Enzymes & the Information that can be obtained from the study of enzyme kinetics

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Synonyms

Key Concepts and Definitions:

Concepts

Catalysis: The process by which the rate of a reaction is enhanced with no effect on the equilibrium position of the reaction.

Catalyst: A substance that speeds up the process of a reaction but is neither consumed during the reaction nor affects the chemical equilibrium of the reaction.

Enzyme: A biological macromolecule [protein or nucleic acid] that acts as a catalyst, and speeds the rate of a chemical (usually) or physical (occasionally) reaction.

Active Site: the region of an enzyme that makes specific interactions with the reactant(s) and participates in catalysis

Rate Limiting Step: the slowest step in the entire process of substrate binding steps, chemical (or physical) transformation or product release steps involved in the catalytic turnover of an enzyme

Synopsis

Enzymes constitute what is probably the largest class of proteins coded for in the genome of an organism and elucidating their structure and function relationships plays a vital role in both understanding the molecular mechanisms of cellular function and designing drugs that can target specific enzymes. To catalyze a reaction an enzyme must bind specifically to its substrates (reactants), lower the activation energy of the transition state of the reaction, and release the products. In biological settings the overall activity of an enzyme may be regulated to allow responses to the needs of the cell. Regulation of the activity of an enzyme must result from alterations in one or more of these three phases. The study of enzyme kinetics provides quantitative information about each of these phases of the overall catalytic pathway of an enzyme to give a framework for interpreting the effects of mutations or understanding the mechanism of regulation of an enzyme. Understanding the information that enzyme kinetics can provide requires knowledge of whether the enzyme is described by either steady state or rapid equilibrium conditions. The resultant equations allow initial rate data to be interpreted in terms of the

order of addition of substrates, whether a “ternary” complex or enzyme substituted mechanism is followed, and the physical significance of the various parameters that describe the kinetic behavior of the enzyme. In combination with the use of inhibitors, environmental variables such as temperature and pH, or information from other experimental approaches, enzyme kinetics provides detailed insight into structure-function relationships of enzymes.

Introduction:

Enzymes are the largest class of proteins, and due to their ease of study (there is often a simple way to follow their biological activity), the most well characterized. Enzymes speed up chemical reactions in the cell often by factors of 10^{12}. For example, the hydrolysis of a peptide bond **(figure 1)** occurs extremely slowly under physiological conditions: at pH 7.0, 37ºC the rate constant for the uncatalyzed reaction is on the order of 1 x 10^{-10}/sec despite the fact that the equilibrium constant for the reaction favors the products (1).

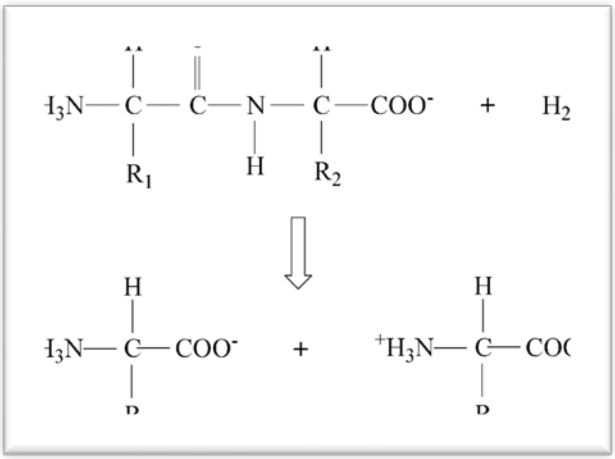
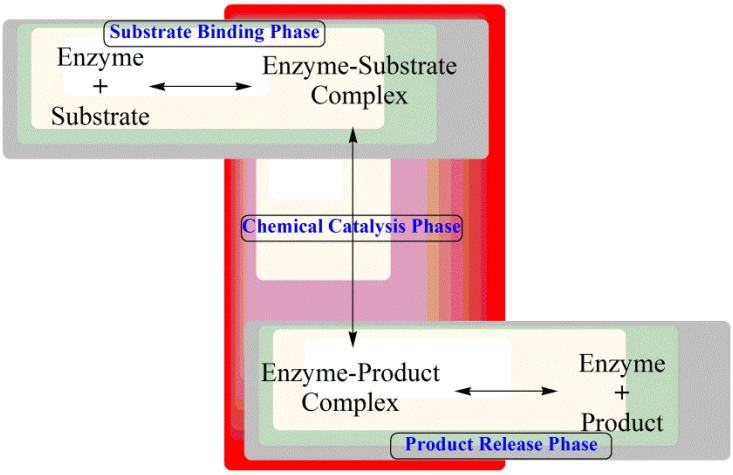


Figure 1: Peptide bond hydrolysis

In the presence of an enzyme such as chymotrypsin, the rate is on the order of 10^2, a rate enhancement of 10^{12}. Although this may seem amazing, the fastest enzyme, in terms of rate enhancement is OMP Decarboxylase, which has a rate enhancement of 1.4 x 10^{17}(2). It is important to distinguish between rate enhancement and rate of the catalyzed reaction. For example, one of the fastest enzymes, carbonic anhydrase has a rate of 1 x 10^6 but only has a rate enhancement of 7.7 x 10^6/second.In some cases enzymes have rate constants as high as 10^8-10^9/second.(3)

Enzymes, like all proteins share a common function: They interact with other molecules. In some cases the other molecule, or "ligand" is unchanged by the interaction but may affect the ability of the enzyme to catalyze its reaction, while in others they are chemically altered by the interaction, (which may involve one or more "reactant" or "substrate" as it is often called). Into the first category fall binding and structural proteins or small molecules which regulate the activity of the enzyme; into the second case fall enzymes the reactants, or substrates, of the reaction(They are still considered to be ligands.) In both cases the enzyme must have specific binding sites recognized by the appropriate ligand. In some instances the ligand is a small molecule and in others it is another protein or some other macromolecule. In many cases proteins interact with either type of ligand at different binding sites, the active site, where catalysis occurs and a regulatory site.

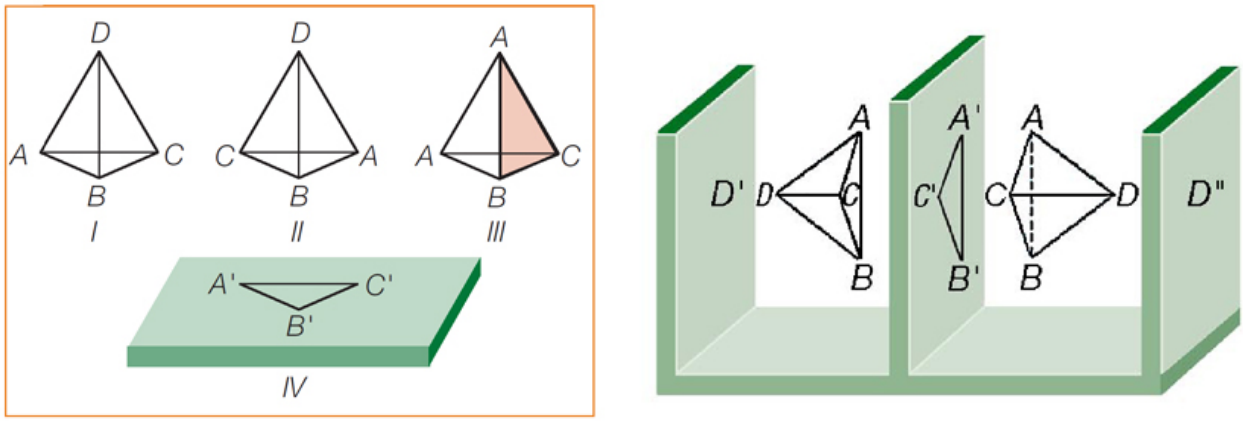


In its simplest formulation, where we consider one substrate and one product, the action of an enzyme can be represented by the three phases shown in **figure 2**:

In the first stage of the process the substrate ligand must recognize and bind to its appropriate binding site- the "active site". In the second stage an interconversion of substrate to product takes place involving, usually, one or more chemical steps where covalent bonds are broken and or created, as in the case of the protease illustrated at the start of this section. These steps some-times involve the formation of covalent intermediates between the substrate (or product) and some amino acid side chain or cofactor on the enzyme. Finally, in the third stage the product must be released from the binding site of the enzyme so that free enzyme is regenerated and the cycle can begin again. As with the first stage, various kinetic and thermodynamic parameters describe this product release phase of the reaction.

What is involved with Substrate Binding?

A binding site, which makes specific interactions with various regions of the substrate, also confers another general property on enzymes; they are specific in their ability to recognize defined substrates. Recognition, by the binding site of the enzyme, of multiple points on the substrate confers stereo selectivity. When an enzyme binding site makes three distinct interactions with a substrate, then stereo-selective reaction is possible even in a symmetrical molecule such as citrate. This is the basis of the three-point attachment theory of enzyme specificity, originally proposed by Ogston **(4)** and subsequently modified to a "four location model" by Mesecar and Koshland. **(5)** (**Figure 3)**



A. **B.**

Figure 3A The original three-point attachment model (Ogston, A. G. *Nature* **163,** 963 (1948).)The ligand binds so that groups A, B, and C of one enantiomer (I) bind to protein sites A8, B8 and C8 of the enzyme, respectively. It is easily seen that the other enantiomer (II) cannot yield an equivalent coincidence of groups A, B and C with A8, B8 and C8. If there are two A groups to produce a prochiral molecule (III), the model can distinguish between the two identical groups. The protein surface to which the chiral molecules bind is shown in IV.

Figure 3B Four-point location model for stereoselectivity of a protein (**Mesecar, A.D. & Koshland, D.E., Jr,** *Nature* **403** , 614-615 (2000), showing how a protein might provide two sites (D8 and D9) in either of two locations for interaction with group D on a chiral carbon atom: D8 would bind one enantiomer and D9 would bind its mirror image

The shape of the binding site may be fixed [lock and key principle] **(6)** with only the correct substrate (ligand) making optimal contact, or may be shaped by the binding step- the substrate causes conformational changes in the binding site that result in optimal interactions with the protein [induced fit] **(7)**. In either model of the interaction of a substrate with an enzyme there can be alternative substrates that can bind and react with varying efficiencies leading to a phenomenon often referred to as enzyme promiscuity. The concepts involved in lock and key, and induced fit have historical significance however with current understanding of the dynamic rather

than static nature of protein structure it is now recognized that the dynamic properties of the enzyme alone, as well as the enzyme substrate or enzyme product complexes play an important role in enzyme kinetics. **(8)**

In addition to the chemical nature of the binding site, this interaction can be characterized by various kinetic and thermodynamic parameters.

For the simple interaction of a ligand with the enzyme:

$$\text{Enzyme + Ligand} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{Enzyme-Ligand Complex}$$

or

$$E + L \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E\text{-}L$$

The two rate constants associated with the reaction, k_1 and $k_2$are related to the dissociation constant for the ligand which is represented by:

$$K_D = [E_f][L_f]/[E:L],$$

where $[E_f]$ and $[L_f]$ are the free concentrations of the enzyme and ligand at equilibrium and [E:L] is the concentration of the enzyme-ligand complex

At equilibrium the rate of formation of E:L is governed by the rate law:

$$\text{rate} = k_1[E_f][L_f]$$

while the rate of breakdown of E:L is governed by the rate law:

$$\text{rate} = k_2[E:L]$$

At equilibrium the two rates are equal, hence

$$k_1[E_f][L_f] = k_2[E:L]$$

Hence:

$$K_D = [E_f][L_f]/[E:L], = k_2/k_1$$

Discussion of enzymes and ligand binding usually involves using the dissociation constant, K_D, as above, which is related to the Equilibrium Constant for the interaction, K_{eq}, by the relationship:

$$K_D = 1/K_{eq}$$

This is an important distinction when it comes to considering thermodynamic parameters related to the interaction, where ΔG and related parameters are calculated using K_{eq}.($\Delta G = -RT\ln Keq$ and $\Delta G = \Delta H - T\Delta S$)

The existence of a specific binding site on the enzyme for the substrate (or any other ligand) is the basis of one of the tenets of enzyme-catalyzed reactions; they are saturable. In its simplest terms, when all the catalytic sites of the enzyme are saturated with substrate, the catalyzed reaction is proceeding at its maximum rate (V_{max}) for that particular concentration of enzyme. The relationship between binding and catalysis are related by the Michaelis-Menten equation, derived to describe the dependence of the measured rate of an enzyme catalyzed reaction, v_o, to the concentration of substrate:

$$V_o = V_{max}.[S]/(K_m + [S])$$

Where K_m and V_{max} are the so-called Michaelis Constant for the substrate, and the maximum velocity respectively at a fixed concentration of enzyme..

The turnover number (often called the catalytic constant, k_{cat}) for an enzyme is usually expressed in terms of moles of product produced per mole of enzyme per unit time (usually seconds). k_{cat} is often the rate of chemical catalysis, as indicated in figure 2, but in terms of the turnover number is in reality the rate constant for the rate limiting step of the overall reaction. V_{max} and $k_{cat}$are related by the expression:

$$k_{cat} = V_{max}/[enzyme]$$

Experimentally a value for the maximum velocity, V_{max}, is determined by measuring the effects of substrate concentration on the initial rate of the reaction **(9),** and plotting a Michaelis-Menten plot, (**figure 4**), or one of a variety of other plots.

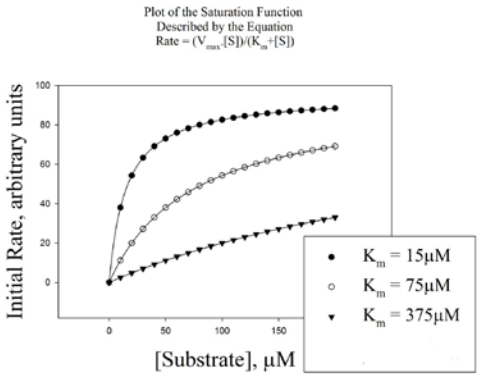


Figure 4

In the second stage an interconversion of substrate to product takes place involving, usually, one or more chemical steps where covalent bonds are broken and or created, as in the case of the protease illustrated at the start of this section.These steps some-times involve the formation of covalent intermediates between the substrate (or product) and some amino acid side chain or cofactor on the enzyme.

