An introduction to enzymes and the kinetics of single-substrate enzyme-catalysed reactions

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A R T I C L E   I N F O

A B S T R A C T

The purpose of this article is not so much to provide an exhaustive treatise on enzymes and enzyme kinetics, as to provide an introduction to new entrants in the field on how to work with enzymes. Biochemists and chemists may require to either directly study enzymes, find small molecule drug candidates to inhibit them or employ enzymes as catalysts in many applications. A basic-level of understanding of the concepts, models and limitations of the analysis used will enable them to better use the biocatalysts as well as to overcome specific challenges they meet along the way. The rather detailed mathematical approach is deliberate. It enables researchers to play with conditions and approximations. It is also hoped that the theoretical and analytical framework provided will be treated as an invitation to explore more advanced topics in the field.

1. Introduction

Enzymes make life possible. Like all catalysts, they control rates of chemical processes without themselves being altered by the reaction. Most biochemical reactions, if carried out in the laboratory, in the absence of enzymes, would either be extremely slow or would require extreme conditions of temperature, pH and pressure. Yet enzymes make them possible under the moderate temperature and pressure conditions of the cell. Thermodynamically unfavourable reactions can be made feasible by coupling them through enzymes to other thermodynamically favourable ones and, thus, whole networks of cellular biochemical pathways are established.

Enzymes may be proteins or RNA (ribozymes). The basic unit of proteins are amino acids, while that of RNA are ribonucleic acids. This article focuses on the former, although the kinetics described here may be also applicable to the latter.

2. Amino acids and proteins

As the name suggests, amino acids possess both amino (-NH$_2$) and carboxylate (-COOH) groups. In biology, just 20 amino acids constitute most of the proteins known. For convenience, each of these amino acids have three letter abbreviations as well as a single letter code that can be used to identify them (Fig. 1). All except one among them are α-amino acids (where the amino group is present on the same α-carbon to which the

-COOH is attached). Proline, the only exception in this group, has a secondary amine and is technically an imino acid. The most general chemical representation of an amino acid is NH$_2$-CH(R)-COOH, where R represents the side-chain of the amino acid. These side chains may be polar, charged or hydrophobic, depending on the amino acids, and are mainly responsible for the properties and functions of proteins. Hence, amino acids are normally classified with reference to the nature of their side-groups (Fig. 1). An alternative approach to building an understanding of them would be to begin from the simplest, glycine, and see how the more complex and bulky ones are built upon this frame. The amino acids in Fig. 1 have been organised so as to enable such an understanding too.

A condensation reaction, between the carboxylate moiety of one amino acid and the amine moiety of the other, produces a peptide (amide) bond and generates a dipeptide molecule. Linear polymers of amino acids formed in this manner are called **oligopeptides** or just **peptides** when the number of amino acid residues in the chain is small (between 2-30) and **polypeptides** when the number of residues exceeds 30. By convention, while representing a polypeptide, the amino acid with the free terminal amine on the α-carbon (N- or amino-terminus) is written to the left and the one with the free carboxylate group (C- or carboxylate terminus) to the right. The dipeptide MY, therefore, is different from YM. In the former, the amino group of methionine (Met or M) is free as is the carboxylate group of tyrosine (Tyr or Y). The

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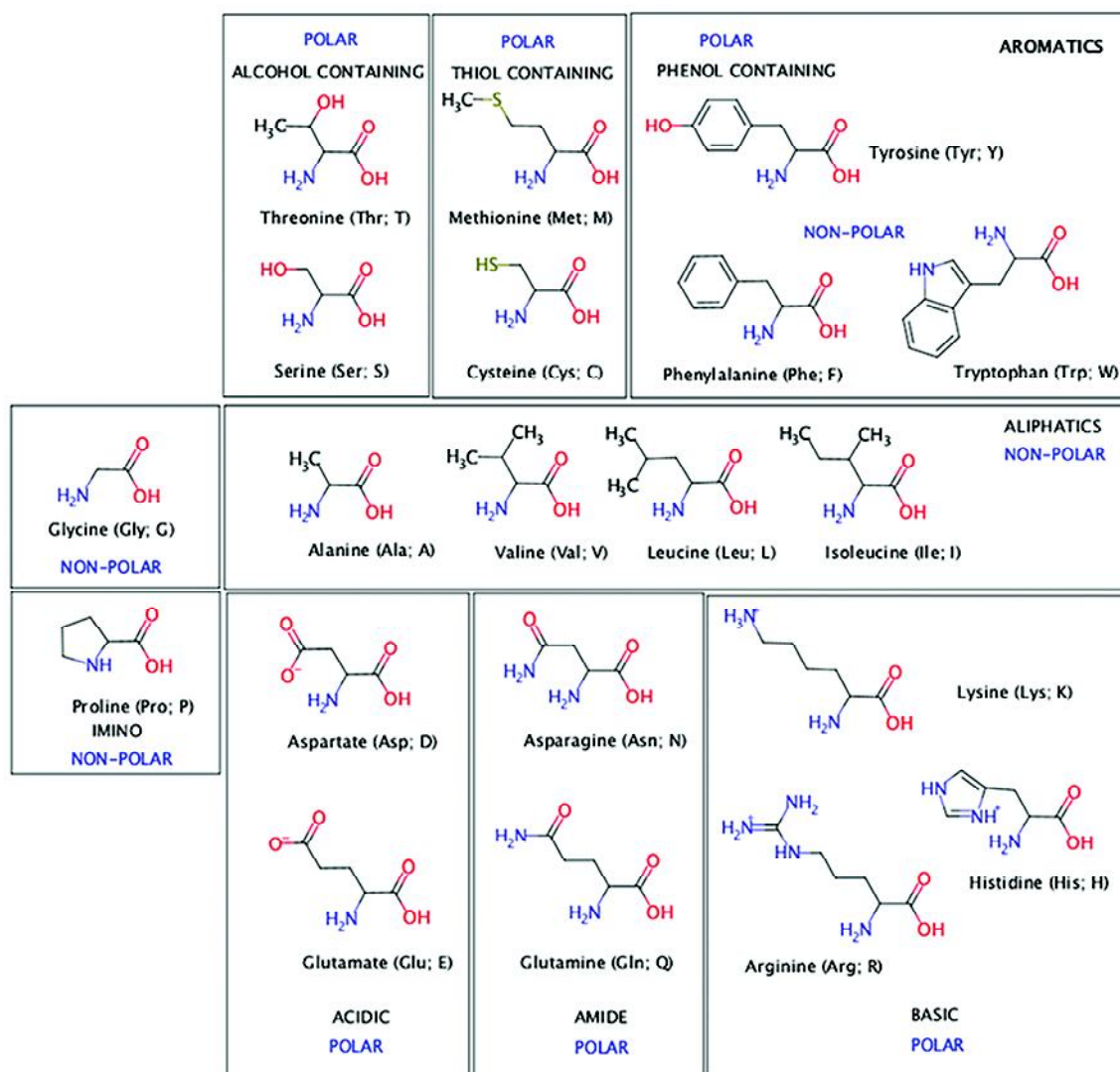*Corresponding author: *E-mail address:* sskomath@mail.jnu.ac.in, sskomath@yahoo.com (S. S. Komath)

Fig. 1. The twenty amino acids which are the main building blocks of proteins: The α-carbon is the one on which the -NH$_2$ and -COOH are attached. The 3-letter as well as single letter abbreviations for each are given in the figure. Gly, the smallest amino acid, is shown in a box all by itself. Pro, the only imino acid, is in a separate box too. The other boxes enable the reader to build each set of amino acids by simple additions/ substitutions in the side chain residues starting from Ala, which itself may be arrived at by replacing a -H in the side chain of Gly with -CH$_3$. Note that except for Gly, all other amino acids are chiral. The functional groups and the nature of the side chains (polar, non-polar, acidic, basic) are mentioned. The charges on the side chains alone are explicitly shown, assuming neutral pH (figure drawn using Marvin Sketch 22.11).

converse is true for YM. Similarly, SPAM and MAPS are very different tetrapeptides. With just 20 amino acids, therefore, it is possible to generate 20^2 dipeptides, 20^4 tetrapeptides and 20^{10} decapeptides. In this manner, an enormous diversity in polypeptide sequences may be generated with even a limited pool of 20 amino acids.

All proteins are made up of one or more polypeptide chains. The linear sequence of amino acids in a polypeptide is its **primary structure**. A polypeptide chain will normally spontaneously fold into a variety of 3-dimensional conformations dictated by the interactions of the amino acid side chains with one another as

well as with the polypeptide backbone. The hydrophobic effect, driven primarily by the tendency to bury hydrophobic side chains and minimize their exposure to water, is a major driver of the folding process. The stability of the resultant structure is maintained by a combination of H-bonding, cation-π, n-π, π-π interactions, salt bridges between acidic and basic residues disulphide bonding between the thiols of appropriately positioned cysteine residues, van der Waals interactions between dipoles, and other electrostatic interactions. Three-dimensional conformations/structures produced within a polypeptide as a result of such folding are their **secondary structures** (for more

details on forces driving secondary structure formation, see [1]).

Different stretches of a single polypeptide can adopt different secondary structures. Two of the most common secondary structures found in proteins are α-**helices** and β-**sheets**. The α-helix is a right-handed spiral, held in place primarily by the H-bonding between a N-H group in the polypeptide backbone with the backbone C=O moiety contributed by an amino acid four residues before. Each turn of the α-helix has 3.6 residues and a rise of 1.5 Å. On the other hand, β-sheets involve H bonding between backbone residues in adjacent chains, with each chain consisting of 3-10 residues in a more-or-less fully extended conformation. Unlike in the α-helix, where the H-bonding is along the axis of the helix, H-bonds in β-sheets align sideways. Depending on whether the polypeptide chains point in the same or in opposite directions, β-sheets may be labelled as "parallel" or "antiparallel". The α-helices and β-sheets are linked together by polypeptide loops and turns, some of which might not possess discernible structure. Such flexible unstructured regions are often referred to as **random coils**.

Different secondary structural elements of a polypeptide can further associate with one another to produce stable three-dimensional spatial arrangements that are called **domains**. Despite differences in the primary sequence, it is possible that domains of two different polypeptides fold into similar overall three-dimensional structures. Such domains are said to have conserved folds, or often simply referred to as **folds** (for more details on domains and folds, see [1,2]). Their presence is used to infer conservation in function. This higher level of the structure of a polypeptide chain is called its **tertiary structure**. Both single-domain and multi-domain proteins are known.

Finally, proteins may be made up of more than one polypeptide chains, each having adopted independent tertiary structures. Proteins may be homo-oligomeric or hetero-oligomeric, depending on whether the same type of polypeptides associate with one another or different polypeptides come together to produce them. These superstructures, formed by the spatial arrangement of two or more folded polypeptide chains, give rise to the **quaternary structures** of proteins (see [1,2]). They also provide an additional degree of thermodynamic stability to the protein.

