EDO UNIVERSITY IYAMHO

Department of Biochemistry

BCH 411 Advanced Enzymology

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General overview of lecture: This course is intended to build on the basic knowledge of enzymology acquired by the student in 300 level. The course covers advanced topics in Enzymology such as enzyme kinetics and enzyme isolation, purification and characterization.

Prerequisites: Students should be familiar with basic knowledge of enzymology such as properties, nomenclature, basic enzyme kinetics, relevance of kinetic parameters and experimental determination of kinetic parameters among others.

Learning outcomes: At the end of the lectures, students should be able to:

1. Demonstrate improved understanding of enzyme kinetics
2. Discuss the chemistry of enzyme catalysis
3. Discuss catalysis in multi-enzyme system
4. Demonstrate an understanding of enzyme isolation, purification, characterization and reconstitution

Assignments: We expect to have at least 3 individual homework and 1 oral presentation throughout the course in addition to a Mid-Term Test and a Final Examination. Home works are due at the beginning of the class on the due date. Home works are organized and structured as preparation for the midterm and final examination, and are meant to be a studying material for both examinations.

Grading: We will assign 10% of this class grade to homework, 10% to oral presentation and 10% for the mid-term test. The final examination, which will be comprehensive, shall be 70%.

Textbook: The recommended textbook for this class are as stated:

Title: *Biochemistry: The Chemical Reactions of Living Cells (Volumes 1 & 2)*

Authors: David E. Metzler

Publisher: Elsevier Academic Press, 2nd edition

Title: *Enzyme Kinetics: Theory and Practice in: Plant Metabolic Networks (Schwender (ed.)*

Authors: Alistair Rogers and Yves Gibon.

Publisher: Springer Science+Business Media, LLC 2009

DOI 10.1007/978-0-387-78745-9_4

ENZYME KINETICS

Enzyme kinetics deals with measuring the speed (reaction rate) of an enzymatic reaction. Its goal is to establish a ñrate equationò which describes the velocity of a reaction interms of kinetic constants and other parameters which are measured experimentally and described mathematically.

In measuring velocity, an enzyme catalysed reaction is started at a definite time by mixing two or more substrates (reactants) together rapidly. Usually, the reaction is kept at a precise constant temperature and pH, and the concentration of the substrate(s) or product(s)is/are measured aftera fixed time interval or at different intervals. The disappearance of the substrate(s) and the appearance of the product(s) with time can then be shown as a progress curve (as shown below).

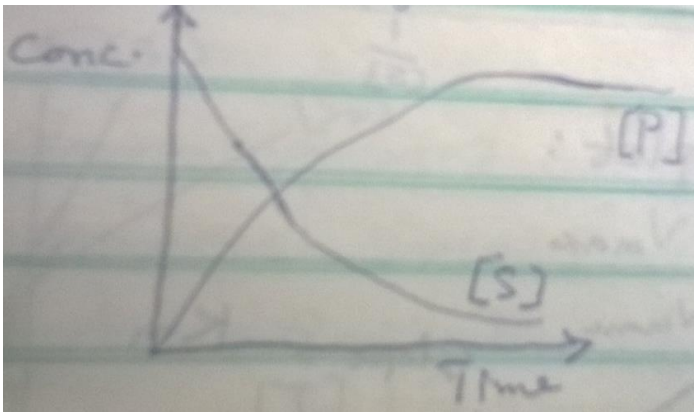

Figure 1: Progress curve showing increased [P] or decreased [S]

For many steps in metabolism, enzyme kinetic properties have been determined, and this information has been collected and organized in publicly available online databases (www.brenda.uni-koeln.de).

VELOCITY OF AN ENZYMATIC REACTION

The velocity (v) of an enzymatic reaction is defined as the rate at which a substrate disappears or at whicha product is formed. The two can be represented mathematically as shown below.

$$v = -d[S]/dt = d[P]/dt$$

The units of velocity is moles per litre per second (M s^{-1}) *but traditionally in enzymology, it is moles per litre per minute.*

STEADY STATE AND TRANSIENT STATE ENZYME KINETICS

When an enzyme is mixed with an excess of substrate, there exists an initial short period of time (few 100 microseconds) during which intermediates leading to the formation of product gradually build up. This state is called *pre-steady state* or *transient state*. Due to the rapid nature of this state, special technique is usually required to study its kinetics.

