Enzyme Classification

Simple Enzymes: composed of whole proteins

Complex Enzymes: composed of protein plus a relatively small organic molecule

holoenzyme = apoenzyme + *prosthetic group* / *coenzyme*

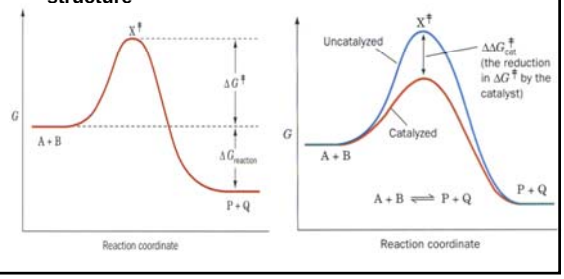
A *prosthetic group* describes a small organic molecule bound to the apoenzyme by covalent bonds.

When the binding between the apoenzyme and non-protein components is non-covalent, the small organic molecule is called a *coenzyme.*

Oxidoreductases	**Act on many chemical groupings to add or remove hydrogen atoms.**
Transferases	**Transfer functional groups between donor and acceptor molecules. Kinases are specialized transferases that regulate metabolism by transferring phosphate from ATP to other molecules.**
Hydrolases	**Add water across a bond, hydrolyzing it.**
Lyases	**Add water, ammonia or carbon dioxide across double bonds, or remove these elements to produce double bonds.**
Isomerases	**Carry out many kinds of isomerization: L to D isomerizations, mutase reactions (shifts of chemical groups) and others.**
Ligases	**Catalyze reactions in which two chemical groups are joined (or ligated) with the use of energy from ATP.**

Enzymes

- **Enzymes are protein catalysts**
- **Catalysts alter the rate of a chemical reaction without undergoing a permanent change in structure**



How can an enzyme reduce the activation energy?

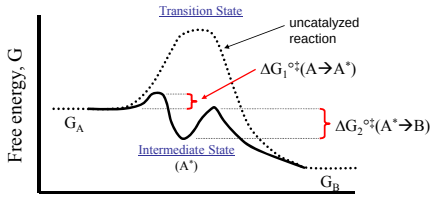
(1) Binding to the substrate can be done such that the formation of the transition state is favored

(2) Orientation and positioning of substrate(s)

(3) Bonds in the substrate can be 'activated' by functional groups in the catalytic site

Enzyme active site

- **Active site is lined with residues and sometimes contains a co-factor**
- **Active site residues have several important properties:**
  - **Charge [partial, dipoles, helix dipole]**
  - **pKa**
  - **Hydrophobicity**
  - **Flexibility**
  - **Reactivity**



- the activation energies for the formation of the intermediate state, and its conversion to the final product are *each* lower than the activation energy for the uncatalyzed reaction

-intermediate state- resembles transition state but with lower energy, (due to interaction with a catalyst)

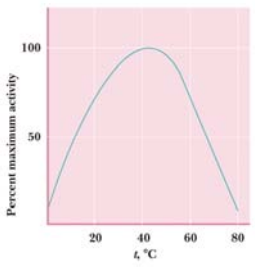
- transition state defines free energy maximum state

Entropic and enthalpic factors in catalysis

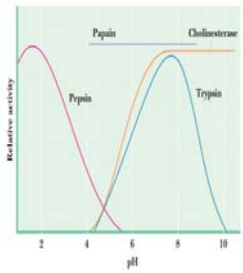
$\Delta G^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger}$

- $\Delta G^{\ddagger}$: activation energy is lowered during catalysis
- $\Delta H^{\ddagger}$: energy required for the reaction; cannot be too large or else reaction will be slow
- $T\Delta S^{\ddagger}$: change in entropy (degree of conformational flexibility) during reaction; molecules often need to go through energy demanding (strained/distorted) conformations for reaction to take place; 'solved' by having an intermediate state that resembles the transition state but is of lower energy because of favourable binding to the catalyst

Effect of Temperature



Effect of pH



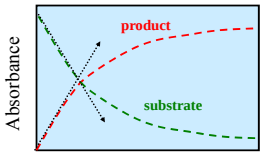
Measuring Enzymatic Rates

-*ideally* done with a system where the **product or substrate** absorb a particular wavelength of light

- this depends on enzyme

- reaction can be monitored with a spectrophotometer by measuring the appearance of product or disappearance of substrate

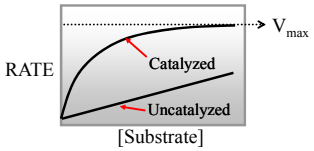
Beer Lambert's law



Abs = εlc ε = extinction Coefficient; l = path length (cm) ~1; c = Concentration (M)

measurement of the initial slope → rate (conc.)/(time)

Why does the enzymatic rate *level* off?