3. Catalysis by enzymes

Reactants of biochemical reactions catalysed by enzymes are called "substrates". One or more substrates may bind to a single enzyme. For example, the formation of a peptide bond between two amino acids requires that the enzyme recognize

and bind to two amino acids. In addition, many enzymes may require metal ions as **cofactors**, or small organic molecules such as vitamins as **coenzymes**, without which the enzyme would be inactive. Such enzymes are called "apoenzymes" in the absence of their cofactors, and "holoenzymes' when bound to them. Cofactors / coenzymes that cannot be reversibly removed from enzymes without damaging them are called "prosthetic groups". The co-factors are crucial to the function of the enzymes that possess them; hence, the prefix 'holo', meaning 'whole' or 'complete'. They may help bind / position the substrate correctly or may participate directly in the catalytic process, as carriers of electric charge and/or donors of specific chemical groups, or as structural moieties that allow the enzyme to attain its catalytic conformation.

The three-dimensional structures of folded proteins (along with their co-factors / coenzymes, where present) provide distinct locations or pockets for substrate binding. These sites are called **active sites**. Substrate binding may involve one or more types of chemical interactions such as, H-bonding, ionic interactions, hydrophobic interactions, van der Waals interactions or even covalent bonding. Fischer suggested that the specificity of the enzyme for a given substrate requires the substrate to 'fit' into a complementary active site, almost like a key fitting into a lock (Fig. 2A).

Building on the Fisher **lock-and-key model**, Koshland argued that the complementarity of the structures probably occurs after, and not before, substrate-binding to the active site. The substrate first binds to an open active site and then induces the active site of the protein to undergo conformational changes in order to attain an optimal fit. This makes the two structures appear to be complementary (Fig. 2B). Such an **induced fit** model takes into account the flexibility of protein chains and also explains how catalytic residues that are placed far apart in space in the unbound-enzyme are able to participate simultaneously in catalysis (for a detailed review, see [3]). Serine proteases, trypsin and chymotrypsin, are some enzymes frequently used to understand these concepts regarding substrate binding. A discussion on them can be found in many standard biochemistry textbooks, some of which are mentioned at the end of this article.

4. Classification of enzymes

The Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (IUBMB) periodically reviews and recommends a rational classification or reclassification of enzymes as required. The term 'ase' is used as a suffix to denote an enzyme. Based on the types of chemical reaction that they
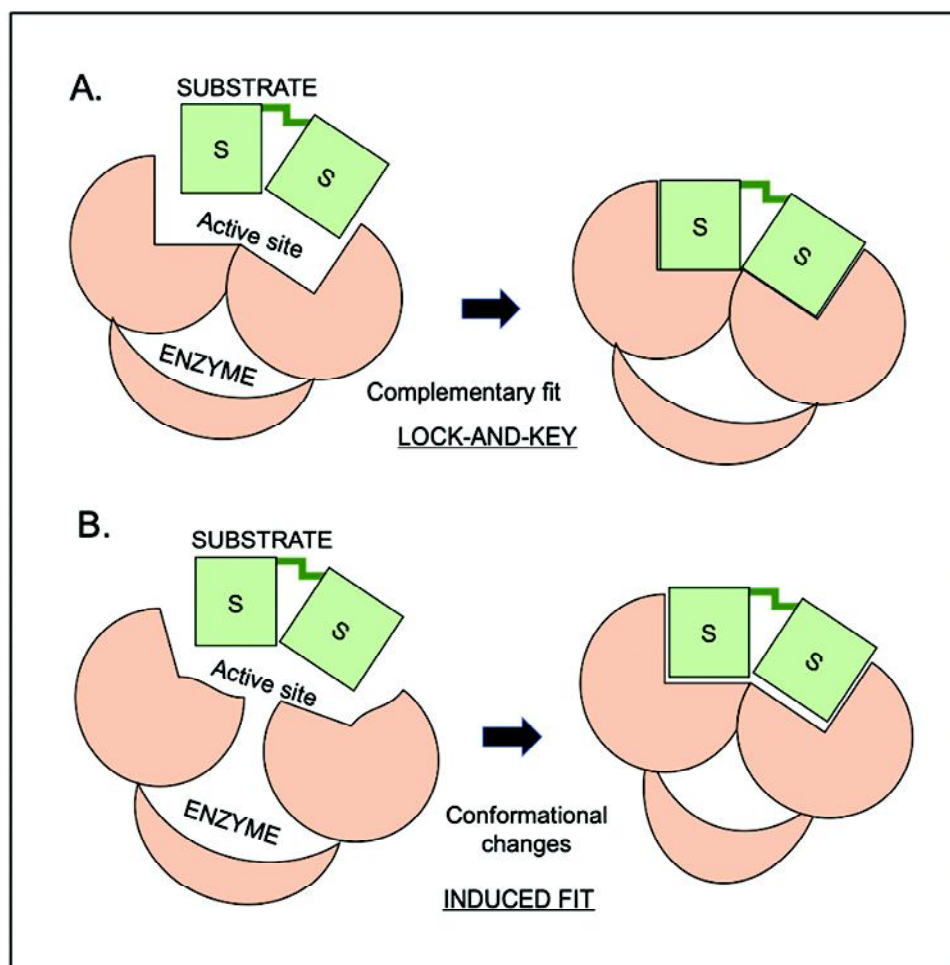
Fig. 2. **Cartoon representations of the mechanisms of substrate recognition. (A) Fischer's lock-and-key model:** In this model, the active site of the enzyme and the substrate molecule are both considered to be rigid entities where the latter fits into the former due to the complementarity in their shapes. **(B) Koshland's induced-fit model:** In this model, the assumption of complementarity of shapes is for the enzyme-substrate complex. It is assumed that the flexible enzyme undergoes conformational changes to wrap itself around a rigid substrate in order to achieve the best fit.

catalyse, enzymes are broadly classified into seven **enzyme classes** (EC) at present:

    i)   Oxidoreductases (EC 1): As the name suggests, these enzymes catalyse oxidation-reduction reactions.

    ii)   Transferases (EC 2): These enzymes catalyse the transfer of a group or moiety from a donor substrate to an acceptor substrate.

    iii) Hydrolases (EC 3): These enzymes catalyse hydrolysis of substrates.

    iv) Lyases (EC 4): These enzymes catalyse the removal of a group and formation of a double bond within a substrate.

    v)   Isomerases (EC 5): These enzymes catalyse conversion of one form of an isomer into another.

    vi) Ligases (EC 6): These enzymes stitch together, or ligate, two large molecules by generating a covalent linkage between them.

    vii) Translocases (EC 7): These enzymes were given a separate class only in 2018. They catalyse the translocation or movement of molecules / ions across membranes. The reactions are frequently coupled with ATP hydrolysis by the same enzymes. So the enzymes could also qualify to be hydrolases (EC 3). However, they are classified as translocases based on the major function that they perform in the cell.

5. Effects of temperature and pH on rates of enzyme-catalysed reactions

Both temperature and pH have notable effects on the rates of chemical reactions. But in the case of enzyme-catalysed reactions, the additional factor to consider is how the enzyme

itself responds to these environmental factors.

5.1. Temperature dependence of enzyme-catalysed reactions:

The temperature-dependence of chemical reactions in general is governed by the Arrhenius equation, $k = Aexp\left(\dfrac{-E_a}{RT}\right)$, where k is the rate constant for the reaction, A is the pre-exponential factor and a constant that depends on the system, R is the universal gas constant, T is the absolute temperature and E_a is the **activation energy** required to cross the potential energy barrier for the conversion of reactants to products (Fig. 3).

It follows from the Arrhenius equation that rates of reactions increase roughly two-fold with every 10 °C increase in temperature. This is expected, since higher the temperature, higher the fraction of reactant molecules with the kinetic energy required to cross the activation barrier, and higher the rate of the reaction (a detailed discussion can be found in standard physical chemistry textbooks); see the list of Reference Textbooks given at the end.

The Arrhenius equation also holds true for enzyme-catalysed reactions. At room temperature, enzymes sample multiple conformations in solution, as do their substrates. The binding and distortion of the substrate to a high-energy transition state, and its stabilisation by the enzyme, are made possible due to this fact. By stabilising the transition state along the reaction coordinate, the enzyme works to lower the activation energy for the process and thereby alter the path or rate of the reaction (Fig. 3). It is worth emphasising that an enzyme does not alter the equilibrium between reactants and products. Indeed, free energy (G) is a state function, independent of path.

However, temperature affects enzyme stability. Most enzymes have evolved to function under physiological conditions (~37 °C), although exceptions occur. Enzymes of thermophilic organisms can function at temperatures close to 100 °C and those in psychrophilic organisms have adapted to sub-zero temperatures. Bond strengths and intermolecular interactions within enzymes are temperature dependent, and hence the stability and structure of proteins are temperature-dependent. Normally, high temperatures result in protein unfolding and enzyme denaturation (loss of activity) due to the disruption of the highly specific intermolecular interactions, especially H-bonding and van der Waals forces within the molecule (although hydrophobic effects increase with temperature). Some enzymes are also cold-labile,
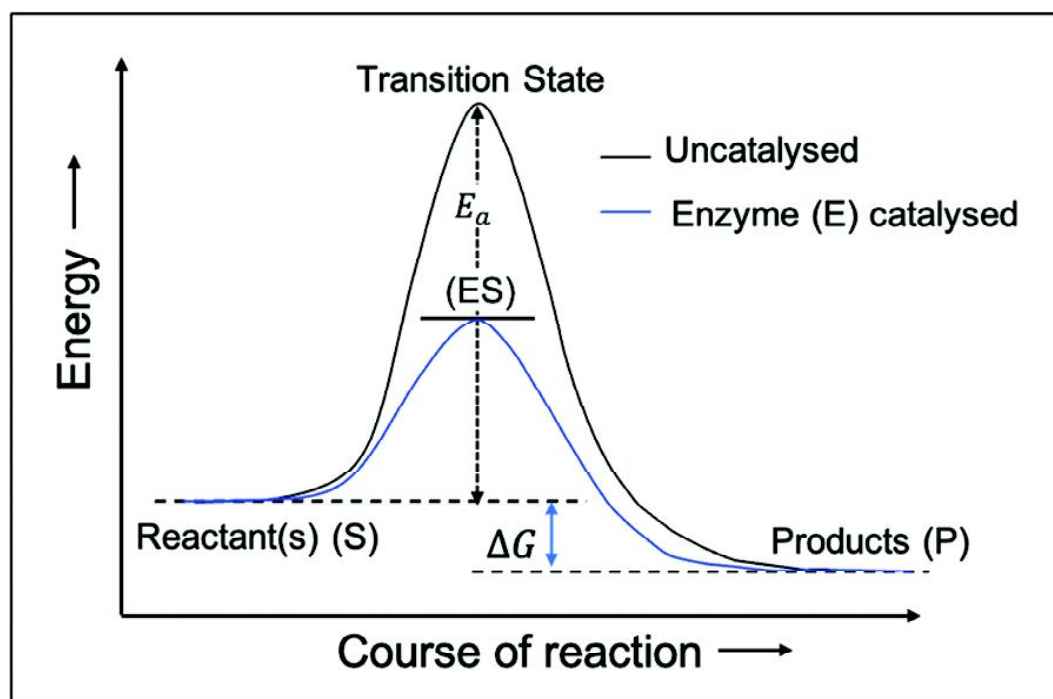

Fig. 3. The effect of an enzyme on the course of a reaction. Enzymes work by stabilizing the transition state and lowering the activation energy (E_a) required for the reaction. They do not alter the equilibrium and therefore do not alter the free energy difference (ΔG) between reactants and products. Since the products have a lower free energy than the reactants, this profile represents an exothermic reaction. ES represents the enzyme-stabilised transition state. (Note: free-hand drawings).

and cold storage can inactivate them. This is driven primarily by the loss of the stabilizing hydrophobic effect [4]. Enzymes that undergo large hydrophobic stabilization during folding are likely to denature more readily at low temperatures. Thus, most enzymes function optimally in a rather narrow temperature range and enzyme-catalysed reactions appear to deviate from the Arrhenius equation outside this range. Some enzymes do refold back correctly and retain function when they are restored to the optimum temperature, but many might undergo irreversible unfolding and denaturation upon a shift in temperature.

