

Enzyme Catalysis

See General Biochemistry Text for assistance and clarification

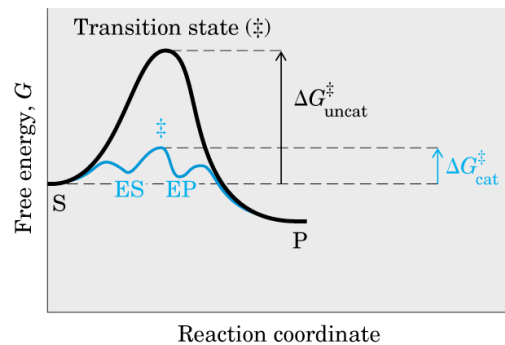
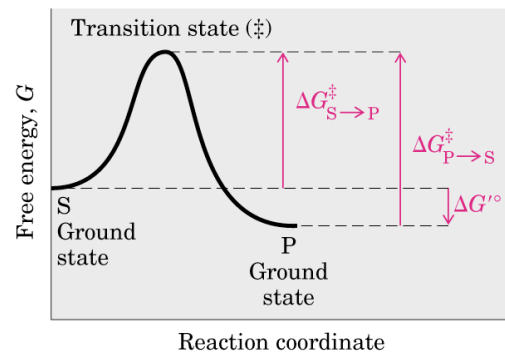
Enzyme Catalysis

- Frequently the interaction between protein and ligand(s) is followed by covalent changes in the ligand(s). The protein is a catalyst for the chemical reaction and is unaltered once the reaction is complete.
- In such cases the protein is known as the enzyme and the ligand(s) its substrate(s).
- In some cases, the catalyzed reaction may be coupled to other processes:
 - Mechanical movement/work.
 - Molecular transport.
 - Other chemical reactions.... etc.
- Enzyme catalysis has been studied extensively through the 20th century and continues to be an area of considerable interest.

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- Begin with introduction to classical enzyme kinetics
 - Then focus on enzyme structural properties and catalytic mechanisms enzymes.

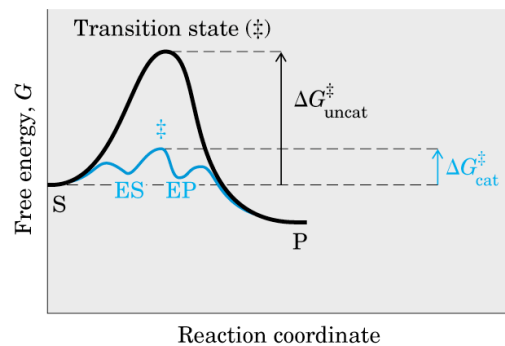
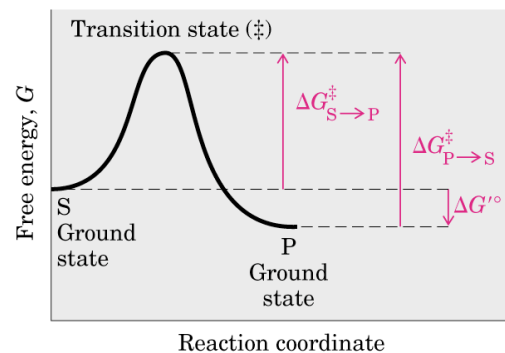
Kinetics of Enzyme Action

- Enzymes like other catalysts only increase reaction rates by lowering the activation energy for a reaction, but do not affect equilibrium between reactants and products.
- Reactions may have multiple steps and distinct reaction intermediates, with the rate-limiting step being the one with the highest activation energy.
- While the enzyme may transiently be modified in the course of the reaction, upon completion of the reaction, the enzyme is restored to its original state.



Kinetics of Enzyme Action

- Classical enzyme studies were carried out at very low enzyme concentrations (relative to substrates).
- Under these conditions, enzyme is in a steady state. The enzyme catalyzes the reaction very rapidly, but does not significantly affect the total substrate concentration.
- Such experiments provided insights into complex reaction schemes and enzyme mechanisms.
- Provided groundwork for directly studying the reactions taking place and the role of the enzyme.



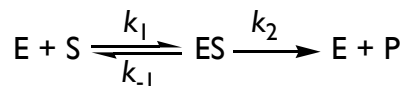
Steady-State Kinetics

Single Substrate and Product:

- Most enzymes catalyze reactions that do not occur readily in their absence.
- The velocity (v) of the reaction is the rate at which substrate disappears or product appears.
- The rate of catalysis by an enzyme is proportional to substrate concentration at low substrate concentrations, and becomes independent higher substrate concentrations.
- Catalysis occurs only after substrate binding. Substrate-enzyme complex (ES) known as the Michaelis complex.
- The substrate concentration at which reaction rate is half-maximal is known as the Michaelis constant (K_m).
- K_m is usually greater than or equal to the equilibrium constant for dissociation of ES.

When enzyme is present at a low concentration relative to substrate(s), the relative concentrations of free substrate and product change slowly, and the enzyme is in a steady state.

$$v = \frac{-d[S]}{dt} = \frac{d[P]}{dt}$$



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

$$V_{\max} = k_{\text{cat}}[E_T]$$

Michaelis-Menten equation:

$$v_0 = \frac{[S]}{K_m + [S]} V_{\max}$$

Steady-State Kinetics

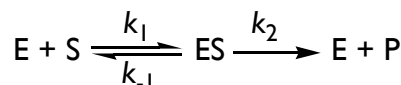
Single Substrate and Product:

- Velocity of enzyme-catalyzed reactions become independent of substrate concentration when the enzyme is saturated with substrate in the steady state.

- Maximal velocity (V_{\max}) is directly proportional to the total enzyme concentration.
- k_{cat} is the rate of breakdown of ES to form product (k_2 in equation).
- k_{cat} expressed in terms of moles of S consumed per unit time per mole enzyme is known as the turnover number of the enzyme. (can vary widely)

- The Michaelis-Menten equation relates velocity (v_0) of an enzyme-catalyzed reaction to substrate and enzyme concentration.

$$v = \frac{-d[S]}{dt} = \frac{d[P]}{dt}$$



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

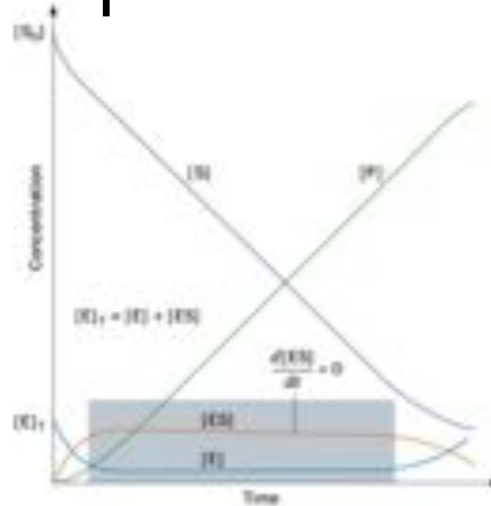
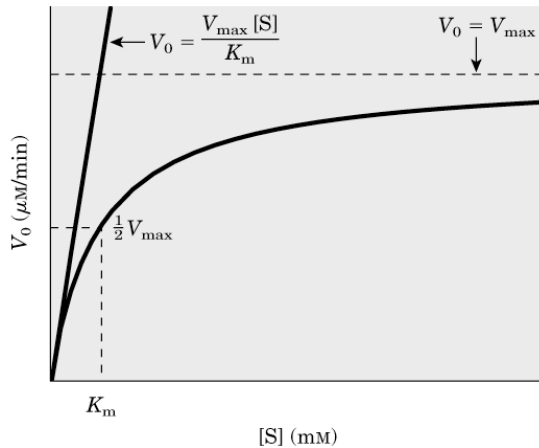
$$V_{\max} = k_{\text{cat}}[E_T]$$

Michaelis-Menten equation:

$$v_0 = \frac{[S]}{K_m + [S]} V_{\max}$$

Initial Velocity Simplification

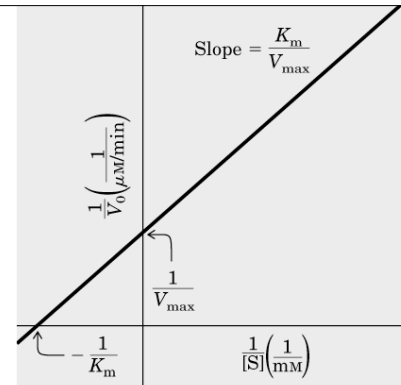
- Velocity of enzyme-catalyzed reactions become independent of substrate concentration when the enzyme is saturated with substrate in the steady state.



- Classical studies use the initial enzyme velocity when changes in total substrate concentration are negligible.
- At very low substrate concentrations, substrate binding becomes rate limiting, and most of the enzyme is not associated with substrate.

Steady-State Kinetics

- Double-reciprocal Lineweaver-Burk plots ($1/v$ vs $1/[S]$) frequently used in enzyme kinetics.
- Based on inverting the Michaelis-Menten equation.
- In a Lineweaver-Burk plot, the y intercept corresponds to $1/V_{\text{max}}$ and the x intercept $-1/K_m$. The slope of the line gives K_m/V_{max} .
- Classical studies use the initial enzyme velocity when changes in total substrate concentration are negligible.
- At very low substrate concentrations, substrate binding becomes rate limiting, and most of the enzyme is not associated with substrate.
- k_{cat}/K_m represents the apparent rate constant for substrate binding, and is an important parameter in determining specificity.
- Because enzyme and substrate cannot combine more rapidly than allowed by diffusion, k_{cat}/K_m has an upper limit of $10^9 \text{s}^{-1} \text{M}^{-1}$.