The Chemical Reaction Phase of the reaction I: The Transition State

From the chemical perspective the reaction proceeds through a "transition state" [see transition state theory] **(10)** and the enzyme acts to stabilize the energy of the transition state by making more productive interactions with the transition state structure than either the substrate or the product.

Any chemical reaction proceeds through a transition state, and the reaction can be visualized in terms of an energy diagram. For a simple one substrate reaction, an energy diagram (**figure 5**) can be shown for the case where both stable E:S and E:P complexes are formed. A diagram for a multisubstrate enzyme can be quite complex and will depend upon the formal kinetic mechanism of the reaction. The energy levels of the various components of the energy diagram relate to a variety of the thermodynamic parameters discussed earlier, while the magnitude of the energy barriers (the activation energy of the step) is related to the appropriate rate constant. Experimentally these values can often be determined from the temperature dependence of various kinetic parameters for the mechanism of the enzyme. In particular the temperature dependence of V_{max} allows the activation energy of the overall reaction to be obtained using an Arrhenius plot. From the temperature dependence of the various equilibrium constants shown in figure 5 (many of which can be calculated from initial rate kinetics data if the formal kinetic mechanism of the enzyme is known) the enthalpy of the reaction

can be obtained from a van't Hoff plot.

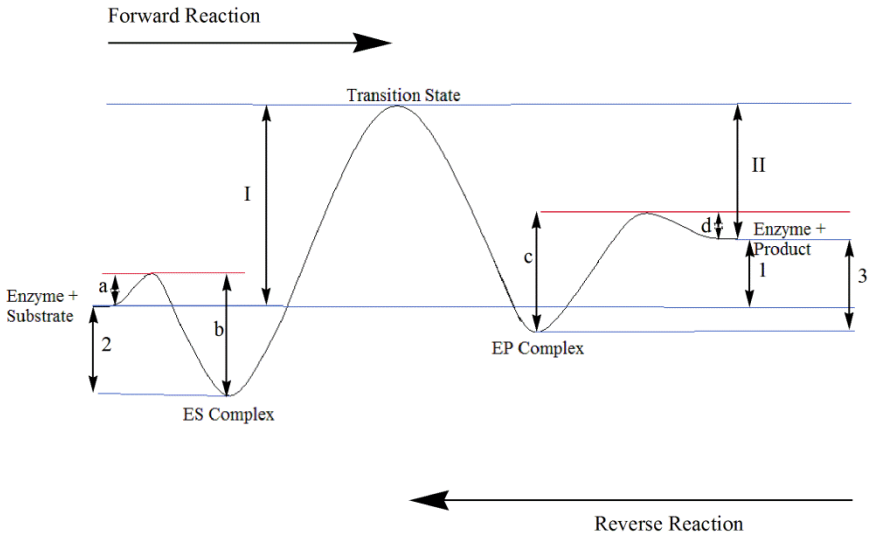


Figure 5: Energy diagram for the forward and reverse reactions of a simple one substrate enzyme catalyzed reaction defining the relationship of the energy to various thermodynamic parameters: I, the activation energy of the forward reaction, related to k_{cat} (forward), II, the activation energy of the reverse reaction, related to k_{cat} (reverse)1, ΔG for the reaction S←→P, 2, ΔG for substrate binding, related to the dissociation constant for the substrate from the ES complex, 3, ΔG for product binding, related to the dissociation constant for the product from the EP complex, a, the activation energy for substrate binding, related to the on velocity constant for substrate binding, b, the activation energy for substrate release from the ES complex, related to the off velocity constant for substrate from the ES complex, c, the activation energy for product release from the EP complex, related to the off velocity constant for product from the EP complex, and d, the activation energy for product binding, related to the on velocity constant for product binding.

The simplest such relationship, the Haldane relationship, (11) allows the overall equilibrium constant of the reaction to be calculated from the forward and reverse kinetic constants for the enzyme.

$$K_{eq} = [P_{eq}]/[S_{eq}] = V_f K_{mp1} K_{mp2} / V_r K_{ms1} K_{ms2}$$

Where V_f and V_r refer to the maximum velocities of the forward and reverse reactions catalyzed by the enzyme, and K_{mp1} and K_{mp2} are the Michaelis contastants for the products (ie the reactants of the reverse reaction while K_{ms1} and K_{ms2} are the Michaelis constants for the two substrates of the forward reaction.

While most enzymes are involved with chemical reactions, in some circumstances, for example proline isomerase, Ref 10 no covalent bonds are made or broken and the reaction involves a conformational change in the "substrate"- a cis-trans isomerization in the case of proline isomerase and the same types of kinetic analysis can be performed.

The Chemical Reaction Phase of the reaction II: Ternary Complex versus Enzyme Substituted Mechanisms

In reality the majority of enzymes in biology have two or more substrates and two fundamentally different types of "transition states" can be envisaged. In the first, all of the substrates must be bound to the active site of the enzyme before the chemical reaction can proceed. There is a single transition state involved in the production of

products, and the energy diagram for such a reaction resembles that if figure x with the exception that there may be several different enzyme substrate complexes formed en route to the transition state. Such mechanisms, for 2 substrates, are referred to as ternary complex mechanisms, or a single displacement mechanism.

In many enzymatic reactions, particularly those involving group transfers or hydrolytic cleavage of a substrate, the overall reaction cycle occurs in two stages and involves a covalent intermediate with a side chain on the enzyme. This is best illustrated using the example of a protease. The overall hydrolysis, illustrated in figure 1, often occurs through a covalent intermediate with either a serine residue (serine proteases) or a cysteine residue (thiol proteases) forming an acyl enzyme intermediate and releasing one product. The acyl enzyme intermediate is then hydrolyzed, releasing the second product. Such enzymes produce a stable enzyme intermediate where part of the substrate is covalently bonded to a side chain of the enzyme. This “enzyme-substituted” species then binds to the next substrate, forms a second transition state and reacts to give the other product of the overall reaction. Enzymes showing this type of behavior are often said to have an “enzyme-substituted” kinetic mechanism, or have a double displacement mechanism. The term “ping-pong mechanism” is also often used to describe such mechanisms.

Product Release Phase of the Reaction

The final phase of the overall reaction cycle is the release of product, and as with the substrate binding phase, this can be described in terms of a dissociation constant for product binding to the enzyme. Since enzyme catalyzed reactions are reversible, the product of a reaction in one direction is the substrate of the reaction in the other direction. When one considers only the conversion of substrate to product, and applies the “initial rate” condition, that there is one product present initially, the product release step becomes uni-directional and the rate of this step is simply the rate of release of product from the enzyme-product complex, and the released enzyme is available for substrate to bind ([S]>>[P]) and initiate the catalytic cycle again.

