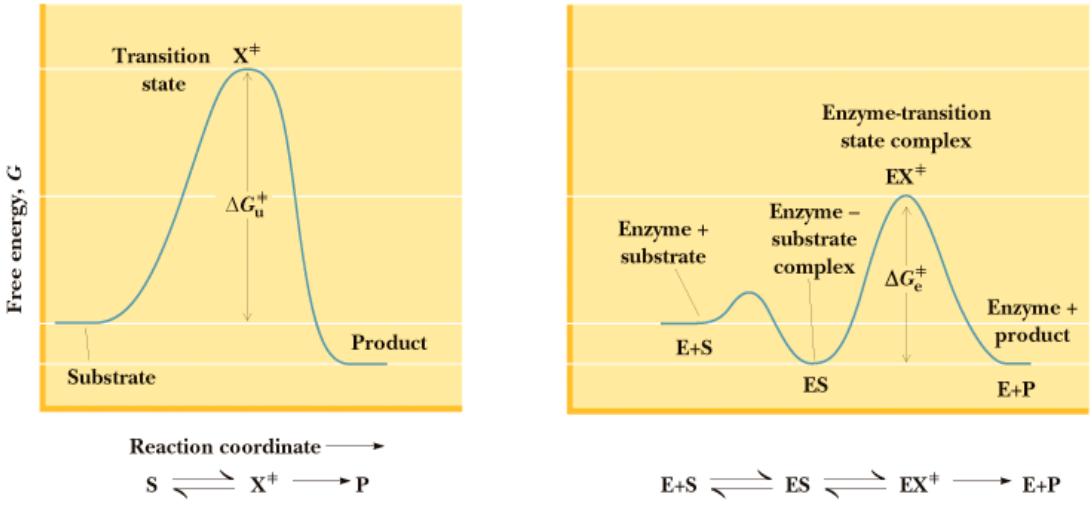
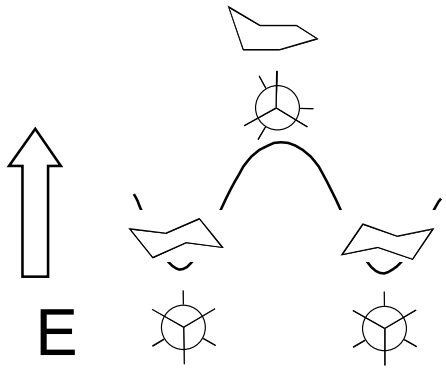
Mechanisms of Enzyme Action

- Stabilization of the Transition State
- Enormous Rate Accelerations
- Binding Energy of ES
- Entropy Loss and Destabilization of ES
- Types of Catalysis
- Serine Proteases
- Aspartic Proteases
- Lysozyme

Stabilizing the Transition State

- Rate acceleration by an enzyme means that the energy barrier between ES and EX‡ must be smaller than the barrier between S and X‡
- This means that the enzyme must stabilize the EX‡ transition state more than it stabilizes ES





A cyclohexane “flippase” would bind more tightly to the eclipsed part of the transition state than to either of the staggered ground state conformers

Rate Acceleration in Enzyme-Catalyzed Reactions

- Mechanisms of catalysis:
  - Entropy loss in ES formation
  - Destabilization of ES
  - Covalent catalysis
  - General acid/base catalysis
  - Metal ion catalysis
  - Proximity and orientation

A Comparison of Enzyme-Catalyzed Reactions and Their Uncatalyzed Counterparts

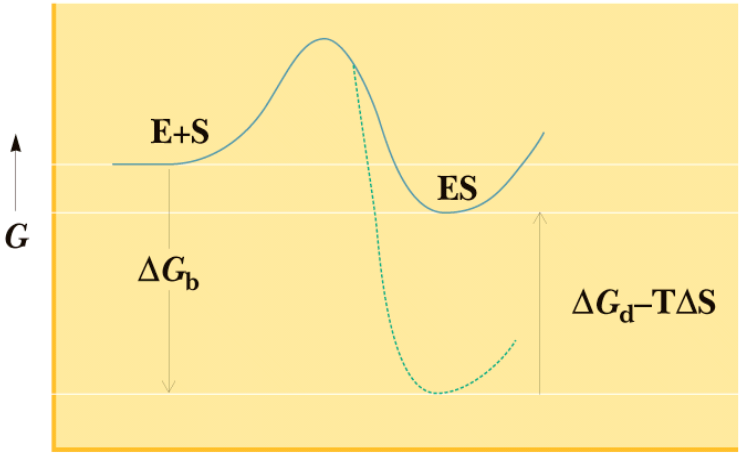
Reaction	Enzyme	Uncatalyzed Rate, v_u (sec^{-1})	Catalyzed Rate, v_c (sec^{-1})	v_c/v_u
$CH_3{-}O{-}PO_3^{2-} + H_2O \longrightarrow CH_3OH + HPO_4^{2-}$	Alkaline phosphatase	1×10^{-15}	14	1.4×10^{16}
$H_2N{-}\overset{\overset{O}{\|}}{C}{-}NH_2 + 2\,H_2O + H^+ \longrightarrow 2\,NH_4^+ + HCO_3^-$	Urease	3×10^{-10}	3×10^{4}	1×10^{14}
$R{-}\overset{\overset{O}{\|}}{C}{-}O{-}CH_2CH_3 + H_2O \longrightarrow RCOOH + HOCH_2CH_3$	Chymotrypsin	1×10^{-10}	1×10^{2}	1×10^{12}
$\underset{(n)}{\text{Glycogen}} + P_i \longrightarrow \underset{(n-1)}{\text{Glycogen}} + \text{Glucose-1-P}$	Glycogen phosphorylase	$< 5 \times 10^{-15}$	1.6×10^{-3}	$> 3.2 \times 10^{11}$
$\text{Glucose} + \text{ATP} \longrightarrow \text{Glucose-6-P} + \text{ADP}$	Hexokinase	$< 1 \times 10^{-13}$	1.3×10^{-3}	$> 1.3 \times 10^{10}$
$CH_3CH_2OH + NAD^+ \longrightarrow CH_3\overset{\overset{O}{\|}}{C}H + NADH + H^+$	Alcohol dehydrogenase	$< 6 \times 10^{-12}$	2.7×10^{-5}	$> 4.5 \times 10^{6}$
$CO_2 + H_2O \longrightarrow HCO_3^- + H^+$	Carbonic anhydrase	10^{-2}	10^{5}	1×10^{7}
$\text{Creatine} + \text{ATP} \longrightarrow \text{Cr-P} + \text{ADP}$	Creatine kinase	$< 3 \times 10^{-9}$	4×10^{-5}	$> 1.33 \times 10^{4}$

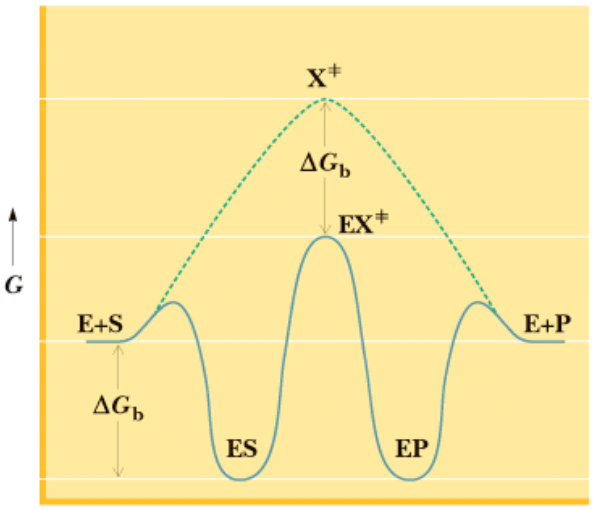
Adapted from Koshland, D., 1956. *Journal of Cellular Comparative Physiology*, Supp. 1, 47:217.

Binding Energy of ES

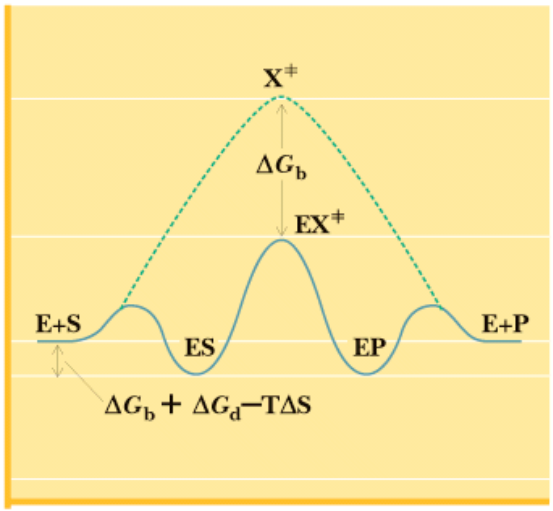
Competing effects determine the position of ES on the energy scale

- Try to mentally decompose the binding effects at the active site into favorable and unfavorable
- The binding of S to E must be favorable
- But not too favorable!
- K_m cannot be "too tight" - goal is to make the energy barrier between ES and $EX^{\ddagger}$ small





No destabilization, thus no catalysis

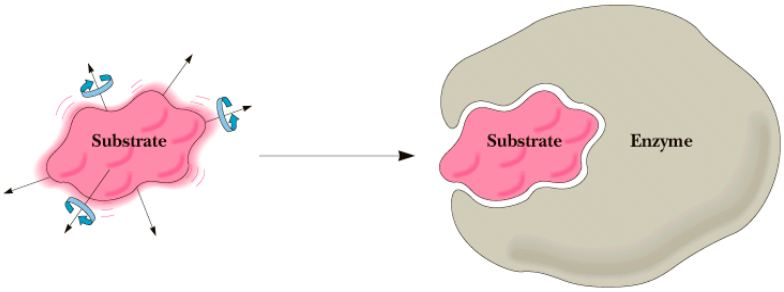


Destabilization of ES facilitates catalysis

Entropy Loss and Destabilization of ES

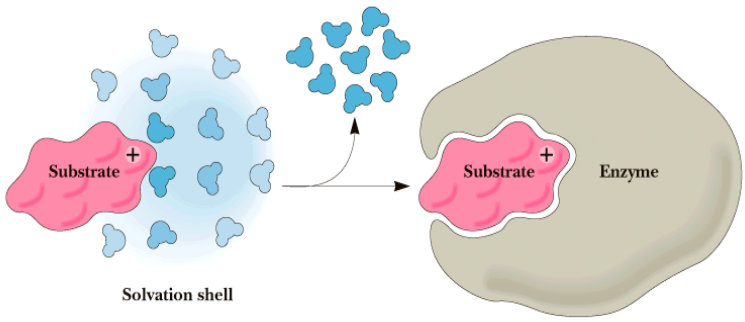
raising the energy of ES raises the rate

- For a given energy of EX‡, raising the energy of ES will increase the catalyzed rate
- This is accomplished by
  - a) loss of entropy due to formation of ES
  - b) destabilization of ES by
    - strain
    - distortion
    - desolvation



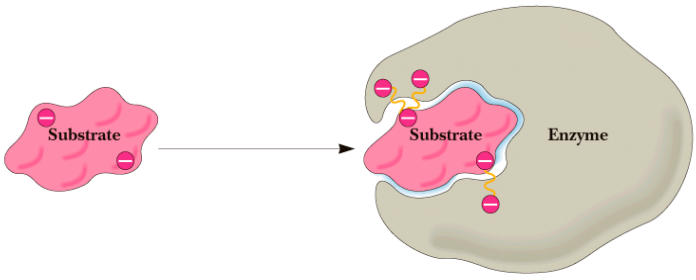
Substrate (and enzyme) are free to undergo translational motion. A disordered, high-entropy situation

The highly ordered, low-entropy complex



Solvation shell

Desolvated ES complex

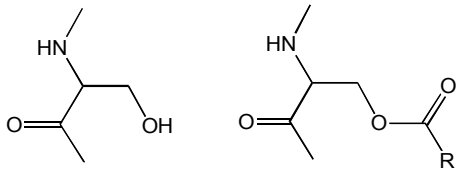


Electrostatic destabilization in ES complex

Covalent Catalysis

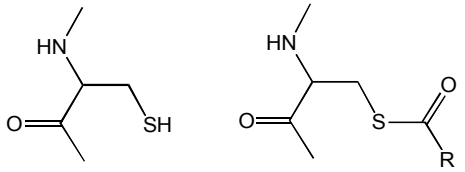
- Enzyme and substrate become linked in a covalent bond at one or more points in the reaction pathway
- The formation of the covalent bond provides chemistry that speeds the reaction

1. Chymotrypsin
   Elastase
   Esterases
   Subtilisin
   Thrombin
   Trypsin



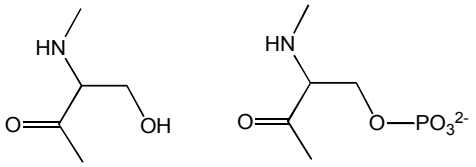
acyl-ser

2. G-3-P dehydrogenase
   Papain



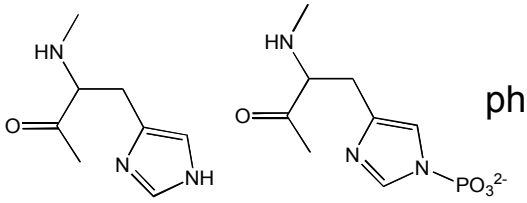
acyl-cys

3. Alkaline phosphatase
   Phosphoglucomutase



phosphoserine

4. Phosphoglycerate mutase
   Succinyl-CoA synthetase

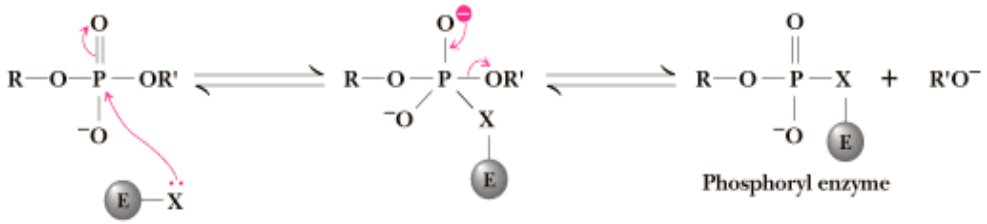


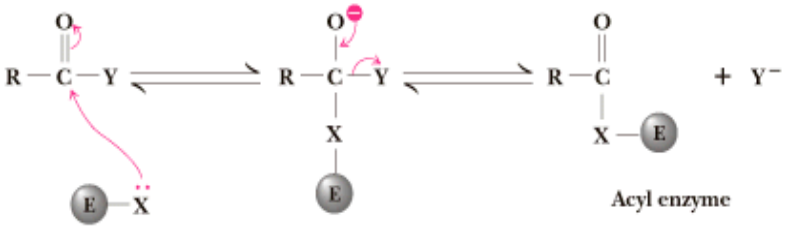
phosphohistidine

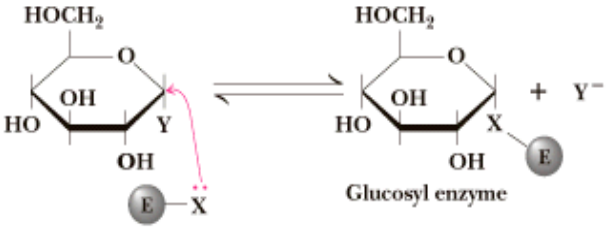
5. Aldolase
   Decarboxylases
   Pyridoxal phosphate-dependent enzymes

