ENZYMES: Binding & Catalysis

A. Binding
B. Catalysis
C. Nomenclature
D. Catalysis
   1. Transition State Theory
   2. Catalytic strategies (What)
   3. Mechanistic strategies (How)

E. Quantifying the Catalytic Power: Kinetics
   1. Review
   2. Enzyme Kinetics
   3. Rate vs. [S] for enzyme catalyzed reaction; initial rate (v_0)
   4. ES complex
      a. Reaction
         i. Binding reaction
         ii. Catalytic reaction
      b. Meaning of rate curve: hyperbolic curve
   5. Rate expression; Michaelis-Menten Kinetics (M-M)
      a. Assumptions
      b. M-M equation derivation
   6. Meaning of rate expression (M-M eqn)
      a. [S] = K_m
      b. [S] >> K_m
      c. [S] << K_m
   7. Collection and manipulation of data
      a. Lineweaver-Burk; double reciprocal; $1/v_0$ vs. $1/[S]$
      b. Eadie-Hofstee; v_0 vs. $v_0/[S]$
      c. Hanes-Woolf; $[S]/v_0$ vs. $1/[S]$

TODAY
•Reading: Ch6; 181-186, 195-196, 203-208
•Homework #16 & #17
NEXT
•Reading: Ch5; 157
Ch12; 413-415

Lecture 16 (10/21/24)

8. Inhibition
   a. Irreversible: protein modification
   b. Reversible
      i. Competitive; like substrate; K_m affected by $(1 + [I]/K_I) = \alpha$
      ii. Uncompetitive; binds only ES; both K_m and V_{max} affected in opposite ways
      iii. Noncompetitive; binds both E & ES (mixed, non-equal binding); V_{max} affected
      iv. Mixed inhibition if I binds E differently than it binds ES

F. Active-site identification
   a. Determine mechanism-distinguish ping-pong *versus* sequential
   b. pH studies; do ionizations match amino acid pK_a's when looking at pH *vs.* activity?
   c. Protein modification; Irreversible
   d. X-ray crystallography structure; cleft, complexes with ligands (inhibitors or substrates)

G. Energetics of Catalysis
   a. The $\Delta\Delta G^{\ddagger}$ is negative
   b. The $\Delta\Delta G^{\ddagger} = \Delta\Delta H^{\ddagger} - T\Delta\Delta S^{\ddagger}$; bonding effects & proximity/position effects
   c. Rate dependent on (**k**T/h)EXP($-\Delta G^{\ddagger}$/RT)
   d. Example of enzyme;
      I. Proline Racemase
      II. HIV protease; tetrahedral t.s. seen in two nM inhibitors (K_I < 1 nM); bioavailability

H. Enzyme Mechanisms
   a. Proteases
   b. Serine Proteases
   c. Other protease mechanisms

Enzymes

ACTIVE SITE

SUMMARY SO FAR:

We have described enzymes in general terms such as:
- catalytic cycle
- binding, even stereo-specific binding
- catalysis, turnover number & proficiency
- nomenclature
- transition state theory
- catalytic strategies (what to do)
- mechanistic strategies (how to do)
- enyzme kinetics and inhibition

ALL of this happens at the ACTIVE SITE

Now, we want to ask what all happens here & how do we determine what happens?

Enzymes

How do your determine what is going on at the active site?

We will discuss FOUR methods for study of the active site

1. Enzyme kinetics
2. pH studies
3. Protein modification
4. Structural studies

Enzymes

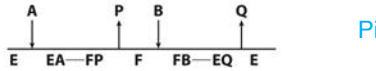
1) Use M-M Kinetics to determine the kinetic mechanism

Steady-state kinetic (SSK) analysis of bi-substrate reactions

- Is it random or ordered?
- With SSK, we cannot easily distinguish sequential from ping-pong (both are ordered).

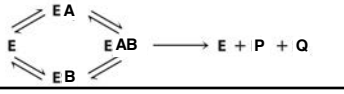
Recall: In enzyme inhibition, if S MUST bind before I, you get parallel lines, and if I can bind to both forms of the enzyme (E and ES) you get x-axis intersecting lines.

Its that same principle for bi-substrate enzymes: if A MUST bind before B, you get parallel lines, and if B can bind to both forms E and EA of the enzyme, you get x-axis intersecting lines.



Ping-pong bi bi

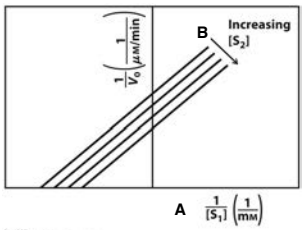
Sequential ordered bi bi



Sequential random bi bi

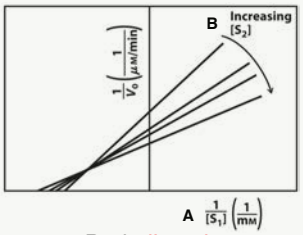
Enzymes

In these double-reciprocal plots, the concentration of A is varied while the concentration of B is held constant (at less than saturating concentrations). This is repeated for several values of [B], generating several separate lines.



Lineweaver-Burk: lines are parallel

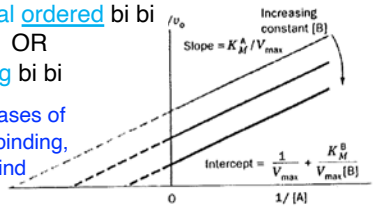
Sequential random bi bi



Lineweaver-Burk: lines intersect

Sequential ordered bi bi
OR
Ping-pong bi bi

In both cases of ordered binding, A must bind before B



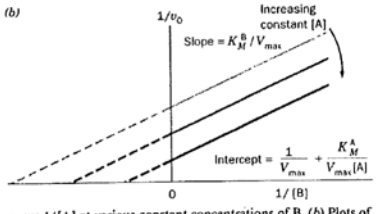
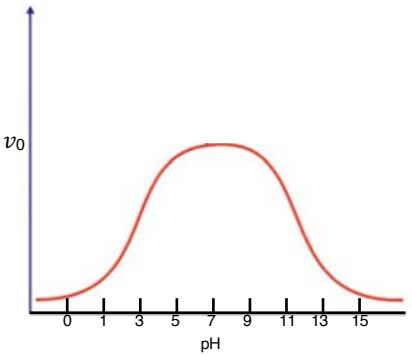


FIGURE 13-17. Double-reciprocal plots for an enzymatic reaction with a Ping Pong Bi Bi mechanism. (*a*) Plots of $1/v_0$ versus $1/[A]$ at various constant concentrations of B. (*b*) Plots of $1/v_0$ versus $1/[B]$ at various constant concentrations of A.

