7

Enzyme Mechanisms

High serum cholesterol can result from diets that are rich in cholesterol-containing foods or from biosynthesis of cholesterol in the liver. Studies have shown that excess serum cholesterol is associated with blockage of arteries and cardiovascular disease. High serum cholesterol is often treated with statin drugs. These drugs function by inhibiting the enzyme 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase, which catalyzes an early step in the cholesterol biosynthetic pathway.

Inhibition of HMG-CoA reductase lowers intracellular cholesterol levels by reducing biosynthesis. Importantly, when intracellular cholesterol is lowered, this triggers increased uptake of cholesterol from the serum and thereby reduces the circulating cholesterol that contributes to arterial plaques and heart disease.

CHAPTER OUTLINE

CREDITS: ATHLETIC WOMAN: ALIN DRAGULIN/ALAMY; FRIED EGG: MIGUEL GARCIA SAAVEDRA/SHUTTERSTOCK; STEAK ON A FORK: FOODCOLLECTION/AGEFOTOSTOCK; ATORVASTATIN: BASED ON PDB FILE 1HWK; WHITE PILLS: GREG WRIGHT/ALAMY.

Life depends on an enormous number of highly integrated and regulated chemical reactions. However, on their own, these reactions do not always happen quickly. Enzymes are the necessary biological catalysts that accelerate the rates of these chemical reactions under physiologic conditions. Indeed, without enzymes, metabolic reactions would be so slow—some requiring millions of years—that life would not be possible.

Enzymes increase the rates of chemical reactions, but they are not consumed by the reactions, nor do they alter the equilibrium concentration ratio of substrates and products. Most enzymes are proteins, although some enzymes, called ribozymes, are composed of RNA (see Chapter 21). Enzyme kinetics, discussed in this chapter, is a quantitative analysis of enzyme function that can be used to probe reaction mechanisms and to compare the efficiency of closely related enzymes under a variety of conditions.

We begin this chapter with an overview of enzymes and their structure and function, then proceed to discuss key concepts in enzyme reaction mechanisms and enzyme kinetics. The chapter concludes with a description of the most common enzyme regulatory mechanisms. Enzymes provide the catalytic power for living cells to perform chemical reactions on a timescale of microseconds to minutes, but enzyme function must be highly regulated to avoid biochemical chaos.

In keeping with the protein biochemistry focus of Part 2 of this book, we present enzyme mechanisms in the context of protein structure and function. Throughout the chapter, we emphasize the chemical properties of functional groups that directly or indirectly contribute to the catalytic efficiency of enzymes.

7.1 Overview of Enzymes

The discovery that proteins can function as enzymes—once called the *ferments of zymes* (from yeast)—was not made until the 1930s, nearly 100 years after proteins were first characterized. The reluctance to accept the notion that proteins can function as biological catalysts was rooted in the way biochemists approached the study of biomolecules. Early biochemists thought of proteins as structural components in cells and focused their research on the most abundant proteins, such as keratin and albumin, which could be readily purified and analyzed by acid hydrolysis. At the same time, other biochemists were interested in measuring rates of chemical reactions in cell-free extracts and isolating organic reaction intermediates formed by mysterious catalytic "enzymes." These two areas of research did not overlap or intersect for quite a long time.

The understanding that most enzymes were proteins did not come about until a controversial report by James Sumner in 1926. Based on his experiments, in which he was able to purify and crystallize the enzyme urease from jack beans, he proposed that urea hydrolysis was mediated by a protein enzyme (**Figure 7.1**). Sumner's work

a.

b.

c.

Figure 7.1 James Sumner provided the first evidence that a protein can function as an enzyme when he purified and crystallized the enzyme urease from jack bean protein extracts. Crystallization of the protein was an important step to demonstrate its purity. **a.** James Sumner (1887–1955) was a biochemist at Cornell University when he first purified urease in 1926. SPL/SCIENCE SOURCE. **b.** Jack beans (*Canavalia ensiformis*) contain high levels of the enzyme urease. SEAN SPRAGUE/ALAMY. **c.** Protein crystals of purified urease isolated from jack beans. A. BALASUBRAMANIAN AND K. PONNURAJ (2009). PURIFICATION, CRYSTALLIZATION, AND PRELIMINARY X-RAY ANALYSIS OF UREASE FROM JACK BEAN *CANAVALIA ENSIFORMIS*. *ACTA CRYSTALLOGRAPHICA*, *65*, 949–951. HTTP://JOURNALS.IUCR.ORG. © 2009 INTERNATIONAL UNION OF CRYSTALLOGRAPHY.

was followed 3 years later by that of John Northrop, who was able to prepare the digestive enzyme pepsin in a highly purified form. With pure preparations of proteins, Sumner's earlier discovery that proteins can function as enzymes could be confirmed. Sumner and Northrop, along with the virologist Wendell Stanley, shared the 1946 Nobel Prize in Chemistry for their combined work on protein purification and characterization.

Our current understanding of how enzymes catalyze biochemical reactions follows from these early studies and relies heavily on analyzing three-dimensional protein structures in the context of enzyme kinetics. However, as described in Chapter 6, the three-dimensional structure of a protein does not by itself reveal the dynamic processes required for it to function. Nothing exemplifies this more than the structure of enzymes. Up until 1958, biochemists thought that the observed high specificity of enzyme-mediated catalysis was best explained by rigid physical and chemical complementarity between the reactant, usually referred to as the substrate, and the enzyme. This older view, called the **lock and key model** of enzyme specificity, was first described by the German chemist Emil Fischer in 1894. However, the lock and key model could not explain how enzymes are regulated or how substrates can bind to sites buried deep within the interior of a protein.

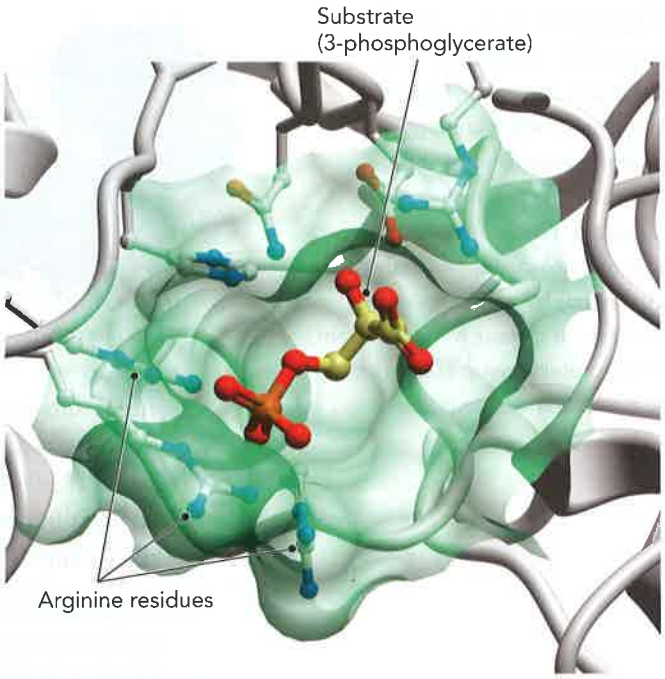


Figure 7.2 Enzyme active sites provide chemical environments that facilitate catalytic reactions by excluding excess solvent and bringing the reactive functional groups of the enzyme into close proximity to the substrate. The molecular structure of the enzyme phosphoglycerate kinase, shown here, highlights the substrate binding site of the enzyme (shaded in green), in which several key amino acid residues help orient the substrate through electrostatic interactions with the phosphate group. BASED ON PDB FILE 3C39.

In 1958, Daniel Koshland proposed the **induced-fit model** of enzyme catalysis. In this model, the enzyme is analogous to a glove that has a three-dimensional shape, but is flexible and able to accommodate an equally flexible hand, which represents the substrate. A major advantage of the induced-fit model is that it permits a much larger number of weak interactions to occur between the substrate and enzyme, as a result of structural adjustments in the enzyme–substrate complex that occur upon binding. These numerous weak interactions between an enzyme and its cognate substrate provide binding energy that contributes to the catalytic activity of enzymes.

Before we discuss enzyme mechanisms in more detail, we want to emphasize three critical aspects of enzyme structure and function.

1. *Enzymes usually bind substrates with high affinity and specificity.* Enzyme active sites are physical pockets or clefts in an enzyme where the substrates bind and catalytic reactions take place. As shown in the structure of the enzyme phosphoglycerate kinase (**Figure 7.2**), the active site provides a protective microenvironment away from bulk solvent. Here, the substrate can bind to the enzyme's functional groups that participate in the catalytic reaction.
2. *Substrate binding to the active site induces structural changes in the enzyme.* These changes result in a large number of weak interactions between the substrate and the enzyme (hydrogen bonds, ionic interactions, and van der Waals interactions) and facilitate the structural changes needed to form the product. A classic example of the induced-fit model of substrate binding occurs in hexokinase, a glycolytic enzyme that phosphorylates glucose using ATP (**Figure 7.3**). Binding of the glucose substrate to the hexokinase active

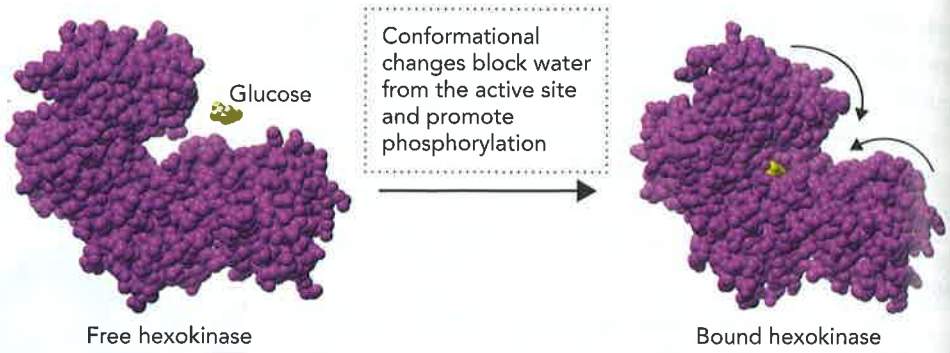


Figure 7.3 An example of the induced-fit model of enzyme catalysis is the glycolytic enzyme hexokinase, which undergoes a large conformational change upon binding of the glucose substrate. The location of the glucose molecule shown in the free (unbound) form of hexokinase is arbitrary, as glucose was not present in the protein crystal. BASED ON PDB FILES 1IG8 (FREE) AND 3B8A (BOUND).

site results in a large conformational change in the enzyme. This change excludes H_2O from the active site and facilitates glucose phosphorylation by ATP. In most enzymes, however, substrate binding leads to more subtle changes in protein structure, with the most significant changes occurring in the vicinity of the enzyme active site.

3. *Enzyme activity is highly regulated in cells.* Enzyme regulation is necessary to maximize energy balance between catabolic and anabolic pathways (see Chapter 2) and to alter cell behavior in response to environmental stimuli. The two primary modes of enzyme regulation are bioavailability and catalytic efficiency. **Bioavailability** refers to the amount of enzyme present in the cell as a result of regulated gene expression and protein turnover. **Catalytic efficiency** is a quantitative measure of enzyme activity. The catalytic efficiency of enzymes can be controlled by the binding of regulatory molecules or by covalent modification—most often phosphorylation of Ser, Thr, or Tyr residues. **Figure 7.4** shows the metabolic enzyme glycogen phosphorylase, whose catalytic efficiency is stimulated both by covalent modification (phosphorylation of a serine residue) and by the noncovalent binding of adenosine monophosphate (AMP), a regulatory molecule. Glycogen phosphorylase is a highly regulated enzyme in liver and muscle cells that controls the amount of glucose released from stored glycogen in response to the energy needs of the cell or the organism.

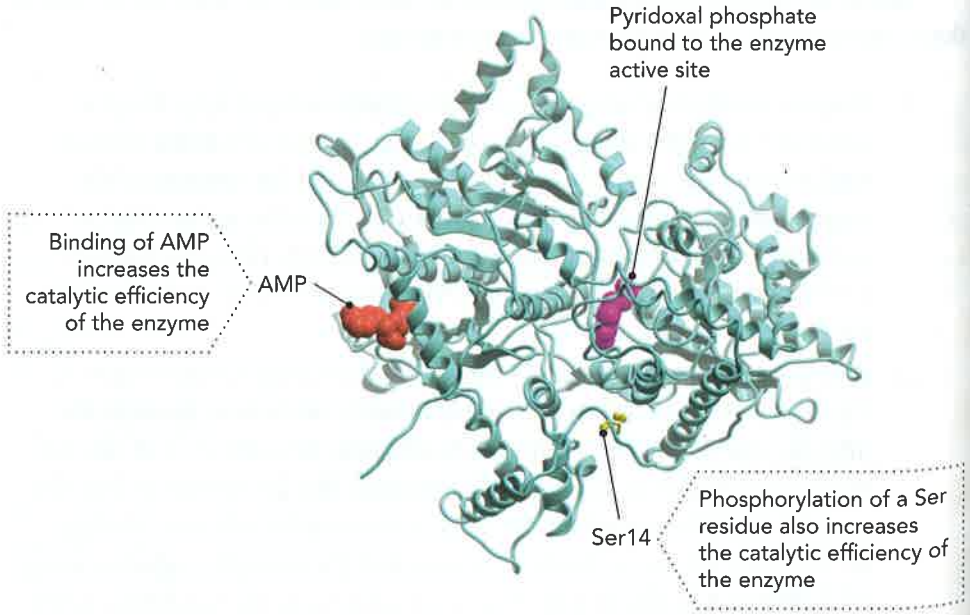


Figure 7.4 The catalytic efficiency of glycogen phosphorylase is increased by noncovalent binding of allosteric regulators, such as AMP, and by covalent attachment of a phosphoryl group to Ser14. The bound coenzyme pyridoxal phosphate is shown in the active site. Note that in glycogen phosphorylase, the regulatory sites are not directly at the active site. However, binding of AMP or phosphate at the regulatory sites causes conformational changes that affect the catalytic efficiency in the active site. BASED ON PDB FILE 8GPB.

Enzymes Are Chemical Catalysts

Like all chemical catalysts, enzymes alter the rates of reactions ($A \rightleftharpoons B$) without changing the ratio of substrates and products at equilibrium. Instead, catalysts simply decrease the time it takes to reach equilibrium. Catalysts increase the rates of reactions in both directions by the same amount ($A \rightarrow B$ and $B \rightarrow A$). Thus, the rates of reactions are affected without changing the final equilibrium or the overall change in free energy (ΔG) of the reaction.

Consider, for example, the thermodynamically favorable conversion of hydrogen peroxide (H_2O_2) to water and oxygen (gas):

$$2\ H_2O_2 \rightleftharpoons 2\ H_2O + O_2$$

The decomposition of H_2O_2 occurs very slowly at room temperature—so slowly that it would take about 3 years for 1 mol of H_2O_2 to decompose to 1 mol of H_2O and 0.5 mol of O_2. This amount of time represents the **half-life**, $t_{1/2}$, of the reaction, meaning the time it takes for half of the reactant to decompose. The $t_{1/2}$ of this reaction can be decreased considerably by adding a small amount of free iron (Fe^{2+}/Fe^{3+}) as a chemical catalyst. Under these conditions, the $t_{1/2}$ is only 11.6 minutes, representing an increase in the rate of decomposition by five orders of magnitude (the rate is 10^5 times faster in the presence of ferric ion).

Now, H_2O_2 is made in small amounts inside cells, where it is a highly reactive and toxic compound that must be rapidly eliminated to avoid damage to cellular components. The enzyme catalase has evolved to play the role of a cellular H_2O_2 detoxifying agent. It catalyzes a very efficient decomposition reaction involving an Fe^{3+} porphyrin ring located at the end of a narrow channel that connects the outside of the enzyme to the internal active site (**Figure 7.5**). The rate of H_2O_2 decomposition in the presence of catalase is amazingly fast, so that millions of molecules of H_2O_2 are decomposed per second per molecule of catalase. The 10^{15}-fold enhancement of the enzyme-catalyzed reaction over the uncatalyzed reaction protects cells from the toxic effects of H_2O_2 through rapid conversion to H_2O and O_2.

How does adding an enzyme catalyst increase reaction rates? To answer this question, we need to introduce the concept of chemical transition states. The **transition state theory** developed by Henry Eyring in the 1930s states that the conversion of substrate to product involves a high-energy transition state in which a molecule can either become a product or remain a substrate. This high-energy state is very

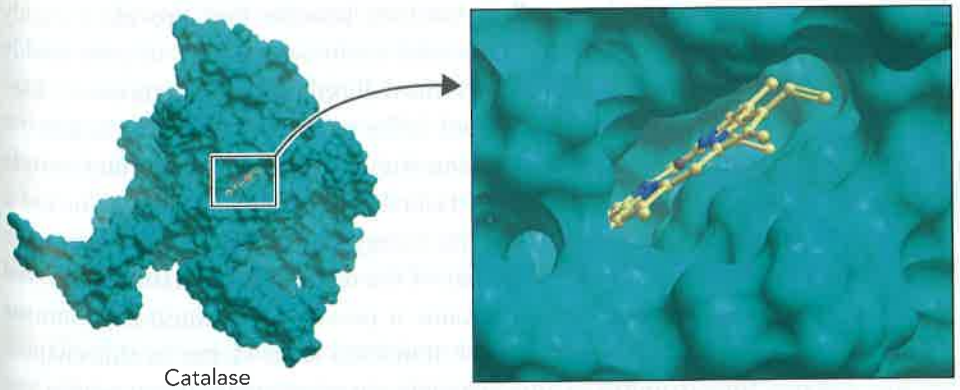


Figure 7.5 The enzyme human erythrocyte catalase, shown in space-filling representation, has a heme group buried within the enzyme active site. The Fe atom in the porphyrin ring is required for an electron transfer step that takes place during the reaction. Amino acid residues in the active site also contribute to the reaction by assisting in proton movement and the oxidation of Fe^{3+} to Fe^{4+} and by interactions with the reaction intermediates. The result is that the catalase reaction is far more efficient than the reaction catalyzed by free iron. BASED ON PDB FILE 1QQW.

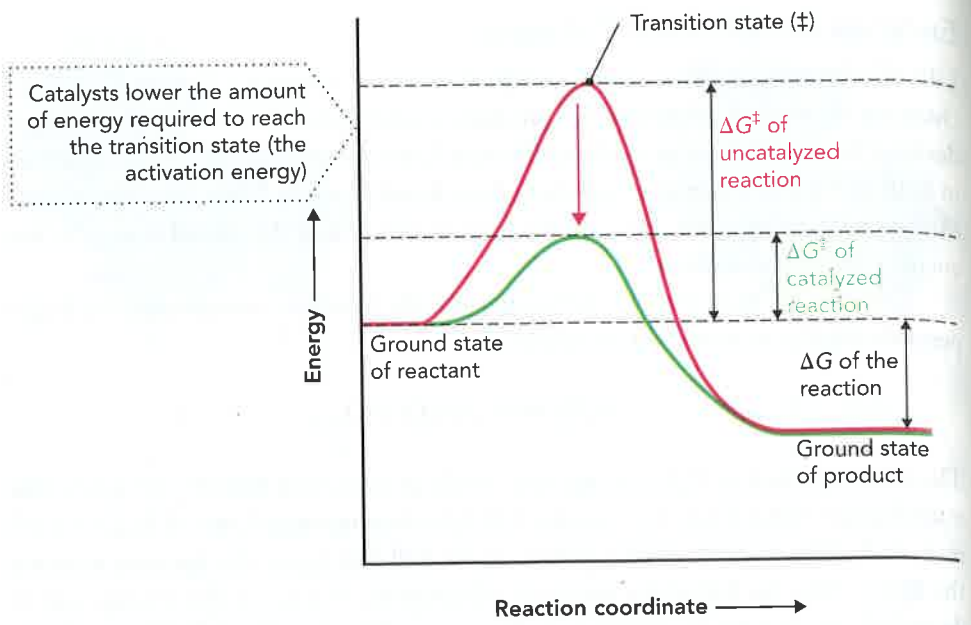


Figure 7.6 A reaction coordinate diagram for a catalyzed chemical reaction and an uncatalyzed chemical reaction is shown here. The difference in energy between the ground state of the reactant and the ground state of the product is ΔG. However, for the reaction to occur, the reactant must first reach the transition state (‡), and the energy required for the reactant to do this is called the activation energy, denoted $\Delta G^{\ddagger}$. Adding a catalyst to the reaction lowers the activation energy by providing an alternative path to product formation.

unstable, and only a few molecules in a reaction mixture are able to achieve this state at any one time. When they do, molecules are only in the transition state for 10^{-15} second—basically the time of a single atomic vibration. Molecules in the transition state are not reaction intermediates that can be physically isolated, but rather molecules that have attained an energy level that must be reached in order to convert from substrate to product.

Figure 7.6 describes the free energy of a chemical reaction, in which we plot A → B using a **reaction coordinate diagram**. As described in Chapter 2, the ΔG of a reaction is the change in free energy between the ground states of the reactant and the product, an energy value that is not changed by the presence of catalyst. Transition state theory dictates that a reactant must first reach an energy level required for the transition state, symbolized by a "double dagger" (‡), before it can form product. For example, a reaction might need enough energy to break a molecular bond so that product can form. As seen in Figure 7.6, the energy required to reach the transition state, also called the **activation energy**, denoted as $\boldsymbol{\Delta G^{\ddagger}}$, is the difference in energy between the ground state of the reactant and the transition state.

The function of a catalyst is to lower $\Delta G^{\ddagger}$ by providing more favorable reaction conditions. This has the effect of lowering the transition energy barrier, thereby increasing the rate of the reaction without altering the overall change in free energy, ΔG. Enzymes like catalase are excellent catalysts because they provide a highly reactive Fe^{3+} porphyrin ring within a protected environment. The enzyme readily promotes product formation as a function of the reduced transition state energy barrier. Catalase reduces $\Delta G^{\ddagger}$ from +71 kJ/mol in the uncatalyzed reaction to just +8 kJ/mol and, in so doing, increases the reaction rate 10^{15}-fold. It is important to note that the decomposition of H_2O_2 is highly favorable regardless of whether or not a catalyst is included in the reaction, as the free energy change (ΔG) of the reaction is approximately −95 kJ/mol. However, the rate of the reaction is dramatically affected by the addition of the enzyme catalase because it provides a chemical environment that makes it easier for H_2O_2 to reach the transition state. (Later in this chapter, we will describe the attributes of this favorable chemical environment within the enzyme active site.)

Table 7.1 REPRESENTATIVE METAL-ION COFACTORS IN ENZYMES

Cofactor	Representative enzymes	Role in catalysis
Fe^{2+}	Cytochrome oxidase	Oxidation–reduction
Mg^{2+}	Hexokinase	Helps bind ATP
Mn^{2+}	Ribonucleotide reductase	Oxidation–reduction
Cu^{2+}	Nitrite reductase	Oxidation–reduction
Zn^{2+}	Alcohol dehydrogenase	Helps bind the substrate
Ni^{2+}	Urease	Required in the catalytic site
K^{+}	Pyruvate kinase	Increases enzyme activity
Se	Glutathione peroxidase	Oxidation–reduction
Mo	Xanthine oxidase	Oxidation–reduction

Cofactors and Coenzymes

Proteins are the primary enzymes in living systems, but they often require small molecules called **cofactors** to aid in the catalytic reaction mechanism within the enzyme active site. Cofactors provide additional chemical groups to supplement the chemistry of the enzyme's amino acid functional groups when they are not sufficient to mediate a particular catalytic mechanism. An enzyme with a bound cofactor is called a **holoenzyme**, whereas removal of the cofactor produces an inactive **apoenzyme** (enzyme without cofactor).

Enzyme cofactors include a variety of inorganic ions such as Fe^{2+}, Mg^{2+}, Mn^{2+}, Cu^{2+}, and Zn^{2+}, which are often required in enzymes that catalyze redox reactions. **Table 7.1** lists the most common metal-ion cofactors found in enzymes and their roles in these reactions. Many nucleic acid metabolizing enzymes, such as RNA and DNA polymerases, require Zn^{2+} in the active site, whereas Mg^{2+} is often associated with ATP hydrolyzing enzymes. In fact, one of the reasons mercury (Hg^{2+}) is so toxic is that it can replace Zn^{2+} in a nonproductive way in a variety of enzymes. Copper (Cu^{2+}) is another important metal-ion cofactor in enzymes. Coordination of a Cu^{2+} ion by His residues in the active site of the *Alcaligenes faecalis* nitrite reductase enzyme positions the metal cofactor so that it can bind nitrite in the correct orientation and thereby mediate its reduction to form nitric oxide (**Figure 7.7**).

Enzyme cofactors with organic components are called **coenzymes**. Coenzyme molecules can be loosely associated with the enzymes or very tightly bound, even covalently attached to the enzyme. Coenzymes provide additional chemical flexibility for facilitating the catalytic reaction, which the protein component of the enzyme may be unequipped to do. Coenzymes that are permanently associated with enzymes, such as the heme group of catalase, are called **prosthetic groups**.

Coenzymes are usually derived from vitamins and were first discovered as biomolecules required for health. One of the coenzymes we will encounter frequently in metabolism

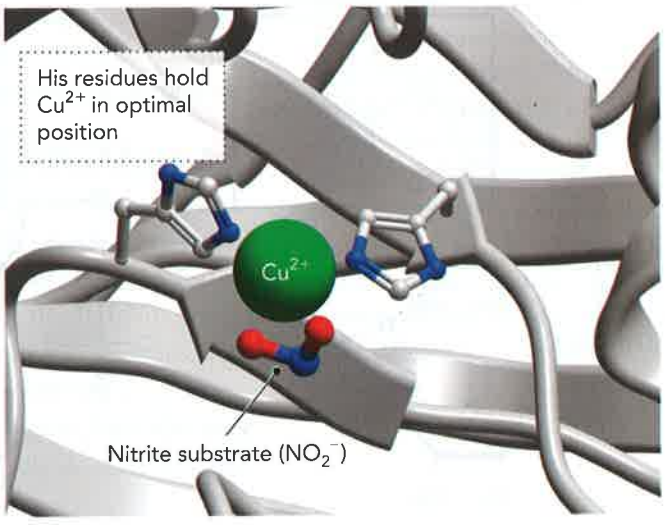


Figure 7.7 Nitrite reductase is an enzyme present in several types of soil bacteria that recycle nitrogen in the environment. As seen here in the molecular structure of the enzyme's active site, the Cu^{2+} ion is coordinated to two histidine residues that function to hold the metal cofactor in an optimal position for catalyzing the reduction of the substrate nitrite (NO_2^-) to form the product, nitric oxide. BASED ON PDB FILE 1SJM.

Table 7.2 REPRESENTATIVE COENZYMES AND THEIR VITAMIN SOURCES

Coenzyme	Vitamin source	Representative enzyme	Role in catalysis
Nicotinamide adenine dinucleotide (NAD^+/NADH)	Vitamin B_3 (niacin)	Lactate dehydrogenase	Oxidation–reduction
Flavin adenine dinucleotide (FAD/$FADH_2$)	Vitamin B_2	Succinate dehydrogenase	Oxidation–reduction
Thiamine pyrophosphate (TPP)	Vitamin B_1	Pyruvate dehydrogenase	Aldehyde group transfer
Biotin	Vitamin B_7	Pyruvate carboxylase	Carboxylation
Coenzyme A (CoA)	Pantothenic acid	Acetyl-CoA carboxylase	Acyl group transfer
Tetrahydrofolate (THF)	Folate	Thymidylate synthase	Single-carbon transfer
Pyridoxal phosphate (PLP)	Vitamin B_6	Aspartate aminotransferase	Amine group transfer
Lipoamide	Lipoic acid	Pyruvate dehydrogenase	Two-carbon transfer
Cobalamin	Vitamin B_{12}	Methylmalonyl-CoA mutase	Alkyl group transfer

is **nicotinamide adenine dinucleotide (NAD^+/NADH)**, which is derived from the vitamin niacin and is a required component in many redox reactions involving dehydrogenase enzymes. Another important coenzyme is **thiamine pyrophosphate**, which is derived from vitamin B_1. A deficiency of thiamine pyrophosphate in the diet results in the human disease beriberi, characterized by neurologic disorders. Thiamine pyrophosphate is a required coenzyme in decarboxylation reactions, such as that catalyzed by the pyruvate dehydrogenase complex (see Chapter 10). Some common coenzymes and their vitamin sources are listed in **Table 7.2**.

Lactate dehydrogenase is an important enzyme in cells. It converts pyruvate to lactate during anaerobic respiration under low O_2 conditions or in cells lacking mitochondria, such as erythrocytes. The reduction of pyruvate to form lactate is a typical metabolic redox reaction in which the coenzyme NADH is concomitantly oxidized to generate NAD^+. In this case, NADH binds to the enzyme near the active site and functions as a transient electron carrier that donates a pair of electrons to the redox reaction, resulting in reduction of the enzyme substrate (**Figure 7.8**). The NAD^+ product formed by the lactate dehydrogenase reaction is used by other enzymatic reactions

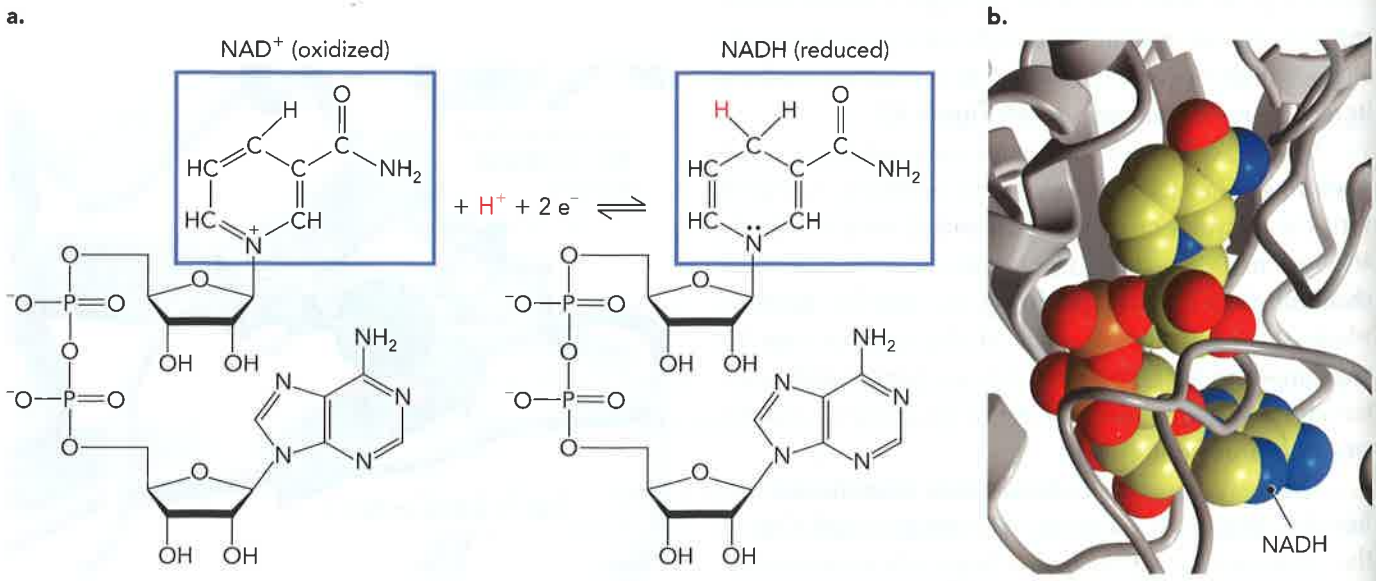


Figure 7.8 Nicotinamide adenine dinucleotide is a coenzyme in the lactate dehydrogenase reaction. **a.** Chemical structure of nicotinamide adenine dinucleotide in the oxidized (NAD^+) and reduced (NADH) forms. **b.** Molecular structure of part of the lactate dehydrogenase enzyme from the organism *Plasmodium falciparum*, with an NADH molecule bound to the active site. BASED ON PDB FILE 1TC2.

in the cell as a coenzyme that accepts a pair of electrons in redox reactions that oxidize enzyme substrates. NAD^+ and NADH are sometimes called co-substrates because they are altered in the course of the reaction through the donation or acceptance of electrons. For another round of catalysis to take place, they need to be regenerated to their original state via another reaction.

