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National Open University
School of Sciences

MBC-004: ENZYMES AND THEIR APPLICATIONS

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MBC-004: ENZYMES AND THEIR APPLICATIONS

Enzymes are biological catalysts that catalyze biochemical reactions in the living organisms. Studies on enzymes are essential to understand their vital role in metabolic processes of living organisms. This course is intended to give a reasonably detailed account of various theoretical and applied aspects of the enzymes. We have also attempted to highlight new developments related to the application of enzymes.

This course is mainly for the students taking master degree courses with substantial biochemistry component. However, it is of immense value to students from other science backgrounds such as chemistry and life sciences. This course is written to meet the aspirations of distance learners firmly in mind.

Enzyme molecules are typically proteins, though some RNA molecules exhibit catalytic activity. Without the presence of non-protein component known as cofactor, enzymes lack catalytic activity. They are highly specific to the substrates and convert them into products. They are active only in certain range of temperature and pH. Enzymes can also be extracted and purified from several sources. They catalyze a wide range of commercially important processes. They also play a key role in analytical devices and enzyme assays have clinical, forensic and environmental applications.

We have attempted to define most of the scientific terms. They have been placed in context when they first appear. We have tried to deal comprehensively with the concept of classification of enzymes, nature of catalysis, kinetics, structures and mechanism of enzymes.

In the first block Block-1, the concept of enzymes has been introduced to the learners (Unit-1). Sections on nomenclature of enzymes are further subdivided into subclasses of enzymes. The concept of activation energy and thermodynamics has been also dealt with in the unit. Fischer's lock and key hypothesis as well as Koshland's theories of catalysis has been discussed in the Unit-2. Several factors affecting the enzyme activity are also discussed in the Unit-2. The principle, theories and their role in explaining the mechanism of catalysis are described in the following unit (Unit-3).

Enzyme kinetics in the second block of this course also involves a basic level of mathematics and some of the equations which are derived may seem complicated at first sight. The derivation of the equations on enzyme kinetics are based on the biochemical assumptions and then followed on logical reasoning and analysis. The kinetics of mono substrate and bisubstrate reactions has been described in Unit-4 and Unit-5 respectively. The derivation of Michaelis-Menten equation has been described in the Unit-4. Several methods of plotting enzyme kinetic data such as Lineweaver-Burk, Hanes-Woolf, Woolf-Augustinsson-Hofstee, Eadie-Scatchard; Direct linear plot have been discussed in the unit. Unit-6 is mainly concerned with the inhibition of single substrate reactions obeying Michaelis Menten kinetics.

Regulation of enzyme activity has been addressed in Block-3. Enzymes can be regulated by several methods to control enzyme activity to coordinate the different metabolic processes. It is also important to maintain cellular homeostasis i.e. to maintain the internal environment of the organism constant. An enzyme's catalytic activity can be directly controlled through structural alterations that control the enzyme's substrate-binding affinity. Catalytic efficiency of enzymes is affected by several methods such as Allosteric regulation, Feedback inhibition, Proenzyme

(zymogen) and Covalent modification. An overview of all these related mechanisms is given in Unit-7. Isozymes and multienzyme complexes have been dealt in Unit-8 and Unit-9 respectively.

Block-4 deals with extraction and purification of enzymes. Application of enzymes and recent advances are also discussed individually in two separate units. The process of enzyme extraction and isolation involve obtaining enzymes from natural sources (such as microorganisms, plants, or animal tissues) and purifying them for various applications (Unit-10). Both the processes employ several steps to ensure that the enzyme is recovered in an active and usable form. Optimization of extraction and isolation methods enables researchers and industries to produce high-quality enzymes for use in various applications, including industrial processes, research, and pharmaceuticals (Unit-12). The concept of diagnostic enzymes has been given in the Unit-11. Advanced topics such as immobilized enzymes and enzyme engineering are covered in the Unit-13.

Expected Learning Outcomes:

After studying this course, you should be able to:

- explain the concept of enzymes, their nomenclature and theories postulated,
- describe the mechanism of catalysis,
- derive equations of enzymes kinetics,
- discuss the mechanism of enzyme regulation,
- explain the role of multienzyme complexes,
- illustrate the multistep procedure of enzyme extraction and purification,
- discuss the wide range of the application of enzymes, and
- understand the role of immobilized enzymes and enzyme engineering.

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MBC-004: ENZYMES AND THEIR APPLICATIONS

Block

1

INTRODUCTION TO ENZYMES

BLOCK 1: INTRODUCTION TO ENZYMES

Living cells contain thousands of different enzymes. They are protein molecules that act as biocatalysts that speed up the rate of biochemical reactions. *Without the presence of enzymes the biochemical reactions would take years to complete.* The rate of biochemical reaction takes place at a relatively low temperature. The enzymes are thus known to lower the energy of activation.

The binding of a substrate to the active site of an enzyme is a kind of precise interaction. These sites are clefts or grooves on the surface of an enzyme, generally constitutes amino acids from different parts of the polypeptide chain that are brought together in the tertiary structure of the folded protein. Substrates initially bind to the active site by noncovalent interactions, including hydrogen bonds, ionic bonds, and hydrophobic interactions.

In the first block we have given an introduction of enzymes and their mechanism of catalysis. In the first unit, the concept of enzymes, their different types, attached components and their precise nature of substrate interaction as well as enzyme classification based on IUPAC have been reasonably described. Several theories such as famous Fischer theory of Lock and Key hypothesis and Induced Fit Model in this regard are described in Unit-2 of the block. The role of several factors affecting the enzyme activity is also explained in the unit. An overview of several theories on mechanism of enzyme catalysis is given in the last unit (Unit-3).

Expected Learning Outcomes:

After studying this block, you should be able to:

- understand enzymes, their specificity and catalytic efficiency,
- describe enzymes classification,
- discuss the factors affecting enzyme activity,
- explain the mechanistic features of enzyme catalysis,
- describe enzyme assays to measure enzyme activity.

UNIT 1

INTRODUCTION TO ENZYMES

Structure

1.1 INTRODUCTION

In 1878, the term 'enzyme' was coined by Friedrich Wilhelm Kuhne to assign 'biological catalysts'. One of the prominent chemical reactions is alcoholic fermentation which leads to the production of ethyl alcohol and carbon dioxide catalyzed by enzyme zymase present in yeast. The name 'enzyme' (en G = in ; zyme G = yeast) literally means 'in yeast'.

In this unit you will learn about the concept of enzymes and different roles played by them as biological catalysts. You are already familiar with the fact that enzymes are **proteins** that catalyzes biochemical transformations effectively and efficiently with very high specificity. Enzymes are large biological molecules which are colloidal and thermolabile in nature. Like catalysts they do not change the state of equilibrium of the reaction when they catalyze a reversible reaction. They only speed up the reaction. The name biological catalysts clearly indicate that these catalysts are produced in the living cells and carried out reactions in them. They are highly selective molecules and their catalytic activity depends on the integrity of their native protein conformation. Enzymes display two key characteristics: catalytic power

and specificity. You will learn more about these two characteristics later in the unit.

Enzymes also employ additional non-protein chemical component known as cofactor or complex organic molecules called as co enzymes to perform their catalytic activity. This additional component may be bound tightly or loosely. The unique feature of an enzyme is the catalytic site or active site. It is a small portion of the enzyme molecule where substrate binding takes place.

With rapid expansion in the knowledge of enzymes several ways were adopted to name and to classify them on one criteria or the other. In this unit we will discuss nomenclature and classification of enzymes.

Most enzymes are proteins but all proteins are not enzymes. Exception is ribozymes where RNA acts as enzyme.

Intracellular and Extracellular Enzymes: Intracellular enzymes are produced and present within cells and they usually act within the cells. Most of the enzymes are plant enzymes. Examples include metabolic enzymes.

Extracellular Enzymes: These enzymes are released by living cells and catalyze reactions outside cells and so they are called as extracellular enzymes. Examples include digestive enzymes.

Expected Learning Outcomes

After studying this unit, you should be able to:

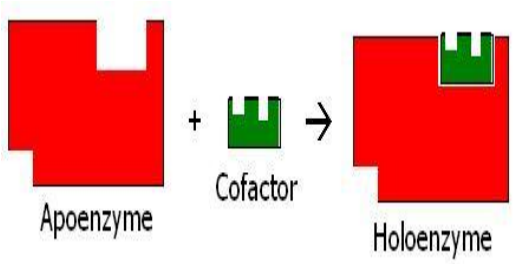
- know about the specificity of enzymes;
- discuss the characteristics of enzymes;
- differentiate between cofactors and coenzymes; and
- describe nomenclature and classification of enzymes.

1.2 NATURE AND STRUCTURE OF ENZYMES

Based on the chemical compositions, enzymes are considered as proteins. Nearly all enzymes are proteins with the exception of ribozymes (RNA acting as enzymes). Proteins as you know are a linear chain of amino acids, which give rise to a three-dimensional structure. The sequence of amino acids specifies the structure of enzyme, which in turn determines the catalytic activity of the enzyme.

Enzymes can be simple of conjugate. Simple enzymes are proteins only. They lack non protein groups. Examples of such enzymes include pepsin, trypsin, lysozyme etc. Conjugate Enzymes possess two parts - a large thermolabile

protein part or apoenzyme and a small dialysable thermostable nonprotein part called cofactor. The conjugate enzymes require cofactors for catalytic activity of enzymes. These cofactors may include inorganic ions, prosthetic groups and coenzymes. A complete, catalytically active enzyme together with its bound coenzyme and/or metal ions is called a **holoenzyme or conjugated enzyme**. The protein part of such an enzyme is called the **apoenzyme** or **apoprotein** (Fig. 1.1).



Apoenzyme/Apoprotein + cofactor/ co-enzyme or prosthetic group = Holoenzyme

Fig. 1.1: Schematic Representation of a Holoenzyme.

Cofactor

Cofactors bind in a temporary dissociable manner to the enzyme or to the substrate. They can be subdivided into two groups: metal ions and small organic molecules. Many enzymes require either one or more inorganic ions, such as Fe^{2+}, Mg^{2+}, Mn^{2+}, or Zn^{2+} for their activity. Enzymes that require these metal ions for their catalytic activity are called as metal activated enzymes. Metal ions such as Mg^{2+}, Mn^{2+}, or Zn^{2+} bind at the enzymes active site as well as with the substrate simultaneously. Examples include cytochrome oxidase. A list of important enzymes and their associated metal ions is given in the Table 1.1

Table 1.1 Enzymes and their associated metals ions (co-factors)

S.No.	Enzyme	Associated Metal ion
1	Cytochrome oxidase	Fe^{2+}/ Fe^{3+}
2	Xanthine oxidase	Fe^{2+}/ Fe^{3+}
3	Peroxidase	Fe^{2+}/ Fe^{3+}
4	Catalase	Fe^{2+}/ Fe^{3+}
5	Phosphofructokinase	Mg^{2+}
6	Hexokinase	Mg^{2+}

Co-enzyme

Cofactors that are **small organic molecules** are called coenzymes. Coenzymes are often

derived from vitamins and can be either tightly or loosely bound to the enzyme. If

coenzyme is tightly bound to the enzyme it is termed as **prosthetic** group. Same coenzyme is used by a variety of enzymes for catalysis. However, enzymes that use the same coenzyme are usually mechanistically similar i.e. their mechanism of catalysis remains same. Examples of well known coenzymes involved in transfer of specific atoms or functional groups are given below in the Table 1.2.

Table 1.2 List of some of the enzymes, coenzymes and their catalytic action.

Enzyme	Coenzyme	Group transferred
Pyruvate carboxylase	Biotin	Carbon dioxide
Acetyl CoA carboxylase	Coenzyme A (CoA)	Acyl group
Methylmalonyl mutase	Coenzyme B_{12} (5'-Deoxyadenosyl cobalamin)	Alkyl groups or hydrogen atoms
Monoamine oxidase	Flavin adenine dinucleotide (FAD)	Hydrogen atoms
Lactate dehydrogenase	Nicotinamide adenine dinucleotide (NAD^+)	Hydride ions (H-)
Glycogen phosphorylase	Pyridoxal phosphate	Amino groups
Thymidylate synthase	Tetrahydrofolate (THF)	One carbon groups other than CO_2
Pyruvate dehydrogenase	Thiamine pyrophosphate (TPP)	Hydroxyl ethyl group

Prosthetic group

A coenzyme or metal ion that is very tightly or even covalently bound to the enzyme protein is called a prosthetic group. Binding of prosthetic group with the enzyme is permanent. Dissociation of the prosthetic group results in an irreversible loss of the catalytic activity of the enzyme. Some of the examples include pyridoxal phosphate, flavin mononucleotide (FMN), flavin dinucleotide (FAD), thiamin pyrophosphate, biotin, and the metal ions of Co, Cu, Mg, Mn, Se, and Zn. Metal ions are the most common prosthetic groups. One third of enzymes contain tightly bound metal ions (metallo-enzymes). They have metal ions as their integral part and are different from the enzymes that need metal ions as cofactor (metal-activated enzyme).

SAQ 1

Tick [√] mark the correct option:

a) Enzymes are made up of proteins and lipids. [True/False]

b) Cofactors that are **small organic molecules** are called coenzymes. [True/False]

c) Binding of prosthetic group with the enzyme is temporary. [True/False]

d) Ribozymes are DNA molecules. [True/False]

1.3 CHARACTERISTICS OF ENZYMES

Enzymes play a very crucial role in the maintenance and progression of life. Unsolved mysteries in biological chemistry include the nature of enzyme catalysis. How does an enzyme specifically enhance the rate of a chemical reaction by 10^6 to 10^{12} times more than the uncatalyzed one? What are the general principles involved?

Several answers to the above questions have been given such as the pathway of changes in chemical bonding, the kinetic order of addition of substrates, the rates of various steps, and the three-dimensional structural aspects of the protein surface.

Catalytic power of the enzyme is its ability to speed up the rate of reaction without them being consumed or permanently altered by the reaction. Another fascinating ability is its ability to increase reaction rates without altering the chemical equilibrium between reactants and products. Enzyme catalyzed reactions are product specific and substrate specific. Catalytic activities of enzymes can be controlled by covalently modifying the enzyme, allosteric regulation and many others which will you study in the later units.

Enzymes are highly selective molecules and their catalytic activity depends on the integrity of their native protein conformation. The two key characteristic properties of enzymes are **catalytic efficiency** and **substrate specificity**.

