CHAPTER 7

Enzymes

STUDY GOALS

After reading this chapter you will:

- appreciate that enzymes play key roles in biochemistry as catalysts of biochemical reactions
- be able to describe examples of both protein and RNA enzymes
- understand the roles of cofactors in enzyme-catalyzed reactions, and be able to give examples of different types of cofactor
- know how enzymes are classified according to the type of biochemical reaction that they catalyze
- be able to describe the free energy changes that occur during a biochemical reaction, distinguishing between exergonic and endergonic reactions
- understand how an enzyme affects the rate of a biochemical reaction by lowering the free energy of the transition state
- know the differences between the thermodynamic terms represented by ΔG and $\Delta G‡$, and be able to explain the biochemical relevance of these terms
- be aware of how energy coupling enables the energy released by an exergonic reaction to drive an endergonic reaction
- understand the general ways in which temperature and pH affect the rate of an enzyme-catalyzed reaction
- be able to understand the effect of substrate concentration on reaction rate, and to explain the meaning of the terms V_{max} and K_m
- understand how the Lineweaver–Burk plot provides a graphical means of assigning V_{max} and K_m to an enzyme-catalyzed reaction
- be able to distinguish between an irreversible and reversible enzyme inhibitor, giving examples of both types
- know the different effects that competitive and non-competitive inhibition have on the kinetics of an enzyme-catalyzed reaction

We now move on to examine the major biochemical reactions in living organisms. These **metabolic** reactions are divided into two groups:

- **Catabolism**, which is the part of metabolism that is devoted to the breakdown of compounds in order to generate energy.
- **Anabolism**, which refers to those biochemical reactions that build up larger molecules from smaller ones.

The biochemical reactions that make up the metabolic activity of a cell are diverse and involve a large variety of compounds. These compounds include not just proteins, nucleic acids, lipids and carbohydrates, but also many smaller molecules that act as substrates, intermediates and products in the reactions that generate energy and participate in synthesis of the larger biomolecules. We will meet the most important of these compounds as we progress through the next seven chapters of this book.

Figure 7.1 **Enzymes catalyze the steps in a biochemical pathway.**
The first three steps in glycolysis are shown. This is the first stage of the energy-generating pathway of living cells. Each step is catalyzed by a different enzyme.

With such a vast range of molecules, and with so many different biochemical reactions serving so many diverse purposes, we might imagine that finding common themes would be difficult. In fact there is one common theme that unifies and underlies all of metabolism, and that is the role of **enzymes**. These are proteins, or very occasionally RNA molecules, that catalyze the individual steps in a biochemical pathway (*Fig. 7.1*). By responding to signals from inside and outside of the cell, enzymes also set the rates at which individual biochemical reactions occur. In this way they coordinate the overall metabolic activity of the cell, and ensure that this activity is appropriate for the cell's environment if it is a unicellular organism, or for its specialized function if the cell is part of a multicellular organism.

We therefore begin Part II of this book by studying enzymes and the way they work.

7.1 What is an enzyme?

The first scientific recognition of what we now refer to as an enzyme was made in 1833. In that year the French chemists Anselm Payen and Jean-François Persoz prepared an aqueous extract from malt, the germinated cereal grains used in brewing. They treated the extract with alcohol and obtained a milky precipitate. This precipitate had the ability to convert starch into sugar, an ability that was lost when the preparation was heated. They called the activity 'diastase', from the Greek for 'separation', because they looked on the activity that they observed as the 'separation' of the sugar from the starch.

Payen and Persoz's work was well ahead of its time, and they had little idea what diastase actually was. Looking back with today's knowledge, we realize that the milky precipitate that they obtained when they added alcohol to their malt extract was made up largely of proteins, which are insoluble in alcohol. We also know that the enzymatic activity of a protein is determined by its tertiary structure, and so is lost when the protein is heated and becomes denatured. Therefore, looking back, we can understand that diastase is a protein, which we now call **amylase**. But we also realize that Payen and Persoz's milky precipitate contained many different proteins, including various other enzymes, as well as a variety of other alcohol-insoluble compounds that are present in malt extract.

It took almost another century before biochemical techniques advanced to the stage where all the proteins and other molecules in an extract could be separated and individual enzymes obtained in pure form. The first person to do this was James Sumner of Cornell University, who in 1926 purified **urease** from jack beans and showed that this enzyme, which converts urea into carbon dioxide and ammonia, is a protein. During the next 10 years, Sumner and other biochemists purified several other enzymes and showed that each one was a protein. By 1946, when Sumner received the Nobel Prize, the fact that enzymes are proteins had become scientific dogma.

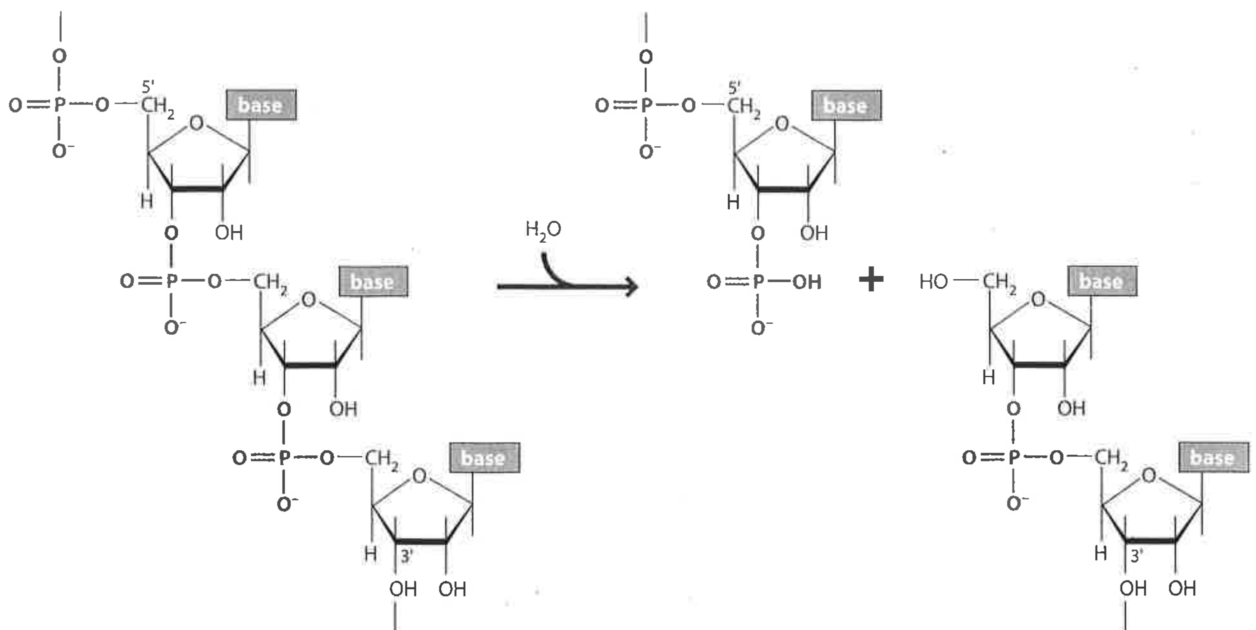
7.1.1 Most enzymes are proteins

Like many scientific dogmas, the one saying that enzymes are proteins turned out only to be partly correct. The vast majority of enzymes are indeed proteins, but a few are RNA molecules.

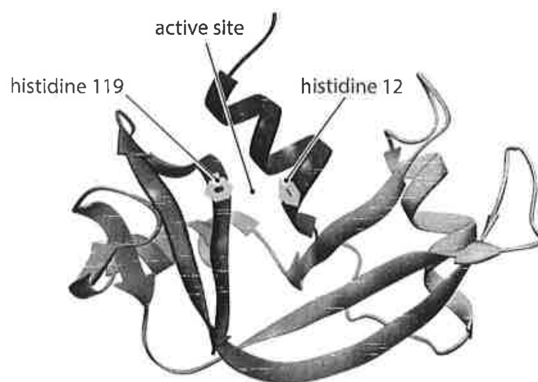
Examples of enzymes made of protein

First we will look at a few examples of the many enzymes that are indeed proteins. Protein enzymes come in all sizes, the smallest ones having fewer than 150 amino acids. **Ribonuclease A**, which we met in *Section 3.4.1* when we studied the denaturation and renaturation of proteins, is a typical small enzyme, with 124 amino acids. The biochemical reaction that it catalyzes is the conversion of a polymeric RNA molecule into two shorter molecules, by cutting one of the internal phosphodiester bonds (*Fig. 7.2A*). Repeated rounds of this reaction will eventually break the RNA

A. the reaction catalyzed by ribonuclease A



B. the two histidines at the active site



C. RNA bound to the enzyme

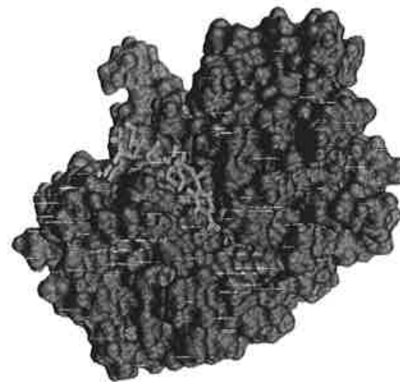
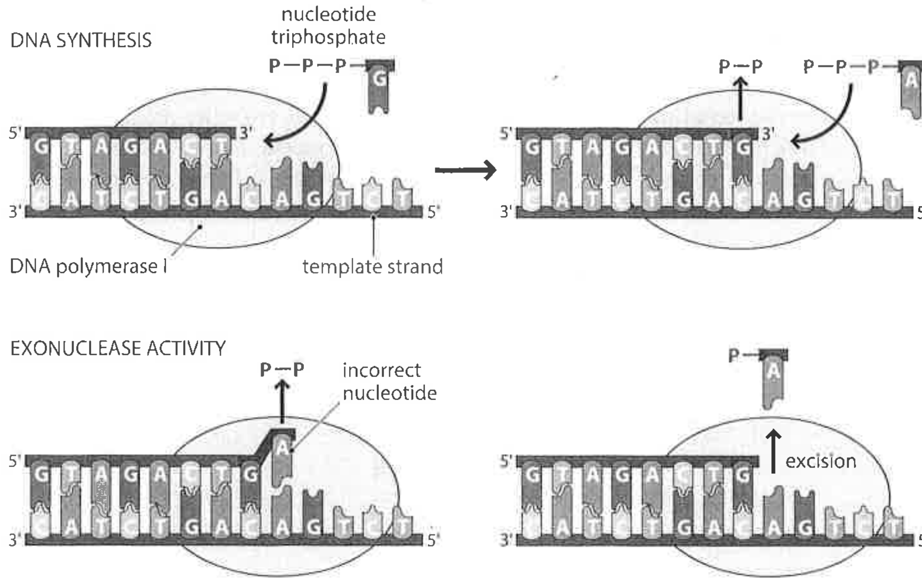


Figure 7.2 Ribonuclease A.
(A) The biochemical reaction catalyzed by ribonuclease A. The reaction requires a molecule of water, and results in a cut being made between a phosphodiester bond and the 5′-carbon of the adjacent nucleotide. (B) A representation of the enzyme structure showing the positions of the two histidine amino acids that flank the active site. (C) A model of the enzyme (blue) with an RNA (green) bound to the active site. This computer generated model shows the actual shape of the enzyme, based on the radii and relative positioning of all of the atoms in the tertiary structure.
Image (B) reproduced from Wikipedia under a CC BY-SA 2.5 license; (C) reprinted with permission from *Journal of Physical Chemistry*, 114:7371. © 2010 American Chemical Society.



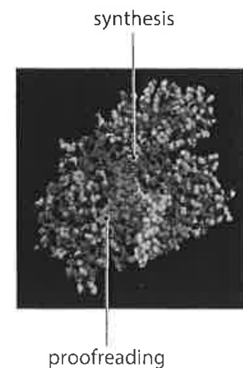


Figure 7.3 **The DNA synthesis and error correction activities of *E. coli* DNA polymerase I.** (A) DNA polymerase I synthesizes a new DNA strand by adding nucleotides to the 3′-end of the polynucleotide that is being made. The enzyme uses nucleoside triphosphates, with two of the phosphates being released when a nucleotide is added. An exonuclease activity enables the enzyme to remove a nucleotide that has been added in error. (B) The polymerase and exonuclease activities are specified by different parts of the DNA polymerase I protein. In this picture, a short piece of DNA is shown attached to the enzyme, with the template strand colored purple and the newly synthesized strand in green. Image (B) produced by David S. Goodsell from The Scripps Research Institute shows the DNA polymerase I from *Escherichia coli*.

down into its constituent nucleotides. When we examine the tertiary structure of ribonuclease A we see a mixture of α-helices and β-sheets that form a U-shape. Two histidine amino acids, located at positions 12 and 119 in the polypeptide (we refer to these amino acids as 'his-12' and 'his-119'), flank the **active site**, the position where the biochemical reaction takes place (*Fig. 7.2B*). The RNA enters the active site and a phosphodiester bond is cut by a chemical reaction that involves the two histidines (*Fig. 7.2C*). Once cut, the two shorter RNA molecules are released from the enzyme.

Ribonuclease A, like most small enzymes, catalyzes a single, clearly defined biochemical reaction. Some larger enzymes have more complex activities, with different parts of the protein catalyzing different reactions. The enzyme called **DNA polymerase I**, from the bacterium *Escherichia coli*, is a good example. Like ribonuclease A, DNA polymerase I is a single polypeptide, but is much longer with a total of 928 amino acids. A DNA polymerase is an enzyme that makes a new DNA molecule by joining together individual nucleotides. The biochemical reaction is therefore synthesis of a phosphodiester bond. DNA polymerase I does this in a template-dependent manner, reading the sequence of nucleotides in an existing DNA strand (the template strand) to determine the sequence of the new polynucleotide, in accordance with the base-pairing rules. Nucleotides are added, one by one, to the 3′-end of the polynucleotide that is being made (*Fig. 7.3A*). DNA polymerase activity is specified by the amino acids between positions 521 and 928 of the DNA polymerase I polypeptide (*Fig. 7.3B*). Copying of the template is very accurate but every now and then, perhaps once for every 9000 nucleotides that are added, the polymerase makes a mistake and attaches an incorrect nucleotide. To correct the error,

See *Section 14.1.2* for further details regarding the actions of DNA polymerases.

the enzyme is able to break the phosphodiester bond that it has just made, releasing the incorrect nucleotide. This error correction is a completely different biochemical reaction, which we refer to as an **exonuclease** activity, removing a nucleotide from the end of a polynucleotide. This exonuclease is specified by amino acids 324–517. So DNA polymerase I is a multifunctional enzyme, with its different activities performed by different parts of its polypeptide.

