

Catalytic hydroxyl/amine dyads within serine proteases

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The 'catalytic triad' mechanism, which involves a serine, histidine and aspartic acid, has become synonymous with serine proteases. However, recently, mechanistically novel serine proteases have been discovered. These proteases use hydroxyl/ ϵ -amine or hydroxyl/ α -amine 'catalytic dyads' as their reactive centers.

PROTEASES ARE UBIQUITOUS in nature and play pivotal roles in both intracellular and extracellular processes, as well as in the regulation of physiological pathways, including the degradation of misfolded proteins, processing short-lived signaling proteins, control of apoptosis and signal peptide cleavage. Most of the proteases fall within four groups: the serine, cysteine, aspartic acid and metallo proteases.

Catalytic triad mechanism

The standard mechanism for the serine proteases, which have evolved both by convergent and divergent evolution¹, involves the catalytic triad. The catalytic triad consists of a histidine general base, which abstracts the proton from the serine hydroxyl sidechain, allowing the serine to act as a nucleophile and attack the carbonyl group of the amide bond within the protein substrate. The third player in the triad, an acidic residue, acts to orient the histidine residue and neutralize the charged histidine intermediate (Fig. 1a). Deacylation of the enzyme involves an activated water molecule acting as the nucleophile².

Catalytic triad dissected

Extensive site-directed mutagenesis studies have been carried out on the classical serine proteases to decipher the importance of each member of the Ser/His/Asp 'catalytic triad'. These investigations indicate that substitution of any of the catalytic triad residues results in large effects on catalysis, but the histidine and serine residues are the most important for catalysis. For instance,

mutation of the catalytic serine or histidine residues within subtilisin³ or trypsin⁴ results in a 10^6 -fold decrease in activity. By contrast, mutation of the catalytic aspartate residue within subtilisin³ or trypsin^{4,5} results in a 10^4 -fold reduction in activity.

Despite being one of the most thoroughly studied class of enzymes, there still remains controversy over the catalytic triad mechanism proposed for the classical serine proteases such as subtilisin and trypsin.

Are some catalytic triads actually dyads?

It is striking that there is a proteolytic catalytic antibody in which the active site contains serine and histidine residues, but not an equivalent aspartic acid residue⁶. Similarly, the structure of an esterase from *Streptomyces scabies* reveals both a serine and a histidine residue in the catalytic site, but instead of the aspartic acid, there is an aromatic tryptophan residue, which cannot function as an acid. The authors suggest that this is an example of a catalytic dyad⁷. Another example of a potential catalytic dyad has been observed in the wheat serine carboxypeptidase; here, the carboxylate of the active-site aspartate is not co-linear with the imidazole of the histidine, which led Liao and co-workers to suggest that the catalytic triad of all serine proteases should be regarded as two dyads, a Ser/His dyad and a His/Asp dyad⁸.

Cysteine proteases, which have been the focus of considerable recent interest owing to their involvement in

apoptosis, usually contain a Ser/His/Asn catalytic triad, which is analogous to the Ser/His/Asp triad in serine proteases. However, the asparagine is not absolutely critical: in papain, if it is replaced with alanine, only a 150-fold reduction in activity is observed⁹. Thus, even with proteases that possess a catalytic triad, the dyads Ser/His (serine proteases) and Cys/His (cysteine proteases) seem to be the most critical residues.

Hydroxyl/ ϵ -amine dyad catalysis: serine and lysine

There is a growing list of serine proteases that contain an essential lysine, but no essential histidines (Table I). These proteases have been grouped into their own ancestral clan of serine peptidases¹⁰. It has been proposed that this group of proteases uses a 'Ser/Lys dyad' mechanism, whereby an ϵ -amino group of a lysine sidechain acts as the general base to increase the nucleophilicity of the active site serine^{11,12} (Fig. 1b). The best-characterized enzymes within this group are those from *Escherichia coli*: leader peptidase¹³, LexA repressor¹¹ and, most recently, Tsp protease¹⁴. Generally, it appears that the Ser/Lys dyad proteases are less efficient than the triad proteases, although leader peptidase, which is very efficient against pre-protein substrates, is an exception to this rule. Among the many interesting unanswered questions about the function of these enzymes, perhaps two of the most critical are (1) do they contain an 'oxyanion hole' like the more classical proteases, and (2) how is the lysine maintained in the unprotonated state so that it can function as the general base? Answers to these questions will hopefully be provided by future experiments.

