



An Example of Enzyme Catalysis: Serine Proteinases

The basic principle underlying enzyme catalysis

An enzyme increases the rate of a chemical reaction by binding and stabilizing the transition state of its specific substrate tighter than the ground state.

Linus Pauling, 1946

The full consequences of Pauling's principle emerged only when it was found that mutants designed to change an enzyme's catalytic rate also changed its substrate specificity and vice versa.

Proteinases – occurrence & function

- Proteinases are widely distributed in nature, where they perform a variety of different functions.
- Viral proteases – cleave the precursor molecules of their coat proteins.
- Bacteria produce many different extracellular proteinases to degrade proteins in their surroundings.
- Higher organisms use proteinases for such different functions as food digestion, cleavage of signal peptides, and control of blood pressure and blood clotting.
- Many proteinases occur as domains in large multifunctional proteins, but others are independent smaller polypeptide chains.
- In vivo* the activity of many proteinases is controlled by endogenous protein inhibitors that complex with the enzymes and block them.

Proteinases form four functional families

-The 3-D structures of a large number of the smaller proteinases and of their complexes with protein inhibitors have been determined, and this wealth of data allows some general conclusions to be drawn.

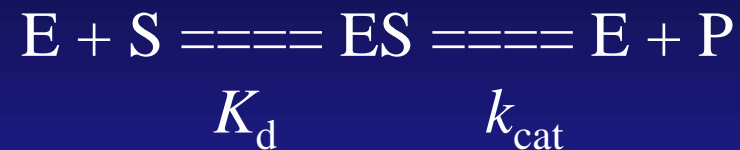
-All the well-characterized proteinases belong to one or other of four families:

- serine proteinases
- cysteine proteinases
- aspartic proteinases
- metallo proteinases

based on a functional criterion, namely, the nature of the most prominent functional group in the active site.

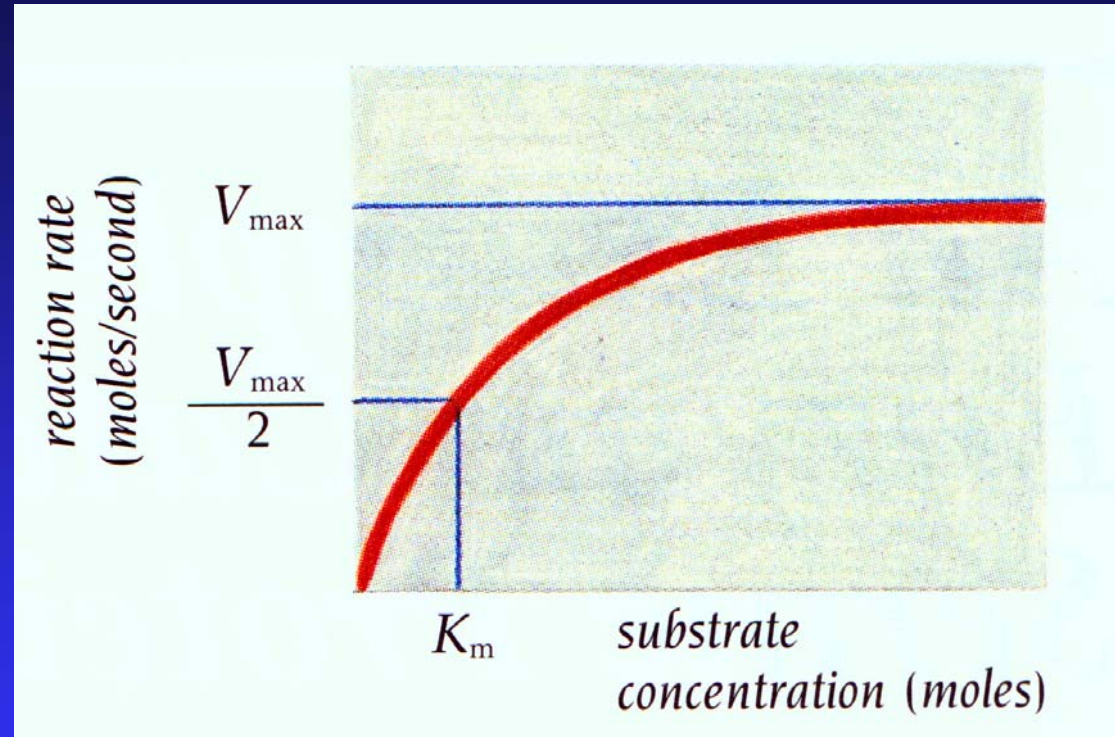
-Two serine proteinases, mammalian **chymotrypsin** and bacterial **subtilisin** are chosen to illustrate one of the catalytic mechanisms leading to proteolysis.

The catalytic properties of enzymes are reflected in K_m and k_{cat} values



- k_{cat} turnover number, which is the maximum number of substrate molecules converted to product per active site of the enzyme per unit time.
- V_{max} a maximum rate that is always observed when the substrate concentration is much higher than the concentration of enzyme.
- K_m the substrate concentration when the half maximal rate is achieved.
- k_{cat}/K_m specificity constant; a rate constant that refers to the overall conversion of substrate into product; useful quantity for comparison of mutant proteins.

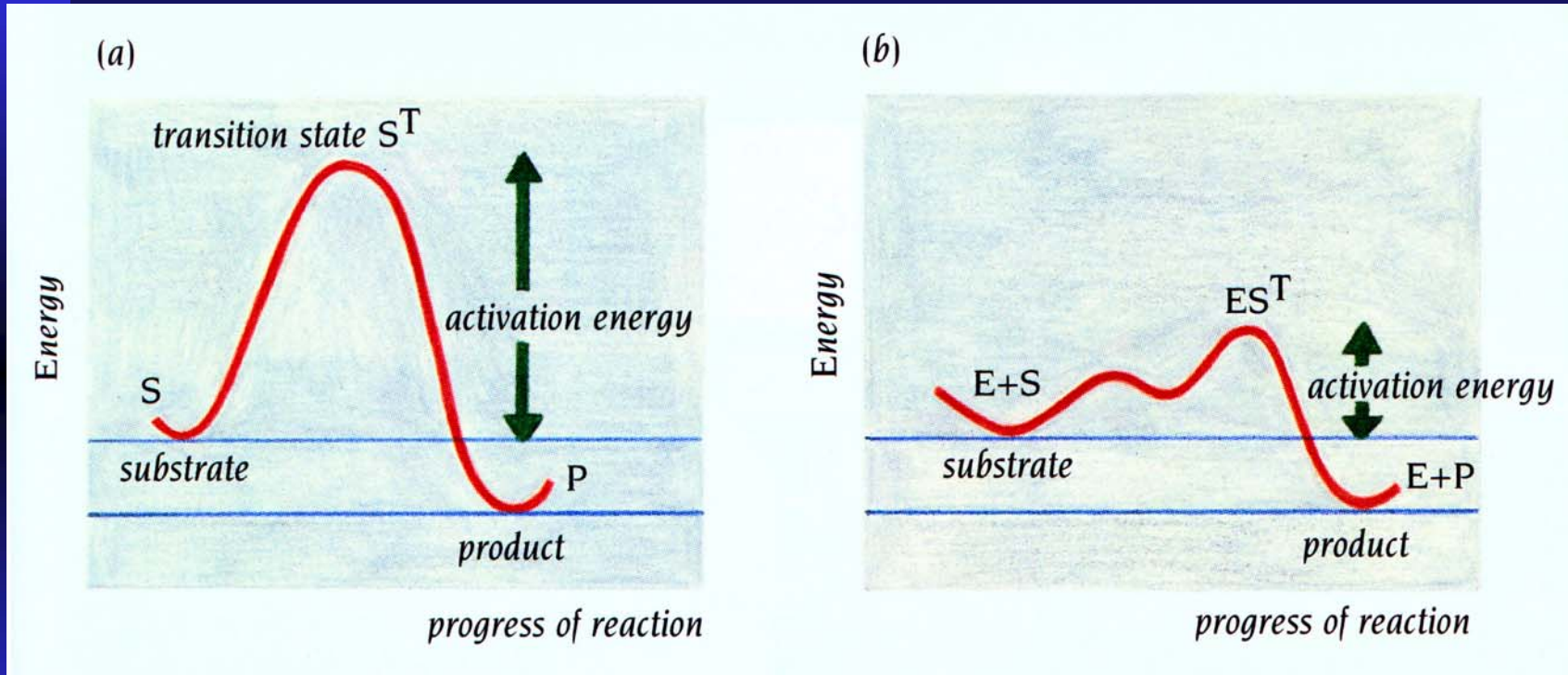
The catalytic properties of enzymes are reflected in K_m and k_{cat} values