Some coenzymes are covalently linked to amino acid functional groups in enzymes and serve as integral components in the catalytic reaction. One example of this is the attachment of lipoic acid to a specific lysine residue in enzymes that catalyze redox and acyl transfer reactions. The oxidized form of this coenzyme is called **lipoamide**, and the reduced form is dihydrolipoamide (**Figure 7.9**). In the pyruvate dehydrogenase complex, which requires three different enzyme subunits in a multistep reaction to decarboxylate pyruvate to form acetyl-coenzyme A (acetyl-CoA), the dihydrolipoyl transacetylase enzyme performs the acyl transfer portion of this reaction with the lipoyl group. (In Chapter 10, we will look at this reaction in some detail.) Remarkably, this highly favorable reaction (essentially irreversible) also requires the participation of three additional coenzymes (flavin adenine dinucleotide, coenzyme A, and thiamine pyrophosphate), all of which are transiently associated with the pyruvate dehydrogenase enzyme complex.

Enzyme Nomenclature

The metabolism of glucose to pyruvate by enzymes in the glycolytic pathway—an important energy conversion pathway in cells—requires 10 enzymatic reactions. Because each reaction step involves a different enzyme, in this one pathway you need to become familiar with 10 different enzyme names. Fortunately, biochemists have developed a systematic nomenclature to name enzymes on the basis of their characterized function.

Most proteins that function as enzymes end with the suffix "-ase" to denote that the protein is an enzyme. In addition, the substrate, or a description of the biochemical function of the enzyme, is usually included in the name. For example, the common name for the protein that converts urea to ammonia and carbon dioxide is *urease*. The term *hexokinase* refers to an enzyme that phosphorylates hexose sugars (a *kinase* is a phosphoryl-transferring enzyme). Not all common names are that useful; for example, the enzyme name *catalase* doesn't reveal anything about hydrogen peroxide decomposition except to signify that this protein is a *catalyst*—like all enzymes.

To improve the usefulness of enzyme nomenclature, a functional classification system has been adopted by the International Union of Biochemistry and Molecular Biology (IUBMB). This classification system is based on six classes of enzymatic reactions, as listed in **Table 7.3**. Each of these six enzyme classes has been further subdivided into subclasses and sub-subclasses. Strictly speaking, the IUBMB system does not distinguish between proteins, but rather between enzymatic reactions. This is apparent from the fact that evolutionarily related proteins from different organisms, which catalyze the same chemical reaction in their respective species, have the same IUBMB number. Other databases have been developed specifically to name proteins, one of which is the Universal Protein Resource (UniProt).

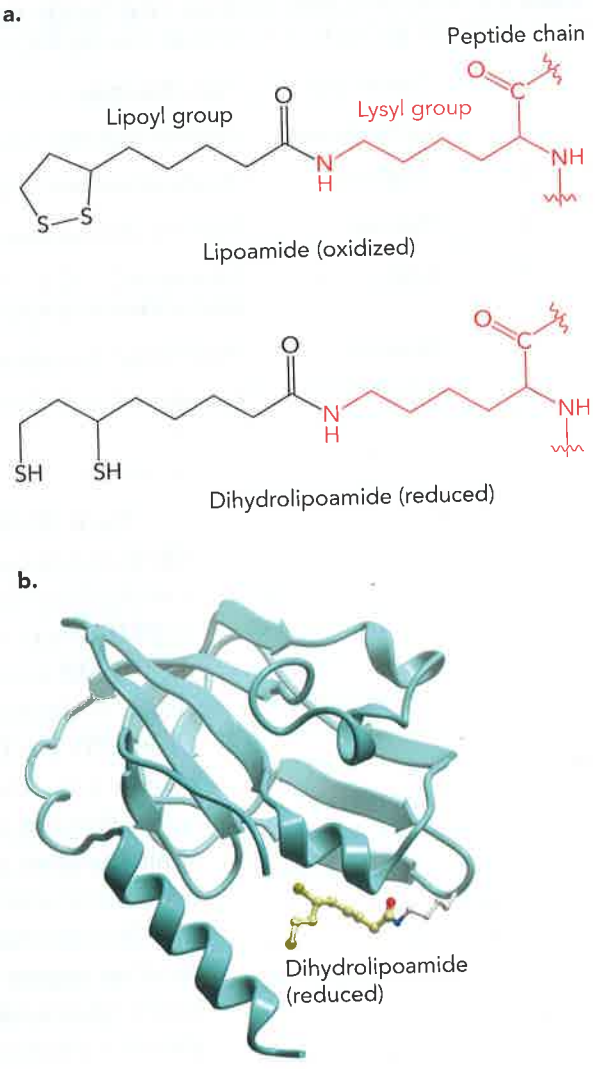


Figure 7.9 Lipoamide is a covalently attached coenzyme that plays a key role in several decarboxylase reactions. **a.** A lipoyl group is attached to the amino group of lysine residues in enzymes, shown here in both the oxidized (lipoamide) and reduced (dihydrolipoamide) forms. **b.** The pea glycine carboxylase enzyme contains a lipoamide coenzyme that protrudes into the enzyme active site. This protein structure contains the reduced form of lipoamide, called dihydrolipoamide. BASED ON PDB FILE 1DXM.

Table 7.3 THE INTERNATIONAL UNION OF BIOCHEMISTRY AND MOLECULAR BIOLOGY ENZYME CLASSIFICATION SYSTEM

Number	Enzyme class	Type of reaction	Generic enzymes
1	Oxidoreductase	Oxidation–reduction, transfer of H or O atoms	Oxidases, dehydrogenases
2	Transferase	Transfer of functional groups; e.g., methyl, acyl, amino, phosphoryl	Kinases, transaminases
3	Hydrolase	Formation of two products by hydrolyzing a substrate	Peptidases, lipases
4	Lyase	Cleavage of C—C, C—O, C—N, and other bonds by means other than hydrolysis or oxidation	Decarboxylases, carboxylases
5	Isomerase	Intramolecular rearrangements, transfer of groups within molecules	Mutases, isomerases
6	Ligase	Formation of C—C, C—O, C—S, or C—N bonds using ATP cleavage	Synthetases

The IUBMB system provides both an official name (systematic name) and a classification number beginning with the letters EC (Enzyme Commission), as well as a method for keeping track of common names (alternative names). To see how the IUBMB system distinguishes between closely related enzyme activities, let's examine the IUBMB number of the enzyme hexokinase. This enzyme catalyzes the phosphorylation of glucose to form glucose-6-phosphate during the first step in glycolysis (see Chapter 9). The IUBMB classification number for hexokinase is EC 2.7.1.1. This EC number is based on classification "2" as a transferase, subclass "7" because it transfers a phosphoryl group, and sub-subclass "1" because the phosphoryl transfer involves an alcohol acceptor group on glucose. The last digit in EC 2.7.1.**1** denotes that it is the first enzyme activity named in this category.

The importance of the IUBMB classification number is illustrated by the example of an enzyme related to hexokinase, called glucokinase, which catalyzes a similar phosphotransferase reaction. However, in the case of glucokinase, its affinity for glucose is much lower than that of hexokinase, and it has a more limited substrate specificity (glucokinase cannot phosphorylate fructose). On the basis of the distinct biochemical properties of glucokinase compared to those of hexokinase, the IUBMB number for glucokinase is EC 2.7.1.**2**. Hexokinase and glucokinase have different amino acid sequences and are encoded by two distinct genes on different chromosomes in the human genome.

Although the IUBMB system is useful in clearing up confusion associated with using historical or "generic" enzyme names, it can become cumbersome when trying to distinguish between enzymes encoded by the same gene, but that differ in amino acid sequence due to alternative mRNA splicing or differential translational start sites. These distinctions are best sorted out by genomic and proteomic databases, as described in Part 5 of this book.

concept integration 7.1

How do enzymes function as biological catalysts?

Enzymes, like all catalysts, lower the activation energy ($\Delta G^{\ddagger}$) of a reaction without affecting the overall change in free energy (ΔG). Enzymes do this by providing an optimal environment for chemical catalysis, called the enzyme active site. Enzymes increase the rates of reactions so that the reactions will happen on a biologically appropriate timescale.

Enzyme functional groups help bind and orient the substrate(s), and in some cases chemical cofactors are used to provide functionality that may be lacking from amino acid side chains. Enzyme activity is regulated by cellular conditions, and enzymes usually have specific cognate substrates. These properties ensure that the appropriate reaction takes place at the appropriate time in the cell.

7.2 Enzyme Structure and Function

The formal definition of a catalyst is that it increases the rate of a chemical reaction without changing the chemical equilibrium. Moreover, a catalyst is not consumed by the reaction. Enzymes increase the rate of a reaction inside cells in three major ways:

1. *They stabilize the transition state*, and thus lower the activation barrier.
2. *They provide an alternative path for product formation*, which could involve formation of stable reaction intermediates that are covalently attached to the enzyme.
3. *They orient the substrates appropriately for the reaction to occur*, thus reducing the entropy change of the reaction.

Enzymes use all of these strategies to some extent—sometimes in combination for the same reaction—with the net result being an increased rate of reaction on a biological timescale.

Raising effective substrate concentrations or elevating the reaction temperature or pressure can also lead to increased reaction rates. In fact, industrial chemical reactions are often made more economical by simply manipulating the reaction conditions within the vessel. However, in nature, where time, space, pressure, and temperature are all rigidly constrained within a cell, enzymes must rely on natural selection to optimize molecular structure and function to achieve increased rates of reactions under physiologic conditions.

To understand how enzymes function as catalysts, it is first necessary to describe the general properties of enzyme active sites. Then we will use this information to examine some of the most common enzyme reaction mechanisms found in cells.

Physical and Chemical Properties of Enzyme Active Sites

Enzymes function as catalysts by providing a physical and chemical environment that promotes product formation. They do this by increasing the local concentration of substrates through selective binding—which also orients substrates in an optimal configuration for functional group interactions—and by providing reactive groups that can participate in the chemistry of the reaction itself.

Figure 7.10 illustrates a reaction in which two substrates form one product, A + B → C. The structure of the enzyme active site provides a geometric and chemical complementarity that favors the binding of substrates in a way that is both selective and productive (substrate reactive

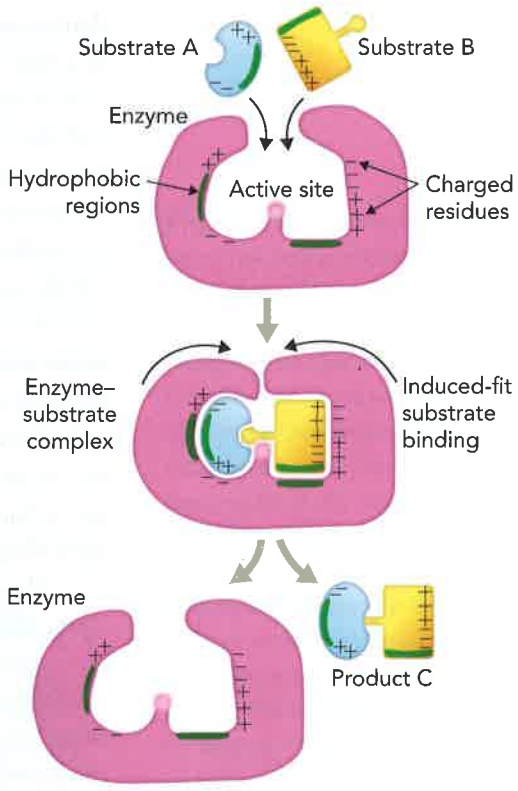


Figure 7.10 The enzyme active site provides an optimal physical and chemical environment that promotes product formation. Random collisions between two substrates are often unproductive due to misalignment of reactive groups required for product formation. The enzyme active site contains binding sites to select substrates and align the reactive groups correctly. This is accomplished by multiple weak interactions through polar and nonpolar regions in the substrates and enzyme. Many substrates bind to enzymes using an induced-fit mechanism, in which the enzyme structure changes to accommodate substrate binding, though not too tightly or products won't be released. Chemical groups present in the enzyme or cofactors are in close proximity to the substrates. Product release frees up the enzyme to bind new substrate molecules.

groups are in close proximity). Moreover, through the cumulative effect of multiple noncovalent interactions between the substrates and enzyme, substrates can have a relatively high binding affinity for the enzyme active site. It is important, however, that this binding not be too tight, especially between the enzyme and the product; otherwise, it will have a negative effect on reaction rates by interfering with product release and binding of new substrate molecules.

Let's now examine three specific physical and chemical properties of enzyme active sites that contribute to their catalytic properties: (1) the sequestered microenvironment of the active site; (2) binding interactions between the substrate and the enzyme that facilitate formation of the transition state; and (3) the presence of catalytic functional groups.

The Active Site Microenvironment The features of an enzyme active site that contribute to a decrease in the $\Delta G^{\ddagger}$ of the reaction are, for the most part, independent of the specific catalytic mechanism:

1. Enzyme active sites provide an optimal orientation of the substrate(s) relative to reactive chemical groups.
2. Enzyme active sites exclude excess solvent (H_2O) that can interfere with the reaction.

An example of the first feature, how enzymes provide an optimal spatial orientation of substrates within the active site, is the metabolic enzyme aldolase. The aldolase reaction in the gluconeogenic pathway converts two phosphorylated three-carbon compounds into one bisphosphorylated six-carbon compound. This reaction is highly favorable under standard conditions with a $\Delta G^{\circ\prime}$ of −23.8 kJ/mol:

Glyceraldehyde-3-phosphate + Dihydroxyacetone phosphate $\rightleftharpoons$ Fructose-1,6-bisphosphate

Amino acid functional groups within the aldolase active site position the substrates in a way that favors aldol condensation. The reaction mechanism involves formation of an enzyme-linked covalent intermediate between a lysine residue in the active site and the substrate dihydroxyacetone phosphate (**Figure 7.11**). Once this intermediate forms, glyceraldehyde-3-phosphate binds to the active site. The condensation reaction is favored because of the proximity of glyceraldehyde-3-phosphate to both the reactive C-3 carbon in the intermediate and to amino acid residues in the enzyme active site, which participate in an acid–base chemical reaction mechanism (described in Section 7.3). After the reaction is complete, the fructose-1,6-bisphosphate product diffuses out of the active site. Note that this same aldolase reaction is favored in the opposite direction (aldol cleavage of fructose-1,6-bisphosphate to form glyceraldehyde-3-phosphate and dihydroxyacetone phosphate) when flux through the glycolytic pathway is high due to elevated levels of glucose in the cell, coupled with a need for ATP production (see Chapter 9). Thus, this same enzyme can catalyze the reaction in either direction, depending on conditions in the cell.

The second general feature of most enzyme active sites is their location in clefts on the protein surface or near the interior of the protein, which functions to sequester the substrates away from excess water. Most H_2O molecules are excluded from the enzyme active site in one of two ways. The first mechanism is through an induced-fit mechanism of substrate binding, in which conformational changes in the protein create significant shape and chemical complementarity between the substrate and enzyme. These conformational changes eject excess H_2O molecules from

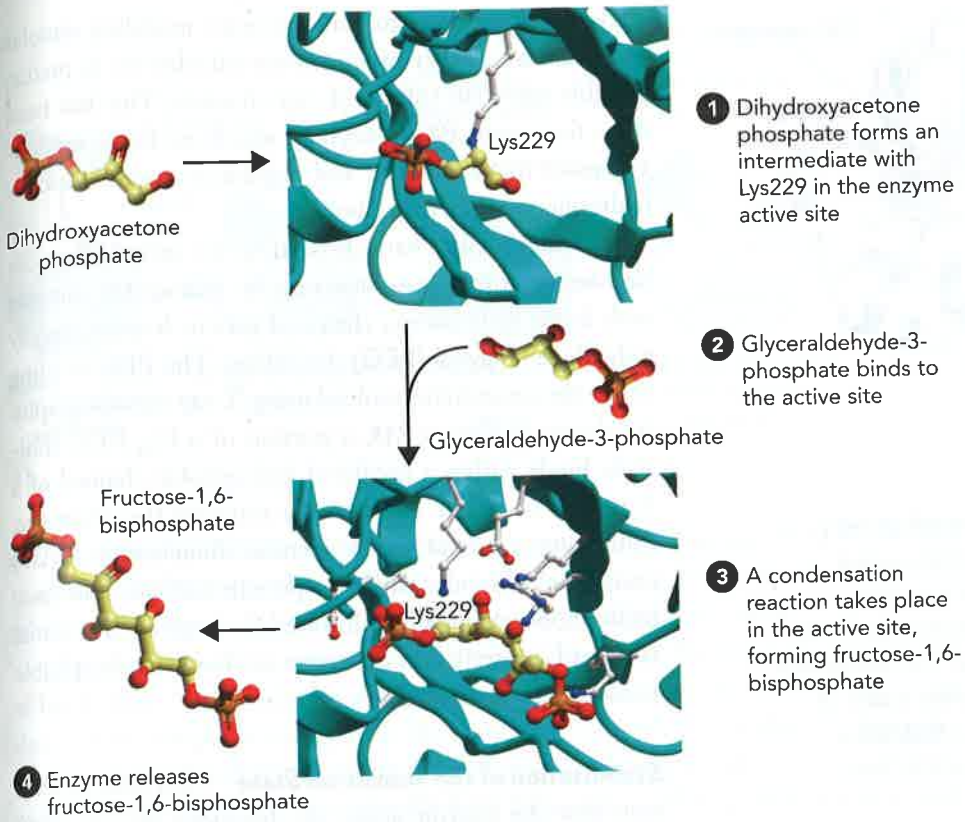


Figure 7.11 The aldolase reaction illustrates the importance of favorable spatial arrangements of substrates within the enzyme active site to promote a reaction. The formation of a covalent substrate–enzyme intermediate in the first step of this reaction contributes to the decreased $\Delta G^{\ddagger}$. BASED ON PDB FILES 1J4E (ALDOLASE WITH COVALENTLY BOUND DIHYDROXYACETONE PHOSPHATE) AND 4ALD (ALDOLASE WITH FRUCTOSE-1,6-BISPHOSPHATE IN THE ACTIVE SITE).

the active site. The binding of glucose by the enzyme hexokinase is a good example of this induced-fit mechanism (see Figure 7.3). A closer look at the hexokinase active site reveals that numerous H_2O molecules occupy the substrate binding site in the absence of glucose because this region of the enzyme is exposed to solvent (**Figure 7.12**). However, upon glucose binding, several polar residues within the active site are brought closer together and form hydrogen bonds with the glucose molecule instead of forming hydrogen bonds with H_2O. Thus, water is displaced from the active site. The importance of this substrate-induced conformational change in the active site is that it prevents phosphoryl transfer from ATP to H_2O, which would be a waste of metabolic energy. Instead, the terminal phosphate of ATP is used to convert glucose to glucose-6-phosphate.

The second mechanism used by enzymes to prevent excess H_2O from entering the active site is to sequester the active site away from the surface, with substrate accessibility controlled by a gated hydrophobic channel. The hydrophobic channel effectively limits H_2O entry to the active site, while still allowing access by hydrophobic substrates. One way to determine how hydrophobic substrates gain access to a buried

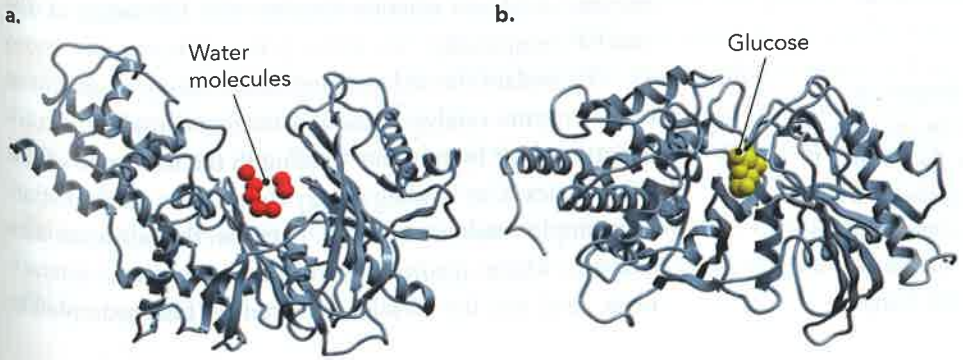


Figure 7.12 The enzyme active site of hexokinase excludes H_2O molecules upon glucose binding, thereby preventing nonproductive phosphoryl transfer from ATP to H_2O. **a.** In the absence of glucose, H_2O molecules (shown as red spheres) occupy the solvent-exposed active site. BASED ON PDB FILE 1IG8. **b.** A conformational change is induced upon glucose binding at the active site. Glucose replaces water in the active site by occupying a similar volume and using similar weak interactions for binding. Therefore, the H_2O molecules are expelled from the active site. BASED ON PDB FILE 3B8A.

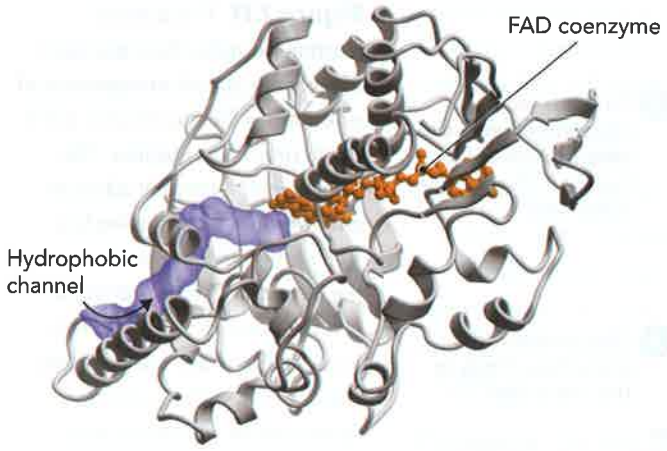


Figure 7.13 Hydrophobic substrate channels prevent H_2O molecules from entering a buried enzyme active site. The structure of a bacterial fatty acid isomerase enzyme is shown with a flavin adenine dinucleotide (FAD) coenzyme bound in the internal active site. The hydrophobic substrate channel is highlighted by the surface outline of a C_{24} PEG molecule. The arrow shows the likely entry pathway of a fatty acid substrate. BASED ON PDB FILE 2B9W.

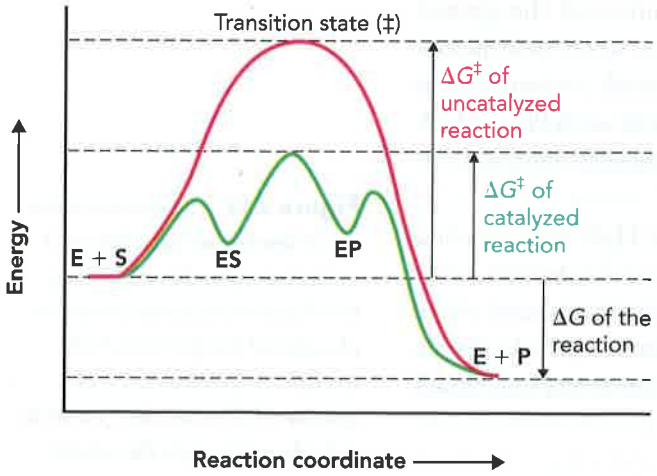


Figure 7.14 In this reaction coordinate diagram, an enzyme-catalyzed reaction is compared to the uncatalyzed reaction. In the enzyme-catalyzed reaction, the ES and EP intermediates are local minima in the energy diagram. Note that the activation energy for the enzyme-catalyzed reaction is less than that for the uncatalyzed reaction, but the free energy change (ΔG) for the reaction S → P does not change.

enzyme active site is to run computer modeling simulations that use energy minimization calculations to predict possible substrate entry and exit channels. This has been done for several P450 enzymes, which are heme monooxygenases that synthesize and degrade a variety of mostly hydrophobic dietary metabolites.

Hydrophobic channels leading to active sites can also be identified in a structure by mixing the enzyme with linear hydrocarbon chains of various lengths, usually **polyethylene glycol (PEG)** derivatives. The PEG binding site in the structure is resolved using X-ray crystallography. As shown in **Figure 7.13**, a portion of a C_{24} PEG molecule binds within a predicted hydrophobic channel of a fatty acid isomerase enzyme that connects the active site, containing a bound flavin adenine dinucleotide (FAD) coenzyme molecule, with the protein surface. This long hydrophobic channel is well suited for entry of the normal fatty acid substrate into the active site but is not hospitable to solvent H_2O molecules.

Stabilization of the Transition State Now that we have seen how the enzyme active site functions, we can study how stabilization of the transition state within the active site leads to increased reaction rates of enzyme-catalyzed reactions. We first consider the equilibrium reactions that define substrate (S) binding to the enzyme (E) and releasing product (P). In this three-step process, substrate binding to the enzyme (E + S) leads to the formation of an enzyme–substrate complex (ES). This is followed by conversion of the enzyme-bound substrate to an enzyme-bound product (EP). Finally, the product is released from the enzyme (E + P):

$$E + S \rightleftharpoons ES \rightleftharpoons EP \rightleftharpoons E + P$$

The reaction coordinate diagram in **Figure 7.14** illustrates the effect of the enzyme on reducing the activation energy ($\Delta G^{\ddagger}$) for the enzyme-catalyzed reaction compared to that for the uncatalyzed reaction. Again, note that the overall free energy of the reaction (ΔG) does not change in the presence of the enzyme, and that the path taken by the enzyme-catalyzed reaction involves the formation of ES and EP complexes.

To explain the reduced activation energy requirement of the enzyme-catalyzed reaction, we first consider the contribution made by substrate binding in the formation of the ES complex. This binding energy comes from the formation of multiple weak interactions between the substrate and enzyme, which involves hydrogen bonds, ionic interactions, and van der Waals interactions. The hydrophobic

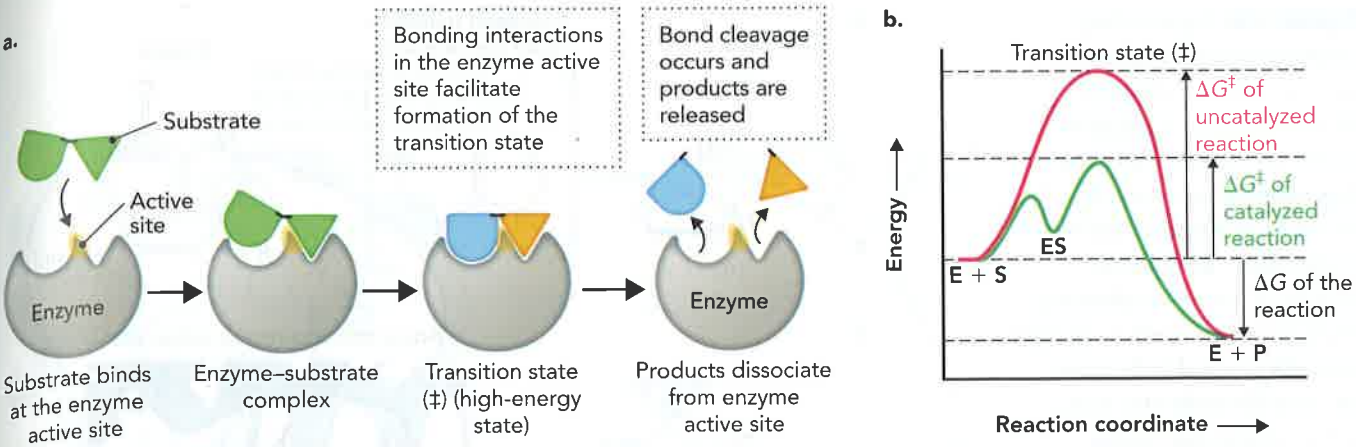


Figure 7.15 The enzyme active site facilitates formation of the transition state. **a.** The substrate binds at the active site of the enzyme. Conformational changes in the substrate aided by bonding interactions in the active site result in the formation of the transition state. Products are released after bond cleavage. **b.** This energy level diagram illustrates how the formation of the ES complex reduces the energy required to attain the transition state.

effect also plays an important role because of an increase in the entropy of H_2O molecules that were released from either the enzyme or substrate upon substrate binding. However, substrate binding is only part of the explanation for a reduced $\Delta G^{\ddagger}$ value.

The next step, the conversion of ES $\rightleftharpoons$ EP, requires the breaking and forming of bonds that distinguish the substrate from the product. If the enzyme active site shares more complementarity with the substrate than with the product (as first proposed in the lock and key model of enzyme action), then it would favor the substrate within the active site and limit the ES $\rightleftharpoons$ EP reaction. Alternatively, if amino acid residues within the enzyme active site make the most contacts (best geometric and chemical complementarity) during the transition state of the reaction, then breaking of bonds during the reaction requires no direct input of energy by the protein. This proposal is called the transition state stabilization model.

One way to think about how binding energies and transition state stabilization work together to lower the activation energy of a reaction is to imagine that formation of the ES complex initiates a series of bond formations that accumulate as the reaction proceeds toward the transition state (the midpoint of the ES $\rightleftharpoons$ EP reaction). The gain in free energy resulting from bond formation between the substrate and the enzyme is used to lower the activation energy required to reach the transition state (**Figure 7.15**).

The transition state stabilization model also explains why product release occurs at the end of the reaction (EP $\rightleftharpoons$ E + P); namely, because the enzyme active site has a higher affinity for the transition state than for either the product or the substrate.

Support for the transition state stabilization model comes from analyzing the binding of stable molecules that mimic the proposed transition state, called **transition state analogs**, to enzyme active sites. **Figure 7.16** shows an example of this idea. Adenosine deaminase is a critical enzyme involved in purine degradation (see Chapter 18). This enzyme deaminates adenosine to form the purine inosine, a process that is proposed to occur through direct addition of water to the purine ring resulting in a tetrahedral transition state. The transition state analog, 6-hydroxy-1,6-dihydropurine ribonucleoside, has a similar tetrahedral arrangement at position 6 of the purine ring, and thus binds very tightly to the enzyme. In fact, the affinity of the enzyme for this transition state analog is estimated to be 10^{-13} M, which exceeds the affinity of the enzyme for substrate or product by approximately a factor of 10^8. As discussed later in this chapter, nonhydrolyzable transition state analogs are often very effective enzyme inhibitors.

