Introduction to Enzyme Kinetics

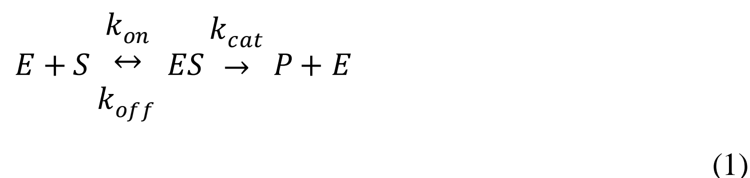
Enzyme kinetics is an increasingly important part of chemistry. Biochemists study the natural functions of enzymes and how they contribute to the cellular processes of life. In chemistry, we study the mechanisms of the reactions in order to develop enzymes for processes. Enzymes are among the best catalysts known. It would be of interest to emulate enzyme function using synthetic molecules. This biomimetic approach is an important area of research. However, many other research groups use enzymes in the native form, or with genetic or chemical modifications to make them appropriate catalysts for synthesis.

Peroxidases are heme enzymes that bind and oxidize a range of substrates near a heme molecule. The typical enzyme mechanism involves one-electron oxidation to form a radical that diffuses into solution. The reaction is completed when two radicals form in this manner combine to form one reactant and one product molecule, a disproportionation reaction. While peroxidase and related cyclooxygenase chemistry are part of numerous intracellular reaction pathways, many peroxidases studied for chemical applications are secretory enzymes. These enzymes are useful for plants and fungi because they degrade complex molecules in the ecosystem and make their molecule components available for uptake by the organism. Horseradish peroxidase is secreted by horseradish (not surprisingly). Our example is the molecule 2, 4, 6-trichlorophenol, which is a common environmental pollutant. However, the product 2, 6-dichloroquinone is not harmful and is readily degraded further.

There are two mechanistic levels we can use to understand this problem. The most general form is Michaelis-Menten kinetics. The specific form involves a three-step peroxidase mechanism, known as the ping-pong mechanism. The two can be shown to be formally equivalent in the derivations that follow.

1. Michaelis-Menten kinetics

The rate of an enzyme-catalyzed reaction in which substrate S is converted into products P depends on the concentration of the enzyme E even though the enzyme does not undergo any net change.



$$E + S \underset{k_{off}}{\overset{k_{on}}{\leftrightarrow}} ES \overset{k_{cat}}{\rightarrow} P + E \quad (1)$$

$$\frac{d[S]}{dt} = -k_{on}[E][S] + k_{off}[ES] \quad (2)$$

$$\frac{d[ES]}{dt} = k_{on}[E][S] - (k_{off} + k_{cat})[ES] \quad (3)$$

$$\frac{d[P]}{dt} = k_{cat}[ES]$$

(4)

These rate equations comprise three processes:

Process 1. Bimolecular formation of the enzyme E and and substrate S: E + S → ES with rate of formation of d[ES]/dt = k_{on}[E][S]

Process 2. Unimolecular decomposition of the complex:

ES →E + S rate of decomposition of ES = -k_{off}[ES]

Process Formation of products and release from the enzyme: ES → P + E with rate of formation of P = k_{cat}[ES]

The rate law of interest is the formation of the product in terms of E and S.The enzyme substrate complex is formed transiently and can be approximated using the steady state approximation.

$$k_{on}[E][S] - (k_{off} + k_{cat})[ES] \approx 0$$

(5)

The result of this approximation is

$$[ES] = \frac{k_{on}[E][S]}{k_{off} + k_{cat}}$$

(6)

In an experiment we know the total enzyme concentration $[E]_0$ and not the unbound enzyme [E].

