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MBC-004: ENZYMES AND THEIR APPLICATIONS

Block

III

ENZYME REGULATION

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Needs and conditions vary from cell to cell and change in individual cells over time. Therefore 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. Because enzymes guide and regulate the metabolism of a cell, they tend to be carefully controlled in order to accomplish balanced partitioning of different precursors among competing pathways to insure a sufficient supply of each metabolite. Regulation of enzyme is needed to ensure homeostasis and to respond to the environmental changes.

The control and coordination of complex metabolic pathways is possible by enzyme regulation. Regulation helps to control the rates of biochemical reactions and ensures that they proceed at appropriate times and under appropriate conditions. Allosteric regulation, feedback regulation, proteolytic cleavage, isozyme and covalent modification are some of the regulatory mechanisms prevalent in the living cells. Enzyme activity can be regulated by several molecules that either enhance or lower their activity. Molecules that enhance the enzyme activity are called activators, while molecules that lower the enzyme activity are known as inhibitors. There are many kinds of molecules that block or promote enzyme function, and that affect enzyme function by different routes.

Unit-7 of the block deals with several regulatory enzyme mechanisms such as allosteric regulation, feedback regulation, proteolytic cleavage and covalent modification. MWC and KNF models of sigmoidal kinetics as well as their significance have also been explained in the unit with relevant examples. Isozymes are used to fine-tune metabolism to meet the needs of different tissues or developmental stages. Different isozymes along with their structural and functional significance are described in Unit-8. 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. The phylogenetic distribution and properties of pyruvate dehydrogenase and fatty acyl synthase have been adequately described in the unit.

Expected Learning Outcomes:

After studying this block, you should be able to:

- determine several regulatory enzyme mechanisms,
- distinguish between MWC and KNF models of sigmoidal kinetics,
- explain the physiological and pharmacological role of isozymes,
- understand clinical importance of isozymes, and
- discuss the significant role of multienzyme complexes in metabolic pathways.

UNIT 7

REGULATION AND CONTROL OF ENZYME ACTIVITY

Structure

7.1 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. 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 regulation of enzyme activity ensures that metabolic processes happen at the right time and place at the proper rate. The necessity for regulation of enzyme arises from the fact that in the complex system of biological transformations concentrations of key metabolites is controlled both in terms of space and time to direct metabolism in a desired direction and not allows drifting. Several mechanisms such as allosteric regulation, feedback regulation, covalent modification, enzyme inhibition to enzyme synthesis and degradation ensure that enzyme activities

are finely tuned to meet the metabolic demands of the cell and organism. The effector molecules regulating the activity of enzymes act as vital elements in the integration of metabolic pathways. 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 discuss some of the principal mechanisms by which enzyme activity is regulated.

This unit will give you an overview about how the metabolic processes are regulated by the regulatory enzymes.

Expected Learning Outcomes

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;
- state the role of allosteric enzymes and feedback inhibition.
- explain the covalent modification of enzymes as regulatory mechanism,
- discuss the role of proteolytic enzymes.

7.2 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. The metabolic efficiency in such a scenario is affected via compartmentation. It 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 of 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 7.1) of the different sites of metabolic pathway is as given below:

Table 7.1: 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. There secondary messenger activate/deactivate the target enzyme. At each step/stage of mechanism, amplification of signal takes place.

7.3 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. Enzyme quantity
2. Availability of Substrate or Cofactor
3. Inhibition
    a) Specific Inhibitor molecules
    b) Reversible Inhibition (Competitive, Non-Competitive and Uncompetitive)
    c) Irreversible Inhibition
    d) Feedback Inhibition
4. Allosteric Regulation
5. Co-valent Modification
6. Proteolytic degradation

7.3.1 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.

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 to 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.

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 ia a small protein having 75 aminoacid residues. It is attached to the protein undergoing degradation in a process termed as ubiquitination catalyzed by 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 under investigation.

7.3.2 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 200μ $moldm^{-3}$. Enzymes operating at their maximal rate will not respond to the increase in substrate concentration but to the decrease in substrate concentration.

SAQ 1

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).

7.3.3 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 α1-antichymotrypsin, α1-antiprotease and α2-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).

d) Feedback Inhibition

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 reaction:

Enz 1 Enz 2 Enz 3

A----------→ B---------→ C ---------→ D

High concentrations of end product D will act as feedback 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 such as one given below also exhibits negative feedback control (Fig. 7.1). The feedback inhibition in series of metabolic pathways is affected at the branch point (solid arrows).

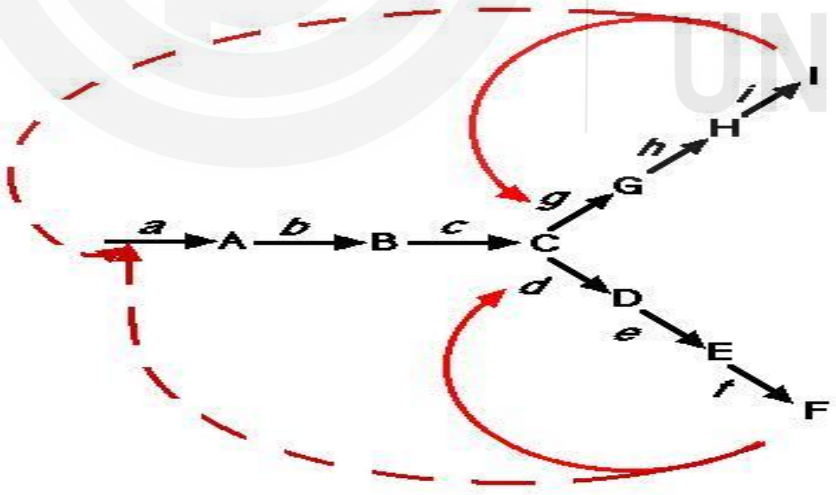


Fig. 7.1: Feedback inhibition and control in a branched pathway.

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. 7.2). This is a branched pathway. There are three separate aspartokinases (a, b, and c) which are regulated by different end product or effector substances.

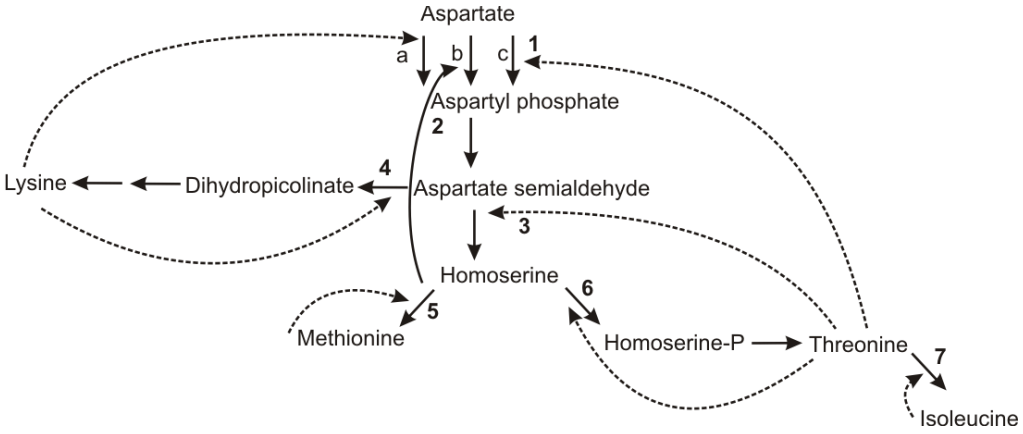


Fig. 7.2: Sites of feedback inhibition in a branched biosynthetic pathway.

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 homoserine.

Aspartyl phosphate is synthesized even in the presence of excess of one of amino acid and 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 (discussed later in the unit, section 7.3.4).

