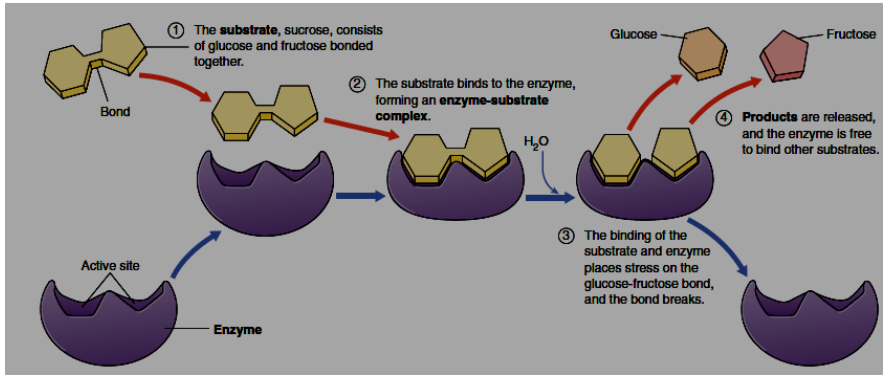
Lect#8: Multi-substrate Reactions and Substrate-Binding Analysis

A. Multi-substrate Reactions
   (1) Cleland Convention
   (2) Ordered and Random Mechanisms
   (3) Sequential and Nonsequential Mechanisms
      (a) Sequential
      (b) Nonsequential



B. Substrate Binding Analysis
   (1) Single Binding Site Model
   (2) Binding Data Plots
      (a) Direct Plot
      (b) Reciprocal Plot
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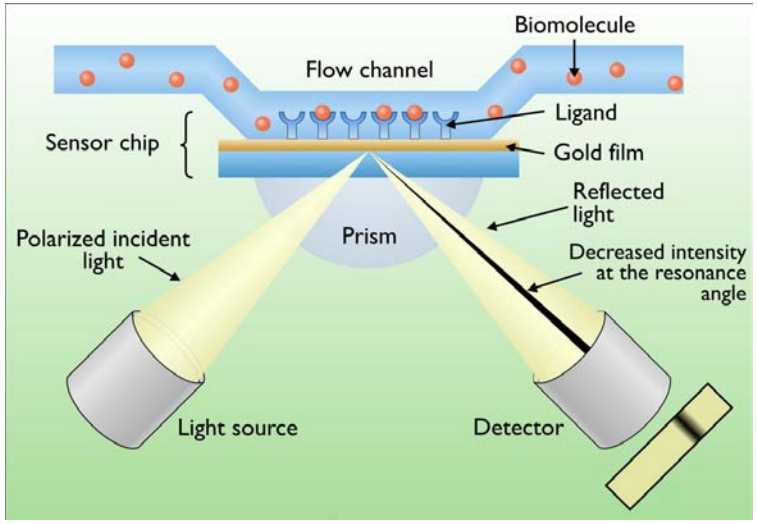
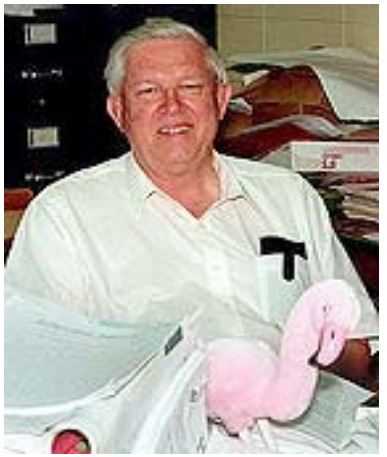
C. Determination of Enzyme-Substrate Dissociation Constants
   (1) Kinetics
   (2) Equilibrium Dialysis
   (3) Equilibrium Gel Filtration
   (4) Ultracentrifugation
   (5) Spectroscopic Methods



Lecture #8: **Multi-substrate Reactions and Substrate Binding Analysis**



A. Multi-substrate Reactions

1. Cleland Convention

- Wallace W. Cleland (Enzyme Institute, Madison, WI)

• most enzymes catalyze reactions between **two or more substrates** to yield **two or more products**

$$E_f + S_1 + S_2 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES_1S_2 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E_f + P_1 + P_2$$

•description of such **multi-substrate enzyme systems** according to the nomenclature and diagrammatic representation proposed by Cleland (BBA **67**, 104-137, 1963)

•**Cleland Convention:** terms **Uni, Bi, Ter, …** are used to identify the number of substrates entering the reaction

•the same terms are used to identify the **number of products formed**

$A \Leftrightarrow P$	Uni Uni
$A \Leftrightarrow P + Q$	Uni Bi
$A + B \Leftrightarrow P$	Bi Uni
$A + B \Leftrightarrow P + Q$	Bi Bi
$A + B + C \Leftrightarrow P + Q$	Ter Bi

•substrates are designated A,B,C, etc in the order in which they bind the enzyme

•the products are designated P,Q,R, etc, in the order in which they leave the enzyme