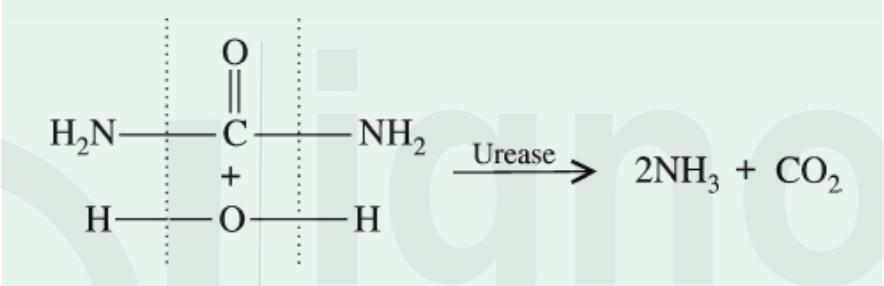
The enzymes increase the rate of a reaction which would otherwise take much longer time for completion. For example, enzyme **carbonic anhydrase** catalyzes the conversion of CO_2 and H_2O into carbonic acid (H_2CO_3). This reaction is very vital for our survival as the CO_2 generated in the tissues must be excluded but uncatalyzed reaction occurs at a very slow pace. Carbonic anhydrase speeds up the formation of carbonic acid. The catalyzed reaction is 10^7 times faster than the uncatalyzed one. Each enzyme molecule can hydrate 10^6 molecules of CO_2 *per second*. This is termed as the **catalytic efficiency** of an enzyme.

Two rationales have found widespread attention regarding catalytic efficiency of enzymes. These are selection of ground state conformations and stabilization of the transition state. The catalytic power of enzymes is due to the precise molecular interactions between enzyme-substrate that occur at the active site, which lower the energy barrier and enable formation of the

transition state. Covalent interactions between substrate and the enzyme lower the activation energy. The second explanation lies in non-covalent interactions between substrate and enzyme such as hydrogen bonding, van der Waal's attraction, hydrophobic and ionic interactions. The energy is released from these interactions and is termed as **binding energy**. Binding energy serves two purposes: it is the driving force for catalysis as well as is responsible for enzyme specificity. We will discuss about the concept of activation energy and transition state later in the unit.

Enzymes may act on one specific substrate molecule or on a group of structurally-related compound. For example carbonic anhydrase and urease enzymes are substrate specific (reactions shown below) while dehydrogenases and transaminases work on structurally related compounds.

$$H_2O + CO_2 \xrightarrow{\text{Carbonic anhydrase}} H_2CO_3$$



Specificity of enzymes lies in the arrangement of functional groups in the enzymes active site. A well defined arrangement of atoms in the active site of enzyme molecule enables the enzyme to discriminate between substrate and other molecules. Specificity means the complementarity between substrate and active site of the enzyme i.e. enzyme-substrate interaction subsequently leading to formation of ES-complex. Emil Fisher proposed that 'enzymes are structurally complementary to their substrates, so that they fit together like a lock and key' in his Lock and Key Hypothesis. However, this theory fails to explain the stabilization of enzyme. It does not visualize the weakening of enzyme substrate bonds proposed in transition state theory. Koshland Jr., in 1958 postulated that "Enzymes are flexible and that the shapes of the active sites can be markedly modified by the binding of substrate'. It has been observed that the active sites of some enzymes assume a shape that is complementary to that of the transition state only *after* the substrate is bound

Similarly, substrates show specificity/complementarity towards specific enzyme. It may be **geometric** (shape) or **electronic** (electrostatic attraction). Geometric specificity can be understood with the help of lock and key hypothesis whereas electronic specificity can be explained with the help of induced fit hypothesis. You will study about these two hypotheses in the next unit.

Let us focus on different types of specificity. **Absolute specificity** refers to enzyme catalysis of reaction with only one substrate for eg.*carbonic anhydrase* which acts only on carbonic acid, *lactase* which acts on lactose etc, while **group specificity** means enzyme catalysis of reactions involving

structurally related group of compounds for eg. *Endopeptidases* like pepsin, trypsin etc. **Optical specificity** is about catalyzing only one of the two optical isomers either D or L form, eg. *L-amino acid oxidase* acting on L-amino acids while **geometrical specificity** is catalyzing only a *cis* or *trans* bond for eg. *fumarase* catalyzes the interconversion of fumaric and malic acids, it does not react with maleic acid which is the *cis* isomer of fumaric acid or with D-malic acid.

SAQ 2

State whether following statements are True or False

a) Specificity of enzymes lies in the arrangement of functional groups in the enzymes active site. [True/False]

b) Enzymes do not alter the point of chemical equilibrium in a reversible reaction. [True/False]

c) Group specificity means enzyme catalysis of reactions involving structurally related group of compounds. [True/False]

d) Urease catalyzed reactions of structurally related compounds. [True/False]

1. 4 ACTIVE SITE OF ENZYMES

The substrate molecules are comparatively smaller than the bigger enzyme molecules. They interact with the enzyme at its active site. The active site constitutes 10-20 % of the enzyme yet it is the most important part of enzyme to catalyze the reaction (Figure 1.2). It is made up of amino acid residues that establish temporary bonds with the substrate (binding site) as well as residues that catalyze that substrate's reaction (catalytic site).

The functional groups of enzymes and substrate/substrates interact with each other and may form a transient covalent bond at the **active site** of enzymes which eventually helps the enzyme to lower their activation energy, thereby increasing the rate of reaction. In the beginning, interactions between the active site and the substrate are non-covalent and transient. Please remember, active site keeps on catalyzing the reaction repeatedly and its amino acid residues are not altered at the end of the reaction. During the reaction some of them might get altered in their interactions with substrate, cofactors or coenzymes but all of them get regenerated at the end of reaction.

Although the enzymes differ widely in their properties, the active site of any enzyme molecule possesses some common features.

- Active site is very small as compared to entire enzyme structure.
- It is three dimensional cleft or crevice.

- Amino acid sequences from different parts of linear protein are present in the active site. For example, chymotrypsin (His 57, Asp 102, Ser 195) [Figure 1.3], carbonic anhydrase II (His 94, His 96, His 119)
- Binding of substrate with the amino acid functional group in the active site is by relatively weak molecular interactions.
- Generally, water is largely excluded from the active sites in the enzyme molecule. The active site contains charged or hydroxy amino acids such as aspartic acid, glutamic acid, lysine serine etc. The side chain groups like -COOH, $-NH_2$, $-CH_2OH$ etc., serve as catalytic groups in the active site. Besides, the crevice creates a micro-environment in which certain polar residues acquire special properties which are essential for catalysis.

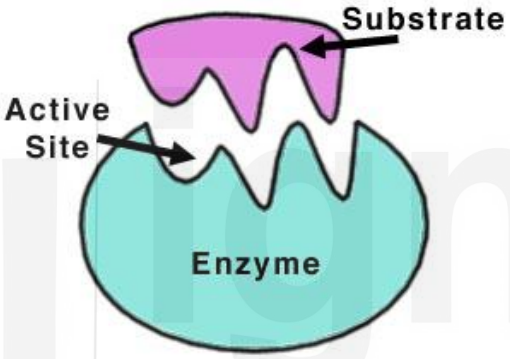


Fig. 1.2: Schematic representation of the active site of an enzyme.

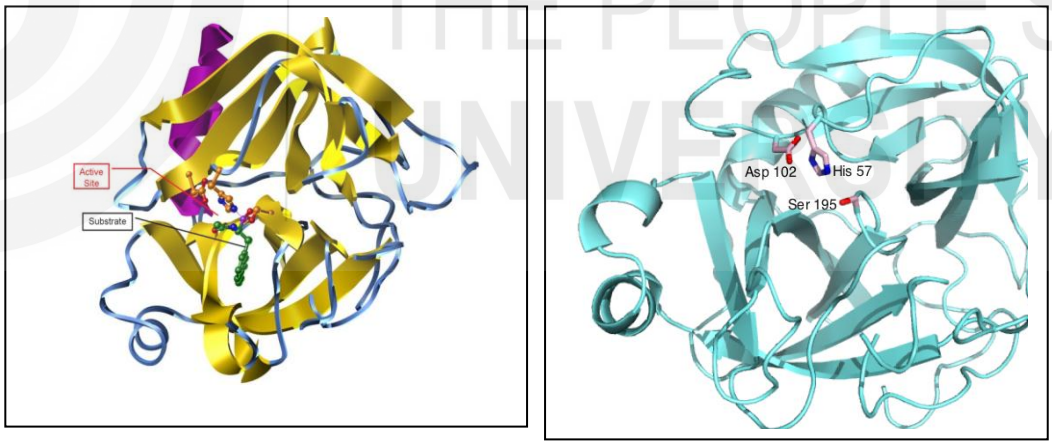


Fig. 1.3: Folded structure of Chymotrypsin revealing amino acids from different positions in the active site.

1.5 NOMENCLATURE AND CLASSIFICATION OF ENZYMES

With rapidly increase in the number of enzymes, several systems has evolved for their nomenclature and classification. These classification systems adopt one or the other criterion as the basis to name the enzyme. These criteria include factors such as composition of enzyme, type of substrate on which the enzyme acts, type of reaction catalyzed and so on. Let us discuss about this

general system of enzyme classification with suitable examples along with the enzyme classification given by Enzyme Commission (EC) which is uniform in nature and followed universally.

1.5.1 General System of Enzyme Classification

Composition of Enzyme: Based on composition, enzymes are classified into three groups:

- enzyme consisting of **protein only**, for example: trypsin, amylase, urease etc
- enzyme consisting of **protein and metal ion** (metalloenzymes): approximately one-third of the enzymes need metal ion for their activity. This metal ion may be a co-factor or covalently linked to the enzyme.
- enzyme consisting of **protein and organic moiety** (commonly known as cofactor or prosthetic group), for example: **Iron porphyrin enzymes** (catalase, cytochrome c peroxidase I and II), **Flavoprotein enzymes** (glycine oxidase, pyruvate oxidase, histamine), **Enzymes requiring other coenzymes** (phosphorylase, amino acid decarboxylase), etc.

Type of substrate on which the enzyme acts: Enzyme derives its name simply by adding the suffix -***ase*** in the name of the substrate catalyzed. For example carbohydrases act upon carbohydrate, proteinases act upon proteins, lipases act upon lipids, maltase act upon maltose, urease act upon urea and so on. The major exceptions to this system are proteolytic enzymes such as Trypsin, Chymotrypsin etc.

Type of reaction catalyzed: Since the enzymes are highly specific, depending on the type of reaction and simply by adding *–ases* to it, enzymes can also be classified. For example, **hydrolases** (catalyzing hydrolysis), **isomerases** (isomerization), **oxidases** (oxidation), **dehydrogenases** (dehydrogenation), **transaminases** (transamination), **transaldolases** (transaldolation), **transketolases** (transketolation), **phosphorylases** (phosphorylation) etc.

More ways of classification leads to several inconsistencies. They tells us about only one aspect of the reaction catalyzed i.e. either only the nature of substrate or type of reaction catalyzed or end product or so on but not over-all chemical reaction. Many enzymes are known by more than one name. Whenever a new enzyme is characterized a great care has to be taken to give it a unique name. In order to bring consistency to the classification of enzymes and to remove ambiguities associated with the use of trivial nomenclature, in 1964, the International Union of Biochemistry and Molecular Biology (IUBMB) established an Enzyme Commission (EC) to develop a logical nomenclature for enzymes which is uniform and universally acceptable. This system should take into consideration of overall reaction.

1.5.2 EC numbers

The first general principle of recommendation was naming of enzymes especially those ending in -ase should be used for single enzymes i.e single

catalytic unit. They should not be used for multienzyme system. The second general principle is that all enzymes should be principally classified and termed according to the reaction they catalyze. The chemical reaction catalyzed is the specific property that differentiates one enzyme from other. The third general principle is that enzymes are classified into groups on the basis of reaction catalyzed and together with the name of substrate provide basis for naming enzymes. It's also the basis for classification and code numbers.

The Enzyme Commission classified enzymes into six main classes. Each class has further 4 to 13 subclasses. Each enzyme name has 2 parts—the first part is the name of the substrate(s) and the second part which ends in the suffix -ase, pointing out the type of reaction catalyzed. Each enzyme has been assigned a systemic code number called Enzyme Commission (E.C.) number. The E.C. number for each enzyme consists of a series of numbers at 4 places. Each major class is divided into sub-class and subclass has numerous sub-subclasses.

Every class, subclass and sub-subclass is assigned a code number. Thus, each enzyme is given a four digit EC number (X1.X2.X3.X4) and a systematic name, which identifies the reaction it catalyzes. First digit (X1) shows the main class to which enzyme belongs. The last place number or the fourth digit represents the serial number of the enzyme within the sub-subclass. Six main classes of enzymes are given below in Table 1.3.

Table 1.3 IUBMB System of Enzyme Classification

First digit	Class	Type of reaction catalyzed	Some examples of subclass with enzyme names
1	Oxidoreductases	Oxidation/ reduction reactions	**Dehydrogenases** (Succinate dehydrogenase, Malate dehydrogenase) **Peroxidases** (Catalase)
2	Transferases	Transfer of an atom or group between two molecules	**Transaldolases** **Transketolases** **Kinases** (Hexokinase)
3	Hydrolases	Hydrolysis reactions (transfer of functional groups to water)	**Esterases** (Acetylcholinesterase) **Peptidases** (Trypsin, Chymotrypsin) **Glycosidases** (Lactase,amylase,

			chitinase, neuraminidase, lysozyme)
4	Lyases	Addition or removal of a group from substrate (not by hydrolysis) to form double bonds	**Decarboxylase** **(**Phosphoenolpyruvate carboxylase,pyruvate decarboxylase, RuBisCO) **Dehydratase** (Serine dehydratase or L-serine ammonia lyase) **Synthase** (ATP synthase, Citrate synthase, Tryptophan synthase, Fatty acid synthase)
5	Isomerases	Isomerization reactions (intramolecular group transfer)	**Isomerases** (Triose phosphate isomerase,) **Mutases** (Bisphosphoglycerate mutase, Phosphoglucomutase)
6	Ligases	Ligation of two substrates at the expense of ATP hydrolysis	**Synthetases** (Aminoacyl tRNA synthetase)

Second (X2) and third digit (X3) in the code corresponds to the subclass and sub-subclass, respectively and further describes the kind of reaction being catalyzed. Fourth number (X4) indicate the actual substrate. As an example, the formal systematic name of the enzyme catalyzing the reaction:

ATP + D-glucose → ADP + D-glucose 6-phosphate

is ATP:glucose phosphotransferase, which indicates that it catalyzes the transfer of a phosphoryl group from ATP to glucose. Its Enzyme Commission number (E.C. number) is 2.7.1.1. The first number (2) denotes the class name (transferase); the second number (7), the subclass (phosphotransferase); the

third number (1), a phosphotransferase with a hydroxyl group as acceptor; and the fourth number (1), D- glucose as the phosphoryl group acceptor.

EC classification system of enzyme brings out clarity and complexities of the reaction catalyzed by the enzyme but it makes the names lengthy and relatively cumbersome. The first enzyme commission after much thought recommended two nomenclatures of enzymes; one systematic and other trivial. The systematic name will be assigned in agreement with definite set of rules. The trivial names are for common use. However, the revised nomenclature decided to give prominence to trivial names called as common names. The systematic names were also retained for the following causes:

1) The code number is useful for the identification of enzyme

2) It reveals the type of reaction catalyzed by the enzyme.