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

i) Concerted Feedback Inhibition: The activity of enzyme is inhibited by the combined feedback by the pair of end products. The end products are required to be bound simultaneously to effect the inhibition. 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 End Product 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.

Cumulative End product 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 % of62.8% 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 feedback inhibitor (Pick one option).

c) During feedback inhibition, Lysine inhibits aspartokinase a/ aspartokinase b/ aspartokinase c (Pick one option).

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.

7.3.4 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. 7.3). 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

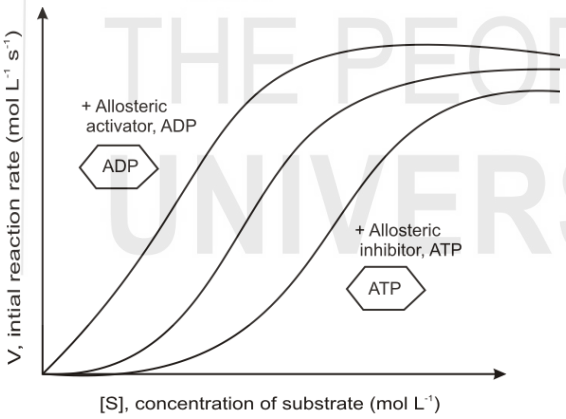


Fig. 7.3: Sigmoidal kinetics of allosteric enzymes.

Cooperativity phenomenon is associated with allosteric enzymes that have generally multiple binding sites. In cooperativity phenomenon when a ligand binds to one subunit of protein conformational changes occur at the other subunits of protein that affects the affinity of these sites. In other words we can say that when a ligand binds to receptor, it alters the binding affinity of second ligand molecule. Cooperativity can be positive or negative, that it becomes more or less likely that following ligand molecules will bind to the receptor molecule.

The controlled change in affinity makes the enzyme more sensitive to changes in substrate concentration (positive cooperativity), or reduces the

enzyme's response to changes in substrate concentration (negative cooperativity). 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 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.

The Michaelis-Menten equation fails to determine the cooperative saturation kinetics of allosteric enzymes. The solution was given by Archibald, V Hill in 1910 by Hill equation. The graphic representation of the Hill equation as given below helps to describe the cooperative binding of O_2 by multi-site, allosteric protein such as haemoglobin. Hill Equation implies that when substrate [S] is low relative to k′, the initial reaction velocity increases as the nth power of [S]. Here the k' is complex constant.

$$\frac{\log v_1}{V_{max} - v_1} = n \log [S] - \log k' \qquad \text{Hill Equation}$$

When a graph was plotted between log $v_1/(V_{max} - v_1)$ and log [S] the sigmoidal curve gets linearized (Figure 7.4). The slope, n of this linear curve is called Hill's coefficient. A perpendicular line drawn on x-axis from the point where the Y axis, log $v_1/(V_{max} - v_1)$ is zero, intersects at a substrate concentration termed S50 i.e the substrate concentration that results in half-maximal velocity.

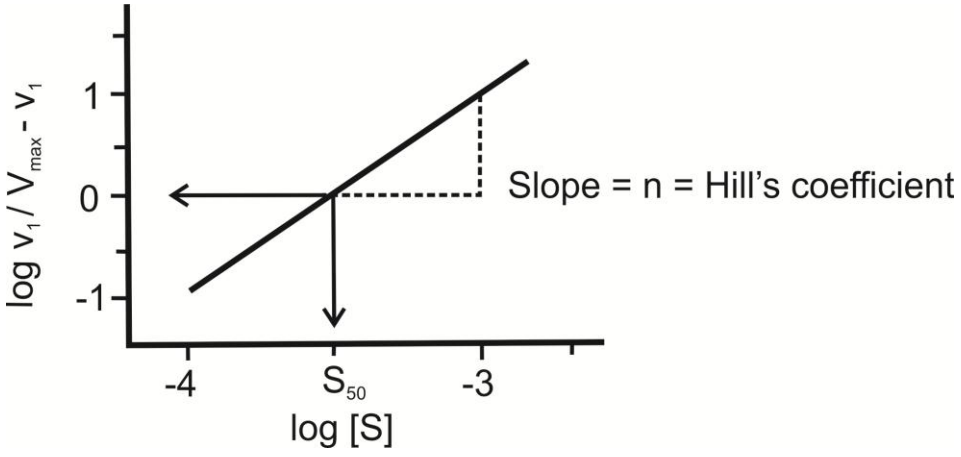


Fig 7.4: Linear representation of the Hill equation and the degree of cooperativity (Hill's coefficient n).

Hill's coefficient is an empirical parameter that determines the degree of interaction between ligand binding sites. Its value is a function of the number, kind, and strength of the interactions of the multiple substrate-binding sites on the enzyme. Now consider the values of Hill's coefficient:

1. If the value of n = 1, then it indicates the reaction is not cooperative, all binding sites conduct themselves independently, and therefore binding of substrate to one site does not affect the binding on the other site.

2. When n>1, it means positive cooperativity; where, binding of the first substrate molecule increases the affinity of the enzyme for binding other substrate molecules. Higher the value for n, the higher the degree of cooperativity and the more sigmoidal will be the plot of v1 versus [S].

3. If n<1, it indicates negative cooperativity which in other words suggests that the binding of one substrate will diminishes the binding on other site.

The binding of oxygen to the haemoglobin is considered to be one of the widely reported models of positive cooperativity. A Danish physician, Christian Bohr was the first who explored the binding of hemoglobin to oxygen under different physiological conditions. A sigmoid curve was obtained on plotting hemoglobin saturation with oxygen as a function of the partial pressure of oxygen. The cooperativity phenomenon was observed that suggest that the oxygen binding to haemoglobin facilitates the binding of more oxygen, until all binding sites gets saturated.

It was also observed that the increased CO_2 concentration in the tissues inhibits the binding of O_2 to haemoglobin. The phenomenon, along with the studies of hemoglobin's affinity for oxygen amplifying with increasing pH, is known as the "Bohr effect". Regarding the haemoglobin, Bohr found out homotropic positive cooperativity (binding of oxygen facilitates binding of more oxygen) as well as heterotropic negative cooperativity (binding of CO_2 reduces hemoglobin's affinity to bind oxygen).

Several models have been proposed to explain the molecular basis of cooperativity. Two main models are concerted model given by Monod-Wyman-Changeux (MWC) model and sequential model given by Koshland-Nemethy-Filmer (KNF) model.

Monod–Wyman–Changeux model (MWC model) is also called as the symmetry model. It tries to explain the allosteric transitions of proteins made up of identical subunits. The model is based on assumption that all the protomers are in the same conformational state i.e. each protomer or allosteric unit can exist in (at least) two conformational states, designated T (tense) and R (relaxed); these states exist in equilibrium whether or not ligand is bound to the oligomer (Fig. 7.5). There are no hybrid states. The ligand can bind to a protomer in either conformation. The R state has a higher affinity for the ligand than the T state. The binding of a ligand will increase the equilibrium in favor of the R state.

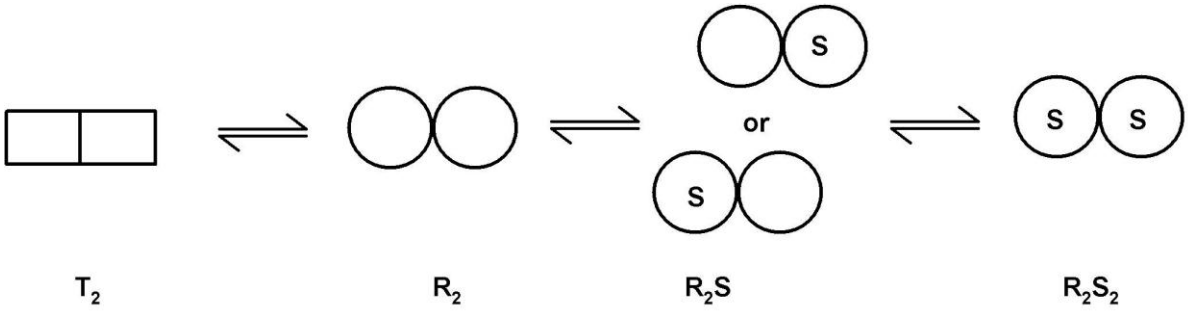


Fig. 7.5: Diagrammatic forms of R and T forms of a dimeric protein with ligand/substrate S in MWC model.

