LECTURE NOTES ON BCH 409: ADVANCED ENZYMOLOGY (3 UNITS)

COURSE OUTLINE:

Steady state enzyme kinetics.
Transient kinetic methods.
Chemistry of enzyme catalysis
Regulatory enzymes
Multienzyme complexes
Enzyme assays
Criteria for determining purity of enzymes
Regulation of enzyme activity and synthesis
(Pre-requisite-BCH 304)
REFER TO THE COLNAS INFORMATION HANDBOOK

NOTES:

INTRODUCTION

Enzymes and Life Processes

The living cell is the site of tremendous biochemical activity called metabolism. This is the process of chemical and physical change which goes on continually in the living organism. Build-up of new tissue, replacement of old tissue, conversion of food to energy, disposal of waste materials, reproduction - all the activities that we characterize as "life."

This building up and tearing down takes place in the face of an apparent paradox. The greatest majority of these biochemical reactions do not take place spontaneously. The phenomenon of catalysis makes possible biochemical reactions necessary for all life processes. Catalysis is defined as the acceleration of a chemical reaction by some substance which itself undergoes no permanent chemical change. The catalysts of biochemical reactions are enzymes and are responsible for bringing about almost all of the chemical reactions in living organisms. Without enzymes, these reactions take place at a rate far too slow for the pace of metabolism.

The oxidation of a fatty acid to carbon dioxide and water is not a gentle process in a test tube - extremes of pH, high temperatures and corrosive chemicals are required. Yet in the body, such a reaction takes place smoothly and rapidly within a narrow range of pH and temperature. In the laboratory, the average protein must be boiled for about 24 hours in a 20% HCl solution to achieve a complete breakdown. In the body, the breakdown takes place in four hours or less under conditions of mild physiological temperature and pH.

It is through attempts at understanding more about enzyme catalysts - what they are, what they do, and how they do it - that many advances in medicine and the life sciences have been brought about.

Enzymology is the study of enzyme and enzyme catalyzed reaction . The comprehensive study of an enzyme involves investigation of:

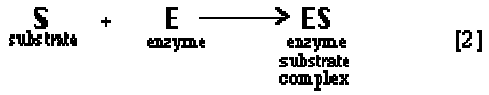
(1) Its molecular structure (i.e 1°, 2°, 3° and 4° structure).
(2) Protein properties (isoelectric point, electrophoretic mobility, pH, temperature, stability and spectroscopic properties).

(3) Enzyme property (specificity and reversibility; kinetic).
(4) Thermodynamic (activation free energy and entropies energy).
(5) Active site (Number, molecular nature of site and the mechanism of catalyzed involved).
(6) Biological properties (cellular location, isoenzymic forms and metabolic relevance of the reaction promoted).

To understand these studies, the enzyme in question has to be isolated in pure form i.e free from other enzymes or contaminants, after which it can be studied *in vitro*. The studied of purified enzyme is fundamental to biochemistry because it generates data that allow biochemist to understand and explain the cellular situation *in vivo*, which could be used as drugs or biocides or in the industrial use to promote specific chemical conversion or diagnosing diseases.

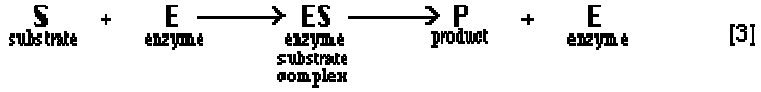
Enzyme Kinetics: The Enzyme Substrate Complex

A theory to explain the catalytic action of enzymes was proposed by the Swedish chemist Savante Arrhenius in 1888. He proposed that the substrate and enzyme formed some intermediate substance which is known as the enzyme substrate complex. The reaction can be represented as:



[2]

If this reaction is combined with the original reaction equation [1], the following results:



[3]

The existence of an intermediate enzyme-substrate complex has been demonstrated in the laboratory, for example, using catalase and a hydrogen peroxide derivative. At Yale University, Kurt G. Stern observed spectral shifts in catalase as the reaction it catalyzed proceeded. This experimental evidence indicates that the enzyme first unites in some way with the substrate and then returns to its original form after the reaction is concluded.

