Chapter 7
Astacins: Proteases in Development and Tissue Differentiation

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7.1 Introduction

The astacins form a versatile family of multi-domain zinc peptidases within the metzincin superfamily (Bond and Beynon 1995; Gomis-Rüth 2003, 2009; Stöcker et al. 1995). To date, the MEROPS database (http://merops.sanger.ac.uk/) of proteolytic enzymes and their inhibitors lists more than a thousand astacin proteases of both prokaryotic and eukaryotic origin. Most of them are secreted proteins; only the members of the meprin subfamily are translated with a membrane anchor. Astacins are generally synthesized as inactive zymogens (pro-enzymes). Thus, their activity relies on the post-translational removal of amino terminal pro-peptides. Thereafter, biological protein inhibitors control their activity.

Eukaryotic astacin proteases are composed of amino terminal signal peptides and pro-segments, zinc-binding protease modules, various carboxy terminal domains, trans-membrane anchors, and cytosolic domains. The X-ray crystal structures of four astacins and one complete zymogen have been solved. The catalytic domain comprises about 200 residues and contains in its center the conserved zinc-binding sequence, HEXXHXXGXXH, typical for metzincin peptidases.

The first family member to be discovered was the digestive metalloprotease astacin from the European fresh water crayfish *Astacus astacus* L.—originally termed 'low molecular weight protease' or '*Astacus* protease' (Stöcker and Zwilling 1995). Generally, the genomes of lower vertebrates and invertebrates

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contain more astacin genes than mammalian genomes. MEROPS lists 7–18 astacins in amphibians and fishes, at least four in cnidarians, 13–25 in insects, and up to 40 in nematodes such as *Caenorhabditis elegans*. The large number in the latter example can at least partially be attributed to the parasitic lifestyle of nematodes, which require an array of proteases to break down host connective tissue. Characterized examples of this phylum are enzymes from *Trichinella spiralis* (Lun et al. 2003) and *Onchocerca volvolus* (Borchert et al. 2007). Astacins with digestive function have been observed in several invertebrate (mostly decapod crustacean) species (Möhrlen et al. 2003; Stöcker and Zwilling 1995). However, most astacins are not involved in digestion, but rather in proteolytic processing of precursors of extracellular matrix constituents and growth factors, including their antagonists and cell surface receptors, during embryonic patterning and cell differentiation.

The first astacin reported to participate in development was bone morphogenetic protein 1 (BMP1, also known as procollagen C-protease), which is co-expressed with TGFβ-like, non-proteolytic growth factors termed BMPs due to their capability to induce ectopic bone formation in mice (Wozney et al. 1988). Since then a variety of astacins have been discovered, which are key players in developmental processes, tissue differentiation, and embryonic hatching, as exemplified by UVS.2 from clawed frog (Sato and Sargent 1990), tolloid from the fruit fly (Shimell et al. 1991), the low (LCE) and high (HCE) choriolytic enzymes from medaka fish (Yasumasu et al. 1992), and SPAN and blastula protein BP10 from sea urchin (Lepage et al. 1992; Reynolds et al. 1992). Consequently, due to these basic regulatory functions, astacins have found to be linked to diseases like cancer, connective tissue disorders, neuro-degenerative disorders like Alzheimer's disease, etc.

This review will focus on the structure and function of astacin proteases involved in various aspects of development, morphogenesis and tissue differentiation.

7.2 Structure of Astacin Proteases

7.2.1 Modular Composition of Astacins

The smallest prokaryote astacins consist only of a catalytic domain of approximately 200 amino acid residues. Eukaryote astacins are larger, at least elongated by an amino terminal pro-peptide, which renders the enzyme inactive (Yiallouros et al. 2002) (Fig. 7.1).

A distinct subfamily of astacins are the BMP-1/tolloid-like proteases (BTPs), named after bone morphogenetic protein 1 and the dorso-ventral patterning protein tolloid, first described in *Drosophila* embryos. The BTPs contain additional downstream domains such as CUB modules (named according to their occurrence in

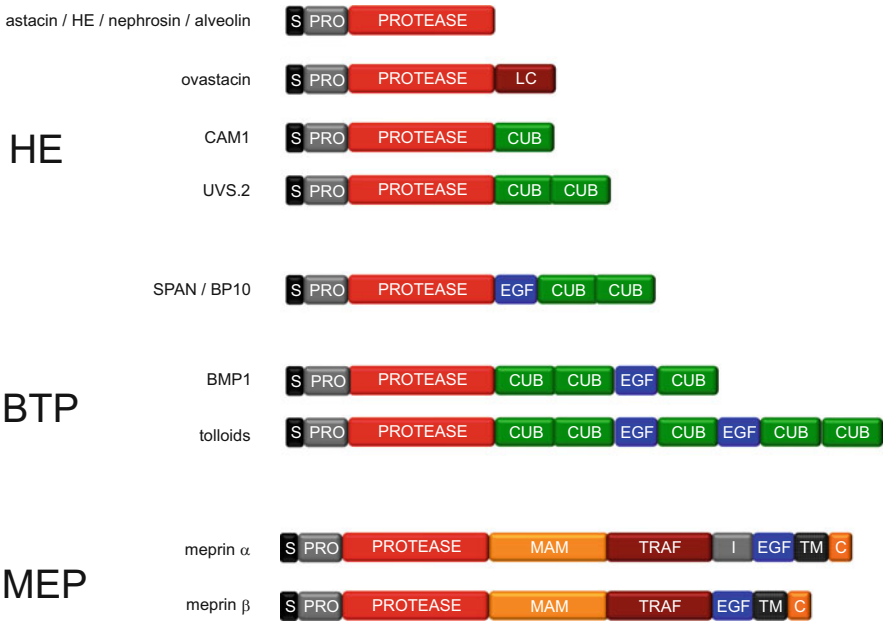

Fig. 7.1 Domain composition of selected astacin proteases. S = (signal peptide); PRO = (pro-peptide); PROTEASE = (catalytic domain); EGF (epidermal growth factor; PFAM PF00008); CUB (domain found in complement C1r/1s, sea urchin Uegf, BMP1; PF00431); MAM (domain found in meprins, A5 receptor protein, and tyrosine phosphatase μ; PF00629); TRAF (tumor necrosis factor receptor-associated factor; PF00917); LC (low complexity domain); C (cytosolic domain); I (inserted domain); TM (transmembrane anchor); HE = (hatching enzymes); BTP = (BMP1/Tolloid-subfamily); MEP = (meprin subfamily)

complement C1s and C1r, sea urchin UEGF and BMP1), EGF (epidermal growth factor like) modules (Bond and Beynon 1995; Stöcker et al. 1995). Some of these domains have been shown to bind calcium and facilitate protein-protein interactions. Especially, they seem to be important for the selective substrate specificity of BTPs (Garrigue-Antar et al. 2004; Hintze et al. 2006; Sieron et al. 2000; Wermter et al. 2007). Sea urchin astacins like SPAN and BP10 are structurally related to tolloids (Lepage et al. 1992; Reynolds et al. 1992), because they also contain CUB and EGF domains.

