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Unconventional serine proteases: Variations on the catalytic Ser/His/Asp triad configuration

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Abstract
Serine proteases comprise nearly one-third of all known proteases identified to date and play crucial roles in a wide variety of cellular as well as extracellular functions, including the process of blood clotting, protein digestion, cell signaling, inflammation, and protein processing. Their hallmark is that they contain the so-called “classical” catalytic Ser/His/Asp triad. Although the classical serine proteases are the most widespread in nature, there exist a variety of “nonclassical” serine proteases where variations to the catalytic triad are observed. Such variations include the triads Ser/His/Glu, Ser/His/His, and Ser/Glu/Asp, and include the dyads Ser/Lys and Ser/His. Other variations are seen with certain serine and threonine peptidases of the Ntn hydrolase superfamily that carry out catalysis with a single active site residue. This work discusses the structure and function of these novel serine proteases and threonine proteases and how their catalytic machinery differs from the prototypic serine protease class.

Keywords: enzymes; active sites; structure/function studies; protein turnover; structure; serine proteases; threonine proteases

Proteases play indispensable functions in all living cells. In mammalian cells, proteases function in angiogenesis, apoptosis, differentiation, immune response, matrix remodeling, and protein activation. They are found in every organelle and compartment of most, if not all, eukaryotic cells. There are over 500 proteases in human cells as determined by sequencing of the human genome (Puente et al. 2005). They are associated with a number of diseases, including cardiovascular and Alzheimer’s disease, cancer, autoimmune diseases, inflammation, and hypertension.

Proteases are typically grouped into four mechanistic classes: the cysteine, serine proteases, metallo, and aspartic acid proteases. In 2004, the protease field was honored by a Nobel Prize in chemistry presented to Avram Hershko, Aaron Ciechanover, and Irwin Rose for their pioneering work on the ubiquitin-proteasome system in which the proteasome is a novel threonine protease. The proteasome, like the proteases in the other four classes, is a target for drug discovery programs. It is essential to understand the specificity, catalytic mechanism, and structure of proteases in order to facilitate drug design efforts.

The best-known class of proteases is the serine protease class (E.C. 3.4.21) that uses the classical Ser/His/Asp catalytic triad mechanism, where serine is the nucleophile, histidine is the general base and acid, and the aspartate helps orient the histidine residue and neutralize the charge that develops on the histidine during the transition states. Well-studied members of this class are chymotrypsin, trypsin, elastase, and subtilisin. The three-dimensional structure of chymotrypsin was first solved in...
1967 (Matthews et al. 1967), and the first trypsin structure was solved in 1974 (Huber et al. 1974; Stroud et al. 1974).

John Northrop made a major advance in the serine protease area in the 1930s by successfully crystallizing proteases, including trypsin and chymotrypsin (Northrop and Kunitz 1931). Other early important discoveries included the identification of the specific amino acid that functioned as the nucleophile by modification with DFP (disopropyl fluorophosphates) in both trypsin and chymotrypsin (Dixon et al. 1958). The determination of the amino acid sequence of trypsinogen (Walsh and Neurath 1964) and chymotrypsinogen (Hartley 1964) revealed they are homologous proteases. In 1969, David Blow proposed the famous charge-relay mechanism where two proton exchanges were thought to occur (from Ser195 to His57 and from His57 to Asp102) (Blow et al. 1969). Later NMR data (Robillard and Shulman 1974; Bachovchin 1985) and neutron diffraction structural data (Kossiakoff and Spencer 1981) suggest a single proton exchange from Ser195 Oγ to the His57Nε2. More recently, ultra-high-resolution structures of serine protease intermediates have been performed to provide mechanistic insights into catalysis (Fodor et al. 2006).

In addition to the Ser/His/Asp serine proteases, there are serine proteases that use catalytic residue arrangements other than the canonical triad (Fig. 1; Table 1). These atypical serine proteases use novel triads such as Ser/His/Glu, Ser/His/His, or Ser/Glu/Asp, dyads such as Ser/Lys or Ser/His, or a single Ser catalytic residue. There are also proteases in which the nucleophilic hydroxyl is derived from threonine rather than a serine residue.

In this work, we describe serine proteases with non-canonical active site arrangements. We will discuss and compare atypical serine proteases with the classical serine protease class in terms of the structure–function and catalytic mechanism. Also, we will present some ideas about why there are different types of active site configurations within serine/threonine proteases.

### Different clans of Ser/Thr proteases

Serine/threonine proteases have been classified into clans and families (see Merops database, http://merops.sanger.ac.uk) (Rawlings et al. 2006). Members of the same clan are proteases that have evolved from a common ancestor and share a common protein fold. Proteases in the same family are related based on the sequence homology of their amino acid sequences. Most, but not all, clans consist of one active site arrangement. The prefix “S” is used for clans formed entirely from families which are serine peptidases, whereas “P” is used for clans in which the homologous families are of several catalytic types (e.g., serine and cysteine), despite the fact that they have diverged from a common ancestor. Table 1 shows that proteases with a Ser/His/Asp triad fall within four of these clans (PA, SB, SC, and SK), while the Ser/Lys proteases fall within five clans (SE, SF, SJ, SK, and SR). This shows that different ancestors can converge on the same Ser/His/Asp or Ser/Lys mechanism.