Enzyme kinetics is a branch of enzymology that deals with the factors affecting the rate of enzyme catalyzed reactions. The most important factors involved among others:

(1) The enzyme concentration
(2) Ligand concentration (substrate, products, inhibitors and activators).
(3) pH
(4) Ionic strength
(5) Temperature

When all these factors are analyzed properly, it is then possible to learn a great deal about the nature of enzyme catalyzed reaction. For instance, by varying the substrate and product concentration, it is possible to deduce the kinetic mechanism of the reaction i.e the order in which substrate adds and product gives up in the course of reaction. It is also possible to determine whether the order is obligate or random. Also, a study of the effects of varying pH and temperature on a kinetic constant can provide information concerning the identity of the amino acid of R-group(s) at the active site.

A kinetic analysis can lead to a model for an enzyme catalyzed reaction and conversely, the principle of the enzyme kinetics can be used to write the velocity equation for an attractive model which can be tested experimentally.

Consider for example, the simplest enzyme catalyzed reaction involving a single substrate going to single product in a process referred to as a uni-uni reaction.

i.e $E + S \rightarrow P$.....(eqn. 1)

The velocity equation for this reaction is

$V = Kp[ES]$..... (eqn. 2)

Can be derived in 2 ways

(1) The simple methods which assumes in rapid equilibrium equation wherein enzyme, substrate and enzyme substrate complex (ES) breaks down to E + P

I.e $ES \rightarrow E + P$

(2) Steady state approach: At a steady state, the concentration of ES is constant i.e the rate at which ES forms is equal to the rate at which ES decomposes.

Consider from equation 1 ;

The rate of decomposition of ES is equal to

$k_{-1}[ES] + Kp[ES\}$

Also, the rate of formation of ES is equal to

$K1[E][S]$

At the steady state, the rate of formation of ES is equal to the rate of its decomposition, therefore, at the steady state; $k1[E][S] = (K-1 + Kp)[ES]$

Substitute for [ES]

$$[ES] = \frac{k_1[E][S]}{k\text{-}1 + Kp} \quad \text{..... (EQ3)}$$

Refer to BCH 304 for the derivation of Michaelis-Menten equation (3)

$$\text{i.e } v = \frac{V_{max}[S]}{K_m + [s]}$$

This shows the mathematical relationship between initial rate and substrate concentration

Note that where $K_m = [s]$ at half maximum velocity.

K_m is the [S] at ½ V_{max}.

Enzyme Kinetics: Energy Levels

Chemists have known for almost a century that for most chemical reactions to proceed, some form of energy is needed. They have termed this quantity of energy, "the energy of activation." It is the magnitude of the activation energy which determines just how fast the reaction will proceed. It is believed that enzymes lower the activation energy for the reaction they are catalyzing. Figure 3 illustrates this concept.

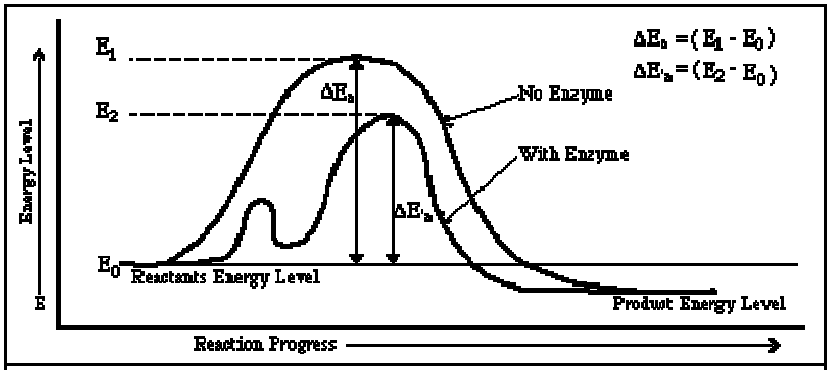


FIGURE 3
Energy of activation ΔE_a is less than $\Delta E'_a$ due to the effect of the enzyme on the substrate.

The enzyme is thought to reduce the "path" of the reaction. This shortened path would require less energy for each molecule of substrate converted to product. Given a total amount of available energy, more molecules of substrate would be converted when the enzyme is present (the shortened "path") than when it is absent. Hence, the reaction is said to go faster in a given period of time.