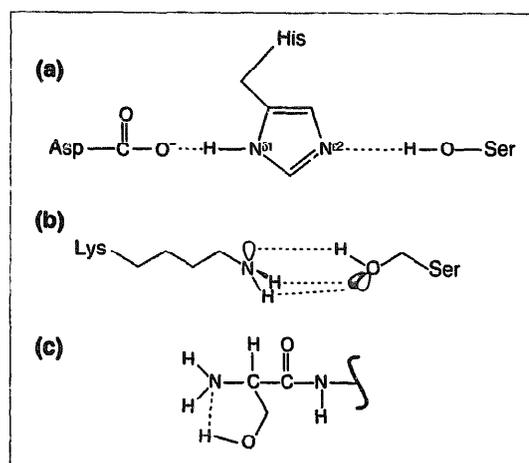


Figure 1
 (a) The classical catalytic triad. (b) The Ser/Lys catalytic dyad. (c) The hydroxyl/ α -amine dyad.

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Leader peptidase. Although leader peptidase, which removes signal peptides from exported proteins, is certainly a serine protease, it is not possible to classify it as such by use of classical protease inhibitors. For instance, leader peptidase is not sensitive to serine protease inhibitors such as di-isopropyl fluorophosphate (DFP) and phenyl methyl sulfonyl fluoride (PMSF)¹⁵. However, it is inhibited by certain β -lactams, which are believed to react with the catalytic serine residue¹⁶. Site-directed mutagenesis studies have shown that within *E. coli* leader peptidase, Ser90 (Ref. 17) and Lys145 (Refs 12, 13) are essential for activity. When Ser90 is replaced with Cys, the resulting active thiol90 leader peptidase can be inactivated by the cysteine-specific reagent *N*-ethyl maleimide¹³. Interestingly, the lysine residue proposed to be involved in the catalytic dyad is conserved only within prokaryotes and the mitochondrial leader peptidases. The homologous subunits within the endoplasmic reticulum signal peptidase do not contain the conserved lysine; rather, they contain a histidine residue at this position¹⁸.

Leader peptidase and the LexA-like enzymes share some sequence similarity in their active-site region, as well as similarity in their cleavage sites. Furthermore, they all show a preference for alanine at the P1 position¹⁰.

LexA, a repressor that can cleave itself in response to activation by the regulatory protein RecA, contains two essential residues, Ser119 and Lys156, which are proposed to be active-site residues¹¹. The main evidence that Lys156 functions as the general base comes from pH-rate profile studies. The pH-rate profile of LexA, in the absence of its activator protein RecA, reveals that a basic group with a pK_a of about 10 is important for catalysis¹⁹. When Lys156 is changed to Arg, the optimal pH for autocatalysis increases to above 11. These results support the hypothesis that the deprotonation of a basic residue at this position is critical for activity²⁰.

Interestingly, the crystal structure of the protein UmuD', which shares significant sequence homology with LexA, revealed that the sidechains of its proposed active-site residues (Ser60 and Lys97) were positioned correctly to form a hydrogen bond²¹. The active-site residues of UmuD' are also superimposable onto the active site residues of the class A β -lactamase (Fig. 2). Although the identity of the general base in class A β -lactamase is still controversial, there

Enzyme	Biological function	Catalytic mechanism	Essential residues	Ref.
<i>Escherichia coli</i> leader peptidase	Signal peptide cleavage	Ser/Lys dyad	S90, K145	13
<i>E. coli</i> LexA	Repressor of SOS regulon	Ser/Lys dyad	S119, K156	30
<i>E. coli</i> UmuD'	SOS mutagenesis response	Ser/Lys dyad	S60, K97	21
<i>E. coli</i> Tsp protease	Tail specific protease	Ser/Lys dyad	S430, K455	14
<i>E. coli</i> β -lactamase	Antibiotic resistance	Ser/Lys dyad	S70, K73	22
<i>Streptomyces</i> D-Ala-D-Ala peptidase	Cell wall biosynthesis	Ser/Lys dyad	S62, K65	23
<i>Pseudomonas</i> 7A glutaminase-asparaginase	Glutamine and asparagine deamidation	Thr/Lys dyad	T100, K173	24
<i>E. coli</i> penicillin acylase	Unknown	Ser/ α -amine dyad	S1, α -amine	25
<i>Thermoplasma acidophilum</i> 20S proteasome	Degradation of short-lived proteins	Thr/ α -amine dyad	T1, α -amine	26

is strong crystallographic evidence for a Ser/Lys dyad mechanism in these enzymes, which hydrolyse β -lactam rings in a manner similar to peptide bond hydrolysis by proteases²². These authors proposed that the buried Lys73 is kept in the neutral state by association with two proximal carbonyl oxygens. These interactions help to align the lysine-sidechain amine with the nucleophilic Ser70 hydroxyl group²². Conversely, NMR and chemical modification studies by the Frere laboratory support the theory that Glu166 working with an intervening water molecule acts as the general base³³. The significance of the connection between the Ser/Lys dyads and the binding of β -lactam type inhibitors and

substrates is still unknown. A model showing the initial step of the novel Ser/Lys catalytic dyad mechanism for leader peptidase and the LexA repressor is shown in Fig. 3.

