CHAPTER 4

Enzyme Kinetics: Basic

Learning Objectives

- Rate if enzyme reaction is dependent on substrate concentration
- First mathematical treatment of kinetic data was provided by Michaelis and Menten
- The velocity versus substrate concentration graph is hyperbolic in shape

INTERESTING FACT

Based on the Einstein's relationship, an enzyme like lactate dehydrogenase, its substrate lactic acid and solvent water molecules could move at a rate of 4 ms^{-1}, 170 ms^{-1} and 370 m s^{-1} respectively. Enzyme and substrate will fly past one another like rifle bullets. In the dense fluid of the cell, however, the movement is more difficult due to a number of obstacles such as ions, metabolites, macromolecules and membranes and, therefore the molecule moves more like a staggering drunkard than in a straight progression. However, this tumbling increases the collision frequency and the probability of distinct molecules meeting one another.

4.1 UNDERSTANDING CHEMICAL KINETICS

In order to begin with enzyme kinetics, it is essential to recap through the high school chemical kinetics, which will enable us to know about the basic treatment of kinetic data and various associated terms. Although the driving force for chemical (or biochemical) reaction is Gibb's free energy, therefore thermodynamics is used to predict the spontaneity of a reaction, however, chemical kinetics allow us to study the speed of various reactions. All steps involved in metabolism, replication, cell division, muscle contraction etc. are subject to the same basic principles of thermodynamics and chemical kinetics, as the elementary reactions of the chemistry. In general, the speed of a chemical reaction is based on the fact that how fast collisions among the reactants are occurring, and this was the basis of Arrhenius collision theory. However, experimental determination of rates of reactions can be done by measurement of loss of reactants with time, or measuring the change in concentration of product with time. We will now discuss more about the measurement of the rate of reactions.

4.1.1 Rate of a Reaction

The rate or velocity (v) of a reaction of a chemical process describes how fast it occurs. Usually, the velocity is expressed as a change in concentration per unit time. For example, in a reaction

$$A + B \qquad C + D$$

The rate of a chemical reaction may be depicted as rate of decrease of reactants or rate of increase of products with time i.e.

$$\text{Rate} = -\frac{dA}{dt} = -\frac{dB}{dt} = +\frac{dC}{dt} = +\frac{dD}{dt}$$

The change in concentration of reactant or products with time may be measured by repeated sampling, but if the reactions are too fast or too slow this method may not be appropriate to determine the rate of reaction. For example, $2H_2 + O_2 \leftrightarrows 2H_2O$ proceeds so slowly as to be unmeasurable. Radioisotopes of some nuclei have very long lifetimes (τ for $^{238}U = 2.3 \times 10^{17}$ s or 4.47 billion years). Other reactions, such as the growth of bacterial cells, are slow ($\tau = 1 \times 10^4$ s) but measurable. The biological reactions on the other hand may be very fast, such as formation of carbonic acid by carbonic anhydrase (millions of molecules formed per second). Hence it may not be feasible to perform sampling experiment and determine the rate of reaction for all biological reactions.

4.1.2 Law of Mass Action

Aforesaid expression for rate is based on the determination of the rate of reaction by performing an experiment and repeated sampling. However, it may not be feasible at the times to perform experiment and determine the rate of reaction. Therefore an alternative approach that involves constants derived from experimental data for theoretical calculation of rate of reactions was devised. This was known as Law of Mass Action (LMA). Law of mass action relates the rate of reaction with that of the initial substrate concentration. For a chemical reaction A + B ⇆ C + D, the law of mass action may be applied for the determination of rate as follows:

$$\text{Rate} = k[A]^m[B]^n$$

Unit of rate of reaction is moles L^{-1} per unit time (e.g. mol L^{-1} s^{-1})

Here k is rate constant; [A] and [B] are concentration of reactants; m and n are the coefficients obtained from experimental observations. Of all the parameters influencing reaction rates, temperature is normally the most important one and is accounted for by the Arrhenius equation.

The magnitude of rate constant is a direct measure of rate of a reaction, higher the value of k, faster will be the reaction and vice versa. The above expression is also known as **rate law**, and the present form is differential form, there can be alternative form of rate law, which is known as integral rate law. Different rate law expressions are described in Table 4.1.