For most enzyme that obeys Michaelis-Menten equation / expression, the initial velocity rate varies hyperbolically with the substrate concentration and could be illustrated graphically as shown below :

However, for regularatory/ allosteric enzyme , the curve is sigmoidal in nature as shown below:

Note that one of the limitations of Michaelis-Menten equation is the difficulty in estimating V_{max} value accurately. Therefore, the best thing to do is to transform this to reciprocal plot of Line- Weaver Burk plot i.e

$$V\square = \frac{V_{max}[S]}{K_m + [S]}$$

Taking the reciprocal of both side of the equation, we have

$$1/v = \frac{K_m + [S]}{} \qquad 1/v = \frac{K_m}{} + \frac{[S]}{}$$

$$\frac{V_{max}[S]}{}$$

$$1/v = \frac{K_m}{V_{max}[S]} + \frac{1}{V_{max}} \qquad \frac{V_{max}}{V_{max}[S]} + \frac{1}{V_{max}}$$

Alternative plots known as Hanes equation could be derived from Lineweaver Burk equation:

$$1/v = \frac{K_m}{V_{max}[S]} + \frac{1}{V_{max}}$$

Multiply both sides by [S]

$$\frac{[S]}{V} = \frac{K_m}{V_{max}} + \frac{[S]}{V_{max}}$$

Rearrange this, we have

$$\frac{[S]}{v} = \frac{1}{V_{max}}[S] + \frac{K_m}{V_{max}}$$

$$y = mx + c$$

We also have Eadie Hofstee plot, which could be derived by multiplying both sides of Lineweaver –Burk plot with vV_{max}

$$1/v_O = \frac{K_m}{V_{max}[S]} + \frac{1}{V_{max}}$$

$$\frac{vV_{max}}{v} = \frac{K_m v V_{max}}{V_{max}[S]} + \frac{vV_{max}}{V_{max}}$$

$$V_{max} = \frac{vK_m}{[S]} + v$$

$$V = V_{max} - \frac{K_m v}{[S]}$$

$$V = -K_m \frac{v}{[S]} + V_{max}$$

$$y = mx + c$$

ENZYME INHIBITION:

There are broadly two types of inhibitors namely

(1) Reversible inhibitor

(2) Irreversible inhibitor

Reversible inhibitior bind with non covalent bond while irreversible inhibitors bind with covalent bond.

Reversible inhibitors are of three types namely

(1) Competitive inhibitor : Binds at the active site

(2) Non competitive inhibitor : Binds at other site
(3) Uncompetitive inhibitor : Binds to ES complex only

Kinetically, these inhibitors can be distinguished by measuring the rate of catalysis at different concentration of substrate and inhibitor is as shown below:

The slope of the graph in the presence of co

mpetitive inhibitor increase by (1 + [I])

Ki

K_i = inhibitor constant

NON COMPETITIVE INHIBITOR

The Km is not affected but the V_{max} increa

ses in the presence of this inhibitor by a fact

or of (1 + [i])
K_i

UNCOMPETITIVE INHIBITOR — Different K_m and V_{max}

MULTISUBSTRATE ENZYME AND KINETIC MECHANISM

In multisubstrate enzyme kinetic mechanism, the km for a particular substrate at one fixed set of co-substrate concentration may not be the real Km but instead an apparent value that changes as the co-substrate concentration varies. Similarly, the observed V_{max} at a saturating concentration of one substrate may not be the same V_{max} observed when another substrate is saturating. The true K_m for a particular substrate is the one that is observed when all other are present at saturating concentration.

Multisubstrate enzyme system may proceed by a number of kinetic mechanisms namely:

(1) **Rapid Equilibrium Random Bi Bi** : In these case, two (2) substrate A and B might add randomly to the enzyme exactly as substrate S and inhibitor I does in classical non-competitive inhibition system and the product P and Q might leave randomly; thus

$E + A \rightleftharpoons EA$

$+$

B

$EB + A \rightarrow EAB \rightleftharpoons$

Substrate A binds to free enzyme with a dissociation constant Ka while B binds to free enzyme with dissociation constant Kb. The binding of one substrate may alter the affinity of the enzyme for the other. Thus, A binds to EB with dissociation constant αKa , conversely the binding of EA to B gives αKb . The only different between the rapid equilibrium BiBi reacting system and non competitive inhibitor is that EAB is catalytically active while ESI is not. If the rate limiting step is the slow conversion of EAB to EPQ, we can derive the velocity equation for the forward reaction in the absence of P and q in usual manner i.e

$$V = Kp[EAB] \text{ and } \frac{v}{[E]} = \frac{kp\,[EAB]}{[E]+[EA]+[EB]+[EAB]}$$

