Metal Ions in Enzymes Catalyze

Introduction

More than a quarter of all known enzymes require the presence of metal atoms for full catalytic activity.

It usually exists as cations and often has more than one oxidation state, as with ferrous (Fe^{2+}) and ferric (Fe^{3+}). This positive change can stabilize transition states by electrostatic interactions, giving one mechanism for metal catalysis.

Therefore,

Metal ions can be involved in enzyme catalysis in a variety of ways:

1- Metal ions may accept or donate electrons to activate electrophiles or nucleophiles.
2- Metal ions may mask nucleophiles to prevent unwanted side reactions.
3- Metal ions may hold reacting groups in the required three-dimensional orientation.

Many enzymes contain one or more metal ions, usually held by coordinate covalent bonds from amino acid side chains but sometimes bound by a prosthetic group like heme. Such enzymes are called **metalloenzymes.**

Metal with **metalloenzymes is** tightly bound and retained by the enzyme through purification, while **metal with metal-activated enzymes,** the binding is less tight and purified. Ternary complexes formed between an enzyme (E), metal ion (M), and substrate (S) may be as follows:

1- enzyme bridge complexes **(M-E-S)**,
2- substrate bridge complexes **(E-S-M)**, or
3- metal bridge complexes. $(E-M-S \text{ or } E\begin{smallmatrix}M\\|\\S\end{smallmatrix})$

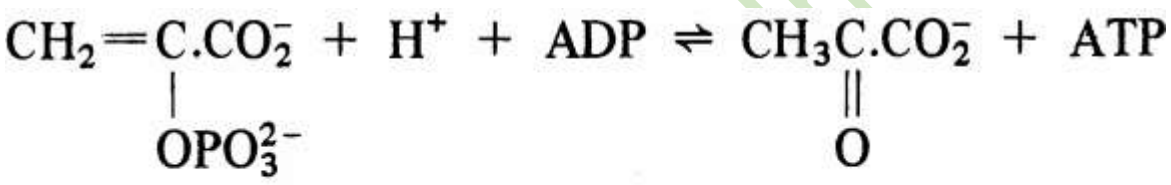
4- Metalloenzymes cannot form substrate bridge complexes because the purified enzyme exists as E-M.

1-Activation of enzymes by alkali metal cations (sodium(Na^+)and potassium(K^+).

Alkali metal cations **bind weakly** to form complexes with enzymes (sodium (Na^+) and potassium (K^+).

Potassium ion (K^+) is the most abundant intracellular cation. It is known to activate many enzymes, particularly those catalyzing phosphoryl transfer or elimination reactions. K^+ appears to **bind to negatively charged groups** on an inactive form of the enzyme and thus causes a change in conformation to a more active form. However, in some cases, **K^+** may also aid substrate binding.

For example, muscle **pyruvate kinase,** a tetrameric enzyme that catalyzes the reaction:



phosphoenolpyruvate (PEP) pyruvate

Pyruvate kinase needs alkali metal cations (K^+), and Mn 2 $^+$ (or $Mg2^+$), alkali metal cations, and **Mn^{2+} bind in the active site region**. The carboxyl group of PEP binds to the enzyme-bound **K^+**. Thus, a conformational change takes place, facilitating the progress of the reaction via an **E-Mn^{2+}- PEP** complex.

2-Activation of enzymes by alkaline earth metal cations (Ca^{2+} and Mg^{2+})

Oxygen atoms are often involved in the bonds of alkali metal and alkaline earth. The cations bonds of alkaline earth metal oxides are stronger than alkali metal oxides. Cations, Ca^{2+} and Mg^{2+} can form six coordinate bonds to produce octahedral complexes.

Mg^{2+} is accumulated by cells in exchange for transport of Ca^{2+} in the opposite direction. As might be expected, therefore, the enzymes requiring Ca^{2+} for activation are mainly be extracellula example., the **salivary and pancreatic *α*-amylases**: the Ca^{2+} appears to play a role in maintaining the structure required for catalytic activity.

In contrast, a variety of intracellular enzymes require Mg^{2+} for activity, and in most cases, this requirement can be replaced in vitro by one for Mn^{2+}. Mn^{2+} is paramagnetic, which helps the system to be more easily investigated. It has been shown that all possible types of ternary bridge complexes involving divalent cations can exist. Most kinases form **E-S-M** complexes, where S (substrate) is the reacting nucleotide.

Example: muscle creatine kinase, the reaction catalyzed:

$$\text{creatine} + \text{MgATP} \rightleftharpoons \text{MgADP} + \text{phosphocreatine} + H^+$$

The true substrate is **Mg-ATP, and the reaction proceeds via the formation of the complex**.

$$E \begin{cases} \text{ATP}-Mg^{2+} \\ \text{creatine} \end{cases}$$

The divalent cation binds to the α- and β-phosphates of the nucleotide but not to the terminal (γ) phosphate transferred to creatine. Therefore, the cation helps in the orientation of the complex and may also assist in breaking the pyrophosphate bond by withdrawing electrons from the β-phosphate.

3- Activation of enzymes by transition metal cations (Cu, Zn, Mo, Fe and Co cations).

Transition metal ions such as **Cu, Zn, Mo, Fe and Co** bind to enzymes much more strongly than metals of alkali and alkaline earth cations and usually form **metalloenzymes.**

Transition metal cations are found in only trace amounts in living organisms, for more significant amounts can be cause toxic. The trace metals Mo and Fe are found in **nitric-oxide reductase,** also, Fe is a component of hemoglobin,

Another trace metal, Co, is found in vitamin B_{12}.

Example: In a little more detail, we will now consider an example of a Cu- and a Zn-metalloenzyme.

1- Superoxide dismutase:

Superoxide dismutase is a copper-metalloenzyme which catalyzes the removal of the highly reactive O_2 produced. The superoxide dismutase reaction is as follows:

$$2O_2^- + 2H^+ \rightleftharpoons H_2O_2 + O_2$$

Bovine erythrocyte **superoxide dismutase is a dimeric protein** containing two. Cu^{2+} ions and two Zn^{2+} ions.

The Zn^{2+} ions appear to have a structural rather than a catalytic role, while the Cu^{2+} ions are involved in the reaction sequence:

$$E - Cu^{2+} + O_2^- \rightarrow E - Cu^+ + O_2$$

$$E - Cu^+ + O_2^- \xrightarrow{+2H^+} E - Cu^{2+} + H_2O_2$$

$$2O_2^- + 2H^+ \rightleftharpoons H_2O_2 + O_2$$

2-Carboxypeptidase-A:

Carboxypeptidase-A zinc metalloenzyme In contrast to the **Superoxide dismutase, the zinc ion** in **carboxypeptidase-A** has a catalytic role in the reaction catalyzed by **Carboxypeptidase A**. Carboxypeptidase-A from the bovine pancreas is a monomeric enzyme containing one zinc atom.

carboxypeptidase-A show that: The carboxypeptidase-A active site contains the zn^{2+} ion attached to histidine-69, glutamate-72, histidine-196, and H2O, as well as a channel for the polypeptide substrate and a hydrophobic region for binding the side chain of the C-terminal amino acid. The terminal carboxyl group of the substrate forms an electrostatic interaction with arginine-145; (Fig. 11.39).

The mechanism of the **carboxypeptidase A** included:

1- The zinc ion (orange circle) binds a water molecule (blue).

2- zinc ion serves as **an electrostatic catalyst** to promote the hydrolysis of **the C-terminal amino acid** from **a peptide substrate (green).**

3- zinc ion **stabilizes the negative charge on oxygen** in the tetrahedral transition state.

Enzyme **active site residues** are indicated by **black coloring,** and the dashed red arrow indicates the bond cleaved**;** Figure 11.39

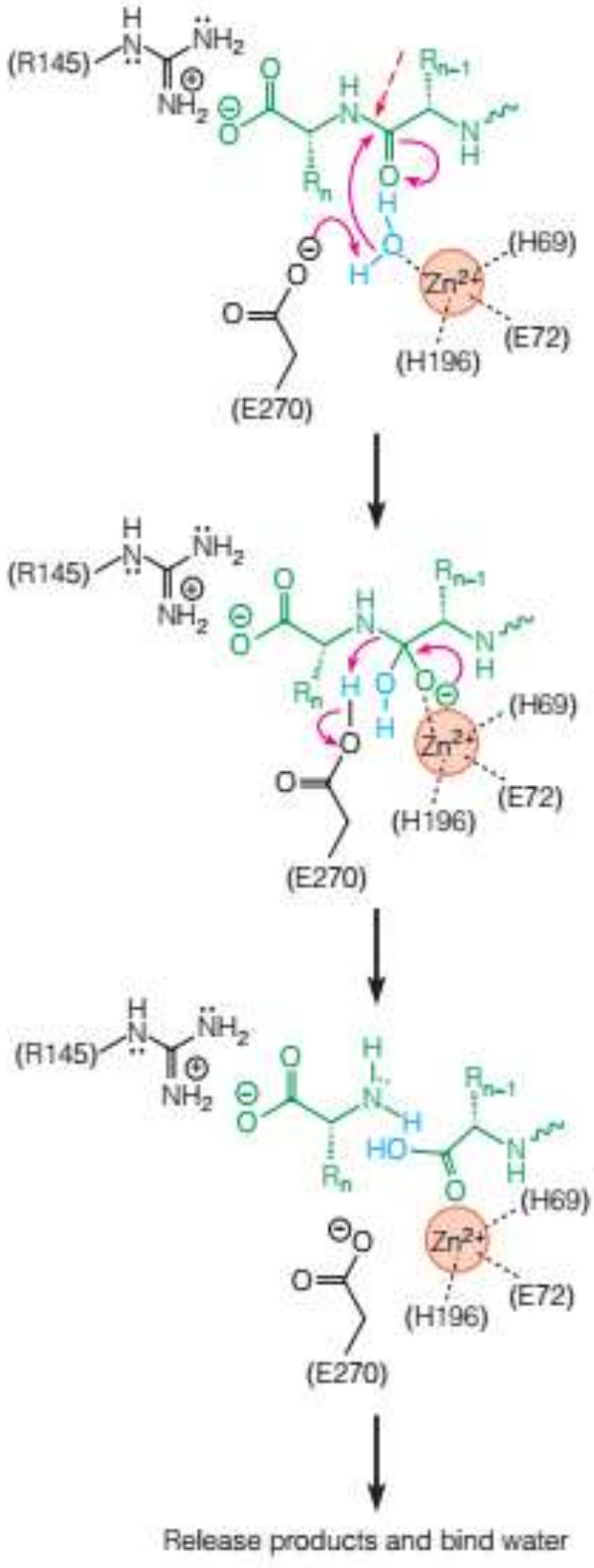


FIGURE 11.39 The mechanism of the **protease carboxypeptidase A**.

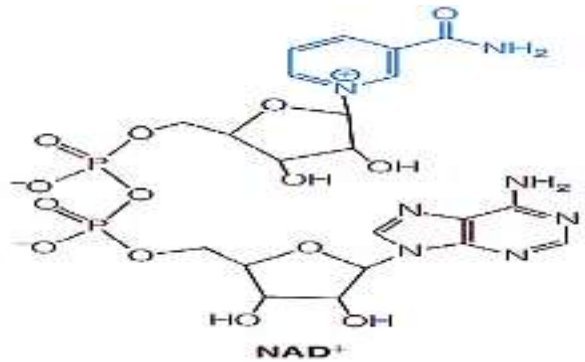
Vitamins in the Enzyme-Catalyzed

- Coenzymes are organic compounds required by many enzymes for catalytic activity; They are often vitamins or derivatives of vitamins. Sometimes, they can act as catalysts in the absence of enzymes but not so effectively as in conjunction with an enzyme.
- As with metal enzyme links, there is a range of strengths for co-enzyme and coenzyme.
- The point of distinction between tightly bound cofactor (prosthetic group) and loosely bound cofactor: Coenzymes, which are prosthetic groups, form an integral part of the active site of an enzyme and undergo no net change as a result of acting as a catalyst; loosely bound coenzymes can be regarded as **co-substrates** since they often bind to the enzyme-protein together with the other substrates at the start of a reaction and are released in an altered form at the end.
- They are regarded as coenzymes since they usually bind to the enzyme Before the other substrates are bound, they participate in many reactions and may be reconverted to their original form by the enzymes present within the cells.

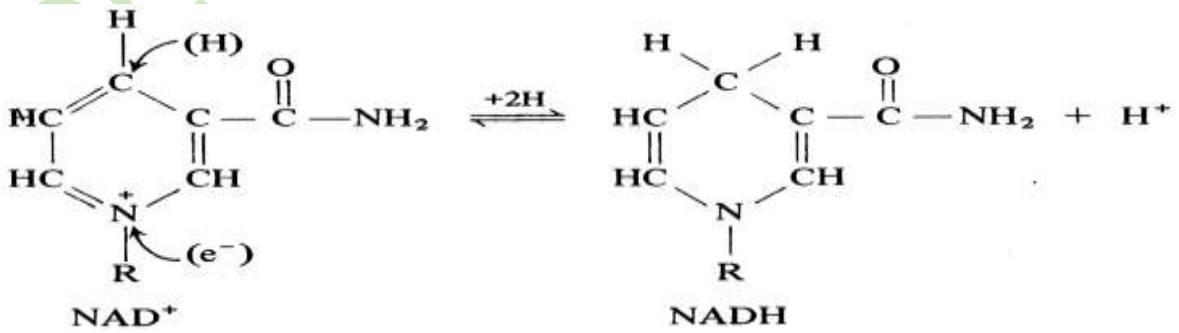
***Some** important coenzymes are discussed below.*

1- Nicotinamide nucleotides (NAD^+ and $NADP^+$)

Nicotinamide nucleotides (NAD^+ and $NADP^+$) are derived from the vitamin **niacin,** which is nicotinamide or nicotinic acid. The structure of nicotinamide adenine dinucleotide (NAD^+) in its oxidized and reduced forms is given below: **Nicotinamide adenine dinucleotide (NAD^+)** is derived from the vitamin **niacin (VIT. B_3).**



It can be seen that the reduction of **NAD^+** to **NADH** requires two reducing equivalents per molecule: one electron (e-) and one hydrogen atom (H=H+ +e-) added to the pyridine ring of nicotinamide as shown below. The pyridine ring is conjugated, so the positive charge may be delocalized, making several points vulnerable to nucleophilic attack. However, the exact mechanism of the reaction is not known.