Example

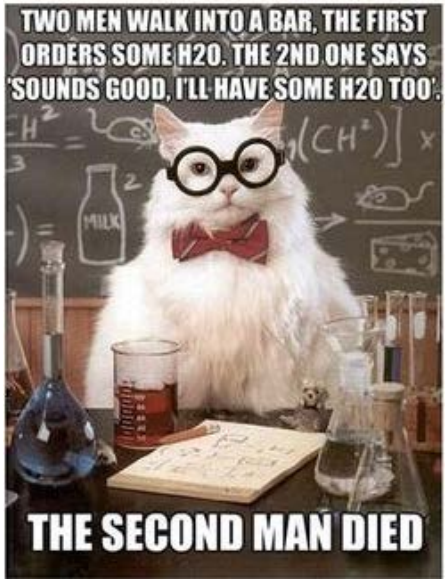
$E + A \Leftrightarrow EA$

$EA + B \Leftrightarrow EAB$

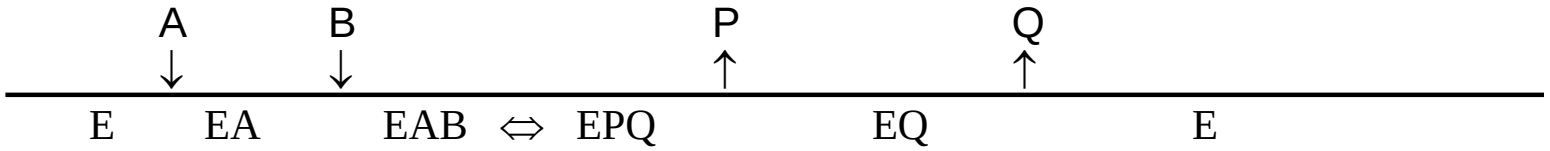
$EAB \Leftrightarrow EPQ$

$EPQ \Leftrightarrow EQ + P$

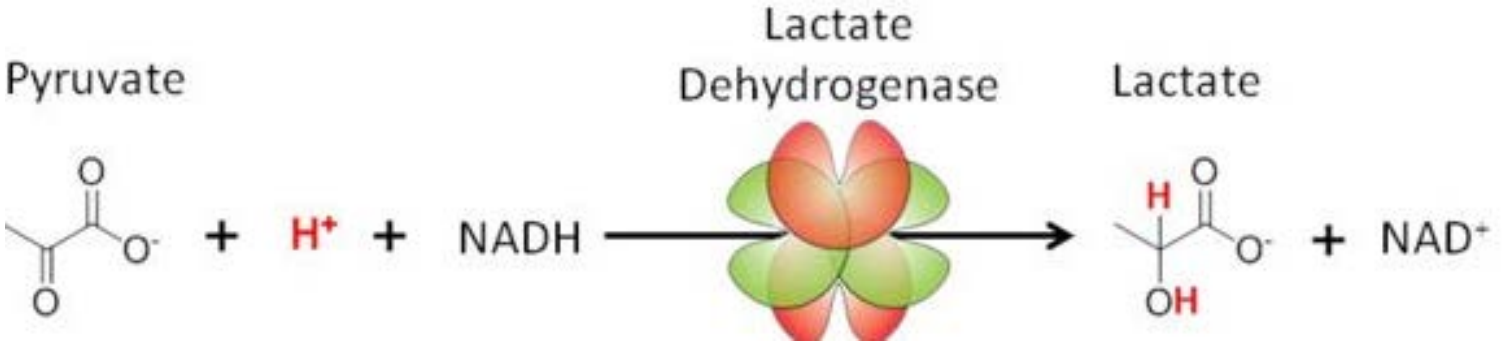
$EQ \Leftrightarrow E + Q$



By means of the Cleland convention this mechanism is represented as follows:



Lactate dehydrogenase



Transitory complex

- enzyme species that undergoes a unimolecular reaction with the release of a **substrate or product**
- may be capable of isomerizing into such an intermediate **or**
- can undergo a bimolecular reaction to **add a substrate** or **release a product**

Central complex

- **transitory complex** that cannot participate in a bimolecular reaction with substrate or product
  - all sites are occupied
  - can only undergo a **unimolecular reaction** with the release of a substrate or product

2. Ordered and Random Mechanisms

- Mechanisms of enzyme reactions are further described by reference to the **manner in which the substrate adds to the enzyme**

- **Ordered Mechanism**
  - the order of addition is **obligatory**

  - substrate A (S1) must add to the enzyme first followed by substrate B (S2)

