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Review

The catalytic triad of serine peptidases

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Abstract. The catalytic action of serine peptidases depends on the interplay of a nucleophile, a general base and an acid. In the classic trypsin and subtilisin families this catalytic triad is composed of serine, histidine and aspartic acid residues and exhibits similar spatial arrangements, but the order of the residues in the amino acid sequence is different. By now several new families have been discovered, in which the nucleophile-base-acid pattern is generally conserved, but the individual components can vary. The variations illustrate how different groups and different protein structures achieve the same reaction.

Key words. Mechanisms of peptidase action; β-lactamase; cytomegalovirus; Ntp-hydrolyses; oxyanion binding site.

Introduction

Proteases, commonly called peptidases, represent approximately 2% of the total number of proteins present in all types of organisms. There are about 500 human genes that encode peptidases and their homolog. Many of these enzymes are of medical importance, and are potential drug targets that originate from the human genome or from the genome of the disease-causing organism. The MEROPS database (http://merops.sanger.ac.uk) provides a rich source of information on peptidases [1, 2]. Storage and retrieval of this information is facilitated by the use of a hierarchical classification system, in which homologous peptidases are divided into clans and families. Families are grouped in a clan if there are indications, principally from tertiary structure comparisons, that they arise from a common ancestor. For every family and clan there is an identifier that shows the catalytic type of the peptidases contained in the group. The identification marks are 'A' (aspartic), 'C' (cysteine), 'M' (metallo), 'S' (serine), 'P' (mixed catalytic type) and 'U' (unknown type). For example, trypsin, chymotrypsin and elastase belong to family S1 of clan PA (formerly SA), subclan PA(S), while papain and its relatives are listed in family C1 of clan CA.

For a long time four distinct mechanisms were known that linked to the serine, cysteine, aspartic and metallopeptidases, respectively. The most conspicuous difference in the mechanisms is the presence or absence of a covalent acyl-enzyme intermediate on the reaction pathway. The catalyses of serine and cysteine peptidases involve the covalent intermediate (ester and thiolester, respectively), whereas the aspartic and the metallopeptidase catalyses do not. During hydrolysis carried out by the latter two groups, the substrate is attacked directly by a water molecule rather than by a serine or cysteine residue. In spite of the differences, the basic catalytic features of the different clans are common [3, 4]. Hydrolysis of the peptide bond is an addition-elimination reaction involving a tetrahedral intermediate. All three heavy atoms of the peptide bond are directly implicated in the catalytic reaction by interacting with appropriate enzymatic groups (fig. 1). The combined action of the catalytic groups is essential, because the peptide bond represents a strong linkage that has a high degree of double-bond character, which results from delocalization of the nitrogen lone pair into the carbonyl group. The strength of the peptide bond weakens in the first step, when a nucleophile, such as water or a serine OH group, attacks the carbonyl carbon atom,
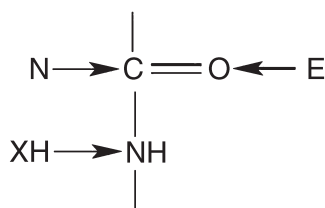
Figure 1. Common features of peptide bond cleavage by peptidases. N, E and XH represent the nucleophile, electrophile and proton donor, respectively.

resulting in a tetrahedral intermediate bearing an oxyanion. The nucleophilic attack is aided by a general base that accepts the proton from the nucleophilic OH group (X in fig. 1). The intermediate is stabilized by electrophilic catalysis, which is provided by hydrogen bonds from the oxyanion binding site to the carbonyl oxygen. This is followed by the expulsion of the leaving group, the amine, from the tetrahedral intermediate. Because the amine is a poor leaving group, it must be protonated by a general acid (XH in fig. 1) to be able to depart. Peptide bond cleavage invariably includes the formation and decomposition of the tetrahedral intermediate. These processes are facilitated by general base-general acid catalysis, mediated by a single proton carrier, which is a histidine side chain in many cases.

The catalytic mechanism

Serine peptidases, named after the catalytic serine residue, constitute the largest group of peptidases. The trypsin-like peptidases of family S1 of clan PA are the most abundant among serine peptidases. They are found in eukaryotes, prokaryotes, archaea and viruses. These enzymes participate in many important physiological processes, including digestion (trypsin, chymotrypsin), immune responses (complement factors B, C, D), blood coagulation (factors VIIa, IXa, Xa, XIIa), fibrinolysis (urokinase, tissue plasminogen activator, plasmin, kallikrein) and reproduction (acrosin).
The first well-characterized mechanism of action of serine peptidases was established primarily by the kinetic studies of chymotrypsin by Bender and his co-workers in the 1960s. Major advances ensued from the determination of the three-dimensional structure of chymotrypsin by Blow and his colleagues. The catalytic features of serine peptidases have been covered in several reviews [3, 5–16].
For a long time only two groups of serine peptidases were known; the trypsin and subtilisin clans, clan PA and clan SB, respectively. With the advent of gene cloning and acceleration of the determination of three-dimensional structures, a variety of other serine peptidases were discovered, which did not fit to these two clans. The active site architecture of the peptidases in clans PA and SB are