Since some members of the same clan can use different active site architectures, this indicates that the tertiary structure is not always related to the active site configuration. For example, clan PB has family members that use a Ser/His/Glu active site configuration or Ser- or Thr-only active site architecture (Table 1). Why do different serine/threonine proteases use different active site configurations? The different active site arrangements may allow for activity in a different cellular environment. For instance, the proteases with Ser/Lys active sites typically carry out catalysis with a pH optimum that is higher than Ser/His/Asp proteases. In contrast, the pH optimum is lower for serine proteases with Ser/Glu/Asp active sites than those with Ser/His/Asp active sites. In large part, the different pH optima reflect differences in the pKₘ values of the different general base residues that are employed in catalysis. In addition, variations in the active site architecture of proteases may influence what cellular inhibitor they are susceptible to. There are other possibilities as well that we discuss below.
### Table 1. Clans and families of serine and threonine proteases with representative PDB accession codes and active site residues

<table>
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Ser/His/Asp triad

Chymotrypsin- and trypsin-like proteases

Two of the best-known serine proteases that utilize the Ser/His/Asp triad are chymotrypsin and trypsin. They share the same protein fold and have their catalytic residues in the order of His/Asp/Ser from the N to C terminus. Their active site regions are composed of (1) the substrate binding groove where nonspecific main-chain hydrogen bond interactions occur between the enzyme and substrate, (2) the substrate specificity binding pockets, (3) the catalytic Ser/His/Asp triad, and (4) the oxyanion hole (for conventions regarding peptides, proteases, protease nomenclature, see http://www.chem.qmul.ac.uk/iubmb/enzyme/EC3/intro.html#EC34) (for a comprehensive review on the catalytic mechanism of classical serine proteases, see Hedstrom [2002]).

The substrate binding pocket of the protease allows the protease to bind its substrates and determines the substrate specificity of the enzyme. For example, trypsin cleaves its substrates at the C-terminal side of arginine or lysine (P1 position, Schechter and Berger nomenclature) (Schechter and Berger 1967). This specificity for a P1 basic side chain of substrates is stabilized by an acidic residue near the bottom of the sterically complementary deep S1 binding pocket. In some proteases, the specificity can go to the opposite side of the cleavage site (scissile bond) as well, P9 side, or also further down the chain of the substrate to the P3, P4, P5 site, etc. The substrate region near the cleavage site needs to be in an extended conformation (not protected by hydrogen bonds within the substrate itself) to bind to the protease in order for the carbonyl of the scissile bond to be attacked by the serine hydroxyl nucleophile of the protease.

Chymotrypsin numbering is conventionally used for sequence and structure comparisons within clan PA such that the catalytic nucleophile is Ser195, the general base is His57, and the aspartate residue is Asp102 (Fig. 2). The backbone amide nitrogens of Gly193 and Ser195 form the oxyanion hole. The serine attacks the trigonal planar scissile carbonyl carbon (Fig. 2A), forming an oxyanion tetrahedral intermediate (Fig. 2B). The tetrahedral intermediate then collapses, which is followed by the loss of the C-terminal peptide. This gives rise to an acyl-enzyme intermediate (Fig. 2C). The acyl intermediate is then attacked by an activated water molecule (the same active site histidine again functioning as the general base), giving rise to the second tetrahedral intermediate (Fig. 2D). This intermediate then collapses leading to the release of the N-terminal peptide.

In the catalytic mechanism, hydrogen bond interactions have been proposed to play important roles. The nucleophilic Ser195 OyH hydrogen bonds to His57 Ne2 and transfers its proton from its hydroxyl group (OyH) to the electrons on the His57 side-chain nitrogen (Nε2). The His57 Nε1/Asp102 Oδ1 hydrogen bond could be classified as a low-barrier hydrogen bond (Frey et al. 1994), although its exact role is still controversial. These hydrogen bonds are present throughout the catalytic cycle, as seen from the hydrogen-bonding network in noncovalent complexes, acyl-enzyme complexes, and transition state analog complexes. In addition, a C–H–O hydrogen bond has been proposed between the Ser214 main-chain carbonyl oxygen of chymotrypsin-like proteases and the hydrogen on the ε1 carbon of the His57 side chain (Derewenda et al. 1994; Ash et al. 2000). It has been postulated that this interaction may function to prevent the localization of the positive charge on Nε1 of His57.

High-resolution crystal structures (at 1.2 Å and better) have provided considerable information about serine protease mechanism and enzyme mechanism in general. Katona et al. (2002) solved the structure of the porcine pancreatic elastase in complex with a peptide at 0.95 Å resolution. This high-resolution structure revealed that the ester bond between the carbonyl of the peptide substrate and the Oy of the nucleophilic serine has a planar geometry. This observation refined the previous theory of Warshel and
Russell (1986), who proposed that the H-bonds between the ester carbonyl oxygen and the oxyanion hole induced strain on the ester bond and distorted it toward sp3 hybridization, which would enhance the formation rate of the second tetrahedral intermediate. A recent comparison of the acyl-enzyme structure and a high-resolution Michaelis complex structure suggests that the β-strand H-bond type interactions between the enzyme binding site and substrate become shorter during the transition from the Michaelis complex to the acyl–enzyme complex (Fodor et al. 2006). This result suggests that the enzyme may be able to utilize the energy released from the shorter, stronger hydrogen bonds to help overcome the energy barrier in the nucleophilic attack steps.

Mutagenesis studies on the related trypsin protein revealed that Ser195 and His57 contribute ~1 million-fold toward the rate of catalysis for trypsin (Corey et al. 1992). The Asp102 contributes ~10,000-fold as determined by mutagenesis experiments (Craik et al. 1987; Corey et al. 1992).