The study of the events that occur when substrate interacts with enzyme and is converted to product is referred to as enzyme kinetics, and gives rise to a detailed description of the “**kinetic mechanism**” of an enzyme which describes the order of addition of the substrates to the enzyme, the overall nature of the catalytic process, does it proceed through a simple transition state bound to the enzyme or does it involve a covalent intermediate with the enzyme, and the nature of the product release- as with substrate binding it could be ordered, where one product must leave first, or random, where either product can leave first.

Initial rate (or steady state, as it is often referred to) kinetics, where the enzyme concentration is much lower than the concentration of reactants, and the reactant concentration does not change effectively (relative to the enzyme concentration) during the measurement, can give much of this information, but cannot givespecific information about discrete steps in the overall reaction since many of them occur at rates faster than the catalytic turnover of the enzyme which limits the observations made in initial rate kinetics. They also occur at the rate much faster than the typical experimental determination of the rate of a reaction can be made. A typical rate measurement using a continuous assay involves adding the enzyme to a solution of substrates and observing either the accumulation of product or the disappearance of substrate over a period of a few seconds to minutes or hours depending upon the type of enzyme. Eventually the reaction will reach a chemical equilibrium, defined by the overall equilibrium constant of the reaction and no further change in substrate or product concentrations occurs. As outlined in **figure 6**, the reaction goes through a number of phases in this process. The initial rate, or steady state, phase is the second of these phases. Prior to this phase there is what is called a **pre-steady state phase** where initial substrate binding and the first turnovers of the enzyme occur. Depending upon where the overall rate limiting step in the reaction cycle is, the so-called pre-steady state rate may be

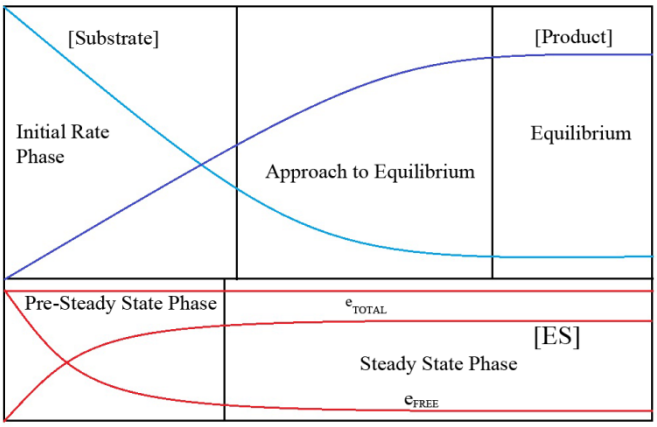
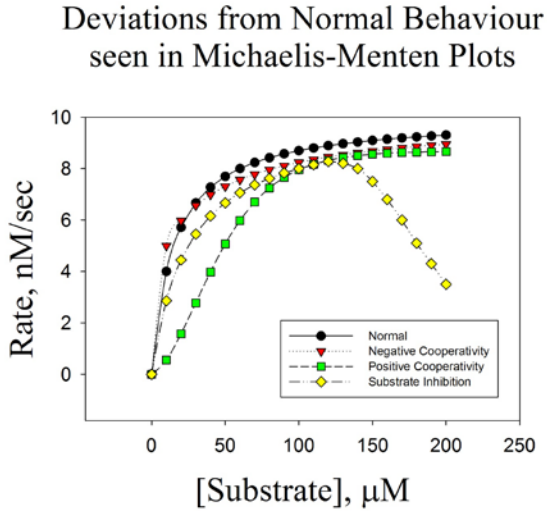


Figure 6: Time dependence of the concentrations of substrate and product (upper panel- time scale seconds to hours) and the various enzyme complexes (lower panel- time scale microseconds to seconds) indicating the various phases of the reaction. The initial rate phase in the upper panel coincides with the stead state phase in the lower panel. During this phase the steady state condition, d[ES]/dt = 0 is obeyed and the initial rate is constant as a function of time while the initial rate condition [S]>>e_{TOTAL} holds.

higher than the initial (steady state) rate. In this case addition information about rate constants involved in various steps in the reaction cycle can be experimentally determined using **"pre-steady state kinetics",** often referred to as rapid reaction kinetics where reactants and enzyme are rapidly mixed and observation of products initiated often on a time frame of milliseconds or less allowing for much faster processes to be observed. To obtain the signal sensitivity necessary much higher enzyme concentrations are used In such experiments that in initial rate kinetics and the steady state assumption is not usually applicable. Pre-steady state kinetics, where measurements are made on the microsecond to second time scale, have the capability of providing information about the discrete rate constants involved in substrate binding, product release and chemical catalysis and this information is often used in conjunction with initial rate kinetic data to help give a full description of the steps involved in the overall catalytic cycle of the enzyme.

In the time range between initial rate measurements and the attainment of chemical equilibrium a number of things are happening which complicate analysis of the data obtained during the "approach to equilibrium" phase of the reaction. Primarily, as time progresses, the concentrations of substrate are also changing, from the initial concentrations (in the initial rate phase of the reaction) to the equilibrium concentrations which apply once equilibrium is reached. As the substrate concentration is decreasing, the product concentration is increasing. In the initial rate phase of the process in effect there is no product and product concentration does not have to be considered. However in the approach to equilibrium phase of the reaction the increasing product concentrations can play a very significant role in the overall process due to **product inhibition** and **abortive complex formation**. The study and analysis of the whole time course of the reaction, from initiation by enzyme addition to equilibrium attainment is complex but offers an alternative way to obtain kinetic data including values for Michaelis constants and Vmax and well as parameters of the product inhibition.

Deviations from normal behavior



Several types of deviations from "normal" behavior can be observed (**Figure 7**) in initial rate kinetics studies and these can best be categorized into three types. 1) **substrate inhibition**, either as a result of non productive substrate binding or abortive complex formation. 2) those involving substrates or products binding to a second distinct location on the enzyme with a resultant effect on the kinetic behavior of the active site as a result of a heterotropic effect, and 3) allosteric phenomena involving substrate binding to multiple active sites within a homopolymer. Typical deviations seen in either Michaelis Menten plots or Lineweaver Burk plots as a result of these phenomena are shown in figure 7. Deviations resulting from either the second or the third cause often give rise to similar deviations in these plots are additional types of experiments are

required to distinguish these phenomena. Before discussing these phenomena in more detail it is necessary to understand the basis of the equations used to describe “normal” behavior.

The Equations of Initial Rate Kinetics

One of the goals of the study of enzymes using enzyme kinetics is to obtain quantitative information about the various steps involved in the overall catalytic cycle. This involves developing the appropriate equations for particular models of the kinetic mechanism and experimentally determining which is the most consistent with observed data. Often this comes down to eliminating particular mechanisms because experimental data is inconsistent with the model. The derivation of equations for enzyme kinetics dependsupon two fundamentally different assumptions, the steady state assumption and the equilibrium assumption. The use of these two assumptions in the derivation of initial rate equations for a formal kinetic mechanism are illustrated here for the simple reaction

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \overset{k_{cat}}{\rightleftharpoons} E + P$$

But the same approaches are used for more complex multi-substrate kinetic mechanisms.

