Indira Gandhi National
Open University
School of Sciences

BBCCT-107
ENZYMES

Block

2
ENZYME KINETICS

Block 2 : Enzyme Knetics

How enzyme catalytic activity is controlled? Enzyme kinetics deals with the measurement of rate of reaction. Our understanding about the *kinetics* of an *enzyme* will help to understand the functional information about its catalytic mechanism, role of enzyme in metabolism and the factors that impact its activity as well as the mechanisms of inhibition.

This block on enzyme kinetics studies—among many things will help the learner to determine how the rate of enzyme-catalyzed reactions depends on the concentration of the compounds directly interacting with the enzyme and what is the highest rate achievable by the enzyme? Further questions of interest include the catalytic mechanism in presence of two or more substrates. How the rate of the catalyzed reaction is affected in presence of different types of inhibitor? All related studies provide pieces of information that serve as input data to establish a mechanistic model of the enzymatic reaction.

Unit 4 of the block discuss the basis of the derivation of Michelis-Menten equation for mono substrate enzyme catalyzed reaction. Since most of the biochemical reactions involve two or more substrates, Unit 5 dwells on bisubstrate enzyme catalyzed reactions. Enzyme activity needs to be firmly regulated to guarantee that levels of the product do not increase to undesired levels. This is accomplished by enzyme inhibition. Unit 6 of the block discusses different types of enzyme inhibition-reversible and irreversible.

Objectives:

After studying this block, you should be able to:

- determine the rate of enzyme catalyzed reaction,
- illustrate bisubstrate enzyme catalyzed reactions,
- explain the mechanism of enzyme catalysis,
- describe different types of inhibitors regulating enzyme activity.

UNIT 4

ENZYME KINETICS

Structure

4.1 INTRODUCTION

The oldest and the important approach to understand enzyme mechanisms is the discipline known as enzyme kinetics. Kinetics is the study of reaction rates, their quantitative measurement and a systematic study of the factors influencing the activity of enzymes. In this unit, you will gain an insight of the enzymatic mechanisms as well as role played by enzyme activity in regulating metabolic pathways. Enzymes convert substrates to products through a series of steps known as enzymatic mechanism. Therefore the effect of substrate concentration on enzyme activity is one of key concepts in enzyme kinetics. Several models have been proposed to explain the kinetics of enzyme catalyzed reactions. Classical experimental work for single enzyme catalysed reactions is Henri-Michaelis-Menten plot, Briggs Haldane equation, Lineweaver-Burk plot, etc.

Objectives

After studying this unit, you should be able to:

- derive Michaelis-Menten equation;
- explain the mechanisms of enzyme catalysis;
- draw Lineweaver-Burk plot; and
- describe K_m and V_{max}.

4.2 ENZYME KINETICS

Kinetic analysis helps to disclose the number and order of the individual steps involved in the transformation of substrates to products. In the past, data generated from the experiments of enzyme catalyzed reactions was collected and analyzed to determine the rate of a reaction. It was found out that at low concentrations of substrate, the reaction was of first-order with respect to the substrate. However, at the higher concentrations of substrate, the reaction became zero-order. Please recall from your chemistry books regarding the zero order or first order enzyme catalyzed reactions. Generally all single substrate enzyme catalyzed reactions and even multi-substrate reactions where concentrations of all but one were kept constant follows the same order. At constant enzyme concentration, graph of initial velocity [v_o] (on y-axis) against substrate [*S*] concentration (on x-axis) exhibit a hyperbolic curve (Fig. 4.1).

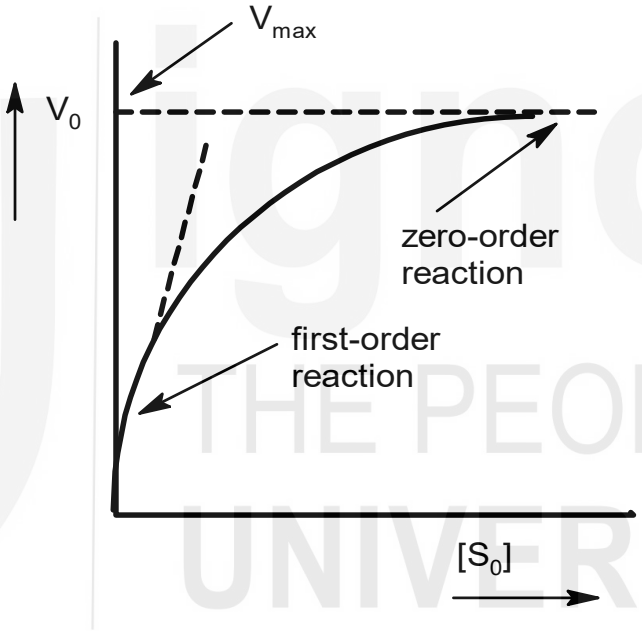


Fig.4.1: Graph of initial velocity against substrate concentration for a single substrate enzyme catalyzed reaction.

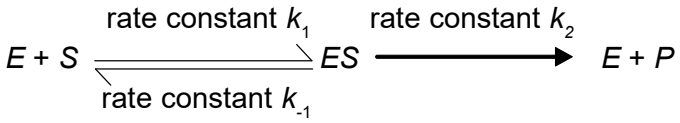
The general equation from the graph is

$$v_o = \frac{V_{max}[S_o]}{[S_o] + b}$$

V_{max} = Maximum velocity = maximum value of v_o

b = constant = value of [S_o] where v_o = ½ V_{max}

In general terms, in a mono substrate enzyme catalyzed reaction and considering just one substrate binding site per enzyme molecule, substrate [*S*] comes in physical contact with enzyme [*E*] to form an enzyme substrate complex [*ES*] complex which eventually undergoes a further reaction and leads to the formation of product [*P*]. It can be represented as:



k_1 = rate constant for the association of substrate and enzyme

k_2 = rate constant for the breakdown of enzyme and product

k_{-1} = rate constant for the dissociation of [*ES*] complex to form free enzyme and substrate

The overall rate of reaction is limited by two factors:

1) The amount of concentration of enzyme

2) The breakdown of enzyme-substrate complex

At low substrate concentrations, the overall rate of reaction is limited by the rate at which enzyme and substrate molecules react to form enzyme-substrate complex. At constant enzyme concentration the rate of reaction is proportional to the substrate concentration (first-order reaction). However, at high substrate concentration enzyme gets saturated with the substrate and therefore no free enzyme remains available. So the overall rate of reaction becomes independent of the substrate concentration. The maximum initial velocity possible is given by the expression.

$V_{max} = k_2 [E_o]$ E_o = Total enzyme concentration

SAQ 1

The rate of reaction is limited by two factors. Name the factors.

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4.2.1 Michaelis-Menten Equation

Kinetic models used to explain above mentioned findings were proposed by Michaelis and Menten (1913). The Michaelis-Menten equation demonstrates the relationship between initial reaction velocity and substrate concentration. Derivation of this equation begins with the generalized scheme of events considering a single substrate enzyme catalyzed reaction as stated earlier;

Substrate [*S*] interacts with enzyme [*E*] to form an enzyme substrate complex [*ES*] complex which eventually breaks down to free enzyme [*E*] and the formation of product [*P*]. The Michaelis and Menten set out the following scheme as given below:

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

The term k_1 denotes the rate constant for the formation of *ES* complex. *ES* complex has two fates, it can dissociate back to enzyme and substrate with rate constant k_{-1}, or proceed to form product and release the free enzyme with a rate constant k_2. In any enzyme catalyzed reaction, the concentration of substrate should be five or six orders higher than that of enzyme. The above model also assumes that $k_2 << k_1$. There is every possibility that reaction can go backward but if we consider only the initial rate of a reaction, we can ignore the backward reaction.

The overall rate of reaction is termed as initial velocity (v_0) and it will depend on two factors – the rate of formation of product (k_2) and the concentration of enzyme bound with the substrate i.e [*ES*]

So $v_o = k_2[ES]$ .....equation 1

Michaelis and Menten made two assumptions in their model. First, the availability of excess substrate [*S*] >> [*E*]. Secondly a rapid equilibrium is established between the reactants ([*E*] + [*S*]) and [*ES*] complex. Moreover, the breakdown of enzyme-substrate complex is too slow to cause any change in equilibrium. Hence Michaelis and Menten model is also known as "Rapid equilibrium model". Thus at the equilibrium state:

$$k_1[E][S] = k_{-1}[ES] \quad \text{..... equation 2}$$

$$\frac{[E][S]}{[ES]} = \frac{k_{-1}}{k_1} = K_s \quad \text{..... equation 3}$$

K_s is the dissociation constant. If E_o is the total enzyme concentration, it is equal to the sum of free enzyme [*E*] and enzyme bound to the substrate [*ES*] as represented in the following equation:

$$E_o = [E] + [ES] \quad \text{.....equation 4}$$

$$E = [E_o] - [ES]$$

Substituting the value of *E* in equation 3

$$\frac{([E_o] - [ES])[S]}{[ES]} = K_s$$

$$([E_o]-[ES])[S]=K_s[ES]$$

$$[E_o][S]-[ES][S]=K_s[ES]$$

$$[E_o][S]=K_s[ES]+[ES][S]$$

$$[E_o][S]=[ES](K_s+[S])$$

$$[ES]=\frac{[E_o][S]}{(K_s+[S])}$$

Substituting the value of [*ES*] in equation 1 we get

So $v_o = k_2 \frac{[E_o][S]}{(K_s+[S])}$ ……..equation 5

Maximum rate of enzyme reaction will be achieved when all the enzyme molecules are bound to the substrate molecules.

So $V_{max} = k_2 [E_o]$ ……..equation 6

Substituting this value in equation 5 we get

$$v_o = \frac{V_{max}[S]}{(K_s+[S])}$$

Michaelis and Menten also made the supposition that initial substrate concentration [S_o] is much higher than the initial enzyme concentration [E_o], in such a scenario the formation of enzyme-substrate complex will have no such big change in free substrate concentration. The expression for v_o will be:

$$v_o = \frac{V_{max}[S_o]}{(K_s+[S_o])}$$ …….equation 7

The above equation equation 7 is well known as Michaelis-Menten equation

Briggs Haldane modified Michaelis-Menten Plot:

The Michaelis-Menten equation is dependent on the assumption of rapid equilibrium approach in any enzyme catalyzed reaction. It limits the applicability of the equation to only rapid kinetic reactions which might not be the case with many of the other enzyme reactions. Most of enzyme catalyzed reactions assume a constant concentration of enzyme-substrate complex [*ES*].

Generally if an enzyme is mixed with high concentration of a substrate, there is an initial period expressed as pre-steady state (lasts in micro seconds) where concentration of [*ES*] slowly builds up. Eventually the concentration of [*ES*] builds up and attains a steady state, remains constant over time. The **steady-state concept** was introduced by Briggs and Haldane in 1925, a modified Michaelis-Menten equation. This concept was considered more valid assumption than the earlier ones. The Michaelis-Menten equation gave

importance to the formation of [*ES*] while Briggs-Haldane method focuses on the consistency of [*ES*] complex, its maintenance at constant concentration and breakdown to products.