A plot of the reaction rate as a function of substrate concentration for an enzyme catalyzed reaction. V_{max} is the maximal velocity. The Michaelis constant, K_m , is the substrate concentration at half V_{max} . The rate v is related to the substrate concentration, $[S]$, by the Michaelis-Menten equation:

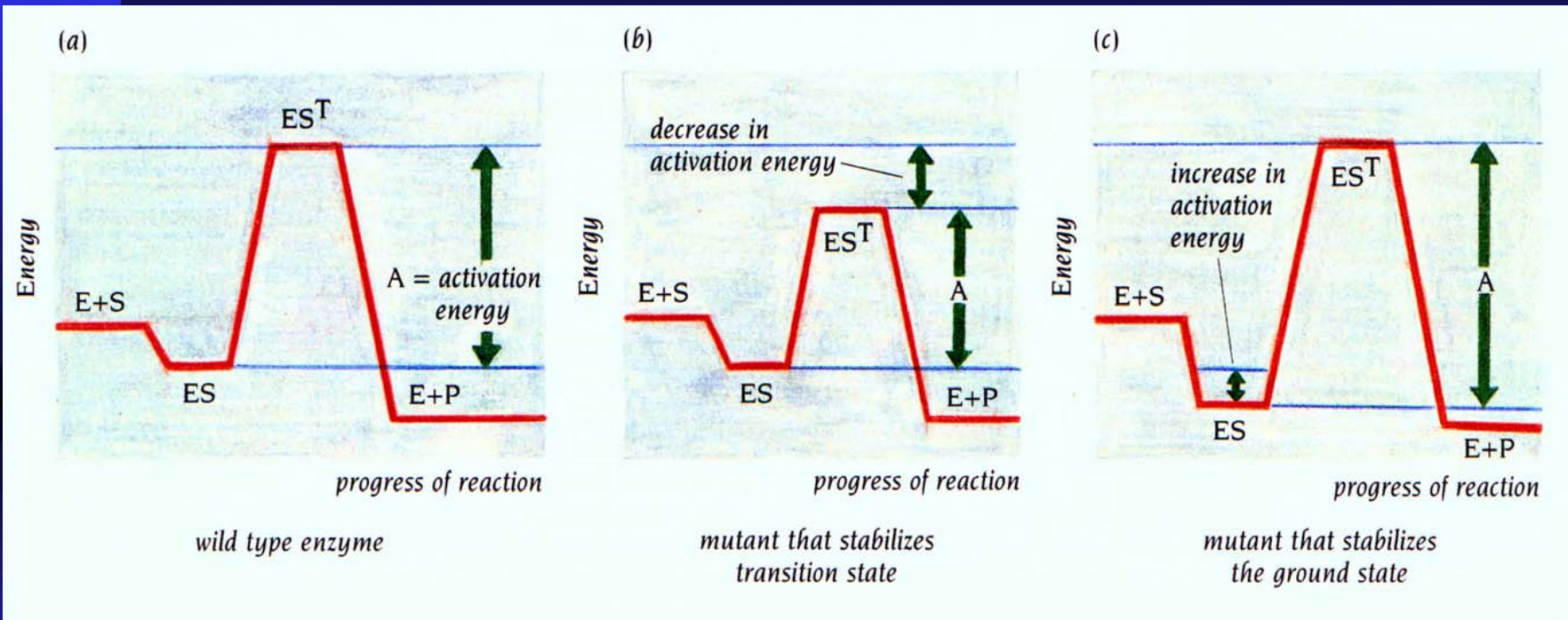
$$V = \{V_{max} \times [S]\} \times \{K_m + [S]\}^{-1}$$

Enzymes decrease the activation energy of chemical reactions



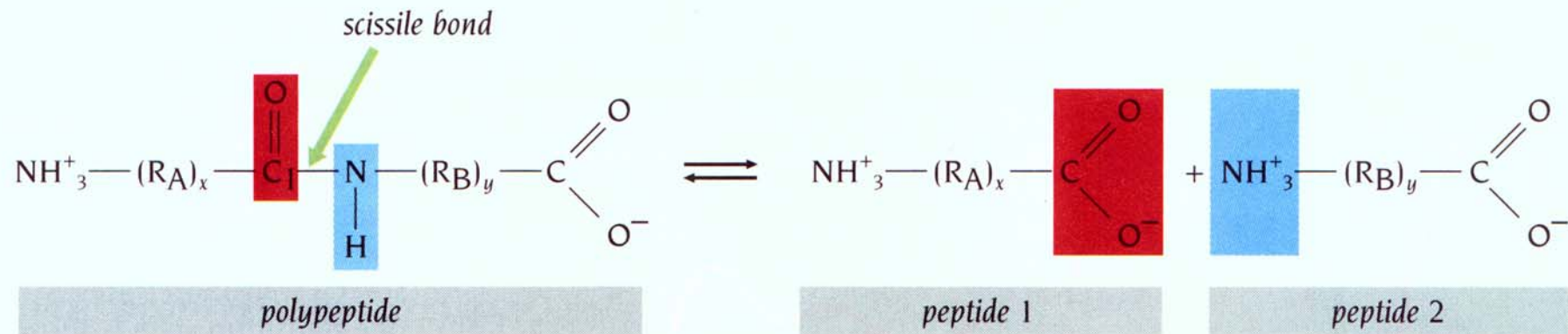
Enzymes accelerate chemical reactions by decreasing the activation energy. The activation energy is higher for a noncatalyzed reaction (a) than for the same reaction catalyzed by an enzyme (b). Both reactions proceed through one or several transition states, S^T . Only one transition state is shown in (a), whereas the two bumps in (b) represent two different transition states.

Enzymes decrease the activation energy of chemical reactions



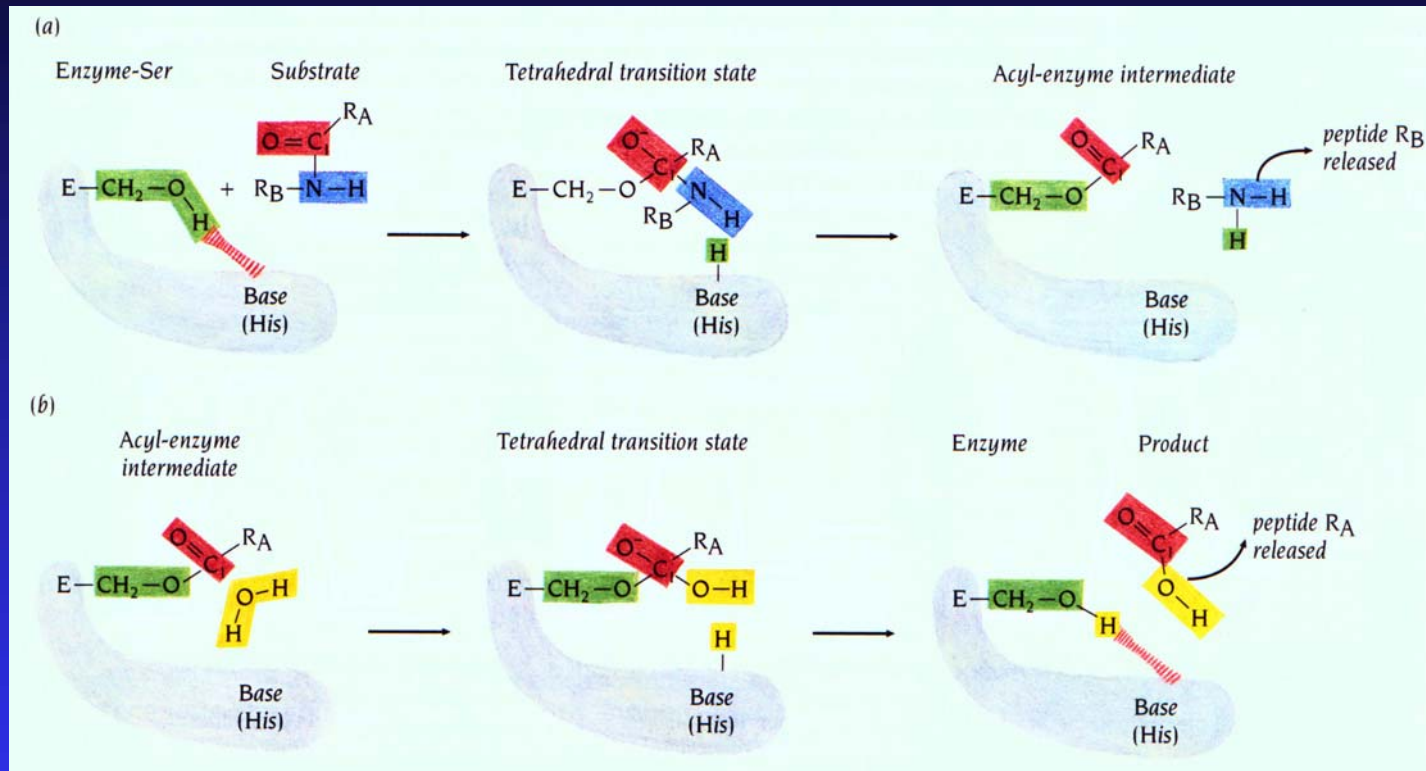
One of the most important factors in enzyme catalysis is the ability of an enzyme to bind substrate more tightly in its transition state than in its ground state. The difference in binding energy between these states lowers the activation energy of the reaction. This is illustrated by energy profiles for an enzyme in its wild-type form (a), for a mutant that stabilizes the substrate in its transition state and therefore decreases the activation energy from ES to the transition state ES^T giving higher rates (b), and for a mutant that stabilizes the substrate in its ground state giving lower rates (c).