Caution

It must be noted here that K (uppercase) represents equilibrium constant and k (lowercase) represents rate constant and both are different terms.

Practice question

Question: In the childhood days, our elderly used to ask, if one cloth needs 2 hours to dry, how many hours will be needed to dry 20 cloths, and the answer would be same. Does that rule also apply to chemical reactions? If you have a vessel with 1mM substrate and other 10 mM, what will be the difference in their reaction rates? Can you predict?

Solution: No! Chemical kinetics is not analogous to drying cloths. Also, we cannot answer this question, without knowing the order of reaction. If the order of reaction is zero, both reaction will have same speed, but if order is 1 second reaction will be 10 times faster than first. If order of the reaction is 2 the second reaction will be 100 times (10^2) times faster than first.

4.1.3 Order of the Reaction

The order of reaction with respect to a given substance (such as reactant, catalyst or product) is defined as the index, or exponent, to which its concentration term in the rate equation is raised. The order of the reaction describes how the velocity of the reaction depends upon the concentration of reactants.

The sum of m and n for a reaction described in previous section is called order of the reaction, and these two constants are not stoichiometric coefficients of the reaction, but experimentally determined values for a reaction. In simple terms we may understand that if the rate of reaction does not change on increasing the concentration of reactants that means m and n are zero and the reaction is zero order. If the rate of reaction doubles on doubling the

Table 4.1 Summary of rate law and formulae for calculating rate, order or half-life of reactions.

Sr. No.	Order of Reaction	Rate law	Integrated rate law	Units of rate constant	Half life
1	Zero	Rate = k	$[A]_t = -k_t + [A]_0$	Mol L^{-1} s^{-1}	$[A]_0$ /2k
2	First	Rate = k [A]	$\ln[A]_t = -k_t + \ln[A]_0$	s^{-1}	ln2/k or 0.693/k
3	Second	Rate = k $[A]^2$	$1/[A]_t = 1/[A]_0 + k_t$	L $Mol^{-1}s^{-1}$	1/ k$[A]_0$

k- rate constant, $[A]_o$ – initial reactant conc., $[A]_t$ –reactant conc. at time t, ln = natural log
It may be noted that the order of reaction can apparently by guessed be looking at the units of the rate constant.

The units of k_1 are time^{-1} (e.g. per second or per minute). There are no concentration units in k_1 so we do not need to know absolute concentrations for determining the rate of reaction, only relative concentrations are needed.

Half-life of a first order reaction is given by

$$t_{1/2} = \frac{\ln 2}{k_1} = \frac{0.693}{k_1}$$

Relation of rate law expression with equation of line and corresponding graph is shown in Fig 4.2

Fig 4.2 Graphical representation of first order kinetics

Some commonly known examples of first order reactions in biological systems include, degradation of chlorinated compounds, microbial growth (bacteria or fungi), oxidation of organic matter, heat denaturation of proteins, degradation of antibiotic in bloodstream, radioactive decay of compounds.

Formulae for calculating percentage of reactant remaining if number of half-lives are given

$$\% \text{ of reactant remaining} = (100/2^n) \text{ — for first order reaction}$$

Second Order Reaction: The second order reaction is much more dependent on substrate concentration, as by doubling the concentration of substrate, the rate of reaction is increased by four times. i.e. the dependence of the rate with the concentration is squared.

$$\text{Rate} = \frac{dC}{dt} = k_1[C]^2 \text{ or } -\frac{dC}{[C]^2} = k_1 dt$$

$$\frac{1}{[C]} = k_2 t + \frac{1}{[C]_0}$$

Half-life of a second order reaction is given by

$$t_{1/2} = \frac{1}{kC_0}$$ where k is rate constant and C_0 is initial substrate concentration.

Relation of rate law expression with equation of line and corresponding graph is shown in Fig 4.3.

Denaturation and renaturation kinetics of DNA is an example of second order kinetics in biological world.

