

## Enzyme Mechanisms

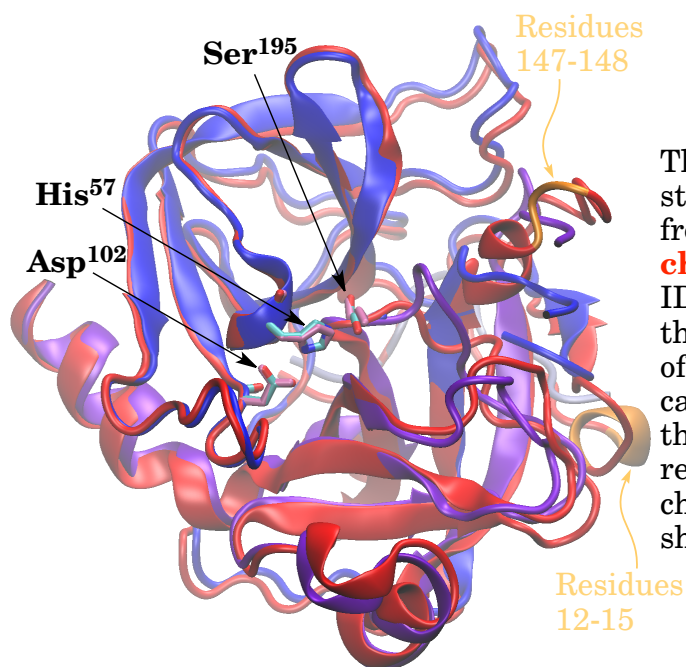
Enzymes function by a wide variety of mechanisms. We will cover a few examples to illustrate the means that enzymes use to catalyze reactions.

### Serine proteases

Peptide bond hydrolysis is a very common process. A wide variety of enzymes can perform proteolytic reactions.

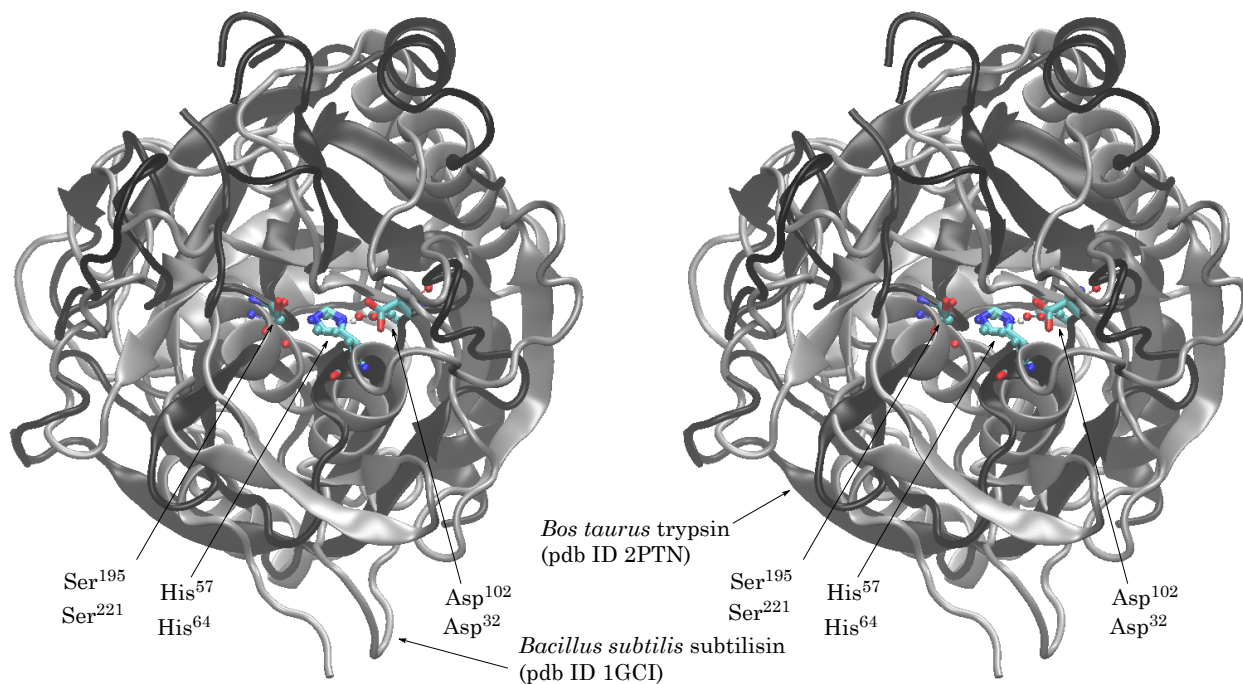
Members of one large family of protease are called “serine proteases” because of the important serine at the active site. All of the serine proteases contain three residues at their active site: a serine, a histidine, and an aspartate. These comprise the characteristic **catalytic triad**. In the numbering scheme for chymotrypsin (a numbering scheme which is typically used in studies of any of the mammalian serine proteases), the residues are Ser<sup>195</sup>, His<sup>57</sup>, and Asp<sup>102</sup>. Note that these residues are distributed throughout the 240 amino acid residues found in chymotrypsin, but are located in close proximity in the active three-dimensional conformation of the enzyme (see figure, below).