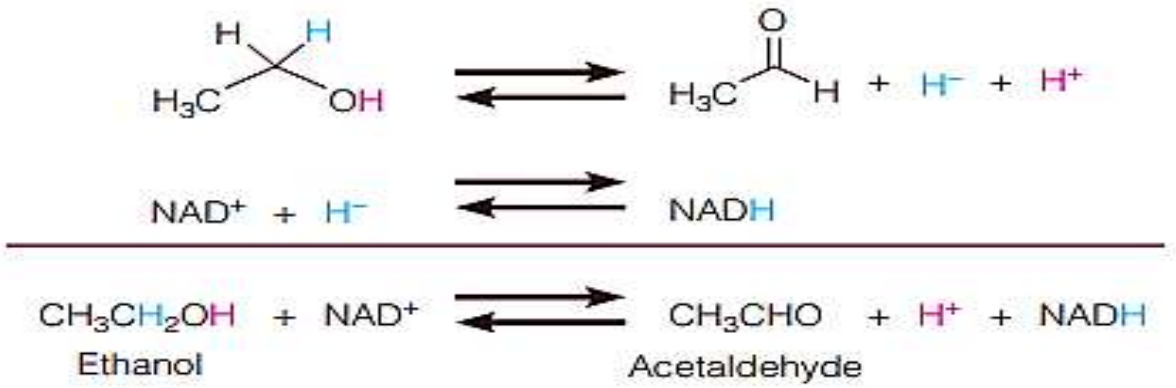


Where: R represents the remainder of the molecule.

Nicotinamide adenine dinucleotide phosphate **($NADP^+$)** is identical to **NAD^+,** except that in the 2'-position of the D-ribose unit attached to adenine is phosphorylated. This does not affect the oxidation/reduction properties but results in NAD^+ and $NADP^+$ acting as coenzymes for different enzymes: enzymes utilizing NAD+ usually have a catabolic function, the NADH produced being an energy source for the cell; anabolic enzymes, in contrast, frequently involve NADPH as a coenzyme.

NAD^+ and **$NADP^+$** act as coenzymes for oxidation/reduction reactions; they are loosely bound and leave the enzyme in a changed form at the end of the reaction.

A typical reaction in which NAD^+ acts as an oxidizing agent is the conversion of alcohols to aldehydes or ketones (for example, by the alcohol dehydrogenase of the liver):



- The C-linked H, not the O-linked H, is transferred to NAD^+, as can be demonstrated by studies using deuterated compounds.
- Sometimes, it isn't easy to distinguish between a true cofactor and the second substrate in a reaction.
- The dehydrogenase enzymes, such as alcohol dehydrogenase, each have a strong binding site for the oxidized form of the cofactor, NAD^+.
- After oxidation of the substrate, the reduced form, NADH, leaves the enzyme and is reoxidised by other electron-acceptor systems in the cells.
- Yet NAD^+ and NADH differ from most substrates in that they are continually recycled in the cell and used repeatedly. Because of this behavior, we consider their cofactors.

An example of NAD^+ behaving as a cofactor is the reaction involving **UDP-galactose 4-epimerase** shown in **Figure 11.38.**

This enzyme facilitates the synthesis of complex polysaccharides by interconverting **UDP-glucose** and **UDP-galactose** (see Chapters 9 and 13) in the following mechanism:

1- **Step 1: UDP-galactose** is bound to the enzyme, which carries the coenzyme NAD^+.

2- **Step 2**: Hydride is transferred to NAD^+ from C4 of the galactose ring to produce the carbonyl intermediate.

3- **Step 3:** Then, it (Hydride) is transferred back to C4 to give the opposite stereochemistry.

4- **Step 4:** The product, **UDP-glucose**, is then released.

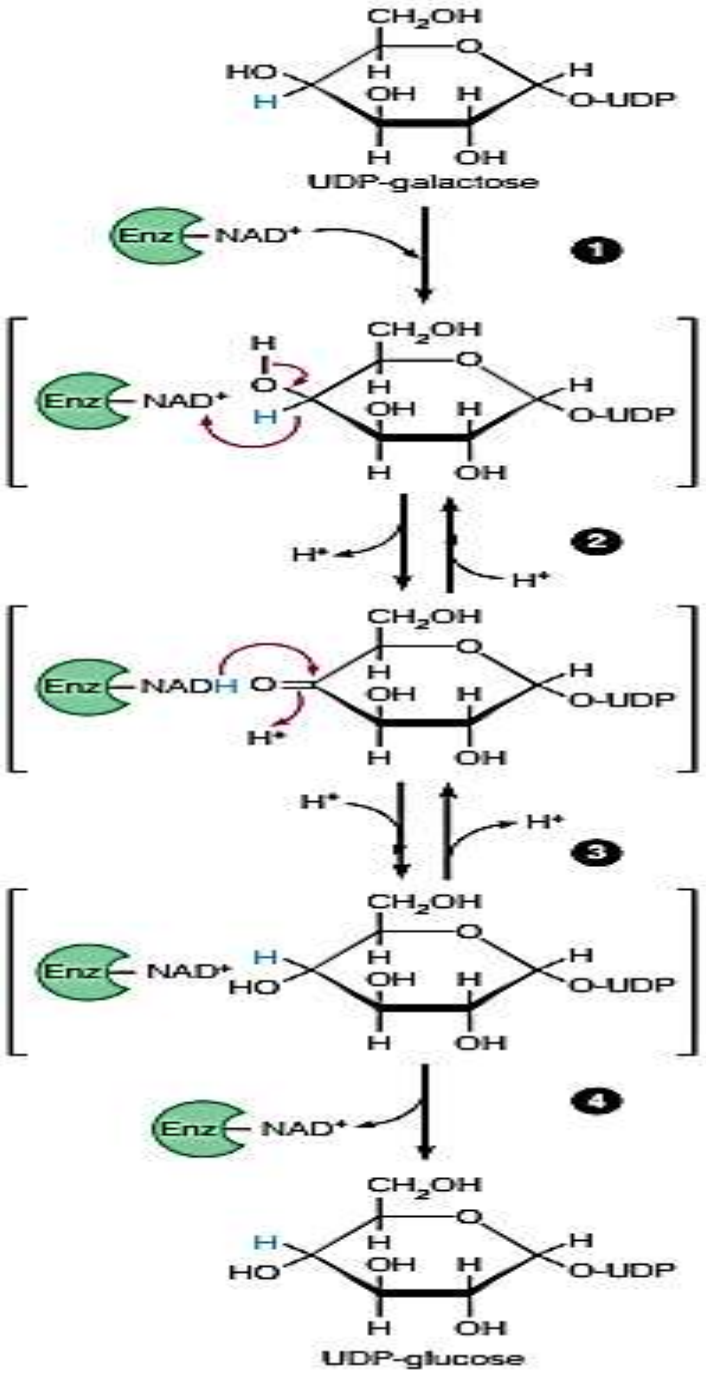
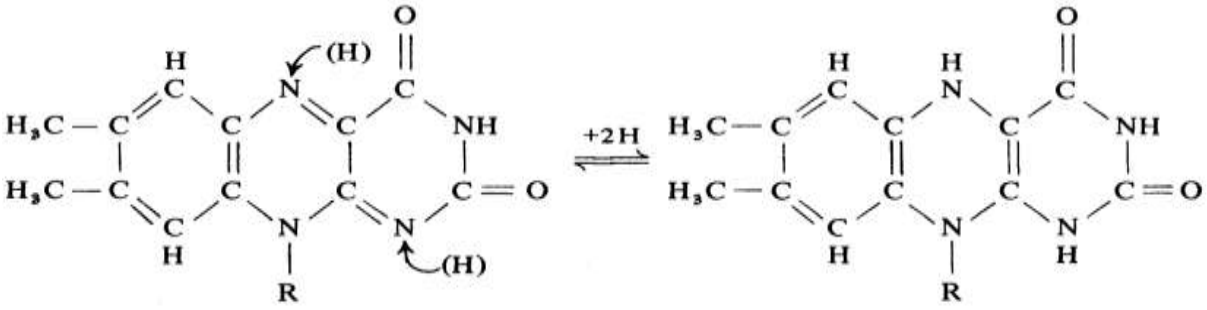


FIGURE 11.38 Proposed mechanism for **UDP-galactose epimerase**.

2- Flavin nucleotides (FMN and FAD)

Flavin nucleotides (FMN and FAD) are derived from riboflavin, vitamin B_2;

Like the nicotinamide nucleotides (NAD, NADP), Flavin nucleotides function in oxidation/reduction reactions, Where the reducing equivalents are carried by the fused three-ringed system of flavin as shown below:



FMN or FAD FMNH$_2$ or FADH$_2$

Where: R represents the remainder of the molecule.

In contrast to NAD^+ and $NADP^+$, FMN and FAD are prosthetic groups and It cannot be separated from the protein (enzyme) without denaturing it: the protein-flavin nucleotide complex is called a **flavoprotein.**

Because the flavin nucleotide does not exist independently, reactions catalyzed by flavoproteins usually involve the transfer of reducing equivalents from a donor via the flavin to some specific external acceptor.

For example, **glucose oxidase**, which catalyzes the reaction

$$\text{D-glucose} + O_2 \rightleftharpoons \text{D-glucono-}\delta\text{-lactone} + H_2O_2$$

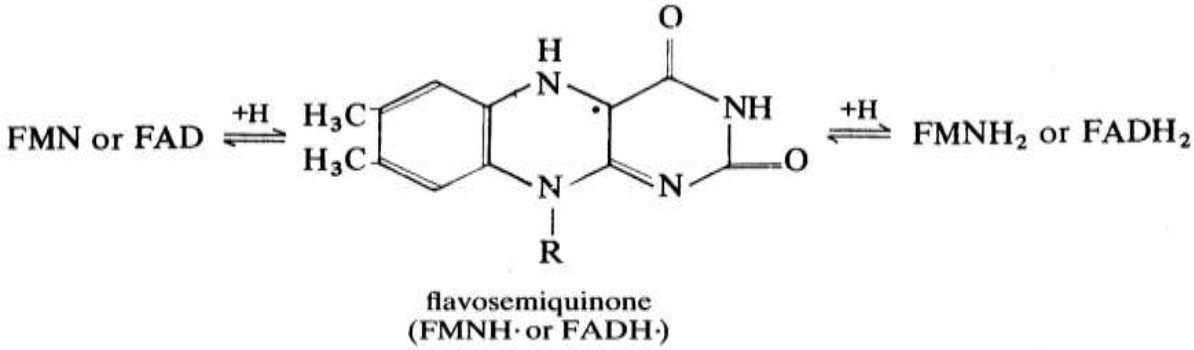
Utilizes FAD as a prosthetic group and O2 as a hydrogen acceptor. That this reaction follows in a two-stage reaction:

$$\text{E–FAD} + \text{D-glucose} \rightleftharpoons \text{E–FADH}_2 + \text{D-glucono-}\delta\text{-lactone}$$

$$\text{E–FADH}_2 + O_2 \rightleftharpoons \text{E–FAD} + H_2O_2$$

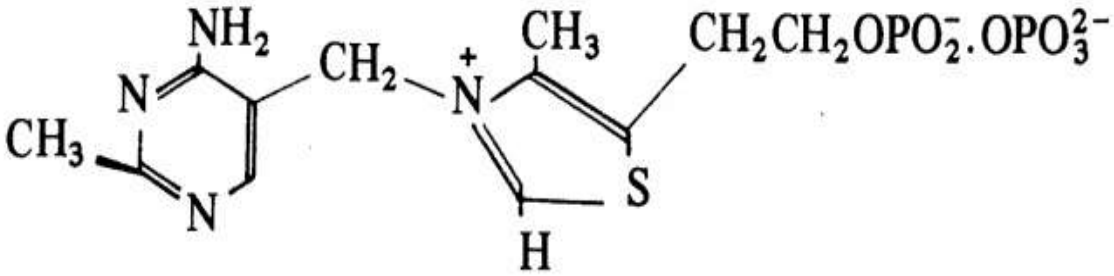
$$\text{D-glucose} + O_2 \rightleftharpoons \text{D-glucono-}\delta\text{-lactone} + H_2O_2$$

With some flavoproteins, the reduction of the flavin has been shown to be a two-step process, involving an unstable free radical **semiquinone** as intermediate:

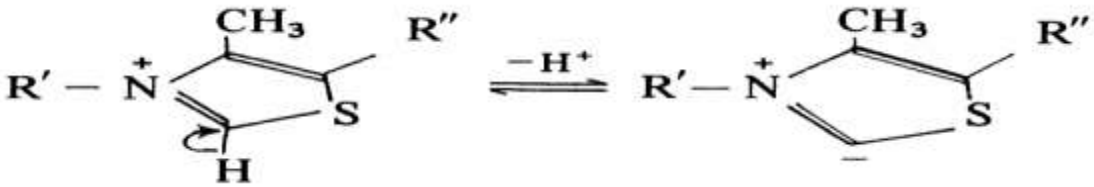


3- Thiamine pyrophosphate (TPP), vitamin B_1

Thiamine pyrophosphate is derived from vitamin B_1 , thiamine, and has the structure:



The **thiazole ring** can lose a proton to produce a negatively charged carbon atom:



This is a potent nucleophile and can participate in covalent catalysis, particularly with α-keto (oxo) acid decarboxylase, α-keto acid oxidase, transketolase and phosphoketolase enzymes.

Table 11.8 lists many important enzyme cofactors, their related vitamins, and the kinds of reactions they are associated with.