Subtilisin-like proteases

Like chymotrypsin (Fig. 3A) and trypsin, subtilisin (Fig. 3B) utilizes a Ser/His/Asp triad (Fig. 3B), but it has no sequence similarity to chymotrypsin-like proteases. In fact, it adopts the α/β-twisted open sheet structure (Wright et al. 1969) rather than the double β-barrel structure seen in the chymotrypsin-like proteases. Subtilisin and chymotrypsin provide excellent examples of convergent evolution (Wallace et al. 1996). The protein folds of these proteases are completely different, although they both converged on a similar Ser/His/Asp mechanism to carry out proteolysis. In the subtilisin-like proteases, the order of the active site residues is Asp/His/Ser in the primary sequence, from N to C terminus, which is different than the chymotrypsin-like proteases (His/Asp/Ser). The serine and histidine residues contribute 10⁶-fold toward catalysis, and the aspartic acid contributes 10⁴-fold residue (Carter and Wells 1988). The active site region of subtilisin is shown in Figure 3B. The subtilisin Asn155, which forms a hydrogen bond to the oxyanion intermediate, contributes ~100- to 1000-fold (Wells et al. 1986; Pantoliano et al. 1987). Subtilisin is engineered for thermal and pH stability for use in laundry detergents. These subtilisin mutants were among the first patents granted for engineered proteins.

Other Ser/His/Asp proteases with different folds than the chymotrypsin- and subtilisin-like proteases include carboxypeptidase Y (Jung et al. 1999) and ClpP (Wang et al. 1997) proteases (Table 1). Interestingly, the ClpP protease is within the SK clan that has members that possess alternative active site arrangements such as the Ser/Lys dyad configuration.

Subtilisin

Asp/His/Glu triad

Aspartyl dipeptidase

A variation of the classical Ser/His/Asp triad is found in the aspartyl dipeptidase protease, where the aspartate is substituted by a glutamate residue (Rawlings and Barrett 1999). Aspartyl dipeptidase is not inactivated by the conventional serine protease inhibitor DFP or PMSF (phenylmethane sulfonyl fluoride) (Conlin et al. 1994). While initial site-directed mutagenesis studies suggested that aspartyl dipeptidase was a classical serine protease, the structure of aspartyl dipeptidase from Salmonella typhimurium revealed that it in fact belongs to a new serine peptidase family with a Ser/His/Glu triad (Hakansson et al. 2000). The Ser120 Oγ atom is 2.7 Å away from the His157 Nε2 atom. The
Glu192 Oe2 is within 2.7 Å of the His Nδ1 atom. This glutamate carboxylate/histidine imidazole hydrogen bond adopts an unusual anti-conformation rather than the syn-conformation usually seen with other serine proteases (Ippolito et al. 1990). Mutagenesis of the glutamate residue of the *S. typhimurium* aspartyl dipeptidase resulted in a 100-fold drop in activity. Typically, mutagenesis of the aspartate residue, the third member of the catalytic triad (Ser/His/Asp), leads to a 10,000-fold loss in activity (Craik et al. 1987; Carter and Wells 1988; Corey et al. 1992). Interestingly, the aspartyl dipeptidase has a similar protein fold as the cysteine proteases γ-glutamyl hydrolase and PfpI peptidase, suggesting they evolved from a common ancestor.

**LD-carboxypeptidase**

The Ser/His/Glu active site arrangement has evolved more than once. The LD-carboxypeptidase from *Pseudomonas aeruginosa* that functions in peptidoglycan recycling uses a Ser/His/Glu triad (Fig. 4A). The crystal structure of the LD-carboxypeptidase from *P. aeruginosa* was solved to 1.5 Å resolution (Korza and Bochtler 2005). Its protein fold consists of an N-terminal β-sheet and a C-terminal β-barrel domain that is different from the aspartyl dipeptidase fold. This indicates that convergent evolution has taken place to give rise to the Ser/His/Glu triad. It is not clear why most serine proteases utilize an aspartate rather than the glutamate observed with the LD-carboxypeptidase. Notably, the Ser/His/Glu active site is observed in lipases (Schrag et al. 1991), and in some esterases as well (Nachon et al. 2005).

**Ser/His/His triad: Cytomegalovirus protease**

Another unconventional active site triad, Ser/His/His, is found in herpes virus proteases that plays a key role in the maturation of the assembly protein. The cytomegalovirus protease is inactivated by DFP (Stevens et al. 1994). Ser132 was identified as the active site serine of the human cytomegalovirus protease as it was modified by the inhibitor. The invariant His63 is critical for activity as determined by site-directed mutagenesis (Welch et al. 1993).

The Ser/His/His catalytic triad was validated by the crystal structure of the cytomegalovirus protease (Qiu et al. 1996; Shieh et al. 1996; Tong et al. 1996). The structure revealed that the protein has a unique fold with the Ser132 Oγ atom and bridging His63 Nε2 nitrogen being within hydrogen-bonding distance (Fig. 4B). In addition, the His63 N81 is within hydrogen-bonding distance to the His157 Ne2 that replaces the aspartate residue within the classic serine proteases.

Site-directed mutagenesis studies showed the non-bridging histidine residue (His157) of the triad contributes only ~10-fold to the catalytic rate of the enzyme (Khayat et al. 2001) in comparison to 1000- to 10,000-fold for the contribution of aspartate in the classical triad protease (Carter and Wells 1988; Corey et al. 1992). Therefore, the cytomegalovirus protease functions more like a Ser/His dyad. Herpes virus proteases, in comparison to Ser/His/Asp proteases such as trypsin and subtilisin, are kinetically less efficient enzymes. This suggests that this alternate active site is a way to tune the catalytic activity of the serine protease.

