

Review

Propeptides as modulators of functional activity of proteases

Ilya V. Demidyuk*, Andrey V. Shubin, Eugene V. Gasanov and Sergey V. Kostrov

Institute of Molecular Genetics, Russian Academy of Sciences, Kurchatov Sq. 2, Moscow 123182, Russia

*Corresponding author
e-mail: duk@img.ras.ru

Abstract

Most proteases are synthesized in the cell as precursor-containing propeptides. These structural elements can determine the folding of the cognate protein, function as an inhibitor/activator peptide, mediate enzyme sorting, and mediate the protease interaction with other molecules and supramolecular structures. The data presented in this review demonstrate modulatory activity of propeptides irrespective of the specific mechanism of action. Changes in propeptide structure, sometimes minor, can crucially alter protein function in the living organism. Modulatory activity coupled with high variation allows us to consider propeptides as specific evolutionary modules that can transform biological properties of proteases without significant changes in the highly conserved catalytic domains. As the considered properties of propeptides are not unique to proteases, propeptide-mediated evolution seems to be a universal biological mechanism.

Keywords: folding; inhibition; protein interaction; protein precursor; sorting.

Introduction

On numerous occasions, proteins substantially change in the period from their synthesis to degradation. Often they are synthesized in the cell as precursors; later, these precursors lose sequence fragments to form new species, each of which can have different physicochemical and biological properties. In some cases, the removed fragments direct their proteins along a secretory pathway. Such fragments share a typical structural organization and are called signal peptides [reviewed in Ref. (1)]. Apart from signal peptides, there are other removed fragments called propeptides, prosequences or proregions.

To date, different functions of propeptides including four major functions are recognized. First, proregions can function as intramolecular chaperones (2) or folding assistants (3) by determining the three-dimensional structure of their protein. Second, they can function as inhibitors or activation peptides by maintaining the proteins (commonly enzymes) that contain them inactive. Third, prosequences can direct

protein sorting into specific cellular compartments or extracellular space. Fourth, they can mediate the precursor interaction with other molecules (such as peptides, proteins, and polysaccharides) or supramolecular structures (e.g., cell walls). It should be noted that a single propeptide can perform several or even all these functions.

At the same time, a growing body of data demonstrates that propeptides can modulate protein functional activity irrespective of their specific role or mechanism of action. They make it possible to substantially alter biological properties of proteins without cardinal changes in the major functional (e.g., catalytic) domains of the molecules. This seems to be the key property of prosequences that allows propeptides to regulate protein activity at the post-translational level and to function as specific evolutionary modules providing for functional variation of protein molecules.

The range of proteins synthesized as propeptide-containing precursors is very wide; it includes structural proteins, hormones, cytokines, various enzymes, and their inhibitors. [A list of examples, although incomplete, can be found in Ref. (4).] Proteases are prominent among such proteins, as their synthesis as a proenzyme is typical of most representatives of this vast group (5). Thus, it is not surprising that proteolytic enzymes considered in the current review have become one of the main models to study the propeptide functions and mechanisms of action.

Propeptides assisting protein folding

The requirement of the propeptide for the active protein formation was originally demonstrated for subtilisin E (SbtE), a secretory serine protease of *Bacillus subtilis* (6). Later, similar data were obtained for another bacterial secretory enzyme of the same catalytic type, *Lysobacter enzymogenes* α -lytic proteinase (7, 8). To date, the involvement of prosequences in the folding has been demonstrated for a variety of proteases of all major catalytic types and different organisms (9–40). At the same time, subtilisin (Sbt) and α -lytic protease (α LP) remain the most thoroughly developed models that contributed most to our understanding of propeptide-assisted folding and the underlying mechanisms. Prosequence-assisted folding of proteins, largely proteases, has been reviewed previously (3, 4, 41–43), and here we will briefly consider its main aspects significant for discussion.

Propeptide-assisted folding means that an unfolded protein without prosequence cannot form the proper biologically active three-dimensional structure. This applies to both *in vitro* denatured mature proteins and proteins synthesized

without propeptides in artificial expression systems. An active protein can be produced after the propeptide is added to the unfolded protein *in trans*, i.e., they are not covalently bound (7, 14, 15, 31, 44–47). For the purpose of completeness, even when the prosequence is a folding assistant, the protein can fold under specific conditions without the propeptide, although it is usually much less efficient (45, 48–52).

