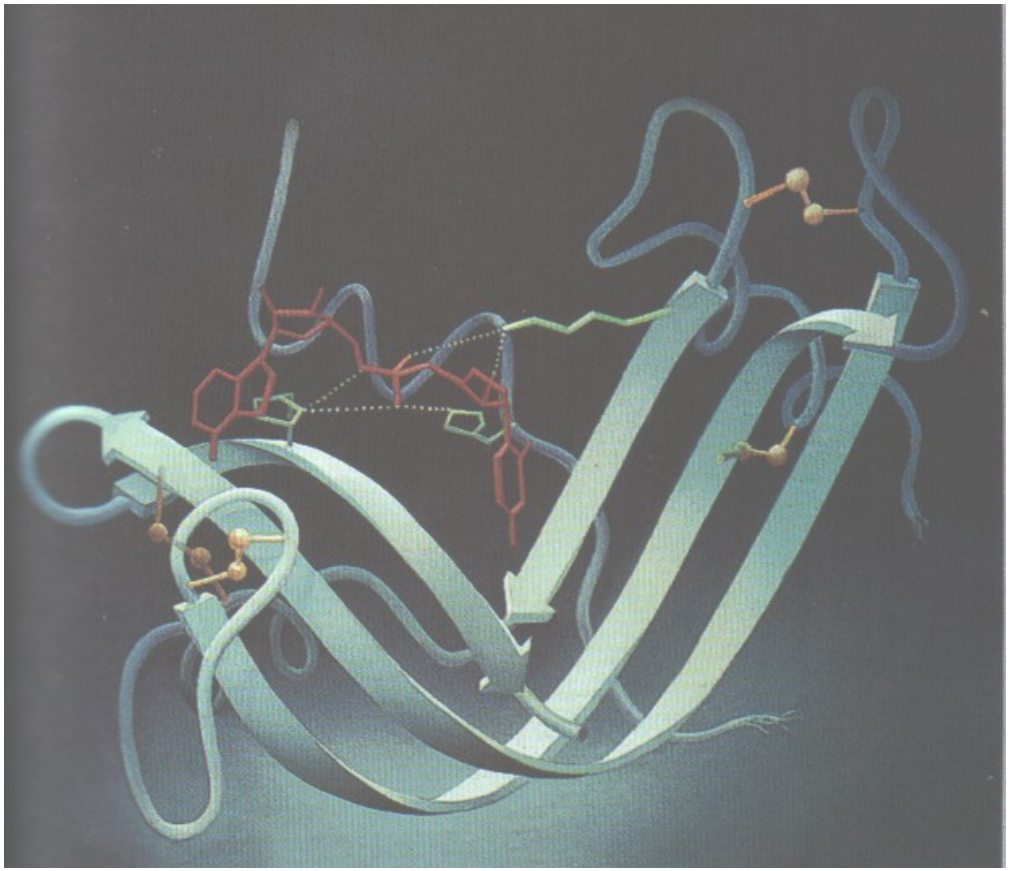
University of Lethbridge

Chapter 13: Introduction to Enzymes

Voet & Voet: Pages 469-481



University of Lethbridge

Introduction

Virtually all biochemical reactions in an organism are mediated by remarkable biological catalysts – enzymes

Enzymes are a category of protein that differ from ordinary chemical catalysts in several respects

(1) Higher reaction rates

- several orders faster than equivalent chemical catalysts
- 10^6-10^{12} rate enhancement

(2) Milder reaction conditions

- low temperature, atmospheric pressure, narrow pH range, *etc.*

(3) Greater reaction specificity

- Narrow range of substrate and product specificities
- stereospecificity, lack of side products

(4) Capacity for regulation

- "fine" control of reaction rate

University of Lethbridge

Substrate Binding

Substrate binding to a protein is governed by the same noncovalent forces that apply to protein structure

Binding sites are:

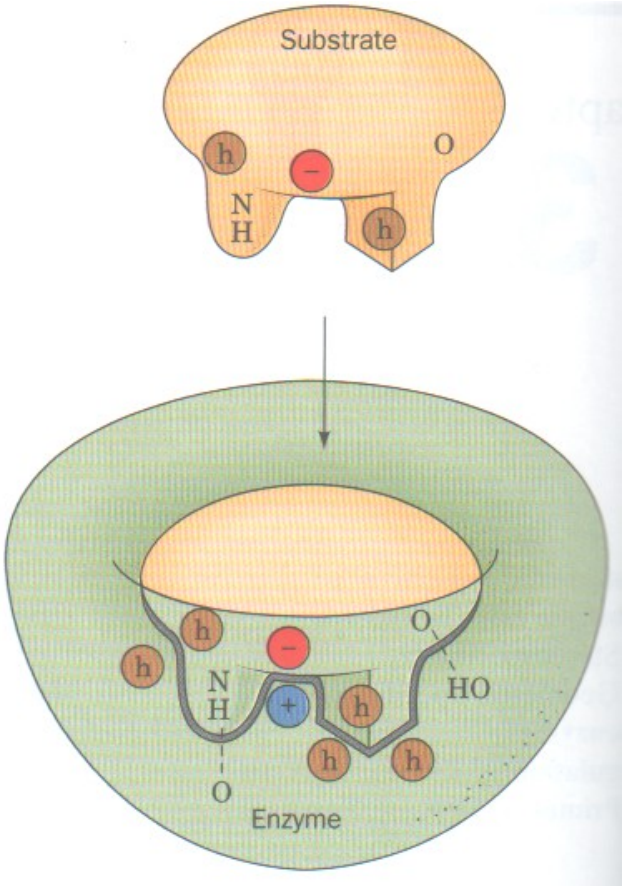
1) generally an indentation or cleft on the surface of the enzyme molecule