The serine proteases are synthesized as larger, inactive, precursors. As an example: chymotrypsinogen is converted to chymotrypsin by two cleavage processes involving excision of residues 12-15 and 147-148. Interestingly the structures of chymotrypsinogen and chymotrypsin (including the catalytic residues) are almost completely superimposable; the conformational change involved in the conversion process appears to be fairly small. The implication is that relatively small structural changes can cause dramatic changes in activity. (Note: some changes in other aspects of the active site are observed. However, because the catalytic triad residues are observed in their normal conformation, the zymogen forms of serine proteases usually exhibit low levels of catalytic activity.)



This figure shows superimposed structures of **chymotrypsin** (in blue, from PDB ID 1CA0) and **chymotrypsinogen** (in red, from PDB ID 2CGA). Note the overall similarity in the structures, especially on the left side of the figure and in the position of the catalytic triad residues. The location of the excised residues (12-15 and 147-148) removed in the conversion of chymotrypsinogen to chymotrypsin is shown in orange.

Some bacterial serine proteases (e.g., *Streptomyces griseus* Protease A) are structurally similar to the mammalian enzymes; the invention of the serine protease structure therefore occurred prior to the divergence of prokaryotes and eukaryotes. On the other hand, the *Bacillus subtilis* protease subtilisin, although it has the same catalytic residues, has a completely different structure, suggesting that serine protease mechanism has been invented more than once during evolution. (The figure below has a superimposition of bovine trypsin and subtilisin; note that the structures are totally different, but that Ser<sup>195</sup>, His<sup>57</sup>, and Asp<sup>102</sup> from trypsin have a very similar arrangement in three-dimensional space to Ser<sup>221</sup>, His<sup>64</sup>, and Asp<sup>32</sup> of subtilisin.)



The serine proteases differ in their sequence and in their substrate specificity: the bacterial protease subtilisin (one of the major enzymes that resulted in the advertising slogan “the cleaning power of enzymes!”) will cleave essentially any substrate, while one of the enzymes in the clotting cascade, Factor Xa, requires a four residue recognition sequence, Ile-Glu-Gly-Arg, in order to uniquely hydrolyze its polypeptide substrate after the arginine.