Ordered

Random mechanism

- the order of substrate addition is not obligatory

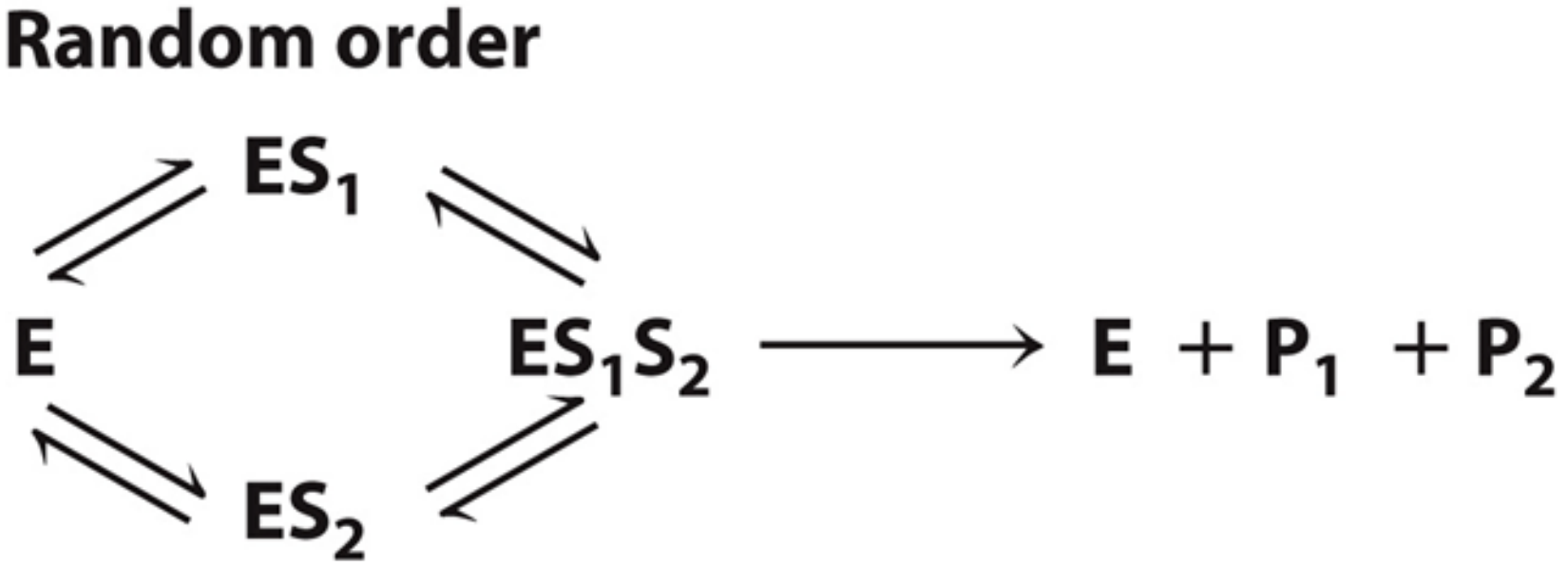


Figure 6-13a
Lehninger Principles of Biochemistry, Seventh Edition
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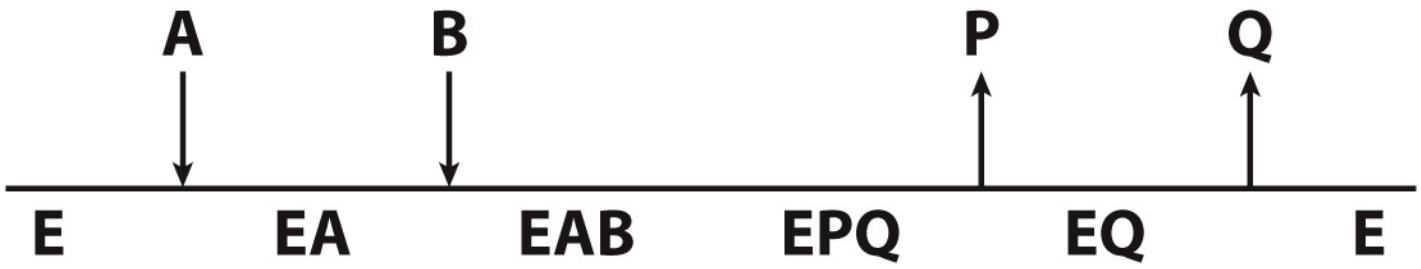
Cleland nomenclature