Enzymes

2) Use SSK to determine if there is acid-base catalysis

Lets suppose you assay enzyme 1 at varying pH values. And, you get this:



What is the difference?
What does this mean in each case?
What other information can you get?

Then on enzyme 2, you get this:

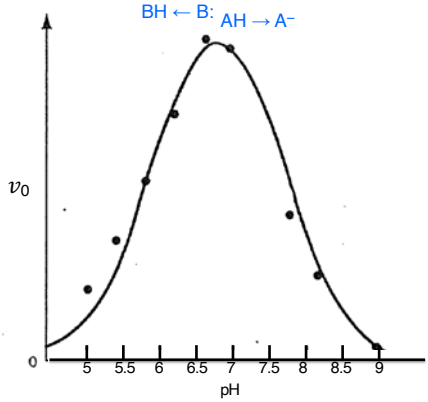


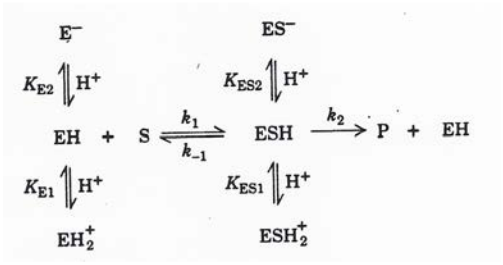
FIGURE 13-14. The effect of pH on the initial rate of the reaction catalyzed by the enzyme fumarase. [After Tanford, C., *Physical Chemistry of Macromolecules*, p. 647, Wiley (1961).]

Enzymes

2) Use SSK to determine if there is acid-base catalysis

Lets take enzyme 2 and determine the values of V_{max} and K_m at varying pH values.

What are you treating protons as?



Do the pK_a ($K_a \approx K_i$) values give your any clue as to what residues are functioning as acid/base catalysts at the active site?

If pK_{E1} = 4.5 ?Glu must be de-protonated for binding
If pK_{ES2} = 9.5 ?Lys must be protonated for catalysis

Now, plot the V_{max} and V_{max}/K_m *versus* $[H^+]$ (i.e., pH)

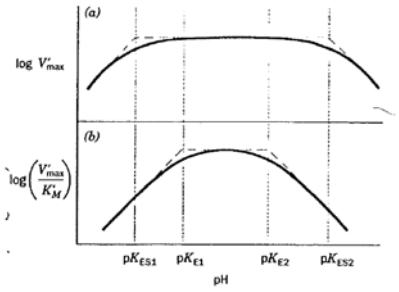


FIGURE 13-15. The pH dependence of (*a*) log V'_{max} and (*b*) log (V'_{max}/K'_M) illustrating how the values of the molecular ionization constants can be determined by graphical extrapolation.

Enzymes

3) Use protein modification to determine what residues might be AT the active site

If you react your enzyme with chemical reagents that are specific to certain amino acid residues, and these residues are at the active site, you might abolish activity.

Certain controls are usually required:
- make sure that reagent doesn't just denature the enzyme
- test to see if substrates or competitive inhibitors will protect
- should measure stoichiometry of reaction

Common Reagents for the Modification of Proteins

Reagent	Residue	Detection
2-hydroxy-5-nitrobenzyl bromide (Koshland's reagent)	Tryptophan	410 nm
N-bromosuccinimide	Tryptophan	260/280 nm
Phenylisothiocyante (Edman's Reagent)	Amino-terminal	Release of a PTH-amino acid
Iodoacetic acid	Cysteine	Carboxylmethyl derivatives
N-ethylmaleimide (NEM)	Cysteine	Derivatives of NEM
5,5'-Dithiobis(2-nitrobenzoic acid) (Ellman's Reagent - DTNB)	Cysteine	412 nm
Diethylpyrocarbonate (DEPC)	Histidine	240 nm
Imidates	Lysine	Derivatives of imidates
2,4,6-trinitrobenzenesulfonic acid (TNBS)	Lysine	420 nm

How can you use this idea and identify WHICH of the many His, Cys, Lys, etc. might be the one at the active site?

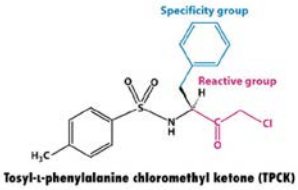
Enzymes

3) Use protein modification to determine what residues might be AT the active site

Use a "Trojan Horse"

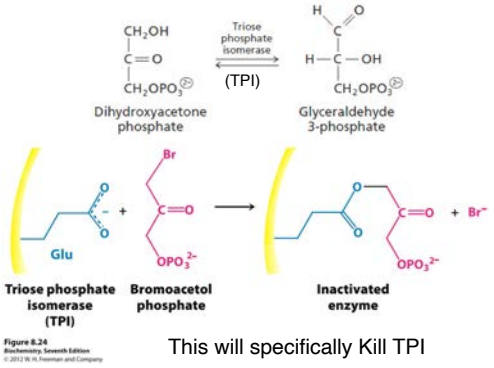
This combines the specificity of binding at the active site with the reactivity of the reagent for certain residues

Examples:



This will specifically Kill chymotrypsin

General term:
Affinity Labeling



This will specifically Kill TPI

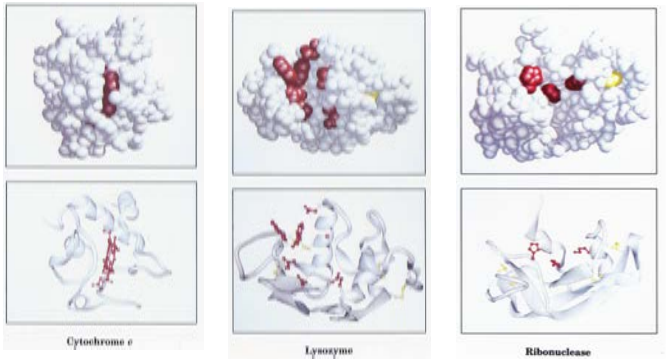
You can then perform protein sequencing studies to find which residue is modified

Enzymes

4) Use structural studies to SEE the active site

X-ray crystallography can often reveal a cleft, which is usually that active site.
Can look in the cleft for metal ions, coenzymes, acid/base groups, and/or nucleophiles

Examples:



You can then test which residues you actually see at the active site by protein modification, pH studies, or site-directed mutagenesis.