Direct transition of an unfolded protein (U) into the native catalytically active form (N) in the absence of the propeptide is thermodynamically forbidden as demonstrated for Sbt, α LP, and protease B of *Streptomyces griseus*. This is due to a higher stability (lower free energy) of the unfolded conformation than the native conformation in such proteins (Figure 1) (53–55). In the absence of the propeptide, they transform into a partially folded stable intermediate (I) with the conformation similar to that of a molten globule and lower free energy than N. In addition, the I is separated from the kinetically trapped N by a high-energy barrier (Figure 1A). After the propeptide (P) is added *in trans*, the I•P complex is formed and the energy barrier is lowered, which allows the fast formation of the thermodynamically stable N•P complex. The metastable native state, protected from the transition into the unfolded conformation by the same energy barrier, is formed after the propeptide degradation, which is usually autocatalytic in active proteases (53, 55, 56). Thus, the propeptide actually catalyzes the protein folding similar to an enzyme [‘foldase’ (57)].

The folding energy profile of the full-length precursor with covalently bound mature and propeptide parts is similar to the *in trans* folding described above (Figure 1B). In the case of Sbt and α LP, the unfolded precursor (Up) was shown to transform into the intermediate (Ip) analogous to the non-covalent I•P complex in the molten globule state. Then, the Ip is folded into the thermodynamically stable propeptide-mature part complex (P-N), which enters the native state after the propeptide is removed (54, 58).

Thus, in all studied cases, the native state of proteases with the propeptide-mediated folding is not a global energy minimum, and the protein folding is under kinetic rather than thermodynamic control (59). This is advantageous in the following respects. As against a thermodynamically stable state, the metastable native state with a high energy barrier of transition to the unfolded form has high rigidity and, consequently, high resistance to harsh environments (54), such as

proteolytic degradation (55, 60, 61), high temperature (62), or low pH (63).

The transition of protein folding from the thermodynamically controlled propeptide-independent pathway (Figure 1C) to the kinetically controlled propeptide-mediated pathway is probably an important evolutionary mechanism. This can be exemplified by bacterial subtilisin-like proteases (subtilases) including proteins both with and without the propeptide. Intracellular and extracellular bacterial subtilases have highly conserved primary structure (more than 50% identity), similar three-dimensional organization of the catalytic domains and closely resembling catalytic activities; however, the intracellular enzymes have no propeptides. A study of folding of two homologous proteins of *B. subtilis*, secretory SbtE and intracellular serine protease 1 (IPS1), has demonstrated substantially different folding pathways and kinetics. SbtE folding requires the propeptide to form a kinetically stable molecule at a local energy minimum. IPS1 folding is more than a million times faster, does not depend on the propeptide, and gives rise to a thermodynamically stable protein (54). Thus, the propeptide makes possible cardinal changes in the energy state of the active enzyme. This requires minimum modifications in the catalytic domain that are largely limited to substitutions of individual surface amino acids without affecting the hydrophobic core so that the catalytic activity is retained. Essentially, the propeptide allows a single protein structure to form two principally different molecules: a high-stability molecule persists in aggressive extracellular environments (42, 61), whereas the other molecule is probably optimal for the intracellular protein turnover (54).

In addition, the kinetic stability make possible the mechanisms of adaptation to harsh environmental conditions unavailable for thermodynamically stable proteins, as demonstrated by comparative analysis of the structure and unfolding behavior of two homologous kinetically stable proteins, acid-resistant *Nocardiopsis alba* protease A (NAPase) and neutrophilic α LP. As the unfolded state is not essential for the stability of kinetically stable proteins, the native state can be arbitrarily destabilized if the intermediate state is equally destabilized at the same time. This is not difficult to realize as demonstrated for the α LP model, as the native and intermediate forms differ in a small number of interactions as compared to the unfolded and native forms in thermodynamically stable proteins. Thus, the kinetic stability allows

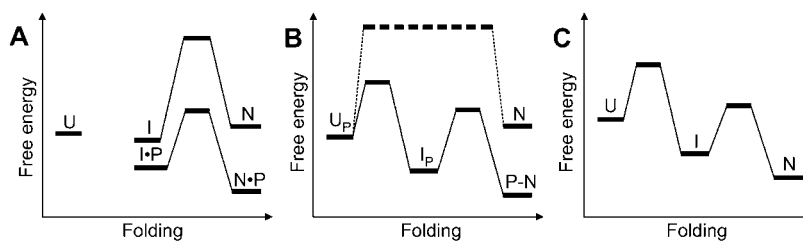


Figure 1 Energy diagrams of protein folding reactions.