Schiff base

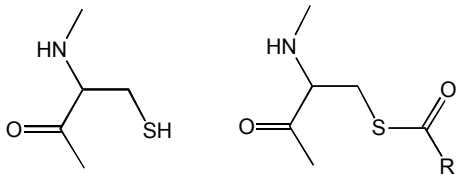
The Enzyme as Nucleophile



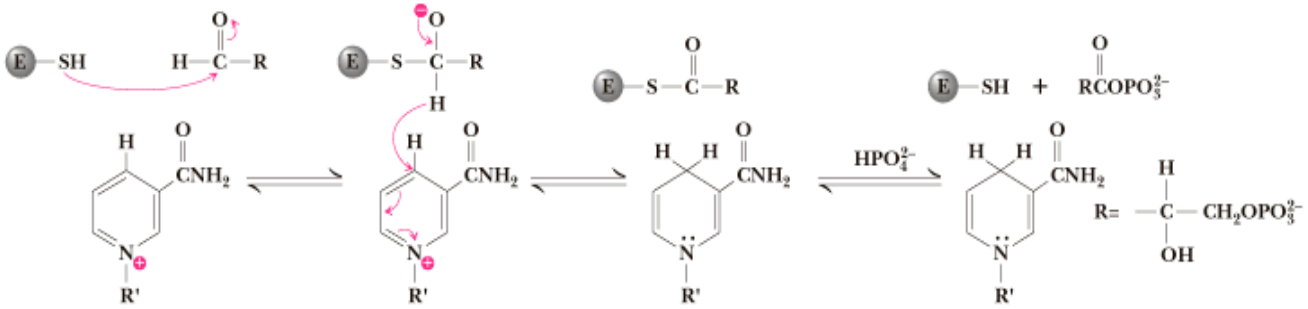




G-3-P dehydrogenase



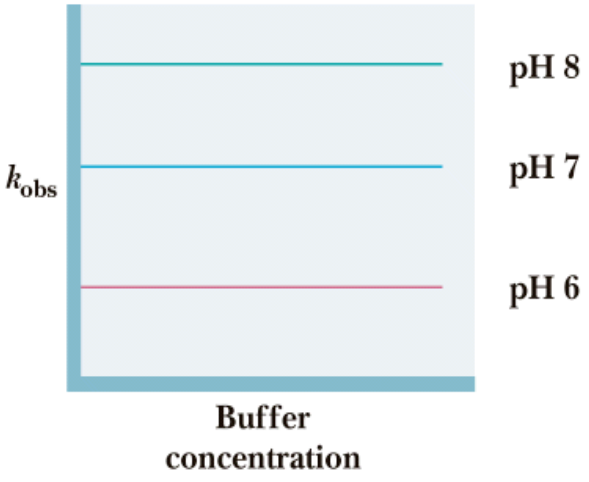
acyl-cys



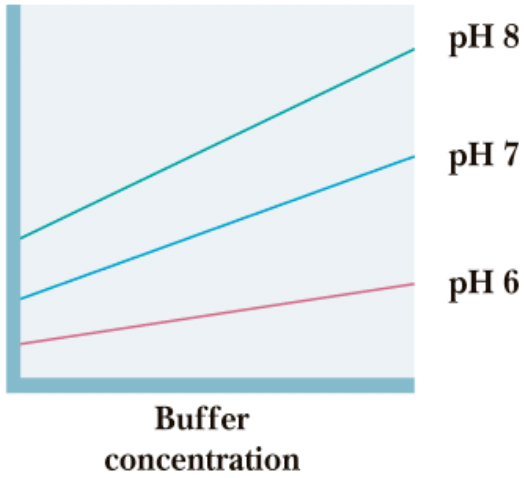
General Acid-base Catalysis

a proton is transferred in the transition state

- "Specific" acid-base catalysis involves H^+ or OH^- that diffuses into the catalytic center
- "General" acid-base catalysis involves acids and bases other than H^+ and OH^-
- These other acids and bases facilitate transfer of H^+ in the transition state



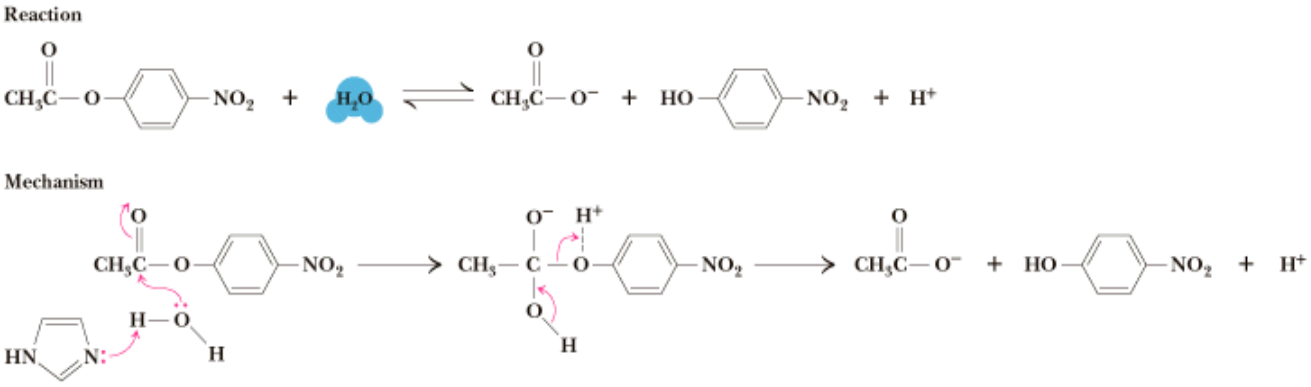
Specific acid-base catalysis



General acid-base catalysis

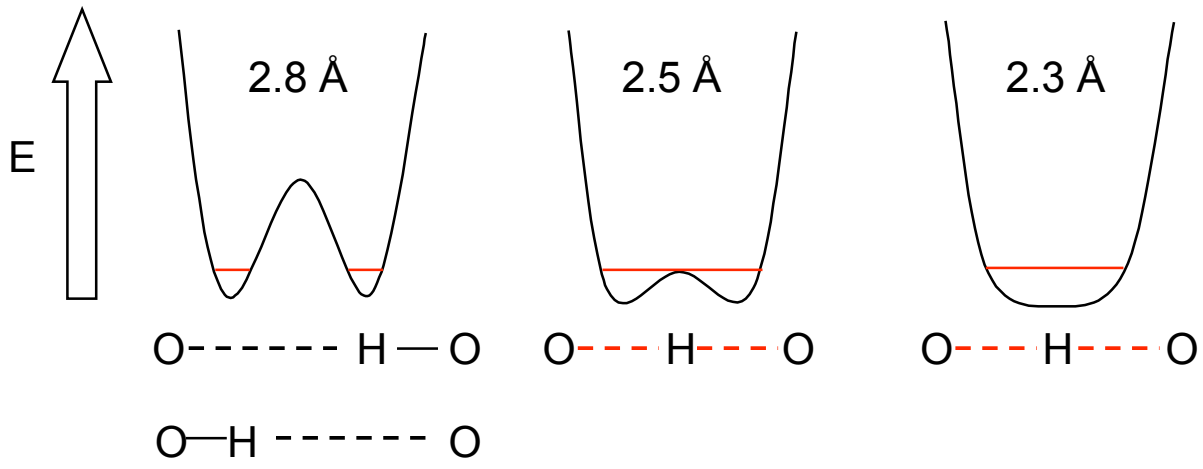
An ionizable group on a protein will be most effective as a H^+ transfer agent at or near its pK_a

Biochemistry usually happens near pH7, where histidine is the most effective general acid or base (imidazole pK_a = 6)



Low-Barrier Hydrogen Bonds

- Typical O - O distance in C=O·····H-O is 2.8 Å
- O-H is 1 Å, H-bond is 1.8 Å
- Bond order ~0.07
- Typical bond strength 10-30 kJ/mol
- Protein structure can constrain H-bond donor and acceptor to be close
- O - O distance may be as low as 2.3 Å
- When there is no barrier to H exchange, the interaction is a low-barrier H-bond
- Typical LBHB strength may be 60 kJ/mol

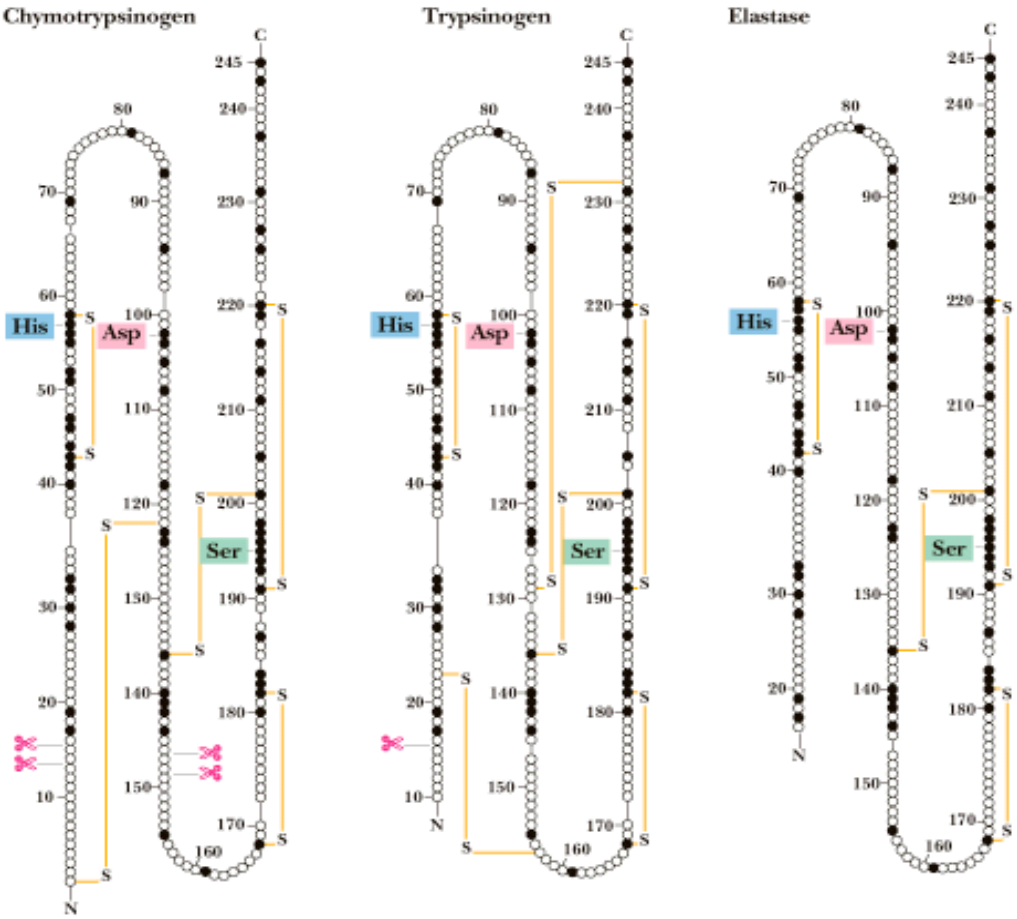


- LBHBs require matched donor/acceptor pK_as
- A weak H-bond in E or ES may become a LBHB in an E'S intermediate or in EX‡

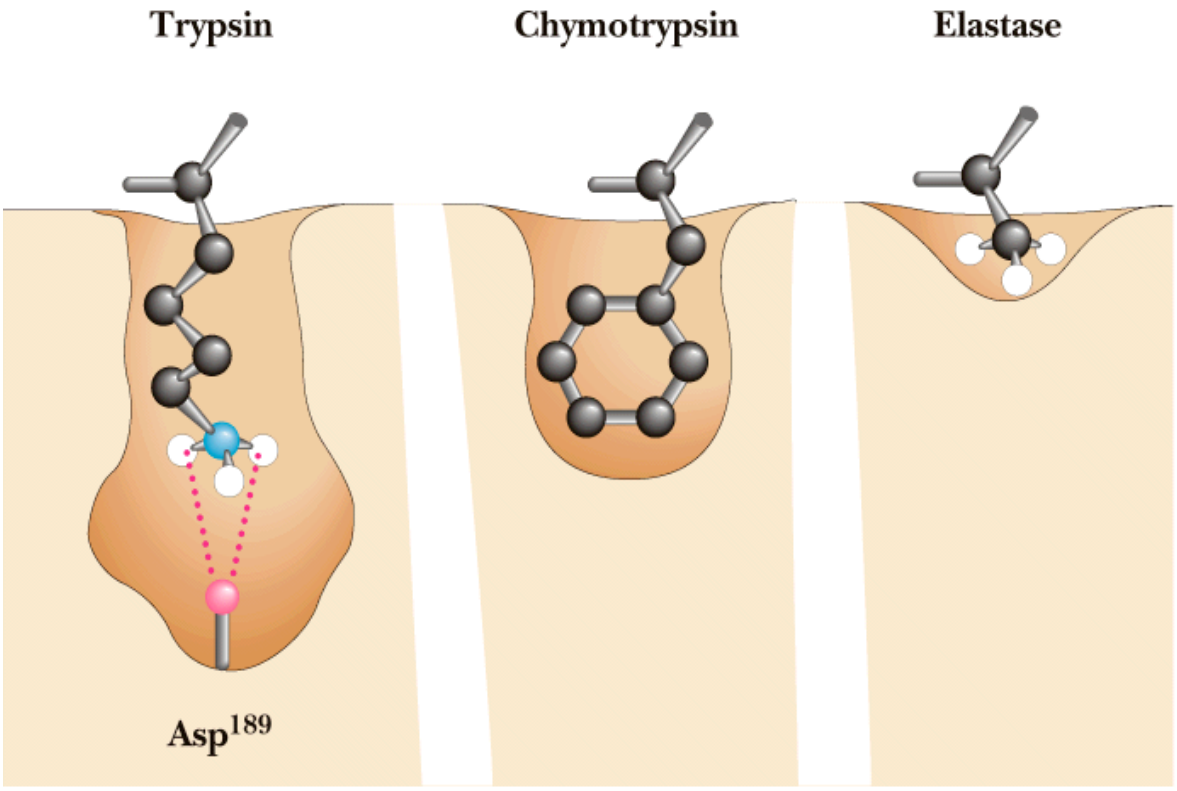
The Serine Proteases

Trypsin, chymotrypsin, elastase, thrombin, subtilisin, plasmin...