Enzymes

TRANSITION-STATE THEORY: Energetics

SUMMARY SO FAR:

We have described enzymes in general terms such as:

- catalytic cycle
- binding, even stereo-specific binding
- catalysis, turnover number & proficiency
- nomenclature
- transition state theory
- catalytic strategies (what to do)
- mechanistic strategies (how to do)
- enyzme kinetics and inhibition
- Deciphering the ACTIVE SITE

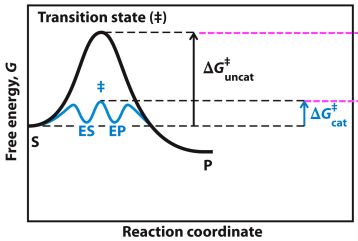
Can we Quantify the energy needed to get the kind of rate enhancements enzymes enjoy?

Enzymes

What enzyme energetics are involved in lowering the activation energy?

Now that we can discover what the "geography" of the active site might be, lets discuss what has to happen there…

Recall Transition State Theory and the 4 Catalytic Strategies:



$\Delta\Delta G^{\ddagger}$ ⊖

$\Delta\Delta G^{\ddagger} = \Delta G^{\ddagger}_{cat} - \Delta G^{\ddagger}_{uncat}$

There is always a difference in activation energies that yield a negative $\Delta\Delta G^{\ddagger}$.

This is the amount of energy that must be supplied somehow to the reaction by the enzyme

Catalytic Strategies

- Position Effects: bind substrates where they need to be for reaction (rather than depending on random collisions)
- Polarization of bonds: make substrates more reactive by polarizing bonds (make better nucleophiles, electrophile, or leaving groups) (Electrostatics)
- Strain of bonds: bind substrates in such a way that they "look" like products (put strain on bonds that are to be broken (sessile)) (Geometry)
- De-solvation: assist in removal of water shell around substrates or adding to products upon release (S & P are usually in direct contact with residues at the active site (no water))

Enzymes

Enzymes organize reactive groups into close proximity and proper orientation.

•Whatever way they do this, they have to have a negative $\Delta\Delta G^{\ddagger}$

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

•This can be from strong polarizing bonds in ES & ES‡

•This can come from differences in energy of the solvated S and ES complex

Catalyzed – Uncatalyzed
(more bonds; lower energy less enthalpy) (fewer bonds; higher energy more enthalpy)

$\Delta\Delta H^{\ddagger}$ Value is: ⊖⊖

• Catalyzed bimolecular and unimolecular reactions MUST use **binding energy** from somewhere to, not only pay the entropic cost of organizing the reactants into a fairly rigid ES complex staged to achieve the transition state, but also to get enough energy to lower the activation barrier.

• Catalyzed bimolecular and unimolecular reactions have to position reactants and/or strain them to reach the transition state in the active site

Catalyzed – Uncatalyzed
(less S) (more S)

$\Delta\Delta S^{\ddagger}$ Value is: ⊖

$-T\Delta\Delta S^{\ddagger}$ value is: ⊕

Thus $\Delta\Delta H^{\ddagger}$ MUST be much more ⊖ than $-T\Delta\Delta S^{\ddagger}$ is ⊕

Enzymes

An easy way to express this is simply to say that:

Enzymes bind transition states best.

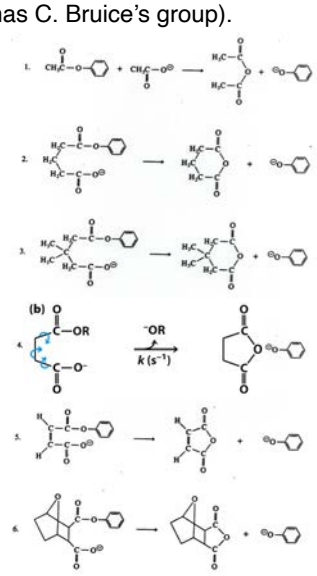
- This idea was proposed by Linus Pauling in 1946.
  - Enzyme active sites are complimentary to the transition state of the reaction.
  - Enzymes bind transition states better than substrates.
  - Stronger/additional interactions with the transition state as compared with the ground state lower the activation barrier.

As shown on last slide, this is largely $\Delta\Delta H^{\ddagger}$ effect

What is an example of using binding energy to increase rates?

Enzymes

The rate of anhydride formation (condensation of two acids) from an esters and carboxylates shows a strong dependence on proximity of two reactive groups, "paid" by covalent bonds (work by Thomas C. Bruice's group).



	Relative Rate
1	1
2	6.0 x 10^2
3	1.2 x 10^3
4	1.0 x 10^5
5	6.0 x 10^6
6	3.0 x 10^7

For an enzyme, how much binding energy is needed to increase the rate by 10^6?