Kinetically controlled propeptide-dependent folding *in trans* (A), *in cis* (B), and thermodynamically controlled propeptide-independent folding (C). See text for explanation.

rapid evolutionary adaptations to environmental changes and provides a flexible longevity strategy in harsh environments (63).

The modulation of protein functional activity by folding-assistant propeptides can also be mediated by protein memory (64). This phenomenon consists of different three-dimensional organization and properties of proteins with identical sequences but folded in the presence of different propeptides. Initially, protein memory was discovered in the study of a SbtE mutant with an Ile(-48)→Val substitution (position +1 corresponds to the first amino acid in the mature enzyme) (64). The resulting protein differed from the wild-type mature enzyme by the secondary structure, thermostability, and substrate specificity. Conformers with properties different from the wild type were also observed in SbtE with an Ile(-48)→Thr mutation in the propeptide (65). Such structural imprinting can be exemplified by cathepsin E, an aspartyl protease, folded in the presence of the propeptide of a highly similar cathepsin D. The resulting protein differs from the wild type in the catalytic efficiency and specificity towards protein inhibitors (37). We have also demonstrated different primary substrate specificity of two derivatives of *Thermoactinomyces* sp. 27a metalloproteinase (66) with identical primary structure folded with the cognate propeptide *in cis* and *in trans* (unpublished observation).

As all current examples of protein memory are available for artificial model systems, the natural significance of this effect remains unclear. However, analysis of published data suggests that protein memory can substantially modulate the functioning of certain proteases *in vivo*, e.g., mammalian proprotein convertases (PCs) homologous to bacterial subtilases. Enzymes of this group hydrolyze peptide bonds in many proteins and peptides localized in different subcellular compartments (67). PC propeptides were shown to assist in protein folding (35, 68). Point mutations in their prosequences can influence the folding and modulate the catalytic properties up to the inactive enzyme formation (68), which can affect processing and, consequently, alter the functioning of the substrate proteins (64).

In summary, folding assistant propeptides can modulate properties of the cognate proteases. Modifications in such prosequences can alter the enzyme functions without or with minor changes in the primary structure of the catalytic domains, which probably underlies one of the mechanisms of protein evolution.

Propeptides maintaining the inactive state of proteins

Maintaining enzymes in inactive state is probably the best known function of propeptides. It is of physiological significance because the controlled activation of latent protease precursors (zymogens) underlies fundamental biochemical processes such as blood clotting, complement activation, and digestion. It is hardly possible to consider all such specific mechanisms of protease suppression by prosequences in the present review. It is of importance that the structure of pro-

peptides maintaining the precursors inactive largely determines the functioning of the cognate proteins.

Propeptides can maintain proteases in catalytically inactive state by at least two mechanisms. The first mechanism can be exemplified by widely known pancreatic serine proteases trypsin and chymotrypsin as well as by structurally related kallikreins. Precursors of all these proteins have a short N-terminal propeptide, an activation domain stabilizing the inactive conformation. Proteolytic cleavage of the peptide bond between precursor residues 15 and 16 (chymotrypsinogen numbering system) induces structural changes in the molecule resulting in a salt bridge between the new N-terminal amino acid 16 (usually Ile) and the carboxyl group of Asp194, which leads to the formation of the substrate-binding site and to enzyme activation (69–73). Although the catalytic domains are highly similar and the activation mechanism is the same, the activation of these enzymes in mammals is regulated in different ways depending on the structure of their propeptides, primarily the processing sites.

The activation domain of trypsin consists of eight amino acids with the DDDDK sequence at the C-terminus. This sequence corresponds to the substrate specificity of enterokinase (enteropeptidase), an enzyme realizing highly selective processing of trypsinogen exclusively after it is released from the intercellular secretory granules to the duodenum (74). In addition to the recognition by enterokinase, the cleavage site structure prevents the precursor hydrolysis by mature trypsin, which allows a strict control of the active enzyme in the intestine. This is due to the presence of four negatively charged amino acids (75) despite a lysine upstream of the bond to cleave [P1 position according to the Schechter and Berger nomenclature (76)]. Next, trypsin concentration determines the removal of the 15-amino acid propeptide of chymotrypsin, the processing site of which is much simpler and contains arginine at the P1 position corresponding to the specificity of the activating enzyme. Such a structure of the site also makes chymotrypsin resistant to autoprocessing (77). Thus, the place and sequence of activation events of pancreatic proteases are determined by the C-terminal amino acid sequence of the propeptides and by its exact conformity to the substrate specificity of the processing enzymes.