Serine proteinases cleave peptide bonds by forming tetrahedral transition states



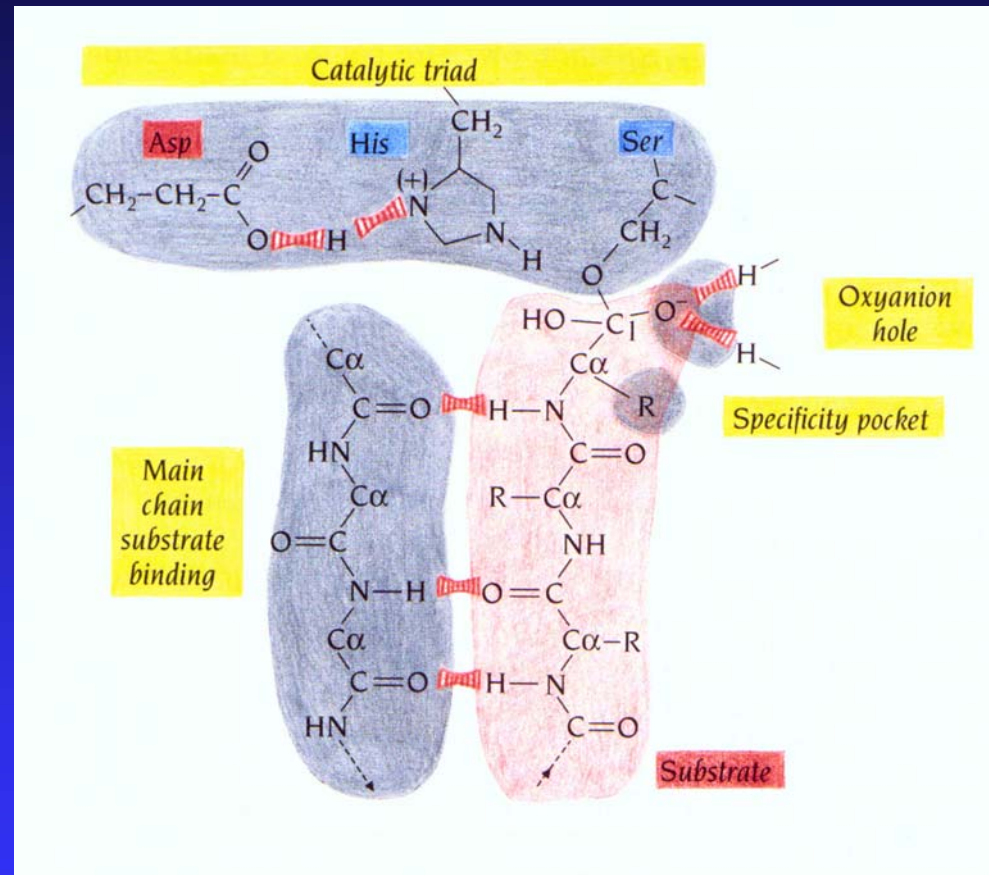
Serine proteinases catalyze the hydrolysis of peptide bonds within a polypeptide chain. The bond is called the scissile bond. $(\text{R}_\text{A})_x$ and $(\text{R}_\text{B})_y$ represent polypeptide chains of varying lengths.

Serine proteinases cleave peptide bonds by forming tetrahedral transition states



(a) Formation of an acyl-enzyme intermediate involving a reactive Ser residue of the enzyme is the first step in hydrolysis of peptide bonds by serine proteinases. First, a transition state is formed where the peptide bond is cleaved in which the C₁ carbon has a tetrahedral geometry with bonds to four groups, including the reactive Ser residue of the enzyme and a negatively charged oxygen atom. (b) Deacylation of the acyl-enzyme intermediate is the second step in hydrolysis. This is essentially the reverse of the acylation step, with water in the role of NH₂ group of the polypeptide substrate. The base shown in the figure is a His residue of the protein that can accept a proton during the formation of the tetrahedral transition state.

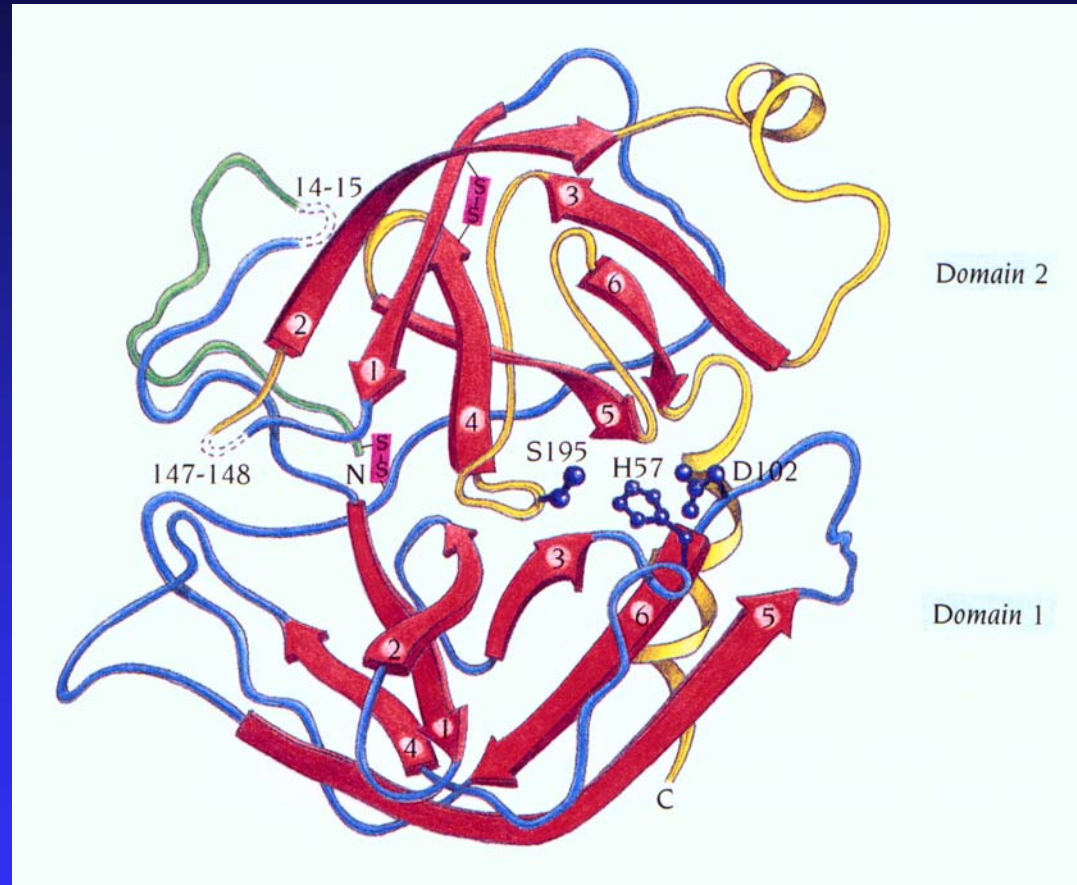
Four important structural features are required for the catalytic action of serine proteinases



A schematic view of the presumed binding mode of the tetrahedral transition state intermediate for the deacylation step. The four essential features of the serine proteinases are highlighted in yellow: the catalytic triad, the oxyanion hole, the specificity pocket, and the unspecific main-chain substrate binding.