Ordered bi bi



Random bi bi

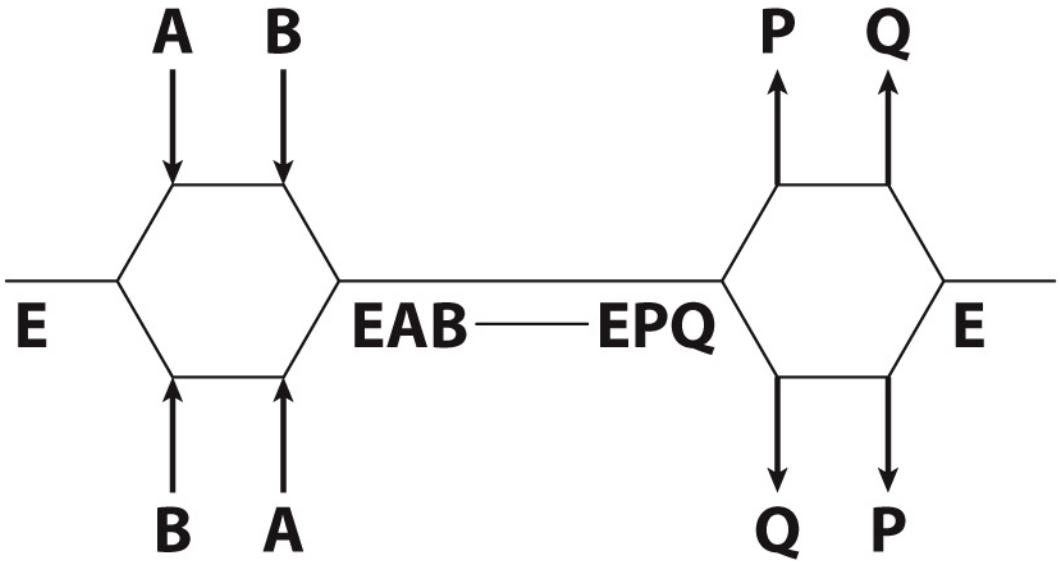


Figure 6-13c
Lehninger Principles of Biochemistry, Seventh Edition
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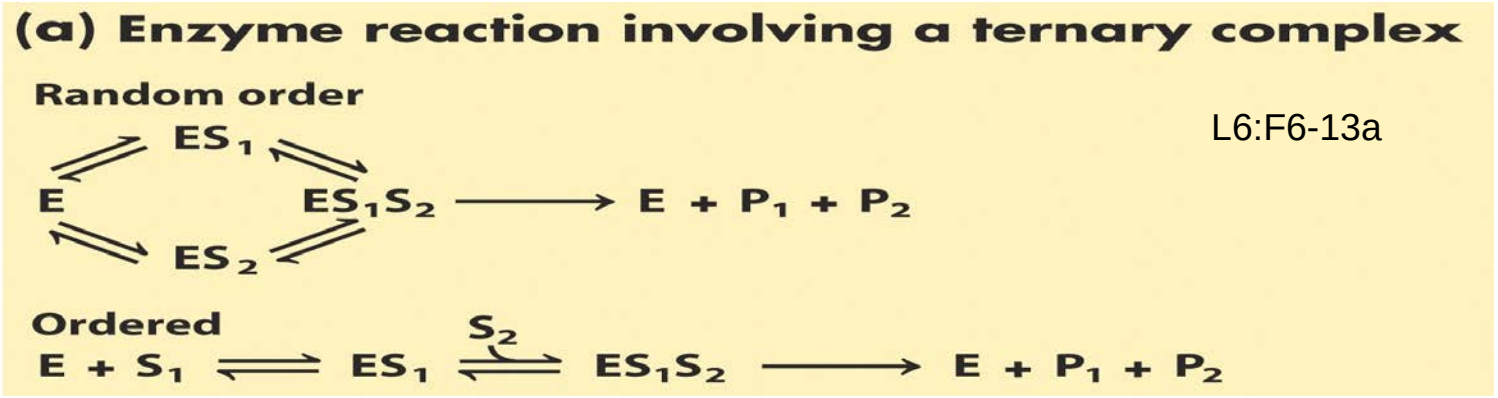
3. Sequential and Nonsequential Mechanisms

(a) Sequential mechanism

- all substrates must bind to the enzyme before any product is released

  -may be either ordered or random



L6:F6-13a

(b) Nonsequential mechanism

- some **substrates bind** to the enzyme and **some products are released** before other substrates bind and then other products are released

- nonsequential mechanisms can only be **ordered**

- the enzyme **exists in two or more** stable forms between which it oscillates during the reaction

-known as **Ping Pong** mechanisms

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

L6:F6-13b

Ping Pong mechanism

- one substrate binds to the enzyme (forms a binary complex)
- then first product is released
- second substrate binds enzyme
- second product is released

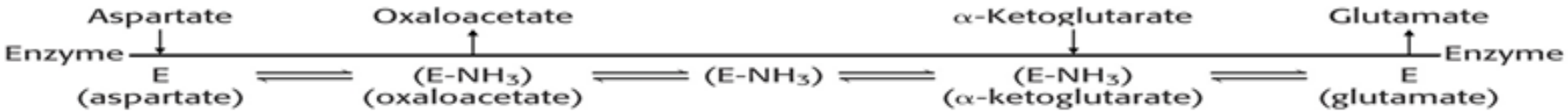
- the enzyme oscillates between the two stable forms, denoted E and F (E or E’)

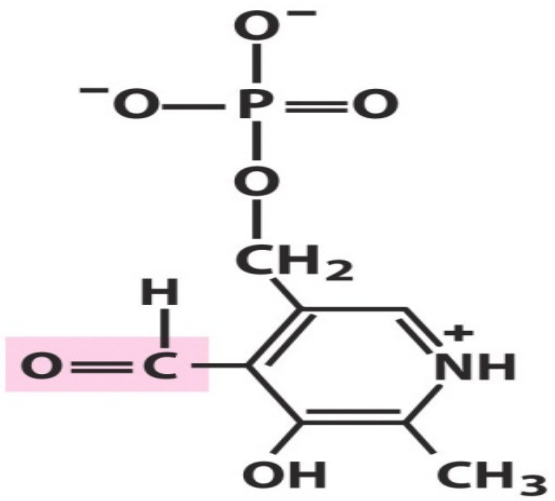
- one enzyme form reacts with one substrate

- the other form reacts with the second substrate

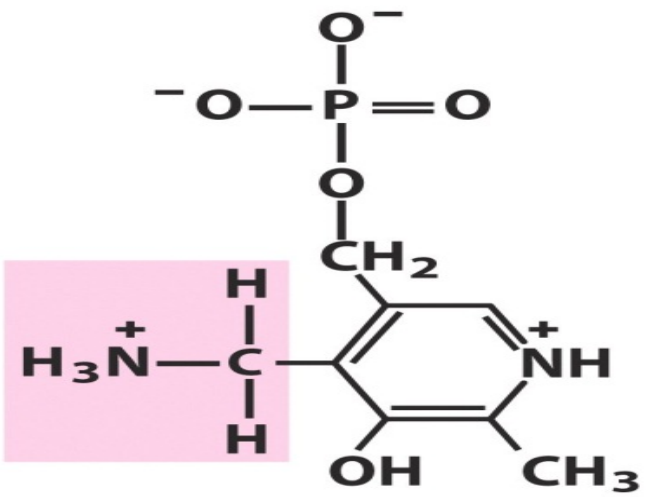
- the modified form of the E may be a phosphorylated protein, or an E in which the coenzyme has been altered