3) Systematic names can be formed for new enzymes by discoverer on application of rules. Individuals cannot allot the code numbers. Only the nomenclature committee of IUBMB should assign a number

4) Common names for new enzymes are formed as condensed version of systematic names.

Trivial or common name of the enzymes are still being used. For instance hexokinase for ATP:glucose phosphotransferase. Some examples of enzymes with their EC number, systematic and common names are given in Table 1.4.

Table 1.4 Systematic and Common names of some of the Enzymes

EC Number	Systematic name	Common or trivial name
1.1.1.1	Alcohol : NAD oxidoreductase	Alcohol dehydrogenase
1.11.1.6	H_2O_2: H_2O_2 oxidoreductase	Catalase
2.7.1.1	ATP : D-hexose-6-phosphotransferase	Hexokinase
3.1.1.3	Glycerol ester hydrolase	Lipase
4.1.2.7	Ketose-1-phosphate aldehyde-lyase	Aldolase
4.2.1.2	L-malate hydro-lyase	Fumarase
5.3.1.9	D-glucose-6-phosphate keto-isomerase	Glucosephosphate isomerase
6.3.1.2	L-glutamate : ammonia ligase (ADP)	Glutamine synthetase

SAQ 3

Match the following:

S.No	Enzyme	S.No	Class
a)	Peptidases	i)	Transferase
b)	Peroxidases	ii)	Isomerase
c)	Intramolecular group transfer	iii)	Hydrolases
d)	Aminoacyl tRNA synthetase	iv)	Oxidoreductase
e)	Kinases	v)	Ligases

1.6 THERMODYNAMICS OF ENZYME CATALYZED REACTION

If you remember in one of first sections of this unit, we told you about the two rationales have found widespread attention regarding catalytic efficiency of enzymes. These are selection of ground state conformations and stabilization of the transition state. Let us discuss them more in detail:

The laws of thermodynamics are equally applicable to biological processes. The first law states in a closed system, energy can neither be lost nor gained, but can be converted into other forms of energy. The second law of thermodynamics states entropy constantly increases in a closed system. Entropy (denoted by S) is a measure of randomness or chaos. This law states that total entropy of the system and surroundings always increase for a spontaneous process.

The second law means that breaking down molecules releases energy and that making new molecules (going against the natural tendency towards disorder) requires energy. Every molecule has an intrinsic energy, and therefore whenever a molecule is involved in a chemical reaction, there will be a change in the energy of the resulting molecule(s). Some of this change in the energy of the system will be utilizable to do work, and that energy is referred to as the free energy of the reaction. The remainder is released as heat.

Enthalpy is defined as the heat content of a system (denoted by H). T is the temperature at which reaction takes place. It is assumed to be constant. In the year 1878, J W Gibbs gave a relation as a function of T and S, which is popularly referred to as free energy or Gibbs free energy equation.

$\Delta \boldsymbol{G} = \Delta \boldsymbol{H} - \boldsymbol{T}\Delta \boldsymbol{S}$ where ΔG represents free energy change.

The free energy change during the reaction process under standard set of conditions (Temperature, 298 K; partial pressure, 1 atmosphere; concentration

of solutes, 1M) is called as standard free energy change $\Delta G°$. As a matter of convention, release of free energy is a negative number, while a requirement for input of energy is denoted with a positive number. **Free energy change must be negative for a reaction to be spontaneous (**also called an exergonic reaction)**.** A reaction is said to be spontaneous if it occurs without being driven by some external force.

Thus, for a reaction, if ΔG is negative, reaction is spontaneous and if ΔG is positive, reaction will not take place spontaneously (also called an endergonic reaction). For a biological reaction, ΔG is the energy available for work (osmotic work, muscular work or biosynthetic work). When $\Delta G = 0$, the system is in equilibrium.

In a simpler system in which there are just two reactants and two products:

$$aA+bB \leftrightharpoons cC+dD$$

the equation for free energy change becomes

$$\Delta G = \Delta G° + RT\ln([C]^c[D]^d/[A]^a[B]^b)$$

For biologists, it's vital to know that cells are not very well suitable to regulate chemical reactions by changing the temperature or the pressure of the reaction conditions. However, they can drive a non-spontaneous reaction ($\Delta G > 0$) forward spontaneously ($\Delta G < 0$) either by escalating substrate concentration (possibly by transporting them into the cell) or by diminishing product concentration. Endergonic reactions can be coupled to exergonic reactions as a series of coupled reactions to proceed forward. The only necessity is that the overall free energy change must be negative ($\Delta G < 0$).

You might know that ATP is called as the most common energy "currency" in cells because its hydrolysis yields about -7.3 kcal/mol free energy change enough to drive many endergonic reactions to proceed forward by coupling, but it is less costly (energetically) to make than other compounds that could potentially release even more energy (e.g. phosphoenolpyruvate, PEP). Also, much of the -14.8 kcal/mol ($\Delta G°'$) from PEP hydrolysis would be wasted because relatively few endergonic reactions are so unfavorable as to need that much free energy.

Even when a reaction is energetically favorable ($\Delta G < 0$), it may not happen without a little "push", These reactions need an extra push to overcome thermodynamic stability. This extra push is called as Activation Energy. Let us discuss more about the activation energy.

For chemical reactions to occur molecules must collide with one another. However, not all colliding molecules react. Not all the colliding molecules of similar types will have equivalent amount of energy. The reactions will take place only when molecules collide in specific orientation and sufficient energy. The molecules must possess energy to overcome the energy barrier to the reaction. This is also known as **activation energy**. The activation energy is an energy barrier between substrates and products. For conversion of substrates to products, energy barrier is the energy required to align reacting molecules

as well as formation of transient unstable intermediates before the formation of products.

Look at fig 1.4, in the coordinate diagram, free energy of the system is plotted against the reaction process. In the figure you will notice that there is an **energy barrier** between S (substrate) and P (product). This energy barrier is known as **activation energy** or **energy of activation (E_A).** E_A is defined as the energy required to overcome the energy barrier so that substrate/s can be converted into product.

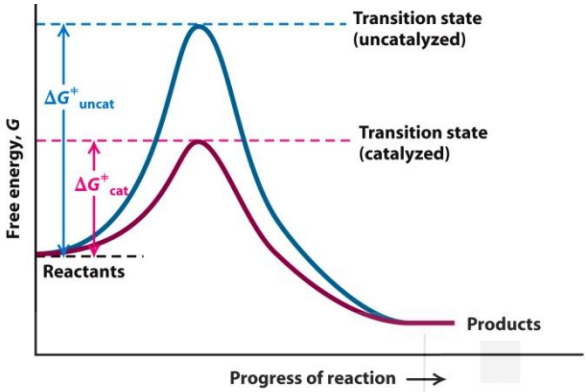


Fig. 1.4: Effect of enzyme on the activation energy. S and P denote the ground state of substrate and product respectively.

Once again look at figure 1.4 and you will notice that the activation energy of an uncatalysed reaction is very high than the catalyzed one. In the catalyzed reaction, a catalyst lowers the activation energy and thus facilitates the achievement of **transition state**, a phase which eventually leads to the formation of product and vice versa. Enzymes help to overcome the energy barrier so that more of the substrate molecule can be converted into product. How does it help the reaction process? The enzyme and the substrate form a reaction intermediate which helps to lower the activation energy than the reaction between reactant molecules without a catalyst. The enzyme substrate interaction creates a new reaction pathway whose **transition state** energy is lower than the reaction taking place without the enzyme. Breaking of older bonds and formation of new bonds require energy for the alignment of reacting groups, formation of transient unstable charges, and this energy comes from the binding energy from enzyme – substrate interaction. The free energy (binding energy) released by the interactions of enzyme and substrate offsets the energy required to reach the top of energy hill and results in lower activation energy.

1.6.1 Transition State Theory

The binding energy is used by enzymes to lower the activation energies of reactions. It adds to the specificity as well as to the catalytic power of the enzyme. In other words, binding energy acts as a driving force for enzyme catalysis. There are several other factors that play a significant role related to the free energy -physical and thermodynamic factors. These include- entropy, solvation etc. The weak bonds between substrate and enzyme in transition state during reaction will reduce the hydrogen bonds between substrate and water (desolvation). The binding energy holds the molecules in precise

alignment so that they can undergo the reaction, thereby reducing the entropy. The reduction in the motion of reactants along with weak interactions between enzyme and the substrate molecules will increase rate of reaction in an enzyme catalyzed reaction. Look at figure 1.5 it is evident from the reaction coordinate, the substrate has to overcome a free energy barrier before the products can be formed. This energy barrier is known as the **activation energy (*E*)** of the reaction. The molecular structure and conformation corresponding to the peak position in this profile is called the **transition state** of the reaction. For an enzyme catalyzed reaction we call it enzyme-substrate complex or ES-complex. In the transition state the chemical bonds are in the process of being formed or broken. It is, therefore, the most activated and hence highly unstable entity in the reaction pathway.

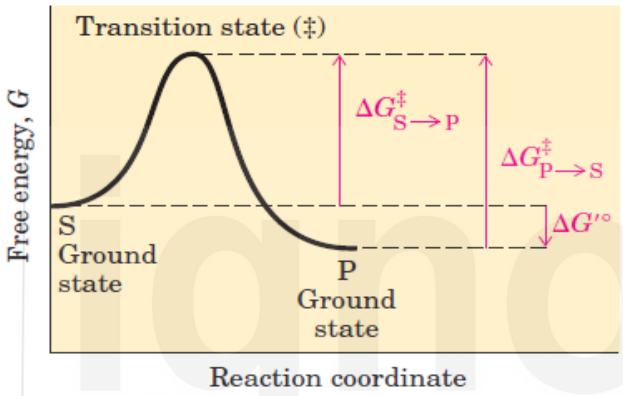


Fig. 1.5: Reaction coordinate showing free energy gap between substrate and product and transition state.

The concept of the transition state helps to express the rate of reaction, in terms of the free energy of activation. Since the molecules in the transition state are the ones which lead to the formation of products, the rate of reaction will depend on the concentration of molecules in the transition state.

Enzymes help in achievement of transition state easily and thus bring about a remarkable increase in the reaction rate. Enzyme only decreases the free energy of activation, so that more and more of reactant molecules pass the energy barrier to form the products. Here you must note that, the enzymes do not change the equilibrium point of the reaction which is related to the free energy of the reaction but merely speed up the rate at which the equilibrium point is reached.

SAQ 4

Do as Directed

a) What is the first law of Thermodynamics?

b) Even when a reaction is energetically favorable (ΔG < 0), it may not happen without a little “push”, what is meant by the push?

c) The substrate has to overcome a …….barrier before the products can be formed. (Fill in the Blanks)

d) The molecular structure and conformation corresponding to the peak position in this profile is called the ……..of the reaction. (Fill in the Blanks)

1.7 ENZYME ACTIVITY AND ITS UNITS

You have read about different aspects of enzyme and its characteristics. But do you know how will you measure enzyme activity?

Enzyme activity: It is the measurement of rate at which an enzyme catalyzes the reaction. The rate of enzyme reaction is measured by the rate of appearance of product or disappearance of reactant per unit time. The rate is measured by change in absorbance at a particular wavelength. So while writing it in formula form it will be represented as:

Enzyme activity = moles of substrate converted per unit time = rate × reaction volume.

The enzyme activity is expressed in terms of units (U) which is defined as:

One unit is the amount of enzyme that catalyses the conversion of 1 micromole of substrate per minute under defined conditions. Since the unit is too large, so the activity can be expressed easily in terms of nmol/min or pmol/min.

Please remember, enzyme activity is a measure of the quantity of active enzyme present and is thus dependent on conditions, ***which should be specified***.

The SI unit of enzyme activity is the katal, 1 katal = 1 mol s^{-1}, but since it is a large unit, for practical purposes commonly-used value is 1 enzyme unit (EU) = 1 µmol min^{-1} (µ = micro, x 10^{-6}). 1 U corresponds to 16.67 nanokatals.

Specific Enzyme Activity: It is another common unit. The purity of an enzyme is expressed in terms of the specific activity, which is the number of enzyme units (U) per milligram of protein. The formula for calculating specific enzyme activity is:

Specific Enzyme Activity = (Enzyme Activity in units/ml) / (Protein concentration mg/ml)

So specific activity values are units/mg or nmol/min/mg.

1.8 SUMMARY

So far you have learned that

- Enzymes speed up chemical reactions in our body. They are highly specialized proteins with a three dimensional structure and may require organic (e.g. biotin) and/or inorganic (e.g. ferric/ferrous ion) cofactors to help in catalysis. A complete, catalytically active enzyme together with its bound coenzyme and/or metal ions is called a holoenzyme or

conjugated enzyme. The protein part of such an enzyme is called the apoenzyme or apoprotein.

- Enzymes can increase reaction rates a billion fold, which enables biochemical reactions to occur under physiological conditions. The reason lies in their catalytic efficiency and high degree of specificity.
- Substrates bind the enzymes at active site to form the product. The active site of an enzyme consists of amino acid side chains, some of which act as binding groups while others act as catalytic groups.
- More ways of classification leads to several inconsistencies. Many enzymes are known by more than one name. In order to bring consistency to the classification of enzymes and to remove ambiguities associated with the use of trivial nomenclature, in 1964, the International Union of Biochemistry and Molecular Biology (IUBMB) established an Enzyme Commission (EC) to develop a logical nomenclature for enzymes which is uniform and universally acceptable.
- The Enzyme Commission classified enzymes into six main classes. Each class has further 4 to 13 subclasses. Each enzyme has been assigned a systemic code number called Enzyme Commission (E.C.) number. The E.C. number for each enzyme consists of a series of numbers at 4 places. Each major class is divided into sub-class and subclass has numerous sub-subclasses. Every class, subclass and sub-subclass is assigned a code number. Thus, each enzyme is given a four digit EC number (X1.X2.X3.X4) and a systematic name, which identifies the reaction it catalyzes.
- In the year 1878, J W Gibbs gave a relation as a function of T and S, which is popularly referred to as free energy or Gibbs free energy equation.

  $\Delta \boldsymbol{G} = \Delta \boldsymbol{H} - \boldsymbol{T}\Delta \boldsymbol{S}$ where ΔG represents free energy change.

  Free energy change must be negative for a reaction to be spontaneous.
- Even when a reaction is energetically favorable ($\Delta G < 0$), it may not happen without a little “push”, These reactions need an extra push to overcome thermodynamic stability. This extra push is called as Activation Energy. There is an **energy barrier** between S (substrate) and P (product). This energy barrier is known as **activation energy** or **energy of activation (E_A).** E_A is defined as the energy required to overcome the energy barrier so that substrate/s can be converted into product.
- Enzyme substrate interaction leads to release of energy. The energy released from these interactions is termed as **binding energy**. Binding energy serves two purposes: it is the driving force for catalysis as well as is responsible for enzyme specificity.
- Enzyme activity is measured by the rate of appearance of product or disappearance of reactant per unit time. The rate is measured by change in absorbance at a particular wavelength.