TABLE 11.8 Some important enzyme cofactors and related vitamins

vitamin	Cofactor	Reactions involving these cofactors
Thiamine (vitamin B_1)	Thiamine pyrophosphate	Activation and transfer of aldehydes
Riboflavin (vitamin B_2)	Flavin mononucleotide; flavin adenine dinucleotide	Oxidation-reduction
Niacin (vitamin B_3)	Nicotinamide adenine dinucleotide (NAD); nicotinamide adenine dinucleotide phosphate (NADP)	Oxidation-reduction
Pantothenic acid (vitamin B_5)	Coenzyme A	Acyl group activation and transfer
Pyridoxine (vitamin B_6)	Pyridoxal phosphate	Various reactions involving amino acid activation
Biotin (vitamin B_7)	Biotin	CO_2activation and transfer
Lipoic acid (vitamin B_1)	Lipoamide Acyl	Acyl group activation; oxidation-reduction
Folic acid (vitamin B_1)	Tetrahydrofolate	Activation and transfer of single carbon functional groups
Cobalamin (vitamin B_{12})	Adenosyl cobalamin; methylcobalamin	Isomerisations and methyl group transfers

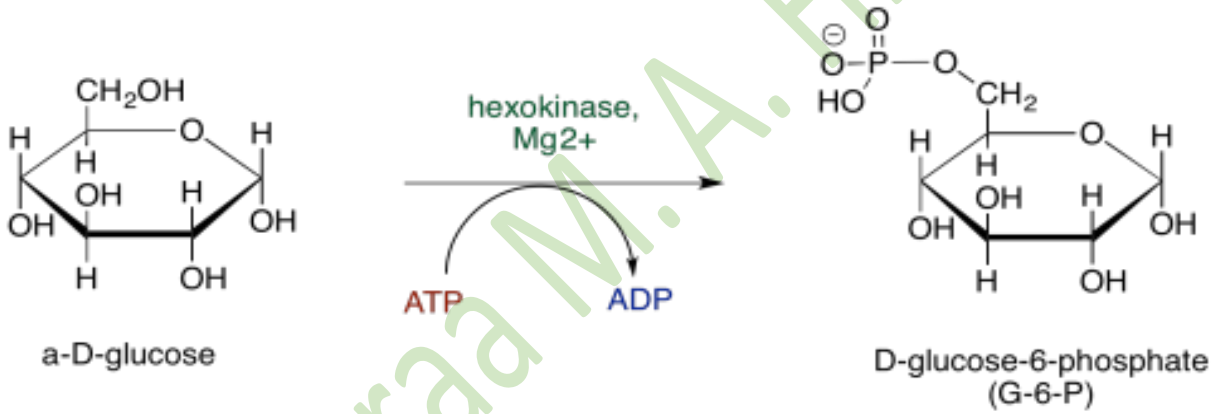
The Regulation of Enzyme Activity:

Regulation of enzyme activity is essential for the metabolism

1- Substrate-Level Control

The substrates and products of each enzyme-catalyzed reaction interact directly with the enzyme to regulate it.
kinetics study shows that increased substrate concentrations accelerate reactions until enzyme saturation.

As an example, consider the first step in glycolysis (see Chapter 13)—the phosphorylation of glucose to yield glucose-6-phosphate (G6P):



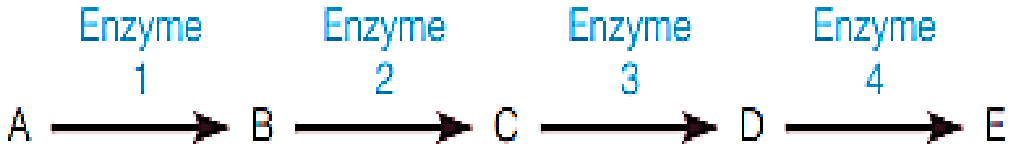
Hexokinase catalyzes this reaction and is inhibited by its product, glucose-6-phosphate (G6P). If subsequent steps in glycolysis are blocked for any reason, G6P will accumulate and bind to hexokinase. This results in the inhibition of hexokinase and slows down further production of G6P from glucose.

In many cases, the reaction product binds the active site for the enzyme and acts as a competitive inhibitor. Hexokinase is an interesting example because the product (G6P) can act as a competitive inhibitor (by binding to the active site for the enzyme) and an uncompetitive inhibitor (by binding at another place for the enzyme).

2- Feedback Control

Feedback control is important in the efficient regulation of complex metabolic pathways.

The metabolic pathways resemble assembly lines. The simplest metabolic assembly line looks like this:

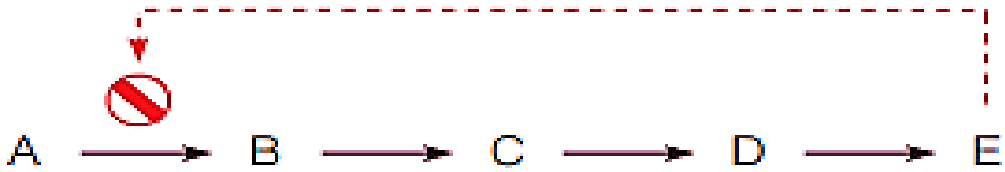


Where **A** represents the initial reactant or raw material (crud), **B**, **C**, and **D** represent intermediate products, and **E** represents the final product.

This pathway's end product **(E)** may be employed in another path, and **A** may be used in other processes. Consider that E's utilization slows. As previously pathway, E would accumulate, and A would be consumed. But this process is inefficient.

A more efficient process would solve this problem by closely monitoring the concentration of E and, when E accumulated, sending a signal back to prevent its production. The cell can control the generation of the final product through activation (⬆) or inhibition (⛔) of a critical step in the pathway.

It would be most efficient to slow the first step—converting A to B ($A \rightarrow B$) So, the "machine" should be regulated by the concentration of E.

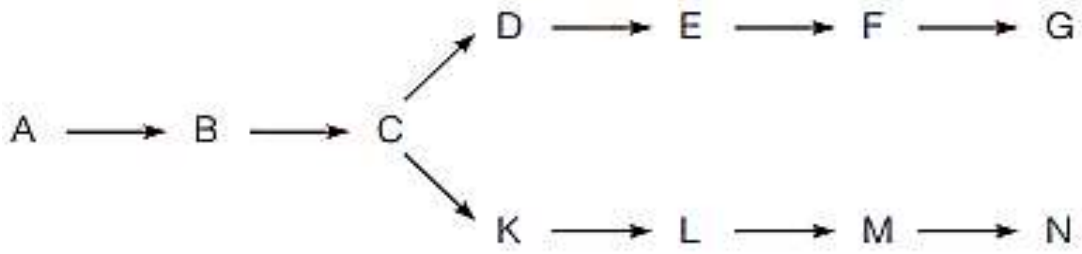


This type of feedback control is called feedback inhibition because an increase in the concentration of E leads to a decrease in its production rate.

Note that inhibiting the first step prevents unwanted utilization of A and accumulation of E.

3- Substrate fed into two pathways

Other metabolic situations require more complicated patterns in which activation and inhibition may be useful. For example, consider a slightly more complex case in which ***A is fed into two pathways***, which leads to two products needed in roughly equivalent amounts. Then, a scheme like the following emerges:



To control the pathways so that **G and N** keep in balance, high concentrations of G might inhibit the C ⟶ D enzyme or activate C ⟶ K enzyme. Conversely, high concentrations of N might inhibit C ⟶ K enzyme or activate the C ⟶ D enzyme.

An example of this kind of control is found in the synthesis of the purine and pyrimidine monomers that go into making DNA because approximately equal quantities of all four deoxyribonucleotides are required for DNA replication.

4- Allosteric Enzyme

Allosteric enzymes show cooperative substrate binding and can respond to a variety of inhibitors and activators

A special class of enzymes capable of allosteric regulation. The term **allosteric** is derived from **Greek words** meaning **"other structure,"** that regulator structures can be very different from either the substrate or the direct product.

Allosteric enzymes are frequently multisubunit proteins with multiple active sites. They exhibit **cooperativity** in substrate binding (**homoallostery**) and regulation of their activity by other **effector** molecules (**heteroallostery**).

An example of allosteric control of protein function. It is a hemoglobin. It is a four-subunit protein with four binding sites for its **"substrate,"** oxygen. Oxygen **"substrate,"** binding is **cooperative** and is influenced by other molecules and ions. The basic ideas presented for the analysis of hemoglobin function apply equally well to allosteric enzymes.

Cooperativity

Cooperativity: If an enzyme (protein) has more than one ligand-binding site, there is a possibility that these sites will interact with each other through the binding process. This is termed **cooperativity.**

Cooperativity include:

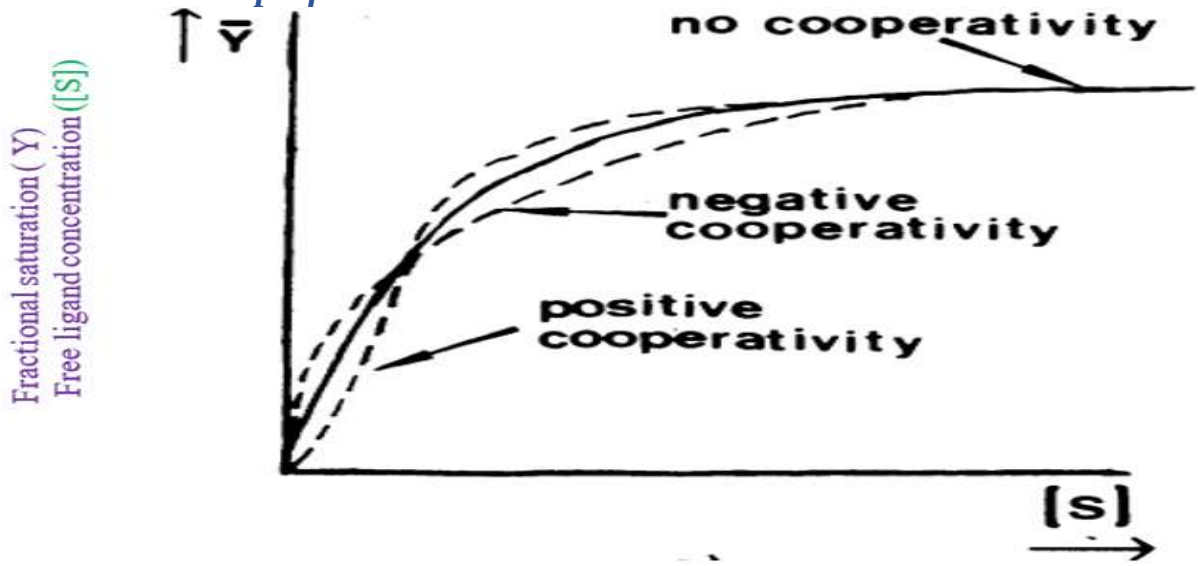
1- Positive cooperativity: Occur when the binding of one molecule of a substrate of ligand **increases the affinity of the enzyme (protein) for other molecules of the same or different substrate or ligand**. If the binding of the first molecule of the substrate increases the affinity of the enzyme for the substrate, the second step of the binding process will be faster than it is in the situation where there is no interaction between the binding sites

2- Negative cooperativity: Occurs when binding one molecule of a substrate or ligand **decreases the affinity of the enzyme (protein) for other molecules of the same or different substrate or ligand**. The second step of the binding process is slower than if there were no interactions between the binding sites.

3- Homotropic cooperativity: This occurs when the binding of one molecule of **a substrate affects the binding to the enzyme (protein) of subsequent molecules of the same substrate or ligand** (i.e., the binding of one molecule of A affects the binding of further molecules of A).

4- Heterotropic cooperativity: Occurs when the binding of **one substrate molecule influences the binding of a different substrate or ligand to the enzyme** (i.e., the binding of one molecule of A impacts the binding of B).

Cooperative effects may be positive and homotropic, positive and heterotropic, negative and homotropic or negative and heterotropic. **Allosteric inhibition** (section 8.2.7) is an example of negative heterotropic cooperativity and **allosteric activation** an example of positive heterotropic cooperativity.



Allosteric-controlled enzymes can be classified into **K-series enzymes** and **V-series enzymes.**

K-series and V-series enzymes refer to two different classifications of enzymes based on their kinetics and the nature of their inhibition.

1- K-series enzymes:

K-series enzymes follow Michaelis-Menten kinetics, which describes the rate of enzymatic reactions concerning substrate concentration. In this model, the relationship between substrate concentration ([S]) and reaction velocity (V)) is hyperbolic.

The term *Km* has no real meaning for an allosteric enzyme, a more appropriate term is **($S_{0.5}$),** which is the substrate (ligand) concentration required to produce 50% saturation of the enzyme (protein).

For a K-series enzyme, ($S_{0.5}$) substrate, i.e. the substrate concentration required to half-saturate of the enzyme, varies with the concentration of modifier.

❖ Allosteric inhibitors, increase the sigmoidal nature of the binding curve for substrate; thus they decrease the fractional saturation of an enzyme with its

substrate at low and moderate substrate concentrations, decreasing the value of *v0* under these conditions (Fig. 13.1).

❖ Allosteric activators, tend to increase the hyperbolic nature of the substrate binding curve. In each case, the degree of allosteric effect depends on the concentration of modifier, but the value of *V*max is not affected.

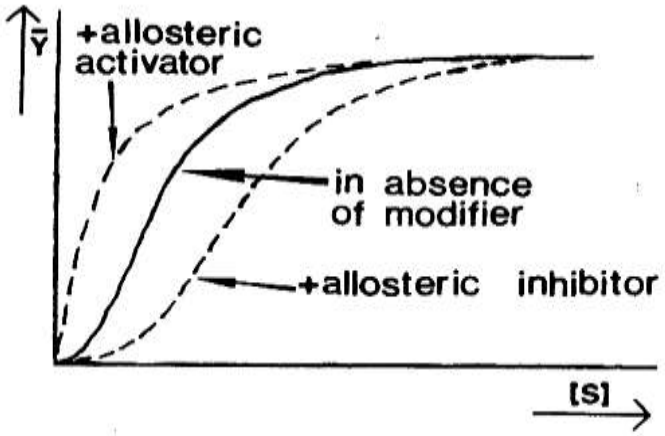


Fig. 13.1 — Effect of allosteric activators and inhibitors on the binding of a substrate to a K-series enzyme, at fixed concentrations of modifier and enzyme.

2- V-series enzymes:

In V-series enzymes, the presence of a modifier results in a change in the Vmax but not in the value of the apparent Km (or $S_{0.5}$) for the substrate .

V-series enzymes typically exhibit a change in Vmax in the presence of a modifier which can be an inhibitor or an activator) without altering the apparent Km (Michaelis constant). In other words, the presence of the modifier affects the maximum rate at which the enzyme can catalyze the reaction (Vmax), but does not change the affinity of the enzyme for the substrate (as measured by Km).

Homoallostery

Homoallosteric effects (cooperative substrate binding). We contrasted binding by the single-subunit protein myoglobin with binding by the multisubunit hemoglobin. Myoglobin gives a hyperbolic binding curve (Figure 7.7); hemoglobin, with its cooperative binding, gives a sigmoidal curve (Figure 7.10d). We find the same contrast when we compare the V vs. [S]

The curve of a single-site enzyme obeying Michaelis–Menten kinetics with that of a multisite enzyme shows cooperative binding (Figure 11.44a). The same applies to an enzyme that binds substrate cooperatively. The enzyme becomes low-activity at low substrate concentration as if it were poor at substrate binding. However, as the substrate levels increase and more substrate is bound, the enzyme becomes more active because it binds substrate more actively in the last sites to be filled (see Figure 11.44b).