5.2. The role of pH in enzyme-catalysed reactions:

The effect of pH on the activity of enzymes is two-fold. Firstly, it can alter the conformational stability of the protein itself. This is because, the folding of the protein, its secondary, tertiary and quaternary structures, can be altered due to the protonation or deprotonation of amino acid side chains. Thus, extremes of pH often result in unfolding of proteins and loss in enzymatic activity. A plot of enzyme stability as a function of pH, therefore, will yield a bell-shaped curve (Fig. 4 A). This is a more general structural effect.

But pH may also specifically affect the active site and the catalytic activity of enzymes. This is because the side chains of specific amino acids within the active site often play a role in the actual process of catalysis. As shown in Fig. 1, the side chains of many amino acids are either polar or charged. In shallow or solvent-exposed active sites, small changes in pH can dramatically alter the protonated or deprotonated states of these catalytic residues and thereby affect activity. For example, enzymes that use a deprotonated Asp residue, will show good catalytic activity at pH > 4.0 (Fig. 4B). Similarly, enzymes that require a protonated His (pK_a ~ 6.0) for catalysis (Fig. 4B), would lose a significant fraction of activity if the pH of the solution increases from 5.0 to 7.0, even when it undergoes no major alteration in the conformational stability of its active site (Fig. 4B).

In order to emphasise how crucial pH is to the activity of an enzyme, it may be important to revisit the concept of pK_a itself. Let *HA* represent an acid, and K_a its dissociation constant in the following reaction:

$$HA \rightleftharpoons H^+ + A^- \hspace{2cm} \textit{Reaction (1)}$$

$$K_a = \frac{[A^-][H^+]}{[HA]}$$

Taking logarithms, $log\ K_a = log\ [H^+] + \frac{[A^-]}{[HA]}$ (V.2)

$$\Rightarrow -log\ K_a = -log\ [H^+] - log\ [A^-] \hspace{1cm} (V.3)$$

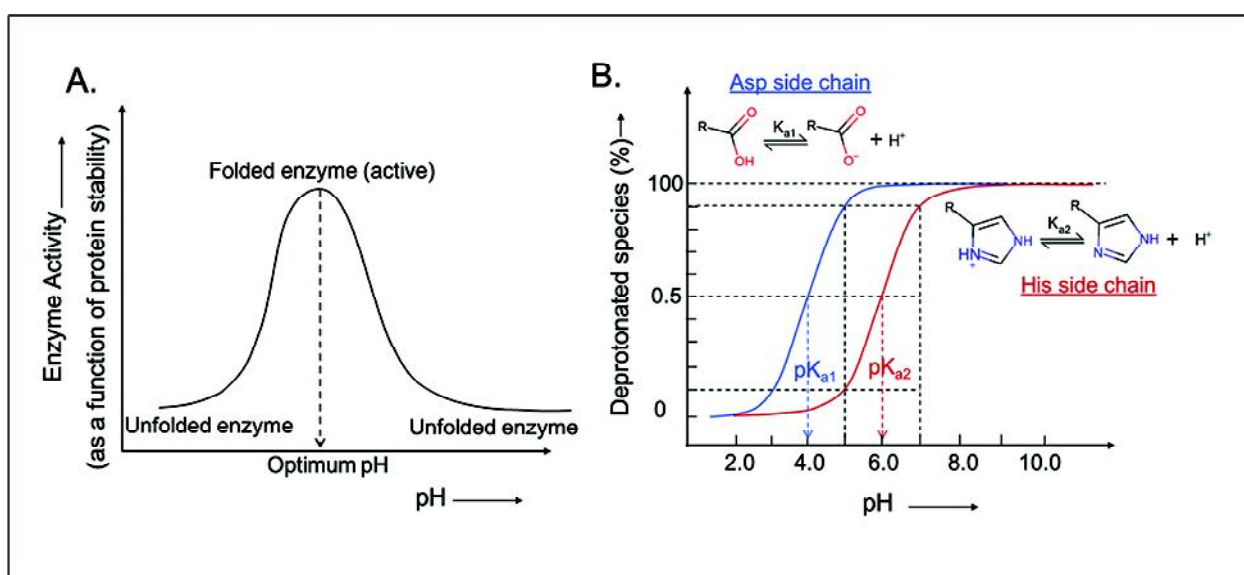$$\Rightarrow pK_a = pH - log\ \frac{[A^-]}{[HA]} \hspace{1.5cm} (V.4)$$

Fig. 4. **The effect of pH on the stability of an enzyme and on the dissociation of amino acid side chains. (A) The pH profile of the activity / stability of an enzyme**. Most enzymes are stable at moderate pH and unfold at extremes of pH. Since the folded state of the enzyme is important for its catalytic activity, the activity of the enzyme correlates with its stability. **(B) Protonation-deprotonation of amino acid side chains as a function of pH**. The dissociation of amino acid side chains of Asp and His and their corresponding pK_a values (the pH at which they show 50% deprotonation). (Note: free-hand drawings; the reactions are drawn using Marvin Sketch 22.11).

Or, $pH = pK_a + log \dfrac{[A^-]}{[HA]}$ (V.5)

(Henderson-Hasselbalch equation)

At 50% dissociation of the acid,

$[A^-] = [HA], \quad \Rightarrow \dfrac{[A^-]}{[HA]} = 1$ and $log \dfrac{[A^-]}{[HA]} = 0$

Hence, from equation (V.5), $pH = pK_a$

In other words, pK_a is the pH at which 50% dissociation of the acid occurs.

Let us rewrite *equation (V.5)* as, $pH - pK_a = log \dfrac{[A^-]}{[HA]}$ (V.6)

What if there is a ten-fold higher concentration of the deprotonated enzyme to the protonated species, i. e., if

$\dfrac{[A^-]}{[HA]} = 10$? This would imply, $log \dfrac{[A^-]}{[HA]} = 1$ and equation (V.6)

yields, $pH - pK_a = 1$.

Thus, at a pH that is 1 unit above the pK_a there is a ten-fold higher amount of dissociated acid to its undissociated form. On the other hand, at a pH that is 1 unit below the pK_a there would be a ten-fold higher amount of undissociated species to the dissociated acid. This implies that for an amino acid side chain that functions as a catalytic residue, a single unit change in pH causes a ten-fold difference in the amount of the charged or uncharged species available for catalysis. In the case of His, for instance, going from pH 5.0 to 7.0 would imply a drop from about 90% to 9% in the fraction of the protonated species available (Fig. 4B), and a corresponding drop in the activity of the enzyme.

The pK_a of the catalytic amino acid is also determined by other residues in its vicinity in 3D space. Thus, the pK_a for the deprotonation of an Asp may be pushed to a higher than normal pH if there are multiple acidic residues within its close proximity. Or the deprotonation of a His residue may be suppressed by an Asp residue in the vicinity. If the charged side chains of two amino acids participate in catalysis, then the optimum pH for the catalysis is likely to be the average of the pK_a of the two. Consider two amino acids, Asp and His in the active site of an enzyme, whose side chains participate in the catalysis and have dissociation curves as shown in Fig. 5. The optimum pH for activity in which a deprotonated Asp and a protonated His act together would be $(4 + 6) / 2$ or 5 (Fig. 5). Thus, the optimum pH
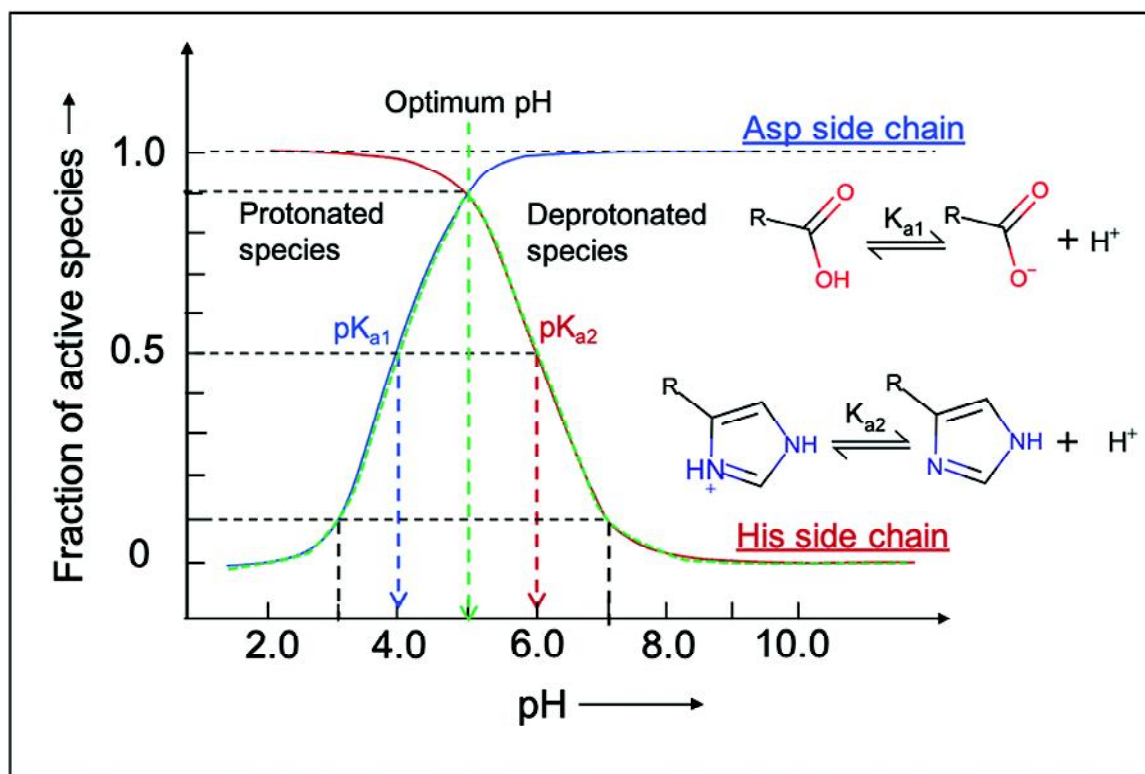

Fig. 5. The pH optimum for activity of an enzyme that uses deprotonated Asp and protonated His as catalytic residues. The blue curve shows the fraction of deprotonated Asp as a function of pH, while the red curve shows the fraction of protonated His species as a function of pH. The blue and red arrows represent, respectively, the pKa of the two amino acids. The green curve represents the optimum pH profile of the active site and the green arrow the optimum pH at which the catalytic activity would be a maximum. (Note: free-hand drawings; the reactions are drawn using Marvin Sketch 22.11).

for enzyme activity is the average of their combined pK_a values. Knowing the optimum pH for catalysis, therefore, often permits us to predict the identity of the catalytic residues involved.

