RECENT ADVANCES IN PHARMACEUTICAL SCIENCES

Volume - 3

Chief Editor

Dr. Chhavi Singla

Principal and Professor, Ram Gopal College of Pharmacy, Sultanpur, Gurugram, Haryana, India

AkiNik Publications

New Delhi

Chapter - 1

Enzyme Inhibition

Dr. Aparoop Das, Dr. Kalyani Pathak, Manash Pratim Pathak, Dr. Pronobesh Chattopadhyay, Riya Saikia, Dr. Supriya Sahu and Urvashee Gogoi

Abstract

Although types of enzyme inhibition are well documented in the scientific arena but their therapeutical exploitation is yet to establish in certain fields. Zymogens and non-specific protein denaturants are some of the new entrant in the fields of enzyme inhibition. Some newly reported clinically useful competitive inhibition drugs are also discussed. Therefore we try to focus on some recent development in the field of enzyme inhibition in a systematic order. As enzyme inhibition is a very vast field and it cannot be concluded in a chapter or two, here we will try to put emphasis on enzyme inhibition and kinetics prevailing in human beings as a whole.

Keywords: enzyme inhibition, therapeutical, zymogens, non-specific protein denaturants and competitive inhibition

1. Introduction

The terminology ‘enzyme inhibition’ itself means inhibition or decrease of enzymes or some processes related to its production or any other enzyme activity [1]. Inhibition by small molecules is often regarded as a control mechanism for various biological systems whose mechanism is exploited for many drug discovery programmes [2]. Cytochrome P450 are a group of enzymes, especially hepatic, plays major role in drug metabolism by way of its inhibition by many drugs. An example of potent adverse effects during enzymatic inhibition is prolongation of QT interval due to reduction of terfenadine (a CYP3A4 substrate) by ketoconazole and drug interaction of grapefruit (a CYP3A4 inhibitor) with midazolam, simvastatin etc. that may cause 15-20 fold increase in plasma concentration to [1, 3]. Enzyme inhibition study have contributed numerous information about many unanswered biological mechanisms such as blood coagulation (hemostasis), activation of complement system, blood clot dissolution (fibrinolysis), turnover of connective tissue and inflammatory reactions [4]. Enzyme inhibition is both reversible or irreversible in which the former an enzyme-inhibitor complex

formed that dissociates easily and the later binds covalently but dissociates slowly [2], or react with residues that involves in catalytical reaction and thereby manipulate them chemically [4]. In toxicological studies, irreversible inhibition is regarded important owing to its dilution contingent to distribution, metabolism and excretion. Irreversible inhibitor is also termed as 'suicide inhibitor' due to its property to metabolise the inhibitor molecule in advance followed by the formation of a reactive intermediate which in many case is quite stable and is not readily reversed [5]. There are different types of reversible enzyme inhibition viz., competitive inhibition, non-competitive inhibition, mixed inhibition etc. [6-7]. Besides these regular types of enzyme inhibitions, allosteric, phosphorylation are some of the type of enzyme inhibition whose mechanisms are quite different then the above mentioned inhibitions. Besides these types of enzyme inhibition, some inactive precursors like 'zymogen' are also broadly discussed in the later part of the chapter. As enzyme inhibition is a very vast field and it cannot be concluded in a chapter or two, here we will try to put emphasis on enzyme inhibition and kinetics prevailing in human beings as a whole.

2. Chapter content

2.1 Types of enzyme inhibition

a) Competitive inhibition

In competitive inhibition, there is only one active binding site (substrate-binding site) where both substrate and competitive inhibitor competes to bind. In case, when substrate concentration is high, substrate binds to the active binding site and forms enzyme-substrate complex (ES) followed by formation of product (P).

b) Non-competitive enzyme inhibition

Non-competitive inhibition can be defined as the phenomenon of an inhibitor that interacts with a group of the enzyme, reversibly, causing inhibition of its normal action, which is important for its activity otherwise. But it does not affect binding of the substrate [8].

c) Mixed enzyme inhibition

Mixed enzyme inhibition comprises of those cases in which the inhibitor may bind to the enzyme whether or not the enzyme has already bound to the substrate but has a greater affinity for one state than the other [9]. The mixed type inhibitor does not have structural similarity to the substrate but it binds to both of the free enzyme and the enzyme-substrate complex. It is termed "mixed" because it can be seen as a conceptual "mixture" of the two main

types of inhibition viz. competitive inhibition, where the inhibitor binds to the enzyme only if the substrate has not already bound, and uncompetitive inhibition, where the inhibitor binds to the enzyme only if the substrate has already bound [10].

d) Allosteric inhibition

It is the type of enzyme inhibition where reactions in a pathway are catalyzed by different enzymes in sequence and the final end-product produced may be responsible for inhibiting the activity of the first enzyme of the series. There is a complete change in structural features of the inhibition caused by the final end product from the substrate molecule. Such an inhibition is known as allosteric inhibition and the enzyme involved is known as allosteric enzyme [11].

e) Phosphorylation

Phosphorylation is an important enzyme inhibition by which the activity of proteins can be altered after they are formed. A phosphate group is added to a protein by specific enzymes called kinases. This phosphate group is usually provided by ATP, the energy carrier of the cell. Phosphorylation is the chemical addition of a phosphoryl group (PO_3^-) to an organic molecule. The removal of a phosphoryl group is called dephosphorylation. Both phosphorylation and dephosphorylation are carried out by enzymes (e.g., kinases, phosphotransferases). Phosphorylation is important in the fields of biochemistry and molecular biology because it's a key reaction in protein and enzyme function, sugar metabolism, and energy storage and release [12].

f) Zymogens

A zymogen also called a proenzyme is an inactive precursor of an enzyme. A zymogen requires a biochemical change to convert an active enzyme. The biochemical change basically takes place in Golgi bodies, wherein a specific part of the precursor enzyme is cleaved so as to activate it. A peptide unit may be cleaved as an inactive piece, or independently folding domains comprising more than 100 residues may be cleaved as inactive [13]. The pancreas secretes zymogens partially so as to prevent enzymes from digesting proteins in the cells whereby they are synthesised. Enzymes like pepsin are designed as pepsinogen, which is its inactive zymogen form. When chief cells releases pepsinogen into the gastric acid it is activated, whose hydrochloric acid partially activates it? Again the partially activated pepsinogen completely activates by removing the peptide, turning the pepsinogen into pepsin [14].

Fungi also secrete digestive enzymes into the environment in the form of zymogens. The pH of external environment is different from inside of the fungal cell and this is important to change the zymogen's structure into its active enzyme form. Another alternative way is that when a cofactor is bound to the enzyme called as coenzyme its inactive form is converted to active form.

2.2 Mechanisms of enzyme inhibition

a) Competitive inhibition

The structural resemblance of the competitor matches that of the substrate and therefore binds to the active site competitively (Fig 1). But when competitive inhibitor's concentration exceeds that of the substrate, competitive inhibitor binds to the active binding site to form enzyme-inhibitor complex (EI) and finally no product is formed [15-16]. It is complete ambiguous information that inhibitors lash out the substrate from the enzyme. Rather, the fact is that the inhibitor would bind to the Enzyme substrate complex and would compel the substrate to dissociate from the enzyme through a thermodynamic principle where the binding between the substrate/inhibitor and the enzyme is governed by the concentration and affinity of the former and the later [16].