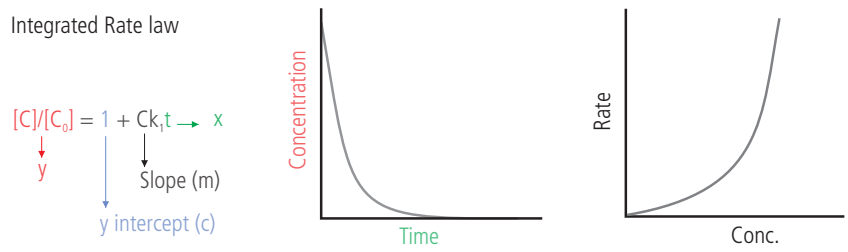

Fig 4.3 Graphical representation of second order kinetics

However, as the calculation was to be performed for initial period of reaction, when no sufficient product was formed, the equation was limited to

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

From the law of mass action, the rate of chemical reaction or rate of formation of product can be represented as

$$v_0 = k_2[ES]$$

LMA

As per the law of mass action (LMA), the rate of product formation is equal to the product of ES-complex concentration and rate constant k_2.

However, it is difficult to measure the magnitude of [ES] at any given time, hence this terms must be substituted. In order to substitute this term, some assumptions were made and finally a substitute of [ES] was found as described below.

The rate of formation of ES at any time t is given by k_1 [E][S], where E is the concentration of enzyme at time t. Similarly, the rate of breakdown of ES is given by k_{-1} [ES]. Another assumption made by Michaelis and Menten was that an equilibrium between enzyme, substrate and enzyme-substrate complex was set up almost immediately. Therefore the rate of formation of ES could be equated to the rate of breakdown of ES. [Therefore, they considered the reaction rate during the initial time period only, when the significant amount of product has not been formed.]

$$k_1[E][S] = k_{-1}[ES]$$

On rearranging the equation, by taking constants on one side, we get

$$\frac{[E][S]}{[ES]} = \frac{k_{-1}}{k_1} = K_s$$

As the total amount of enzymes [E] at any given time, can be represented by total enzyme present initially [E_0] minus the enzyme that has turned into enzyme substrate complex [ES]. Replacing these values in the above equation we get

$$([E_0] - [ES])[S] = K_S[ES]$$

On further solving with an objective to get [ES] on one side,

$$[E_0][S] - [ES][S] = K_S[ES] \text{ or } [E_0][S] = K_S[ES] + [ES][S]$$

$$[ES] = \frac{[E_0][S]}{[S] + K_S}$$

Finally, placing this value in the equation stated in the beginning for the rate of enzymatic reaction obtained from law of mass action ($v_o = k_2$ [ES].

$$v_o = \frac{k_2[E_0][S]}{[S] + K_s}$$

Note

As most the biological reaction do not acheive equilibrium, original equation given by Michaelis and Menten was less realistic. However, complete biochemical milieu of cell attains a steady state, suggestion of Brigg's and Haldane made the equation more realistic.

This was the original Michaelis-Menten equation. It is similar to an equation that is encountered in most of the text books, however this equation was little unsatisfactory because, general assumption of equilibrium made by Michaelis and Menten could not be applied, as many enzymes work rapidly, and therefore an improvement was desired. This improvement was performed by Briggs and Haldane to finally shape the equation.

4.2.3 Haldane – Briggs – Michaelis and Menten Equation

George Briggs and John Haldane in 1925, introduced a more generalized valid assumption, called steady state, rather than equilibrium. It was argued that, since the concentration of

Practice Question

Question: A metabolic pathway containing four sequential steps that are catalysed by enzymes a, b, c and d respectively with K_m values 10^{-2}, 10^5, 10^{-4}, 10^{-3} respectively. Which of the enzyme must be catalysing the rate limiting step

Solution: A rate limiting step is the slowest step of a chemical process. From the kinetic equation of enzyme discussed above, it can be deduced that the rate is inversely proportional to the Km value. Hence, a biochemical step catalysed by enzyme with highest K_m value shall have least velocity. Here, the highest value of K_m is 10^5, hence, enzyme b represents the rate limiting step of a biochemical pathway.