Enzymes

- Enzymes increase reaction rates (v_0) by decreasing $\Delta G^{\ddagger}$.

$$v_0 = k_{cat}\,[E]_T$$

The change in rate constant from the uncatalyzed:

$$\Delta k_{cat} = \left(\frac{k_B T}{h}\right) e^{\left(\frac{-\Delta\Delta G^{\ddagger}}{RT}\right)}$$

k_B = Boltzmann's constant (J/K)
h = Plank's constant (J•sec)
T = Temperature (K)
R = Gas constant (J•K^{-1}•mol^{-1})

TWO points about this equation:

1) the relationship between $\Delta\Delta G^{\ddagger}$ and rate is negative; the higher the negative value, the larger the rate [recall we are taking – or $-\Delta\Delta G^{\ddagger}$].
2) the relationship between $\Delta\Delta G^{\ddagger}$ and rate is exponential; a small change in energy, a large change in rate

$\Delta\Delta G^{\ddagger}$ (kcal/mole)	Δk_{cat} (s^{-1})
–1.4	10^1
–2.8	10^2
–5.6	10^4
–8.0	10^6

Enzymes

Transition-State Analogs Are Potent Inhibitors of Enzymes

Binding Energy is the free energy released upon interaction of the enzyme and substrate.

Binding Energy need NOT be just in the interactions directly with the substrate; it could be that Binding Energy (bonds) is gained from the entire protein (enzyme dynamics) in the ES complex.

It has been proposed that the ES complex is a high-energy state and the ES‡ complex is a low-energy state. Upon S binding, the ES complex is sort of a "wound-up" protein, and Binding Energy helps force the ES → ES‡ reaction, i.e., ES‡ is more easily achieved as the whole protein finds a lower energy state.

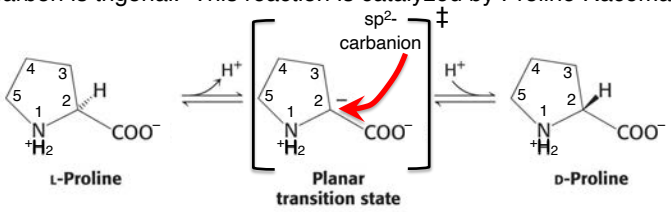
In other words, it seems clear now that Binding Energy is greatest when the enzyme interacts as it approaches the transition state, thus facilitating the formation of the transition state.

EXAMPLE: The racemization of proline proceeds through a transition state in which the α-carbon is trigonal. This reaction is catalyzed by Proline Racemase.

Enzymes

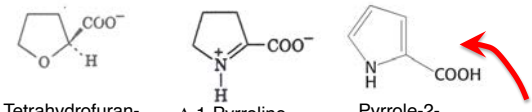
Transition-State Analogs Are Potent Inhibitors of Enzymes

EXAMPLE: The racemization of proline proceeds through a transition state in which the α-carbon is trigonal. This reaction is catalyzed by Proline Racemase.



HA-E-B: HA-E-BH $^{-}$A-E-BH

Other substrates/inhibitors that have a trigonal geometry (sp^2) might look more like the transition state, so called transition-state analogs. These might bind better than S.



	L-Proline	Tetrahydrofuran-2-carboxylate	Δ-1-Pyrroline-2-carboxylate	Pyrrole-2-carboxylate	A potent inhibitor of proline racemase.
Relative K_d values:	1	2	0.006	0.006	

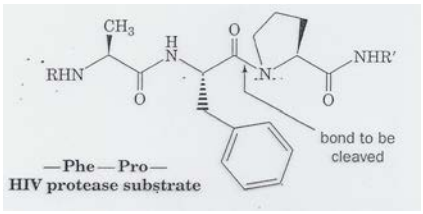
Enzymes

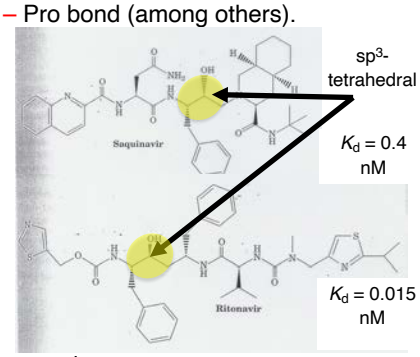
Transition-State Analogs Are Potent Inhibitors of Enzymes

EXAMPLE: HIV protease

This protease is important in the processing of the viral proteins and is encoded by the HIV genome. It was the first successful target for treatment of HIV. Saved millions of lives to date.

This is a protease that uses an Asp at the active site (Asp-protease or acid protease), but it has a specificity for cleavage at a Tyr/Phe – Pro bond (among others).





What are all these other substituents doing?

In drug development, issues of Bioavailability are paramount:
survival in the gut, absorption, half-life, membrane permeability, off-target minimization

Enzymes

ENZYME MECHANISMS

Enzymes

What is an Enzyme Mechanism?

- In organic chemistry, there is a chemical mechanism.
- In biochemistry, there is a chemical mechanism performed by the enzyme.
  - This mechanism may not have the same pathway as one in organic chemistry (think covalent catalysis).
  - An Enzyme Mechanism is a description of step-by-step what goes on at the active site, what are the intermediates, what order do they occur, and what are the rate constants (energetics and rate-limiting steps, etc.).

Enzyme Mechanism

Protease (Peptide hydrolase)

- During digestion, dietary proteins must be broken down into small peptides by proteases.
- During protein turnover in the cell, proteins must be broken down by proteases (lysosomal cathepsins, proteosome, etc.)
- During regulation, protein processing:
  - Pro-collagen → collagen
  - Generation of endorphins
  - Blood clotting
- During development, from fertilization (acrosome) onward

Enzyme Mechanism

Protease (Peptide hydrolase)

- Proteases are classified by their mechanism
- Although there are hundreds of different proteases, there are only a few standard mechanisms on which these proteins have converged.

