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The expanding diversity of serine hydrolases

Istvan Botos[1] and Alexander Wlodawer[2]

Serine hydrolases use a hydroxyl of a serine, assisted by one or more other residues, to cleave peptide bonds. They belong to several different families whose general mechanism is well known. However, the subtle structural differences that have recently been observed across a variety of families shed light on their functional diversity, including variations in mechanism of action, differences in the modes of substrate binding, and substrate-assisted orientation of catalytic residues. Of particular interest are the Rhomboid family serine proteinases that are active within the plasma membrane, for which several new structures have been reported. Because these enzymes are involved in biological and pathological processes, many are becoming important targets of drug design.

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Introduction

Proteinases (peptidases, proteases) are enzymes that catalyze the hydrolysis of peptide bonds. They are classified into six classes on the basis of their catalytic mechanism: serine, threonine, cysteine, aspartate, glutamate, and metalloproteases. Serine proteinases (SP) cleave the peptide bond by nucleophilic attack of the serine hydroxyl group on the scissile carbonyl bond (some enzymes with nucleophiles such as threonine or cysteine share the fold with serine proteases; they will not be further discussed here). Typical SPs have an active site catalytic triad consisting of the Oγ atom of the serine, the imidazole ring of the histidine, which serves as a general acid/base, and the carboxylate of an aspartic acid, which helps to orient the imidazole ring. In addition, an oxyanion hole provides electrophilic assistance to the nucleophilic attack by the serine Oγ on the carbonyl atom of the scissile bond. The goal of this review is to highlight some key structural features of the serine proteinase structures published in the past two years (Table 1).

Structures of trypsin-like and chymotrypsin-like proteinases

The terms 'trypsin-like proteinases' and 'chymotrypsin-like proteinases' are sometimes used interchangeably to not only identify members of MEROPS [1] (also see http://merops.sanger.ac.uk/) clan PA (mixed catalytic type), family S1 (chymotrypsin), but also less strictly to more distant families in that clan. All these SPs share the same basic fold in their catalytic domains (although they may or may not also have other domains) but vary widely in their specificity and regulation. A number of them will be discussed in this section.

Kallikreins are trypsin-like proteases that cleave kininogen; they are grouped into two major categories, plasma and tissue. Plasma kallikrein is a multidomain, glycosylated protein that is more homologous to factor XI than to tissue kallikrein with a single protease domain. The structural basis for the specificity of plasma kallikrein was revealed by its crystal structure [2]. Human tissue kallikreins (hK) belong to a closely related 15-member family. Its best functionally characterized member, hK1, is involved in blood pressure regulation. Structural studies of hK1 revealed that binding of a peptide substrate induces structural rearrangement of the catalytic Ser that optimally positions it for interactions with the catalytic His [3]. The highly specific hK4, which activates hK3/PSA, is normally expressed in the prostate but is overexpressed in prostate and ovarian cancer [4]. The hK4 structure uncovered a novel metal-binding site formed by a 10-residue loop linked to the N-terminal segment, specific for this enzyme. hK4 can exist in a Zn^{2+}-free high activity form and a Zn^{2+}-bound low activity form [4]. The prostatic fluid contains micromolar concentrations of zinc that can inhibit hK4.

Component B of earthworm fibrinolytic enzyme (EFE-b) is a two-chain glycoprotein that exhibits strong fibrinolytic activity [5]. It has a novel conserved structural motif that is formed by two consecutive Cys residues linked by a *cis* peptide bond and a disulfide bond. This ring may play a similar role to Tyr151 in u-PA, t-PA, or DSPA and may be related to the selectivity for the P2′ of its substrate.

SplC proteinase from *Staphylococcus aureus*, structurally most similar to V8 proteinase, another trypsin-like enzyme [6], is possibly involved in staphylococcal virulence. The protease is latent until cleaved by signal peptidase during secretion. Its structure reveals a V8-like fold, a well-defined S1 pocket, and a possible mechanism of substrate-induced conformational switch [7].