1.9 TERMINAL QUESTIONS

1. Distinguish between cofactor, coenzyme and prosthetic group of an enzyme.
2. Discuss the features of an active site of enzyme.
3. Explain the concept of Activation Energy and Transition State Theory.
4. Describe nomenclature and IUBMB system of the classification of enzymes.
5. Are all enzymes protein?

1.10 ANSWERS

Self Assessment Questions

1. a) False, b) True, c) False, d) False
2. a) True, b) True, c) True, d) False
3. a) iii), b) iv), c) ii), d) v), e) i)
4. a) The first law states in a closed system, energy can neither be lost nor gained, but can be converted into other forms of energy.

   b) Activation Enenrgy

   c) Free energy

   d) Transition state

Terminal Questions

1. Refer to section 1.2
2. Refer to section 1.4
3. Refer to section 1.6
4. Refer to Section 1.5

   Class is divided into sub-class and subclass has many sub-subclasses. Thus, each enzyme has a unique four digit EC number (X1.X2.X3.X4) and a systematic name, which identifies the enzyme on the basis of reaction it catalyzes. Refer section 1.6

5. Yes but there are some exceptions such as Ribozymes. Ribozymes are RNA molecules that have the ability to catalyze biochemical reactions such as RNA splicing in gene expression, transfer RNA biosynthesis, and viral replication.

   First Ribozyme was discovered in 1980. They might have played a very crucial role during the evolutionary phase.

1.11 FURTHER READINGS

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UNIT 2

MECHANISM OF CATAYSIS–1

Structure

2.1 INTRODUCTION

The magnificent history of the enzyme as a molecule is entrenched in the contribution of renowned scientists and noble laureates that help to the development of enzymology as a distinct entity. Two prominent scientists Koshland and Fischer elucidated the nature of enzyme as a molecule and try to explain its common associated characteristic specificity and flexibility respectively. Enzymes activity is influenced by a number of factors such as temperature, pH, susbstrate concentration etc. So that is why enzyme work best within specific temperature and pH ranges, and sub-optimal conditions can be the reason an enzyme to lose its ability to bind to a substrate. Several factors affect the rate at which enzymatic reactions proceed.

Activity of some enzymes gets influenced by compounds that do not seem to be directly involved the reaction, like metal ions, detergents, and hydrophobic molecules.

All enzyme assays measure either the disappearance of the substrate or production of product over time. Measurement of enzyme activity by different enzyme assays is vital for studying biological processes, diagnosing diseases, and designing pharmaceuticals. A large number of different methods of measuring the concentrations of substrates and products exist and many

enzymes can be assayed in different ways. Two main categories of enzyme assays-continuous and discontinuous assay have distinct methodologies and applications. We are going to discuss more about these assays later in the unit.

Expected Learning Outcomes

After studying this unit, you should be able to:

- elucidate Fischer views on enzyme catalysis;
- differentiate between Koshland and Fischer views on enzyme-substrate interaction;
- factors influencing enzyme activity;
- distinguish between continuous and discontinuous assays; and
- optimization of enzyme assays.

2.2 FISHER LOCK AND KEY HYPOTHESIS

To explain the fundamental action of single substrate enzyme, in 1894, Emil Fisher proposed that 'enzymes are structurally complementary to their substrates, so that they fit together like a lock and key' (Figure 2.1). This model is also known as template model. According to this model enzyme-substrate interaction is like a key fitting into its lock. Enzyme represents the lock and substrate is the key. Only the right key will be able to open the lock. Other keys that are too small or too big or are wrongly positioned will not be able to open the lock. As we have different set of keys for different locks, similarly different enzymes possess unique active sites for variable substrates. This theory also accounts for enzyme-substrate specificity. This analogy proved valuable and has vastly influenced the knowledge and development of biochemistry, since such interactions lie at the heart of many biochemical processes.

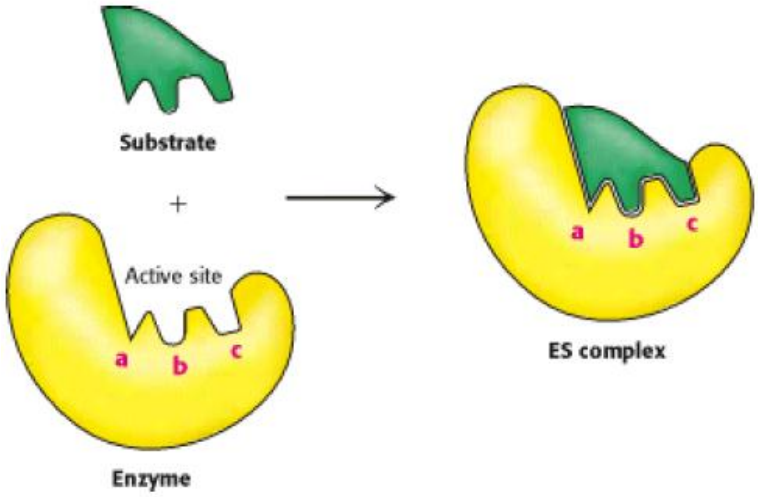


Fig. 2.1: Representation of the enzyme–substrate interaction in Lock and Key model.

Since the substrate must fit into the active site of the enzyme before catalysis can happen, only correctly devised molecules can serve as substrates for a specific enzyme; in many cases, an enzyme will react with only one naturally occurring molecule. The theory explains the enzyme specificity as lock and key hypothesis is focused on the active site of enzyme. Enzyme specificity is important because it helps to maintain and separate the multiple interlinked metabolic pathways, involving hundreds of enzymes.

However, this theory fails to explain the stabilization of enzyme. The active site of enzyme as per Lock and Key mechanism seems rigid (lock) and matches with the structure of substrate (key). It does not visualize the weakening of enzyme substrate bonds proposed in transition state theory. It does not take into account of intermediate shape of the enzyme structure. Active site seems to be more static and a single entity with no separate catalytic group. The observations from X-ray diffraction studies reveal tight fitting of the substrate to a pocket (active site) in the enzyme with complementary structure which suggests that the active site of enzyme is a much more flexible structure than postulated by Lock and Key model of enzyme catalysis.

2.3 INDUCED FIT MODEL

In view of above mentioned limitations of Fischer lock and key hypothesis, a second model was proposed by Koshland Jr., in 1958. He postulated that "Enzymes are flexible and that the shapes of the active sites can be markedly modified by the binding of substrate'. In the Fisher's lock and key model, the active sites are considered to be rigid structures. But this is not the case always. It has been observed that the active sites of some enzymes assume a shape that is complementary to that of the transition state only *after* the substrate is bound. That is, the proximity of the substrate induces a conformational change in the enzyme molecule aligning the groups for both substrate binding and catalysis. This process of dynamic recognition is called *induced fit* (Figure 2.2). The active site continuously modifies itself until and unless the substrate binds to it perfectly which is required for the further reaction. It has also been postulated that enzyme induced reciprocal changes in substrate and harness the binding energy to facilitate the transformation of substrates to products. The interactions between substrate and the enzyme and transition state formation as well as weakening of interactive bonds by nucleophilic or electrophilic attack are well explained in the induced fit mechanism. The induced fit model clarifies the characteristic features of the enzyme; specificity and flexibility. Therefore in comparison to lock and key model, the later model gained much more acceptance.

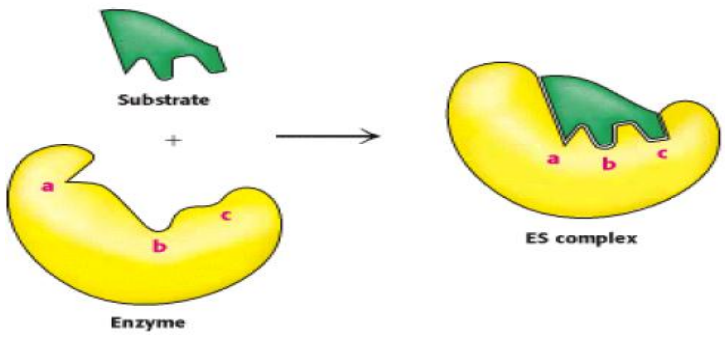


Fig. 2.2: Pictorial representation of enzyme–substrate interaction in the induced fit model.

Generally, an enzyme catalyzed reaction is 10^6 to 10^{12} times faster than any non-catalyzed reaction. The enzyme-substrate interactions occur at the molecular level. So it will be difficult to find the exact and accurate nature of these processes. Even enzymes differ in their specificity; some of the enzymes are compatible with several substrates- similar side chains or functional groups. There are several other factors that affect enzyme activity such as temperature and pH. Let is discuss more about these factors in subsequent sections of the unit.

SAQ 1

a) Which one is the lock and which one is the key in the proposed model of Fischer Lock and Key hypothesis?

b) In the Fisher's lock and key model, the active sites are rigid structures [True/False].

c) According to Induced fit hypothesis, enzymes are flexible [True/False].

d) Out of the two models (Induced Fit and Lock and Key) of enzyme catalysis, which one gained more acceptances?

2.4 FACTORS AFFECTING ENZYME ACTIVITY

You have learnt that the three dimensional structure of proteins (enzymes) is important for their catalytic activity. Apart from the peptide bond which links amino acids together in a chain, a protein is stabilized by various intermolecular forces like hydrogen bonding, vander Waals attraction, electrostatic interaction and so on. Changes in the physical or chemical environment of an enzyme affect its structure, which in turn affects its activity. In this section you will study about various factors like temperature, pH, concentration of substrate, concentration of enzyme, concentration of product and how these factors affects the enzyme activity.

2.4.1 Effect of Temperature

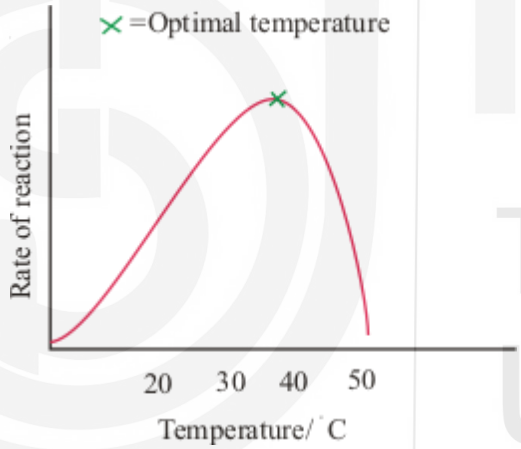
Enzymes are biological catalysts. So how does change in temperature affects the enzyme activity? Every enzyme has an optimum range of temperature at which enzyme shows its maximum activity. Generally, most of the enzymes have optimal temperature of 40^0 C, which is close to the physiological body temperature (37.5^0C). At high temperature typically between 42-70^0 C enzymes get irreversibly denatured.

At temperatures above and below the optimum range, activity of enzyme declines. This is because, at very low temperature enzyme activity gets arrested while at very high temperatures the three dimensional structure of the enzyme is lost. High temperature destroys the intermolecular attractions like hydrogen bonding which lead to loss of functional structure of enzyme, hence the affinity of enzyme for substrate diminishes and the its activity declines.

Look at the temperature versus activity graph (Fig. 2.3), it's a bell shaped curve, depicting maximum enzyme activity at the optimum temperature. In this context, one important fact which you must note is that increase in rate of reaction with increase in temperature is due to increase in the number of collisions between the enzyme and substrate molecules as well as also due to increase in the energy of these collisions. Rise in temperature is followed by increase in the velocity and kinetic energy and with faster velocities, molecules reaching activation energy will increase and that leads to increase in rate of reactions. Generally ten degree increase in temperature leads to increase in enzyme activity by 50 % to 100 %.

The change in rate of reaction for any 10^0 rise in temperature is known as its **Q_{10}, or temperature coefficient value**. This value is close to 2 for most of the chemical reactions. However, for enzymatic reactions, Q_{10} values are lower.

The increase in temperature also causes rise in the internal energy of molecules which may include translational, vibrational and rotational energy of molecules, the energy engaged in chemical bonding of molecules as well as energy engaged in nonbonding interactions. However, too much heat can lead to decrease in enzyme activity due to denaturation and inactivity of either enzyme or substrate.



The activity of most of the enzymes is usually destroyed by heat. However, some exceptions are the enzymes of bacteria, living in hot springs at temperature of 60-80^o C.

Fig. 2.3: Plot of temperature versus rate of reaction.

2.4.2 Effect of pH

Enzyme activity is influenced by pH. What is a pH value? All of you are familiar with this term right from school days. pH i.e. negative logarithm of the reciprocal of hydrogen ion concentration. Enzymes in general work within a small range and most of them have a particular pH at which they exhibit optimal activity. The particular pH of enzymes is the pH value at which the bonds within enzyme are influenced by the H^+ and OH^- ions in such a way that the shape of the active site of enzyme is most complementary to the shape of its substrate. As you know enzymes are proteins and their molecular interactions is determined by the interactions among the charges displayed by amino acids present in the proteins. The interactions may involve hydrogen bonding that can be altered by changes in pH.

Any change in pH most likely will alter the state of ionization of acidic or basic amino acids (e.g amino acids Asp, Lys) that plays a crucial role in the substrate binding and/ or the catalytic action or else the three dimensional structure of proteins will change. Therefore, alteration in pH leads to changes in protein function or inactivation of enzymes leading to the loss of enzyme structure and its activity.

Each enzyme, thus, acts best in a working pH range which is specific to it and its activity slows down with any substantial change i.e. increase or decrease in the H^+ ion concentration. At optimum pH enzymes show maximum activity (Fig. 2.4). Most of the enzymes have optimal pH of 7.4 which is also the physiological pH. The activity generally falls off on either side of this value. Deviations above 0.5 pH unit from the normal blood pH of 7.4 leads to protein denaturation. Although the covalent bonds are not broken but even disruption of some hydrogen bonding leads to loss of enzymatic activity.

Generally optimum enzyme activities are observed at or near the isoelectric point (pI) of the enzymes. The pH optimum of some enzymes is given in Table 2.1. For example, trypsin, whose pI value is 10.1, shows maximum activity at pH ranges between 7 and 9.

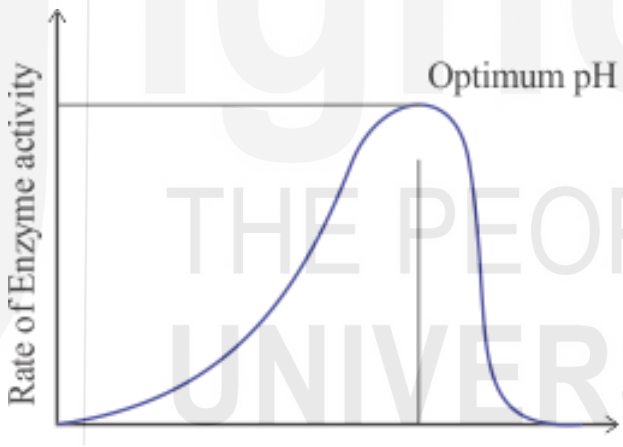


Fig. 2.4: Plot of pH versus enzyme activity.