The total concentration of enzyme $[E]_0$ = [E] + [ES].

$$[ES] = \frac{k_{on}([E]_0 - [ES])[S]}{k_{off} + k_{cat}}$$

(7)

which rearranges to

$$[ES] = \frac{k_{on}[E]_0[S]}{k_{off} + k_{cat} + k_{on}[S]}$$

(8)

The rate of formation of product can be written where K_M is the Michaelis constant and k_{cat} is the maximum turnover number.

$$\frac{d[P]}{dt} = k_{eff}[E]_0$$

(8)

Where

$$k_{eff} = \frac{k_{cat}[S]}{K_M + [S]}$$

(9)

The Michaelis constant is:

$$K_M = \frac{k_{off} + k_{cat}}{k_{on}}$$

(10)

Often the Michaelis-Menten equation is plotted as the rate, V, which is equal to d[P]/dt. In this form we have,

$$V = \frac{V_{max}[S]}{K_M + [S]}$$

(11)

There are several special regimes that can be useful to understand the Michaelis-Menten equation:

Maximal rate: If there is excess substrate present the rate is limited by the rate at which the ES complex falls apart. The rate of formation of products is a maximum and $V_{max} = k_{cat}[E]_0$ is called the maximum velocity.

Half-maximal rate: If $V = V_{max}/2$, then $[S] = K_M$.

Second order regime: If $[S] << K_M$ then the rate of formation of products is $d[P]/dt = k_{cat}/K_M [E]_0[S]$. The rate depends on [S] as well as $[E]_0$.

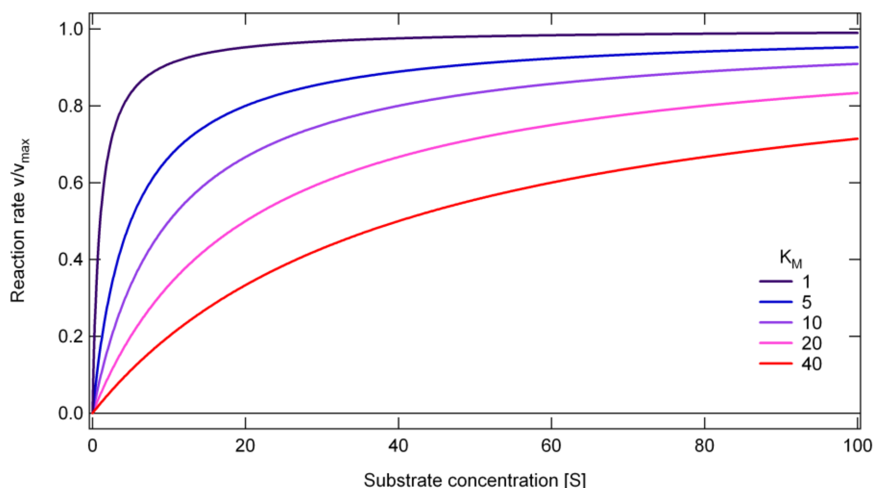


Figure 1. Michaelis-Menten curves showing the saturation of the kinetics at high [S]

A plot of 1/k yields k_{cat} and K_M but not the rate constants k_{on} and k_{off}. The latter rate constants can be obtained from stopped-flow experiments.

1.1 Lineweaver-Burke plots

The Michaelis-Menton expression is non-linear. The Lineweaver-Burke plot is linearized plot of data obtained by inverting both side of the Michaelis-Menten equation.

$$\frac{1}{V} = \frac{K_M + [S]}{V_{max}[S]} = \frac{1}{V_{max}} + \left(\frac{K_M}{V_{max}}\right)\frac{1}{[S]} \tag{12}$$

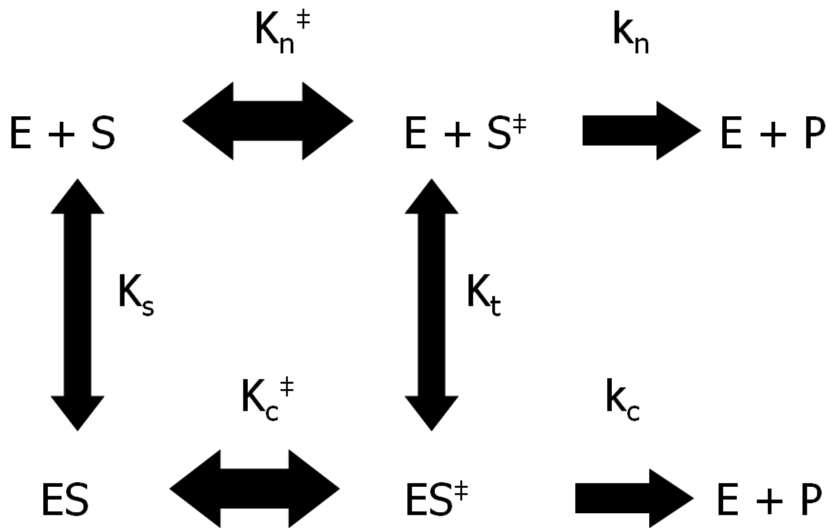
This expression has the form of an equation for a line:

$$y = \text{intercept} + \text{slope}\ x$$

Such plots are not necessary today with common non-linear fitting programs. However, they have historically played an important role in the application of the Michaelis-Menten equation.

1.2 Transition state stabilization

The original idea of the enzyme having maximum complementarity to the transition state (TS) was put forward by Linus Pauling in 1946. It wasn't until the early 70's that the idea was put on a more solid foundation. As put forward by Lienhard and Wolfenden the idea is as follows: the substrate binds to the enzyme more tightly in the transition state than in the equilibrium geometry. This concept is shown in Scheme 1.



Scheme 1. Transition state stabilization. The top pathway involves activation of the substrate not bound to the enzyme. The non-catalyzed transition state equilibrium is ${K_n}^‡$. The bottom pathway is the catalyzed pathway with equilibrium constant ${K_c}^‡$ for the transition state. The transition complex is $ES^‡$ in this case. The transition state hypothesis suggests that the substrate is much more tightly bound in the transition state geometry $ES^‡$ than the original geometry, ES.

Defining the equilibrium constants as association constants:

$$ {K_n}^‡ = \frac{[S^‡]}{[S]} \ , K_t = \frac{[ES^‡]}{[E][ES^‡]} \tag{13} $$

from transition state theory:

$$ \Delta G^‡ = -RT ln\, K^‡ $$

and

$$ k_{obs} = \frac{k_B T}{h} e^{-\Delta G^‡/RT} $$

Thus,

$$k_n = \frac{k_B T}{h} K_n^{\ddagger}\ , \qquad k_c = \frac{k_B T}{h} K_c^{\ddagger}$$

(14)

where c means catalyzed and n means uncatalyzed.

From the scheme you can see that

$$K_s K_c^{\ddagger} = K_n^{\ddagger} K_t$$

hence

$$\frac{K_t}{K_s} = \frac{K_c^{\ddagger}}{K_n^{\ddagger}}$$

However,

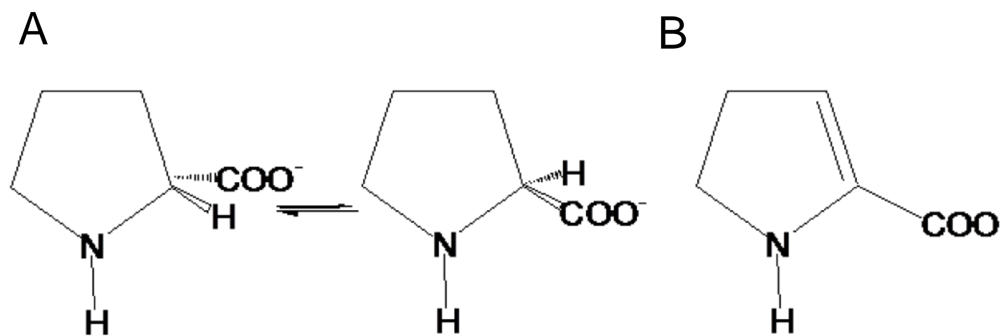
$$\frac{k_c}{k_n} = \frac{K_c^{\ddagger}}{K_n^{\ddagger}}$$

The observed rate enhancement is:

$$\frac{k_c}{k_n} = \frac{K_t}{K_s} \gg 1$$

(15)

Therefore, we can conclude that the transition state geometry $S^{\ddagger}$ must bind more tightly than the substrate S in its equilibrium geometry! The transition state stabilization hypothesis was tested by designing so-called transition state analogs, molecules which mimic the real TS as closely as possible. One of the first enzymes examined was proline racemase, which catalyzes the isomerization shown in Scheme 2.