-at low [substrate], the enzyme 'active site' is occupied at a frequency ∝ [substrate]

- at a certain point, **saturation** is reached
  - enzyme active site is always occupied
  - it is operating as maximum rate, V_{max}

*- most cellular enzymes **do not operate at V_{max}***
- however, under certain conditions the cell needs more of particular enzymes

Michaelis-Menten Enzyme kinetics

Reaction Scheme

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \xrightarrow{k_2} E + P$$

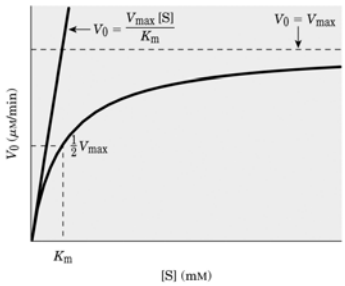
Michaelis-Menten Equation

$$v = \frac{V_m[S]}{K_m + [S]}$$

where $V_m = k_2[E_t]$ and $Km = \frac{k_{-1} + k_2}{k_1}$

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

Michaelis – Menten Kinetics

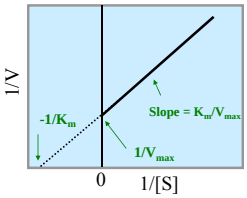


low [S], v is proportional to [S] - first order
high [S], v is independent of [S] - zero order

In order to change this equation to a form we can use in our analysis of enzymatic rate constants, we invert both sides of the equation:

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

Lineweaver-Burk Plot



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

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

Michaelis-Menten Enzyme kinetics

For the binding reaction:

E + S <===> ES

ΔG°′ = - RTlnK where: $K = \left(\frac{[ES]}{[E][S]} \right)_{eq}$ K_A

K is thus an equilibrium association constant (units: M^{-1})
An equilibrium dissociation constant (units: M).

~~which is~~ $\left(\frac{[E][S]}{[ES]} \right)_{eq} = \frac{1}{K}$ K_D

Tight binding implies a *low* dissociation constant and a *high* association constant.

Turnover number (k_{cat})

The k_{cat} is a direct measure of the conversion of substrate to product

The number of substrate molecules turned over per enzyme molecule per second, hence "turnover number".

The overall rate of a reaction is limited by its slowest step

In this case k_{cat} will be equal to the rate constant for the rate determining step

For the Michaelis-Menten system this is k_2

K_m — High K_m means strength of binding is low

Relates to how strongly an enzyme binds its substrate

k_{cat} — High k_{cat} means high speed of catalysis

Relates to how rapid a catalyst the enzyme is

V_{max} — High V_{max} means high rate of catalysis

Related to k_{cat} and [E] by: $V_{max}=k_{cat}[E]$

- **k_{cat} = turnover number; $k_{cat} = V_{max}/[E]_T$**
- **k_{cat}/K_m is a measure of activity, catalytic efficiency**

K_M is a useful indicator of the affinity of an enzyme for the substrate

A low K_M indicates a high affinity for the substrate

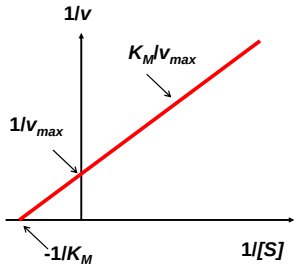
A high k_{cat}/K_M ratio implies an efficient enzyme

This could result from: **Large k_{cat}**
Small K_M

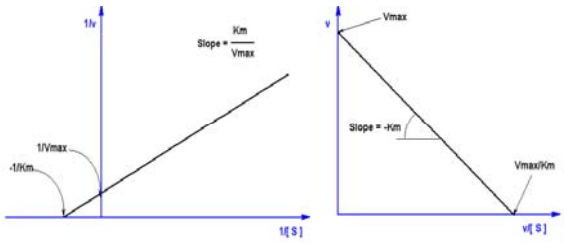
Double-reciprocal Lineweaver-Burke Plot

$$\frac{1}{v} = \frac{1 + K_M/[S]}{v_{max}}$$

$$\frac{1}{v} = \frac{1}{v_{max}} + \frac{K_M}{v_{max}} \cdot \frac{1}{[S]}$$



Linear plots for determination of V_{max} and K_m



Lineweaver-Burke plot **Eadie-Hofstee plot**

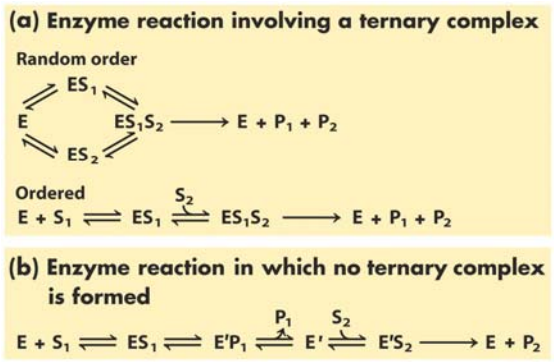
Bisubstrate Reactions

$$S1 + S2 \xrightleftharpoons{E} P1 + P2$$

$$A\text{-}X + B \xrightleftharpoons{E} A + B\text{-}X \text{ (in transferase reactions)}$$