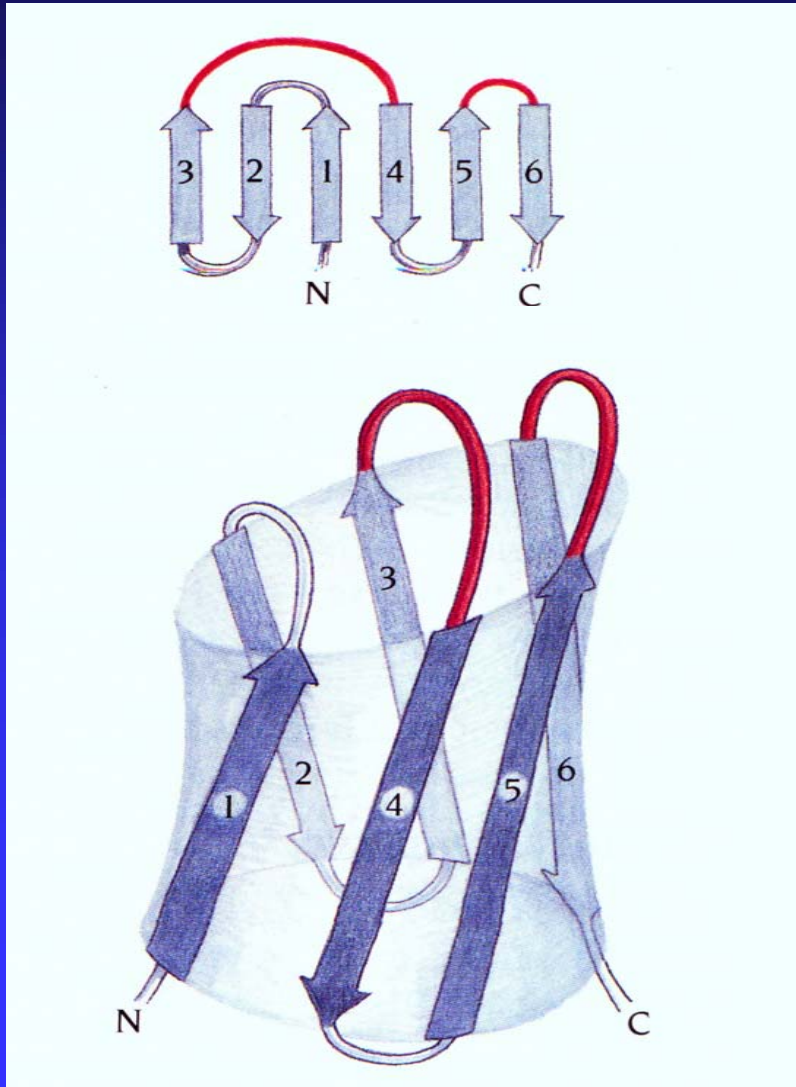
The chymotrypsin structure has two antiparallel β -barrel domains

Chymotrypsin is folded into two antiparallel β domains. The six β strands of each domain are red, the side chains of the catalytic triad are dark blue, and the disulfide bridges that join the three polypeptide chains are marked violet. Chain A (green, residues 1-13) is linked to chain B (blue, residues 16-146) by a disulfide bridge between Cys 1 and Cys 122.



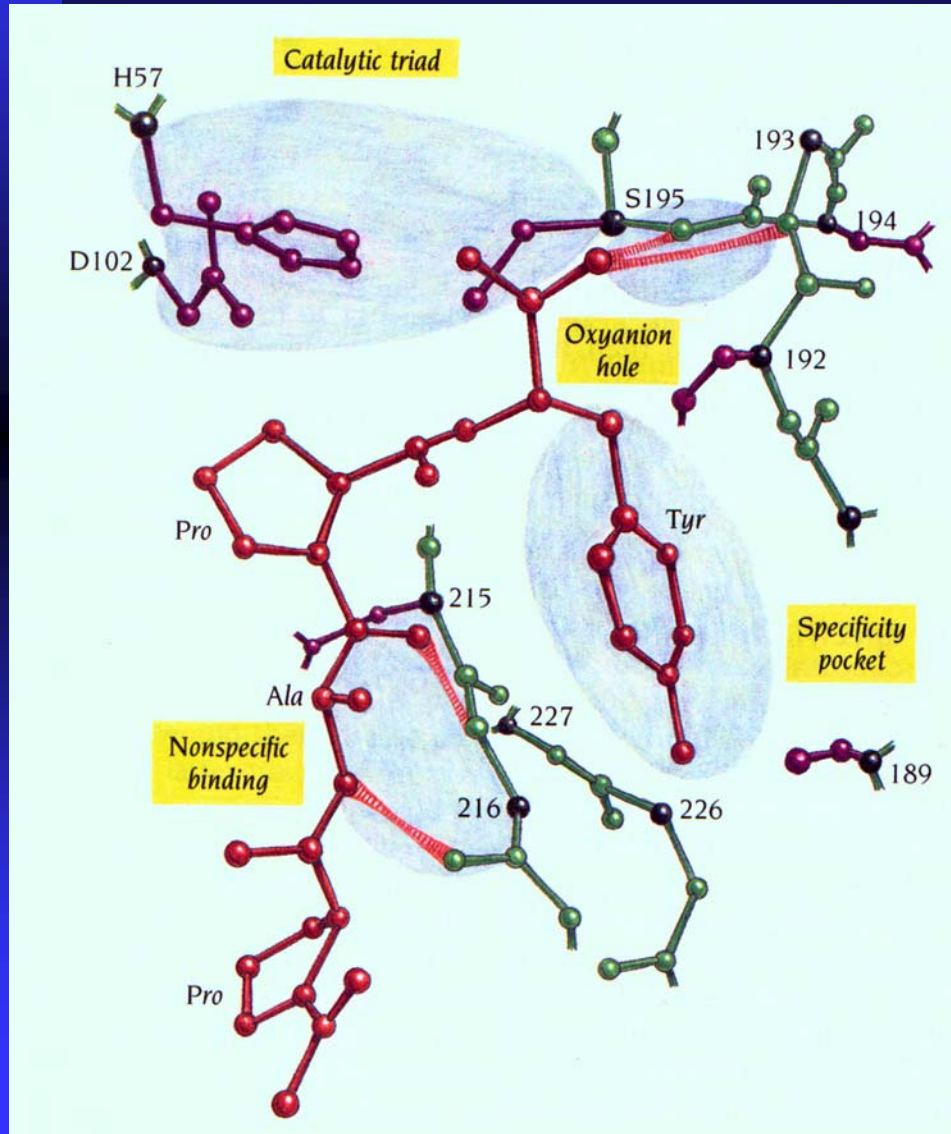
Chain B is in turn linked to chain C (yellow, residues 149-245) by disulfide bridge between Cys 136 and Cys 201. Dotted lines indicate residues 14-15 and 147-148 in the inactive precursor, chymotrypsinogen. These residues are excised during the conversion of chymotrypsinogen to the active enzyme chymotrypsin. The chymotrypsinogen comprises of 245 amino acid residues.

The chymotrypsin structure has two antiparallel β -barrel domains



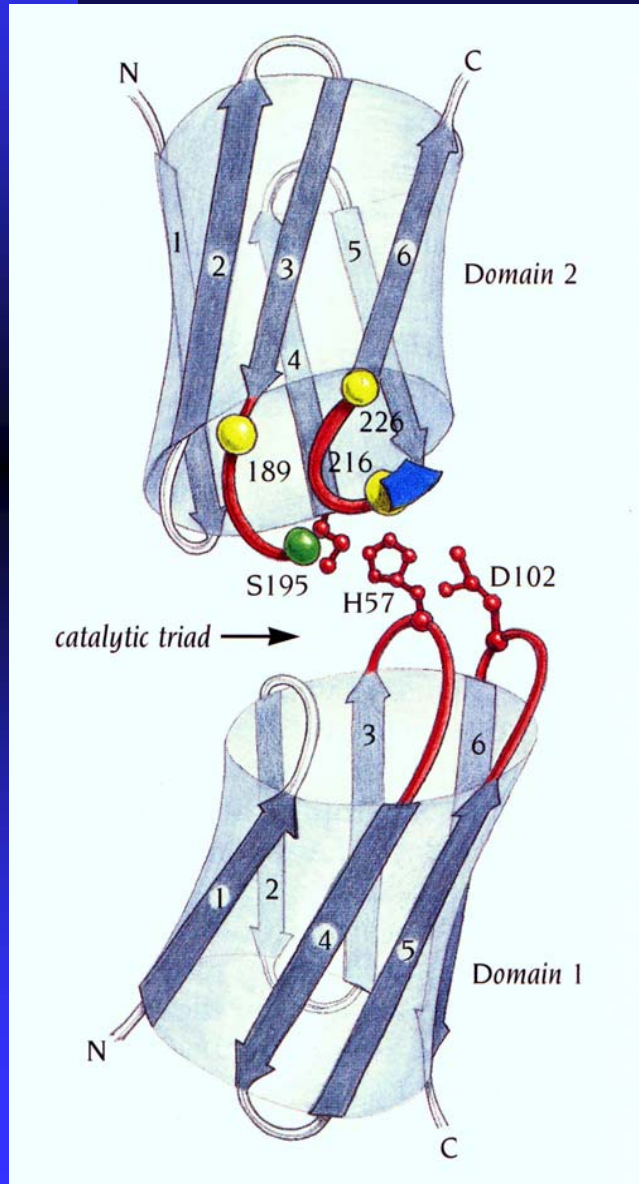
Topology diagrams of the domain structure of chymotrypsin. The chain is folded into a six-stranded anti-parallel β barrel arranged as a Greek key motif followed by a hairpin motif.

