Concepts in Metabolism

4

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Though the vitalist group initially used the term "organic" for compounds produced only by organisms, it was later on used for carbon compounds. Wohler (1928) discovered that urea, which otherwise was thought to be produced only in the living beings, could also be produced in the laboratory from ammonia and bicarbonate. In 1897, German chemists Eduard Buchner and Hans Buchner demonstrated that fermentation could be carried out by the **cell-free extract** of yeast. These observations lead to the development of the science of biochemistry. In the early twentieth century, due to discovery of various metabolic pathways, biochemistry was dominated by organic chemistry, followed by enzymology and **bioenergetics**. Some of the analytical techniques which made study of biochemistry possible included isolation of organelles, high-performance liquid chromatography, electrophoresis, use of radioactive tracers, plant transformation techniques using *Agrobacterium tumefaciens,* gene silencing, forward genetics, reverse genetics, mass spectrometry, and DNA microarray, among others. With computational technology, it is now possible to have complete understanding of the interconnectivity of metabolic pathway.

Autotrophs are able to synthesize organic material from CO_2 and H_2O, deriving energy either from the chemical reactions (chemoautotrophs) or by utilizing light energy (photoautotrophs). Heterotrophs, including mammals, have to depend on the autotrophs for the availability of complex carbon containing organic substances. Sum total of all chemical reactions occurring in a living being are called as **metabolism**. These occur through enzyme-catalyzed reactions that constitute metabolic pathways. Metabolic pathways include precursors, which are converted to products. Various intermediates are called metabolites. Combined activity of all metabolic pathways involved in interconversion of precursors, metabolites, and products is called **intermediary metabolism**. **Primary metabolites** are the intermediates or the products of a pathway, which are used for growth, development,

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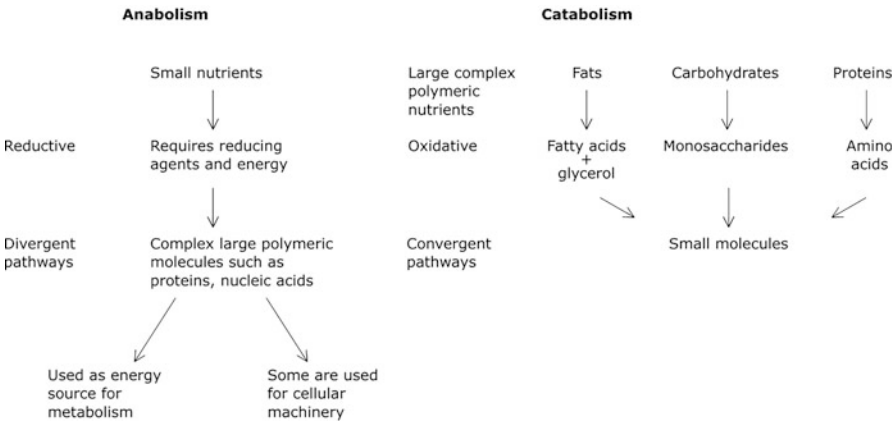


Fig. 4.1 Comparison of anabolism and catabolism

and reproduction of the organism. S**econdary metabolites** are bioactive specialized compounds produced in a metabolic pathway which are used to protect plants against herbivory and microbial pathogen infection or to attract pollinators or seed dispersal animals**.** Metabolism includes both anabolic and catabolic reactions. The terms anabolism and catabolism were first used by the physiologist Gasket in 1886. Anabolism includes all the reactions involved in conversion of simpler molecules to complex ones. This requires input of energy and the pathway involved is a divergent pathway (Fig. 4.1). On the contrary, catabolism involves conversion of complex substances into simpler molecules, which is coupled with release of energy. The catabolic pathways are convergent pathways since many of the metabolic reactions converge to join the pathway involved with release of simpler molecules. The energy transitions in these pathways are mediated through two high-energy molecules which are reduced form of nicotinamide dinucleotide (NADH), and adenosine triphosphate (ATP). ATP is a high-energy phosphate compound, which mediates energy transfer, while NADH is the donor for high-energy electron transfer (Fig. 4.2).

All living organisms have the unique ability to adjust to the changing environment through alteration in their metabolism even though maintaining their internal cellular environment. Unlike animals, plants are sessile and are exposed to harsher conditions. They have more robust metabolism, which is evident from flexibility in their metabolism and **metabolic redundancy.** Not only that, **metabolic flux** (rate of movement of metabolites in a pathway) should also be regulated according to the need of the cell, tissue, or the organism. The metabolic flux is achieved through regulation of metabolism by pacemaker enzymes which are responsible for

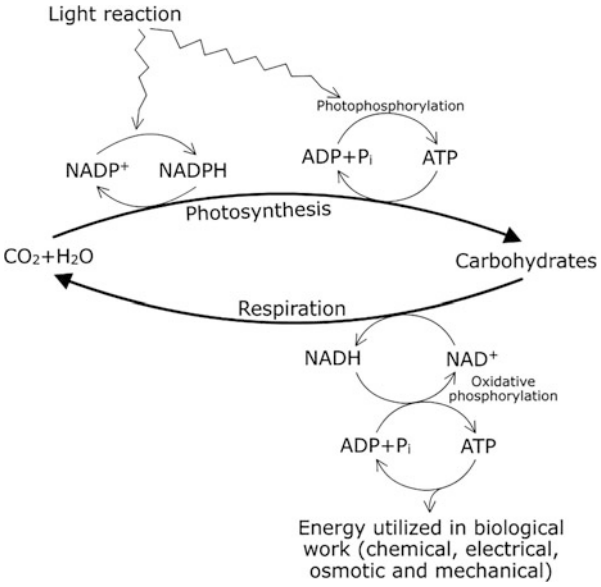


Fig. 4.2 Role of mobile electron carriers NAD^+/$NADP^+$ and ATP (the energy currency of the cell)

catalyzing rate-determining steps of metabolic pathways. Understanding the regulation of such enzymes at the expression of gene level or at the level of protein degradation would help in producing plants with altered metabolism (**metabolic engineering**). Many enzymes involved in a pathway have been proposed to exist as multi-enzyme complexes (**metabolons**) as means of **metabolic channeling**. Metabolic channeling allows direct transfer of biosynthetic intermediates from one enzyme to another in a pathway, minimizing their loss due to diffusion. Each cell compartment provides optimal conditions for specific metabolic pathways to occur at the optimal level (Fig. 4.3). Exchange of metabolites between the compartments is regulated by the transporters localized in the membranes. Plants are unique in having plastids which house the enzymes for photosynthesis. Besides photosynthesis, the enzymes for lipid and terpenoid biosynthesis, for biosynthesis of chlorophyll and related pigments, and for starch biosynthesis as well as many enzymes of nitrogen metabolism are also present. Both plastids and cytosol contain enzymes of glycolysis as well as for oxidative pentose phosphate pathway. Besides occurrence of various metabolic activities among cell organelles, various metabolic processes are also compartmentalized between soluble phase and the membranes. Thus, enzymes required for CO_2 reduction are present in stroma of the chloroplasts, while those involved in harvesting the solar energy and electron transport process are localized in the thylakoids.

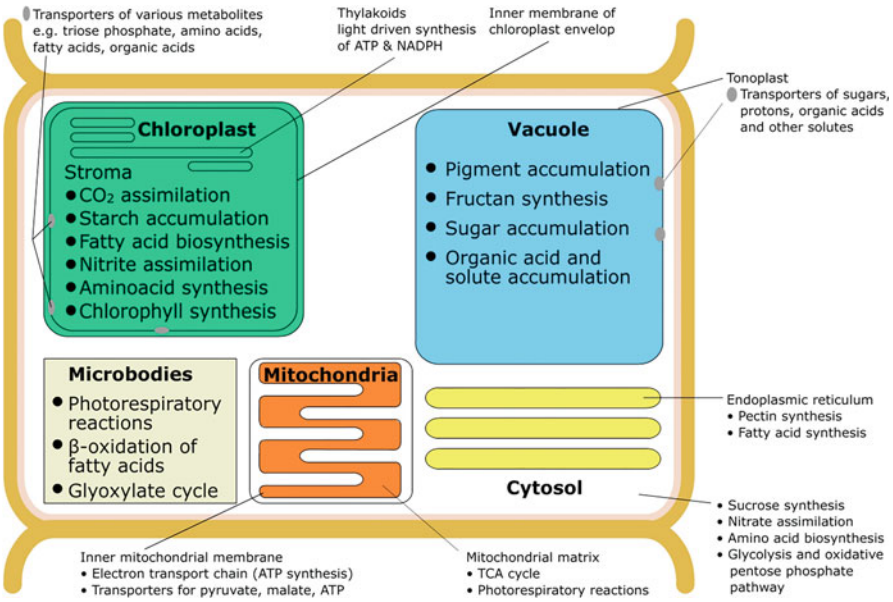


Fig. 4.3 Compartmentalization of metabolic pathways in a plant cell

4.1 Basic Energetic Principles that Govern Metabolism

Sun is the ultimate form of energy for most carbon-based life forms. Thermonuclear fusion reactions in sun convert four protons ($4H^+$) to one helium (He). During this conversion, there is 0.72% loss in total mass of H^+ (atomic weight of H^+ is 1.0079, while helium has an atomic weight of 4.0026). The missing mass is converted into energy in the form of electromagnetic radiations. Flow of energy is central to maintenance of life (Fig. 4.4 and 4.5). A living cell is a **system** in which all the reactants and products of a reaction are present along with the solvent. It is neither an **isolated** nor a **closed** system since there a continuous exchange of energy and matter with the surroundings, making it an **open** system. The science which deals with energy transduction within a living system is called **bioenergetics**. An understanding of integration of bioenergetics with biochemical reaction is central for understanding cell physiology. The energy released during reactions is utilized by the cell to perform work, e.g., for creation of proton gradient across the membrane. To understand the energy transduction within a cell, we need to revise the laws of thermodynamics (Box 4.1).

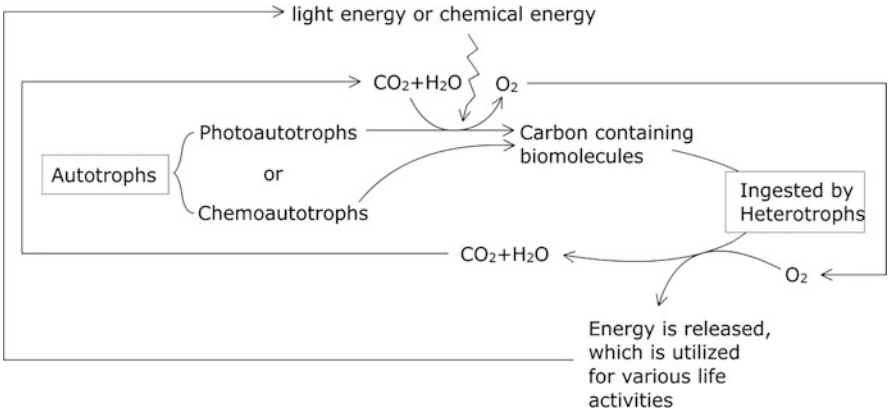


Fig. 4.4 Global energy cycle

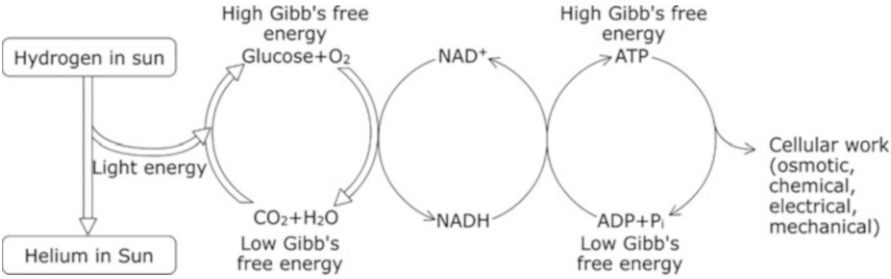


Fig. 4.5 Sun is the ultimate source of energy. Autotrophs are able to convert solar energy into organic compounds. These compounds are oxidized and are the source of ATP, which primarily is used for cellular work

Box 4.1: Laws of Thermodynamics

First law of thermodynamics states that the total energy of the universe is constant and it can neither be created nor destroyed. Different forms of energy such as light energy, chemical energy, thermal energy, mechanical energy, etc. are interconvertible. A living cell is an open system which can exchange energy and the matter with the surroundings. According to the second law of thermodynamics, there is increase in disorder during any spontaneous reaction which is coupled with release in energy. Energy is required to put the system in order. Extent of disorder of any system is measured by **entropy**, a term which was coined by the German physicist Rudolf Clausius. It is a thermodynamic quantity which represents the amount of energy that is no longer available for doing mechanical work. Higher is value of entropy, high is the disorder of the

(continued)