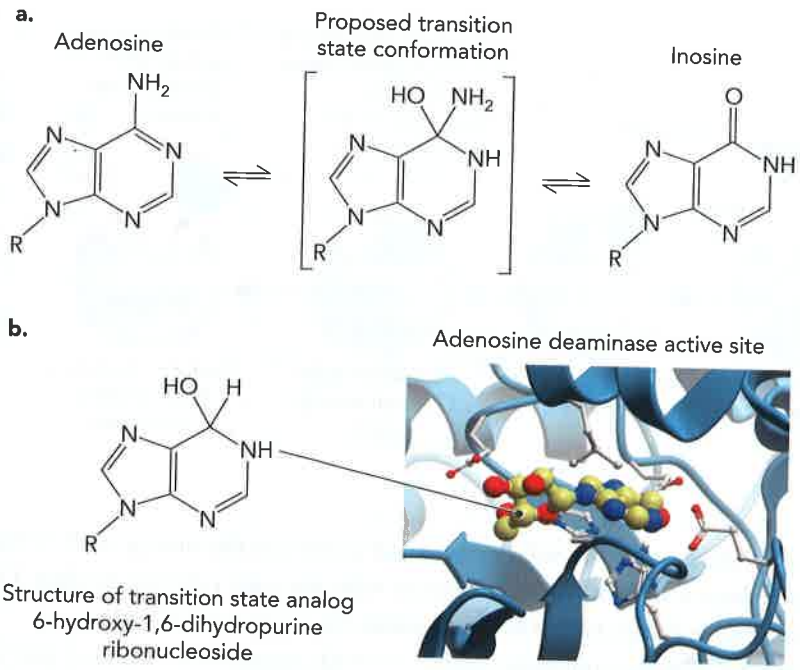


Figure 7.16 Tight binding of transition state analogs to enzyme active sites supports the transition state stabilization model. **a.** Proposed adenosine deaminase reaction, showing the predicted transition state conformation. **b.** Molecular structure of the mouse adenosine deaminase enzyme, with a transition state analog bound to the active site. Note the similar structures of the transition state analog 6-hydroxy-1,6-dihydropurine ribonucleoside and the proposed transition state shown in panel **a**. BASED ON PDB FILE 1KRM.

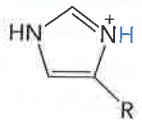
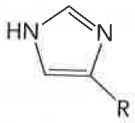
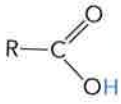
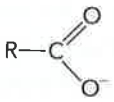
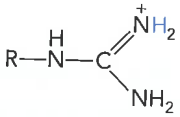
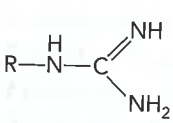
Amino acid	General acid form	General base form
His		
Asp, Glu		
Ser, Thr	R—OH	R—O⁻
Tyr		
Cys	R—SH	R—S⁻
Lys	R—$\overset{+}{N}H_3$	R—NH_2
Arg		

Figure 7.17 Amino acid groups commonly involved in enzyme-mediated acid–base catalysis. The acid form is the proton donor (shown in blue), and the base form is the proton acceptor.

Presence of Catalytic Functional Groups Once substrates are bound to the protected enzyme active site away from solvent, often through a mechanism that involves stabilization of the transition state, it is the job of nearby reactive chemical groups on amino acids or coenzymes to catalyze the reaction. The three most common catalytic reaction mechanisms in the enzyme active site are (1) acid–base catalysis; (2) covalent catalysis; and (3) metal-ion catalysis.

Acid–base catalysis. Many enzyme reactions involve proton transfer through acid–base mechanisms that involve addition or removal of a proton. Two types of acid–base catalysis can occur in enzyme reactions: (1) specific acid–base catalysis, which involves water, or (2) general acid–base catalysis, in which the proton transfer involves a functional group. General acid catalysis refers to the donation of a proton by the enzyme, whereas general base catalysis refers to the removal of a proton by the enzyme. **Figure 7.17** shows amino acid residues that commonly function in general acid–base catalysis.

The histidine side chain is often involved in enzyme-mediated acid–base catalysis because the imidazole ring has a pK_a near ~7 in most proteins and can therefore be found in both the protonated and deprotonated states. This means that depending on the local charge distribution in the enzyme active site, histidine can function as either a proton donor or a proton acceptor at physiologic pH. **Figure 7.18** illustrates the role of two histidine residues (His12 and His119) within the active site of the enzyme pancreatic ribonuclease. The residues are involved in promoting the cleavage of an RNA substrate molecule through a general acid–base catalysis mechanism.

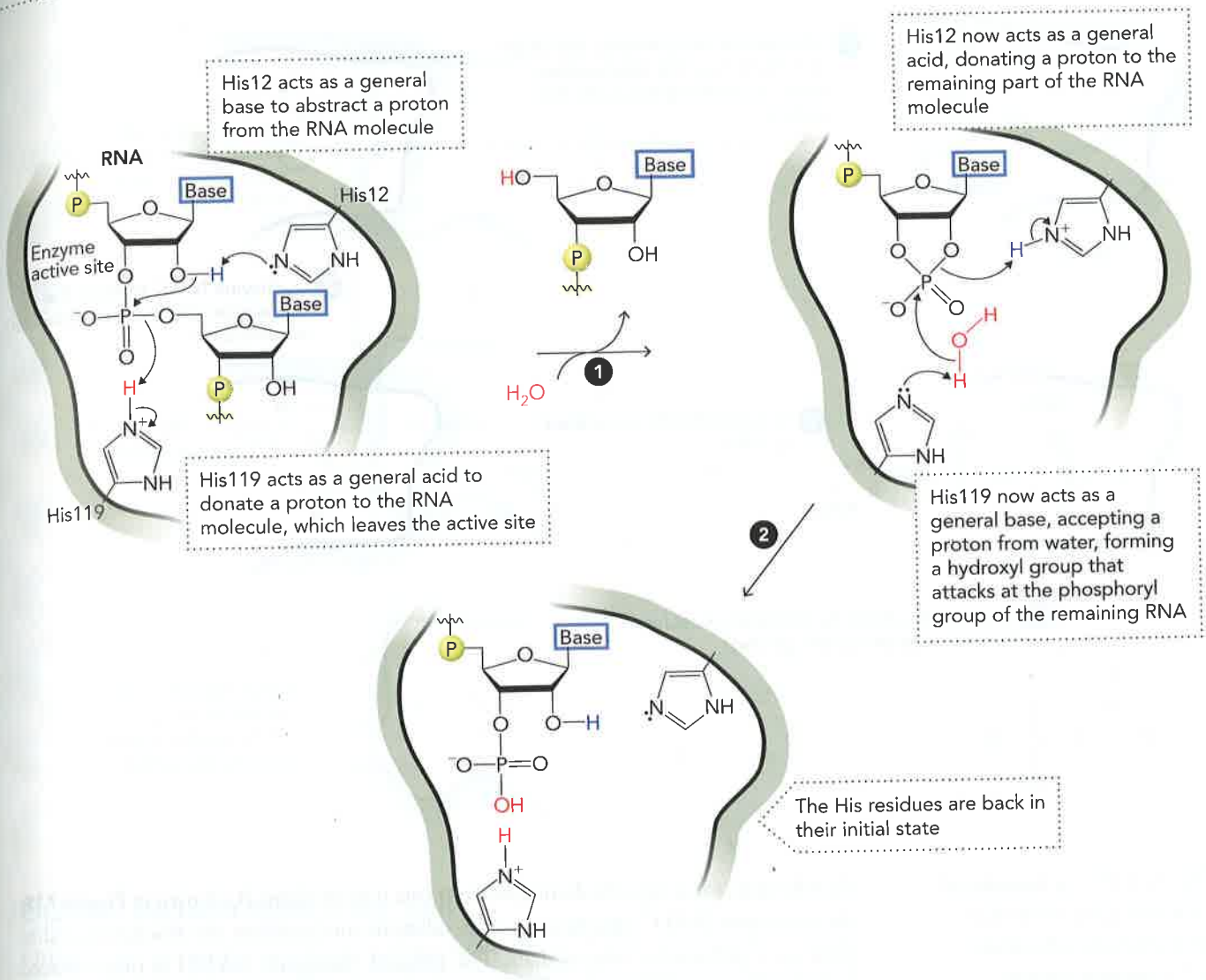


Figure 7.18 The mechanism of RNA cleavage by pancreatic ribonuclease is shown here. Two histidine residues function in a general acid–base catalysis mechanism that requires the addition of H_2O.

Covalent catalysis. Formation of a transient covalent bond between the substrate and the enzyme can be used to create an unstable intermediate that promotes the catalytic reaction. Usually a nucleophilic (electron-rich) group on the enzyme attacks an electrophilic (electron-deficient) center on the substrate to form a covalent enzyme–substrate intermediate. Consider the following two-step reaction:

$$\begin{aligned} &\textit{Step 1} && \text{A–X + Enzyme} \rightarrow \text{Enzyme–X + A} \\ &\textit{Step 2} && \text{Enzyme–X + Y} \rightarrow \text{X–Y + Enzyme} \\ &\textit{Net reaction} && \text{Enzyme + A–X + Y} \rightarrow \text{Enzyme + X–Y + A} \end{aligned}$$

In this case, Y can be a second substrate (or H_2O) that reacts more strongly with the unstable enzyme–X intermediate than it would with A–X. In other words, the enzyme uses two faster reactions instead of one slower reaction to achieve an overall increased rate. Note that the active enzyme is regenerated after the formation of the X–Y product.

One example of covalent catalysis is the formation of 1,3-bisphosphoglycerate from glyceraldehyde-3-phosphate by the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase. This important reaction in carbohydrate metabolism illustrates the central role of the coenzyme NAD^+ in mediating product formation. Substrate binding initiates a nucleophilic attack by a sulfhydryl group in the enzyme on

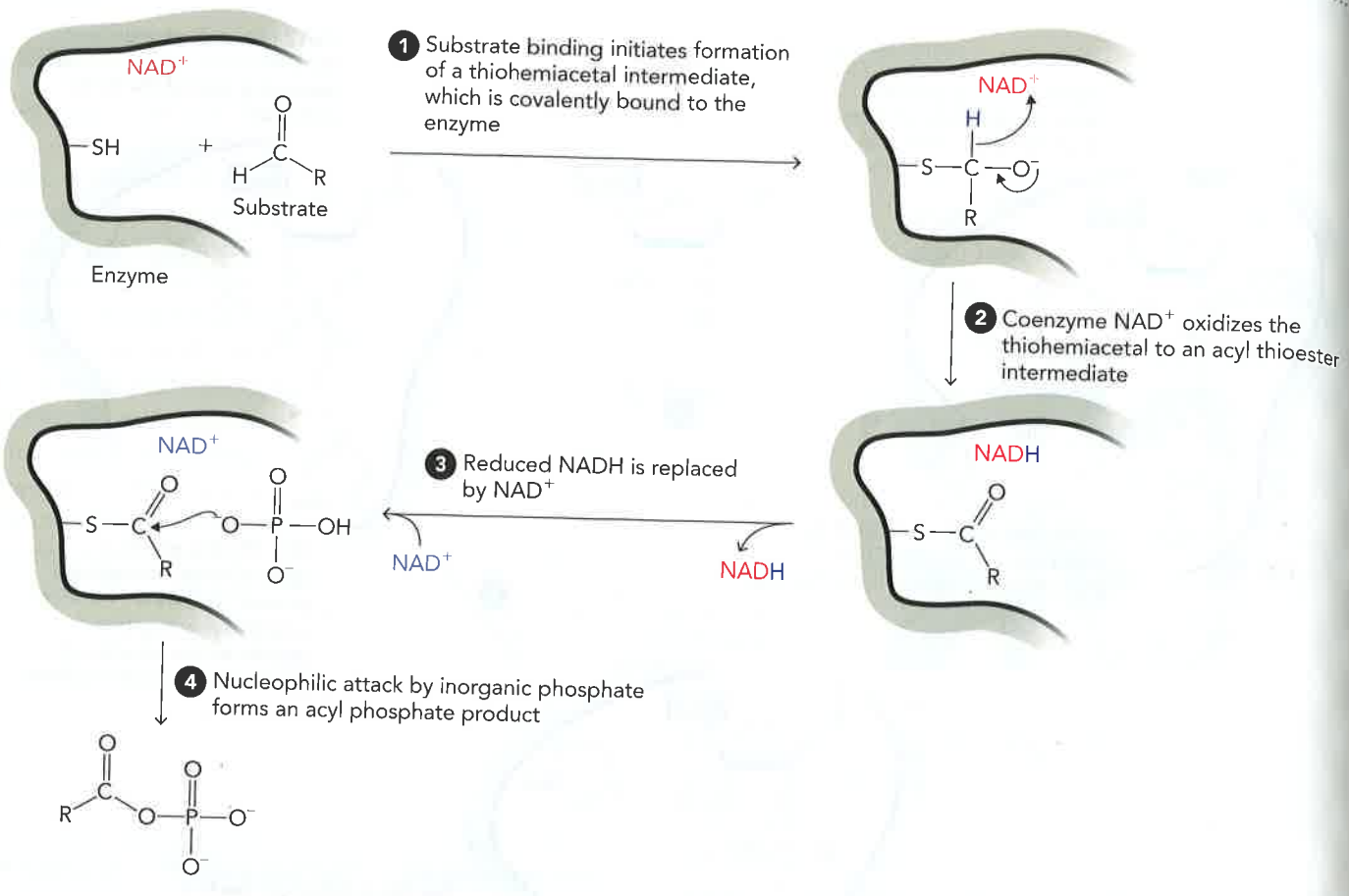


Figure 7.19 The formation of 1,3-bisphosphoglycerate from glyceraldehyde-3-phosphate by the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase is an example of covalent catalysis involving the coenzyme NAD^+. The sulfhydryl group from a cysteine residue of the enzyme forms a covalent bond with glyceraldehyde-3-phosphate to form the thiohemiacetal intermediate.

the substrate, forming a thiohemiacetal enzyme intermediate. As shown in **Figure 7.19**, the coenzyme NAD^+ functions as a co-substrate and oxidizes the thiohemiacetal to form an acyl thioester intermediate. The reduced coenzyme NADH is then replaced by NAD^+ in the active site, and inorganic phosphate (HPO_4^{2-}) attacks the thioester intermediate, resulting in the formation of the acyl phosphate product. We will look at this reaction in more detail in Chapter 9 when we discuss glycolytic enzymes.

Metal-ion catalysis. A large number of enzymes require metal ions to function as enzyme cofactors in the catalytic reaction (see Table 7.1). Positively charged metal ions function to promote proper orientation of bound substrates and to shield or stabilize negative charges through electrostatic interactions. Some metal ions mediate redox reactions through reversible changes in oxidation state. Enzymes with loosely bound metal ions are called metal-activated enzymes, whereas enzymes with tightly bound metal ions are called **metalloenzymes**. Metal-activated enzymes bind alkali and alkaline earth metals present in solution, most often Mg^{2+}, Ca^{2+}, Na^+, and K^+. Metalloenzymes usually contain Zn^{2+}, Fe^{2+}, Fe^{3+}, Mn^{2+}, Cu^{2+}, or Co^{2+}.

Carbonic anhydrase, an important enzyme in gas exchange and blood pH balance, is a metalloenzyme that uses a Zn^{2+} ion to catalyze the reversible reaction of bicarbonate formation from carbon dioxide, as shown in **Figure 7.20**. Through metal ion coordination bonds, the Zn^{2+} lowers the pK_a of water and facilitates formation of a nucleophilic hydroxyl group (OH^-) that attacks the substrate (CO_2). The active enzyme is regenerated by the binding of another H_2O molecule. The molecular structure of carbonic anhydrase reveals the positions of three conserved histidine residues in the substrate binding pocket that help coordinate the Zn^{2+} ion (**Figure 7.21**).

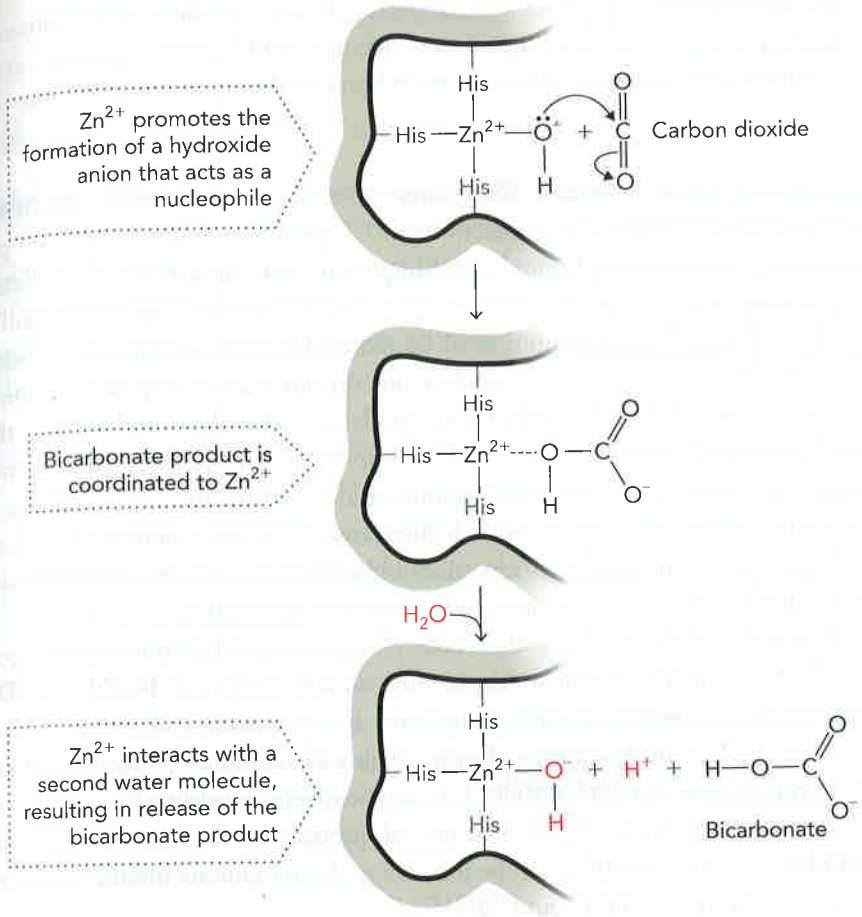


Figure 7.20 The carbonic anhydrase reaction uses a coordinated Zn^{2+} ion as a catalytic group that supports the nucleophilic attack on the CO_2 substrate, yielding the bicarbonate product.

Enzymes Perform Work in the Cell

Enzymes are essential to life because they enable chemical reactions in cells to occur on a useful timescale and under physiologic conditions characterized by neutral pH (pH 7–8), low pressure (1 atm), and relatively low temperature (25–40 °C). The thousands of enzyme-mediated reactions that take place inside cells can be grouped into three general categories on the basis of the work they accomplish:

- *Coenzyme-dependent redox reactions* are responsible for much of the energy conversion that takes place in cells. These essential redox reactions are central components of three major metabolic pathways described later in the book: (1) the citrate cycle, (2) the electron transport system, and (3) the photosynthetic light reactions.
- *Metabolite transformation reactions* are essential steps in all biosynthetic (anabolic) and degradative (catabolic) pathways. These reactions include a variety of isomerization and condensation reactions, as well as hydrolysis and dehydration reactions.
- *Reversible covalent modification reactions* attach or remove molecular tags to biomolecules to alter their recognition properties and regulate cellular processes. Two of the most common

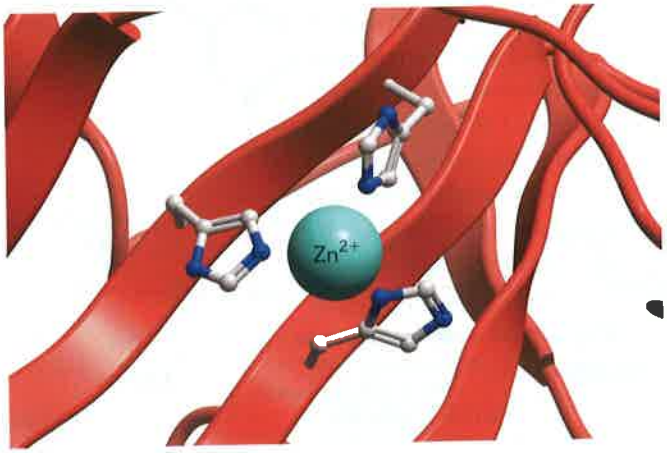


Figure 7.21 Human carbonic anhydrase uses three histidine residues to coordinate the Zn^{2+} ion (blue sphere) in the active site. BASED ON PDB FILE 5CAC.

of these reversible reactions in eukaryotic cells are the phosphorylation and dephosphorylation of signaling molecules and the methylation and demethylation of DNA at cytosine bases in the regulatory regions of genes.

Coenzyme-Dependent Redox Reactions We first introduced biochemical oxidation and reduction (redox) reactions in Chapter 2, when we discussed principles of bioenergetics (see Figure 2.3). Simply put, oxidation is a loss of electrons and reduction is a gain of electrons. Moreover, when one compound is oxidized by an enzyme, another compound must be reduced, meaning that redox reactions are coupled. The reason is that free electrons do not exist stably in solution, but instead pass from compounds that have a tendency to be electron donors to those that function as electron acceptors. Most important, the physical and chemical properties of amino acid functional groups located within the enzyme active site can alter the redox potential of bound substrates. However, common amino acid side chains, which are unable to reversibly bind or accept electrons, are not sufficient to catalyze the redox reaction, and coenzymes are required.

In general, redox reactions that take place at C—O bonds use the coenzymes NAD^+/NADH (nicotinamide adenine dinucleotide) and **$NADP^+$/NADPH (nicotinamide adenine dinucleotide phosphate)**. Redox reactions at C—C bonds are usually mediated by the coenzymes **flavin adenine dinucleotide ($FAD/FADH_2$)** and **flavin mononucleotide ($FMN/FMNH_2$)**. Redox reactions requiring these coenzymes involve the transfer of 2 e^-, either as a pair of electrons in the case of NADH and NADPH or 1 e^- at a time through the formation of semiquinone intermediates, as in the free-radical forms $FADH^\bullet$ and $FMNH^\bullet$.

Figure 7.22 illustrates two enzymatic reactions that use coenzymes as electron carriers during redox reactions that are involved directly or indirectly in energy conversion. The enzyme lactate dehydrogenase catalyzes a redox reaction that interconverts pyruvate, the product of the glycolytic pathway, and lactate, a metabolite that accumulates during anaerobic respiration. The reaction requires the conjugated redox pair NAD^+/NADH. In the direction shown, pyruvate is reduced to form lactate, while at the same time, NADH is oxidized to generate NAD^+. Similarly, the citrate cycle

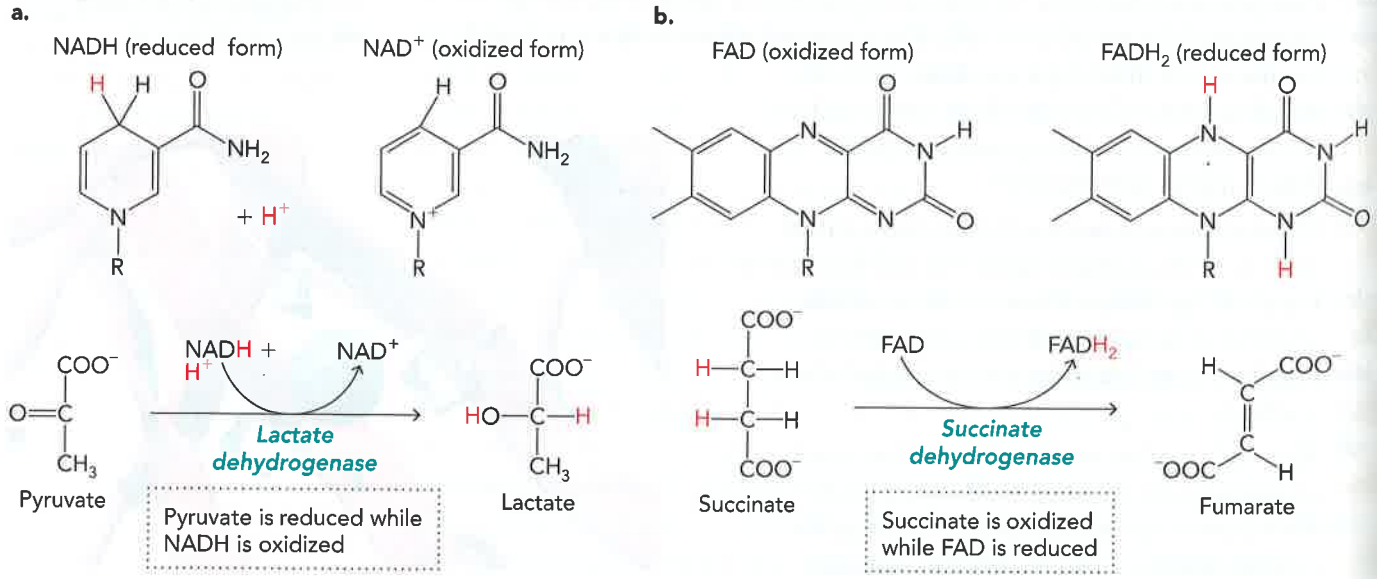


Figure 7.22 Enzyme-mediated redox reactions involved in energy conversion often require coenzymes, which function as electron carriers. **a.** Lactate dehydrogenase uses the coenzyme NADH to form lactate. NADH is oxidized while pyruvate is reduced. **b.** The enzyme succinate dehydrogenase forms fumarate in a redox reaction requiring the coenzyme FAD. FAD is reduced while succinate is oxidized.

enzyme succinate dehydrogenase uses the conjugated redox pair $FAD/FADH_2$ to interconvert succinate and fumarate. In the citrate cycle, the oxidation of succinate forms fumarate, while the reduction of FAD generates $FADH_2$. Note that each reaction involves the transfer of a pair of electrons.

Metabolite Transformation Reactions An important biochemical reaction in cells is the chemical transformation of metabolites to generate reactive intermediates, which are necessary components of anabolic and catabolic pathways. The variety of enzyme-mediated reactions in this category reflects the chemical diversity of organic compounds, but three types of these reactions are the most common in metabolic pathways:

1. **Isomerization** reactions do not change the molecular formula of the product compared to that of the substrate.
2. **Condensation** reactions combine two substrates to form a larger molecule, with the loss of a smaller molecule, usually water.
3. **Hydrolysis** or **dehydration** reactions cleave a substrate to two products by the addition or removal of water.

For example, **Figure 7.23a** illustrates the isomerization of dihydroxyacetone phosphate to form glyceraldehyde-3-phosphate in a reaction catalyzed by the glycolytic enzyme triose phosphate isomerase. The molecular formula of these two metabolites, $C_3H_7O_6P$, is identical, and under physiologic conditions in the cell, this isomerization reaction is readily reversible.

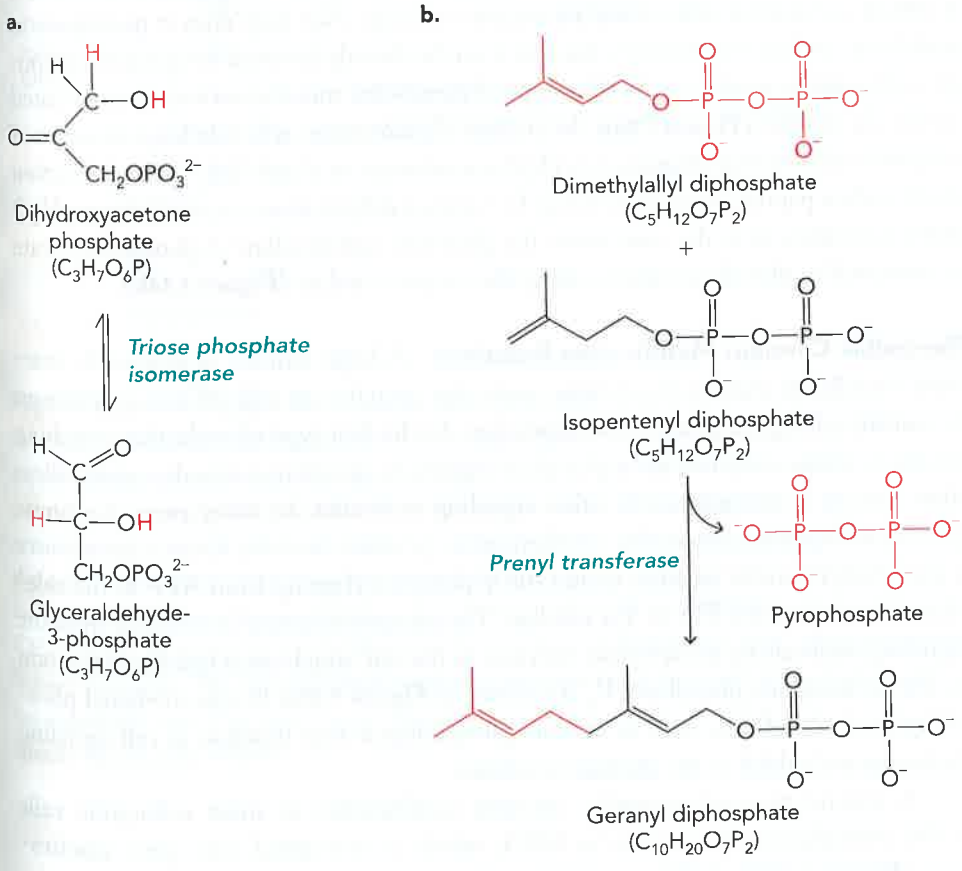


Figure 7.23 Metabolite transformation reactions are essential components of anabolic and catabolic pathways. **a.** Triose phosphate isomerase catalyzes an isomerization reaction that interconverts the phosphorylated C_3 metabolites dihydroxyacetone phosphate and glyceraldehyde-3-phosphate. Isomerization reactions generate a product that has the same molecular formula as the substrate. **b.** Condensation reactions in metabolic pathways are often characterized by the combination of two substrates with the same molecular formula, which function as building blocks for the synthesis of larger biomolecules. The prenyl transferase reaction shown here is a step in the cholesterol biosynthetic pathway.

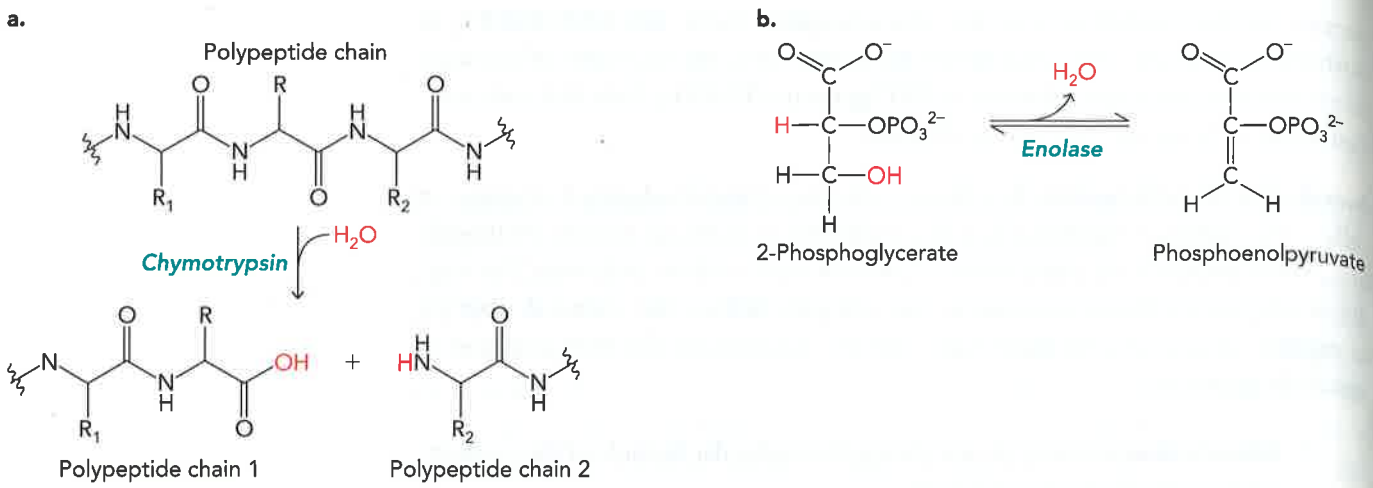


Figure 7.24 Numerous reactions in metabolic pathways involve H_2O directly as a substrate or product. **a.** Hydrolysis reactions often result in chemical cleavage, as seen here in the reaction catalyzed by the protease enzyme chymotrypsin. **b.** The removal of H_2O from 2-phosphoglycerate to form phosphoenolpyruvate, an enzymatic reaction in the glycolytic pathway, is a dehydration reaction catalyzed by the enzyme enolase.