An allosteric protein such as hemoglobin is an oligomer of protomers that are symmetrically related. The tetramer hemoglobin transforms conformation together (R state) after four oxygen molecules join to all four monomers. The transition from the T state to the R state occurs in one step. At each step of oxygen binding, equilibrium exists between the T-state and R-state. The equilibrium shifts from robustly favoring the T state (no oxygen bound) to strongly favoring the R-state (fully loaded with oxygen). Overall, oxygen binding shifts the equilibrium toward the R state. This means that at high oxygen levels, the R form will be widespread and at lower oxygen levels, the T form will be prevalent.

For a protein consisting of n protomers, each with a binding site for the substrate or ligand (S), the MWC equation is

$$\bar{Y} = \frac{\frac{[S]}{K_R}\left(1+\frac{[S]}{K_R}\right)^{n-1}}{L+\left(1+\frac{[S]}{K_R}\right)^{n}}$$

According to this equation the higher value of L indicates a more sigmoidal nature of plot of Y against [S]. If L=0, a hyperbolic curve is obtained.

As per MWC model, allosteric inhibitors bind to T-form of protomer thereby increasing the value of L. They increase the sigmoidal nature of binding curve of substrate with the enzyme. These inhibitors decrease the fractional saturation of enzyme with its substrate at low or moderate concentrations and decrease the value of v_o. On the other hand allosteric activators have opposite effect; they bind to R-form and lower the L value. These activators tend to increase the hyperbolic nature of enzyme substrate binding curve. The degree of allosteric effect depends on the modifier concentration but the V_{max} value is not changed. The binding of modifier to protomers disturbs the R/T equilibrium. Enzymes that exhibit such allosteric control falls in one of the two categories K-series or V series respectively.

In K-series enzymes the presence of modifier alters the substrate binding characteristics to enzyme but does not affect the V_{max} of the reaction. For a K-

series enzyme S50 i.e the substrate concentration needed to half saturate the enzyme varies with the concentration of modifier. The substrates prefer to bind to the R-form of the protomer.

V-series enzymes are those series of enzymes in which modifier change the V_{max} but do not change the K_m or S50 for the substrate. The binding curve of substrate at constant modifier concentration is a rectangular hyperbola but the binding curve for modifier itself is sigmoidal. V-series enzymes are less common than K-series enzymes.

Sequential Model or KNF Model: In this model subunits are not connected in such a way that a conformational change in one induces a similar change in others. Its not essential that all subunits of enzyme posses the same conformation in multi subunit structure. The sequential model states that molecules of substrate bind through an induced fit. In this model subunits of the enzyme can change sequentially from the T form to the R form and back again.

The major difference between MWC and KNF model is that KNF model does not exclude hybrids between two conformational forms of protein. If you recall in MWC model in a dimeric protein, only R and T forms of protomer are allowed (Fig. 7.5). So these following species states exist:

T_2, R_2, R_2S and R_2S_2

Secondly, the binding of one molecule does not change the affinity for binding site for S. However, in KNF model a dimeric protein, the protomer can exist in T and R forms and the species R_2, T_2, R_2S, R_2S_2, R.TS, RSTS, T_2S and T_2S_2 can all exist (Figure 7.6). Another difference is that in the sequential model, the T states do not have to convert to R states all at one time. The ligand will change the conformation of the subunit that it is bound to and induce changes in the neighboring subunits.

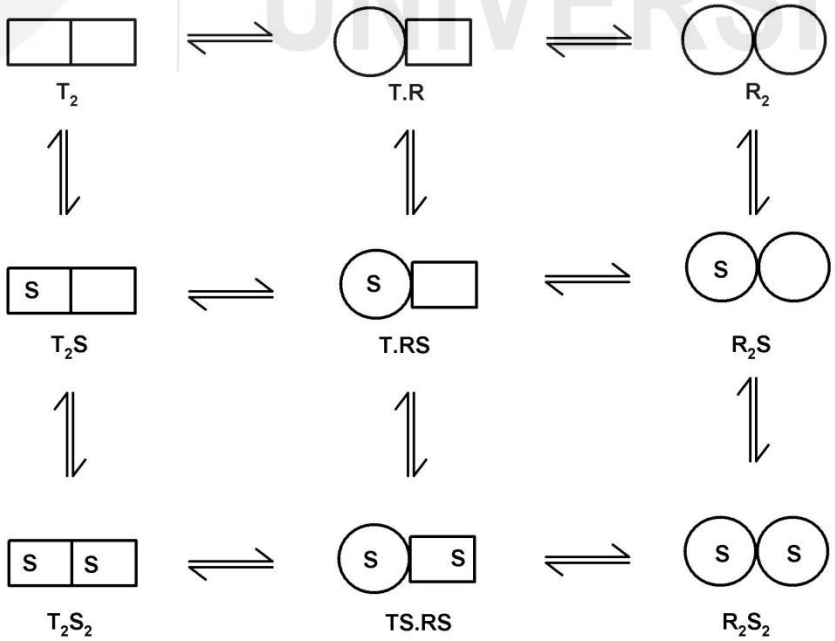
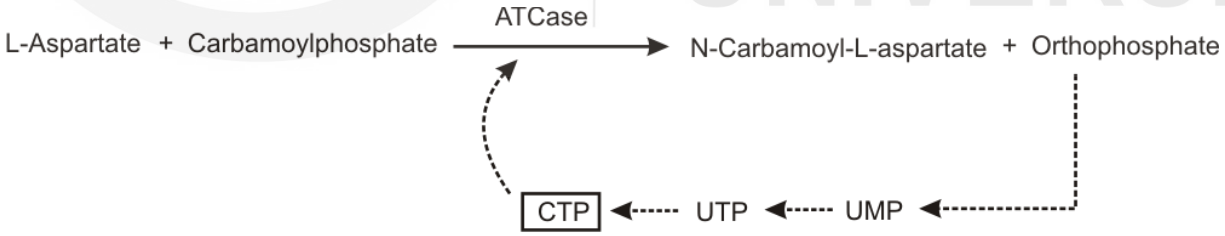


Fig. 7.6: Binding of ligand (S) to a dimeric protein who's each protomer exists in two conformational forms in the KNF sequential model.

Both models agree that the affinity of protein for a given ligand changes upon binding of the ligand. The MWC model proposed that this happens by a quaternary conformational change that engages the entire protein, transitioning from T state in favor of the R state. On the other hand, the KNF model proposed that the conformational changes take place at the tertiary structure level within the protein, as neighboring subunits change conformation with successive ligand binding. In the sequential model, the change in the conformation begins with binding of inhibitor or activator to one subunit and it affects the conformations of other subunits. The net result is to favour the R state when activator is present and to favour the T form when inhibitor, I, is present. In other words, we can also say that the binding of one molecule of substrate to one subunit promotes the transition of the subunit to the R form, which then promotes another subunit to change to the R form. However, the binding of inhibitor to one subunit induces a change in the other sub-units to a form with lower affinity for the substrate. Binding of an activator to one subunit induces a shift in the other subunits to a form that has a high affinity for substrate.

