




## Chapter 4. Enzyme Kinetics

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### Enzyme Engineering



### 4.1 Why is enzyme kinetics important?

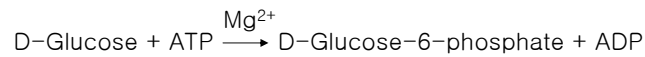
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1. It provides valuable information for enzyme mechanism
2. It gives an insight into the role of an enzyme under physiological conditions
3. It can help show how the enzyme activity is controlled and regulated



## 4.2 How to obtain enzyme kinetics?

- Measure the formation of product or disappearance of substrate



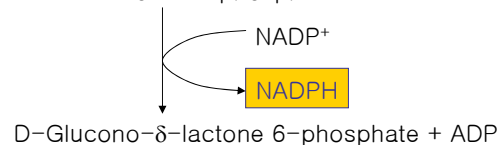
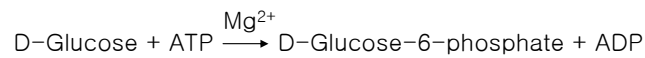
### 1. Discontinuous method

- Add the enzyme, then stop the reaction at different time by adding acid to the sample
- Measure the concentration of substrate or product using HPLC or other chromatography



## 4.2 How to obtain enzyme kinetics?

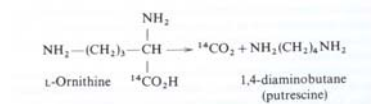
### 2. Continuous method



- NADPH absorb at 340nm, while NADP+ does not
- If a coupled reaction is used, the second reaction should not be rate-limiting (sufficient amount of the enzyme must be provided)
- Continuous method is not always available

## 4.2 How to obtain enzyme kinetics?

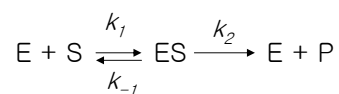
- Radioactive or fluorescent-labeling is useful



- Precautions
  - The substrate, buffers, etc. must be extremely pure
  - Enzyme should be pure
  - Enzyme should be stable as the assay is proceeded
  - Temp, pH\* must be maintained carefully
  - Once steady state is achieved, reaction rate must be constant and proportional to the enzyme added
  - Initial rate of reaction should be measured to avoid possible complexity

## 4.3 How to analyze kinetic data?

### 4.3.1 One substrate reactions



- Equilibrium assumption (1913)
  - Second reaction is slower than first reverse reaction ( $k_{-1} \gg k_2$ )

$$V_0 = \frac{V_{\max}[\text{S}]}{K_s + [\text{S}]} \quad \text{Michaelis-Menten eqn}$$

$$V_{\max} = k_2[\text{E}]_0 \quad K_s = \frac{[\text{E}][\text{S}]}{[\text{ES}]}$$



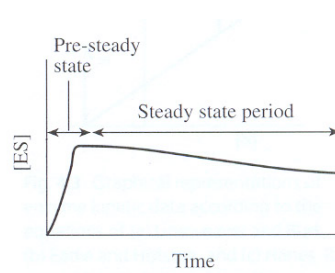
## 4.3 How to analyze kinetic data?

- Steady state assumption (1925)
  - [ES] is constant

$$V_0 = \frac{V_{\max} [S]}{K_m + [S]}$$

$$V_{\max} = k_2 [E]_0$$

$$K_m = \frac{k_2 + k_{-1}}{k_1}$$



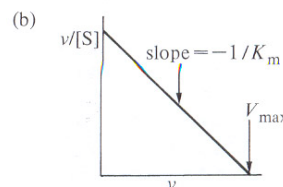
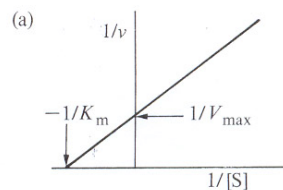
### 4.3.1 One substrate reactions

- Lineweaver-Burk equation

$$\frac{1}{v} = \frac{K_m}{[S] V_{\max}} + \frac{1}{V_{\max}}$$

- Eadie-Hofstee equation

$$\frac{v}{[S]} = \frac{V_{\max}}{K_m} - \frac{v}{K_m}$$





### 4.3.1 One substrate reactions

- Significance of the result
1.  $k_{cat}/K_m$  is the substrate specificity

$$v_{S_i} = \left( \frac{k_{cat}}{K_m} \right)_{S_i} [E][S_i]$$

2.  $k_{cat}/K_m$  can be used for the applicability of equilibrium or steady state assumption