similar, but their three-dimensional structures are different, indicating that they arose by convergent evolution. The trypsin-like enzymes (clan PA) exhibit a double β-barrel fold, whereas the subtilisin-like enzymes (clan SB) have a parallel β-sheet structure. The peptidases of clan SC, which were discovered considerably later [17], display an α,β-hydrolase fold with the same catalytic triad as found with the classic serine peptidases, chymotrypsin and subtilisin (serine, histidine and aspartic acid), but with an opposite handedness [18]. Apart from some structural changes, such as found at the oxyanion binding site, the chemical mechanisms of action for the three clans are the same. However, in recently identified clans (SE, SF, SH, SJ, SK, SP, SR) the members of the catalytic triad and thus the mechanism of action changed to some extent.
Figure 2 illustrates the basic features of the mechanism of action of the classic serine peptidases [5]. It is shown that the nucleophilic attack by the serine hydroxyl group on the carbonyl carbon atom of the substrate is catalyzed by a histidine imidazole group as a general base. This leads to formation of a tetrahedral intermediate and an imidazolium ion (addition reaction). The tetrahedral intermediate breaks down by general acid catalysis to an acyl-enzyme, an imidazole base and an amine product (elimination reaction). During the acylation step, the imidazole group transfers the proton of the serine hydroxyl to the amine leaving group. The acyl-enzyme is then deacylated through the reverse reaction pathway of acylation, but in the second addition-elimination reaction a water molecule instead of the serine residue is the attacking nucleophile.

The classical catalytic triad

Determination of the three-dimensional structure of chymotrypsin, the first structure reported for a peptidase [19], allowed a deeper insight into the mechanism of action of serine peptidases. The data from the X-ray diffraction measurements have shown that the serine and histidine residues are in the proper position to function according to the mechanism implied by figure 2. As illus-
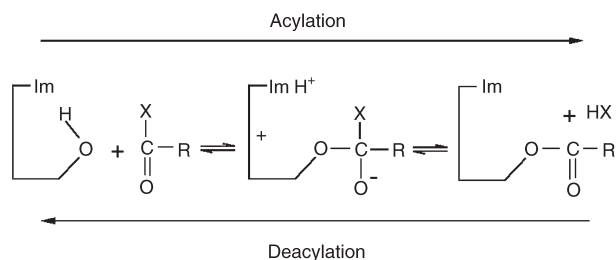

Figure 2. Scheme of the basic feature of the mechanism of action of serine peptidases. In acylation X stands for OR and NHR groups in the hydrolysis of esters and peptides, respectively, and for OH in deacylation.
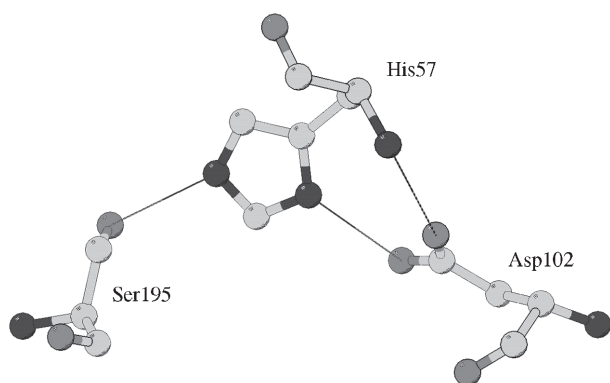
Figure 3. The catalytic triad of chymotrypsin (PDB code 4CHA).

trated in figure 3, the OG of Ser195 and the NE2 of His57 are within hydrogen bonding distance. The other nitrogen atom (ND1) of the imidazole ring is hydrogen bonded to the carboxyl group of Asp102, so that the hydrogen bond is shielded from water by several amino acid residues. The geometric relation of Asp102, His57 and Ser195 led to the postulation that His57 serves for transferring the proton from Ser195 to Asp102 in a charge relay mechanism [20]. However, a proton relay from the highly basic serine OH group to the acidic aspartate is chemically unlikely [21]. It is a more reliable assumption that Asp102 may be involved in the stabilization of the ion-pair generated between the imidazoliun ion and the negatively charged-tetrahedral intermediate, and that Asp102 may participate in the orientation of the correct tautomer of His57 relative to Ser195 [21–24]. Nuclear magnetic resonance (NMR) [25–28] and neutron diffraction studies [29] have then confirmed that it is the imidazole and not the aspartate that is protonated. The interaction between the aspartate and the imidazole is more important in the transition state of the formation of the tetrahedral intermediate than in the ground state of the reaction. This was indicated by the very low field resonance observed in ^{1}H NMR studies of subtilisin and thiolsubtilisin [27]. Specifically, thiolsubtilisin contains a thiolate-imidazolium ion pair at its active site, which resembles the charge distribution of the negative tetrahedral intermediate and the positive imidazolium ion. The results have shown that while the proton was still on the imidazole group, the hydrogen bond with the aspartate was much stronger in this transition state-like structure than in the native subtilisin.

Engineering the aspartic acid of the catalytic triad

Replacement of the catalytic Asp102 of trypsin with the neutral Asn resulted in an enzyme variant (D102N) that displayed a specificity rate constant (k_{cat}/K_m) about 10^4 times lower than that of the native enzyme, using suc-Ala-

Ala-Pro-Arg-SBzl substrate at neutral pH [30]. Determination of the three-dimensional structure of the enzyme variant indicated that the catalytic His57 was present in the tautomer that was unable to accept the proton from Ser195 [31]. The kinetic analysis has also shown that the mutation greatly reduced k_{cat}, and caused only minor alteration in K_m. Specifically, the NE2 of His57 was protonated and the non-protonated, basic ND1 formed a hydrogen bond with the NH$_2$ group of Asn102. When both the ND1 and the NE2 atoms were protonated, His57 adopted an alternative conformation and interacted with a solvent water molecule rather than with the Asn102 residue.