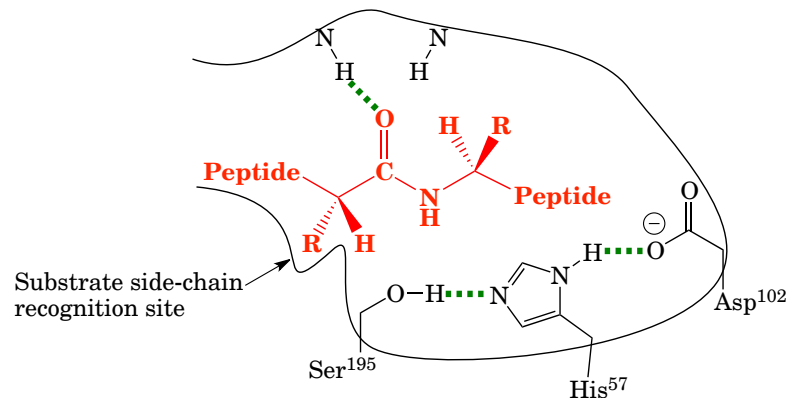
Trypsin is specific for cleavage after lysine and arginine. Trypsin contains Asp<sup>189</sup> in the substrate side-chain binding pocket, and this residue forms an electrostatic interaction with basic residues. However, the specificity is not completely dependent on a single residue; mutating Asp<sup>189</sup> to serine (the corresponding residue in chymotrypsin) converts trypsin into a non-specific protease, rather than into an enzyme with chymotrypsin-like specificity. (Even incorporating other mutations in nearby residues is only partially effective at producing a chymotrypsin-like enzyme; proteins are complex entities!)

### Catalytic mechanism of serine proteases

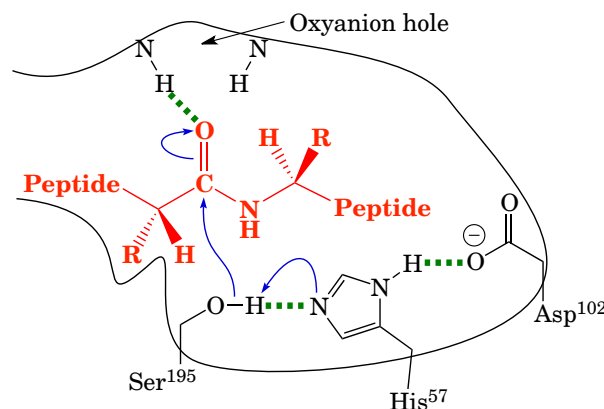
Serine proteases increase the rate of peptide bond hydrolysis by  $\sim 10^{10}$  compared to the uncatalyzed reaction. A variety of structural features are responsible for the catalytic effectiveness of these enzymes. As mentioned above, the serine proteases all have three residues that are critical for catalysis: a serine, a histidine, and an aspartic acid. These are conserved in all of the serine proteases, and are superimposable in the crystal structures of these proteins.

#### Steps in the catalytic process:

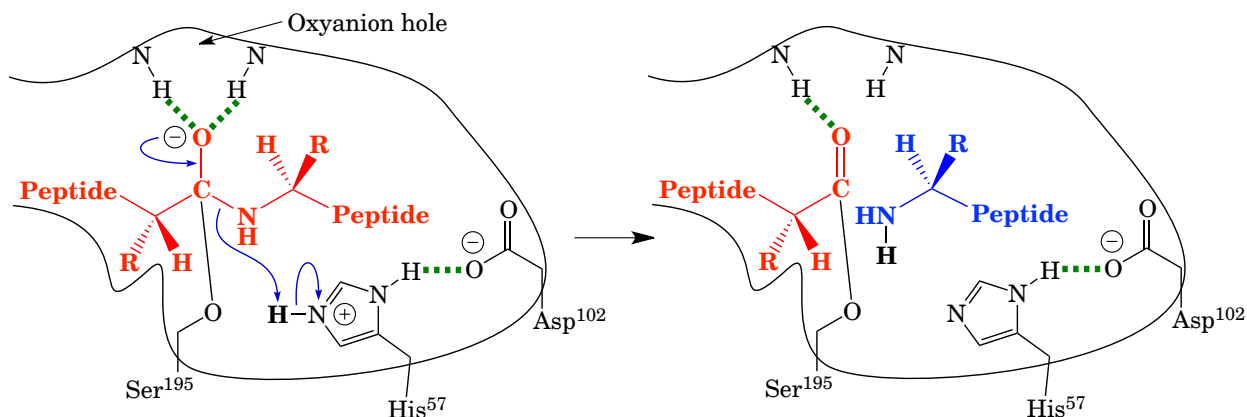
- 1) **Substrate binding.** Note that for the substrate peptide, the side-chain of the amino acid residue immediately before the scissile peptide bond can bind to the recognition site on the enzyme.