The sequential KNF model explains the concept of negative cooperativity unlike the MWC model. In negative co-operativity, there is decrease in the affinity of the remaining other binding sites of a protein for a ligand after the binding of one or more of the ligand to its subunits. Several biological molecules such as tyrosyl-tRNA synthetase and glyceraldehyde-3-phosphate dehydrogenase exhibit negative cooperativity. The MWC model only allows for positive cooperativity, where a single conformational switch from the T to R states led to an increase in the affinity for the ligand at unligated binding sites. Ligand binding to the T state does not enhance the amount of the protein in the T or low-affinity, state.

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. 7.7).



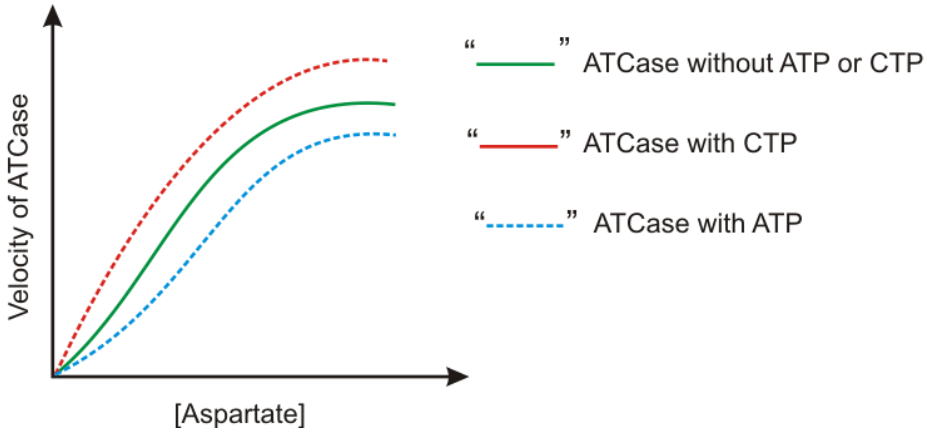


Fig. 7.7: Allosteric regulation of enzyme aspartate transcarbamoylase.

CTP: cytidine triphosphate

ATP: adenosine triphosphate

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

Structure: Intact ATCase is comprised 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 is shifted towards higher substrate concentration (Km increase, Vmax 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, Km of ATCase for aspartate decreases, Vmax 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→ R transition, catalytic trimers detach alongside the molecule threefold 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.

7.3.5 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 contain 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.

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

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.

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

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.

7.3.6 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 in 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 (Table 7.2). Activation segments are sometimes seen to be inserted into 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). In retroviruses, protienases are essentially required for cleaving large polypeptide into functional units.

Table 7.2: Some examples of enzymes and their zymogens along with their functions.

Enzyme	Precursor	Function
Trypsin	Trypsinogen	pancreatic secretion
Chymotrypsinogen	Chymotrypsin	pancreatic secretion
Carboxypeptidase procarboxypeptidase		pancreatic secretion
Elastase proelastase		pancreatic secretion
Phosphalipase A_2 prophospholipase A_2		pancreatic secretion
Pepsin	Pepsinogen	Secreted in gastric juice (Most active in pH range 1-5)
Thrombin	Prothrombin	Part of first component of complement system
Clr·	Clr·	Part of first component of complement system
Chitin Synthase	Zymogen	Involved in formation of septum during budding and cell division in yeast.

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. This coordination is achieved by a common factor that all zymogens are activated by action of trypsin.

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 (Fig 7.8). Enteropeptidase (Enterokinase) produced by brush border of epithelial cells in small intestine initially activates trypsinogen. Activated trypsin further activates trypsin and other zymogens (Fig 7.9).

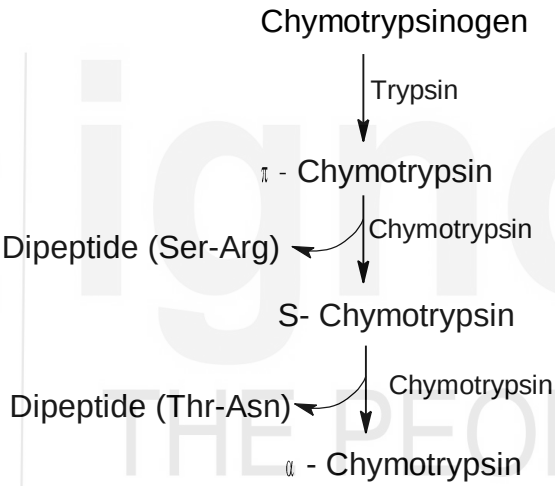


Fig 7.8: Activation of Trypsinogen.

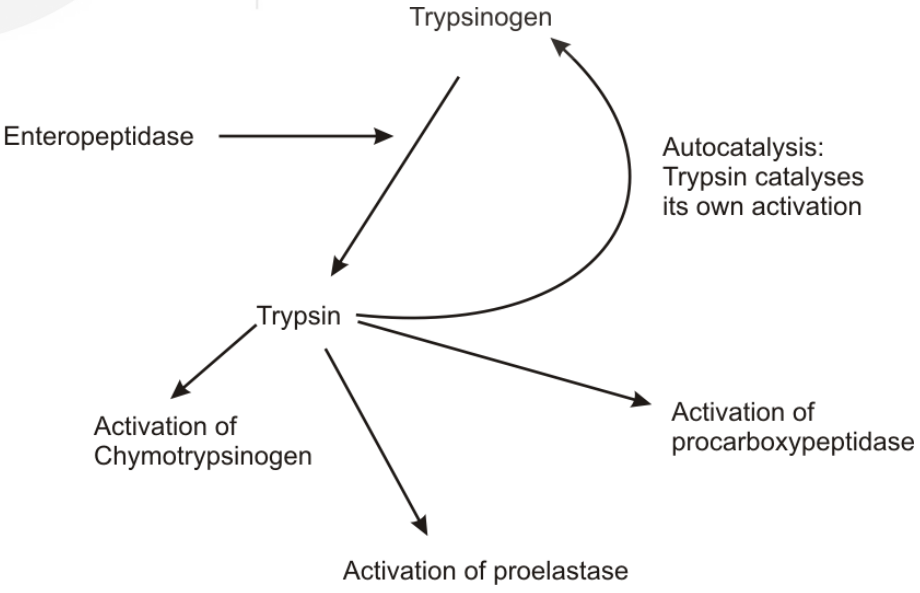


Fig 7.9: Principle of signal amplification and coordinated action of pancreatic proteases.

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

Blood coagulation and complement activation system also illustrate similar amplification system. 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.

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.

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. 7.10). The active enzyme inturns acts on two more dipeptide bonds of other π-chymotrypsin molecules to give fully active and stable α-chymotrypsin enzymes.

