Indira Gandhi National
Open University
School of Sciences

BBCCT-107
ENZYMES

Block

3

MECHANISMS AND REGULATION OF ENZYME ACTIVITY

Block 3 : Mechanisms and Regulation of Enzyme Activity

The rates and direction of the metabolic processes must be managed in such a way as to make available an orderly supply of energy and metabolites for the biochemical reactions. So the rate of metabolic processes must be controlled in order to accomplish balanced partitioning of different precursors among competing pathways to ensure a sufficient supply of each metabolite. Enzymes play a significant role in managing metabolic processes and their regulation ensures smooth organization of the metabolic system in the cell.

Unit-7 of the block determines several types of interactions involved in enzyme-substrate binding by giving relevant examples. Unit-8 describes how balanced coordinated adjustments in metabolism are feasible through efficient management and organization of enzymes. The unit discusses several regulatory mechanisms of enzymes for the directionality of metabolic pathways. As you know enzymes in a cell do not act as single isolated enzyme, rather there are several enzymes with different membranous structures and therefore it is very much likely that they are associated with other enzymes to different extents. Unit-9 enlists the properties of multienzyme complexes and determines their role as regulatory enzymes.

Objectives:

After studying this block, you should be able to:

- determine mechanisms that makes enzymes significantly better catalysts;
- illustrate interactions between substrate and enzyme;
- explain the regulatory mechanism of enzyme catalysis; and
- discuss the significant role of multi-enzyme complexes in metabolic pathways.

UNIT 7

MECHANISM OF ACTION OF ENZYMES

Structure

7.1 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.

Objectives

After studying this unit, you should be able to:

- determine different types of interactions involved in enzyme-substrate binding;
- explain acid – base catalysis;
- discuss the examples of enzymes such as chymotrypsin and lysozyme; and
- determine the precise nature of interactions between enzyme and substrate.

7.2 GENERAL FEATURES

In the previous units, you have come across several aspects of the enzyme, its regulation and inhibition. How do enzymes work? What do you think? Due to their 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. The 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.

7.2.1 Proximity and Orientation

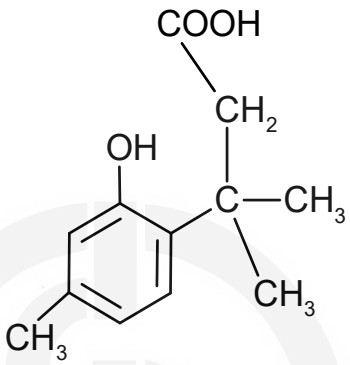
The efficiency of enzymes arises from the physical conditions at the enzyme catalytic site that advance biochemical reactions. Major physical and thermodynamic barriers to reaction include: (1) the relative motion of reacting molecules in solution, entropy; (2) the solvated shell of hydrogen-bonded water 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 reduces entropy loss for the subsequent formation of a transition – state; this is known as **proximation** effect. Even when reactions involve 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

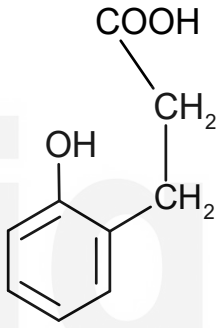
holds them in proper orientation and alignment so as to increase the number of effective collisions. In the transition state, these groups have little relative motion. Such type of effects can increase rate of reaction up to ~10^7.

Undoubtedly orientation of the reacting molecules with respect to each other can greatly influence the rate of reaction. An example of rate enhancement is provided by the rate of lactone (internal ester) formation.

The rate of lactone formation varies in compounds I and II (Fig. 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 4 x 10^5. Rate of lactone formation is slow in structure II.



Structure I



Structure II

Fig. 7.1: 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. Rotational entropy is 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 an increase in the reaction rate.

Enzymes catalyze reactions on account 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 proceeds towards the formation of product.

It was estimated by two renowned scientists Page and Jencks that each factor i.e. proximity and orientation increases reaction rate by 10^4 fold for a bimolecular reaction. The rate enhancement is by 10^8 fold 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)

7.2.2 Strain and Distortion

Binding energy is the major driving force in enzyme catalyzed mechanisms. It can be used to overcome transition state 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.

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

7.2.3 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.

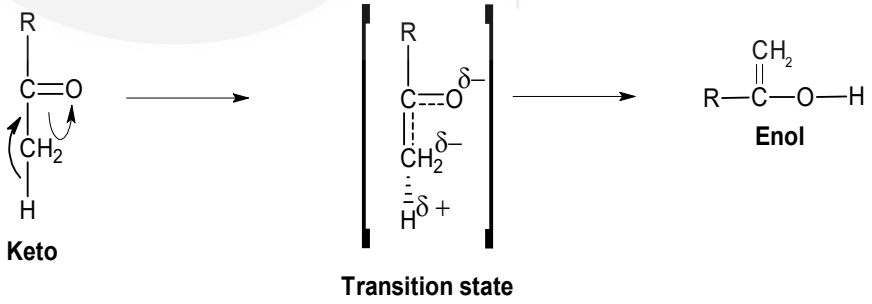
Acid catalyses the reaction by donating the proton and bases do the same by accepting a proton. Acid-base catalysis is broadly classified as:

i) **Specific acid base catalysis:** Specific acid or base catalysis means only H^+ (H_3O^+) 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 H_3O^+ 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.

a)



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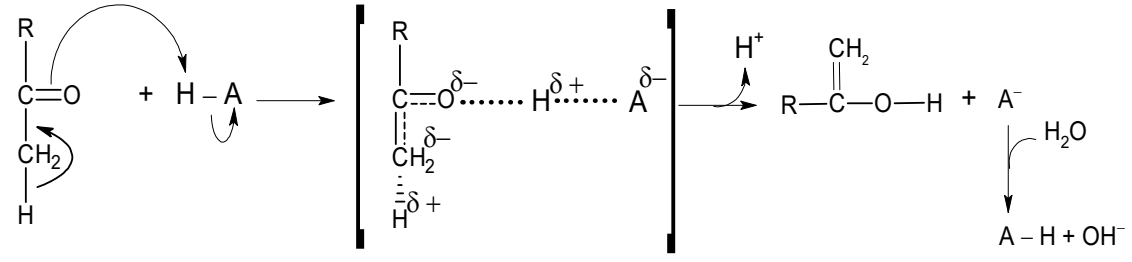


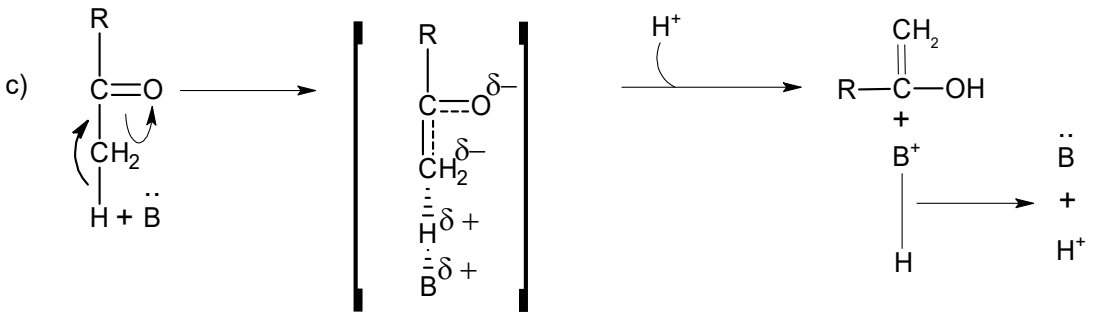


Fig. 7.2: Enzyme catalyzed mechanism of keto-enol tautomerization. a) uncatalyzed b) general acid catalysis c) general base catalysis enzyme reaction.

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. The side chains of the amino acid residues such as Asp, Glu, His, Cys, Tyr and Lys whose pK's lies near the physiological pH range helps them to act as acid/or base catalysts. These groups are positioned very precisely in enzyme's active site so as to allow proton transfer, thereby providing rate enhancement by the order 10^2 to 10^5. 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 pK_a. This alteration of pK_a is possible through local environment of the residue.

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

SAQ 2

Do as Directed

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)

7.2.4 Covalent Catalysis

In covalent catalysis, the transition-state is stabilized by changes involving covalent bonds. It is also called alternative pathway catalysis. In covalent catalysis, 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)

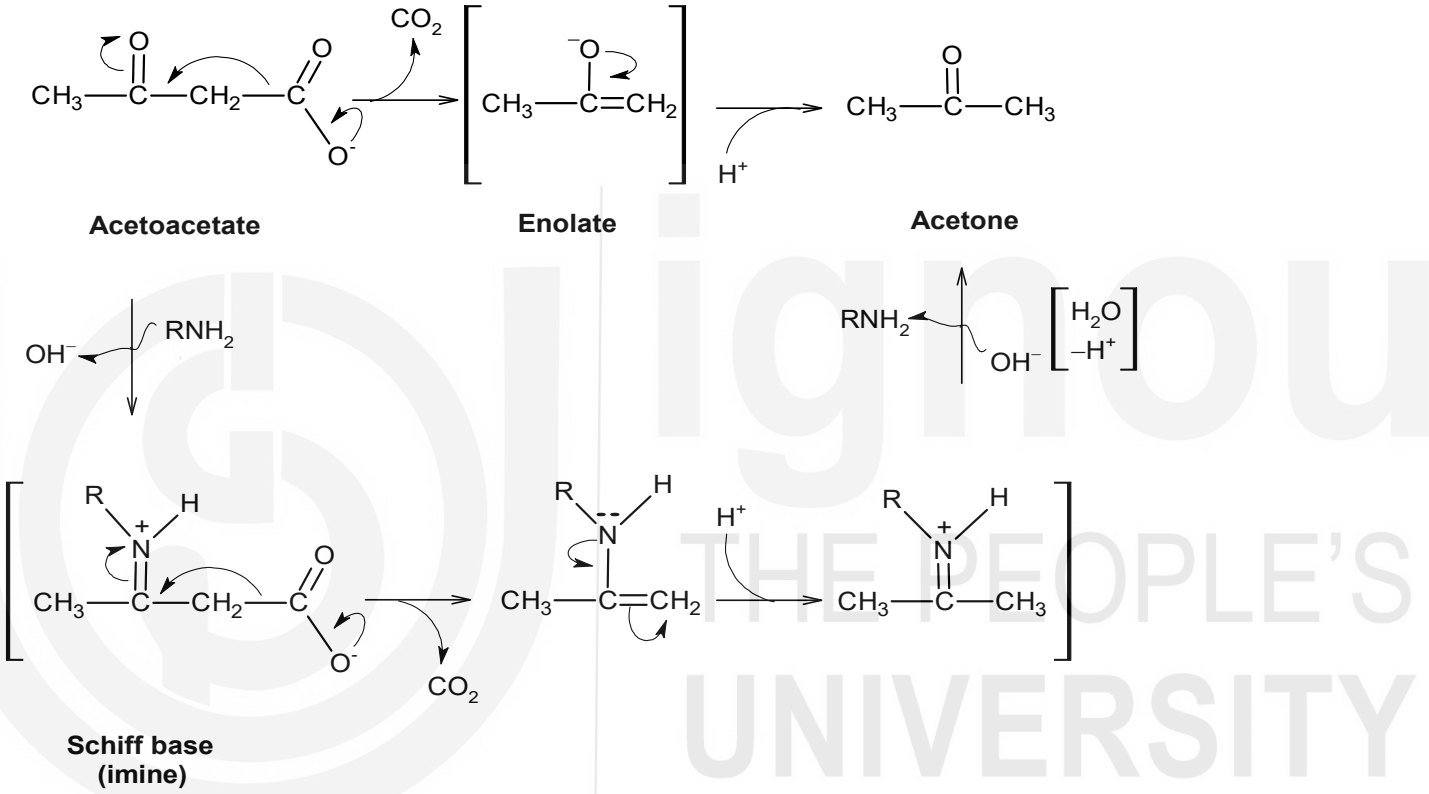


Fig. 7.3: Decarboxylation of Acetoacetate Nucleophilic catalysis by primary amines is shown at the bottom while uncatalyzed reaction is shown at the top.

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.

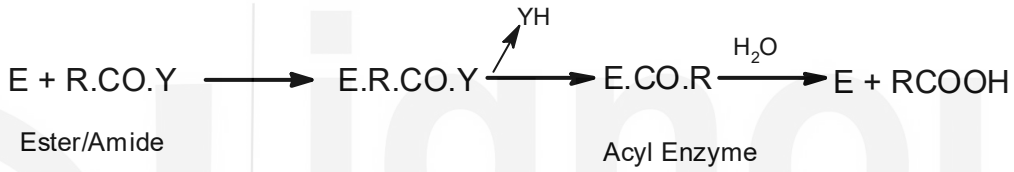
3) Elimination of the catalyst.

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

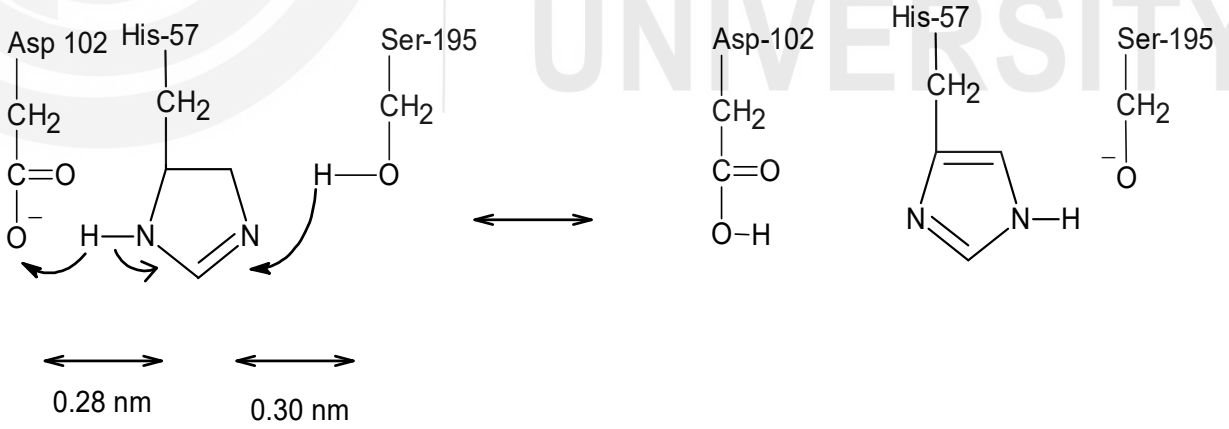
covalent catalysts. Several coenzymes such as thiamine pyrophosphate and pyridoxal phosphate function in connection with their apoenzymes in covalent catalysis.

7.2.5 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 proceeds via formation of an acylenzyme. This is also true for hydrolysis of amides.



X-ray studies showed a hydrophobic binding pocket at the 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 (Fig. 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, 7.4: Catalytic triad at active site of enzyme Chymotrypsin

His-57 acts as a 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 helped 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 facilitated by polarizing efffect of Asp-102 on His-57. Look at the Fig. 7.5 and you will notice that amine group (RNH_2) 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.