**Ser/Glu/Asp triad: Sedolisin proteases**

The sedolisin proteases carry out catalysis using a novel Ser/Glu/Asp triad (Wlodawer et al. 2001a). These proteases take their name from their catalytic groups serine (S), glutamate (E), and aspartate (D). Due to the low pKₐ
of the carboxylate that plays the role of the general base, these proteases are active at low pH, which is unusual for serine proteases. Proteases in this clan include kumamolysin that is found in acidic hot springs and human tripeptidyl peptidase that is found in the low pH environment of the lysosome. This is an excellent demonstration of how the different active site architectures allow the protease to be active in different cellular environments.

Because they operate at low pH, these proteases were thought to be aspartic acid proteases. However, these proteases are not inactivated by pepstatin, which is a natural inhibitor of the aspartic acid class of proteases. Rather they are inactivated by inhibitors with aldehyde functional groups such as chymostatin, which typically modify the serine nucleophile within serine proteases (Wlodawer et al. 2001b).

The X-ray structure of the sedolisin protease from *Pseudomonas* revealed that these proteases have the subtilisin protein fold (Wlodawer et al. 2001a). Structures of the enzyme with the iodotyrostatin inhibitor revealed that Ser287 is the catalytic residue. Ser287 is covalently attached to the inhibitor and forms a hemiacetal linkage to the Ser278 Oγ side chain. Within hydrogen-bonding distance of the Ser278 side chain is the side-chain carboxylate of Glu80 (Fig. 4C), suggesting that the acidic residue acts as the general base in catalysis. The third member of the triad is Asp84, which interacts with the Glu80 side chain. It is the Glu80/Asp84 pair that allows this protease class to have optimum activity at acidic pH. The active site geometry was also confirmed in a 1.4 Å X-ray structure of kumamolysin, a serine-carboxyl-type protease from *Bacillus* (Comellas-Bigler et al. 2002). Site-directed mutagenesis studies confirmed the importance of these active site serine, glutamate, and aspartate residues (Oyama et al. 1999, 2005).

**Ser/Lys dyad**

**Type 1 signal peptidases**

The prototypic protease of the Ser/Lys class is the type I signal peptidase found in the SF clan (Table 1). These proteases function to proteolytically remove N-terminal signal peptides from exported proteins after they have been translocated across the membrane (for review, see Paetzel et al. 2002b). Signal peptidases are integral membrane proteins with their catalytic domain on the extracytoplasmic side of the membrane.

Early studies pointed to the fact that signal peptidases were novel proteases. First, these proteases failed to be inhibited by inhibitors against the aspartic acid, metallo, serine, and cysteine protease groups (Zwizinski et al. 1981; Black et al. 1992; Kuo et al. 1993). Second, the *Escherichia coli* signal peptidase had a critical serine (Ser90) and lysine (Lys145) residue (Sung and Dalbey 1992; Black 1993; Tschantz et al. 1993) but no critical histidine residue (Sung and Dalbey 1992), and both residues are absolutely conserved in evolution within the bacterial, chloroplast, and mitochondrial signal peptidase family members (Paetzel et al. 2002b).

The 1.9 Å X-ray structure of the catalytic domain of the *E. coli* signal peptidase with a covalently bound inhibitor provided direct proof of a Ser/Lys dyad operating within the signal peptidase family (Paetzel et al. 1998). The Ser90 was covalently linked to the 5S-penem inhibitor, indicating that the Ser90 is the nucleophile. The Lys145 was within 2.8 Å of the Ser90 (Fig. 5A), supporting the idea that it does indeed function as the general base. No other ionizable groups were located within 7.5 Å of the Ser90 Oγ residue. The Lys145 is completely buried within the active site, which presumably lowers the lysine’s pKₐ, explaining how the lysine can serve as a general base at biological pH. Interestingly, Ser278 Oγ forms a hydrogen bond to Lys145 Nζ, helping to orient the lysine residue. The structure of the signal peptidase apoenzyme is consistent with Ser88 Oγ participating in the formation of the oxyanion hole (Paetzel et al. 2002a).

**Figure 5.** Structures of serine proteases that use active site dyads. (A) The Type 1 signal peptidase from *E. coli* (PDB 1b12) uses a Ser/Lys catalytic dyad. (B) The rhomboid protease GlpG from *E. coli* (PDB 2ic8) uses a Ser/His dyad.
Mutagenesis studies have shown Ser88 contributes ~1000-fold to catalysis by stabilizing the oxyanion intermediate (Carlos et al. 2000).

The active site lysine residue has an apparent pK_a of 8.7 as determined from the pH rate profile of the E. coli signal peptidase (Paetzel et al. 1997). Typically, Ser/Lys proteases show optimum activity at a higher pH such as 9 compared with Ser/His/Asp proteases that have optimum activity near pH 7.

The UmuD family of peptidases

Other Ser/Lys dyad proteases are found within the UmuD family of peptidases that include UmuD, LexA, and the λ repressor (Slilaty and Little 1987; Little 2004; Paetzel and Woodgate 2004). These proteases are involved in the SOS response and function as repressors. These proteins undergo autoproteolysis in vivo in a manner dependent on RecA, a protein involved in homologous recombination. The biological function of autocleavage is to inactivate these proteins (Mustard and Little 2000).