In the condensation reaction, two compounds function as building blocks for larger biomolecules. One example is the condensation of dimethylallyl diphosphate ($C_5H_{12}O_7P_2$) and isopentenyl diphosphate ($C_5H_{12}O_7P_2$) to form geranyl diphosphate ($C_{10}H_{20}O_7P_2$), a reaction catalyzed by the enzyme prenyl transferase (**Figure 7.23b**). Multiple condensation reactions containing C_5-derivative compounds are a hallmark of the cholesterol biosynthetic pathway. Notably, the dihydroxyacetone phosphate and glyceraldehyde-3-phosphate isomers in the triose phosphate isomerase reaction can also be combined in a condensation reaction mediated by the enzyme aldolase (see Figure 7.11). This reaction leads to the formation of fructose-1,6-bisphosphate, an intermediate in the gluconeogenic pathway.

Water is present in cells at very high concentrations and behaves as if it were a pure substance. One of the reasons for the central role of H_2O in life processes is that it is a critical component of so many enzymatic reactions. Not only does it participate in acid–base catalysis mechanisms, but also it can be directly involved in enzyme reactions as a substrate or product—the third type of metabolite transformation reactions listed earlier. As shown in **Figure 7.24a**, the enzyme chymotrypsin, which belongs to a class of enzymes called **endoproteases**, uses H_2O as a substrate in a hydrolytic cleavage reaction that breaks a peptide bond in proteins. In contrast, dehydration reactions remove H_2O from substrates, as is the case when the glycolytic intermediate 2-phosphoglycerate is converted to phosphoenolpyruvate by the enzyme enolase (**Figure 7.24b**).

Reversible Covalent Modification Reactions A large number of enzymatic reactions in cells are involved in turning molecular switches on and off as a mechanism to control cell signaling and gene expression. In the first type of molecular switching reactions, kinase enzymes add a phosphoryl group to signaling molecules, which alters their activity or recognition by other signaling molecules. In many cases, the phosphorylated signaling molecules are themselves proteins, and the kinase enzyme uses a phosphoryl transfer reaction to add the γ-phosphoryl group from ATP to the side-chain hydroxyl of Ser, Thr, or Tyr residues. The phosphoryl group is removed from the signaling molecule by phosphatase enzymes in the cell, which use a hydrolysis reaction to release inorganic phosphate, P_i. As shown in **Figure 7.25a**, kinase-mediated phosphorylation can also be used to activate phospholipids that function as cell signaling molecules embedded in the plasma membrane.

A second type of reversible covalent modification in most eukaryotic cells is the methylation of cytosine in DNA, which is associated with gene inactivation (**Figure 7.25b**). DNA methyltransferase enzymes transfer a methyl group

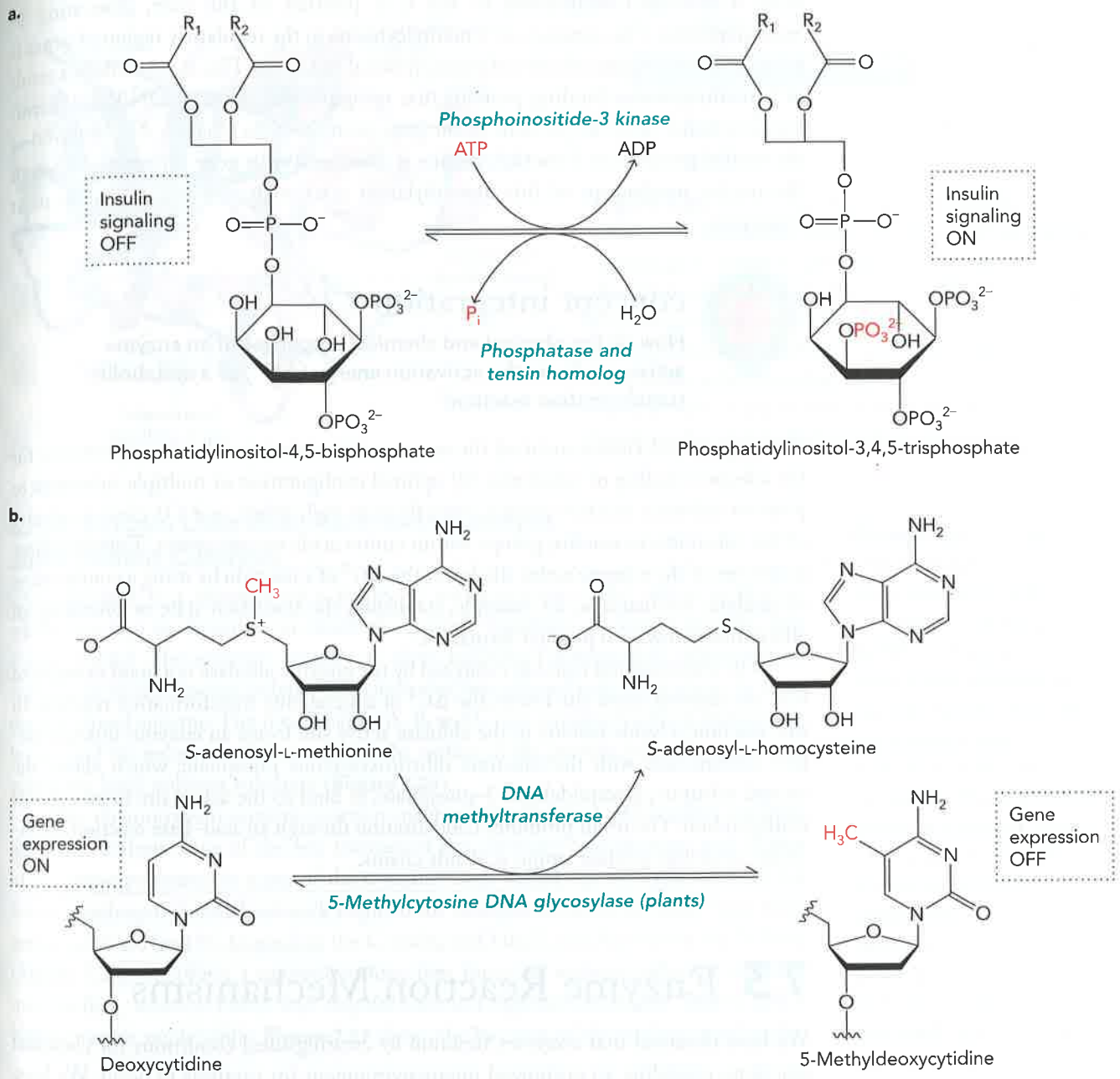


Figure 7.25 Reversible covalent modifications of biomolecules by enzymes in the cell function as molecular switching signals that control a variety of cellular processes. **a.** Insulin signaling leads to stimulation of phosphoinositide-3 kinase activity. This enzyme then phosphorylates phosphatidylinositol-4,5-bisphosphate on C-3 of inositol to generate phosphatidylinositol-3,4,5-trisphosphate. The insulin signaling pathway is turned off when the enzyme phosphatase and tensin homolog (PTEN) removes this phosphate. **b.** DNA methyltransferases use the metabolite *S*-adenosyl-L-methionine as a methyl donor to add a CH_3 group to deoxycytidine in DNA on the C-5 position of the cytosine base, which generates 5-methylcytosine. The demethylation reaction is associated with gene activation. In plants, the base containing the CH_3 group is removed through a DNA repair mechanism that involves a specific glycosylase enzyme.

from **S-adenosyl-L-methionine** to the C-5 position of the base, generating **5-methylcytosine**. The presence of 5-methylcytosine in the regulatory region of genes is associated with decreased rates of transcriptional initiation. This is most likely a result of 5-methylcytosine binding proteins that recognize this form of DNA and recruit transcriptional silencing proteins to the gene promoter (see Chapter 23). Removal of the methyl group from 5-methylcytosine is associated with gene activation, although the precise mechanism of this demethylation reaction is not understood in most organisms.

concept integration 7.2

How do the physical and chemical properties of an enzyme active site lower the activation energy ($\Delta G^{\ddagger}$) of a metabolite transformation reaction?

The sequestered environment of the enzyme active site provides an opportunity for (1) selective binding of substrates; (2) optimal configuration of multiple substrates to position substrate reactive groups correctly near each other; and (3) close proximity of the substrates to reactive groups within amino acids or coenzymes. Together, these properties of the enzyme active site lower the $\Delta G^{\ddagger}$ of a reaction by using a combination of catalytic mechanisms; for example, stabilizing the transition state or providing an alternative pathway to product formation.

The condensation reaction catalyzed by the enzyme aldolase is a good example of how the enzyme active site lowers the $\Delta G^{\ddagger}$ of a metabolite transformation reaction. In this reaction, a lysine residue in the aldolase active site forms an enzyme-linked covalent intermediate with the substrate dihydroxyacetone phosphate, which allows the second substrate, glyceraldehyde-3-phosphate, to bind to the active site in an optimal configuration. The result promotes condensation through an acid–base reaction mechanism involving aldolase amino acid side chains.

7.3 Enzyme Reaction Mechanisms

We have observed that enzymes function by creating ideal conditions for chemical reactions, providing an optimized microenvironment for catalysis to occur. We have also observed that enzymes stabilize transition states or provide an alternative pathway to product formation, thus lowering the activation energy. In this section, we examine in more detail three proposed enzyme reaction mechanisms. These models are based on molecular analysis of protein structures and on results of *in vitro* kinetic studies using a variety of high-affinity inhibitors and nonhydrolyzable substrates. The use of X-ray crystallography to determine the structures of enzymes in the presence and absence of bound pseudosubstrates has permitted biochemists to view enzymes "caught in the act" of catalysis.

The enzyme reaction mechanisms used by chymotrypsin, enolase, and HMG-CoA reductase provide classic examples of multistep reactions. Together, these examples reinforce two core concepts in enzymology: (1) substrates bind to enzyme active sites through weak noncovalent interactions, which orient amino acid functional groups within close proximity to substrate reactive centers; and (2) enzymes use conventional catalytic reaction mechanisms that follow basic principles of organic chemistry.

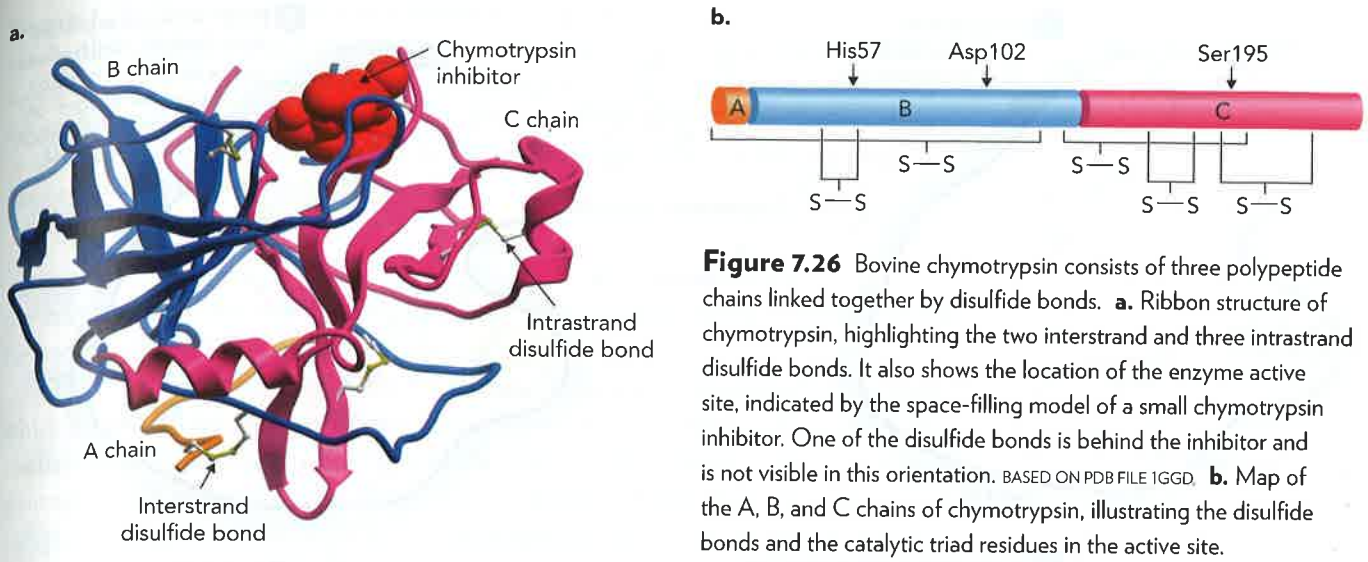


Figure 7.26 Bovine chymotrypsin consists of three polypeptide chains linked together by disulfide bonds. **a.** Ribbon structure of chymotrypsin, highlighting the two interstrand and three intrastrand disulfide bonds. It also shows the location of the enzyme active site, indicated by the space-filling model of a small chymotrypsin inhibitor. One of the disulfide bonds is behind the inhibitor and is not visible in this orientation. BASED ON PDB FILE 1GGD. **b.** Map of the A, B, and C chains of chymotrypsin, illustrating the disulfide bonds and the catalytic triad residues in the active site.

Chymotrypsin Uses Both Acid–Base Catalysis and Covalent Catalysis

Chymotrypsin is a member of a family of endoproteases called **serine proteases,** which function in digestion to cleave the peptide backbone of dietary proteins (see Figure 7.24a). The enzyme consists of three individual polypeptide chains, which started out as one nascent polypeptide chain that was cleaved into three pieces to form the functional enzyme. The three chains (A, B, and C) are covalently linked by disulfide bonds, and the enzyme active site sits on the surface of the protein, where it can readily access the large polymer substrate (**Figure 7.26**).

The chymotrypsin enzyme reaction mechanism involves both covalent catalysis and acid catalysis. One of the key features of the chymotrypsin reaction, and indeed of all serine proteases, is a set of three amino acids called the **catalytic triad,** which forms a hydrogen-bonded network required for catalysis. In chymotrypsin, these three amino acids are Ser195, located on the C chain, and His57 and Asp102 on the B chain (Figure 7.26). Ser195 is a catalytic residue that forms an enzyme–substrate covalent intermediate, whereas His57 and Asp102 function together to convert Ser195 into a highly reactive nucleophile. **Figure 7.27** shows the location of the catalytic triad within

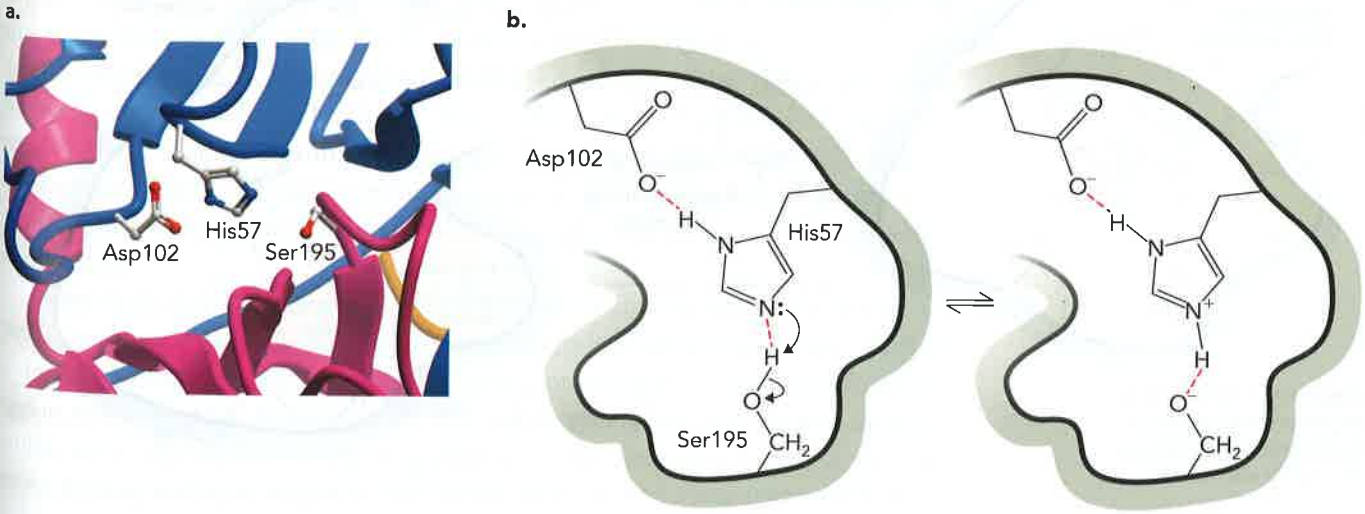


Figure 7.27 The catalytic triad in the chymotrypsin enzyme active site consists of the amino acid residues Asp102, His57, and Ser195. **a.** Molecular structure of the bovine chymotrypsin active site, with the three amino acids in the catalytic triad shown in stick representation. BASED ON PDB FILE 1GGD. **b.** A hydrogen-bond network is established among the Asp102, His57, and Ser195 residues of the chymotrypsin catalytic triad. Through an interaction with Asp102, the proton from Ser195 is transferred to the His57 imidazole ring as the nucleophilic reaction occurs.

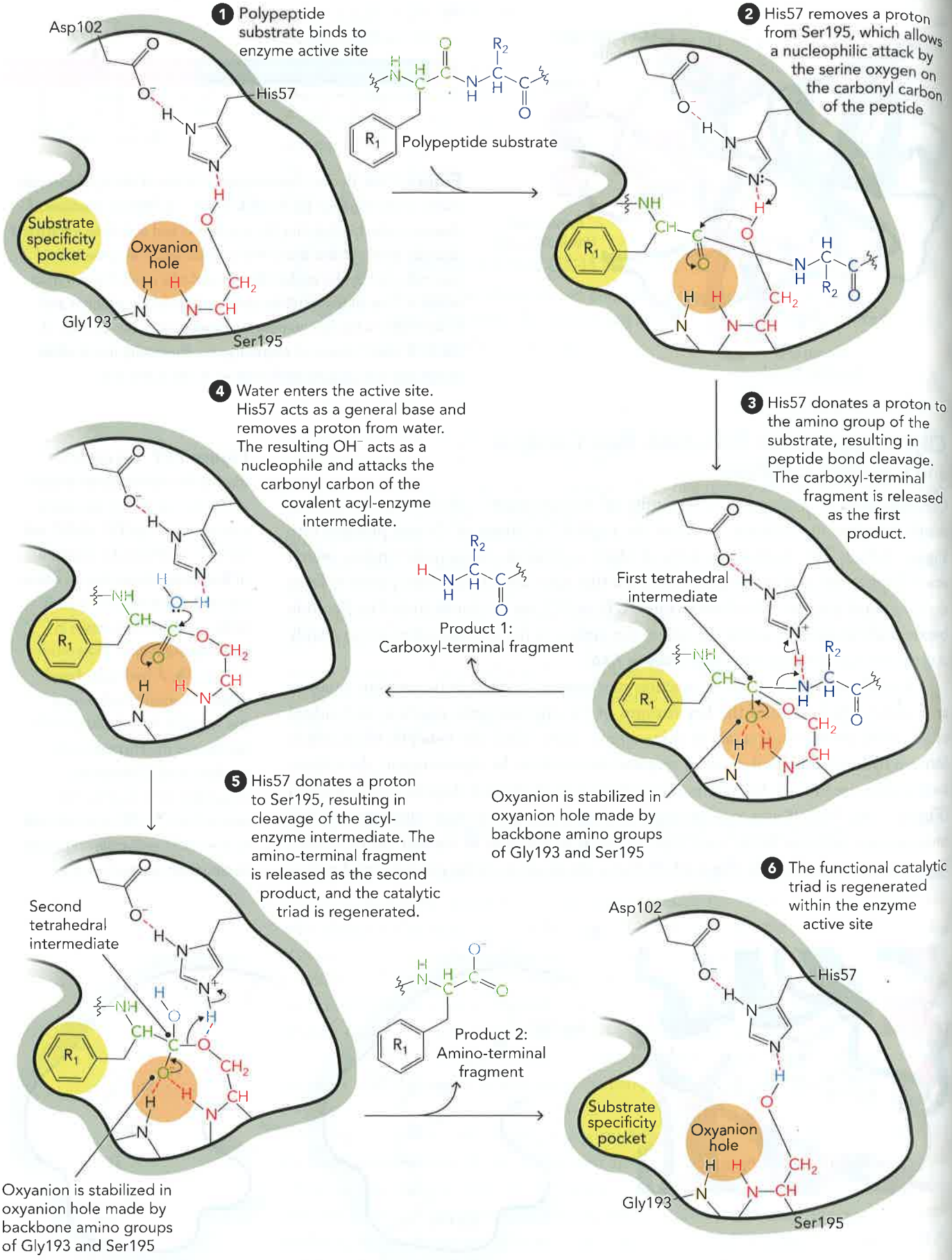

1 Polypeptide substrate binds to enzyme active site
Asp102
His57
Substrate specificity pocket
Oxyanion hole
Gly193
Ser195
Polypeptide substrate
2 His57 removes a proton from Ser195, which allows a nucleophilic attack by the serine oxygen on the carbonyl carbon of the peptide
3 His57 donates a proton to the amino group of the substrate, resulting in peptide bond cleavage. The carboxyl-terminal fragment is released as the first product.
First tetrahedral intermediate
Product 1: Carboxyl-terminal fragment
4 Water enters the active site. His57 acts as a general base and removes a proton from water. The resulting OH⁻ acts as a nucleophile and attacks the carbonyl carbon of the covalent acyl-enzyme intermediate.
Oxyanion is stabilized in oxyanion hole made by backbone amino groups of Gly193 and Ser195
5 His57 donates a proton to Ser195, resulting in cleavage of the acyl-enzyme intermediate. The amino-terminal fragment is released as the second product, and the catalytic triad is regenerated.
Second tetrahedral intermediate
Product 2: Amino-terminal fragment
6 The functional catalytic triad is regenerated within the enzyme active site
Asp102
His57
Substrate specificity pocket
Oxyanion hole
Gly193
Ser195
Oxyanion is stabilized in oxyanion hole made by backbone amino groups of Gly193 and Ser195

Figure 7.28 The proposed catalytic mechanism of chymotrypsin can be broken down into two discrete phases. Phase 1 includes the first three steps of the reaction, which includes formation of an acyl-enzyme intermediate and cleavage of the scissile peptide bond, releasing the carboxyl-terminal polypeptide fragment. In phase 2, steps 4 and 5 lead to cleavage of the covalent bond between the substrate and Ser195 to release the amino-terminal polypeptide fragment and regenerate the enzyme (step 6).

the enzyme active site and the organization of the hydrogen-bond network formed between Asp102, His57, and Ser195.

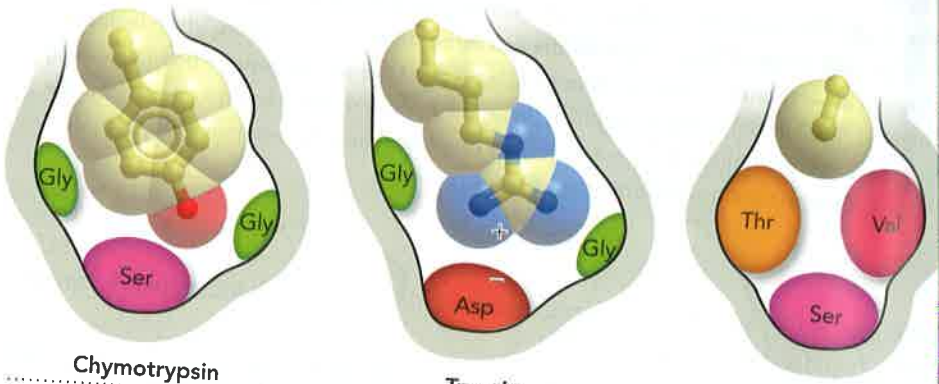
The proposed enzyme reaction mechanism of chymotrypsin consists of six steps, which can be divided into two phases. In the first phase, a covalent acyl-enzyme intermediate is formed between Ser195 and the polypeptide substrate, which promotes cleavage of the **scissile peptide bond** (site of cleavage). Then the carboxyl-terminal polypeptide fragment is released. In the second phase, the enzyme is regenerated after a series of steps that results in deacylation and release of the amino-terminal polypeptide fragment. Let's now look at each of these six steps individually, as illustrated in **Figure 7.28**.

Step 1 of the reaction begins with binding of the substrate to the enzyme active site after a productive interaction between the aromatic side chain (R_1 in Figure 7.28) of the polypeptide substrate and the nearby specificity pocket, which functions to properly align the substrate with the catalytic triad. In *step 2*, a proton is transferred from Ser195 to His57, which allows a nucleophilic attack by the oxygen of Ser195 to occur on the carbonyl carbon of the polypeptide backbone. The result is the formation of a covalent, transient **tetrahedral intermediate**, which is thought to resemble the transition state conformation. This tetrahedral intermediate has a C—O bond that is longer than a double bond, which allows the negatively charged oxygen (called the oxyanion) to fit into a region of the active site called the **oxyanion hole**. Here, the oxyanion forms hydrogen bonds with the backbone NH groups of Ser195 and Gly193. In *step 3* of the reaction, the imidazole ring of His57 functions as an acid catalyst by donating a proton to the nitrogen of the peptide bond, facilitating cleavage. The carboxyl-terminal fragment of the polypeptide (product 1) leaves the active site, and the amino-terminal fragment of the polypeptide remains bound to the enzyme as a covalent acyl-enzyme intermediate.

Once the carboxyl-terminal fragment of the polypeptide substrate exits the active site, H_2O is able to enter and donate a proton to His57. This results in the generation of a free OH^- in *step 4* that attacks the carbonyl carbon on the acyl-enzyme and leads to the formation of a second tetrahedral intermediate stablized by the oxyanion hole. *Step 5* occurs when the now protonated imidazole ring of His57 donates a proton that cleaves the covalent bond of the acyl-enzyme intermediate. Then, the amino-terminal fragment of the polypeptide (product 2) is released, and the functional catalytic triad is regenerated within the enzyme active site (*step 6*).

One of the important features of serine proteases is that the size of the enzyme active site of three closely related proteins—chymotrypsin, trypsin, and elastase—imparts a degree of substrate specificity. Chymotrypsin, for example, contains a hydrophobic region in the substrate binding pocket that can accommodate proteins with aromatic amino acids adjacent to the scissile peptide bond. In contrast, a region of the substrate binding pocket of trypsin is much deeper and contains a negatively charged Asp

Figure 7.29 Regions of the substrate binding pockets in serine proteases reflect specificity for amino acids adjacent to the scissile bond. Note that the Ser and Asp residues shown in this figure are different amino acids from the ones in the catalytic triad.



Chymotrypsin
Large substrate binding pocket accommodates aromatic residues such as tyrosine

Trypsin
Binding pocket with a negatively charged residue at the bottom accommodates residues with a positive charge such as arginine

Elastase
Thr and Val residues close off the binding pocket so that only small residues such as alanine can be accommodated

residue at the bottom (**Figure 7.29**). This substrate specificity pocket explains why trypsin cleaves proteins at peptide bonds bordering positively charged lysine and arginine residues. Elastase does not contain a functional substrate specificity pocket because instead of the glycine residues located at the opening of the chymotrypsin and trypsin substrate specificity pockets, elastase has amino acids, such as threonine and valine, that effectively close off this region. This structure is consistent with the observation that the natural substrate of elastase, the fibrous protein elastin, is rich in glycine and alanine residues. These relatively small amino acids do not require a separate substrate binding pocket for proper alignment of the scissile bond with the serine of the catalytic triad.

Enolase Uses Metal Ions in the Catalytic Mechanism

Enolase is an enzyme that catalyzes the dehydration of 2-phosphoglycerate (2-PG) to form phosphoenolpyruvate in the glycolytic pathway (see Figure 7.24b). It also catalyzes the reverse reaction in gluconeogenesis. The formation of phosphoenolpyruvate is the penultimate step of the glycolytic pathway, as phosphoenolpyruvate—whose phosphoryl group has high transfer energy—serves as the phosphoryl group donor to produce ATP from ADP.

Enolase functions as a dimer with each monomer containing one active site. The active site is located within a cleft of the alpha/beta domain of the monomer, as shown in **Figure 7.30**. Enolase is a metalloenzyme: Each active site contains two divalent metal ions, which are absolutely required for the reaction.

Enolase, like chymotrypsin and many other enzymes, uses a combination of catalytic strategies in order to carry

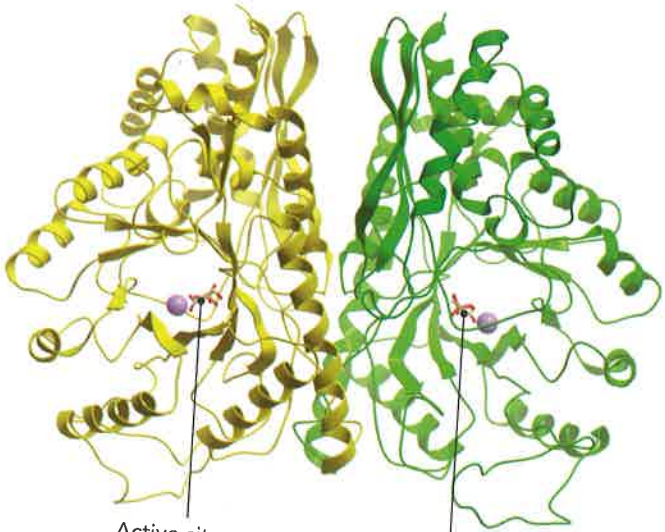


Figure 7.30 Enolase is a dimeric enzyme with one active site per monomer. In the monomer on the left (yellow), the substrate 2-phosphoglycerate is shown in stick representation, and one Mg^{2+} ion is shown as a lavender sphere. In the monomer on the right (green), the product phosphoenolpyruvate is shown in stick representation, and one Mg^{2+} ion is shown as a lavender sphere. The second Mg^{2+} ion in each active site is not shown. BASED ON PDB FILE 2ONE.

out its reaction. Mechanistic studies have shown that enolase uses general acid–base catalysis as well as metal-ion catalysis in its reaction mechanism.

The dehydration reaction takes place in two steps, as illustrated in **Figure 7.31**. In the first step, Lys345 functions as a general base, which removes the proton at the C-2 position of the substrate, generating an intermediate with a carbanion at C-2. The negative charge of the carbanion is delocalized to the carboxylate group, which is stabilized by ionic interactions with the Mg^{2+} ions. In the second step, Glu211 functions as a general acid, donating a proton to the OH leaving group, resulting in the elimination of H_2O and formation of phosphoenolpyruvate.

The divalent Mg^{2+} ions play several important roles in this reaction. First, they serve to bind and orient the substrate in the active site. Furthermore, while normally the proton at the C-2 position is not acidic and would be difficult to remove in the first step of the reaction, the strong ionic interactions of the substrate with the Mg^{2+} ions serve to make the proton at the C-2 position more acidic, facilitating its removal by Lys345. After abstraction of this proton, the Mg^{2+} ions also stabilize the increased negative charge on the intermediate.

