Multiple architectures and mechanisms of latency in metallopeptidase zymogens

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ABSTRACT

Metallopeptidases cleave polypeptides bound in the active-site cleft of catalytic domains through a general base/acid-mechanism. This involves a solvent molecule bound to a catalytic zinc and general regulation of the mechanism through zymogen-based latency. Sixty reported structures from eleven metallopeptidase families reveal that pro-segments, mostly N-terminally of the catalytic domain, block the cleft regardless of their size. Pro-segments may be peptides (5-14 residues), which are only structured within the zymogens, or large moieties (<227 residues) of one or two folded domains. While some pro-segments globally shield the catalytic domain through a few contacts, others specifically run across the cleft in the same or opposite direction of a substrate, making numerous interactions. Some pro-segments block the zinc by replacing the solvent with particular side chains, others use terminal α -amino or carboxylate groups. Overall, metallopeptidase zymogens employ disparate mechanisms that diverge even within families, which supports that latency is less conserved than catalysis.

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1. INTRODUCTION

Proteolytic enzymes cleave proteins and peptides by hydrolyzing peptide bonds, and “peptidases” is the general term that encompasses proteases, proteinases and peptide hydrolases ¹. Peptidases are found across the kingdoms of life participating in all major biological processes. They function as indiscriminate degraders during digestion and catabolism of proteins and during tissue turnover associated with wound healing, embryogenic development, menstruation and pregnancy ². They also exert fine-tuned regulation through the cleavage of a single or a few bonds, dubbed “limited proteolysis” ^{2,3}. This is a post-translational modification observed during removal of signal peptides; activation or inactivation of enzymes, proteins and bioactive peptides, as found in the blood coagulation and fibrinolysis cascades; cleavage of inactive polypeptides to liberate competent individual domains; assembly of multimeric quaternary structures such as collagen fibers, fibrin clots and viral capsids; and shedding of protein ectodomains from the membrane-bound precursors ^{2,4-8}.

Seven chemical classes of peptidases have been described, among them the metallopeptidases (MPs)¹. These are divided into metalloendopeptidases, which cleave in the middle of peptides, and metalloexopeptidases, which cleave at peptide termini and are divided into aminopeptidases and carboxypeptidases among others. MPs can be mono- or bimetallic, thus binding one or two catalyst metals, which are usually zinc cations. In monometallic MPs, the zinc is mostly tetrahedrally liganded by three atoms from protein side chains and a catalytic solvent molecule at the bottom of an active-site cleft. The solvent is further linked to a general base/acid, which is generally a glutamate. The peptidic substrate is bound in the cleft, so that residue side chains upstream of the scissile bond nestle into non-primed cleft sub-sites S₁, S₂, S₃, etc. and those downstream of the bond into primed cleft sub-sites S₁['], S₂['], S₃['], etc.^{9,10}. In general, S₁['] is the main driver of specificity in MPs¹⁰. Subsequently, the general base/acid polarizes and poises the solvent for nucleophilic attack on the scissile bond carbonyl, which results in a tetrahedral reaction intermediate and subsequent peptide bond cleavage (see Scheme 1, I-IV;¹¹⁻¹⁴). This mechanism explains why mutants, in which the glutamate is replaced with alanine or glutamine, exhibit only residual activity and, therefore, have often been to prevent autolysis during structural studies (see Table 1).

Scheme 1 – Consensus catalytic mechanism of monometallic MPs. In the unbound enzyme (I), the catalytic metal ion, generally a zinc (grey sphere), binds the catalytic solvent molecule, which is further bound by the general base/acid, mostly a glutamate, in the active site. Upon substrate binding (II), the polarized solvent molecule attacks the carbonyl group of the scissile bond (Ⓢ), which leads to a tetrahedral reaction intermediate (III). The reaction results in bond breakage and double proton transfer to the newly formed α-amino group to give rise to a double-product complex with an upstream (left) and a downstream product (right) (IV), which eventually fall apart to start a new round of catalysis.

Monometallic MPs include the peptidase tribes of the αβα-exopeptidases; the LAS MPs (from LytM/lysostaphins, D-alanine-D-alanine MPs and sonic hedgehog, see^{15,16}); and the zincins, among others (Scheme 2 and¹³). The αβα-exopeptidases encompass the clan of the funnelins, which englobes metallocarboxypeptidases that use a general base/acid glutamate and bind the zinc through a histidine and a glutamate within a short HXXE motif (amino-acid one-letter code;

Scheme 2 — Classification of mononuclear MPs of reported zymogenic structures. Within the MP class of enzymes, mononuclear MPs are a subclass that contains several tribes, including the zincins, LAS MPs and αβα-exopeptidases. Tribes subdivide into clans and these into families. Catalytic zinc-binding residues are in green, general base/acid residues in magenta, and residues occupying the position of the Met-turn or Ser/Gly-turn beneath the metal site in zincins¹³ are in orange (within gluzincins, Z=A/F/S/G/T). Other residues engaged in substrate or product binding, stabilization of the reaction intermediate, and/or catalysis are further shown in black. X stands for any residue. MEROPS nomenclature is provided in blue when it matches the groups of the present classification. The clan aspzincins so far contains a single homonymous family. See also¹³ and references therein.

X stands for any residue) *plus* a second downstream histidine. LAS MPs bind the zinc through a histidine and an aspartate from one motif (HX₃₋₁₂D) *plus* a second histidine from a second downstream motif (EXXH or HXH). In these MPs, the general base/acid is proposed to be either glutamate or histidine from this second motif, although a further histidine may likewise be involved¹⁶. Finally, zincins contain the short motif HEXXH within an “active-site helix”, which encompasses two zinc-binding histidines *plus* the general base/acid glutamate¹⁷. Zincins split into three clans: metzincins^{13,17-20}, gluzincins^{13,21,22} and aspzincins^{13,23,24}. These are characterized by respective extended zinc-binding motifs and/or additional downstream motifs, which provide a histidine, glutamate or aspartate as third zinc ligand (Scheme 2).

2. REGULATION OF METALLOPEPTIDASE ACTIVITY

Due to the irreversibility of peptide bond cleavage by MPs *in vivo*—exceptions have been reported *in vitro*²⁵—these enzymes must be exquisitely regulated to prevent aberrant activity, which commonly leads to pathology. Control of MPs is achieved through transcriptional regulation²⁶, cellular compartmentalization²⁷ and specific or broad-spectrum protein inhibitors²⁸, among which multi-domain α_2 -macroglobulins block prey peptidases through pan-peptidase Venus-flytrap or snap-trap mechanisms²⁹⁻³². Other inhibitor examples are the tissue inhibitors of metalloproteinases^{33,34}, the bacterial bifunctional serine- and metalloproteinase inhibitor sermetstatin³⁵, the insect metallopeptidase inhibitor²⁵, and several metallocarboxypeptidase inhibitors^{21,36}. Some MPs are regulated by the transit between non-functional closed conformations with occluded active sites and competent open conformations through hinge motions. This is the case for cowrans within the $\alpha\beta$ -exopeptidases and other metallocarboxypeptidases^{21,37}, which are grouped into the M32 family of the MEROPS database of peptidases and inhibitors (www.ebi.ac.uk/merops,³⁸; MP families are characterized by the prefix “M,” see Scheme 2 for families described in this study). Insulinase and other inverzincins (M16) behave similarly³⁹. An alternative regulatory mechanism is self-compartmentalization into large multimeric hollow particles, which contain the active sites inside, and thus restrict activity to substrates entering through dedicated openings or channels. This is observed in bimetallic zinc MPs such as the tetrahedral dodecameric ApAp-subfamily of archaeal metalloaminopeptidases (M42;⁴⁰) and barrel-shaped decameric *Bacillus subtilis* D-aminopeptidase DppA (M55;⁴¹). Finally, MP activity may be regulated through latency exerted by zymogens^{2,42,43}.