Box 4.1 (continued)
system and lesser energy will be available to do the work. **Enthalpy** (H) refers to the total potential energy of a molecule which is determined by its chemical structure. It includes the number and the type of chemical bonds which make up the molecule. During any spontaneous reaction, a complex molecule (having more ordered structure) is converted to the simpler molecules (having less ordered structure), which is coupled with release of energy. In a living cell, which is an isothermal system, released energy may be utilized for doing work. Thus, in an isothermal system out of total energy of a system (H), only some amount of energy is available for doing work, which is called as **Gibbs free energy**, in the, honor of J. Willard Gibbs, who developed the theory of energy exchanges during chemical reactions. Relationship between these thermodynamic quantities at the absolute temperature (T) can be expressed as,

$$G = H - TS$$

Since measurement of absolute values is not possible, changes in these three thermodynamic quantities during a reaction are expressed as

$$\Delta G = \Delta H - T\Delta S$$

where ΔG refers to change in free energy during a chemical reaction, ΔH is the change in enthalpy, and ΔS is change in entropy. Since conditions which occur in a biological system include constant temperature and pressure, *free energy is defined as the energy isothermally available to do work.* ΔG refers to the difference in free energy of the products and the free energy of the reactants during a reaction. In a spontaneous reaction, ΔG value is negative, i.e., energy is released during reaction (exergonic). On the contrary, a positive value of ΔG indicates the reaction to be endergonic and would require input of energy. In case value of ΔG is 0, the reaction will be at equilibrium and will occur in either forward or backward direction depending upon concentrations of reactants and the products. ΔG values are expressed in terms of calories (cal) or joules (J) per mole (1 cal = 4.184 J). Joule is the official term used now. The magnitude of free energy change is also determined by the conditions in which the reaction is taking place, which include the molar concentrations of the reactants, pH, and temperature of the medium. **Standard free energy** change refers to free energy change during a reaction that occurs at physiological pH (7.0), at 25 °C and under conditions when both reactants and products are at unit concentrations (1 M) and is expressed as ΔG^0. Relationship between ΔG and ΔG^0 in a reaction, which is not at equilibrium, is expressed as

(continued)

Box 4.1 (continued)

$$\Delta G = \Delta G^{0\prime} + RT \ln K_{eq}$$

where R is the universal gas constant, T is the absolute temperature, and K_{eq} is the equilibrium constant of a reaction. The standard free energy changes are directly related to the equilibrium constant. For a reaction at equilibrium, value of ΔG will be 0. The relationship between ΔG^0 and K_{eq} is expressed as

$$\Delta G^{0\prime} = -RT \ln K_{eq}$$

The standard free energy change ($\Delta G^{0\prime}$) is a constant value which tells us a characteristic unchanging value for a given reaction, while actual free energy change (ΔG) is a function of reactants and products concentrations in the cell and the temperature prevailing at the time of occurrence of chemical reactions. Value of ΔG changes with the reaction proceeding spontaneously toward equilibrium and becomes 0 at the point of equilibrium. Thus, criterion for spontaneity of the reaction is ΔG and not $\Delta G^{0\prime}$. Equilibrium of the reaction plays a very important role. Value of $\Delta G^{0\prime}$ of a reaction at equilibrium is zero. It is important to maintain a reaction far from equilibrium since amount of work done depends on how far a reaction is maintained away from equilibrium. Thus, maintaining a disequilibrium is key to all the life processes.

The standard free energy changes occurring during sequential reactions in a metabolic pathway are additive. The overall change in standard free energy ($\Delta G^{0\prime}_{total}$) in two sequential reactions having standard free energy change values of $\Delta G^{0\prime}_1$ and $\Delta G^{0\prime}_2$, respectively, sharing common intermediate will be $= \Delta G^{0\prime}_1 + \Delta G^{0\prime}_2$. This explains how a thermodynamically unfavorable reaction (endergonic) is driven forward by its coupling with the thermodynamically favorable reaction (exergonic) through a common intermediate.

$$\text{Glucose} + P_i \rightarrow \text{Glucose 6-phosphate} + H_2O \qquad \Delta G^{0\prime}_1 = 13.8 \text{ kJ/mol}$$

$$ATP + H_2O \rightarrow ADP + P_i \qquad \Delta G^{0\prime}_2 = -30.5 \text{ kJ/mol}$$

These two reactions share the common intermediates P_i and H_2O. Overall reaction is the sum of these reactions, which can be written as,

$$\text{Glucose} + ATP \rightarrow ADP + \text{Glucose 6-phosphate}$$

Overall standard free energy change ($\Delta G^{0\prime}_{total}$) is obtained by adding the values of $\Delta G^{0\prime}_1$ and $\Delta G^{0\prime}_2$.

(continued)

Box 4.1 (continued)

$$\Delta G^{0\prime}_{total} = \Delta G^{0\prime}_{1} + \Delta G^{0\prime}_{2}$$
$$= 13.8\ \text{kJ/mol} + (-30.5\ \text{kJ/mol}) = -16.7\ \text{kJ/mol}$$

Overall reaction is exergonic. Exergonic ATP hydrolysis is coupled to the endergonic reaction involving synthesis of glucose 6-phosphate. The common intermediate-strategy is used by all of living cells.

There are many cellular reactions, which cannot occur spontaneously without input of required energy. These reactions are coupled to the energy releasing reactions. This is possible as long as the net ΔG (free energy change) value of the combined reactions is negative. Such reactions are known as coupled reactions. Coupled reactions occur simultaneously since one reaction is necessary for the other one to occur. These are reactions, which share common intermediates, and the product of one reaction becomes the reactant for another. For example, the product of ATP hydrolysis is the reactant for phosphorylation of glucose. Coupled reactions can be either energy-coupled reactions or oxidation-reduction reactions.

4.2 Energy Coupled Reactions

Adenosine triphosphate (ATP) is synthesized from ADP and P_i during exergonic reactions and is hydrolyzed to provide energy for reactions requiring energy. It was first isolated from muscles in 1929 by Cyrus H. Fiske in the USA and Yellapragada Subbarao and Karl Lohman in Germany independently. Fritz Lipmann along with Herman Kalckar proposed in 1941 the possible involvement of ATP in bioenergetic processes in cells. Lipmann was awarded Nobel Prize in 1953 for his work. He introduced the "squiggle" notation (~) for the energy-rich bonds of biomolecules such as ATP and ADP. A high-energy bond generally refers to unstable or labile bond. For the sake of simplicity, high-energy bond refers to ATP or any other phosphate compound with large, negative, standard free energy. The P-O bond itself does not contain energy. Free energy that is released from hydrolysis of P-O bond does not come from breaking of specific bond. It results from the products of the reaction that have lower free energy content than the reactants. ATP, ADP, AMP are charged molecules which are not able to diffuse through cell membrane. Since cells cannot get them from outside, each cell synthesizes the entire molecules by itself. Intracellular ATP/ADP exchange occurs between different compartments of the cell.

4.2.1 Structure of ATP

The phosphate groups attached to 5′ hydroxyl group of a nucleoside result in formation of trinucleoside phosphates, which include UTP, GTP, CTP, and ATP. ATP is the nucleoside triphosphate most widely used as a high-energy phosphate

compound. The three phosphates are labeled as α, β, and γ (Fig. 4.6). The bond between ribose and α-phosphate is an ester bond, while α-β and β-γ linkages are **phosphoanhydrides.** Hydrolysis of ester bond yields about 14 kJ/mole under standard conditions, while phosphoanhydride bonds yield 30.5 kJ/mole. However, actual free energy change during the hydrolysis of phosphoanhydride bonds in cellular conditions also known as **phosphorylation potential** (ΔG_p) is very different since concentrations of ATP, ADP, and P_i are not identical and is very much lower than 1.0 M. Secondly since Mg^{2+} forms complex with ATP, it is the Mg-ATP which is the true substrate for enzyme-catalyzed reactions (Fig. 4.6). ATP is not complexed with Mg^{2+} when being transported across membranes within the cell.

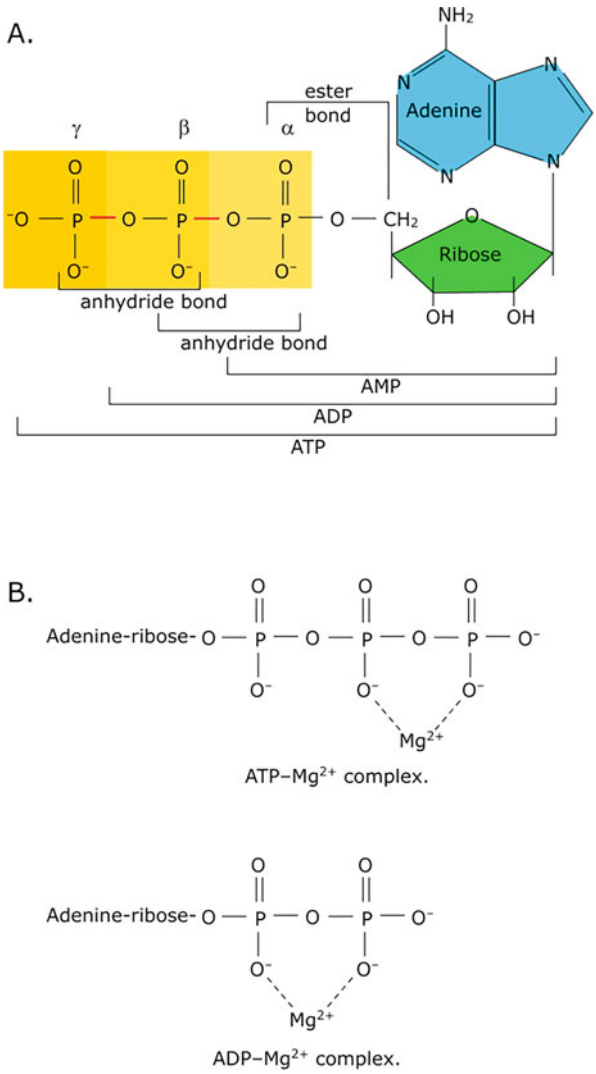


Fig. 4.6 (**A**) Structure of ATP; the two phosphoanhydride bonds γ-β and β-α are high-energy bonds. (**B**) Mg^{2+} is complexed with ATP and ADP. Mg^{2+} partially shields the negative charge and influences the conformation of phosphate groups in molecules, such as ATP and ADP

4.2.2 ATP Is the High-Energy Molecule

Standard free energy change ($\Delta G^{\circ\prime}$) is determined by unstability or stability of the reactants/products of the chemical reaction and also by subsequent fate of the products. Large negative value of standard free energy ($\Delta G^{\circ\prime}$) of ATP hydrolysis is associated with unstability of the reactant (ATP) and stability of its products (ADP + P_i) of hydrolysis. The electronegative oxygen in the P=O bond of ATP molecule attracts electrons creating a partial negative (δ^-) charge on oxygen atom and a partial positive (δ^+) charge on phosphorus atom. As a result, two strongly electron withdrawing groups must compete for the lone pair of electrons of its bridging oxygen making the molecule less stable than its hydrolysis products. At the physiological of around pH 7.0, ATP molecule has four negative charges because of which an electrostatic repulsion is established between the adjacent oxygen atoms causing a strain on the phosphoanhydride bond (Fig. 4.6). A sufficient internal energy is required to overcome this repulsion like charges. At the time of hydrolysis of ATP, the internal energy which is required to maintain the strained phosphoanhydride bonds is released resulting in large negative value of $\Delta G^{\circ\prime}$ of the reaction. Low $\Delta G^{\circ\prime}$ associated with hydrolysis of ester bond of AMP is due to fewer electrostatic repulsion forces associated with it. On the contrary, at the time of ATP synthesis, electrostatic repulsion between two negatively charged groups needs to be overcome, which requires expenditure of energy. The phosphoanhydride bond formation can be compared with the analogy of compressing a spring, which requires work to be done, but as soon as the hand is removed, energy is released in the form of popping up of the spring. Another reason for ATP to be a high-energy molecule is that the products of hydrolysis of ATP, i.e., ADP and P_i, are **resonance stabilized**. Stability of the products increases with increase in resonance, which results increase in entropy and decrease in the energy level of the products of the reaction. As a result, there is release of energy coupled with ATP hydrolysis (Fig. 4.7). Probability of reverse reaction decreases due to stability of the products. In aqueous environment of the cell, hydration of the reactants and products also plays a significant role in ΔG of the reaction. Thus, large negative value of $\Delta G^{\circ\prime}$ of ATP hydrolysis is due to electrostatic repulsion in the molecule and resonance and hydration of the products.