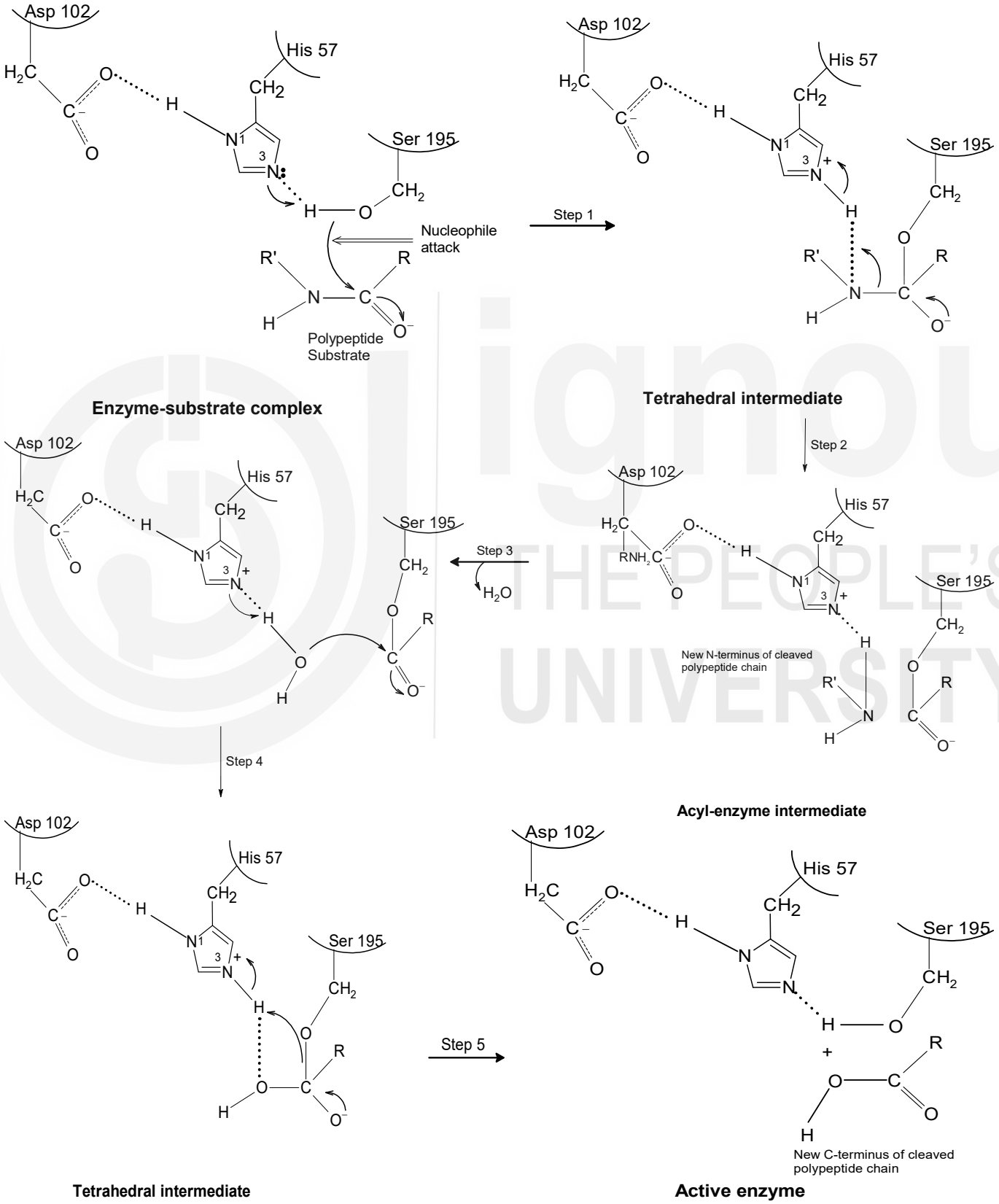


Fig. 7.5: Catalytic mechanism of Chymotrypsin based on chemical and structural data.

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

7.2.6 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. Lysozymes are secreted abundantly in saliva, human milk, tears and occur widely in cells. Hen egg white lysozyme is one of the most widely studied enzyme. Do you know that lysozyme catalyzed substrate hydrolysis is 10^8 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 Fig. 7.6, these six units are designated as A-F).

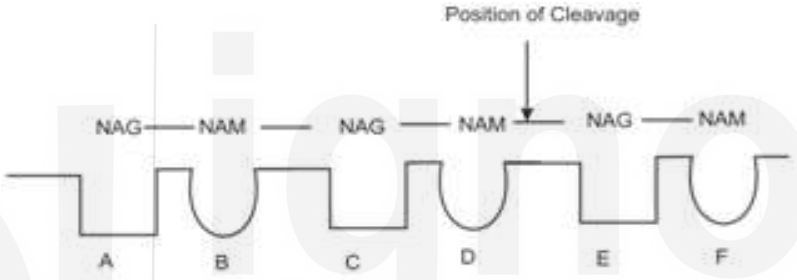
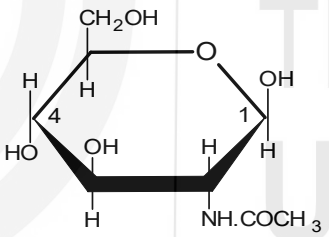
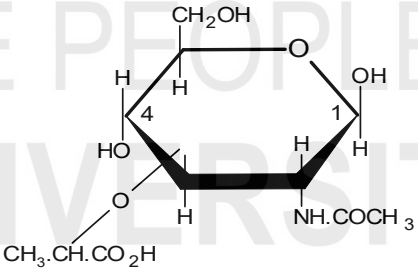


Fig. 7.6: Cleft of Lysozyme

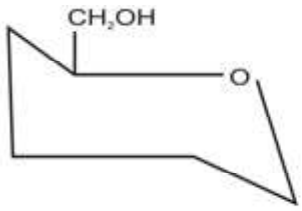


NAG



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 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).



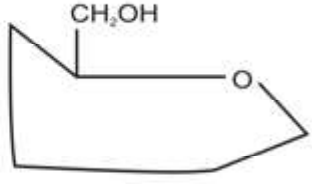


Fig. 7.7 a): Chair Conformation **b): Half-Chair Conformation**

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 (Fig. 7.8) 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 towards the half chair conformation.

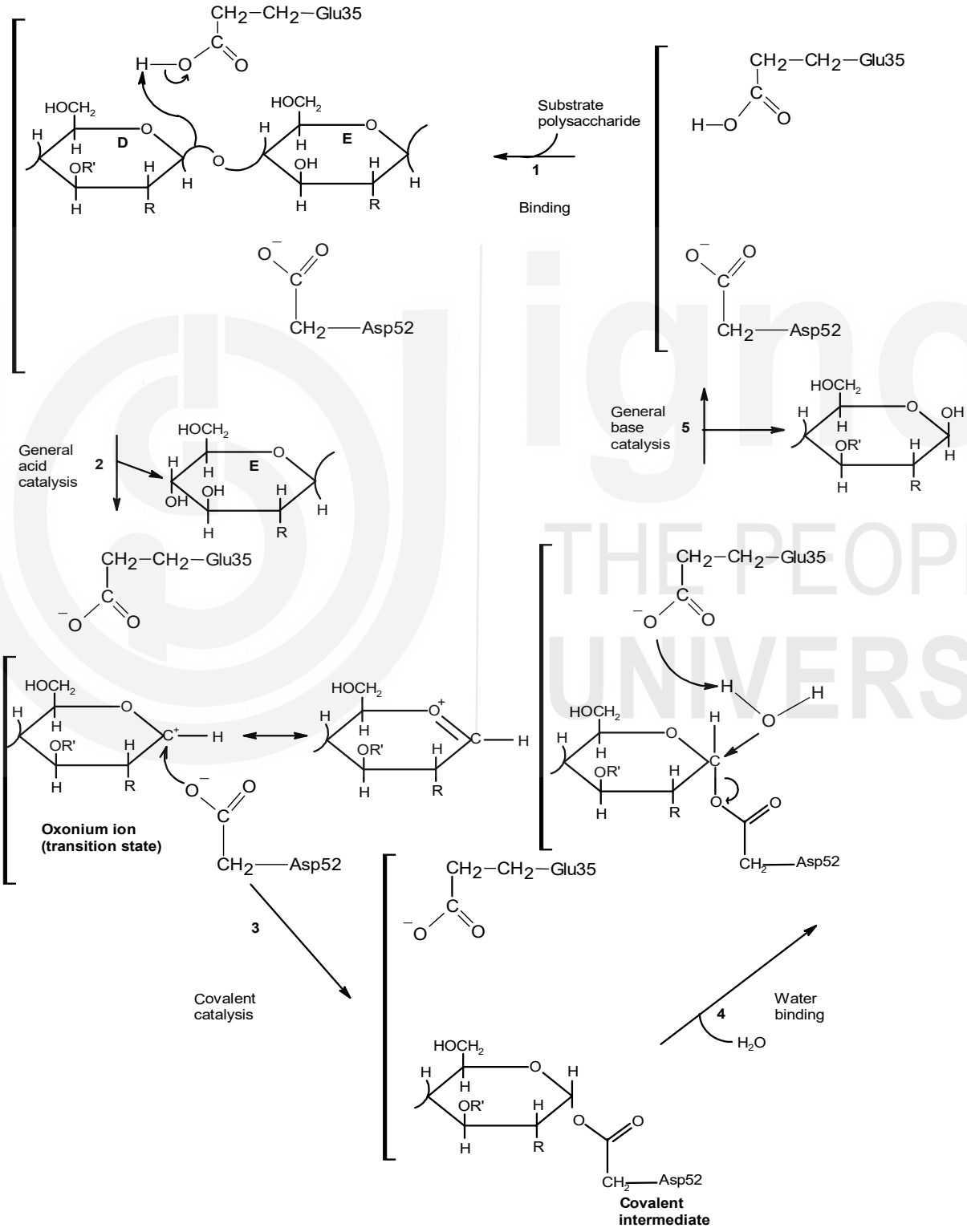


Fig. 7.8: Catalytic mechanism of Lysozyme

Step 2: Glu-35, in non-polar environment gets 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 it is negatively charged at pH 5.0. Therefore, it can stabilize the carbonium ion by electrostatic interaction. The first product leaves and the reaction gets completed by nucleophilic attack on the carbonium ion by water.

SAQ 3

Do as Directed

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 substrate amino-sugar units can be accommodated in the cleft of enzyme lysozyme?

7.2.7 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 (Fe^{2+}) and Ferric (Fe^{3+}), the presence of positive charge can stabilize the transition states by electrostatic interactions. Therefore metals are also one of the possible mechanism of enzyme catalysis. 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 should 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) They 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 needs to be activated by the 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 Zn^{2+} and urease has Ni^{2+}.

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). A 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 Ca^{2+} and Mg^{2+} 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.

7.2.8 Transition State Analogues

Enzymes bind the transition states with greater affinity than substrates or products. In other words enzyme is more suited to make favourable interactions with the transition state for the reaction than with the substrate in its normal conformation. Enzymes mechanically strained their reacting substrates towards the transition position geometry. The induced strain promotes the reaction at a faster rate. 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 the geometry and electronic structure and thus these

analogues bind more tightly to enzyme in comparison to its substrate e.g. 3,4-dihydrouridine binds $2x10^{9-}$ 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:

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

ii) Since transition state analogs resemble the transition state they bind to the enzyme and serve as potential enzyme inhibitors. They are short lived compounds (10^{13} seconds).

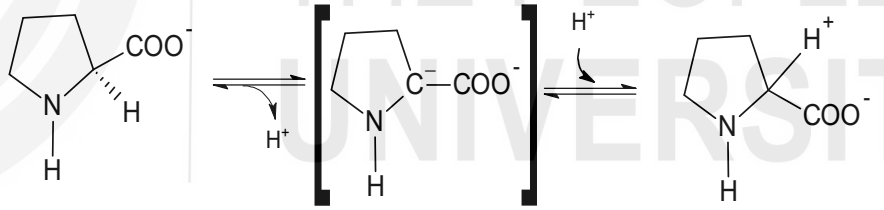
iii) They can function as antimetabolites.

iv) They are used in structure determination i.e studies on transformation of the original substrate.

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

vi) They are also used to design drugs.

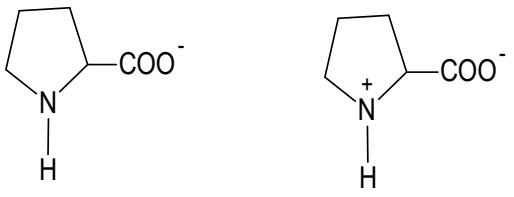
Enzyme-analogue complexes have slow rates of dissociation. Some of the examples of enzyme reactions and their transition state analogues are given in Fig. 7.9 and 7.10.



L-Proline Planar transition state D-Proline

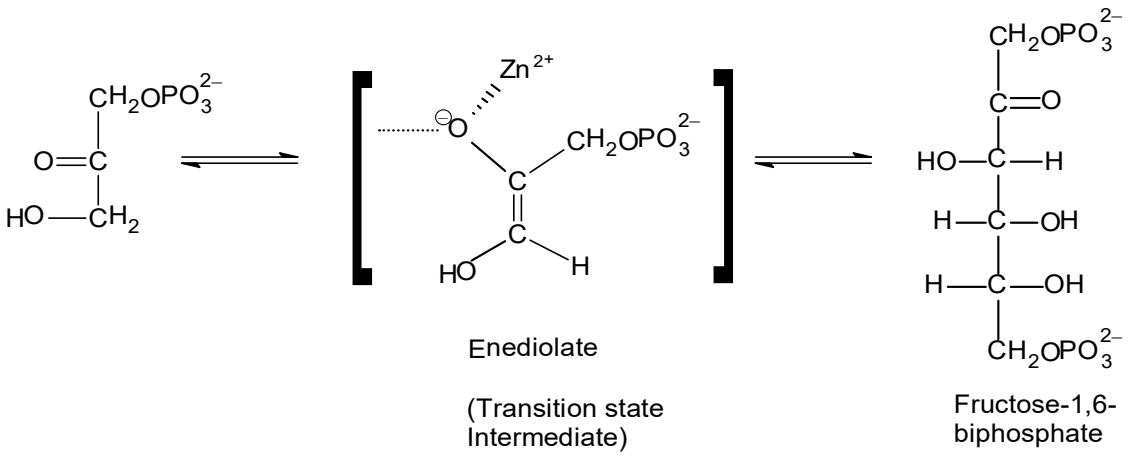
Enzymatic reaction of Proline Racemase

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.



Pyrrole-2-carboxylate Δ-1-Pyrroline-2-carboxylate

Fig. 7.9: Analogue inhibitors of enzyme proline racemase.



Reaction catalyzed by Yeast Aldolase

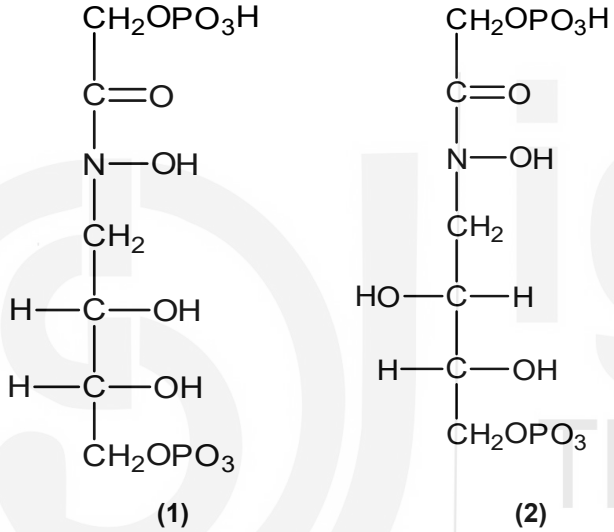


Fig. 7.10: Designated analogue inhibitors (1 and 2) of aldolase reaction.

SAQ 4

Do as Directed:

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

b) Enzyme carboxypeptidase contains Zn^{2+} / Cu^{2+}ion. (Pick one option)

c) Name two transition metal ions.

d) Which analogues are used to design drug analogues?

7.3 SUMMARY

1) 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.

2) The main reason of increase in the rate of enzyme catalyzed reaction lies in their lowering of activation energy.