Table 1

Summary of the structures covered in this review

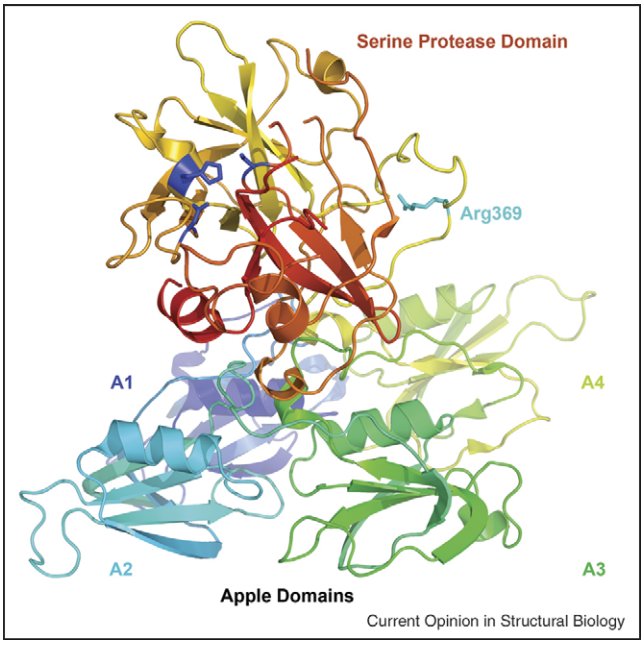
Enzyme	Family	Peptidase type	MEROPS ID	PDB accession code	Reference
Complement factor B	S1A	Chymotrypsin-A	S01.196	2OK5	[13•]
Complement component C2a			S01.194	2ODP,2ODQ	[14]
MASP-2 zymogen R444Q mutant fragment			S01.229	1ZJK	[15]
Human tissue kallikrein 1			S01.160	1SPJ	[3]
Human tissue kallikrein 4			S01.251	2BDG,2BDH,2BDI	[4]
Human plasma kallikrein			S01.212	2ANW,2ANY	[2]
Prophenoloxidase-activating factor I			S01.204	2OLG	[11•]
Prophenoloxidase-activating factor II			S01.960	2B9L	[12]
Earthworm fibrinolytic enzyme component B			S01.243	1YM0	[5]
Factor XI zymogen			S01.213	2F83	[10]
A. actus venom serine proteinase I and II			S01.x	1OP0,1OP2	[8]
A. contortrix contortrix protein C activator			S01.466	2AIP,2AIQ	[9]
SplC proteinase			S01.283	AS9	[7]
Hemoglobin protease	S6	IgA1-specific serine peptidase	S06.003	1WXR	[16••]
NS3 protease	S7	Flavivirin	S07.001	2FOM,2FP7	[17•]
Subtilase cytotoxin protease SubA	S8A	Subtilisin Carlsberg	S08.121	2IY9	[22]
Subtilisin-like protease *Vibrio*			S08.x	1SH7	[24•]
Serratia proteinase K			S08.x	2B6N	[23]
Fibroblast activation protein α	S9A	Dipeptidyl-peptidase IV	S09.007	1Z68	[19]
Prolyl tripeptidyl peptidase			S09.017	2D5L,2DCM	[20]
Prolyl endopeptidase	S9B	Prolyl oligopeptidase	S09.001	1YR2,2BKL	[21]
Archaeoglobus fulgidus LonB	S16	Lon-A	S16.005	1ZOW,1ZOC,1ZOB, 1ZOG,1ZOE	[28•]
Sesbania mosaic virus polyprotein protease	S39A	Sobemovirus peptidase	S39.001	1ZYO	[18]
Membrane protease 1510-N	S49	Signal-peptide peptidase A	S49.005	2DEO	[29]
Birnavirus VP4	S50	Infectious pancreatic necrosis birnavirus Vp4 peptidase	S50.001	2GEF	[30•]
E. coli GlpG	S54	Rhomboid-1	S54.016	2IC8,2NRF,2IRV	[31••,32•,33]
H. influenzae hiGlpG			S54.024	2NR9	[35]

Trypsin-like *Agkistrodon actus* snake venom serine proteinases I and II are identical except for one amino acid. An interesting feature observed in these structures is the conserved *N*-glycosylation site at Asn35. This oligosaccharide, close to the active site, can sterically restrict access of some inhibitors, such as soybean trypsin inhibitor (STI) [8]. *Agkistrodon contortrix* protein C activator activates protein C without relying on thrombomodulin. Structures of the native and inhibited enzyme reveal three carbohydrate moieties positioned around the active site that are involved in substrate recognition [9]. These carbohydrates, together with the positive charge on the interfacial surface, may be crucial for activation—protein C does not bind the macromolecular trypsin inhibitors BPTI and STI.