It is not only the intrinsic property of ATP molecule that determines high value of ΔG of its hydrolysis but also the cellular reactions, which are responsible for holding high cellular ATP concentrations far above than required to maintain the equilibrium of the hydrolysis reactions. ΔG of a reaction is also determined by how far the rate constant of the reaction is from the **equilibrium constant** at a given time. Potency of the hydrolysis of ATP is lost at the equilibrium of the reaction; thus it is necessary that effective intracellular concentration of ATP should be maintained high so as to keep the rate of the reaction higher than the equilibrium rate constant. When the ATP level is dropped, not only the amount of fuel decreases, but there is also a decline in phosphorylation potential of the molecule. Thus, living cells have developed effective mechanisms to maintain high intracellular ATP concentrations. Even though

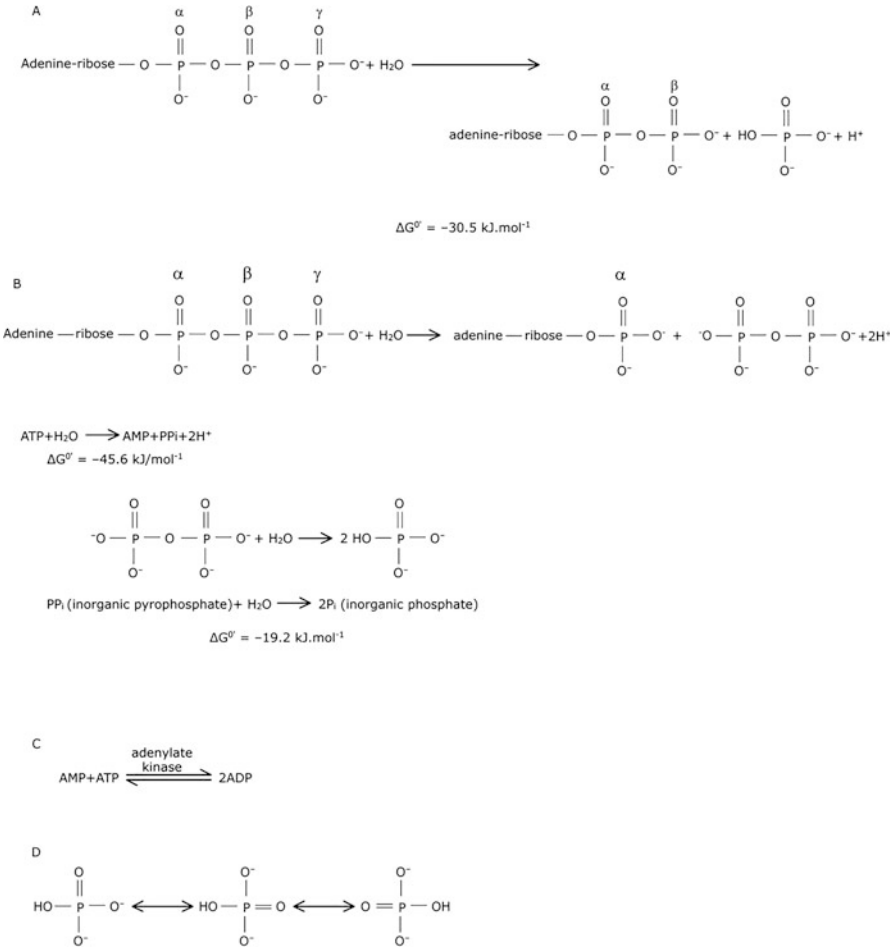


Fig. 4.7 (**a**) ATP hydrolysis leading to formation of ADP and P_i; (**b**) ATP hydrolysis leading to formation of AMP and P_i; (**c**) conversion of AMP to ADP in a reaction catalyzed by adenylate kinase. (**d**) Products of hydrolysis of ATP (P_i) are resonance stabilized. This increases entropy and therefore decreases the energy level of the products so that on breaking of the bond there is larger yield of energy. In an inorganic phosphate ion, all the P-O bonds are partially double-bonded in character rather than proton being associated with any one oxygen, resulting in increase in entropy and a lowering of their energy level. ↔ symbol indicates the structure which exists in an intermediate one in which all oxygen have partial negative charge and proton is not associated with any form

ATP hydrolysis is a highly exergonic reaction, it is kinetically stable because high amount of activation energy is required for uncatalyzed hydrolysis (200–400 kJ/mol) of the phosphoanhydride bonds of the molecule. Enzymes lower the requirement of activation energy, and phosphoryl group transfer occurs either to water or to any other acceptor of the group.

4.2.3 ATP Is the Energy Currency of the Cell

Besides ATP, there are other phosphoryl group carrying compounds which can be divided into two categories. One category of compounds includes those which have $\Delta G^{\circ\prime}$ of hydrolysis larger than −25 kJ/mol. These compounds are high-energy phosphate compounds, while the other category of compounds are low-energy phosphate compounds whose hydrolysis is associated with negative $\Delta G^{\circ\prime}$ value of about 9–20 kJ/mol. ATP serves as the energy currency in the cell since it has $\Delta G^{\circ\prime}$ value—30.5 kJ/mol. It occupies intermediate position in phosphoryl group transfer potential. It can carry energy from high-energy phosphate compounds, which are produced during catabolism (such as phosphoenolpyruvate) to compounds such as glucose, converting them into more reactive compounds, such as glucose 6-phosphate (Fig. 4.8). Transfer of phosphoryl group results in adding more free energy to a molecule, which is given up during subsequent metabolic reactions. This activation is called "priming" of the molecule. ATP itself can be synthesized by coupling with exergonic reactions of hydrolysis of compounds which have higher value of $\Delta G^{\circ\prime}$ of hydrolysis than ATP itself. ATP synthesis by this means is referred to as **substrate-level phosphorylation** and has been dealt in the chapter dealing with ATP synthesis.

During group transfer all three phosphate groups of ATP molecule can take part. Depending on the site of action of **nucleophilic** group (e.g., –OH group of attacking molecule) on ATP molecule, it can be phosphoryl (attack at γ-phosphate), pyrophosphoryl (attack at β-phosphate), or adenylyl moiety (attack at α-phosphate) transfer. It is the phosphoryl group ($-PO_3^{2-}$) of ATP which is transferred and not the phosphate (PO_4^{2-}) since oxygen (-O-) that bridges the group with the attacking molecule does not come from ATP; rather it comes from the attacking molecule. Free energy release during pyrophosphoryl (PP_i) transfer (hydrolysis of α-β

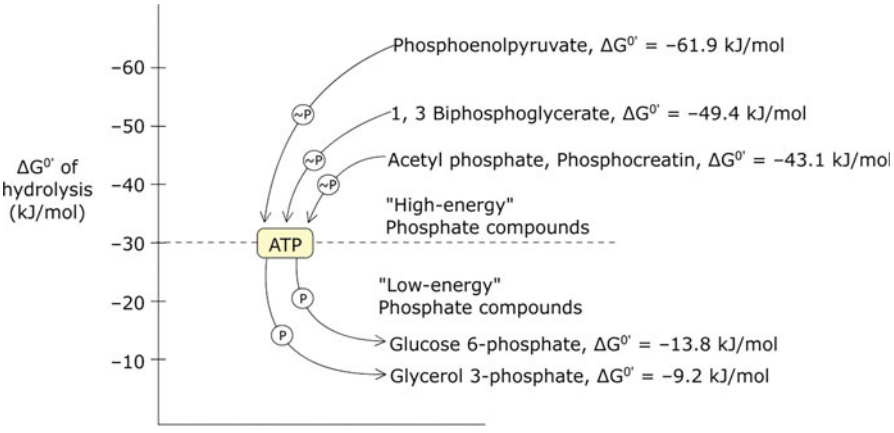


Fig. 4.8 ATP is the "energy currency" of the cell since free energy of hydrolysis ($\Delta G^{\circ\prime}$) of ATP is between the "high-energy" and "low-energy" phosphate compounds. Transfer of phosphoryl group from "high-energy" compounds to "low-energy" acceptor compounds occurs via ATP-ADP system

phosphoanhydride bond) is much more (~46 kJ/mol) than the hydrolysis of β-γ bond (~31 kJ/mol), making the reaction irreversible. PP_i formed is hydrolyzed to two P_i by the ubiquitous enzyme inorganic **pyrophosphatase**, resulting in further release of energy (19 kJ/mol). The adenylation reaction is particularly useful to drive thermodynamically unfavorable reactions, e.g., fatty acid activation. During fatty acid activation, first adenylyl (AMP) is transferred from ATP to carboxylic group of the fatty acids resulting in the formation of fatty acid adenylate and PP_i. The adenyl group is replaced by thiol group of Coenzyme A, resulting in the formation of thioester. Net free energy change in these two reactions is negative and energetically equivalent to hydrolysis of ATP to AMP and PP_i (−45.6 kJ/mol). AMP has to be converted to ADP since it the ADP, which is required for conversion to ATP (Fig. 4.7).

$$\mathrm{AMP} + \mathrm{ATP} \rightarrow 2\mathrm{ADP}$$

The reaction is catalyzed by **adenylate kinase** (or AMP kinase). Kinase is the term used for the enzymes which transfer the phosphoryl group from ATP to other molecules. Such reactions, in which hydrolysis of ATP is not involved, occur frequently during the metabolic pathway.

4.3 Reduction-Oxidation Coupled Reactions

Photoautotrophs utilize radiant energy from the sun to remove electrons from water (oxidation of water) and transfer them to CO_2 resulting in its reduction. As a result, solar energy is trapped in the form of reduced organic molecules:

$$6CO_2 + 12\ H_2O \rightarrow C_6H_{12}O_6 + 6O_2 + 6H_2O$$

Chemoautotrophs derive energy from the oxidation of the chemical compounds. For doing work, organisms obtain energy by removing electrons from organic molecules (oxidation) and recycle them to O_2 (reduction) resulting in synthesis of water.

$$C_6H_{12}O_6 + 6O_2 \rightarrow \quad 6CO_2 + 6H_2O$$

Thus, there is global recycling of O_2 and CO_2 accompanied with the **oxidation-reduction** reactions which are responsible for energy release and energy conservation. In a cell, many reactions involved in energy transductions require electron flow from one molecule to another. These are called **reduction-oxidation reactions or redox reactions** (Box 4.2). In cells oxidation-reduction reactions are part of metabolic pathways. Electrons removed during hundreds of oxidative reactions are channeled through only just a few types of universal electron carriers such as NAD^+, $NADP^+$, FMN, and FAD (Figs. 4.9, 4.10 and 4.11). These undergo reversible oxidation and reduction in many of the redox reactions of cellular metabolism.

Box 4.2: Redox Potential and Redox Couples
In an oxidation-reduction reaction,

$$AH_2 + B \rightarrow A + BH_2$$

AH_2 is oxidized to A and B is reduced to BH_2. The reaction can be understood as two half reactions; oxidation of AH_2 (removal of electrons coupled with removal of protons) and reduction of B (acceptance of electrons and protons) separately,

$$AH_2 \rightarrow A + 2e^- + 2H^+$$

$$B + 2e^- + 2H^+ \rightarrow BH_2$$

AH_2 is the reductant (electron donor), while B is the oxidant (electron acceptor) in this redox reaction. In any redox reaction electron transfer may or may not be coupled with proton transfer. The following redox reaction involves transfer of electrons only,

$$Fe^{3+} + Cu^+ \rightarrow Fe^{2+} + Cu^{2+}$$

Cu^+ is the reductant since it is the electron donor while Fe^{3+} as an oxidant as it is the electron acceptor.

The two half reactions can be written as,

$$Cu^+ \rightarrow Cu^{2+} + e^- \quad \text{(oxidation)}$$

$$Fe^{3+} + e^- \rightarrow Fe^{3+} \quad \text{(reduction)}$$

Both reactions occur simultaneously as oxidation of Cu^+ to Cu^{2+} is coupled with reduction of Fe^{3+} to Fe^{2+}. Cu^+ and Cu^{2+} are called conjugate **redox couple** since Cu^+ serves as electron donor while Cu^{2+} will serve as conjugate electron acceptor. In the similar way Fe^{3+}/Fe^{2+} will be another redox couple of the reaction.

There are four ways in reduction/oxidation, i.e., involving only electron transfer, as hydrogen atom (hydrogen atom consists of a proton and an electron), as **hydride** ion (H^-) (hydride ion has two electrons; net charge on hydrogen atom will be negative), e.g., hydride ion transfer in the reduction of $NAD^+/NADP^+$, involvement of oxygen in the redox reaction which is covalently incorporated in the product. **Reducing equivalent** term is used to express transfer of single electron, which participates in the redox reaction either as electron or hydrogen atom or hydride ion. Direction of electron flow is determined by affinity of the compounds for electrons. Electron will flow

(continued)

Box 4.2 (continued)
from the compounds having lower affinity to those which have higher affinity for the electrons. Relative affinity of compounds for the electrons is expressed as their **redox potential. Standard reduction potential (E^0)** is a measure of this affinity, which is expressed in volts and standard of reference is half-cell where hydrogen ion in aqueous solution is in equilibrium with hydrogen gas. The standard reduction potential (E^0) of the conjugate redox pair represents the potential difference at 1M concentration, 25 °C and pH 7.0 with reference to the standard (pH 0) hydrogen electrode.

The oxidized form of a redox couple with a large positive standard reduction potential has a high affinity for electrons and is a strong oxidizing agent, while its conjugate reductant is a weak electron donor. Electron flow occurs from the redox couple with less positive reduction potential to the redox couple with more positive values. Thus, direction of the electron flow in between electron donor of a redox couple to electron acceptor of another redox couple is determined by difference in their standard reduction potential (ΔE^0). It is measured in volts (V).

$$\Delta E^0 = E^0_{(e^- \text{ acceptor})} - E^0_{(e^- \text{ donor})}$$

Standard reduction potential is used to calculate free energy change during the electron transfer which can be calculated by the following formula,

$$\Delta G = -nF\Delta E \text{ or } \Delta G^{0'} = -nF\Delta E^{\mathbf{0}}$$

where n is the number of electrons transferred in the reaction and F is Faraday's constant (a proportionality constant that converts volts to joules (F = 96,480 J/V.mol)).

NAD^+ and $NADP^+$ are loosely bound with enzyme protein. So, they can move from one enzyme protein to another enzyme protein, while FMN and FAD are tightly bound to the enzyme protein and form prosthetic group of the enzyme. Besides these iron-sulfur proteins, cytochromes also have tightly bound prosthetic groups that undergo reversible reduction and oxidation on accepting and removal of electron. Quinones, such as ubiquinone and plastoquinone, serve as the mobile carriers of electrons as they become lipid soluble on being reduced. NADH produced in mitochondria during catabolic oxidative reactions is oxidized, and electrons which are removed are finally accepted by O_2. Since O_2 accepts one electron at a time, electrons removed in pairs (from a metabolite to NADH) are transferred further to other intermediate carriers that can undergo both two-electron and one-electron redox reactions. O_2 accepts electrons from cytochromes which facilitate one-electron transfer. Electrons move through various electron carriers in order of their increasing positive $\Delta E^{0'}$ values.