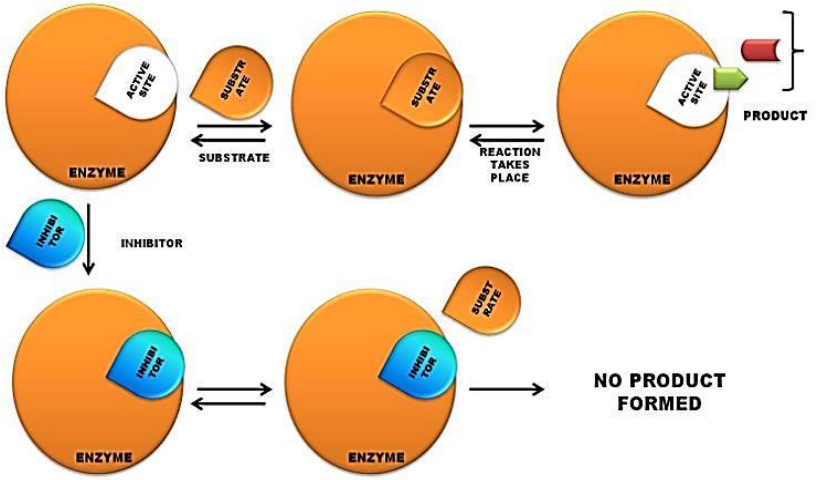


Fig 1: Mechanism of competitive inhibition

In a normal enzymatic activity, Vmax is the maximum velocity of the reaction while Km (or Michaelis-Menten constant) is the substrate concentration which is halfway to Vmax. Km is a suitable measuring unit to measure the rate of reaction with increasing concentration of substrate.

Lower the Km, higher is the affinity for the substrate (dissociation constant increases K_I) and vice-versa. A plateau occurs in the graph because all the enzyme molecules are saturated with available substrates and no enzymes other than substrates left for further binding. That means, the extra available substrates left out is due to unavailability of enzymes which may be a rate limiting factor for the rate of the reaction. But in competitive inhibition, Vmax remain unchanged or the reaction reaches its normal Vmax but to reach that point, higher concentration of Km is required. Here a question arises as why extra concentration of substrate is required? And the most appropriate answer to the question is that the extra concentration of substrate enables the substrate entity to thrash the competitive inhibitor to complete the reaction. Allosteric competitive inhibition is another type of competitive inhibitor in which due to the presence of an allosteric site in the enzyme, the inhibitor not necessarily binds to the active site, it may bind to former site too (Fig. 2). If inhibitor binds to the allosteric site before the substrate binds to the active site, there will be slight conformational of the active site due to which the substrate cannot bind at the active site of the enzyme [17].

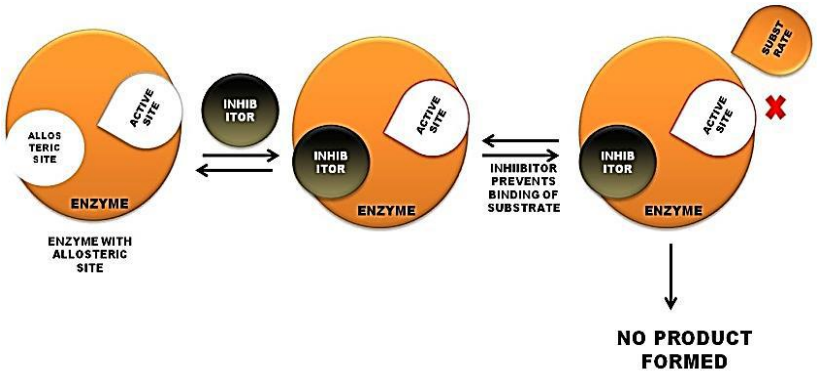


Fig 2: Allosteric competitive inhibition

Causes of competitive inhibition

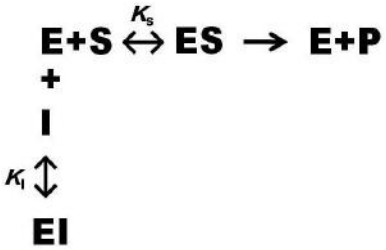
i) Causes when substances that are structurally and electrostatically related to the substrate

Inhibitors which have the same structure as of the substrate will only competitively bind to the active site of the enzyme. These bindings are specific to each other, forms either enzyme-substrate [ES] or enzyme-inhibitor [EI] complex but do not form a tertiary complex [18]. 'Substrate channeling' is a hypothesis that states that products of an enzyme-catalyzed reaction which are utilized as substrates for the next enzyme may be

transferred directly (channeled) between two enzymes without break out to the bulk solution. A recent work compared the simulated electrostatic substrates channeling of substrates and inhibitors to an experimental model. Experimental results shows similarity to those obtained from an simulated experiment that includes electrostatic interaction [19].

ii) Causes when a product functions as an inhibitor.

Competitive inhibition is reversible in nature, but in some reactions that are not readily reversible, product acts as an inhibitor owing to overtaking of an irreversible step just before product formation. Alkaline phosphatase reaction fits the example where hydrolysis of a variety of organic monophosphates esters to inorganic phosphates and alcohols occurs where inorganic phosphates plays role as a competitive inhibitor. In this type of reaction, both substrate and the inhibitor have the same binding affinities towards the active site of the enzyme [20].



Enzyme kinetics deals with the chemicals reaction by measuring the reaction rate catalyzed by respective enzymes. Studying the enzyme kinetics of a particular enzyme reveals its role in metabolism, its regulation and manipulation by agonists or inhibitors. Michaelis-Menten kinetics and Line weaver-Burk plot are some of the well-known enzyme kinetics model.

1) Michaelis menten hyperbolic plot

During competitive inhibition, according to Michaelis-Menten enzyme kinetics (Fig. 3) the initial velocity of the rate of reaction is slow, but on increasing the substrate concentration, reaction ultimately reaches normal Vmax.

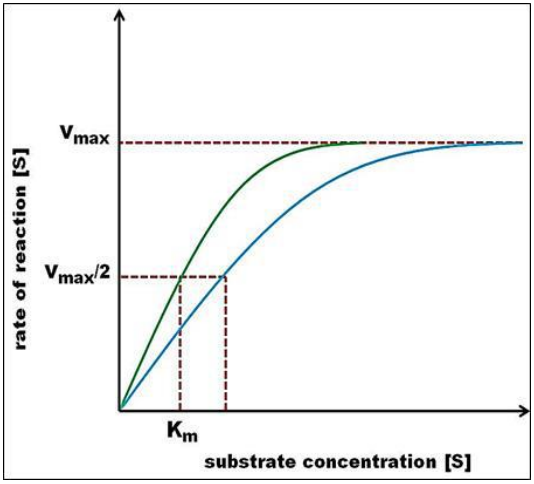


Fig 3: Michaelis-Menten plot on the reaction velocity of normal enzyme activity compared to enzyme activity with a competitive inhibitor. Green curve represents normal enzyme activity and Blue curve represents presence of competitive inhibitor

2) Lineweaver-burk plot

As Lineweaver-Burk plot is reciprocal to the Michaelis-Menten plot, according to the former plot (Fig. 4), during competitive inhibition, the Vmax remains same in the y intercept at the cost of increasing Km at the X intercept.

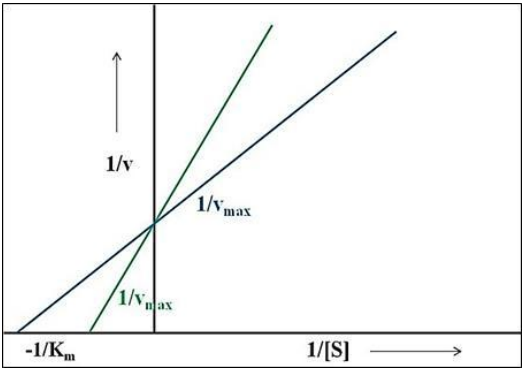
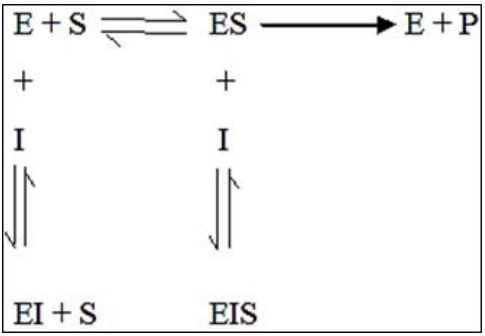


Fig 4: Lineweaver-Burk plot of normal enzyme activity compared to enzyme activity with a competitive inhibitor. Green curve represents normal enzyme activity and Blue curve represents presence of competitive inhibitor.