The propeptide structure is also important for the activation of human kallikreins found in diverse tissues and biological fluids (78). However, the situation is not so unambiguous in this case. The processing sites of most kallikreins contain arginine or lysine at P1 excluding kallikrein 4 with P1 glutamine (79). The substrate specificity varies between kallikreins. Kallikreins 2, 4–6, 8, and 12–14 have trypsin-like substrate specificity (80–83). Kallikreins 1, 10, and 11 can hydrolyze both trypsin and chymotrypsin substrates (81, 84). The substrate specificity of kallikreins 3 and 7 (81) and, possibly, 9 and 15 is similar to that of chymotrypsin. In contrast to pancreatic proteases, this situation allows autoprocessing in most cases. This can be significant for the activation signal amplification and, considering the co-expression of kallikreins in many tissues, assumes a complex activation cascade (79, 85) involving other proteolytic enzymes apart from kallikreins (86).

Activation peptides are not exclusive for eukaryotic enzymes. An activation mechanism similar to that of mammalian chymotrypsin-like proteases is probably utilized by some bacterial glutamyl endopeptidases, which belong to the same structural family. Their precursors have no detectable proteolytic activity (39); the substrate-binding site is formed after propeptide removal (87, 88); and the cellular production of active glutamyl endopeptidases largely depend on the processing site structure (89, 90).

These examples of the chymotrypsin family of serine proteases indicated that the local modifications in the structure of the propeptide activation modules can substantially change the functioning of the corresponding proteins in the living systems. However, we have already mentioned another mechanism to maintain proteases in inactive state; moreover, it can be more common. Many propeptides are inhibitors of the cognate proteolytic enzymes. The precursors with such prosequences are inactivated as a result of blocked active site rather than of changed conformation of the catalytic domain. This provides for a functional distinction: in the case of activation modules, a breakage of the covalent bond between the prosequence and catalytic domain is the only event required for the activation; whereas in the case of propeptide inhibitors, the noncovalent inhibitory complex should also be broken.

The inhibition of the cognate propeptide was demonstrated for many proteases from diverse organisms. Such enzymes include serine (68, 91–101), cysteine (32, 40, 102–118), aspartyl (119), and glutamate (120) proteases as well as metalloproteinases (23, 33, 34, 94, 121–125). The molecular details of the contacts between propeptide inhibitors and their cognate proteases significantly vary. Consequently, the inhibition constants also widely vary (Table 1). Apparently, the realization of biological functions in different proregion-catalytic domain pairs requires highly different interaction-forces. However, the effect of many propeptides is highly selective. The prosequence inhibition of the cognate mature protease and closely related catalytic domains of other proteins can vary by several orders of magnitude (23, 40, 96, 97, 99, 101, 103, 105–110, 114, 119, 123, 127) and even involve different mechanisms (96, 127). Mutations in the propeptides including point mutations can have a profound impact on inhibition efficiency (32, 100, 101, 120, 127–131). Overall, this suggests that prosequence modifications can modulate their inhibitory capacity and, thus, the functioning of proteolytic enzymes.

Apparently, the main function of propeptide inhibitors is the same as the function of activation domains: to avoid undesirable activation of the protease and to provide the mature enzyme formation in the right place and/or time. However, it is not easy to obtain data on the effect of altered inhibitory capacity of propeptides on the function of individual proteins. On the one hand, this can only be done *in vivo* unlike studies on the proper inhibitory effect. On the other hand, propeptide inhibitors can be folding assistants and mediate secretion at the same time (9, 23, 27, 46, 91, 122, 127), and it is hardly possible to identify the contribution of each component to the observed total effect. Nevertheless,

some detailed studies clearly demonstrate the relationship between the prosequence inhibitory capacity and the functioning of the associated protease.

Pseudomonas aeruginosa elastase (PAE), a zinc-containing metalloproteinase of the thermolysin structural family, is encoded by the *lasB* gene and is synthesized as a preproprotein (132). The prosequence is a signal peptide directing the enzyme through the inner cell membrane (133). The propeptide provides for the PAE folding in the periplasm (19, 46), which leads to proenzyme autocatalytic processing (134). However, the propeptide not covalently bound to the protein remains in complex with the catalytic domain (133) and blocks its proteolytic activity (122). Then, the propeptide-enzyme complex is secreted and dissociates during or after the translocation across the outer membrane. In the extracellular space, the propeptide is degraded, apparently, by the proper PAE (135, 136). Thus, the propeptide folding and inhibitory activities are separated in this system. After the folding, the propeptide only inhibits the enzyme activity, which exposed the effect of the strength of the propeptide-elastase inhibitory complex on the active enzyme production by bacteria.

