A standard orientation for metallopeptidases.

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ABSTRACT

Visualization of three-dimensional structures is essential to the transmission of information to the general reader and the comparison of related structures. Therefore, it would be useful to provide a common framework. Based on the work of Schechter and Berger, and the finding that most peptidases bind their substrates in extended conformation, we suggest a “standard orientation” for the overall description of metallopeptidases (MPs) as done before for peptidases of other classes. This entails a frontal view of the horizontally-aligned active-site cleft. A substrate is bound N- to C-terminally from left (on the non-primed side of the cleft) to right (on the primed side), and the catalytic metal ion resides at the cleft bottom at roughly half width. This view enables us to see that most metalloendopeptidases are bifurcated into an upper and a lower sub-domain by the cleft, whose back is framed by a nearly horizontal “active-site helix”. The latter comprises a short zinc-binding consensus sequence, either HEXXH or HXXEH, which provides two histidines to bind the single catalytic metal and the general-base/acid glutamate required for catalysis. In addition, an oblique “backing helix” is observed behind the active-site helix, and a mixed β -sheet of at least three strands is positioned in the upper sub-domain paralleling the cleft. The lowermost “upper-rim” strand of the sheet runs antiparallel to the substrate bound in the cleft and therefore contributes both to delimitating the cleft top and to binding of the substrate main-chain on its non-primed side through β -ribbon-like interactions. In contrast, in metalloexopeptidases, which chop off N- or C-terminal residues only, extensive binding on both sides of the cleft is not required and a different overall scaffold is generally observed. This consists of an $\alpha\beta\alpha$ -sandwich, which is reminiscent of, but clearly distinct from, the archetypal α/β -hydrolase fold. Metalloexopeptidases have their active sites at the C-terminal end of a central, eight-stranded twisted β -sheet, and can contain one or two catalytic metal ions. As the zinc-binding site and the residues engaged in substrate binding and catalysis are mainly provided by loops connecting the β -sheet strands and the helices on either side, the respective standard orientations vary with respect to the position of the sheets. The standard orientation of eight prototypic MP structures is presented and discussed.

On the 56th anniversary of the discovery of the lysosome in 1955 [1], it would seem appropriate to commemorate the nearly 45 years of the pioneering work of Schechter, Berger and colleagues on the topological analysis of the active-site cleft of peptidases [2, 3]. Based on the hydrolytic activity of the endopeptidase papain on alanine peptides of varying length, these authors hypothesized that the active site of the enzyme had seven “subsites” interacting with the side chains of the residues flanking the scissile bond (Fig. 1a). They subdivided the cleft into an N-terminal or upstream region with respect to the scissile bond, the “non-primed” side, and a C-terminal or downstream region, the “primed” side. Consequently, the substrate side chains on the non-primed side away from the scissile bond are now termed P₁, P₂, P₃, etc. and their cognate enzyme subsites S₁, S₂, S₃, etc. On the primed side, substrate side chains P₁′, P₂′, P₃′, etc. are accommodated in subsites S₁′, S₂′, S₃′, etc. These findings arise from the fact that peptidases must provide a scaffold that is large enough to bind a substrate efficiently and then to exert the cleavage reaction. This also entails that the reactivity of a scissile peptide bond depends not only on the flanking residues in S₁ and S₁′ but also on the side chains of residues further upstream and downstream [3]. In the exopeptidase bovine pancreatic carboxypeptidase A, Schechter, Berger, and colleagues stated that the C-terminal residue to be excised had to be placed in S₁′ and that residues upstream in the substrate occupied subsites S₁, S₂, etc., thus giving rise to a shorter active-site cleft than in the endopeptidase papain. They further underlined the importance of the nature of the substrate side chains up to S₄ for cleavage by this model metallopeptidase (MP) [2]. Members of the mostly zinc-dependent class of MPs are characterized by a catalytic metal ion, which cleaves peptide bonds following a common mechanism with the participation of a solvent molecule and a polarizing general base/acid, usually a glutamate [4-7]. Upon binding of the substrate, the metal simultaneously coordinates the scissile carbonyl oxygen and the solvent molecule, and the glutamate activates the latter for attack on the peptide bond. MPs are subdivided into endopeptidases and exopeptidases. The former accommodate substrate side chains on both the non-primed and the primed side of the cleft as described above for the cysteine proteinase papain, while the latter only bind single or few residues on the non-primed side (aminopeptidases, dipeptidyl peptidases, tripeptidyl peptidases, etc.) or the primed side (carboxypeptidases, peptidyl dipeptidases, peptidyl tripeptidases, etc.).