We imagine this happening, as with hemoglobin, because as more substrate is bound, the enzyme undergoes a transition from a **lower affinity state (T state) to a higher affinity state (R state)**, which has a lower K_M..

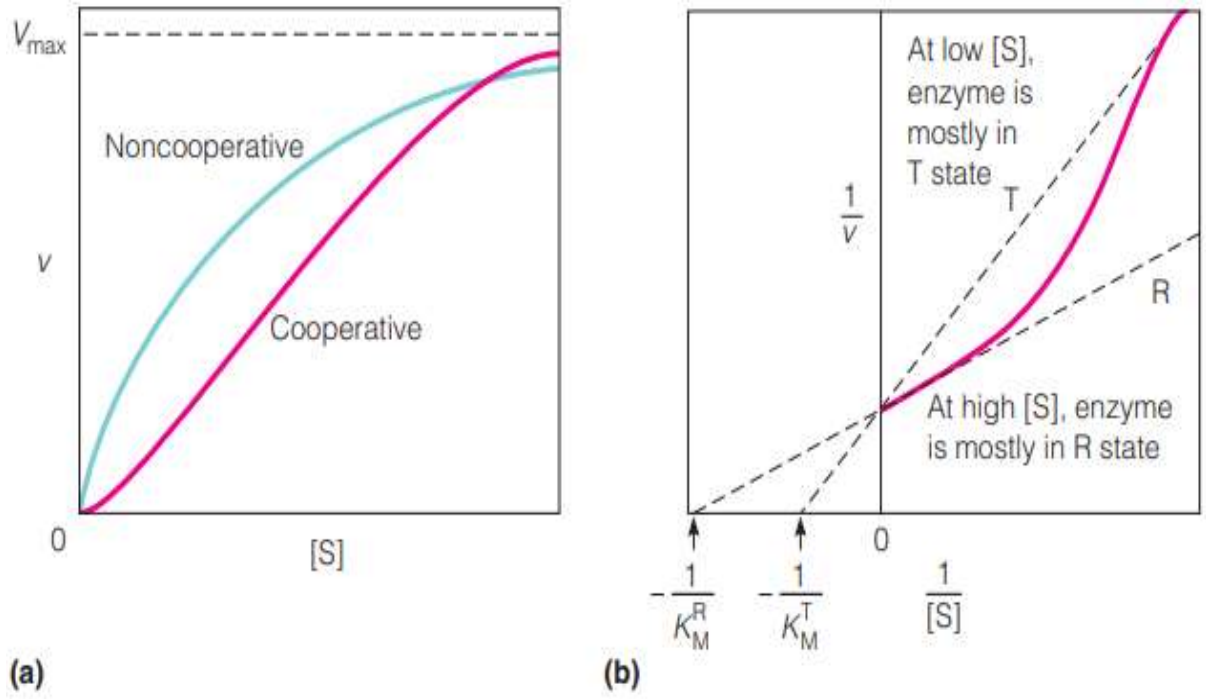


FIGURE 11.44 Effect of cooperative substrate binding on enzyme kinetics.

What physiological function does sigmoidal kinetics fulfill?

- Enzymes obeying sigmoidal kinetics can regulate substrate levels to relatively constant levels
- Substrate can easily accumulate up to the critical level at which the enzyme is inactive at lower [S], allowing [S] to increase up to [S]c, as shown in Figure 11.45.
- However, any further increase of the substrate [S] leads to a greatly increased enzyme activity so that the substrate will be more rapidly consumed.
- Although natural allosteric enzymes rarely exhibit curves as sigmoidal as in Figure 11.45, the principle remains: Multi-subunit enzymes help maintain substrate-level control.

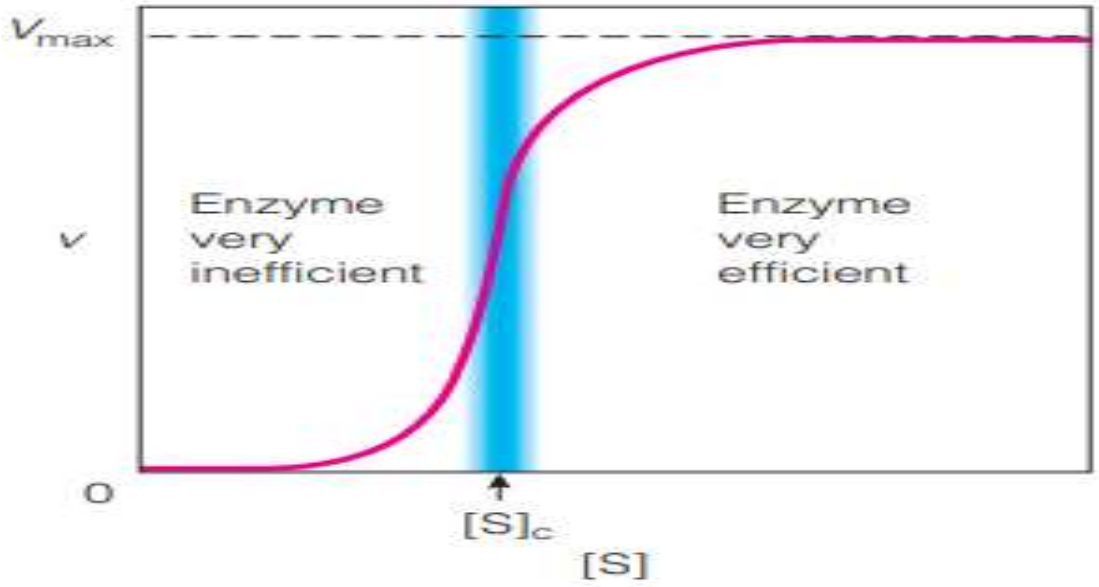


FIGURE 11.45 Effect of extreme homoallostery. The v vs. [S] curve. At concentrations below the enzyme is almost inactive; above this concentration, it is very active

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Heteroallostery

The major allosteric control is found in the role of hetero-allosteric effectors, which may be either inhibitors or activators. The activation and inhibition of enzymes by allosteric effectors are the keys to feedback control.

Suppose an enzyme molecule exists in two conformational states (T and R) that differ in the strength with which the substrate is bound or in the catalytic rate. In that case, its kinetics can be controlled by any substance that alters the equilibrium binding to the

protein. **Allosteric inhibitors shift the equilibrium toward T, and activators shift it toward R as shown in** Figure 11.46, The V vs. [S] curve is sigmoidal without activation or inhibitors. Activators shift the system toward the R state; inhibitors shift the system toward T state. [S]c represents the homeostatic concentration range for S. Some enzymes are regulated by multiple inhibitors and activators, allowing subtle metabolic control.

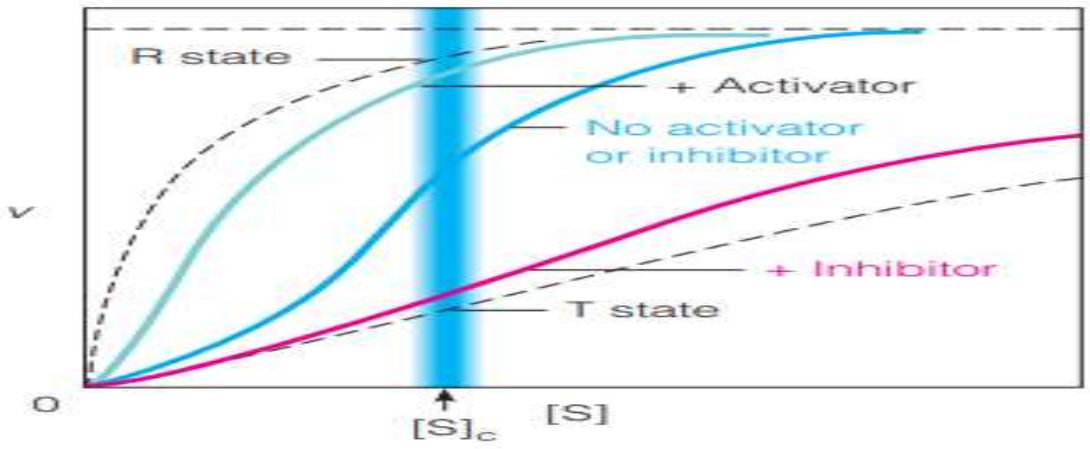


FIGURE 11.46: Heteroallostery (Hetero-allosteric control of an enzyme). [S]c represents the homeostatic concentration range for S.

An Example of an Allosteric Enzyme

Aspartate Carbamoyltransferase:

An excellent example of allosteric regulation is provided by the aspartate carbamoyltransferase (also known as aspartate transcarbamoylase or ATCase), a key enzyme in pyrimidine synthesis (Chapter 22).

As described in Fig 11.47, ATCase is at a crossing point in biosynthetic pathways.

Glutamine, glutamate, and aspartate are also used in protein synthesis, but once aspartate has been carbamoylated to form N-carbamoyl-L-aspartate (CAA), the molecule is committed to pyrimidine synthesis.

Thus, the enzyme (ATCase) that controls this step must be sensitive to pyrimidine needs.

In bacteria like E. coli, the activity of ATCase is regulated to respond to this need. This enzyme is inhibited by cytidine triphosphate (CTP) and activated by

ATP. Both responses make physiological sense; when CTP levels are already high, more pyrimidines are not needed.

On the other hand, high ATP indicates a purine-rich state (indicating a requirement for higher pyrimidine synthesis) and an energy-rich cell condition where DNA and RNA synthesis will be active.

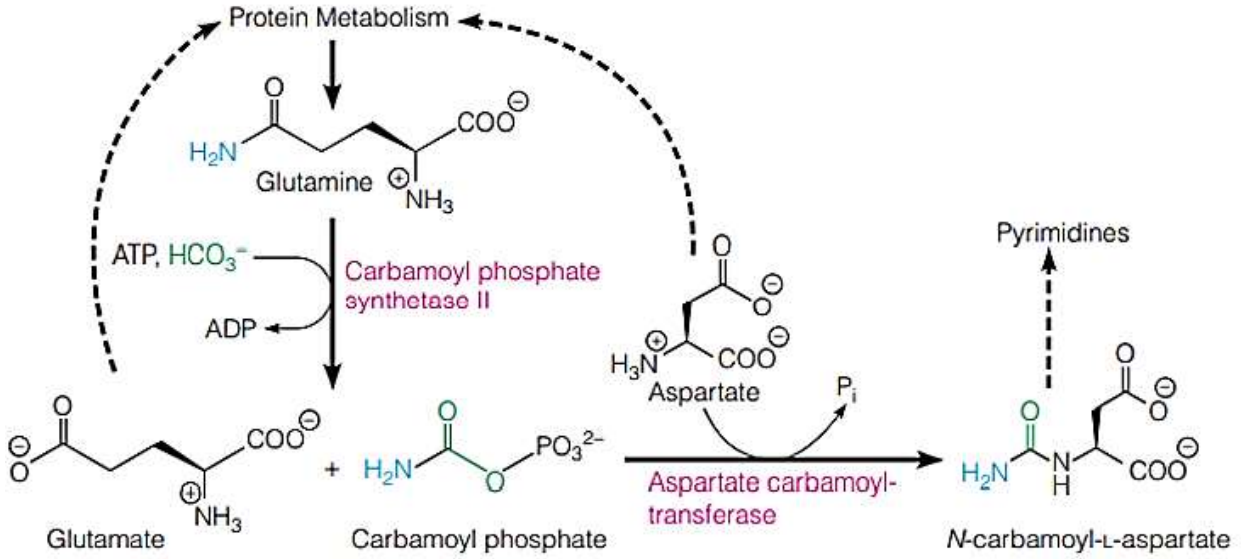


FIGURE 11.47 Control points in pyrimidine synthesis.

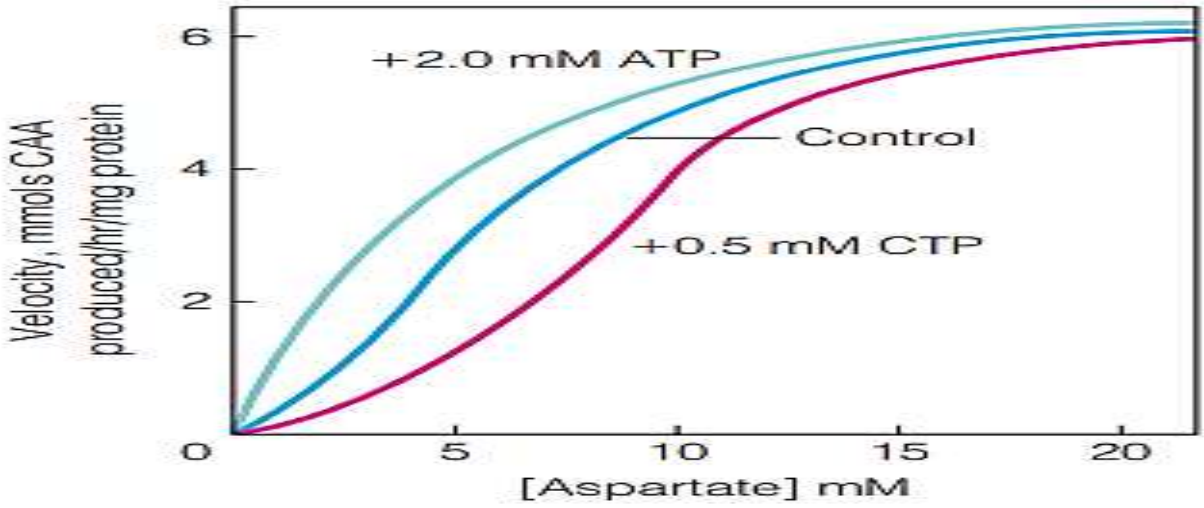


FIGURE 11.48: Regulation of aspartate carbamoyltransferase by ATP and CTP.

In mammals, the carbamoyl phosphate synthetase II is inhibited by UDP, UTP, CTP, dUDP, and UDP-glucose. These compounds all inhibit the binding of the ATP substrate.

Like most allosteric enzymes, ATCase is a multi-subunit protein. Figure 11.49 a. The of ATCase has six catalytic subunits distributed in two levels. These six (catalytic) subunits combine with six regulatory subunits. So it appears that pairs of regulatory subunits link catalytic subunits at the two levels.