The **steady-state assumption** used by Briggs and Haldane(12) is that the intermediates, in this case the ES complex, are always present in very small quantities compared to the reactants and products. Thus after an initial "pre-steady-state" phase, the concentrations of the intermediates remain constant, that is,

$$d[ES]/dt = 0$$

The initial velocity, Vo, is defined by:

$$V_o = k_{cat}[ES]$$

Combining these equations we get:

$$d[ES]/dt = 0 = k_1[E][S] - [ES](k_2 + k_1)$$

However, since the total enzyme concentration, e, is equal to E + ES (the enzyme conservation equation), we get

$$E = e - ES$$

Substituting into this equation and rearranging, we find

$$k_1[E][S] = [ES](k_2 + k_1)$$

Therefore

$$k_1[e][S] = [ES](k_1[S] + k_2 + k_1)$$

and

$$[ES] = k_1[e][S]/(k_1[S] + k_2 + k_1)$$

Combining this equation with the one for the initial velocity results in the following equations:

$$v_o = k_{cat}k_1[e][S]/(k_1[S] + k_2 + k_1)$$

$$= k_{cat}[e][S]/([S] + (k_2 + k_1)/k_1)$$

and

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

where$V_{max} = k_{cat}[e]$ and is the maximum rate of the enzyme-catalyzed reaction,

and

$$K_m = (k_2 + k_1)/k_1$$

where K_m is the Michaelis Constant for the substrate.

In the **equilibrium assumption (13)**, which can replace the steady-state assumption, k_{cat} is very much slower than k_2 and as a result the ES complex is in an effective equilibrium with E and S. Thus we can define an equilibrium constant, K_{eq}:

$$K_{eq} = k_1/k_2 = [ES]/[E][S]$$

Substituting, the enzyme conservation equation, into this equation we get

$$K_{eq} = [ES]/([e][S] - [ES][S])$$

Rearranging this equation, we find the equations

$$K_{eq}[e][S] - K_{eq}[ES][S] = [ES]$$

and

$$K_{eq}[e][S] = [ES] + K_{eq}[ES][S] = [ES](1 + K_{eq}[S])$$

This equation can be put in terms of [ES]:

$$[ES] = K_{eq}[e][S]/(1 + K_{eq}[S])$$

As before, $V_o = k_{cat}[ES]$, and

$$v_o = k_{cat}K_{eq}[e][S]/(1 + K_{eq}[S])$$

Dividing by K_{eq} we get an equation of similar form to the equation derivedusing the steady state assumption:

$$= k_{cat}[e][S]/(1/K_{eq} + [S])$$

As before:

$$V_{max} = k_{cat}[e]$$

and

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

where

$$K_m = 1/K_{eq} = k_2/k_1$$

The difference between this equation and the one obtained using the steady state assumptionlies in the definitions of K_m. In the earlier, steady-state case, K_m is equal to $(k_2 + k_{cat})/k_1)$ whereas in the equilibrium case Km is a true dissociation constant. This points to an important difference between steady-state and equilibrium assumptions. In the equilibrium assumption the Michaelis Constant, Km, is a true equilibrium constant, whereas in the steady-state assumption this is not the case. In steady-state mechanisms as k_{cat} gets slower (relative to k_2), we approach the equilibrium situation. At this point the difference may seem inconsequential; however it is important in establishing the formal kinetic mechanism of a multi-substrate enzyme.

The Michaelis-Menten equation **(13)** describes the dependence of the initial velocity, Vo, on the substrate concentration, and is in the format of a hyperbolic saturation function. From this dependence two parameters, V_{max} and K_m, can be determined.

The MichaelisMenten equation can be transformed into a linear equation:

$1/v_o = 1/V_{max} + K_m/V_{max} . 1/[S]$.the so-called Lineweaver Burk equation (14) used in the double reciprocal Lineweaver-Burk plot.

The Linewaver-Burk equation can be put in a generalized format (15):

$$e/V_o = \Phi_0 + \Phi_1/[S]$$

In reality the majority of enzymes require two or more substrates to function and in some cases a covalent intermediate of one or other of the substrates with the enzyme might be involved.

When all of the substrates bind non-covalently to give an enzyme-substrates complex prior to any chemical reaction, the overall reaction can be represented by a "Generalized" rate equation:

$$e/v_o = \Phi_0 + \Phi_1/[S_1] + \Phi_2/[S_2] + \Phi_{12}/[S_1][S_2]$$

where S_1 and S_2 are the concentrations of the two substrates, and the formal kinetic mechanism of the enzyme is referred to as a "complex" mechanism- ternary complex in the case of two substrates, quaternary complex in the case of three substrates and so forth.

When a covalent intermediate is formed the generalized rate equation becomes:

$$e/v_o = \Phi_0 + \Phi_1/[S_1] + \Phi_2/[S_2]$$

and the reaction is referred to as an "enzyme-substituted" or "ping-pong" mechanism.

The format of the generalized rate equation predicts the effects of varied concentrations of one substrate on a Lineweaver-Burk plot with the other substrate varied and such experiments can be used to give information as to the 'formal" kinetic mechanism of an enzyme catalyzed reaction.

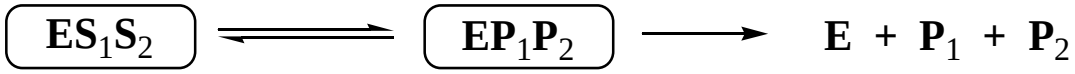
Both of these types of mechanisms and their generalized rate equations can be put in either Michaelis-Mentenformat or Lineweaver Burk format and appropriate Km values and Vmax calculated from experimental data. With 2 or more substrates it is important to differentiate the order the substrates bind in. The substrates might bind where either S_1 or S_2 in the scheme above can bind first followed by the other in which case the mechanism is known as a "Random Order" mechanism. If one substrate is required to bind before the other, the mechanism is referred to as "Compulsory Order"

The formal kinetic mechanism of an enzyme also includes a consideration of the nature of the interaction of the substrates or products with the enzyme and involves either the steady state assumption or the rapid equilibrium assumption. These assumptions have an impact on the physical interpretation of K_m.

In situations with two or more substrates, one also has to consider the impact that one substrate has on the binding of another substrate, or on the release of a product. In the case of a multi-substrate enzyme, the binding of one substrate might enhance or interfere with the binding of the other substrate, or could have no impact at all on the binding of the other substrate. Such effects will manifest themselves as an impact of one substrate on the Michaelis Constant for the other substrate.