- 2) **Nucleophilic attack.** Serine<sup>195</sup> acts as a nucleophile, facilitated by Histidine<sup>57</sup>, which abstracts a proton from Ser<sup>195</sup>. The result of the nucleophilic attack is a covalent bond between the Ser<sup>195</sup> side-chain oxygen and the substrate. The negative charge that develops on the peptide carbonyl oxygen is stabilized by hydrogen bonds formed from two protease backbone amide protons. This region of the protein is called the "oxyanion hole", because it stabilizes the negative charge on the oxygen; the oxyanion hole is critical for catalysis. (Note: the oxyanion hole includes the backbone amide proton from Ser<sup>195</sup>; the diagrams illustrating this discussion are thus somewhat distorted from the three-dimensional structure.)

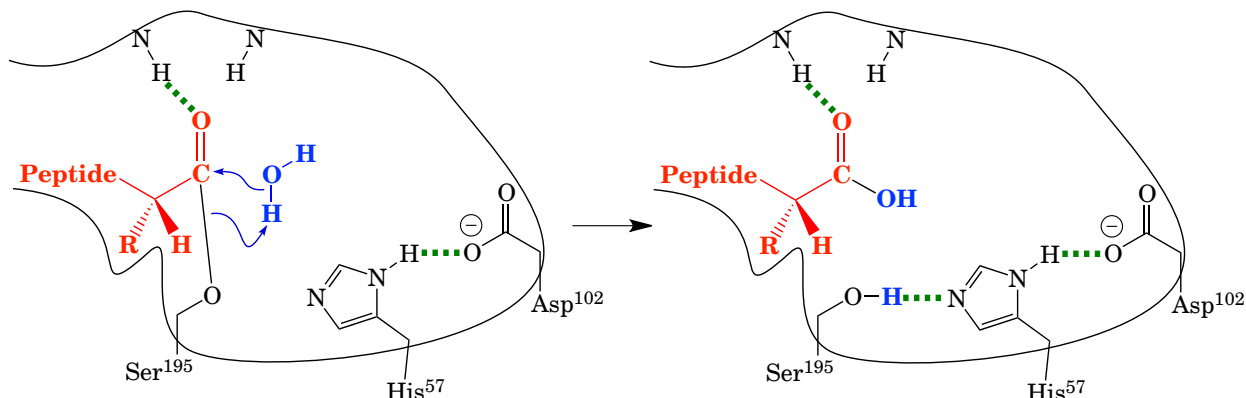


- 3) **Protonation.** His<sup>57</sup> donates a proton to the substrate amide nitrogen, allowing release of the C-terminal part of the substrate as a free peptide.



- 4) **Ester hydrolysis.** The final step is an attack by water on the ester bond between the peptide and the Ser<sup>195</sup> oxygen. This forms the second product peptide with a normal carboxyl group, and regenerates the serine hydroxyl. The second peptide then dissociates from the enzyme to allow another catalytic cycle to begin.

5)



As is apparent in the series of drawings above, Ser<sup>195</sup> is the residue that performs the actual catalysis, while the other residues seem to be important for positioning of the serine and for stabilizing the intermediate states. Aspartate<sup>102</sup> is relatively far from the substrate; Asp<sup>102</sup> helps position His<sup>57</sup>, and raises the pK<sub>a</sub> of His<sup>57</sup> to allow the histidine to act as a base. This is important to the catalytic process: mutation of Asp<sup>102</sup> to asparagine decreases the  $k_{cat}$  by ~10,000-fold.