Astacins of the meprin subfamily are distinguished by TRAF domains (from tumor necrosis factor receptor-associated factor) (Rothe et al. 1994; Zapata et al. 2001) and MAM domains initially identified in meprins, A5 protein, and receptor tyrosine phosphatase μ (Beckmann and Bork 1993). MAM domains alone are also present in HMP2 from hydra (Yan et al. 2000a, b), LAST-MAM from the horseshoe crab (Becker-Pauly et al. 2009) and in the so-called myosinases from squids (Tamori et al. 1999). A considerable number of astacin proteases contain less characterized additional modules often termed LC (regions of low complexity) with little similarity to other protein modules. Such regions have been identified in *Caenorhabditis elegans*

astacins, sea urchin astacins SPAN/BP10 (Lepage et al. 1992; Reynolds et al. 1992) and ovastacin (Quesada et al. 2004). Likewise, there are other uncharacterized protein domains such as the EB module found in some *C. elegans* proteins and the TT domain of bacterial astacins, which has been named after the viral ORF2 (open reading frame) of the TT virus. Several *C. elegans* astacins have thrombospondin type 1 repeats, which are also found in other metzincins, such as the ADAMTS peptidases (Apte 2009). Further extracellular domains seen in astacins are ShK toxin moieties, i.e. six-cysteine (SXC) domains, which were originally observed in metridin, a toxin from sea anemone, and several hypothetical *C. elegans* proteins. For more information on astacin domain confer to http://merops.sanger.ac.uk (Rawlings et al. 2010).

Based on the sequences of their catalytic domains, the astacins can be grouped in several major subfamilies (Fig. 7.1). There is the distinct BTP cluster present in all animal phyla. On the other hand, the meprin cluster is as yet only present in vertebrates. Another cluster is formed by the hatching enzymes, which in amphibians and fishes have evolved in several divergent lineages. A different scenario becomes evident in cnidarians, nematodes, insects, and mollusks, where specific clusters not seen outside the respective phyla have evolved (Gomis-Rüth et al. 2012b).

Most structural and functional details are known of the pro-peptide regions and catalytic domains (Fig. 7.2). In the pro-domain, there is a unique conserved motif termed 'aspartate-switch'; in the middle of the catalytic domain there is the zinc-binding consensus sequence; 40 residues downstream, the 'Met-turn' is found; a further 25 residues later, the absolutely crucial S_1'-subsite shaping "170-loop" is found engaged in substrate recognition and cleavage specificity within the catalytic domain.

7.2.2 *Structure of Catalytic Domains and Metal Binding Sites*

The three dimensional structures of five astacins have been solved by X-ray crystallography so far. These are crayfish astacin (Bode et al. 1992; Gomis-Rüth et al. 1993; Grams et al. 1996), human BMP1 and Tll1 (Mac Sweeney et al. 2008), the zebrafish hatching enzyme ZHE1 (Okada et al. 2010) and the human meprin beta dimer (Arolas et al. 2012) (Fig. 7.2). Astacin catalytic domains are compact kidney-shaped ellipsoids with dimensions of about 55 × 45 × 35 Å (Fig. 7.3). If viewed in standard orientation (Gomis-Rüth et al. 2012a), the active-site cleft divides the catalytic domain into an upper N-terminal and a lower C-terminal sub-domain. Superposition of the catalytic domains of BMP1, ZHE1, meprin and astacin reveals high topological equivalence.

In all cases, the catalytic domain is composed of a twisted five-stranded β-sheet of four parallel strands and one antiparallel strand, which forms the upper edge of the active site cleft. After passing the sheet, the polypeptide chain enters the active-site

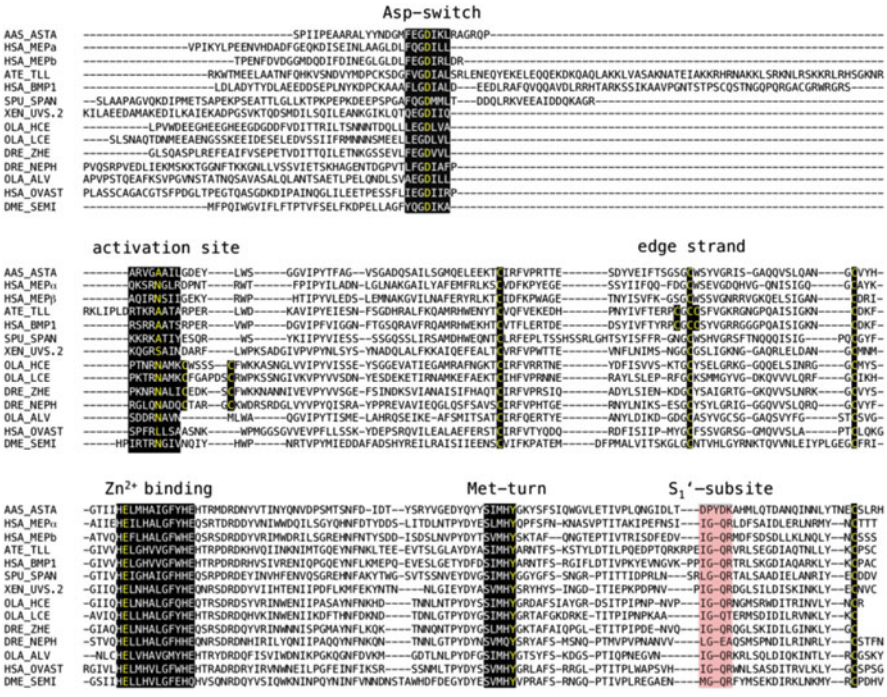

Fig. 7.2 Alignment of pro- and catalytic domains of selected astacins. On *black* background: the aspartate-switch residue in the pro-peptide, the activation site, the zinc binding motif, the Met-turn, and disulfide-forming cysteines; in *pink*: residues shaping the S_1'-subsite. Proteins and UniProt database accession numbers: AAS_AST (P07584) astacin from the crayfish *Astacus astacus* (Titani et al. 1987); ATE_TLL (Q75UQ6) tolloid from house spider *Achaearanea tepidariorum*; CJA_CAM1 (P42662) hatching enzyme from Japanese quail *Coturnix japonica* (Elaroussi and DeLuca 1994); DME_SEMI (CG11864, Q9VJN9) seminase from the fruitfly *Drosophila melanogaster* (LaFlamme et al. 2012); DRE_ZHE (Q75NR9) zebrafish hatching enzyme from *Danio rerio* (Okada et al. 2010); DRE_NEPH (Q8AYF4) zebrafish nephrosin from *Danio rerio* (Hung et al. 1997); HSA_BMP1 (P13497) human BMP1 (Wozney et al. 1988); HSA_MEPa (Q16819) human meprin α (Dumermuth et al. 1991); HSA_MEPb (Q16820) human meprin β (Dumermuth et al. 1991); HSA_OVAST (Q6HA08) human ovastacin (Quesada et al. 2004); OLA_HCE (P31580) medaka fish high choriolytic enzyme from *Oryzias latipes* (Yasumasu et al. 1992); OLA_LCE (P31579) medaka fish low choriolytic enzyme from *Oryzias latipes* (Yasumasu et al. 1992); OLA_ALV (Q9VJN9) alveolin from *Oryzias latipes* (Shibata et al. 2003); SPU_SPAN (P98068) sea urchin blastula protease from *Strongylocentrotus purpuratus* (Reynolds et al. 1992); and XLA_UVS.2 (P42664) hatching enzyme from the clawed frog *Xenopus laevis* (Sato and Sargent 1990)