The role of catalytic histidine in the catalysis was also investigated with the prolyl oligopeptidase variant, D641N. Prolyl oligopeptidase of clan SC has an α/β-hydrolase fold, and its catalytic triad (Ser554, Asp641, His680) is covered by a β-propeller domain [18]. In addition to the D641N variant, the D641A variant was also investigated, because the alanine residue cannot form a hydrogen bond with the catalytic histidine [32]. The similar reductions in k_{cat}/K_m for these two enzyme variants suggested that the rate decrease couldn't be ascribed to the stabilization of an incompetent histidine tautomer.

As for the D102N variant of trypsin, it was claimed that the activity of the mutant enzyme towards a variety of substrates [31], specifically towards a thiolester and a nitroanilide [30], was equally reduced by four orders of magnitude. In contrast, with the prolyl oligopeptidase variant the k_{cat}/K_m was highly dependent upon the substrate employed. The change was virtually zero for the weak nitrophenyl ester bond and six orders of magnitude for the octapeptide substrate, Abz-Gly-Phe-Gly-Pro-Phe-Gly-Phe(NO$_2$)-Ala-NH$_2$. This huge difference implies that the contribution of the catalytic triad is needed more when a stronger peptide bond is to be cleaved.

A further difference between the corresponding variants of trypsin and prolyl oligopeptidase was found in the alkaline pH region. With the trypsin variant the hydrolysis substantially augmented with the increase in pH [30], whereas with the prolyl oligopeptidase variants the rate constants decreased at high pH, as observed with the wild-type enzyme [32]. The reason for the distinct behavior is not clear.

Crystal structure determinations of D641A and D641N mutants of prolyl oligopeptidase indicated that His680 occupied the same position as in the wild-type enzyme. In the D641A variant, a water molecule resided in place of the mutated carboxylate group and was hydrogen bonded to the ND1 atom of His680 and to other groups [32]. In the D641N variant, Asn641 was pushed out of hydrogen bonding distance from ND1 of His680 to a van der Waals distance, suggesting that ND1 was protonated and couldn't accept a proton from the NH$_2$ group of Asn641. The similar catalytic properties of the D641A and D641N

variants of prolyl oligopeptidase point out the importance of the negative charge of Asp641 and discount the catalytic significance of an incompetent tautomer of His680. This is consistent with the earlier suggestion that a major function of the aspartate residue is to stabilize the transition state on the way to the ion-pair formation between the tetrahedral intermediate and the imidazolium ion [21, 22].

The role in catalysis of the other two members of the triad, serine and histidine, is quite obvious. Serine forms the acyl-enzyme intermediate, and histidine transfers the proton from the serine OH to the leaving group of the substrate to produce the acyl-enzyme. In a similar way, the histidine also operates in the hydrolysis of the acyl-enzyme. The catalytic contributions of these two groups were studied with trypsin [33] and subtilisin [34]. When an alanine residue was substituted for either Ser195 or His57, the value of k_{cat}/K_m decreased 10^5-fold using Z-Gly-Pro-Arg-7-amino-4-methylcoumarin substrate. However, the change in k_{cat}/K_m varied to some extent if leucine, aspartic acid, asparagine, glutamic acid, lysine or arginine was used instead of alanine. Also, the k_{cat}/K_m was affected by the nature of the substrate, so that the natural peptide substrate was much worse than the activated amide substrate. However, when all three members of the triad were modified, the rate decrease was 5×10^5. [33]. Kinetic analyses of the multiple mutants of subtilisin (D32A/H64A/S221A) demonstrated that the residues of the triad accelerated the amide bond hydrolysis by a factor of approximately 2×10^6.

It is significant that a single mutation of serine or histidine destroys the enzyme activity to an extent similar to that of the double mutant. This implies that only one mutation abolishes the normal hydrolytic reaction. However, some activity remains, because the enzymes impart a total rate enhancement of at least 10^9–10^{10} times the non-enzymatic hydrolysis of amide bonds [34]. Accordingly, the catalytic triad is not the sole source of the peptidase activity since with the catalytic triad disabled, the mutant peptidases hydrolyze substrates at three orders of magnitude faster relative to the uncatalyzed reaction rate. The remaining activity may arise from the contribution of binding, desolvation (entropy effects) and the oxyanion binding site (electrophilic catalysis). Of course, the mechanism of the remaining hydrolytic activity is different from that achieved by the normal enzymatic machinery; the peptide bond is presumably directly attacked by a water molecule.

The oxyanion binding site

A further mechanistically crucial outcome of X-ray crystallographic studies on chymotrypsin has been the discovery of the oxyanion binding site. The negatively charged oxyanion of the tetrahedral intermediate is generated from the carbonyl oxygen of the scissile bond, and this high-energy species is stabilized by two peptide backbone NH groups from Gly193 and Ser195, respectively [35]. The oxyanion binding site, as a general feature of serine peptidases, was termed the oxyanion hole, when a similar site was also observed in subtilisin [36]. However, in subtilisin the backbone NH group of the catalytic Ser221 and the side chain amide group of Asn155 constitute the oxyanion binding site.

