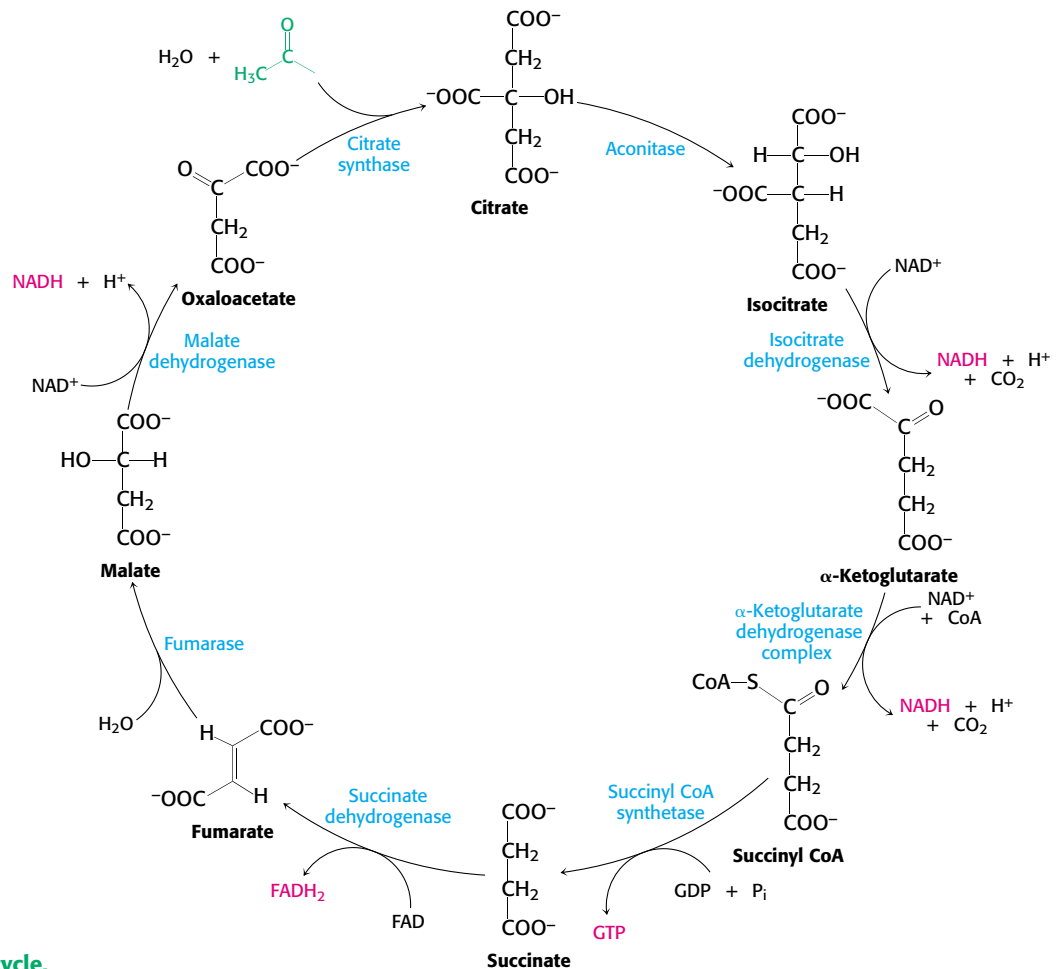


FIGURE 17.15 The citric acid cycle.

TABLE 17.2 Citric acid cycle

Step	Reaction	Enzyme	Prosthetic group	Type*	$\Delta G^{\circ'}$	
					kcal mol ⁻¹	kJ mol ⁻¹
1	Acetyl CoA + oxaloacetate + H ₂ O \longrightarrow citrate + CoA + H ⁺	Citrate synthase		a	-7.5	-31.4
2a	Citrate \rightleftharpoons <i>cis</i> -aconitate + H ₂ O	Aconitase	Fe-S	b	+2.0	+8.4
2b	<i>cis</i> -Aconitate + H ₂ O \rightleftharpoons isocitrate	Aconitase	Fe-S	c	-0.5	-2.1
3	Isocitrate + NAD ⁺ \rightleftharpoons α -ketoglutarate + CO ₂ + NADH	Isocitrate dehydrogenase		d + e	-2.0	-8.4
4	α -Ketoglutarate + NAD ⁺ + CoA \rightleftharpoons succinyl CoA + CO ₂ + NADH	α -Ketoglutarate dehydrogenase complex	Lipoic acid, FAD, TPP	d + e	-7.2	-30.1
5	Succinyl CoA + P _i + GDP \rightleftharpoons succinate + GTP + CoA	Succinyl CoA synthetase		f	-0.8	-3.3
6	Succinate + FAD (enzyme-bound) \rightleftharpoons fumarate + FADH ₂ (enzyme-bound)	Succinate dehydrogenase	FAD, Fe-S	e	~0	0
7	Fumarate + H ₂ O \rightleftharpoons L-malate	Fumarase		c	-0.9	-3.8
8	L-Malate + NAD ⁺ \rightleftharpoons oxaloacetate + NADH + H ⁺	Malate dehydrogenase		e	+7.1	+29.7

*Reaction type: (a) condensation; (b) dehydration; (c) hydration; (d) decarboxylation; (e) oxidation; (f) substrate-level phosphorylation.

1. Two carbon atoms enter the cycle in the condensation of an acetyl unit (from acetyl CoA) with oxaloacetate. Two carbon atoms leave the cycle in the form of CO₂ in the successive decarboxylations catalyzed by isocitrate dehydrogenase and α -ketoglutarate dehydrogenase. Interestingly, the results of isotope-labeling studies revealed that the two carbon atoms that enter each cycle are not the ones that leave.

2. Four pairs of hydrogen atoms leave the cycle in four oxidation reactions. Two molecules of NAD⁺ are reduced in the oxidative decarboxylations of isocitrate and α -ketoglutarate, one molecule of FAD is reduced in the oxidation of succinate, and one molecule of NAD⁺ is reduced in the oxidation of malate.

3. One compound with high phosphoryl transfer potential, usually GTP, is generated from the cleavage of the thioester linkage in succinyl CoA.

4. Two molecules of water are consumed: one in the synthesis of citrate by the hydrolysis of citryl CoA and the other in the hydration of fumarate.

Recall also that NADH is generated in the formation of acetyl CoA from pyruvate by the pyruvate dehydrogenase reaction.

The efficiency of the citric acid cycle may be enhanced by the arrangement of the constituent enzymes. Evidence is accumulating that the enzymes are physically associated with one another to facilitate substrate channeling between active sites. The word *metabolon* has been suggested as the name for such multienzyme complexes.

As will be discussed in Chapter 18, the electron-transport chain oxidizes the NADH and FADH₂ formed in the citric acid cycle. The transfer of electrons from these carriers to O₂, the ultimate electron acceptor, leads to the generation of a proton gradient across the inner mitochondrial membrane. This proton-motive force then powers the generation of ATP; the net

stoichiometry is about 2.5 ATP per NADH, and 1.5 ATP per FADH_2 . Consequently, 9 high-transfer-potential phosphoryl groups are generated when the electron-transport chain oxidizes 3 molecules of NADH and 1 molecule of FADH_2 , and 1 high-transfer-potential phosphoryl group per acetyl unit is directly formed in the citric acid cycle. Thus, 1 acetate unit generates approximately 10 molecules of ATP. In dramatic contrast, only 2 molecules of ATP are generated per molecule of glucose (which generates 2 molecules of acetyl CoA) by anaerobic glycolysis.

Recall that molecular oxygen does not participate directly in the citric acid cycle. However, the cycle operates only under aerobic conditions because NAD^+ and FAD can be regenerated in the mitochondrion only by the transfer of electrons to molecular oxygen. *Glycolysis has both an aerobic and an anaerobic mode, whereas the citric acid cycle is strictly aerobic.* Glycolysis can proceed under anaerobic conditions because NAD^+ is regenerated in the conversion of pyruvate into lactate.

17.2 ENTRY TO THE CITRIC ACID CYCLE AND METABOLISM THROUGH IT ARE CONTROLLED

The citric acid cycle is the final common pathway for the aerobic oxidation of fuel molecules. Moreover, as we will see shortly (Section 17.3) and repeatedly elsewhere in our study of biochemistry, the cycle is an important source of building blocks for a host of important biomolecules. As befits its role as the metabolic hub of the cell, entry into the cycle and the rate of the cycle itself are controlled at several stages.

17.2.1 The Pyruvate Dehydrogenase Complex Is Regulated Allosterically and by Reversible Phosphorylation

As we saw earlier, glucose can be formed from pyruvate (Section 16.3). However, the formation of acetyl CoA from pyruvate is an irreversible step in animals and thus they are unable to convert acetyl CoA back into glucose. The oxidative decarboxylation of pyruvate to acetyl CoA commits the carbon atoms of glucose to two principal fates: oxidation to CO_2 by the citric acid cycle, with the concomitant generation of energy, or incorporation into lipid (Figure 17.16). As expected of an enzyme at a critical branch point in metabolism, the activity of the pyruvate dehydrogenase complex is stringently controlled by several means (Figure 17.17). High concentrations of reaction products of the complex inhibit the reaction: acetyl CoA inhibits the transacetylase component (E_2), whereas NADH inhibits the dihydrolipoyl

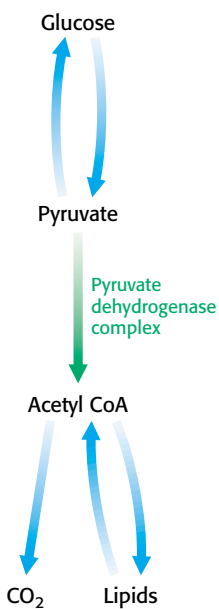
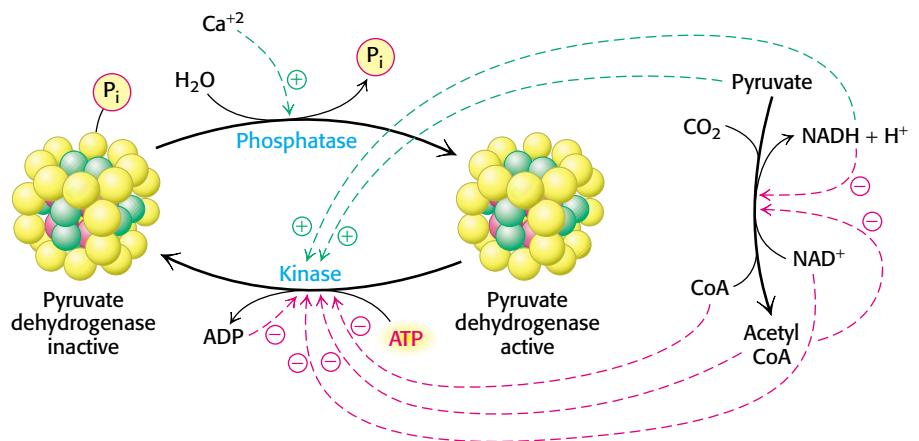


FIGURE 17.16 From glucose to acetyl CoA. The synthesis of acetyl CoA by the pyruvate dehydrogenase complex is a key irreversible step in the metabolism of glucose.

FIGURE 17.17 Regulation of the pyruvate dehydrogenase complex. The complex is inhibited by its immediate products, NADH and acetyl CoA. The pyruvate dehydrogenase component is also regulated by covalent modification. A specific kinase phosphorylates and inactivates pyruvate dehydrogenase, and a phosphatase activates the dehydrogenase by removing the phosphoryl. The kinase and the phosphatase also are highly regulated enzymes.



dehydrogenase (E_3). However, the key means of regulation in eukaryotes is covalent modification of the pyruvate dehydrogenase component. *Phosphorylation of the pyruvate dehydrogenase component (E_1) by a specific kinase switches off the activity of the complex. Deactivation is reversed by the action of a specific phosphatase.* The site of phosphorylation is the transacetylase component (E_2), again highlighting the structural and mechanistic importance of this core. Increasing the NADH/NAD^+ , acetyl CoA/CoA, or ATP/ADP ratio promotes phosphorylation and, hence, deactivation of the complex. In other words, high concentrations of immediate (acetyl CoA and NADH) and ultimate (ATP) products inhibit the activity. Thus, *pyruvate dehydrogenase is switched off when the energy charge is high and biosynthetic intermediates are abundant.* On the other hand, pyruvate as well as ADP (a signal of low energy charge) activate the dehydrogenase by inhibiting the kinase.

In contrast, α_1 -adrenergic agonists and hormones such as vasopressin stimulate pyruvate dehydrogenase by triggering a rise in the cytosolic Ca^{2+} level (Section 15.3.4), which in turn elevates the mitochondrial Ca^{2+} level. The rise in mitochondrial Ca^{2+} activates the pyruvate dehydrogenase complex by stimulating the phosphatase. Insulin also accelerates the conversion of pyruvate into acetyl CoA by stimulating the dephosphorylation of the complex. In turn, glucose is funneled into pyruvate.



The importance of this covalent control is illustrated in people with a phosphatase deficiency. Because pyruvate dehydrogenase is always phosphorylated and thus inactive, glucose is processed to lactic acid. This condition results in unremitting lactic acidosis (high blood levels of lactic acid), which leads to the malfunctioning of many tissues, most notably the central nervous system (Section 17.3.2).

17.2.2 The Citric Acid Cycle Is Controlled at Several Points

The rate of the citric acid cycle is precisely adjusted to meet an animal cell's needs for ATP (Figure 17.18). The primary control points are the allosteric enzymes isocitrate dehydrogenase and α -ketoglutarate dehydrogenase.

Isocitrate dehydrogenase is allosterically stimulated by ADP, which enhances the enzyme's affinity for substrates. The binding of isocitrate, NAD^+ , Mg^{2+} , and ADP is mutually cooperative. In contrast, NADH inhibits isocitrate dehydrogenase by directly displacing NAD^+ . ATP, too, is inhibitory. It is important to note that several steps in the cycle require NAD^+ or FAD, which are abundant only when the energy charge is low.

A second control site in the citric acid cycle is α -ketoglutarate dehydrogenase. Some aspects of this enzyme's control are like those of the pyruvate dehydrogenase complex, as might be expected from the homology of the two enzymes. α -Ketoglutarate dehydrogenase is inhibited by succinyl CoA and NADH, the products of the reaction that it catalyzes. In addition, α -ketoglutarate dehydrogenase is inhibited by a high energy charge. Thus, *the rate of the cycle is reduced when the cell has a high level of ATP.*

In many bacteria, the funneling of two-carbon fragments into the cycle also is controlled. *The synthesis of citrate from oxaloacetate and acetyl CoA carbon units is an important control point in these organisms.* ATP is an allosteric inhibitor of citrate synthase. The effect of ATP is to increase the value of K_M for acetyl CoA. Thus, as the level of ATP increases, less of this enzyme is saturated with acetyl CoA and so less citrate is formed.

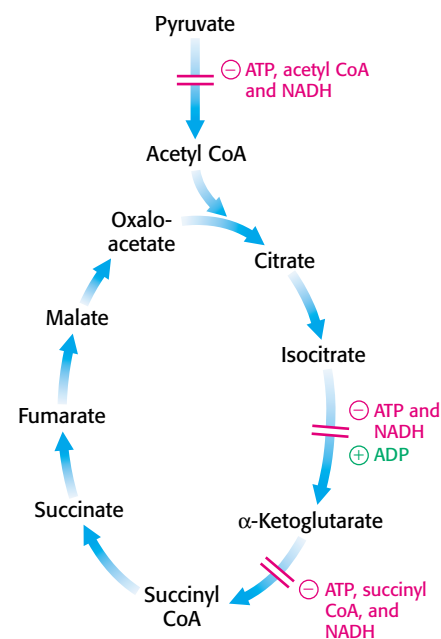


FIGURE 17.18 Control of the citric acid cycle. The citric acid cycle is regulated primarily by the concentration of ATP and NADH. The key control points are the enzymes isocitrate dehydrogenase and α -ketoglutarate dehydrogenase.

17.3 THE CITRIC ACID CYCLE IS A SOURCE OF BIOSYNTHETIC PRECURSORS

Thus far, discussion has focused on the citric acid cycle as the *major degradative pathway for the generation of ATP*. As a major metabolic hub of the cell, the citric acid cycle also *provides intermediates for biosyntheses* (Figure 17.19). For example, most of the carbon atoms in porphyrins come from *succinyl CoA*. Many of the amino acids are derived from α -*ketoglutarate* and *oxaloacetate*. These biosynthetic processes will be discussed in subsequent chapters.

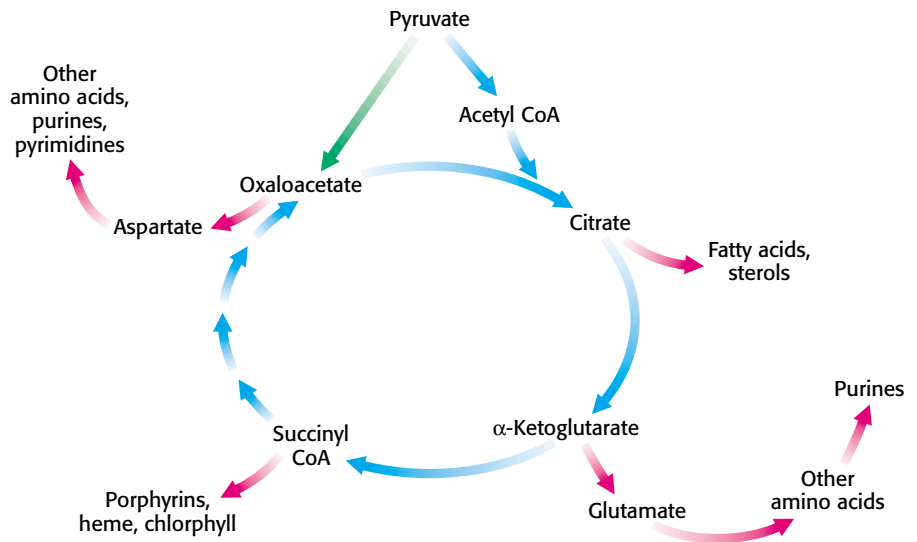
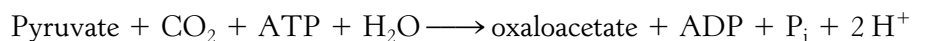


FIGURE 17.19 Biosynthetic roles of the citric acid cycle. Intermediates drawn off for biosyntheses (shown by red arrows) are replenished by the formation of oxaloacetate from pyruvate.

17.3.1 The Citric Acid Cycle Must Be Capable of Being Rapidly Replenished


The important point now is that *citric acid cycle intermediates must be replenished if any are drawn off for biosyntheses*. Suppose that much oxaloacetate is converted into amino acids for protein synthesis and, subsequently, the energy needs of the cell rise. The citric acid cycle will operate to a reduced extent unless new oxaloacetate is formed, because acetyl CoA cannot enter the cycle unless it condenses with oxaloacetate. Even though oxaloacetate is recycled, a minimal level must be maintained to allow the cycle to function.

How is oxaloacetate replenished? Mammals lack the enzymes for the net conversion of acetyl CoA into oxaloacetate or any other citric acid cycle intermediate. Rather, oxaloacetate is formed by the carboxylation of pyruvate, in a reaction catalyzed by the biotin-dependent enzyme pyruvate carboxylase.



Recall that this enzyme plays a crucial role in gluconeogenesis (Section 16.3.2). It is active only in the presence of acetyl CoA, which signifies the need for more oxaloacetate. If the energy charge is high, oxaloacetate is converted into glucose. If the energy charge is low, oxaloacetate replenishes the citric acid cycle. The synthesis of oxaloacetate by the carboxylation of pyruvate is an example of an *anaplerotic reaction* (of Greek origin, meaning to “fill up”), a reaction that leads to the net synthesis, or replenishment, of pathway components. Note that, because the citric acid cycle is a cycle, it can be replenished by the generation of any of the intermediates.

17.3.2 The Disruption of Pyruvate Metabolism Is the Cause of Beriberi and Poisoning by Mercury and Arsenic

 *Beriberi*, a neurologic and cardiovascular disorder, is caused by a dietary deficiency of thiamine (also called *vitamin B₁*). The disease has been and continues to be a serious health problem in the Far East because rice, the major food, has a rather low content of thiamine. This deficiency is partly ameliorated if the whole rice grain is soaked in water before milling—some of the thiamine in the husk then leaches into the rice kernel. The problem is exacerbated if the rice is polished, because only the outer layer contains significant amounts of thiamine. Beriberi is also occasionally seen in alcoholics who are severely malnourished and thus thiamine deficient. The disease is characterized by neurologic and cardiac symptoms. Damage to the peripheral nervous system is expressed as pain in the limbs, weakness of the musculature, and distorted skin sensation. The heart may be enlarged and the cardiac output inadequate.

Which biochemical processes might be affected by a deficiency of thiamine? *Thiamine pyrophosphate is the prosthetic group of three important enzymes: pyruvate dehydrogenase, α -ketoglutarate dehydrogenase, and transketolase.* Transketolase functions in the pentose phosphate pathway, which will be discussed in Chapter 20. *The common feature of enzymatic reactions utilizing TPP is the transfer of an activated aldehyde unit.* In beriberi, the levels of pyruvate and α -ketoglutarate in the blood are higher than normal. The increase in the level of pyruvate in the blood is especially pronounced after the ingestion of glucose. A related finding is that the activities of the pyruvate and α -ketoglutarate dehydrogenase complexes in vivo are abnormally low. The low transketolase activity of red cells in beriberi is an easily measured and reliable diagnostic indicator of the disease.

Why does TPP deficiency lead primarily to neurological disorders? The nervous system relies essentially on glucose as its only fuel. In contrast, most other tissues can use fats as a source of fuel for the citric acid cycle. The product of aerobic glycolysis, pyruvate, can enter the citric acid cycle only through the pyruvate dehydrogenase complex.

Symptoms similar to those of beriberi arise if an organism is exposed to mercury or arsenite (AsO_3^{3-}). Both elements have a high affinity for neighboring sulfhydryls, such as those in the reduced dihydrolipoyl groups of the dihydrolipoyl dehydrogenase component of the pyruvate dehydrogenase complex (Figure 17.20). The binding of mercury or arsenite to the dihydrolipoyl groups inhibits the complex and leads to central nervous system

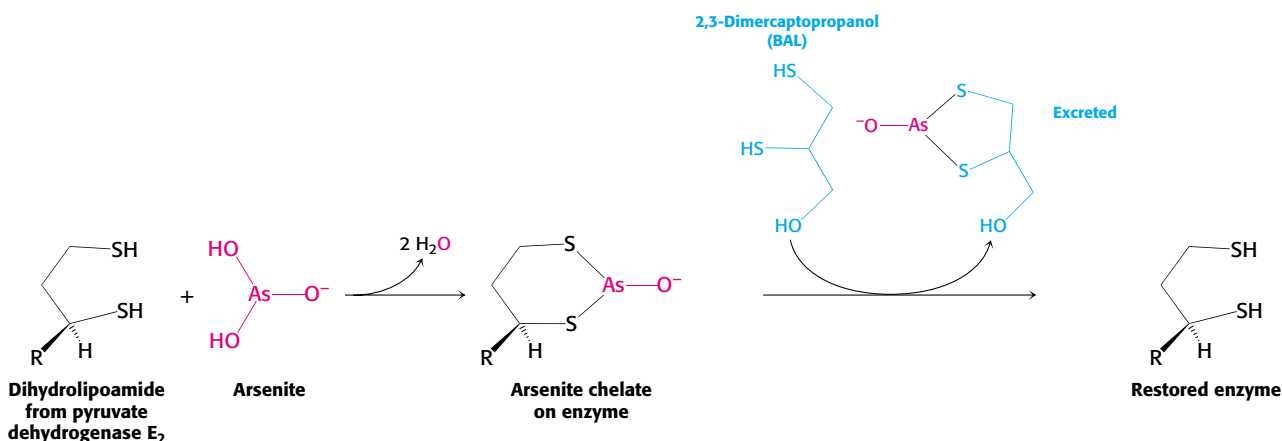
Beriberi—

A vitamin-deficiency disease first described in 1630 by Jacob Bonitus, a Dutch physician working in Java:

“A certain very troublesome affliction, which attacks men, is called by the inhabitants Beriberi (which means sheep). I believe those, whom this same disease attacks, with their knees shaking and the legs raised up, walk like sheep. It is a kind of paralysis, or rather Tremor: for it penetrates the motion and sensation of the hands and feet indeed sometimes of the whole body.”

FIGURE 17.20 Arsenite poisoning.

Arsenite inhibits the pyruvate dehydrogenase complex by inactivating the dihydrolipoamide component of the transacetylase. Some sulfhydryl reagents, such as 2,3-dimercaptopropanol, relieve the inhibition by forming a complex with the arsenite that can be excreted.





[The Granger Collection.]

The manuscript proposing the citric acid cycle was submitted for publication to *Nature* but was rejected. It was subsequently published in *Enzymologia*. Dr. Krebs proudly displayed the rejection letter throughout his career as encouragement for young scientists.

“June 1937

The editor of NATURE presents his compliments to Dr. H. A. Krebs and regrets that as he has already sufficient letters to fill the correspondence columns of NATURE for seven or eight weeks, it is undesirable to accept further letters at the present time on account of the time delay which must occur in their publication.


If Dr. Krebs does not mind much delay the editor is prepared to keep the letter until the congestion is relieved in the hope of making use of it.

He returns it now, in case Dr. Krebs prefers to submit it for early publication to another periodical.”

pathologies. The proverbial phrase “mad as a hatter” refers to the strange behavior of poisoned hat makers who used mercury nitrate to soften and shape animal furs. This form of mercury is absorbed through the skin. Similar problems afflicted the early photographers, who used vaporized mercury to create daguerreotypes.

Treatment for these poisons is the administration of sulfhydryl reagents with adjacent sulfhydryl groups to compete with the dihydrolipoyl residues for binding with the metal ion, which is then excreted in the urine. Indeed, 2,3-dimercaptopropanol (see Figure 17.20) was developed after World War I as an antidote to lewisite, an arsenic-based chemical weapon. This compound was initially called BAL, for British anti-lewisite.

17.3.3 Speculations on the Evolutionary History of the Citric Acid Cycle

 How did the citric acid cycle come into being? Although definitive answers are elusive, it is nevertheless instructive to speculate how this complicated central hub of metabolism developed. We can perhaps begin to comprehend how evolution might work at the level of biochemical pathways.

It is most likely that the citric acid cycle was assembled from preexisting reaction pathways. As noted earlier, many of the intermediates formed in the citric acid cycle are used in biosynthetic pathways to generate amino acids and porphyrins. Thus, compounds such as pyruvate, α -ketoglutarate, and oxaloacetate were likely present early in evolution for biosynthetic purposes. The oxidative decarboxylation of these α -ketoacids is quite favorable thermodynamically. The elegant modular structures of the pyruvate and α -ketoglutarate dehydrogenase complexes reveal how three reactions (decarboxylation, oxidation, and thioester formation) can be linked to harness the free energy associated with decarboxylation to drive the synthesis of both acyl CoA derivatives and NADH. These reactions almost certainly formed the core of processes that preceded the citric acid cycle evolutionarily. Interestingly, α -ketoglutarate can be directly converted into oxaloacetate by transamination of the respective amino acids by aspartate aminotransferase, another key biosynthetic enzyme. Thus, cycles comprising smaller numbers of intermediates could have existed before the present form evolved to harvest the electrons from pyruvate or other compounds more efficiently.

17.4 THE GLYOXYLATE CYCLE ENABLES PLANTS AND BACTERIA TO GROW ON ACETATE

Many bacteria and plants are able to subsist on acetate or other compounds that yield acetyl CoA. They make use of a metabolic pathway absent in most other organisms that converts two-carbon acetyl units into four-carbon units (succinate) for energy production and biosyntheses. This reaction sequence, called the *glyoxylate cycle*, bypasses the two decarboxylation steps of the citric acid cycle. Another key difference is that two molecules of acetyl CoA enter per turn of the glyoxylate cycle, compared with one in the citric acid cycle.

The glyoxylate cycle (Figure 17.21), like the citric acid cycle, begins with the condensation of acetyl CoA and oxaloacetate to form citrate, which is then isomerized to isocitrate. Instead of being decarboxylated, isocitrate is cleaved by *isocitrate lyase* into succinate and glyoxylate. The subsequent steps regenerate oxaloacetate from glyoxylate. Acetyl CoA condenses with

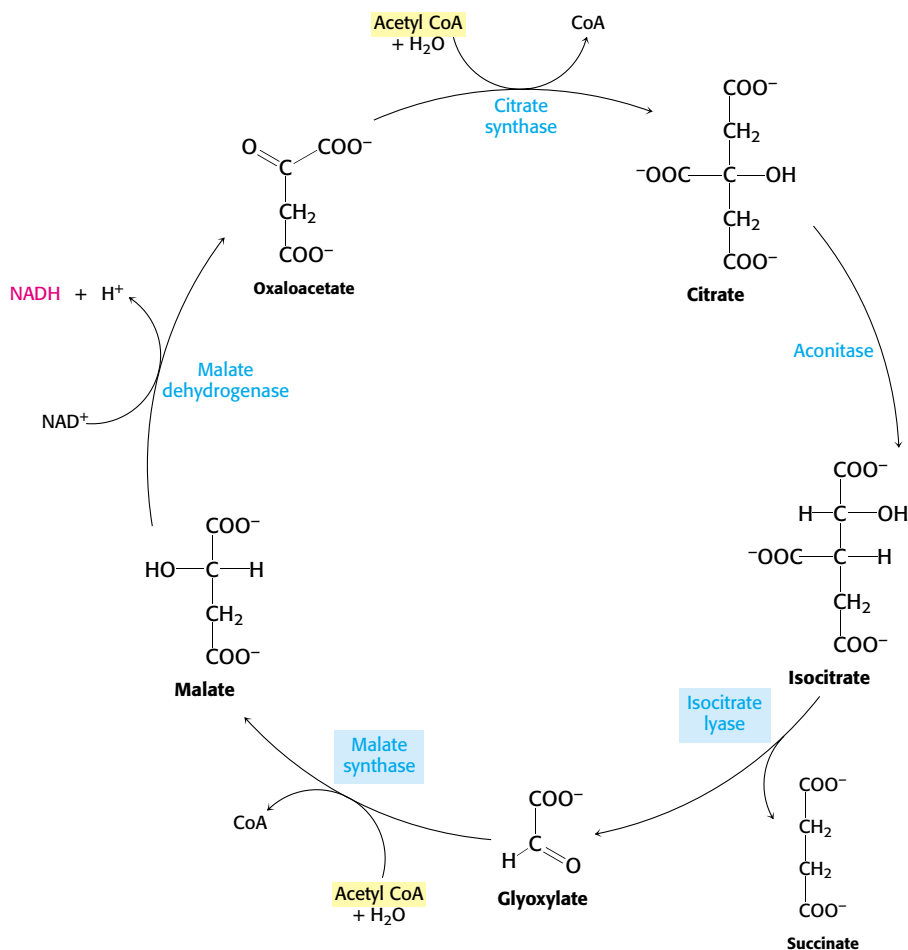
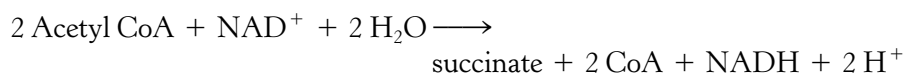


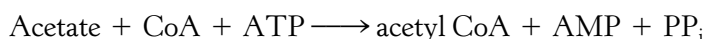
FIGURE 17.21 The glyoxylate pathway. The glyoxylate cycle allows plants and some microorganisms to grow on acetate because the cycle bypasses the decarboxylation steps of the citric acid cycle. The enzymes that permit the conversion of acetate into succinate–isocitrate lyase and malate synthase—are boxed in blue.

glyoxylate to form malate in a reaction catalyzed by *malate synthase*, which resembles citrate synthase. Finally, malate is oxidized to oxaloacetate, as in the citric acid cycle. The sum of these reactions is:



In plants, these reactions take place in organelles called *glyoxysomes*. Succinate, released midcycle, can be converted into carbohydrates by a combination of the citric acid cycle and gluconeogenesis. Thus, organisms with the glyoxylate cycle gain a metabolic versatility.

Bacteria and plants can synthesize acetyl CoA from acetate and CoA by an ATP-driven reaction that is catalyzed by *acetyl CoA synthetase*.



Pyrophosphate is then hydrolyzed to orthophosphate, and so the equivalents of two compounds having high phosphoryl transfer potential are consumed in the activation of acetate. We will return to this type of activation reaction in fatty acid degradation (Section 22.2.2), where it is used to form fatty acyl CoA, and in protein synthesis, where it is used to link amino acids to transfer RNAs (Section 29.x.x).

SUMMARY

The citric acid cycle is the final common pathway for the oxidation of fuel molecules. It also serves as a source of building blocks for biosyntheses. Most fuel molecules enter the cycle as acetyl CoA. The link between glycolysis and the citric acid cycle is the oxidative decarboxylation of pyruvate to form acetyl CoA. In eukaryotes, this reaction and those of the cycle take place inside mitochondria, in contrast with glycolysis, which takes place in the cytosol.

- **The Citric Acid Cycle Oxidizes Two-Carbon Units**

The cycle starts with the condensation of oxaloacetate (C_4) and acetyl CoA (C_2) to give citrate (C_6), which is isomerized to isocitrate (C_6). Oxidative decarboxylation of this intermediate gives α -ketoglutarate (C_5). The second molecule of carbon dioxide comes off in the next reaction, in which α -ketoglutarate is oxidatively decarboxylated to succinyl CoA (C_4). The thioester bond of succinyl CoA is cleaved by inorganic phosphate to yield succinate, and a high phosphoryl transfer potential compound in the form of GTP is concomitantly generated. Succinate is oxidized to fumarate (C_4), which is then hydrated to form malate (C_4). Finally, malate is oxidized to regenerate oxaloacetate (C_4). Thus, two carbon atoms from acetyl CoA enter the cycle, and two carbon atoms leave the cycle as CO_2 in the successive decarboxylations catalyzed by isocitrate dehydrogenase and α -ketoglutarate dehydrogenase. In the four oxidation–reduction reactions in the cycle, three pairs of electrons are transferred to NAD^+ and one pair to FAD. These reduced electron carriers are subsequently oxidized by the electron-transport chain to generate approximately 9 molecules of ATP. In addition, 1 molecule of a compound having a high phosphoryl transfer potential is directly formed in the citric acid cycle. Hence, a total of 10 molecules of compounds having high phosphoryl transfer potential are generated for each two-carbon fragment that is completely oxidized to H_2O and CO_2 .

- **Entry to the Citric Acid Cycle and Metabolism Through It Are Controlled**

The citric acid cycle operates only under aerobic conditions because it requires a supply of NAD^+ and FAD. The irreversible formation of acetyl CoA from pyruvate is an important regulatory point for the entry of glucose-derived pyruvate into the citric acid cycle. The activity of the pyruvate dehydrogenase complex is stringently controlled by reversible phosphorylation. The electron acceptors are regenerated when $NADH$ and $FADH_2$ transfer their electrons to O_2 through the electron-transport chain, with the concomitant production of ATP. Consequently, the rate of the citric acid cycle depends on the need for ATP. In eukaryotes, the regulation of two enzymes in the cycle also is important for control. A high energy charge diminishes the activities of isocitrate dehydrogenase and α -ketoglutarate dehydrogenase. These mechanisms complement each other in reducing the rate of formation of acetyl CoA when the energy charge of the cell is high and when biosynthetic intermediates are abundant.

- **The Citric Acid Cycle Is a Source of Biosynthetic Precursors**

When the cell has adequate energy available, the citric acid cycle can also provide a source of building blocks for a host of important biomolecules, such as nucleotide bases, proteins, and heme groups. This use depletes the cycle of intermediates. When the cycle again needs to metabolize fuel, anaplerotic reactions replenish the cycle intermediates.

• The Glyoxylate Cycle Enables Plants and Bacteria to Grow on Acetate

The glyoxylate cycle enhances the metabolic versatility of many plants and bacteria. This cycle, which uses some of the reactions of the citric acid cycle, enables these organisms to subsist on acetate because it bypasses the two decarboxylation steps of the citric acid cycle.

KEY TERMS

citric acid (tricarboxylic acid, TCA; Krebs) cycle (p. 465)
oxidative phosphorylation (p. 467)
acetyl CoA (p. 467)
pyruvate dehydrogenase complex (p. 467)
flavoprotein (p. 470)

citrate synthase (p. 472)
iron–sulfur (nonheme iron) protein (p. 474)
isocitrate dehydrogenase (p. 474)
 α -ketoglutarate dehydrogenase (p. 475)
metabolon (p. 479)

anaplerotic reaction (p. 482)
beriberi (p. 483)
glyoxylate cycle (p. 484)
isocitrate lyase (p. 484)
malate synthase (p. 485)
glyoxysome (p. 485)

SELECTED READINGS

Where to start

- Reed, L. J., and Hackert, M. L., 1990. Structure-function relationships in dihydrolipoamide acyltransferases. *J. Biol. Chem.* 265:8971–8974.
Mattevi, A., Obmolova, G., Schulze, E., Kalk, K. H., Westphal, A. H., De Kok, A., and Hol, W. G., 1992. Atomic structure of the cubic core of the pyruvate dehydrogenase multienzyme complex. *Science* 255:1544–1550.

Pyruvate dehydrogenase complex

- Izard, T., Evarsson, A., Allen, M. D., Westphal, A. H., Perham, R. N., De Kok, A., and Hol, W. G., 1999. Principles of quasi-equivalence and Euclidean geometry govern the assembly of cubic and dodecahedral cores of pyruvate dehydrogenase complexes. *Proc. Natl. Acad. Sci. USA* 96:1240–1245.
Evarsson, A., Seger, K., Turley, S., Sokatch, J. R., and Hol, W. M. J., 1999. Crystal structure of 2-oxoisovalerate and dehydrogenase and the architecture of 2-oxo acid dehydrogenase multiple enzyme complexes. *Nat. Struct. Biol.* 6:785–792.
Domingo, G. J., Chauhan, H. J., Lessard, I. A., Fuller, C., and Perham, R. N., 1999. Self-assembly and catalytic activity of the pyruvate dehydrogenase multienzyme complex from *Bacillus stearothermophilus*. *Eur. J. Biochem.* 266:1136–1146.
Jones, D. D., Horne, H. J., Reche, P. A., and Perham, R. N., 2000. Structural determinants of post-translational modification and catalytic specificity for the lipoyl domains of the pyruvate dehydrogenase multienzyme complex of *Escherichia coli*. *J. Mol. Biol.* 295:289–306.
McCartney, R. G., Rice, J. E., Sanderson, S. J., Bunik, V., Lindsay, H., and Lindsay, J. G., 1998. Subunit interactions in the mammalian α -ketoglutarate dehydrogenase complex: Evidence for direct association of the α -ketoglutarate dehydrogenase and dihydrolipoamide dehydrogenase components. *J. Biol. Chem.* 273:24158–24164.

Structure of citric acid cycle enzymes

- Chapman, A. D., Cortes, A., Dafforn, T. R., Clarke, A. R., and Brady, R. L., 1999. Structural basis of substrate specificity in malate dehydrogenases: Crystal structure of a ternary complex of porcine cytoplasmic malate dehydrogenase, α -ketomalonnate and tetrahydroNAD. *J. Mol. Biol.* 285:703–712.
Fraser, M. E., James, M. N., Bridger, W. A., and Wolodko, W. T., 1999. A detailed structural description of *Escherichia coli* succinyl-CoA

synthetase. *J. Mol. Biol.* 285:1633–1653. [Published erratum appears in May 7, 1999, issue of *J. Mol. Biol.* 288(3):501.]

- Lloyd, S. J., Lauble, H., Prasad, G. S., and Stout, C. D., 1999. The mechanism of aconitase: 1.8 Å resolution crystal structure of the S642a: citrate complex. *Protein Sci.* 8:2655–2662.
Remington, S. J., 1992. Structure and mechanism of citrate synthase. *Curr. Top. Cell. Regul.* 33:209–229.
Rose, I. A., 1998. How fumarase recycles after the malate \rightarrow fumarate reaction: Insights into the reaction mechanism. *Biochemistry* 37:17651–17658.
Johnson, J. D., Muhonen, W. W., and Lambeth, D. O., 1998. Characterization of the ATP- and GTP-specific succinyl-CoA synthetases in pigeon: The enzymes incorporate the same subunit. *J. Biol. Chem.* 273:27573–27579.
Karpusas, M., Branchaud, B., and Remington, S. J., 1990. Proposed mechanism for the condensation reaction of citrate synthase: 1.9-Å structure of the ternary complex with oxaloacetate and carboxymethyl coenzyme A. *Biochemistry* 29:2213–2219.
Lauble, H., Kennedy, M. C., Beinert, H., and Stout, C. D., 1992. Crystal structures of aconitase with isocitrate and nitroisocitrate bound. *Biochemistry* 31:2735–2748.

Organization of the citric acid cycle

- Velot, C., Mixon, M. B., Teige, M., and Srere, P. A., 1997. Model of a quinary structure between Krebs TCA cycle enzymes: A model for the metabolon. *Biochemistry* 36:14271–14276.
Barnes, S. J., and Weitzman, P. D., 1986. Organization of citric acid cycle enzymes into a multienzyme cluster. *FEBS Lett.* 201:267–270.
Haggie, P. M., and Brindle, K. M., 1999. Mitochondrial citrate synthase is immobilized in vivo. *J. Biol. Chem.* 274:3941–3945.
Morgunov, I., and Srere, P. A., 1998. Interaction between citrate synthase and malate dehydrogenase: Substrate channeling of oxaloacetate. *J. Biol. Chem.* 273:29540–29544.

Regulation

- Huang, B., Gudi, R., Wu, P., Harris, R. A., Hamilton, J., and Popov, K. M., 1998. Isoenzymes of pyruvate dehydrogenase phosphatase: DNA-derived amino acid sequences, expression, and regulation. *J. Biol. Chem.* 273:17680–17688.
Bowker-Kinley, M., and Popov, K. M., 1999. Evidence that pyruvate dehydrogenase kinase belongs to the ATPase/kinase superfamily. *Biochem. J.* 1:47–53.

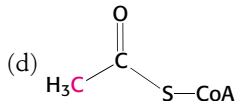
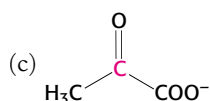
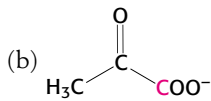
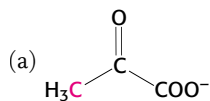
- Jitrapakdee, S., and Wallace, J. C., 1999. Structure, function and regulation of pyruvate carboxylase. *Biochem. J.* 340:1–16.
- Hurley, J. H., Dean, A. M., Sohl, J. L., Koshland, D. J., and Stroud, R. M., 1990. Regulation of an enzyme by phosphorylation at the active site. *Science* 249:1012–1016.

Evolutionary aspects

- Meléndez-Hevia, E., Waddell, T. G., and Cascante, M., 1996. The puzzle of the Krebs citric acid cycle: Assembling the pieces of chemically feasible reactions, and opportunism in the design of metabolic pathways in evolution. *J. Mol. Evol.* 43:293–303.
- Baldwin, J. E., and Krebs, H., 1981. The evolution of metabolic cycles. *Nature* 291:381–382.

PROBLEMS

1. *Flow of carbon atoms.* What is the fate of the radioactive label when each of the following compounds is added to a cell extract containing the enzymes and cofactors of the glycolytic pathway, the citric acid cycle, and the pyruvate dehydrogenase complex? (The ^{14}C label is printed in red.)



(e) Glucose 6-phosphate labeled at C-1.

2. $\text{C}_2 + \text{C}_2 \longrightarrow \text{C}_4$.

- (a) Which enzymes are required to get *net synthesis* of oxaloacetate from acetyl CoA?
- (b) Write a balanced equation for the net synthesis.
- (c) Do mammalian cells contain the requisite enzymes?

3. *Driving force.* What is the ΔG° for the complete oxidation of the acetyl unit of acetyl CoA by the citric acid cycle?

4. *Acting catalytically.* The citric acid cycle itself, which is composed of enzymatically catalyzed steps, can be thought of essentially as the product of a supramolecular enzyme. Explain.

5. *Probing stereospecificity.* A sample of deuterated reduced NAD was prepared by incubating $\text{H}_3\text{C}-\text{CD}_2-\text{OH}$ and NAD^+ with alcohol dehydrogenase. This reduced coenzyme was added to a solution of 1,3-BPG and glyceraldehyde 3-phosphate dehydrogenase. The NAD^+ formed by this second reaction contained one atom of deuterium, whereas glyceraldehyde 3-phosphate, the other product, contained none. What does this experiment reveal about the stereospecificity of glyceraldehyde 3-phosphate dehydrogenase?

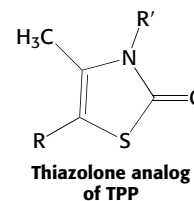
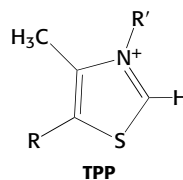
6. *A potent inhibitor.* Thiamine thiazolone pyrophosphate binds to pyruvate dehydrogenase about 20,000 times as strongly

- Gest, H., 1987. Evolutionary roots of the citric acid cycle in prokaryotes. *Biochem. Soc. Symp.* 54:3–16.
- Weitzman, P. D. J., 1981. Unity and diversity in some bacterial citric acid cycle enzymes. *Adv. Microbiol. Physiol.* 22:185–244.

Discovery of the citric acid cycle

- Krebs, H. A., and Johnson, W. A., 1937. The role of citric acid in intermediate metabolism in animal tissues. *Enzymologia* 4:148–156.
- Krebs, H. A., 1970. The history of the tricarboxylic acid cycle. *Perspect. Biol. Med.* 14:154–170.
- Krebs, H. A., and Martin, A., 1981. *Reminiscences and Reflections*. Clarendon Press.

as does thiamine pyrophosphate, and it competitively inhibits the enzyme. Why?



7. *Lactic acidosis.* Patients in shock will often suffer from lactic acidosis due to a deficiency of O_2 . Why does a lack of O_2 lead to lactic acid accumulation? One treatment for shock is to administer dichloroacetate, which inhibits the kinase associated with the pyruvate dehydrogenase complex. What is the biochemical rationale for this treatment?

8. *Coupling reactions.* The oxidation of malate by NAD^+ to form oxaloacetate is a highly endergonic reaction under standard conditions [$\Delta G^\circ = +7 \text{ kcal mol}^{-1}$ ($+29 \text{ kJ mol}^{-1}$)]. The reaction proceeds readily under physiological conditions.

- (a) Why?
- (b) Assuming an $[\text{NAD}^+]/[\text{NADH}]$ ratio of 8 and a pH of 7, what is the lowest $[\text{malate}]/[\text{oxaloacetate}]$ ratio at which oxaloacetate can be formed from malate?

9. *Synthesizing α -ketoglutarate.* It is possible, with the use of the reactions and enzymes discussed in this chapter, to convert pyruvate into α -ketoglutarate without depleting any of the citric acid cycle components. Write a balanced reaction scheme for this conversion, showing cofactors and identifying the required enzymes.

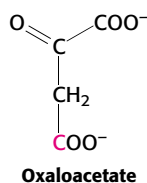
Chapter Integration Problem

10. *Fats into glucose?* Fats are usually metabolized into acetyl CoA and then further processed through the citric acid cycle. In Chapter 16, we learned that glucose could be synthesized from oxaloacetate, a citric acid cycle intermediate. Why, then, after a long bout of exercise depletes our carbohydrate stores, do we need to replenish those stores by eating carbohydrates? Why do we not simply replace them by converting fats into carbohydrates?

Mechanism Problems

11. *Theme and variation.* Propose a reaction mechanism for the condensation of acetyl CoA and glyoxylate in the glyoxylate cycle of plants and bacteria.

12. *Symmetry problems.* In experiments carried out in 1941 to investigate the citric acid cycle, oxaloacetate labeled with ^{14}C in the carboxyl carbon atom farthest from the keto group was introduced to an active preparation of mitochondria.



Analysis of the α -ketoglutarate formed showed that none of the radioactive label had been lost. Decarboxylation of α -ketoglutarate then yielded succinate devoid of radioactivity. All the label was in the released CO_2 . Why were the early investigators of the citric acid cycle surprised that *all* the label emerged in the CO_2 ?

13. *Symmetric molecules reacting asymmetrically.* The interpretation of the experiments described in problem 12 was that citrate (or any other symmetric compound) cannot be an intermediate in the formation of α -ketoglutarate, because of the asymmetric fate of the label. This view seemed compelling until Alexander Ogston incisively pointed out in 1948 that “it is possible that an asymmetric enzyme which attacks a symmetrical compound can distinguish between its identical groups.” For simplicity, consider a molecule in which two hydrogen atoms, a group X, and a different group Y are bonded to a tetrahedral carbon atom as a model for citrate. Explain how a symmetric molecule can react with an enzyme in an asymmetric way.

Data Interpretation

14. *A little goes a long way.* As will become clearer in Chapter 18, the activity of the citric acid cycle can be monitored by monitoring the amount of O_2 consumed. The greater the rate of O_2 consumption, the faster the rate of the cycle. Hans Krebs used this assay to investigate the cycle in 1937. He used as his experimental system minced pigeon breast muscle, which is rich in mitochondria. In one set of experiments, Krebs measured the O_2 consumption in the presence of carbohydrate only and in the presence of carbohydrate and citrate. The results are shown in the following table.

Effect of citrate on oxygen consumption by minced pigeon breast muscle

Time (min)	Micromoles of oxygen consumed	
	Carbohydrate only	Carbohydrate plus 3 μmol of citrate
10	26	28
60	43	62
90	46	77
150	49	85

(a) How much O_2 would be absorbed if the added citrate were completely oxidized to H_2O and CO_2 ?

(b) Based on your answer to part a, what do the results given in the table suggest?

15. *Arsenite poisoning.* The effect of arsenite on the experimental system of problem 14 was then examined. Experimental data (not presented here) showed that the amount of citrate present did not change in the course of the experiment in the absence of arsenite. However, if arsenite was added to the system, different results were obtained, as shown in the following table.

Disappearance of citric acid in pigeon breast muscle in the presence of arsenite

Micromoles of citrate added	Micromoles of citrate found after 40 minutes	Micromoles of citrate used
22	0.6	21
44	20	24
90	56	34

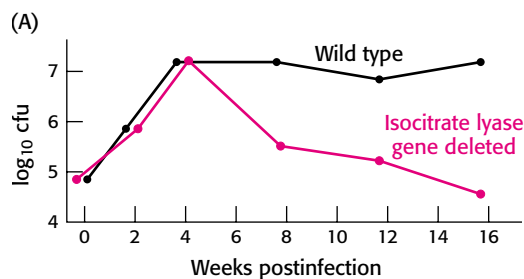
(a) What is the effect of arsenite on the disappearance of citrate?

(b) How is the arsenite's action altered by the addition of more citrate?

(c) What do these data suggest about the site of action of arsenite?

16. *Isocitrate lyase and tuberculosis.* The bacterium *Mycobacterium tuberculosis*, the cause of tuberculosis, can invade the lungs and persist in a latent state for years. During this time, the bacteria reside in granulomas—nodular scars containing bacteria and host-cell debris in the center and surrounded by immune cells. The granulomas are lipid-rich, oxygen-poor environments. How these bacteria manage to persist is something of a mystery. The results of recent research suggest that the glyoxylate cycle is required for the persistence. The following data show the amount of bacteria [presented as colony-forming units (cfu)] in mice lungs in the weeks after an infection.

In graph A, the black circles represent the results for wild-type bacteria and the red circles represent the results for bacteria from which the gene for isocitrate lyase was deleted.



(a) What is the effect of the absence of isocitrate lyase?

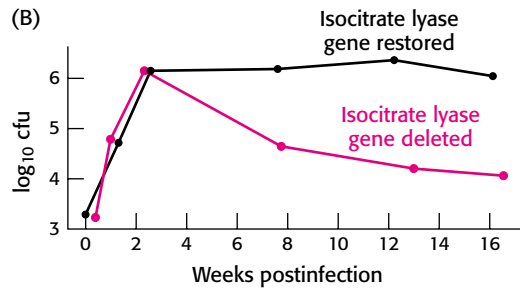
The techniques discussed in Chapter 6 were used to reinsert the gene encoding isocitrate lyase into bacteria from which it had previously been deleted.

In graph B, black circles represent bacteria into which the gene was reinserted and red circles bacteria from which the gene was still missing.

- (b) Do these results support those obtained in part *a*?
 (c) What is the purpose of the experiment in part *b*?
 (d) Why do these bacteria perish in the absence of the glyoxylate cycle?

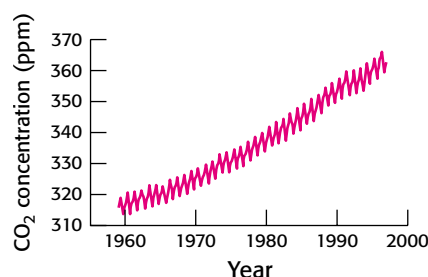


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[Data after McKinney et al., 2000. *Nature* 406:735–738.]

The Calvin Cycle and the Pentose Phosphate Pathway



Atmospheric carbon dioxide measurements at Mauna Loa, Hawaii. These measurements show annual cycles resulting from seasonal variation in carbon dioxide fixation by the Calvin cycle in terrestrial plants. Much of this fixation takes place in rain forests, which account for approximately 50% of terrestrial fixation. [Dennis Potokar/Photo Researchers.]

Photosynthesis proceeds in two parts: the light reactions and the dark reactions. The light reactions, which were discussed in Chapter 19, transform light energy into ATP and biosynthetic reducing power, NADPH. The dark reactions, which constitute the Calvin cycle, named after Melvin Calvin, the biochemist who elucidated the pathway, reduce carbon atoms from their fully oxidized state as carbon dioxide to the more reduced state as a hexose. The components of the Calvin cycle and called the dark reactions because, in contrast with the light reactions, these reactions do not directly depend on the presence of light.

In addition to ATP, the dark reactions require reducing power in the form of NADPH, *the currency of readily available reducing power in cells*. The phosphoryl group on the 2'-hydroxyl group of one of the ribose units of NADPH distinguishes NADPH from NADH. There is a *fundamental distinction between NADPH and NADH in biochemistry: NADH is oxidized by the respiratory chain to generate ATP, whereas NADPH serves as a reductant in biosynthetic processes*.

The second half of this chapter examines a pathway common to all organisms, known variously as the pentose phosphate pathway, the hexose monophosphate pathway, the phosphogluconate pathway, or the pentose shunt. The pathway provides a means by which glucose can be oxidized to generate NADPH and is the source of much of the NADPH that is needed for the biosynthesis of many biomolecules, most notably fats. We will observe the use of

OUTLINE

- 20.1 The Calvin Cycle Synthesizes Hexoses from Carbon Dioxide and Water
- 20.2 The Activity of the Calvin Cycle Depends on Environmental Conditions
- 20.3 The Pentose Phosphate Pathway Generates NADPH and Synthesizes Five-Carbon Sugars
- 20.4 The Metabolism of Glucose 6-phosphate by the Pentose Phosphate Pathway Is Coordinated with Glycolysis
- 20.5 Glucose 6-phosphate Dehydrogenase Plays a Key Role in Protection Against Reactive Oxygen Species

NADPH in many of the biosynthetic reactions considered in Part III of this text. The pentose phosphate pathway can also be used for the catabolism of pentose sugars from the diet, the synthesis of pentose sugars for nucleotide biosynthesis, and the catabolism and synthesis of less common four- and seven-carbon sugars. The pentose phosphate pathway and the Calvin cycle have in common several enzymes and intermediates that attest to an evolutionary kinship. Like glycolysis and gluconeogenesis (Chapter 16), these pathways are mirror images of one another: the Calvin cycle uses NADPH to reduce carbon dioxide to generate hexoses, whereas the pentose phosphate pathway breaks down glucose into carbon dioxide to generate NADPH. The Calvin cycle is sometimes referred to as the *reductive pentose phosphate pathway*.

20.1 THE CALVIN CYCLE SYNTHESIZES HEXOSES FROM CARBON DIOXIDE AND WATER

We saw in Chapter 16 that glucose could be formed from noncarbohydrate precursors, such as lactate and amino acids, by gluconeogenesis. The synthesis of glucose from these compounds is simplified because the carbons are already incorporated into relatively complex organic molecules. In contrast, the source of the carbon atoms in the Calvin cycle is the simple molecule carbon dioxide. In this extremely important process, *carbon dioxide gas is trapped in a form that is useful for many processes*. The Calvin cycle brings into living systems the carbon atoms that will become constituents of nucleic acids, proteins, and fats. Photosynthetic organisms are called *autotrophs* (literally “self-feeders”) because they can synthesize glucose from carbon dioxide and water, by using sunlight as an energy source, and then recover some of this energy from the synthesized glucose through the glycolytic pathway and aerobic metabolism. Organisms that obtain energy from chemical fuels only are called *heterotrophs*, which ultimately depend on autotrophs for their fuel. The Calvin cycle also differs from gluconeogenesis in where it takes place in photosynthetic eukaryotes. Whereas gluconeogenesis takes place in the cytoplasm, the Calvin cycle takes place in the stroma of chloroplasts, the photosynthetic organelles.

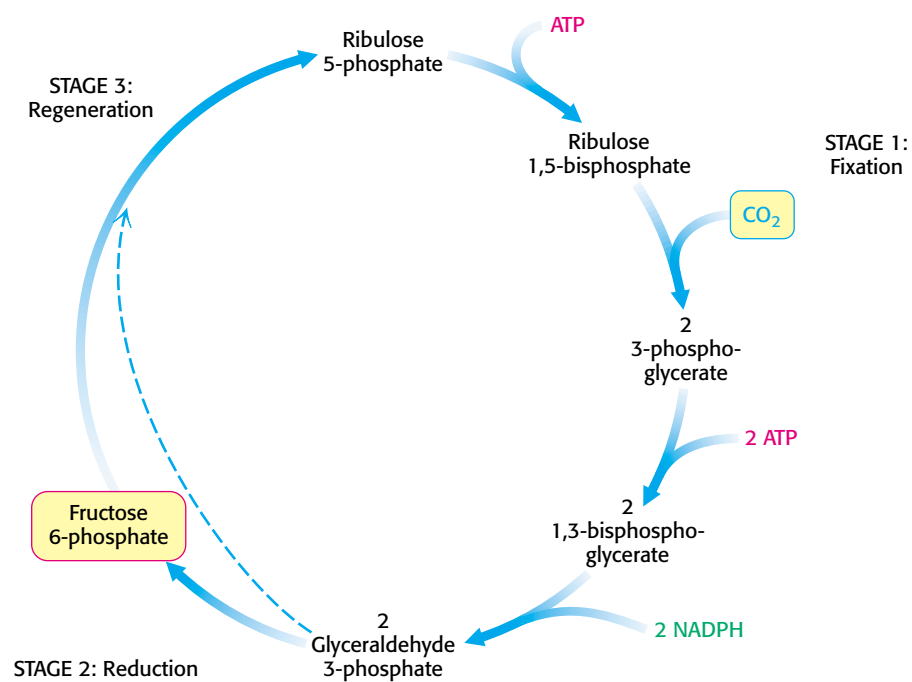


FIGURE 20.1 Calvin cycle. The Calvin cycle consists of three stages. Stage 1 is the fixation of carbon by the carboxylation of ribulose 1,5-bisphosphate. Stage 2 is the reduction of the fixed carbon to begin the synthesis of hexose. Stage 3 is the regeneration of the starting compound, ribulose 1,5-bisphosphate.

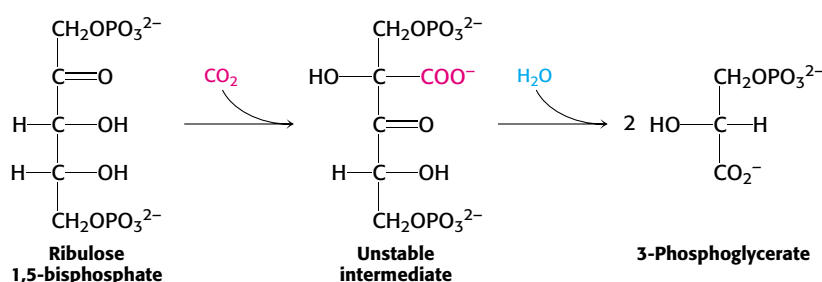
The Calvin cycle comprises three stages (Figure 20.1):

1. The fixation of CO_2 by ribulose 1,5-bisphosphate to form two molecules of 3-phosphoglycerate.
2. The reduction of 3-phosphoglycerate to form hexose sugars.
3. The regeneration of ribulose 1,5-bisphosphate so that more CO_2 can be fixed.

Although we will focus on the Calvin cycle, other means of fixing carbon dioxide into hexose sugars exist in the photosynthetic world, notably a version of the citric acid cycle running in reverse.

20.1.1 Carbon Dioxide Reacts with Ribulose 1,5-bisphosphate to Form Two Molecules of 3-Phosphoglycerate

The first step in the Calvin cycle is the fixation of CO_2 . The CO_2 molecule condenses with ribulose 1,5-bisphosphate to form an unstable six-carbon compound, which is rapidly hydrolyzed to two molecules of 3-phosphoglycerate.



The initial incorporation of CO_2 into 3-phosphoglycerate was revealed through the use of a carbon-14 radioactive tracer (Figure 20.2). This highly exergonic reaction [$\Delta G^{\circ} = -12.4 \text{ kcal mol}^{-1}$ ($-51.9 \text{ kJ mol}^{-1}$)] is catalyzed by *ribulose 1,5-bisphosphate carboxylase/oxygenase* (usually called *rubisco*), an enzyme located on the stromal surface of the thylakoid membranes of chloroplasts. This important reaction is the rate-limiting step in hexose synthesis. Rubisco in chloroplasts consists of eight large (L, 55-kd) subunits and eight small (S, 13-kd) ones (Figure 20.3). Each L chain contains a catalytic site and a regulatory site. The S chains enhance the catalytic

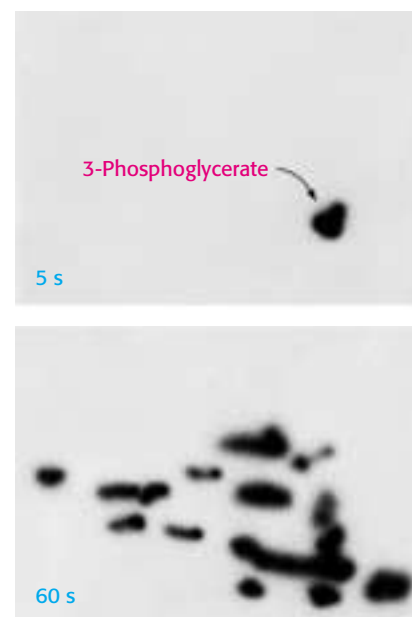
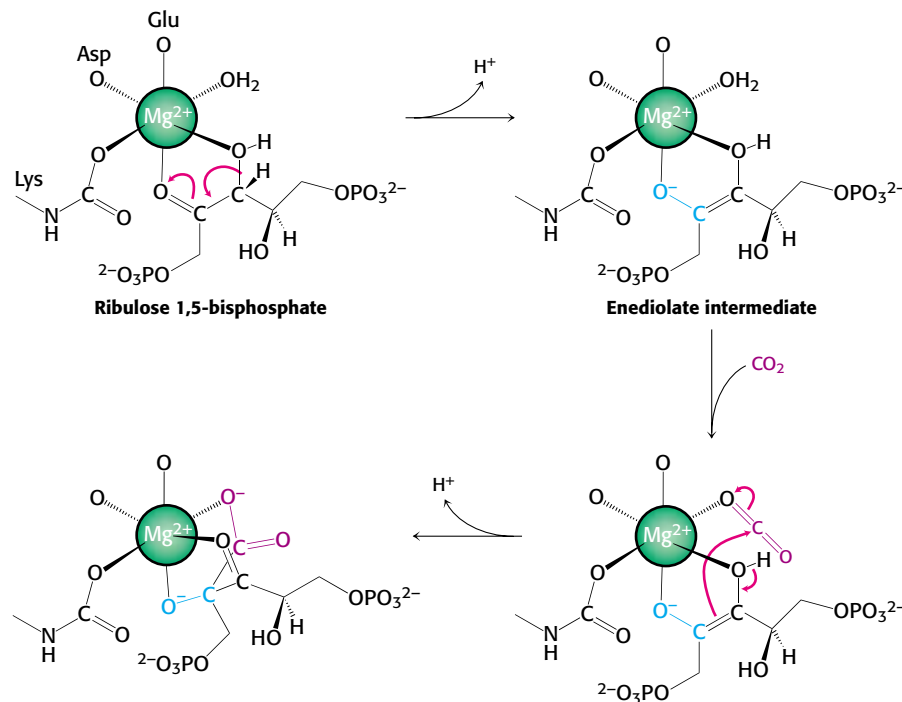
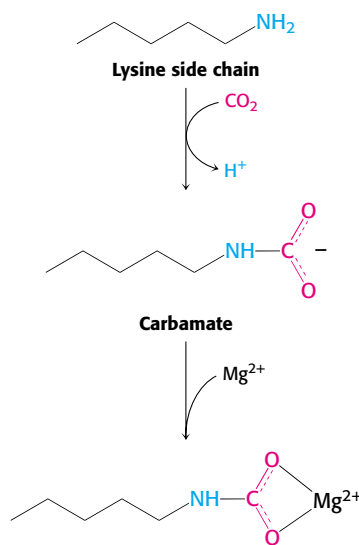


FIGURE 20.2 Tracing the fate of carbon dioxide. Radioactivity from $^{14}\text{CO}_2$ is incorporated into 3-phosphoglycerate within 5 s in irradiated cultures of algae. After 60 s, the radioactivity appears in many compounds, the intermediates within the Calvin cycle. [Courtesy of Dr. J. A. Bassham.]

FIGURE 20.3 Structure of rubisco. The enzyme ribulose 1,5-bisphosphate carboxylase/oxygenase (rubisco) comprises eight large subunits (one shown in red and the others in yellow) and eight small subunits (one shown in blue and the others in white). The active sites lie in the large subunits.

**FIGURE 20.4** Role of the magnesium ion in the rubisco mechanism.

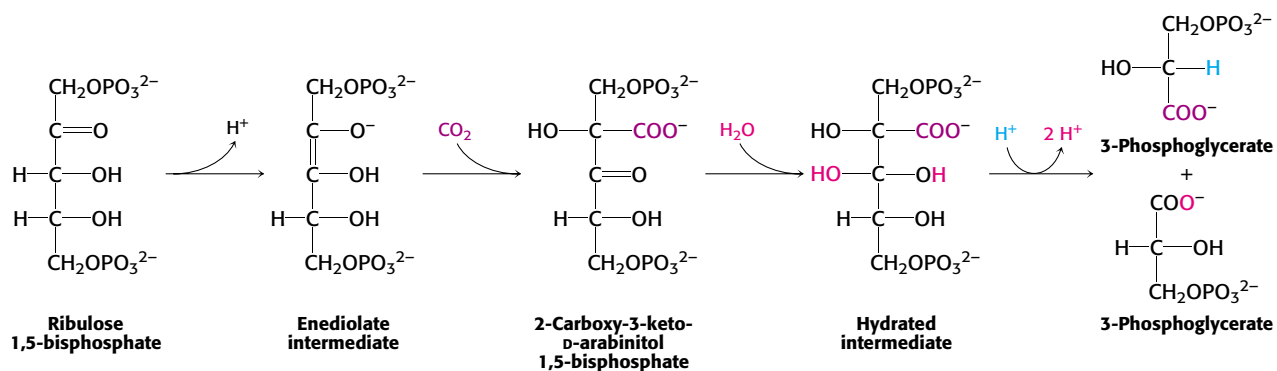
Ribulose 1,5-bisphosphate binds to a magnesium ion that is linked to rubisco through a glutamate residue, an aspartate residue, and the lysine carbamate. The coordinated ribulose 1,5-bisphosphate gives up a proton to form a reactive enediolate species that reacts with CO_2 to form a new carbon-carbon bond.



activity of the L chains. This enzyme is very abundant in chloroplasts, constituting more than 16% of their total protein. In fact, rubisco is the most abundant enzyme and probably the most abundant protein in the biosphere. Large amounts are present because rubisco is a slow enzyme; its maximal catalytic rate is only 3 s^{-1} .

Rubisco requires a bound divalent metal ion for activity, usually magnesium ion. Like the zinc ion in the active site of carbonic anhydrase (Section 9.2.1), this metal ion serves to activate a bound substrate molecule by stabilizing a negative charge. Interestingly, a CO_2 molecule other than the substrate is required to complete the assembly of the Mg^{2+} binding site in rubisco. This CO_2 molecule adds to the uncharged ϵ -amino group of lysine 201 to form a *carbamate*. This negatively charged adduct then binds the Mg^{2+} ion. The formation of the carbamate is facilitated by the enzyme *rubisco activase*, although it will also form spontaneously at a lower rate.

The metal center plays a key role in binding ribulose 1,5-bisphosphate and activating it so that it will react with CO_2 (Figure 20.4). Ribulose 1,5-bisphosphate binds to Mg^{2+} through its keto group and an adjacent hydroxyl group. This complex is readily deprotonated to form an enediolate intermediate. This reactive species, analogous to the zinc-hydroxide species in carbonic anhydrase (Section 9.2.2), couples with CO_2 , forming the new

**FIGURE 20.5** Formation of 3-phosphoglycerate. The overall pathway for the conversion of ribulose 1,5 bisphosphate and CO_2 into two molecules of 3-phosphoglycerate. Although the free species are shown, these steps take place on the magnesium ion.

carbon-carbon bond. The resulting product is coordinated to the Mg^{2+} ion through three groups, including the newly formed carboxylate. A molecule of H_2O is then added to this β -ketoacid to form an intermediate that cleaves to form two molecules of 3-phosphoglycerate (Figure 20.5).

20.1.2 Catalytic Imperfection: Rubisco Also Catalyzes a Wasteful Oxygenase Reaction

The reactive intermediate generated on the Mg^{2+} ion sometimes reacts with O_2 instead of CO_2 . Thus, rubisco also catalyzes a deleterious oxygenase reaction. The products of this reaction are *phosphoglycolate* and *3-phosphoglycerate* (Figure 20.6). The rate of the carboxylase reaction is four times

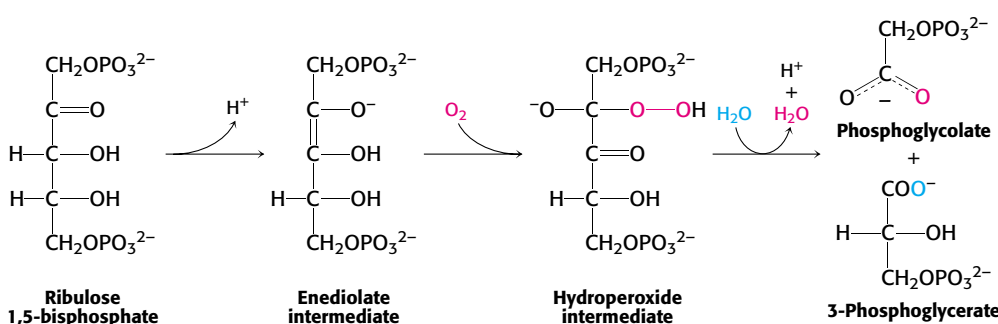


FIGURE 20.6 A wasteful side reaction. The reactive enediolate intermediate on rubisco also reacts with molecular oxygen to form a hydroperoxide intermediate, which then proceeds to form one molecule of 3-phosphoglycerate and one molecule of phosphoglycolate.

that of the oxygenase reaction under normal atmospheric conditions at $25^\circ C$; the stromal concentration of CO_2 is then $10 \mu M$ and that of O_2 is $250 \mu M$. The oxygenase reaction, like the carboxylase reaction, requires that lysine 201 be in the carbamate form. Because this carbamate forms only in the presence of CO_2 , this property would prevent rubisco from catalyzing the oxygenase reaction exclusively when CO_2 is absent.

Phosphoglycolate is not a versatile metabolite. A salvage pathway recovers part of its carbon skeleton (Figure 20.7). A specific phosphatase converts phosphoglycolate into *glycolate*, which enters *peroxisomes* (also called

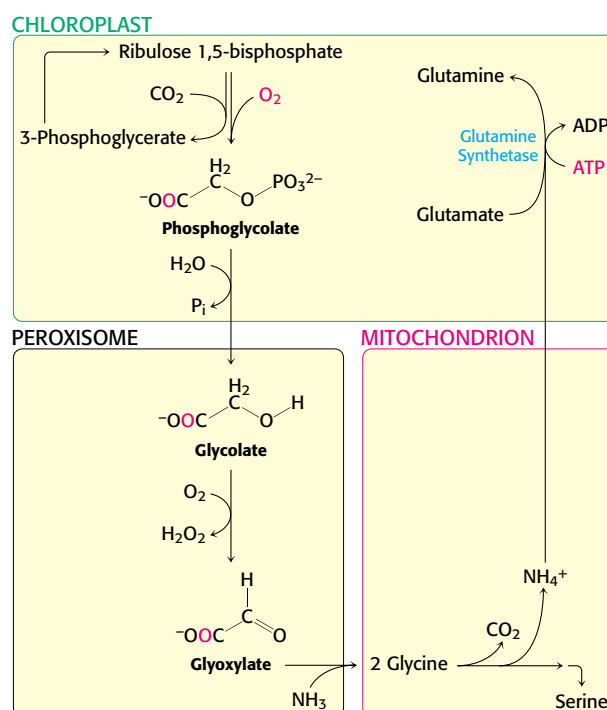


FIGURE 20.7 Photorespiratory reactions. Phosphoglycolate is formed as a product of the oxygenase reaction in chloroplasts. After dephosphorylation, glycolate is transported into peroxisomes where it is converted into glyoxylate and then glycine. In mitochondria, two glycines are converted into serine, after losing a carbon as CO_2 and ammonia. The ammonia is salvaged in chloroplasts.

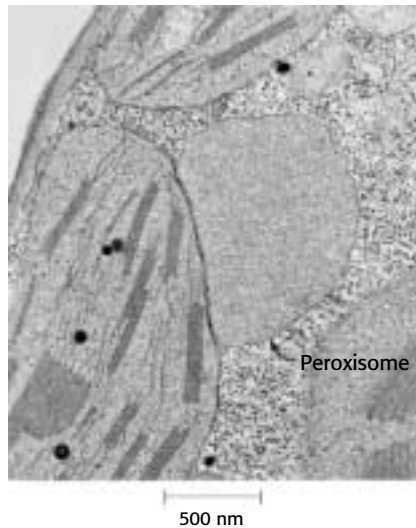


FIGURE 20.8 Electron micrograph of a peroxisome nestled between two chloroplasts. [Courtesy of Dr. Sue Ellen Frederick.]

microbodies; Figure 20.8). Glycolate is then oxidized to *glyoxylate* by glycolate oxidase, an enzyme with a flavin mononucleotide prosthetic group. The H_2O_2 produced in this reaction is cleaved by catalase to H_2O and O_2 . Transamination of glyoxylate then yields *glycine*. Two glycine molecules can be used to form serine, a potential precursor of glucose, with the release of CO_2 and ammonia (NH_4^+). The ammonia, used in the synthesis of nitrogen-containing compounds, is salvaged by glutamine synthetase reaction.

This salvage pathway serves to recycle three of the four carbon atoms of two molecules of glycolate. However, one carbon atom is lost as CO_2 . This process is called *photorespiration* because O_2 is consumed and CO_2 is released. Photorespiration is wasteful because organic carbon is converted into CO_2 without the production of ATP, NADPH, or another energy-rich metabolite. Moreover, the oxygenase activity increases more rapidly with temperature than the carboxylase activity, presenting a problem for tropical plants (Section 20.2.3). Evolutionary processes have presumably enhanced the preference of rubisco for carboxylation. For instance, the rubisco of higher plants is eightfold as specific for carboxylation as that of photosynthetic bacteria.

20.1.3 Hexose Phosphates Are Made from Phosphoglycerate, and Ribulose 1,5-bisphosphate Is Regenerated

The 3-phosphoglycerate product of rubisco is next converted into three forms of hexose phosphate: glucose 1-phosphate, glucose 6-phosphate, and fructose 6-phosphate. Recall that these isomers are readily interconvertible (Sections 16.1.2 and 16.1.11). The steps in this conversion (Figure 20.9) are like those of the gluconeogenic pathway (Section 16.3.1), except that glyceraldehyde 3-phosphate dehydrogenase in chloroplasts, which generates glyceraldehyde 3-phosphate (GAP), is specific for NADPH rather than NADH. Alternatively, the glyceraldehyde 3-phosphate can be transported to the cytosol for glucose synthesis. These reactions and that catalyzed by rubisco bring CO_2 to the level of a hexose, converting CO_2 into a chemical fuel at the expense of NADPH and ATP generated from the light reactions.

The third phase of the Calvin cycle is the regeneration of ribulose 1,5-bisphosphate, the acceptor of CO_2 in the first step. The problem is to construct a five-carbon sugar from six-carbon and three-carbon sugars. A transketolase and an aldolase play the major role in the rearrangement of the carbon atoms. The *transketolase*, which we will see again in the pentose phosphate pathway (Section 20.2.3), requires the coenzyme thiamine pyrophosphate (TPP) to transfer a two-carbon unit ($\text{CO}-\text{CH}_2\text{OH}$) from a ketose to an aldose.

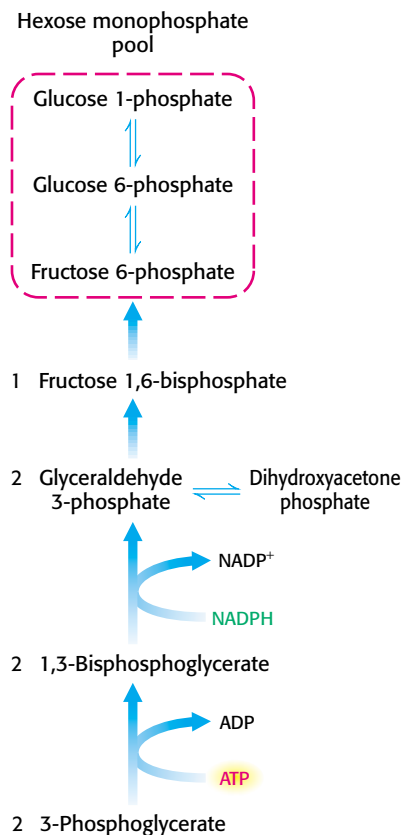
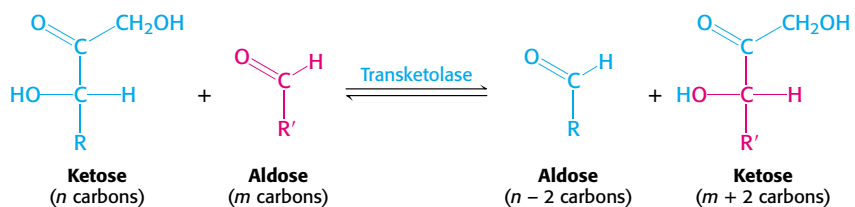
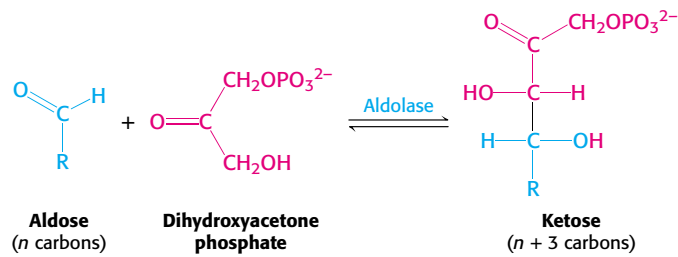


FIGURE 20.9 Hexose phosphate formation. 3-Phosphoglycerate is converted into fructose 6-phosphate in a pathway parallel to that of gluconeogenesis.



We will consider the mechanism of transketolase when we meet it again in the pentose phosphate pathway (Section 20.3.2). *Aldolase*, which we have already encountered in glycolysis (Section 16.1.3), catalyzes an aldol condensation between dihydroxyacetone phosphate and an aldehyde. This enzyme is highly specific for dihydroxyacetone phosphate, but it accepts a wide variety of aldehydes.



With these enzymes, the construction of the five-carbon sugar proceeds as shown in Figure 20.10.

Finally, ribose-5-phosphate is converted into ribulose 5-phosphate by *phosphopentose isomerase* while xylulose 5-phosphate is converted into ribulose 5-phosphate by *phosphopentose epimerase*. Ribulose 5-phosphate is converted into ribulose 1,5-bisphosphate through the action of *phosphoribulose kinase* (Figure 20.11). The sum of these reactions is

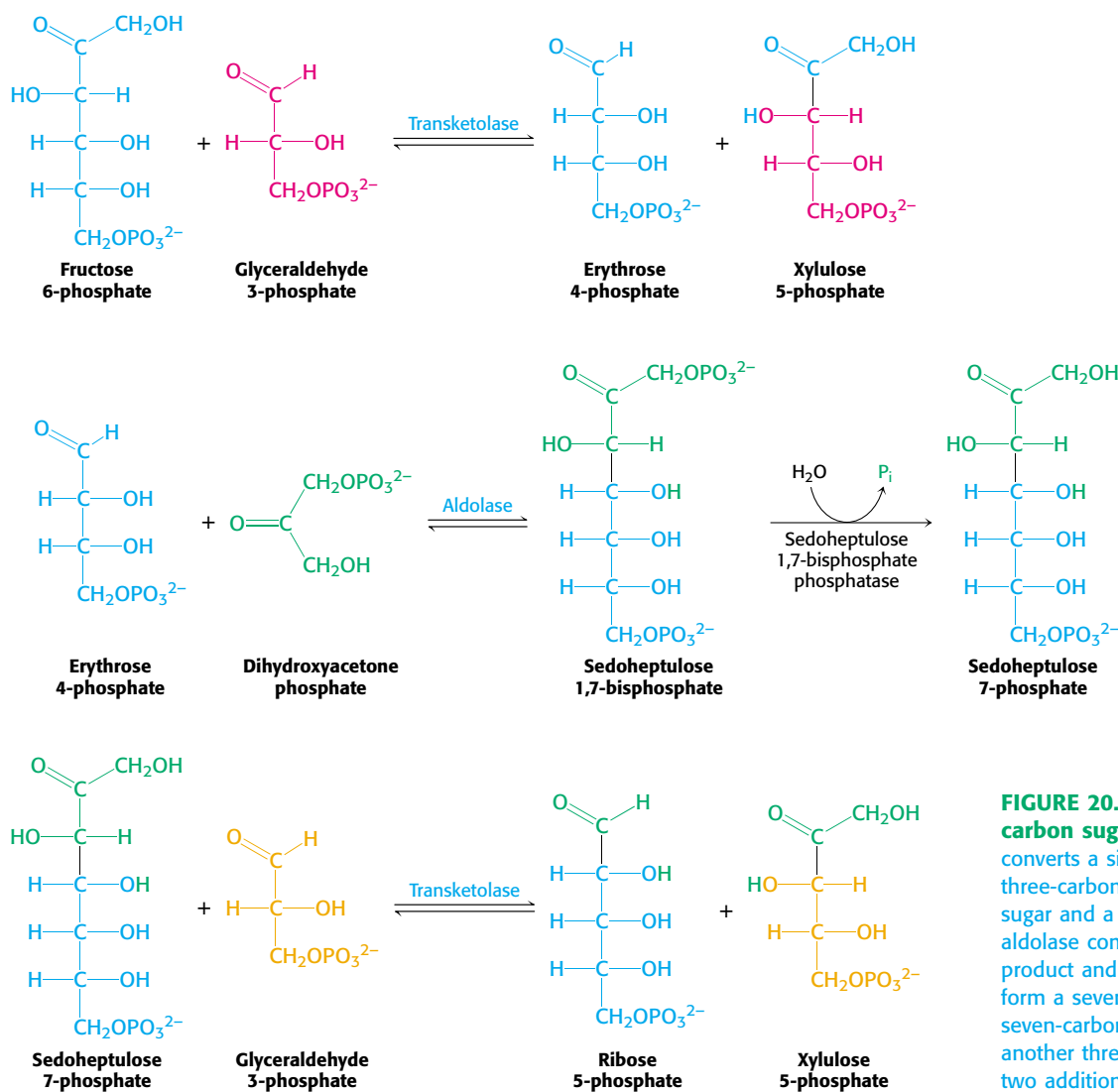
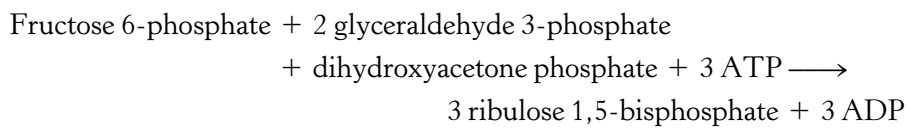


FIGURE 20.10 Formation of five-carbon sugars. First, transketolase converts a six-carbon sugar and a three-carbon sugar into a four-carbon sugar and a five-carbon sugar. Then, aldolase combines the four-carbon product and a three-carbon sugar to form a seven-carbon sugar. Finally, this seven-carbon fragment combines with another three-carbon fragment to form two additional five-carbon sugars.

FIGURE 20.11 Regeneration of ribulose 1,5-bisphosphate. Both ribose 5-phosphate and xylulose 5-phosphate are converted into ribulose 5-phosphate, which is then phosphorylated to complete the regeneration of ribulose 1,5-bisphosphate.

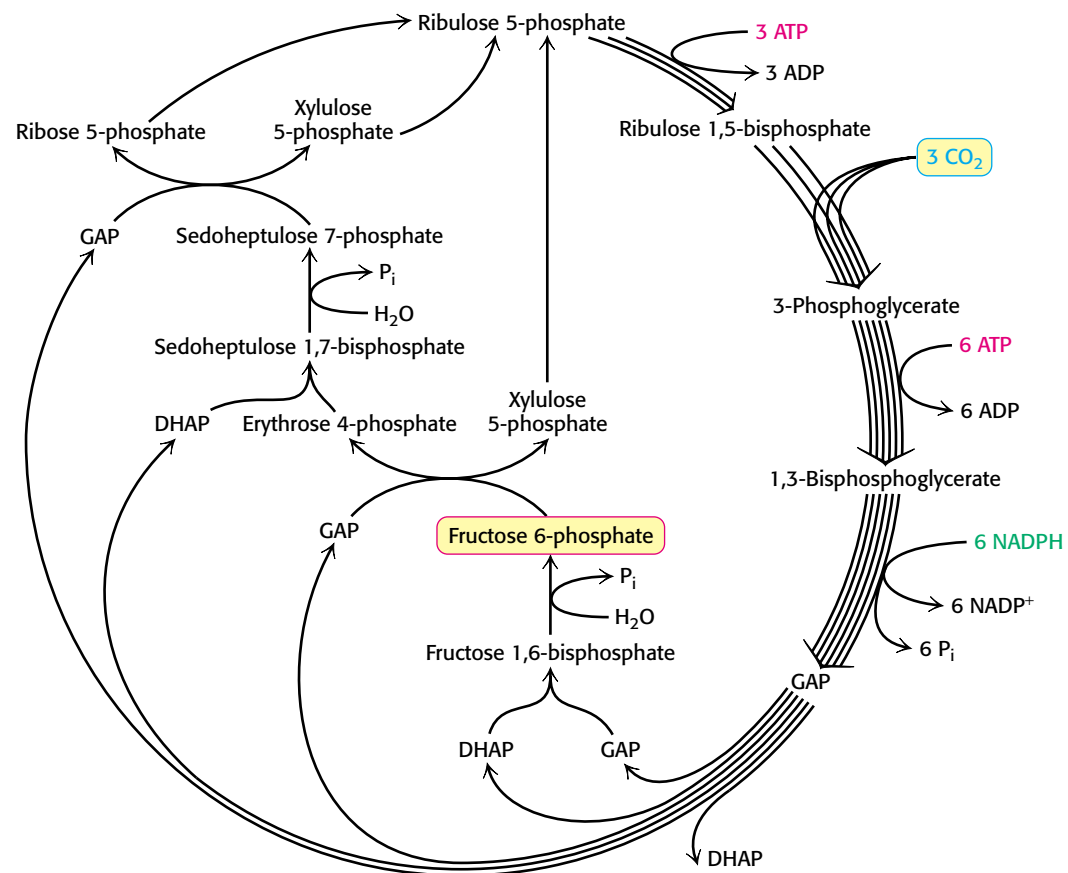
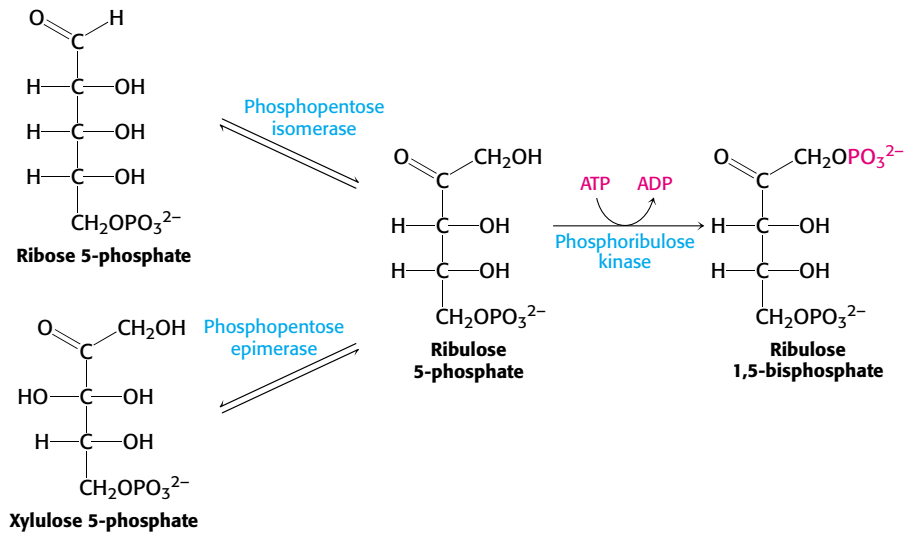
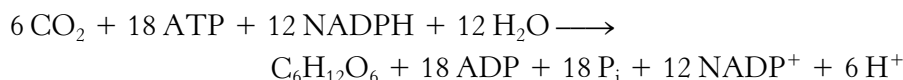


FIGURE 20.12 Calvin cycle. The diagram shows the reactions necessary with the correct stoichiometry to convert three molecules of CO₂ into one molecule of DHAP. The cycle is not as simple as presented in Figure 20.1; rather, it entails many reactions that lead ultimately to the synthesis of glucose and the regeneration of ribulose 1,5-bisphosphate. [After J. R. Bowyer and R. C. Leegood. "Photosynthesis," in *Plant Biochemistry*, P. M. Dey and J. B. Harborne, Eds. (Academic Press, 1997), p. 85.]

This series of reactions completes the Calvin cycle (Figure 20.12). The sum of all the reactions results in the generation of a hexose and the regeneration of the starting compound, ribulose 5-phosphate. In essence, ribulose 1,5-bisphosphate acts catalytically, similarly to oxaloacetate in the citric acid cycle.

20.1.4 Three Molecules of ATP and Two Molecules of NADPH Are Used to Bring Carbon Dioxide to the Level of a Hexose

What is the energy expenditure for synthesizing a hexose? Six rounds of the Calvin cycle are required, because one carbon atom is reduced in each round. Twelve molecules of ATP are expended in phosphorylating 12 molecules of 3-phosphoglycerate to 1,3-bisphosphoglycerate, and 12 molecules of NADPH are consumed in reducing 12 molecules of 1,3-bisphosphoglycerate to glyceraldehyde 3-phosphate. An additional six molecules of ATP are spent in regenerating ribulose 1,5-bisphosphate. We can now write a balanced equation for the net reaction of the Calvin cycle.



Thus, three molecules of ATP and two molecules of NADPH are consumed in incorporating a single CO_2 molecule into a hexose such as glucose or fructose.

20.1.5 Starch and Sucrose Are the Major Carbohydrate Stores in Plants

Plants contain two major storage forms of sugar: *starch* and *sucrose*. Starch, like its animal counterpart glycogen, is a polymer of glucose residues, but it is less branched than glycogen because it contains a smaller proportion of α -1,6-glycosidic linkages (Section 11.2.2). Another difference is that ADP-glucose, not UDP-glucose, is the activated precursor. Starch is synthesized and stored in chloroplasts.