3) 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.

4) The driving force in enzyme catalyzed mechanisms is the binding energy.

5) The principal effect of induced fit binding is the strain and distortion as one of the probable mechanisms of enzyme catalyzed reaction.

6) 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.

7) In covalent catalysis, the rate of reaction is increased by forming catalyst-substrate covalent bond.

8) Chymotrypsin and lysozyme are the two well characterized enzymes whose catalytic mechanism reveal basis for catalysis in these enzymes.

9) Many enzymes need metal ions for their catalytic activity and are known as metalloenzymes.

10) Transition state analogues are unstable molecules that act as potent enzyme inhibitors. They are very helpful in understanding the enzyme mechanisms.

7.4 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.

7.5 ANSWERS

Self-Assessment Questions

1) a) Free energy, b) specific, c) stabilize, d) False

2) a) False, b) False, c) True, d) Desolvation, e) True

3) a) Nucleophilic, b) True, c) True, d) saliva, human milk, tears, e) Six

4) a) True, b) Zn^{2+} c) Cu, Zn, Mo, Fe and Co cations, d) Transition state analogues

Terminal Questions

1) Refer to section 7.2.2
2) Refer to section 7.2.3
3) Refer to section 7.2.6
4) Refer to section 7.2.7

7.6 SUGGESTED READINGS

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2) Robert K. Murray, Daryl K. Granner, Victor W. Rodwell Harper's Illustrated Biochemistry, 27th edition. 2006, McGraw-Hill.
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UNIT 8

REGULATION OF ENZYME ACTIVITY

Structure

8.1 INTRODUCTION

The cell metabolism behaves in an extremely balanced fashion in its response to several challenges in the internal or external environment. These balanced coordinated adjustments in the metabolism are feasible through an effective control of enzymes. In the previous unit you have been introduced to the mechanism of action of enzymes. The catalytic efficiency of enzymes is effectively controlled to ensure proper homeostasis. Several different mechanisms are prevalent in the cell to regulate the activity of enzymes. The effector molecules regulating the activity of enzymes act as vital elements in the integration of metabolic pathways. The necessity for regulation of enzyme arises from the fact that the complex system of biological transformations has to be regulated so that concentrations of key metabolites are controlled both in terms of space and time to direct metabolism in a desired direction and not allow drifting. Any abnormalities in the metabolic pools of enzymes or their regulation will lead to severe disorders or diseases such as cancer, diabetes or neurodegeneration. In this unit we will look at some of the principal mechanisms by which enzyme activity is regulated.

Objectives

This unit will give you an overview about how the metabolic processes are regulated by the regulatory enzymes. After studying this unit, you should be able to:

- determine the need to control enzyme activity;
- describe the need to control metabolic pathways via compartmentation;
- determine different regulatory mechanisms for directionality of metabolic pathways; and
- state the role of allosteric enzymes and feedback inhibition.

8.2 COMPARTMENTATION - *Control of Metabolic Pathways*

Living states exist in a dynamic steady state. The metabolic concentrations remain constant over time. Except few, most of the enzyme catalyzed reactions in the living body are reversible. Enzyme reactions of the metabolic pathways are linked via substrate and product. The enzyme reactions are tightly regulated to ensure the necessary reactions are carried out in the cell and the unnecessary reactions are stopped by inhibition. The biosynthesis and degradation of biomolecules such as carbohydrates, proteins and fatty acids involve interconnectivity. Compartmentation ensures specific distinct sites of organelles or its sub cellular compartments for catabolism or anabolism for example fatty acid synthesis takes place in cytosol and degradation occurs by beta oxidation in the mitochondria. Segregation of metabolic flows in distinct sub cellular compartments (nucleus, cytosol, mitochondria etc) despite having many common intermediates between them, permits even two opposing pathways to co-exist. It increases metabolic efficiency and as well as ensures regulatory efficacy of the cellular processes. Certain enzymes are found to be predominantly present in one organelle and are used as marker enzyme. Tabulated presentation (Table 8.1) of the different sites of metabolic pathway are as given below:

Table 8.1: Localization of Metabolic Pathway

S.No.	Organelle site	Metabolic Pathway	Marker Enzyme
1.	Cytosol	Glycolysis, HMP Pathway, Fatty acid synthesis, Purine and Pyrimidine catabolism	Lactate dehydrogenase, Glucose-6 phosphate dehydrogenase
2.	Mitochondria	TCA, ETC, Fatty acid oxidation, Urea synthesis	Succinate dehydrogenase Cytochrome c oxidase
3.	Nucleus	DNA and RNA synthesis	DNA polymerase
4.	Plasma Membrane	Amino acid transport systems, Na^+-K^+ ATPase	5-Nucleotidase
5.	Endoplasmic Reticulum	Protein Synthesis, Glycosylation, Detoxification	Glucose 6-phosphatase

SAQ 1

What are the marker enzymes?

.....

.....

.....

.....

Metabolic pathways are under two types of control:

1. **Intrinsic (internal) control:** The enzyme activity in metabolic pathway is regulated by the concentration of metabolites e.g. substrates, products, end product of pathways or adenine nucleotides. This type of regulation is predominantly seen in unicellular organisms.

2. **Extrinsic (external) control:** In multicellular organism, the cell's metabolism is related to the requirements of the whole organism. So, in addition to intrinsic regulation as in unicellular organism, an extra level of control is exerted by hormones and nervous stimulation. These signals act via secondary messengers to affect the activities of target enzyme(s). The first messengers act on target cell through specific receptors present on them. The secondary messenger activate/deactivate the target enzyme. At each step/stage of mechanism, amplification of signal takes place.

8.3 ENZYME REGULATION

You have been introduced briefly about the concept of enzyme inhibitor for regulation of enzyme activity in the previous unit on enzyme inhibition (Block-2, Unit-6). The given below sections gives a much broader perspective of enzyme regulation for the learners of biochemistry along with relevant examples.

The metabolic processes involve sequence of pathways or steps catalyzed by enzymes. An active control of these pathways can be achieved by the regulation of only select set of enzymes. These set of enzymes include enzymes whose catalytic efficiency or quantity directly influences the rate of reaction or if it's catalytic step is slowest relative to others in the pathway. Regulatory enzymes are at or near the starting steps in a pathway, or part of a branch point or cross-over point between pathways (where a metabolite can be potentially converted into several products in different pathways). The slowest step in the metabolic pathway is called as the rate limiting step and the enzyme catalyzing this step effectively controls the metabolic flux through the entire pathway. An increase or decrease in the concentration or activity of these enzymes will affect the reaction in a similar manner.

Have you thought of ways by which enzyme activity can be regulated? There are various mechanisms of enzyme regulation as listed below:

1. Quantity
2. Availability of Substrate or Cofactor
3. Inhibition
    a) Specific Inhibitor molecules
    b) Reversible Inhibition (Competitive, Non-Competitive and Uncompetitive)
    c) Irreversible Inhibition
4. Feedback Regulation
5. Control by ligand induced conformational changes
6. Control by changes in the covalent structure of enzymes.

8.3.1 Quantity

How is the quantity of enzymes regulated?

The quantity of enzymes or turnover number is determined by the overall rate of synthesis and rate of degradation of the enzyme. Any change in its quantity can be affected by a change in rate constant for the overall synthesis and degradation processes or both. Radiolabeled studies using radiolabel ^{15}N have demonstrated that the proteins are continuously synthesized or degraded. The concentration of proteins or enzymes remained essentially constant in a state of 'dynamic equilibrium'. It gets influenced by a wide range of physiologic, hormonal or dietary factors. The turnover number of the enzymes can vary from minutes to hours to days for different enzymes.

8.3.1.1 Genetic Control: Enzyme synthesis at the gene level

Genes involved in the synthesis of enzymes can be induced or repressed. Induction of enzymes can be done at the gene expression, RNA translation or at the level of post-translational modifications. Hormones or growth factors signal cascade may lead to an increase in the expression or translation of enzyme not present before the signal. Inducers induce the synthesis of enzymes while repressors decrease the production of enzyme. Inducers are generally substrates or structurally similar molecules that initiate the synthesis of enzymes. Inducible enzymes in humans include tryptophan pyrrolase, threonine dehydratase, HMG-CoA reductase and cytochrome P-450. On the other hand, a metabolite or repressor when produced in excess inhibits the synthesis of enzymes involved in its formation. These regulatory molecules block the transcription of mRNA by binding to a part of DNA termed as operator. The operator region of DNA lies to the downstream of the promoter region. Binding of the repressor on the operator region prevents RNA polymerase to transcribe the coding sequences for the enzymes. Repressors are allosteric proteins to which specific molecules can bind to alter their shape and ability to bind DNA. Inducer molecules bind to the repressor molecule and prevent its binding to the operator region of DNA. It

allows the transcription of the coding sequences for the enzymes. For example, ***lac*** operon in ***E.coli***, lactose acts an inducer to transcribe the synthesis of three enzymes (β-galactosidase, permease and transacetylase) involved in its degradation if it is present in the surrounding environment.

8.3.1.2 Enzyme Degradation

You should know that enzymes are continuously degraded in the cell. Enzymes are proteins and their degradation take place in the lysosomes (non-specific) or in the macromolecular complexes called proteasomes. Lysosomes contain hydrolytic enzymes that degrade proteins by endocytosis. Selective protein degradation also takes place in the proteasome located in nucleus or cytosol of the cell. It is one of the essential mechanisms by which cellular processes are regulated. The 26S proteasome in eukaryotes contains more than 30 polypeptide subunits arranged in the form of cylinder. Proteins are targeted to the proteasome by ubiquitination. Ubiquitin is a small protein having 75 aminoacid residues. It is attached to the protein undergoing degradation in a process termed as ubiquitination catalyzed by the family of enzymes called E3 ligases. These enzymes are highly selective and competent to discriminate between different physical or conformational states of a target protein. Ubiquitin-proteasome pathway is highly conserved in eukaryotes. Damaged, oxidatively modified or defective proteins are degraded by this pathway. Accumulating evidence from the ongoing research work suggests that dysfunction in this pathway may contribute to the development of several neurodegenerative diseases. The potential use of proteasome inhibitors in treatment of cancer is also being explored.

8.3.2 Availability of Substrate or Co-factor

We know that cells contain a number of organelles. These organelles are membranes having selective permeability as well as distinct composition; it is quite likely that intracellular concentration of the metabolite acting as substrates for different enzyme catalyzed reactions may vary under different set of conditions. According to Michaelis – Menten equation, rate of an enzyme catalyzed reaction depends on the substrate concentration. For many enzymes, intracellular substrate concentration is significantly lower than their K_m values. If the intracellular concentration of substrates is lower than that of Michaelis constant (K_m) the rate of enzyme catalyzed reaction will depend on it. The corresponding change will also be reflected in the metabolic flux for example D-Glyceraldehyde 3-phosphate dehydrogenase enzyme in muscle has much lower concentration of its substrate glyceraldehyde 3-phosphate. High substrate concentration will have generally little or no effect on the rate of reaction e.g. fructose bisphosphate aldolase of mouse brain has K_m of 12 $\mu moldm^{-3}$ for its substrate fructose bisphosphate. The intracellular concentration of the substrate is 200 $\mu moldm^{-3}$. Enzymes operating at their maximal rate will not respond to the increase in substrate concentration but to the decrease in substrate concentration.

SAQ 2

Do as Directed:

a) The concentration of proteins or enzymes remained essentially constant in a state of '..... equilibrium'. (Fill in the blank)

b) Degradation of enzymes takes place in the lysosomes. (True/False)

c) Give two examples of inducible enzymes in humans.

d) Repressors are ..... proteins to which specific molecules can bind to alter their shape and ability to bind DNA. (Fill in the blank)

8.3.3 Inhibition

a) Specific inhibitor molecules

Some proteins act as inhibitors of intracellular or secreted enzymes and regulate enzyme activity. These regulatory proteins are called regulatory subunits (not antizymes). e.g. bovine trypsin inhibitor and soybean trypsin inhibitor mimic the structure of tetrahedral intermediate of trypsin and bind to it and thus inhibit the enzyme's activity. Other examples are α_1-antichymotrypsin, α_1-antiprotease and α_2-macroglobulin present in blood plasma. Though, the details of binding of these inhibitors to enzyme are not clear but these interactions can be of great physiological significance in regulating enzyme's activity.

b) Reversible Enzyme inhibition: This section has been dealt in the previous unit (Block 2, Unit 6).

c) Irreversible Enzyme inhibition: This section has been dealt in the previous unit (Block 2, Unit 6).

8.3.4 Feedback Regulation

In this type of enzyme regulation, the product of an enzyme catalysed reaction acts as inhibitor of the reaction. This generally happens when product accumulates and thereafter it inhibits the enzyme to decrease its formation.

1) Another interesting aspect of enzyme regulation is the inhibition of an enzyme in the biosynthetic pathway by the end product of the pathway. This type of inhibition is termed as feedback inhibition and it develops when metabolic demand for the end product of the pathway gets declined. The end product binds to the regulatory site of the enzyme at the start of metabolic pathway and suppresses its activity. Feedback inhibitors do not bear any structural similarity to the substrates of the enzymes. For example, consider the following reactions:

$$A \xrightarrow{\text{Enz 1}} B \xrightarrow{\text{Enz 2}} C \xrightarrow{\text{Enz 3}} D$$

High concentrations of end product D will act as feed back inhibitor of enzyme Enz 1. The kinetics of feedback inhibition may be competitive, non-competitive or uncompetitive or mixed. Small molecules such as amino acids or nucleotides act as feedback inhibitors in several biosynthetic pathways. In the bacterial enzyme system, L-threonine is converted to L-isoleucine in a sequence of five steps metabolic pathway. Isoleucine binds to the first enzyme in the pathway threonine dehydratase in a non-covalent manner and inhibits its own production.

Branched chain of metabolic pathways (shown in Fig. 8.1) also exhibits negative feedback control. The feed back inhibition in series of metabolic pathways is affected at the branch point (solid arrows).

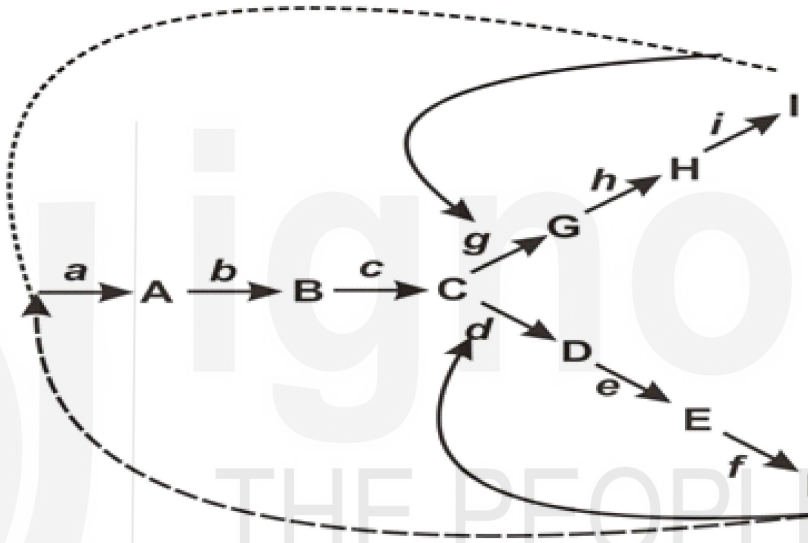

Fig. 8.1: Feedback inhibition and control in a branched pathway

The metabolic control as shown by broken arrows can be affected by number of ways. Let us begin with such control mechanism in *E.coli*.