Class of Protease	Examples
Serine	Trypsin, Chymo… …stase
Thiol	Papain, Cathepsin B, Caspases
Acid	HIV protease, Pepsin, Cathepsin D, Renin, Chymosin
Metal	Carboxylpeptidase A, Thermolysin

Enzyme Mechanism

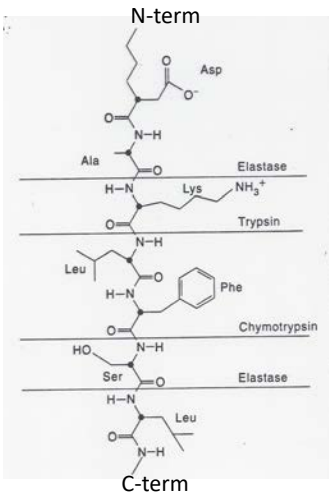
Serine Proteases

TABLE 14-4. A SELECTION OF SERINE PROTEASES

Enzyme	Source	Function
Trypsin	Pancreas	Digestion of proteins
Chymotrypsin	Pancreas	Digestion of proteins
Elastase	Pancreas	Digestion of proteins
Thrombin	Vertebrate serum	Blood clotting
Plasmin	Vertebrate serum	Dissolution of blood clots
Kallikrein	Blood and tissues	Control of blood flow
Complement C1	Serum	Cell lysis in the immune response
Acrosomal protease	Sperm acrosome	Penetration of ovum
Lysosomal protease	Animal cells	Cell protein turnover
Cocoonase	Moth larvae	Dissolution of cocoon after metamorphosis
α-Lytic protease	*Bacillus sorangium*	Possibly digestion
Proteases A and B	*Streptomyces griseus*	Possibly digestion
Subtilisin	*Bacillus subtilus*	Possibly digestion

Source: Stroud, R.M., *Sci. Am.* 231(1): 86 (1974).

Serine proteases have varying specificities

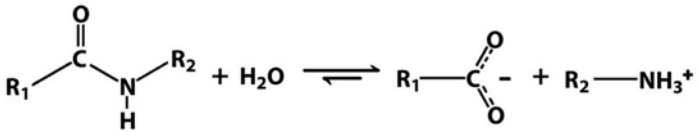


Enzyme Mechanism

Serine Proteases

- Serine proteases are among the best studied enzymes.
- Illustrate charge delocalization and transition-state stabilization by general acid/base catalysis
- Also, illustrates how enzymes are regulated (for the first time for us)….. Zymogen activation.

What is the reaction?



This is a bi bi reaction. The specificity comes from R_1 and R_2 (mostly R_1)

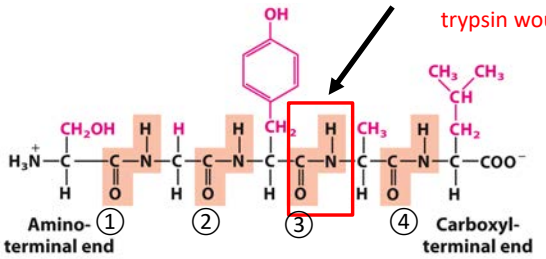
Enzyme Mechanism

Chymotrypsin

- This protease is able to cleave the peptide bond adjacent to aromatic amino acids.

Chymotrypsin cuts this bond (the sessile bond).

trypsin would **not** cut this peptide.



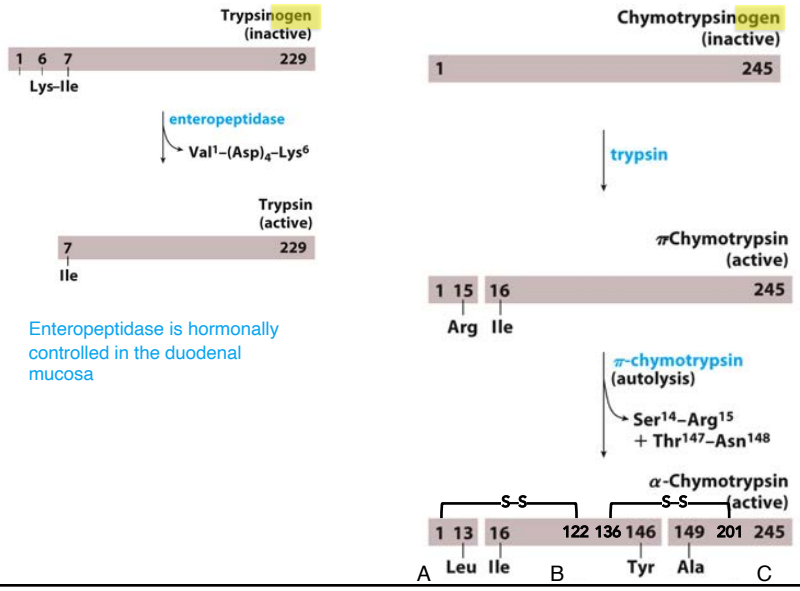
OUTLINE

- Regulation
- Kinetic mechanism
- Enzyme intermediates from protein modification studies
- Enzyme intermediates from pH studies
- Enzyme intermediates from structural studies
- Enzyme mechanism and binding energy

Enzyme Mechanism

• *Regulation*

Zymogens are Activated by Irreversible Covalent Modification

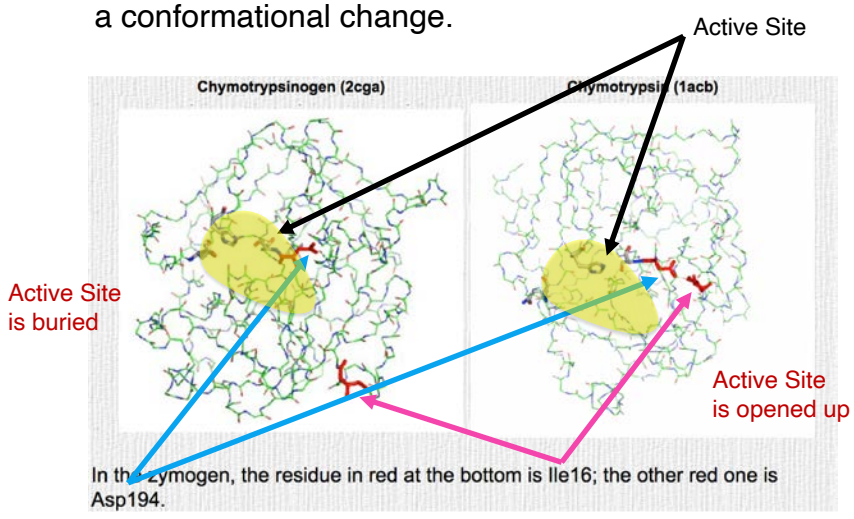


Enzyme Mechanism

• *Regulation*

Chymotrypsin

- This protease is activated by proteolysis, which leads to a conformational change.