Tsp protease. Recent studies with the Tsp protease have revealed that alanine substitutions of Ser430 and Lys455 inactivate the enzyme, suggesting that the serine and lysine are involved in the catalytic mechanism of this protease. Similar to leader peptidase, substitution of Ser430 with Cys results in a partially active thiol-protease that can be inhibited with cysteine-specific modifying reagents¹⁴.

Other enzymes that appear to use a hydroxyl/amine dyad are highlighted in

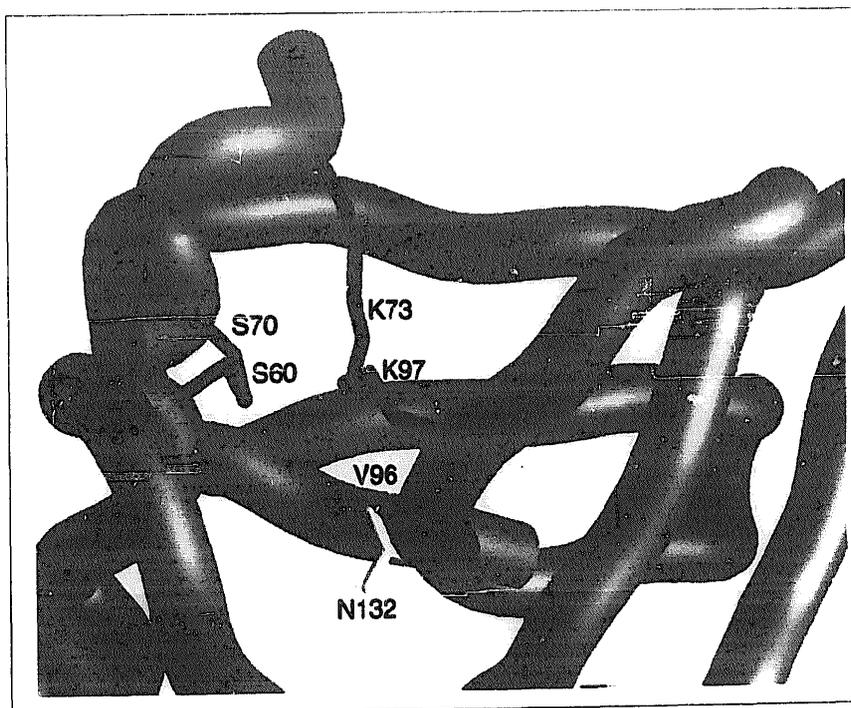


Figure 2

The backbone structure of UmuD' (blue) showing the active-site residues, Ser60 (S60; red) and Lys97 (K97; blue). Superimposed are two segments of the TEM1 β -lactamase (magenta), which contain the active-site residues, Ser70 (S70; red) and Lys73 (K73; blue). Figure reproduced from Ref. 21, with kind permission.