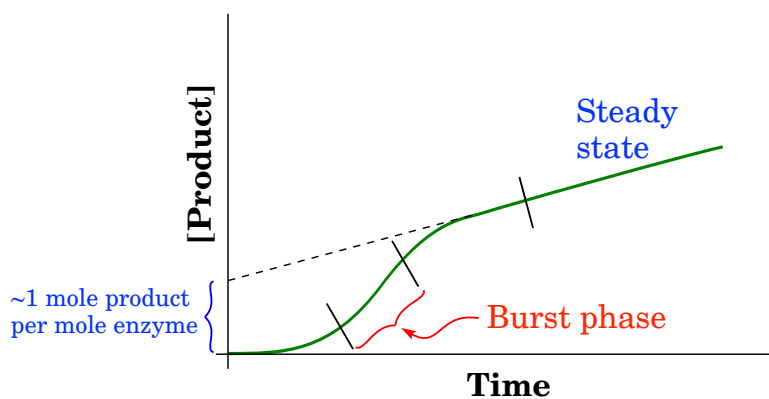
The serine protease reaction mechanism involves a covalent intermediate between Ser<sup>195</sup> and the substrate. Although polypeptides do (very slowly) hydrolyze in aqueous solution, the hydrolysis reaction does not involve the formation of a covalent bond with a passing serine side-chain. Thus, the serine protease mechanism involves the **use of an alternative pathway** from that used in the uncatalyzed reaction.

The serine protease mechanism does, however, involve **stabilization of the transition state**. This has been observed in mutant studies in which all of the catalytic triad residues were mutated. Although the reaction mechanism might be interpreted to predict that the loss of the active site serine would inactivate the enzyme, in fact, the triple mutant (where all three of the catalytic triad residues are changed to other residues) retains some ability to catalyze the reaction, and increases the rate of hydrolysis by  $\sim 5 \times 10^4$ -fold over the uncatalyzed reaction. This appears to be the result of the binding of the transition state to the enzyme, and in particular to the precise positioning of the “oxyanion hole” which allows the stabilization of the charged transition state. (Although all proteins contain peptide backbones, in general proteins lack the precise three-dimensional structure to catalyze reactions, which is why all proteins do not act as proteases.)

Serine proteases are very useful as model enzymes for studying catalytic processes, because they use a variety of techniques for accelerating the reaction rate. They use acid-base catalysis (the histidine abstraction of the serine proton, and the histidine donation of the proton to allow release of the first peptide). Serine proteases also use charge stabilization (in particular, the oxyanion hole) and geometric factors (in particular the better fit of the tetrahedral alkoxide intermediate than the planar carbonyl structures) to lower the energy of the transition state, and use covalent catalysis to assist in the reaction.

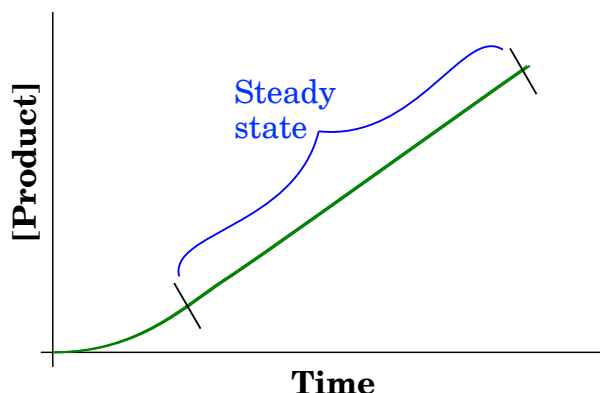
### How do we know all this?

The serine proteases have been heavily studied. We will return to the serine protease mechanism later when we discuss inhibition, but one feature of research into the mechanism deserves some emphasis. The formation of the covalent enzyme-substrate intermediate was first proposed after a series of studies by B.S. Hartley and B.A. Kilby in 1954. They used a model substrate (*p*-nitrophenylacetate) to examine the kinetics of the reaction. Chymotrypsin has a low  $k_{cat}$  for *p*-nitrophenylacetate, which simplifies some of the analysis. The model substrate also has the advantage of forming a colored product *p*-nitrophenolate. They discovered that some *p*-nitrophenolate was released rapidly (burst phase kinetics) prior to the establishment of the steady state.