Table 2.1: pH optimum of some common enzymes

S.No.	Enzyme	pH optimum
1	Pepsin	1.5-1.6
2	Lipase (Stomach)	4.0-5.0
3	Lipase (Pancreas)	8.0
4	Catalase	7.0
5	Trypsin	7.8-8.7

Small changes in the pH above or below the pH optimum do not lead to a lasting transformation in the enzyme, since the bonds can be renewed. However, acute changes in pH i.e highly acidic or highly alkaline may eventually cause denaturation of enzymes and they will lose their function irreversibly. Enzymes in dissimilar locations may have diverse optimum pH values since their environmental settings may be different.

2.4.3 Effect of Substrate Concentration

The enzyme activity is measured by determining the rate of disappearance of substrate or appearance of product. Therefore any changes in the substrate concentration will affect the rate of an enzyme catalyzed reaction. Therefore, in such condition, an increase in substrate concentration will lead to an increase in the rate of reaction.

In the presence of a given amount of enzyme, at the low concentration of substrate the rate of enzyme reaction will increase with increasing substrate concentration. In this condition, the catalytic sites of enzymes are vacant and substrate can bind most of time so the rate of reaction is limited by the availability of substrate. The rate of reaction increases linearly until it reaches a **maximum known as "point of saturation**" (Figure 2.5). The point of saturation is where maxima is attained and is denoted by V_{max}. V_{max} is the maximum possible rate of reaction. On reaching this stage an increase in substrate concentration will not produce any significant change in the rate of reaction. At this stage, substrate is present in excess and all the enzyme molecules are saturated with the substrate and all the enzyme active sites are occupied by substrate molecules. The increase in substrate will not have any effect until the bound substrate molecules gets released. The rate of formation of product is dependent on the enzyme activity itself.

The shape of the curve depicting the effect of substrate concentration on rate of reaction / enzyme activity is a hyperbola and well explained with a mathematical equation known as Michaelis- Menten equation.

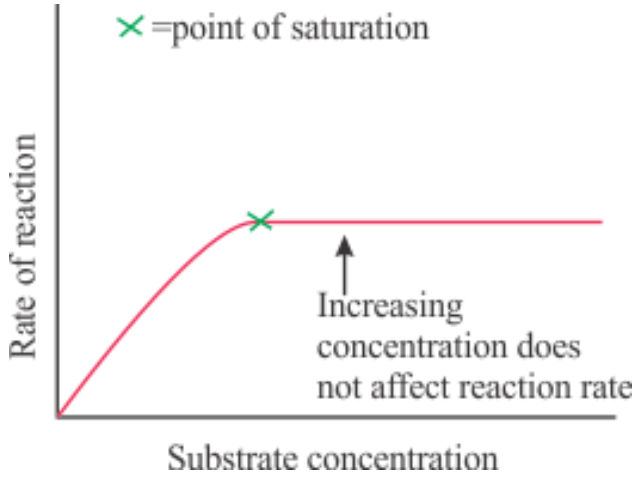


Fig. 2.5: Saturation curve for an enzyme obeying Michaelis-Menten equation, showing relation between substrate concentration and rate of reaction.

SAQ 2

a) Generally, most of the enzymes have optimal temperature of 40^0 C (True/False).

b) At high temperature typically between 42-70^0 C enzymes get irreversibly denatured (True/False).

c) Pepsin has pH Optima at 7.8 (True/False).

d) V_{max} is the maximum possible rate of reaction (True/False).

2.5 TYPES OF ENZYME ASSAYS

Enzyme activity serves as a vital parameter for determining biochemical processes and catalytic reactions within biological systems. Enzyme assays are indispensable tools used for assessing enzyme activity. Enzymatic activity assays are performed to determine the presence or amount of a selected protein in an organism, tissue, or sample. The following question may arise in the mind of learner.

How does an enzyme assay work?

The answer to the above question is that an enzyme assay is a laboratory technique used to measure enzyme activity. The assay involves preparation of reagents and samples and setting up of optimal reaction conditions to make the reaction feasible. Several key factors such as are temperature, pH, ionic strength and the proper concentrations of the essential components like substrates and enzymes must be considered for assaying enzymes. It is always advisable to standardize the parameters however the vast diversity of the characteristic features of different enzymes prevents unification of assay conditions. Nonetheless several enzymes, especially those from mammalian sources, possess a pH optimum near the physiological pH of 7.5, and the body temperature of about 37 °C can serve as assay temperature.

Once the enzyme reaction is initiated and monitoring of substrate or product concentration is done over a period of time to determine the changes. The collected data helps to determine the reaction rate. It also serves to controls different parameters as well as ensures calibration accuracy. These assays provide insights into enzyme behavior and kinetics which is very much important to assess their role in various biological pathways.

Rules for assaying enzyme activity include appropriate handling, methodical aspects, preparation of assay mixtures and blanks as well as choice of the assay time. Several other particulars of complex enzyme assays, including reversible reactions and coupled tests also need to be considered.

Enzyme assays can be divided into two groups based to their sampling method: **continuous assays**, where the assay gives a continuous reading of activity, and **discontinuous assays**, where samples are taken, the reaction stopped and then the concentration of substrates/products is determined. Let us discuss them more in detail.

Continuous enzyme assay: As the name suggests continuous assays are those assays where the assay gives a continuous reading of activity till completion. So these are often termed as endpoint assays. The enzyme activity is measured via the quantity of substrate consumed, or the amount of product formed during the reaction over a fixed period of time. Both values are directly proportional to the concentration of enzymes present in the sample.

Examples of continuous assays include spectrophotometry, calorimetry, chemiluminescence and fluorimetry. In these methods, the enzyme activity is determined by measuring the progress of reactions by light or heat.

Spectrophotometric enzyme assays:

In spectrophotometric assays, the course of the reaction is determined by measuring a change in the absorption of assay solution. If the absorption falls in the visible region then the change in the color of the assay can be determined. Such assays are known as **spectrophotometric assays**.
The MTT assay, a redox assay using a tetrazolium dye as substrate is an example of a colorimetric assay. In several oxidation reduction reactions, NADH or NADPH is extensively used as coenzymes. These coenzymes absorb UV light in their reduced forms but not in their oxidized forms so UV light can also be used to measure enzyme activity in oxidoreductive reactions. An oxidoreductase using NADH as a substrate could therefore be assayed by following the decrease in UV absorbance at 340 nm as it consumes the coenzyme.

Direct versus Coupled assays: There are several enzyme reactions which do not result in the change in the absorption of fluorescence. In such a scenario, it is possible to measure enzyme activity indirectly using spectrophotometric or flurometric assay for the enzyme by using a **coupled assay**. In coupled assay, the product of one reaction is used as the substrate of another, easily-detectable reaction. For example, hexokinase catalyses addition of phosphate group to glucose to form glucose-6-phosphate. Its enzyme activity can be measured by coupling the production of glucose-6-phosphate to NADPH production, using glucose-6-phosphate dehydrogenase. So coupled assays allow measurement of enzyme indirectly.

Fluorimetric enzyme assays

You are already familiar with fluorescence and fluorometry. When a molecule emits light of one wavelength after absorbing light of a different wavelength it is called fluorescence. Fluorometric assays are based on the difference in the fluorescence of substrate from product to determine the enzyme activity. These assays are considered to be much more sensitive than spectrophotometric assays. However, several limitations include external and intrinsic fluorescence as well as the instability of several fluorescent compounds when exposed to light.

The examples of these assays include fluorescent dyes, NADH and NADPH. Since NADH and NADPH are reduced forms they are fluorescent while their oxidized forms are non- fluorescent. The increase of decrease in fluorescence in enzymatic reactions will help to determine enzyme activity. Synthetic

substrates that release a fluorescent dye in an enzyme-catalyzed reaction includes 4-methylumbelliferyl-β-D-glucuronide for assaying β-galactosidase.

Calorimetric

In the calorimetric enzyme assays the heat released or absorbed by chemical reactions is measured. These assays are very general, since many reactions involve some change in heat and with use of a microcalorimeter, not much enzyme or substrate is required. The calorimetric enzyme assay offers several advantages over other conventional enzyme assay techniques such as it allows the use of natural substrates and it remain unaffected by the spectroscopic properties of the sample.

Chemiluminescent Enzyme Assays

Chemiluminescence is the emission of light by a chemical reaction. When the excited intermediates in enzyme reactions return back to their stable ground state, a photon is released, which is detected by the luminescent signal instrument to measure product formation. These types of assay can be extremely sensitive than colorimetric, fluorimetric, or radiometric assays, since the light produced can be captured by photographic film over days or weeks. Examples of such reactions includes detection of horseradish peroxidase by enzymatic chemiluminescence, a very common method of detecting antibodies in western blotting Another example of such reactions is enzyme luciferase found in fireflies and it naturally produces light from its substrate luciferin.

Discontinuous enzyme assays: In such enzyme assays, the reaction is stopped and then the concentration of substrates/products is determined to measure the enzyme activity. In contrast to continuous enzyme assays, discontinuous assays are performed when samples are taken at set intervals of time. Therefore, such form of enzyme assay directly or indirectly measures changes in substrate or products over time, to determine the reaction rate changes. These assays involve radiometric assays as well as chromatographic assays such as HPLC or TLC.

Radiometric enzyme assays

You have read about radioactivity in course bioanalytical tools and techniques. Radiometric assays measure the incorporation of radioactivity into substrates or its release from substrates. The radioactive isotopes most frequently used in these assays are ^{14}C, ^{32}P, ^{35}S and ^{125}I. These assays are both extremely sensitive and specific. Radioactivity is usually measured in these procedures using a scintillation counter.

Chromatographic enzyme assays

As the name suggests, these chromatographic assays make use of high-performance liquid chromatography (HPLC) technique to measure product formation by separating the reaction mixture into its components. These assays are more complex and its sensitivity can be increased by labelling the substrates/products with a radioactive or fluorescent tag.

A comparison of two methods reveals that the continuous enzyme assay method is generally easy to perform while discontinuous enzyme assays are used in scenario where higher precision or complex sample matrices are required.

SAQ 3

a) Continuous assays are those assays where the assay gives a continuous reading of activity till……?

b) In coupled enzyme assay, enzyme activity is measured …….

c) Give two examples of continuous enzyme assays.

d) Name two discontinuous enzyme assays.

2.6 OPTIMIZATION OF ENZYME ASSAYS

Thus, the development of reproducible sensitive enzyme assays is essential for so many metabolic processes. It has wider applications in the industrial and pharma sector. The development of an in vitro enzyme assay requires consideration of multiple factors such as purity of the enzyme, pH, viscosity and ionic strength of the reaction mixture, and the addition of co-factors or other buffer additives. It is important to determine the stability of reagents under storage and assay conditions.

During the optimization of an enzyme assay, the effects of the following factors are normally examined:

(1) Buffer composition,

(2) pH,

(3) Temperature,

(4) Concentration of divalent ions (e.g., Mg^{2+}, Ca^{2+}),

(5) Concentration of ion salts (e.g., NaCl, KCl),

(6) Type and concentration of reducing agents (e.g., DTT, β-mercaptoethanol), and

(7) Solvent concentration (e.g., DMSO, polyethylene glycol [PEG]).

Apart from the above listed factors, the choice of detection method for following an enzymatic reaction and the size of the reaction volume are also critical. Nowadays a lot of efforts are concentrated on miniaturization of enzymes assays to reduce the consumption of reagents (and therefore the cost). However, these efforts need to skillfully determine the potential effect of miniaturization on the assay performance.

2.8 SUMMARY

Let us summarize our unit:

- In Lock and Key paradigm. Emil Fischer proposed model depicts inflexible interactions. The shapes of the interaction between enzyme and substrate are complimentary, and binding induces relatively minimal conformational change.
- The induced fit model proposed by Koshland described about the conformational alterations that happen during enzyme substrate binding and these interactions enable several degrees of shape complementarity in the unbound state. The model helps to explain the dynamic structure of proteins.
- On binding a ligand, an enzyme alters its conformation just as a glove changes its shape when a hand slides inside it. In the beginning, a weak initial complex is formed which is followed by intermediates that gradually reorganize to make new interactions until the final high-affinity state is accomplished.
- Several factors affect or alter the activity of enzymes for example temperature, pH and concentration of substrate etc. Every enzyme has an optimum temperature at which it shows its maximum activity. Generally, most of the enzymes have optimal temperature of 40^0 C.
- Generally most of the enzymes have optimal pH of 7.4 which is also the physiological pH. The activity generally falls off on either side of this value.
- Enzyme activity is also affected by substrate concentration. Shape of the curve depicting the effect of substrate concentration on rate of reaction / enzyme activity is a hyperbola and well explained with a mathematical equation known as Michaelis- Menten equation.
- Enzyme assays are crucial tools used for assessing enzyme activity. These assays are established to determine the activity or concentration of enzymes. These enzymatic assays are of different types such as based on the detection of fluorescent, luminescent, or spectrophotometric endpoint signal.

2.7 TERMINAL QUESTIONS

1. Explain induced fit theory proposed by Koshland.
2. Explain the difference between lock and key hypothesis and induced fit model.
3. How temperature influences enzyme activity?
4. Describe different types of enzyme assays.
5. Write note on optimization of enzyme assays.
6. Have you ever thought why metabolic rate increases during fever?

2.8 ANSWERS

Self-Assessment Questions

1. a) Enzyme is the lock, substrate is the key,
   b) True
   c) True
   d) Induced Fit
2. a) True
   b) True
   c) False
   d) True
3. a) Completion
   b) Indirectly
   c) Spectrophotometry, calorimetry, chemilumin escence and fluorimetry, d- Radiometric and chromatographic enzyme assays

Terminal Questions

1. Refer to section 2.3.
2. Refer to section 2.2 and 2.3.
3. Refer to section 2.4
4. Refer section 2.5
5. Refer section 2.6
6. The metabolic rate increases partially due to increased temperature and also because of effect of temperature on enzyme.

2.9 FURTHER READINGS

1. Albert L.Lehninger: Principles of Biochemistry, Worth Publishers, Inc. New York, 1984.
2. Donald J Voet Principles of Biochemistry, John Wiley and Sons, Inc, USA.
3. Eric E Conn, Paul K Stumpf: Outlines of Biochemistry, John Wiley and Sons, Inc, USA.
4. S. Shanmugan and T. Sathishkumar: Enzyme Technology, I K International Publishing House Pvt Ltd, New Delhi.
5. Nicholas C Price and Lewis Stevens: Fundamentals of Enzymology, Oxford University Press, Oxford, New York, USA.