After this initial state (i.e. transient state), a steady state is reached where reaction rates and concentration of intermediate change relatively slowly with time.Measurement of the progress of reaction during the steady state is characterised by reduction in [S] with an equivalent increase in [P] as shown in Figure 1.

Steady state kinetics is based mainly on Michaelis-Menten equation which rests on 2 assumptions:

1. Equilibrium assumption

The rate of formation of enzyme substrate (ES) complex from free enzyme and substrate is equal to the rate of dissociation of ES complex to free enzyme and substrate i.e.*The rate of formation of ES = Rate of its disappearance.* $E + S \rightleftharpoons ES$

In this state, backward and forward reactions are at equilibrium.

2. Steady state assumption

The concentration of ES complex remains relatively constant over a period of rate measurement until S is almost totally exhausted. In this state, the rate of synthesis of ES complex is equal to the rate of its breakdown. $E + S \rightleftharpoons ES \longrightarrow E + P$

REACTION RATES AND REACTION ORDER

Reactions can be of different types. Some of these types include First-Order Irreversible Reaction, First-Order Reversible Reaction and Second-Order Reaction.

First-order reactions

First-order kinetics is observed for unimolecular processes in which a molecule of A (reactant) is converted into product P in a given time interval with a probability that does not depend on interaction with another molecule e.g. radioactive decay.

In ýrst-order reaction, the rate depends on the ýrst power of the concentration i.e. the rate of decrease of the concentration of a given reactant [A] is experimentally directlyproportional to the concentration of that reactant atany given time.

A. First-Order Irreversible Reaction

The simplest possible reaction is the irreversible conversion of substance A to product

P $\mathrm{A} \xrightarrow{k_1} \mathrm{P}$ Eqn 1

The arrow in the reaction above signifies that the equilibrium lies far to the right, and the reverse reaction is *infinitesimallysmall*. As discussed earlier, the reaction rate or velocity (*v*) of the reaction can be defined in terms of the time (*t*)-dependent production of product P. Alternatively, the reaction rate or *v* can also be defined in terms of the time-dependent consumption of substance A, since formation of P involves the loss of A.

First-Order Irreversible Reaction is mathematically expressed as:

$$v = \frac{\delta[\mathrm{P}]}{\delta t} = -\frac{\delta[\mathrm{A}]}{\delta t} = k_1[\mathrm{A}]$$ Eqn 2 where [A] and [P] are the concentrations of the substance and product, respectively.

As A is transformed to P, there is less of A to undergo the transformation, and therefore the concentration of A will decrease exponentially with time.

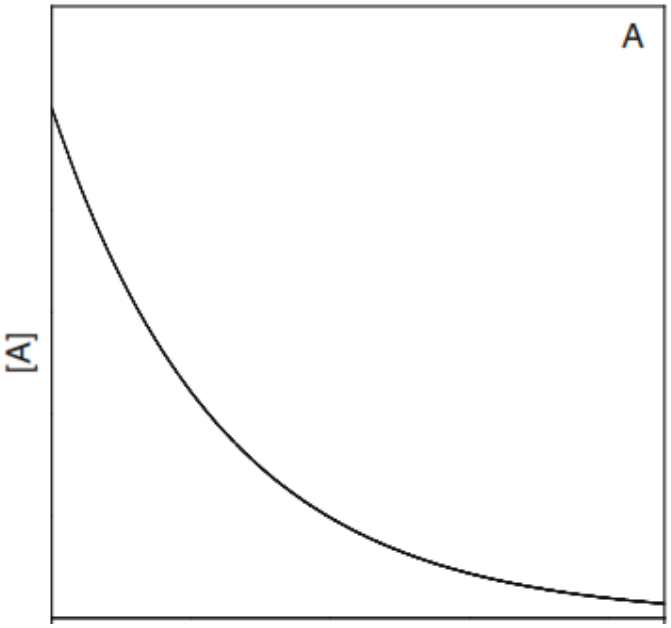

Figure 2: First-order reaction showing the decrease of A over time

The rate constant (k_1) of this reaction is proportional to the concentration of A and has the unit s^{-1}.