The chymotrypsin active site is formed by two loop regions from each domain






A diagram of the active site of chymotrypsin illustrates how an inhibitor (Ac-Pro-Ala-Pro-Tyr-COOH; red) binds in relation to the catalytic triad, the substrate specificity pocket, the oxyanion hole and the nonspecific substrate binding region. Hydrogen bonds between inhibitor and enzyme are striped.

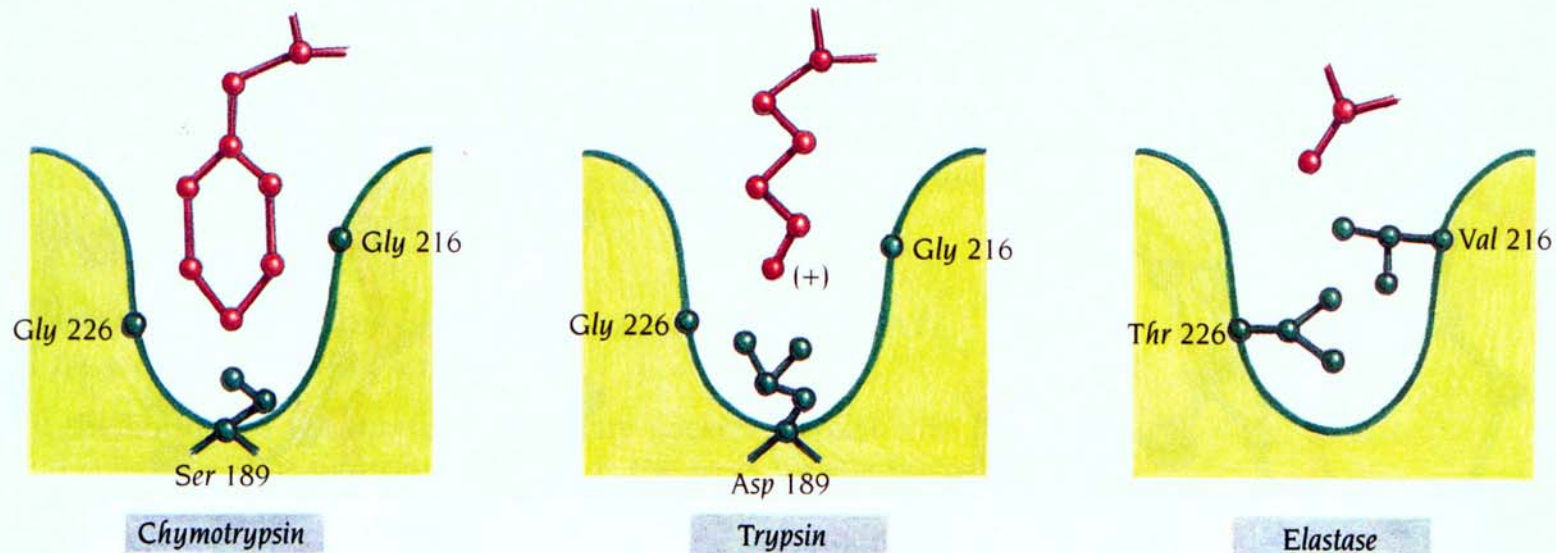
The chymotrypsin active site is formed by two loop regions from each domain



Topological diagram of the two domains of chymotrypsin illustrates that the essential active-site residues are part of the same two loop regions (3-4 and 5-6, red) of the two domains. These residues form the catalytic triad, the oxyanion hole (green), and the substrate binding regions (yellow and blue) including essential residues in the specificity pocket.

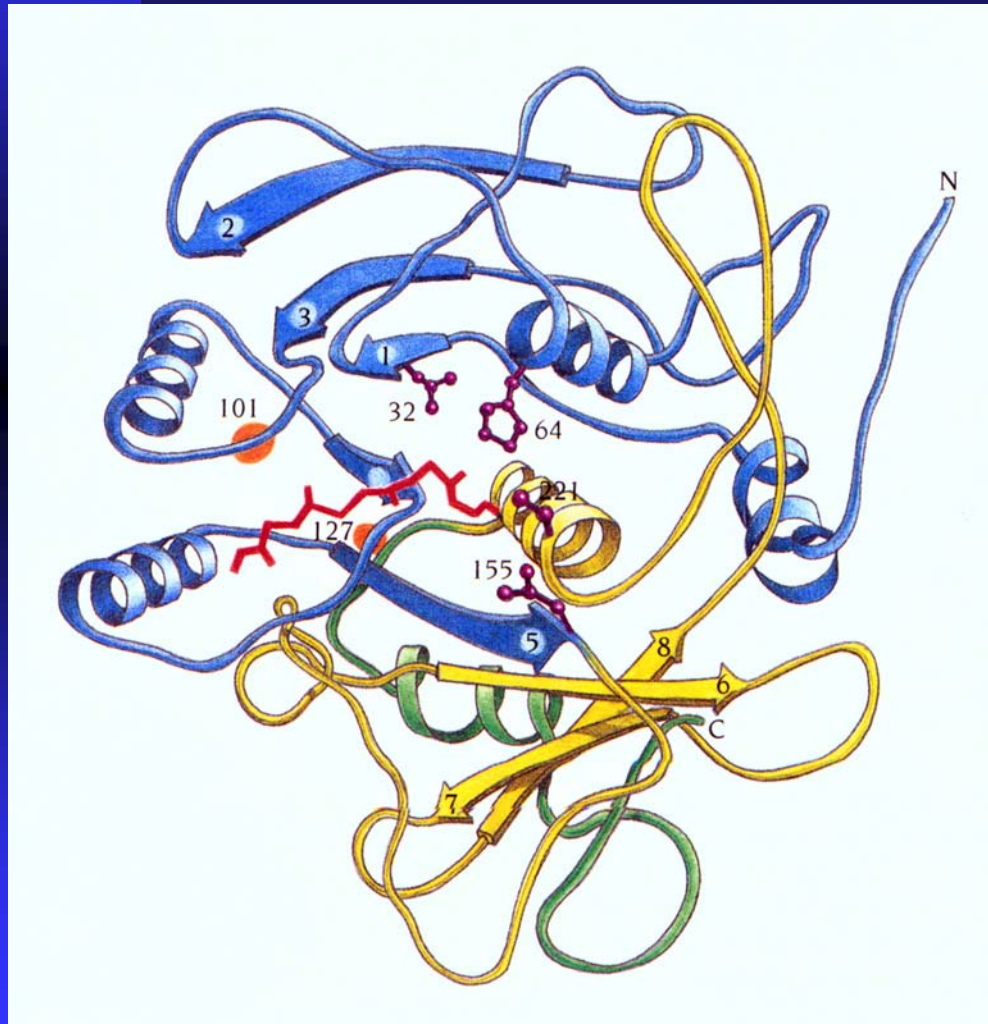
-  oxyanion hole
(res. 193-195)
-  main-chain
substrate binding
(res. 214-216)
-  substrate specificity pocket
(res. 189, 216, 226)

Different side chains in the substrate specificity pocket confer preferential cleavage