3) Non-competitive inhibition

In non-competitive inhibition, there is no similarity between the structure of the substrate and the inhibitor. The inhibitors bind with the

enzyme at sites other than the substrate binding site leading to the formation of both enzyme-inhibitor (EI) and enzyme-inhibitor-substrate (EIS) complexes. [21] (Fig. 5). The inhibitor forms non-covalent bonding with the enzyme and so the enzyme inhibition can be reversed by simply removing the inhibitor. The catalysis is still stopped; the reason behind this may be distortion in the enzyme conformation [20].



In simple non-competitive inhibition, both enzyme and enzyme-inhibitor complexes have similar affinity for the substrate whereas the enzyme-inhibitor-substrate complex produces product in a very small amount. More complex non-competitive inhibition occurs when inhibitor binding affect the apparent affinity of the enzyme for substrate [21].

The inhibitor binds to some other site located in the same enzyme and changes the total shape of that site for the substrate to fit into as earlier, which ultimately slows down the reaction that is taking place. The reaction gets slowed down but never stopped. Non-competitive inhibition minimizes the turnover rate of enzyme instead of interfering with the quantity of substrate binding to the enzyme [23]. As the inhibitor is not competing with the substrate, the inhibitor's effect cannot be withdrawn by increasing substrate levels [22-23]. Some authors name this sort of inhibition as specific non-competitive enzyme inhibition [24].

Heavy metal ions for example Ag^{+}, Pb^{++}, Hg^{++} etc. can inhibit the enzymes by binding non-competitively with cysteinyl sulfhydryl groups [22]. e.g.

$$E\text{-}SH + Hg^{++} \rightleftharpoons E\text{-}S.....Hg^{+} + H^{+}$$

Since enzymes are protein in nature factors that causes protein denaturation like acids, alkalis, agitation, freezing and thawing will inhibit enzyme activity. This sort of enzyme inhibition is known as Non-specific non-competitive inhibition [24].

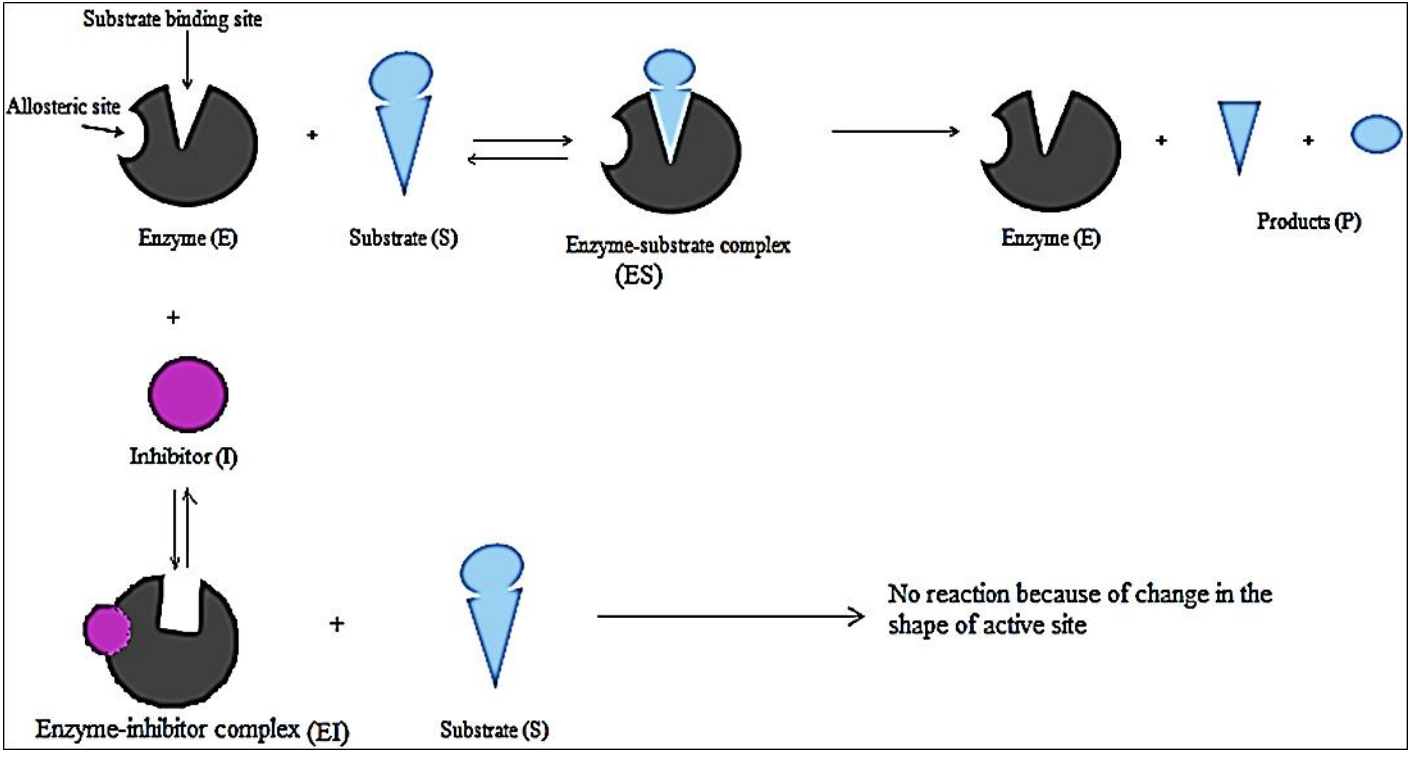


Fig 5: Diagrammatic representation of non-competitive inhibition

Enzyme behaviour in presence of non-competitive inhibitor

- V_{max} is decreased [22].
- Michaelis Menten constant (K_m) remains unchanged [22].
- Michaelis Menten hyperbolic plot (Fig: 6) shows [21-22] the initial velocity (v_i) of the reaction catalyzed by enzyme increases more slowly in presence of non-competitive inhibitor and becomes stagnant at reduced V_{max}.

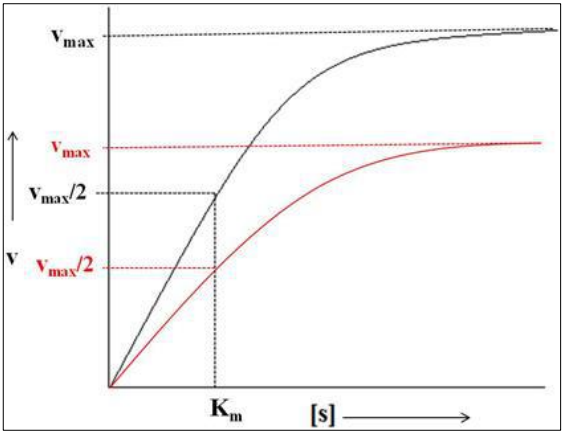


Fig 6: Michaelis-Menten hyperbolic plot. Black curve represents normal enzyme activity and Red curve represents presence of non-competitive inhibitor

- Lineweaver-Burk plot (Fig: 7) [21-22] shows that K_m remains same for non-competitive inhibition. Y-intercept and slope increase due to the inverse relation with V_{max}, which decreases.