2) complementary in shape (includes chirality) to the substrate (geometric complementarity)

3) complementary in electrostatic surface potentials to the substrate (electronic complementarity)

- **charge or partial charge associated with each point along the surface of a protein**

Only complementary (geometric and electronic) substrates bind to an enzyme binding site (via noncovalent forces)



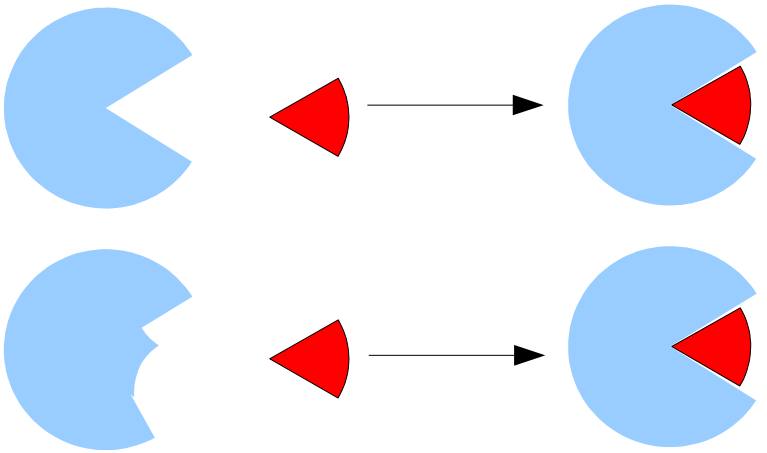
University of Lethbridge

Enzyme Binding Sites
(substrate binding)

Two major hypothesis

(1) **Lock-and-key**

- Enzyme active site is **preformed** in the absence of substrate
- Enzyme is the lock and the substrate is the key



(2) **Induced fit**

- Enzyme active site only **forms in the presence of substrate**
- Substrate provides stabilizing energetic interactions that are otherwise unavailable

Structural data suggest enzyme active sites are largely preformed with most demonstrating at least some induced fit

University of Lethbridge

Specificity

Enzyme specificity – the range of compounds that can be used as substrates

Specificity of enzymes vary widely

- a few enzymes are specific for a single compound
- most enzymes catalyze the reactions of a small range of related compounds
  - *eg.* YADH can utilize methanol (40%) and propanol (4%) at reduced efficiency compared to the natural substrate (ethanol; 100%)
- some enzymes (eg. digestive enzymes) are so 'promiscuous' it is more appropriate to talk of preference than specificity
  - *eg.* cleave all peptide bonds except before proline

High specificity

Low specificity

Enzymes are strictly stereospecific

eg. Proteases only hydrolyze polypeptides composed of L-amino acids, Glycolysis is specific for D-glucose, ...

University of Lethbridge

Cofactors

Protein functional groups are not suited to all types of reactions

- proteins have evolved binding sites for (small molecule) cofactors that participate in catalysis
- range in size from ions (*eg.* Zn^{2+}, Ca^{2+}) to large organic molecules known as coenzymes (transiently associate) and prosthetics (permanent association)

Nomenclature for enzymes that bind cofactors

Holoenzyme: enzyme plus cofactor (active)

Apoenzyme: enzyme without cofactor (inactive)

Vitamin	Coenzyme	Reaction Mediated
Biotin	Biotin	Carboxylation
Cobalamin (B_{12})	Cobalamin (B_{12}) coenzymes	Alkylation
Pantothenate	Coenzyme A	Acyl transfer
Riboflavin (B_2)	Flavin coenzymes	Oxidation–reduction
	Lipoic acid	Acyl transfer
Nicotinamide	Nicotinamide coenzymes	Oxidation–reduction
Pyridoxine (B_6)	Pyridoxal phosphate	Amino group transfer
Folic acid	Tetrahydrofolate	One-carbon group transfer
Thiamine (B_1)	Thiamine pyrophosphate	Aldehyde transfer

Many vitamins are organic cofactors

University of Lethbridge

Regulation of Enzyme Activity

Cells must regulate catalytic activity of enzymes in order to coordinate its metabolic processes

Two general approaches

(1) Cells can regulate catalytic activity by **varying the amount of enzyme**

- synthesis of new enzyme or degradation of existing enzyme (eg. constant activity, variable amount)

(2) The activity of an enzyme may be **regulated directly**

- subtle conformational changes increase or decrease affinity for substrate and alter catalytic activity
- structural alterations result from either:

  a) non-covalent binding of regulatory molecule (eg. steric inhibition, effector binding)

  b) covalent modification of enzyme (eg. zymogen activation, modification)

University of Lethbridge

Allostery & Cooperativity

Regulation of enzyme activity through conformational change

(1) allostery – binding at effector site alters binding affinity for different ligand at the functional site (eg. catalytic)

- **eg. ATP binding to ATCase enhances binding affinity for normal substrates**

(2) cooperativity – binding of a ligand at functional site (eg. binding site) alters binding affinity for same ligand at other similar functional sites.

- **eg. O_2 binding to Hemoglobin enhances binding affinity for O_2**

In each case, the activity of enzyme is regulated by a binding induced conformational change that is transmitted throughout the quaternary structure.

University of Lethbridge

Nomenclature

Effector molecule – any molecule that regulates the binding affinity of a protein or enzyme.