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UNIT 3

MECHANISM OF CATALYSIS–II

Structure

3.1 INTRODUCTION

As you know enzymes are substantially better catalysts and surpass all chemical catalysts in relation to substrate and reaction specificities. Several types of interactions are involved in the binding of enzyme with the substrate such as covalent bonding, hydrogen bonding, ionic interactions, ion-dipole and dipole-dipole interactions, charge transfer interactions, hydrophobic interactions, and van der Waals interactions. After binding of the substrate with the active site, the enzyme converts the substrate into the product by employing various types of catalytic mechanisms. These methods are classified as acid-base catalysis, covalent catalysis, metal ion catalysis, proximity and orientation, strain and distortion and transition state analogues. These mechanisms of enzyme catalysis hold the key to accelerate the rate of enzyme reaction.

Expected Learning Outcomes

After studying this unit, you should be able to:

- determine several type of interactions involved in enzyme-substrate binding;

- ❖ explain acid – base catalysis;
- ❖ discuss the examples of chymotrypsin and lysozyme; and
- ❖ determine the precise nature of interactions between enzyme and substrate.

3.2 GENERAL FEATURES

In the previous units, you must have come across several aspects of the enzyme, its regulation and inhibition. How do enzymes work? What do you think? Due to larger size and variety of properties enzymes are able to enforce their presence on a reaction to a far greater extent than most catalysts. As you know all catalysts act by reducing activation energy of the reaction being catalyzed, free energy of an enzyme catalyzed reaction consists of both enthalpy and entropy components as $\Delta G = \Delta H - T \Delta S$, where ΔH is the enthalpy and ΔS is the entropy. Enzymes accomplish lowering of activation energy through various mechanisms that depend on the arrangement of functional groups in the enzyme's active site. Active site of an enzyme is the region where catalysis occurs. Three- dimensional structure of active site guard substrates from solvent and help in catalysis. It is quite likely that number of catalytic processes may be involved.

3.2.1 Proximity and Orientation

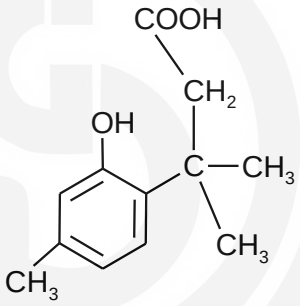
The efficiency of enzymes arises from the physical conditions at the enzyme catalytic site that advance biochemical reactions. Major physical and thermodynamic barriers to reaction include: (1) the relative motion of reacting molecules in solution, entropy; (2) the solvated shell of hydrogen-bonded water that surrounds and assist to stabilize most biomolecules in the aqueous solution; (3) the electronic or structural distortion of substrates in reactions; and (4) to attain proper alignment of relevant catalytic functional groups on the enzyme.

Proximity and orientation illustrate the significance of the specific arrangement of the catalytic groups at the active site. In an enzyme catalyzed reaction, binding of all the necessary groups (substrate and co-factors) molecules in close proximity to each other on the enzyme surface effectively increases their concentrations and reduced entropy loss for the subsequent formation of a transition – state; this is known as **proximation** effect. Even when reactions involved more than one substrate, enzymes tend to increase the rate of reaction by binding the substrates at adjacent sites and then bring them in close proximity to each other. Simple model calculations suggested that such proximity effects can increase the reaction rates by factor ~5. Orientation is important because side chains in proteins are relatively fixed and geometric consideration become important in catalysis. Molecules are not equally reactive in all directions. The enzyme must ensure that the reacting groups of the bound substrates approach each other with their electronic orbital's correctly oriented thus ensuring that the reaction takes place under optimal conditions. The presence of charged groups helps to stabilize transition state of the reaction. Enzymes drive away the relative

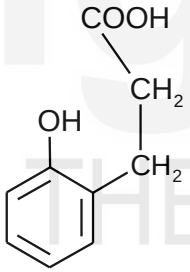
translational and rotational motions of the reactive groups. The reduction in the relative motion of reacting molecules or entropy reduction helps them to bind to the enzyme. The binding energy holds them in proper orientation and alignment so as to increase the number of effective collisions. In the transition state, these groups have little relative motion. Such type of effects can increase rate of reaction up to ~107.

Intramolecular reactions between groups present in a single molecule are faster than their corresponding intermolecular reactions between two independent molecules, e.g. cyclization of succinic acid to form succinyl anhydride. This is due to difference between the entropy changes that accompany the inter-intramolecular reaction. Product formation involves much larger loss of translational and rotational entropy in the intermolecular reaction. Negative change in entropy results in increase of overall free energy change in the reaction and activation of free energy for the formation of transition state.

During intramolecular reaction, much of the entropy decrease take place during preparation of reagent. The rate of lactone formation varies in compounds I and II (Figure 7.1) e.g. presence of two methyl groups on C-atom adjacent to the aromatic ring increases the rate of lactone formation by a factor of 4xl 05. Rate of lactone formation is slow in structure Il.



Structure I



Structure II

Fig. 3.1: Structures engaged in the intramolecular reaction for lactone formation.

Rate of reaction is high in structure I because

i) Bulky substituents place restrictions on rotation about single bonds in carboxylate side chain and thus ensure preferred orientation of reacting groups. This orientation resembles the transition state of reaction.

ii) Molecules will spend a greater proportion of time in a conformation that leads to reaction. Due to this loss rotational entropy will be lost when reaction proceeds to transition state (Less rotational entropy means a fewer degrees of freedom).

iii) The smaller negative entropy of activation leads to increased reaction rate.

Enzymes that catalyze intermolecular reactions take advantage of proximity effect by binding the reactants close together in active site, so that the

reactive groups are oriented appropriately for the reaction. Once the substrates are fixed, the reaction kinetically proceeds like intramolecular process. The decrease in entropy is associated with the formation of transition state that has been moved to an earlier step, the binding of the substrate to form E-S complex.

It was estimated by Page and Jencks that each factor i.e. proximity and orientation increases reaction rate by 104 fold for a bimolecular reaction. The rate enhancement is by 108fold when two factors operate together.

SAQ 1

Do as directed :

a) In the equation $\Delta G = \Delta H - T \Delta S$, G stands for ……(Fill in the Blank).

b) Proximity and orientation demonstrate the significance of the general/specific arrangement of the catalytic groups at the active site (Pick one option).

c) The presence of charged groups helps to stabilize/destabilize the transition state of the reaction (Pick one option).

d) Entropy increases during the formation of transition state (True/False).

3.2.2 Strain and Distortion

Binding energy is the major driving force in enzyme catalyzed mechanisms. It can be used to overcome all of above mentioned barriers for a reaction to take place. The formation of weak bonds between substrate and enzyme results in **desolvation** of the substrate. The interactions between enzyme and substrate swap most or all of the hydrogen bonds that may exist between the substrate and water in solution. Therefore, these interactions facilitate to compensate thermodynamically for any strain or distortion that substrate may go through to react. The substrate distortion may be electrostatic or structural.

Strain and distortion explain the probable mechanism of enzyme catalyzed reaction. This is the principal effect of induced fit binding, where the enzyme's affinity to the transition state is greater than the substrate itself. The structure of the active site is almost but not exactly complementary to the substrate. If active site is rigid, then in order to bind to the enzyme, the substrate needs to be slightly distorted. This induces structural rearrangement and distortion might result in stretching and thus weakening of bond which is subsequently cleaved. This assists the forward reaction.

The structural rearrangement (due to strain) brings substrate into a position closer to the conformation of transition state, so lowering the energy difference between the substrate and transition state and thus helping the reaction catalysis.

Strain and distortion effect is more of a ground state destabilization effect, rather than the transition state stabilization effect. As enzymes are very flexible, they cannot apply large strain effect. In addition to substrate, bond strain may also be induced within the enzyme itself to activate residue in the active site.

3.2.3 Acid Base Catalysis

The ionizable functional groups of aminoacyl side chains and prosthetic groups contribute to catalysis by acting acids or bases. The charged intermediates are stabilized by transfer to proton to or from substrate to intermediate to form a species that readily breaks down to products. For non-enzymatic reactions, the transfer of proton can either involve the constituents of only water or other weak proton donors or acceptors.

Acid catalyses the reaction by donating the proton and bases do the same by accepting a proton. Acid-base catalysis is broadly classified as:

i) **Specific acid base catalysis:** Specific acid or base catalysis means means only H+ (H30+) or OH- ions. Catalysis by acids or bases in solution is said to be specific only when catalysis is carried out due to ions formed from the solvent. The rate of reaction is susceptible to the changes in the concentration of H+ and/ or H30+ ions but it is independent of all concentrations of other acids in the solution or at the active site.

ii) **General acid-base catalysis:** In general acid-base catalysis, rate of reaction changes with all acids or bases present in the solution. In this type of catalysis, proton transfer is mediated by other classes of molecules. For non-enzymatic reactions in aqueous solution, this occurs only when the unstable reaction intermediate breaks down to reactants faster than protons can be transferred to or from water.

The general acid catalytic process involves lowering of free energy of the reaction transition state by transfer of a proton from an acid. Reaction involving keto-enol tautomerization in the absence of any catalysis is very slow due to high free energy of its carbanion like transition state (Fig 7.2 a). You will notice in the next reaction that in the presence of acid, transfer of proton to the oxygen atom increases the rate of reaction by reducing the carbanion character of the transition state (Fig 7.2 b). Similarly rate of reaction can also be increased by base (Fig 7.2 c). This mechanism of reaction is known as general base catalysis.

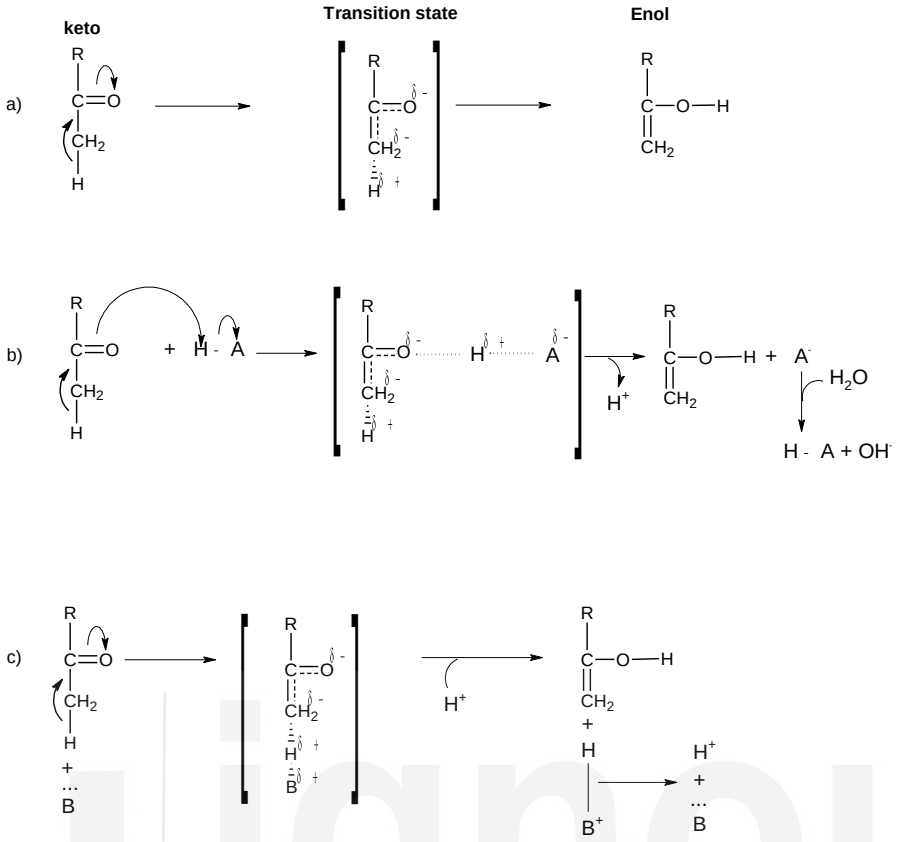


Fig. 3.2: Mechanism of keto-enol tautomerization. a) uncatalyzed b) general acid catalysis c) general base catalysis

Weak organic acids can supplement water as proton donors or weak organic bases can serve as proton acceptors. At enzyme's active site, side chains of a number of amino acids can act as proton donors and acceptors. These groups are positioned very precisely in enzyme's active site so as to allow proton transfer, thereby providing rate enhancement by the order 102 to 105• Side chains of active site amino acid residues can either donate or retrieve H+ to or from substrate molecule respectively during catalytic reaction.

Many reactions involving acid/base catalysis assume a substantially altered pKa. This alteration of pKa is possible through local environment of the residue.

Conditions of active site	**Acids**	**Bases**
Hydrophobic environment	pKa increase	pKa decrease
Adjacent residues of like charge	pKa increase	pKa decrease
Salt bridge (and H-bond	pKa decrease	pKa increase formation

SAQ 2

Answer the following :

a) The structure of the active site is not complementary to the substrate (True/False).

b) Strain brings substrate into a position farther from the conformation of transition (True/False).

c) Bonding interactions compensate thermodynamically for any strain or distortion (True/False).

d) The formation of weak bonds between substrate and enzyme results in .....of the substrate. (Fill in the Blank).

e) Weak organic acids can supplement water as proton donors or weak organic bases can serve as proton acceptors (True/False).

3.2.4 Covalent Catalysis

In covalent catalysis, the transition-state is stabilized by changes involving covalent bonds. It is also called alternative pathway catalysis. In this, a transient covalent bond is formed between the enzyme and substrate. The covalent bond is formed between the nucleophilic group on the catalyst and an electrophilic group on the substrate. So this type of catalysis is also known as nucleophilic catalysis. A well known example of this type of catalysis is decarboxylation of acetoacetate by primary amines (Fig. 7.3)

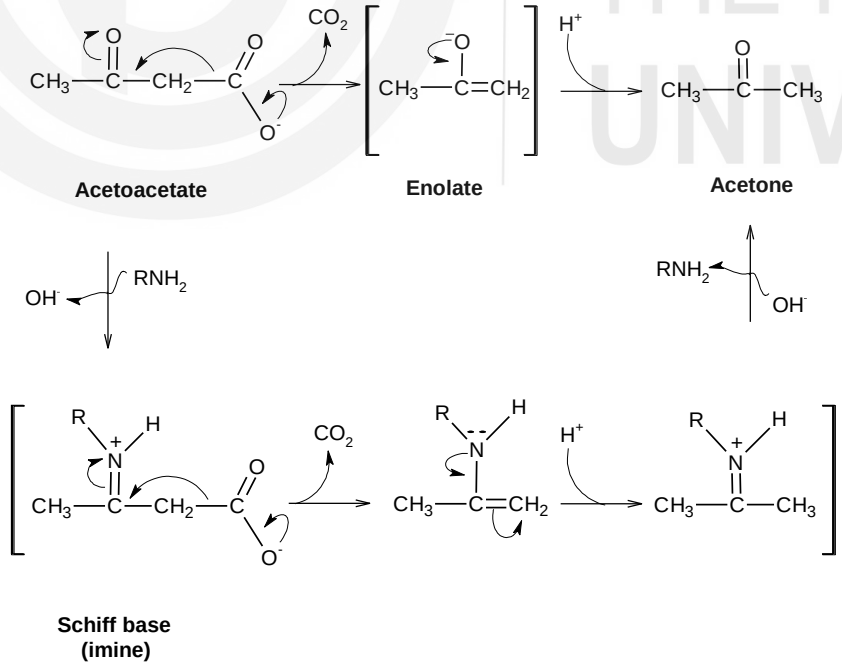


Fig. 3.3: Decarboxylation of Acetoacetate.