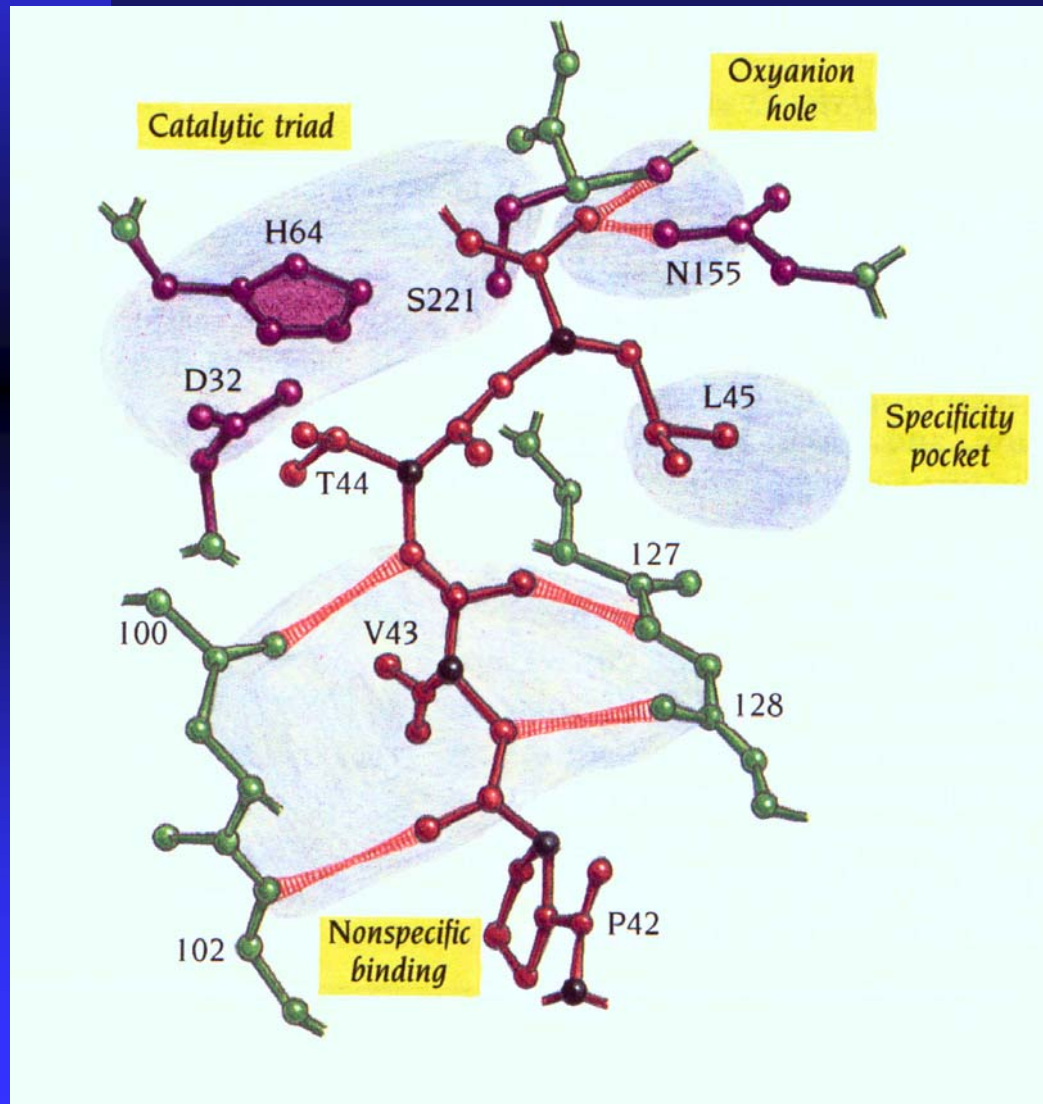
Schematic diagrams of the specificity pockets of chymotrypsin, trypsin and elastase illustrate the preference for a side chain adjacent to the scissile bond in polypeptide substrates. Chymotrypsin prefers aromatic side chains and trypsin prefers positively charged side chains that can interact with Asp 189 at the bottom of the specificity pocket. The pocket is blocked in elastase, which therefore prefers small uncharged side chains.

The structure of the serine proteinase subtilisin is of the α/β type



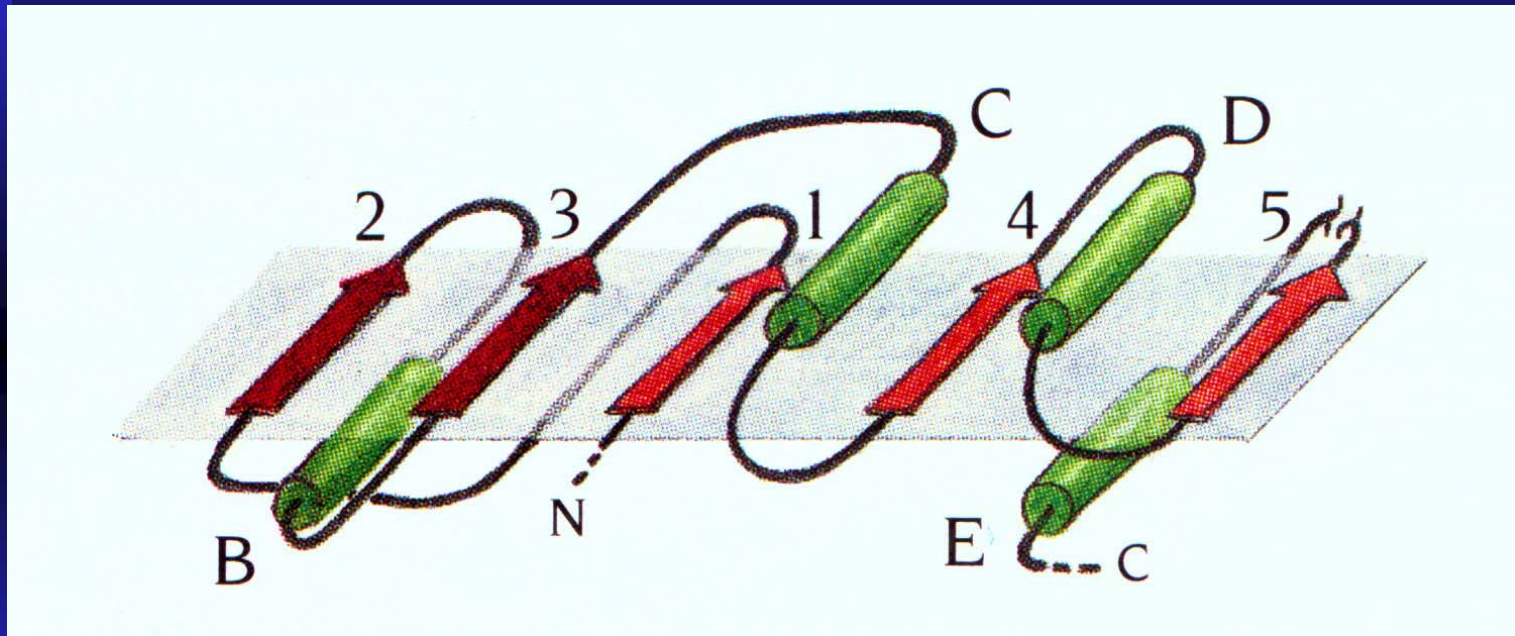
Schematic structure of the 3-D structure of subtilisin viewed down the central parallel β sheet. The N-terminal region that contains the α/β structure is blue. It is followed by a yellow region, which ends with the fourth α helix of the α/β structure. The C-terminal part is green. The catalytic triad Asp 32, His 64, and Ser 221 as well as Asn 155, which forms part of the oxyanion hole are shown in purple. The main chain of part of a polypeptide inhibitor is shown in red. Main-chain residues around 101 and 127 (orange circles) form the nonspecific binding regions of peptide substrate.

The active sites of subtilisin and chymotrypsin are similar



Schematic diagram of the active site of subtilisin. A region (residues 42-45) of a bound polypeptide inhibitor, eglin, is shown in red. The four essential features of the active site – the catalytic triad, the oxyanion hole, the specificity pocket, and the region for non-specific binding of substrate – are highlighted in yellow. Important hydrogen bonds between enzyme and inhibitor are striped.

A structural anomaly in subtilisin has functional consequences



Topological diagram of the α/β region of subtilisin illustrates that β_2 - α_B - β_3 has a different hand than other β - α - β units.

Transition-state stabilization in subtilisin is dissected by protein engineering

- Two essential features are required to stabilize the tetrahedral transition state in serine proteinases:
 - the oxyanion hole, which provides hydrogen bonds to the negatively charged oxygen atom in the transition state;
 - the histidine residue of the catalytic triad, which provides a positive charge.
- The charge on this histidine is, in turn, stabilized by the aspartic acid side chain of the catalytic triad.
- The histidine residue also plays a second role in the catalytic mechanism by accepting a proton from the reactive serine residue and then donating that proton to the nitrogen atom of the leaving group.

Transition-state stabilization in subtilisin is dissected by protein engineering

-The effects on the catalytic rate of the different side chains involved in the catalytic triad and the oxyanion hole have been examined by analyses of mutants of subtilisin with one or several of these side chains have been changed.

Transition-state stabilization in subtilisin is dissected by protein engineering

- Asn155Thr k_{cat} reduced by a factor of about 10^3
- Asn 155 provides one of the two hydrogen bonds to the substrate transition state in the oxyanion hole of subtilisin.
- Model building shows that the OH group of Thr in the mutant is too far away to provide such a hydrogen bond.
- The loss of this feature of the stabilization of the transition state thus reduces the rate by more than thousand-fold.