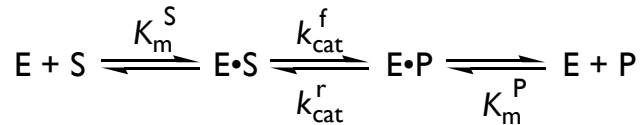
$$v_0 = \frac{[S]}{K_m + [S]} V_{\text{max}}$$

$$\frac{1}{v_0} = \left(1 + \frac{K_m}{[S]}\right) \frac{1}{V_{\text{max}}}$$

$$v_0 = \frac{k_{\text{cat}}}{K_m} [E_T][S] \quad ([S] \ll K_m)$$

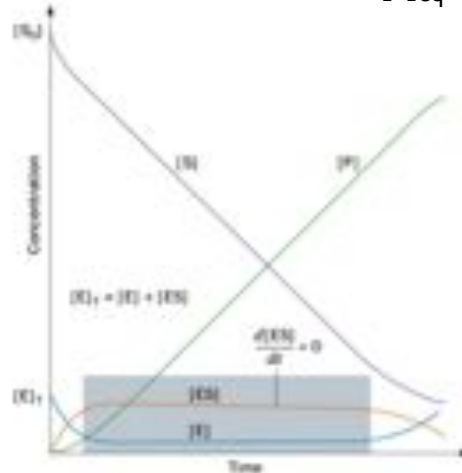
$$v_0 = \frac{k_{\text{cat}}}{K_m} [E][S] \quad (\text{at very low } [S])$$

Steady-State Kinetics



- Because enzymes do not alter the equilibrium between S and P, it must also catalyze the reverse reaction.
- An enzyme-product complex must exist, and there must be a K_m (K_m^P) for the product, as well as a k_{cat}^f .
- The values of K_m^P and k_{cat}^r are not independent of their counterparts in the forward reaction (K_m^S and k_{cat}^f). [Expressed in Haldane relationship]
- The equilibrium ratio of product to substrate on enzyme is given by k_{cat}^f / k_{cat}^r , which is different from K_{eq} (S/P equilibrium in solution) when the substrate and product have different K_m values.

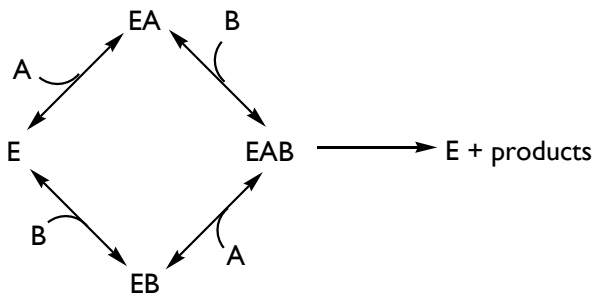
$$\frac{k_{cat}^f / K_m^S}{k_{cat}^r / K_m^P} = K_{eq} = \frac{[P]_{eq}}{[S]_{eq}}$$



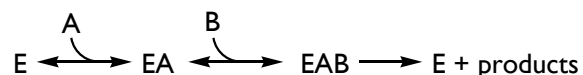
Steady-State Kinetics

Multiple Substrates and Products

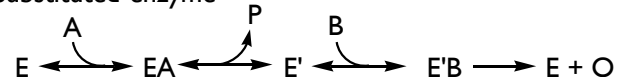
- Many enzyme catalyzed reactions involve multiple substrates and/or multiple products.
- A number of reaction mechanisms are possible, and they can be distinguished by steady-state kinetic measurements.
- Measuring the effects of independently varied substrate and product concentrations.
- Varying one of the substrates (keeping others constant) usually yields normal Michaelis-Menten kinetics.
- ...But the involvement of multiple substrates and products allows for many potential mechanisms.
- Therefore, anticipate that each substrate/product may have different impacts on the apparent K_m and V_{max} .



Large differences in affinity (A and B)

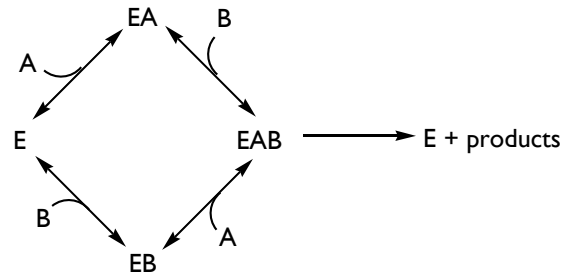


Substituted-enzyme

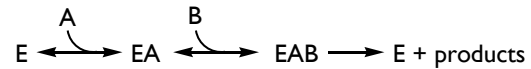


Steady-State Kinetics

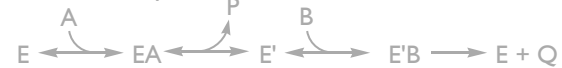
- Sequential reactions are those in which all the involved substrates are bound by the enzyme before the first product is formed.
- The order of binding or release of products may be essentially random.
- Binding of one substrate/product may alter the affinity for others.
- In cases where there exists a large difference in affinity between substrates, ordered substrate binding may be observed.



Large differences in affinity (A and B)

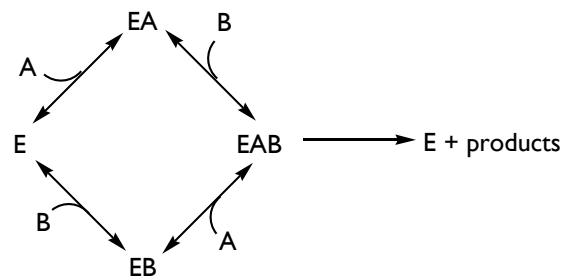


Substituted-enzyme



Steady-State Kinetics

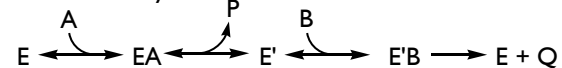
- An alternative strategy involves the formation and release of one or more products by the enzyme before the other substrates bind. (substituted-enzyme, ping-pong or double-displacement mechanism)
- Usually occurs when the reaction involves the transfer of part of one substrate to another in forming the product.
- Group to be transferred is usually held transiently by an intermediate form of the enzyme (E').
- E' can often be isolated by adding substrate A in the absence of other substrates.
- Water may be one of the substrates/products.



Large differences in affinity (A and B)



Substituted-enzyme



Catalytic Power of Enzymes

- Enzymes can enhance reaction rates by 5-17 orders of magnitude.
- Enzymes are very discriminating in their choice of substrate... and products.
- Where does the energy and the specificity come from?
 - One answer is the rearrangement of covalent bonds during the reaction.
 - Another involves noncovalent interactions between the enzyme and substrate.

table 8-4

Relationship between K'_{eq} and $\Delta G'^{\circ}$
(see Eqn 8-3)

K'_{eq}	$\Delta G'^{\circ}$ (kJ/mol)
10^{-6}	34.2
10^{-5}	28.5
10^{-4}	22.8
10^{-3}	17.1
10^{-2}	11.4
10^{-1}	5.7
1	0.0
10^1	-5.7
10^2	-11.4
10^3	-17.1

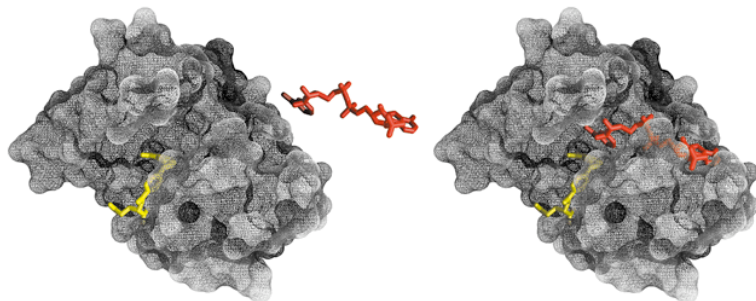
table 8-5

Some Rate Enhancements Produced by Enzymes

Cyclophilin	10^5
Carbonic anhydrase	10^7
Triose phosphate isomerase	10^9
Carboxypeptidase A	10^{11}
Phosphoglucomutase	10^{12}
Succinyl-CoA transferase	10^{13}
Urease	10^{14}
Orotidine monophosphate decarboxylase	10^{17}

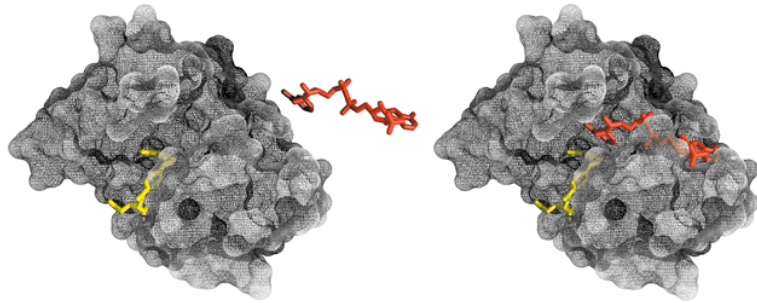
Binding Energy and Weak Interactions

- Energy released upon formation of the ES complex is called the binding energy.
- Binding energy contributes in lowering activation energy of the catalyzed reaction.
- Weak interactions between enzyme and substrate contribute to substrate specificity and catalysis.
- The active site is structurally complementary to substrate(s), but not completely complementary.

