Chapter 5
Enzymes: Core Concepts and Kinetics

Much of life is motion, whether at the macroscopic level of our daily life or at the molecular level of a cell. Stop-motion photography captures the motion of this dancer in frames separated by only about a quarter of a second. In biochemistry, kinetics (derived from the Greek *kinesis*, meaning "movement") is used to measure the extremely fast dynamics of enzyme activity. The enzyme carbonic anhydrase, one of the fastest known, can catalyze 250,000 reactions in the time elapsed between each frame shown in the image of the dancer.

Outline

Learning Goals

By the end of this chapter, you should be able to:

1. Explain the thermodynamics of catalyzed and uncatalyzed reactions using Gibbs free energy diagrams.
2. Describe the central role of the formation of the transition state in enzyme catalysis.
3. Explain how reaction velocity is determined and used to characterize enzyme activity in terms of the fundamental kinetic parameters of an enzyme, K_M and k_{cat}.
4. Differentiate between types of reversible and irreversible inhibitors by mechanisms of action and using kinetics data.

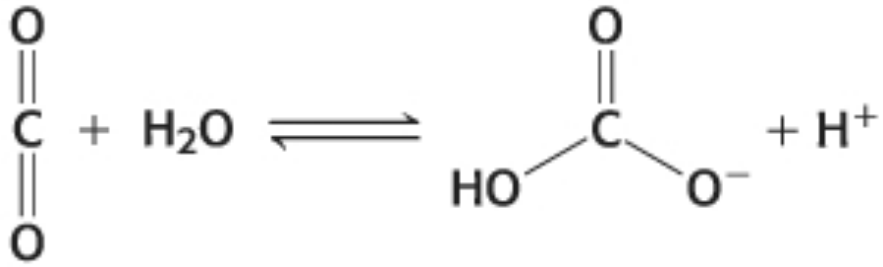
Enzymes like carbonic anhydrase are biological catalysts that determine the patterns of chemical transformations and mediate the transformation of one form of energy into another. Enzymes are also the target of many drugs; in fact, the majority of all pharmaceuticals ever created have been molecules that reduce enzyme function. For instance, a host of drugs used to manage pain, inflammation, and fever are inhibitors of a class of enzymes called cyclooxygenases. One of the most widely used medications in the world, acetylsalicylic acid, commonly known as aspirin, is among this group. About a quarter of the genes in the human genome encode enzymes, a testament to their importance to life. In this chapter, you will learn how enzymes accelerate reactions, how the

speed of their effects is measured, and how their action can be inhibited.

5.1 Enzymes Are Powerful and Highly Specific Catalysts

Agents called **catalysts** speed up chemical reactions without being consumed by them. **Enzymes** are powerful biological catalysts — made by all living organisms — that dramatically enhance and control the rates of chemical reactions. Almost all enzymes are proteins, which are highly effective catalysts for an enormous diversity of chemical reactions because of their capacity to specifically bind a very wide range of molecules. Using the full repertoire of intermolecular forces, enzymes optimally orient reactant molecules to make and break chemical bonds. However, proteins do not have an absolute monopoly on catalysis; the discovery of catalytically active RNA molecules, called **ribozymes**, provides compelling evidence that RNA was a biocatalyst early in evolution. Two remarkable properties of enzymes to consider from the start are their speed and their specificity.

- *Speed*. Enzymes can accelerate reactions by factors of a billion or more (**Table 5.1**). 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.



The transfer of CO_2 from the tissues to the blood and then to the air in the alveolae of the lungs 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 10^6 molecules of CO_2 per second. This catalyzed reaction is 10^7 times as fast as the uncatalyzed one. We will consider the mechanism of carbonic anhydrase catalysis in Chapter 6.

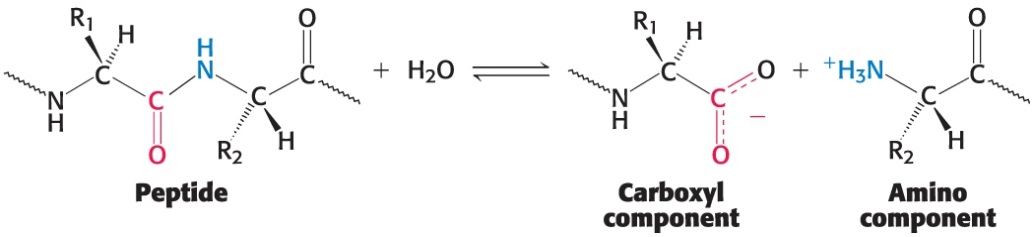
TABLE 5.1 Rate enhancement by selected enzymes

Enzyme	Nonenzymatic half-life	Uncatalyzed rate ($k_{un}s^{-1}$)	Catalyzed rate ($k_{cat}s^{-1}$)	Rate enhancement ($k_{cat}s^{-1}/k_{un}s^{-1}$)
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}

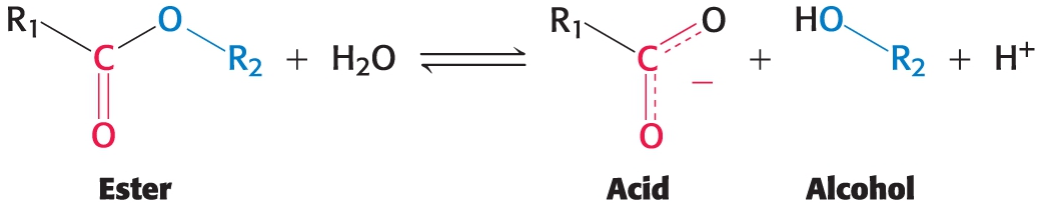
Triose phosphate isomerase	1.9 days	4.3×10^{-6}	4300	1.0×10^{9}
Chorismate mutase	7.4 hours	2.6×10^{-5}	50	1.9×10^{6}
Carbonic anhydrase	5 seconds	1.3×10^{-1}	1×10^{6}	7.7×10^{6}

Abbreviations: OMP, orotidine monophosphate; AMP, adenosine monophosphate.
Source: After A. Radzicka and R. Wolfenden, *Science* 267:90–93, 1995.

- *Specificity*. Enzymes are highly specific both in the reactions that they catalyze and in the reactants they bind, which are called **substrates**. An enzyme usually catalyzes a single chemical reaction or a set of closely related reactions. For example, enzymes known as **proteases** 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.



Proteolytic enzymes differ markedly in their degree of substrate specificity. Papain, which is found in papaya plants, is quite undiscriminating: it will cleave any peptide bond with little regard to the identity of the adjacent side chains. The digestive enzyme trypsin, on the other hand, is quite specific and catalyzes the splitting of peptide bonds only on the carboxyl side of lysine and arginine residues (**Figure 5.1A**). Thrombin, an enzyme that participates in blood clotting (Section 10.4), is even more specific than trypsin. It catalyzes the hydrolysis of Arg–Gly bonds in particular peptide sequences only (**Figure 5.1B**). The specificity of an enzyme is due to the precise interaction of the substrate with the enzyme, which is a result of the intricate three-dimensional structure of the enzyme.

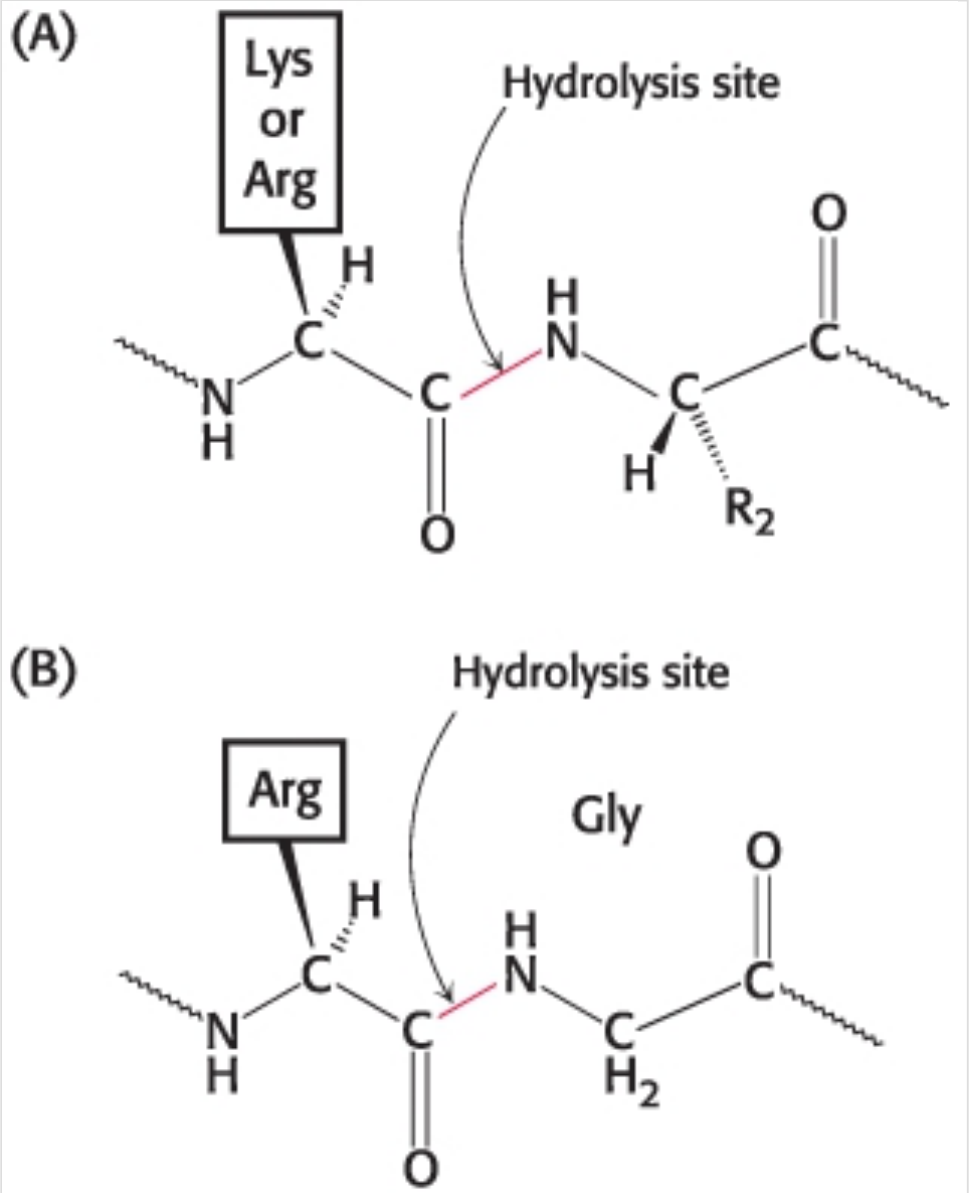


FIGURE 5.1 Enzymes are highly specific to particular substrates and chemical reactions. (A) Trypsin cleaves on the carboxyl side of arginine and lysine residues, whereas (B) thrombin cleaves Arg–Gly bonds in particular sequences only.

Most enzymes are classified by the types of reactions they catalyze

While some enzymes have common names — like papain, trypsin, and thrombin — that provide little information regarding their function, most enzymes are named for one of their substrates and for the reactions that they catalyze, with the suffix "-ase" added. Thus, a peptide hydrolase is an enzyme that hydrolyzes peptide bonds, whereas ATP synthase is an enzyme that synthesizes ATP. It is important to note, however, that enzymes catalyze chemical reactions in both the forward and reverse directions, yet only one direction of the reaction is typically denoted in the name.

To bring some consistency to enzyme nomenclature, a classification system for enzymes — developed by the International Union of Biochemistry — divides reactions into seven major groups (**Table 5.2**). These groups are further subdivided so that a four-number code preceded by the letters *EC* could precisely identify all enzymes. Consider as an example nucleoside monophosphate (NMP) kinase, an enzyme that we will examine in detail in Section 9.4. It catalyzes the following reaction:

$$\mathrm{ATP} + \mathrm{NMP} \rightleftharpoons \mathrm{ADP} + \mathrm{NDP}$$

NMP kinase transfers a phosphoryl group from ATP to any NMP to form a nucleoside diphosphate (NDP) and ADP. Consequently, it is a transferase, or member of group 2. Transferases that shift a phosphoryl group are designated 2.7. If a phosphate is the acceptor,

the transferase is designated 2.7.4, and most precisely EC 2.7.4.4 if a nucleoside monophosphate is the acceptor. Although the common names are used routinely, the classification number is used when the precise identity of the enzyme is not clear from the common name alone.

TABLE 5.2 Seven 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)	6
3. Hydrolases	Hydrolysis reactions (transfer of functional groups to water)	Chymotrypsin	6
4. Lyases	Addition or removal of groups to form double bonds	Fumarase	17
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	30
7. Translocases	Movement of ions or molecules across membranes	Na^+–K^+ATPase	12

	or within membranes

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. Generally, these cofactors are able to execute chemical reactions that cannot be performed by the standard set of twenty amino acids. An enzyme without its cofactor is referred to as an **apoenzyme**; the complete, catalytically active enzyme is called a **holoenzyme**.

$$\text{apoenzyme} + \text{cofactor} = \text{holoenzyme}$$

Cofactors can be subdivided into two groups: (1) metals, whose importance to enzymatic activity we will explore in Chapter 6, and (2) small organic molecules called **coenzymes** (**Table 5.3**). Often derived from vitamins, coenzymes either can be tightly or loosely bound to the enzyme.

TABLE 5.3 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′-Deoxyadenosylcobalamin	Methylmalonyl mutase
Tetrahydrofolate	Thymidylate synthase
Metal	
Zn^{2+}	Carbonic anhydrase
Zn^{2+}	Carboxypeptidase
Mg^{2+}	*Eco*RV
Mg^{2+}	Hexokinase
Ni^{2+}	Urease
Mo	Nitrogenase
Se	Glutathione peroxidase

Mn	Superoxide dismutase
K^+	Acetyl CoA thiolase

Tightly bound coenzymes are called **prosthetic groups**. Prosthetic groups are catalytic in that they are unchanged in the overall chemical reaction. In contrast, loosely associated coenzymes often behave more like second substrates (cosubstrates) because they bind to the enzyme, are changed by it, and then are released from it. Thus, these are also sometimes called *stoichiometric coenzymes* because they must be present in stoichiometric ratios with other substrates. The use of the same loosely associated coenzyme by a variety of enzymes sets these coenzymes apart from normal substrates, however, as does their source in vitamins (Table 5.3 and Section 15.4). Enzymes that use the same coenzyme usually perform catalysis by similar mechanisms. Throughout the book, we will see how coenzymes and their enzyme partners operate in their biochemical context.

Self-Check Question

Which terms (cofactor, coenzyme, and/or prosthetic group) correctly describe the heme from cytochrome c oxidase, which doesn't dissociate from the protein?

Enzymes can transform energy from one form into another

A key activity in all living systems is the conversion of one form of energy into another. For example, in photosynthesis, light energy is converted into chemical potential energy. In cellular respiration, which takes place 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. Given their centrality to life, it should come as no surprise that enzymes play vital roles in energy transformation.

After enzymes perform fundamental roles in photosynthesis and cellular respiration, other enzymes can then use the chemical potential energy of ATP in diverse ways. For instance, the enzyme myosin converts the energy of ATP into the mechanical energy of contracting muscles (Section 6.5). Pumps in the membranes of cells and organelles, which can be thought of as enzymes that move substrates rather than chemically alter them, use the energy of ATP to transport molecules and ions across the membrane (Chapter 13). The chemical and electrical gradients resulting from the unequal distribution of these molecules and ions are themselves forms of potential energy that can be used for a variety of purposes, such as sending nerve impulses (Section 13.4).

Recent developments show that the power of enzymes may be harnessed to generate energy for entire communities, as well as reducing landfill. Unsorted municipal waste can be treated with a cocktail of enzymes that includes an array of proteases as well as carbohydrate- and lipid-degrading enzymes, turning much of the waste into a bioliquid of sugars, amino acids, and other biomolecules. The bioliquid can then be used to fuel the growth of

methane-producing bacteria, and the methane harvested and burned to generate electricity. Any waste not degraded by the enzyme cocktail is recycled or incinerated to produce electricity.

5.2 Gibbs Free Energy Is a Useful Thermodynamic Function for Understanding Enzymes

Enzymes speed up the rate of chemical reactions, but the properties of the reaction — whether it can take place at all and the degree to which the enzyme accelerates the reaction — depend on energy differences between reactants (the initial state) and products (the final state). Gibbs free energy (G), which was discussed in Section 1.3, is a thermodynamic property that is a measure of useful energy, or the energy that is capable of doing work. To understand how enzymes operate, we need to consider only 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 into products. The former determines whether the reaction will take place spontaneously, whereas the latter determines the rate of the reaction. Enzymes affect only the rate.

The free-energy change provides information about the spontaneity but not the rate of a reaction

The free-energy change of a reaction (ΔG) tells us if the reaction can take place spontaneously:

- *A reaction can take place spontaneously only if* ΔG *is negative*. Such reactions are said to be *exergonic*.
- *A system is at equilibrium and no net change can take place if* ΔG *is zero*.
- *A reaction cannot take place spontaneously if* ΔG *is positive*. An input of free energy is required to drive such a reaction. These reactions are termed *endergonic*.
- *The* ΔG *of a reaction depends only on the free energy of the products minus the free energy of the reactants*. The ΔG of a reaction is independent of the molecular mechanism of the transformation. For example, the ΔG for the oxidation of glucose to CO_2 and H_2O is the same whether it takes place by combustion 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 take place spontaneously, but it does not signify whether it will proceed at a perceptible rate.

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 to know whether the reaction is spontaneous or requires an input of energy, measured in kilojoules (kJ) or kilocalories (kcal); one kilojoule is equivalent to 0.239

kilocalories. 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

$$A + B \rightleftharpoons C + D$$

The ΔG of this reaction is given by

$$\Delta G = \Delta G^\circ + RT \ln \frac{[C][D]}{[A][B]} \quad \textbf{(1)}$$

in which ΔG° is the standard free-energy change, R is the gas constant, T is the absolute temperature, and [A], [B], [C], and [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 bar, which is very close to 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, in which 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 2. 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 $\Delta G^{\circ\prime}$, will be used throughout this book. A simple way to determine $\Delta G^{\circ\prime}$ is to measure the concentrations of reactants and products when the reaction has reached equilibrium. At equilibrium, there is no net change in reactants and products; in essence, the reaction has stopped and $\Delta G = 0$. At equilibrium, equation 1 then becomes

$$0 = \Delta G^{\circ\prime} + RT \ln \frac{[C]_{eq}[D]_{eq}}{[A]_{eq}[B]_{eq}}$$

and so

$$\Delta G^{\circ\prime} = -RT \ln \frac{[C]_{eq}[D]_{eq}}{[A]_{eq}[B]_{eq}} \quad \textbf{(2)}$$

The equilibrium constant under standard conditions, K'_{eq}, is defined as

$$K'_{eq} = \frac{[C]_{eq}[D]_{eq}}{[A]_{eq}[B]_{eq}} \tag{3}$$

Substituting equation 3 into equation 2 gives

$$\Delta G^{\circ\prime} = -RT \ln K'_{eq} \tag{4}$$

which can be rearranged to give

$$K'_{eq} = e^{-\Delta G^{\circ\prime}/RT}$$

Substituting $R = 8.315 \times 10^{-3}\ \text{kJ mol}^{-1}\ \text{K}^{-1}$ and $T = 298\ \text{K}$ (corresponding to 25°C) gives

$$K'_{eq} = e^{-\Delta G^{\circ\prime}/2.48} \tag{5}$$

where $\Delta G^{\circ\prime}$ is here expressed in kilojoules per mole because of the choice of the units for R in equation 4. 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 $-5.69\ \text{kJ mol}^{-1}$ or $-1.36\ \text{kcal mol}^{-1}$ at 25°C (Table 5.4). Note that, for each 10-fold change in the equilibrium constant, the $\Delta G^{\circ\prime}$ changes by $5.69\ \text{kJ mol}^{-1}$ or $1.36\ \text{kcal mol}^{-1}$.

TABLE 5.4 Relationship between $\Delta G^{\circ\prime}$ and K'_{eq} (at 25°C)

	$\Delta G^{\circ\prime}$	
K'_{eq}	kJ mol^{-1}	kcal mol^{-1}
10^{-5}	28.53	6.82
10^{-4}	22.84	5.46
10^{-3}	17.11	4.09
10^{-2}	11.42	2.73
10^{-1}	5.69	1.36
1	0.00	0.00
10	−5.69	−1.36
10^{2}	−11.42	−2.73

10^3	−17.11	−4.09
10^4	−22.84	−5.46
10^5	−28.53	−6.82

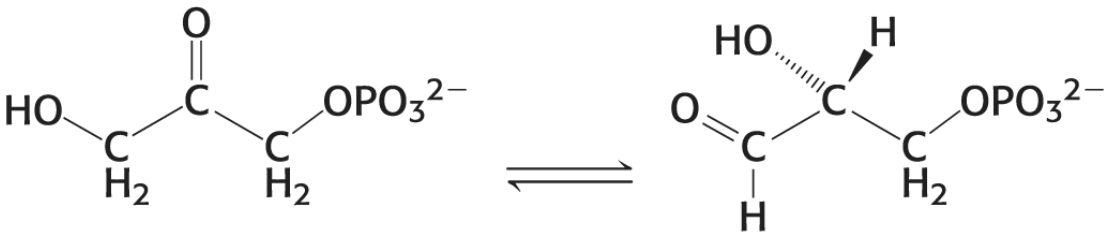
It is important to stress that whether the ΔG for a reaction is larger, smaller, or the same as $\Delta G^{\circ\prime}$ depends on the concentrations of the reactants and products. The criterion of spontaneity for a reaction under real conditions is ΔG, not $\Delta G^{\circ\prime}$. This point is important because reactions that are not spontaneous based on $\Delta G^{\circ\prime}$ can be made spontaneous by adjusting the concentrations of reactants and products. This principle is commonly observed in enzyme-catalyzed reactions in metabolic pathways (Chapter 15).

Example

Calculating and Comparing $\Delta G^{\circ\prime}$ and ΔG

Problem:

Calculate $\Delta G^{\circ\prime}$ and ΔG for a specific reaction that occurs during glucose metabolism:



Dihydroxyacetone phosphate (DHAP) ⇌ **Glyceraldehyde 3-phosphate (GAP)**

At equilibrium, the ratio of GAP to DHAP is 0.0475 at 25°C (298 K) and pH 7; hence, $K'_{eq} = 0.0475$. Typical concentrations in the cell are 2×10^{-4} M for DHAP and 3×10^{-6} M for GAP.

Getting Started:

Locate the equations used to calculate $\Delta G^{\circ\prime}$ and ΔG (equations 1 and 4). Besides the values given in the problem, is there anything else we need to know to solve these equations? Recall that the gas constant, *R*, is 8.315×10^{-3} kJ mol^{-1} K^{-1}

Calculate:

Let's begin by calculating $\Delta G^{\circ\prime}$, the standard free-energy change for this reaction, using equation 4 and substituting in the values for

R, the temperature in Kelvin, and the value of K'_{eq}:

$$\begin{aligned}\Delta G^{\circ\prime} &= -RT \ln K'_{eq} \\ &= -8.315 \times 10^{-3} \times 298 \times \ln (0.0475) \\ &= +7.53 \text{ kJ mol}^{-1} \text{or} +1.80 \text{ kcal mol}^{-1}\end{aligned}$$

What does $\Delta G^{\circ\prime}$ tell us? We can see from this result that under standard conditions the reaction is endergonic and DHAP will not spontaneously convert into GAP. However, this pertains only to standard conditions, not actual physiological conditions. For those we need to calculate ΔG, by substituting the real cellular concentrations into the equation for ΔG (equation 1):

$$\begin{aligned}\Delta G &= 7.53 \text{ kJ mol}^{-1} - RT \ln \frac{3 \times 10^{-6} \text{ M}}{2 \times 10^{-4} \text{ M}} \\ &= 7.53 \text{ kJ mol}^{-1} - (10.42 \text{ kJ mol}^{-1}) \\ &= -2.89 \text{ kJ mol}^{-1} \text{ or } -0.69 \text{ kcal mol}^{-1}\end{aligned}$$

Reflect:

What is the significance of the difference between ΔG and $\Delta G^{\circ\prime}$? Note that ΔG for this reaction is negative, even though $\Delta G^{\circ\prime}$ is positive. The negative value for ΔG indicates that the conversion of

DHAP to GAP is exergonic and can take place spontaneously when these species are present at the concentrations given.

Enzymes alter only the reaction rate and not the reaction equilibrium

We have established that enzymes are excellent catalysts, but it is equally important to understand what they cannot do: an enzyme cannot alter the laws of thermodynamics and consequently cannot alter the equilibrium of a chemical reaction. Consider an enzyme-catalyzed reaction, the conversion of substrate, S, into product, P. **Figure 5.2** shows the rate of product formation with time in the presence and absence of enzyme. Note that the amount of product formed is the same whether or not the enzyme is present but, in the current example, the amount of product formed in seconds when the enzyme is present might take hours (or centuries; see Table 5.1) to form if the enzyme were absent.

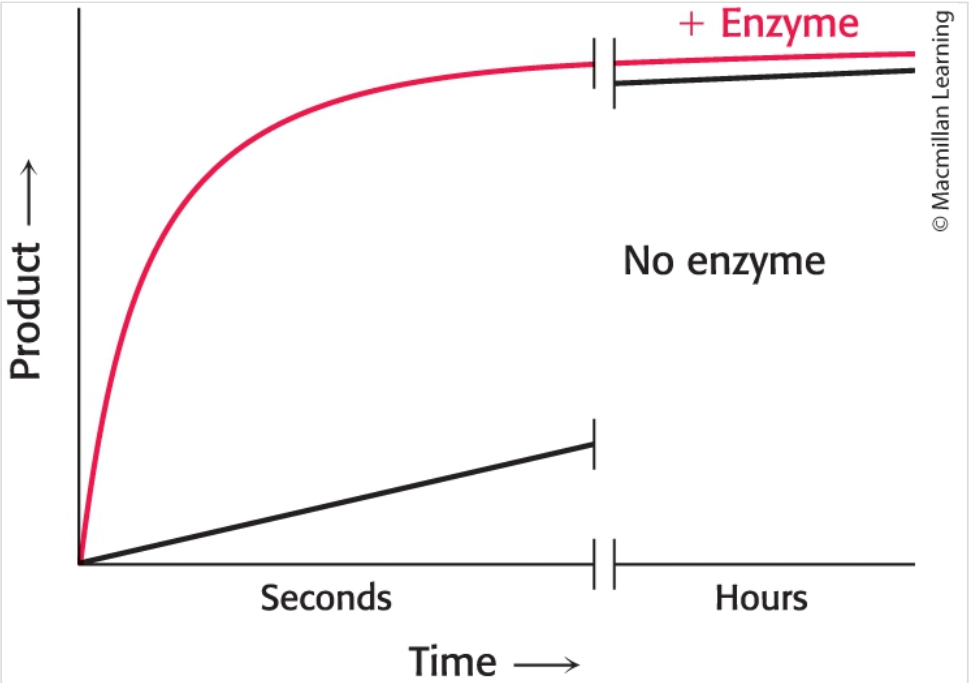


FIGURE 5.2 Enzymes do not affect thermodynamics and therefore do not change the equilibrium point. The same equilibrium point is reached much more quickly in the presence of an enzyme.

Why does the rate of product formation level off with time? The reaction has reached equilibrium. Substrate S is still being converted into product P, but P is being converted into S at a rate such that the amount of P present stays the same.

Let us examine the equilibrium in a more quantitative way. Suppose that, in the absence of enzyme, the forward rate constant (k_F) for the conversion of S into P is $10^{-4}\ s^{-1}$ and the reverse rate constant (k_R) for the conversion of P into S is $10^{-6}\ s^{-1}$. Remember: enzymes accelerate the rate of both the forward and reverse reactions. So,

also suppose that in the presence of an enzyme these values are $10^5\ s^{-1}$ and $10^3\ s^{-1}$ for k_F and k_R, respectively. The equilibrium constant *K* is given by the ratio of these rate constants in either case:

$$S \underset{10^{-6}\ s^{-1}}{\overset{10^{-4}\ s^{-1}}{\rightleftharpoons}} P \qquad K_{Uncatalyzed} = \frac{[P]}{[S]} = \frac{k_F}{k_R} = \frac{10^{-4}}{10^{-6}} = 100$$

$$S \underset{10^{3}\ s^{-1}}{\overset{10^{5}\ s^{-1}}{\rightleftharpoons}} P \qquad K_{Catalyzed} = \frac{[P]}{[S]} = \frac{k_F}{k_R} = \frac{10^{5}}{10^{3}} = 100$$

Notice that the equilibrium concentration of P is 100 times that of S, whether or not enzyme is present. However, it might take a very long time to approach this equilibrium without enzyme, whereas equilibrium would be attained rapidly in the presence of a suitable enzyme (Table 5.1).

By catalyzing both the forward and reverse directions of reactions, 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.

5.3 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** $X^{\ddagger}$ that has a higher free energy than does either S or P.

$$S \rightarrow X^{\ddagger} \rightarrow P$$

The double dagger $(^{\ddagger})$ denotes the transition state. The transition state is a transitory molecular structure that is no longer the substrate but is not yet the product. The transition state is the least-stable and 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 $\Delta G^{\ddagger}$ (**Figure 5.3**).

$$\Delta G^{\ddagger} = G_{X}{}^{\ddagger} - G_{S}$$

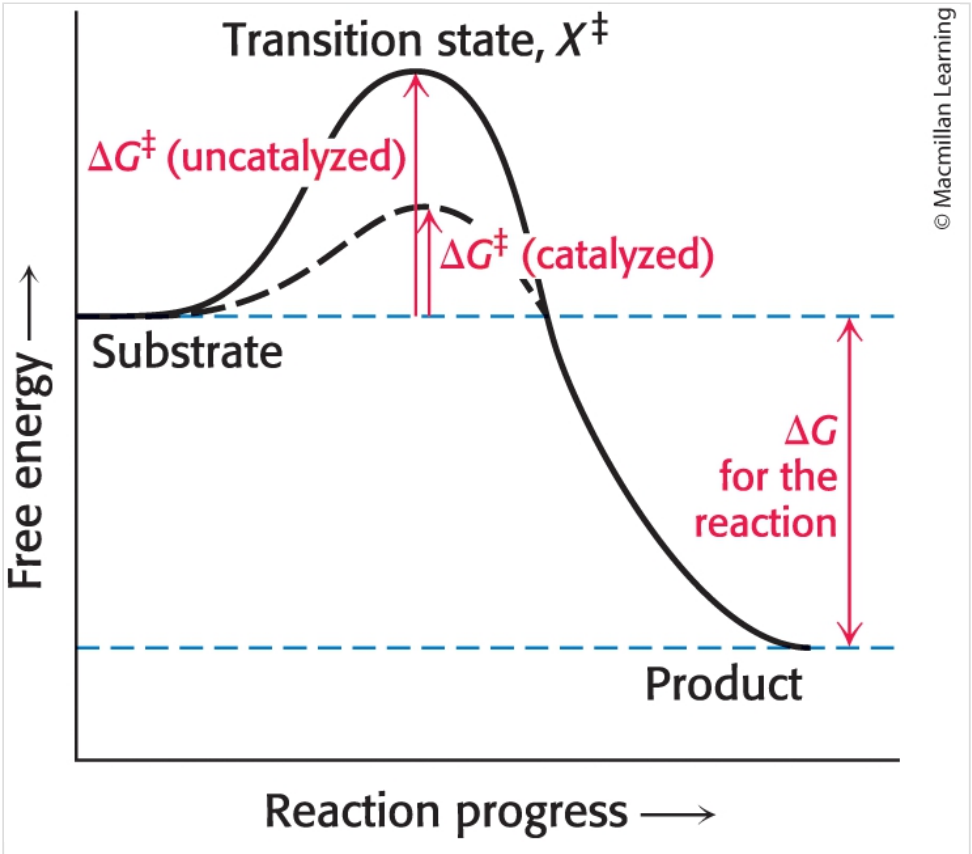


FIGURE 5.3 Enzymes accelerate reactions by decreasing $\Delta G^{\ddagger}$, the free energy of activation.

Note that $\Delta G^{\ddagger}$ does not enter into the final ΔG calculation for the reaction, because the energy required to generate the transition state is released when the transition state forms the product. This is also true when we consider the reaction from the reverse direction.

The activation-energy barrier immediately suggests how an enzyme enhances the reaction rates in both directions without altering ΔG of the reaction: enzymes accelerate reactions by decreasing $\Delta G^{\ddagger}$, the activation energy, thus facilitating the formation of the transition state from either substrate or product.

The combination of substrate and enzyme creates a reaction pathway whose transition-state energy is lower than that of the reaction in the absence of enzyme (Figure 5.3). Because the activation energy is lower, more molecules have the energy required 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 formation of an enzyme–substrate complex is the first step in enzymatic catalysis

Much of the catalytic power of enzymes comes from their binding to and then altering the structure of the substrate to promote the formation of the transition state. Thus, the first step in catalysis is the formation of an enzyme–substrate (ES) complex. Substrates bind 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?

- 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 5.4), suggesting that a discrete ES complex has formed. In contrast, uncatalyzed reactions do not show this saturation effect. At a sufficiently high substrate concentration, all the catalytic sites are filled, or saturated, and so the reaction rate cannot increase. Although indirect, the ability to saturate an enzyme with substrate is the most general evidence for the existence of ES complexes.

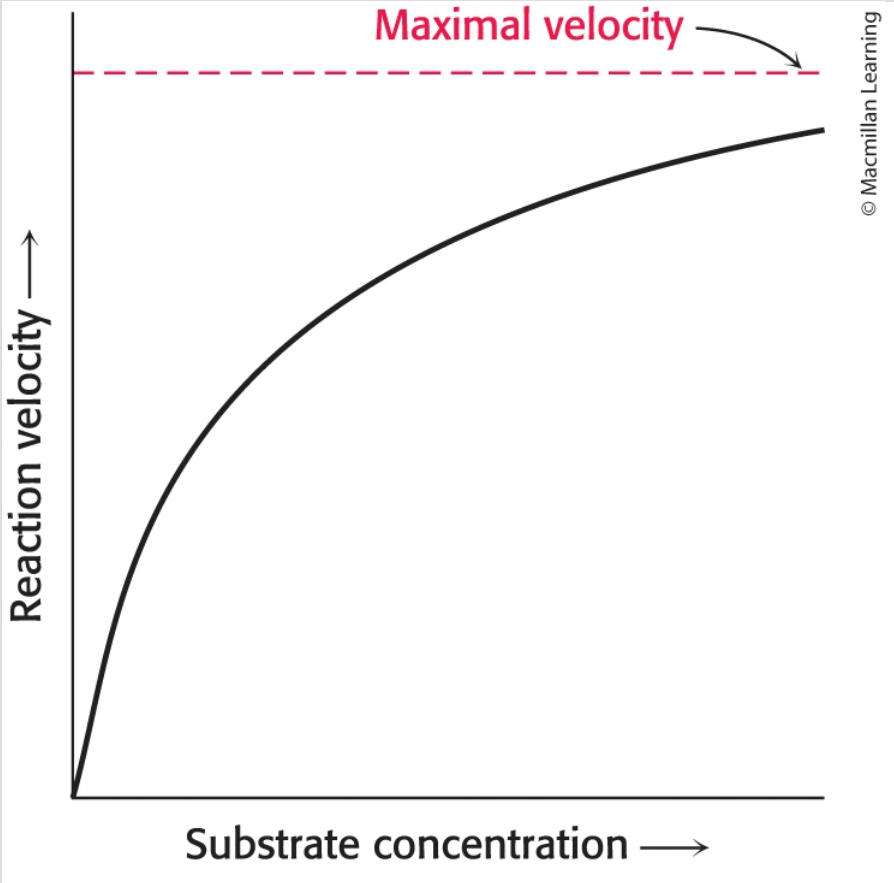


FIGURE 5.4 As substrate concentration increases, an enzyme-catalyzed reaction asymptotically approaches a maximal velocity.

- The spectroscopic characteristics of many enzymes and substrates change on the formation of an ES complex. These changes are particularly striking if the enzyme contains a colored prosthetic group.
- X-ray crystallography has provided high-resolution images of substrates and substrate analogs bound to the active sites of many enzymes (**Figure 5.5**). In Chapter 6, we will take a close look at several of these complexes.

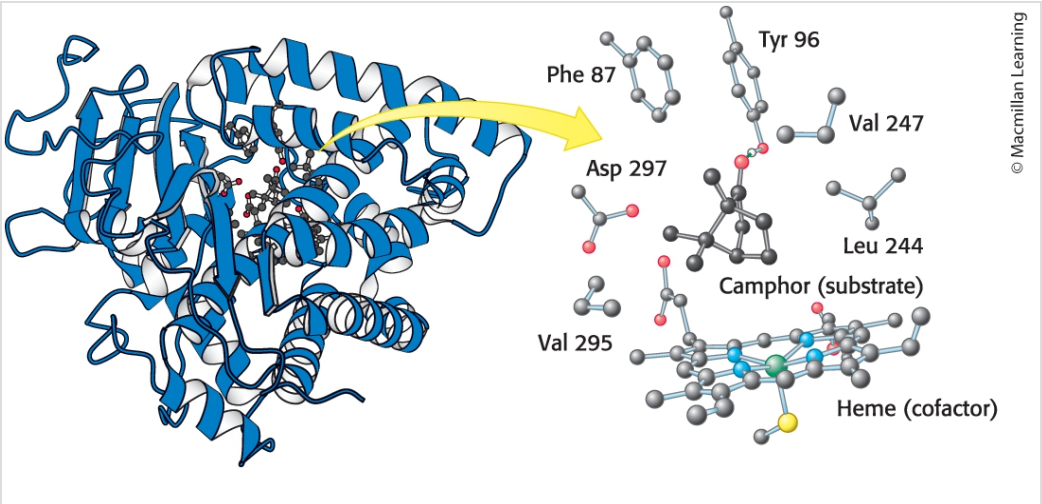


FIGURE 5.5 In the active site, substrates are surrounded by enzyme residues and sometimes cofactors. (Left) The structure of the complex between the enzyme cytochrome P450 and its substrate camphor. (Right) The substrate sits in the active site, which is a cavity formed by residues from the enzyme and the heme prosthetic group.

[Drawn from 2CPP.pdb.]

INTERACT with this model in Achieve

The active sites of enzymes have some common features

Recall from Chapter 2 that proteins are not rigid structures, but are flexible and exist in an array of conformations. Thus, the interaction of the enzyme and substrate at the active site and the formation of the transition state is a dynamic process. Although enzymes differ widely in structure, specificity, and mode of catalysis, at least five generalizations can be made concerning their active sites:

1. *The active site is a three-dimensional cleft, or crevice, formed by groups that come from different parts of the amino acid sequence.* Indeed, residues far apart in the amino acid sequence may interact more strongly than adjacent residues in the sequence, which may be sterically constrained from interacting with one another. For example, in the enzyme lysozyme, the residues that contribute important groups to the active site are numbered 35, 52, 62, 63, 101, and 108 in the sequence of 129 amino acids (**Figure 5.6**). Lysozyme, found in a variety of organisms and tissues, including human tears, degrades the cell walls of some bacteria.

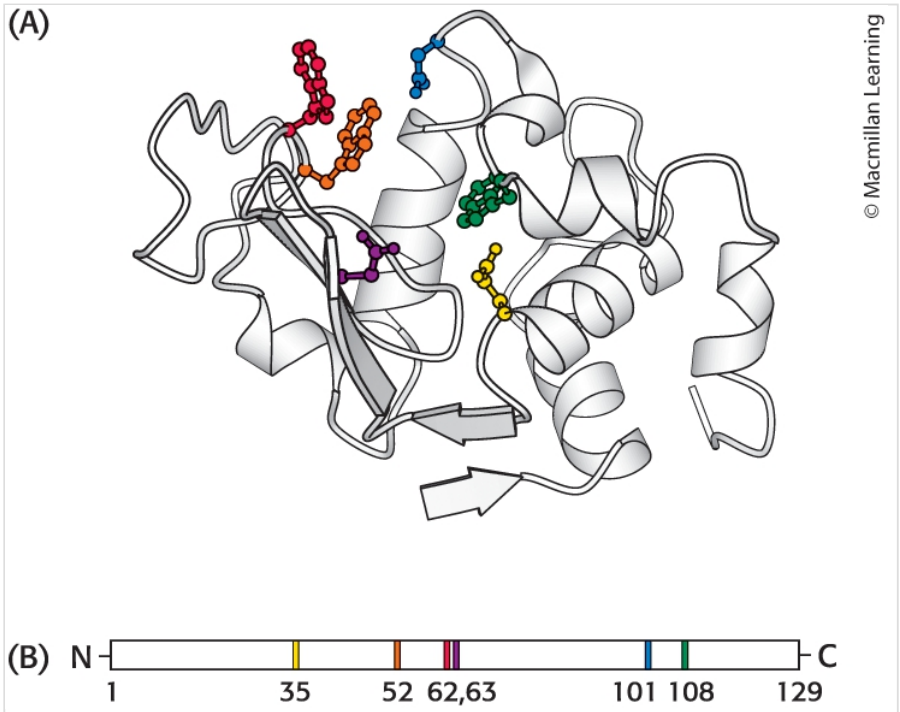


FIGURE 5.6 Amino acids that are far apart in sequence can be close together in the active site of the folded protein. (A) A ribbon diagram of the enzyme lysozyme with several components of the active site shown in color. (B) A schematic representation of the primary structure of lysozyme shows that the active site is composed of residues that come from different parts of the polypeptide chain.

[Drawn from 6LYZ.pdb.]

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2. *The active site takes up a small part of the total volume of an enzyme*. Although residues in an enzyme are not in contact with the substrate, the cooperative motions of the entire enzyme help to correctly position the catalytic residues at the active site. Experimental attempts to reduce the size of a catalytically active enzyme show that the minimum size requires about 100

residues. In fact, nearly all enzymes are larger than this, which gives them a mass normally greater than 10 kDa and a diameter of more than $25\ \text{Å}$, suggesting that all residues in a protein, not just those at the active site, are ultimately required to form a functional enzyme.

3. *Active sites are unique microenvironments*. Water is usually excluded from the active site cleft unless it is a reactant. Often a nonpolar microenvironment in the cleft enhances the binding of substrates as well as catalysis. The cleft may also contain polar residues, some of which may 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 located on the surface of proteins, exposed to water.

4. *Substrates are bound to enzymes by multiple weak attractions*. The noncovalent interactions in ES complexes are much weaker than covalent bonds, which have energies between -210 and $-460\ \text{kJ mol}^{-1}$ (between -50 and $-110\ \text{kcal mol}^{-1}$). In contrast, ES complexes usually have equilibrium constants that range from 10^{-2} to $10^{-8}\ \text{M}$ corresponding to free energies of interaction ranging from about -13 to $-50\ \text{kJ mol}^{-1}$ (from -3 to $-12\ \text{kcal mol}^{-1}$). As discussed in Section 1.3, these weak reversible contacts are mediated by ionic interactions, hydrogen bonds, van der Waals forces, and the hydrophobic effect. Van der Waals forces become significant in binding only when numerous substrate and enzyme atoms simultaneously come into close contact. Hence, the enzyme and substrate should have complementary 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 5.7).

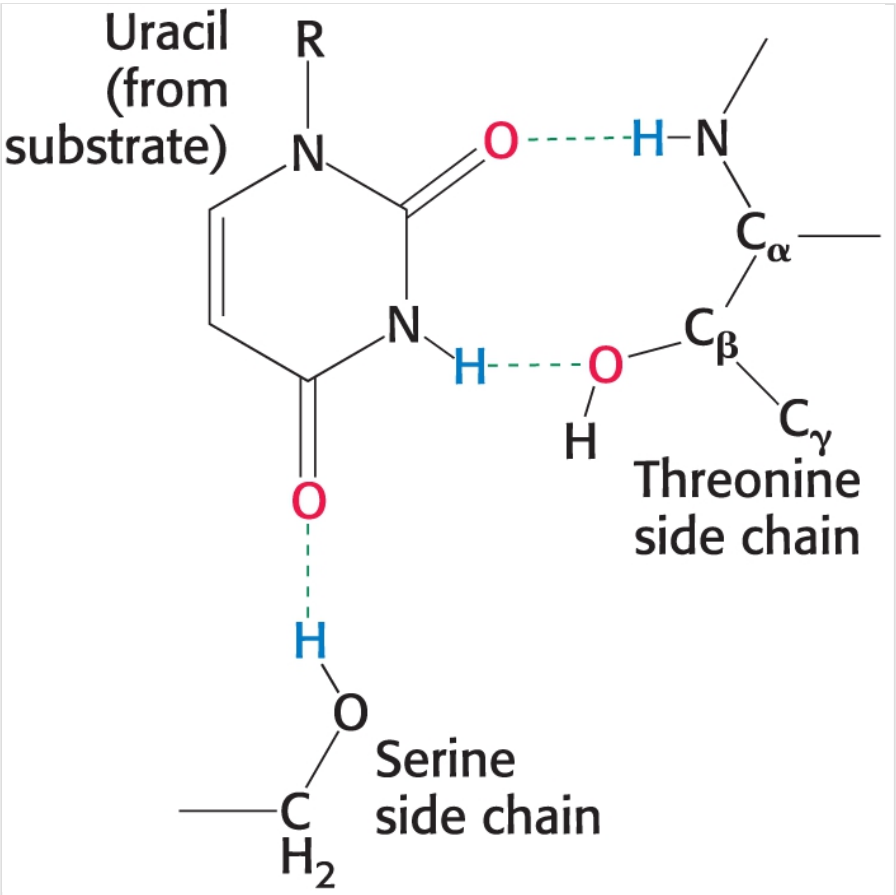


FIGURE 5.7 The geometry of hydrogen bonds often creates specificity between an enzyme and its substrate. The enzyme ribonuclease forms hydrogen bonds with the uridine component of the substrate.

[Information from F. M. Richards, H. W. Wyckoff, and N. Allewell, In *The Neurosciences: Second Study Program*, F. O. Schmidt, Ed. (Rockefeller University Press, 1970), p. 970.]