Aspartate aminotransferase



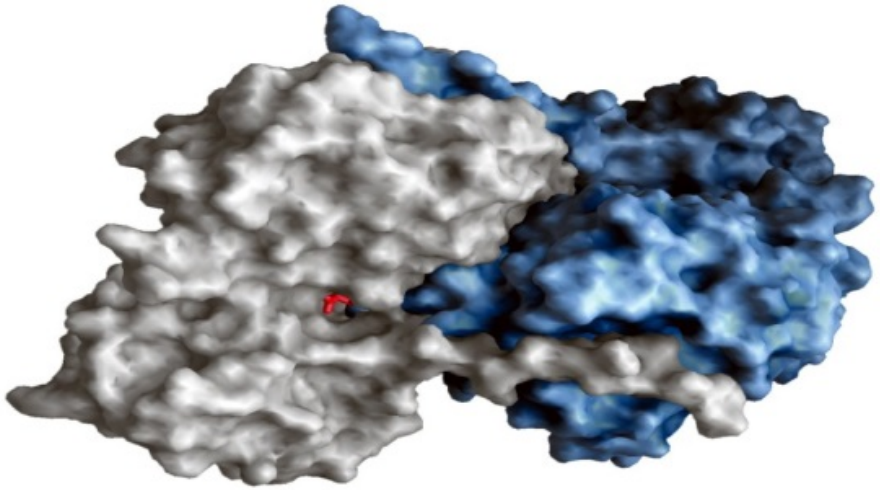


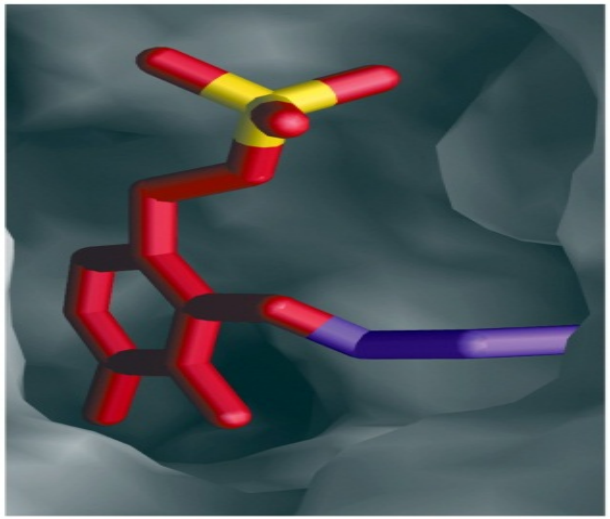
Pyridoxal phosphate (PLP)

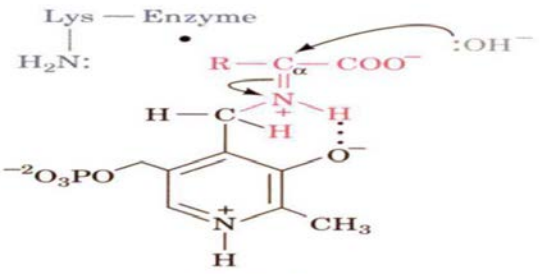


Pyridoxamine phosphate

L7:F18-5






Lys — Enzyme
H2N:
:OH⁻
R — Cα — COO⁻
H — C
N
H
H
O⁻
⁻2O3PO
N
H
CH3
Ketimine

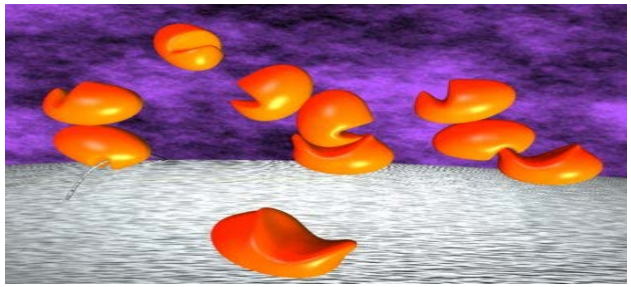
O⁻
⁻O — P = O
O
CH2
H
O = C
NH
OH
CH3
Enz
Lys — NH2
H2O
O⁻
⁻O — P = O
O
CH2
H
H
Enz
Lys — N = C
NH
Schiff base
OH
CH3