6. Studying the kinetics of single substrate enzyme-catalysed reactions

As already explained above, an enzyme can bind to one or more substrates during a catalytic cycle and give rise to products. The simplest case involves binding of a single substrate molecule by the enzyme and its conversion to product. Not every binding event leads to a productive interaction, and it is possible that sometimes the substrate is released from the active site without conversion to product. Thus, one could assume that the first step, namely, the binding of the enzyme (E) to the substrate (S) to form the enzyme-substrate (ES) intermediate is a reversible process with two different rate constants, k_1 and k_{-1}. (Note, the finer distinction between a transition state and an intermediate is immaterial here). ES can also release the product in a reversible reaction as shown below:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} P + E \qquad \text{\textit{Reaction} (2)}$$
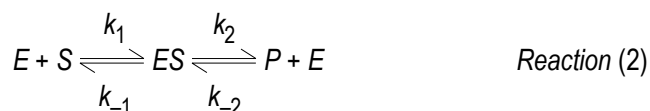
At early time points in the reaction, the concentration of S is high and that of P is negligible. In such conditions, the formation of ES from P will also be insignificant and can be ignored. Hence, one may further simplify the above equation **for early stages of the reaction**, by assuming that release of P from ES occurs in an irreversible step, such that the only rate constant we need to consider is k_2 as shown below:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \overset{k_2}{\longrightarrow} P + E \qquad \text{\textit{Reaction} (3)}$$
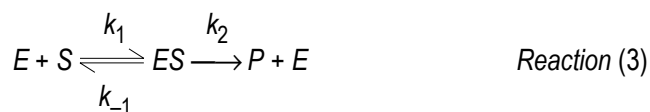
The initial rate of product formation ($\frac{dP}{dt}$, or, v) may then be written simply as,

$$v = k_2 [ES] \qquad (VI.1))$$

The rate equation now is dependent on $[ES]$ alone, and has a single rate constant k_2 (assuming a single pathway by which ES goes to E + P) corresponding to the unimolecular reaction.

Normally, the intermediate ES is transient and it would be hard to estimate $[ES]$ available at any point during the progress of the reaction. This makes it difficult to also predict the amount of product that may be formed at any given time point in the reaction, which is the purpose of developing a mathematical

model. One way to get around this challenge would be to make some more simplifying assumptions.

6.1. The equilibrium approximation:

A possible approximation, one that Michaelis and Menten used, is to assume that E and S establish an instantaneous equilibrium with the enzyme-substrate intermediate, ES. This is possible, only if $k_2 << k_{-1}$. In other words, we could presume an equilibrium exists for the first part of *Reaction* (3) above, such that the rates for the forward and reverse reactions may be represented as follows:

Rate of the forward reaction, $r_f = k_1[E][S]$ $\qquad (VI.2)$

Rate of the backward reaction, $r_b = k_{-1}[ES]$ $\qquad (VI.3)$

And the equilibrium condition would be represented by

$$r_f = r_b \qquad (VI.4)$$

Hence, $k_1[E][S] = k_{-1}[ES]$ $\qquad (VI.5)$

Or, $\dfrac{k_{-1}}{k_1} = \dfrac{[E][S]}{[ES]}$ $\qquad (VI.6)$

Thus, the dissociation constant for the binding of *S* to *E* is given by,

$$K_{ES} = \frac{[E][S]}{[ES]} \qquad (VI.7)$$

where $\qquad K_{ES} = \dfrac{k_{-1}}{k_1}$ $\qquad (VI.8)$

Rearranging equation ($VI.7$) gives

$$[ES] = \frac{[E][S]}{K_{ES}} \qquad (VI.9)$$

The term $[E]$ in equation ($VI.9$) refers to the concentration of E that is not bound to S, or the amount of free E available in solution. Determining $[E]$ at any time point in the reaction would normally be as difficult as determining $[ES]$ in solution. Hence, it would be better to try and express $[ES]$ in terms of total concentration of E i.e. $[E]_0$ rather than in terms of $[E]$.

So, we write: $[E] = [E]_0 - [ES]$ $\qquad (VI.10)$

Substituting for $[E]$ in equation ($VI.9$) gives

$$[ES] = \frac{([E]_0 - [ES])[S]}{K_{ES}} \qquad (VI.11)$$

Rearranging, $[E]_0 [S] = [ES] (K_{ES} + [S])$ $\qquad (VI.12)$

$$[ES] = \frac{[E]_0 [S]}{K_{ES} + [S]} \qquad (VI.13)$$

Substituting for [ES] from *equation* (*VI*.13) in *equation* (*VI*.1) yields

$$v = \frac{k_2[E]_0 \, [S]}{K_{ES} + [S]} \qquad (VI.14)$$

Now, consider the case for *Reaction* 3 where the concentration of free or unbound E in solution is negligible, for example, when [S] is high (by Le Chatelier's principle) and when $k_1 \gg k_{-1}$ (substrate binds very tightly to the enzyme and does not dissociate readily). In such a case, $[ES] \approx [E]_0$ and corresponds to the condition where maximum rate of product formation is achieved. This rate is often referred to as the "limiting velocity" or "maximum velocity" (v_{max}) for the reaction. Thus, *equation* (*VI*.1) becomes

$$v_{max} = k_2[E]_0 \qquad (VI.15)$$

This converts *equation* (*VI*.14) to the original form of the rate equation derived by Michaelis and Menten:

$$v = \frac{v_{max} \, [S]}{K_{ES} + [S]} \qquad (VI.16)$$

6.2. The steady-state hypothesis and its comparison with the equilibrium approximation:

An alternative model for the study of enzyme-catalysed reactions, is the steady state hypothesis proposed by Briggs and Haldane. Here too, using the same initial arguments as above, we may begin once again with the simplified form of the chemical reaction (*Reaction* (3)):

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} P + E$$
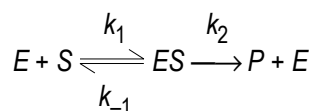
However, we no longer need to assume that a pre-equilibrium exists in the first half of the reaction. Instead, we assume that very early in the reaction process, a steady concentration of [ES] is established. Mathematically, this would be written as

$$\frac{d[ES]}{dt} = 0 \qquad (VI.17)$$

i.e. the rate of change of [ES] is zero during the early period of the reaction.

One cannot reiterate two points enough: 1) The steady state is not a condition of equilibrium. 2) The steady state approximation holds only at early time points in the reaction, when [S] is high enough to ensure that whenever ES breaks down to either E + P, or to E+ S, another molecule of S is available to take its place in the active site of E and convert it back to ES. At late time points in the reaction, as [S] drops, the steady state condi-

tion no longer prevails. Thus, the time at which a reaction is being studied is an important aspect of the steady state model. As long as we are considering initial rates of reactions, the steady state approximation can be used.

The steady state approximation, represented by *equation* (*VI*.17), leads us to the following equation:

Rate of the formation of ES = Rate of breakdown of ES into either E + S or E + P.

Mathematically this is written as,

$$k_1[E][S] = k_{-1}[ES] + k_2[ES] \qquad (VI.18)$$

$$k_1[E][S] = (k_{-1} + k_2) \, [ES] \qquad (VI.19)$$

Rearranging, gives $[ES] = \dfrac{k_1}{(k_{-1} + k_2)} [E][S] \qquad (VI.20)$

Briggs and Haldane defined the constant, $\dfrac{k_{-1} + k_2}{k_1}$, as K_M, the Michaelis-Menten constant, to honour their seminal contributions to the field of enzyme kinetics.

Therefore, using $K_M = \dfrac{k_{-1} + k_2}{k_1} \qquad (VI.21)$

equation (*VI*.20) becomes $[ES] = \dfrac{[E][S]}{K_M} \qquad (VI.22)$

And *equation* (*VI*.1) may be written as,

$$v = \frac{k_2}{K_M} [E][S] \qquad (VI.23)$$

(an equation that we will return to in section 6.5 of this article).

Using the same arguments as in the Section above, the definition of [E] from *equation* (*VI*.10) allows us to rewrite equation (*VI*.22) as,

$$[ES] = \frac{([E]_0 - [ES])[S]}{K_M} \qquad (VI.24)$$

This equation may be rearranged to

$$[ES] = \frac{[E]_0[S]}{K_M + [S]} \qquad (VI.25)$$

Now, substituting for [ES] in *equation* (*VI*.1) gives

$$v = \frac{k_2[E]_0[S]}{K_M + [S]} \qquad (VI.26)$$

Using the definition of v_{max} from *equation* (*VI*.15), we arrive

at the Briggs-Haldane version of the Michaelis-Menten equation that is now more commonly used for the study of enzyme kinetics:

$$v = \frac{v_{max}\,[S]}{K_M + [S]} \qquad (VI.27)$$

A point to be noted here is that, like $[E]$, $[S]$ too refers to the concentration of free substrate in solution and the relation $[S] = [S]_0 - [ES]$ should apply (where $[S]_0$ is the original concentration of S). But normally at early time points of the reaction, the initial substrate concentration is much higher than the initial enzyme concentration ($[S]_0 \gg [E]_0$). Therefore, $[ES]$ is insignificant in comparison to $[S]_0$ even when $[ES] \approx [E]_0$. So, we can assume that $[S] \approx [S]_0$. This is why, $[S]$ in *equation* (*VI*.27) is often assumed to be the initial substrate concentration itself. This assumption is not valid at late time points in the reaction, and must be kept in mind for any analysis of enzyme kinetics data.