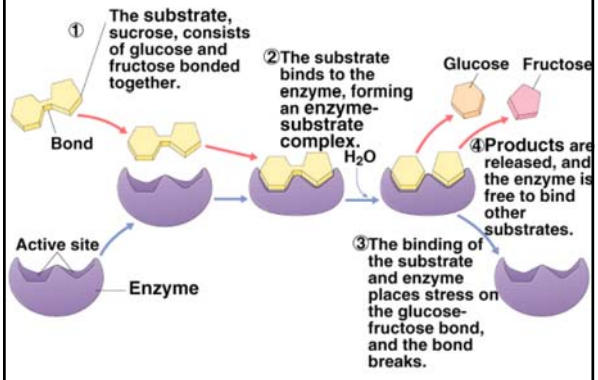
- Sequential binding of S1 and S2 before catalysis:
  - Random substrate binding - Either S1 or S2 can bind first, then the other binds.
  - Ordered substrate binding - S1 must bind before S2.
- Ping Pong reaction - first S1 → P1, P1 released before S2 binds, then S2 → P2.

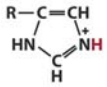
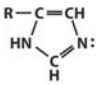
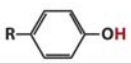
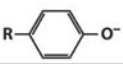
Bisubstrate Reactions



Active Site

- The area of an enzyme that binds to the substrate
- Structure has a unique geometric shape that is designed to fit the molecular shape of the substrate
- Each enzyme is substrate specific
- Thus the active site that is complementary to the geometric shape of a substrate molecule



Amino acid residues	General acid form (proton donor)	General base form (proton acceptor)
Glu, Asp	R—COOH	R—COO⁻
Lys, Arg	R—⁺NH₂—H	R—NH₂
Cys	R—SH	R—S⁻
His		
Ser	R—OH	R—O⁻
Tyr		

Proteases

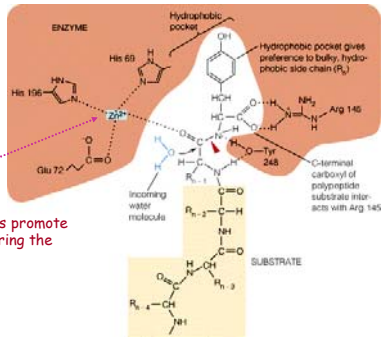
- To maintain protein turnover;
- To digest diet proteins;
- To regulate certain enzyme activities (zymogens):
- General hydrolysis reaction:

- A class of proteases whose catalytic mechanism is based on an active-site serine residue – serine proteases;
- Include trypsin, chymotrypsin, elastase, thrombin, subtilisin, plasmin, tissue plasminogen activator etc.

Another type of peptidase: carboxypeptidase A

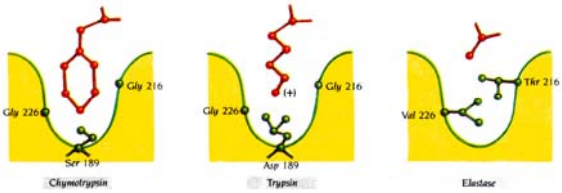
This is an example of a metallo-enzyme

- metallo-enzymes are a class of enzymes which utilize metal cofactors



Zn^{2+} stabilizes and helps promote the formation of O^- during the reaction

Enzyme active site



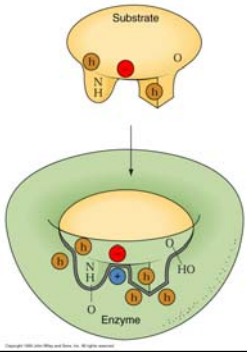
- Chymotrypsin (Cuts next to Hydrophobic Groups)
- Trypsin (Cuts next to Arg & Lys)
- Elastase (Cuts next to Val & Thr)

Substrate Binding specificity

Complementarity

- **Geometric**
- **Electronic (electrostatic)**
- **Stereospecificity (enzymes and substrates are chiral)**