Effector molecules are further subdivided based upon:

(I) activator/inhibitor

- **activators increase binding affinity for substrate**
- **inhibitors decrease binding affinity for substrate**

(II) homotropic/heterotropic

- **homotropic effectors are the same as the ligand or substrate**
- **heterotropic effectors are different from the ligand or substrate**

Cooperativity is always associated with homotropic effectors while allostery is typically associated with heterotropic effectors

Feedback inhibition of pathway

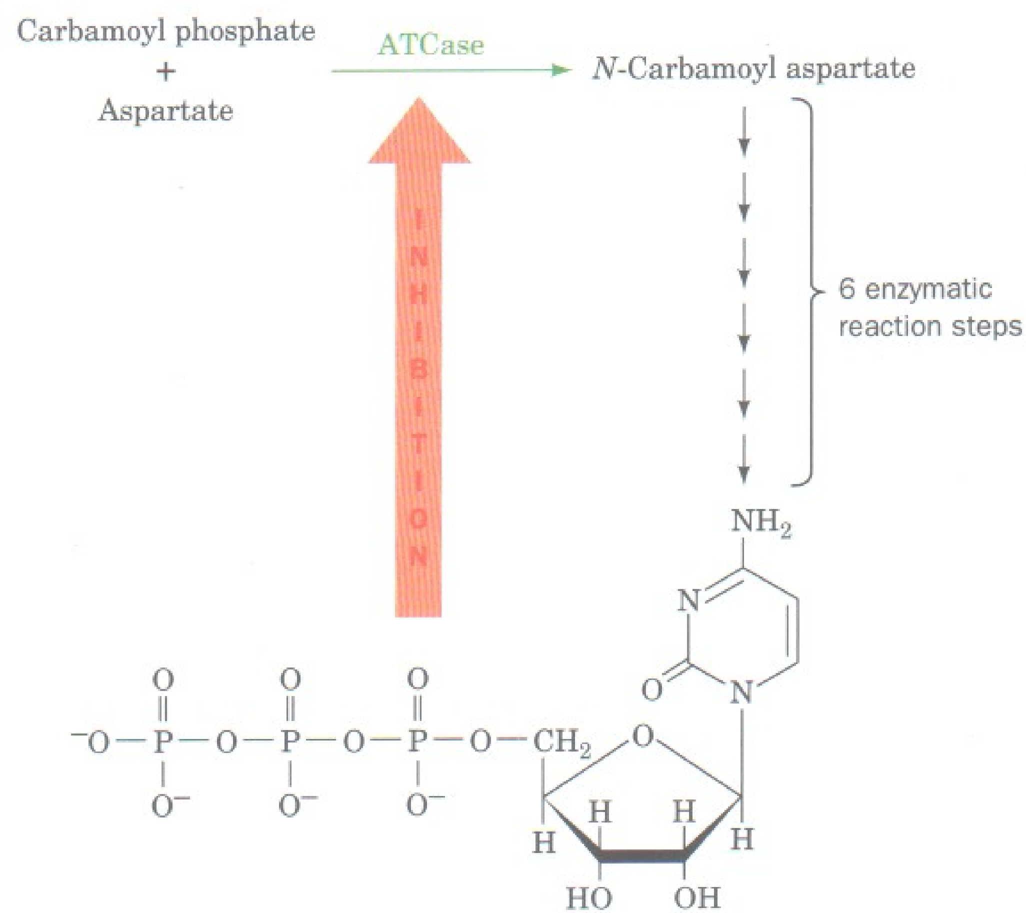
Feedback inhibition - pathway product is heterotropic inhibitor of the enzyme catalyzing the first committed step of the pathway

Example:

ATCase catalyzes the committed step for pyrimidine biosynthesis

CTP is the product of the pyrimidine biosynthesis pathway

- CTP is also a heterotropic inhibitor (allosteric inhibitor) of ATCase



Cytidine triphosphate (CTP)

University of Lethbridge

Regulation of ATCase

I) Feedback inhibition

(Normal case) ATCase funnels aspartate and carbamoyl phosphate from intracellular pools into pyrimidine biosynthesis (committed step)

- **at sufficiently high [CTP], CTP will bind to ATCase and heterotropically inhibit the enzyme**
- **low [CTP] lead to dissociation of CTP and removal of heterotropic inhibition**