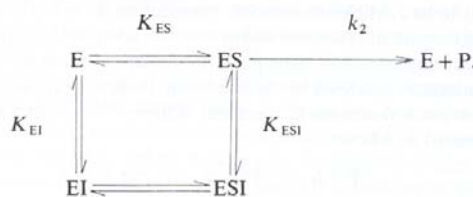
If  $k_{cat}/K_m$  is near  $10^9/\text{mol dm}^{-3}$ , this is steady state mechanism ( $k_2 \gg k_{-1}$ )

3.  $K_m$  is  $[S]$  where the rate is  $\frac{1}{2} V_{max}$   
 $K_m$  may be the affinity of an enzyme to the substrate (not always)



### 4.3 How to analyze kinetic data?

- 4.3.2 Enzyme inhibition gives information on the active site of an enzyme



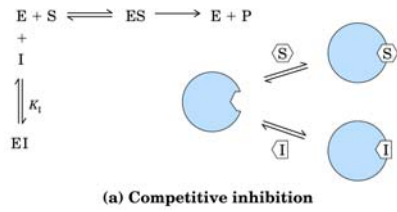
$$v = V_{max} \frac{\frac{[S]}{K_{ES}}}{1 + \frac{[S]}{K_{ES}} + \frac{[I]}{K_{EI}} + \frac{[S][I]}{K_{ESI} \cdot K_{ES}}}$$

$$\frac{1}{v} = \frac{1}{V_{max}} \left[ 1 + \frac{[I]}{K_{ESI}} \right] + \frac{K_{ES}}{V_{max}} \left[ 1 + \frac{[I]}{K_{EI}} \right] \frac{1}{[S]}$$



## 4.3.2 Enzyme Inhibition

### 1. Competitive inhibition



$$V_0 = \frac{V_{\max}[S]}{\alpha K_m + [S]}$$

where  $\alpha = 1 + \frac{[I]}{K_i}$

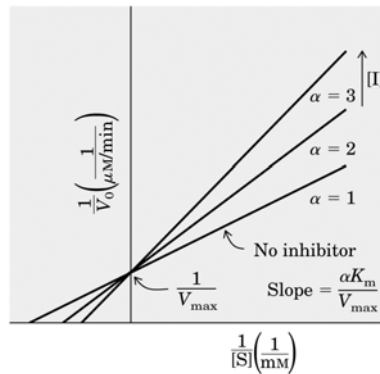
$$K_i = \frac{[E][I]}{[EI]}$$



## 4.3.2 Enzyme Inhibition

### 4.3.2 Enzyme Inhibition

$$\frac{1}{V_0} = \left(\frac{\alpha K_m}{V_{\max}}\right) \frac{1}{[S]} + \frac{1}{V_{\max}}$$



### 4.3.2 Inhibition of the enzyme

$E + S \rightleftharpoons ES \rightarrow E + P$   
 $+ \quad +$   
 $I \quad I$   
 $\uparrow K_i \quad \uparrow K_i'$   
 $EI + S \rightleftharpoons ESI$

Non-competitive inhibition

$$V_0 = \frac{V_{\max} [S]}{\alpha K_m + \alpha' [S]}$$

$$\frac{1}{V_0} = \left( \frac{\alpha K_m}{V_{\max}} \right) \frac{1}{[S]} + \frac{\alpha'}{V_{\max}}$$

$\frac{1}{[S]} \left( \frac{1}{\text{mM}} \right)$

### 4.3.2 Inhibition of the enzyme

$E + S \rightleftharpoons ES \rightarrow E + P$   
 $+ \quad +$   
 $I \quad I$   
 $\uparrow K_i' \quad \uparrow K_i'$   
 $ESI$

(b) Uncompetitive inhibition

$$V_0 = \frac{V_{\max} [S]}{K_m + \alpha' [S]}$$

$$\alpha' = 1 + \frac{[I]}{K_i'} \quad K_i' = \frac{[ES][I]}{[ESI]}$$

$$\frac{1}{V_0} = \left( \frac{K_m}{V_{\max}} \right) \frac{1}{[S]} + \frac{\alpha'}{V_{\max}}$$