- All involve a serine in catalysis - thus the name
- Ser is part of a catalytic triad of ser, his, asp
- Serine proteases are homologous, but locations of the three crucial residues differ somewhat
- Enzymologists agree, however, to number them always as his57, asp102, ser195

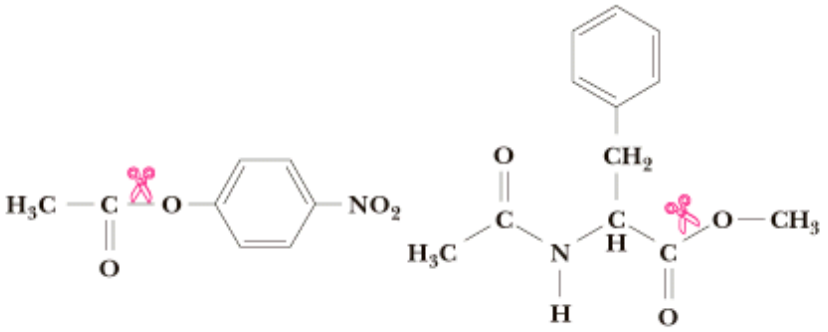


SubstrateSpecificity in the Serine Proteases



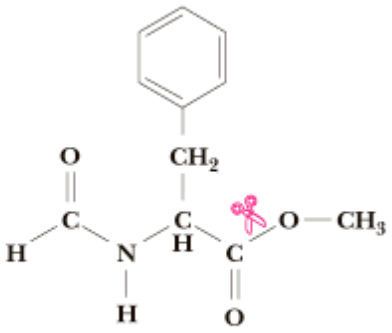
Experimental Evidence for Mechanism

- Most studies use artificial substrates
- p-nitrophenylacetate cleaved to p-nitrophenolate (λ_{max} = 400 nm)
- At high [E], a rapid burst of p-nitrophenolate is observed
- Followed by slower, steady-state hydrolysis

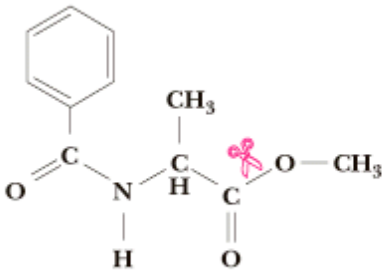


p-Nitrophenylacetate

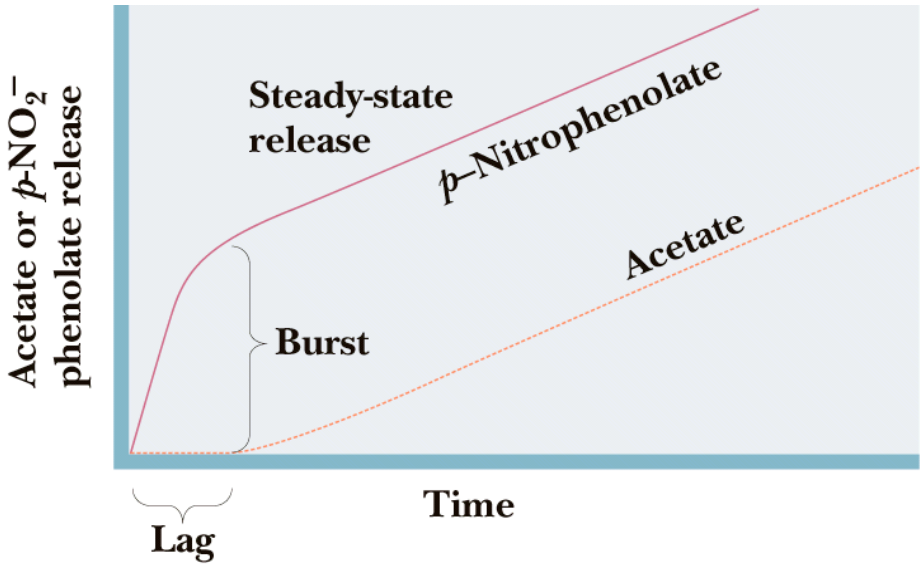
Acetylphenylalanine methyl ester



Formylphenylalanine methyl ester



Benzoylalanine methyl ester

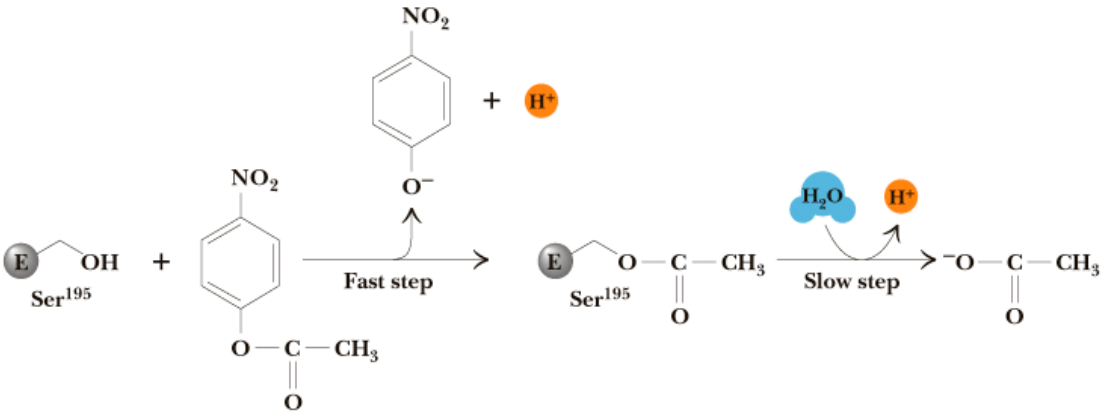


Burst-phase kinetics

- Evidence for a 2-step mechanism
- Fast first step
- Slower second step

- $E + A \rightleftharpoons E'P + H_2O \overset{rds}{\rightleftharpoons} Q + E$

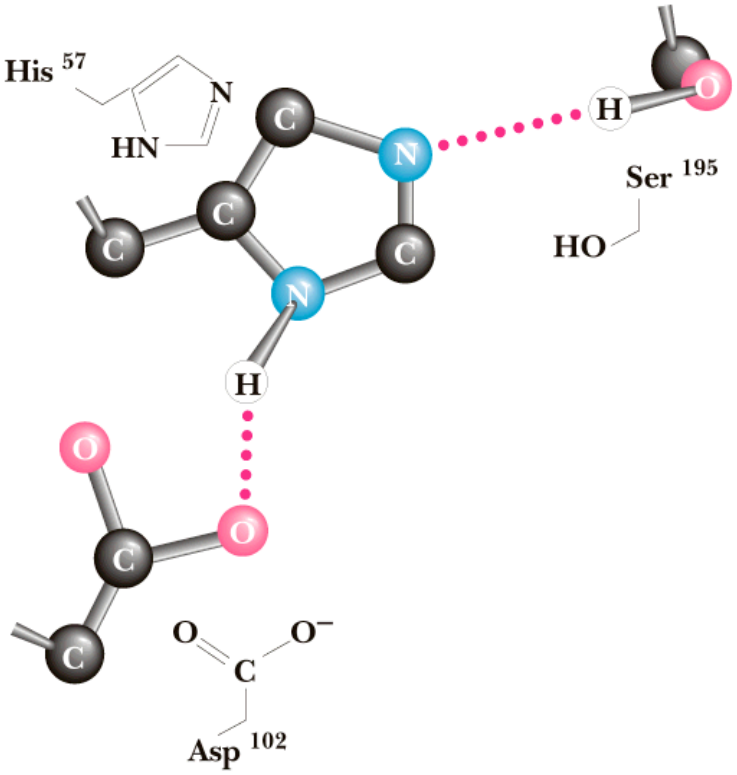
- Fast when [E'P] is v. small
- Slows down until E is saturated by E'P



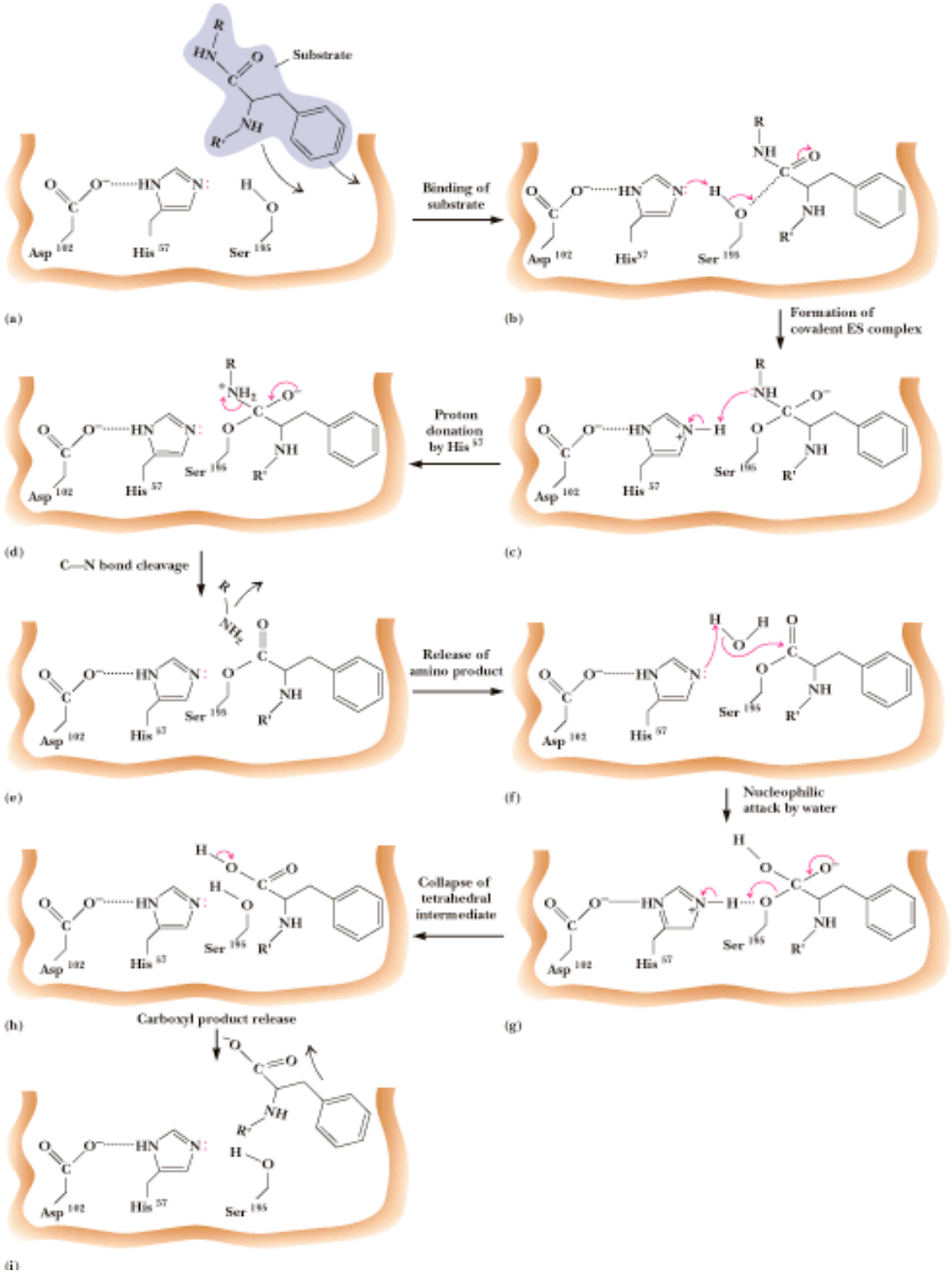
Serine Protease Mechanism

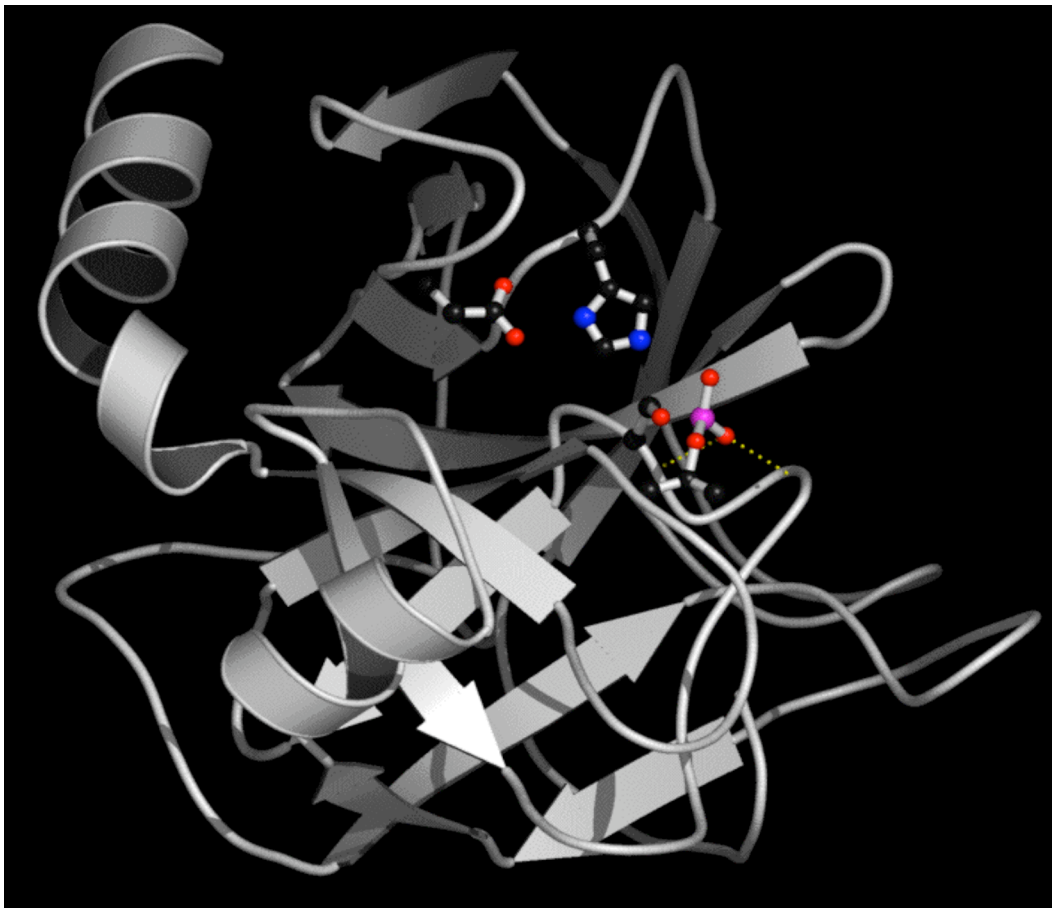
A mixture of covalent and general acid-base catalysis

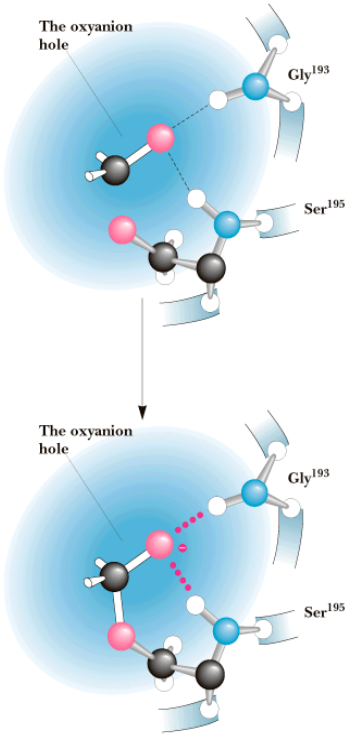
- Asp102 functions only to orient his57
- His57 acts as a general acid and base
- Ser195 forms a covalent bond with peptide to be cleaved
- Covalent bond formation turns sp^2 C into sp^3
- The tetrahedral oxyanion intermediate is stabilized by NH of gly193 and ser195