There are several methods to classify MPs, but the MEROPS database (<http://merops.sanger.ac.uk>; [8]) probably provides the most comprehensive and widespread system. It is based on catalytic types and amino-acid-sequence similarity within peptidase moieties, and MPs are currently split into 62 families (M1-M85 with several unassigned numbers; as of March, 2011). Optionally, other classifications are conceivable considering three-dimensional structural criteria [7, 9-14]. Fig. 2 provides a scheme showing part of such a tree, which is under continuous evolution as new structural analyses and comparisons become available [7, 10, 11, 13, 15, 16]. An important subset of MPs comprises a short zinc-binding consensus sequence, HEXXH—first reported by McKerrow in 1987 [17]—, which includes two metal-binding histidines and the general-base/acid glutamate for catalysis. These enzymes give rise to the single-metal zincin tribe of MPs [10, 15]. On the same hierarchy level are the inverzincins, which contain an inverted consensus sequence of identical residues, HXXEH, and the αβα-

exopeptidases, which comprise at least two clans, the funnelins and the EEM2-MPs, each with conserved sequence signatures and structural features. While the funnelins are single-zinc enzymes, EEM2-MPs harbor two (co)catalytic metal ions. Zincins, in turn, divide into metzincins, gluzincins, aspzincins, and S2P-zincins, depending on the nature of the third zinc-binding residue and other structural features. Finally, metzincins split into at least nine families, which have been structurally characterized, and gluzincins bifurcate into thermolysins and cownrins, among others. Zincins and inverzincins generally recognize their substrates in an extended β -strand-like backbone conformation, at least with respect to the positions immediately flanking the scissile peptide bond [18]. In addition, MPs generally bind their substrates and inhibitors through deep S_1' pockets and this contrasts with serine proteinases of the trypsin/chymotrypsin type, which mostly use their S_1 pockets for specificity. This entails that, in the first case, the side chain of the substrate at P_1' points into the enzyme body, while the P_1 residue does this in the trypsin-like serine proteinases.

In order to improve the way we visualize the active-site cleft, the catalytic metal-binding site, the distinct enzyme subsites, the conformation of bound substrates, reaction intermediates and products, as well as inhibitors, we propose a “standard orientation” for the general description of structural features of MPs as also used for trypsin-like, subtilisin-like, and α/β -hydrolase-type serine peptidases. The term was first used back in 1993 for thrombin [19]. For MPs, this orientation implies a frontal view into the cleft, with a substrate running from left (non-primed side) to right (primed side), and the catalytic metal residing at the base of the cleft. This view is compatible with the intuitive scheme proposed nearly 45 years ago by Schechter and Berger (Fig. 1a,b,c), and it is outlined for eight structural MP prototypes (Figs. 3 and 4).

***Bacillus thermoproteolyticus* thermolysin (BtTL)** — This endopeptidase is a prototype of the zincin tribe of MPs and of one of the two gluzincin families discussed here, the thermolysins. It has been the subject of many crystallographic studies since the first report by Matthews and colleagues back in 1972 [20-22]. Together with the structure of the funnelin, bovine pancreatic carboxypeptidase A1 (see below), first reported by Lipscomb and co-workers in 1967 [23], BtTL was the gold standard to study fold and catalysis of MPs for 20 years, until the structure of astacins was reported in 1992 [24]. As BtTL continues to be the paradigm for the zincins, its structural features will be discussed in more detail and subsequent structures will be referred to it. Viewed in the standard orientation, BtTL is split horizontally into two roughly equal-sized sub-domains by its elongated active-site cleft (Fig. 3a). The upper sub-domain comprises a horizontal five-stranded β -sheet on the front surface, whose three central strands run parallel to a substrate bound in the cleft, while the two flanking strands are antiparallel (Fig. 3a, ②). The lowermost is the “upper-rim strand” that frames the top of the active-site cleft and binds the main chain of substrates in the non-primed side of the cleft. This is best shown by the complex with a reaction-intermediate analog, Z-Phe^P-Leu-Ala, which spans subsites S_2 - S_2' (Fig. 3a; [25]). On top of this sheet, a five-stranded open β -barrel is inserted. The open face of the barrel and the concave side of the sheet accommodate a long helix, the “backing helix” (Fig. 3a, ③). The last strand of the sheet leads to the “active-site helix”, which contains the short zinc-binding consensus sequence, HEXXH (Fig. 3a, ③), which is characteristic

for zincins and therefore also for gluzincins (see above and Fig. 2). The lower sub-domain of BtTL contains two helices: the “glutamate helix” (Fig.3a, ④), which provides the third protein zinc ligand, a glutamate, and thus gives the name to the gluzincin clan [10]; and the “histidine helix” (Fig.3a, ⑤), which contains a histidine for catalysis [4]. In addition, the lower sub-domain contains an antiparallel two-stranded β -sheet, a β -ribbon, and a C-terminal three-helix bundle (Fig. 3a).