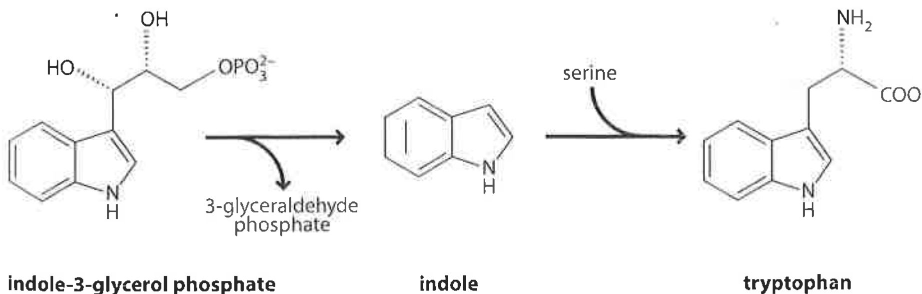
The synthesis pathways for phenylalanine, tyrosine and tryptophan are described in *Section 13.2.1*.

Other multifunctional enzymes have multiple subunits, each one responsible for a different enzymatic reaction. An example, again in *E. coli*, is the enzyme **tryptophan synthase**. As its name implies, tryptophan synthase is involved in the anabolic pathway that results in synthesis of the amino acid tryptophan. This pathway begins with the aromatic compound called **chorismate**, and has branches leading to phenylalanine and tyrosine as well as the one leading to tryptophan. The last two steps in the tryptophan branch are catalyzed by tryptophan synthase. In the first of these reactions, indole-3-glycerol phosphate is converted to indole by removing the glycerol phosphate side-chain attached to carbon number 3, and in the second reaction a serine is attached at this position to form tryptophan (*Fig. 7.4A*). Tryptophan synthase is made up of four subunits, two called α and two β. An α-subunit has a barrel-like structure made up of an eight-stranded β-sheet surrounded by eight α-helices. A molecule of indole-3-glycerol phosphate enters this barrel and attaches to a glutamic acid and an aspartic acid at positions 49 and 60, respectively, in the α polypeptide. It is then cleaved to produce indole and 3-glyceraldehyde phosphate. The 3-glyceraldehyde phosphate molecule is ejected and the indole passed down a tunnel that leads to the active site of the β-subunit, some 2.5 nm away (*Fig. 7.4B*). A serine molecule is now attached to the indole, giving tryptophan. Channeling of the intermediate in a two-step biochemical reaction, from one subunit to another in a multifunctional enzyme, is a common way of ensuring that the intermediate does not diffuse away from the enzyme, and immediately enters the next step of the pathway.

Some enzymes are RNA molecules

Until the early 1980s it was believed that all enzymes were proteins. This rule was overturned when the first **ribozyme** was discovered, by Sidney Altman of Yale University and Thomas Cech of the University of Colorado. A ribozyme is an enzyme that is made of RNA.

A. tryptophan synthesis pathway



B. tryptophan synthase α and β subunits

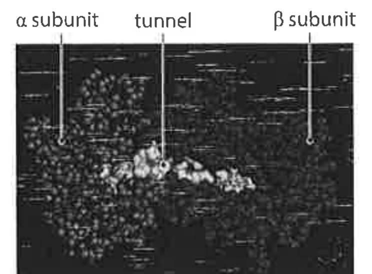


Figure 7.4 **Tryptophan synthase.**
(A) The last two steps of the biosynthetic pathway that results in synthesis of tryptophan. (B) The structure of the α- and β-subunits of *E. coli* tryptophan synthase, showing the tunnel between the two subunits along which indole travels. Note that the tryptophan synthase enzyme has four subunits, two α and two β. Two simultaneous reactions can therefore occur, one in each of the αβ dimers.
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Many ribozymes work in conjunction with proteins to carry out their enzymatic function but, in all the examples known at present, the catalytic activity itself is specified by the RNA component. A good example is the bacterial enzyme **ribonuclease P**. This is a different enzyme to the protein ribonuclease A that we studied above. Rather than making random cuts in an RNA molecule, ribonuclease P has a more specialized function, making single cuts at specific positions in a small number of cellular RNA molecules. These molecules include tRNAs, which are initially made as precursor RNAs that are longer than the mature tRNAs that assist in protein synthesis. The pre-tRNA molecules are processed by ribonuclease processing enzymes that remove the additional stretches of polynucleotide to either side of the cloverleaf structure. One of these processing enzymes is ribonuclease P, which makes a single cut at the 5′-end of the mature tRNA (*Fig. 7.5A*).

We will look at the role of ribonuclease P in tRNA processing in *Section 15.2.1*.

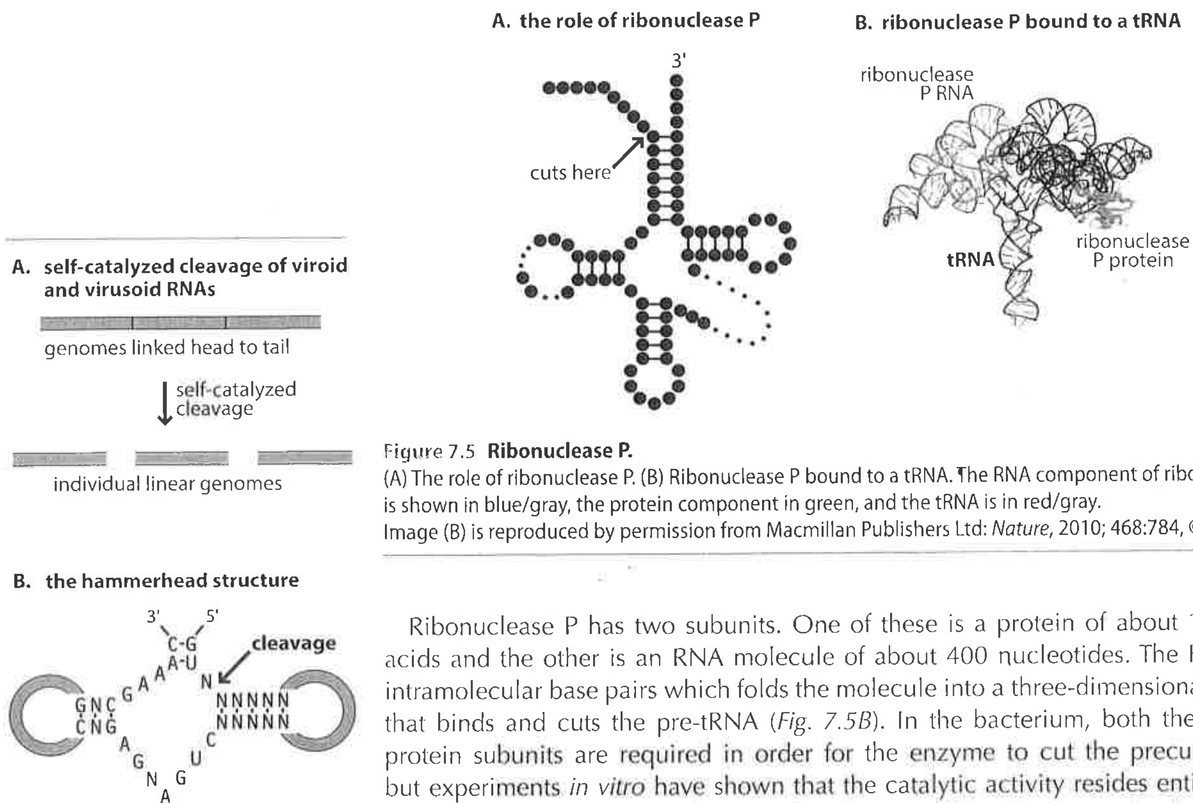


Figure 7.5 **Ribonuclease P.**
(A) The role of ribonuclease P. (B) Ribonuclease P bound to a tRNA. The RNA component of ribonuclease P is shown in blue/gray, the protein component in green, and the tRNA is in red/gray.
Image (B) is reproduced by permission from Macmillan Publishers Ltd: *Nature*, 2010; 468:784, © 2010.

B. the hammerhead structure

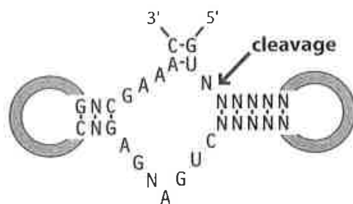


Figure 7.6 **Self-catalyzed cleavage of linked genomes during replication of viroids and virusoids.**
(A) The replication pathway. (B) The hammerhead structure, which forms at each cleavage site and which has enzymatic activity. N, any nucleotide.

Ribonuclease P has two subunits. One of these is a protein of about 120 amino acids and the other is an RNA molecule of about 400 nucleotides. The RNA forms intramolecular base pairs which folds the molecule into a three-dimensional structure that binds and cuts the pre-tRNA (*Fig. 7.5B*). In the bacterium, both the RNA and protein subunits are required in order for the enzyme to cut the precursor tRNA, but experiments *in vitro* have shown that the catalytic activity resides entirely in the RNA subunit. The protein subunit is thought to stabilize the interaction between the enzyme and the pre-tRNA, but cannot itself carry out the ribonuclease activity.

Many of the ribozymes that have been discovered so far are ribonucleases. They are thought to have originated in the **RNA world**, the very early stage in evolution before DNA and proteins existed and when all biochemical systems were thought to have been centered on RNA. According to the theories, these early RNA molecules included some that carried genes. In today's world, the genes of all cellular organisms are contained in DNA molecules, but a few viruses still have genomes made of RNA. These include the **virusoids** and **viroids**, whose genomes are RNA molecules some 200–400 nucleotides in length. The replication process for some virusoids and viroids results in a series of genome copies joined end-to-end in a single long RNA molecule. This RNA molecule is a ribozyme, and is able to cut itself up, releasing the individual genome copies, by a self-catalyzed reaction (*Fig. 7.6A*). As with ribonuclease P, the enzymatic activity is contained within a three-dimensional structure that is formed by intramolecular base pairs. This structure is not the same in all virusoids and viroids, but a common type is the **hammerhead** (*Fig. 7.6B*).

Theories about the origins of the first cells, which are thought to have contained self-replicating RNA molecules, were described in *Section 2.2.1*.

7.1.2 Some enzymes require cofactors

We have seen how certain amino acids in an enzyme play a central role in the biochemical reaction catalyzed by that enzyme. For example, in ribonuclease A the two histidines at positions 12 and 119 in the polypeptide participate in the biochemical reaction that results in breakage of phosphodiester bonds. Similarly, glu-49 and asp-60 in the α-subunit of tryptophan synthase are involved in the cleavage of indole-3-glycerol phosphate to indole and 3-glyceraldehyde phosphate. With these and many other enzymes, the catalytic activity is provided solely by chemical groups present on particular amino acids within the protein. This is not always the case though, and many enzymes require additional ions or molecules, called **cofactors**, in order to perform their catalytic function.

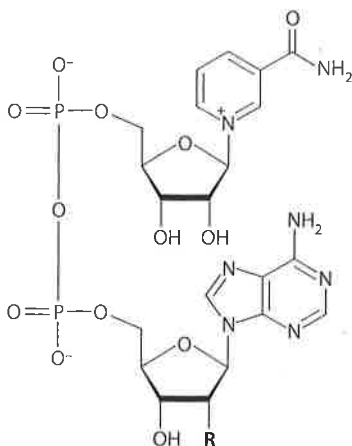
The commonest cofactors are metal ions, which are required by about one-third of all known enzymes (*Table 7.1*). These ions include Cu^{2+}, Fe^{2+}, Fe^{3+}, Mg^{2+}, Mn^{2+}, Ni^{+} and Zn^{2+}. They form attachments at specific sites within the enzyme, usually at or close to the active site so that they can participate directly in the catalytic process. An enzyme that contains a metal ion is called a **metalloenzyme**, and their ubiquity in human cells is the reason why we require trace elements in our diet.