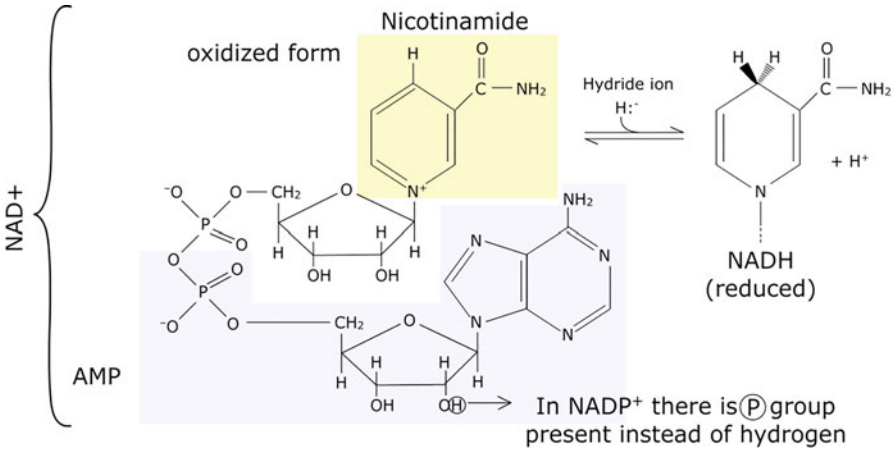


Fig. 4.9 Reduction of NAD^+ to NADH requires two-electron transfer. Only nicotinamide ring is affected. Hydride ion (a proton with two electrons) transfer results in reduction of NAD^+ to NADH. $NADP^+$ differs from NAD^+ only in presence of phosphoryl group on the 2′-hydroxyl group of ribose sugar. Plus (+) sign on NAD^+ and $NADP^+$ indicates that nicotinamide ring is in oxidized form and there is positive charge on the nitrogen atom of the ring. In many cells ratio of NAD^+ to NADH is high which favors hydride transfer to NAD^+ to form NADH. Contrary to this ratio of NADPH to $NADP^+$ is high which favors hydride transfer from NADPH to substrates. In most of the cells, total concentration of NAD^+ + NADH is about 10^{-5} M, while that of NADPH + $NADP^+$ is 10^{-6} M

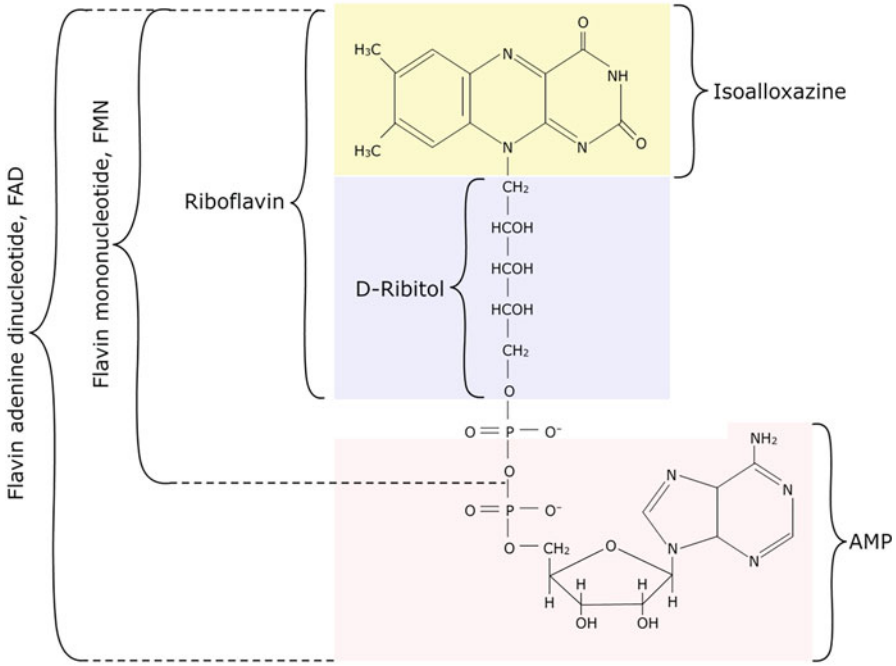


Fig. 4.10 Figure shows structures of riboflavin, flavin mononucleotide (FMN), and flavin adenine dinucleotide (FAD). Flavin coenzymes are stronger oxidizing agents than NAD^+ and $NADP^+$. These can be reduced both by single electron and two electron pathways. These are reoxidized by molecular oxygen

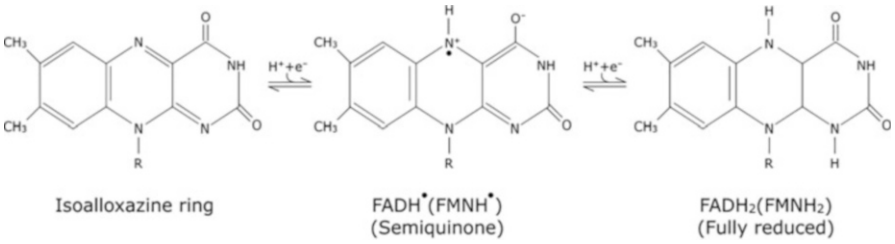


Fig. 4.11 Isoalloxazine ring of flavin nucleotide (FMN and FAD) undergoes reversible reduction. Unlike reduction of NAD^+ and $NADP^+$, reduction of these nucleotides occurs on accepting either one or two electrons in the form of one or two hydrogen atoms (each atom is in the form of an electron and one proton). On accepting one hydrogen atom, semiquinone form of isoalloxazine ring is formed. These are abbreviated as FADH (FMNH) which on accepting one more hydrogen atom is fully reduced to $FADH_2$ ($FMNH_2$). Since these nucleotides can participate in either one or two electron transfer reactions, flavoproteins are involved in a greater diversity of reactions

4.4 Enzymes

Enzymes are central to metabolism since these are the biocatalysts catalyzing almost all cellular reactions. These include slow but thermodynamically feasible reactions at ambient cellular conditions, which include biological pH, temperature, as well as the molar concentrations of the reactants and the products. The metabolic pathways need to be modified in response to the cellular needs. This occurs through regulation of the activity of various enzymes. Discovered in yeast for the first time, the biocatalysts were called "enzyme" by Wilhelm Kuhne in 1878 which in Greek means "in yeast" (*en,* in; *zyme,* yeast). Earlier Louis Pasteur had called these vital factors present in intact yeast cells as "ferments," since these were thought to be responsible for carrying out fermentation. Chemical nature of enzymes was not established till the time Sumner crystallized urease from jack beans and established their proteinaceous nature in 1926. Earlier enzymes were thought to be small biologically active molecules analogous to hormones. Sumner postulated that all enzymes were proteins. It was after John Northrop and Moses Kunitz had crystallized trypsin and pepsin in 1930 that Sumner's conclusions were widely accepted. Sumner was awarded the Nobel Prize in 1946. At the same time in a treatise entitled "enzymes", J.B.S. Haldane postulated that the weak bonding between enzyme and substrate might be responsible for the reactions catalyzed by them. Since then thousands of enzymes have been isolated and characterized. A new science called "enzymology" developed, which dealt with study of enzymes. Barring ribozymes (catalytic RNA molecules), all enzymes are proteins. While some of the enzymes consist of proteins only (simple proteins), in others a non-protein part is also part of their structures

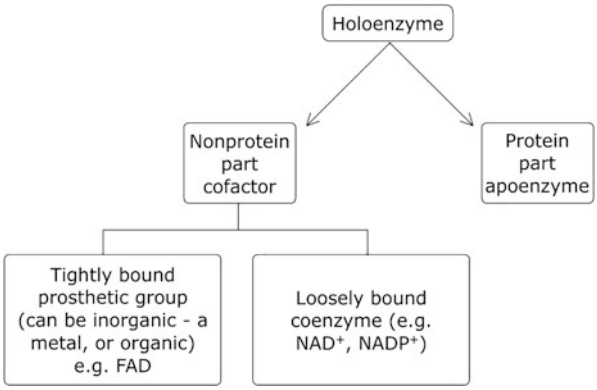


Fig. 4.12 Enzymes consist of non-protein part in addition to the proteinaceous structure

(conjugated proteins). The non-protein part of these enzyme-conjugated proteins is called **cofactor**. In case where cofactor is inorganic, such as metals (Mg^{2+}, Zn^{2+}, Fe^{2+}), the enzymes are called as metalloenzymes. The organic cofactors are called **coenzymes**. Cofactors may be loosely bound with the enzyme proteins or may be tightly associated through a covalent bond. Cofactors, which are tightly associated with the protein part of the enzymes, are called **prosthetic group**, which may be inorganic or organic in nature (Fig. 4.12). Sometimes both metal and the organic molecules are required as the cofactors for enzyme activity. In case of cytochromes, the prosthetic group **heme**, along with a metal ion (Fe^{3+}), is bound to enzyme protein through hydrogen bonding, hydrophobic interactions, and the covalent bonding to a specific site of the enzyme protein. The functionally active enzyme, in case of conjugated proteins, is called **holoenzyme,** and the protein portion of the enzyme is called **apoenzyme**. Loosely bound coenzymes, such as NAD^+ or $NADP^+$, are transiently associated with enzyme proteins. These function as **co-substrates**, which need to be regenerated maybe through independent reactions. Contrary to this in case of prosthetic groups, regeneration of the group occurs as a part of enzyme-catalyzed reaction. Catalytic activity of enzymes depends on the integrity of constituent protein conformation, which is determined by the primary, secondary, and tertiary protein structures. Besides, in case of enzyme molecule requiring two or more than two subunits, the intact quaternary structure is also important for the catalytic activity. Any factor, which is responsible for destroying the conformation, would lead to loss in their activity.

4.4.1 Nomenclature and Classification of Enzymes

After thousands of enzymes had been discovered, different strategies were adopted, namely:

(i) By adding suffix "-ase" to the name of the substrate: Substrate is the substance on which the enzyme acts upon. For example, enzymes acting upon proteins

were called proteinases, those acting upon lipids were called as "lipases," and the ones acting on nucleic acid were named as "nucleases." Specific names were also given to enzymes acting on specific substrates, such as "urease," "lecithinase," or "maltase," for the enzymes acting on urea, lecithine, or maltose, respectively.

(ii) Another strategy adopted to name the enzymes was to add suffix "-ase" to the kind of reaction catalyzed by the enzymes, e.g., isomerases (which catalyze isomerization), hydrolases (catalyzing hydrolysis reactions), transaminases (catalyzing transamination), etc.

(iii) Both of the above systems of naming the enzymes appeared inadequate since the naming was either based on the type of molecules on which the enzyme acted upon or the type of reaction catalyzed by them. Another system was adopted in which some of the enzymes were named both on the basis of substrate utilized and the reaction catalyzed by them. For example, succinic acid dehydrogenase signifies both the name of the substrate succinic acid as well as the reaction catalyzed by them dehydrogenation.

To maintain uniformity in naming enzymes the International Union of Biochemistry and Molecular Biology (IUBMB) set up an Enzyme Commission (EC) on enzyme nomenclature, which gave its first recommendations in 1961. Some of the recommendations given by EC are as follows:

1. Each enzyme can have a **trivial name**, which is short and is easy to use. The **systematic name** of the enzyme however should be formed according to the definite rules showing the action of the enzyme as much as possible. It should have two parts: the first name denotes the substrate, the second one with the suffix "-ase" which specifies the reaction catalyzed by them. Additional information, if there is any, is given in parenthesis. For example, malate dehydrogenase which catalyzes the following reaction:

$$\text{L} - \text{Malate} + \text{NAD}^+ \rightarrow \quad \text{Pyruvate} + \text{CO}_2 + \text{NADH} + \text{H}^+$$

The enzyme can be called L-malate: NADH oxidoreductase (decarboxylating).

2. All enzymes were classified into six classes depending upon the type of reaction catalyzed by them (Table 4.1). Each enzyme is given a **classification number**. The classification number is known as Enzyme Commission (EC) number assigned by the nomenclature committee of IUBMB. The classification number has four digits, e.g., if the classification number of an enzyme is a, b, c, and d, a stands for the number of the class given in the classification number, b is the

Table 4.1 Major classes of enzymes

Class No.	Name of the class	Nature of the reaction catalyzed
1.	Oxidoreductases	Catalyze transfer of hydrogen or oxygen atoms or electrons from one substrate to another, also called oxidases, dehydrogenases, or reductases. Substrate that is oxidized is electron donor. Systematic name is based on *donor: acceptor oxidoreductase*. Common name will be dehydrogenase except where electron acceptor is oxygen, then called oxidases
2.	Transferases	Catalyze group transfer reactions. Systematic names are formed according to the scheme *donor: acceptor group transferase*. Common name according to acceptor group transferase or donor group transferase
3.	Hydrolases	Catalyze hydrolytic cleavage of C-C, C-O, and C-N bonds and some other bonds including phosphoanhydride bonds. Common name in many cases formed by the name of the substrate with suffix "-ase"
4.	Lyases	Catalyze cleavage of C-C, C-O, C-N, or other bonds by elimination, leaving double bonds or rings, or catalyze addition of groups to double bonds. Systematic name is formed according to the pattern substrate group-lyase. Hyphen is important part of the name
5.	Isomerases	Catalyze transfer of groups within molecule to yield isomeric form. According to type of isomerism, these can be accordingly called isomerases, epimerases, mutases, etc. Subclass is formed according to type of isomerism and sub-subclass according to type of substrate
6.	Ligases	Catalyze joining together two molecules forming C-C, C-O, C-S, and C-N bonds by condensation reactions coupled with hydrolysis of ATP or similar triphosphate

number of subclass, c is the number of sub-subclass, while d represents the number of sub-sub-subclass which specifies the actual substrate of the enzyme which distinguishes it from other enzymes catalyzing similar reactions. In the following enzyme-catalyzed reaction:

$$\mathrm{ATP} + \mathrm{D} - \mathrm{glucose} \rightarrow \mathrm{ADP} + \mathrm{glucose}\ 6 - \mathrm{phosphate}$$

The trivial name of the enzyme is hexokinase/glucokinase, which is commonly used. The systematic name of the enzyme catalyzing the reaction is ATP: glucose phosphotransferase which indicates that the enzyme catalyzes transfer of phosphoryl group from ATP to glucose. The classification number (Enzyme Commission number) is E.C 2.7.1.1. The first number 2 signifies class number (transferase); the

second number 7 is about the phosphate group transferred; the third number 1 is about the number of the sub-subclass which signifies a phosphotransferase with a hydroxyl group as an acceptor, while the last digit 1 is the number of sub-sub-subclass which includes D-glucose as the phosphoryl group acceptor.