UmuD was the first protease in the UmuD family whose structure was solved to high resolution. Its protein fold consists of a β-sheet structure (Peat et al. 1996), and the critical serine and lysine residues that were implicated in autocatalysis (McDonald et al. 1998) are within hydrogen-bonding distance. The likely role of the RecA binding in the in vivo autoproteolysis reaction is to decrease the pK_a of the general base by burying the ε-amino group of the lysine in a hydrophobic environment.

LexA protease is proposed to use an active site Ser/Lys dyad that is not inactivated by a classical protease inhibitor (Slilaty and Little 1987). Autodigestion of LexA is pH-dependent. In the absence of RecA, a basic residue with a pK_a of 10 is necessary for autodigestion (Slilaty et al. 1986). A likely candidate for this basic group is a lysine residue. Further evidence came from structural studies. Briefly, the structure of LexA was solved at 2.0 Å with two conformations of the protein present (Luo et al. 2001). One of the conformations, the “cleavable form (C),” revealed the scissile bond of LexA within the active site region. The other conformation, the “noncleavable form (NC)” of the protein had the scissile bond of LexA located ~20 Å from the Ser/Lys dyad. A 1.8 Å resolution crystal structure revealed that the catalytic serine and lysine are within hydrogen-bonding distance. Consistent with the lysine functioning as a general base in the proteolytic mechanism, the lysine side chain is completely buried in a hydrophobic pocket in the cleaved form. Similar to signal peptidase, this hydrophobic environment is expected to lower the pK_a of the catalytic lysine. The residues that contribute to the oxyanion hole are the main-chain amide nitrogens of Met118 and Ser119.

The λ repressor, which also has a Ser/Lys dyad, has a Thr190 residue that may also help orient the catalytic Lys192 residue as it is hydrogen bonded to this residue (Bell et al. 2000). Most of the Ser/Lys proteases (such as UmuD, LexA, Lon, VP4, signal peptidase I, and signal peptide peptidase SppA) have a second (non-nucleophilic) hydroxyl group coordinating to the lysine, suggesting this interaction has a functional role (Paetzel et al. 2002a). This seems to be a common theme with Ser/Lys proteases, in general.

Comparison of the active site regions of the signal peptidase and UmuD family proteases reveals that the orientation of the serine and lysine are quite similar (Paetzel and Strynadka 1999; Paetzel et al. 2002b). In these proteases, the serine hydroxyl groups attack the si-face of the scissile amide bond. In contrast, serine proteases that contain the Ser/His/Asp triad attack the re-face of the substrate (James 1994).

Lon protease

Another Ser/Lys dyad serine protease is the Lon protease, which is involved in quality control and degradation of misfolded proteins. It has a peptidase and an AAA domain. The latter domain uses the energy of ATP hydrolysis to actively unfold proteins (Chung 2004). However, ATP hydrolysis is not required for cleavage of short peptides.

In 2003, the E. coli Lon protease was shown to contain an essential serine and lysine residue (Rotanova et al. 2003). Like many Ser/Lys proteases (Tschantz and Dalbey 1994), the Lon protease is not inhibited by the classical serine protease inhibitors such as PMSF or DFP. The X-ray crystal structure of the peptidase domain was achieved using a mutant with the catalytic serine residue mutated to an alanine. Modeling studies where the Ala679 was replaced with Ser679 are consistent with a Ser/Lys dyad mechanism and revealed that the ε-amino group of Lys722 is in position to hydrogen bond to the catalytic Ser679 residue. In addition, the conserved Thr704 residue could hydrogen bond to the Lys722 side chain, helping to position the general base. The protein fold of the E. coli Lon protease domain is completely different from that found in the signal peptidase and UmuD family members and contain 10 β-strands and six α-helices (Botos et al. 2004).

C-terminal processing peptidases

The Ser/Lys dyad C-terminal processing peptidases (Cpps) cleave precursor proteins in eubacteria and plants and show no sequence homology with signal peptidases and UmuD members or to the D-Ala-D-Ala
carboxypeptidases. *E. coli* tail specific protease (Tsp) exhibits C-terminal processing activity and is implicated in the degradation of truncated proteins that are tagged by the SsrA pathway (Keiler et al. 1996). Tsp is located in the periplasm of Gram-negative bacteria and in the extracellular/cell wall region of Gram-positive bacteria.

The *E. coli* Tsp, as seen with the *E. coli* signal peptidase, is not inactivated by inhibitors against the four groups of proteases (Silber et al. 1992). Mutagenesis studies showed that the protein had a critical serine and lysine residue (Keiler and Sauer 1995). Sequence alignment studies of Cpp members revealed that these residues are absolutely conserved (Keiler and Sauer 2001). Interestingly, N-ethylmaleimide could inactivate Tsp when the critical serine is changed to a cysteine, supporting the notion that the serine is an active site residue (Keiler and Sauer 1995). Tsp has activity over a broad pH range, from pH 5 to 9.

Currently, the only Cpp whose structure is solved is the photosystem II D1 C-terminal processing protease from the green algae *Scenedesmus obliquus* (Liao et al. 2000), which is essential for the assembly of the manganese cluster involved in water oxidation (Taylor et al. 1988).

The structure reveals the C-terminal protease domain with the active site Ser372 and Lys397 residues, a middle PDZ-like domain, and a mainly α-helical N-terminal domain. The active site Lys397 is hydrogen bonded to a threonine residue. The active site Ser372 interacts with several water molecules. In order for Lys397 to act as a general base, a conformational change has to take place upon substrate binding. In addition, substrate binding most likely would cause the Lys397 to become buried in a hydrophobic environment so that its pKₐ is lowered.