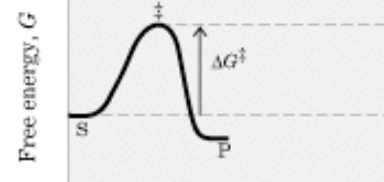
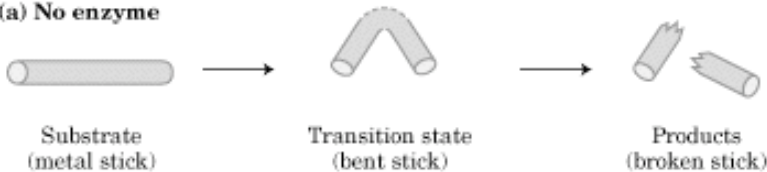


Binding Energy and Weak Interactions

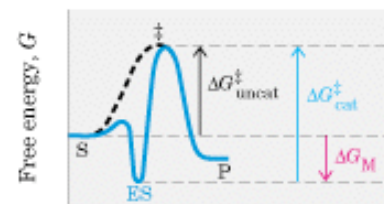
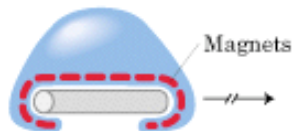
- The active site is structurally complementary to substrate(s), but not completely complementary.
- Perfect substrate complementarity would result in an extremely stable ES complex.
- Complementation of the transition state, however, promotes catalysis.
- The energy required to destabilize the substrate is offset by favorable interactions with the transition state.



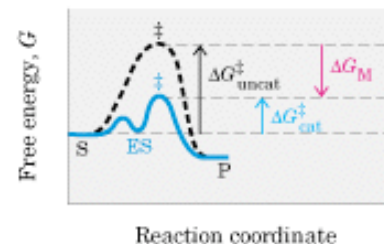
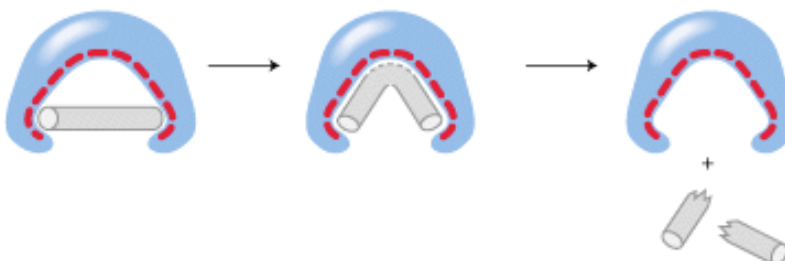
(a) No enzyme



(b) Enzyme complementary to substrate



(c) Enzyme complementary to transition state



Catalytic Strategies

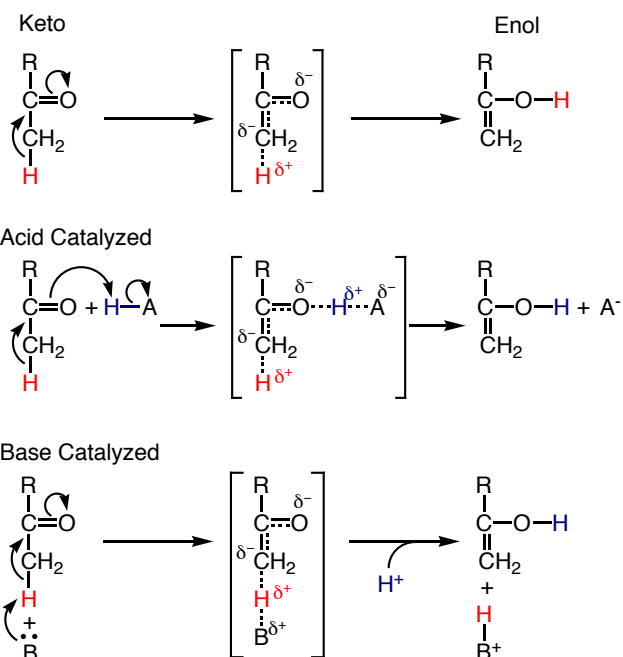
Enzymes use a number of strategies to accelerate reactions:

1. Acid-Base catalysis
2. Covalent catalysis
3. Metal Ion catalysis
4. Electrostatic effects
5. Proximity and Orientation
6. Preferential binding of transition state

Reactions on the Enzyme (catalytic mechanisms)

Acid-Base Catalysis

- Acid catalysis: donation of a proton by an acid group on the enzyme lowers the free energy of the transition state.
- Base catalysis: abstraction of a proton by a basic group of the enzyme lowers the free energy of the transition state.
- Common method used to increase reactivity.
- Proteins have a diverse range of side chain functional groups that can be used in acid-base catalysis (Arg, Asp, Cys, Glu, His, Lys and Tyr).
- Active site may contain many of these groups arranged around the bound substrate.
- Keto-enol tautomerization and RNase A good examples.

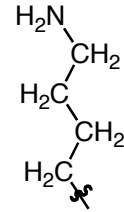
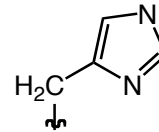
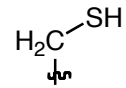
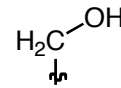


Reactions on the Enzyme (catalytic mechanisms)

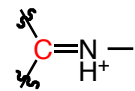
Covalent Catalysis

- Transient formation of covalent bonds between an enzyme and substrate can be used to accelerate a reaction.
- Frequently utilizes nucleophilic groups on the enzyme to form bonds with electrophilic centers on the substrate.
- Common nucleophiles in proteins are: HO-, HS-, H₂N- and unprotonated imidazole groups.
- Generally proceeds in three phases:
 - Nucleophilic reaction between the enzyme and the substrate.
 - Leads to withdrawal of electrons from the reaction center.
 - Elimination of the nucleophilic group provided by enzyme.
- Common electrophiles include groups with unfilled orbitals and are bonded to electronegative atoms such as oxygen (i.e. carbonyl carbon or carbon atom in Schiff base)

Nucleophiles



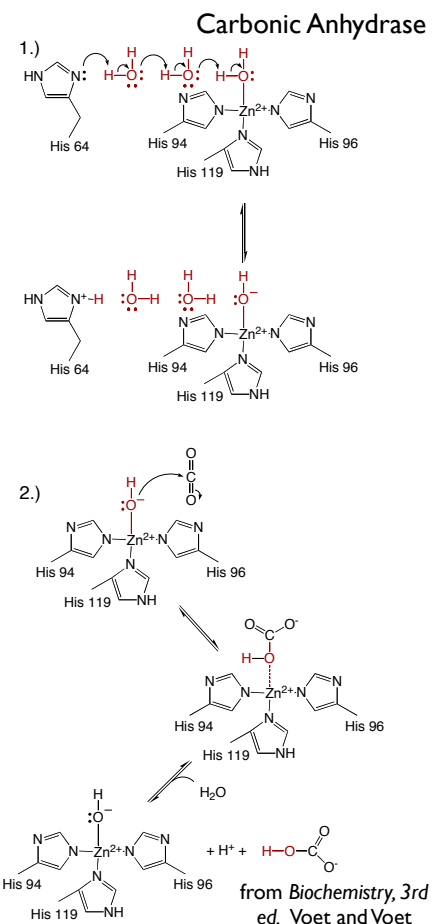
Electrophilic Groups



Reactions on the Enzyme (catalytic mechanisms)

Metal-ion Catalysis

- Nearly 1/3 of known enzymes require a bound metal ion of catalytic activity.
- Two classes of enzymes requiring metal ions:
 - Metalloenzymes utilize tightly bound metal ions such as Fe²⁺/Fe³⁺, Cu⁺/Cu²⁺, Zn²⁺, Mn²⁺ and Co²⁺ to catalyze reactions.
 - Metal-activated enzymes require loosely bound metal ions such as Na⁺, K⁺, Mg²⁺ and Ca²⁺.
- The bound metal ion may be used for:
 - Binding and proper orientation of substrates.
 - Mediate redox reactions through reversible changes in the metal ion oxidation state.
 - Electrostatic shielding/stabilization of negative charges.



Reactions on the Enzyme (catalytic mechanisms)

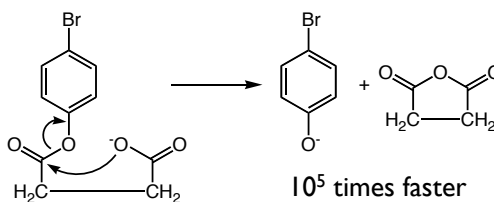
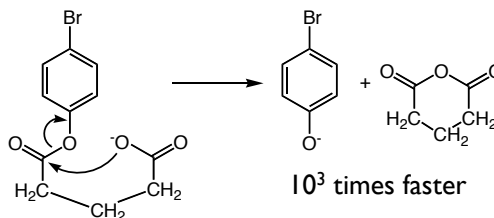
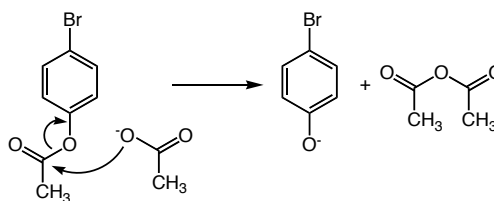
Electrostatic Catalysis

- Substrate binding is often mediated by charged and polar groups positioned in an active site which is rich in non-polar side chains.
- The abundance of hydrophobic groups in the active site effectively shifts the pK_a of ionizable groups in the active site and increases the potential for electrostatic interactions and catalysis.
- Direct involvement in catalysis hard to prove, but likely plays a role in drawing in substrate and stabilizing the transition state (“circe” effect).
- The frequent presence of charged and polar groups in enzyme active sites suggests their involvement in the catalytic process.

Catalysis via Proximity and Orientation

- Enzymes catalytic mechanisms resemble those of organic reactions, but they are far more efficient than would be expected.
- Two important parameters affecting catalysis are the proximity and orientation of the reacting species.
- For a reaction to occur, reactants must come together in the correct spatial relationship.
- An enzyme active site brings substrates together in close proximity and specific orientation significantly increasing their effective concentrations.