A Detailed Mechanism for Chymotrypsin





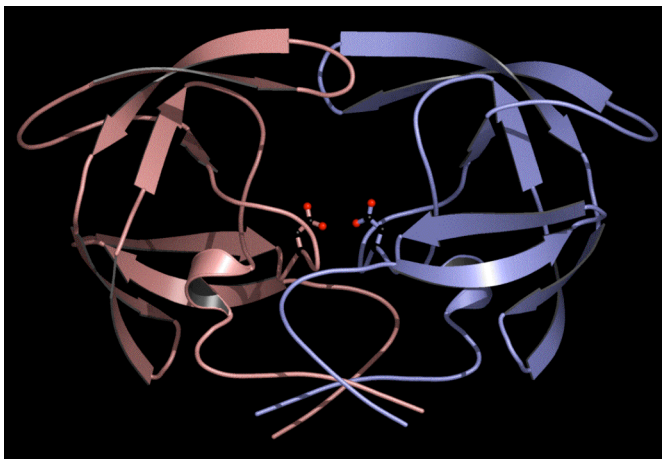

The oxyanion hole
Gly193
Ser195
The oxyanion hole
Gly193
Ser195

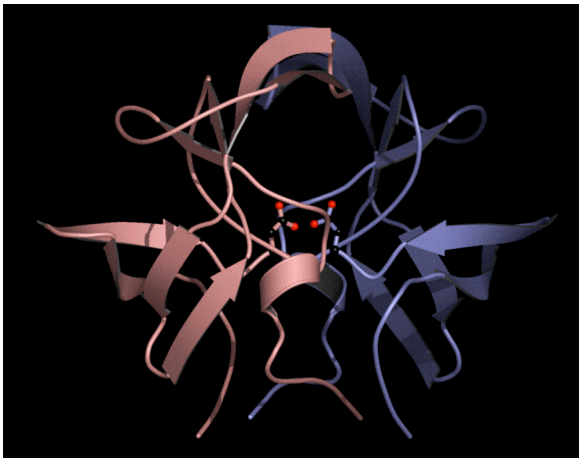
The Aspartic Proteases

pepsin, chymosin, cathepsin D, renin and HIV-1 protease

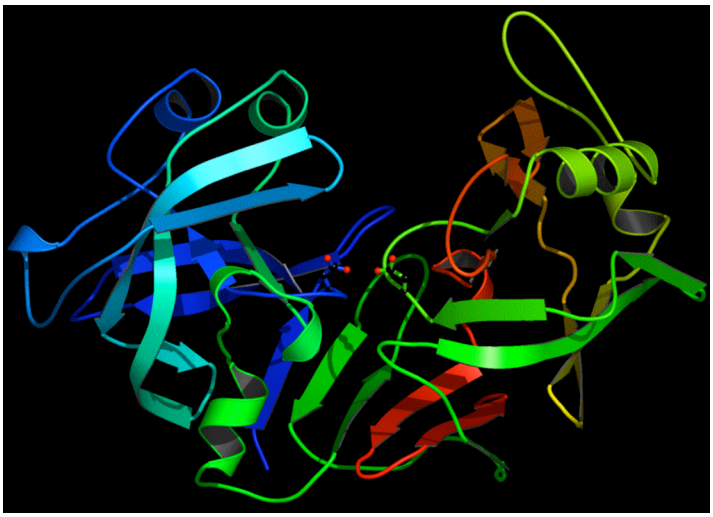
- All involve two asp residues at the active site
- Two asps work together as general acid-base catalysts
- Most aspartic proteases have a tertiary structure consisting of two lobes (N-terminal and C-terminal) with approximate two-fold symmetry
- HIV-1 protease is a homodimer

HIV Protease





Pepsin

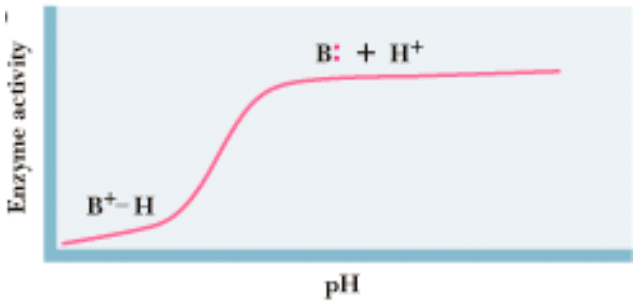


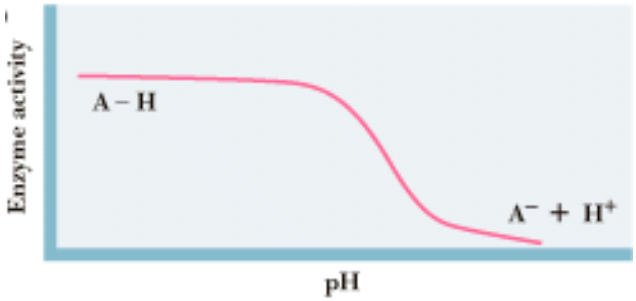
Aspartic Protease Mechanism

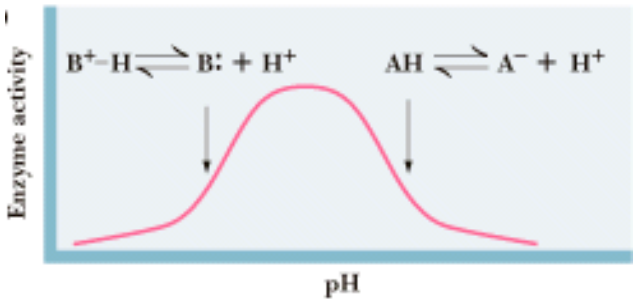
the pK_a values of the asp residues are crucial

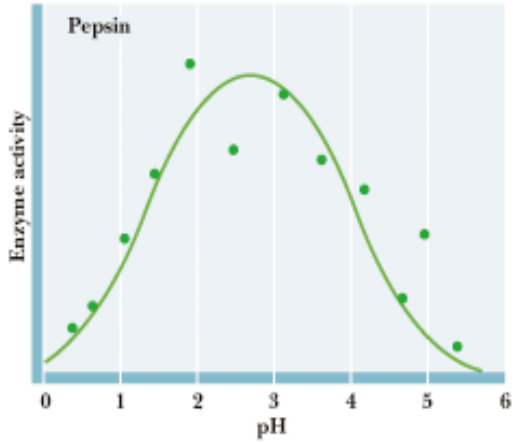
- One asp has a relatively low pK_a, other has a relatively high pK_a
- Deprotonated asp acts as general base, accepting a proton from H_2O, forming OH^- in the transition state
- Other asp (general acid) donates a proton, facilitating formation of tetrahedral intermediate

- What evidence exists to support the hypothesis of different pK_a values for the two asp residues?
- If activity increases with increasing pH, there is likely a general base at the active site
  - –can't function when protonated (low pH)
- If activity decreases with increasing pH, there is likely a general acid at the active site
  - –can't function when deprotonated (high pH)
- If both, we get a bell-shaped activity profile



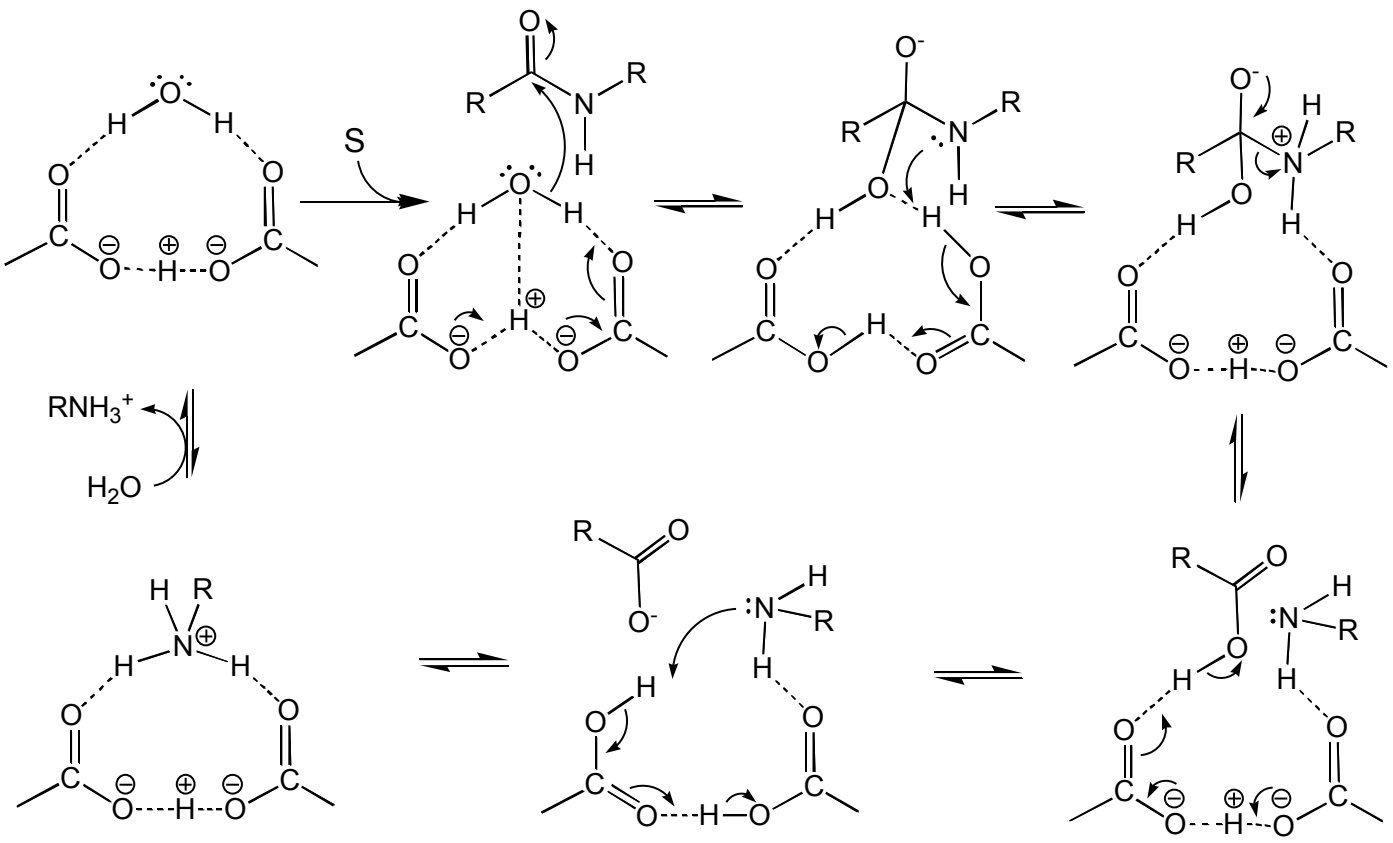






- Curve fitting allows an estimate of pK_as
- In pepsin, one asp has pK_a of 1.4, the other 4.3
- This simple model was modified in 2000…

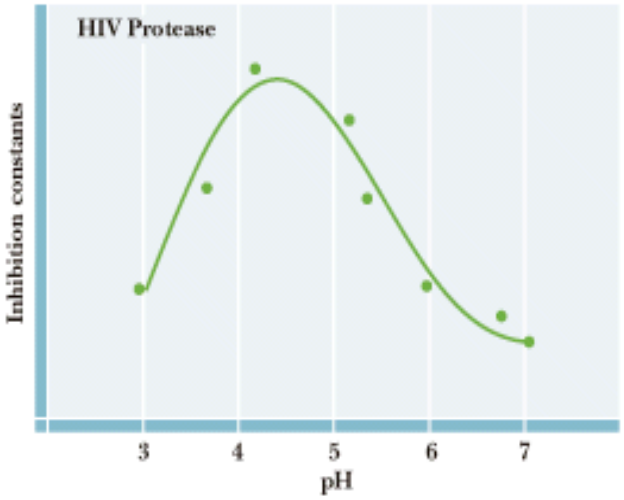
A Mechanism for Asp Proteases



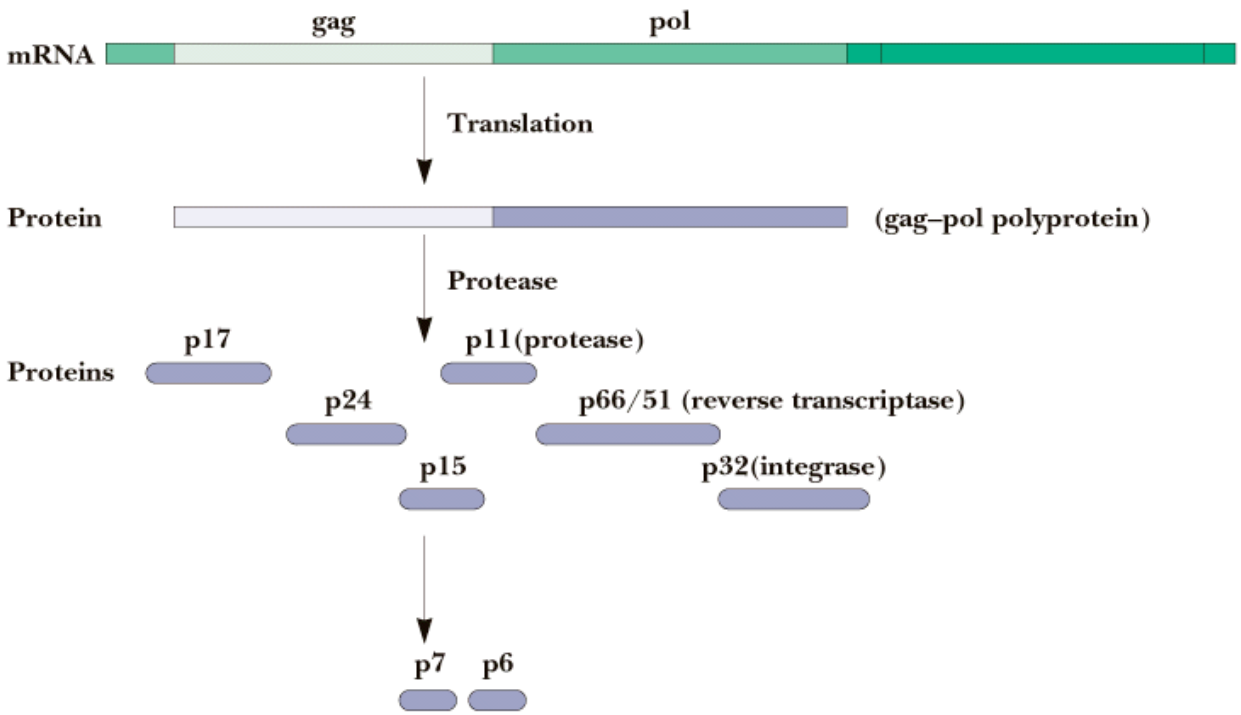
HIV-1 Protease

a novel aspartic protease

- HIV-1 protease cleaves the polyprotein products of the HIV genome
- This is a remarkable imitation of mammalian aspartic proteases
- HIV-1 protease is a homodimer - more genetically economical for the virus
- Active site is two-fold symmetric
- Mechanism doesn't need different pK_as



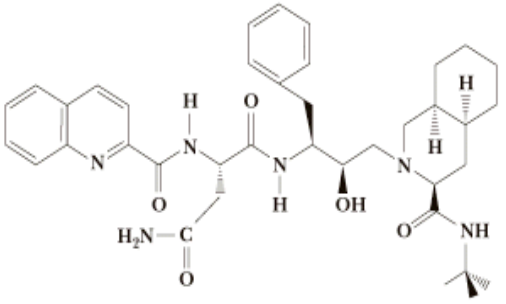
What does HIV Protease do?