4.4.2 General Characteristics of Enzyme-Catalyzed Reactions

Most of the chemical reactions require presence of a catalyst and generally occur in extreme conditions, such as high temperatures or a low or high pH, or may require organic solvents. However, enzymes enable the chemical reactions to occur in ambient cellular conditions, i.e., at temperature 37 °C, biological pH of 6.5–7.5, and in aqueous medium. Enzymes are very efficient in catalyzing the reactions 10^6–10^{14} times faster than those not catalyzed by enzymes. One of the most catalytically potent enzymes, carbonic anhydrase has a **turnover number** of 600,000 per second. Even in a spontaneous thermodynamically feasible reaction where free energy of the product is less than that of reactants, i.e. $\Delta G^{\circ\prime}$ of the reaction is negative, the reaction does not start by itself. A substrate needs to be converted into an intermediate state before being converted to products. The intermediate state known as **transition state** refers to energy requiring molecular arrangement in a substrate molecule which makes it easy for the substrate to get converted to product. Free energy of transition state is higher than either substrate or the product. The starting point for either in forward or reverse direction is known as **ground state**. Difference in free energy of ground state and transition state is known as **energy of activation** of that reaction. During interconversion of substrate (S) and the product (P), change in free energy is plotted against progress of a reaction in a reaction coordinate diagram (Fig. 4.13). Substrate exists for a very short period in transition state, i.e., 10^{-14} to 10^{-13} of a second, after which it is converted to product. Energy of activation refers to the energy required to initiate a reaction. It constitutes the barrier to any chemical reaction. Higher activation energy of a reaction corresponds with slower reaction rate. Enzymes do not alter equilibrium constant but enhance reaction rates by lowering activation energies. Though activation energy is lowered, there is no alteration of $\Delta G^{\circ\prime}$ of enzyme-catalyzed reaction. Interconversion of two sequential reaction intermediates constitutes a reaction step. In case there are several reaction steps in a pathway, the one which requires highest activation energy is the rate-limiting step.

Transition state is achieved when enzymes bind with the substrates to form enzyme-substrate (ES) complex. The region of an enzyme molecule by which it binds with substrate is called the **active site**. Active site of an enzyme molecule is a three-dimensional structure formed due to the folding of constituent polypeptides leading to specific conformation of the molecule. Though active site occupies only a very small fraction of the large structure of an enzyme, it is needed to keep the interacting groups properly positioned so as to prevent active site from collapsing. Residues which constitute active site are responsible both for binding with substrate and holding it in specific orientation (binding residues), and also for carrying out

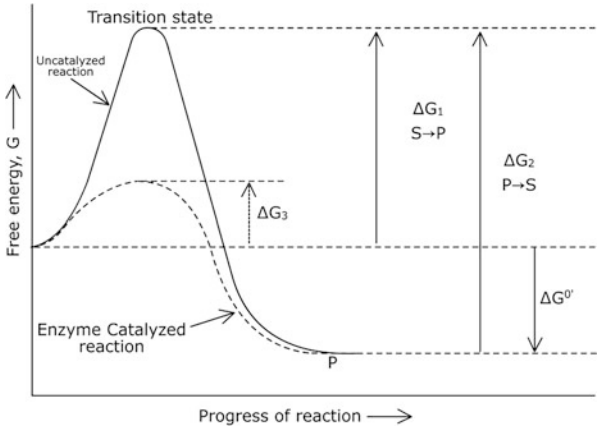


Fig. 4.13 Reaction coordinate diagram showing changes in free energy during uncatalyzed and enzyme-catalyzed reaction. Changes in free energy during reaction is plotted against progress of reaction. ΔG_1 is the energy of activation for uncatalyzed conversion of substrate to product ($S \rightarrow P$) which is required for bond breakage and formation. ΔG_2 is energy of activation for reversible reaction $P \rightarrow S$. $\Delta G^{o'}$ is the overall change in standard free energy in a spontaneous exergonic reaction during $S \rightarrow P$. ΔG_3 is the energy of activation for enzyme-catalyzed reaction

catalysis (catalytic residues). In some enzymes, binding and catalytic residues may be same. Any change in protein conformation will result in alteration of the structure of active site, and enzyme will not be able to carry out catalysis. Enzymes differ from other catalysts in being highly specific for a particular substrate. Specificity is derived from formation of many weak interactions between the active site of the enzyme and the substrate. Specific groups of R-side chains of both binding and catalytic residues interact with specific substrate, which provides specificity to the enzyme-catalyzed reactions. For example, if a hydroxyl group of a substrate interacts with a specific residue of the active site, any compound lacking a hydroxyl group will be a poor substrate for that enzyme. Many enzymes act only on one biological substrate (absolute substrate specificity); others act on broader range of substrates, which are structurally similar (relative group specificity). Glucose 6-phosphatase catalyzes hydrolysis of only glucose 6-phosphate, while acid and alkaline phosphatase can act on various phosphorylated substrates, thus displaying absolute substrate specificity and relative group specificity, respectively. Hexokinase adds a phosphate group to D-glucose and not to its optical isomer (L-glucose) displaying stereospecificity. Stereospecificity makes them unique and highly useful in pharmacology industry. This property of enzyme is due to their inherent chirality (proteins consist of only L-amino acids), which leads to formation of asymmetric active site.

Binding of substrate with active site of the enzyme involves non-covalent bonds such as ionic, hydrogen, and hydrophobic bonds and van der Waals interactions. It is the **binding energy** which is responsible for lowering of activation energy. Two models have been proposed to describe the binding process. According to **lock and key** model proposed by Emil Fischer in 1894, there is a structural similarity between

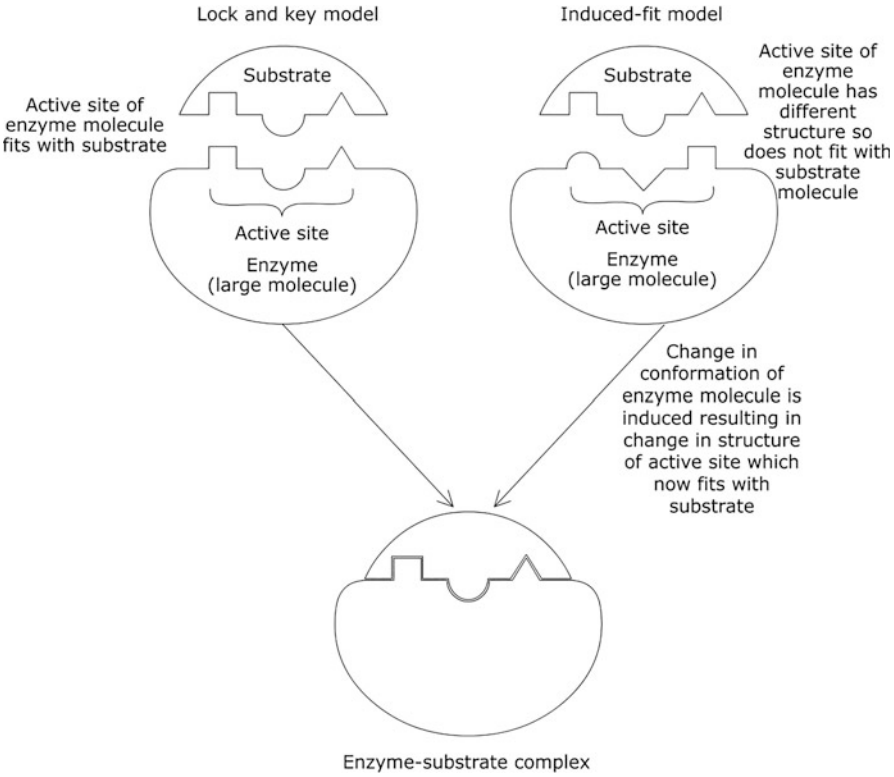


Fig. 4.14 Two proposed models of enzyme action: lock and key and induced fit

active site of the enzyme and the substrate. As there is a specific key, which fits into the grooves of a lock, a compound having a unique structure, which fits into the active site, will be the substrate of the enzyme (Fig. 4.14). Compounds having structural similarity to the substrate have been found to inhibit the enzyme activity (**competitive inhibition**). A competitive inhibitor binds with the active site of the enzyme forming enzyme-inhibitor complex, thus preventing the binding of the substrate molecule, which does not get dissociated to form products. However, the model suggests structural rigidity for the enzyme, which is otherwise a dynamic structure. Change in protein conformation is possible due to formation and breaking of non-covalent bonds giving flexibility to the enzyme molecule. X-ray studies have indicated that the active sites undergo conformational changes on binding with substrate. In 1959, Daniel E. Koshland proposed **induced-fit** model, according to which the active site of an enzyme is flexible. Presence of a substrate induces a conformational change resulting in alteration of the active site, which can now bind with the substrate. "Induced-fit" model is more attractive since it provides flexibility and dynamicity to enzyme molecule. The model was first established for the enzyme **hexokinase**. Hexokinase catalyzes transfer of phosphoryl group from ATP to

glucose. Active site of enzymes may be complimentary not to the substrate rather to transition state of the substrate. Substrate in transition state binds more tightly with the active site, resulting in lowering of activation energy requirement in enzyme-catalyzed reactions. **Transition state analogs** (molecules which have structure similar to transition state) have been found to bind with active site of the enzyme more tightly than either the substrate or the product. The fact that active site is less perfect fit to the substrate than to the transition state, binding of substrate will cause strain on the substrate molecule to fit properly into the active site which favor formation of its transition state resulting in lowering of activation energy. The products are released since these bind less tightly with the active site resulting in enhancement of rate. A very small reduction in activation energy can increase rate of the reaction many times. Decrease in activation energy by 84 kJ/mole by urease can result in increase in reaction rate by a factor of 10^{14}.

4.4.3 Enzyme Kinetics

Enzyme kinetics is the study of enzymes by determining their reaction rates. Laboratory measurement of rate of enzyme-catalyzed reaction is called enzyme assay. Enzyme assays are developed either to measure the amount of substrate used up during the reaction or amount of products formed in a unit time. Measuring amount of products formed is preferred since it is a direct method. In case the product formed is colored or produces colored compounds on reacting with some chemical, colorimetric assay is possible. Enzyme assay by spectrophotometric method is possible for the enzymes which utilize NAD^+ or NADH during a reaction. Since NADH (not NAD^+) has absorption peak at 340 nm, change in absorption of that wavelength will indicate appearance or disappearance of NADH. Rate of an enzyme-catalyzed reaction decreases with time because of either depletion of substrate or accumulation of products while enzyme is kept constant. The decrease may also be due to denaturation of protein in case of a sensitive enzyme. The most rapid reaction rate is observed at the start of reaction and is known as **initial velocity** (v_0). v_0 is used in enzyme kinetic studies. Adrian Brown had started enzyme kinetic studies in 1902. Substrate concentration is one of key factors affecting velocity of enzyme-catalyzed reaction. In case initial velocity of a reaction (v_0) is plotted against substrate concentration [S], the graph obtained is hyperbolic (Fig. 4.15). At low substrate concentration, v_0 is considered as function of [S]. Increase in v_0 becomes smaller with increasing [S] until a plateau-like region for v_0 is achieved which is close to maximum velocity (V_{max}), beyond which substrate concentrations do not increase the reaction rate substantially. V_{max} is the function of the amount of enzyme present in a given experiment. Briggs and Haldane introduced the concept of steady state in 1925 when P is produced at the same rate at which S is consumed. Lower region of the graph displays first-order kinetics since increase in v_0 is proportional to increase in substrate concentration. At lower substrate concentrations, active sites of the enzyme molecules are not saturated and are free to bind with the substrate molecules. It is this transient pre-steady phase when the concentration of ES builds

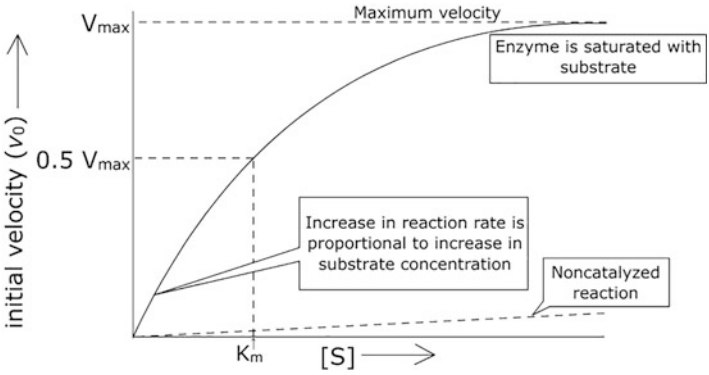


Fig. 4.15 Effect of substrate concentration on the reaction rate catalyzed by an enzyme following Michaelis-Menten kinetics. K_m (Michaelis constant) is a constant, which is the substrate concentration at which velocity of the reaction is half of the maximum. The dashed line which is below represents the reaction rate of a non-catalyzed reaction which has been given for comparison

up for a very short duration and generally lasts microseconds. Steady state is achieved when all of the enzyme molecules are saturated and the reaction becomes independent of further increase in substrate concentrations. Since pre-steady state lasts only for a very short time, v_0 generally reflects steady state, and analysis of these reaction rates refers to steady-state kinetics. The reaction displays mixed order in the intermediate portion of the curve when there is increase in reaction rate with increasing substrate concentration, but increase is not proportional with substrate concentration. Victor Henri in 1903 had proposed the idea of formation of enzyme-substrate complex as an explanation for the kinetic pattern of the enzyme-catalyzed reactions. This was further expanded in 1913 by Leonor Michaelis and Maud L. Menten into a general theory of enzyme action. An enzyme displaying this kinetics is referred as **Michaelis-Menten enzyme** and the kinetics as hyperbolic kinetics or **Michaelis-Menten kinetics**. In 1913, Michaelis and Menten proposed a general theory of enzyme action and enzyme kinetics. Hyperbolic kinetics of enzymes can be expressed algebraically by Michelis-Menten equation.