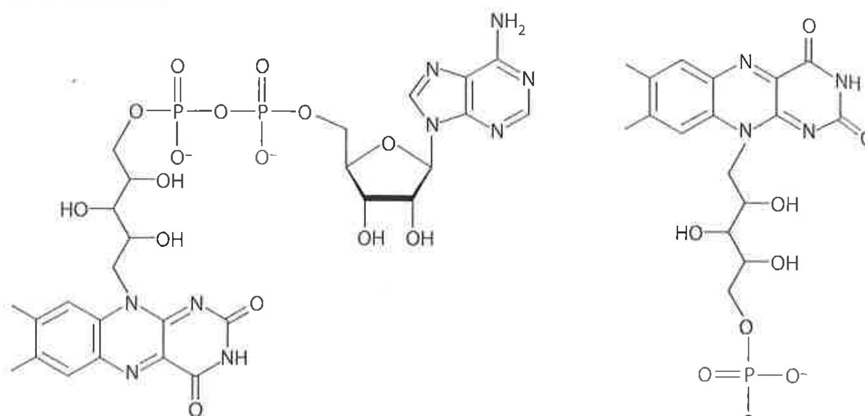
Table 7.1. **Examples of cofactors**

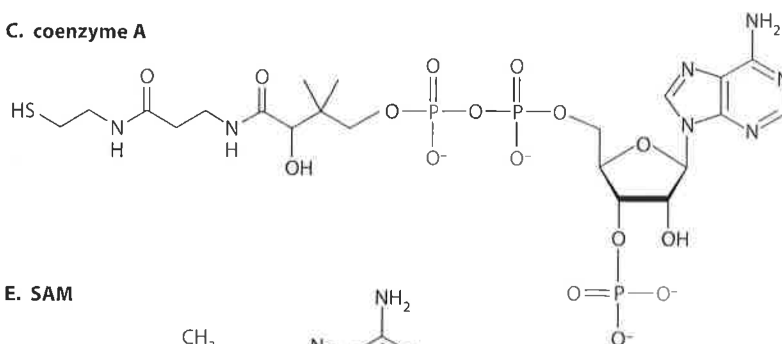
Cofactor	Enzymes requiring the cofactor
Metal ion cofactors	
Cu^{2+}	Cytochrome oxidase
Fe^{2+}, Fe^{3+}	Catalase, nitrogenase
Mg^{2+}	Hexokinase
Mn^{2+}	Arginase
Ni^{+}	Urease
Zn^{2+}	Carboxypeptidase, carbonic anhydrase
Organic cofactors (coenzymes)	
NAD^{+}	Oxidoreductases
$NADP^{+}$	Fatty acid synthesis enzymes
FAD	Succinate dehydrogenase
FMN	NADH dehydrogenase
Coenzyme A	Fatty acid synthesis enzymes
Ascorbic acid	Antioxidative defense enzymes

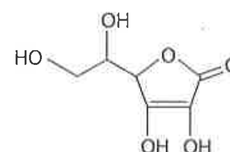
Other cofactors are organic compounds. Many of these are derived from dietary vitamins. These vitamins include:

- **Niacin** (vitamin B_3), which is the precursor of the cofactors **nicotinamide adenine dinucleotide (NAD^{+})** and **nicotinamide adenine dinucleotide phosphate ($NADP^{+}$)** (*Fig. 7.7A*). NAD^{+} and $NADP^{+}$ are required by several enzymes involved in energy generation and anabolism, respectively.
- **Riboflavin** (vitamin B_2) is the precursor of **flavin adenine dinucleotide (FAD)** and **flavin mononucleotide (FMN)** (*Fig. 7.7B*). These have a similar function to NAD^{+} and $NADP^{+}$.
- **Pantothenic acid** (vitamin B_5) is converted into **coenzyme A** (*Fig. 7.7C*), which is involved in energy generation as well as lipid metabolism.
- **Ascorbic acid** (vitamin C; *Fig. 7.7D*) is a cofactor in its own right, in particular in enzymatic reactions that modify amino acids on collagen polypeptides, enabling the polypeptides to form the triple helix structure that collagen adopts in









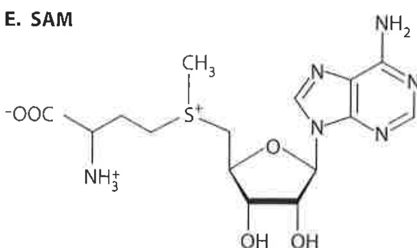


Figure 7.7 The structures of various organic cofactors.

connective tissues such as tendons, ligaments, cartilage and bones. When there is a deficiency of vitamin C in the diet, the action of these enzymes is limited and collagen triple helices cannot be formed correctly. The defective collagen fibrils have a severe effect on the connective tissues, leading to the disease called scurvy.

Other cofactors for human proteins can be made in our cells. These cofactors include the modified amino acid **S-adenosyl methionine** or **SAM** (*Fig. 7.7E*).

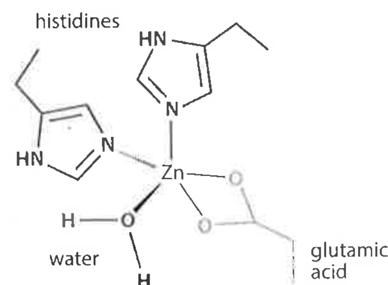
Organic compounds that are cofactors for enzymatic reactions are sometimes called **coenzymes**. Some coenzymes participate in catalysis by forming a transient attachment to the enzyme they are assisting. Others form a permanent or semi-permanent linkage and so become an inherent part of the enzyme's structure. The latter are called **prosthetic groups**. Most metal ion cofactors are an inherent part of the enzyme structure and these are also classed as prosthetic groups.

Box 7.1 Metalloproteins and metalloenzymes

Metal ions are present in many different types of **metalloprotein**, including ones that have no catalytic function and hence are not enzymes. In most metalloproteins, the metal ion is held in place by one or more **coordinate bonds**. A coordinate bond is a special type of covalent bond that forms between the metal ion and the nitrogen, oxygen or sulfur-containing group present in the side-chains of amino acids such as histidine, cysteine and glutamic acid. For example, the Zn^{2+} ion in carboxypeptidase (a protein-degrading enzyme secreted by the pancreas) is held in place by coordinate bonds to two histidines and one glutamic acid, and also makes an attachment to a water molecule, as shown in the diagram to the right.

The resulting structure is called a **coordination sphere**, with the metal ion forming the **coordination center**.

In some metalloproteins, the metal ion forms part of a non-protein organic compound, the commonest example being the iron-containing porphyrin called heme (see *Fig. 3.25*). A heme molecule is made up of four pyrrole subunits linked together in a circle, with an Fe^{2+} ion coordinated in the center. Heme is found in hemoglobin, the oxygen-carrying protein of red blood cells (see *Section 3.3.2*), and in the cytochrome proteins, which are components of the electron transport chain (see *Section 9.2.2*).



An enzyme plus its cofactor is called the **holoenzyme**. When the cofactor is absent what is left is known as the **apoenzyme**. Finally, we should note that it is not just protein enzymes that require cofactors. Some ribozymes, including ribonuclease P, need Mg^{2+} ions in order to carry out their enzymatic reactions.

7.1.3 Enzymes are classified according to their function

How many different enzymes are there? There are billions if we consider each species separately, and so, for example, count the amylase enzyme of barley malt, the one discovered by Payen and Persoz in 1833, as different from the amylase enzymes of other cereals such as wheat or rye. But how many are there if we only consider the different enzyme *activities* known in nature, and so count all these amylases, whether from barley, wheat or rye, as a single enzyme? Using this criterion, there are about 3200 different enzymes.

Over the years, various ways of classifying all these different enzymes have been proposed. Today, everybody uses a single standard scheme which was first agreed by the International Union of Biochemistry and Molecular Biology in 1961. In this classification, all 3200 types of enzyme are initially divided into six broad groups:

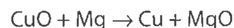
- Enzyme Commission group 1 (EC 1) comprises the **oxidoreductases**. These are enzymes that catalyze oxidation or reduction reactions.
- EC 2 are the **transferases**, enzymes that transfer a chemical group from one compound to another.
- EC 3 are **hydrolases**, which carry out hydrolysis reactions in which a chemical bond is cleaved by the action of water.
- EC 4 are **lyases**, enzymes that break chemical bonds by processes other than oxidation and hydrolysis.
- EC 5 comprises enzymes that rearrange the atoms within a molecule. This rearrangement is called **isomerization** and the enzymes are called **isomerases**.
- EC 6 are the **ligases**, which join molecules together.

PRINCIPLES OF CHEMISTRY

Box 7.2 Oxidation and reduction reactions

The oxidation and reduction reactions catalyzed by oxidoreductase enzymes are important in many areas of biochemistry. In particular, oxidation and reduction underlie the biochemical processes that release energy from carbohydrates and lipids, as we will see in *Chapters 8* and *9*.

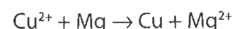
In chemistry, oxidation is often defined as the gain of oxygen by a substance, and reduction is the loss of oxygen. For example, when copper oxide is heated with magnesium metal, the following reaction occurs:



$$CuO + Mg \rightarrow Cu + MgO$$

The magnesium metal gains an oxygen and so is oxidized, whereas the copper oxide loses oxygen and is reduced. Most oxidation and reduction reactions are linked in this way, with the oxygen being lost by one compound and gained by another. We therefore refer to them as **redox reactions.**

In biochemistry, we usually look on redox reactions in a slightly different way. Rather than focusing on the oxygen transfer, we consider the gain and loss of electrons that occurs during the reaction. For example, leaving out the oxide part, we can rewrite the reaction between copper oxide and magnesium as:



$$Cu^{2+} + Mg \rightarrow Cu + Mg^{2+}$$

This representation of the reaction shows us that:

- The oxidation part of the reaction involves loss of two electrons by magnesium, converting the Mg atom to an Mg^{2+} ion.
- The reduction involves gain of two electrons by the Cu^{2+} ion, converting it to a Cu atom.

There is a useful mnemonic:

OIL RIG
Oxidation Is Loss, Reduction Is Gain.

Each of these groups is further subdivided in such a way that every individual enzyme has its own four-part **EC number**. For example, the amylase discovered by Payen and Persoz is referred to as EC 3.2.1.2. This number indicates the precise nature of the enzyme's activity (*Fig. 7.8*):

- EC 3 tells us that amylase is a member of EC group 3, being a hydrolase that uses water to break a chemical bond. This is the underlying nature of the biochemical reaction that results in the conversion of starch to sugar, as observed by Payen and Persoz with their malt extract.
- EC 3.2 indicates that amylase is a **glycosidase** enzyme, one that breaks glycosidic bonds. A glycosidic bond is any bond that links two sugar units or a sugar and another molecule. This part of the EC number distinguishes amylase from other types of hydrolase that work on, for example, ester bonds (which form EC Group 3.1 and include the ribonucleases which break the phosphodiester bonds in RNA) or peptide bonds (EC Group 3.4, this group containing the proteases which break peptide bonds in proteins).

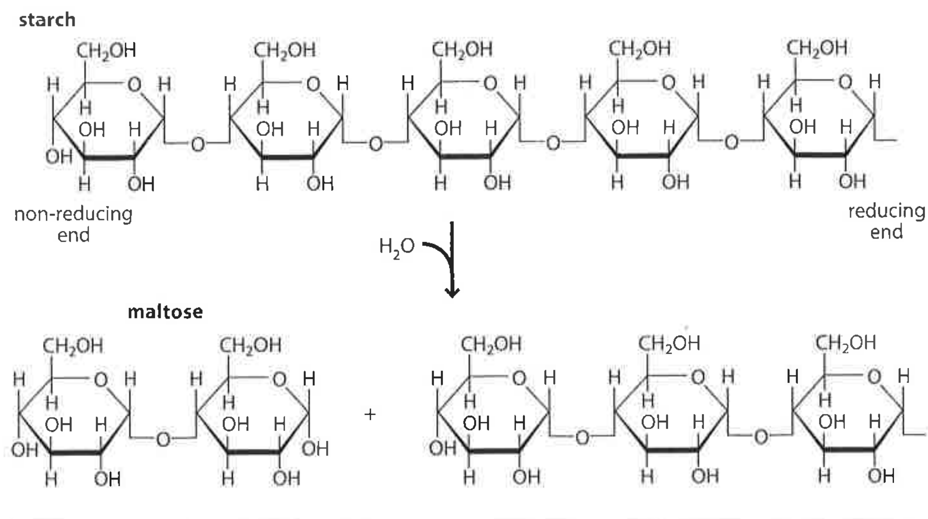


Figure 7.8 The β-amylase reaction (EC 3.2.1.2).

- EC 3.2.1 indicates that amylase is a member of a subgroup of glycosidases that hydrolyze *O*- or *S*-glycosidic bonds. These are glycosidic bonds in which the linkage between the sugar and the second molecule includes an oxygen or sulfur atom. The second subgroup at this level, 3.2.2, comprises enzymes that work on *N*-glycosidic bonds, in which the linkage is via a nitrogen atom. Enzymes in subgroup 3.2.2 therefore include ones that break the bond between the sugar and base in a nucleotide.
- EC 3.2.1.2 is the specific number for β-amylase, the enzyme which hydrolyzes the α(1→4) *O*-glycosidic bonds between glucose units in starch, glycogen and related polysaccharides, in such a way as to release maltose disaccharide units from the non-reducing ends of the polymers.

There are 135 enzymes altogether in the EC 3.2.1 subgroup, all of them hydrolases that break *O*- or *S*-glycosidic bonds between pairs of sugar units or between a sugar and another molecule. The first in the list, EC 3.2.1.1, is α-amylase, which also hydrolyzes α(1→4) *O*-glycosidic bonds in starch and other α(1→4) glucose polysaccharides, but cleaves these bonds randomly within the polymeric chains, rather than just at the non-reducing ends. This type of amylase is found in the salivary and pancreatic secretions of humans and other mammals, and enables us to digest oligo- and polysaccharides in our diet.

The distinction between α- and β-amylase illustrates the importance of the EC classification scheme. It enables us not only to distinguish between different enzymes in a single organism, but also to recognize **homologous** enzymes – ones with identical functions – from different organisms. The β-amylases are considered to be homologous regardless of which type of cereal they are obtained from. There are also enzymes with β-amylase activity in bacteria, and these are also assigned to EC 3.2.1.2. The α-amylases are looked on as a separate type of enzyme because the biochemical reaction that they catalyze is different. They therefore have a different EC number, and again that number is used for α-amylases from any species.

7.2 How enzymes work

Now that we know what enzymes are and how they are classified according to their biochemical activity, we can move on to study the way in which enzymes work in living cells. First, we will examine the fundamental property of an enzyme, which is its ability to act as a biological catalyst.

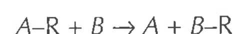
7.2.1 Enzymes are biological catalysts

We have already established that an enzyme is a biological catalyst. A catalyst is a substance that increases the rate of a chemical reaction but is not itself used up as a result of that reaction. An enzyme is no different from any other type of catalyst except that the reaction that it catalyzes is a biochemical one.