Other Ser/Lys peptidases include the penicillin binding proteins 5 D-Ala-D-Ala carboxypeptidase B (Davies et al. 2001), the viral VP4 protease of the Birnaviridae family (Feldman et al. 2006; Lee et al. 2007), signal peptide peptidase A (Kim et al. 2007), and Lactoferrin (Table 1; Anderson et al. 1989). The Ser/Lys dyad active site arrangement has arisen many times during evolution.

**Ser/His Dyad**

**Rhomboid protease**

Rhomboid proteases have a Ser/His active site dyad. These proteases play roles in signal transduction and participate in regulated intramembrane proteolysis (Selkoe and Kopan 2003; Wolfe and Kopan 2004). They have six transmembrane helices, and their catalytic groups are localized within the membrane plane (Wolfe and Kopan 2004; Maegawa et al. 2005). Initial site-directed mutagenesis studies suggested that the rhomboid protease utilized a Ser/His/Asn catalytic triad (Urban et al. 2001). However, further studies showed that the asparagine, the third member of the proposed triad, is not always necessary for catalysis (Lemberg et al. 2001; Maegawa et al. 2005).

Recently, the X-ray structure of the *E. coli* and *Haemophilus influenzae* rhomboid protease GlpG was solved (Wang et al. 2006; Wu et al. 2006; Ben-Shem et al. 2007; Lemieux et al. 2007)). The *E. coli* structure revealed that the protease does use a Ser/His dyad in catalysis where Ser201 and His254 are within hydrogen-bonding distance (Fig. 5B). The structure showed that its catalytic groups are in a hydrophilic environment, ~10 Å below the periplasmic membrane surface. In addition, the GlpG protease has a proposed gate that controls access to the hydrophilic active site region of GlpG (Baker et al. 2007; Wang and Ha 2007). Although the gating mechanism is controversial because none of the structures contain an extended substrate, this mechanism allows for the substrate to enter the substrate binding site of the protease laterally from the lipid phase.

**Nucleoporin**

Another Ser/His peptidase is the nucleoporin proteins that undergo autoprocessing to produce a dimer. The crystal structure of the human nucleoporin Nup98 revealed an active site Ser/His dyad (Hodel et al. 2002). The structure was determined using a catalytic deficient mutant where the serine nucleophile was substituted with an alanine. The overall structure revealed a β-sandwich-like protein fold that is half open. Modeling studies where the catalytic Ser864 hydroxyl side chain is modeled from the solved mutant Ser864Ala structure show that it can form a hydrogen bond to the His862. Surprisingly, mutagenesis of the His862 to alanine only slightly inhibits the enzyme with over 50% activity remaining. This is very unusual for serine proteases as a similar mutation in subtilisin causes a million-fold inhibition (Hodel et al. 2002).

**“Ser-only” configuration**

**L-aminopeptidase D-Ala-esterase/amidase**

The simplest active site arrangement uses a “Ser-only” configuration. Proteases that use this configuration are made in a precursor form and undergo autoprocessing to generate the active site residue at position 1 in the mature protein. This active site arrangement is found in the *Ochrobactrum anthropi* L-aminopeptidase D-Ala-esterase/amidase. After autocleavage, Ser250 becomes the first residue of the mature protein and the α-amino group of the newly generated N terminus of the protein functions as the general base in catalysis abstracting a proton from the
Ser250 hydroxyl group. The protein fold of this protease is novel and characterized by a four-layered αβαα topology (Bompard-Gilles et al. 2000) called the N-terminal nucleophile hydrolase fold (Brannigan et al. 1995).

**Penicillin G acylase precursor**

The penicillin G acylase precursor uses a “Ser-only” catalytic configuration to undergo autoproteolysis. It undergoes self-processing using Ser264 as a nucleophile (Fig. 6A) in order to produce the enzymatically active mature acylase. The autoproteolysis generates a dimeric enzyme with the serine nucleophile located at the N terminus of one of the subunits (Ser264 becomes Ser1 in one of the newly generated protein subunits). This penicillin G acylase protein has an αβαα-core protein fold (Duggleby et al. 1995), similar to the L-amino peptidase D-Ala-esterase/amidase L. This fold is also found in the proteasome and glutamine PRPP amido transferase that contain a catalytic threonine located at residue 1 in the polypeptide chain (Brannigan et al. 1995; Lowe et al. 1995; Oinonen and Rouvinen 2000). In the glutamine PRPP enzyme, a cysteine nucleophile is found at this position (Smith et al. 1994). Therefore, proteins that have the N-terminal nucleophile hydrolase fold always carry out catalysis with an N-terminal active site residue (Brannigan et al. 1995), although the specific active site residue (serine, threonine, or cysteine) can vary.

Insights into the catalytic mechanism of these peptidases emerged from the structure of the penicillin G acylase precursor from *E. coli* (Hewitt et al. 2000). The structure revealed two conformations of the active site. One of the conformations revealed that Ser264 in the active site is positioned such that its Oγ group could attack the appropriate carbonyl carbon atom of the scissile bond. Moreover, this active site Ser264 is absolutely essential for activity (Choi et al. 1992). Strikingly, from the structure, there was no obvious candidate for the general base in the proteolytic reaction (Hewitt et al. 2000).