Derivation of the Michaelis-Menten equation has been given in the Box 4.3. Enzymes initially interact with the substrate in a relatively faster reaction forming enzyme-substrate complex [ES]. This (ES) then breaks down in a slower second step to yield free enzyme and the product. Both reactions are considered reversible reactions. The substrate concentration at which the velocity of an enzyme-catalyzed reaction is half of its maximum is defined as **Michaelis constant** (K_m). K_m is expressed in mM. For many enzymes, K_m is in the range of 10^{-4} to 10^{-6} M. Measured values of K_m can provide an estimate of intracellular concentration of the substrate. Generally, most of the enzymes function at subsaturating levels in the cell. In case enzymes can use different substrates, their K_m values can be used for differences in their relative affinity for the substrates. Greater K_m value indicates lesser affinity of enzyme for the substrate and vice versa. An enzyme's biological

Box 4.3: Derivation of Michaelis-Menten Equation

Michaelis-Menten theory explains the course of enzyme catalyzed reaction as follows:

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

Assuming that reverse reaction P→S is negligible, v_0 can be determined by breakdown of [ES]:

$v_0 = k_2[ES]$ (i)

Since neither k_2 nor [ES] can be measured in a reaction, an alternative expression was found.

- Rate of formation of [ES]

$$\frac{d[ES]}{dt} = k_1([Et] - [ES])[S]$$

- Rate of breakdown of [ES]

$$-\frac{d[ES]}{dt} = k_{-1}[ES] + k_2[ES]$$

- Since initial rate (v_0) represents steady state, i.e., in which [ES] is constant—i.e., rate of formation of ES is equal to rate of its breakdown,

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

- Equation is simplified to find value of [ES]

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

Since rate constants can be combined into one expression Km, which is defined as Michaelis constant.

By substituting value of [ES] in equation (i),

$v_0 = \frac{k_2[E_t][S]}{[S]+Km}$ (ii)

Maximum velocity (V_{max}) occurs when the enzyme is saturated, i.e.,

$[ES] = [E_t]$

$V_{max} = k_2[E_t]$

Substituting the value in equation (ii),

(continued)

Box 4.3 (continued)

$v_0 = \frac{V_{max}[S]}{Km+[S]}$

This is rate equation for one-substrate enzyme catalyzed reaction (Michaelis-Menten equation)

In case v_0 is exactly one-half of V_{max}.

$\frac{V_{max}}{2} = \frac{V_{max}[S]}{Km+[S]}$

On dividing by V_{max}, the equation will be,

$\frac{1}{2} = \frac{[S]}{Km+[S]}$

i.e., $Km = [S]$, when v_0 is 1/2 V_{max}.

Thus Km (Michaelis constant) can be defined as the substrate concentration at which velocity is half of the maximum. The term is sometimes used as an indicator of the affinity of the enzyme for its substrate.

function can be estimated from the K_m value for its substrate. For example, K_m values of glutamate dehydrogenase and glutamine synthetase with reference to utilization to NH_4^+ were found to be 30 mM and 0.015 mM, respectively, during an experiment conducted with *Lemna*. Tissue concentration of NH_4^+ was estimated to be 1/30 of those required for glutamate dehydrogenase but was saturating for glutamine synthetase, indicating that the enzyme glutamine synthetase may be having a primary role in NH_4^+ assimilation. Function of glutamate dehydrogenase predominantly is to release NH_4^+ from glutamate by the reverse of assimilatory reaction. V_{max} is related to turnover number, which refers to the number of moles of substrate that react to form product per mole of enzyme per unit time. This assumes that the enzyme is fully saturated with substrate and the reaction is proceeding at maximum rate. It is also expressed as K_{cat}. A straight line obtained, when reciprocal of v_0 is plotted against reciprocal of [S], is known as **Lineweaver-Burk double reciprocal plot** which is used to study enzyme kinetics (Box 4.4).

4.4.4 Factors Affecting Enzyme-Catalyzed Reactions

Effect of pH Protein conformation is influenced by the state of its ionizable groups, thereby affecting the structure and function of active site of the enzyme. pH of the medium influences ionic status of the R-side chains of the amino acid residues of proteins affecting the non-covalent bonds responsible for holding the protein molecule in correct conformation. Furthermore, residues present at the active site need to be in appropriate ionic status required for binding with the substrate as well as for catalyzing the reaction. pH also affects the ionic status of the substrate. Generally, a typical bell-shaped plot is obtained on studying the effect of pH on enzyme activity.

Box 4.4: Lineweaver-Burk Double Reciprocal Plot

It is quite difficult to estimate V_{max} in the hyperbolic curve which describes the rate of non-allosteric enzymatic reaction. V_{max} value is never reached with any finite substrate concentration that could be used in lab and it becomes difficult to determine Km of the enzyme. Michaelis-Menten equation can be algebraically transformed into equation by which a straight line is obtained instead of hyberbolic curve which becomes more useful. Michaelis-Menten equation,

$$v_0 = \frac{V_{max}[S]}{Km+[S]}$$

By taking reciprocals on both sides of equation:

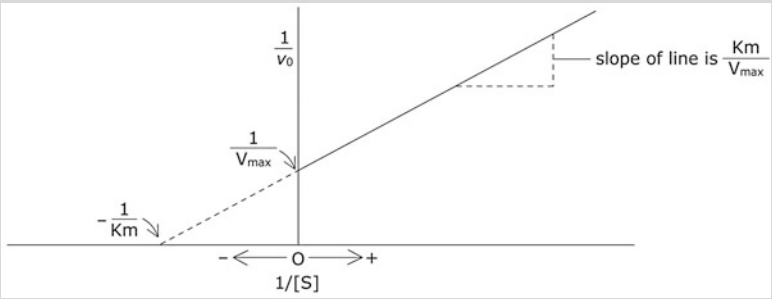
$$\frac{1}{v_0} = \frac{Km+[S]}{V_{max}[S]}$$

The reaction is simplified to give:

$$\frac{1}{v_0} = \frac{Km}{V_{max}}\frac{1}{[S]} + \frac{1}{V_{max}}$$

This equation now has a form of a straight line equation, y = mx+b, where $\frac{1}{v_0}$ is plotted on y-axis and $\frac{1}{[S]}$ on x-axis; it gives straight line, where $\frac{Km}{V_{max}}$ is represented by slope of reaction. Intercept of straight line on y-axis represents $\frac{1}{V_{max}}$, while on the horizontal axis it gives value of $-\frac{1}{Km}$.

This form of the Michaelis-Menten equation is called Lineweaver-Burk equation and the graphic representation of same is called Lineweaver-Burk double reciprocal plot.



Effect of Temperature Increase in temperature is responsible for increase in kinetic energy of the reactant molecules as it increases their chances of collision and therefore increasing the rate of any chemical reaction. In an enzyme-catalyzed reaction, temperature adversely affects enzyme structure because of the thermolabile nature of the non-covalent bonds maintaining the protein structure. An optimum temperature for the enzyme-catalyzed reaction is the balance of the two. Optimum temperature is also determined by the time of exposure of enzyme to that temperature. Temperatures above 50 °C are generally destructive for the enzyme protein. However, some enzymes are stable even at high temperatures.

4.4.5 Role of Inhibitors

Various compounds inhibit or alter the activity on binding with enzyme molecules. Inhibitors act through a variety of mechanisms. An inhibitor which binds reversibly to the enzyme molecule thereby lowering its activity causes reversible inhibition. On the contrary, the inhibitor which results in permanent damage to enzyme molecule results in irreversible inhibition. Removal of such inhibitor does not result in resumption of the enzyme activity. **Reversible inhibitors** generally bind with the enzyme molecules through non-covalent bonds, altering their conformation temporarily or they bind with the active sites due to similarity of their structure with the substrate molecules. Reversible inhibition can be competitive, uncompetitive, or noncompetitive. In presence of competitive inhibitors, availability of free enzyme to bind with the substrate is reduced since, unlike ES, EI complex does not break down to form product. This type of inhibition can be reversed by increasing the substrate concentration since it increases the possibility of the substrate binding to the active sites of enzyme molecules rather than with the inhibitor. V_{max} of the reaction is not altered; however, K_m increases (Fig. 4.16). This demonstrates a decrease in sensitivity of the enzyme for the substrate in presence of competitive inhibitor. Inhibition of succinic acid dehydrogenase by malonate is an example of competitive inhibition. Succinic acid dehydrogenase catalyzes conversion of succinate to fumarate. Malonate competes for binding with the active site of the enzyme since it resembles succinate in its structure. Inhibition of Rubisco by CO_2 and O_2 is also an example of competitive inhibition in plants. These two gases compete with each other for binding with the active site of the enzyme. Oxygenase activity of the enzyme can be reduced by increasing concentration of CO_2. Transition state analogues are specifically effective competitive inhibitors since active site of the enzyme specifically catalyzes the reaction on binding with transition state of the substrate. Noncompetitive inhibition is reversible inhibition. It is also known as "mixed type" of inhibition as the inhibitor binds either with free enzyme or with the enzyme-substrate complex. Inhibitor binds to a site of the enzyme distinct from the active site. As a result, active site of the enzyme is not blocked for binding with the substrate, but subsequent reaction is inhibited, resulting in decrease in V_{max} of the reaction. Since affinity of the enzyme for substrate is not reduced, K_m does not change. This kind of inhibition is not reversed by an increase in substrate concentration because inhibitor and substrate are not competing for the same active site. In the **uncompetitive inhibition**, inhibitor binds only with enzyme-substrate complex and not with free enzyme. As a result of this, inhibition increases with increase in substrate concentration. Both V_{max} and K_m are affected. V_{max} is reduced while there is increase in K_m (Fig. 4.16).