Reactions on the Enzyme (catalytic mechanisms)

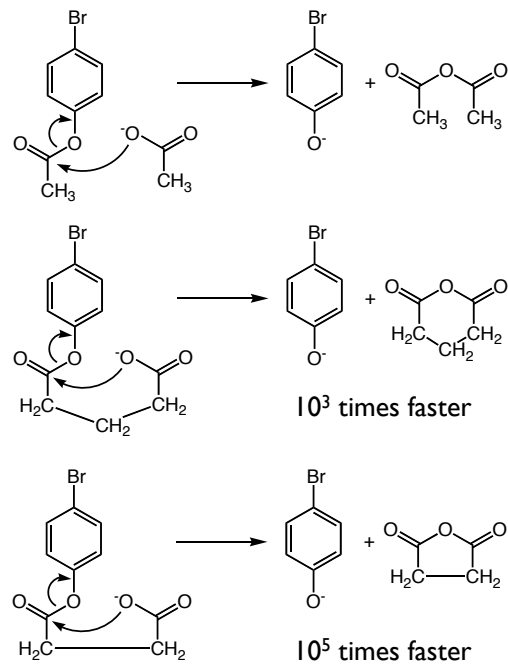


Enzymes achieve reaction rate increases that cannot be accounted for based solely on proximity and orientation.

Reactions on the Enzyme (catalytic mechanisms)

Catalysis via Proximity and Orientation

- Binding and orienting the substrates in the proper spatial arrangement and reducing internal motions promotes reaction.
- Impact of proximity demonstrated in experiments involving the non-enzymatic hydrolysis of *p*-bromo-phenylacetate.
 - First study intermolecular reaction.
 - Compare to intramolecular reactions in which the bridging segment exerts increasing conformation restriction.



Enzymes achieve reaction rate increases that cannot be accounted for based solely on proximity and orientation.

Reactions on the Enzyme

- In enzyme catalyzed reactions, the reaction occurs with the reactants complexed with the enzyme in an essentially unimolecular process.
- The greater the number of reactants involved the greater the advantage provided by enzymatic high effective concentration and substrate orientation in active site.
- At first glance, would not appear to be a factor for unimolecular reactions.

But... virtually every chemical reaction can be catalyzed in solution, and the observed rate of the reaction depends on the concentration of catalysts (i.e. nucleophiles, electrophiles, acid-base catalysis).

Enzymes position numerous functional groups in the active site that could potentially play roles as nucleophilic and acid-base catalysts. Result in very high effective concentrations relative to the substrates.

Reactions on the Enzyme

● **Relative to the uncatalyzed reactions:**

- Acid-base and covalent catalytic mechanisms can be estimated to increase reaction rates by one or two orders of magnitude.
- Orientation and proximity can account for up to 10^8 fold increase in reaction rate (often much less).
- Some enzymes achieve 10^{14} - 10^{17} fold increases in rates of reaction.

Reactions on the Enzyme

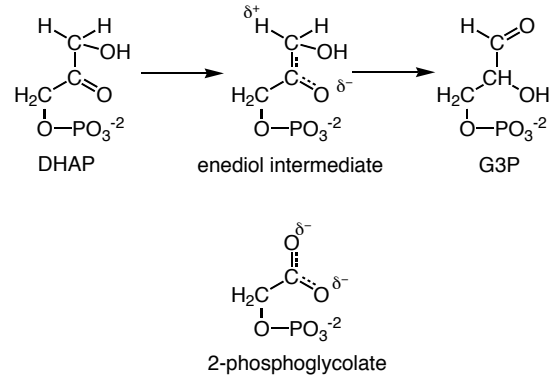
Transition State Binding

- One of the most important catalytic mechanisms employed by enzymes is preferential binding and stabilization of the reaction transition state.
- Enzymes believed to strain/distort substrates into transition state geometries through binding sites that optimally bind the transition state.
- By this mechanism, a 10^6 fold rate increase would require a 10^6 fold enhancement in transition state binding relative to substrate (~ 8.1 Kcal/mol at RT).
- Very large rate enhancements could be achieved with the formation of a few new bonds/interactions between the transition state and the enzyme.

Reactions on the Enzyme

Transition State Binding

- Transition state binding may also contribute to enzyme specificity.
 - While molecules similar to the substrate may be bound by the enzyme, they are incapable of achieving the transition state, and no reaction occurs.
- Supported by the observation that transition state analogs bind tightly to the enzyme active site and make good inhibitors of enzyme activity.
 - 2-phosphoglycolate is an inhibitor of triose phosphate isomerase (normal substrate is dihydroxyacetone phosphate and product is glyceraldehyde-3-phosphate).
 - The reaction involves general acid-base catalysis and formation of enediol/enediolate intermediates.
 - 2-phosphoglycolate reproduces the partial charge on oxygen in the enediol intermediate and the transition state.



Coenzymes and Cofactors

- The Functional groups provided by amino acid side chains can readily participate in acid-base reactions, certain types of transient covalent bonds and electrostatic interactions.
- For the catalysis of oxidation-reduction reactions and many types of group transfer reactions enzymes frequently rely on coenzymes and cofactors.
- Cofactors may be metal ions (i.e. Fe²⁺/Fe³⁺, Cu⁺/Cu²⁺, Zn²⁺, Mn²⁺ and Co²⁺) or small organic molecules such as NAD⁺.
- Some cofactors are transiently associated with the enzyme and can be thought of as co-substrates (NAD⁺). Other cofactors (prosthetic groups) are tightly bound (Heme), sometimes by covalent bonds (considered part of the enzyme).
- Some cofactors are tightly bound at the enzyme active site and directly interact with the substrate, others are loosely associated with protein surface and have remote roles in enzyme function.
- Coenzymes are chemically changed during the reaction (i.e. NAD⁺ to NADH), and must be regenerated before they can participate in a second reaction.
- An enzyme without its cofactor is called an “apoenzyme”, with its cofactor it is known as a “holoenzyme”.

table 8-1**Some Inorganic Elements That Serve as Cofactors for Enzymes**

Cu^{2+}	Cytochrome oxidase
Fe^{2+} or Fe^{3+}	Cytochrome oxidase, catalase, peroxidase
K^+	Pyruvate kinase
Mg^{2+}	Hexokinase, glucose 6-phosphatase, pyruvate kinase
Mn^{2+}	Arginase, ribonucleotide reductase
Mo	Dinitrogenase
Ni^{2+}	Urease
Se	Glutathione peroxidase
Zn^{2+}	Carbonic anhydrase, alcohol dehydrogenase, carboxypeptidases A and B

table 8-2**Some Coenzymes That Serve as Transient Carriers of Specific Atoms or Functional Groups***

Coenzyme	Examples of chemical groups transferred	Dietary precursor in mammals
Biotin	CO_2	Biotin
Coenzyme A	Acyl groups	Pantothenic acid and other compounds
5'-Deoxyadenosylcobalamin (coenzyme B_{12})	H atoms and alkyl groups	Vitamin B_{12}
Flavin adenine dinucleotide	Electrons	Riboflavin (vitamin B_2)
Lipoate	Electrons and acyl groups	Not required in diet
Nicotinamide adenine dinucleotide	Hydride ion ($:\text{H}^-$)	Nicotinic acid (niacin)
Pyridoxal phosphate	Amino groups	Pyridoxine (vitamin B_6)
Tetrahydrofolate	One-carbon groups	Folate
Thiamine pyrophosphate	Aldehydes	Thiamine (vitamin B_1)

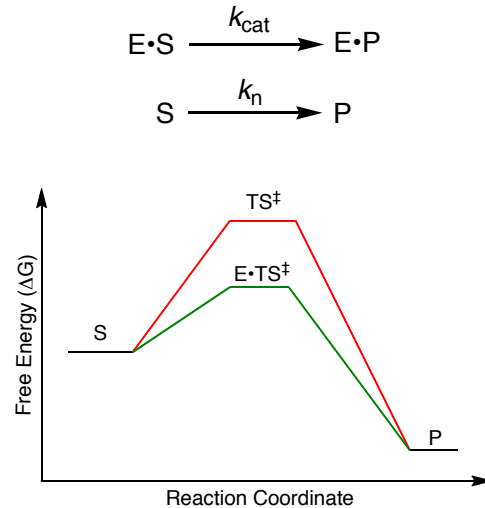
*The structure and mode of action of these coenzymes are described in Part III of this book.

Enzyme Catalysis

- The rate of a reaction depends on the relative free energies of the reactants, any stable intermediates and the transition state.
- A catalyst can increase the rate of a reaction by many mechanisms:
 - Destabilization of reactants.
 - Stabilization of the transition state.
 - Or alter the reaction mechanism.
- Magnitude of rate enhancement given by the relative rates of the catalyzed (k_{cat}) and the uncatalyzed (k_n) reactions (enhancement ranges from 10^6 - 10^{14}).
- Comparison is straightforward for unimolecular reactions, but in reactions involving 2 or more reactants comparison is more complicated.

Rate constants for reactions involving 2 or more reactants are second order or higher, but the enzyme catalyzed reaction k_{cat} is always first order.

In enzyme catalyzed reactions, the reaction occurs with the reactants complexed with the enzyme in an essentially unimolecular process.