The contribution of the oxyanion binding site to catalysis was examined by replacing Asn155 of subtilisin with the isosteric leucine [37] or glycine [38]. This lowered the turnover number (k_{cat}) for a specific substrate 150–300-fold with virtually no change in the Michaelis constant (K_m). It was concluded from these data that the oxyanion binding site only contributed to the transition state and left the binding unaffected [37, 38].

The oxyanion binding site is precisely tailored for the oxygen atom. Thionoester substrates, which contain the slightly bigger sulfur atom in place of oxygen, are not hydrolyzed by chymotrypsin and subtilisin, although the chemical reactivities of esters and thionoesters do not differ significantly [39].

A novel oxyanion binding site was recently found in prolyl oligopeptidase [18]. This enzyme has a tyrosine OH group (Tyr473) at the oxyanion binding site. Kinetic studies with the Y473F variant lacking the critical OH group have shown that the k_{cat}/K_m for the charged succinyl-Gly-Pro-4-nitroanilide was impaired to a much greater extent than that for the neutral benzyloxycarbonyl-Gly-Pro-2-naphthylamide, although the binding modes of the two substrates were similar, as shown by X-ray crystallography [32, 40]. This effect could be ascribed to the unfavorable electrostatic interaction of the succinyl group with Arg643. Unlike most enzyme reactions, catalysis by the wild-type enzyme exhibited positive activation entropy. In contrast, the activation entropy for the Y473F variant was negative, suggesting that the tyrosine OH group is involved in stabilizing both the transition state and the water shell at the active site. Importantly, Tyr473 is also implicated in the formation of the enzyme-substrate complex. The non-linear Arrhenius plot suggested a greater significance of the oxyanion binding site at physiological temperature. Thus, the oxyanion binding site showed multiple effects on catalysis. The results indicated that the mechanism of transition state stabilization was markedly dependent upon the nature of the substrate and that Tyr473 was more needed at high pH and at high temperature [32, 40].

An interesting lesson of site-specific mutagenesis arises from modification of the oxyanion binding site of subtilisin. While the replacement of Asn155 by glycine lowers k_{cat} by 150-fold with virtually no change in K_m, for the double mutant (N155G/S221A), in which the active site serine is eliminated, k_{cat} is 5-fold greater than for the S221A variant [38]. It is likely that in the double mutant

the water molecule attacks the carbonyl carbon of the substrate from the face opposite Ala221, and Asn155 appears to sterically interfere with solvent attack. Hence, when a mutation causes a gross change in mechanism, the role of otherwise important catalytic groups can be deleterious to the enzyme.

Additional aspects of the catalytic triad

Low-barrier hydrogen bonds
The theory of low-barrier hydrogen bonds (LBHB) has emerged in recent years and implies short (<2.5 Å) and very strong (40–80 kJ/mol) hydrogen bonds [41–43]. Such hydrogen bonds are formed if the donor and acceptor atoms are close to one another and display similar pK_a values. In LBHBs the proton is shared by the donor and acceptor in a single, low-potential energy well, which implies that the barrier for proton transfer is eliminated. Several arguments were raised in support of LBHBs between the catalytic histidine and aspartic acid of serine peptidases [44–47], like the unusually downfield proton NMR signal of ND1 of His57 [25] or the low D/H fractionation factor of the ND1 proton [47, 48]. However, these data have little diagnostic value; for example, the downfield NMR signal implies that the proton belongs primarily to the ND1 atom of His57. Moreover, the signal is not visible in some instances [27]. On the other hand, theory and experimental evidence regarding the aspartic acid-histidine hydrogen bond are inconsistent with fundamental tenets of the LBHB hypothesis [49–51]. Thus, both ^{15}N–H spin couplings and ^{15}N chemical shifts indicate that about 85% of the proton is localized on His 57 [51, 52].
The location of the hydrogen between the donor and acceptor atoms was observed by ultra-high resolution X-ray diffraction at 100 K and 0.78 Å resolution [53]. The bonding distance was 2.6 Å between Asp32 and His64 of *Bacillus lentus* subtilisin, and the hydrogen atom extended 1.2 Å from His64 and 1.5 Å from Asp32, indicating that the hydrogen atom is partially shared by the two residues. However, this short bond was not considered as LBHB, because it is not shielded from solvent, and the pK_a values of the donor and acceptor residues are not matched [53]. Indeed, the short hydrogen bond is not characteristic of all serine peptidases [54]. That the position of the proton depends on the nature of serine peptidase and the experimental conditions employed follows from neutron diffraction studies, which demonstrated that the deuterium is associated with the histidine and not with the aspartic acid [29]. Also, the very low field NMR signal characteristic of chymotrypsin is not visible in subtilisin BPN, indicating that the stability of the hydrogen bond is lower in subtilisin than in chymotrypsin and implies that the histidine has more motional freedom in the former pep-

tidase than in the latter one [27]. However, the low field resonance is present in subtilisin derivatives bearing a negative charge near the catalytic histidine, as found with thiolsubtilisin, which possesses a thiolate-imidazolium ion pair at the active site, and with the transition state-like phenylboronate-serine adduct [55]. This observation suggests that the hydrogen bond between the histidine and the aspartic acid is greatly stabilized by the presence of a negative charge, such as the oxyanion of the catalytic tetrahedral intermediate, and that the stable hydrogen bond should be more important during catalysis than in the substrate-free enzyme [27].