If we express the concentration of each enzyme species in term of free E, we will obtain :

$$\frac{V}{Vmax} = \frac{[A][B]}{\alpha K\, Kb}$$

$$1+ \frac{[A]}{Ka} + \frac{[B]}{Kb} + \frac{[A][B]}{\alpha KaKb}$$

Where $Vmax = Kp[E]$

(2) ORDERED Bi Bi REACTION: In this case, it may be impossible for B to bind until A binds and promote a conformational changes in the enzyme such that exposes the binding site. The reaction sequence is represented thus

If the conversion of EAB to EPQ is the rate limiting step, such that E, A, EA, EAB are at equilibrium, the velocity equation will give

(3) **PING PONG Bi Bi REACTION**: This is a mechanism of an enzymatic reaction in which two substrate and two products participate. The enzyme shuttles backward and front between its original and its modified form. According to this mechanism, after the binding of the first substrate by the enzyme, a product is released and the enzyme is converted to a modified form. The second substrate then binds to the modified form of the enzyme. This is followed by the release of the second products and regeneration of the original form of the enzyme .It is called ping-pong because the enzyme oscillates between two stable forms and could be represented as shown below:

Where f is the modified form of the enzyme. Typical of these is the transfer of phosphate from mgATP to the enzyme, followed by decrease of mgATP before the glucose binds and pick up the phosphate.

A= mgATP	B= GLUCOSE
EA= ENZ-mgATP	FB=ENZ-PO_4glucose
FP= ENZ-PO_4-MGADP	EQ= ENZ-GLU-6-PO_4
P= mgADP	Q= GLU-6-PO_4
E= HEXOKINASE/GLUCOKINASE	

CHEMISTRY OF ENZYME CATALYSIS

The folding of macromolecules, binding of substrate to enzyme, the interactions of cells and in fact all molecular interactions in biological systems require the interplay of at least four (4) different kind of forces or interactions namely:

(1) Electrostatic bond/ interaction
(2) Hydrogen bond
(3) Vander Waals force
(4) Hydrogen bonding

These four fundamental non covalent bonds differ in their geometric requirement, strength and specificity. More so, they are affected in different ways by the presence of water molecules.

ELECTROSTATIC INTERACTION: A charged group on a substance can interact with oppositely charged group on an enzyme and the force of such electrostatic interaction is given by coulombs law, which states that:

$$F = \frac{q_1 q_2}{r^2 D}$$

wherein $q_1 q_2$ are the charges of the 2 groups

r = distance between them

D = dielectric constant of the medium

Note that electrostatic interaction is strongest in a vaccum (where D=1) and weakest in medium such as (where D=80).

A typical example is the binding of glycyl-l-tyrosine carboxypeptidase A, a proteolytic enzyme that cleaves carboxyl terminal residue. In this case, the negatively charged terminal carboxyl group of the dipeptide substrate interacts with the positively charged guanidinium group of a arginine residue.

This type of interaction is also called ionic bond, salt bridge or salt linkage or ion pair. Also, the imidazole group of histidine and positively charged group of lysine are also potential binding site for negatively substrate.

HYDROGEN BONDING: Many substrates are uncharged, yet they bind to enzyme with affinity and specificity. The significant interactions for this substrate and indeed for most charged substrate are H-bonding. The atom to which hydrogen is more tightly linked is called hydrogen donor whereas the atom is the hydrogen acceptor. The acceptor has a partial negatively charge that attracts the hydrogen atom. For instance,

Meaning that the donor atom in H-bonding in biological system could be oxygen or nitrogen atom that has a covalently attached hydrogen atom while the acceptor is either oxygen or nitrogen. The strongest bond occur in a situation whereby the donor, hydrogen and acceptor atom are co-linear. If the acceptor is at an angle to the line joining the donor atom and hydrogen atom, the bond becomes weaker with increasing angle.