Substrate Inhibition and Abortive Complex Formation

As discussed earlier a variety of deviations from ‘normal” behavior can be observed when the rate is measured as a function of substrate concentration. A common phenomenon is substrate inhibition where after reaching a plateau the rate starts to decrease as the concentration of substrate continues to be raised (figure 7). There are two causes of such an effect that are usually considered. In the first, termed “**non-productive binding**” the substrate binding site can be considered as consisting of several “subsites” and while at low concentrations of substrate the substrate orients so that each subsite is filled by a single substrate molecule and the substrate binds productively, as the concentration of the substrate is increased there might be competition between different substrate molecules for the various subsites and two substrate molecules end up binding to separate subsites in the binding site. As a result neither is in the correct orientation to allow catalysis to proceed and the binding is “non productive”. The result is that as the substrate concentration is raised the rate decreases and substrate inhibition is observed. The second, and likely more prevalent, mechanism for substrate inhibition involves the formation of an “abortive complex” where one substrate and one product is bound to the active site. This mechanism requires the enzyme to normally have two or more substrates and two or more products and follow a ternary complex mechanism where an ES_1S_2 complex is interconverted to an EP_1P_2 which under normal circumstances would dissociate both products (in either an ordered or a random fashion) to regenerate free enzyme:



In such circumstances one of the products often resembles one of the substrates and binds to the same subsite in the active site. If, for example, P_2 resembles S_2 and is the first product to dissociate, an EP_1 complex exists and at high concentrations of S_2, S_2 can bind to form an EP_1S_2 complex. If P_1 dissociates from this complex at a rate slower than the rate limiting step under normal substrate conditions, then the presence of the high concentration of S_2 has, by forming the EP_1S_2 complex, effectively changed the overall rate limiting step of the catalytic cycle, slowing the turn-over of the enzyme, and inhibition at high concentrations of S_2 is observed. The EP_1S_2 complex is a so-called abortive complex and leads to substrate inhibition. The existence of potential abortive complexes in an enzyme pathway leads to a variety of possibilities for the regulation of the activity of the enzyme. These will be explored in the section on Regulation of enzyme activity.

Activation, Inhibition and Allostery

So far we have considered only the interactions of the substrate(s) with an enzyme. In many circumstances one must also consider the impact that a regulatory ligand may have on the overall reaction. A regulatory ligand might affect substrate binding, catalysis or product release regulatory and could either speed up or slow down one or more of these steps, with resultant activation or inhibition of the overall turnover of the enzyme
If we assume that the second regulatory ligand is not a substrate, it could affect some parameter related to any or all of the three stages mentioned. A description of the effects of this second ligand requires a knowledge not only of its binding site and related parameters, but also of the effects its binding has on the other stages of the reaction. An understanding of the effects of this second ligand requires a detailed knowledge of the protein structure connecting the various sites and the way in which the protein structure changes in response to ligand binding. Such effects are often referred to as **heterotropic** regulation. When the interaction of the heterotropic ligand affects the binding of substrates or products the regulation is referred to as a K type effect. When the overall rate limiting step of the reaction is affected it is referred to as a V type effect.

When there is a second, non-active site that binds the substrate, one of two effects can be envisaged- binding of substrate to this second site could lead to an activation or an inhibition of catalysis by enhancing or weakening substrate binding to the active site (K type effects), or speeding or slowing the overall rate limiting effect (V type effect)

In an enzyme with 2 or more subunits and active sites or regulatory sites, interactions between between identical sites (active or regulatory) can lead to "**allosteric**" phenomena where binding of ligand to one of the sites affects binding to the other "identical" sites in the oligomer. Such interactions are often referred to as "**Homotropic**" interactions and can be either positive, giving a sigmoidal saturation curve (Figure 7) or negative, giving a saturation curve that is too steep below half saturation and not steep enough above half saturation. These effects can involve either substrate binding or regulatory ligand binding and are distinct from the heterotropic effects described above. Allosteric phenomena play an important role in the regulation of enzyme activity and are discussed in detail in section 17 of this encyclopedia.

In the case of inhibitors of the overall reaction two types of interaction with the protein are possible, binding at a separate site as discussed above, or binding to the active site. When an inhibitor binds at the active site in place of the substrate it is known as a competitive inhibitor and the inhibition can be overcome by raising the concentration of the substrate. This simple case of inhibition is shown below in scheme A, and the interaction of the inhibitor with the enzyme is described by an "inhibition constant, Ki" that represents binding of the inhibitor to the active site:

For the scheme shown, we can write the expression:

K_i = [E][I]/[EI], and where [I] is the concentration of free inhibitor and [EI] the concentration of the complex.

The same effect can be achieved by an inhibitor binding at a separate site and triggering a conformational change that occludes the substrate binding site. If this site is conformationally linked with the substrate binding site such that substrate binding also precludes inhibitor binding the effect will again be competitive as shown in scheme B and again the interaction of the inhibitor with the enzyme can be described by K_i.

Rate equations describing the expected velocity in the presence of inhibitor in these cases of competitive inhibition can easily be derived using approaches similar to those described earlier for cases where no inhibitor is present. The only changes made are the inclusion of the appropriate terms describing the interaction ofthe enzyme and the inhibitor, which are manifested in the enzyme conservation equation which becomes

e = [ES] + [E] + [EI]

which, since [EI] = [E][I]/K_i, becomes

e = [ES] + [E] + [E][I]/K_i = [ES] + [E](1 + [I]/K_i)

Using this enzyme conservation in either the steady state or equilibrium approach to deriving the initial rate equation for the enzyme give a Michaelis-Menten equation in the form:

Vo =V_{max}[S]/ ([S] + K_m[1 + [I]/K_i)

where K_m and V_{max} have the same meanings as previously.

A second mode of interaction of an inhibitor with an enzyme, B, involves a site other than the active site. As in the first case, interaction of inhibitor with protein can be described by a dissociation constant, K_i', whichhas a similar form to that of K_i. Since this type of inhibitor (the same applies to an activator) does not directly involve the active site, we must consider what effects it might have: (a) as a result of a conformational change the ability of the active site to bind substrate may be altered but not prevented; (b) as a result of a conformational change the ability of the enzyme to bind the substrate may not be affected, but the ability of the enzyme to catalyze the reaction is affected; and (c) the site of inhibitor binding, although not at the active site, may be so close to it that there isdirect physical interaction between inhibitor and substrate binding, leading to distortion of the enzyme-substrate complex. Although mechanisms a and b can lead to either decreased activity (inhibition) or increased activity (activation), the third type is conceptually quite different from the other two - no conformational change induced by inhibitor binding is required.

In this second category of interaction the inhibitor (or activator) and the substrate can be bound to the enzyme at the same time, giving an ESI complex. Where an ESI complex can be formed, the inhibitor is referred to as a category II inhibitor.