Transition state stabilization and transition state analogs
Rate enhancement brought about by serine peptidases is primarily achieved by the catalytic triad. On the reaction pathway from reactant to product, the transition state has the highest energy. The transition state converts to a high energy tetrahedral intermediate bearing the oxyanion, which is still of high energy, but is stabilized by the imidazolium cation. Since the lifetimes of both the transition state and the tetrahedral intermediate are too short to study with the available methods, transition state analogs are used that resemble the tetrahedral intermediate and bind much more tightly to the enzyme than the substrates and do not transform into products. Transition state analogs often serve as potent inhibitors. Their stable complexes with the target enzyme are suitable for X-ray crystallographic studies. Both the analogs and the intermediate are true covalent species, whereas the transition state possesses partially broken bonds.
Some widely used serine peptidase inhibitors, such as diisopropyl fluorophosphate and phenylmethanesulfonyl fluoride form tetrahedral intermediates at the active site. However, because of the bulkiness of the heteroatoms, phosphorus and sulfur, these inhibitors are not ideal models of the catalytic species. The tetrahedral adducts
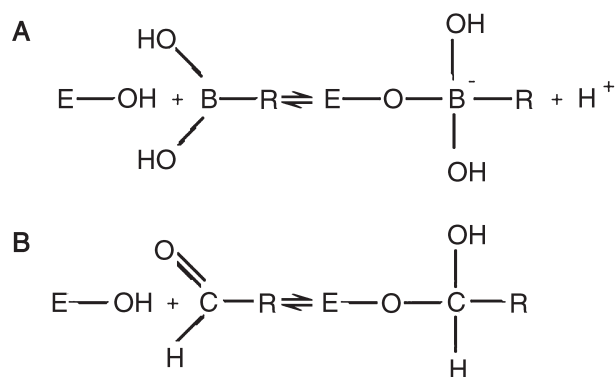

Figure 4. The reaction of transition state analog inhibitors with serine peptidases. (*A*) boronic acid derivative; (*B*) aldehyde derivative.

generated from boronic acid derivatives and peptide alde-hydes bear a closer relationship to the structure of the true intermediate. There is, however, an important difference between these two analogs. The boronic acid derivative possesses a negative charge (fig. 4A), whereas the hemi-acetal adduct is a neutral species (fig. 4B). Hence, the boronic acid adduct is a better transition state analog than the hemiacetal, although the negative charge is on the boron atom, whereas in the true intermediate it is located on the carbonyl oxygen (fig. 2). Some peptide boronic acids are very good inhibitors, with K_i values in the nanomolar range and with the potential of their use as drugs [56–58].

Ser-His-His catalytic triad

Herpesviruses encode a serine peptidase called assemblin that specifically cleaves the assembly protein precursor, a process necessary for replication. Human cytomegalovirus peptidase, a member of the herpesvirus group (clan SH), contains 256 amino acid residues. Its three-dimensional structure has been independently determined in several laboratories [59–62]. This was followed by the structures of varicella-zoster virus and herpes simplex virus pepti-dases types 1 and 2 [63, 64]. The backbone fold of these proteases is different from those of the other known peptidase structures. It consists of a seven-stranded, mostly antiparallel β-barrel, which is surrounded by seven helices. The structural, catalytic, biological and pharma-cological features of herpesvirus peptidases have been reviewed [65–67].

Ser132 and His63 are found in close proximity in the active site of the cytomegalovirus peptidase (fig. 5A). The structure suggests that the third member of the triad is His157. The ND1 atom of His63 and the NE2 atom of His157 are hydrogen bonded. However, the contribution of His157 to the catalysis is much smaller than that of the aspartate in the classic serine peptidases [68]. Owing to the presence of two histidine residues in the catalytic triad, zinc is an active site inhibitor of herpesvirus pepti-dases [60].