Rate Limiting Step

In a process a step that consumes maximum amount of time decides the rate of a process, hence called rate limiting step. Example in a car industry all the parts are assembled in 2 days but fitting the engine takes 5 days. The fitting of engine would be rate limiting step. Hence, it represents the slowest step.

4.3 ASSESSMENT OF ENZYME EFFICIENCY

Enzyme efficiency, is the measure of performance of an enzyme that can be assessed by various parameters, such as how rapidly the product is being formed or how efficiently the product is being formed. The rate-limiting step of an enzymatic reaction is generally the formation of the product, which predict the net velocity of the enzymatic reaction. Nevertheless, the affinity between enzyme-substrate also matters in explaining the enzyme efficiency. Michaelis-Menten constant, turnover number and specificity constant are the three are few commonly used terms to represent the performance of an enzyme and its inherent properties.

4.3.1 Michaelis Menten Constant (K_m) and its Meaning

Michaelis-Menten (K_m) is a constant used in Michaelis Menten equation of enzyme kinetics, its meaning can be understood in three simple ways.

K_m is the ratio of rate constants: In terms of rate constants K_m is defined as ratio of rate constants for the dissociation of ES with rate constant of formation of ES. i.e.

$$K_m = \frac{k_{-1} + k_2}{k_1} \qquad | \; K_m \text{ has the units of concentration [Molar]}$$

K_m is measure of affinity: K_m is the measure of affinity of substrate and enzyme when the formation of product represent the rate limiting step. Considering that k_2 is rate limiting step then $k_2 << k_{-1}$. Therefore,

$$K_m = \frac{k_{-1}}{k_1}$$

If this condition satisfies then K_m may be taken as a measure of affinity of substrate. (i.e. how rapidly ES complex is dissociating back to E and S)

It must be however noted that, **K_m is inversely proportional to affinity**, therefore higher the K_m value lower will be affinity.

Note

If an enzyme can utilize more than one substrate, then enzyme can have different K_m values with respect to each substrate.

K_m is the substrate concentration at half V_{max}: Using Standard MM equation we can prove this definition if we replace v_0 as ½ V_{max}. (Place, $v_o = V_{max}/2$ in MM equation), we get

$$\frac{V_{max}}{2} = \frac{V_{max}[S_0]}{K_m + [S_0]} \qquad | V_{max} \text{ on both side gets cancelled } |$$

$$K_m + [S_0] = 2[S_0]$$

$$K_m = [S_0]$$

The third interpretation of MM constant is also its standard definition which states that Michaelis Menten constant is the substrate concentration at velocity equal to half of maximum, or $K_m = [S]$ when $v_o = ½ V_{max}$.

4.4.4 Edie Hofstee Plot

George Eadie and Barend Hofstee, further modified the double reciprocal equation, by multiplying it on both the sides by $v_o.V_{max}$ and obtained a new equation as below commonly known as Eadie- Hofstee equation. This equation overcomes the shortcomings of lineweaver burk plot. Following is the mathematical conversion of double reciprocal equation into Eadie Hofstee equation.

Original double reciprocal equation

$$\frac{1}{v_o} = \frac{K_m + [S_0]}{V_{max}[S_0]}$$

Multiplied on both sides by $v_0\ V_{max}$

$$\frac{1}{v_o} \times\ v_o V_{max} = \frac{K_m}{V_{max}[S_0]} \times\ v_o V_{max} + \frac{[S_0]}{V_{max}[S_0]} \times\ v_o V_{max}$$

On rearranging we get

$$V_{max} = \frac{K_m}{[S_0]} \times\ v_o + v_o$$

Rearranging to bring variable into other side (in the format of y = mx + c)

$$v_o = -\frac{v_o}{[S_0]} \times\ K_m + V_{max}$$

This equation, can now be compared with equation of line y = mx + c,

$$\text{Where, } y = v_o\ ,\ x = \frac{v_o}{[S_0]}\ ,\ m = -K_m,\ c = V_{max}$$

Now, if we plot the graph between rate of reaction (v_0) on y axis and v_o/ [S] on x axis, a straight line is obtained. It must be noted that point at which the line intersects y axis (c) represents V_{max} and the slope of the line represents $-K_m$. So there is no absolute requirement of extrapolation to determine the value of K_m using this equation. Also, the point at which the same line intersects x axis represents V_{max}/K_m.