6.3. Comparison between K_M and K_{ES} :

Comparing *equations* (*VI*.27) and (*VI*.16), we see that the two appear to be very similar, with K_M replacing K_{ES} in the former. Indeed, a simple look at $K_M = \dfrac{k_{-1} + k_2}{K_1}$ (from *equation*

(*VI*.21)), and $K_{ES} = \dfrac{k_{-1}}{K_1}$ (from *equation* (*VI*.8)) shows that

when $k_{-1} \gg k_2$, $K_M = K_{ES}$ $\qquad$ (VI. 28)
and the two equations would become identical.

Thus, at very early times in the reaction, or when product formation is low, the dissociation constant for the enzyme-substrate complex, K_{ES}, may substitute for K_M. It would be apparent why K_M is often used to obtain a measure of the affinity of the enzyme for its substrate. The higher the value of K_M, the poorer is its affinity for the substrate and greater the chance of unproductive dissociation of ES. However, larger the value of k_2, greater is the deviation of K_M from K_{ES}. It should also be noted, that K_M is always higher than K_{ES}.

Both kinds of Michaelis-Menten equations yield rectangular hyperbola plots for *v* versus $[S]$ when $[E]$ is fixed. From the initial phase of the plot in Fig. 6A, we note the linear first-order relationship between the rate of the reaction and $[S]$. However, beyond a certain value of $[S]$ the rate of the reaction tends to reach a constant value, or becomes independent of the amount of S in the reaction. In other words, the order of the reaction becomes zero, when $[S]$ is far in excess of $[E]_0$.

Depending on whether the equilibrium or the steady state approximation is used, we can obtain K_M or K_{ES} from these plots

as the concentration of the substrate at which half the maximal rate or velocity (v_{max}) is obtained ($K_M = [S]$ or $K_{ES} = [S]$). This is easily derived theoretically by setting $v = \dfrac{v_{max}}{2}$ in *equation* (*VI*.27) or *equation* (*VI*.16).

The units of K_M or K_{ES} are the same as that of $[S]$. They are convenient to work with, especially when setting up enzyme-based assays, since one can directly estimate the concentrations of substrate required. This probably explains why biochemists conventionally prefer to use dissociation constants in all their analysis, even though the terms are inversely related to affinity of the enzyme / protein for its substrate / ligand, unlike the association constants which most chemists favour.

6.4. Linear plots for enzyme kinetics:

The Michaelis-Menten plots are often difficult to fit accurately, especially when saturation is hard to reach. In such cases extrapolating the graph to obtain v_{max} might often lead to erroneous results, although several excellent computational programs with different fitting algorithms exist. In Fig. 6A(ii), for example, only line (a) corresponds to the value of v_{max} obtained using computational fitting programs (listed in Fig. 6A(iii)). This would have been hard to predict in a manual fit of the data. Easy mathematical methods for linearisation of the Michaelis-Menten equation exist, which can help circumvent the problem. Each comes with its own sets of advantages and limitations, which must be kept in mind while using them. Three such methods are discussed below in more detail.

6.4.1. *The Lineweaver-Burk Equation:* The Michaelis-Menten equation is linearised simply by inverting it as below:

$$\frac{1}{v} = \frac{K_M}{v_{max}} \cdot \frac{1}{[S]} + \frac{1}{v_{max}} \qquad (VI.29)$$

A plot of $\dfrac{1}{v}$ vs. $\dfrac{1}{[S]}$ produces a linear plot with $\dfrac{1}{v_{max}}$ as the

y-intercept and $\dfrac{K_M}{v_{max}}$ as the slope (Fig. 6B). K_M can also be

obtained from the intercept, $\dfrac{-1}{K_M}$, on the x-axis.

While the Lineweaver-Burk is an extremely useful plot, it does suffer from the common problems of all double-reciprocal plots, including uneven spacing of data. The initial data points from the Michaelis-Menten plots (at low $[S]$) appear widely spaced in the Lineweaver-Burk plots while the data points close to saturation appear bunched together (compare Fig. 6A(ii) with Fig. 6B).
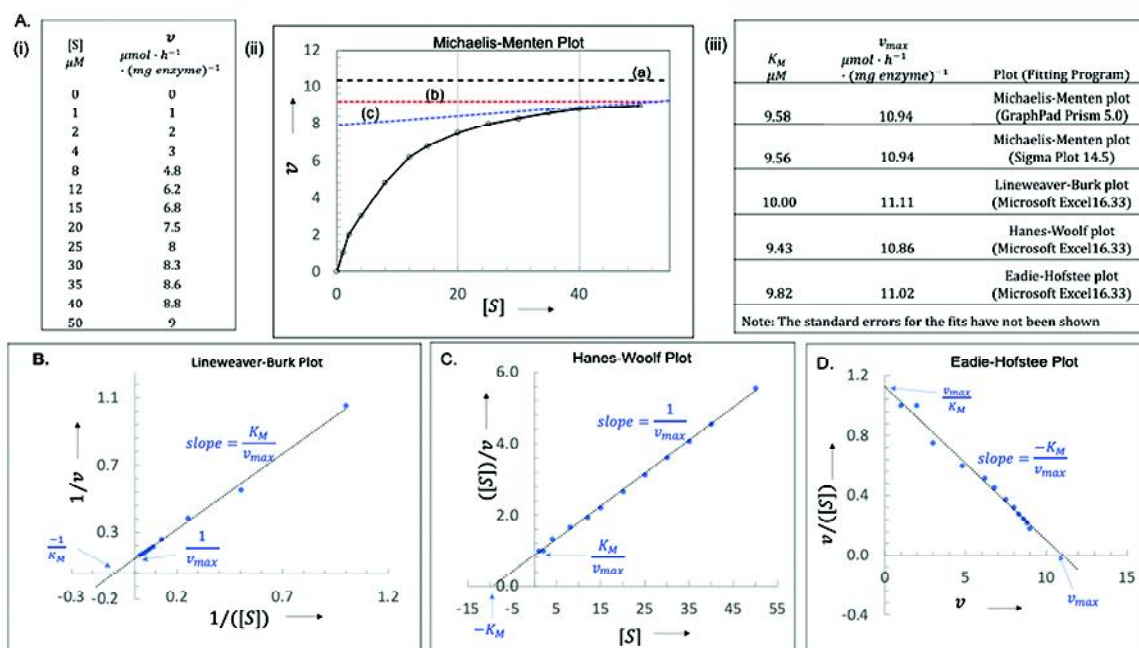
Fig. 6. A. Michaelis-Menten Plot: Data (shown in Table A(i)) for a single-substrate enzyme-catalysed reaction is fit to the Michaelis-Menten equation. It yields a rectangular hyperbola plot irrespective of whether the equilibrium or steady-state approximations are used. The lines (a), (b) and (c) represent possible extrapolations of the fits to obtain v_{max} when fitting the data manually. Since the value of v_{max} in the graph determines $\frac{v_{max}}{2}$, and therefore our estimate of K_M, the manner in which the extrapolation is done is important to understand. The values for K_M and v_{max} obtained computationally, from two different non-linear regression analysis software, is given in A(iii). **B. Lineweaver-Burk Plot:** For the data given in Table A(i), the Lineweaver-Burk plot was obtained. Unlike in the Michaelis-Menten plot where the data points are evenly spaced, here we notice the uneven spacing of data points and the bunching of data points close to saturation. The v_{max} and K_M obtained from the graph are given in A(iii). **C. Hanes-Woolf Plot:** For the same set of data, the Hanes-Woolf plot was obtained. Unlike in the Lineweaver-Burk plot here the spacing of data points is better. The v_{max} and K_M values obtained from the graph are listed in A (iii). **D. Eadie-Hofstee Plot:** For the same data, the Eadie-Hofstee plot is drawn. Here too the spacing of data points is better than in the Lineweaver-Burk plot. The v_{max} and K_M values obtained are listed in A (iii).

Deciding on the best possible linear fit in such cases becomes challenging and the number of data points corresponding to low substrate concentrations become very crucial in determining the slopes for these plots. At low [S] when product formation is low, the sensitivity of detection and accuracy of the measurement may be lower than at high [S], which can lead to very serious errors in the estimation of K_M and v_{max}.

6.4.2. The Hanes-Woolf Equation: The Hanes-Woolf equation is arrived at by a simple rearrangement of the Lineweaver-Burk equation (*VI*.29) to:

$$\frac{[S]}{v} = \frac{[S]}{v_{max}} + \frac{K_M}{v_{max}} \qquad (VI.30)$$

The graph obtained, for $\frac{[S]}{v}$ vs. [S] will be a semi-reciprocal linear one now, with $\frac{1}{v_{max}}$ as the slope, $\frac{K_M}{v_{max}}$ as the y-inter-

cept and $-K_M$ as the x-intercept (Fig. 6C). Since only values of v are reciprocal and not of [S], the data points are more evenly spaced than in Fig. 6B. However, having [S] on both axes implies that any error in the estimation of [S] will affect the data on both axes.

6.4.3. The Eadie-Hofstee Equation: The Eadie-Hofstee plot too is a semi-reciprocal plot obtained by a rearrangement of either the Lineweaver-Burk or the Hanes-Woolf equations. It has the same strengths and weaknesses as the Hanes-Woolf plot. Multiplying *equation* (*VI*.30) with ($v \cdot v_{max}$), and rearranging, gives the popular form of the Eadie-Hofstee equation:

$$v = -K_M \cdot \frac{v}{[S]} + v_{max} \qquad (VI.31)$$

A plot of v versus $\frac{v}{[S]}$ will have $-K_M$ as the slope, v_{max} as y-intercept and $\frac{v_{max}}{K_M}$ as the x-intercept (Fig. 6D). Since this plot

has v on both axes, any error in the estimation of v affects the data on both axes.

6.5. Catalytic efficiency:

For a single-substrate enzyme-catalysed reaction of the kind that we began with in *Reaction* (3), the rate equation for catalysis is given by *equation* (*VI*.1). Two factors determine the rate of the reaction: [*ES*] and k_2. But the concentration of ES is determined by K_M. The rate constant, k_2, is called the catalytic rate constant and frequently replaced by k_{cat} in discussions about the catalytic efficiency of the enzyme. The units of k_{cat} are s^{-1}, and it represents the number of molecules of product formed per second per molecule of enzyme. Thus, k_{cat} is also called the **turnover number**. It can range from as low as 1 s^{-1} to as high as 10^4 s^{-1}. In order to understand how the two factors, K_M and k_{cat}, determine the final catalytic efficiency of the enzyme, we return to *equation* (*VI*.23), where we replace k_2 by k_{cat} to obtain:

$$v = \frac{k_{cat}}{K_M} [E][S] \qquad (VI.32)$$

The ratio $\dfrac{k_{cat}}{K_M}$ will determine the efficiency of the process, and is called the **catalytic efficiency** of the enzyme.