Factor XI is an essential coagulation protein that circulates as a disulfide-linked homodimer [10] and cleaves factor IX. Each monomer has four homologous apple domains (A1–A4) that mediate essential protein–protein interactions, followed by a chymotrypsin-like serine protease domain (Figure 1). Each apple domain is formed by seven β strands that cradle a single α helix. Thrombin binds the A1 domain through its exosite I, whereas the platelet surface receptor glycoprotein Ib-V-IX (GpIb) binds A3 through its leucine-rich repeats. These two apple domain sites are diametrically positioned in factor XI, separated by the centrally positioned protease domain. Thrombin cleaves after Arg369, and the free N-terminus moves 20 Å and inserts into the activation pocket of factor XIa, generating an oxyanion hole [10].

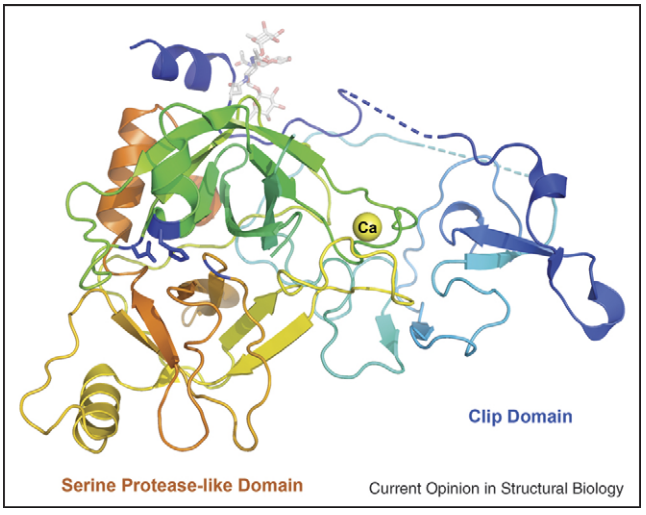
The easter-type SPs, prophenoloxidase-activating factors (PPAF) I and II are involved in the insect immune response. The structures of the SP domain of PPAF-I [11•] and the whole PPAF-II from *Holotrichia diomphalia* [12] reveal a chymotrypsin-like fold of their proteinase domains (Figure 2). PPAF-I belongs to the catalytic group of clip domain SPs, in contrast to PPAF-II, which belongs to the non-catalytic group (the latter contains Gly353 in place of the catalytic serine). The clip domain of PPAF-II is a protein-interaction module with a novel fold; it is essential to the binding and activation of the 76 kDa phenoloxidase via its central cleft [12]. After cleavage by PPAF-III, the clip domain remains tightly attached to the SP domain, and the enzyme oligomerizes into two hexameric rings that serve as a hub for phenoloxidase binding. In contrast to PPAF-II, the clip domain of PPAF-I is separated from the SP domain during activation.

Figure 1



Crystal structure of Factor XI (2f83). The four homologous apple domains are colored blue to yellow, the serine proteinase domain orange-red, and the catalytic triad is shown in blue stick. Thrombin cleaves after Arg369 (cyan stick). The blue to red color gradient in the ribbon diagrams indicates the direction of the chain from the N to C terminus. This and all other figures were prepared with PyMol [34].