In contrast, sucrose (common table sugar), a disaccharide, is synthesized in the cytosol. Plants lack the ability to transport hexose phosphates across the chloroplast membrane, but an abundant phosphate translocator mediates the transport of triose phosphates from chloroplasts to the cytosol in exchange for phosphate. Fructose 6-phosphate formed from triose phosphates joins the glucose unit of UDP-glucose to form sucrose 6-phosphate (Figure 20.13). Hydrolysis of the phosphate ester yields sucrose, a readily

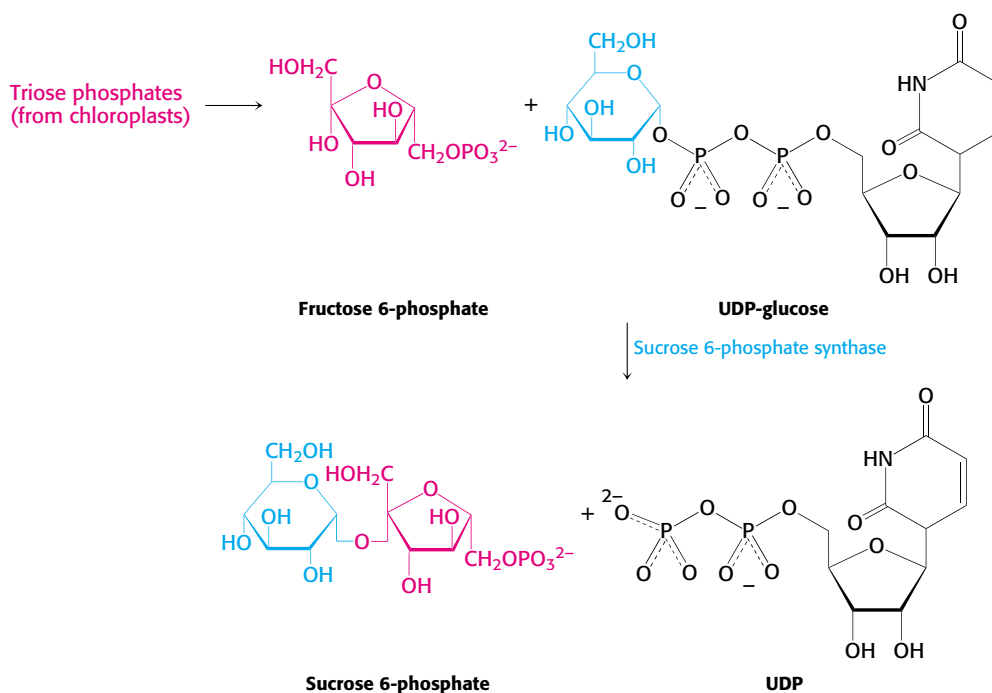


FIGURE 20.13 Synthesis of sucrose. Sucrose 6-phosphate is formed by the reaction between fructose 6-phosphate and the activated intermediate uridine diphosphate glucose (UDP-glucose).

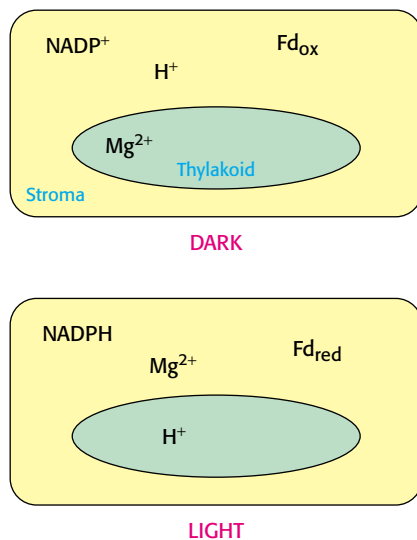


FIGURE 20.14 Light regulation of the Calvin cycle. The light reactions of photosynthesis transfer electrons out of the thylakoid lumen into the stroma and they transfer protons from the stroma into the thylakoid lumen. As a consequence of these processes, the concentrations of NADPH, reduced ferredoxin (Fd), and Mg²⁺ in the stroma are higher in the light than in the dark, whereas the concentration of H⁺ is lower in the dark. Each of these concentration changes helps couple the Calvin cycle reactions to the light reactions.

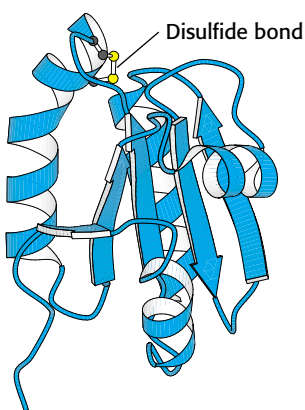


FIGURE 20.15 Thioredoxin. The oxidized form of thioredoxin contains a disulfide bond. When thioredoxin is reduced by reduced ferredoxin, the disulfide bond is converted into two free sulfhydryl groups. Reduced thioredoxin can cleave disulfide bonds in enzymes, activating certain Calvin cycle enzymes and inactivating some degradative enzymes.

transportable and mobilizable sugar that is stored in many plant cells, as in sugar beets and sugar cane.

20.2 THE ACTIVITY OF THE CALVIN CYCLE DEPENDS ON ENVIRONMENTAL CONDITIONS

Carbon dioxide assimilation by the Calvin cycle operates during the day, whereas carbohydrate degradation to yield energy takes place primarily at night. How are synthesis and degradation coordinately controlled? The light reactions lead to changes in the stroma—namely, an increase in pH and in Mg²⁺, NADPH, and reduced ferredoxin concentration—all of which contribute to the activation of certain Calvin cycle enzymes (Figure 20.14).

20.2.1 Rubisco Is Activated by Light-Driven Changes in Proton and Magnesium Ion Concentrations

As stated earlier, the rate-limiting step in the Calvin cycle is the carboxylation of ribulose 1,5-bisphosphate to form two molecules of 3-phosphoglycerate. *The activity of rubisco increases markedly on illumination.* The addition of CO₂ to lysine 201 of rubisco to form the carbamate is essential for Mg²⁺ coordination and, hence, catalytic activity (Section 20.1.1). Carbamate formation is favored by alkaline pH and high concentrations of Mg²⁺ ion in the stroma, both of which are consequences of the light-driven pumping of protons from the stroma into the thylakoid space. Magnesium ion concentration rises because Mg²⁺ ions from the thylakoid space are released into the stroma to compensate for the influx of protons.

20.2.2 Thioredoxin Plays a Key Role in Regulating the Calvin Cycle

Light-driven reactions lead to electron transfer from water to ferredoxin and, eventually, to NADPH. Both reduced ferredoxin and NADPH regulate enzymes from the Calvin cycle. One key protein in these regulatory processes is *thioredoxin*, a 12-kd protein containing neighboring cysteine residues that cycle between a reduced sulfhydryl and an oxidized disulfide form (Figure 20.15). The reduced form of thioredoxin activates many biosynthetic enzymes by reducing disulfide bridges that control their activity and inhibits several degradative enzymes by the same means (Table 20.1). In chloroplasts, oxidized thioredoxin is reduced by ferredoxin in a reaction catalyzed by *ferredoxin-thioredoxin reductase*. This enzyme contains

TABLE 20.1 Enzymes regulated by thioredoxin

Enzyme	Pathway
Rubisco	Carbon fixation in the Calvin cycle
Fructose 1,6-bisphosphatase	Gluconeogenesis
Glyceraldehyde 3-phosphate dehydrogenase	Calvin cycle, gluconeogenesis, glycolysis
Sedoheptulose bisphosphatase	Calvin cycle
Glucose 6-phosphate dehydrogenase	Pentose phosphate pathway
Phenylalanine ammonia lyase	Lignin synthesis
Ribulose 5'-phosphate kinase	Calvin cycle
NADP ⁺ -malate dehydrogenase	C ₄ pathway

a 4Fe-4S cluster that couples two one-electron oxidations of reduced ferredoxin to the two-electron reduction of thioredoxin. Thus, *the activities of the light and dark reactions of photosynthesis are coordinated through electron transfer from reduced ferredoxin to thioredoxin and then to component enzymes containing regulatory disulfide bonds* (Figure 20.16). We shall return to thioredoxin when we consider the reduction of ribonucleotides (Section 25.3).

Other means of control also exist. For instance, phosphoribulose kinase and glyceraldehyde 3-phosphate dehydrogenase also are regulated by NADPH directly. In the dark, these enzymes associate with a small protein called CP12 to form a large complex in which the enzymes are inactivated. NADPH generated in the light reactions binds to this complex, leading to the release of the enzymes. Thus, the activity of these enzymes depends first on reduction by thioredoxin and then on the NADPH-mediated release from CP12.

20.2.3 The C_4 Pathway of Tropical Plants Accelerates Photosynthesis by Concentrating Carbon Dioxide

Recall that the oxygenase activity of rubisco increases more rapidly with temperature than does its carboxylase activity. How then do plants, such as sugar cane, that grow in hot climates prevent very high rates of wasteful photorespiration? Their solution to this problem is to achieve a high local concentration of CO_2 at the site of the Calvin cycle in their photosynthetic cells. The essence of this process, which was elucidated by M. D. Hatch and C. R. Slack, is that *four-carbon (C_4) compounds such as oxaloacetate and malate carry CO_2 from mesophyll cells, which are in contact with air, to bundle-sheath cells, which are the major sites of photosynthesis* (Figure 20.17). Decarboxylation of the four-carbon compound in a bundle-sheath cell maintains a high concentration of CO_2 at the site of the Calvin cycle. The three-carbon compound pyruvate returns to the mesophyll cell for another round of carboxylation.

The C_4 pathway for the transport of CO_2 starts in a mesophyll cell with the condensation of CO_2 and phosphoenolpyruvate to form *oxaloacetate*, in a reaction catalyzed by *phosphoenolpyruvate carboxylase*. In some species, oxaloacetate is converted into *malate* by an $NADP^+$ -linked malate dehydrogenase. Malate goes into the bundle-sheath cell and is oxidatively decarboxylated within the chloroplasts by an $NADP^+$ -linked malate dehydrogenase. The released CO_2 enters the Calvin cycle in the usual way by condensing with ribulose 1,5-bisphosphate. Pyruvate formed in this decarboxylation reaction returns to the mesophyll cell. Finally, phosphoenolpyruvate is formed from pyruvate by *pyruvate- P_i dikinase*.

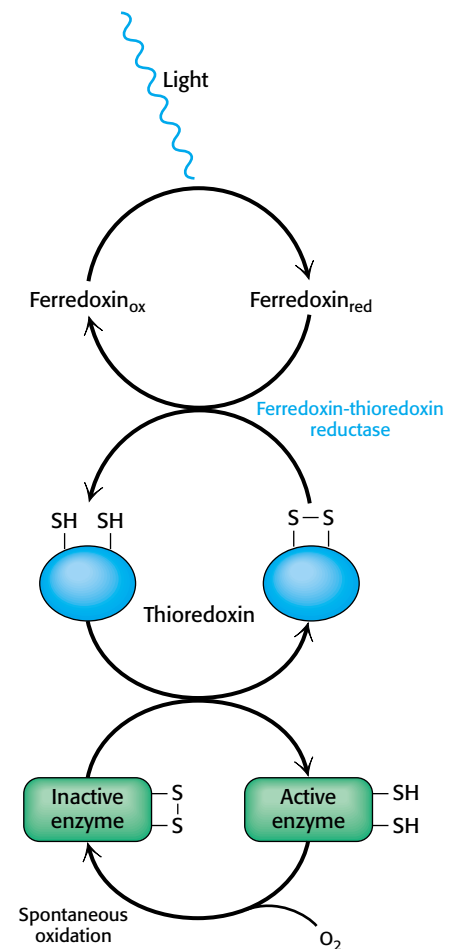


FIGURE 20.16 Enzyme activation by thioredoxin. Reduced thioredoxin activates certain Calvin cycle enzymes by cleaving regulatory disulfide bonds.

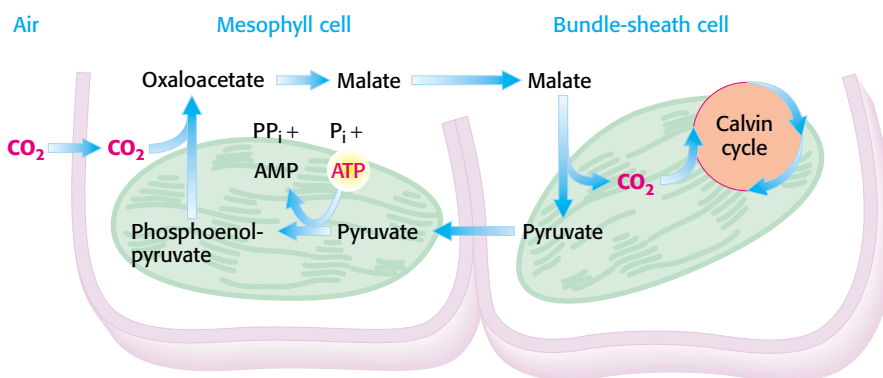
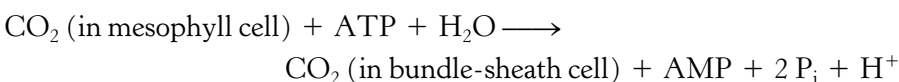


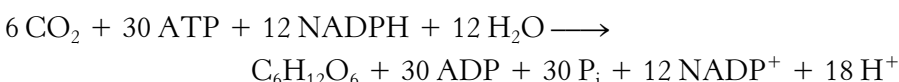
FIGURE 20.17 C_4 pathway. Carbon dioxide is concentrated in bundle-sheath cells by the expenditure of ATP in mesophyll cells.

The net reaction of this C_4 pathway is




Thus, *the energetic equivalent of two ATP molecules is consumed in transporting CO_2 to the chloroplasts of the bundle-sheath cells.* In essence, this process is active transport: the pumping of CO_2 into the bundle-sheath cell is driven by the hydrolysis of one molecule of ATP to one molecule of AMP and two molecules of orthophosphate. The CO_2 concentration can be 20-fold as great in the bundle-sheath cells as in the mesophyll cells.

When the C_4 pathway and the Calvin cycle operate together, the net reaction is



Note that 30 molecules of ATP are consumed per hexose molecule formed when the C_4 pathway delivers CO_2 to the Calvin cycle, in contrast with 18 molecules of ATP per hexose molecule in the absence of the C_4 pathway. The high concentration of CO_2 in the bundle-sheath cells of C_4 plants, which is due to the expenditure of the additional 12 molecules of ATP, is critical for their rapid photosynthetic rate, because CO_2 is limiting when light is abundant. A high CO_2 concentration also minimizes the energy loss caused by photorespiration.

Tropical plants with a C_4 pathway do little photorespiration because the high concentration of CO_2 in their bundle-sheath cells accelerates the carboxylase reaction relative to the oxygenase reaction. This effect is especially important at higher temperatures. The geographic distribution of plants having this pathway (C_4 plants) and those lacking it (C_3 plants) can now be understood in molecular terms. C_4 plants have the advantage in a hot environment and under high illumination, which accounts for their prevalence in the tropics. C_3 plants, which consume only 18 molecules of ATP per hexose molecule formed in the absence of photorespiration (compared with 30 molecules of ATP for C_4 plants), are more efficient at temperatures of less than about 28°C , and so they predominate in temperate environments.

 Rubisco is found in bacteria, eukaryotes, and even archaea, though other photosynthetic components have not been found in archaea. Thus, rubisco emerged early in evolution, when the atmosphere was rich in CO_2 and almost devoid of O_2 . The enzyme was not originally selected to operate in an environment like the present one, which is almost devoid of CO_2 and rich in O_2 . Photorespiration became significant about 60 million years ago, when the CO_2 concentration fell to present levels. The C_4 pathway is thought to have evolved in response to this selective pressure no more than 30 million years ago and possibly as recently as 7 million years ago. It is interesting to note that none of the enzymes are unique to C_4 plants, suggesting that this pathway was created using existing enzymes.

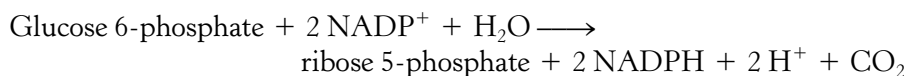
20.2.4 Crassulacean Acid Metabolism Permits Growth in Arid Ecosystems

Crassulacean acid metabolism (CAM) is yet another adaptation to increase the efficiency of the Calvin cycle. Crassulacean acid metabolism, named after the genus *Crassulacea* (the succulents), is a response to drought as well as warm conditions. In CAM plants, the stomata of the leaves are closed in

the heat of the day to prevent water loss (Figure 20.18). As a consequence, CO_2 cannot be absorbed during the daylight hours when it is needed for glucose synthesis. When the stomata open at the cooler temperatures of night, CO_2 is fixed by the C_4 pathway into malate, which is stored in vacuoles. During the day, malate is decarboxylated and the CO_2 becomes available to the Calvin cycle. In contrast with C_4 plants, CO_2 accumulation is separated from CO_2 utilization temporally in CAM plants rather than spatially.

20.3 THE PENTOSE PHOSPHATE PATHWAY GENERATES NADPH AND SYNTHESIZES FIVE-CARBON SUGARS

The pentose phosphate pathway meets the need of all organisms for a source of NADPH to use in reductive biosynthesis (Table 20.2). This pathway consists of two phases: the oxidative generation of NADPH and the non-oxidative interconversion of sugars (Figure 20.19). In the oxidative phase, NADPH is generated when glucose 6-phosphate is oxidized to ribose 5-phosphate. This five-carbon sugar and its derivatives are components of RNA and DNA, as well as ATP, NADH, FAD, and coenzyme A.



In the nonoxidative phase, the pathway catalyzes the interconversion of three-, four-, five-, six-, and seven-carbon sugars in a series of nonoxidative reactions that can result in the synthesis of five-carbon sugars for nucleotide biosynthesis or the conversion of excess five-carbon sugars into intermediates of the glycolytic pathway. All these reactions take place in the cytosol. These interconversions rely on the same reactions that lead to the regeneration of ribulose 1,5-bisphosphate in the Calvin cycle.

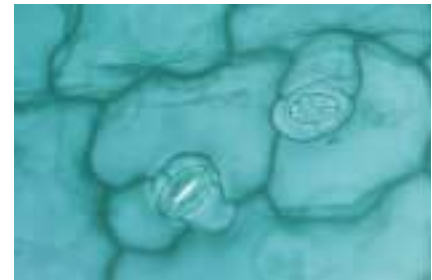
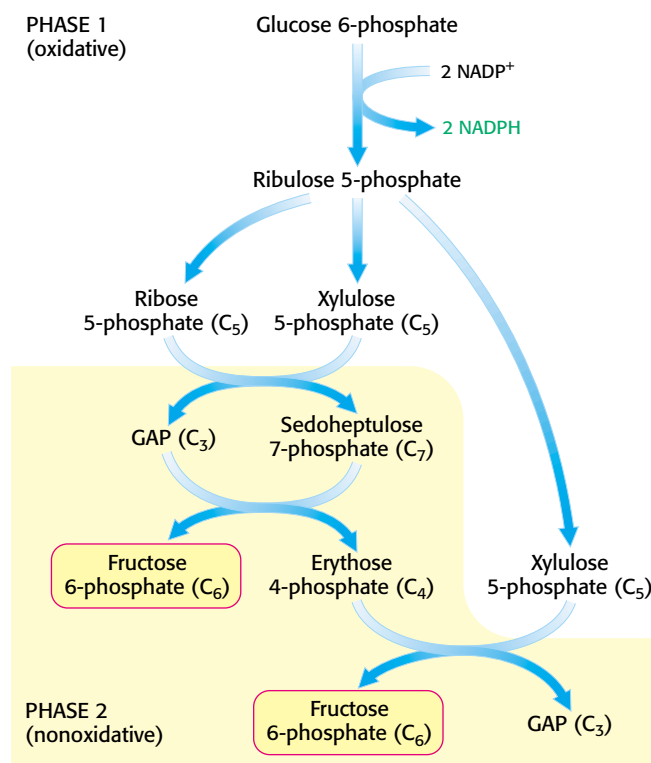


FIGURE 20.18 Electron micrograph of an open stoma and a closed stoma. [Herb Charles Ohlmeyer/Fran Heyl Associates.]

TABLE 20.2 Pathways requiring NADPH

Synthesis

- Fatty acid biosynthesis
- Cholesterol biosynthesis
- Neurotransmitter biosynthesis
- Nucleotide biosynthesis

Detoxification

- Reduction of oxidized glutathione
- Cytochrome P450 monooxygenases

FIGURE 20.19 Pentose phosphate pathway. The pathway consists of (1) an oxidative phase that generates NADPH and (2) a nonoxidative phase that interconverts phosphorylated sugars.

20.3.1 Two Molecules of NADPH Are Generated in the Conversion of Glucose 6-phosphate into Ribulose 5-phosphate

The oxidative phase of the pentose phosphate pathway starts with the dehydrogenation of glucose 6-phosphate at carbon 1, a reaction catalyzed by *glucose 6-phosphate dehydrogenase* (Figure 20.20). This enzyme is highly specific for NADP^+ ; the K_M for NADP^+ is about a thousand times as great as that for NAD^+ . The product is *6-phosphoglucono- δ -lactone*, which is an intramolecular ester between the C-1 carboxyl group and the C-5 hydroxyl group. The next step is the hydrolysis of 6-phosphoglucono- δ -lactone by a specific *lactonase* to give *6-phosphogluconate*. This six-carbon sugar is then oxidatively decarboxylated by *6-phosphogluconate dehydrogenase* to yield *ribulose 5-phosphate*. NADP^+ is again the electron acceptor. The final step in the synthesis of ribose 5-phosphate is the isomerization of ribulose 5-phosphate by phosphopentose isomerase (see Figure 20.11)

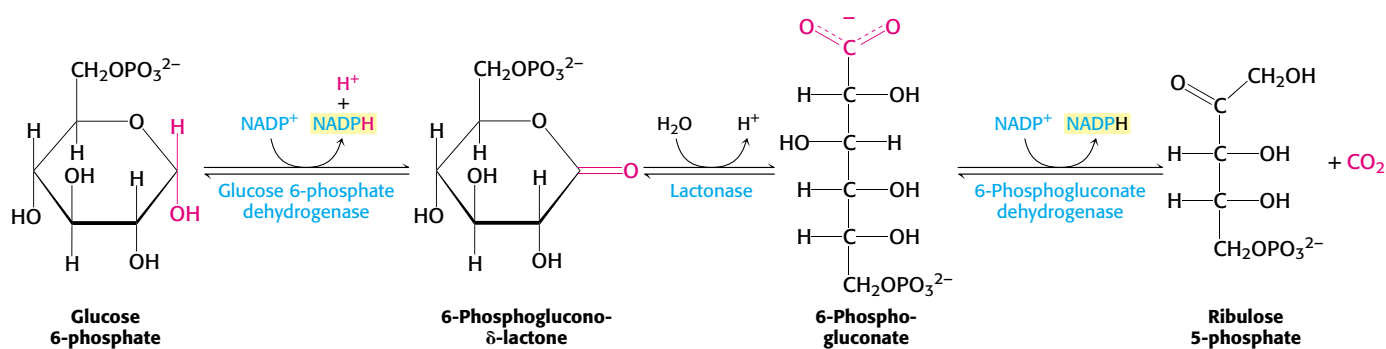
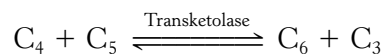
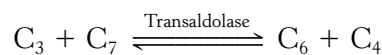
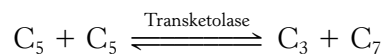


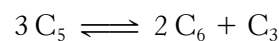
FIGURE 20.20 Oxidative phase of the pentose phosphate pathway. Glucose 6-phosphate is oxidized to 6-phosphoglucono- δ -lactone to generate one molecule of NADPH . The lactone product is hydrolyzed to 6-phosphogluconate, which is oxidatively decarboxylated to ribulose 5-phosphate with the generation of a second molecule of NADPH .

20.3.2 The Pentose Phosphate Pathway and Glycolysis Are Linked by Transketolase and Transaldolase

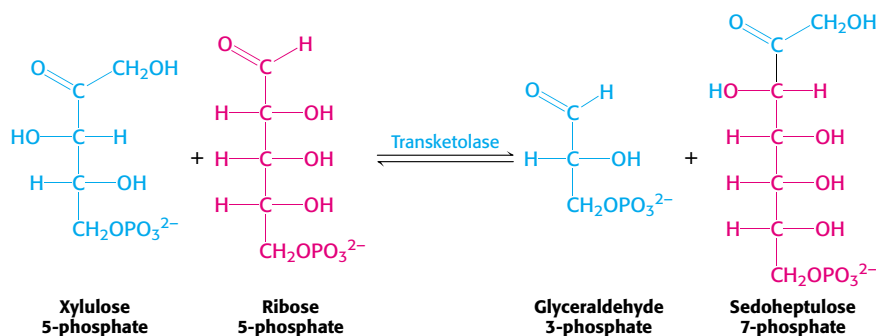
The preceding reactions yield two molecules of NADPH and one molecule of ribose 5-phosphate for each molecule of glucose 6-phosphate oxidized. However, many cells need NADPH for reductive biosyntheses much more than they need ribose 5-phosphate for incorporation into nucleotides and nucleic acids. In these cases, ribose 5-phosphate is converted into glyceraldehyde 3-phosphate and fructose 6-phosphate by *transketolase* and *transaldolase*. These enzymes create a reversible link between the pentose phosphate pathway and glycolysis by catalyzing these three successive reactions.



The net result of these reactions is the formation of two hexoses and one triose from three pentoses:

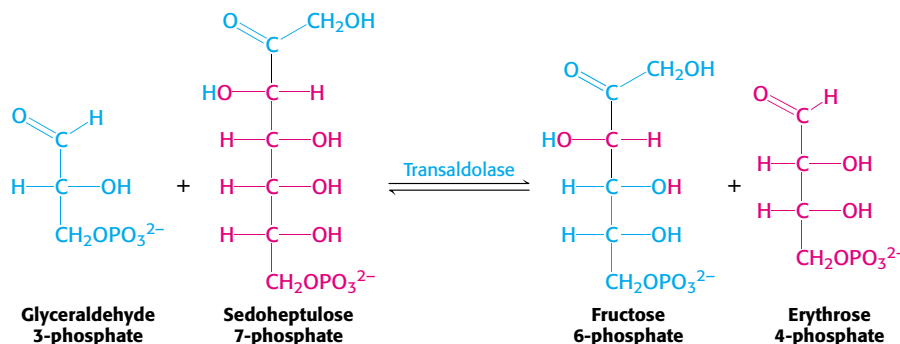


The first of the three reactions linking the pentose phosphate pathway and glycolysis is the formation of *glyceraldehyde 3-phosphate* and *sedoheptulose 7-phosphate* from two pentoses.



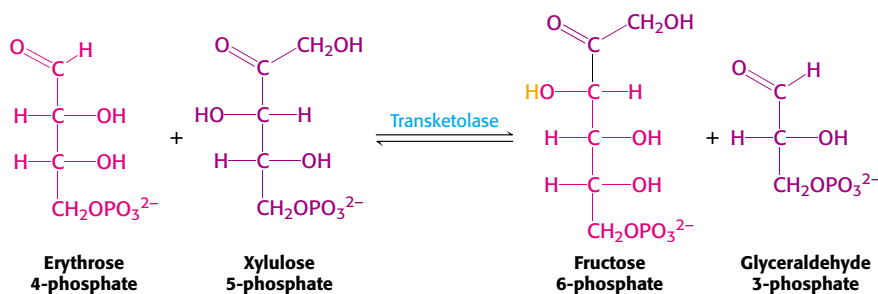
The donor of the two-carbon unit in this reaction is xylulose 5-phosphate, an epimer of ribulose 5-phosphate. A ketose is a substrate of transketolase only if its hydroxyl group at C-3 has the configuration of xylulose rather than ribulose. Ribulose 5-phosphate is converted into the appropriate epimer for the transketolase reaction by *phosphopentose epimerase* (see Figure 20.11) in the reverse reaction of that which occurs in the Calvin cycle.

Glyceraldehyde 3-phosphate and sedoheptulose 7-phosphate generated by the transketolase then react to form *fructose 6-phosphate* and *erythrose 4-phosphate*.

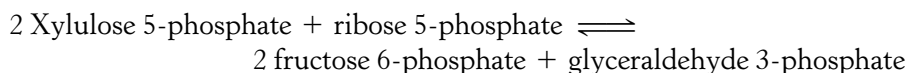


This synthesis of a four-carbon sugar and a six-carbon sugar is catalyzed by *transaldolase*.

In the third reaction, transketolase catalyzes the synthesis of *fructose 6-phosphate* and *glyceraldehyde 3-phosphate* from *erythrose 4-phosphate* and *xylulose 5-phosphate*.



The sum of these reactions is



Xylulose 5-phosphate can be formed from ribose 5-phosphate by the sequential action of *phosphopentose isomerase* and *phosphopentose epimerase*, and so the net reaction starting from ribose 5-phosphate is

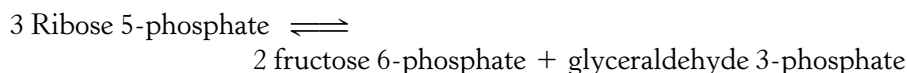


TABLE 20.3 Pentose phosphate pathway

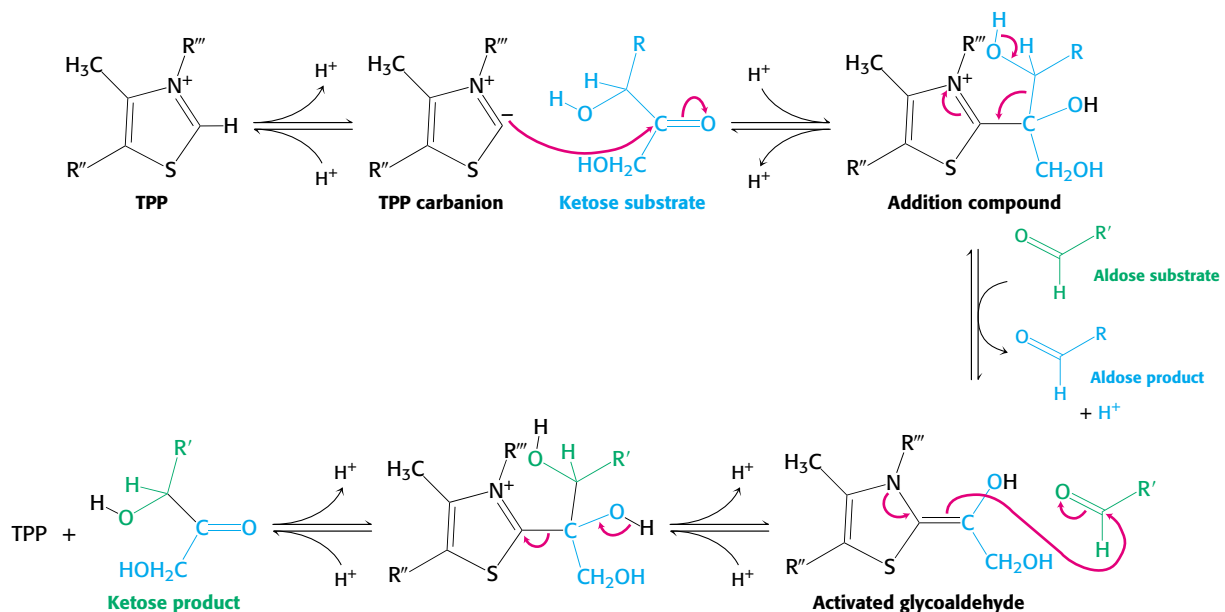
Reaction	Enzyme
Oxidative phase	
Glucose 6-phosphate + NADP ⁺ → 6-phosphoglucono-δ-lactone + NADPH + H ⁺	Glucose 6-phosphate dehydrogenase
6-Phosphoglucono-δ-lactone + H ₂ O → 6-phosphogluconate + H ⁺	Lactonase
6-Phosphogluconate + NADP ⁺ → ribulose 5-phosphate + CO ₂ + NADPH	6-Phosphogluconate dehydrogenase
Nonoxidative Phase	
Ribulose 5-phosphate ⇌ ribose 5-phosphate	Phosphopentose isomerase
Ribulose 5-phosphate ⇌ xylulose 5-phosphate	Phosphopentose epimerase
Xylulose 5-phosphate + ribose 5-phosphate ⇌ sedoheptulose 7-phosphate + glyceraldehyde 3-phosphate	Transketolase
Sedoheptulose 7-phosphate + glyceraldehyde 3-phosphate ⇌ fructose 6-phosphate + erythrose 4-phosphate	Transaldolase
Xylulose 5-phosphate + erythrose 4-phosphate ⇌ fructose 6-phosphate + glyceraldehyde 3-phosphate	Transketolase

Thus, *excess ribose 5-phosphate formed by the pentose phosphate pathway can be completely converted into glycolytic intermediates*. Moreover, any ribose ingested in the diet can be processed into glycolytic intermediates by this pathway. It is evident that the carbon skeletons of sugars can be extensively rearranged to meet physiologic needs (Table 20.3).

20.3.3 Transketolase and Transaldolase Stabilize Carbanionic Intermediates by Different Mechanisms

FIGURE 20.21 Transketolase mechanism. The carbanion of thiamine pyrophosphate (TPP) attacks the ketose substrate. Cleavage of a carbon-carbon bond frees the aldose product and leaves a two-carbon fragment joined to TPP. This activated glycoaldehyde intermediate attacks the aldose substrate to form a new carbon-carbon bond. The ketose product is released, freeing the TPP for the next reaction cycle.

The reactions catalyzed by transketolase and transaldolase are distinct yet similar in many ways. One difference is that transketolase transfers a two-carbon unit, whereas transaldolase transfers a three-carbon unit. Each of these units is transiently attached to the enzyme in the course of the reaction. In transketolase, the site of addition of the unit is the thiazole ring of the required coenzyme thiamine pyrophosphate. Transketolase is homologous to the E₁ subunit of the pyruvate dehydrogenase complex (Section 17.1.1) and the reaction mechanism is similar (Figure 20.21).



The C-2 carbon atom of bound TPP readily ionizes to give a *carbanion*. The negatively charged carbon atom of this reactive intermediate attacks the carbonyl group of the ketose substrate. The resulting addition compound releases the aldose product to yield an *activated glycoaldehyde unit*. The positively charged nitrogen atom in the thiazole ring acts as an *electron sink* in the development of this activated intermediate. The carbonyl group of a suitable aldose acceptor then condenses with the activated glycoaldehyde unit to form a new ketose, which is released from the enzyme.

Transaldolase transfers a three-carbon *dihydroxyacetone* unit from a ketose donor to an aldose acceptor. Transaldolase, in contrast with transketolase, does not contain a prosthetic group. Rather, a *Schiff base* is formed between the carbonyl group of the ketose substrate and the ϵ -amino group of a lysine residue at the active site of the enzyme (Figure 20.22). This kind of covalent enzyme–substrate intermediate is like that formed in fructose 1,6-bisphosphate aldolase in the glycolytic pathway (Section 16.1.3) and, indeed, the enzymes are homologous. The Schiff base becomes protonated, the bond between C-3 and C-4 is split, and an aldose is released. The negative charge on the Schiff-base carbanion moiety is stabilized by resonance. The positively charged nitrogen atom of the protonated Schiff base acts as an electron sink. The Schiff-base adduct is stable until a suitable aldose becomes bound. The dihydroxyacetone moiety then reacts with the carbonyl group of the aldose. The ketose product is released by hydrolysis of the Schiff base. The nitrogen atom of the protonated Schiff base plays the same role in transaldolase as the thiazole-ring nitrogen atom does in transketolase. In each enzyme, a group within an intermediate reacts like

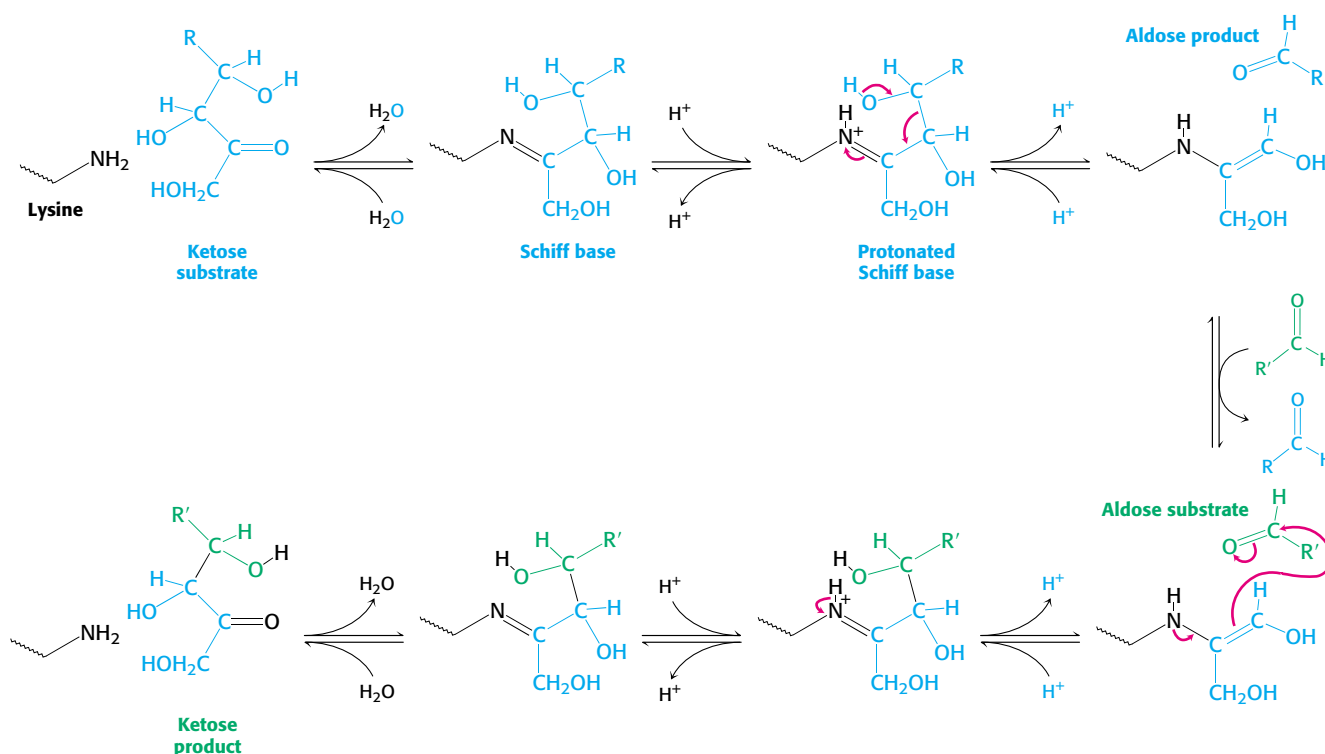


FIGURE 20.22 Transaldolase mechanism. The reaction begins with the formation of a Schiff base between a lysine residue in transaldolase and the ketose substrate. Protonation of the Schiff base leads to release of the aldose product, leaving a three-carbon fragment attached to the lysine residue. This intermediate adds to the aldose substrate to form a new carbon–carbon bond. The reaction cycle is completed by release of the ketose product from the lysine side chain.

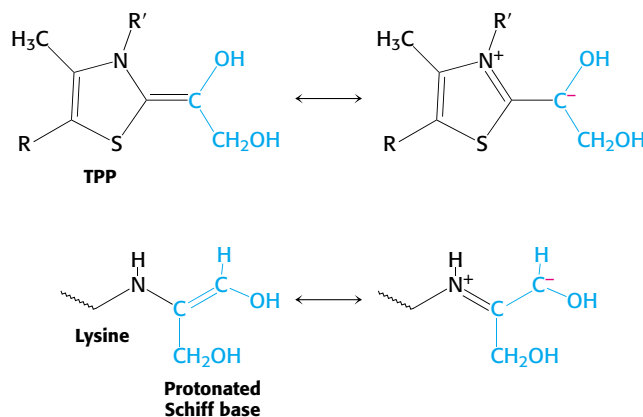


FIGURE 20.23 Carbanion intermediates. For transketolase and transaldolase, a carbanion intermediate is stabilized by resonance. In transketolase, TPP stabilizes this intermediate; in transaldolase, a protonated Schiff base plays this role.

a carbanion in attacking a carbonyl group to form a new carbon–carbon bond. In each case, the charge on the carbanion is stabilized by resonance (Figure 20.23).

20.4 THE METABOLISM OF GLUCOSE 6-PHOSPHATE BY THE PENTOSE PHOSPHATE PATHWAY IS COORDINATED WITH GLYCOLYSIS

Glucose 6-phosphate is metabolized by both the glycolytic pathway (Chapter 16) and the pentose phosphate pathway. How is the processing of this important metabolite partitioned between these two metabolic routes? The cytoplasmic concentration of NADP⁺ plays a key role in determining the fate of glucose 6-phosphate.

20.4.1 The Rate of the Pentose Phosphate Pathway Is Controlled by the Level of NADP⁺

The first reaction in the oxidative branch of the pentose phosphate pathway, the dehydrogenation of glucose 6-phosphate, is essentially irreversible. In fact, this reaction is rate limiting under physiological conditions and serves as the control site. The most important regulatory factor is the level of NADP⁺, the electron acceptor in the oxidation of glucose 6-phosphate to 6-phosphoglucono- δ -lactone. The inhibitory effect of low levels of NADP⁺ is exacerbated by the fact that NADPH competes with NADP⁺ in binding to the enzyme. The ratio of NADP⁺ to NADPH in the cytosol of a liver cell from a well-fed rat is about 0.014, several orders of magnitude lower than the ratio of NAD⁺ to NADH, which is 700 under the same conditions. The marked effect of the NADP⁺ level on the rate of the oxidative phase ensures that NADPH generation is tightly coupled to its utilization in reductive biosyntheses. The nonoxidative phase of the pentose phosphate pathway is controlled primarily by the availability of substrates.

20.4.2 The Flow of Glucose 6-phosphate Depends on the Need for NADPH, Ribose 5-phosphate, and ATP

We can grasp the intricate interplay between glycolysis and the pentose phosphate pathway by examining the metabolism of glucose 6-phosphate in four different metabolic situations (Figure 20.24).

Mode 1. *Much more ribose 5-phosphate than NADPH is required.* For example, rapidly dividing cells need ribose 5-phosphate for the synthesis of nucleotide precursors of DNA. Most of the glucose 6-phosphate is converted



CONCEPTUAL INSIGHTS, Overview of Carbohydrate and Fatty Acid Metabolism. View this media module to gain a “bigger picture” understanding of the roles of the pentose phosphate pathway in the context of other metabolic pathways (glycolysis, citric acid cycle, glycogen and fatty acid metabolism).

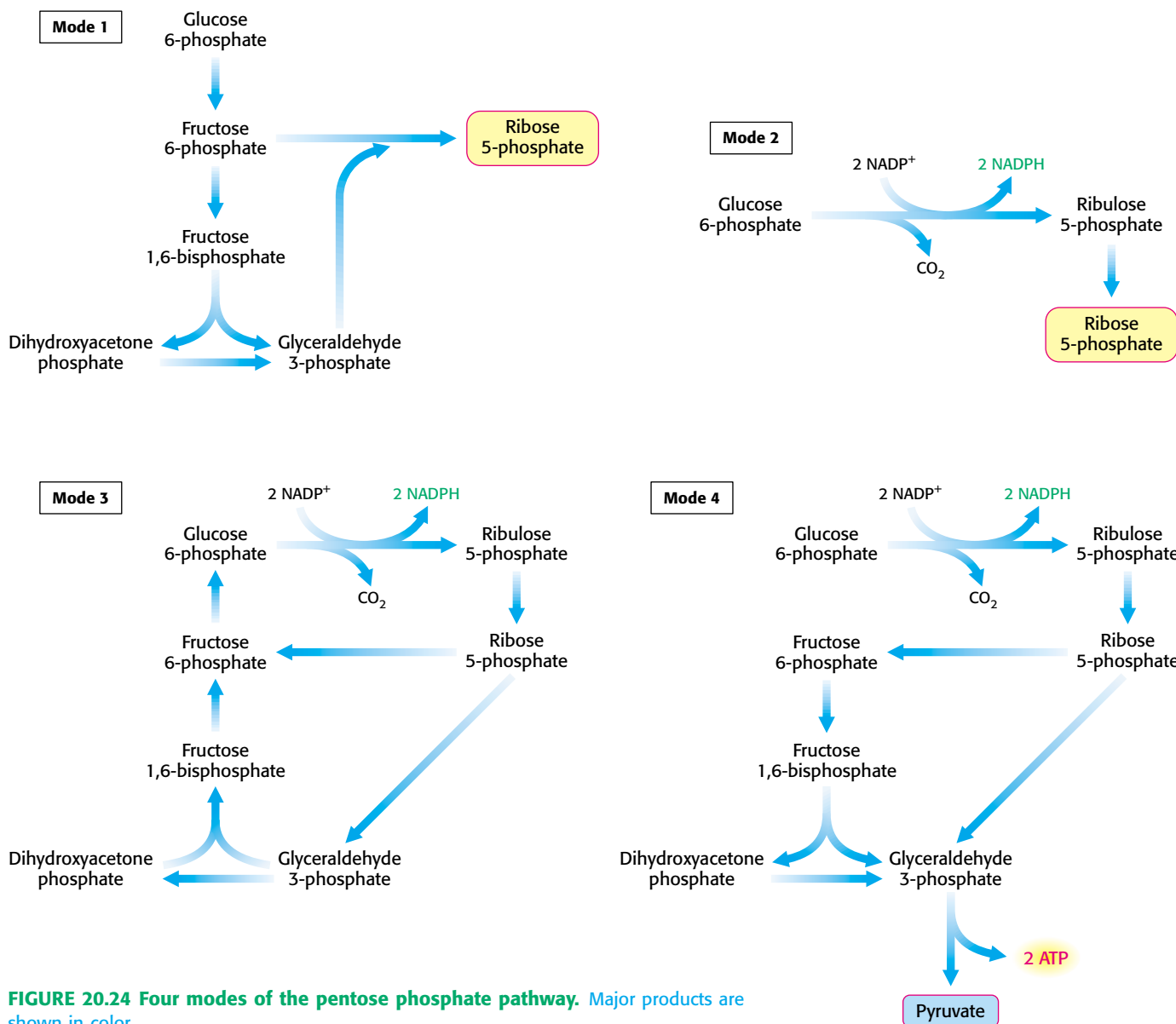
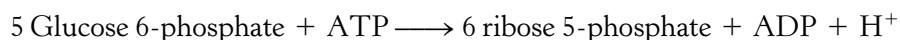
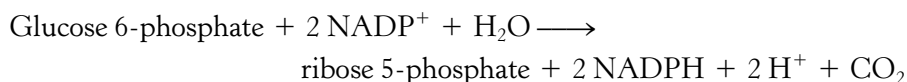


FIGURE 20.24 Four modes of the pentose phosphate pathway. Major products are shown in color.

into fructose 6-phosphate and glyceraldehyde 3-phosphate by the glycolytic pathway. Transaldolase and transketolase then convert two molecules of fructose 6-phosphate and one molecule of glyceraldehyde 3-phosphate into three molecules of ribose 5-phosphate by a reversal of the reactions described earlier. The stoichiometry of mode 1 is



Mode 2. *The needs for NADPH and ribose 5-phosphate are balanced.* The predominant reaction under these conditions is the formation of two molecules of NADPH and one molecule of ribose 5-phosphate from one molecule of glucose 6-phosphate in the oxidative phase of the pentose phosphate pathway. The stoichiometry of mode 2 is

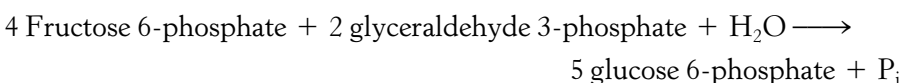
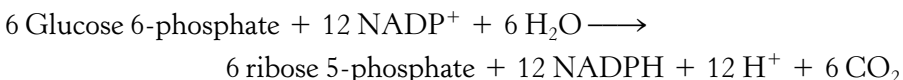


Mode 3. *Much more NADPH than ribose 5-phosphate is required.* For example, adipose tissue requires a high level of NADPH for the synthesis of

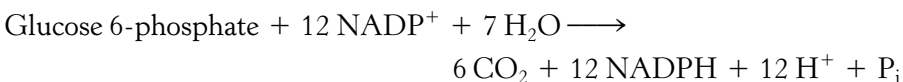
TABLE 20.4 Tissues with active pentose phosphate pathways

Tissue	Function
Adrenal gland	Steroid synthesis
Liver	Fatty acid and cholesterol synthesis
Testes	Steroid synthesis
Adipose tissue	Fatty acid synthesis
Ovary	Steroid synthesis
Mammary gland	Fatty acid synthesis
Red blood cells	Maintenance of reduced glutathione

fatty acids (Table 20.4). In this case, glucose 6-phosphate is completely oxidized to CO_2 . Three groups of reactions are active in this situation. First, the oxidative phase of the pentose phosphate pathway forms two molecules of NADPH and one molecule of ribose 5-phosphate. Then, ribose 5-phosphate is converted into fructose 6-phosphate and glyceraldehyde 3-phosphate by transketolase and transaldolase. Finally, glucose 6-phosphate is resynthesized from fructose 6-phosphate and glyceraldehyde 3-phosphate by the gluconeogenic pathway. The stoichiometries of these three sets of reactions are

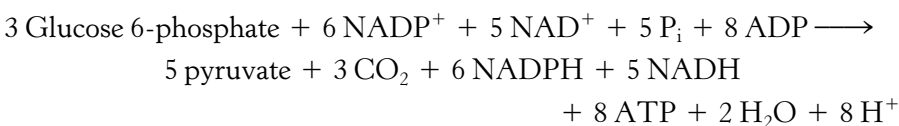


The sum of the mode 3 reactions is



Thus, *the equivalent of glucose 6-phosphate can be completely oxidized to CO_2 with the concomitant generation of NADPH.* In essence, ribose 5-phosphate produced by the pentose phosphate pathway is recycled into glucose 6-phosphate by transketolase, transaldolase, and some of the enzymes of the gluconeogenic pathway.

Mode 4. *Both NADPH and ATP are required.* Alternatively, ribose 5-phosphate formed by the oxidative phase of the pentose phosphate pathway can be converted into pyruvate. Fructose 6-phosphate and glyceraldehyde 3-phosphate derived from ribose 5-phosphate enter the glycolytic pathway rather than reverting to glucose 6-phosphate. In this mode, *ATP and NADPH are concomitantly generated, and five of the six carbons of glucose 6-phosphate emerge in pyruvate.*



Pyruvate formed by these reactions can be oxidized to generate more ATP or it can be used as a building block in a variety of biosyntheses.


20.4.3 Through the Looking Glass: The Calvin Cycle and the Pentose Phosphate Pathway

The complexities of the Calvin cycle and the pentose phosphate pathway are easier to comprehend if we consider them mirror images of each other. The Calvin cycle begins with the fixation of CO_2 and goes on to use NADPH in the synthesis of glucose. The pentose phosphate pathway begins with the oxidation of a glucose-derived carbon atom to CO_2 and concomitantly generates NADPH. The regeneration phase of the Calvin cycle converts C_6 and C_3 molecules back into the starting material—the C_5 molecule ribulose 1,5-bisphosphate. The pentose phosphate pathway converts a C_5 molecule, ribose 5-phosphate, into C_6 and C_3 intermediates of the glycolytic pathway. Not surprisingly, in photosynthetic organisms, many enzymes are common to the two pathways. We see the economy of evolution: the use of identical enzymes for similar reactions with different ends.

20.5 GLUCOSE 6-PHOSPHATE DEHYDROGENASE PLAYS A KEY ROLE IN PROTECTION AGAINST REACTIVE OXYGEN SPECIES

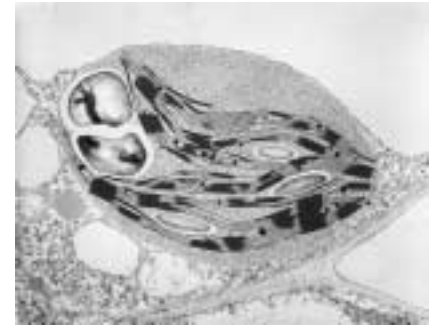
Reactive oxygen species (ROS) generated in oxidative metabolism inflict damage on all classes of macromolecules and can ultimately lead to cell death. Indeed, ROS are implicated in a number of human diseases (Section 18.3.6). Reduced *glutathione* (GSH), a tripeptide with a free sulfhydryl group, is required to combat oxidative stress and maintain the normal reduced state in the cell. Oxidized glutathione (GSSG) is reduced by NADPH generated by glucose 6-phosphate dehydrogenase in the pentose phosphate pathway. Indeed, cells with reduced levels of glucose 6-phosphate dehydrogenase are especially sensitive to oxidative stress. This stress is most acute in red blood cells because, lacking mitochondria, they have no alternative means of generating reducing power.

20.5.1 Glucose 6-phosphate Dehydrogenase Deficiency Causes a Drug-Induced Hemolytic Anemia

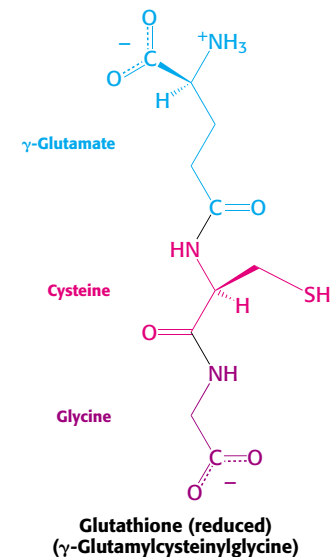
 At its introduction in 1926, an antimalarial drug, pamaquine, was associated with the appearance of severe and mysterious ailments. Most patients tolerated the drug well, but a few developed severe symptoms within a few days after therapy was started. The urine turned black, jaundice developed, and the hemoglobin content of the blood dropped sharply. In some cases, massive destruction of red blood cells caused death.

This drug-induced hemolytic anemia was shown 30 years later to be caused by a *deficiency of glucose 6-phosphate dehydrogenase*, the enzyme catalyzing the first step in the oxidative branch of the pentose phosphate pathway. This defect, which is inherited on the X chromosome, is the most common enzymopathy, affecting hundreds of millions of people. The major role of NADPH in red cells is to reduce the disulfide form of glutathione to the sulfhydryl form. The enzyme that catalyzes the regeneration of reduced glutathione, the flavoprotein *glutathione reductase*, a dimer of 50-kd subunits, is homologous to ferredoxin-NADP⁺ reductase, which we encountered in photosynthesis (Section 19.3.4). The reduced form of glutathione serves as a *sulfhydryl buffer* that maintains the cysteine residues of hemoglobin and other red-blood-cell proteins in the reduced state. Normally, the ratio of the reduced to oxidized forms of glutathione in red blood cells is 500.

How is GSH regenerated from GSSG and NADPH by glutathione reductase? The electrons from NADPH are not directly transferred to the



Electron micrograph of a chloroplast. The thylakoid membranes course throughout the stroma of a chloroplast from a cell of *Phleum pratense*, a grass. The dark areas of stacked thylakoid membrane are grana. Several large starch granules, which store the newly synthesized glucose, are also obvious. [Biophoto Associates/Photo Researchers.]





Vicia faba. The Mediterranean plant *Vicia faba* is a source of fava beans that contain the purine glycoside vicine. [Inga Spence/Visuals Unlimited.]

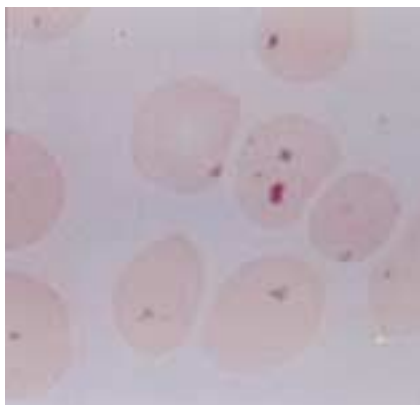
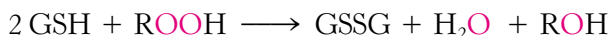


FIGURE 20.25 Red blood cells with Heinz bodies. The light micrograph shows red blood cells obtained from a person deficient in glucose 6-phosphate dehydrogenase. The dark particles, called Heinz bodies, inside the cells are clumps of denatured protein that adhere to the plasma membrane and stain with basic dyes. Red blood cells in such people are highly susceptible to oxidative damage. [Courtesy of Dr. Stanley Schrier.]


disulfide bond in oxidized glutathione. Rather, they are transferred from NADPH to a tightly bound flavin adenine dinucleotide (FAD) on the reductase, then to a disulfide bridge between two cysteine residues in the enzyme subunit, and finally to oxidized glutathione.

Reduced glutathione is essential for maintaining the normal structure of red blood cells and for keeping hemoglobin in the ferrous state. The reduced form also plays a role in detoxification by reacting with hydrogen peroxide and organic peroxides.



Cells with a lowered level of reduced glutathione are more susceptible to hemolysis. How can we explain this phenomenon biochemically? The presence of pamaquine, a purine glycoside of fava beans, or other nonenzymatic oxidative agents leads to the generation of peroxides, reactive oxygen species that can damage membranes as well as other biomolecules. Peroxides are normally eliminated by glutathione peroxidase with the use of glutathione as a reducing agent (Section 24.4). Moreover, in the absence of the enzyme, the hemoglobin sulfhydryl groups can no longer be maintained in the reduced form and hemoglobin molecules then cross-link with one another to form aggregates called *Heinz bodies* on cell membranes (Figure 20.25). Membranes damaged by the Heinz bodies and reactive oxygen species become deformed and the cell is likely to undergo lysis. In the absence of oxidative stress, however, the deficiency is quite benign. The occurrence of this dehydrogenase deficiency also clearly demonstrates that *atypical reactions to drugs may have a genetic basis*.

20.5.2 A Deficiency of Glucose 6-phosphate Dehydrogenase Confers an Evolutionary Advantage in Some Circumstances

 The incidence of the most common form of glucose 6-phosphate dehydrogenase deficiency, characterized by a tenfold reduction in enzymatic activity in red blood cells, is 11% among Americans of African heritage. This high frequency suggests that the deficiency may be advantageous under certain environmental conditions. Indeed, *glucose 6-phosphate dehydrogenase deficiency protects against falciparum malaria*. The parasites causing this disease require reduced glutathione and the products of the pentose phosphate pathway for optimal growth. Thus, glucose 6-phosphate dehydrogenase deficiency is a mechanism of protection against malaria, which accounts for its high frequency in malaria-infested regions of the world. We see here once again the interplay of heredity and environment in the production of disease.

SUMMARY

- **The Calvin Cycle Synthesizes Hexoses from Carbon Dioxide and Water**

ATP and NADPH formed in the light reactions of photosynthesis are used to convert CO_2 into hexoses and other organic compounds. The dark phase of photosynthesis, called the Calvin cycle, starts with the reaction of CO_2 and ribulose 1,5-bisphosphate to form two molecules of 3-phosphoglycerate. The steps in the conversion of 3-phosphoglycerate into fructose 6-phosphate and glucose 6-phosphate are like those of gluconeogenesis, except that glyceraldehyde 3-phosphate dehydrogenase in chloroplasts is specific for NADPH rather than NADH. Ribulose 1,5-bisphosphate is regenerated from fructose 6-phosphate, glyceraldehyde 3-phosphate, and dihydroxyacetone phosphate by a complex

series of reactions. Several of the steps in the regeneration of ribulose 1,5-bisphosphate are like those of the pentose phosphate pathway. Three molecules of ATP and two molecules of NADPH are consumed for each molecule of CO₂ converted into a hexose. Starch in chloroplasts and sucrose in the cytosol are the major carbohydrate stores in plants.

- **The Activity of the Calvin Cycle Depends on Environmental Conditions**

Reduced thioredoxin formed by the light-driven transfer of electrons from ferredoxin activates enzymes of the Calvin cycle by reducing disulfide bridges. The light-induced increase in pH and Mg²⁺ level of the stroma is important in stimulating the carboxylation of ribulose 1,5-bisphosphate by ribulose 1,5-bisphosphate carboxylase. This enzyme also catalyzes a competing oxygenase reaction, which produces phosphoglycolate and 3-phosphoglycerate. The recycling of phosphoglycolate leads to the release of CO₂ and further consumption of O₂ in a process called photorespiration. This wasteful side reaction is minimized in tropical plants, which have an accessory pathway—called the C₄ pathway—for concentrating CO₂ at the site of the Calvin cycle. This pathway enables tropical plants to take advantage of high levels of light and minimize the oxygenation of ribulose 1,5-bisphosphate. Plants in arid ecosystems employ Crassulacean acid metabolism (CAM) to prevent dehydration. In CAM plants, the C₄ pathway is active during the night when the plant exchanges gases with the air. During the day, gas exchange is eliminated and CO₂ is generated from malate stored in vacuoles.

- **The Pentose Phosphate Pathway Generates NADPH and Synthesizes Five-Carbon Sugars**

Whereas the Calvin cycle is present only in photosynthetic organisms, the pentose phosphate pathway is present in all organisms. The pentose phosphate pathway generates NADPH and ribose 5-phosphate in the cytosol. NADPH is used in reductive biosyntheses, whereas ribose 5-phosphate is used in the synthesis of RNA, DNA, and nucleotide coenzymes. The pentose phosphate pathway starts with the dehydrogenation of glucose 6-phosphate to form a lactone, which is hydrolyzed to give 6-phosphogluconate and then oxidatively decarboxylated to yield ribulose 5-phosphate. NADP⁺ is the electron acceptor in both of these oxidations. The last step is the isomerization of ribulose 5-phosphate (a ketose) to ribose 5-phosphate (an aldose). A different mode of the pathway is active when cells need much more NADPH than ribose 5-phosphate. Under these conditions, ribose 5-phosphate is converted into glyceraldehyde 3-phosphate and fructose 6-phosphate by transketolase and transaldolase. These two enzymes create a reversible link between the pentose phosphate pathway and glycolysis. Xylulose 5-phosphate, sedoheptulose 7-phosphate, and erythrose 4-phosphate are intermediates in these interconversions. In this way, 12 molecules of NADPH can be generated for each molecule of glucose 6-phosphate that is completely oxidized to CO₂.

- **The Metabolism of Glucose 6-phosphate by the Pentose Phosphate Pathway Is Coordinated with Glycolysis**

Only the nonoxidative branch of the pathway is significantly active when much more ribose 5-phosphate than NADPH needs to be synthesized. Under these conditions, fructose 6-phosphate and glyceraldehyde 3-phosphate (formed by the glycolytic pathway) are converted into ribose 5-phosphate without the formation of NADPH. Alternatively, ribose 5-phosphate formed by the oxidative branch can be converted into pyruvate through fructose 6-phosphate and glyceraldehyde 3-phosphate. In this mode, ATP and NADPH are generated, and five

of the six carbons of glucose 6-phosphate emerge in pyruvate. The interplay of the glycolytic and pentose phosphate pathways enables the levels of NADPH, ATP, and building blocks such as ribose 5-phosphate and pyruvate to be continuously adjusted to meet cellular needs.

• **Glucose 6-phosphate Dehydrogenase Plays a Key Role in Protection Against Reactive Oxygen Species**

NADPH generated by glucose 6-phosphate dehydrogenase maintains the appropriate levels of reduced glutathione required to combat oxidative stress and maintain the proper reducing environment in the cell. Cells with diminished glucose 6-phosphate dehydrogenase activity are especially sensitive to oxidative stress.

KEY TERMS

Calvin cycle (dark reactions) (p. 552)	sucrose (p. 559)	glucose 6-phosphate dehydrogenase (p. 564)
autotroph (p. 552)	thioredoxin (p. 560)	transketolase (p. 564)
heterotroph (p. 552)	C ₄ pathway (p. 561)	transaldolase (p. 564)
rubisco (ribulose 1,5-bisphosphate carboxylase/oxygenase) (p. 553)	C ₄ plant (p. 562)	glutathione (p. 571)
peroxisome (p. 555)	C ₃ plant (p. 562)	
photorespiration (p. 556)	Crassulacean acid metabolism (CAM) (p. 562)	
starch (p. 559)	pentose phosphate pathway (p. 563)	

SELECTED READINGS

Where to start

- Horecker, B. L., 1976. Unravelling the pentose phosphate pathway. In *Reflections on Biochemistry* (pp. 65–72), edited by A. Kornberg, L. Cornudella, B. L. Horecker, and J. Oro, Pergamon.
- Levi, P., 1984. Carbon. In *The Periodic Table*. Random House.
- Melendez-Hevia, E., and Isidoro, A., 1985. The game of the pentose phosphate cycle. *J. Theor. Biol.* 117:251–263.
- Barber, J., and Andersson, B., 1994. Revealing the blueprint of photosynthesis. *Nature* 370:31–34.
- Rawsthorne, S., 1992. Towards an understanding of C₃-C₄ photosynthesis. *Essays Biochem.* 27:135–146.

Books and general reviews

- Wood, T., 1985. *The Pentose Phosphate Pathway*. Academic Press.
- Buchanan, B. B., Gruissem, W., and Jones, R. L., 2000. *Biochemistry and Molecular Biology of Plants*. American Society of Plant Physiologists.
- Schneider, G., Lindqvist, Y., and Branden, C., 1992. Rubisco: Structure and mechanism. *Annu. Rev. Biophys. Biomol. Struct.* 21:119–143.
- Spreitzer, R. J., 1993. Genetic dissection of rubisco structure and function. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 44:411–434.

Enzymes and reaction mechanisms

- Harrison, D. H., Runquist, J. A., Holub, A., and Mizioro, H. M., 1998. The crystal structure of phosphoribulokinase from *Rhodobacter sphaeroides* reveals a fold similar to that of adenylate kinase. *Biochemistry* 37:5074–5085.
- Mizioro, H. M., 2000. Phosphoribulokinase: Current perspectives on the structure/function basis for regulation and catalysis. *Adv. Enzymol. Relat. Areas Mol. Biol.* 74:95–127.
- Thorell, S., Gergely, P., Jr., Banki, K., Perl, A., and Schneider, G., 2000. The three-dimensional structure of human transaldolase. *FEBS Lett.* 475:205–208.

- Lindqvist, Y., Schneider, G., Ermler, U., and Sundstrom, M., 1992. Three-dimensional structure of transketolase, a thiamine diphosphate dependent enzyme, at 2.5 Å resolution. *EMBO J.* 11:2373–2379.
- Robinson, B. H., and Chun, K., 1993. The relationships between transketolase, yeast pyruvate decarboxylase and pyruvate dehydrogenase of the pyruvate dehydrogenase complex. *FEBS Lett.* 328:99–102.

Carbon dioxide fixation and rubisco

- Sugawara, H., Yamamoto, H., Shibata, N., Inoue, T., Okada, S., Miyake, C., Yokota, A., and Kai, Y., 1999. Crystal structure of carboxylase reaction-oriented ribulose 1,5-bisphosphate carboxylase/oxygenase from a thermophilic red alga, *Galdieria partita*. *J. Biol. Chem.* 274:15655–15661.
- Hansen, S., Vollan, V. B., Hough, E., and Andersen, K., 1999. The crystal structure of rubisco from *Alcaligenes eutrophus* reveals a novel central eight-stranded beta-barrel formed by beta-strands from four subunits. *J. Mol. Biol.* 288:609–621.
- Knight, S., Andersson, I., and Branden, C. I., 1990. Crystallographic analysis of ribulose 1,5-bisphosphate carboxylase from spinach at 2.4 Å resolution: Subunit interactions and active site. *J. Mol. Biol.* 215:113–160.
- Taylor, T. C., and Andersson, I., 1997. The structure of the complex between rubisco and its natural substrate ribulose 1,5-bisphosphate. *J. Mol. Biol.* 265:432–444.
- Cleland, W. W., Andrews, T. J., Gutteridge, S., Hartman, F. C., and Lorimer, G. H., 1998. Mechanism of rubisco: The carbamate as general base. *Chem. Rev.* 98:549–561.
- Buchanan, B. B., 1992. Carbon dioxide assimilation in oxygenic and anoxygenic photosynthesis. *Photosynth. Res.* 33:147–162.
- Hatch, M. D., 1987. C₄ photosynthesis: A unique blend of modified biochemistry, anatomy, and ultrastructure. *Biochim. Biophys. Acta* 895:81–106.

Regulation

- Rokka, A., Zhang, L., and Aro, E.-M., 2001. Rubisco activase: An enzyme with a temperature-dependent dual function? *Plant J.* 25:463–472.
- Zhang, N., and Portis, A. R., Jr., 1999. Mechanism of light regulation of rubisco: A specific role for the larger rubisco activase isoform involving reductive activation by thioredoxin-f. *Proc. Natl. Acad. Sci. USA* 96:9438–9443.
- Wedel, N., Soll, J., and Paap, B. K., 1997. CP12 provides a new mode of light regulation of Calvin cycle activity in higher plants. *Proc. Natl. Acad. Sci. USA* 94:10479–10484.
- Avilan, L., Lebreton, S., and Gontero, B., 2000. Thioredoxin activation of phosphoribulokinase in a bi-enzyme complex from *Chlamydomonas reinhardtii* chloroplasts. *J. Biol. Chem.* 275:9447–9451.
- Irihimovitch, V., and Shapira, M., 2000. Glutathione redox potential modulated by reactive oxygen species regulates translation of rubisco large subunit in the chloroplast. *J. Biol. Chem.* 275:16289–16295.

Glucose 6-phosphate dehydrogenase

- Au, S. W., Gover, S., Lam, V. M., and Adams, M. J., 2000. Human glucose-6-phosphate dehydrogenase: The crystal structure reveals a structural NADP(+) molecule and provides insights into enzyme deficiency. *Structure Fold. Des.* 8:293–303.
- Salvemini, F., Franze, A., Iervolino, A., Filosa, S., Salzano, S., and Ursini, M. V., 1999. Enhanced glutathione levels and oxidoresistance mediated by increased glucose-6-phosphate dehydrogenase expression. *J. Biol. Chem.* 274:2750–2757.
- Tian, W. N., Braunstein, L. D., Apse, K., Pang, J., Rose, M., Tian, X., and Stanton, R. C., 1999. Importance of glucose-6-phosphate dehydrogenase activity in cell death. *Am. J. Physiol.* 276:C1121–C1131.

- Tian, W. N., Braunstein, L. D., Pang, J., Stuhlmeier, K. M., Xi, Q. C., Tian, X., and Stanton, R. C., 1998. Importance of glucose-6-phosphate dehydrogenase activity for cell growth. *J. Biol. Chem.* 273:10609–10617.
- Ursini, M. V., Parrella, A., Rosa, G., Salzano, S., and Martini, G., 1997. Enhanced expression of glucose-6-phosphate dehydrogenase in human cells sustaining oxidative stress. *Biochem. J.* 323:801–806.

Evolution

- Coy, J. F., Dubel, S., Kioschis, P., Thomas, K., Micklem, G., Delius, H., and Poustka, A., 1996. Molecular cloning of tissue-specific transcripts of a transketolase-related gene: Implications for the evolution of new vertebrate genes. *Genomics* 32:309–316.
- Schenk, G., Layfield, R., Candy, J. M., Duggleby, R. G., and Nixon, P. F., 1997. Molecular evolutionary analysis of the thiamine-diphosphate-dependent enzyme, transketolase. *J. Mol. Evol.* 44: 552–572.
- Notaro, R., Afolayan, A., and Luzzatto L., 2000. Human mutations in glucose 6-phosphate dehydrogenase reflect evolutionary history. *FASEB J.* 14:485–494.
- Wedel, N., and Soll, J., 1998. Evolutionary conserved light regulation of Calvin cycle activity by NADPH-mediated reversible phosphoribulokinase/CP12/glyceraldehyde-3-phosphate dehydrogenase complex dissociation. *Proc. Natl. Acad. Sci. USA* 95:9699–9704.
- Martin, W., and Schnarrenberger, C., 1997. The evolution of the Calvin cycle from prokaryotic to eukaryotic chromosomes: A case study of functional redundancy in ancient pathways through endosymbiosis. *Curr. Genet.* 32:1–18.
- Ku, M. S., Kano-Murakami, Y., and Matsuoka, M., 1996. Evolution and expression of C₄ photosynthesis genes. *Plant Physiol.* 111:949–957.
- Pereto, J. G., Velasco, A. M., Becerra, A., and Lazzano, A., 1999. Comparative biochemistry of CO₂ fixation and the evolution of autotrophy. *Int. Microbiol.* 2:3–10.

PROBLEMS

- Variation on a theme.* Sedoheptulose 1,7-bisphosphate is an intermediate in the Calvin cycle but not in the pentose phosphate pathway. What is the enzymatic basis of this difference?
- Total eclipse.* An illuminated suspension of *Chlorella* is actively carrying out photosynthesis. Suppose that the light is suddenly switched off. How would the levels of 3-phosphoglycerate and ribulose 1,5-bisphosphate change in the next minute?
- CO₂ deprivation.* An illuminated suspension of *Chlorella* is actively carrying out photosynthesis in the presence of 1% CO₂. The concentration of CO₂ is abruptly reduced to 0.003%. What effect would this reduction have on the levels of 3-phosphoglycerate and ribulose 1,5-bisphosphate in the next minute?
- A potent analog.* 2-Carboxyarabinitol 1,5-bisphosphate (CABP) has been useful in studies of rubisco.
 - Write the structural formula of CABP.
 - Which catalytic intermediate does it resemble?
 - Predict the effect of CABP on rubisco.
- Salvage operation.* Write a balanced equation for the transamination of glyoxylate to yield glycine.
- When one equals two.* In the C₄ pathway, one ATP molecule is used in combining the CO₂ with phosphoenolpyruvate to form oxaloacetate (Figure 20.17), but, in the computation of energetics bookkeeping, two ATP molecules are said to be consumed. Explain.

- Dog days of August.* Before the days of pampered lawns, most homeowners practiced horticultural Darwinism. A result was that the lush lawns of early summer would often convert into robust cultures of crabgrass in the dog days of August. Provide a possible biochemical explanation for this transition.
- Global warming.* C₃ plants are most common in higher latitudes and become less common at latitudes near the equator. The reverse is true of C₄ plants. How might global warming affect this distribution?
- Tracing glucose.* Glucose labeled with ¹⁴C at C-6 is added to a solution containing the enzymes and cofactors of the oxidative phase of the pentose phosphate pathway. What is the fate of the radioactive label?
- Recurring decarboxylations.* Which reaction in the citric acid cycle is most analogous to the oxidative decarboxylation of 6-phosphogluconate to ribulose 5-phosphate? What kind of enzyme-bound intermediate is formed in both reactions?
- Carbon shuffling.* Ribose 5-phosphate labeled with ¹⁴C at C-1 is added to a solution containing transketolase, transaldolase, phosphopentose epimerase, phosphopentose isomerase, and glyceraldehyde 3-phosphate. What is the distribution of the radioactive label in the erythrose 4-phosphate and fructose 6-phosphate that are formed in this reaction mixture?
- Synthetic stoichiometries.* What is the stoichiometry of synthesis of (a) ribose 5-phosphate from glucose 6-phosphate with-

576 CHAPTER 20 • The Calvin Cycle and the Pentose Phosphate Pathway

out the concomitant generation of NADPH; (b) NADPH from glucose 6-phosphate without the concomitant formation of pentose sugars?

13. *Trapping a reactive lysine.* Design a chemical experiment to identify the lysine residue that forms a Schiff base at the active site of transaldolase.

14. *Reductive power.* What ratio of NADPH to NADP⁺ is required to sustain [GSH] = 10 mM and [GSSG] = 1 mM? Use the redox potentials given in Table 18.1.

Mechanism Problems

15. *An alternative approach.* The mechanisms of some aldolases do not include Schiff-base intermediates. Instead, these enzymes require bound metal ions. Propose such a mechanism for the conversion of dihydroxyacetone phosphate and glyceraldehyde 3-phosphate into fructose 1,6-bisphosphate.

16. *A recurring intermediate.* Phosphopentose isomerase interconverts the aldose ribose 5-phosphate and the ketose ribulose 5-phosphate. Propose a mechanism.

Chapter Integration Problems

17. *Catching carbons.* Radioactive-labeling experiments can yield estimates of how much glucose 6-phosphate is metabolized by the pentose phosphate pathway and how much is metabolized by the combined action of glycolysis and the citric acid cycle. Suppose that you have samples of two different tissues as well as two radioactively labeled glucose samples, one with glucose labeled with ¹⁴C at C-1 and the other with glucose labeled with ¹⁴C at C-6. Design an experiment that would enable you to determine the relative activity of the aerobic metabolism of glucose compared with metabolism by the pentose phosphate pathway.

18. *Photosynthetic efficiency.* Use the following information to estimate the efficiency of photosynthesis.

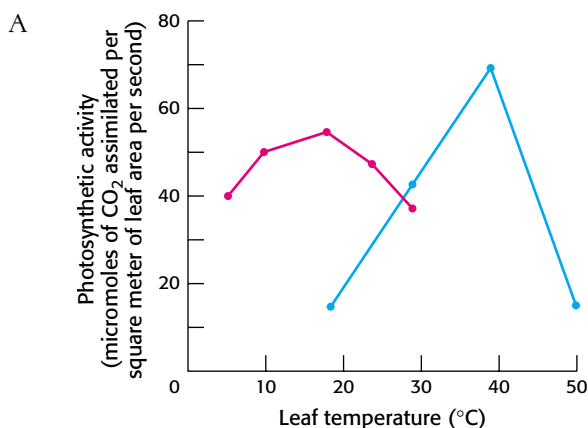
The ΔG° for the reduction of CO₂ to the level of hexose is +114 kcal mol⁻¹ (+477 kJ mol⁻¹).

A mole of 600-nm photons has an energy content of 47.6 kcal (199 kJ).

Assume that the proton gradient generated in producing the required NADPH is sufficient to drive the synthesis of the required ATP.

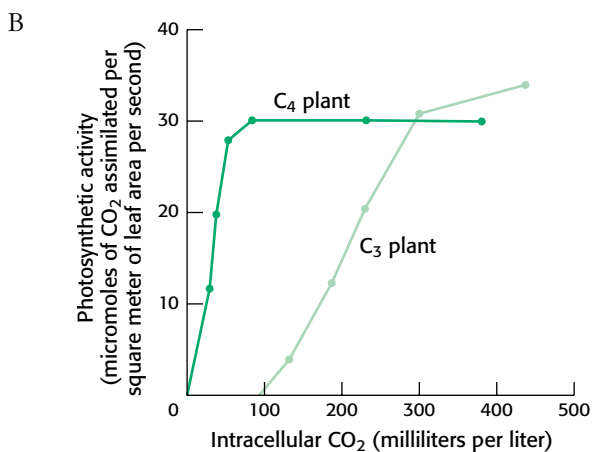
Date Interpretation Problem

19. Graph A shows the photosynthetic activity of two species of plant, one a C₄ plant and the other a C₃ plant, as a function of leaf temperature.



- (a) Which data were most likely generated by the C₄ plant and which by the C₃ plant? Explain.
 (b) Suggest some possible explanations for why the photosynthetic activity falls at higher temperatures.

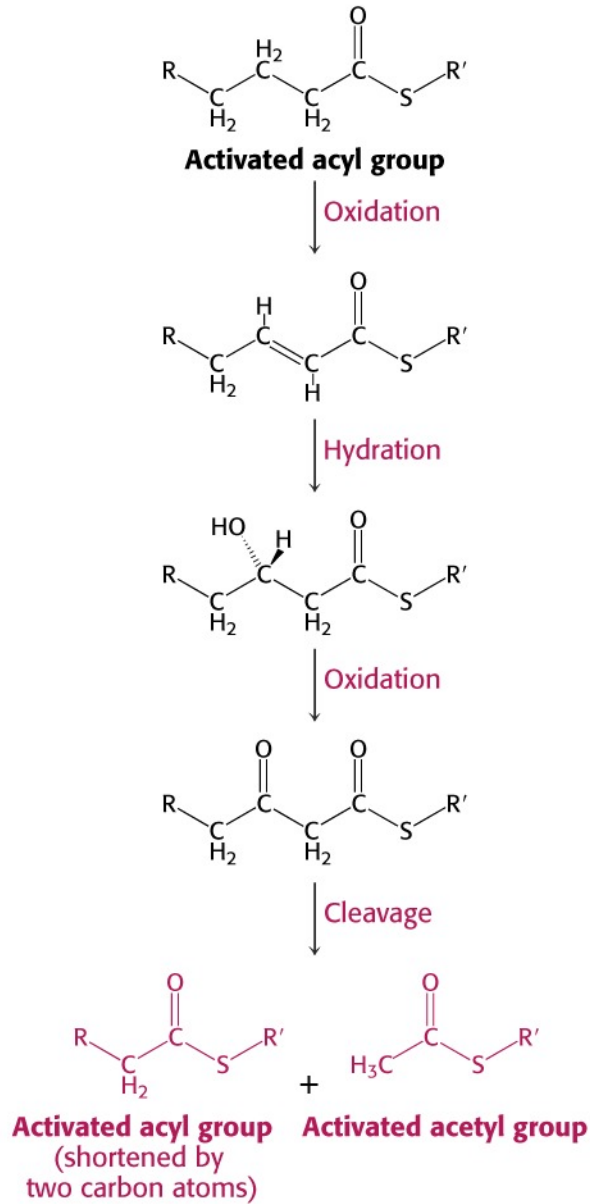
Graph B illustrates how the photosynthetic activity of C₃ and C₄ plants varies with CO₂ concentration when temperature (30°C) and light intensity (high) are constant.



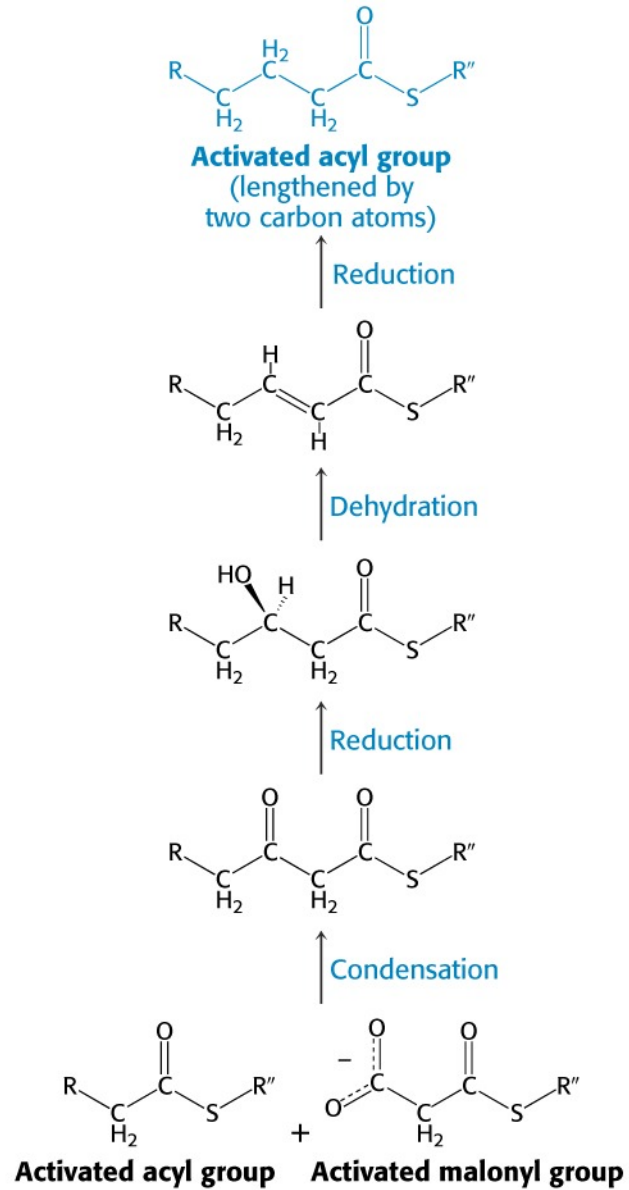
- (c) Why can C₄ plants thrive at CO₂ concentrations that do not support the growth of C₃ plants?
 (d) Suggest a plausible explanation for why C₃ plants continue to increase photosynthetic activity at higher CO₂ concentrations, whereas C₄ plants reach a plateau.