The *lasB* gene was introduced into *Pseudomonas putida* cells but, unexpectedly, no extracellular activity of the enzyme could be detected (137). Immunoblotting and co-immunoprecipitation analysis using antibodies against mature elastase and its propeptide demonstrated that the bulk of the enzyme is localized in the cell as a noncovalent complex with the propeptide. Thus, the PAE maturation including protein transport across the inner membrane, folding, and autocatalytic processing was not affected in *P. putida*, but no efficient secretion of the enzyme from the cell took place. However, substantial elastase quantities were detected in the extracellular space also in a complex with the propeptide. Thus, active enzyme was not produced after heterologous expression owing to a very strong inhibitory complex, whereby its dissociation probably requires a specific host cell factor. Point mutations Ala(-15)→Val or Thr(-153)→Ile destabilized the complex, which resulted in efficient propeptide degradation and active extracellular elastase production with no changes in its maturation, intercellular accumulation, or secretion rate (130).

Another example illustrating the significance of changes in the propeptide inhibitory properties for the cognate protein functioning is tripeptidyl-peptidase I (TPPI), a mammalian serine protease of the sedolisin family. The enzyme is active in lysosomes, where it cleaves off N-terminal tripeptides of small unstructured polypeptides. TPPI is synthesized as a precursor with a signal peptide and a propeptide (138, 139). The latter is removed autocatalytically by the intramolecular mechanism (140). TPPI propeptide proved to be an efficient inhibitor of the mature enzyme (Table 1) (100). The interest in this protein is largely due to classic late-infantile neuronal ceroid lipofuscinosis, a severe hereditary disease caused by natural mutations in the TPPI gene [reviewed in (141)]. To date, two of disease-causing mutations have been found in the prosequence: Gly77→Arg and Ser153→Pro. *In vitro* analysis demonstrated that the Gly77→Arg mutation caused

Table 1 Parameters of inhibition of some proteases by their cognate propeptides.

Protease	K_i or IC_{50}^a , nM	References
Aspartic/glutamic proteases		
Cathepsin D (human)	30	(119)
Aspergilloglutamic peptidase (<i>Aspergillus niger</i>)	27	(120)
Pepsin (chicken)	<10	(119)
Serine proteases		
Serine protease (<i>Aspergillus fumigatus</i>)	5300	(94)
Subtilisin E (<i>Bacillus subtilis</i>)	540	(92)
Kex2p (<i>Saccharomyces cerevisiae</i>)	160 ^a	(98)
Cucumis (Cucumis melo L.)	6.2	(101)
Proprotein convertase PC1/3 (mouse)	6	(96)
Furin (human)	4 ^a	(97)
	2	(99)
Tripeptidyl peptidase I (human)	3.55	(100)
Proprotein convertase PC5/6 (human)	0.8	(99)
Subtilisin BPN' (<i>Bacillus amyloliquefaciens</i>)	~0.5	(93)
Proprotein convertase LPC/PC7/8 (human)	1.3	(99)
Proprotein convertase LPC/PC7/8 (rat)	0.4 ^a	(97)
α -Lytic protease (<i>Lysobacter enzymogenes</i>)	0.05–0.2	(91)
Cysteine proteases		
Proteinase IV (papaya)	860	(103)
Cathepsin L (<i>Paramecium tetraurelia</i>)	60 ^a	(106)
	20.9	(126)
Cathepsin V (human)	10.2	(115)
Protease Der p 1 (<i>Dermatophagoides pteronyssinus</i>)	7	(116)
Cathepsin K (human)	5.5	(109)
	2.61	(110)
	0.630	(40)
Papain (papaya)	1.89	(103)
Cathepsin L1 (<i>Fasciola hepatica</i>)	1.73	(108)
Cathepsin B (rat)	0.4	(102)
Falcpain-2 (<i>Plasmodium falciparum</i>)	0.3	(111)
Cathepsin S (human)	7.6	(109)
	2.5	(117)
	0.27	(107)
	0.05	(32)
	0.049	(40, 126)
Cathepsin L (human)	0.12	(109)
	0.088	(105)
	0.018	(40)
Cruzipain (<i>Trypanosoma cruzi</i>)	0.018	(114)
Metalloproteases		
TNF- α -converting enzyme (human)	70 ^a	(124)
PA protease (<i>Aeromonas caviae</i>)	69	(34)
Thermolysin (<i>Bacillus thermoproteolyticus</i>)	6	(23)
Metalloprotease (<i>Aspergillus fumigatus</i>)	3	(94)
Carboxypeptidase A (pig)	1.9	(121)
Metalloprotease (<i>Brevibacillus brevis</i>)	0.17	(123)