Phosphorylation of aspartate to aspartyl phosphate by aspartokinase is the first step for biosynthesis of lysine, methionine, threonine and isoleucine (Fig. 8.2). This is a branched pathway. There are three separate aspartokinases (a, b, and c) which are regulated by different end product or effector substances.

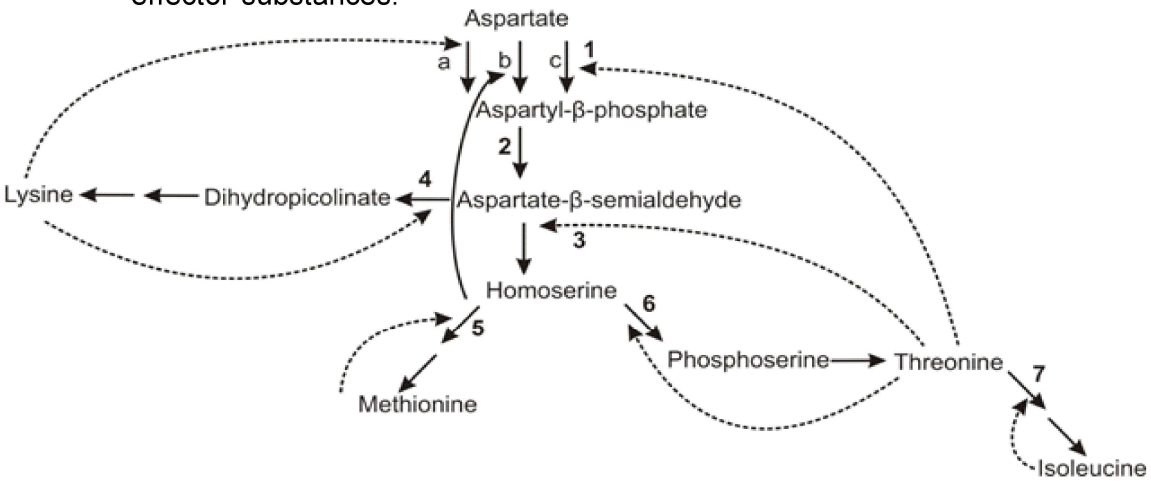

Fig. 8.2: Sites of feedback inhibition in a branched biosynthetic pathyway

Aspartokinase a: It is specifically and completely inhibited by lysine. Its synthesis is also repressed by lysine.

Aspartokinase b: It is specifically inhibited by homoserine.

Aspartokinase c: It is specifically and completely inhibited by threonine.

Aspartyl phosphate is synthesized even in the presence of excess of one of amino acid end product. Overall rate of aspartyl-phosphate synthesis depends on the extent to which all the three pathways are turned on or off.

Further controls are exerted by the selective inhibition of first step diverging from the common pathways (branching step) by the amino acid end product e.g. methionine inhibition of metabolic step (5) and lysine inhibition of metabolic step (4), threonine inhibition of metabolic step (6). In this way metabolic directionality of the pathways are controlled. These are allosteric inhibitions.

Some of the different modes of feedback inhibition are as follows:

i) **Concerted feedback Inhibition:** In this case, total inhibition is observed when two or more end products in excess are simultaneously present. e.g. aspartokinase of *Rhodopseudomonas capsulatus* and *Bacillus polymyxa* exist as a single enzyme which is not inhibited by lysine, threonine, methionine or isoleucine alone. But when lysine and threonine are present together, the enzyme is inhibited. This regulation permits the metabolic flow to continue in the presence of an excess of only one end product.

ii) **Cooperative feedback Inhibition:** In this type of inhibition, individual end products are weakly inhibitory or result in partial inhibition of enzyme. However, when the two weakly inhibitors are together, the inhibitory effect of each of them becomes additive. So presence of two or more end products result in cooperative inhibition in such a way that total inhibition is greater than the simple sum of their individual effects. e.g. amidophosphoribosyl transferase catalyses the first step of purine biosynthesis. It is inhibited by nucleotide end products. Let us assume that a particular concentration of GMP results in 10% inhibition of enzyme and similarly AMP also causes 10% inhibition at a particular concentration. When AMP+GMP are simultaneously present at these concentrations, the enzyme's inhibition is not a simple algebraic sum (10% + 10% of 90% =19%) but a much higher value, such as 50% inhibition. The cooperative feedback inhibition is not observed with every combination of nucleotides. GMP+IMP and AMP+ADP e.g. show simple additive effects.

iii) **Cumulative feedback Inhibition:** In this type of inhibition, individual end product is able to inhibit the enzyme by a given amount irrespective of the presence or absence of other end products (effects not more than additive). Inhibition of key enzyme by each product is separate and independent e.g. Glutamine is synthesized by the activity of enzyme glutamine synthetase. Glutamine is a precursor of eight end

products i.e. tryptophan, AMP, GMP, glucosamine-6-P, histidine, carbamoylphosphate, glycine and alanine. In *E.coli,* glutamine synthetase is partially inhibited by saturating concentration of each end product. The concentrations of end products inhibit independently e.g. we assume that separately tryptophan inhibits 16%, CTP-14%, Carbamoyl-P-13% and AMP-41 %. If all the four are present together then total inhibition is calculated as follows - tryptophan inhibits 16% so activity remained is 84%, CTP inhibits 14%, So inhibition is 14% of 84% i.e. 11.8 and remaining activity is 84-11.8 = 72.2%. Carbamoyl-P inhibits 13% and 13% of 72.2% is 9.4% so remaining activity is 72.2-9.4 i.e. 62.8%. AMP inhibits 41 % and 41 % of 62.8% is 25.8, so the remaining activity is 62.8 - 25.8=37% of original activity. In the presence of all the eight end products, the enzyme activity is completely turned off.

SAQ 3

Do as Directed:

a) Feedback inhibitors do not bear any structural similarity to the substrates of the enzymes (True/False).

b) High/Low concentrations of end product will act as feed back inhibitor (Pick one option).

c) Name the type of feedback inhibition which occurs when two or more end products in excess are simultaneously present.

d) When an individual end product inhibits the enzyme by a given amount irrespective of the presence or absence of other end products, name the type of inhibition.

8.3.5 Control by Ligand Induced Conformational Changes

The role of conformational changes in controlling enzyme activity got developed based on the observations on biosynthetic pathway in microorganisms. The ligands or effectors regulating enzyme catalysed reactions are generally distinct from the substrates or products of the enzyme. Scientists Monod, Chaneux and Jacob proposed that these regulatory molecules bind enzyme at a site different other than the active site or an indirect or allosteric site. Such enzymes are called as allosteric enzymes.

Allosteric Enzymes

Allosteric means ‘other structure or different site’. Allosteric enzymes are the enzymes whose catalytic activity can be modulated by the effector molecules. These molecules do not participate in catalysis directly. They bind at a site other than the active site on the enzyme and can cause activation or inhibition of the enzyme. The binding is reversible, non-covalent and brings about the conformational change in the active site. These conformational changes occur at the tertiary and quaternary levels of protein organization. Allosteric enzymes

are generally bigger and composed of multiple subunits having distinct active site and allosteric site. Several studies on X-ray crystallography and site directed mutagenesis have confirmed the existence of two separate sites on variety of enzymes.

Allosteric enzymes exhibit a characteristic sigmoidal saturation curve rather than hyperbolic curve [Michaelis-Menten curve] when v_o is plotted versus [S] on account of cooperativity of structural changes between enzyme subunits (Fig. 8.3). Allosteric enzymes behave in a cooperative system manner with both the substrate as well as modulator. A small change in substrate, inhibitor or activator concentrations brings about a huge change in the rate of reaction. The effectors that increase the catalytic activity are called positive effectors and those that reduce or inhibit the catalytic activity are called negative effectors.

For example, Phosphofructokinase (PFK) is regulated by

a) Negative effectors: High levels of ATP and citrate

b) Positive effectors: High levels of ADP and AMP

Effectors molecules that are not identical to the substrate and bind at the allosteric site of a multi-subunit enzyme to affect the binding of substrate are called heterotropic effectors. Small inorganic molecules to complex nucleotides can assume the role of allosteric effector. The substrate molecule can also induce distant allosteric effects when it binds to the catalytic site. Substrates of the enzyme that can also act as allosteric modulator are known as homotropic effectors. These molecules affect the binding of another substrate molecule to the same enzyme. The cooperative effect of oxygen binding to hemoglobin is a well-known example of this type of allosteric regulation. Oxygen molecules bind sequentially and more tightly than the previous bound molecule to hemoglobin. The progressive binding of subunits (oxygen) to the hemoglobin brings about conformational change and leads to sigmoidal kinetics. The binding of hormones to the cell surface causes alterations in the enzyme activity in the target cell by inducing the release or synthesis of specific effector molecules known as secondary messengers.

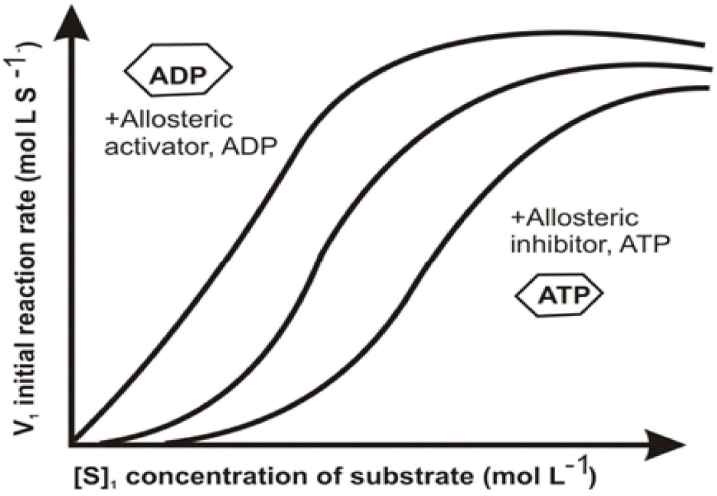

Fig. 8.3: Sigmoidal kinetics of allosteric enzymes

Considering the fact that allosteric enzymes are generally large and more complex, let us consider example of one of the well-known allosteric enzyme-aspartate transcarbamoylase (ATCase) from *E.coli* (Fig. 8.4).

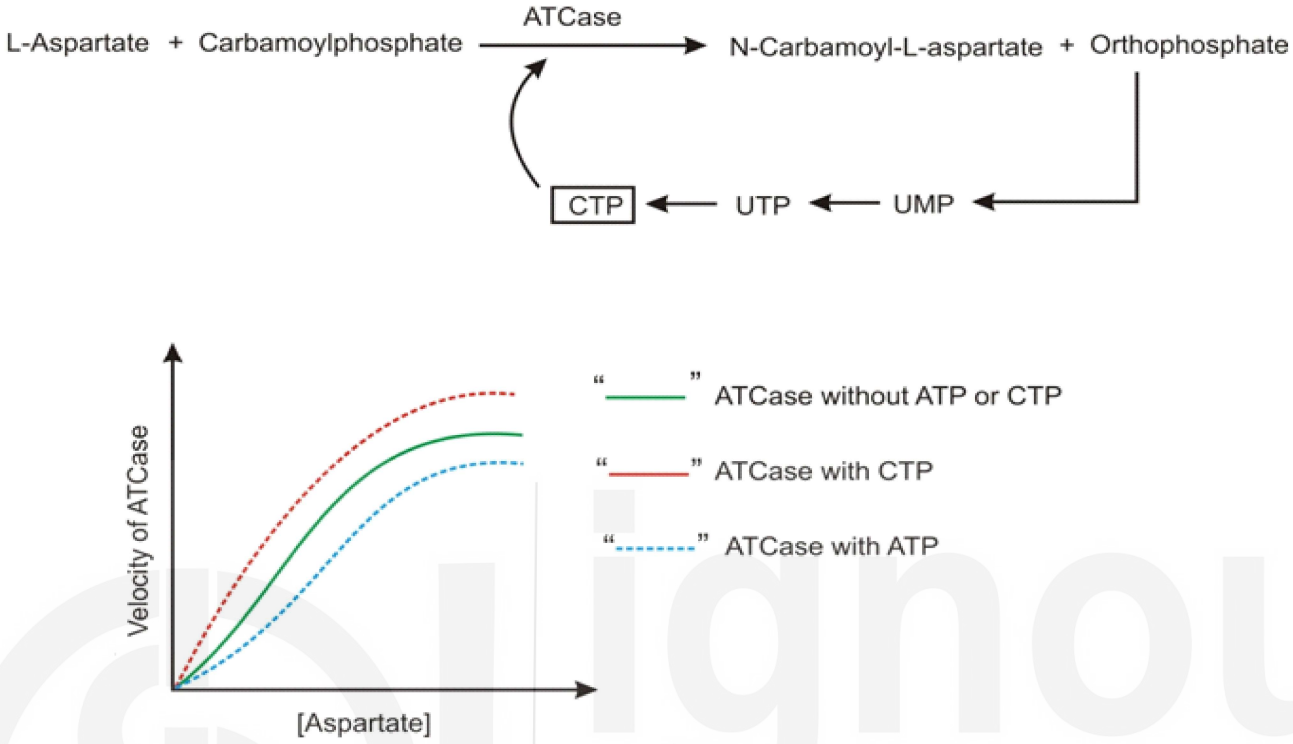

Fig. 8.4: Allosteric regulation of enzyme aspartate transcarbamoylase

CTP: cytidine triphosphate

ATP: adenosine triphosphate

The above reaction is the first step leading to the biosynthesis of pyrimidine.

Structure: Intact ATCase enzyme comprises of twelve subunits i.e. six regulatory and six catalytic subunits. The enzyme structure consists of two catalytic trimers being separated by three regulatory dimers. The kinetics of ATCase with substrate aspartate is sigmoidal i.e. binding of one molecule of aspartate facilitates the binding of subsequent aspartate molecules.

Regulatory subunits are binding sites for CTP and ATP. CTP is an end product competitive inhibitor with aspartate. In the presence of excess CTP, CTP binds to ATCase and reduces the formation of CTP synthesis. The sigmoid aspartate curve shifts towards higher substrate concentration (K_m increase, V_{max} remains unchanged and sigmoidal character is still present). So the CTP inactivates ATCase (inactive T-state;low substrate affinity) whereas ATP, reverses CTP inhibition and activates ATCase and thus acts as an allosteric activator. At high ATP concentration, K_m of ATCase for aspartate decreases, V_{max} remains unchanged and kinetic shifts from sigmoid to hyperbolic. ATP activates enzyme ATCase to R-state (high substrate affinity). Both CTP and ATP (allosteric effectors) tend to coordinate the synthesis of purine and pyrimidine nucleotides. As you know purine and pyrimidine nucleotides are needed in almost equal amounts in nucleic acid biosynthesis.

Isolated catalytic trimers of ATCase exhibit catalytic rate much higher than the intact ATCase and are unaffected by the presence of ATP or CTP. The isolated regulatory dimers bind the allosteric effectors but do not show any enzymatic activity. This shows that the regulatory subunits allosterically decrease the activity of catalytic subunits of the intact enzyme. The structural studies using substrate analogues on ATCase enzyme shows that during its T $\rightarrow$ R transition, catalytic trimers detach alongside the molecule three fold axis by ~ 11 A^{o} and reorient around this axis comparative to each other by 12^{o}. The regulatory dimers turn around in a clockwise manner by 15^{o} around the twofold axes and detach by ~4A^{o} along the threefold axes. Each catalytic subunit of the enzyme contains a carbamoyl phosphate-binding domain and an aspartate –binding domain. The binding of substrate induces a conformational change that swings both domains together so as to form the product. Thus the binding of one substrate to one catalytic subunit of the enzyme increases its substrate binding affinity and catalytic activity for other subunits. This shows that ATCase enzyme exhibits co-operative substrate binding.