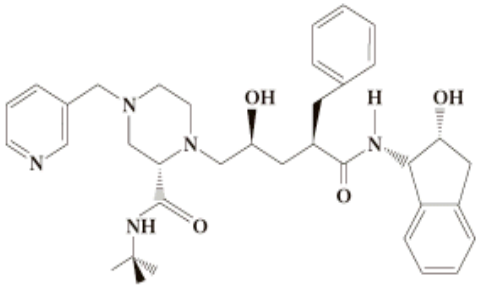
Therapy for HIV?

protease inhibitors as AIDS drugs

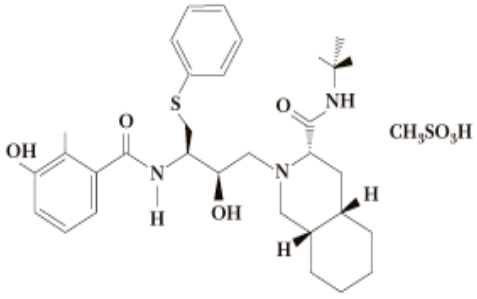
- If the HIV protease can be selectively inhibited, then new HIV particles cannot form
- Several novel protease inhibitors are currently marketed as AIDS drugs
- Many such inhibitors work in a culture dish
- However, a successful drug must be able to kill the virus in a human subject without blocking other essential proteases in the body



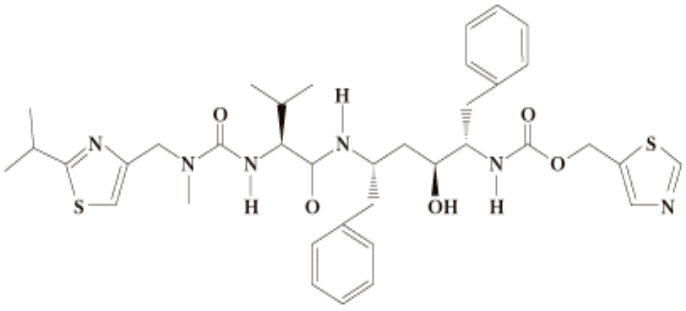
Invirase (Saquinavir)



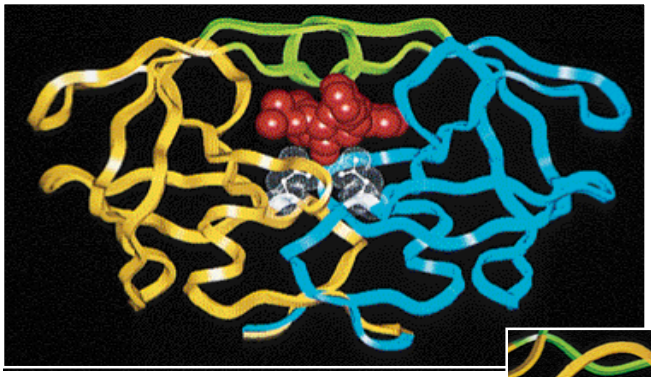
Crixivan (Indinavir)

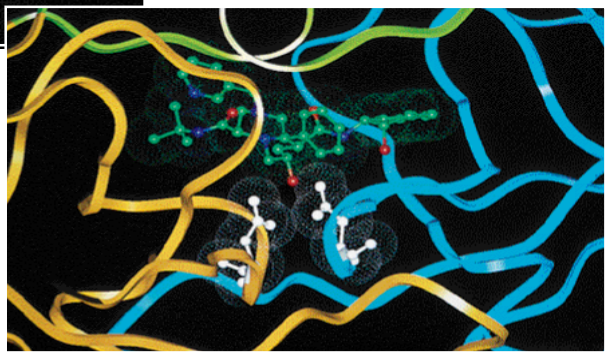


Viracept (Nelfinavir mesylate)



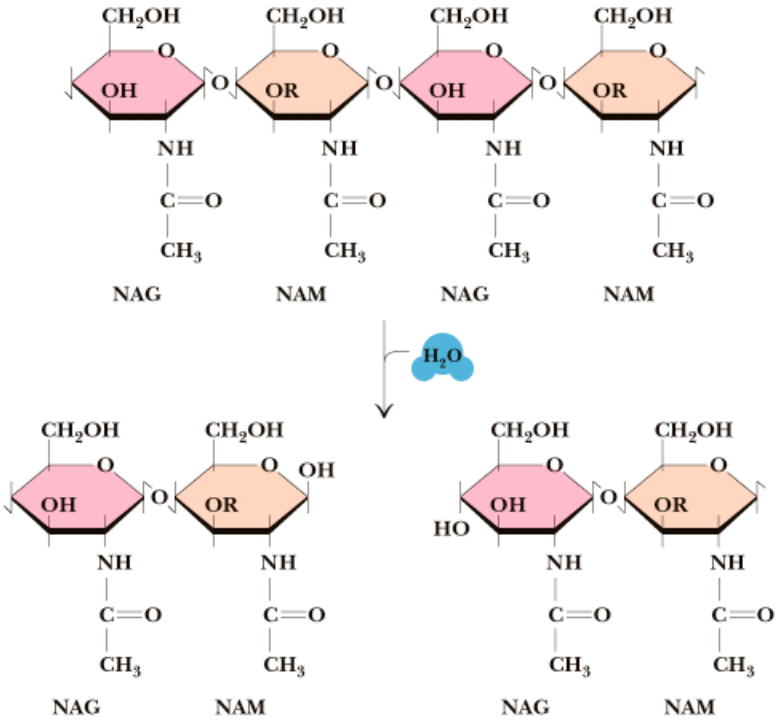
Norvir (Ritonavir)

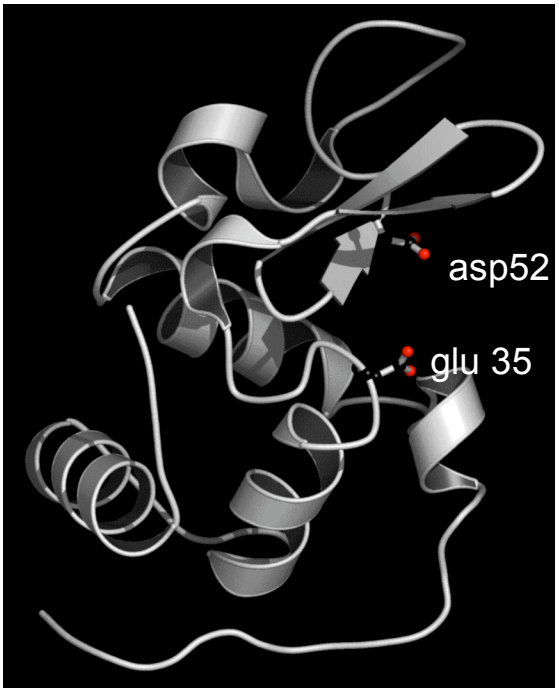




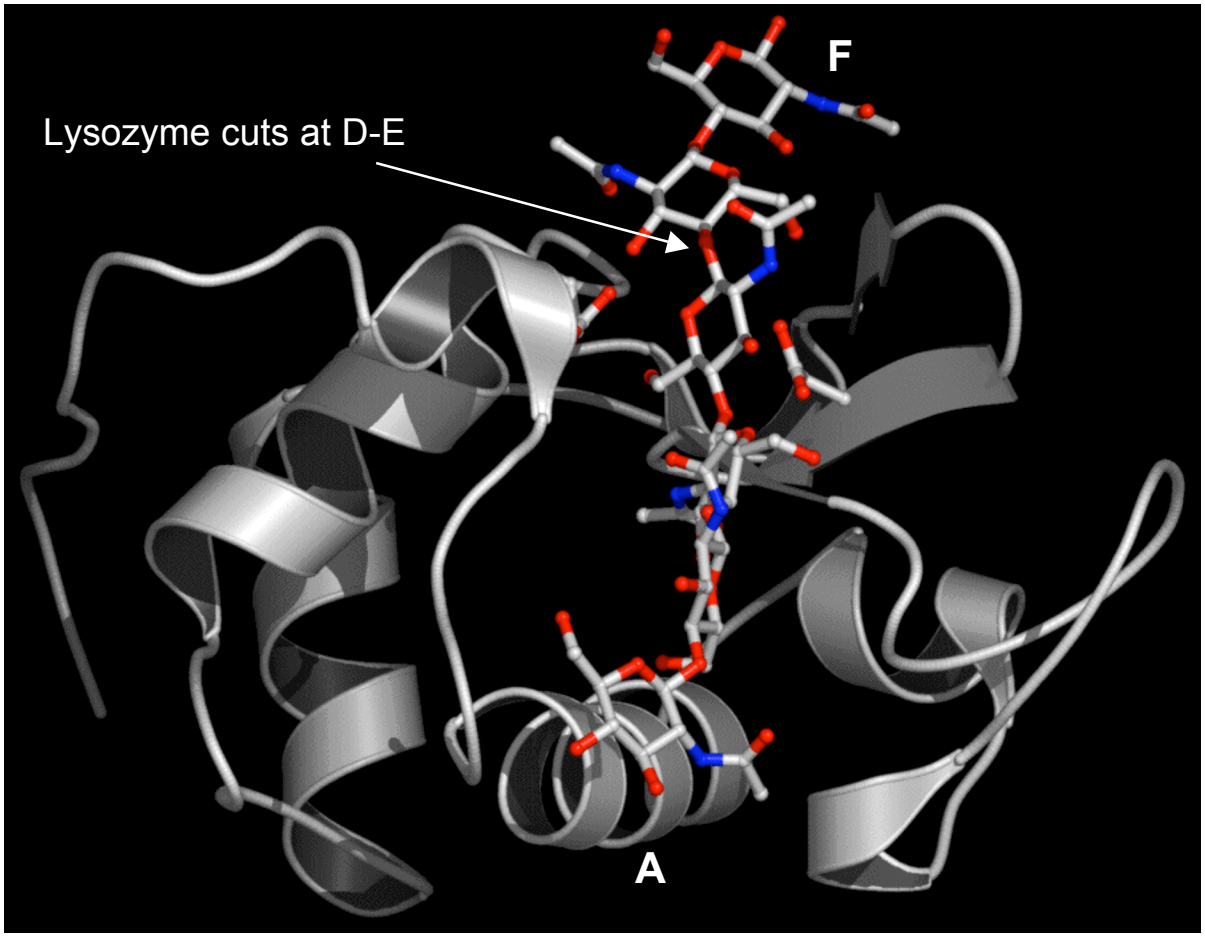
Lysozyme

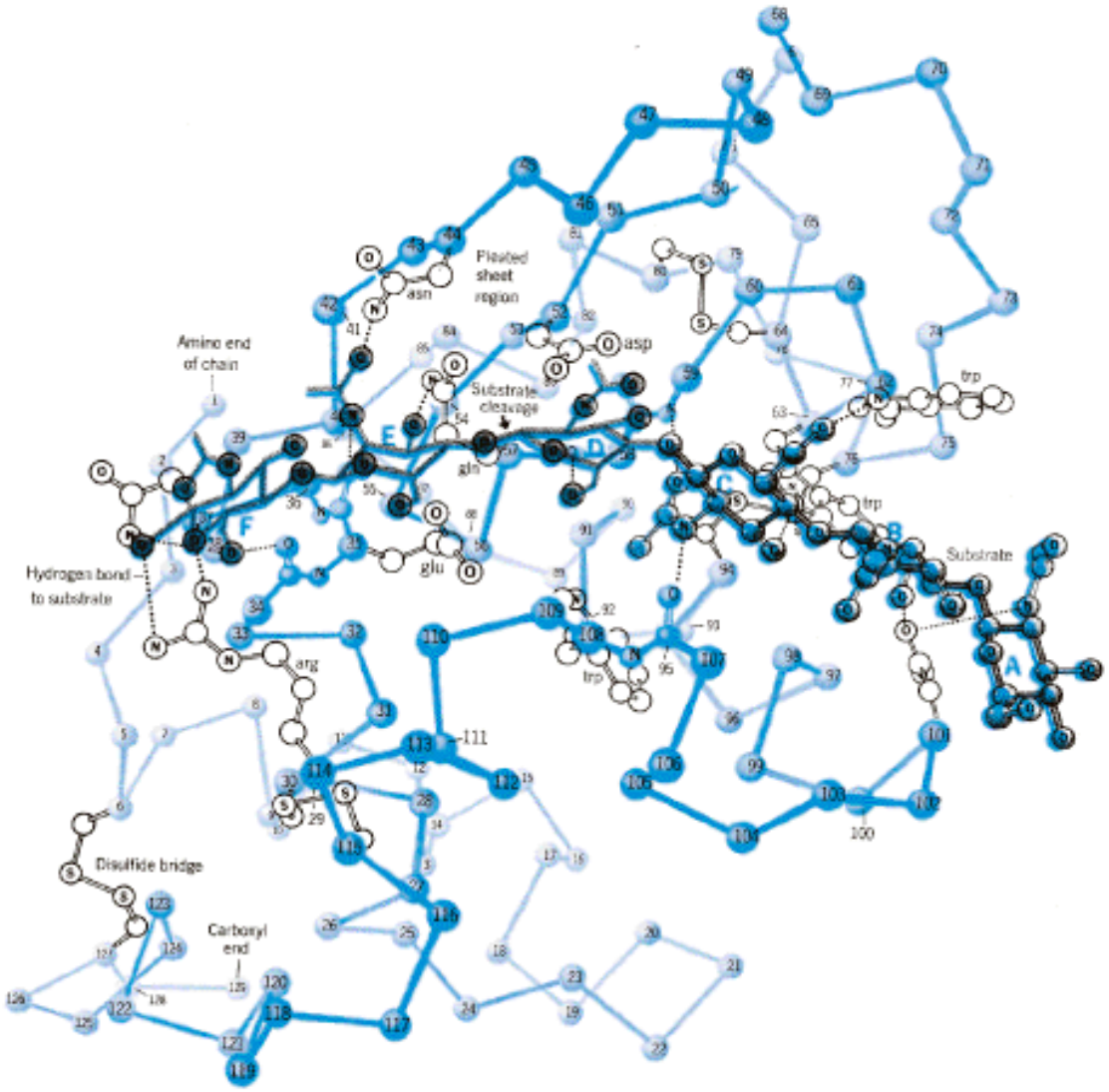
- Lysozyme hydrolyzes polysaccharide chains and ruptures certain bacterial cells by breaking down the cell wall
- Hen egg white enzyme has 129 residues with four disulfide bonds
- The first enzyme whose structure was solved by X-ray crystallography (by David Phillips in 1965)