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 proposed the lock-and-key analogy in 1890, which was the model for enzyme–substrate interaction for several decades (**Figure 5.8A**). We now know that enzymes are flexible and that the shapes of the active sites can be markedly modified by the binding of substrate, a process of dynamic recognition called **induced fit** (**Figure 5.8B**). Moreover, the substrate may bind to only certain conformations of the enzyme, in what is called *conformation selection*. Thus, the mechanism of catalysis is dynamic, involving structural changes in both the substrates and the enzyme until the substrate reaches the transition state.

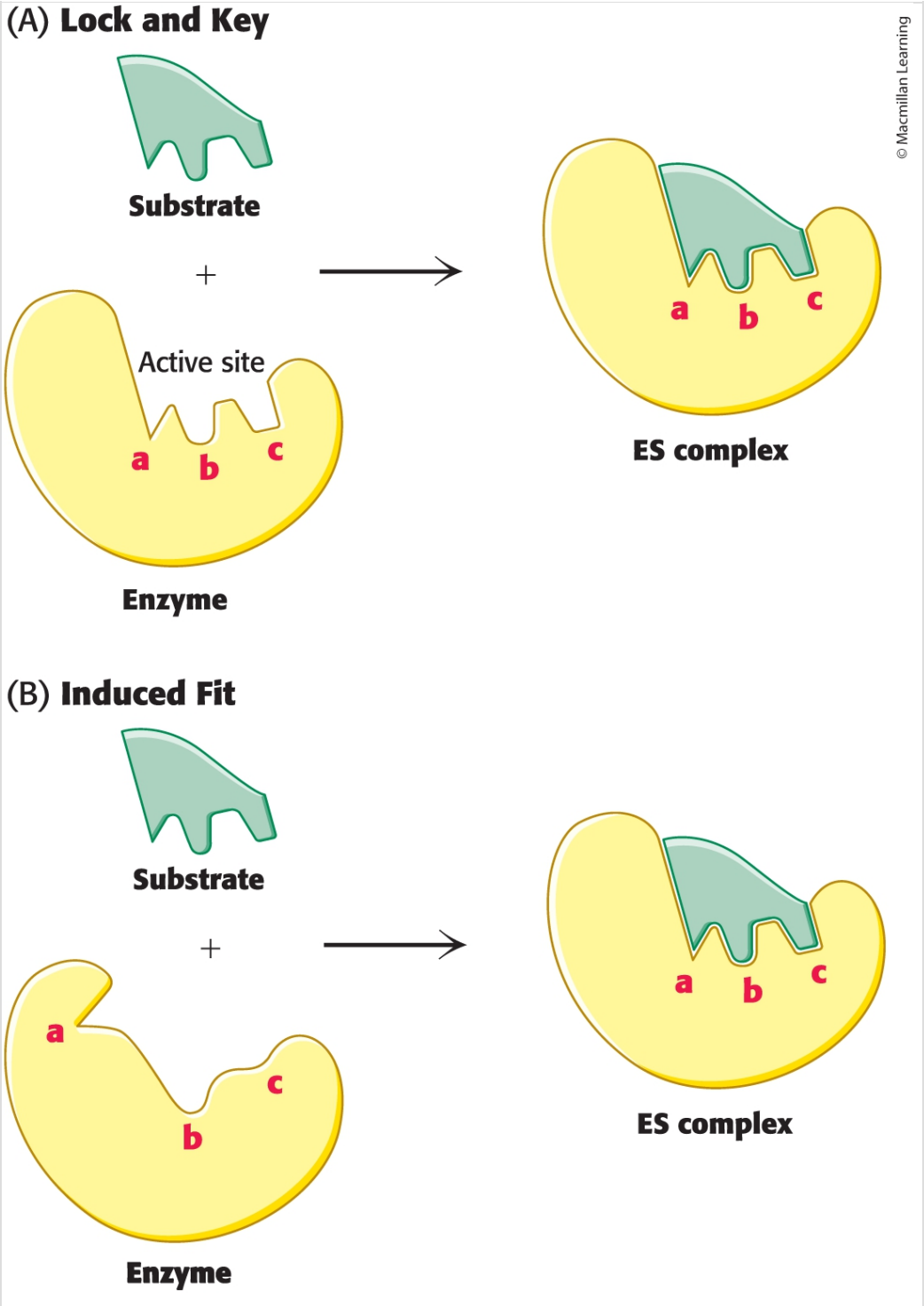


FIGURE 5.8 The old lock-and-key model of enzyme binding has been replaced by an induced-fit model. (A) In the lock-and-key model of enzyme–substrate binding, the

active site of the unbound enzyme was proposed to be complementary in shape to the substrate, whereas in (B) the current induced-fit model of enzyme–substrate binding, the enzyme changes shape on substrate binding, with the active site forming a shape complementary to the substrate only after the substrate has been bound. The enzyme then continues to change shape as it induces changes in the substrate, converting it into the transition state.

The binding energy between enzyme and substrate is important for catalysis

Enzymes lower the activation energy, but where does the energy to lower the activation energy come from? Free energy is released by the formation of a large number of weak interactions between a complementary enzyme and its substrate. The free energy released on binding is called the **binding energy**. 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 — and the maximal binding energy is released — only when the substrate is converted into the transition state. The energy released by the interaction between the enzyme and the substrate can be thought of as lowering the activation energy.

Because the transition state collapses randomly, the activation energies

determine the accumulation of either product or substrate

Molecular movements resulting in the optimal alignment of functional groups at the active site occur fleetingly. The transition state is too unstable to exist for long, collapsing randomly to either substrate or product. A consideration of the biochemical timescale, in which molecule motion is occurring on the order of microseconds to nanoseconds, is once again illuminating: a substrate may reach the transition state and collapse back to substrate again many times in a single active site within a fraction of a second before collapsing to product.

Whether substrate or product ultimately accumulates is determined only by the energy difference between the substrate and the product — that is, by the ΔG of the reaction, but why? If the direction of the collapse from the transition state is random, then the accumulation of either product or substrate can be explained by the activation energy in either direction because it dictates how frequently substrates or products reach the transition state. By lowering this activation energy, enzymes accelerate reactions in both directions by increasing the rate at which both products and substrates reach the transition state.

5.4 The Michaelis–Menten Model Accounts for the Kinetic Properties of Many Enzymes

The study of the rates of chemical reactions is called *kinetics*, and the study of the rates of enzyme-catalyzed reactions is called *enzyme kinetics*. A kinetic description of enzyme activity will help us understand how enzymes function.

Kinetics is the study of reaction rates

What do we mean when we say the "rate" of a chemical reaction? Consider a simple reaction:

$$A \rightarrow P$$

The rate V is the quantity of A that disappears in a specified unit of time. It is equal to the rate of the appearance of P, or the quantity of P that appears in a specified unit of time.

$$V = -d[A]/dt = d[P]/dt$$

If A is yellow and P is colorless, we can follow the decrease in the concentration of A by measuring the decrease in the intensity of yellow color with time. Consider only the change in the

concentration of A for now. The rate of the reaction is directly related to the concentration of A by a proportionality constant, *k*, called the *rate constant*.

$$V = k[A]$$

Reactions that are directly proportional to the reactant concentration are first-order reactions. First-order rate constants have the units of s^{-1}.

Many important biochemical reactions include two reactants. For example,

$$2A \rightarrow P$$

or

$$A + B \rightarrow P$$

They are called *bimolecular reactions*, and the corresponding rate equations often take the form

$$V = k[A]^2$$

and

$$V = k[A][B] \quad (6)$$

The rate constants, called second-order rate constants, have the units $M^{-1} s^{-1}$.

Sometimes, second-order reactions can appear to be first-order reactions. For instance, in the reaction described by equation 6, if B is present in excess and A is present at low concentrations, the reaction rate will be first order with respect to A and will not appear to depend on the concentration of B. These reactions are called *pseudo-first-order reactions*, and we will see them a number of times in our study of biochemistry.

Interestingly, under some conditions, a reaction can be zero order. In these cases the rate is independent of reactant concentrations. Enzyme-catalyzed reactions can approximate zero-order reactions under some circumstances, as we will soon see.

The steady-state assumption aids a description of enzyme kinetics

The simplest way to investigate the reaction rate is to follow the increase in reaction product as a function of time. First, the extent of product formation is determined as a function of time for a series of substrate concentrations (**Figure 5.9A**). 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 vice versa, but the reaction equilibrium has been attained.

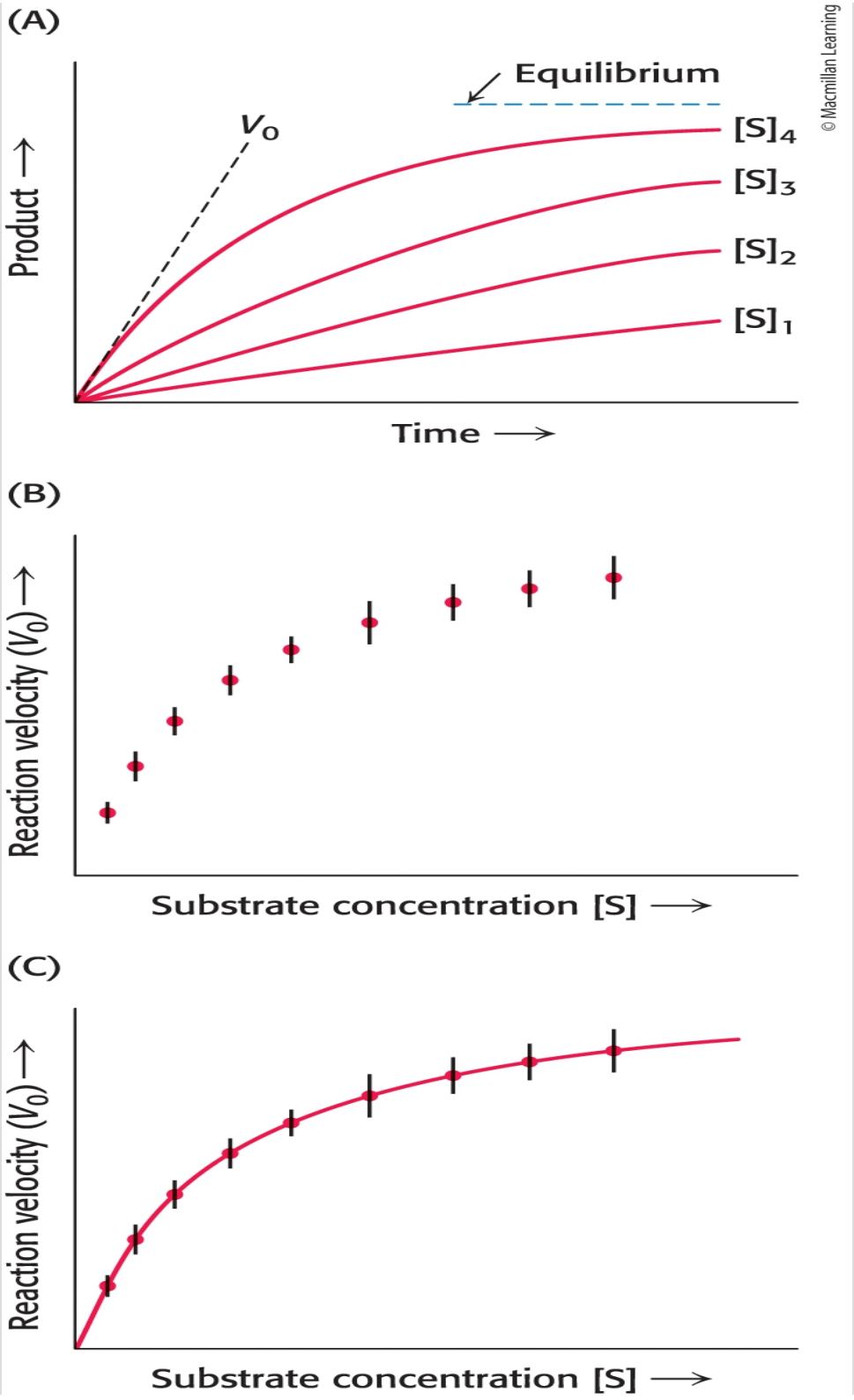


FIGURE 5.9 The relation between initial velocity and substrate concentration can be determined by a series of assays. (A) The amount of product formed at different substrate concentrations is plotted as a function of time. The initial velocity (V_0) for each substrate concentration is determined from the slope of the curve at the beginning of a reaction, when the reverse reaction is insignificant. (B) The values for initial velocity determined in part A are then plotted, with error bars, against substrate concentration. (C) The data points are connected to clearly reveal the relationship of initial velocity to substrate concentration.

However, enzyme kinetics is more readily comprehended if we consider only the forward reaction, which is most closely described by the initial rates of the reaction. Note that the rate of the reaction (the slope of the tangent line in Figure 5.9A) decreases over time. Additionally, only at the beginning of the reaction is the exact concentration of the substrate known because it is being converted to product as the reaction progresses. For these two reasons, it is often most useful to focus on initial rate enzyme kinetics. We can define the rate of catalysis V_0, or the initial rate of catalysis, as the number of moles of product formed per second when the reaction is just beginning — that is, when $t \approx 0$ and $[P] \approx 0$, so the rate of the reverse reaction is negligible (Figure 5.9A). These experiments are repeated three to five times with each substrate concentration to ensure the accuracy of and assess the variability of the values attained.

Next, we plot V_0 versus the initial substrate concentration used in each experiment, [S], assuming a constant amount of enzyme and showing the data points with error bars (**Figure 5.9B**). Finally, the data points are connected, yielding the results shown in **Figure 5.9C**. The rate of catalysis rises linearly as substrate concentration increases and then begins to level off and approach a maximum at

higher substrate concentrations. For convenience, we will show idealized data without error bars throughout the text, but it is important to keep in mind that in reality, all experiments are repeated multiple times.

The Michaelis–Menten model explains many observations of enzyme kinetics

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 they proposed is

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E + P$$

An enzyme E (here representing the “free enzyme”) combines with substrate S (“free substrate”) 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. The ES complex can also be reformed from E and P by the reverse reaction with a rate constant k_{-2}. However, as before, we can simplify these reactions by considering the rate of

reaction at times close to zero (hence, V_0) when there is negligible product formation and thus no back reaction $(k_{-2}[E][P] \approx 0)$.

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

(7)

Thus, for the graph in **Figure 5.10**, V_0 is determined for each substrate concentration by measuring the rate of product formation at early times before P accumulates (Figure 5.9A).

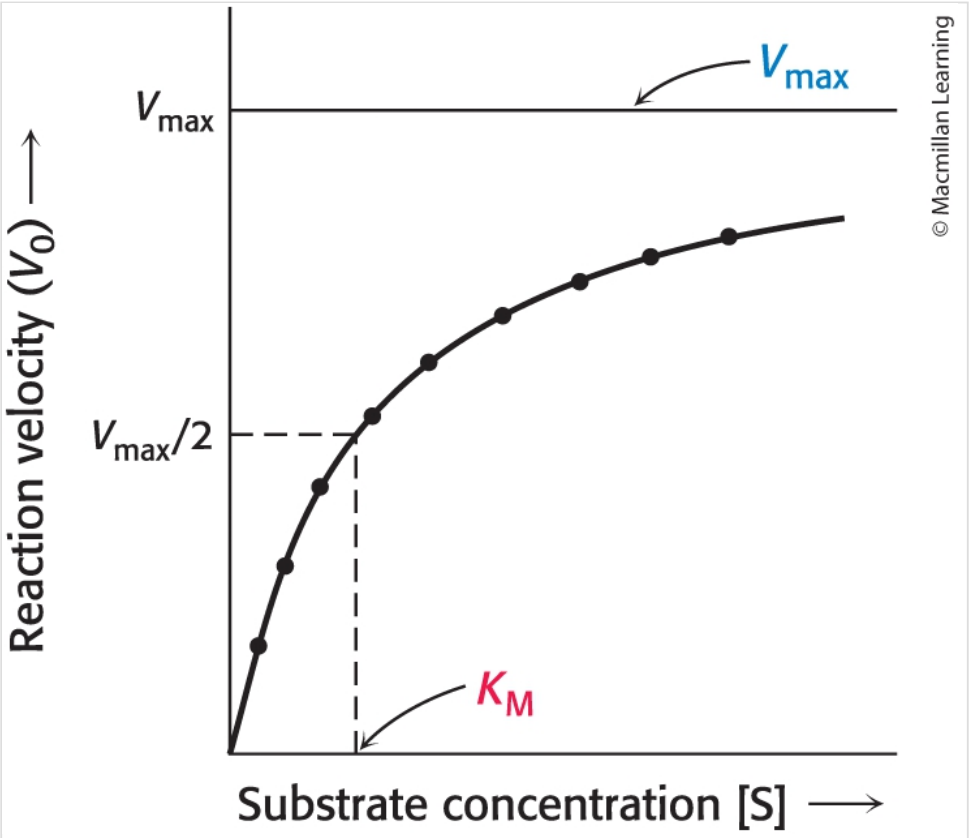


FIGURE 5.10 Michaelis–Menten kinetics are marked by a hyperbolic relationship between initial velocity and substrate concentration. A plot of the reaction velocity (V_0) as a function of the substrate concentration [S] for an enzyme that obeys Michaelis–Menten kinetics shows that the maximal velocity (V_{max}) is approached asymptotically, which is to say, V_{max} will be attained only at an infinite substrate concentration. The Michaelis constant (K_M) is the substrate concentration yielding a velocity of $V_{max}/2$.

The Michaelis–Menten equation describes the relationship between initial velocity and substrate concentration

We want an expression that relates the rate of catalysis to the concentrations of substrate and enzyme and the rates of the individual steps. For any chemical reaction, the overall reaction rate is determined only by the slowest, or rate-determining step. Thus, our starting point is the assumption that catalysis, resulting from the ES complex, is slow relative to the rate of substrate binding, i.e., the formation of the ES complex. Thus, the overall reaction rate can be expressed solely as the rate of this one step, which is the product of the concentration of the ES complex and k_2.

$$V_0 = k_2[\mathrm{ES}]$$

(8)

Now we need to express [ES] in terms of known quantities. The rates of formation and breakdown of ES are given by

$$\text{Rate of formation of ES} = k_1[\mathrm{E}][\mathrm{S}]$$

(9)

$$\text{Rate of breakdown of ES} = (k_{-1} + k_2)[\mathrm{ES}]$$

(10)

We will use what biochemists term the *steady-state assumption* to simplify matters. In a steady-state system, the concentrations of intermediates — in this case, [ES] — stay the same even though the concentrations of starting materials and products are changing and the system is not in equilibrium. An analogy is a sink filled with water that has the tap open just enough to match the loss of water down the drain: the level of the water in the sink never changes even though water is constantly flowing from the faucet through the sink and out through the drain. This steady state is reached when the rates of formation and breakdown of the ES complex are equal. Setting the right-hand sides of equations 9 and 10 equal gives

$$k_1[E][S] = (k_{-1} + k_2)[ES] \tag{11}$$

By rearranging equation 11, we obtain

$$[E][S]/[ES] = (k_{-1} + k_2)/k_1 \tag{12}$$

Equation 12 can be simplified by defining a new constant, K_M, called the **Michaelis constant**:

$$K_M = \frac{k_{-1} + k_2}{k_1} \tag{13}$$

Note that K_M has the units of concentration and is independent of enzyme and substrate concentrations. As will be explained, K_M is an important characteristic of enzyme–substrate interactions.

Inserting equation 13 into equation 12 and solving for [ES] yields

$$[ES] = \frac{[E][S]}{K_M} \tag{14}$$

Now let us examine the numerator of equation 14. Because the substrate is usually present at a much higher concentration than that of the enzyme, the concentration of uncombined (free) substrate [S] is very nearly equal to the total substrate concentration. The concentration of uncombined (free) enzyme [E] is equal to the total enzyme concentration $[E]_T$ minus the concentration of the ES complex:

$$[E] = [E]_T - [ES]$$

Substituting this expression for [E] in equation 14 gives

$$[ES] = \frac{([E]_T - [ES])[S]}{K_M}$$

Solving the above equation for [ES] gives

$$[ES] = \frac{[E]_T[S]/K_M}{1 + [S]/K_M}$$

or

$$[ES] = [E]_T \frac{[S]}{[S] + K_M}$$

By substituting this expression for [ES] into equation 8, we obtain

$$V_0 = k_2[E]_T \frac{[S]}{[S] + K_M}$$

(15)

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,

$$V_{max} = k_2[E]_T$$

(16)

Substituting equation 16 into equation 15 yields the **Michaelis–Menten equation**:

$$V_0 = V_{max} \frac{[S]}{[S] + K_M}$$

(17)

This equation accounts for the kinetic data given in Figure 5.10. At very low substrate concentration, when [S] is much less than K_M, $V_0 = (V_{max} / K_M)[S]$; that is, the reaction is first order with the rate 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. The reaction is zero order, independent of substrate concentration.

Notice that because V_0 is a function of [S], then if the K_M value is known, the equation gives the velocity as a fraction of V_{max} at any concentration of substrate. Thus, the velocity can be calculated at any [S], using the Michaelis–Menten equation, and expressed either in real units (if V_{max} is known) or as a fraction of V_{max}. Likewise, if the fraction of V_{max} is known, then the relationship between [S] and K_M can be calculated.

The significance of K_M is clear when we set $[S] = K_M$ in equation 17. 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. As we will see, K_M is an important characteristic of an enzyme-catalyzed reaction and is significant for its biological function.

Smithsonian Institution Archives, Accession 90-105, Science Service Records, Image No. SIA2008-5999

MAUD MENTEN Paving new career paths for women at the dawn of the 20th century, Maud Menten was among the first Canadian women to obtain an MD degree, in 1911; she then earned a PhD in 1916. Although she is most famous for her groundbreaking work on enzyme kinetics as a graduate student, she had a long and productive scientific career at the University of Pittsburgh and the British Columbia Medical Research Institute, teaching and publishing over 70 papers on a variety of subjects. She invented a still-used histochemical assay for

alkaline phosphatase, characterized bacterial toxins and early vitamin C deficiency, and pioneered the use of electrophoresis for the study of proteins.

Example

Applying the Michaelis–Menten Equation

Problem:

Assume that the initial velocity of an enzyme is 80% of V_{max}. What is the ratio of [S] to K_M under these conditions?

Getting Started:

The Michaelis–Menten equation relates the three terms discussed in the problem, so let's begin with it:

$$V_0 = V_{max} \frac{[S]}{[S] + K_M}$$

What do we already know? The question states the $V_0 = 80\%\ V_{max}$. This is the only value we are provided, so we should be able to solve the problem with that value and the equation.

SOLVE:

First, for convenience, let's convert 80% to 0.8 and substitute the $0.8\ V_{max}$ for V_0 in the equation:

$$0.8V_{max} = V_{max}\frac{[S]}{[S] + K_M}$$

Now we see that we can divide both sides of the equation by V_{max}, which yields

$$0.8 = \frac{[S]}{[S] + K_M}$$

Now we can solve the equation for $[S]/K_M$.

$$\begin{aligned} 0.8[S] + 0.8K_M &= [S] \\ 0.8K_M &= [S] - 0.8[S] \\ 0.8K_M &= 0.2[S] \\ 4 &= \frac{[S]}{K_M} \end{aligned}$$

Thus, when [S] is fourfold greater than K_M, $V_0 = 0.8\ V_{max} = 80\%\ V_{max}$.

Reflect:

You can confirm this answer by solving the Michaelis–Menten equation using $[S] = 4K_M$ and solving for V_0 as a fraction of V_{max}.

Variations in K_M can have physiological consequences

The physiological consequence of K_M is illustrated by the sensitivity of some persons 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 (CH_3CH_2OH) into acetaldehyde (CH_3CHO).

$$CH_3CH_2OH + NAD^+ \underset{}{\overset{\text{Alcohol dehydrogenase}}{\rightleftharpoons}} CH_3CHO + NADH + H^+$$

Normally, the acetaldehyde, which is the cause of the symptoms when present at high concentrations, is processed to acetate by aldehyde dehydrogenase.

$$CH_3CHO + NAD^+ + H_2O \xrightleftharpoons{\text{Aldehyde dehydrogenase}} CH_3COO^- + NADH + 2H^+$$

Most people have two forms of the aldehyde dehydrogenase, a low K_M mitochondrial form and a high K_M cytoplasmic form. In susceptible persons, the mitochondrial enzyme is less active owing to the substitution of a single amino acid, and acetaldehyde is processed only by the cytoplasmic enzyme. Because this enzyme has a high K_M, it achieves a high rate of catalysis only at very high concentrations of acetaldehyde. Consequently, less acetaldehyde is converted into acetate; excess acetaldehyde escapes into the blood and accounts for the physiological effects.

K_M and V_{max} values can be determined by several means

K_M is equal to the substrate concentration that yields $V_{max}/2$; however, like perfection, V_{max} is approached but never attained. How, then, can we experimentally determine K_M and V_{max}, and how do these parameters enhance our understanding of enzyme-catalyzed reactions? 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 17. The derivation of K_M and V_{max} is most commonly achieved with the use of curve-fitting programs on a computer. However, an older method,

although rarely used because the data points at high and low concentrations are weighted differently and thus sensitive to errors, is a source of further insight into the meaning of K_M and V_{max}.

Before the availability of computers, the determination of K_M and V_{max} values required algebraic manipulation of the Michaelis–Menten equation. The Michaelis–Menten equation is transformed into one that gives a straight-line plot that yields values for V_{max} and K_M. Taking the reciprocal of both sides of equation 17 gives

$$\frac{1}{V_0} = \frac{K_M}{V_{max}} \times \frac{1}{[S]} + \frac{1}{V_{max}} \quad \textbf{(18)}$$

A plot of $1/V_0$ versus $1/[S]$, called a **Lineweaver–Burk plot** or sometimes just called a *double-reciprocal plot*, yields a straight line with a *y*-intercept of $1/V_{max}$ and a slope of K_M/V_{max} (**Figure 5.11**). The intercept on the *x*-axis is $-1/K_M$.

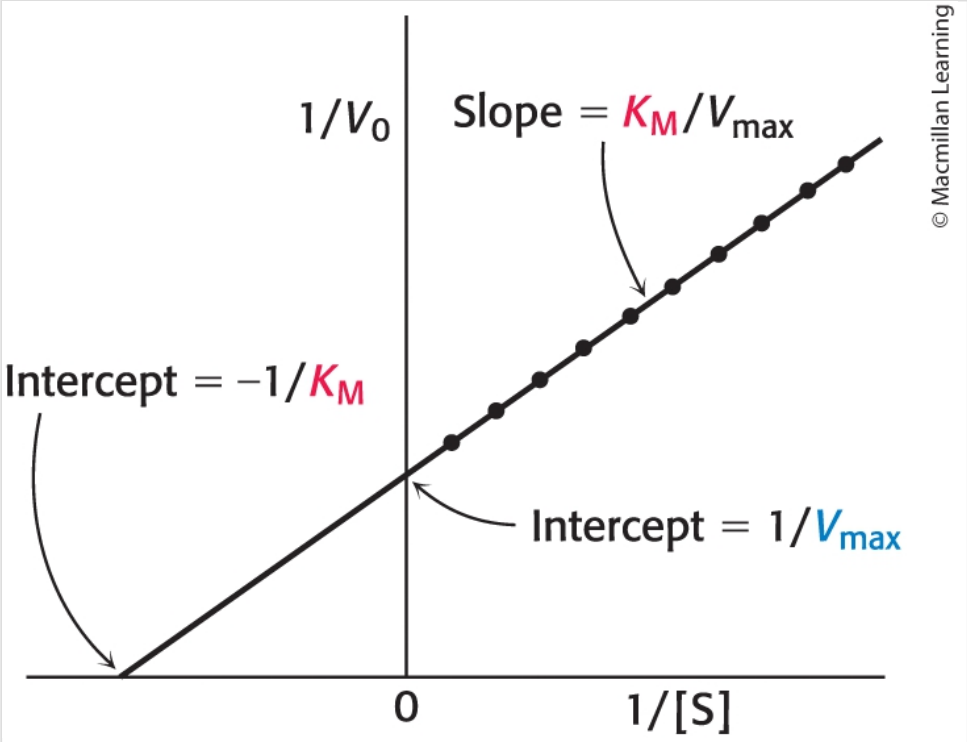


FIGURE 5.11 A Lineweaver–Burk plot shows $1/V_0$ as a function of $1/[S]$. The slope is K_M/V_{max}, the intercept on the vertical axis is $1/V_{max}$, and the intercept on the horizontal axis is $-1/K_M$.

K_M and k_{cat} values are important enzyme characteristics

The K_M values of enzymes range widely (Table 5.5). 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, being equal to the concentration of substrate at which half the active sites are filled, thus provides a measure of the substrate concentration required for significant catalysis to take place.

TABLE 5.5 K_M values of some enzymes

Enzyme	Substrate	K_M (μM)
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

For many enzymes, experimental evidence suggests that the K_M value provides an approximation of the substrate concentration in vivo, which in turn suggests that most enzymes evolved to have a K_M approximately equal to the substrate concentration commonly available. Why might it be beneficial to have a K_M value approximately equal to the commonly available substrate concentration? If the normal concentration of substrate is near K_M, the enzyme will display significant activity, and yet the activity will be sensitive to changes in environmental conditions — that is, changes in substrate concentration. This property is known as **elasticity**. At values below K_M, enzymes are very sensitive to changes in substrate concentration — in other words, they are elastic — but display little activity. At substrate values well above K_M, enzymes have greater catalytic activity but are insensitive — or inelastic — to changes in substrate concentration. Thus, with the normal substrate concentration being approximately K_M, the enzymes have significant activity ($1/2\ V_{max}$) but are still elastic to changes in substrate concentration.

Under certain circumstances, K_M reflects the strength of the enzyme–substrate interaction. In equation 13, K_M is defined as $(k_{-1} + k_2)/k_1$. Consider a 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, ($k_{-1} \gg k_2$)

$$K_M \approx \frac{k_{-1}}{k_1}$$

(19)

Equation 19 describes the dissociation constant, K_d (Chapter 3), of the ES complex.

$$K_{ES} = \frac{[E][S]}{[ES]} = \frac{k_{-1}}{k_1}$$

In other words, K_M is equal to the dissociation constant (K_d) 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. For a single active site, it is equal to the rate 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

$$V_{max} = k_{cat}[E]_T$$

and thus

$$k_{cat} = V_{max}/[E]_T \tag{20}$$

For example, a 10^{-6} M solution of carbonic anhydrase catalyzes the formation of 0.6 M H_2CO_3 per second when the enzyme is fully saturated with substrate. Hence, k_{cat} is 6×10^5 s^{-1}. This turnover number is one of the largest known. Each catalyzed reaction takes place in a time equal to, on average, $1/k_{cat}$, which is 1.7 μs for carbonic anhydrase. The turnover numbers of most enzymes with their physiological substrates range from 1 to 10^6 per second (Table 5.6).

TABLE 5.6 Turnover numbers of some enzymes

Enzyme	Turnover number (per second)
Carbonic anhydrase	600,000
3-Ketosteroid isomerase	280,000
Acetylcholinesterase	25,000
Penicillinase	2000
Lactate dehydrogenase	1000
Chymotrypsin	100

DNA polymerase I	15
Tryptophan synthetase	2
Lysozyme	0.5

K_M and V_{max} also permit the determination of f_{ES}, the fraction of active sites filled. This relation of f_{ES} to K_M and V_{max} is given by the following equation:

$$f_{ES} = \frac{V_0}{V_{max}} = \frac{[S]}{[S] + K_M} \quad \textbf{(21)}$$

Self-Check Question

Explain why K_M is an intrinsic property of an enzyme while V_{max} is not?

k_{cat}/K_M is a measure of catalytic efficiency

Recall that when the substrate concentration is much greater than K_M, the rate of catalysis is equal to V_{max}, which is a function of k_{cat}, the turnover number. 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 $[S] = K_M$, 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 8 and 14 to give

$$V_0 = \frac{k_{cat}}{K_M}[E][S]$$

When $[S] \ll K_M$, the concentration of free enzyme [E], is nearly equal to the total concentration of enzyme $[E]_T$; so

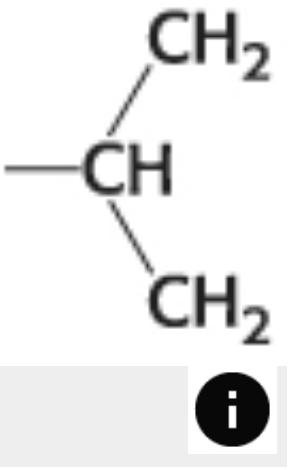
$$V_0 = \frac{k_{cat}}{K_M}[S][E]_T$$

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.

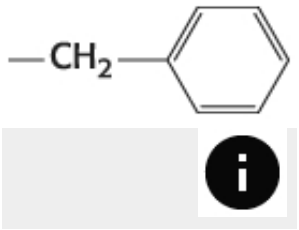
The rate constant $\boldsymbol{k_{cat}/K_M}$, called the **specificity constant**, is a measure of catalytic efficiency because it takes into account both the rate of catalysis with a particular substrate (k_{cat}) and the nature of the enzyme–substrate interaction (K_M). For instance, by using

k_{cat}/K_M values, we can compare an enzyme's preference for different substrates. Table 5.7 shows the k_{cat}, K_M, and k_{cat}/K_M values for several different substrates of chymotrypsin. Chymotrypsin clearly has a preference for cleaving next to bulky, hydrophobic side chains.

TABLE 5.7 Substrate preferences of chymotrypsin

Amino acid in ester	Amino acid side chain	k_{cat}/K_M ($s^{-1}\,M^{-1}$)
Glycine	—H	1.3×10^{-1}
Valine		2.0
Norvaline	$-CH_2CH_2CH_3$	3.6×10^{2}
Norleucine	$-CH_2CH_2CH_2CH_3$	3.0×10^{3}

Phenylalanine	1.0×10^5



Source: Information from 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.

How efficient can an enzyme be? We can approach this question by determining whether there are any physical limits on the value of k_{cat}/K_M. Note that the k_{cat}/K_M ratio depends on k_1, k_{-1}, and k_{cat}, as can be shown by substituting for K_M.

$$k_{cat}/K_M = \frac{k_{cat}k_1}{k_{-1}+k_{cat}} = \left(\frac{k_{cat}}{k_{-1}+k_{cat}}\right)k_1 < k_1$$

Note that the value of k_{cat}/K_M is always less than k_1. 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_{cat}/K_M then approaches k_1. Thus, the ultimate limit on the value of k_{cat}/K_M is set by k_1, the rate of formation of the ES complex, which is limited by the diffusion-controlled encounter of an enzyme and its substrate. Because diffusion limits the value of k_1, it cannot be higher than between 10^8 and $10^9\ s^{-1}M^{-1}$.

Enzymes that have k_{cat}/K_M ratios in the range of these upper limits, such as superoxide dismutase, acetylcholinesterase, and triose phosphate isomerase, have attained what is called *kinetic perfection*. Their catalytic velocity is restricted only by the rate at which they encounter substrate in the solution (Table 5.8). Any further gain in catalytic rate can come only by decreasing the time for diffusion of the substrate into the enzyme's immediate environment.

TABLE 5.8 Enzymes with k_{cat}/K_M near the diffusion-controlled limit

Enzyme	k_{cat}/K_M ($s^{-1}\,M^{-1}$)
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: Information from 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.

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*, named for the figure from Greek mythology known for her powers of seduction and transformation.

The diffusion of a substrate throughout a solution can also be partly overcome by confining substrates and products in the limited volume of a multienzyme complex, such 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.

Self-Check Question

If a mutation occurs in the gene encoding an enzyme which decreases k_{cat} for a specific substrate by a factor of 2 and decreases K_M for that substrate by a factor of 3, did the mutation make the enzyme a more or less efficient catalyst with respect to that substrate?

Most biochemical reactions include multiple substrates

Most reactions in biological systems are bisubstrate reactions, which start with two substrates and yield two products, represented by:

$$A + B \rightleftharpoons P + Q$$

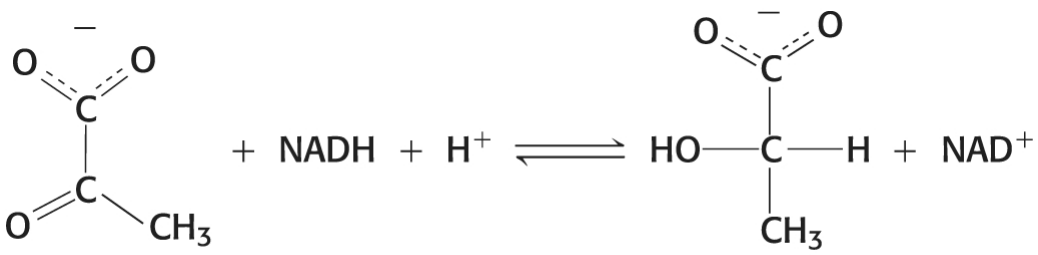
Many bisubstrate reactions transfer a functional group, such as a phosphoryl or an ammonium group, from one substrate to the other. Those that are oxidation–reduction reactions transfer electrons between substrates. Multiple substrate reactions can be divided into two classes: sequential reactions and double-displacement reactions.

Sequential reactions

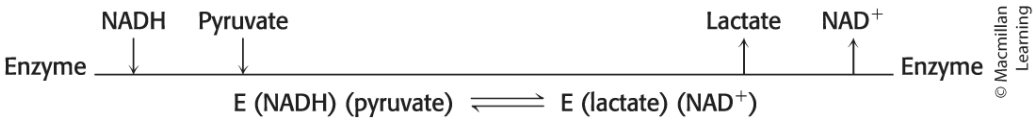
In **sequential reactions**, all substrates must bind to the enzyme before any product is released. Specifically, in a bisubstrate reaction, a ternary complex — composed of the enzyme and both substrates — forms.

- *In ordered sequential mechanisms, the substrates bind the enzyme in a defined sequence*. For example, many enzymes that have NAD^+ or NADH as a substrate exhibit the ordered sequential mechanism. Consider lactate dehydrogenase, an important

enzyme in glucose metabolism (Section 16.1). This enzyme reduces pyruvate to lactate while oxidizing NADH to NAD^+.

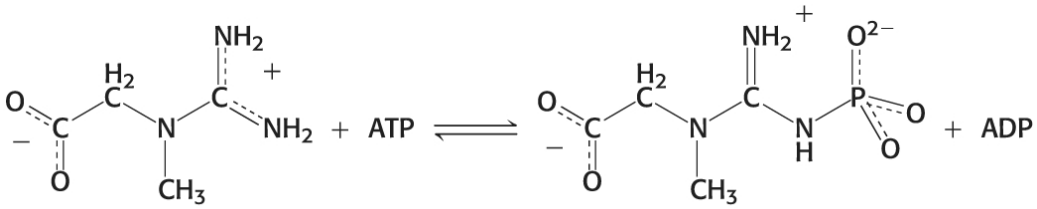


In the ordered sequential mechanism, the coenzyme always binds first, and the lactate is always released first. This sequence can be represented by using a notation developed by W. Wallace Cleland:

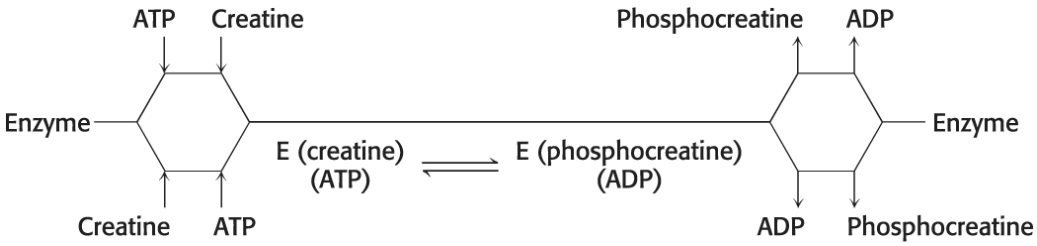


The enzyme exists as a ternary complex consisting of, first, the enzyme and substrates and, after catalysis, the enzyme and products.

- *In the random sequential mechanism, the order of the addition of substrates and the release of products is arbitrary*. An example of a random sequential reaction is the formation of phosphocreatine and ADP from creatine and ATP which is catalyzed by creatine kinase (Section 15.2).



Either creatine or ATP may bind first, and either phosphocreatine or ADP may be released first. Phosphocreatine is an important energy source in muscle. Random sequential reactions also can be depicted in the Cleland notation.

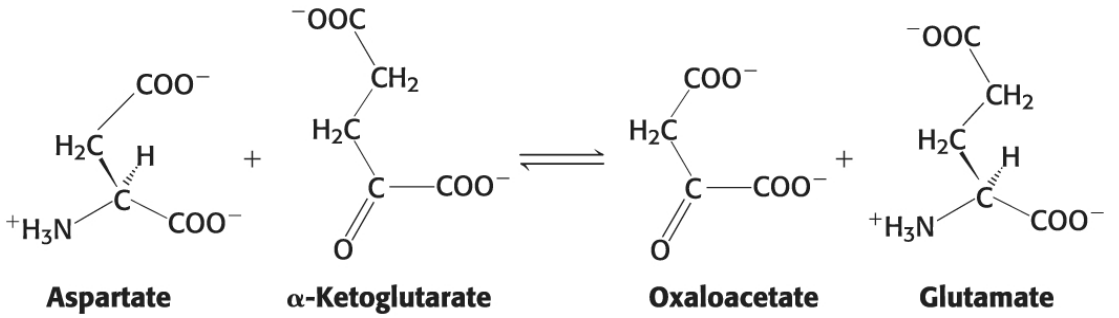


Although the order of certain events is random, the reaction still passes through the ternary complexes, including the substrates first and then the products.

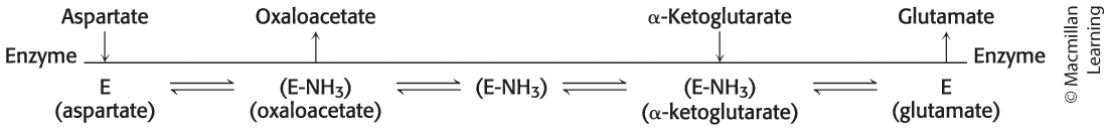
Double-displacement reactions

In **double-displacement reactions** (also called **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 an enzyme intermediate in which the enzyme is temporarily modified.

Reactions that shuttle amino groups between amino acids and α-ketoacids are classic examples of double-displacement mechanisms. The enzyme aspartate aminotransferase catalyzes the transfer of an amino group from aspartate to α-ketoglutarate.



The sequence of events can be portrayed as the following Cleland notation:



After aspartate binds to the enzyme, the enzyme accepts 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 much as a Ping-Pong ball bounces on a table. Note that for double-displacement reactions, substrate binding order is always fixed (ordered) and never random.

Allosteric enzymes often do not obey Michaelis–Menten kinetics

The Michaelis–Menten model has greatly assisted the development of enzymology. 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 often do not obey Michaelis–Menten kinetics are **allosteric enzymes**, which consist of multiple subunits and multiple active sites. The binding of substrate to one active site can alter the properties of other active sites in the same enzyme molecule (an effect known as *allostery*, Chapter 3). Allosteric enzymes often display sigmoidal plots of the reaction velocity V_0 versus substrate

concentration [S] (**Figure 5.12**), rather than the hyperbolic plots predicted by the Michaelis–Menten equation (see Figure 5.10).

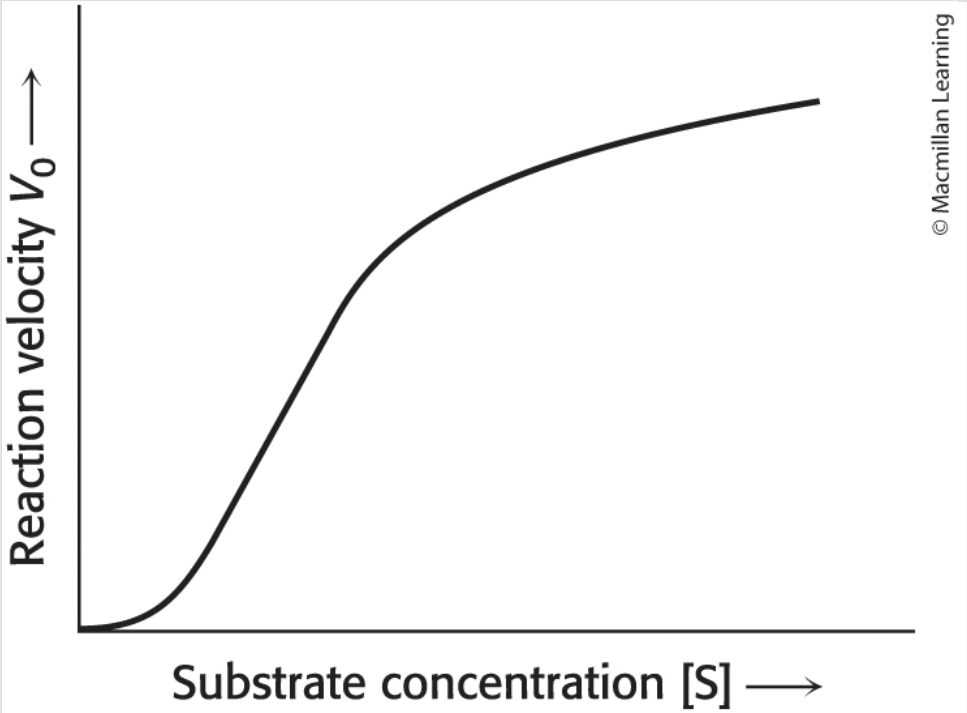


FIGURE 5.12 Allosteric enzymes often display a sigmoidal dependence of reaction velocity on substrate concentration. Shown are the kinetics for an allosteric enzyme exhibiting positive cooperativity.