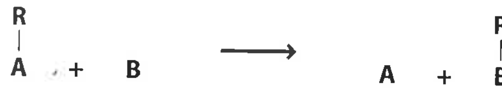
To understand how enzymes work we must therefore study some of the principles of catalysis. In order to do this, we will examine the events that occur during a biochemical reaction, in particular those events relating to the **energetics** of the reaction.

Most biochemical reactions result in a change in free energy

We will look at a typical biochemical reaction, catalyzed by a transferase enzyme (a member of EC group 2), and resulting in transfer of a chemical group from one compound to another (*Fig. 7.9*). The chemical equation for this reaction could be written as:



$$A\text{–R} + B \rightarrow A + B\text{–R}$$



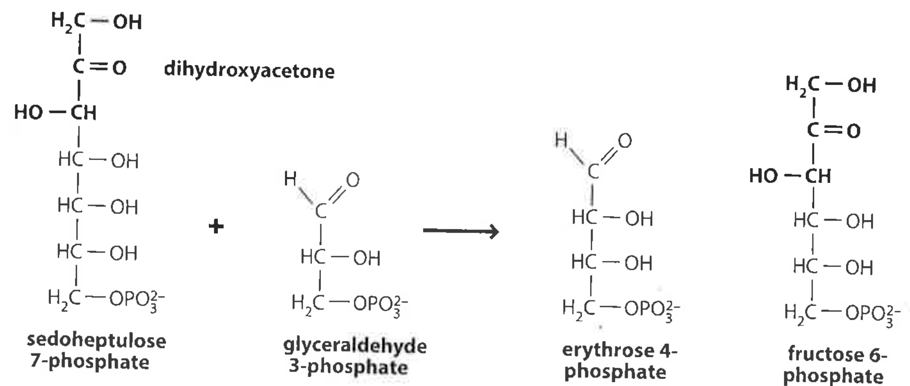


Figure 7.9 Transferase reactions. (A) The general formula for a transferase reaction. (B) An example of a transferase reaction, occurring in the pentose phosphate pathway (see *Section 11.3*). Transaldolase catalyzes the transfer of dihydroxyacetone from sedoheptulose 7-phosphate to glyceraldehyde 3-phosphate.

In this equation, the chemical group 'R' is transferred from compound '*A*' to compound '*B*'. The starting compounds, *A*–R and *B*, are the **substrates** for the reaction, and *A* and *B*–R are the **products**.

The chemical equation that we have just written summarizes the reaction that takes place, but it does not tell us a great deal about what actually happens. To begin to delve more deeply into the nature of the reaction we need to consider whether it results in a change in **free energy**. The Gibbs free energy, referred to as *G*, is a very useful thermodynamic function invented by the American scientist Josiah Willard Gibbs in 1873. In simple terms, it is a measure of the energy content of a 'system'. A system with a low energy content and therefore a low value for *G* is more stable than one with a higher energy content and higher *G* value. In our typical biochemical reaction, the two 'systems' are the substrates *A*–R + *B* and the products *A* + *B*–R.

When considering a biochemical reaction, what interests us is not so much the actual values for *G* for the substrates and products but the difference between these values. We use the Greek letter Δ (an upper case 'delta') to denote the difference between two values for *G*. The change in free energy that occurs during a biochemical reaction is therefore expressed as ΔG.

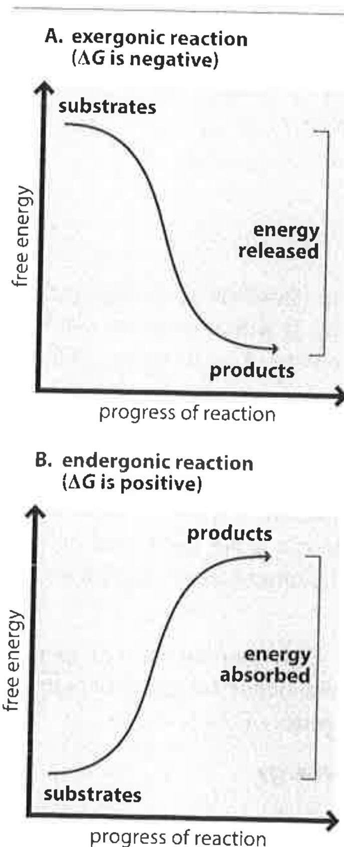


Figure 7.10 The difference between an exergonic and endergonic reaction.

If the ΔG for a reaction is negative, then the free energy of the products is less than that of the substrates (*Fig. 7.10A*). This reaction can occur spontaneously, releasing an amount of energy equivalent to the ΔG value. This is called an **exergonic** reaction. Some chemical reactions have high negative ΔG values and are highly exergonic. The reaction of sodium with water, a favorite of high school chemistry classes, is an example. The products are sodium hydroxide, hydrogen gas and a great deal of energy released as heat, so much that the hydrogen ignites and the sodium metal speeds around the surface of a beaker of water emitting flames. Many biochemical reactions have negative ΔG values and hence release energy when they occur. This is the basis of catabolism, the part of metabolism that results in the generation of energy.

What if the free energy of the products is greater than that of the substrates (*Fig. 7.10B*)? In this case, ΔG is positive and the reaction is energy-requiring or **endergonic**. In biochemistry, many anabolic reactions are endergonic. These are the reactions that result in synthesis of larger products from smaller molecules. Endergonic reactions cannot occur spontaneously because they always need an input of energy.

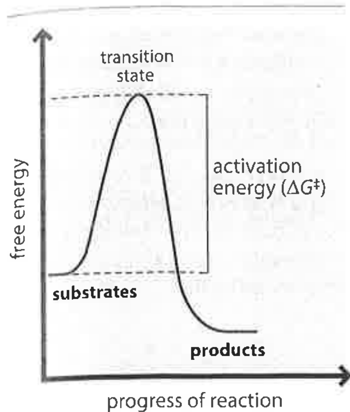
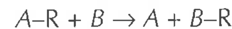


Figure 7.11 The transition state and its energetic implications.
The activation energy is needed to push the reaction over the barrier represented by the transition state.

There is an energy barrier between substrates and products

We have seen how the difference in the free energy values for the substrates and products of a chemical reaction are expressed as a ΔG value. The next issue that we must consider is the free energy of any intermediate structures that are formed during the course of a reaction. By 'intermediate structure' we do not mean actual compounds such as those that are formed during a multistep process, and which might be individually purified. Instead we mean a structure formed part way during a single reaction.

To clarify this important point, we will look more closely at the typical transferase reaction from the previous section. We drew the equation for this reaction as:



$$A\text{–R} + B \rightarrow A + B\text{–R}$$

If we think more carefully about this reaction then we will realize that it probably involves an intermediate structure that is formed at the very instant that group R is being passed from a molecule of *A* to a molecule of *B*. To indicate the existence of this structure we should therefore redraw the equation as:

$$A\text{–R} + B \rightarrow A\cdots\text{R}\cdots B \rightarrow A + B\text{–R}$$

Here, we use dotted lines '···' to indicate that the bonds connecting group R to molecules *A* and *B* are in the process of being broken (in the case of *A*···R) or formed (R···*B*). This intermediate structure will be very unstable and hence will have a high free energy content. We call the point in the reaction pathway at which this structure is formed the **transition state**.

The existence of a transition state means that when considering the free energy changes that occur during a reaction, we must look beyond the ΔG value obtained simply by comparing the substrates and products. We must also consider a second ΔG, between the substrates and the transition state (*Fig. 7.11*). We call this free energy difference the **activation energy** or **ΔG‡**. Often ΔG‡ is much greater than ΔG and forms a significant barrier that must be overcome in order for the reaction to proceed. It is the existence of this energy barrier that limits the rate of most biochemical reactions.

Enzymes lower the free energy of the transition state

Now we begin to understand how an enzyme, or any other type of catalyst, can speed up the rate of a chemical reaction. A catalyst has no influence on the free energy values of the substrates and products and hence does not change ΔG. Instead, a catalyst reduces ΔG‡, usually by stabilizing the intermediate structure formed at the transition state (*Fig. 7.12*). A catalyst therefore decreases the size of the energy barrier that has to be crossed in order to convert the substrates into the products. Because the energy barrier is easier to cross, the rate of the reaction increases.

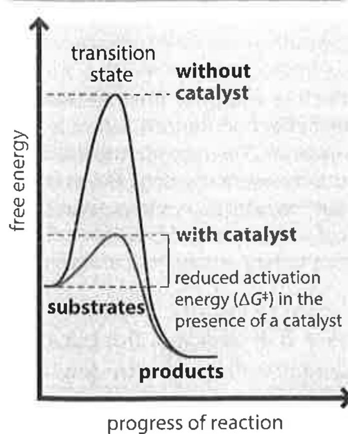


Figure 7.12 A catalyst reduces the free energy of the transition state.

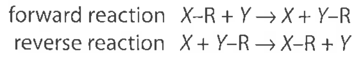
How does an enzyme stabilize the transition state of the reaction that it catalyzes? The answer is by reducing **entropy**. In thermodynamics, entropy is a measure of the degree of disorder of a system. Entropy contributes to the free energy value, so by reducing the entropy of the transition state an enzyme reduces the value of ΔG‡. This might sound complicated but in fact the underlying principle is quite straightforward. Entropy is reduced by bringing order to a system. In our favorite biochemical reaction

$$A\text{–R} + B \rightarrow A\cdots\text{R}\cdots B \rightarrow A + B\text{–R}$$

the 'system' at the start of the reaction is *A*–R + *B*. There might be 100 molecules of *A*–R in the cell, and 100 molecules of *B*, all floating quite close together in the aqueous cytoplasm. But the *A*–R and *B* molecules have no natural attraction for one another and, in the absence of the enzyme, diffuse in random directions according to chance collisions with other molecules (*Fig. 7.13A*). Occasionally a molecule of *A*–R might encounter a molecule of *B* in just the correct manner needed to form the

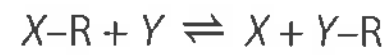
Box 7.3 Reversible reactions

Many biochemical reactions are reversible. This means that the products of the reaction can react with one another to re-form the substrates. A reversible transferase reaction would therefore be made up of two parts, which we call the **forward** and **reverse reactions**:



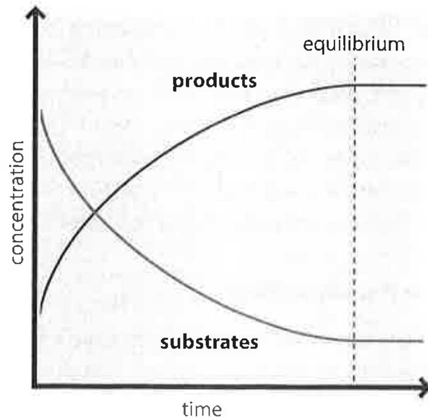
forward reaction $X\text{–R} + Y \rightarrow X + Y\text{–R}$
reverse reaction $X + Y\text{–R} \rightarrow X\text{–R} + Y$

Usually, we combine these two part-reactions into a single equation, using a bidirectional arrow to indicate that the overall reaction is reversible:



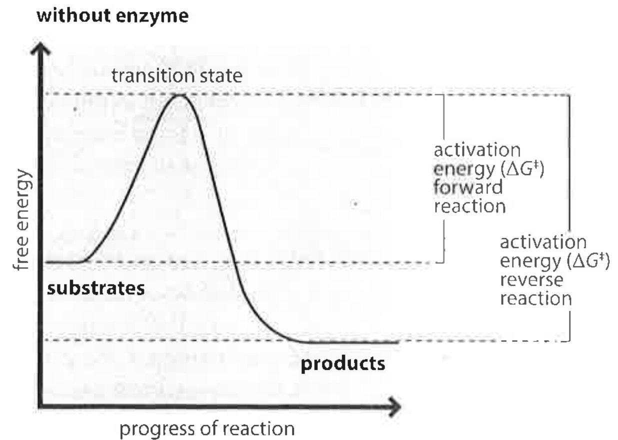
$$X\text{–R} + Y \rightleftharpoons X + Y\text{–R}$$

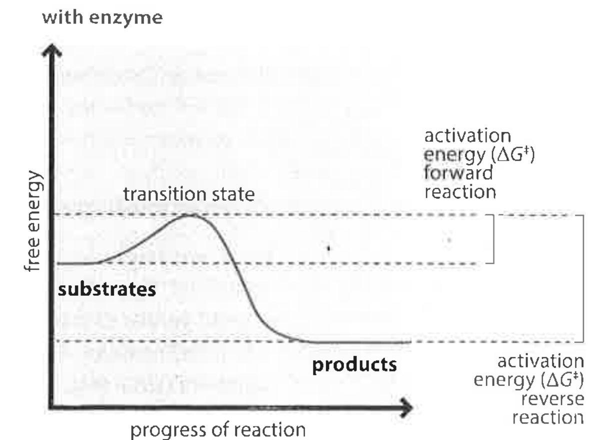
For a reversible reaction, the rate at which the products are formed is counterbalanced by the rate at which the products are converted back to the substrates. As the reaction proceeds, an equilibrium point is reached. After this point, the number of substrate molecules that are converted into products during a particular time period equals the number of substrate molecules that are re-formed by the reverse reaction. The forward and reverse reactions continue to take place, but the relative concentrations of substrate and product no longer change.



The ratio of products to substrates at the equilibrium point depends on the relative rates of the forward and reverse reactions. If the forward reaction is much more rapid than the reverse, then the substrate concentration at the equilibrium point will be small. On the other hand, if there is little difference between the forward and reverse reaction rates, then the substrates and products will have similar concentrations at the equilibrium point. Most reversible reactions are ones where the difference between the free energy values for the substrates and products is relatively small, so in energetic terms the forward reaction is only slightly favored over the reverse one.

What effect will addition of an enzyme have on a reversible reaction? It is important to recognize that the enzyme does *not* affect the equilibrium point. This is because the forward and reverse reactions have the same transition state, even though the $\Delta G‡$ values for the two reactions are different.