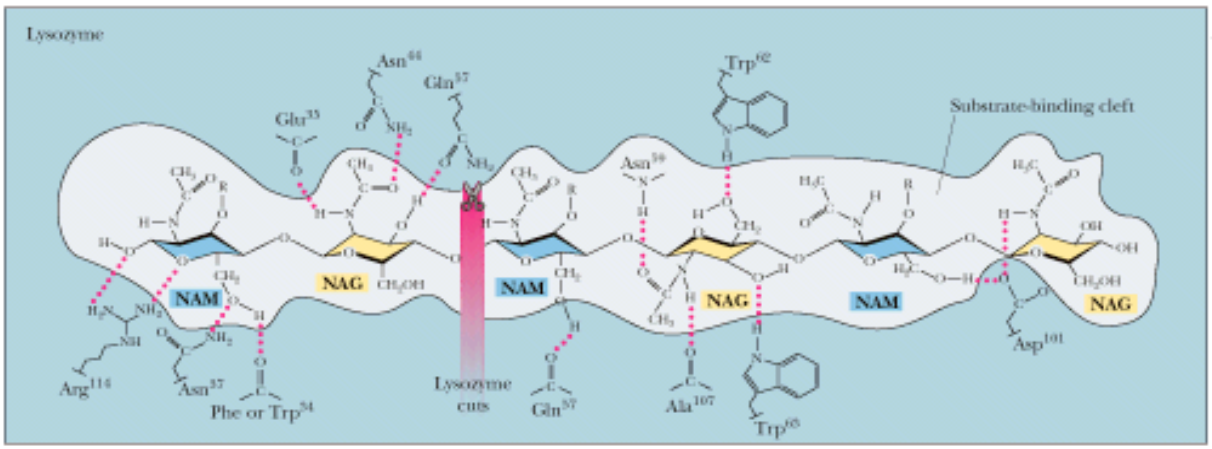




NAG - N-acetylglucosamine
NAM - N-acetylmuraminic acid

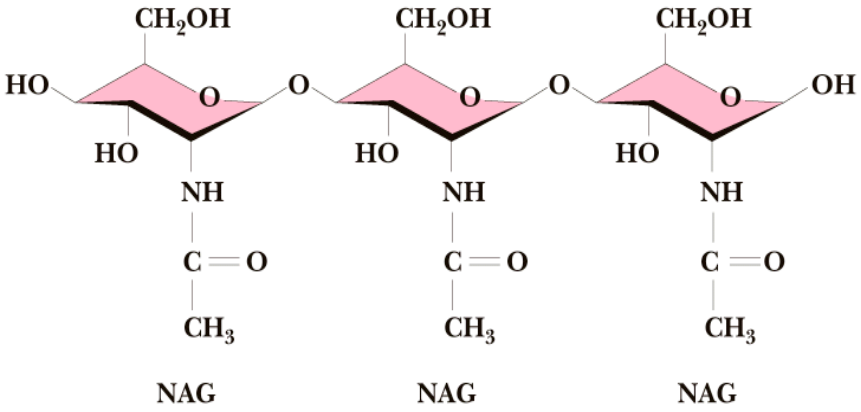

Lysozyme cuts at D-E
F
A


Pleated sheet region
Amino end of chain
Substrate cleavage
Hydrogen bond to substrate
Disulfide bridge
Carboxyl end
Substrate
asn
asp
glu
gln
arg
trp
A
B
C
D
E
F


Lysozyme
Substrate-binding cleft
NAM
NAG
Lysozyme cuts
Phe or Trp54
Asp101

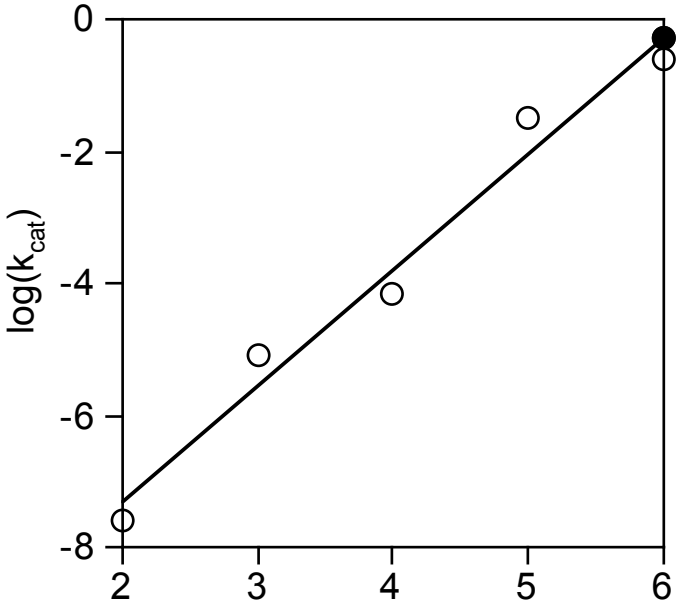
Substrate Analog Studies

- Natural substrates are not stable in the active site for structural studies
- But analogs can be used - like $(NAG)_3$
- Fitting a NAG into the D site requires a distortion of the sugar
- This argues for stabilization of a transition state via destabilization (distortion and strain) of the substrate



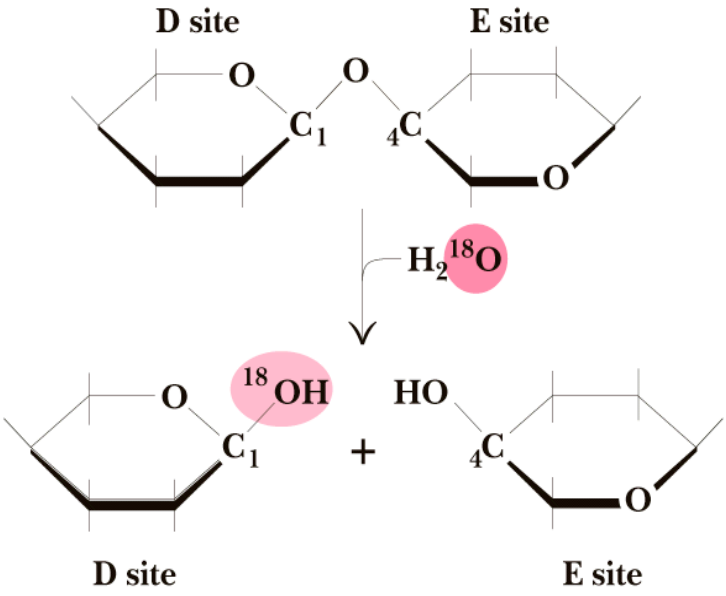
Hydrolysis Rates for Model Oligosaccharides

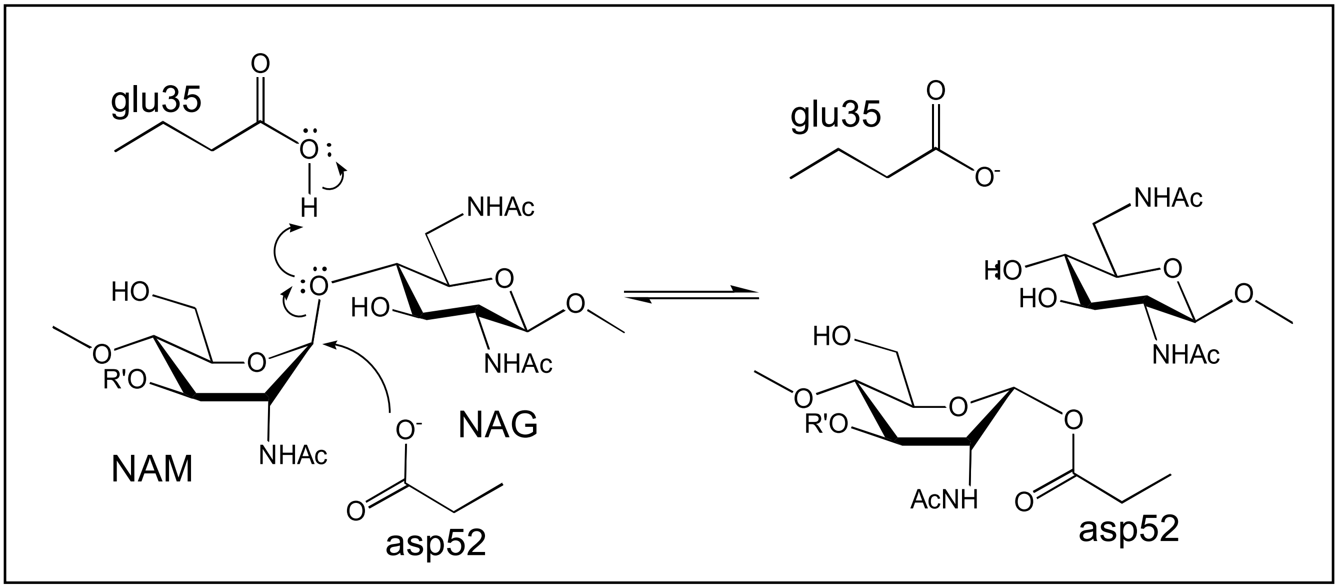
Oligosaccharide	k_{cat} (s^{-1})
$(NAG\text{-}NAM)_3$	0.5
$(NAG)_6$	0.25
$(NAG)_5$	0.033
$(NAG)_4$	7×10^{-5}
$(NAG)_3$	8×10^{-6}
$(NAG)_2$	2.5×10^{-8}

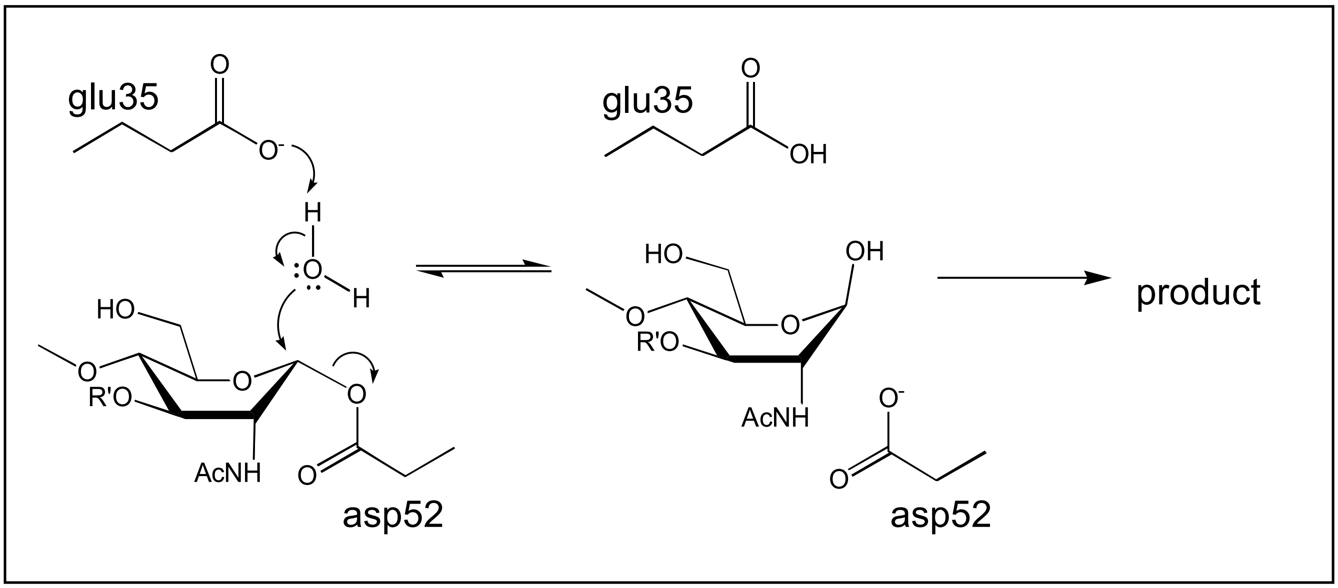


The Lysozyme Mechanism

- Studies with ^{18}O-enriched water show that the C_1-O bond is cleaved on the substrate between the D and E sites
- This incorporates ^{18}O into C_1
- Glu^{35} acts as a general acid
- Asp^{52} forms a covalent intermediate




glu35
O
H
NHAc
HO
HO
O
NAM
R'O
NHAc
O-
NAG
O
asp52
glu35
O-
NHAc
HO
HO
HO
R'O
AcNH
NHAc
asp52


glu35
O
O-
H
O
H
HO
R'O
AcNH
asp52
glu35
OH
HO
OH
R'O
AcNH
O-
asp52
product

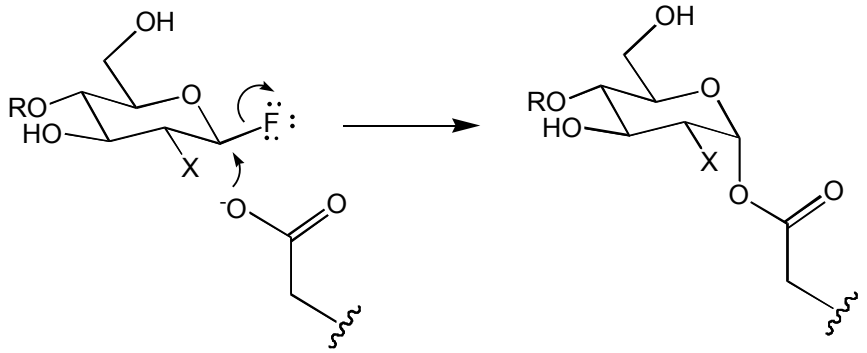
Mechanistic Evidence

can a covalent intermediate be observed?

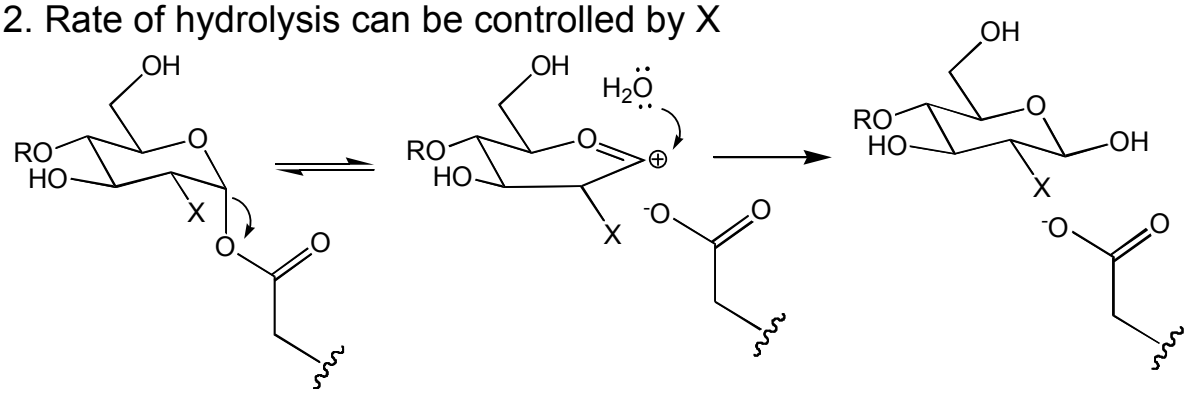
- How to make the rate of formation of the covalent intermediate faster than its breakdown?
- Mutate glu52 to gln
- Slows the reaction enough to see intermediates br mass spectrometry
- Deactivate the glycosidic C_1 to slow hydrolysis of the intermediate sufficiently for crystallography

Modification of the Substrate Slows Step 2 Even More

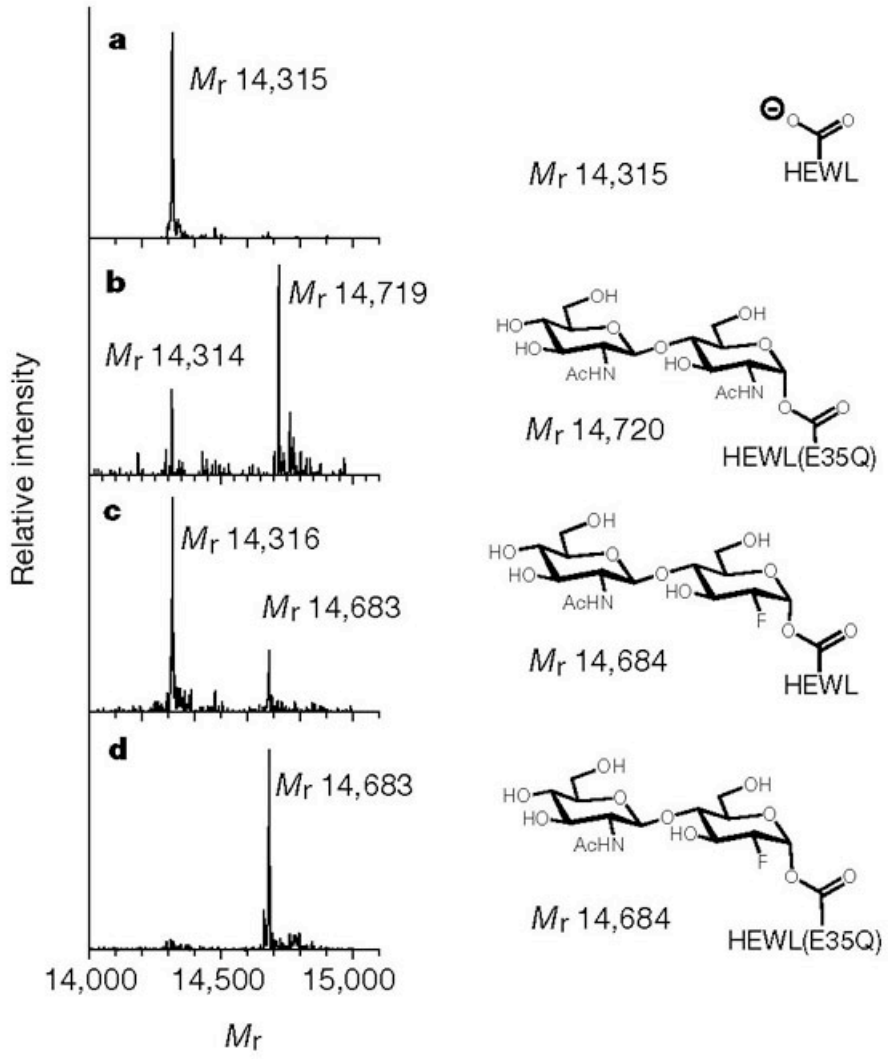
1. Formation of the covalent intermediate