1. **Lock and Key model**
2. **Induced Fit model**



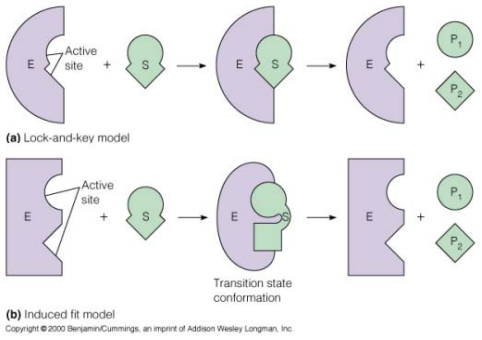
Lock and Key Model

- **An enzyme binds a substrate in a region called the active site**
- **Only certain substrates can fit the active site**
- **Amino acid R groups in the active site help substrate bind**

Induced Fit Model

- **Enzyme structure flexible, not rigid**
- **Enzyme and active site adjust shape to bind substrate**
- **Increases range of substrate specificity**
- **Shape changes also improve catalysis during reaction - transition-state like configuration**

Enzyme-Substrate Interaction



Enzyme Inhibition

- **Inhibitors: compounds that decrease activity of the enzyme**
- **Can decrease binding of substrate (affect K_M), or turnover # (affect k_{cat}) or both**
- **Most drugs are enzyme inhibitors**
- **Inhibitors are also important for determining enzyme mechanisms and the nature of the active site.**
- **Important to know how inhibitors work – facilitates drug design, inhibitor design.**

- **Antibiotics inhibit enzymes by affecting bacterial metabolism**
- **Nerve Gases cause irreversible enzyme inhibition**
- **Insecticides – choline esterase inhibitors**
- **Many heavy metal poisons work by irreversibly inhibiting enzymes, especially cysteine residues**

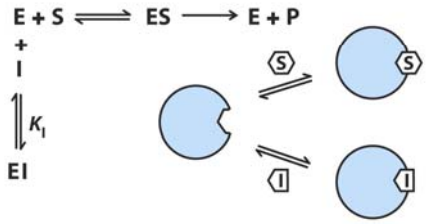
Types of Enzyme Inhibition

- **Reversible inhibition**
  reversibly bind and dissociate from enzyme, activity of enzyme recovered on removal of inhibitor - usually non-covalent in nature
  - **Competitive**
  - **Noncompetitive (Mixed)**
  - **Uncompetitive**

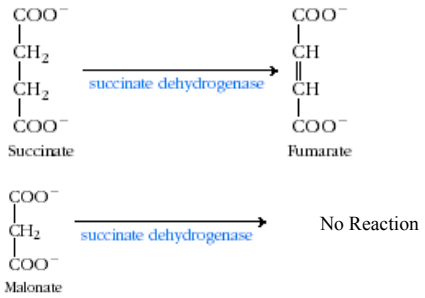
- **Irreversible inhibition**
  inactivators that irreversibly associate with enzyme
  activity of enzyme not recovered on removal - usually covalent in nature

Competitive Inhibition

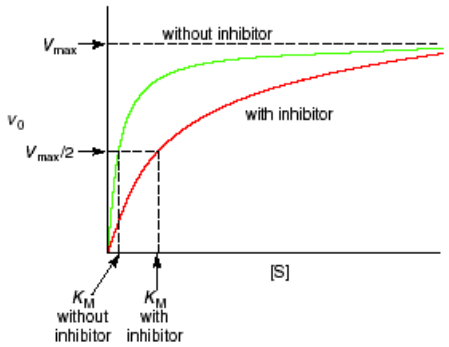
Inhibitor competes for the substrate binding site – most look like substrate
substrate mimic / substrate analogue