In addition to the essential Mg^{2+} ions, other residues at the active site also contribute to the overall mechanism (**Figure 7.32**). Many of these residues are important for the formation of weak interactions. Lys396 and Gln167, for example, act in concert

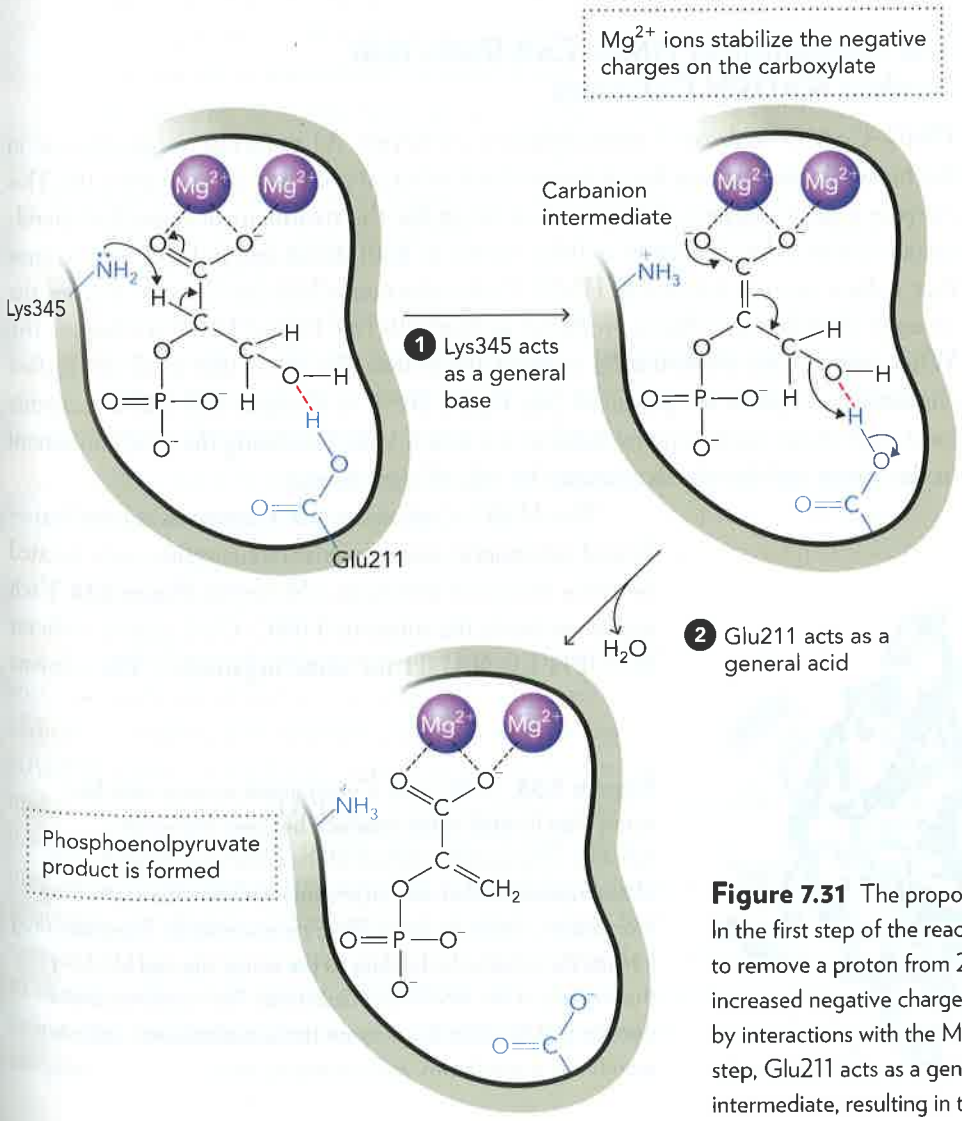


Figure 7.31 The proposed enolase reaction mechanism. In the first step of the reaction, Lys345 acts as a general base to remove a proton from 2-phosphoglycerate. The resulting increased negative charge on the carboxylate group is stabilized by interactions with the Mg^{2+} ions in the enzyme. In the second step, Glu211 acts as a general acid, donating a proton to the intermediate, resulting in the formation of phosphoenolpyruvate.

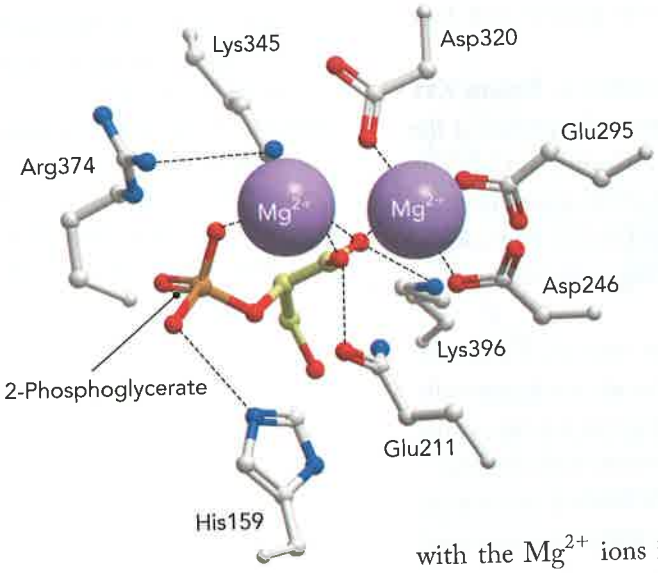


Figure 7.32 Residues in the active site of enolase are precisely arranged for recognition of the substrate and to promote catalysis. Lys396 and Gln167 form ionic interactions with the carboxylate group of the substrate 2-phosphoglycerate. His159 makes a hydrogen bond with the phosphoryl group of the substrate. Arg374 interacts with Lys345 to help produce Lys345 as a general base. Negatively charged aspartate and glutamate residues help bind the positively charged metal ions, which are shown as lavender spheres. Because this illustration is a two-dimensional representation of a three-dimensional structure, the lengths of the dashed lines indicating the bonds vary greatly. BASED ON PDB FILE 2ONE.

with the Mg^{2+} ions in making ionic interactions with the carboxylate group of the substrate. Other residues participate in the activation of acid–base groups. The formation of a hydrogen bond between His159 and the phosphate group of the substrate contributes to the acidification of the C-2 proton. As another example, interactions between Arg374 and the amino group of Lys345 serves to set up Lys345 as a general base for the start of the reaction.

The Mechanism of HMG-CoA Reductase Involves NADPH Cofactors

HMG-CoA (3-hydroxy-3-methylglutaryl-coenzyme A) reductase is an enzyme in the biosynthetic pathway for cholesterol and other isoprenoids (see Chapter 16). This enzyme is an important pharmaceutical target for the treatment of hypercholesterolemia because reduced enzyme activity results in both direct and indirect mechanisms that reduce serum cholesterol. HMG-CoA reductase inhibition directly reduces the amount of cholesterol that is produced in liver cells (see Figure 16.37 in Chapter 16). When intracellular cholesterol is reduced, the indirect benefit is that more cell surface cholesterol receptors are produced (see Figure 16.50 in Chapter 16). These receptors bind cholesterol-containing particles in the serum, thus decreasing the total cholesterol in the serum and thereby decreasing the risk of heart disease.

The HMG-CoA reductase enzyme is a membrane-bound tetrameric enzyme with four active sites located between monomer interfaces as shown in **Figure 7.33**. Each active site binds the substrate HMG-CoA and the cofactor NADPH (or NADH for some organisms). The enzyme

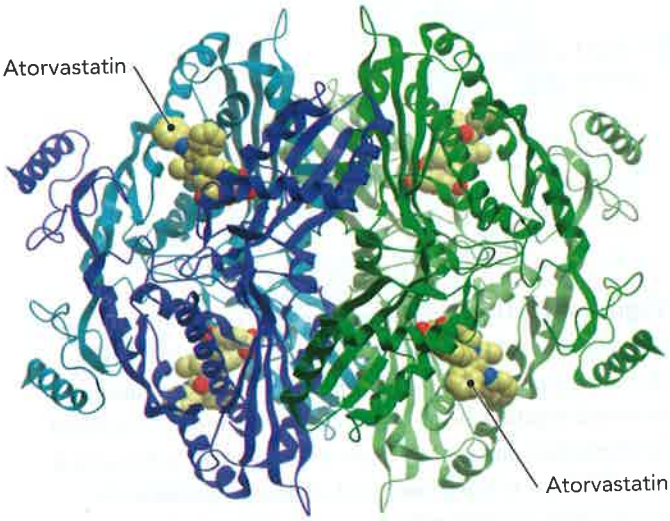


Figure 7.33 HMG-CoA is a tetrameric enzyme with four active sites located at the interface between monomeric subunits. The catalytic portion of the enzyme is shown here in ribbon representation with an enzyme inhibitor, the statin drug atorvastatin, shown in space-filling representation. The statin drug inhibits the enzyme by binding to the active site and blocking the binding of the HMG-CoA substrate. The transmembrane domain that localizes the enzyme to the endoplasmic reticulum membrane is not shown. BASED ON PDB FILE 1HWK.

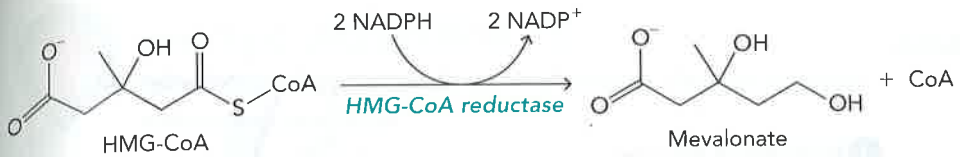


Figure 7.34 HMG-CoA reductase catalyzes the four-electron reduction of HMG-CoA to mevalonate and CoA using two NADPH molecules as cofactors.

catalyzes the four-electron reduction of HMG-CoA into CoA and mevalonate (**Figure 7.34**). Mevalonate is a precursor in the production of terpenes and steroids, such as cholesterol.

The HMG-CoA reductase mechanism has been elucidated from numerous kinetic studies and crystallographic structures, aided by computational studies. The reduction of the HMG-CoA thioester is achieved by two hydride transfer steps involving two NADPH cofactors. The active site of the protein is important for both binding and orienting HMG-CoA and NADPH so hydride transfer can occur, and it also provides functional groups to stabilize the transition state.

The overall reaction mechanism of HMG-CoA reductase can be described in four steps as shown in **Figure 7.35**:

1. The hydride ion from NADPH attacks the carbonyl carbon of HMG-CoA, developing an oxyanion transition state, which is stabilized by a lysine side chain in the active site. A glutamate residue acts as a general acid to protonate the oxyanion, resulting in the formation of a hydroxyl group in the intermediate, mevaldyl-CoA. At the end of the first step, the thioester of HMG-CoA has been reduced to a mevaldyl-CoA hemithioacetal.
2. Protein conformational changes trigger the exchange of $NADP^+$ for NADPH. The second NADPH is now in position for another hydride transfer reaction.
3. The glutamate side chain in the active site now acts as a general base to deprotonate the hydroxyl group producing an aldehyde, mevaldehyde. As the thiolate bond is broken, the active site histidine donates a proton to CoA forming the reduced CoA molecule (CoA-SH).
4. The hydride from the second NADPH molecule attacks the carbonyl center of the aldehyde, and the glutamate residue donates a proton to the oxygen of the aldehyde. This step results in the formation of the reduced mevalonate species and $NADP^+$.

The HMG-CoA reductase mechanism presented here is largely consistent with the available data, but there are still outstanding questions that will require additional studies. For example, there has been some debate over whether the NADPH cofactor exchange step happens before or after the hemithioacetal is broken down. If the NADPH exchange happens after mevaldehyde is formed, then it would be expected that conformational changes that promote cofactor exchange may allow the aldehyde species to be released from the active site to some extent. Moreover, the details of the enzyme conformational changes that take place during cofactor exchange are also an active area of research. It is anticipated that answers to these mechanistic details of HMG-CoA reductase structure and function will aid in the development of more specific inhibitors of this biomedically relevant enzyme.

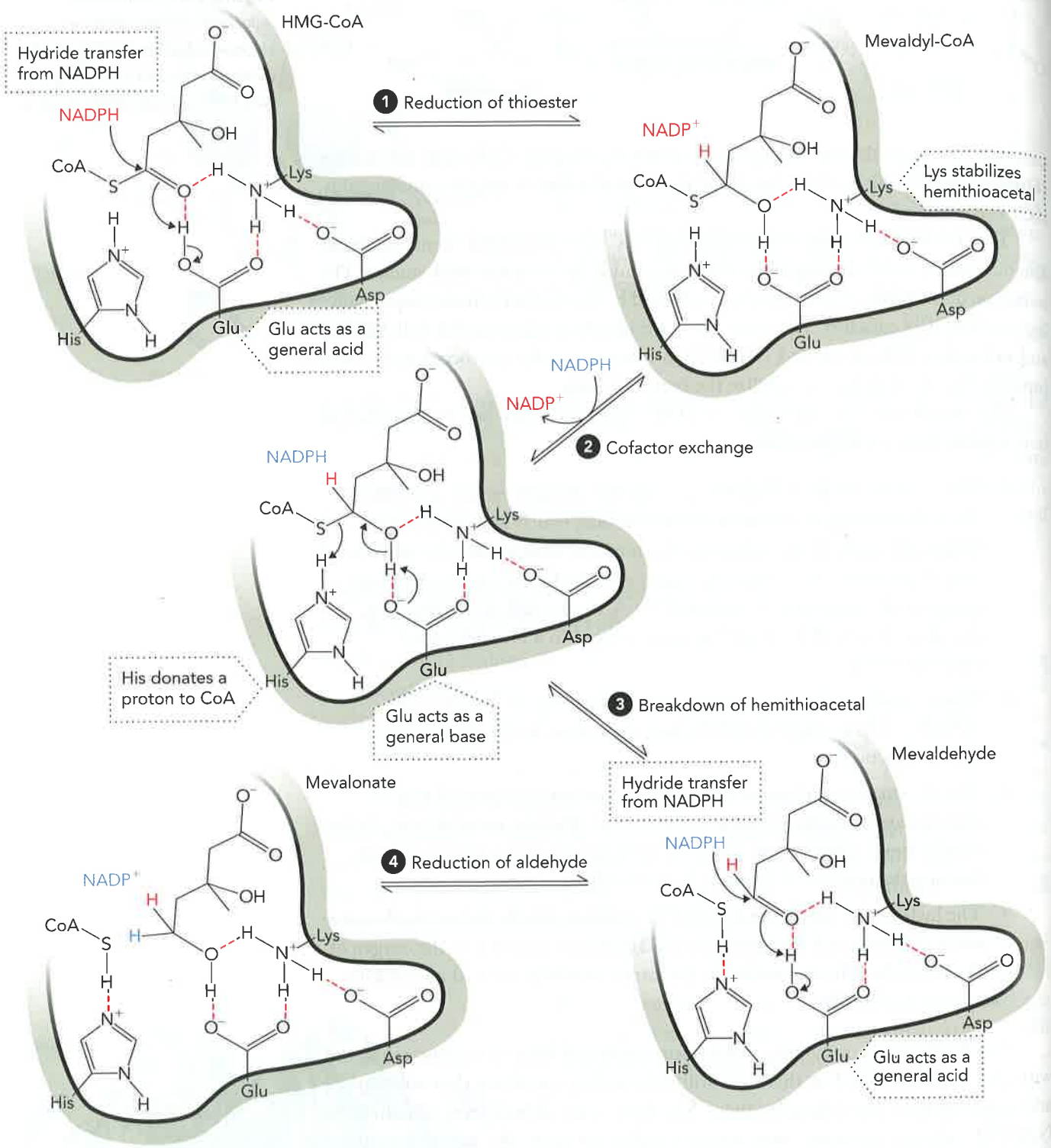


Figure 7.35 HMG-CoA reductase converts HMG-CoA to mevalonate in a four-step reaction using two NADPH cofactors. The residues shown are Glu83, Lys267, Asp283, and His381 of the soluble HMG-CoA reductase enzyme from *Pseudomonas mevalonii*. The details of each step are described in the text.

concept integration 7.3

What mechanisms of catalysis are shared by the chymotrypsin and enolase reactions?

The reaction mechanisms for both enzymes involve general acid–base catalysis. The chymotrypsin mechanism involves the formation of a covalent intermediate, which does not occur in the enolase reaction. Enolase requires divalent metal ions for catalysis, whereas the chymotrypsin mechanism relies solely on functional groups from the enzyme active site. Both reactions may also use transition state stabilization, although this is clearer in the case of the chymotrypsin reaction than it is for enolase.

7.4 Enzyme Kinetics

Enzyme kinetics is the quantitative study of the rates of chemical reactions performed by enzymes. An understanding of enzyme kinetics is important for determining the mechanism by which a reaction takes place, the effects of regulatory molecules (including metabolites or toxins), and the effects of enzyme variants under a defined set of conditions.

Enzyme kinetics is best characterized as the quantitative analysis of reaction rate data obtained with purified enzymes under defined laboratory conditions. For example, to determine how efficient a mutant enzyme is under various conditions of temperature, pH, and substrate concentration or to understand mechanisms of enzyme inhibition, we need to measure enzyme reaction rates quantitatively. Enzyme kinetics involves relating reaction rates to free energy and equilibrium. In Chapter 2, we introduced the concept of the change in free energy (ΔG) and showed how it relates to the concentrations of reactants and products of a reaction. We now describe enzyme kinetics in terms of reaction rates (v, velocity) and substrate concentration ([S]). With a quantitative understanding of how enzyme activity relates to protein function, we can define the catalytic efficiency of different enzymes to gain insight into their role as biological catalysts.

Enzymes, like all catalysts, function by increasing the rate at which a reaction reaches equilibrium. As already mentioned, enzymes catalyze reactions by lowering the activation energy ($\Delta G^{\ddagger}$) without altering the change in free energy of the reaction (ΔG). It is important to note that $\Delta G^{\ddagger}$ is not directly related to ΔG because catalyzed and uncatalyzed reactions have different $\Delta G^{\ddagger}$ values without affecting ΔG (see Figure 7.6).

Relationship between $\Delta G^{\ddagger}$ and the Rate Constant k

There are many parameters to consider when studying enzyme kinetics and numerous variations in types of reactions. As a starting point, we will first consider how the initial substrate concentration, [S], relates to the activation energy, $\Delta G^{\ddagger}$, for a **first-order reaction** (a reaction in which the rate varies as the first power of the reactant concentration) in which a single substrate is converted to a single product, $S \rightarrow P$. To do this, we define the **velocity of the reaction**, v, as the product of the **rate constant of a reaction**, k, and [S]:

$$v = k[S] \tag{7.1}$$

The rate constant k reflects how quickly a substrate molecule is converted to product (S → P) as a function of time under a defined set of conditions. For a first-order reaction, k has units of second^{-1} (s^{-1}). At a given substrate concentration, the velocity is directly related to the rate constant and refers to the units of product formed per unit time; for example, molarity per second ($M\ s^{-1}$). This will be important in the next section when we describe Michaelis–Menten enzyme kinetics.

In the description of enzyme mechanisms earlier in the chapter, we stated that the activation energy of a reaction, $\Delta G^{\ddagger}$, is lowered by the presence of a catalyst, resulting in an increased reaction rate. This relationship can be expressed mathematically by relating velocity v to activation energy:

$$v = \frac{k_B T}{h}[S]e^{-\Delta G^{\ddagger}/RT}$$

In this equation, k_B is the Boltzmann constant (1.38×10^{-23} J K^{-1}), and h is Planck's constant (6.63×10^{-34} J s). The value of $k_B T/h$ at 25 °C is equal to $6.2 \times 10^{12}\ s^{-1}$. We can rewrite this equation in terms of the rate constant, $k = v/[S]$:

$$k = \frac{v}{[S]} = \frac{k_B T}{h} e^{-\Delta G^{\ddagger}/RT} \tag{7.2}$$

On the basis of this equation, we can see why an enzyme-catalyzed reaction has a higher reaction rate, k, than that of an uncatalyzed reaction. Specifically, a lower $\Delta G^{\ddagger}$ value produces a higher (inverse and exponential) reaction velocity v, as defined by k and [S].

Many reactions in metabolism are bimolecular and involve two substrates or two molecules of the same substrate. These reactions are **second-order reactions** in which the reaction rate is proportional to the product of the substrate concentrations. The units of a second-order rate constant are molarity^{-1} second^{-1} ($M^{-1}\ s^{-1}$). A bimolecular reaction consisting of two substrates, S and Y, can be written as

$$S + Y \rightarrow P$$

The rate equation for this reaction is

$$v = k[S]\,[Y]$$

By experimentally measuring the disappearance of the substrates S and Y independently, we can see that S → P and Y → P are each first-order reactions, but that the overall conversion of S + Y → P is a second-order reaction.

Michaelis–Menten Kinetics

To characterize the catalytic properties of an enzyme under experimental conditions, it is necessary to know the substrate concentration during the course of the reaction. However, because substrate is constantly being consumed to generate product, substrate concentration is not constant, which affects the rate of the reaction over time. A common simplifying approach is to measure the reaction rate at the beginning of the reaction (**initial velocity, v_0**) before the substrate concentration has changed significantly. If working under conditions where [S] >> [E], at the beginning of the reaction (approximately within the first 60 seconds in typical reactions) the substrate concentration changes only minimally and can thus be considered constant.

We can measure initial velocities from plots of product formation versus time by taking the slope of the tangent at the beginning of each reaction (**Figure 7.36a**). The initial velocities vary with the substrate concentration when substrate concentrations

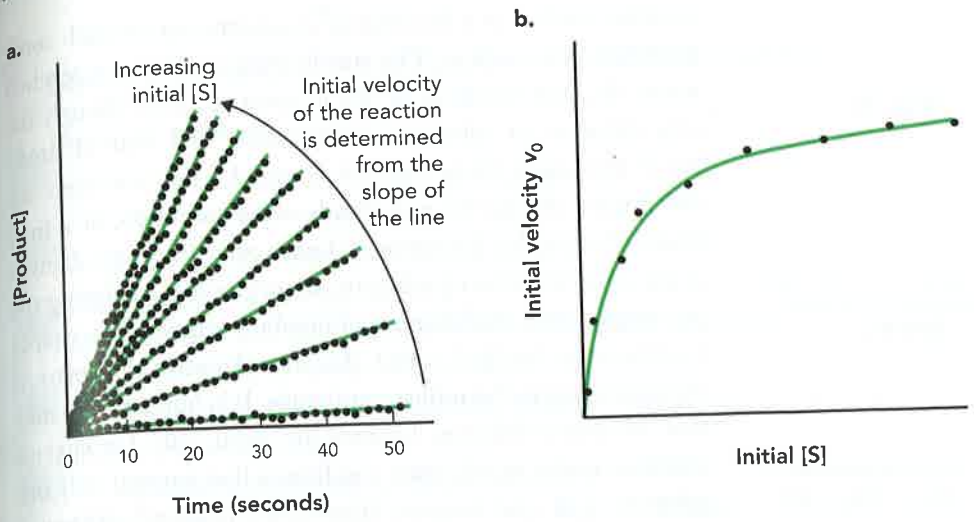


Figure 7.36 Michaelis–Menten kinetic parameters are obtained from experimental data under steady-state conditions. **a.** From a plot of product [P] formation versus time for different substrate concentrations [S], the initial velocity v_0 for each reaction is determined from the slope of the tangent at the early part of the reaction. **b.** Plotting v_0 versus [S] produces a hyperbolic curve if the enzyme reaction is first order and follows Michaelis–Menten kinetics.

ANIMATION

are relatively low. When substrate concentrations become sufficiently high, the initial velocities no longer show significant changes. At this point, the reaction is said to have reached the **maximum velocity, v_{max}**, where the reaction velocity cannot increase any further, even with the addition of more substrate. A plot of initial velocity versus substrate concentration shows the curve leveling off as v_{max} is approached (**Figure 7.36b**).

For an enzyme to convert a substrate (S) to a product (P), an enzyme–substrate (ES) complex must first be formed. Then the chemistry occurs to convert substrate to product, and finally product is released. This process can be described as

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EP \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E + P$$

We can make several simplifying assumptions to facilitate the study of enzyme-catalyzed reactions of this type, which are described in the kinetic model developed by Leonor Michaelis and Maud Menten in 1913. In the **Michaelis–Menten kinetic model**, first we consider the reaction at an early time when no appreciable product has been generated. Therefore the back reaction, where ES forms from enzyme-bound product (EP) with a rate constant k_{-2}, is negligible. Second, product released is assumed to be a rapid step in the process, so the conversion of EP to E + P does not need to be explicitly considered. Thus the reaction scheme can be reduced to

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P \qquad (7.3)$$

Another assumption in Michaelis–Menten kinetics, as defined in 1925 by G. E. Briggs and James Haldane, is that the **steady-state condition** is reached quickly, such that the concentration of ES is relatively constant after an initial reaction time. By working under conditions where [S] >> [E], the concentration of ES remains approximately constant. **Figure 7.37** plots the time course of an

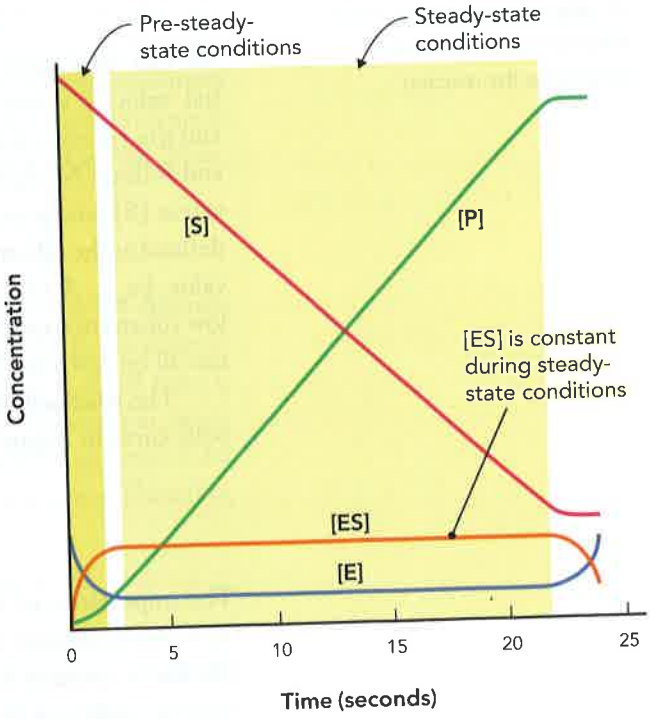


Figure 7.37 The relative concentrations of reaction components change over the course of an enzyme reaction. After the initial burst of ES complex formation during the pre-steady-state period, which often occurs on a timescale of milliseconds, the reaction reaches the steady-state phase, characterized by constant levels of [ES] and free [E], with a linear increase in [P]. The accumulation of [P] during the steady-state phase provides rate data as a function of initial [S].

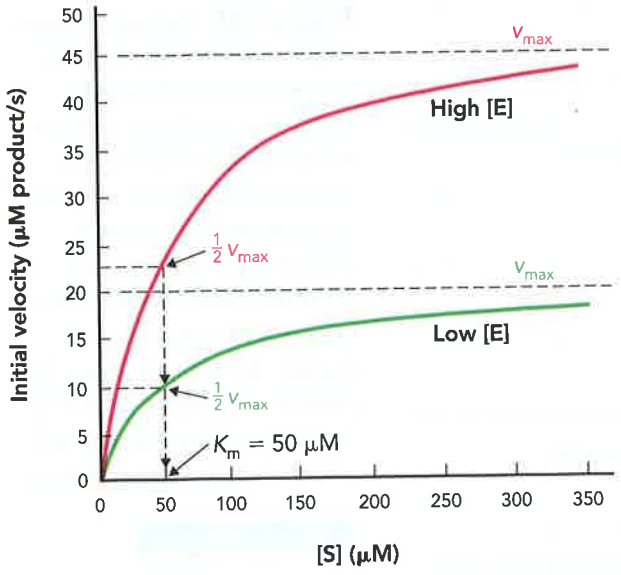


Figure 7.38 We can determine the Michaelis–Menten parameters K_m, v_{max}, and $\frac{1}{2}v_{max}$ by plotting kinetic data derived from enzyme activity assays. The K_m for any given enzyme is independent of enzyme concentration when enzyme concentration [E] is limiting for the reaction.

enzyme reaction as a function of concentration for each component in the reaction. The steady-state condition is reached when the concentration of ES is constant, even though the concentration of substrate is decreasing and that of product is increasing. Note that the k_1 and k_{-1} rate constants are determined during the **pre-steady-state condition**, at a time when ES complex formation is linear over time, and changes in the concentration of substrate are negligible. Analyzing the pre-steady-state condition experimentally requires a very rapid enzyme assay method called **stop-flow kinetics** that permits measurements in the millisecond range. It is important to note that Michaelis–Menten kinetics are valid only for enzyme reactions under steady-state conditions that contain only one substrate and one enzyme (first-order reaction). Although most enzymes have more than one substrate, these enzymes can still be studied using the Michaelis–Menten approach, as long as only one substrate is varied at a time.

To understand how we can use Michaelis–Menten kinetics to analyze enzyme function, we first need to define the **Michaelis constant, K_m**, which relates the rate constants k_1, k_{-1}, and k_2 (describing the rates of breakdown and formation of the ES complex) for a given enzyme reaction:

$$K_m = \frac{k_{-1} + k_2}{k_1} \tag{7.4}$$

We can determine the K_m value for an enzyme experimentally from a plot of initial velocity versus substrate concentration under conditions where [S] >> [E], and the reaction is at steady state (**Figure 7.38**). If the enzyme reaction is first order and follows Michaelis–Menten kinetics, then plotting v_0 data (concentration/time) versus [S] produces a hyperbolic curve. The experimentally determined value of K_m is defined as the substrate concentration at which the reaction rate is half of its maximum value, $\frac{1}{2}v_{max}$. A low value of K_m means that an enzyme has high catalytic activity at low substrate concentration. As shown in Figure 7.38, the K_m, v_{max} and $\frac{1}{2}v_{max}$ values can all be extracted from a plot of initial velocity versus substrate concentration.

The **Michaelis–Menten equation** (see derivation in **Box 7.1**) describes the hyperbolic curve in Figure 7.38 using [S], v_{max}, and K_m to define v_0 as follows:

$$v_0 = \frac{v_{max}[S]}{K_m + [S]} \tag{7.5}$$

The importance of the Michaelis–Menten equation is that it relates initial velocity v_0 to the maximum velocity v_{max} as a function of the substrate concentration and the Michaelis constant K_m. These values are very useful in describing the properties of an enzyme under a defined set of conditions.

For enzyme reactions that follow Michaelis–Menten kinetics, v_0 does not increase appreciably at high substrate concentration, and moreover, v_0 is proportional to enzyme concentration, as shown in Figure 7.38. This makes sense because the reaction rate is directly related to the amount of enzyme (when enzyme concentration is limiting), whereas the K_m of an enzyme is an inherent property of the geometric and chemical complementarity between amino acids in the enzyme active site and the chemical structure of the substrate.

Box 7.1 DERIVATION OF THE MICHAELIS–MENTEN EQUATION

For an uncatalyzed reaction

$$S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} P$$

the rate of product formation is proportional to the substrate concentration, so that the rate of production of [P] is k_1[S]. Thus for an uncatalyzed reaction, increases in the substrate concentration always result in increases in the rate, and no maximum velocity is reached. In contrast, enzyme-catalyzed reactions reach a maximum velocity at high substrate concentration, giving rise to the characteristic curves as shown in Figure 7.38.