Considering the single substrate enzyme catalyzed reaction one more time. In this reaction at steady state rate of formation of [*ES*] will be equal to the rate of its decomposition to products. Therefore

$$k_1[E][S] = k_{-1}[ES] + k_2[ES] \qquad \text{.....equation 8}$$

Separating the constant from variables:

$$\frac{[E][S]}{[ES]} = \frac{k_{-1} + k_2}{k_1} = K_m \qquad \text{.....equation 9}$$

Where K_m = Michaelis constant

Substituting K_s with K_m in the above equations (4-7)

$$E = [E_o] - [ES]$$

Substituting the value of *E* in equation 9

$$\frac{([E_o] - [ES])[S]}{[ES]} = K_m$$

$$([E_o] - [ES])[S] = K_m[ES]$$

$$[E_o][S] - [ES][S] = K_m[ES]$$

$$[E_o][S] = K_m[ES] + [ES][S]$$

$$[E_o][S] = [ES](K_m + [S])$$

$$[ES] = \frac{[E_o][S]}{(K_m + [S])}$$

Substituting the value of [*ES*] in equation 1 we get

So $v_o = k_2 \frac{[E_o][S]}{(K_m + [S])}$

Since $V_{max} = k_2 [E_o]$

Substituting this value in above equation

$$v_o = \frac{V_{max}[S]}{(K_m + [S])}$$

Since substrate concentration is much higher than the enzyme concentration [*S*]~ [S_o] so

$$v_o = \frac{V_{max}[S_o]}{(K_m + [S_o])} \qquad \text{.....equation 10}$$

The above equation equation 10 is similar to well known Michaelis-Menten equation. The only change is the K_m instead of K_s in the denominator. So the equation retains its previous name of Michaelis-Menten equation and constant K_m is known as Michaelis-Menten constant.

A graph of v_o at y-axis vs substrate concentration [*S*] at x-axis will be in the form of a hyperbolic curve (Fig. 4.2).

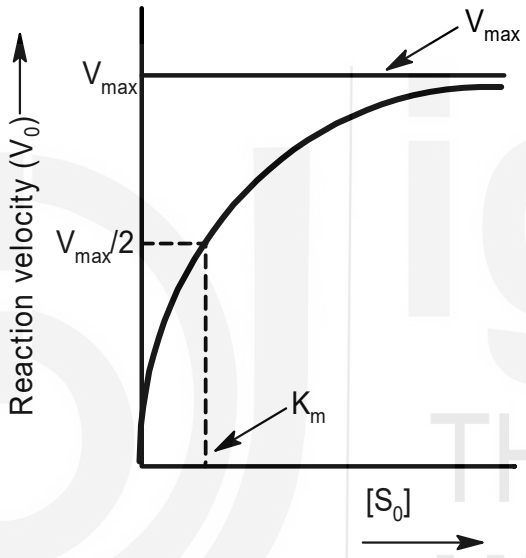

Fig. 4.2: Michaelis-Menten plot of a single substrate catalyzed enzyme reaction

SAQ 2

Explain how the rate of an enzyme-catalyzed reaction reaches a maximum value at high substrate concentration in a Michaelis-Menten equation?

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4.2.2 Lineweaver Burk Plot

If you look at the Michaelis-Menten plot, you will observe that v_o approaches V_{max} in a tangential manner at higher substrate concentrations. So if you want to determine V_{max} and K_m from the plot, it will be difficult and unsatisfactory. A hyperbolic curve nature of the graph makes it difficult to determine the accurate

value of V_{max} and K_m. Therefore, to overcome this difficulty, Lineweaver and Burk (1934) suggested a straight line graph for enzyme catalyzed reactions obeying Michaelis-Menten equation. They did not made any new assumptions and derive the Lineweaver-Burk plot, which is also known as double reciprocal plot. Lineweaver and Burk took the Michaelis-Menten equation and inverted it.

$$v_o = \frac{V_{max}[S_o]}{(K_m + [S_o])}$$

$$\frac{1}{v_o} = \frac{(K_m + [S_o])}{V_{max}[S_o]}$$

$$\frac{1}{v_o} = \frac{[S_o]}{V_{max}[S_o]} + \frac{K_m}{V_{max}[S_o]}$$

$$\frac{1}{v_o} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}[S_o]}$$

This equation is known as Lineweaver-Burk equation. It is in the form of

y = mx + c ..... equation 11

which is the equation of a straight line graph. Plot of $1/v$ against $1/S_o$ is linear and obeys Michaelis-Menten equation (Fig. 4.3). It is known as Lineweaver-Burk plot or double reciprocal plot.

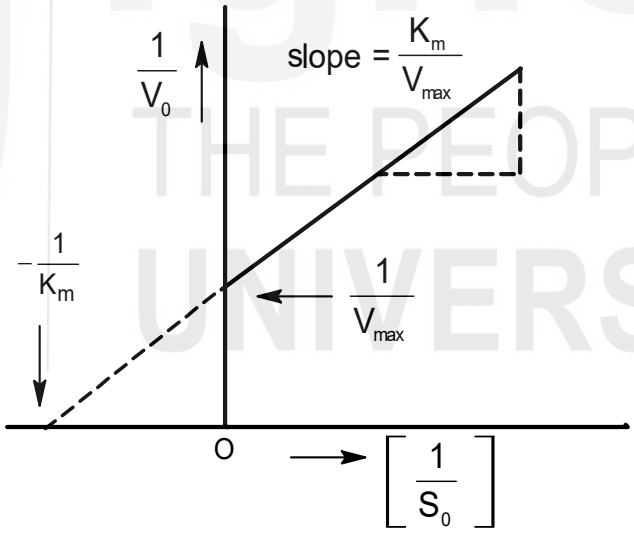

Fig. 4.3: Double reciprocal plot of the Michaelis-Menten equation.

4.2.3 Significance of K_m and V_{max}

K_m is also called as Michaelis constant. It is expressed as the substrate concentration at which velocity is half of V_{max} (maximal velocity) (Fig. 4.2). Here you must note that K_m has the units of concentration but it is independent of the enzyme and substrate concentration. As you know K_m is equal to the substrate concentration at ½ V_{max}. And since at V_{max} all the enzyme molecules are bound with substrate to form *ES* complex, thus the substrate concentration (K_m) required to convert half of the enzyme molecules into *ES* complex signifies the affinity of the enzyme for the substrate. When K_m value is small, it signifies that enzyme has very high affinity for that substrate i.e. low concentration of

substrate is needed to saturate the enzyme. Similarly, a large value of K_m indicates a relatively high concentration of substrate required to saturate the enzyme, thus signifying a low affinity of the enzyme for substrate. This is why K_m is also known as **affinity constant**.

V_{max}, the maximal rate represents the rate at which number of substrate molecules is being converted into product by an enzyme molecule in a unit time when the enzyme is fully saturated with substrate.

SAQ 3

Do as Directed

a) Michaelis-Menten equation relates the rate of an enzyme-catalyzed reaction to substrate concentration/product concentration. (Choose one option)

b) A hyperbolic curve gives an accurate value of V_{max} and K_m (True/False)

c) Lineweaver-Burk plot is linear/hyperbolic (Choose one option)

d) K_m is equal/more to the substrate concentration at ½ V_{max} (Choose one option)

4.2.4 k_{cat} and Turnover number

To determine the enzyme efficiency in enzyme kinetics, we are interested to determine how many maximum molecules of substrate can be converted into product per catalytic site of a given concentration of enzyme per unit time.

$$k_{cat} = V_{max}/E_t$$

where

k_{cat} = Turnover number,

V_{max} = Maximum rate of reaction when enzyme catalytic site is saturated with substrate E_t =Total enzyme concentration or concentration of total enzyme catalytic sites.

The k_{cat} is a direct measure of catalytic production of product under optimal conditions. The units of **Turn over number (k_{cat})** = (moles of product/sec)/ (moles of enzyme) or sec^{-1}.

Metalloenzyme carbonic anhydrase catalyzes interconversion of carbon dioxide and water to form bicarbonate ions and protons. A turnover number of 400,000 to 600,000 s^{-1} of carbonic anhydrase enzyme suggests that each enzyme molecule can produce up to 600,000 molecules of product (bicarbonate ions) per second.

SAQ 4

Do as Directed:

a) K_m is independent of the enzyme and substrate concentration (True/False).

b) What are the units of turnover number?

c) K_{cat} is known as turnover number. (True/False)

d) Each enzyme generally catalyzes ..... reaction. (Specific/Any).

4.3 SUMMARY

1) Enzyme kinetics is the basis for enzyme catalyzed biochemical reactions. Therefore, understanding of enzyme kinetics is important to understand the biological processes as well as several enzyme assays being carried out.

2) Kinetics provides a rationale for the complex behavior of enzymes. The rational is based on simple chemical principles.

3) For single substrate reactions, at constant *E*, increasing *S* results in increased product formation to a point where product formation no longer increases. This saturation is presumed to reflect the fact that all *E* is now in the form of *ES*.

4) Kinetic model proposed by Michaelis-Menten used equilibrium assumption for deriving Michaelis-Menten equation.

5) The equilibrium assumption was later modified to introduce a more valid steady state assumption. The equation remains the same except the definition of Michaelis constant (K_m).

6) The substrate concentration at which velocity is half of V_{max} (maximal velocity) is known as Michaelis constant (K_m). This constant gives an idea about the affinity of the enzyme for its substrate.

7) The hyperbolic graph of *v* versus *S* obtained by Michaelis-Menten equation proved inadequate for determining the accurate value of V_{max} and K_m. Lineweaver and Burk plot provides the solution by inverting the Michaelis-Menten equation to obtain double reciprocal plot.

4.4 TERMINAL QUESTIONS

1) Why is the rate of an enzyme-catalyzed reaction proportional to the amount of *ES* complex?

2) Explain the maximal velocity V_{max} in the v_o vs *S* graph?

3) Derive Lineweaver-Burk equation from Michaelis-Menten equation and give its importance.

4) How a value for K_m can be obtained from the v_o vs *S* graph when v_o = 1/2 V_{max}?

4.5 ANSWERS

Self-Assessment Questions

1) The rate of reaction is determined by
   i) The amount of the concentration of enzyme.
   ii) The breakdown of enzyme-substrate complex.

2) At high substrate concentration S_o, *Km* <<<< S_o (numerically), so the term $K_m + S_o$ in the Michaelis-Menten equation becomes equal to S_o. $v_o = (V_{max} S_o)/S_o$, and S_o cancels. Therefore, at high S_o, $v_o = V_{max}$.

3) a) Substrate concentration, b) False, c) Linear, d) Equal.

4) a) True, b) sec^{-1}, c) True, d) specific.

Terminal Questions

1) The product formation takes place after *ES* complex formation in an enzyme catalyzed reaction [Refer to section 4.2].

2) At high substrate concentrations, enzyme *E* will be bound to substrate *S*. So the maximum amount of *E.S* is formed under these conditions. Since the rate is proportional to the amount of *ES*, the rate is at a maximum value under these conditions.

3) Refer to section 4.2.2

4) When $v_o = V_{max}/2$, then $V_{max}/2 = V_{max}.S/K_m + S$

   cancelling V_{max},

   $1/2 = S/(K_m + S)$

   $K_m + S = 2S$

   or $K_m = S$ at $v_o = V_{max}/2$

4.6 SUGGESTED READINGS

1) David L. Nelson and Michael M. Cox: Lehninger Principles of Biochemistry6th Ed., W.H. Freeman.

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

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

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

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

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

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UNIT 5

BISUBSTRATE REACTIONS

Structure

5.1 INTRODUCTION

In the previous unit you learnt about the enzyme kinetics of single substrate enzyme catalyzed reactions. The enzyme catalyzed reactions involving two or more substrates and yielding two or more products are more common than the single substrate reaction. Biochemical reactions with two substrates account for ~60 % of the known biochemical reactions. Therefore it is necessary to consider the kinetics of “bisubstrate reactions”.