Figure 2

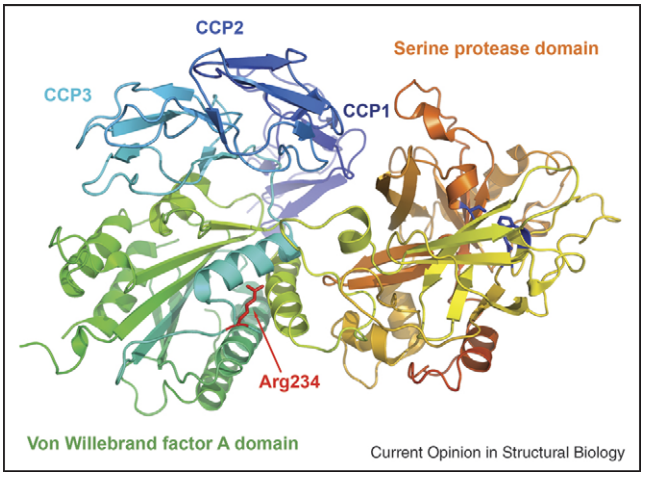


Prophenoloxidase-activating factor II (2b9l). In the active enzyme the N-terminal clip domain (blue-cyan) remains attached to the C-terminal serine proteinase-like domain (green-red), with the catalytic triad shown in blue stick (H200, D252, G353). The bound calcium ion is a yellow sphere and the paucimannose glycosyl moiety on Asn32 is shown in white stick. The clip domain is a protein-interaction module important in the binding and activation of the 76 kDa prophenoloxidase through its central cleft.

The plasma protein factor B is a very specific SP whose activation is tightly controlled. It has a central role in adaptive immunity: it regulates the amplification step of complement activation and triggers cell lysis, phagocytosis, inflammatory responses, and B cell stimulation [13•]. The inactive factor B proenzyme has five domains: three complement control protein (CCP)s, a von Willebrand factor A (VWA), and a C-terminal SP domain. The CCP domains, with their typical β-sandwich fold that is stabilized by disulfide bonds, form a tightly packed triad that provides an important binding site for C3b (Figure 3). The CCP3-VWA linker contains a short loop, an α helix (αL), and a partially disordered long loop. The VWA domain, with its α/β/α fold, contains a metal ion-dependent adhesion site (MIDAS) that is Mg^{2+}-dependent. The human factor B proenzyme structure shows a 'locked' or inactive conformation of VWA with α helix 7 displaced by helix αL and a distorted conformation MIDAS with low affinity for ligands. Linker helix αL binds Arg234 of the scissile bond and prevents proteolytic activation. The conformation of the oxyanion hole is identical between the factor B and fragment Bb structures, suggesting that the proteolytic activity of factor B is probably not controlled directly as in trypsinogens but rather through rearrangement of the domains [13•].

Another pathway of complement activation involves the cleavage of C2 from the C4b–C2 complex, which gives rise to the C4b2a C3 convertase. C2 is the structural and functional homolog of factor B, and both have identical domain organization. The structure of C2a consists of a VWA and an atypical chymotrypsin-like SP domain and is

Figure 3



The structure of Factor B (2ok5). The structural domains CCP1–CCP3 are shown in blue-cyan, VWF domain in green, and serine protease domain yellow-red. The catalytic triad is shown in blue stick. Arg234 of the scissile bond is shown in red, right under the linker helix αL (cyan).

very similar to Bb [14]. The active site is in an inactive, zymogen-like conformation.

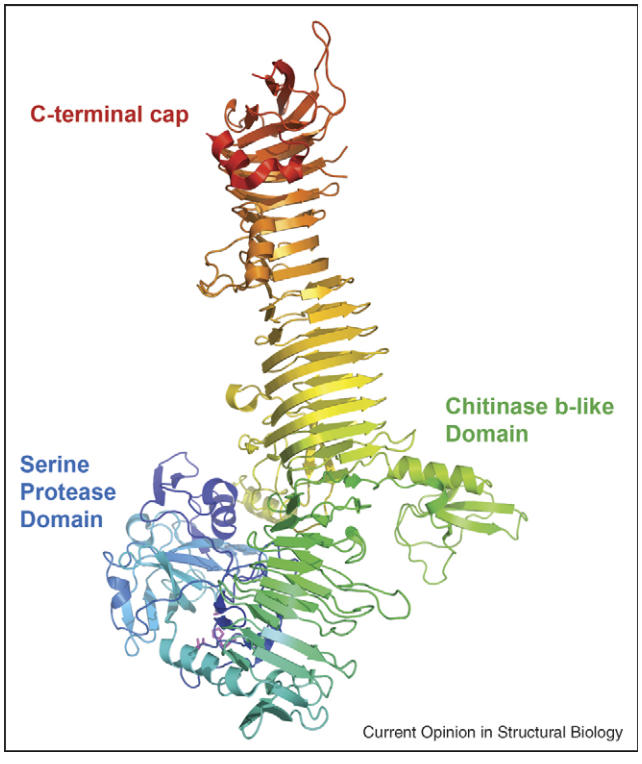
The third lectin pathway of complement activation involves mannose-binding lectin-associated SP-2 (MASP-2). This is a truly autoactivating enzyme, for which no external factors are required to initiate the reaction—it can directly initiate the complement cascade. The structure of a stable, MASP-2 zymogen CCP1–CCP2-SP fragment, together with the available active MASP-2 revealed the mechanism of autoactivation [15]. The zymogen can still cleave its natural substrate C4 with low efficiency. In addition to the activation domain, a series of loops of the serine protease domain can undergo conformational changes, yielding an active enzyme.