helix, which provides the first two His residues of the typical metzincin-type zinc-binding motif, $H^{92}EXXHXXG^{99}XXH^{102}$ (mature astacin numbering, single letter amino-acid code; X = any residue) (Bode et al. 1993; Gomis-Rüth 2003, 2009; Stöcker and Bode 1995; Stöcker et al. 1993, 1995). At G^{99} the active-site helix is terminated, and the chain is bent sharply thereby placing the third zinc-binding residue

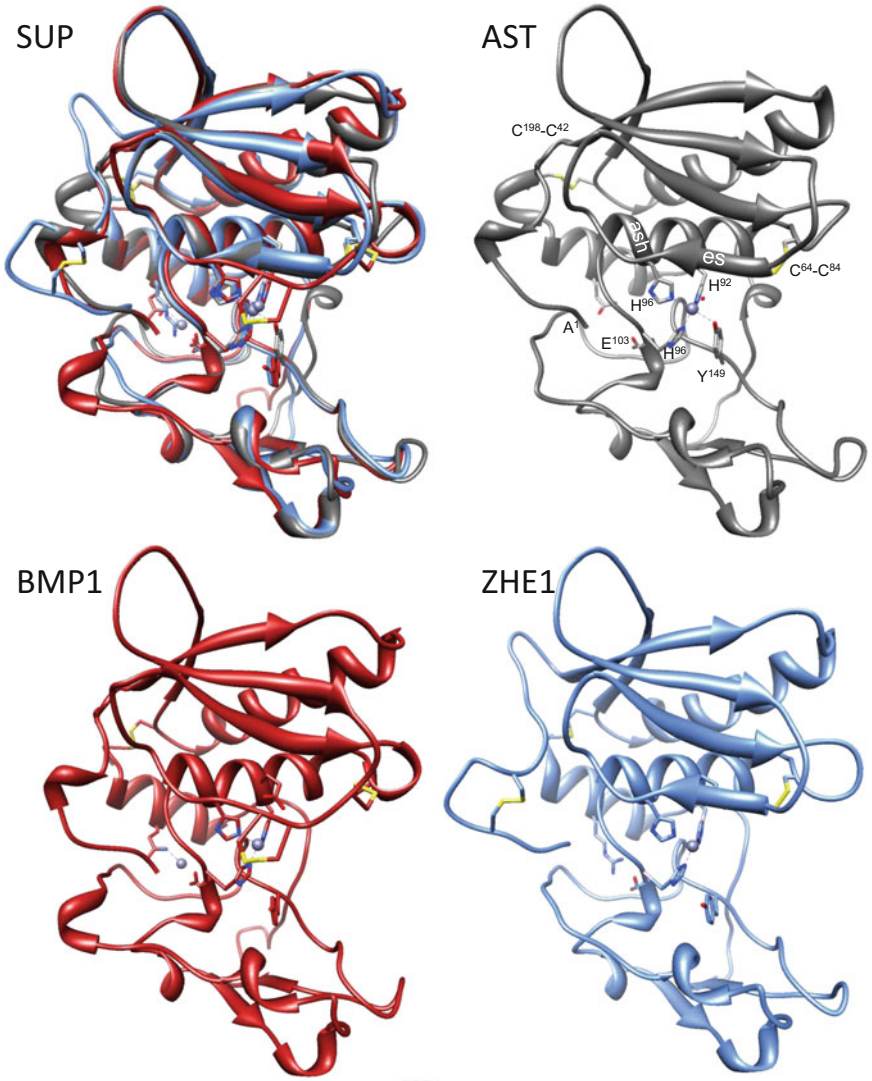

Fig. 7.3 Structure of astacin proteases. Shown are ribbon representations based on the X-ray crystal structures of the mature catalytic domains of crayfish astacins in *grey* (AST) (PDB accession code: 1AST), human BMP1 (PDB accession code 3LQB) in *red* and zebrafish ZHE1 (PDB accession code 3EDH) modeled with CHIMERA (http://www.cgl.ucsf.edu/chimera/) in *blue*. Labeled in AST are the side chains of the three zinc-binding histidines, Y^{149} of the Met-turn, the two conserved disulfide bonds (between C^{42}–C^{198} and C^{64}–C^{84}) and the zinc-bound water symbolized by a *red dot*. (SUP) superposition of BMP1 and ZHE1 onto the structure of astacin (*es* edge strand, *ash* active site helix)

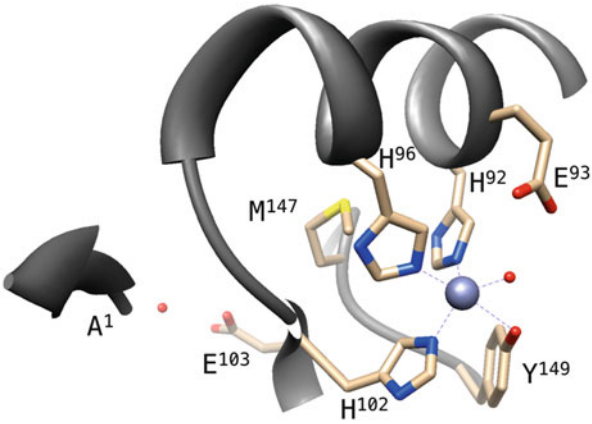

Fig. 7.4 Zinc-binding region of astacin. Shown are the zinc ligands H^{92}, H^{96}, H^{102}, Y^{149}, the catalytic base E^{93}, the water-mediated (*red dot*) salt bridge between A^1 and E^{103}, and the methionine residue M^{147} of the Met-turn backing the zinc site. Modeling of PDB 1AST was performed with CHIMERA (http://www.cgl.ucsf.edu/chimera/)

H^{102} in a competent position, and the astacin family-specific E^{103} (Stöcker et al. 1993), which is salt bridged to the mature amino terminus (see below).

The subsequent part of the lower sub-domain contains little regular secondary structure except for a short 3_{10}-helix and two short strands before the chain enters the prominent C-terminal helix. However, this rather unstructured C-terminal half contains a unique 1,4-β-turn immediately beneath the zinc-site. This turn is characterized by a methionine residue (M^{147}), shown in a close-up view of the zinc site in Fig. 7.4, which is conserved even for its side-chain conformation in all metzincins of known structure (Gomis-Rüth 2009; Goulas et al. 2010; Waltersperger et al. 2010), although its ultimate functional and structural implications are still under debate (Boldt et al. 2001; Butler et al. 2004; Hege and Baumann 2001; Oberholzer et al. 2009; Perez et al. 2007; Pieper et al. 1997; Tallant et al. 2010; Walasek and Honek 2005). Also located in the Met-turn is Y^{149}, which is engaged in zinc binding and catalysis.