L7:F18-5

B. Substrate Binding Analysis

1. One Binding Site per Enzyme Molecule

- simplest case in which one site of the enzyme binds one molecule of ligand (substrate)



$$\mathbf{P + L \Leftrightarrow PL}$$

Units= M^{-1}

$$K_a = \frac{[PL]}{[P][L]} \qquad [B1]$$

- frequently it is more convenient to refer to the equilibrium process in terms of the dissociation constant K_d

$$K_d = \frac{[P][L]}{[PL]} \qquad [B2]$$

Units = M

- the smaller the K_d , the "tighter" is the complex PL
- the K_a and K_d are related: $K_a \times K_d = 1.0$
- we can define a quantity Y
- where Y = (concn of L bound to P)/(total concn of all forms of P)

$$Y = \frac{[PL]}{[P] + [PL]} \qquad [B3]$$

- from equation [B2] we have:

$$[PL] = \frac{[P][L]}{K_d} \qquad [B4]$$

- so that $$Y = \frac{[P][L]/K_d}{[P] + [P][L]/K_d} \qquad [B5]$$

Multiply by

(K_d/[P])/ (K_d/[P])

and $$Y = \frac{[L]}{[L] + K_d} \qquad [B6]$$

- the quantity, Y, is called the "fractional saturation"
- its value ranges from zero (when [PL] = 0) to 1 (when [P] = 0)
- when Y = 0.5, half of the P molecules are complexed with L and half of the P molecules are in the free form and [L] = K_d

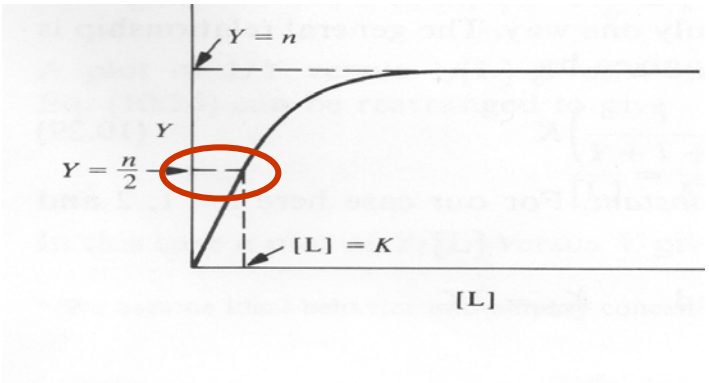
For the general case of n equivalent sites then:

$$Y = \frac{n[L]}{[L] + K_d} \qquad [B7]$$

2. Binding Data Plots

a) The Direct Plot

consists of a plot of Y versus [L]



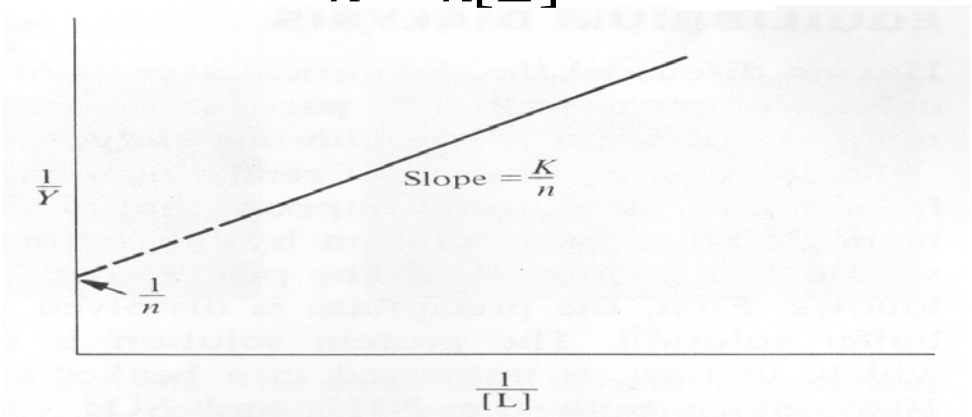
- direct plot yields a hyperbolic curve which is characteristic of simple binding

- when [L] = K_d then Y = n/2, i.e., 50% saturation

b) Reciprocal Plot

- take the reciprocal of [B7] then we get:

$$1/Y = \frac{1}{n} + \frac{K_d}{n[L]} \qquad [\ B8\ ]$$



- also known as the **Hughes-Klotz** plot

https://www.youtube.com/watch?v=HLU0srQ6sjc
(2:14 min)

c) Scatchard Plot

Recall $$Y = \frac{n[L]}{[L] + K_d} \qquad [\ B7\ ]$$

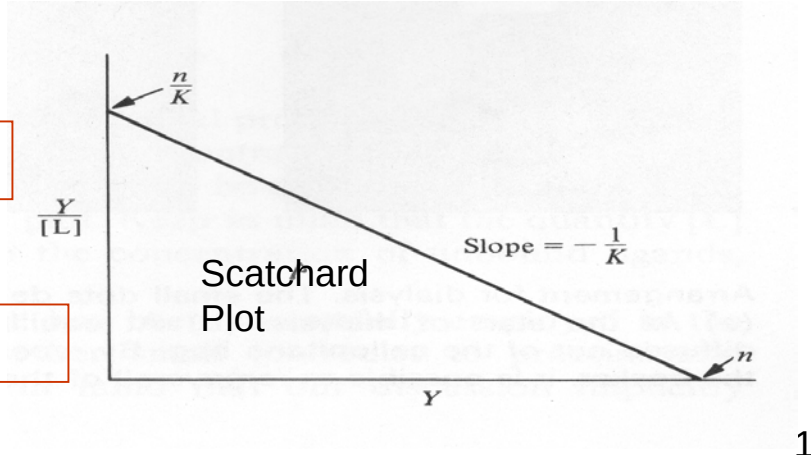
so: $Y[L] + K_d Y = n[L]$ [B9]

x $1/K_d$

$$\frac{Y[L]}{K_d} + Y = \frac{n[L]}{K_d} \qquad [B10]$$

x $1/[L]$

or $$\frac{Y}{[L]} = \frac{n}{K_d} - \frac{Y}{K_d} \qquad [B11]$$



C. Determination of Enzyme-Substrate Dissociation Constants

(Ref: Fersht Ch6: pp202-209)