E. coli hemoglobin protease (Hbp) is an autotransporter that degrades hemoglobin and delivers heme to the symbiotic *E. coli* and *Bacteroides fragilis* involved in peritonitis. The 110 kDa passenger domain (Figure 4) is the largest parallel β-helical structure solved and the first structure from this class of SPs [16••]. The N-terminal SP domain has a trypsin-like fold, followed by 24 turns of right-handed β helices that form three parallel β sheets similar to pertactin. This β-helical structure is interrupted between residues 481–556 by a chitin binding like domain that possibly binds extracellular matrix proteins. The β helix is capped by a conserved C-terminal domain that seems more flexible and may be involved in the passage across the outer membrane [16••].

The structures of the West Nile virus and dengue NS3 proteases in the presence of the catalytically important NS2B cofactor fragment reveal the catalytically relevant conformation of the NS2B-NS3 complexes. The chymotrypsin-like viral protease NS3 is required for the correct processing of the flaviviral polyprotein and is activated by a 47-residue region of the NS2B cofactor. The N-terminal and C-terminal barrels of NS3 are stabilized by NS2B, which provides additional β strands and completes the substrate-binding site with its C-terminal region [17•].

The *Sesbania mosaic virus* polyprotein is processed by its N-terminal SP domain. The trypsin-like SP domain has a catalytic triad identical to cellular SPs and different from other viral proteases. The enzyme cleaves Glu-Thr bonds between the protease and the following VPg domain and Glu-Ser bonds between the subsequent p10 and RdRP domains of the polyprotein [18].

Figure 4



The entire passenger region of hemoglobin protease (1wxr). The N-terminal serine proteinase domain (blue) is followed by an extensive right-handed β helix, interrupted by a chitinase b-like domain (green). Passage through the membrane is facilitated by the flexible C-terminal capping motif. The catalytic triad is shown in magenta stick.

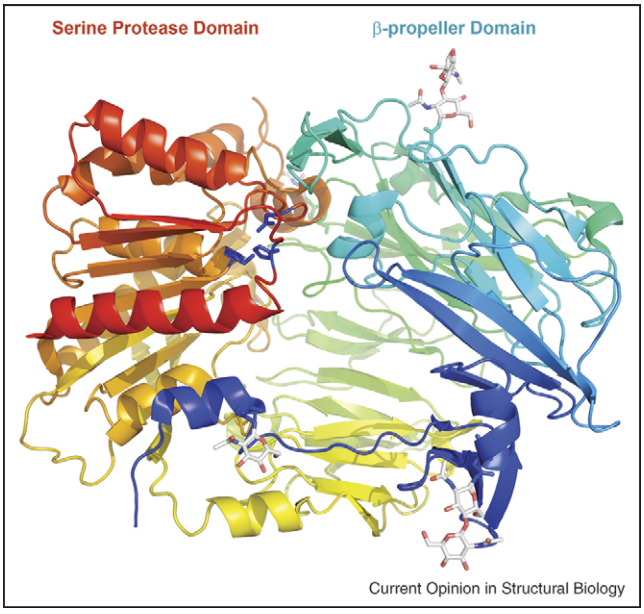
Dipeptidyl peptidase IV (DPPIV)-like structures

Fibroblast activation protein α (FAPα) is a type II transmembrane protease expressed in epithelial cancers and is implicated in extracellular matrix remodeling, tumor growth, and metastasis. The FAPα extracellular domain has an identical fold to DPPIV, with an α/β-hydrolase and an eight-bladed β-propeller domain (Figure 5). FAPα has a decreased catalytic efficiency for cleaving DPPIV dipeptides because it has an Ala657 in its active site instead of the corresponding Asp in DPPIV, reducing the acidity in the pocket and lowering the affinity for N-terminal amines [19].