***Methanosarcina acetivorans* ulilysin** — This peptidase represents the pappalysin family within the metzincin clan of MPs. Metzincins share the short zinc-binding consensus sequence with BtTL. They also share the general architecture of the upper sub-domain, including the four lowermost strands of a likewise five-stranded β -sheet—with its upper-rim strand—, the backing helix, and the active-site helix (Fig. 3a,b; ①-③). In contrast to BtTL, however, in metzincins the short zinc-binding consensus sequence is elongated C-terminally to render HEXHXXG/NXXH/D, with two additional key positions for shaping the metal-binding site [13-16, 26, 27]: (i) mostly a glycine is found three residues downstream of the second zinc-binding histidine and it accounts for a sharp change in the direction of the polypeptide chain at the junction between the upper and the lower sub-domains; and (ii) the last residue of the motif, generally a histidine, is the third metal ligand. Further to these earmarks, metzincins possess a spatially conserved tight 1,4-turn containing a methionine—the “Met-turn”—, which provides a hydrophobic base required for structural integrity of the metal-binding site [28]. Moreover, metzincins contain a C-terminal helix at the end of their lower sub-domains (Fig. 3b; ⑥). Within metzincins, the polypeptide chains vary greatly in both sequence and length, in the loops connecting the common features and between the elongated zinc-binding consensus sequence and the Met-turn, thus giving rise to catalytic domains spanning ~130-260 residues. In addition, specific structural elements—structural and regulatory metal-binding sites, helices and β -strands—characterize the distinct families: astacins, adamalysins/ADAMs, serralysins, matrixins, snapalysins, leishmanolysins, pappalysins, archaemetzincins, and fragilysins.

***Trypanosoma cruzi* metallocarboxypeptidase 1 (TcMCP-1)** — This is a carboxypeptidase, which belongs to the cowrin family within the gluzincin clan (Fig. 2; [7]). Cowrins comprise large (~500-700 residues) ellipsoidal endo- and exopeptidases, and all are characterized by a horizontal extended narrow cleft, optimally shaped to accommodate and cleave oligopeptides and unstructured proteins. They share a common core of 17 helices (Fig. 3c) and a three-stranded β -sheet in the upper sub-domain that frames the active-site cleft on its non-primed side. The latter element is shared with thermolysins (Fig. 3c; ②), as are the backing helix (Fig. 3c; ③); the HEXXH-containing active-site helix (Fig. 3c; ①); the glutamate helix, which provides the third glutamate metal ligand (Fig. 3c; ④); and the histidine helix (Fig. 3c; ⑤), termed “tyrosine helix” in cowrins due to the replacement of the BtTL histidine with a tyrosine [7].

***Grifola fondosa* peptidyl-lysine metalloendopeptidase (GfMEP)** — This is the prototype of the aspzincins, a clan within the zincins [29]. GfMEP is an elongated ~165-residue endopeptidase with an extended cleft separating two sub-domains as described in previous zincins. Viewed in standard orientation, the upper sub-

domain has a four-stranded β -sheet, which is topologically equivalent to the four lowermost strands in thermolysins and metzincins; an active-site helix with the HEXXH motif; and a backing helix (Fig. 3d; ①-③). The lower sub-domain is entirely helical and shows two helices that could be topologically equivalent to the glutamate helix and the histidine helix of BtTL (Fig. 3d; ④-⑤). However, the third protein zinc ligand, an aspartate, is provided not by the former helix but by a loop following the active-site helix, in a similar fashion as in metzincins [14].