Addition of an enzyme lowers the free energy of the transition state, and hence has an equivalent effect on the $\Delta G‡$ values for both the forward and reverse reactions. The enzyme therefore increases the rates of the forward and reverse reactions, but does not affect the equilibrium between the substrates and products.

transition state structure and enable the conversion to A + B–R to occur. But because of the disorder of the system (its high entropy) these chance encounters are few and far between. The rate of the reaction – the conversion of A–R + B to A + B–R – is very slow.

Now we will introduce into the system the transferase enzyme that catalyzes this reaction. The enzyme reduces entropy by binding one molecule of A–R and one

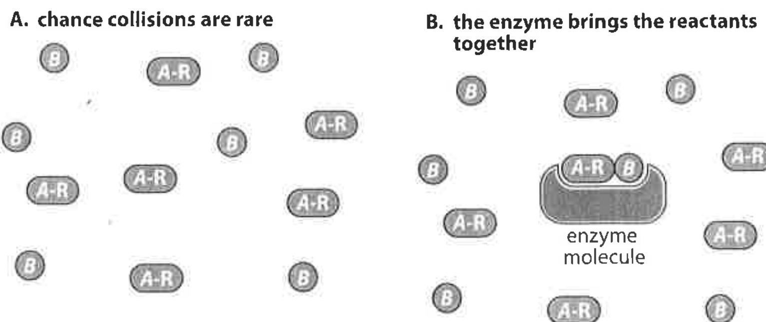


Figure 7.13 An enzyme stabilizes the transition state of a reaction. (A) In a random mixture of reactants, chance collisions are needed in order for the transition state to form. (B) By binding the reactants, the enzyme lowers the entropy of the system, increasing the rate of conversion of reactants to products.

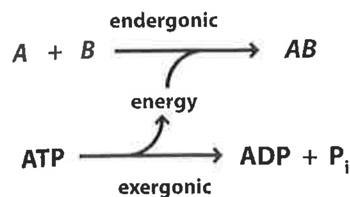


Figure 7.14 Energy coupling. In this example, the free energy required by the endergonic reaction, in which reactants A and B are combined to form product AB, is provided by hydrolysis of ATP to ADP and inorganic phosphate (P_i).

We will learn more about how ATP stores energy in *Section 8.1.1*.

molecule of *B* in precisely the correct relative positioning and orientation needed for these two substrates to form the transition state structure (*Fig. 7.13B*). By binding the substrates in this way the enzyme introduces order into the system, reducing the entropy and hence also reducing the value of $\Delta G\ddagger$. The rate of conversion of *A*–R + *B* to *A* + *B*–R is therefore increased.

An enzyme therefore lowers the energy barrier between the substrates and the transition state, but does not alter the free energy values for the substrates and products. In thermodynamic terms, the enzyme reduces $\Delta G\ddagger$ but has no effect on ΔG. If the reaction is exergonic, with a negative ΔG, then it will proceed with the release of energy. But what if the reaction is endergonic, with the products having a higher free energy content than the substrates? Endergonic reactions require an input of energy in order to reach completion. Many enzymes obtain this energy by coupling the endergonic reaction with a second reaction that generates energy. This is called **energy coupling**. Often the second reaction is hydrolysis of the nucleotide ATP, which gives ADP and inorganic phosphate (*Fig. 7.14*). The free energy released by this exergonic reaction is used by the enzyme to drive the coupled endergonic reaction.

Box 7.4 The specificity of substrate binding

Most enzymes have high specificity for their substrates and are able to distinguish between molecules with very similar structures, binding only those that are the correct substrates for the reaction that the enzyme catalyzes. What is the basis to this specificity?

During the early years of biochemistry, Emil Fischer suggested that the interaction between a substrate and an enzyme is similar to the way in which a key fits into a lock. According to this **lock and key model**, the binding pocket on the surface of an enzyme has a shape that precisely matches that of its substrate. Specificity is achieved because only the substrate, and no other compound, has the shape needed to fit into the binding pocket.

A more recent suggestion is that the enzyme binding site is not a rigid structure, but instead has some flexibility. Attachment of the substrate induces a change in the binding pocket, so that the substrate becomes more precisely enclosed by the enzyme. Only the correct substrate can induce the necessary change to the structure of the binding pocket, increasing the specificity of the enzyme for the substrate. This is called the **induced fit model**.

7.2.2 Factors influencing the rate of an enzyme-catalyzed reaction

Our study of the principles of catalysis has shown us that an enzyme increases the rate of a reaction by reducing the energy barrier between the substrates and the products. Without catalysis, most biochemical reactions occur at a negligible rate and product synthesis is extremely slow. When catalyzed by their enzymes, the same reactions proceed much more rapidly and the products are produced at rates sufficient to satisfy the requirements of the cell. This does not mean, however, that a biochemical reaction has only two possible rates: either switched off because no enzyme is present, or switched on because it is being catalyzed by its enzyme. The rate of the catalyzed reaction is affected by various factors which together determine precisely how rapidly the products are made at any given time. We must now examine these factors and look at the effects that each one has on the rate of an enzyme-catalyzed biochemical reaction.

Temperature and pH affect the rates of enzyme-catalyzed reactions

All chemical reactions are affected by heat, occurring more rapidly at higher temperatures. This is because heating results in an increase in thermal energy, which causes the substrates to move about more rapidly, increasing the frequency with which they come into contact with one another. In thermodynamic terms, the addition of thermal energy helps to push the substrates over the energy barrier of the transition state. In this regard, enzyme-catalyzed reactions are no different to any other chemical reaction, and occur more rapidly when the temperature is increased. However, this is only true at relatively low temperatures, because at higher temperatures a second factor comes into play. This is the effect that temperature has on the stability of chemical bonds, in particular the relatively weak hydrogen bonds that hold together the secondary structures within a protein molecule. As the temperature rises, hydrogen bonds break and the secondary structure of the protein unfolds (denatures) causing a loss of enzyme activity. High temperature therefore denatures proteins in the same way as a chemical denaturant such as urea. The rate of a typical enzyme-catalyzed reaction therefore gradually increases as the temperature is raised, reaching an optimum beyond which the activity declines, possibly quite rapidly because small additional temperature increments cause a relatively large disruption to the enzyme's structure (*Fig. 7.15A*).

We examined the effects of urea on protein activity in *Section 3.4.1*.

In vertebrates, most enzymes have a **temperature optimum** of around 37°C, this being the temperature within the tissues of these warm-blooded animals. The enzymes

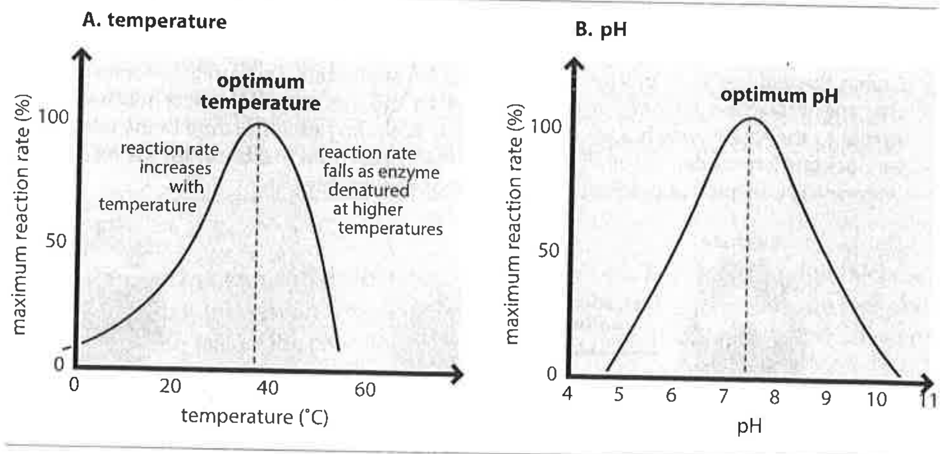


Figure 7.15 The effect of (A) temperature and (B) pH on the rate of an enzyme-catalyzed reaction.

of many of the bacteria that live in or on the bodies of vertebrates have a similar optimum temperature, but bacteria that live in other environments might be quite different in this regard. Thermophilic bacteria, which live naturally in hot springs, are a good example. The temperatures within hot springs can approach boiling point, so the proteins in these bacteria must be able to withstand high temperatures. The **thermostable** enzymes present in these bacteria typically have temperature optima of 75–80°C.

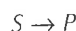
Proteins will also denature at extreme pH values, but the effect of pH on a reaction rate is rather more subtle than a simple disruption of the enzyme's structure. Often the amino acids at the active site of an enzyme are ones that have ionizable side-chains, these side-chains participating in the enzymatic reaction in some way. If we recall how the ionizable groups of an amino acid are affected by the pH then we will immediately appreciate how critical a pH change can be to the rate of an enzymatic reaction. Most enzymes have **pH optima** of 6.8–7.4, matching the physiological pH found in living cells and tissues (*Fig. 7.15B*). As with the effects of temperature, there are exceptions. Pepsin, one of the enzymes that breaks down proteins in the stomach, has a pH optimum of about 2.0, reflecting the highly acid environment in which this enzyme has to function.

The effect of pH on ionization of amino acids was described in *Section 3.1.2*.

Substrate concentration has an important effect on reaction rate

Although pH and temperature have important effects on the rates of enzyme-catalyzed reactions, most organisms have mechanisms for ensuring that the pH within their cells stays constant, and vertebrates also control their internal temperatures so that these rarely vary far from 37°C. So, temperature and pH are not themselves important determinants of the actual rate at which individual biochemical reactions take place. Of much greater importance is the availability of the substrates for the reaction.

To illustrate the important effects of substrate concentration we will consider the simplest type of biochemical reaction, in which there is a single substrate and a single product. This is the type of reaction catalyzed by the isomerase enzymes of EC group 5. We could write this reaction as:



$$S \rightarrow P$$

where S is the substrate, and P is the product. Imagine that we have purified this isomerase enzyme and mixed it with its substrate. The enzyme begins to catalyze the conversion of the substrate into the product, and we are following the reaction by measuring the amount of product that is present at successive time intervals. We plot the results as a graph and see the pattern shown in *Figure 7.16*. The shape of this curve tells us that initially the reaction proceeds at a linear rate, which we refer to as the **initial velocity** or $\mathbf{V_0}$. Gradually, however, the rate of the reaction decreases until at some point the graph forms a plateau because no additional product is being produced, indicating that the reaction rate is now zero.

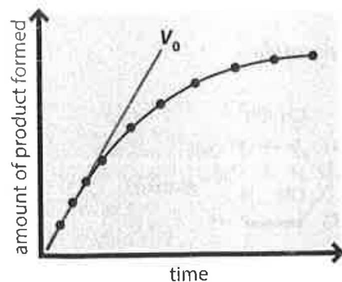


Figure 7.16 The time course for a typical enzyme-catalyzed reaction. The initial velocity (V_0) is shown as an extrapolation of the linear part of the reaction.

The simplest explanation for the leveling out of the curve shown in *Figure 7.16* is that product synthesis stops when all the substrate has been used up. This is not a complete explanation, but for the time being it is enough of an answer to suit our purpose. The important point is that the graph shows that the reaction rate gradually slows down as the amount of substrate decreases. In other words, the reaction rate is dependent on the substrate concentration.

The effect of substrate concentration reveals features of an enzyme's mode of action

The relationship between substrate concentration and reaction rate forms the basis of **enzyme kinetics**. This is an important subject because we can use the kinetics of an enzyme-catalyzed reaction to make deductions about the way that the enzyme works.

RESEARCH HIGHLIGHT

Box 7.5 Exploiting thermostable enzymes in biofuel production

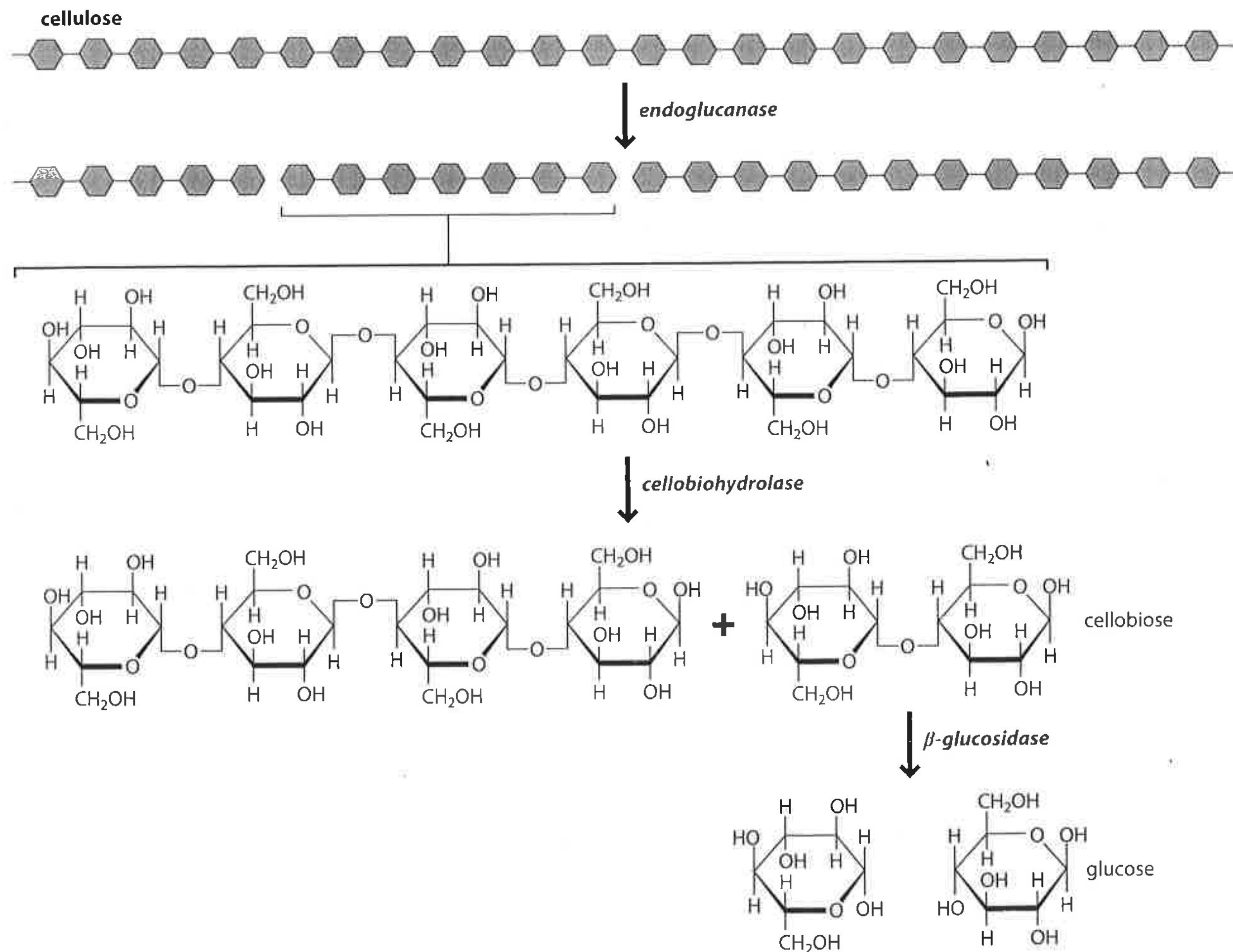
Purified enzymes have been used in industrial processes for decades. Examples include chymosin (also called rennin), a protease obtained from the stomach lining of calves, which is used in cheese-making, and invertase from yeast, which breaks sucrose into glucose and fructose and is used to make syrup for the production of toffee and other candy. In recent years, biotechnologists have started to explore the possible applications of thermostable enzymes in industrial processes that involve high temperatures, ones that would denature most proteins. An example is in the production of **biofuel** from plant material.