The structure of a prolyl tripeptidyl aminopeptidase from *P. gingivalis*, revealed another DPPIV fold with an eight-bladed β-propeller domain. The membrane-anchoring α helix was removed for structure determination [20]. The active site is virtually identical to DPPIV, but some β strands are shorter in the β-propeller domain, giving rise to a wider opening between the two domains.

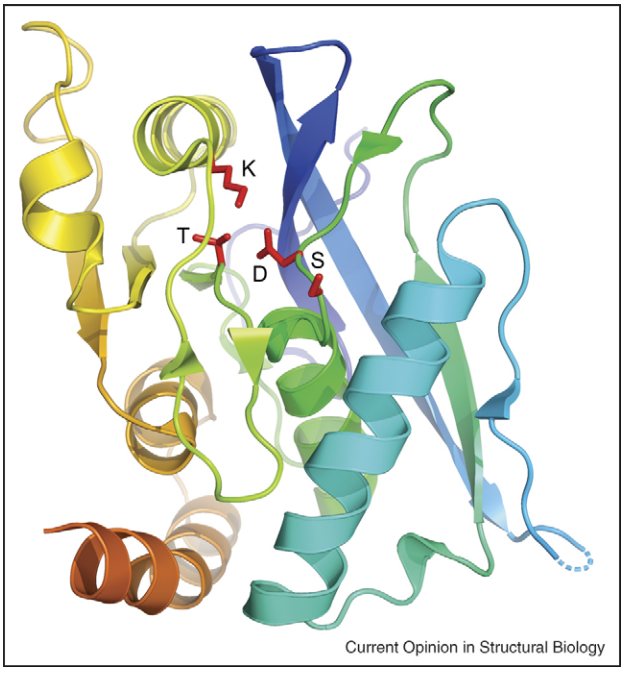
Two prolyl enopeptidase (PEP) structures from *Sphingomonas capsulata* and *Myxococcus xanthus* capture an open and a closed conformation, respectively, for these DPPIV-like enzymes [21]. In contrast to DPPIV, their β-propeller domains have only seven blades. Electrostatic points of interaction may provide the 'latches' for domain opening and closing. Domain opening could select the size of oligopeptides and protect larger structures from proteolysis.

Figure 5



Extracellular domain of fibroblast activation protein α (1z68). The N-terminal eight-bladed propeller domain to the right (blue to yellow) is separated by a central cavity from the serine protease domain (orange to red) with the catalytic triad (in blue stick). *N*-glycosyl moieties are shown as white stick.

Figure 6



Proteolytic domain of B-type Lon (1z0w). The catalytic Ser509 is facing away from the lysine and the adjacent Asp508 and Thr534 residues (shown in red) were shown not to be essential for catalysis. The Lys552 NZ to Ser509 OG distance is 9.44 Å. These results suggest that a functional catalytic site in Lon proteinases may only be created in the presence of a substrate.

Enzymes with subtilisin-like structures (subtilases)

Subtilase cytotoxin SubAB is produced by a highly infectious Shiga toxigenic *E. coli* strain, which is responsible for the hemolytic uremic syndrome. Its A subunit, SubA, is similar to subtilisin Carlsberg [22]. The catalytic triad lies at the bottom of a deep cleft, where the S′ side of the active site is restricted in a similar way as in thrombin. Unlike other bacterial cytotoxins that are translocated from the endoplasmic reticulum (ER) lumen into the cytoplasm, SubAB can rapidly and specifically cleave BiP (or GRP78), a highly conserved, essential ER chaperone [22].

Two other new subtilase-like structures are those of *Serratia* sp. proteinase K [23] and a cold-adapted SP from *Vibrio* sp. [24•]. Minor structural differences in their surface loops account for their different substrate specificities and binding affinities. *Serratia* proteinase has one calcium-binding site, whereas the *Vibrio* proteinase has three. The cold-adapted enzyme has more extensive hydrogen-pair and ion-pair interactions, and its surface has more apolar regions exposed and is abundant in uncompensated negatively charged residues [24•].