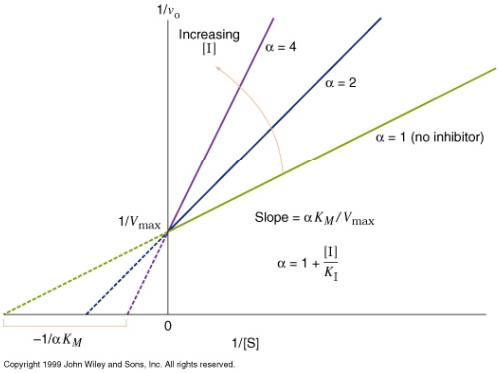
Competitive Inhibition



Competitive Inhibition

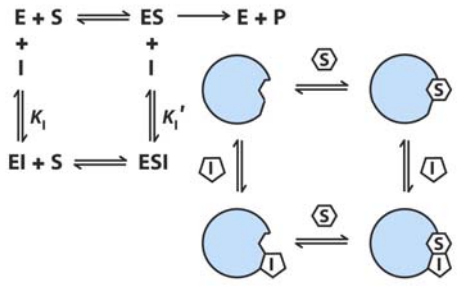


Competitive Inhibition

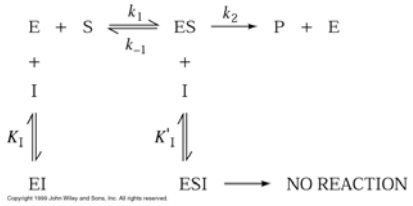


Noncompetitive Inhibition

- **Inhibitor can bind to either E or ES**

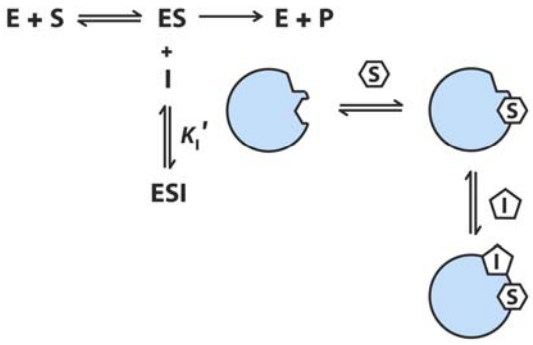


Noncompetitive Inhibition

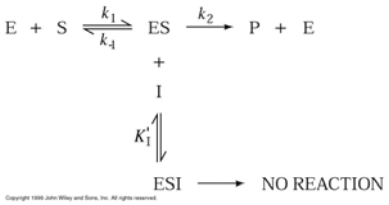


- **Vo = Vmax[S]/(αK$_M$ + α'[S])**
- **Vmax decreases; K$_M$ can go up or down.**

Uncompetitive Inhibition

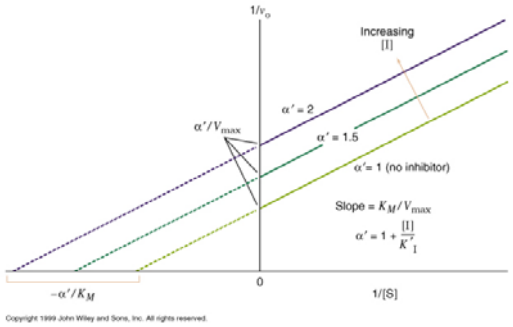


Uncompetitive Inhibition

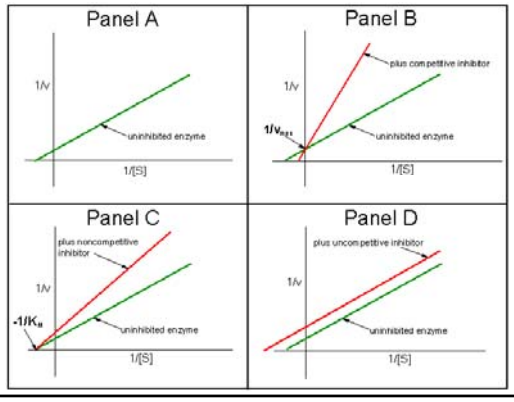


- **Active site distorted after binding of S (usually occurs in multisubstrate enzymes) Decreases both K$_M$ and kcat**
- **Vo = Vmax[S]/(K$_M$ + α'[S]) K$_I$ = [ES][I]/[ESI]**
- **Cannot be reversed by increasing [S] – available enzyme decreases**

Uncompetitive Inhibition



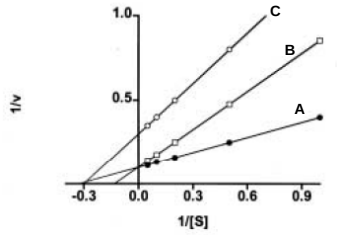
Lineweaver-Burke plots



Enzyme Kinetics

- **Solve for [ES] gives [ES] = [E]$_T$[S]/(K$_M$+[S])**
- **Initial velocity (<10% substrate used)**

  $v_o = (dP/dt)_{t=0} = k_2[ES] = k_2[E]_T[S]/(K_M + [S])$
- **Et and S known, at t close to 0, assume irreversible**
- **Maximal velocity at high S (S >> K$_M$)**
  $V_{max} = k_2[E]_T$
- **$v_o = V_{max}[S]/(K_M + [S])$**



Allosteric regulation

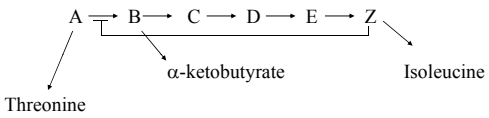
When a small molecule can act as an *effector* or *regulator* to *activate or inactivate* an action of a protein

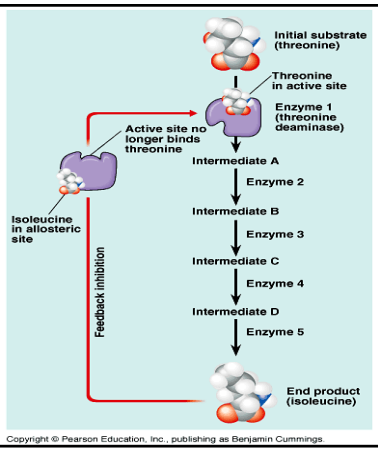
- the protein is said to be under allosteric control. The binding of the small ligand is distant from the protein's active site and regulation is a result of a *conformational change* in the protein when the ligand is bound