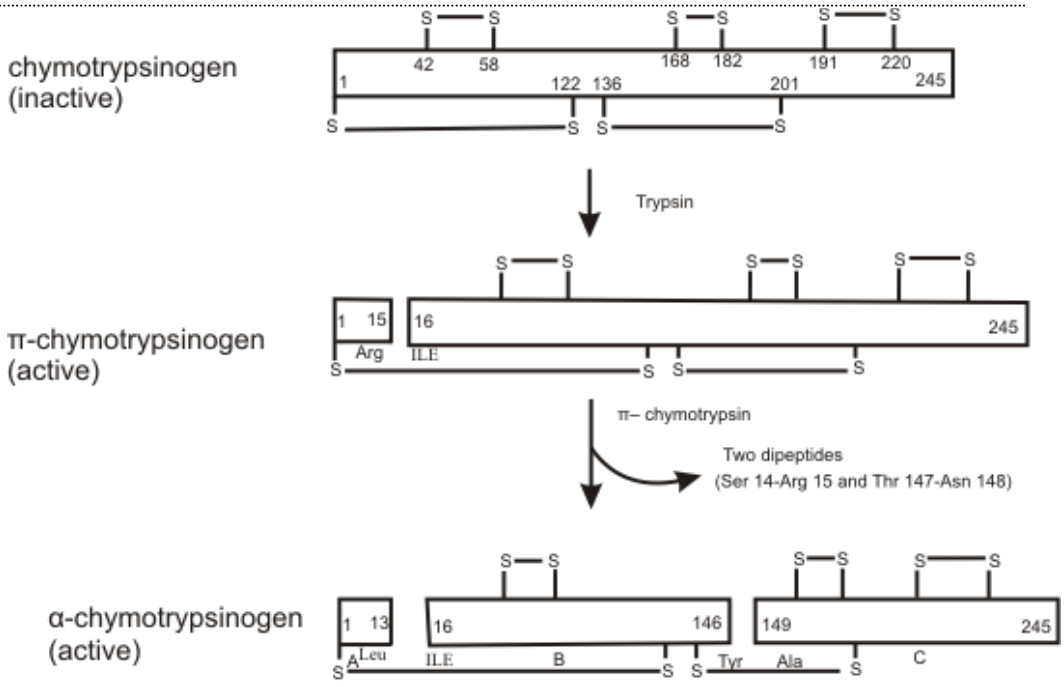


Fig. 7.10: Proteolytic activation of chymotrypsinogen.

Activation of Pepsinogen

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

Activation of Firbrinogen I (Factor I)

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

During blood coagulation, coagulation cascade activates prothrombic (zymogen) to active serine protease thrombin. Thrombin converts soluble fibrinogen into insoluble fibrin strands which are cross linked by factor XIII to form blood clot.

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-terminus residues.

7.4 SUMMARY

Let us summarize

- 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.
- Tight integration of metabolic pathways is possible using regulatory enzymes. Regulatory enzymes generally catalyzed 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.
- Feedback inhibition 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.
- Allosteric means 'other structure or different site'. Allosteric enzymes are the enzymes whose catalytic activity can be modulated by the effector molecules. 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.
- Both MWC and KNF models agree that the affinity of protein for a given ligand changes upon binding of the ligand. The MWC model proposed that this happens by a quaternary conformational change that engages the entire protein, transitioning from T state in favor of the R state. On the other hand, the KNF model proposed that the conformational changes take place at the tertiary structure level within the protein, as neighboring subunits change conformation with successive ligand binding.
- Phosphorylation and dephosphorylation are the most common but not the only means of covalent modification. 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.
- 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 in active forms at the particular site. Precursor of pepsin is pepsinogen, trypsin is synthesized as trypsinogen and procarboxypeptidase is zymogen of carboxypeptidase.

- Regulation of enzyme activity by inhibitors, allosteric and covalent modifiers has wide range of applications in the fields of medicine and agriculture.

7.5 TERMINAL QUESTIONS

1. How compartmentation simplifies regulation?
2. Describe allosteric regulation of the enzyme activity.
3. Discuss the different modes of feedback inhibition.
4. Distinguish between MWC and KNF models.
5. What are proteolytic enzymes? Discuss any two of them.

7.6 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 as 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. stabilize
   d) Allosteric
3. a) True
   b) High
   c) Aspartokinase a
   d) Cumulative End product 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 to 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 in the catalytic activity of the enzymes. There are two types of allosteric modulation-positive allosteric modulation when binds 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 7.3.4).

3. Refer to section 7.3.3 subsection (d).

4. Refer to section 7.3.4

5. Refer to section 7.3.6 subsection proteolytic enzymes

7.7 FURTHER READINGS

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UNIT 8

ISOZYMES

Structure

8.1 INTRODUCTION

A surprising discovery in biochemistry was 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 'isozymes' 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. Isozymes allow the fine-tuning of

metabolism to meet the specific requirements of a given tissue or developmental stage.

In this unit we will read more about isozymes, their physiological role as well as clinical importance.

Expected Learning Outcomes

After studying this unit, you should be able to:

- define isozymes;
- determine the physiological role;
- determine their origin; and
- discuss their clinical importance.

8.2 ORIGIN OF ISOZYMES

Isoenzymes (or isozymes) are referred to as group of enzymes that catalyze the same reaction but have dissimilar enzyme forms and varying catalytic efficiencies. Generally they also vary in regard to kinetic parameters and are regulated differently.

It is well known that different genes can make different variations of enzymes. Most of times, isozymes are encoded by homologous genes that have diverged over time. You will be surprised to know that most of the recombinant enzymes that have arisen on account of deletion, insertion, and/or other mutations at the genetic level fall into the class of isozymes. There are several processes which have been accounted to contribute to the generation of isozymes. Some of these are:

Isozymes due to Multiple Alleles: The occurrence of more than one allele at a particular gene locus is a major origin of isozymes. Variant alleles may be relatively uncommon in the general population or they may be fairly widespread and could give rise to genetic polymorphism. Each allele codes for a structurally separate polypeptide chain; thus the primary structure of the enzyme protein may vary from individual to individual according to the particular alleles which are carried. If an individual is heterozygous for two such alleles, eg, Al and A2 which determine αl and α2 polypeptides respectively, at a given enzyme locus, then more than one molecular form of that enzyme is expected to happen, though the exact number of isozymes observed in such heterozygotes will depend on the basic subunit structure of the enzyme.

Gene Duplication: This process is also considered to be the most common origin of isozymes. As the name suggests, original gene encoding an enzyme may replicate that can cause multiple copies of the gene. These replicas can further build up mutations over time that could lead to enzymes with somewhat different structures but similar functions. These alterations may be favored by natural selection as they can prove to be beneficial by permitting fine-tuning of the properties of enzyme to rally diverse physiological needs.

Alternative Splicing: This is another process leading to the formation of isozymes. In eukaryotic organisms, alternative splicing of precursor mRNA can generate different isoforms of an enzyme from a distinct gene. Hence a single gene can encode multiple protein variants, which chose to function diversely in several tissues or at different stages of developmental.

Gene Families: In the evolutionary process, some enzyme families may arise where multiple genes with similar sequences and functions evolve to produce isozymes. Due to divergent evolution, these gene families may lead to generation of isozymes with distinct properties.

Post-Translational Modifications: It is considered to be the most uncommon primary method for the production of isozymes which arise by secondary changes in the structure of an enzyme protein subsequent to its primary synthesis on the ribosomes. The post-translational modifications such as phosphorylation, glycosylation, or acetylation can sometimes produce functionally diverse forms of an enzyme from a single polypeptide chain.

The presence of isozymes allows for a multipurpose and adaptable biochemical system within organisms, which allows them to regulate metabolic processes more precisely according to tissue-specific needs or environmental conditions.

8.3 NOMENCLATURE OF ISOZYMES

Although isozymes are quite roughly identical functionally, they are dissimilar in several ways. Since enzymes are made up of proteins, any amino acid substitutions may lead to alterations in the electric charge. Therefore these isozymes which differ on account of amino acid substitutions can be identified by gel electrophoresis.

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, and etc. the most widely accepted criteria are electrophoresis 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, yet it is fairly incompetent 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 ($1_a1_b1_c2_a2_b$).

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

8.3.1 Lactate Dehydrogenase (LDH) Isoenzyme

The most widely studied isoenzyme is lactate dehydrogenase (LDH), which has been investigated in numerous laboratories including Markert, Kaplan, Pfleiderer, Wieland, Vessel and Bearn. These studies have provided a rich catalogue of data on the biological and clinical significance of LDH.