The role of hydrogen bonding interaction of substrate with enzyme could be illustrated by the binding of the uridine of the substrate to pancreatic ribonuclease (an enzyme that cleaves ribonucleic acid). Three hydrogen bonding capacity are involved

The hydrogen bond capacity of lysine, aspartate , glutamine, tyrosine, histidine, varies with pH, thus they can serve as both hydrogen bond donor or acceptor over a certain range of Ph.

VANDER WAALS INTERACTIONS: This is weaker and less specific (but not less important in biological system) than electrostatic and hydrogen bonds. The basis of VanderWaals interaction is that the distribution of an electrostatic charge around an atom charges with time and at any instant, the charge distribution is not perfectly symmetric. Thus, thin transient asymmetric in the electronic charge will alter the electronic distribution around its neighboring atom. The attraction between a pair of atom increases as they come closer until they are separated by Vander Waals constant distance. At a shorter, distance, very strong repulsive forces become dominant because the outer electron cloud overlaps.

HYDROPHOBIC INTERACTION: It is another major driving force in the folding of macromolecules and it exist when substrate bind to an enzyme or when other macromolecules interact in biological system. It is more prominent when non-polar molecule or group cluster together in an aqueous medium. The presence of water diminishes the strength of electrostatic and hydrogen bonding capacity between molecules or ionic concern. However, the binding of substrate to the active site of an enzyme tends to exclude water from it thereby strengthen the interaction between the enzyme and the substrate .

MECHANISM OF ENZYME ACTION

In this section, we would consider the mechanism of carboxypeptidase A, lysozyme, and chymotrypsin.

CARBOXYPEPTIDASE A : This is a proteolytic enzyme that hydrolyses the carboxyl terminal peptide bond in polypeptide hydrolysis occur most readily

if the carboxyl terminal residue has an aromatic or bulky aliphatic side chain.

Carboxypeptidase A is a single polypeptide chain of 307 amino acids residues. It has a compact shape containing region of α-helix of about 28% and a beta-helical portion of about 17% (beta-pleated sheet). It has a tightly bond zinc ion which is essential for enzymatic activity. The zinc ion is located in a groove near the surface of the molecule where it is co-ordinate to the tetrahedral side chain and a water molecule. There is always a large pocket where the zinc ion accommodated the side chain of the terminal residue of the peptide substrate. The mechanism of action of this enzyme involves 2 aspects:

(1) Induced fit
(2) Electronic strain

The binding of substrate e.g glycyl-tyrosine induces large structural changes at the active site of carboxylpeptidase A such that its binding to the active site could be described on the basis of 5 interactions :

(1) The positively charged terminal carboxylase of glycyl-tyrosyine interact electrostatically with the positively charge side chain of Arg 145.
(2) The tyrosine side chain of the substrate tends to binds with the non polar or pocket region of the enzyme.
(3) The NH hydrogen of the peptide bond to be cleaved id hydrogen bonded to the hydroxyl group of the aromatic side chain of tyrosine-248.

(4) The carboxyl-oxygen of the peptide bond to be cleaved is co-ordinated to the zinc ion.
(5) The terminal amino acid of substrate is hydrogen bonded through, thus the mechanism of catalytic action of this enzyme as based on x-ray crystallographic study is explained thus

In this mechanism, the OH group on tyr 248 donates a proton to the NH of the peptide bond to be cleaved, the c=o carbon atom of this peptide is attacked by the carboxylate group of glu-270. The resulting anhydride of glu-270 and the acid component of the substrate is hydrolysed in the subsequent step. The role of zinc is this catalytic scheme is to render the carbonyl atom more vulnerable to nucleophilic attack, thus, it does by pointing towards the susceptible carbonyl group, thus inducing a dipole which may be further enhanced by the non-polar end of the zinc ion. The proximity of the negatively charge on glu-270 also contribute to the induction of a large dipole in the c=o group, thus carboxypeptidase A induces electronic strain in its substrate to accelerate catalysis.

MECHANISM OF ACTION OF LYSOZYME

Lysozyme is often refered to as bacteriolytic enzyme i.e an enzyme that lyse or dissolves bacteria by cleaving the polysaccharide that occur in the glycopeptides component or layer of their cell wall. It is a relatively small enzyme made up of a single polypeptide chain of 129 amino acid. It has a mass of approximately 14.6kDalton. the enzyme is cross-linked by 4 disulfide bridges which are between the cysteine residue at positions 6&127, 30&115, 64&80, and 76&94.