During **irreversible inhibition,** an inhibitor binds covalently to the enzyme protein forming a complex which does not dissociate to release free enzyme from the product. Organophosphorus compound—diisopropylfluorophosphate (DIFP)—is an irreversible inhibitor of the enzyme acetylcholine esterase which catalyzes hydrolysis of ester bond in acetylcholine, producing inactive molecules, acetate and choline. DIFP covalently binds to the seryl residue of active site of the enzyme,

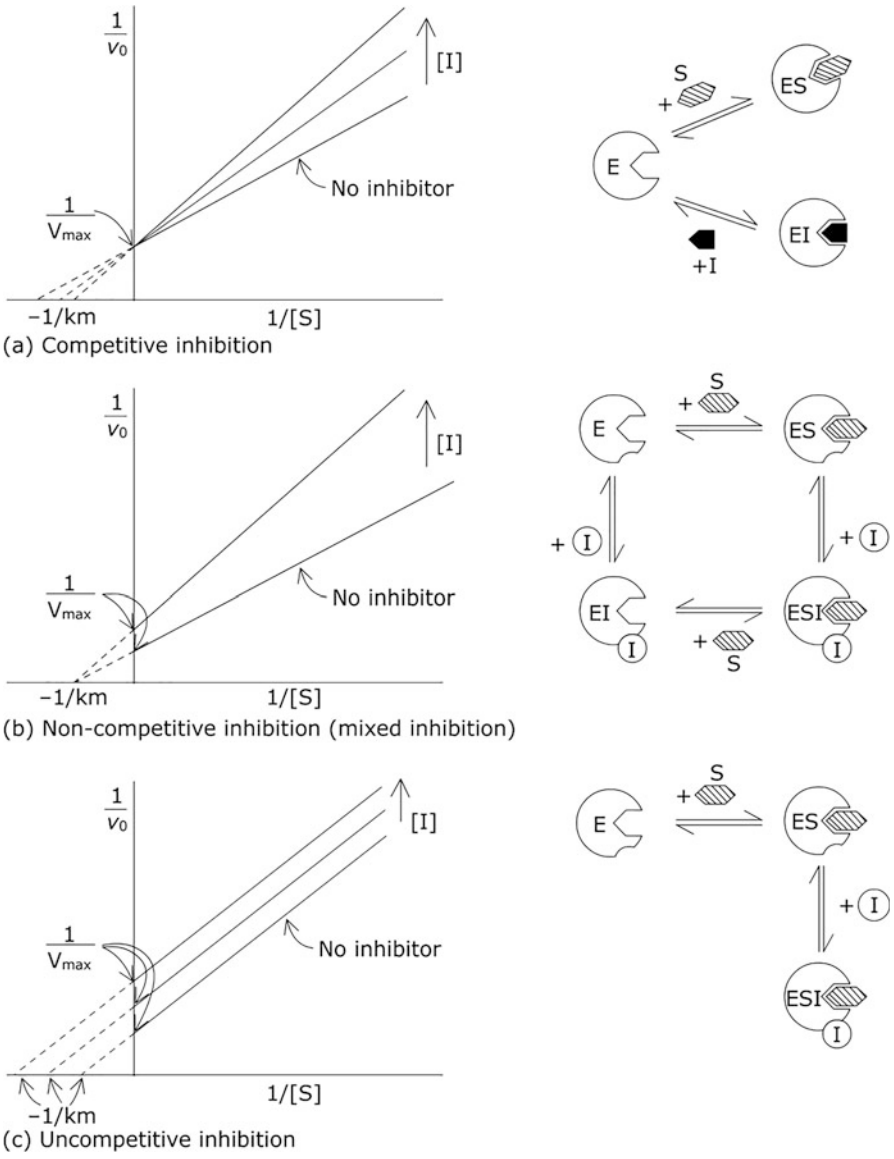


Fig. 4.16 Double reciprocal plot showing three types of reversible inhibition of enzyme activity

thereby inhibiting its activity (Fig. 4.17). Irreversible inhibitor may not bind covalently in some cases, but binding is strong enough so that the inhibitor does not get dissociated easily from the enzyme, e.g., transition state analogues. Though bonding is non-covalent, these compounds bind with active site of the enzyme so tightly that the two rarely get dissociated, thus inhibiting the enzyme activity. Transition state

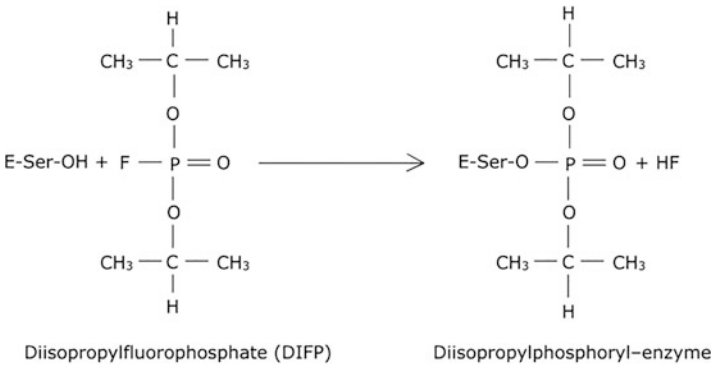


Fig. 4.17 Irreversible inactivation of enzyme acetylcholine esterase. Active serine residue of the enzyme binds covalently with DIFP, and the resulting complex of the enzyme is not reactive toward its own substrate

analogues cannot perfectly mimic the transition state. Even then they bind to the target enzyme 10^2–10^8 times more tightly. This concept of enzyme inhibition by transition state analogs is important in pharmaceutical industry for designing new drugs. Another class of irreversible enzyme inhibitors includes **suicide inactivators.** After allowing first few reactions to happen normally, instead of being converted to product, these compounds are converted to highly reactive molecules which combine irreversibly with the enzyme and inhibit its activity.

4.4.6 Regulatory Enzymes

Metabolic pathways in a cell are regulated according to the requirement of a cell. One of the mechanisms for regulation of metabolism is determined by the amount and availability of a particular enzyme, indicating control being at the transcription or translational level. This is a slower process and regulation through this mechanism will require a longer time period. A quicker regulation of the metabolic pathway occurs through regulation of activity of enzymes. Regulatory enzymes catalyze the slowest reactions of a pathway and the occurrence and pace of that pathway is determined by activity of these enzymes. Generally, it the first reaction which is regulatory besides other steps in a pathway. Metabolic pathways may be linear or branched. In addition to the first step, the reactions at the branching point of the pathway are also regulated since conversion of common metabolite to the products may depend upon the need of the cell for a particular end product (Fig. 4.18). In case the end product of a particular branched pathway is not required and its production in the cell is stopped and the enzyme at the branching of the pathway is inactivated, activity of the regulatory enzymes may increase or decrease in case of positive or negative regulation, respectively, which in turn influences the metabolic reactions

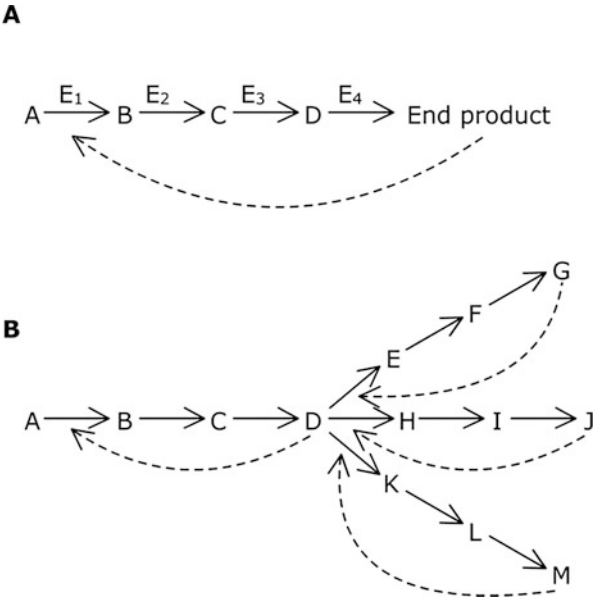


Fig. 4.18 Feedback regulation in (**a**) a linear metabolic and (**b**) branched pathway. Linear pathway is regulated by the end product of the pathway if accumulated. In a branched metabolic pathway, regulation may occur at the branching or at the starting reaction of the pathway in case D accumulates

accordingly. There are different mechanisms in the cell by which the activity of an enzyme is regulated.

4.4.6.1 Allosteric Regulation

Allosteric regulation of enzyme activity is important in control of metabolism. The term allosteric is derived from Greek word *allos* which means "other" and *stereos* meaning "three-dimensional." Activity of allosterically regulated enzymes is determined by the metabolites known as **allosteric modulators**. These modulators act by inducing change in the conformation of the enzyme upon binding to a site other than the active site by non-covalent bonds. Enzyme activity may be either inhibited or activated on binding with the allosteric modulator which are called allosteric inhibitor or allosteric activator, respectively. **Ligand** refers to the end product of a reaction (in case of feedback regulation) or a metabolite. Feedback inhibition is instantaneous and can be reversed quickly. Enzyme activity is inhibited in case the product of a reaction accumulates and will start functioning when the concentration of the product falls down. NADH/NAD^+ and ADP/ATP are some of the important allosteric modulators of enzyme activity. For example, ADP may act as positive modulator for several enzymes which are involved in the oxidation of sugars, thereby stimulating the conversion of more ADP to ATP. Allosteric enzymes are much larger and complex in structure. They generally consist of more than two subunits. The catalytic sites present on subunits are different from the modulator sites. Different subunits communicate with each other through change in conformation. Binding of a modulator to the modulator site of the enzyme subunit induces change

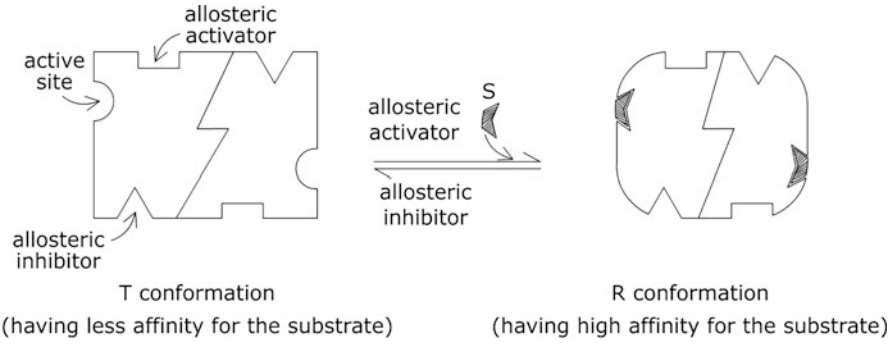


Fig. 4.19 Subunits of allosteric enzymes can exist in two conformations, T (tense) conformation, which has less affinity for substrate, or R (relaxed) conformation, which has more affinity for binding with substrate. Substrate may act as allosteric activator (homotropic allosteric enzymes) or other metabolite may act as allosteric activator or allosteric inhibitor (heterotropic allosteric enzymes)

in the conformation, resulting in increased or decreased affinity of the catalytic site for the substrate in case of positive or negative regulation, respectively. Generally, modulators have shapes different from that of the substrates. Besides enzymes, there are non-enzymatic proteins which alter their conformation on binding with ligand. The protein subunits can exist in two conformations, one which has lesser affinity for the substrate, i.e. T (tense) conformation, and the one having more affinity for the substrate R (relaxed) conformation (Fig. 4.19). Two principal models have been proposed to explain the behavior of the enzyme subunits upon binding with the modulators. These are the "concerted model" and "sequential model." Both of these models are used as the basis for interpreting experimental results. The "concerted model" was proposed by Jacques Monod, Jeffries Wyman, and Jean-Pierre Changeux in 1965. According to this model, conformation of all the subunits of an enzyme changes from T state to R state simultaneously on binding with the positive modulator, and vice versa on binding with negative modulator. Binding of positive modulator to the subunit is cooperative and stabilizes all the subunits in R conformation resulting in shifting of equilibrium, while reverse happens when negative modulator is bound. This results in T conformation of all the subunits simultaneously (Fig. 4.20). Positive cooperativity is also seen on binding with substrate in case of homotropic allosteric enzymes. The "sequential model" was proposed by Daniel Koshland in 1966 based on "induced-fit theory" of substrate binding. According to this model, binding of substrate induces conformation change in the subunit of allosteric enzyme from T to R which makes conformational change in other subunits of the enzyme easier. Similarly, binding of activators or inhibitors also takes place by induced-fit mechanism. Conformation change in one subunit influences the conformation change in other subunits also. In presence of an inhibitor, substrate is less likely to bind to the active site in T conformation, thus affecting the sensitivity of the subunits. Similarly, in presence of an activator, sensitivity of the

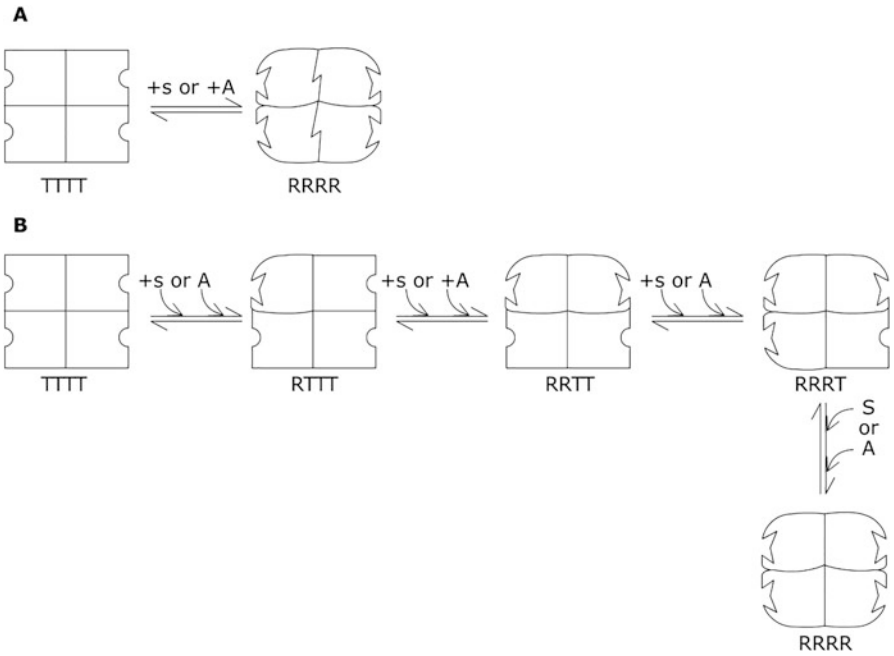


Fig. 4.20 Hypothetical models given for allosterically regulated enzymes. (**a**) Concerted model: on binding with substrate S (homotropic) or activator (A) other than substrate (heterotropic) affinity of all the subunits is increased for binding with substrate. (**b**) Sequential model: on binding with the substrate (homotropic) or activator other than substrate (heterotropic) affinity of other subunits to bind with substrate changes one by one as a result of which all intermediate species are present

subunits to bind with the substrate increases substantially. Thus, conformational change is passed to all other subunits making them more or less likely to bind with substrate in presence of activator or inhibitor, respectively. Sequential model has also been able to incorporate negative cooperativity, which did not find any provision in concerted model (Fig. 4.20).