[CTP] directly regulates CTP production via inhibition of ATCase

II) ATP activation

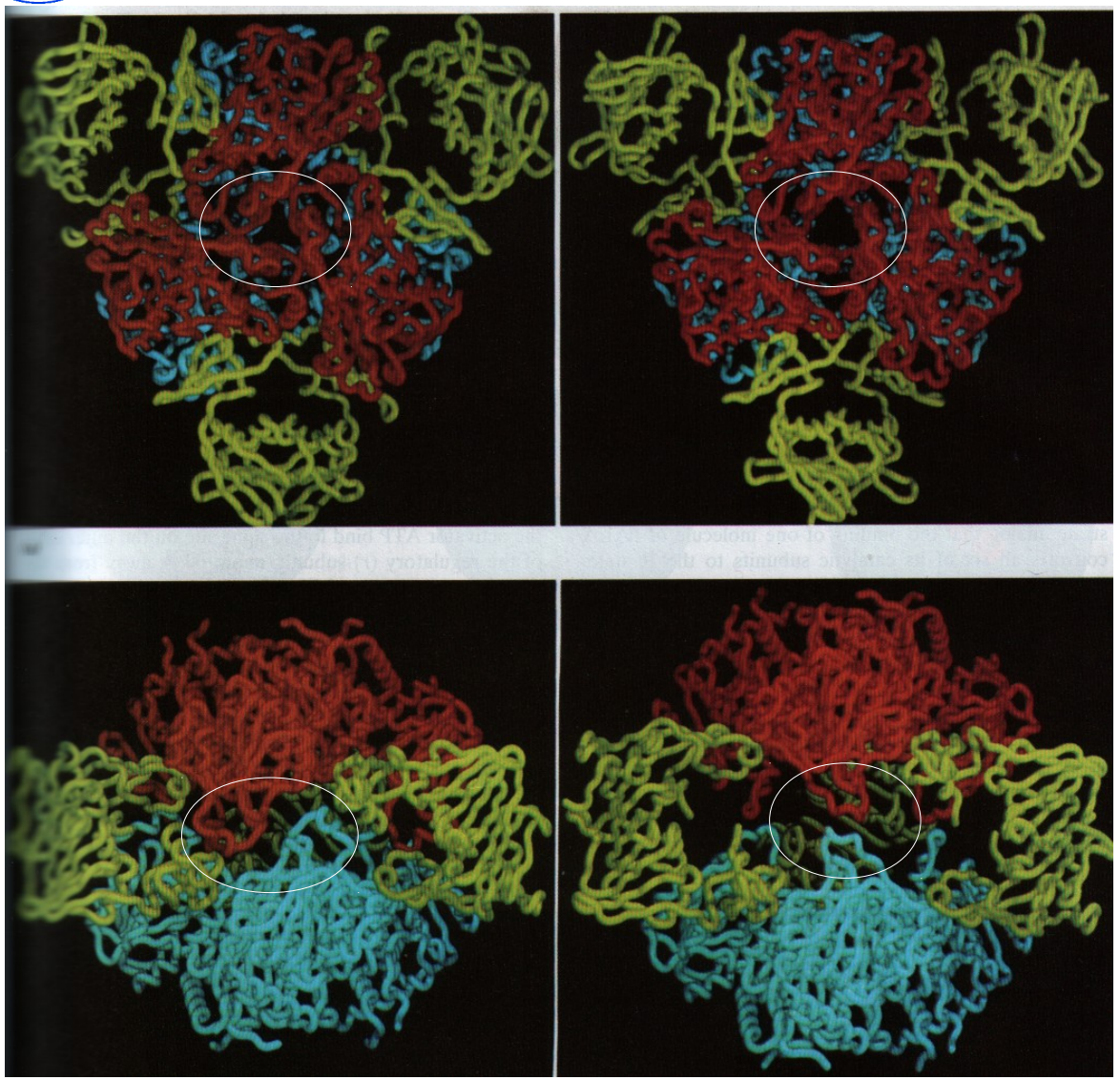
(Excess purine) The relative [ATP] and [CTP] represent the amounts of purine and pyrimidine nucleotides in the cell and must be regulated

- **if [purine] and [pyrimidine] are out of balance, ATP binding as a heterotropic activator increases pyrimidine biosynthesis to limit excess [purine]**

[ATP] regulates CTP (pyrimidine):ATP (purine) ratio via activation of ATCase

University of Lethbridge

ATCase: Structure & Regulation



T state (inactive) R state (active)

Regulatory chains – yellow; Catalytic chains – red & blue

R state has red and blue trimers separated (~10 Å) and rotated (12°) relative to T state

- **subunit contacts are sufficiently large and tightly coupled that the T --> R transition occurs simultaneously throughout ATCase**

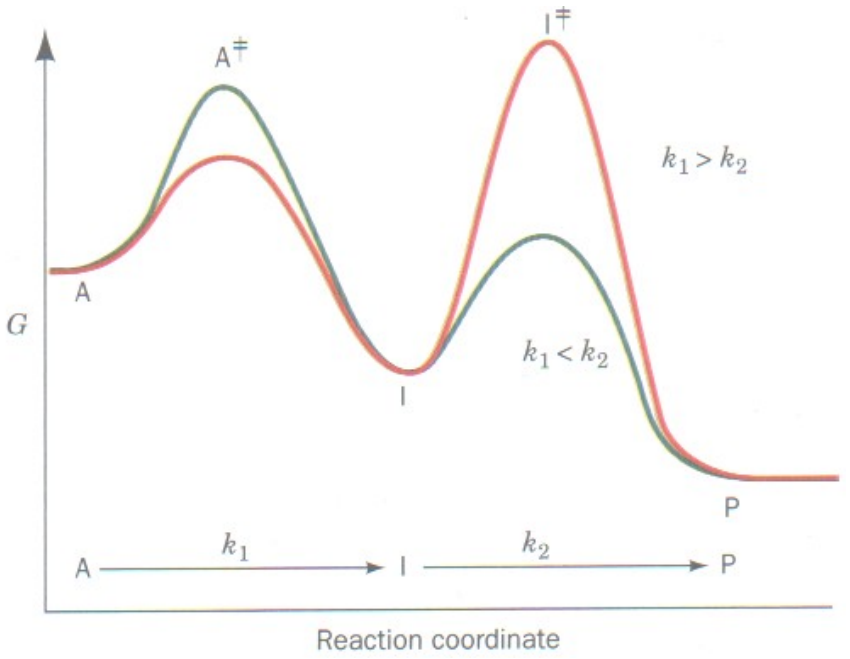
ATP binding stabilizes the R state and CTP binding stabilizes the T state

- **bind to overlapping site on edge of regulatory chain over 60 Å from active site**

University of Lethbridge

Chapter 14: Rates of Enyzmatic Reactions

Voet & Voet: Pages 482-506



University of Lethbridge

Introduction

Kinetics - study of the rates at which chemical reactions occurs

Major purpose is to understand reaction mechanism
(the sequence of steps that result in catalysis)

Enzyme Kinetics have enormous practical importance:

(1) catalytic rate and binding affinities of substrates & inhibitors

(2) determination of catalytic mechanism

- **especially powerful in combination with structural information**
- **involves examining catalytic rate under a variety of conditions**

(3) role in metabolism

- **role within pathway**

(4) assays

- **clinical tests and research experiments**

University of Lethbridge

Chemical Kinetics

- **Overall reaction of a reactant (A) forming a product (P) may actually involve a sequence of elementary reactions**
  - **elementary reactions create and/or utilize reaction intermediates (I)**
- **Reaction mechanism involves a description of all elementary reactions in an overall reaction**
- **At constant T, reaction rates vary with [substrate] in a simple manner expressed as the rate equation**
  - **k is the rate constant**
  - **lower case letters are coefficients from a balanced chemical equation and indicate stoichiometry**
  - **[upper case letters] are substrate concentrations**

Overall Reaction

$$A \longrightarrow P$$

(Possible) Elementary Reactions

$$A \longrightarrow I_1 \longrightarrow I_2 \longrightarrow P$$

General Single Product Reaction

$$aA + bB + \ldots + zZ \longrightarrow P$$

Rate equation

$$\text{Rate} = k\,[A]^a\,[B]^b \ldots [Z]^z$$

University of Lethbridge

Reaction Order

In the general chemical reaction for a process with a single product

- **typically, the sum of (a + b + ... + z) is the reaction order**

  corresponds to the number of molecules that must simultaneously collide in an elementary reaction

Elementary reactions in biological systems are 1st order (unimolecular) or 2nd order (bimolecular)

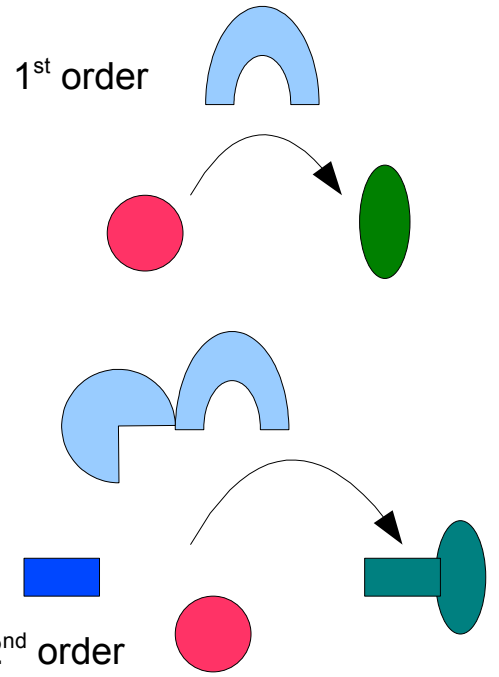
  - **3rd order (termolecular) elementary reactions are unusual and higher order are unknown**

General Single Product Reaction

$$aA + bB + ... + zZ \longrightarrow P$$

Rate equation

$$Rate = k [A]^a [B]^b ... [Z]^z$$



University of Lethbridge

Reaction Rates

Reaction rates can be experimentally determined by following the concentrations of reactants and/or products over time

First order reaction rate equation (A --> P)

- ***v*** **is the instantaneous rate or velocity of a reaction**

$$v = -d\frac{[A]}{dt} = k[A] \qquad \ln[A] = \ln[A_o] - kt$$

Second order reaction rate equation (2A --> P)

$$v = -d\frac{[A]}{dt} = k[A]^2 \qquad \frac{1}{[A]} = \frac{1}{[A_o]} + kt$$

Second order reaction rate equation (A + B --> P)

$$v = -d\frac{[A]}{dt} = -d\frac{[B]}{dt} = k[A][B]$$

Note: rate constant, k, has different units for 1st (s^{-1}) and 2nd order ($M^{-1}s^{-1}$) reactions

University of Lethbridge

Transition State

Consider a bimolecular elementary reaction,

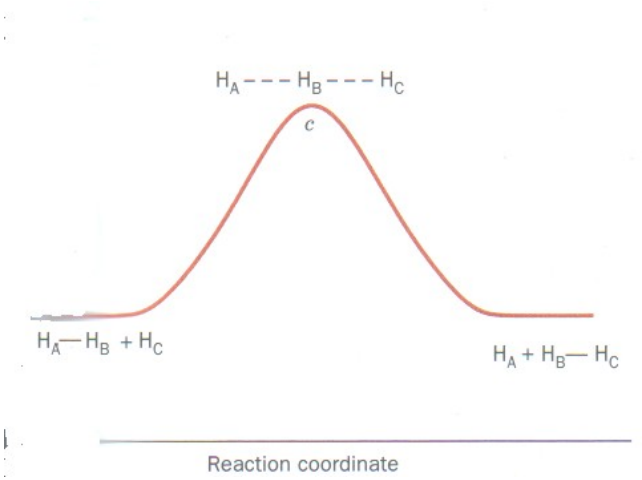
$$H_a - H_b + H_c \rightarrow H_a + H_b - H_c$$

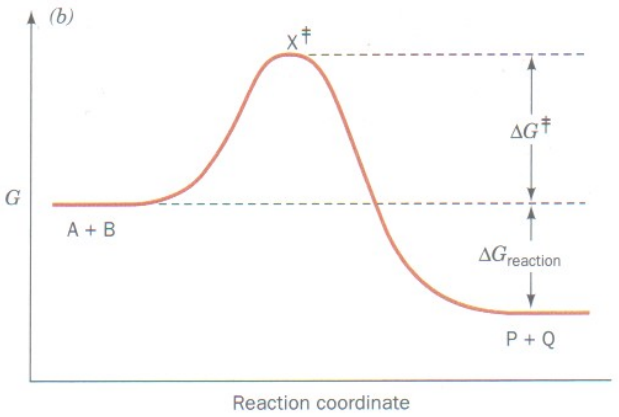
- **H_c must approach H_a-H_b sufficiently closely to form a bond with H_b as the H_a-H_b bond breaks**