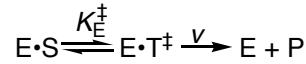
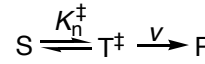
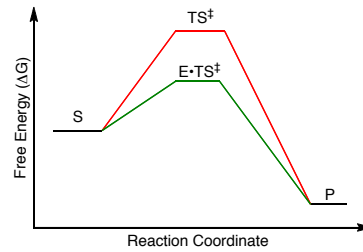


Enzyme Kinetics

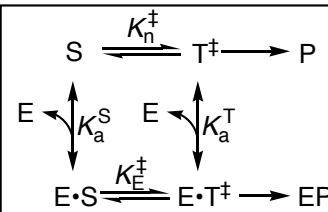
- The presence of multiple substrate molecules and/or catalytic groups in an enzyme-substrate complex suggests that the catalyzed reactions may be somewhat different from comparable reactions in solution.
- Such complex reactions in solution become entropically problematic (such higher order encounters in solution are improbable).
- Interaction of multiple groups in the enzyme-substrate complex are essentially unimolecular and are not burdened by such an entropic restriction on encounters.

Enzyme Kinetics

- Enzyme catalyzed reactions proceed more rapidly primarily because the difference in the free energies of the substrate (S) and the transition state (T^\ddagger) is not so great when bound to the enzyme.
- Could be the result of destabilization of the substrate or stabilization of the transition state.
- Most enzymes catalyze reactions by lowering the free energy of the transition state.
- The enzyme should bind the transition state more tightly than the substrate by a factor corresponding to the increase in reaction rate.
- Enzymes are not expected to have extremely high affinities for their substrates.



$$\frac{k_{\text{cat}}}{k_n} = \frac{K_E^\ddagger}{K_n^\ddagger}$$



$$\frac{K_a^T}{K_a^S} = \frac{K_E^\ddagger}{K_n^\ddagger} = \frac{k_{\text{cat}}}{k_n}$$

Substrate Specificity and Induced Fit

- Koshland proposed that substrate binding that induces a change in the protein conformation in order to produce the active form of the enzyme (induced fit) could account for the extreme substrate specificity demonstrated by some enzymes.
- Hexokinase which catalyzes glucose phosphorylation provides a good example.
 - Have “lobed” structure... common to other kinases.
 - Binding of glucose drives the two domains of hexokinase to rotate by 12° relative to each other.
 - As a result the bound glucose is almost entirely shielded from water.
 - ~50 fold increase in affinity for ATP. Also appears to activate the enzyme for ATP hydrolysis.
 - ATP binding likely results in additional conformational changes to facilitate transfer of the phosphoryl group to glucose.
- The induced structural changes in the enzyme associated with substrate binding may be required for the enzyme to position the required functional groups appropriately, constraining the transition state and excluding water.
 - Such changes are observed in tyrosyl tRNA synthetase.

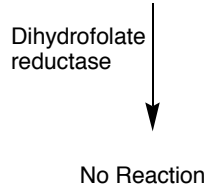
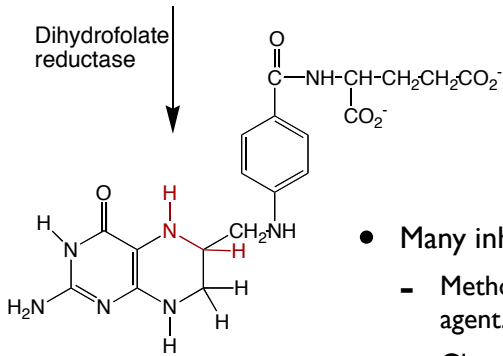
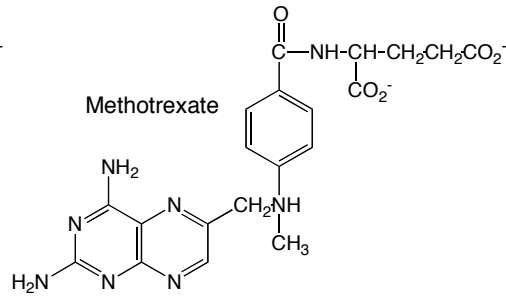
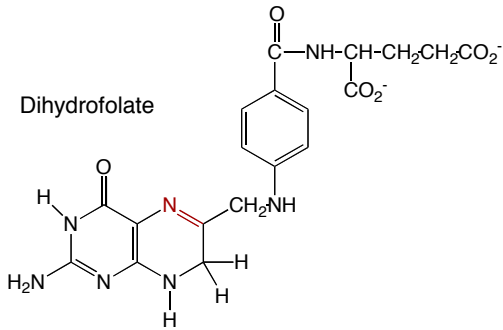
Substrate Specificity and Induced Fit

- While the enzyme must be able to bind to substrates and the transition state it must also be able to release the products.
- The release of product is often observed to be the rate-limiting step.
- Structural changes in the enzyme that occur in the course of catalysis result in the state of the enzyme following release of product to be different from the enzyme at the start of the reaction.

Enzyme Inhibition and Regulation

- An organism must be able to regulate the catalytic activity of its enzymes.
- Two general schemes:
 - ★ Controlling enzyme availability by controlling expression and degradation.
 - ★ Controlling activity:
 - ❖ Reversible covalent modification of enzymes resulting in inactivation (often phosphorylation of Ser residues).
 - ❖ Expression and storage of enzymes in nonfunctional proforms (zymogens) that require proteolytic processing for activation.
 - ❖ Direct control of enzyme activity through the use of inhibitors and enhancers that directly affect the enzyme.

Inhibition

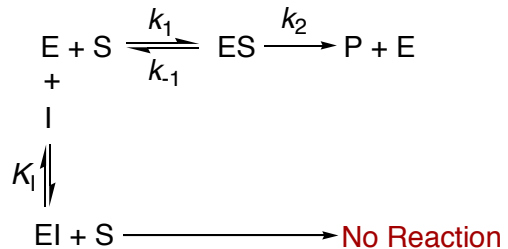


- Substances that reduce the activity of an enzyme are known as inhibitors.
- Inhibitors can alter the binding of a substrate with the enzyme or they can affect the turnover number of an enzyme.

- Many inhibitors are used in medicine:
 - Methotrexate is an inhibitor of DHFR and is a potent anticancer agent.
 - Gleevec is a tyrosine kinase inhibitor and is used to treat some forms of leukemia.

Competitive Inhibition

- A competitive inhibitor is a substance that competes directly with the substrate for binding to the active site.
- Structurally, competitive inhibitors generally resemble the natural substrate, but are unreactive.
- Binding of the inhibitor is reversible and is in rapid equilibrium.
- Competitive inhibitors effectively reduce the concentration of free enzyme.
- The Michaelis-Menten equation is modified to account for the effects of competitive inhibition.
 - ★ The primary impact of competitive inhibition is on the K_M .
 - ★ A competitive inhibitor does not change the turnover number.
 - ★ Makes K_M appear larger than it actually is.
- A competitive inhibitor is most effective at low substrate concentrations.



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

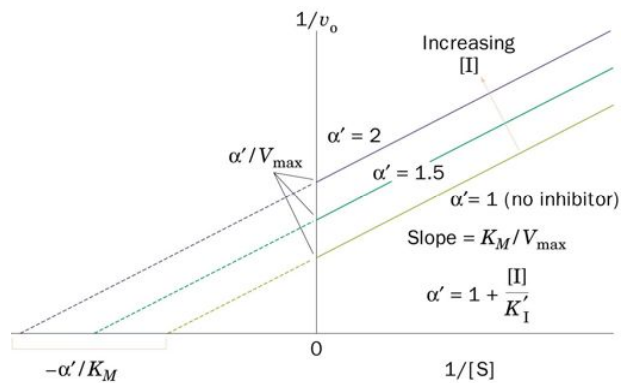
$$v_0 = \frac{V_{\max}[S]}{\alpha K_M + [S]}$$

$$\alpha = (1 + [I]/K_I)$$

Uncompetitive Inhibition

- The double reciprocal form of the adjusted Michaelis-Menten equation indicates that inhibition does not affect the slope of a Lineweaver-Burke plot, but does affect the Y-intercept.

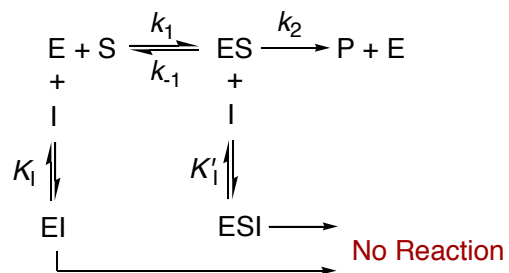
$$\frac{1}{v_0} = \left(\frac{K_M}{V_{\max}} \right) \frac{1}{[S]} + \frac{\alpha'}{V_{\max}}$$



from *Biochemistry*, 3rd ed. Voet and Voet

Mixed Inhibition

- Mixed inhibition occurs when the inhibitor binds both the free enzyme and the enzyme-substrate complex. (sometimes known as noncompetitive inhibition.)
- In adjusting the Michaelis-Menten equation to accommodate mixed inhibition, both the K_M and $[S]$ terms in the denominator are modified.
- Such inhibitors are effective at both high and low substrate concentrations.