Integration of Eqn 2 fromtime zero (t_0) to time t gives $$\ln \frac{[A]}{[A]_0} = -k_1 t$$ Eqn 3

OR $$\frac{[A]}{[A]_0} = e^{-k_1 t}$$

OR $\log[A_0] - \log[A] = kt / 2.303$ (equation of exponential decay) where $[A]_0$ is the starting concentration at t_0

Whenln[A] is plotted against t, a ýrst-order reaction will yield a straight line, where the gradient is equal to $-k_1$ (Figure 3).

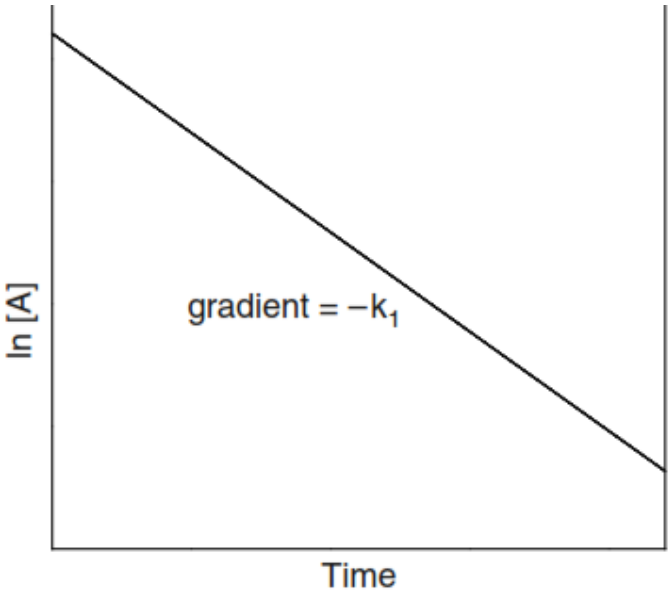

Figure 3: First-order reaction showing the decrease of ln[A] over time

B. First-Order Reversible Reaction

In Biochemistry, few reactions are as simple as the ýrst-order reaction. In most cases, reactions are reversible and equilibrium does not lie far to one side. $[A] \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [P]$ Eqn 4

Therefore, the corresponding rate equation is given as

$$v = -\frac{\delta[A]}{\delta t} = k_1[A] - k_{-1}[P]$$ Eqn 5 where where k_1 and k_{-1} are the rate constants for the ýrst-order, forward and reverse, reactions respectively.

When the rates of the forward and reverse reactions are equal, consumption of A will stop and the overall reaction rate is zero, i.e., a stateof equilibrium is attained.

$$0 = -k_1[A]_{eq} + k_{-1}[P]_{eq}$$ Eqn 6 where [A]eq and [P]eq are the substrate concentrations at equilibrium.

It should be noted that in catalyzed reactions, the position of equilibrium is not altered by the presence of an enzyme but the enzyme increases the rate at which equilibrium is attained.

When the forward and reverse reactions are both ýrst order, the equilibrium constant (K_{eq}) is equal to the ratio of the rate constants for the forward and reverse reactions. For a reaction to precede in the direction of product (P) formation, the equilibrium constant must be large.

$$K_{eq} = \frac{k_1}{k_{-1}} = \frac{[P]_{eq}}{[A]_{eq}}$$ Eqn 7

PSEUDO-FIRST ORDER REACTION

A reaction is considered pseudo-first order; when substance A actually reacts with a second molecule such as water, which is present in such excess that its concentration does not change during the experiment. Consequently, the velocity is apparently proportional only to [A].