3. SYNTHESIS AND ACTIVATION OF ZYMOGENS

Most MPs are synthesized as inactive or latent zymogens with a pro-segment, which is either a protein domain or a peptide. The pro-segment is removed during activation within the subcellular compartment or the extracellular space in which the mature catalytic domain performs its physiological function². Pro-segments are usually located at the N-terminus of the catalytic domains to prevent activity before the zymogen is fully synthesized, although exceptions are found, e.g. within the minigluzincins (see section 11 and Scheme 2;^{44,45}). In general, the catalytic domains are already preformed or in a close-to-competent conformation in the zymogens, so latency is mainly exerted by sterically blocking access of substrates to the active site.

In some instances, the pro-segment is a short peptide, which does not fold independently of the catalytic domain. It only blocks the active site until maturation and is discarded once cleaved. Examples are found in the astacin family (section 7;⁴⁶). In other instances, pro-segments are large and fold into separate domains, which once cleaved may still inhibit their cognate MPs *in trans*. They often also contribute to folding, thus chaperoning the downstream mature catalytic domain, to stability and intracellular sorting of the zymogen and to binding of other proteins^{4,42,47-51}. Examples of such large pro-segments include funnelin metallocarboxypeptidases (section 4;^{50,52,53}); LytM/lysostaphin/LasA-MPs (section 5;⁵⁴) within LAS MPs; several metzincin families, e.g. the adamalysins/ADAMs (M12B;⁵⁵), matrix metalloproteinases (MMPs; section 6;⁵⁶), fragilysins (section 8;⁵⁷) and thuringilysins (section 9;⁵⁸); thermolysins (section 10;⁵⁹) and fungalysins (section 12;⁶⁰) within gluzincins (Scheme 2); and aspzincins (section 14;²⁴).

Pro-segment removal occurs through single- or multi-step limited proteolysis, which may be autocatalytic and triggered by external reagents, changes in pH or simple incubation at high concentration^{61,62}. Pro-segment cleavage can also be heterolytic through other peptidases, as described for pro-MMPs⁶³. Some zymogens are activated through association with proteins, which induce allosteric changes that liberate activity without removal of the pro-segment. Examples include pro-MMP-2 and pro-MMP-9⁶⁴⁻⁶⁶, as well as members of the anthrax lethal factor-type MPs (section 13). A case apart is selease, a minimal MP that exhibits reversible concentration-dependent latency (section 11;⁴⁴). Yet other zymogens—such as those of some MMPs—are activated by chemicals that react with pro-segment residues and thus release the active-site cleft without pro-segment cleavage⁶⁷.

Currently, three-dimensional structures of MP zymogens or zymogenic complexes are available for monometallic enzymes only, and the structural determinants of their mechanisms of latency are discussed hereafter (see also Schemes 2 and 3 *plus* Table 1).

4. A/B-TYPE FUNNELINS

Funnelins—*alias* M14 metallocarboxypeptidases—include the A/B-type (M14A) and E/D-type (M14B) members, which are mainly extracellular enzymes from animals^{21,36,50,52,68,69}. They are engaged in the digestion of proteins and peptides but also in pro-hormone and neuropeptide processing and in several other roles in different mammalian extra-pancreatic tissues and fluids⁶⁹. One protein was reported from the bacterium *Lysinibacillus sphaericus*, which is involved in the metabolism of the bacterial cell wall (M14C). More recently, the intracellular cytosolic carboxypeptidases (M14D) were discovered in mammals and classified into a distinct group^{70,71}. Porcine pancreatic pro-carboxypeptidase B1 was the

first MP, for which the zymogen structure was solved in 1991 ⁷², and to date sixteen more pro-funnelin structures have been published, all from A/B-type zymogens (Table 1). As to the cytosolic carboxypeptidases, the structure of an inactive potential orthologue from *Pseudomonas aeruginosa* was reported ⁷³, which likely is an active form whose substrate has not yet been determined, rather than a zymogen.

As exemplified by prototypic human pro-carboxypeptidase A4 ⁷⁴, A/B-type zymogens consist of highly similar ~310-residue catalytic domains of α_8/β_8 topology and α/β -hydrolase fold (Scheme 3A; ^{75,76}). Framed by loops connecting the core helices and strands, these domains have a compact globular shape and a funnel-like opening, at whose base the active-site cleft resides ²¹. The catalytic domains, which are already in an active conformation in the zymogen, are preceded by an N-terminal pro-segment of 90-95 residues, which inhibits the cognate mature catalytic domain *in trans* in several family members ^{50,52,53}. The pro-segment consists of an ~80-residue globular part and a C-terminal mostly helical connecting segment. Zymogen activation occurs stepwise through limited proteolysis at the end of this segment in a region that is α -helical in A-type zymogens, but not in B-type. As peptidases normally cleave substrates in extended conformation ⁷⁷, this entails that the polypeptide must unwind before the cut. After cleavage, the pro-segment is released and degraded *in vivo*.

Scheme 3 — Structures of prototypic MP zymogens (I). Normally, MP structures are best displayed following the established standard orientation, which provides a frontal view of the horizontally-aligned active-site cleft ¹⁰. However, given that pro-segments block the cleft mostly frontally, specific orientations for each zymogen were chosen here and in Schemes 4 and 5 to provide the best view into the distinct latency mechanisms. The pro-segments are shown in sandy brown, the catalytic domains in aquamarine, the catalytic zincs as magenta spheres, the zinc-binding residues and the general base/acid residues as yellow sticks, and selected relevant pro-segment and catalytic-domain residues as red sticks. The N- and C-termini of the zymogens are labelled. Non-catalytic structural calcium and zinc ions are depicted as dark red and magenta spheres, respectively, within catalytic domains only for clarity. Flexible segments connecting the pro-segments with the catalytic domains are pinpointed by dotted lines. For PDB access codes of the structures and references, see Table 1. **(A)** Human pro-carboxypeptidase A4. **(B)** *Klebsiella pneumoniae* pro-MtfA. **(C)** *Staphylococcus aureus* pro-LytM. **(D)** *Helicobacter pylori* pro-Csd3. The catalytic domain is inserted into an intermediate domain (in white) downstream of the pro-segment. **(E)** Human pro-MMP-2. The three fibronectin-type-II domains inserted within the catalytic domain are shown in white. **(F)** *Tannerella forsythia* pro-karilysin.

The globular part of the pro-segment is an open-sandwich with a twisted four-stranded antiparallel β -sheet, packed through its face distal to the enzyme against two nearly parallel α -helices. These are inserted between the first two and the last two strands, respectively, and their axes roughly parallel the sheet β -strands. The pro-segment covers, but not completely shields, the catalytic domain through a large interface, which is created by a β -ribbon from the pro-segment made up of the second and third sheet strands and by catalytic-domain funnel-rim loops. The pro-segment prevents

substrate access but does not approach the active site, and only few direct contacts are made with the catalytic domain⁷⁴. These include a conserved electrostatic interaction between a pro-segment aspartate/asparagine and an active-site proximal arginine, and hydrophobic contact of a pro-segment tryptophan with a catalytic-domain phenylalanine/tyrosine (Scheme 3A). Most B-type zymogens contain an additional salt bridge between a pro-segment aspartate and a catalytic-domain arginine. This contributes to a tighter inhibitory complex and explains why B-type zymogens are inert, while A-type zymogens have residual activity in front of small substrates⁶⁹. All these interactions are missing in what is probably the physiologically most relevant A/B-type metallocarboxypeptidase due to its key role in blood coagulation, *viz.* thrombin activatable fibrinolysis inhibitor. This explains why this zymogen is active even against larger peptides^{78,79}.

5. LYTM/LYSOSTAPHIN/LASA-METALLOPEPTIDASES

LAS enzymes (families M15 and M23) are a disparate group of peptidases engaged in turnover of the bacterial cell envelope¹⁵. Their catalytic domains contain a four-stranded antiparallel β -sheet of conserved topology and connectivity, which provides the zinc ligands, within otherwise diverging overall architectures (Scheme 3C,D). Within these enzymes, extracellular *Staphylococcus aureus* LytM is a representative of the LytM/lysostaphin/LasA-MPs found in bacteriophages, both Gram-positive and Gram-negative bacteria, and the malaria mosquito, *Anopheles gambiae*⁵⁴. In addition to LytM¹⁶, structurally-characterized mature proteins include *Staphylococcus simulans* lysostaphin⁸⁰, *Pseudomonas aeruginosa* LasA⁸¹ and *Escherichia coli* MepA⁸².