Many types of proteins show allosteric control:

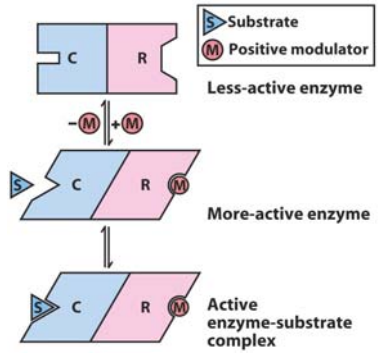
- **haemoglobin (*NOT* myoglobin)**
- **various enzymes**
- **various gene-regulating proteins**

Feedback Inhibition





Allosteric regulation

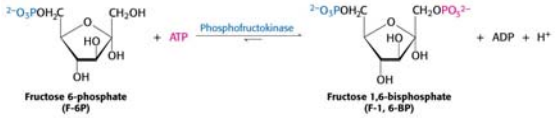


Example: Phosphofructokinase and ATP

Substrate: Fructose-6-phosphate

Reaction: **fructose-6-phosphate + ATP → fructose-1,6-bisphosphate + ADP**

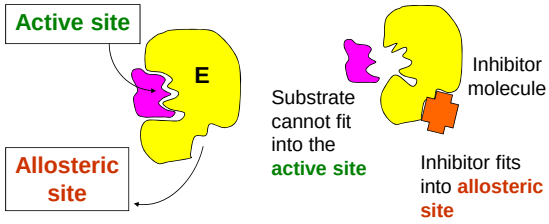
ENZYME: phosphofructokinase



- This reaction lies near the beginning of the respiration pathway in cells
- The end product of respiration is **ATP**
- If there is a lot of ATP in the cell this enzyme is inhibited
- Respiration slows down and less ATP is produced
- As ATP is used up the inhibition stops and the reaction speeds up again

Allosteric inhibition

Allosteric means **"other site"**

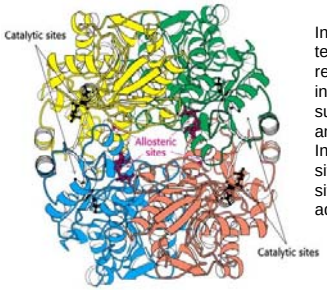


- These enzymes have **two receptor sites**
- One site fits the substrate like other enzymes
- The other site fits an inhibitor molecule

Phosphofructokinase

- This enzyme has an **active site** for fructose-6-phosphate molecules to bind with another phosphate group
- It has an **allosteric site** for ATP molecules, the inhibitor
- When the cell consumes a lot of ATP the level of ATP in the cell falls
- No ATP binds to the **allosteric site** of phosphofructokinase
- The enzyme's conformation (shape) changes and the **active site** accepts substrate molecules
- The respiration pathway accelerates and ATP (the final product) builds up in the cell
- As the ATP increases, more and more ATP fits into the **allosteric site** of the phosphofructokinase molecules
- The enzyme's conformation changes again and stops accepting substrate molecules in the **active site**
- Respiration slows down

Allosteric sites in Phosphofructokinase (**PFK**)



In mammals, PFK is a 340 kDa tetramer, which enables it to respond allosterically to changes in the concentrations of the substrates fructose 6-phosphate and ATP
In addition to the substrate-binding sites, there are multiple regulatory sites on the enzyme, including additional binding sites for ATP

Enzyme assays

- Enzyme assay – method to detect and quantitate the presence of an enzyme
  - Often used to determine the purity of an enzyme
  - Used to determine mechanism and kinetic parameters of a reaction
- Features of a good assay
  - Fast, convenient, and cost effective
  - Quantitative, specific, and sensitive

Enzyme Assays

A useful enzyme assay must meet four criteria:
(a) absolute specificity
(b) high sensitivity
(c) high precision & accuracy
(d) convenience

Most enzyme assays monitor disappearance of a substrate or appearance of a product

Ensure that only one enzyme activity is contributing to the monitored effect

High sensitivity and precision

For purification, specific activities of most enzymes are very low. Therefore, the assay must be highly sensitive.

The accuracy and precision of an enzyme assay usually depend on the underlying chemical basis of techniques that are used.

For example, if an assay is carried out in buffer of the wrong pH, the observed rates will not accurately reflect the rate of enzymatically produced products

Factors affecting an assay

- pH
- Temperature
- Buffer
- Cofactors
- Inhibitors
- Activators
- Other substrates
- Allosteric effects
- Stabilizing agents (detergent, salt, reducing agent, etc)

pH

pH values yielding the highest reaction rates are not always those at which the enzyme is most stable. It is advisable to determine the pH optima for enzyme assay and stability separately.