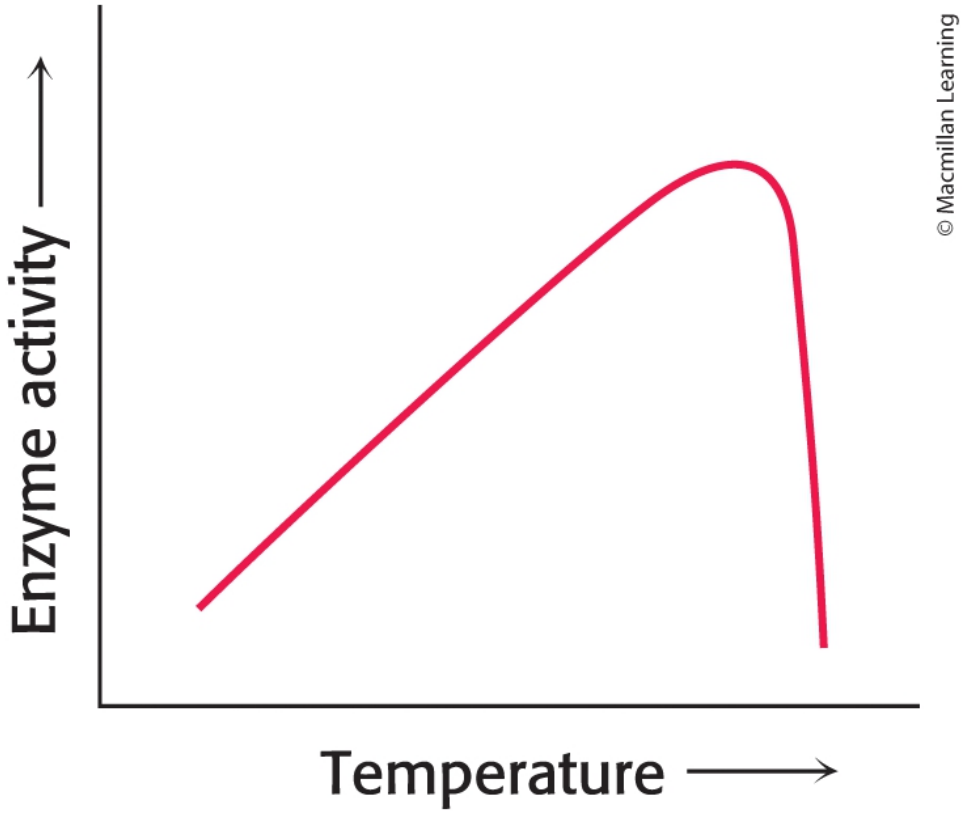
The interaction between an allosteric enzyme's multiple subunits can exhibit positive cooperativity. In other words, the binding of substrate to one active site facilitates the binding of substrate to the other active sites, akin to the behavior of oxygen binding to the multiple subunits of the allosteric protein hemoglobin (see Section 3.3). Such positive 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 reversibly bind to specific sites

other than the catalytic sites. Furthermore, the enzymes themselves can often interconvert between more- or less-active quaternary states, typically denoted as “R” and “T,” respectively, by the conventions established for hemoglobin.

The complexities of allosteric enzymes often require more-elaborate models than the Michaelis–Menten model to account for multiple active sites and quaternary state transitions. The catalytic properties of allosteric enzymes make them key regulators of metabolic pathways in ways we will explore more fully in Chapter 7.

Temperature affects enzymatic activity

As the temperature rises, the rate of most reactions, including enzyme-catalyzed reactions, increases. For most enzymes, there is a temperature at which the increase in catalytic activity ceases and there is a precipitous loss of activity.

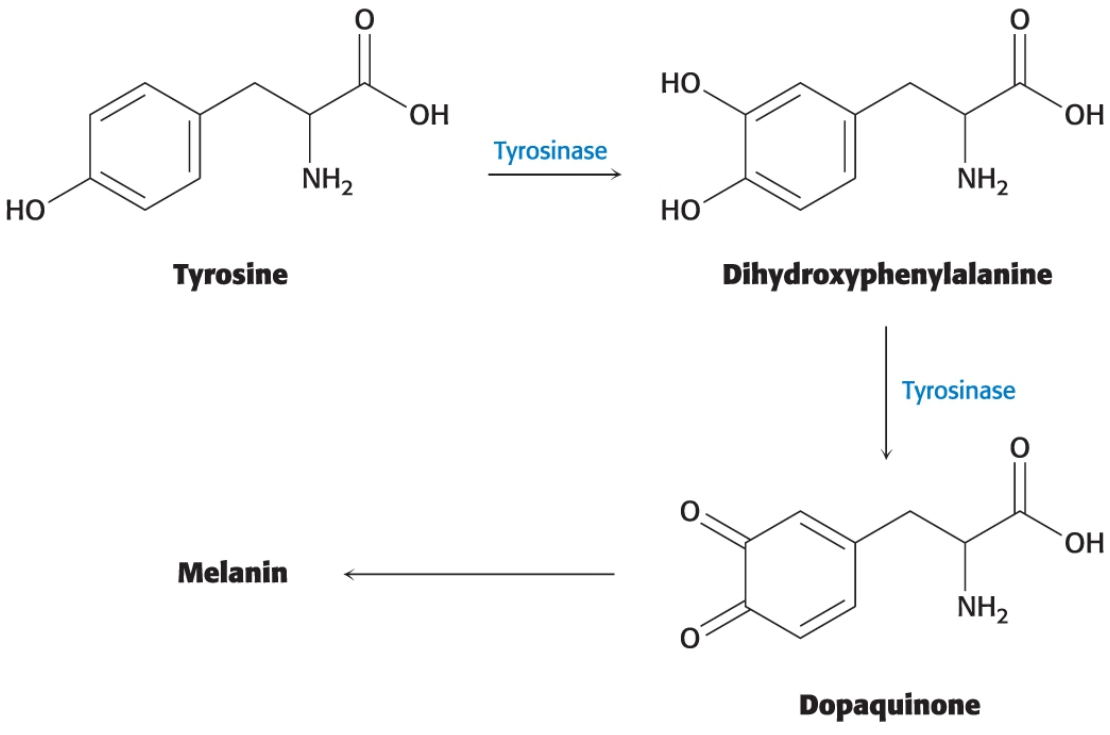


What is the basis of this loss of activity? Recall from Chapter 2 that proteins have a complex three-dimensional structure that is held together by weak interactions. When the temperature increases beyond a certain point, the favorable interactions maintaining the three-dimensional structure are not strong enough to withstand the polypeptide chain's thermal jostling, and the protein loses the structure required for activity. The protein is said to be *denatured* (Section 2.7).

In endotherms, organisms such as ourselves that maintain a constant body temperature, the effect of outside temperature on enzyme activity is minimized. However, in ectotherms, organisms

that assume the temperature of their environment, temperature is an important regulator of biochemical and, indeed, biological activity. Lizards, for instance, are most active in warmer temperatures — a behavioral manifestation of biochemical activity.

Although endotherms are not as sensitive to ambient temperature as ectotherms, slight tissue temperature alterations are sometimes important and can even have dramatic effects. For example, the characteristic coloration of Siamese cats can be explained by variation in enzyme sensitivity due to temperature. Their fur color is due to the presence of the pigment melanin, the same pigment that is responsible for human skin color. The first steps in the synthesis of melanin are catalyzed by the enzyme tyrosinase:



Most Siamese cats are born with very little coloration; sometimes they are even white. As they mature, their extremities — tips of the ears, snout, paws, and end of the tail — darken to black. A hint to understanding this phenomenon comes from the knowledge that a cat's skin temperature is coolest at the extremities. Biochemists have established that there is a mutation in the Siamese tyrosinase that results in a loss of activity above 37-39°C. The temperature of a Siamese kitten is too warm for the enzyme to be active, but as the kitten grows, the temperature at its extremities cools enough to allow the enzyme to become active and pigment to form. The core of the cat's body remains above the threshold temperature, however, and thus remains pale in color.

5.5 Enzymes Can Be Studied One Molecule at a Time

Most experiments performed to determine an enzyme's characteristics use an enzyme preparation in a buffered solution. Even a few microliters of such a solution will contain millions of enzyme molecules. Experiments on this scale, called *ensemble studies*, make the basic assumption that all of the enzyme molecules are the same or very similar. For example, when we determine an enzymatic property such as the value of K_M in ensemble studies, we assume that value is an average value of all of the enzyme molecules present.

Keep in mind, however, that individual enzyme molecules behave stochastically, as we saw for DNA double helix formation in Chapter 1. New methods, dubbed *single-molecule experiments*, now allow one individual molecule to be examined at a time, yielding a great deal of new information but with a potential pitfall: how can we be certain that the molecule is representative and not an outlier? We can overcome this challenge by studying enough individuals to satisfy statistical analysis for validity.

Let us consider a biochemical situation. **Figure 5.13A** shows a hypothetical enzyme that displays molecular heterogeneity, with three active forms that catalyze the same reaction but at different rates. These forms have slightly different stabilities, but thermal motion is sufficient to interconvert them. Each form is present as a fraction of the total enzyme population, as indicated. If we were to perform an ensemble method experiment to determine enzyme activity under a particular set of conditions, we would get a single

value, which would represent the average of the heterogeneous assembly (Figure 5.13B). However, were we to perform a sufficient number of single-molecule experiments, we would discover that the enzyme has three different molecular forms with very different activities (Figure 5.13C). Moreover, these different forms would most likely correspond to important biochemical differences.

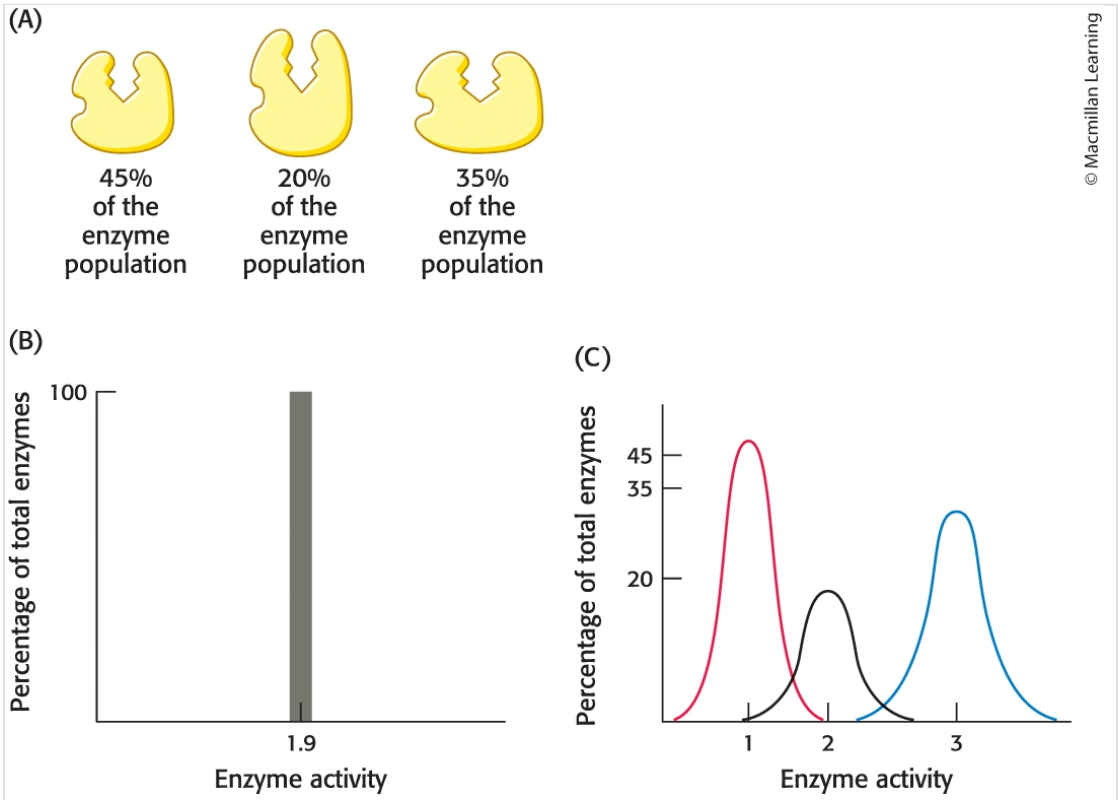


FIGURE 5.13 Single-molecule studies can reveal molecular heterogeneity. (A) Complex biomolecules, such as enzymes, display molecular heterogeneity. (B) When measuring an enzyme property using ensemble methods, an average value of all of the enzymes present can be obtained. (C) Single-enzyme studies reveal molecular heterogeneity, with the various forms showing different properties.

Single-molecule kinetics confirm results obtained from ensemble studies

Now suppose that we watch an individual enzyme molecule in action, monitoring every time it produces a molecule of product. Figure 5.14 illustrates what we would expect to see, with each line representing a single reaction event. Because individual enzyme molecules behave stochastically, the time between consecutive reaction events appears to vary randomly. However, if we watch an individual enzyme molecule turn over many thousands of times, we find a pattern in the distribution of the time intervals separating consecutive events that allows us to state the anticipated relationship between the average time increments at different substrate concentrations as

$$1/(\text{Time increment})_{\text{average}} = k_2[S]/([S] + K_M)$$

The output from this equation, called the *single-molecule Michaelis–Menten equation*, reveals the same saturation behavior for reaction rates that we observe for ensemble studies of enzymes. In fact, plotting the reciprocal of the average time increment at different substrate concentrations produces a Michaelis–Menten saturation curve, which can also be visualized as a Lineweaver–Burk plot, as we will see shortly. This illustrates a reassuring finding of single-molecule methods: they often reaffirm observations made by ensemble methods.

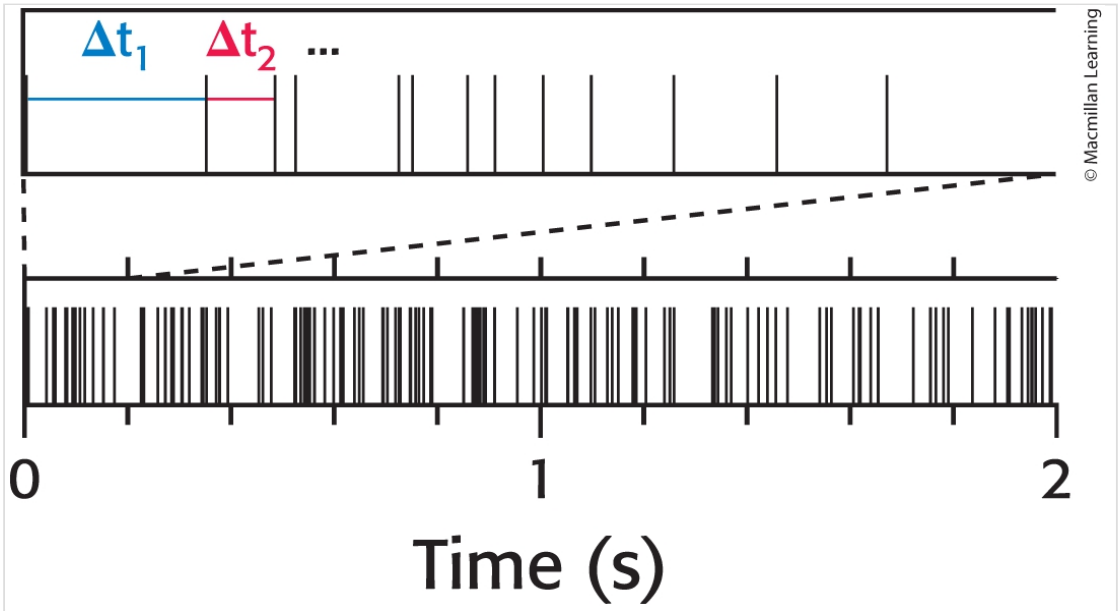
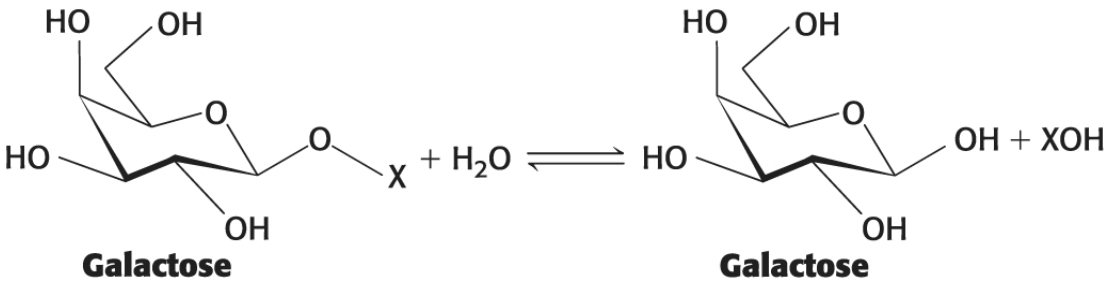


FIGURE 5.14 An anticipated time course for a single-enzyme molecule reveals stochastic behavior. Each line represents an enzyme molecule turning a substrate molecule into product. These events occur stochastically, with variable time increments between consecutive events.

Up to this point, we have discussed monitoring hypothetical enzymes. We now turn to a real example, the enzyme β-galactosidase from *E. coli*, which allows this bacterium to utilize certain sugars. Studies of this enzyme played a central role in elucidating fundamental mechanisms in gene regulation, to be discussed in Chapter 31. This enzyme catalyzes reactions of the form



While the enzyme is quite specific for the carbohydrate galactose (to be discussed in Chapter 11), substantial variations in the other half of the substrate can be accommodated, permitting the binding of probe substrates such as Substrate G-R (**Figure 5.15**). Cleavage of this substrate by β-galactosidase releases a fluorescent product.

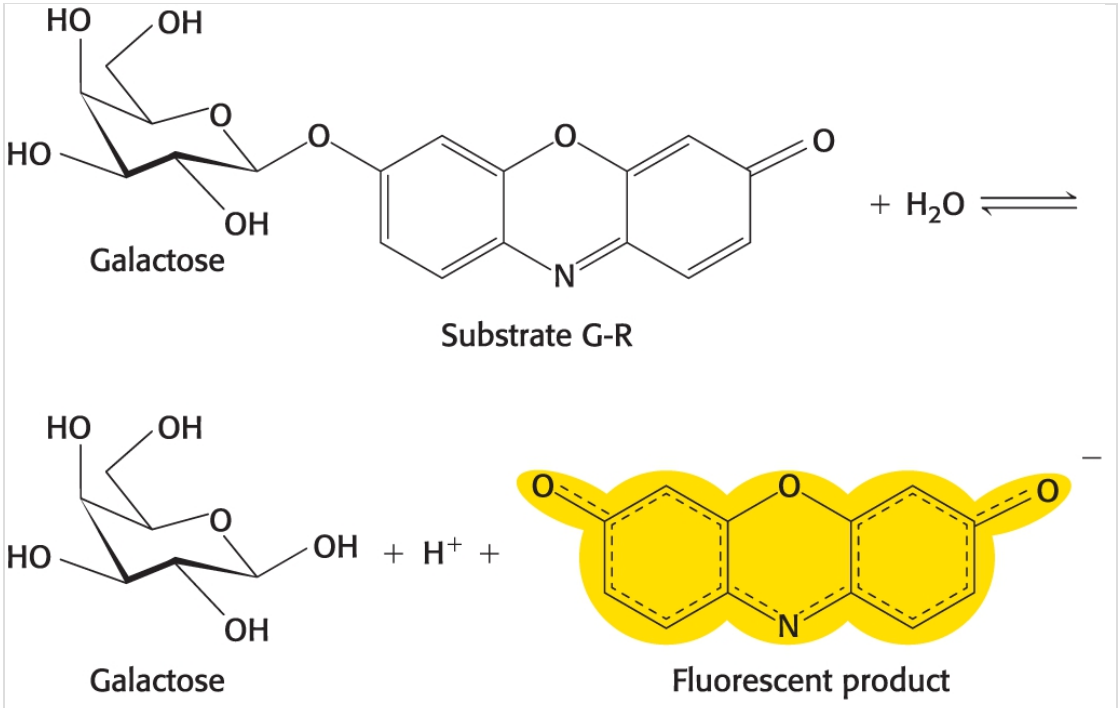


FIGURE 5.15 Upon reaction with β-galactosidase, Substrate G-R produces a highly fluorescent product. The formation of individual molecules of this product can be detected,

allowing for the observation of single-enzyme turnovers to be tracked.

Using immobilized enzyme molecules and a fluorescence microscope, product release (and, hence, enzyme activity) can be monitored directly at the single-molecule level. With this system, experiments were performed at a range of substrate concentrations and the results compared with those obtained from ensemble studies using the same enzyme and substrate (**Figure 5.16**).

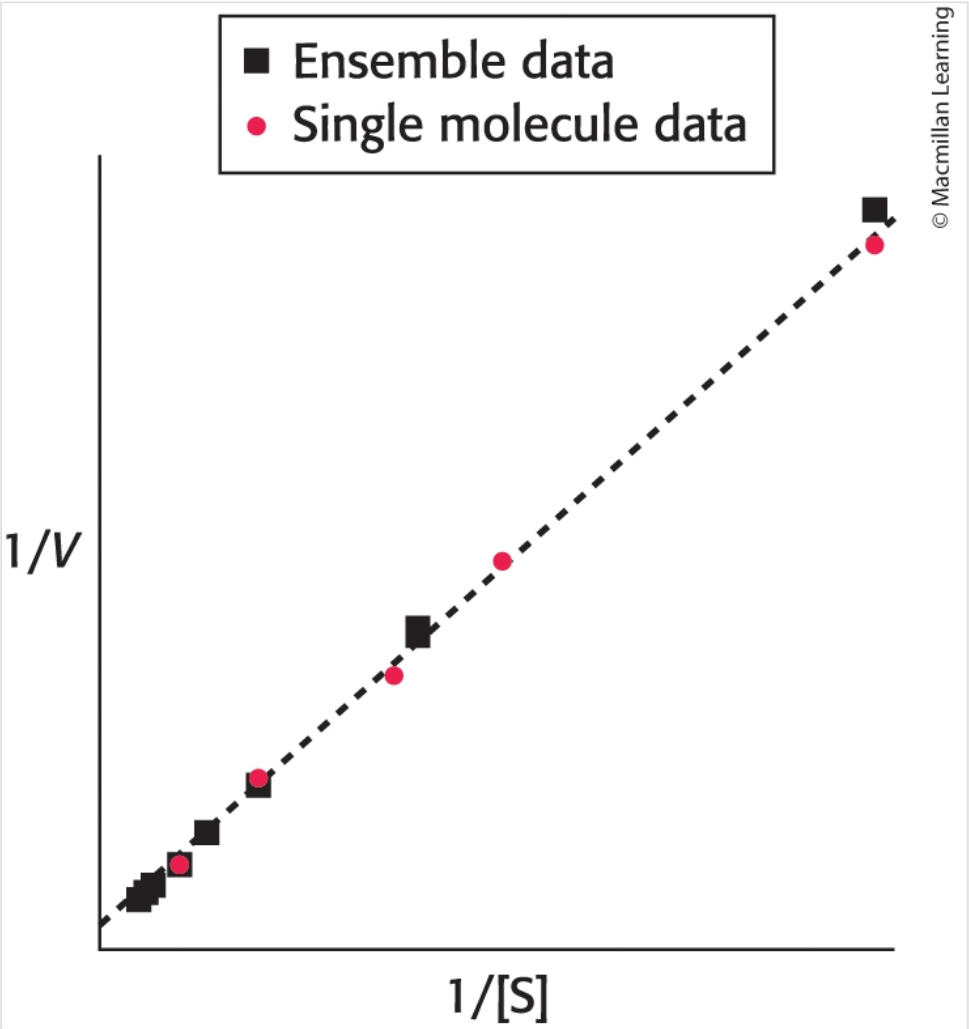


FIGURE 5.16 Lineweaver–Burk plots of single-molecule data compare favorably to those from ensemble studies. The results from single-molecule kinetic studies of β-galactosidase (red) are nearly indistinguishable from those from ensemble studies of the same enzyme at the same substrate concentration (black).

[Adapted from B. P. English et al., *Nature Chemical Biology* 2:87–94, 2006.]

Single-molecule studies continue to reveal new information about enzyme molecular dynamics

While it is quite reassuring to see in a real example that the results of technically challenging single-molecule studies agree so well with those of ensemble studies, the single-molecule results don't tell us anything new about enzyme behavior. However, depicting the distribution of time increments on a log plot does reveal an important new insight (**Figure 5.17**). At a low substrate concentration of $10\ \mu M$, this plot is relatively linear. However, at a higher concentration of $100\ \mu M$, this plot is decidedly nonlinear, changing from a relatively steep slope at short time increments to a more gradual slope at longer time increments.

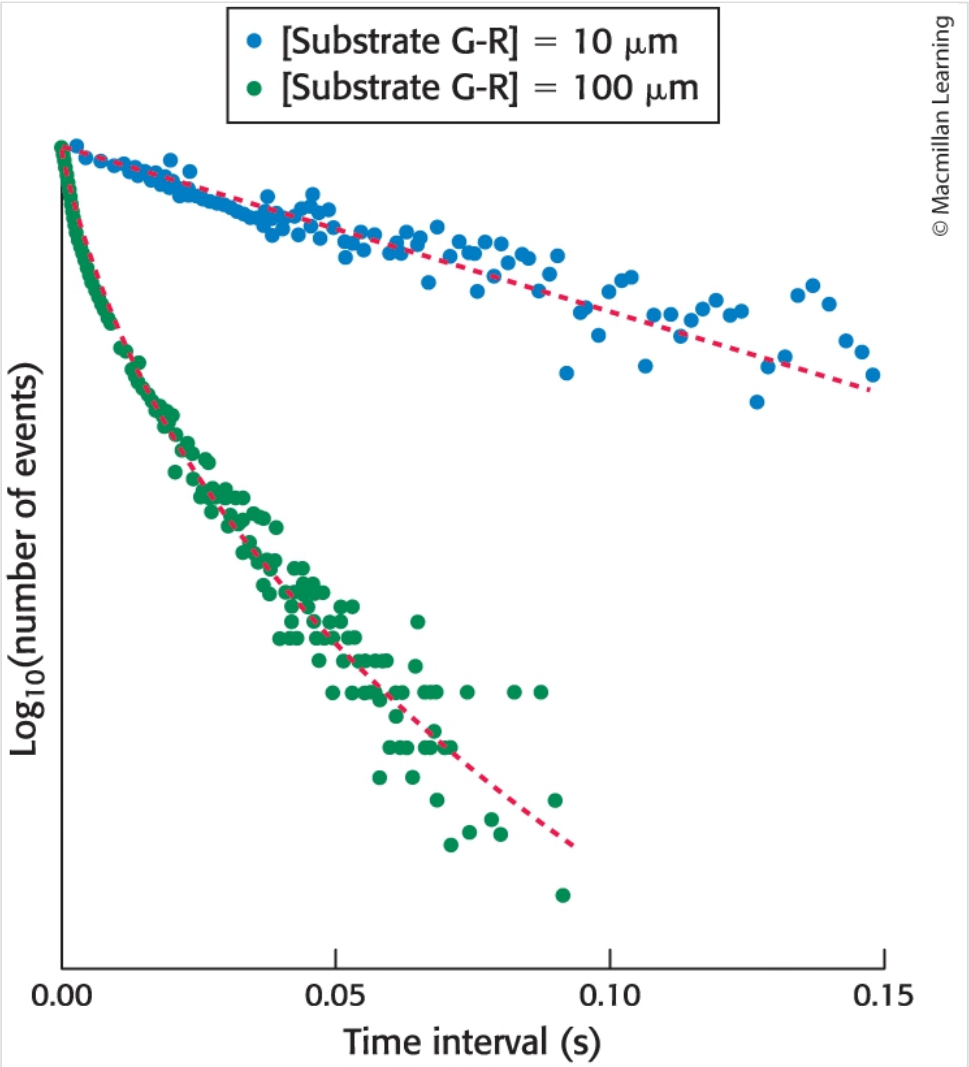


FIGURE 5.17 Details of single-molecule time increment distributions for β-galactosidase indicate heterogeneity at the molecular level. At a relatively high substrate concentration (100 μM), a plot of the logarithm of the number of events in each time interval is not linear, indicated by the distribution not following a simple exponential fall off. This observation reveals that the enzyme cannot be modeled as a single species at the single-molecule level.

[Adapted from B. P. English et al., *Nature Chemical Biology* 2:87–94, 2006.]

We can explain this observation in terms of the dynamic nature of individual enzyme molecules. Each molecule can exist and convert between multiple conformational states, as we envisioned hypothetically in Figure 5.13, each conformation having distinct catalytic properties. The structural differences of these conformational states could be as simple as the orientations of particular side chains or as complex as large, interdomain movements. Further studies are required to gain such structural information, but regardless of the structural details, single-molecule experiments provide direct evidence for a critical aspect of enzymes: their dynamic nature.

Using the many powerful single-molecule techniques that now exist, we can observe events at a molecular level to reveal rare or transient structures and fleeting events in a reaction sequence, as well as to measure mechanical forces affecting or generated by an enzyme. Single-molecule studies open a new vista on the function of enzymes in particular and all large biomolecules in general. We will examine additional single-molecule studies in future chapters.

5.6 Enzymes Can Be Inhibited by Specific Molecules

The activity of many enzymes can be inhibited by the binding of specific small molecules and ions. Enzyme inhibition serves as a major control mechanism in biological systems, especially by regulating the activity of allosteric enzymes. In addition, many drugs and toxic agents act by inhibiting enzymes (Chapter 32). These inhibitors are often designed by scientists or result from a chance discovery of an inhibitory molecule. Examining inhibition can give insight into the mechanism of enzyme action; for example, specific inhibitors are often used to identify residues critical for catalysis. Enzyme inhibition can be either irreversible or reversible.

- *An irreversible inhibitor covalently bonds to its target enzyme and thus does not dissociate at any appreciable rate*. The mode of action of some important drugs is irreversible inhibition. For example, as we will discuss shortly, penicillin acts by covalently modifying the enzyme transpeptidase, thereby preventing the synthesis of bacterial cell walls and thus killing the bacteria. Aspirin acts by covalently modifying the enzyme cyclooxygenase, reducing the synthesis of signaling molecules in inflammation.

- *Reversible inhibition is characterized by the dissociation of the enzyme–inhibitor complex, which is formed by noncovalent interactions*. Depending on their target and their effect, reversible inhibitors are classified as competitive, uncompetitive, or noncompetitive.

In **competitive inhibition**, the inhibitor competes with the substrate for binding to the enzyme and thus reduces the proportion of enzyme molecules bound to substrate. The enzyme can bind the substrate (**Figure 5.18A**) or an inhibitor (**Figure 5.18B**), but not both at the same time. Competitive inhibitors can bind anywhere on the enzyme, but

they most often resemble the substrate and bind to the active site. The substrate is thereby prevented from binding to the same active site. At any given inhibitor concentration, competitive inhibition can be relieved by increasing the substrate concentration. Under these conditions, the substrate successfully competes with the inhibitor for the enzyme.

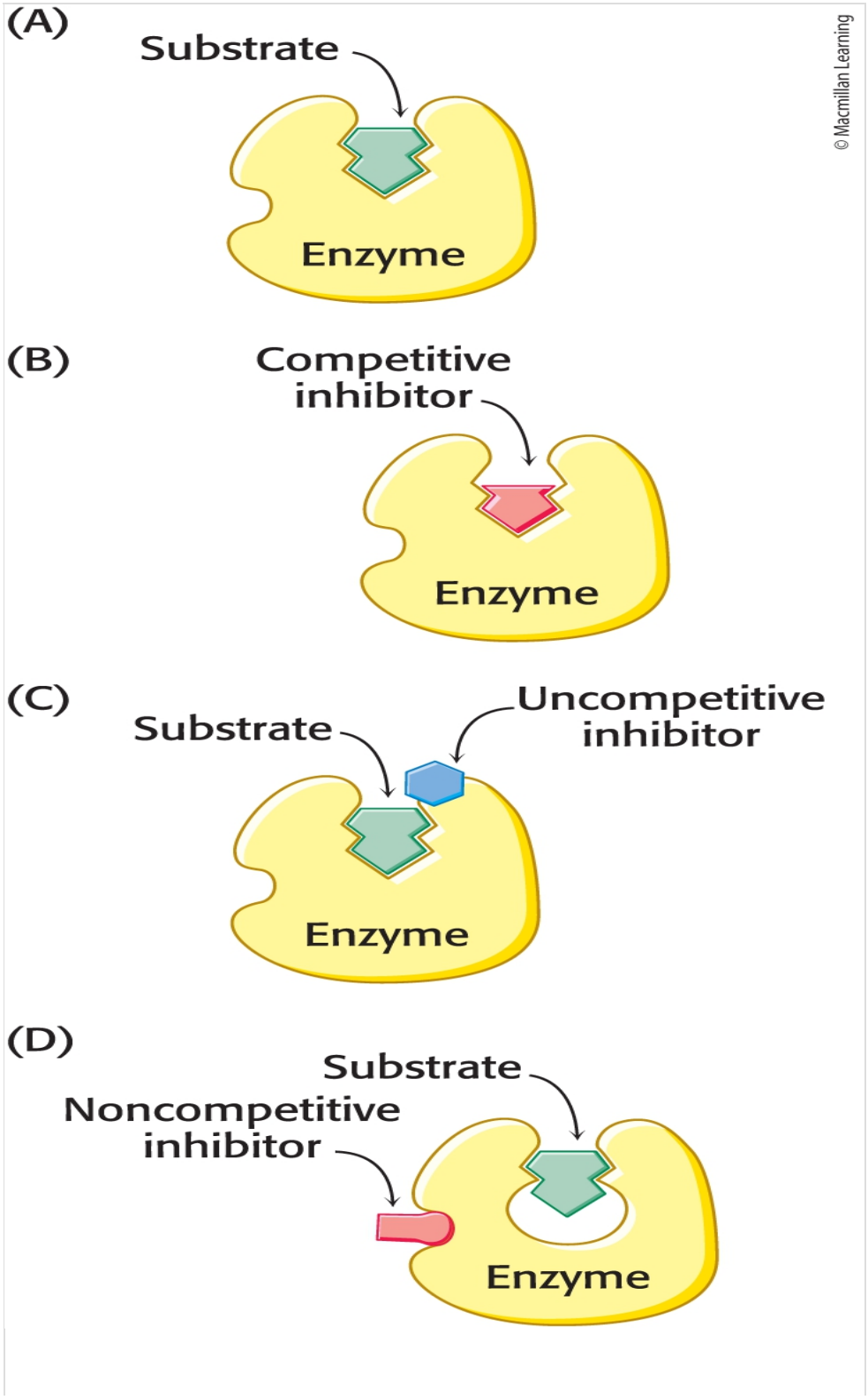
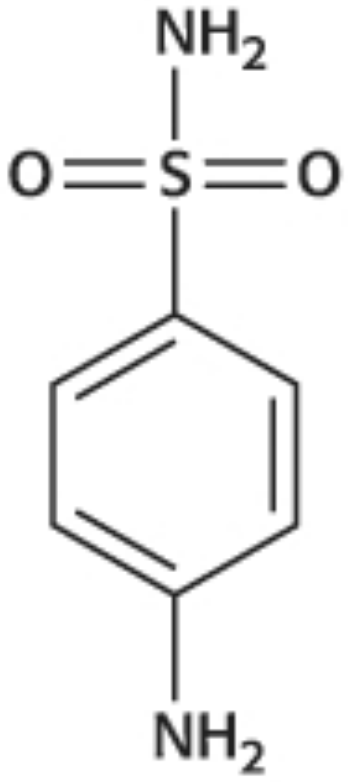
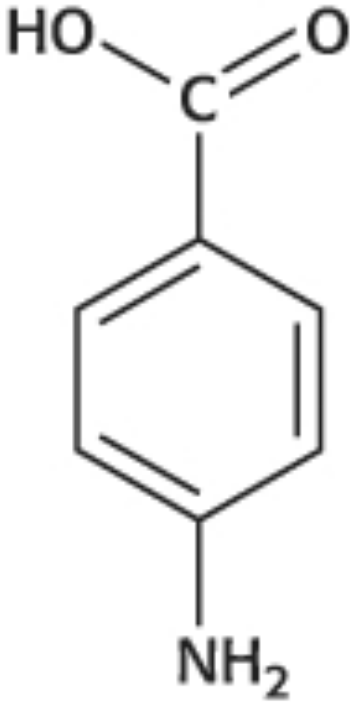


FIGURE 5.18 Reversible inhibitors can be distinguished by whether they bind to the enzyme with or without the substrate present. (A) An enzyme–substrate complex; (B) competitive inhibitors prevent the substrate from binding, usually by themselves binding at the active site; (C) an uncompetitive inhibitor binds only to the enzyme–substrate complex; (D) a pure noncompetitive inhibitor does not affect the binding of substrate.

Many competitive inhibitors are useful drugs. Drugs such as ibuprofen are competitive inhibitors of enzymes that participate in signaling pathways in the inflammatory response. Statins are drugs that reduce high cholesterol levels by competitively inhibiting a key enzyme in cholesterol biosynthesis (Section 26.3). One of the earliest examples was the use of sulfanilamide as an antibiotic. Sulfanilamide is an example of a sulfa drug, a sulfur-containing antibiotic. Structurally, sulfanilamide mimics *p*-aminobenzoic acid (PABA), a metabolite required by bacteria for the synthesis of the coenzyme folic acid. Sulfanilamide binds to the enzyme that normally metabolizes PABA and competitively inhibits it, preventing folic acid synthesis. Human beings, unlike bacteria, absorb folic acid from the diet and are thus unaffected by the sulfa drug.





Sulfanilamide **PABA**

In **uncompetitive inhibition**, the inhibitor binds not to the enzyme itself but to the enzyme–substrate complex; it is therefore substrate-dependent. The binding site of an uncompetitive inhibitor is created only on interaction of the enzyme and substrate (**Figure 5.18C**). Uncompetitive inhibitors inhibit catalysis, rather than prevent substrate binding, and cannot be overcome by the addition of more substrate. The herbicide glyphosate, also known as Roundup, is an uncompetitive inhibitor of an enzyme in the biosynthetic pathway for aromatic amino acids.

In **pure noncompetitive inhibition**, the inhibitor and substrate can bind simultaneously to an enzyme molecule at two different binding sites (**Figure 5.18D**). Unlike competitive or uncompetitive inhibition, a noncompetitive inhibitor can bind free enzyme or the enzyme–substrate complex, but in either case it decreases the rate catalysis.

Noncompetitive inhibitors can be either pure or mixed. A pure noncompetitive inhibitor binds equally well to the enzyme with or without substrate bound, and the effect is that only turnover number is decreased. Thus, pure noncompetitive inhibitors act by decreasing the concentration of functional enzyme rather than by altering the proportion of enzyme molecules that are bound to substrate. Noncompetitive inhibition, like uncompetitive inhibition, cannot be overcome by increasing the substrate concentration. **Mixed noncompetitive inhibition** is more complex in that the inhibitor binds preferentially to either the free enzyme or the enzyme–substrate complex, resulting in a pattern of inhibition that alters both substrate binding and turnover number simultaneously. The commonly prescribed antibiotic doxycycline, which is used to treat periodontal disease, functions at low concentrations as a noncompetitive inhibitor of a proteolytic enzyme (collagenase).

The different types of reversible inhibitors are kinetically distinguishable

How can we determine whether a reversible inhibitor acts by competitive, uncompetitive, or noncompetitive inhibition? Considering only enzymes that exhibit Michaelis–Menten kinetics, we can use measurements of the rates of catalysis at different concentrations of substrate and inhibitor and Lineweaver–Burk plots as clues to which type of reversible inhibition is occurring.

Competitive inhibition

In competitive inhibition, where the inhibitor competes with the substrate for the enzyme, the dissociation constant for the inhibitor is given by

$$K_i = [E][I]/[EI]$$

The smaller the K_i, the more potent the inhibition. The hallmark of competitive inhibition is that it can be overcome by a sufficiently high concentration of substrate (**Figure 5.19**). The effect of a competitive inhibitor is to increase the apparent value of K_M, meaning that more substrate is needed to obtain the same reaction rate. This new apparent value of K_M, called K_M^{app}, is numerically equal to

$$K_M^{app} = K_M(1 + [I]/K_i)$$

where [I] is the concentration of inhibitor and K_i is the dissociation constant for the enzyme–inhibitor complex. In the presence of a competitive inhibitor, an enzyme will have the same V_{max} as in the absence of an inhibitor. At a sufficiently high concentration, virtually all the active sites are filled with substrate, and the enzyme is fully operative.

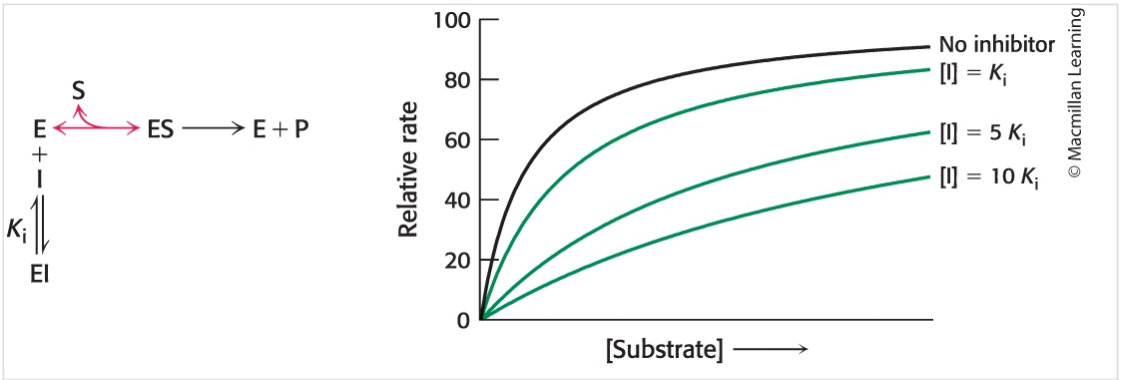


FIGURE 5.19 Michaelis–Menten plots of a competitive inhibitor reveal changes in the apparent K_M but not V_{max}. As the concentration of a competitive inhibitor increases, higher concentrations of substrate are required to attain a particular reaction velocity. The reaction pathway suggests how sufficiently high concentrations of substrate can completely relieve competitive inhibition resulting in the same V_{max} value at infinite substrate concentration.

A Lineweaver–Burk plot can also reveal competitive inhibition. 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 5.20).

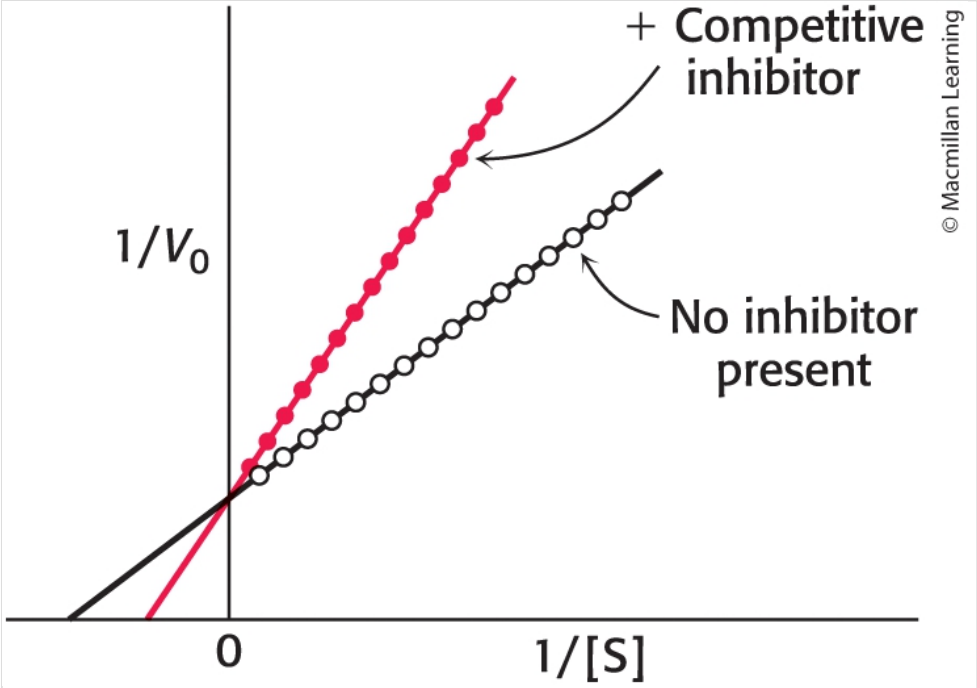


FIGURE 5.20 Competitive inhibition is easily recognized using a Lineweaver–Burk plot. A Lineweaver–Burk plot of enzyme kinetics in the presence and absence of a competitive inhibitor illustrates that the inhibitor has no effect on V_{max} but increases the apparent K_M.

The intercept is unchanged because a competitive inhibitor does not alter V_{max}. The increase in the slope of the $1/V_0$ versus $1/[S]$ plot indicates the strength of binding of a competitive inhibitor. In the presence of a competitive inhibitor, equation 18 is replaced by

$$\frac{1}{V_0} = \frac{1}{V_{max}} + \frac{K_M}{V_{max}}\left(1 + \frac{[I]}{K_i}\right)\left(\frac{1}{[S]}\right) \quad \textbf{(22)}$$

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, when $V_0 = V_{max}/2$, $[S] = 10^{-4}$ M. In the presence of a 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 $K_M(1 + [I]/K_i)$, or 3×10^{-4} M. Substitution of these values into equation 22 gives $V_0 = V_{max}/4$, when $[S] = 10^{-4}$ M. Thus, the presence of the competitive inhibitor cuts the reaction rate in half at this substrate concentration.

Uncompetitive inhibition

In uncompetitive inhibition, where the inhibitor binds only to the ES complex, the resulting enzyme–substrate–inhibitor (ESI) complex does not go on to form any product. Because some unproductive ESI complex will always be present, V_{max} will be lower in the presence of inhibitor than in its absence (**Figure 5.21**). The uncompetitive inhibitor lowers the apparent value of K_M because the inhibitor binds to ES to form ESI, depleting ES. To maintain the equilibrium between E and ES, more S binds to E, increasing the apparent value of k_1 and thereby reducing the apparent value of K_M (see equation 13). Thus, a lower concentration of S is required to form half of the maximal concentration of ES.