Nucleophilic catalysis by primary amines is shown at the bottom while uncatalyzed reaction is shown at the top.

Covalent catalysis is carried out in three stages:

1) Formation of covalent bond by nucleophilic reaction of catalyst with the substrate.

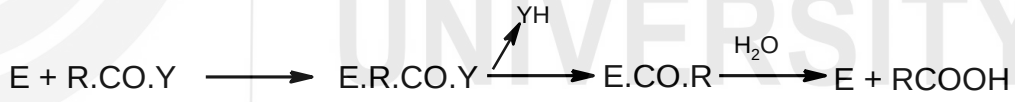
2) Extraction of electrons from the reaction center by now electrophilic catalyst.

3) Elimination of the catalyst.

The presence of nucleophile alters the pathway of reaction. Catalysis results only if new pathway has lower energy of activation than uncatalysed pathway. Covalent intermediates are both rapidly formed and broken down. In nucleophilic catalysis, the catalyst is more nucleophilic than normal attacking groups. So intermediate is rapidly formed and broken down to form the products. Highly polar groups such as imidazole and thiol groups are good covalent catalysts. Several coenzymes such as thiamine pyrophosphate and pyridoxal phosphate function in connection with their apoenzymes as covalent catalysis.

3.2.5 Mechanism of Action of Chymotrypsin

Several peptide bonds are cleaved in the inactive monomeric protein called as chymotrypsinogen to produce the enzyme chymotrypsin. Chymotrypsinogen is produced and secreted by pancreas. The active enzyme chymotrypsin contains three non-identical polypeptide chains. It catalyses protein hydrolysis-cleaves peptide bonds at the carboxyl side of aromatic amino acid (phenylalanine, tyrosine or tryptophan) residues. It also hydrolyses a range of amides and esters. Evidence suggests that chymotrypsin catalyzed hydrolysis of an ester that proceeds via formation of an acylenzyme. This is also true for hydrolysis of amides.



X-ray studies revealed a hydrophobic binding pocket at active site for aromatic side chains. Asp-102 is buried in the interior of the molecule and is linked to action of His-57 and Ser-195 by forming charge-relay system. These three amino acid residues form collectively catalytic triad of the enzyme (Figure 7.4). Asp-102 removes a proton from His-57 and His-57 removes a proton from Ser-195 during the path of enzyme reaction.

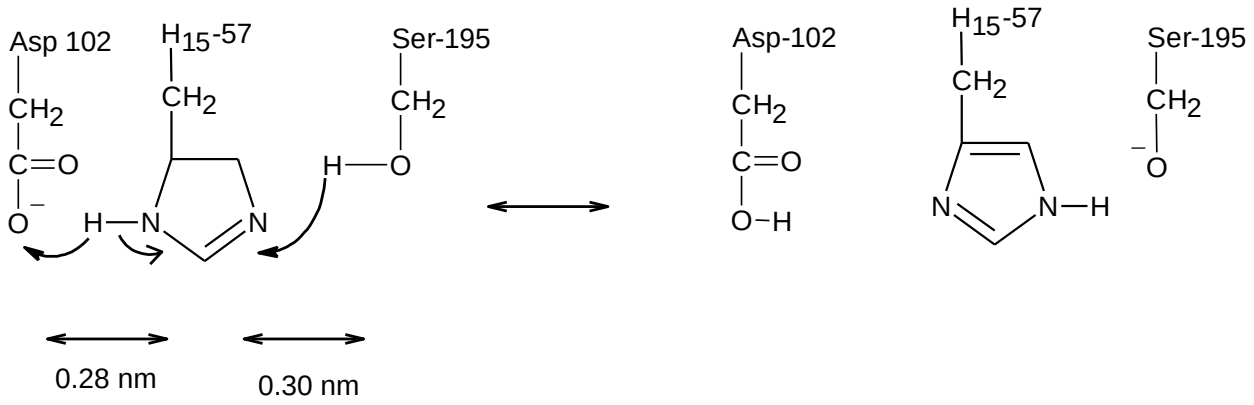
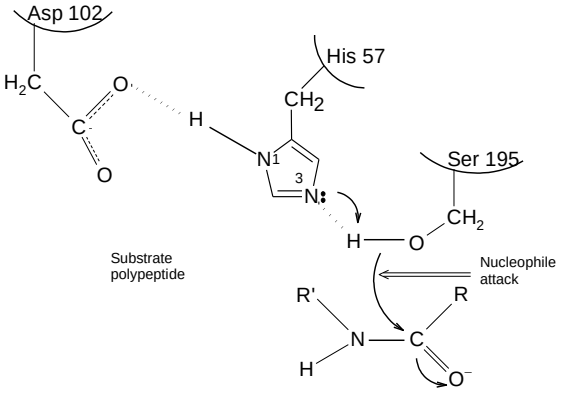
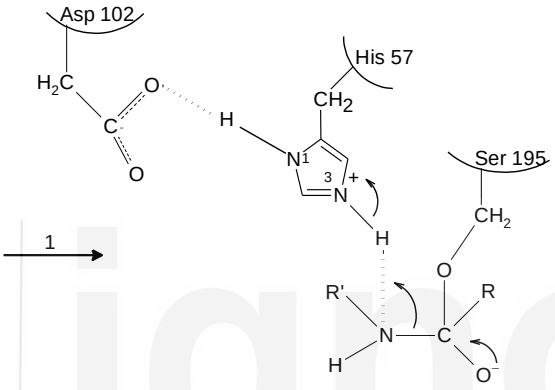


Fig. 3.4: Catalytic triad at active site of enzyme Chymotrypsin.

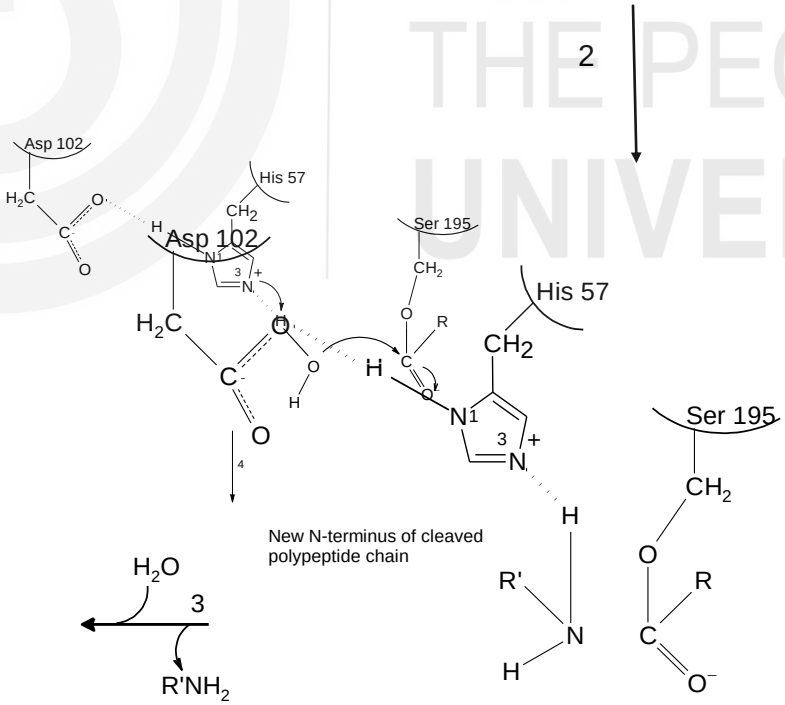
His-57 acts as base catalyst and enables the oxygen of Ser-195 to make a nucleophilic attack on the carboxyl group of the enzyme bound substrate. The nucleophilic attack is assisted by the transfer of proton to the imidazole ring of His-57 to form an imadazolium ion. The catalysis is further aided by the polarizing effect of unsolvated carboxylate ion of Asp-102 which is hydrogen bonded to His-57 (electrophilic catalysis). An unstable tetrahedral intermediate is formed with transient existence. The unstable intermediate crumbles to acyl-enzyme intermediate due to the driving forces on account of acid hydrolysis of Asp-102 on His-57. Please observe the figure 3.5 and you will notice that amine group (RNH2) leaves the enzyme and is replaced by water from the solvent. The next step involves hydrolytic cleavage and formation of tetrahedral intermediate by the addition of water. The reversal of first step leads to regeneration of active enzyme.



Enzyme-substrate complex



Tetrahedral intermediate



Acyl-enzyme intermediate

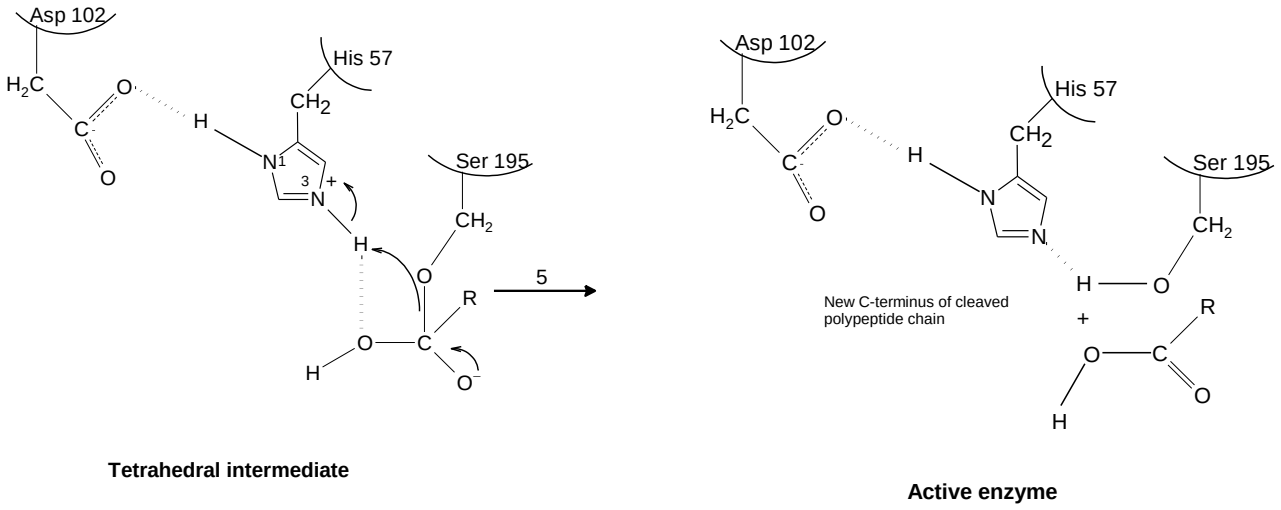


Fig. 3.5: Catalytic Mechanism of Chymotrypsin based on chemical and structural data.

Similar mechanisms operate for peptide substrates R'NH.C(R'').CO.NH.R''.

3.2.6 Mechanism of Lysozyme Action

Lysozyme is well known as an antimicrobial enzyme N-acetylmuramide glycanhydrolase. It derives its name by hydrolysing the polymer consisting of alternating N-acetylglucosamine (NGA) and N-acetylmurarnic acid (NAM) units which gives shape and rigidity to bacterial cell walls. So bacterial cell wall loses its integrity and gets destroyed. Lysozymes are secreted abundantly in saliva, human milk, tears and occur widely in cells. Hen egg white lysozyme serves as one of the most widely studied enzyme. Do you know that lysozyme catalyzed substrate hydrolysis is 108 folds greater than that of uncatalysed reaction? Let us study the mechanistic pathway of this enzyme.

Lysozyme has large cleft which can accommodate six of the substrate's amino-sugar units (look at the figure 7.6, these six units are designated as A-F).

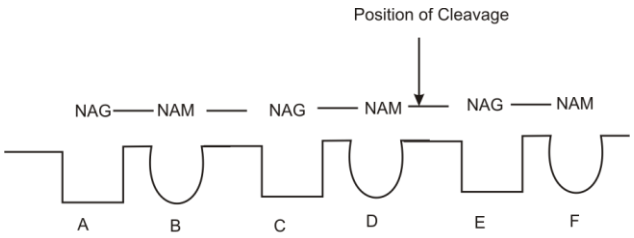
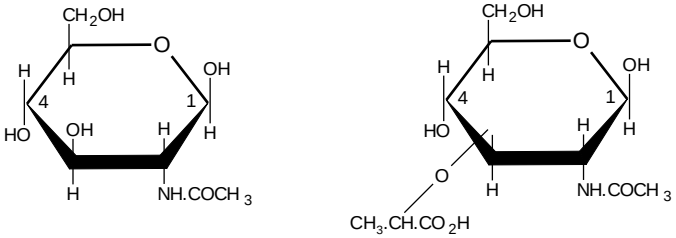


Fig. 7.6: Cleft of Lysozyme.



NAG **NAM**

The cleft is one of the most striking features as a substrate binding site of the enzyme molecule. Every second residue of the substrate of enzyme is an NAM. Each of six amino-sugars is bound to the enzyme by H-bonds, e.g. those in sites A and B to aspartate 101, that in site B to Trp-62 and Trp-63, and in site F to Asn-37. NAM, has large lactyl side chain thus it cannot bind to sites A,C or E, due to restricted space, and during or immediately after binding to site D its hexose ring is distorted from the chair to the less stable half-chair conformation (Fig. 7.7).

Fig. 3.7 a): Chair Conformation b): Half-Chair Conformation.

Hydrolysis takes place between units D and E, and therefore must always involve the β(1->4) glycosidic link between Cl (the reducing end) of NAM and C4 of NAG. The amino acids near this bond are Glu-35 and Asp-52. The reaction proceeds as shown in the figure 3.8 is as follows:

Step 1: Substrate binds to enzyme and ring D is distorted to the half-chair conformation; it facilitates the formation of a carbonium ion also having the half chair conformation.

Step 2: Glu-35, which is in non-polar environment and thus protonated at pH 5.0, it can act as a general acid catalyst and donates its proton to the 0-of glycosidic bond, causing the bond to break.

Step 3: Asp-52, is in a more polar environment than Glu-35 and is negatively charged at pH 5.0. Therefore, it can stabilize the carbonium ion by electrostatic interaction. The first product leaves and the reaction get completed by nucleophilic attack on the carbonium ion by water.