The transition state is an *unstable* chemical structure that represents a free energy maxima on a reaction coordinate diagram

- **transition state formation and breakdown are the rate determining processes for most reactions**

Note: reaction coordinate is the minimum free energy pathway of a reaction





University of Lethbridge

Rate determining step

Multistep chemical reactions are common

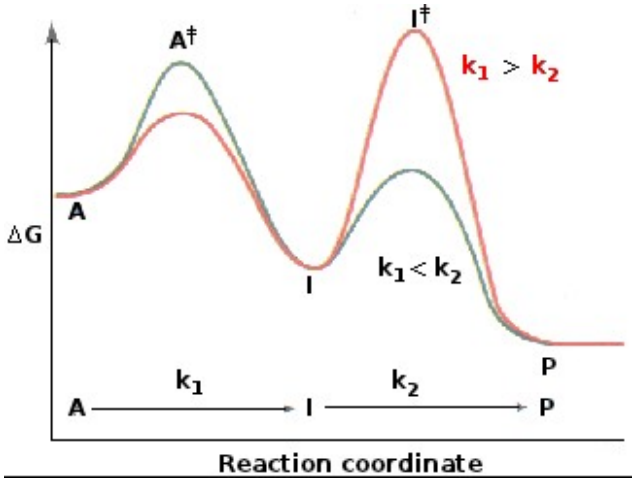
- **Rates of the elementary reactions are often different**
  - **if one step is significantly slowly than the others it will form a bottleneck in the overall reaction**
  - **the slowest step in the overall reaction is referred to as the rate determining step**

$$A \rightarrow I \rightarrow P$$

$$k_1 = [I]/[A]$$
$$k_2 = [P]/[I]$$

Reaction coordinate diagram for an overall reaction with two elementary reaction steps

- **Transition state theory tells us the free energy difference between reactant and transition state reflect rate and the slowest step has the largest difference**
- **two cases – step 1 slow (green); step 2 slow (red)**



University of Lethbridge

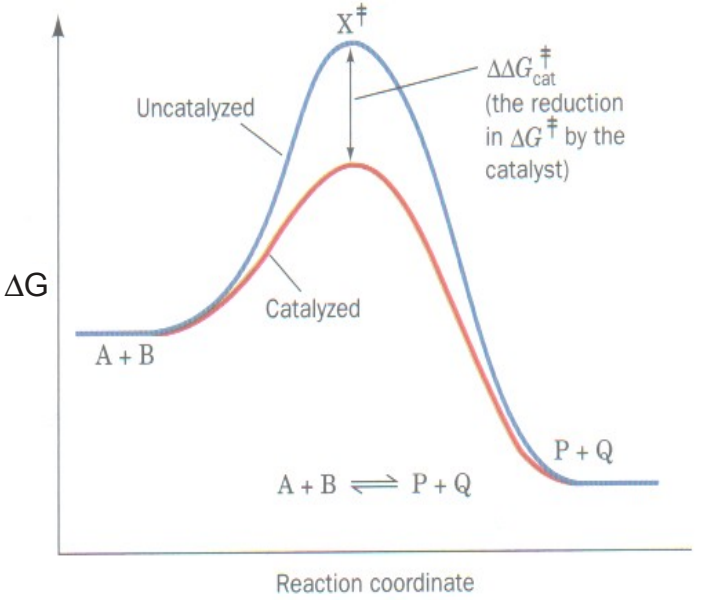
Catalysts (Enzymes)

Catalysts enhance reaction rates by lowering the activation barrier, $\Delta G^{\ddagger}$ for the reaction they catalyze

- **rate enhancement is $\exp(-\Delta\Delta G^{\ddagger}/RT)$, where $\Delta\Delta G^{\ddagger}_{cat}$ is the difference in activation energy between catalyzed and uncatalyzed reactions**

  Thermodynamics: 10 fold rate enhancement results from a 5.7 kJ/mol decrease in $\Delta\Delta G^{\ddagger}_{cat}$

  (10^6 fold enhancement requires 34.2 kJ/mol decrease)



Catalysts enhance the rates of both forward and backwards reactions equally

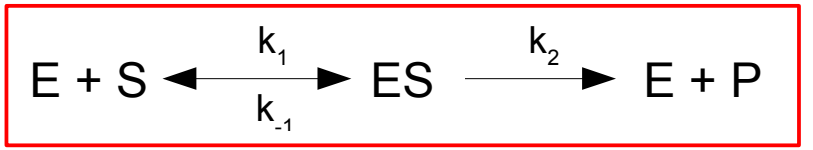
- **Catalysts cannot change the equilibrium**

University of Lethbridge

Enzyme Substrate (ES) Complex

Enzyme catalyzed reactions are independent of substrate concentration at high substrate concentrations

Catalyst has binding sites that can be saturated



- **Once saturated, further increase of [substrate] will not increase rate of product formation**
- **ES ---> E + P (ie. k_2) is rate determining and reaction rate depends upon the [ES]**

$$d\frac{[P]}{dt} = k_2[ES]$$

Note: E is enzyme, S is substrate, P is product and ES is the enzyme substrate complex

$$d\frac{[ES]}{dt} = k_1[E][S] - k_{-1}[ES] - k_2[ES]$$

This rate equation is not directly useful as we require rate constants for each step AND the [ES] as a function of time

University of Lethbridge

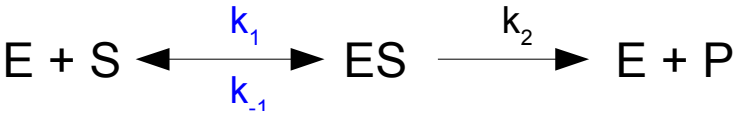
Assumptions

Using two assumptions, the reaction rate equation can be rewritten in terms that are experimentally measurable

(1) Steady State Assumption – [ES] stays constant during the measurement. Only valid when $k_2 <<< k_1$ or k_{-1}.