***Methanocaldococcus jannaschii* site-2 protease (S2P-MP)** — This is a representative of the site-2 proteases, the only integral-membrane MPs structurally characterized to date [30]. These enzymes participate in regulated intramembrane proteolysis, i.e. they cleave substrates imbedded within the lipid bilayer. The structure of S2P-MP is similar to BtTL if displayed in standard orientation, which entails showing the plane of the membrane vertically (Fig. 4a) rather than horizontally (see Fig. 1 in [30]). This view reveals that the enzyme has an elongated horizontal cleft, with the catalytic zinc ion about 14Å below the membrane surface on its cytosolic side. The cleft is ideally suited to bind substrates in extended conformation within the membrane. The presence of an active-site helix with the HEXXH motif, a four-stranded β -sheet with an upper-rim strand, and a helix equivalent to the glutamate helix providing the third zinc-binding protein ligand, an aspartate, ascribes S2P-MP to the zincins. We propose that these membrane MPs should be considered a distinct clan within the zincins (Fig. 2).

***Escherichia coli* pitrilysin** — Pitrilysin is a structural representative of the inverzincin tribe of MPs, which is characterized by an inverted signature, HXXEH [31]. Inverzincins are multimodular proteins, into which a metal-dependent catalytic domain has been inserted. The catalytic domain can be displayed in a similar manner to the preceding zincins (Fig. 4b) and this view likewise reveals a subdivision into two sub-domains by an elongated cleft. In the upper sub-domain, there is a frontal seven-stranded horizontal β -sheet, which shares strand direction with the sheet of zincins for its two lowermost strands only (Fig. 4b; ②). The upper-rim strands run in the same direction as in zincins, suggesting that the substrates are similarly bound from left to right. In addition, a backing helix nestles into the concave side of the sheet as in zincins (Fig. 4b; ③). The most important difference is that the active-site helix, which is almost horizontal, runs in the opposite direction to that of zincins (Fig. 3a-d, 4a). This provides a structural explanation for the inverted signature, as the latter places the two histidines and the glutamate in a similar position as in zincins. A third zinc-ligand, a glutamate, is provided by a glutamate helix as in zincins (Fig. 4b; ④). Accordingly, zincins and inverzincins share several overall structural elements, as well as elongated clefts, which are consistent with their overall endopeptidase function.

***Staphylococcus aureus* HmrA** — This endopeptidase, like the funnelins (see below), has a different architecture from zincins and inverzincins. HmrA is a double-metal or co-catalytic zinc MP that shows structural similarity with a series of general amidohydrolases, which include carboxypeptidase G₂ (PDB 1CG2; [32]) or peptidase T (PDB 1FNO; [33]). These structural relatives comprise a catalytic domain consisting of a central twisted mostly eight-stranded β -sheet of equivalent strand polarity and connectivity (Fig. 4c,e), which together

with helices on either face of the sheet give rise to a three-layer ($\alpha\beta\alpha$)-sandwich. The β -sheet is mixed parallel/antiparallel and shows strand connectivity +1,+2,-1x,+2x,+2,+1x,-2 (according to [34]), i.e. distinct from that found in α/β -hydrolases (+1,+2,-1x,+2x,+1x,+1x,+1x; [35, 36]): although the five leftmost strands coincide both in topology and connectivity, the remaining three differ (Fig. 4e). An oligomerisation domain is inserted between the fifth and sixth β -strands of the catalytic domain of HmrA and consists of an open α/β -sandwich with four antiparallel β -strands and two α -helices. The active-site cleft is placed on the C-terminal end of the central β -sheet, and contributing residues are provided by loops connecting these strands and the flanking helices, as well as by the oligomerisation domain. So there is variation in the exact location of the cleft with respect to the sheet between HmrA and its structural relatives. These amidohydrolases have been grouped into the aminoacylase-1 family [37], for which three conserved amino acid motifs encompassing residues engaged in zinc binding and catalysis were identified as the hallmark: (S/G/A)HXDXV+GXXD+XEE. However, recent structural data, such as those on HmrA and others, narrow the common signature down to a pair of consecutive glutamate residues engaged, respectively, in zinc binding and acting as the general base/acid, so that the term Glu-Glu-containing double-metal MPs, briefly EEM2-MPs, is suggested (Fig.2). When such EEM2-MPs are displayed in standard orientation, the β -sheet is seen in edge view (Fig. 4c). As EEM2-MPs are mostly exopeptidases—HmrA being the only endopeptidase described—, substrate binding does not require extensive contacts with the substrate on either side of the cleft. Accordingly, no upper-rim strand is found here to bind the substrate main chain.