Biofuels are becoming increasing attractive in the search for greener types of energy, ones that are not derived from fossil fuels and which generate fewer pollutants. Various biofuels are being produced in different parts of the world, but the most widely used type is based on ethanol obtained by breakdown of carbohydrates from plant material. Production of this biofuel involves the initial conversion of the plant's cellulose into glucose, followed by the breakdown of the glucose into ethanol and carbon dioxide. We will study the latter pathway in more detail in *Section 8.2.2*.

Conversion of cellulose into glucose is achieved by adding an enzyme preparation called cellulase to the plant material. Cellulase is a mix of different enzymes, the most important being:

- An endoglucanase, which breaks internal β-glycosidic bonds in cellulose, breaking the polymer into smaller fragments.
- A cellobiohydrolase, which removes cellobiose units sequentially from the ends of the fragments created by endoglucanase treatment. Cellobiose is a disaccharide comprising two glucoses linked by a β(1→4) bond.
- β-glucosidase, which cleaves the β(1→4) bond, converting cellobiose into glucose.

These three enzymes therefore work together to release glucose from cellulose.



The cellulases currently used in biofuel production are obtained from fungi, and are not heat-resistant. They are therefore denatured at temperatures above 60°C. This complicates the industrial process because the plant material has to be heated to 75°C in order to release the cellulose content from other plant biopolymers such as lignin, which cannot be broken down into glucose. The conventional technology therefore requires two stages, each carried out in a different bioreactor. In the first

stage the plant material is heated to liberate its cellulose and, in the second, cellulase is added to the cooled extract to convert the cellulose to glucose. Having two stages lengthens the time needed to complete the process and, importantly, increases the overall cost.

A thermostable cellulase would therefore reduce the costs of this type of biofuel production by enabling both the release and breakdown of cellulose to be carried out as a single-stage process. Suitable thermostable enzymes do not appear to be common among thermophilic bacteria, but a few examples are known and these are being investigated as alternatives to the fungal enzymes. The main question is whether the costs of growing the thermophilic bacteria and extracting their enzymes will make their use uneconomic.

Another possibility is to use **protein engineering** to increase the heat stability of a fungal cellulase. Protein engineering involves making changes to the amino acid sequence of a protein, using techniques that we will study in *Box 19.2*. The intention would be to change the amino acid sequences of a set of fungal cellulose-degrading enzymes in order to make these enzymes more heat resistant. The problem with this approach is that we do not yet understand exactly why a thermostable enzyme is able to withstand high temperatures without being denatured. A variety of structural innovations have been identified in different thermostable enzymes that might explain their heat tolerance, but none of these features are present in all enzymes of this type. The innovations include a more compact conformation, with a relatively high percentage of the polypeptide folded into α-helices and β-sheets, rather than unfolded in loops and turns. Often the different secondary structural units are held together, and attached to one another, by a higher number of hydrogen bonds and van der Waals interactions than would be present in a non-thermostable protein. The surface features of a thermostable protein are also likely to be important, as these will dictate how the protein interacts with the surrounding water molecules, which in turn will influence the ease with which the protein is unfolded at higher temperatures. Even if the key features of a thermostable enzyme can be identified, it will be difficult to work out what changes should be made to the amino acid sequence of a non-thermostable enzyme in order to bring about these types of structural change.

Because it is difficult to predict what amino acid changes should be made to fungal cellulase enzymes, biotechnologists are exploring a different type of protein engineering called **directed evolution**. In this approach, random changes are made to the amino acid sequence of a protein, and the resulting variants then tested to see which ones have improved properties. For biofuel production, we would make random changes to one of the fungal cellulase enzymes, and then test each new variant to identify any that, by chance, displayed increased heat resistance. The increase might be small, but if a variant was then subjected to further rounds of random alteration, with the most heat-resistant versions carried forward at each stage, then eventually we might obtain a cellulase with sufficient thermostability to be used in a single-stage biofuel production process.

First, we need a consistent way of comparing the rate of a reaction at different substrate concentrations. The experiment we depicted in *Figure 7.16* indicates how we can do this. To compare reaction rates at different substrate concentrations we simply set up a series of experiments with identical amounts of enzyme but different amounts of substrate, and measure the V_0 for each of these substrate concentrations (*Fig. 7.17A*). Plotting these results on a graph will give a hyperbolic curve (*Fig. 7.17B*). This curve reveals two key parameters relating to the activity of the enzyme:

- The maximum velocity or $\boldsymbol{V_{max}}$ is achieved when the curve eventually reaches a plateau. This parameter indicates the maximum rate at which the enzyme can carry out the reaction.
- The $\boldsymbol{K_m}$ or **Michaelis constant** is the substrate concentration at which the reaction rate is half of the maximum value (i.e. $0.5 \times V_{max}$). This gives us a numerical value for K_m for any enzyme. But what does K_m tell us about an enzyme? The K_m is a measure of the stability of the enzyme–substrate complex, or to be more precise the 'affinity' of the enzyme for its substrate. This is a reciprocal relationship; a low K_m indicates high affinity and a high K_m indicates low affinity.

The precise relationship between the substrate concentration, V_{max} and K_m, was first worked out by Leonor Michaelis and Maud Menten in 1913. The **Michaelis–Menten equation** states that:

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

In this equation, the square brackets indicate 'concentration of', so [S] refers to the substrate concentration.

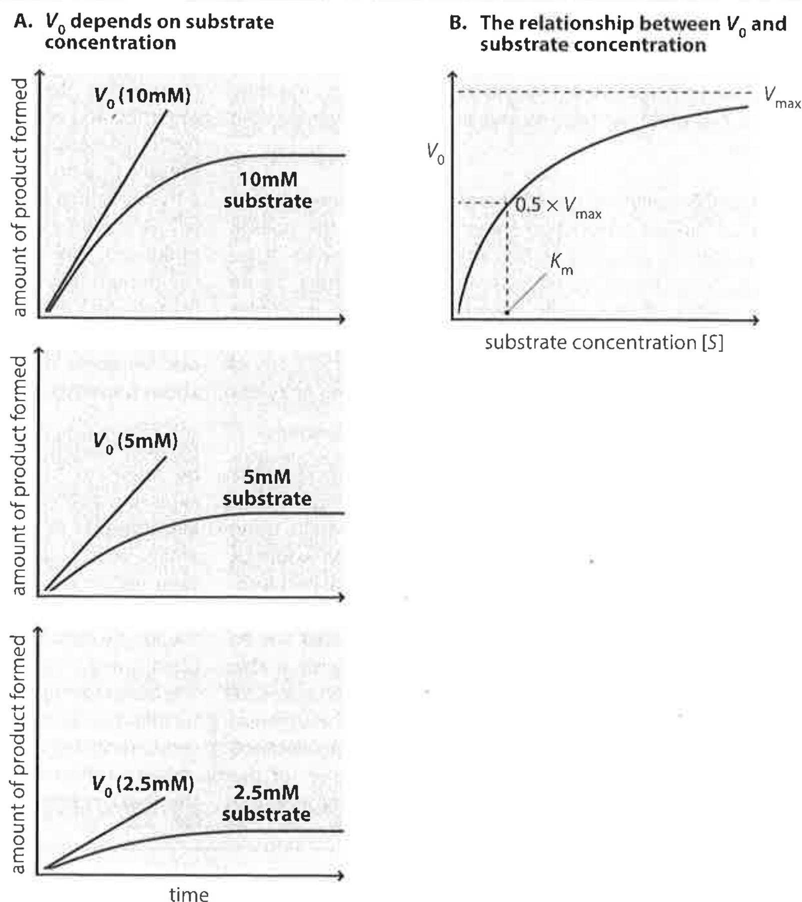


Figure 7.17 Comparing the rate of an enzyme-catalyzed reaction at different substrate concentrations. (A) Measuring the V_0 at different substrate concentrations (2.5 mM, 5 mM and 10 mM substrate). (B) Using the V_0 value to calculate the V_{max} and K_m for the enzyme.

How do we measure the V_{max} and K_m of an enzyme experimentally? The graph shown in *Figure 7.17B* does not allow us to do this because the curve does not quite reach the V_{max} – we had to "guesstimate" it from the way the curve was proceeding. As the numerical value of K_m is half V_{max} we had to "guesstimate" this value too. We could continue the experiment with more and more substrate, but a completely accurate measure of V_{max} would require an infinite substrate concentration, which of course is impossible to achieve. Fortunately, we can convert the curve shown in *Figure 7.17B* into a straight line by plotting the reciprocals of the initial velocity and the substrate concentration (*Fig. 7.18*). This is the **Lineweaver–Burk plot**. The advantage of a straight line plot is that you can extrapolate it as far as you like, in our case to the point where the line crosses the x axis, which enables the value of the K_m to be calculated. The intercept with the y axis gives us the V_{max}.

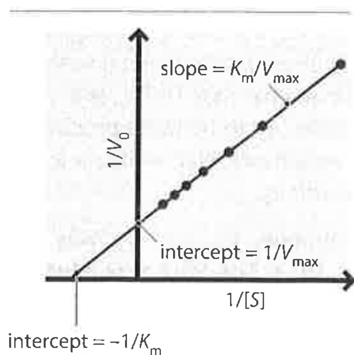


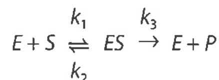
Figure 7.18 The Lineweaver–Burk plot.

7.2.3 Inhibitors and their effects on enzymes

To complete our study of the way that enzymes work, we must end this chapter by examining how enzymes are affected by **inhibitors**. An inhibitor is a compound that interferes with the activity of an enzyme, reducing its catalytic rate. There is a vast range of compounds that affect the activities of different enzymes in this way, but we can place all of these compounds into two broad groups, depending on whether or not their inhibitory action can be reversed. We will look first at **irreversible inhibitors**, whose effects are permanent.

Box 7.6 The Michaelis–Menten equation

The Michaelis–Menten equation is central to the study of enzymes and it is important that we understand how it is derived. The Michaelis–Menten equation is based on the following concept of enzyme catalysis.



$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P$$

In this scheme, the enzyme E combines with its substrate S to form an enzyme–substrate complex ES. The ES complex can dissociate again to form $E + S$, or can proceed to form E and the product P. The symbols k_1, k_2 and k_3 are **rate constants**, which describe the rates associated with each step of the process. We assume that there is no significant rate for the backward reaction of $E + P \rightarrow ES$.

According to this model, the concentration of the enzyme–substrate complex, which we represent by $[ES]$, remains approximately constant until nearly all the substrate is used. This means that the rate of synthesis of ES equals the rate of its consumption over most of the course of the reaction. In other words, $[ES]$ maintains a **steady state**.

We know that the initial velocity (V_0) at low substrate concentrations is directly proportional to the concentration of substrate, $[S]$, while at high substrate concentrations the velocity becomes independent of $[S]$, eventually reaching its maximum value, V_{max}. The Michaelis–Menten equation describes the hyperbolic curve obtained when (V_0) is plotted against $[S]$ (see *Fig. 7.17B*). The equation is:

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

In deriving the equation, Michaelis and Menten defined a new constant, K_m, the Michaelis constant:

$$K_m = \frac{k_2 + k_3}{k_1}$$

K_m is therefore equal to the rate of breakdown of ES ($k_2 + k_3$) divided by its rate of formation (k_1). This means that the K_m of an enzyme indicates the stability of the ES complex. However, for many enzymes k_2 is much greater than k_3. If this is the case then K_m becomes dependent on the relative values of k_1 and k_2, which are the rate constants for ES formation and dissociation, respectively. Under these circumstances, the K_m becomes a measure of the degree of affinity of an enzyme for its substrate:

- If an enzyme has a weak affinity for its substrate then k_2 (dissociation of ES into E and S) will be predominant over k_1 (association of E and S to form ES). The K_m value will therefore be high.
- Conversely, an enzyme with a strong affinity for its substrate will have a low K_m, because for this enzyme k_1 will be predominant over k_2.