1. Kinetics

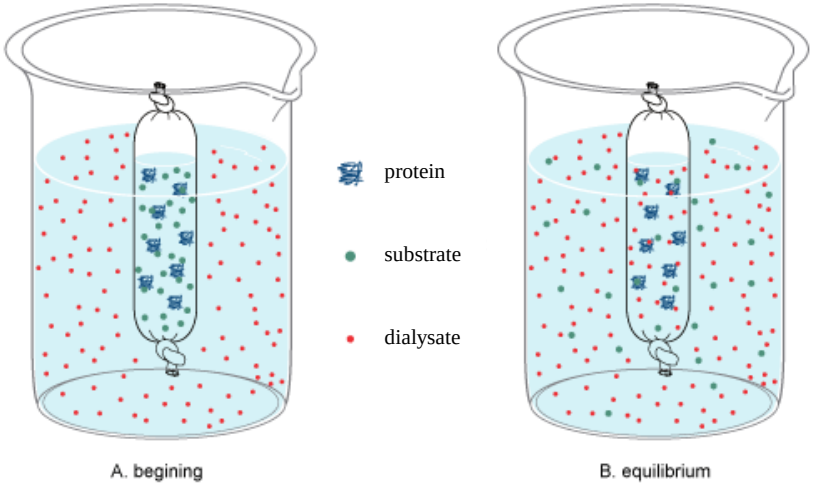
• the K_i (the dissociation constant for competitive inhibitors) can be determined from kinetic inhibition studies

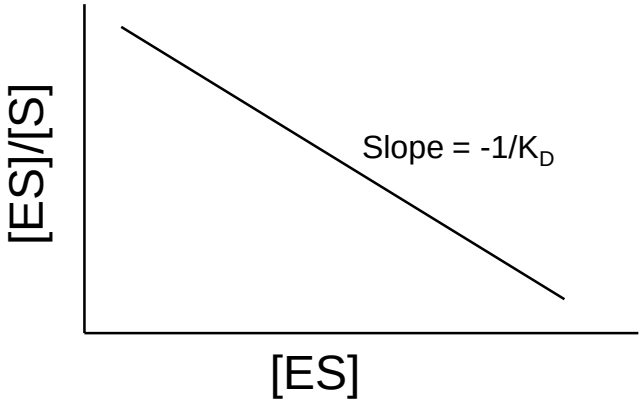
- need a good **competitive inhibitor** for a given substrate against the enzyme
- Perform a competitive inhibition kinetic study and determine the K_i for the inhibitor and relate this to the affinity of the substrate
- $K_i = [E_f]\,[I]/\,[EI]$

2. Equilibrium Dialysis

• method directly measures the concentrations of free enzyme and enzyme-bound ligand

• a solution of the enzyme and ligand is separated from a solution of the ligand by a dialysis membrane, which only permits the ligand to flow across it





(i) Measure total ligand concentration inside dialysis bag

([ES] + [S])

(ii) Measure ligand concentration in buffer solution outside dialysis bag ≡ ([S])