The structure of prototypic pro-LytM⁵⁴ reveals an N-terminal pro-segment of ~160 residues connected through a flexible C-terminal segment with the already preformed ~130-residue catalytic domain. The pro-segment consists of a core with a four-stranded antiparallel β -sheet *plus* a short helix, which is loosely attached to the catalytic domain far from the active-site cleft, and a second small bihelical core. These two moieties are connected by a coiled segment that enters the active-site cleft, so that asparagine N¹¹⁷ blocks activity by binding the catalytic zinc in replacement of the catalytic solvent (Scheme 3C). Consistently, removal of the pro-segment yielded activity *in vitro*⁵⁴. This latency mechanism was termed the “asparagine switch”⁵⁴ by analogy with the “cysteine switch” of MMPs (see section 6).

Due to the poor conservation of the asparagine in LytM/lysostaphin/LasA-MPs, diverse residues likely take over its role in other family members, so that the latency mechanism would rather correspond to a “zinc-ligand switch”⁵⁴. Indeed, *Helicobacter pylori* Csd3, which shares the catalytic domain core with LytM, has a different ~95-residue pro-segment with a five-stranded antiparallel β -sheet and three short α -helices inserted between the first two strands *plus* an extra ~100-residue downstream domain, into which the catalytic domain is inserted (Scheme 3D and Table 1). Here, the pro-segment provides the scaffold for a zinc-blocking glutamate (E⁷⁴) inserted within the third helix⁸³. A similar three-domain architecture to Csd3 was found in proteins NMB0315 from *Neisseria meningitidis*⁸⁴ and VC0503 from *Vibrio cholera*⁸⁵, in which segments coming from an N-terminal domain likewise enter the active-site cleft. However, they do not disturb the zinc coordination sphere, which is potentially functional. Thus, these two forms could be zymogens⁸³, but also active enzymes with short-substrate specificities or exopeptidase activity. Finally, large variability among LytM/lysostaphins/LasA-MPs further implies that not even zymogenicity is conserved: some members lack a pro-segment and are active as full-length proteins, as shown for *Staphylococcus aureus* LytU⁸⁶.

6. MATRIX METALLOPROTEINASES

The zymogens of the MMP family were the first to be structurally analyzed within the metzincin clan of MPs (Table 1; ⁸⁷). MMPs are mostly secreted enzymes and broadly process extracellular matrix components, but they also shed membrane-bound substrates and activate other peptidases through limited proteolysis^{8,88}. They are involved in chronic inflammatory diseases and degrade basement membrane components during several pathologies, including cancer and metastasis processes⁷. There are 23 MMP paralogs in humans, which mostly consist of several modules: an ~80-residue pro-segment; a ~165-residue zinc- and calcium-dependent catalytic domain; a linker region; a ~200-residue hemopexin-like domain with binding functions; and other C-terminal domains⁸⁷. Human MMPs are kept latent through a pro-segment, which is generally not required for (re)folding of the downstream catalytic domain⁸⁹.

To date, sixteen zymogen structures have been reported for human MMP-1, -2, -3, -7 and -9, and bacterial karilysin from *Tannerella forsythia*, an oral-microbiome member of several animal hosts (Table 1). By comparison with mature forms, the human zymogen structures reveal that the catalytic domains are already in competent conformations in the zymogens⁸⁷. Like other metzincins catalytic domains, which generally span 132-309 residues^{13,18,58,90}, MMPs consist of an upper and a lower sub-domain separated by a central active-site cleft. The upper sub-domain contains a “backing helix”, an active-site helix, and a mostly five-stranded β -sheet, in which all strands except the lowermost are parallel to a substrate bound in the cleft. The latter strand is antiparallel, forms the cleft’s “upper rim” and binds substrates through β -ribbon-like interactions between the respective main chains. The lower sub-domain includes a conserved methionine-containing “Met-turn” immediately beneath the catalytic cation and a “C-terminal helix” (see Scheme 3E,F and ⁸⁷). Uniquely, the catalytic domains of MMP-2 and MMP-9 have three fibronectin type-II-related domains inserted between the fourth strand of the β -sheet and the active-site helix (Scheme 3E). The latter helix and the downstream segment, which leads to the interface between the sub-domains, contain the residues of the extended zinc-binding sequence characteristic for metzincins, HEXXHXXGXXH/D (Scheme 2). The architecture of human MMP catalytic domains is shared with that of bacterial karilysin (Scheme 3F), which is probably the result of horizontal gene transfer from an animal host to *T. forsythia*⁹¹.

Structurally characterized MMP pro-segments from mammals comprise 66-91 residues⁸⁷ and frontally shield the active-site clefts. As exemplified by pro-MMP-2 (Scheme 3E; ⁹²), the 80-residue pro-segment has an extended N-terminus that is initially flexible and thereafter interacts with the third fibronectin-type-II insertion of the catalytic domain before shaping a globular moiety. This moiety has a hydrophobic core made up by side chains from a three-helix bundle, which

forms a scaffold to place the downstream segment in extended conformation to block the active-site cleft. This peptide encompasses a conserved cysteine-switch or “velcro” motif” (PRCGXPD, ⁹³⁻⁹⁵), and runs in the opposite direction to a substrate, so that it cannot produce a productive Michaelis complex. Thus, this is a strategy to prevent autolytic cleavage of the pro-segment ⁴² and it is found also in other MPs (see the sections below). The cysteine of the motif binds the catalytic zinc ion *via* its S γ atom, thus replacing the catalytic solvent molecule and yielding the zymogen inactive. A further key element of the pro-segment structure is an invariant salt bridge between the arginine and the aspartate of the motif. Exceptionally, this element is absent from the motif of pro-MMP-26 (PHCGXXD), which is thus not functional and apparently not involved in the activation mechanisms of this MMP ⁹⁶.

Scheme 4 — Structures of prototypic MP zymogens (II). (A) Enterotoxigenic *Bacteroides fragilis* pro-fragilysin-3. (B) *Astacus astacus* pro-astacin. (C) *Bacillus anthracis* pro-BaInhA2, with the inserted VEG and MAM domains in white and in gray, respectively. (D) *Serratia proteamaculans* pro-protealysin. (E) *Pseudomonas* sp. vibriolysin in complex with its pro-segment in an orientation of its catalytic domain similar to pro-protealysin in (D). The C-terminus of the pro-segment and the N-terminus of the catalytic domain are pinpointed by blue and purple arrows, respectively. Protein domains and features are rendered and colored as described in the legend of Scheme 3.

Activation of pro-MMPs is produced *in vitro* through chemicals that pull out the cysteine from the active-site cleft and give rise to a functional catalytic solvent-zinc pair, but this does not remove the pro-segment. Pro-segment removal is observed during activation through multistep limited proteolysis by trypsin, plasmin, and other MMPs ⁹⁷, for example. Depending on the final activation cleavage reaction, mature MMP catalytic domains have different N-termini. Variants that are most stable and physiologically relevant are obtained by cleavage of an X-F/Y bond (N¹⁰⁹-Y¹¹⁰ in MMP-2), because they can make a salt-bridge between the new N-terminus and an aspartate from the C-terminal helix ⁸⁷. Other MMPs such as the membrane-type variants possess a specific recognition sequence for activation through Golgi-associated Kex-2/furin-like convertases.