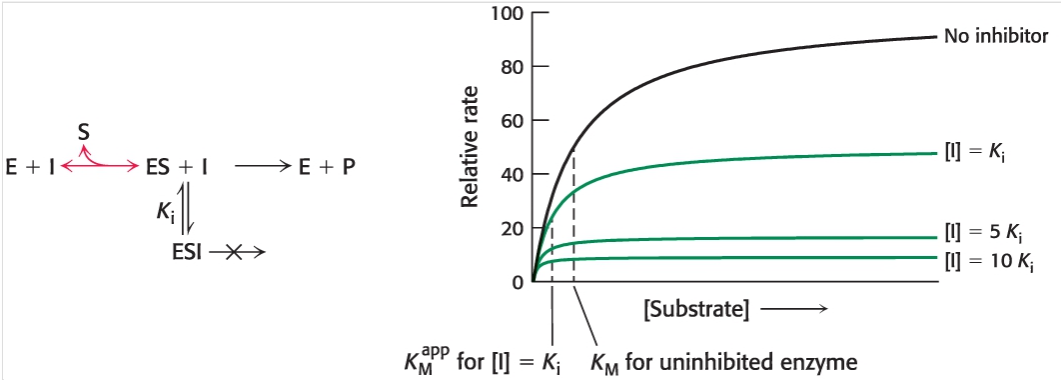


FIGURE 5.21 Michaelis–Menten plots of an uncompetitive inhibitor reveal decreases in both the apparent K_M and V_{max}. The reaction pathway shows that the inhibitor binds only to the enzyme–substrate complex. Consequently, V_{max} cannot be attained, even at high substrate concentrations. The apparent value for K_M is lowered, becoming smaller as more inhibitor is added.

The equation that describes the Lineweaver–Burk plot for an uncompetitive inhibitor is

$$\frac{1}{V_0} = \frac{K_M}{V_{max}}\left(\frac{1}{[S]}\right) + \frac{1}{V_{max}}\left(1 + \frac{[I]}{K_i}\right)$$

The slope of the line, K_M / V_{max}, is the same as that for the uninhibited enzyme, but the intercept on the *y*-axis will be increased by $1 + [I]/K_i$. Consequently, the lines in Lineweaver–Burk plots will be parallel (Figure 5.22).

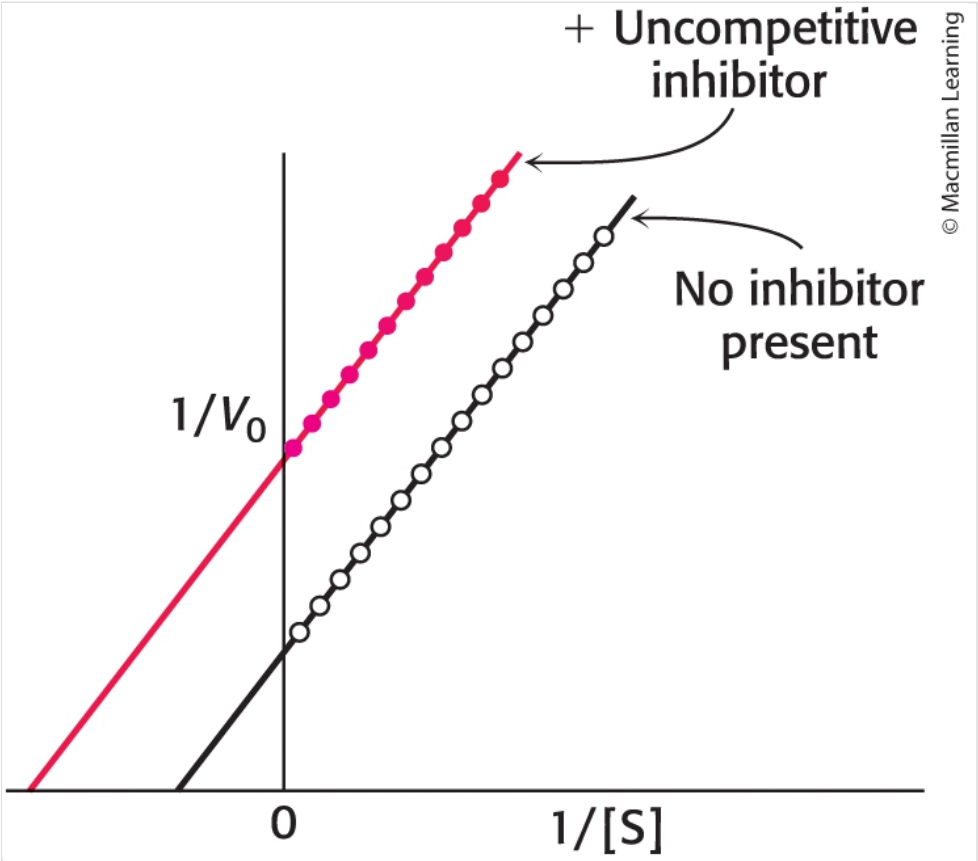


FIGURE 5.22 Uncompetitive inhibition is easily distinguished by parallel lines in a Lineweaver–Burk plot. An uncompetitive inhibitor does not affect the slope of the Lineweaver–Burk plot. V_{max} and K_M are reduced by equivalent amounts.

Noncompetitive inhibition

Recall that in noncompetitive inhibition, the inhibitor can bind at a different site from the substrate either the enzyme or the enzyme–substrate complex; in either case, the enzyme–inhibitor–substrate complex does not proceed to form product. In pure noncompetitive

inhibition, the K_i for the inhibitor binding to E is the same as for binding to ES complex (Figure 5.23).

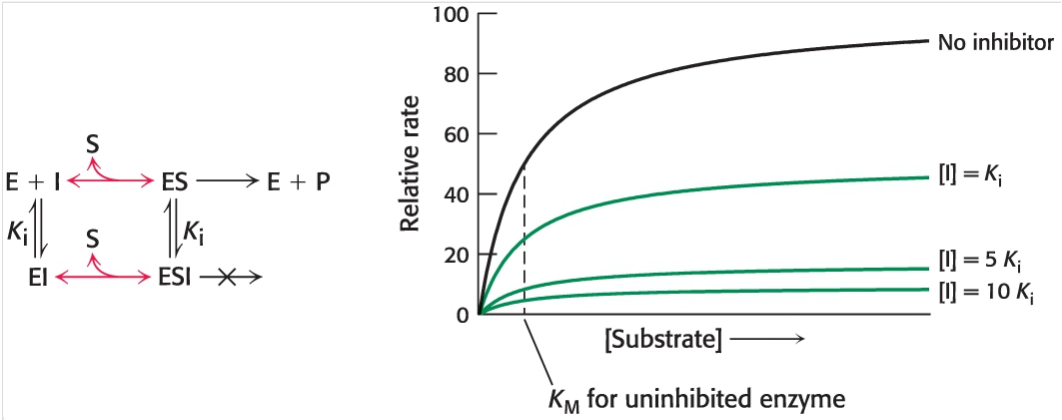


FIGURE 5.23 Michaelis–Menten plots of a pure noncompetitive inhibitor reveal a decrease in V_{max}, while K_M remains unchanged. The reaction pathway shows that the inhibitor binds both to free enzyme and to an enzyme–substrate complex. Consequently, as with uncompetitiv inhibition, V_{max} cannot be attained. In pure noncompetitive inhibition, K_M remains unchanged, and so the reaction rate increases more slowly at low substrate concentrations tha is the case for uncompetitive competition.

The apparent value of V_{max} is decreased to a new value called V_{max}^{app}, whereas the value of K_M is unchanged. The maximal velocity in the presence of a pure noncompetitive inhibitor, V_{max}^{app}, is given by

$$V_{max}^{app} = \frac{V_{max}}{1 + [I]/K_i}$$

(23)

Why is V_{max} lowered though K_M remains unchanged? In essence, the inhibitor simply lowers the concentration of functional enzyme because some quantity of enzyme is always bound to the inhibitor and inactive at any given moment, regardless of the substrate concentration. Because the inhibitor and the substrate do not affect the binding of one another, pure noncompetitive inhibition cannot be overcome by increasing the substrate concentration.

In the Lineweaver–Burk plot for pure noncompetitive inhibition (**Figure 5.24**), the value of V_{max} is decreased to the new value V_{max}^{app}, and so the intercept on the vertical axis is increased (equation 23). The new slope, which is equal to K_M / V_{max}^{app}, is larger by the same factor. In contrast with V_{max}, K_M is not affected by pure noncompetitive inhibition.

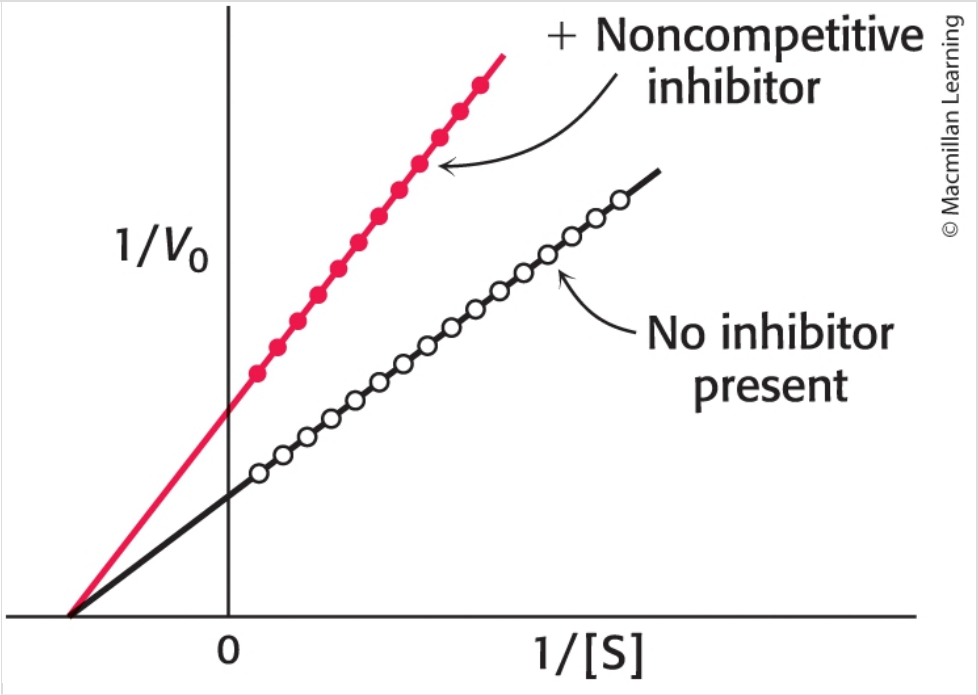


FIGURE 5.24 Pure noncompetitive inhibition can be distinguished on a Lineweaver–Burk plot by lines converging at the *x*-axis. A Lineweaver–Burk plot of enzyme kinetics in the presence and absence of a pure noncompetitive inhibitor shows that K_M is unaltered and V_{max} is decreased.

Finally, in mixed noncompetitive inhibition, the inhibitor binds preferentially to either E or ES, but still binds both, and thus behaves as a blend of a noncompetitive inhibitor and either a competitive or an uncompetitive inhibitor, depending upon the binding preference. Thus, for a mixed noncompetitive inhibitor V_{max} always decreases but the apparent value of K_M can either increase or decrease; the Lineweaver–Burk plot resembles that of a pure noncompetitive inhibitor but with lines converging either above or below the *x*-axis.

Transition-state analogs are potent competitive inhibitors

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 (**Figure 5.25**). 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. Pauling's hypothesis was supported by the finding that the inhibitor pyrrole 2-carboxylate binds to the racemase active site 160 times as

tightly as does proline. The α-carbon atom of this inhibitor, like that of the transition state, is trigonal.

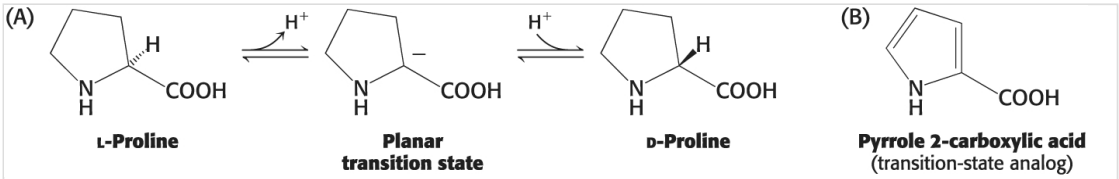


FIGURE 5.25 Transition-state analogs are potent competitive inhibitors because they structurally resemble the transition state of a catalyzed reaction. (A) The isomerization of L-proline to D-proline by proline racemase proceeds through a planar transition state in which the α-carbon atom is trigonal rather than tetrahedral. (B) Pyrrole 2-carboxylic acid, a transition-state analog because of its trigonal geometry, is a potent competitive inhibitor of proline racemase. An analog that also carries a negative charge on C_α would be expected to bind even more tightly.

In general, highly potent and specific competitive inhibitors of enzymes can be produced by synthesizing compounds that more closely resemble the transition state than the substrate itself. Because only molecules that are structurally similar to the real transition state will bind tightly to the active site, potent competitive inhibition — or its absence — by these molecules can be used to support or refute hypotheses regarding the structure of the transition state for a given enzyme-catalyzed reaction, providing evidence for particular chemical mechanisms. The inhibitory power of transition-state analogs underscores the essence of enzyme catalysis: catalysis is accomplished by selective binding of the transition state structure by the active site.

Self-Check Question

An inhibitor binds noncovalently to an allosteric site and induces a change in protein structure such that the enzyme can no longer bind substrate in the active site. Conversely,

when the substrate is bound in the active site, the allosteric site for the inhibitor is closed such that the inhibitor cannot bind. What type of inhibition is being described here?

Irreversible inhibitors can be used to map the active site

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 what these functional groups are? X-ray crystallography of the enzyme bound to its substrate or substrate analog provides one approach. Irreversible inhibitors that covalently bond to the enzyme provide an alternative and often complementary approach: the inhibitors modify the functional groups, which can then be identified. Irreversible inhibitors can be divided into three categories: group-specific reagents, affinity labels, and suicide inhibitors.

- **Group-specific reagents** inhibit enzymes by reacting with specific side chains of the enzyme's amino acids. An example of a group-specific reagent is diisopropylphosphofluoridate (DIPF). DIPF modifies only 1 of the 28 serine residues in the proteolytic enzyme chymotrypsin and yet inhibits the enzyme, implying that this serine residue is especially reactive. We will see in Chapter 6 that this serine residue is indeed located at the active site. DIPF also revealed a reactive serine residue in acetylcholinesterase, an enzyme important in the transmission of nerve impulses (**Figure 5.26**). Thus, DIPF and similar compounds that bind and inactivate acetylcholinesterase are potent nerve gases. Most group-specific reagents do not display the exquisite specificity shown by DIPF. Consequently, more specific means of modifying the active site are required.

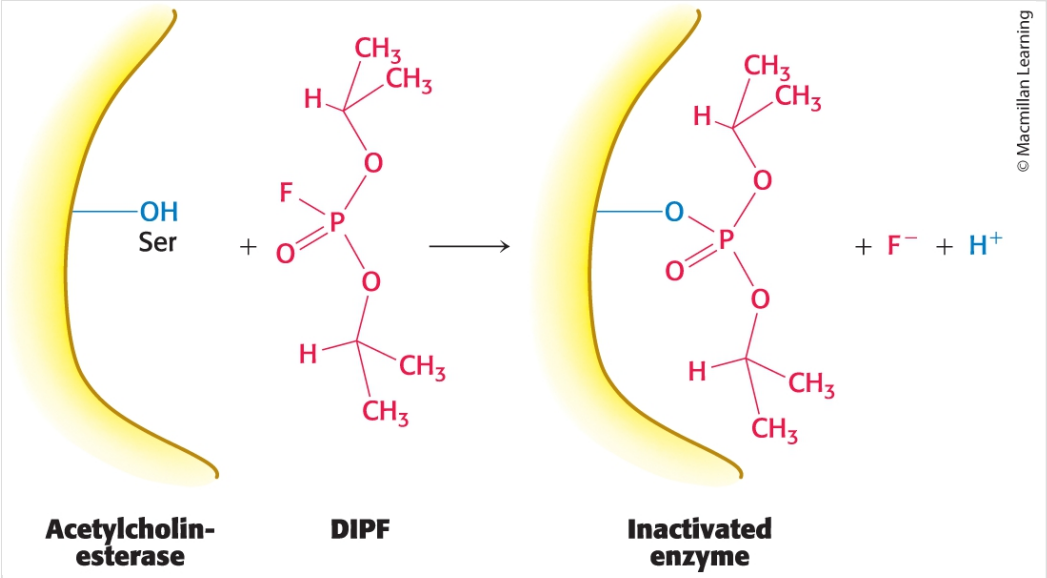


FIGURE 5.26 A group-specific reagent irreversibly inhibits enzymes by reacting with specific side chains. For example, DIPF can inhibit an enzyme by covalently modifying a crucial serine residue.

- **Affinity labels (reactive substrate analogs)** inhibit enzymes by being structurally similar to the substrate and covalently bonding to active-site residues. They are thus more specific for the enzyme's active site than are group-specific reagents. Tosyl-L-phenylalanine chloromethyl ketone (TPCK) is a substrate analog for chymotrypsin (Figure 5.27). TPCK binds at the active site and then reacts irreversibly with a histidine residue at that site, inhibiting the enzyme.

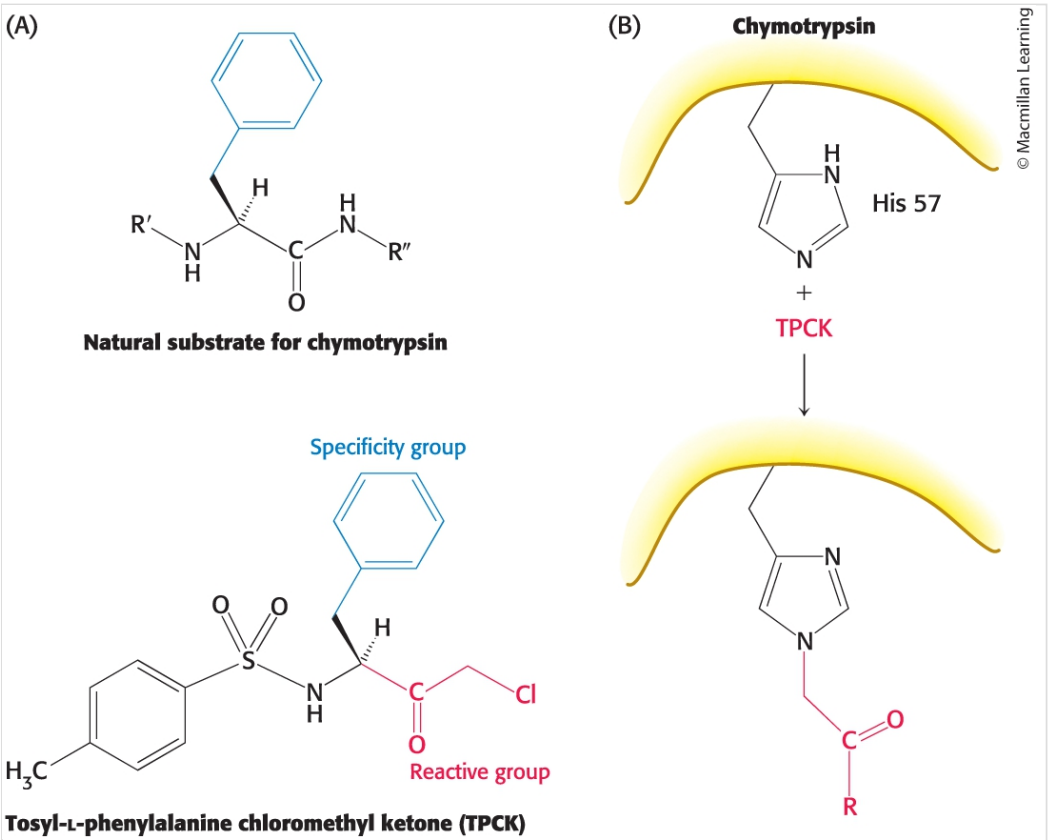


FIGURE 5.27 Affinity labels are highly specific irreversible inhibitors because they are structurally similar to the enzyme substrate. (A) Tosyl-L-phenylalanine chloromethyl ketone (TPCK) is a reactive analog of the normal substrate for the enzyme chymotrypsin. (B) TPCK binds at the active site of chymotrypsin and modifies an essential histidine residue.

- **Mechanism-based (suicide) inhibitors** are modified substrates that provide the most specific means for modifying an enzyme's 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 vital for catalysis.

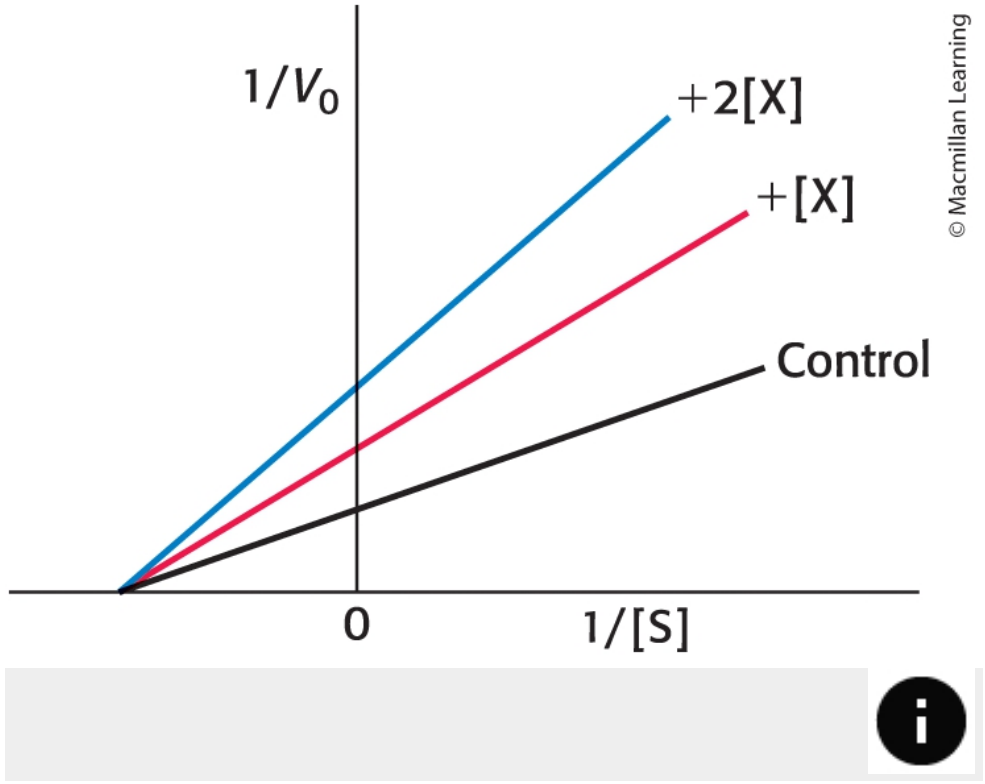
How would a molecule like TPCK — or other types of irreversible inhibitors, for that matter — affect the kinetic parameters V_{max} and K_M that we have discussed throughout this chapter? We will address that question in the context of all the inhibitors discussed in this chapter in the example below.

Example

Determining Inhibitor Type from Data

Problem:

The graph shows the effect of two different concentrations of a molecule X on an enzyme. What kind of molecule is X, and how is it affecting enzyme activity?



Getting Started:

What kind of plot is this? The first step is to examine the axes: the *x*-axis shows 1/[S], while the *y*-axis displays $1/V_0$. Note that these are two reciprocal values, so this is a double reciprocal plot, specifically a Lineweaver–Burk plot. Next, consider what we know about these plots: they can reveal how molecules affect the apparent values of the kinetic parameters K_M and V_{max} based on where the line crosses the two axes. Recall that the point where the line crosses the *y*-axis is $1/V_{max}$, while the intersection with the *x*-axis is $-1/K_M$.

Analyze:

How is the molecule affecting the enzyme? If we first consider the *x*-axis, we see that all of the lines converge there. Therefore, the apparent K_M is not altered in the presence of X. However, when X is present, the lines cross the *y*-axis at increasing values, indicating that $1/V_{max}$ increases with increasing amounts of X. If $1/V_{max}$ increases in the presence of X, then the apparent V_{max} must be decreasing.

Let's summarize these effects and then consider the possibilities. The apparent K_M is unchanged, but the apparent V_{max} is reduced. This latter observation means that X must be some type of an inhibitor of the enzyme. Let's consider some possibilities: competitive inhibitors, including transition-state analogs, alter the apparent K_M but don't affect V_{max}. Uncompetitive inhibitors decrease both the apparent values of K_M and V_{max}. Finally, mixed noncompetitive inhibitors may raise or lower the apparent value of K_M but do not leave it unchanged. None of these descriptions fit the data. The only type of reversible inhibitor that affects an enzyme this way is a pure noncompetitive inhibitor. Recall that pure noncompetitive inhibitors lower the apparent V_{max} without altering K_M.

However, this is not the end of the problem! One more possibility exists that we have not considered: irreversible inhibitors. Because irreversible inhibitors permanently inactivate enzyme molecules, essentially removing a stoichiometric amount of them from pool of active enzyme molecules involved in the observed reaction, the effect is the same as if there were less enzyme originally added to the reaction mixture. That is, the apparent V_{max} is lowered because there are fewer active enzyme molecules, but the K_M is unaffected because those excess enzyme molecules which do not react with the inhibitor are completely unaffected by it.

Reflect:

All irreversible inhibitors give rise to the same pattern as a pure noncompetitive inhibitor with respect to the observed changes to the apparent values of K_M and V_{max}; so, in this case, additional information is required to distinguish whether molecule X is an irreversible or a pure noncompetitive inhibitor. The effects of various types of inhibitors are summarized in Table 5.9.

Table 5.9 Summary of effects of enzyme inhibitors on kinetic parameters

Type of inhibition	K_M	V_{max}
Competitive	Increased	Unchanged
Uncompetitive	Decreased	Decreased
Pure noncompetitive	Unchanged	Decreased
Mixed noncompetitive	Increased or decreased	Decreased
Irreversible	Unchanged	Decreased

Penicillin irreversibly inactivates a key enzyme in bacterial cell-wall synthesis

Penicillin, the first antibiotic ever discovered, is a clinically useful suicide inhibitor. Penicillin consists of a thiazolidine ring fused to a β-*lactam* ring to which a variable R group is attached by a peptide bond (Figure 5.28A). In benzylpenicillin, for example, R is a benzyl group (Figure 5.28B). The β-lactam ring is very labile due to significant strain in the four-membered ring; this instability is closely tied to the antibiotic action of penicillin.

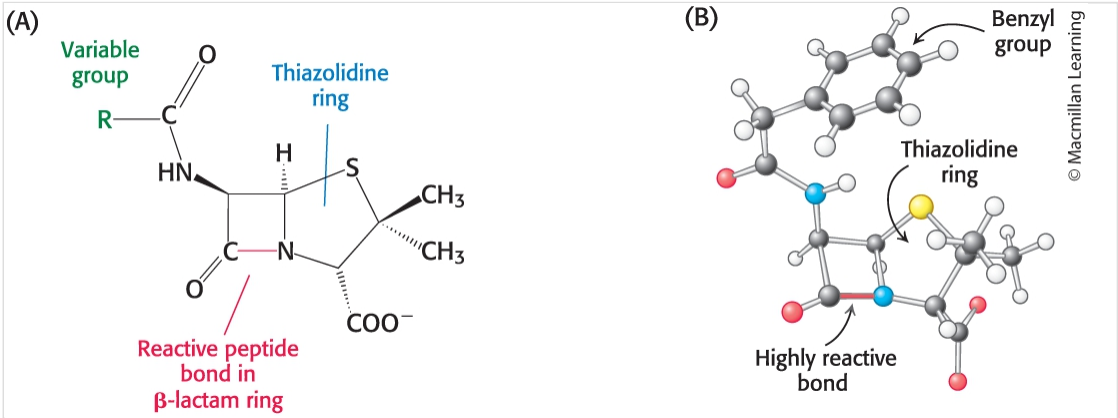


FIGURE 5.28 The reactive site of penicillin is the peptide bond of its β-lactam ring. (A) Structural formula of penicillin. (B) Representation of benzylpenicillin.

How does penicillin inhibit bacterial growth? Let us consider *Staphylococcus aureus*, the most common cause of staph infections. Like the vast majority of all bacteria, the cell wall of *S. aureus* is made up of a macromolecule, called *peptidoglycan* (Figure 5.29), which consists of linear polysaccharide chains that are cross-linked by short peptides. The enormous peptidoglycan molecule provides a continuous, covalently linked mesh that confers mechanical support and prevents bacteria from bursting in response to their high internal osmotic pressure. Glycopeptide transpeptidase catalyzes the formation of the cross-links that make the peptidoglycan so stable. Note that bacterial

cell walls are distinctive in containing D amino acids, which form cross-links by a mechanism different from that used to synthesize proteins.

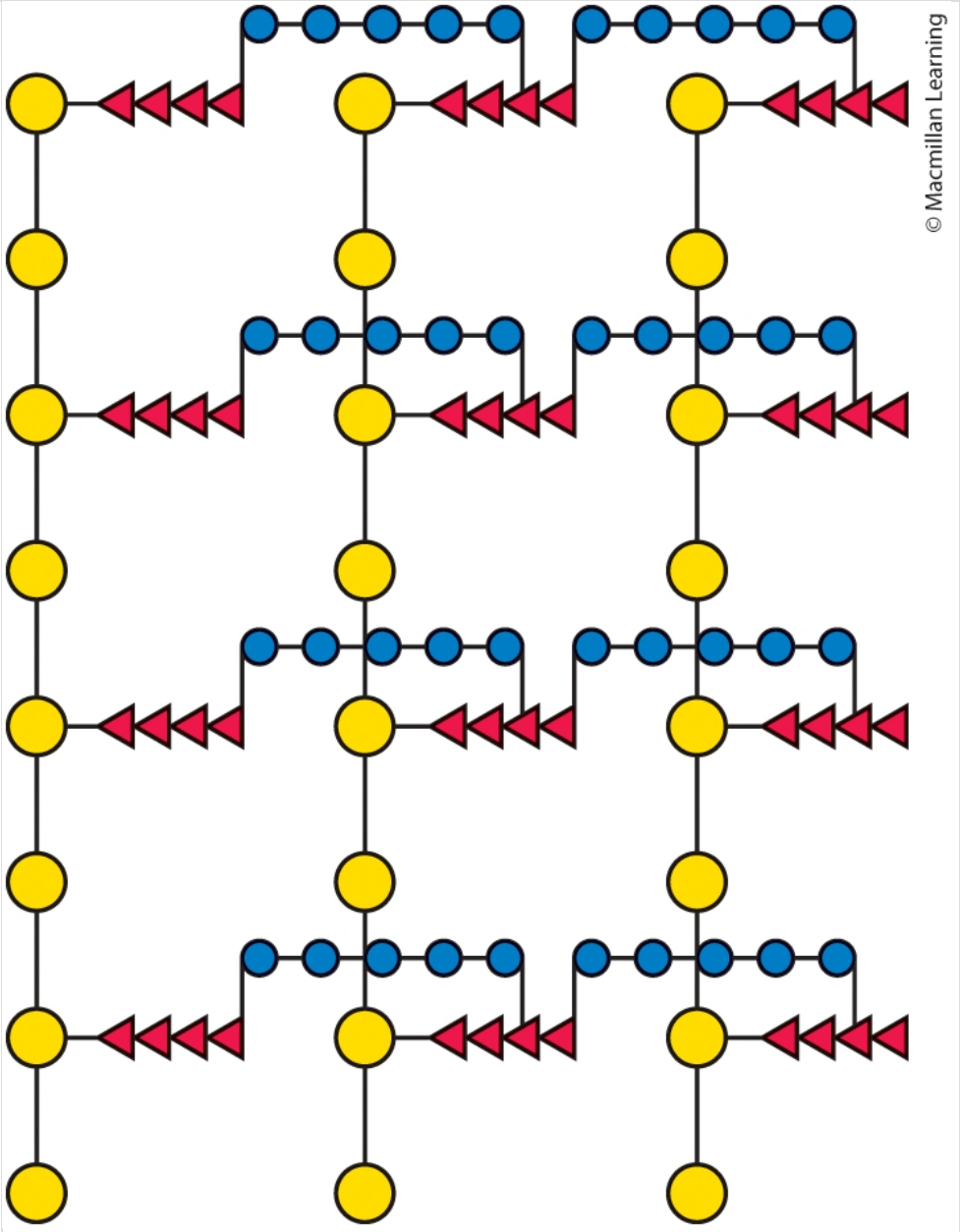


FIGURE 5.29 A schematic representation of a peptidoglycan section in *S. aureus* shows the crosslinking between sugar fibers. The sugars are shown in yellow, the tetrapeptides in red, and the pentaglycine bridges in blue. Only a small section is represented; the cell wall is a single, enormous, bag-shaped macromolecule because of extensive crosslinking.

Penicillin is an effective antibiotic because it interferes with the synthesis of the bacterial cell wall by irreversibly inhibiting the cross-linking transpeptidase. The transpeptidase normally forms an acyl intermediate with the penultimate D-alanine residue of the D-Ala-D-Ala peptide, which then reacts with the amino group of the terminal glycine in another peptide to form the cross-link. But penicillin can be accepted into the active site of the transpeptidase instead, because it mimics the D-Ala-D-Ala moiety of the normal substrate (Figure 5.30). Bound penicillin then forms a covalent bond with an active-site serine, permanently inactivating the enzyme and preventing the completion of cell-wall synthesis.

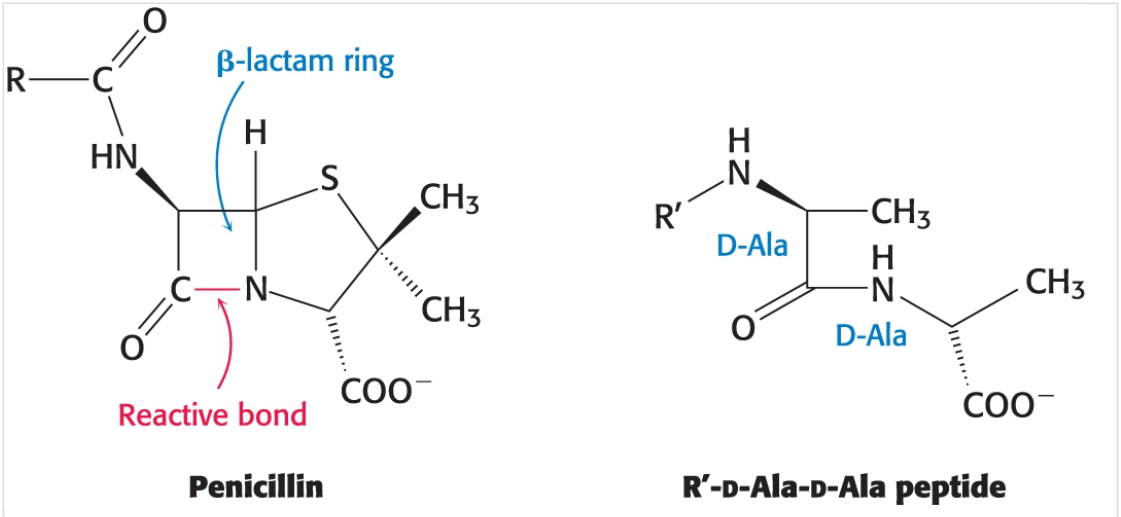


FIGURE 5.30 Penicillin resembles the transition state of the transpeptidase reaction. In the vicinity of its reactive peptide bond, the conformation of penicillin resembles the postulated conformation of the transition state of R'-D-Ala-D-Ala in the transpeptidation reaction.

[Information from B. Lee, *J. Mol. Biol.* 61:463–469, 1971.]

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 5.31). Because the peptidase participates in its own inactivation, penicillin acts as a suicide inhibitor.

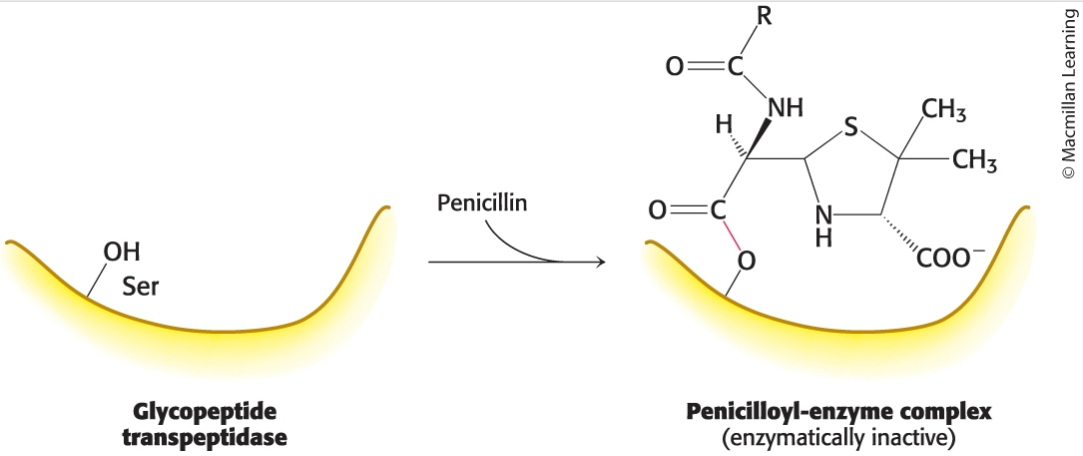


FIGURE 5.31 The formation of a penicilloyl-enzyme derivative is irreversible. Penicillin reacts irreversibly with the transpeptidase to inactivate the enzyme.

Chapter 5 Summary

5.1 Enzymes Are Powerful and Highly Specific Catalysts

- Enzymes are biological catalysts, most of which are proteins.
- Enzymes are highly specific and can enhance reaction rates by factors as great as 10^{17}.
- Many enzymes require cofactors for activity which can be metal ions or small, vitamin-derived organic molecules called coenzymes.

5.2 Gibbs Free Energy Is a Useful Thermodynamic Function for Understanding Enzymes

- A reaction can take place spontaneously only if the change in free energy (ΔG) is negative.
- The free-energy change of a reaction under standard conditions is called the standard free-energy change (ΔG°) while biochemists use $\Delta G^{\circ\prime}$, the standard free-energy change at pH 7.
- Enzymes do not alter reaction equilibria; rather, they increase the rate at which equilibrium is attained.

5.3 Enzymes Accelerate Reactions by Facilitating the Formation of the Transition State

- Enzymes decrease the free energy of activation of a chemical reaction 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 result is that the reaction is accelerated in both directions.
- The first step in catalysis is the formation of an enzyme–substrate complex in which substrates are bound at active-site

clefts from which water is largely excluded.
- The specificity of enzyme–substrate interactions arises mainly from weak reversible contacts mediated by ionic interactions, hydrogen bonds, and van der Waals forces, and from the shape of the active site, which rejects molecules that do not have a sufficiently complementary shape and charge.
- Enzymes facilitate formation of the transition state by a dynamic process in which the substrate binds to specific conformations of the enzyme, accompanied by conformational changes at active sites that result in catalysis.

5.4 The Michaelis–Menten Model Accounts for the Kinetic Properties of Many Enzymes

- The kinetic properties of many enzymes are described by the Michaelis–Menten model in which 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.

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

- The rate of formation of product V_0 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 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. The ratio of k_{cat}/K_M provides a measure of enzyme efficiency and specificity.
- Allosteric enzymes constitute an important class of enzymes whose catalytic activity can be regulated. These enzymes have multiple active sites which often display cooperativity, as evidenced by a sigmoidal dependence of reaction velocity on substrate concentration.

5.5 Enzymes Can Be Studied One Molecule at a Time

- Single-molecule methods reaffirm the key findings from ensemble studies but also reveal a distribution of enzyme characteristics rather than an average value.

5.6 Enzymes Can Be Inhibited by Specific Molecules

- Irreversible inhibitors bind covalently to enzymes and can provide a means of mapping the enzyme's active site, while reversible inhibition is characterized by a more rapid and less stable interaction between enzyme and inhibitor.
- Competitive inhibitors prevent the substrate from binding to the active site and thereby reduce the reaction velocity by diminishing the proportion of enzyme molecules that are bound to substrate. Competitive inhibition can be overcome by raising the substrate concentration.
- Uncompetitive inhibitors bind only to the enzyme–substrate complex, decreasing both the turnover number and the apparent K_M.
- Pure noncompetitive inhibitors decrease only the turnover number, while mixed noncompetitive inhibitors can have a

variety of effects.
- Transition-state analogs are stable compounds that mimic key features of the transition state and are potent and specific competitive inhibitors of enzymes.

Key Terms

catalyst
enzyme
ribozyme
substrate
protease
cofactor
apoenzyme
holoenzyme
coenzyme
prosthetic group
transition state
Gibbs free energy of activation (activation energy)
active site
induced fit
binding energy
K_M (Michaelis constant)
V_{max} (maximal rate)
Michaelis–Menten equation
Lineweaver–Burk plot
elasticity
turnover number
k_{cat}/K_M (specificity constant)
sequential reaction
double-displacement (Ping-Pong) reaction
allosteric enzyme

competitive inhibition
uncompetitive inhibition
pure noncompetitive inhibition
mixed noncompetitive inhibition
transition-state analog
group-specific reagent
affinity label (reactive substrate analog)
mechanism-based (suicide) inhibitor

Problems

1. What does an apoenzyme require to become a holoenzyme?

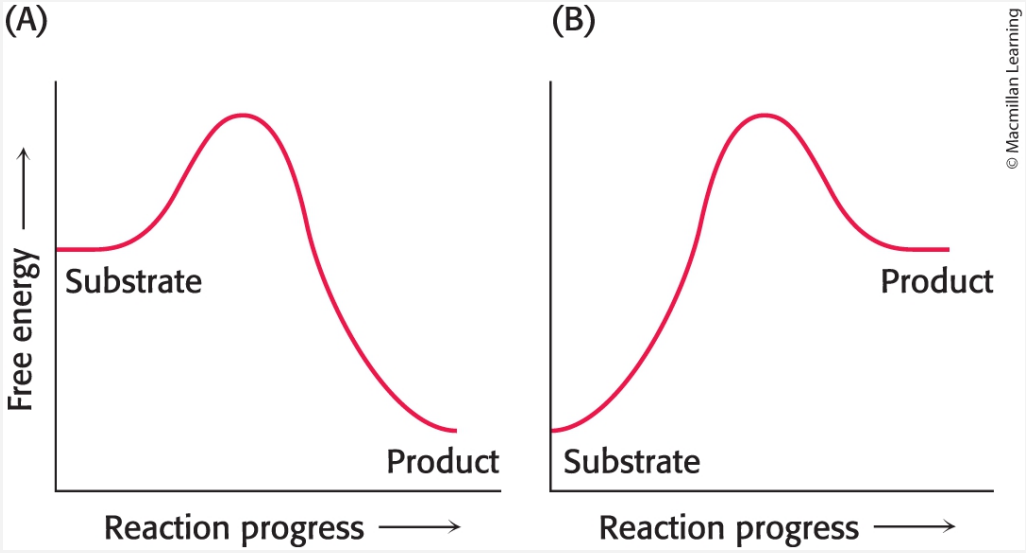
2. Consider the reaction: $S \rightleftharpoons P$. Which of the following effects are produced by an enzyme on the general reaction? 1, 2

a. The reaction equilibrium is shifted away from the products.
b. The concentration of the products is increased.
c. ΔG for the reaction increases.
d. The activation energy for the reaction is lowered.
e. The formation of the transition state is promoted.
f. The rate constant for the forward reaction increases.

3. Why does the activation energy of a reaction not appear in the final ΔG of the reaction? 1

4. The illustrations below show the reaction-progress curves for two different reactions. Indicate the activation energy as well as

the ΔG for each reaction. Which reaction is endergonic? Exergonic? 1



5. Suppose that, in the absence of enzyme, the forward rate constant (k_F) for the conversion of S into P is $10^{-4}\ s^{-1}$ and the reverse rate constant (k_R) for the conversion of P into S is $10^{-6}\ s^{-1}$. 1, 2

$$S \underset{10^{-6}\ s^{-1}}{\overset{10^{-4}\ s^{-1}}{\rightleftharpoons}} P$$

a. What is the equilibrium for the reaction? What is the $\Delta G^{\circ\prime}$?

b. Suppose an enzyme enhances the rate of the reaction 100-fold. What are the rate constants for the enzyme-catalyzed reaction? What is the equilibrium constant? The $\Delta G^{\circ\prime}$?

6. What would be the result of an enzyme having a greater binding energy for the substrate than for the transition state?

2

7. Match the K'_{eq} values with the appropriate $\Delta G^{\circ\prime}$ values.

1

K'_{eq}	$\Delta G^{\circ\prime}$ (kJ mol^{-1})
1. 1	28.53
2. 10^{-5}	-11.42
3. 10^{4}	5.69
4. 10^{2}	0

-22.84

5. 10^{-1}

---

8. Assume that you have a solution of 0.1 M glucose 6-phosphate (G 6-P). To this solution, you add the enzyme phosphoglucomutase, which catalyzes the following reaction:

$$\text{G 6-P} \underset{}{\overset{\text{Phosphoglucomutase}}{\rightleftharpoons}} \text{G 1-P}$$

The $\Delta G^{\circ\prime}$ for the reaction is $+7.5\ \text{kJ mol}^{-1}\ (+1.8\ \text{kcal mol}^{-1})$. 1

a. Does the reaction proceed as written? If so, what are the final concentrations of G 6-P and G 1-P?
b. Under what cellular conditions could you produce G 1-P at a high rate?

9. Consider the following reaction:

$$\text{G 6-P} \underset{}{\overset{\text{Phosphoglucomutase}}{\rightleftharpoons}} \text{G 1-P}$$

After reactant and product were mixed and allowed to reach equilibrium at 25°C, the concentration of each compound was measured:

$$[\text{Glucose 1-phosphate}]_{eq} = 0.01\ \text{M}$$
$$[\text{Glucose 6-phosphate}]_{eq} = 0.19\ \text{M}$$

Calculate K_{eq} and $\Delta G^{\circ\prime}$. 1

10. The affinity between a protein and a molecule that binds to the protein is frequently expressed in terms of a dissociation constant K_d. 3

$$\text{Protein} + \text{small molecule} \rightleftharpoons \text{Protein} - \text{small molecule complex}$$

$$K_d = \frac{[\text{protein}][\text{small molecule}]}{[\text{protein} - \text{small molecule complex}]}$$

Does K_M measure the affinity of the enzyme complex? Under what circumstances might K_M approximately equal K_d?