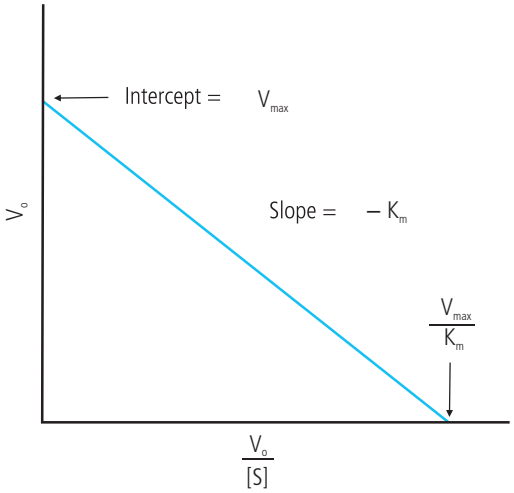

Fig 4.7 Eadie Hofstee Plot

4.4.5 Hanes Wolf Plot

Similar to Eadie-Hofstee transformation, another transformation was performed by Charles Hanes, by multiplying the double reciprocal equation on both the sides by $[S_0]$. Following is the mathematical conversion of double reciprocal equation into Hanes Wolf equation.

Original Double reciprocal equation

$$\frac{1}{v_o} = \frac{K_m + [S_0]}{V_{max}[S_0]}$$

Multiplied on both sides by $[S_0]$, we get,

$$\frac{1}{v_o} \times [S_0] = \frac{K_m}{V_{max}[S_0]} \times [S_0] + \frac{[S_0]}{V_{max}[S_0]} \times [S_0]$$

On rearranging we get,

$$\frac{[S_0]}{v_o} = \frac{K_m}{V_{max}} + \frac{1}{V_{max}} \times [S_0]$$

This equation, can now be compared with equation of line $y = mx + c$,

$$\text{Where, } y = \frac{[S_0]}{v_o},\ x = [S_0],\ m = \frac{1}{V_{max}} \text{ and } c = \frac{K_m}{V_{max}}$$

Now, if we plot the graph between rate of reaction $[S_0]/ v_o$ on y axis and $[S_0]$ on x axis, a straight line is obtained. It must be noted that point at which the line intersects y axis (c) represents $\frac{K_m}{V_{max}}$ and the slope of the line represents $\frac{1}{V_{max}}$. So, there is no absolute requirement of extrapolation to determine the value of K_m using this equation. Also, the point at which the same line intersects x axis represents $-K_m$.

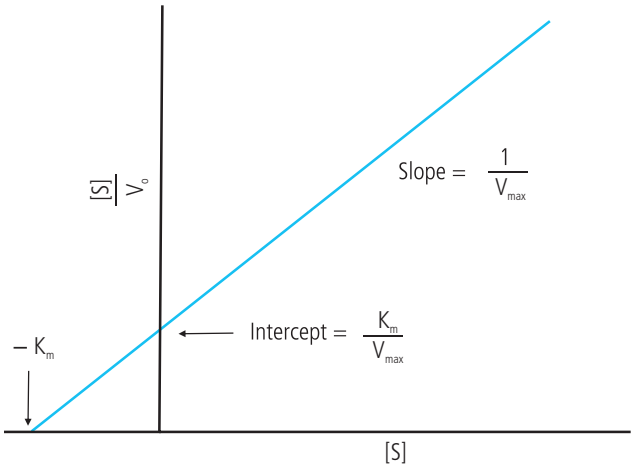

Fig 4.8 Hanes wolf Plot

4.4.6 Eisenthal - Cornish - Bowden plot

One more approach, which was quite unusual and different, was developed by Robert Eisenthal and Cornish-Bowden in 1974. This was an unusual approach because the graph that was proposed, was between two constants V_{max} and K_m, which is usually not observed in standard mathematics text. However, the equation holds good mathematically,