The active site of the cytomegalovirus peptidase is situated in the only region of the core of the β-barrel that is not sheltered by helices and flanking loops. The active site region is very shallow, in accord with the small P1-P1′ residues (Ala-Ser). The residues of the triad superimpose very well on the Ser195, His57, Asp102 triad of trypsin. Overlays of the catalytic triad of the herpesvirus pepti-dases with that of trypsin suggest that the highly conserved Arg165 and Arg166 are involved in the stabilization of the oxyanion intermediate (fig. 5A). Specifically, the Arg165 backbone atoms correspond to those of Gly193, the NH group of which is known to donate the hydrogen bond to the oxyanion. The other hydrogen bond comes
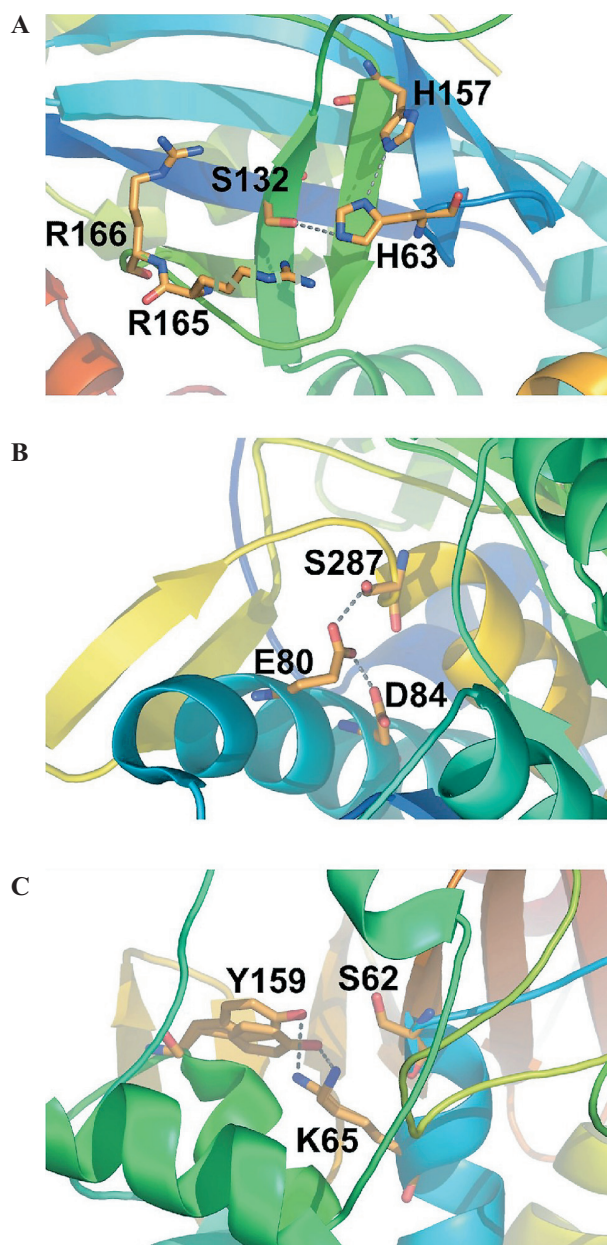

Figure 5. Active sites of peptidases. The ribbon diagrams are color-ramped blue to red from the N to the C terminus. The catalytic residues are shown in ball-and-stick representation. (*A*) Cytome-galovirus assemblin (PDB code 1LAY). The side chain of Arg165 is disordered and shown by a broken line. (*B*) Sedolisin from *Pseudomonas* sp. 101 (PDB code 1GA6). (*C*) D-Ala-D-Ala carboxypeptidase B (PDB code 3PTE). The Tyr159 occurs in two conformations.

from a water molecule held by the side chain of Arg166 [60, 63, 64]. These roles of Arg165 and Arg166 are further supported by the fact that the cytomegalovirus peptidase Arg165Ala mutant still has 30% of the wild-type activity, while the Arg166Ala mutant is four orders of magnitude less active [69].

The herpesvirus proteases are only active as homodimers. Kinetic and structural studies showed that communication occurs between the active sites and the dimer interface [70, 71].

Carboxyl serine peptidases: Asp-Glu-Ser catalytic triads

Sedolisins, carboxyl serine peptidases, constitute a novel family of serine peptidases (clan SB, family S53), whose folding resembles that of subtilisin. However, they are considerably larger, the catalytic domains containing approximately 375 amino acid residues [72]. Crystal structure determination of a peptidase has defined the first structure with a catalytic triad consisting of Glu80, Asp84 and Ser287 (fig. 5B) [73, 74]. Another important member of this family is the product of the human CNL2 gene, mutation of which causes fatal neurodegenerative disease. It was recently identified as tripeptidyl-peptidase I and predicted to be a serine peptidase [75–77].

Glu80 is in a stereochemically equivalent position to His64 of subtilisin, and so should play the same role as a general base. One oxygen atom of the carboxyl group accepts the proton from Ser287, while the other oxygen atom is hydrogen bonded to Asp84, which helps to hold the carboxyl group of glutamic acid in the correct position for function. However, Asp84 is not structurally related to Asp32 of the catalytic triad of subtilisin. Presumably, the active site of these enzymes resulted from separate evolutionary events [73]. With this new catalytic triad, the mechanism is similar to that of the classic serine peptidases. The reaction proceeds through an acyl-enzyme, and the formation and decomposition of the tetrahedral intermediates are aided by general base and general acid catalysis, respectively. However, the oxyanion binding site is unique, containing Asp170, which is equivalent to Asn155 in subtilisin [73]. Of course, Asp170 can fulfill its role in an acidic environment only, when the carboxyl group is in the protonated form. The low pH is also necessary for the hydrogen bonding interaction between Glu80 and Asp84, because in alkaline medium the charged carboxylate ions would repulse one another. Indeed, the enzymes of this family are active in the pH range of 3–5 [76, 78].

Kumamolisin-As from a thermophilic bacterium, *Bacillus novo* sp. MN-32 is a collagenase that belongs to the sedolisin family. Its catalytic triad contains Glu78, Asp82 and Ser278 [79]. The E78H mutant, in which the catalytic triad was engineered to resemble that of subtilisin, showed only 0.01% activity of the wild-type enzyme, and its structure revealed that the members of the triad do not interact with each other; thus the catalytic triad is disrupted.