LDH of human tissues can be separated into five distinct forms by applying starch gel electrophoresis, LDH-1 being closest to the anode and LDH-5 being nearest to cathode. In adult human liver and muscle, LDH-5 is the dominant band whereas, in kidneys and heart, the dominant bands 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 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.

SAQ 1

Do as directed:

a) On the basis of electrophoretic mobility, the individual isoenzymes should be numbered and distinguished. (True/False).

b) Name two processes which have been accounted to contribute to the generation of isozymes.

b) Most of times, isozymes are encoded by homologous genes that have not diverged over time. (True/False).

c) In LDH isoenzymes, it appears that each isoenzyme consists of four polypeptide chains. (True/False).

8.4 PHYSIOLOGICAL ROLE OF ISOZYMES

Human, plant and animal cells also contain many isozymes that are involved in various metabolic pathways. These enzymes permit differential regulation of metabolic enzyme such as different subcellular localizations and different allosteric regulations. Isoenzymes also hold potential usefulness in the study of differentiation and gene regulation.

During the development of several tissues, from a variety of species, characteristic alterations in isoenzyme patterns have been detected. There were apprehensions regarding the existence of five forms of the LDH 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 are fewer requirements 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 permitting 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' 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 collects and 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 the aspartokinase isoenzyme, in *E. coli.* It is responsible for catalyzing the first step of reactions that lead 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 P450 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 and regulation of cerebral vascular tone by arachidonic acid metabolites. The regulation of corticosteroids and progesterone in the brain are believed to affect the mood and state of excitement; and also control the intracellular concentration of cholesterol. The pathways modulated by the enzyme P450 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, thought to have a role in determining the psychological state and behavior of an individual.

Serum and tissue isoenzyme patterns are being used for diagnostic purposes and associated explorations in the inborn errors of metabolism. Alternate enzyme forms have been found in patients suffering with hereditary deficiencies of erythrocyte-6-phosphogluconate dehydrogenase, glucose-6-phosphate de-hydrogenase and pseudoCholinesterase.

Several isoenzymes contain two types of subunit which are controlled by separate genes and are regulated independently by a variety of environmental factors. These findings are of significant interest to the geneticist, as research based applications will have significant implications in studies on growth and development.

SAQ 2

Fill in the Blanks:

a) ……..is key isoenzyme in breast muscles of hummingbird and storm petrel bird which excel in their ability for long and persistent flights ……...

b) The isoenzyme ……..is involved in degradation and biosynthesis of endogenous compounds like steroids, fatty acids and cholesterol.

c) ……. isoenzyme, in *E. coli* is responsible for catalyzing the first step of reactions that lead to formation of lysine, threonine and methionine.

d) Several isoenzymes contain two types of subunit which are controlled by separate ……and are regulated independently by a variety of environmental factors.

8.5 PHARMACOLOGICAL ROLE OF ISOZYMES

The study of activity of enzymes in serum has been useful in diagnostic procedures from a very long time. In medical diagnostics, the presence of certain isoenzymes in the blood can be indicative of damage to specific organs. Isozymes are also extremely useful in the study for differentiation and gene regulation. During the development of several tissues from a variety of species, characteristic alterations in the isozyme patterns have been detected. Isozymes are therefore amongst the most widely used molecular markers for the study of genetics.

The specificity of information gained from Isozyme 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. 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 P450 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, the cytochrome P450 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 P450s. 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 formation of drug interactions and toxic metabolites. Cytochrome isozymes also play a vital role in the biosynthesis of steroid hormones and bile acids.

Characteristic changes in the creatine kinase isoenzymes have been observed in a number of these tissues during development and these alterations have been found to be correlated with biochemical and morphological events.

Clinical Importance of Isozymes

1. Diagnostic Markers:

1. **Cardiac Enzymes**: Isozymes such as lactate dehydrogenase (LDH) and creatine kinase (CK) are vital in diagnosing myocardial infarction (heart attack). Unlike LDH, creatine kinase activity appears to be restricted to muscular and nervous tissues. For example, CK-MB is a cardiac isozyme that is released into the blood when heart muscle cells are damaged.
2. **Liver Function Tests**: Isozymes of alkaline phosphatase (ALP) and gamma-glutamyl transferase (GGT) aids in distinguishing between bone and liver diseases, as well as other conditions thereby affecting ALP levels.
3. **Disease Monitoring**:

- **Cancer**: Serum acid phosphatase isoenzymes have been investigated by electrophoresis in patients with multiple myeloma, prostatic carcinoma and Gaucher's disease. For instance, prostatic acid phosphatase (PAP) and prostate-specific antigen (PSA) are used to monitor prostate cancer.
- **Enzyme Replacement Therapy**: Isozymes can play a significant role in determining the effectiveness of enzyme replacement therapies in several genetic disorders, such as Gaucher's disease or Fabry disease, where specific enzyme deficiencies are present.

4. **Metabolic Disorders**: Isozymes can be used to diagnose and monitor inherited metabolic disorders. For example, variations in hexokinase isozymes are related with certain types of glycogen storage diseases.
5. **Tissue-Diagnosis**: Tissue-specific isozymes such as LDH are expressed in several different tissues such as muscle, liver and heart. Their expression can help to identify the origin of tissue damage or disease.
6. **Pharmacogenomics and Drug metabolism**: Different isozymes metabolize drugs at diverse rates. Cytochrome P450 isozymes plays a vital role in drug metabolism, and any variations in these isozymes can influence an individual's response to medication and risk of adverse effects.

Isozymes provide vital key information for diagnosing diseases, monitoring their progression as well as aiding in their treatments. Their role helps to determine the genetic conditions of individual and population in general.

SAQ 3

Do as Directed:

a) Isozymes are also extremely useful in the study for differentiation and gene regulation (True/False).

b) Which cardiac isozyme is released into the blood when heart muscle cells get damaged?

c) Name two diseases in which serum acid phosphatase isozymes needs to be investigated.

d) Cytochrome P450 isozymes plays a vital role in drug metabolism (True/False).

8.6 SUMMARY

Let us summarize

- 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.
- There are several processes which have been accounted to contribute to the generation of isozymes. Multiple alleles, gene duplication, alternative splicing and post translational modifications are the processes that contribute to their generation.
- Out of all the other criteria available to specify the properties of isozymes such as chromatography, electrophoresis, chemical structure, kinetics, and etc. the most widely accepted criteria are electrophoresis.

- Lactate dehydrogenase was one of the first enzymes to be identified as isozyme. Activity of LDH isoenzyme emerges to be more definite and long-lasting indication of tissue damage. LDH of human tissues can be separated into five distinct forms by applying starch gel electrophoresis, LDH-1 being closest to the anode and LDH-5 being nearest to cathode.
- Isoenzymes also hold potential usefulness in the study of differentiation and gene regulation. During the development of several tissues, from a variety of species, characteristic alterations in isoenzyme patterns have been detected.
- The pharmacological role of isozymes is extremely useful in diagnostic procedures. In medical diagnostics, the presence of certain isoenzymes in the blood can be indicative of damage to specific organs.
- The clinical applications of isozymes are diverse and continue to evolve with advances in medical science.

8.7 TERMINAL QUESTIONS

1. What are isozymes? Describe their physiological role.
2. Discuss the origin of isozymes.
3. Write a short note on Lactate Dehydrogenase (LDH).
4. Explain the clinical importance of isozymes.