Scheme 2. A. Racemization of proline. B. The transition state analog is a planar molecule.

The compound on the right is a planar TS state analog. This molecule was found to be a good inhibitor, with K_i some two orders of magnitude smaller than K_m.

1.3 The role of entropy

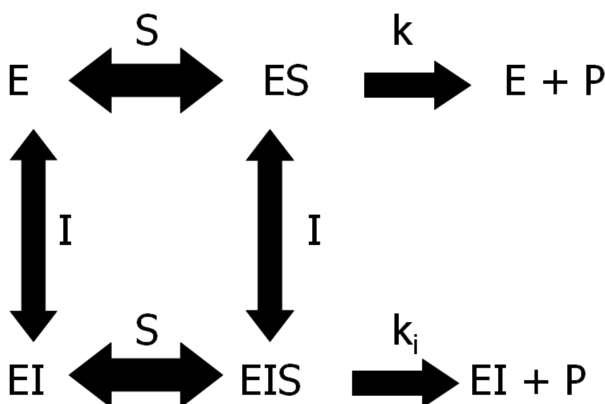
In a seminal paper Page and Jencks showed that the loss in entropy in going from a bimolecular to a unimolecular reaction, i.e. $E + S \leftrightarrow ES$, could account for as much as 10^8 of the observed rate enhancement. In other words, this much free energy would come from the intrinsic binding energy. The entropy loss arises from the loss of translational and rotational degrees of freedom when the substrate is bound. The configurational entropy is:

$$S = k_B \ln W$$

where W is the number of degrees of freedom available to a molecule.

1.4 Inhibition

An inhibitor is any compound that causes a decrease in the catalytic rate. We will consider non-covalent ligands that can bind to the enzyme. The general scheme is shown below:



Competitive inhibition kinetic scheme

Although the scheme does not reveal the binding site of the inhibitor, there are a number of different possibilities. If the inhibitor binds in exactly the same site as the substrate, then it competes for that site, leading to competitive inhibition. On the hand, if the inhibitor binds to a remote site, but reduces the affinity of substrate binding, then binding is called uncompetitive. We contrast the solution of the equations for these two cases.

Competitive inhibition

Competitive inhibition results from the direct competition between the I and S for the substrate binding site. There is an additional equilibrium constant:

$$E + I \leftrightarrow EI \tag{16}$$

With dissociation constant

$$K_I = \frac{[E][I]}{[EI]} \tag{17}$$

The velocity under these conditions turns out to be:

$$V = \frac{V_{max}[S]}{\alpha K_M + [S]} \tag{18}$$

where

$$\alpha = 1 + \frac{[I]}{K_I} \tag{19}$$

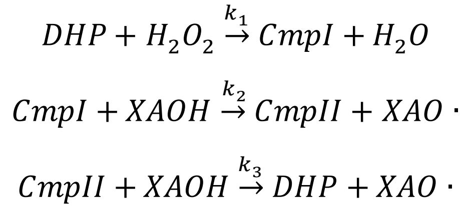
Uncompetitive inhibition

Uncompetitive inhibition arises when the inhibitor, I, can bind at site that is not the same as the substrate binding site. The equation for the binding of the inhibitor is identical to that for competitive inhibition, Eqns. 16, 17 and However, for uncompetitive inhibition, the complex EI indicates that the inhibitor does not bind in the same site as the substrate. The velocity under these conditions is:

$$V = \frac{V_{max}[S]}{K_M + \alpha[S]} \tag{20}$$

2. Specific case of the ping-pong mechanism

The peroxidase rate scheme, also known as the ping-pong mechanism, is given as:

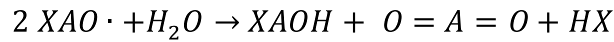


$$DHP + H_2O_2 \xrightarrow{k_1} CmpI + H_2O$$

$$CmpI + XAOH \xrightarrow{k_2} CmpII + XAO\cdot$$

$$CmpII + XAOH \xrightarrow{k_3} DHP + XAO\cdot$$

Where $XAOH$ and $XAO\cdot$ are the para-halophenol substrate and para-halophenoxyl radical intermediate, respectively. X represents a halogen, usually a Cl or Br. In a typical peroxidase reaction scheme the product, $O = A = O$, is a quinone formed by disproportionation of the radical intermediates. $CmpI$ and $CmpII$ are intermediates of HRP, known as compound I and compound II, respectively.



$$2\,XAO\cdot + H_2O \rightarrow XAOH + \; O = A = O + HX$$

To determine a rate equation for this system, we write rate equations for each component in the peroxidase rate scheme. We have defined P as a shorter form of $O = A = O$, as the product, and HRP as the ferric form of the enzyme in the rate equations below.

$$\frac{d[HRP]}{dt} = -k_1[HRP][H_2O_2]$$

$$\frac{d[CmpI]}{dt} = k_1[HRP][H_2O_2] - k_2[CmpI][XAOH]$$

$$\frac{d[CmpII]}{dt} = k_2[CmpI][XAOH] - k_3[CmpII][XAOH]$$

$$\frac{d[P]}{dt} = (k_2[CmpI] + k_3[CmpII])[XAOH]$$

Next we apply the steady state approximation to both the CmpI and CmpII intermediates.

$$0 \approx k_1[HRP][H_2O_2] - k_2[CmpI][XAOH]$$

$$k_1[HRP][H_2O_2] \approx k_2[CmpI][XAOH]$$

and

$$0 \approx k_2[CmpI][XAOH] - k_3[CmpII][XAOH]$$

$$k_2[CmpI][XAOH] \approx k_3[CmpII][XAOH]$$

Thus,

$$[CmpI] \approx \frac{k_1[HRP][H_2O_2]}{k_2[XAOH]}$$

and

$$[CmpII] \approx \frac{k_1[HRP][H_2O_2]}{k_3[XAOH]}$$

Substituting the equations for CmpI and CmpII back into the rate equation for product formation, we have,

$$v_o \approx \frac{d[P]}{dt} = (k_2[CmpI] + k_3[CmpII])[XAOH]$$

$$v_o \approx \left(k_2\left[\frac{k_1[HRP][H_2O_2]}{k_2[XAOH]}\right] + k_3\left[\frac{k_1[HRP][H_2O_2]}{k_3[XAOH]}\right]\right)XAOH$$

$$v_o \approx 2k_1[HRP][H_2O_2]$$

For the peroxidase scheme presented above,

$$[E]_o = [HRP] + [CmpI] + [CmpII]$$

Substituting the equations for CmpI and CmpII into the above expression, we have,

$$[E]_o = [HRP] + \frac{k_1[HRP][H_2O_2]}{k_2[XAOH]} + \frac{k_1[HRP][H_2O_2]}{k_3[XAOH]}$$

$$[E]_o = [HRP]\left(1 + \frac{k_1[H_2O_2]}{k_2[XAOH]} + \frac{k_1[H_2O_2]}{k_3[XAOH]}\right)$$

$$[E]_o = [HRP]\left(1 + \frac{k_1[H_2O_2]}{[XAOH]}\left(\frac{1}{k_2} + \frac{1}{k_3}\right)\right)$$