The astacin catalytic domains contain two or three internal disulfide bonds. Conserved in most astacins are C^{42}–C^{198} and C^{64}–C^{84} first observed in crayfish astacin (Figs. 7.2 and 7.3). The first connects the end of the C-terminal helix to the body of the protein. The second links the so-called edge strand (es) to the active-site helix (ash) and thus contributes to shaping the active-site cleft at its primed side and to substrate binding (Figs. 7.2 and 7.5) (Stöcker et al. 1993). The hatching enzymes like ZHE1 contain an additional pair of cysteines within the amino terminal segment (Fig. 7.2). As seen in ZHE1 (Fig. 7.3), these cysteines are cross-linked, thus locking the amino terminus distant from E^{103} (Fig. 7.3). BMP1 and TLL1, likewise contain an additional disulfide bond, albeit in a different position compared to the hatching enzymes. Interestingly, this additional pair of cysteines rearranges the conserved disulfide pattern (Figs. 7.2 and 7.3) by introducing another unique link between the side chains of two consecutive cysteines of the edge strand (C^{64}–C^{65}; BMP1 numbering), whereas the remaining C^{62} in the edge strand is now linked to C^{84}. This 'cysteine-rich loop', is unique for the tolloid subfamily (Fig. 7.2) and has implications for substrate binding, because the two consecutive cysteines

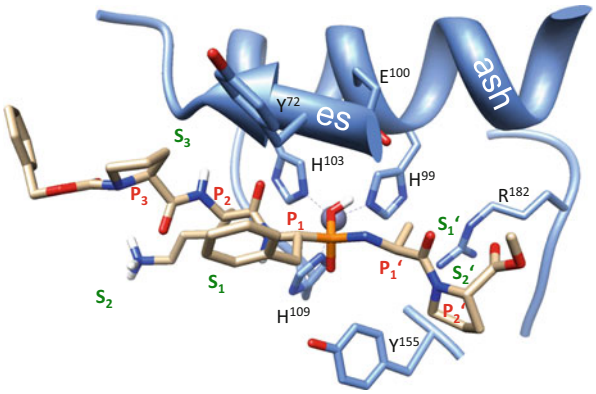

Fig. 7.5 Active site of astacins. Superposition of the structure of the zebrafish hatching enzyme ZHE1 (PDB accession code 3LQB, in *blue*) (Okada et al. 2010) onto the complex of astacin with the phosphinic pseudopeptide inhibitor, BOC-PKRΨ(PO_2CH_2)AP-OCH_3 (PDB accession code 1QJI) (BOC = benzoyl-oxycarbonyl) (K_i = 14 μM against astacin) (Grams et al. 1996; Yiallouros et al. 1998). Shown are the inhibitor in *grey*, the zinc-binding histidine imidazoles, Y^{155} (ZHE1 numbering) in hydrogen-bonding distance to the lower phosphinyl oxygen, the catalytic base E^{100} and, finally, R^{182}, which is thought to be responsible for the specificity of ZHE1 for acidic side chains in P_1' and P_2' of the substrate. The substrate positions from P_3 to P_2' and their corresponding binding sites in the enzyme, S_3 to S_2', are labeled according to (Schechter and Berger 1967). The metal-chelating phosphinyl group adopts the geometry of a tetrahedrally coordinated carbon as visualized with CHIMERA, http://www.cgl.ucsf.edu/chimera/, during peptide bond cleavage

generate a flap occluding the active site. Substrate binding requires opening of this flap (Mac Sweeney et al. 2008).

7.2.3 Buried N-Terminal Region in Mature Astacins

In mature astacin, the three N-terminal residues A^1-A^2-I^3 are plugged into a cavity and the A^1-ammonium integrates in a buried hydrogen-bonding network together with eight solvent molecules (one of them shown as a red dot in Fig. 7.4) and the carboxylate of E^{103}, the direct neighbor of zinc ligand H^{102} (Fig. 7.4) (Stöcker et al. 1993, 1995). The solvent molecule is further hydrogen bonded to the side chain of Q^{189}. These interactions are crucial for the structural stability in mature astacin catalytic domains. Replacement of E^{103} by Q or A did not diminish catalytic efficiency, but caused thermal instability (Yiallouros et al. 2002). In both BMP1 and TLL1, the amino terminal alanine is acetylated and the carbonyl oxygen of the acetyl group is integrated in the aforementioned network of hydrogen bonds. There is also electron density indicating a second metal ion (Fig. 7.3), which is inserted between Q^{189} of the terminal helix and E^{103} (Mac Sweeney et al. 2008).

A different scenario is seen in ZHE1, where the amino terminus is more than 8 Å apart from the conserved family-specific glutamate, which, instead, is linked to

R^{189} (rather than Q^{189}) provided by the carboxy terminal helix (Fig. 7.3). This distinct arrangement can in part be attributed to the additional disulfide bond, which locks the amino terminus in a glutamate-distal position (see above). Alignment and modeling of the mature amino termini of representative astacin family members (Figs. 7.2 and 7.3) (Stöcker et al. 1993) suggests that most of them are arranged in structures similar to astacin/BMP1/TLL1 or the hatching enzymes like ZHE1. This also implicates that variability in the length of the amino terminal segment is restricted. This is a structural analogy to the salt bridged amino terminus of trypsin-like serine proteinases to an aspartate that neighbors the catalytic serine residue (Fehlhammer et al. 1977; Huber and Bode 1978).

7.2.4 Active-Site Cleft and Substrate Specificity

The catalytic mechanism suggested for astacin-like zinc-peptidases relies on the polarization of the metal-bound water between the zinc(II)-ion, which acts as a Lewis-acid, and the glutamic acid residue (E^{93}), which acts as the general base (Grams et al. 1996). Since the catalytic water is also bound to Y^{149} (Figs. 7.3, 7.4, and 7.5), both residues were mutated to assay their significance in catalysis. The $Y^{149}F$ mutant still retained low activity, whereas the $E^{93}A$ mutant was completely inactive (Yiallouros et al. 2000). This supports the role of E^{93} as a general base and proton shuttle to the leaving amino group during catalysis in analogy to E^{143} of thermolysin (Matthews 1988) or E^{270} of carboxypeptidase A (Christianson and Lipscomb 1989). Y^{149} on the other hand seems to stabilize the transition state similarly to H^{231} in thermolysin and Y^{248} of carboxypeptidase A.