For protein purifications:
Buffer must have an appropriate pKa and not adversely affect the protein(s) of interest.

Temperature

Not all proteins are most stable at 0 °C, e.g. Pyruvate carboxylase is cold sensitive and may be stabilized only at 25 °C.

Freezing and thawing of some protein solutions is quite harmful. If this is observed, addition of glycerol or small amounts of dimethyl sulfoxide to the preparation before freezing may be of help.

Storage conditions must be determined by trial and error for each protein.

Proteins requiring a more hydrophobic environment may be successfully maintained in solutions whose polarity has been decreased using sucrose, glycerol, and in more drastic cases, dimethyl sulfoxide or dimethylformamide. Appropriate concentrations must usually be determined by spectroscopic methods with a knowledge of the extinction coefficient, ε.

A few proteins, on the other hand, require a polar medium with high ionic strength to maintain full activity. For these infrequent occasions, KCl, NaCl, NH_4Cl, or $(NH_4)_2SO_4$ may be used to raise the ionic strength of the solution.

Types of assays

- Time resolved
  - continuous
- Single point (“fixed time”) assay
  - Incubate each sample with substrate for a fixed time
  - Quench rxn and detect product formation

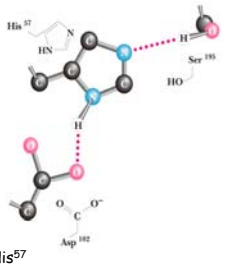
Proteases

- To maintain protein turnover;
- To digest diet proteins;
- To regulate certain enzyme activities (zymogens):
- General hydrolysis reaction:

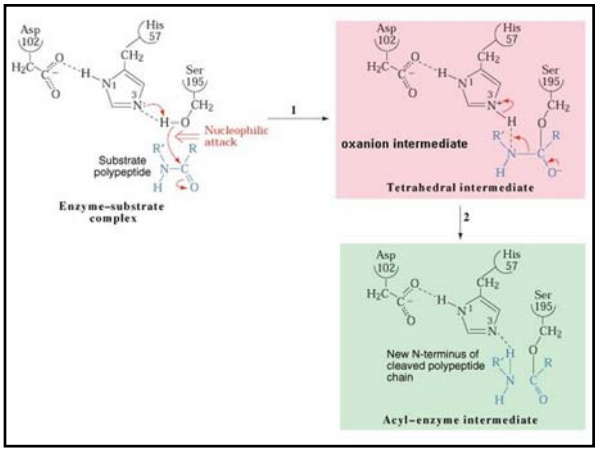
- A class of proteases whose catalytic mechanism is based on an active-site serine residue – serine proteases;
- Include trypsin, chymotrypsin, elastase, thrombin, subtilisin, plasmin, tissue plasminogen activator etc.

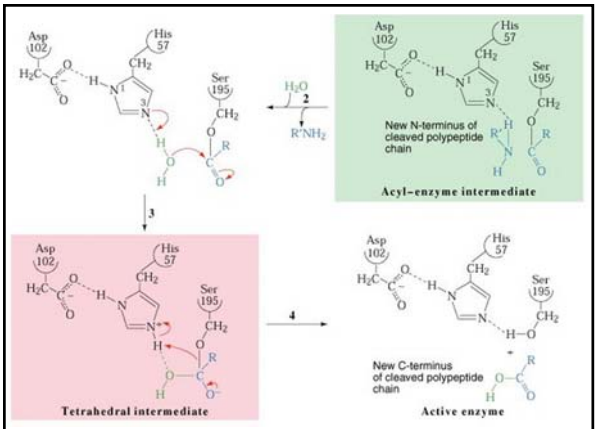
Outline of Catalytic Mechanism of Serine Proteases

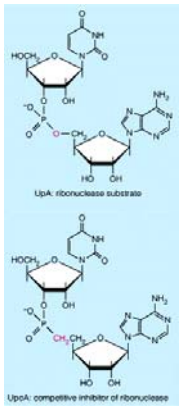
- Chymotrypsin cleaves after hydrophobic aromatic residues Phe, Trp, sometimes Met);
- The active site of chymotrypsi contains three conserved resid His^{57}, Aps^{102}, and Ser^{195};
- catalytic strategy: covalent intermediate.