8.8 ANSWERS

Self -Assessment Questions:

1. a) True,
   b) Gene duplication, alternative splicing and post translational modifications.
   c) False
   d) True
2. a) LDH-1
   b) Cytochrome P450
   c) Aspartokinase
   d) Genes
3. a) True
   b) CK-MB
   c) Multiple myeloma, prostatic carcinoma and Gaucher's disease
   d) True

Terminal Questions

1. Refer to section 8.4
2. Refer to section 8.2
3. Refer to section 8.3.1
4. Refer to section 8.5

8.9 FURTHER READINGS

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9. **https://www.jstor.org/stable/43419693?seq=9**
10. **https://www.sciencedirect.com/science/article/abs/pii/S0074769608602065#:~:text=Most%20isozymes%20can%20be%20classified,these%20are%20in%20vitro%20artifacts.**

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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 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 extents. You will also notice that organized system of cell enzyme system is more complex. Let us introduce a new term multienzyme complex- stable gathering of several enzymes with multiple catalytic domains. Multienzyme complexes are aggregates of different enzymes that work together as a single functional unit. These complexes catalyze two or more steps in a metabolic pathway. Hence, this type of organization permits more efficient and coordinated processing of substrates in biochemical pathways. Generally these complexes are composed of two to six functionally related enzymes and have molecular

weights ranging from few hundred thousand to several million. In this unit we will read more about these complexes to understand the regulation of enzyme activity.

Expected Learning Outcomes

After studying this unit, you should be able to:

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

9.2 MULTIENZYME COMPLEXES AS REGULATORY ENZYMES

In free solution, the rate of enzyme catalyzed reaction is dependent upon 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.

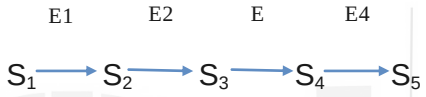
A multienzyme complex is a stable assembly of more than one enzyme, which are generally involved in sequential catalytic transformations. The organization of functionally related enzymes in a complex is expected to influence the efficiency and regulation or both features of the enzyme system. In the large assembly of enzymes, spatial proximity of the enzyme active sites results in a substrate channeling effect that streamlines the cascade reaction and increases the overall efficiency of the metabolic pathway. In several cases, activity of one component of a multienzyme complex gets changed on account of its association with others for example E.Coli tryptophan synthase compoenets are modified by complex formation; the reactions involving substrates of the tryptophan pathway are catalyzed more efficiently while serine deaminase activity of B protein is diminished. Similarly activity of regulatory enzymes pyruvate dehydrogenase kinase and pyruvate dehydrogenase phosphatase amplified significantly by associating with dihydrolipoyl transacetylase.

Multienzyme complexes can be regulated more effortlessly than individual enzymes. The complex's activity can be altered as a whole, which permits tighter control over metabolic pathways.

Multienzyme complexes are different from multienzyme polypeptides, which have multiple catalytic domains found in single polypeptide. 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 above-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 yield 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). An example of 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 OF MULTIENZYME PROTEINS AND THEIR 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. Pyruvate dehydrogenase complex and the fatty acid synthase complex in bacteria are adapted to several environmental conditions thereby contributing to metabolic versatility and survival in diverse habitats.

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 in 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.

Overall, the phylogenetic distribution of multienzyme proteins echoes their fundamental role in metabolism across different domains of life, with deviations reflecting evolutionary adaptations and functional specializations.

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 ------→ B-------→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 evidently observed. 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 …… than in a prokaryotic cell. (Fill the blanks with right choices) [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 . (Fill the blanks with right choices) [more/less]

d) …… reduces loss of pathway flux by leakage or instability of free intermediates (Fill in the Blanks).

9.4.1 Pyruvate Dehydrogenase Multienzyme 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.

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

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 cerevisiae, Arabidopsis, Neurospora crassa, Enterococcus faecalis*, mammalian heart, kidney and liver and avian tissues. It has been cloned and sequenced because of playing 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 (Figure 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 (Figure 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.

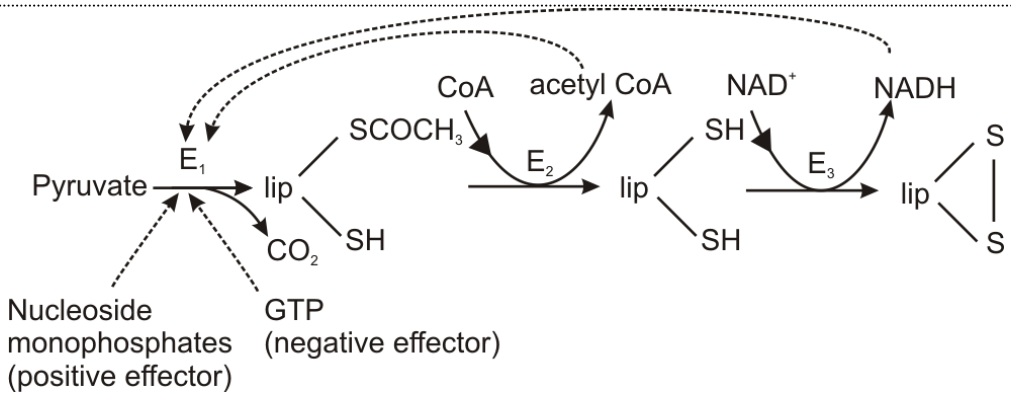


Fig. 9.1: Regulation of pyruvate dehydrogenase complex in *E.coli.*

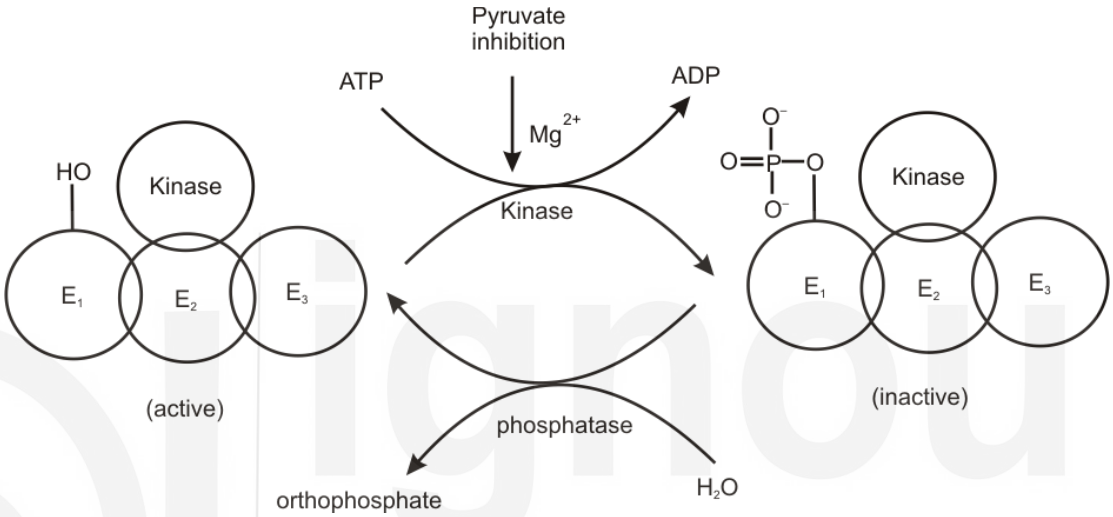


Fig. 9.2: Regulation of mammalian pyruvate dehydrogenase complex.