Ser, Lys dyad catalysis

In clan SE the catalytic residues occur within the motif SerXaaXbbLys, and the tertiary fold of the enzymes is shared with that of class C β-lactamases. These enzymes are mostly involved in the biosynthesis and remodeling of the bacterial cell wall. They are also known for being penicillin-binding proteins, and exhibit carboxypeptidase and transpeptidase activities. The three-dimensional structures are known for *Streptomyces* R61 D-Ala-D-Ala carboxypeptidase B (fig. 5C) [80–83] and *Citrobacter* class C β-lactamase [84]. These molecules are composed of an all-α and an α/β domain, with the catalytic serine and lysine on a helix that traverses the cleft between the two lobes. It is suggested that a tyrosine residue is also involved in the catalysis.

In clan SF the dyad is Ser, Lys, sometimes Ser, His, which are more distant in the primary structure than they are in clan SE, and the tertiary structures display no similarity to the members of clan SE. The enzymes have different biological functions, and they mostly prefer alanine in the P1 position of the peptide substrate. The clan includes repressor proteins, like the LexA protein from *Escherichia coli*, which undergo autocatalysis. This clan also comprises signal or leader peptidases, and the tail-specific (Tsp) endopeptidase of *E. coli* which removes nonpolar residues from the C-terminus of proteins. The tertiary structure of the UmuD′ protein has been reported [85]. This protein is generated from the UmuD peptidase by autocatalysis. The inactive UmuD' protein contains mainly β-structure, and it is thus unrelated to the enzymes of clan SE. The active site residues, Ser60 and Lys97, are poised properly for hydrogen bond formation [85]. The C-terminal domains of λ repressor [86] and LexA protein [87] displayed similar structures and active sites. In *E. coli* leader peptidase Ser90 [88] and Lys145 [89] are the catalytic residues, forming an active site comparable to that of UmuD peptidase [90]. Substitution of a cysteine for Ser90 does not inactivate the enzyme, but the thiol enzyme can be inactivated by the thiol-specific N-ethylmeleimide [89]. The catalytic lysine is conserved in the prokaryote and mitochondrial leader peptidases. In the endoplasmic reticulum signal peptidase the corresponding residue is histidine [91]. The functional significance of this apparent substitution is not known.

The catalyses by the enzymes of clans SE and SF follow the classical acyl-enzyme pathway of serine peptidases. The X-ray structure of a deacylation-defective mutant of RTEM-1 β-lactamase from *E. coli* complexed with penicillin G has shown that the substrate is covalently bound to Ser70 as an acyl-enzyme intermediate [92]. A major issue concerns the role of the lysine residue, which has a pK_a too high to function as a general base around neutrality. LexA cleaves itself in response to activation by the regulatory protein RecA. In the absence of RecA,

LexA exhibited some activity at mildly alkaline pH, and the pH-rate profile demonstrated that a basic group with a pK_a of about 10 was required for catalysis [93]. This supports the mechanism in which deprotonation of the catalytic Lys156 is critical for activity [94]. Apparently, in the presence of RecA, the pK_a of Lys156 decreases considerably. In principle, the pK_a can be depressed by the nearby positive charge of another lysine or arginine, while a carboxylate ion stabilizes the protonated form. Indeed, Glu152 of LexA repressor is the closest charge to the catalytic lysine (~8 Å), and the Glu152Ala mutation produces a 0.3 decrease in the pK_a and a 7-fold increase in the autocatalytic activity at pH 10 [95]. It appears that in contrast to the catalytic triad of the classic serine peptidases, the Ser-Lys dyad does not require a carboxylate ion. In the free enzyme there is strong hydrogen bonding interaction between the serine and lysine [85, 92], which could also decrease the pK_a of the lysine residue. However, the key factor in LexA is the hydrophobic microenvironment [87], which can also diminish the pK_a value. Unlike the anticipated low pK_a of the lysine residue in LexA, the Lys73 of the TEM-1 β-lactamase exhibited a normal pK_a value of 10–10.5, using direct titration of the amino group. To explain the activity, the contribution of Glu166 was suggested directly or through an intervening water molecule [96].The crystal structure of LexA defined the oxyanion binding site, which comprises the main chain amides of Ser119 and Met118 [87].

In the λ repressor Ser149 OG is hydrogen bonded to the side-chain amino group of Lys192. The structure suggests a potential role for Thr190, which is also hydrogen bonded to the Lys192 nitrogen through its side-chain oxygen atom. This could facilitate deprotonation of Lys192 and help to orient its side-chain to interact with Ser149 [86].

The ATP-dependent Lon peptidase degrades specific short-lived regulatory proteins as well as defective and abnormal proteins in the cell (clan SJ, family S16). The crystal structure of the C-terminal proteolytic domain of the *E. coli* Lon protease revealed a unique fold, which assembled into hexameric rings that likely mimic the oligomerization state of the holoenzyme. The catalytic site involves a Ser679-Lys722 dyad [97, 98].