Although the cysteine-switch mechanism was derived from mammalian MMPs, sequence analyses revealed that plant MMPs likely also contain a pro-segment with a cysteine-switch motif, which, however, may occasionally diverge from the consensus ^{98,99}. Accordingly, this mechanism of latency seems to be widely conserved across eukaryotic MMPs ⁹⁹. Furthermore, the more distantly related members of the gametolysin family of MPs from green algae, such as unicellular *Chlamydomonas reinhardtii* and multicellular *Volvox carteri*, may likewise operate through a cysteine switch ⁹⁹⁻¹⁰². Finally, similar mechanisms may also occur in other metzincin families with conserved cysteine-containing motifs in their pro-segments, such as the ADAMs/adamalysin (motif PKMCGV), leishmanolysin (motif HRCIHD) and pappalysin families (motif CG) ^{87,103-106}.

A different architecture and, thus, mechanism within MMPs has been described for *T. forsythia* pro-karilysin. This 472-residue protein just contains a 14-residue pro-segment—the shortest N-terminal pro-segment for a structurally analyzed pro-MP—without cysteines upstream of a 161-residue MMP-type catalytic domain and a further 277-residue C-terminal extension unrelated to any domain of eukaryotic MMPs. Latency is exerted by the pro-segment running in an extended conformation across the cleft in the opposite direction to a substrate and acting through an “aspartate-switch” mechanism—first described for astacins (section 7; ¹⁰⁷)—in which aspartate D²⁵ replaces the cysteine of mammalian MMPs and binds the zinc in a monodentate manner (Scheme 3F).

7. ASTACINS

This family encompasses secreted or membrane-bound multi-domain MPs with manifold biological functions across animals. Among others, they are involved in digestion, developmental processes, tissue differentiation and embryonic hatching ⁴⁶. The family’s eponym is the digestive enzyme astacin from the European freshwater crayfish *Astacus astacus* ^{46,108}. Astacins include pro-segments of variable length (from 34 residues in astacin to 483 residues in *Drosophila melanogaster* tolkin), ~200-residue catalytic domains with overall metzincin architecture (see section 6) that are crosslinked by two or three disulfide bonds, and further downstream extracellular domains, trans-membrane anchors, and cytosolic domains ⁴⁶.

The pro-segment of pro-astacin, which does not have an intramolecular chaperone function ⁴⁶, contains an aspartate embedded in a conserved motif, FE/QGDI, which acts as an aspartate switch ¹⁰⁷. To prevent premature autolysis, the pro-segment both runs along the active-site cleft in the opposite direction to a substrate (see also section 6) and adopts a non-cleavable helical conformation on the primed side of the cleft (Scheme 4B). Thereafter, the chain forms a large loop protruding from the surface. This gives rise to a small globular nucleus that poises the aspartate of the pro-segment motif (D^{21P}; ¹⁰⁷) to occupy the position of the catalytic solvent and bidentately bind the catalytic zinc through its side chain. The loop structure is further stabilized by an electrostatic interaction between the glutamate/glutamine of the pro-segment motif and the penultimate residue of the pro-segment, an arginine. The pro-segment runs in extended conformation along the cleft after the aspartate and binds the upper-rim strand. Thereafter, a short β -hairpin includes the final maturation cleavage point, which is buried in the structure. Activation entails stepwise heterologous and autologous limited proteolysis, which makes this bond eventually accessible ^{107,109}.

In contrast to funnelins and MMPs but similarly to trypsin-like serine endopeptidases ^{46,107,108,110}, astacins undergo rearrangement upon activation within an “activation domain”, and the mature N-terminal segment becomes inserted and buried for its first six residues in an internal solvent-filled cavity of the catalytic domain. The N-terminus makes a solvent-mediated salt bridge with a glutamate immediately after the third zinc-binding residue (Scheme 2), the so-called “family specific residue” of astacins ¹⁹. In addition to the residues of the zinc-binding motif of metzincins (see section 1 and Scheme 2), mature unbound astacins display a conserved tyrosine as a fourth proteinaceous zinc ligand (Scheme 4B), which is swung out following a “tyrosine switch” motion upon substrate or inhibitor binding, as well as in the zymogen ¹⁰⁷.

Other reported zymogen structures of the family are those of pro-meprin β and pro-myroilysin (Table 1). The former has a very similar pro-segment to astacin, which is just ten residues longer and partially runs along the front surface of vicinal TRAF domain (found in tumor necrosis factor receptor-associated factors) before covering the catalytic-domain cleft ¹¹¹. The aspartate-switch residue of pro-meprin β is D⁵². Similarly, pro-myroilysin has a 37-residue pro-segment that runs along the molecular surface of the upper sub-domain of the catalytic domain instead of a vicinal domain. Notably, the aspartate-switch aspartate is here replaced by a cysteine (C²⁶; ¹¹²), thus mimicking mammalian MMPs (see section 6).

8. FRAGILYSINS

The fragilysins are a metzincin family restricted to four members: three closely-related isoforms (fragilysin-1, -2 and -3; >95% sequence identity) and metalloproteinase II (~25% identity). They are virulence factors exclusively encoded by a single pathogenicity island of enterotoxigenic *Bacteroides fragilis* and are not found in commensal strains or any other organism. Enterotoxigenic *Bacteroides fragilis* is the most frequent disease-causing anaerobe in the intestinal tract of humans and livestock ¹¹³ and fragilysins cleave E-cadherin, which is crucial for cell-to-cell contacts, thus facilitating bacterial penetration of the intestinal epithelium. This causes abscesses and inflammation and increases the risk of gastrointestinal cancer ¹¹⁴. Pro-fragilysins contain a catalytic domain preceded by a pro-segment, which plays a role in both the latency and folding stability of the catalytic domain ^{57,115}. The latter contains the characteristic structural element of the metzincins (see section 6) and is closest to ADAMs/adamalysins, despite low sequence similarity ⁵⁷. Taken together with the extremely limited distribution of fragilysins, this suggests that the catalytic domain of these MPs may derive from an adamalysin/ADAM xenolog coopted by *B. fragilis*, which colonizes the gut of mammals ⁵⁷.

The 194-residue pro-segment of pro-fragilysin-3 consists of a globular part with a central antiparallel twisted seven-stranded β -sheet, which laterally attaches to the 186-residue catalytic domain on its right-hand side through one face (Scheme 4A). This interface further contains two helices upstream and one helix downstream of the last β -strand. Attached to the opposite face of the sheet is an N-terminal three-stranded mixed β -sheet, whose strands are orthogonal to those of the long sheet, and an extra helix inserted between the first two strands. After this globular part, the pro-segment chain adopts an extended conformation for its C-terminal segment, which runs in the opposite orientation to a substrate and performs an antiparallel β -strand interaction with the upper-rim strand of the catalytic domain on the non-primed side of the cleft. A prominent bulge is found where the pro-segment chain passes over the catalytic zinc. This causes the Y¹⁹¹ and D¹⁹⁴ side chains to penetrate the catalytic moiety and the latter to coordinate the catalytic zinc ion from the top in a bidentate manner in substitution of the catalytic solvent ⁵⁷. Accordingly, the mechanism of latency in fragilysins follows an aspartate

switch as described for astacins (section 7) and karilysin (section 6). Eight residues of the pro-segment/catalytic domain linker are flexible in pro-fragilysin-3, which likely facilitates proteolytic activation to yield the mature MP starting at A²¹². This zymogenic mechanism was confirmed by the very similar structure of pro-metalloproteinase II¹¹⁶.

9. THURINGILYSINS

The name of this bacterial metzincin family was dubbed based on immune inhibitor A1 (InhA1) peptidase from *Bacillus thuringiensis*¹⁹, which was reported in the 1970s to interfere with the humoral defense system of saturniid insects by degrading antibacterial host proteins upon infection¹¹⁷. Close orthologs and paralogs, which include InhA1, InhA2 and InhA3 variants, are found within bacteria of the *Bacillus cereus* group, and more distant relatives are the PrtV peptidases from several *Vibrio* species. In particular, *Bacillus anthracis* produces BaInhA2 and BaInhA1, which modulates the secretome of the causal agent of anthrax¹¹⁸.