Catalysis occurs without a catalytic triad

- Ser221Ala k_{cat} reduced by a factor of about 10^6
- His64Ala k_{cat} reduced by a factor of about 10^6
- Asp32Ala k_{cat} reduced by a factor of about 10^4

- S221A+H64A k_{cat} reduced by a factor of about 10^6
- S221A+D32A k_{cat} reduced by a factor of about 10^6
- indicates that the reaction mechanism that involves the catalytic triad is no longer in operation.

- However, the enzyme still has an appreciable catalytic effect; peptide hydrolysis is still about 10^3 - 10^4 times the non-enzymatic rate.

Catalysis occurs without a catalytic triad

Whatever the reaction mechanism used by these mutants, it is apparent that the remaining parts of the active site must bind more tightly to the substrate in its transition state than in its initial state and thereby increase the reaction rate above that in the absence of enzyme.

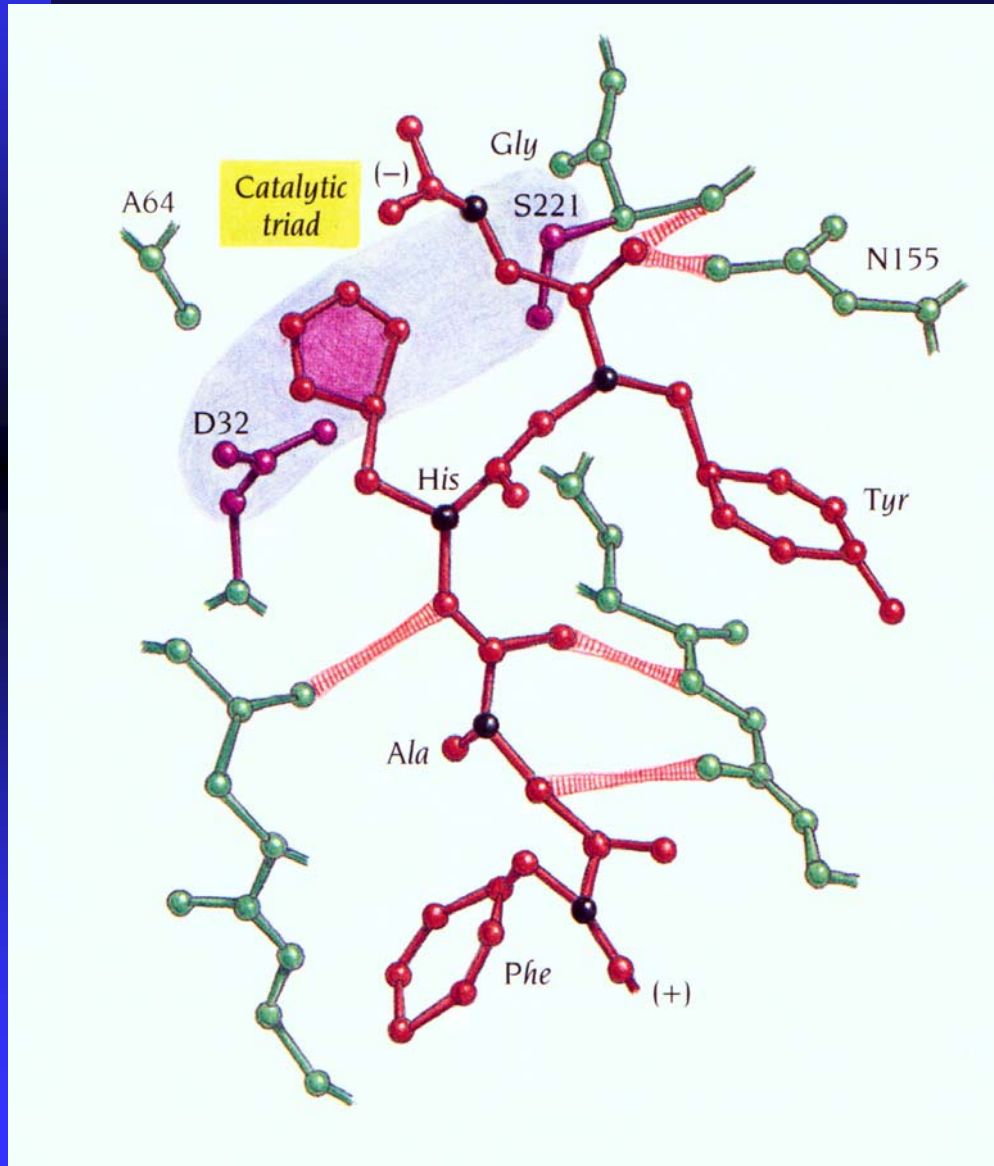
Substrate molecules provide catalytic groups in substrate-assisted catalysis

-His64Ala k_{cat} reduced by a factor of about 10^6

-His64 seems to be as important as Ser221 for the formation of a covalent tetrahedral intermediate.

-However, model building suggested that it might be possible at least partly to compensate for the loss of this histidine in the catalytic triad of the mutant protein with a histidine side chain from a peptide substrate.

Substrate molecules provide catalytic groups in substrate-assisted catalysis



Schematic diagram from model building of a substrate, NH₂-Phe-Ala-His-Tyr-Gly-COOH (red), bound to the subtilisin mutant His 64-Ala illustrates that the His residue of the substrate can occupy roughly the same position in this mutant as His 64 in wild-type subtilisin and thereby partly restore the catalytic triad.

Substrate molecules provide catalytic groups in substrate-assisted catalysis

-Experiment:

-His64Ala catalyzes hydrolysis of a peptide substrate about 400 times faster when the peptide has histidine at the appropriate position in its sequence.

-however, the rate is still several orders of magnitude below the rate of the wild-type enzyme, presumably because of the slightly different position and orientation of the histidine side chain.

Substrate molecules provide catalytic groups in substrate-assisted catalysis

- Nevertheless, the principle of substrate-assisted catalysis has been demonstrated: an essential group that is lacked by a mutant enzyme can be replaced by a similar group from the substrate.
- One consequence of substrate-assisted catalysis is that the mutant enzyme is highly specific for substrates containing the essential group.
- The His64Ala mutant of subtilisin, for example, has a specificity factor ($k_{\text{cat}}/K_{\text{m}}$) of about 200-fold higher for substrates containing histidine.

Enzymes can be made more stable by engineering

Protein engineering, via site-directed mutagenesis, can be used to answer very specific questions about protein stability, and the results of these studies are being used to increase the stability of industrially important enzymes.

Factors of importance for protein stability were extensively analyzed in the enzyme lysozyme from bacteriophage T4.

Enzymes can be made more stable by engineering

Lysozyme from bacteriophage T4:

- 164 amino acid polypeptide chain
- two domains
- no S-S bridges
- Cys in positions 54 and 97 are far apart in folded structure