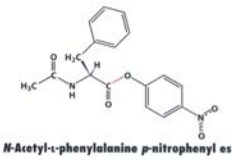
Enzyme Mechanism

• *Kinetic mechanism*

Chymotrypsin

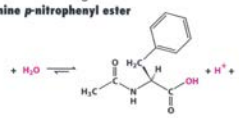
- This protease cleaves peptide bonds with k_{cat} value of ~50 s^{-1}.
- This is very fast and difficult to study
- This protease cleaves ester bonds with slower k_{cat} values (~0.1 s^{-1}), but also the products (alcohols) are more easily measured using a spectrophotometer.
- This is easier to study.

What is the kinetic mechanism? Calculate K_m & V_{max} by measuring the rate of PNP formation versus [NAcPhe-PNPE], then at varying concentrations of water…..... wha wha



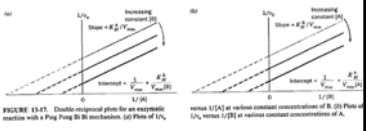
N-Acetyl-L-phenylalanine *p*-nitrophenyl ester

NAcPhe-PNPE



p-Nitrophenolate

PNP



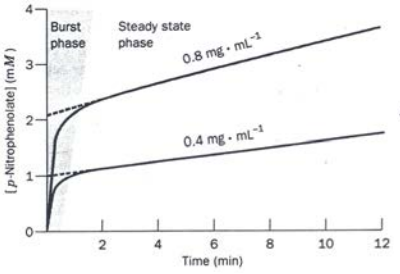
Schwert, G.W. *et al.* (1948) *J Biol Chem* **172**:221-239

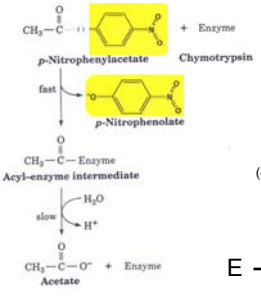
Enzyme Mechanism

• *Kinetic mechanism*

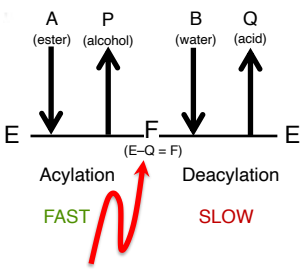
Chymotrypsin

- React with *p*-nitrophenylacetate or *N*-acetyl-L-phenylalanine *p*-nitrophenyl ester (**NAcPhe-PNPE**).
- Monitor *p*-nitrophenolate (**PNP**) as a function of time
- There are two rates: a fast "burst" rate and a slower steady-state rate.





Ping-pong bi bi



This is called pre-steady state kinetics

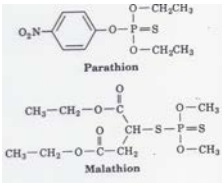
What is the nature of this enzyme intermediate (F)?

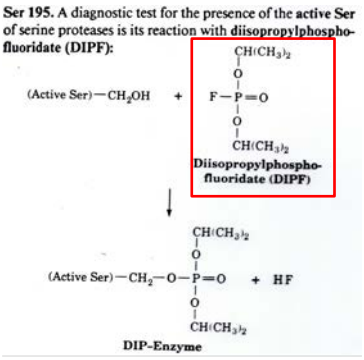
• *Protein modification studies*

Enzyme Mechanism

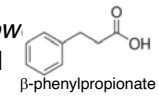
Chymotrypsin

- React with *acylating reagent*





- Denature the enzyme; *no reaction*
- Add substrate or inhibitor (β-phenylpropionate); *reaction blocked/slow*
- Use as an inhibitor of the whole class of Serine Proteases and Esterases



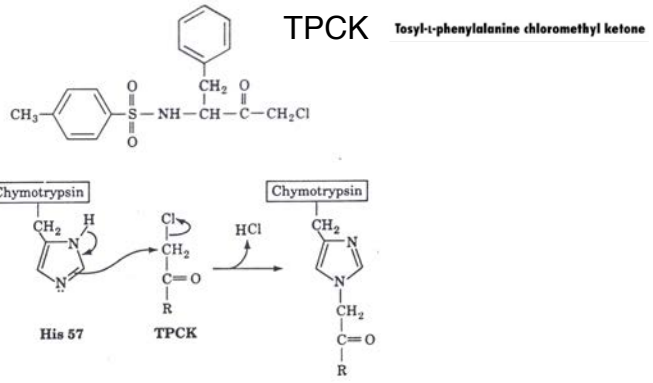
The nature of this enzyme intermediate (F) is an acylated enzyme at Ser-195

• *Protein modification studies*

Enzyme Mechanism

Chymotrypsin

- React with *Affinity Label*



- Denature the enzyme; *no reaction*
- Add substrate or inhibitor; *reaction blocked/slowed*

The enzyme must utilize a His-57 in the active site, near Ser-195. Does it activate the Ser (acting as a base)?

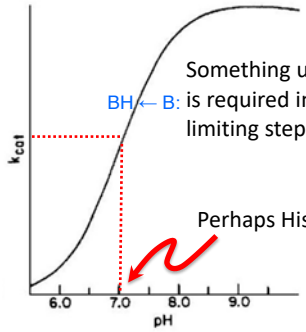
Enzyme Mechanism

• *pH studies*

Chymotrypsin

- Measure activity and enzyme constants as a function of pH

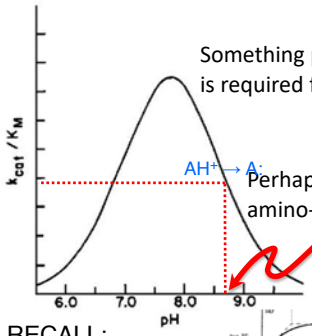
1) pH *vs.* k_{cat} (V_{max}) [catalysis]



BH ← B: Something unprotonated is required in the rate-limiting step.

Perhaps His-57

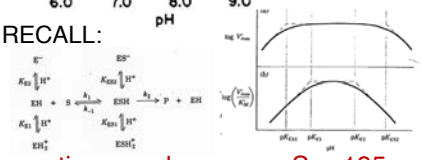
2) pH *vs.* k_{cat}/K_m [binding]



Something protonated is required for binding.