The only zymogenic thuringilysin structure reported to date is that of pro-BaInhA2. The secreted protein has a 767-residue four-domain architecture containing a 309-residue catalytic domain, the longest for a metzincin (see section 6). It is preceded by an 84-residue pro-segment, which wraps around and interacts with the catalytic domain through a large surface (Scheme 4C). A VEG domain (found in viral envelope glycoproteins) and a MAM domain (reported from meprin α and β , A5 protein, and receptor-type tyrosine-protein phosphatase μ) grafted into the latter are found downstream of the catalytic domain (Scheme 4C;⁵⁸).

The first 18 residues of the pro-segment of pro-BaInhA2 are cleaved off after protein synthesis to yield a functional zymogen, which starts at residue N⁵⁰ and binds to the primed side of the active-site cleft like a downstream reaction product (see Scheme 1, IV). Its deprotonated α -amino group ligands the catalytic zinc ion instead of the catalytic solvent, and its side chain points into cleft sub-site S₁'. Thereafter, the pro-segment runs in extended conformation for its first four residues along the cleft and shapes a small globular moiety containing two perpendicular helices. This moiety is attached to the right side of the catalytic domain, and then passes across the non-primed side of the cleft and forms a third helix at its leftmost end (Scheme 4C). This helix runs downward along the left catalytic domain surface and leads to the final maturation cleavage point (E¹³³-Q¹³⁴) in the lower back part of the molecule, on top of the VEG domain. Overall, this mechanism of latency maintenance in MPs through the pro-segment N-terminus is probably shared with other thuringilysins, including more distant relatives from *Vibrio* sp.⁵⁸.

10. THERMOLYSINS

Thermolysins are found across Gram-positive and -negative bacteria, as well as in archaea and fungi, and they have high biotechnological interest. As they are produced as virulence factors by several pathogens, they have also been identified as promising drug targets¹¹⁹. Thermolysins are the structurally most extensively characterized MPs and belong to the gluzincin clan^{13,21,22,120}. Gluzincins share with metzincins the gross architecture of the upper sub-domain of the catalytic domain, including an at least three-stranded β -sheet with an antiparallel upper-rim strand, the backing helix and the active-site helix with the short HEXXH zinc-binding motif. However, they differ in the lower sub-domains, which contain five helices in thermolysins¹²⁰ and include a characteristic third zinc-liganding glutamate within a “glutamate helix” that runs below and oblique to the active-site helix in an antiparallel manner (Scheme 4D,E). Thermolysins split into a majority with large pro-segments spanning ~160-260 residues, which are intramolecular chaperones, inhibit cognate mature peptidases *in trans* and influence their secretion; and a minority with short pro-segments of ~50-60 residues^{47,59,121}. In the former, the catalytic domains encompass ~320 residues and contain structural calcium ions; in the latter, the catalytic domains span ~290 residues, have extended primed sub-sites and lack calcium ions. The zymogen structure of one representative of each group has been published (see Table 1).

Pro-protealysin from *Serratia proteomaculans* has a short 50-residue pro-segment, which forms a separate moiety of two antiparallel helices that is placed on top of the primed-side of the active-site cleft (Scheme 4D;¹²²). From this scaffold, an N-terminal segment runs like a substrate in extended conformation above the catalytic zinc—but does not contact the metal—and binds the upper-rim strand on the non-primed side. Inspection of the flexible junction between the pro-segment and the catalytic domain (Scheme 4D) suggests that activation cleavage and pro-segment removal should not cause significant rearrangement of the mature N-terminus.

Vibriolysin from *Pseudoalteromonas* sp., which was reported in a zymogenic complex obtained by autolysis, has a long 180-residue pro-segment laterally attached through a large interface to the lower sub-domain of the catalytic domain (Scheme 4E;⁵⁹). The pro-segment is hinge-shaped and consists of two domains: an FTP domain, as found in fungilysin and thermolysin pro-peptides; and a PepSY domain, present in peptidase pro-peptides and *Bacillus subtilis* YpeB sporulation protein. FTP is an α/β -sandwich reminiscent of cystatins (cysteine peptidase inhibitors) and ferredoxins, which comprises a twisted antiparallel five-stranded β -sheet, whose concave side accommodates two consecutive helices inserted between the first two sheet strands. Perpendicular to FTP and connected by a linker containing a short helix, downstream PepSY has a similar architecture to FTP, but the sheet is four-stranded. PepSY makes fewer interactions with the catalytic domain than FTP—mainly through the loop connecting the two central strands of the sheet. It serves as a scaffold for the introduction of the last three pro-segment residues into the non-primed side of the active-site cleft, where they occupy sub-sites S₃-S₁ and block them for substrate binding. The main determinant of inhibition is the C-terminal carboxylate of the last pro-segment residue, H²⁰⁴, which replaces the catalytic solvent molecule in the zinc-coordination sphere in a monodentate manner, similarly to an upstream cleavage product (see Scheme 1, IV and Scheme 4E).

The catalytic domain in the zymogenic complex is in overall competent conformation, as revealed by comparison with isolated mature vibriolysin⁵⁹. The N-terminal residue A²⁰⁵ is ~32 Å apart from H²⁰⁴, which indicates that autolysis of the intact zymogen to yield the zymogenic complex caused large conformational rearrangement under

displacement of the mature N-terminus out of the cleft (see Scheme 2). The zymogen, of unknown structure, is likely in a high-energy state, so that activation may lead to a relaxed conformation of high thermal stability⁵⁹. The first eight N-terminal residues of the mature catalytic domain form a β -strand, which contributes to a three-stranded β -sheet within the upper sub-domain that is part of the ~ 30 -residue segment absent from thermolysins with short pro-segments (¹²² and Scheme 4D,E). This sheet is also found in the structure of mature family prototype, thermolysin of *Bacillus thermoproteolyticus* (Protein Data Bank access code [PDB] 4TLN; ¹²⁰) and the other mature structures reported to date ¹²¹, which are all forms with long pro-segments. Thus, the rearrangement of the mature N-terminus may be a general mechanism for these thermolysins. Overall, the latency mechanisms of thermolysins with short and long pro-segments are markedly different, which is striking given the close similarity of the downstream catalytic domains. Moreover, some species encode both types of thermolysins, e.g. *Streptomyces avermitilis*, *Streptomyces coelicolor*, *Xanthomonas axonopodis* and *Methanosarcina acetivorans* ¹²¹.

11. MINIGLUZINCINS

With only ~ 105 residues, members of this family are exclusively present in thermophilic prokaryotes. They have the minimal scaffold required for MP activity and contain a glutamate helix, as in gluzincins. However, the zinc-binding glutamate is here replaced by a histidine (Scheme 2 and ⁴⁵). Among minigluzincins, 110-residue selease from *Methanocaldococcus jannaschii* selectively cleaved casein at a single site ⁴⁴. Uniquely for peptidases, this MP showed vanishing enzymatic activity when its concentration increased. This owed to oligomerization as proven by structural and biophysical studies. Only the monomer was active, and dimers, tetramers and octamers corresponded to self-inhibiting latent species. Even more strikingly, inactive concentrated protein reversibly became active by simple dilution with buffer, for inactive oligomers dissociated to active monomers. Two more minigluzincins, abylysin from *Pyrococcus abyssi* and jannalysin from *M. jannaschii*, were exclusively isolated as zymogens and solved for their crystal structures ⁴⁵.

The structure of 105-residue pro-abilylsin shows the overall competent moiety of an MP catalytic domain, including a zinc site and active-site cleft with reasonable geometry. It contains the common structural elements of competent gluzincins: an upper sub-domain consisting of a three-stranded β -sheet with an upper-rim strand, a backing helix and an active-site helix with the HEXXH motif; and a lower sub-domain with a glutamate helix and a C-terminal helix (Scheme 5B). The latter is followed by a segment in extended conformation from Q¹⁰¹ onwards, which runs across the primed side of the cleft in reverse orientation to a substrate. It interacts with the upper-rim strand and binds the zinc through the C-terminal carboxylate of V¹⁰⁵ in a monodentate fashion. Accordingly, latency is here uniquely exerted by a C-terminal pro-segment of five residues.