Michaelis and Menten developed an equation to describe the kinetic characteristics of enzyme-catalyzed reactions. This equation describes the relationship of substrate concentrations to initial velocity, as the simple model for an uncatalyzed reaction is inadequate to explain these data.

Consider the following reaction, in which substrate (S) is converted to product (P) via an enzyme (E)-catalyzed reaction:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EP \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E + P$$

Michaelis and Menten made several simplifying assumptions that facilitate the treatment of enzyme-catalyzed reactions. The first of these assumptions is that when working at the early stages of the reaction, no appreciable enzyme–product complex (EP) has yet accumulated. Thus, the back reaction of EP to enzyme–substrate complex (ES) can be neglected. Furthermore, Michaelis and Menten assumed that once substrate is converted to product, the release of product from enzyme is fast. So this backward step to form ES from E + P can be neglected as well. Thus, the reaction above simplifies to

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

The three ways in which the concentration of ES can change are through the breakdown of ES to E and P (which occurs with a rate k_2[ES]), the dissociation of ES to E and S (rate = k_{-1}[ES]), or through the formation of ES from E and S (rate = k_1[E][S]). Michaelis and Menten used a further assumption, originally developed by Briggs and Haldane, that the reaction is being measured under steady-state conditions, where the concentration of ES is not changing. Therefore, the rates of formation and breakdown of ES must be equal.

$$\text{Rate of formation of ES} = \text{Rate of breakdown of ES}$$

$$k_1[E][S] = k_{-1}[ES] + k_2[ES]$$

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

Under these conditions, where the concentration of ES is not changing, they defined the Michaelis constant K_m, which balances the rates of formation and breakdown of ES:

$$K_m = \frac{k_{-1} + k_2}{k_1}$$

Another assumption in Michaelis–Menten kinetics is that the total amount of enzyme (E_t) stays constant throughout a reaction. Though the total amount of enzyme in a reaction does not change, once the reaction starts some free enzyme

[E] binds to substrate to become [ES], so the total amount of enzyme is $[E_t] = [E] + [ES]$. Rearranging this equation in terms of [E] gives

$$[E] = [E_t] - [ES]$$

Substitute this expression for [E] into

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

to get

$$k_1([E_t] - [ES])[S] = (k_{-1} + k_2)[ES]$$

Distribute the term:

$$k_1[E_t][S] - k_1[ES][S] = (k_{-1} + k_2)[ES]$$

Add $k_1[ES][S]$ to both sides:

$$k_1[E_t][S] - k_1[ES][S] + k_1[ES][S] = (k_{-1} + k_2)[ES] + k_1[ES][S]$$

$$k_1[E_t][S] = k_{-1}[ES] + k_2[ES] + k_1[ES][S]$$

$$k_1[E_t][S] = (k_{-1} + k_2 + k_1[S])[ES]$$

Divide by $(k_{-1} + k_2 + k_1[S])$ to yield

$$\frac{k_1[E_t][S]}{(k_{-1} + k_2 + k_1[S]} = [ES]$$

Divide both numerator and denominator by k_1:

$$\frac{(k_1/k_1)[E_t][S]}{\{(k_{-1}+k_2)/k_1\} + (k_1[S]/k_1)} = \frac{[E_t][S]}{(k_{-1}+k_2)/k_1 + [S]} = [ES]$$

$$[ES] = \frac{[E_t][S]}{(k_{-1} + k_2)/k_1 + [S]}$$

Recall that

$$K_m = \frac{(k_{-1} + k_2)}{k_1}$$

Therefore, K_m can be substituted into the equation above to yield

$$[ES] = \frac{[E_t][S]}{K_m + [S]}$$

The only way product can be formed is through [ES], and thus the initial velocity of the reaction is

$$v_0 = k_2[ES]$$

Substituting the expression obtained for [ES] into $v_0 = k_2[ES]$ gives

$$v_0 = \frac{k_2[E_t][S]}{K_m + [S]}$$

However, the reaction is limited by the amount of enzyme. The maximum velocity v_{max} occurs when all the enzyme is in the form [ES] such that

$$v_{max} = k_2[E_t]$$

By substitution of this equation for v_{max} into the initial velocity expression, we obtain the *Michaelis–Menten equation*:

$$v_0 = \frac{v_{max}[S]}{K_m + [S]}$$

This expression relates the initial velocity of a reaction to the substrate concentration, maximum velocity, and the Michaelis constant.

For the simple reaction shown earlier, where

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

under the special condition where product formation is slow, such that $k_{-1} >> k_2$, we can reduce K_m to

$$K_m = \frac{k_{-1}}{k_1} = K_d$$

This is defined as the dissociation constant, K_d, of the ES complex (see Chapter 6). A high K_d value, measured in units of molar concentration (M; typically micromolar to millimolar), means that the enzyme has a low affinity for the substrate because a higher substrate concentration is required to reach K_m. However, it is important to remember that experimentally determined values for v_{max} and K_m do not provide information about the number of discrete steps involved in the reaction or the rates of individual steps. In fact, for most enzymes, the reaction steps are not as simple as defined here, and K_m is not a measure of the enzyme affinity for substrate. For example, when $k_2 >> k_{-1}$, making $K_m = k_2/k_1$, then K_m is not equal to K_d. However, even though K_m values may not represent K_d for a particular enzyme, K_m is a value that can be experimentally determined and is therefore a useful comparative measure for enzymes under various conditions.

Although Michaelis–Menten parameters are commonly derived computationally by analyzing experimental data directly, it is sometimes useful to use an algebraic transformation of the Michaelis–Menten equation, called the **Lineweaver–Burk equation**, to draw a double reciprocal plot of the enzyme data. The Lineweaver–Burk equation is derived from the Michaelis–Menten equation by taking the reciprocal of both sides of the equation and rearranging:

$$\frac{1}{v_0} = \frac{K_m}{v_{max}[S]} + \frac{1}{v_{max}} \tag{7.6}$$

This rearrangement is useful because we can fit the data points with a straight line. As shown in **Figure 7.39**, we can obtain Michaelis–Menten parameters from a **Lineweaver–Burk plot** by determining the slope of the line to obtain the ratio of K_m/v_{max}; the y-axis intercept to find $1/v_{max}$; and the x-axis intercept to determine the value of $-1/K_m$. Note that using the Lineweaver–Burk plot allows a determination of v_{max} (through extrapolation) even under conditions of nonsaturating concentrations of substrate. You will see the usefulness of Lineweaver–Burk plots for enzyme kinetic analysis later in this chapter when we describe mechanisms of enzyme inhibition.

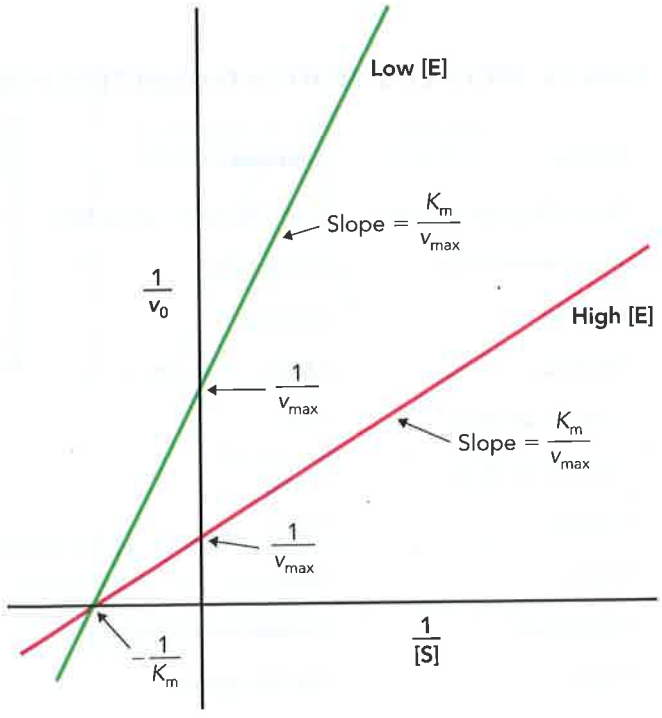


Figure 7.39 We can plot enzyme kinetic data as a straight line by using the Lineweaver–Burk equation. Note that the x-axis intercept ($-1/K_m$) is the same for both sets of enzyme reactions, confirming that K_m is not affected by enzyme concentration under these conditions.

Enzymes Have Different Kinetic Properties

Michaelis–Menten kinetics helps us determine how an enzyme functions and what its reaction rate is. However, other comparisons of enzyme properties can be helpful in putting together the picture of how efficiently enzymes function under different conditions of pH and temperature.

Catalytic Efficiency of Enzymes Another way to compare enzymes is to determine the maximum catalytic activity under saturating levels of substrate. This value, called the **turnover number**, is defined as k_{cat}. The k_{cat} for a reaction is the value of the rate-limiting step. In the Michaelis–Menten equation, $k_{cat} = v_{max}/[E_t]$, where $[E_t]$ equals the total amount of enzyme in the reaction ([E] + [ES]). Substituting k_{cat} into the Michaelis–Menten equation gives

$$v_0 = \frac{k_{cat}[E_t][S]}{K_m + [S]}$$

The turnover number k_{cat} provides information about the maximum catalytic rate, but it does not reveal how well the enzyme works. Two enzymes may have the same k_{cat} value, but the efficiency of the reaction can be different. For example, consider two enzymes E1 and E2 that have the same k_{cat} value. If E1 increases the rate of reaction 10^{12}-fold over the uncatalyzed reaction, whereas E2 only increases the reaction rate 10^8-fold, you can conclude that E1 has a higher catalytic efficiency than E2.

The catalytic efficiency of two enzyme reactions or of the same enzyme with two different substrates can be represented by the **specificity constant**, defined as k_{cat}/K_m. Under conditions of low substrate concentrations ($[S] << K_m$), we can rewrite the rate equation as

$$v_0 = \frac{k_{cat}[E_t][S]}{K_m} \tag{7.7}$$

This expression makes k_{cat}/K_m an apparent second-order rate constant because v_0 depends on the concentrations of two reactants: E_t and S. It is apparent here that there is an upper limit to the rate of the reaction, which is the rate of encounter of E and S. In aqueous solution, this limit is approximately 10^8 to 10^9 M^{-1} s^{-1}, which is the diffusion-controlled limit. **Table 7.4** lists K_m, k_{cat}, and specificity constants for several enzymes.

As shown in Table 7.4, catalase has an extremely high turnover rate (4×10^7 s^{-1}); however, it also has a high K_m (1.1 M) and is therefore not as catalytically efficient as some other enzymes with a much lower turnover rate. The data in Table 7.4 also illustrate the difference in kinetic properties when the same enzyme reacts with different

Table 7.4 MICHAELIS–MENTEN PARAMETERS FOR REPRESENTATIVE ENZYMES AND SUBSTRATES

Enzyme	Substrate	k_{cat} (s^{-1})	K_m (M)	k_{cat}/K_m (M^{-1} s^{-1})
Triose phosphate isomerase	Glyceraldehyde-3-phosphate	4.3×10^3	4.7×10^{-4}	2.4×10^8
Acetylcholinesterase	Acetylcholine	1.4×10^4	9.0×10^{-5}	1.5×10^8
Fumarase	Fumarate	8.0×10^2	5.0×10^{-6}	1.6×10^8
Fumarase	Malate	9.0×10^2	2.5×10^{-5}	3.6×10^7
Carbonic anhydrase	CO_2	1.0×10^6	1.2×10^{-2}	8.3×10^7
Carbonic anhydrase	HCO_3^-	4.0×10^5	2.6×10^{-2}	1.5×10^7
Catalase	H_2O_2	4.0×10^7	1.1	4.0×10^7
Urease	Urea	1.0×10^4	2.5×10^{-2}	4.0×10^5
Ribonuclease	Cytidine cyclic phosphate	8.0×10^2	8.0×10^{-3}	1.0×10^5
Pepsin	Phe-Gly peptide	5.0×10^{-1}	3.0×10^{-4}	1.7×10^3

substrates. In the case of the enzyme fumarase, the K_m for fumarate is fivefold lower than it is for malate, yet the k_{cat} values for these two substrates are nearly the same. This results in a specificity constant for fumarate that is about four times higher for the enzyme than when malate is the substrate. These kinetic properties of fumarase are consistent with the fact that fumarate is the preferred substrate of the enzyme in the citrate cycle reactions (malate is the product of this reaction), as described in Chapter 10.

Effect of pH and Temperature on Enzyme Activity Rates of enzymatic reactions are affected by both pH and temperature, reflecting changes in the structure and chemistry of the active site under differing conditions. With regard to pH, seven amino acids have ionizable side chains that gain or lose a proton depending on the local environment and effective pK_a. As a result, changes in pH can alter the chemistry of the active site and disrupt critical tertiary structures within the protein, potentially resulting in denaturation. **Figure 7.40** shows that the optimal pH for an enzyme reflects the normal physiologic conditions in which it operates. Pepsin, the pancreatic protease found in the acidic environment of the stomach, has an optimal pH around 1.6, whereas the liver enzyme glucose-6-phosphatase has an optimal pH of 7.8. Arginase, an important liver enzyme involved in the urea cycle, has an optimal pH of 9.7.

Figure 7.41 shows the activity curves for two enzymes as a function of reaction temperature. Temperature affects both the catalytic properties of the enzyme and the stability of the protein structure. Increased temperatures lower the activation energy by increasing the free energy of the ground state; however, the protein structure may be destabilized at high temperature. Thus, the temperature optimum for a reaction balances catalytic efficiency and structural stability. Similar to the natural difference in pH optimums of pepsin and arginase, the DNA polymerase from a thermophilic bacterium, which normally lives at temperatures as high as 80 °C, has a much higher temperature optimum than the DNA polymerase of *Escherichia coli,* which lives in the human intestine at a comfortable 37 °C.

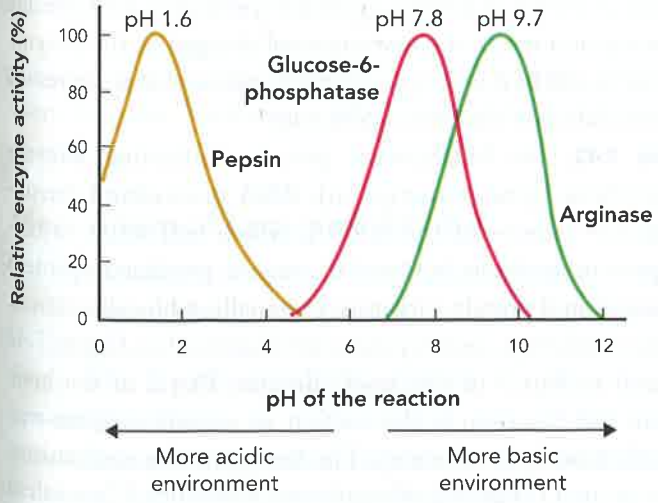


Figure 7.40 The optimal pH of an enzyme reaction reflects the chemical environment of the active site and is consistent with the physiologic role of the enzyme.

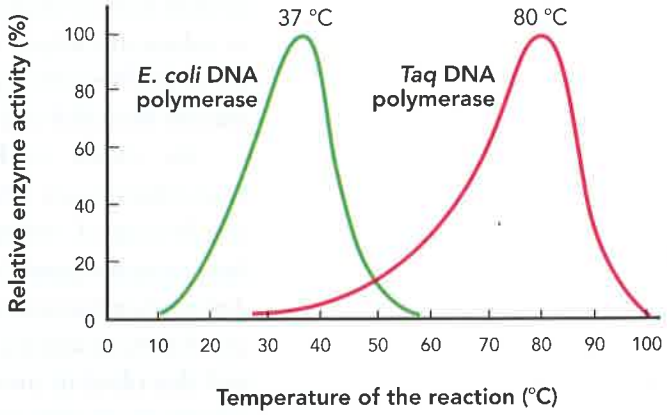


Figure 7.41 *E. coli* DNA polymerase and *Taq* DNA polymerase from the thermophilic bacterium *Thermus aquaticus* have different optimal temperatures for activity, reflecting their environmental niches.

concept integration 7.4

What can enzyme kinetics reveal about enzyme mechanisms?

Enzyme kinetics is an experiment-based approach used to study enzyme function and provides critical insight into the mechanism of an enzyme-catalyzed reaction. The primary goal in enzyme kinetics is to use quantitative analysis directly to compare the rates of reactions under various conditions of substrate concentration, temperature, and pH to understand catalytic mechanisms. Enzyme kinetics can also be used to discover the molecular basis for diseases in which a mutant enzyme is expressed in cells, but it is not known what accounts for decreased product formation. Michaelis–Menten kinetics is a mathematical description of enzyme reactions that take place at steady state. Measurements of maximum reaction velocity (v_{max}), substrate concentration at half-maximal velocity (K_m), and turnover number (k_{cat}) are parameters that can be used to compare related enzymes and to determine the potency of enzyme inhibitors. In the case of a disease caused by a defective enzyme, quantitative differences in v_{max}, K_m, and k_{cat} between the wild-type and mutant enzymes can provide clues that could lead to the development of new treatment strategies.

7.5 Regulation of Enzyme Activity

One of the reasons enzymes make such great biological catalysts is that they are proteins, which means they are subject to multiple levels of biochemical regulation. This regulation could be stimulatory, resulting in an overall increase in enzyme activity, or it could be inhibitory, resulting in a decrease in enzyme activity.

Enzyme regulation is mediated by two primary mechanisms: (1) bioavailability with regard to the amount of enzyme in different tissues and cellular compartments, and (2) control of catalytic efficiency through protein modification (covalent and noncovalent chemical bonds). For example, a positive regulatory mechanism could involve an increase in enzyme activity as a result of increased enzyme synthesis or could be due to enhanced catalytic efficiency as a result of conformational changes in the enzyme active site. Negative regulation would be due to opposite mechanisms; that is, decreased enzyme bioavailability or disturbance of the protein structure.

As shown in **Figure 7.42**, the biochemical processes affecting enzyme bioavailability are RNA synthesis (gene transcription); RNA processing (alternative joining of coding regions—exons—of the mRNA, which will result in different protein products); protein synthesis (amount of enzyme produced); protein degradation (protein turnover); and protein targeting (organelle sublocalization or membrane insertion). These regulatory control points are illustrated in Figure 7.42 and described in more detail in Part 5 of this book. Because Part 2 of the book focuses on protein structure and function, in this section we present enzyme regulatory mechanisms that affect catalytic efficiency. The three primary mechanisms that affect catalytic efficiency are (1) binding of regulatory molecules, (2) covalent modification, and (3) proteolytic processing (Figure 7.42). We will discuss control of activity by the binding of regulatory molecules from two different perspectives: First, we discuss molecules that function as enzyme inhibitors; then, we discuss how an enzyme can be activated or inhibited by allosteric regulators and use the bacterial

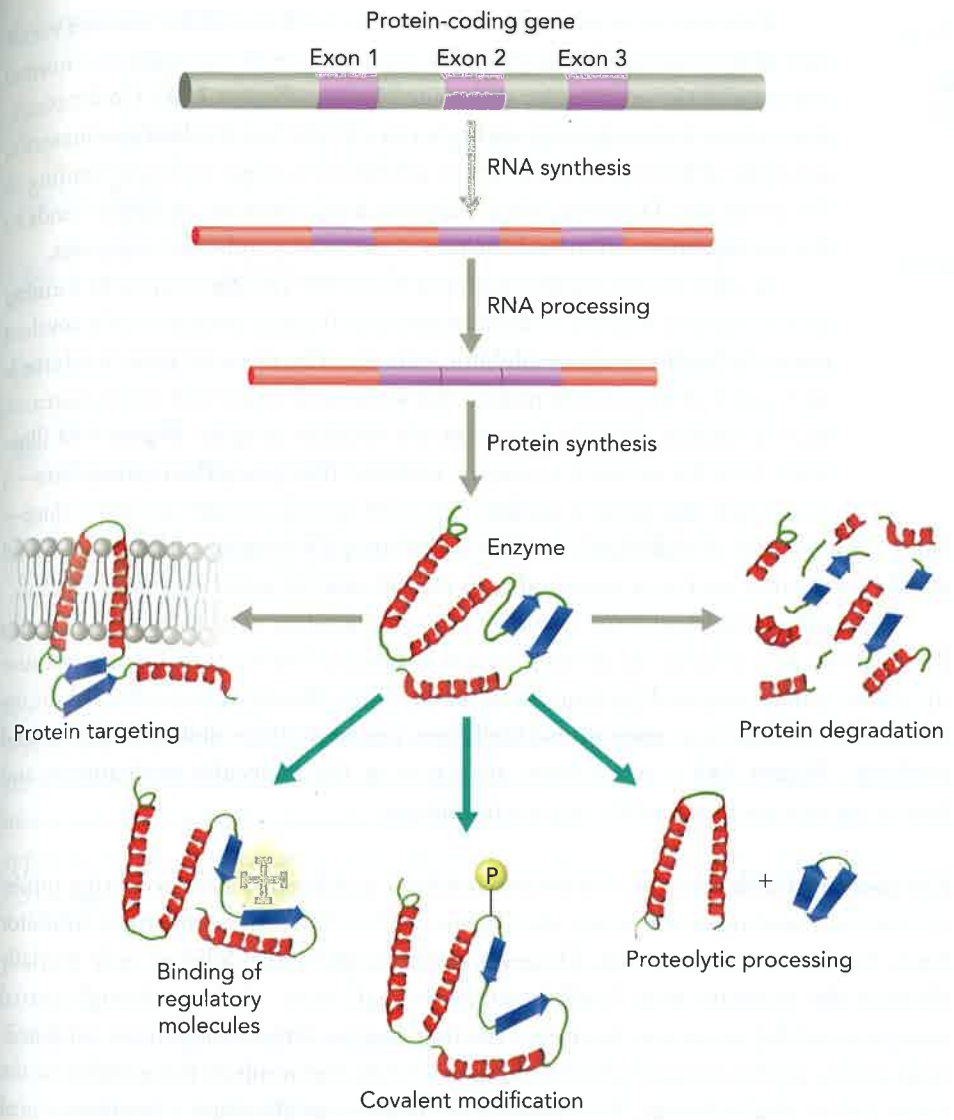


Figure 7.42 Enzyme bioavailability and catalytic efficiency are the two main enzyme regulatory mechanisms. Enzyme bioavailability is controlled by biochemical processes involved in protein synthesis and localization (gray arrows), whereas catalytic efficiency is determined by protein modifications (teal arrows).

enzyme aspartate transcarbamoylase as an example. Most enzymes are regulated by more than one mechanism, which function together to provide a fine-tuned level of control.

Mechanisms of Enzyme Inhibition

Enzyme inhibition is used in cells as a regulatory mechanism to control enzyme activity. Enzyme inhibitors are also used in *in vitro* studies to learn about the catalytic mechanisms of enzymes. Enzymes are subject to both **reversible inhibition**, due to the noncovalent binding of small biomolecules or proteins to the enzyme subunit, and **irreversible inhibition**, in which the inhibitory molecule forms a covalent bond (or, less commonly, very strong noncovalent interactions) with catalytic groups in the enzyme active site. These two types of inhibition can be distinguished by analyzing the effect of enzyme dilution on the level of inhibition. Specifically, the effect of reversible inhibitors can be decreased by diluting the enzyme reaction, whereas irreversible inhibitors are not affected by dilution because the covalent bond remains intact independent of enzyme and inhibitor concentrations.

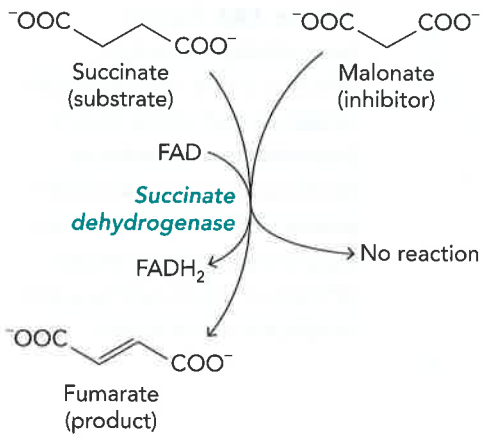


Figure 7.43 Malonate is structurally related to succinate and functions as a reversible inhibitor by competing with succinate for binding to the succinate dehydrogenase active site. Because malonate has only one methylene group, the oxidation reaction cannot proceed, and the enzyme is inhibited.

Malonate is an example of a reversible inhibitor of the enzyme succinate dehydrogenase. It functions by competing with succinate, the normal substrate of the enzyme, for active site binding (**Figure 7.43**). Under conditions where malonate levels are higher in the cell than levels of succinate, the succinate dehydrogenase reaction is inhibited by excess malonate binding to the active site. However, when malonate levels decrease, succinate binds to the enzyme more often, and the rate of fumarate production increases.

In contrast, irreversible inhibitors effectively "kill" the enzyme by forming a tight complex with the enzyme, sometimes through formation of a covalent nonhydrolyzable enzyme-inhibitor complex. The α_1-antitrypsin inhibitor is an example of a regulatory protein that irreversibly inactivates serine proteases, such as elastase, through formation of a covalent complex. **Figure 7.44** illustrates how the irreversible enzyme inhibitor **diisopropylfluorophosphate**—a compound that forms a covalent link with specific reactive serine residues—blocks protease and phospholipase enzymes. Diisopropylfluorophosphate is not found in cells but is often used in *in vitro* studies to identify reactive active site serine residues.

Reversible inhibition is the more common mechanism used in cells. It can also be exploited as a strategy to develop structure-based pharmaceutical drugs because the effects can be reversed by drug withdrawal. Three classes of reversible inhibitors have been characterized: **competitive inhibitors, uncompetitive inhibitors,** and **mixed inhibitors. Figure 7.45** is a schematic depiction of the molecular mechanisms and kinetic parameters of reversible enzyme inhibitors.

Competitive Inhibitors A competitive inhibitor is defined as a molecule that inhibits substrate binding at the active site (Figure 7.45a). A classic competitive inhibitor binds directly to the active site. However, some competitive inhibitors only partially obstruct the substrate from binding, either through steric clash or through partial occupancy of the active site by similar binding groups. Other competitive inhibitors bind at sites on the enzyme other than the active site, but result in the inability of the active site to bind substrate. For example, an inhibitor might cause a conformational change that closes the active site.

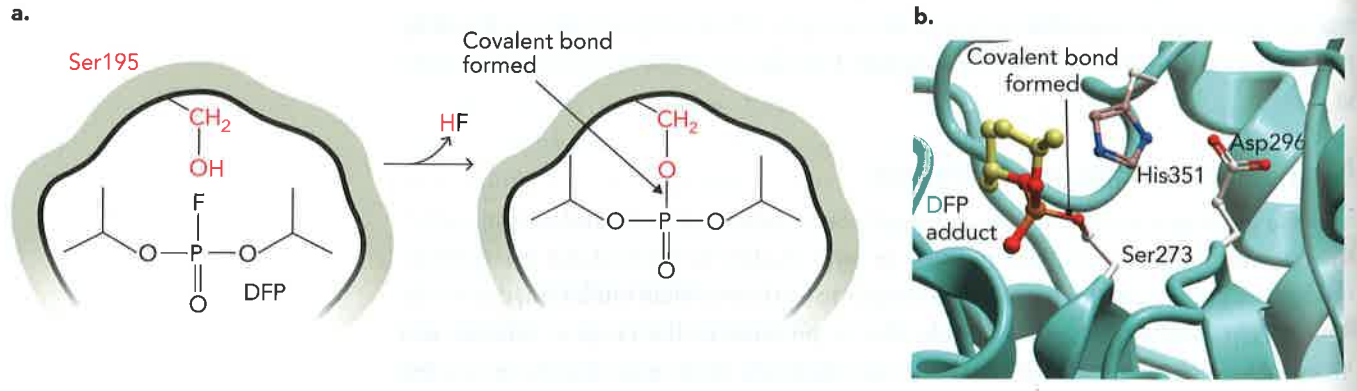


Figure 7.44 Diisopropylfluorophosphate (DFP) is an irreversible inhibitor of enzymes that contain catalytic serine residues in the active site. **a.** Schematic representation of a covalent link that forms between diisopropylfluorophosphate and the catalytic Ser195 residue located in the active site of the serine protease chymotrypsin. **b.** Molecular structure of the human phospholipase A_2 enzyme, showing the location of a diisopropylfluorophosphate adduct formed with Ser273 in the active site. The other two residues contributing to the catalytic triad in this enzyme active site are His351 and Asp296. BASED ON PDB FILE 3F9C.

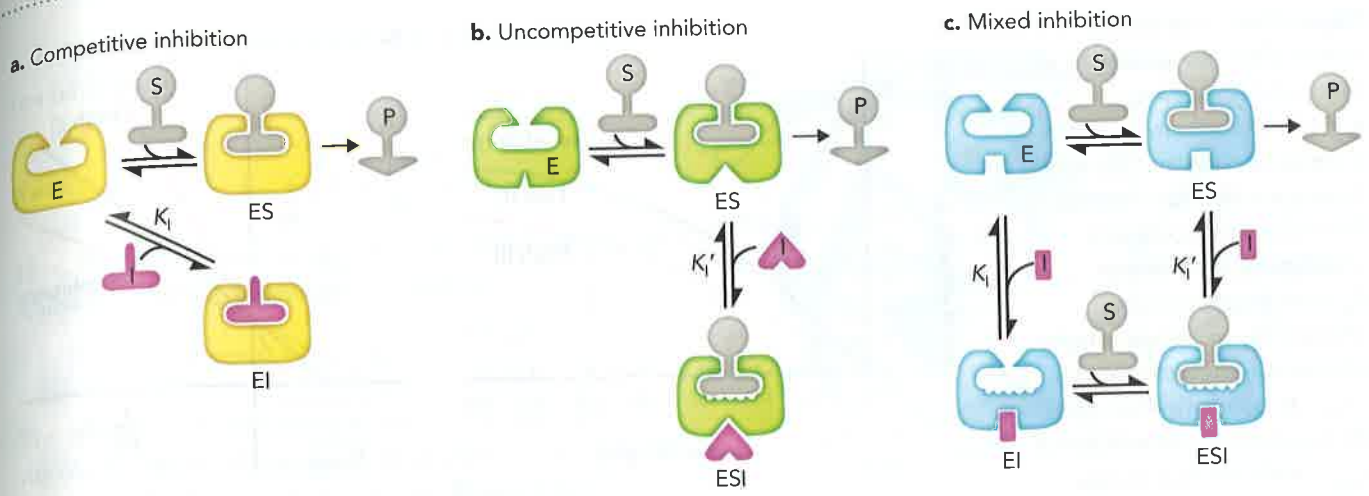


Figure 7.45 Three distinct classes of reversible enzyme inhibitors have been characterized. **a.** Competitive inhibition is characterized by inhibitors that bind to the free enzyme and inhibit substrate binding at the active site. Competitive inhibitors often bind to the active site or to a site that overlaps the active site on the enzyme. The equilibrium dissociation constant for inhibitor binding is defined by K_I (see text). **b.** Uncompetitive inhibition is characterized by inhibitors that bind only to the enzyme–substrate complex. **c.** Mixed inhibitors bind to both the enzyme and the enzyme–substrate complex. In all cases, inhibitor-bound complexes do not catalyze product formation. E = enzyme; I = inhibitor; P = product; S = substrate; EI = enzyme–inhibitor complex; ES = enzyme–substrate complex; ESI = enzyme–substrate–inhibitor complex.