AH⁺ → A: Perhaps the amino-terminus at Ile-16

Log k_{cat} and k_{cat}/K_m gives a better measurement; RECALL:

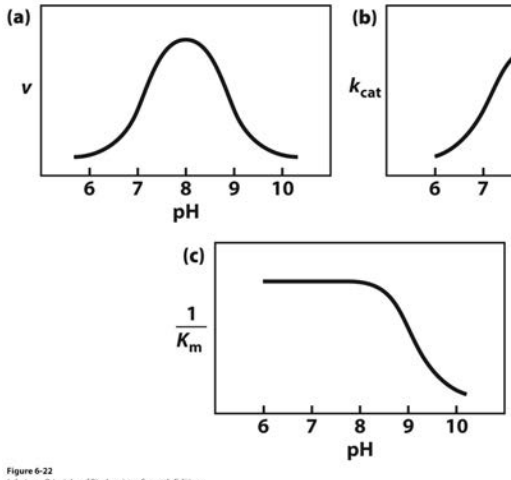


This is consistent with His-57 in the active site, acting as a base near Ser-195.

Enzyme Mechanism

• *pH studies*

From the book.....



His-57 must be uncharged to perform at the active site (act as a base)

Ile-16 at N-term must be + to keep active site open

Figure 6-22
Lehninger Principles of Biochemistry, Seventh Edition
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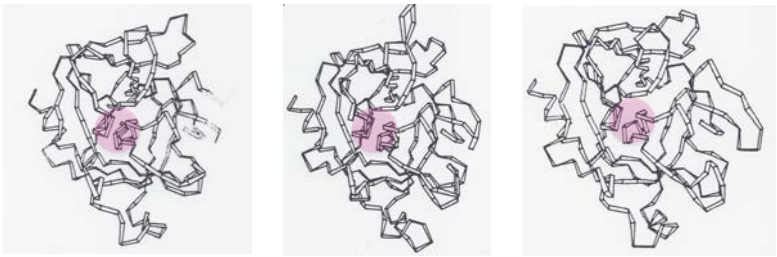
Enzyme Mechanism

• *structural studies*

Chymotrypsin

- *X-ray crystallography*



Chymotrypsin Elastase Trypsin

In the active site, near Ser-195 & His-57, there is ALWAYS an Asp (Asp-102). This is called the "Catalytic Triad."

Ser/His/Asp

Enzyme Mechanism

• *structural studies*

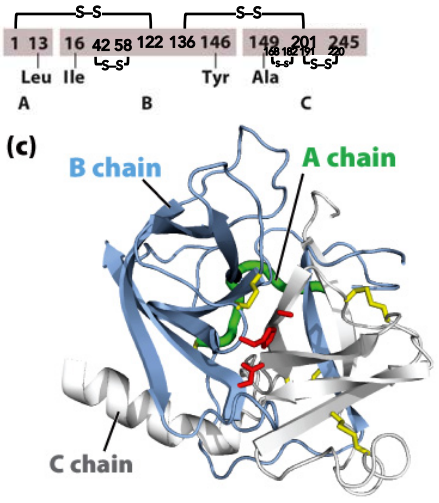
Chymotrypsin

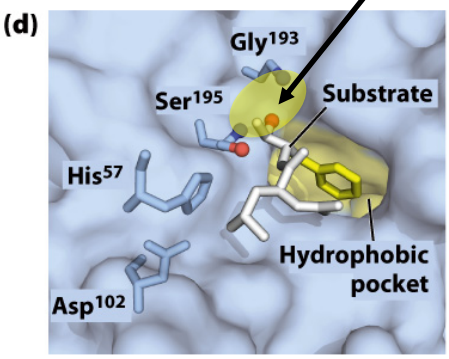
- *X-ray crystallography*

Chymotrypsin Uses Two of our the Mechanistic Strategies

covalent
acid/base

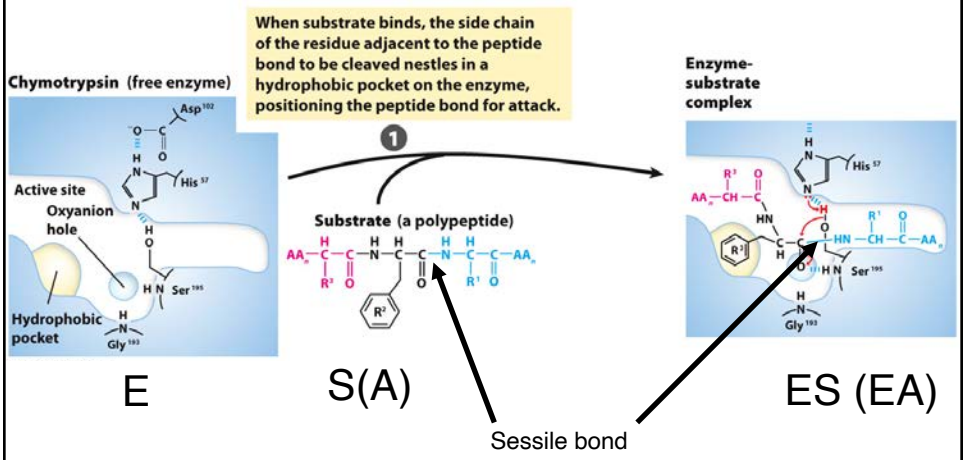
Anion "Hole"