In contrast, 110-residue pro-jannalysin, with a practically overlapping polypeptide chain with pro-abilylsin (see Fig. 3B in ⁴⁵), lacks the C-terminal extension and is N-terminally elongated instead. This segment, however, does not account for latency. This is exerted by the last residue of the C-terminal helix (I¹¹⁰), which projects away from the molecular body and approaches the zinc-binding site—though not the metal—of a neighboring molecule through its C-terminal carboxylate, thus blocking the primed side of the cleft *in trans* (Scheme 5C). Accordingly, consistent with studies in solution, pro-jannalysin is a homodimer, in which latency is symmetrically exerted crosswise through respective C-terminal α -helices. This accounts for a different mechanism of latency than in proabilylsin despite substantial sequence identity among the two proteins (31%).

Strikingly, yet other mechanisms were found for selease ⁴⁴. Consistently with an active species, monomeric selease is in a competent and functional conformation, which is very similar to the catalytic domains of pro-jannalysin and pro-abilylsin, except that the active-site cleft is not blocked. In contrast, in dimeric selease latency is symmetrically exerted through major rearrangement of the metal-binding sites, so that the third zinc-binding histidine (H⁸⁰) is pulled out of the active site. This ligand is replaced by H¹⁰⁷ from the C-terminal helix of the symmetric molecule, thus yielding an incompetent active site (compare Scheme 5B,C with Scheme 5D). Furthermore, two point mutants of selease show yet diverse dimeric quaternary structures, and thus zymogenic mechanisms ⁴⁴. Finally, the structure of tetrameric selease reveals that the glutamate helix and the preceding segment undergo drastic conformational rearrangement to give rise to a canonical β -ribbon structure, which removes the third histidine from the zinc coordination sphere. Four such monomers assemble to yield an almost spherical self-inhibitory particle of ~ 70 Å in diameter, in which the distorted active sites are in the particle lumen. Thus, selease represents a unique case of metamorphic protein that reversibly exerts zymogenicity through oligomerization mediated by molecular plasticity. It has the capacity to adopt several different structures, which are all dysfunctional except for one ⁴⁴.

12. FUNGALYSINS

The archetype of this family of the gluzincin clan (Scheme 1) is *Aspergillus fumigatus* fungalysin *alias* AfuMep, a secreted 42-kDa MP that degrades extracellular matrix proteins and is considered a virulence factor in fungal diseases ¹²³. Pro-fungalysin contains a pro-segment of 227 residues, which is the longest for a structurally-characterized MP zymogen, followed by a 389-residue catalytic domain. Similar to vibriolysin (see section 10), the structure ¹²⁴ corresponds to an autolytic cleavage product, a zymogenic complex in which the last residue of the pro-segment (E²⁴⁵) is ~ 31 Å apart from the N-terminus of the catalytic domain. Also like in vibriolysin, the N-terminal segment of the catalytic domain is engaged in a small β -sheet (Scheme 5A) and the rest of the catalytic domain evinces most of the structural elements characteristic of thermolysins with long pro-segments. This includes helices and strands of the upper sub-domain and the five helices of the lower sub-domain, as well as a preference to cleave before hydrophobic residues ¹²⁴.

Scheme 5 — Structures of prototypic MP zymogens (III). (A) *Aspergillus fumigatus* fungalysin in complex with its pro-segment. The C-terminus of the pro-segment and the N-terminus of the catalytic domain are pinpointed by blue and purple arrows, respectively. (B) Monomeric *Pyrococcus abyssi* pro-abylysin. (C) Dimeric pro-jannalysin from *Methanocaldococcus jannaschii*. Zymogenicity is symmetrically exerted within a dimer by the C-terminal helix of one molecule acting as the pro-segment (in sandy brown) and the catalytic domain of the other molecule (in aquamarine). The rest of the dimer is depicted in white for clarity. (D) Dimeric latent selecase from *M. jannaschii*. For coloring, see (C). (E) *Aeromonas salmonicida* pro-AsaP1. Protein domains and features are rendered and colored as in Scheme 3.

The fungalysin pro-segment consists of two α/β -sandwiches, which are very similar to FTP and PepSY from the vibriolysin pro-segment, connected by a linker likewise encompassing a short helix. They are arranged with respect to the catalytic domain similarly to the MP from *Vibrio*, merely the C-terminal sandwich of fungalysin lacks one of the helices of PepSY and shows two extra β -strands. Its C-terminus likewise penetrates the active-site cleft, here for its four last residues, so that E²⁴⁵ binds the catalytic zinc ion through its C-terminal carboxylate in a monodentate manner, similarly to an upstream reaction product (see Scheme 1, IV). Accordingly, the architectures of the catalytic domains and the latency mechanisms of fungalysins from fungi and thermolysins with large pro-segments from bacteria are equivalent, which suggests that fungalysins may be grouped with thermolysins. Both families likely share a common origin, with one family potentially resulting from horizontal gene transfer from the other, followed by subsequent spreading within a distinct kingdom.

13. ANTHRAX LETHAL FACTOR-TYPE METALLOPEPTIDASES

The archetypal domain of this family is the catalytic domain (residues P⁵⁵¹-I⁷⁷³) of multi-domain *B. anthracis* lethal factor (PDB 1J7N; ¹²⁵), which is essential for the pathogenesis of anthrax ¹²⁵. Lethal factor specifically cleaves mitogen-activated protein kinase kinase family members, which causes fatal inhibition of several cellular signaling pathways ¹²⁵. The mature lethal factor structure conforms to the architecture of gluzincins and includes a glutamate-helix with a glutamate as the third protein ligand of the catalytic zinc (see section 10 and Scheme 2).

Among the several structural relatives of the lethal factor catalytic domain is the 265-residue bacterial protein MtfA (from Mlc titration factor A), which participates in the control of the glucose-phosphotransferase sensory and regulatory system by inactivating transcriptional repressor Mlc (from makes large colonies; ^{126,127}). The structure of an incompetent full-length variant of MtfA from *Klebsiella pneumoniae* with only poor aminopeptidase activity reveals that the upper-rim strand and the preceding segment within the upper sub-domain β -sheet found in lethal factor are replaced by a helical peptide (G¹⁰⁹-Q¹¹⁸) flanked by ten- and five-residue flexible segments, respectively. The helical peptide covers

the non-primed side of the active-site cleft by running in the reverse direction of a substrate and H¹¹² atom Nε2 coordinates the catalytic zinc replacing the solvent in a kind of “histidine-switch” reminiscent of minigluzincins (section 11 and Scheme 3B). Competent lethal factor has a helical segment (H⁶⁴⁵-A⁶⁴⁰) within the loop linking the upper-rim strand with the preceding strand and H⁶⁴⁵ could correspond to H¹¹² of MtfA, so competent lethal factor might be a valid model for functional MtfA. This suggests an unheard hypothetical latency mechanism, which would entail rearrangement of incompetent 30-residue segment P⁹⁵-Q¹²⁴ of MtfA to match the competent conformation of D⁶³³-E⁶⁶² of lethal factor. In addition, other segments would undergo rearrangement in MtfA, in particular loop R¹⁵⁸-A¹⁷⁵, which connects the active-site helix with the downstream helix and partially occludes the non-primed side of the cleft when compared with the equivalent segment from lethal factor (Y⁶⁹⁵-K⁷¹²). However, this conformation may also be authentic in active MtfA and account for its aminopeptidase activity, which requires a small non-primed side of the cleft. Thus, in this mechanism of latency, activation would probably not be proteolytic, but rather an internal metamorphic rearrangement reminiscent of minigluzincins (section 11) through interaction with a yet unknown factor such as Mlc¹²⁷.