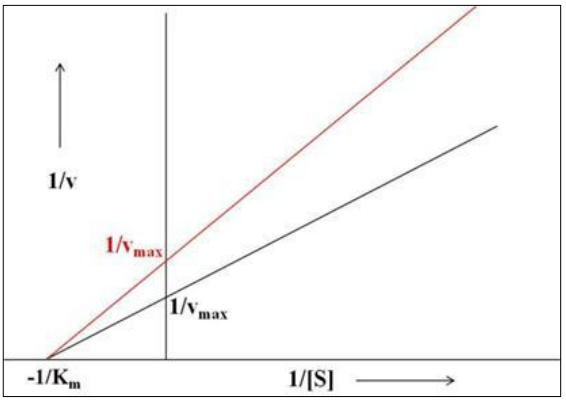


Fig 7: Lineweaver-Burk plot. Black curve represents normal enzyme activity and Red curve represents presence of non-competitive inhibitor

b) Causes of Non-competitive inhibition

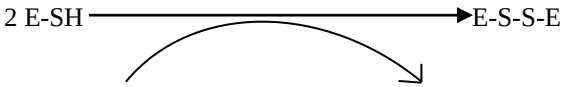
This may be caused by [24]

1. Inhibition of sulfhydryl group
2. Inhibition of cofactors
3. Inhibition of metal ion activator

1. Inhibition of sulfhydryl (-SH) group

The free sulfhydryl group present in many enzymes is responsible for its activity. Inhibition of this cluster results in loss of the protein activity which can be achieved by:

i) Oxidizing agents like Potassium Ferricyanide [24]

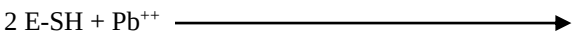


Potassium Ferricyanide Potassium Ferrocyanide

ii) Alkylating agents like iodoacetic acid (I-CH_2-COOH) and iodoacetamide [24]

I-CH_2-COOH HI

iii) Effect of heavy metals: Heavy metal ions for example Mercury (Hg^{++}) and Lead (Pb^{++}) block sulfhydryl group present in the enzymes by forming mercaptides [24].



Where E-SH represents enzyme containing free or open sulfhydryl group.

2. Inhibition of cofactors

The inhibitors block active group present in coenzymes or block the prosthetic group:

i) Coenzyme inhibition: Pyridoxal phosphate is a coenzyme required for decarboxylation, desulfhydration and transamination of amino acids. This can be blocked by hydrazine and hydroxylamine by interacting with the aldehyde group present in it [24]

ii) Inhibitors of prosthetic group: Iron is present in the haem moiety as prosthetic group present in cytochrome oxidase enzyme, which

can be blocked by carbon monoxide (CO), bisulphates and cyanide [24]

3. Inhibition of metal ion activator

Ca^{++} is required to activate the thrombokinase enzyme which causes the conversion of inactivate prothrombin to active thrombin responsible for blood clotting ultimately preventing blood coagulation [24].

c) Mixed inhibitions

In this type of inhibition, the inhibitor binds to an allosteric site, i.e. a site other than the enzyme's active site where the substrate normally binds. However, not all inhibitors that bind at allosteric sites can be called mixed inhibitors.

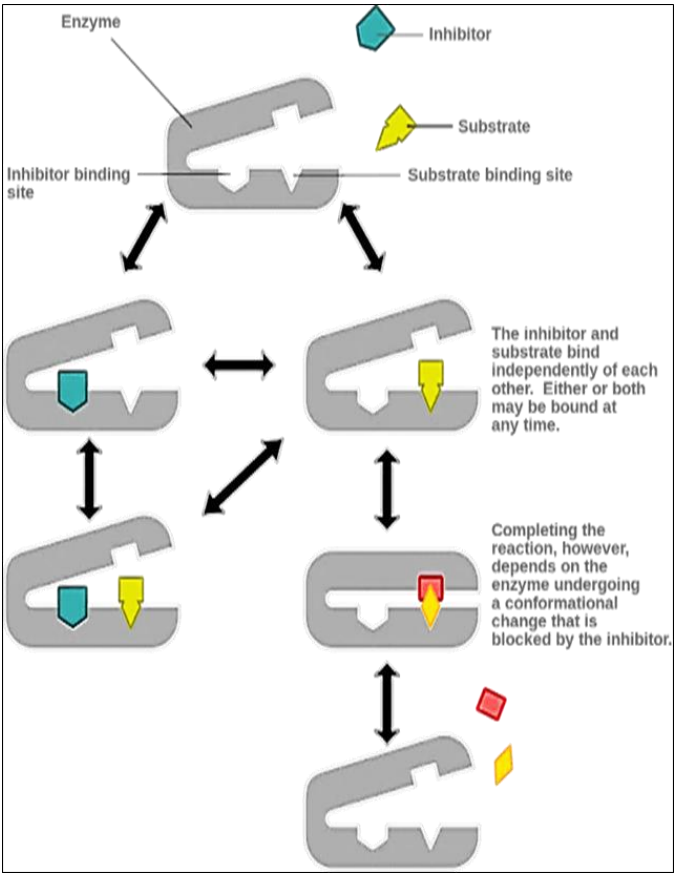
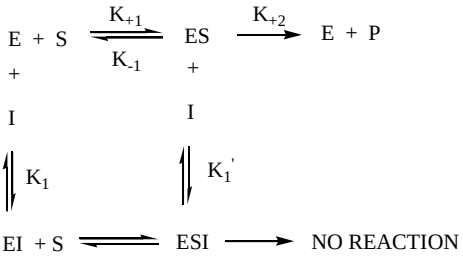


Fig 8: Example of Non-competitive inhibition, which is a special case of mixed enzyme inhibition

The scheme of mixed inhibitor can be represented as,



This results in an additional term in the reaction scheme. It is however to be noted that the dissociation constant for binding the free enzyme may differ from the dissociation constant for binding the ES complex. EI and ESI complexes are non-productive and increasing substrate to a saturating concentration does not reverse the inhibition leading to unaltered K_m but reduced V_{max}. Reversal of the inhibition requires a special treatment, e.g., dialysis or pH adjustment. Both of the inhibitor-binding steps are assumed to be at equilibrium but with different dissociation constants:

$$K_I = \frac{[E][I]}{[EI]} \quad (1)$$

$$K_I' = \frac{[ES][I]}{[ESI]} \quad (2)$$

Note: When the dissociation constants of I for E and ES are the same, the inhibition comes under a special case of mixed inhibition called non-competitive inhibition. It is quite rare as it would be practically inconceivable to imagine an inhibitor so large that inhibits the turnover of bound substrate but has no effect on binding of S to E.

Since inhibition occurs, we can hypothesize that ESI cannot form product. It is a dead end complex which has only one fate, to return to ES or EI as illustrated in the reaction scheme above. Here, we've made the assumption that binding of substrate doesn't affect the equilibrium of binding to inhibitor and vice versa. Derivation of the rate equation then follows:

Rate of product formation in the reaction is given by,

$$\frac{d[P]}{dT} = v = k_2 [E.S] \quad (3)$$

And from the Michaelis-Menten kinetics,

$$K_m = \frac{[E][S]}{[ES]} \quad (4)$$

$$[ES] = \frac{[E][S]}{K_m} \quad (5)$$

For the present purposes, it is assumed that neither EI nor ESI may react to form product. Equilibrium between EI and ESI is allowed, but makes no net contribution to the rate equation as it must be equivalent to the equilibrium established through:

$$EI + S \rightleftharpoons E + S + I \rightleftharpoons ES + I \rightleftharpoons ESI \quad (6)$$

Binding of inhibitors may change with the pH of the solution and result in the independent variation of both K_1 and K_1' with pH.

In order to simplify the analysis substantially, it is necessary that the rate of product formation (K_{+2}) is slow relative to the establishment of the equilibria between the species. (25).

Therefore,

$$K_m = \frac{K_{-1}}{K_{+1}} = \frac{[E][S]}{[ES]} \quad (7)$$

$$\text{Also, } \frac{V}{V_{max}} = \frac{[ES]}{[E]_0} \quad (8)$$

$$\text{Where, } [E]_0 = [E] + [EI] + [ES] + [ESI] \quad (9)$$

$$\text{Therefore, } \frac{V}{V_{max}} = \frac{[ES]}{[E]+[EI]+[ES]+[ESI]} \quad (10)$$

Substituting from equations (1), (2) and (4), followed by simplification, gives:

$$\frac{V}{V_{max}} = \frac{1}{\frac{K_m}{[S]} + \frac{K_m\,[I]}{[S]K_1} + 1 + \frac{[I]}{K_1'}} \quad (11)$$

$$\text{Therefore, } v = \frac{V_{max}\,[S]}{K_m\left(1+\frac{[I]}{K_1}\right)+[S]\left(1+\frac{[I]}{K_1'}\right)} \quad (12)$$

If the total enzyme concentration is much less than the total inhibitor concentration (i.e. $[E]_0 << [I]_0$), then

$$v = \frac{V_{max}\,[S]}{K_m\left(1+\frac{[I]_0}{K_1}\right)+[S]\left(1+\frac{[I]_0}{K_1'}\right)} \quad (13)$$

$$\text{Or, } V = \frac{V_{max}\,[S]}{K_m\,\alpha + [S]\alpha'} \quad (14)$$

$$\text{Where, } \alpha = \left(1+\frac{[I]_0}{K_1}\right) \text{ and } a' = \left(1+\frac{[I]_0}{K_1'}\right)$$

This is the equation used generally for mixed inhibition involving both EI and ESI complexes.