Objectives

After studying this unit, you should be able to:

- determine nomenclature of enzyme catalyzed reactions based on substrate or products.
- distinguish two different types of bisubstrate kinetic mechanisms;
- write kinetic mechanisms for different types of bisubstrate reactions using Cleland terminology; and
- explain *sequential* and *ping-pong* kinetic mechanisms based on the patterns observed on double reciprocal (Lineweaver-Burk) plots.

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5.2 BISUBSTRATE REACTIONS

The nomenclature formulated for multisubstrate kinetics is given in the Table 5.1.

Table 5.1: Nomenclature of enzyme catalyzed reactions depending on substrates or products involved in the reaction.

S.No.	*Reaction*	*Nomenclature*
1.	$A \longrightarrow P$	Uni uni
2.	$A + B \longrightarrow P$	Bi uni
3.	$A + B \longrightarrow P_1 + P_2$	Bi bi
4.	$A + B + C \longrightarrow P$	Ter uni
5.	$A + B + C \longrightarrow P_1 + P_2$	Ter bi

The derivation of velocity equations for multisubstrate reactions is difficult as compared to single substrate reactions. Kinetic equations for non-rapid multisubstrate systems can be derived algebraically with the aid of steady state assumptions. However, as the number of substrates increases the algebraic manipulations becomes more complicated. In bisubstrate systems, isomerization of the central complex and subsequent product release steps may be so rapid that (free enzyme) E, EA (enzyme-substrate A), EAB (enzyme-substrate A and substrate B complex), EPQ (enzyme - product P- product Q complex) and EQ (enzyme-product Q) may never attain equilibrium. The kinetic analysis may depend on the distribution of enzyme in different states as per the rate constants of all the steps along with the release of products.

Considering the complexity of reactions, we will begin with two substrate two product reactions taking reactions in a series of steps for example serine proteases such as chymotrypsin that catalyses the reaction involving two substrates and yield two products. In the first step, peptide substrate is hydrolyzed to form two peptide fragment molecules and in the second step, the water molecule as substrate indirectly supplies the proton and hydroxyl groups required to complete the hydrolysis. Similarly ATP dependent kinases catalyses the phosphorylation of protein utilizing ATP as energy molecule and yielding ADP and phosphoprotein as two product molecules.

Bisubstrate reactions involving two substrate two product (bi-bi) reactions are generally transfer reactions (including oxidation/reduction reactions) and are represented as:

$$AX + B \rightleftharpoons BX + A$$

Such type of reactions are of two types viz., sequential and non sequential.

5.2.1 Sequential Reactions

In these types of reactions, both the substrates (reactants) bind to the enzyme before the product is released. If you observe carefully, you will notice that even in sequential reactions, there could be two ways of reaction mechanisms. It may be ordered or random.

Ordered Mechanism: In this type of enzyme mechanism, substrate molecules bind to the enzyme thereby forming a ternary complex and then the products are released in a defined ordered sequential manner. In other words the order of binding and leaving the enzyme is compulsory. The order is well précise and well specified. So this mechanism is also known as Compulsory order mechanism. Assuming two substrates A and B binds to the enzyme E, the mechanism is represented as:

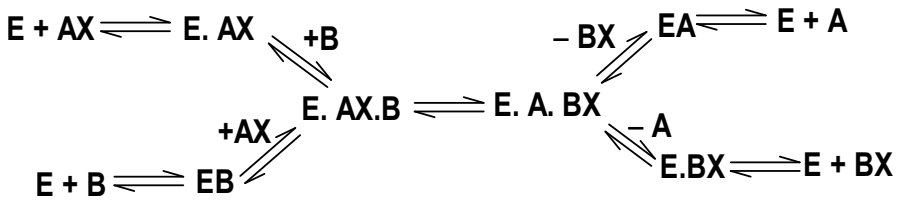
$$E + AX \rightleftharpoons E.AX \overset{+B}{\rightleftharpoons} E.AX.B \rightleftharpoons E.A.BX \overset{-BX}{\rightleftharpoons} EA \rightleftharpoons E + A$$

$$E + B \rightleftharpoons EB \overset{+AX}{\rightleftharpoons} E.B.AX \rightleftharpoons E.BX.A \overset{-A}{\rightleftharpoons} E.BX \rightleftharpoons E + BX$$

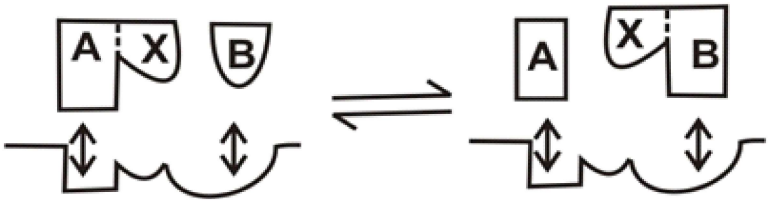
The diagrammatical summary of the sequences of reactions is also represented in the form of Cleland plot in which the enzyme is represented as a horizontal line and the arrows are used to represent the arrival and departure of substrates and products.

Some of the well known examples include enzymes such as citrate synthase, lactate dehydrogenase and aspartate transcarbamoyalase (ATCase). ATP sensitive K channels and H, K-pumps are also some of the other examples of compulsory order sequential reactions.

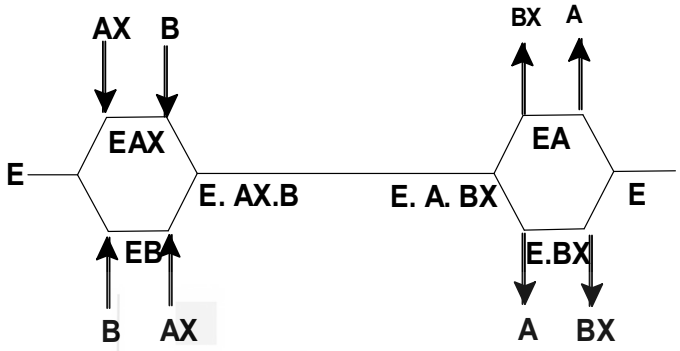
Random Sequential Mechanism: In this type of bisubstrate reactions, there is no specified order. Any substrate can bind first to the enzyme and any product may be released first. The formation of ternary complex (enzyme with two substrates) in this type of mechanism is similar to the ordered mechanism. The reaction mechanism is represented as:



In this type of reaction mechanism, there are two separates binding sites on the enzyme, one for A/AX and other for B/BX.



The difference between two types of sequential mechanism – Ordered and Random lies in the fact that enzyme may follow ordered sequential pathway if first substrate causes a conformational change in the enzyme. However, in case of random order mechanism there will be separate binding sites on the enzyme, one for one substrate and another site for another substrate. Random sequential mechanism is typical in multienzyme complexes such as hemoglobin, ligand operated channels and tyrosine kinase receptors. The Cleland plot for such type of reaction mechanism is as follows:



SAQ 1

Do as Directed.

a) Chymotrypsin is an example of an enzyme that catalyzes the ..... type of reactions (single substrate/bisubstrate). (Choose one option)

b) Multisubstrate reactions are more common than the single substrate reactions. (True/False)

c) Ternary complex is formed in ..... reactions. (sequential/non-sequential)

d) Give one example of the enzyme following compulsory order sequential mechanism.

5.2.2 Non- Sequential Reactions

Ping-Pong Bi-Bi Reactions: These reactions follow non-sequential mechanism and are also known as double displacement reactions. In such reactions at least one product gets released from the enzyme before both substrates bound to the enzyme. A pictorial representation is given below:

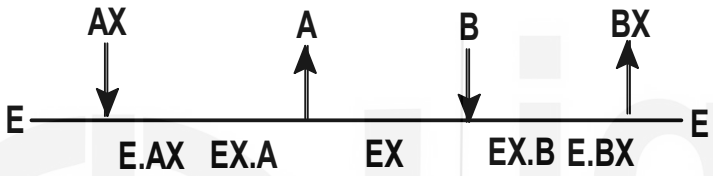
$$AX + E \rightleftharpoons E.AX \rightleftharpoons EX.A \rightleftharpoons EX + A$$

$$EX + B \rightleftharpoons EX.B \rightleftharpoons E.BX \rightleftharpoons E + BX$$

Firstly, AX binds to the enzyme E forming a binary complex. E.AX. The X represents a small group which on its own cannot participate in the reaction. The active site of the enzyme is full as this substrate (AX) gets converted to product on account of intramolecular reorganization in the active site where the bond E-X is formed and the bonding between X-A is broken. The first

product (A) is also formed which is then released from the enzyme E before second substrate (B) binds to the enzyme. The second substrate will not bind free enzyme E but will bind in its modified form E-X. In this type of reaction mechanism, enzyme has only one binding site since only one substrate binds at a time. There is no room for the second substrate. The release of first product exposes the binding site on the enzyme to the second substrate. Another phase of intermolecular reorganization takes place in the active site of enzyme where bond B-X is formed and bond E-X breaks down. The release of second product (BX) follows leaving behind the enzyme in its original form. Several enzymes such as co-transporters and pumps work as per double displacement reaction mechanism. Examples include serine proteases such as trypsin, chymotrypsin and amino transferases.

Cleland plot for ping-pong bi-bi enzyme catalyzed reaction is:



SAQ 2

What are two characteristics of an enzyme that catalyzes a reaction through the ping-pong mechanism?

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5.3 ALBERTY RATE EQUATION

Bisubstrate enzyme catalyzed reactions observe Michaelis-Menten equation when one of the two substrates is at constant concentration with respect to the other substrate. We would like to remind the learner that it will be applicable only when enzyme has one binding site per substrate or multiple binding sites per substrate provided there is no interaction between the binding sites. Alberty derived equation for such reactions is:

$$v_o = \frac{V_{max}\,[AX_o][B_o]}{K_m^B[AX_o] + K_m^{AX}[B_o] + [AX_o][B_o] + K_s^{AX}K_m^B}$$

Where V_{max} is the maximum v_o when both the substrates *AX* and *B* are saturating K^{AX}_m is concentration of substrate *AX* which gives ½ V_{max} when substrate *B* is saturating

K^B_m is concentration of substrate *B* which gives ½ V_{max} when substrate *AX* is saturating K^{AX}_s is the dissociation constant for $E + AX \longrightarrow EAX$

The total enzyme concentration is much less than the concentrations of two substrates. At saturating concentration of $[B_o]$ the Alberty equation will be

$$v_o = \frac{V_{max}[AX_o]}{K_m^{AX}+[AX_o]}$$

Similarly at the saturating concentration of $[AX_o]$ the Alberty equation will be

$$v_o = \frac{V_{max}\ [B_o]}{K_m^{B}+[B_o]}$$

From the above equations, you will observe that two substrate reactions will also obey Michaelis-Menten equation with respect to one substrate when the concentration of other substrate is fixed. So, the corresponding Lineweaver-Burk plot or double reciprocal plot will also be linear.

Several sequential bisubstrate enzyme catalyzed reactions (random order and compulsory order) will observe Alberty equation when the rate limiting step is interconversion of the ternary complex ($E.AX.B \longrightarrow E.A.BX$). If you remember, the rate limiting step in single substrate reactions is the interconversion of $ES \longrightarrow EP$.