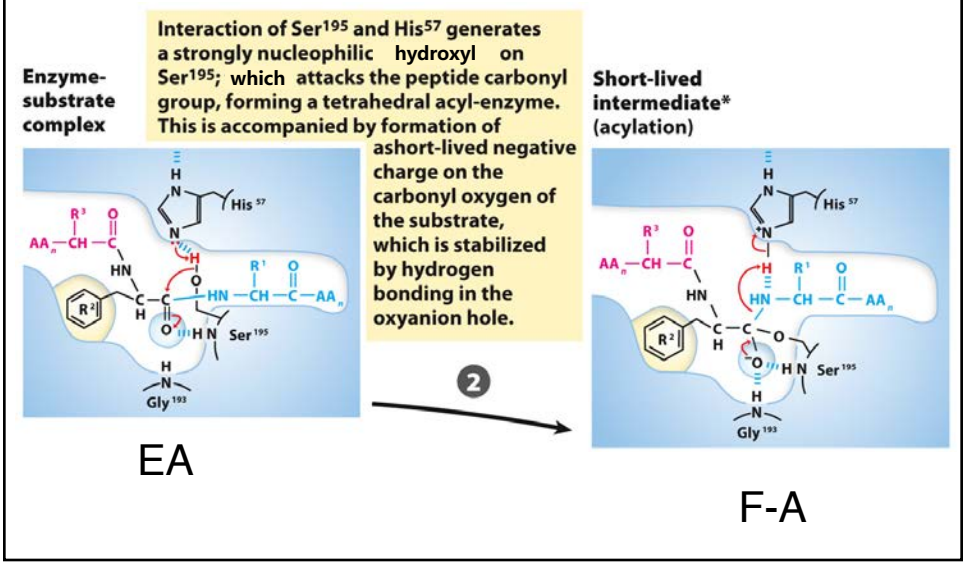
Chymotrypsin Mechanism

Step 1: Substrate Binding



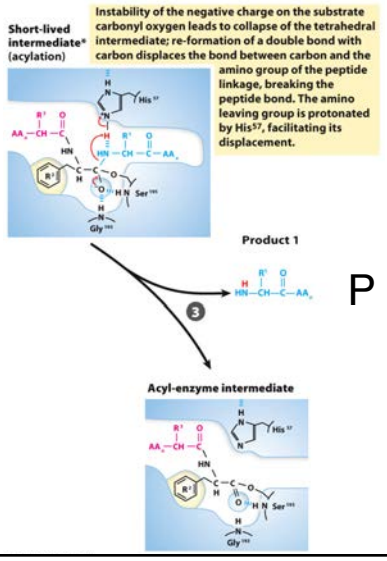
Chymotrypsin Mechanism

Step 2: Nucleophilic Attack



Chymotrypsin Mechanism

Step 3: Substrate Cleavage



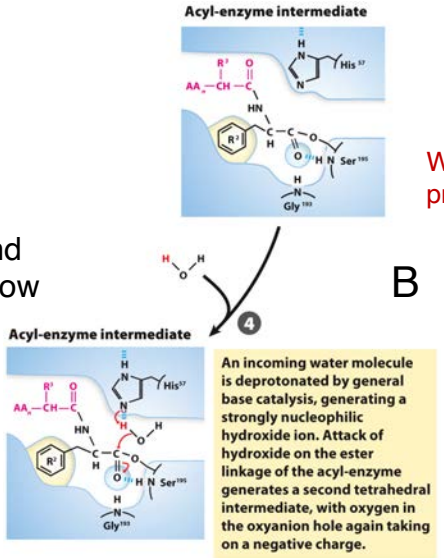
F-A⇌F-QP

P

F-Q’

Chymotrypsin Mechanism

Step 4: Water Comes In



F-Q’

What if His: were protonated to His:H⁺?

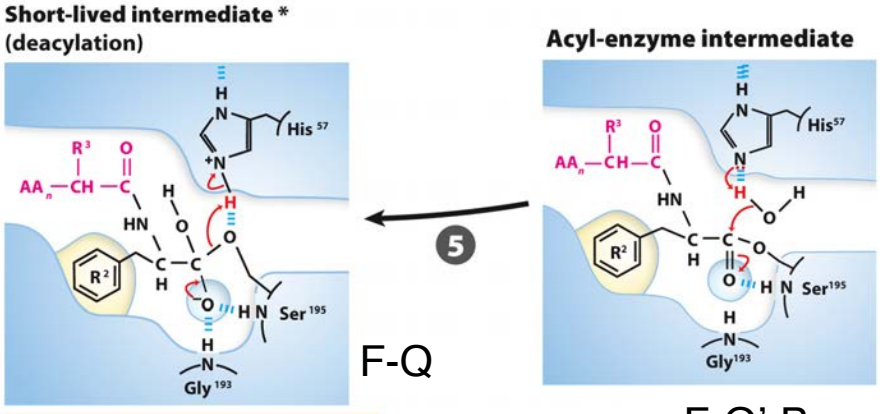
Recall, the second half-reaction is slow

B

F-Q’•B

Chymotrypsin Mechanism

Step 5: Water Attacks



F-Q

F-Q'-B

F-Q'-B⇌F-Q

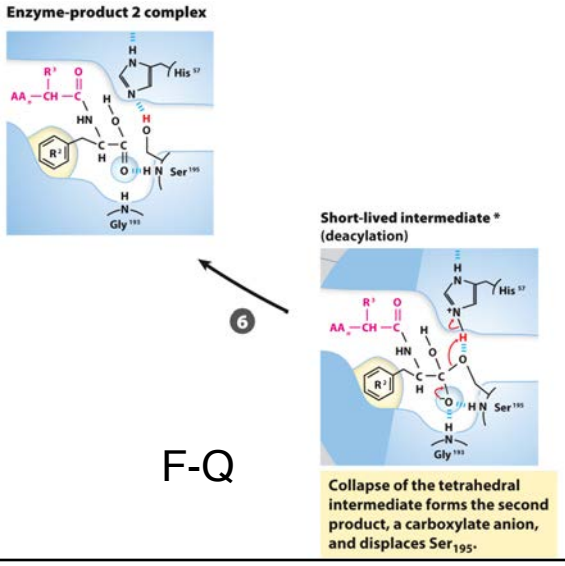
Collapse of the tetrahedral intermediate forms the second product, a carboxylate anion, and displaces Ser_{195}.

What if this His:H^+ were deprotonated?

Chymotrypsin Mechanism

Step 6: Break-off from the Enzyme

E•Q



F-Q

Collapse of the tetrahedral intermediate forms the second product, a carboxylate anion, and displaces Ser_{195}.

Chymotrypsin Mechanism

Step 7: Product Dissociates

E