Finally, we will examine what happens if we take the reciprocal of the Michaelis–Menten equation. This would give us:

$$\frac{1}{V_0} = \frac{K_m + [S]}{V_{max}[S]} = \frac{K_m}{V_{max}}\frac{1}{[S]} + \frac{1}{V_{max}}$$

This is the equation presented by Hans Lineweaver and Dean Burk in 1934. It tell us that a plot of $1/V_0$ against $1/[S]$ will give us a straight line. The slope of this line will equal K_m/V_{max}, the intercept with the y axis ($1/[S] = 0$) will indicate the value of $1/V_{max}$, and the intercept with the x-axis ($1/V_0 = 0$) will give $-1/K_m$. This graph is called the Lineweaver–Burk plot (see *Fig. 7.18*).

An irreversible inhibitor causes a permanent reduction in an enzyme's activity

Most irreversible inhibitors are compounds that alter the active site of an enzyme in such a way that the enzyme is no longer able to bind to its substrate. Often the inhibitor compound simply forms a covalent bond with one of the amino acids at the active site, blocking the active site so that the substrate cannot enter. This is usually a permanent, irreversible change, because the inhibitor can only be removed from the active site by cleaving the covalent bond that now attaches it to the enzyme's polypeptide chain. Those amino acids with hydroxyl (–OH) or sulfydryl (–SH) groups in their side-chains are often the targets of irreversible inhibitors, so enzymes with serine, threonine, tyrosine or cysteine at their active sites are particularly susceptible to this type of inhibition.

Diisopropyl fluorophosphate (DIFP) is an example of an irreversible inhibitor. DIFP reacts with many compounds that contain a hydroxyl group, including serine (*Fig. 7.19*). Attachment of DIFP to a serine side-chain at an active site is likely to block entry of the substrate, and will also prevent the serine side-chain from playing its role in the biochemical reaction catalyzed by the enzyme. The activity of the enzyme molecule to which DIFP is attached is therefore inhibited, totally and irreversibly. DIFP inhibits many proteases (enzymes that cleave peptide bonds and so break polypeptides into

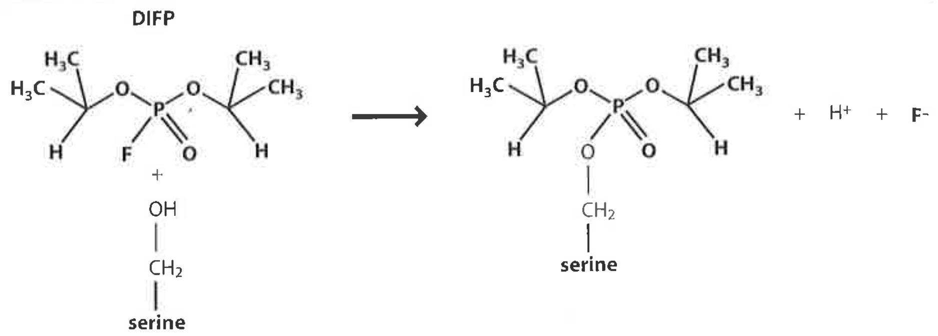


Figure 7.19 The reaction between diisopropyl fluorophosphate (DIFP) and serine.

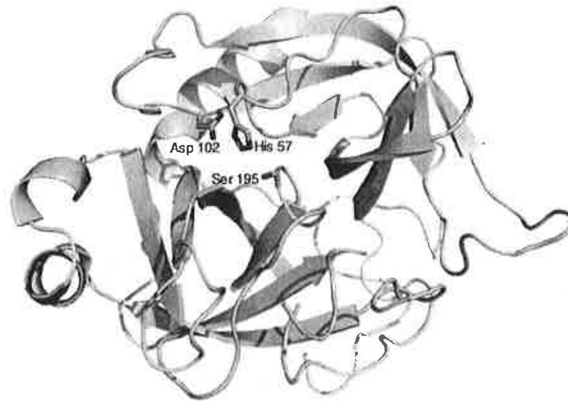
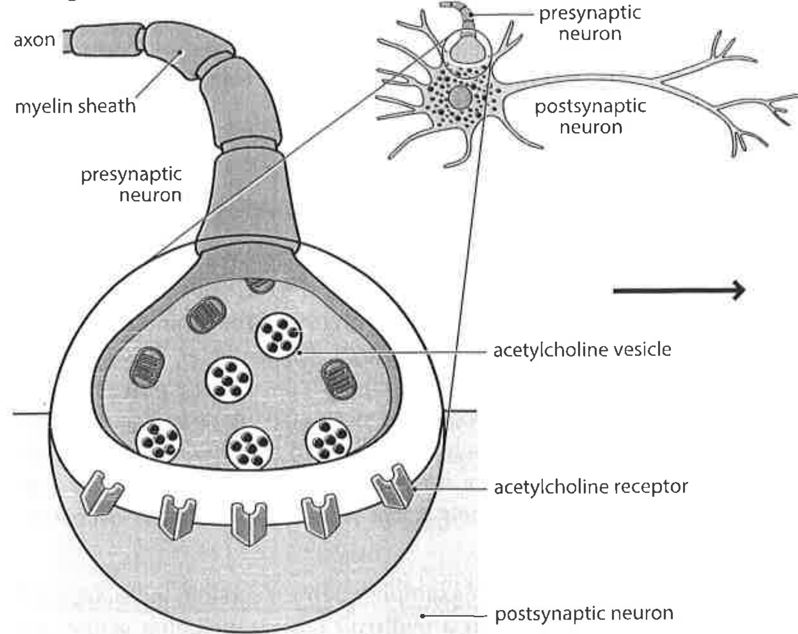


Figure 7.20 Chymotrypsin.
The chymotrypsin active site comprises a 'catalytic triad' of three amino acids: a serine, a histidine and an aspartic acid. Reaction between the serine and DIFP results in irreversible inhibition of chymotrypsin. Image reproduced from Wikimedia under a CC BY-SA 3.0 license.



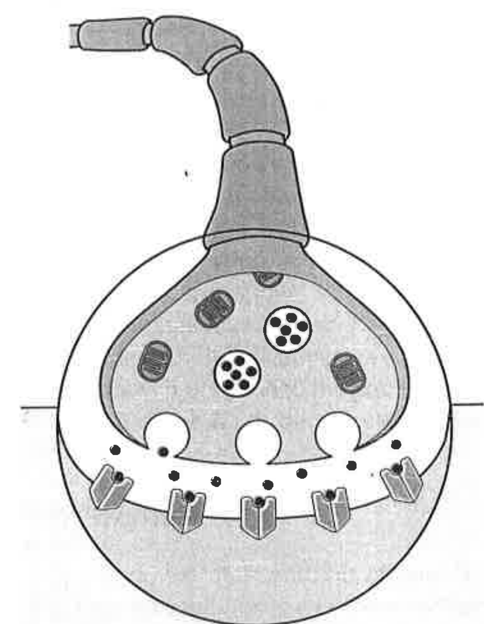


Figure 7.21 A cholinergic synapse.
Arrival of a nerve impulse stimulates release of the neurotransmitter acetylcholine from the presynaptic neuron. Binding of the acetylcholine molecules to receptor proteins on the surface of the postsynaptic neuron results in transmission of the impulse across the synapse. Immediately after transmission, the acetylcholine molecules are broken down by acetylcholinesterase, so the synapse returns to its resting state.

amino acids) because many of these enzymes have a serine at their active site. An example is **chymotrypsin** (*Fig. 7.20*), which is secreted by the pancreas and is involved in the digestive breakdown of proteins in the duodenum.

Diisopropyl fluorophosphate also inhibits the enzyme called **acetylcholinesterase,** which is present in nerve cells and degrades acetylcholine. Acetylcholine is a neurotransmitter, a compound that passes nerve impulses across the **synapses** between adjacent nerve cells or **neurons** (*Fig. 7.21*). Once a nerve impulse has passed, the neurotransmitter must be broken down, otherwise the nerve cells continue to signal to one another. Inhibition of acetylcholinesterase by DIFP therefore disrupts the nervous system by preventing acetylcholine being broken down in those synapses in which it acts as the neurotransmitter. DIFP is notorious as a component of some types of nerve gas.

Before we move on we must make a careful distinction between irreversible inhibition and the more general inactivation of enzyme activity caused by heat, pH and those chemicals that act as denaturants. Both types of event have the same result, this being a substantial or complete loss of enzyme activity. The difference is that heat, pH and chemical denaturants are non-specific in their action. They affect all enzymes because their mode of action is to disrupt the non-covalent chemical bonds that stabilize a protein's three-dimensional structure. An inhibitor, on the other hand, has a specific effect on a single enzyme, or group of similar enzymes, with which it is able to react because of the structure of the active site. The same compound will be unable to react with other enzymes, whose active sites have different structures, and hence will display no inhibitory effect with those other enzymes.

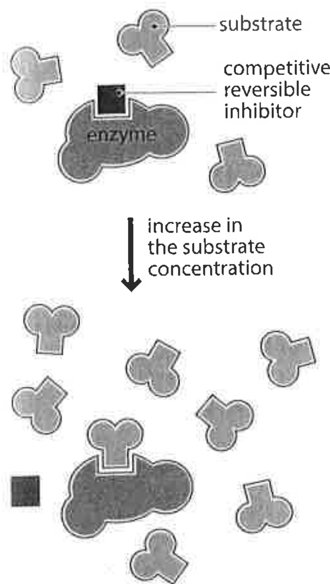
Reversible inhibition can be competitive or non-competitive

A **reversible inhibitor** is a compound whose inhibitory effects can be reversed, at least to some extent, by the presence of the substrate. Different types of reversible inhibition can be distinguished, the most common being described as **competitive reversible inhibition** and **non-competitive reversible inhibition.**

In competitive reversible inhibition, the inhibitor binds to the active site, but not in a permanent fashion as is the case with an irreversible inhibitor. Instead, the reversible inhibitor forms only relatively weak non-covalent attachments with the amino acids in the active site. Because the attachment is not via covalent bonds, it is possible for the enzyme substrate to displace the inhibitor. The substrate and inhibitor therefore *compete* for access to the active site. This means that the rate at which the enzymatic reaction proceeds depends on the relative amounts of substrate and inhibitor that are present. With a relatively large amount of inhibitor, the reaction rate will be slow, but this inhibition can be overcome by increasing the substrate concentration (*Fig. 7.22A*). This relationship has a specific effect on the kinetics of the reaction. The V_{max} of the reaction is unchanged, because the enzyme is still capable of achieving its maximum catalytic activity, if enough substrate is added to completely displace the inhibitor. However, the K_m is increased because the presence of the inhibitor decreases the affinity of the enzyme for its substrate. Whether or not a reversible inhibitor is acting in this competitive manner can therefore be determined by examining its effect on the Lineweaver–Burk plot for the enzyme-catalyzed reaction (*Fig. 7.22B*). Presence of the inhibitor will not change the intercept of the plot with the *y* axis, which corresponds to the V_{max}, but does move the position of the intercept with the *x* axis, which gives the K_m.

A **non-competitive reversible inhibitor** does not compete directly with the substrate, usually because it binds to some other part of the enzyme, away from the active site. This is called **allosteric inhibition** and the binding position for the inhibitor is called the **allosteric site**. Binding of the inhibitor to the allosteric site will still cause an alteration in the active site, and hence affect substrate binding, but this will be

A. the effect of substrate concentration



B. the effect on V_{max} and K_m

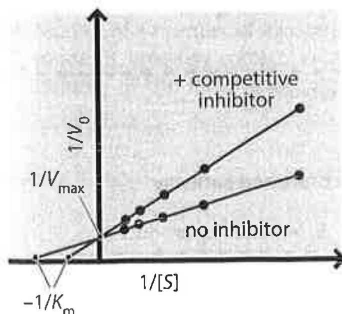
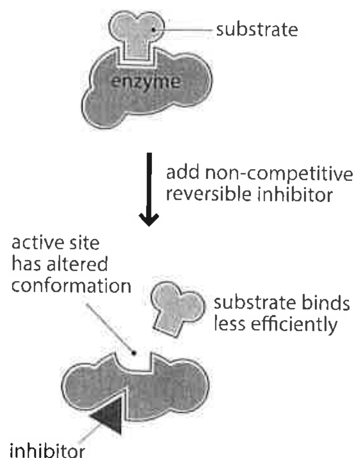


Figure 7.22 Competitive reversible inhibition.
(A) The substrate and inhibitor compete for access to the active site, so the inhibition can be overcome by increasing the substrate concentration. (B) The effect on the V_{max} and K_m of the reaction, as revealed by the Lineweaver–Burk plot.



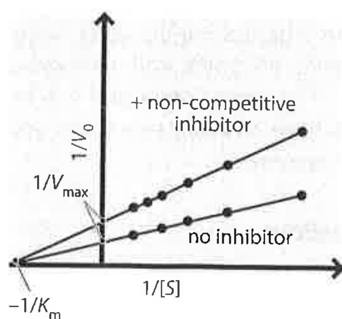


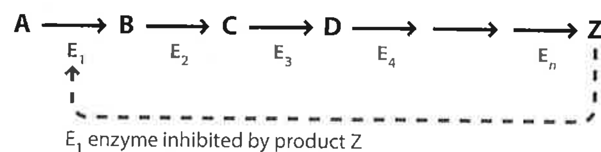
Figure 7.23 Non-competitive reversible inhibition.
(A) Binding of the inhibitor to some other part of the enzyme results in an alteration in the active site, and hence affects substrate binding. (B) The effect on the V_{max} and K_m of the reaction, as revealed by the Lineweaver–Burk plot.

by changing the enzyme's structure in some way rather than by entering the active site (*Fig. 7.23A*). Increasing the amount of substrate will increase the reaction rate, but there is no direct competition between substrate and inhibitor, because only the former is able to enter the active site. The kinetics of a non-competitively inhibited reaction are therefore different to the kinetics resulting from competitive inhibition. The V_{max} of the reaction is reduced, because addition of substrate does not displace the inhibitor, which means that there will always be some inhibitory effect however much substrate is added. Because substrate binding is not affected by the inhibitor, the K_m, which indicates the affinity of the enzyme for its substrate, is unaltered. Once again, the Lineweaver–Burk plot gives a diagnostic result from which this type of inhibition can be identified (*Fig. 7.23B*).