On rearranging double reciprocal equation as follows

$$\frac{1}{v_o} = \frac{K_m + [S_0]}{V_{max}[S_0]}$$, multiplying both sides by V_{max}, we get,

$$\frac{V_{max}}{v_o} = \frac{K_m + [S_0]}{[S_0]} = \frac{K_m}{[S_0]} + 1$$

to alpha 1-antitrypsin (Owen et al, 1983). Deficiency of antithrombin III is associated with thromboembolic disorders. Yet another evidence of the use of term anti-enzyme comes from the work of Kummerling in 1943, who prepared killed suspensions of Bacterium coli, Proteus X 19 and certain vibrios which possessed tryptophanase action. He showed that the tryptophanase from Bacterium could be inhibited by an anti-coli serum, and that the Proteus X 19 tryptophanase could be similarly inhibited by the serum of typhus patients which give a positive Weil Felix reaction. As the term anti-enzyme is very general and does not represent a specific class of molecule, the use of anti-trypsin is not common.

4.5.6 Isotopic substitution

Isotopic substitution, or substitution of isotopes (usually radioactive) has been used to detect and measure the rate of enzymatic reactions. Isotopic substitution is also known to affect the rate of enzymatic reaction. Substitution of isotopes, leads to a quantum phenomenon due to different energy states of interacting electrons, known as isotopic effect. There are three types of isotopic effects that exits in enzymes. primary, secondary and solvent isotope effects. Primary isotope effects are associated with isotopic substitution of an atom subtending a covalent bond broken or made during the course of the reaction. Secondary isotope effects are associated with isotopic substitution of an atom subtending a covalent bond which may be changed but not broken or made during the course of the reaction. Solvent isotope effects are changes in the rate or equilibrium of the reaction due to substitution of deuterium for hydrogen in the solvent water in which the reaction takes place.

Isotope effects can be measured using the competition between isotopic and non-isotopic substrate together, and the ratio of the two isotopes is measured at two different time points, one early time point and one at late time point. The ratios of these two rate constants is represented as k/k_i and used as an indicative of primary isotopic effect. This ratio for some of the commonly used isotopes is 6.82 for H^2, 16.04 for H^3, 1.054 for C^{13}, 1.044 for N^{15} and 1.068 for O^{18}.

4.5.7 Enzyme inhibitors

Certain chemical compounds inhibit activity of enzyme molecules either permanently or temporarily. These compounds may fall into different categories, sometime they destroy the active site or occupy it making dead end complex, and in other cases they may completely denature the protein itself. Poisons such as cyanide and radiation destroy the tertiary structure of the enzymes, making them ineffective. Compounds such as aspirin inhibits enzymes by destroying its active site catalysing hydrolysis of ester linkage. A more detailed discussion on the enzyme inhibition is provided in the chapter 6.

QUICK REVISION

- Kinetics of enzymes is best studied using Michaelis-Menten equation modified by Haldane.
- A curve between velocity of reaction and substrate concentration is hyperbolic.
- In order to make graph linear, conventional equation was converted to double reciprocal and plot between inverse of velocity and inverse of substrate was named Lineweaver Burk plot.
- Several more modifications were made to the lineweaver Burk equation.
- All enzymes do not follow Michaelis Menten kinetics

QUESTIONS

1. Comment on how enzyme kinetics is different from equilibrium kinetics and what is the disadvantage of equilibrium kinetics for studying enzymes.
2. The K_m of a Michaelis-Menten enzyme for a substrate is 1.0×10^{-4} M. At a substrate concentration of 0.20 M, $v_o = 43$ μM/min for a certain enzyme concentration. What is the value of v_o for this enzyme at a substrate concentration of 0.02 M?
3. Three different rate constant are generally assumed during the derivation of kinetic equation of enzymes, k_1 representing the formation of ES complex from E and S, k_{-1} representing the breakdown of ED back into the E and S, while k_2 represents the rate constant of formation of product from ES. Are the units of all these rate constants same?
4. What is the difference between K_s and K_m, for an enzymatic reaction $E + S \leftrightarrows ES \rightarrow E + P$, the values of k_1, k_{-1} and k_2 are 10^8, 10^3 and 2×10^3 respectively. Determine the values of K_s and K_m.
5. What fraction of V_{max} is observed at $[S] = 6\ K_m$?