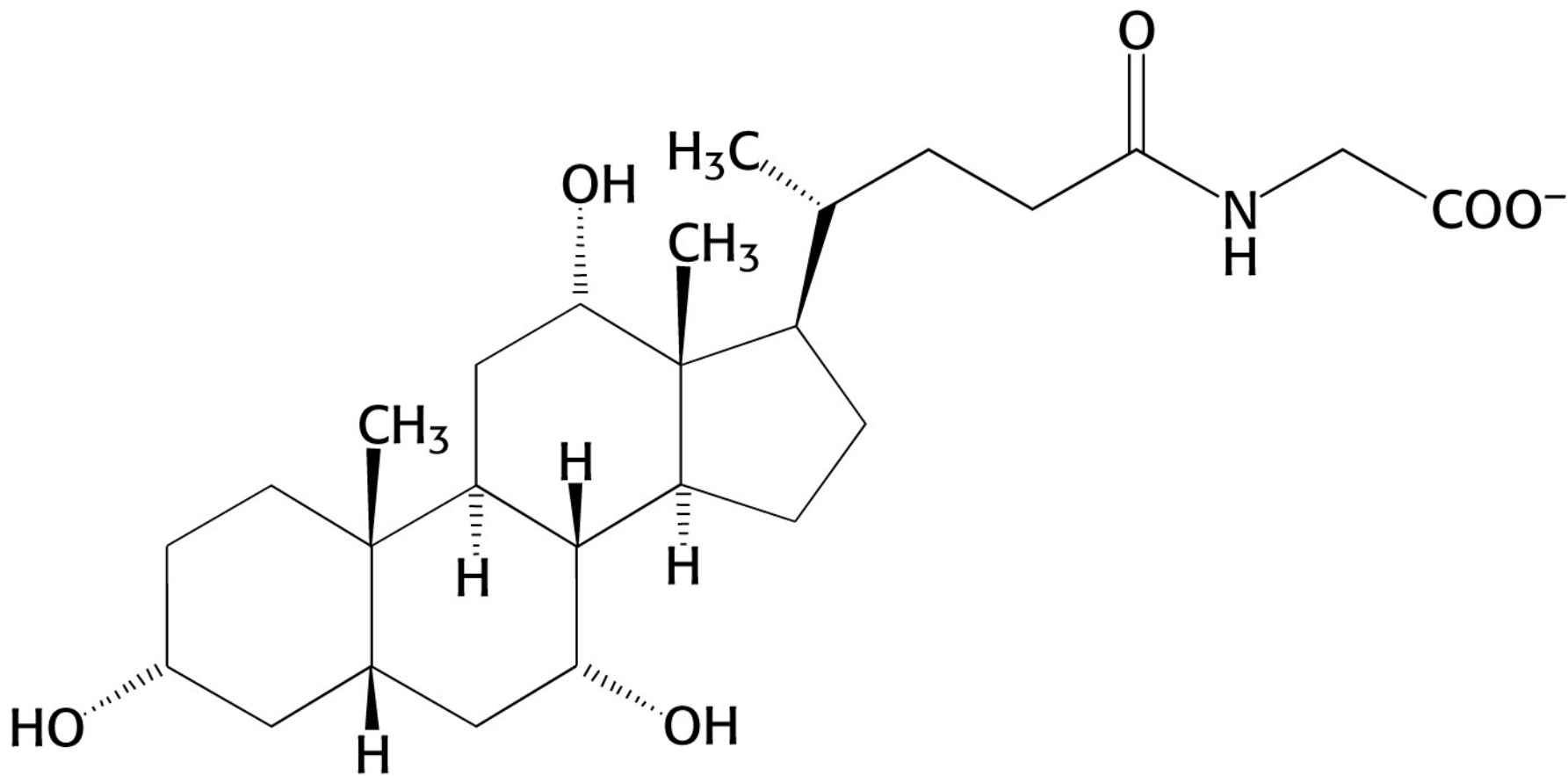


FATTY ACID DEGRADATION

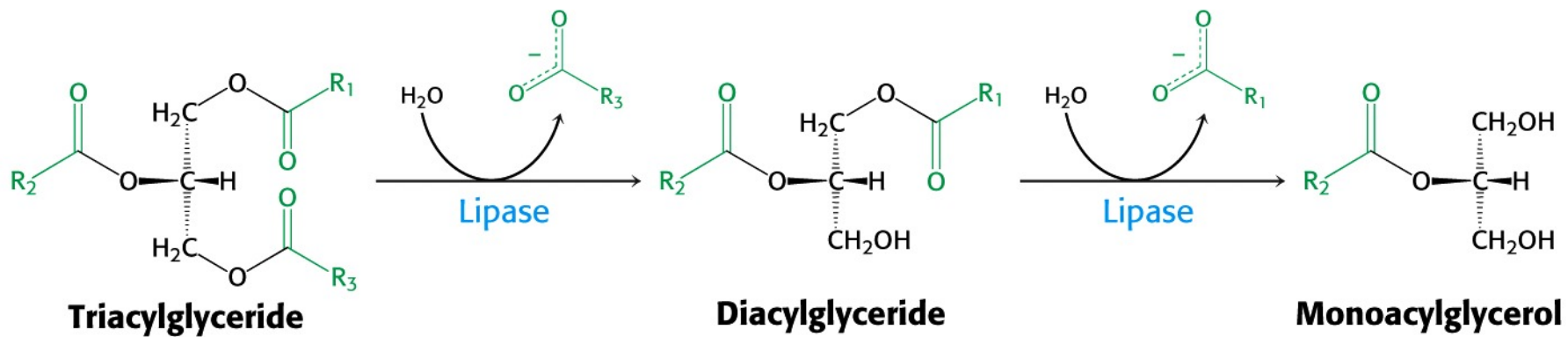


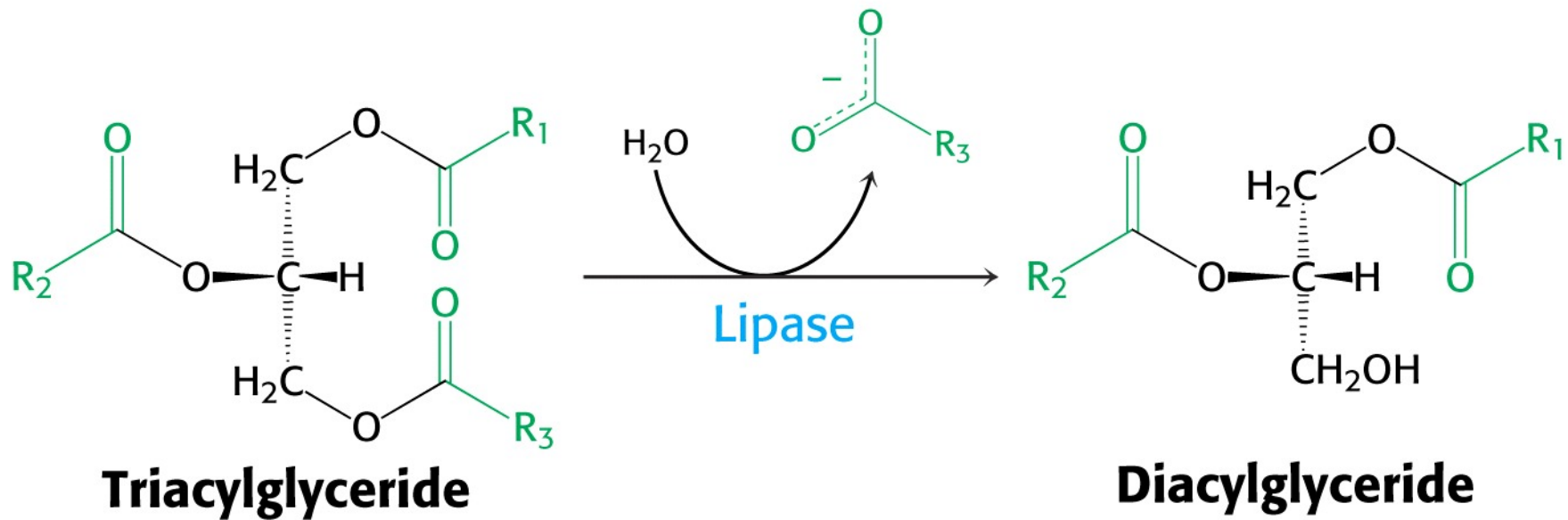
FATTY ACID SYNTHESIS

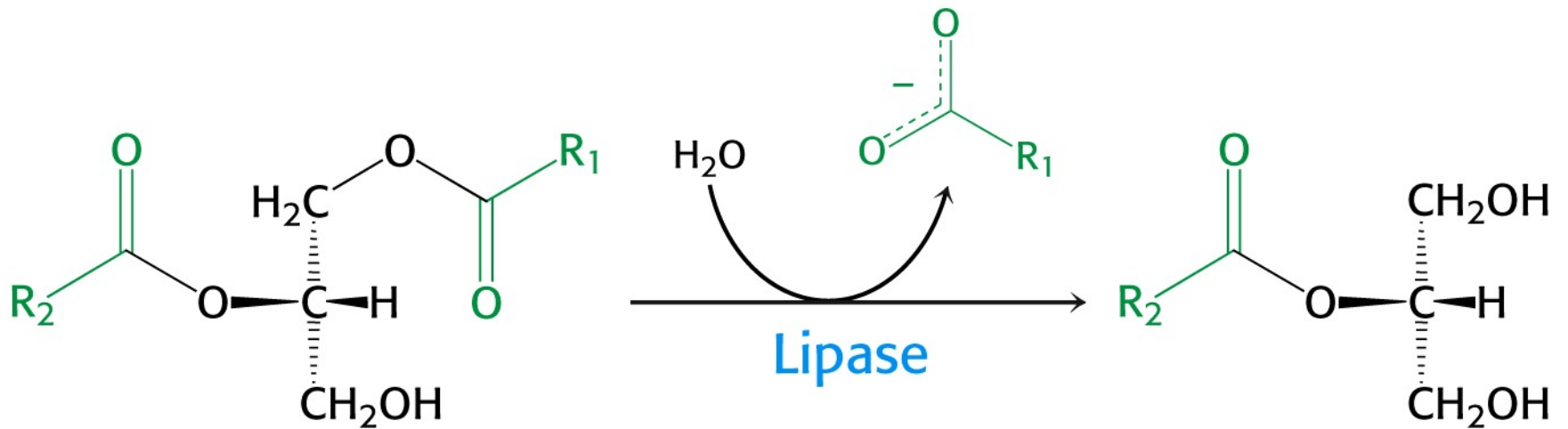




Glycocholate

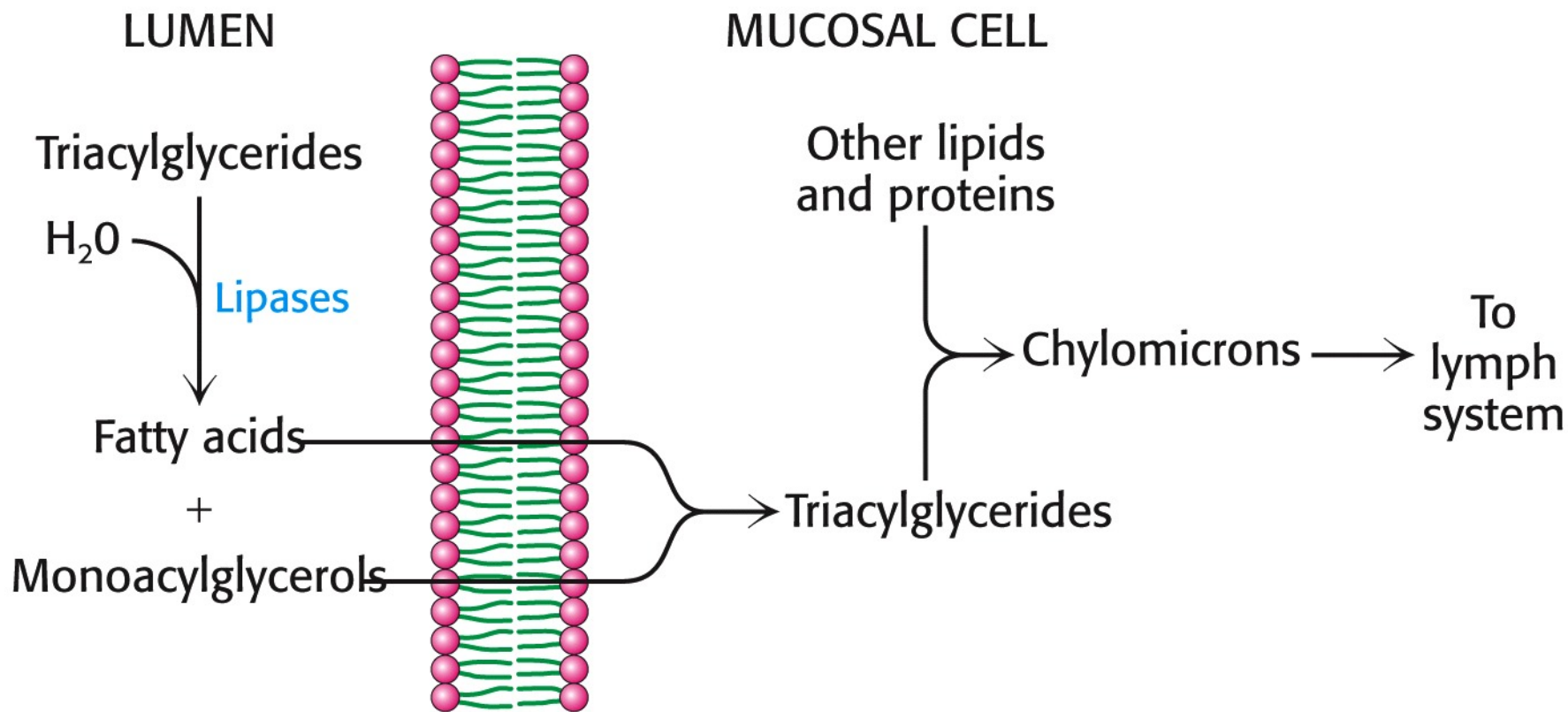


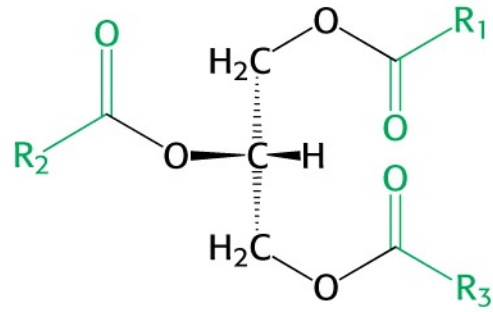




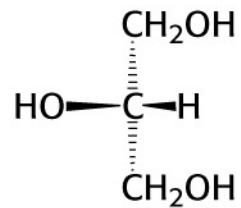
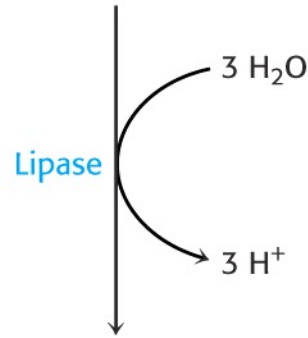
Diacylglyceride

Monoacylglycerol



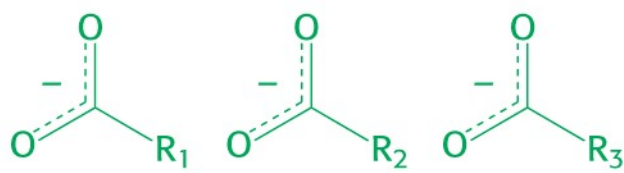


Triacylglyceride

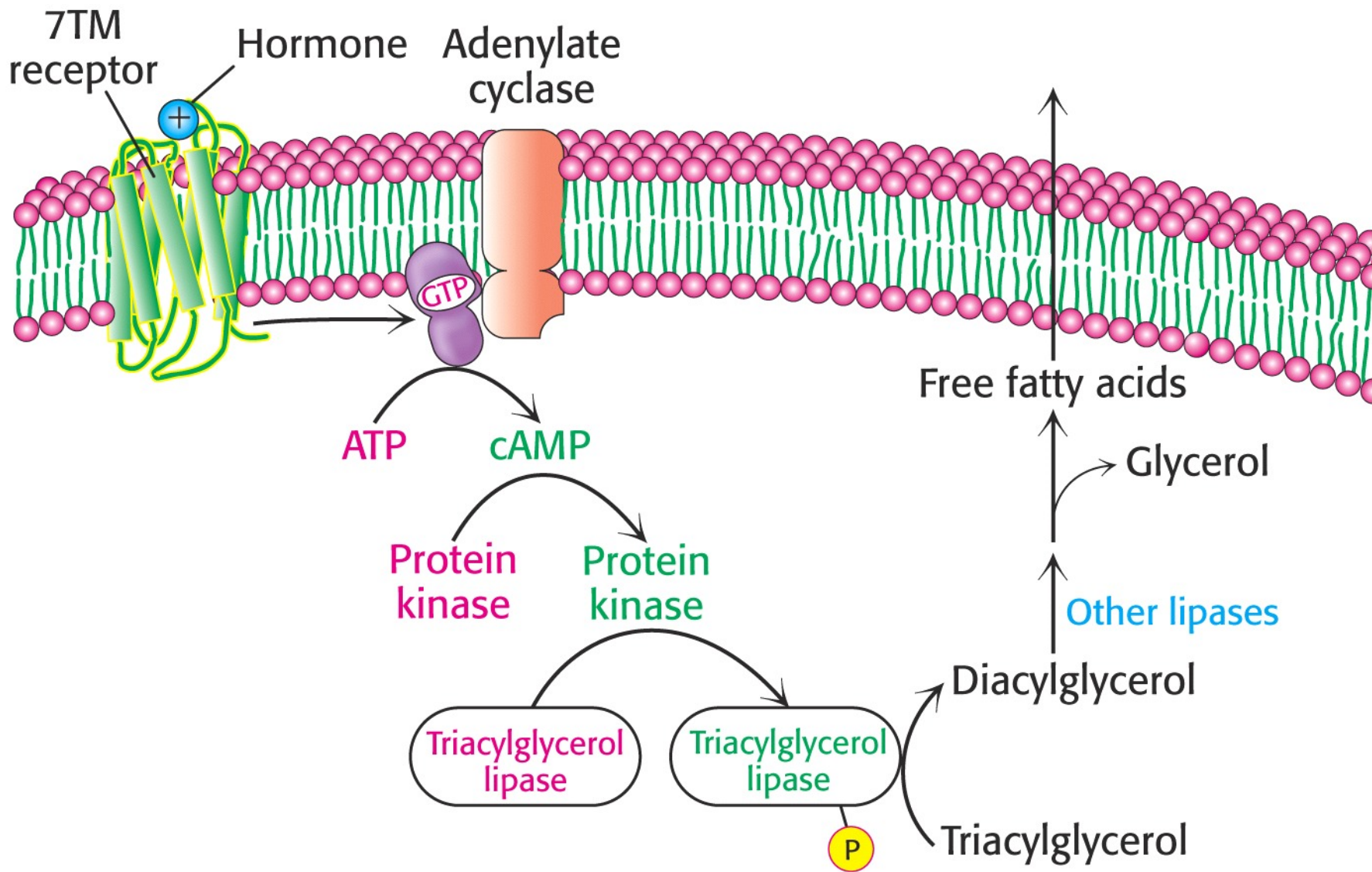


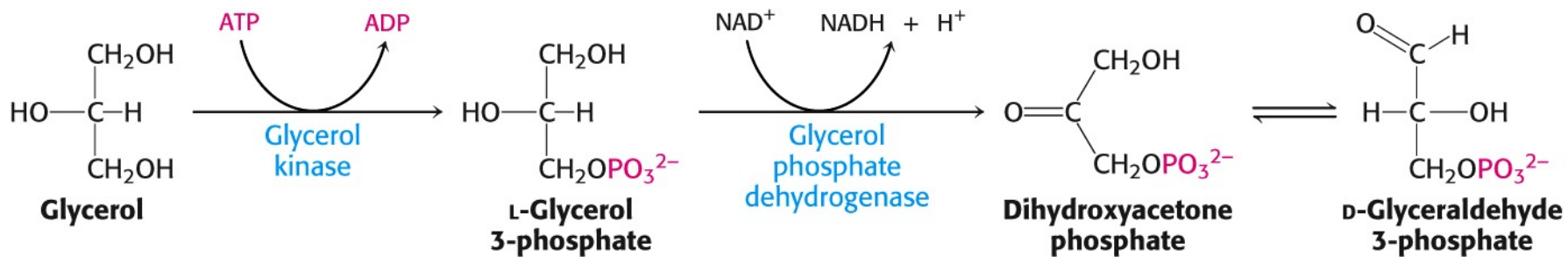
Glycerol

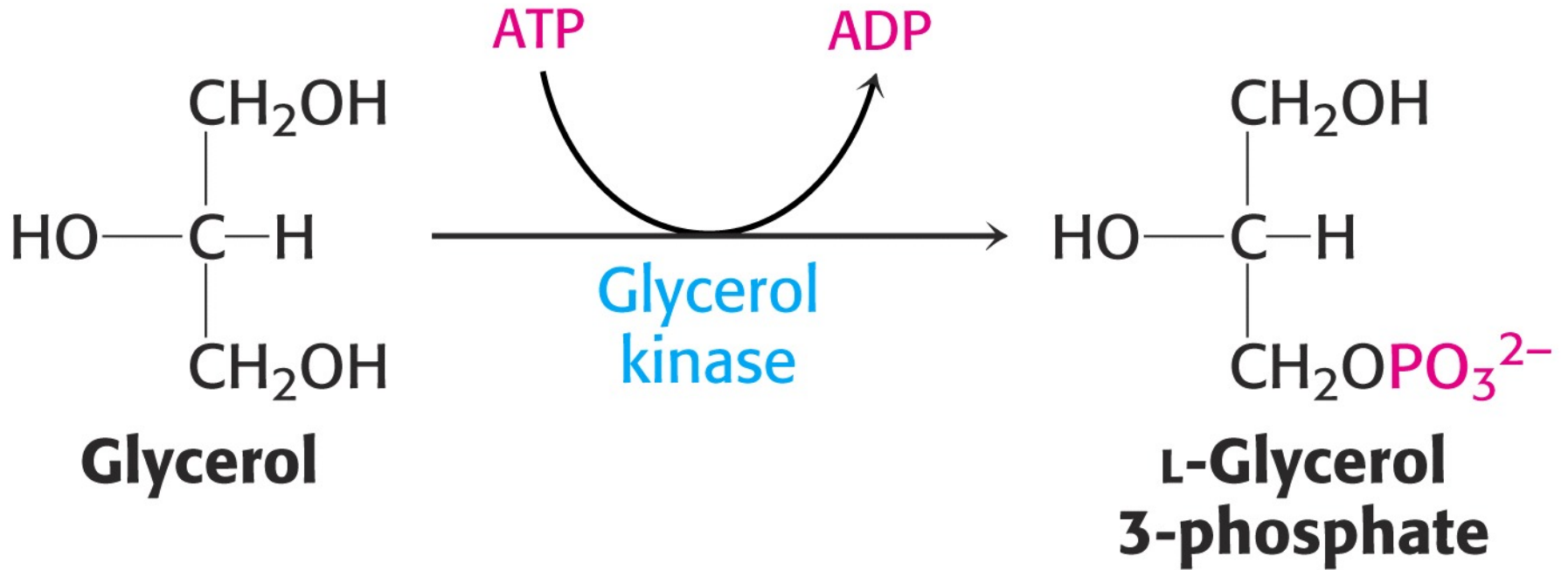
+

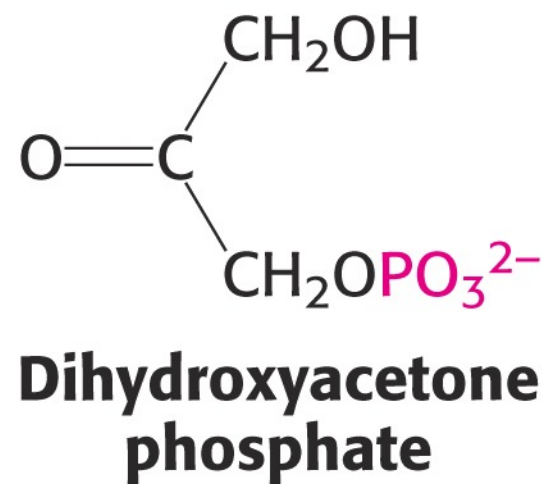
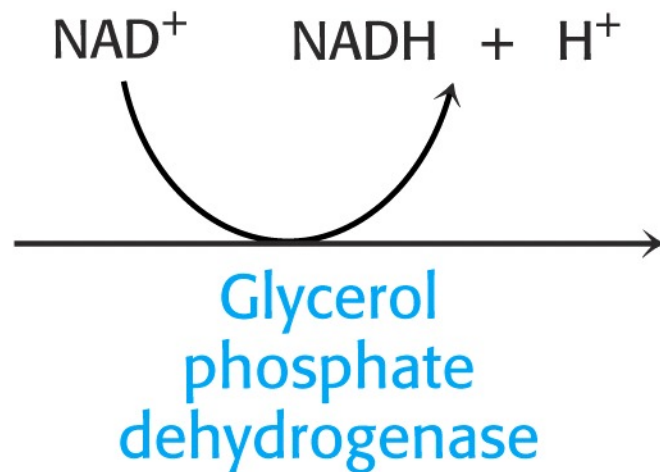
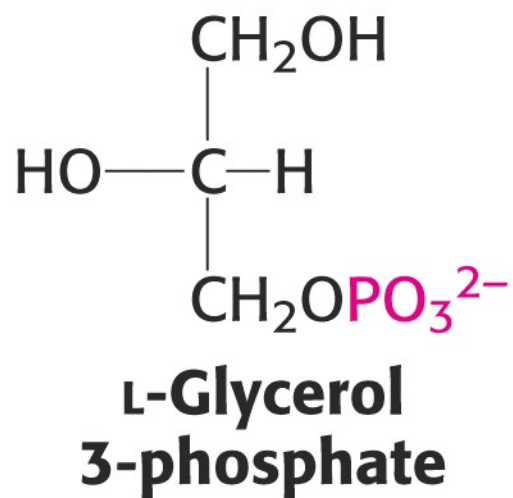


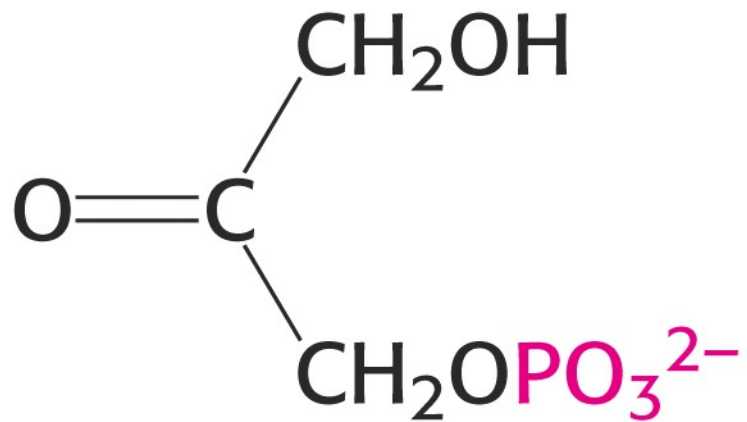
Fatty acids



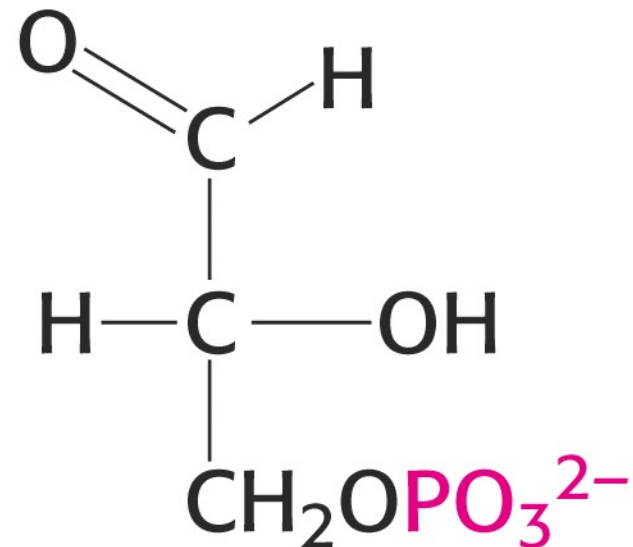




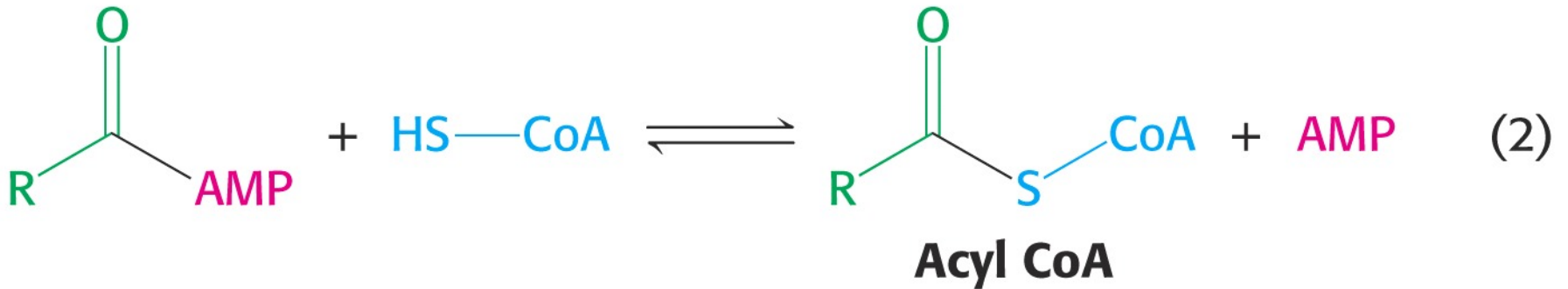
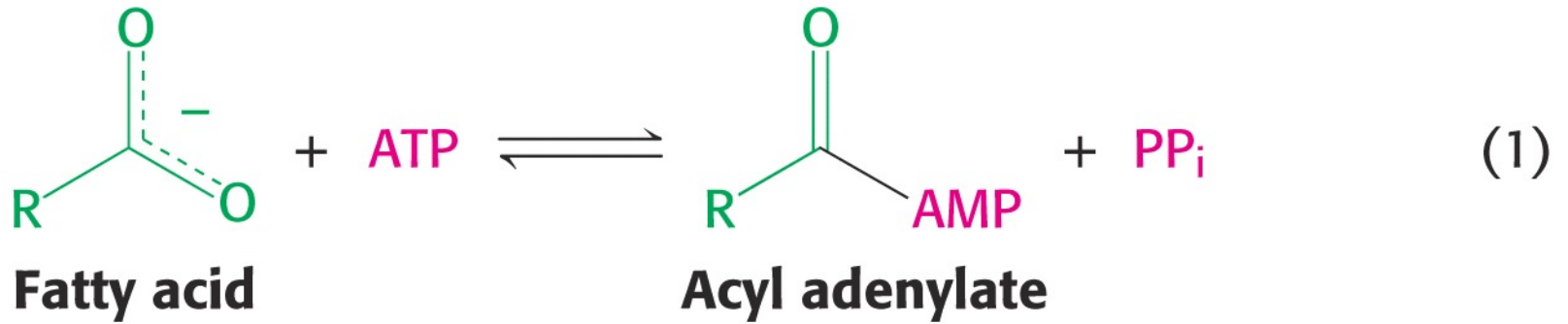


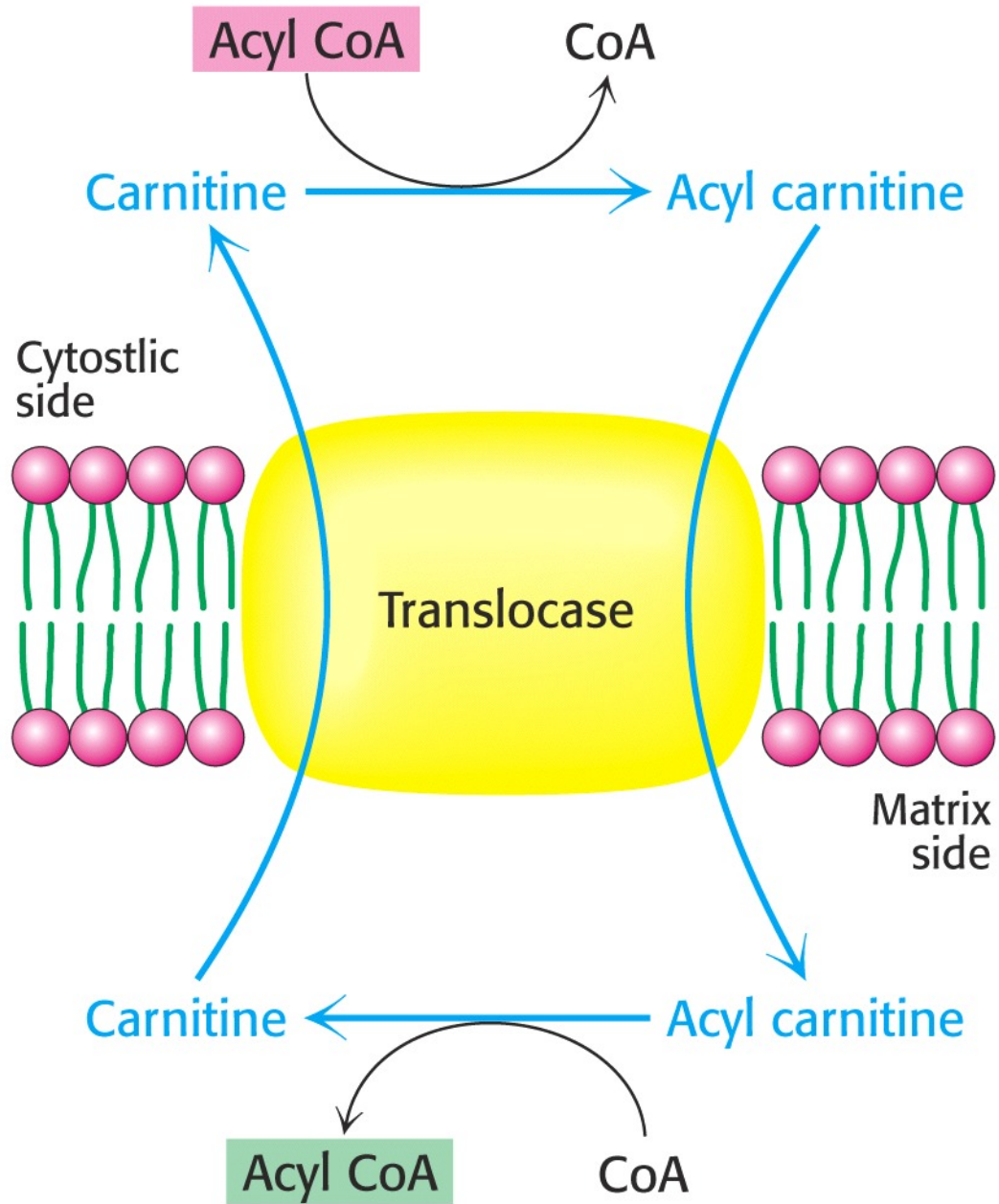


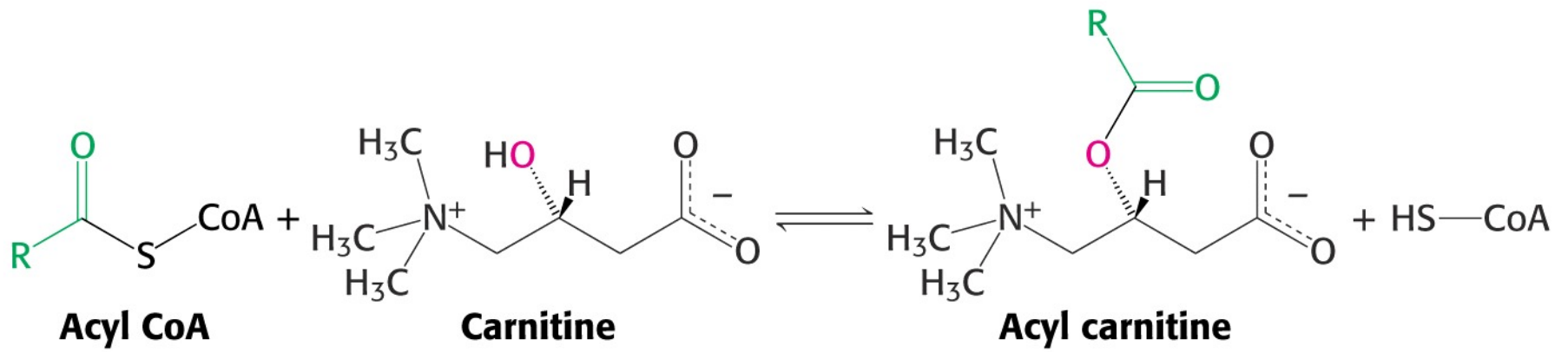
**Dihydroxyacetone
phosphate**

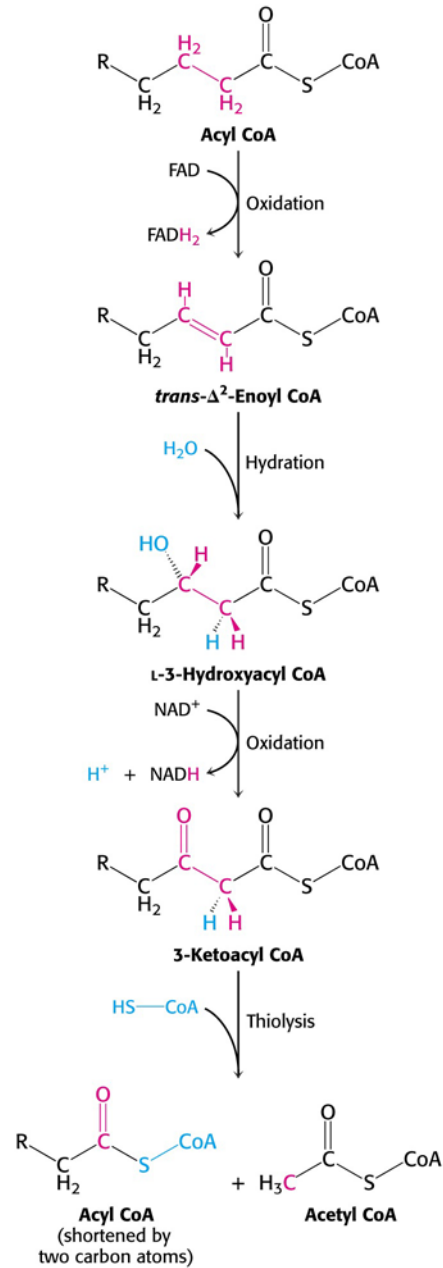


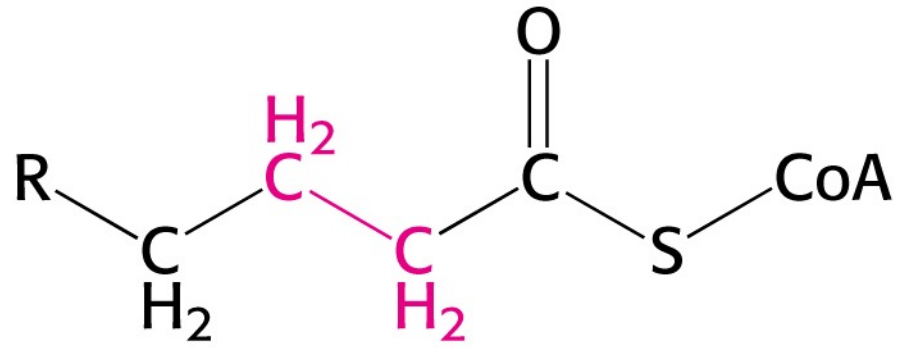
**D-Glyceraldehyde
3-phosphate**



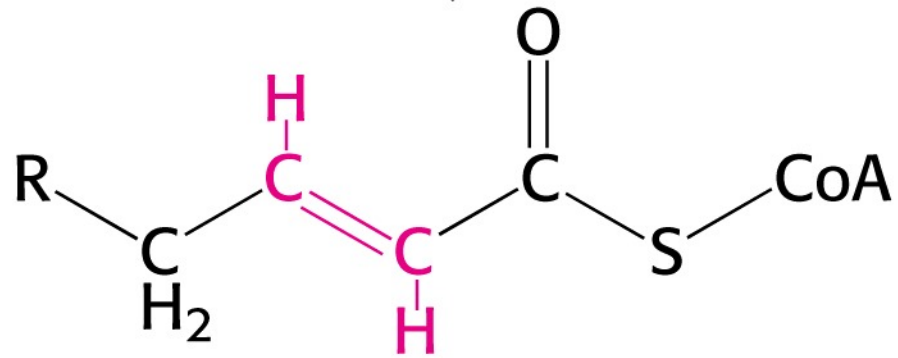
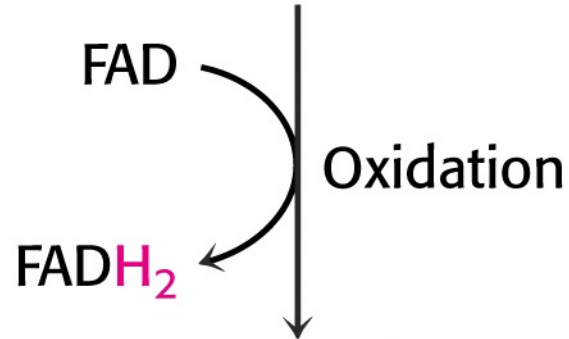




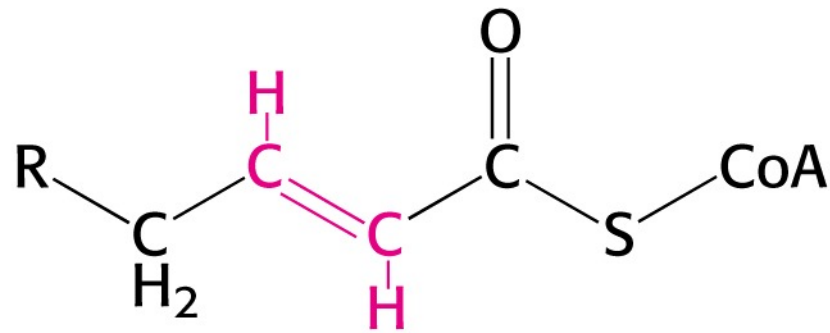




Acyl CoA



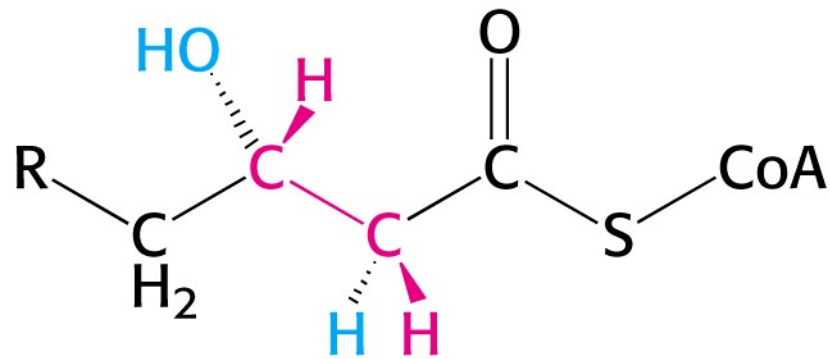
***trans*-Δ²-Enoyl CoA**



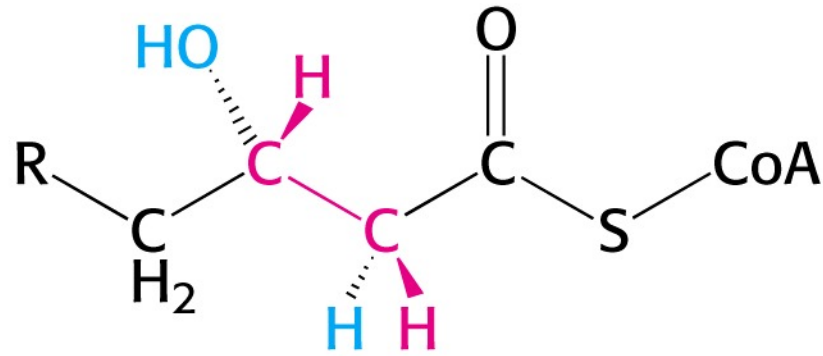
***trans*- Δ^2 -Enoyl CoA**



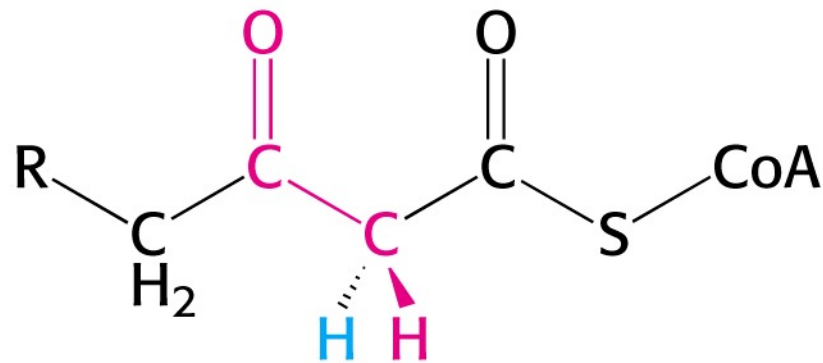
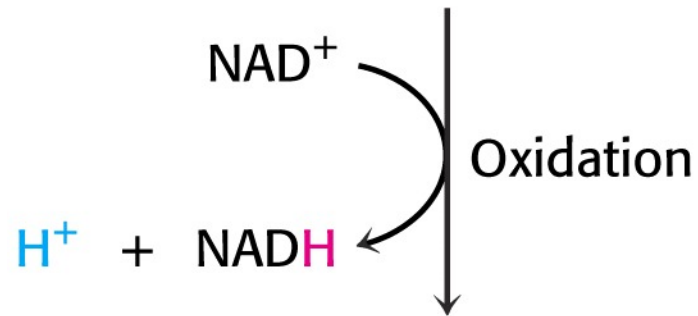
Hydration



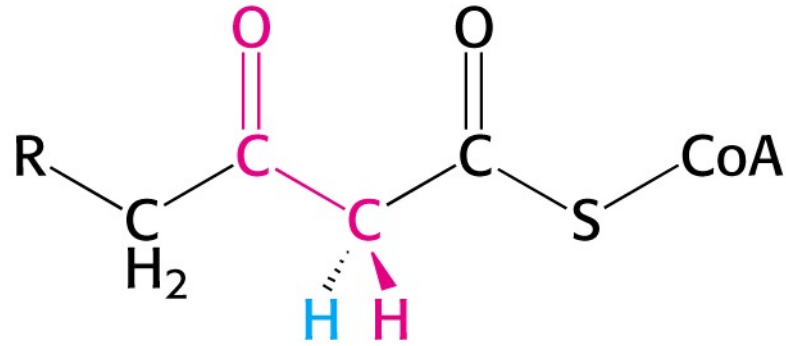
L-3-Hydroxyacyl CoA



L-3-Hydroxyacyl CoA



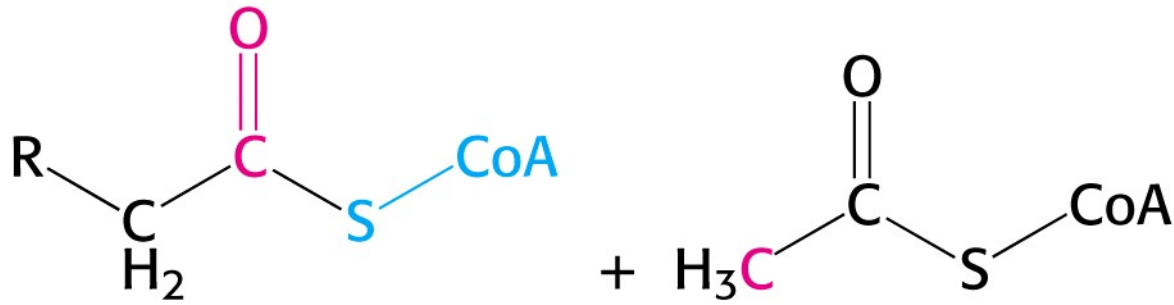
3-Ketoacyl CoA



3-Ketoacyl CoA



Thiolysis

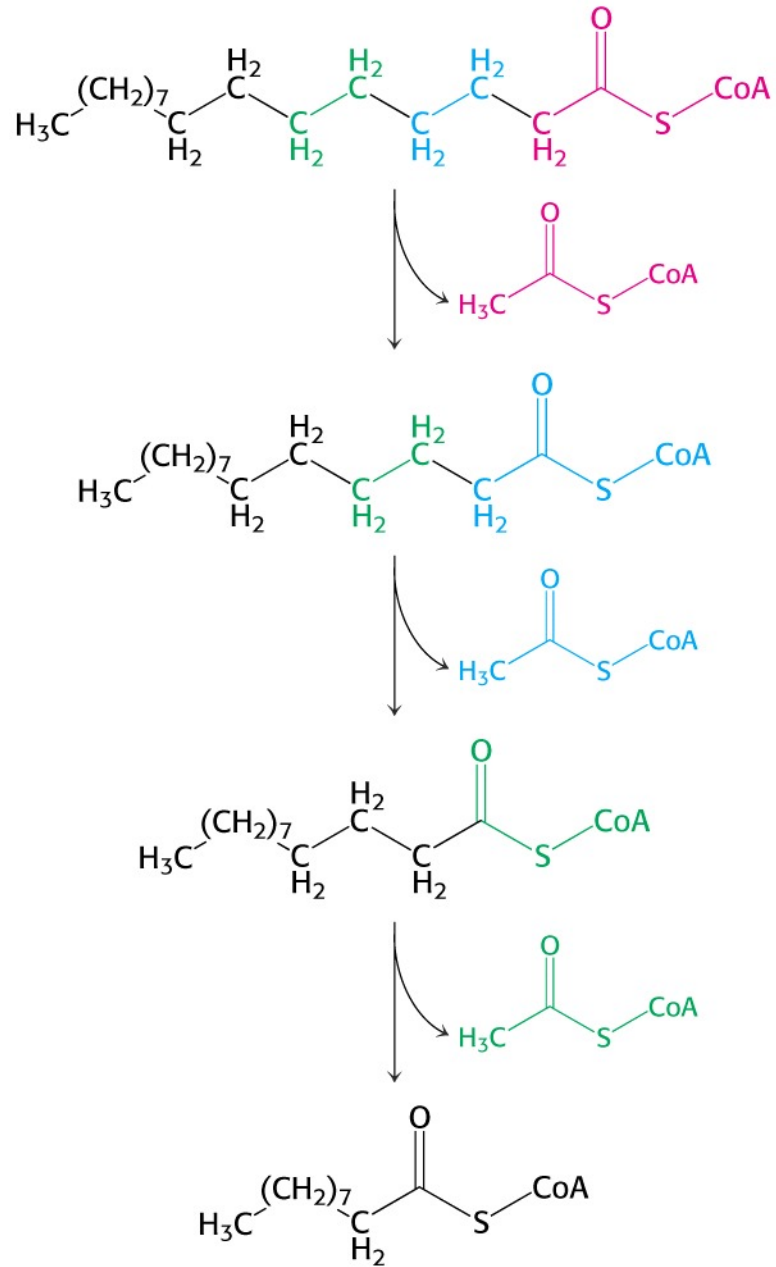


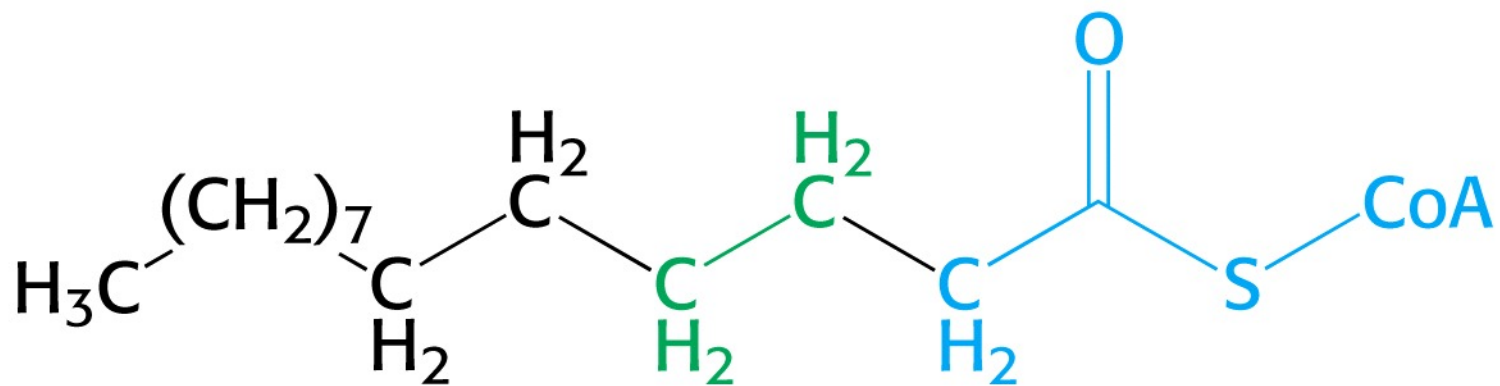
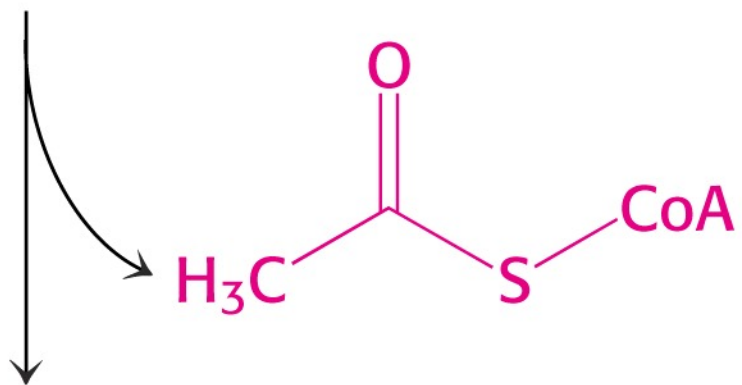
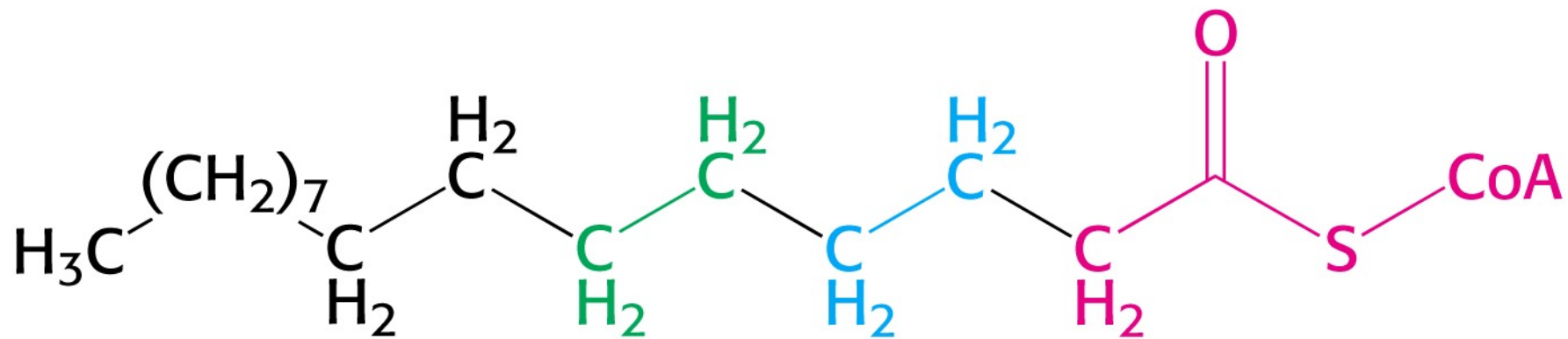
Acyl CoA
(shortened by
two carbon atoms)

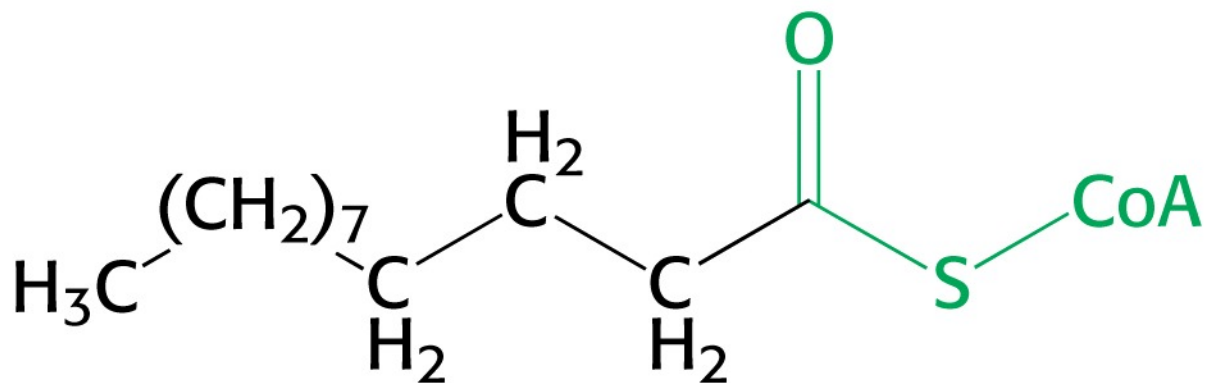
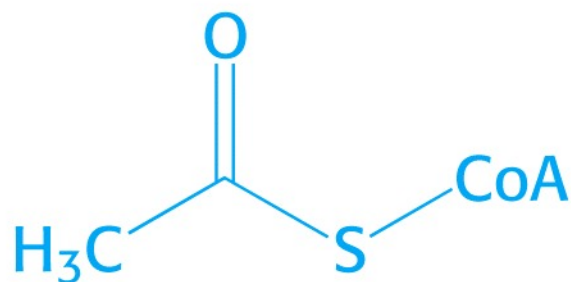
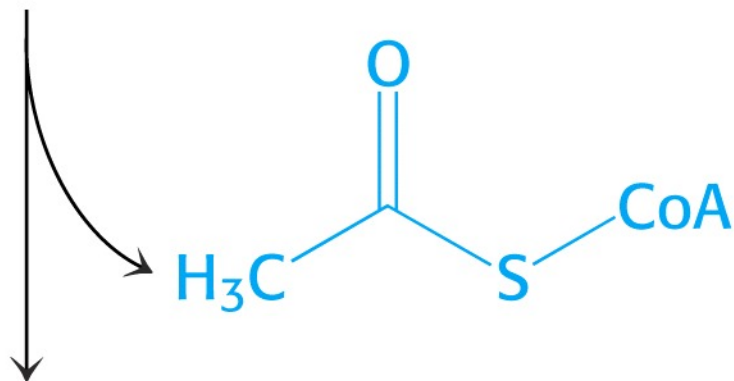
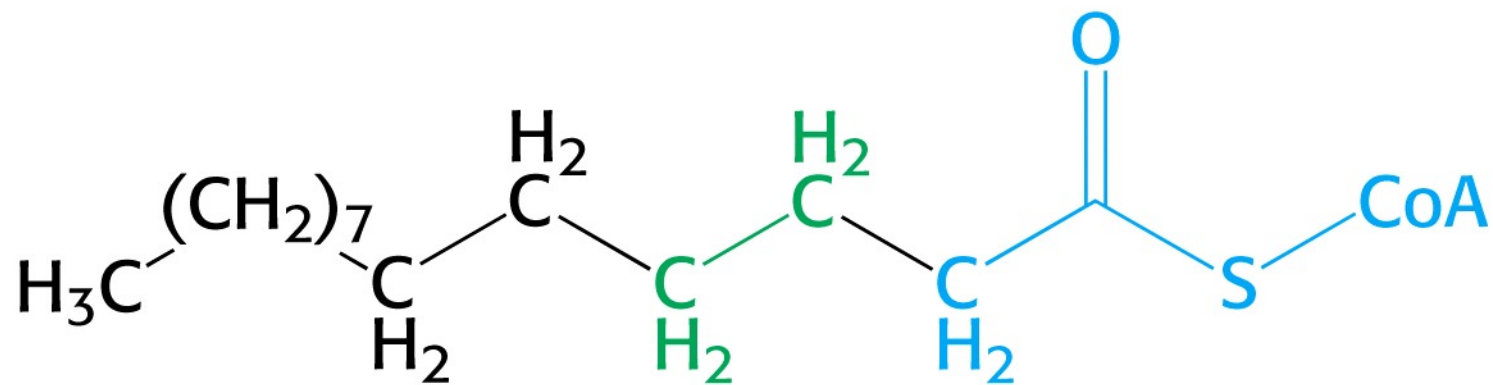
Acetyl CoA

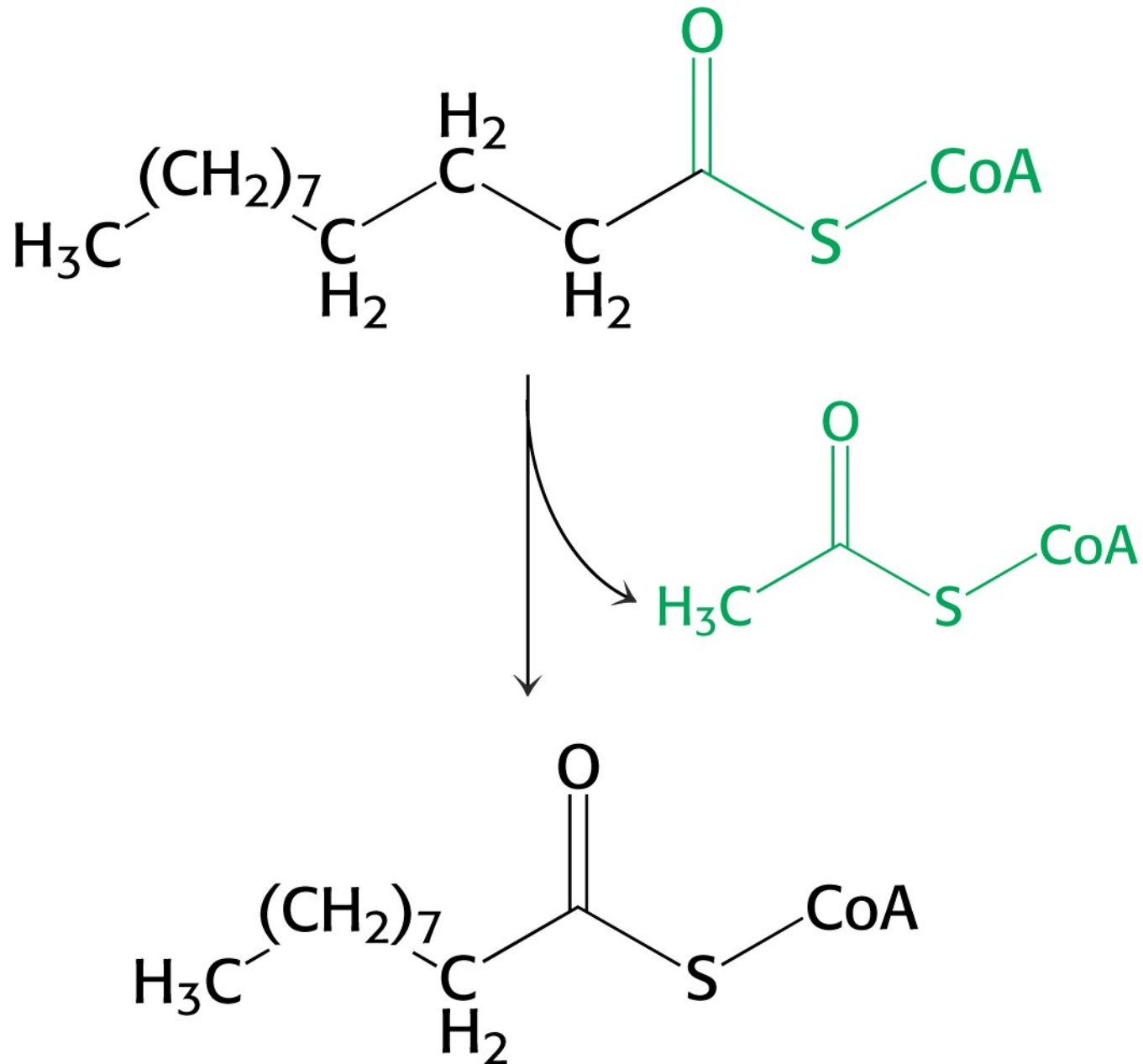
TABLE 22.1 Principal reactions in fatty acid oxidation

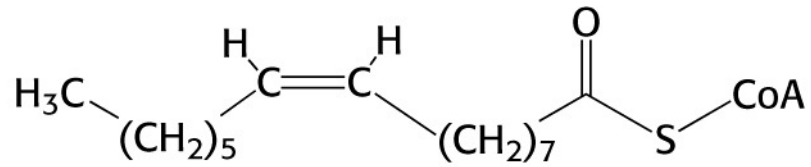
Step	Reaction	Enzyme
1	Fatty acid + CoA + ATP \rightleftharpoons acyl CoA + AMP + PP _i	Acyl CoA synthetase [also called fatty acid thiokinase and fatty acid:CoA ligase (AMP)]
2	Carnitine + acyl CoA \rightleftharpoons acyl carnitine + CoA	Carnitine acyltransferase (also called carnitine palmitoyl transferase)
3	Acyl CoA + E-FAD \rightarrow <i>trans</i> - Δ^2 -enoyl CoA + E-FADH ₂	Acyl CoA dehydrogenases (several isozymes having different chain-length specificity)
4	<i>trans</i> - Δ^2 -Enoyl CoA + H ₂ O \rightleftharpoons L-3-hydroxyacyl CoA	Enoyl CoA hydratase (also called crotonase or 3-hydroxyacyl CoA hydrolyase)
5	L-3-Hydroxyacyl CoA + NAD ⁺ \rightleftharpoons 3-ketoacyl CoA + NADH + H ⁺	L-3-Hydroxyacyl CoA dehydrogenase
6	3-Ketoacyl CoA + CoA \rightleftharpoons acetyl CoA + acyl CoA (shortened by C ₂)	β -Ketothiolase (also called thiolase)



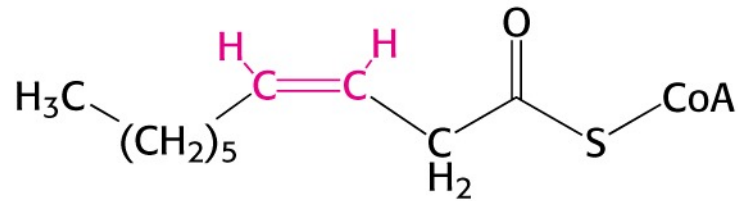






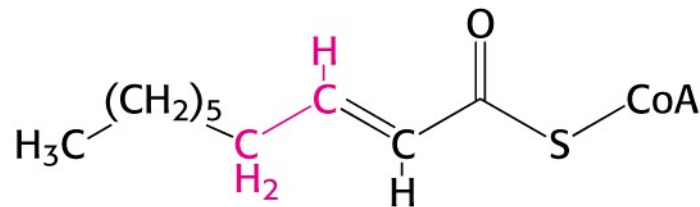
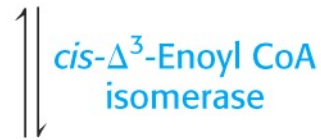


Palmitoyl CoA



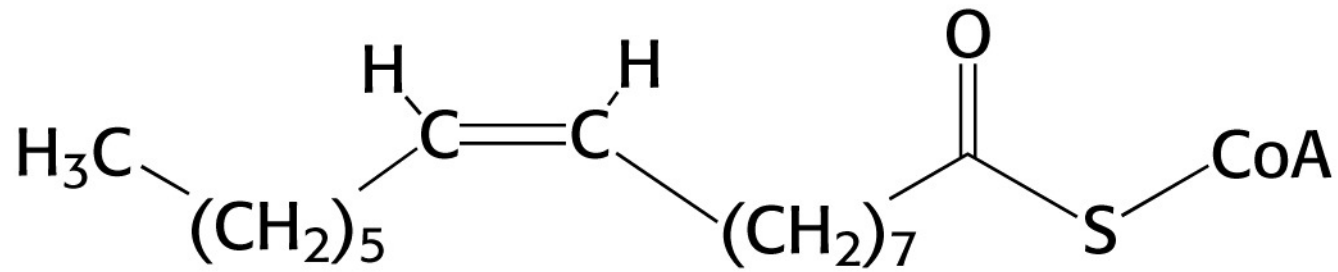
4 3 2 1

cis-Δ³-Enoyl CoA

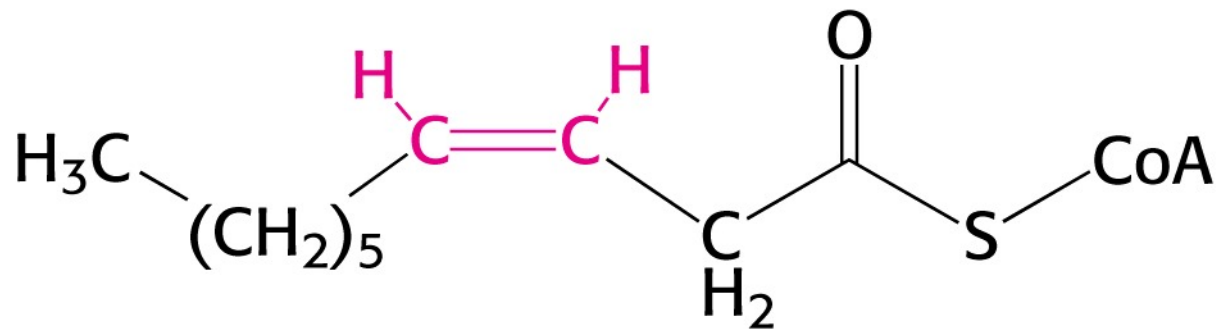


4 3 2 1

trans-Δ²-Enoyl CoA

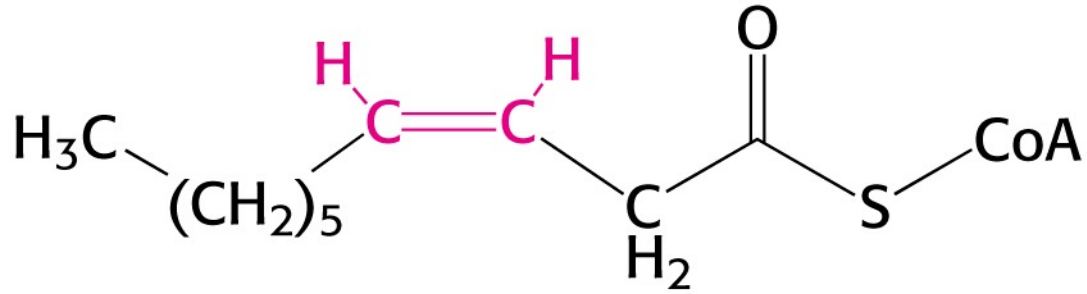


Palmitoyl CoA



4 3 2 1

***cis*-Δ³-Enoyl CoA**

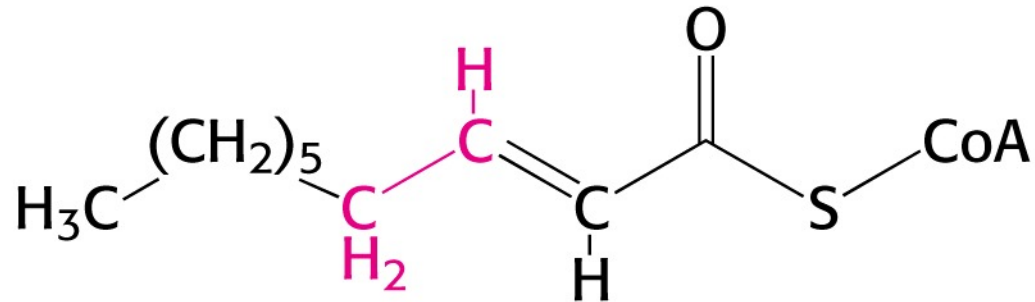


4 3 2 1

***cis*- Δ^3 -Enoyl CoA**

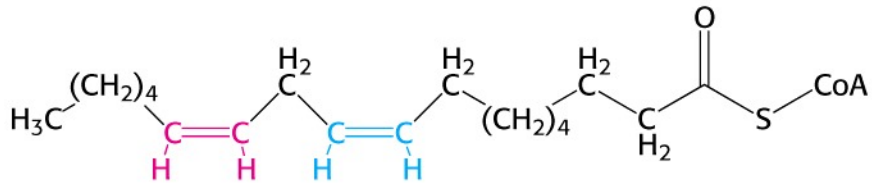


cis- Δ^3 -Enoyl CoA
isomerase

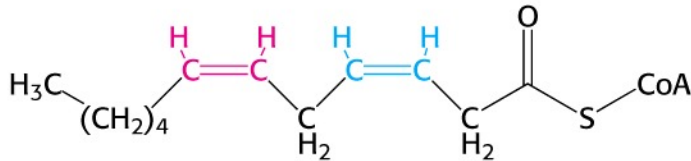
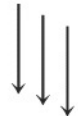


4 3 2 1

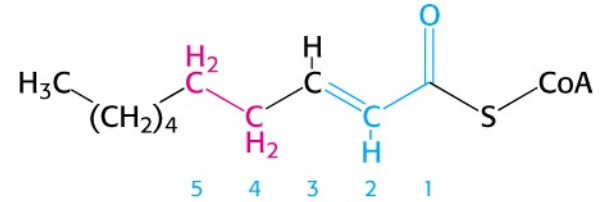
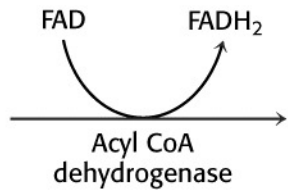
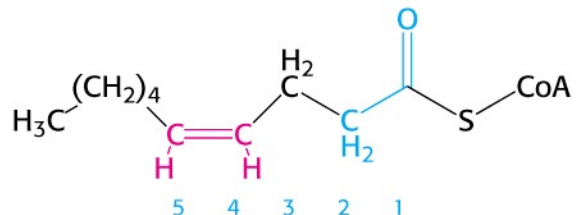
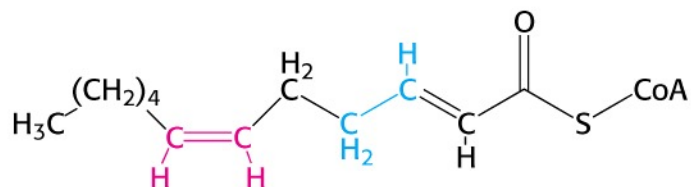
***trans*- Δ^2 -Enoyl CoA**



Linoleoyl CoA



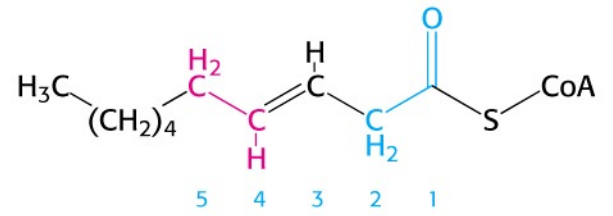
cis- Δ^3 -Enoyl CoA isomerase



trans- Δ^2 -Enoyl CoA

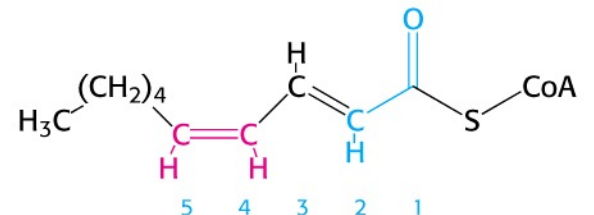
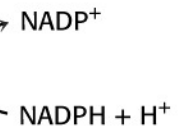


cis- Δ^3 -Enoyl CoA isomerase

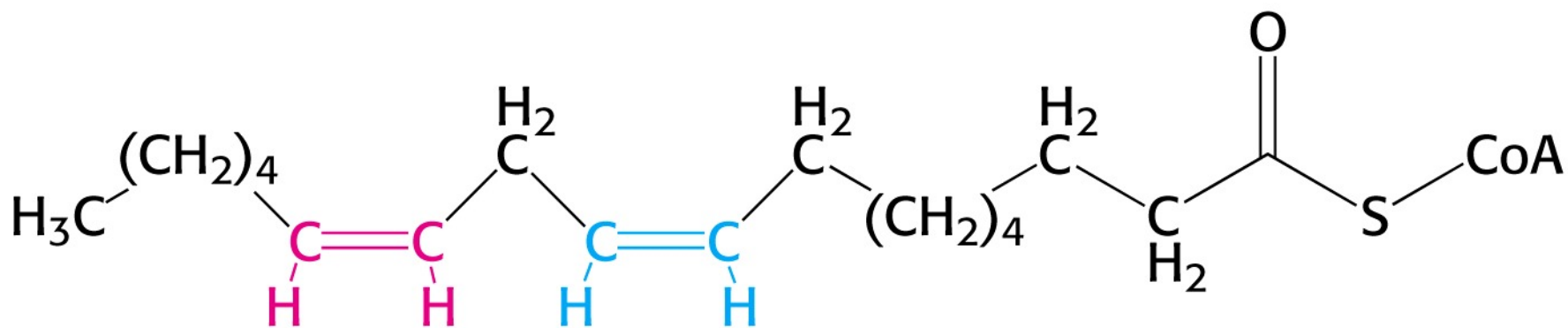


trans- Δ^3 -Enoyl CoA

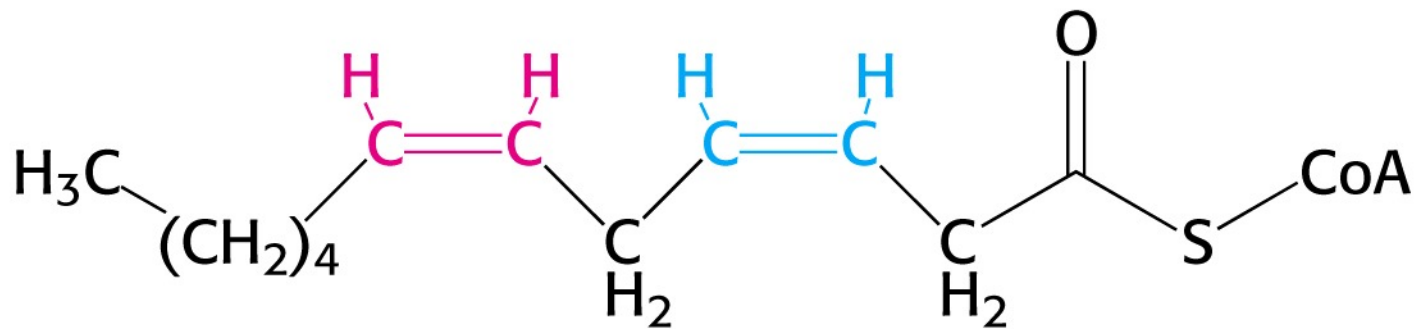
2,4 Dienoyl CoA reductase

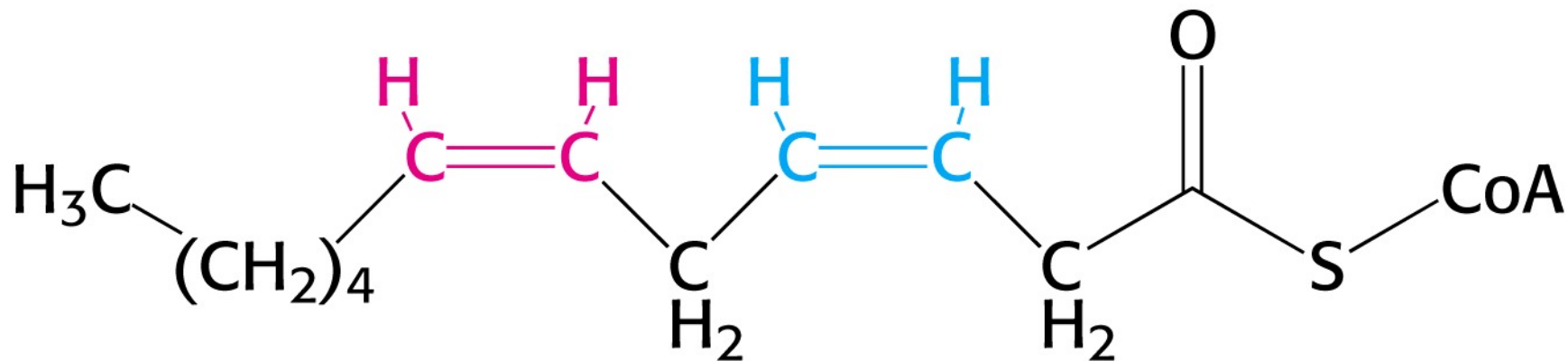


2,4-Dienoyl CoA

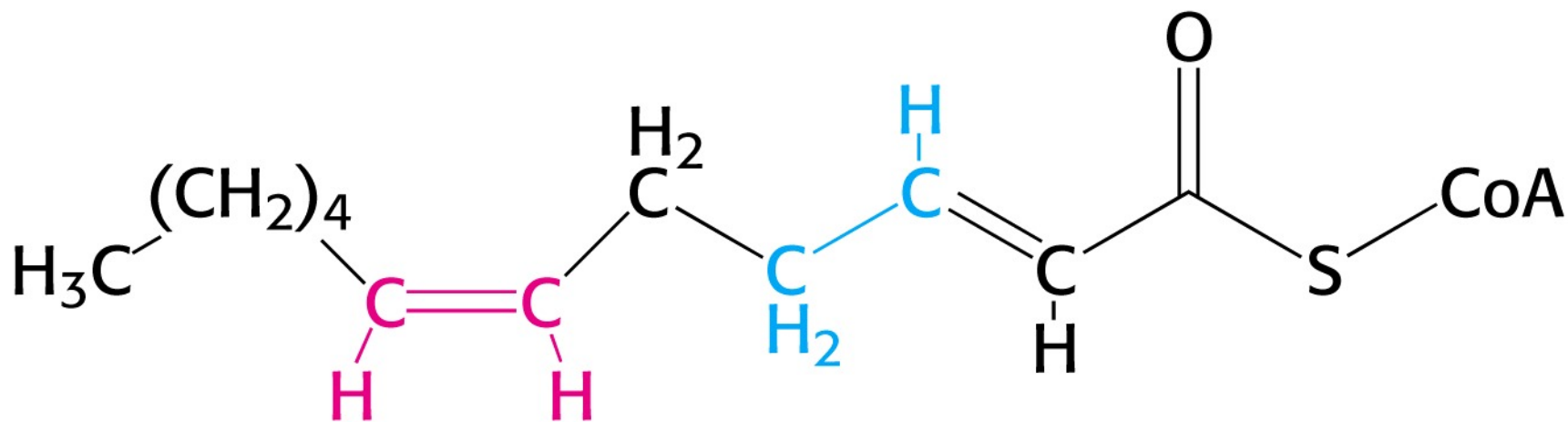


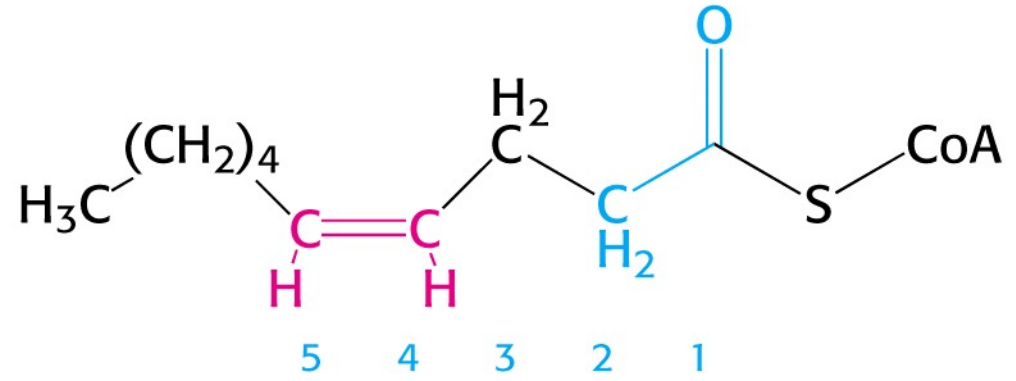
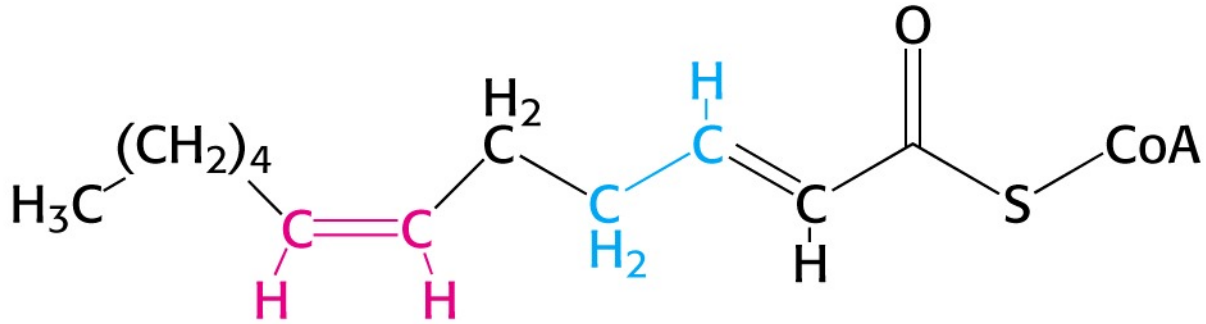
Linoleoyl CoA

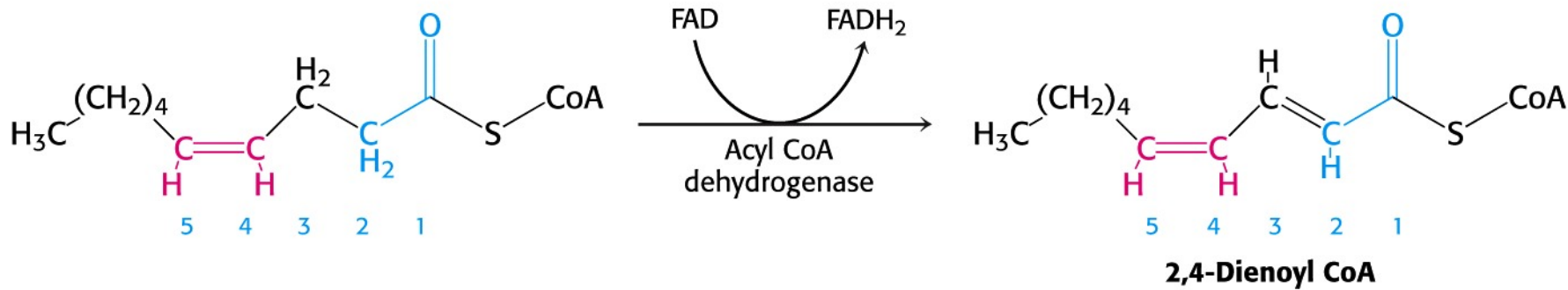


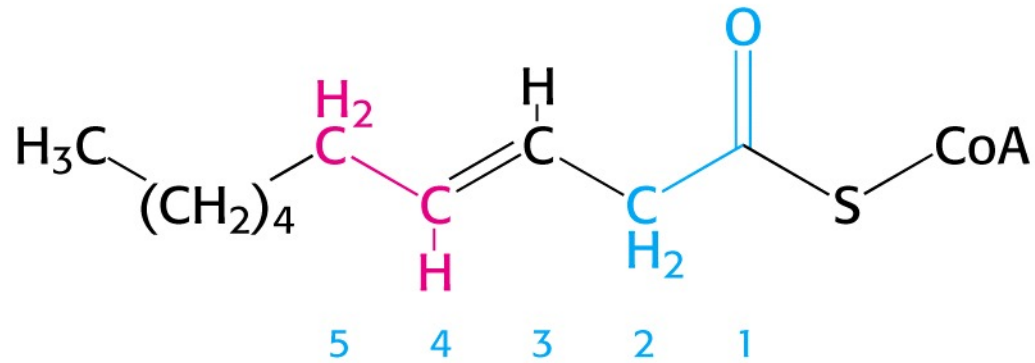


cis- Δ^3 -Enoyl CoA
isomerase

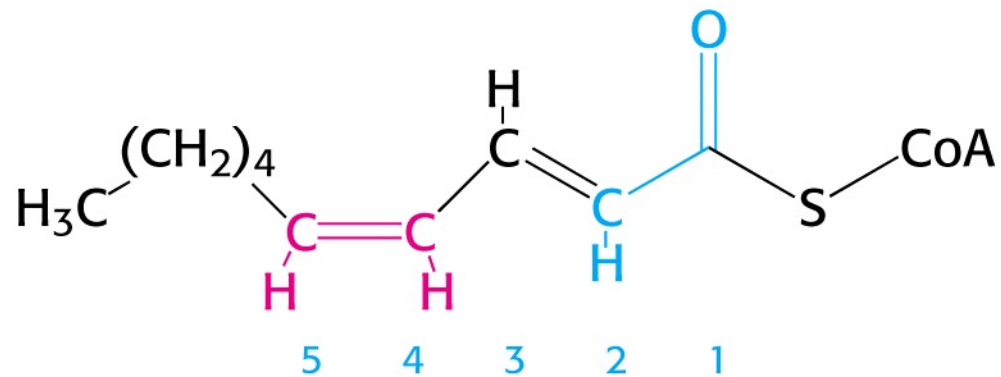
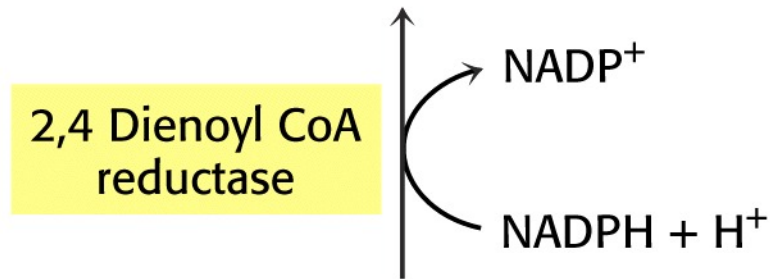




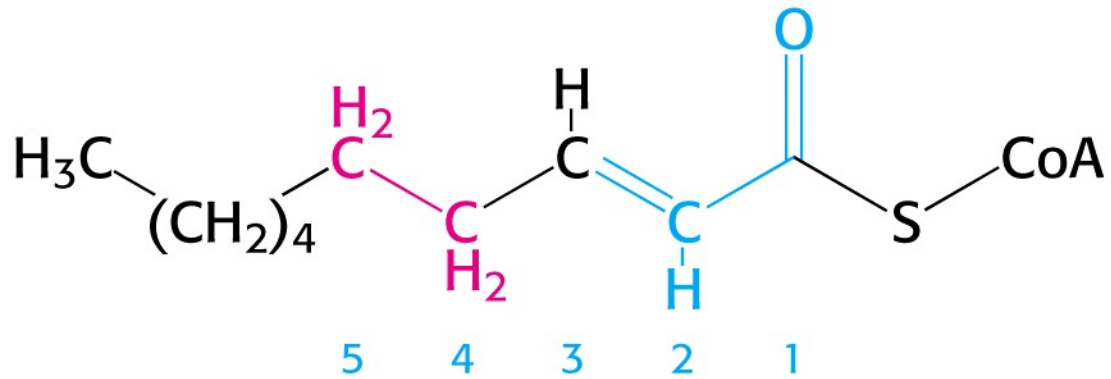




***trans*- Δ^3 -Enoyl CoA**



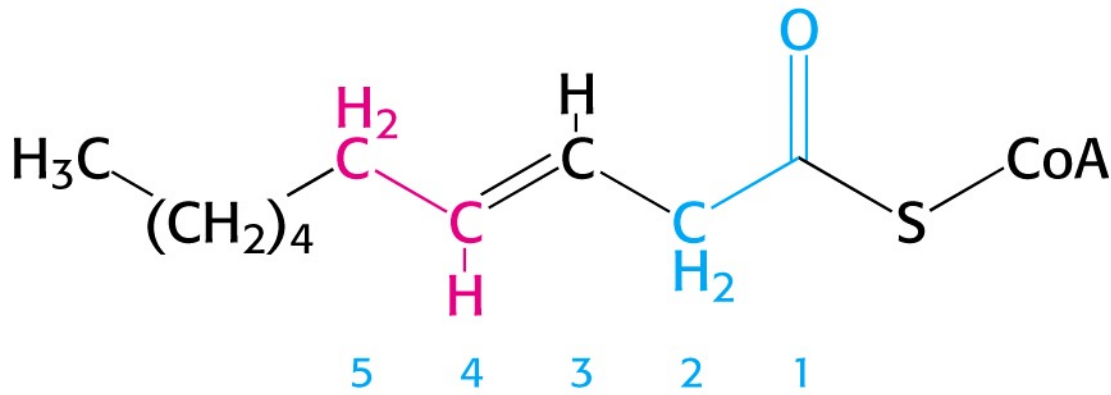
2,4-Dienoyl CoA



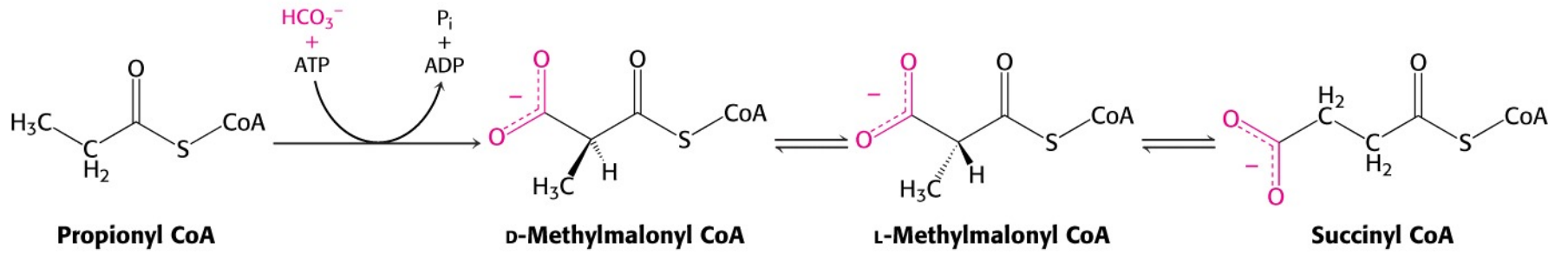
***trans*- Δ^2 -Enoyl CoA**

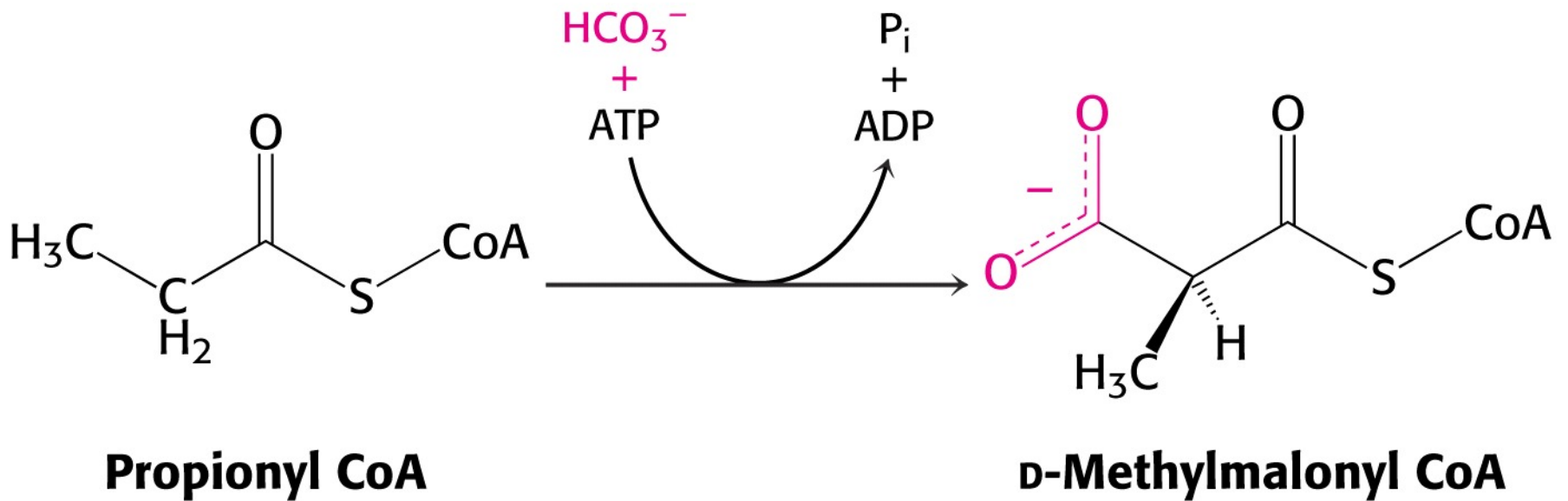


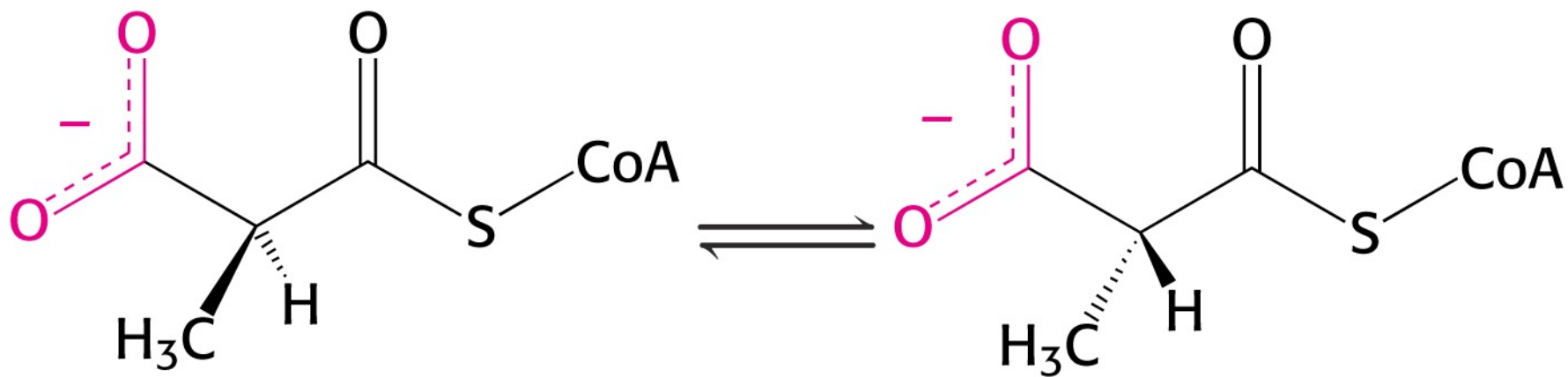
cis- Δ^3 -Enoyl CoA
isomerase



***trans*- Δ^3 -Enoyl CoA**

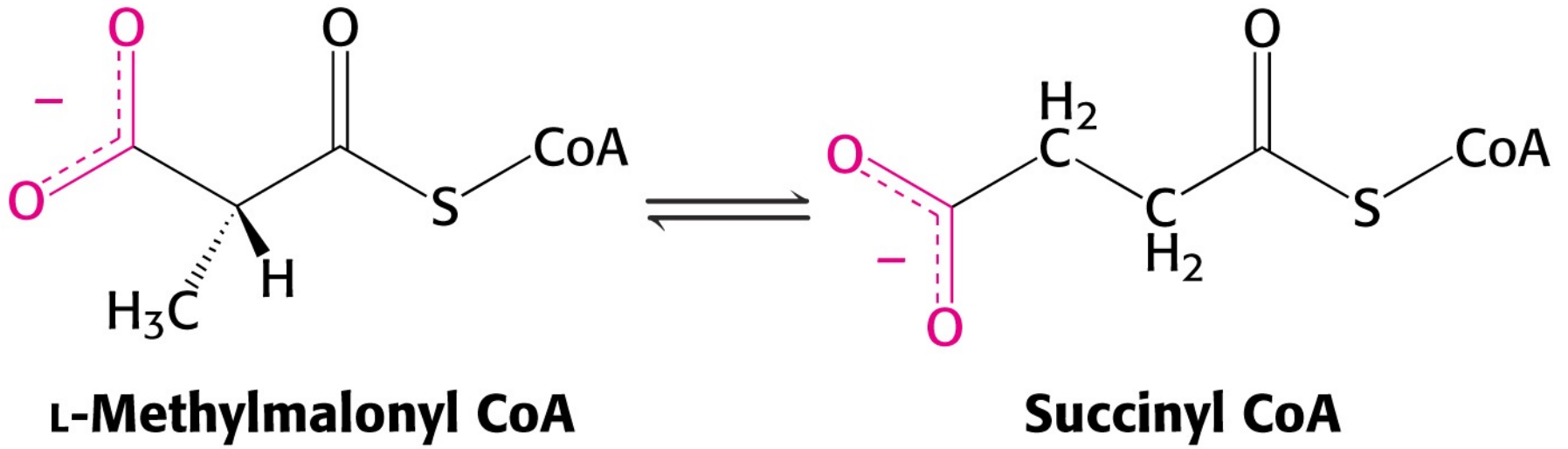


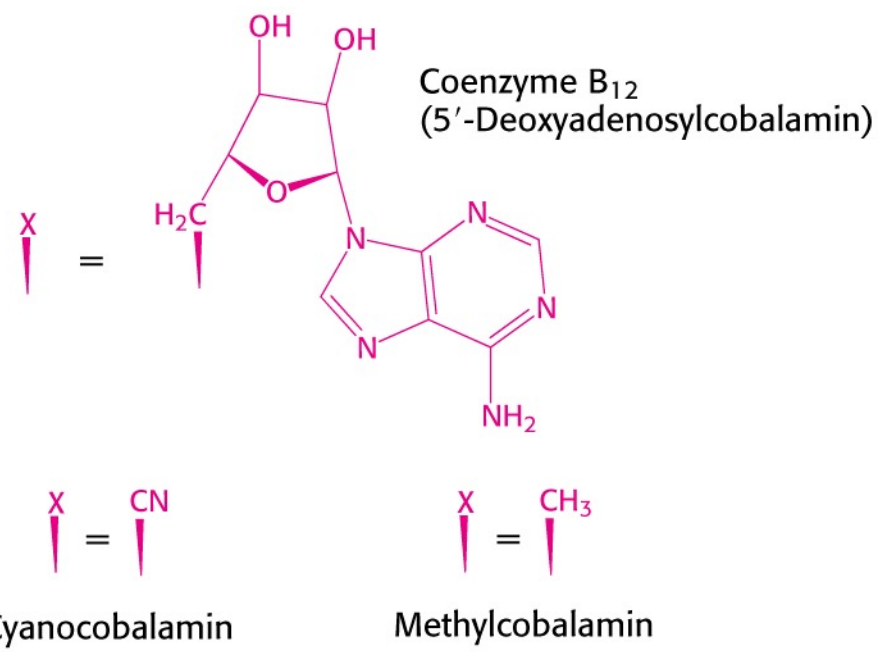
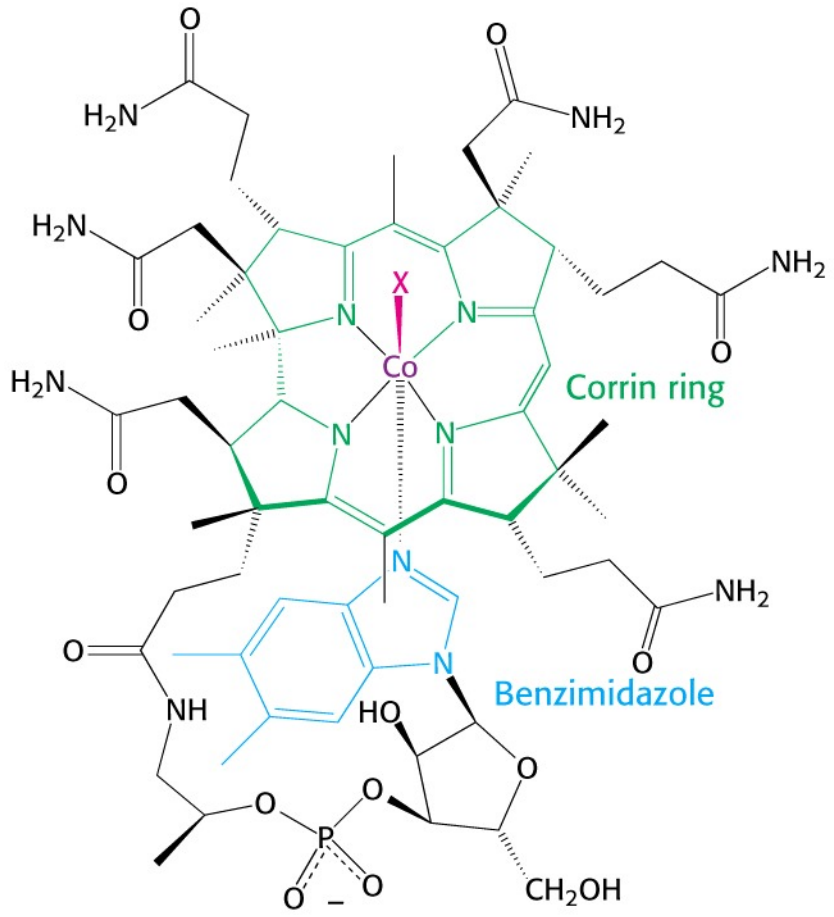