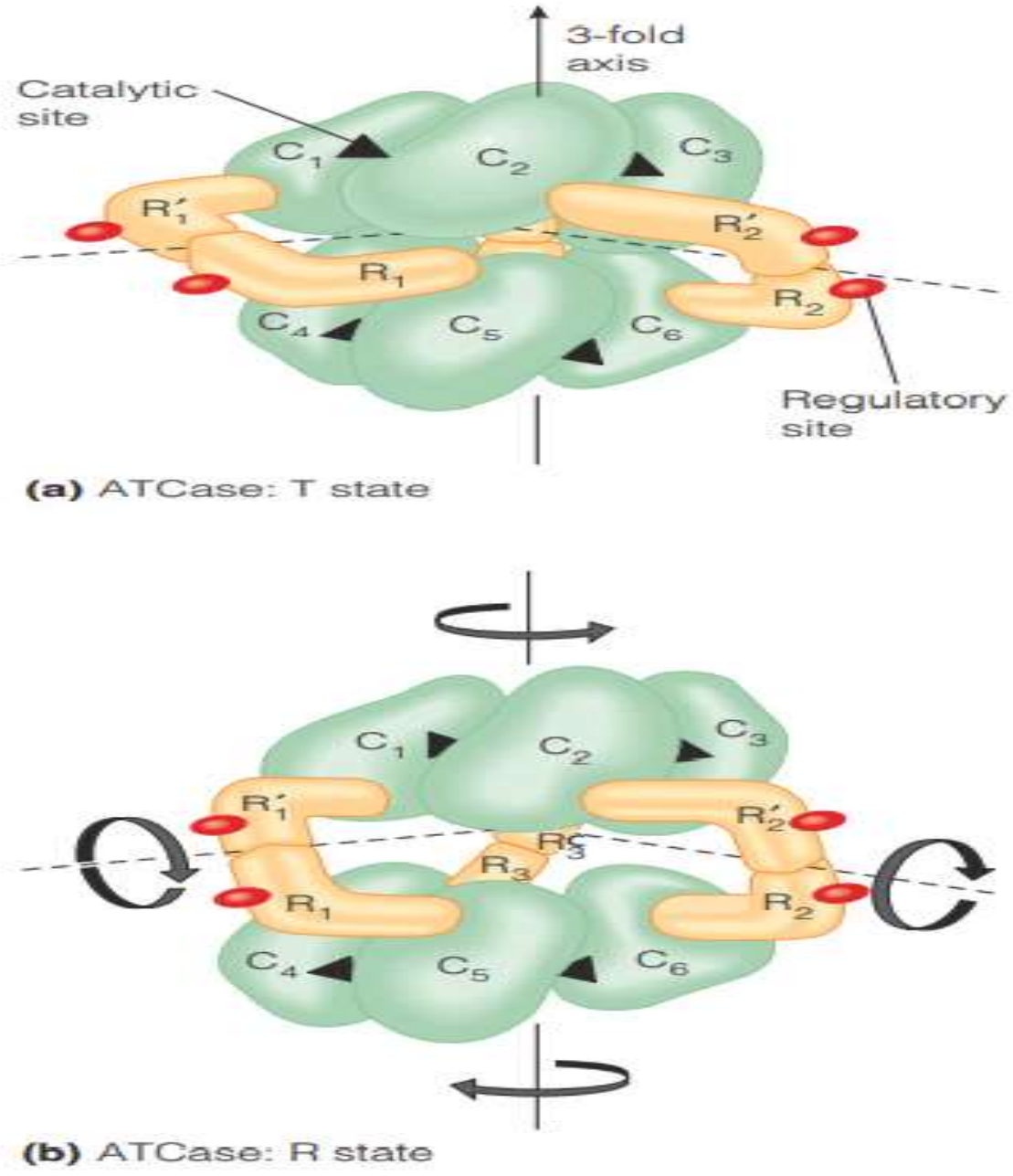


FIGURE 11.49 Quaternary structure of aspartate carbamoyltransferase (ATCase).

The structure of ATCase's catalytic and regulatory subunits (Figure 11.50)

- The active region sits between the aspartate-binding and carbamoyl-phosphate-binding - sites of the catalytic subunit.
- The regulatory subunit has two parts: zinc and allosteric. The first binds a structurally essential zinc atom; the second has an ATP/CTP binding site.
- ATP and CTP compete for the same site. Therefore, the ratio of ATP to CTP in the cell regulates the activity of ATCase.

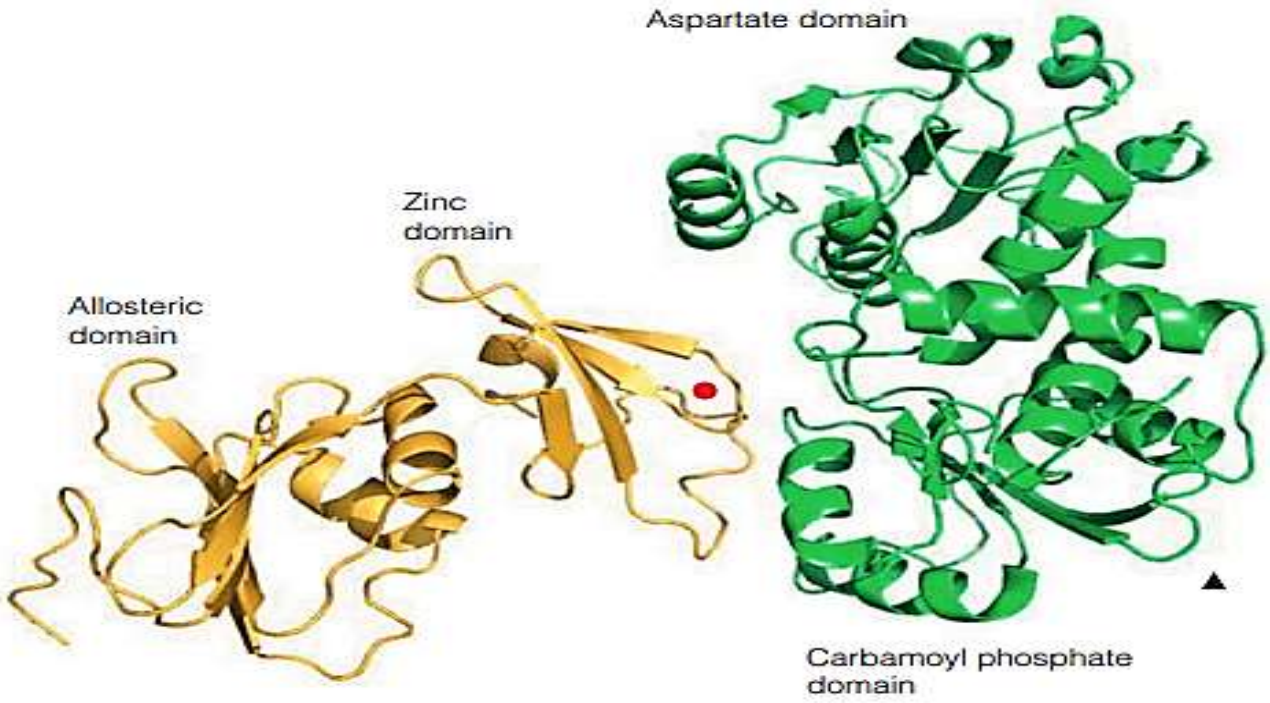


FIGURE 11.50 The detailed structureof ATCase.

Note: The ability of organisms to control metabolism in subtle and complex ways through allosteric enzymes should now be obvious; even so, this type of regulation is insufficient for all requirements.

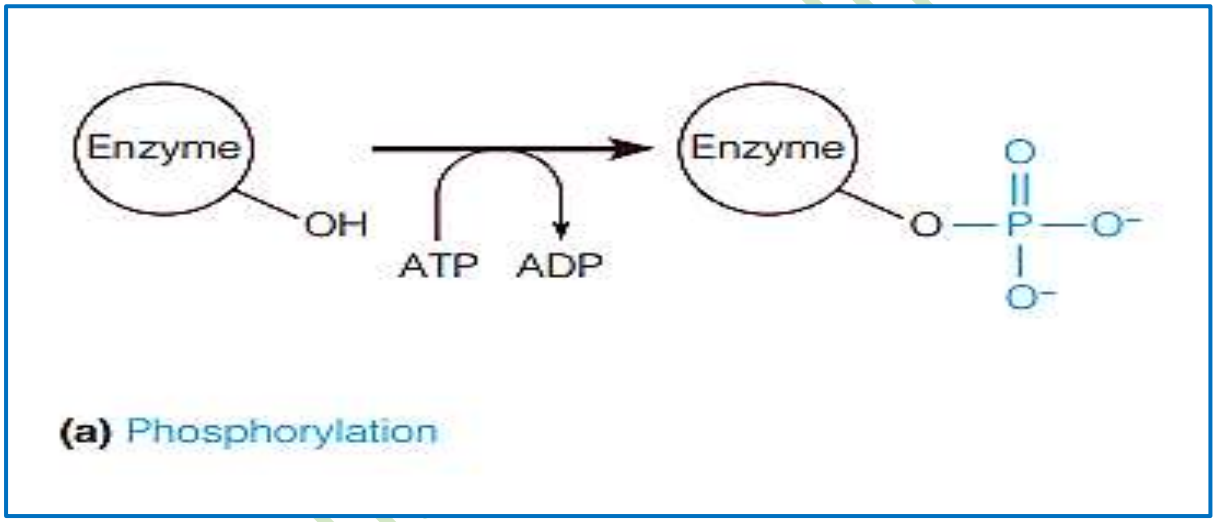
We now move on to covalent modification, a completely different type of regulatory mechanism.

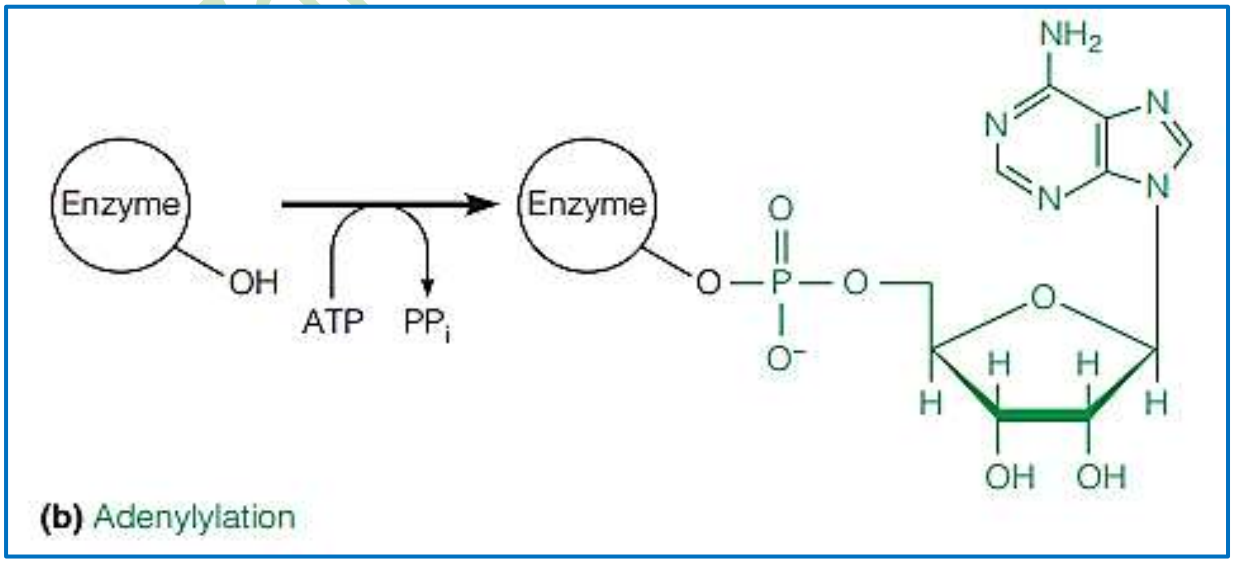
Covalent Modifications Used to *Regulate Enzyme Activity*

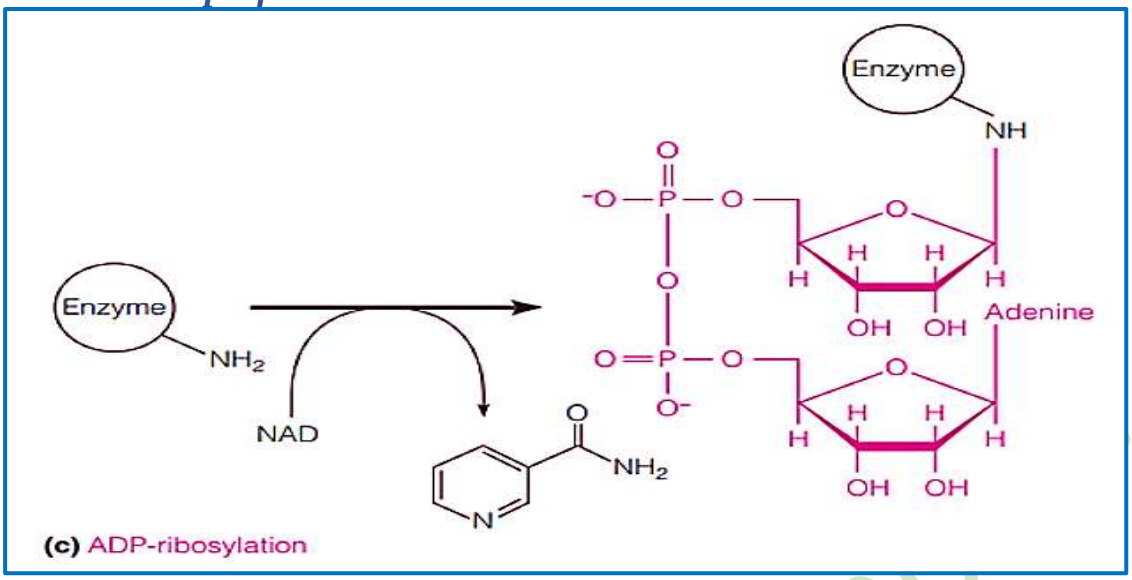
A number of kinds of covalent modifications are commonly used to regulate enzyme activity (Figure 11.51).

The most widespread is phosphorylation or dephosphorylation of various amino acid side chains (serine, threonine, tyrosine, and histidine, for example).

Other covalent modifications include adenylation, the transfer of an adenylate moiety from ATP; ADP-ribosylation, the transfer of an ADP-ribosyl moiety from and acetylation, the transfer of an acetyl group from acetyl-coenzyme A (see Table 11.5).