If k_{cat} is low, the efficiency of the enzyme acting on a substrate is low. If K_M is high, then too the catalytic efficiency is low. An enzyme with extremely high catalytic efficiency must have high k_{cat} and low K_M. For K_M this is only possible when k_1 is high too and k_{-1} is very small in *equation* (*VI*.21). In other words, every encounter between the enzyme and its substrate must be a productive one. Several such enzymes are known, which act almost instantaneously upon their substrates, converting them to products. This was first reported for the decomposition of urea by urease [5]. Catalase, acetylcholine esterase and carbonic anhydrase are some other enzymes that show rapid turnover numbers, and catalytic efficiencies approaching the limit of diffusion (10^8 to 10^9 mol·l^{-1}·s^{-1}). It is worth noting that the condition $k_2 > k_{-1}$, is the second special case of the Briggs-Haldane model and corresponds to $K_M \neq K_{ES}$.

7. Inhibition and regulation of enzyme activity in single-substrate enzyme-catalysed reactions

The activity of enzymes may be regulated in many ways. For example, the concentration of substrate can determine the rate of catalysis, as shown above. As the concentration of the product begins to build-up, it may begin to compete for the substrate binding site. There are also substrate-mimetics, transition state analogues, drugs, toxins, or allosteric regulators that can alter catalytic rates.

Inhibitors, as the name suggests, work to inhibit the activity of the enzyme. Many naturally occurring reversible and irreversible inhibitors of enzymes are known. Reversible inhibitors establish an equilibrium in solution, either directly with the enzyme, or with the ES intermediate or with both.

7.1. Competitive inhibitors:

Inhibitors that compete directly with the substrate for binding to an enzyme are called competitive inhibitors (Fig. 7A(i)). The effect of such an inhibition is to reduce the concentration of the enzyme-substrate intermediate (ES) available for catalysis. In such cases, increasing the concentration of S will help out-compete the inhibitor. In other words, when [S] is very high, the inhibitor binding will become negligible and the maximal velocity can still be attained. This implies that v_{max} is not affected. However, since higher [S] is used, it will appear as though the affinity of the enzyme is reduced or the equilibrium has been altered. Hence, the new apparent K_M (or K'_M) will be higher ($K'_M > K_M$).

This qualitative argument can also be developed mathematically as follows. For the model given in Fig. 7A(i), the total enzyme concentration is given by

$$[E]_0 = [E] + [ES] + [EI] \qquad (VII.1)$$

$$\Rightarrow [E]_0 = [E] + [ES] + \frac{[E][I]}{K_{EI}} \qquad (VII.2)$$

where, K_{EI} is the dissociation constant for the enzyme-inhibitor complex.

Rearranging *equation* (*VII*.2) to obtain [*E*] in terms of [*E*]$_0$, [*ES*] and [*I*], leads to

$$[E] = \frac{[E]_0 - [ES]}{\left(1 + \dfrac{[I]}{K_{EI}}\right)} \qquad (VII.3)$$

From *equation* (*VI*.22) we know, $[ES] = \dfrac{[E][S]}{K_M}$, hence, we substitute for [*E*] from *equation* (*VII*.3) to get

$$[ES] = \frac{([E]_0 - [ES])[S]}{K_M \left(1 + \dfrac{[I]}{K_{EI}}\right)} \qquad (VII.4)$$

$$\Rightarrow [ES] = \frac{[E]_0[S]}{K_M \left(1 + \dfrac{[I]}{K_{EI}}\right) + [S]} \qquad (VII.5)$$
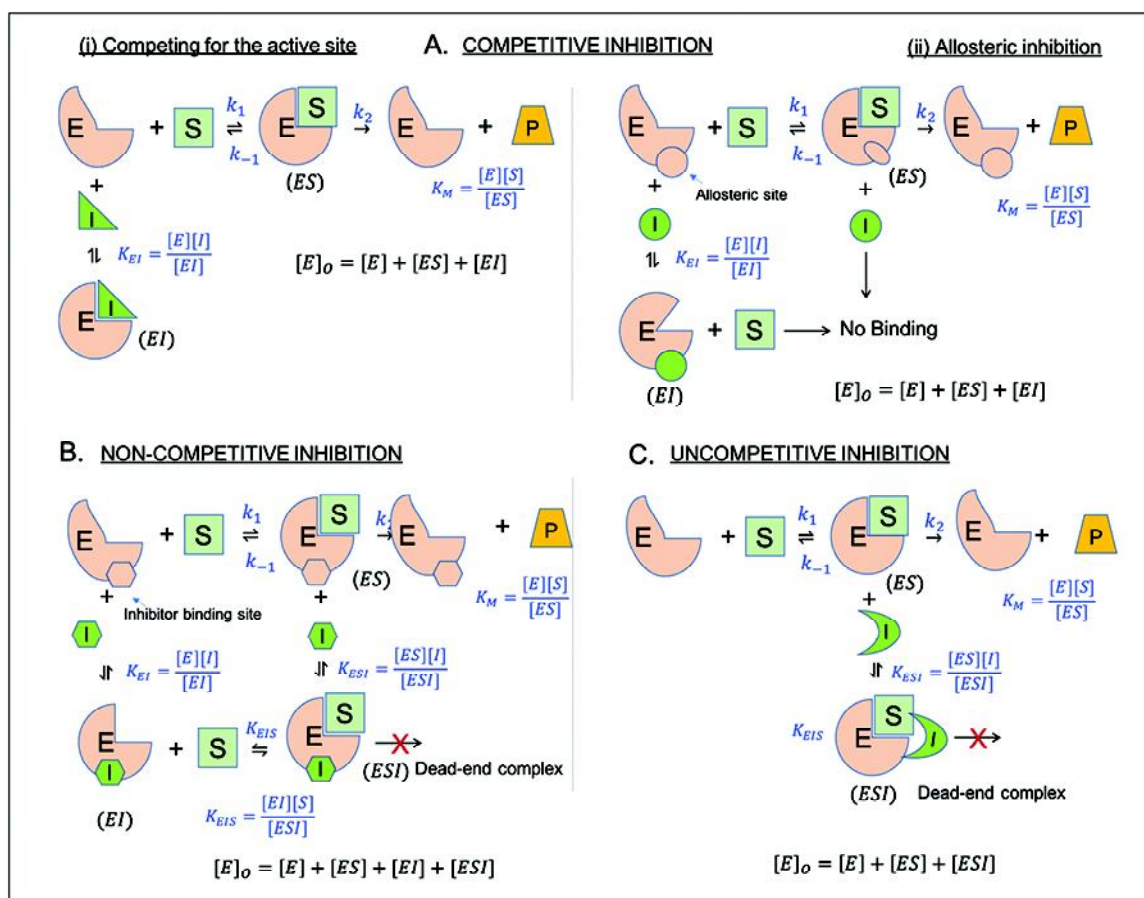
Fig. 7. Models for reversible inhibition of an enzyme: A. Competitive Inhibitor. (i) The inhibitor (I) competes with the substrate (S) for the active site of the enzyme (E), thus the binding of one of them blocks the other. (ii) Inhibitor binds at an allosteric site, 'A', on E, and causes a conformational change due to which S is unable to access the active site. The converse may also occur. Thus, binding of either S or I prevents the other from binding to E, hence they still appear to be competitive in their binding. **B. Non-competitive inhibition.** The binding of I and S can occur independent of each other and at different sites. However, once I is bound at site 'B', the enzyme can no longer process the bound S and the resultant complex is a dead-end complex. **C. Uncompetitive inhibition.** The inhibitor does not bind to the native enzyme or S directly, but binds to the enzyme-substrate intermediate (ES). The inhibitor may bind to sites on either E or S or both (as shown here), such that the enzyme is unable to catalyse conversion of S to P.

Let us assume, $K'_M = K_M \left(1 + \dfrac{[I]}{K_{EI}}\right)$ (*VII*.6)

(Note, if $[I] = 0$, then $K'_M = K_M$ and when $[I] \neq 0$, $K'_M > K_M$) Now we can write *equation* (*VI*.1) as

$v = k_2 [ES] = \dfrac{k_2 [E]_0 [S]}{K'_M + [S]}$ (*VII*.7)

From earlier arguments, it follows that

$v = \dfrac{v_{max} [S]}{K'_M + [S]}$ (*VII*.8)

Since [I] must be of the same order of magnitude as [S] in order

to be able to compete for the active site, and they should both be much higher than $[E]_0$ for establishing a steady state concentration of ES, we can also assume that $[S] \approx [S]_0$ and $[I] \approx [I]_0$. *Equation* (*VII*.8) very closely resembles the Michaelis-Menten equation (*equation* (*VI*.27)), except that K_M is replaced by K'_M.

A special case of competitive inhibition is when an inhibitor binds to a site away from the substrate binding site (**allosteric site**) but still inhibits substrate binding (the converse, would be true too: the binding of S to E would inhibit the binding of the inhibitor). Such inhibitors are competitive allosteric inhibitors (Fig. 7A(ii)). The equation for this will remain the same as above (*equation* (*VII*.8)).

7.2. Non-competitive inhibitors:

Some inhibitors may bind to an enzyme at a site away from the substrate binding site and may not affect substrate binding *per se*. Instead, they inhibit the processing of the substrate into product(s). Such inhibitors are called non-competitive inhibitors. Since they do not affect substrate binding, the K_M for the substrate will remain unaltered and increasing the concentration of S cannot out-compete the inhibitor. However, the apparent v_{max} (or v'_{max}) will be reduced. As with competitive inhibitors, we could begin building a mathematical formalism for such a model by assuming that the total enzyme concentration,

$$[E]_0 = [E] + [ES] + [EI] + [ESI] \tag{VII.9}$$

Writing for $[EI]$ and $[ESI]$ in terms of their dissociation contants we get

$$[E]_0 - [ES]\left(1 + \frac{[I]}{K_{ESI}}\right) = [E]\left(1 + \frac{[I]}{K_{EI}}\right) \tag{VII.10}$$

If the inhibitor binding is not affected by the binding of the substrate, we can assume that its affinity for E and ES are the same. Therefore, $K_{ESI} = K_{EI}$

$$\Rightarrow [E] = \frac{[E]_0}{\left(1 + \frac{[I]}{K_{EI}}\right)} - [ES] \tag{VII.11}$$

Substituting for $[E]$ in $[ES] = \dfrac{[E][S]}{K_M}$ (from *equation* (*VI*.22))

and rearranging, we get

$$[ES] = \frac{[E]_0[S]}{K_M\left(1 + \frac{[I]}{K_{EI}}\right)\left(1 + \frac{[S]}{K_M}\right)} \tag{VII.12}$$

Substituting this in *equation* (*VI*.1),

$$v = k_2[ES] = \frac{\dfrac{k_2[E]_0}{\left(1 + \dfrac{[I]}{K_{EI}}\right)}[S]}{K_M + [S]} \tag{VII.13}$$

This leads us to the modified Michaelis-Menten equation

$$v = \frac{v'_{max}[S]}{K_M + [S]} \tag{VII.14}$$

where, $v'_{max} = \dfrac{v_{max}}{\left(1 + \dfrac{[I]}{K_{EI}}\right)}$ $\qquad(VII.15)$

(Note, $v'_{max} = v_{max}$ if $[I] = 0$; $v'_{max} < v_{max}$ if $[I] \neq 0$)

7.3. Uncompetitive inhibitors:

Inhibitors that don't bind to the enzyme but to the ES intermediate and prevent catalysis are called uncompetitive inhibitors. This happens because substrate binding causes a conformational change that exposes site(s) for inhibitor binding either on the protein or on the substrate, or both. Since the inhibitor doesn't compete with the substrate for binding, the inhibition can't be overcome by increasing the substrate concentration and apparent v_{max} is less than expected ($v'_{max} < v_{max}$). But because it binds to the ES intermediate, it also shifts the equilibrium between E and S more towards ES, making it seem as though the enzyme has a greater affinity for S. In other words, K'_M appears to be lower than K_M.