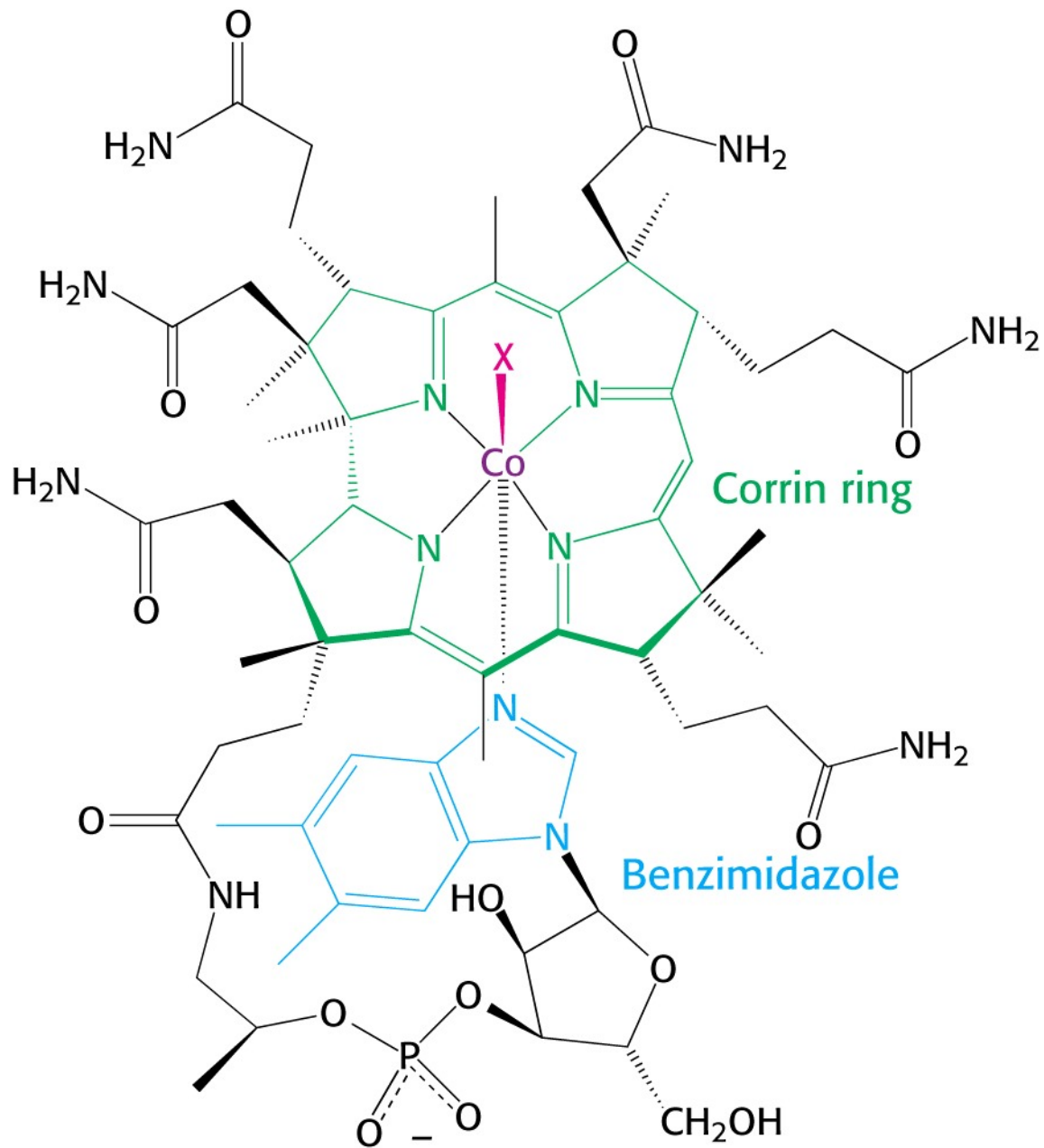


D-Methylmalonyl CoA

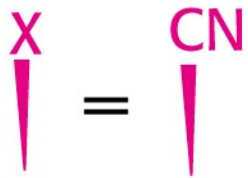
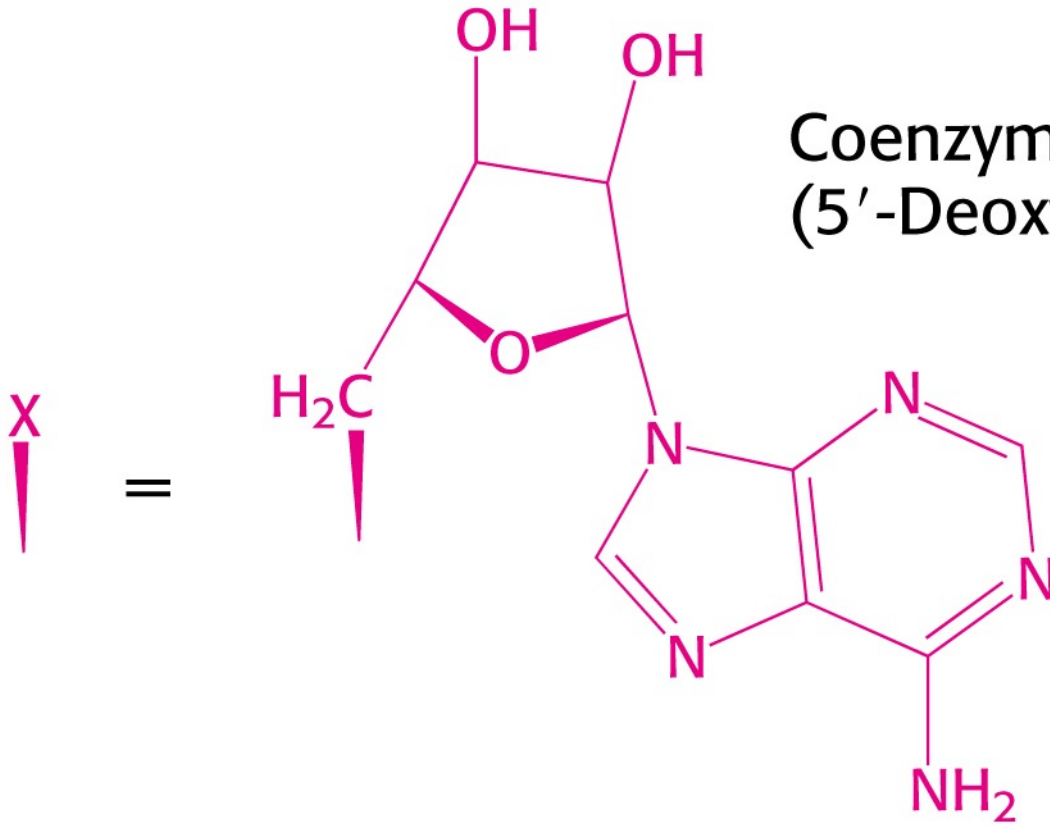
L-Methylmalonyl CoA



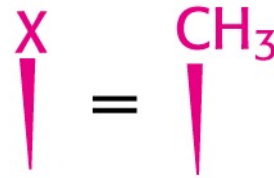




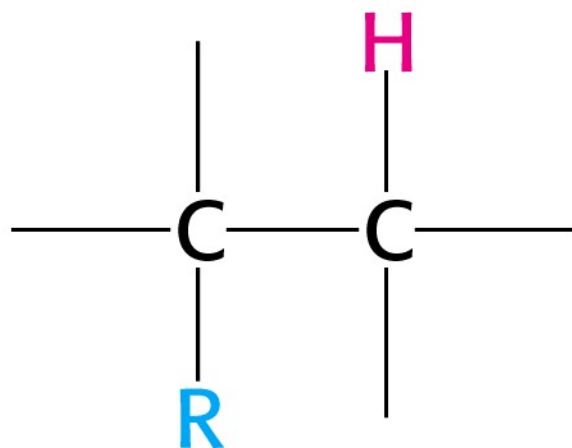
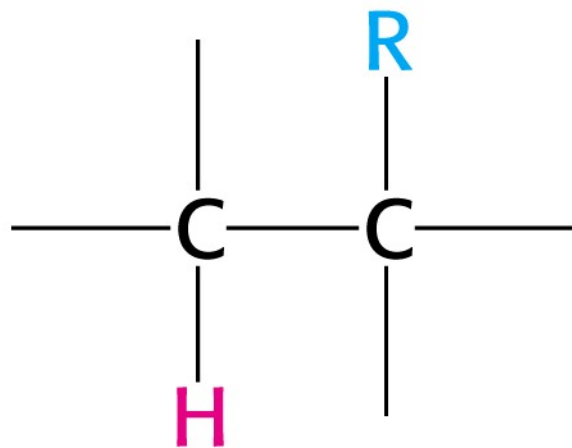
Coenzyme B₁₂
(5'-Deoxyadenosylcobalamin)

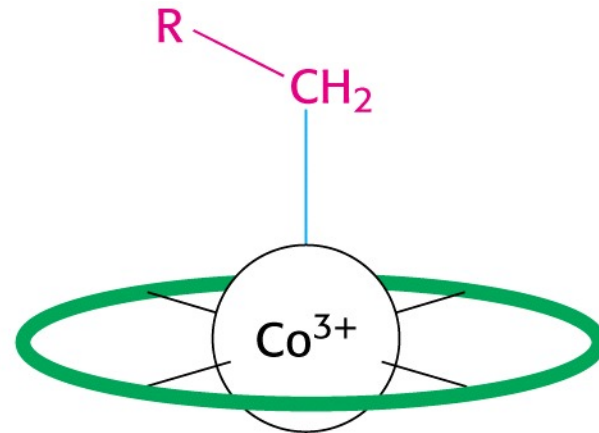


Cyanocobalamin

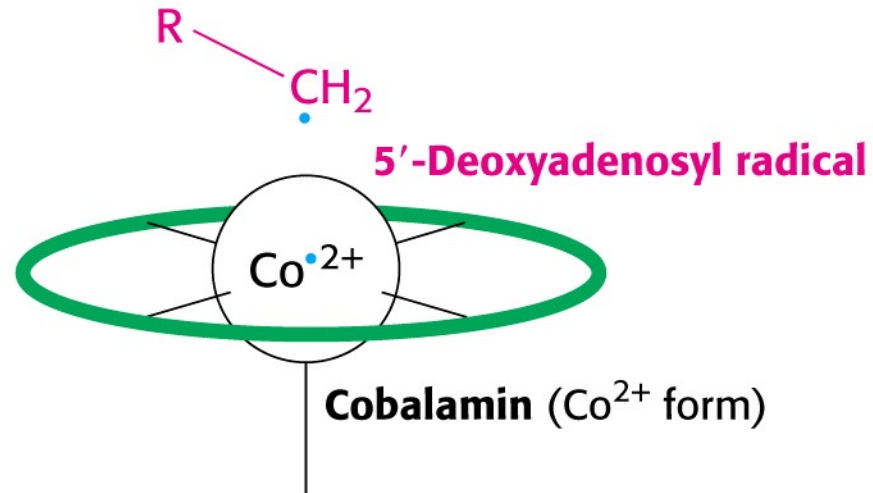


Methylcobalamin

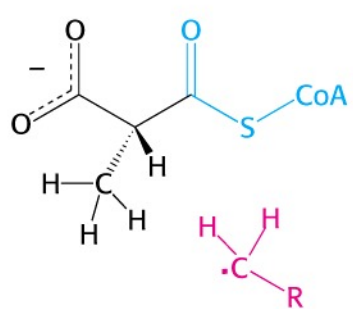




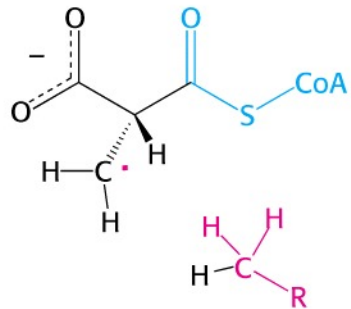
Homolytic
bond cleavage



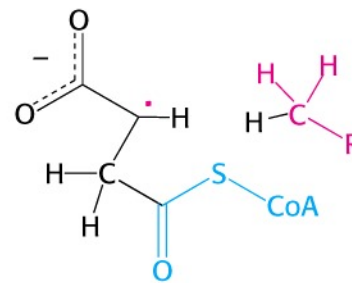
L-Methylmalonyl CoA



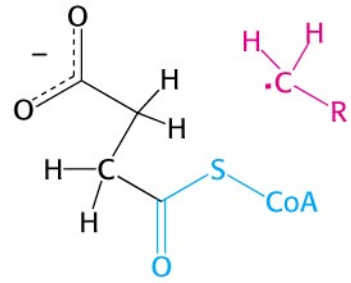
5'-Deoxyadenosyl radical



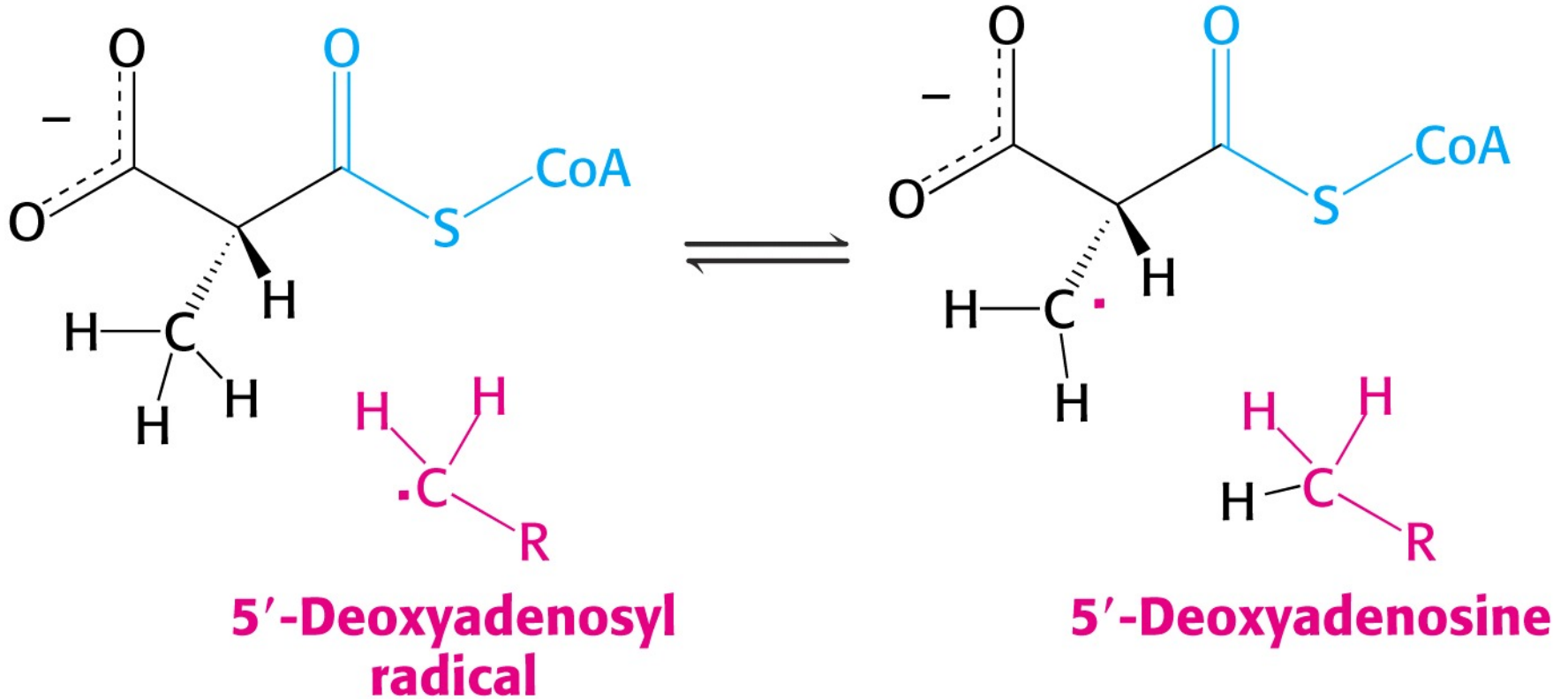
5'-Deoxyadenosine

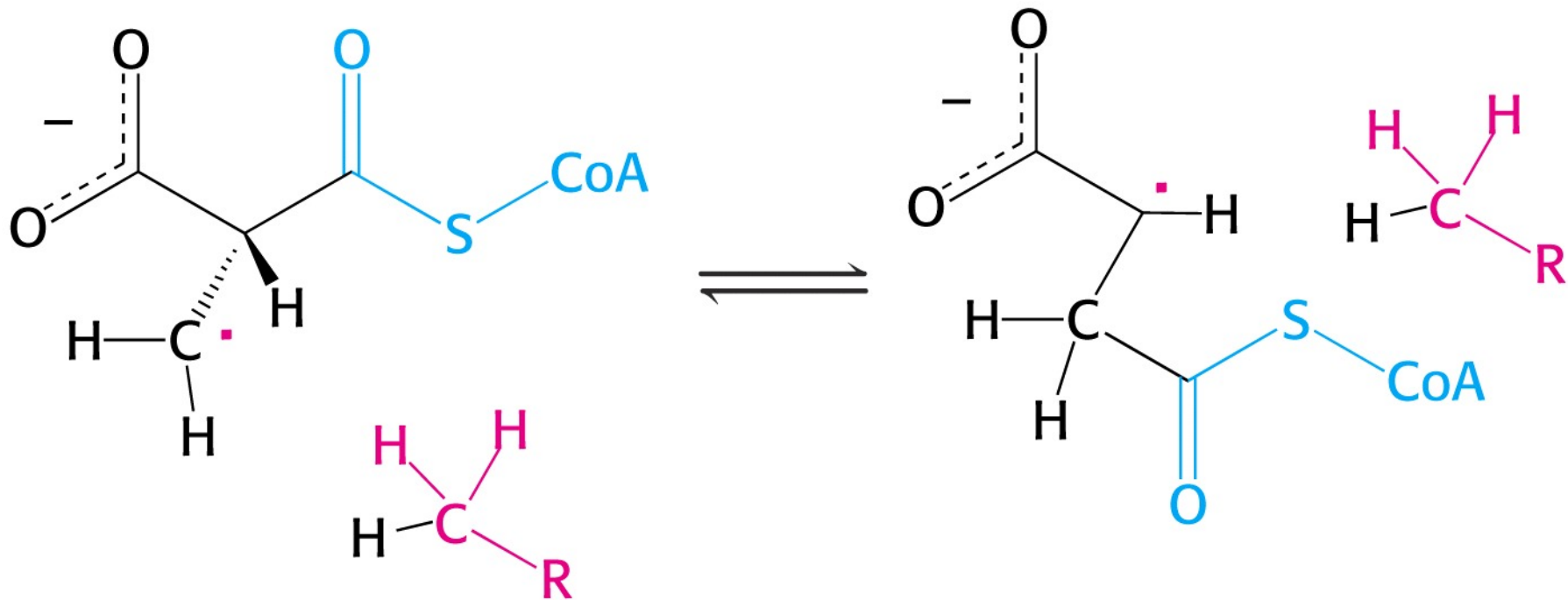


Succinyl CoA

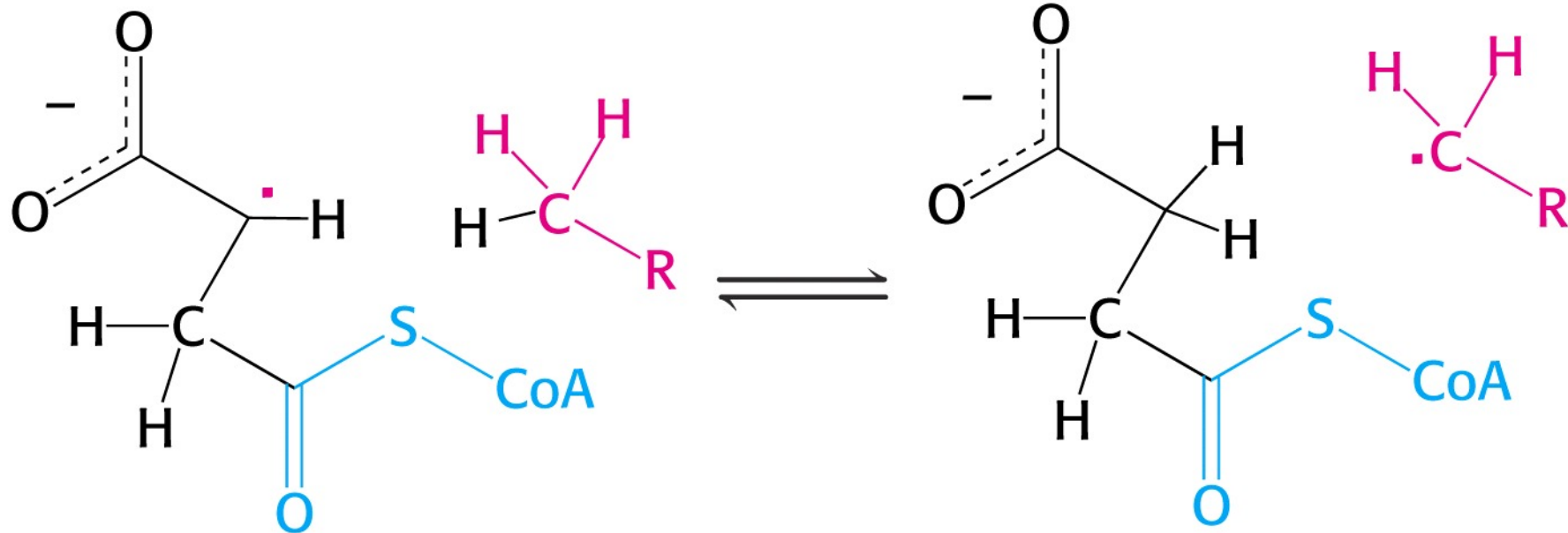


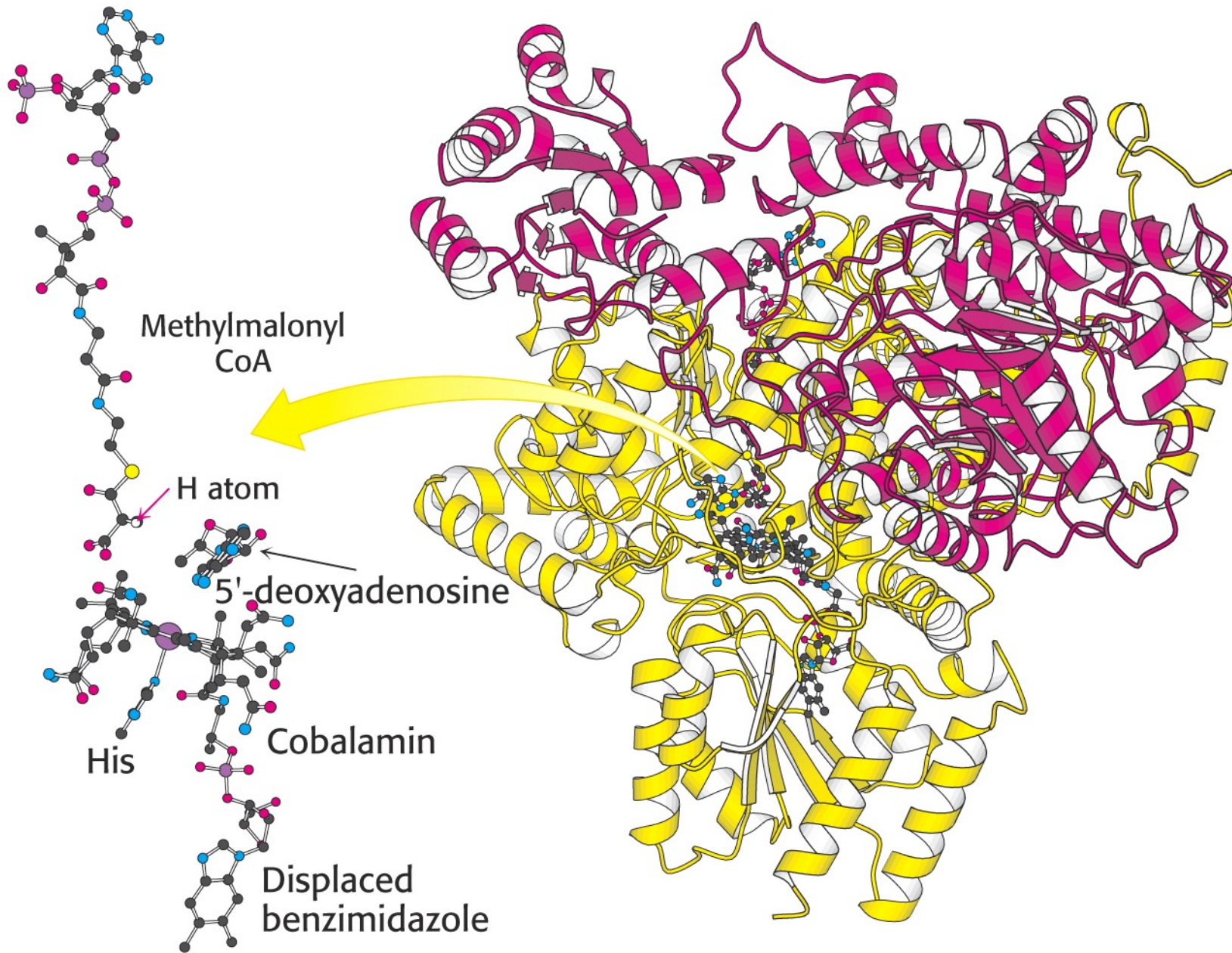
L-Methylmalonyl CoA

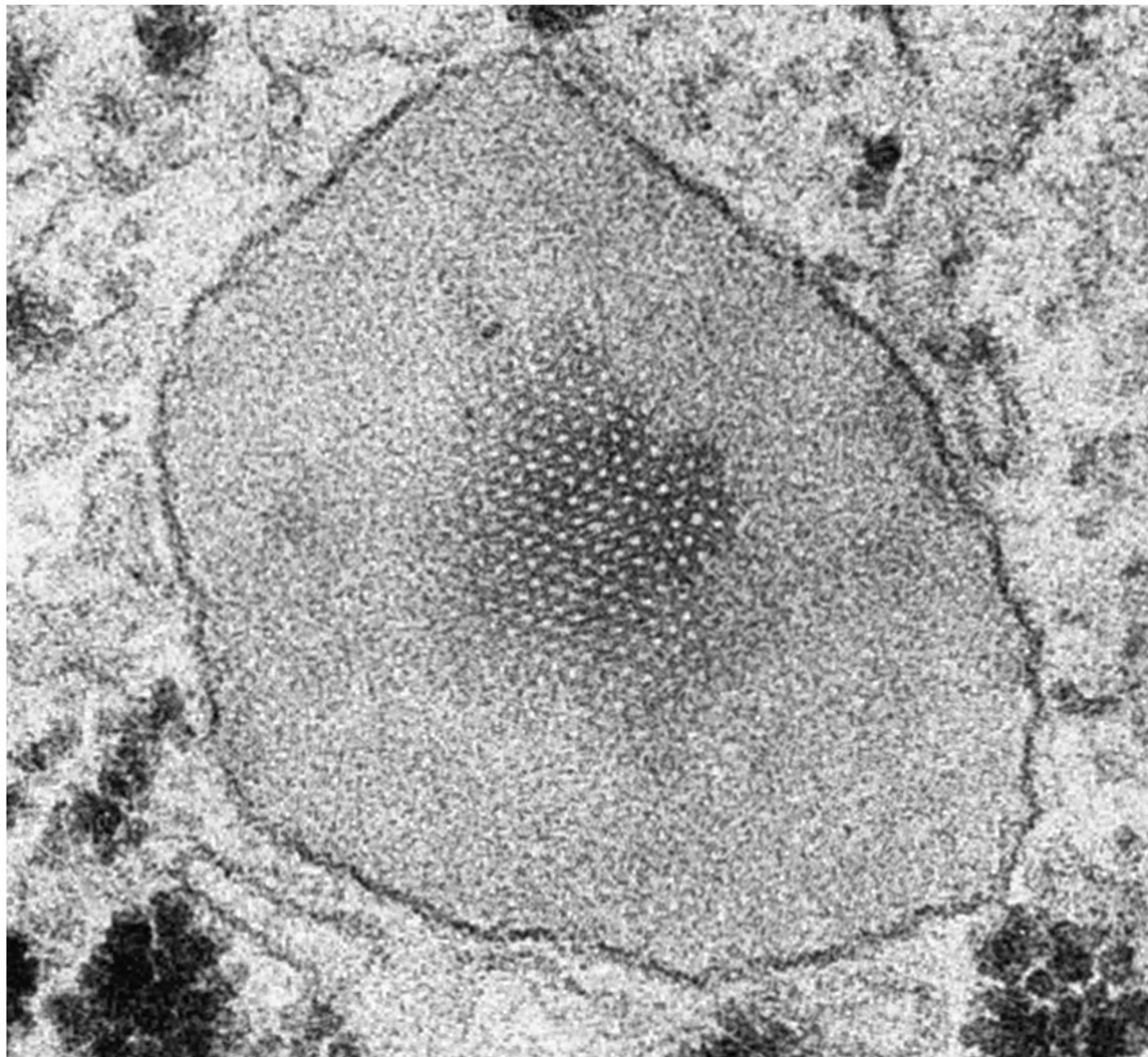


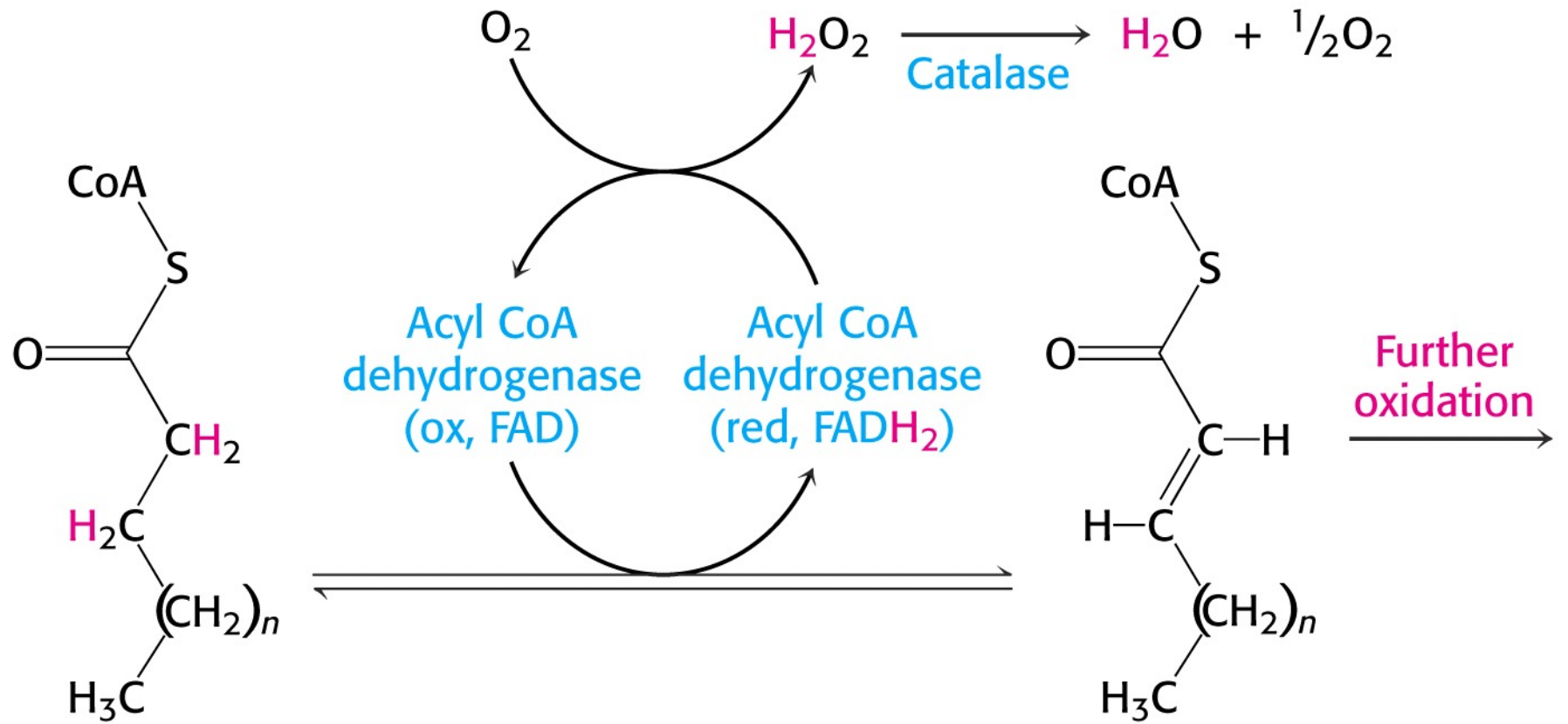


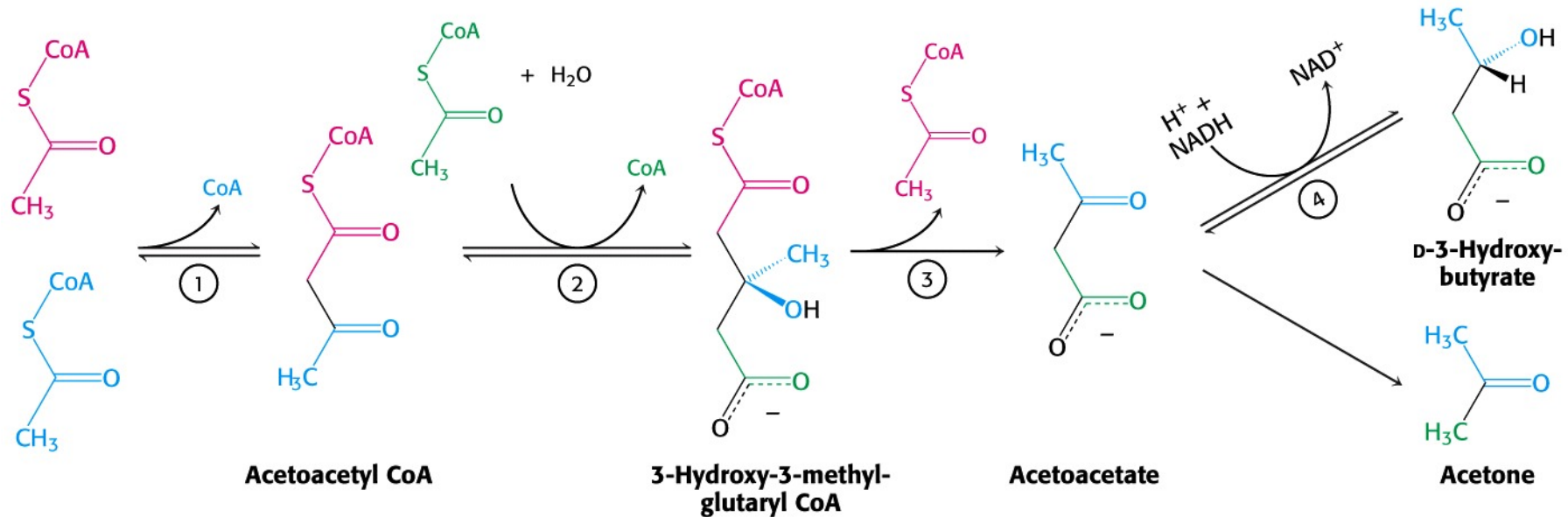
5'-Deoxyadenosine

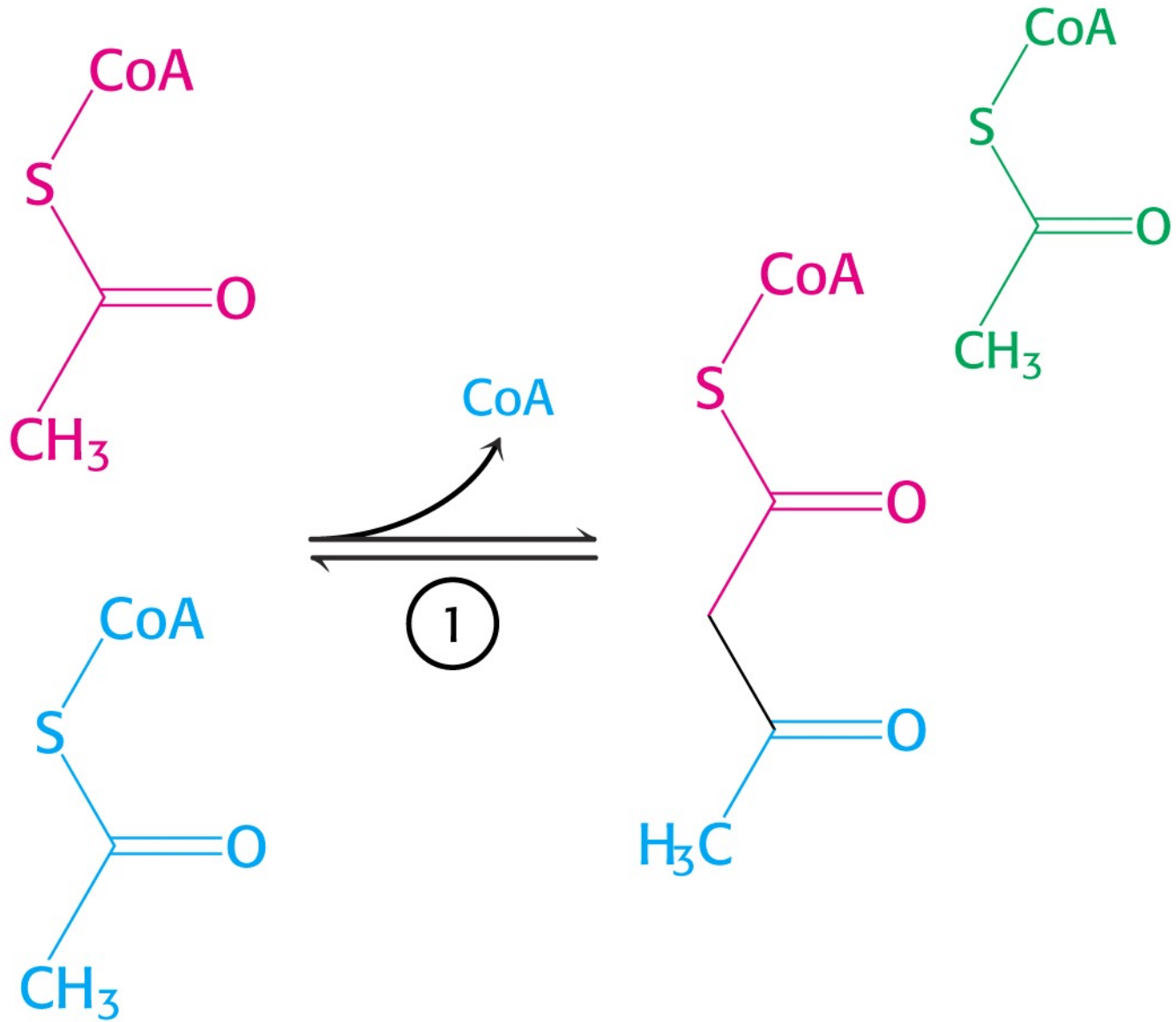




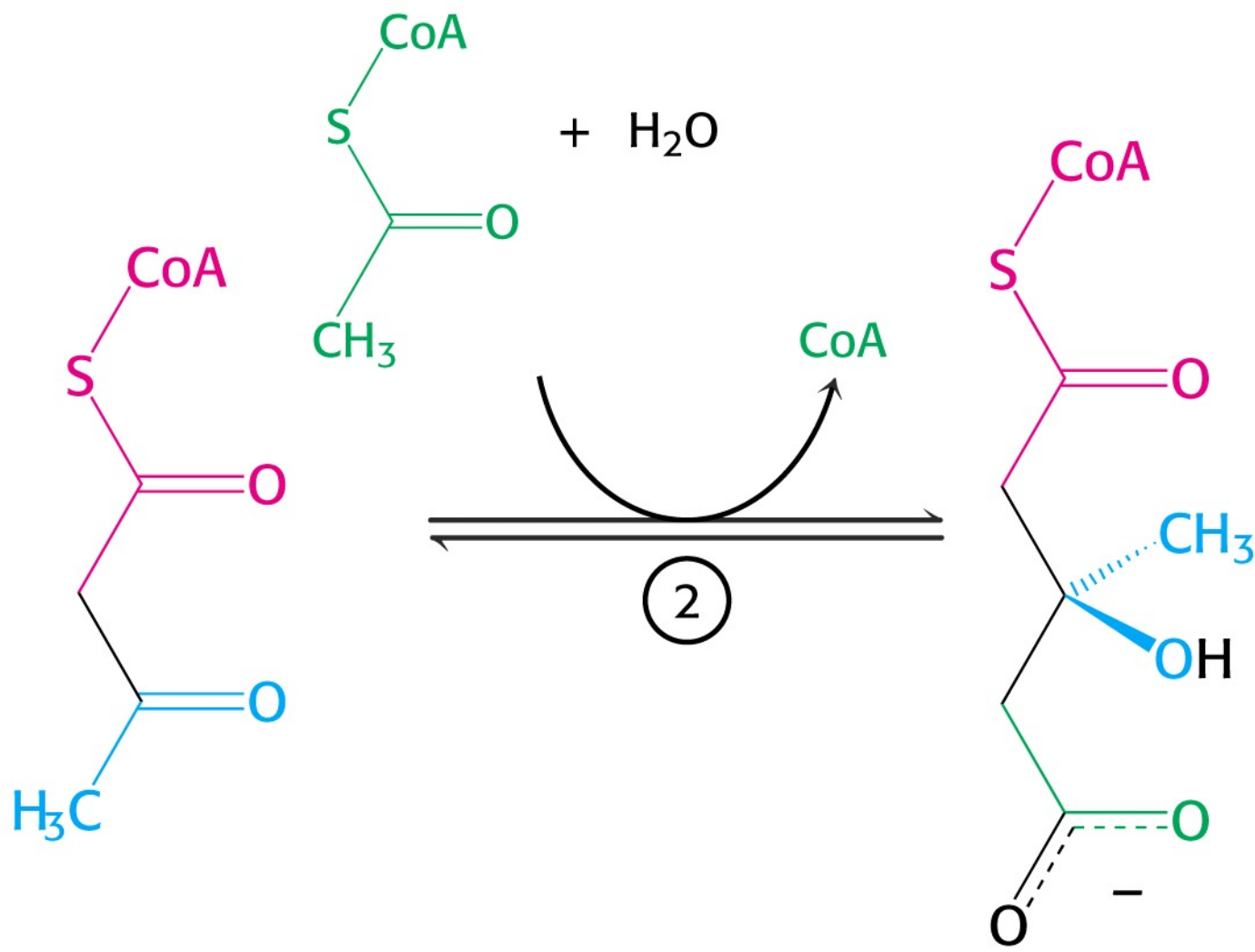






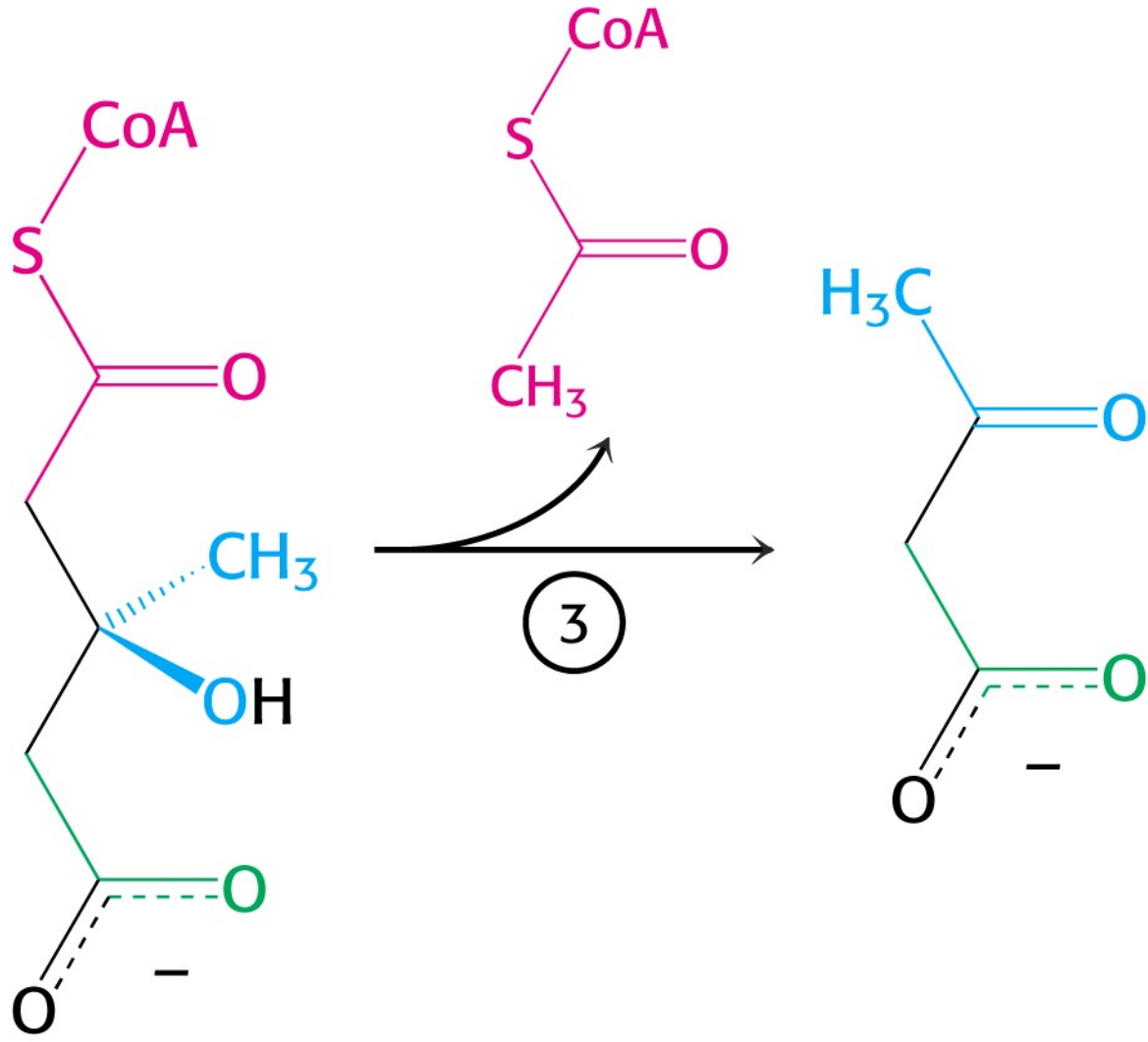


Acetoacetyl CoA



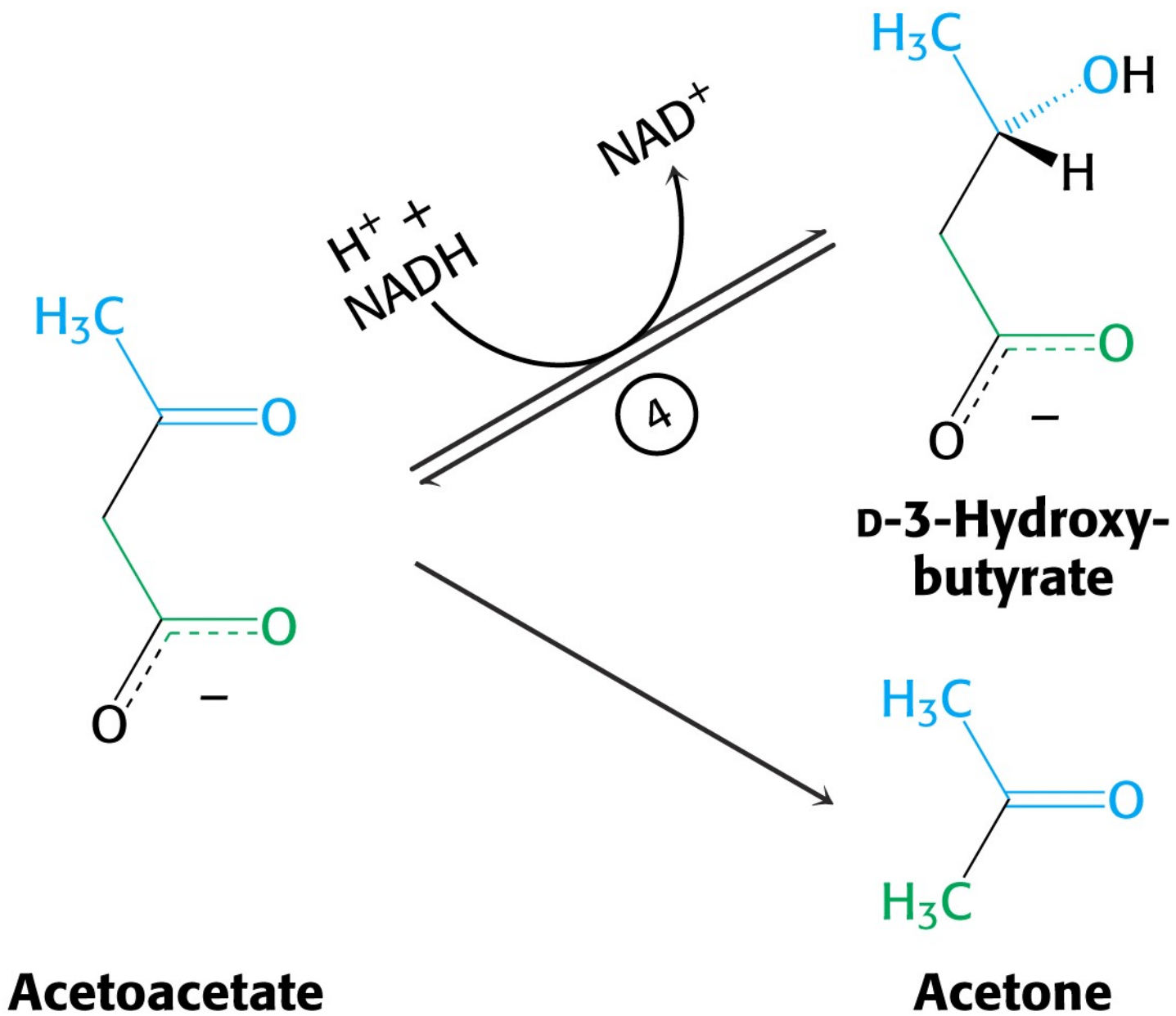
Acetoacetyl CoA

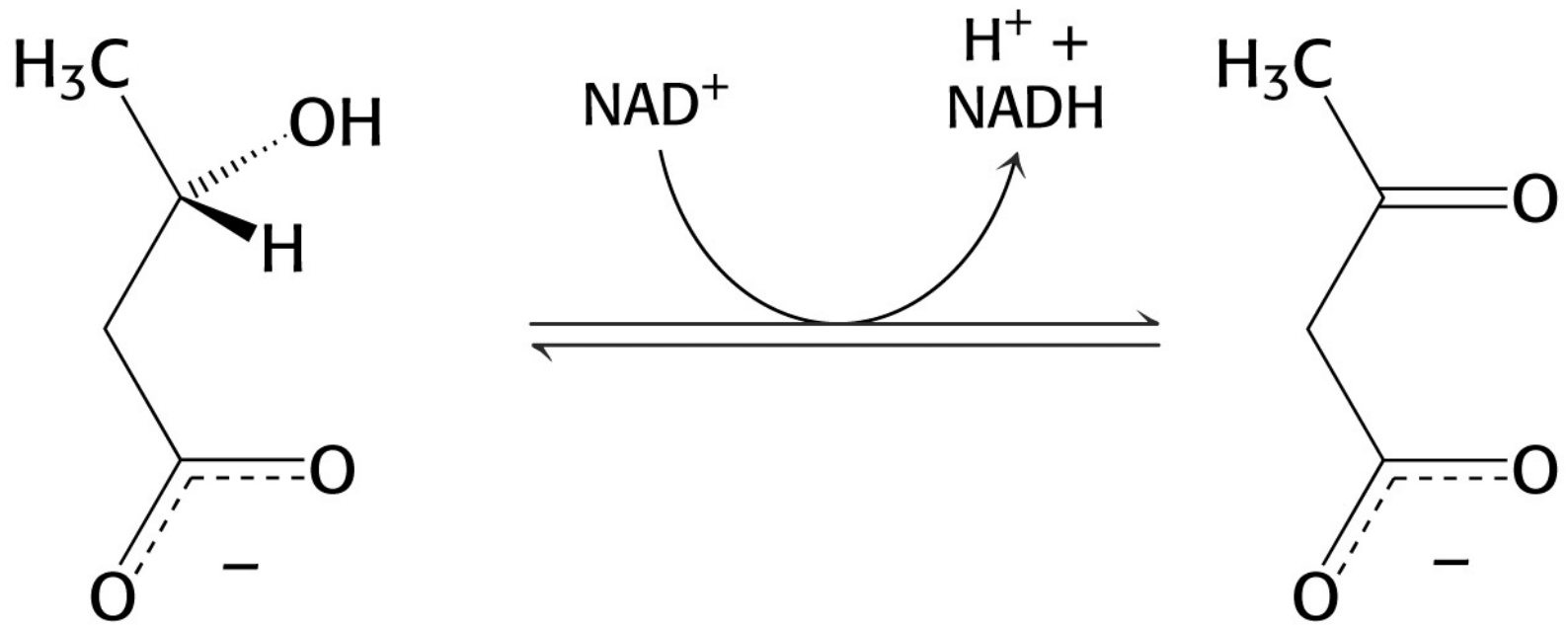
3-Hydroxy-3-methylglutaryl CoA



3-Hydroxy-3-methyl-glutaryl CoA

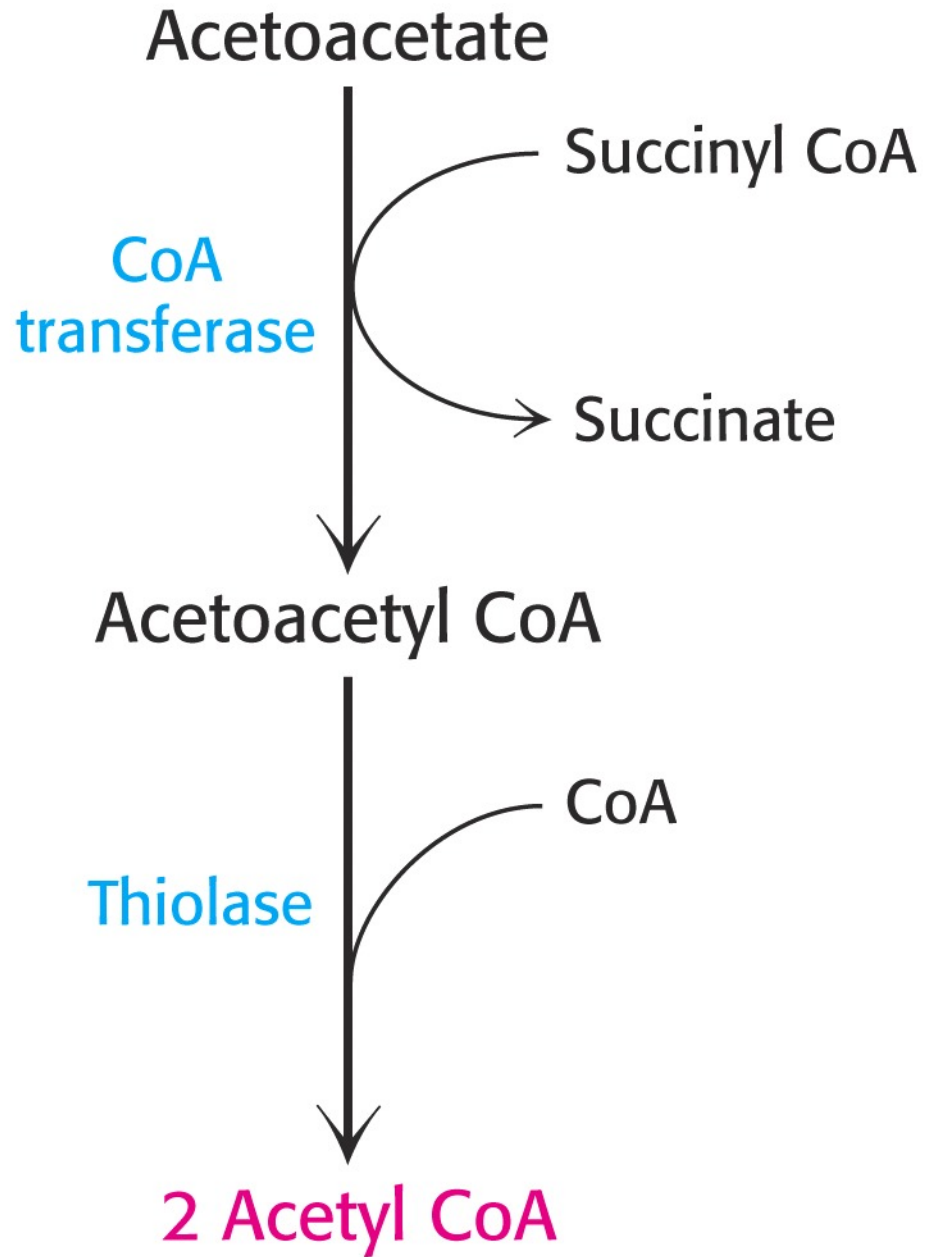
Acetoacetate





D-3-Hydroxybutyrate

Acetoacetate



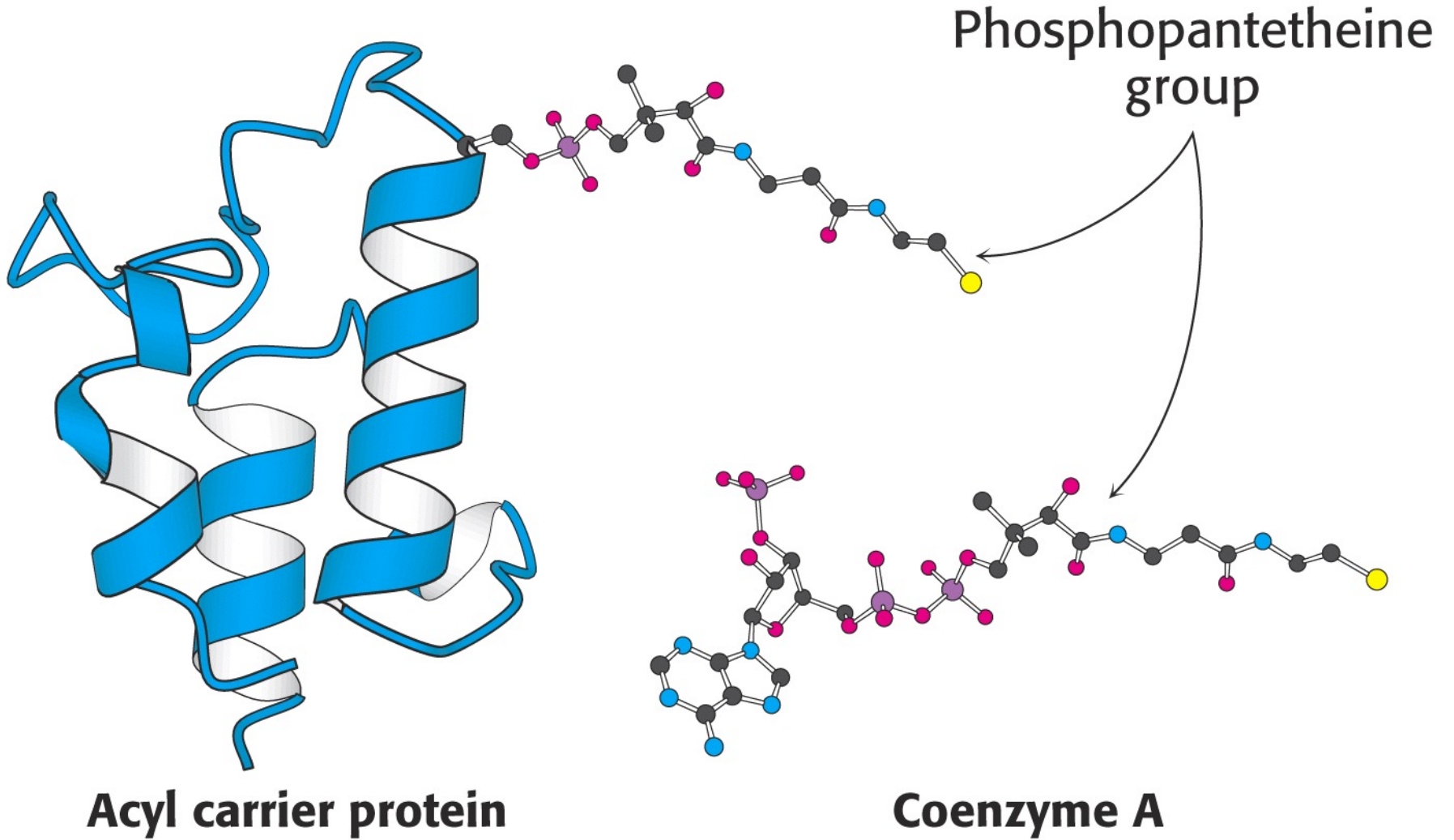
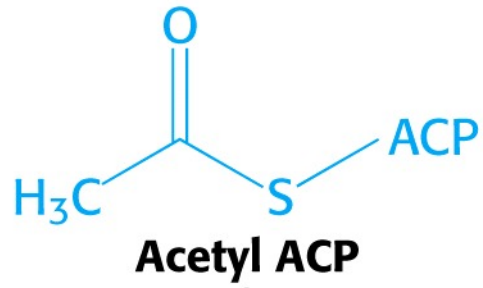


TABLE 22.2 Principal reactions in fatty acid synthesis in bacteria

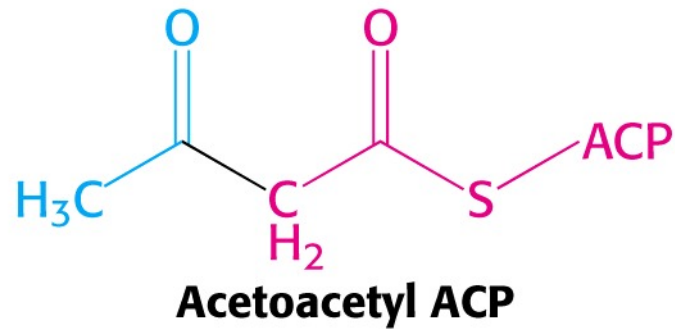
Step	Reaction	Enzyme
1	$\text{Acetyl CoA} + \text{HCO}_3^- + \text{ATP} \longrightarrow$ $\text{malonyl CoA} + \text{ADP} + \text{P}_i + \text{H}^+$	Acetyl CoA carboxylase
2	$\text{Acetyl CoA} + \text{ACP} \rightleftharpoons \text{acetyl ACP} + \text{CoA}$	Acetyl transacylase
3	$\text{Malonyl CoA} + \text{ACP} \rightleftharpoons \text{malonyl ACP} + \text{CoA}$	Malonyl transacylase
4	$\text{Acetyl ACP} + \text{malonyl ACP} \longrightarrow$ $\text{acetoacetyl ACP} + \text{ACP} + \text{CO}_2$	Acyl-malonyl ACP condensing enzyme
5	$\text{Acetoacetyl ACP} + \text{NADPH} + \text{H}^+ \rightleftharpoons$ $\text{D-3-hydroxybutyryl ACP} + \text{NADP}^+$	β -Ketoacyl ACP reductase
6	$\text{D-3-Hydroxybutyryl ACP} \rightleftharpoons$ $\text{crotonyl ACP} + \text{H}_2\text{O}$	3-Hydroxyacyl ACP dehydratase
7	$\text{Crotonyl ACP} + \text{NADPH} + \text{H}^+ \longrightarrow$ $\text{butyryl ACP} + \text{NADP}^+$	Enoyl ACP reductase

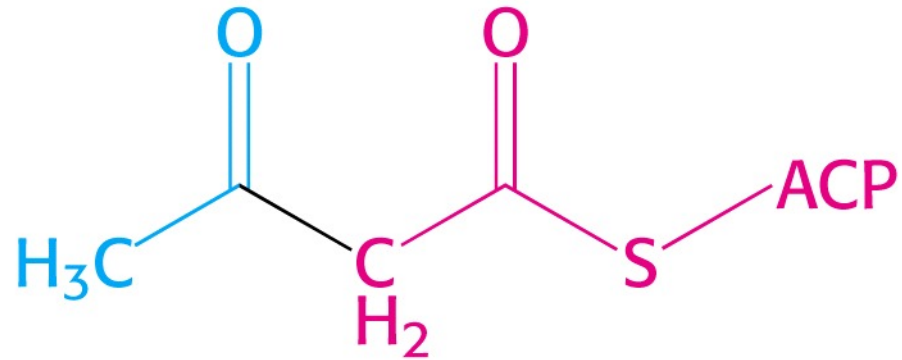


+

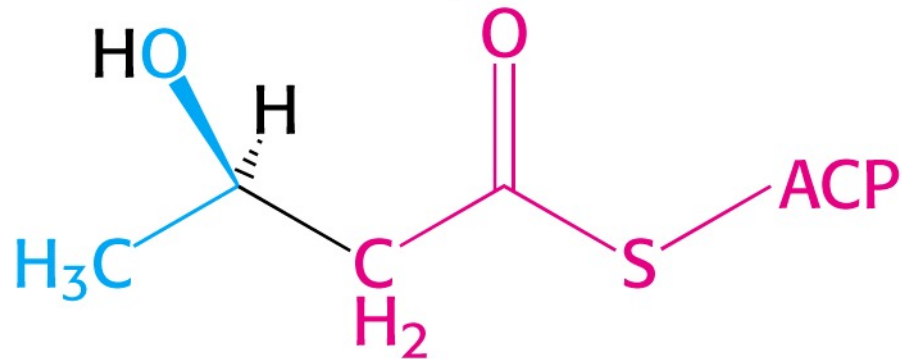
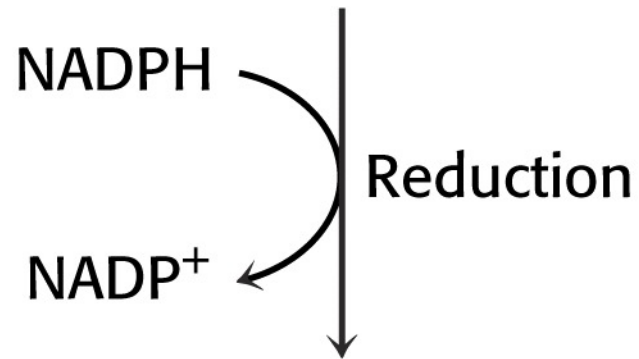


Condensation

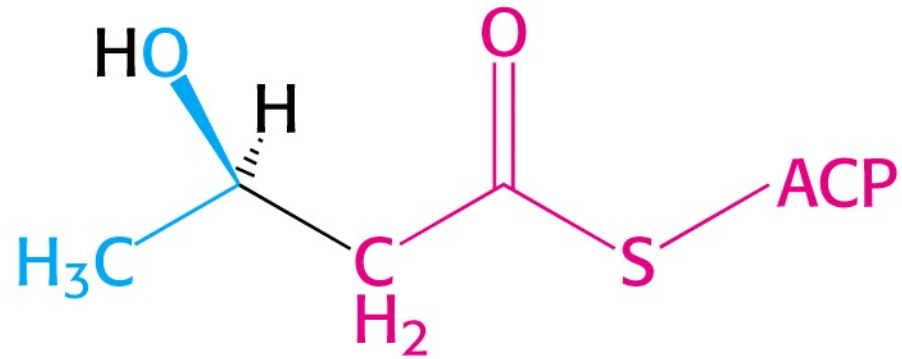




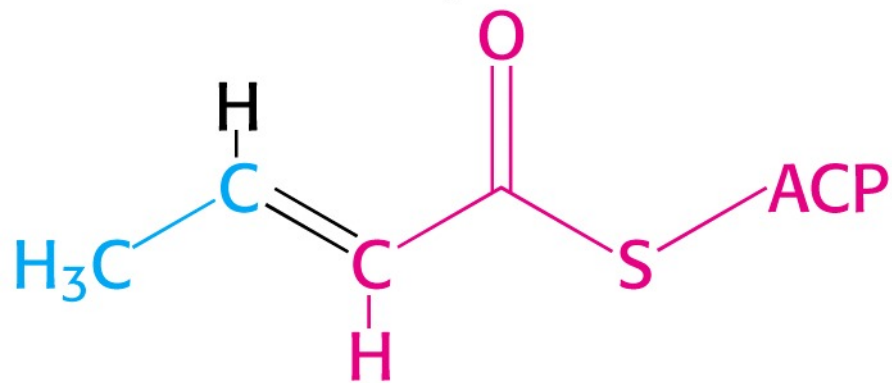
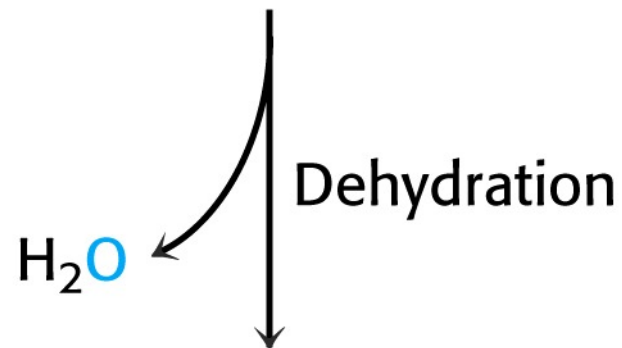
Acetoacetyl ACP



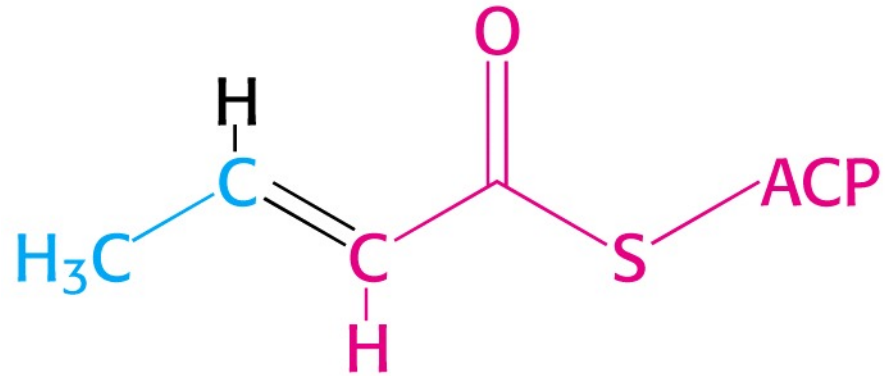
d-3-Hydroxybutyryl ACP



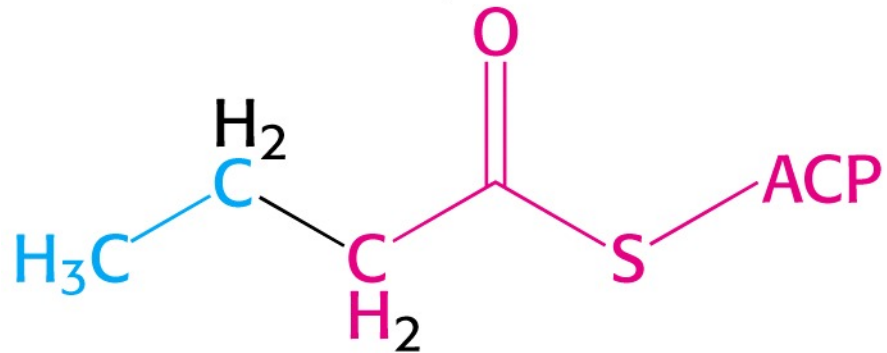
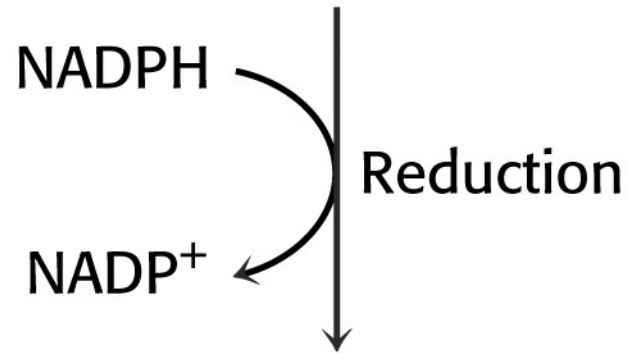
D-3-Hydroxybutyryl ACP



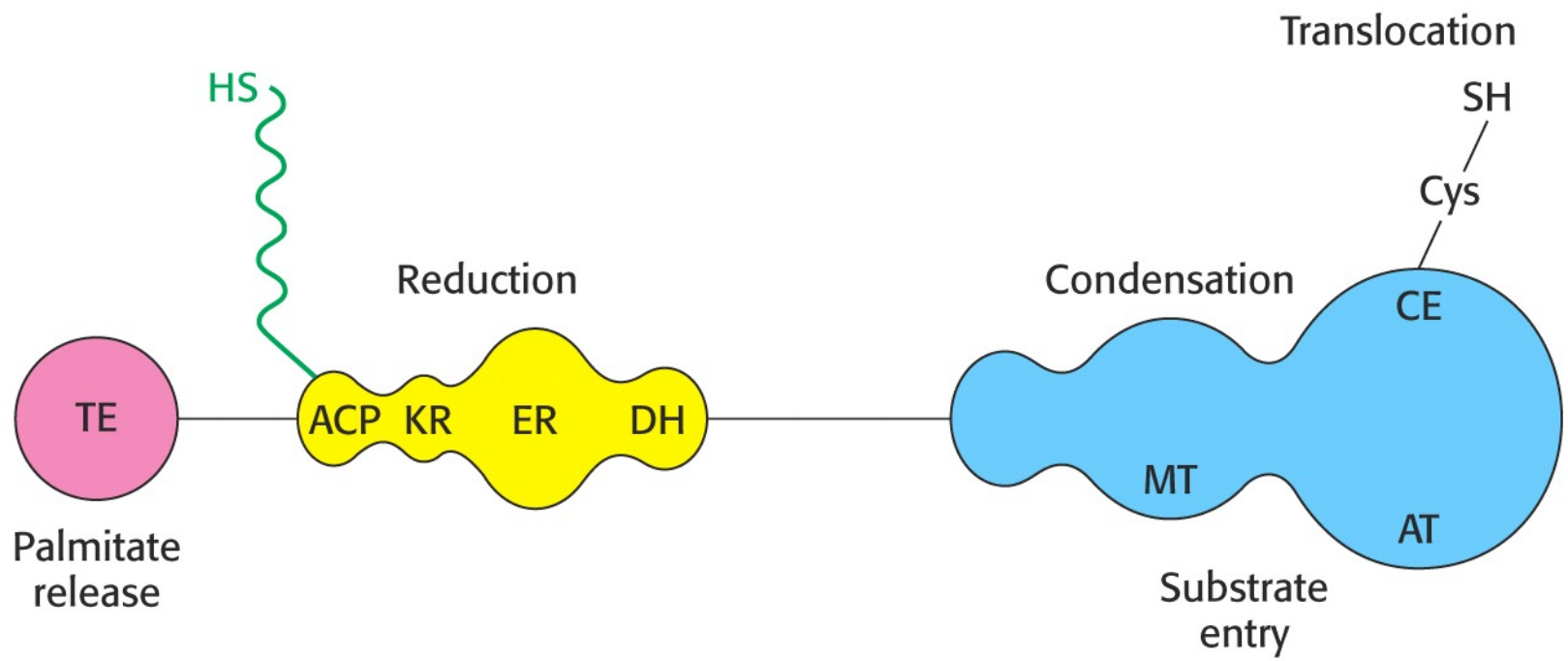
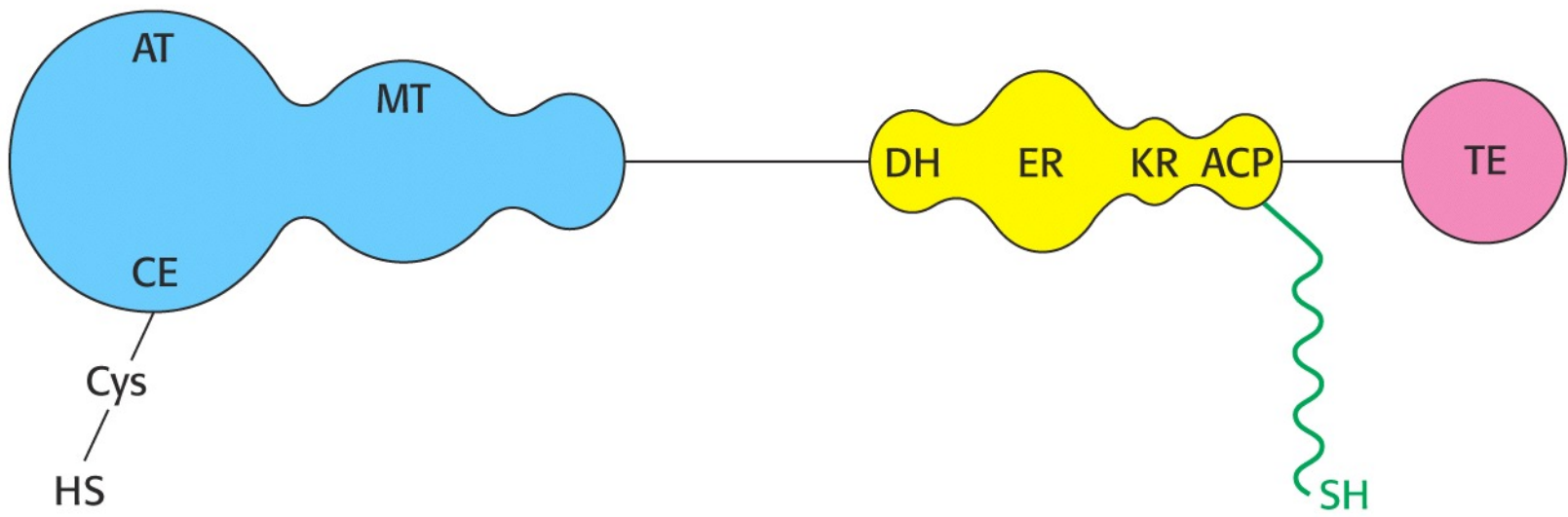
Crotonyl ACP

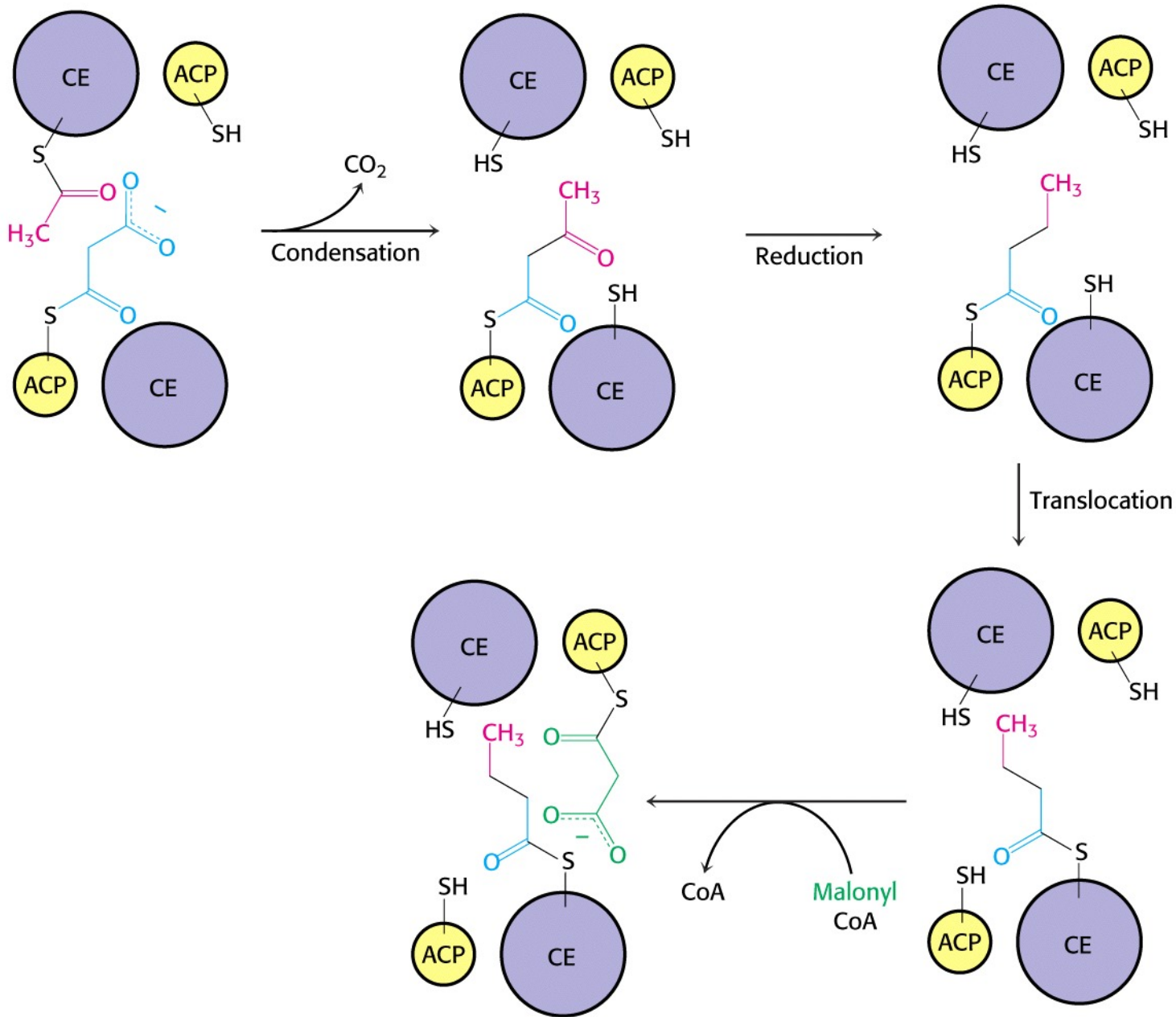


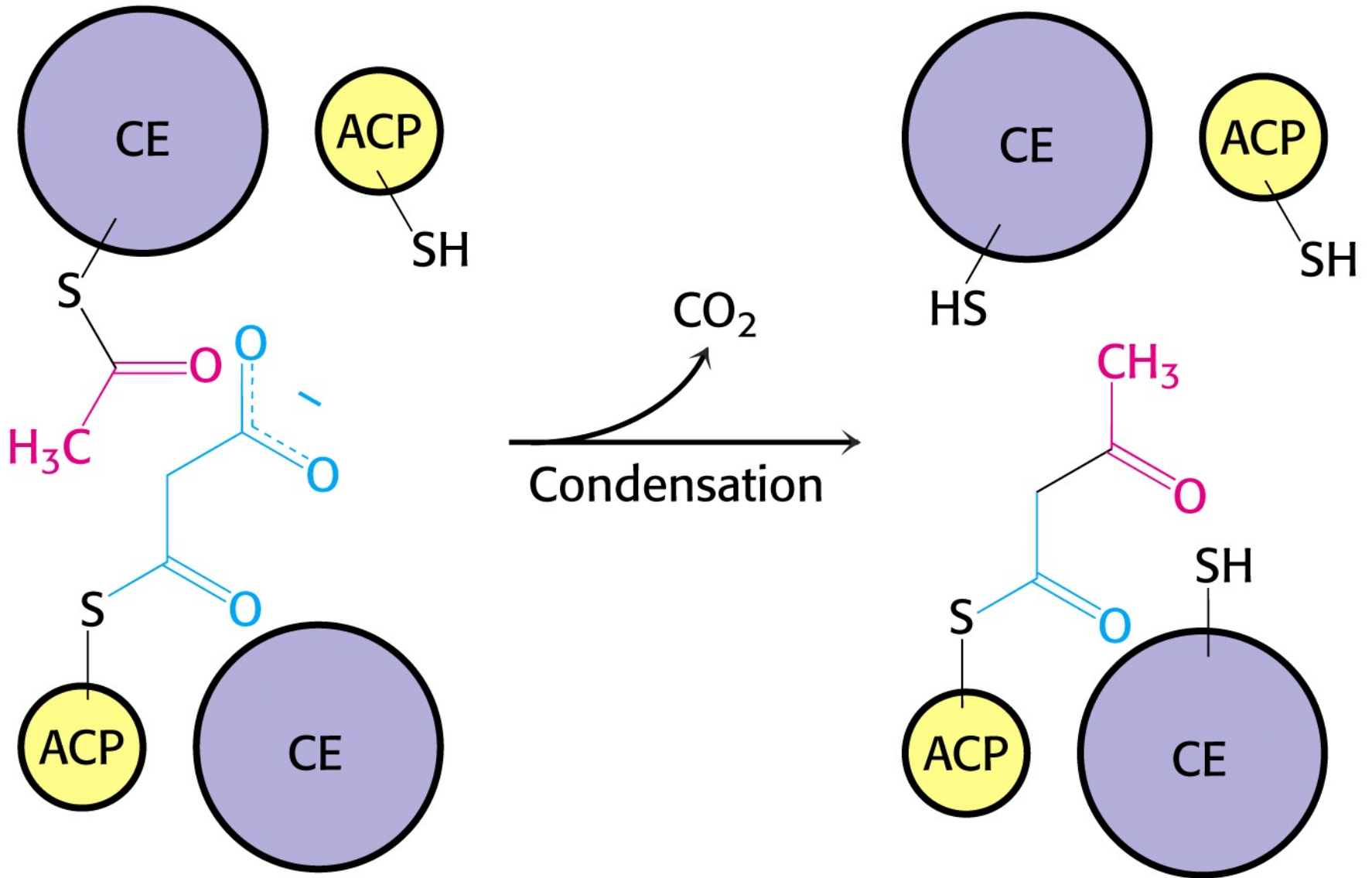
Crotonyl ACP

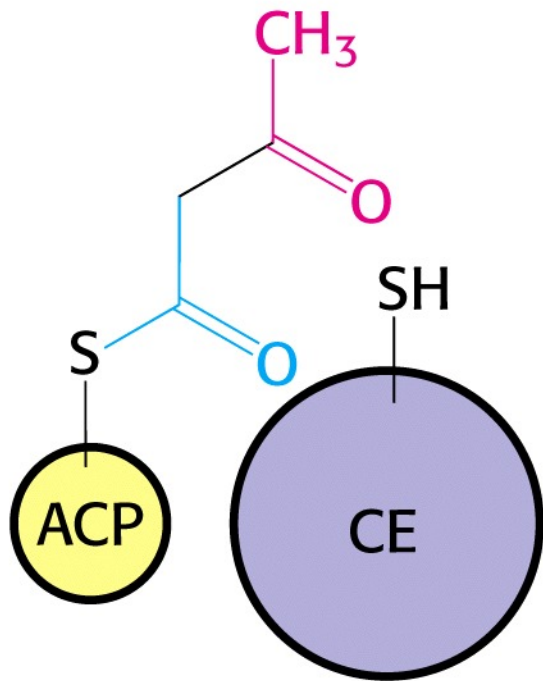
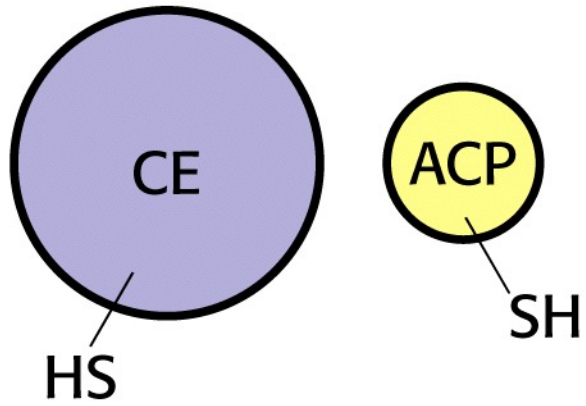


Butyryl ACP

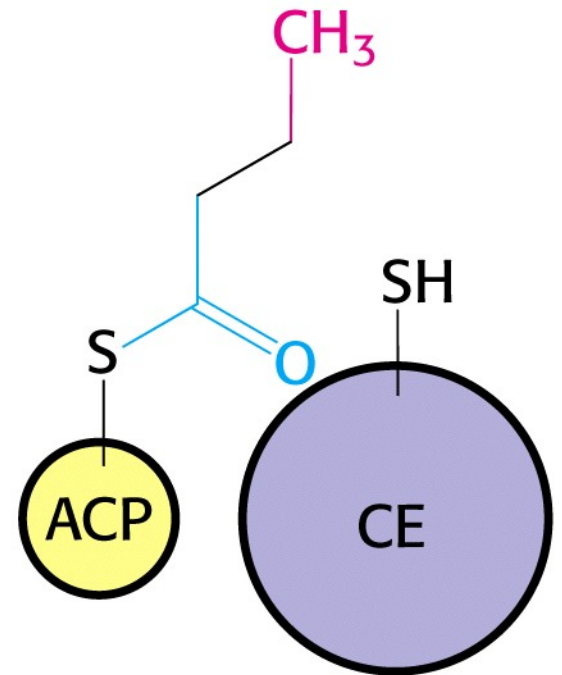
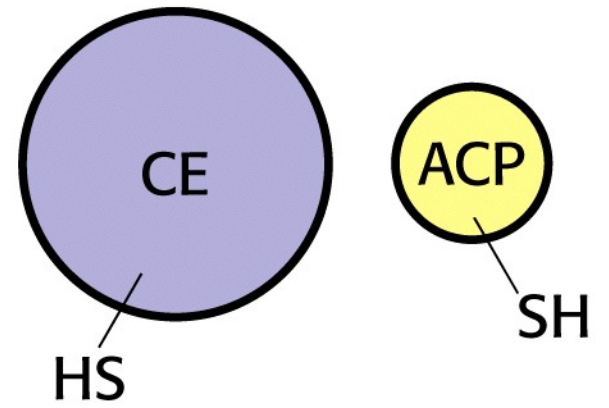


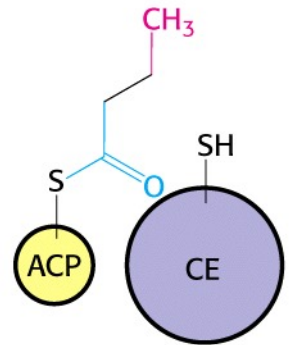
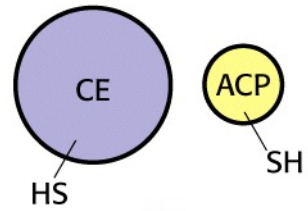




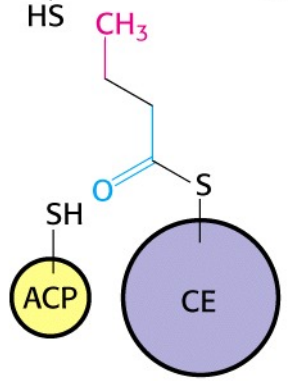
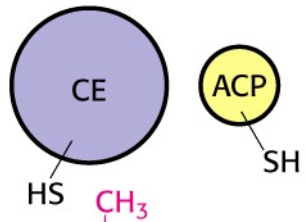