The specific interactions of astacin with the reaction-intermediate analog inhibitor, BOC-PKRΨ(PO_2CH_2)AP-OCH_3, have provided insight into the catalytic mechanism of astacin-like enzymes (Grams et al. 1996) (Fig. 7.5). In this mimic, the scissile peptide bond is replaced by a phosphinic pseudo peptide bond Ψ(PO_2CH_2), in which the PO_2 mimicks the tetrahedrally coordinated carbon of the intermediate during peptide bond cleavage.

Astacins bind protein substrates in an extended conformation by antiparallel alignment to their upper-rim edge β-strand (es) (Figs. 7.3 and 7.5). The non-primed part (Schechter and Berger 1967) is hydrogen bonded via backbone carbonyl and amide groups (Fig. 7.5). The two phosphinyl oxygens of the PO_2 group chelate the metal ion. Most importantly, upon inhibitor binding, the tyrosine side chain moves into a position about 5.0 Å removed from the metal, and becomes hydrogen-bonded with the PO_2 group, which mimics a water-attacked peptide bond (Fig. 7.5). This 'tyrosine switch' is a unique feature of the astacin-like proteinases and also of serralysins. In the structures of BMP1 and ZHE1 dimethyl sulfoxide and sulfate were found, respectively, to bind the zinc ion. This is probably the reason, why in these structures the tyrosine is shifted into this remote position even in the absence of an inhibitor, for sterical reasons. The overlay of ZHE1 and BOC-PKRΨ

(PO_2CH_2)AP-OCH_3 depicted in Fig. 7.5 therefore most likely resembles the 'inhibited' conformation of ZHE1.

The side chain carboxy terminally of the cleavage site is an alanine methyl group in BOC-PKRΨ(PO_2CH_2)AP-OCH_3 (Fig. 7.5). For the subsite specificity of zinc endopeptidases, this is the most important side chain, since it points directly into the specificity pocket. According to Schechter and Berger (1967), this is the P_1' position of the substrate, which is harbored in the corresponding S_1' subsite of the enzyme. Remarkably, the great majority of astacin proteases in the MEROPS database are very similar in their S_1' regions (see Fig. 7.2). Particularly, the consensus motif highlighted in Fig. 7.2 contains a conserved arginine residue, which forms the bottom of the S_1'-pocket in the structures of ZHE1, TLL1, and BMP1 as seen in Fig. 7.5. Crayfish astacin belongs to the minority of astacins, which do not share this arginine but instead have a rather shallow S_1' subsite. This is probably the reason for the preference of acidic side chains of aspartate and glutamate in P_1' and P_2' by most astacins, whereas astacin itself prefers small aliphatic residues, as shown recently on a quantitative basis in a proteomics approach covering cleavage sites in complete cellular proteomes employing several astacins. This preference is even more pronounced in meprin β and BTP astacins (e.g. BMP1), which have additional basic side chains for recognition of acidic substrates in their S_2' subsites (Becker-Pauly et al. 2011).

In astacin and ZHE1 the upper-rim strand lines the top of the cleft on its primed side, together with the conserved disulfide bond between Cys^{64} and Cys^{84} (astacin numbering; Figs. 7.2 and 7.3). By contrast, in BMP1 and TLL1 this disulfide bond is slightly displaced and a further, unique SS-bridge is found between two consecutive cysteines within a cysteine-rich loop that replaces the upper-rim strand in astacin and ZHE1. This gives rise to an eight-membered, largely hydrophobic ring above the S_1 pocket (Fig. 7.3), which prevents substrate binding to the cleft and causes the upper rim to deviate from a regular β-strand. This cysteine-rich loop is disordered in the inhibitor free structures, and it has been proposed to act as a mobile flap clamping substrates into a competent position for a Michaelis complex (Mac Sweeney et al. 2008). On its non-primed side, the cleft is limited in astacins by the end of the edge strand. At its bottom, the cleft is constrained on its non-primed side by I^4-G^5 and the loops following the active-site helix and connecting the 3_{10}-helix with the Met-turn, and, on its primed side, by the Met-turn and the downstream segment up to W^{158}.

7.2.5 Zymogen Structure and Activation Mechanism

Eukaryotic astacins are synthesized as inactive pro-proteases (zymogens), which require proteolytic removal of an amino terminal pro-peptide (Guevara et al. 2010). The pro-segments of distinct family members are aligned in Fig. 7.2. They differ in length (from 34 to 393 residues), but they share a unique consensus sequence $F^{18P}XGD^{21P}$ (pro-peptide residues are labeled with the suffix 'P'). The only

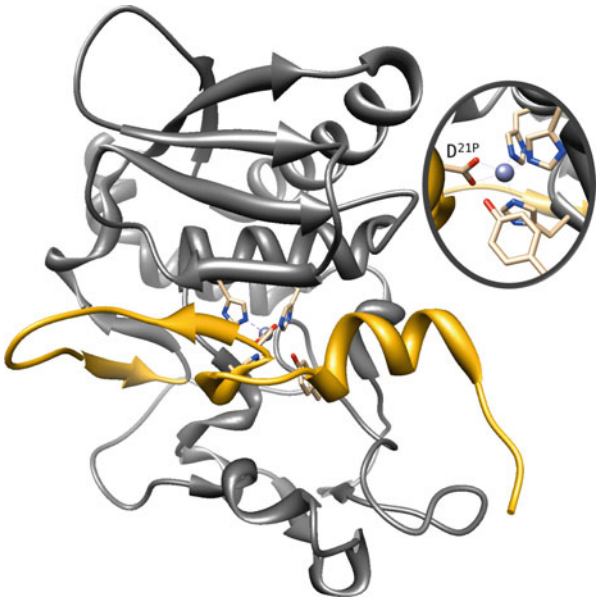

Fig. 7.6 Structure of pro-astacin. Ribbon model of pro-astacin (standard view) shown in *grey* with the pro-peptide in *yellow*. The ellipsoid shows the interaction of D^{21P} with the metal. Modeling of PDB 3LQ0 was performed with CHIMERA (http://www.cgl.ucsf.edu/chimera/)

structurally characterized zymogen of the astacin family is that of crayfish pro-astacin (Guevara et al. 2010) (Fig. 7.6). Pro-astacin could be purified from *Escherichia coli* inclusion bodies and correctly folded as a potentially activatable zymogen (Reyda et al. 1999; Yiallouros et al. 2000, 2002). It contains a short pro-peptide of 34 residues, which is not required for folding as an intramolecular chaperon (Reyda et al. 1999). However, this possibility cannot been excluded for pro-domains, which are even larger than the catalytic domain, as it is the case in *Drosophila* tolloid-related (Nguyen et al. 1994).

The pro-segment of the astacin zymogen runs across the front surface of the catalytic domain in the inverse direction of a substrate (Fig. 7.6). This prevents self-cleavage, as observed in cysteine-protease and matrix metalloproteinase zymogens (Khan and James 1998). Most remarkable is a Z-shaped loop directly in front of the zinc site, which posts D^{21P} as a bidentate chelator of the catalytic zinc ion (Figs. 7.2 and 7.6). This loop contains two tight 1,4-β-turns, which also explains the conservation of a glycine next to the aspartate, and it also contains conserved hydrophobic residues that facilitate the formation of a compact globular moiety backing D^{21P} (Fig. 7.5).