Isolation and separation of subunits of pyruvate dehydrogenase multienzyme complex

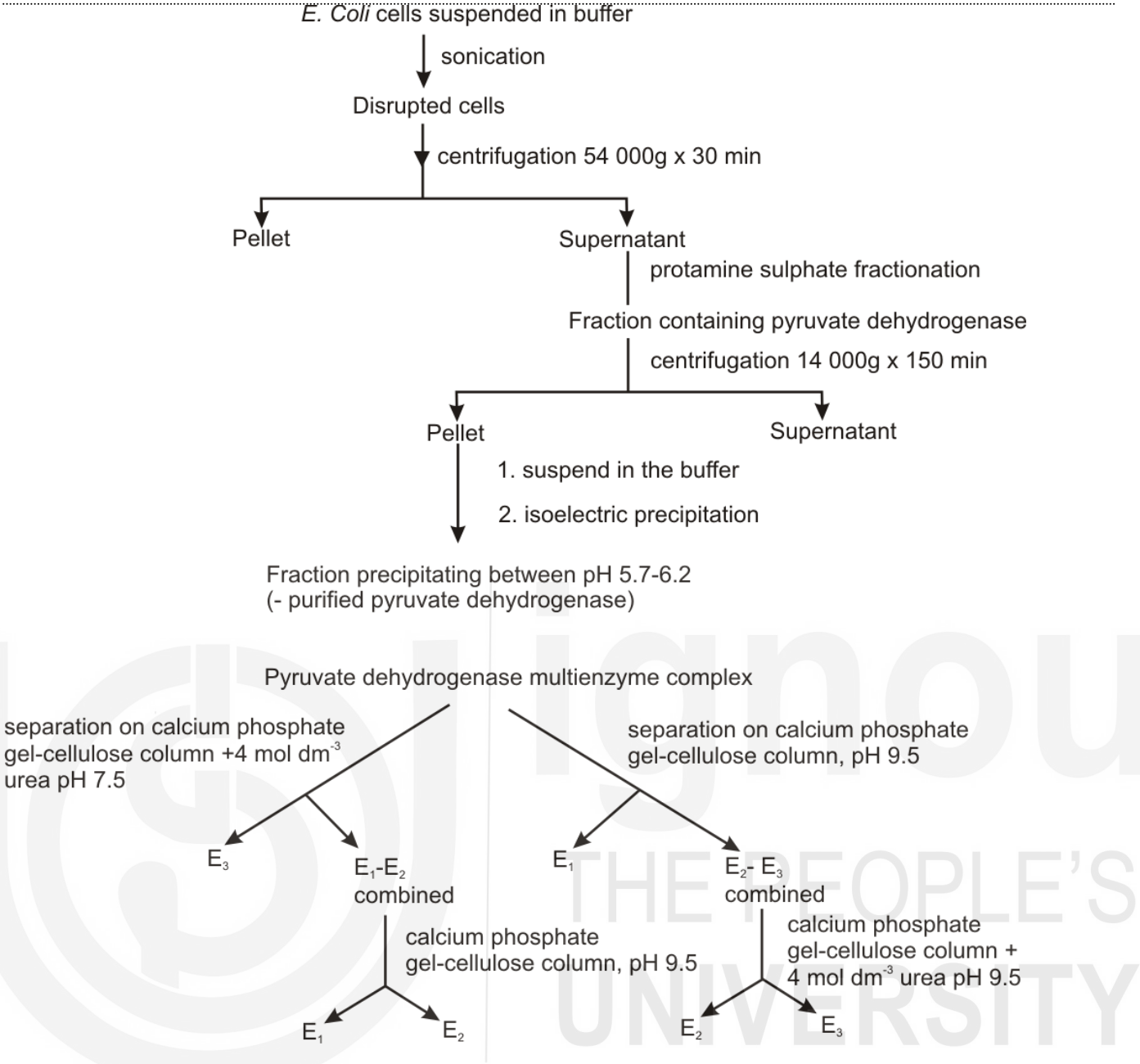


Fig. 9.3: Isolation of pyruvate dehydrogenase multienzyme complex from *E.coli* cells.

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 are 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 Multienzyme Polypeptide

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 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 1×10^6 to 2.4×10^6 and subunit structure α_6-α_8 or $\alpha_6\beta_6$. The type IA are found in animal tissues and type IB occur in fungi, algae and certain bacteria such as *Corynebacteria, Mycobacteria and Streptomyces*. Figure 9.4 displays a model proposed for the organization of domains in type I fatty acid synthase.

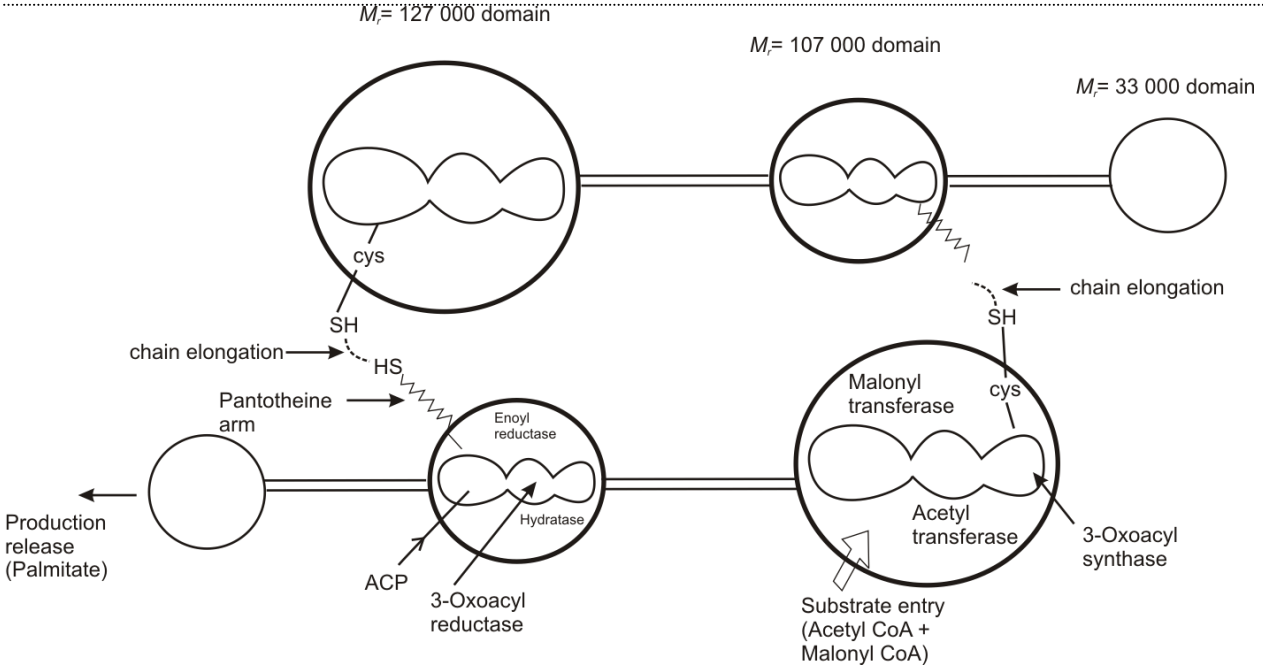


Fig. 9.4: Proposed domain structure of animal fatty acid synthase (type 1A).

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*, which 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

Do as directed:

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 SUMMARY

Let us summarize:

- Ordered association of various enzymes catalyzing successive steps in a reaction sequence are called as multienzyme complex. Several examples of these complexes include pyruvate dehydrogenase complex, glycine decarboxylase, tryptophan synthase etc.
- 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. Instances of coordinate activation of whole multienzyme protein have also been observed.
- However, 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.
- 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.
- Pyruvate dehydrogenase was the first enzyme to be purified. The complex catalyzes an important step regulating the flow of acetyl groups into the tricarboxylic acid (TCA) cycle. It has three multienzyme complexes, branched chain oxoacid dehydrogenase, 2- oxoglutarate dehydrogenase and pyruvate dehydrogenase that display common properties, as they employ the same five cofactors, demonstrate

structural and mechanical similarities and have three catalytic centres catalyzing a dehydrogenation, a decarboxylation and a transacylation.

- Fatty acyl synthase multienzyme polypeptide in the organisms has the ability to synthesize long- chain fatty acids. 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.

9.6 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. Discuss the structure and function of fatty acyl synthase multienzyme polypeptide.

9.7 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.

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 9.4.2

9.8 FURTHER 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.