For double displacement reactions or ping-pong bi-bi enzyme catalyzed non-sequential reactions, the release of one product from the enzyme in the initial period of reaction will be irreversible. Hence $K^{AX}_s = 0$ and $K^{AX}_s . K^B_m = 0$. The rate of reaction will be

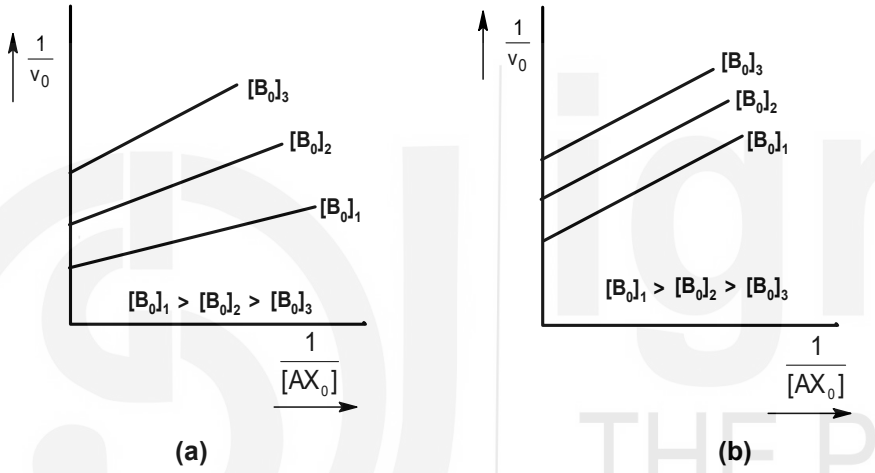
$$v_o = \frac{V_{max}\ [AX_o][B_o]}{K_m^{B}[AX_o]+ K_m^{AX}[B_o]+[AX_o][B_o]}$$

5.4 DIFFERENTIATING BI SUBSTRATE MECHANISMS

5.4.1 Primary Plots

The distinguishment of the bisubstrate enzyme catalyzed reactions can be made from the primary plots of reaction mechanisms observing Alberty rate equation. The reaction mechanisms of bisubstrate reaction obeying Alberty rate equation will give linear plots of $1/v_o$ vs $1/[AX_o]$ at constant concentration of

other substrate [B_o]. Similarly $1/v_o$ vs $1/[B_o]$ at constant concentration of other substrate [AX_o] will also be a linear plot. The slopes and intercept of these linear plots includes concentration of the fixed substrate at non-saturating concentrations. Therefore, the slope as well as intercept of these plots will change if the experiments are repeated with fixed substrate concentration at a different value. The linear plots of compulsory or random order sequential enzyme catalyzed reactions are shown in the Fig. 5.1 a. The double displacement or Ping-Pong bi bi enzyme catalyzed reaction is also shown in Fig. 5.1 b. The learner can see from the graph that the linear plot in figures is independent of the concentration of the fixed substrate. Recall from the last section, you will notice that $K^{AX}_s . K^{B}_m = 0$ in ping-pong bi-bi reaction mechanism. The linear plot of ping-pong bi bi will show several parallel lines if the experiments are repeated at the different concentrations of the fixed substrate.



Fig, 5.1: Graph of plot taken at non-saturating concentrations of both substrates of the enzyme:

a) for compulsory-order and random –order ternary complex

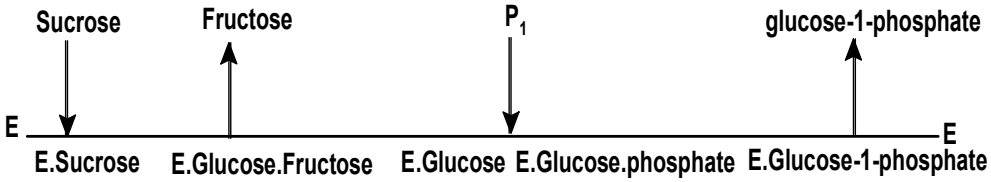
b) for ping-pong bi-bi reaction mechanisms

The limitation of these plots is that compulsory-order and random order can be distinguished from the ping pong bi bi reaction mechanism but not from each other.

5.4.2 Non-steady State Methods-Isotope Exchange

If you recall from your school books regarding radioisotopes, it is one of the finest technique which can be effectively used to determine enzyme reaction mechanism. Let us see how? The isotope exchange at chemical equilibrium helps to determine the reaction mechanism. In ping pong non sequential mechanism, one of the products is released before the joining of another substrate to the enzyme. Therefore, exchange of isotope between reactant and product in a reaction in the absence of other reactants and products suggests a ping-pong enzyme catalyzed reaction mechanism. Consider the bisubstrate reaction catalyzed by enzyme sucrose phosphorylase converting orthophosphate to glucose-1-phosphate. The other substrate in this reaction is sucrose and other product is fructose. The isotope exchange between

orthophosphate (substrate) and glucose-1-phosphate (product) in sucrose catalyzed reaction in the absence of sucrose (other substrate) and fructose (other product) suggests a ping pond mechanism. Similarly, isotope exchange between sucrose and fructose in the absence of orthophosphate and glucose-1-phosphate also suggests a ping pong bi bi enzyme catalyzed reaction mechanism.



Another useful application of isotopes is in distinguishment of compulsory order sequential mechanism from random order sequential mechanism. The perceived change in the rate of equilibrium isotope exchange on account of changes in the concentration of reactants and products without changing the concentration of reactants and products will help to determine whether the bisubstrate enzyme catalyzed reaction mechanism is compulsory or random order.

The general representation of bisubstrate reaction is:

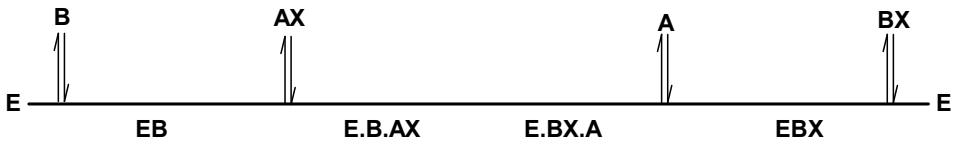
$$AX + B \rightleftharpoons BX + A$$

When the rate of forward reaction is equal to the rate of backward reaction, the equilibrium will be:

$$K_{eq} = \frac{[BX][A]}{[AX][B]}$$

The addition of a small amount of radiolabel molecule will not affect the equilibrium significantly. Therefore, if we add a small amount of radiolabel molecule *B* in the reaction and then measure the rate of formation of radiolabel *BX*, we can trace the reaction mechanism. In the next step we will increase the concentrations of *A* and *AX*, keeping their ratio [*A*] : [*AX*] constant as well as keeping the equilibrium unchanged. This will lead to change in the rate of isotope exchange between the reactants and products.

Now consider a compulsory order sequential mechanism:



In this bisubstrate reaction, substrate *B* binds first to the enzyme leading to the formation of *EB*. The second substrate *AX* joins the *EB* complex to form a ternary complex *E.B.AX*. An interchange is followed by the release of first product *A* and second product *BX* in compulsory order. A little increase in the concentration of one substrate *AX* and one product *A* will increase the isotope exchange between *B* and *BX*. The isotope exchange will increase with the increase in the concentration of *A* and *AX* keeping [*A*] : [*AX*] constant. You should see the graph on isotope exchange in Fig. 5.2. If you can recall, there is formation of ternary complex in compulsory order reactions. Therefore substantial increase in the concentrations of *A* and *AX* will make it difficult for the substrate *B* to dissociate from *E.B* and product *BX* to dissociate from *EBX*. So there will be decrease in the rate of isotope exchange between *B* and *BX*.

Let us look again at the compulsory order mechanism with the first substrate *AX* binding the enzyme *E* to form *E.AX* and then joined by *B* to form *E.AX.B*. An interchange is followed by the release of first product *BX* and second product *A* in a compulsory order. An increase in the concentrations of *AX* and *A* will lead to the free enzyme *E* forming *E.AX* and *EA* complexes. *E.AX* reacts with second substrate *B* and *EA* doesn't affect release of *BX* from *E.A.BX*. Therefore the rate of isotope exchange will increase in a hyperbolic manner on further increase in the concentrations of *A* and *AX*. So you will notice a hyperbolic graph as shown in Fig. 5.3

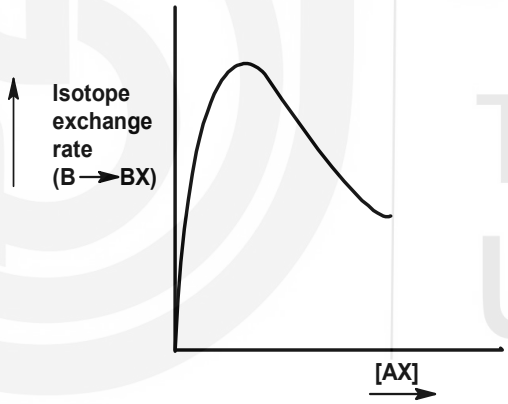


Fig. 5.2: Graph of plot for the isotope exchange from *B* → *BX* against [*AX*] in a compulsory order bisubstrate enzyme catalyzed reaction when *B* binds first.

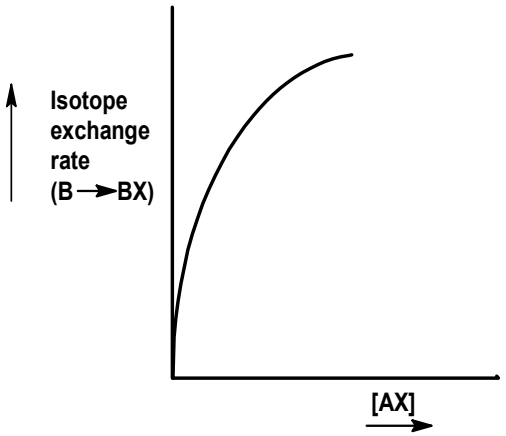


Fig. 5.3: Graph of plot for the isotope exchange from *B* → *BX* against [*AX*] in a compulsory order bisubstrate enzyme catalysed reaction when *AX* binds first.

Similar results will be obtained in Random order enzyme mechanism.

Rapid Reaction Studies:

Many biochemical processes are too fast for fitting observation using a standard spectrophotometer. Sometimes these processes can be avoided by alternative of conditions, other than for enzyme reactions that occur in 0.01 - 100 second timescales, stopped flow methods are helpful. Theorell and Chance used stopped flow techniques with a double beam detector for studying enzyme reaction of horse liver alcohol dehydrogenase. The enzyme catalyzes the formation of formaldehyde from ethanol. The entire reaction is represented as:

$$CH_3CH_2OH + NAD^+ \rightleftharpoons CH_3CHO + NADH\ (+H^+)$$

The coenzyme *NADH* shows absorbance at 350 nm and not at 328nm. So measuring the differences in the absorbance at 350nm and 328nm will help to determine the formation of *NADH* from *E.NADH* complex (*E.NADH* $\longrightarrow$ *E* + *NADH*). The rate of absorbance at 328 measures the rate of reduction of NAD^+ (*E.NAD*$^+$ $\longrightarrow$ *E.NADH*).

In this type of compulsory order reaction, Chance found out that interconversion of ternary complex is too fast and the rate limiting step of the reaction is the release of *NADH* from *E.NADH* complex.