The activation site (Figs. 7.2 and 7.6), $G^{34P}{*}A^{1}$, is buried like the amino-terminus of the mature form and is located at the tip of a sharp turn. Activation occurs through successive cleavages by trypsin and mature astacin, which liberate the mature N-terminus at A^{1} (Guevara et al. 2010; Yiallouros et al. 2002) and enable formation of the salt bridge to E^{103} and the release of the pro-peptide's D^{21P} from the zinc ion. In a similar fashion, the amino terminus of matrix metalloproteinases (MMPs) is trimmed by successive cleavage events to become finally

engaged in a hydrogen bond with a conserved aspartate of the C-terminal helix (Nagase 1997; Reinemer et al. 1994). In analogy to the cysteine-switch of MMPs the removal of the zinc-blocking aspartate has been termed 'aspartate-switch' (Guevara et al. 2010). This causes major rearrangement of the 'activation domain' beneath the mature amino terminus, which adopts a rigid and competent conformation in the mature enzyme only (Fig. 7.6). This increase in rigidity and stability is another analogy to trypsin-like serine proteinases (Bode and Huber 1978; Khan and James 1998).

7.2.6 Protein Inhibitors and Enhancers of Astacins

Astacins are resistant to inhibition by tissue inhibitors of metalloproteinases (TIMPs), which are effective against MMPs ad ADAMs. However, the general protein scavenger and regulator of vascular and interstitial proteolysis, α_2-macroglobulin, is a potent inactivator of members of the astacin family. This holds true not only for small single domain astacins, but also for middle-sized multi-domain proteins like BMP1 (Marrero et al. 2012; Meier et al. 1994; Stöcker et al. 1991; Zhang et al. 2006). However, α_2-macroglobulin does not inhibit larger oligomeric astacins such as meprins (Kruse et al. 2004).

Another natural protein inhibitor of astacins was discovered in complex with nephrosin, an astacin-like protease from the carp, *Cyprinus carpio* (Hung et al. 1997; Tsai et al. 2004). The enzyme was termed nephrosin after its site of biosynthesis, the head kidney, which is a hematopoietic organ in fishes. The nephrosin inhibitor turned out to be the fish homolog of fetuin, a large mammalian plasma protein with many functions (Jahnen-Dechent et al. 2011; Schäfer et al. 2003). These fetuins contain cystatin-like domains and are related to cystatin C-like inhibitors of papain-like cysteine proteases. It has been shown recently that the plasma proteins fetuin and cystatin C act as physiological inhibitors of human astacin proteases such as ovastacin and meprins (Hedrich et al. 2010; Dietzel et al. 2013).

Other protein inhibitors of astacin proteases have been discovered in the context of body axis formation during early embryogenesis in amphibians and fishes. In these lower vertebrates secreted frizzled like proteins (sFRPs) are potent antagonists of BTPs (Lee et al. 2006). sFRPs are composed of a carboxy terminal netrin-like domain and an amino terminal frizzled domain. Frizzled is a cell surface receptor of the Wnt-signalling pathway. Interestingly, there are several sFRPs in *Xenopus*, which are expressed in different regions of the embryo. One of these, the dorsally expressed crescent protein enables cross talk between Wnt- and BMP-pathways, since it is an inhibitor of BTPs and can also trigger Wnt signalling (Ploper et al. 2011). By contrast, the mammalian sFRPs do not inhibit BTS proteases (Bijakowski et al. 2012) but may act as BTS protease enhancers (Kobayashi et al. 2009), albeit this enhancing function has yet to be corroborated (von Marschall and Fisher 2010b).

However, there are true enhancers, which regulate BTPs in a highly substrate specific manner. These enhancers have been termed procollagen C-proteinase enhancers (PCPE1 and PDCPE2), because they bind selectively to the C-propeptides of fibrillar collagens and increase the activity of BTS proteases by about 20-fold. PCPEs are composed of two CUB domains and a netrin-like domain (Kessler et al. 1990; Kronenberg et al. 2009; Moali et al. 2005; Steiglitz et al. 2002; Vadon-Le Goff et al. 2011). Studies with PCPE$^{-/-}$ mice also indicate a role of these proteins for proper collagen fiber assembly (Steiglitz et al. 2006).

7.3 Distribution and Physiological Role of Astacins

In the human and mouse genomes (see http://degradome.uniovi.es/met.html), there are six genes encoding astacin proteases, namely *bmp1*, *tll1*, *tll2*, *mep1a*, *mep1b* and *astl*. The first three code for the BTPs, which include protein BMP1 and its major splice variant, mammalian tolloid, and the mammalian tolloid-like proteins mTll1 and mTll2 (Muir and Greenspan 2011). Genes *mep1a* and *mep1b* encode the multi-domain proteins meprin α and meprin β, respectively (Sterchi et al. 2008). The third subgroup of astacins in vertebrates and invertebrates comprises the so-called hatching enzymes, which degrade embryonic envelopes during the free water developmental stage of crustaceans, echinoderms, fishes, and frogs (Kawaguchi et al. 2010a).

7.3.1 BMP1/Tolloid Proteases (BTPs)

BTPs cleave precursors of fibrillar procollagens for proper matrix assembly. They also process other matrix proteins including proteoglycans, laminins, and anchoring fibrils. In addition, tolloids also cleave growth factors and their antagonists, which are crucial for dorso-ventral patterning during gastrulation in the embryo (for reviews, see Ge and Greenspan 2006b; Hopkins et al. 2007; Muir and Greenspan 2011). In vertebrates, the four major BTPs are all expressed during embryonic development in the gastrula (Ploper et al. 2011). They are crucial for dorso-ventral patterning through cleavage of chordin, an antagonist of the transforming growth factor-like bone morphogenetic proteins BMP2 and BMP4. In later development and in the adult, BMP1, mTLD, and mTLL1 are further required for bone formation and connective tissue differentiation, because they specifically activate and trim a variety of procollagens and therefore are also termed 'procollagen C-proteases' (Kessler et al. 1996; Li et al. 1996). mTLL2, by contrast, localizes to skeletal muscle in later development (Scott et al. 1999). However, it has been found in bmp1$^{-/-}$ mice that it also may be involved in procollagen VII processing (Rattenholl et al. 2002). Validated BTP substrates are listed in Table 7.1.