Human carboxypeptidase A4 (CPA4) — This is a member of the funnelin clan of single-zinc MPs [7, 38], which comprises structural relatives of the archetypal bovine carboxypeptidase A1 and feature mammalian, insect and bacterial proteins with strict carboxypeptidase activity. Their ~300-residue globular catalytic domains evince a consensus central eight-stranded β -sheet, with identical topology and connectivity to EEM2-MPs, i.e. it does not conform to the α/β -hydrolase fold as previously reported (e.g. in [7]). The sheet is flanked on either side by a total of eight helices. Funnelins contain a characteristic set of conserved residues engaged in metal and substrate binding as well as in catalysis (HXXE+R+NR+H+Y+E), and their active-site clefts are rather shallow and lie at the bottom of a funnel-like cavity. Therefore, these enzymes act on a large variety of well-folded proteins by cleaving off the C-terminal residues only. The complex of CPA4 with a cleaved hexapeptide (Fig. 4d; [6]) revealed the standard orientation of funnelins, which shows the cleft running in extended conformation from S_3 to S_1' (right; the specificity pocket of funnelins) as suggested by Schechter, Berger and colleagues [2]. At P_4 , the chain folds back along the molecular surface (Fig. 4d). In contrast to zincins and similarly to EEM2-MPs, the substrate main chain is bound by enzyme side chains rather than by a segment in extended conformation. In this clan, the standard orientation gives an almost lateral view on the central β -sheet (Fig. 4d).

Conclusions — Structural studies of MPs have accumulated over the past fifty years and have significantly contributed to our understanding of function and mechanism of this class of enzymes. In this sense, it is essential to transmit structural details to the interested reader, and therefore a common orientation of the catalytic domains

may be helpful to compare structures and discuss both similarities and differences. The detailed analysis of representative structures reveals that most MPs bind their substrates in extended conformation through extended active-site clefts, so that a frontal view of the cleft running horizontally in the plane of the paper provides such a common framework., a “standard orientation”.

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FIGURE LEGENDS

Figure 1. Active-site subsites. (A) Original Figure 1 with legend reprinted from “On the size of the active site in proteases. I. Papain.”, Vol. 27, No. 2, Israel Schechter and Arie Berger, Copyright (1967), with permission from Elsevier. (B) (Left) Plastic three-dimensional model of a metallopeptidase (cyan ellipsoid) showing the active-site cleft with the metal ion at its bottom center (magenta sphere). The enzyme subsites flanking the ion are labeled according to (A). (Center) model of a peptidic substrate in extended conformation depicting the side chains on both sides of the scissile bond labeled as in (A). (Right) Model of the Michaelis complex poised for reaction. The side chains of the peptide substrate reside in the corresponding enzyme subsites. Scissors pinpoint the cleavage point. (C) Detail of the active-site cleft of human matrix metalloproteinase 8 superimposed with its Connolly solid surface. The cyan surface earmarks the position of the catalytic zinc ion. A stick model of a substrate, which was prepared on the basis of experimental inhibitor structures [39, 40], highlights subsites S_3 - S_2' of the enzyme. A curled arrow points to the scissile bond.

Figure 2. Classification of MPs. Structure-based tree depicting a possible subdivision of the metallopeptidase class of enzymes. Four hierarchy levels are proposed: class, tribe, clan, and family. Consensus motifs containing amino acids participating in metal binding, substrate binding, and /or catalysis are shown for some groups.

Figure 3. Standard orientation (I). Ribbon-plots in stereo of selected structural prototypes of metzincins, gluzincins, and aspzincins are shown in standard orientation. (A) *Methanosarcina acetivorans* ulilysin (PDB 2CKI; [41]); (B) *Bacillus thermoproteolyticus* thermolysin (BtTL) in complex with Z-Phe^P-Leu-Ala (PDB 4TMN; [25]); (C) *Trypanosoma cruzi* TcMCP-1 (PDB ; [42]); and (D) *Grifola frondosa* peptidyl-Lys MP (GfMEP; PDB 1G12; [29]).

Figure 4. Standard orientation (II). Ribbon-plots in stereo of selected structural prototypes of S2P-zincins, inverzincins, and $\alpha\beta\alpha$ -exopeptidases are shown in standard orientation. (A) *Methanocaldococcus jannaschii* site-2 protease (S2P-MP; PDB 3B4R; [30]); (B) *Escherichia coli* pitrilysin (PDB 1Q2L; [31]); (C) *Staphylococcus aureus* HmrA in complex with glycerol (PDB 3RAM; [43]); and (D) human carboxypeptidase A4 in complex with a cleaved hexapeptide (PDB 2PCU; [6]). (E) Topology and connectivity of the central eight-stranded β -sheet in $\alpha\beta\alpha$ -exopeptidases (left) and α/β -hydrolases (right).

Fig.1

Hierarchy level

Fig.2

Fig.4