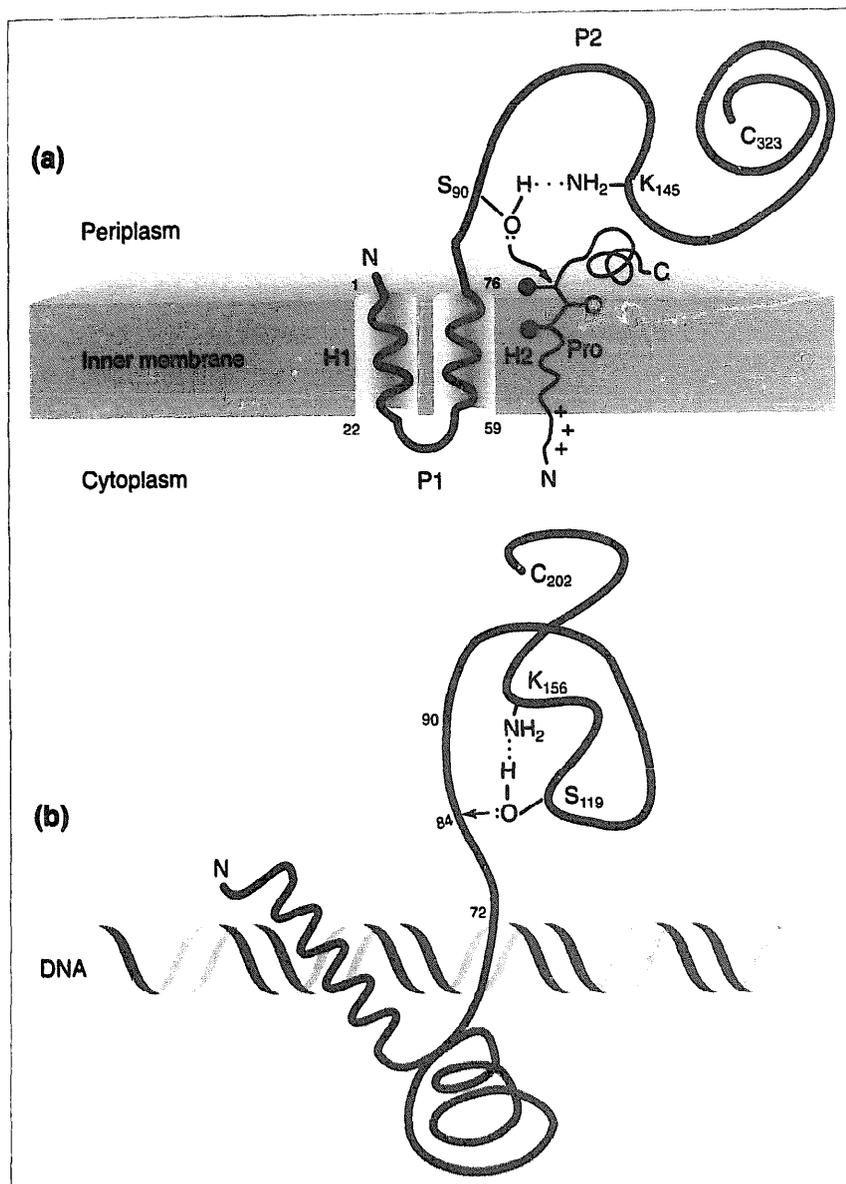


Figure 3

(a) A model of the membrane topology of the *Escherichia coli* leader peptidase and its first step in the cleavage of a leader peptide from an exported protein. The ϵ -amino group of Lys145 serves as a general base to abstract the proton from the hydroxyl sidechain of Ser90, which then acts as the nucleophile to attack the scissile peptide bond of the translocated preprotein substrate. Leader peptidase has a substrate specificity for preproteins that contain a small, uncharged residue at the P1 position (seen as a black dot in the figure) and a small uncharged or aliphatic residue at the P3 position (seen as a black dot in the figure) in the leader sequence. (b) The first step in the intramolecular cleavage of the *E. coli* LexA repressor. The ϵ -amino group of Lys156 serves as a general base to abstract the proton from the sidechain hydroxyl of Ser119, which then acts as the nucleophile to attack the scissile peptide bond in this intramolecular cleavage reaction. The cleavage site for LexA is the peptide bond between Ala84 and Gly85, which lie in the hinge region (red). The amino-terminal region (green) is the DNA-binding domain. The carboxy-terminal region (blue) contains the active-site residues involved in the specific cleavage.

Table I. These include D-Ala-D-Ala transpeptidase from *Streptomyces*²³ and *Pseudomonas* 7A glutaminase-asparaginase²⁴.

Hydroxyl/ α -amine dyad catalysis: variations on a theme

Another type of hydroxyl/amine catalytic dyad, similar to the Ser/Lys dyad,

is found at the active-site of penicillin acylase²⁵. This enzyme contains an amino-terminal serine residue that appears to use its own α -amino group as a general base (Fig. 1c). Although not a protease, penicillin acylase hydrolyses an amide bond in substrates such as penicillin G.

The 20S proteasome from *Thermoplasma acidophilum* also appears to employ an α -amino group as a general base in its proteolytic mechanism²⁶. The proteasome is unique in that instead of a serine hydroxyl it uses a threonine hydroxyl as the nucleophile. Huber and co-workers²⁶ propose, from their crystal structure of the proteasome, that Thr1 uses its own α -amino group in a hydrogen-bonded, five-membered ring structure to activate its own sidechain hydroxyl group (Fig. 1c). Alternatively, they propose that Thr1 uses Lys33 as a general base.