Kinetics of Allosteric Enzymes When effect of [S] on v_0 of enzyme-catalyzed reaction is studied, allosteric enzymes do not display Michelis-Menten kinetics. Instead of hyperbolic graph, the allosteric enzymes usually produce sigmoid saturation curve (Fig. 4.21). Sigmoid kinetics usually displays cooperative interactions between multiple subunits. Change in conformation in one subunit triggers change in all other subunits mediated by non-covalent interactions at the interface between subunits. Sigmoid kinetic behavior of allosteric enzymes is explained by subunit interactions in both concerted and sequential models. Value of v_0 at half of [S] is not referred as K_m because enzymes do not follow hyperbolic kinetics, rather it is expressed as $K_{0.5}$. One characteristic of sigmoid curve is that a small change in the concentration of modulator can bring about large changes in the velocity of the

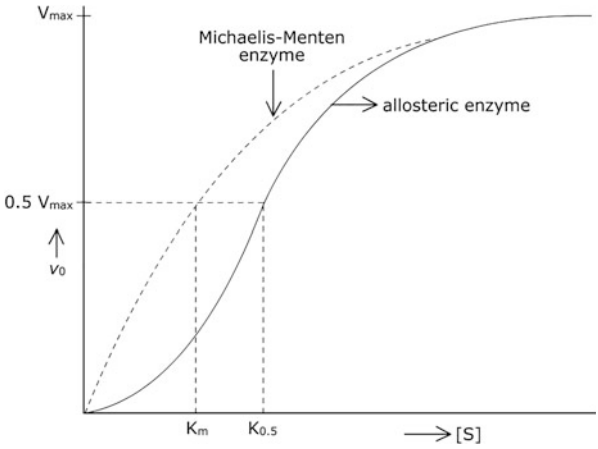


Fig. 4.21 An allosterically regulated enzyme displays a sigmoid curve when effect of [S] is studied on initial velocity (v_0) of the reaction, contrary to hyperbolic curve obtained in case of a Michaelis-Menten enzyme

reaction. For heterotropic allosteric enzymes, a positive modulator may change the sigmoid curve to more of a hyperbolic curve with a decrease in $K_{0.5}$. On the contrary, presence of a negative modulator may increase sigmoid nature of the curve with an increase in $K_{0.5.}$ There are some heterotropic modulators which increase or decrease V_{max} with little changes in $K_{0.5}$ (Fig. 4.22).

4.4.6.2 Covalently Modulated Enzymes

Another way by which activity of enzymes is regulated is by reversible covalent attachment of a group such as phosphoryl, adenylyl, adenosine, ribosyl, etc. to specific amino acid residues of the enzyme protein. Covalent attachment of a protein such as ubiquitin may also alter the activity of an enzyme. The most significant group which alters the enzyme activity on being covalently attached is phosphoryl group. Phosphoryl group is generally attached reversibly to a specific serine, threonine, or tyrosine residue of the enzyme protein, resulting in alteration of structural and functional properties of the molecule. Since phosphoryl group carries two negative charges, it will attract positively charged amino acids of the molecule while repelling amino acids with negatively charged side chains. As a result, conformation of the enzyme protein is altered. Phosphoryl group bound to the enzyme protein may also influence interaction with substrate molecule. Removal of phosphoryl group reverses the effect of phosphorylation. Phosphorylation and dephosphorylation of enzyme protein are catalyzed by protein kinases and phosphoprotein phosphatases, respectively. Phosphorylation may result in activation or inhibition of enzyme activity which will depend on a particular enzyme. However, reverse will be true on dephosphorylation of that enzyme (Fig. 4.23). One example is the regulation of pyruvate dehydrogenase activity, which is a component of pyruvate dehydrogenase complex and catalyzes conversion of pyruvate to acetyl-CoA. Phosphorylation of pyruvate dehydrogenase makes it inactive, while removal of

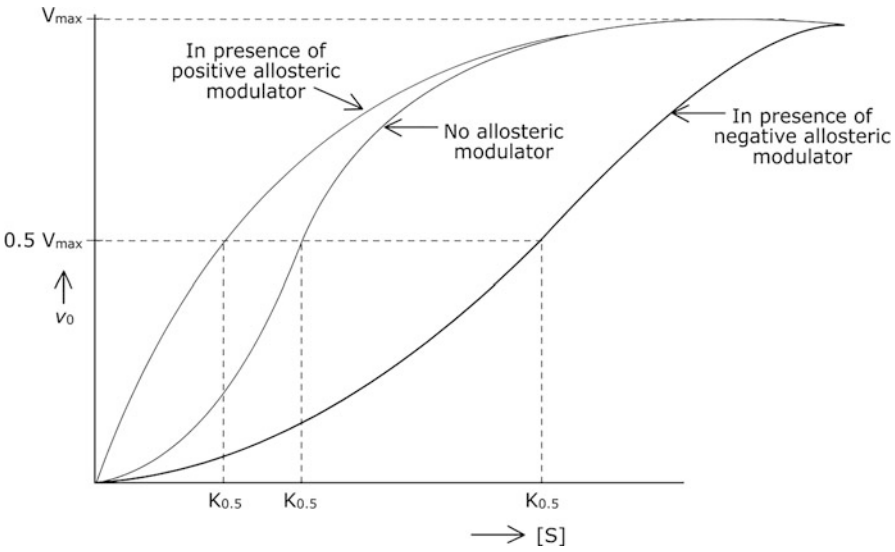


Fig. 4.22 Effect of a modulator on the kinetics of an allosterically regulated enzyme. Curves are drawn arbitrarily

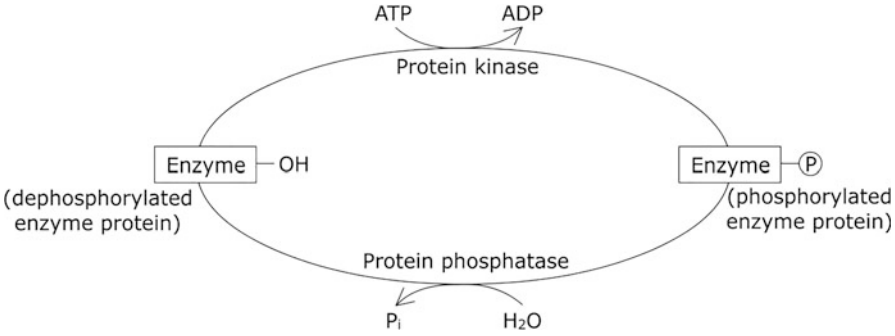


Fig. 4.23 Interconversion of phosphorylated and dephosphorylated forms of an enzyme through the action of protein kinase and protein phosphatase. Some enzymes are active when they are phosphorylated while others are active when they are dephosphorylated

the phosphate group restores the active enzyme. Phosphorylation and dephosphorylation of the enzyme protein are carried out by ATP-dependent pyruvate dehydrogenase kinase and phosphopyruvate dehydrogenase phosphatase, respectively. Additionally, covalently modulated enzymes include enzymes in which protein conformation changes in response to reversible reduction and oxidation of sulfur-containing groups of cysteine residues, which occurs in response to the redox status of the cell. Interconversion of sulfur-containing groups of the cysteine residues

between dithiol (-SH SH-) and disulfide (–S-S-) is mediated by thioredoxin. Activities of four of the Calvin cycle enzymes are regulated through this type of covalent modification. Alternate oxidase activity is also regulated through reversible oxidation and reduction of sulfur-containing groups of cysteine. Covalent addition of a hydrophobic group may also affect conformation of some of the enzymes altering their activity. Enzyme regulation also occurs through proteolytic cleavage or through calcium-mediated calmodulins.

Summary

- A sum total of all the chemical reaction occurring in a cell is called as metabolism and study of metabolites of a cell is called metabolomics. Since plants are sessile organisms and are exposed to harsher environmental conditions, they have more flexible and diverse metabolic pathways. Metabolic pathways are compartmentalized in a cell and regulated movements of metabolites occur through the cell membranes.
- Metabolism is classified as anabolism and catabolism. Anabolism includes biosynthetic reactions which involve synthesis of complex molecules from simple ones requiring input of energy. On the contrary complex molecules are broken down into simpler forms during catabolism releasing energy in the process.
- Spontaneous reactions are exergonic reactions with a negative free energy change during the reaction, while free energy change during endergonic reactions is positive. Exchange of energy occurs through ATP, which is the energy currency of the cell. ATP is synthesized during exergonic reactions, while endergonic reactions occur at the expense of ATP. Another kind of reactions involves removal and acceptance of electrons which are mediated by electron carriers such as NAD^+/$NADP^+$ and FAD besides others.
- All enzymes, except ribozymes, are proteins. Additionally, some enzymes consist of non-protein part also, which is called cofactor. A conjugated enzyme protein is called holoenzyme, while the protein part is called apoenzyme. A covalently bound cofactor is called prosthetic group, while a loosely bound cofactor is called coenzyme. All enzymes are classified into six major classes and named according to guidelines given by International Union of Biochemists.
- Enzymes are very efficient catalysts. Active site of the enzyme refers to the part of protein molecule with which substrate binds. It is a three-dimensional structure and consists of grooves and crevices. Substrate binds with the active site by non-covalent bonds. Enzymes act by lowering the activation energy since they bind with the transition state of the substrates.
- Michaelis and Menten proposed the equation known as Michaelis-Menten equation, which explains the relationship between the substrate concentration and velocity of the reaction. A new kinetic constant, Michaelis constant, was derived which refers to the substrate concentration at which velocity of the enzyme-catalyzed reaction is half of the maximum. Michaelis-Menten equation is

rearranged to get a straight-line equation which is used to get Lineweaver-Burk double reciprocal plot. Enzyme activity is altered by various factors, including pH and temperature of the medium. Enzyme activity is also inhibited by the compounds known as inhibitors. Inhibition can be reversible or irreversible.

- There are regulatory steps in a metabolic pathway, which are catalyzed by regulatory enzymes. Enzymes can either be allosterically regulated or covalently modulated besides other means. Kinetics of allosterically regulated enzymes is different from that of a typical enzyme following Michaelis-Menten kinetics. One of the most important groups which is responsible for regulating the enzyme activity of covalently modulated enzymes is the phosphoryl group.

Multiple-Choice Questions

1. Bioenergetics refers to:
   (a) Energy exchange in between the cell and the surroundings
   (b) Science which deals with energy transductions within the cell
   (c) Energy release during a chemical reaction
   (d) None of the above
2. A living cell is an open system because:
   (a) It neither exchanges energy nor matter with the surroundings.
   (b) It can exchange both energy and matter with the surroundings.
   (c) It can exchange energy with the surroundings but not the matter.
   (d) It can exchange matter but not energy with the surroundings.
3. According to second law of thermodynamics, spontaneous reaction will occur:
   (a) When less complex molecules will be converted to more complex ones.
   (b) When there is absorption of energy from the surroundings.
   (c) Molecules having higher entropy are converted to molecules having lesser entropy.
   (d) Molecules having lesser entropy are converted to molecules having high entropy.
4. ΔG of a cellular reaction will be negative if:
   (a) Products of the reaction have lesser entropy than the reactants.
   (b) Products of the reaction have more entropy than the reactants.
   (c) The reaction is non-spontaneous.
   (d) There is requirement of input of energy for the reaction to occur.
5. Which of the following statement is true?
   (a) In a living cell, equilibrium constant is maintained at 0.
   (b) Free energy is the total energy present in a molecule.
   (c) Free energy is the energy isothermally available to do work.
   (d) $\Delta G^{\circ\prime}$ is defined as the change in free energy during a reaction which is not at equilibrium.

6. High-energy bond (~) of ATP indicates:
   (a) Formation of this bond requires energy.
   (b) Hydrolysis of this bond releases energy.
   (c) Products of hydrolysis have lesser energy than the molecule itself.
   (d) Products of hydrolysis have more energy than the molecule itself.
7. ATP is the high-energy molecule because:
   (a) It is nucleoside triphosphate.
   (b) ATP is more resonance stabilized than the products of its hydrolysis.
   (c) ATP is present as Mg-ATP complex in the cell.
   (d) The products of its hydrolysis are stabilized by resonance.
8. In a redox reaction, electrons move:
   (a) From the compounds having more positive redox potential to compounds having lesser positive redox potential
   (b) From compounds having lesser positive redox potential to more positive redox potential
   (c) From compounds having lesser negative redox potential to compounds have more negative redox potential
   (d) None of the above
9. In an enzyme molecule consisting of a protein and non-protein structure, the protein part is known as:
   (a) Cofactor
   (b) Holoenzyme
   (c) Apoenzyme
   (d) Coenzyme
10. A non-protein structure covalently attached to the protein part of an enzyme molecule is called:
   (a) Coenzyme
   (b) Cofactor
   (c) Apoenzyme
   (d) Prosthetic group
11. Michaelis constant (K_m) of an enzyme is:
   (a) The substrate concentration at which enzyme is fully saturated
   (b) The substrate concentration at which V_{max} is half of the maximum
   (c) The enzyme concentration at V_{max}
   (d) The enzyme concentration at which V_{max} is half of the maximum

Answers

1. b 2. b 3. d 4. b 5. c 6. c
7. d 8. b 9. c 10. d 11. b

Suggested Further Readings

Jones RL, Ougham H, Thomas H, Waaland S (2013) The molecular life of plants. Wiley-Blackwell, Chichester, pp 42–70

Nelson DL, Cox MM (2017) Lehninger principles of biochemistry, 7th edn. W.H. Freeman, New York, pp 495–525

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