11. Match the term with the description or compound. 3, 4

1. Competitive inhibition _____
2. Uncompetitive inhibition _____
3. Noncompetitive inhibition _____

1. Inhibitor and substrate can bind simultaneously
2. V_{max} remains the same but the K_M^{app} increases
3. Sulfanilamide
4. Binds to the enzyme–substrate complex only
5. Lowers V_{max} and K_M^{app}

6. Roundup

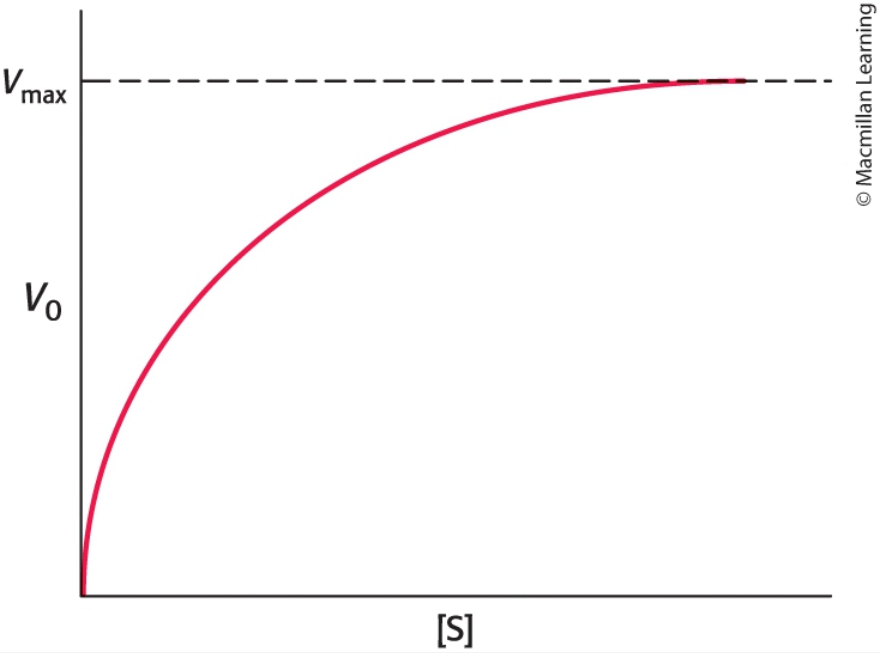
7. K_M remains unchanged but V_{max} is lower

8. Doxycycline

9. Prevents S from binding to the active site

---

12. Many biochemists go bananas, and justifiably, when they see a Michaelis–Menten plot like the one shown below. To see why, determine the V_0 as a fraction of V_{max} when the substrate concentration is equal to 10 K_M and 20 K_M. Please control your outrage. 3



13. 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 *E. coli* by a pyrophosphatase that has a mass of 120 kDa 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. 3

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 sites 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.

14. Penicillin is hydrolyzed and thereby rendered inactive by penicillinase (also known as β-lactamase), an enzyme present in some penicillin-resistant bacteria. The mass of this enzyme in *Staphylococcus aureus* is 29.6 kDa. 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. 3

[Penicillin] μM	Amount hydrolyzed (nmol)

1	0.11
3	0.25
5	0.34
10	0.45
30	0.58
50	0.61

a. Plot V_0 versus [S] and $1/V_0$ versus 1/[S] for these data. Does penicillinase appear to obey Michaelis–Menten kinetics? If so, what is the value of K_M?
b. What is the value of V_{max}?
c. What is the turnover number of penicillinase under these experimental conditions? Assume one active site per enzyme molecule.

15. The kinetics of an enzyme is measured as a function of substrate concentration in the presence and absence of $100\ \mu M$ inhibitor. 3, 4

	Velocity $(\mu mol\ minute^{-1})$	
[S] (μM)	**No inhibitor**	**Inhibitor**
3	10.4	2.1

5	14.5	2.9
10	22.5	4.5
30	33.8	6.8
90	40.5	8.1

a. What are the values of V_{max} and K_M in the presence of this inhibitor?
b. What type of inhibition is it?
c. What is the dissociation constant of this inhibitor?
d. If $[S] = 30\ \mu M$, what fraction of the enzyme molecules has a bound substrate in the presence and in the absence of $100\ \mu M$ inhibitor?

16. The plot of $1/V_0$ versus 1/[S] is sometimes called a Lineweaver–Burk plot. Another way of expressing the kinetic data is to plot V_0 versus $V_0/[S]$, which is known as an Eadie–Hofstee plot. 3, 4

a. Rearrange the Michaelis–Menten equation to give V_0 as a function of $V_0/[S]$.
b. What is the significance of the slope, the *y*-intercept, and the *x*-intercept in a plot of V_0 versus $V_0/[S]$?
c. Sketch a plot of V_0 versus $V_0/[S]$ in the absence of an inhibitor, in the presence of a competitive inhibitor, and in the presence of a noncompetitive inhibitor.

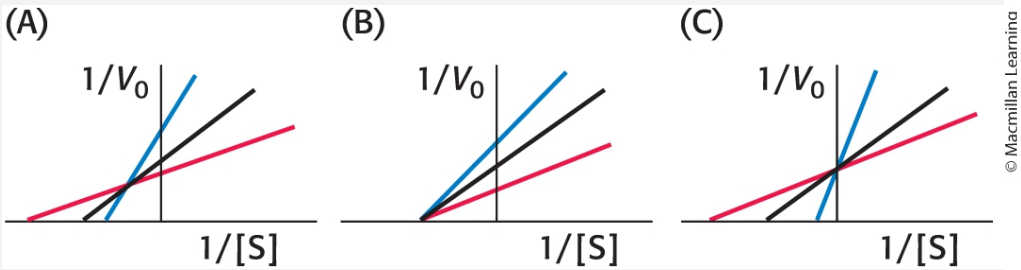
17. What is the defining characteristic for an enzyme catalyzing a sequential reaction? A double-displacement reaction?

18. You have isolated two versions of the same enzyme, a wild type and a mutant differing from the wild type at a single amino acid. Working carefully but expeditiously, you then establish the following kinetic characteristics of the enzymes. 1, 3

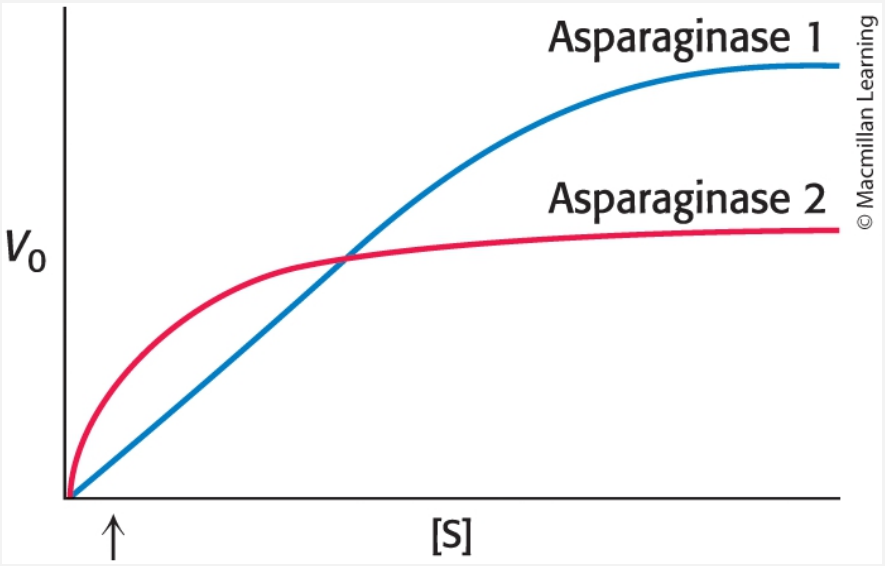
	Maximum velocity	K_M
Wild type	100 μmol/min	10 mM
Mutant	1 μmol/min	0.1 mM

a. With the assumption that the reaction occurs in two steps in which k_{-1} is much larger than k_2, which enzyme has the higher affinity for substrate?
b. What is the initial velocity of the reaction catalyzed by the wild-type enzyme when the substrate concentration is 10 mM?
c. Which enzyme alters the equilibrium more in the direction of product?

19. For a one-substrate, enzyme-catalyzed reaction, double-reciprocal plots were determined for three different enzyme concentrations. Which of the following three families of curve would you expect to be obtained? Explain. 3



20. The amino acid asparagine is required by cancer cells to proliferate. Treating patients with the enzyme asparaginase is sometimes used as a chemotherapy treatment. Asparaginase hydrolyzes asparagine to aspartate and ammonia. The below illustration shows the Michaelis–Menten curves for two asparaginases from different sources, as well as the concentration of asparagine in the environment (indicated by the arrow). Which enzyme would make a better chemotherapeutic agent? 3



21. Picture in your mind the velocity-versus-substrate concentration curve for a typical Michaelis–Menten enzyme.

Now, imagine that the experimental conditions are altered as described below. For each of the conditions described, fill in the table indicating precisely (when possible) the effect on V_{max} and K_M of the imagined Michaelis–Menten enzyme. 3, 4

Experimental condition	V_{max}	K_M
1. Twice as much enzyme is used.		
2. Half as much enzyme is used.		
3. A competitive inhibitor is present.		
4. An uncompetitive inhibitor is present.		
5. A pure noncompetitive inhibitor is present.		

22. 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 and the enzymes have approximately the same V_{max}, which step will be rate limiting and why? 3

$$A \underset{E_A}{\rightleftharpoons} B \underset{E_B}{\rightleftharpoons} C \underset{E_C}{\rightleftharpoons} D$$

$K_M =$ 10^{-2} M 10^{-4} M 10^{-6} M

23. Proteins are thermodynamically unstable. The ΔG of the hydrolysis of proteins is quite negative, yet proteins can be quite

stable. Explain this apparent paradox. What does it tell you about protein synthesis? 1

24. Transition-state analogs, which can be used as enzyme inhibitors and to generate catalytic antibodies, are often difficult to synthesize. Suggest a reason. 2

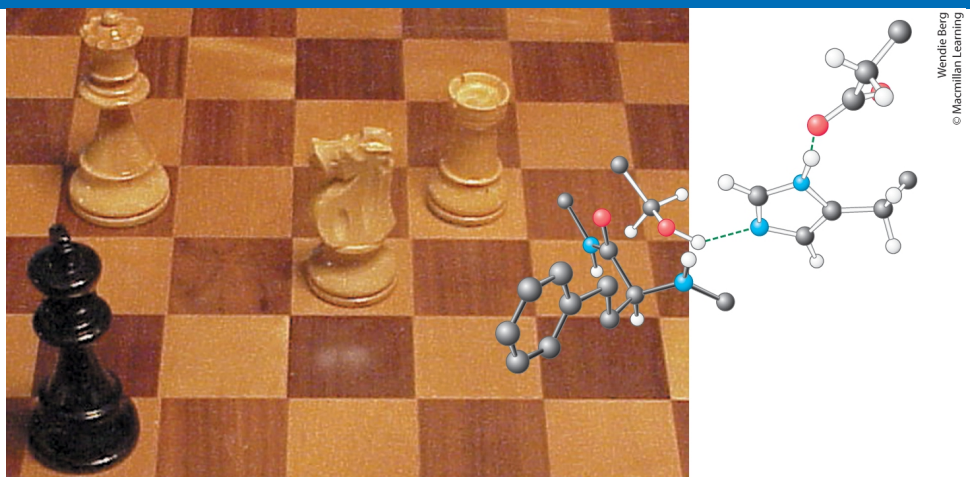
25. What is the biochemical advantage of having a K_M approximately equal to the substrate concentration normally available to an enzyme? 3

26. Succinylcholine is a fast-acting, short-duration muscle relaxant that is used when a tube is inserted into a patient's trachea or when a bronchoscope is used to examine the trachea and bronchi for signs of cancer. Within seconds of the administration of succinylcholine, the patient experiences muscle paralysis and is placed on a respirator while the examination proceeds. Succinylcholine is a competitive inhibitor of acetylcholinesterase, a nervous system enzyme, and this inhibition causes paralysis. However, succinylcholine is hydrolyzed by blood-serum cholinesterase, which shows a broader substrate specificity than does the nervous system enzyme. Paralysis lasts until the succinylcholine is hydrolyzed by the serum cholinesterase, usually several minutes later. 3

a. As a safety measure, serum cholinesterase is measured before the examination takes place. Explain why this measurement is good idea.
b. What would happen to the patient if the serum cholinesterase activity were only 10 units of activity per liter rather than the normal activity of about 80 units?
c. Some patients have a mutant form of the serum cholinesterase that displays a K_M of 10 mM, rather than

the normal 1.4 mM. What will be the effect of this mutation on the patient?

Chapter 6
Enzyme Catalytic Strategies



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, represent a particular chemical strategy for catalyzing a reaction that is normally very slow. This strategy relies on the properties of the different amino acids, much as chess strategy relies on different rules for moving each type of piece.

Outline

Learning Goals

By the end of this chapter, you should be able to:

1. Discuss four general strategies used by enzymes to accelerate particular reactions.
2. Give examples of specific chemical features of enzyme active sites that facilitate increasing the rates of specific reactions.
3. Give an example of when high absolute rate acceleration is physiologically important and how an enzyme achieves this acceleration.
4. Understand when high specificity is important for an enzyme and how this specificity is achieved.
5. Describe an example of when large conformational changes that occur during an enzymatic reaction cycle are used to drive other processes.
6. Discuss some of the experimental approaches biochemists use to elucidate enzymatic mechanisms.

What are the sources of the catalytic power and specificity of enzymes? This chapter presents the catalytic strategies used by four classes of enzymes, each of which catalyze reactions that involve the addition of water to a substrate. Scientists have revealed the mechanisms of these enzymes using incisive experimental probes, including protein structure determination, site-directed mutagenesis, detailed kinetic studies, and the use of isotope labels.

Each of the four classes of enzymes addresses a different challenge. Serine proteases promote a reaction that is almost immeasurably slow at neutral pH in the absence of a catalyst; carbonic anhydrases

achieve a high absolute rate of reaction, suitable for integration with other rapid physiological processes; and restriction enzymes attain a high degree of specificity. Finally, molecular motor proteins such as myosin harness the free energy associated with the hydrolysis of ATP to drive other processes.

6.1 Enzymes Use a Core Set of Catalytic Strategies

In Chapter 5, 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. The use of this binding energy is the first common strategy used by enzymes. This binding energy serves two purposes: it establishes substrate specificity and increases catalytic efficiency. Often only the correct substrate can participate in most or all of the interactions with the enzyme and thus optimize binding energy, accounting for the exquisite substrate specificity exhibited by many enzymes.

Furthermore, the full complement of such interactions is formed only when the combination of enzyme and substrate is in the transition state. Thus, interactions between the enzyme and the substrate stabilize the transition state, thereby lowering the free energy of activation. 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**.

In addition to the first strategy involving binding energy, enzymes commonly employ one or more of the following four additional strategies to catalyze specific reactions:

1. *Covalent catalysis*. In **covalent catalysis**, the active site contains a reactive group that becomes temporarily covalently attached to a part of the substrate in the course of catalysis. The proteolytic enzyme chymotrypsin provides an excellent example of this strategy (Section 6.2).

2. *General acid–base catalysis*. In **general acid–base catalysis**, a molecule other than water plays the role of a proton donor or acceptor. These include histidine residues in chymotrypsin and carbonic anhydrase, an aspartate residue in EcoRV, and a phosphate group of the ATP substrate for myosin and kinesin.

3. *Catalysis by approximation*. Many reactions have two distinct substrates, including all four classes of hydrolases considered in detail in this chapter. In such cases, the reaction rate may be considerably enhanced by bringing the two substrates together along a single binding surface on an enzyme, a process called **catalysis by approximation**. For example, carbonic anhydrase binds carbon dioxide and water in adjacent sites to facilitate their reaction.

4. *Metal ion catalysis*. In **metal ion catalysis**, metal ions function catalytically in several ways. For instance, a metal ion may facilitate the formation of reactive species such as hydroxide ion by direct coordination. A zinc(II) ion serves this purpose in catalysis by carbonic anhydrase. Alternatively, a metal ion may serve to stabilize a negative charge on a reaction intermediate, a role played by magnesium(II) ion in EcoRV. Finally, a metal ion may serve as a bridge between enzyme and substrate, increasing the binding energy and holding the substrate in a conformation appropriate for catalysis. This strategy is used by myosin and kinesin and, indeed, by essentially all enzymes that use ATP as a substrate.

Although we shall not consider catalytic RNA molecules explicitly in this chapter, the principles described for protein enzymes also apply to these catalysts.

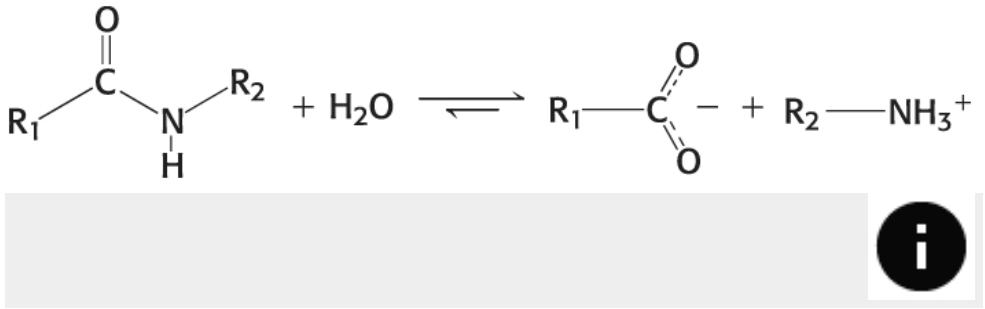
Self-Check Question

The enzyme adenylate kinase can convert two molecules of ADP to one molecule of AMP and one molecule of ATP. Suggest at least one catalytic strategy likely used by this enzyme.

6.2 Proteases Facilitate a Fundamentally Difficult Reaction

Peptide bond hydrolysis is an important process in living systems. Proteins that have served their purpose must be degraded so that their constituent amino acids can be recycled for the synthesis of new proteins; for example, 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 7, proteolytic reactions are important in regulating the activity of certain enzymes and other proteins.

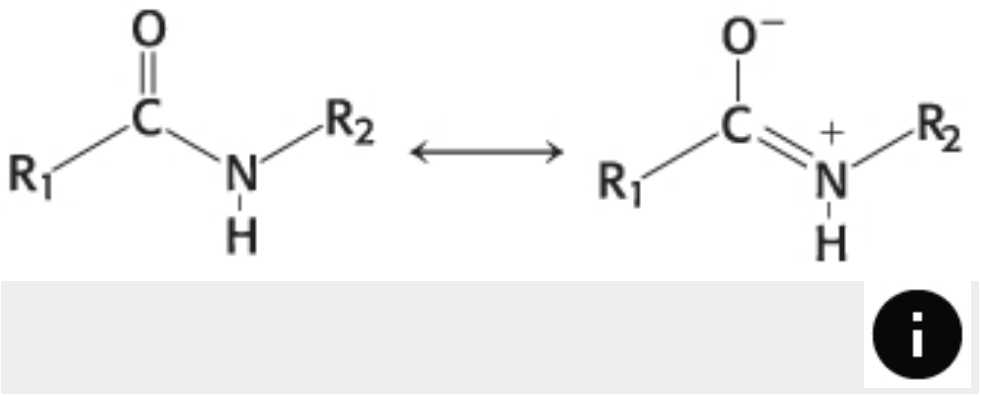
Proteases cleave proteins by a hydrolysis reaction — the addition of a molecule of water to a peptide bond:



Although the hydrolysis of peptide bonds is thermodynamically favorable, such 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 nature of peptide bonds is responsible for their kinetic stability. Specifically, the resonance structure that accounts for the

planarity of peptide bonds also makes them resistant to hydrolysis. This resonance structure endows them with partial double-bond character:



The carbon–nitrogen bond is strengthened by its double-bond character. More importantly, the carbonyl carbon atom is less **electrophilic** and less susceptible to **nucleophilic** attack than are the carbonyl carbon atoms in more reactive compounds such as carboxylate esters. Consequently, to promote peptide-bond cleavage, an enzyme must facilitate nucleophilic attack at a normally unreactive carbonyl group.

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 6.1**). Chymotrypsin is a good example of the use of covalent catalysis. The enzyme employs a powerful nucleophile to attack the unreactive carbonyl carbon atom of the substrate. This nucleophile becomes covalently attached to the substrate transiently in the course of catalysis.

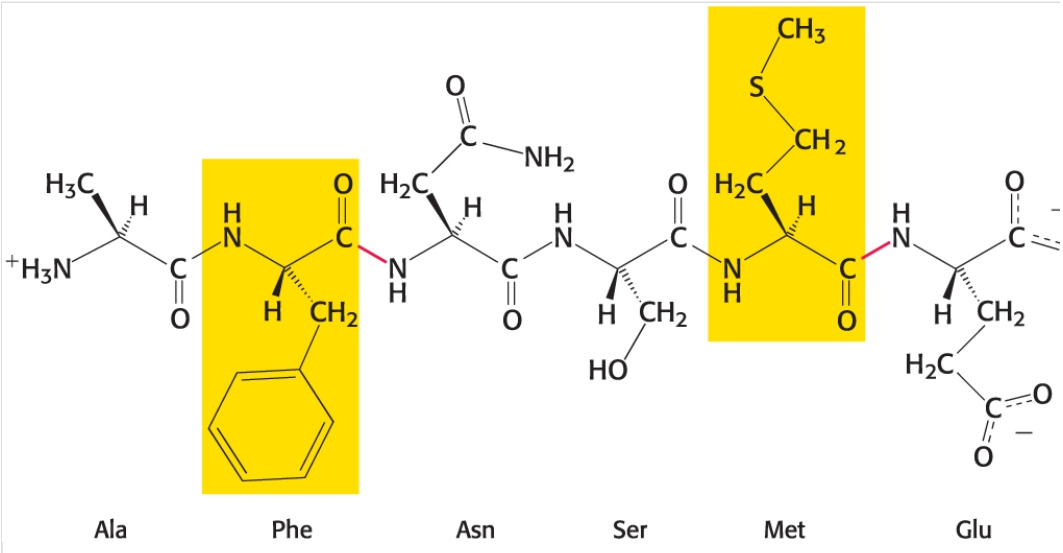


FIGURE 6.1 Chymotrypsin cleaves proteins specifically on the carboxyl side of aromatic or large hydrophobic amino acids. The targeted amino acids are highlighted, and the bonds cleaved by chymotrypsin are indicated in red.

What is the nucleophile that chymotrypsin employs to attack the substrate carbonyl carbon atom? A clue comes from the fact that chymotrypsin contains an extraordinarily reactive serine residue. Chymotrypsin molecules treated with organofluorophosphates such as diisopropylphosphofluoridate (DIPF) lose all activity irreversibly (Figure 6.2). Only a single residue, serine 195, is modified. This chemical modification reaction suggests that this unusually reactive serine residue plays a central role in the catalytic mechanism of chymotrypsin.

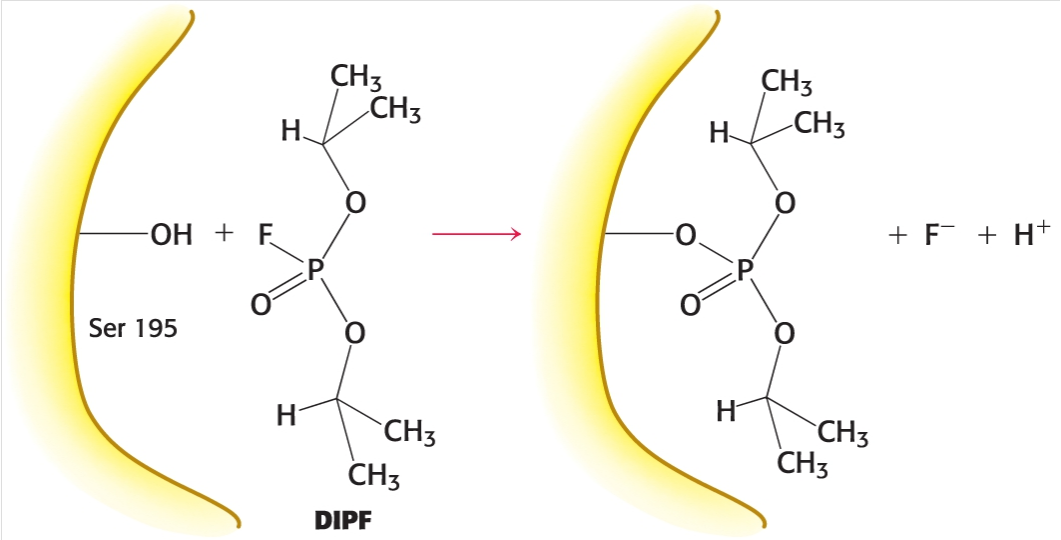
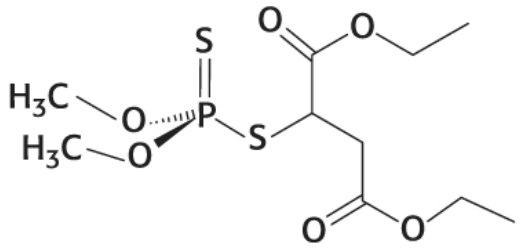
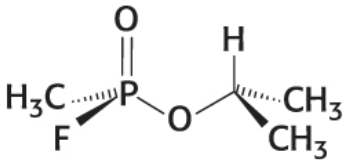


FIGURE 6.2 Chymotrypsin is inactivated by treatment with diisopropylphosphofluoridate (DIPF). DIPF reacts only with one serine among 28 possible serine residues, revealing the unusu reactivity of serine 195.

Phosphorus-based agents that modify reactive serine residues can be potent toxins. Compounds such as malathion, used to kill pest insects, and nerve agents such as sarin, developed as a chemical weapon, each modify a reactive serine residue in a key enzyme in the nervous system and disrupt nerve function.





Chymotrypsin action proceeds in two steps linked by a covalently bound intermediate

A study of the kinetics of chymotrypsin provides a second clue to its catalytic mechanism. Enzyme kinetics is often monitored by having the enzyme act on a substrate analog, called a **chromogenic substrate**, that forms a colored product. For chymotrypsin, one such substrate is *N*-acetyl-L-phenylalanine *p*-nitrophenyl ester. You might notice that this substrate is an ester rather than an amide, but many proteases will also hydrolyze esters. One of the products formed cleavage of this substrate by chymotrypsin is *p*-nitrophenolate, which has a yellow color (Figure 6.3). Measurements of the absorbance of light reveal the amount of *p*-nitrophenolate being produced.

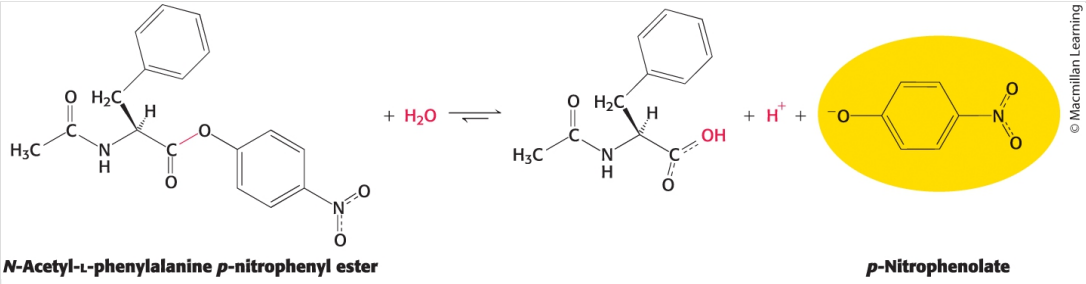


FIGURE 6.3 The action of chymotrypsin can be monitored by using a substrate that forms a colored product. The substrate *N*-Acetyl-L-phenylalanine *p*-nitrophenyl ester is cleaved to produce a yellow-colored product, *p*-nitrophenolate. *p*-Nitrophenolate forms by deprotonation of *p*-nitrophenol at pH 7.

Under steady-state conditions, the cleavage of this substrate obeys Michaelis–Menten kinetics with K_M of $20\ \mu M$ and k_{cat} of $77\ s^{-1}$. More insight into the mechanism can be gained by monitoring the initial phase of the reaction by using the stopped-flow method, which makes it possible to mix enzyme and substrate and monitor the results within a millisecond. This method reveals an initial rapid burst of colored product, followed by its slower formation as the reaction reached the steady state (Figure 6.4), confirming that hydrolysis proceeds in two phases. In the first reaction cycle that takes place on each enzyme molecule immediately after mixing, only the first phase must take place before the colored product is released. In subsequent reaction cycles, both phases must take place. Note that the burst is observed because the first phase is substantially more rapid than the second phase for this substrate.

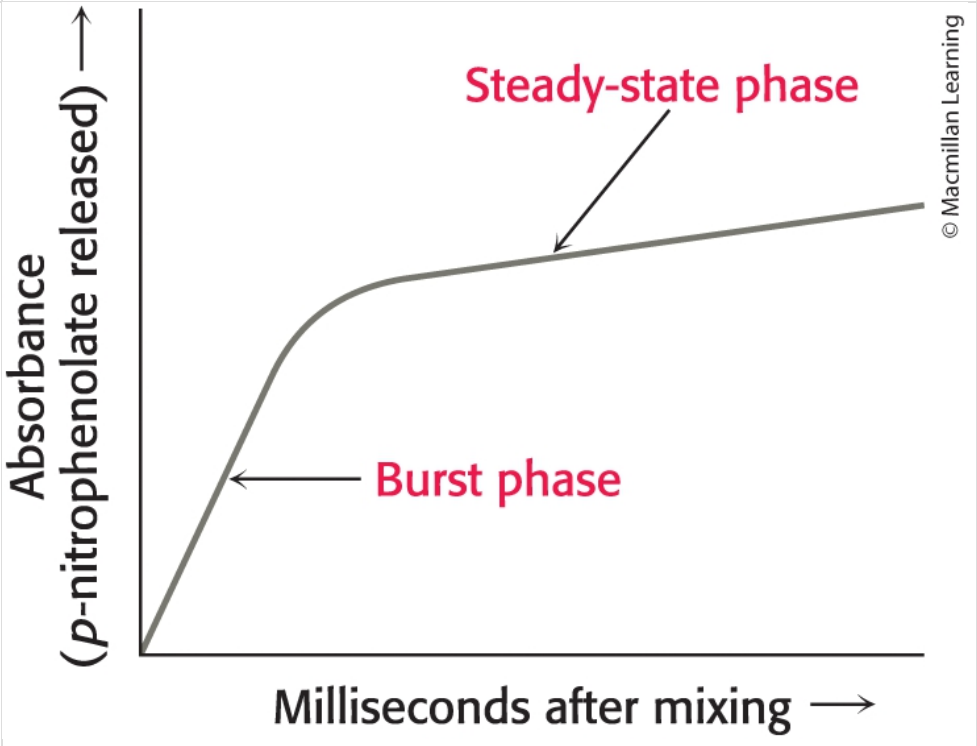


FIGURE 6.4 Two kinetic phases are evident by monitoring the initial action of chymotrypsin. When chymotrypsin is rapidly mixed with a solution of *N*-acetyl-L-phenylalanine *p*-nitrophenyl ester, a rapid burst phase of colored product is

observed followed by a steady-state phase. This observation provides insight into the chymotrypsin mechanism.

The two phases are explained by the formation of a covalent enzyme–substrate intermediate (Figure 6.5). First, the acyl group of the substrate becomes covalently attached to the enzyme as *p*-nitrophenolate (or an amine if the substrate is an amide rather than an ester) is released. The enzyme–acyl group complex is called the acyl-enzyme intermediate. Second, the acyl-enzyme intermediate is hydrolyzed to release the carboxylic acid component of the substrate and regenerate the free enzyme. Thus, one molecule of *p*-nitrophenolate is produced rapidly from each enzyme molecule as the acyl-enzyme intermediate is formed. However, it takes longer for the enzyme to be reset by the hydrolysis of the acyl-enzyme intermediate, and both phases are required for enzyme turnover.

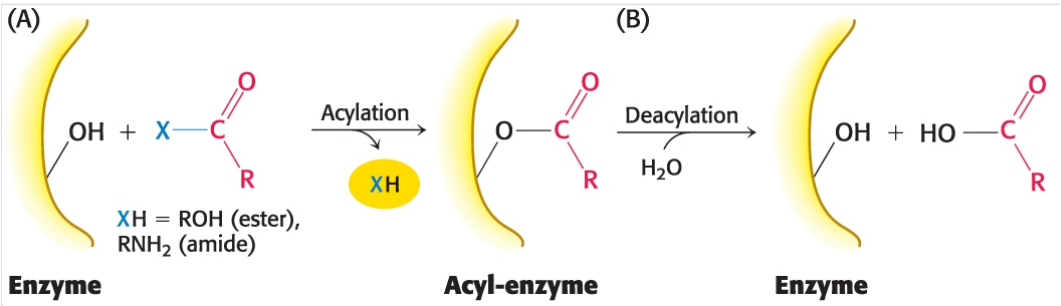


FIGURE 6.5 Hydrolysis by chymotrypsin involves covalent catalysis. In step (A), the enzyme is covalently modified to form an acyl-enzyme intermediate. In step (B), the enzyme is freed by deacylation.

Serine is part of a catalytic triad that also includes histidine and aspartate

The three-dimensional structure of chymotrypsin reveals that this enzyme is roughly spherical and comprises three polypeptide chains, linked by disulfide bonds (Figure 6.6). Note that the active site of chymotrypsin, marked by serine 195, lies in a cleft on the surface of the enzyme. The structure of the active site explains the special reactivity of serine 195 (Figure 6.7). 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**.

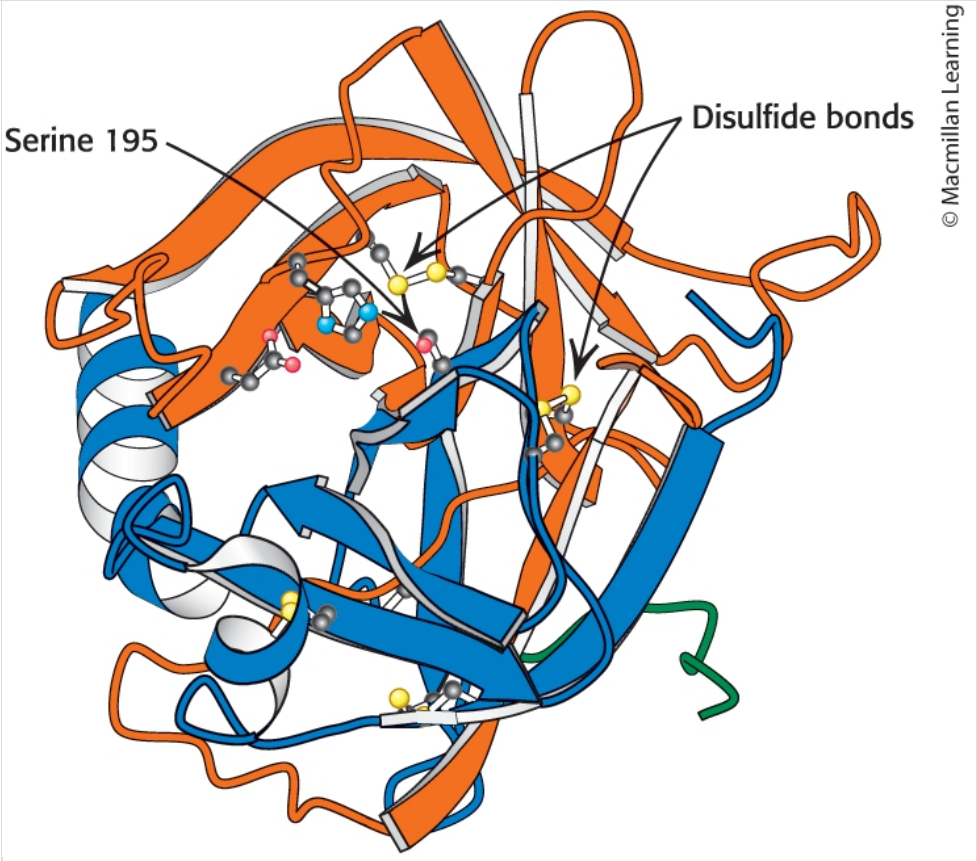


FIGURE 6.6 The active site serine 195 sits in a cleft on the surface of chymotrypsin. In the ribbon diagram shown here, chymotrypsin's three chains are indicated in

orange, blue, and green. Two intrastrand and two interstrand disulfide bonds are also shown.

[Drawn from 1GCT.pdb.]

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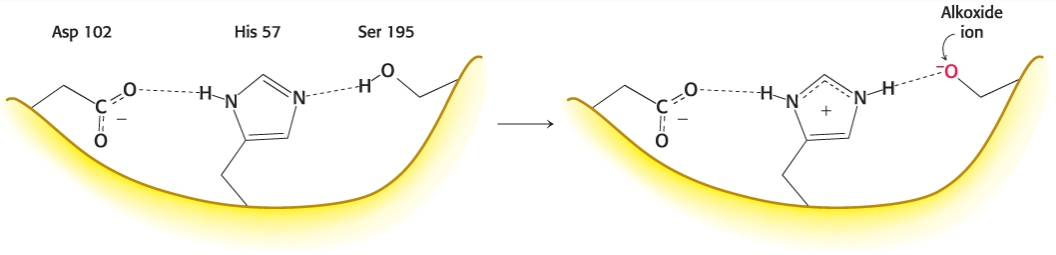

FIGURE 6.7 The catalytic triad increases the reactivity of serine 195. The catalytic triad poises this residue for formation of an alkoxide ion, a much stronger nucleophile than a hydroxyl grou

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 so that it is poised for deprotonation. In the presence of the substrate, the histidine residue accepts the proton from the serine 195 hydroxyl group. In doing so, the histidine acts as a general base catalyst. The withdrawal of the proton from the hydroxyl group generates an alkoxide ion, which is a much more powerful nucleophile than is an alcohol. The aspartate residue helps orient the histidine residue and make it a better proton acceptor through hydrogen bonding and electrostatic effects.

These observations suggest a mechanism for peptide hydrolysis (Figure 6.8). After substrate binding (step 1), the reaction begins with the oxygen atom of the side chain of serine 195 making a nucleophilic attack on the carbonyl carbon atom of the target peptide bond (step 2).

There are now four atoms bonded to the carbonyl carbon, arranged as a tetrahedron, instead of three atoms in a planar arrangement. This inherently unstable tetrahedral intermediate 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 termed the **oxyanion hole** (Figure 6.9). These interactions also help stabilize the transition state that precedes the formation of the tetrahedral intermediate. This tetrahedral intermediate collapses to generate the acyl-enzyme (step 3), facilitated by the transfer of the proton being held by 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), completing the first stage of the hydrolytic reaction — acylation of the enzyme. Such acyl-enzyme intermediates have been observed using x-ray crystallography by trapping them through adjustment of conditions such as the nature of the substrate, pH, or temperature.

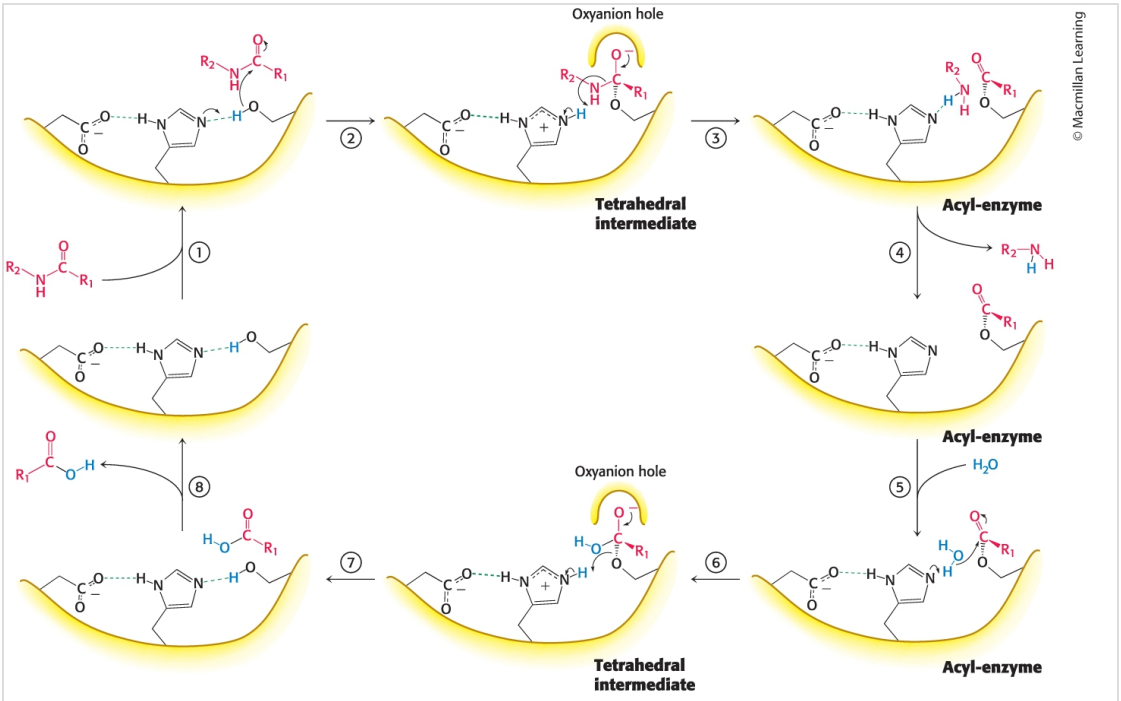

FIGURE 6.8 The mechanism of peptide hydrolysis illustrates the principles of covalent and acid–base catalysis. The mechanism proceeds in eight steps: (1) substrate binding, (2) nucleophilic attack of serine on the peptide carbonyl group, (3) collapse of the tetrahedral

intermediate, (4) release of the amine component, (5) water binding, (6) nucleophilic attack of water on the acyl-enzyme intermediate, (7) collapse of the tetrahedral intermediate, and (8) release of the carboxylic acid component. The dashed green lines represent hydrogen bonds.

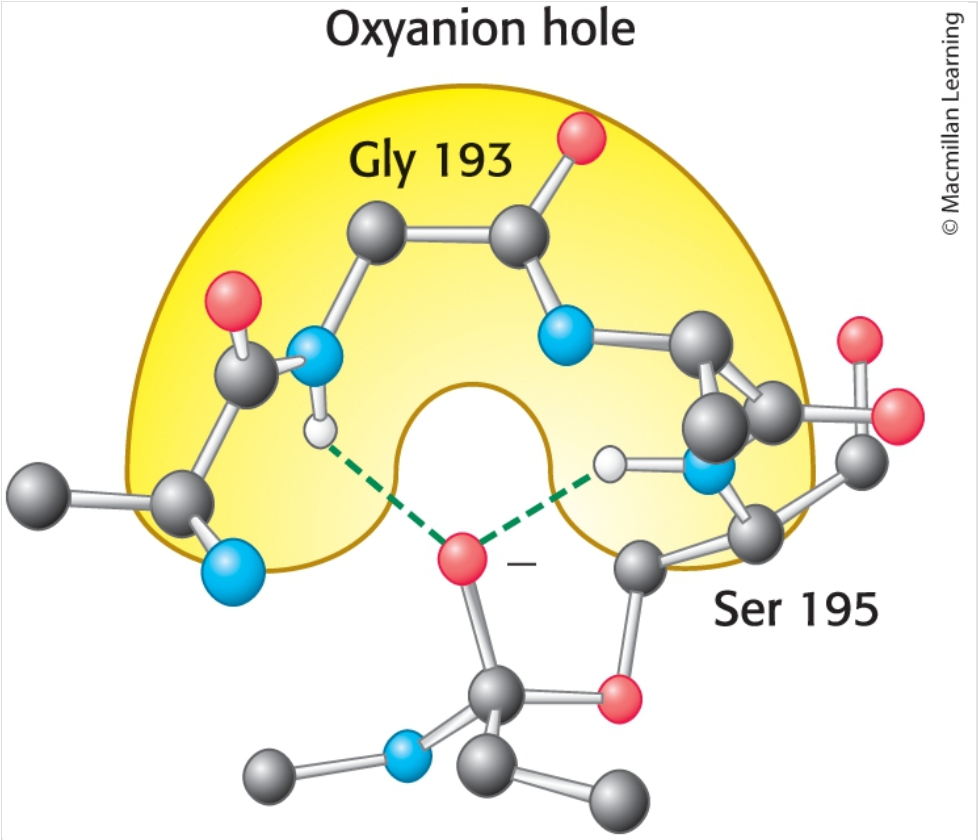

FIGURE 6.9 The oxyanion hole stabilizes the tetrahedral intermediates that occur during the chymotrypsin reaction. Note the hydrogen bonds (shown in green) that link peptide NH groups and the negatively charged oxygen atom of the intermediate.

The next stage — deacylation — begins when a water molecule takes the place occupied earlier by the amine component of the substrate (step 5).

The ester group of the acyl-enzyme is now hydrolyzed by a process that essentially repeats steps 2 through 4. Again acting as a general base catalyst, histidine 57 draws a proton away from the water molecule. The resulting OH^- ion attacks the carbonyl carbon atom of the acyl group, 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 reveals the presence of a deep 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 6.10**). The bond to be cleaved is called the **scissile bond**.