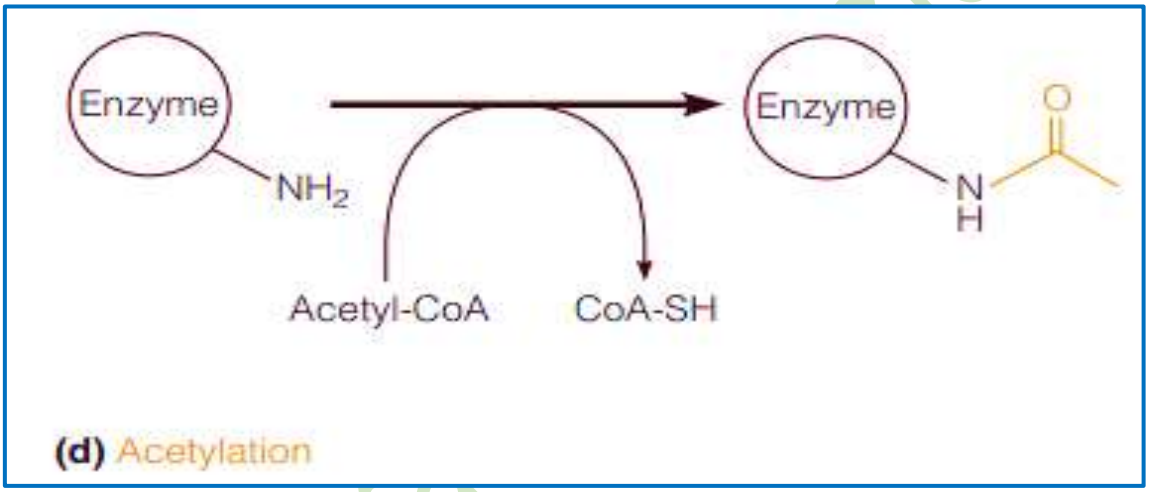


FIGURE 11.51 ***Four types of covalent modifications that control the activities of enzymes.*** The target residue for phosphorylation oadenylation is usually serine, threonine, or tyrosine, whereas ADP-ribosylation can involve arginine, glutamate, aspartate, or a modified histidine residue. N-acetylation involves a reaction between a lysine side chain and acetyl-coenzyme A.

The majority of enzymes, and their associated metabolic and signaling pathways, are regulated by reversible phosphorylation.

Protein kinases are ATP-dependent enzymes that add a phosphoryl group to the group of Tyr, Ser, or Thr on some target proteins (Figure 11.52).

This process is made reversible by the second class of enzymes, called phosphatases, which hydrolyze the resulting side chain phosphate esters, releasing.

Protein phosphorylation and acetylation are part of complex regulatory pathways, frequently under hormonal control. The majority of enzymes, and their associated metabolic and signaling pathways, are regulated by reversible phosphorylation. Protein kinases are ATP-dependent enzymes that add a phosphoryl group to the group of Tyr, Ser, or Thr on some target proteins (Figure 11.52).

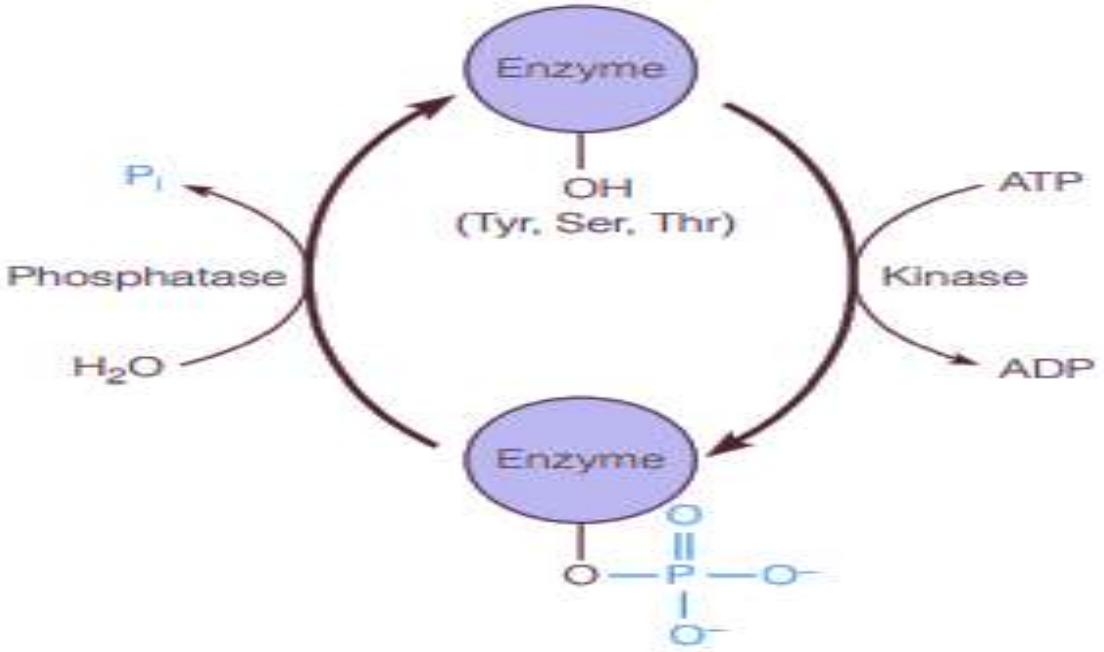


FIGURE 11.52 ***Reversible covalent modification by kinases/ phosphatases.*** The target residues for ATP-dependent phosphorylation by kinases are serine, threonine, or tyrosine. The phosphoprotein is dephosphorylated by a phosphatase-catalyzed hydrolysis reaction.

Pancreatic Proteases: *Activation by Cleavage*

Some enzymes, such as pancreatic proteases, are irreversibly switched on by proteolytic cleavage

An important example of covalent enzyme activation, proteolytic cleavage. These include a number of enzymes—for example, trypsin, chymotrypsin, elastase, and carboxypeptidase

They are secreted through the pancreatic duct into the duodenum of the small intestine in response to a hormone signal generated when food passes from the stomach.

They are not synthesized in active form because potent proteases free in the pancreas would digest the pancreatic tissue.

They are made as slightly longer, called **zymogens.** Included: chymotrypsinogen, proelastase, and procarboxypeptidase, respectively. The zymogens must be cleaved proteolytically in the intestine to yield the active enzymes.

The cleavage of zymogens to active enzymes is diagrammed in Figure 11.53.

The first step is the activation of trypsin removed from the N-terminal end of trypsinogen by enteropeptidase, a protease secreted by duodenal cells. This action yields the active trypsin, which then activates the other zymogens by specific proteolytic cleavages.

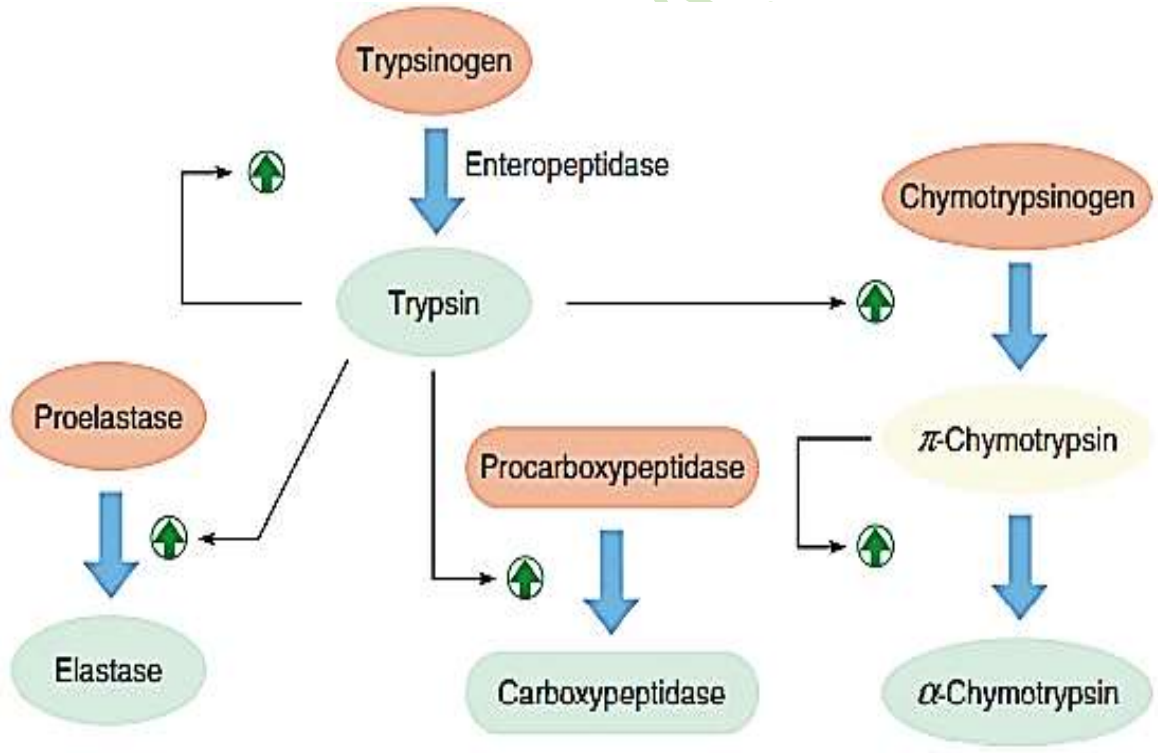


FIGURE 11.53 ***Zymogen activation by proteolytic cleavage.*** This schematic view shows the activation of pancreatic zymogens, molecules that become catalytically active when cleaved. Zymogens are shown in orange and active proteases are in yellow or green. The difference between -chymotrypsin and -chymotrypsin is shown in Figure 11.54.

TOOLS OF BIOCHEMISTRY

How to measure the rates of enzyme-catalyzed reactions?

There are essentially two approaches to enzyme kinetic analysis. The first and simplest is to make measurements of rates under conditions in which the steady-state approximation holds (see page 432). Under these conditions, the Michaelis–Menten equation is often applicable, and determination of the reaction velocity as a function of substrate and enzyme concentrations will yield Almost all enzymatic studies at least start in this way. However if the experimenter wishes to learn more of the details of the mechanism, it is often important to carry out studies before the steady state has been attained. Such pre-steady-state experiments require the use of special fast techniques. On pages 437–438, we described how a combination of such approaches can be used to dissect a complex enzymatic process and to understand it in detail. Here we describe some of the experimental techniques that can be employed.

Analysis of the Steady State

The steady state in most enzymatic reactions is established within seconds or a few minutes and persists for many minutes or even hours thereafter.

Therefore, extreme rapidity of measurement is not important, and many techniques are available to the experimenter wishing to follow the reaction. Descriptions of the most commonly used techniques follow.

Spectrophotometry

Spectrophotometric methods are simple and accurate (see Tools of Biochemistry 6A). However, an obvious requirement is that either a substrate or a product of the reaction must absorb light in a spectral region where other substrates or products do not.

Classic examples are reactions that generate or consume NADH. NADH absorbs quite strongly at 340 nm but does not absorb in this region. Thus we could, for example, follow the oxidation of ethanol to acetaldehyde, as catalyzed by alcohol dehydrogenase, by measuring the formation of NADH spectrophotometrically.

Fluorescence

The applications of fluorescence are similar to those of spectrophotometry, and the problems are similar:

A substrate or a product must have a distinctive fluorescence emission spectrum (see Tools of Biochemistry 6A).

However, fluorescence often has the advantage of high sensitivity, so extremely dilute solutions may be employed, enabling an experimenter to greatly extend the concentration range (i.e., [S]) over which studies are practicable.

Automatic Titration

If the reaction produces or consumes acid or base, it can be followed by using a device called a pH-stat. A glass elects the pH of the solution, and its signal is used to actuate a motor-driven syringe that titrates acid or base into the reaction vessel to keep the pH constant. The time-based record of acid or base consumed is then a record of the progress of the enzymatically catalyzed reaction.

Radioactivity Assays

If a substrate is labeled with a radioactive isotope that will be lost or transferred during the reaction to be studied, measurement of changes in radioactivity can be an extremely sensitive kinetic method. This procedure

requires that the labeled compound can be separated quickly at different, precisely defined times during the reaction.

An example is a method often used with radioactive ATP. The ATP can be adsorbed on charcoal-impregnated filter disks by very fast filtration of aliquots from the reaction mixture. The radioactivity can then be measured in a scintillation counter (see Tools of Biochemistry 12A).

Another example of the use of radioisotopes comes from measuring the rates of peptide-bond cleavage (by a protease), or protein biosynthesis (e.g., ribosomal protein synthesis). Peptides are most commonly labeled with radioactive amino acids that contain or

The rate of a peptide cleavage or synthesis reaction can be monitored by rapidly precipitating the peptide (or peptide fragments) from the reaction solution using cold trichloroacetic acid and collecting the precipitate on filter paper. As described above, the radioactivity present on the filter paper can be quantitated using a scintillation counter

Analysis of Very Fast Reactions Reactions

Reactions that are extremely rapid require special techniques to investigate the pre-steady-state processes. Two major methods are currently employed to cover the rapid time scales shown in Figure 11A.1.

Stopped Flow

Figure 11A.2 shows a stopped-flow apparatus, first described by Quentin Gibson in the 1950s. Enzyme and substrate are initially in separate syringes. The syringes are driven, within a few milliseconds, to deliver their contents through a mixing chamber and into a third, “stopping” syringe. This step triggers a detector

to begin observing (for example, by light absorption or fluorometry) the solution in the tube connecting the mixer to the stopping syringe.

Flow rates can easily be made as high as 1000 cm/s. If the mixture was moving at this rate when the flow was stopped, and if the observation point is 1 cm from the mixer, the detection system first sees a mixture that is 1 ms "old." The reaction can then be followed for as long as desired—often for a period of only a few seconds. The limitations of the method are imposed only by the initial "dead time" (i.e., the time it takes the mixed solutionsto arrive at the detector—in the example above, 1 ms) and the rapidity of the detection system.