It can be seen from Eq. (14) that the name "mixed inhibition" arises from the fact that the denominator has the factor α multiplying K_m as in competitive inhibition and the factor α′ multiplying [S] as in uncompetitive inhibition. Mixed inhibitors are therefore effective at both high and low substrate concentrations.

The Lineweaver–Burk equation for mixed inhibition is,

$$\frac{1}{V} = \left(\frac{\alpha K_m}{V_{max}}\right)\frac{1}{[S]} + \frac{\alpha'}{V_{max}} \qquad (15)$$

The plot of this equation consists of lines that have slope $\alpha K_M/V_{max}$ with a 1/v intercept of α'/V_{max} and a 1/[S] intercept of $-\alpha/\alpha' K_M$ (Fig. 2). Algebraic manipulation of Eq. (15) for different values of [I] reveals that this equation describes a family of lines that intersect to the left of the 1/v axis (Fig. 9). For the special case in which $K_1 = K_1$ (α=α), the intersection is, in addition, on the 1/[S] axis, a situation which, in an ambiguity of nomenclature, is sometimes described as noncompetitive inhibition.

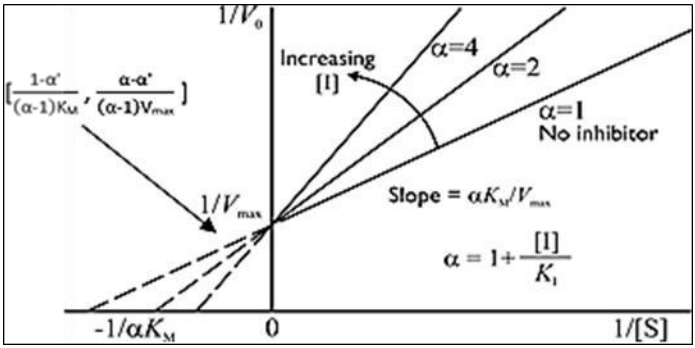


Fig 9: Lineweaver-Burk plot of a simple Michaelis-Menten enzyme in the presence of a mixed inhibitor. Note that the lines all intersect to the left of the 1/v axis. The coordinates of this intersection point are given in brackets. When $K_1 = K_1$ (α=α), the lines intersect on the 1/[S] axis at $-1/K_M$

Mixed enzyme inhibition may result in either of the following

a) A decrease in the apparent affinity of the enzyme for the substrate (K_m value appears to increase; $K_m^{app} > K_m$) seen in cases where the inhibitor favours binding to the free enzyme. More closely mimics competitive binding

b) An increase in the apparent affinity of the enzyme for the substrate (K_m value appears to decrease; $K_m^{app} < K_m$) seen in cases where the inhibitor favors binding to the enzyme-substrate complex. More closely mimics uncompetitive binding

In either case the inhibition decreases the apparent maximum enzyme reaction rate ($V_{max}^{app} < V_{max}$.

d) Allosteric inhibition

Allosteric inhibition occurs due to the presence of allosteric site on the surface of the allosteric enzyme. This site is totally far from the active site of the enzyme. The accumulated final end-product perfectly fits to the allosteric site. It brings a structural change of the enzyme in such a way that the active site of the enzyme is unable to fit for making complex with its substrate (Fig. 10). The allosteric inhibition is a reversible phenomenon. When the concentration of the final end product in the cell decreases, it leaves the allosteric site. Then the activity of the allosteric enzyme is restored in cells [25].

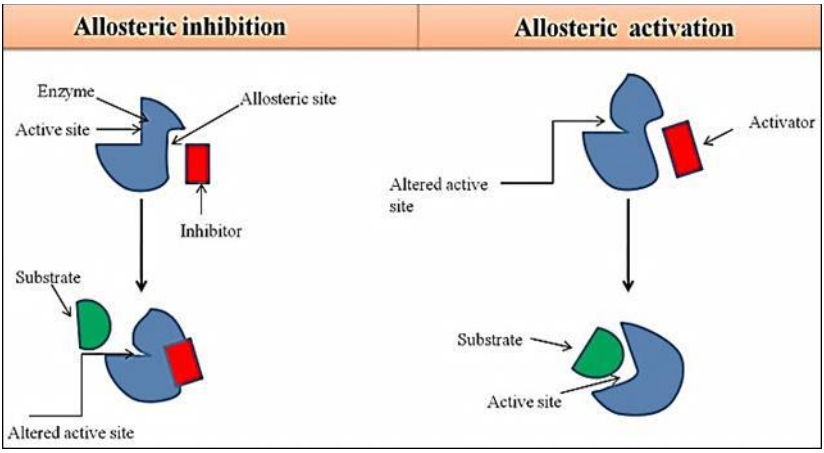


Fig 10: Allosteric inhibition

Structural biochemistry/enzyme/allosteric enzymes

Allosteric enzymes are an exception case of Michaelis-Menten model. They are having two or more than two subunits and active sites. They follow sigmoidal kinetics instead of obey the Michaelis-Menten kinetics. Allosteric enzymes are cooperative, a sigmoidal plot of V_0 versus [S] results.

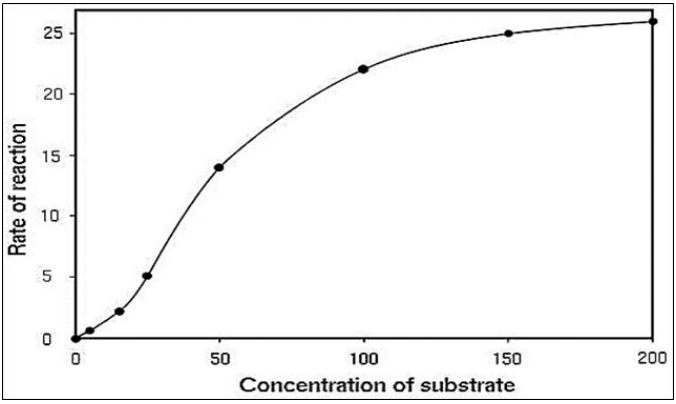


Fig 11: Sigmoidal plot representing concentration of substrate Vs rate of reaction

A sigmoidal plot is always having an S curve obtained from the combination of the T state and R state curves. The T state curve would be lower than the curve shown above and the R state curve would be higher. Thus, allosteric enzyme represents a sigmoidal curve as shown in Fig 11. The activity (v_0) versus substrate concentration from a hyperbole (Michaelis-Menten kinetics) transformed into an "S" shape curve.

Sigmoidal nature can be explained with the Hill equation as below mentioned equation

$$v_0 = V_{MAX} * [s]^n / ([s]^n + K_{0.5}^n)$$

The interaction between the different subunits can be expressed by a cooperatively coefficient n (Hill coefficient).

When n = 1, there is no interaction between the subunits, the enzyme follow Michaelis-Menten kinetics.

The $K_{0.5}$ value, a second important parameter signifies the shape of the curve, the substrate concentration at which the enzyme shows half of its maximum activity [27].

Properties of allosteric enzymes

There are some distinct properties of Allosteric Enzymes which makes them different from other enzymes [28-30].

1. Allosteric enzymes do not follow the Michaelis-Menten Kinetics as they have multiple active sites. All these active sites exhibit the co-operatively property in the enzyme. Binding of one active site influences the affinity of other active sites on the enzyme.
2. Concentration of the substrate molecule effects on the affinity of the Allosteric Enzymes. For example, more enzymes are found in the R

state at high concentrations of substrate whereas T state gets favor when insufficient amount of substrate is available to bind to the enzyme. Both T and R state are equilibrium depends on the concentration of the substrate.