Allosteric inhibition is important in the regulation of metabolic pathways

Reversible inhibition is an important part of the natural control processes by which the metabolic pathways within a cell are regulated so that the correct amounts of the end products are synthesized. Many pathways are controlled by a type of **feedback regulation**, in which the end product controls the rate of its own synthesis by acting as a reversible inhibitor of one of the enzymes that catalyzes an early step in the pathway (*Fig. 7.24A*). Usually the structure of the product of a pathway is quite different from that of the substrate for the first step, so the product is unable to enter the active site of the first enzyme and cannot exert competitive reversible inhibition. This type of control is therefore almost always exerted by an allosteric effect.

Feedback regulation usually operates at the **commitment step** of a pathway. This is the first step in the pathway that produces an intermediate that is unique to that pathway, so synthesis of this intermediate only affects the pathway in question and has no effect on any other part of the metabolic network of the cell. In energy terms, this is the most economic strategy, because it means that no energy is wasted in synthesizing intermediates that are not needed.



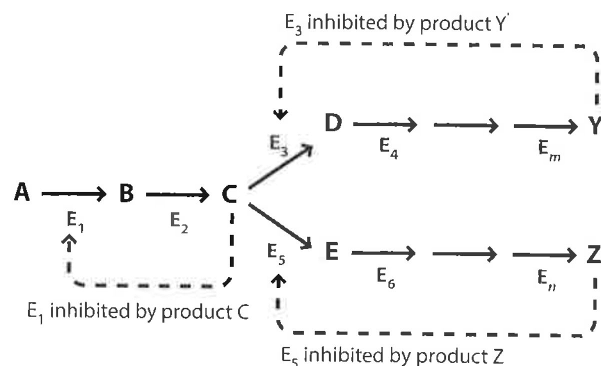


Figure 7.24 Feedback inhibition of a biochemical pathway.
(A) Regulation of a linear pathway. The end product Z controls the rate of its own synthesis by acting as a reversible inhibitor of enzyme E_1, which catalyzes an early step in the pathway. (B) Regulation of a branched pathway. End product Y controls the rate of its own synthesis by acting as a reversible inhibitor of enzyme E_3, and end-product Z regulates its synthesis by acting on enzyme E_5. If there are sufficient amounts of both Y and Z then intermediate C accumulates, which inhibits enzyme E_1, at the commitment step for the entire branched pathway.

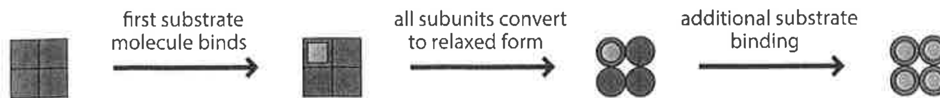
Box 7.7 Allosteric enzymes

Allosteric inhibition is one facet of a broader set of regulatory processes that are mediated by binding of an **effector** molecule to an enzyme. Many effectors have a negative impact on the activity of the target enzyme, as we have seen with non-competitive reversible inhibition, but other effectors have positive effects, stimulating enzyme activity when attached to an allosteric site. An **allosteric enzyme** is any enzyme whose activity is influenced by allosteric effectors, irrespective of whether the effect is to stimulate (activate) the enzyme (**positive allosteric control**) or inhibit it (**negative allosteric control**).

Positive allosteric control is used by some enzymes to enhance their sensitivity to small changes in the availability of a substrate. With these enzymes, the binding of a substrate molecule to one active site induces a conformational change that facilitates substrate binding at other active sites in the enzyme. Substrate binding is therefore **cooperative**. Two models have been proposed to explain this effect. Both assume that the allosteric enzyme is a multisubunit protein:

- The **concerted model** was first proposed by Jacques Monod, Jeffries Wyman and Jean-Pierre Changeux. In this model, each subunit of the enzyme can take up either of two conformations. One of these is a 'tensile' conformation which has low affinity for the substrate, whereas the other is a 'relaxed' conformation, which has higher substrate affinity. When there is no substrate, each subunit is in the tensile conformation. Binding of a substrate molecule to one of the subunits induces the immediate conversion of all the subunits to their relaxed conformations. Binding of the first substrate molecule therefore increases the affinity of the enzyme for other substrate molecules.
- The **sequential model**, first proposed by Daniel Koshland, also assumes that there are tensile and relaxed conformations. The difference is that in this model the binding of the first substrate molecule only influences the substrate affinity of neighboring subunits, rather than all the subunits of the enzyme.

concerted model



sequential model

tensile conformation, no substrate bound
tensile conformation, substrate bound
relaxed conformation, no substrate bound
relaxed conformation, substrate bound

Studies of a number of allosteric enzymes suggest that neither model is precisely correct, with most enzymes responding to binding of the initial substrate molecule in a manner that is intermediate between the predictions of the concerted and sequential processes.

Feedback regulation is particularly useful if a metabolic pathway contains branches, the initial substrate being converted into more than one end product. Allosteric inhibition can then switch off one branch of the pathway when that particular end product is present in adequate amounts, so that the substrate is directed entirely towards synthesis of the product of the second branch (*Fig. 7.24B*). If the end products of both branches of a pathway are present in sufficient amounts, then the intermediate immediately before the branchpoint accumulates. This intermediate might be able to inhibit an earlier commitment step so now the entire pathway is shut down. We will encounter several examples of feedback regulation when we examine individual metabolic pathways in the next few chapters.

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Self-assessment questions

Multiple choice questions

Only one answer is correct for each question. Answers can be found on the website: www.scionpublishing.com/biochemistry.

1. What was the first enzyme to be shown to be a protein?
- **(a)** Amylase
- **(b)** Diastase
- **(c)** Ribonuclease A
- **(d)** Urease

2. The active site of ribonuclease A contains two copies of which amino acid?
- **(a)** Glycine
- **(b)** Histidine
- **(c)** Isoleucine
- **(d)** Leucine

3. Which one of the following statements is **correct** with regard to tryptophan synthase?
- **(a)** An intermediate in the biochemical reaction is channeled between two subunits of the enzyme
- **(b)** It is a dimer of two identical subunits
- **(c)** It possesses an exonuclease activity
- **(d)** It uses chorismate as its substrate

4. What is an RNA enzyme called?
- **(a)** Ribosome
- **(b)** Ribozyme
- **(c)** Transfer RNA
- **(d)** This is a trick question, all enzymes are made of protein

5. Which is the metal ion cofactor in cytochrome oxidase?
- **(a)** Cu^{2+}
- **(b)** Fe^{2+}
- **(c)** Mg^{2+}
- **(d)** Zn^{2+}

6. Riboflavin (vitamin B_2) is the precursor of which organic cofactors?
- **(a)** Coenzyme A
- **(b)** FAD and FMN
- **(c)** NAD^+ and $NADP^+$
- **(d)** *S*-adenosyl methionine

7. What is the term used to describe the combination of an enzyme with its cofactor?
- **(a)** Apoenzyme
- **(b)** Holoenzyme
- **(c)** Multisubunit enzyme
- **(d)** Ribozyme

8. Which one of the following statements is **correct** with regard to redox reactions?
- **(a)** Both oxidation and reduction result in gain of electrons
- **(b)** Both oxidation and reduction result in loss of electrons
- **(c)** Oxidation is loss of electrons, reduction is gain
- **(d)** Reduction is loss of electrons, oxidation is gain

9. What are enzymes with identical functions from different organisms called?
- **(a)** Allosteric enzymes
- **(b)** Homologous enzymes
- **(c)** Isozymes
- **(d)** Paralogous enzymes

10. What is the term used to describe an enzymatic reaction that releases energy?
- **(a)** Endergonic
- **(b)** Energy coupled
- **(c)** Exergonic
- **(d)** Reversible

11. What term is used to denote the energy difference between the substrates of an enzymatic reaction and the transition state?
- **(a)** ΔG
- **(b)** $\Delta G‡$
- **(c)** $\Delta G'$
- **(d)** $\Delta G^{0'}$

12. Which one of the following statements is **incorrect**?
- **(a)** An enzyme changes the ΔG values for substrates and products
- **(b)** An enzyme increases the reaction rate
- **(c)** An enzyme reduces the free energy of the transition state
- **(d)** None of the above statements is incorrect

13. Which thermodynamic term is a measure of the degree of disorder of a system?
- **(a)** Chaos
- **(b)** Enthalpy
- **(c)** Entropy
- **(d)** Free energy

14. The lock and key and induced fit models refer to which aspect of enzyme behavior?
- **(a)** Cooperative substrate binding
- **(b)** Irreversible inhibition
- **(c)** Reduction of the free energy of the transition state
- **(d)** Specificity of substrate binding

15. Which one of the following statements is **incorrect** regarding thermostable enzymes?
- **(a)** They are able to withstand high temperatures without denaturing
- **(b)** They are obtained from thermophilic bacteria
- **(c)** They have a temperature optimum of 75–80°C
- **(d)** All of the above statements are incorrect

16. What is the term used to denote the substrate concentration at which the rate of an enzymatic reaction is half of the maximum value?
- **(a)** k_1
- **(b)** K_m
- **(c)** $[S]$
- **(d)** V_0

17. In the Lineweaver–Burk plot, what does the intercept with the x axis give?
- **(a)** K_m
- **(b)** $\frac{1}{V_{max}}$
- **(c)** $-\frac{1}{K_m}$
- **(d)** $\frac{1}{K_m}$

18. Diisopropyl fluorophosphate (DIFP) is an example of what type of enzyme inhibitor?
- **(a)** Allosteric
- **(b)** Competitive
- **(c)** Irreversible
- **(d)** Non-competitive

19. What is the name of the enzyme, inhibited by DIFP, that is involved in transmission of nerve impulses?
- **(a)** Acetylcholinesterase
- **(b)** Chymotrypsin
- **(c)** Neuraminidase
- **(d)** Synapsase

20. In which type of inhibition does V_{max} stay the same, but K_m is increased?
- **(a)** Competitive reversible
- **(b)** Irreversible
- **(c)** Non-competitive reversible
- **(d)** The scenario described never occurs

21. In which type of inhibition is V_{max} reduced, but K_m stays the same?
- **(a)** Competitive reversible
- **(b)** Irreversible
- **(c)** Non-competitive reversible
- **(d)** The scenario described never occurs

22. An allosteric site is the part of an enzyme that does what?
- **(a)** Binds an inhibitor or other effector molecule
- **(b)** Binds the substrate
- **(c)** Binds the product prior to its release by the enzyme
- **(d)** Channels an intermediate between two subunits of the enzyme

23. What is the name given to the first step in a metabolic pathway that produces an intermediate that is unique to that pathway?
- **(a)** Allosteric step
- **(b)** Commitment step
- **(c)** Concerted step
- **(d)** Cooperative step

24. The concerted and sequential models refer to which aspect of enzyme behavior?
- **(a)** Cooperative substrate binding
- **(b)** Irreversible inhibition
- **(c)** Reduction of the free energy of the transition state
- **(d)** Specificity of substrate binding

Short answer questions

These questions do not require additional reading.

1. Compare the structures of ribonuclease A, DNA polymerase I and tryptophan synthase, in each case explaining how the structure relates to the enzymatic activity.
2. What is unusual about the structure of ribonuclease P?
3. Giving as many examples as possible, describe the main categories of enzyme cofactor.
4. Outline the EC enzyme classification system.
5. Explain what is meant by the term 'free energy' and describe the free energy differences between the substrates and products of exergonic and endergonic biochemical reactions.
6. Why is the free energy of the transition state central to any discussion of enzyme-catalyzed reactions?
7. How does substrate concentration affect the rate of an enzyme-catalyzed reaction?
8. Describe how the Lineweaver–Burk plot is derived from the Michaelis–Menten equation, and draw examples of the Lineweaver–Burk plots expected in the presence or absence of (a) a competitive reversible inhibitor, and (b) a non-competitive reversible inhibitor.
9. Explain why diisopropyl fluorophosphate disrupts the transmission of nerve impulses.
10. Define the term 'allosteric inhibition' and describe why allosteric inhibition is important in the control of metabolic pathways.

Self-study questions

These questions will require calculation, additional reading and/or internet research.

1. The existence of ribozymes is looked upon as evidence that RNA evolved before proteins and therefore at one time, during the earliest stages of evolution, all enzymes were made of RNA. Assuming that this hypothesis is correct, explain why some ribozymes persist to the present day.
2. Identify the EC numbers for (a) ribonuclease A, (b) DNA polymerase I, and (c) tryptophan synthase.
3. The rate constants for the reactions catalyzed by two different enzymes are given below. Calculate the K_m for each enzyme and identify which one has the strongest affinity for its substrate.

	k_1	k_2	k_3
Enzyme A	$5 \times 10^6\ M^{-1}\ sec^{-1}$	$2 \times 10^3\ sec^{-1}$	$5 \times 10^2\ sec^{-1}$
Enzyme B	$2 \times 10^7\ M^{-1}\ sec^{-1}$	$5 \times 10^3\ sec^{-1}$	$2 \times 10^2\ sec^{-1}$

4. Explain why k_1, the rate constant for formation of the enzyme–substrate complex, is expressed as $M^{-1}\ sec^{-1}$, whereas k_2 and k_3, which are the rate constants for breakdown of the enzyme–substrate complex, are expressed as sec^{-1}.

5. The initial velocity was measured for an enzyme-catalyzed reaction at different substrate concentrations, with and without the presence of two different inhibitors. Using the data in the table opposite, determine V_{max} and K_m values for the enzyme with and without the inhibitors, and identify the type of inhibition that is occurring in each case.

Substrate concentration	Initial velocity (μM sec⁻¹)		
(mM)	no inhibitor	inhibitor 1	inhibitor 2
1.0	2.0	1.1	1.0
2.0	3.3	2.0	1.7
5.0	5.9	4.0	3.0
10.0	7.7	5.9	4.0
20.0	10.0	8.3	5.0