Typically valid for a short duration (initial velocity, v_o) after the reaction starts and before significant amounts of substrate (10%) have been converted to product (do not have to consider k_{-2})

(2) Equilibrium Assumption – bind/release substrate is fast compared to catalysis ($k_{-1} >>> k_2$) and reaches equilibrium.



[ES] depends upon k_1/k_{-1} (note: $K_{eq,association} = k_1/k_{-1} = [P]/[S]$)

University of Lethbridge

Michaelis-Menten Equation

The rate of product formation is $k_2[ES]$

$$d\frac{[P]}{dt} = k_2[ES]$$

From the steady state assumption d[ES]/dt = 0

- Allows us to solve for the [ES] term in our rate equation

$$d\frac{[ES]}{dt} = k_1[E][S] - k_{-1}[ES] - k_2[ES]$$

- Substituting [E] = [E_T] – [ES] (E_T is total Enzyme)

By definition, the maximum velocity of the enzyme catalyzed reaction occurs when [E_{total}] = [ES]

$$v = d\frac{[P]}{dt} = k_2[ES] = k_2\frac{([E_T][S])}{(K_M + [S])}$$

- Allows us to define $V_{max} = k_2 [E_T]$

$$V_{max} = k_2[E_T]$$

- Allows us to define $K_M = (k_{-1} + k_2)/k_1$

Result is the Michaelis-Menten equation; the basic equation of enzymology

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

Note: You are not responsible for deriving this equation. You do need to know the equation and those for V_{max} and K_M

University of Lethbridge

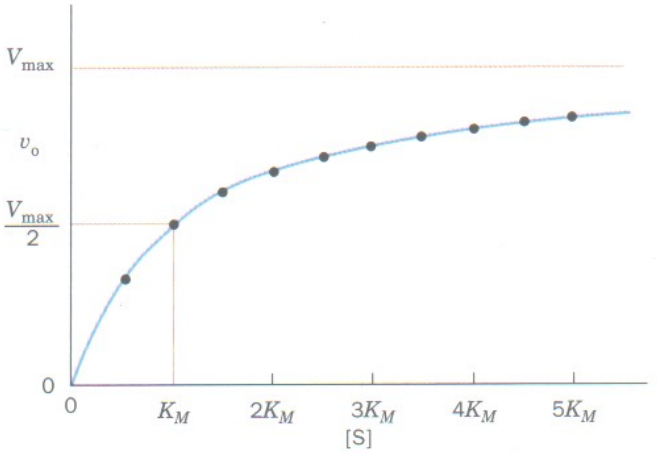
K_M – Michaelis constant

Plots of initial velocity vs. substrate concentration yield hyperbolic curves for Michaelis-Menten reactions

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

K_M is the substrate concentration at which the reaction velocity is ½ maximal

- **assuming K_M = [S], the Michaelis-Menten equation reduces to $v_o = V_{max}/2$**
- **small K_M is associated with efficient catalysis at low [S]**



When $k_2 <<< k_{-1}$, K_M is a measure of substrate affinity

$$K_M = \frac{(k_{-1} + k_2)}{k_1} = \frac{(breakdown\ of\ ES)}{(formation\ of\ ES)}$$ **≈ (k_{-1}/k_1) = ($1/K_{eq,assoc}$) = ($K_{eq,dissoc}$)**

University of Lethbridge

Analysis of Kinetic Data

Several methods exist for determining the kinetic constants in the Michaelis-Menten equation

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

1) Initial velocity (v_o vs [S]) plots require extrapolation of V_{max}

2) Lineweaver-Burke or double reciprocal plot

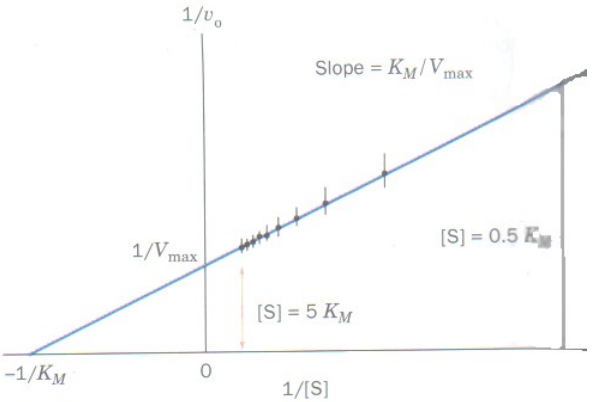
- **Reciprocal of Michaelis-Menten equation ($1/v_o$ vs $1/[S]$)**

$$\frac{1}{v_o} = \frac{(K_M + [S])}{(V_{max}[S])}$$

slope = K_M/V_{max} y intercept = $1/V_{max}$ x intercept = $-1/K_M$

$$\frac{1}{v_o} = \frac{K_M}{V_{max}}\frac{1}{[S]} + \frac{1}{V_{max}}$$

- **Better approach but nature of 1/[S] means most measurements are near the y axis. Small errors can skew calculation of constants**



<u>Other methods exist</u> for treating kinetic data, each with its own strengths and weaknesses

University of Lethbridge

k_{cat} (catalytic constant or turnover number)

k_{cat} is the rate constant for the overall reaction at saturating substrate

$$k_{cat} = \frac{V_{max}}{[E]_T}$$

- varies widely (10^8 times) among enzymes

Relationship between k_{cat} and rate constants (k_1, k_{-1}, k_2, etc.) depends upon the reaction mechanism

1) Michaelis-Menten mechanism (simplest case)

$k_{cat} = k_2$

2) More complicated mechanisms

$k_{cat} \leq k_2$ AND may depend on other rate constants

University of Lethbridge

k_{cat} / K_M (catalytic efficiency)

Catalytic efficiency depends upon both reaction rate and substrate concentration.