3. Allosteric Enzymes are regulated by some other agents. This is noticed in the condition, the molecules 2, 3-BPG, pH, and CO_2 modulates the binding affinity of hemoglobin to oxygen. 2, 3-BPG reduces the binding affinity of O_2 to hemoglobin by stabilizing the T- state. In this Lowering the pH from physiological pH=7.4 to 7.2 (pH in the muscles and tissues) helps in the release of O_2. Hemoglobin often releases oxygen in CO_2 rich areas in the body.

e) Phosphorylation

Types of Phosphorylation

Many types of molecules can undergo phosphorylation and dephosphorylation. Three of the most important types of phosphorylation are glucose phosphorylation, protein phosphorylation, and oxidative phosphorylation [31-35].

1. Glucose phosphorylation

Phosphorylation of sugars is often the first stage in their catabolism. Phosphorylation allows cells to accumulate sugars because the phosphate group prevents the molecules from diffusing back across their transporter. Phosphorylation of glucose is a key reaction in sugar metabolism because many sugars are first converted to glucose before they are metabolized further.

The chemical equation for the conversion of D-glucose to D-glucose-6-phosphate in the first step of glycolysis is given by

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

$\Delta G° = -16.7$ kJ/mol (°indicates measurement at standard condition)

Researcher D.G. Walker of the University of Birmingham determined the presence of two specific enzymes in adult guinea pig liver, both of which catalyze the phosphorylation of glucose to glucose 6 phosphate. The two enzymes have been identified as a specific glucokinase (ATP-D-glucose 6-phosphotransferase) and non-specific hexokinase (ATP-D-hexose 6-phosphotransferase).

Hepatic cell is freely permeable to glucose, and the initial rate of phosphorylation of glucose is the rate-limiting step in glucose metabolism by

the liver (ATP-D-glucose 6-phosphotransferase) and non-specific hexokinase (ATP-D-hexose 6-phosphotransferase).

The role of glucose 6-phosphate in glycogen synthase: High blood glucose concentration causes an increase in intracellular levels of glucose 6 phosphate in liver, skeletal muscle and fat (adipose) tissue. (ATP-D-glucose 6-phosphotransferase) and non-specific hexokinase (ATP-D-hexose 6-phosphotransferase). In liver, synthesis of glycogen is directly correlated by blood glucose concentration and in skeletal muscle and adipocytes, glucose has a minor effect on glycogen synthase. High blood glucose releases insulin, stimulating the Trans location of specific glucose transporters to the cell membrane.

The liver's crucial role in controlling blood sugar concentrations by breaking down glucose into carbon dioxide and glycogen is characterized by the negative delta G value, which indicates that this is a point of regulation with. The hexokinase enzyme has a low Km, indicating a high affinity for glucose, so this initial phosphorylation can proceed even when glucose levels at nanoscopic scale within the blood. The phosphorylation of glucose can be enhanced by the binding of Fructose-6-phosphate, and lessened by the binding fructose-1-phosphate. Fructose consumed in the diet is converted to F1P in the liver. This negates the action of F6P on glucokinase, which ultimately favors the forward reaction. The capacity of liver cells to phosphorylate fructose exceeds capacity of metabolize fructose-1-phosphate. Consuming excess fructose ultimately results in an imbalance in liver metabolism, which indirectly exhausts the liver cell's supply of ATP.

Allosteric activation by glucose 6 phosphate, which acts as an effector, stimulates glycogen synthase, and glucose 6 phosphate may inhibit the phosphorylation of glycogen synthase by cyclic AMP-stimulated protein kinase. Phosphorylation of glucose is imperative in processes within the body. For example, phosphorylating glucose is necessary for insulin-dependent mechanistic target of rapamycin pathway activity within the heart. This further suggests a link between intermediary metabolism and cardiac growth.

2. Protein Phosphorylation

Reversible phosphorylation of proteins is an important regulatory mechanism that occurs in both prokaryotic and eukaryotic organisms. It is estimated that 230,000, 156,000 and 40,000 phosphorylation sites exist in human, mouse and yeast, respectively. Kinases phosphorylate proteins and phosphatases dephosphorylate proteins. Many enzymes and receptors are switched "on" or "off" by phosphorylation and dephosphorylation.

Reversible phosphorylation results in a conformational change in the structure in many enzymes and receptors, causing them to become activated or deactivated. Phosphorylation usually occurs on serine, threonine, tyrosine and histidine residues in eukaryotic proteins. Histidine phosphorylation of eukaryotic proteins appears to be much more frequent than tyrosine phosphorylation. In prokaryotic proteins phosphorylation occurs on the serine, threonine, tyrosine, histidine or arginine or lysine residues. The addition of a phosphate (PO_4) molecule to a non-polar R group of an amino acid residue can turn a hydrophobic portion of a protein into a polar and extremely hydrophilic portion of a molecule. In this way protein dynamics can induce a conformational change in the structure of the protein via long-range allostery with other hydrophobic and hydrophilic residues in the protein.

One such example of the regulatory role that phosphorylation plays is the p53 tumor suppressor protein. The p53 protein is heavily regulated and contains more than 18 different phosphorylation sites. Activation of p53 can lead to cell cycle arrest, which can be reversed under some circumstances, or apoptotic cell death. This activity occurs only in situations where in the cell is damaged or physiology is disturbed in normal healthy individuals. Upon the deactivating signal, the protein becomes dephosphorylated again and stops working. This is the mechanism in many forms of signal transduction, for example the way in which incoming light is processed in the light-sensitive cells of the retina.

3. Oxidative phosphorylation

Oxidative phosphorylation is how a cell stores and releases chemical energy. In a eukaryotic cell, the reactions occur within the mitochondria. Oxidative phosphorylation consists of the reactions of the electron transport chain and those of chemiosmosis. In summary, redox reaction pass electrons from proteins and other molecules along the electron transport chain in the inner membrane of the mitochondria, releasing energy that is used to make adenosine triphosphate (ATP) in chemiosmosis. In this process, NADH and $FADH_2$ deliver electrons to the electron transport chain. Electrons move from higher energy to lower energy as they progress along the chain, releasing energy along the way. Part of this energy goes to pumping hydrogen ions (H^+) to form an electrochemical gradient. At the end of the chain, electrons are transferred to oxygen, which bond with H^+ to form water. H^+ ions supply the energy for ATP synthase to synthesize ATP. When ATP is dephosphorylated, cleaving the phosphate group releases energy in a form the cell can use.

Adenosine is not the only base that undergoes phosphorylation to form AMP, ADP, and ATP. For example, guanosine may also form GMP, GDP, and GTP.

f) Zymogens

Some of the examples of zymogens are [36-43]

1. Pepsinogen

Pepsinogen is the inactive precursor form of pepsin that is released by Chief cells in the stomach. Pepsinogen is activated by Hydrochloric acid (secretion from Parietal cells) because Hydrochloric acid provides acidic environment where pepsin works best. Once pepsinogen activates to pepsin, it breaks down food. The difference between pepsinogen and pepsin is that it has an additional 44 amino acids in its primary structure.

2. Trypsinogen

Trypsinogen, inactive form of trypsin is found in pancreatic juice and released by the pancreas. Trypsin cleaves peptide chains at the carboxyl side of the amino acids lysine and arginine unless they are preceding a proline after it is activated in duodenum. The significance of trypsin is its potential to cleave other zymogens such as chymotrypsinogen and procarboxypeptidase.

3. Chymotrypsinogen

Chymotrypsin is established in the digestive system of mammals, and other organisms. It is produced when chymotrypsinogen is splitted by trypsin and subsequently reacts with chymotrypsin producing fully activates enzyme chymotrypsin. It produce action by splitting peptides at the carboxyl end of aromatic amino acids (Tryptophan, Tyrosine and Phenylalanine).