The affinity of an enzyme for inhibitor can be described by the equilibrium dissociation constant K_I as follows:

$$E + I \rightleftharpoons EI \qquad K_I = \frac{[E][I]}{[EI]}$$

Note that this is similar to the equilibrium dissociation constant K_d discussed for general protein–ligand complexes in Chapter 6; however, K_I specifically refers to the equilibrium dissociation constant for an enzyme–inhibitor complex. In competitive inhibition, the substrate concentration required to reach $\frac{1}{2}v_{max}$ increases (an increase in apparent K_m; $K_{m\text{-}app}$), because more substrate is required to form the same amount of ES complex. This can be stated as

$$K_{m\text{-}app} = K_m\left(1 + \frac{[I]}{K_I}\right)$$

This equation takes into account both the concentration of inhibitor, [I], and the dissociation constant of the enzyme–inhibitor complex, K_I. In the presence of a competitive inhibitor, the Michaelis–Menten equation becomes

$$v_0 = \frac{v_{max}[S]}{K_m\left(1 + \frac{[I]}{K_I}\right) + [S]}$$

Here you can see that the effectiveness of a competitive inhibitor depends on the concentrations of inhibitor and substrate and the relative affinities of the enzyme for inhibitor and substrate.

A plot of reaction velocity versus substrate concentration shows the effect of adding a competitive inhibitor (**Figure 7.46a**). Note that because the inhibitor competes for binding to the active site, the apparent K_m value for the enzyme is shifted to the right as a function of increasing inhibitor concentration. With addition of sufficiently high concentrations of substrate, the effects of the inhibitor can be overcome, and therefore competitive inhibitors do not affect the maximum velocity, v_{max}, of the reactions. The effect of a competitive inhibitor on enzyme kinetics can be seen more easily by plotting the data as a Lineweaver–Burk plot (**Figure 7.46b**). In this double reciprocal plot, it is clear that v_{max} is not affected by inhibitor concentration, whereas the apparent K_m of the enzyme increases.

Structure-based drug design is an active area of research in which knowledge of an enzyme's structure is used to devise an inhibitor with shape and chemical

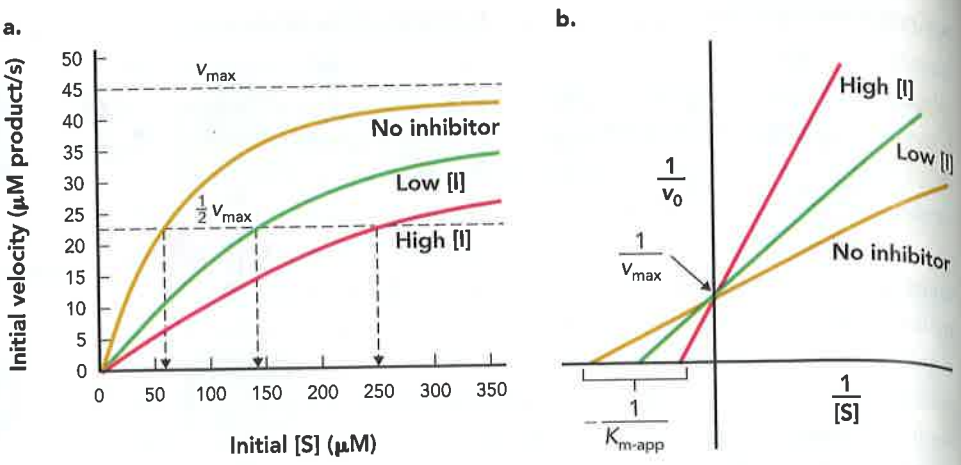
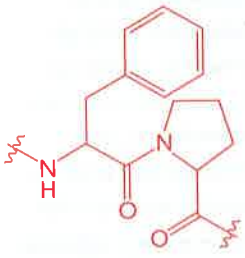
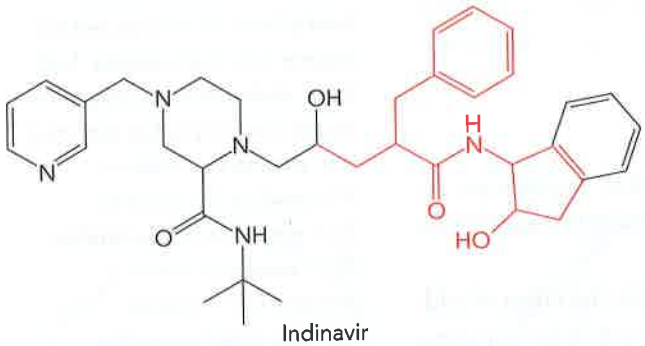


Figure 7.46 A competitive inhibitor affects the apparent K_m of an enzyme but not the v_{max}. **a.** Plot of initial velocity (v_0) versus substrate concentration ([S]) in the absence and presence of inhibitor. With increasing concentration of inhibitor ([I]), the apparent K_m of the enzyme increases, reflecting the requirement of higher substrate concentration to reach $\frac{1}{2}v_{max}$. **b.** Lineweaver–Burk plot of the data shown in panel **a**. Note that v_{max} is unaffected by an increase in inhibitor concentration (*y*-axis intercept remains constant), whereas the apparent K_m increases, as shown by the decrease in the $-1/K_{m\text{-}app}$ value at the *x*-axis intercept.

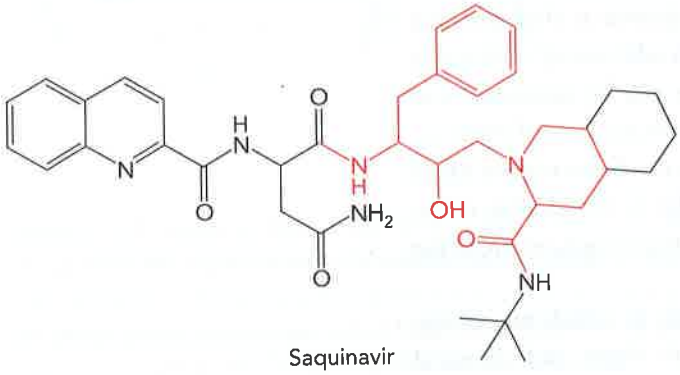
complementarities to the enzyme's active site. An example of structure-based drug design using these ideas is the development of competitive inhibitors for the aspartate protease enzyme in human immunodeficiency virus (HIV). This viral enzyme is required for proper maturation of infectious particles. The enzyme cleaves viral proteins at specific sites between Phe-Pro or Tyr-Pro residues, using a pair of catalytic Asp residues in the active site. As shown in **Figure 7.47**, the HIV protease inhibitors indinavir and saquinavir contain a component in their structures that mimics the natural Phe-Pro dipeptide substrate. Both of these competitive inhibitors bind tightly and reversibly to the enzyme active site, which is located at the dimer interface (**Figure 7.48**).



Phe-Pro



Indinavir



Saquinavir

Uncompetitive Inhibitors Uncompetitive inhibitors bind to enzyme–substrate complexes and alter the active site conformation, thus rendering the enzyme less catalytically active (see Figure 7.45b). An uncompetitive inhibitor does not bind to the free enzyme.

Uncompetitive inhibitors for enzymes with a single substrate are rare. More often, uncompetitive inhibitors act upon enzymes with multiple substrates that are added sequentially. Even in situations with high substrate concentrations, some of the enzyme remains in a nonproductive ESI form. Furthermore, because some ES complex is used to make the enzyme–substrate–inhibitor (ESI) complex, the apparent K_m decreases as well. Thus, the net effect of an uncompetitive inhibitor is to decrease both K_m and v_{max}. However, because K_m and v_{max} decrease by the same factor, the slope of the line K_m/v_{max} in a Lineweaver–Burk plot is unaffected.

Figure 7.47 Indinavir and saquinavir are competitive inhibitors of the HIV aspartate protease enzyme required for maturation of viral proteins. The regions of the chemical structures of these inhibitors that mimic the HIV protease substrate Phe-Pro dipeptide are highlighted.

The Michaelis–Menten equation for an uncompetitive inhibitor is

$$v_0 = \frac{v_{max}[S]}{K_m + \left(1 + \frac{[I]}{K_I'}\right)[S]}$$

Here, the dissociation constant K_I' reflects the affinity of inhibitor to the enzyme–substrate complex:

$$ES + I \rightleftharpoons ESI \qquad K_I' = \frac{[ES][I]}{[ESI]}$$

The effect of uncompetitive inhibition on enzyme activity can be seen in a Lineweaver–Burk plot over a range of inhibitor and substrate concentrations (**Figure 7.49**).

Mixed Inhibitors Mixed inhibitors are similar to uncompetitive inhibitors in that they bind to sites distinct from the active site. The main difference is that mixed inhibitors can bind to both the enzyme and the enzyme–substrate complex (see Figure 7.45c), which can be seen as a mixture of both competitive and uncompetitive inhibition and thus is termed *mixed*. An unproductive EI or ESI complex is formed in which the enzyme's catalytic activity is decreased, potentially through structural alterations of the catalytic residues. As with uncompetitive inhibition, because some enzyme remains in an unproductive ESI complex, high concentrations of substrate cannot overcome the presence of a mixed inhibitor, resulting in a decreased v_{max}. Because some enzyme is bound to inhibitor, the overall effect is that less enzyme appears to be present. The Michaelis–Menten equation for a mixed inhibitor is

$$v_0 = \frac{v_{max}[S]}{K_m\left(1 + \frac{[I]}{K_I}\right) + [S]\left(1 + \frac{[I]}{K_I'}\right)}$$

A mixed inhibitor decreases v_{max} and may increase or decrease K_m, depending on the relative K_I and K_I' values (relative inhibitor affinity for free enzyme and ES complex, respectively). **Figure 7.50a** shows the Lineweaver–Burk plots for two mixed inhibitors.

A special rare case of mixed inhibition is called **noncompetitive inhibition**, where the inhibitor has equal affinity for both the free enzyme and the ES complex ($K_I = K_I'$). In the case of noncompetitive inhibition, the Michaelis–Menten equation becomes

$$v_0 = \frac{v_{max}[S]}{(K_m + [S])\left(1 + \frac{[I]}{K_I}\right)}$$

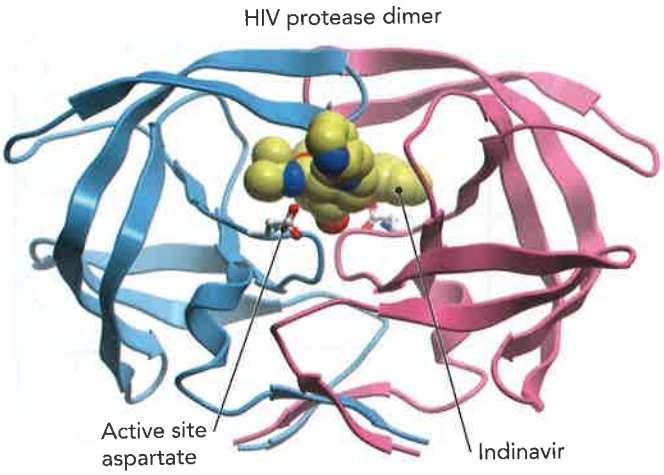


Figure 7.48 Structure of an HIV protease dimer with the competitive inhibitor indinavir bound to the active site. The catalytic aspartate residues located in each monomer are observed in the enzyme active site. BASED ON PDB FILE 1K6C.

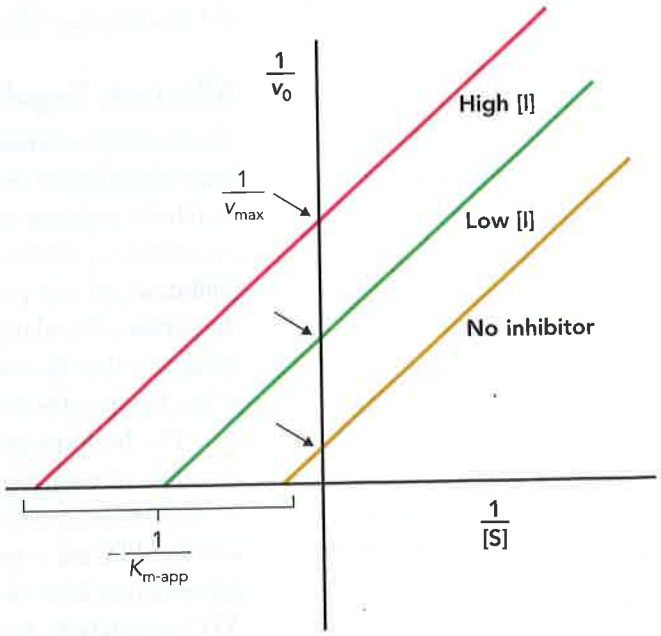


Figure 7.49 Uncompetitive inhibitors decrease both the v_{max} and apparent K_m kinetic parameters by the same factor. Note that unlike competitive inhibition, uncompetitive inhibition is not overcome by increasing substrate concentration.

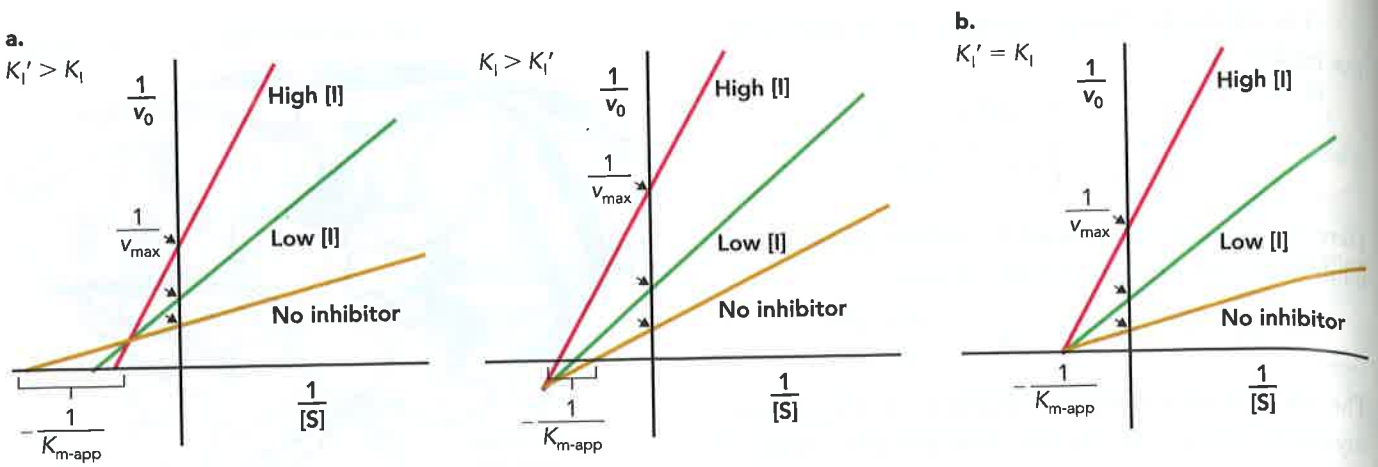


Figure 7.50 **a.** Mixed inhibitors decrease v_{max} and increase or decrease K_m, depending on the relative values of K_I and K_I'. In the plot on the left, where K_I' is greater than K_I, the K_m is increased, indicating decreased affinity of enzyme for substrate in the presence of inhibitor. **b.** Noncompetitive inhibitors decrease v_{max}, though the K_m value is unaffected.

Figure 7.50b shows the Lineweaver–Burk plot for a noncompetitive inhibitor. The K_m value is unaffected, as both the free enzyme and the enzyme–inhibitor complex can bind to substrate.

Both uncompetitive and mixed inhibitors affect multi-substrate enzymes and often can be used to distinguish different types of enzyme reaction mechanisms. For example, an inhibitor that binds to a site occupied by one substrate (S1) may influence binding of another substrate (S2) to its binding site. This would be an example of uncompetitive or mixed inhibition with regard to S1 binding. By altering the concentration of substrates and inhibitors, enzyme kinetics can be used to determine if substrate binding is ordered (S1 binds before S2) or random (either S1 or S2 can bind first).

Allosteric Regulation of Catalytic Activity

The first step in a metabolic pathway is often controlled by a regulated enzyme to maximize the efficient use of metabolic intermediates. For example, if the end product of a metabolic pathway accumulates in the cell, it is prudent to decrease production of the metabolite by inhibiting the first step in the pathway. In the simplest case of **feedback inhibition**, the end product of a pathway functions as an inhibitor of the first enzyme in the pathway. Similarly, if metabolic products of a particular pathway are in short supply, molecules that function as metabolic activators can bind to and stimulate the activity of key regulated enzymes.

The bacterial enzyme aspartate transcarbamoylase (ATCase) is one of the best examples of regulation of an enzyme by metabolic products of its associated pathway or related metabolites. It is also a classic example of allosteric regulation of catalytic activity. ATCase is one of the key regulated enzymes in the pyrimidine biosynthetic pathway that leads to the production of cytidine triphosphate (CTP; see Chapter 18). ATCase catalyzes the formation of *N*-carbamoyl-L-aspartate from carbamoyl phosphate and aspartate, which is an early step in the CTP synthesis pathway (**Figure 7.51**). ATCase is feedback inhibited by the product of the pathway, CTP, and is activated by ATP, as described shortly. Both CTP and ATP are allosteric regulators of enzyme activity. (Recall that allosteric regulation was first described in Chapter 6 in the context of oxygen binding to hemoglobin and the role of 2,3-bisphosphoglycerate [2,3-BPG]

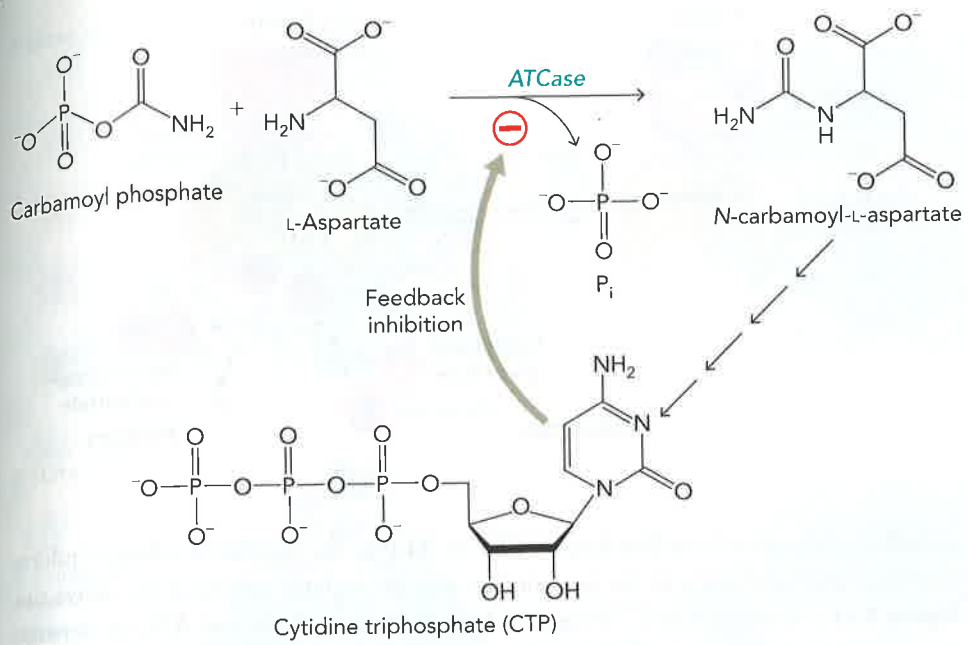


Figure 7.51 ATCase activity is feedback inhibited by CTP, the end product of the pyrimidine pathway.

in shifting the equilibrium between the T state [low oxygen affinity] and R state [high oxygen affinity].) Allosteric regulation allows for a higher degree of control of enzyme activity.

Accumulation of CTP in the cell is an indication that nucleic acid biosynthesis is saturated, which results in feedback inhibition of ATCase enzyme activity by CTP. In contrast, ATP is a purine-containing nucleotide, and the cell must maintain equivalent amounts of purine-containing and pyrimidine-containing nucleotides for DNA synthesis. Moreover, high levels of ATP increase the energy charge of the cell and favor DNA synthesis (purine-containing and pyrimidine-containing nucleotides are needed for DNA synthesis; see Chapter 18). Therefore, ATP-mediated stimulation of ATCase activity ensures that sufficient amounts of CTP will be available.

ATCase is a multi-subunit protein containing both catalytic (C) and regulatory (R) subunits with an overall organization of C_6R_6. ATCase is an enzyme that shows cooperativity, in that the binding of the reaction substrates carbamoyl phosphate and aspartate to an active site in one catalytic subunit of the enzyme increases the affinity of the other active sites for their substrates. (This situation is analogous to the cooperative effects that O_2 binding has on oxygen saturation of the hemoglobin tetramer; see Chapter 6.) The substrates carbamoyl phosphate and aspartate can also be called homotropic allosteric activators, as they affect the binding of the same molecules at other active sites. Cooperative enzymes such as ATCase do not follow standard Michaelis–Menten kinetics because substrate binding by one subunit changes the substrate affinity of other subunits. This is apparent in the sigmoidal shape of the activity curve, as shown in **Figure 7.52**. This sigmoidal shape shows that enzyme activity can increase or decrease significantly over a relatively small range of substrate concentrations.

CTP and ATP, which are not substrates for the ATCase-catalyzed reaction, bind to the regulatory subunits

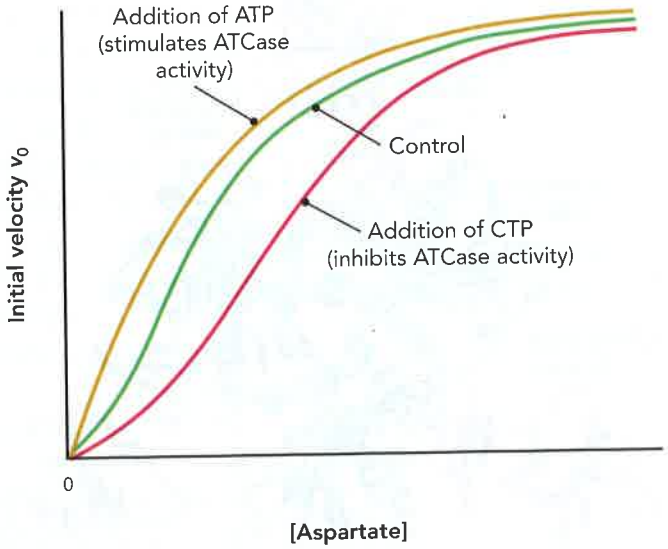


Figure 7.52 ATCase is a cooperative enzyme and is allosterically regulated by ATP and CTP. The initial velocity (v_0) versus substrate concentration curve of ATCase activity shifts in the presence and absence of allosteric effectors. In the absence of both regulators, the behavior of the enzyme is shown by the curve marked "control." Note that the shape of the activity curves are sigmoidal (reflecting cooperatively in the enzyme) and therefore not amenable to analysis by Michaelis–Menten kinetics.

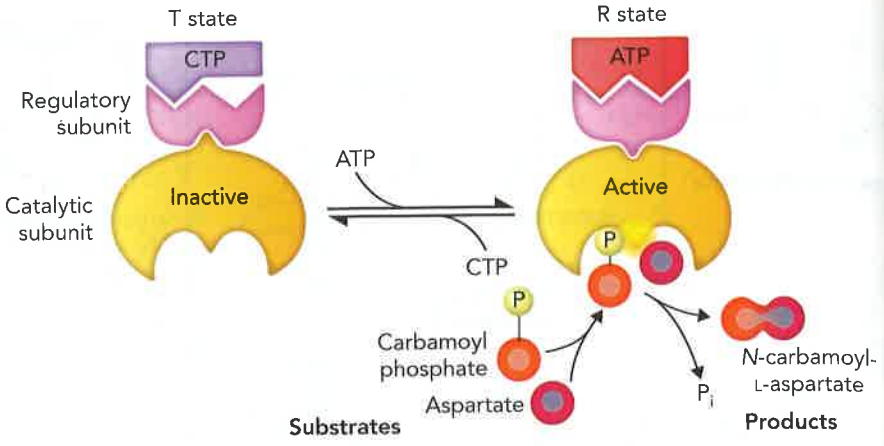


Figure 7.53 Allosteric regulation of ATCase activity by CTP and ATP. Binding of ATP to the regulatory subunit shifts ATCase to the activated R state, which catalyzes the formation of *N*-carbamoyl-L-aspartate from carbamoyl phosphate and aspartate. CTP binding to the regulatory subunit stabilizes the inactive T-state conformation.

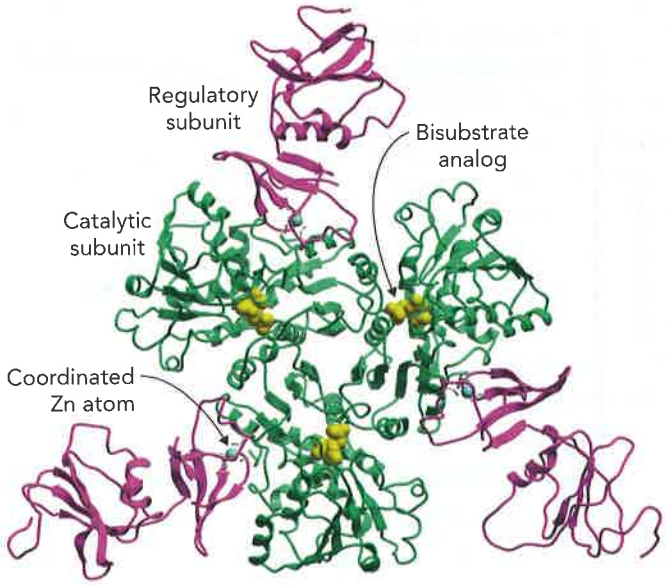
and affect enzyme activity. Binding of CTP or ATP to the regulatory subunits induces a conformational change in the protein that affects catalytic activity at the active site. **Figure 7.53** illustrates how CTP and ATP shift the equilibrium of ATCase between the inactive state (T state) and the active state (R state). CTP and ATP are considered heterotropic allosteric regulators (similar to 2,3-BPG for hemoglobin) because their binding affects the binding of different molecules (the substrates) to other sites. The addition of ATP or CTP to the reaction mixture will shift the enzyme activity curve to the left or right, respectively, reflecting changes in enzyme activity (see Figure 7.52).

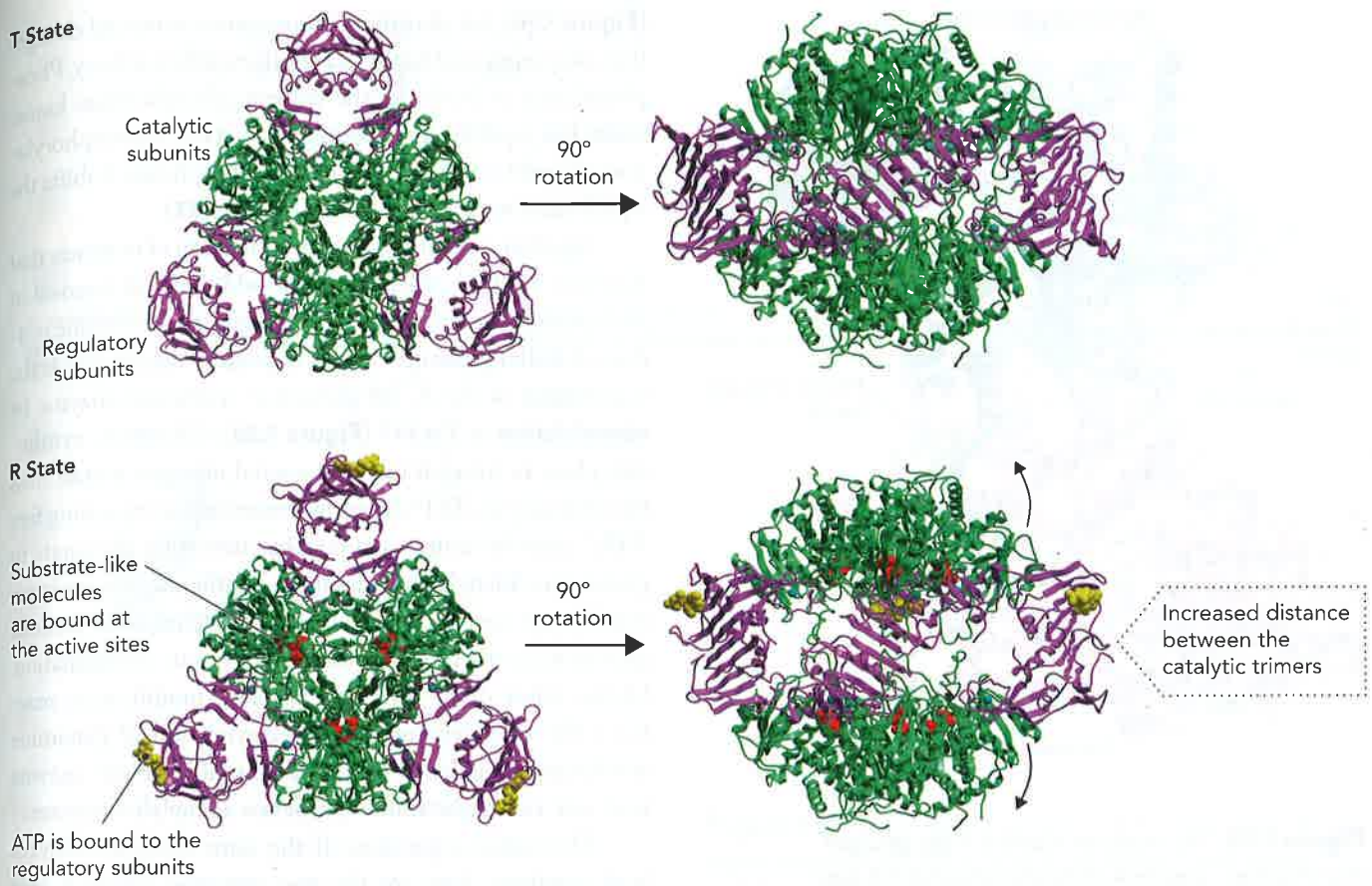
Figure 7.54 shows the structure and organization of three ATCase dimers, each containing one catalytic subunit (C) and one regulatory subunit (R), forming a C_3R_3 trimeric protein complex. This structure illustrates the relationship between the regulatory nucleotide binding site and the active site. Though the regulatory and catalytic sites reside in separate subunits and are separated from each other in the assembled protein, regulation is achieved through binding-induced conformational changes that are propagated through the subunits. Two C_3R_3 complexes associate to form the functional C_6R_6 ATCase complex, which is shown in **Figure 7.55** in both the T-state and R-state conformations. The most notable difference in these two conformations is the increased separation of catalytic subunits in the R conformation. This rearrangement eliminates a region of steric interference between two catalytic subunits in the T conformation. Careful inspection reveals that the catalytic subunits have also rotated in opposite directions relative to each other. Although not evident from the T-state and R-state conformations shown in Figure 7.55, the geometry of the active site in the R-state conformation stimulates catalysis by bringing the carbamoyl phosphate and aspartate substrates into close proximity.

As you have now seen, regulatory molecules can either activate or inhibit enzyme activity. For example, in the case of ATCase, CTP is a negative heterotropic allosteric regulator, which could also be called an enzyme inhibitor. But CTP is not a competitive inhibitor, as it does not bind at the active site. Positive and negative regulators can also be called activators or inhibitors, respectively.

ANIMATION

Figure 7.54 In this molecular structure of an ATCase trimer (C_3R_3), a bisubstrate analog (*N*-phosphonacetyl-L-aspartate) is bound in each of the three catalytic subunits. The regulatory subunits each contain a coordinated zinc ion, which has been shown to be required for stability of the tertiary structure. In ATCase, the regulatory molecules bind at a site distant from the active site. Ligand binding at the regulatory sites induces conformational changes that affect activity at the catalytic sites. BASED ON PDB FILE 1Q95.