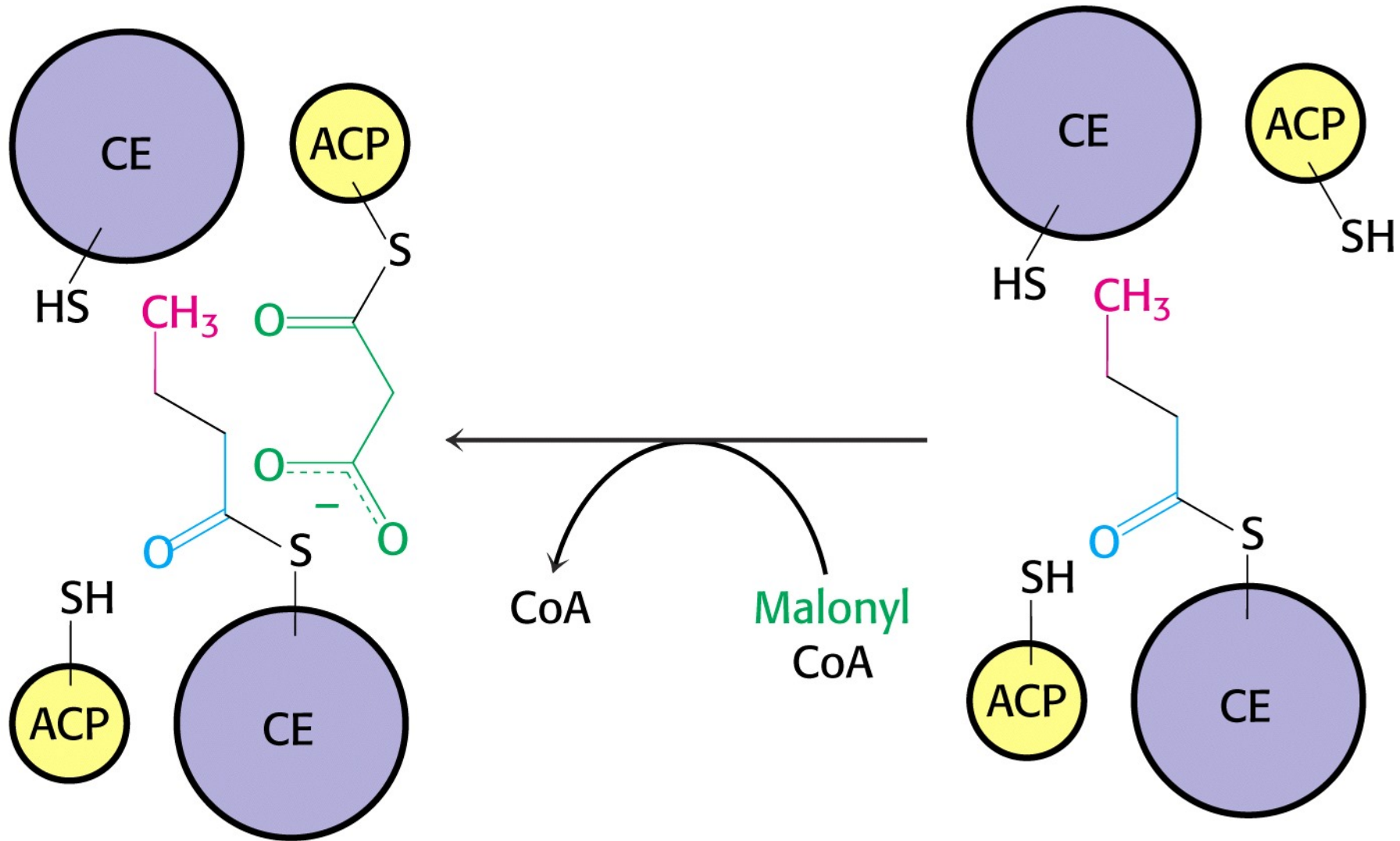
Reduction

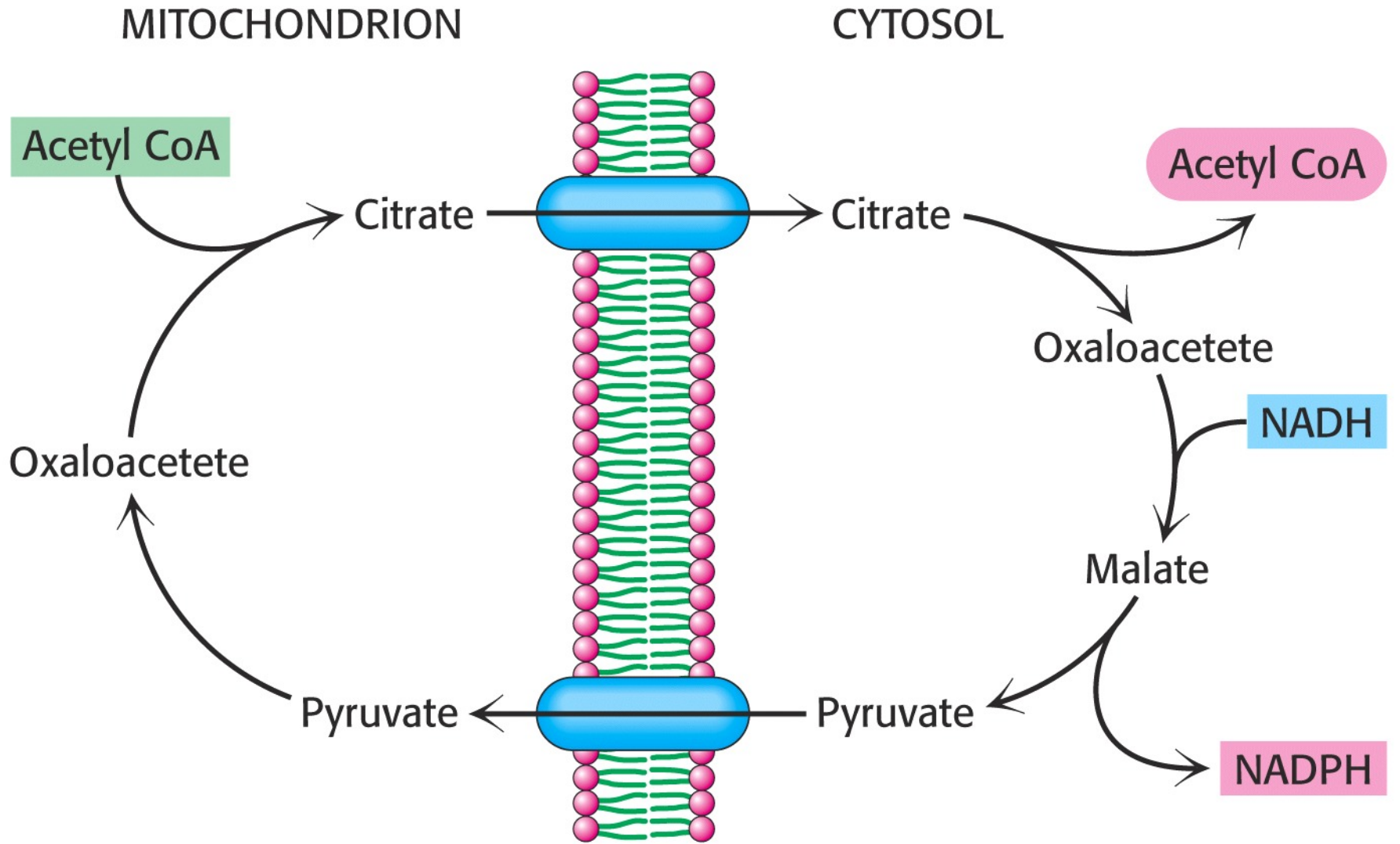


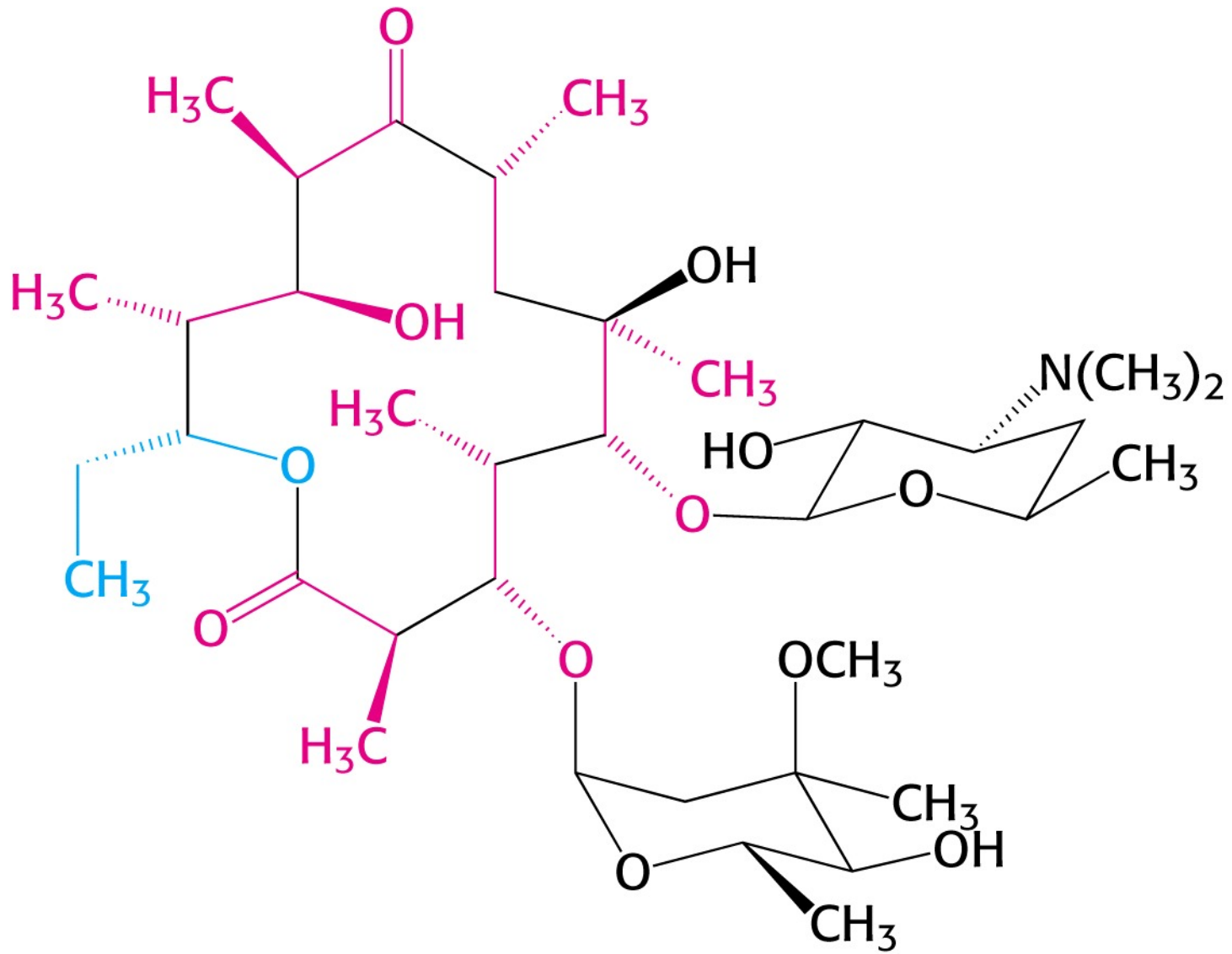


↓
Translocation

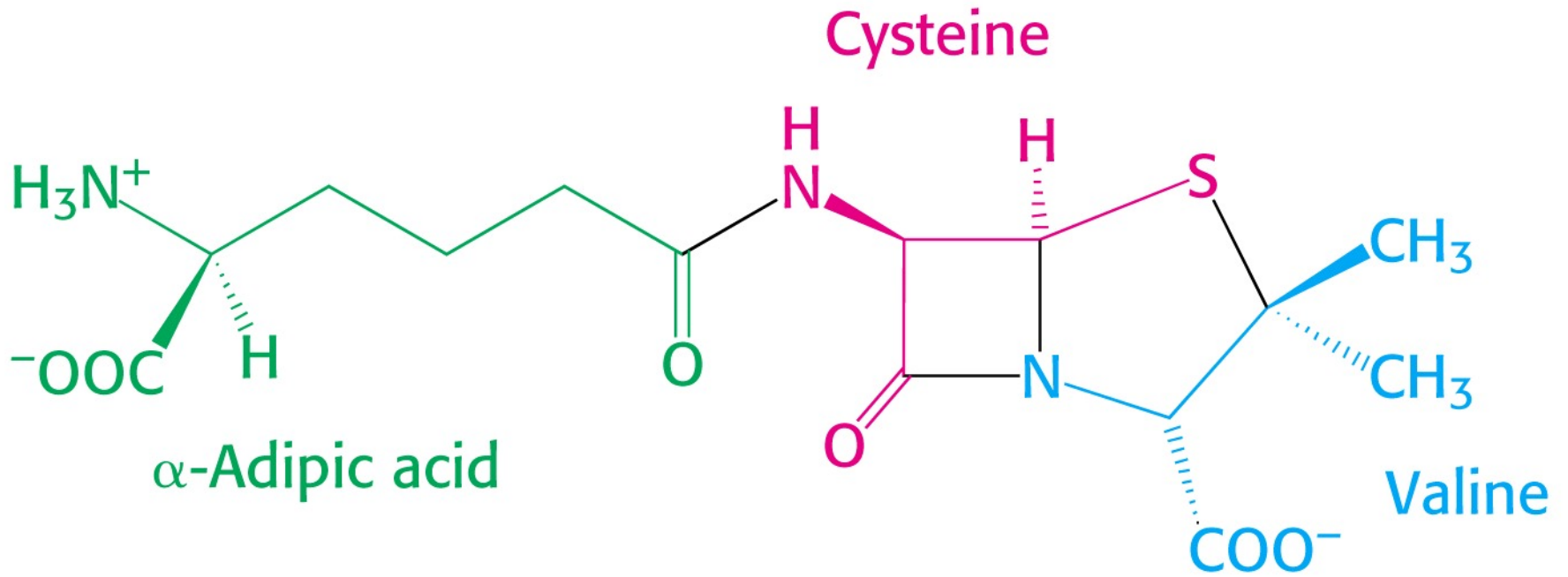




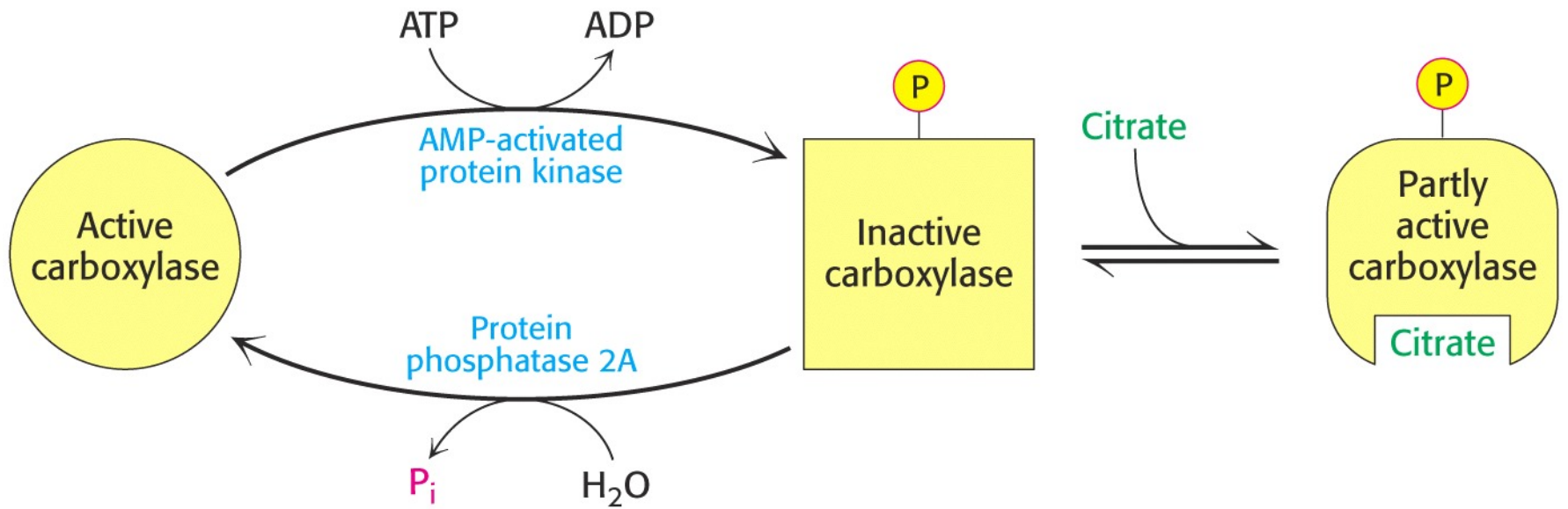


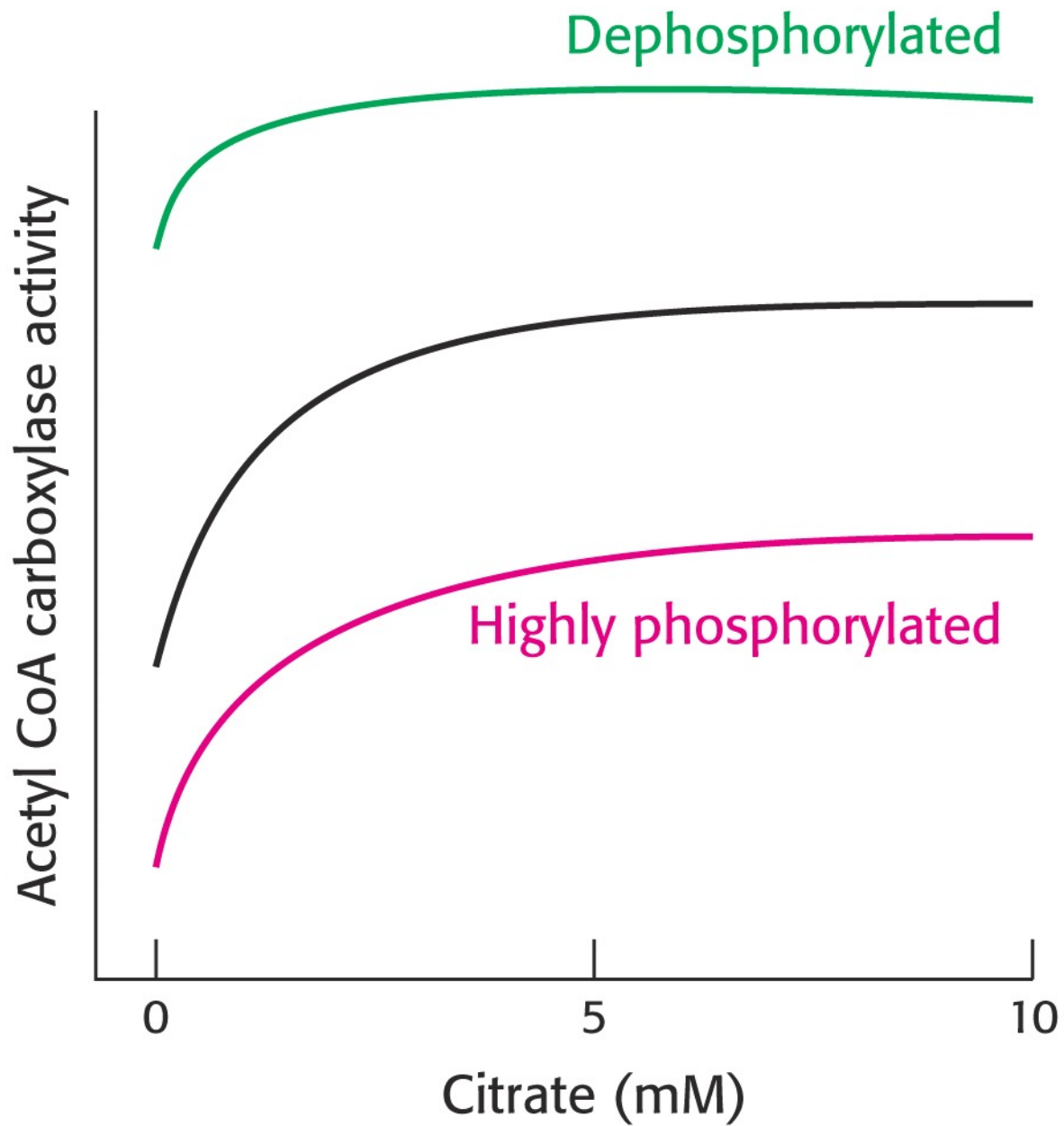


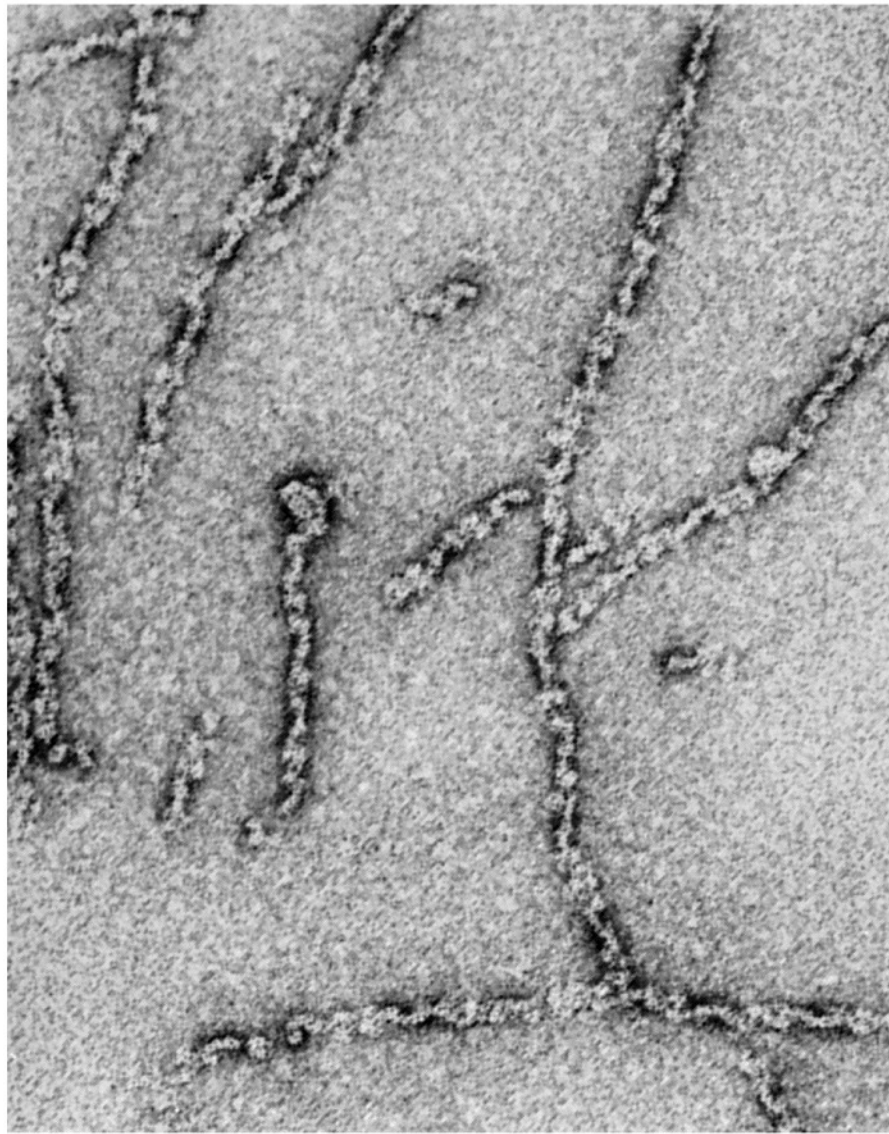
Erythromycin



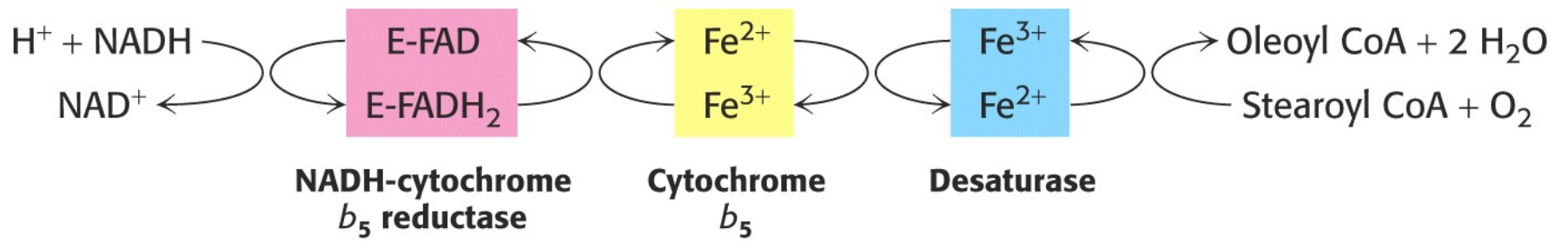
Penicillin







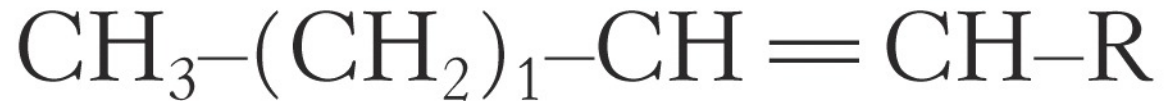
100 nm



Precursor

Formula

Linolenate (ω -3)



Linoleate (ω -6)

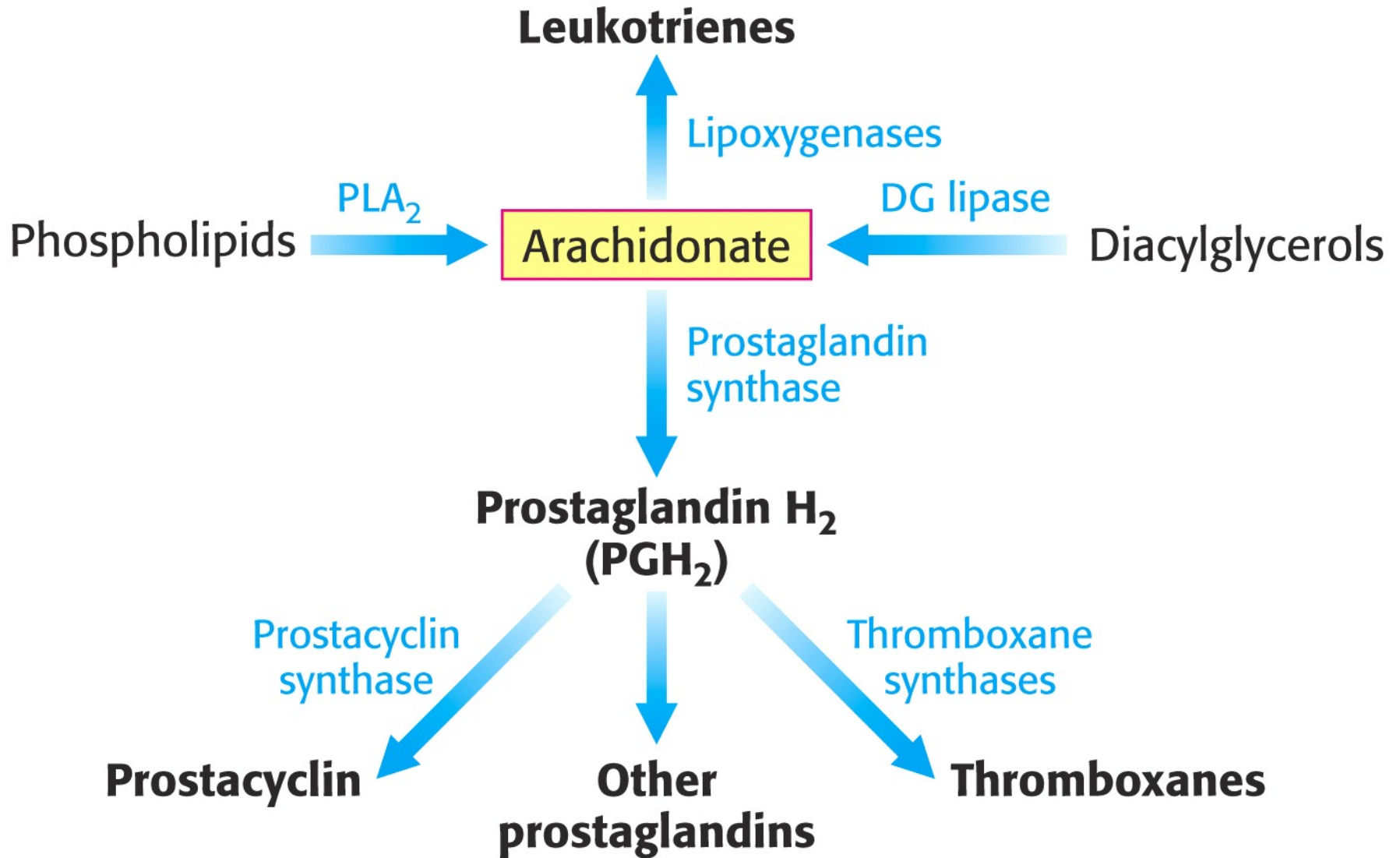


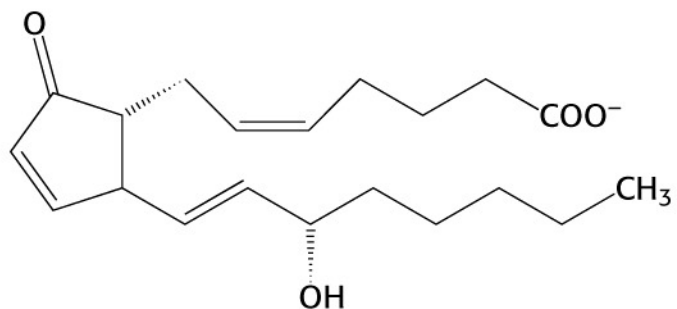
Palmitoleate (ω -7)



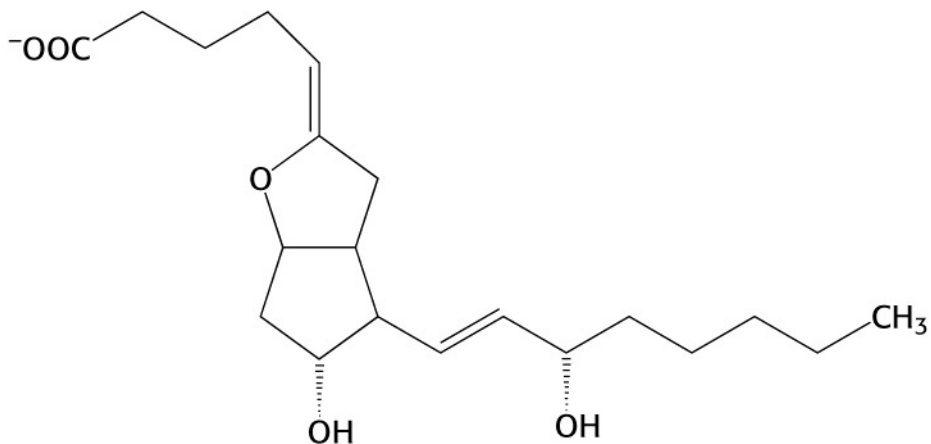
Oleate (ω -9)



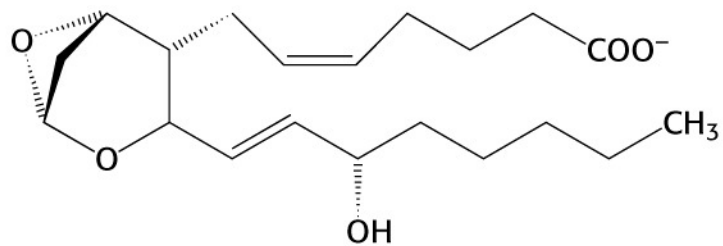




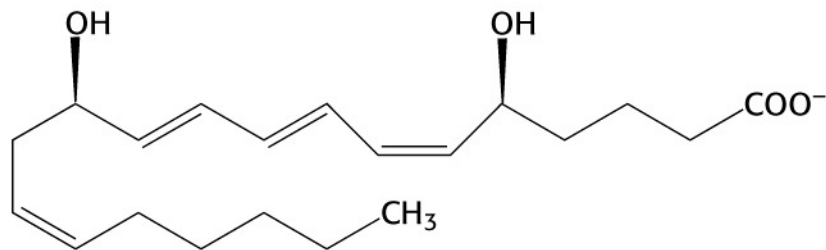
Prostaglandin A₂



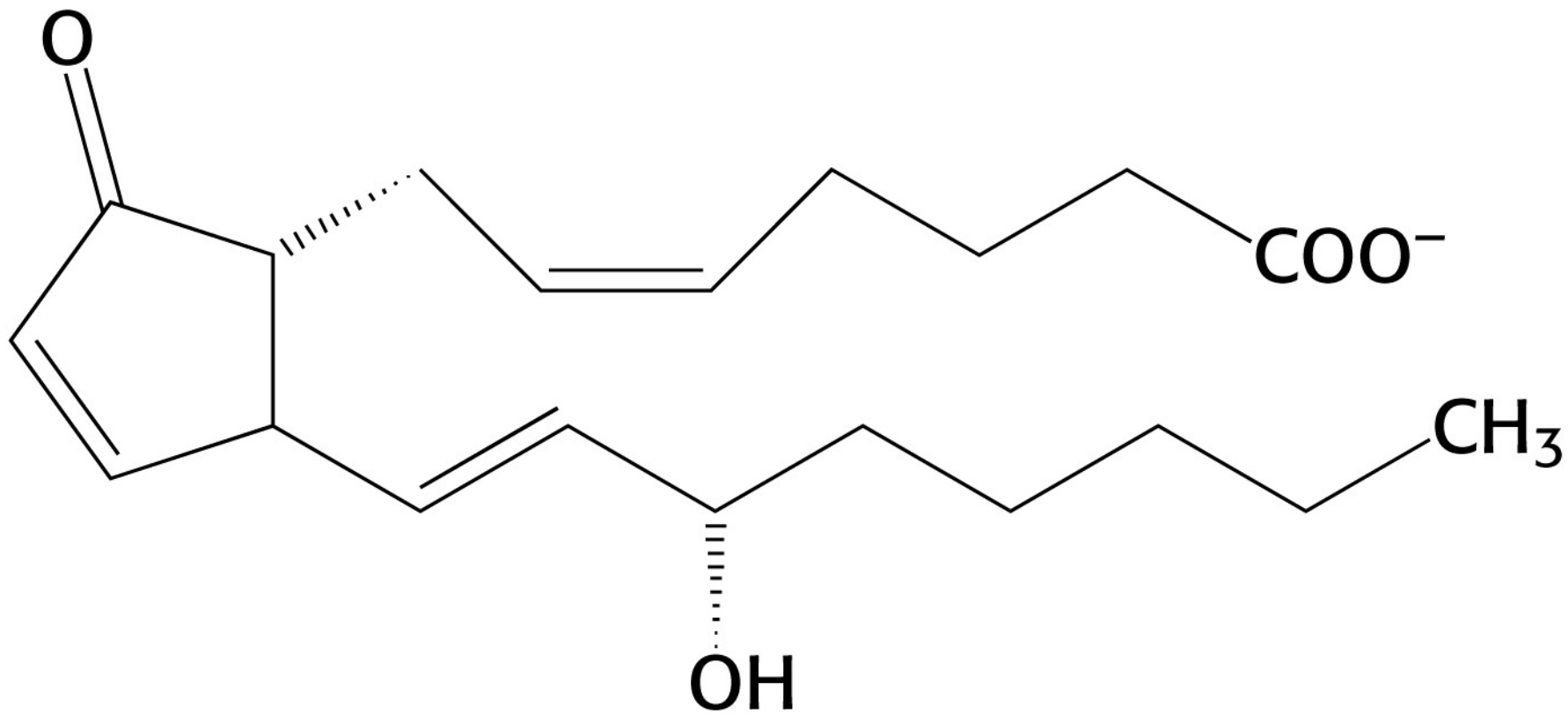
Prostacyclin (PGI₂)



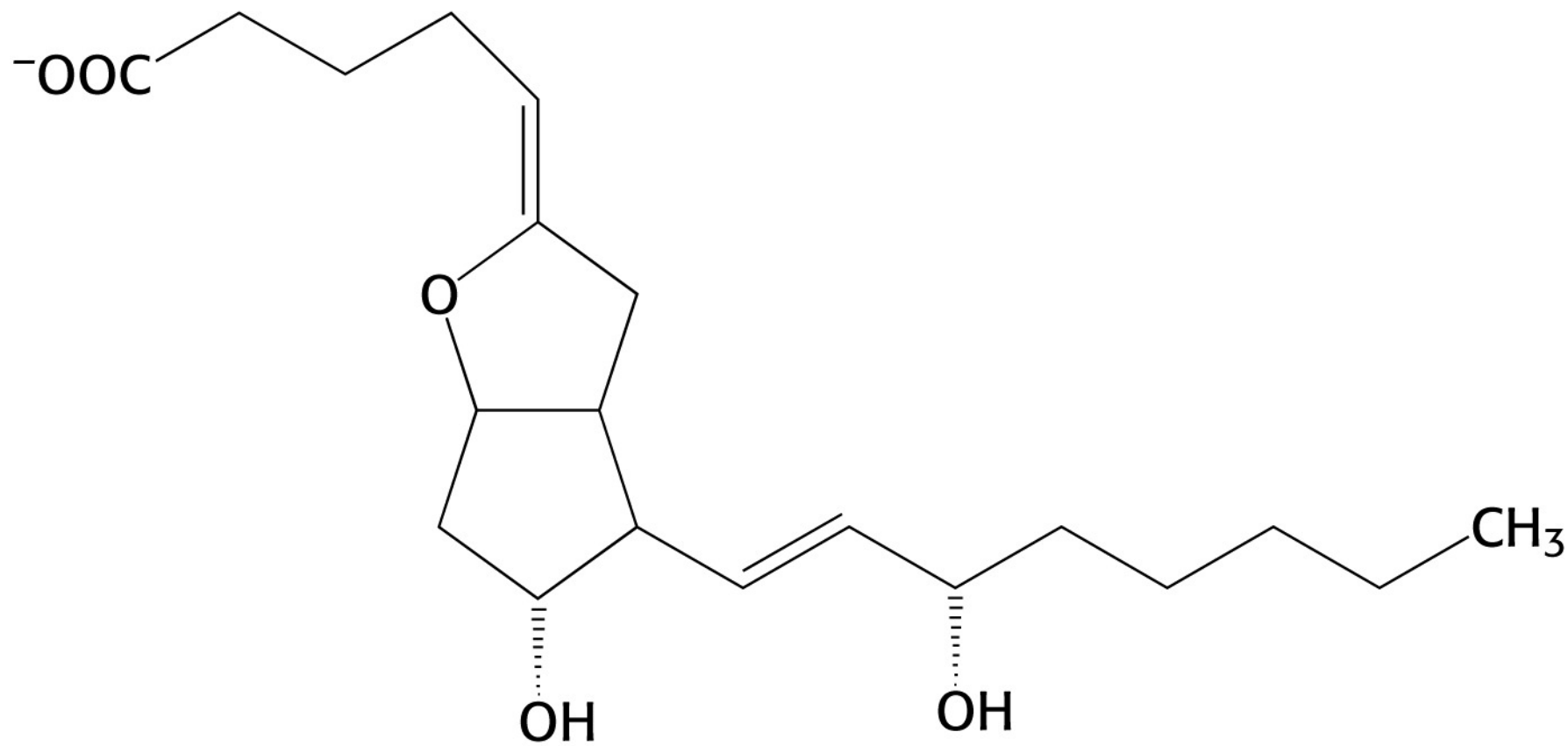
Thromboxane A₂ (TXA₂)



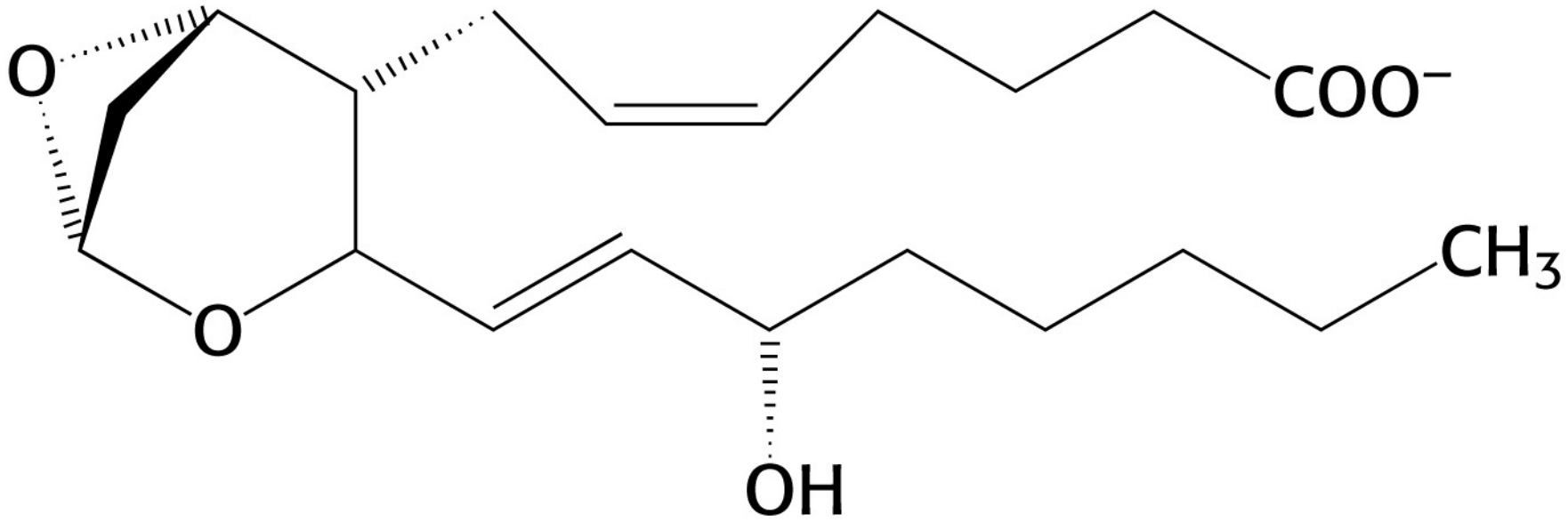
Leukotriene B₄



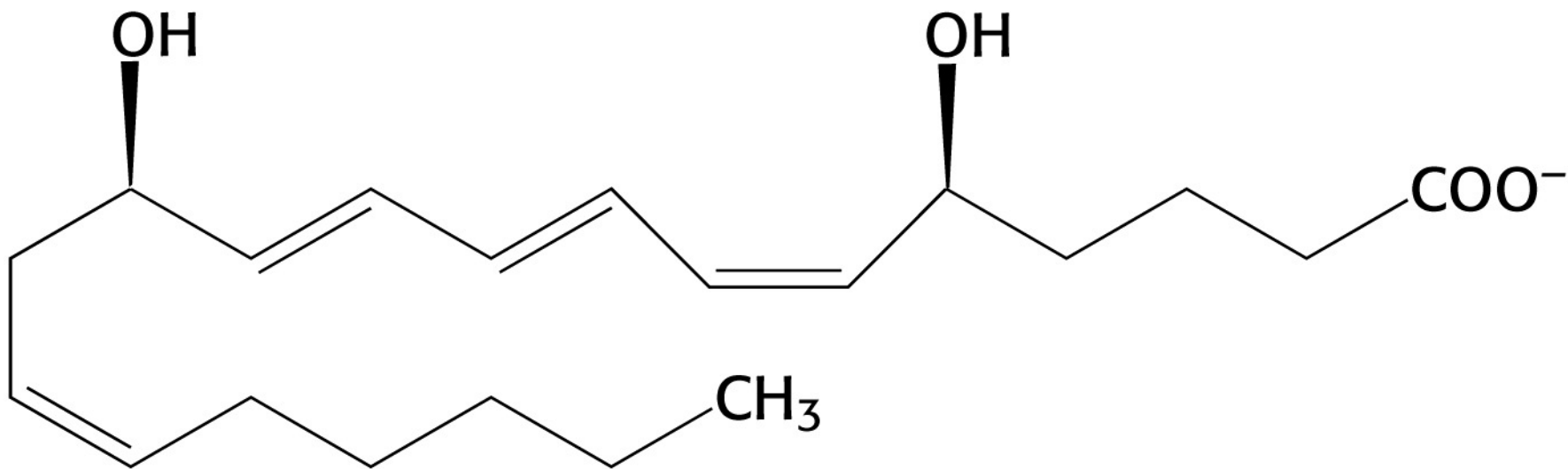
Prostaglandin A₂



Prostacyclin (PGI₂)



Thromboxane A₂ (TXA₂)



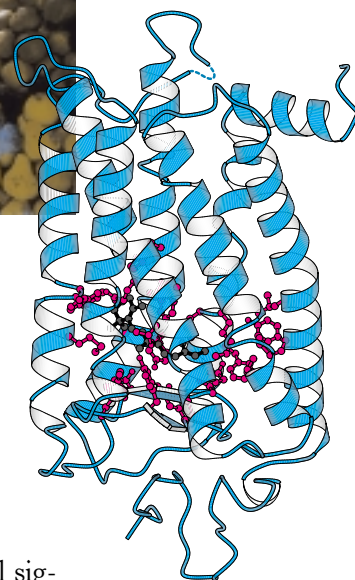
Leukotriene B₄

Sensory Systems



Color perception. The photoreceptor rhodopsin (right), which absorbs light in the process of vision, consists of the protein opsin and a bound vitamin A derivative, retinal. The amino acids (shown in red) that surround the retinal determine the color of light that is most efficiently absorbed. Individuals lacking a light-absorbing photoreceptor for the color green will see a colorful fruit stand (left) as mostly yellows (middle).

[Photographs from L. T. Sharpe, A. Stockman, H. Jagle, and J. Nathans. (1999) -Opsin genes, cone photopigments, color vision, and color blindness, in *Color Vision: from Genes to Perception*, pp. 3–51. K. Gegenfurtner, L. T. Sharpe, eds. Cambridge University Press.]



Our senses provide us with means for detecting a diverse set of external signals, often with incredible sensitivity and specificity. For example, when fully adapted to a darkened room, our eyes allow us to sense very low levels of light, down to a *limit of less than ten photons*. With more light, we are able to distinguish millions of colors. Through our senses of smell and taste, we are able to detect thousands of chemicals in our environment and sort them into categories: pleasant or unpleasant? healthful or toxic? Finally, we can perceive mechanical stimuli in the air and around us through our senses of hearing and touch.

How do our sensory systems work? How are the initial stimuli detected? How are these initial biochemical events transformed into perceptions and experiences? We have previously encountered systems that sense and respond to chemical signals—namely, receptors that bind to growth factors and hormones. Our knowledge of these receptors and their associated signal-transduction pathways provides us with concepts and tools for unraveling some of the workings of sensory systems. For example, 7TM receptors (seven-transmembrane receptors, Section 15.x.x) play key roles in olfaction, taste, and vision. Ion channels that are sensitive to mechanical stress are essential for hearing and touch.

In this chapter, we shall focus on the five major sensory systems found in human beings and other mammals: olfaction (the sense of smell; i.e., the detection of small molecules in the air), taste or gustation (the detection of selected organic compounds and ions by the tongue), vision (the detection of light), hearing (the detection of sound, or

OUTLINE

- 32.1 A Wide Variety of Organic Compounds Are Detected by Olfaction
- 32.2 Taste Is a Combination of Senses That Function by Different Mechanisms
- 32.3 Photoreceptor Molecules in the Eye Detect Visible Light
- 32.4 Hearing Depends on the Speedy Detection of Mechanical Stimuli
- 32.5 Touch Includes the Sensing of Pressure, Temperature, and Other Factors

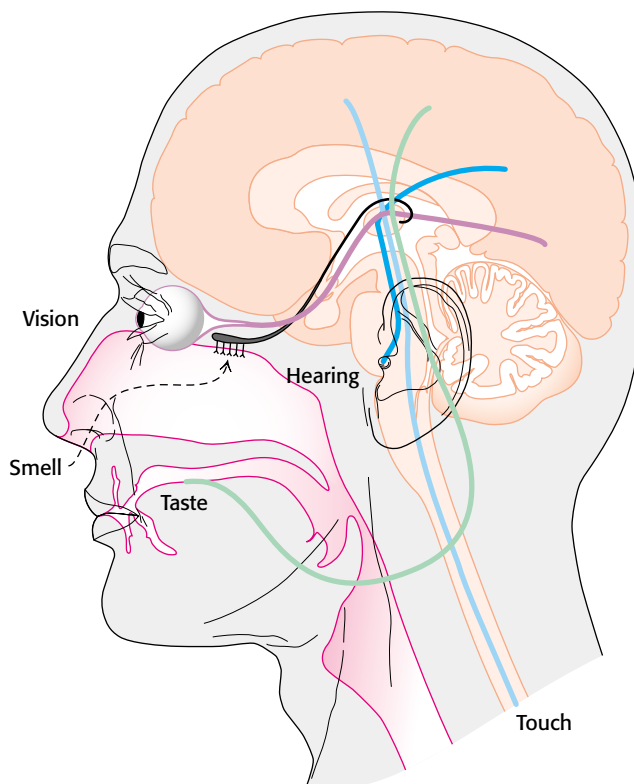
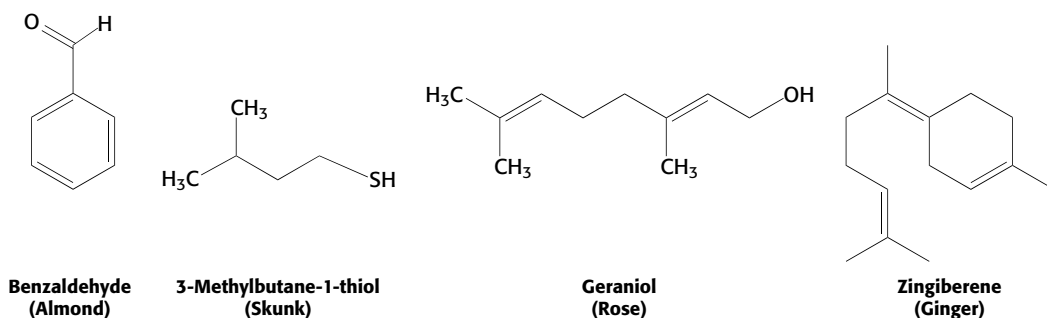


FIGURE 32.1 Sensory connections to the brain. Sensory nerves connect sensory organs to the brain and spinal cord.

pressure waves in the air), and touch (the detection of changes in pressure, temperature, and other factors by the skin). Each of these primary sensory systems contains specialized sensory neurons that transmit nerve impulses to the central nervous system (Figure 32.1). In the central nervous system, these signals are processed and combined with other information to yield a perception that may trigger a change in behavior. By these means, our senses allow us to detect changes in our environments and to adjust our behavior appropriately.

32.1 A WIDE VARIETY OF ORGANIC COMPOUNDS ARE DETECTED BY OLFACTION

Human beings can detect and distinguish thousands of different compounds by smell, often with considerable sensitivity and specificity. Most odorants are relatively small organic compounds with sufficient volatility that they can be carried as vapors into the nose. For example, a major component responsible for the smell of almonds is the simple aromatic compound benzaldehyde, whereas the sulfhydryl compound 3-methylbutane-1-thiol is a major component of the smell of skunks.



What properties of these molecules are responsible for their smells? First, *the shape of the molecule rather than its other physical properties is crucial*. We can most clearly see the importance of shape by comparing molecules such as those responsible for the smells of spearmint and caraway. These compounds are identical in essentially all physical properties such as hydrophobicity because they are exact mirror images of one another. Thus, the smell produced by an odorant depends not on a physical property but on the compound's interaction with a specific binding surface, most likely a protein receptor. Second, some human beings (and other animals) suffer from *specific anosmias*; that is, they are incapable of smelling specific compounds even though their olfactory systems are otherwise normal. Such anosmias are often inherited. These observations suggest that mutations in individual receptor genes lead to the loss of the ability to detect a small subset of compounds.

32.1.1 Olfaction Is Mediated by an Enormous Family of Seven-Transmembrane-Helix Receptors

Odorants are detected in a specific region of the nose, called the *main olfactory epithelium*, that lies at the top of the nasal cavity (Figure 32.2). Approximately 1 million sensory neurons line the surface of this region. Cilia containing the odorant-binding protein receptors project from these neurons into the mucous lining of the nasal cavity.

Biochemical studies in the late 1980s examined isolated cilia from rat olfactory epithelium that had been treated with odorants. Exposure to the odorants increased the cellular level of cAMP, and this increase was observed only in the presence of GTP. On the basis of what was known about signal-transduction systems, *the participation of cAMP and GTP strongly suggested the involvement of a G protein and, hence, 7TM receptors*. Indeed, Randall Reed purified and cloned a G protein α subunit, termed $G_{(olf)}$, which is uniquely expressed in olfactory cilia. The involvement of 7TM receptors suggested a strategy for identifying the olfactory receptors themselves. cDNAs were sought that (1) were expressed primarily in the sensory neurons lining the nasal epithelium, (2) encoded members of the 7TM receptor family, and (3) were present as a large and diverse family to account for the range of odorants. Through the use of these criteria, cDNAs for odorant receptors from rats were identified in 1991 by Richard Axel and Linda Buck.

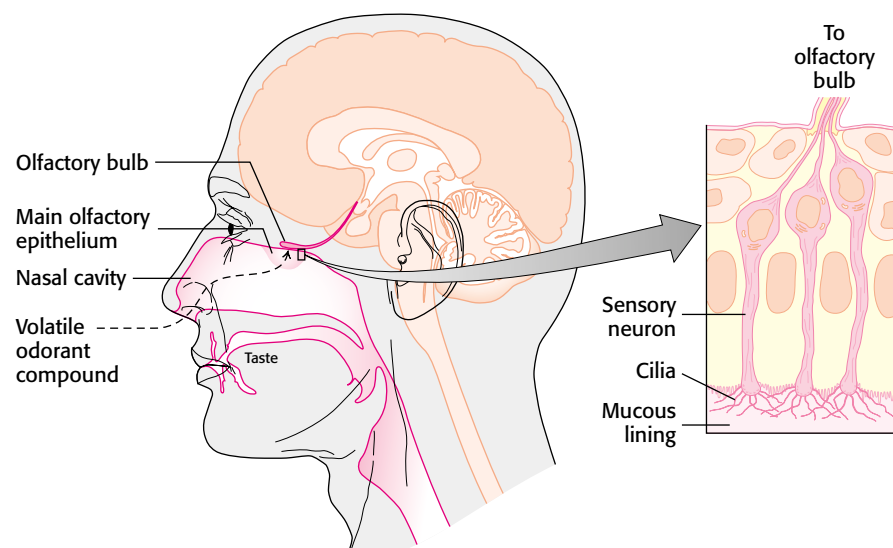
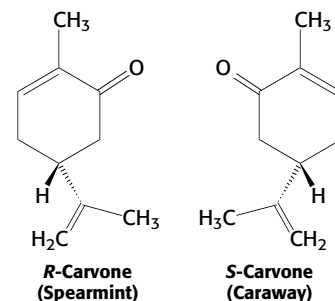


FIGURE 32.2 The main nasal epithelium. This region of the nose, which lies at the top of the nasal cavity, contains approximately 1 million sensory neurons. Nerve impulses generated by odorant molecules binding to receptors on the cilia travel from the sensory neurons to the olfactory bulb.



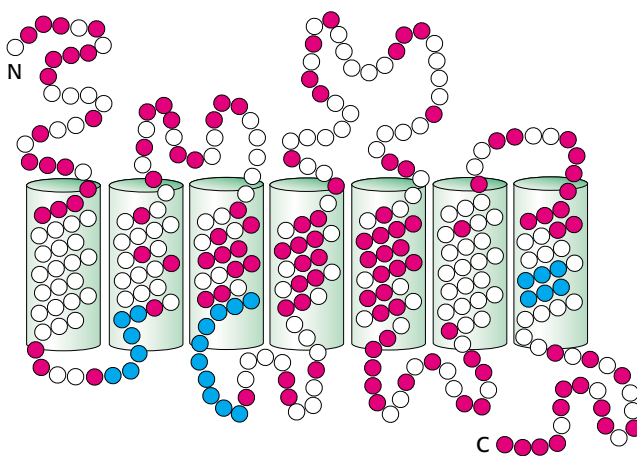
FIGURE 32.3 Evolution of odorant receptors. Odorant receptors appear to have lost function through conversion into pseudogenes in the course of primate evolution. The percentage of OR genes that appear to be functional for each species is shown in parentheses.



The odorant receptor (hereafter, OR) family is even larger than expected: *more than 1000 OR genes are present in the mouse and the rat, whereas the human genome encodes between an estimated 500 and 750 ORs.* The OR family is thus one of the largest gene families in human beings. However, more than half the human odorant receptor genes appear to be pseudogenes (Section 31.x.x); that is, they contain mutations that prevent the generation of a full-length, proper odorant receptor. In contrast, essentially all rodent OR genes are fully functional. Further analysis of primate OR genes reveals that the fraction of pseudogenes is greater in species more closely related to human beings (Figure 32.3). Thus, we may have a glimpse at the evolutionary loss of acuity in the sense of smell as higher mammals presumably became less dependent on this sense for survival.

The OR proteins are typically 20% identical in sequence to the β -adrenergic receptor (Section 15.x.x) and from 30 to 60% identical with each other. Several specific sequence features are present in most or all OR family members (Figure 32.4). The central region, particularly transmembrane helices 4 and 5, is highly variable, suggesting that this region is the site of odorant binding. That site must be different in odorant receptors that bind distinct odorant molecules.

FIGURE 32.4 Conserved and variant regions in odorant receptors. Odorant receptors are members of the 7TM receptor family. The green cylinders represent the seven presumed transmembrane helices. Strongly conserved residues characteristic of this protein family are shown in blue, whereas highly variable residues are shown in red.



What is the relation between OR gene expression and the individual neuron? Interestingly, *each olfactory neuron expresses only a single OR gene, among hundreds available.* Apparently, the precise OR gene expressed is determined largely at random. The mechanism by which all other OR genes are excluded from expression remains to be elucidated. The binding of an odorant to an OR on the neuronal surface initiates a signal-transduction cascade that results in an action potential (Figure 32.5). The ligand-bound OR activates $G_{(olf)}$, the specific G protein mentioned earlier. $G_{(olf)}$ is initially in its GDP-bound form. When activated, it releases GDP, binds GTP, and releases its associated $\beta\gamma$ subunits. The α subunit then activates a specific adenylate cyclase, increasing the intracellular concentration of cAMP. The rise in the intracellular concentration of cAMP activates a nonspecific cation channel that allows calcium and other cations into the cell. The flow of cations through the channel depolarizes the neuronal membrane and initiates an action potential. This action potential, combined with those from other olfactory neurons, leads to the perception of a specific odor.



FIGURE 32.8 Converging olfactory neurons. This section of the nasal cavity is stained to reveal processes from sensory neurons expressing the same olfactory receptor. The processes converge to a single location in the olfactory bulb.

[From P. Mombaerts, F. Wang, C. Dulac, S. K. Chao, A. Nemes, M. Mendelsohn, J. Edmondson, and R. Axel. *Cell* 87(1996):675–689.]



FIGURE 32.9 The Cyranose 320. The electronic nose may find uses in the food industry, animal husbandry, law enforcement, and medicine. [Courtesy of Cyrano Sciences.]

receptors. *Almost every odorant activates a number of receptors* (usually to different extents) and *almost every receptor is activated by more than one odorant*. Note, however, that each odorant activates a unique combination of receptors. In principle, this combinatorial mechanism allows even a relatively small array of receptors to distinguish a vast number of odorants.

How is the information about which receptors have been activated transmitted to the brain? Recall that each neuron expresses only one OR and that the pattern of expression appears to be largely random. A substantial clue to the connections between receptors and the brain has been provided by the creation of mice that express a gene for an easily detectable colored marker in conjunction with a specific OR gene. Olfactory neurons that express the OR–marker protein combination were traced to their destination in the brain, a structure called the olfactory bulb (Figure 32.8). The processes from neurons that express the same OR gene were found to connect to the same location in the olfactory bulb. Moreover, this pattern of neuronal connection was found to be identical in all mice examined. Thus, *neurons that express specific ORs are linked with specific sites in the brain*. This property creates a spatial map of odorant-responsive neuronal activity within the olfactory bulb.

Can such a combinatorial mechanism truly distinguish many different odorants? An “electronic nose” that functions by the same principles provides compelling evidence that it can (Figure 32.9). The receptors for the electronic nose are polymers that bind a range of small molecules. Each polymer binds every odorant, but to varying degrees. Importantly, the electrical properties of these polymers change on odorant binding. A set of 32 of these polymer sensors, wired together so that the pattern of responses can be evaluated, is capable of distinguishing individual compounds such as *n*-pentane and *n*-hexane as well as complex mixtures such as the odors of fresh and spoiled fruit.

32.1.3 Functional Magnetic Resonance Imaging Reveals Regions of the Brain Processing Sensory Information

Can we extend our understanding of how odorants are perceived to events in the brain? Biochemistry has provided the basis for powerful methods for examining responses within the brain. One method, called *functional magnetic resonance imaging* (fMRI), takes advantage of two key observations. The first is that, when a specific part of the brain is active, blood vessels relax to allow more blood flow to the active region. Thus, a more active region of the brain will be richer in oxyhemoglobin. The second observation is that the iron center in hemoglobin undergoes substantial structural changes on binding oxygen (Section 10.4.1). These changes are associated with a rearrangement of electrons such that the iron in deoxyhemoglobin acts as a strong magnet, whereas the iron in oxyhemoglobin does not. The difference between the magnetic properties of these two forms of hemoglobin can be used to image brain activity.

Nuclear magnetic resonance techniques (Section 4.5.1) detect signals that originate primarily from the protons in water molecules but are altered by the magnetic properties of hemoglobin. With the use of appropriate techniques, images can be generated that reveal differences in the relative amounts of deoxy- and oxyhemoglobin and thus the relative activity of various parts of the brain.

These noninvasive methods reveal areas of the brain that process sensory information. For example, subjects have been imaged while breathing air that either does or does not contain odorants. When odorants are pres-

ent, the fMRI technique detects an increase in the level of hemoglobin oxygenation (and, hence, brain activity) in several regions of the brain (Figure 32.10). Such regions include those in the primary olfactory cortex as well as other regions in which secondary processing of olfactory signals presumably takes place. Further analysis reveals the time course of activation of particular regions and other features. Functional MRI shows tremendous potential for mapping regions and pathways engaged in processing sensory information obtained from all the senses. Thus, *a seemingly incidental aspect of the biochemistry of hemoglobin has yielded the basis for observing the brain in action.*

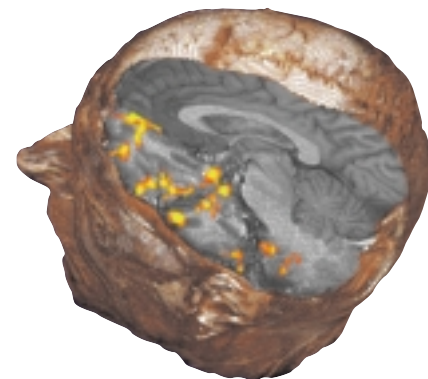


FIGURE 32.10 Brain response to odorants. A functional magnetic resonance image reveals brain response to odorants. The light spots indicate regions of the brain activated by odorants. [N. Sobel et al., *J. Neurophysiol.* 83:537–551 2000 537; courtesy of Nathan Sobel.]

32.2 TASTE IS A COMBINATION OF SENSES THAT FUNCTION BY DIFFERENT MECHANISMS

The inability to taste food is a common complaint when nasal congestion reduces the sense of smell. Thus, smell greatly augments our sense of taste (also known as *gustation*), and taste is, in many ways, the sister sense to olfaction. Nevertheless, the two senses differ from each other in several important ways. First, we are able to sense several classes of compounds by taste that we are unable to detect by smell; salt and sugar have very little odor, yet they are primary stimuli of the gustatory system. Second, whereas we are able to discriminate thousands of odorants, discrimination by taste is much more modest. Five primary tastes are perceived: *bitter*, *sweet*, *sour*, *salty*, and *umami* (the taste of glutamate from the Japanese word for “deliciousness”). These five tastes serve to classify compounds into potentially nutritive and beneficial (sweet, salty, umami) or potentially harmful or toxic (bitter, sour). Tastants (the molecules sensed by taste) are quite distinct for the different groups (Figure 32.11).

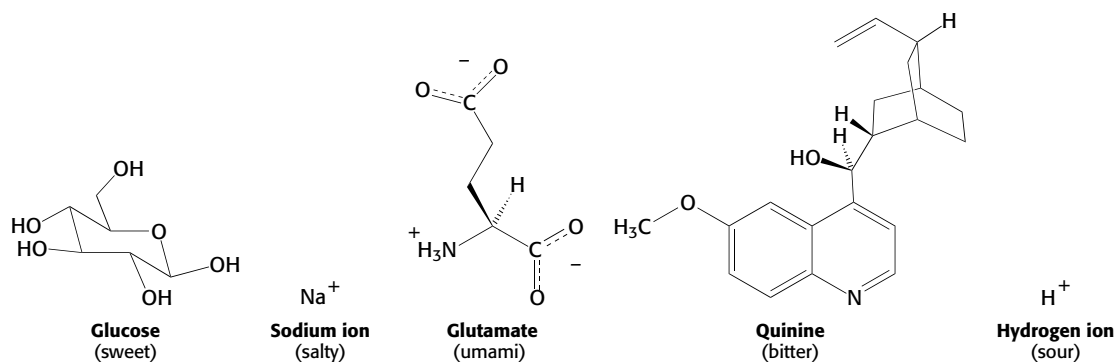


FIGURE 32.11 Examples of tastant molecules. Tastants fall into five groups: sweet, salty, umami, bitter, and sour.

The simplest tastant, the hydrogen ion, is perceived as sour. Other simple ions, particularly sodium ion, are perceived as salty. The taste called umami is evoked by the amino acid glutamate, often encountered as the flavor enhancer monosodium glutamate (MSG). In contrast, *tastants perceived as bitter or sweet are extremely diverse.* Many bitter compounds are alkaloids or other plant products of which many are toxic. However, they do not have any common structural elements or other common properties. Carbohydrates such as glucose and sucrose are perceived as sweet, as are other compounds including some simple peptide derivatives, such as aspartame, and even some proteins.

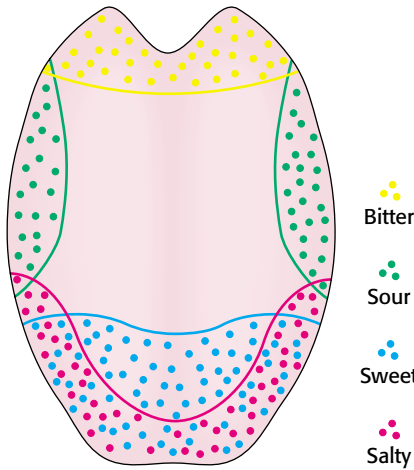
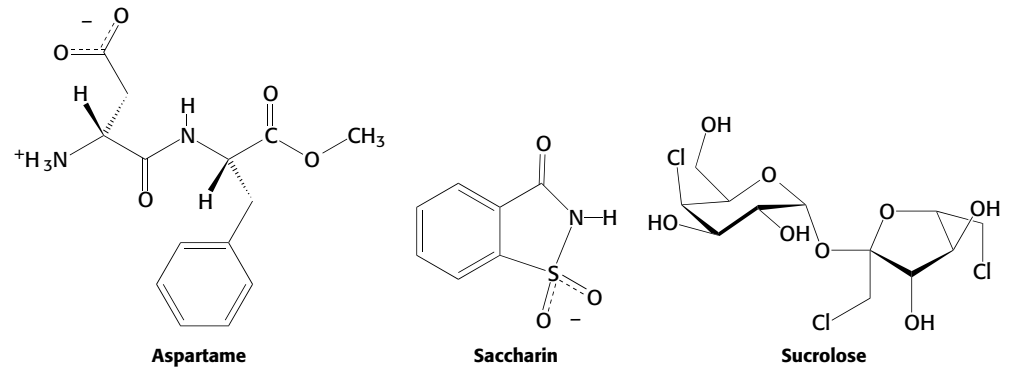


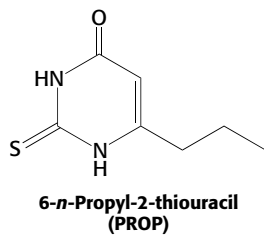
FIGURE 32.12 Taste-sensitive areas of the tongue. The tongue contains overlapping regions that are particularly sensitive to specific taste stimuli.

These differences in specificity among the five tastes are due to differences in their underlying biochemical mechanisms. The sense of taste is, in fact, a number of independent senses all utilizing the same organ, the tongue, for their expression.

Tastants are detected by specialized structures called *taste buds*, which contain approximately 150 cells, including sensory neurons. Projections called *taste papillae* contain numerous taste buds. Papillae somewhat specialized to detect different taste types are concentrated in distinct regions across the surface of the tongue. Consequently, different regions of the tongue are particularly sensitive to specific classes of tastants (Figure 32.12).

32.2.1 Sequencing the Human Genome Led to the Discovery of a Large Family of 7TM Bitter Receptors

Just as in olfaction, a number of clues pointed to the involvement of G proteins and, hence, 7TM receptors in the detection of bitter and sweet tastes. The evidence included the isolation of a specific G protein α subunit termed *gustducin*, which is expressed primarily in taste buds (Figure 32.13). How could the 7TM receptors be identified? The ability to detect some compounds depends on specific genetic loci in both human beings and mice. For instance, the ability to taste the bitter compound 6-*n*-propyl-2-thiouracil (PROP) was mapped to a region on human chromosome 5 by comparing DNA markers of persons who vary in sensitivity to this compound.



This observation suggested that this region might encode a 7TM receptor that responded to PROP. Approximately 450 kilobases in this region had been sequenced early in the human genome project. This sequence was searched by computer for potential 7TM receptor genes, and, indeed, one was detected and named *T2R-1*. Additional database searches for sequences similar to this one detected 12 genes encoding full-length receptors as well as 7 pseudogenes within the sequence of the human genome known at

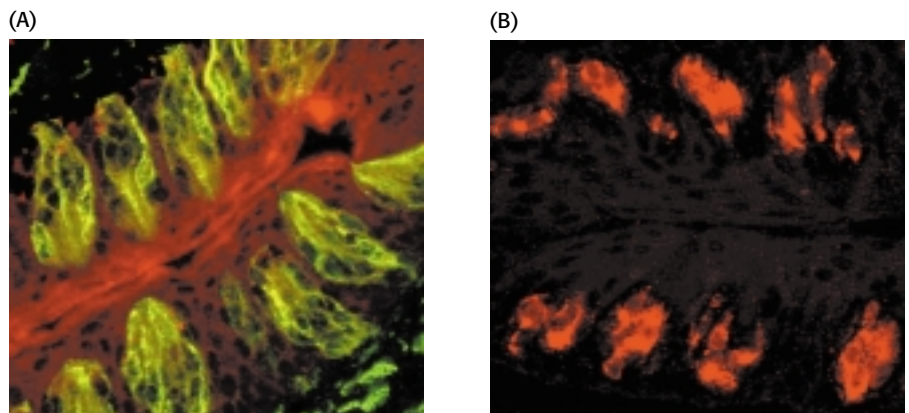


FIGURE 32.13 Expression of gustducin in the tongue. (A) A section of tongue stained with a fluorescent antibody reveals the position of the taste buds. (B) The same region stained with an antibody directed against gustducin reveals that this G protein is expressed in taste buds. [Courtesy of Charles S. Zuker.]

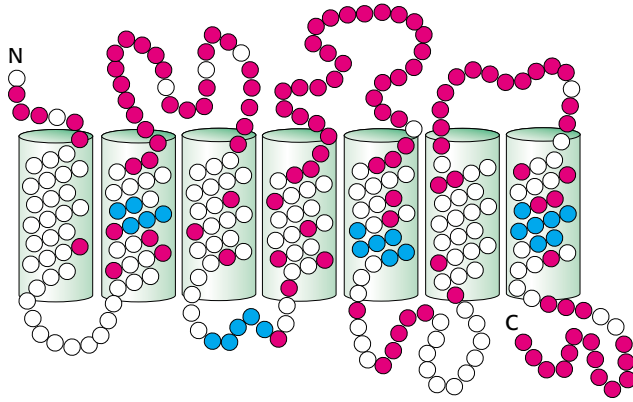


FIGURE 32.14 Conserved and variant regions in bitter receptors. The bitter receptors are members of the 7TM receptor family. Strongly conserved residues characteristic of this protein family are shown in blue, and highly variable residues are shown in red.

the time. The encoded proteins were between 30 and 70% identical with T2R-1 (Figure 32.14). Further analysis suggests that there are from 50 to 100 members of this family of 7TM receptors in the entire human genome. Similar sequences have been detected in the mouse and rat genomes.

Are these proteins, in fact, bitter receptors? Several lines of evidence suggest that they are. First, their genes are expressed in taste-sensitive cells—in fact, in many of the same cells that express gustducin. Second, cells that express individual members of this family respond to specific bitter compounds. For example, cells that express a specific mouse receptor (mT2R-5) responded when exposed specifically to cycloheximide. Third, mice that had been found unresponsive to cycloheximide were found to have point mutations in the gene encoding mT2R-5. Finally, cycloheximide specifically stimulates the binding of GTP analogs to gustducin in the presence of the mT2R-5 protein (Figure 32.15).

Importantly, each taste receptor cell expresses many different members of the T2R family. This pattern of expression stands in sharp contrast to the pattern of one receptor type per cell that characterizes the olfactory system (Figure 32.16). The difference in expression patterns accounts for the much greater specificity of our perceptions of smells compared with tastes. *We are able to distinguish among subtly different odors because each odorant stimulates*

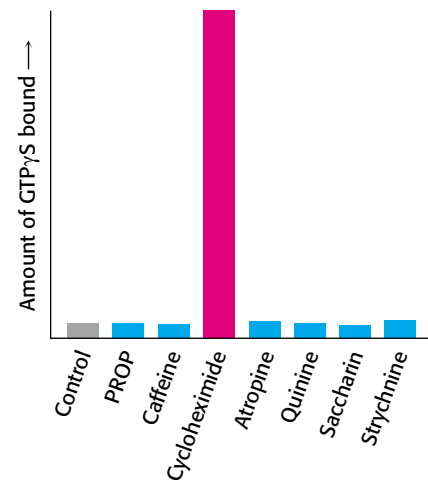


FIGURE 32.15 Evidence that T2R proteins are bitter taste receptors. Cycloheximide uniquely stimulates the binding of the GTP analog GTP γ S to gustducin in the presence of the mT2R protein. [Adapted from J. Chandrashekar, K. L. Mueller, M. A. Hoon, E. Adler, L. Feng, W. Guo, C. S. Zuker, and N. J. Ryba. *Cell* 100(2000):703.]

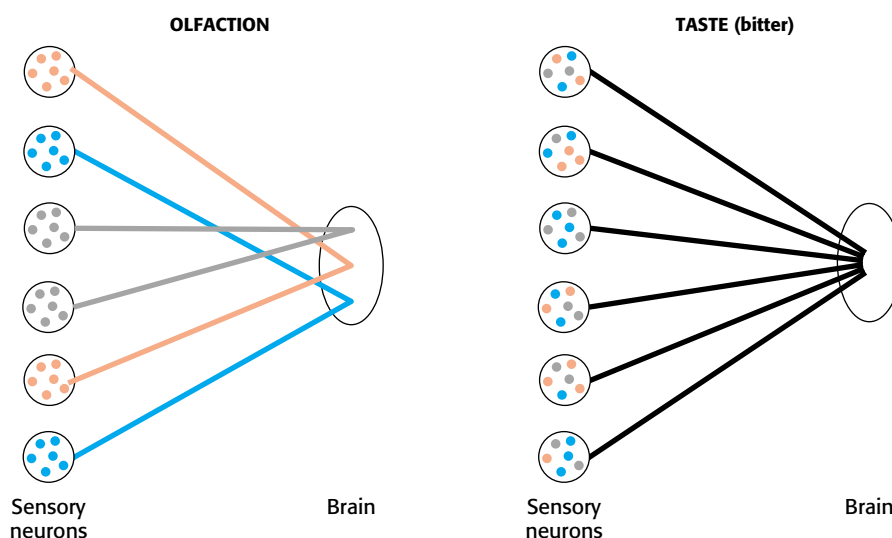


FIGURE 32.16 Differing gene expression and connection patterns in olfactory and bitter taste receptors. In olfaction, each neuron expresses a single OR gene, and the neurons expressing the same OR converge to specific sites in the brain, enabling specific perception of different odorants. In gustation, each neuron expresses many bitter receptor genes, so the identity of the tastant is lost in transmission.

a unique pattern of neurons. In contrast, many tastants stimulate the same neurons. Thus, we perceive only “bitter” without the ability to discriminate cycloheximide from quinine.

32.2.2 A Family of 7TM Receptors Almost Certainly Respond to Sweet Compounds

Most sweet compounds are carbohydrates, energy rich and easily digestible. However, as noted in Chapter 11, carbohydrates are structurally diverse. Moreover, some noncarbohydrate compounds such as aspartame also taste sweet. The structural diversity among sweet-tasting compounds, though less than that among bitter compounds, strongly suggests that a family of receptors detects these compounds. The *sweet receptors are very likely coupled to the G protein gustducin and, hence, are members of the 7TM superfamily*. This likelihood was most sharply revealed in studies that disrupted the gene for gustducin in mice. These mice lost much of their ability to sense both bitter and sweet compounds. Genes for candidate receptors have been cloned but have not yet been proved to be functional sweet receptors.

32.2.3 Salty Tastes Are Detected Primarily by the Passage of Sodium Ions Through Channels

Salty tastants are not detected by 7TM receptors. Rather, they are detected directly by their passage through ion channels expressed on the surface of cells in the tongue. Evidence for the role of these ion channels comes from examining known properties of sodium channels characterized in other biological contexts. One class of channels, characterized first for their role in salt reabsorption, are thought to be important in salt taste detection because they are sensitive to the compound *amiloride*, which mutes the taste of salt and significantly lowers sensory neuron activation in response to sodium.

An *amiloride-sensitive sodium channel* comprises four subunits that may be either identical or distinct but in any case are homologous. An individual subunit ranges in length from 500 to 1000 amino acids and includes two presumed membrane-spanning helices as well as a large extracellular domain in between them (Figure 32.17). The extracellular region includes two (or, sometimes, three) distinct regions rich in cysteine residues (and, presumably, disulfide bonds). A region just ahead of the second membrane-spanning helix appears to form part of the pore in a manner analogous to the structurally characterized potassium channel (Section 13.5.6). The members of the amiloride-sensitive sodium-channel family are numerous and diverse in their biological roles. We shall encounter them again in the context of the sense of touch.

Sodium ions passing through these channels produce a significant transmembrane current. Amiloride blocks this current, accounting for its effect on taste. However, about 20% of the response to sodium remains even in the presence of amiloride, suggesting that other ion channels also contribute to salt detection.

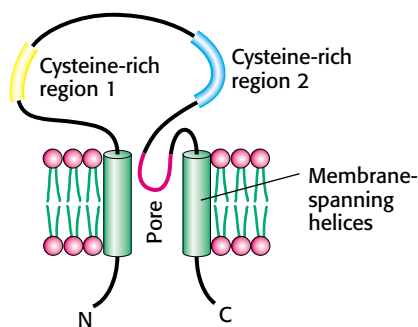
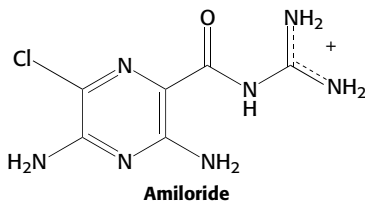


FIGURE 32.17 Schematic structure of the amiloride-sensitive sodium channel. Only one of the four subunits that constitute the functional channel is illustrated. The amiloride-sensitive sodium channel belongs to a superfamily having common structural features, including two hydrophobic membrane-spanning regions, intracellular amino and carboxyl termini; and a large, extracellular region with conserved cysteine-rich domains.


32.2.4 Sour Tastes Arise from the Effects of Hydrogen Ions (Acids) on Channels

Like salty tastes, *sour tastes are also detected by direct interactions with ion channels*, but the incoming ions are hydrogen ions (in high concentrations) rather than sodium ions. For example, in the absence of high concentrations of sodium, hydrogen ion flow can induce substantial transmembrane currents through amiloride-sensitive sodium channels. However, hydrogen ions are also sensed by mechanisms other than their direct passage through

membranes. Binding by hydrogen ions blocks some potassium channels and activates other types of channels. Together, these mechanisms lead to changes in membrane polarization in sensory neurons that produce the sensation of sour taste.

32.2.5 Umami, the Taste of Glutamate, Is Detected by a Specialized Form of Glutamate Receptor

Glutamate is an abundant amino acid that is present in protein-rich foods as well as in the widely used flavor enhancer monosodium glutamate. This amino acid has a taste, termed *umami*, that is distinct from the other four basic tastes. Adults can detect glutamate at a concentration of approximately 1 mM. Glutamate is also a widely used neurotransmitter, and thus, not surprisingly, several classes of receptors for glutamate have been identified in the nervous system. One class, called *metabotropic glutamate receptors*, are 7TM receptors with large amino-terminal domains of approximately 600 amino acids. Sequence analysis reveals that the first half of the amino-terminal region is most likely a ligand-binding domain, because it is homologous to such domains found in the Lac repressor (Section 31.x.x) and other bacterial ligand-binding proteins.

 One glutamate receptor gene, encoding a protein called the metabotropic glutamate receptor 4 (mGluR4), has been found to be expressed in taste buds. Further analysis of the mRNA that is expressed in taste buds reveals that this mRNA lacks the region encoding the first 309 amino acids in brain mGluR4, which includes most of the high-affinity glutamate-binding domain (Figure 32.18). The glutamate receptor found in taste buds shows a lowered affinity for glutamate that is appropriate to glutamate levels in the diet. Thus, *the receptor responsible for the perception of glutamate taste appears to have evolved simply by changes in the expression of an existing glutamate-receptor gene.* We shall consider an additional receptor related to taste, that responsible for the “hot” taste of spicy food, when we deal with mechanisms of touch perception.

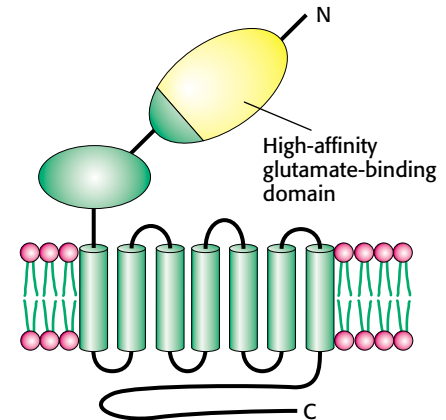


FIGURE 32.18 Schematic structure of a metabotropic glutamate receptor. The umami receptor is a variant of a brain glutamate receptor. A substantial part of the high-affinity glutamate-binding domain (shown in yellow) is missing in the form expressed in the tongue.

32.3 PHOTORECEPTOR MOLECULES IN THE EYE DETECT VISIBLE LIGHT

Vision is based on the absorption of light by photoreceptor cells in the eye. These cells are sensitive to light in a relatively narrow region of the electromagnetic spectrum, the region with wavelengths between 300 and 850 nm (Figure 32.19). Vertebrates have two kinds of photoreceptor cells, called *rods* and *cones* because of their distinctive shapes. Cones function in bright light and are responsible for color vision, whereas rods function in dim light but do not perceive color. A human retina contains about 3 million cones and 100 million rods. Remarkably, a rod cell can respond to a single photon, and the brain requires fewer than 10 such responses to register the sensation of a flash of light.

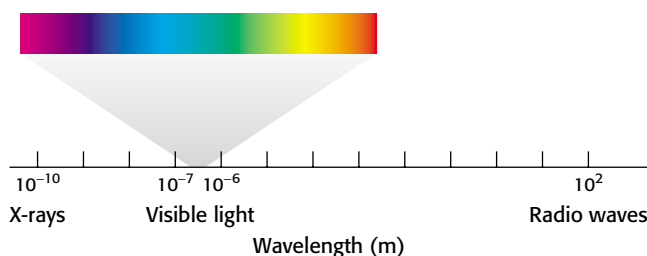
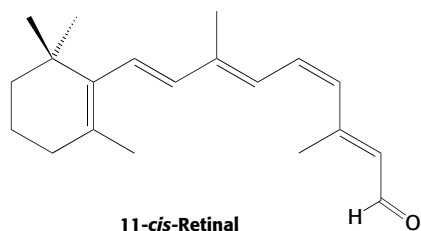


FIGURE 32.19 The electromagnetic spectrum. Visible light has wavelengths between 300 and 850 nanometers.



32.3.1 Rhodopsin, a Specialized 7TM Receptor, Absorbs Visible Light

Rods are slender elongated structures; the outer segment is specialized for photoreception (Figure 32.20). It contains a stack of about 1000 discs, which are membrane-enclosed sacs densely packed with photoreceptor molecules. The photosensitive molecule is often called a *visual pigment* because it is highly colored owing to its ability to absorb light. The photoreceptor molecule in rods is *rhodopsin* (Section 15.x.x), which consists of the protein *opsin* linked to 11-*cis*-retinal, a prosthetic group.

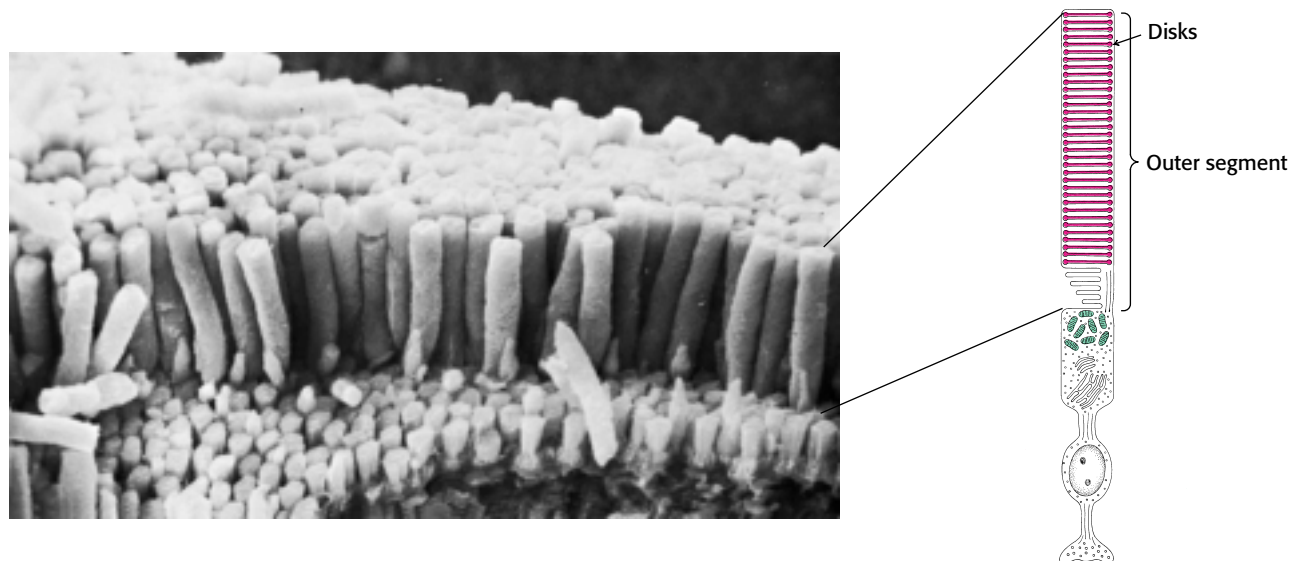


FIGURE 32.20 The rod cell. (Left) Scanning electron micrograph of retinal rod cells. (Right) Schematic representation of a rod cell. [Photograph courtesy of Dr. Deric Bownds.]

Rhodopsin absorbs light very efficiently in the middle of the visible spectrum, its absorption being centered on 500 nm, which nicely matches the solar output (Figure 32.21). A rhodopsin molecule will absorb a high percentage of the photons of the correct wavelength that strike it, as indicated by the extinction coefficient of $40,000 \text{ M}^{-1} \text{ cm}^{-1}$ at 500 nm. The extinction coefficient for rhodopsin is more than an order of magnitude greater than that for tryptophan, the most efficient absorber in proteins that lack prosthetic groups.

Opsin, the protein component of rhodopsin, is a member of the 7TM receptor family. Indeed, rhodopsin was the first member of this family to be purified, its gene was the first to be cloned and sequenced, and its three-dimensional structure was the first to be determined. The color of rhodopsin and its responsiveness to light depend on the presence of the light-absorbing group (*chromophore*) 11-*cis*-retinal. This compound is a powerful absorber of light because it is a polyene; its six alternating single and double bonds constitute a long, unsaturated electron network. Recall that alternating single and double bonds account for the chromophoric properties of chlorophyll (Section 20.x.x). The aldehyde group of 11-*cis*-retinal forms a Schiff base (Figure 32.22) with the ϵ -amino group of lysine residue 296, which lies in the center of the seventh transmembrane helix. Free retinal absorbs maximally at 370 nm, and its unprotonated Schiff-base adduct absorbs at 380 nm, whereas the protonated Schiff base absorbs at 440 nm or longer wavelengths. Thus, the 500-nm absorption maximum for rhodopsin strongly suggests that the Schiff base is protonated; additional interactions

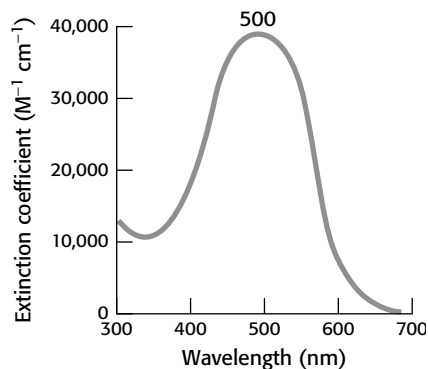


FIGURE 32.21 Rhodopsin absorption spectrum.

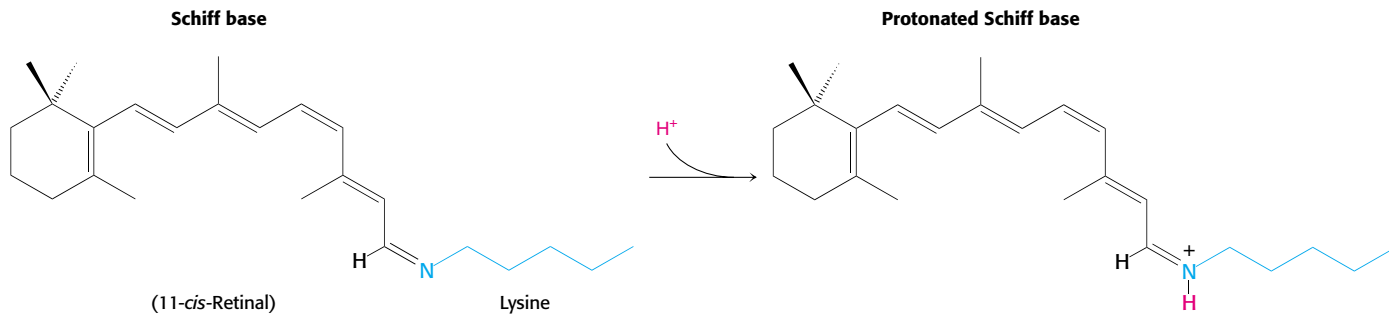


FIGURE 32.22 Retinal–lysine linkage. Retinal is linked to lysine 296 in opsin by a Schiff-base linkage. In the resting state of rhodopsin, this Schiff base is protonated.

with opsin shift the absorption maximum farther toward the red. The positive charge of the protonated Schiff base is compensated by the negative charge of glutamate 113 located in helix 2; the glutamate residue closely approaches the lysine–retinal linkage in the three-dimensional structure of rhodopsin.



STRUCTURAL INSIGHTS, Rhodopsin: A G Protein Coupled 7TM Receptor

offers a more detailed look at rhodopsin structure and function (Figure 32.5).

32.3.2 Light Absorption Induces a Specific Isomerization of Bound 11-*cis*-Retinal

How does the absorption of light by the retinal Schiff base generate a signal? George Wald and his coworkers discovered that *light absorption results in the isomerization of the 11-*cis*-retinal group of rhodopsin to its all-*trans* form* (Figure 32.23). This isomerization causes the Schiff-base nitrogen atom to move approximately 5 Å, assuming that the cyclohexane ring of the retinal group remains fixed. In essence, *the light energy of a photon is converted into atomic motion*. The change in atomic positions, like the binding of a ligand to other 7TM receptors, sets in train a series of events that lead to the closing of ion channels and the generation of a nerve impulse.

The isomerization of the retinal Schiff base takes place within a few picoseconds of a photon being absorbed. The initial product, termed *bathorhodopsin*, contains a strained all-*trans*-retinal group. Within approximately 1 millisecond, this intermediate is converted through several additional intermediates into *metarhodopsin II*. In *metarhodopsin II*, the Schiff base is deprotonated and the opsin protein has undergone significant reorganization.