**Glutaryl 7-aminocephalosporanic acid acylase precursor (GCA precursor)**

There is another member of the acylase family called GCA, glutaryl 7-aminocephhalosporanic acid acylase with a Ser-only catalytic configuration. The structure of the precursor GCA at 2.5 Å resolution suggests that a water molecule is the general base that activates the active site serine residue (Kim et al. 2002; Yoon et al. 2004). The water molecule is coordinated by the main chain NHs of Thr69 and Gly168 and by the amide side chain of Asn244.

**“Thr-only” configuration**

**Proteasome**

The proteasome is a threonine protease that plays a key role in protein degradation and quality control. Proteolysis involves a Thr/α-amine configuration. In archaea and prokaryotes, the proteasome forms a 20S structure comprised of four 7-membered rings (α7β7α7β7α7) (for review, see Baumeister et al. 1998; Voges et al. 1999). The two outer rings are comprised of identical α-subunits and the two inner rings contain the identical β-subunits. The β-subunits contain the active site. In eukaryotic cells, the 20S proteolytic core complex associates with a regulatory domain complex, forming a 26S proteasine particle. This 26S particle functions in the ubiquitin-dependent degradation pathway. It uses ATP hydrolysis to unfold protein substrates and promoting translocation of the substrate peptide chain into the protease degradation chamber.
In contrast to eubacterial and archaeal proteasomes, the eukaryotic enzyme contains two copies each of seven distinct α- and β-subunits. The protease chamber contains 28 subunits that form the 20S particle. The α-subunits are located on the outside of the 20S particle, and the β polypeptides are on the inside. Briefly, of the seven different types of β-subunits, three of them are proteolytically active (for review, see Voges et al. 1999). The eukaryotic proteasome exhibits several distinct substrate specificities cleaving after basic, hydrophobic, or acidic amino acids.

The active site of the proteasome is comprised of an N-terminal threonine residue. To form the proteasome active site, it is necessary for the β-subunit to be autoprocessed such that the N-terminal propeptide is removed from the precursor polypeptide as the 20S proteasome particle is being assembled (Seemuller et al. 2001). Evidence that the active site threonine is involved in catalysis comes from site-directed mutagenesis and protease inhibitor studies. Mutagenesis of the threonine to an alanine residue blocks processing of the archaeal (Seemuller et al. 1995) and yeast proteasome (Chen and Hochstrasser 1996; Arendt and Hochstrasser 1997; Heinemeyer et al. 1997). Modification of the threonine residue of the proteasome by protease inhibitors completely inactivates the protease (Fenteany et al. 1995; Bogyo et al. 1997; Groll et al. 1997).

The crystal structure of the Thermoplasma acidophilum proteasome showed that the catalytic machinery is composed of the Thr1, Glu17, and Lys33 (Fig. 6B; Lowe et al. 1995). The structure was solved in the presence of the calpain inhibitor I, acetyl-Leu-Leu-norleucinal (LLnL). The structure revealed that the Thr1 hydroxyl group is localized very close to the aldehyde functional group of LLnL and may form a hemiacetal. Thus, the structure supports the notion that the Thr1 Oy residue is the nucleophile involved in catalysis. Also in proximity to the Thr1 residue is the amino group of Lys33 and the Glu17 carboxylate. The Lys33 ε-amino group is coordinated to the Glu17 carboxylate and the threonine side-chain oxygens. Based on the coordination partners, it is likely that the Lys33 is positively charged. Initially, the amino group of Thr1 was proposed to function as the general base. However, a bound water molecule, located in between the Thr1 amino group and its alcohol side-chain hydroxyl most likely acts as the general base to deprotonate the Thr1 hydroxyl group. The N terminus is coordinated to the carbonyl groups of Arg19 and Ala168 residues. A similar active site architecture is seen within the enzymatically active β subunit of the yeast proteasome solved at 2.4 Å resolution (Groll et al. 1997).

Proteasome precursor

A “Thr-only” catalytic mechanism is involved in autoproteolysis to generate mature proteasome (Seemuller et al. 1996). A water molecule bound to the proteasome precursor β-subunit is proposed to function as a general base in activating the threonine hydroxyl group (Ditzel et al. 1998; for review, see Seemuller et al. 2001).

Glycosylasparaginase and γ-glutamyltransferase I protein precursors

Other “Thr-only” active site peptidases are the glycosylasparaginase and γ-glutamyltransferase I protein in which a threonine residue is the catalytic nucleophile involved in autocleavage. A threonine residue is implicated in autoproteolysis of the glycosylasparaginase (Arsonson 2004). The inhibitor 5-diazo-4-oxonorvaline has been shown to react with the N-terminal threonine amino acid of the β subunit (Kaartinen et al. 1991). The γ-glutamyltransferase I protein (Sakai et al. 1996), which undergoes self processing, also requires a threonine residue (Suzuki and Kumagai 2002; Okada et al. 2007). Both the glycosylasparaginase and γ-glutamyltransferase I protein have the N-terminal nucleophile hydrolase fold (Brannigan et al. 1995; Suzuki and Kumagai 2002; Okada et al. 2007). To date, the structural studies did not clearly determine the general base that activates the threonine hydroxyl group in the precursor.

Variation on a theme: Ser/Ser/Lys triad

A general base lysine residue is not only used in serine proteases but also employed in certain amidases that contain a Ser/Ser/Lys triad active site. The amidases are found in archaea, eubacteria, fungi, plants, and mammals and rival the number of proteins in the Ser/His/Asp serine protease families (for review, see McKinney and Cravatt 2005). The signature domain is ~130 residues in length and includes the conserved motif with the active site Ser/Ser/Lys residues. Initially, it was unclear what type of mechanism was used by the amidases since they were inactivated by both serine and cysteine types of protease inhibitors (Ueda et al. 1995; Patterson et al. 1996).