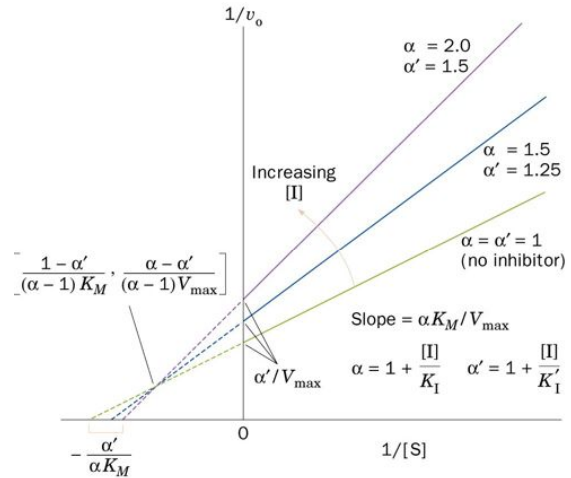
$$K_1 = \frac{[E][I]}{[EI]} \quad K'_1 = \frac{[ES][I]}{[ESI]}$$

$$v_0 = \frac{V_{\max}[S]}{\alpha K_M + \alpha'[S]}$$

Mixed Inhibition

- The double reciprocal of Michaelis-Menten adjusted for mixed inhibition indicates that the inhibitor affects both the slope of the Lineweaver-Burk plot and the Y-intercept.
- Plots at varied inhibitor concentrations intersect at a point to the left of the Y-axis.

$$\frac{1}{v_0} = \left(\frac{\alpha K_M}{V_{\max}} \right) \frac{1}{[S]} + \frac{\alpha'}{V_{\max}}$$



from *Biochemistry, 3rd ed.* Voet and Voet

Impact of Various forms of Inhibition

Type of Inhibition	V_{\max} (apparent)	K_M (apparent)
No Inhibition	V_{\max}	K_M
Competitive Inhibition	V_{\max}	αK_M
Uncompetitive Inhibition	V_{\max}/α'	K_M/α'
Mixed Inhibition	V_{\max}/α'	$\alpha K_M/\alpha'$

Enzyme Mechanisms (Examples)

- Serine Proteases: hydrolyze peptide bonds, via the formation of a covalent substrate-enzyme intermediate.
- Tyrosyl tRNA Synthetase: catalyzes the charging of the appropriate tRNA with a Tyr residue.
- **tRNA Synthetases are not on Test 2 (Nov. 5), but will be included on the final exam.**

Serine Proteases

- Proteolytic enzymes that cleave peptide bonds (endopeptidases):
 - Chymotrypsin cleaves peptide bonds C-terminal to amino acids with aromatic side chains (Trp, Phe and Tyr).
 - Trypsin cleaves peptide bonds C-terminal to amino acids with basic side chains (Arg and Lys).
 - Elastase cleaves peptide bonds C-terminal to amino acids with small hydrophobic side chains (Gly, Ala and Val).
- Enhance bond hydrolysis at least 10^9 fold.
- Water ultimately is added across the peptide bond, but a covalent acyl-enzyme intermediate is formed.
- The reaction can be separated into two parts, formation of the acyl-enzyme and hydrolysis by water.

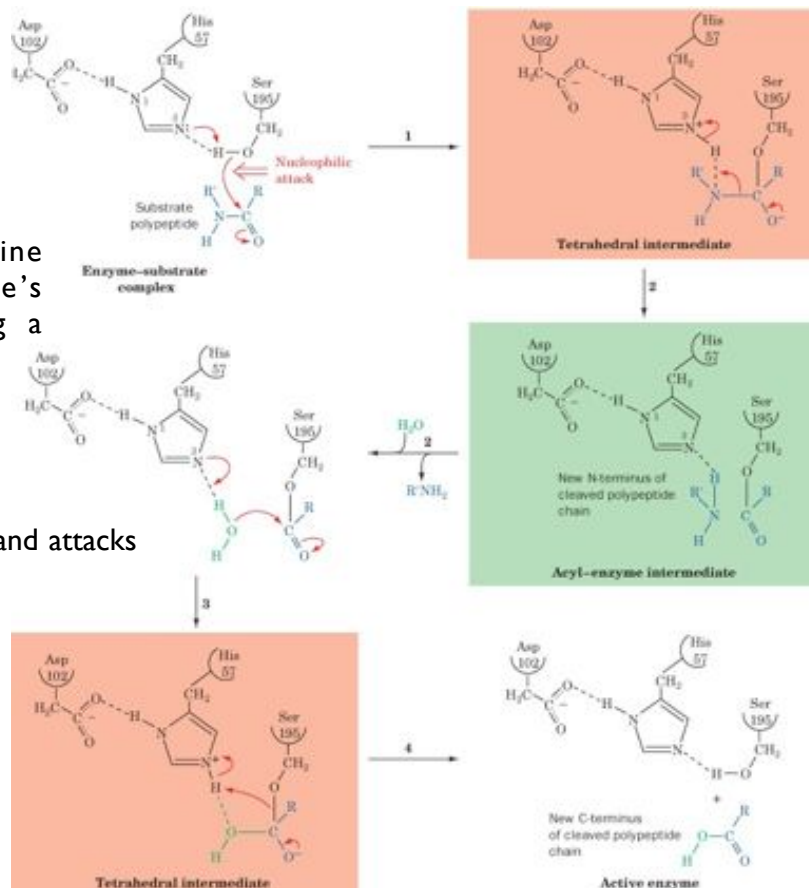
Chymotrypsin and the Catalytic Triad

- Trypsin, Chymotrypsin and Elastase have similar structures.
- All utilize and active site Ser and catalytically essential His residue.
- Folded structures incorporate two domains w/ extensive regions of antiparallel β -sheets.
- Domain interface forms binding cleft.



The catalytic mechanism occurs in four steps:

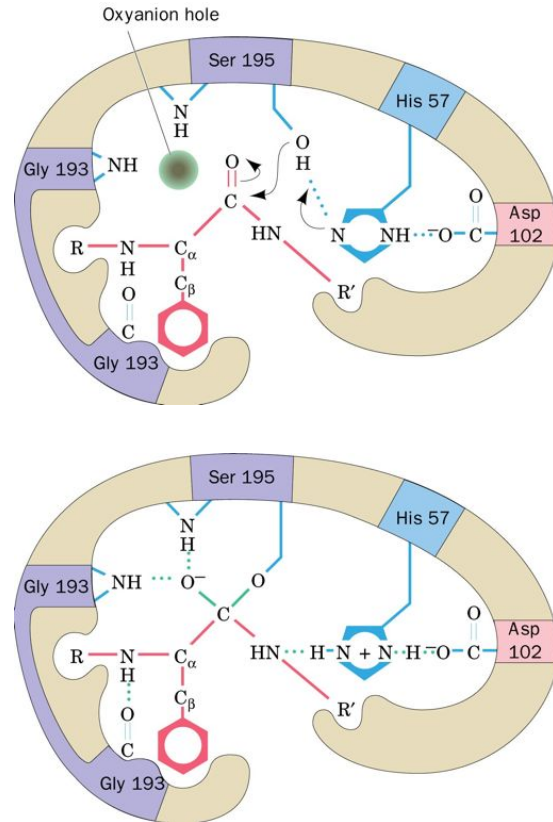
1. The nucleophilic serine attacks the peptide's carbonyl group, forming a tetrahedral intermediate
2. The tetrahedral intermediate decomposes to form an acylated enzyme
3. A water molecule binds and attacks the acylated enzyme
4. The tetrahedral intermediate decomposes to generate free enzyme, releasing the second peptide product



Serine Protease Mechanism

Serine Protease Mechanism

- Upon formation of the tetrahedral intermediate, the carbonyl oxygen moves deeper into the active site and occupies an oxyanion hole.
- Two new hydrogen bonds are formed between the enzyme and the intermediate.
- A third new hydrogen bond is formed between the enzyme and the backbone NH group of the residue preceding the scissile peptide.



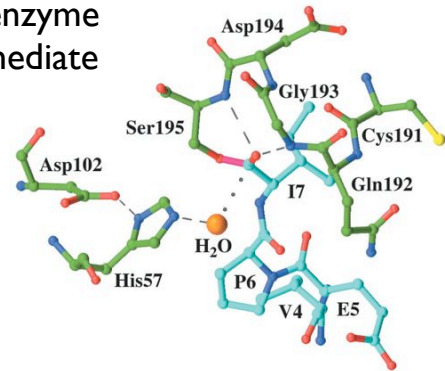
Intermediate Stabilization

- Attack by the serine hydroxyl forms a negatively charged intermediate which moves into the oxyanion hole.
- This intermediate is stabilized by hydrogen bonds from two amides of the peptide backbone.
- A new hydrogen bond is formed only in the transition state between an enzyme backbone carbonyl group and a substrate amide. This extra interaction further stabilizes the transition state.
- Interactions between the enzyme and substrate are maximized in the transition state and they decrease once the acyl-enzyme is formed.

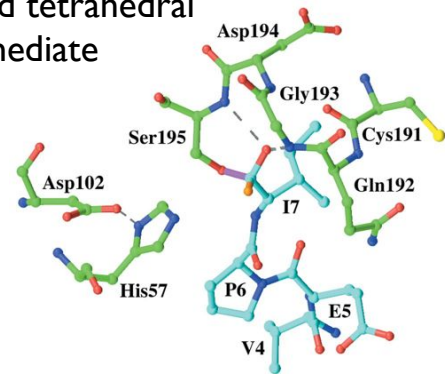
Serine Protease Mechanism

- The X-ray structure of elastase with a complexed seven residue peptide (BCM7 = YPFVEPI) (@ pH5) has been solved.
- enzyme intermediate with a covalent bond between Ser-195 and the peptide C-terminal acyl group.
- Moreover, a bound water found in the active site (~3.1Å from BCM7's C-terminal C atom) was properly oriented for direct attack on the carbonyl bond of the intermediate
- Activation of the BCM7-enzyme crystal for one minute (by soaking in pH9 buffer) resulted in formation of a tetrahedral intermediate.