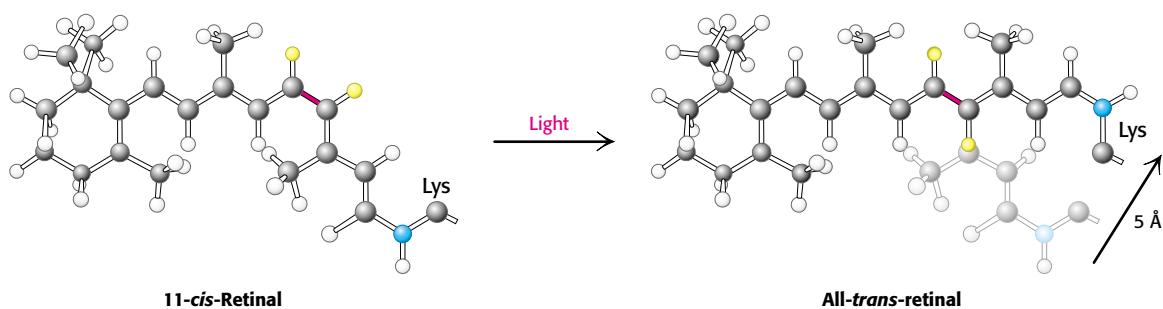


FIGURE 32.23 Atomic motion in retinal. The Schiff-base nitrogen atom moves 5 Å as a consequence of the light-induced isomerization of 11-*cis*-retinal to all-*trans*-retinal by rotation about the bond shown in red.

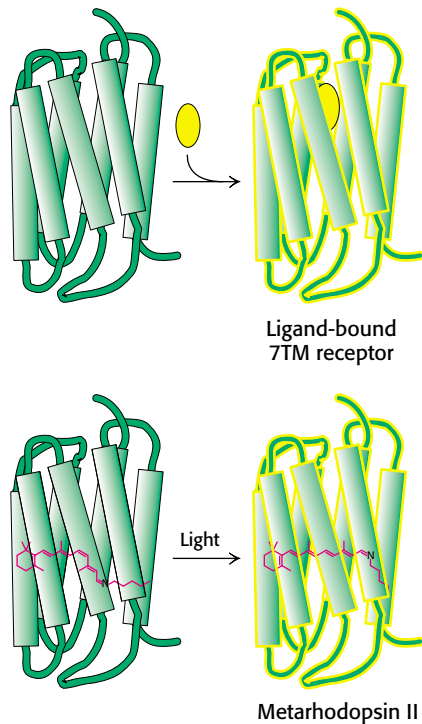


FIGURE 32.24 Analogous 7TM receptors. The conversion of rhodopsin into metarhodopsin II activates a signal-transduction pathway analogously to the activation induced by the binding of other 7TM receptors to appropriate ligands.

Metarhodopsin II (also referred to as R^*) is analogous to the ligand-bound state of 7TM receptors such as the β_2 -adrenergic receptor (Section 15.x.x) and the odorant and tastant receptors heretofore discussed (Figure 32.24). Like these receptors, this form of rhodopsin activates a heterotrimeric G protein that propagates the signal. The G protein associated with rhodopsin is called *transducin*. Metarhodopsin II triggers the exchange of GDP for GTP by the α subunit of transducin (Figure 32.25). On the binding of GTP, the $\beta\gamma$ subunits of transducin are released and the α subunit switches on a *cGMP phosphodiesterase* by binding to and removing an inhibitory subunit. The activated phosphodiesterase is a potent enzyme that rapidly hydrolyzes cGMP to GMP. The reduction in cGMP concentration causes cGMP-gated ion channels to close, leading to hyperpolarization of the membrane and neuronal signaling. *At each step in this process, the initial signal—the absorption of a single photon—is amplified so that it leads to sufficient membrane hyperpolarization to result in signaling.*

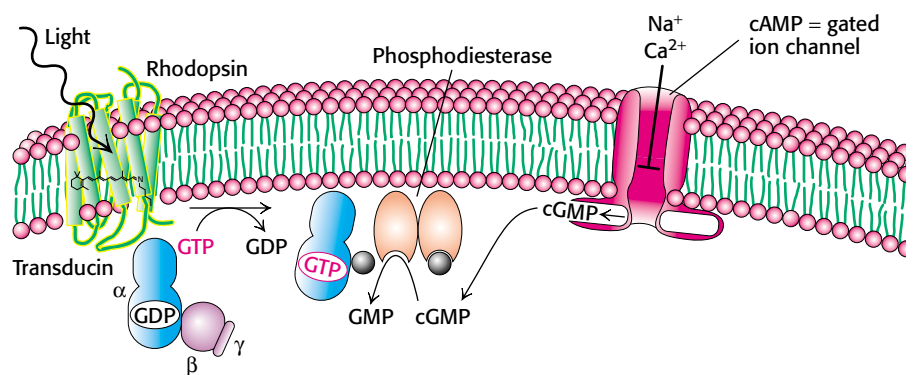


FIGURE 32.25 Visual signal transduction. The light-induced activation of rhodopsin leads to the hydrolysis of cGMP, which in turn leads to ion channel closing and the initiation of an action potential.

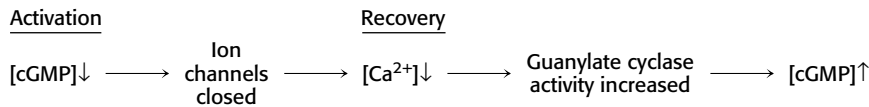
CONCEPTUAL INSIGHTS, Signaling Pathways: Response and Recovery presents an animated version of Figure 32.25 and a comparison to olfactory signal transduction (Figure 32.5).

32.3.3 Light-Induced Lowering of the Calcium Level Coordinates Recovery

As we have seen, the visual system responds to changes in light and color within a few milliseconds, quickly enough that we are able to perceive continuous motion at nearly 1000 frames per second. To achieve a rapid response, the signal must also be terminated rapidly and the system must be returned to its initial state. First, activated rhodopsin must be blocked from continuing to activate transducin. *Rhodopsin kinase* catalyzes the phosphorylation of the carboxyl terminus of R^* at multiple serine and threonine residues. *Arrestin*, an inhibitory protein (Section 15.x.x), then binds phosphorylated R^* and prevents additional interaction with transducin.

Second, the α subunit of transducin must be returned to its inactive state to prevent further signaling. Like other G proteins, the α subunit possesses built-in GTPase activity that hydrolyzes bound GTP to GDP. Hydrolysis takes place in less than a second when transducin is bound to the phosphodiesterase. The GDP form of transducin then leaves the phosphodiesterase and reassociates with the $\beta\gamma$ subunits, and the phosphodiesterase returns to its inactive state. Third, the level of cGMP must be raised to reopen the cGMP-gated ion channels. *The action of guanylate cyclase accomplishes this third step by synthesizing cGMP from GTP.*

Calcium ion plays an essential role in controlling guanylate cyclase because it markedly inhibits the activity of the enzyme. In the dark, Ca^{2+} as well as Na^+ enter the rod outer segment through the cGMP-gated channels. Calcium ion influx is balanced by its efflux through an exchanger, a transport system that uses the thermodynamically favorable flow of four Na^+ ions into the cell and one K^+ ion out of the cell to extrude one Ca^{2+} ion. After illumination, the entry of Ca^{2+} through the cGMP-gated channels stops, but its export through the exchanger continues. Thus, the cytosolic Ca^{2+} level drops from 500 nM to 50 nM after illumination. This drop markedly stimulates guanylate cyclase, rapidly restoring the concentration of cGMP to reopen the cGMP-gated channels.



By controlling the rate of cGMP synthesis, Ca^{2+} levels govern the speed with which the system is restored to its initial state.

32.3.4 Color Vision Is Mediated by Three Cone Receptors That Are Homologs of Rhodopsin

Cone cells, like rod cells, contain visual pigments. Like rhodopsin, these photoreceptor proteins are members of the 7TM receptor family and utilize 11-*cis*-retinal as their chromophore. In human cone cells, there are three distinct photoreceptor proteins with absorption maxima at 426, 530, and ~560 nm (Figure 32.26). These absorbances correspond to (in fact, define) the blue, green, and red regions of the spectrum. Recall that the absorption maximum for rhodopsin is 500 nm.

The amino acid sequences of the cone photoreceptors have been compared with each other and with rhodopsin. The result is striking. Each of the cone photoreceptors is approximately 40% identical in sequence with rhodopsin. Similarly, the blue photoreceptor is 40% identical with each of the green and red photoreceptors. The green and red photoreceptors, however, are > 95% identical with each other, differing in only 15 of 364 positions (Figure 32.27).

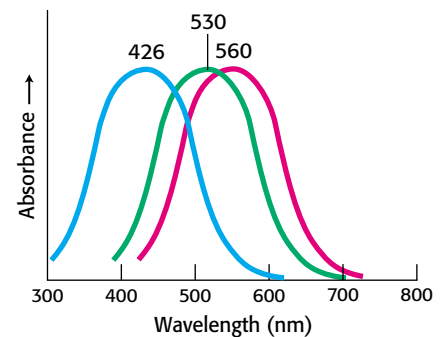


FIGURE 32.26 Cone-pigment absorption spectra. The absorption spectra of the cone visual pigment responsible for color vision.

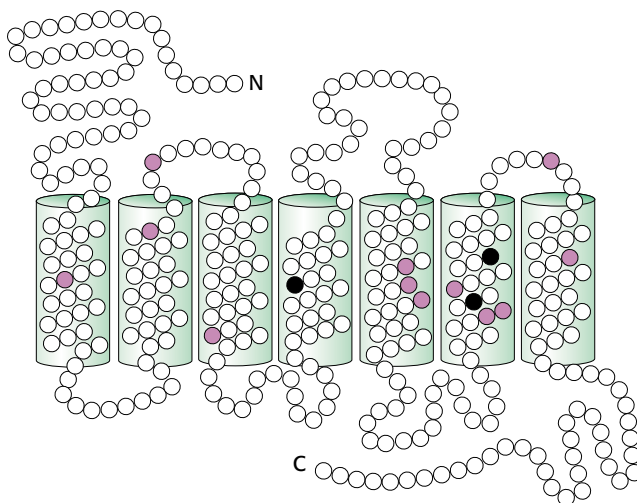
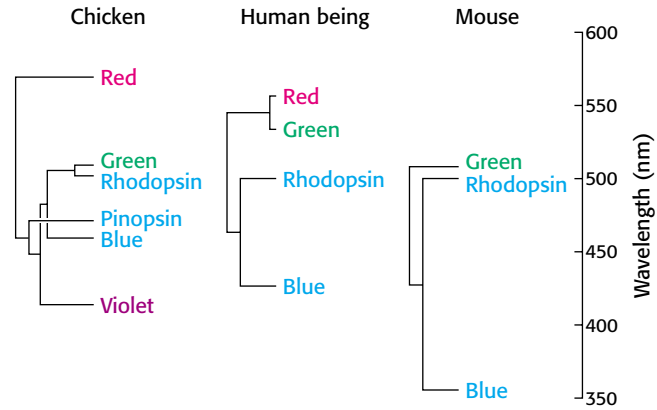


FIGURE 32.27 Comparison of the amino acid sequences of the green and red photoreceptors. Open circles correspond to identical residues, whereas colored circles mark residues that are different. The differences in the three black positions are responsible for most of the difference in their absorption spectra.

FIGURE 32.28 Evolutionary relationships among visual pigments. Visual pigments have evolved by gene duplication along different branches of the animal evolutionary tree. The branch lengths of the “trees” correspond to the percentage of amino acid divergence. [Adapted from Nathans, J. *Neuron* 24(1999):299; by permission of Cell Press.]



These observations are sources of insight into photoreceptor evolution. First, the green and red photoreceptors are clearly products of a recent evolutionary event (Figure 32.28). The green and red pigments appear to have diverged in the primate lineage approximately 35 million years ago. Mammals, such as dogs and mice, that diverged from primates earlier have only two cone photoreceptors, blue and green. They are not sensitive to light as far toward the infrared region as we are, and they do not discriminate colors as well. In contrast, birds such as chickens have a total of six pigments: rhodopsin, four cone pigments, and a pineal visual pigment called *pinopsin*. Birds have highly acute color perception.

Second, the high level of similarity between the green and red pigments has made it possible to identify the specific amino acid residues that are responsible for spectral tuning. Three residues (at positions 180, 277, and 285) are responsible for most of the difference between the green and red pigments. In the green pigment, these residues are alanine, phenylalanine, and alanine, respectively; in the red pigment, they are serine, tyrosine, and threonine. A hydroxyl group has been added to each amino acid in the red pigment. The hydroxyl groups can interact with the photoexcited state of retinal and lower its energy, leading to a shift toward the lower-energy (red) region of the spectrum.



STRUCTURAL INSIGHTS, Rhodopsin: A G Protein Coupled 7TM Receptor
explores the structural basis of color vision and night blindness in more detail.

32.3.5 Rearrangements in the Genes for the Green and Red Pigments Lead to “Color Blindness”



The genes for the green and red pigments lie adjacent to each other on the human X chromosome. These genes are more than 98% identical in nucleotide sequence, including introns and untranslated regions as well as the protein-coding region. Regions with such high similarity are very susceptible to unequal homologous recombination.

Recombination can take place either between or within transcribed regions of the gene (Figure 32.29). If recombination takes place between transcribed regions, the product chromosomes will differ in the number of pigment genes that they carry. One chromosome will lose a gene and thus may lack the gene for, say, the green pigment; the other chromosome will gain a gene. Consistent with this scenario, approximately 2% of human X chromosomes carry only a single color pigment gene, approximately 20% carry two, 50% carry three, 20% carry four, and 5% carry five or more. A person lacking the gene for the green pigment will have trouble distinguishing red and green color, characteristic of the most common form of color blindness.

Homologous recombination—

The exchange of DNA segments at equivalent positions between chromosomes with substantial sequence similarity.

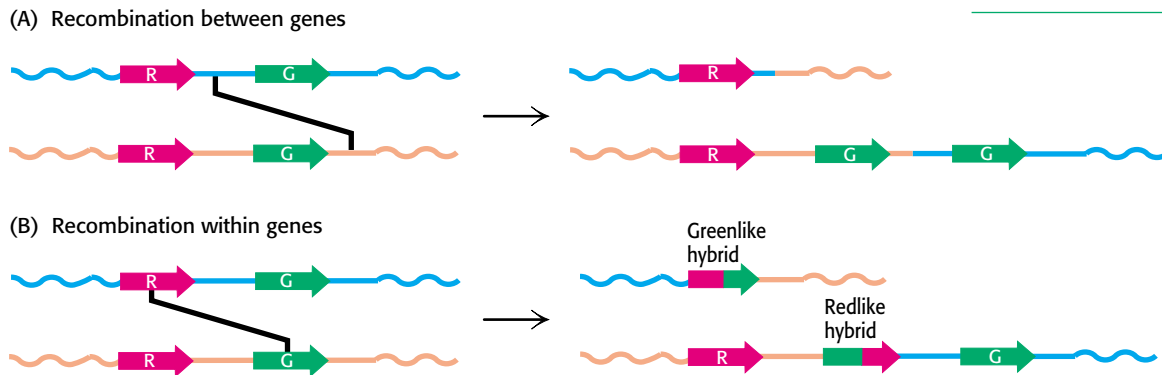


FIGURE 32.29 Recombination pathways leading to color blindness. Rearrangements in the course of DNA replication may lead to (A) the loss of visual pigment genes or (B) the formation of hybrid pigment genes that encode photoreceptors with anomalous absorption spectra. Because the amino acids most important for determining absorption spectra are in the carboxyl-terminal half of each photoreceptor protein, the part of the gene that encodes this region most strongly affects the absorption characteristics of hybrid receptors. [Adapted from J. Nathans. *Neuron* 24(1999):299–312; by permission of Cell Press.]

Approximately 5% of males have this form of color blindness. Recombination can also take place within the transcription units, resulting in genes that encode hybrids of the green and red photoreceptors. The absorption maximum of such a hybrid lies between that of the red and green pigments. A person with such hybrid genes who also lacks either a functional red or a functional green pigment gene does not discriminate color well.

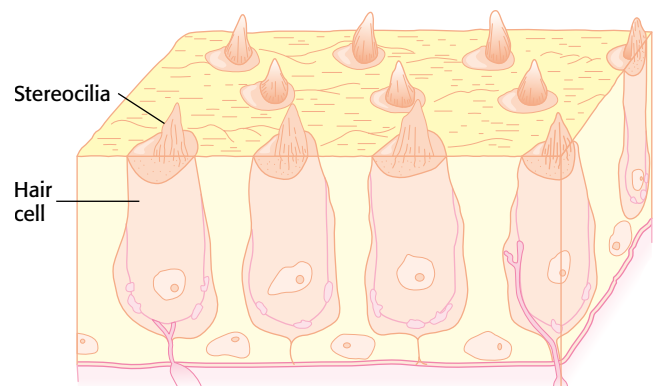
32.4 HEARING DEPENDS ON THE SPEEDY DETECTION OF MECHANICAL STIMULI

Hearing and touch are based on the detection of mechanical stimuli. Although the proteins of these senses have not been as well characterized as those of the senses already discussed, anatomical, physiological, and biophysical studies have elucidated the fundamental processes. *A major clue to the mechanism of hearing is its speed.* We hear frequencies ranging from 200 to 20,000 Hz (cycles per second), corresponding to times of 5 to 0.05 ms. Furthermore, our ability to locate sound sources, one of the most important functions of hearing, depends on the ability to detect the time delay between the arrival of a sound at one ear and its arrival at the other. Given the separation of our ears and the speed of sound, we must be able to accurately sense time differences of 0.7 ms. In fact, human beings can locate sound sources associated with temporal delays as short as 0.02 ms. This high time resolution implies that hearing must employ direct transduction mechanisms that do not depend on second messengers. Recall that, in vision, for which speed also is important, the signal-transduction processes take place in milliseconds.

32.4.1 Hair Cells Use a Connected Bundle of Stereocilia to Detect Tiny Motions

Sound waves are detected inside the cochlea of the inner ear. The *cochlea* is a fluid-filled, membranous sac that is coiled like a snail shell. The primary detection is accomplished by specialized neurons inside the cochlea called *hair cells* (Figure 32.30). Each cochlea contains

FIGURE 32.30 Hair cells, the sensory neurons crucial for hearing. [Adapted from Hudspeth, A. J. *Nature* 341(1989):397.]



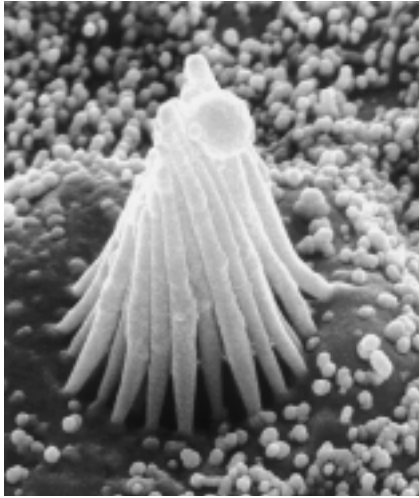


FIGURE 32.31 An electron micrograph of a hair bundle. [Courtesy of A. Jacobs and A. J. Hudspeth.]

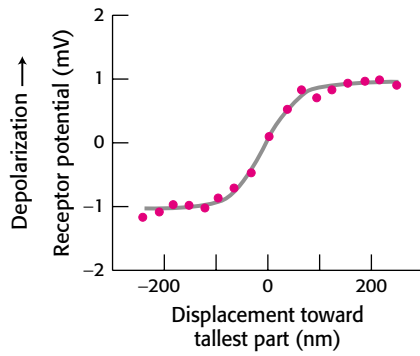


FIGURE 32.32 Micromanipulation of a hair cell. Movement toward the tallest part of the bundle depolarizes the cell as measured by the microelectrode. Movement toward the shortest part hyperpolarizes the cell. Lateral movement has no effect. [Adapted from Hudspeth, A. J. *Nature* 341(1989):397.]

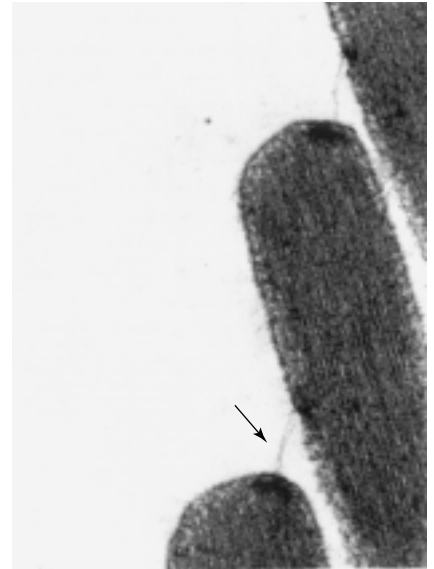


FIGURE 32.33 Electron micrograph of tip links. The tip link between two hair fibers is marked by an arrow. [Courtesy of A. Jacobs and A. J. Hudspeth.]

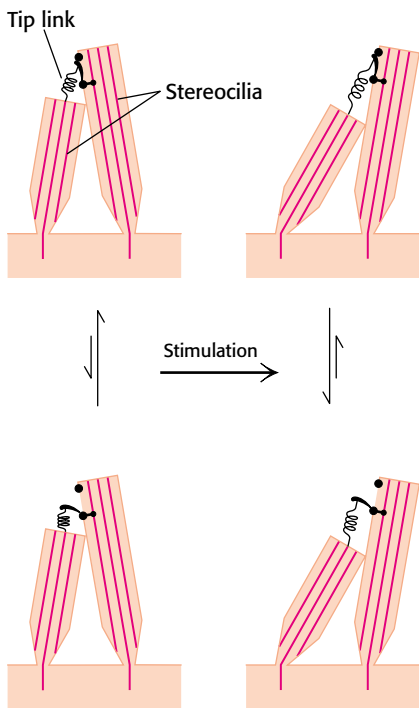


FIGURE 32.34 Model for hair-cell transduction. When the hair bundle is tipped toward the tallest part, the tip link pulls on and opens an ion channel. Movement in the opposite direction relaxes the tension in the tip link, increasing the probability that any open channels will close. [Adapted from A. J. Hudspeth. *Nature* 341(1989):397.]

approximately 16,000 hair cells, and each hair cell contains a hexagonally shaped bundle of 20 to 300 hairlike projections called *stereocilia* (Figure 32.31). These stereocilia are graded in length across the bundle. Mechanical deflection of the hair bundle, as occurs when a sound wave arrives at the ear, creates a change in the membrane potential of the hair cell.

Micromanipulation experiments have directly probed the connection between mechanical stimulation and membrane potential. Displacement toward the direction of the tallest part of the hair bundle results in depolarization of the hair cell, whereas displacement in the opposite direction results in hyperpolarization (Figure 32.32). Motion perpendicular to the hair-length gradient does not produce any change in resting potential. Remarkably, *displacement of the hair bundle by as little as 3 Å (0.3 nm) results in a measurable (and functionally important) change in membrane potential.* This motion of 0.003 degree corresponds to a 1-inch movement of the top of the Empire State Building.

How does the motion of the hair bundle create a change in membrane potential? The rapid response, within microseconds, suggests that the movement of the hair bundle acts on ion channels directly. An important observation is that adjacent stereocilia are linked by individual filaments called *tip links* (Figure 32.33).

The presence of these tip links suggests a simple mechanical model for transduction by hair cells (Figure 32.34). The tip links are coupled to ion channels in the membranes of the stereocilia that are gated by mechanical stress. In the absence of a stimulus, approximately 15% of these channels are open. When the hair bundle is displaced toward its tallest part, the stereocilia slide across one another and the tension on the tip links increases, causing additional channels to open. The flow of ions through the newly opened channels depolarizes the membrane. Conversely, if the displacement is in the opposite direction, the tension on the tip links decreases, the open channels close, and the membrane hyperpolarizes. *Thus, the mechanical motion of the hair bundle is directly converted into current flow across the hair-cell membrane.*

32.4.2 A Candidate Mechanosensory Channel Has Been Identified in *Drosophila*

A likely ortholog of the transduction channel in hearing has been identified in fruit flies. *Drosophila* have sensory bristles used for detecting small air currents. These bristles respond to mechanical displacement in ways similar to those of hair cells; displacement of a bristle in one direction leads to substantial transmembrane current. To isolate the gene encoding the transduction channel, investigators isolated 27 different strains of mutant fruit flies that showed uncoordinated motion and clumsiness and then examined their electrophysiological responses to displacement of the sensory bristles. In one set of strains, transmembrane currents were dramatically reduced. The mutated gene in these strains was found to encode a protein of 1619 amino acids, called NompC for *no mechanoreceptor potential*.

The carboxyl-terminal 469 amino acids of NompC resemble a class of ion channel proteins called TRP (transient receptor potential) channels. This region includes six putative transmembrane helices with a porelike region between the fifth and sixth helices. The amino-terminal 1150 amino acids consist almost exclusively of 29 *ankyrin repeats* (Figure 32.35). Ankyrin repeats are structural motifs formed by 33 amino acids folded into a hairpin loop followed by a helix-turn-helix. Importantly, in other proteins, regions with tandem arrays of these motifs mediate protein–protein interactions. Although the role of the ankyrin repeats in NompC has not yet been established, they likely mediate interactions with other components to couple mechanical motion to conformational changes in the channel. The *C. elegans* genome encodes an orthologous protein that also is expressed in mechanosensory neurons. Given the mechanistic parallels, homologous sequences from the human genome are strong candidates for genes encoding the transduction channel in hearing.

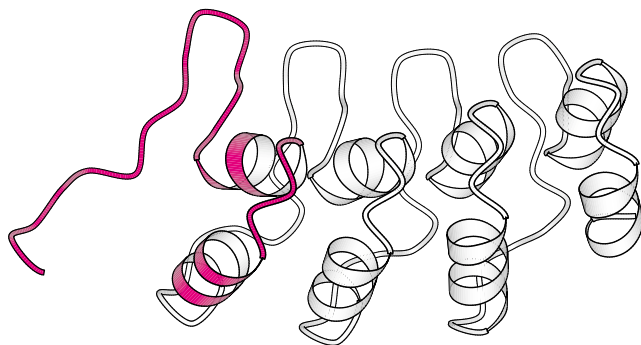


FIGURE 32.35 Ankyrin repeat structure. Four ankyrin repeats are shown with one shown in red. These domains interact with other proteins, primarily through their loops.

32.5 TOUCH INCLUDES THE SENSING OF PRESSURE, TEMPERATURE, AND OTHER FACTORS

Like taste, touch is a combination of sensory systems that are expressed in a common organ—in this case, the skin. The detection of pressure and the detection of temperature are two key components. Amiloride-sensitive sodium channels, homologous to those of taste, appear to play a role. Other systems are responsible for detecting painful stimuli such as high temperature, acid, or certain specific chemicals. Although our understanding of this sensory system is not as advanced as that of the other sensory systems, recent work has revealed a fascinating relation between pain and taste sensation, a relation well known to anyone who has eaten “spicy” food.

32.5.1 Studies of Capsaicin, the Active Ingredient in “Hot” Peppers, Reveal a Receptor for Sensing High Temperatures and Other Painful Stimuli

Our sense of touch is intimately connected with the sensation of pain. Specialized neurons, termed *nociceptors*, transmit signals to pain-processing centers in the spinal cord and brain in response to the onset of tissue damage. What is the molecular basis for the sensation of pain? An intriguing clue came from the realization that *capsaicin*, the chemical responsible for the “hot” taste of spicy food, activates nociceptors.

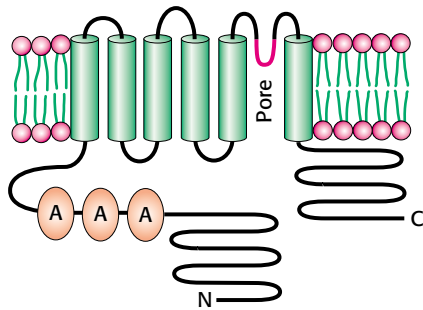
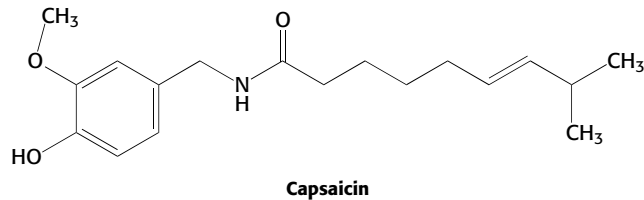


FIGURE 32.36 The membrane topology deduced for VR1, the capsaicin receptor. The proposed site of the membrane pore is indicated in red, and the three ankyrin (A) repeats are shown in orange. The active receptor comprises four of these subunits. [Adapted from Caterina, M. J., Schumacher, M. A., Tominaga, M., Rosen, T. A., Levine, J. D., and Julius, D. *Nature* 389 (1997):816.]

Early research suggested that capsaicin would act by opening ion channels that are expressed in nociceptors. Thus, a cell that expresses the capsaicin receptor should take up calcium on treatment with the molecule. This insight led to the isolation of the capsaicin receptor with the use of cDNA from cells expressing this receptor. Such cells had been detected by their fluorescence when loaded with the calcium-sensitive compound Fura-2 and then treated with capsaicin or related molecules. Cells expressing the capsaicin receptor, which is called VR1 (for *vanilloid receptor 1*), respond to capsaicin below a concentration of $1 \mu\text{M}$. The deduced 838-residue sequence of VR1 revealed it to be a member of the TRP channel family (Figure 32.36). The amino-terminal region of VR1 includes three ankyrin repeats.

Currents through VR1 are also induced by temperatures above 40°C and by exposure to dilute acid, with a midpoint for activation at pH 5.4 (Figure 32.37). Temperatures and acidity in these ranges are associated with infection and cell injury. The responses to capsaicin, temperature, and acidity are not independent. The response to heat is greater at lower pH, for example. Thus, *VR1 acts to integrate several noxious stimuli*. We feel these responses as pain and act to avoid the potentially destructive conditions that caused the unpleasant sensation. Mice that do not express VR1 suggest that this is the case; such mice do not mind food containing high concentrations of capsaicin and are, indeed, less responsive than control mice to normally noxious heat. Plants such as chili peppers presumably gained the ability to synthesize capsaicin and other “hot” compounds to protect themselves from being consumed by mammals. Birds, which play the beneficial role of spreading pepper seeds into new territory, do not appear to respond to capsaicin.

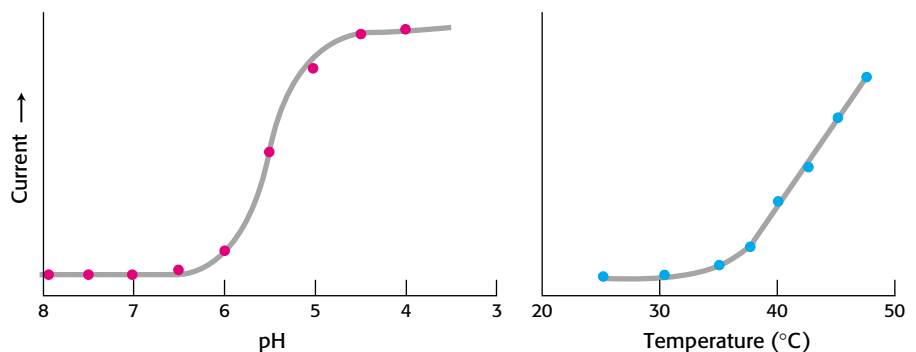


FIGURE 32.37 Response of the capsaicin receptor to pH and temperature. [Adapted from Tominaga, M., Caterina, M. J., Malmberg, A. B., Rosen, T. A., Gilbert, H., Skinner, K., Raumann, B. E., Basbaum, A. I., and Julius, D. *Neuron* 21(1998):531.]



Because of its ability to simulate VR1, capsaicin is used in pain management for arthritis, neuralgia, and other neuropathies. How can a compound that induces pain assist in its alleviation? Chronic exposure to capsaicin overstimulates pain-transmitting neurons, leading to their desensitization.

32.5.2 Subtle Sensory Systems Detect Other Environmental Factors Such as Earth's Magnetic Field

In addition to the five primary senses, human beings may have counterparts to less-familiar sensory systems characterized in other organisms. These sensory systems respond to environmental factors other than light, molecular shape, or air motion. For example, some species of bacteria are magnetotactic; that is, they move in directions dictated by Earth's magnetic field (Figure 32.38). In the Northern Hemisphere, Earth's magnetic field points northward but also has a component directed downward, toward Earth's center. Magnetotactic bacteria not only swim northward but also swim downward, away from the surface and the presence of high levels of oxygen, toxic to these bacteria. Remarkably, these bacteria synthesize intracellular chains of small particles containing a magnetic ore called magnetite (Fe_3O_4) that run through the center of each bacterium. Such chains are called *magnetosomes*. The magnetic force exerted by these particles is sufficiently strong in relation to the size of the bacterium that it causes the bacterium to become passively aligned with Earth's magnetic field. Intriguingly, similar magnetite particles have been detected in the brains of birds, fish, and even human beings, although their role in sensing magnetic fields has not yet been established.

There may exist other subtle senses that are able to detect environmental signals that then influence our behavior. The biochemical basis of these senses is now under investigation. One such sense is our ability to respond, often without our awareness, to chemical signals called pheromones, released by other persons. Another is our sense of time, manifested in our daily (circadian) rhythms of activity and restfulness. Daily changes in light exposure strongly influence these rhythms. The foundations for these senses have been uncovered in other organisms; future studies should reveal to what extent these mechanisms apply to human beings as well.

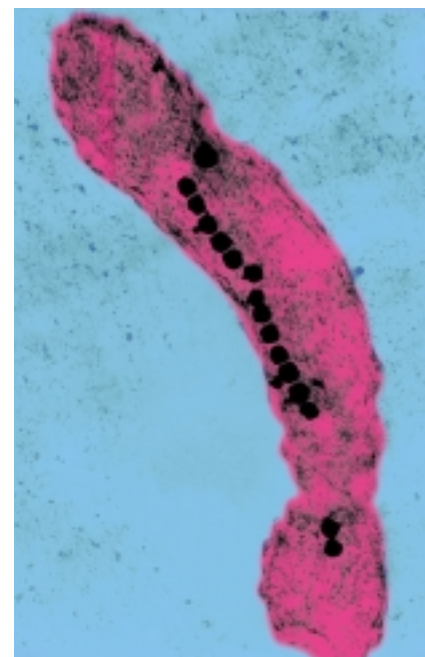


FIGURE 32.38 Magnetotactic bacterium. The magnetosome, visible as a chain of opaque membrane-bound magnetite crystals, acts as a compass to orient the bacteria with the earth's magnetic field. The bacterium is artificially colored. [Courtesy of Richard B. Frankel, California Polytechnic State University, San Luis Obispo, California.]

SUMMARY

- **Smell, taste, vision, hearing, and touch are based on signal-transduction pathways activated by signals from the environment.**

These sensory systems function similarly to the signal-transduction pathways for many hormones. These intercellular signaling pathways appear to have been appropriated and modified to process environmental information.

- **A Wide Variety of Organic Compounds Are Detected by Olfaction**

The sense of smell, or olfaction, is remarkable in its specificity—it can, for example, discern stereoisomers of small organic compounds as distinct aromas. The 7TM receptors that detect these odorants operate in conjunction with $G_{(olf)}$, a G protein that activates a cAMP cascade resulting in the opening of an ion channel and the generation of a nerve impulse. An outstanding feature of the olfactory system is its ability to detect a vast array of odorants. Each olfactory neuron expresses only one type of receptor and connects to a particular region of the olfactory

bulb. Odors are decoded by a combinatorial mechanism—each odorant activates a number of receptors, each to a different extent, and most receptors are activated by more than one odorant.

- Taste Is a Combination of Senses That Function by Different Mechanisms**
We can detect only five tastes: bitter, sweet, salt, sour, and umami. The transduction pathways that detect taste are, however, diverse. Bitter and sweet tastants are experienced through 7TM receptors acting through a special G protein called gustducin. Salty and sour tastants act directly through membrane channels. Salt tastants are detected by passage through sodium channels, whereas sour taste results from the effects of hydrogen ions on a number of types of channels. The end point is the same in all cases—membrane polarization that results in the transmission of a nerve impulse. Umami, the taste of glutamate, is detected by a receptor that is a modified form of a brain receptor that responds to glutamate as a neurotransmitter rather than as a tastant.
- Photoreceptor Molecules in the Eye Detect Visible Light**
Vision is perhaps the best understood of the senses. Two classes of photoreceptor cells exist: cones, which respond to bright lights and colors, and rods, which respond only to dim light. The photoreceptor in rods is rhodopsin, a 7TM receptor that is a complex of the protein opsin and the chromophore 11-*cis*-retinal. Absorption of light by 11-*cis*-retinal changes its structure into that of all-*trans*-retinal, setting in motion a signal-transduction pathway that leads to the breakdown of cGMP, to membrane hyperpolarization, and to a subsequent nerve impulse. Color vision is mediated by three distinct 7TM photoreceptors that employ 11-*cis*-retinal as a chromophore and absorb light in the blue, green, and red parts of the spectrum.
- Hearing Depends on the Speedy Detection of Mechanical Stimuli**
The immediate receptors for hearing are found in the hair cells of the cochleae, which contain bundles of stereocilia. When the stereocilia move in response to sound waves, cation channels will open or close, depending on the direction of movement. The mechanical motion of the cilia is converted into current flow and then into a nerve impulse.
- Touch Includes the Sensing of Pressure, Temperature, and Other Factors**
Touch, detected by the skin, senses pressure, temperature, and pain. Specialized nerve cells called nociceptors transmit signals that are interpreted in the brain as pain. A receptor responsible for the perception of pain has been isolated on the basis of its ability to bind capsaicin, the molecule responsible for the hot taste of spicy food. The capsaicin receptor, also called VR1, functions as a cation channel that initiates a nerve impulse.

KEY TERMS

main olfactory epithelium (p. 32-3)

$G_{(olf)}$ (p. 32-3)

functional magnetic resonance imaging (fMRI) (p. 32-6)

gustducin (p. 32-8)

amiloride-sensitive sodium channel (p. 32-10)

metabotropic glutamate receptor (p. 31-11)

rod (p. 32-11)

cone (p. 32-11)

rhodopsin (p. 32-12)

opsin (p. 32-12)

retinal (p. 32-12)

chromophore (p. 32-12)

transducin (p. 32-14)

cGMP phosphodiesterase (p. 32-14)

rhodopsin kinase (p. 32-14)

arrestin (p. 32-14)

guanylate cyclase (p. 32-14)

cGMP-gated calcium channel (p. 32-15)

hair cell (p. 32-17)

stereocilium (p. 32-18)

tip link (p. 32-18)

nociceptor (p. 32-20)

capsaicin receptor (p. 32-20)

SELECTED READINGS

Where to start

- Axel, R., 1995. The molecular logic of smell. *Sci. Am.* 273(4):154–159.
- Dulac, C., 2000. The physiology of taste, vintage 2000. *Cell* 100: 607–610.
- Stryer, L., 1996. Vision: From photon to perception. *Proc. Natl. Acad. Sci. U. S. A.* 93:557–559.
- Hudspeth, A. J., 1989. How the ear's works work. *Nature* 341:397–404.

Olfaction

- Buck, L., and Axel, R., 1991. A novel multigene family may encode odorant receptors: A molecular basis for odor recognition. *Cell* 65:175–187.
- Malnic, B., Hirono, J., Sato, T., and Buck, L. B., 1999. Combinatorial receptor codes for odors. *Cell* 96:713–723.
- Mombaerts, P., Wang, F., Dulac, C., Chao, S. K., Nemes, A., Mendelsohn, M., Edmondson, J., and Axel, R., 1996. Visualizing an olfactory sensory map. *Cell* 87:675–686.
- Mombaerts, P., 1999. Molecular biology of odorant receptors in vertebrates. *Annu. Rev. Neurosci.* 22:487–509.
- Belluscio, L., Gold, G. H., Nemes, A., and Axel, R., 1998. Mice deficient in G(olf) are anosmic. *Neuron* 20:69–81.
- Vosshall, L. B., Wong, A. M., and Axel, R., 2000. An olfactory sensory map in the fly brain. *Cell* 102:147–159.

Taste

- Herness, M. S., and Gilbertson, T. A., 1999. Cellular mechanisms of taste transduction. *Annu. Rev. Physiol.* 61:873–900.
- Adler, E., Hoon, M. A., Mueller, K. L., Chandrashekar, J., Ryba, N. J., and Zuker, C. S., 2000. A novel family of mammalian taste receptors. *Cell* 100:693–702.
- Chandrashekar, J., Mueller, K. L., Hoon, M. A., Adler, E., Feng, L., Guo, W., Zuker, C. S., and Ryba, N. J., 2000. T2Rs function as bitter taste receptors. *Cell* 100:703–711.
- Mano, I., and Driscoll, M., 1999. DEG/ENaC channels: A touchy superfamily that watches its salt. *Bioessays* 21:568–578.
- Benos, D. J., and Stanton, B. A., 1999. Functional domains within the degenerin/epithelial sodium channel (Deg/ENaC) superfamily of ion channels. *J. Physiol. (Lond.)* 520(part 3):631–644.
- McLaughlin, S. K., McKinnon, P. J., and Margolskee, R. F., 1992. Gustducin is a taste-cell-specific G protein closely related to the transducins. *Nature* 357:563–569.
- Chaudhari, N., Landin, A. M., and Roper, S. D., 2000. A metabotropic glutamate receptor variant functions as a taste receptor. *Nat. Neurosci.* 3:113–119.

Vision

- Stryer, L., 1988. Molecular basis of visual excitation. *Cold Spring Harbor Symp. Quant. Biol.* 53:283–294.

- Wald, G., 1968. The molecular basis of visual excitation. *Nature* 219:800–807.
- Ames, J. B., Dizhoor, A. M., Ikura, M., Palczewski, K., and Stryer, L., 1999. Three-dimensional structure of guanylyl cyclase activating protein-2, a calcium-sensitive modulator of photoreceptor guanylyl cyclases. *J. Biol. Chem.* 274:19329–19337.
- Nathans, J., 1994. In the eye of the beholder: Visual pigments and inherited variation in human vision. *Cell* 78:357–360.
- Nathans, J., 1999. The evolution and physiology of human color vision: Insights from molecular genetic studies of visual pigments. *Neuron* 24:299–312.
- Palczewski, K., Kumasaka, T., Hori, T., Behnke, C. A., Motoshima, H., Fox, B. A., LeTrong, I., Teller, D. C., Okada, T., Stenkamp, R. E., Yamamoto, M., and Miyano, M., 2000. Crystal structure of rhodopsin: A G protein-coupled receptor. *Science* 289:739–745.

Hearing

- Hudspeth, A. J., 1997. How hearing happens. *Neuron* 19:947–950.
- Pickles, J. O., and Corey, D. P., 1992. Mechano-electrical transduction by hair cells. *Trends Neurosci.* 15:254–259.
- Walker, R. G., Willingham, A. T., and Zuker, C. S., 2000. A *Drosophila* mechanosensory transduction channel. *Science* 287:2229–2234.

Touch and pain reception

- Franco-Obregon, A., and Clapham, D. E., 1998. Touch channels sense blood pressure. *Neuron* 21:1224–1226.
- Caterina, M. J., Schumacher, M. A., Tominaga, M., Rosen, T. A., Levine, J. D., and Julius, D., 1997. The capsaicin receptor: A heat-activated ion channel in the pain pathway. *Nature* 389:816–824.
- Tominaga, M., Caterina, M. J., Malmberg, A. B., Rosen, T. A., Gilbert, H., Skinner, K., Raumann, B. E., Basbaum, A. I., and Julius, D., 1998. The cloned capsaicin receptor integrates multiple pain-producing stimuli. *Neuron* 21:531–543.
- Caterina, M. J., and Julius, D., 1999. Sense and specificity: A molecular identity for nociceptors. *Curr. Opin. Neurobiol.* 9:525–530.

Other sensory systems

- Frankel, R. B., 1984. Magnetic guidance of organisms. *Annu. Rev. Biophys. Bioeng.* 13:85–103.
- Kirschvink, J. L., Kobayashi-Kirschvink, A., and Woodford, B. J., 1992. Magnetite biomineralization in the human brain. *Proc. Natl. Acad. Sci. U. S. A.* 89:7683–7687.
- Dulac, C., and Axel, R., 1995. A novel family of genes encoding putative pheromone receptors in mammals. *Cell* 83:195–206.

PROBLEMS

- Of mice and rats.* As noted in Section 32.1.2, one of the first odorant receptors to be matched with its ligand was a rat receptor that responded best to *n*-octanal. The sequence of the corresponding mouse receptor differed from the rat receptor at 15 positions. Surprisingly, the mouse receptor was found to respond best to *n*-heptanal rather than *n*-octanal. The substitution of isoleucine at position 206 in the mouse for valine at this position in the rat receptor was found to be important in determining the specificity for *n*-heptanal. Propose an explanation.
- Olfaction in worms.* Unlike the olfactory neurons in the mammalian systems discussed herein, olfactory neurons in the nem-

atode *C. elegans* express multiple olfactory receptors. In particular, one neuron (called AWA) expresses receptors for compounds to which the nematode is attracted, whereas a different neuron (called AWB) expresses receptors for compounds that the nematode avoids. Suppose that a transgenic nematode is generated such that one of the receptors for an attractant is expressed in AWB rather than AWA. What behavior would you expect in the presence of the corresponding attractant?

- Odorant matching.* A mixture of two of the compounds illustrated in Figure 32.6 is applied to a section of olfactory epithelium. Only receptors 3, 5, 9, 12, and 13 are activated,

according to Figure 32.7. Identify the likely compounds in the mixture.

4. *Timing.* Compare the aspects of taste (bitter, sweet, salty, sour) in regard to their potential for rapid time resolution.

5. *Two ears.* Our ability to determine the direction from which a sound is coming is partly based on the difference in time at which our two ears detect the sound. Given the speed of sound (350 meter/second) and the separation between our ears (0.15 meter), what difference is expected in the times at which a sound arrives at our two ears? How does this difference compare with the time resolution of the human hearing system? Would a sensory system that utilized 7TM receptors and G proteins be capable of adequate time resolution?

6. *Constitutive mutants.* What effect within the olfactory system would you expect for a mutant in which adenylate cyclase is always fully active? What effect within the visual system would you expect for a mutant in which guanylate cyclase is always fully active?

7. *Bottle choice.* A widely used method for quantitatively monitoring rodent behavior with regard to taste is the bottle-choice assay. An animal is placed in a cage with two water bottles, one of which contains a potential tastant. After a fixed period of time (24–48 hours), the amount of water remaining in each bottle is measured. Suppose that much less water remains in the bottle with the tastant after 48 hours. Do you suspect the tastant to be sweet or bitter?

8. *It's better to be bitter.* Some nontoxic plants taste very bitter to us. Suggest one or more explanations.

9. *Unexpected consequences.* Sildenafil (Viagra) is a drug widely used to treat male impotence. Sildenafil exerts its effect by inhibiting a cGMP phosphodiesterase isozyme (PDE5) that is especially prevalent in smooth muscle. Interestingly, certain airlines restrict pilots from flying for 24 hours after using sildenafil. Suggest a reason for this restriction.

Chapter Integration Problem

10. *Energy and information.* The transmission of sensory information requires the input of free energy. For each sensory system (olfaction, gustation, vision, hearing, and touch), identify mechanisms for the input of free energy that allow the transmission of sensory information.

Mechanism Problem

11. *Schiff-base formation.* Propose a mechanism for the reaction between opsin and 11-*cis*-retinal.



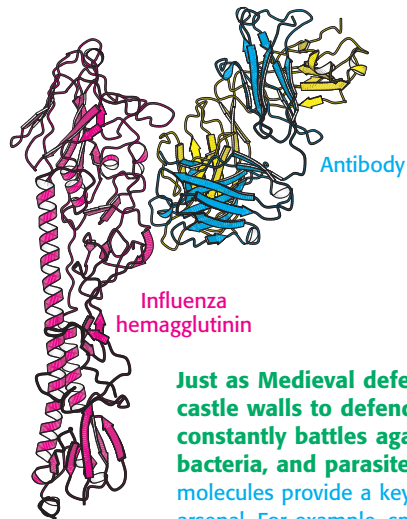
Media Problems

12. *Homologous proteins, analogous binding?* Odorants bind to 7TM receptors, but where they bind (and whether all bind in the same way) is unclear. Odorants might, for example, bind on the extracellular surface, or, like retinal, they might bind in the interior of the transmembrane region. Problem 1 of this chapter presents evidence for the direct involvement of residue 206 in odorant binding in receptors from mouse and rat. While these receptors' structures are not known in detail, their sequences are similar enough to rhodopsin's that the rhodopsin structure can be used to infer the approximate location of residue 206. To see the likely location, look in the **Structural Insights** module on rhodopsin. Where do you think the mouse and rat receptors bind their odorants?

13. *Deodorant?* A cAMP phosphodiesterase has been discovered that is found predominantly in olfactory sensory neurons (Yan et al., 1995, Proc. Natl. Acad. Sci. 10:9677). The enzyme is activated by Ca^{2+} . What do you think this enzyme does, and why do you think it is regulated by calcium? (Hint: Study the response and recovery animations in the **Conceptual Insights** module on signaling pathways.)

The Immune System

Dedicated to the memory of Don Wiley, a pioneer in unraveling the structural basis of immune-system function



Just as Medieval defenders used their weapons and the castle walls to defend their city, the immune system constantly battles against foreign invaders such as viruses, bacteria, and parasites to defend the organism. Antibody molecules provide a key element in the immune system's defensive arsenal. For example, specific antibodies can bind to molecules on the surfaces of viruses and prevent the viruses from infecting cells. Above, an antibody binds to one subunit on hemagglutinin from the surface of influenza virus. [(Left) The Granger Collection.]

We are constantly exposed to an incredible diversity of bacteria, viruses, and parasites, many of which would flourish in our cells or extracellular fluids were it not for our immune system. Remarkably, we are often even able to defend ourselves against organisms that we have never before encountered. How does the immune system protect us? The key is our ability to produce more than 10^8 distinct *antibodies* and more than 10^{12} *T-cell receptors*, each of which presents a different surface for specifically binding a molecule from a foreign organism and initiating the destruction of the invader.

The presence of this remarkable repertoire of defensive molecules poses a challenge. What prevents the immune system from attacking cells that express molecules normally present in our bodies; that is, *how does the immune system distinguish between nonself and self?* We shall examine these questions, focusing first on the structures of the proteins participating in the molecular recognition processes and then on the mechanisms for selecting cells that express molecules useful for protecting us from a specific pathogen. Emphasis will be on the modular construction of the proteins of the immune system—identifying structural motifs and considering how spectacular diversity can arise from modular construction.

33.0.1 The Immune System Adapts, Using the Principles of Evolution

The immune system comprises two parallel but interrelated systems. In the *humoral immune response*, soluble proteins

OUTLINE

- **33.1 Antibodies Possess Distinct Antigen-Binding and Effector Units**
- **33.2 The Immunoglobulin Fold Consists of a Beta-Sandwich Framework with Hypervariable Loops**
- **33.3 Antibodies Bind Specific Molecules Through Their Hypervariable Loops**
- **33.4 Diversity Is Generated by Gene Rearrangements**
- **33.5 Major-Histocompatibility-Complex Proteins Present Peptide Antigens on Cell Surfaces for Recognition by T-Cell Receptors**
- **33.6 Immune Responses Against Self-Antigens Are Suppressed**

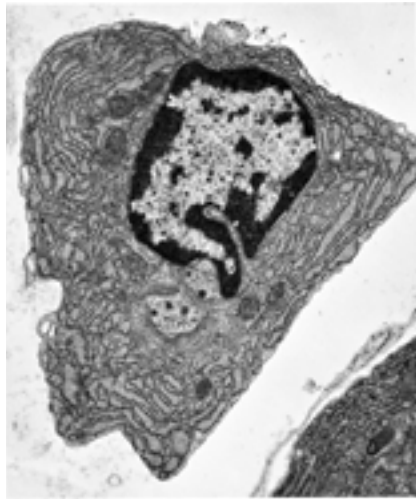


FIGURE 33.1 Immunoglobulin production. An electron micrograph of a plasma cell shows the highly developed rough endoplasmic reticulum necessary for antibody secretion. [Courtesy of Lynne Mercer.]

called *antibodies (immunoglobulins)* function as recognition elements that bind to foreign molecules and serve as markers signaling foreign invasion (Figure 33.1). Antibodies are secreted by *plasma cells*, which are derived from *B lymphocytes (B cells)*. A foreign macromolecule that binds selectively to an antibody is called an *antigen*. In a physiological context, if the binding of the foreign molecule stimulates an immune response, that molecule is called an *immunogen*. The specific affinity of an antibody is not for the entire macromolecular antigen but for a particular site on the antigen called the *epitope* or *antigenic determinant*.

In the *cellular immune response*, cells called *cytotoxic T lymphocytes* (also commonly called *killer T cells*) kill cells that display foreign motifs on their surfaces. Another class of T cells called *helper T lymphocytes* contributes to both the humoral and the cellular immune responses by stimulating the differentiation and proliferation of appropriate B cells and cytotoxic T cells. The cellular immune response is mediated by specific receptors that are expressed on the surfaces of the T cells.

The remarkable ability of the immune system to adapt to an essentially limitless set of potential pathogens requires a powerful system for transforming the immune cells and molecules present in our systems in response to the presence of pathogens. *This adaptive system operates through the principles of evolution, including reproduction with variation followed by selection of the most well suited members of a population.*

If the human genome contains, by the latest estimates, only 40,000 genes, how can the immune system generate more than 10^8 different antibody proteins and 10^{12} T-cell receptors? The answer is found in a novel mechanism for generating a highly diverse set of genes from a limited set of genetic building blocks. Linking different sets of DNA regions in a combinatorial manner produces many distinct protein-encoding genes that are not present in the genome. A rigorous selection process then leaves for proliferation only cells that synthesize proteins determined to be useful in the immune response. The subsequent reproduction of these cells without additional recombination serves to enrich the cell population with members expressing a particular protein species.

Critical to the development of the immune response is the selection process, which determines which cells will reproduce. The process comprises several stages. In the early stages of the development of an immune response, cells expressing molecules that bind tightly to self-molecules are destroyed or silenced, whereas cells expressing molecules that do not bind strongly to self-molecules and that have the potential for binding strongly to foreign molecules are preserved. The appearance of an immunogenic invader at a later time will stimulate cells expressing immunoglobulins or T-cell receptors that bind specifically to elements of that pathogen to reproduce—in evolutionary terms, such cells are selected for. Thus, the immune response is based on the selection of cells expressing molecules that are specifically effective against a particular invader; the response evolves from a population with wide-ranging specificities to a more-focused collection of cells and molecules that are well suited to defend the host when confronted with that particular challenge.

33.1 ANTIBODIES POSSESS DISTINCT ANTIGEN-BINDING AND EFFECTOR UNITS

Antibodies are central molecular players in the immune response, and we examine them first. A fruitful approach in studying proteins as large as antibodies is to split the protein into fragments that retain activity. In 1959,

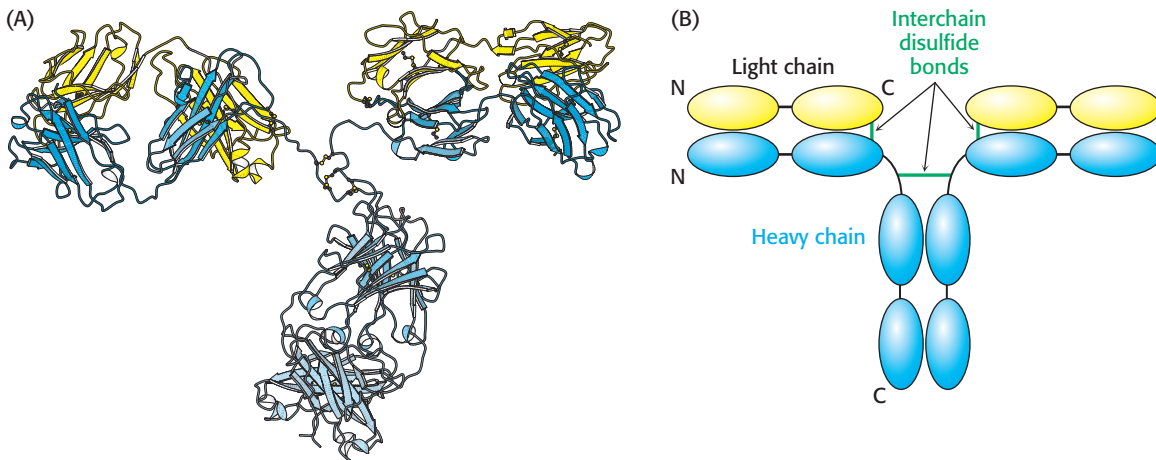


FIGURE 33.2 Immunoglobulin G structure. (A) The three-dimensional structure of an IgG molecule showing the light chains in yellow and the heavy chains in blue. (B) A schematic view of an IgG molecule indicating the positions of the interchain disulfide bonds. N, amino terminus; C, carboxyl terminus.

Rodney Porter showed that *immunoglobulin G* (IgG), the major antibody in serum, can be cleaved into three 50-kd fragments by the limited proteolytic action of papain. Two of these fragments bind antigen. They are called F_{ab} (F stands for fragment, *ab* for antigen binding). The other fragment, called F_c because it crystallizes readily, does not bind antigen, but it has other important biological activities, including the mediation of responses termed *effector functions*. These functions include the initiation of the *complement cascade*, a process that leads to the lysis of target cells. Although such effector functions are crucial to the functioning of the immune system, they will not be discussed further here.

How do these fragments relate to the three-dimensional structure of whole IgG molecules? Immunoglobulin G consists of two kinds of polypeptide chains, a 25-kd *light* (L) chain and a 50-kd *heavy* (H) chain (Figure 33.2). The subunit composition is L_2H_2 . Each L chain is linked to an H chain by a disulfide bond, and the H chains are linked to each other by at least one disulfide bond. Examination of the amino acid sequences and three-dimensional structures of IgG molecules reveals that each L chain comprises two homologous domains, termed *immunoglobulin domains*, to be described in detail in Section 33.2. Each H chain has four immunoglobulin domains. Overall, the molecule adopts a conformation that resembles the letter Y, in which the stem, corresponding to the F_c fragment obtained by cleavage with papain, consists of the two carboxyl-terminal immunoglobulin domains of each H chain and in which the two arms of the Y, corresponding to the two F_{ab} fragments, are formed by the two amino-terminal domains of each H chain and the two amino-terminal domains of each L chain. The linkers between the stem and the two arms consist of relatively extended polypeptide regions within the H chains and are quite flexible.

Papain cleaves the H chains on the carboxyl-terminal side of the disulfide bond that links each L and H chain (Figure 33.3). Thus, each F_{ab} consists of an entire L chain and the amino-terminal half of an H chain, whereas F_c consists of the carboxyl-terminal halves of both H chains. Each F_{ab} contains a single antigen-binding site. Because an intact IgG molecule contains two F_{ab} components and therefore has two binding sites, it can cross-link

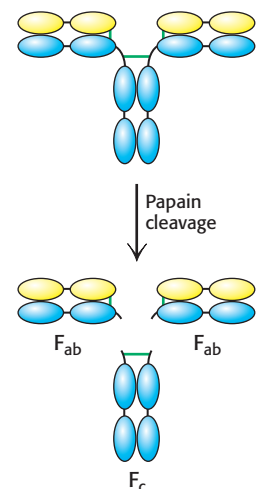


FIGURE 33.3 Immunoglobulin G cleavage. Treatment of intact IgG molecules with the protease papain results in the formation of three large fragments: two F_{ab} fragments that retain antigen-binding capability and one F_c fragment that does not.

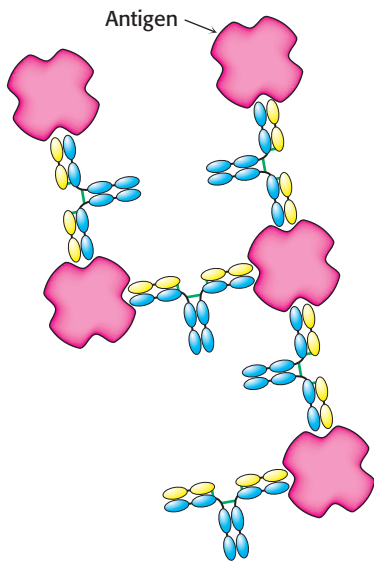


FIGURE 33.4 Antigen cross-linking. Because IgG molecules include two antigen-binding sites, antibodies can cross-link multivalent antigens such as viral surfaces.

multiple antigens (Figure 33.4). Furthermore, the F_c and the two F_{ab} units of the intact IgG are joined by flexible polypeptide regions that allow facile variation in the angle between the F_{ab} units through a wide range (Figure 33.5). This kind of mobility, called *segmental flexibility*, can enhance the formation of an antibody–antigen complex by enabling both combining sites on an antibody to bind an antigen that possesses multiple binding sites, such as a viral coat composed of repeating identical monomers or a bacterial cell surface. The combining sites at the tips of the F_{ab} units simply move to match the distance between specific determinants on the antigen.

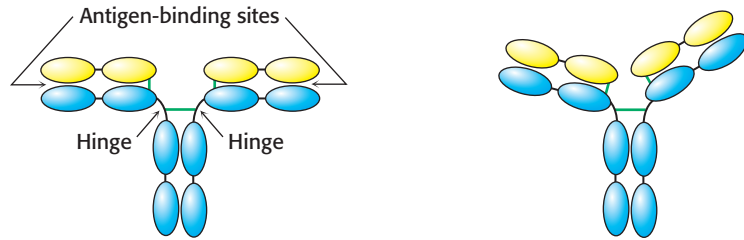


FIGURE 33.5 Segmental flexibility. The linkages between the F_{ab} and the F_c regions of an IgG molecule are flexible, allowing the two antigen-binding sites to adopt a range of orientations with respect to one another. This flexibility allows effective interactions with a multivalent antigen without requiring that the epitopes on the target be a precise distance apart.

Immunoglobulin G is the antibody present in highest concentration in the serum, but other classes of immunoglobulin also are present (Table 33.1). Each class includes an L chain (either κ or λ) and a distinct H chain (Figure 33.6). The heavy chains in IgG are called γ chains, whereas those in immunoglobulins A, M, D, and E are called α , μ , δ , and ϵ , respectively. *Immunoglobulin M (IgM)* is the first class of antibody to appear in the serum after exposure to an antigen. The presence of 10 combining sites enables IgM to bind especially tightly to antigens containing multiple identical epitopes. The strength of an interaction comprising multiple independent binding interactions between partners is termed *avidity* rather than affinity, which denotes the binding strength of a single combining site. The presence of 10 combining sites in IgM compared with 2 sites in IgG enables IgM to bind many multivalent antigens that would slip away from IgG.

Immunoglobulin A (IgA) is the major class of antibody in external secretions, such as saliva, tears, bronchial mucus, and intestinal mucus. Thus, IgA serves as a first line of defense against bacterial and viral antigens. The role of *immunoglobulin D (IgD)* is not yet known. *Immunoglobulin E (IgE)* is

TABLE 33.1 Properties of immunoglobulin classes

Class	Serum concentration (mg/ml)	Mass (kd)	Sedimentation coefficient(s)	Light chains	Heavy chains	Chain structure
IgG	12	150	7	κ or λ	γ	$\kappa_2\gamma_2$ or $\lambda_2\gamma_2$
IgA	3	180–500	7, 10, 13	κ or λ	α	$(\kappa_2\alpha_2)_n$ or $(\lambda_2\alpha_2)_n$
IgM	1	950	18–20	κ or λ	μ	$(\kappa_2\mu_2)_5$ or $(\lambda_2\mu_2)_5$
IgD	0.1	175	7	κ or λ	δ	$\kappa_2\delta_2$ or $\lambda_2\delta_2$
IgE	0.001	200	8	κ or λ	ϵ	$\kappa_2\epsilon_2$ or $\lambda_2\epsilon_2$

Note: $n = 1, 2,$ or 3 . IgM and oligomers of IgA also contain J chains that connect immunoglobulin molecules. IgA in secretions has an additional component.

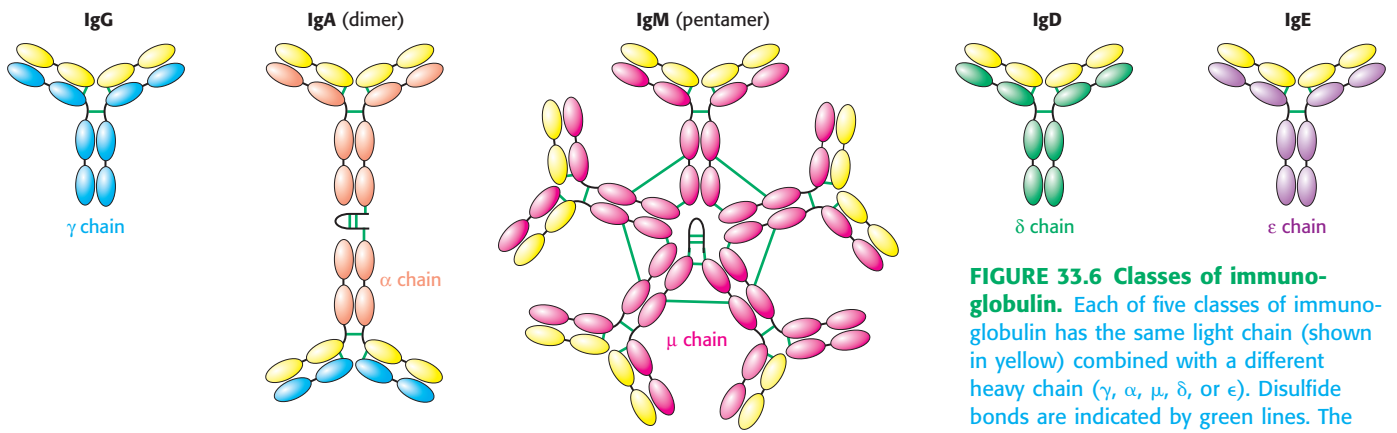


FIGURE 33.6 Classes of immunoglobulin. Each of five classes of immunoglobulin has the same light chain (shown in yellow) combined with a different heavy chain (γ , α , μ , δ , or ϵ). Disulfide bonds are indicated by green lines. The IgA dimer and the IgM pentamer have a small polypeptide chain in addition to the light and heavy chains.

important in conferring protection against parasites, but IgE also causes allergic reactions. IgE-antigen complexes form cross-links with receptors on the surfaces of mast cells to trigger a cascade that leads to the release of granules containing pharmacologically active molecules. Histamine, one of the agents released, induces smooth muscle contraction and stimulates the secretion of mucus.

A comparison of the amino acid sequences of different IgG antibodies from human beings or mice shows that the carboxyl-terminal half of the L chains and the carboxyl-terminal three-quarters of the H chains are very similar in all of the antibodies. Importantly, the amino-terminal domain of each chain is more variable, including three stretches of approximately 7 to 12 amino acids within each chain that are hypervariable, as shown for the H chain in Figure 33.7. The amino-terminal immunoglobulin domain of each chain is thus referred to as the *variable region*, whereas the remaining immunoglobulin domains are much more similar in all antibodies and are referred to as *constant regions* (Figure 33.8).

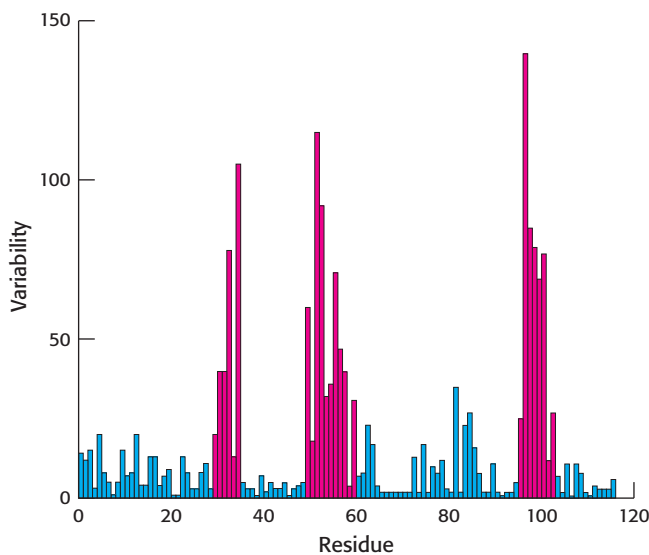


FIGURE 33.7 Immunoglobulin sequence diversity. A plot of sequence variability as a function of position along the sequence of the amino-terminal immunoglobulin domain of the H chain of human IgG molecules. Three regions (in red) show remarkably high levels of variability. These hypervariable regions correspond to three loops in the immunoglobulin domain structure. [After R. A. Goldsby, T. J. Kindt, and B. A. Osborne, *Kuby Immunology*, 4th ed. (W. H. Freeman and Company, 2000), p. 91.]

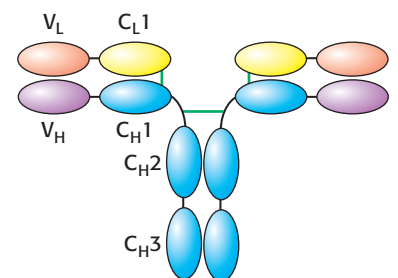


FIGURE 33.8 Variable and constant regions. Each L and H chain includes one immunoglobulin domain at its amino terminus that is quite variable from one antibody to another. These domains are referred to as V_L and V_H . The remaining domains are more constant from one antibody to another and are referred to as constant domains (C_L1 , C_H1 , C_H2 , and C_H3).

33.2 THE IMMUNOGLOBULIN FOLD CONSISTS OF A BETA-SANDWICH FRAMEWORK WITH HYPERVARIABLE LOOPS

An IgG molecule consists of a total of 12 immunoglobulin domains. These domains have many sequence features in common and adopt a common structure, the *immunoglobulin fold* (Figure 33.9). Remarkably, this same structural domain is found in many other proteins that play key roles in the immune system.

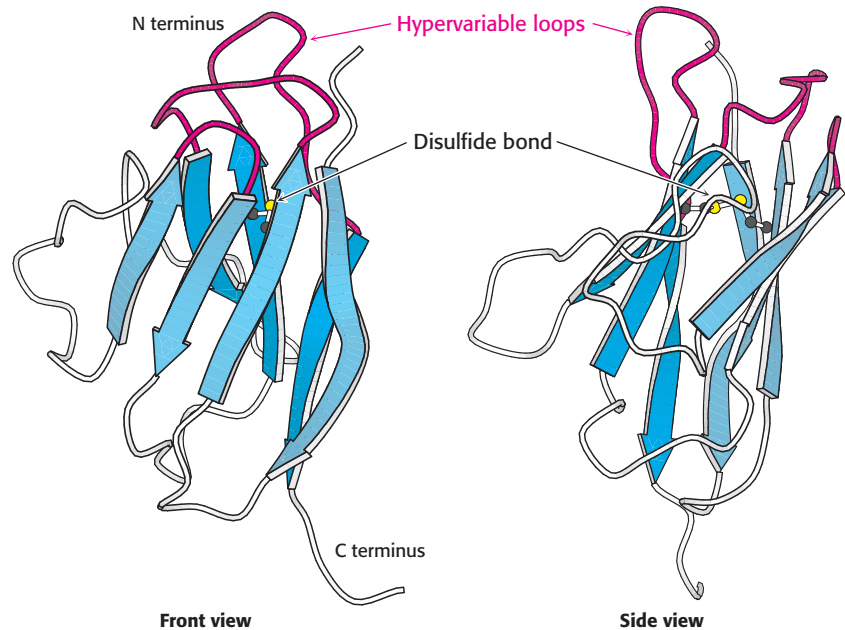



FIGURE 33.9 Immunoglobulin fold. An immunoglobulin domain consists of a pair of β -sheets linked by a disulfide bond and hydrophobic interactions. Three hypervariable loops lie at one end of the structure.

The immunoglobulin fold consists of a pair of β sheets, each built of antiparallel β strands, that surround a central hydrophobic core. A single disulfide bond bridges the two sheets. Two aspects of this structure are particularly important for its function. First, three loops present at one end of the structure form a potential binding surface. These loops contain the hypervariable sequences present in antibodies and in T-cell receptors (see Sections 33.3 and 33.5.2). Variation of the amino acid sequences of these loops provides the major mechanism for the generation of the vastly diverse set of antibodies and T-cell receptors expressed by the immune system. These loops are referred to as *hypervariable loops* or *complementarity-determining regions (CDRs)*. Second, the amino terminus and the carboxyl terminus are at opposite ends of the structure, which allows structural domains to be strung together to form chains, as in the L and H chains of antibodies. Such chains are present in several other key molecules in the immune system.

 The immunoglobulin fold is one of the most prevalent domains encoded by the human genome—more than 750 genes encode proteins with at least one immunoglobulin fold recognizable at the level of amino acid sequence. Such domains are also common in other multicellular animals such as flies and nematodes. However, from inspection of amino acid sequence alone, immunoglobulin-fold domains do not appear to be present in yeast or plants. However, structurally similar domains are present in these organisms, including the key photosynthetic electron-transport protein plastocyanin in plants (Section 19.3.2). Thus, the immunoglobulin-fold family appears to have expanded greatly along evolutionary branches leading to animals—particularly, vertebrates.

33.3 ANTIBODIES BIND SPECIFIC MOLECULES THROUGH THEIR HYPERVARIABLE LOOPS

For each class of antibody, the amino-terminal immunoglobulin domains of the L and H chains (the variable domains, designated V_L and V_H) come together at the ends of the arms extending from the structure. The positions of the complementarity-determining regions are striking. These hypervariable sequences, present in three loops of each domain, come together so that all six loops form a single surface at the end of each arm (Figure 33.10). Because virtually any V_L can pair with any V_H , a very large number of different binding sites can be constructed by their combinatorial association.

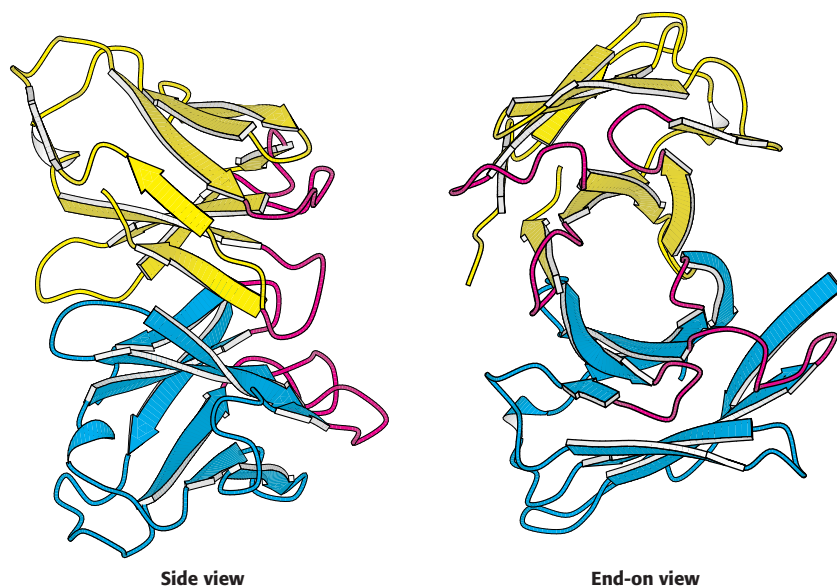


FIGURE 33.10 Variable domains.

Two views of the variable domains of the L chain (yellow) and the H chain (blue); the complementarity-determining regions (CDRs) are shown in red. The six CDRs come together to form a binding surface. The specificity of the surface is determined by the sequences and structures of the CDRs.

33.3.1 X-Ray Analyses Have Revealed How Antibodies Bind Antigens

The results of x-ray crystallographic studies of many large and small antigens bound to F_{ab} molecules have been sources of much insight into the structural basis of antibody specificity. The binding of antigens to antibodies is governed by the same principles that govern the binding of substrates to enzymes. The apposition of complementary shapes results in numerous contacts between amino acids at the binding surfaces of both molecules. Numerous hydrogen bonds, electrostatic interactions, and van der Waals interactions, reinforced by hydrophobic interactions, combine to give specific and strong binding.

A few aspects of antibody binding merit specific attention, inasmuch as they relate directly to the structure of immunoglobulins. The binding site on the antibody has been found to incorporate some or all of the CDRs in the variable domains of the antibody. Small molecules (e.g., octapeptides) are likely to make contact with fewer CDRs, with perhaps 15 residues of the antibody participating in the binding interaction. Macromolecules often make more extensive contact, interacting with all six CDRs and 20 or more residues of the antibody. Small molecules often bind in a cleft of the antigen-binding region. Macromolecules such as globular proteins tend to interact across larger, fairly flat apposed surfaces bearing complementary protrusions and depressions.

A well-studied case of small-molecule binding is seen in an example of phosphorylcholine bound to F_{ab} . Crystallographic analysis revealed

FIGURE 33.11 Binding of a small antigen. The structure of a complex between an F_{ab} fragment of an antibody and its target—in this case, phosphorylcholine. Residues from the antibody interact with phosphorylcholine through hydrogen bonding and electrostatic and van der Waals interactions.

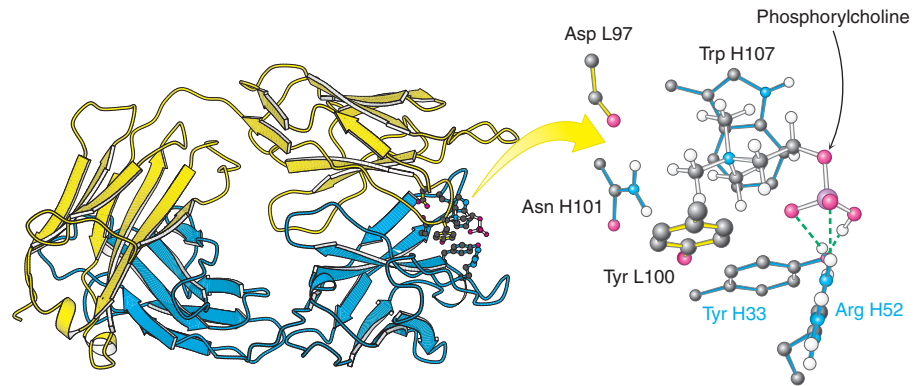
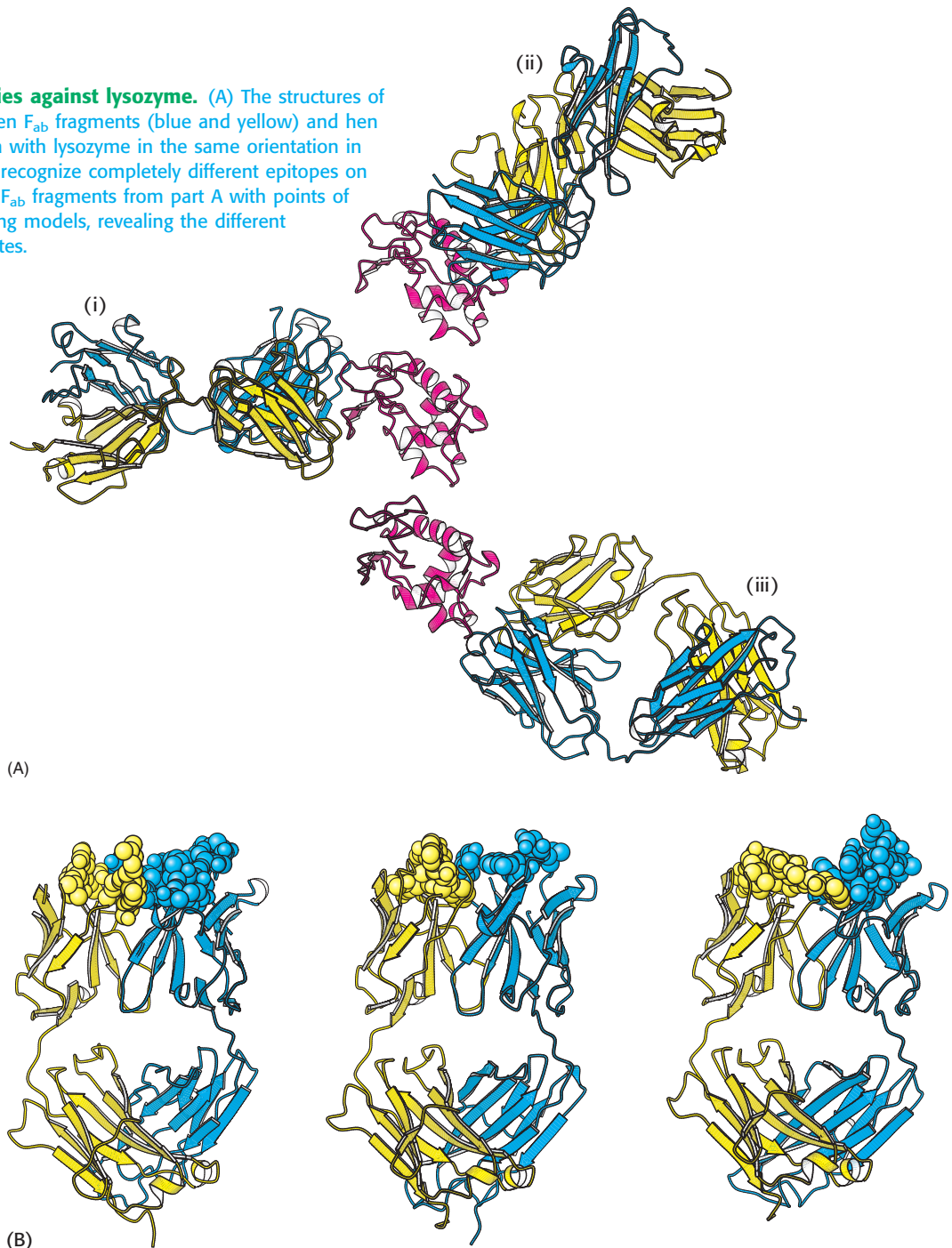


FIGURE 33.12 Antibodies against lysozyme. (A) The structures of three complexes (i, ii, iii) between F_{ab} fragments (blue and yellow) and hen egg-white lysozyme (red) shown with lysozyme in the same orientation in each case. The three antibodies recognize completely different epitopes on the lysozyme molecule. (B) The F_{ab} fragments from part A with points of contact highlighted as space-filling models, revealing the different shapes of the antigen-binding sites.



phosphorylcholine bound to a cavity lined by residues from five CDRs—two from the L chain and three from the H chain (Figure 33.11). The positively charged trimethylammonium group of phosphorylcholine is buried inside the wedge-shaped cavity, where it interacts electrostatically with two negatively charged glutamate residues. The negatively charged phosphate group of phosphorylcholine binds to the positively charged guanidinium group of an arginine residue at the mouth of the crevice and to a nearby lysine residue. The phosphate group is also hydrogen bonded to the hydroxyl group of a tyrosine residue and to the guanidinium group of the arginine side chain. Numerous van der Waals interactions, such as those made by a tryptophan side chain, also stabilize this complex.

The binding of phosphorylcholine does not significantly change the structure of the antibody, yet induced fit plays a role in the formation of many antibody–antigen complexes. A malleable binding site can accommodate many more kinds of ligands than can a rigid one. Thus, induced fit increases the repertoire of antibody specificities.

33.3.2 Large Antigens Bind Antibodies with Numerous Interactions

How do large antigens interact with antibodies? A large collection of antibodies raised against hen egg-white lysozyme has been structurally characterized in great detail (Figure 33.12). Each different antibody binds to a distinct surface of lysozyme. Let us examine the interactions present in one of these complexes in detail. This antibody binds two polypeptide segments that are widely separated in the primary structure, residues 18 to 27 and 116 to 129 (Figure 33.13).

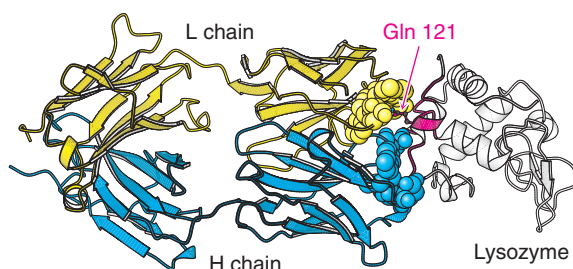


FIGURE 33.13 Antibody–protein interactions. The structure of a complex between an F_{ab} fragment and lysozyme reveals that the binding surfaces are complementary in shape over a large area. A single residue of lysozyme, glutamine 121, penetrates more deeply into the antibody combining site.

All six CDRs of the antibody make contact with this epitope. The region of contact is quite extensive (about $30 \times 20 \text{ \AA}$). The apposed surfaces are rather flat. The only exception is the side chain of glutamine 121 of lysozyme, which penetrates deeply into the antibody binding site, where it forms a hydrogen bond with a main-chain carbonyl oxygen atom and is surrounded by three aromatic side chains. The formation of 12 hydrogen bonds and numerous van der Waals interactions contributes to the high affinity ($K_d = 20 \text{ nM}$) of this antibody–antigen interaction. Examination of the F_{ab} molecule without bound protein reveals that the structures of the V_L and V_H domains change little on binding, although they slide 1 \AA apart to allow more intimate contact with lysozyme.

33.4 DIVERSITY IS GENERATED BY GENE REARRANGEMENTS

A mammal such as a mouse or a human being can synthesize large amounts of specific antibody against virtually any foreign determinant within a matter of days of being exposed to it. We have seen that antibody specificity is determined by the amino acid sequences of the variable regions of both light

and heavy chains, which brings us to the key question: How are different variable-region sequences generated?

The discovery of distinct variable and constant regions in the L and H chains raised the possibility that the genes that encode immunoglobulins have an unusual architecture that facilitates the generation of a diverse set of polypeptide products. In 1965, William Dreyer and Claude Bennett proposed that multiple *V* (*variable*) genes are separate from a single *C* (*constant*) gene in embryonic (germ-line) DNA. According to their model, one of these *V* genes becomes joined to the *C* gene in the course of differentiation of the antibody-producing cell. A critical test of this novel hypothesis had to await the isolation of pure immunoglobulin mRNA and the development of techniques for analyzing mammalian genomes. Twenty years later, Susumu Tonegawa found that *V* and *C* genes are indeed far apart in embryonic DNA but are closely associated in the DNA of antibody-producing cells. Thus, immunoglobulin genes are rearranged in the differentiation of lymphocytes.

33.4.1 J (Joining) Genes and D (Diversity) Genes Increase Antibody Diversity

Sequencing studies carried out by Susumu Tonegawa, Philip Leder, and Leroy Hood revealed that *V* genes in embryonic cells do not encode the entire variable region of L and H chains. Consider, for example, the region that encodes the κ light-chain family. A tandem array of 40 segments, each of which encodes approximately the first 97 residues of the variable domain of the L chain, is present on human chromosome 2 (Figure 33.14).



FIGURE 33.14 The κ light-chain locus. This part of human chromosome 2 includes an array of 40 segments that encode the variable (*V*) region (approximately residues 1–97) of the light chain, an array of 5 segments that encode the joining (*J*) region (residues 98–110), and a single region that encodes the constant (*C*) region.

However, the variable region of the L chain extends to residue 110. Where is the DNA that encodes the last 13 residues of the *V* region? For L chains in undifferentiated cells, this stretch of DNA is located in an unexpected place: near the *C* gene. It is called the *J* gene because it joins the *V* and *C* genes in a differentiated cell. In fact, a tandem array of five *J* genes is located near the *C* gene in embryonic cells. In the differentiation of an antibody-producing cell, a *V* gene becomes spliced to a *J* gene to form a complete gene for the variable region (Figure 33.15). RNA splicing generates

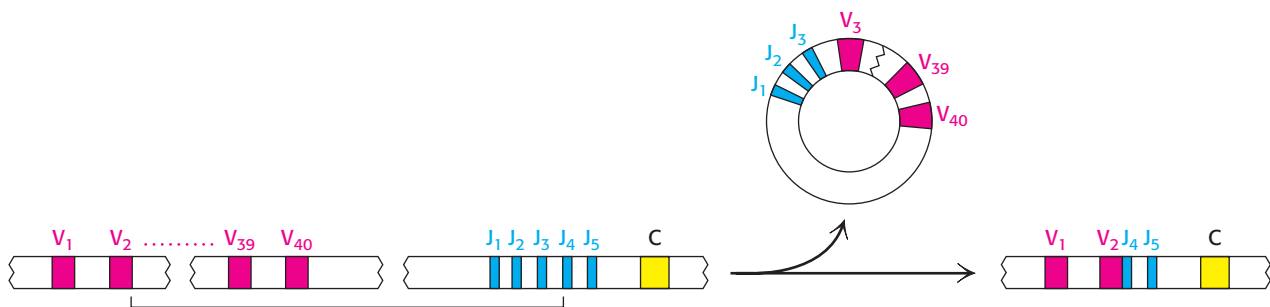


FIGURE 33.15 VJ recombination. A single *V* gene (in this case, V_2) is linked to a *J* gene (here, J_4) to form an intact VJ region. The intervening DNA is released in a circular form. Because the *V* and *J* regions are selected at random and the joint between them is not always in exactly the same place, many VJ combinations can be generated by this process.

an mRNA molecule for the complete L chain by linking the coding regions for the rearranged VJ unit with that for the C unit (Figure 33.16).

J genes are important contributors to antibody diversity because they encode part of the last hypervariable segment (CDR3). In forming a continuous variable-region gene, any of the 40 V genes can become linked to any of five J genes. Thus, somatic recombination of these gene segments amplifies the diversity already present in the germ line. The linkage between V and J is not precisely controlled. Recombination between these genes can take place at one of several bases near the codon for residue 95, generating additional diversity. A similar array of V and J genes encoding the λ light chain is present on human chromosome 22. This region includes 30 V_λ gene segments and four J_λ segments. In addition, this region includes four distinct C genes, in contrast with the single C gene in the κ locus.

In human beings, the genes encoding the heavy chain are present on chromosome 14. Remarkably, the variable domain of heavy chains is assembled from *three* rather than two segments. In addition to V_H genes that encode residues 1 to 94 and J_H segments that encode residues 98 to 113, this chromosomal region includes a distinct set of segments that encode residues 95 to 97 (Figure 33.17). These gene segments are called D for *diversity*. Some 27 D segments lie between 51 V_H and 6 J_H segments. The recombination process first joins a D segment to a J_H segment; a V_H segment is then joined to DJ_H . A greater variety of antigen-binding patches and clefts can be formed by the H chain than by the L chain because the H chain is encoded by three rather than two gene segments. Moreover, CDR3 of the H chain is diversified by the action of terminal deoxyribonucleotidyl transferase, a special DNA polymerase that requires no template. This enzyme inserts extra nucleotides between V_H and D. The $V(D)J$ recombination of both the L and the H chains is executed by specific enzymes present in immune cells. These proteins, called *RAG-1* and *RAG-2*, recognize specific DNA sequences called *recombination signal sequences (RSSs)* adjacent to the V, D, and J segments and facilitate the cleavage and religation of the DNA segments.

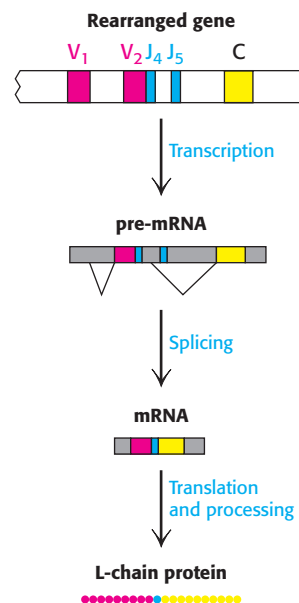


FIGURE 33.16 Light-chain expression. The light-chain protein is expressed by transcription of the rearranged gene to produce a pre-mRNA molecule with the VJ and C regions separated. RNA splicing removes the intervening sequences to produce an mRNA molecule with the VJ and C regions linked. Translation of the mRNA and processing of the initial protein product produces the light chain.

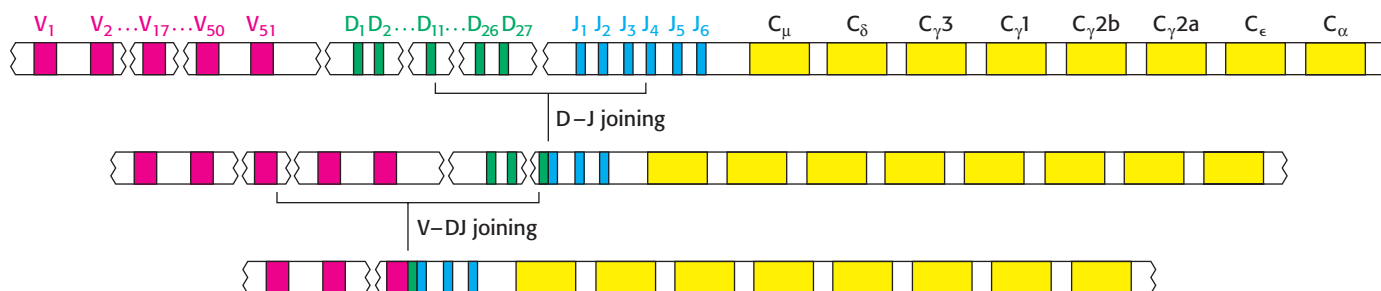


FIGURE 33.17 V(D)J recombination. The heavy-chain locus includes an array of 51 V segments, 27 D segments, and 6 J segments. Gene rearrangement begins with D-J joining, followed by further rearrangement to link the V segment to the DJ segment.