Covalent Modification of Enzymes

As we described earlier in the context of reversible covalent modifications (see Section 7.2), a large number of enzymes catalyze reactions involving the covalent linkage of functional groups to target biomolecules in the cell. In some cases, enzymes are themselves the target of covalent modification, and the addition or removal of functional groups on these enzymes can regulate their catalytic efficiency. The functional groups involved in this form of enzyme regulation can be phosphoryl groups, methyl or acetyl groups, and adenylyl or uridylyl groups.

The most common of these covalent modifications in enzymes is the phosphorylation of Ser, Thr, and Tyr residues by **kinase** enzymes, which add a large, negatively charged group to the enzyme through the addition of a phosphoryl group (PO_3^{2-}). This can alter the chemistry or structure of the enzyme active site and thereby regulate catalytic activity. Phosphoryl groups are removed from proteins by phosphatase enzymes, which hydrolyze the ester linkage between the phosphate and the amino acid side chain. Some enzymes are activated by phosphorylation, whereas other enzymes are inactivated by phosphorylation. This process of covalent modification by phosphorylation–dephosphorylation results in a functional on/off switch for enzyme activity in much the same way it does for signal transduction (see Figure 7.25).

An example of how phosphorylation controls enzyme activity is the regulation of glycogen phosphorylase. This enzyme degrades glycogen—a storage form of glucose in liver and muscle cells—to generate glucose for energy conversion reactions associated with the glycolytic pathway. In the unphosphorylated T-state conformation, glycogen phosphorylase is inactive, whereas it is active in the phosphorylated R-state conformation. Similar to ATCase, the regulatory site in glycogen phosphorylase is not directly at the active site

Figure 7.55 The molecular structures of ATCase in the T-state and R-state conformations are shown here in two orthogonal views. The six regulatory subunits are shown in magenta, and the six catalytic subunits are shown in green. In the R-state conformation (bottom), the allosteric activator ATP (yellow) is shown bound to three of the six regulatory sites. The R-state molecule has substrate analogs (phosphonoacetamide and malonate) bound in each of the six active sites. The transition between the T and R states involves significant movements between the subunits, resulting in conformational changes at the active site that affect reactivity. BASED ON PDB FILES 6AT1 (T-STATE CONFORMATION) AND 7AT1 (R-STATE CONFORMATION).

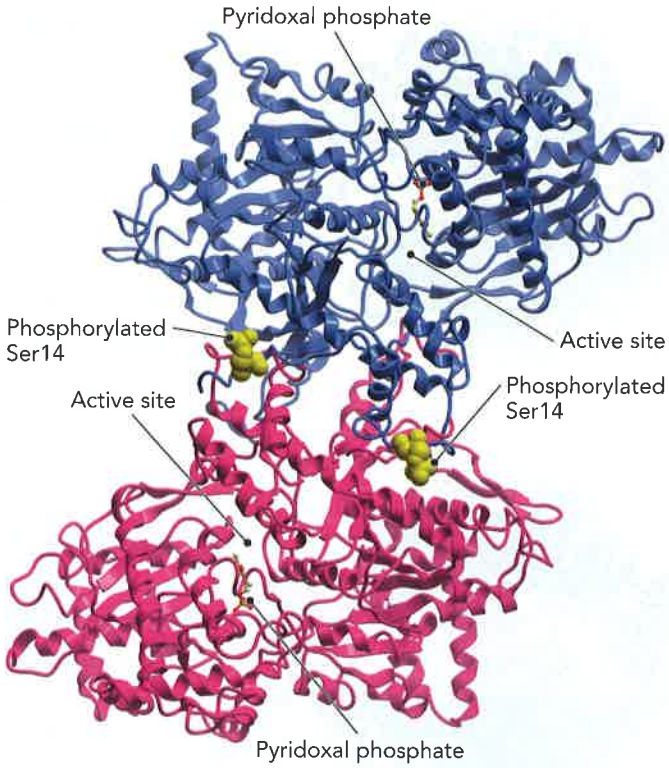


Figure 7.56 The molecular structure of the glycogen phosphorylase homodimer in the phosphorylated R-state conformation is shown here. The coenzyme pyridoxal phosphate is bound in the enzyme active site. The phosphorylated Ser residues in each monomer, shown in yellow, are at the subunit interface and are distinct from either active site. See also Figure 7.4. BASED ON PDB FILE 1GPA.

(**Figure 7.56**), but phosphorylation causes structural changes that are propagated to the active site to affect activity. Phosphorylation of Ser14 by the enzyme phosphorylase kinase shifts the equilibrium toward the R state; dephosphorylation of Ser14 by the enzyme protein phosphatase 1 shifts the equilibrium toward the T state (**Figure 7.57**).

Another type of covalent modification of enzymes that regulates catalytic efficiency is the addition and removal of nucleoside monophosphate (NMP) groups on tyrosine residues. A striking example of this regulatory mechanism is the inactivation of the *E. coli* glutamine synthetase enzyme by **adenylylation** of Tyr397 (**Figure 7.58**). Glutamine synthetase plays an integral role in bacterial nitrogen metabolism by catalyzing an ATP-dependent reaction incorporating free NH_4^+ into the amino acid pool by converting glutamate to glutamine. Adenylylated glutamine synthetase is in the inactive T-state conformation, and the deadenylylated form of glutamine synthetase is in the active R-state conformation. Unlike many other reversible covalent modification reactions, the adenylylation and deadenylylation of glutamine synthetase is catalyzed by the same multi-subunit enzyme in *E. coli*, called glutamine synthetase adenylyltransferase.

This raises a question: If the same enzyme catalyzes both reactions, how are the two opposing activities regulated to prevent futile cycling? The answer is through **uridylylation** of glutamine synthetase adenylyltransferase on Tyr51 by another bifunctional enzyme called uridylyltransferase. Under conditions of high ATP and elevated levels of the metabolite α-ketoglutarate, the *uridylylation activity* of uridylyltransferase is stimulated, resulting in uridylylation of glutamine synthetase adenylyltransferase and activation of its *deadenylylation activity* (**Figure 7.59**). However, when P_i and glutamine levels are elevated in the cell, the *deuridylylation activity* of uridylyltransferase is stimulated, leading to the deuridylylation of glutamine

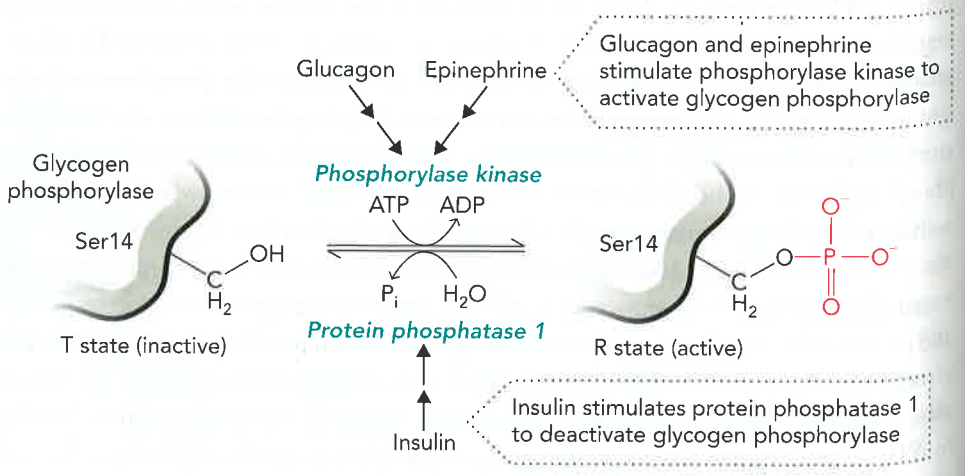


Figure 7.57 Covalent modification can act as an on/off switch for enzyme activity. Phosphorylase kinase catalyzes the phosphorylation reaction at Ser14 that stimulates the catalytic efficiency of glycogen phosphorylase (active R-state conformation). Protein phosphatase 1 catalyzes the dephosphorylation reaction that shifts the equilibrium toward the inactive T-state conformation. The hormones glucagon and epinephrine result in stimulation of the activity of phosphorylase kinase, whereas the hormone insulin results in stimulation of the activity of protein phosphatase 1 (see Chapter 14).

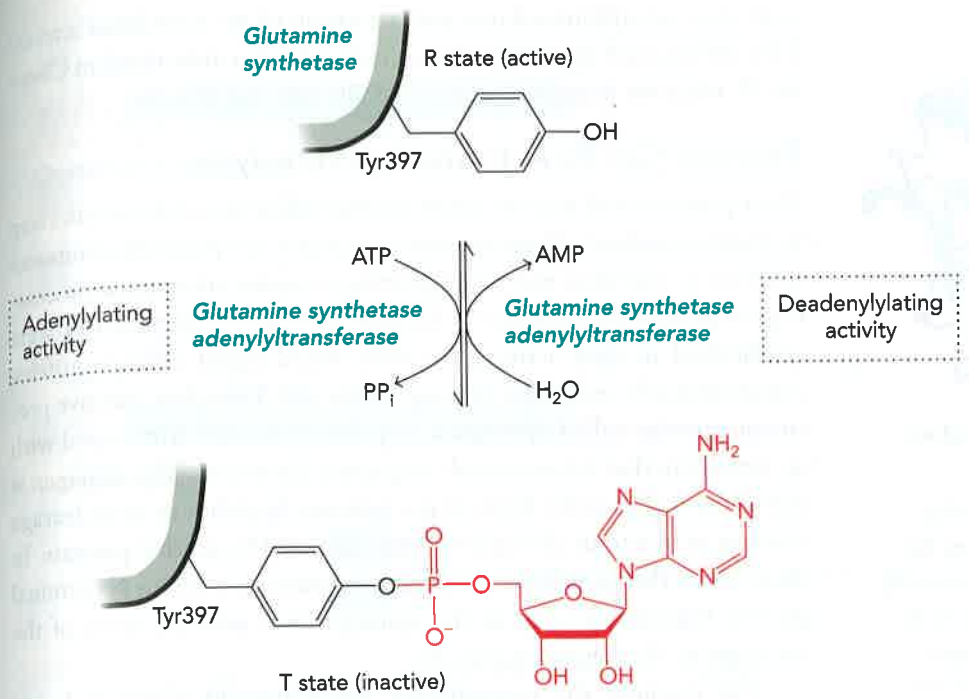


Figure 7.58 The glutamine synthetase adenylyltransferase protein complex catalyzes a reaction that covalently attaches an AMP moiety to a tyrosine residue on glutamine synthetase, which inhibits enzyme activity. A different subunit in the glutamine synthetase adenylyltransferase protein complex catalyzes the reverse reaction, which deadenylylates glutamine synthetase to activate the enzyme.

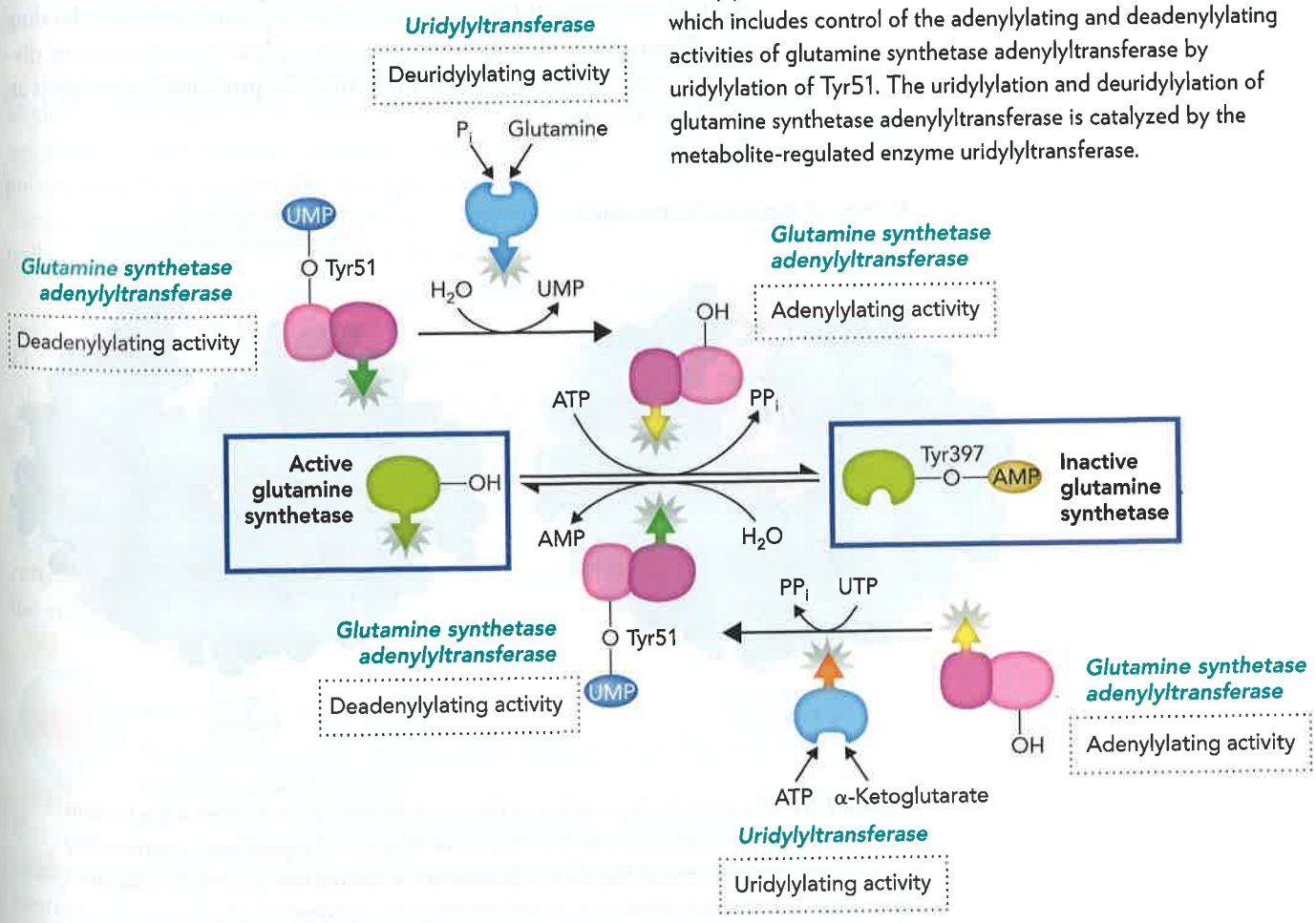


Figure 7.59 Regulation of glutamine synthetase activity by adenylylation on Tyr397 involves a multilayered mechanism, which includes control of the adenylylating and deadenylylating activities of glutamine synthetase adenylyltransferase by uridylylation of Tyr51. The uridylylation and deuridylylation of glutamine synthetase adenylyltransferase is catalyzed by the metabolite-regulated enzyme uridylyltransferase.

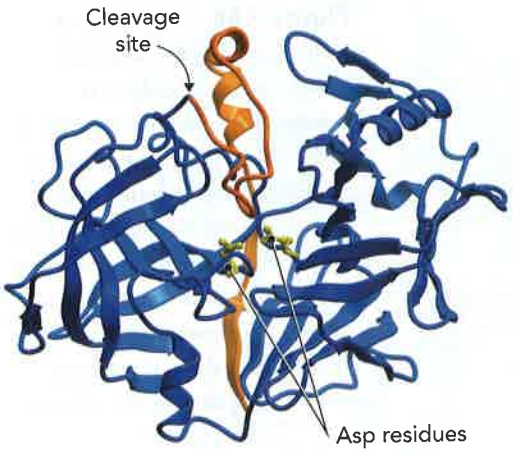


Figure 7.60 Pepsinogen is a zymogen that catalyzes an autocleavage reaction to create the active form of the enzyme pepsin. The N-terminal 44 amino acids (orange) that block access to the active site must be removed from pepsinogen by autocleavage to generate the active enzyme. The two catalytic Asp residues (yellow) in the active site are shown. BASED ON PDB FILE 2PSG.

synthetase adenylyltransferase and activation of its *adenylation activity*. This multilayered regulatory scheme is described in more detail in Chapter 17, when we describe regulation of nitrogen metabolism.

Enzymes Can Be Activated by Proteolysis

Most proteins fold into an active conformation as a concomitant step in protein synthesis. However, some enzymes—in particular, proteases involved in digestion and blood clotting—require subsequent processing by proteolytic cleavage to become fully active. If proteases were synthesized in their active form, they would digest cellular proteins indiscriminately and cause damage to the cell. Therefore, inactive precursor proteins called **zymogens**, or proenzymes, are synthesized with an active site that is inaccessible to protein substrates. The zymogen is converted to the active form of the protease by either an autocleavage reaction or by a trans cleavage reaction mediated by another protease. In many cases, the proteolytic processing reaction removes an N-terminal protein fragment present in the zymogen that prevents entry of the substrate to the enzyme active site.

For example, the removal of a 44-amino-acid N-terminal segment of pepsinogen, the zymogen, generates the proteolytically active form of the enzyme pepsin (**Figure 7.60**). This autocleavage reaction of pepsinogen, which occurs as both an intramolecular and intermolecular reaction, is stimulated by the acidic environment of the stomach (pH 1.5) and leads to the formation of an accessible substrate binding cleft (**Figure 7.61**). Unlike the reversible regulatory mechanisms discussed earlier, regulation of enzyme activity by proteolytic cleavage is an irreversible process.

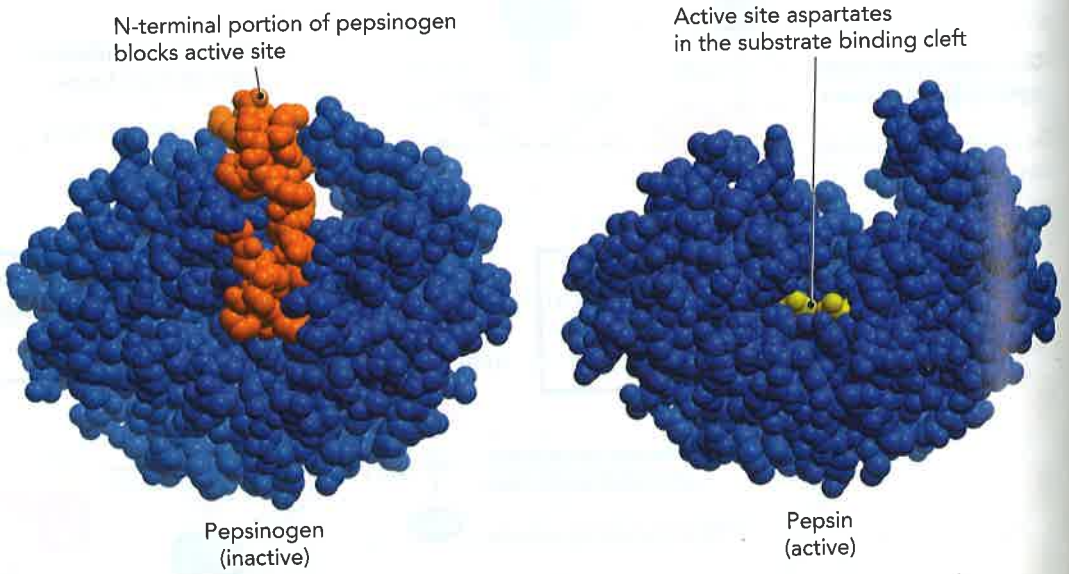


Figure 7.61 The catalytic Asp residues in the pepsin active site are located at the bottom of a large cleft that is accessible after the N-terminal fragment of pepsinogen is removed by autocleavage. The N-terminal fragment in pepsinogen is colored orange, and the catalytic Asp residues are colored yellow. BASED ON PDB FILES 2PSG (PEPSINOGEN) AND 5PEP (PEPSIN).

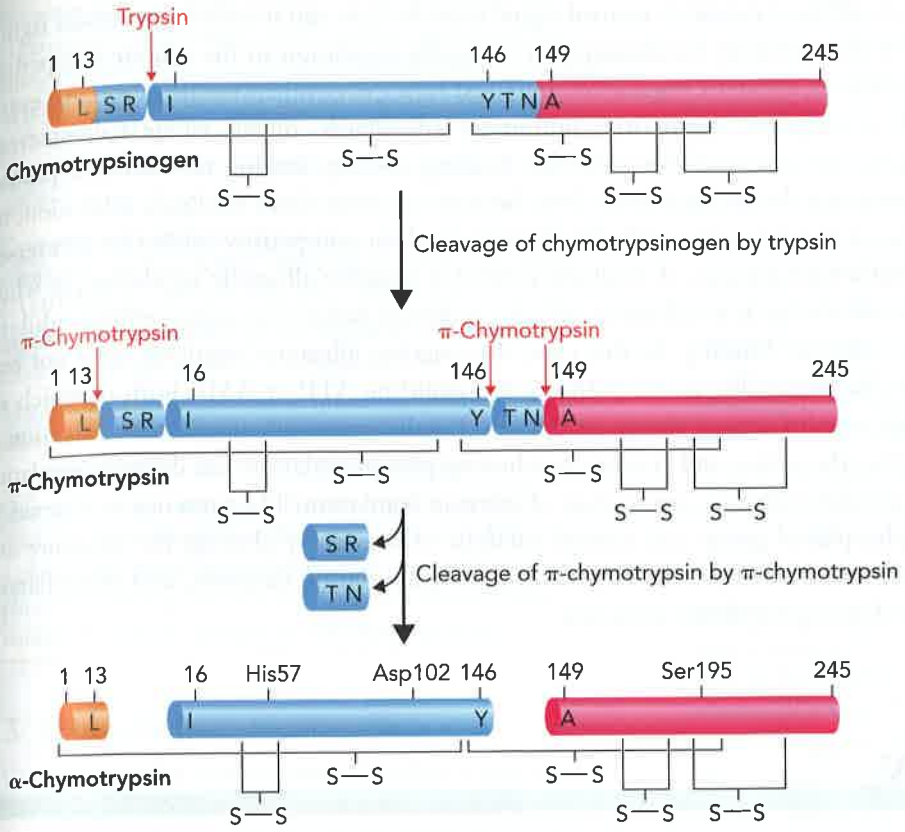


Figure 7.62 Chymotrypsin is generated by proteolytic processing of chymotrypsinogen into three polypeptide chains as shown by the colored bars. Trypsin cleaves chymotrypsinogen (inactive zymogen) to create π-chymotrypsin, which cleaves other π-chymotrypsin molecules to generate the fully active α-chymotrypsin enzyme. The relative positions of the five disulfide bonds and the three amino acids in the catalytic triad are shown (see also Figure 7.26).

Another example of enzyme regulation by protein processing is the conversion of chymotrypsinogen to its active form chymotrypsin, after four proteolytic cleavage events. These cleavages generate a serine protease consisting of three separate polypeptides held together by disulfide bonds (see Figure 7.26). Chymotrypsin is a digestive enzyme produced in the pancreas and secreted into the small intestine, where it digests small polypeptides that exit the stomach.

As shown in **Figure 7.62**, the first chymotrypsinogen cleavage reaction is catalyzed by the enzyme trypsin, which breaks the peptide bond on the C-terminal side of Arg15. The product of this trypsin cleavage reaction is a moderately active serine protease called π-chymotrypsin, which then functions in trans to cleave other π-chymotrypsin proteins on the C-terminal side of Leu13, Tyr146, and Asn148. The final product of these proteolytic processing events is the fully active form of the enzyme, called α-chymotrypsin. The key to this regulatory mechanism is the site-specific cleavage of chymotrypsinogen by trypsin, which is itself activated by a serine protease called enterokinase. Enterokinase is released into the duodenum after proteolytic cleavage of the membrane-bound enterokinase zymogen.

concept integration 7.5

How is the catalytic activity of an enzyme reversibly regulated to facilitate on/off control?

Enzyme activity in the cell is regulated by both bioavailability and catalytic efficiency. Regulating enzyme bioavailability can be a rather slow process, but in contrast, reversibly regulating the catalytic efficiency of an enzyme can be rapid and

is an efficient means to control signal transduction and metabolic processes tightly. The three primary mechanisms of reversible regulation in the cell are competitive inhibition, allosteric regulation, and most forms of covalent modification. An example of reversible competitive inhibition is feedback control, where a downstream metabolite competes for substrate binding, thereby leading to decreased product formation. As product levels drop because of competitive feedback inhibition, the level of downstream metabolite decreases and the competitive inhibition is reversed. Another mechanism of feedback control is negative allosteric regulation, in which the metabolite is an allosteric effector molecule rather than a competitive inhibitor for substrate binding. In this case, the negative allosteric regulator need not be a specific metabolite in the pathway, but could be ATP or AMP, both of which are indicators of energy charge in the cell. Finally, reversible covalent modification of serine, threonine, and tyrosine residues by phosphorylation and dephosphorylation is another common mechanism of enzyme regulation. The presence or absence of a phosphoryl group can control catalytic efficiency by altering the structure and function of the enzyme. Kinases are phosphorylating enzymes, and phosphatases are dephosphorylating enzymes.

chapter summary

7.1 Overview of Enzymes

- Enzymes are biological catalysts that alter reaction rates without changing the overall ΔG or K_{eq} and are not consumed by the reaction.
- Substrates often bind with high affinity and specificity to an enzyme's active site, which is a cleft or pocket in the protein structure where the catalyzed reaction takes place.
- Induced fit is a term that describes how substrate binding to enzymes is often associated with structural changes in the enzyme. These changes maximize the number of weak noncovalent interactions between the substrate and amino acid residues in the enzyme active site.
- Enzyme activity is highly regulated in cells to maximize energy balance between anabolic and catabolic pathways and to alter cell behavior in response to environmental stimuli.
- According to transition state theory, a reactant must first reach an energy level required for chemical transformation before the product can be formed.
- The activation energy ($\Delta G^{\ddagger}$) is the difference between the ground state energy of the reactant and the transition state energy. Enzymes lower $\Delta G^{\ddagger}$ by providing a favorable physical and chemical environment in the active site to promote catalysis.
- Cofactors and coenzymes provide additional reactive groups to the enzyme active site that complement the limited chemistry of amino acid side chains. Cofactors are inorganic ions, whereas coenzymes are small organic molecules originally discovered as vitamins.
- The IUBMB enzyme classification system provides a standard nomenclature for enzymes. It is based on a hierarchical numbering system beginning with one of six classes of reactions (redox reactions, transferase reactions, hydrolase reactions, lyase reactions, isomerase reactions, and ligase reactions).

7.2 Enzyme Structure and Function

- Enzymes lower the activation energy ($\Delta G^{\ddagger}$) of a reaction in three different ways: (1) stabilizing the transition state, which lowers the activation barrier; (2) providing an alternative path for product formation through reaction intermediates; and (3) orienting the substrates appropriately for the reaction to occur.
- Stabilizing the transition state is one of the key mechanisms of enzyme catalysis and is the molecular basis for tight binding of transition state analogs, which often function as enzyme inhibitors.
- Functional groups in the active site mediate three main types of catalytic reaction mechanisms: (1) acid–base catalysis, (2) covalent catalysis, and (3) metal-ion catalysis.
- Metal ions are important enzyme cofactors and promote catalysis by aiding in substrate orientation, mediating redox reactions, and shielding or stabilizing negative charges through electrostatic interactions.

- Enzymes perform three main types of work in the cell: (1) coenzyme-dependent redox reactions associated with energy conversion; (2) metabolite transformation reactions to interconvert metabolites in anabolic and catabolic pathways; and (3) reversible covalent modification reactions to control cell signaling processes and enzyme activity.
- Enzyme-catalyzed redox reactions in the cell often require coenzymes such as NAD^+/NADH, $NADP^+$/NADPH, $FAD/FADH_2$, or $FMN/FMNH_2$. These redox reactions involve the transfer of a pair of electrons or a single electron through a radical intermediate.
- Metabolite transformations in metabolic pathways most often involve isomerization reactions, condensation reactions, or hydrolysis or dehydration reactions.
- One of the most common types of reversible covalent modification reactions in cells is the addition and removal of a phosphoryl group in biomolecules. Enzymes that attach phosphoryl groups are called kinases, and enzymes that remove phosphoryl groups are called phosphatases.

7.3 Enzyme Reaction Mechanisms

- Chymotrypsin is a serine protease that cleaves peptide bonds using a combination of acid–base and covalent catalysis. In addition, a tetrahedral intermediate is formed that resembles the transition state conformation.
- A key feature of serine proteases is the presence in the enzyme active site of three amino acids called the catalytic triad, which consists of a catalytic serine residue plus histidine and aspartate residues that function to convert the serine residue into a highly reactive nucleophile.
- Enolase catalyzes the dehydration of 2-phosphoglycerate to form phosphoenolpyruvate in a two-step mechanism that involves both acid–base and metal-ion catalysis. The metal ions in this reaction are necessary for ionic interactions with the substrate and intermediate.
- HMG-CoA reductase, an enzyme that catalyzes an early step in cholesterol biosynthesis, uses two NADPH coenzymes to achieve catalysis.

7.4 Enzyme Kinetics

- Enzyme kinetics is the quantitative analysis of reaction rate data obtained with purified enzymes and defined laboratory conditions. We can use enzyme kinetic parameters to compare the catalytic efficiency of related enzymes under a variety of conditions.
- The velocity v of an enzyme reaction is the product of the rate constant k and substrate concentration [S], where k refers to the rate at which S → P under standard conditions.
- Michaelis–Menten enzyme kinetics provides a way to analyze a first-order reaction under steady-state conditions in order to relate the initial velocity v_0 to the maximum velocity v_{max}, substrate concentration [S], and Michaelis constant K_m. K_m is experimentally determined as the concentration of substrate required to attain $\frac{1}{2}v_{max}$.
- The values of v_{max} and K_m for an enzyme reaction are obtained from experiments in which data are collected under steady-state conditions when the concentration of the enzyme–substrate complex [ES] is minimally changing (substrate binding to enzyme is rate limiting). Product formation is measured over time for several different initial substrate concentrations.
- Plotting experimental rate data as initial velocity v_0 (which is the slope of the line [P]/time) versus initial [S] produces a Michaelis–Menten plot that is hyperbolic if the enzyme reaction follows simple Michaelis–Menten kinetics.
- A Lineweaver–Burk plot is a double reciprocal plot of enzyme kinetic data that transforms the Michaelis–Menten plot into a linear plot that can be used to estimate values for v_{max} and K_m.
- The calculated efficiency of an enzyme is called the turnover number, k_{cat}, which is a measure of how well an enzyme functions in the reaction. Turnover number is defined as $k_{cat} = v_{max}/[E_t]$.
- Enzyme reaction rates are affected by pH and temperature, which reflect physical and chemical changes in the active site under suboptimal conditions.

7.5 Regulation of Enzyme Activity

- Enzyme regulation is mediated by both enzyme bioavailability (amount of enzyme in the cell and where it is located) and catalytic efficiency (how well an enzyme works).
- Catalytic efficiency of an enzyme is regulated by reversible and irreversible inhibition, allosteric control, covalent modification, and proteolytic processing.
- The three types of reversible and irreversible inhibition are (1) competitive inhibition, (2) uncompetitive, and (3) mixed inhibition, which can be distinguished from each other using enzyme kinetic data.
- The three most common ways that enzymes are regulated by covalent modification are the addition and removal of (1) phosphoryl groups, (2) methyl or acetyl groups, and (3) NMP groups, primarily adenylyl and uridylyl groups.
- Zymogens are inactive proenzymes that are irreversibly processed by proteolysis to generate the active form of the enzyme.