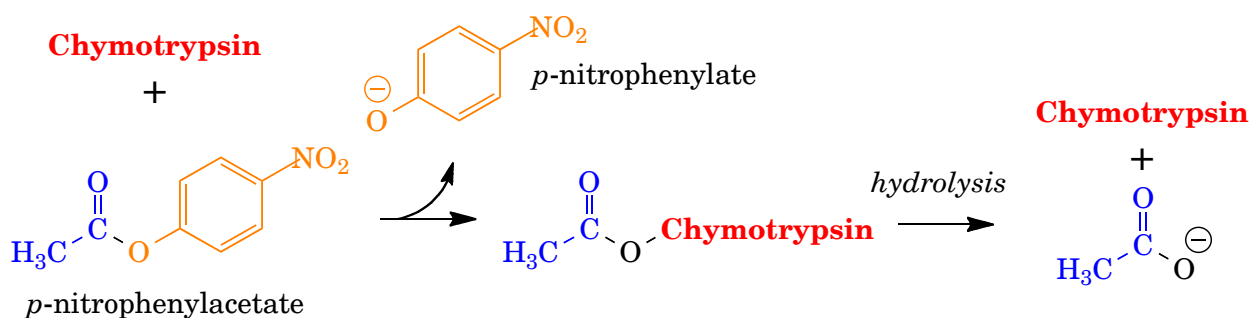
Normal enzyme kinetic measurements involve a brief period following addition of

the enzyme, in which the enzyme-substrate complex forms. This pre-steady state condition is followed by the linear formation of product with time characteristic of steady state conditions. (The length of time required for steady state formation is somewhat exaggerated in the graph below).



In the case of the serine proteases, a brief period of rapid product formation also occurs prior to establishment of steady state conditions. The amount of product formed in this burst phase corresponded to about 1 mole of product per mole of enzyme, suggesting that the enzyme rapidly formed a product, but then became trapped in a non-catalytic state that cycled back to the catalytic state more slowly.

The mechanism that Hartley and Kilby proposed to explain the burst phase kinetics was that a covalent intermediate was formed between the enzyme and the acetate from the substrate, rapidly releasing the colored *p*-nitrophenolate. The acetylated-enzyme intermediate then slowly hydrolyzed to regenerate active enzyme. The initial burst of *p*-nitrophenolate production occurred at a high rate because it preceded the rate-limiting step for the reaction. Once the majority of the enzyme had become acetylated, the release of *p*-nitrophenolate became dependent on the rate-limiting step, which is the hydrolysis of the covalent bond formed to the enzyme.