Stopped-flow is used to measure rates of rapid enzymatic reactions as well as ligand binding events, such as binding to, or release from, hemoglobin (see Chapter 7)

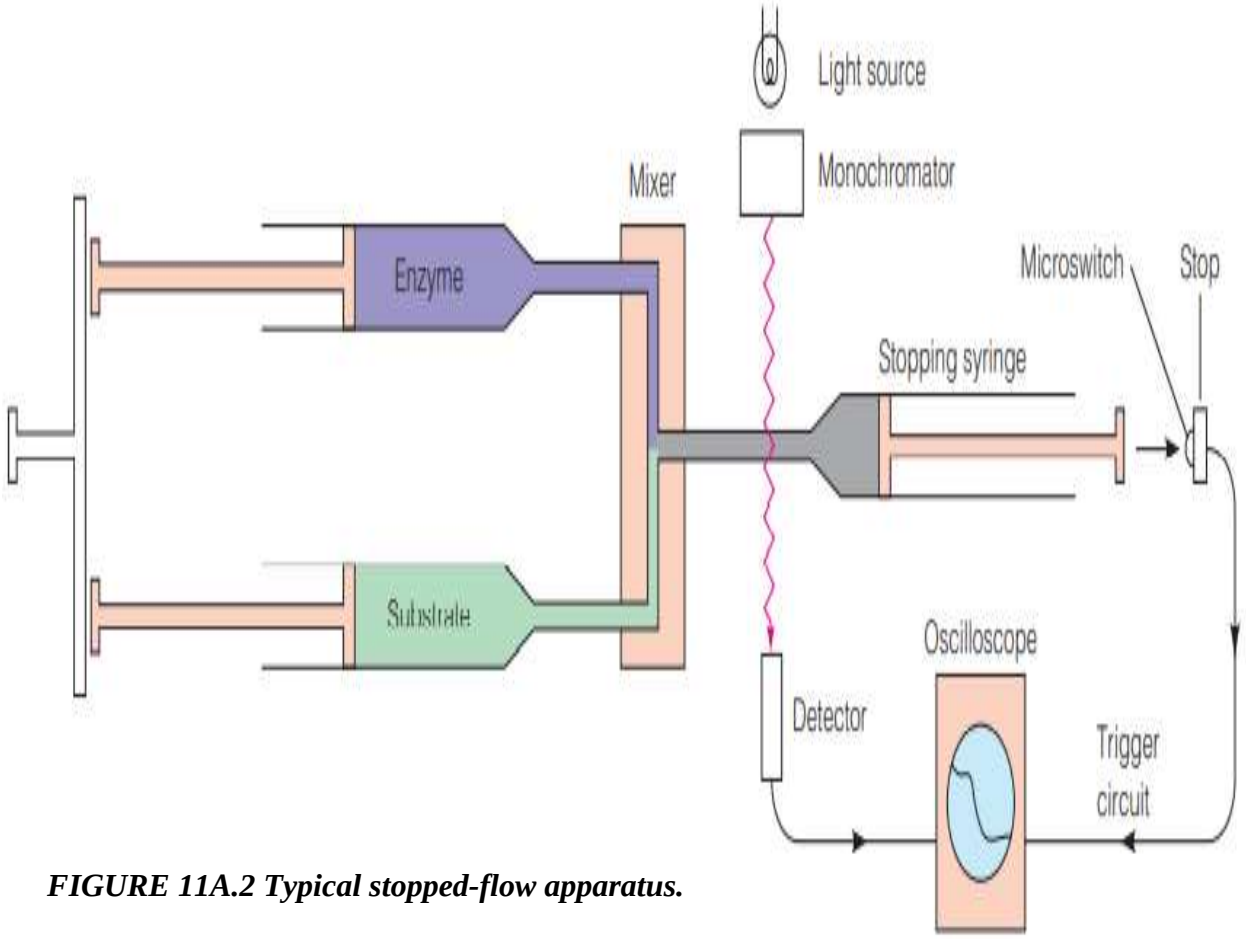


FIGURE 11A.2 Typical stopped-flow apparatus.

Temperature Jump

Some processes are so fast that they are essentially completed in the dead time of a stopped-flow apparatus. The experimenter may then turn to temperature jump (T-jump) methods. The basic apparatus and principle of the method are shown in Figure 11A.3a and b, respectively. A reaction mixture that is at equilibrium at a temperature is suddenly jumped to a temperature Because chemical equilibria are typically temperature-dependent, the position of equilibrium will shift, and the system must now react to attain this new equilibrium. A rapid jump in temperature (5–10 °C in) can be obtained by passing a large burst of electrical current between electrodes immersed in the reaction mixture. Even more rapid jumps (10–100 ns) can be obtained if a pulsed infrared laser is used to heat the mixture. The relaxation (approach) to anew equilibrium, monitored by absorption or fluorescence measurements, is an exponential process. For a simple reaction, the change in reactant concentration is given by

$$\Delta[A] = (\Delta[A]_{total})e^{-t/\tau} \qquad (11.A1)$$

Where ***T*** is called the relaxation time and can be related to the rate constants for the reaction. For example, for the simple reversible reaction

$$A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} B$$

we have

$$\frac{1}{\tau} = k_1 + k_{-1} \qquad (11.A2)$$

More complex reactions involve multiple relaxation times and more complex curves than expressed by equation (11A.1). ***T*** -jump experiments are appropriate for reactions with ***T*** values as low as about Although a number of other techniques are employed for even faster reactions, including some newly developed NMR 10^{-5} s.

Although a number of other techniques are employed for even faster reactions, including some newly developed NMR methods and pulsed laser techniques, the methods described here are widely used. If we consider the variety of techniques available to the experimenter, we can see that they cover a wide time range. Altogether, times from nanoseconds to hours can be studied.

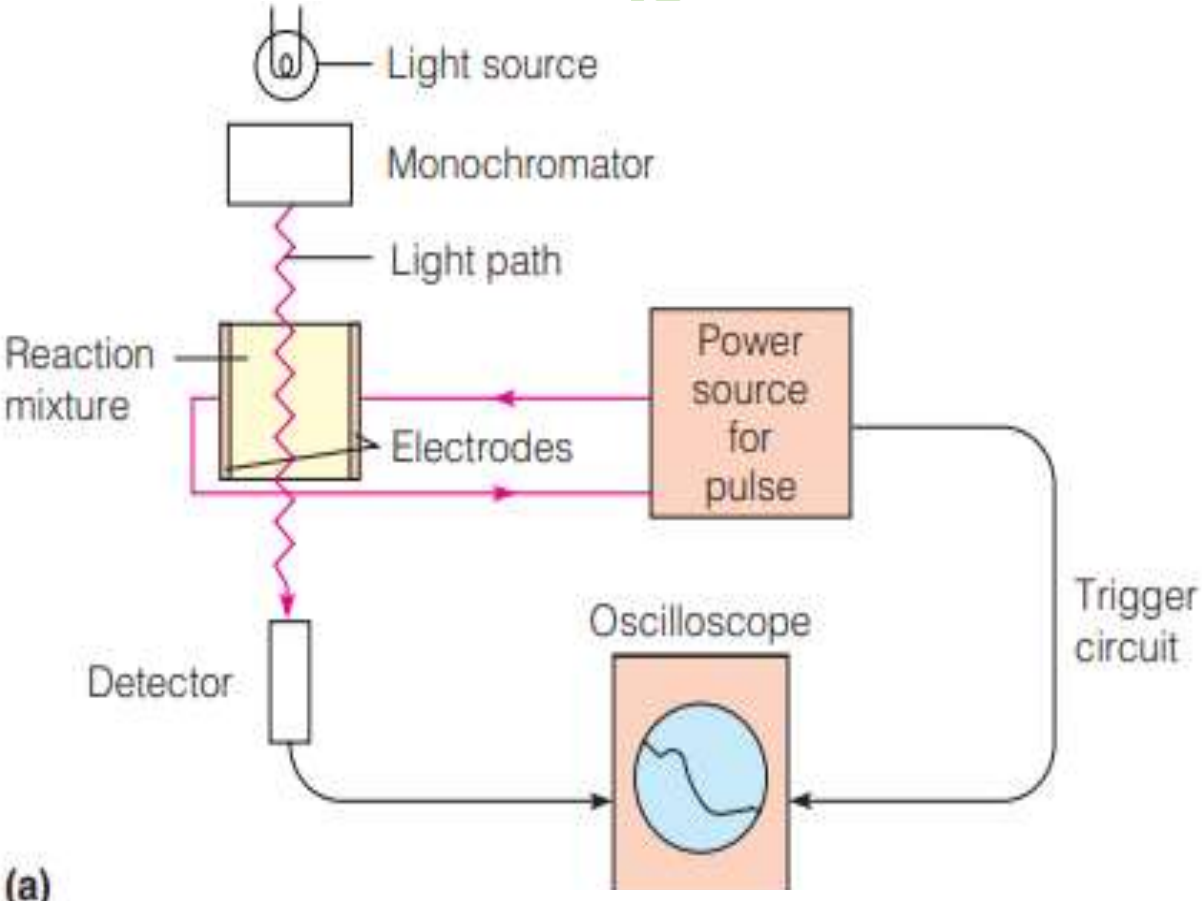


FIGURE 11A.3 The temperature jump method

Non-protein Biocatalysts:

Catalytic antibodies (Abzyme)

Antibodies show remarkably high specificity in binding to their antigens.

Enzymes bind most strongly to the transition state in a reaction.

What happens if we make antibodies against molecules that are structurally analogous to the transition state of a particular substrate?

The answer is that:

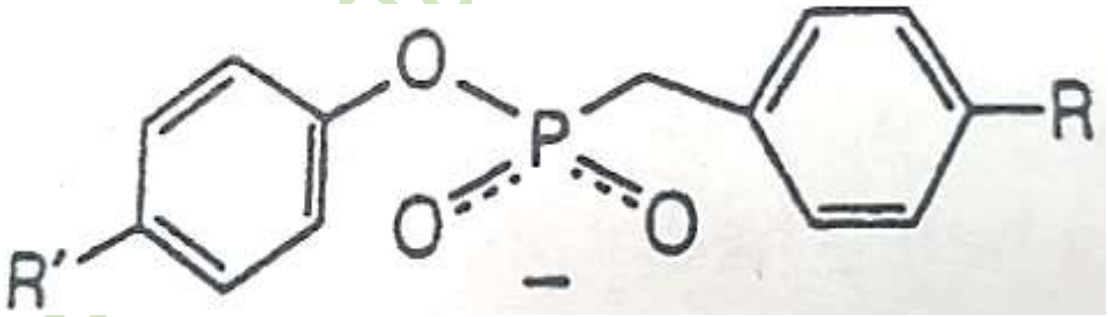
These antibodies act like enzymes, so they are now often called **abzymes.**

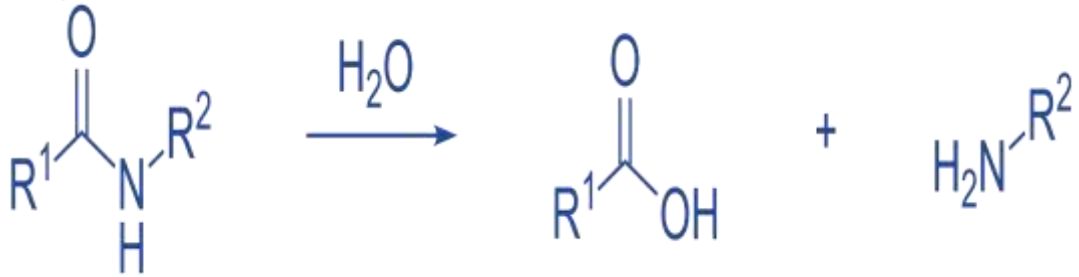
Suppose, for example, we wish to make an antibody that will function as a catalyst for the hydrolysis of esters.

Example:

Hydrolysis of amides, a tetrahedral transition state is required in ester hydrolysis.

A kind of molecule that mimics such a structure is the following:





By using various kinds of molecules as antigens, it has been possible to produce abzymes catalyzing a number of the classes of reactions in Table 11.7. in some cases, reaction rates as much as 10^7 times the uncatalyzed rate have been obtained.

Abzyme (from antibody and enzyme), also called catmab (from catalytic monoclonal antibody), and most often called catalytic antibody or sometimes catab,[is a monoclonal antibody with catalytic activity.

For many years, the major difficulty in generating catalytic antibodies directed toward specific compounds or functional groups was the necessity of using the immune system of some animals to make the selection.

More recently, however, selection systems have been developed that circumvent this requirement.

The basic idea is that randomly rearranged Fab fragments (see Chapter 7) are cloned and the mixture is subjected to selection by chemical affinity to the desired molecule or structure.

Such techniques have allowed the development of abzymes. directed toward synthetic molecule substrates that would be very difficult to present as antigens to an in vivo system because of toxic effects.

Catalytic antibodies are becoming of considerable importance in synthetic organic chemistry.

A major problem in the in vitro synthesis of complex organic molecules is that of obtaining the correct stereochemistry. The remarkable stereospecificity exhibited by enzymes (including abzymes) has enormously aided in some such syntheses. It seems at this point that biochemists have only begun to explore the possibilities of engineering enzymes for specific purposes.

Catalytic Nucleic Acids (Ribozyme , DNAzymes)

Ribozymes, a class of ribonucleic acids, function as biological catalysts.

Ribozymes are catalytically active RNA molecules or RNA–protein complexes,

in which solely the RNA provides the catalytic activity.

The term ribozyme refers to the enzymatic activity and ribonucleic acid nature at the same time.

Ribozymes are found in the genomes of species from all kingdoms of life.