33.4.2 More Than 10⁸ Antibodies Can Be Formed by Combinatorial Association and Somatic Mutation

Let us recapitulate the sources of antibody diversity. The germ line contains a rather large repertoire of variable-region genes. For κ light chains, there are about 40 V-segment genes and five J-segment genes. Hence, a total of $40 \times 5 = 200$ kinds of complete V_κ genes can be formed by the combinations of V and J. A similar analysis suggests that at least 120 different λ light chains can be generated. A larger number of heavy-chain genes can be formed because of the role of the D segments. For 51 V, 27 D, and 6 J gene segments, the number of complete V_H genes that can be formed is 8262.

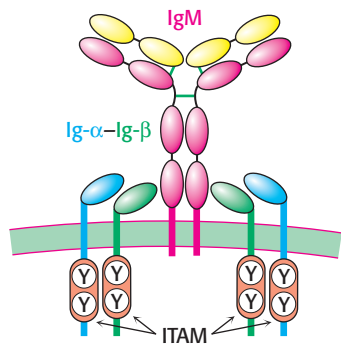


FIGURE 33.18 B-cell receptor. This complex consists of a membrane-bound IgM molecule noncovalently bound to two Ig- α -Ig- β heterodimers. The intracellular domains of each of the Ig- α and Ig- β chains include an immunoreceptor tyrosine-based activation motif (ITAM).

The association of 320 kinds of L chains with 8262 kinds of H chains would yield 2.6×10^6 different antibodies. Variability in the exact points of segment joining and other mechanisms increases this value by at least two orders of magnitude.

Even more diversity is introduced into antibody chains by *somatic mutation*—that is, the introduction of mutations into the recombined genes. In fact, a 1000-fold increase in binding affinity is seen in the course of a typical humoral immune response, arising from somatic mutation, a process called *affinity maturation*. The generation of an expanded repertoire leads to the selection of antibodies that more precisely fit the antigen. Thus, nature draws on each of three sources of diversity—a germ-line repertoire, somatic recombination, and somatic mutation—to form the rich variety of antibodies that protect an organism from foreign incursions.

33.4.3 The Oligomerization of Antibodies Expressed on the Surface of Immature B Cells Triggers Antibody Secretion

The processes heretofore described generate a highly diverse set of antibody molecules—a key first step in the generation of an immune response. The next stage is the selection of a particular set of antibodies directed against a specific invader. How does this selection occur? Each immature B cell, produced in the bone marrow, expresses a monomeric form of IgM attached to its surface (Figure 33.18). Each cell expresses approximately 10^5 IgM molecules, but *all of these molecules are identical in amino acid sequence and, hence, in antigen-binding specificity*. Thus, the selection of a particular immature B cell for growth will lead to the amplification of an antibody with a unique specificity. The selection process begins with the binding of an antigen to the membrane-bound antibody.

Associated with each membrane-linked IgM molecule are two molecules of a heterodimeric membrane protein called Ig- α -Ig- β (see Figure 33.18). Examination of the amino acid sequences of Ig- α and Ig- β is highly instructive. The amino terminus of each protein lies outside the cell and corresponds to a single immunoglobulin, and the carboxyl terminus, which lies inside the cell, includes a sequence of 18 amino acids called an *immunoreceptor tyrosine-based activation motif (ITAM)* (see Figure 33.18). As its name suggests, each ITAM includes key tyrosine residues, which are subject to phosphorylation by particular protein kinases present in immune-system cells.

A fundamental observation with regard to the mechanism by which the binding of antigen to membrane-bound antibody triggers the subsequent steps of the immune response is that *oligomerization or clustering of the antibody molecules is required* (Figure 33.19). The requirement for oligomerization is reminiscent of the dimerization of receptors triggered by growth hormone and epidermal growth factor encountered in Sections 15.4 and 15.4.1; indeed, the associated signaling mechanisms appear to be quite similar. The oligomerization of the membrane-bound antibodies results in the phosphorylation of the tyrosine residues within the ITAMs by protein tyrosine kinases including Lyn, a homolog of Src (Section 15.5). The phosphorylated ITAMs serve as docking sites for a protein kinase termed spleen tyrosine kinase (Syk), which has two SH2 domains that interact with the pair of phosphorylated tyrosines in each ITAM.

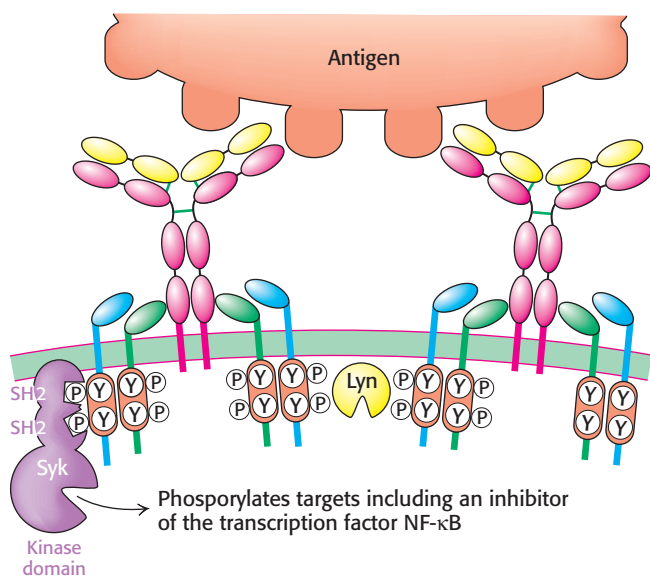

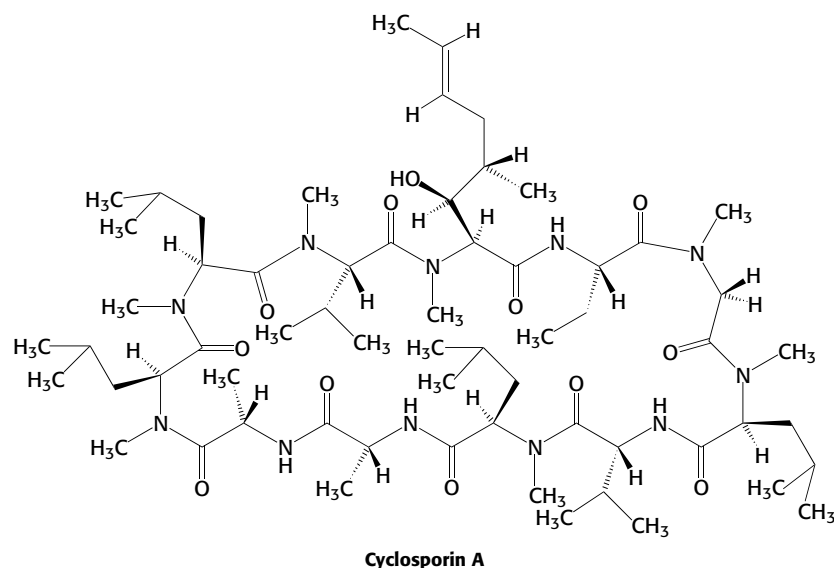


FIGURE 33.19 B-cell activation. The binding of multivalent antigen such as bacterial or viral surfaces links membrane-bound IgM molecules. This oligomerization triggers the phosphorylation of tyrosine residues in the ITAM sequences by protein tyrosine kinases such as Lyn. After phosphorylation, the ITAMs serve as docking sites for Syk, a protein kinase that phosphorylates a number of targets, including transcription factors.

The oligomerization of the membrane-bound antibodies results in the phosphorylation of the tyrosine residues within the ITAMs by protein tyrosine kinases including Lyn, a homolog of Src (Section 15.5). The phosphorylated ITAMs serve as docking sites for a protein kinase termed spleen tyrosine kinase (Syk), which has two SH2 domains that interact with the pair of phosphorylated tyrosines in each ITAM.

Syk, when activated by phosphorylation, proceeds to phosphorylate other signal-transduction proteins including an inhibitory subunit of a transcription factor called NF- κ B and an isoform of phospholipase C. The signaling processes continue downstream to activate gene expression, leading to the stimulation of cell growth and initiating further B-cell differentiation.

 Drugs that modulate the immune system have served as sources of insight into immune-system signaling pathways. For example, *cyclosporin*, a powerful suppressor of the immune system, acts by blocking a phosphatase called *calcineurin*, which normally activates a transcription factor called NF-AT by dephosphorylating it.



The potent immune suppression that results reveals how crucial the activity of this transcription factor is to the development of an immune response. Without drugs such as cyclosporin, organ transplantation would be extremely difficult because transplanted tissue expresses a wide range of foreign antigens, which causes the immune system to reject the new tissue.

The role of oligomerization in the B-cell signaling pathway is illuminated when we consider the nature of many antigens presented by pathogens. The surfaces of many viruses, bacteria, and parasites are characterized by arrays of identical membrane proteins or membrane-linked carbohydrates. Thus, most pathogens present multiple binding surfaces that will naturally cause membrane-associated antibodies to oligomerize as they bind adjacent epitopes. In addition, the mechanism accounts for the observation that most small molecules do not induce an immune response; however, coupling multiple copies of the small molecule to a large oligomeric protein such as key-hole limpet hemocyanin (KLH), which has a molecular mass of close to 1 million daltons or more, promotes antibody oligomerization and, hence, the production of antibodies against the small-molecule epitope. The large protein is called the *carrier* of the attached chemical group, which is called a *haptenic determinant*. The small foreign molecule by itself is called a *hapten*. Antibodies elicited by attached haptens will bind unattached haptens as well.

33.4.4 Different Classes of Antibodies Are Formed by the Hopping of V_H Genes

The development of an effective antibody-based immune response depends on the secretion into the blood of antibodies that have appropriate effector functions. At the beginning of this response, an alternative mRNA splicing

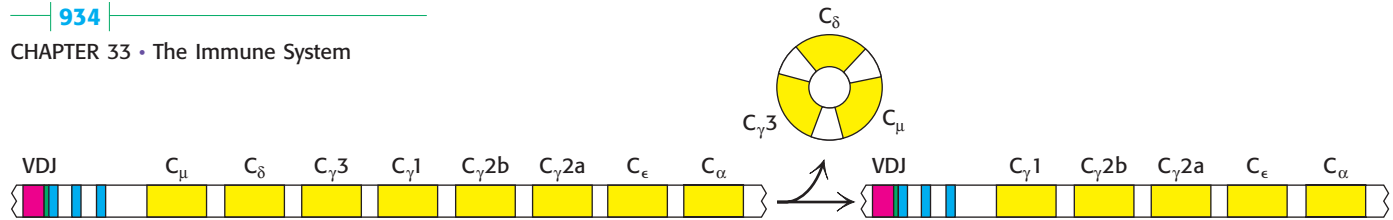


FIGURE 33.20 Class switching. Further rearrangement of the heavy-chain locus results in the generation of genes for antibody classes other than IgM. In the case shown, rearrangement places the VDJ region next to the $C_{\gamma 1}$ region, resulting in the production of IgG1. Note that no further rearrangement of the VDJ region takes place, so the specificity of the antibody is not affected.

pathway is activated so that the production of membrane-linked IgM is supplanted by the synthesis of secreted IgM. As noted in Section 33.1, secreted IgM is pentameric and has a relatively high avidity for multivalent antigens. Later, the antibody-producing cell makes either IgG, IgA, IgD, or IgE of the same specificity as the initially secreted IgM. In this switch, the light chain is unchanged, as is the variable region of the heavy chain. Only the constant region of the heavy chain changes. This step in the differentiation of an antibody-producing cell is called *class switching* (Figure 33.20). In undifferentiated cells, the genes for the constant region of each class of heavy chain, called C_{μ} , C_{δ} , C_{γ} , C_{ϵ} , and C_{α} , are next to each other. There are eight in all, including four genes for the constant regions of γ chains. A complete gene for the heavy chains of IgM antibody is formed by the translocation of a V_H gene segment to a DJ_H gene segment.

How are other heavy chains formed? Class switching is mediated by a gene-rearrangement process that moves a VDJ gene from a site near one C gene to a site near another C gene. Importantly, *the antigen-binding specificity is conserved in class switching because the entire V_HDJ_H gene is translocated in an intact form*. For example, the antigen-combining specificity of IgA produced by a particular cell is the same as that of IgM synthesized at an earlier stage of its development. The biological significance of C_H switching is that a whole recognition domain (the variable domain) is shifted from the early constant region (C_{μ}) to one of several other constant regions that mediate different effector functions.

33.5 MAJOR-HISTOCOMPATIBILITY-COMPLEX PROTEINS PRESENT PEPTIDE ANTIGENS ON CELL SURFACES FOR RECOGNITION BY T-CELL RECEPTORS

Soluble antibodies are highly effective against extracellular pathogens, but they confer little protection against microorganisms that are predominantly intracellular, such as viruses and mycobacteria (which cause tuberculosis and leprosy). These pathogens are shielded from antibodies by the host-cell membrane (Figure 33.21). A different and more subtle strategy, *cell-mediated*



FIGURE 33.21 Intracellular pathogen. An electron micrograph showing mycobacteria (arrows) inside an infected macrophage. [Courtesy of Dr. Stanley Falkow.]

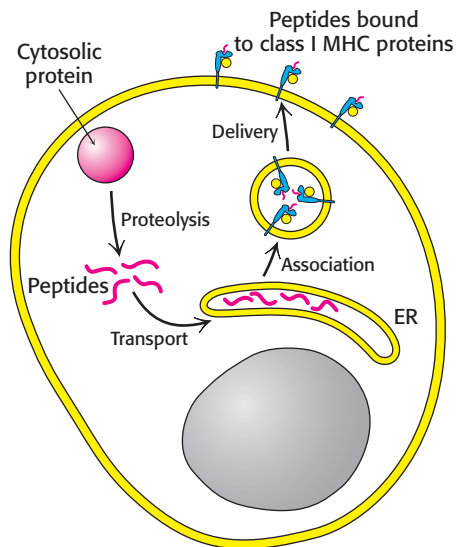


FIGURE 33.22 Presentation of peptides from cytosolic proteins. Class I MHC proteins on the surfaces of most cells display peptides that are derived from cytosolic proteins by proteolysis.

immunity, evolved to cope with intracellular pathogens. *T cells* continually scan the surfaces of all cells and kill those that exhibit foreign markings. The task is not simple; intracellular microorganisms are not so obliging as to intentionally leave telltale traces on the surface of their host. Quite the contrary, successful pathogens are masters of the art of camouflage. Vertebrates have devised an ingenious mechanism—cut and display—to reveal the presence of stealthy intruders. Nearly all vertebrate cells exhibit on their surfaces a sample of peptides derived from the digestion of proteins in their cytosol. These peptides are displayed by integral membrane proteins that are encoded by the *major histocompatibility complex (MHC)*. Specifically, peptides derived from cytosolic proteins are bound to *class I MHC proteins*.

How are these peptides generated and delivered to the plasma membrane? The process starts in the cytosol with the degradation of proteins, self proteins as well as those of pathogens (Figure 33.22). Digestion is carried out by proteasomes (Section 23.2.2). The resulting peptide fragments are transported from the cytosol into the lumen of the endoplasmic reticulum by an ATP-driven pump. In the ER, peptides combine with nascent class I MHC proteins; these complexes are then targeted to the plasma membrane.

MHC proteins embedded in the plasma membrane tenaciously grip their bound peptides so that they can be touched and scrutinized by T-cell receptors on the surface of a killer cell. Foreign peptides bound to class I MHC proteins signal that a cell is infected and mark it for destruction by cytotoxic T cells. An assembly consisting of the foreign peptide–MHC complex, the T-cell receptor, and numerous accessory proteins triggers a cascade that induces apoptosis in the infected cell. Strictly speaking, infected cells are not killed but, instead, are triggered to commit suicide to aid the organism.

33.5.1 Peptides Presented by MHC Proteins Occupy a Deep Groove Flanked by Alpha Helices

The three-dimensional structure of a large fragment of a human MHC class I protein, *human leukocyte antigen A2 (HLA-A2)*, was solved in 1987 by Don Wiley and Pamela Bjorkman. Class I MHC proteins consist of a 44-kd α chain noncovalently bound to a 12-kd polypeptide called β_2 -microglobulin. The α chain has three extracellular domains (α_1 , α_2 , and α_3), a transmembrane segment, and a tail that extends into the cytosol (Figure

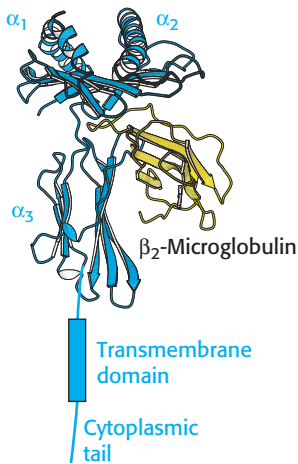


FIGURE 33.23 Class I MHC protein. A protein of this class consists of two chains. The α chain begins with two domains that include α helices (α_1 , α_2), an immunoglobulin domain (α_3), a transmembrane domain, and a cytoplasmic tail. The second chain, β_2 -microglobulin, adopts an immunoglobulin fold.

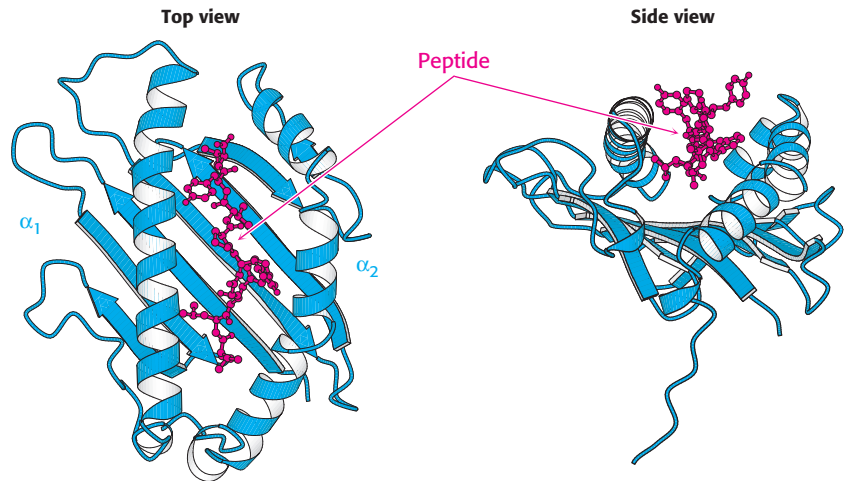
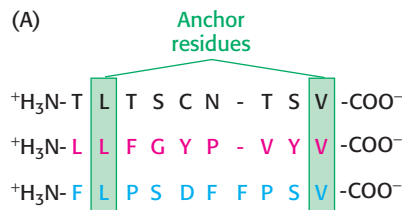


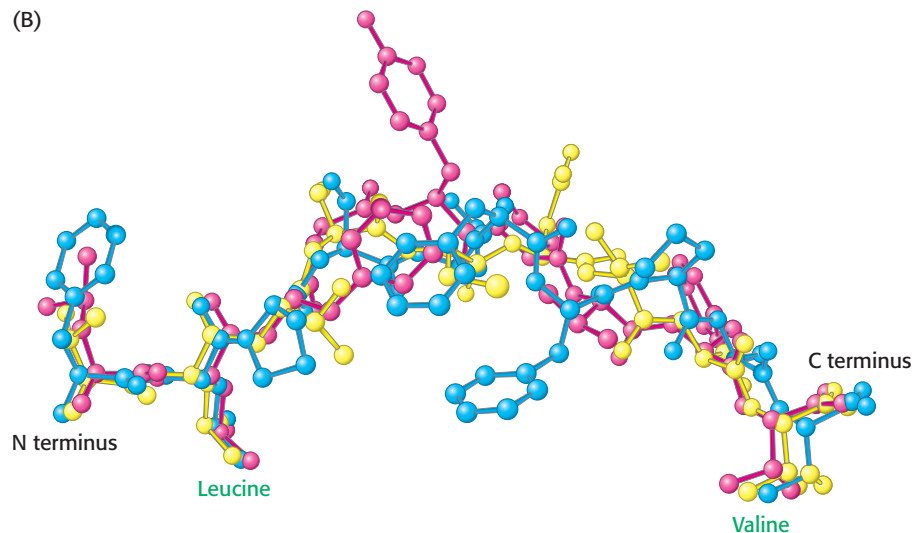
FIGURE 33.24 Class I MHC peptide-binding site. The α_1 and α_2 domains come together to form a groove in which peptides are displayed. The two views shown reveal that the peptide is surrounded on three sides by a β sheet and two α helices, but it is accessible from the top of the structure.

FIGURE 33.25 Anchor residues. (A) The amino acid sequences of three peptides that bind to the class I MHC protein HLA-A2 are shown. Each of these peptides has leucine in the second position and valine in the carboxyl-terminal position. (B) Comparison of the structures of these peptides reveals that the amino and carboxyl termini as well as the side chains of the leucine and valine residues are in essentially the same position in each peptide, whereas the remainder of the structures are quite different.



33.23). Cleavage by papain of the HLA α chain several residues before the transmembrane segment yielded a soluble heterodimeric fragment. The β_2 -microglobulin subunit and the α_3 domains have immunoglobulin folds, although the pairing of the two domains differs from that in antibodies. The α_1 and α_2 domains exhibit a novel and remarkable architecture. They associate intimately to form a deep groove that serves as the peptide-binding site (Figure 33.24). The floor of the groove, which is about 25 Å long and 10 Å wide, is formed by eight β strands, four from each domain. A long helix contributed by the α_1 domain forms one side, and a helix contributed by the α_2 domain forms the other side. *This groove is the binding site for the presentation of peptides.*

The groove can be filled by a peptide from 8 to 10 residues long in an extended conformation. As we shall see (Section 33.5.6), MHC proteins are remarkably diverse in the human population; each person expresses as many as six distinct class I MHC proteins and many different forms are present in different people. The first structure determined, HLA-A2, binds peptides that almost always have leucine in the second position and valine in the last position (Figure 33.25). Side chains from the MHC molecule in-



teract with the amino and carboxyl termini and with the side chains in these two key positions. These residues are often referred to as the *anchor residues*. The other residues are highly variable. Thus, many millions of different peptides can be presented by this particular class I MHC protein; the identities of only two of the nine residues are crucial for binding. Each class of MHC molecules requires a unique set of anchor residues. Thus, a tremendous range of peptides can be presented by these molecules. Note that *one face of the bound peptide is exposed to solution where it can be examined by other molecules, particularly T-cell receptors*. An additional remarkable feature of MHC–peptide complexes is their kinetic stability; once bound, a peptide is not released, even over a period of days.

33.5.2 T-Cell Receptors Are Antibody-like Proteins Containing Variable and Constant Regions

We are now ready to consider the receptor that recognizes peptides displayed by MHC proteins on target cells. The *T-cell receptor* consists of a 43-kd α chain (T_{α}) joined by a disulfide bond to a 43-kd β chain (T_{β} ; Figure 33.26). Each chain spans the plasma membrane and has a short carboxyl-terminal region on the cytosolic side. A small proportion of T cells express a receptor consisting of γ and δ chains in place of α and β . T_{α} and T_{β} , like immunoglobulin L and H chains, consist of *variable* and *constant* regions. Indeed, *these domains of the T-cell receptor are homologous to the V and C domains of immunoglobulins*. Furthermore, hypervariable sequences present in the V regions of T_{α} and T_{β} form the binding site for the epitope.

The genetic architecture of these proteins is similar to that of immunoglobulins. The variable region of T_{α} is encoded by about 50 V-segment genes and 70 J-segment genes. T_{β} is encoded by two D-segment genes in addition to 57 V and 13 J-segment genes. Again, the diversity of component genes and the use of slightly imprecise modes of joining them increase the number of distinct proteins formed. *At least 10^{12} different specificities could arise from combinations of this repertoire of genes*. Thus, T-cell receptors, like immunoglobulins, can recognize a very large number of different epitopes. All the receptors on a particular T cell have the same specificity.

How do T cells recognize their targets? The variable regions of the α and β chains of the T-cell receptor form a binding site that recognizes a combined epitope—foreign peptide bound to an MHC protein (Figure 33.27). Neither the foreign peptide alone nor the MHC protein alone forms a complex with the T-cell receptor. Thus, fragments of an intracellular pathogen are presented in a context that allows them to be detected, leading to the initiation of an appropriate response.

33.5.3 CD8 on Cytotoxic T Cells Acts in Concert with T-Cell Receptors

The T-cell receptor does not act alone in recognizing and mediating the fate of target cells. Cytotoxic T cells also express a protein termed *CD8* on their surfaces that is crucial for the recognition of the class I MHC–peptide complex. The abbreviation CD stands for *cluster of differentiation*, referring to a cell-surface marker that is used to identify a lineage or stage of differentiation. Antibodies specific for particular CD proteins have been invaluable

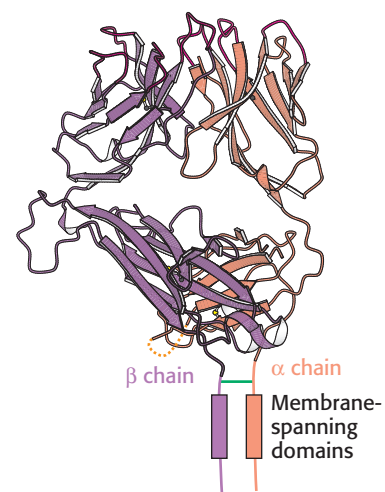


FIGURE 33.26 T-cell receptor. This protein consists of an α chain and a β chain, each of which consists of two immunoglobulin domains and a membrane-spanning domain. The two chains are linked by a disulfide bond.

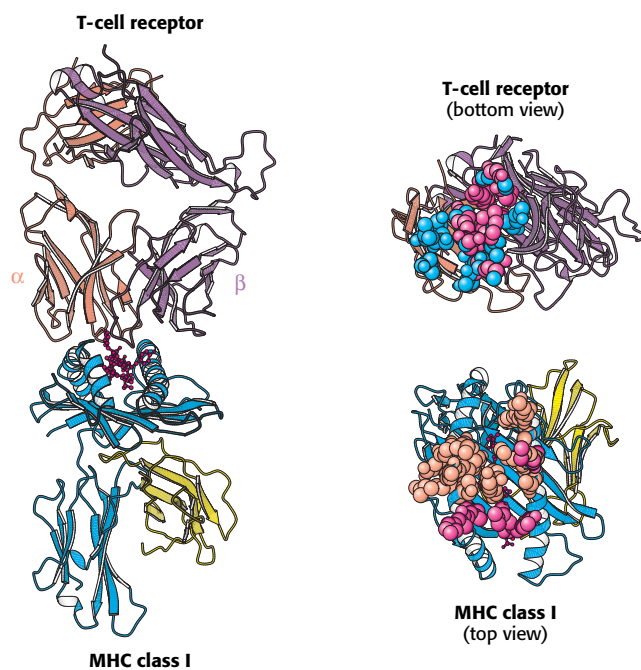


FIGURE 33.27 T-cell receptor–Class I MHC complex. The T-cell receptor binds to a class I MHC protein containing a bound peptide. The T-cell receptor contacts both the MHC protein and the peptide as shown by surfaces exposed when the complex is separated (right). These surfaces are colored according to the chain that they contact.

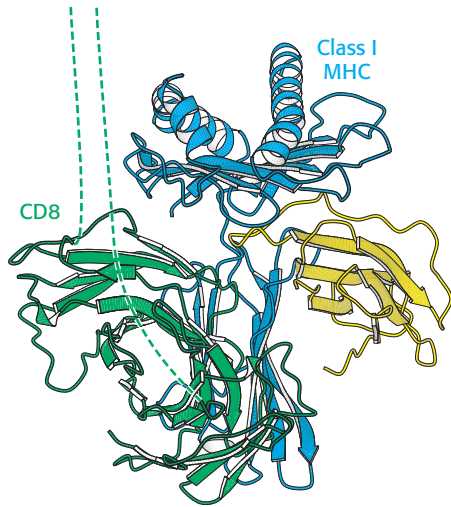


FIGURE 33.28 The coreceptor CD8. This dimeric protein extends from the surface of a cytotoxic T cell and binds to class I MHC molecules that are expressed on the surface of the cell that is bound to the T cell. The dashed lines represent extended polypeptide chains that link the immunoglobulin domain of CD8 to the membrane.

in following the development of leukocytes and in discovering new interactions between specific cell types.

Each chain in the CD8 dimer contains a domain that resembles an immunoglobulin variable domain (Figure 33.28). CD8 interacts primarily with the relatively constant α_3 domain of class I MHC proteins. This interaction further stabilizes the interactions between the T cell and its target. The cytosolic tail of CD8 contains a docking site for Lck, a cytosolic tyrosine kinase akin to Src. The T-cell receptor itself is associated with six polypeptides that form the CD3 complex (Figure 33.29). The γ , δ , and ϵ chains of CD3 are homologous to Ig- α and Ig- β associated with the B-cell receptor (Section 33.4.3); each chain consists of an extracellular immunoglobulin domain and an intracellular ITAM region. These chains associate into CD3 $\gamma\epsilon$ and CD3 $\delta\epsilon$ heterodimers. An additional component, the CD3 ζ chain, has only a small extracellular domain and a larger intracellular domain containing three ITAM sequences.

On the basis of these components, a model for T-cell activation can be envisaged that is closely parallel to the pathway for B-cell activation (Section 33.3; Figure 33.30). The binding of the T-cell receptor with the class I MHC-peptide complex and the concomitant binding of CD8 from the T-cell with the MHC molecule results in the association of the kinase Lck with the ITAM substrates of the components of the CD3 complex. Phosphorylation of the tyrosine residues in the ITAM sequences generates docking sites for a protein kinase called ZAP-70 (for 70-kd zeta-associated protein) that is homologous to Syk in B cells. Docked by its two SH2 domains, ZAP-70 phosphorylates downstream targets in the signaling cascade. Additional molecules, including a membrane-bound protein phosphatase called CD45 and a cell-surface protein called CD28, play ancillary roles in this process.

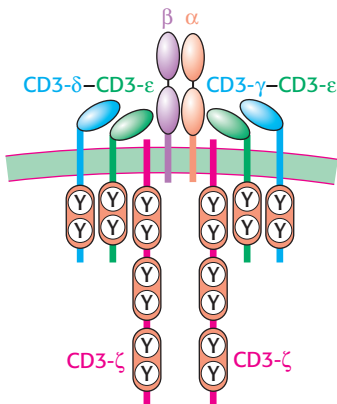


FIGURE 33.29 T-cell receptor complex. The T-cell receptor is associated with six CD3 molecules: a CD3- γ -CD3- ϵ heterodimer, a CD3- δ -CD3- ϵ heterodimer, and two chains of CD3- ζ . Single ITAM sequences are present in the cytoplasmic domains of CD3- γ , CD3- δ , and CD3- ϵ whereas three such sequences are found in each CD3- ζ chain.

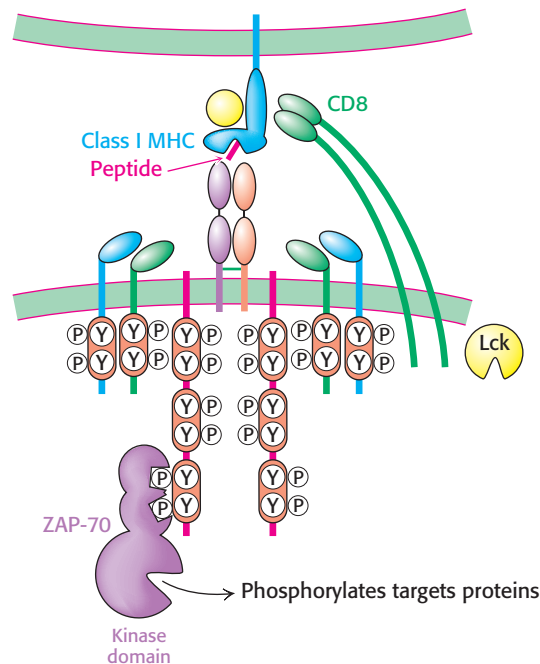


FIGURE 33.30 T-cell activation. The interaction between the T-cell receptor and a class I MHC-peptide complex results in the binding of CD8 to the MHC protein, the recruitment of the protein tyrosine kinase Lck, and the phosphorylation of tyrosine residues in the ITAM sequences of the CD3 chains. After phosphorylation, the ITAM regions serve as docking sites for the protein kinase ZAP-70, which phosphorylates protein targets to transmit the signal.

T-cell activation has two important consequences. First, the activation of cytotoxic T cells results in the secretion of *perforin*. This 70-kd protein makes the cell membrane of the target cell permeable by polymerizing to form transmembrane pores 10 nm wide (Figure 33.31). The cytotoxic T cell then secretes proteases called *granzymes* into the target cell. These enzymes initiate the pathway of apoptosis (Section 18.6.6), leading to the death of the target cell and the fragmentation of its DNA, including any viral DNA that may be present. Second, after it has stimulated its target cell to commit suicide, the activated T cell disengages and is stimulated to reproduce. Thus, additional T cells that express the same T-cell receptor are generated to continue the battle against the invader after these T cells have been identified as a suitable weapon.

33.5.4 Helper T Cells Stimulate Cells That Display Foreign Peptides Bound to Class II MHC Proteins

Not all T cells are cytotoxic. *Helper T cells*, a different class, stimulate the proliferation of specific B lymphocytes and cytotoxic T cells and thereby serve as partners in determining the immune responses that are produced. The importance of helper T cells is graphically revealed by the devastation wrought by AIDS, a condition that destroys these cells. Helper T cells, like cytotoxic T cells, detect foreign peptides that are presented on cell surfaces by MHC proteins. However, the source of the peptides, the MHC proteins that bind them, and the transport pathway are different.

Helper T cells recognize peptides bound to MHC molecules referred to as class II. Their helping action is focused on B cells, macrophages, and dendritic cells. *Class II MHC proteins* are expressed only by these *antigen-presenting cells*, unlike class I MHC proteins, which are expressed on nearly all cells. The peptides presented by class II MHC proteins do not come from the cytosol. Rather, they arise from the degradation of proteins that have been internalized by endocytosis. Consider, for example, a virus particle that is captured by membrane-bound immunoglobulins on the surface of a B cell (Figure 33.32). This complex is delivered to an endosome, a membrane-enclosed acidic compartment, where it is digested. The resulting peptides become associated with class II MHC proteins, which move to the cell surface. Peptides from the cytosol cannot reach class II proteins, whereas peptides from endosomal compartments cannot reach class I proteins. This segregation of displayed peptides is biologically critical. The association of

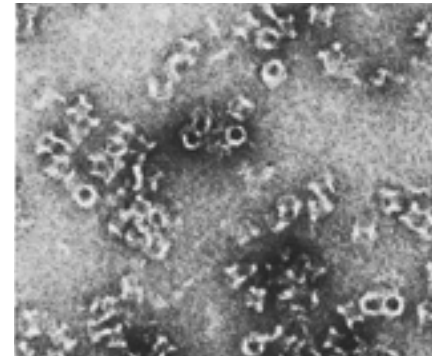


FIGURE 33.31 Consequences of cytotoxic-T-cell action. An electron micrograph showing pores in the membrane of a cell that has been attacked by a cytotoxic T cell. The pores are formed by the polymerization of perforin, a protein secreted by the cytotoxic T cell. [Courtesy of Dr. Eckhard Podock.]

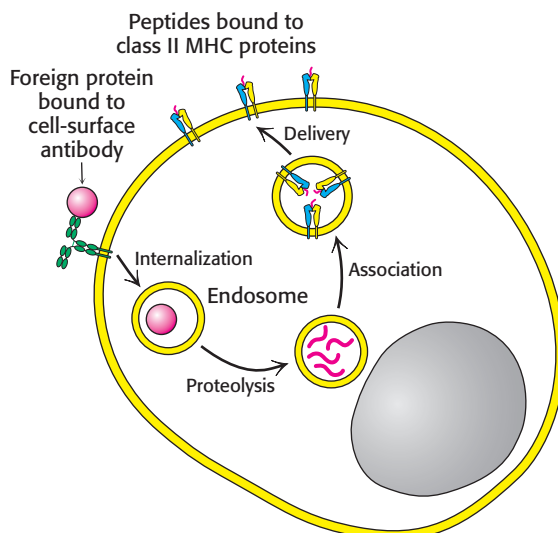


FIGURE 33.32 Presentation of peptides from internalized proteins. Antigen-presenting cells bind and internalize foreign proteins and display peptides that are formed from the digestion of these proteins in Class II MHC proteins.

a foreign peptide with a class II MHC protein signals that a cell has *encountered* a pathogen and serves as a call for *help*. In contrast, association with a class I MHC protein signals that a cell has *succumbed* to a pathogen and is a call for *destruction*.

33.5.5 Helper T Cells Rely on the T-Cell Receptor and CD4 to Recognize Foreign Peptides on Antigen-Presenting Cells

The overall structure of a class II MHC molecule is remarkably similar to that of a class I molecule. Class II molecules consist of a 33-kd α chain and a noncovalently bound 30-kd β chain (Figure 33.33).

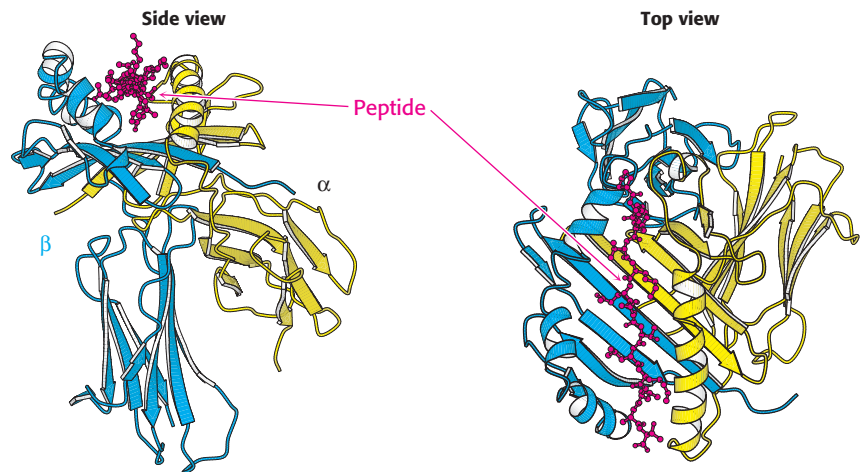


FIGURE 33.33 Class II MHC protein. A class II MHC protein consists of homologous α and β chains, each of which has an amino-terminal domain that constitutes half of the peptide-binding structure, as well as a carboxyl-terminal immunoglobulin domain. The peptide-binding site is similar to that in class I MHC proteins except that it is open at both ends, allowing class II MHC proteins to bind longer peptides than those bound by class I.



FIGURE 33.34 Coreceptor CD4. This protein comprises four tandem immunoglobulin domains that extend from the surface of helper T cells.

Each contains two extracellular domains, a transmembrane segment, and a short cytosolic tail. The peptide-binding site is formed by the α_1 and β_1 domains, each of which contributes a long helix and part of a β sheet. Thus, the same structural elements are present in class I and class II MHC molecules, but they are combined into polypeptide chains in different ways. Class II MHC molecules appear to form stable dimers, unlike class I molecules, which are monomeric. The peptide-binding site of a class II molecule is open at both ends, and so this groove can accommodate longer peptides than can be bound by class I molecules; typically, peptides between 13 and 18 residues long are bound. The peptide-binding specificity of each class II molecule depends on binding pockets that recognize particular amino acids in specific positions along the sequence.

Helper T cells express T-cell receptors that are produced from the same genes as those on cytotoxic T cells. These T-cell receptors interact with class II MHC molecules in a manner that is analogous to T-cell-receptor interaction with class I MHC molecules. Nonetheless, helper T cells and cytotoxic T cells are distinguished by other proteins that they express on their surfaces. In particular, helper T cells express a protein called CD4 instead of expressing CD8. CD4 consists of four immunoglobulin domains that extend from the T-cell surface, as well as a small cytoplasmic region (Figure 33.34). The amino-terminal immunoglobulin domains of CD4 interact with the base on the class II MHC molecule. Thus, helper T cells bind cells expressing class II MHC specifically because of the interactions with CD4 (Figure 33.35).

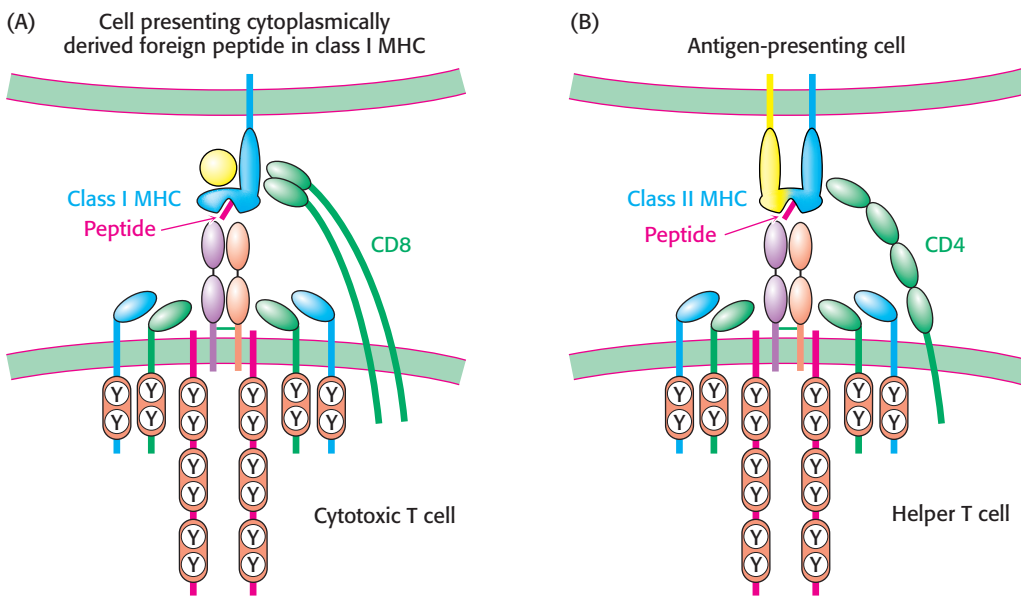



FIGURE 33.35 Variations on a theme. (A) Cytotoxic T cells recognize foreign peptides presented in class I MHC proteins with the aid of the coreceptor CD8. (B) Helper T cells recognize peptides presented in class II MHC proteins by specialized antigen-presenting cells with the aid of the coreceptor CD4.

When a helper T cell binds to an antigen-presenting cell expressing an appropriate class II MHC–peptide complex, signaling pathways analogous to those in cytotoxic T cells are initiated by the action of the kinase Lck on ITAMs in the CD3 molecules associated with the T-cell receptor. However, rather than triggering events leading to the death of the attached cell, *these signaling pathways result in the secretion of cytokines from the helper cell.* Cytokines are a family of molecules that include, among others, interleukin-2 and interferon- γ . Cytokines bind to specific receptors on the antigen-presenting cell and stimulate growth, differentiation, and in regard to plasma cells, which are derived from B cells, antibody secretion (Figure 33.36). Thus, the internalization and presentation of parts of a foreign pathogen help to generate a local environment in which cells taking part in the defense against this pathogen can flourish through the action of helper T cells.

33.5.6 MHC Proteins Are Highly Diverse

 MHC class I and II proteins, the presenters of peptides to T cells, were discovered because of their role in *transplantation rejection*. A tissue transplanted from one person to another or from one mouse to another is usually rejected by the immune system. In contrast, tissues transplanted from one identical twin to another or between mice of an inbred strain are accepted. Genetic analyses revealed that rejection occurs when tissues are transplanted between individuals having different genes in the major histocompatibility complex, a cluster of more than 75 genes playing key roles in immunity. The 3500-kb span of the MHC is nearly the length of the

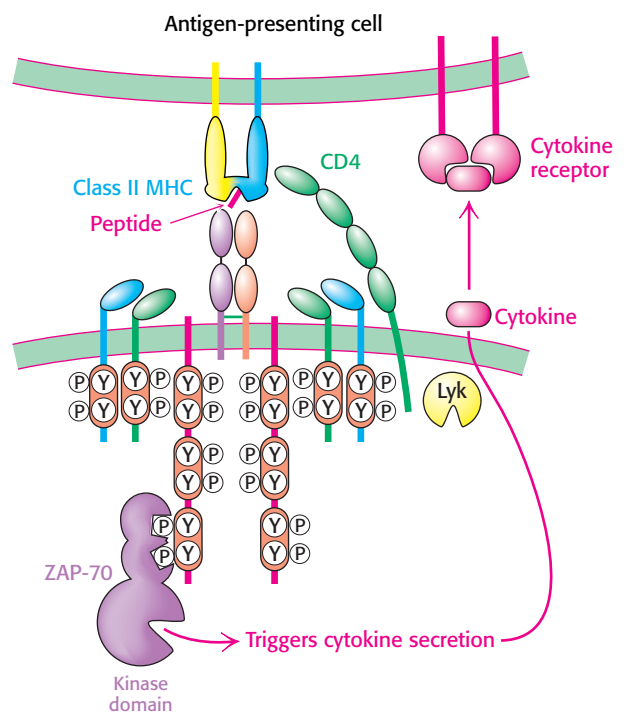


FIGURE 33.36 Helper T cell action. The engagement of the T-cell receptor in helper T cells results in the secretion of cytokines. These cytokines bind to cytokine receptors expressed on the surface of the antigen-presenting cell, stimulating cell growth, differentiation, and, in regard to a B cell, antibody secretion.

entire *E. coli* chromosome. The MHC encodes class I proteins (presenters to cytotoxic T cells) and class II proteins (presenters to helper T cells), as well as class III proteins (components of the complement cascade) and many other proteins that play key roles in immunity.

Human beings express six different class I genes (three from each parent) and six different class II genes. The three loci for class I genes are called HLA-A, -B, and -C; those for class II genes are called HLA-DP, -DQ, and -DR. These loci are *highly polymorphic*: many alleles of each are present in the population. For example, more than 50 each of HLA-A, -B, and -C alleles are known; the numbers discovered increase each year. Hence, the likelihood that two unrelated persons have identical class I and II proteins is very small ($<10^{-4}$), accounting for transplantation rejection unless the genotypes of donor and acceptor are closely matched in advance.

Differences between class I proteins are located mainly in the α_1 and α_2 domains, which form the peptide-binding site (Figure 33.37). The α_3 domain, which interacts with a constant β_2 -microglobulin is largely conserved. Similarly, the differences between class II proteins cluster near the peptide-binding groove. Why are MHC proteins so highly variable? *Their diversity makes possible the presentation of a very wide range of peptides to T cells.* A particular class I or class II molecule may not be able to bind any of the peptide fragments of a viral protein. The likelihood of a fit is markedly increased by having several kinds (usually six) of each class of presenters in each individual. If all members of a species had identical class I or class II molecules, the population would be much more vulnerable to devastation by a pathogen that had evolved to evade presentation. The evolution of the diverse human MHC repertoire has been driven by the selection for individual members of the species who resist infections to which other members of the population may be susceptible.

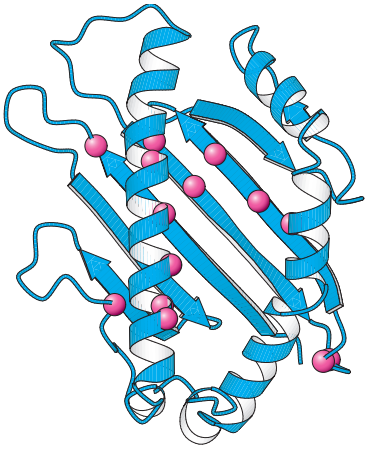


FIGURE 33.37 Polymorphism in class I MHC proteins. The positions of sites with a high degree of polymorphism in the human population are displayed as red spheres on the structure of the amino-terminal part of a class I MHC protein.

33.5.7 Human Immunodeficiency Viruses Subvert the Immune System by Destroying Helper T Cells

In 1981, the first cases of a new disease now called *acquired immune deficiency syndrome (AIDS)* were recognized. The victims died of rare infections because their immune systems were crippled. The cause was identified two years later by Luc Montagnier and coworkers. AIDS is produced by *human immunodeficiency virus (HIV)*, of which two major classes are known: HIV-1 and the much less common HIV-2. Like other *retroviruses*, HIV contains a single-stranded RNA genome that is replicated through a double-stranded DNA intermediate. This viral DNA becomes integrated into the genome of the host cell. In fact, viral genes are transcribed only when they are integrated into the host DNA.

The HIV virion is enveloped by a lipid bilayer membrane containing two glycoproteins: gp41 spans the membrane and is associated with gp120, which is located on the external face (Figure 33.38). The core of the virus contains two copies of the RNA genome and associated transfer RNAs, and several molecules of reverse transcriptase. They are surrounded by many copies of two proteins called p18 and p24. *The host cell for HIV is the helper T cell.* The gp120 molecules on the membrane of HIV bind to CD4 molecules on the surface of the helper T cell (Figure 33.39). This interaction allows the associated viral gp41 to insert its amino-terminal head into the host-cell membrane. The viral membrane and the helper-cell membrane fuse, and the viral core is released directly into the cytosol. Infection by HIV leads to the destruction of helper T cells because the permeability of the host plasma membrane is markedly increased by the insertion of viral

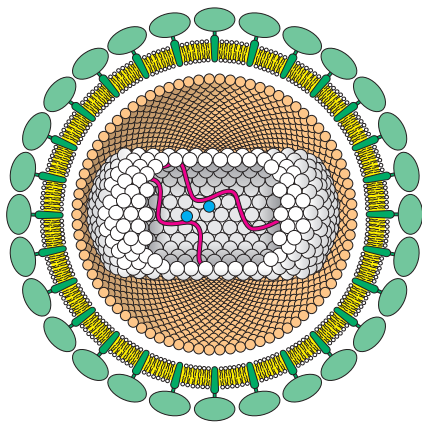


FIGURE 33.38 Human immunodeficiency virus. A schematic diagram of HIV reveals its proteins and nucleic acid components. The membrane-envelope glycoproteins gp41 and gp120 are shown in dark and light green. The viral RNA is shown in red, and molecules of reverse transcriptase are shown in blue. [After R. C. Gallo. *The AIDS virus*. Copyright © 1987 by Scientific American, Inc. All rights reserved.]

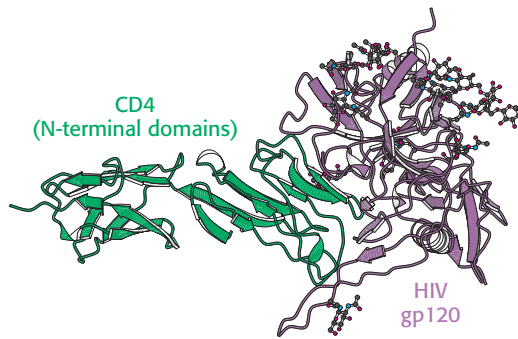


FIGURE 33.39 HIV receptor. A complex between a modified form of the envelope glycoprotein gp120 from HIV and a peptide corresponding to the two amino-terminal domains from the helper T-cell protein CD4 reveals how viral infection of helper T cells is initiated.

glycoproteins and the budding of virus particles. The influx of ions and water disrupts the ionic balance, causing osmotic lysis.



The development of an effective AIDS vaccine is difficult owing to the antigenic diversity of HIV strains. Because its mechanism for replication is quite error prone, a population of HIV presents an ever-changing array of coat proteins. Indeed, the mutation rate of HIV is more than 65 times as high as that of influenza virus. A major aim now is to define relatively conserved sequences in these HIV proteins and use them as immunogens.

33.6 IMMUNE RESPONSES AGAINST SELF-ANTIGENS ARE SUPPRESSED

The primary function of the immune system is to protect the host from invasion by foreign organisms. But how does the immune system avoid mounting attacks against the host organism? In other words, how does the immune system distinguish between self and nonself? Clearly, proteins from the organism itself do not bear some special tag identifying them. Instead, selection processes early in the developmental pathways for immune cells kill or suppress those immune cells that react strongly with self-antigens. The evolutionary paradigm still applies; immune cells that recognize self-antigens are generated, but selective mechanisms eliminate such cells in the course of development.

33.6.1 T Cells Are Subject to Positive and Negative Selection in the Thymus

T cells derive their name from the location of their production—the thymus, a small organ situated just above the heart. Examination of the developmental pathways leading to the production of mature cytotoxic and helper T cells reveals the selection mechanisms that are crucial for distinguishing self from nonself. These selection criteria are quite stringent; approximately 98% of the thymocytes, the precursors of T cells, die before the completion of the maturation process.

Thymocytes produced in the bone marrow do not express the T-cell-receptor complex, CD4, or CD8. On relocation to the thymus and rearrangement of the T-cell-receptor genes, the immature thymocyte expresses all of these molecules. These cells are first subjected to *positive selection*

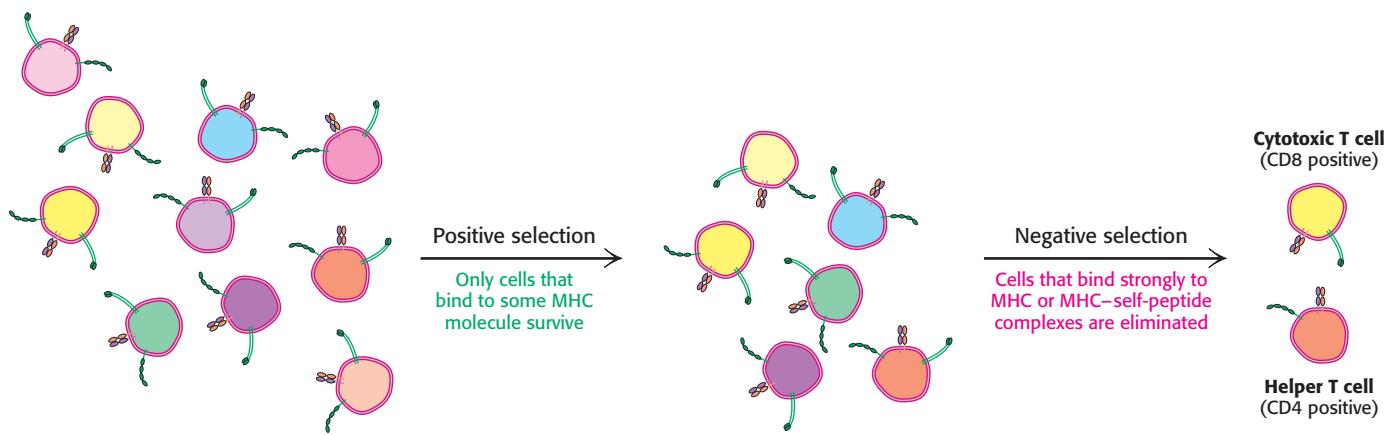


FIGURE 33.40 T-cell selection. A population of thymocytes is subjected first to positive selection to remove cells that express T-cell receptors that will not bind to MHC proteins expressed by the individual organism. The surviving cells are then subjected to negative selection to remove cells that bind strongly to MHC complexes bound to self-peptides.

(Figure 33.40). Cells for which the T-cell receptor can bind with reasonable affinity to either class I or class II MHC molecules survive this selection; those for which the T-cell receptor does not participate in such an interaction undergo apoptosis and die. The affinities of interaction required to pass

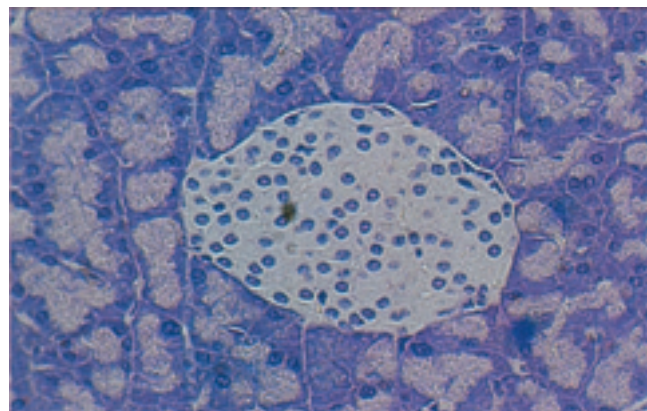
this selection are relatively modest, and so contacts between the T-cell receptor and the MHC molecules themselves are sufficient without any significant contribution from the bound peptides (which will be derived from proteins in the thymus). *The role of the positive selection step is to prevent the production of T cells that will not bind to any MHC complex present, regardless of the peptide bound.*

The cell population that survives positive selection is subjected to a second step, *negative selection*. Here, T cells that bind with high affinity to MHC complexes bound to self-peptides expressed on the surfaces of antigen-presenting cells in the thymus undergo apoptosis or are otherwise suppressed. Those that do not bind too avidly to any such MHC complex complete development and become mature cytotoxic T cells (which express only CD8) or helper T cells (which express only CD4). The negative selection step leads to *self tolerance*; cells that bind an MHC–self-peptide complex are removed from the T-cell population. Similar mechanisms apply to developing B cells, suppressing B cells that express antibodies that interact strongly with self-antigens.

33.6.2 Autoimmune Diseases Result from the Generation of Immune Responses Against Self-Antigens

Although thymic selection is remarkably efficient in suppressing the immune response to self-antigens, failures do occur. Such failures result in *autoimmune diseases*. These diseases include relatively common illnesses such as insulin-dependent diabetes mellitus, multiple sclerosis, and rheumatoid arthritis. In these illnesses, immune responses against self-antigens result in damage to selective tissues that express the antigen (Figure 33.41).

(A)



(B)

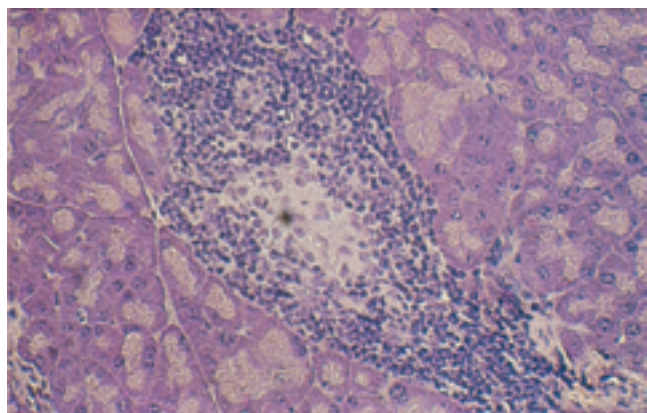



FIGURE 33.41 Consequences of autoimmunity. Photomicrographs of an islet of Langerhans (A) in the pancreas of a normal mouse and (B) in the pancreas of a mouse with an immune response against pancreatic β cells, which results in a disease resembling insulin-dependent diabetes mellitus in human beings. [From M. A. Atkinson and N. K. Maclaren. What causes diabetes? Copyright © 1990 by Scientific American, Inc. All rights reserved.]

In many cases, the cause of the generation of self-reactive antibodies or T cells is unclear. However, in other cases, infectious organisms such as bacteria or viruses may play a role. Infection leads to the generation of antibodies and T cells that react with many different epitopes from the infectious organism. If one of these antigens closely resembles a self-antigen, an autoimmune response can result. For example, *Streptococcus* infections sometimes lead to rheumatic fever owing to the production of antibodies to streptococcal antigens that cross-react with exposed molecules in heart muscle.

33.6.3 The Immune System Plays a Role in Cancer Prevention

 The development of immune responses against proteins encoded by our own genomes can be beneficial under some circumstances. Cancer cells have undergone significant changes that often result in the expression of proteins that are not normally expressed. For example, the mutation of genes can generate proteins that do not correspond in amino acid sequence to any normal protein. Such proteins may be recognized as foreign, and an immune response will be generated specifically against the cancer cell. Alternatively, cancer cells often produce proteins that are expressed during embryonic development but are not expressed or are expressed at very low levels after birth. For example, a membrane glycoprotein protein called *carcinoembryonic antigen (CEA)* appears in the gastrointestinal cells of developing fetuses but is not normally expressed at significant levels after birth. More than 50% of patients with colorectal cancer have elevated serum levels of CEA. Immune cells recognizing epitopes from such proteins will not be subject to negative selection and, hence, will be present in the adult immune repertoire. These cells may play a cancer surveillance role, killing cells that overexpress antigens such as CEA and preventing genetically damaged cells from developing into tumors.

SUMMARY

- To respond effectively to a vast array of pathogens, the immune system must be tremendously adaptable. Adaptation by the immune system follows the principles of evolution: an enormously diverse set of potentially useful proteins is generated; these proteins are then subjected to intense selection so that only cells that express useful proteins flourish and continue development, until an effective immune response to a specific invader is generated.
- **Antibodies Possess Distinct Antigen-Binding and Effector Units**
The major immunoglobulin in the serum is immunoglobulin G. An IgG protein is a heterotetramer with two heavy chains and two light chains. Treatment of IgG molecules with proteases such as papain produces three fragments: two F_{ab} fragments that retain antigen-binding activity and an F_c fragment that retains the ability to activate effector functions such as the initiation of the complement cascade. The F_{ab} fragments include the L chain and the amino-terminal half of the H chain; the F_c domain is a dimer consisting of the carboxyl-terminal halves of two H chains. Five different classes of antibody—IgG, IgM, IgA, IgD, and IgE—differ in their heavy chains and, hence, in their effector functions.
- **The Immunoglobulin Fold Consists of a Beta-Sandwich Framework with Hypervariable Loops**
One particular protein fold is found in many of the key proteins of the immune system. The immunoglobulin fold consists of a pair of β sheets

that pack against one another, linked by a single disulfide bond. Loops projecting from one end of the structure form a binding surface that can be varied by changing the amino acid sequences within the loops. Domains with immunoglobulin folds are linked to form antibodies and other classes of proteins in the immune system including T-cell receptors.

- **Antibodies Bind Specific Molecules Through Their Hypervariable Loops**

Two chains come together to form the binding surface of an antibody. Three loops from each domain, the complementarity-determining regions, form an essentially continuous surface that can vary tremendously in shape, charge, and other characteristics to allow particular antibodies to bind to molecules ranging from small molecules to large protein surfaces.

- **Diversity Is Generated by Gene Rearrangements**

The tremendous diversity of the amino acid sequences of antibodies is generated by segmental rearrangements of genes. For antibody κ light chains, one of 40 variable regions is linked to one of five joining regions. The combined VJ unit is then linked to the constant region. Thousands of different genes can be generated in this manner. Similar arrays are rearranged to form the genes for the heavy chains, but an additional region called the diversity region lies between the V and the J regions. The combination L and H chains, each obtained through such rearranged genes, can produce more than 10^8 distinct antibodies. Different classes of antibodies are also generated by gene rearrangements that lead to class switching. Oligomerization of membrane-bound antibody molecules initiates a signal-transduction cascade inside B cells. Key steps in this signaling process include the phosphorylation of specific tyrosine residues in sequences termed immunoreceptor tyrosine-based activation motifs (ITAMs), present in proteins that associate with the membrane-bound antibodies.

- **Major-Histocompatibility-Complex Proteins Present Peptide Antigens on Cell Surfaces for Recognition by T-Cell Receptors**

Intracellular pathogens such as viruses and mycobacteria cannot be easily detected. Intracellular proteins are constantly being cut into small peptides by proteasomes and displayed in class I major-histocompatibility-complex proteins on cell surfaces. Such peptides lie in a groove defined by two helices in the class I MHC proteins. The combination of MHC protein and peptide can be bound by an appropriate T-cell receptor. T-cell receptors resemble the antigen-binding domains of antibodies in structure, and diversity in T-cell-receptor sequence is generated by V(D)J gene rearrangements. The T-cell receptor recognizes features of both the peptide and the MHC molecule that presents it. Cytotoxic T cells initiate apoptosis in cells to which they bind through T-cell receptor–class I MHC–peptide interactions aided by interactions with the coreceptor molecule CD8. Helper T cells recognize peptides presented in class II MHC proteins, a distinct type of MHC protein expressed only on antigen-presenting cells such as B cells and macrophages. Helper T cells express the coreceptor CD4, rather than CD8. CD4 interacts with class II MHC proteins present on antigen-presenting cells. Signaling pathways, analogous to those in B cells, are initiated by interactions between MHC–peptide complexes and T-cell receptors and the CD8 and CD4 coreceptors. Human immunodeficiency virus damages the immune system by infecting cells that express CD4, such as helper T cells.

● Immune Responses Against Self-Antigens Are Suppressed

In principle, the immune system is capable of generating antibodies and T-cell receptors that bind to self-molecules; that is, molecules that are normally present in a healthy and uninfected individual organism. Selection mechanisms prevent such self-directed molecules from being expressed at high levels. The selection process includes both positive selection, to enrich the population of cells that express molecules that have the potential to bind foreign antigens in an appropriate context, and negative selection, which eliminates cells that express molecules with too high an affinity for self-antigens. Autoimmune diseases such as insulin-dependent diabetes mellitus can result from amplification of a response against a self-antigen.

KEY TERMS

humoral immune response (p. 921)	immunoglobulin E (p. 924)	human leukocyte antigen (HLA) (p. 935)
B lymphocyte (B cell) (p. 922)	variable region (p. 925)	β_2 -microglobulin (p. 935)
antigen (p. 922)	constant region (p. 925)	T-cell receptor (p. 937)
antigenic determinant (epitope) (p. 922)	immunoglobulin fold (p. 926)	CD8 (p. 937)
cellular immune response (p. 922)	hypervariable loop (p. 926)	perforin (p. 939)
cytotoxic T lymphocyte (killer T cell) (p. 922)	complementarity-determining region (CDR) (p. 926)	granzymes (p. 939)
helper T lymphocyte (p. 922)	V(D)J recombination (p. 931)	helper T cell (p. 939)
immunoglobulin G (p. 923)	immunoreceptor tyrosine-based activation motif (ITAM) (p. 932)	class II MHC protein (p. 939)
F _{ab} (p. 923)	cyclosporin (p. 933)	CD4 (p. 940)
F _c (p. 923)	hapten (p. 933)	human immunodeficiency virus (HIV) (p. 940)
light chain (p. 923)	class switching (p. 934)	positive selection (p. 943)
heavy chain (p. 923)	T cell (p. 935)	negative selection (p. 944)
segmental flexibility (p. 924)	major histocompatibility complex (MHC) (p. 935)	autoimmune disease (p. 944)
immunoglobulin M (p. 924)	class I MHC protein (p. 935)	carcinoembryonic antigen (CEA) (p. 945)
immunoglobulin A (p. 924)		
immunoglobulin D (p. 924)		

SELECTED READINGS

Where to start

- Nossal, G. J. V., 1993. Life, death, and the immune system. *Sci. Am.* 269(3): 53–62.
- Tonegawa, S., 1985. The molecules of the immune system. *Sci. Am.* 253 (4): 122–131.
- Leder, P., 1982. The genetics of antibody diversity. *Sci. Am.* 246(5): 102–115.
- Bromley, S. K., Burack, W. R., Johnson, K. G., Somersalo, K., Sims, T. N., Sumen, C., Davis, M. M., Shaw, A. S., Allen, P. M., and Dustin, M. L., 2001. The immunological synapse. *Annu. Rev. Immunol.* 19:375–396.

Books

- Goldsby, R. A., Kindt, T. J., Osborne, B. A. 2000. *Kuby Immunology* (4th ed.). W. H. Freeman and Company.
- Abbas, A. K., Lichtman, A. H., and Pober, J. S., 1992. *Cellular and Molecular Immunology* (2d ed). Saunders.
- Cold Spring Harbor Symposia on Quantitative Biology, 1989. Volume 54. Immunological Recognition.
- Nisimoff, A., 1985. *Introduction to Molecular Immunology* (2d ed.). Sinauer.

Weir, D. M. (Ed.), 1986. *Handbook of Experimental Immunology*. Oxford University Press.

Structure of antibodies and antibody–antigen complexes

- Davies, D. R., Padlan, E. A., and Sheriff, S., 1990. Antibody-antigen complexes. *Annu. Rev. Biochem.* 59:439–473.
- Poljak, R. J., 1991. Structure of antibodies and their complexes with antigens. *Mol. Immunol.* 28:1341–1345.
- Davies, D. R., and Cohen, G. H., 1996. Interactions of protein antigens with antibodies. *Proc. Natl. Acad. Sci. USA* 93:7–12.
- Marquart, M., Deisenhofer, J., Huber, R., and Palm, W., 1980. Crystallographic refinement and atomic models of the intact immunoglobulin molecule Kol and its antigen-binding fragment at 3.0 Å and 1.9 Å resolution. *J. Mol. Biol.* 141:369–391.
- Silverton, E. W., Navia, M. A., and Davies, D. R., 1977. Three-dimensional structure of an intact human immunoglobulin. *Proc. Natl. Acad. Sci. USA* 74:5140–5144.
- Padlan, E. A., Silverton, E. W., Sheriff, S., Cohen, G. H., Smith, G. S., and Davies, D. R., 1989. Structure of an antibody-antigen complex: Crystal structure of the HyHEL-10 Fab lysozyme complex. *Proc. Natl. Acad. Sci. USA* 86:5938–5942.

- Rini, J., Schultze-Gahmen, U., and Wilson, I. A., 1992. Structural evidence for induced fit as a mechanism for antibody-antigen recognition. *Nature* 255:959–965.
- Fischmann, T. O., Bentley, G. A., Bhat, T. N., Boulot, G., Mariuzza, R. A., Phillips, S. E., Tello, D., and Poljak, R. J., 1991. Crystallographic refinement of the three-dimensional structure of the FabD1.3-lysozyme complex at 2.5-Å resolution. *J. Biol. Chem.* 266: 12915–12920.
- Burton, D. R., 1990. Antibody: The flexible adaptor molecule. *Trends Biochem. Sci.* 15:64–69.
- Generation of diversity**
- Tonegawa, S., 1988. Somatic generation of immune diversity. *Biosci. Rep.* 8:3–26.
- Honjo, T., and Habu, S., 1985. Origin of immune diversity: Genetic variation and selection. *Annu. Rev. Biochem.* 54:803–830.
- Gellert, M., and McBlane, J. F., 1995. Steps along the pathway of VDJ recombination. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 347:43–47.
- Harris, R. S., Kong, Q., and Maizels, N., 1999. Somatic hypermutation and the three R's: Repair, replication and recombination. *Mutat. Res.* 436:157–178.
- Lewis, S. M., and Wu, G. E., 1997. The origins of V(D)J recombination. *Cell* 88:159–162.
- Ramsden, D. A., van Gent, D. C., and Gellert, M., 1997. Specificity in V(D)J recombination: New lessons from biochemistry and genetics. *Curr. Opin. Immunol.* 9:114–120.
- Roth, D. B., and Craig, N. L., 1998. VDJ recombination: A transposase goes to work. *Cell* 94:411–414.
- Sadofsky, M. J., 2001. The RAG proteins in V(D)J recombination: More than just a nuclease. *Nucleic Acids Res.* 29:1399–1409.
- MHC proteins and antigen processing**
- Bjorkman, P. J., and Parham, P., 1990. Structure, function, and diversity of class I major histocompatibility complex molecules. *Annu. Rev. Biochem.* 59:253–288.
- Goldberg, A. L., and Rock, K. L., 1992. Proteolysis, proteasomes, and antigen presentation. *Nature* 357:375–379.
- Madden, D. R., Gorga, J. C., Strominger, J. L., and Wiley, D. C., 1992. The three-dimensional structure of HLA-B27 at 2.1 Å resolution suggests a general mechanism for tight binding to MHC. *Cell* 70:1035–1048.
- Brown, J. H., Jardetzky, T. S., Gorga, J. C., Stern, L. J., Urban, R. G., Strominger, J. L., and Wiley, D. C., 1993. Three-dimensional structure of the human class II histocompatibility antigen HLA-DR1. *Nature* 364:33–39.
- Saper, M. A., Bjorkman, P. J., and Wiley, D. C., 1991. Refined structure of the human histocompatibility antigen HLA-A2 at 2.6 Å resolution. *J. Mol. Biol.* 219:277–319.
- Madden, D. R., Gorga, J. C., Strominger, J. L., and Wiley, D. C., 1991. The structure of HLA-B27 reveals nonamer self-peptides bound in an extended conformation. *Nature*. 353:321–325.
- Cresswell, P., Bangia, N., Dick, T., and Diedrich, G., 1999. The nature of the MHC class I peptide loading complex. *Immunol. Rev.* 172: 21–28.
- Madden, D. R., Garboczi, D. N., and Wiley, D. C., 1993. The antigenic identity of peptide-MHC complexes: A comparison of the conformations of five viral peptides presented by HLA-A2. *Cell* 75: 693–708.
- T-cell receptors and signaling complexes**
- Hennecke, J., and Wiley, D. C., 2001. T cell receptor-MHC interactions up close. *Cell* 104:1–4.
- Ding, Y. H., Smith, K. J., Garboczi, D. N., Utz, U., Biddison, W. E., and Wiley, D. C., 1998. Two human T cell receptors bind in a similar diagonal mode to the HLA-A2/Tax peptide complex using different TCR amino acids. *Immunity* 8:403–411.
- Reinherz, E. L., Tan, K., Tang, L., Kern, P., Liu, J., Xiong, Y., Hussey, R. E., Smolyar, A., Hare, B., Zhang, R., Joachimiak, A., Chang, H. C., Wagner, G., and Wang, J., 1999. The crystal structure of a T cell receptor in complex with peptide and MHC class II. *Science* 286:1913–1921.
- Cochran, J. R., Cameron, T. O., and Stern, L. J., 2000. The relationship of MHC-peptide binding and T cell activation probed using chemically defined MHC class II oligomers. *Immunity* 12:241–250.
- Cochran, J. R., Cameron, T. O., Stone, J. D., Lubetsky, J. B., and Stern, L. J., 2001. Receptor proximity, not intermolecular orientation, is critical for triggering T-cell activation. *J. Biol. Chem.* 276:28068–28074.
- Garcia, K. C., Teyton, L., and Wilson, I. A., 1999. Structural basis of T cell recognition. *Annu. Rev. Immunol.* 17:369–397.
- Gaul, B. S., Harrison, M. L., Geahlen, R. L., Burton, R. A., and Post, C. B., 2000. Substrate recognition by the Lyn protein-tyrosine kinase: NMR structure of the immunoreceptor tyrosine-based activation motif signaling region of the B cell antigen receptor. *J. Biol. Chem.* 275:16174–16182.
- Kern, P. S., Teng, M. K., Smolyar, A., Liu, J. H., Liu, J., Hussey, R. E., Spoerl, R., Chang, H. C., Reinherz, E. L., and Wang, J. H., 1998. Structural basis of CD8 coreceptor function revealed by crystallographic analysis of a murine CD8 α ectodomain fragment in complex with H-2Kb. *Immunity* 9:519–530.
- Konig, R., Fleury, S., and Germain, R. N., 1996. The structural basis of CD4-MHC class II interactions: Coreceptor contributions to T cell receptor antigen recognition and oligomerization-dependent signal transduction. *Curr. Top. Microbiol. Immunol.* 205:19–46.
- Krummel, M., Wulfiging, C., Sumen, C., and Davis, M. M., 2000. Thirty-six views of T-cell recognition. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 355:1071–1076.
- Janeway, C. J., 1992. The T cell receptor as a multicomponent signalling machine: CD4/CD8 coreceptors and CD45 in T cell activation. *Annu. Rev. Immunol.* 10:645–674.
- Podack, E. R., and Kupfer, A., 1991. T-cell effector functions: Mechanisms for delivery of cytotoxicity and help. *Annu. Rev. Cell Biol.* 7:479–504.
- Davis, M. M., 1990. T cell receptor gene diversity and selection. *Annu. Rev. Biochem.* 59:475–496.
- Leahy, D. J., Axel, R., and Hendrickson, W. A., 1992. Crystal structure of a soluble form of the human T cell coreceptor CD8 at 2.6 Å resolution. *Cell* 68:1145–1162.
- Lowin, B., Hahne, M., Mattmann, C., and Tschopp, J., 1994. Cytolytic T-cell cytotoxicity is mediated through perforin and Fas lytic pathways. *Nature* 370:650–652.
- HIV and AIDS**
- Fauci, A. S., 1988. The human immunodeficiency virus: Infectivity and mechanisms of pathogenesis. *Science* 239:617–622.
- Gallo, R. C., and Montagnier, L., 1988. AIDS in 1988. *Sci. Am.* 259(4): 41–48.
- Kwong, P. D., Wyatt, R., Robinson, J., Sweet, R. W., Sodroski, J., and Hendrickson, W. A., 1998. Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. *Nature* 393:648–659.
- Discovery of major concepts**
- Ada, G. L., and Nossal, G., 1987. The clonal selection theory. *Sci. Am.* 257(2):62–69.
- Porter, R. R., 1973. Structural studies of immunoglobulins. *Science* 180:713–716.
- Edelman, G. M., 1973. Antibody structure and molecular immunology. *Science* 180:830–840.
- Kohler, G., 1986. Derivation and diversification of monoclonal antibodies. *Science* 233:1281–1286.
- Milstein, C., 1986. From antibody structure to immunological diversification of immune response. *Science* 231:1261–1268.
- Janeway, C. A., Jr., 1989. Approaching the asymptote? Evolution and revolution in immunology. *Cold Spring Harbor Symp. Quant. Biol.* 54:1–13.

PROBLEMS

1. *Energetics and kinetics.* Suppose that the dissociation constant of an F_{ab} -hapten complex is 3×10^{-7} M at 25°C .

- What is the standard free energy of binding?
- Immunologists often speak of affinity (K_a), the reciprocal of the dissociation constant, in comparing antibodies. What is the affinity of this F_{ab} ?
- The rate constant of release of hapten from the complex is 120 s^{-1} . What is the rate constant for association? What does the magnitude of this value imply about the extent of structural change in the antibody on binding hapten?

2. *Sugar niche.* An antibody specific for dextran, a polysaccharide of glucose residues, was tested for its binding of glucose oligomers. Maximal binding affinity was obtained when the oligomer contained six glucose residues. How does the size of this site compare with that expected for the binding site on the surface of an antibody?

3. *A brilliant emitter.* Certain naphthalene derivatives exhibit a weak yellow fluorescence when they are in a highly polar environment (such as water) and an intense blue fluorescence when they are in a markedly nonpolar environment (such as hexane). The binding of ϵ -dansyl-lysine to specific antibody is accompanied by a marked increase in its fluorescence intensity and a shift in color from yellow to blue. What does this finding reveal about the hapten-antibody complex?

4. *Avidity versus affinity.* The standard free energy of binding of F_{ab} derived from an antiviral IgG is -7 kcal mol^{-1} (-29 kJ mol^{-1}) at 25°C .

- Calculate the dissociation constant of this interaction.
- Predict the dissociation constant of the intact IgG, assuming that both combining sites of the antibody can interact with viral epitopes and that the free-energy cost of assuming a favorable hinge angle is $+3 \text{ kcal mol}^{-1}$ (12.6 kJ mol^{-1}).

5. *Miniantibody.* The F_{ab} fragment of an antibody molecule has essentially the same affinity for a monovalent hapten as does intact IgG.

- What is the smallest unit of an antibody that can retain the specificity and binding affinity of the whole protein?
- Design a compact single-chain protein that is likely to specifically bind antigen with high affinity.

6. *Turning on B cells.* B lymphocytes, the precursors of plasma cells, are triggered to proliferate by the binding of multivalent antigens to receptors on their surfaces. The cell-surface receptors are transmembrane immunoglobulins. Univalent antigens, in contrast, do not activate B cells.

- What do these findings reveal about the mechanism of B-cell activation?
- How might antibodies be used to activate B cells?

7. *An ingenious cloning strategy.* In the cloning of the gene for the α chain of the T-cell receptor, T-cell cDNAs were hybridized with B-cell mRNAs. What was the purpose of this hybridization step? Can the principle be applied generally?

8. *Instruction.* Before the mechanism for generating antibody diversity had been established, a mechanism based on protein folding around an antigen was proposed, primarily by Linus Pauling. In this model, antibodies that had different specificities had the same amino acid sequence but were folded in different ways. Propose a test of this model.

9. *Dealing with nonsense.* Cells, including immune cells, degrade mRNA molecules in which no long open reading frame is present. The process is called nonsense-mediated RNA decay. Suggest a role for this process in immune cells.

10. *Crystallization.* The proteolytic digestion of a population of IgG molecules isolated from human serum results in the generation of F_{ab} and F_c fragments. Why do F_c fragments crystallize more easily than F_{ab} fragments generated from such a population?

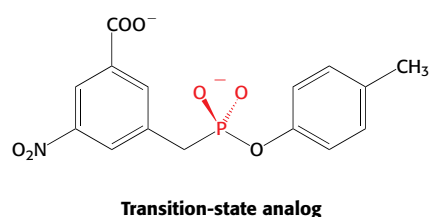
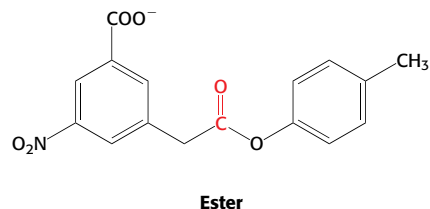
11. *Presentation.* The amino acid sequence of a small protein is:

MSRLASKNLRSDHAGGLLQATYSAVSS-
IKNTMSFGAWSNAALNDSRDA

Predict the most likely peptide to be presented by the class I MHC molecule HLA-A2.

Mechanism Problem

12. *Catalytic antibody.* Antibody is generated against a transition state for the hydrolysis of the following ester.



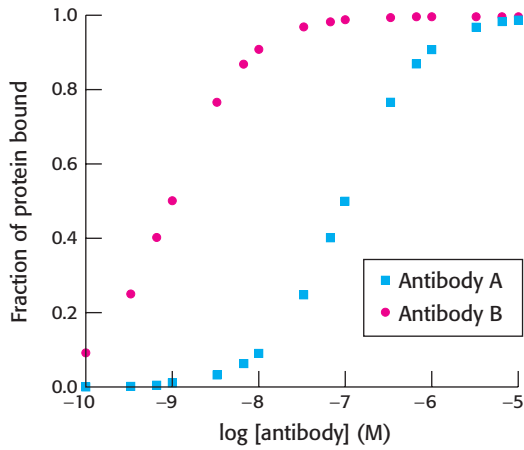
Some of these antibodies catalyze the hydrolysis of the ester. What amino acid residue might you expect to find in the binding site on the antibody?

Chapter Integration Problem

13. *Signaling.* Protein tyrosine phosphatases, such as the molecule CD45 expressed in both B cells and T cells, play important roles in activating such protein tyrosine kinases as Fyn and Lck, which are quite similar to Src. Suggest a mechanism for the activation of such protein kinases by the removal of a phosphate from a phosphotyrosine residue.

Data Interpretation Problem

14. *Affinity maturation.* A mouse is immunized with an oligomeric human protein. Shortly after immunization, a cell line



that expresses a single type of antibody molecule (antibody A) is derived. The ability of antibody A to bind the human protein is assayed with the results shown in the adjoining graph. After repeated immunizations with the same protein, another cell line is derived that expresses a different antibody (antibody B). The results of analyzing the binding of antibody B to the protein also are shown. From these data, estimate

- the dissociation constant (K_d) for the complex between the protein and antibody A.
- the dissociation constant for the complex between the protein and antibody B.

Comparison of the amino acid sequences of antibody A and antibody B reveals them to be identical except for a single amino acid. What does this finding suggest about the mechanism by which the gene encoding antibody B was generated?

33.2 THE IMMUNOGLOBULIN FOLD CONSISTS OF A BETA-SANDWICH FRAMEWORK WITH HYPERVARIABLE LOOPS

An IgG molecule consists of a total of 12 immunoglobulin domains. These domains have many sequence features in common and adopt a common structure, the *immunoglobulin fold* (Figure 33.9). Remarkably, this same structural domain is found in many other proteins that play key roles in the immune system.

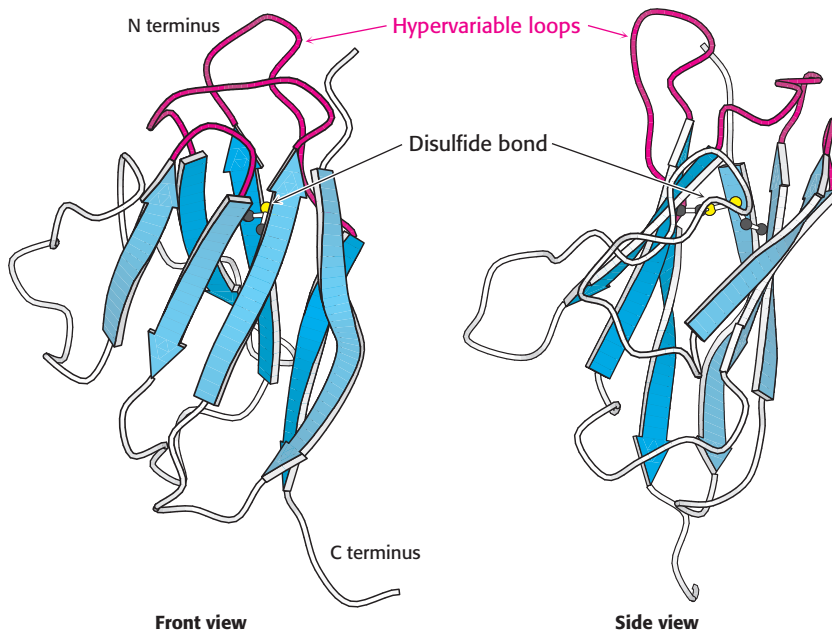



FIGURE 33.9 Immunoglobulin fold. An immunoglobulin domain consists of a pair of β -sheets linked by a disulfide bond and hydrophobic interactions. Three hypervariable loops lie at one end of the structure.

The immunoglobulin fold consists of a pair of β sheets, each built of antiparallel β strands, that surround a central hydrophobic core. A single disulfide bond bridges the two sheets. Two aspects of this structure are particularly important for its function. First, three loops present at one end of the structure form a potential binding surface. These loops contain the hypervariable sequences present in antibodies and in T-cell receptors (see Sections 33.3 and 33.5.2). Variation of the amino acid sequences of these loops provides the major mechanism for the generation of the vastly diverse set of antibodies and T-cell receptors expressed by the immune system. These loops are referred to as *hypervariable loops* or *complementarity-determining regions (CDRs)*. Second, the amino terminus and the carboxyl terminus are at opposite ends of the structure, which allows structural domains to be strung together to form chains, as in the L and H chains of antibodies. Such chains are present in several other key molecules in the immune system.

 The immunoglobulin fold is one of the most prevalent domains encoded by the human genome—more than 750 genes encode proteins with at least one immunoglobulin fold recognizable at the level of amino acid sequence. Such domains are also common in other multicellular animals such as flies and nematodes. However, from inspection of amino acid sequence alone, immunoglobulin-fold domains do not appear to be present in yeast or plants. However, structurally similar domains are present in these organisms, including the key photosynthetic electron-transport protein plastocyanin in plants (Section 19.3.2). Thus, the immunoglobulin-fold family appears to have expanded greatly along evolutionary branches leading to animals—particularly, vertebrates.

33.3 ANTIBODIES BIND SPECIFIC MOLECULES THROUGH THEIR HYPERVARIABLE LOOPS

For each class of antibody, the amino-terminal immunoglobulin domains of the L and H chains (the variable domains, designated V_L and V_H) come together at the ends of the arms extending from the structure. The positions of the complementarity-determining regions are striking. These hypervariable sequences, present in three loops of each domain, come together so that all six loops form a single surface at the end of each arm (Figure 33.10). Because virtually any V_L can pair with any V_H , a *very large number of different binding sites can be constructed by their combinatorial association.*

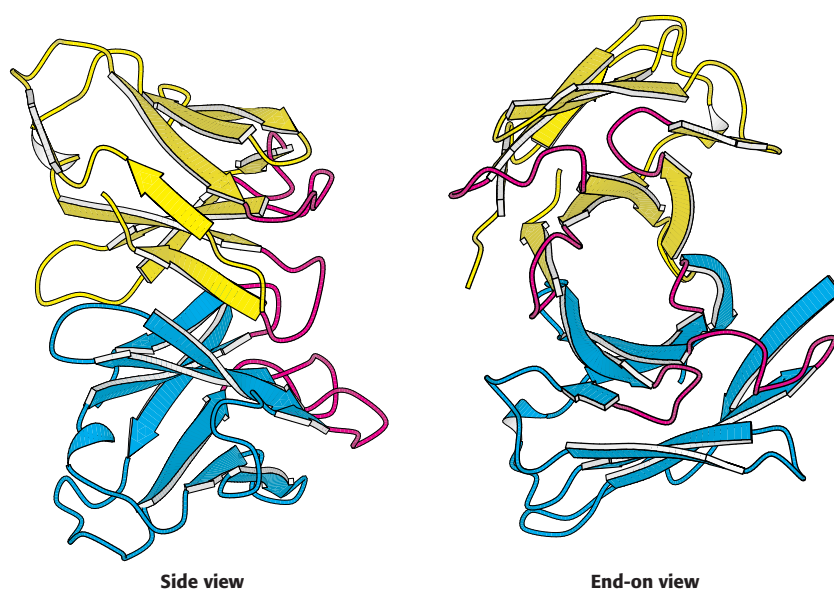


FIGURE 33.10 Variable domains. Two views of the variable domains of the L chain (yellow) and the H chain (blue); the complementarity-determining regions (CDRs) are shown in red. The six CDRs come together to form a binding surface. The specificity of the surface is determined by the sequences and structures of the CDRs.

33.3.1 X-Ray Analyses Have Revealed How Antibodies Bind Antigens

The results of x-ray crystallographic studies of many large and small antigens bound to F_{ab} molecules have been sources of much insight into the structural basis of antibody specificity. The binding of antigens to antibodies is governed by the same principles that govern the binding of substrates to enzymes. The apposition of complementary shapes results in numerous contacts between amino acids at the binding surfaces of both molecules. Numerous hydrogen bonds, electrostatic interactions, and van der Waals interactions, reinforced by hydrophobic interactions, combine to give specific and strong binding.

A few aspects of antibody binding merit specific attention, inasmuch as they relate directly to the structure of immunoglobulins. The binding site on the antibody has been found to incorporate some or all of the CDRs in the variable domains of the antibody. Small molecules (e.g., octapeptides) are likely to make contact with fewer CDRs, with perhaps 15 residues of the antibody participating in the binding interaction. Macromolecules often make more extensive contact, interacting with all six CDRs and 20 or more residues of the antibody. Small molecules often bind in a cleft of the antigen-binding region. Macromolecules such as globular proteins tend to interact across larger, fairly flat apposed surfaces bearing complementary protrusions and depressions.

A well-studied case of small-molecule binding is seen in an example of phosphorylcholine bound to F_{ab} . Crystallographic analysis revealed



FIGURE 33.11 Binding of a small antigen. The structure of a complex between an F_{ab} fragment of an antibody and its target—in this case, phosphorylcholine. Residues from the antibody interact with phosphorylcholine through hydrogen bonding and electrostatic and van der Waals interactions.

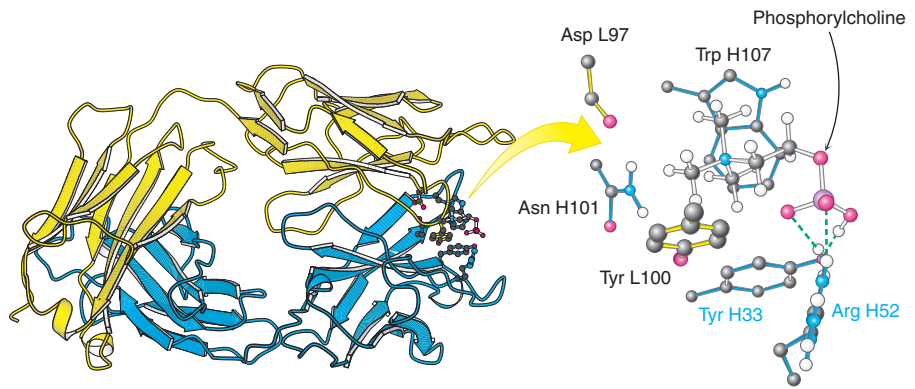


FIGURE 33.12 Antibodies against lysozyme. (A) The structures of three complexes (i, ii, iii) between F_{ab} fragments (blue and yellow) and hen egg-white lysozyme (red) shown with lysozyme in the same orientation in each case. The three antibodies recognize completely different epitopes on the lysozyme molecule. (B) The F_{ab} fragments from part A with points of contact highlighted as space-filling models, revealing the different shapes of the antigen-binding sites.



phosphorylcholine bound to a cavity lined by residues from five CDRs—two from the L chain and three from the H chain (Figure 33.11). The positively charged trimethylammonium group of phosphorylcholine is buried inside the wedge-shaped cavity, where it interacts electrostatically with two negatively charged glutamate residues. The negatively charged phosphate group of phosphorylcholine binds to the positively charged guanidinium group of an arginine residue at the mouth of the crevice and to a nearby lysine residue. The phosphate group is also hydrogen bonded to the hydroxyl group of a tyrosine residue and to the guanidinium group of the arginine side chain. Numerous van der Waals interactions, such as those made by a tryptophan side chain, also stabilize this complex.

The binding of phosphorylcholine does not significantly change the structure of the antibody, yet induced fit plays a role in the formation of many antibody–antigen complexes. A malleable binding site can accommodate many more kinds of ligands than can a rigid one. Thus, induced fit increases the repertoire of antibody specificities.