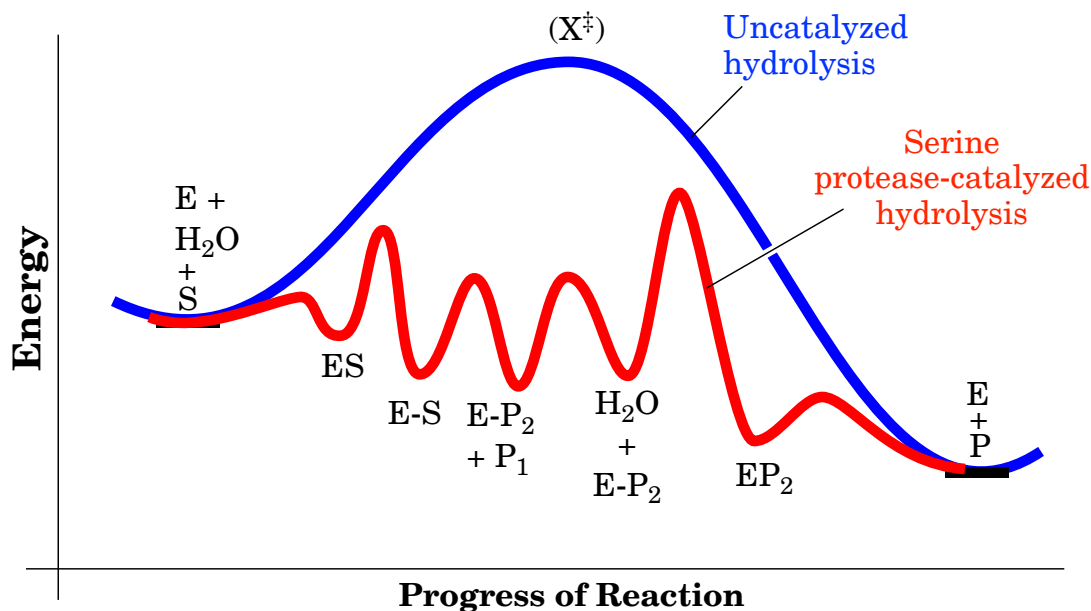


When using normal polypeptide substrates, this burst phase kinetics can also occur. For most peptide substrates, however, the burst phase tends to be less obvious, because chymotrypsin cleaves polypeptides more effectively than it cleaves the model substrate *p*-nitrophenylacetate. These types of kinetic studies, when properly performed, allow insights into the enzyme mechanism, and have been the source of



much of the available information regarding enzyme catalysis.

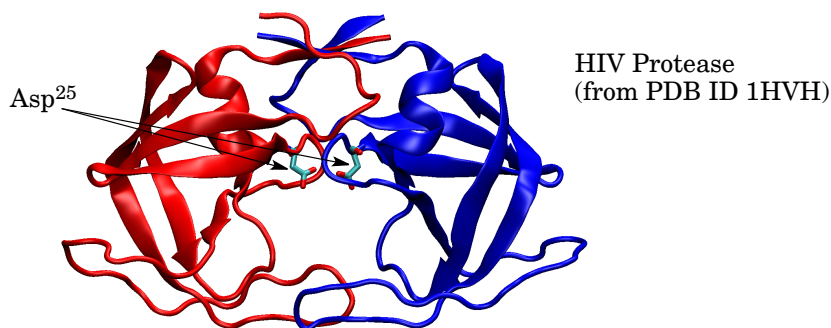
The diagram below summarizes the reaction pathway for serine protease peptide bond cleavage. Note that the highest energy peak in the serine protease reaction pathway corresponds to the covalent intermediate hydrolysis process, which is the slowest step in the reaction.



### Aspartyl proteases

Although serine proteases constitute a large family of enzymes with differing specificity, the serine-dependent catalytic triad is only one of many possible mechanisms for hydrolyzing peptide bonds. We will examine one other mechanism for proteolysis (a number of others exist).

Aspartyl proteases comprise another family of proteolytic enzymes. This family includes the stomach digestive enzyme **pepsin**, the highly specific protease **renin** (the plasma enzyme that converts the circulating precursor angiotensinogen to the hormone angiotensin), and the **HIV protease** (the enzyme that cleaves HIV proteins during construction of the viral particle).

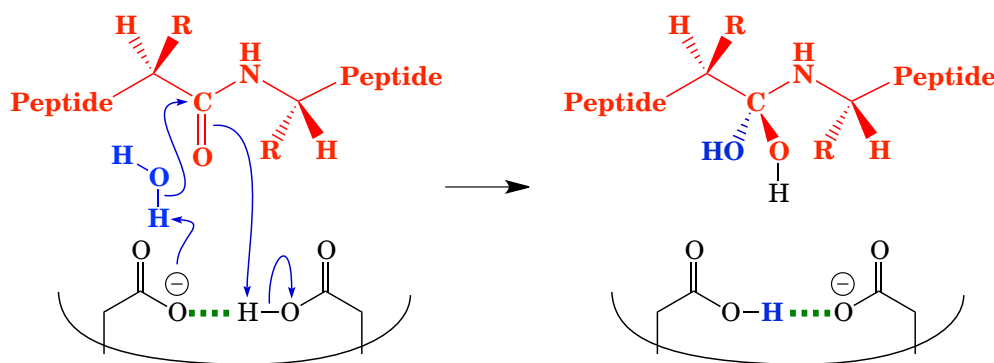


Aspartyl proteases contain two aspartate residues at the active site; in some cases, as in the HIV protease structure shown above, each monomer provides one of the

two aspartate residues in the dimeric protein complex. While their catalytic mechanism differs considerably from that of the serine proteases, the end result, the hydrolysis of a peptide bond within the substrate to release two polypeptide products, is the same.