The mechanism of allosteric binding of effector molecules to the enzyme shows that the inhibitor CTP and activator ATP binds to the same site on the outer edge of regulatory subunit ~ 60 A^{o} away from the catalytic subunit. The binding of CTP to R-state ATCase induces contraction in the regulatory dimer that causes the catalytic trimers to come together by 0.5 A^{o}. It leads to reorientation of the key residues in the active site of enzyme and reduces the catalytic activity of the enzyme. The other allosteric effector ATP exhibits an opposite effect on binding T-state of ATCase enzyme, it causes catalytic trimers to move apart by 0.4 A^{o} and reorient key residues in the active site of enzyme to increase its catalytic activity.

8.3.6 Control by Changes in the Covalent Structure of Enzymes

Reversible Covalent Modification

Covalent modification is also a means of regulating enzyme activity. The reversible covalent modification process of enzyme regulation involves many target proteins and membrane channels. Modified groups are attached to the enzyme by covalent bond. The covalent modification activates some enzymes as well as inactivates others. Most modifications are reversible. Phosphorylation and dephosphorylation are the most common but not the only means of covalent modification. Enzyme regulation by phosphorylation-dephosphorylation plays a key role in cell signaling. It allows the cell to respond to a signal at its surface and transmits its effect to intracellular enzymes. Phosphorylation cascade is highly selective and sophisticated. Seryl, Threonyl or Tyrosyl residues on the regulatory enzymes are phosphorylated by specific protein kinases. The very nature of protein folding determines whether protein kinase has access to the substrate undergoing phosphorylation. The removal of phosphoryl groups is catalyzed by protein phosphatases. Mammalian cell possesses many phosphorylated proteins and several protein kinases and phosphatases that catalyses their interconversion for regulatory control. Phosphorylation influences the

functional properties of affected enzyme by altering its three-dimensional structure. Phosphorylation of one enzyme can lead to phosphorylation of a different enzyme which in turn acts on another enzyme, and so on.

Let us consider well known example of glycogen phosphorylase. Glycogen phosphorylase in muscle and liver catalyzes the breakdown of polysaccharide glycogen.

$$(\text{Glucose})_n + P_i \rightarrow (\text{glucose})_{n-1} + \alpha\text{-D-glucose-1-phosphate}$$

Glycogen phosphorylase is a dimer of two identical subunits. It exist in two forms, phosphorylase a and phosphorylase b.

a) **Phosphorylase b:** It requires AMP (or few other ligands for activity).

b) **Phosphorylase a:** It is active in the absence of AMP.

Phosphorylase 'a' and 'b' differ in their covalent structure.

Phosphorylase a is an active form containing a phosphorylated serine residue required for its maximal activity. The conformation of first 19-amino acids at N-terminus differs markedly. This is flexible segment in phosphorylase b form, but ordered in phosphorylase-a form. (In phosphorylase-a form, phosphorylated side chain of Ser-14 interacts with positively charged side chain of Arg-69). Protein phosphatase enzyme removes the phosphoryl group from phosphorylase a and converts it to less active phosphorylase b. Orthophosphate is liberated in this reaction. In overall cyclic reaction, net reaction requires hydrolysis of ATP to ADP and orthophosphate (Fig. 8.5).

Phosphorylase a + 4 H_2O → 2 Phosphorylase b + 4 Pi

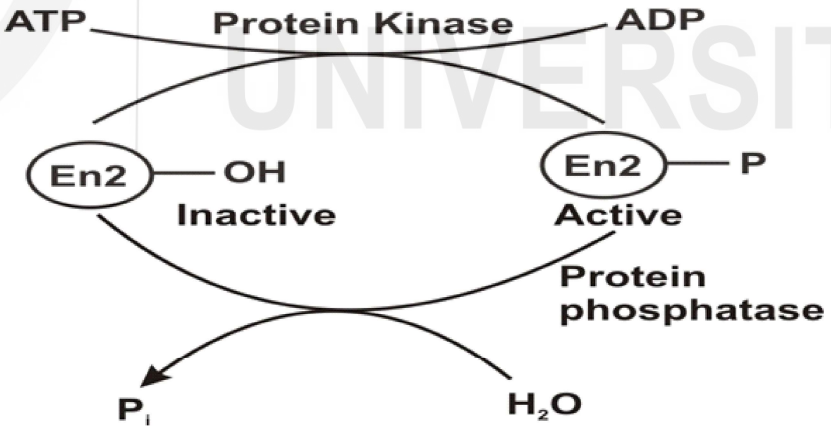

Fig. 8.5: Covalent modification by phosphorylation and dephosphorylation

Phosphorylase kinase can further reconvert the inactive Phosphorylase b to the active form of enzyme Phosphorylase a. The enzyme requires ATP and Mg^{2+} for the reaction. Phosphorylase kinase catalyzes the transfer of phosphoryl groups from ATP to the serine residues in phosphorylase b.

Other covalent modifications such as glycosylation, hydroxylation, fatty acid acylation, palmitoylation and prenylation are unique changes that shape structure and localization of enzyme for its lifetime. Hydrophobic acylations can cause the target protein to be associated with a membrane rather than the cytosol.

Irreversible Covalent Modification—Proteolytic Enzymes

Certain enzymes are secreted as precursors in an inactive form and are known as proenzymes or zymogens. Proenzymes help the proteins to be transported or stored in inactive forms that can be converted to active forms at the particular site. Precursor of pepsin is pepsinogen, trypsin is synthesized as trypsinogen and procarboxypeptidase is zymogen of carboxypeptidase. Several other examples include blood clotting enzymes, procollagen and proinsulin. Zymogens underlie the mechanism whereby the levels of enzymes can be readily increased at the post translational level by proteolytic cleavage, an irreversible modification. Generally, cellular and bacterial proteolytic enzymes are synthesized as inactive precursor (zymogen) to prevent undesired protein degradation. Conversion of zymogens to active form either requires accessory molecules or the process is autocatalytic in response to drop in pH.

Zymogens possess N-terminal extension of mature enzyme (prosegments) that prevents access of substrate to the active site. Activation segments seen to lie in the primary sequence of mature enzyme, between two catalytic residues. Activation segment is never found at C-terminus of a zymogen. It precludes the risk of active site gaining activity before polypeptide is complete. In some zymogens, activation segments play additional role in protein folding or intracellular sorting (e.g. prosubtilisin, yeast procarboxypeptidase Y and yeast procarboxypeptidase A).

Some examples of enzymes and their zymogens are given in the Table 8.2:

Table 8.2: Enzymes and their Precursor

Enzyme	Precursor	Function
Trypsin	Trypsinogen	pancreatic secretion
Chymotrypsin	Chymotrypsinogen	pancreatic secretion
Carboxypeptidase	Procarboxypeptidase	pancreatic secretion
Elastase	Proelastase	pancreatic secretion
Phosphalipase A_2	Prophospholipase A_2	pancreatic secretion
Pepsin	Pepsinogen	Secreted in gastric juice (Active pH range 1-5)
Thrombin	Prothrombin	Part of blood coagulation System
Clr·	Clr·	Part of first component of complement system

In retroviruses, proteinases are essentially required for cleaving large polypeptide into functional units.

Such enzymes include serine proteases because of presence of serine residue at the active site. Trypsin, chymotrypsin, carboxypeptidase and elastase are synthesized as zymogens in the acinar cells of pancreas. They are stored in zymogen granules and their release in duodenum is under hormonal control (cholecystokinin-pancreozymin and secretin). All enzymes are involved in protein digestion in a coordinated manner.

SAQ 4

Do as Directed:

a) Allosteric enzymes exhibit a characteristic hyperbolic curve rather than sigmoidal saturation curve. (True/False)

b) Name two types of covalent modifications for regulating enzyme activity.

c) Glycogen phosphorylase is a dimer of two identical/different subunits. (Pick one option)

d) Trypsin, chymotrypsin, carboxypeptidase and elastase are synthesized as zymogens. (True/False)

Brief account of some of the well-known proenzymes/zymogens and their activation:

Activation of Trypsinogen: Trypsinogen is a precursor of trypsin. Activation of trypsinogen involves removal of hexapeptide (Val-Asp-Asp-Asp-Asp-Lys) from N-terminus of polypeptide chain to active trypsin. Enteropeptidase (Enterokinase) produced by brush border epithelial cells in small intestine initially activates trypsinogen. Activated trypsin further activates trypsin and other zymogens (Fig 8.6).

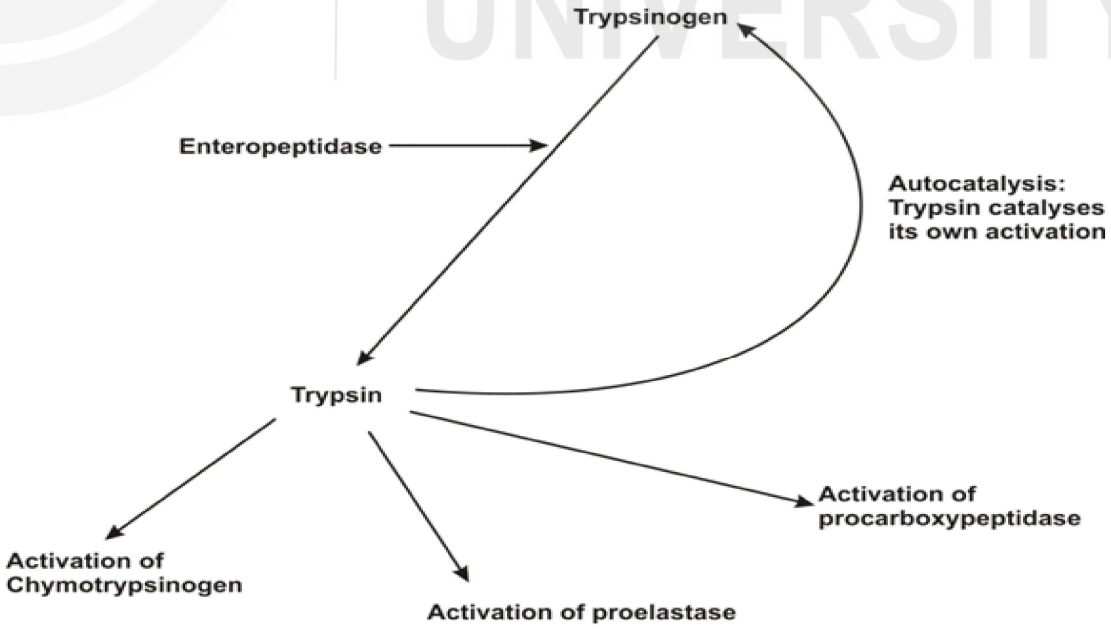

Fig. 8.6: Principle of signal amplification and coordinated action of pancreatic proteases

In each case, activation of zymogen lies on C-terminus of Lys or Arg i.e. in accordance to the specificity of trypsin.

Significance: Initial trigger is given by the enzyme enteropeptidase at a site different from site of production of zymogens. So there is no premature activation of zymogens (otherwise it could damage pancreas). Release of zymogen in duodenum is under hormonal control. Control of enteropeptidase is not well understood. Presence of trypsin inhibitor protein in pancreatic secretion also prevents premature activation of trypsinogen.

Activation of Chymotrypsinogen

Exocrine cells of the pancreas secrete chymotrypsinogen, an inactive precursor form. It is single polypeptide chain with 245 aminoacid residues with five intrachain disulphide bridges. Chymotrypsinogen is inactive until it gets to digestive tract. Trypsin breaks the peptide bond between arginine and isoleucine and converts the inactive form into an active form Π-chymotrypsin (Fig. 8.7). The active enzyme inturns acts on two more dipeptide bonds of other Π-chymotrypsin molecules to give fully active and stable α-chymotrypsin enzymes.

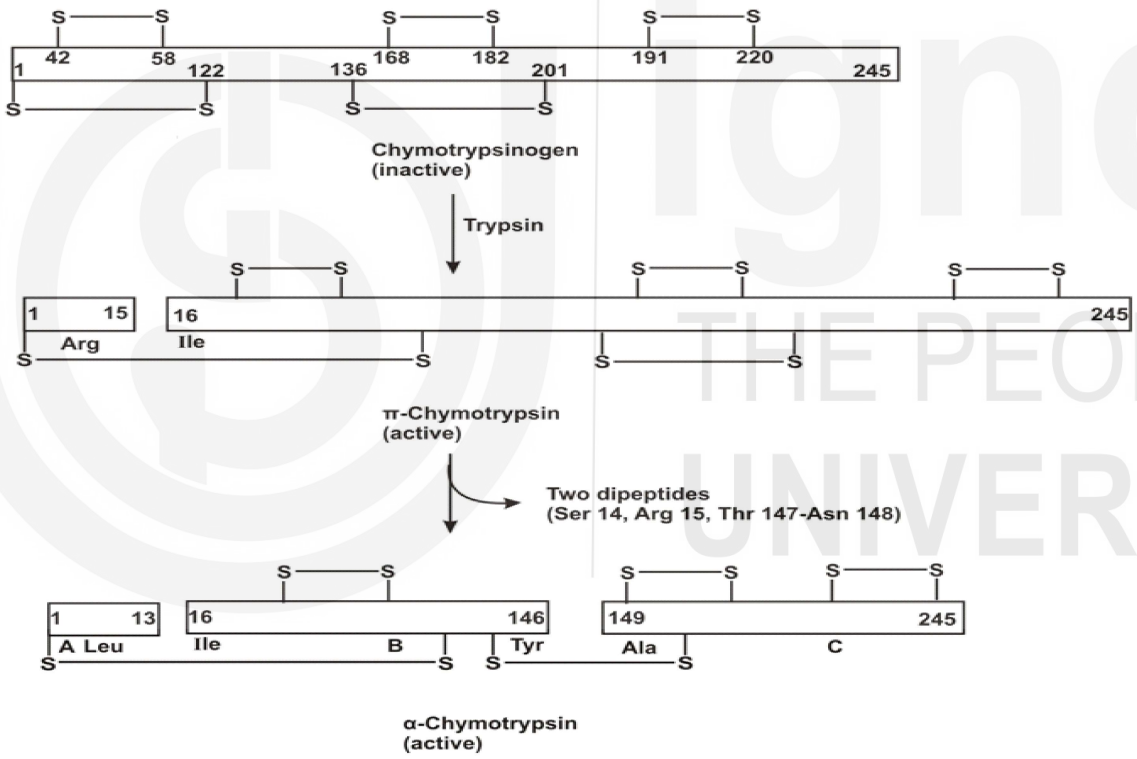

Fig. 8.7: Proteolytic activation of Chymotrypsinogen

Complement System: It is comprised of series of blood plasma proteins that damage and lyse invading microorganisms. They also work in cascade manner. After activated zymogen (proteases) performs their functions, they are degraded and not converted back to zymogens. System is switched off at an appropriate time e.g. fibrin is formed only at the injury site and not in entire blood stream.

During blood coagulation, different enzymes and proteins factor act on each other in sequential fashion i.e. cascade and finally lead to activation of zymogen prothrombin to thrombin. Thrombin further catalyses hydrolysis of Arg-Gly bonds of fibrinogen to produce fibrin, which forms insoluble clot that is strengthened by cross-linking between fibrin molecules.

Activation of Fibrinogen I (Factor I)

Fibrinogen is a large, soluble and complex plasma glycoprotein of 340 kDa. Thrombin converts fibrinogen to fibrin during blood clot formation.

Activation of Pepsinogen

Pepsinogen is a precursor of pepsin, released by chief cells of stomach. It is activated by HCl released from parietal cells lining of stomach. In acidic environment, pepsinogen undergoes autocatalytic hydrolysis and cleaves 44 amino acids from zymogen resulting in formation of pepsin.