33.3.2 Large Antigens Bind Antibodies with Numerous Interactions

How do large antigens interact with antibodies? A large collection of antibodies raised against hen egg-white lysozyme has been structurally characterized in great detail (Figure 33.12). Each different antibody binds to a distinct surface of lysozyme. Let us examine the interactions present in one of these complexes in detail. This antibody binds two polypeptide segments that are widely separated in the primary structure, residues 18 to 27 and 116 to 129 (Figure 33.13).

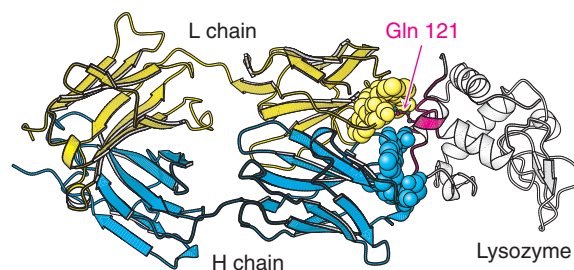


FIGURE 33.13 Antibody–protein interactions. The structure of a complex between an F_{ab} fragment and lysozyme reveals that the binding surfaces are complementary in shape over a large area. A single residue of lysozyme, glutamine 121, penetrates more deeply into the antibody combining site.

All six CDRs of the antibody make contact with this epitope. The region of contact is quite extensive (about $30 \times 20 \text{ \AA}$). The apposed surfaces are rather flat. The only exception is the side chain of glutamine 121 of lysozyme, which penetrates deeply into the antibody binding site, where it forms a hydrogen bond with a main-chain carbonyl oxygen atom and is surrounded by three aromatic side chains. The formation of 12 hydrogen bonds and numerous van der Waals interactions contributes to the high affinity ($K_d = 20 \text{ nM}$) of this antibody–antigen interaction. Examination of the F_{ab} molecule without bound protein reveals that the structures of the V_L and V_H domains change little on binding, although they slide 1 \AA apart to allow more intimate contact with lysozyme.

33.4 DIVERSITY IS GENERATED BY GENE REARRANGEMENTS

A mammal such as a mouse or a human being can synthesize large amounts of specific antibody against virtually any foreign determinant within a matter of days of being exposed to it. We have seen that antibody specificity is determined by the amino acid sequences of the variable regions of both light

and heavy chains, which brings us to the key question: How are different variable-region sequences generated?

The discovery of distinct variable and constant regions in the L and H chains raised the possibility that the genes that encode immunoglobulins have an unusual architecture that facilitates the generation of a diverse set of polypeptide products. In 1965, William Dreyer and Claude Bennett proposed that multiple *V* (*variable*) genes are separate from a single *C* (*constant*) gene in embryonic (germ-line) DNA. According to their model, one of these *V* genes becomes joined to the *C* gene in the course of differentiation of the antibody-producing cell. A critical test of this novel hypothesis had to await the isolation of pure immunoglobulin mRNA and the development of techniques for analyzing mammalian genomes. Twenty years later, Susumu Tonegawa found that *V* and *C* genes are indeed far apart in embryonic DNA but are closely associated in the DNA of antibody-producing cells. Thus, immunoglobulin genes are rearranged in the differentiation of lymphocytes.

33.4.1 J (Joining) Genes and D (Diversity) Genes Increase Antibody Diversity

Sequencing studies carried out by Susumu Tonegawa, Philip Leder, and Leroy Hood revealed that *V* genes in embryonic cells do not encode the entire variable region of L and H chains. Consider, for example, the region that encodes the κ light-chain family. A tandem array of 40 segments, each of which encodes approximately the first 97 residues of the variable domain of the L chain, is present on human chromosome 2 (Figure 33.14).



FIGURE 33.14 The κ light-chain locus. This part of human chromosome 2 includes an array of 40 segments that encode the variable (*V*) region (approximately residues 1–97) of the light chain, an array of 5 segments that encode the joining (*J*) region (residues 98–110), and a single region that encodes the constant (*C*) region.

However, the variable region of the L chain extends to residue 110. Where is the DNA that encodes the last 13 residues of the *V* region? For L chains in undifferentiated cells, this stretch of DNA is located in an unexpected place: near the *C* gene. It is called the *J* gene because it joins the *V* and *C* genes in a differentiated cell. In fact, a tandem array of five *J* genes is located near the *C* gene in embryonic cells. In the differentiation of an antibody-producing cell, a *V* gene becomes spliced to a *J* gene to form a complete gene for the variable region (Figure 33.15). RNA splicing generates

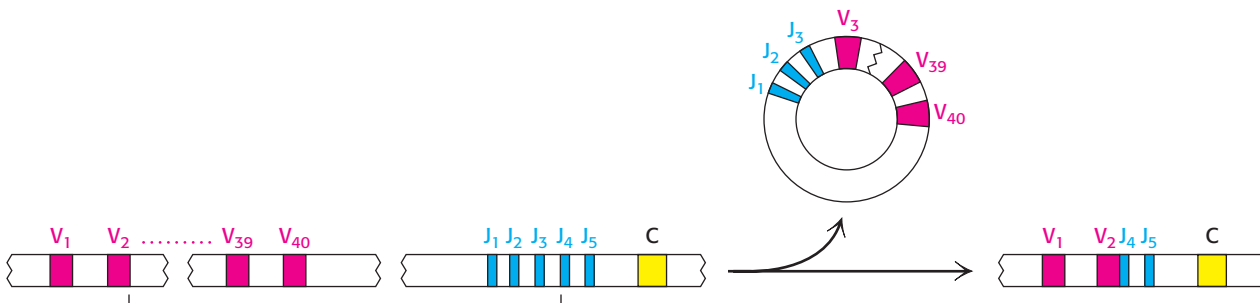


FIGURE 33.15 VJ recombination. A single *V* gene (in this case, *V*₂) is linked to a *J* gene (here, *J*₄) to form an intact *VJ* region. The intervening DNA is released in a circular form. Because the *V* and *J* regions are selected at random and the joint between them is not always in exactly the same place, many *VJ* combinations can be generated by this process.

an mRNA molecule for the complete L chain by linking the coding regions for the rearranged VJ unit with that for the C unit (Figure 33.16).

J genes are important contributors to antibody diversity because they encode part of the last hypervariable segment (CDR3). In forming a continuous variable-region gene, any of the 40 V genes can become linked to any of five J genes. Thus, somatic recombination of these gene segments amplifies the diversity already present in the germ line. The linkage between V and J is not precisely controlled. Recombination between these genes can take place at one of several bases near the codon for residue 95, generating additional diversity. A similar array of V and J genes encoding the λ light chain is present on human chromosome 22. This region includes 30 V_λ gene segments and four J_λ segments. In addition, this region includes four distinct C genes, in contrast with the single C gene in the κ locus.

In human beings, the genes encoding the heavy chain are present on chromosome 14. Remarkably, the variable domain of heavy chains is assembled from *three* rather than two segments. In addition to V_H genes that encode residues 1 to 94 and J_H segments that encode residues 98 to 113, this chromosomal region includes a distinct set of segments that encode residues 95 to 97 (Figure 33.17). These gene segments are called D for *diversity*. Some 27 D segments lie between 51 V_H and 6 J_H segments. The recombination process first joins a D segment to a J_H segment; a V_H segment is then joined to DJ_H . A greater variety of antigen-binding patches and clefts can be formed by the H chain than by the L chain because the H chain is encoded by three rather than two gene segments. Moreover, CDR3 of the H chain is diversified by the action of terminal deoxyribonucleotidyl transferase, a special DNA polymerase that requires no template. This enzyme inserts extra nucleotides between V_H and D. The $V(D)J$ recombination of both the L and the H chains is executed by specific enzymes present in immune cells. These proteins, called *RAG-1* and *RAG-2*, recognize specific DNA sequences called *recombination signal sequences (RSSs)* adjacent to the V, D, and J segments and facilitate the cleavage and religation of the DNA segments.

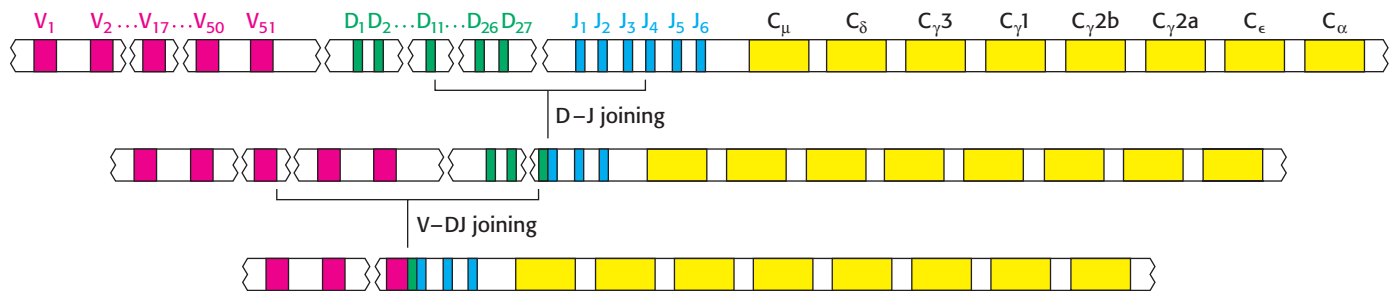


FIGURE 33.17 V(D)J recombination. The heavy-chain locus includes an array of 51 V segments, 27 D segments, and 6 J segments. Gene rearrangement begins with D–J joining, followed by further rearrangement to link the V segment to the DJ segment.

33.4.2 More Than 10^8 Antibodies Can Be Formed by Combinatorial Association and Somatic Mutation

Let us recapitulate the sources of antibody diversity. The germ line contains a rather large repertoire of variable-region genes. For κ light chains, there are about 40 V-segment genes and five J-segment genes. Hence, a total of $40 \times 5 = 200$ kinds of complete V_κ genes can be formed by the combinations of V and J. A similar analysis suggests that at least 120 different λ light chains can be generated. A larger number of heavy-chain genes can be formed because of the role of the D segments. For 51 V, 27 D, and 6 J gene segments, the number of complete V_H genes that can be formed is 8262.

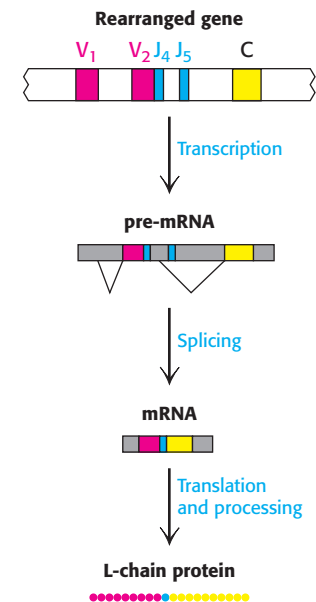


FIGURE 33.16 Light-chain expression.

The light-chain protein is expressed by transcription of the rearranged gene to produce a pre-mRNA molecule with the VJ and C regions separated. RNA splicing removes the intervening sequences to produce an mRNA molecule with the VJ and C regions linked. Translation of the mRNA and processing of the initial protein product produces the light chain.

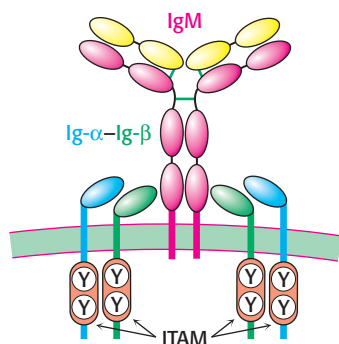


FIGURE 33.18 B-cell receptor. This complex consists of a membrane-bound IgM molecule noncovalently bound to two Ig- α -Ig- β heterodimers. The intracellular domains of each of the Ig- α and Ig- β chains include an immunoreceptor tyrosine-based activation motif (ITAM).

The association of 320 kinds of L chains with 8262 kinds of H chains would yield 2.6×10^6 different antibodies. Variability in the exact points of segment joining and other mechanisms increases this value by at least two orders of magnitude.

Even more diversity is introduced into antibody chains by *somatic mutation*—that is, the introduction of mutations into the recombined genes. In fact, a 1000-fold increase in binding affinity is seen in the course of a typical humoral immune response, arising from somatic mutation, a process called *affinity maturation*. The generation of an expanded repertoire leads to the selection of antibodies that more precisely fit the antigen. Thus, nature draws on each of three sources of diversity—a germ-line repertoire, somatic recombination, and somatic mutation—to form the rich variety of antibodies that protect an organism from foreign incursions.

33.4.3 The Oligomerization of Antibodies Expressed on the Surface of Immature B Cells Triggers Antibody Secretion

The processes heretofore described generate a highly diverse set of antibody molecules—a key first step in the generation of an immune response. The next stage is the selection of a particular set of antibodies directed against a specific invader. How does this selection occur? Each immature B cell, produced in the bone marrow, expresses a monomeric form of IgM attached to its surface (Figure 33.18). Each cell expresses approximately 10^5 IgM molecules, but *all of these molecules are identical in amino acid sequence and, hence, in antigen-binding specificity*. Thus, the selection of a particular immature B cell for growth will lead to the amplification of an antibody with a unique specificity. The selection process begins with the binding of an antigen to the membrane-bound antibody.

Associated with each membrane-linked IgM molecule are two molecules of a heterodimeric membrane protein called Ig- α -Ig- β (see Figure 33.18). Examination of the amino acid sequences of Ig- α and Ig- β is highly instructive. The amino terminus of each protein lies outside the cell and corresponds to a single immunoglobulin, and the carboxyl terminus, which lies inside the cell, includes a sequence of 18 amino acids called an *immunoreceptor tyrosine-based activation motif (ITAM)* (see Figure 33.18). As its name suggests, each ITAM includes key tyrosine residues, which are subject to phosphorylation by particular protein kinases present in immune-system cells.

A fundamental observation with regard to the mechanism by which the binding of antigen to membrane-bound antibody triggers the subsequent steps of the immune response is that *oligomerization or clustering of the antibody molecules is required* (Figure 33.19). The requirement for oligomerization is reminiscent of the dimerization of receptors triggered by growth hormone and epidermal growth factor encountered in Sections 15.4 and 15.4.1; indeed, the associated signaling mechanisms appear to be quite similar. The oligomerization of the membrane-bound antibodies results in the phosphorylation of the tyrosine residues within the ITAMs by protein tyrosine kinases including Lyn, a homolog of Src (Section 15.5). The phosphorylated ITAMs serve as docking sites for a protein kinase termed spleen tyrosine kinase (Syk), which has two SH2 domains that interact with the pair of phosphorylated tyrosines in each ITAM.

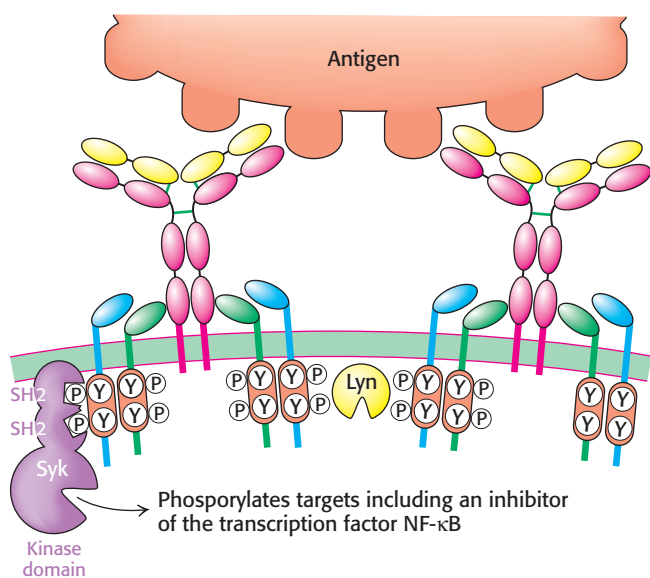

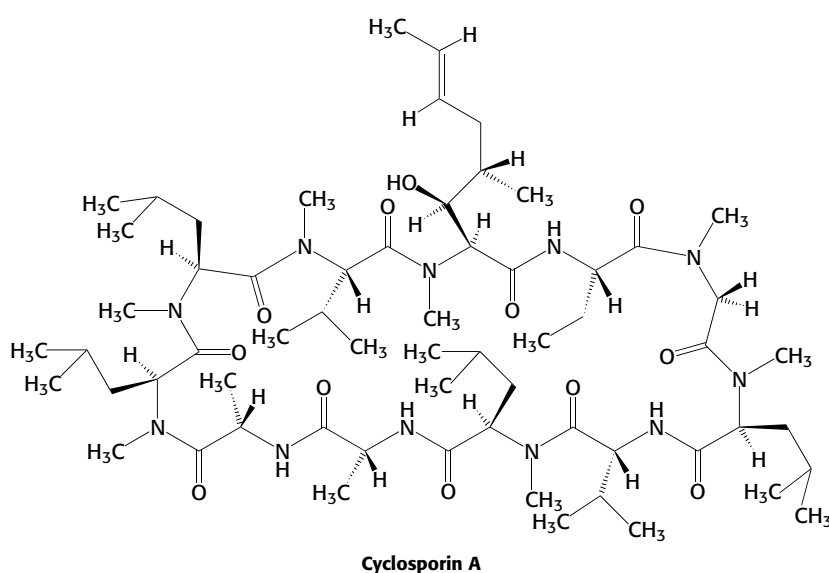


FIGURE 33.19 B-cell activation. The binding of multivalent antigen such as bacterial or viral surfaces links membrane-bound IgM molecules. This oligomerization triggers the phosphorylation of tyrosine residues in the ITAM sequences by protein tyrosine kinases such as Lyn. After phosphorylation, the ITAMs serve as docking sites for Syk, a protein kinase that phosphorylates a number of targets, including transcription factors.



Syk, when activated by phosphorylation, proceeds to phosphorylate other signal-transduction proteins including an inhibitory subunit of a transcription factor called NF- κ B and an isoform of phospholipase C. The signaling processes continue downstream to activate gene expression, leading to the stimulation of cell growth and initiating further B-cell differentiation.

 Drugs that modulate the immune system have served as sources of insight into immune-system signaling pathways. For example, *cyclosporin*, a powerful suppressor of the immune system, acts by blocking a phosphatase called *calcineurin*, which normally activates a transcription factor called NF-AT by dephosphorylating it.



The potent immune suppression that results reveals how crucial the activity of this transcription factor is to the development of an immune response. Without drugs such as cyclosporin, organ transplantation would be extremely difficult because transplanted tissue expresses a wide range of foreign antigens, which causes the immune system to reject the new tissue.

The role of oligomerization in the B-cell signaling pathway is illuminated when we consider the nature of many antigens presented by pathogens. The surfaces of many viruses, bacteria, and parasites are characterized by arrays of identical membrane proteins or membrane-linked carbohydrates. Thus, most pathogens present multiple binding surfaces that will naturally cause membrane-associated antibodies to oligomerize as they bind adjacent epitopes. In addition, the mechanism accounts for the observation that most small molecules do not induce an immune response; however, coupling multiple copies of the small molecule to a large oligomeric protein such as keyhole limpet hemocyanin (KLH), which has a molecular mass of close to 1 million daltons or more, promotes antibody oligomerization and, hence, the production of antibodies against the small-molecule epitope. The large protein is called the *carrier* of the attached chemical group, which is called a *haptenic determinant*. The small foreign molecule by itself is called a *hapten*. Antibodies elicited by attached haptens will bind unattached haptens as well.

33.4.4 Different Classes of Antibodies Are Formed by the Hopping of V_H Genes

The development of an effective antibody-based immune response depends on the secretion into the blood of antibodies that have appropriate effector functions. At the beginning of this response, an alternative mRNA splicing

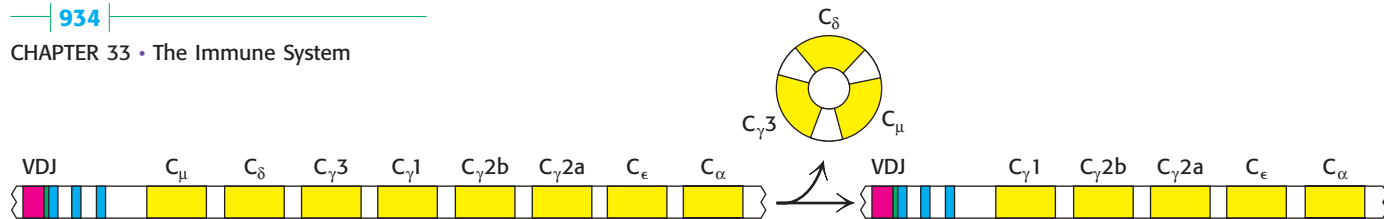


FIGURE 33.20 Class switching. Further rearrangement of the heavy-chain locus results in the generation of genes for antibody classes other than IgM. In the case shown, rearrangement places the VDJ region next to the $C_{\gamma 1}$ region, resulting in the production of IgG1. Note that no further rearrangement of the VDJ region takes place, so the specificity of the antibody is not affected.

pathway is activated so that the production of membrane-linked IgM is supplanted by the synthesis of secreted IgM. As noted in Section 33.1, secreted IgM is pentameric and has a relatively high avidity for multivalent antigens. Later, the antibody-producing cell makes either IgG, IgA, IgD, or IgE of the same specificity as the initially secreted IgM. In this switch, the light chain is unchanged, as is the variable region of the heavy chain. Only the constant region of the heavy chain changes. This step in the differentiation of an antibody-producing cell is called *class switching* (Figure 33.20). In undifferentiated cells, the genes for the constant region of each class of heavy chain, called C_{μ} , C_{δ} , C_{γ} , C_{ϵ} , and C_{α} , are next to each other. There are eight in all, including four genes for the constant regions of γ chains. A complete gene for the heavy chains of IgM antibody is formed by the translocation of a V_H gene segment to a DJ_H gene segment.

How are other heavy chains formed? Class switching is mediated by a gene-rearrangement process that moves a VDJ gene from a site near one C gene to a site near another C gene. Importantly, *the antigen-binding specificity is conserved in class switching because the entire V_HDJ_H gene is translocated in an intact form*. For example, the antigen-combining specificity of IgA produced by a particular cell is the same as that of IgM synthesized at an earlier stage of its development. The biological significance of C_H switching is that a whole recognition domain (the variable domain) is shifted from the early constant region (C_{μ}) to one of several other constant regions that mediate different effector functions.

33.5 MAJOR-HISTOCOMPATIBILITY-COMPLEX PROTEINS PRESENT PEPTIDE ANTIGENS ON CELL SURFACES FOR RECOGNITION BY T-CELL RECEPTORS

Soluble antibodies are highly effective against extracellular pathogens, but they confer little protection against microorganisms that are predominantly intracellular, such as viruses and mycobacteria (which cause tuberculosis and leprosy). These pathogens are shielded from antibodies by the host-cell membrane (Figure 33.21). A different and more subtle strategy, *cell-mediated*

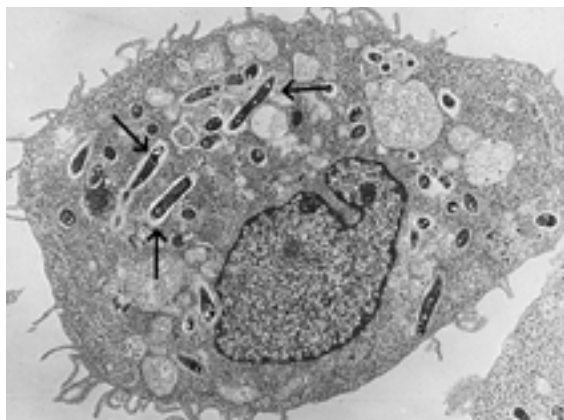


FIGURE 33.21 Intracellular pathogen. An electron micrograph showing mycobacteria (arrows) inside an infected macrophage. [Courtesy of Dr. Stanley Falkow.]

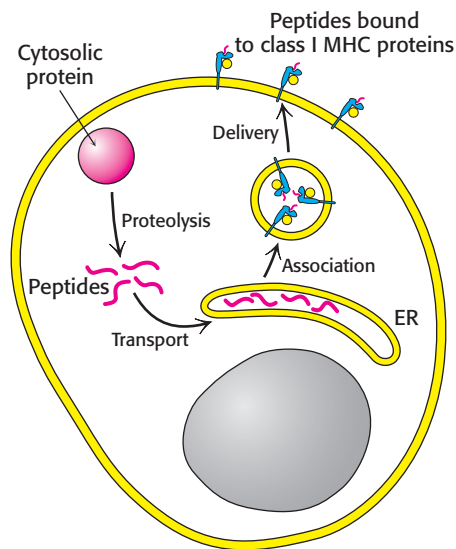


FIGURE 33.22 Presentation of peptides from cytosolic proteins. Class I MHC proteins on the surfaces of most cells display peptides that are derived from cytosolic proteins by proteolysis.

immunity, evolved to cope with intracellular pathogens. *T cells* continually scan the surfaces of all cells and kill those that exhibit foreign markings. The task is not simple; intracellular microorganisms are not so obliging as to intentionally leave telltale traces on the surface of their host. Quite the contrary, successful pathogens are masters of the art of camouflage. Vertebrates have devised an ingenious mechanism—cut and display—to reveal the presence of stealthy intruders. Nearly all vertebrate cells exhibit on their surfaces a sample of peptides derived from the digestion of proteins in their cytosol. These peptides are displayed by integral membrane proteins that are encoded by the *major histocompatibility complex (MHC)*. Specifically, peptides derived from cytosolic proteins are bound to *class I MHC proteins*.

How are these peptides generated and delivered to the plasma membrane? The process starts in the cytosol with the degradation of proteins, self proteins as well as those of pathogens (Figure 33.22). Digestion is carried out by proteasomes (Section 23.2.2). The resulting peptide fragments are transported from the cytosol into the lumen of the endoplasmic reticulum by an ATP-driven pump. In the ER, peptides combine with nascent class I MHC proteins; these complexes are then targeted to the plasma membrane.

MHC proteins embedded in the plasma membrane tenaciously grip their bound peptides so that they can be touched and scrutinized by T-cell receptors on the surface of a killer cell. Foreign peptides bound to class I MHC proteins signal that a cell is infected and mark it for destruction by cytotoxic T cells. An assembly consisting of the foreign peptide–MHC complex, the T-cell receptor, and numerous accessory proteins triggers a cascade that induces apoptosis in the infected cell. Strictly speaking, infected cells are not killed but, instead, are triggered to commit suicide to aid the organism.

33.5.1 Peptides Presented by MHC Proteins Occupy a Deep Groove Flanked by Alpha Helices

The three-dimensional structure of a large fragment of a human MHC class I protein, *human leukocyte antigen A2 (HLA-A2)*, was solved in 1987 by Don Wiley and Pamela Bjorkman. Class I MHC proteins consist of a 44-kd α chain noncovalently bound to a 12-kd polypeptide called β_2 -microglobulin. The α chain has three extracellular domains (α_1 , α_2 , and α_3), a transmembrane segment, and a tail that extends into the cytosol (Figure

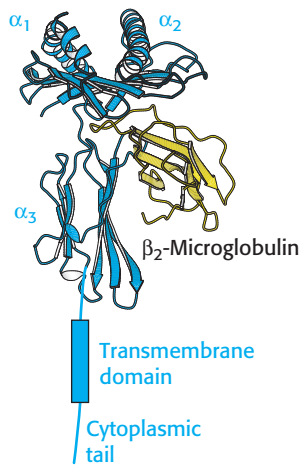


FIGURE 33.23 Class I MHC protein. A protein of this class consists of two chains. The α chain begins with two domains that include α helices (α_1 , α_2), an immunoglobulin domain (α_3), a transmembrane domain, and a cytoplasmic tail. The second chain, β_2 -microglobulin, adopts an immunoglobulin fold.

FIGURE 33.25 Anchor residues. (A) The amino acid sequences of three peptides that bind to the class I MHC protein HLA-A2 are shown. Each of these peptides has leucine in the second position and valine in the carboxyl-terminal position. (B) Comparison of the structures of these peptides reveals that the amino and carboxyl termini as well as the side chains of the leucine and valine residues are in essentially the same position in each peptide, whereas the remainder of the structures are quite different.

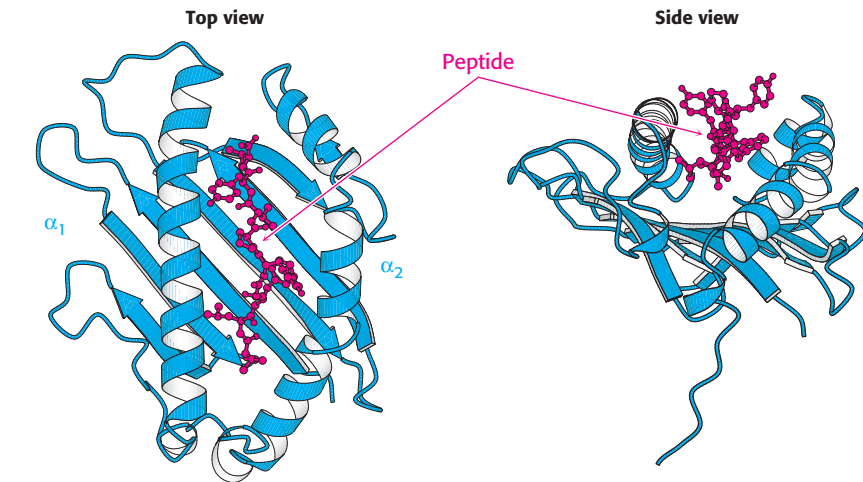
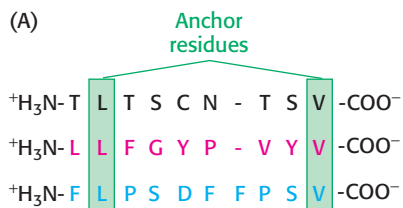
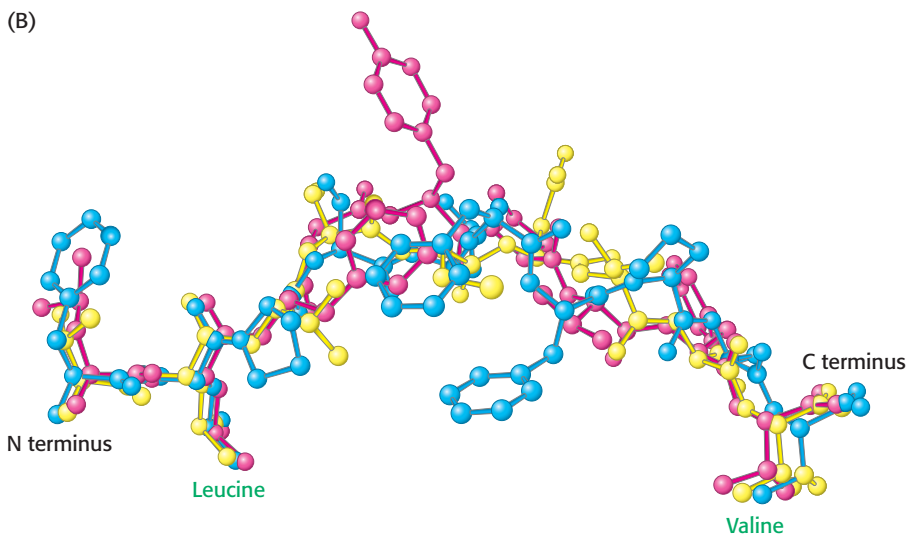


FIGURE 33.24 Class I MHC peptide-binding site. The α_1 and α_2 domains come together to form a groove in which peptides are displayed. The two views shown reveal that the peptide is surrounded on three sides by a β sheet and two α helices, but it is accessible from the top of the structure.

33.23). Cleavage by papain of the HLA α chain several residues before the transmembrane segment yielded a soluble heterodimeric fragment. The β_2 -microglobulin subunit and the α_3 domains have immunoglobulin folds, although the pairing of the two domains differs from that in antibodies. The α_1 and α_2 domains exhibit a novel and remarkable architecture. They associate intimately to form a deep groove that serves as the peptide-binding site (Figure 33.24). The floor of the groove, which is about 25 Å long and 10 Å wide, is formed by eight β strands, four from each domain. A long helix contributed by the α_1 domain forms one side, and a helix contributed by the α_2 domain forms the other side. *This groove is the binding site for the presentation of peptides.*

The groove can be filled by a peptide from 8 to 10 residues long in an extended conformation. As we shall see (Section 33.5.6), MHC proteins are remarkably diverse in the human population; each person expresses as many as six distinct class I MHC proteins and many different forms are present in different people. The first structure determined, HLA-A2, binds peptides that almost always have leucine in the second position and valine in the last position (Figure 33.25). Side chains from the MHC molecule in-



teract with the amino and carboxyl termini and with the side chains in these two key positions. These residues are often referred to as the *anchor residues*. The other residues are highly variable. Thus, many millions of different peptides can be presented by this particular class I MHC protein; the identities of only two of the nine residues are crucial for binding. Each class of MHC molecules requires a unique set of anchor residues. Thus, a tremendous range of peptides can be presented by these molecules. Note that *one face of the bound peptide is exposed to solution where it can be examined by other molecules, particularly T-cell receptors*. An additional remarkable feature of MHC–peptide complexes is their kinetic stability; once bound, a peptide is not released, even over a period of days.

33.5.2 T-Cell Receptors Are Antibody-like Proteins Containing Variable and Constant Regions

We are now ready to consider the receptor that recognizes peptides displayed by MHC proteins on target cells. The *T-cell receptor* consists of a 43-kd α chain (T_α) joined by a disulfide bond to a 43-kd β chain (T_β ; Figure 33.26). Each chain spans the plasma membrane and has a short carboxyl-terminal region on the cytosolic side. A small proportion of T cells express a receptor consisting of γ and δ chains in place of α and β . T_α and T_β , like immunoglobulin L and H chains, consist of *variable* and *constant* regions. Indeed, *these domains of the T-cell receptor are homologous to the V and C domains of immunoglobulins*. Furthermore, hypervariable sequences present in the V regions of T_α and T_β form the binding site for the epitope.

The genetic architecture of these proteins is similar to that of immunoglobulins. The variable region of T_α is encoded by about 50 V-segment genes and 70 J-segment genes. T_β is encoded by two D-segment genes in addition to 57 V and 13 J-segment genes. Again, the diversity of component genes and the use of slightly imprecise modes of joining them increase the number of distinct proteins formed. *At least 10^{12} different specificities could arise from combinations of this repertoire of genes*. Thus, T-cell receptors, like immunoglobulins, can recognize a very large number of different epitopes. All the receptors on a particular T cell have the same specificity.

How do T cells recognize their targets? The variable regions of the α and β chains of the T-cell receptor form a binding site that recognizes a combined epitope–foreign peptide bound to an MHC protein (Figure 33.27). Neither the foreign peptide alone nor the MHC protein alone forms a complex with the T-cell receptor. Thus, fragments of an intracellular pathogen are presented in a context that allows them to be detected, leading to the initiation of an appropriate response.

33.5.3 CD8 on Cytotoxic T Cells Acts in Concert with T-Cell Receptors

The T-cell receptor does not act alone in recognizing and mediating the fate of target cells. Cytotoxic T cells also express a protein termed *CD8* on their surfaces that is crucial for the recognition of the class I MHC–peptide complex. The abbreviation CD stands for *cluster of differentiation*, referring to a cell-surface marker that is used to identify a lineage or stage of differentiation. Antibodies specific for particular CD proteins have been invaluable

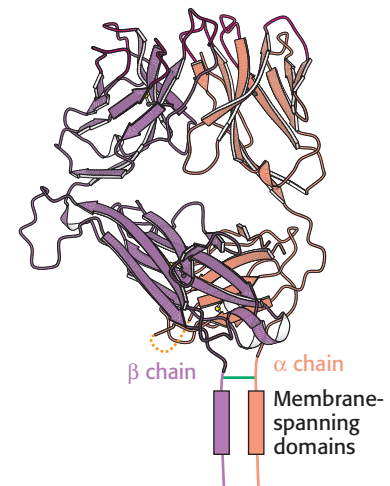


FIGURE 33.26 T-cell receptor.

This protein consists of an α chain and a β chain, each of which consists of two immunoglobulin domains and a membrane-spanning domain. The two chains are linked by a disulfide bond.

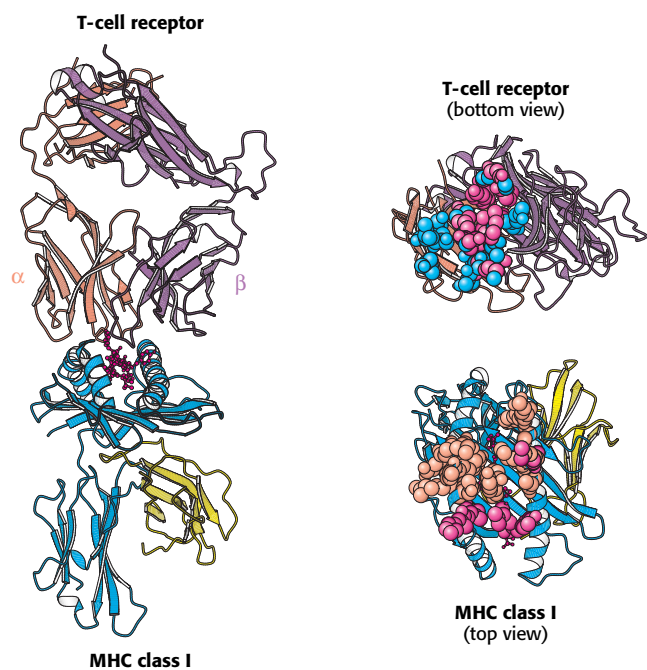


FIGURE 33.27 T-cell receptor–Class I MHC complex.

The T-cell receptor binds to a class I MHC protein containing a bound peptide. The T-cell receptor contacts both the MHC protein and the peptide as shown by surfaces exposed when the complex is separated (right). These surfaces are colored according to the chain that they contact.

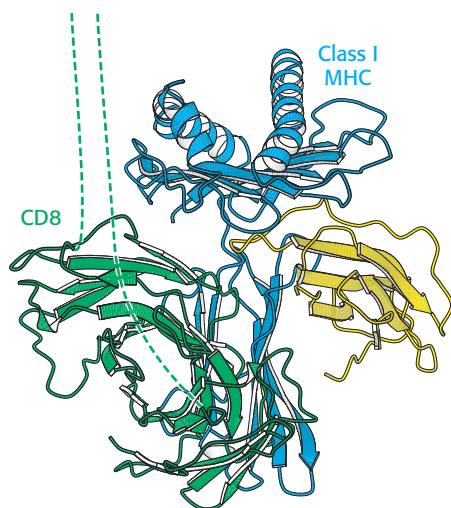


FIGURE 33.28 The coreceptor CD8. This dimeric protein extends from the surface of a cytotoxic T cell and binds to class I MHC molecules that are expressed on the surface of the cell that is bound to the T cell. The dashed lines represent extended polypeptide chains that link the immunoglobulin domain of CD8 to the membrane.

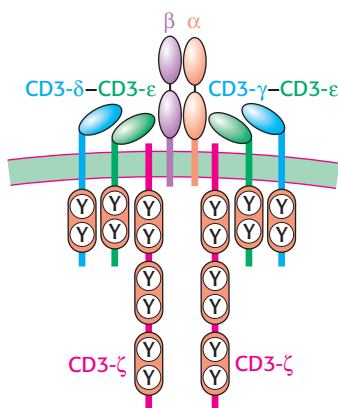


FIGURE 33.29 T-cell receptor complex. The T-cell receptor is associated with six CD3 molecules: a CD3- γ -CD3- ϵ heterodimer, a CD3- δ -CD3- ϵ heterodimer, and two chains of CD3- ζ . Single ITAM sequences are present in the cytoplasmic domains of CD3- γ , CD3- δ , and CD3- ϵ whereas three such sequences are found in each CD3- ζ chain.

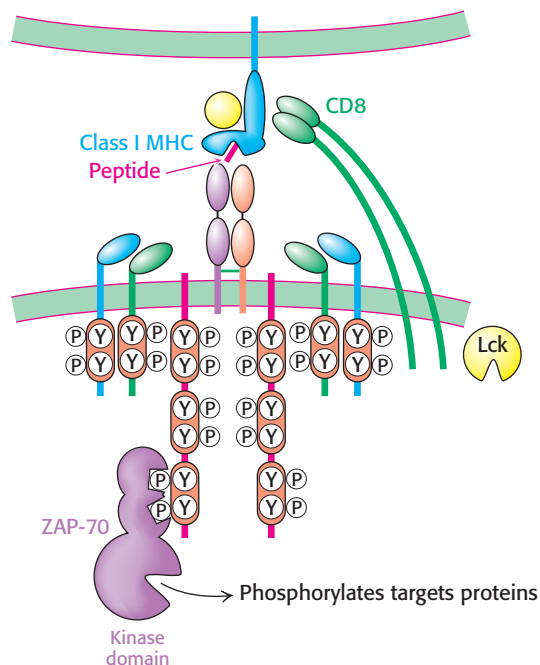


FIGURE 33.30 T-cell activation. The interaction between the T-cell receptor and a class I MHC-peptide complex results in the binding of CD8 to the MHC protein, the recruitment of the protein tyrosine kinase Lck, and the phosphorylation of tyrosine residues in the ITAM sequences of the CD3 chains. After phosphorylation, the ITAM regions serve as docking sites for the protein kinase ZAP-70, which phosphorylates protein targets to transmit the signal.

in following the development of leukocytes and in discovering new interactions between specific cell types.

Each chain in the CD8 dimer contains a domain that resembles an immunoglobulin variable domain (Figure 33.28). CD8 interacts primarily with the relatively constant α_3 domain of class I MHC proteins. This interaction further stabilizes the interactions between the T cell and its target. The cytosolic tail of CD8 contains a docking site for Lck, a cytosolic tyrosine kinase akin to Src. The T-cell receptor itself is associated with six polypeptides that form the CD3 complex (Figure 33.29). The γ , δ , and ϵ chains of CD3 are homologous to Ig- α and Ig- β associated with the B-cell receptor (Section 33.4.3); each chain consists of an extracellular immunoglobulin domain and an intracellular ITAM region. These chains associate into CD3 $\gamma\epsilon$ and CD3 $\delta\epsilon$ heterodimers. An additional component, the CD3 ζ chain, has only a small extracellular domain and a larger intracellular domain containing three ITAM sequences.

On the basis of these components, a model for T-cell activation can be envisaged that is closely parallel to the pathway for B-cell activation (Section 33.3; Figure 33.30). The binding of the T-cell receptor with the class I MHC-peptide complex and the concomitant binding of CD8 from the T-cell with the MHC molecule results in the association of the kinase Lck with the ITAM substrates of the components of the CD3 complex. Phosphorylation of the tyrosine residues in the ITAM sequences generates docking sites for a protein kinase called ZAP-70 (for 70-kd zeta-associated protein) that is homologous to Syk in B cells. Docked by its two SH2 domains, ZAP-70 phosphorylates downstream targets in the signaling cascade. Additional molecules, including a membrane-bound protein phosphatase called CD45 and a cell-surface protein called CD28, play ancillary roles in this process.

T-cell activation has two important consequences. First, the activation of cytotoxic T cells results in the secretion of *perforin*. This 70-kd protein makes the cell membrane of the target cell permeable by polymerizing to form transmembrane pores 10 nm wide (Figure 33.31). The cytotoxic T cell then secretes proteases called *granzymes* into the target cell. These enzymes initiate the pathway of apoptosis (Section 18.6.6), leading to the death of the target cell and the fragmentation of its DNA, including any viral DNA that may be present. Second, after it has stimulated its target cell to commit suicide, the activated T cell disengages and is stimulated to reproduce. Thus, additional T cells that express the same T-cell receptor are generated to continue the battle against the invader after these T cells have been identified as a suitable weapon.

33.5.4 Helper T Cells Stimulate Cells That Display Foreign Peptides Bound to Class II MHC Proteins

Not all T cells are cytotoxic. *Helper T cells*, a different class, stimulate the proliferation of specific B lymphocytes and cytotoxic T cells and thereby serve as partners in determining the immune responses that are produced. The importance of helper T cells is graphically revealed by the devastation wrought by AIDS, a condition that destroys these cells. Helper T cells, like cytotoxic T cells, detect foreign peptides that are presented on cell surfaces by MHC proteins. However, the source of the peptides, the MHC proteins that bind them, and the transport pathway are different.

Helper T cells recognize peptides bound to MHC molecules referred to as class II. Their helping action is focused on B cells, macrophages, and dendritic cells. *Class II MHC proteins* are expressed only by these *antigen-presenting cells*, unlike class I MHC proteins, which are expressed on nearly all cells. The peptides presented by class II MHC proteins do not come from the cytosol. Rather, they arise from the degradation of proteins that have been internalized by endocytosis. Consider, for example, a virus particle that is captured by membrane-bound immunoglobulins on the surface of a B cell (Figure 33.32). This complex is delivered to an endosome, a membrane-enclosed acidic compartment, where it is digested. The resulting peptides become associated with class II MHC proteins, which move to the cell surface. Peptides from the cytosol cannot reach class II proteins, whereas peptides from endosomal compartments cannot reach class I proteins. This segregation of displayed peptides is biologically critical. The association of

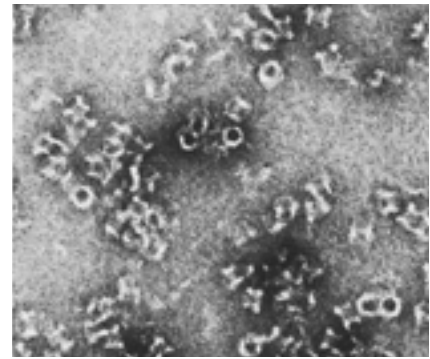
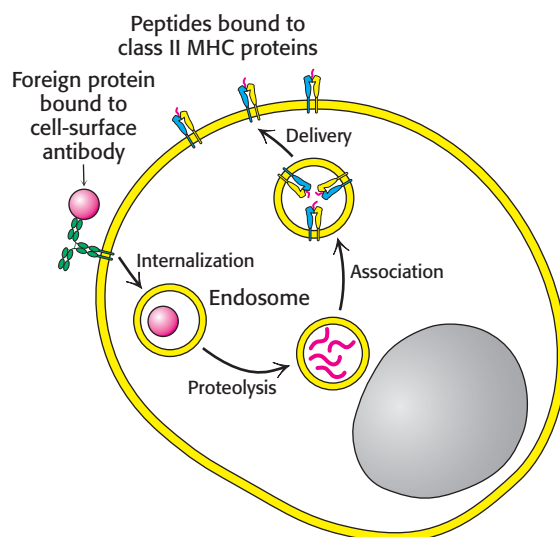


FIGURE 33.31 Consequences of cytotoxic-T-cell action. An electron micrograph showing pores in the membrane of a cell that has been attacked by a cytotoxic T cell. The pores are formed by the polymerization of perforin, a protein secreted by the cytotoxic T cell. [Courtesy of Dr. Eckhard Podock.]

FIGURE 33.32 Presentation of peptides from internalized proteins. Antigen-presenting cells bind and internalize foreign proteins and display peptides that are formed from the digestion of these proteins in Class II MHC proteins.

a foreign peptide with a class II MHC protein signals that a cell has *encountered* a pathogen and serves as a call for *help*. In contrast, association with a class I MHC protein signals that a cell has *succumbed* to a pathogen and is a call for *destruction*.

33.5.5 Helper T Cells Rely on the T-Cell Receptor and CD4 to Recognize Foreign Peptides on Antigen-Presenting Cells

The overall structure of a class II MHC molecule is remarkably similar to that of a class I molecule. Class II molecules consist of a 33-kd α chain and a noncovalently bound 30-kd β chain (Figure 33.33).

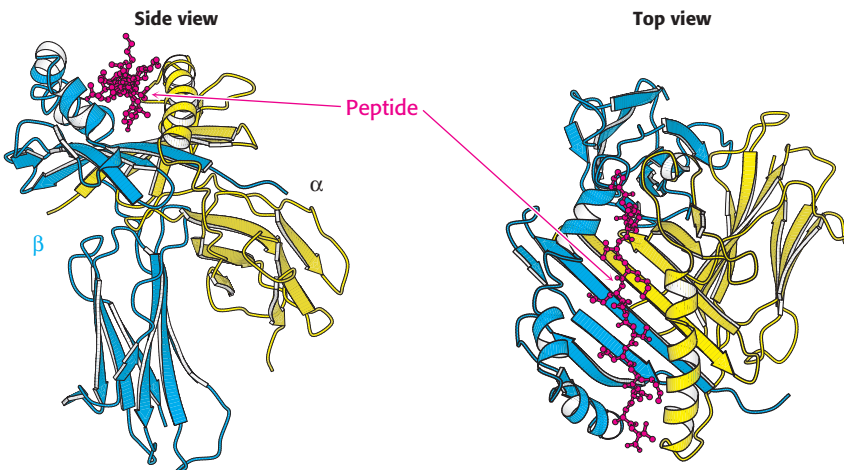


FIGURE 33.33 Class II MHC protein. A class II MHC protein consists of homologous α and β chains, each of which has an amino-terminal domain that constitutes half of the peptide-binding structure, as well as a carboxyl-terminal immunoglobulin domain. The peptide-binding site is similar to that in class I MHC proteins except that it is open at both ends, allowing class II MHC proteins to bind longer peptides than those bound by class I.



FIGURE 33.34 Coreceptor CD4. This protein comprises four tandem immunoglobulin domains that extend from the surface of helper T cells.

Each contains two extracellular domains, a transmembrane segment, and a short cytosolic tail. The peptide-binding site is formed by the α_1 and β_1 domains, each of which contributes a long helix and part of a β sheet. Thus, the same structural elements are present in class I and class II MHC molecules, but they are combined into polypeptide chains in different ways. Class II MHC molecules appear to form stable dimers, unlike class I molecules, which are monomeric. The peptide-binding site of a class II molecule is open at both ends, and so this groove can accommodate longer peptides than can be bound by class I molecules; typically, peptides between 13 and 18 residues long are bound. The peptide-binding specificity of each class II molecule depends on binding pockets that recognize particular amino acids in specific positions along the sequence.

Helper T cells express T-cell receptors that are produced from the same genes as those on cytotoxic T cells. These T-cell receptors interact with class II MHC molecules in a manner that is analogous to T-cell-receptor interaction with class I MHC molecules. Nonetheless, helper T cells and cytotoxic T cells are distinguished by other proteins that they express on their surfaces. In particular, helper T cells express a protein called CD4 instead of expressing CD8. CD4 consists of four immunoglobulin domains that extend from the T-cell surface, as well as a small cytoplasmic region (Figure 33.34). The amino-terminal immunoglobulin domains of CD4 interact with the base on the class II MHC molecule. Thus, helper T cells bind cells expressing class II MHC specifically because of the interactions with CD4 (Figure 33.35).

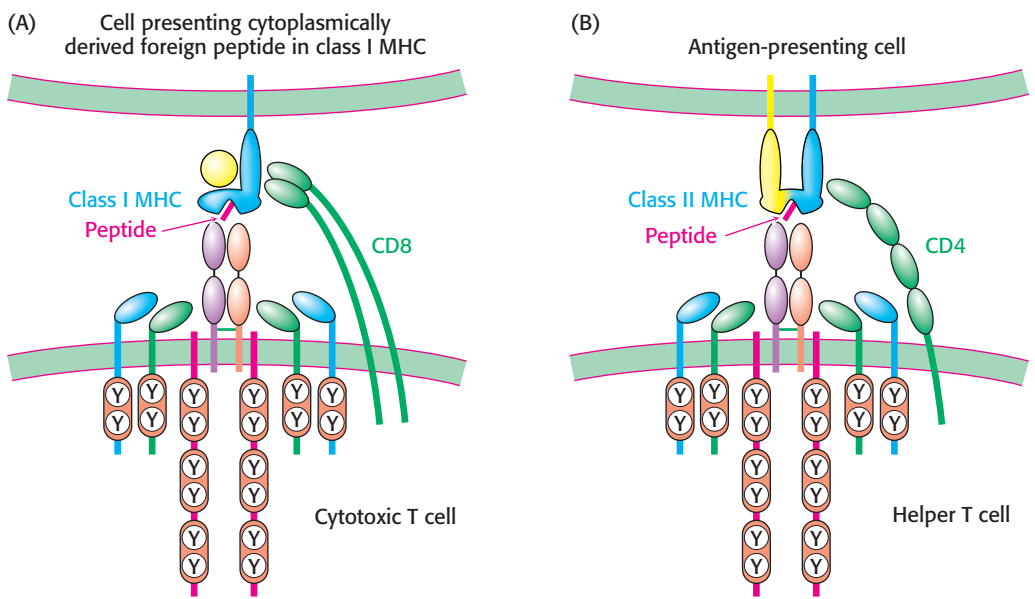



FIGURE 33.35 Variations on a theme. (A) Cytotoxic T cells recognize foreign peptides presented in class I MHC proteins with the aid of the coreceptor CD8. (B) Helper T cells recognize peptides presented in class II MHC proteins by specialized antigen-presenting cells with the aid of the coreceptor CD4.

When a helper T cell binds to an antigen-presenting cell expressing an appropriate class II MHC–peptide complex, signaling pathways analogous to those in cytotoxic T cells are initiated by the action of the kinase Lck on ITAMs in the CD3 molecules associated with the T-cell receptor. However, rather than triggering events leading to the death of the attached cell, *these signaling pathways result in the secretion of cytokines from the helper cell.* Cytokines are a family of molecules that include, among others, interleukin-2 and interferon- γ . Cytokines bind to specific receptors on the antigen-presenting cell and stimulate growth, differentiation, and in regard to plasma cells, which are derived from B cells, antibody secretion (Figure 33.36). Thus, the internalization and presentation of parts of a foreign pathogen help to generate a local environment in which cells taking part in the defense against this pathogen can flourish through the action of helper T cells.

33.5.6 MHC Proteins Are Highly Diverse

 MHC class I and II proteins, the presenters of peptides to T cells, were discovered because of their role in *transplantation rejection*. A tissue transplanted from one person to another or from one mouse to another is usually rejected by the immune system. In contrast, tissues transplanted from one identical twin to another or between mice of an inbred strain are accepted. Genetic analyses revealed that rejection occurs when tissues are transplanted between individuals having different genes in the major histocompatibility complex, a cluster of more than 75 genes playing key roles in immunity. The 3500-kb span of the MHC is nearly the length of the

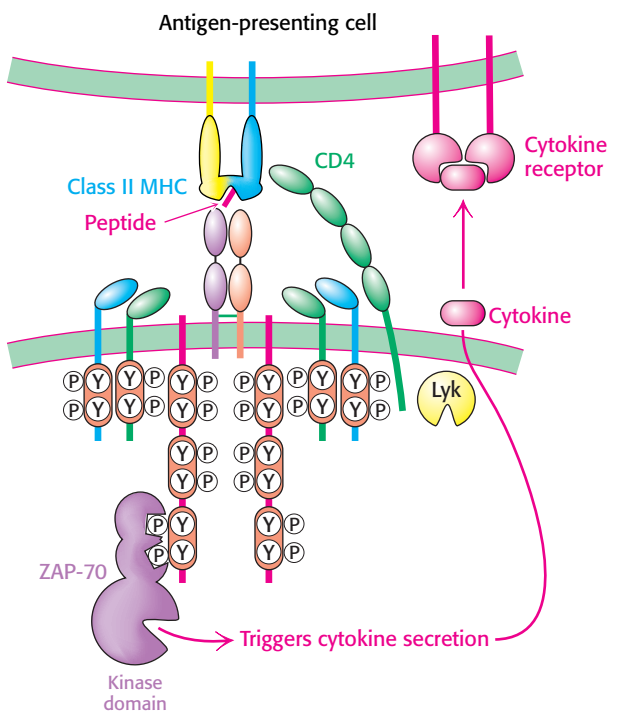


FIGURE 33.36 Helper T cell action. The engagement of the T-cell receptor in helper T cells results in the secretion of cytokines. These cytokines bind to cytokine receptors expressed on the surface of the antigen-presenting cell, stimulating cell growth, differentiation, and, in regard to a B cell, antibody secretion.

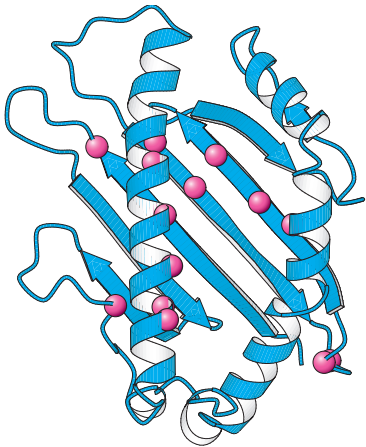


FIGURE 33.37 Polymorphism in class I MHC proteins. The positions of sites with a high degree of polymorphism in the human population are displayed as red spheres on the structure of the amino-terminal part of a class I MHC protein.

entire *E. coli* chromosome. The MHC encodes class I proteins (presenters to cytotoxic T cells) and class II proteins (presenters to helper T cells), as well as class III proteins (components of the complement cascade) and many other proteins that play key roles in immunity.

Human beings express six different class I genes (three from each parent) and six different class II genes. The three loci for class I genes are called HLA-A, -B, and -C; those for class II genes are called HLA-DP, -DQ, and -DR. These loci are *highly polymorphic*: many alleles of each are present in the population. For example, more than 50 each of HLA-A, -B, and -C alleles are known; the numbers discovered increase each year. Hence, the likelihood that two unrelated persons have identical class I and II proteins is very small ($<10^{-4}$), accounting for transplantation rejection unless the genotypes of donor and acceptor are closely matched in advance.

Differences between class I proteins are located mainly in the α_1 and α_2 domains, which form the peptide-binding site (Figure 33.37). The α_3 domain, which interacts with a constant β_2 -microglobulin is largely conserved. Similarly, the differences between class II proteins cluster near the peptide-binding groove. Why are MHC proteins so highly variable? *Their diversity makes possible the presentation of a very wide range of peptides to T cells.* A particular class I or class II molecule may not be able to bind any of the peptide fragments of a viral protein. The likelihood of a fit is markedly increased by having several kinds (usually six) of each class of presenters in each individual. If all members of a species had identical class I or class II molecules, the population would be much more vulnerable to devastation by a pathogen that had evolved to evade presentation. The evolution of the diverse human MHC repertoire has been driven by the selection for individual members of the species who resist infections to which other members of the population may be susceptible.

33.5.7 Human Immunodeficiency Viruses Subvert the Immune System by Destroying Helper T Cells

In 1981, the first cases of a new disease now called *acquired immune deficiency syndrome (AIDS)* were recognized. The victims died of rare infections because their immune systems were crippled. The cause was identified two years later by Luc Montagnier and coworkers. AIDS is produced by *human immunodeficiency virus (HIV)*, of which two major classes are known: HIV-1 and the much less common HIV-2. Like other *retroviruses*, HIV contains a single-stranded RNA genome that is replicated through a double-stranded DNA intermediate. This viral DNA becomes integrated into the genome of the host cell. In fact, viral genes are transcribed only when they are integrated into the host DNA.

The HIV virion is enveloped by a lipid bilayer membrane containing two glycoproteins: gp41 spans the membrane and is associated with gp120, which is located on the external face (Figure 33.38). The core of the virus contains two copies of the RNA genome and associated transfer RNAs, and several molecules of reverse transcriptase. They are surrounded by many copies of two proteins called p18 and p24. *The host cell for HIV is the helper T cell.* The gp120 molecules on the membrane of HIV bind to CD4 molecules on the surface of the helper T cell (Figure 33.39). This interaction allows the associated viral gp41 to insert its amino-terminal head into the host-cell membrane. The viral membrane and the helper-cell membrane fuse, and the viral core is released directly into the cytosol. Infection by HIV leads to the destruction of helper T cells because the permeability of the host plasma membrane is markedly increased by the insertion of viral

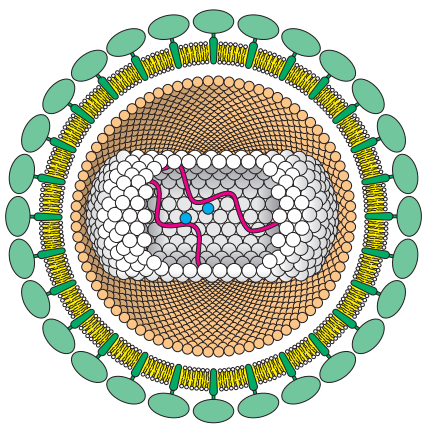


FIGURE 33.38 Human immunodeficiency virus. A schematic diagram of HIV reveals its proteins and nucleic acid components. The membrane-envelope glycoproteins gp41 and gp120 are shown in dark and light green. The viral RNA is shown in red, and molecules of reverse transcriptase are shown in blue. [After R. C. Gallo. *The AIDS virus*. Copyright © 1987 by Scientific American, Inc. All rights reserved.]

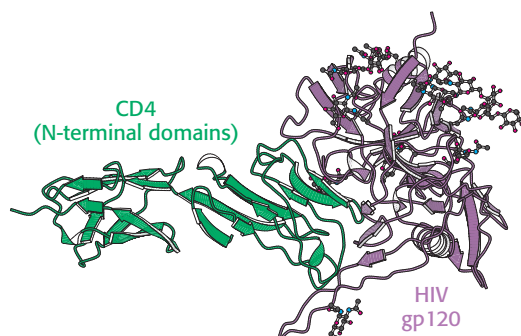


FIGURE 33.39 HIV receptor. A complex between a modified form of the envelope glycoprotein gp120 from HIV and a peptide corresponding to the two amino-terminal domains from the helper T-cell protein CD4 reveals how viral infection of helper T cells is initiated.

glycoproteins and the budding of virus particles. The influx of ions and water disrupts the ionic balance, causing osmotic lysis.

The development of an effective AIDS vaccine is difficult owing to the antigenic diversity of HIV strains. Because its mechanism for replication is quite error prone, a population of HIV presents an ever-changing array of coat proteins. Indeed, the mutation rate of HIV is more than 65 times as high as that of influenza virus. A major aim now is to define relatively conserved sequences in these HIV proteins and use them as immunogens.

33.6 IMMUNE RESPONSES AGAINST SELF-ANTIGENS ARE SUPPRESSED

The primary function of the immune system is to protect the host from invasion by foreign organisms. But how does the immune system avoid mounting attacks against the host organism? In other words, how does the immune system distinguish between self and nonself? Clearly, proteins from the organism itself do not bear some special tag identifying them. Instead, selection processes early in the developmental pathways for immune cells kill or suppress those immune cells that react strongly with self-antigens. The evolutionary paradigm still applies; immune cells that recognize self-antigens are generated, but selective mechanisms eliminate such cells in the course of development.

33.6.1 T Cells Are Subject to Positive and Negative Selection in the Thymus

T cells derive their name from the location of their production—the thymus, a small organ situated just above the heart. Examination of the developmental pathways leading to the production of mature cytotoxic and helper T cells reveals the selection mechanisms that are crucial for distinguishing self from nonself. These selection criteria are quite stringent; approximately 98% of the thymocytes, the precursors of T cells, die before the completion of the maturation process.

Thymocytes produced in the bone marrow do not express the T-cell-receptor complex, CD4, or CD8. On relocation to the thymus and rearrangement of the T-cell-receptor genes, the immature thymocyte expresses all of these molecules. These cells are first subjected to *positive selection*

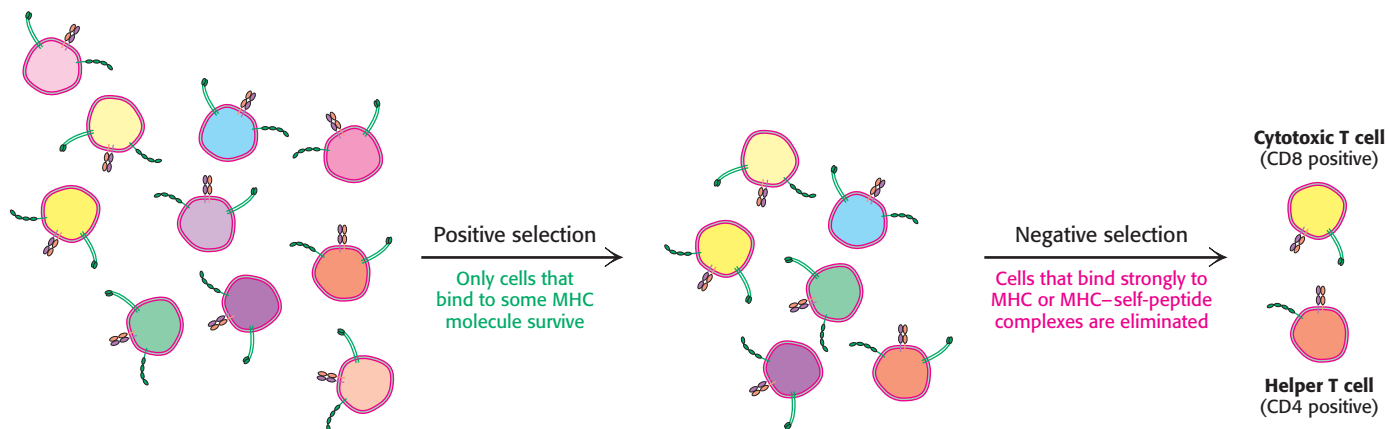



FIGURE 33.40 T-cell selection. A population of thymocytes is subjected first to positive selection to remove cells that express T-cell receptors that will not bind to MHC proteins expressed by the individual organism. The surviving cells are then subjected to negative selection to remove cells that bind strongly to MHC complexes bound to self-peptides.

(Figure 33.40). Cells for which the T-cell receptor can bind with reasonable affinity to either class I or class II MHC molecules survive this selection; those for which the T-cell receptor does not participate in such an interaction undergo apoptosis and die. The affinities of interaction required to pass

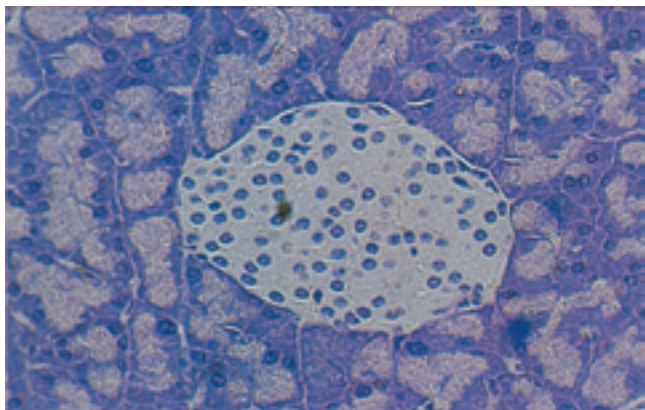
this selection are relatively modest, and so contacts between the T-cell receptor and the MHC molecules themselves are sufficient without any significant contribution from the bound peptides (which will be derived from proteins in the thymus). *The role of the positive selection step is to prevent the production of T cells that will not bind to any MHC complex present, regardless of the peptide bound.*

The cell population that survives positive selection is subjected to a second step, *negative selection*. Here, T cells that bind with high affinity to MHC complexes bound to self-peptides expressed on the surfaces of antigen-presenting cells in the thymus undergo apoptosis or are otherwise suppressed. Those that do not bind too avidly to any such MHC complex complete development and become mature cytotoxic T cells (which express only CD8) or helper T cells (which express only CD4). The negative selection step leads to *self tolerance*; cells that bind an MHC-self-peptide complex are removed from the T-cell population. Similar mechanisms apply to developing B cells, suppressing B cells that express antibodies that interact strongly with self-antigens.

33.6.2 Autoimmune Diseases Result from the Generation of Immune Responses Against Self-Antigens

 Although thymic selection is remarkably efficient in suppressing the immune response to self-antigens, failures do occur. Such failures result in *autoimmune diseases*. These diseases include relatively common illnesses such as insulin-dependent diabetes mellitus, multiple sclerosis, and rheumatoid arthritis. In these illnesses, immune responses against self-antigens result in damage to selective tissues that express the antigen (Figure 33.41).

(A)



(B)

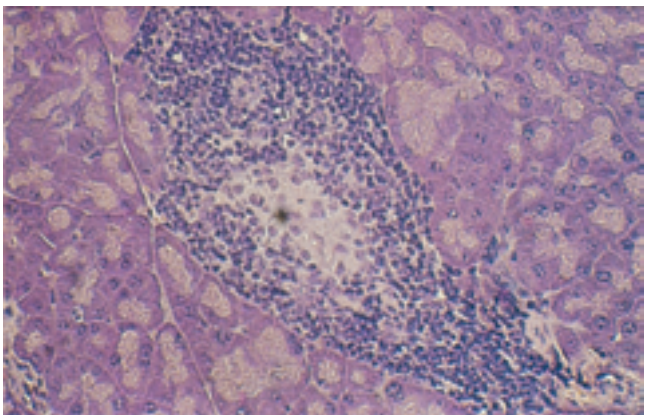



FIGURE 33.41 Consequences of autoimmunity. Photomicrographs of an islet of Langerhans (A) in the pancreas of a normal mouse and (B) in the pancreas of a mouse with an immune response against pancreatic β cells, which results in a disease resembling insulin-dependent diabetes mellitus in human beings. [From M. A. Atkinson and N. K. Maclaren. *What causes diabetes?* Copyright © 1990 by Scientific American, Inc. All rights reserved.]

In many cases, the cause of the generation of self-reactive antibodies or T cells is unclear. However, in other cases, infectious organisms such as bacteria or viruses may play a role. Infection leads to the generation of antibodies and T cells that react with many different epitopes from the infectious organism. If one of these antigens closely resembles a self-antigen, an autoimmune response can result. For example, *Streptococcus* infections sometimes lead to rheumatic fever owing to the production of antibodies to streptococcal antigens that cross-react with exposed molecules in heart muscle.

33.6.3 The Immune System Plays a Role in Cancer Prevention

 The development of immune responses against proteins encoded by our own genomes can be beneficial under some circumstances. Cancer cells have undergone significant changes that often result in the expression of proteins that are not normally expressed. For example, the mutation of genes can generate proteins that do not correspond in amino acid sequence to any normal protein. Such proteins may be recognized as foreign, and an immune response will be generated specifically against the cancer cell. Alternatively, cancer cells often produce proteins that are expressed during embryonic development but are not expressed or are expressed at very low levels after birth. For example, a membrane glycoprotein protein called *carcinoembryonic antigen (CEA)* appears in the gastrointestinal cells of developing fetuses but is not normally expressed at significant levels after birth. More than 50% of patients with colorectal cancer have elevated serum levels of CEA. Immune cells recognizing epitopes from such proteins will not be subject to negative selection and, hence, will be present in the adult immune repertoire. These cells may play a cancer surveillance role, killing cells that overexpress antigens such as CEA and preventing genetically damaged cells from developing into tumors.

SUMMARY

- To respond effectively to a vast array of pathogens, the immune system must be tremendously adaptable. Adaptation by the immune system follows the principles of evolution: an enormously diverse set of potentially useful proteins is generated; these proteins are then subjected to intense selection so that only cells that express useful proteins flourish and continue development, until an effective immune response to a specific invader is generated.
- **Antibodies Possess Distinct Antigen-Binding and Effector Units**
The major immunoglobulin in the serum is immunoglobulin G. An IgG protein is a heterotetramer with two heavy chains and two light chains. Treatment of IgG molecules with proteases such as papain produces three fragments: two F_{ab} fragments that retain antigen-binding activity and an F_c fragment that retains the ability to activate effector functions such as the initiation of the complement cascade. The F_{ab} fragments include the L chain and the amino-terminal half of the H chain; the F_c domain is a dimer consisting of the carboxyl-terminal halves of two H chains. Five different classes of antibody—IgG, IgM, IgA, IgD, and IgE—differ in their heavy chains and, hence, in their effector functions.
- **The Immunoglobulin Fold Consists of a Beta-Sandwich Framework with Hypervariable Loops**
One particular protein fold is found in many of the key proteins of the immune system. The immunoglobulin fold consists of a pair of β sheets

that pack against one another, linked by a single disulfide bond. Loops projecting from one end of the structure form a binding surface that can be varied by changing the amino acid sequences within the loops. Domains with immunoglobulin folds are linked to form antibodies and other classes of proteins in the immune system including T-cell receptors.

- **Antibodies Bind Specific Molecules Through Their Hypervariable Loops**

Two chains come together to form the binding surface of an antibody. Three loops from each domain, the complementarity-determining regions, form an essentially continuous surface that can vary tremendously in shape, charge, and other characteristics to allow particular antibodies to bind to molecules ranging from small molecules to large protein surfaces.

- **Diversity Is Generated by Gene Rearrangements**

The tremendous diversity of the amino acid sequences of antibodies is generated by segmental rearrangements of genes. For antibody κ light chains, one of 40 variable regions is linked to one of five joining regions. The combined VJ unit is then linked to the constant region. Thousands of different genes can be generated in this manner. Similar arrays are rearranged to form the genes for the heavy chains, but an additional region called the diversity region lies between the V and the J regions. The combination L and H chains, each obtained through such rearranged genes, can produce more than 10^8 distinct antibodies. Different classes of antibodies are also generated by gene rearrangements that lead to class switching. Oligomerization of membrane-bound antibody molecules initiates a signal-transduction cascade inside B cells. Key steps in this signaling process include the phosphorylation of specific tyrosine residues in sequences termed immunoreceptor tyrosine-based activation motifs (ITAMs), present in proteins that associate with the membrane-bound antibodies.

- **Major-Histocompatibility-Complex Proteins Present Peptide Antigens on Cell Surfaces for Recognition by T-Cell Receptors**

Intracellular pathogens such as viruses and mycobacteria cannot be easily detected. Intracellular proteins are constantly being cut into small peptides by proteasomes and displayed in class I major-histocompatibility-complex proteins on cell surfaces. Such peptides lie in a groove defined by two helices in the class I MHC proteins. The combination of MHC protein and peptide can be bound by an appropriate T-cell receptor. T-cell receptors resemble the antigen-binding domains of antibodies in structure, and diversity in T-cell-receptor sequence is generated by V(D)J gene rearrangements. The T-cell receptor recognizes features of both the peptide and the MHC molecule that presents it. Cytotoxic T cells initiate apoptosis in cells to which they bind through T-cell receptor–class I MHC-peptide interactions aided by interactions with the coreceptor molecule CD8. Helper T cells recognize peptides presented in class II MHC proteins, a distinct type of MHC protein expressed only on antigen-presenting cells such as B cells and macrophages. Helper T cells express the coreceptor CD4, rather than CD8. CD4 interacts with class II MHC proteins present on antigen-presenting cells. Signaling pathways, analogous to those in B cells, are initiated by interactions between MHC–peptide complexes and T-cell receptors and the CD8 and CD4 coreceptors. Human immunodeficiency virus damages the immune system by infecting cells that express CD4, such as helper T cells.

● Immune Responses Against Self-Antigens Are Suppressed

In principle, the immune system is capable of generating antibodies and T-cell receptors that bind to self-molecules; that is, molecules that are normally present in a healthy and uninfected individual organism. Selection mechanisms prevent such self-directed molecules from being expressed at high levels. The selection process includes both positive selection, to enrich the population of cells that express molecules that have the potential to bind foreign antigens in an appropriate context, and negative selection, which eliminates cells that express molecules with too high an affinity for self-antigens. Autoimmune diseases such as insulin-dependent diabetes mellitus can result from amplification of a response against a self-antigen.

KEY TERMS

humoral immune response (p. 921)	immunoglobulin E (p. 924)	human leukocyte antigen (HLA) (p. 935)
B lymphocyte (B cell) (p. 922)	variable region (p. 925)	β_2 -microglobulin (p. 935)
antigen (p. 922)	constant region (p. 925)	T-cell receptor (p. 937)
antigenic determinant (epitope) (p. 922)	immunoglobulin fold (p. 926)	CD8 (p. 937)
cellular immune response (p. 922)	hypervariable loop (p. 926)	perforin (p. 939)
cytotoxic T lymphocyte (killer T cell) (p. 922)	complementarity-determining region (CDR) (p. 926)	granzymes (p. 939)
helper T lymphocyte (p. 922)	V(D)J recombination (p. 931)	helper T cell (p. 939)
immunoglobulin G (p. 923)	immunoreceptor tyrosine-based activation motif (ITAM) (p. 932)	class II MHC protein (p. 939)
F _{ab} (p. 923)	cyclosporin (p. 933)	CD4 (p. 940)
F _c (p. 923)	hapten (p. 933)	human immunodeficiency virus (HIV) (p. 940)
light chain (p. 923)	class switching (p. 934)	positive selection (p. 943)
heavy chain (p. 923)	T cell (p. 935)	negative selection (p. 944)
segmental flexibility (p. 924)	major histocompatibility complex (MHC) (p. 935)	autoimmune disease (p. 944)
immunoglobulin M (p. 924)	class I MHC protein (p. 935)	carcinoembryonic antigen (CEA) (p. 945)
immunoglobulin A (p. 924)		
immunoglobulin D (p. 924)		

SELECTED READINGS

Where to start

- Nossal, G. J. V., 1993. Life, death, and the immune system. *Sci. Am.* 269(3): 53–62.
- Tonegawa, S., 1985. The molecules of the immune system. *Sci. Am.* 253(4): 122–131.
- Leder, P., 1982. The genetics of antibody diversity. *Sci. Am.* 246(5): 102–115.
- Bromley, S. K., Burack, W. R., Johnson, K. G., Somersalo, K., Sims, T. N., Sumen, C., Davis, M. M., Shaw, A. S., Allen, P. M., and Dustin, M. L., 2001. The immunological synapse. *Annu. Rev. Immunol.* 19:375–396.

Books

- Goldsby, R. A., Kindt, T. J., Osborne, B. A. 2000. *Kuby Immunology* (4th ed.). W. H. Freeman and Company.
- Abbas, A. K., Lichtman, A. H., and Pober, J. S., 1992. *Cellular and Molecular Immunology* (2d ed.). Saunders.
- Cold Spring Harbor Symposia on Quantitative Biology, 1989. Volume 54. Immunological Recognition.
- Nisino, A., 1985. *Introduction to Molecular Immunology* (2d ed.). Sinauer.

Weir, D. M. (Ed.), 1986. *Handbook of Experimental Immunology*. Oxford University Press.

Structure of antibodies and antibody–antigen complexes

- Davies, D. R., Padlan, E. A., and Sheriff, S., 1990. Antibody-antigen complexes. *Annu. Rev. Biochem.* 59:439–473.
- Poljak, R. J., 1991. Structure of antibodies and their complexes with antigens. *Mol. Immunol.* 28:1341–1345.
- Davies, D. R., and Cohen, G. H., 1996. Interactions of protein antigens with antibodies. *Proc. Natl. Acad. Sci. USA* 93:7–12.
- Marquart, M., Deisenhofer, J., Huber, R., and Palm, W., 1980. Crystallographic refinement and atomic models of the intact immunoglobulin molecule Kol and its antigen-binding fragment at 3.0 Å and 1.9 Å resolution. *J. Mol. Biol.* 141:369–391.
- Silverton, E. W., Navia, M. A., and Davies, D. R., 1977. Three-dimensional structure of an intact human immunoglobulin. *Proc. Natl. Acad. Sci. USA* 74:5140–5144.
- Padlan, E. A., Silverton, E. W., Sheriff, S., Cohen, G. H., Smith, G. S., and Davies, D. R., 1989. Structure of an antibody-antigen complex: Crystal structure of the HyHEL-10 Fab lysozyme complex. *Proc. Natl. Acad. Sci. USA* 86:5938–5942.

- Rini, J., Schultze-Gahmen, U., and Wilson, I. A., 1992. Structural evidence for induced fit as a mechanism for antibody-antigen recognition. *Nature* 255:959–965.
- Fischmann, T. O., Bentley, G. A., Bhat, T. N., Boulot, G., Mariuzza, R. A., Phillips, S. E., Tello, D., and Poljak, R. J., 1991. Crystallographic refinement of the three-dimensional structure of the FabD1.3-lysozyme complex at 2.5-Å resolution. *J. Biol. Chem.* 266:12915–12920.
- Burton, D. R., 1990. Antibody: The flexible adaptor molecule. *Trends Biochem. Sci.* 15:64–69.
- Generation of diversity**
- Tonegawa, S., 1988. Somatic generation of immune diversity. *Biosci. Rep.* 8:3–26.
- Honjo, T., and Habu, S., 1985. Origin of immune diversity: Genetic variation and selection. *Annu. Rev. Biochem.* 54:803–830.
- Gellert, M., and McBlane, J. F., 1995. Steps along the pathway of VDJ recombination. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 347:43–47.
- Harris, R. S., Kong, Q., and Maizels, N., 1999. Somatic hypermutation and the three R's: Repair, replication and recombination. *Mutat. Res.* 436:157–178.
- Lewis, S. M., and Wu, G. E., 1997. The origins of V(D)J recombination. *Cell* 88:159–162.
- Ramsden, D. A., van Gent, D. C., and Gellert, M., 1997. Specificity in V(D)J recombination: New lessons from biochemistry and genetics. *Curr. Opin. Immunol.* 9:114–120.
- Roth, D. B., and Craig, N. L., 1998. VDJ recombination: A transposase goes to work. *Cell* 94:411–414.
- Sadofsky, M. J., 2001. The RAG proteins in V(D)J recombination: More than just a nuclease. *Nucleic Acids Res.* 29:1399–1409.
- MHC proteins and antigen processing**
- Bjorkman, P. J., and Parham, P., 1990. Structure, function, and diversity of class I major histocompatibility complex molecules. *Annu. Rev. Biochem.* 59:253–288.
- Goldberg, A. L., and Rock, K. L., 1992. Proteolysis, proteasomes, and antigen presentation. *Nature* 357:375–379.
- Madden, D. R., Gorga, J. C., Strominger, J. L., and Wiley, D. C., 1992. The three-dimensional structure of HLA-B27 at 2.1 Å resolution suggests a general mechanism for tight binding to MHC. *Cell* 70:1035–1048.
- Brown, J. H., Jardetzky, T. S., Gorga, J. C., Stern, L. J., Urban, R. G., Strominger, J. L., and Wiley, D. C., 1993. Three-dimensional structure of the human class II histocompatibility antigen HLA-DR1. *Nature* 364:33–39.
- Saper, M. A., Bjorkman, P. J., and Wiley, D. C., 1991. Refined structure of the human histocompatibility antigen HLA-A2 at 2.6 Å resolution. *J. Mol. Biol.* 219:277–319.
- Madden, D. R., Gorga, J. C., Strominger, J. L., and Wiley, D. C., 1991. The structure of HLA-B27 reveals nonamer self-peptides bound in an extended conformation. *Nature* 353:321–325.
- Cresswell, P., Bangia, N., Dick, T., and Diedrich, G., 1999. The nature of the MHC class I peptide loading complex. *Immunol. Rev.* 172:21–28.
- Madden, D. R., Garboczi, D. N., and Wiley, D. C., 1993. The antigenic identity of peptide-MHC complexes: A comparison of the conformations of five viral peptides presented by HLA-A2. *Cell* 75:693–708.
- T-cell receptors and signaling complexes**
- Hennecke, J., and Wiley, D. C., 2001. T cell receptor-MHC interactions up close. *Cell* 104:1–4.
- Ding, Y. H., Smith, K. J., Garboczi, D. N., Utz, U., Biddison, W. E., and Wiley, D. C., 1998. Two human T cell receptors bind in a similar diagonal mode to the HLA-A2/Tax peptide complex using different TCR amino acids. *Immunity* 8:403–411.
- Reinherz, E. L., Tan, K., Tang, L., Kern, P., Liu, J., Xiong, Y., Hussey, R. E., Smolyar, A., Hare, B., Zhang, R., Joachimiak, A., Chang, H. C., Wagner, G., and Wang, J., 1999. The crystal structure of a T cell receptor in complex with peptide and MHC class II. *Science* 286:1913–1921.
- Cochran, J. R., Cameron, T. O., and Stern, L. J., 2000. The relationship of MHC-peptide binding and T cell activation probed using chemically defined MHC class II oligomers. *Immunity* 12:241–250.
- Cochran, J. R., Cameron, T. O., Stone, J. D., Lubetsky, J. B., and Stern, L. J., 2001. Receptor proximity, not intermolecular orientation, is critical for triggering T-cell activation. *J. Biol. Chem.* 276:28068–28074.
- Garcia, K. C., Teyton, L., and Wilson, I. A., 1999. Structural basis of T cell recognition. *Annu. Rev. Immunol.* 17:369–397.
- Gaul, B. S., Harrison, M. L., Geahlen, R. L., Burton, R. A., and Post, C. B., 2000. Substrate recognition by the Lyn protein-tyrosine kinase: NMR structure of the immunoreceptor tyrosine-based activation motif signaling region of the B cell antigen receptor. *J. Biol. Chem.* 275:16174–16182.
- Kern, P. S., Teng, M. K., Smolyar, A., Liu, J. H., Liu, J., Hussey, R. E., Spoerl, R., Chang, H. C., Reinherz, E. L., and Wang, J. H., 1998. Structural basis of CD8 coreceptor function revealed by crystallographic analysis of a murine CD8 α alpha ectodomain fragment in complex with H-2Kb. *Immunity* 9:519–530.
- Konig, R., Fleury, S., and Germain, R. N., 1996. The structural basis of CD4-MHC class II interactions: Coreceptor contributions to T cell receptor antigen recognition and oligomerization-dependent signal transduction. *Curr. Top. Microbiol. Immunol.* 205:19–46.
- Krummel, M., Wulfing, C., Sumen, C., and Davis, M. M., 2000. Thirty-six views of T-cell recognition. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 355:1071–1076.
- Janeway, C. J., 1992. The T cell receptor as a multicomponent signalling machine: CD4/CD8 coreceptors and CD45 in T cell activation. *Annu. Rev. Immunol.* 10:645–674.
- Podack, E. R., and Kupfer, A., 1991. T-cell effector functions: Mechanisms for delivery of cytotoxicity and help. *Annu. Rev. Cell Biol.* 7:479–504.
- Davis, M. M., 1990. T cell receptor gene diversity and selection. *Annu. Rev. Biochem.* 59:475–496.
- Leahy, D. J., Axel, R., and Hendrickson, W. A., 1992. Crystal structure of a soluble form of the human T cell coreceptor CD8 at 2.6 Å resolution. *Cell* 68:1145–1162.
- Lowin, B., Hahne, M., Mattmann, C., and Tschopp, J., 1994. Cytolytic T-cell cytotoxicity is mediated through perforin and Fas lytic pathways. *Nature* 370:650–652.
- HIV and AIDS**
- Fauci, A. S., 1988. The human immunodeficiency virus: Infectivity and mechanisms of pathogenesis. *Science* 239:617–622.
- Gallo, R. C., and Montagnier, L., 1988. AIDS in 1988. *Sci. Am.* 259(4):41–48.
- Kwong, P. D., Wyatt, R., Robinson, J., Sweet, R. W., Sodroski, J., and Hendrickson, W. A., 1998. Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. *Nature* 393:648–659.
- Discovery of major concepts**
- Ada, G. L., and Nossal, G., 1987. The clonal selection theory. *Sci. Am.* 257(2):62–69.
- Porter, R. R., 1973. Structural studies of immunoglobulins. *Science* 180:713–716.
- Edelman, G. M., 1973. Antibody structure and molecular immunology. *Science* 180:830–840.
- Kohler, G., 1986. Derivation and diversification of monoclonal antibodies. *Science* 233:1281–1286.
- Milstein, C., 1986. From antibody structure to immunological diversification of immune response. *Science* 231:1261–1268.
- Janeway, C. A., Jr., 1989. Approaching the asymptote? Evolution and revolution in immunology. *Cold Spring Harbor Symp. Quant. Biol.* 54:1–13.

PROBLEMS

1. *Energetics and kinetics.* Suppose that the dissociation constant of an F_{ab} -hapten complex is 3×10^{-7} M at 25°C .

- What is the standard free energy of binding?
- Immunologists often speak of affinity (K_a), the reciprocal of the dissociation constant, in comparing antibodies. What is the affinity of this F_{ab} ?
- The rate constant of release of hapten from the complex is 120 s^{-1} . What is the rate constant for association? What does the magnitude of this value imply about the extent of structural change in the antibody on binding hapten?

2. *Sugar niche.* An antibody specific for dextran, a polysaccharide of glucose residues, was tested for its binding of glucose oligomers. Maximal binding affinity was obtained when the oligomer contained six glucose residues. How does the size of this site compare with that expected for the binding site on the surface of an antibody?

3. *A brilliant emitter.* Certain naphthalene derivatives exhibit a weak yellow fluorescence when they are in a highly polar environment (such as water) and an intense blue fluorescence when they are in a markedly nonpolar environment (such as hexane). The binding of ϵ -dansyl-lysine to specific antibody is accompanied by a marked increase in its fluorescence intensity and a shift in color from yellow to blue. What does this finding reveal about the hapten-antibody complex?

4. *Avidity versus affinity.* The standard free energy of binding of F_{ab} derived from an antiviral IgG is -7 kcal mol^{-1} (-29 kJ mol^{-1}) at 25°C .

- Calculate the dissociation constant of this interaction.
- Predict the dissociation constant of the intact IgG, assuming that both combining sites of the antibody can interact with viral epitopes and that the free-energy cost of assuming a favorable hinge angle is $+3 \text{ kcal mol}^{-1}$ (12.6 kJ mol^{-1}).

5. *Miniantibody.* The F_{ab} fragment of an antibody molecule has essentially the same affinity for a monovalent hapten as does intact IgG.

- What is the smallest unit of an antibody that can retain the specificity and binding affinity of the whole protein?
- Design a compact single-chain protein that is likely to specifically bind antigen with high affinity.

6. *Turning on B cells.* B lymphocytes, the precursors of plasma cells, are triggered to proliferate by the binding of multivalent antigens to receptors on their surfaces. The cell-surface receptors are transmembrane immunoglobulins. Univalent antigens, in contrast, do not activate B cells.

- What do these findings reveal about the mechanism of B-cell activation?
- How might antibodies be used to activate B cells?

7. *An ingenious cloning strategy.* In the cloning of the gene for the α chain of the T-cell receptor, T-cell cDNAs were hybridized with B-cell mRNAs. What was the purpose of this hybridization step? Can the principle be applied generally?

8. *Instruction.* Before the mechanism for generating antibody diversity had been established, a mechanism based on protein folding around an antigen was proposed, primarily by Linus Pauling. In this model, antibodies that had different specificities had the same amino acid sequence but were folded in different ways. Propose a test of this model.

9. *Dealing with nonsense.* Cells, including immune cells, degrade mRNA molecules in which no long open reading frame is present. The process is called nonsense-mediated RNA decay. Suggest a role for this process in immune cells.

10. *Crystallization.* The proteolytic digestion of a population of IgG molecules isolated from human serum results in the generation of F_{ab} and F_c fragments. Why do F_c fragments crystallize more easily than F_{ab} fragments generated from such a population?

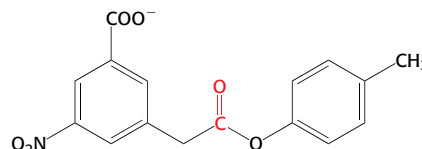
11. *Presentation.* The amino acid sequence of a small protein is:

MSRLASKNLRSDHAGGLLQATYSAVSS-
IKNTMSFGAWSNAALNDSRDA

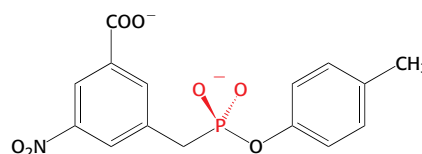
Predict the most likely peptide to be presented by the class I MHC molecule HLA-A2.

Mechanism Problem

12. *Catalytic antibody.* Antibody is generated against a transition state for the hydrolysis of the following ester.



Ester



Transition-state analog

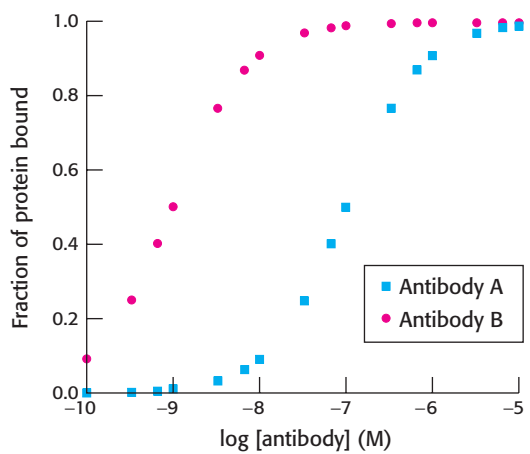
Some of these antibodies catalyze the hydrolysis of the ester. What amino acid residue might you expect to find in the binding site on the antibody?

Chapter Integration Problem

13. *Signaling.* Protein tyrosine phosphatases, such as the molecule CD45 expressed in both B cells and T cells, play important roles in activating such protein tyrosine kinases as Fyn and Lck, which are quite similar to Src. Suggest a mechanism for the activation of such protein kinases by the removal of a phosphate from a phosphotyrosine residue.

Data Interpretation Problem

14. *Affinity maturation.* A mouse is immunized with an oligomeric human protein. Shortly after immunization, a cell line



that expresses a single type of antibody molecule (antibody A) is derived. The ability of antibody A to bind the human protein is assayed with the results shown in the adjoining graph. After repeated immunizations with the same protein, another cell line is derived that expresses a different antibody (antibody B). The results of analyzing the binding of antibody B to the protein also are shown. From these data, estimate

- the dissociation constant (K_d) for the complex between the protein and antibody A.
- the dissociation constant for the complex between the protein and antibody B.

Comparison of the amino acid sequences of antibody A and antibody B reveals them to be identical except for a single amino acid. What does this finding suggest about the mechanism by which the gene encoding antibody B was generated?