Hydrophobic interactions plays an important role in the folding of lysozyme , although the interior of the enzymeis almost entirely non polar. The hen egg white is a rich source of the lysozyme, althoughalso present in saliva and tears. It does not contain prosthetic

group. Lysozyme breaks theglycosidic bond between N-acetyl glucosamineand N-acetyl muramic acid. X-ray crystallographic studies have shown that hexa-NAG & higher oligoomers are good substrate for lysozyme while tri-NAG or di-NAG are also hydrolysed. NAG-NAG-NAG-NAG-NAG-NAG

A B C D E F

Lysozyme mechanism of action involves general acid catalysis where a proton is transferred from glu-35 which is unionized and optimally located at about 3 Armstrong from the glycosidic o ato. Promotion of formation of carbanion intermediate is stabilized by 2 factors

(a) Electronic strain

(b) Geometric factor

It has been reported that hexa-NAG fit best in to the active site cleft if sugar residue D is distorted out of its customary chair conformation in to the hair-chair form. This distortion enhances catalysis because the half chair geometry greatly enhances/ promotes carbonion ion formation.

The essential step in its catalysis is shown below:

The carbanion intermediate then reacts with OH from the solvent, thus

The glu-35 also becomes protonated and the enzyme is ready for another round of catalysis. N.B ASP-52 helps in stabilizing the carbonium ion.

CATALYTIC MECHANISM OF CHYMOTRYPSINOGEN

Chymotrypsinogen is a digestive enzyme that hydrolysis protein in the small intestine. It is secreted in an inactive form called chymotrypsinogen (zymogen form). It is activated by the action of ∏- chymotrpsinogen on it. It has 245 amino acid residues, 3 residues namely His-57, Asp-102 & Ser 195 interact greatly to enhance the catalytic power of chymotrypsin. There exists a charge relay network among the residues mentioned above. This serves as (proton shuttle)

On addition of substrate, Asp-102 & His-57 transiently bind to the product proton thus :

Further substrate hydrolysis by this enzyme involves 2 stages i.e (1) Acylation (2) deacylation

Acylation involves the formation of a tetrahedral intermediate mediated by the transfer of a proton from serine-195 to His-57, Asp-102 partially neutralizes the charge on this ring that is developed during the transition state. The proton stored by His-Asp complex of this network is then donated to the N-atom of the susceptible peptide bond. At this stage, the amine component is hydrogen bonded to His-57 whereas the acid component of the substrate is esterifies by Ser-195. Thereby, completing the acylation stage of hydrolysis.

During deacylation, the OH ion simultaneously attacks a carbonyl carbon atom of the acyl group that is attached to Ser-195, His-57,then donate a proton to the oxygen atom of Ser-195, which result in the release of the acid component of substrate. The acid component then diffuse away and the enzyme is ready for another round of catalysis.

MULTIENZYME COMPLEX/ SYSTEM

This is the structural and functional entity that is formed by the association of several different enzymes which catalyze a sequence of closely related reactions. The aggregate may contain one or more molecules of the given enzymes. In some cases, the enzyme of a sequence of a reaction may tightly bind to form such a multi-enzyme complex. Examples include

(1) Pyrvate dehydrogenase complex
(2) Pyruvate carboxylase
(3) Fatty acid synthase

Pyruvat dehydrogenase complex (P.D.C)

This en zyme catalyze the oxidative decarboxylationof pyruvate to acetylCoA. It is an organized assembly of 3 types of enzyme. The reaction catalyzed is summarized thus,

Pyruvate + CoA → Acetyl C_oA +CO_2 + NADH

It has cofactors namely, C_oA, NAD, thiamine PPO_4 and FAD.

Structural assembly of E-coli PDC studies have these properties:

ENZYME	ABBREVIATION	NO OFCHAINS	PROSTHETICGROUP	REACTION CATALYSED
PYRUVATE DH COMPONENT	A or E1	24	TPP	DECARBOXYLATION OF PYRUVATE
DIHYDROLIPOYL TRANSACETYLASE	B or E2	12	LIPOAMIDE	OXIDATION OF CARBON 2 UNITS
DIHYDROLIPOYL TRANSACETYLASE	C or E3	12	FAD	REGENERATION OF THE OXIDIZED FORM OF LIPOAMIDE

MECHANISM OF ACTION OF PYRUVATE DEHYDROGENASE COMPLEX

There are 4 steps in the conversion of pyruvate to acetyl CoA .