SAQ 3

State whether the following statements are True or False

a) The rate equation of Alberty bears resemblance to the Michaelis-Menten equation (True/False).

b) In the Alberty rate equation for bisubstrate reactions, V_{max} denotes the maximum v_o when only one substrate is saturating (True/False).

c) NADH shows absorbance at 350 nm. (True/False)

d) Compulsory-order and random order reaction mechanisms can be distinguished from ping pong bi bi mechanisms but not from each other by the primary plots. (True/False)

e) Isotope exchange technique can be used for investigating reaction mechanisms. (True/False)

5.5 SUMMARY

1) Enzyme kinetics can be complex especially in case of bisubstrate reactions but can be very informative. Two substrate enzyme reactions can proceed by several different mechanisms leading to the release of two products.

2) The bisubstrate reaction mechanism can be sequential (single displacement reactions) or non-sequential (double displacement reactions or ping-pong bi-bi).

3) Single displacement reactions can also be either of two subtypes: ordered sequential or random sequential.

4) The Alberty rate equation for bisubstrate reactions obeys Michaelis-Menten equation with respect to one substrate at fixed concentration of the other.

5) The kinetic mechanism of bisubstrate reactions can be determined by steady-state methods and by non-steady state methods such as isotope exchange and rapid-reaction techniques.

5.6 TERMINAL QUESTIONS

1) What is the difference between random sequential and ordered sequential reaction mechanisms?

2) Discuss Alberty rate equation?

3) Draw primary plot for distinguishing sequential and non-sequential rate mechanisms.

4) Discuss rapid-reaction techniques for bisubstrate reactions.

5.7 ANSWERS

Self Assessment Questions

1) a) Bisubstrate, b) True, c) Sequential, d) Lactate Dehydrogenase,

2) Answers:

The characteristics of enzyme catalyzing ping-pong bi bi reaction mechanism are:

a) A product is formed before the second substrate binds to the enzyme.

b) The binding of the first substrate causes the enzyme to change into an intermediate form that will bind the second substrate.

c) The plot of $1/v$ vs. $1/[A]$ as $[B]$ changes will be parallel lines.

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

Terminal Questions

1) Refer to section 5.2.1
2) Refer to section 5.3
3) Refer to section 5.4.1
4) Refer to section 5.4.2 Rapid Reaction Studies

5.8 SUGGESTED READINGS

1) David L. Nelson and Michael M. Cox: Lehninger Principles of Biochemistry6th Ed., W.H. Freeman.
2) Robert K. Murray, Daryl K. Granner, Victor W. Rodwell Harper's Illustrated Biochemistry, 27th edition. 2006, McGraw-Hill.
3) Donald J Voetand Judith G. Voet: Principles of Biochemistry 4th ed., John Wiley and Sons, Inc, USA.
4) Eric E Conn, Paul K Stumpf: Outlines of Biochemistry, John Wiley and Sons, Inc, USA.
5) S. Shanmugan and T. Sathish Kumar: Enzyme Technology, I K International Publishing House Pvt Ltd, New Delhi.
6) Nicholas C Price and Lewis Stevens: Fundamentals of Enzymology, Oxford University Press, Oxford, New York, USA.

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UNIT 6

ENZYME INHIBITION

Structure

6.1 INTRODUCTION

In the previous unit, you have learnt about the kinetics of enzyme catalyzed reactions. In this unit we will introduce you to important class of molecules called “enzyme inhibitors”. Enzyme inhibitors are the molecules that regulate the activity of enzymes in the cell. Inhibitors alter the catalytic action of the enzyme; as a result slows down the enzyme activity, or in some cases, close the catalysis. The study of these inhibitors provides wealth of information on the working of enzymes and their mechanism. The blockage of enzyme activity can lead to several changes such as correction of metabolic imbalance or killing of a bacteria or pathogen. Therefore, many of the drug molecules are enzyme inhibitors and their discovery as well as improvement has been a major area of research for biochemistry and pharmacology.

Objectives

This unit will give you an overview about different types of enzyme inhibitors. After studying this unit, you will be able to:

- determine types of enzyme inhibitor;
- find out how inhibitor interacts with the enzyme;
- state the role of inhibitor affecting enzyme kinetic parameters;
- distinguish between reversible and irreversible inhibitors; and
- explain the role of enzyme inhibitors and their classification according to nature and function.

6.2 ENZYME INHIBITION-REVERSIBLE AND IRREVERSIBLE

Molecules that bind to the enzymes and cause a decrease in their activity are called enzyme inhibitors. These molecules either bind at the active site of enzyme thereby preventing the substrate molecule to bind to the enzyme or they may inhibit the catalytic activity of enzyme. You should know that many of these molecules perform several regulatory roles in the metabolism. Some of them are used as herbicides or pesticides. Most of the drug molecules also act as enzyme inhibitors. Natural enzymes inhibitors e.g. poison are a part of the defense mechanisms in wild life animals.

Enzyme inhibitions are mainly classified into two types:

a) Reversible Inhibition

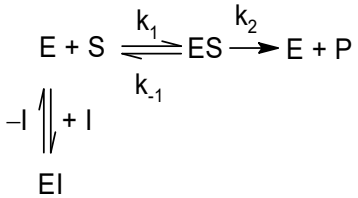
b) Irreversible Inhibition

6.3 REVERSIBLE INHIBITION

In this unit, we will discuss about the inhibition of simple single substrate enzyme catalyzed reactions. In reversible enzyme inhibition, loss of the enzyme activity due to inhibitory molecule is reversible. Enzyme activity gets restored on the removal of inhibitor. These inhibitors bind non-covalently and give rise to different kinds of inhibition. Multiple weak bonds between the inhibitor and the enzyme combine to give strong binding which prevents the formation of product. They can be easily removed by dilution or dialysis to restore full enzyme activity. Reversible inhibitors tend to form equilibrium with an enzyme leading to certain level of inhibition.

6.3.1 Competitive Inhibition

There is a direct competition between the substrate and the inhibitor for the binding site of enzyme in a competitive inhibition. If inhibitory molecule is bound to the enzyme then substrate will not be able to bind it and vice versa. In such kind of scenario, inhibition can be overcome by increasing substrate concentration in comparison to inhibitor. Therefore, the maximum velocity (V_{max}) of the reaction will remain unchanged while K_m will be decreased in a competitive inhibition. Most of the competitive inhibitors are structurally similar to the substrate molecules.



The dissociation constant (K_i) or inhibitor constant for the reaction is

$$\frac{[E][I]}{[EI]} = K_i \; or \; \frac{[E][I]}{[K_i]} = [EI] \qquad \text{..... equation 1}$$

I = Inhibitor

EI = Enzyme bound to Inhibitor

E = Free Enzyme

E_o = Total enzyme

ES = Enzyme bound to substrate

As you know in the previous unit, using steady state assumption, Michaelis-Menten constant for the rate of reaction is given by the following equation.

$$\frac{[E][S]}{[ES]} = \frac{k_{-1} + k_2}{k_1} = K_m \qquad \text{..... equation 2}$$

$$E_o = [E] + [ES] + [EI] \qquad \text{..... equation 3}$$

Substituting the value of [*EI*] as given in equation 1

$$E_o = [E] + [ES] + \frac{[E][I]}{K_i}$$

$$E_o = [E](1 + \frac{[I]}{K_i}) + [ES]$$

$$E = \frac{[E_o] - [ES]}{(1 + \frac{[I]}{K_i})}$$

Substituting the value of [*E*] in equation 2:

$$\frac{([E_o] - [ES])[S]}{(1 + [\frac{I}{K_i}])[ES]} = K_m$$

$$\frac{([E_o][S])}{[ES]} - [S] = K_m(1 + [\frac{I}{K_i}])$$

$$\frac{([E_o][S])}{[ES]} = K_m(1 + [\frac{I}{K_i}]) + [S]$$

$$\frac{([E_o][S])}{K_m(1 + [\frac{I}{K_i}]) + [S]} = [ES]$$

$$v_o = k_2[ES] \qquad \text{..... equation 4}$$

(Ref: Michaelis Menten equation in the previous Unit-4, Block-2)

Putting the value of [*ES*] in equation 4

$$v_o = \frac{k_2[E_o][S]}{K_m(1 + [\frac{I}{K_i}]) + [S]}$$

$$V_{max} = k_2 [E_0]$$ ..... equation 5

(Ref: Michaelis Menten equation in the previous Unit-4, Block-2)

$$v_o = \frac{V_{max}[S]}{K_m(1+[\frac{I}{K_i}])+[S]}$$

Since inhibitor concentration is generally of the same order of magnitude as the substrate concentration and much higher than the enzyme concentration [*I*] ~ [I_o] just as [*S*] ~ [S_o]. The above equation can be expressed as

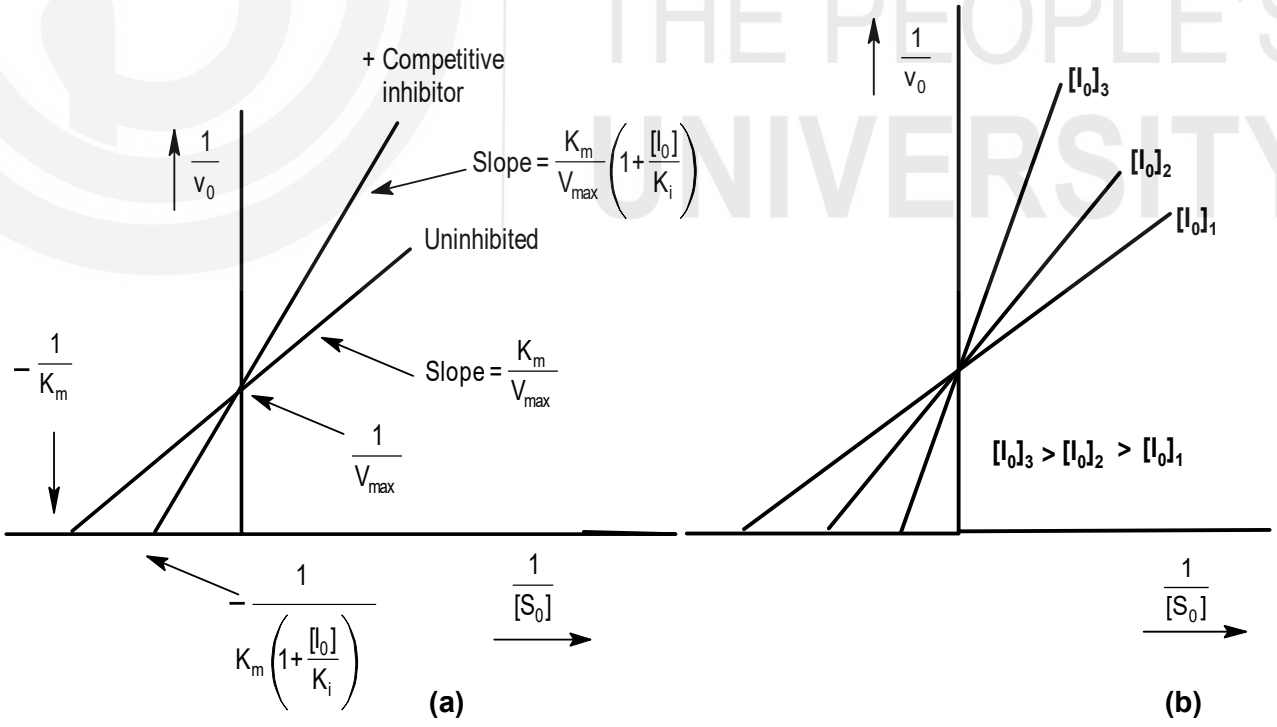
$$v_o = \frac{V_{max}[S_o]}{K_m(1+[\frac{I_o}{K_i}])+[S_o]}$$ ..... equation 6

This equation is similar to Michaelis- Menten equation and the K_m is increased by a factor

$$K_m(1+[\frac{I_o}{K_i}]) \text{ or } \quad K'_m = K_m(1+[\frac{I_o}{K_i}])$$

The Lineweaver- Burk equation and plot (Fig. 6.1) of competitive inhibition will be:

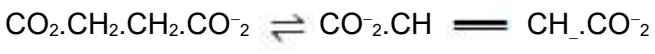
$$\frac{1}{v_o} = \frac{K'_m}{V_{max}[S_o]} + \frac{1}{V_{max}}$$



Fig,6.1(a): Lineweaver-Burk plot for competitive inhibitor (b) Plot at fixed enzyme concentration but different inhibitor concentrations

Examples of Competitive Inhibition: Succinate dehydrogenase (SDH) enzyme converts succinate to fumarate. Succinate is competitively inhibited by

malonate. Malonate ($CO^{-}_{2}.CH_2.CO^{-}_{2}$) is structurally similar to fumarate having two carboxyl groups. Similarly, inhibition of xanthine oxidase enzyme by allopurinol leads to decreased formation of uric acid and act as a remedy for gout.