Acyl-enzyme intermediate



Second tetrahedral intermediate



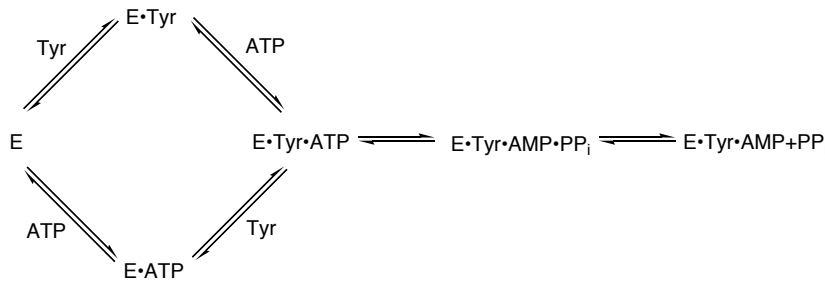
Aminoacyl tRNA Synthetase



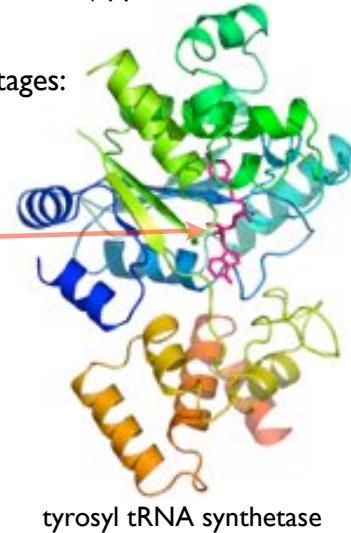
- Attachment of amino acids to the correct t-RNA molecule is an essential step for translation and protein synthesis.
- Aminoacyl tRNA synthetases generate activated amino acids and mediate their transfer to the 3' end of the acceptor arm of the appropriate t-RNA molecule. (Acceptor arm contain a conserved -CCA-3' sequence)
- They are amino acid specific and pair amino acid with correct tRNA molecule.
- An error in this process could be disastrous. Therefore, the error rate for most aminoacyl tRNA synthetases is extremely low.
- Aminoacyl tRNA synthetases link amino acids to the 3' end of the acceptor arm of tRNA molecules. Characterized by a conserved -CCA-OH 3' sequence.
- Aminoacyl t-RNA synthetases are grouped into two families (Class I and Class II).
 - Class I synthetases have utilize a Rossmann fold motif in binding ATP/AA-AMP. Class II enzymes utilize an antiparallel β -sheet.
 - Most Class I synthetases are monomeric (Tyr tRNA synthetase is an exception). Class II synthetases more commonly dimeric.
 - Class I enzymes bind at the minor groove of the acceptor stem. Class II synthetases bind the major groove.

Tyrosyl tRNA Synthetase

- Tyrosyl tRNA synthetase is a dimeric protein with each monomer being predominantly α -helical with a central 6-stranded β -sheet core (contains Rossmann fold).
- The C-terminal portion tends to be disordered in the absence of tRNA.
- The error rate for most aminoacyl tRNA synthetases is very low. (in the case of Tyr tRNA synthetase erroneous incorporation of Phe is on the order of 1 in 5×10^4).
- Tyrosyl tRNA synthetase binds Tyr with a K_D of $\sim 2 \times 10^{-6}$ M (approx. 5 orders of magnitude greater than for Phe).
- Charging of tRNA with Tyr can be broken down into two stages:
 - Tyr activation with formation of Tyr-AMP.
 - Transfer of Tyr to specific tRNA molecule.

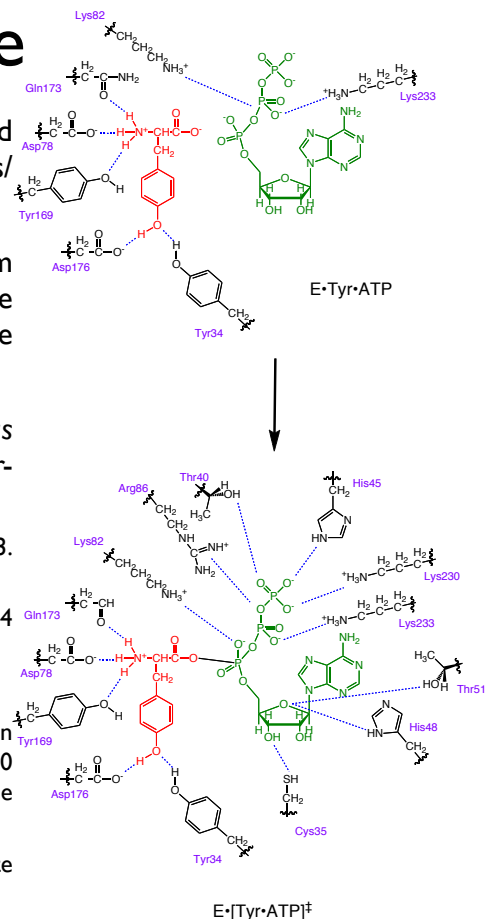


Bound Tyr-adenylate



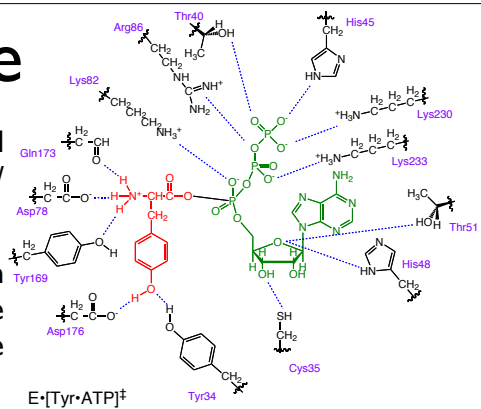
Tyrosyl tRNA Synthetase

- ATP and Tyr intermediates have been isolated, and interactions between tRNA synthetase and substrates/transition state have been investigated.
- Substrate binding to Tyr tRNA synthetase is a random process, but the enzyme binding of Tyr appears to be much stronger than the binding of ATP. (results in some ordering of addition, Tyr usually binds before ATP)
- Structure of Tyr tRNA synthetase from *Bacillus stearothermophilus* has been solved (with bound Tyr-AMP).
 - The α -amino group of Tyr is bound by Asp78, Tyr169 and Glu173. (contributes to affinity but not catalysis).
 - Side chain phenol -OH group forms hydrogen bonds with Tyr34 and Asp176 (contributes to affinity and specificity).
 - Cys35, Thr51 and His48 appear to interact with the ribose ring.
 - Mutation of Thr40 and His45 resulted in significant decreases in rates of Tyr-Amp formation. (Thr40 to Ala resulted in a ~ 7000 fold drop. His45 to Gly resulted in ~ 200 fold decrease. Double mutant $\sim 10^5$ fold decrease.)
 - Thr40 and His45 appear to be critical to transition state stabilization through interactions with the PP_i group.

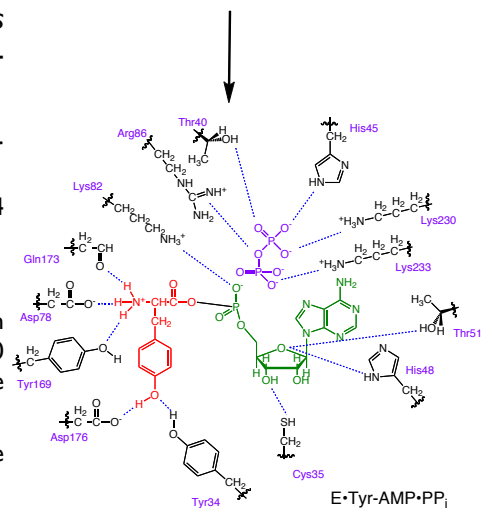


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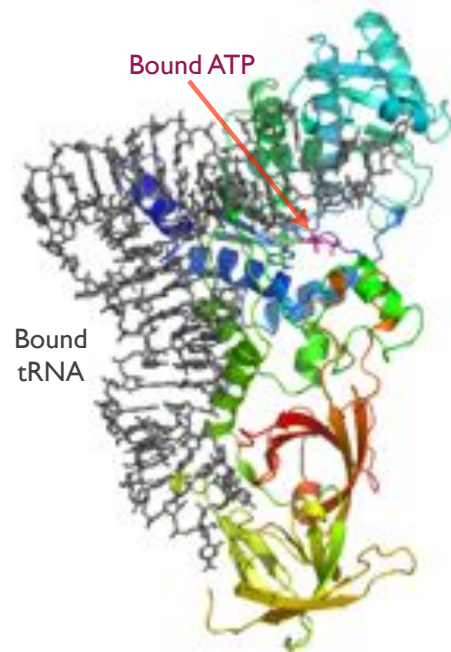


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Tyrosyl tRNA Synthetase

- Structure of Tyr tRNA synthetase from *Bacillus stearothermophilus* has been solved (with bound Tyr-AMP).
- Enzyme binding pocket is designed to constrain the substrate molecules in an extended geometry which lowers the activation energy for the formation of the Tyr-AMP complex intermediate. (true for aminoacyl tRNA synthetases in general).
- Involves two motifs with sequences of His-Ile-Gly-His (HIGH) and Met-Ser-Lys (MSK)-(characteristic in Class I aminoacyl tRNA synthetases).
- These groups interact with carboxyl group of the bound amino acid and the α -phosphate group of ATP.
- The phosphate group acts as a leaving group.
- Less detail is known about the transfer of the amino acid to tRNA.
- Crystal structures of other Class I (and Class II) synthetases provide insights.
 - Suggest that the tRNA is bound in a tight complex, positioning the acceptor arm (C₇₄C₇₅A₇₆) in close proximity of the ATP in the active site.
 - The arrangement observed in structure of Gln tRNA synthetase with bound Gln-adenylate analogs and tRNA.