Catalytic mechanism of serine-carboxyl proteases

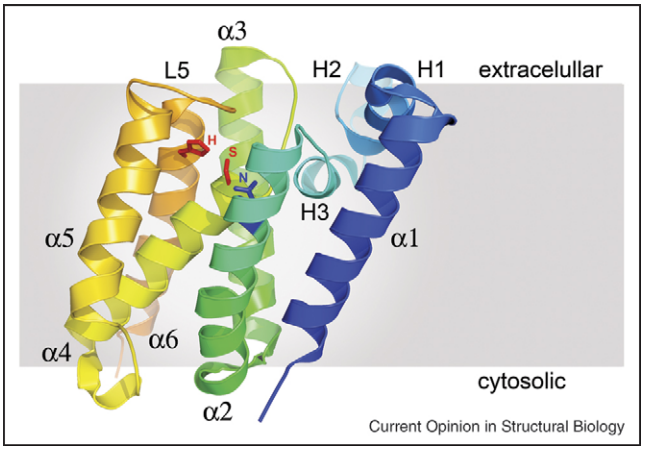
Serine-carboxyl proteases (sedolisins) have a fold similar to subtilisin, but their catalytic triad contains a Glu instead of a His, and the oxyanion hole an Asp instead of an Asn. Recent investigation of their catalytic mechanism by quantum mechanical/molecular mechanical molecular dynamics and free energy simulations indicated that these enzymes might not act as typical serine proteinases, in that the aspartate located in the oxyanion hole might play a role of a general base during the formation of the acyl-enzyme from the tetrahedral intermediate [25]. Recent analysis of the prokaryotic genome databases predicted the existence of sedolisin-like enzymes with the catalytic aspartate located in a different segment of the polypeptide chain and with an asparagine in the oxyanion hole [26].

Enzymes with a Ser-Lys catalytic dyad

Lon proteases are involved in protein quality control in the cell. The atomic-resolution structure of the proteolytic domain of B-type *Archaeoglobus fulgidus* Lon is very similar to the *M. janaschii* LonB [27], though significant differences are found in the architecture of the active site (Figure 6), suggesting that a functional catalytic site in Lon proteases may only be created in the presence of a substrate. The enzyme oligomerizes into ring-shaped hexamers, similar to *E. coli* A-type Lon [28•].

The N-terminal segment of *Pyrococcus horikoshii* putative membrane protease PH1510 is a Ser-Lys dyad protease

Figure 7



Intramembrane protease GlpG (2ic8). The core of the GlpG molecule is formed by six transmembrane helices (α1–α6) of varying lengths with a loop region inserted between the first two. This loop contains a novel structural motif of three short helices (H1–H3) that stack against hydrophobic residues on the third transmembrane helix [31••,32•,33]. Ser201 and His254 of the catalytic dyad (shown in red) are located at the bottom of a water-filled cavity, toward the periplasmic side of the membrane. Asn154 (blue) was initially believed to be the third residue of the catalytic triad, but it is now suggested to create the oxyanion hole, at least in *E. coli* GlpG. In *H. influenzae* GlpG, an equivalent role is performed by His65 [35]. Intramembrane substrate entry to the V-shaped active site is gated by a small conformational change of transmembrane helix 5 that also carries loop L5 with it. GlpG in the 'open' state can accommodate and cleave a polypeptide chain from the unwound end of a transmembrane substrate helix. However, it is not clear how substrate entry is initiated.

that is structurally similar to *E. coli* ClpP, but it forms a dimer only through helices α7–α8 and the β9 strand, which are not present in ClpP. The catalytic lysine is located in a disordered loop region (L2), suggesting that substrate binding might induce correct positioning of this residue [29].