Succinate **Fumarate**

SAQ 1

Draw Lineweaver- Burk plot for different inhibitory concentrations of a competitive inhibitor at the fixed enzyme concentrations.

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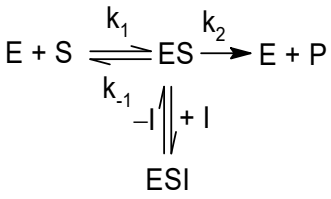
6.3.2 Un-Competitive Inhibition

Uncompetitive inhibitors are those inhibitors who do not compete with the substrate and do not bind to the free enzyme. They bind to the enzyme substrate complex thereby forming a dead end complex. It is quite likely that binding of substrate to the enzyme may cause some conformational changes and reveals an inhibitor binding site where inhibitor can bind. This type of inhibition cannot be overcome by excess of substrate. Both K_m and V_{max} gets changed in this type of inhibition pattern.



The inhibitor constant K_i is as given below:

$$\frac{[ES][I]}{[ESI]} = K_i \quad \text{or} \quad \frac{[ES][I]}{Ki} = [ESI] \qquad \text{.....equation 7}$$

I = Inhibitor

ESI = Enzyme bound to Inhibitor and substrate

E = Free Enzyme

ES = Enzyme bound to substrate

E_o = Total enzyme

Using steady state assumption, Michaelis- Menten constant for the rate of reaction is given by

$$\frac{[E][S]}{[ES]} = \frac{k_{-1} + k_2}{k_1} = K_m \qquad \text{..... equation 2}$$

$$E_o = [E] + [ES] + [ESI] \qquad \text{..... equation 8}$$

Substituting the value of [*ESI*] as given in equation 7

$$E_o = [E] + [ES] + \frac{[ES][I]}{K_i}$$

$$E_o = [E] + [ES](1 + \frac{[I]}{K_i})$$

$$E = [E_o] - [ES](1 + \frac{[I]}{K_i})$$

Substituting the value of *E* in equation 2

$$\frac{[E_o] - [ES](1 + \frac{[I]}{K_i})][S]}{[ES]} = K_m$$

$$\frac{[E_o][S]}{[ES]} - (1 + \frac{I}{K_i})[S] = K_m$$

$$\frac{[E_o][S]}{[ES]} = K_m + (1 + \frac{I}{K_i})[S]$$

$$\frac{[E_o][S]}{K_m + (1 + \frac{I}{K_i})[S]} = [ES]$$

$$v_o = k_2[ES] \qquad \text{..... equation 4}$$

(Ref: Michaelis Menten equation section in the previous Unit 4, Block 2)

Putting the value of [*ES*] in equation 4

$$v_o = k_2 \frac{[E_o][S]}{K_m + (1 + \frac{I}{K_i})[S]}$$

$$V_{max} = k_2[E_0]$$ …… equation 5

(Ref: Michaelis Menten equation section in the previous Unit-4, Block-2)

Since inhibitor concentration is generally of the same order of magnitude as the substrate concentration and much higher than the enzyme concentration [*I*] ~ [I_o] just as [*S*] ~ [S_o]. Thus continuing similarly, above equation can be expressed as

$$v_o = \frac{V_{max}[S_o]}{K_m + (1 + \frac{I_o}{K_i})[S_o]}$$ …… equation 9

Dividing the numerator and denominator by $(1 + \frac{[I_o]}{K_i})$ gives

$$v_o = \frac{\frac{V_{max}[S_o]}{(1 + \frac{I_o}{K_i})}}{\frac{Km}{(1 + \frac{I_o}{K_i})} + [S_o]}$$

The above equation is similar to Michaelis-Menten equation. However, the constants K_m and V_{max} are changed as following:

$$V'_{max} = \frac{V_{max}}{(1 + \frac{I_o}{K_i})}$$

and

$$K'_m = \frac{K_m}{(1 + \frac{I_o}{K_i})}$$

The Lineweaver-Burk equation in the presence of an uncompetitive inhibitor is:

$$\frac{1}{v_o} = \frac{K'_m}{V'_{max}[S_o]} + \frac{1}{V_{max}}$$

The slope of a Lineweaver-Burk plot will remain unchanged as given by the following equation:

$$\frac{K'_m}{V'_{max}} = \frac{\frac{K_m}{(1 + \frac{[I_o]}{K_i})}}{\frac{V_{max}}{(1 + \frac{[I_o]}{K_i})}} = \frac{K_m}{V_{max}}$$

The x-intercept as well as y-intercept of the Lineweaver-Burk plot in the presence of an uncompetitive inhibitor (Fig. 6.2) will change but the slope will remain unaltered.

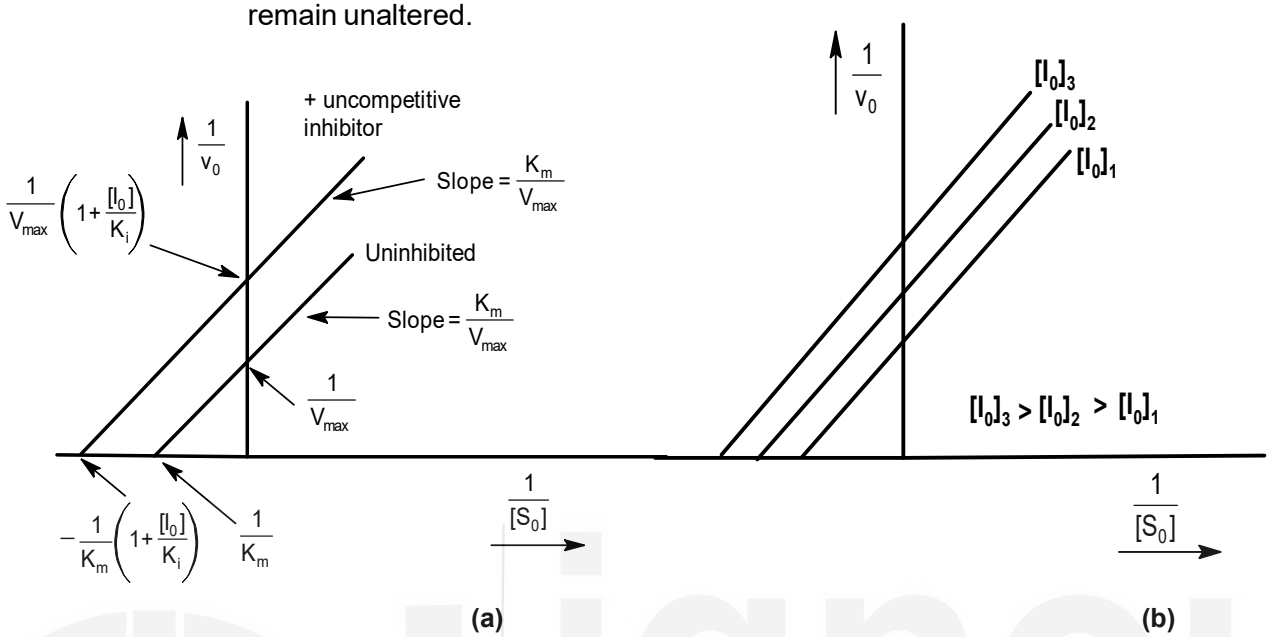


Fig.6.2 (a): Lineweaver-Burk plot of enzyme inhibited by an uncompetitive inhibitor (b) Plot at fixed enzyme concentration but different inhibitor concentrations.

Example of Uncompetitive Inhibitor: Enzyme aryl sulphatase is uncompetitively inhibited by hydrazine. Generally uncompetitive inhibition pattern is not seen in single substrate reactions but several bi-substrate reactions shows this kind of pattern.

SAQ 2

Draw Lineweaver- Burk plot for different inhibitory concentrations of an uncompetitive inhibitor at the fixed enzyme concentrations.

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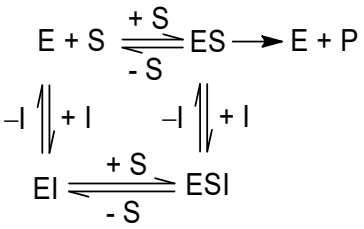
6.3.3 Non-Competitive Inhibition

Non-competitive inhibitory molecules react with the enzyme at a site other than the active site. The inhibitor can bind to the free enzyme or when it is bounded to the substrate molecule. It destroys the catalytic activity of the enzyme. The impact of inhibitor can not be overcome by increasing the substrate concentration. Apparently, K_m value will not be changed but V_{max} will change. Let us consider a simple situation involving single substrate reaction



Since *ES* can be formed via two alternative routes and considering the fact that inhibitory rates could vary in binding the free enzyme or when it is bound to the substrate the situation will become quite complex. In order to simplify the above model, we assume that substrate binding will have no effect on the binding of inhibitor. The dissociation constant (inhibitor constant) for both the reactions $E + I \rightleftharpoons EI$ and $ES + I \rightleftharpoons ESI$ will then be the same. The total enzyme concentration will be reduced in the presence of inhibitor and it will lead to decrease in V_{max}. The value of K_m will not change because binding of inhibitor does not affect the binding of substrate to the enzyme or vice versa.