Activation of Angiotensinogen

It is a α-2 globulin constitutively produced and released in circulation by liver.

Angiotensin I: It is formed by action of renin (produced by kidney) on angiotensinogen. Renin hydrolyses peptide bond between Leu and Val residue, thus producing a decapeptide angiotensin I.

Angiotensin II: Angiotensin converting enzyme converts angiotensin I to angiotensin II by removing two C-terminal residues.

8.4 SUMMARY

1) Tight integration of metabolic pathways is possible using regulatory enzymes.
2) Regulatory enzymes generally catalyze the slowest step in the metabolic pathway. These enzymes tend to maintain the homeostasis in spite of numerous changes in the external environment of the cell.
3) Enzyme can be regulated by the end product of a biosynthetic pathway. The final end product inhibits the first enzyme of pathway by binding to an allosteric site which is distinct from the active site.
4) The enzyme inhibition may result from a conformational change in the oligomeric structure of enzyme.
5) In another type of regulation, amino acid chains of the enzyme are reversibly modified, mainly by phosphorylation and dephosphorylation to make an active or inactive enzyme.
6) Regulation of enzyme activity by inhibitors, allosteric and covalent modifiers has wide range of applications in the fields of medicine and agriculture.

8.5 TERMINAL QUESTIONS

1) How compartmentation simplifies regulation?

2) Describe allosteric regulation of the enzyme activity?

3) Discuss the different modes of feedback inhibition.

4) What are proteolytic enzymes? Discuss any two of them.

8.6 ANSWERS

Self-Assessment Questions

1) Cells have many specialized structures which perform diverse roles. The purity of these structures can be assessed by biochemical and histological methods. Predominant enzymes in these structures are referred as marker enzymes as they help to determine the extent of contamination of a particular organelle or subcellular fraction during the separation of these organelles.

2) a) dynamic, b) True, c) Tryptophan pyrrolase, Threonine dehydratase, HMG-CoA reductase and cytochrome P-450, d) allosteric.

3) a) True, b) High, c) Concerted Feedback Inhibition, d) Cumulative Feedback Inhibition.

4) a) False, b) glycosylation, hydroxylation, fatty acid acylation, c) identical, d) True.

Terminal Questions

1) Biological processes take place at a specific time and specific location and at a specific speed. Compartmentation ensures metabolic efficiency and simplifies regulation. It also ensures maintenance of a specific ordered state in a timely fashion without wasting resources. Anabolic and catabolic pathways that interconvert common products takes place in specific sub cellular compartments for examples proteins are degraded in lysosomes and fatty acid oxidation takes place in mitochondria. Active control allows rapid adjustment in response to environmental changes and ensure homeostasis. It is achieved by the regulation of a select subset of enzymes.

2) Allosteric control is one of the important mechanisms of enzyme regulation. In an allosteric enzyme, activity of the enzyme is controlled by the binding of molecule at a site other than the active site. This other site is known as allosteric site. The binding of an allosteric modulator induces conformational changes which results in the alterations of the catalytic activity of enzymes. There are two types of allosteric modulation-

positive allosteric modulation when binding of modulator to the enzyme increases the rate of reaction. On the other hand negative allosteric modulator decreases the activity of enzyme (for details refer to section 8.3.4).

3) Refer to section 8.3.4.

4) Refer to section 8.3.6 subsection proteolytic enzymes

8.7 SUGGESTED READINGS

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2) Robert K. Murray, Daryl K. Granner, Victor W. Rodwell Harper's Illustrated Biochemistry, 27th edition. 2006, McGraw-Hill.

3) Donald J Voet and Judith G. Voet:Principles of Biochemistry 4th ed., John Wiley and Sons, Inc, USA.

4) Eric E Conn, Paul K Stumpf: Outlines of Biochemistry, Jophn Wiley and Sons, Inc, USA.

5) S. Shanmugan and T. Sathishkumar: Enzyme Technology, I K International Publishing House Pvt Ltd, New Delhi.

6) Nicholas C Price and Lewis Stevens: Fundamentals of Enzymology, Oxford University Press, Oxford, New York, USA.

UNIT 9

MULTIENZYME COMPLEXES

Structure

9.1 INTRODUCTION

If you are given two choices- a single enzyme or an enzyme complex with two or more enzymes, what do you think will be suitable to study about the structure, kinetics and mechanism of action of enzymes? Single enzyme can be studied more easily because it will be free from any interference from competing reactions. But now you should look into the situation in an intact cell. Enzymes in a cell do not act as single isolated enzyme doing catalysis of reaction with single or multiple substrates in buffer solutions. It means that the operation of enzyme *in vivo* depends on the nature of conditions of cell and are more likely to be different then its functioning *in vitro*. In an intact cell with different subcellular membranous structures, several enzymes compete with one another for substrates and effectors. Therefore it is very much likely that the enzymes are physically associated with other enzymes to different extent. You will also notice that organized system of cell enzyme system is more complex. If you will recall from unit (Unit-1, Block-1) about the Enzyme Commission for nomenclature of enzymes, enzymes are classified on the basis of catalysis of reaction and not on their physical organization. Let us introduce a new term, multienzyme complex- stable gathering of several enzymes with multiple catalytic domains. In this unit we will read more about these complexes to understand the regulation of enzyme activity.

Objectives

After studying this unit, you will be able to:

- define multi enzyme complexes;
- determine their role as regulatory enzymes;
- discuss the major role played by key multienzyme complexes;
- enlist their properties; and
- explain the function of isoenzymes.

9.2 MULTIENZYME COMPLEXES AS REGULATORY ENZYMES

In free solution, the rate of enzyme catalyzed reaction is dependent on the enzyme concentration and the substrate concentration. In a metabolic pathway, the product of one reaction acts as the substrate for the next enzyme to act upon in the pathway. An enzyme- catalyzed reaction is said to be diffusion- limited at suboptimal concentrations, since it is dependent on the random collisions of enzyme and substrate. The direct metabolite transfer from one enzyme to other avoids dilution of the metabolite in aqueous environment of the cell and the rate of reaction would increase.

In the cells, for such metabolic channeling to occur, the enzymes of a certain pathway are spatially organized. Some enzymes associate with other enzymes involved in a certain pathway to give multienzyme complexes. For enzymes participating in such complexes, the substrate diffusion is not rate-limiting. In eukaryotes, most enzymes don't diffuse freely in cytoplasm but are concentrated in particular parts of the cell beside other enzymes or proteins of related process.

A multienzyme complex is a stable assembly of more than one enzyme, which are generally involved in sequential catalytic transformations. These are different from multienzyme polypeptides, which have multiple catalytic domains found in single polypeptide. Such examples include: pyruvate dehydrogenase, a complex of three different enzymes that collectively catalyze the oxidation of pyruvate into acetyl-CoA by the process of pyruvate decarboxylation; fatty acyl CoA synthase, a complex of six enzymes that assemble in the shape of a barrel to generate fatty acids.

9.3 OCCURRENCE AND ISOLATION

During metabolic processes, a number of enzymes catalyze the sequence of reactions in such a way that the product of one enzyme- catalyzed reaction becomes the substrate for the next enzyme as shown in the following sequence.

The before-mentioned reaction represents a sequential metabolic pathway in which S_1, S_2, S_3 and S_4 are the substrates for the enzymes E1, E2, E3 and E4. The overall rate of reaction for conversion of S_1 to S_5 will depend fairly on the coordination between these four enzymes. It has been known that for some metabolic pathways, certain enzymes are physically associated with each other to form multienzyme complex.

The isolation and characterization of multienzyme complexes is comparatively more difficult than that of a single enzyme for example- pyruvate dehydrogenase complex and yeast fatty-acid synthase complex. Isolation of multienzyme complex, yeast fatty- acid synthase was found to be difficult as it involves separation of the polypeptide chains for the seven catalytic activities of the whole enzyme. Later it was revealed that the whole complex consists of two multifunctional polypeptide chains and the smaller fragments observed earlier were due to limited proteolysis during isolation of the enzyme complex. For another such complex- pyruvate dehydrogenase complex, it is well established that it comprises of three different enzyme activities, it has been difficult to determine the exact number of polypeptide chains present in the complex. The reason might be (a) disparity in isolation procedures yielding complexes with slightly different compositions (b) intact cell complexes with slight difference in composition also exist (c) dissociation during isolation. The existence of a multienzyme complex can be deduced when a component of the enzyme complex is being isolated and also found to be purified along with another enzyme from the same metabolic pathway. The presence of a multienzyme complex can further be confirmed if the ratio of the enzyme activities remains constant during isolation e.g. carbamoyl phosphate synthase, aspartate carbamoyltransferase, dihydro-orotase (CAD). Examples include enzymes having kinase and phosphatase activities present in the same polypeptide is isocitrate dehydrogenase kinase and isocitrate dehydrogenase phosphatase.

SAQ 1

Do as Directed:

a) In the cells, for metabolic channeling to occur, the enzymes of a certain pathway are spatially organized. (True/False)

b) A multienzyme complex is an unstable/stable assembly of more than one enzyme. (Pick one option)

c) The isolation of multienzyme complexes is comparatively simpler than that of a single enzyme. (True/False)

d) A multienzyme complex is similar to multienzyme polypeptide. (True/False)

9.4 PHYLOGENETIC DISTRIBUTION AND PROPERTIES

Generally, multienzyme polypeptides and multienzyme proteins are both more complex and more common in eukaryotes as compared to prokaryotes. This can be observed with the examples of fatty acid synthase, pyruvate dehydrogenase and the enzymes of tryptophan biosynthesis. If you can recall from your school textbooks the difference between prokaryotes and eukaryotes, you will understand that even after the intracellular compartmentalization, the volume of the eukaryotic cell in which free diffusion of substrate and enzyme can occur is much greater than a prokaryotic cell. Therefore, if the intracellular concentration of catalytic centers and substrates are similar in both types of cells, even then the diffusion time is possibly rate limiting in eukaryotes. Available information on multienzyme complexes such as carbamoyl phosphate synthase, aspartate carbamoyl transferase, fatty acid synthase and several others suggest that evolution process consequently leads to tightly associated multienzyme complexes. Genetic evidence also points out clustering or fusion of genes coding for these proteins.

Multienzyme complexes exhibit characteristic properties. Less time will be required for a product of one enzyme to diffuse to the catalytic site of the next enzyme in the case of a physically associated multienzyme. There are two instances in which transit time plays an important role (a) when the intermediate has short half-life e.g. the carbamoyl phosphate probably has a short half- life, because there are enzymes capable of degrading it. Its association with aspartate carbamoyl transferase, probably prevents its degradation (b) when intermediate has high M_r and hence diffuse slowly e.g. the second step in the reaction sequence of the pyruvate dehydrogenase system involves two enzyme bound intermediates hydroxylethyl-thiaminepyrophosphate-pyruvate dehydrogenase and dihydrolipoamide acetyltransferase.

In addition to the transit time, the transient time i.e. the time required to change from one steady- state to another also gets reduced in a multienzyme protein. Let us consider a system with two enzymes (E_1 & E_2):

$$A \xrightarrow{E_1} B \xrightarrow{E_2} C$$

In a steady state in the cell, if the concentration of A is increased then in multienzyme protein, the transient time i.e the time required to reach the new steady- state will be much less.

Multienzyme complex system also makes channeling or compartmentalization possible. After the formation of an intermediate in the multienzyme protein, it is not available for the other enzymes outside the complex to be acted upon. This means that the concentration of such metabolites inside the cell will be lower. The solvent capacity of water is limited due to the relatively high concentrations

of solute in the cell compartments. Lowering the concentrations of metabolites would spare this capacity. The loss of pathway flux by leakage or instability of free intermediates would also be reduced by channeling.

Coordinate activation of whole multienzyme protein is also observed evidently. There is possibility that one domain may exert an allosteric effect on adjacent domain as in the ribulose bisphosphate carboxylase- oxygenase which acts together along with four other enzymes namely phosphoribulokinase, phosphoglycerate kinase, phosphoribose isomerase and glyceraldehyde phosphate dehydrogenase to catalyze five successive reactions of the Benson- Calvin cycle. The free enzyme is made up of small (S) and large (L) subunits possessing the structure L_8S_8, but when available as the five enzyme complex has a L_2S_4 structure. Another multienzyme is the arom complex in which substrate of the first enzyme has the ability to activate all five catalytic activities of the whole multienzyme protein.

The tryptophan synthase system and pyruvate dehydrogenase system are examples of multienzyme complexes whereas fatty acid synthase and the arom complex are the examples of multienzyme polypeptides.

SAQ 2

Fill in the blanks.

a) Multienzyme polypeptides and multienzyme proteins are both more complex and more common in eukaryotes as compared to .....

b) The volume of the eukaryotic cell in which free diffusion of substrate and enzyme can occur is ..... than in a prokaryotic cell. [more/less]

c) ..... time is required for a product of one enzyme to diffuse to the catalytic site of the next enzyme in multienzyme complex. [more/less]

d) ..... reduces loss of pathway flux by leakage or instability of free intermediates.

9.4.1 Pyruvate Dehydrogenase Complex

Pyruvate dehydrogenase was the first enzyme to be purified. Earlier, in 1950s it was known that the oxidation of pyruvate was catalyzed by large homogenous enzyme preparation and that the reaction involves more than one catalytic step. The system catalyzes an important step regulating the flow of acetyl groups into the tricarboxylic acid (TCA) cycle. At that time, it was understood that 2 oxoglutarate dehydrogenase was closely related to pyruvate dehydrogenase catalyzing an oxidative decarboxylation and has same cofactor requirements. It also catalyzes a step in TCA cycle. In the mid-1970s a third multienzyme system was identified which was capable of oxidizing branched chain keto acids derived from amino acids valine, isoleucine and leucine.

These three multienzyme complexes, branched chain oxoacid dehydrogenase, 2- oxoglutarate dehydrogenase and pyruvate dehydrogenase display common properties, as they:

a) employ the same five cofactors, lipoate, coenzyme A, FAD, NAD^+ and thiamine pyrophosphate,

b) demonstrate structural and mechanical similarities, with a transacylase at the core of the complex and dehydrogenase and decarboxylase on the edge,

c) have three catalytic centres catalyzing a dehydrogenation, a decarboxylation and a transacylation,

d) share similar dihydrolipoamide dehydrogenase component excluding in *Pseudomonas putida*.

Apart from sharing similarities, these complexes also vary from each other, as:

a) some are based on icosahedral symmetry, others on octahedral,

b) a supplementary polypeptide component is there in mammals, yeast and probably other eukaryotes, which is vital for appropriate assembly and functioning of the complex,

c) the decarboxylase constituent may consist of homodimers (α_2) or heterotetramers ($\alpha_2\beta_2$) of two polypeptides,

d) the catalytic property of some eukaryote complexes is regulated by phosphorylation of the decarboxylase.

The complex enables pyruvate to move in the TCA cycle, by catalyzing its decarboxylation. It also utilizes another coenzyme lipoic acid for the oxidation step and lastly, coenzyme A (CoASH) which reacts to the acetyl lipoamide complex, producing acetyl CoA as the product.

$$\text{Pyruvate} + \text{CoASH} + NAD^+ \rightarrow \text{acetyl-CoA} + CO_2 + \text{NADH}$$

Lester Reed and coworkers, in 1968 reported that the *E.coli* pyruvate dehydrogenase multienzyme complex consists of 60 polypeptide chains having a molecular weight of about 4,600,000. The complex comprises of: pyruvate dehydrogenase (E_1); dihydrolipoyl acetyltransferase (E_2) and dihydrolipoyl dehydrogenase (E_3). The catalytic reaction takes place with enzyme bound substrate, which may be directly or via cofactors thiamine pyrophosphate (TPP) and lipoate. TPP is associated with E_1 and the side chain of lipoate is covalently bound to lysyl residue of E_2. FAD acts as the prosthetic group for E_3. The enzyme complex is about 300 Angstroms in diameter and may easily undergo dissociation because of being held by non-covalent interactions. The cubical core complex comprises of 24 subunits of E_2 associated as trimers around which there is a symmetrical arrangement of E_1 and E_3. A dimer of E_1 and E_3 is present on each of the 12 edges and 6 faces of the cube respectively.