SECOND-ORDER REACTION

For a chemical reaction to occur between two molecules, A and B, they must meet and collide.The velocity of a second-order process is characterized by a bimolecular rate constant and is proportional to the product of the concentrations of A and B.

Whenever two reactants come together to form a product, the reaction is considered second order, e.g., $A + B \xrightarrow{k_1} P$ Eqn 8

In addition to being reversible, most reactions are second order or greater in their complexity. The rate of reaction in Eqn 8 is proportional to the consumption of A and B and to the formation of P. A reaction is described as second order because the rate is proportional to the second power of the concentration.

The rate constant k_1 has the unit $s^{-1} M^{-1}$

$$v = -\frac{\delta[A]}{\delta t} = -\frac{\delta[B]}{\delta t} = \frac{\delta[P]}{\delta t} = k_1[A][B]$$ Eqn 9

Integration of Eqn 9 yields an equation where *t* is dependent on the two variables,

A and B. To solve this equation, an assumption that either A or B is constant must be made. This is achieved experimentally by using a concentration of B that is far

in excess of requirements such that only a tiny fraction of B is consumed during the reaction and therefore the concentration can be assumed not to change. The reaction can then be considered as pseudo-ýrst order.

$$v = k_1[A][B]_0 = k_1'[A]$$ Eqn 10

On the other hand, if the concentration of both A and B at time zero are the same, i.e., [A0] = [B0], then Eqn 10 can be simply written as:

$$v = -\frac{\delta[\mathrm{A}]}{\delta t} = k_1[\mathrm{A}]^2$$ Eqn 11

THE MICHAELISï MENTEN EQUATION

The Michaelisï Menten equation was derived by Michaelis and Menten but was further developed by Briggs and Haldane. The equation is fundamentally importantto enzyme kinetics and it is characterized by two constants: theMichaelisï Menten constant (K_m) and the indirectly obtained catalytic constant, k_{cat}.

Derivation of M-M Equation

As you will recall, a step-by-step derivation of the M-M equation was done in 300 level (BCH 312). However, a simple revision is provided here using the simple conversion of substrate (A) into product (P) catalyzed by the enzyme (E).

As explained in induced-ýt hypothesis, the ýrst step is substrate binding and the second step is the catalytic step.

$$\mathrm{E+A} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{EA} \xrightarrow{k_2} \mathrm{E+P}$$ Eqn 12

Referring to Eqn 2, $v = \frac{\delta[\mathrm{P}]}{\delta t} = -\frac{\delta[\mathrm{A}]}{\delta t} = k_1[\mathrm{A}]$, formation of P can be defined in terms of the dissociation rate (k_2) of the EA complex, commonly denoted as k_{cat}, and the concentration of the enzymeï substrate complex ([EA])

$$v = k_{\mathrm{cat}}[\mathrm{EA}]$$ Eqn 13

Assumption

The dissociation rate (k_{cat} in Eqn 13 or k_2 in Eqn 12) of the EA complex is assumed to be slow compared to association (k_1) and redissociation (k_{-1}) reactions and that the reverse reaction (PŸ A) is negligible.

Change in substrate (A), product (P), free enzyme (E), enzymeï substrate complex (EA), and total enzyme (E_t) concentration over time

Using a simple reaction such as Eqn12 $E+A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EA \xrightarrow{k_2} E+P$, the interaction between A and E, formation of EA and the eventual formation of P can be divided into 3 distinct phases.

The first phase is a very brief initial period during which the concentration of enzymeï substrate complex [EA] reaches a steady state in which consumption and formation of EA complex are balanced.

During the second phase, the [EA] remains almost constant for a considerable time; this period is known as the steady state, this is the condition described by Michaelisï Menten equation.

The third phase of the reaction is characterized by substrate depletion in which [EA] gradually falls as Ais consumed, this facilitates a rise in the concentration of the free enzyme since there is no A to bind to.