In the presence of non-competitive inhibitor

$$\frac{[E][I]}{[EI]} = K_i = \frac{[ES][I]}{[ESI]} \qquad \text{..... equation 10}$$

$$E_o = [E] + [ES] + [EI] + [ESI] \qquad \text{..... equation 11}$$

Substituting the value of [*EI*] and [*ESI*] as given in equation 10

$$E_o = [E] + [ES] + \frac{[E][I]}{K_i} + \frac{[ES][I]}{K_i}$$

$$E_o = [E] + \frac{[E][I]}{K_i} + [ES] + \frac{[ES][I]}{K_i}$$

$$E_o = [E](1 + \frac{[I]}{K_i}) + [ES](1 + \frac{[I]}{K_i})$$

$$E_o = ([E] + [ES])(1 + \frac{[I]}{K_i})$$

$$E = \frac{[E_o]}{(1 + \frac{[I]}{K_i})} - [ES]$$

Substituting the value of *E* in equation 2

$$\frac{(\frac{[E_o]}{(1 + \frac{[I]}{K_i})} - [ES])[S]}{[ES]} = K_m$$

$$\frac{(\frac{[E_o][S]}{(1+\frac{[I]}{K_i})} - [ES][S])}{[ES]} = K_m$$

$$\frac{\frac{[E_o][S]}{(1+\frac{[I]}{K_i})} - [ES][S](1+\frac{[I]}{K_i})}{[ES]} = K_m$$

$$\frac{[E_o][S]}{[ES]} - [S](1+\frac{[I]}{K_i}) = K_m(1+\frac{[I]}{K_i})$$

$$\frac{[E_o][S]}{[ES]} = K_m(1+\frac{[I]}{K_i}) + [S](I+\frac{[I]}{K_i})$$

$$\frac{[E_o][S]}{[ES]} = (K_m + S)(I+\frac{[I]}{K_i})$$

$$\frac{[E_o][S]}{(K_m+[S])(1+\frac{[I]}{K_i})} = [ES]$$

$v_o = k_2[ES]$ ……. equation 4

(Ref: Michaelis Menten equation section in the previous Unit-4, Block-2)

Putting the value of [*ES*] in equation 4

$$v_o = k_2 \frac{[E_o][S]}{(K_m+[S])(1+\frac{[I]}{K_i})}$$

$V_{max} = k_2[E_o]$ ……. equation 5

(Ref: Michaelis Menten equation section in the previous Unit-4, Block-2)

Continuing similarly as in previous sections,

$$v_o = \frac{V_{max}[S_o]}{(K_m+[S_o])(1+\frac{[I_o]}{K_i})}$$ ……. equation 12

This is similar to the Michaelis-Menten equation with V_{max} divided by a factor

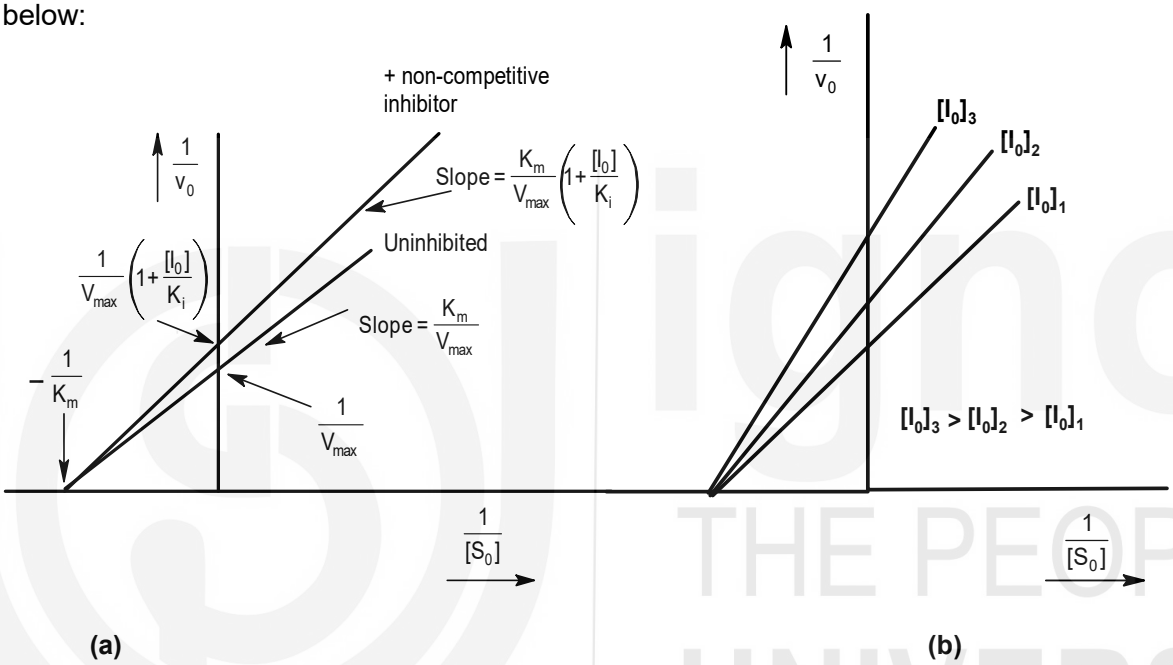
$$(1+\frac{[I_o]}{K_i})$$

$$V'_{max} = \frac{V_{max}}{\left(1+\frac{I_o}{K_i}\right)}$$

The Lineweaver-Burk equation in the presence of a noncompetitive inhibitor is:

$$\frac{1}{v_o} = \frac{K_m}{V'_{max}[S_o]} + \frac{1}{V'_{max}}$$

The Lineweaver-Burk plot (Fig. 6.3) of a non-competitive inhibitor is as given below:



Fig, 6.3 (a): Lineweaver-Burk plot for non-competitive inhibitor (b) Plot at fixed enzyme concentration but different inhibitor concentrations.

Example of Noncompetitive Inhibition: Chymotrypsin is noncompetitively inhibited by H^+. Heavy metal ions and small organic molecules bound to the –SH groups of the cysteine moiety in an enzyme can also lead to the noncompetitive inhibition.

SAQ 3

Draw Lineweaver- Burk plot for different inhibitory concentrations of a noncompetitive inhibitor at the fixed enzyme concentrations.

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6.3.4 Mixed Inhibition

Mixed inhibition occurs when an inhibitor may binds to the enzyme as well as enzyme substrate complex. However, the affinity with which inhibitor binds to two different states may varies. It may bind with greater affinity when the enzyme is free than the enzyme substrate complex or vice versa. The inhibition is a mixture of competitive inhibition and uncompetitive inhibition. If the inhibitor has equal affinity for both the states of enzyme (free as well as bound to the substrate), mixed inhibition will become non-competitive inhibition. In mixed inhibition, inhibitor binds to the enzyme at a site different from the active site, the site where substrate binds. As you know, there are two processes by which inhibitor can bind to the enzyme.

$E + I \rightleftharpoons EI$ (inhibitor constant K_i)

and

$ES + I \rightleftharpoons ESI$ (inhibitor constant K_1)

Therefore

$$\frac{[E][I]}{[EI]} = K_i$$

and

$$K_1 = \frac{[ES][I]}{[ESI]}$$

As you know in a single substrate reaction,

$$K_m = \frac{[E][S]}{[ES]}$$

$$E_o = [E] + [ES] + [EI] + [ESI] \quad \text{.....equation 13}$$

Since K_i and K_1 are not similar, they are different. So

$$E_o = [E] + [ES] + \frac{[E][I]}{K_i} + \frac{[ES][I]}{K_1}$$

$$E_o = [E] + \frac{[E][I]}{K_i} + [ES] + \frac{[ES][I]}{K_1}$$

$$E_o = [E](1 + \frac{[I]}{K_i}) + [ES](1 + \frac{[I]}{K_1})$$

$$\frac{\left([E_0] - [ES]\left(1 + \frac{[I]}{K_1}\right)\right.}{\left(1 + \frac{[I]}{K_i}\right)} = [E]$$

Substituting for [*E*] in equation 2, the expression for K_m :

$$\frac{\left([\mathrm{E}_0]-[\mathrm{ES}]\left(1+\frac{[\mathrm{I}]}{K_\mathrm{I}}\right)\right)[S]}{\left(1+\frac{[\mathrm{I}]}{K_\mathrm{i}}\right)[\mathrm{ES}]} = K_m$$

$$\therefore [\mathrm{E}_0][\mathrm{S}]\text{-}[\mathrm{S}]\,[\mathrm{ES}]\left(1+\frac{[\mathrm{I}]}{K_\mathrm{I}}\right) = K_\mathrm{m}[\mathrm{ES}]\left(1+\frac{[\mathrm{I}]}{K_\mathrm{i}}\right)$$

$$\therefore [\mathrm{ES}]\left([\mathrm{S}]\left(1+\frac{[\mathrm{I}]}{K_\mathrm{I}}\right)+K_\mathrm{m}\left(1+\frac{[\mathrm{I}]}{K_\mathrm{i}}\right)\right)=[\mathrm{E}_0][\mathrm{S}]$$

$$\therefore [\mathrm{ES}]=\frac{[\mathrm{E}_0][\mathrm{S}]}{[\mathrm{S}]\left(1+\frac{[\mathrm{I}]}{K_\mathrm{I}}\right)+K_m\left(1+\frac{[I]}{K_\mathrm{i}}\right)}$$

$v_o = k_2[ES]$ ……. equation 4

(Ref: Michaelis Menten equation section in the previous Unit-4, Block-2 of BBCCT-107)

Putting the value of [*ES*] in equation 4

$$v_0 = \frac{k_2[\mathrm{E}_0][S]}{[\mathrm{S}]\left(1+\frac{[\mathrm{I}]}{K_\mathrm{I}}\right)+K_m\left(1+\frac{[I]}{K_\mathrm{i}}\right)}$$

$V_{\max} = k_2[E_o]$ ……. equation 5

(Ref: Michaelis Menten equation section in the previous Unit-4, Block-2 of BBCCT-107) Continuing similarly as in previous sections:

$$v_0 = \frac{V_{\max}[S_o]}{[\mathrm{S}]\left(1+\frac{[\mathrm{I}_0]}{K_\mathrm{I}}\right)+K_m\left(1+\frac{[I_0]}{K_\mathrm{i}}\right)}$$ ……. equation 14

Dividing the numerator and denominator by $(1 + ([I_0]/K_i))$,

$$v_0 = \frac{\frac{V_{\max}}{\left(1+\frac{[\mathrm{I}_0]}{K_\mathrm{I}}\right)}[S_o]}{[\mathrm{S}_o]+\frac{K_m\left(1+\frac{[I_0]}{K_\mathrm{i}}\right)}{\left(1+\frac{[\mathrm{I}_0]}{K_\mathrm{I}}\right)}}$$

The above equation assumes the same form as the Michaelis-Menten equation and can be written as:

$$v_0 = \frac{V'_{max}\ [S_o]}{[S_o] + K'_m}$$

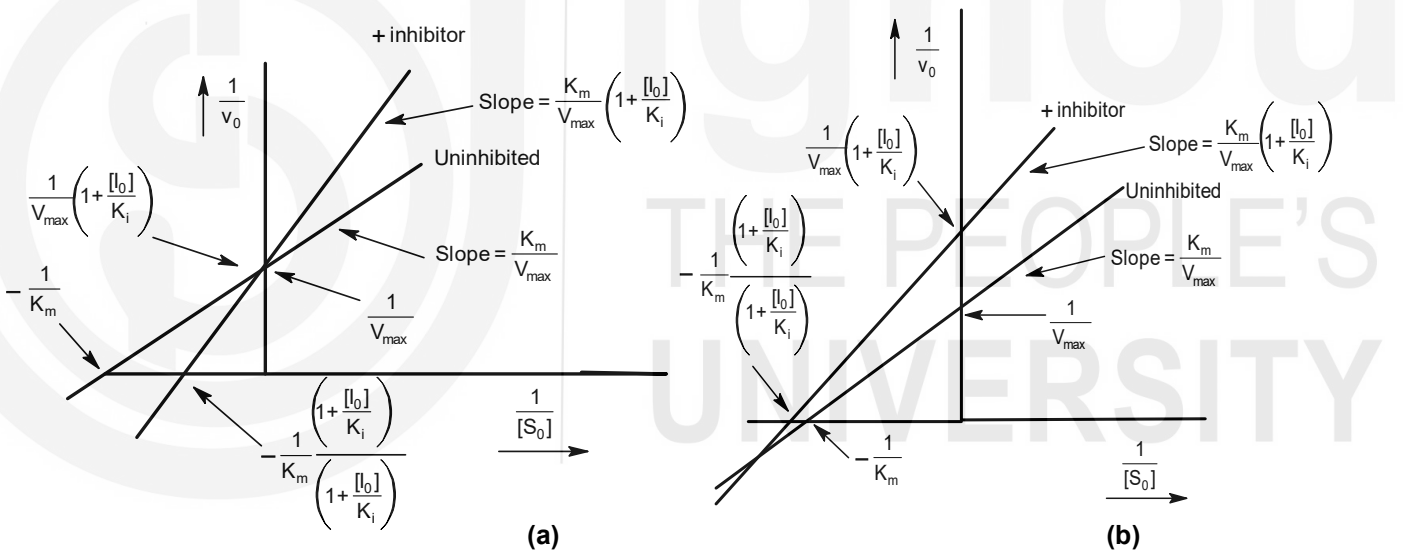
where

$$V'_{max} = \frac{V_{max}}{\left(1 + \frac{[I_0]}{K_I}\right)} \text{ and } K'_m = K_m \frac{\left(1 + \frac{[I_0]}{K_i}\right)}{\left(1 + \frac{[I_0]}{K_I}\right)}$$

The Lineweaver-Burk equation will be:

$$\frac{1}{v_0} = \frac{K'_m}{V'_{max}} \frac{1}{[S_o]} + \frac{1}{V'_{max}}$$

From the equation, it appears that the Lineweaver-Burk plot will be linear. However, K_m, V_{max} and slope will be affected by the inhibitor. The Lineweaver-Burk plot will be represented as shown in the Fig. 6.4.