$\frac{1}{[S]} \left( \frac{1}{\text{mM}} \right)$



## 4.3 How to analyze kinetic data?

### 4.3.3 The effect of changes in pH

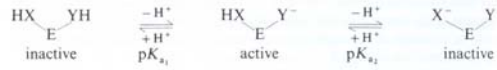
$K_m$  and  $V_{max}$  are dependent on pH since the side chain of enzyme is ionized by pH change

$$K_a = \frac{[E][H^+]}{[EH^+]}, \quad \therefore [EH^+] = \frac{[E][H^+]}{K_a}$$

$$V_{max} = (V_{max})_m \cdot \frac{K_a}{K_a + [H^+]} \quad (1)$$

$$F = \frac{[E]}{[E] + [EH^+]} = \frac{K_a}{K_a + [H^+]}$$

$$V_{max} = (V_{max})_m \cdot F$$



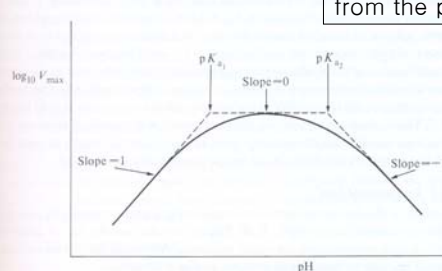
$$V_{max} = \frac{(V_{max})_m}{1 + \frac{[H^+]}{K_{a_1}} + \frac{K_{a_2}}{[H^+]}} \quad (2)$$



### 4.3.3 The effect of changes in pH

(1)   $\left. \begin{array}{l} \text{pH} > \text{pK}_a + 1.5 \\ \text{pH} < \text{pK}_a - 1.5 \end{array} \right\} \text{Linear range}$

$\text{pK}_a$  can be quite different from the  $\text{pK}_a$  of free amino acids

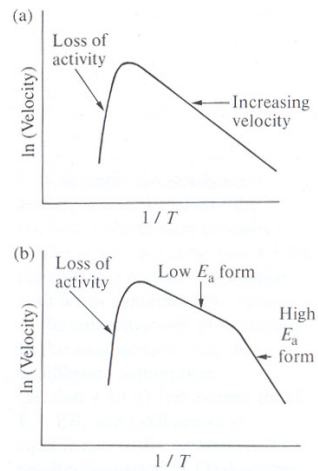
(2) 



### 4.3.4 The effect of changes in Temp

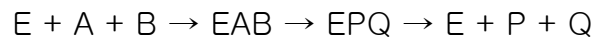
$$k = Ae^{-E_a/RT}$$

- Generally  $E_a$  of enzyme is smaller than that of others
- Over 50°C, enzymes are denatured and lose their activity
- 'Optimal temp' must be avoided
- There may be more than one form of enzyme
- Enzymes working in cold condition may have no temp dependency

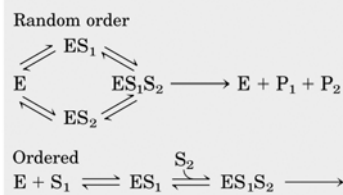


### 4.3.5 Two-substrate reactions

- Many enzymes (oxidoreductase, transferase etc.) use more than one substrate
  - The same form of equation can be extended for these reactions
1. With ternary complex



(a) Enzyme reaction involving a ternary complex





### 4.3.5 Two-substrate reactions

$$v = \frac{V_{\max} [A][B]}{K'_A K_B + K_B [A] + K_A [B] + [A][B]}$$

$$v = \frac{V_{\max} [A]}{\frac{K'_A K_B}{[B]} + \frac{K_B [A]}{[B]} + K_A + [A]}$$

When [B] goes to infinite,

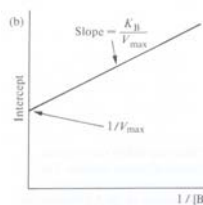
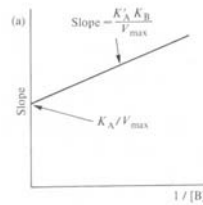
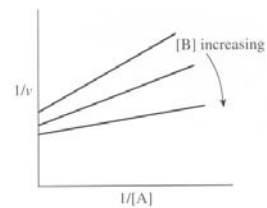
$$v = \frac{V_{\max} [A]}{K_A + [A]}$$



$$\frac{1}{v} = \left[ 1 + \frac{K_A}{[A]} + \frac{K_B}{[B]} + \frac{K'_A K_B}{[A][B]} \right] \frac{1}{V_{\max}}$$

$$\text{slope} = \frac{1}{V_{\max}} \left[ K_A + \frac{K'_A K_B}{[B]} \right]$$

$$\text{y-interception} = \frac{1}{V_{\max}} \left[ 1 + \frac{K_B}{[B]} \right]$$