For traditional Michaelis Menton kinetics,

$$\frac{v_o}{[E]_o} = \frac{k_{cat}[S]}{\frac{k_{off} + k_{cat}}{k_{on}} + [S]}$$

$$v_o = \frac{k_{cat}[E]_o[S]}{\frac{k_{off} + k_{cat}}{k_{on}} + [S]}$$

$$v_o = \frac{V_{max}[S]}{K_M + [S]}$$

$$V_{max} = k_{cat}[E]_o$$

$$K_M = \frac{k_{off} + k_{cat}}{k_{on}}$$

Mapping the peroxidase rate scheme onto the Michaelis Menton equation yields,

$$\frac{v_o}{[E]_o} = \frac{k_1[HRP][H_2O_2]}{[HRP]\left(1 + \frac{k_1[H_2O_2]}{[XAOH]}\left(\frac{1}{k_2} + \frac{1}{k_3}\right)\right)}$$

$$\frac{v_o}{[E]_o} = \frac{k_1[H_2O_2]}{\left(1 + \frac{k_1[H_2O_2]}{[XAOH]}\left(\frac{1}{k_2} + \frac{1}{k_3}\right)\right)}$$

$$\frac{v_o}{[E]_o} = \frac{k_1[H_2O_2]}{\left(1 + \frac{k_1[H_2O_2]}{[XAOH]}\left(\frac{1}{k_2} + \frac{1}{k_3}\right)\right)}$$

$$\frac{v_o}{[E]_o} = \frac{1}{\left(\frac{1}{k_1[H_2O_2]} + \frac{1}{[XAOH]}\left(\frac{1}{k_2} + \frac{1}{k_3}\right)\right)}$$

$$\frac{v_o}{[E]_o} = \frac{1}{\left(\frac{[XAOH]}{k_1[H_2O_2][XAOH]} + \frac{k_1[H_2O_2]}{k_1[H_2O_2][XAOH]}\left(\frac{1}{k_2} + \frac{1}{k_3}\right)\right)}$$

$$\frac{v_o}{[E]_o} = \frac{1}{\left(\frac{[XAOH] + k_1[H_2O_2]}{k_1[H_2O_2][XAOH]}\left(\frac{1}{k_2} + \frac{1}{k_3}\right)\right)}$$

$$\frac{v_o}{[E]_o} = \frac{k_1[H_2O_2][XAOH]}{\left([XAOH] + k_1[H_2O_2]\left(\frac{1}{k_2} + \frac{1}{k_3}\right)\right)}$$

Rearranging,

$$v_o = \frac{k_1[H_2O_2][E]_o[XAOH]}{\left(\left(\frac{1}{k_2} + \frac{1}{k_3}\right) k_1[H_2O_2] + [XAOH]\right)}$$

By analogy to the traditional Michaelis Menton equation,

$$V_{max} = k_1[H_2O_2][E]_o$$

and

$$K_M = \left(\frac{1}{k_2} + \frac{1}{k_3}\right) k_1[H_2O_2]$$

Thus,

$$v_o = \frac{V_{max}[XAOH]}{(K_M + [XAOH])}$$

3. Pre-class preparation

Please answer the following questions.

1. What is the rate-limiting step in a typical enzyme reaction described by the Michaelis-Menten equation?

2. Why is it not possible to neglect the concentration of the ES intermediate in Michaelis-Menten kinetics?

3. We derived the equation
$$v_o = \frac{V_{max}[XAOH]}{(K_M + [XAOH])}$$
which is valid at a fixed concentration of $[H_2O_2]$. Please write an analogous equation that holds at constant $[XAOH]$.

4. Given extinction coefficient of 2, 4, 6-trichlorophenol, what is the highest concentration of this substrate that can reasonably be studied? Consider the fact that substrate absorbance at 316 nm should be less 1.5.

5. Britten Chance first presented horseradish peroxidase kinetics in an article published in 1950. He described three forms of HRP. The ferric form of the protein, the resting state of the enzyme, has a brownish color. After adding H_2O_2 alone he observed that the protein solution turned a pale green color. If he added substrate to this form the solution turned red. As the reaction proceeded to completion and all of the H_2O_2 was consumed, the solution became brown again. What are the green and red forms of HRP in terms of the peroxidase reaction scheme.

6. In the experiment conducted in class, we will use a short-time approximation (method of initial rates). That means that we will only measure the chance in absorbance of product formation for a few seconds. This has the advantage that the change is linear. Why do we use this approach? Why not fit the entire kinetic trace to a function?