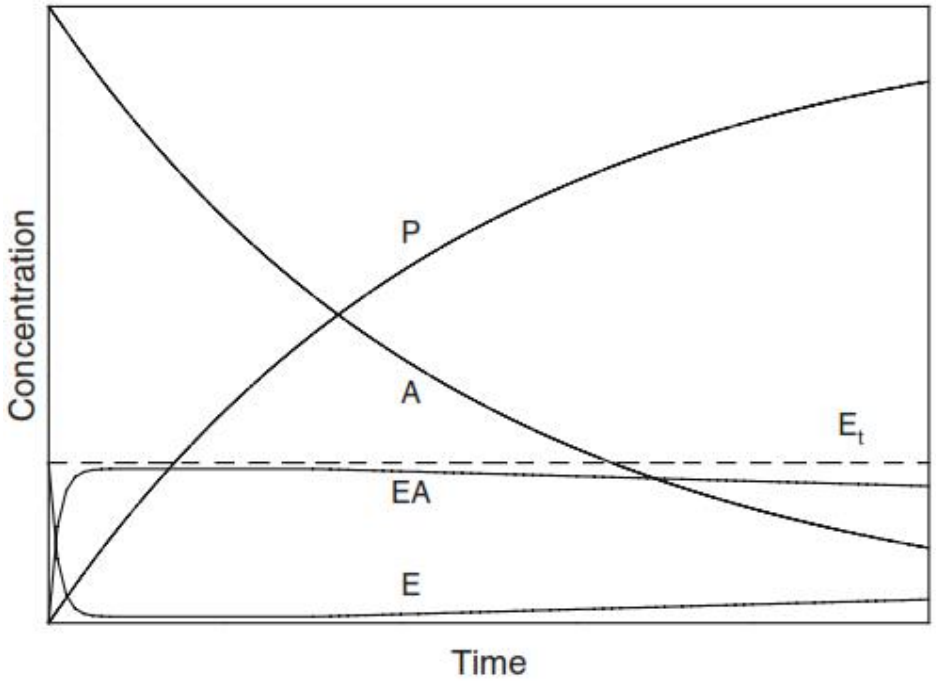

Figure 4: Change in A, P, free enzyme (E), EA, and total enzyme (E_t) concentration over time

At steady state, [EA] is stable, i.e., $\frac{\delta[\mathrm{EA}]}{\delta t} = 0$ Eqn 14

Therefore, the association reaction (formation of ES complex) and the sum of the redissociation and dissociation reactions (the breakdown of ES complex) are equal.

$$k_1[\mathrm{E}][\mathrm{A}] = k_{-1}[\mathrm{EA}] + k_{\mathrm{cat}}[\mathrm{EA}]$$ Eqn 15

Rearrangement of Eqn 15 by making EA the subject of the formula yields

$$\frac{k_1[\mathrm{E}][\mathrm{A}]}{k_{-1} + k_{\mathrm{cat}}} = [\mathrm{EA}]$$ Eqn 16

The three rate constants can now be combined as one term. This new constant, K_m, is known as the Michaelisï Menten constant

$$K_{\mathrm{m}} = \frac{k_{-1} + k_{\mathrm{cat}}}{k_1}$$ Eqn 17

Rewriting Eqn 16, you have

$$\frac{[\mathrm{E}][\mathrm{A}]}{K_{\mathrm{m}}} = [\mathrm{EA}]$$ Eqn 18

But the amount of free enzyme (E) and enzyme that is bound to the substrate (EA) variesover the course of a reaction, while the total amount of enzyme (E_t) is constant

$$\mathrm{E} = \mathrm{E_t} - \mathrm{EA}$$ Eqn 19

Substituting Eqn 19 into Eqn 18, you have

$$\frac{([\mathrm{E_t}] - [\mathrm{EA}])[\mathrm{A}]}{K_{\mathrm{m}}} = [\mathrm{EA}]$$ Eqn 20