Apart from *E.coli*, the complex has also been studied in various other organisms and tissues which include: *Bacillus stearothermophilus, Azotobacter vinelandii, Pseudomonas spp, Saccharomyces cerevisviae,*

Arabidopsis, Neurospora crassa, Enterococcus faecalis, mammalian heart, kidney liver and avian tissues. It has been cloned and sequenced because it plays an important role in genetic deficiencies.

If you can recall from the school text books, you will remember that the pyruvate dehydrogenase multienzyme complex links glycolytic pathway with the tricarboxylic acid cycle. How the activity of this multienzyme complex in prokaryotes *E.coli* can be controlled or regulated? The answer is the product acetyl CoA formed in the reaction catalyzed by pyruvate dehydrogenase complex. Acetyl- CoA is competitive inhibitor with respect of CoA and another coenzyme NADH (Fig. 9.1). Both NADH and acetyl-CoA may also hinder acetylation of the bound lipoamide. The pyruvate dehydrogenase complex is also inhibited by GTP and activated by nucleoside monophosphates. The regulation in the mammalian system is similar but much complex (Fig. 9.2): covalent modification is involved while kinase and phosphatase enzymes are present within the complex. The kinase bound to E_2, catalyze a serine residue phosphorylation in the E_1 which inactivates the complex when the intracellular ratio of [ATP]/[ADP] is high. The kinase is inhibited by ADP and pyruvate and activated itself by acetyl-CoA and NADH. The phosphatase is activated by Ca^{2+} and Mg^{2+}which is responsible for the removal of this phosphate.

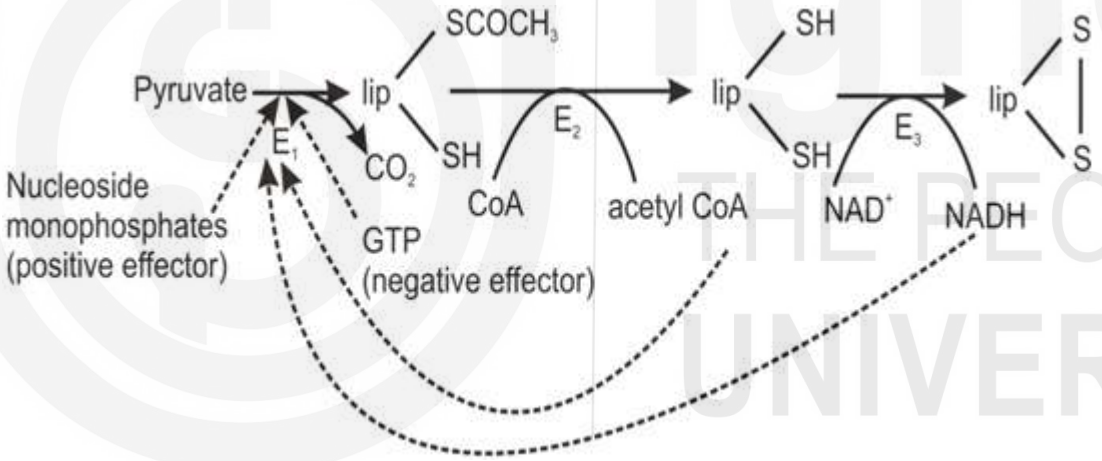

Fig. 9.1: Regulation of pyruvate dehydrogenase complex in *E.coli*

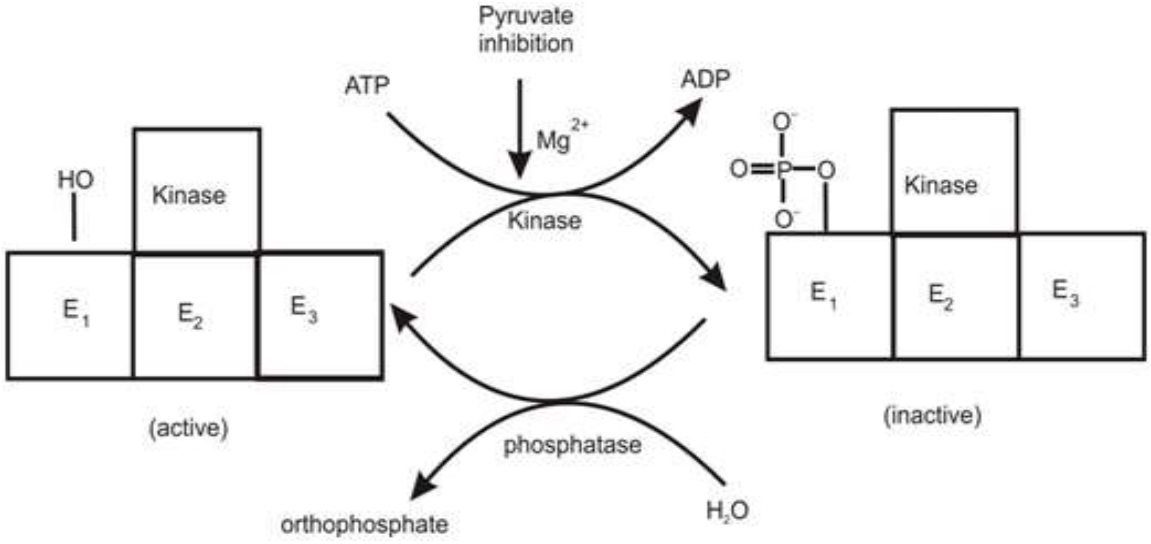

Fig. 9.2: Regulation of mammalian pyruvate dehydrogenase complex

Isolation and separation of subunits of pyruvate dehydrogenase multienzyme complex

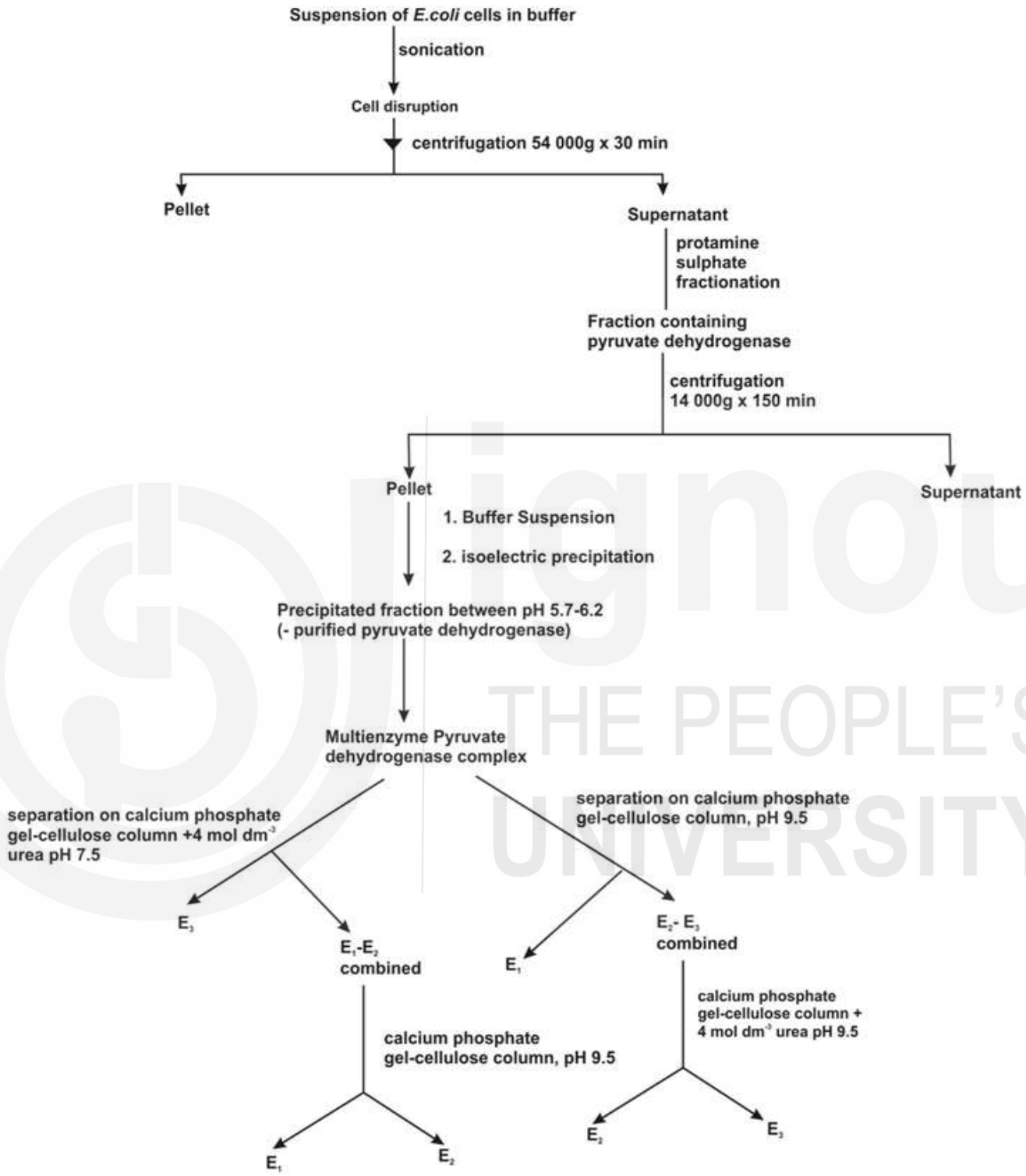

Fig. 9.3: Isolation of pyruvate dehydrogenase multienzyme complex from *E.coli* cells.

The enzyme is often expressed as ‘housekeeping enzyme’ that catalyzes a fundamental metabolic pathway being managed in almost all cell types and therefore present in reasonable quantity inside the cells and tissues. The process for the isolation and purification of this enzyme complex are based on the formerly developed procedures for the *E.coli* complex (See Fig: 9.3) or the process of poly (ethylene glycol) fractionation followed by isoelectric

precipitation and gel filtration adopted for mammalian pyruvate dehydrogenase complex. The genes for human and *Saccharomyces* pyruvate dehydrogenase have been cloned and expressed in *E.coli* enabling large quantities of the complex to be isolated and purified for structural studies. The isolated enzyme can be dissociated by use of 4 mol dm^{-3} urea, calcium phosphate gel and high pH or alternatively by using high salt concentrations. The complex can again be reconstituted from the subunits and the whole enzyme activity can be restored. The dissociation and reconstitution studies reveal that E_2 has binding sites for both E_1 and E_3 but $E_1$and E_3 do not bind when E_2 is absent. The M_r value of the polypeptide chains can be predicted by conventional methods and in many cases, have been established from those inferred by the DNA sequence. In yeast and mammalian pyruvate dehydrogenase complex, an additional polypeptide X has been found attached to the E_2 core after E_1 and E_3 have been dissociated. Chaotropic agents such as urea is required to dissociate the E_2-X subcomplex. The polypeptide X is structurally similar to the N-terminal region of E_2, but differs in C-terminal region.

9.4.2 Fatty Acyl Synthase Complex

Organisms have the ability to synthesize long- chain fatty acids and show similar basic metabolic pathway, though certain modifications do exist. The synthesis of fatty acids occurs from acetyl CoA and malonyl CoA as the precursor and the overall process of acyl chain extension occurs by addition of two methylene groups and requires transacylations, two reductions, a dehydration and a condensation and thus involves six catalytic activities. For the formation of malonyl CoA, an additional enzyme acetyl CoA carboxylase is necessary for the catalysis of the reaction.

The predominant chain length of fatty acids synthesized and the mechanism of release of the long- chain acyl carboxylate varies among organisms, but the sequence of enzyme reaction is alike in all cases. Despite the similarities, the organization of the enzyme system may be different.

There are two principal classes of fatty acid synthases:

In many animal tissues, avian tissues, *Neurospora*, and in some prokaryotes fatty-acid synthase system is found to exist as an aggregated multienzyme protein. This type of system is referred to as type I fatty acid synthase system. It was thought that these multienzyme proteins comprise of several tightly bound polypeptides with each chain having a catalytic activity, but with proper purification, in which proteolysis was avoided, revealed that they exist as multienzyme polypeptides. The type 1 fatty- acid synthase may further be divided on the basis of their size as type IA, having M_r of about 500 000 and subunit structure α_2, and type IB, having $M_r$value from 1 x 10^6 to 2.4 x 10^6 and subunit structure α_6-α_8 or $\alpha_6\beta_6$. The type IA is found in animal tissues and type IB occur in fungi, algae and certain bacteria such as *Corynebacteria, Mycobacteria and Streptomyces*. Fig. 9.4 displays a model proposed for the organization of domains in type I fatty acid synthase.

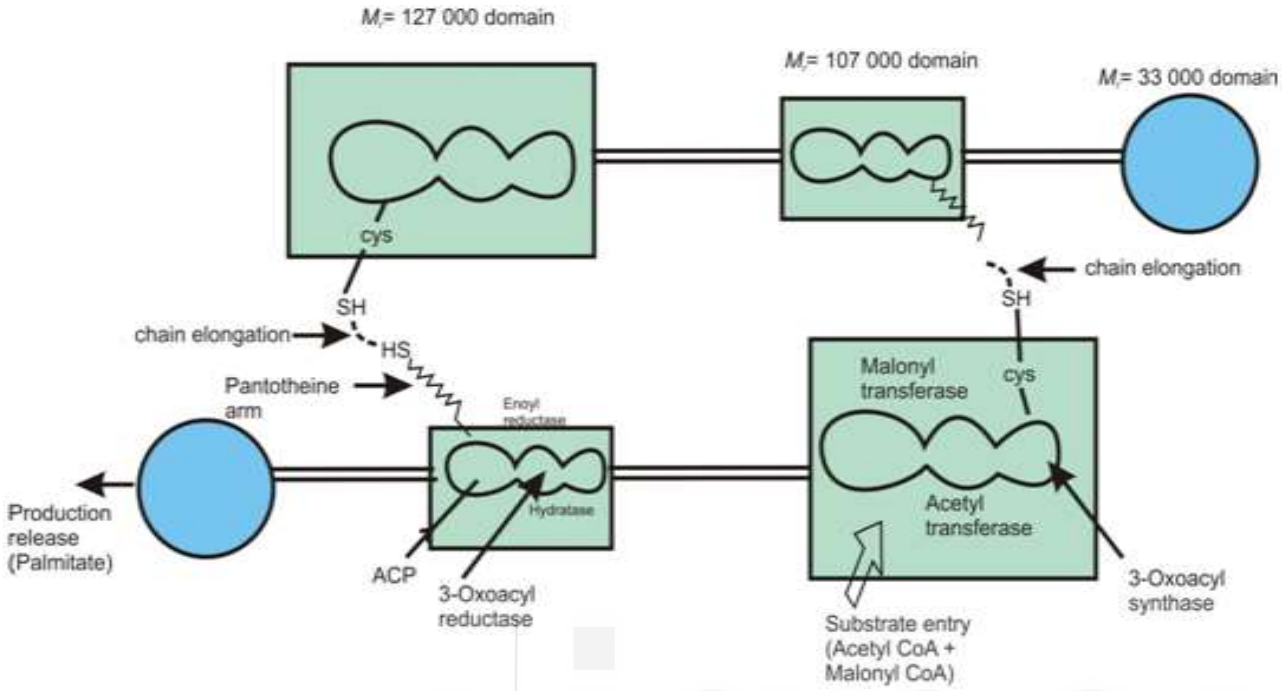

Fig. 9.4: Proposed domain structure of animal fatty acid synthase (type 1A)

type II is found in archaea and bacteria, and is characterized by the use of discrete, monofunctional enzymes for fatty acid synthesis. In *E.coli* six separate enzymes: ACP acetyltransferase, ACP malonyltransferase, 3-oxoacyl ACP synthase, 3-oxoacyl ACP reductase, crotonyl ACP hydratase and enoyl ACP reductase, together with the acyl carrier protein have been isolated and purified. Altogether, these enzymes are known to catalyze fatty- acid synthesis and there is no evidence of their physical association with one another. This non-aggregated type of system is also known as type II fatty acid synthase system. A similar system has been found in other bacteria, *Phormidium luridum, Euglena*, Avocardo mesocarp, *Chlamydomonas* and in the chloroplast from lettuce and spinach.