14. ASPZINCINS

The name aspzincins arises from the fungal prototype, deuterolysin from *Aspergillus oryzae*, which was identified as a zincin with an aspartate as third zinc ligand^{23,24}. All currently described aspzincins originate from fungi, apart from two bacterial specimens from the genus *Aeromonas*²⁴. Thus, the clan aspzincins consists to date of a single MP family (Scheme 2). The only currently available zymogen structure is that of AsaP1, an extracellular toxic MP from the Gram-negative bacterium infecting salmonids and other fish, *Aeromonas salmonicida*²⁴. AsaP1 is a major exotoxin that has been linked with skin ulcers and lesions, degradation of subjacent muscles and necrosis, pallor gills and anaemia²⁴. Secreted pro-AsaP1 has an N-terminal pro-segment of ~140-residues followed by a ~170-residue catalytic domain. The exact physiological activation site, the proteolytic activators and the substrate specificity are not known²⁴. Within the zymogen structure, which lacks 13 residues at the interface between the pro-segment and the catalytic domain, the catalytic domain shows the competent fold of mature aspzincins such as deuterolysin (PDB 1EB6;¹²⁸) and peptidyl-lysine MP from *Grifola frondosa* (PDB 1G12;¹²⁹). This indicates that activation cleavage does not lead to significant conformational rearrangement of the mature N-terminus in aspzincins. The catalytic domain contains a mixed four-stranded β-sheet with an upper-rim strand and five helices, which includes an active-site helix, in a large upper sub-domain that extends into the space occupied by the back of the lower sub-domains in other zincins (Scheme 5E). Three helices are found within a small lower sub-domain. One of them is topologically similar to the glutamate helix of gluzincins but it corresponds to the C-terminal helix of the catalytic domain and does not provide the third protein zinc ligand. Instead, a zinc-binding aspartate is provided by a loop region downstream of the active-site helix as in metzincins (Scheme 2). The pro-segment, which acts as an intramolecular chaperone²⁴, is an elongated open β-barrel of immunoglobulin fold, which consists of two three-stranded antiparallel β-sheets capped on one side by a small two-stranded antiparallel sheet. Connecting one strand of either three-stranded sheet, a long 26-residue loop (G⁷⁴-A⁹⁹) projects away from the barrel surface and inserts into the cleft of AsaP1. In particular, segment A⁸²-A⁸⁷ is inserted in extended conformation mimicking a productive Michaelis complex. It occupies sub-sites S₃-S₃' and interacts with the upper-rim strand above the cleft, both on the primed and non-primed sides. In particular, L⁸³ inserts into S₂ and K⁸⁵ penetrates specificity pocket S₁', which matches the specificity of mushroom family members^(24; Scheme 5E).

Because the structure of pro-AsaP1 was obtained with an inert mutant replacing the general base/acid glutamate, which was unable to cleave the pro-segment, it was suggested that the crystallized complex would correspond to a trapped intermediate²⁴. In this case, physiological self-cleavage of the wild-type MP, i.e. upstream of K⁸⁵, would yield a functional zymogen operating similarly to pro-*BaInhA2* (see section 9) or thermolysins with long pro-segments and fungolysins (sections 10 and 12), in that either side of the cleft would be blocked and the zinc coordinated by the deprotonated α-amino group of K⁸⁵ or the carboxylate of I⁸⁴. However, such a mechanism would be much more plausible if an N- or C-terminal peptide from the pro-segment were cleaved, as found in the aforementioned three families, rather than a peptide within an independent folding unit.

Thus, the structure might also correspond to a true zymogen, which would be unique for all the MPs reviewed here and rather reminiscent of “standard-mechanism” inhibitors of serine endopeptidases¹³⁰⁻¹³² and some MPs²⁵. Similarly, the pro-segment of pro-AsaP1 would form an operative Michaelis complex with the catalytic domain, so that it should actually be cleaved following thermodynamic criteria (see Scheme 1, II). However, this cleavage would occur only very slowly as the intact and cleaved pro-segment would be equally potent inhibitors of the catalytic domain. Thus, the pro-segment would act as an energy sink: the system would behave as if it were in an equilibrium between the complex and the free pro-segment (cleaved or uncleaved) *plus* the catalytic domain^{130,132}. Consistent with this hypothesis, the closest structural relative of the pro-AsaP1 pro-segment is an intracellular peptidase inhibitor of subtilisin-type serine endopeptidases from *Bacillus subtilis*, which was suggested to follow the standard mechanism of inhibition¹³³. This points to a possible evolutionary origin of the pro-segment of bacterial aspzincins as a peptidase inhibitor co-opted from another bacterium to function as a latency-maintaining pro-segment.

Finally, as to the general validity of either mechanism for aspzincins, the pro-segment sequences of the two *Aeromonas* species reported do not share significant sequence identity with those from fungi²⁴, so that variable mechanisms of latency are conceivable.

15. SUMMARY AND OUTLOOK

Zymogenicity is a widespread strategy for the regulation of peptidase activity. Structural studies of 60 proteins from eleven MP families reveal that while catalytic domains share the gross architecture and a set of common structural

features, a wide range of latency mechanisms is observed. These involve physically blocking or distorting the active site, and thus inevitably affect the access of substrates. Short or long, mostly N-terminal pro-segments, spanning from peptides without defined tertiary structure outside the zymogen to one or two independently folded domains, carry out the function. The N-termini of the mature catalytic domains after activation may or may not undergo large rearrangement. In particular, the pro-segments of funnelins shield, but do not enter, the active site. Those of LytM/lysostaphin/LasA-MPs *plus* thermolysins with short pro-segments and those of MMPs, fragilysins *plus* astacins run across the active-site cleft in the same or opposite directions to a substrate, respectively. With the exception of thermolysins with short pro-segments, the five latter MPs employ particular residues (cysteine, aspartate, glutamate and asparagine) as switches to replace the catalytic solvent. In astacins, an aspartate switch is the rule and a cysteine switch is the exception, the opposite holds for MMPs. In LytM/lysostaphin-MPs, asparagine and glutamate switches have been described but low conservation of these residues points to other potential binders in other family members.

In bacterial apzincins, the standard mechanism of certain protein inhibitors of peptidases might have been adapted for zymogenicity: a loop protruding from the pro-segment moiety would be inserted across the entire active-site cleft like a substrate, but effectively not cleaved. Alternatively, a first cleavage here would generate the competent aspzincin zymogen, in which either side of the cleft would be blocked and the zinc coordinated by the new α -amino or carboxylate group, as found in thuringilysins and long-pro-segment thermolysins *plus* fungalysins, respectively. In the latter two, the mature N-terminus of the catalytic domain is rearranged and positioned widely apart from the last pro-segment residue, possibly following the transition from a high-energy metastable zymogen to a low-energy stable mature catalytic domain. In anthrax lethal factor-type MPs, latency may be exerted by an intramolecular segment above the active-site cleft. This segment blocks the zinc in the zymogen through a histidine and becomes rearranged in the mature form without proteolytic cleavage, possibly through interactions with a binding partner. Moreover, within minigluzincins, latency of only three different MPs is exerted by C-terminal peptides or helices according to at least six different mechanisms, which involve oligomerization. Altogether, the plethora of mechanisms of zymogenicity described for MPs, even within the same family, predicts that yet more architectures and mechanisms of latency can be expected in the future, as already anticipated 20 years ago⁴².

Finally, the lessons learned from the zymogenic structures are susceptible to contribute to the development of new drugs to modulate their activity *in vivo*. In particular, pro-segments have been assayed as inhibitors *in trans* (see also section 3) in members of the adamalysin/ADAM family^{134,135}. Other recent approaches contemplate the usage of small molecules or monoclonal antibodies to prevent zymogen activation through pro-segment removal, as recently shown for MMP-9¹³⁶⁻¹³⁸. Thus, zymogen-based strategies are likely to provide orthogonal approaches to conventional drug discovery strategies, which target active-site clefts or exosites.

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Notes

The authors declare no competing financial interest.