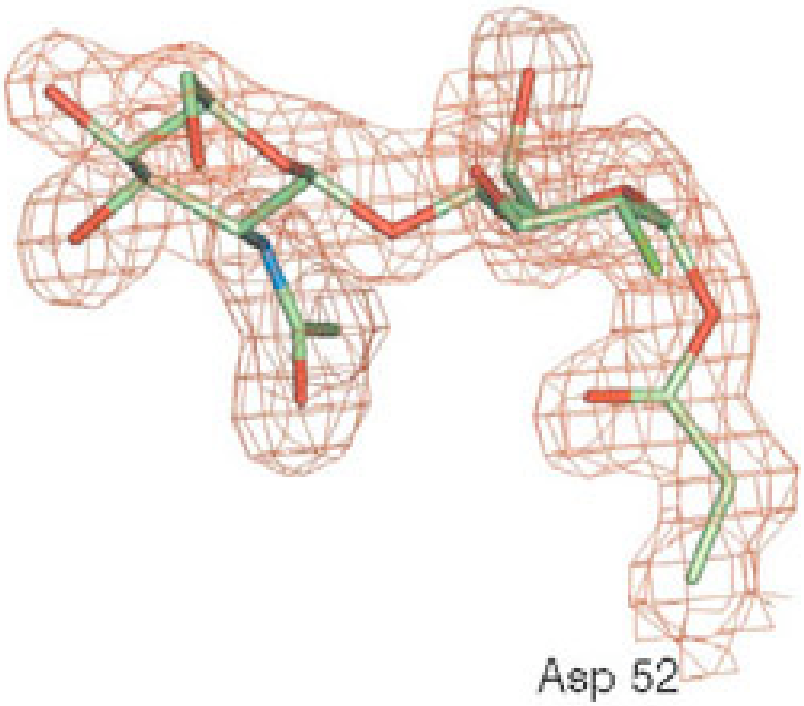


2. Rate of hydrolysis can be controlled by X



Electronegative X destabilizes oxocarbenium intermediate


a
M_r 14,315
b
M_r 14,719
M_r 14,314
c
M_r 14,316
M_r 14,683
d
M_r 14,683
Relative intensity
14,000
14,500
15,000
M_r
M_r 14,315
HEWL
M_r 14,720
HEWL(E35Q)
M_r 14,684
HEWL
M_r 14,684
HEWL(E35Q)


Asp 52

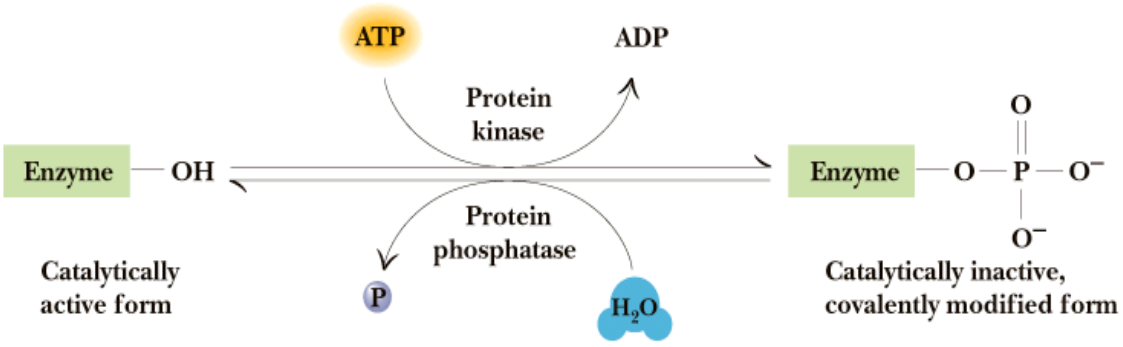
Controls over Enzyme Activity

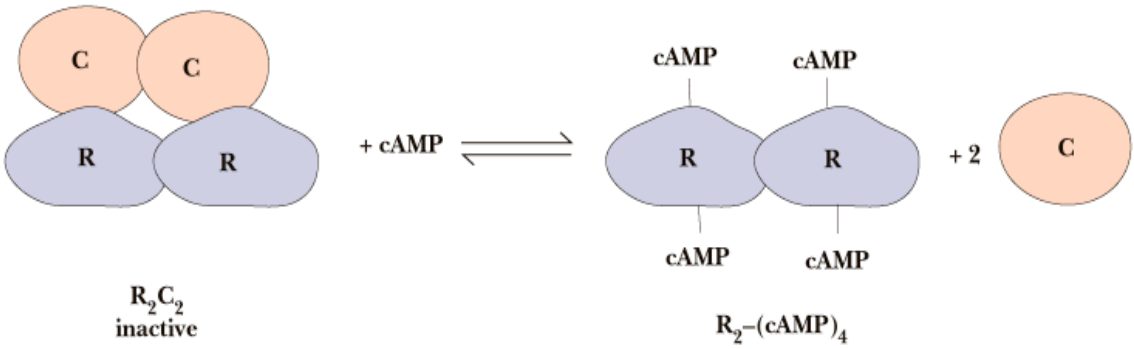
- Rate slows as product accumulates
- Rate depends on substrate availability
- Genetic controls - induction and repression
- Enzymes can be modified covalently
- Zymogens, isozymes and modulator proteins
- Allosteric effectors and inhibitors

Interconvertible Enzymes

Enzymes regulated by covalent modification

- Converter enzymes
  - –Protein kinase, protein phosphatase
  - –Phosphorylation at S,Y,T modulates enzyme activity



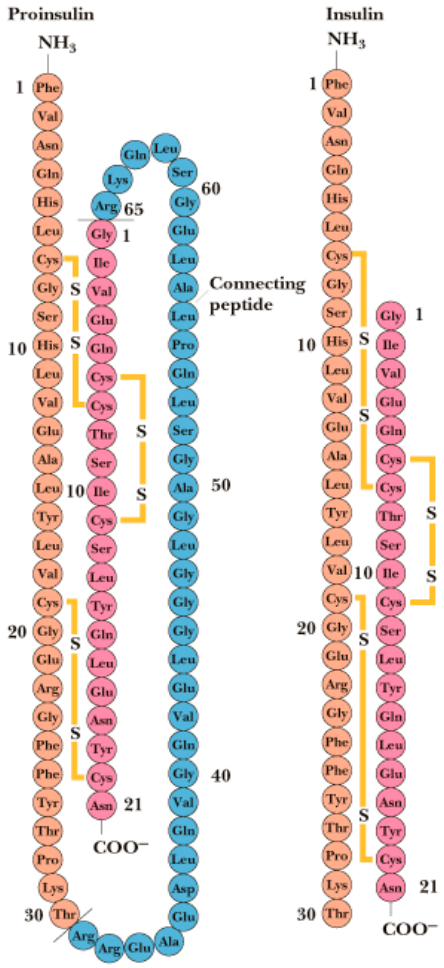


- Cyclic AMP-dependent protein kinase (PKA) is a R_2C_2 tetramer
- Regulatory (R) subunits bind cAMP (K_D = 30 nM)
- cAMP binding releases R subunits from C (catalytic) subunits

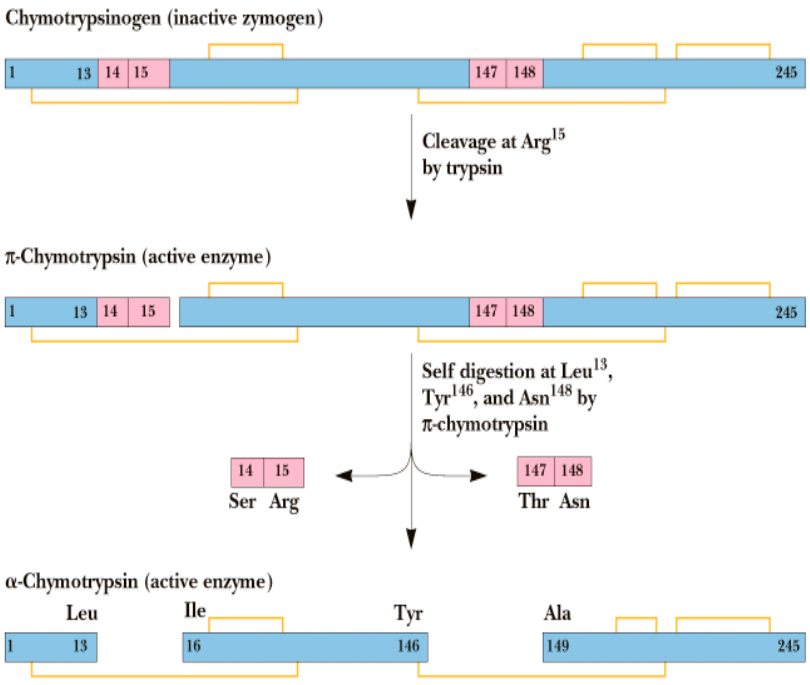
Zymogens - Inactive Enzyme Precursors

Enzymes regulated by covalent modification

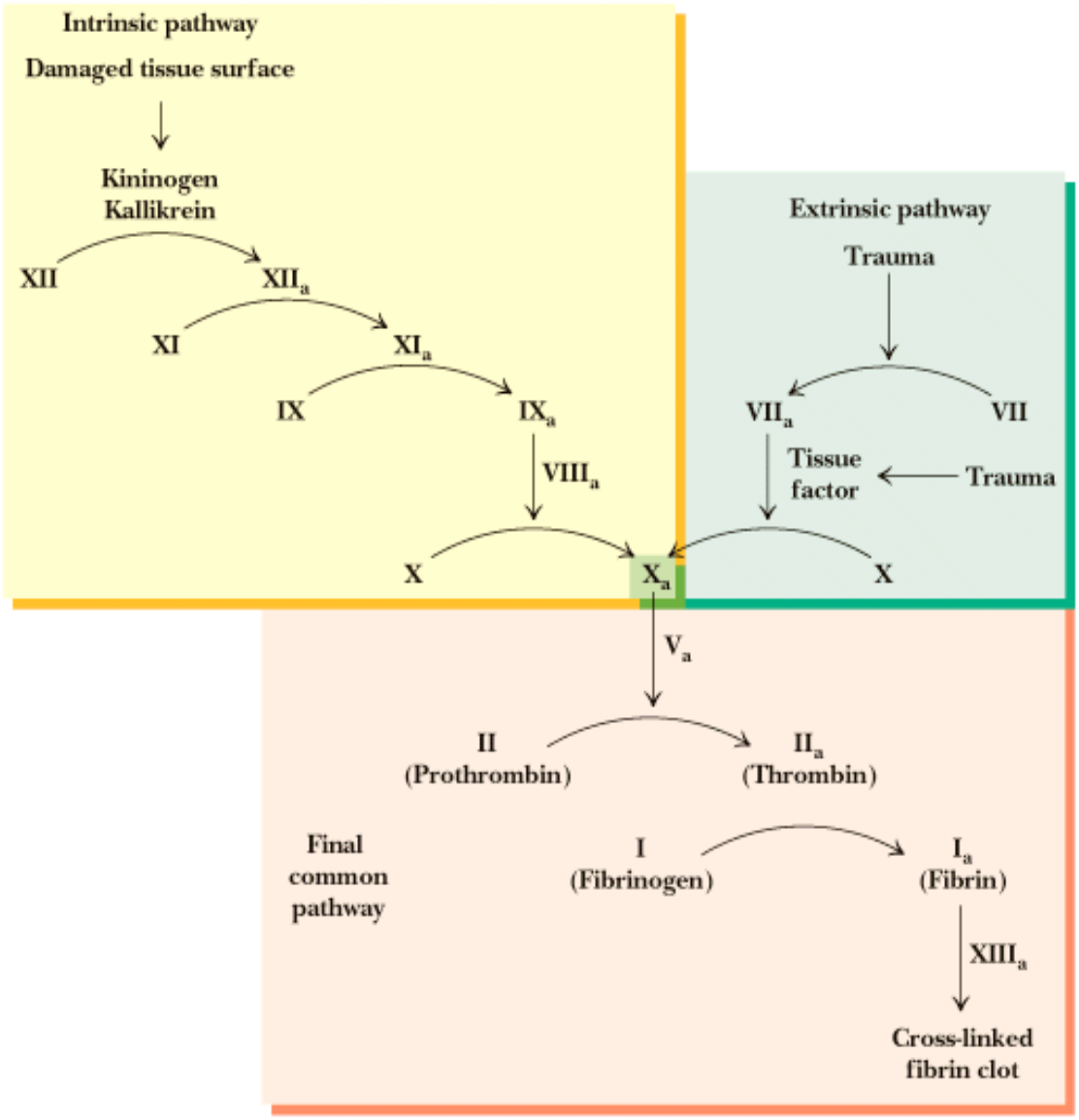
- Zymogens, or proenzymes, are synthesized as inactive proteins
- Activated by proteolysis
- Irreversible (unlike allosteric regulation or covalent modification)
  - –insulin
  - –digestive tract enzymes
  - –blood clotting factors
  - –caspases