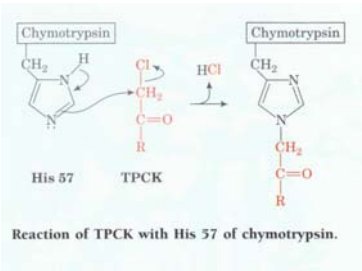


- Asp^{102} functions only to orient His^{57}
- His^{57} acts as a general acid and base
- Ser^{195} forms a covalent bond with peptide to be cleaved
- Covalent bond formation turns a trigonal C into a tetrahedral C
- The tetrahedral oxyanion intermediate is stabilized by NHs of Gly^{193} and Ser^{195}



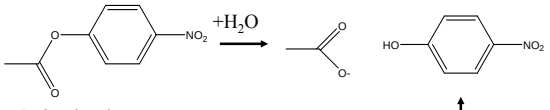




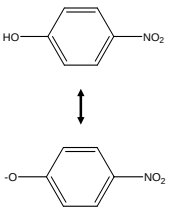


Reaction of TPCK with His 57 of chymotrypsin.

Chymotrypsin can also cleave ESTER linkages.



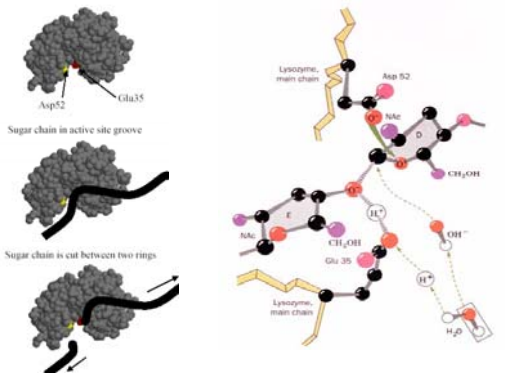
P-nitrophenyl acetate (Colorless)



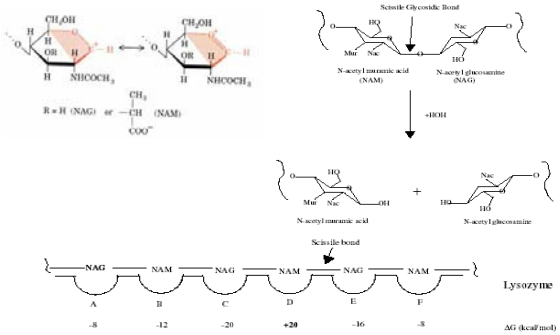
P-nitrophenolate (Yellow)

This is not biologically relevant but a convenient way to study the reaction

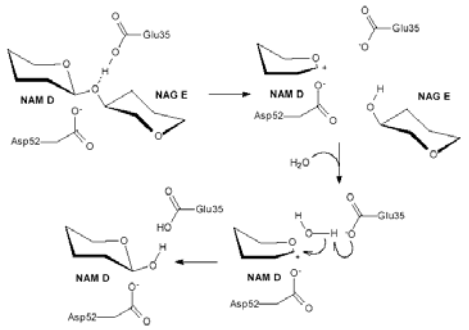
Lysozyme mechanism



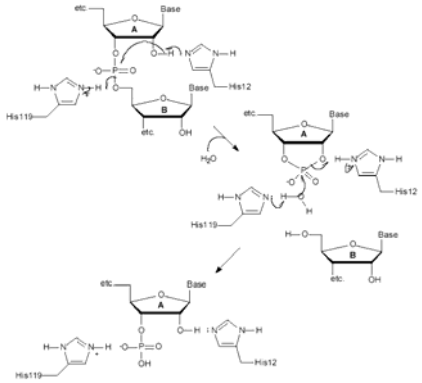
The reaction catalyzed by lysozyme is the hydrolysis of the glycosidic bond of the (NAM-NAG)$_n$ heteropolymer that is the backbone of the bacterial cell wall. The enzyme is specific for NAM-NAG glycosidic bonds (β-1,4 conformation).

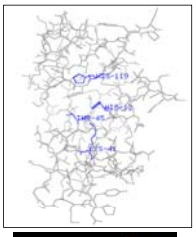


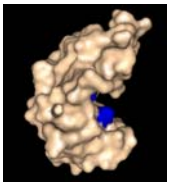
Mechanism



Ribonuclease mechanism



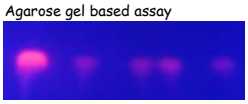




Ribonuclease assay

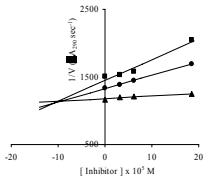
Ribonucleolytic activity

Agarose gel based assay



Enzyme kinetic assay

Dixon plot for inhibition of RNase A



Inhibition of Ribonuclease A

Agarose gel based assay for the inhibition of RNase A.
Lane 1: tRNA;
Lane 2: RNase A + tRNA;
Lane 3: GTC, RNase A + tRNA;
Lane 4: EGCG, RNase A + tRNA

RNase A concentration: 13.42 μM
Concentration of substrate: 0.1866 mM (■), 0.2554 mM (▲) and 0.4464 mM
Concentration of EGCG: 0-0.94 mM
Inhibition Constant: 77.35 μM