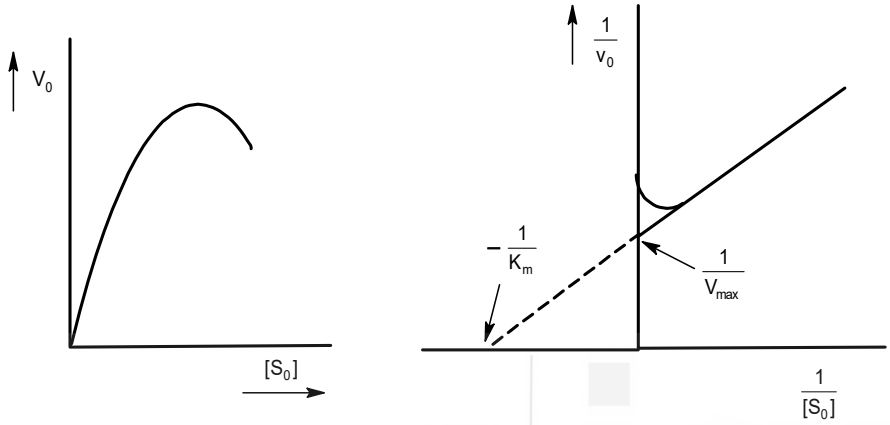


Fig, 6.4 : Lineweaver-Burk plots showing the effect of mixed inhibition: (a) $K_1 > K_i$; (b) $K_1 < K_i$.

6.3.5 Substrate Inhibition

In enzyme catalyzed reactions, when the amount of enzyme is kept constant and substrate concentration is increased gradually the rate of reaction will increase and the velocity curve will reach a maximum. A further increase in substrate concentration does not increase the rate of reaction and the velocity curve falls. This event occurs on account of substrate inhibition whenever a dead-end-enzyme substrate complex is formed thereby inhibiting its own conversion to the product. Several enzymes are inhibited by their own substrates. For example, enzyme succinate dehydrogenase catalyzes dehydrogenation of substrate succinate. If you will look at the structure of succinate, you will see two carboxyl groups at the two ends of the molecule.

The enzyme reaction takes place when both the carboxyl groups of the substrate bind to the enzyme. Excess concentration of succinate will lead to an increased possibility of carboxyl groups from two different molecules of succinate binding to the same enzyme, thereby stopping the enzyme reaction due to the formation of a dead-end complex. The graphs of characteristic substrate inhibition are shown in the Fig. 6.5a and 6.5b.



a) Michaelis-Menten plot **b) Lineweaver-Burk plot**

Fig. 6.5: Graphs on Substrate inhibition

The biological significance of substrate inhibition of phosphofructokinase ensures resources are not dedicated to the formation of ATP when it is in plenty. Another enzyme DNA metyltransferases catalyze transfer of methyl group to DNA. Their substrate inhibition leads to faithfully copy of DNA methylation patterns when cells divide while preventing de novo methylation of methyl-free promoter regions.

Regarding the enzyme kinetics, if you recall from the uncompetitive inhibition

$$v_o = \frac{\frac{V_{max}[S_o]}{(1+\frac{I_o}{K_i})}}{\frac{K_m}{(1+\frac{I_o}{K_i})}+[S_o]}$$

If the inhibitor is identical to the substrate, the equation will become:

$$v_0 = \frac{\frac{V_{max}}{\left(1+\frac{[S_o]}{K_i}\right)}\ [S_o]}{[S_o]+\frac{K_m}{\left(1+\frac{[S_o]}{K_i}\right)}} = \frac{V_{max}[S_o]}{[S_o]\left(1+\frac{[S_o]}{K_i}\right)+K_m}$$

When the substrate concentration is low [*S*] the term [*S*]/K_i becomes negligible and the equation will be the normal Michaelis-Menten equation. However, when the substrate concentration is high, then

$$[S_o]\left(1+\frac{[S_o]}{K_i}\right) + K_m \simeq [S_o]\left(1+\frac{[S_o\ ]}{K_i}\right)$$

The rate of equation will be

$$v_0 = \frac{V_{max}}{1+\frac{[S_o]}{K_i}} \qquad \text{.....equation 15}$$

As shown in the above equation, v_o will decrease on an increase of substrate concentration due to substrate inhibition.

SAQ 4

Do as Directed:

a) Mixed inhibition is a mixture of competitive inhibition and uncompetitive inhibition. (True/False)

b) If the inhibitor has equal affinity for both the states of enzyme (free as well as bound to the substrate), mixed inhibition will become non-competitive inhibition. (True/False)

c) The substrate inhibition of phosphofructokinase ensures resources are not dedicated to the formation of ATP when it is in less/excess. (Pick one option)

d) Succinate has one/two carboxyl groups. (Pick one option)

6.4 MECHANISM BASED INHIBITORS- ANTIBIOTICS AS INHIBITORS

Irreversible Enzyme Inhibition

Irreversible inhibition is different from temporary enzyme inactivation by the reversible inhibitor. The enzyme activity is lost on the binding of inhibitor molecule to enzyme and its activity cannot be recovered afterwards. These inhibitory molecules are highly specific and can modify enzyme 3D structure. The enzyme gets inactive or there is time dependent loss of enzyme concentration. Inhibition cannot be removed by dilution or dialysis without losing enzyme activity. Inhibitors generally form or break covalent bonds with the amino acid residues essential for substrate binding, catalysis or maintenance of enzyme conformation.

Examples: Heavy metal ions such as mercury, lead, aldehydes and haloalkanes. Alkylating agents such as iodoacetate and iodoacetamide forms covalent linkages with –*SH* groups of the enzyme.

$$E\text{-}SH + ICH_2.CO^-_2 \longrightarrow E\text{-}S\text{-}CH_2.CO^-_2 + HI$$

The **mechanism-based inhibitors** are modified substrates that are also known as suicide inhibitors or suicide inactivators. They modify the active site of enzyme irreversibly. These suicide inactivators or inhibitors are generally inactive unless they bound to the enzyme at its active site. The inhibitor binds to the enzyme as substrate and is catalyzed in a similar manner. The mechanism of catalysis generates a chemically reactive intermediate that leads to the inactivation of enzyme by covalent modification. These inactivators are used to unravel the mechanism of enzyme reaction and substrate reactivity. This mechanism has proved to be a boon for *rational drug* designing by pharmaceutical companies to determine the molecules which could be synthesized by the chemists as pharmaceutical agents.

Several important drugs are well known examples of irreversible inhibitors. Widely used drugs such as penicillin act by covalently inhibiting the enzyme transpeptidase, thereby preventing the synthesis of bacterial cell walls and thus killing the bacteria. You must have heard of tablet aspirin. The drug aspirin inhibits the enzyme cyclooxygenase thereby reducing the synthesis of inflammatory signals. Enzyme monoamine oxidase deaminates neurotransmitters such as dopamine and serotonin, and lowers the levels of these hormones in the brain. A neurodegenerative disease such as Parkinson's disease is linked with low levels of dopamine, while depression is related with low levels of serotonin. Suicide inhibitor such as drug (-)deprenyl, is widely used to treat Parkinson disease and depression.

6.5 SUMMARY

1) Enzyme inhibition can be either reversible or irreversible. The different modes of reversible enzyme inhibition can be distinguished by their effects on the kinetic behavior of enzymes: competitive, uncompetitive or non-competitive.
2) Competitive inhibitors compete with the substrates for the same binding site available on the enzyme. Therefore the graphs of these inhibitors show that K_m is increased but V_{max} remain unchanged in the presence of an inhibitor.
3) The uncompetitive inhibitor does not compete with the substrate. However, it binds at a site other than the substrate binding site on the enzyme-substrate complex. The graphs of these inhibitors show that K_m and V_{max} is altered in the presence of an inhibitor but the slope remains unchanged.
4) Non-competitive inhibitors also bind at a different site other than the substrate binding site on the enzyme and enzyme substrate complex. In the presence of such inhibitors, K_m remains unchanged, however V_{max} is decreased.
5) Mixed inhibition is a mixture of competitive inhibition and uncompetitive inhibition.
6) Substrate inhibition seems to be a type of uncompetitive inhibition, the extra substrate molecule acts as an inhibitor.

7) Irreversible inhibitors can be used to map the active site of enzyme. They bind to the active site of enzyme and modify it covalently and hence cannot dissociate from the enzyme. They reduce the concentration of enzyme present.

8) Mechanism based inhibitors or suicide inhibitors are processed by the enzyme in a catalytic mechanism resulting in the formation of a reactive compound that inactivates or inhibits the enzyme. These inhibitors are widely used for rational drug designing.

6.6 TERMINAL QUESTIONS

1) Discuss uncompetitive inhibition and derive the equation for an uncompetitive inhibitor.
2) Distinguish between reversible and irreversible inhibition?
3) Explain the substrate inhibition.
4) What are the mechanism-based inhibitors? How they help in drug designing?

6.7 ANSWERS

Self-Assessment Questions

1) Refer to Fig. 6.1 b
2) Refer to Fig. 6.2 b
3) Refer to Fig. 6.3 b
4) a) True, b) True, c) Excess, d) Two

Terminal Questions

1. Refer to section 6.3.2
2. Refer to section 6.2
3. Refer to section 6.3.5
4. Refer to section 6.4

6.8 SUGGESTED READINGS

1) David L. Nelson and Michael M. Cox: Lehninger Principles of Biochemistry 6th Ed., W.H. Freeman.
2) Robert K. Murray, Daryl K. Granner, Victor W. Rodwell Harper's Illustrated Biochemistry, 27th edition. 2006, McGraw-Hill.
3) Donald J Voet and Judith G. Voet:Principles of Biochemistry4th ed., John Wiley and Sons, Inc, USA.
4) Eric E Conn, Paul K Stumpf: Outlines of Biochemistry, John Wiley and Sons, Inc, USA.
5) S. Shanmugan and T. Sathish Kumar: Enzyme Technology, I K International Publishing House Pvt Ltd, New Delhi.
6) Nicholas C Price and Lewis Stevens: Fundamentals of Enzymology, Oxford University Press, Oxford, New York, USA.