Mathematically, we would write the following equations for this model:

$$[E]_0 = [E] + [ES] + [ESI] \tag{VII.16}$$

$$[E]_0 = [E] + [ES] + \frac{[ES][I]}{K_{ESI}} \tag{VII.17}$$

$$[E] = [E]_0 - [ES]\left(1 + \frac{[I]}{K_{ESI}}\right) \tag{VII.18}$$

Once again, we substitute for $[E]$ in *equation* (*VI*.22) and obtain

$$[ES] = [E]_0\frac{[S]}{[K_M]} - [ES]\left(1 + \frac{[I]}{K_{ESI}}\right)\frac{[S]}{[K_M]} \tag{VII.19}$$

And hence,

$$[ES] = \frac{[E]_0[S]}{K_M\left(1 + \frac{[S]}{K_M}\left(1 + \frac{[I]}{K_{ESI}}\right)\right)} \tag{VII.20}$$

Substituting in *equation* (*VI*.1) as before

$$v = \frac{k_2[E]_0[S]}{K_M + [S]\left(1 + \frac{[I]}{K_{ESI}}\right)} \tag{VII.21}$$

Writing for v_{max} as before and dividing numerator and denominator by $\left(1 + \dfrac{[I]}{K_{ESI}}\right)$ yields

$$v = \frac{\dfrac{v_{max}}{\left(1 + \dfrac{[I]}{K_{ESI}}\right)}[S]}{\dfrac{K_M}{\left(1 + \dfrac{[I]}{K_{ESI}}\right)} + [S]} \qquad (VII.22)$$

Replacing $v'_{max} = \dfrac{v_{max}}{\left(1 + \dfrac{[I]}{K_{ESI}}\right)}$, and $K'_M = \dfrac{K_M}{\left(1 + \dfrac{[I]}{K_{ESI}}\right)}$,

we get

$$v = \frac{v'_{max}[S]}{K'_M + [S]} \qquad (VII.23)$$

(Note, $v'_{max} = v_{max}$ and $K'_M = K_M$ if $[I] = 0$; $v'_{max} < v_{max}$ and $K'_M < K_M$ if $[I] \neq 0$).

It is quite straightforward now to linearise by inversion and arrive at the Lineweaver-Burk equations and plots (Fig. 8) for all the modified Michaelis-Menten equations for reversible inhibition. The equations are also given in the legends to Fig. 8.

7.4. Irreversible inhibitors:

These inhibitors are also sometimes called **suicide inhibitors**. They covalently bind to and inhibit the enzyme, such that it is no longer able to process its substrates. Effectively, this is equivalent to reducing the concentration of enzyme available for the reaction and plots for these kinds of inhibitions resemble the non-competitive inhibition reactions.

7.5. Allosteric regulators of enzyme activity:

Unlike inhibitors which shut down enzyme activity, some regulators of enzymes only modulate catalytic rates, either positively or negatively. In fact, many of those that reduce activity, are comparable to non-competitive inhibitors. They bind at sites away from substrate binding and induce a conformational change which reduces the affinity of the enzyme for its substrate. Allosteric activators of enzymes, on the other hand, induce a conformational change upon binding that activates the enzyme. Thus, we have both negative and positive allosteric regulators which co-operate either positively or negatively in substrate binding. They
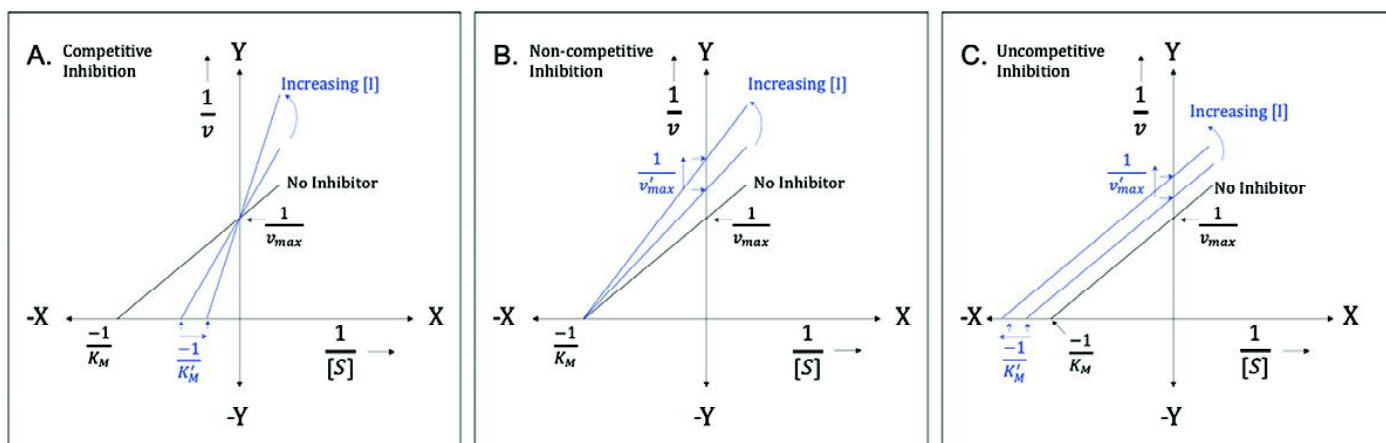

Fig. 8. Lineweaver-Burk plots for reversible inhibition of an enzyme. A. Competitive Inhibitor. The Lineweaver-Burk equation for this plot is given by, $\dfrac{1}{v} = \dfrac{K'_M}{v_{max}} \cdot \dfrac{1}{[S]} + \dfrac{1}{v_{max}}$. In the presence of the inhibitor, only K_M increases to K'_M, but v_{max} is unaltered. Hence, increasing $[S]$ out-competes the inhibitor. **B. Non-competitive Inhibitor.** The Lineweaver-Burk equation for this plot is given by $\dfrac{1}{v} = \dfrac{K_M}{v'_{max}} \cdot \dfrac{1}{[S]} + \dfrac{1}{v'_{max}}$. In the presence of the inhibitor, v_{max} decreases to v'_{max}, but K_M is unaffected. Hence, increasing $[S]$ cannot out-compete the inhibitor. **C. Uncompetitive Inhibitor.** The Lineweaver-Burk equation for this plot is given by, $\dfrac{1}{v} = \dfrac{K'_M}{v'_{max}} \cdot \dfrac{1}{[S]} + \dfrac{1}{v'_{max}}$. In the presence of the inhibitor, v_{max} reduces to v'_{max} and K_M increases to K'_M. Increasing $[S]$ cannot out-compete the inhibitor.
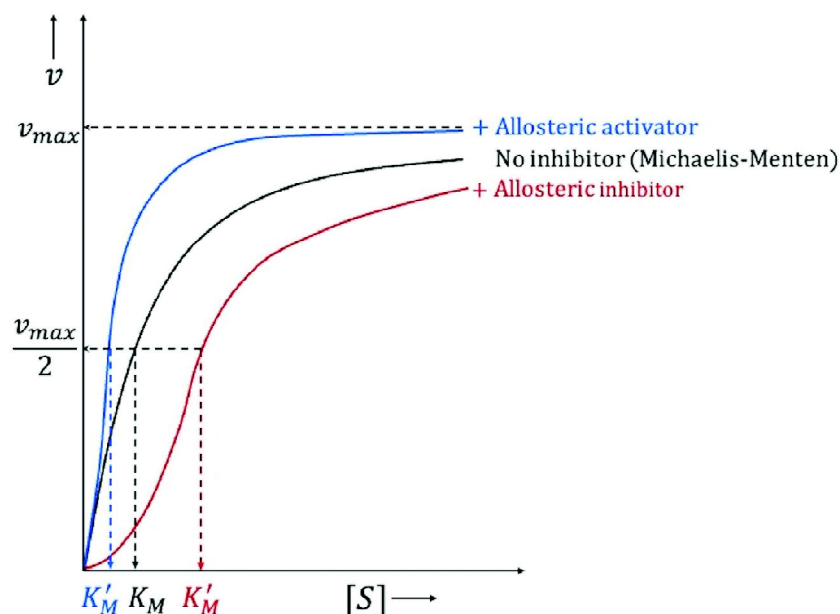
Fig. 9. Alteration of the Michaelis-Menten plot due to allosteric regulation of an enzyme: An allosteric regulator binds to a site away from the active site of the enzyme and induces a conformational change in it which might enhance or reduce catalytic activity. In the presence of a fixed concentration of an allosteric activator the binding of the substrate to the enzyme improves (positive cooperativity). This reduces K_M but does not alter v_{max}. However, v_{max} is attained at a lower [S]. In the presence of an allosteric inhibitor K_M is increased and it is harder to reach v_{max} (negative cooperativity). (Note: free-hand drawings).

produce Michaelis-Menten graphs with positive or negative **cooperativity** as shown in Fig. 9.

Many allosteric proteins and enzymes have multiple substrate-binding sites or multiple subunits, where binding of a substrate molecule at one site / subunit may increase (or decrease) the affinity of the other sites / subunits for the ligand. Thus, the interaction between substrate binding sites may be the reason for cooperative effects. In order to obtain rectangular hyperbolic plots for allosteric reactions, the Michaelis-Menten model will not do. We need to use other approximations / models, that result in equations such as the Hill's (for the simplest model), Monod-Wyman-Changeux, Koshland-Nemethy-Filmer or the Adair (for a more comprehensive model) equations. The interested reader is referred to the list of Reference Textbooks at the end of this article for further reading.