(1) Decarboxylation of pyruvate after its combination with TPP in a reaction catalyzed by E1 (pyruvate dehydrogenase complex)

(2) The hydroxyl ethyl ether group attached to TPP is oxidized to form an acetyl group concomitantly transferred to lipoamide.

(3) The acetyl group is transferred from acetyl lipoamide to CoA to form acetyl CoA.

(4) The oxidized form of lipoamide is regenerated to complete the reaction. NAD is the oxidant in this reaction catalyzed by E3.

CONTROL OF PYRUVATE DEHYDROGENASE COMPLEX

(1) CONTROL OF PRODUCT INHIBITION: Acetyl CoA and NADH inhibit the enzyme complex and this is reversible by AMP.

(2) FEED BACK REGULATION BY NUCLEOTIDES: The enzyme is inhibited by GTP and AMP.

(3) REGULATION BY COVALENT MODIFICATION: The enzyme complex becomes enzymatically inactive when a specific serine residue of pyruvate dehydrogenase complex is phosphorylated by ATP.

MECHANISM OF ACTION: PYRUVATE CARBOXYLASE (I.e CATALYTIC ACTIVITY).

Pyruvate carboxylase catalyses the conversion of pyruvate to oxaloacetate. It has both catalytic and allosteric properties. It contain a prosthetic group (biotin) which serves as a carrier of activated co_2. this biotin is linked to the epsilon amino group of specific lysine residue by an amide bond. The carboxylation processes occur in two (2) stages thus:

(1) Biotin-energy + ATP + $HCO_3 \rightleftharpoons CO_2$-biotin-enz + ADP + pi

(2) Co_2-biotin-enz + pyruvate ⇌ -biotin-enz + oxaloacetate

The carboxyl group in the carboxy-biotin enzyme intermediate is bounded to N-1 of biotin ring. The carboxyl group Is activated thus:

Co_2-biotin-enz + H ⇌ CO_2 + biotin-enz

The activated carboxyl group is then tranfered from the carboxyl –biotin to pyruvate to form oxaloacetate. The long flexible chain enables this prosthetic group to rotate from one active site of the enzyme (the ATP- biocarbonate site) to other (the pyruvate site).

CONTROL OF PYRUVATE CARBOXYLASE

The activity of pyruvate carboxylase is dependent largely on the presence of acetyl CoA because biotin will not be carboxylated unless acetyl coA (or a closely related acyl CoA) is bound to the enzyme. A high level of acetyl CoA signals the need for more oxaloacetate. If there is surplus of ATP , oxaloacetate will be cosumed in

gluconeogenesis. If there is deficiency of ATP, oxaloacetate will enter into TCA cycle upon condensing with acetyl CoA to form citrate.

ENZYME ASSAY TECHNIQUES

Assay simply means measurement of the enzymatic activity that is based on the determination of either the rate of formation of the product or the rate of utilization or disappearance of reactant or substrate under control conditions. Most assays are carried out at 30°C-37°C. Adequate buffering capacity is always being ensured. Apparatus used must be clean.

Analytical assays may be classified as (1) continous (2) discontinuous.

In order to standardize the report on enzyme activities, the commission of enzyme of the international union of biochemistry defined a standard unit i.e International unit (I.U) of enzyme as the amount of enzyme that catalysed the formation of 1 micro mole of product per minute under defined condition. The concentration of enzyme in an impure preparation is expressed in terms of units/ml while the specificity activity is expressed as units/mg protein.

In most cases, as the enzyme is being purified, its specific activity is expected to increase to maximum.

K_{cat}: is defined as the amount of enzyme that catalyze I mole of substrate per second.

Turnover number: is defined as the number of moles of substrate transformed per minute per second.

There exist different enzyme assay techniques, however, irrespective of the principle of the chosen method, the enzyme assay requires the use of excess substrate (zero order kinetics at least equal to $10K_m$) and an appropriate control is required. This control is in all respect the same as the test assay but lacks either the enzyme or substrate. Both the test and the control must be subjected to the same experimental condition.