By using the inhibitor ethoxy oleoyl fluorophosphonate, it was shown that serine is the nucleophilic residue in this amidase family (Patricelli et al. 1999). Ser241 was established to be the active site nucleophile of the fatty acid amidase hydrolase (FAAH). While no histidine residues were found to be required for activity, the invariant Ser217, Ser241, and Lys142 residues were discovered to be indispensable for catalysis (Patricelli and Cravatt 1999; Patricelli et al. 1999).

The pH rate profiles for the FAAH enzyme indicated an increase in activity going from pH 5 to 9, revealing a titratable group with a pKₐ of ~7.9. This titratable group most likely corresponds to the Lys142. Mutation
of the lysine creates altered pH rate profiles that are similar to those of histidine mutants in classical Ser/His/Asp proteases (Patricelli et al. 1999). The combined data suggested that the amidase family employed a Ser/Lys dyad mechanism (Patricelli and Cravatt 2000).

The Ser/Ser/Lys triad active site was established by the crystal structures of several amidases (Fig. 7; Bracey et al. 2002; Shin et al. 2002). The structure of the malonamidase E2 demonstrated that the catalytic serine residue was separated from the lysine by a bridging serine residue (Shin et al. 2002). The bridging serine was in an unusual conformation, in which the serine hydroxyl side chain and the amide nitrogen polarize the catalytic serine. The lysine ε-amino group was hydrogen bonded to the bridging serine residue hydroxyl. The Ser/Ser/Lys triad was also seen in the structure of the FAAH enzyme (Bracey et al. 2002). The Ser241 was covalently attached to a phosphonate inhibitor while the bridging Ser217 was hydrogen bonded to Lys142.

The catalytic mechanism of the FAAH amidases is unusual. In addition to using a lysine as a general base, the residue (Ser217) that normally removes the proton from the catalytic nucleophile (Ser241) is not the “catalytic base.” It is Lys217 that interacts only with the bridging serine that initiates the entire proton transfer process (McKinney and Cravatt 2003).

**Conclusion**

The results collected over the past two decades indicate that there are a wide variety of active site configurations in serine and threonine proteases. Variations are observed in each of the residues that comprise the Ser/His/Asp triad. Serine can be substituted by a threonine residue; histidine can be replaced with glutamic acid and lysine; and the aspartic acid can be substituted by a glutamic acid or a histidine residue or in some cases eliminated all together. Ser/Glu/Asp, Ser/His/Glu, and Ser/His/His triads are found in serine proteases, and a Ser/Ser/Lys triad is found in the amidase family. Glutamate and even serine (activated by a lysine) can function as a general base residue within serine proteases/amidases. Furthermore, the third member of the triad can include a histidine, glutamate, or a lysine residue. Here histidine and glutamate are proposed to function in the orientation of the catalytic general base similar to the way aspartic acid orients histidine in the conventional serine proteases. However, in the amidases, the general base lysine is believed to function via a bridging serine residue to activate the serine hydroxyl oxygen nucleophile. The lysine also assists in the protonation step of the substrate, allowing it to break down and release one of the products.

In addition to catalytic triads, active site dyads are found to function in serine and threonine proteases. The most common dyad is the Ser/Lys dyad, where lysine functions as a general base. Some of these proteases also contain a third residue (usually a serine or threonine) that helps to orient the lysine side chain such that it can function properly as a general base.

The simplest catalytic center that was discovered among serine/threonine peptidases utilizes a single catalytic residue. A Thr/ε-amine configuration, which is generated by autoproteolysis, is utilized in the proteasome. In the proteasome, a water molecule that is activated by the amine may function as the general base. A serine-only catalytic residue is found in the glutaryl 7-aminocephalosporanic acid acylase precursor. The serine is the nucleophile, and a bound water is believed to be the general base. The mechanism is not fully understood. A similar single catalytic residue is found in threonine protease precursors that undergo self processing. The active site reveals a threonine catalytic residue with no candidate general base protein residue except for a bound water. How the threonine nucleophile is activated such that it can catalyze the reaction is not yet certain.

So far, serine and threonine peptidases have been found in 15 clans. Some of these clans contain mixed catalytic types. The mixed catalytic types include the PB clan with serine or threonine proteases, the PC clan with serine and cysteine peptidases, and the PA clan that contain the serine, cysteine, or threonine peptidases. All peptidase

![Figure 7. Structure of the Ser/Ser/Lys amidase FAAH from Rattus norvegicus (PDB 1mt5). This enzyme exists as homodimers in solution; therefore, one monomer is shown in white and the other monomer is shown in black.](image-url)
members of a clan have similar protein folds. While many of the clans contain peptidase families that carry out catalysis using the same active site catalytic residues, some family members within a clan use different active site residues. This indicates that there is not necessarily a connection between clans and the catalytic residues. Why are different active site arrangements used in serine proteases across the three kingdoms of life? One possibility is that they allow these proteases to work in different environments as seen with the sedolisin protease with the Ser/Glu/Asp triad that can function at low pH. Also, alternate active site geometries might allow the cell to regulate proteases that use specific types of active site geometries without interfering with other configurations. Future studies will provide insight into the reasons why alternative active site constellations have arisen during evolution.

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Unconventional serine proteases


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