The blotched snakehead virus (BSNV) encodes a polyprotein NH_2-pVP2-X-VP4-VP3-COOH that is processed by its own VP4 protease to yield pVP2 and VP3. pVP2 is further processed by VP4 into the capsid protein VP2 and four structural peptides. VP4 contains a catalytic serine/lysine dyad similar to other Ser/Lys proteases, such as Lon, LexA, and signal peptidase [30•]. Domain I of VP4 consists of predominantly β sheet, whereas domain II has an α/β structure. When a substrate binds to VP4, the N^{ζ} of Lys729 probably becomes completely buried, lowering its pK_a value and acting as a general base. The location of the oxyanion hole is not certain but probably involves Ser692 and Gln691.

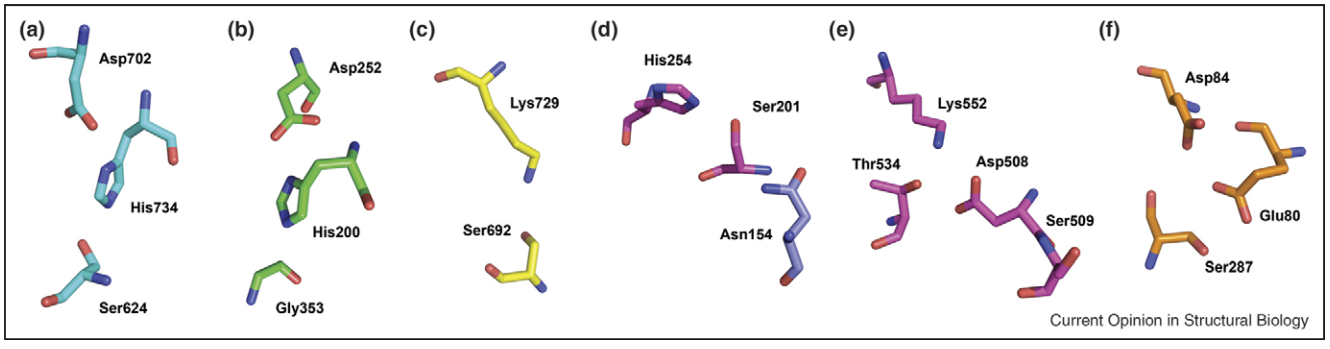
Intramembrane protease GlpG (Rhomboids)

The function of GlpG from *E. coli* is unknown, but this enzyme is homologous to *Drosophila* rhomboid-1 that was shown to cleave the transmembrane domain of the epidermal growth factor (EGF)-like protein Spitz, thus regulating EGFR signaling. Three recent structures of *E. coli* GlpG and one of *H. influenzae* GlpG depict the same features (Figure 7) but with some minor differences [31••,32•,33]. GlpG cleaves single-span transmembrane substrates a few residues inward from the extracellular side. This cleavage mechanism may be used by other families of intramembrane proteases, such as presenilin, site-2 protease, and signal-peptide peptidase [32•].

Conclusions

Many structures of serine proteinases have been published in the past two years. The present review attempted to capture a snapshot of this vast new information. Not surprisingly, given the general abundance of this class of enzymes, most of the newly characterized enzymes exhibit a trypsin-like fold. However, aside from the common scaffold, the diverse structural details uncovered novel mechanisms of activation and substrate recognition. A variety of the catalytic site configurations have been

Figure 8



Diversity of the constellations of residues located in or near the active sites of serine proteinases. **(A)** Classical Ser-His-Asp triad (cyan) from the fibroblast activation protein α. **(B)** Inactive Gly-His-Asp triad (green) from phenoloxidase-activating factor II. **(C)** Ser-Lys dyad (yellow) from birnavirus VP4 protease. **(D)** Ser-His dyad (magenta) from rhomboid GlpG protease. Asn154 (blue) creates the oxyanion hole. **(E)** Inactive Ser-Lys dyad (magenta) observed in LonB protease. In the absence of the substrate, Asp508, rather than the catalytic Ser509, interacts with Lys552. **(F)** Active site of sedolisin with the Ser-Glu-Asp triad (orange).

observed for the active and inactive enzymes (Figure 8). In particular, new structures of the serine/lysine dyad proteinases indicate that the active sites might not be fully formed in the absence of substrates. The most interesting new structures appear to be those of the Rhomboid family of intramembrane proteinases as they reveal their novel mode of action. Because most of these enzymes are involved in important physiological and pathological processes, the new structural details should permit structure-based inhibitor design.

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Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

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