In terms of deriving the initial rate equation, the enzyme conservation equation becomes:

e = [ES] + [ESI] + [E]

and the MichaelisMenten equation becomes

vo = (V_{max}[S](1 + [I]/K_i'))/([S] + K_m(1 + [I]/K_i')

When both types of interaction can occur, the enzyme conservation equation becomes

e = [E] + [ES] + [EI] + [ESI]

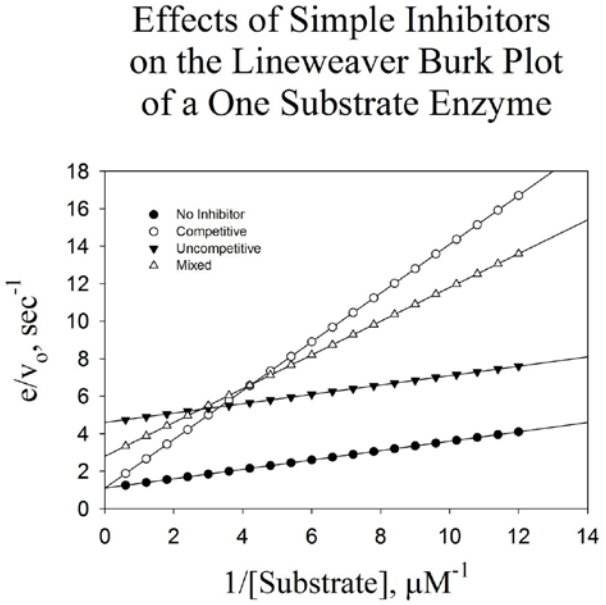
and the MichaelisMenten equation becomes

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

Under the circumstances where $K_i = K_i'$ this reduces to:

$V_o = (V_{max}[S]/(1 + [I]/K_i'))/([S] + K_m)$

In this category, category II, the presence of the inhibitor(or activator) can affect either the ability of the enzyme to bind substrate or the ability of the enzyme to catalyze the reaction once the enzyme has bound substrate. From the earlier discussion of basic kinetics in the absence of inhibitors, it is apparent that either K_m or V_{max} can be affected in these cases, whereas only K_m is affected in category I situations.



As with the equations derived in the absence of inhibitors, it is convenient to express these equations in double-reciprocal (Lineweaver-Burk) form and consider the effects in Lineweaver-Burk plots(Figure 8). Each of the three equations is shown in Lineweaver-Burk format in the following table. From these double reciprocal equations it is apparent that in terms of Lineweaver-Burk plots, three types of inhibitors can be distinguished. Category I inhibitors affect only the slope,(often referred to as Competitive Inhibition), while category II inhibitors affect the intercept,(often referred to as Uncompetitive Inhibition). The slope may also be affected in cases where an EI and an ESI complex are formed and this type of inhibition is often called Non-Competitive or Mixed Inhibition..

Table

Effects of Category I and II Inhibitors on Lineweaver Burk Equation

Type of Inhibitor	$1/v_o$ = slope x 1/[S]	+ Intercept
No Inhibitor	K_m/V_{max}	$1/V_{max}$
Category I Inhibitor- EI complex	$(K_m/V_{max})(1 + [I]/K_i)$	$1/V_{max}$
Category II Inhibitor- ESI complex	K_m/V_{max}	$(1/V_{max})(1 + [I]/K_i')$
EI and ESI Complexes formed	$(K_m/V_{max})(1 + [I]/K_i)$	$(1/V_{max})(1 + [I]/K_i')$

In some enzymes the protein molecule consists of a number of polypeptide chains: sometimes homopolymers such as malate dehydrogenase where there are two chemically identical polypeptide chains in the quaternary structure of the protein, sometimes heteropolymers such as lactose synthase where two distinctly different

polypeptide chains are involved.In the extreme are complex enzymes such as the pyruvate dehydrogenase complex where a variety of different polypeptide chains and catalytic activities are involved in an overall reaction.

In either type of "reaction", the catalysis almost always involves the interaction of a variety of amino acid side chains in the active site of the enzyme with various stages of the substrate to product conversion.

Where is the Rate Limiting Step in the Overall Catalytic Cycle?

Although an enzyme catalyzed reaction can be distinguished by these three distinct phases it is not always clear which of the phases is the overall rate determining step. The rate limiting step of the overall reaction governs the catalytic rate of the enzyme, as distinct from the rate of the chemical step. As discussed in subsection a), the balance between these various facets of the enzyme catalyzed reaction can be represented by the "catalytic efficiency" of the enzyme, k_{cat}/K_m

Factors that Effect Enzyme Kinetic Parameters and Enzyme Activity

Detailed analysis of enzyme kinetic data can give rise, depending upon the kinetic mechanism, to values for either individual rate constants or individual equilibrium constants (remembering that KD = 1/Keq). The temperature dependence of such constants gives rise to various thermodynamic parameters, as described by either the van't Hoff equation:

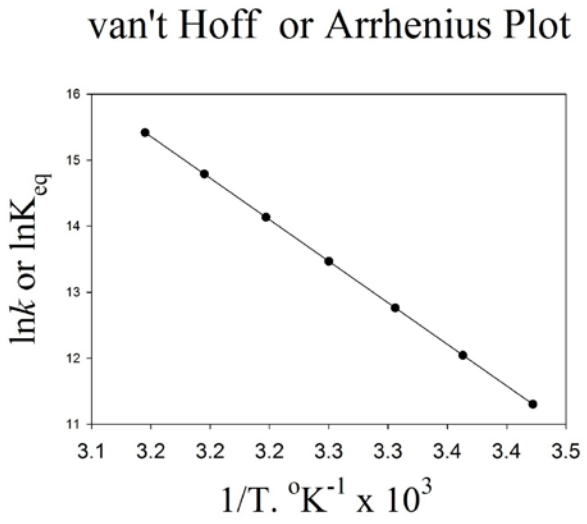
$$\ln K_{eq} = -\Delta H^o/RT + \Delta S^o/R$$

or the Arrhenius equation:

$$\ln k = (-E_a/R)(1/T) + \ln(A)$$

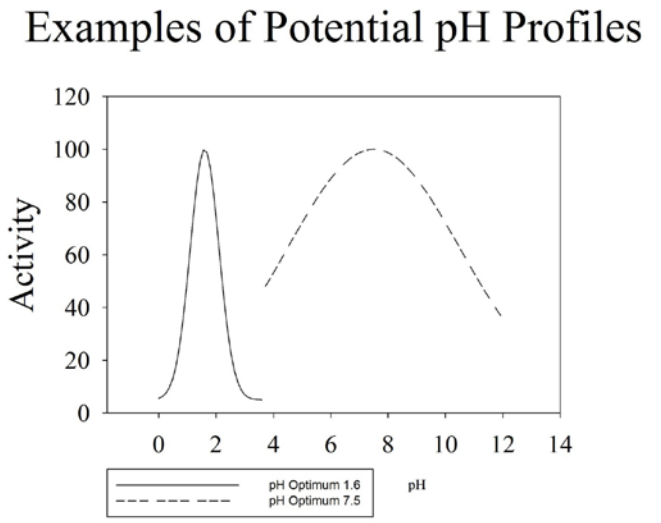
which give rise to linear plots of either $\ln K_{eq}$ or $\ln k$ versus 1/T (figure 9) allowing the appropriate thermodynamic parameters to be calculated.