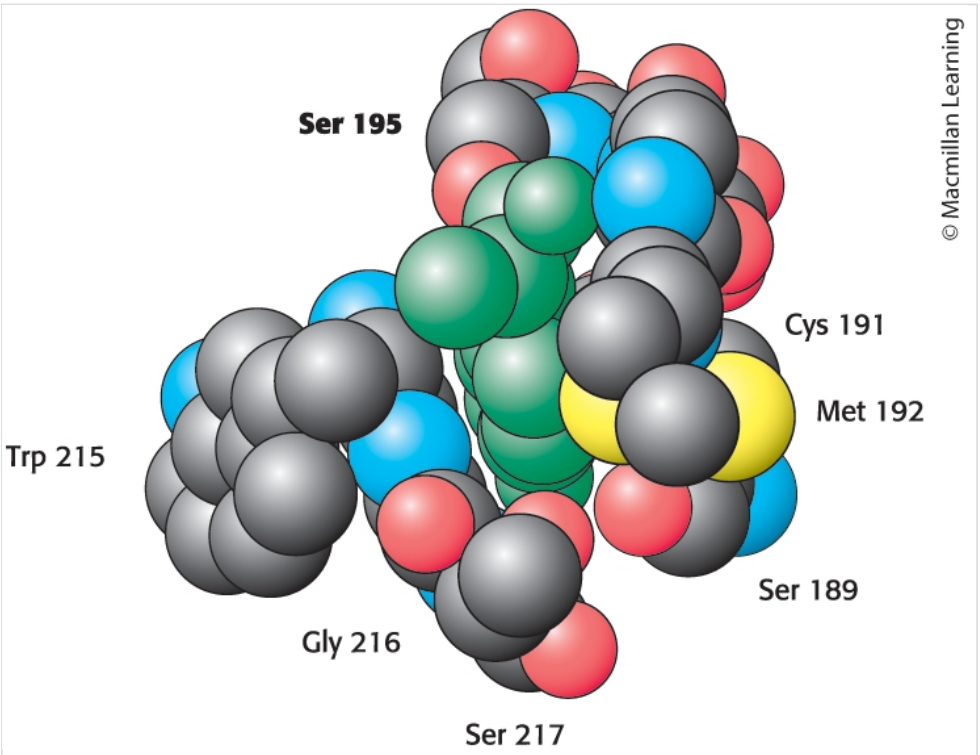

FIGURE 6.10 The specificity pocket of chymotrypsin favors the binding of residues with long hydrophobic side chains. The pocket is deep and lined with hydrophobic residues, with active-site serine residue (serine 195) positioned to cleave the peptide backbone between the residue bound in the pocket (in this case, phenylalanine, shown in green) and the next residue in the sequence.

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, but other proteases have more-complex specificity patterns. Such enzymes have additional pockets on their surfaces for the recognition of other residues in the substrate. Figure 6.11 shows a generic example of the nomenclature used in diagramming protease-substrate interactions. Residues on the amino-terminal side of the scissile bond are labeled P_1, P_2, P_3, and so forth, heading away from 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.

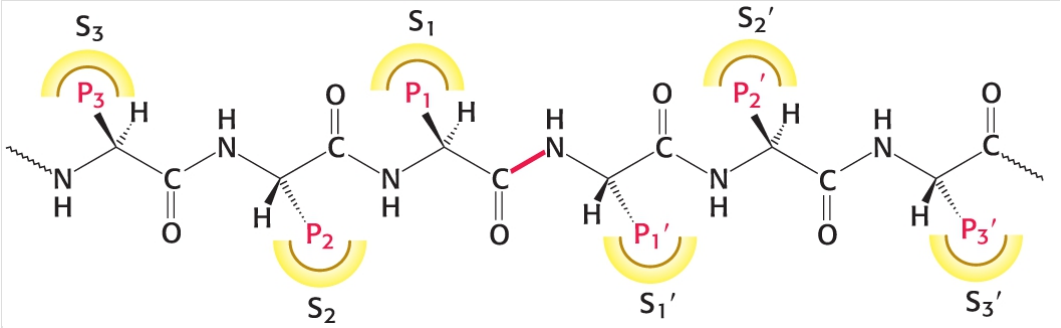

FIGURE 6.11 The specificity nomenclature for protease–substrate interactions shows the potential sites of interaction. The protease site is designated P, and the corresponding binding sites on the enzyme are designated S. The scissile bond (shown in red) is the reference point.

Catalytic triads are found in other hydrolytic enzymes

Many other peptide-cleaving 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; that is, they clearly evolved from a common ancestor. The sequences of these proteins are approximately 40% identical with that of chymotrypsin, and their overall structures are quite similar (Figure 6.12). These proteins operate by mechanisms identical with that of chymotrypsin. However, the three enzymes differ markedly in substrate specificity. Chymotrypsin cleaves at the peptide bond after residues with an aromatic or long, nonpolar side chain. Trypsin cleaves at the peptide bond after residues with long, positively charged side

chains — namely, arginine and lysine. Elastase cleaves at the peptide bond after amino acids with small side chains — such as alanine and serine.

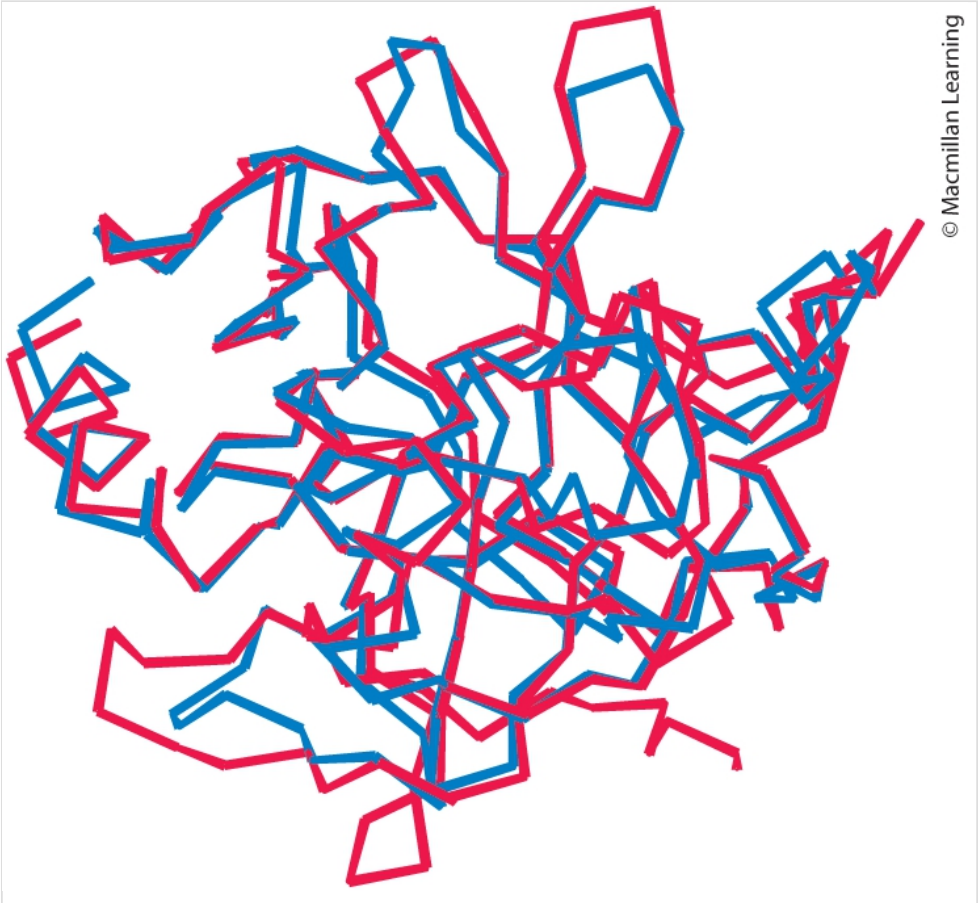

FIGURE 6.12 Chymotrypsin and trypsin are structurally similar. This similarity is revealed by overlaying the structures of chymotrypsin (red) and trypsin (blue). Only α-carbon-atom positions are shown. The mean deviation in position between corresponding α-carbon atoms is quite small, only 1.7 Å.

[Drawn from 5PTP.pdb and 1GCT.pdb.]

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Comparison of the S_1 pockets of these enzymes reveals that these different specificities are due to small structural differences. 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 by much bulkier valine residues (Val 190 and Val 216). These residues close off the mouth of the pocket so that only small side chains can enter (Figure 6.13).

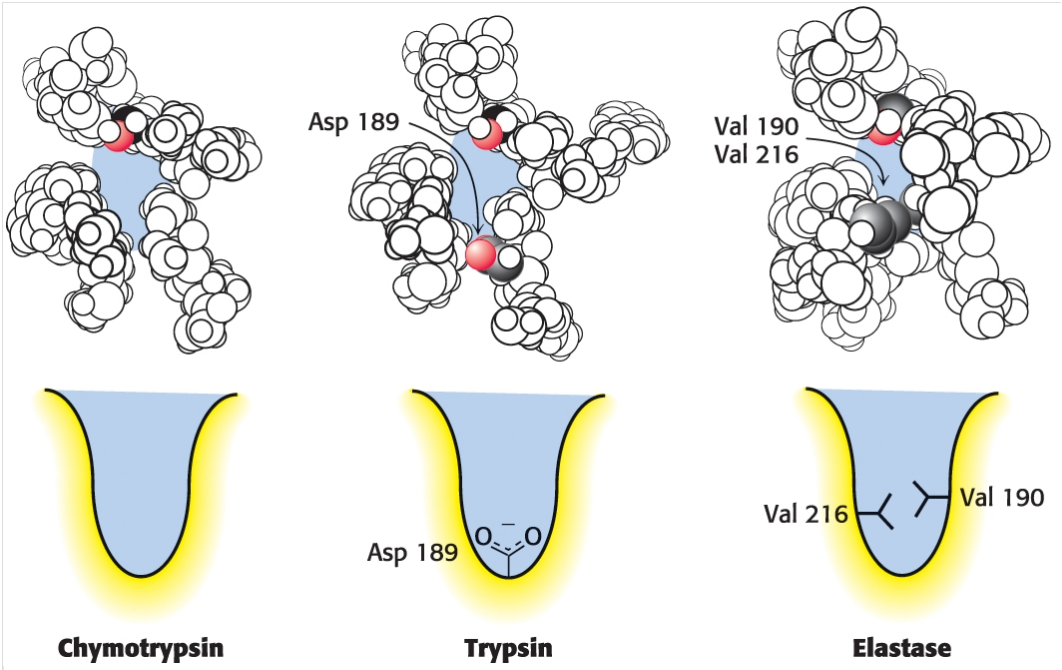

FIGURE 6.13 The S_1 pockets of chymotrypsin, trypsin, and elastase show the residues that play key roles in determining enzyme specificity. The side chains of these residues, as well as those of the active-site serine residues, are shown in color, and schematic depictions are shown below.

Other members of the chymotrypsin family include a collection of proteins that take part in blood clotting, to be discussed in Chapter 7, as well as the tumor marker protein prostate-specific antigen (PSA). In addition, a wide range of proteases found in bacteria, viruses, and plants belong to this clan.

Other enzymes that are not homologs of chymotrypsin have been found to contain very similar active sites to that of chymotrypsin, not because of a shared common ancestor, but because a similar active evolved independently. 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 6.14**). Subtilisin is the founding member of another large family of proteases. Still 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.

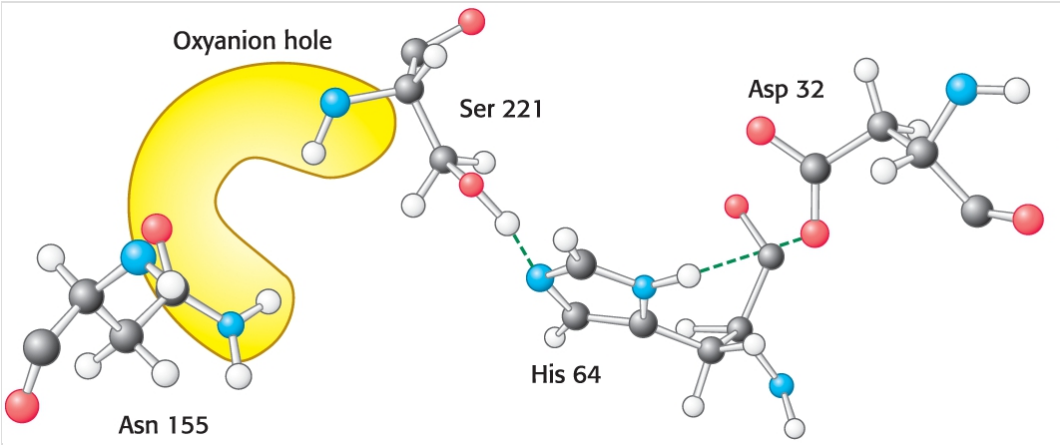

FIGURE 6.14 The catalytic triad and oxyanion hole of subtilisin includes two NH groups, one from the backbone and one from the side chain of Asn 155. The NH groups will stabilize a negative charge that develops on the peptide bond attacked by nucleophilic serine 221 of the catalytic triad.

The catalytic triad in proteases has clearly emerged several 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.

Scientists have dissected the catalytic triad using site-directed mutagenesis

How can we test the validity of the mechanism proposed for the catalytic triad? One way is to test the contribution of individual amino acid residues to the catalytic power of a protease by using site-directed mutagenesis. 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 6.15).

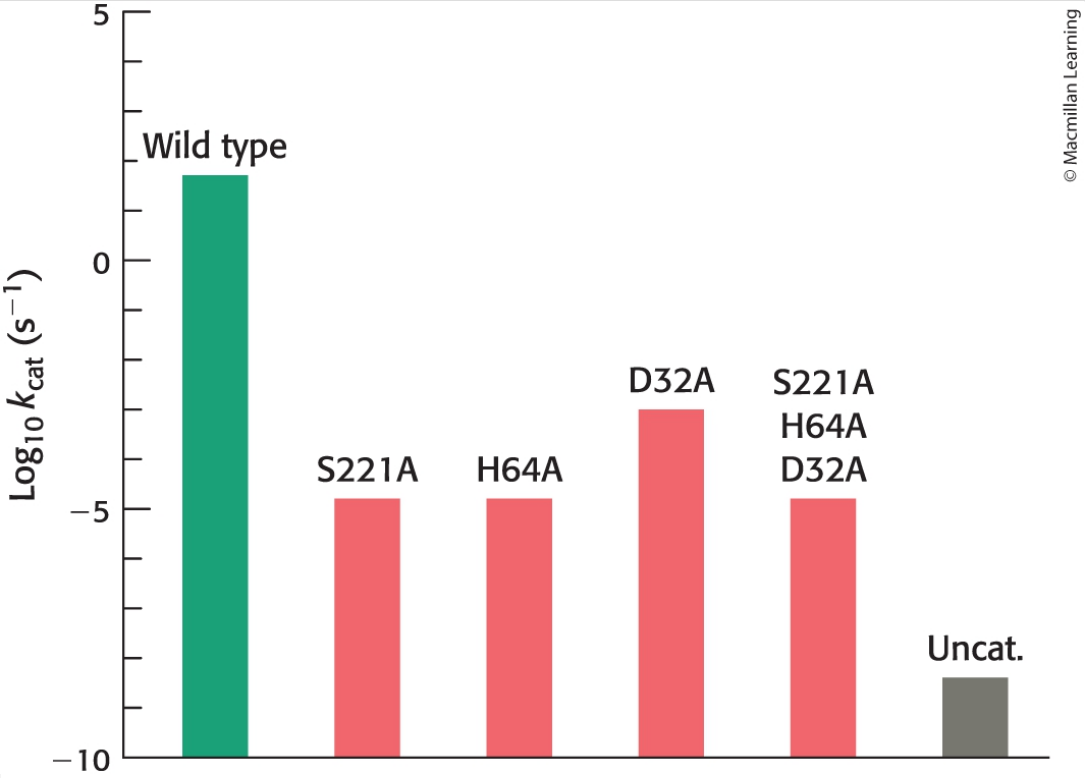

FIGURE 6.15 Mutating the residues of the catalytic triad to alanine allows the importance of each residue for catalytic activity to be quantified. Note that the activities are displayed on a logarithmic scale. The mutations are identified as follows: the first letter is the one-letter abbreviation for 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. *Uncat.* refers to the estimated rate for the uncatalyzed reaction.

As expected, the conversion of active-site serine 221 into alanine dramatically reduces catalytic power; the value of k_{cat} falls to less than one millionth of its value for the wild-type enzyme. The value of K_M is essentially unchanged; its increase by no more than a factor of two indicates that substrate continues to bind normally. The mutation of histidine 64 to alanine reduces catalytic power to a similar degree. The conversion of aspartate 32 into alanine reduces catalytic power by less, although the value of k_{cat} still falls to less than 0.005% of its wild-type

value. The simultaneous conversion of all three residues into alanine is no more deleterious than the conversion of serine or histidine alone. These observations support the notion that the catalytic triad and, particularly, the serine–histidine pair act together to generate a nucleophile of sufficient power to attack the carbonyl carbon atom of a peptide bond. Despite the reduction in their catalytic power, the mutated enzymes still hydrolyze peptides a thousand times as fast as buffer at pH 8.6.

Site-directed mutagenesis also offers a way to probe the importance of the oxyanion hole for catalysis. The mutation of asparagine 155 to glycine eliminates the side-chain NH group from the oxyanion hole of subtilisin. The elimination of the NH group reduces the value of k_{cat} to 0.2% of its wild-type value but increases 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.

Some proteases cleave peptides at other locations besides serine residues

Not all proteases use strategies based on activated serine residues. Classes of proteins have been discovered that employ three alternative approaches to peptide-bond hydrolysis. In each case, the strategy is to generate a nucleophile that attacks the peptide carbonyl group (**Figure 6.16**).

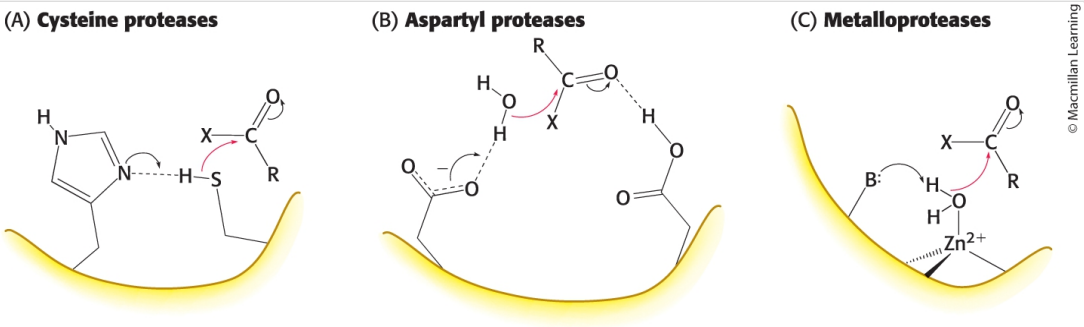

FIGURE 6.16 The key steps employed by the three classes of proteases reveal three different catalytic strategies. The peptide carbonyl group is attacked by (A) a histidine-activated cysteine in the cysteine proteases, (B) an aspartate-activated water molecule in the aspartyl proteases, and (C) a metal-activated water molecule in the metalloproteases. For the metalloproteases, the letter B represents a base (often glutamate) that helps deprotonate the metal-bound water.

Cysteine proteases

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 in a manner quite analogous to that of the serine residue in serine proteases (Figure 6.16). Because the sulfur atom in cysteine is inherently a better nucleophile than is the oxygen atom in serine, cysteine proteases appear to require only this histidine residue in addition to cysteine and not the full catalytic triad.

Aspartyl proteases

The central feature of the active sites in aspartyl proteases 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 poising it for deprotonation. The other aspartic acid residue (in its protonated form)

polarizes the peptide carbonyl group so that it is more susceptible to attack (Figure 6.16).

Metalloproteases

The active site of a metalloprotease contains a bound metal ion, most often zinc, that activates a water molecule to act as a nucleophile to attack the peptide carbonyl group. In each of these three classes of enzymes, the active site includes features that act to (1) activate a water molecule or another nucleophile, (2) polarize the peptide carbonyl group, and (3) stabilize a tetrahedral intermediate (Figure 6.16).

Self-Check Question

Write out a mechanism for a cysteine protease analogous with that for chymotrypsin.

Protease inhibitors are important drugs

Because of the fundamental biological roles of proteases, these enzymes are important drug targets. Drugs that block protease activity are called **protease inhibitors**. For example, captopril, a drug used to regulate blood pressure, is one of many inhibitors of the angiotensin-converting enzyme (ACE), a metalloprotease. Indinavir (Crixivan), retrovir, and many other compounds used in the treatment of AIDS are inhibitors of HIV protease, an aspartyl protease. HIV protease cleaves multidomain

viral proteins into their active forms; blocking this process completely prevents the virus from being infectious. HIV protease inhibitors, in combination with inhibitors of other key HIV enzymes, have dramatically reduced deaths due to AIDS, assuming that the cost of the treatment could be covered. In many cases, these drugs have converted AIDS from a death sentence to a treatable chronic disease.

Indinavir, one of the first HIV protease inhibitors developed but not one that is widely used at present, reveals an important strategy for developing enzyme inhibitors. Indinavir was designed to resemble a peptide substrate of the enzyme. X-ray crystallographic studies revealed that indinavir adopts a confirmation that approximates the twofold symmetry of the enzyme (Figure 6.17). The active site of HIV protease is covered by two flexible flaps that fold down on top of the bound inhibitor. The OH group of the central alcohol interacts with the two aspartate residues of the active site and was intended to mimic a water molecule observed in crystal structures of HIV protease but generally absent in cellular aspartyl proteases. This OH group was intended to contribute to the specificity of indinavir for HIV protease. To prevent side effects, enzyme inhibitors used as drugs should be relatively specific for one enzyme, minimally inhibiting other similar enzymes within the body.

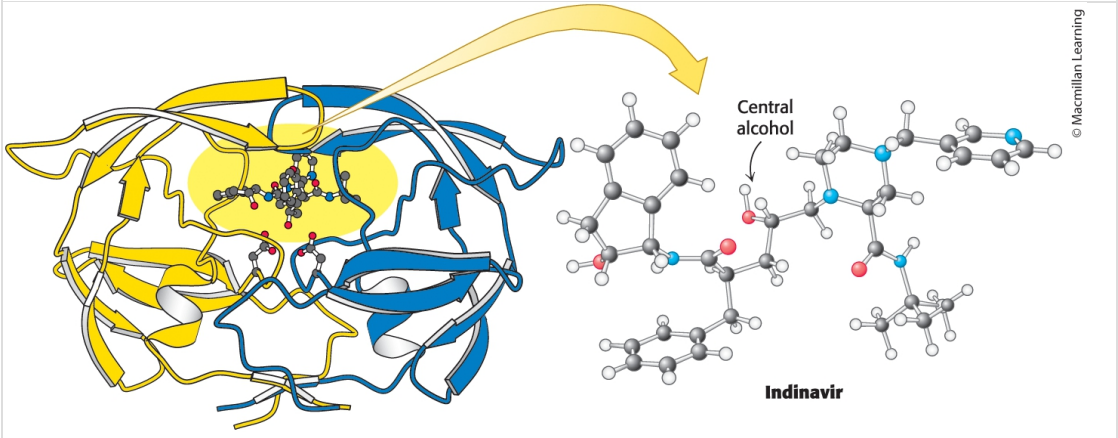

FIGURE 6.17 HIV protease has twofold symmetry. (Left) The three-dimensional structure of HIV protease is shown with the inhibitor indinavir bound in the active site. (Right) The drug has been rotated to reveal its approximately twofold symmetric conformation.

[Drawn from 1HSH.pdb.]

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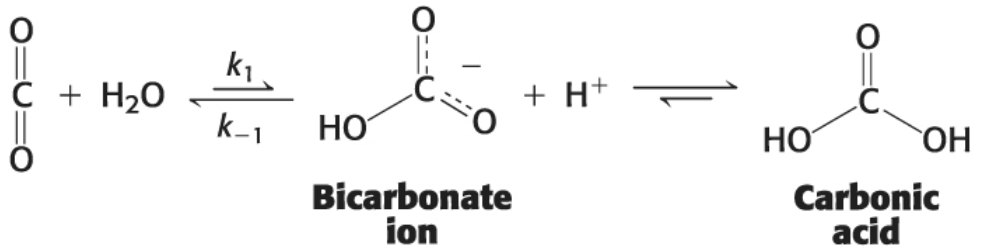
6.3 Carbonic Anhydrases Make a Fast Reaction Faster

Carbon dioxide is a major end product of aerobic metabolism. In mammals, this carbon dioxide is released into the blood and transported to the lungs for exhalation. While in the red blood cells, carbon dioxide reacts with water. The products of this reaction are bicarbonate ion (HCO_3^-) and a proton. These are a form of carbonic acid, H_2CO_3, a moderately strong acid, with $pK_a = 3.5$.



Even in the absence of a catalyst, this hydration reaction proceeds at a moderately fast pace. At 37°C near neutral pH, the second-order rate constant k_1 is $0.0027\ M^{-1}\ s^{-1}$. This value corresponds to an effective first-order rate constant of $0.15\ s^{-1}$ in water $([H_2O] = 55.5\ M)$. The reverse reaction, the dehydration of HCO_3^-, is even more rapid, with a rate constant of $k_{-1} = 50\ s^{-1}$. These rate constants correspond to an equilibrium constant of $K_1 = 5.4 \times 10^{-5}$ and a ratio of $[CO_2]$ to $[H_2CO_3]$ of 340:1 at equilibrium.

Carbon dioxide hydration and HCO_3^- dehydration are often coupled to rapid processes, particularly transport processes. Thus, almost all organisms contain enzymes, referred to as **carbonic anhydrases**, that increase the rate of reaction beyond the already relatively high spontaneous rate. For example, carbonic anhydrases dehydrate HCO_3^- in the blood to form CO_2 for exhalation as the blood passes through the lungs. Conversely, they convert CO_2 into HCO_3^- to generate the aqueous humor of the eye and other secretions. For this reason, one of the drugs used for treating glaucoma, a condition associated with too much pressure inside the eye, acts by inhibiting carbonic anhydrase. 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.

Carbonic anhydrases accelerate CO_2 hydration dramatically. The most-active enzymes hydrate CO_2 at rates as high as $k_{cat} = 10^6\ s^{-1}$, or a million times a second per enzyme molecule. Fundamental physical processes such as diffusion and proton transfer ordinarily limit the rate of hydration, and so the enzymes employ special strategies to attain such high rates.

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 a bound zinc ion. Moreover, the zinc ion appeared to be necessary for catalytic activity. This discovery, remarkable at the time, made carbonic anhydrase the first known zinc-containing enzyme. At present, thousands 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.

Metal ions have several properties that increase chemical reactivity: their positive charges, their ability to form strong yet kinetically labile bonds, and, in some cases, their capacity to be stable in more than one oxidation state. The chemical reactivity of metal ions explains why catalytic strategies that employ metal ions have been adopted throughout evolution.

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 sequence identity. Carbonic anhydrase II, a major protein component of red blood cells, has been the most extensively studied (Figure 6.18). It is also one of the most active carbonic anhydrases.

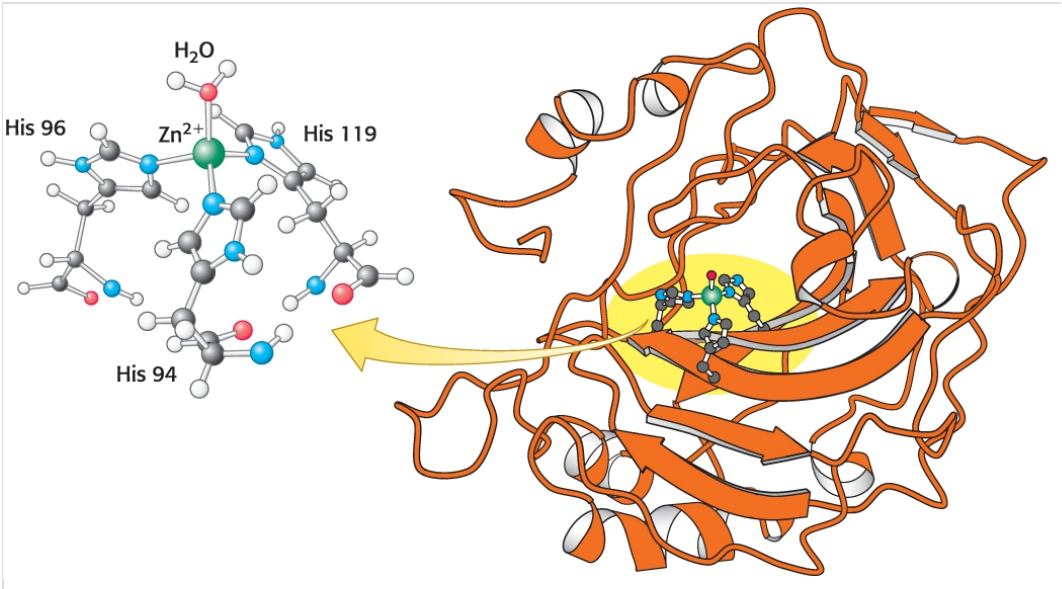


FIGURE 6.18 The active site of human carbonic anhydrase II includes a bound zinc ion. The active site includes a zinc ion that is bound to the imidazole rings of three histidine residues as well as to a water molecule in a cleft near the center of the enzyme.

[Drawn from 1CA2.pdb.]

INTERACT with this model in Achieve

Zinc is found only in the $^{+2}$ state in biological systems. A zinc atom is essentially always bound to four or more specific molecules or groups, called **ligands**. In carbonic anhydrase, three coordination sites are occupied by nitrogen ligands from the imidazole rings of three histidine residues, and an additional coordination site is occupied by a water molecule (or a hydroxide ion, depending on pH). Because the ligands occupying the coordination sites are neutral, the overall charge on the $Zn(His)_3$ unit remains $^{+2}$.

Catalysis involves zinc activation of a water molecule

How does this zinc complex facilitate carbon dioxide hydration? A major clue comes from the pH profile of enzymatically catalyzed carbon dioxide hydration (**Figure 6.19**). 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 that loses a proton at pH 7 $(pK_a = 7)$ plays an important role in the activity of carbonic anhydrase. Moreover, the curve suggests that the deprotonated (high pH) form of this group participates more effectively in catalysis. Although some amino acids, notably histidine, have pK_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.

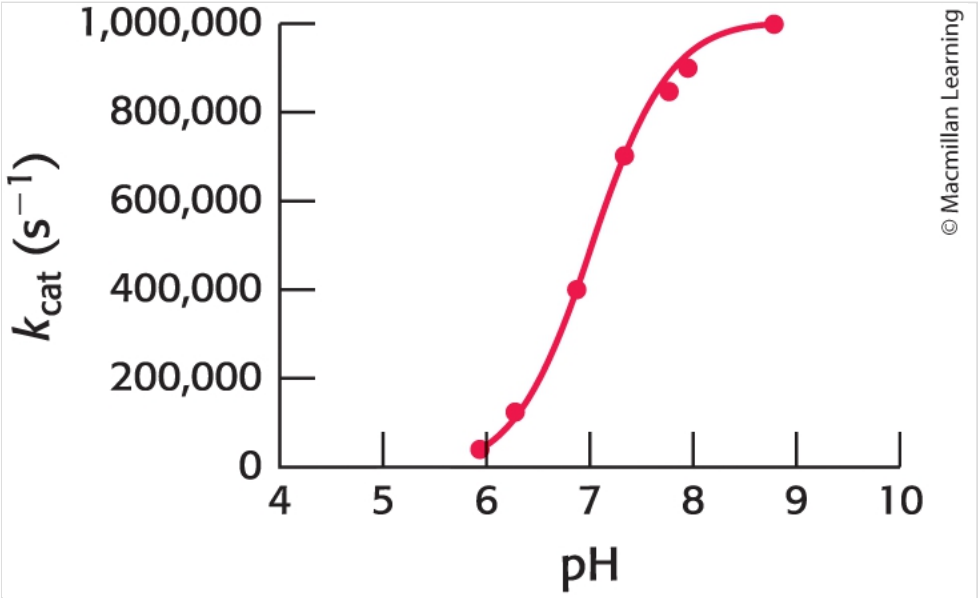


FIGURE 6.19 The activity of carbonic anhydrase varies with pH. The enzyme is maximally active at pH values near 9.

The binding of a water molecule to the positively charged zinc center reduces the pK_a of the water molecule from 15.7 to 7 (Figure 6.20). With the pK_a lowered, the water molecule can more easily lose a proton at neutral pH, generating a substantial concentration of hydroxide ion (bound to the zinc atom). A zinc-bound hydroxide ion (OH^-) is a potent nucleophile able to attack carbon dioxide much more readily than water does. Adjacent to the zinc site, carbonic anhydrase also possesses a hydrophobic patch that serves as a binding site for carbon dioxide (Figure 6.21). Based on these observations, a simple mechanism for carbon dioxide hydration can be proposed (Figure 6.22):

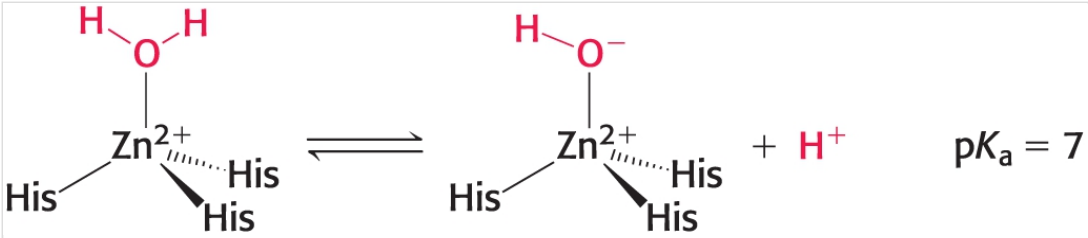


FIGURE 6.20 Upon binding to zinc in carbonic anhydrase, the pK_a of water decreases from 15.7 to approximately 7.

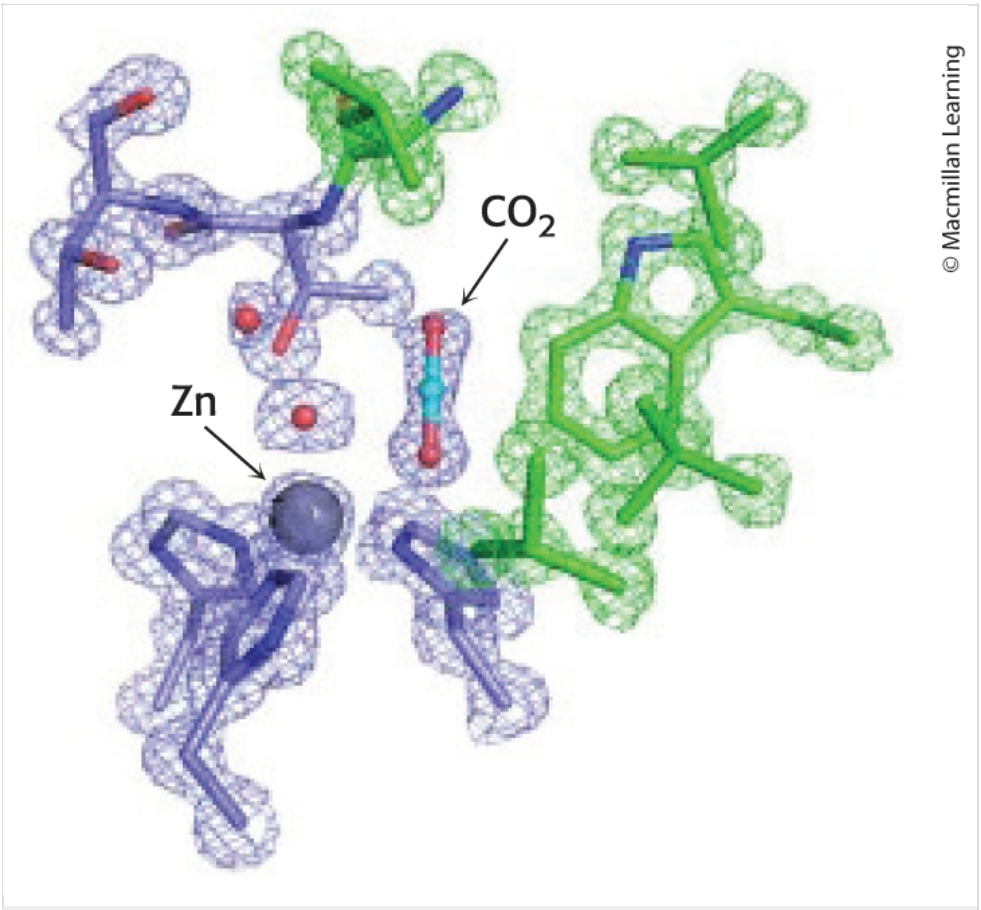


FIGURE 6.21 Carbonic anhydrase has a carbon dioxide binding site adjacent to the active site zinc ion. Crystals of carbonic anhydrase were exposed to carbon dioxide gas at high pressure and low temperature, and x-ray diffraction data were collected.

The electron density (shown as cages) for carbon dioxide, clearly visible adjacent to the zinc and its bound water, reveals the carbon dioxide binding site. Hydrophobic amino acids are shaded green, and the purple ones are hydrophilic.

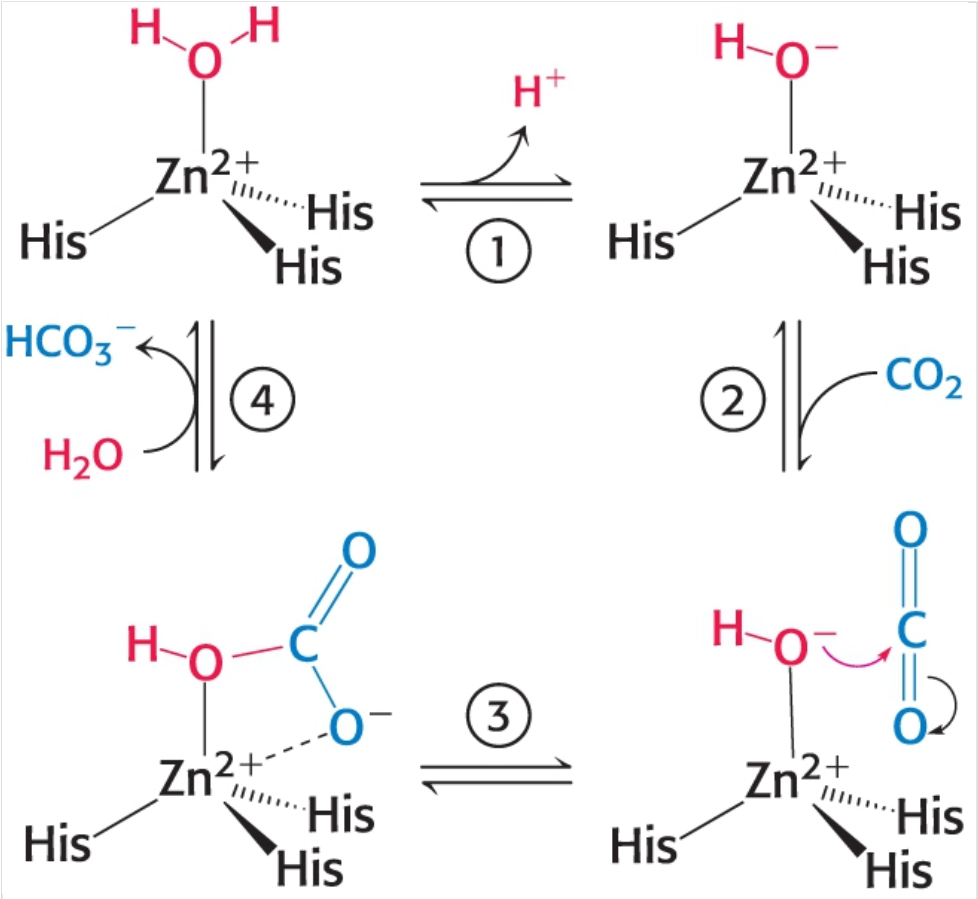


FIGURE 6.22 The zinc-bound hydroxide mechanism for the hydration of carbon dioxide proceeds in four steps. These are (1) water deprotonation, (2) carbon dioxide binding, (3) nucleophilic attack by hydroxide on carbon dioxide, and (4) displacement of bicarbonate ion by water. This mechanism provides a good example of one type of metal ion catalysis.

1. The zinc ion 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, HCO_3^-.
4. The catalytic site is regenerated with the release of HCO_3^- and the binding of another molecule of water.

Thus, the binding of a water molecule to the zinc ion favors the formation of the transition state by facilitating proton release and by positioning the water molecule to be in close proximity to the other reactant.

Rapid regeneration of the active form of carbonic anhydrase depends on proton availability

In the first step of a carbon dioxide hydration reaction, the zinc-bound water molecule must lose a proton to regenerate the active form of the enzyme (Figure 6.23). Let use consider this in detail:

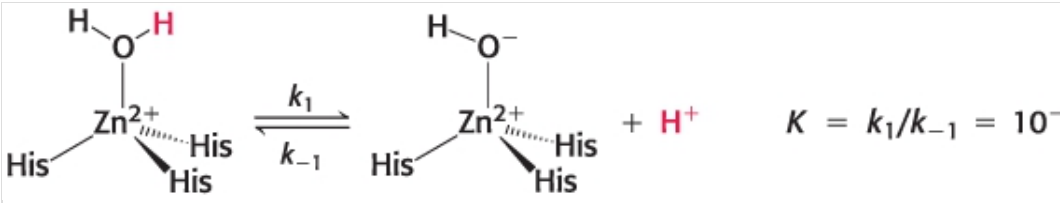


FIGURE 6.23 The ratio of the rate constant for water deprotonation to that for hydroxide protonation must equal 10^{-7}. This is true since the pK_a of this water molecule in carbonic anhydrase is 7.

- The equilibrium constant for this process is $K = 10^{-7}\ \mathrm{M}$ (since $\mathrm{p}K_a = 7$) and $K = k_1/k_{-1}$.
- 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-order rate constants near $10^{11}\ \mathrm{M}^{-1}\,\mathrm{s}^{-1}$.
- The forward rate constant is given by $k_1 = K \times k_{-1}$. Since $k_{-1} \leq 10^{11}\ \mathrm{M}^{-1}\,\mathrm{s}^{-1}$, k_1 must be less than or equal to $10^4\ \mathrm{s}^{-1}$.

As noted earlier, some carbonic anhydrases can hydrate carbon dioxide at rates as high as a million times a second $(10^6\ \mathrm{s}^{-1})$. This rate is so high that it seems to violate the analysis above. If carbon dioxide is hydrated at a rate of $10^6\ \mathrm{s}^{-1}$, then every step in the mechanism (Figure 6.22) must take place at least this fast. How is this apparent paradox resolved?

The role of buffers

The answer became clear with the observation 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 $10^{-7}\ \mathrm{M}$ at neutral pH, the concentration of buffer components can be much higher, of the order of several millimolar. Let us consider the role of buffer components in more detail:

- If the buffer component BH^+ has a pK_a of 7 (matching that for the zinc-bound water molecule), then the equilibrium constant for the reaction in Figure 6.24 is 1.

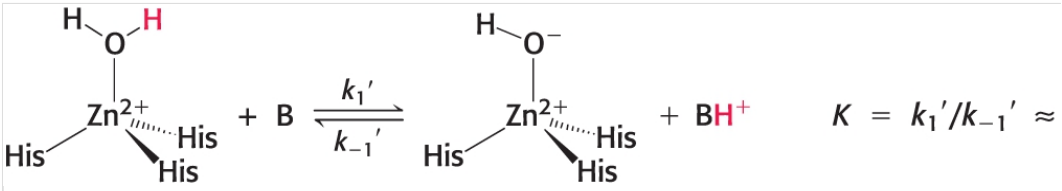


FIGURE 6.24 The deprotonation of the zinc-bound water molecule in carbonic anhydrase can be aided by buffer component B. This is optimized if the pK_a of the buffer is near 7 and if the buffer is present at a sufficiently high concentration.

- The second-order rate constants k_1' and k_{-1}' will be limited by buffer diffusion to values less than approximately $10^9\ M^{-1}\ s^{-1}$.
- The rate of proton abstraction is given by $k_1' \times [B]$.
- If buffer concentrations greater than $[B] = 10^{-3}\ M$ (or 1 mM) are used, then carbon dioxide hydration rates $\geq 10^6\ M^{-1}\ s^{-1}$ may be possible because

$$k_1' \times [B] = (10^9\ M^{-1}\ s^{-1}) \times (10^{-3}\ M) = 10^6\ s^{-1}$$

The prediction that the rate increases with increasing buffer concentration has been confirmed experimentally (Figure 6.25).

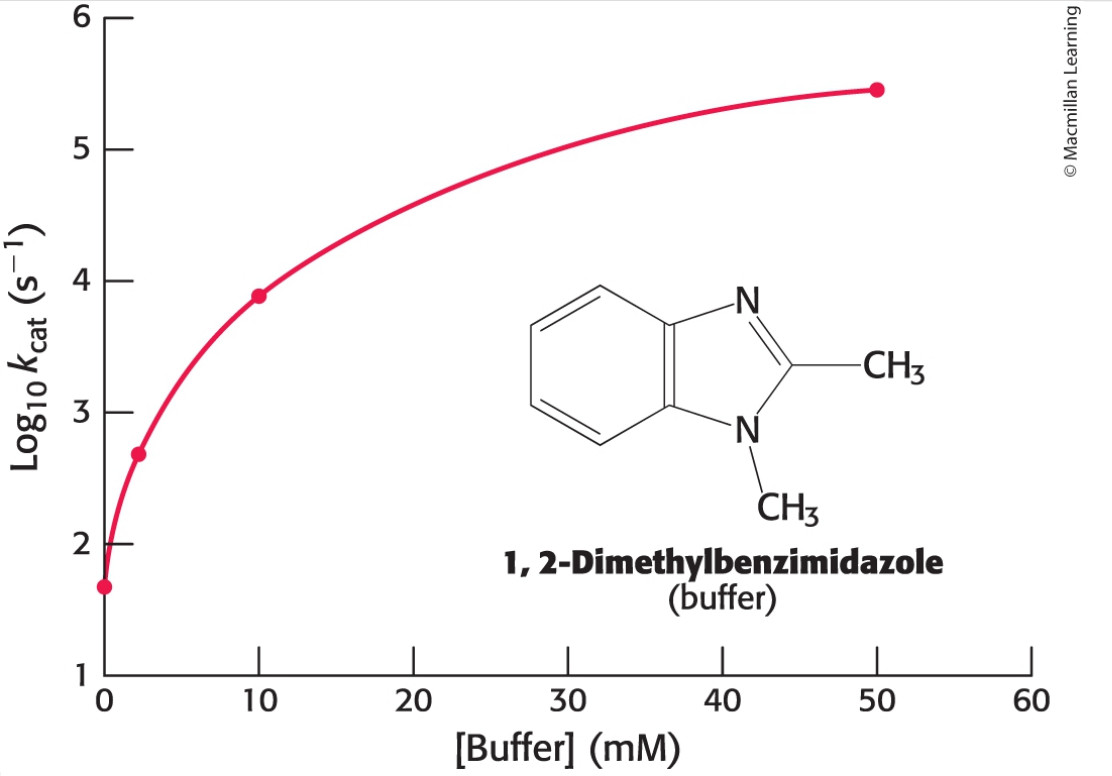


FIGURE 6.25 The rate of carbon dioxide hydration by carbonic anhydrase increases with the concentration of the buffer 1,2-dimethylbenzimidazole. If present at high enough concentrations, such buffers enable the enzyme to achieve its highest catalytic rates.