Rearrangement of Eqn 20 yields

$$\frac{[E_t][A]}{K_m + [A]} = [EA]$$ Eqn 21

Substituting into Eqn13 $v = k_{cat}[EA]$, you have

$$v = \frac{k_{cat}[E_t][A]}{K_m + [A]}$$ Eqn 22

But the maximum possible reaction rate (v_{max}) would be achieved when all the available enzyme is bound to A and involved in catalysis, i.e.,

$$[EA] = [E_t]$$ Eqn 23

Substituting Eqn 23 into Eqn 13 under conditions of saturating [A] yields

$$v_{max} = k_{cat}[E_t]$$ Eqn 24

Substituting Eqn 24 into Eqn 22 yields the Michaelisï Menten equation

$$v = \frac{v_{max}[A]}{K_m + [A]}$$ Eqn 25

Key Parameters of the Michaelisï Menten Equation

There are four key parameters of the M-M equation. These include K_m, k_{cat}, K_m/k_{cat} ratio and v_{max}.

A. K_m (mol.l^{-1})

The K_m for a given enzyme is constant provided a stable pH, temperature, and redox state is assumed. $K_m$provides an indication of the binding strength of an enzyme to its substrate.

M-M kinetics assumes that k_{cat} is very low when compared to k_1 and k_{-1}. Therefore, a high K_m indicates that the redissociation rate (k_{-1}) is markedly greater than the

association rate and that the enzyme binds the substrate weakly. Conversely, a low K_m indicates a higher afýnity for the substrate.

It must be noted however, that a large K_m could also be the result of very large k_{cat} (see Eqn 17) therefore, caution must be taken when using K_m as a proxy for the dissociation equilibrium constant of the EA complex.

$$K_{\mathrm{m}} = \frac{k_{-1} + k_{\mathrm{cat}}}{k_1} \qquad \text{Eqn 17}$$

B. k_{cat} ($s^{ï\,1}$)

k_{cat}, is a measure of the maximum catalytic production of P under saturating substrate conditions per unit time per unit enzyme. It is also referred to as the turnover number of the enzyme.

The larger the value of k_{cat}, the more rapidly catalyticevents occur. Values of k_{cat}vary markedly from one enzyme to the other.

C. Enzyme Efýciency ($s^{ï\,1}(mol.l^{ï\,1})^{ï\,1}$)

The ratio of k_{cat}/K_m is deýned as the catalytic efýciency or a measure of substrate speciýcity. When the$k_{cat}$is markedly greater than $k_{ï\,1}$, it indicates that the catalytic process is extremely fast sequel to the efýciency of the enzyme to bind the substrate. Based on the laws of diffusion, the upper limit forsuch rates, as determined by the frequency of collisions between the substrate andthe enzyme, is between $10^8$and 10^9. Some enzymes are very efficient with their catalytic rate approaching this range, e.g., fumarase,$2.3 \times 10^8 s^{ï\,1}(mol.l^{ï\,1})^{ï\,1}$

D. v_{max}

$v_{max}$is the maximum velocity that an enzyme could achieve. Measurement of $v_{max}$is theoretical because at a given time, it would require all enzyme molecules to be tightly bound to their substrates. v_{max} is approached at high [A] but never reached as can be seen in Figure 5.

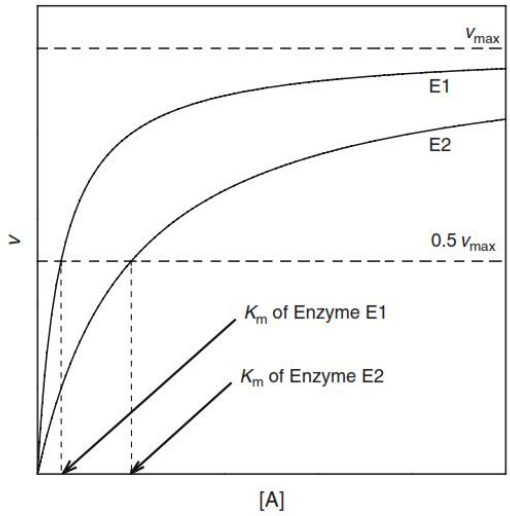

Figure 5: Change in velocity with concentration of substrate

DETERMINATION OF MICHAELISï MENTEN PARAMETERS

Determination of M-M kinetic parameters is usually done graphically.