While the temperature dependence of these various kinetic parameters allows information about the energy diagram to be obtained (16) it is important to remember that other factors can also contribute to the temperature dependence of an enzyme, both of which can give anomalous temperature dependence. The first involves the stability of the enzyme being investigated: if during the experimental determination of the rate of the reaction the effective enzyme concentration is changing as a result of incubation at an elevated temperature anomalous kinetic data will be obtained leading to anomalous points on the Arrhenius, or van't Hoff plot. The second type of "anomaly" is more accurately a reflection of real properties of the enzyme itself. If an enzyme can exist in two or more conformations at a particular temperature that are rapidly interconverted whichever is the more active will in effect be the only form seen, and all of the enzyme will behave as if it were that form. In reality, the energy barrier between the two forms is such that the thermal energy of the system is sufficient to overcome it (**figure 9**), At this temperature, and at temperatures above it the enzyme behaves as one



form and this region of an Arrhenius plot or van't Hoff plot will be linear and characterized by the parameters of this one form. At lower temperatures however the energy barrier between the two forms is not overcome by the prevailing thermal energy and two discrete forms of the enzyme, with different properties exist and the slope of the van't Hoff or Arrhenius plot reflects this. In effect the two different forms have different rate limiting steps in the overall catalytic cycle which are reflected in the different slopes of the non-linear Arrhenius plot.**(17,18)**

The enzyme, and possibly the substrates or products, contain many protonatable groups and as a result the structure, and function of the protein will have a pH dependence. Physiologically, the activity of the enzyme under a specific set of substrate concentrations will have a **pH Optimum**, which can be experimentally determined by measuring the rate as a function of pH. The pH optimum for a particular enzyme usually correlates with the pH of its physiological environment- for example the protease pepsin, which operates at very acidic pH values in the stomach has a pH optimum of 1.6-1.7 while the protease trypsin, catalyzing the same chemical reaction, has a pH optimum around 8-8.5. As the pH either increases or decreases away from the pH optimum of the enzyme the activity falls, resulting in the typical "bell-shaped" curve for activity as a function of pH (**figure 10**). **(19).** As is discussed later in this subsection the pH dependence of various kinetic parameters can allow conclusions to be drawn regard the pKa of various amino acid sidechains on the enzyme. If the substrate of the reaction can undergo a protonation-deprotonation reaction and only one form of the substrate effectively bids to the enzyme, this alone can affect the pH dependence of the overall reaction. Likewise, if the chemistry of the reaction involves a group that can be protonated this agan is likely to be reflected in the pH dependence of the reaction. Even with reactions where the substrates or products are not capable of being protonated/deprotonated, the effects of pH on the enzyme are complex and will result in a pH dependence of the rate of the enzyme catalyzed reaction. The enzyme contains many amino acids with side-chains that can be protonated/deprotonated and usually either the protonated or the deprotonated form of a particular side chain in the protein is required for some aspect of structure or function of the protein. Hence as the pH changes away from the optimum their individual contributions to the correct structure or function of the protein will change resulting in decreased activity. These contributions can be to any phase of the enzyme catalyzed reaction, substrate binding, chemical catalysis, or product release and the ability of enzyme kinetics to dissect these different phases makes the study of the **pH dependence of kinetic parameters** a powerful tool in the investigation of structure function relationships of enzymes.



Finally, in the third stage the product must be released from the binding site of the enzyme so that free enzyme is regenerated and the cycle can begin again. As with the first stage, various kinetic and thermodynamic parameters describe this product release phase of the reaction.

Do Enzymes Behave the Same Way in Cells as they do in Vitro?

Over the years the study of enzymes and enzyme kinetics has given much information about structure-function relationships in a wide variety of enzymes and has given insight into the catalytic steps involved as well as the effects of regulation on their activity. As detailed above much of the experimental work on enzymes over the years has required the use of "initial rate" conditions where the effective substrate concentration is very much higher that the enzyme concentration, and the use of purified proteins in vitro. In recent years there has been much interest in what happens inside the cell as compared to in vitro conditions in processes such as protein

folding and protein-protein interactions. These considerations are also being discussed in the context of enzyme kinetics and regulation. Two issues are forefront in these discussions: the effects of viscosity, and the impact of "molecular crowding"(20-22). In vitro the viscosity of the solution is essentially that of an aqueous milleux, while the plethora of other molecules in the in vivo environment give rise to a much higher viscosity inside the cell. This will effect diffusion processes involved in enzyme-substrate interactions and may impact the overall catalytic cycle, depending upon the rates of various steps involved. Second, the total macromolecule concentration inside a cell - estimates range upto 200-400mg/mL inside a mitochondrion or e coli – are very different from protein concentrations use in a typical enzyme kinetics experiment- often in the μg/mL range. Recent studies suggest that "molecular crowding" may well effect kinetic parameters such as Km and Vmax, as well as impact the roles that the dynamic properties of the protein may play. Such impacts are of particular importance when using the various parameters obtained from enzyme kinetics to model potential pathways and impacts of substrate/product concentration changes or regulation of in vivo pathways in systems biology.

Terms:

Pre-Steady State: The pre-steady state phase of an enzyme catalyzed reaction is that phase immediately following mixing of the enzyme and its substrates that occurs prior to the overall reaction reaching a "steady state" and may involve binding steps of chemical steps in the overall reaction.

Steady State: The steady state is defined by the general equation:

$$d[E:S]/ dt = 0$$

Under steady state conditions there is no change in the concentration of any enzyme-substrate, or enzyme product complexes and hence the catalyzed reaction proceeds at a constant rate, V_o, often called the initial rate. The steady state persists until there is a significant change in the total substrate concentration such that the concentration of E:S is changed.

Formal Kinetic Mechanism: the formal kinetic mechanism of an enzyme is a description of whether the reaction proceeds through a "complex" mechanism where all substrates are present on the enzyme surface prior to reaction occuring or through an enzyme substituted mechanism where one substrate reacts with the enzyme to form some covalent interaction with the enzyme or a tightly bound cofactor and a product is released prior to the second substrate binding and reacting with the covalently bound intermediate

Substrate Inhibition: At high concentrations of a substrate, substrate inhibition is observed if more than one substrate molecule can bind to the active site region resulting in non productive substrate binding or if substrate can bind to an enzyme product complex slowing the release of a product to create a new rate limiting step in the overall reaction.

Abortive Complex Formation: When one or more substrate and product bind in a single enzyme complex with the result that an overall slower rate limiting step in the catalytic cycle is created, the complex is termed an abortive complex. Such complexes result in substrate inhibition and frequently plan important roles in heterotropic regulation of an enzyme's activity.

Allosteric: Allosteric sites on proteins (from the greek, *allos* (other) and *stereos* (solid object)) refer to sites that are physically distinct from the active site of the protein and give rise to either activation or inhibition of processes occurring at the active site. Such effects may be heterotropic or homotropic and involve interactions across subunit interfaces.

Heterotropic: When one ligand impacts the effects of another chemically different ligand at a physically separate binding site on the protein the effect is said to be a heterotropic effect. Such effects can be positive or

negative.

Homotropic: When one ligand, binding to one type of site in a multimeric protein impacts the binding (or resultant effects) of the same type of ligand binding to subsequent sites of the same type in the multimeric protein the effects are said to be homotropic. Such effects can be positive or negative.

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