N-terminal nucleophile peptidases

An interesting class of hydrolases are the so-called Ntn-hydrolases, the members of which are very different from the enzymes of the other serine peptidases. They share a unique fold and a novel catalytic mechanism with an N-terminal catalytic threonine, in some cases serine or cysteine [99]. This group includes glutamine 5-phosphoribosyl-1-pyrophosphate (PRPP) amidotransferase from *Bacillus subtilis* [100], penicillin acylase [101] and the

proteasomes [102]. The hallmark of the protein fold is a four-layer structure composed of two sheets of antiparallel β-strands sandwiched between two layers of α-helices. The proteasome is the most intriguing in this clan (clan PB, subclan PB(T), family T1). It is a multisubunit complex comprising four stacked rings each containing seven (eukaryotes and archaea) or six (bacteria) subunits. Their characteristics have been reviewed [103–105]. In the general $\alpha\beta\beta\alpha$ architecture, the α-subunits are not catalytically active. They constitute the outer rings and share the fold with the β-subunits of the inner rings. Proteasomes recognize, unfold and digest protein substrates that have been marked for degradation by ubiquitin.

That the N-terminal threonine (Thr1) serves as the catalytic nucleophile is supported (i) by the loss of activity if alanine is substituted for Thr1 [106, 107], and (ii) by the modification of the hydroxyl group of Thr1 by irreversible inhibitors [102, 108–111]. In the vicinity of the proteasome active site there are conserved residues, such as Asp17 (Glu17 in *Thermoplasma acidophilum* proteasome), Lys33, Ser129 and Ser169 [102, 108]. The latter two residues may contribute to the structural integrity of the protein. The lysine and glutamic acid could play the role that histidine and aspartic acid do in the classic serine peptidases. Although Lys33 is essential for catalysis, it is not found in other Ntn-hydrolases, such as penicillin acylase and aspartylglucosaminidase. This absence indicates that the N-terminal amino group is the proton acceptor in the catalysis of this group of enzymes. The protonated Lys33 may decrease the pK_a of the amino group of Thr1 by electrostatic effects. The oxyanion of the tetrahedral transition state may be stabilized by the main chain amide of Gly47, which points toward the oxygen of a covalently bound aldehyde inhibitor [102].

The formation of mature Ntn-hydrolases requires a processing step, which liberates the N-terminal amino group. This is an autocatalytic process [106, 112, 113] which cannot use the blocked amino group of Thr1. Instead, a water molecule is seen in the high-resolution structures of penicillin acylase [101] and in all three active subunits of the yeast proteasome [108], and it is suggested that this water molecule fulfills the role of the general base catalyst. However, it is clear that the mechanisms of catalysis and intramolecular processing related to the Ntn-hydrolases are far from our knowledge acquired for the classic serine peptidases.

Further variations in the members of the catalytic triad

In the previous sections we have seen how the members of the triad may vary in the different peptidases, and that in some cases the triad reduces to a dyad. While this review concentrates on peptidases, it should be mentioned that

other hydrolytic enzymes have similar and altered catalytic triads. Thus, some lipases share the fold and the catalytic triad with the oligopeptidases of clan SC [114]. On the other hand, the lipase from *Geotrichum candidum* and acetylcholinesterase from *Torpedo californica* contain a glutamate residue in place of the catalytic aspartate residue [115]. Although glutamate is chemically equivalent to aspartate, it does not occur in the classic serine peptidases. Furthermore, in an esterase from *Streptomyces scabies* a main chain carbonyl oxygen atom forms a hydrogen bond with the NH group of the histidine residue instead of the aspartate or glutamate [116]. This is further evidence against the LBHB discussed in a previous section because the pK_a difference between the backbone oxygen and the imidazole group is of considerable magnitude.

Asparaginases, which catalyze the hydrolysis of L-asparagine to L-aspartate and ammonia, contain a threonine nucleophile instead of serine. While the crystal structure was determined for the *E. coli* [117, 118] and the *Erwinia chrysanthemi* [119, 120] L-asparaginases, the enzymatic mechanism is unclear [121]. The enzyme displays an unusual active site that exhibits two essential threonine residues, and either appears to be a good candidate for functioning as nucleophile. In the *E. coli* enzyme Thr89 seems to be the genuine nucleophile because Lys162 can act as the general base to activate it. However, in the Thr89Val variant, the deacylation of which is impaired, Thr12 becomes acylated, but the mechanism of activation of this nucleophile remains hidden in the crystal structure. It may be that a significant conformational change brings together the required catalytic groups.

The catalytic triad of picornains is of particular interest. Picornaviruses are small RNA viruses, such as poliovirus, hepatitis A virus, rhinovirus and others. They produce cysteine peptidases named picornains 2A and 3C. These adopt a two-domain β-barrel fold similar to that of chymotrypsin, indicating that the structures of the virus peptidases are basically different from that of the classical cysteine peptidases of the papain family. The catalytic triad of poliovirus 3C is composed of His40, Glu71 and Cys147 [122]. Cysteine peptidases are thought to attack the substrate by a thiolate-imidazolium ion pair, as demonstrated with papain. However, poliovirus 3C peptidase contains an ordinary thiol group, rather than its ionized form [123]. Therefore, the imidazole assistance in the hydrolysis is very likely general base catalysis, as found with serine peptidases. The acidic component of the catalytic triad is essential for activity, but its negative charge does not influence the ionization of the thiol group, as demonstrated with the E71Q variant [124]. On the other hand, in the hepatitis A virus 3C peptidase the side chains of His44 and Asp84 (corresponding to His57 and Asp102 in chymotrypsin) are not in contact. The aspartate residue is replaced by a water molecule [125, 126].

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