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Theodoros Goulas performed his Ph.D. thesis in 2008 on protein biochemistry and biotechnology under the supervision of G.R. Gibson at the University of Reading (UK). In 2009, he joined the group of F.X. Gomis-Rüth at the Institute of Molecular Biology of Barcelona, where he is senior research associate since 2014. He is author of 23 publications in the field of structural and biochemical characterization of host-microbiome interactions.

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ACKNOWLEDGEMENTS

This study was funded in part by grants from Spanish and Catalan public agencies (BFU2015-64487-R; MDM-2014-0435 and 2017SGR00003). T.Go. acknowledges a “Juan de la Cierva” research contract (JCI-2012-13573) from the Spanish Ministry of Economy and Competitiveness. The Structural Biology Unit of IBMB is a “María de Maeztu” Unit of Excellence of the Spanish Ministry of Economy, Industry and Competitiveness.

ABBREVIATIONS

ADAM	name of a family of metallopeptidases (a disintegrin and a metallopeptidase)
FTP	domain found in fungalsin and thermolysin pro-peptides
InhA[1,2,3]	immune inhibitor A[1,2,3]
MAM	domain found in meprin α and β , A5 protein, and receptor-type tyrosine-protein phosphatase μ
Mlc	protein associated with making large colonies
MMP	matrix metalloprotease
MP	metallopeptidase
MtfA	Mlc-titration factor A
PDB	protein data bank at www.pdb.org
PepSY	domain present in peptidase pro-peptides and <i>Bacillus subtilis</i> sporulation protein YpeB
TRAF	domain of tumor necrosis factor receptor-associated factors
VEG	domain found in viral envelope glycoproteins

Table 1 – Protein Data Bank entries of metallopeptidase zymogens containing at least the pro- and catalytic domains.		
Peptidase	Organism	PDB codes ^a ; UniProt code; Ref.
Metzincins		
<i>Astacins</i>		
Astacin (E ¹⁴² A) ^b	<i>A. astacus</i> (European freshwater crayfish; Animalia)	3LQ0; Q9U918; ¹⁰⁷
Meprin β	<i>H. sapiens</i> (Animalia)	4GWM; Q16820; ¹¹¹
Myroilysin	<i>M. profund</i> i (Bacteria)	5CZW,5GWD; B5B0E6; ¹¹²
<i>Matrix metalloproteinases</i>		
MMP-1 (E ²¹⁹ A)	<i>H. sapiens</i> (Animalia)	1SU3; P03956; ¹³⁹
MMP-2 (E ⁴⁰⁴ Q/A)	<i>H. sapiens</i> (Animalia)	1EAK,1CK7,1GXD; P08253; ^{92,140}
MMP-3	<i>H. sapiens</i> (Animalia)	1SLM; P08254; ¹⁴¹
MMP-7 (E ²¹⁵ A)	<i>H. sapiens</i> (Animalia)	2MZE,2MZH,2MZI,5UE2,5UE5; P09237; ^{142,143}
MMP-9 ^c	<i>H. sapiens</i> (Animalia)	1L6J,5TH6,5TH9,5UE3,5UE4; P14780; ^{137,138,144}
Karilysin (E ¹⁵⁶ A)	<i>T. forsythia</i> (Bacteria)	4R3V; D0EM77; ¹⁴⁵
<i>Fragilysins</i>		
Fragilysin-3	Enterotoxigenic <i>B. fragilis</i> (Bacteria)	3P24; O86049; ^{57,115}
Metalloproteinase II (E ³⁵² A)	Enterotoxigenic <i>B. fragilis</i> (Bacteria)	4ON1; O68424; ¹¹⁴
<i>Thuringilysins</i>		
Immune inhibitor A2 (E ³⁸⁰ A)	<i>B. anthracis</i> (Bacteria)	4YU5,4YU6; WP_001253006.1 ^d ; ⁵⁸
Gluzincins		
<i>Thermolysins</i>		
Protealysin (E ¹⁶³ A)	<i>S. proteomaculans</i> (Bacteria)	2VQX; Q5MJ80; ¹²²
Vibriolysin MCP-02 (E ³⁴⁶ A)	<i>Pseudoalteromonas</i> sp. (Bacteria)	3NQY,3NQZ; A1DRD5; ⁵⁹
<i>Minigluzincins</i>		
Abylysin	<i>P. abyssi</i> (Archaea)	4JIU; Q9V1Y2; ⁴⁵
Jannalysin	<i>M. jannaschii</i> (Archaea)	4JIX; Q57587; ⁴⁵
Selecase	<i>M. jannaschii</i> (Archaea)	4QHG,4QHH,4QHI,4QHJ; Q58610; ⁴⁴
<i>Fungalysins</i>		
Fungalysin	<i>A. fumigatus</i> (Fungi)	4K90; P46075; ¹²⁴
<i>Anthrax lethal factor-type MPs</i>		
MtfA	<i>K. pneumoniae</i> (Bacteria)	3KHI; A6TB83; ¹²⁷
Aspzincins		
<i>Aspzincins</i>		
AsaP1 (E ²⁹⁴ Q/A)	<i>A. salmonicida</i> (Bacteria)	2X3A,2X3B,2X3C; Q8GMV9; ²⁴
LytM/lysostaphin-MPs		
LytM	<i>S. aureus</i> (Bacteria)	1QWY; O33599; ⁵⁴
Csd3	<i>H. pylori</i> (Bacteria)	4RNY, 4RNZ; O25247; ⁸³
NMB0315	<i>N. meningitidis</i> (Bacteria)	3SLU; E0N688; ⁸⁴
VC0503	<i>V. cholera</i> (Bacteria)	2GU1; Q9KUL5; ⁸⁵
Funnelins		
<i>A/B-type Funnelins</i>		

PCPA1	<i>S. scrofa</i> (swine; Animalia)	1PCA ^e ; P09954; ¹⁴⁶
	<i>H. sapiens</i> (Animalia)	4UF4; P15085
	<i>B. taurus</i> (cow; Animalia)	1PYT; P00730; ¹⁴⁷
PCPA2	<i>H. sapiens</i> (Animalia)	1AYE; P48052; ¹⁴⁸
PCPA4	<i>H. sapiens</i> (Animalia)	2BOA ; Q9UI42; ⁷⁴
HaPCPA	<i>H. armigera</i> (cotton bollworm; Animalia)	1JQG; O97389; ¹⁴⁹
PCPB1	<i>S. scrofa</i> (swine; Animalia)	1NSA,3GLJ; P09955; ^{72,150}
	<i>H. sapiens</i> (Animalia)	1KWM; P15086; ¹⁵¹
PCPB2/TAFI	<i>H. sapiens</i> (Animalia)	3D66,3D67,3D68,4P10,5HVF,5HVG,5HVH; Q96IY4; ¹⁵²⁻¹⁵⁴
	<i>B. taurus</i> (cow; Animalia)	3DGV; Q2KIG3; ¹⁵⁵

^a See the Protein Data Bank (PDB) at www.pdb.org; codes in bold correspond to structures discussed in the text and depicted in Schemes 3-5.

^b In parenthesis, general base/acid positions that are mutated in any of the structures deposited.

^c All structures except that of PDB 1L6J lack the three fibronectin type-II domains inserted into the catalytic moiety.

^d GenBank accession number.

^e This structure was obtained before the protein sequence was available and thus contains 41 mutations.

Search completed on 14 November 2017.

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TABLE OF CONTENTS GRAPHICS

In the center, mature metalloproteases (*in turquoise*) cleave (*scissors*) protein and peptide substrates (*in red*) bound to an active-site cleft (*in light green*) containing a catalytic zinc ion (*in magenta*) in a particular N-to-C orientation. Given the irreversibility of this cleavage, metalloproteases must be kept latent until reaching their physiological site of action. Several latency mechanisms have been documented through structural studies, represented by the zymogens surrounding the mature enzyme. Zymogen pro-segments are depicted as orange ellipses and other ancillary domains are in gray. Particular pro-segment residues binding the catalytic zinc are shown in one-letter code within white ellipses, N- or C-termini of the zymogens are labeled *in brown*. Numbers refer to the corresponding sections in the text.