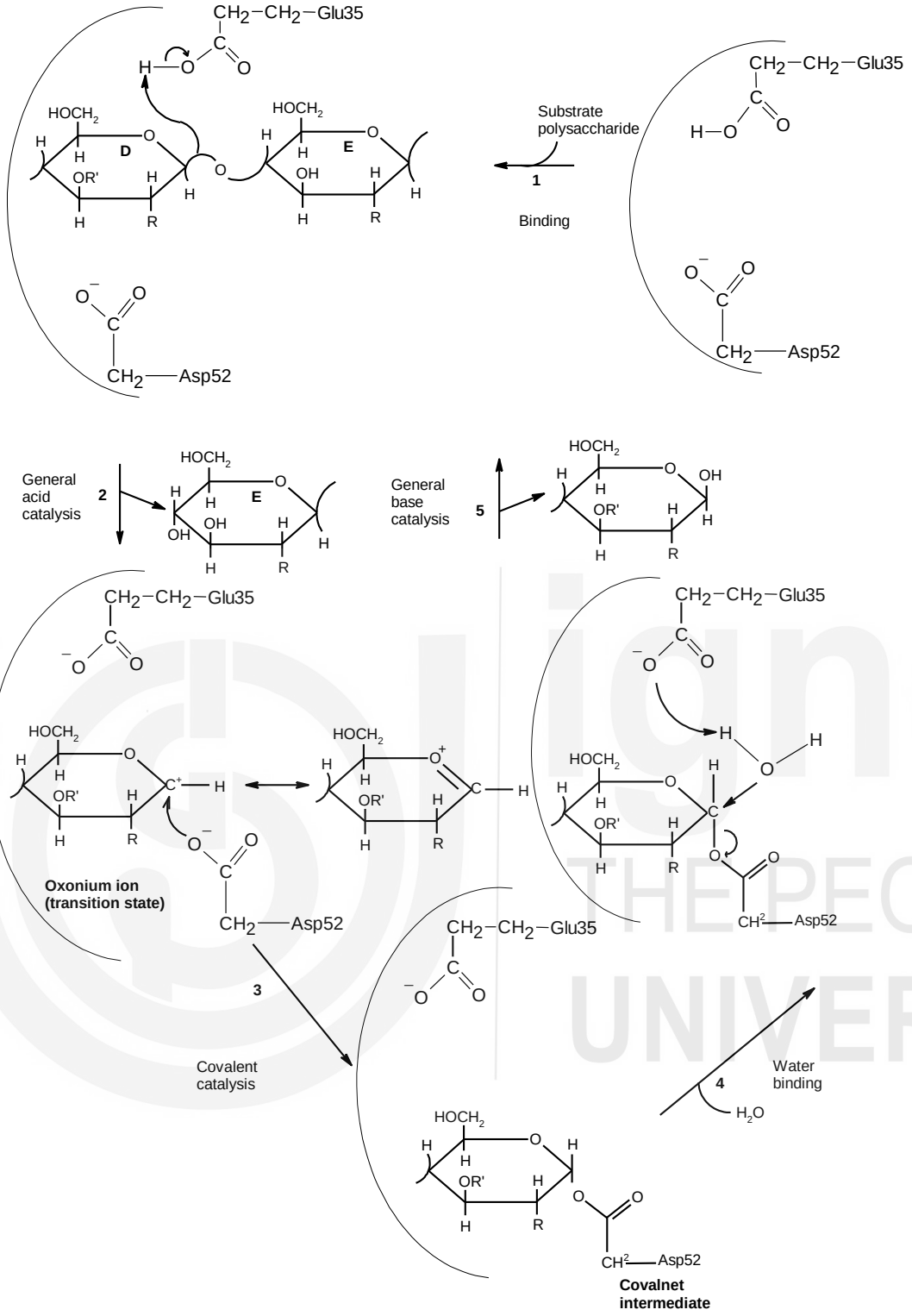


Fig. 3.8: Catalytic Mechanism of Lysozyme.

SAQ 3

Answer the following :

a) In nucleophilic catalysis, the catalyst is more electrophilic/nucleophilic than normal attacking groups. (Pick one option).

b) Polar groups such as imidazole and thiol groups are good covalent catalysts (True/False).

c) Chymotrypsinogen is secreted by pancreas (True/False).

d) Name two sources of enzyme lysozyme.

e) How many substrates' amino-sugar units can be accommodated in the cleft of enzyme lysozyme?

3.2.7 Metal-Activated Enzymes and Metalloenzymes

More than one fourth of all known enzymes require the presence of metal ion for their catalytic activity. As metal exist in more than one oxidation state (e.g. ferrous (Fe2+) and Ferric (Fe3+), the presence of positive charge can stabilize the transition states by electrostatic interactions, this suggests one possible mechanism of catalysis by metals. Additionally, irrespective of the oxidation state and net charge on metal ion, it can bind to a particular number of ligands (groups) by forming coordinate bond (by accepting free pairs of electrons) in specific orientations. So, you will come to know that metals ions are involved in several ways in enzyme catalysis.

Involvement of metal ions in enzyme catalysis

1) Metals may accept or donate electrons to activate electrophiles or nucleophiles even in neutral solution.

2) Metals themselves may act as electrophiles.

3) Metals may prevent unwanted side reactions by masking nucleophiles.

4) By forming coordinate bonds, metals may bring enzyme and substrate together.

5) They may grasp reacting groups in specific orientations.

6) Metals may stabilize catalytically active conformations.

Metal activated Enzymes

In metal activated enzymes, the binding of metal to the enzyme is not so firm. The catalytic enzyme may have to be activated by addition of metal ions e.g. Hexokinase is activated by Mg^{2+}.

Metalloenzymes: The metal binds to the enzyme so tightly and it is retained by the enzyme even during purification e.g. carboxypeptidase has Zn2+ and urease has Ni2+.

However, there is no sharp distinction between metal-activated and metalloenzymes. A ternary complex is formed between enzyme (E), substrate (S) and metal ion (M). Mildvan (1970) suggested that ternary complex formed by metal activated enzymes can be enzyme-bridge-complexes (M-E-S), substrate bridge complexes (E-S-M) or metal bridge complexes (E-M-S).

Metalloenzymes, when purified exist as E-M, so they cannot form substrate bridge complexes. Both M-E-S and E-M-S are formed by metalloenzymes.

Let us consider some of the examples of metal activated enzymes:

1) Potassium ion is one of the richly found intracellular cation. It helps in catalysis by activating many enzymes involved in phosphoryl transfer or elimination reactions for example pyruvate kinase. The carboxyl group of PEP binds to the enzyme bound K+ to bring conformational change thereby catalyzing the reaction.

2) The divalent cations Ca2+ and Mg2+ can form six coordinate bonds to generate octahedral complexes. Calcium ion plays a crucial role in maintaining the structure of enzymes such as salivary and pancreatic α-amylases. Most kinases that require divalent cations form E-S-M complexes for example muscle creatine kinase. It catalyzes phosphorylation of creatine to phosphocreatine via formation of complex creatine-E-ATP-Mg.

3) Transition metal ions such as Cu, Zn, Mo, Fe and Co cations bind to the enzyme more firmly than the metal ions thereby forming metalloenzymes. These metal ions are generally found in trace amounts such as Mo and Fe in enzyme nitric-oxide reductase.

3.2.8 Transition State Analogues

One of the important mechanisms of enzyme catalysis is "Enzymes bind the transition states with greater affinity than substrates or products'. The concept evolves from the fact that enzymes mechanically strained their reacting substrates towards the transition position geometry all the way through the binding sites. The induced strain promotes reaction faster. Considering these facts, what would you expect how the transition state analogues will behave? The answer is transition state analogues resemble the transition state (intermediate state) of a substrate in an enzyme catalyzed reaction. The resemblance of the transition state lies in geometry and electronic structure and thus these analogues bind more tightly to enzyme in comparison to its substrate e.g. 3,4-dihydrouridine binds 2x109-fold more tightly to cytidine deaminase as compared to uridine.

Transition state analogues are the unstable chemical compounds. These molecules act as potent inhibitors of the enzyme. There are several hundred of transition state analogues. Some of their uses are as given below:-

Uses:

i) They are generally useful in understanding the mechanism of action.

ii) They serve as potential enzyme inhibitor. They are short lived compounds (1013 seconds).

iii) They are important in understanding the kinetics and enzyme catalysis.

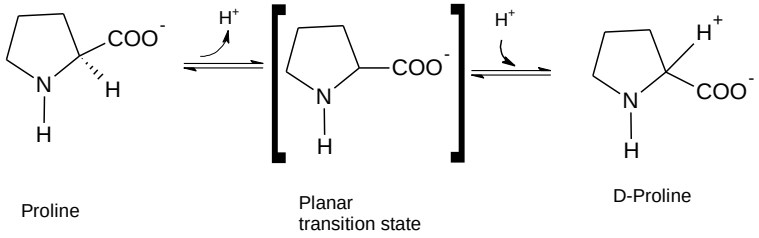
iv) They can function as antimetabolites.

v) These are used in structure determination and study of transformation of original substrate.

vi) These can help to identify the binding determinants of active site and they are able to generate immunogens which display catalytic nature.

vii) They are also used to design drugs.

Enzyme-analogue complexes have slow rates of dissociation. Some of the examples of transition state analogues are as given below (Figure 7.9 and 7.10):



Proline racemase enzyme from Clostridium sticklandii catalyzes the above reaction via. planar transition state. However its activity is inhibited by the planar analogues of proline, Pyrrole-2-carboxylate and Δ-1-pyrroline-2-carboxylate.

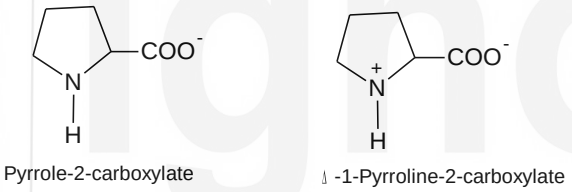
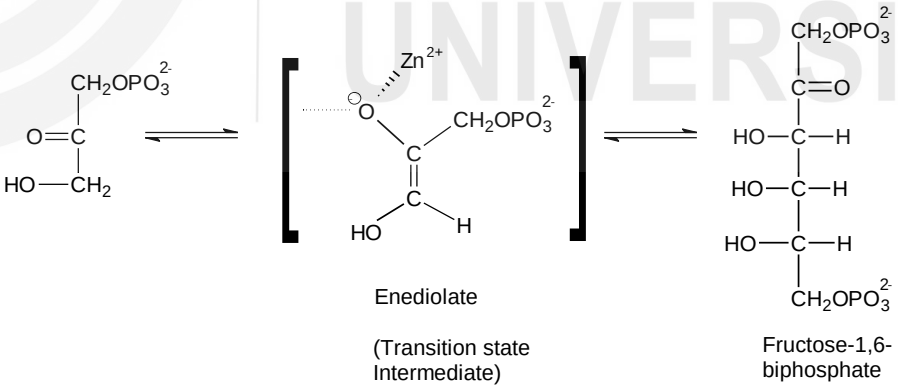


Fig. 3.9: Analogue inhibitors of enzyme proline racemase.

Yeast aldolase Reaction



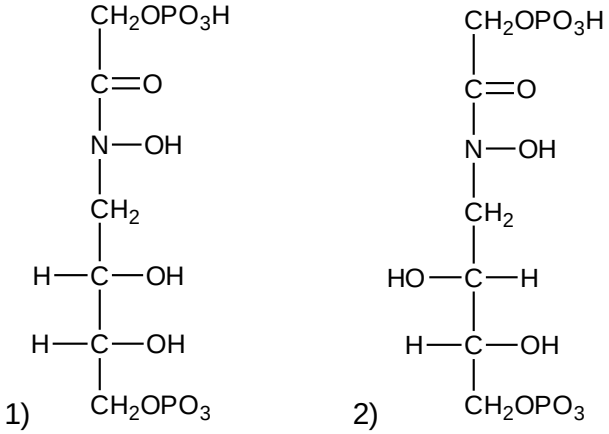


Fig. 3.10: Designated inhibitors (1 and 2) of aldolase reaction.

Transition state analogues are designed by the following methods.

i) Kinetic isotope effect

ii) Computational simulation.

Transition state analogues of two substrate reaction have also been studied. These bind to binding determinants of both the substrates. Therefore, they possess large entropic advantage over binding of separate substrates e.g. N-phosphono acetyl-L-asparate is substrate analogue of asparate carbomoyl-transferase. It is a powerful inhibitor with Kd of 25 nmol dm-3.

SAQ 4

Do as directed :

a) Metals themselves may act as electrophiles (True/False).

b) Enzyme carboxypeptidase contains Zn^{2+} / Cu^{2+} ion.

c) Name two transition metal ions.

d) Which analogues are used to design drugs?

3.3 SUMMARY

- There are several mechanisms of catalysis in enzyme catalyzed reactions. Enzyme because of their shape, size and range of properties enforce their existence on a reaction to a greater extent. The main reason of increasing the rate of reaction lies in their lowering of activation energy. The binding of substrate molecules in close proximity of enzyme surface reduces the entropy leading to the formation of a transition state also known as proximation effect. The correct orientation of reacting molecules ensures that the enzyme reaction takes place under optimal conditions.
- It increases the number of effective collisions.

- The driving force in enzyme catalyzed mechanisms is the binding energy. The principal effect of induced fit binding is the strain and distortion as one of the probable mechanisms of enzyme catalyzed reaction. This mechanism is more of ground state destabilization effect. Acid catalyses the reaction by providing the proton and bases do the same by gaining a proton. This mechanism of catalysis is classified as specific acid base catalysis or general acid base catalysis. In covalent catalysis increases the rate of reaction by forming catalyst-substrate covalent bond. Chymotrypsin and lysozyme are the two well characterized enzymes whose catalytic mechanism reveal basis for catalysis in these enzymes. Many enzymes need metal ions for their catalytic activity and are known as metalloenzymes. Transition state analogues are unstable molecules that act as potent enzyme inhibitors. They are very helpful in understanding the enzyme mechanisms.

3.4 TERMINAL QUESTIONS

1. Justify the statement 'Binding energy is the major driving force in enzyme catalyzed mechanisms'.
2. What is the difference between specific acid base catalysis and general acid base catalysis?
3. Explain the mechanism of enzyme lysozyme.
4. Distinguish between metal activated enzymes and metalloenzymes.

3.5 ANSWERS

Self-Assessment Questions (SAQ):

1. a) Free energy, b) Specific, c) Stabilize, d) False
2. a) False, b) False, c) True, d) Desolvation e) False
3. a) Nucleophilic, b) True, c) True, d) Saliva, human milk, tears e) Six
4. a) True, b) Zn2+ c) Cu, Zn, Mo, Fe and Co cations, d) Transition state analogues

Terminal Questions

1. Refer to section 3.2.2
2. Refer to section 3.2.3
3. Refer to section 3.2.6
4. Refer to section 3.2.7

3.6 FURTHER READINGS

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