Indeed, it was assumed that all biochemical catalysis was carried out by proteins for many years. But biochemistry is full of surprises, a research performed in the 1980s revealed something wholly unexpected: ***Some RNA molecules***, called ***ribozymes***, can ***act as enzymes.***

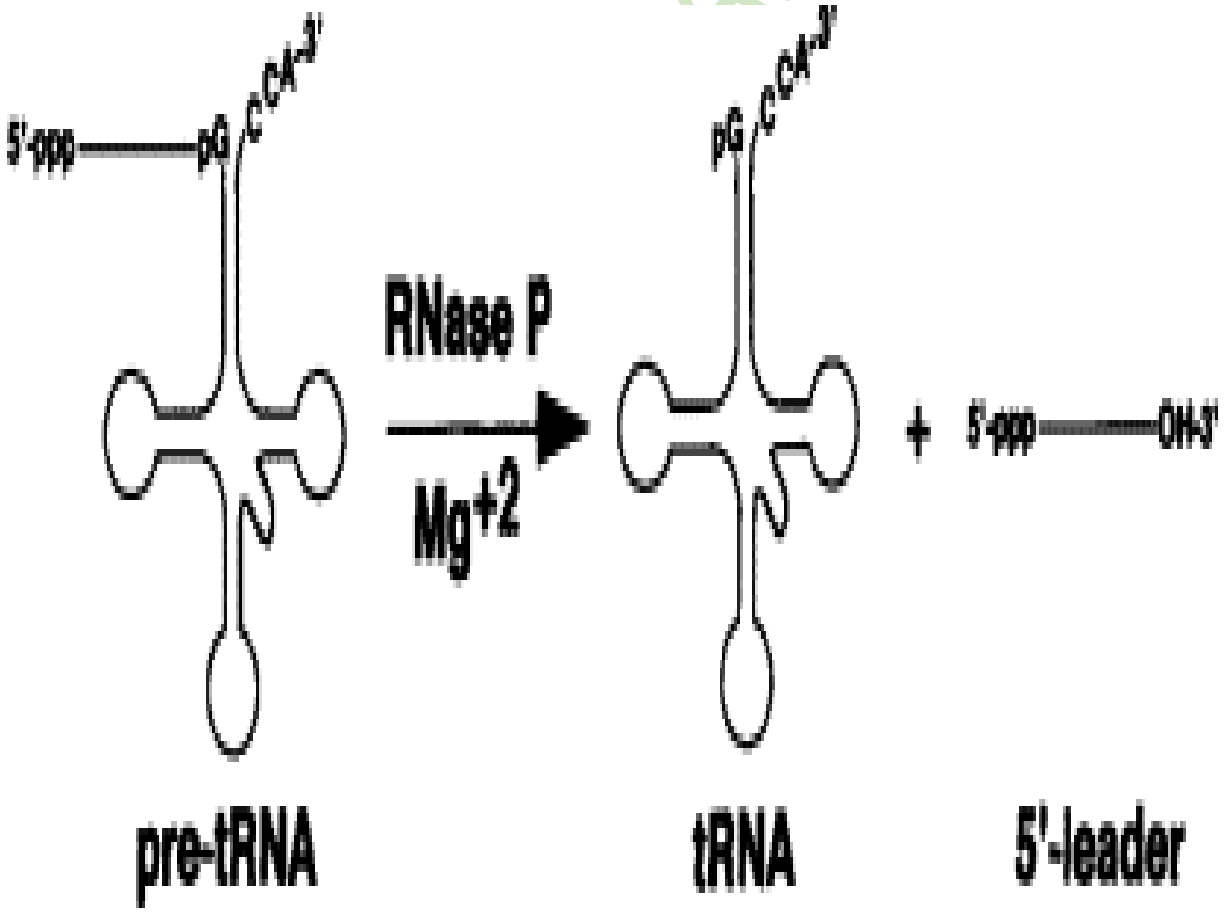
It had been known for some time that active **ribonuclease P** contained - **a protein and RNA** but it was widely assumed that the active site resided on the protein portion.

The first hint that RNA might have catalytic activity came from studies of ribonuclease P, an enzyme that cleaves the precursors of tRNAs to yield the functional tRNAs (Figure 11.40 and Chapter 27).

Ribonuclease P (**EC 3.1.26.5, RNase P**) is an RNA-cleaving ribonuclease. RNase P differs from other RNases in that it is a ribozyme, which is a ribonucleic acid that functions as a catalyst in the same way that as an enzyme that is made of a protein. Its (RNase P) function is to act on the maturation of tRNA by clipping an extra or primary RNA sequence in its molecules.

However, careful studies of the isolated components by Sidney Altman and coworkers in 1983 revealed a fact: Whereas the protein component alone was wholly inactive, the RNA by itself, if provided with either a sufficiently high concentration of magnesium ion or a small amount of magnesium ion plus the small basic molecule ***spermine****, was capable of catalyzing the specific cleavage of ***pre-tRNAs.***

Furthermore, the RNA acted like a true enzyme, being unchanged in the process and obeying Michaelis–Menten kinetics. The addition of the protein portion of ribonuclease P does enhance the activity (K_{cat} is markedly increased) but is in no way essential for either substrate binding or cleavage.



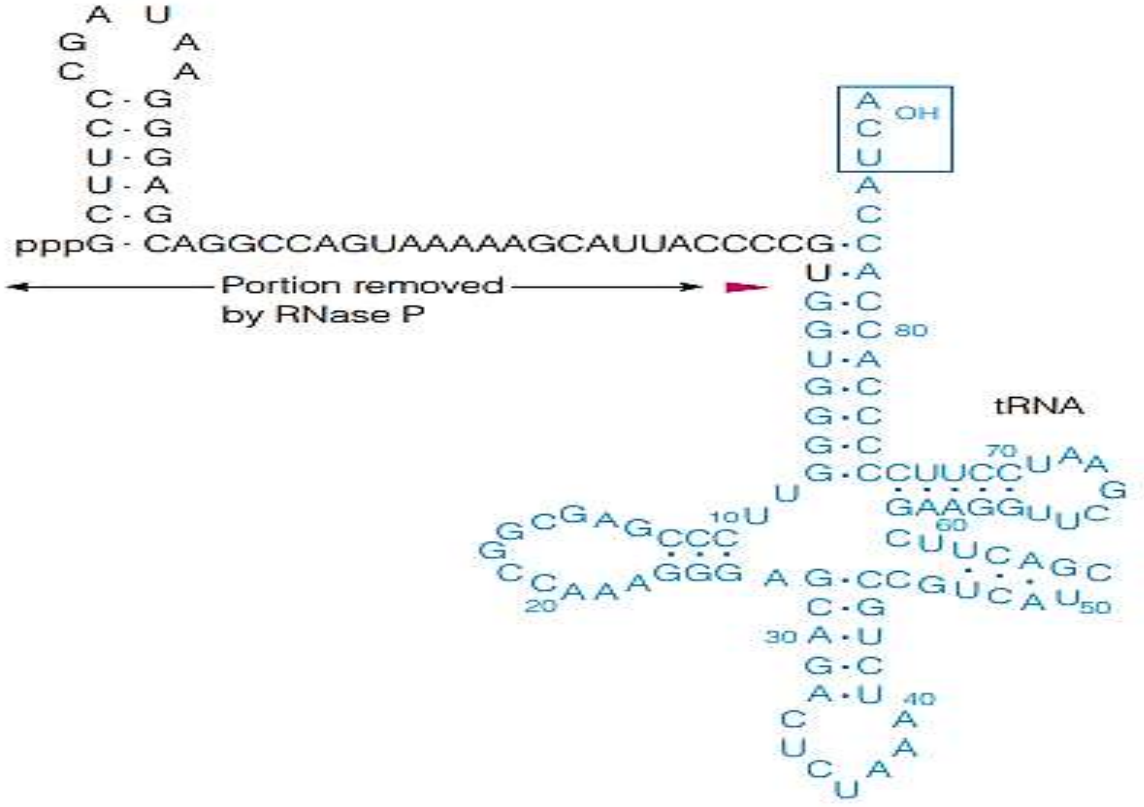


FIGURE 11.40 Cleavage of pre-tRNA by ribonuclease P. The production of tRNA from pre-tRNA is catalyzed by an **RNA–protein complex** called ribonuclease P (RNase P). The portion removed from tRNA is shown in **black**, and the resulting tRNA is in blue. The RNA portion of ribonuclease P can, by itself catalyze the hydrolysis of the specific **phosphodiester bond** indicated by the magenta wedge. The 3' terminal-OH group is shown as a subscript to the 3' terminal-OH adenosine.

DNAzymes

Like RNA, single-stranded DNA can form complex tertiary structures required for specific binding to a ligand/ (or) substrate and catalytic activity. Given the chemical similarities between DNA and RNA, it is reasonable to ask whether DNA can carry out meaningful biological catalysis. So far, no naturally occurring catalytic DNA or ***DNAzyme*** has been discovered in cells.

Like ribozymes, ***DNAzymes*** have been shown to catalyze a diverse set of reactions, with significant rate enhancements (Table 11.11). For example, the types of reactions catalyzed by DNAzymes include hydrolysis (RNA and DNA cleavage), bond cleavage, and photolytic repair of damaged DNA. Because DNA has greater chemical stability than do RNA and peptides, there is much current interest in developing DNAzymes as therapeutics, diagnostics, and biosensors.

TABLE 11.11 Examples of reaction types and rate enhancements

Reaction Type	k_{cat} (min^{-1})	Rate Enhancement
Various RNA transesterifications	0.007–4.3	10^5–10^8
DNA cleavage	0.05–0.2	10^7–10^8
Porphyrin metallation	1.3	10^3
DNA ligation	0.0001–0.07	10^2–10^5
Adenylylation	0.005	10^{10}
N-Glycosyl cleavage	0.2	10^6
Phosphorylation	0.012	10^9

From Cellular and Molecular Life Sciences 59:596–607, G. M. Emilsson and R. R. Breaker, Deoxyribozymes: New activities and new applications, © 2002, with kind permission from Springer Science+Business Media B.V

The Diversity of Enzymatic Function Classification of Protein Enzymes

By this point, it should be clear that an enormous number of different proteins act as enzymes. Many of these enzymes were given common names, especially during the earlier years of enzymology.

Some enzyme names, like triose phosphate isomerase, are descriptive of the enzyme's function; others, like trypsin, are not.

To reduce confusion, a rational naming and numbering system has been devised by the Enzyme Commission of the International Union of Biochemistry and Molecular Biology (IUBMB).

Enzymes are divided into six major classes, with subgroups and sub-subgroups to define their functions more precisely. The major classes are as follows:

1- **Oxidoreductases catalyze** oxidation–reduction reactions.
2- **Transferases catalyze** the transfer of functional groups from one molecule to another.
3- **Hydrolases catalyze** hydrolytic cleavage.
4- **Lyases catalyze** the removal of a group from or the addition of a group to a double bond, or other cleavages involving electron rearrangement.
5- **Isomerases catalyze** intramolecular rearrangement.
6- **Ligases catalyze** reactions in which two molecules are joined.

International Union of Biochemistry and Molecular Biology (IUBMB) Enzyme Commission (EC)

The IUBMB Enzyme Commission (EC) has given each enzyme a number with four parts, such as:

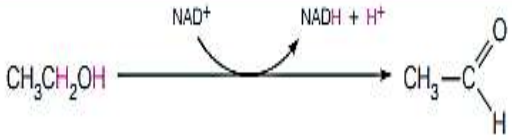
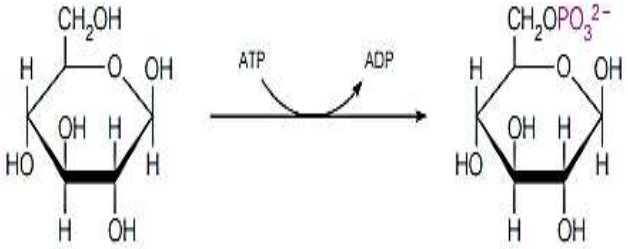
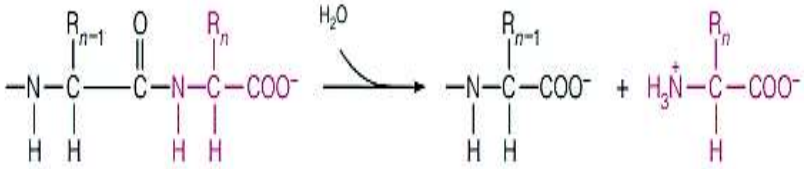
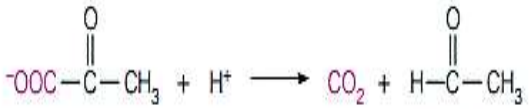
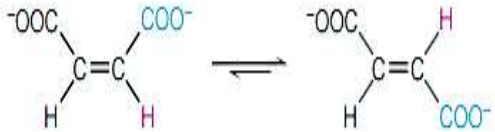
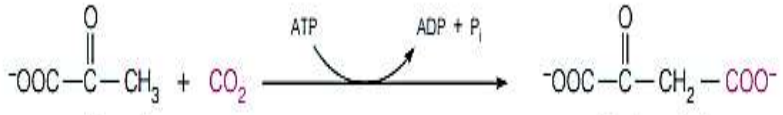
EC 3.4.21.5. The first three numbers define major class, subclass, and sub-subclass, respectively. The last is a serial number in the sub-subclass, indicating the order in which each enzyme is added to the list, which is continually growing.

Listings for almost all currently recognized enzymes, together with information on each enzyme and literature references, can be found in online databases such as BRENDA (Braunschweig ENzyme DAtabase; http://www.brenda-enzymes.info) or ExPASy (Expert Protein Analysis System; http://expasy.org/enzyme). The 5000+ entries in these databases do not include all enzymes; more are being discovered all the time.

Indeed, it has been estimated that the typical cell contains many thousands of different kinds of enzymes.

Table 11.10 lists one example enzyme and reaction from each of the major classes. We will discuss each of these reactions later in this book. The main point here is the enormous diversity of enzymatic functions and how their nomenclature has been rationalized.

TABLE 11.10 Examples of each of the major classes of enzymes

Class	Example (reaction type)	Reaction Catalyzed
1. Oxidoreductases	Alcohol dehydrogenase (EC 1.1.1.1) (oxidation with NAD^+)	 Ethanol — Acetaldehyde
2. Transferases	Hexokinase (EC 2.7.1.2) (phosphorylation)	 D-Glucose — D-Glucose-6-phosphate
3. Hydrolases	Carboxypeptidase A (EC 3.4.17.1) (peptide bond cleavage)	 C-terminus of polypeptide — Shortened polypeptide — C-terminal residue
4. Lyases	Pyruvate decarboxylase (EC 4.1.1.1) (decarboxylation)	 Pyruvate — Acetaldehyde
5. Isomerases	Maleate isomerase (EC 5.2.1.1) (*cis–trans* isomerization)	 Maleate — Fumarate
6. Ligases	Pyruvate carboxylase (EC 6.4.1.1) (carboxylation)	 Pyruvate — Oxaloacetate

Molecular Engineering of New and Modified Enzymes

NOTE: New or radically modified enzymes can be created by "protein engineering," which includes a number of techniques such as site-directed mutagenesis, protein domain fusion, selection from randomly generated libraries, and computational design.

Despite the variety of enzymatic functions available in nature, modern biotechnology continually faces the need for substances with new catalytic abilities, or enzymes that function with different specificities or under unusual conditions.

These needs have generated a field of enzyme design and engineering, which has enormous potential in the design of industrial catalysts as well as biopharmaceuticals.

Several approaches are being taken to achieve the goal of generating suchas:

1- Tailor-made catalysts. These include site-directed mutagenesis,
2- Fusion of one or more functional domains,
3- Selection of a sequence with the desired activity from a large pool of randomly generated protein sequences,
4- Generation of "catalytic antibodies," and
5- Computational design.

NOTE: Tools of Biochemistry 11B provides a brief introduction to these techniques.