Self-Check Question

Suppose that the rate of CO_2 hydration is measured in 1 mM buffer at pH 7.0. What is the maximum expected rate?

A “proton shuttle” for large buffers

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 6.26). 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.

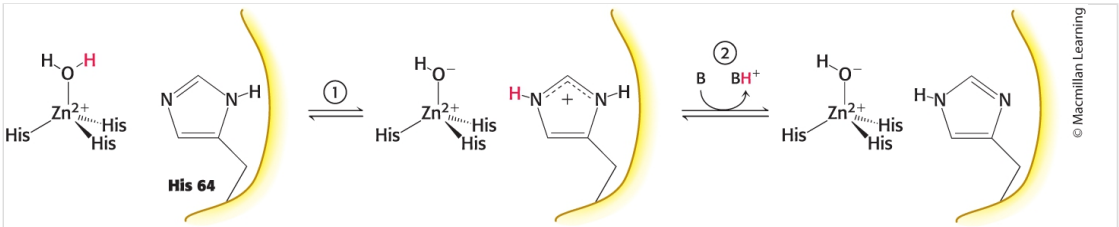


FIGURE 6.26 Histidine 64 acts as a proton shuttle. Histidine 64 (1) removes a proton from the zinc-bound water molecule and (2) transfers the proton to buffer molecules in solution.

6.4 Restriction Enzymes Catalyze Highly Specific DNA-Cleavage Reactions

We 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 cell.

A major protective strategy for the host is to use **restriction enzymes** (also called **restriction endonucleases**) to degrade the viral DNA on its introduction into a cell. These enzymes recognize particular base sequences, called **recognition sequences** or recognition sites, in DNA molecules and cleave such molecules (hereafter referred to as *cognate DNA*) at defined positions. Restriction enzymes are best known because they are powerful tools for characterizing and manipulating DNA molecules, and their discovery was a key step that led to a revolution in molecular biology and genetic engineering. The most well-studied class of restriction enzymes comprises the 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 enzymes must show tremendous specificity at two levels:

1. *Restriction enzymes must cleave only DNA molecules that contain recognition sites, 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 4000-fold higher than the concentration of sites that should be cleaved. Thus, to keep from damaging host DNA, restriction enzymes must cleave cognate DNA molecules much more than 4000 times as efficiently as they cleave nonspecific sites. We shall return to the mechanism used to achieve the necessary high specificity after considering the chemistry of the cleavage process.

2. *Restriction enzymes must not degrade host DNA containing the recognition sequences.* How do these enzymes manage to degrade viral DNA while sparing their own? In *E. coli*, the restriction endonuclease EcoRV cleaves double-stranded viral DNA molecules that contain the sequence $5'-GATATC-3'$, but it leaves intact host DNA containing hundreds of such sequences. We shall return to the strategy by which host cells protect their own DNA at the end of this section.

Cleavage is by direct displacement of $3'$-oxygen from phosphorus by magnesium-activated water

A restriction enzyme catalyzes 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 at the cleavage site (Figure 6.27). This reaction proceeds by nucleophilic attack at the phosphorus atom. We will consider two alternative mechanisms, suggested by analogy with the proteases.

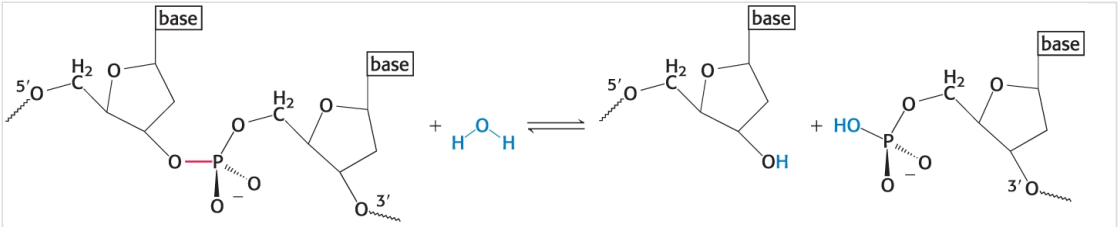
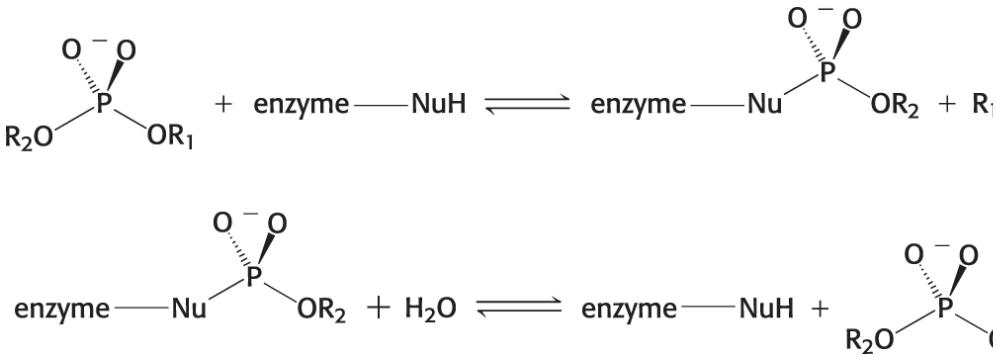
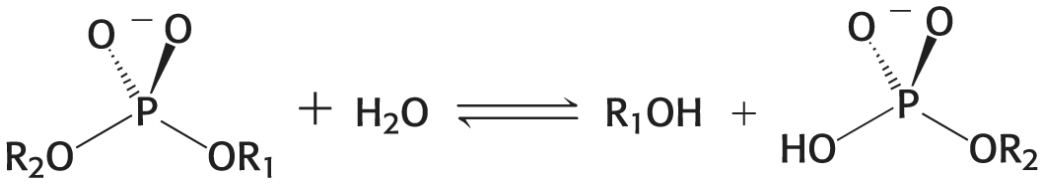


FIGURE 6.27 Restriction enzymes catalyze the hydrolysis of DNA phosphodiester bonds. The reaction leaves a hydroxyl group at the 3′ end of one product fragment and a phosphoryl group attached to the 5′ end of the other. The bond that is cleaved is shown in red.

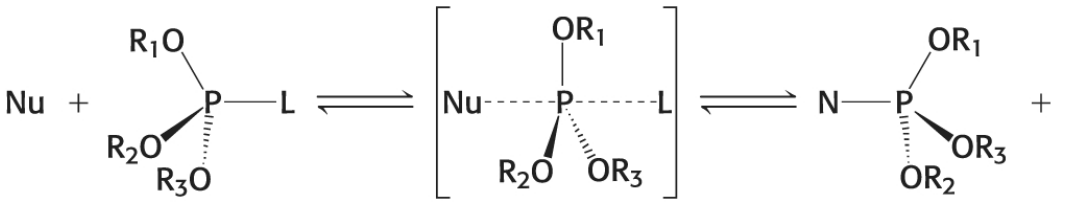
1. The restriction enzyme might cleave DNA through a covalent intermediate, employing a potent nucleophile (Nu):
   Mechanism 1 (covalent intermediate)



2. Alternatively, the restriction enzyme might cleave DNA through direct hydrolysis:
   Mechanism 2 (direct hydrolysis)



Each mechanism postulates a different nucleophile to attack the phosphorus atom. In either case, each reaction takes place by in-line displacement, in which the incoming nucleophile attacks the phosphorus atom, and a pentacoordinate transition state is formed:



The transition state has a trigonal bipyramidal geometry centered at the phosphorus atom, with the incoming nucleophile at one apex of the two pyramids and the group that is displaced (the leaving group, L) at the other apex. Note that the displacement inverts the stereochemical conformation at the tetrahedral phosphorous atom, analogous to the interconversion of stereoisomers around a tetrahedral carbon center.

The two mechanisms differ in the number of times that the displacement takes place in the course of the reaction:

1. In the first type of mechanism, a nucleophile in the enzyme (analogous to serine 195 in chymotrypsin) attacks the phosphate

group to form a covalent intermediate. Next, this intermediate is hydrolyzed to produce the final products. In this case, two displacement reactions take place at the phosphorus atom. Consequently, the stereochemical configuration at the phosphorus atom would be inverted and then inverted again, and the overall configuration would be retained.

2. 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 at the phosphorus atom is inverted after cleavage.

One approach to determine which mechanism is correct involves examining potential changes in the stereochemistry at the phosphorus atom over the course of cleavage. However, this stereochemical change is not easily observed, because two of the groups bound to the phosphorus atom are simply oxygen atoms, identical with each other. This challenge can be overcome by replacing one oxygen atom with sulfur (producing a species 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 is to synthesize an appropriate substrate for EcoRV containing phosphorothioates at the sites of cleavage (**Figure 6.28**). The reaction is then performed in water that has been greatly enriched in ^{18}O to allow the incoming oxygen atom to be tagged. The location of the ^{18}O label with respect to the sulfur atom indicates whether the reaction proceeds with inversion or retention of stereochemistry. This experiment reveals that the stereochemical configuration at the phosphorus atom is inverted only once with cleavage. This result is consistent with a direct attack by

water at the phosphorus atom and rules out the formation of any covalently bound intermediate (Figure 6.29).

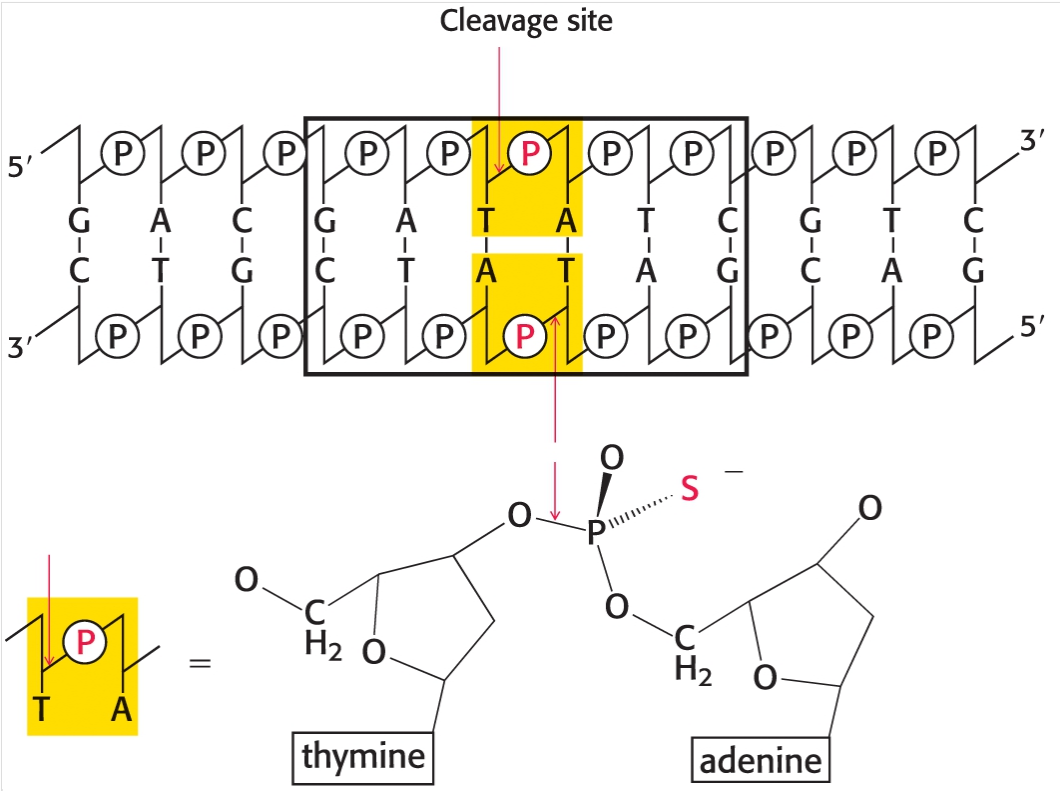


FIGURE 6.28 Phosphorothioate groups can be used to determine the overall stereochemical course of a displacement reaction. A phosphorothioate group has one of the nonbridging oxygen atoms replaced by a sulfur atom. Here, a phosphorothioate is placed at sites of cleavage by EcoRV endonuclease.

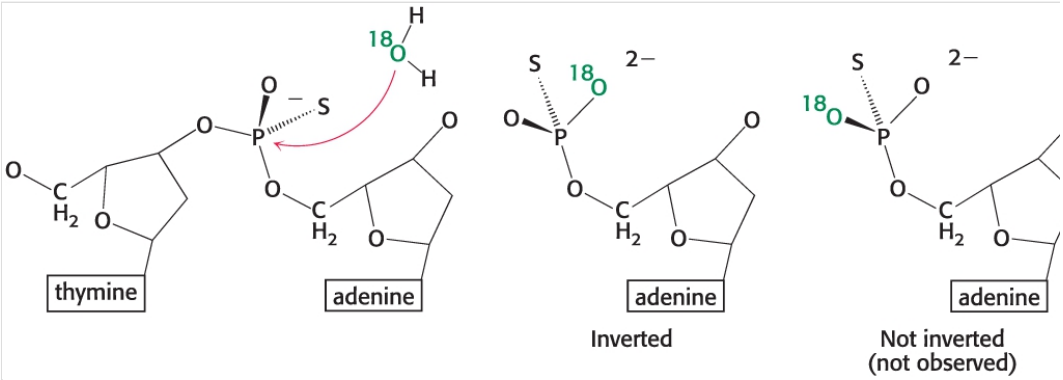


FIGURE 6.29 Cleavage of DNA by EcoRV endonuclease results in overall inversion of the stereochemical configuration at the phosphorus atom. This is indicated by the stereochemistı of the phosphorus atom bound to one bridging oxygen atom, one ^{16}O, one ^{18}O, and one sulfur atom. Two possible products are shown, only one of which is observed, indicating direct attack of water at the phosphorous atom.

Restriction enzymes require magnesium for catalytic activity

Many enzymes that act on phosphate-containing substrates require Mg^{2+} or some other similar divalent cation for activity. One or more Mg^{2+} (or similar) cations are essential to the function of restriction endonucleases. What are the functions of these metal ions?

Direct visualization of the complex between EcoRV endonuclease and cognate DNA molecules in the presence of Mg^{2+} by crystallization has been challenging, because the enzyme cleaves the substrate under these circumstances. Nonetheless, metal ion complexes can be visualized through several approaches. In one approach, crystals of

EcoRV endonuclease are prepared bound to oligonucleotides that contain the enzyme's recognition sequence. These crystals are grown in the absence of magnesium to prevent cleavage; after their preparation, the crystals are soaked in solutions containing the metal ion. Alternatively, crystals have been grown with the use of a mutated form of the enzyme that is less active. Finally, Mg^{2+} can be replaced by metal ions such as Ca^{2+} that bind but do not result in much catalytic activity. In all cases, no cleavage takes place, and so the locations of the metal ion-binding sites are readily determined.

As many as three metal ions per active site have been found. One ion-binding site is occupied in essentially all structures. This metal ion is coordinated to the protein through two aspartate residues and to one of the phosphate-group oxygen atoms at the site of cleavage. This metal ion binds the water molecule that attacks the phosphorus atom, helping to position and activate the water molecule in a manner similar to that for the Zn^{2+} ion of carbonic anhydrase (Figure 6.30).

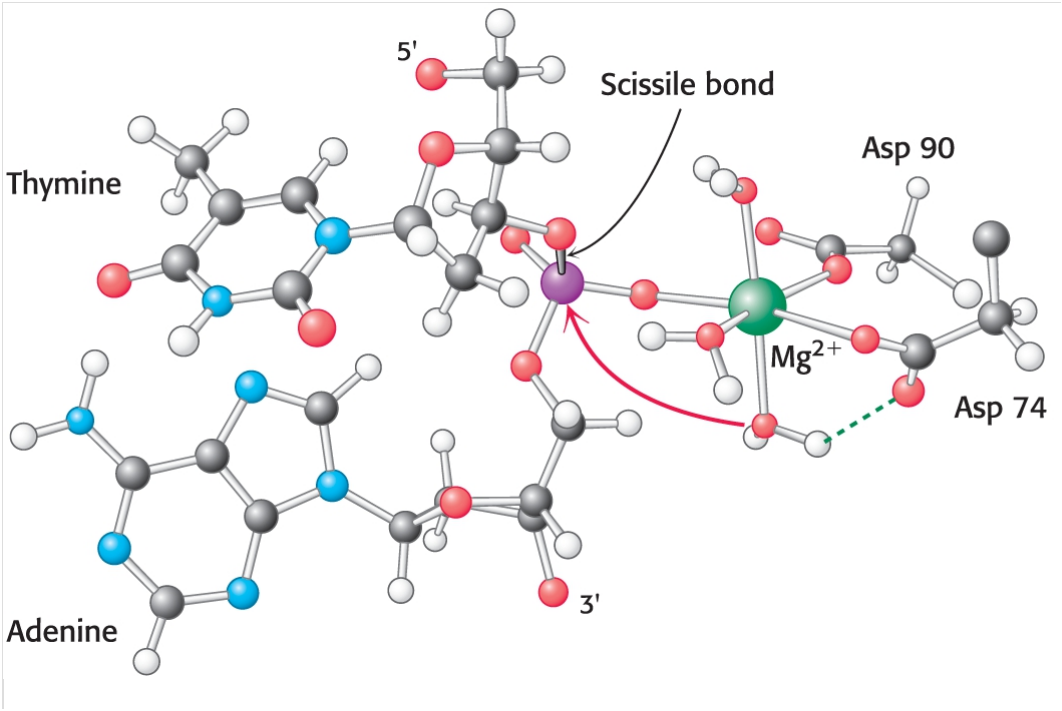


FIGURE 6.30 A magnesium ion forms a bridge between the enzyme and the DNA substrate. In this position, the ion helps activate a water molecule and position it so that it can attack the phosphorus atom at the cleavage site.

The complete catalytic apparatus is assembled only within complexes of cognate DNA molecules, ensuring specificity

We now return to the question of cleavage site-specificity, the defining feature of restriction enzymes.

The recognition sequences for most restriction enzymes are inverted repeats, giving the three-dimensional structure of the recognition site a twofold rotational symmetry, meaning that the structure is the same if rotated by $360°/2 = 180°$ (Figure 6.31). The restriction enzymes display a corresponding symmetry: they are dimers whose two subunits are related by an appropriate 180° rotation. The matching symmetry of the recognition sequence and the enzyme facilitates the recognition of cognate DNA by the enzyme. This similarity in structure has been confirmed by the determination of the structure of the complex between EcoRV endonuclease and DNA fragments containing its recognition sequence (Figure 6.32). The enzyme surrounds the DNA in a tight embrace.

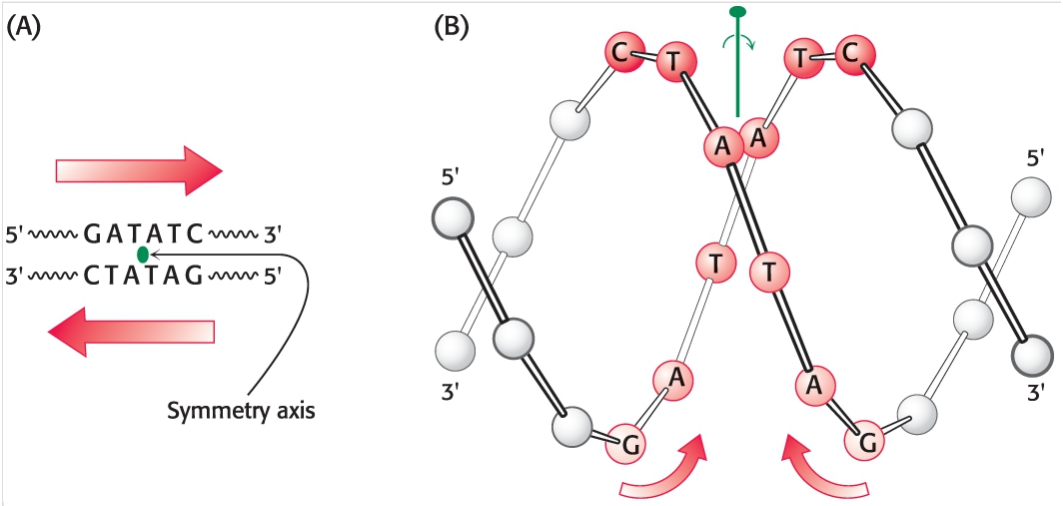


FIGURE 6.31 The recognition site for EcoRV displays twofold rotational symmetry. (A) The sequence of the recognition site, which is symmetric around the axis of rotation designated in green. (B) The inverted repeat within the recognition sequence of EcoRV (and most other restriction endonucleases) endows the DNA site with twofold rotational symmetry.

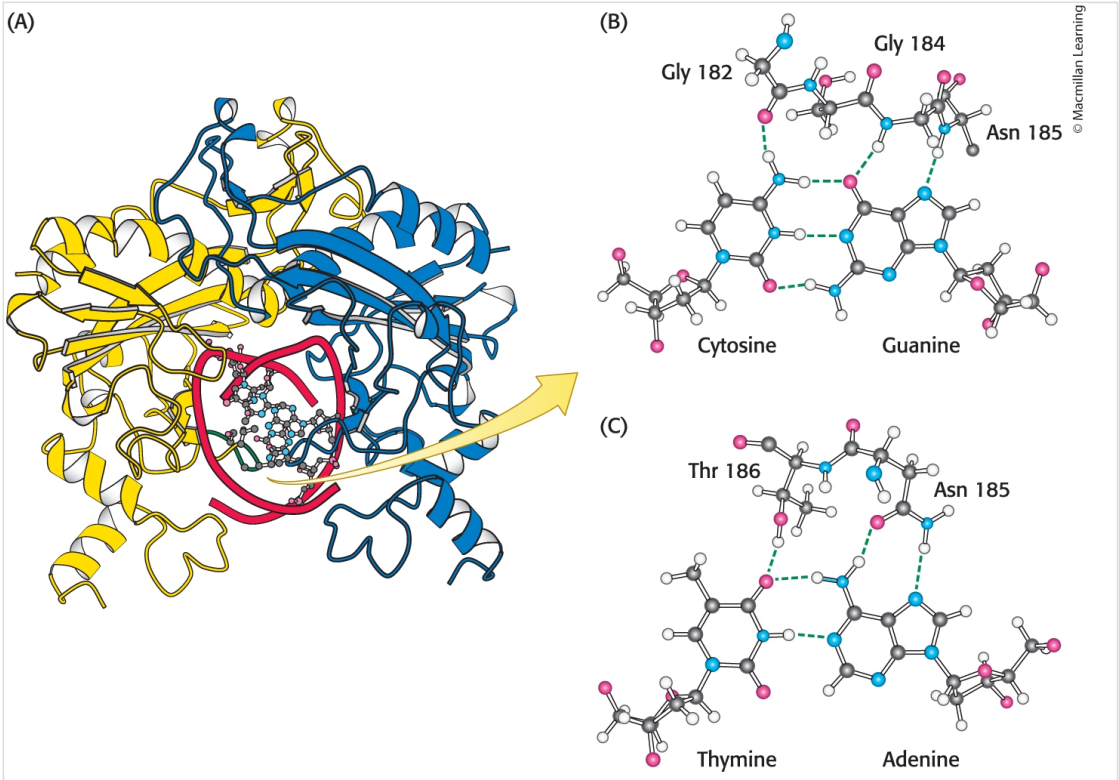


FIGURE 6.32 The three-dimensional structure of the complex between EcoRV and a cognate DNA molecule reveals interactions responsible for the sequence specificity. (A) This view of the structure of EcoRV endonuclease bound to a cognate DNA fragment is down the helical axis of the DNA. The two protein subunits are in yellow and blue, and the DNA backbone is in red. One of the DNA-binding loops 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.

[Drawn from 1RVB.pdb.]

INTERACT with this model in Achieve

Surprisingly, binding studies performed in the absence of magnesium have demonstrated that the EcoRV endonuclease binds to all sequences, both cognate and noncognate, with approximately equal affinity. Why, then, does the enzyme cleave only cognate sequences? The answer lies in a unique set of interactions 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 via hydrogen bonds with residues that are located in two loops, one projecting from the surface of each enzyme subunit (Figure 6.32). The most striking feature of this complex is the distortion of the DNA, which is substantially kinked in the center (**Figure 6.33**), whereas double-stranded DNA is normally straight. The central two TA base pairs in the recognition sequence play a key role in allowing the kink. They do not make contact with the enzyme but appear to be required because of their ease of distortion. The $5'-TA-3'$ sequence is known to be among the most easily deformed base-pair steps.

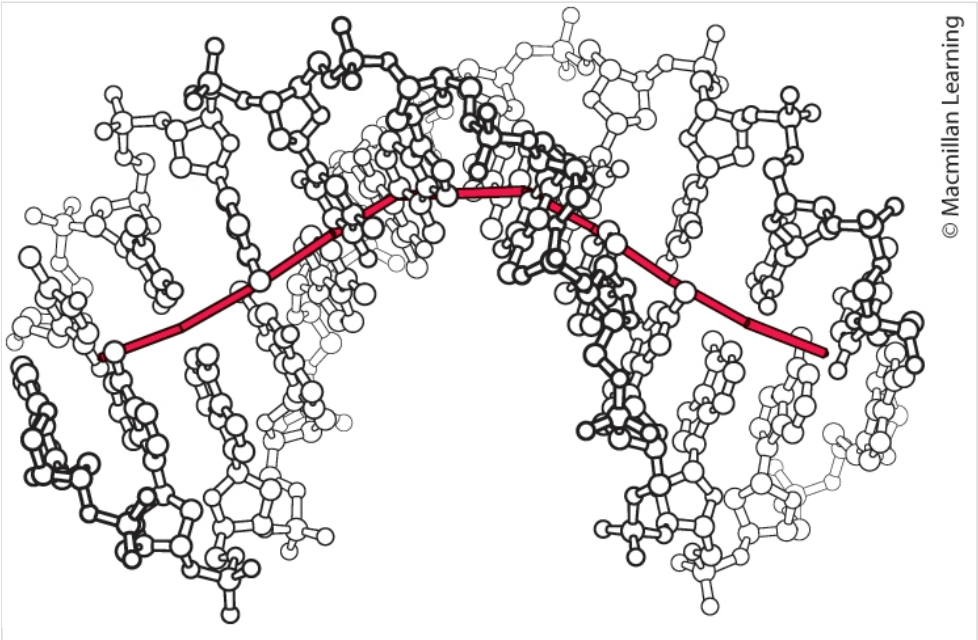


FIGURE 6.33 The cognate DNA from the EcoRV complex is substantially bent. The DNA is represented as a ball-and-stick model. The path of the DNA helical axis, shown in red, is substantially distorted on binding to the enzyme. For B form DNA, the axis is normally straight (not shown).

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 6.34**). This lack of distortion has important consequences with regard to catalysis. No phosphate is positioned sufficiently close to the active-site aspartate residues to complete a magnesium ion-binding site (Figure 6.30). Hence, the nonspecific complexes do not bind the magnesium ions 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 a million-fold that is observed for EcoRV endonuclease. Thus, enzyme specificity may be determined not by the specificity of substrate binding but rather by the specificity of forming a structure capable of DNA cleavage.

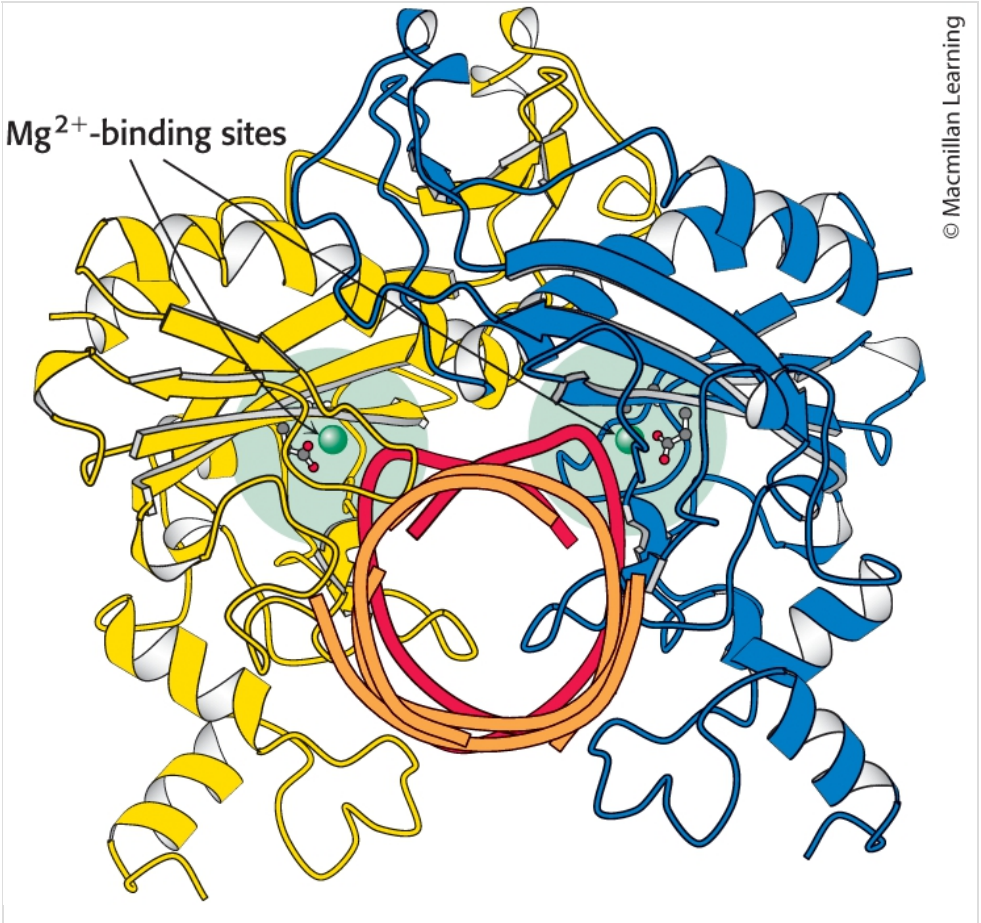


FIGURE 6.34 A comparison of the positions of the nonspecific and the cognate DNA within EcoRV shows the absence and presence of substantial DNA distortion. Note that, in the nonspecific complex (shown in orange compared to the specific complex in red), the DNA backbone is too far from the enzyme to complete the magnesium ion-binding sites required for DNA cleavage.

[Drawn from 1RVB.pdb.]

INTERACT with this model in Achieve

We can now see the role of binding energy in this strategy for attaining catalytic specificity. The distorted DNA makes additional contacts with the enzyme, increasing the binding energy, but the increase in binding energy is canceled by the energetic cost of distorting the DNA from its relaxed conformation into one that is catalytically competent, that is, into a conformation that allows DNA cleavage to proceed (Figure 6.35). 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 DNA hydrolysis by completing the magnesium ion-binding site. This example illustrates how enzymes can use available binding energy to deform substrates and position them for chemical transformation. Interactions that take place within the distorted substrate complex stabilize the transition state leading to DNA hydrolysis.

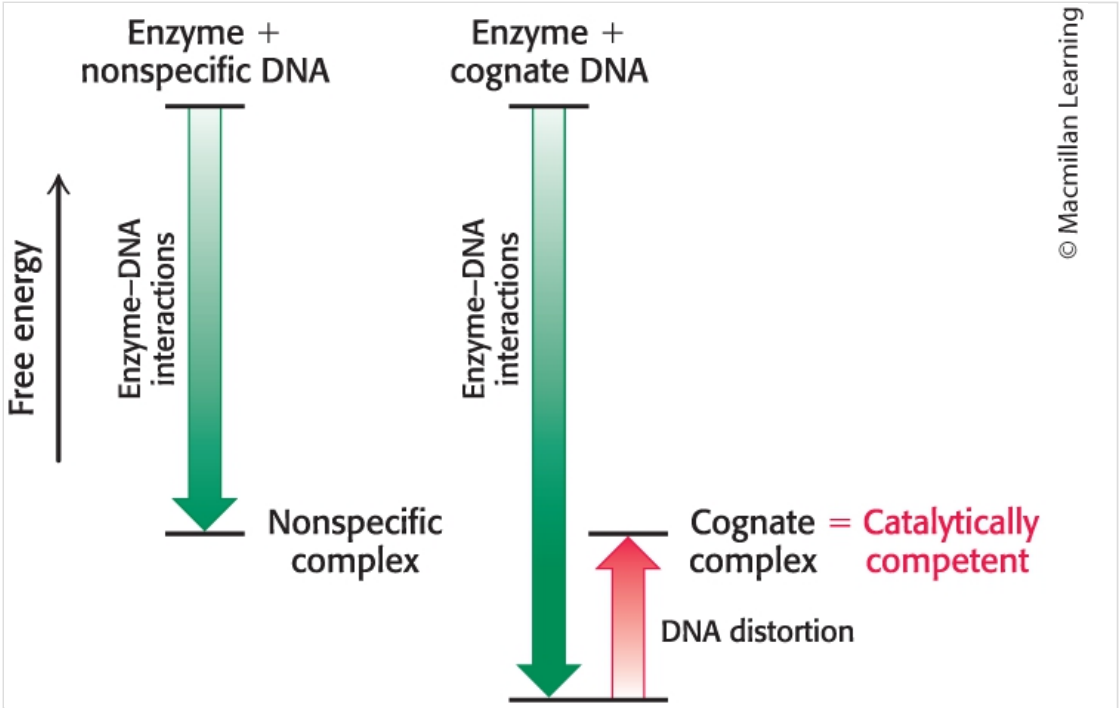


FIGURE 6.35 The binding energy drives DNA distortion. The additional interactions between EcoRV endonuclease and cognate DNA increase the binding energy, driving distortions that form a complex that is catalytically competent for DNA cleavage.

Self-Check Question

The restriction enzyme EcoRI binds DNA fragments containing its recognition site very tightly and specifically; changing even a single base results is a loss of approximately 1000-fold in binding affinity. How does this compare with EcoRV?

Host-cell DNA is protected by the addition of methyl groups to specific bases

How does a host cell harboring a restriction enzyme protect its own DNA? The host DNA is methylated on specific adenine bases within host recognition sequences by other enzymes called **DNA methylases** (**Figure 6.36**). A restriction enzyme will not cleave DNA if its recognition sequence is methylated. For each restriction enzyme, the host cell produces a corresponding methylase that marks the host DNA at the appropriate methylation site. These pairs of enzymes are referred to as **restriction-modification systems**.

FIGURE 6.36 Methylation of recognition sequences for EcoRV endonuclease protects these DN sites from cleavage by EcoRV. The specific sites are indicated by asterisks; the structure of a methylated adenine is shown on the right.

The distortion of the DNA explains how methylation blocks catalysis and protects host-cell DNA. The host *E. coli* adds a methyl group to the amino group of the adenine nucleotide at the 5′ end of the recognition sequence for EcoRV endonuclease. The presence of the methyl group blocks the formation of a hydrogen bond between the amino group and the side-chain carbonyl group of asparagine 185 in the endonuclease

(Figure 6.37). This asparagine residue is closely linked to the other amino acids that would 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. Thus, these added methyl groups prevent cleavage and protect the host DNA; the methylation reactions are slower than the DNA-cleavage reactions so that injected viral DNA is cleaved before it can be protected.

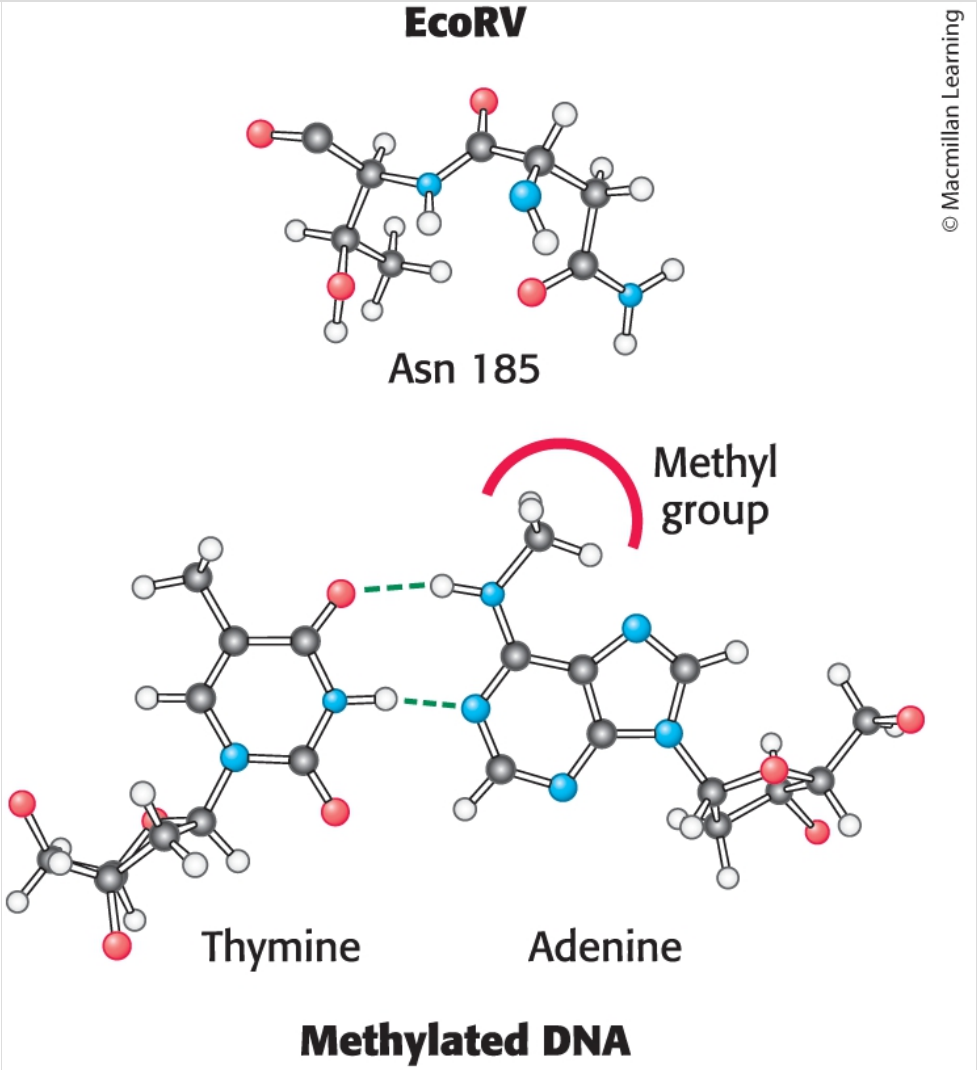
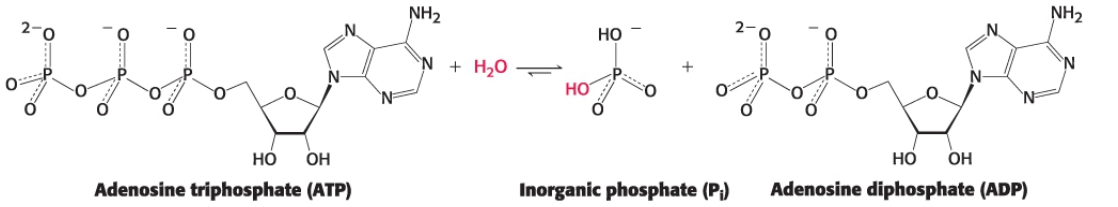


FIGURE 6.37 The methylation of adenine blocks the formation of hydrogen bonds between EcoRV endonuclease and cognate DNA molecules. This blockage prevents

the hydrolysis of methylated DNA.

6.5 Molecular Motor Proteins Harness Changes in Enzyme Conformation to Couple ATP Hydrolysis to Mechanical Work

The final enzymes that we will consider are a class of molecular motor proteins called **myosins**. These enzymes catalyze the hydrolysis of adenosine triphosphate (ATP) to form adenosine diphosphate (ADP) and inorganic phosphate (P_i) and use the energy associated with this thermodynamically favorable reaction to drive the motion of molecules within cells.



For example, when we lift a book, the energy required comes from ATP hydrolysis catalyzed by myosin in our muscles. Myosins are found in all eukaryotes, and the human genome encodes more than 40 different myosins, defined by large proteins termed myosin heavy chains. Myosins have elongated structures with globular domains that actually carry out ATP hydrolysis at one end, extended α-helical structures that promote dimer formation, and ancillary associate proteins termed light chains (**Figure 6.38**). We will focus on the globular **ATPase** domains, particularly the strategies that allow myosins to hydrolyze ATP in a

controlled manner and to use the free energy associated with this reaction to promote substantial conformational changes within the myosin molecule. These conformational changes are amplified by other structures in the elongated myosin molecules to transport proteins or other cargo substantial distances within cells.

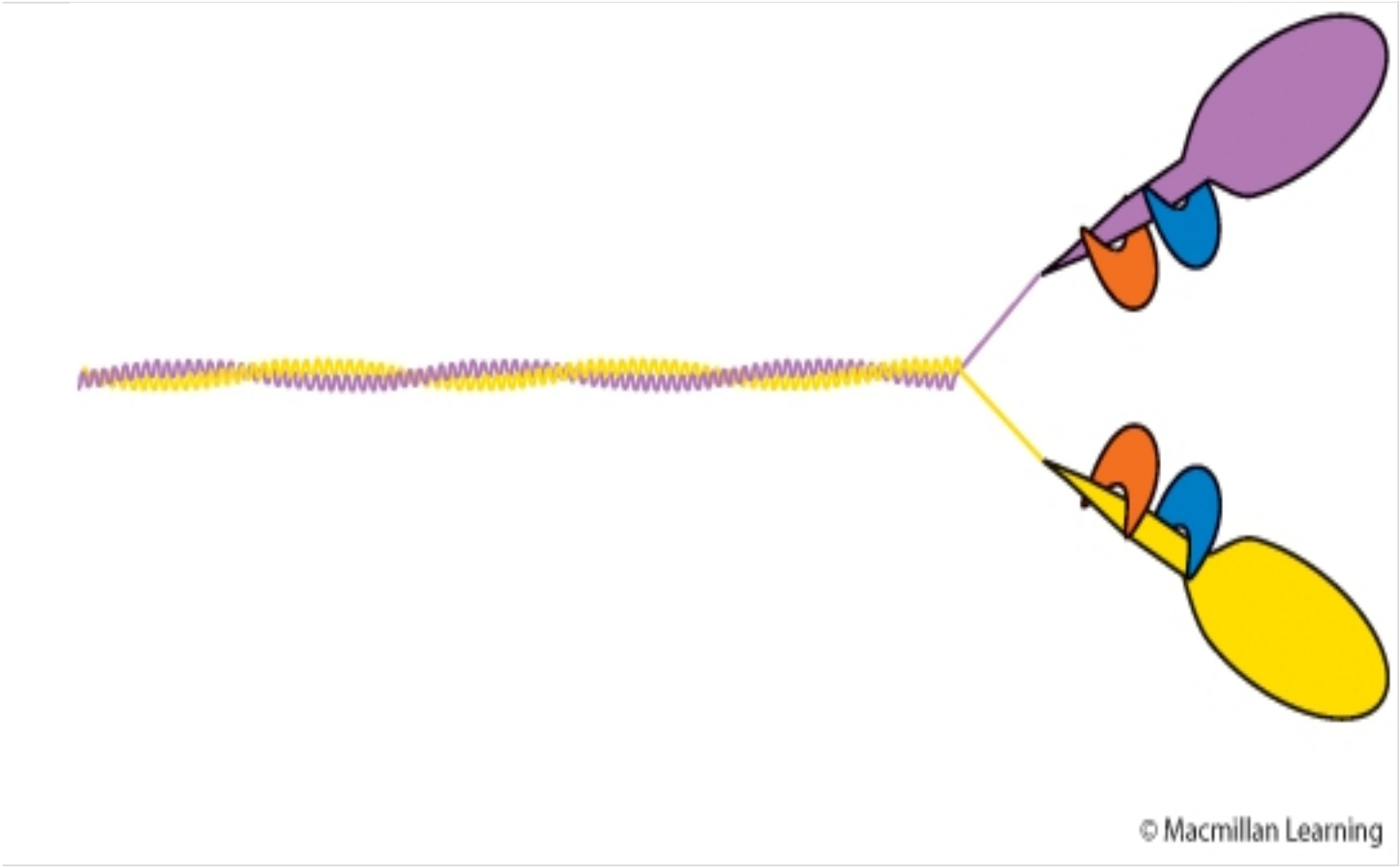

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FIGURE 6.38 A schematic view of a myosin molecule reveals an elongated dimeric structure. The two heavy chains are shown in purple and yellow, and the light chains are shown in blue and orange.

ATP is used as the major currency of energy inside cells. Many enzymes use ATP hydrolysis to drive other reactions and processes. In almost all cases, an enzyme that hydrolyzed ATP without coupling the reaction to other processes would simply drain the energy reserves of a cell without benefit.

ATP hydrolysis proceeds by the attack of water on the gamma phosphoryl group

In our examination of the mechanism of restriction enzymes, we learned that an activated water molecule performs a nucleophilic attack on phosphorus to cleave the phosphodiester backbone of DNA. The cleavage of ATP by myosins follows an analogous mechanism. To understand the myosin mechanism in more detail, we must first examine the structure of the myosin ATPase domain.

The structures of the ATPase domains of several different myosins have been examined. One such domain, that from the soil-living amoeba *Dictyostelium discoideum*, an organism that has been extremely useful for studying cell movement and molecular-motor proteins, has been studied in great detail. The crystal structure of this protein fragment in the absence of nucleotides reveals a single globular domain comprising approximately 750 amino acids. A water-filled pocket is present toward the center of the structure, suggesting a possible nucleotide-binding active site (Figure 6.39). However, when crystals of this protein were soaked in a solution containing ATP, the resulting structure reveals intact ATP bound in the proposed active site, but with very little change

in the overall structure and without evidence of significant hydrolysis. The ATP is also bound to a Mg^{2+} ion.