Table 7.1 Cleavage sites of BMP1 in extracellular matrix proteins

ProCOLL α_1(I)	YYRA*DDAN	Kessler et al. (1996), Li et al. (1996)
ProCOLL α_2(I)	FYRAA*DQPR	Kessler et al. (1996), Li et al. (1996)
ProCOLL α_1(II)	YMRAA*DQAA	Kessler et al. (1996), Li et al. (1996)
ProCOLL α_1(III)	PYYGA*DEPM	Kessler et al. (1996), Li et al. (1996)
ProCOLL α_1(V)C-Pro	QLLDA*DGNG	Pappano et al. (2003), Unsöld et al. (2002)
ProCOLL α_2(V)C-Pro	EFTEA*DQAA	Pappano et al. (2003), Unsöld et al. (2002)
ProCOLL α_1(VII)	SYAAA*DTAG	Rattenholl et al. (2002)
ProCOLL α_3(V)N-Pro	SFQQA*AQAQ	Gopalakrishnan et al. (2004)
ProCOLL α_1(V)N-Pro	TPQSA*QDPN	Pappano et al. (2003), Unsöld et al. (2002)
IGFBP3	ESQSA*TDTQ	Kim et al. (2011)
α_1(XI)COLL	AAQAA*QEPQ	Pappano et al. (2003), Unsöld et al. (2002)
α_2(XI)COLL	RPQNA*QQPH	Pappano et al. (2003), Unsöld et al. (2002)
Pro-LOX	RMVGA*DDPY	Borel et al. (2001), Uzel et al. (2001)
Pro-LOX-like	VAVGA*DSTG	Borel et al. (2001), Uzel et al. (2001)
Pro-LOX-like	VRSSA*DAPP	Borel et al. (2001), Uzel et al. (2001)
Laminin 332 γ_2	CYSGA*DENP	Veitch et al. (2003)
Laminin 332 α_3	QEPKA*DSSP	Veitch et al. (2003)
Probiglycan	FMMNA*DEEA	Scott et al. (2000a)
Decorin	FLMEA*DEAS	von Marschall and Fisher (2010a)
DMP1	EMQSA*DDPE	Steiglitz et al. (2004)
DSPP	SMQGA*DDPN	Tsuchiya et al. (2011), von Marschall and Fisher (2010b)
Myostatin	DVQRA*DDSS	Wolfman et al. (2003)
GDF11	DFQGA*DALQ	Ge et al. (2005)
LTBP		
N-term	IPSLA*DQEK	Ge and Greenspan (2006a)
C-term	YFIQA*DRFL	Ge and Greenspan (2006a)
Perlecan	SGGNA*DAPG	Gonzalez et al. (2005)
Chordin		
N-term	RSYSA*DRGE	Piccolo et al. (1997)
C-term	PMQAA*DGPR	Piccolo et al. (1997)
Osteoglycin	QLQKA*DEVI	Ge et al. (2004)
Gliomedin	AIPNA*DDTL	Maertens et al. (2007)

Cleavage sites with four adjacent amino acid residues (single-letter code) on either side of the scissile bond (marked by an asterisk *) are indicated
Procoll procollagen, *coll* collagen, *IGFBP3* insulin-like growth factor binding protein III, *LOX* lysyloxidase, *DMP1* dentin matrix protein 1, *DSPP* dentin sialophosphoprotein, *GDF11* growth and differentiation factor 11, *LTBP* latent TGFβ binding protein

In lower vertebrates BTPs have been studied in the zebrafish *Danio rerio* (Blader et al. 1997) and the frog *Xenopus laevis* (Holley et al. 1996; Ploper et al. 2011). Basic insight on BTPs has been gained for *Drosophila* tolloid, which cleaves the chordin homolog short gastrulation, the antagonist of the BMP2/4 homolog decapentaplegic (DPP) in fly embryos (Shimell et al. 1991), and for tolloid related (tolkin) from *Drosophila* larvae (Serpe and O'Connor 2006). BTPs are not only expressed in the ECM, but also in the developing and adult nervous system of both

invertebrates as the sea hare *Aplysia* (Liu et al. 1997) or the fruitfly *Drosophila* (Serpe and O'Connor 2006) and mammals (Clark et al. 1999; Scott et al. 2000b; Takahara et al. 1994, 1996).

Related to BTPs are the sea urchin proteases SPAN from *Strongylocentrotus purpuratus* (Reynolds et al. 1992) and blastula protein 10 (BP10) from *Paracentrotus lividus* (Lepage et al. 1992). They are expressed in the blastula stage and as BTPs they play roles in early gastrulation. For example they are involved in spicule formation, the larval skeleton of echinoderms. Their domain composition is similar to that of vertebrate BTPs (Fig. 7.1).

bmp1$^{-/-}$ mice have skeletal abnormalities and incomplete ventral body closure due to abnormal collagen fibrils; however, they do have bony skeleton because of the partially compensating effect of mTLL1 (Suzuki et al. 1996). mTLL1 deficiency, on the other hand, causes defects in heart compartmentalization (Clark et al. 1999). *mtll2*$^{-/-}$ mice have less muscle tissue (Lee 2008). mTld, the longer splice form derived from the *bmp1* gene is thought to circulate in the blood and to play a role in bone fracture healing (Grgurevic et al. 2011) and kidney fibrosis (Grgurevic et al. 2007).

Other matrix proteins like lysyl oxidase and lysyl oxidase like enzymes (Borel et al. 2001; Uzel et al. 2001), dentin matrix protein 1, and dentin sialophosphoprotein, osteoglycin and leucin-rich proteoglycans like biglycan (Scott et al. 2000a) and decorin (von Marschall and Fisher 2010a) are also activated by BTS proteases, which thereby promote extracellular matrix assembly in manifold ways. Likewise, BTPs proteolytically modify basement membrane-laminin-332 (Veitch et al. 2003) and perlecan (Gonzalez et al. 2005) (Table 7.1).

Transforming growth factor β (TGFβ, an important regulator of cell functions is released from latent TGFβ-binding proteins by BTPs (Ge and Greenspan 2006a). TGFβ-related factors like GDF8 and 11 (Ge et al. 2005) (Table 7.1) as well as myostatin acting as negative regulators of skeletal muscle growth are also activated by BTS proteases, which adds an intriguing facet to BTP function (Wolfman et al. 2003).

Other BTP substrates are IGFBPs (insulin growth factor binding proteins), as shown for IGFBP3, which regulate cell differentiation and development (Kim et al. 2011).

7.3.2 Meprin Proteases

Meprins are membrane-bound or soluble astacin proteases (Barnes et al. 1989; Beynon et al. 1981; Bond and Beynon 1995; Corbeil et al. 1993; Dumermuth et al. 1991; Johnson and Hersh 1992, 1994; Milhiet et al. 1994; Sterchi et al. 1982, 1988a, 2008; Broder and Becker-Pauly 2013). They are composed of an amino terminal pro-domain, followed by the astacin-like catalytic domain. Typical meprins also contain a MAM domain (meprin, A5 protein, and receptor protein tyrosine phosphatase μ (Beckmann and Bork 1993) (Sterchi et al. 1988b), a

TRAF domain (tumor necrosis factor receptor-associated factor) (Rothe et al. 1994; Zapata et al. 2001), an EGF-like domain, a transmembrane segment, and a cytosolic domain (Fig. 7.1).