4. Procarboxypeptidase

Procarboxypeptidase, is the idle form of carboxypeptidase; trypsin and enteropeptidase is responsible for transforming its inactive form to active form and is basically secreted by the pancreas. Only a few forms are produced in the inactive form initially. The advantage of this mechanism is to ensure that the enzymes are promptly not exhausted before digestion.

5. Nuclease

Nuclease breakdown phosphodiester bonds between nucleotides in a DNA sequence and includes enzymes such as endonuclease. They vary in the DNA sequences and split as phosphodiester bonds that are symmetrical.

6. Pancreatic amylase

Pancreatic Amylase converts complex sugars such as starches and polysaccharides of carbohydrates into simpler sugars during digestion. Amylase hydrolyzes starch, glycogen, and dextrin to form glucose, maltose, and dextrins. Amylase is released by the salivary glands, but some may also be found in the pancreas that also helps aid in digestion.

7. Lipase

Lipase is the active form of prolapse. Once activated, the water soluble enzyme acts as the catalyst that breaks down ester bonds in water-insoluble lipid substrates. It cleaves fats into monoglycerides, fatty acids, and glycerols. The enzyme is essential in the process of digestion, as well as the transferring and proceeding of dietary lipids in organisms.

8. Proelastase

Proelastase is the idle form of enzyme elastase. Activation of proelastase is done by splitting of multiple sub-unit residues that binds to the central structure of the protein.

9. Enteropeptidase

Enteropeptidase is produced within the walls of the small intestines and is released by the duodenum glands. This enzyme proteolytically converts trypsinogen to trypsin which consecutively activates other digestive enzymes. Enteropeptidase breaks at the C-terminal end of trypsinogen and potentiates the enzyme, converting trypsinogen into trypsin.

10. Caspase

Caspases are enzymes that play important roles in apoptosis, necrosis, and inflammation. It plays vital role in cells for apoptosis (programmed death) during the progressing and other stages of adult life.

11. Prothrombin

Prothrombin is the inactive form of enzyme Thrombin that converts fibrinogen in to fibrin. During tissue rupture, fibrin is responsible for blood clotting and tissue restoration. Fibrinogen has a linearly symmetrical structure, containing a central cleavage site.

12. Angiotensin

Angiotensin is an oligopeptide and responsible for constricting blood vessels thereby increasing blood pressure. It also accelerates the secretion of aldosterone from the adrenal cortex. It is a hormone also a powerful dipsogen.

Zymogen granules

Proteases inside the cells of pancreas and salivary glands can stimulate the digestive enzymes. The cell uses special holding rooms called granules to confirm that inside the cell protease can't change into the zymogen form before they are released into digestive system. Zymogen granules are little rooms in the cell mostly found in acinar cells that protects zymogens from exposure of proteases inside the cell. Most zymogen granules start its formation in the endoplasmic reticulum (ER). ER is the factory of cell wherein proteins are prepared and from here, they are sent to the Golgi wherefrom products are packed and shipped to different parts of the cell [44-45].

2.3 Effect of inhibition on enzyme activities

a) Competitive inhibition

Unlike substrates and other irreversible inhibitors, competitive inhibitors are basically reversible inhibitors that bind non-covalently to the enzymes through ionic and hydrogen bonds as well as through hydrophobic interactions [46]. Although effect of irreversible inhibitors are long lasting but from the experimental point of view, many diseases requires reversible inhibitors [47-48]. A classical example of competitive reversible inhibitor in pharmaceutical is reserpine owing to its hydrophobic property [49]. ML-236A and ML-236B, two fungal metabolites isolated from Penicillium citrinum are two potent competitive inhibitors that regulated the hepatic cholesterol production by inhibiting 3-hydroxy-3-methylglutaryl-CoA reductase [50]. Zaragozic acid family are potent competitive inhibitor of squalene synthase that are reported to be effective against hypercholesterolemia [51]. Carboxyalkanoyl and mercaptoalkanoyl amino acids are potent competitive inhibitors of angiotensin-converting enzyme that competitively binds with the active site containing a carboxyl binding group, a tightly zinc bond and a group with affinity of c-terminal peptide bond [52]. An example of non-drug related competitive inhibition is nitric oxide that inhibits oxygen combustion in the respiratory chain of mitochondria by competing with oxygen in cultured cells and tissues, mitochondria, cytochrome oxidase and nerve terminals [53]. Glutamate, a potent excitatory neurotransmitter, is reported to be a positive allosteric modulator of glycine receptor which was evident in cultured and sliced spinal neurons as well as in HEK293 cells [54]. BMS-345541 (4(2'-Aminoethyl) amino-1, 8-dimethylimidazo (1,2-a) quinoxaline), is reported to be an highly selective competitive allosteric inhibitor of IkB kinase that blocks NF-kB-dependent transcription by binding to the allosteric

site of the enzyme [55]. p38 MAP kinase, a crucial regulator of inflammatory pathways is reported to be inhibited by a highly potent and selective synthetic inhibitor, BIRB 796, through a conformational change via allosteric inhibition in the highly conserved Asp-Phe-Gly motif within the active site of the enzyme [56].

b) Non-competitive inhibition

Cyanide is a poison which stops the production of ATP by aerobic respiration, ultimately leading to eventual death. It forms boding with allosteric site of cytochrome oxidase which is an important enzyme of the renowned electron transport chain. Cyanide changes the geometry of the active site of cytochrome oxidase because of which electrons can no more pass to the final oxygen (acceptor). As a result, the electron transport chain is not able to function and production of ATP through aerobic respiration is stopped [22, 24].

c) Mixed Inhibition

An example of mixed-type inhibition is inhibition of xanthine oxidase enzyme by palladium (Pd^{+2}) ions. Examples of noncompetitive inhibitors includes poisons because of the crucial role of the targeted enzymes. The ions cyanide and azide are found to inhibit enzymes containing iron or copper as a component of the active site or the prosthetic group, e.g., cytochrome c oxidase. They include the inhibition of an enzyme by H^+ ion at the acidic side and by the OH^- ion at the alkaline side of its optimum pH. They also include inhibition of; carbonic anhydrase by acetazolamide; cyclooxygenase by aspirin; and, fructose-1,6-diphosphatase by AMP. Cyanide binds to the Fe^{3+} in the heme of the cytochrome aa3 component of cytochrome c oxidase and prevents electron transport to O_2. Mitochondrial respiration and energy production cease, and cell death rapidly occurs. The central nervous system is the primary target for cyanide toxicity. Acute inhalation of high concentrations of cyanide (e.g., smoke inhalation during a fire and automobile exhaust) produces a rapid but brief central nervous system stimulation followed by convulsion, coma, and death. Acute exposure to lower amounts can result in lightheadedness, breathlessness, dizziness, numbness, and headaches. Cyanide ion is found in the air as hydrogen cyanide (HCN), in soil and water as cyanide salts (e.g., NaCN), and in foods as cyanoglycosides [10, 57].

d) Allosteric inhibition

One of the important and well known examples of allosteric inhibition is equipped by the bacterial enzyme system of E. coli. Which catalyses the

conversion of L-Threonine into L-Isoleucine involving 5 different enzymes in the pathway i.e.