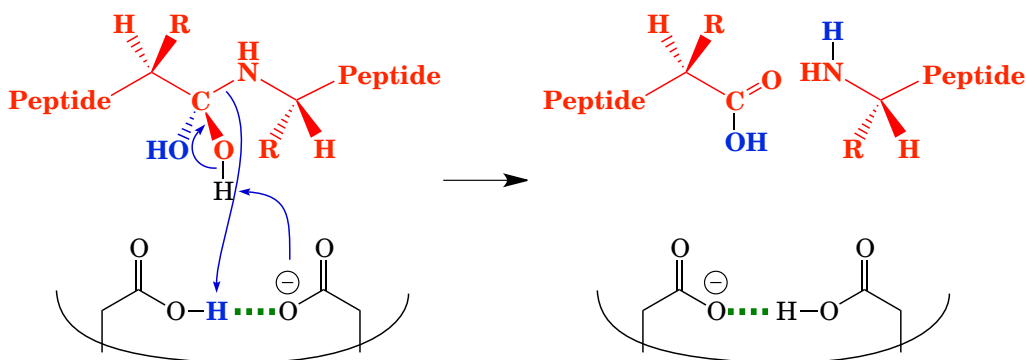
### Catalytic mechanism

The mechanism is different from that of the serine proteases. In the aspartyl proteases, the substrate never forms a covalent bond with the enzyme. Instead, the first step of the reaction (following substrate binding) is a nucleophilic attack by a water molecule on the substrate due to proton abstraction from the water by one of the aspartate from the enzyme.



One aspartate therefore acts as a base, while the water forms a covalent bond to the substrate. Note that while one aspartate removes a proton from the water molecule; the other aspartate acts as an acid, by releasing a proton to the carbonyl oxygen of the polypeptide substrate.

In the next step, one aspartate residue acts as a base again, removing a proton from one of the hydroxyls that have been formed on the former carbonyl carbon. At the same time, the carbon-nitrogen bond breaks, with the nitrogen taking a proton from the other active site aspartate.



This results in the formation of the carboxylic acid at one end of the new peptide, and the free amino group at the end of the other new peptide.

The aspartyl protease mechanism requires that the environment of the protein



result in the two aspartate residues being 50% protonated. Since the typical  $pK_a$  of aspartate residues is about 4, this means that either the external pH must be fairly low (as is the case for pepsin, a stomach enzyme that is most active at pH of about 2), or the environment of the active site must alter the  $pK_a$  of the aspartate residues enough to allow the partial protonation of the aspartate residues required for activity (as is the case for the renin and the HIV protease, which are active at pH of 7.4).

## Summary

In order to recognize a substrate, the enzyme must have a structure that is capable of binding to the substrate. However, many enzymes alter their structure upon substrate binding.

Enzymes alter reaction rates by a variety of methods. These methods include increasing the local concentration of the substrate molecules by confining them to a small volume, positioning the substrates in appropriate orientations for the reaction to occur, inducing strain in the substrate, donating or abstracting protons, stabilizing charge development, and interacting covalently with the substrate.

The variety of methods used result in enhanced reaction rates because of a decreased energy of the most energetic transition state in the pathway. This is possible because the enzyme binds to the transition state more tightly than to its substrate or products.

Serine proteases, and to a lesser extent, aspartyl proteases, have been used as models for studying the effect of protein structure on specific catalytic processes. Serine proteases and aspartyl proteases catalyze peptide bond hydrolysis by different mechanisms; both mechanisms take advantage of acid-base catalysis, but the serine proteases form a covalent intermediate with their substrate, while the aspartyl proteases do not.