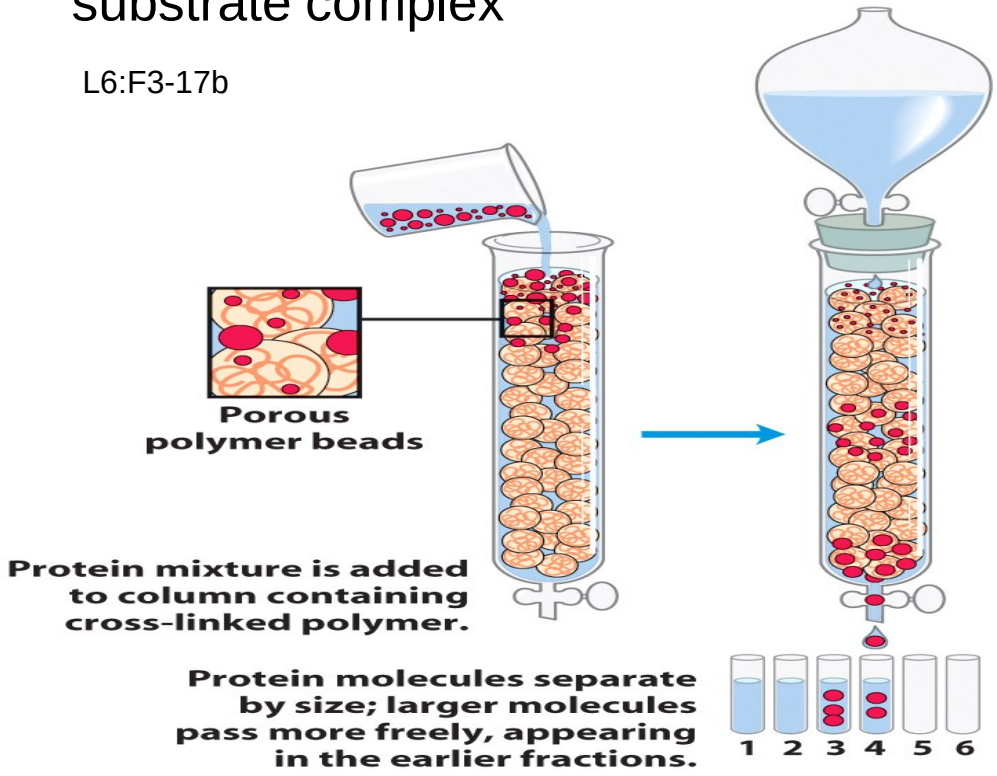
Need to know the [E] in sample

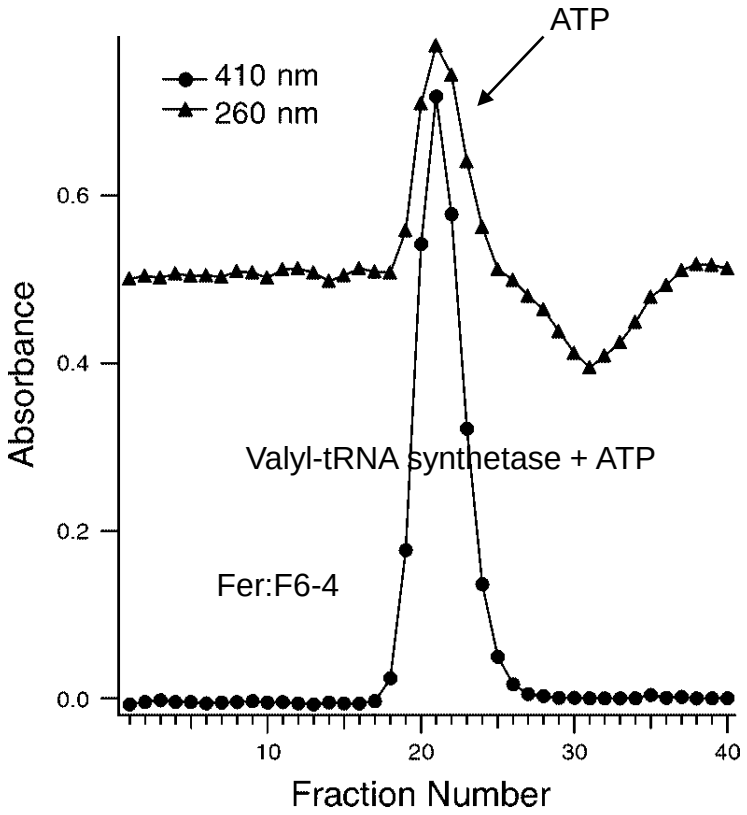
(iii) Use (i) - (ii) = ([ES] + [S]) - [S] = [ES]

3. ***Equilibrium Gel Filtration***

•use a size-exclusion resin such as Sephadex, Sephacryl, or Biogel and can separate the free ligand (substrate) from the enzyme-substrate complex

L6:F3-17b



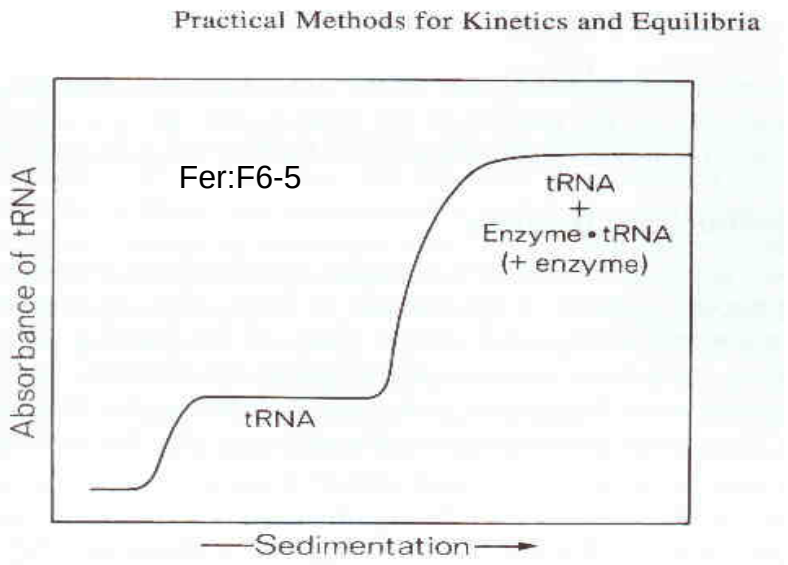


4. Ultracentrifugation

•binding of a small polymer such as the binding of tRNA to an aminoacyl tRNA synthetase cannot be determined by equilibrium dialysis

•in an **analytical ultracentrifuge**, the cell is filled with tRNA and aminoacyl-tRNA synthetase and the absorbances of the bound tRNA and the free tRNA are directly measured by the ultraviolet optics

•the higher MW complex of the enzyme with tRNA sediments faster than the free tRNA



5. ***Spectroscopic Methods***

• in NMR experiments, the concentration of free and bound ligands (substrates) can often be measured directly

•most spectroscopic techniques **do not allow** direct measurement of bound and free substrate (ligand)

•in these cases, the [PL] is usually directly proportional to the change in the spectroscopic signal being observed, eg., fluorescence

$$\Delta F = \Delta F_{\max} - K_d \frac{\Delta F}{[L]}$$