Glutamyl tRNA synthetase with bound tRNA and ATP

Tyrosyl tRNA Synthetase

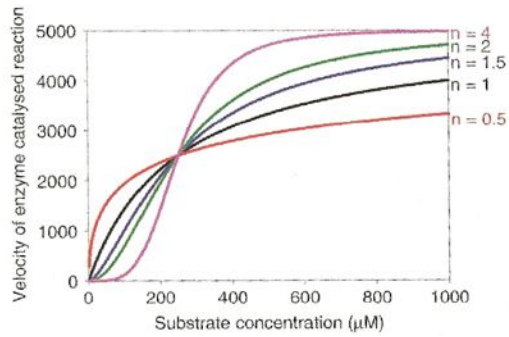
- High fidelity of tRNA aminoacylation not only the result of specificity in amino acid binding.
- In the case of Tyr tRNA synthetase the binding preferential binding Tyr (over Phe) can account for much of the specificity exhibited by the enzyme (the enzyme binds Tyr 1×10^3 times more tightly than it does Phe).
- Depending on the substrate, there are limits to the specificity than can be achieved solely through preferred substrate binding.
 - In charging tRNA, Ile tRNA synthetase must differentiate between Ile and Val, which differ by only a methylene.
 - Ile tRNA synthetase binds Ile with ~ 100 - 200 fold greater affinity than it does Val.
 - Would translate into an error rate of 2-5%, but the actual error rate is only 0.03%.
- Specificity is improved by incorporating an editing mechanism, which results in hydrolysis of the incorrect adenylate directly or after transfer to the tRNA.
- The rate of hydrolysis of the correct adenylate or charged-tRNA is much slower.

Allosteric Control

- The activity of enzymes can be regulated by means of interactions with effector molecules that are unrelated to substrate.
- These molecules regulate and modulate enzyme activity through allosteric interactions.
- They bind to sites on the protein that are distinct from the enzyme active site.
- Allosteric enzymes are multimeric proteins incorporating at least two subunits/ polypeptide chains (quaternary structure) with multiple catalytic and binding sites.
 - ★ They can be composed of identical or combinations of non-identical subunits.
 - ★ In enzymes composed of identical subunits, each polypeptide chain contains at least one catalytic site and regulatory site.
 - ★ In enzymes assembled from non-identical subunits the catalytic and regulatory sites may be located on different polypeptide chains.
- Binding of effector molecules at sites that are distinct from the active site favor the active or inactive conformations of the enzyme.
- Effector molecules are frequently components of the metabolic pathway, and their presence serves to stimulate or inhibit activity, regulating flow in the pathway.

Allosteric Regulation

- Allosteric modulators, effectors, bind non-covalently to the enzyme and affect either K_M or V_{max} of the enzyme.
- Allosteric enzymes can be identified by plotting initial velocity (v_0) versus substrate concentration $[S]$. The correlation between v_0 and the concentration of at least one substrate will demonstrate a sigmoidal profile.
 - Traces back to the Hill equation, cooperativity and the Hill coefficient. (v_0 vs $[S]$ defined by Hill equation)
 - Hill coefficient (n) of 1 results in a hyperbolic plot (v_0 vs $[S]$).
 - Hill coefficients of >1 or <1 will result in curves indicative of cooperativity.
- Many enzymes demonstrate allosteric regulation, but it is particularly prevalent in enzymes that are components of long metabolic pathways (i.e. biosynthetic processes, glycolysis, glycogenesis and β -oxidation).



Proteins: Structure and Function, 2005, by David Whitford, John Wiley & Sons publishers, Ltd., p231.

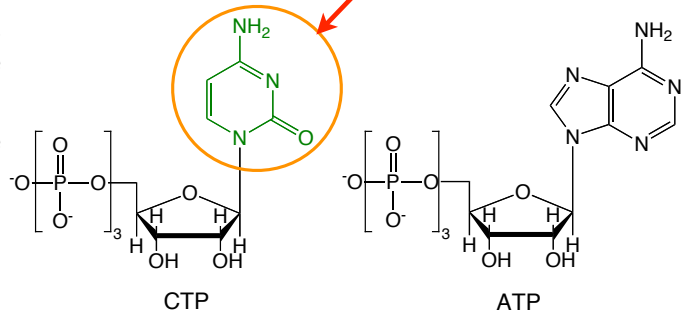
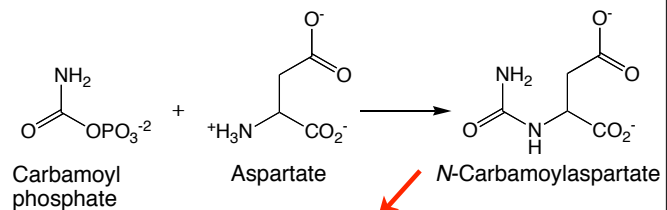
Hill equation

$$v = \frac{V_{max}[S]^n}{K_{0.5}^n + [S]^n}$$

$K_{0.5}^n$ = substrate concentration at half V_{max} .

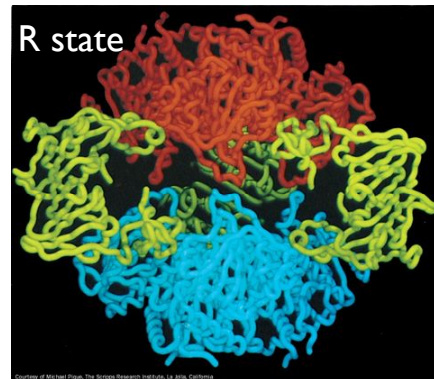
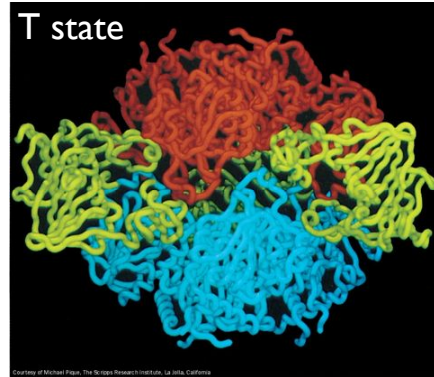
Allosteric Regulation and Aspartate Transcarbamylase

- Aspartate transcarbamylase (ATCase) catalyzes the carbamylation of the α -amino group of aspartic acid. (the first unique step in pyrimidine biosynthesis)
- The activity of ATCase is influenced by the presence of substrate and downstream products.
 - The binding of substrates (Carbamoyl phosphate and aspartate) increases enzyme activity in a cooperative manner.
 - Binding of CTP decreases enzyme activity, while ATP has opposite affect.
 - CTP and ATP bind to same site in regulatory dimer.
- ATCase consists of 12 protein subunits: 6 catalytic and 6 regulatory (C_6R_6).



Allosteric Regulation and ATCase

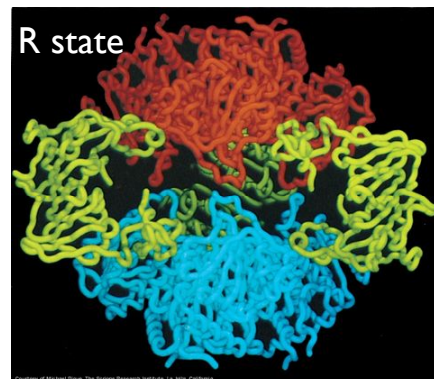
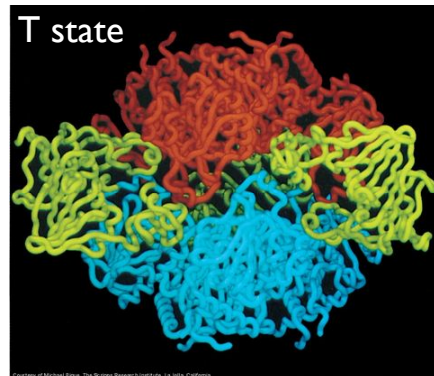
- ATCase is involved with the first unique step in pyrimidine biosynthesis.
- CTP is a downstream product of this synthetic pathway.
 - When the concentration of CTP becomes high, CTP binds to ATCase and inhibits enzyme activity.
 - At lower CTP concentrations, CTP dissociates and the enzyme is in its activated form.
 - Similarly, ATP functions as a regulator and helps to coordinate purine and pyrimidine biosynthesis (cellular ATP conc. is generally higher than the conc. of CTP.)
- The X-ray structure of ATCase indicates that it consists of a pair of c_3 trimers and three r_2 dimers.
- When the catalytic dimers are separated from the regulatory dimers, they retain catalytic activity but are unaffected by the presence of either ATP or CTP.
- The regulatory dimers bind both ATP and CTP, but have no catalytic activity.



from *Biochemistry*, 3rd ed. Voet and Voet

Allosteric Regulation and ATCase

- ATCase has an active (R state) conformation and an inactive (T state) conformation.
 - ATP is preferentially bound by ATCase in the R state.
 - CTP is preferentially bound by ATCase in the T state.
- Conversion from T to R involves separation of the two catalytic trimers by $\sim 11\text{\AA}$ and a slight reorientation about their axis. Associated with clockwise rotation of the regulatory dimers.
- As in other allosteric systems, tertiary and quaternary structures are tightly coupled. Minor shifts in tertiary structure induce larger changes at the quaternary level.
- Carbamoyl phosphate and aspartate bind to separate domains of the catalytic subunit, which induces active site closure. Favored in R state.
- Appears to be all or nothing. Do not see one subunit in R state while others in T (symmetry model of allostery).



from *Biochemistry*, 3rd ed. Voet and Voet