There is a significant difference between the α subunit and the β subunit. Meprin α contains an additional I-domain inserted between the TRAF and the EGF-like domain, which can be cleaved by furin like enzymes (Fig. 7.1) during the passage through the endoplasmic reticulum and Golgi (Dumermuth et al. 1993; Gorbea et al. 1993; Grünberg et al. 1992; Hahn et al. 2000; Jiang et al. 1992; Kounnas et al. 1991; Milhiet et al. 1995; Sterchi et al. 2008). For this reason, meprin α is secreted as a soluble enzyme and forms high-molecular weight multimers up to megadalton size (Becker et al. 2003; Ishmael et al. 2001). Therefore it appears to be the largest extracellular protease reported so far. By contrast, meprin β homodimers and αβ heterodimers remain cell-surface-bound unless shed proteolytically (Hahn et al. 2003). Meprins are important for tissue differentiation and pericellular signaling. In this context, a variety of meprin substrates have been reported (Bertenshaw et al. 2001) including biologically active peptides such as gastrin and cholecystokinin, substance P, cytokines, and chemokines (for a review see Sterchi et al. 2008). Of special interest is the fact that meprins cleave components of the extracellular matrix (Kruse et al. 2004; Walker et al. 1998), in particular the basal lamina but also adhesion proteins at the cell-cell interface (Ambort et al. 2010; Huguenin et al. 2008; Sterchi et al. 2008; Vazeille et al. 2011).

Recent proteomics approaches have identified previously known and new physiologically relevant *in vivo* substrates such as vascular endothelial growth factor (Schütte et al. 2010), amyloid precursor protein (Jefferson et al. 2011), procollagens I and III (Kronenberg et al. 2010), interleukin-1β (Herzog et al. 2005), interleukin 18 (Banerjee and Bond 2008), pro-kallikrein 7 (Ohler et al. 2010), and fibroblast growth factor 19 (Becker-Pauly et al. 2011). Their ability to cleave procollagens at exactly the same sites as BTPs sheds additional light on the differential function of meprins in health and disease (Becker-Pauly et al. 2007) (Kronenberg et al. 2010; Broder et al. 2013).

Meprins have been found to be implicated in various pathological situations. The processing of interleukins by meprins might explain the observation that meprin$^{-/-}$ mice have deficiencies in their immune system (Bylander et al. 2008; Crisman et al. 2004; Sun et al. 2009). Meprins are also linked to intestinal disorders like inflammatory bowel disease (Banerjee et al. 2009), Crohn's disease (Vazeille et al. 2011), and celiac disease (Lottaz et al. 2007). Furthermore they are linked to atherosclerosis (Gao et al. 2009), kidney disorders (Bylander et al. 2008; Carmago et al. 2002; Herzog et al. 2007; Mathew et al. 2005; Oneda et al. 2008; Red Eagle et al. 2005; Takayama et al. 2008; Yura et al. 2009) and tumor metastasis (Heinzelmann-Schwarz et al. 2007; Matters et al. 2005; Rösmann et al. 2002).

Recent observations of meprins in Alzheimer's disease support their basic regulatory signaling functions. The amyloid precursor protein is obviously processed by meprin β at several sites including the β-secretase site (Jefferson et al. 2011; Bien et al. 2012).

7.3.3 Hatching Enzymes

The embryonic development of many aquatic invertebrates, e.g. crustaceans, fishes, amphibians and reptiles, takes place in free ambient water. The eggs and embryos of these organisms are protected by a durable envelope of extracellular matrix (also termed *zona pellucida* or vitelline membrane), which has to be cleaved off after a certain stage of embryogenesis to release the young larva. This process is called 'hatching' and is frequently triggered by proteolytic enzymes.

Among the members of the astacin family there is a considerable number of so-called 'hatching enzymes' (Figs. 7.1 and 7.2). Some of these are composed only of a pro-domain and an astacin-like catalytic domain, others additionally contain carboxy terminal CUB domains or other protein modules. Examples include crayfish embryonic astacin (Geier and Zwilling 1998), and the 'low choriolytic enzymes' (LCE) and 'high chorolytic enzymes' (HCE), first described in teleost fishes (Yasumasu et al. 1996), or corresponding proteases from amphibians like the UVS.2 gene product from the frog *Xenopus laevis* (Fan and Katagiri 2001; Katagiri et al. 1997; Sato and Sargent 1990).

The hatching process has been studied most intensively in the medaka fish *Oryzias latipes* and other euteleosts. In these organisms, the hatching seems to be organized in a conserved fashion. Enzymes of the HCE type cleave *zona pellucida* proteins at specific sites, which allow for swelling of the envelope. In a second step, enzymes of the LCE type then completely digest the egg envelope (Hiroi et al. 2004; Kawaguchi et al. 2006, 2010a, b; Sano et al. 2010; Yasumasu et al. 2010a, b). LCE and HCE hatching enzymes are distinguished by an additional pair of cysteine residues in the amino terminal segments of their catalytic astacin-like domains (Figs. 7.2 and 7.3).

Closely related to hatching enzymes is nephrosin from carp head kidney (Hung et al. 1997). The physiological function of this protease is not clarified yet. However, since it is expressed in the head kidney, which is an ancient hematopoetic organ, it might be involved in immune defense and/or general blood cell differentiation.

Also sequentially related to hatching enzymes is bird CAM1 (chorio allantoic membrane protein 1) from the Japanese quail, which is important for calcification of the egg shell (Elaroussi and DeLuca 1994).

Other hatching-type enzymes are alveolin fom medaka fish (Shibata et al. 2000) and ovastacin from mammals (Quesada et al. 2004). Both are involved in *zona pellucida* hardening (ZPH). Ovastacin cleaves *zona pellucida* protein 2 at a highly conserved diacidic site (Burkart et al. 2012), typical for astacins (Becker-Pauly et al. 2011),

which results in ZPH immediately after sperm penetration in order to prevent polyspermy; this is supported by the presence of ovastacin in cortical granules of the egg, which are released into the *zona pellucida* upon sperm penetration (Burkart et al. 2012). As observed recently, ovastacin is controlled by fetuin B, a cystatin-like plasma protein, in order to prevent premature ZPH (Dietzel et al. 2013). Another report on ovastacin suggested also a role in sperm-egg interaction (Sachdev et al. 2012).

7.3.4 Seminase Activated Astacin-Like Protease

The seminal fluid of many animal taxa contains proteases and protease inhibitors. In mammals these are important for regulating seminal clot liquefaction, for example. An intriguing protolytic network has recently been discovered in the seminal fluid of the fruitfly *Drosophila melanogaster*. During mating of fruit flies, seminal fluid proteases are transferred from males to females (Ravi Ram et al. 2006). These enzymes form a cascade and one of them is an astacin-like metalloprotease (Ayroles et al. 2011; Sirot et al. 2009). Obviously, this protease cascade is required for post-mating responses in the female. In the first step, a serine protease termed 'seminase' (CG10586) activates the astacin protease CG11864, which subsequently cleaves the sex peptide ovulin and other seminal plasma proteins. These observations indicate an important role for this protease cascade to temporally regulate many responses in females after mating (LaFlamme et al. 2012).

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