TYPES OF ENZYME ASSAY TECHNIQUES

(1) VISIBLE AND UV SPECTROPHOTOMETRIC METHOD

(2) SPECTROFLUORIMETRY

(3) RADIOISOTOPE

(4) IMMUNOCHEMICAL METHOD

(5) MACRO CALORIMETRIC METHOD

(6) MANOMETRIC METHOD

(7) COUPLE ASSAY METHOD/TECHNIQUES

REFERENCES

CONTACT: Keith, W and John.W (1995) Practical Biochemistry: Principle and technique. 4th edition; Cambridge University Press, pp 209-216.

ENZYME PURIFICATION

Enzymes are purified by employing successive chemical or physical fractionation procedures**.** However, it should be noted that there is no fast and hard rule in respect of the protocol to be controlled. It is usually by a trial and error study based on a pilot study.

The utmost idea of each step is to get rid of the contaminants as possible and retain much desired enzyme. The efficiency of each step is given by

(1) The yield or recovery (i.e percentage of the total enzyme activity originally present that is retained.

(2) The purification factor (the factor by which the specific activity of the preparation has increased).

The preparation of the cell free extract may be prepared by several means depending on the nature of the starting material (tissue, or cell, or organism) and the size of the preparation. For instance, cell breakage method may be by autolysis freeze thaw, mechanical grinding and holistic homogenization e.t.c. the resulting homogenate is usually centrifuged to remove unbroken cells and large debris. At times, differential centrifugation for mitochondria, chloroplast, microsome, ribosome, or nucleic acids could be carried out.

The purity of the final preparation should be checked by several methods before one can conclude if the final preparation is homogenous. Some of the criteria are

(1) Homogenous enzyme preparation should elute from an ion-exchange or gel filtration column as a single symmetrical activity and protein peak with a constant specific activity throughout.
(2) Probably give a single bond (if not a dimer or polymer without artifacts or background noise).

REGULATION OF ENZYME ACTIVITY

In every metabolic pathway, the activity of at least one enzyme is subject to regulation so that the flux of material through the pathway can be controlled.

There are at least 4 means of control namely:

(1) Allosteric control
(2) Feedback inhibition
(3) Control by reversible covalent modification
(4) Control by irreversible covalent modification

ALLOSTERIC CONTROL: Some enzymes are reversibly inhibited or activated by the presence of metabolites that are not their substrate or product. These metabolites, if inhibitory are normally distant products of the pathway, thereby providing negative feedback for the activity of the pathway.

The enzymes controlled in this way usually have additional binding site other than the active or substrate binding site. The binding of inhibitor or activators at distant site from active site often brought about conformational changes in the enzyme molecule which may decrease or increase its catalytic activity. Thus, allosteric enzymes usually compose of subunits and use to have multiple interacting active centres and often shows sigmoidal graph of initial rate versus [S] and therefore do not obey Michaelis Menten kinetics.

FEEDBACK INHIBITION: This is a means by which biosynthetic pathways are regulated and involves the process whereby and products or near end products control the metabolic flux by inhibiting one or more of the enzyme at the early part of the pathway. Often maximum feedback inhibition is attained only by the combined actions of multiple end products.

Feedback inhibition could be (1) sequential (2) concerted (3)cumulative (4) co-operative

ILLUSTRATION

CONTROL BY REVERSIBLE COVALENT MODIFICATION:

This regulation is often the response to a signal coming from outside the cells such as response to hormone. In this case, the enzyme is itself the substrate of other enzymes. One of these modifies the enzyme making it active while the other reverses the modification thereby is inactive. A typical example is the control of glycogen phosphorylase through phosphorylation and dephosphorylation of specific serine residue. The enzyme exist in two forms i.e glycogen phosphorylase a (active) and glycogen phosphorylase b (inactive)

CONTROL BY IRREVERSIBLE COVALENT MODIFICATION

The best known example of the enzyme that exhibit /undergo this mechanism or modification is a protease enzyme named chymotrypsinogen. The enzyme, when reacted with diisopropyl phosphofluoridate becomes inactivated because of the reaction of serine-195 with chemical coupled with the formation of covalent linkage and elimination of HF. Also, activation of zymogen is another example of this type of modification. For instance, activation of trypsinogen by enteropeptidase will result in formation of trypsin, which also acts on chymotrypsinogen and converts its to chymotrypsin.

ILLUSTRATION