The mechanism of FAS I and FAS II elongation and reduction is the same, as the domains of the FAS II enzymes are basically homologous to their domain counterparts in FAS I multienzyme polypeptides. However, the differences in the organization of the enzymes - integrated in FAS I, discrete in FAS II - gives rise to many significant biochemical differences.

The evolutionary history of fatty acid synthases is very much intertwined with that of polyketide synthases (PKS). Polyketide synthases use a related mechanism and homologous domains to generate secondary metabolite lipids. Furthermore, polyketide synthases also display a Type I and Type II organization. FAS I in animals is thought to have arisen through modification of PKS I in fungi, whereas FAS I in fungi and the CMN group of bacteria (corynebacteria, mycobacteria, and nocardia) seem to have arisen separately through the fusion of FAS II genes.

The yeast fatty- acid synthase system has been systematically explored from a genetic as well as biochemical standpoint. Genetic mapping has revealed that there are two unlinked genetic loci on the chromosomes designated as *fas 1* and *fas 2*, are accountable for the whole fatty- acid synthase system.

Modification of its free amino groups using maleic anhydride results in reversible dissociation of the fatty- acid synthase.

SAQ 3

State whether following statements are True or False:

a) Acetyl- CoA acts as a competitive inhibitor of CoA for regulating the activity of pyruvate dehydrogenase complex. (True/False).

b) In pyruvate dehydrogenase complex, E_2 subunit, catalyze a serine residue phosphorylation in the E_1 which inactivates the complex when the intracellular ratio of [ATP]/[ADP] is low. (True/False).

c) In type II fatty acid synthase system, six separate enzymes known to catalyze fatty - acid synthesis are physically associated with each other. (True/False).

d) Type I fatty acid synthase system are multienzyme polypeptides. (True/False).

9.5 ISOENZYMES

A surprising discovery in biochemistry was the finding that a single enzyme can occur in several different forms within a single tissue. Markert and Møller proposed that 'multiple enzyme forms' in a single species should be known as isoenzymes or isozymes. However, numerous biochemists felt that the term 'isoenzymes' should be limited to those forms rising from genetic control of primary structure of protein. Due to the differences in opinions, it was finally recommended that:

The term 'multiple forms of enzymes' should be used as a wide term which covers all proteins occurring naturally in single species and also catalyzing the same reaction.

The term 'isoenzymes' or 'isozymes' should be applicable to only those various forms of enzymes arising from genetically determined disparity in protein primary structure but not to those which are resulting by alteration of the same primary sequence. These isozymes may be present in the identical species, in the similar tissues or even in the same cell. These isozymes usually vary in the kinetic nature or mode of regulation or usage of cofactor or even in their subcellular distribution.

Nomenclature of isoenzymes

In 1964, it was also recommended that based on the electrophoretic mobility, the individual isoenzymes should be numbered and distinguished, with the number 1 representing the form possessing highest mobility towards anode. Out of all the other criteria available to specify the properties of isoenzymes such as chromatography, electrophoresis, chemical structure, kinetics etc.

electrophoresis is the most widely accepted criteria due to the following reasons:

a) In electrophoresis, the resolution of individual proteins is not improperly inclined by their application in the original state, as tissue homogenate, or likewise.

b) The degree of resolution by electrophoresis is generally more effective than any other approaches of protein separations.

c) It tenders broad applicability and rapidity.

Although, kinetic data are helpful to explore the enzyme multiplicity, they are not competent to present evidence on the degree of heterogeneity. With respect to chemical structures, the final goal is to define the interrelationships of enzymes' multiple forms in chemical terms, and it is not useful to convey general approvals of nomenclature of isoenzyme based on structural data because such data is obtainable for a smaller number of enzyme systems. Once, structural data becomes available, notations in upper case roman letters, as assigned for aldolase and lactate dehydrogenase, may be used.

Therefore, it was recommended that:

(a) The normal enzyme name followed by a number should be used, which should be allotted successively on the basis of their electrophoretic mobility under definite conditions. The lower number being given to the forms having high mobility in the direction of anode.

(b) In a complex isoenzyme pattern, the number may be used to represent major groups, with lower case alphabets in subscript applied serially for individual subzones ($1_a\ 1_b\ 1_c\ 2_a\ 2_b$).

(c) Additional characteristics such as molecular weight, subunit structure, stability should be provided. Subunits may be represented by Roman upper-case letters or lower-case Greek letters.

The most widely studied isoenzyme is lactate dehydrogenase (LDH), which has been investigated in numerous laboratories. Several studies have provided a rich catalogue of data on the biological and clinical significance of LDH.

9.5.1 Lactate Dehydrogenase (LDH) isoenzyme

LDH of human tissues can be separated into five distinct forms by the application of starch gel electrophoresis. LDH-1 is closest to the anode and LDH-5 is nearest to the cathode. In adult human liver and muscle, LDH-5 is the dominant forms whereas, in kidneys and heart, the dominant forms are LDH-1, LDH-2, LDH-3. Examination of a variety of human tissues has revealed that the isoenzyme pattern in each case is unique.

After the elucidation of the structure of LDH isoenzyme, it appears that each isoenzyme consists of four polypeptide chains which are arranged at random from two separate polypeptide subunits A and B. The polypeptide composition

of each isoenzyme can be represented as:

LDH_1 $BBBB (A_0B_4)$

LDH_2 $ABBB (A_1B_3)$

LDH_3 $AABB (A_2B_2)$

LDH_4 $AAAB (A_3B_1)$

LDH_5 $AAAA (A_4B_0)$

This formulation was gained by a series of experiments. Firstly, it was shown that each isoenzyme has a molecular weight of 135,000 and can bind four molecules of DPN (diphosphopyridine nucleotide). When proteolytic reagents were added to the solutions, the molecules split into four polypeptide chains of equal lengths (M.W. 34,000), and these can be separated by electrophoresis into two distinct forms A and B. Finally, an extensive analysis of tissues from many sources revealed five isoenzymes almost in all of them. The arrangement of mixture of two kinds of subunits A and B, into five different molecules, each containing four subunits was elucidated by Markert (1963). The molecular structures of LDH and hemoglobin share many similarities. Each molecule is a tetramer comprising of four polypeptide chains, and each chain is associated with a catalytic site. Another important difference among isoezymes is the manner in which the molecules are assembled from their subunits.

Shaw and Barto (1963) observed the genetic evidence for the subunit hypothesis for LDH isoenzyme synthesis. They discovered an electrophoretic variant of polypeptide B in the deer mouse, and were able to show that the production of A and B subunits is managed by two different non- allelic genes. Further experiments showed that similar genetic mechanism control the LDH isoenzyme synthesis in human tissues. Pedigrees listed by Kraus and Neely (1964) suggested that the LDH variants are inherited as autosomal codominant characters. If only two genetic loci are involved in the synthesis of the LDH isoenzyme, then it is hard to interpret the presence of a sixth isoenzyme ‘band X’, in post-pubertal testis and sperm. The dissociation and recombination experiments suggest that the ‘band X’ isoenzyme is a tetramer composed of polypeptides different from A and B. Therefore, the complement of LDH isoenzyme can be elucidated on the basis of the activity of gene at three loci A, B and C where each of them is accountable for synthesis of corresponding polypeptide.

9.5.2 Physiological Role of Isoenzymes

There were apprehensions regarding the existence of five forms of the enzyme when each of them catalyzes the same reaction. The explanation is based on the pathway of glycolysis. During the breakdown of glucose, the fate of pyruvate can be (1) conversion to lactate, and (2) conversion to carbon dioxide and water. In the skeletal muscles, the energy results from anaerobic glycolysis with the generation of ATP. Reduced diphosphopyridine nucleotide;

(DPNH) is also produced during ATP production, and if DPNH does not get dehydrogenated to DPN, glycolysis would stop. The chief reaction for oxidation of DPNH is the transformation of pyruvate to form lactate, thus, it is essential that LDH of skeletal muscle should be able to handle the huge quantity of pyruvate formed at the time of strong muscular activity. Whereas, in the heart tissue, there is less requirement for anaerobic energy, maximum amount of the pyruvate enters citric acid cycle. A particular type of LDH in heart is the one which is inhibited by small amounts of pyruvate. The major isoenzymes in heart, comprising mainly polypeptide B, are powerfully inhibited by low concentrations of pyruvate and permits the metabolism of pyruvate to proceed via citric acid cycle. On the contrary, the muscle enzyme, having polypeptide A is inhibited only by very high concentrations of pyruvate, which favors the transformation of pyruvate to lactate. Therefore, it is ostensible that the catalytic features of the isoenzymes are apt according to metabolic needs of the respective tissues.

Wilson, Cahn and Kaplan (1962) studied the LDH isoenzyme present in birds and breast muscles. Birds which fly infrequently like grouse, pheasants and domestic fowls have largely LDH-5. Their patterns are like human skeletal muscle. While performing muscular activities, lactate gets accumulated with frequent activity fatigue arises. LDH-1 is key isoenzyme in breast muscles of hummingbird and storm petrel bird which excel in their ability for long and persistent flights.

In the fetal mouse, there is a dominance of isoenzymes in the LDH-5 end of the spectrum. This condition allows the tissue function in relatively anaerobic environment. In chick embryo, LDH-1 is predominant, suggesting that chick tissues rely more on aerobic metabolism.

Stadtman and coworkers (1961) investigated aspartokinase isoenzyme, in *E. coli.* It is responsible for catalyzing the first step of reactions that leads to formation of lysine, threonine and methionine. They were successful in separating the aspartokinase activity of whole homogenates into three fractions. One fraction is found to be inhibited by lysine, another by threonine and the third by homoserine which is a precursor of methionine.

The isoenzyme cytochrome P_{450} is involved in degradation and biosynthesis of endogenous compounds like steroids, fatty acids and cholesterol. It is also found to perform physiological roles in brain, for example: signal transduction by arachidonic acid metabolites; the regulation of cerebral vascular tone by arachidonic acid metabolites, the regulation of corticosteroids and progesterone in the brain which are believed to affect the mood and state of excitement; and also control the intracellular concentration of cholesterol. The pathways modulated by the enzyme P_{450} enzymes results in formation of metabolites (1A1, 1A2, 2B1, 2B2, 2C11) that may be responsible in regulating the ion permeability, enzyme activity and turnover of the membranes. A well-known polymorphic isoenzyme is debrisoquine hydroxylase enzyme also known as CYP2D6. The enzyme plays a pivotal role in the metabolism of many endogenous substances such as neurosteroids, monoaminergic neurotransmitters, neurotransmitters such as serotonin and dopamine and

hence, believed to have a role in determining the psychological state and behavior of an individual.

9.5.3 Pharmacological Role of Isoenzymes

The study of isoenzymes in serum has been useful in diagnostic procedures from a very long time. The specificity of information gained from such assays is dependent upon the tissue distribution of the enzymes. The LDH isoenzyme in normal serum are present in the proportion of LDH-2 > LDH-1 > LDH-3 > LDH-4 > LDH-5. Myocardial infractions have suggested changes in serum levels as compared to normal. There is a marked increase in LDH-1 and LDH-2 levels. Determination of LDH isoenzyme activity appears to be more specific and long-lasting indication of tissue damage. Patients with hepatitis exhibit increase in LDH-5 activity, which is normally present in trace amounts.

The isoenzyme cytochrome P_{450} in the liver performs important role in metabolism of drug by transforming them from a hydrophobic state to a much more easily excretable form. The reaction is grouped into two forms; the cytochrome P_{450} dependent phase I and phase II conjugation reactions. Enzyme inhibition, polymorphism, enzyme induction and various other physiological factors may lead to alterations in the activity of cytochrome P_{450}. These factors have many clinical consequences as they may change the drug pharmacokinetics leading to its reformed efficacy. This may also result in amplified toxicity due to reduction in metabolism or increase in the formation of drug interactions and toxic metabolites.

SAQ 4

Do as Directed

a) Isozymes or Isoenzymes are proteins with ..... structure which catalyze the same reaction. (similar/different) [Fill in the blank].

b) The degree of resolution by electrophoresis is generally ineffective than any other approaches of protein separations. (True/False).

c) From the physiological point of view, isozymes are similar enzymes with different characteristics, “customized” to specific tissue requirements or metabolic conditions. (True/False).

d) Arrange in the decreasing order of the proportion of five LDH isoenzymes present in serum LDH-1, LDH-2, LDH-3, LDH-4, LDH-5.

9.6 SUMMARY

1) Ordered association of various enzymes catalyzing successive steps in a reaction sequence are called as multienzyme complexes. Several examples of these complexes include pyruvate dehydrogenase complex, glycine decarboxylase, tryptophan synthase etc.

2) There are several advantages of enzymes being physically associated with one another in an enzyme complex. Transit time is reduced along with channeling and compartmentation in multienzyme complexes.

3) Instances of coordinate activation of whole multienzyme proteins have also been observed.

4) Isolation and characterization of multienzyme complexes is reasonably more complicated than that of a single enzyme. These complexes are more common in eukaryotes as compared to prokaryotes.

5) Multiple forms of same enzyme are called as isozymes. The distribution of isozymes of a given enzyme shows different metabolic patterns in different organs. They may have variable metabolic and regulatory roles. Lactate dehydrogenase was one of the first enzymes to be identified as isozyme. The pharmacological role of isoenzymes is extremely useful in diagnostic procedures. Activity of LDH isoenzyme emerges to be more definite and long-lasting indication of the tissue damage.

9.7 TERMINAL QUESTIONS

1) What are the characteristic properties of multienzyme complexes?

2) Enumerate the role of pyruvate dehydrogenase multienzyme complex.

3) Distinguish between multienzyme complex and multienzyme polypeptides.

4) Explain the physiological and pharmacological role of isozyme lactate dehydrogenase.

9.8 ANSWERS

Self-Assessment Questions

1) a) True, b) Stable, c) False, d) False

2) a) prokaryotes, b) more, c) less, d) Channeling

3) a) True, b) False, c) False, d) True.

4) a) different, b) False c) True, d) LDH-2 > LDH-1 > LDH-3 > LDH-4 > LDH-5.

Terminal Questions

1) Refer to section 9.4

2) Refer to section 9.4.1

3) Refer to section 9.2

4) Refer to section 9.5.1

9.9 SUGGESTED READINGS

1) David L. Nelson and Michael M. Cox: Lehninger Principles of Biochemistry 6th Ed., W.H. Freeman.

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8) Organized Multienzyme Systems: Catalytic Properties by G. Rickey Welch, Academic Press, 28th January 1985.