8. Applications of Enzymes in Medicine, Biology and Chemistry

Enzyme assays and enzyme-based assays are the cornerstone of clinical biochemistry (for more information, please see the list of Reference Textbooks given). From standard blood biochemistry panels that involve activity assays for several enzymes, to identifying rare genetic disorders involving mutations in a single enzyme, clinical biochemistry has come to routinely rely on enzyme assays as a diagnostic tool. For example, a

routine test for liver function involves assaying the activity of lactate dehydrogenase (LDH; EC 1), serum glutamate-pyruvate transaminase (SGPT) or what is now more preferably called alanine transaminase (EC 2), serum glutamic-oxaloacetic transaminase (SGOT; or aspartate transaminase; EC 2), gamma-glutamyltransferase (GGT; EC 2) and alkaline phosphatase (ALP; EC 3). Tests for trypsin (EC 3) and lipases (EC 3) are used to screen for pancreatic disorders. Some haemolytic disorders involve deficiencies in enzymes such as glucose-6-phosphate dehydrogenase (G-6-PD; EC 1) or pyruvate kinase (PK; EC 2). Phenylketonuria, a rare inherited genetic disorder is identified by detecting the levels of phenylalanine in the blood by using the enzyme phenylalanine dehydrogenase (EC 1). Enzyme-linked immunosorbent assays (ELISA) to identify infections frequently use peroxidases (EC 1) with chromogenic reporter molecules. Host and viral proteases have been of great interest in recent times because of the prominent role they play in the pathogenesis of SARS-Cov2, the organism responsible for the COVID-19 pandemic (see for example, [6]). Enzymes are also used for the treatement of many medical conditions. Common examples include, the dissolution of blood clots due to a stroke or a heart attack and to assist in digestion [7].

Enzyme inhibitors too have much clinical significance. They are normally the first candidates to be examined when seeking

new drugs. Many inhibitors are already much-celebrated drugs. Penicillin, and penicillin-like antibiotics, are classic examples. They block bacterial growth by inhibiting the D,D-transpeptidases (EC 3) required for their cell wall peptidoglycan synthesis. Similarly, nonsteroidal anti-inflammatory drugs (NSAID) like ibuprofen are used for treatment of inflammation and pain. They work by blocking the production of prostaglandins by cyclooxygenases (COX; EC 1). The triazole drugs currently used for antifungal treatment are inhibitors of lanosterol 14-α-demethylase, a key enzyme the ergosterol biosynthetic pathway of fungi. Dinitrophenol (DNP) was once touted as a "safe" weight-control pill, even though a small difference in concentrations was all that separated its efficacy from lethality! It works by uncoupling oxidative phosphorylation (the synthesis of ATP from ADP and inorganic phosphate) from the electron transport chain of respiration, so that the energy generated during respiration is not funnelled into ATP production [8]. This forces the body to compensate by using up stored carbohydrates / fats to produce ATP. The drug was later labelled as dangerous as its many side effects became known.

Biochemists routinely use enzymes in the laboratory for a number of applications. Some common examples include use of peroxidases (EC 1) in ELISA and western blot assays, reverse transcriptase (EC 2) for reverse transcription-polymerase chain reaction (RT-PCR), DNA polymerases (EC 2) for gene amplification in PCR, endonucleases or restriction digestion enzymes (EC 3) for gene cloning and ligases (EC 6) for inserting a DNA fragment into a larger vehicle / vector. In mass spectrometric applications, glycosidases (EC 3) are used to remove protein glycosylation and lyases (EC 4) to obtain detailed structural information of complex carbohydrates such as chondroitin sulphate. Similarly, trypsin or other proteases (EC 3) are used for protein fragmentation and peptide analysis. Inhibitors enable the identification of transition states and reaction mechanisms. For example, vanadate may be used to trap the transition state of ATP hydrolysis to obtain mechanistic details of maltose transport by the *E. coli* maltose transporter, an EC 7 enzyme [9].

Despite the fact that chemists were at the forefront of the discovery and studies of enzymes, they have not been used as extensively in the chemistry lab as they have been in biochemistry. Nevertheless, their applications are picking up. Chemists use "biocatalysis" for obtaining reaction products with strict regio / stereo-specificity and to produce active compounds (for a review, see [10]). This is particularly true for industrial level applications. For example, laccases (EC 1) produced by different bacteria, have been very popular for dealing with the waste

material in the textile and paper-pulp industry. Similarly, alcohol dehydrogenases (EC 1) that catalyse interconversion between alcohols and aldehydes / ketones are used for obtaining enantiomerically pure molecules / substrates and oxygenases (EC 1) are used in the oxidation of hydrocarbons with high stereoselectivity. Transferases (EC 2) are used in the polymer industry, for the synthesis of carbohydrates, peptides, polyesters, polyisoprene and other complex longchain molecules. From amongst the hydrolase (EC 3) family, acylases, lipases, glycosidases and proteases have been all been used either to break specific bonds or to synthesise new C-C bonds to produce fatty acids, esters, carbohydrates and peptides. For example, keratinases are used to soften leather and α-amylases are required for the breakdown of starch in the brewing of beer, or in the generation of industrial ethanol. Many proteases and lipases are components of household detergents, and work as stain removers. Lyases (EC 4) can produce a double bond by removing a group or can add groups at the double bond. So, they find applications in the generation of carbocycles, deoxy sugars, amino alcohols or other natural products. Of the isomerases (EC 5), a very popular example is that of glucose isomerase which is employed in the large-scale production of high fructose corn syrup from glucose.

These applications of enzymes, novel synthesis protocols, diagnostic tools, ready-to-use kits and automation of techniques, have been made possible by a finer appreciation and understanding of enzyme specificities and kinetics.

9. Summarizing the main concepts discussed

A quick summary of the major points discussed in this article must read as follows:

- Enzymes are catalysts that reduce the activation energy for a reversible reaction and increase its reaction rate (v) without affecting the substrate-product equilibrium.

- They are made of one or more polypeptide chains, which can be viewed as possessing multiple levels of organisation: primary (amino acid sequence), secondary (eg. α-helices, β-sheets, coils and turns), tertiary (domains and folds) and quaternary (superstructure due to spatial arrangement of two or more folded polypeptide chains) structures.

- Electrostatic interactions, H-bonding, van der Waals forces, covalent disulphide bonds, and the tendency to bury hydrophobic patches dictate protein structure and give rise to enzyme function. Conserved domains frequently share conserved functions.

- Koshland's induced fit model is a modified version of Fischer's lock-and-key hypothesis for making sense of substrate binding. Substrate binding often cause conformational changes in enzymes which are important for the catalytic activity.

- Enzyme-catalysed reactions appear to deviate from the Arrhenius equation, because enzymes unfold and denature at high temperatures. Many enzymes exhibit cold denaturation as well, making them optimally active in a limited temperature range.

- Enzyme stability as well as catalytic activity at the active site are dictated by pH.

- The simplest models for studying the kinetics of single-substrate enzyme-catalysed reactions are the equilibrium approximation and the steady state hypothesis.

- The steady state hypothesis is only valid at early time points, when there is sufficiently high substrate concentration to ensure that the concentration of the enzyme-substrate (ES) complex is maintained at a steady level.

- The equilibrium model is a special case of the steady-state hypothesis. It works well in cases where product formation is low.

- The steady state hypothesis gives rise to two constants, K_M (the Michaelis-Menten constant), and v_{max} (maximal velocity or rate of the reaction), and is the basis for the Michaelis-Menten equation.

- K_M is used as a measure of the affinity of an enzyme for a specific substrate. However, it is not a true dissociation constant. It can be equated to the dissociation constant (K_{ES}) only when product formation is low, i.e. where the equilibrium hypothesis is valid. The greater the rate constant (k_2 or k_{cat}) for the step determining product formation, greater the deviation of K_M from K_{ES}.

- Only for a fixed concentration of enzyme, v_{max} is a constant.

- The ratio k_{cat}/K_M measures the catalytic efficiency of an enzyme for a given substrate.

- The Michaelis-Menten equation yields a rectangular hyperbola when the catalytic rate (v) is plotted as a function of increasing substrate concentration ([S]).

- Linearization of the Michaelis-Menten equation can be achieved in different ways: the Lineweaver-Burk, Hanes-Woolf, and Eadie-Hofstee equations are discussed. Each has its advantages and limitations.

- Inhibitors inhibit enzyme activity. These may alter either K_M or v_{max} or both, depending on the nature of the inhibition.

- Allosteric modulators of enzyme activity may either cause activation or inhibition of the enzyme.

- Enzymes have a wide range of applications, but to successfully apply them a good understanding of enzyme kinetics is essential.

10. Conclusions and future perspectives

While much of the discussion in this article has been on a simplified model, that assumes a single substrate and an irreversible conversion of the ES intermediate to E + P, it should be kept in mind that the enzymes are actually bi-directional catalysts. The same enzyme can catalyse the backward reaction too, converting P to S. It is important to emphasise here, that it was our choice of approximations that simplified the reaction to a model that appeared to be irreversible. In the living cell, directionality of processes is achieved in many ways. For example, there may be a constant high supply of substrate to push the reaction towards product formation. Alternatively, the product formed may be rapidly transported away, or be liberated as a gas. Or, the reactions may be coupled to other reactions, each using the product of the previous step. Sometimes, compartmentalisation is what ensures that a specific substrate is used and a specific product is formed. Many of these conditions can be designed for laboratory experiments as well. A knowledge of regulators is also extremely useful in designing and analysing experimental data. Is the product formed in a reaction a possible feedback inhibitor of the enzyme, or does one component of the buffer act as a modulator of enzyme activity?

No doubt, it is also possible to study enzymes by including the reversible step with a rate constant of k_{-2}, as in Reaction (2). While this is beyond the scope of the present article, a mathematical analysis of this and more complex reactions, including bi- and multi-substrate reactions, allosteric regulation, mixed inhibition and feedback inhibition by the product formed are all available in standard enzymology books. It is also not necessary that steady state conditions have to be established in order to study rates of enzyme-catalysed reactions. It is possible to design pre-steady state experiments and to study their kinetics using continuous-flow, stopped flow, and relaxation techniques.

One major advantage of enzyme-based catalysis is the fact that they occur in aqueous conditions and do not require environmentally detrimental organic solvents. In other words, they would enable "green chemistry". However, many organic molecules are poorly soluble in water. This presents a challenge, reducing the efficiency of enzyme-catalysed reactions. For polar

and hydrophilic compounds, enzyme-based catalysis are more readily used at present. The narrow range of pH and temperature at which enzymes function are also problematic issues for chemical synthesis, which most biochemists perhaps do not need to grapple with.

Since molecular biology now allows us to manipulate enzymes at the genetic level, to improve their stabilities, specificities and catalytic efficiencies, this is a field that is waiting to be explored for new and novel applications.

11. Conflict of Interest and Funding

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