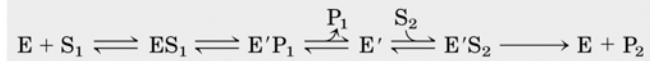




### 4.3.5 Two-substrate reactions

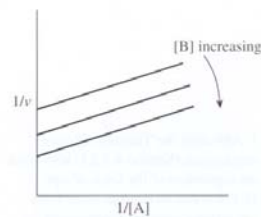
#### 2. Without ternary structure

**(b) Enzyme reaction in which no ternary complex is formed**



$$v = \frac{V_{\max}[A][B]}{K_B[A] + K_A[B] + [A][B]}$$

$$\frac{1}{v} = \left[ 1 + \frac{K_A}{[A]} + \frac{K_B}{[B]} \right] \frac{1}{V_{\max}}$$

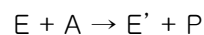


### 4.3.5 Two-substrate reactions

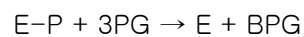
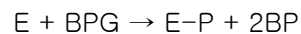
- The data must be highly accurate to distinguish from the former to later

- Additional method is needed

- Demonstration of partial reaction



- Isolation of E' using radioactive label





### 4.3.5 Two-substrate reactions

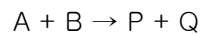
- How to distinguish between random and ordered ternary complex mechanism?

#### 1) Substrate binding experiments



Enzyme bound with  $\text{NAD}^+$  were found, but not with lactate

#### 2) Product inhibition pattern



Enzyme is competitively inhibited by Q in random mech,  
while mixed-type inhibition was shown in ordered mech.



### 4.3.5 Two-substrate reactions

#### 3) Isotope exchange at equilibrium

- Enzyme with more than two substrates : Basically the same as two-substrate reaction with more complicated algebra

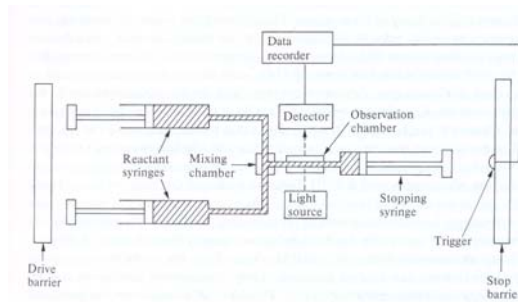
*Glutamate dehydrogenase*



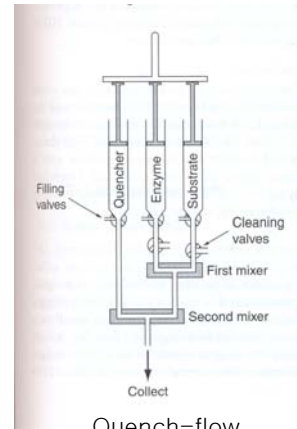
Making quaternary structure in random order

## 4.4 Pre-Steady State Kinetics

- Pre-steady state gives us a lot of information.
- A special apparatus is needed



Stopped flow



Quench-flow

## 4.4 Pre-Steady State Kinetics

- In steady-state study, very limited amount of enzyme is used: Manual addition of enzyme or substrate is OK
- Faster mixing and detection required for pre-steady state kinetic study
- Stopped-flow method has dead time from input to stop of flow (1 ms)
- If there is no convenient optical method available, quench flow method is used : A lot more amount of enzyme and substrate required



## 4.4 Pre-Steady State Kinetics

1. Determination of rate constant
2. Identification of transient species
3. Temperature jump method



Enzyme	Substrate	$k_1$ ( $s^{-1} M^{-1}$ )	$k_{-1}$ ( $s^{-1}$ )
<i>Protein-small ligands</i>			
Lactate dehydrogenase (pig heart)	NADH	$5.5 \times 10^7$	39
Liver alcohol dehydrogenase	Oxamate	$8.1 \times 10^6$	17
Lysozyme	NADH	$2.5 \times 10^7$	9
Malate dehydrogenase	(NAG) <sub>2</sub>	$4 \times 10^7$	$1 \times 10^5$
Pyruvate carboxylase–Mn <sup>2+</sup>	NADH	$5 \times 10^8$	50
Ribonuclease	Pyruvate	$4.5 \times 10^6$	$2.1 \times 10^4$
Tyrosyl-tRNA synthetase	Uridine 3'-phosphate	$7.8 \times 10^7$	$1.1 \times 10^4$
	Uridine 2', 3'-cyclic phosphate	$1 \times 10^7$	$2 \times 10^4$
Tyrosyl-tRNA synthetase	Tyrosine	$2.4 \times 10^6$	24