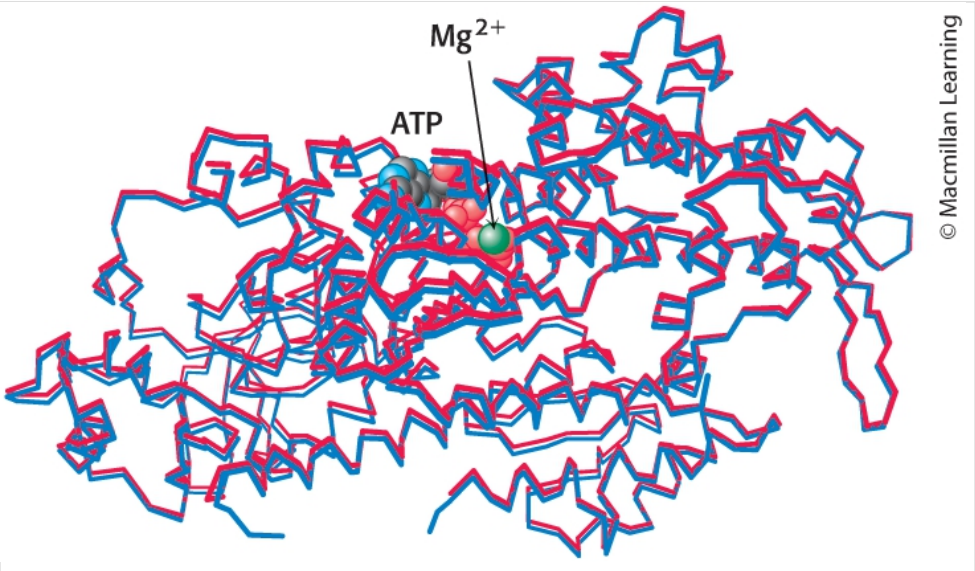


FIGURE 6.39 ATP binding to myosin does not dramatically alter its structure. An overlay of the structures of the ATPase domain from *Dictyostelium discoideum* myosin with nothing bound (blue) and the complex of this protein with ATP and magnesium bound (red) reveals that these two structures are quite similar to one another.

[Drawn from 1FMV.pdb and 1FMW.pdb.]

INTERACT with this model in Achieve

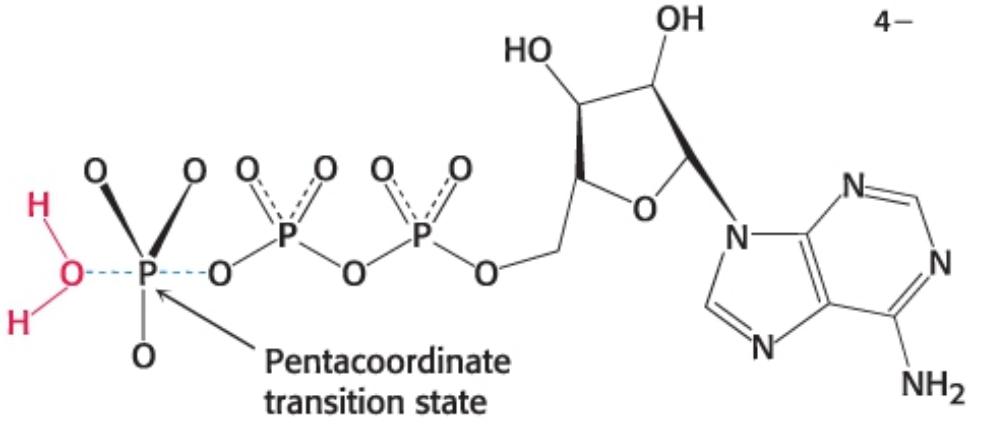
Kinetic studies of myosins, as well as many other enzymes having ATP or other nucleoside triphosphates as a substrate, reveal that these enzymes are essentially inactive in the absence of divalent metal ions such as magnesium (Mg^{2+}) or manganese (Mn^{2+}) but acquire activity on the addition of these 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 (NTP) are present as NTP–Mg^{2+} complexes. Magnesium or manganese complexes of nucleoside triphosphates are the true substrates for essentially all NTP-dependent enzymes.

Since the ATP–Mg^{2+} complex was present in the crystallography study discussed above, why was there no evidence for hydrolysis? The nucleophilic attack by a water molecule on the γ-phosphoryl group requires some mechanism to activate the water, such as a basic residue or a bound metal ion. Examination of the myosin–ATP complex structure shows no basic residue in an appropriate position and reveals that the bound Mg^{2+} ion is too far away from the phosphoryl group to play this role. These observations suggest why the ATP complex is relatively stable: The enzyme is not in a conformation that is suitable for promoting the ATP-hydrolysis reaction. This conclusion suggests that the domain must undergo a conformational change in order to carry out catalysis.

Formation of the transition state for ATP hydrolysis is associated with a substantial conformational change

The catalytically competent conformation of the myosin ATPase domain must bind and stabilize the transition state of the reaction. In analogy with restriction enzymes, we expect that ATP hydrolysis includes a pentacoordinate transition state.



Such pentacoordinate structures based on phosphorus are too unstable to be readily observed. However, transition-state analogs in which other atoms replace phosphorus are more stable. The transition metal vanadium, in particular, forms similar structures. After crystallizing the myosin ATPase domain in the presence of ADP and vanadate, VO_4^{3-}, a complex forms that closely matches the expected transition-state structure (Figure 6.40).

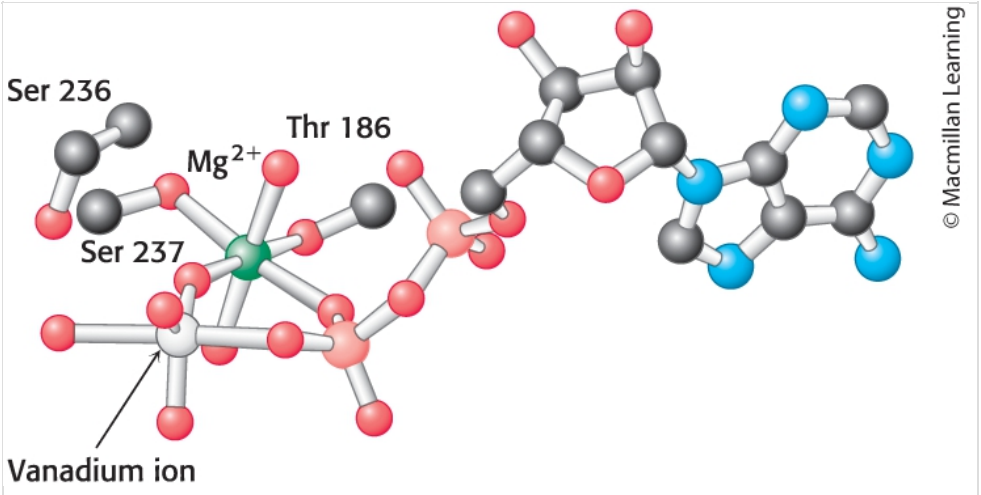


FIGURE 6.40 A stable structural analog of the myosin transition state can be prepared. The transition-state analog formed by treating the myosin ATPase domain

with ADP and vanadate (VO_4^{3-}) in the presence of magnesium shows the vanadium ion (analogous to phosphorus) coordinated to five oxygen atoms including one from ADP. The positions of two residues that bind magnesium as well as Ser 236, a residue that appears to play a direct role in catalysis, are shown.

[Drawn from 1VOM.pdb.]

As expected, the vanadium atom is coordinated to five oxygen atoms, including one oxygen atom from ADP diametrically opposite an oxygen atom that is analogous to the attacking water molecule in the transition state. The Mg^{2+} ion is coordinated to one oxygen atom from the vanadate, one oxygen atom from the ADP, two hydroxyl groups from the enzyme, and two water molecules. In this position, this ion does not appear to play any direct role in activating the attacking water. However, an additional residue from the enzyme, Ser 236, is well positioned to play a role in catalysis (Figure 6.41).

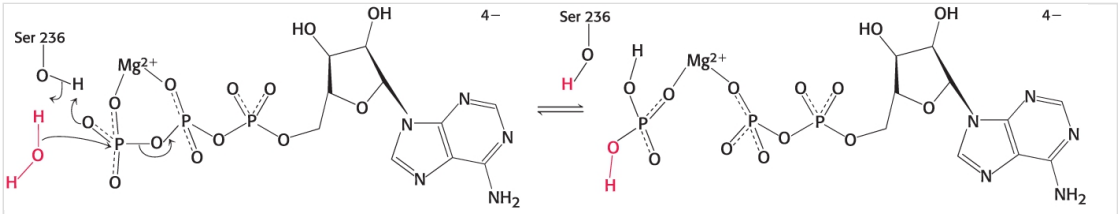


FIGURE 6.41 Water attack is facilitated by Ser 236. The attack of the water molecule on the γ-phosphoryl group of ATP is facilitated by Ser 236, which helps to deprotonate the water molecule. The serine sidechain, in turn, is deprotonated by one of the oxygen atoms of the γ-phosphoryl group forming the $H_2PO_4^-$ product.

In the proposed mechanism of ATP hydrolysis based on this structure, the water molecule attacks the γ-phosphoryl group, with the hydroxyl

group of Ser 236 facilitating the transfer of a proton from the attacking water to the hydroxyl group of Ser 236, which, in turn, is deprotonated by one of the oxygen atoms of the γ-phosphoryl group. Thus, in effect, the ATP serves as a base to promote its own hydrolysis.

Comparison of the overall structures of the myosin ATPase domain complexed with ATP and with the ADP–vanadate reveals some remarkable differences. Relatively modest structural changes occur in and around the active site. In particular, a stretch of amino acids moves closer to the nucleotide by approximately 2 Å and interacts with the oxygen atom that corresponds to the attacking water molecule. These changes aid the hydrolysis reaction by stabilizing the transition state. However, examination of the overall structure shows even more striking changes.

A region comprising approximately 60 amino acids at the carboxyl-terminus of the domain adopts a different configuration in the ADP–vanadate complex, displaced by as much as 25 Å from its position in the ATP complex (**Figure 6.42**). This displacement tremendously amplifies the relatively subtle changes that take place in the active site, and it even affects locations beyond from the ATPase domain, because the carboxyl-terminus is connected to other atoms within the elongated structures typical of myosin molecules (Figure 6.38). Thus, the conformation that is capable of promoting the ATP hydrolysis reaction is substantially different from other conformations that are present in the course of the catalytic cycle.

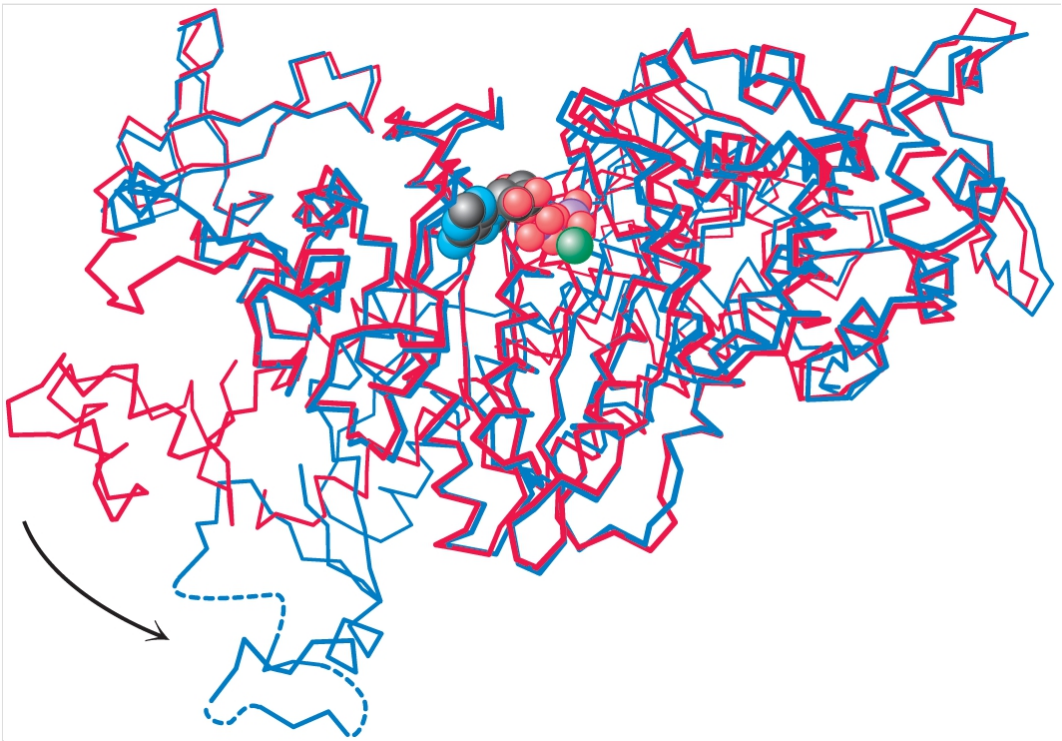


FIGURE 6.42 A substantial conformation change is associated with the formation of the myosin transition state. A comparison of the overall structures of the myosin ATPase domain with ATP bound (shown in red) and that with the transition-state analog ADP–vanadate (shown blue) reveals dramatic conformational changes of a region at the carboxyl-terminus of the domain (see arrow), some parts of which move as much as 25 Å.

[Drawn from 1FMW.pdb and 1VOM.pdb.]

The altered conformation of myosin persists for a substantial period of time

Myosins are slow enzymes, typically turning over approximately once per second. What steps limit the rate of turnover? In a particularly revealing experiment, the hydrolysis of ATP was catalyzed by the

myosin ATPase domain from mammalian muscle. The reaction took place in water labeled with ^{18}O to track the incorporation of solvent oxygen into the reaction products; then the fraction of oxygen in the phosphate product was analyzed. In the simplest case, the phosphate would be expected to contain one oxygen atom derived from water and three initially present in the terminal phosphoryl group of ATP. Instead, between two and three of the oxygen atoms in the phosphate were found, on average, to be derived from water. These observations indicate that the ATP hydrolysis reaction within the enzyme active site is reversible. Each molecule of ATP is cleaved to ADP and P_i and then re-formed from these products several times before the products are released from the enzyme (Figure 6.43).

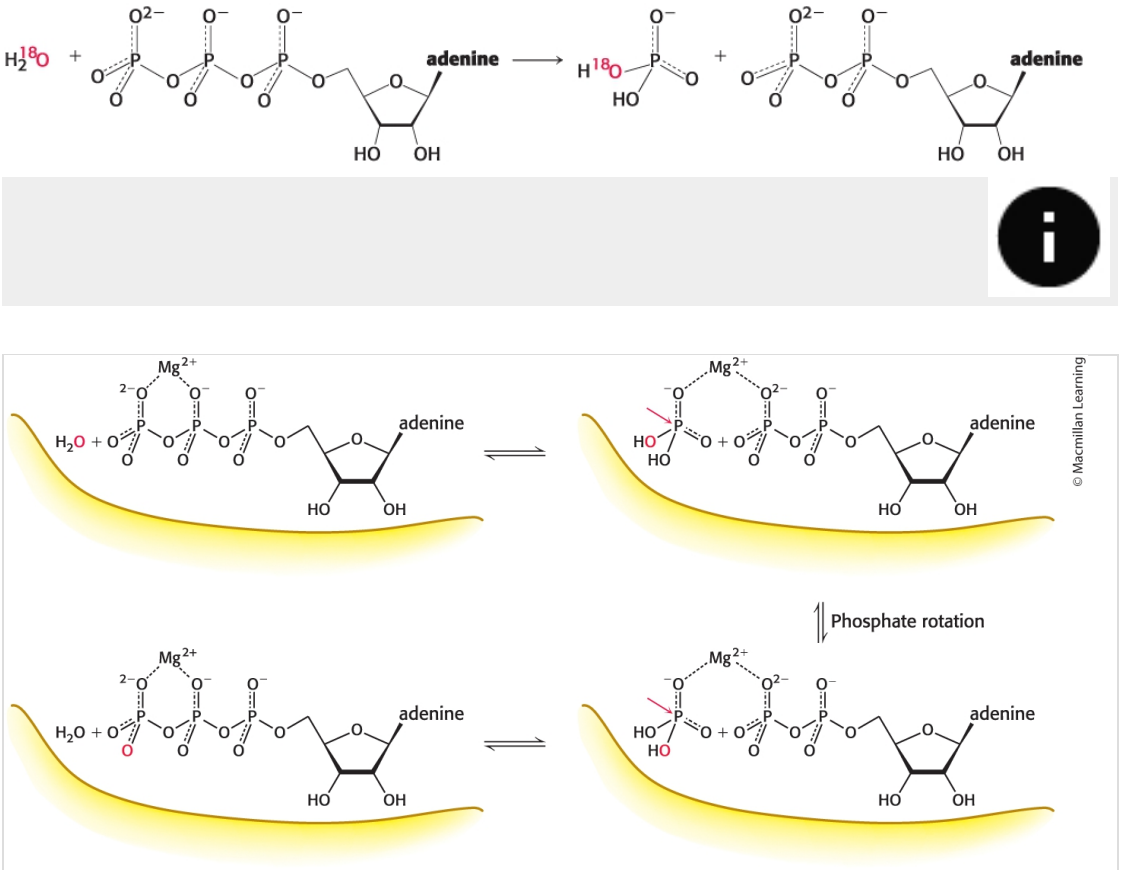


FIGURE 6.43 The hydrolysis of ATP is reversible within the active site of myosin. This is revealed by the fact that more than one atom of oxygen from water is incorporated in inorganic

phosphate. The oxygen atoms are incorporated in cycles of hydrolysis of ATP to ADP and inorganic phosphate, phosphate rotation within the active site, and reformation of ATP now containing oxygen from water.

At first glance, this observation is startling because ATP hydrolysis is a very favorable reaction with an equilibrium constant of approximately 140,000. However, this equilibrium constant applies to the molecules free in solution, not within the active site of an enzyme. Indeed, further analysis suggests that this equilibrium constant on the enzyme is approximately 10.

This result illustrates a general strategy used by enzymes: Enzymes catalyze reactions by stabilizing the transition state. The structure of this transition state is intermediate between the enzyme-bound reactants and the enzyme-bound products. Many of the interactions that stabilize the transition state will help equalize the stabilities of the reactants and the products. Thus, the equilibrium constant between enzyme-bound reactants and products usually 10 or below and is often close to 1, regardless of the equilibrium constant for the reactants and products free in solution.

These observations reveal that the hydrolysis of ATP to ADP and P_i is not the rate-limiting step for the reaction catalyzed by myosin. Instead, the release of the products, particularly P_i, from the enzyme is rate limiting. The fact that a conformation of myosin with ATP hydrolyzed but still bound to the enzyme persists for a significant period of time is critical for coupling conformational changes that take place in the course of the reaction to other processes.

Self-Check Question

Myosin is treated with ATP in the presence of water labelled with $H_2{}^{18}O$. After a period of time, the remaining ATP is isolated and found to contain ^{18}O. Explain.

Actin forms filaments along which myosin can move

Myosin molecules use the free energy of hydrolysis of ATP to drive their own macroscopic movement along a filamentous protein termed *actin*, a 42-kDa protein that very abundant in eukaryotic cells, typically accounting for as much as 10% of the protein. Actin monomers come together to form actin filaments (**Figure 6.44**). Actin filaments have helical structures; each monomer is related to the preceding one by a translation of $27.5\ \text{Å}$ and a rotation of 166 degrees around the helical axis. Because the rotation is nearly 180 degrees, F-actin resembles a two-stranded cable. Note that each actin monomer is oriented in the same direction along the actin filament, and so the structure is polar, with discernibly different ends. One end is called the barbed (plus) end, and the other is called the pointed (minus) end. Each actin monomer contains a bound nucleotide, ATP or ADP.

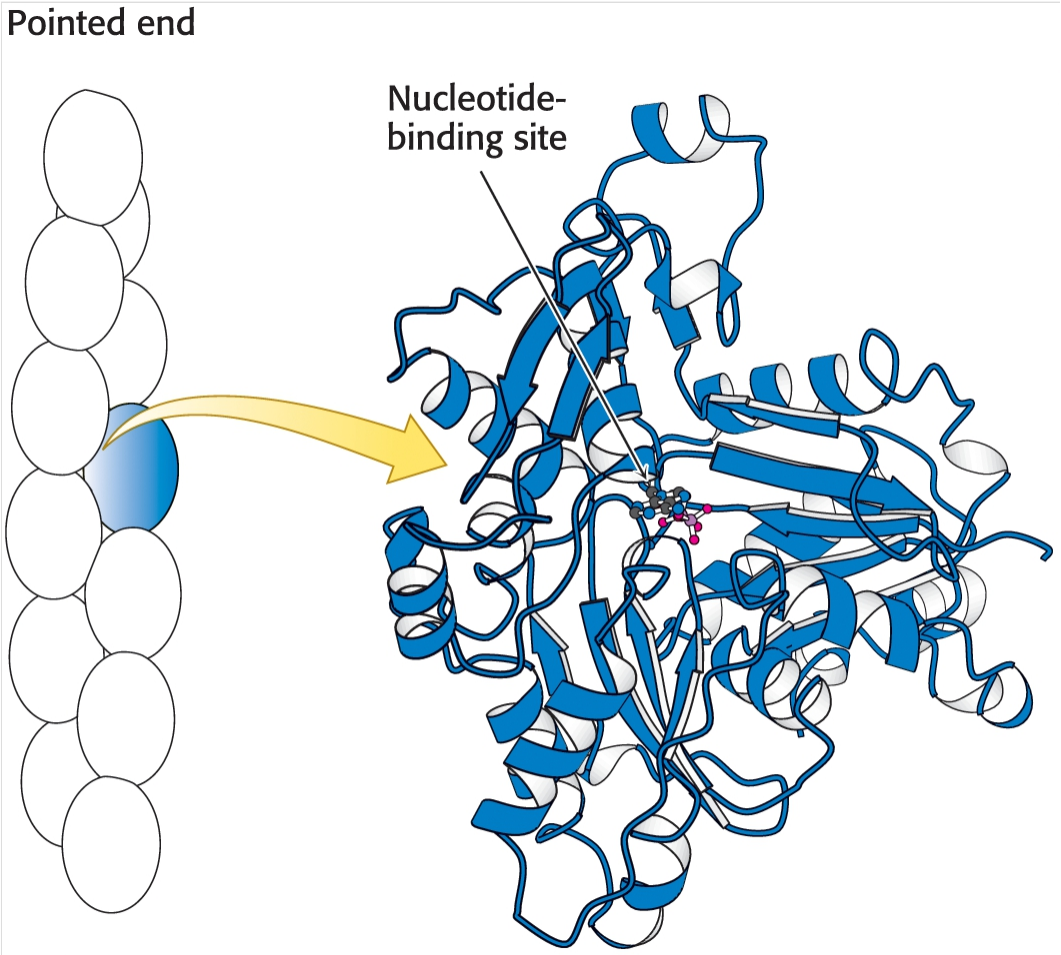


FIGURE 6.44 An actin filament has a polymeric structure. In the schematic view at left, actin monomers are depicted as ovals. The detailed structure of one actin monomer (blue) is shown a right.

[Drawn from 1J6Z.pdb.]

INTERACT with this model in Achieve

Using a variety of physical methods, scientists have been able to watch single myosin molecules moving along actin filaments. For example, a myosin family member termed myosin V can be labeled with

fluorescent tags so that it can be localized when fixed on a surface with a precision of less than 15 Å. When this myosin in the absence of ATP is placed on a surface coated with actin filaments, each molecule remains in a fixed position. However, when ATP is added, each molecule moves along the surface. Tracking individual molecules reveals that each moves in steps of approximately 74 nm (Figure 6.45). The observation of steps of a fixed size as well as the determination of this step size helps reveal details of the mechanism of action of these tiny molecular machines.

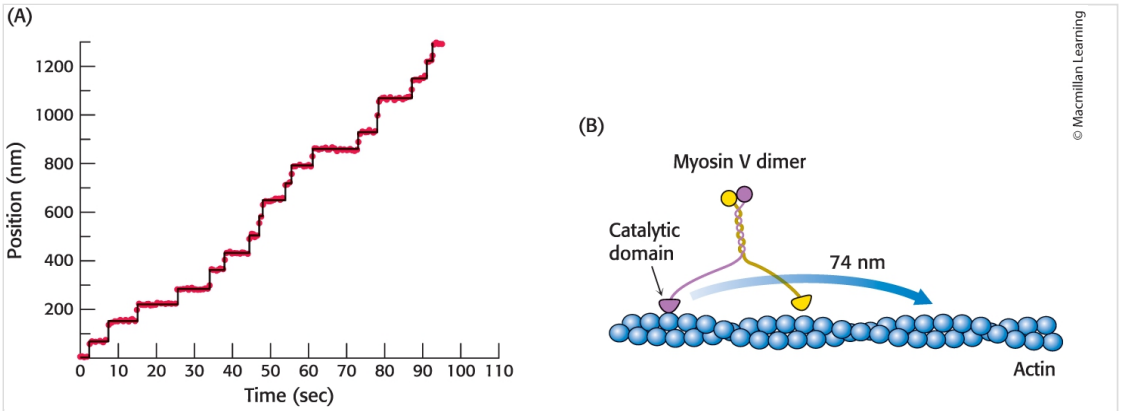


FIGURE 6.45 A molecular motor such as myosin V moves in discrete steps. (A) A trace of the position of a single dimeric myosin V molecule as it moves across a surface coated with actin filaments. (B) A model of how the dimeric molecule moves in discrete steps with an average size of 74 ± 5 nm.

How does ATP hydrolysis drive this motion? A key observation is that the addition of ATP to a complex of myosin and actin results in the dissociation of the complex. Thus, ATP binding and hydrolysis cannot be directly responsible for the power stroke. We can combine this fact with observations from three-dimensional structures of myosin in various forms to construct a mechanism for the motion of myosin along actin (Figure 6.46).

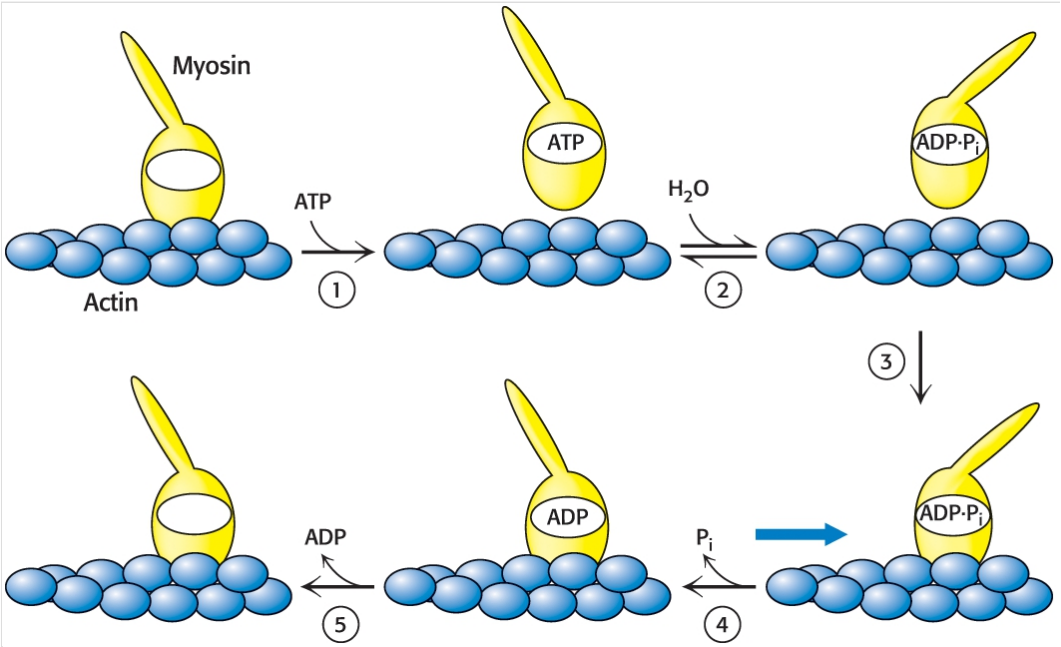


FIGURE 6.46 The mechanism by which myosin moves along an actin filament involves five steps. The binding of ATP (1) results in the release of myosin (yellow) from actin (blue). The reversible hydrolysis of ATP bound to myosin (2) can result in the reorientation of the lever arm. With ATP hydrolyzed but still bound to actin, myosin can bind actin (3). The release of P_i (4) results in the reorientation of the lever arm and the concomitant motion of actin relative to myosin. The release of ADP (5) completes the cycle.

Let us begin with nucleotide-free myosin bound to actin. The binding of ATP to actin results in the dissociation of myosin from actin. With ATP bound and free of actin, the myosin domain can undergo the conformational change associated with the formation of the transition state for ATP hydrolysis. This conformational change results in the reorientation of the lever arm. In this form, the myosin head can dock onto the actin filament; phosphate is released with an accompanying motion of the lever arm. This conformational change represents the power stroke and moves the body of the myosin molecule relative to the actin filament. The release of ADP completes the cycle.

The most well-studied actin-myosin motor system is that from skeletal muscle (Figure 6.47). Muscle contains organized assemblies of actin molecules, referred to as thin filaments, together with multiheaded assemblies of myosin molecules that form thick filaments. Upon activation, the myosin motors move along the actin filaments to cause the combined assembly to contract.

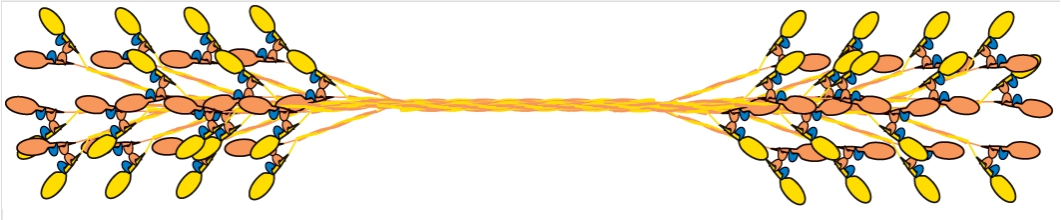


FIGURE 6.47 A myosin assembly from muscle has a two-headed structure. A schematic view shows how multiple myosin molecules come together to form the thick filament.

Other cells contain elaborate meshworks of actin filaments that form an internal cellular skeleton or **cytoskeleton**, which plays a major role in determining cell shape. Two techniques for observing the cytoskeleton have traditionally been used, but each has shortcomings: Electron microscopy can produce clear images but cannot be performed on living cells, whereas light microscopy that visualizes fluorescent tags attached to actin can be performed on living cells, but the clarity of the images is limited by the relatively long wavelengths of visible light.

Recently, scientists have developed new microscopic methods based on the principle that the position of an isolated fluorescent molecule can be determined much more precisely than the image produced by monitoring the fluorescence. These methods have produced beautiful pictures of the intricate tracks inside living cells along which molecular motors can move (Figure 6.48). With these new tools, scientists can now ask new questions about the elaborate movements that occur within living cells driven by molecular motors.

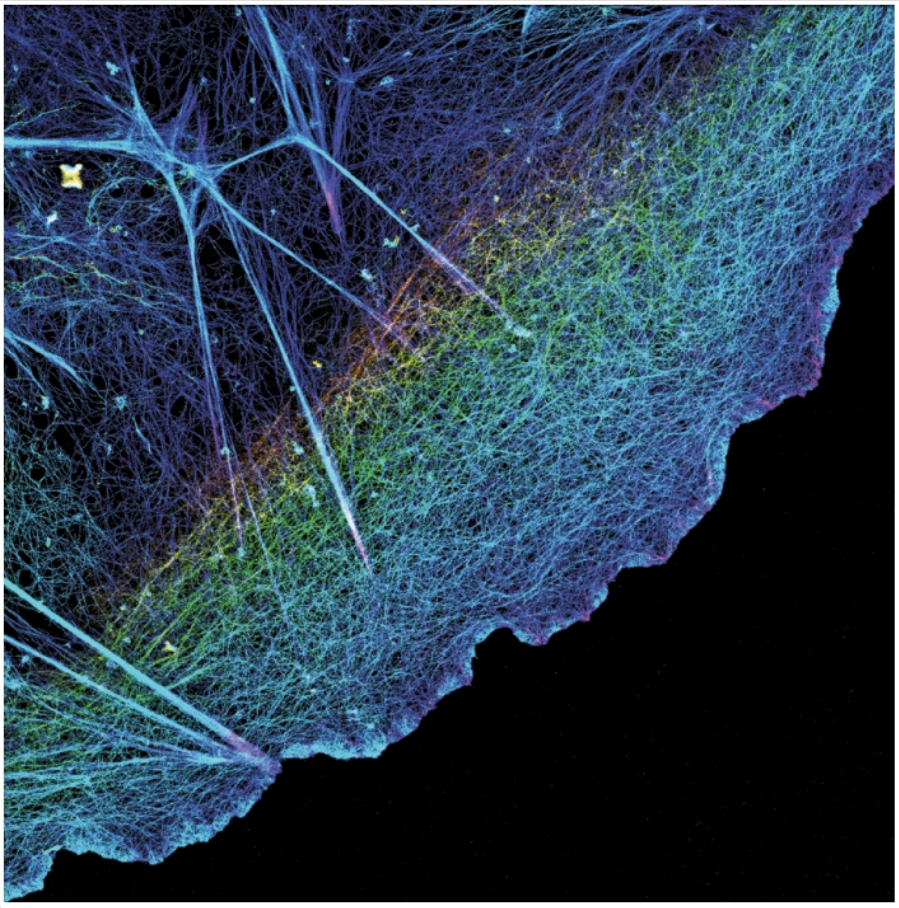


Image Courtesy of Ke Xu, University of California, Berkeley, and Xiaowei Zhuang, Harvard University

FIGURE 6.48 Actin networks can be imaged in great detail using modern fluorescence microscopy methods. A section of a live mammalian fibroblast cell with tagged actin clearly reveals individual actin filaments.

Chapter 6 Summary

6.1 Enzymes Use a Core Set of Catalytic Strategies

- Enzymes adopt conformations that are structurally and chemically complementary to the transition states of the reactions that they catalyze.
- Enzymes use five basic strategies to form and stabilize the transition state: the use of binding energy to promote both specificity and catalysis, covalent catalysis, general acid–base catalysis, catalysis by approximation, and metal ion catalysis.

6.2 Proteases Facilitate a Fundamentally Difficult Reaction

- The cleavage of peptide bonds by chymotrypsin is initiated by the attack by a serine residue on the peptide carbonyl group with the serine activated by a catalytic triad of Ser-His-Asp.
- The product of this initial reaction is a covalent intermediate formed by the enzyme and an acyl group derived from the bound substrate that is subsequently hydrolyzed.
- Tetrahedral intermediates that occur during these reactions have a negative charge on the peptide carbonyl oxygen atom that is stabilized by interactions with peptide NH groups in a region on the enzyme termed the oxyanion hole.
- Some other proteases employ the same catalytic strategy based on a similar catalytic triad, including homologs of chymotrypsin such as trypsin and elastase and other proteins such as subtilisin that evolved the same catalytic apparatus independently.

6.3 Carbonic Anhydrases Make a Fast Reaction Faster

- Carbonic anhydrases catalyze the reaction of water with carbon dioxide at rates as high as 1 million times per second to generate bicarbonate ion which can be protonated to form carbonic acid.
- A tightly bound zinc ion binds a water molecule, promotes its deprotonation to generate a hydroxide ion at neutral pH, and uses this nucleophile to attack carbon dioxide.
- To overcome limitations imposed by the rate of proton transfer from the zinc-bound water, the most-active carbonic anhydrases have evolved a proton shuttle to transfer protons to buffer components in solution.

6.4 Restriction Enzymes Catalyze Highly Specific DNA-Cleavage Reactions

- Restriction enzymes that cleave DNA at specific recognition sequences discriminate between molecules that contain these recognition sequences and those that do not.
- Restriction enzymes use magnesium ions to bind and activate a water molecule to attacks the phosphodiester backbone and facilitate DNA cleavage.
- EcoRV distorts DNA molecules containing the proper sequence are in a manner that allows magnesium ion binding and, hence, DNA cleavage.
- Restriction enzymes are prevented from acting on the DNA of a host cell by the methylation of key sites within its recognition sequences, blocking specific interactions between the enzymes and the DNA.

6.5 Molecular Motor Proteins Harness Changes in Enzyme Conformation to Couple ATP Hydrolysis to Mechanical Work

- Molecular motors called myosins catalyze the hydrolysis of adenosine triphosphate, using the free energy generated to drive large-scale molecular movement.

- Myosin associated with the transition state for ATP hydrolysis has a distinct structure indicating a large-scale conformational change.
- The rate of ATP hydrolysis by myosin is relatively low and is limited by the rate of product release from the enzyme.
- Myosins move along a filamentous protein called actin, binding and releasing actin filaments over the course of its catalytic cycle to facilitate movement.
- Actin filaments occur in muscle and also form a cytoskeleton inside eukaryotic cells along which myosins can move; the cytoskeleton also functions like scaffolding, giving the cell its shape.

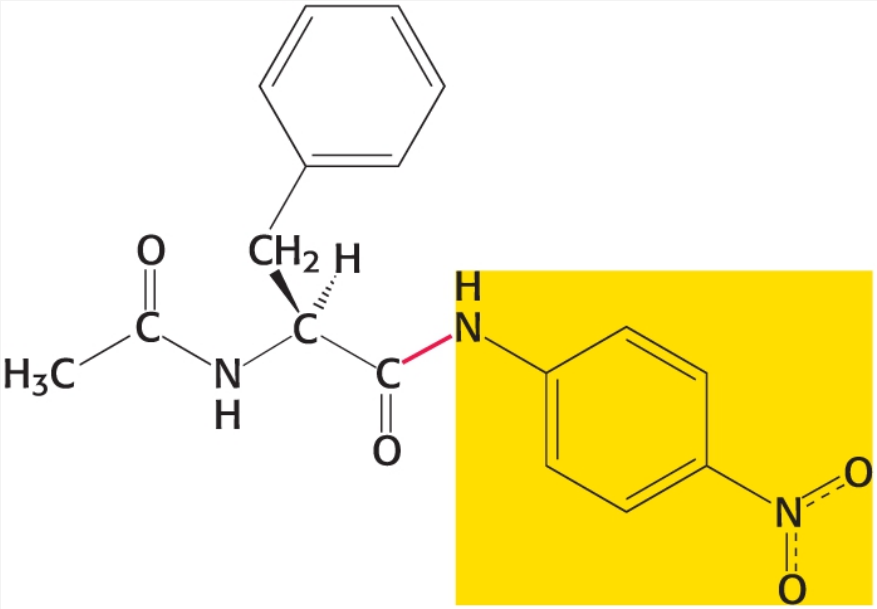
Key Terms

binding energy
induced fit
covalent catalysis
general acid–base catalysis
catalysis by approximation
metal ion catalysis
protease
electrophilic
nucleophilic
chemical modification reaction
chromogenic substrate
catalytic triad
oxyanion hole
scissile bond
protease inhibitor
carbonic anhydrase
ligand

proton shuttle
restriction enzyme (restriction endonuclease)
recognition sequence
DNA methylase
restriction-modification system
myosin
ATPase
cytoskeleton

Problems

1. Examination of the cleavage of the amide substrate, A, by chymotrypsin with the use of stopped-flow kinetic methods reveals no burst. The reaction is monitored by noting the color produced by the release of the amino part of the substrate (highlighted in orange). Why is no burst observed? 1, 6



2. Consider the subtilisin substrates A and B.

Phe-Ala-Gln-Phe-X	Phe-Ala-His-Phe-X
A	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. 2

3. 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 decrease. 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. 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 mutation?

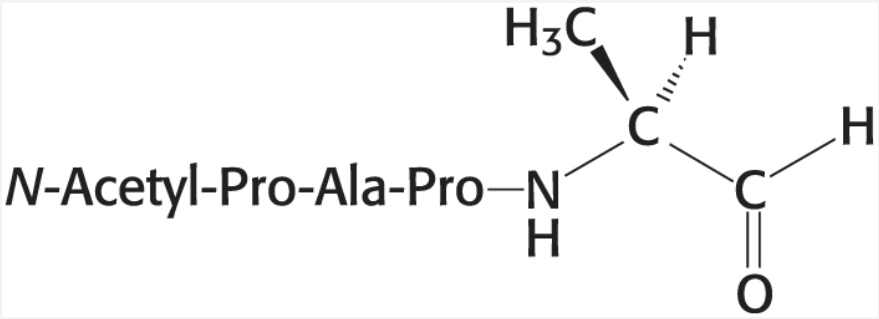
5. In carbonic anhydrase II, mutation of the proton-shuttle residue His 64 to Ala was expected to result in a decrease in the maximal catalytic rate. However, in buffers such as imidazole

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. 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 such that they act faster than do methylases. Would this increased rate be beneficial to host cells, assuming that the fast enzymes have similar levels of specificity?

7. Treatment of carbonic anhydrase with high concentrations of the metal chelator EDTA (ethylenediaminetetraacetic acid) results in the loss of enzyme activity. Propose an explanation.
2, 3

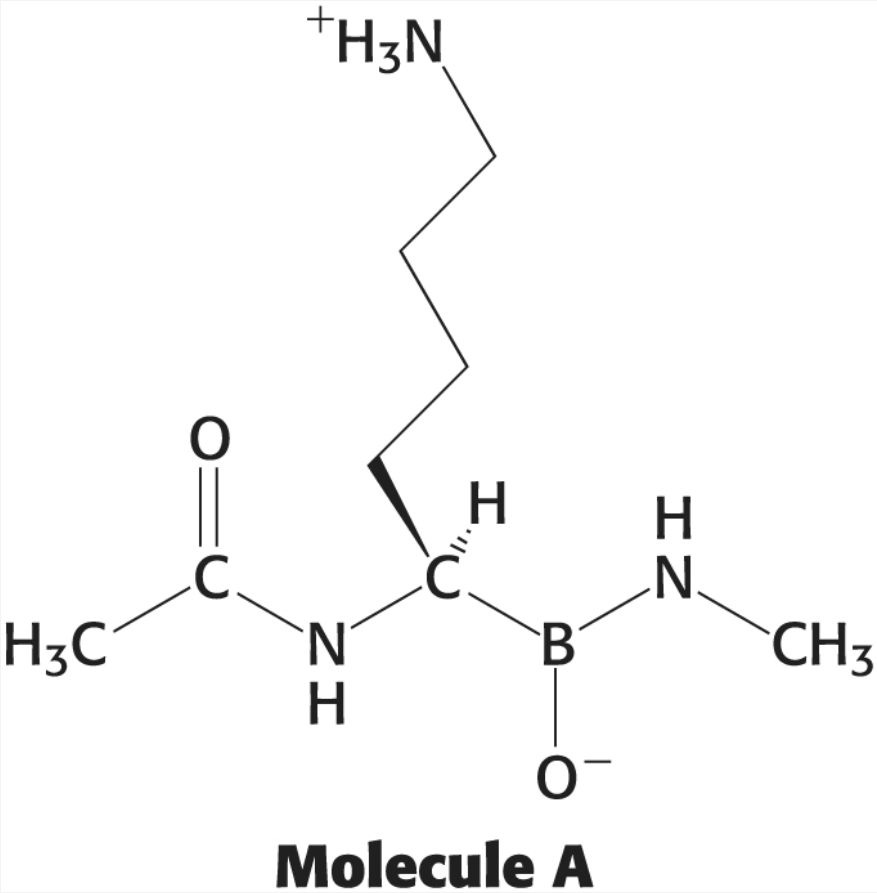
8. Elastase is specifically inhibited by an aldehyde derivative of one of its substrates:



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?

9. Use the provided structure of molecule A to complete the passage.

Molecule A contains a side chain similar to that of _____. Molecule A effectively inhibits _____ by binding to the _____ residue in the enzyme's S_1 pocket.
2



Molecule A

10. At pH 7.0, carbonic anhydrase exhibits a k_{cat} of $60{,}000\ s^{-1}$. Estimate the value expected for k_{cat} at pH 6.0.

11. To terminate a reaction in which a restriction enzyme cleaves DNA, researchers often add high concentrations of the metal chelator EDTA (ethylenediaminetetraacetic acid). Why does the addition of EDTA terminate the reaction?

12. Many patients become resistant to HIV protease inhibitors with the passage of time, owing to mutations in the HIV gene that encodes the protease. Mutations are not found in the aspartate residue characteristic of aspartyl proteases. Why not? 1, 2

13. Serine 236 in *Dictyostelium discoideum* myosin has been mutated to alanine. The mutated protein showed modestly reduced ATPase activity. Analysis of the crystal structure of the mutated protein revealed that a water molecule occupied the position of the hydroxyl group of the serine residue in the wild-type protein. Propose a mechanism for the ATPase activity of the mutated enzyme. 1, 2, 6

14. The catalytic power of an enzyme can be defined as the ratio of the rate of the enzyme catalyzed reaction to that for the uncatalyzed reaction. Using the information in Figure 6.15 for subtilisin and in Figure 6.19 for carbonic anhydrase, calculate the catalytic powers for these two enzymes.

15. Would a reaction catalyzed by a version of subtilisin with all three residues in the catalytic triad mutated have less or more

activity compared to the uncatalyzed reaction (see Figure 6.15)? Propose an explanation for any remaining catalytic power.

16. On the basis of the information provided in Figure 6.16, complete the mechanisms for peptide-bond cleavage by

a. a cysteine protease,
b. an aspartyl protease, and
c. a metalloprotease. 1, 2

17. Most enzymes are quite specific, catalyzing a particular reaction on a set of substrates that are structurally quite similar to one another. Discuss why this is advantageous from a biological perspective. Suggest why this is likely to be true from a chemical perspective. Why would it be difficult to evolve an enzyme with high catalytic activity but low specificity? 4

18. In the absence of actin, myosin will hydrolyze ATP, but very slowly. Based on the mechanism shown in Figure 6.46, propose an explanation for this slow rate.