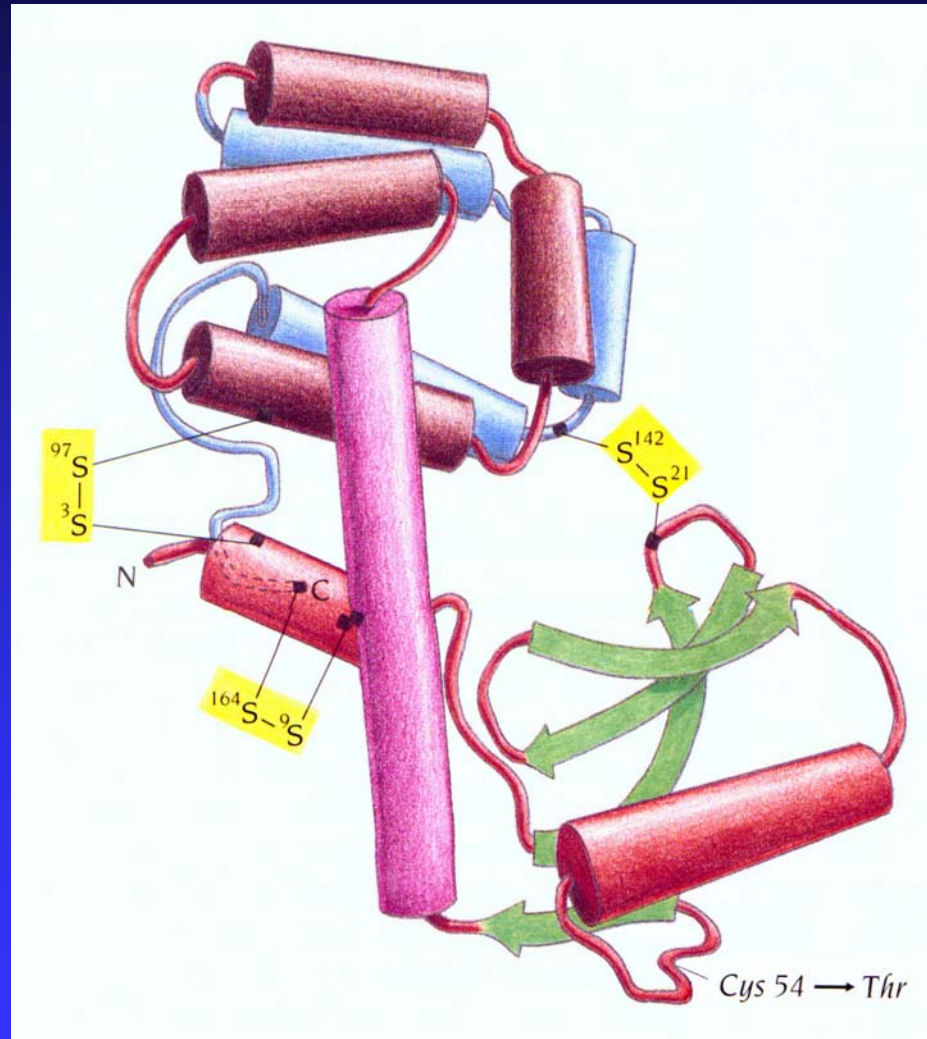
The stability of both wild-type and mutant proteins is expressed as the melting temperature, T_m , which is obtained by studying reversible heat denaturation. For the wild-type T4 lysozyme the T_m is 41.9°C.

Enzymes can be made more stable by engineering

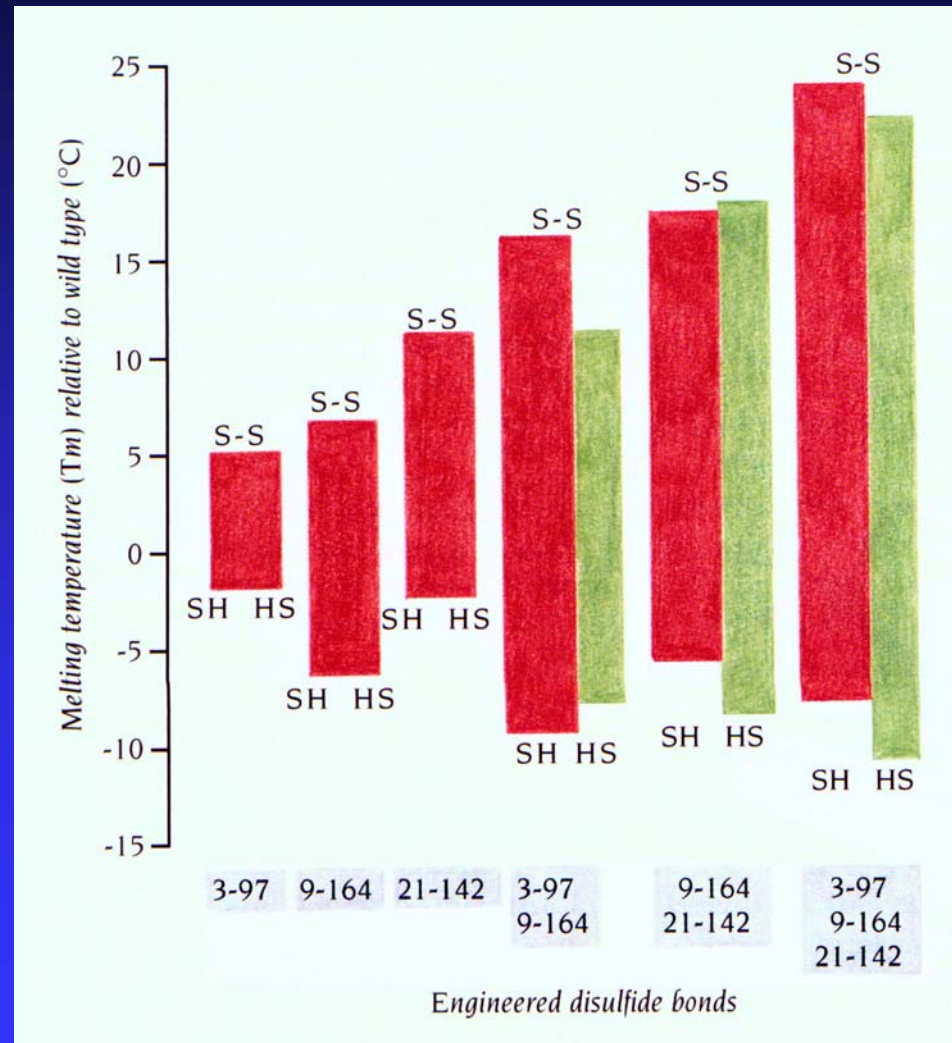
Different approaches to engineer a more thermostable protein than wild-type T4 lysozyme:

- reducing the difference in entropy between folded and unfolded protein, which in practice means reducing the number of conformations in the unfolded state
- stabilizing the α helices.

Disulfide bridges increase protein stability



Disulfide bridges increase protein stability

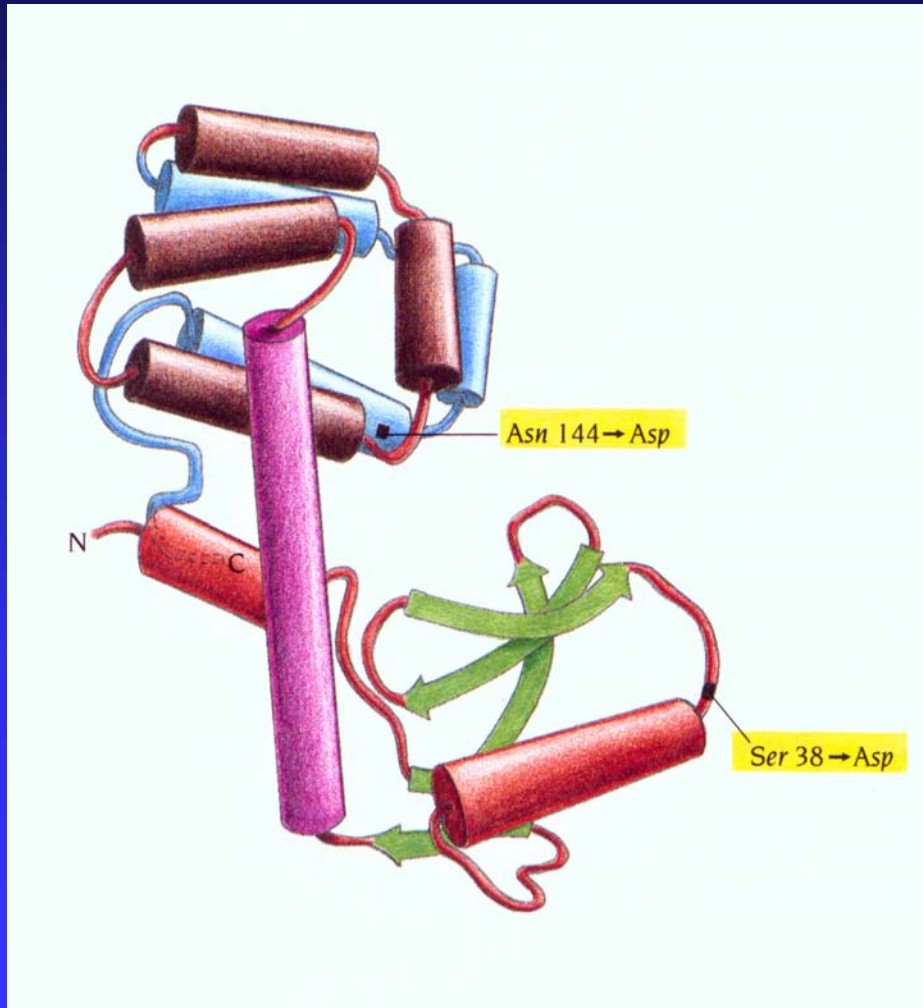


Glycine and proline have opposite effects on stability

mutation	increase in T _m
Gly77Ala	1°C
Ala82Pro	2°C

Such effects are expected to be additive, so even though each mutation makes only a small contribution to increased stability, the combined effect of a number of such mutations should significantly increase a protein's stability.

Stabilizing the dipoles of α helices increases stability



mutation	increase in T_m
Ser38Asp	2°C
Asn144Asp	2°C
Ser38Asp + Asn144Asp	4°C