1. Threonine dehydratase
2. Acetolactate synthase
3. Ketol acid reductoisomerase
4. Dihydroxy acid dehydratase
5. Transaminase (Fig. 12)

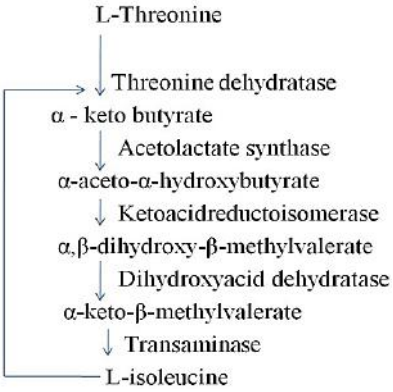


Fig 12: Schematic representation of biosynthesis of Isoleucin from Threonine and allosteric inhibition of Threonine dehydratase by Isoleucin E. coli

1. In the above sequence, only the first enzyme Threonine dehydratase is inhibited by the end-product of this sequence i.e. Isoleucine. The activity of this enzyme is neither inhibited by intermediates of the sequence, nor any other enzyme of this sequence. This is inhibited by Isoleucine. The inhibition of the first enzyme Threonine dehydratase is a reversible process. Increase in concentration of isoleucine in the cells decreases the activity of this enzyme, so as to decrease the production of isoleucine. Decrease in isoleucine concentration causes increase activity of threonine dehydratase and the production of isoleucine in the cells is restored [58]
2. Another example of an Allosteric enzyme is Aspartate transcarbamoylase. The enzyme causes catalysis of the first step of synthesis of pyrimidines. It also catalyzes the condensation of aspartate and carbamoyl phosphate to produce N carbamoyl aspartate and orthophosphate. The enzyme mainly catalyzes the reaction which yields cytidine triphosphate (CTP). This allosteric enzyme is unique in nature, as it leads to higher production of the

final product CTP. In such case the enzyme activity is low. For low concentrations of the final product, the enzymatic activity is high. The allosteric nature has a odd configuration or shape which is not like the substrates. CTP binds to the allosteric site of the enzyme rather than binding to the active site. Thus, CTP plays an important role in allosteric inhibitor for decreasing the enzymatic activity of the particular enzyme [59]. In some cases CTP concentrations remain high and cells in the body requires more numbers of enzymes. This situation occurs when different allosteric molecule ATP fits to the allosteric site and acts as enzyme activator to enhancing enzyme activity. This type of inhibition provides the benefits of allosteric control and the enzymes ability to adapt difficult conditions of the environment [60].

e) Phosphorylation

Regulatory roles of phosphorylation [61-64]

- Biological thermodynamics of energy-requiring reactions
- Phosphorylation of Na^+/K^+-ATPase during the transport of sodium (Na^+) and potassium (K^+) ions across the cell membrane in osmoregulation to maintain homeostasis of the body's water content
- Mediates enzyme inhibition
- Phosphorylation of the enzyme GSK-3 by AKT (Protein kinase B) as part of the insulin signaling pathway
- Phosphorylation of Src tyrosine kinase (pronounced "sarc") by C-terminal Src kinase (Csk) induces a conformational change in the enzyme, resulting in a fold in the structure, which masks its kinase domain, and is thus shut "off"
- Important for protein-protein interaction via "recognition domains"
- Phosphorylation of the cytosolic components of NADPH oxidase, a large membrane-bound, multi-protein enzyme present in phagocytic cells, plays an important role in the regulation of protein-protein interactions in the enzyme
- Important in protein degradation
- In the late 1990s, it was recognized that phosphorylation of some proteins causes them to be degraded by the ATP-dependent ubiquitin/proteasome pathway. These target proteins

become substrates for particular E3 ubiquitin ligases only when they are phosphorylated

- **Importance in glycolysis:** Glycolysis is an essential process of glucose degrading into two molecules of pyruvate, through various steps, with the help of different enzymes. It occurs in ten steps and proves that phosphorylation is a much required and necessary step to attain the end products. Phosphorylation initiates the reaction in step 1 of the preparatory step (first half of glycolysis), and initiates step 6 of payoff phase (second phase of glycolysis). Phosphorylation functions as an extremely vital component of glycolysis, for it helps in transport, control and efficiency.

Importance of phosphorylation in signaling networks [65-67]

Elucidating complex signaling pathway phosphorylation events can be difficult. In cellular signaling pathways, protein A phosphorylates protein B, and B phosphorylates C. However, in another signaling pathway, protein D phosphorylates A, or phosphorylates protein C. Global approaches such as phosphoproteomics, the study of phosphorylated proteins, which is a sub-branch of proteomics, combined with mass spectrometry-based proteomics, have been utilized to identify and quantify dynamic changes in phosphorylated proteins over time. These techniques are becoming increasingly important for the systematic analysis of complex phosphorylation networks. They have been successfully used to identify dynamic changes in the phosphorylation status of more than 6000 sites after stimulation with epidermal growth factor. Another approach for understanding Phosphorylation Network, is by measuring the genetic interactions between multiple phosphorylating proteins and their targets. This reveals interesting recurring patterns of interactions – network motifs. Computational methods have been developed to model phosphorylation networks and predict their responses under different perturbations.

Detection and characterization [68-70]

Antibodies can be used as powerful tool to detect whether a protein is phosphorylated at a particular site. Antibodies bind to and detect phosphorylation-induced conformational changes in the protein. Such antibodies are called phospho-specific antibodies; hundreds of such antibodies are now available. They are becoming critical reagents both for basic research and for clinical diagnosis. More recently large-scale mass spectrometry analyses have been used to determine sites of protein

phosphorylation. Over the last 4 years, dozens of studies have been published, each identifying thousands of sites, many of which were previously undescribed. Mass spectrometry is ideally suited for such analyses using HCD or ETD fragmentation, as the addition of phosphorylation results in an increase in the mass of the protein and the phosphorylated residue. Advanced, highly accurate mass spectrometers are needed for these studies, limiting the technology to labs with high-end mass spectrometers. However, the analysis of phosphorylated peptides by mass spectrometry is still not as straightforward as for "regular", unmodified peptides. Recently EThcD has been developed combining electron-transfer and higher-energy collision dissociation. Compared to the usual fragmentation methods, EThcD scheme provides more informative MS/MS spectra for unambiguous phosphosite localization. A detailed characterization of the sites of phosphorylation is very difficult, and the quantitation of protein phosphorylation by mass spectrometry requires isotopic internal standard approaches. A relative quantitation can be obtained with a variety of differential isotope labeling technologies. There are also several quantitative protein phosphorylation methods, including fluorescence immunoassays, Microscale thermophoresis, FRET, TRF, fluorescence polarization, fluorescence-quenching, mobility shift, bead-based detection, and cell-based formats.

Purposes of phosphorylation [71-73]

Phosphorylation plays a critical regulatory role in cells. Its functions include.

- Important for glycolysis
- Used for protein-protein interaction
- Used in protein degradation
- Regulates enzyme inhibition
- Maintains homeostasis by regulating energy-requiring chemical reactions

f) Zymogen

Zymogens are activated by breaking the bonds between two or more amino acids. When the bonds are cut, the enzyme its conformation, such that the active site is free or it is able to become active. Upon activation, parts of the protein completely leave the enzyme. Zymogens can also be stimulated by autocatalysis [74]. Autocatalysis is self-activation process, and takes place

when some stimulus in the environment allows the zymogen to break its own chemical bonds. Pepsinogen converts to pepsin when the pH is around 2-3 [75].

1. Activation of pepsinogen in the stomach

The peptidase enzyme in the stomach is known as pepsin. Pepsin works optimally in the pH 2-3, and becomes inactive when pH is greater than 5. The chief cells releases the zymogen, which is called pepsinogen. When hydrochloric acid (HCl) is secreted by the parietal cells activation of pepsinogen begins partially. This partially active enzyme now breaks the peptide to form pepsin [76].

2. Activation of pancreatic zymogens in the small intestine

When the pancreatic zymogens reach the small intestine they gets stimulated. An enzyme present in the microvilli called enterokinase, cleaves a peptide from trypsinogen, forming the active enzyme trypsin. Trypsin then stimulates rest of the enzymes simultaneously. When there is inappropriate production of trypsin in the pancreas then it leads to development of a dangerous situation called pancreatitis. Acinar cells synthesize and secrete a trypsin inhibitor that acts as a protection against activation of trypsin in the pancreas. Another safety mechanism is autolysis (self-digestion) [77]. Another important mechanism to prevent pancreatitis is secretion of fluids by duct cells. If there is occlusion of the pancreatic duct it will prevent flow out of the pancreas and result a state called acute pancreatitis. Secretion of fluid ih the pancreas depends upon the chloride channel CFTR (as does fluid secretion in the lungs and small intestine). Patients having mutations in the CFTR gene (which causes cystic fibrosis) have a high risk for the development of pancreatitis. Two other factors that enhance the risk of pancreatitis are excessive alcohol consumption and hyperlipidemia [78].

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