Proteolytic Activation of Chymotrypsinogen



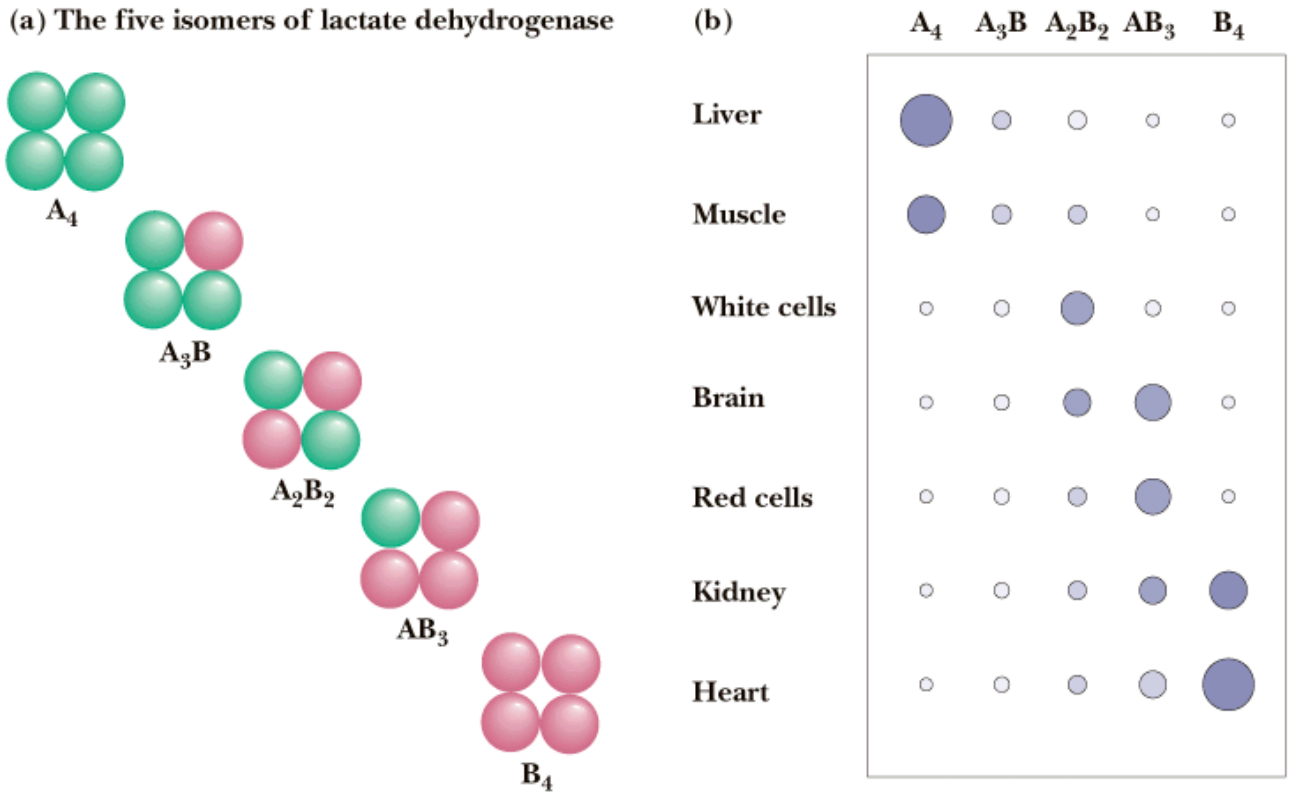
The Blood Clotting Cascade



Isoenzymes

multimeric enzymes with slightly different subunits

- Lactate dehydrogenase (LDH) exists as 5 different isoenzymes
  - A_4, A_3B, A_2B_2, AB_3, B_4
- Cells in different tissues express different levels of A and B and so control the isomeric composition according to their metabolic requirements

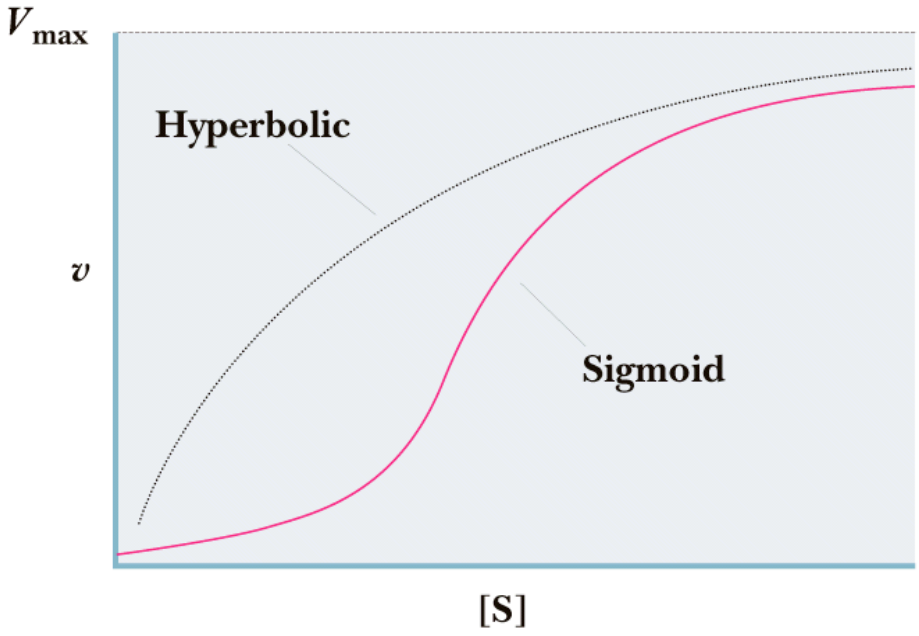


Allosteric Regulation

$$A \xrightarrow{1} B \xrightarrow{2} C \xrightarrow{3} D \xrightarrow{4} E \xrightarrow{5} F$$

- Feedback regulation:
- If F, the essential end product, inhibits enzyme 1, 2, 3 or 4, it blocks its own synthesis (negative feedback)
- If F is an activator of enzyme 1, 2 etc it will accelerate its own synthesis (positive feedback)

- Regulatory enzymes (subject to feedback regulation)
  - Do not obey Michaelis-Menten kinetics
- Behavior of substrates S
  - v_0 vs [S] plots are S-shaped (sigmoidal)
  - v_0 is proportional to $[S]^n$ where n > 1 (power law)
  - Binding of one S to a subunit increases binding of a second S
  - This is positive cooperativity
- Regulation by feedback inhibitors
  - Does not conform to normal inhibition patterns
- Regulatory effects are achieved by conformational changes when effector molecules bind

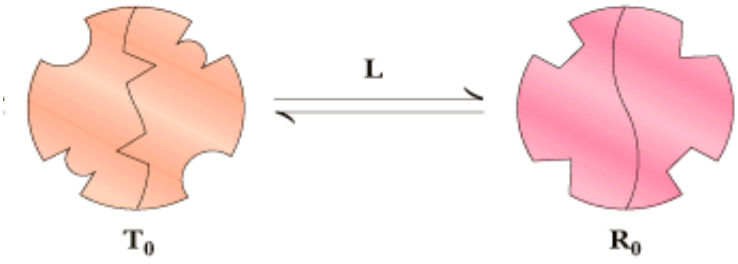


A Model for Allosteric Behavior

- Monod, Wyman, Changeux (MWC) model: allosteric proteins can exist in two states R (relaxed) and T (taut)
- In this model:
  - –all the subunits of an oligomer are in the same state
  - –T state predominates in the absence of substrate S
  - –S binds much tighter to R than to T

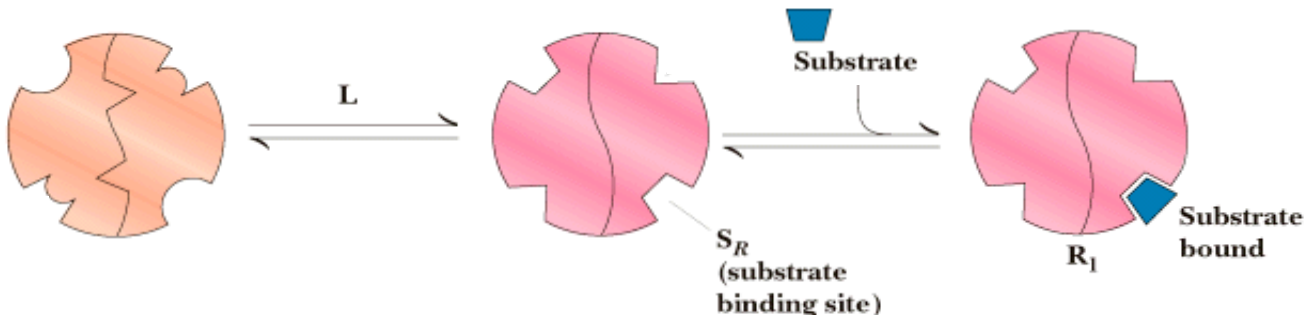
- Cooperativity is achieved because S binding increases the population of R, which increases the sites available to S
- Ligands such as S are positive homotropic effectors
- Molecules that influence the binding of something other than themselves are heterotropic effectors

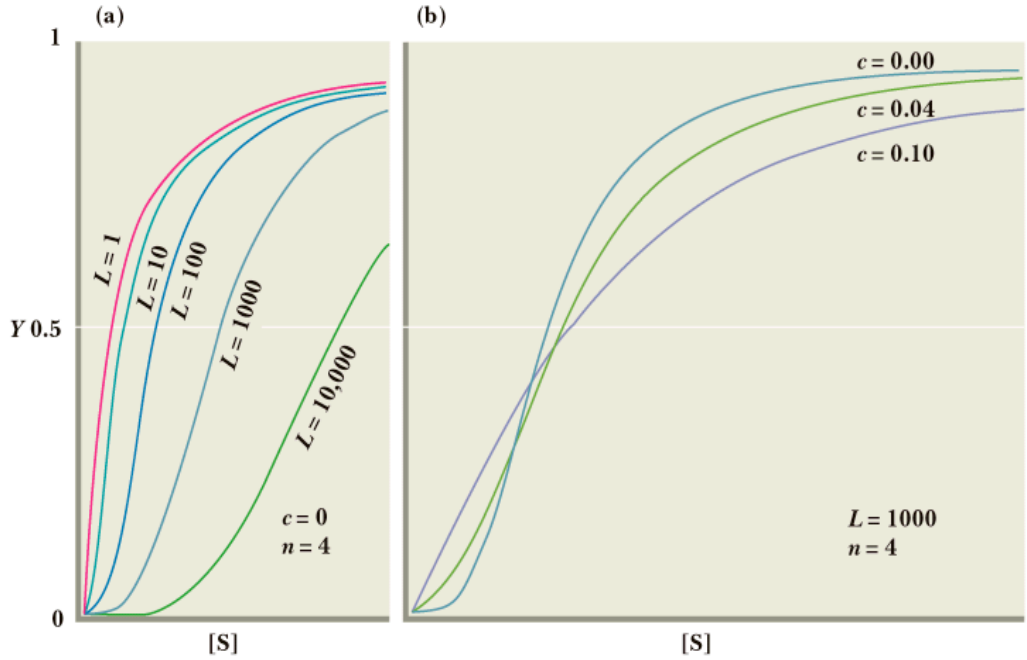
A dimeric protein can exist in either of two conformational states at equilibrium.



$$L = \frac{T_0}{R_0}$$ L is large. ($T_0 >> R_0$)

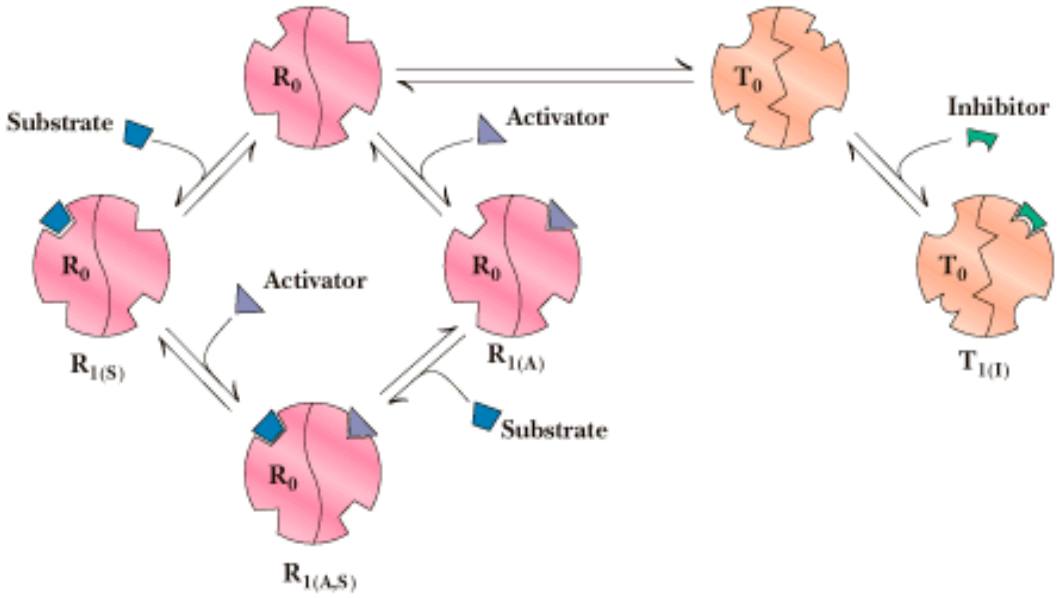
Substrate binding shifts equilibrium in favor of R.





$c = K_R/K_T$ n = number of monomers

$L = T_0/R_0$ Y = [occupied sites]/[total sites]



A dimeric protein which can exist in either of two states R_0 and T_0.
This protein can bind 3 ligands:

1) Substrate (S): A positive homotropic effector that binds only to R at site S

2) Activator (A): A positive heterotropic effector that binds only to R at site F

3) Inhibitor (I): A negative heterotropic effector that binds only to T at site F

Effects of A:
$A + R_0 \rightarrow R_{1(A)}$
Increase in number of R-conformers shifts $R_0 \rightleftharpoons T_0$ so that $T_0 \rightarrow R_0$

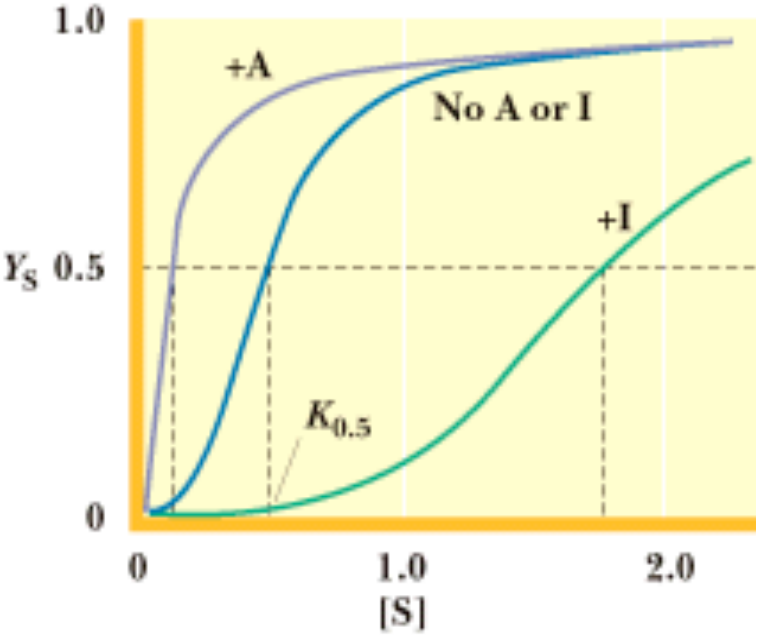
1) More binding sites for S made available

2) Decrease in cooperativity of substrate saturation curve. Effector A lowers the apparent value of *L*.

Effects of I:
$I + T_0 \rightarrow T_{1(I)}$
Increase in number of T-conformers (decrease in R_0 as $R_0 \rightarrow T_0$ to restore equilibrium).

Thus, I inhibits association of S and A with R by lowering R_0 level. I increases cooperativity of substrate saturation curve. I raises the apparent value of *L*.

- This is a K system
- [S] required for half-maximum velocity $K_{0.5}$ changes in response to effectors
- V_{max} is constant for A, I, and no A or I



- V systems are less common
- $K_{0.5}$ remains constant in response to effectors
- V_{max} changes
- v_0 vs [S] plots are hyperbolic, not sigmoidal
- R and T have the same affinity for S but different catalytic efficiencies