How lysine can function as a general base in catalytic dyads

The ϵ -amino group of lysine must reside in the deprotonated state for lysine to act as a general base in catalytic dyads. The pK_a of free lysine in water is 10.5. Therefore, the enzyme must provide a local environment for the lysine that will enable it to remain deprotonated.

There are many examples in the literature of enzymes that contain lysine residues with significantly depressed pK_a values. These low pK_a values are usually explained from either an electrostatic or a polarity viewpoint. In the electrostatic situation, a lysine with a low pK_a is generated when a positive charge, such as another lysine or an arginine, is immediately proximal to the lysine. This electrostatic effect in enzymes was first investigated in detail by Westheimer's laboratory in their study of the enzyme acetoacetate decarboxylase²⁷.

An increase in a lysine sidechain pK_a can occur when there is a carboxylate nearby. In this scenario, the pK_a of the ϵ -amino group increases, as a result of its greater affinity for protons, in order to neutralize the interaction with the negatively charged carboxylate. Sliatay and Vu have shown that one of the two conserved negative charges within the LexA repressor family (Glu152) might be positioned near the proposed lysine general base²⁸. They demonstrate that removal of this negatively charged residue actually increases the rate of the LexA cleavage reaction, and propose that a negative charge in the active site would impede the activity of the LexA reaction by stabilizing the positively charged lysine of the transition state and, as a result, slow the protonation of the leaving amino group. They also point out that the ϵ -amino group of lysine, serving as a general base, is different from the imidazole sidechain of histidine in that

it does not require a counter charge to effect a proton transfer²⁸. Therefore, in the absence of the histidine general base, an active-site negative charge appears to hinder the catalytic efficiency, and thus a dyad should be sufficient for catalysis. This was first observed when a tenfold increase in activity was seen upon removing a negative charge (Asp32) from the active site of a subtilisin mutant that lacked the histidine general base³.

A hydrophobic microenvironment is another situation that produces a lowered lysine pK_a . There are examples of buried lysines with pK_a values as low as 6.5 (Ref. 29).

In the case of the LexA repressor, the binding of the regulatory protein RecA is required *in vivo* for the intramolecular proteolysis to occur. In the absence of RecA, proteolysis will occur only at elevated pH (>10). It is proposed that the RecA reduces the pK_a of the Lys156 so that it is deprotonated at physiological pH³⁰. There is no evidence to show whether the effect that RecA has on the pH at which autocatalysis occurs is a result of electrostatic, polar or steric effects.

As the serine (hydroxyl) and lysine (amine) interactions are being investigated in the biochemical field, the physical organic chemists are exploring, with great interest, the triple hydrogen-bond arrangement that can occur between these functional groups³¹ (Fig. 1b). The additional stability of this hydrogen-bonding arrangement might contribute to low lysine pK_a values in some cases. But, it is possible that such hydrogen bonds would form only upon binding of the substrate, if they are similar at all to the classical serine proteases. It has been shown for the classical serine proteases that a strong hydrogen bond between the catalytic serine hydroxyl group and the catalytic histidine imidazole group usually only forms upon binding of the substrate. Conversely, the proposed catalytic residues of the free RTEM-1 β -lactamase (Lys73 and Ser70), as well

as the catalytic residues of UmuD' (Lys97 and Ser60), have been shown to be within hydrogen-bonding distance^{22,21}. Investigation into the mechanism of intramolecular general base catalysis is still under way at the small-molecule level and might lead to insights regarding these reactions within enzymes³².

Concluding remarks

Recent developments have shown that serine proteases can function without the landmark Ser/His/Asp catalytic triad. It has been proposed that these proteases carry out catalysis, in some instances, employing an active-site Ser/Lys dyad. This novel mechanism, where lysine is believed to function as a general base, might help to explain why such proteases are resistant to inhibitors against the classical serine protease group. Other variations on the classical catalytic triad theme also exist, such as that found in the penicillin acylase, which seems to use just one residue, the amino-terminal serine, as its active center.

So far, catalytic hydroxyl/amine dyads are found predominantly in enzymes from prokaryotic sources or sources that are believed to have evolved from prokaryotes. Thorough study of these enzymes will lead to a deeper understanding of the full structural and functional repertoire of the hydroxyl/amine interactions in proteins and will be instrumental in the rational design of novel antibiotics.

Acknowledgements

This work was supported by the American Heart Association Grant-in-Aid grant (94011570) and the NIH (GM48805). M. P. would like to thank Procter & Gamble for a one year graduate fellowship. We would like to thank T. Peat and N. Strynadka for their help in the preparation of Figs 2 and 3. We would also like to acknowledge all those whose work has not been cited owing to a limit in the number of references permitted.

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