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

When [S] << K_M,

very little ES is formed and the Michaelis-Menten equation reduces to

$$v_o = \frac{(V_{max}[S])}{(K_M)} = (\frac{(k_{cat})}{(K_M)})[E_T][S]$$

- second order rate equation with an apparent rate constant, (k_{cat}/K_M)

The **apparent rate constant (k_{cat}/K_M)** under these conditions is a measure of catalytic efficiency

University of Lethbridge

k_{cat} / K_M Limits

What is the upper limit to catalytic efficiency?

$$\frac{k_{cat}}{K_M} = \frac{k_2}{K_M} = \frac{(k_2 k_1)}{(k_{-1} + k_2)}$$

- **efficiency is highest when $k_2 >> k_{-1}$, and $k_{cat}/K_M \approx k_1$**
  (Note: if $k_2 >>> k_{-1}$, the enzyme does not have a Michaelis-Menten mechanism)
- **rate constant (k_1) for formation of ES limits catalytic efficiency**

Theoretically, k_1 can be no larger than the frequency with which enzyme and substrate encounter one another

- **diffusion rates define limits of enzyme efficiency and yield values in the 10^8-10^9 $M^{-1}s^{-1}$ range (Diffusion-controlled limit)**
- **several enzymes have k_{cat}/K_M values in this range and must catalyze a reaction almost every time they encounter a substrate**
  - **Sometimes called 'catalytically perfect'**

University of Lethbridge

Inhibition

Inhibitors are compounds that bind to enzyme in such a way that the activity of the enzyme is reduced

- many inhibitors resemble the enzyme's natural substrate but react slowly or not at all

- **Commonly used to investigate the nature of the substrate-binding site and to elucidate enzyme mechanism**

- many (most) clinical drugs are enzyme inhibitors produced synthetically or isolated from natural sources

- **recently the first rationally 'designed' drugs have been utilized**

Large portion of pharmacy is medicinal use of inhibitors

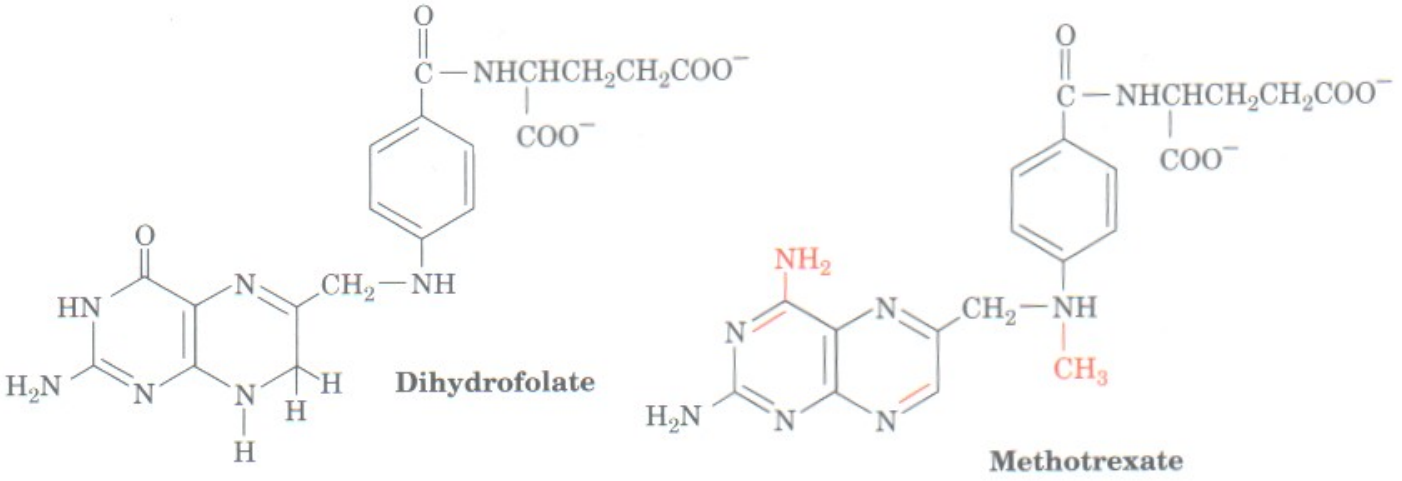
- **must consider many issues over and above binding and inhibition**
  - **dosage, solubility, side effects, cost, *etc***

University of Lethbridge

Inhibitor - methotrexate

Example of enzyme inhibitor used as chemotherapeutic agent

- dihydrofolate reductase catalyzes an essential step in the synthesis of dTMP
- methotrexate binds to and inhibits dihydrofolate reductase preventing dTMP biosynthesis
  - preferentially kills actively dividing cells (*eg* cancer cells)
  - clearly, methotrexate is structurally related to the normal substrate and is known to bind in the enzyme active site in a conformation similar but not identical to the substrate



(Red) Structural differences between natural substrate (dihydrofolate) and methotrexate