^a IC_{50} is the concentration of an inhibitor at which half of the maximum enzyme activity is observed.

an 80-fold decrease in the efficiency of propeptide binding to the TPPI catalytic domain and a significant change in the inhibition mechanism: in contrast to the intact propeptide, a slow-binding inhibitor of the enzyme, the mutant propeptide rapidly reaches equilibrium. *In vivo*, this mutation leads to a substantial retention of the proenzyme in the endoplasmic reticulum, suppresses its secretion, and significantly (almost 10-fold) decreases mature TPPI activity in the lysosomes (100).

These examples suggest that, similar to folding assistants, prosequences that maintain the protein in inactive state (both activation peptides and inhibitors) can modulate the functional activity of the associated proteases. Minor variations in the propeptides (e.g., point mutations) can change the activation place and time of the cognate catalytic domains. On the one hand, this can cause functional disorders; on the other hand, it is a possible pathway of functional evolution of proteins.

Propeptides mediating protein sorting

Propeptides are attributes of most secretory proteases and numerous publications report their involvement in the sorting of cognate enzymes in the cell (11, 15, 17–20, 25, 32, 35, 46, 47, 142–164). In some cases, this effect is due to the requirement of correct protein folding for the translocation across cell membranes, i.e., to the folding assistant function of propeptides discussed above. However, the dependence of protein migration in the cell on the specific interaction between the prosequence and the sorting system has been demonstrated for a variety of proteases.

The propeptide-mediated mechanisms of secretion of bacterial proteolytic enzymes are not clearly understood. Apparently, the propeptides primarily determine the protein conformation required for its secretion. Accordingly, the removal of the prosequence without affecting the signal peptide usually inhibits the secretion (17, 19, 20, 46, 158). It is of interest that the coexpression of such deletion variants with the artificial genes encoding for the propeptide fused to the signal sequence restores protein release from the cell (19, 20, 47). Finer modifications of propeptides also significantly affect the enzyme secretion by bacteria. For instance, a C-terminal fragment deletion in the propeptide (23 out of 222 amino acids) delayed the release of *Bacillus cereus* neutral proteinase by several hours and decreased the protein production by 75% (150). N-terminal deletions in the propeptide of *Streptomyces griseus* protease B (4, 10, 15, and 20 out of 76 amino acids) decreased the extracellular protein quantities by 40–99%; protease secretion correlated with the length of the propeptide (25). Point mutations in the prosequences can change active protease quantities in culture medium (159, 163) and lead to the enzyme accumulation in the periplasm of Gram-negative bacteria (159). Propeptide modifications can also be positive and increase the secretory protein production by the cell (163). Thus, structural changes in prosequences of bacterial proteases can change the secreted enzyme quantities as well as the time or rate of their release from the cell.

A demonstrative example of prosequences of bacterial proteases as a factor of cognate enzyme functioning *in vivo* is the propeptide-mediated regulation of *Listeria monocytogenes* metalloproteinase (Mpl) zymogen location during intercellular infection. This enzyme together with broad-range phospholipase C (PC-PLC) underlie *L. monocytogenes* escape from the vacuole, where the pathogen resides after the entry into the host cell, to the cytosol (which is crucial for the bacterial ability to replicate in eukaryotic cells) (165). In all probability, PC-PLC hydrolyzes phospholipids of the vacuolar membrane, whereas Mpl controls phospholipase translocation across the bacterial cell wall. Both enzymes are synthesized as precursors with N-terminal prosequences. In both cases, the propeptides hold inactive zymogens at the membrane-cell wall interface but are not essential for the formation of active enzymes (164, 166). The colocalization of the proteins allows the bacterial cell to rapidly release large quantities of phospholipase. At low pH typical for vacuoles, Mpl is autocatalytically activated (167). The metal-

loproteinase processes PC-PLC, which consequently leads to vacuolar membrane degradation, and *L. monocytogenes* escape to the cytosol (166).

Correct three-dimensional structure is also important for the sorting of eukaryotic proteases. Several publications demonstrate that deletions or modifications of the folding assistant propeptide affect protein transport (11, 12, 15, 32, 35, 37, 149, 152, 155, 161). Nonetheless, prosequences of eukaryotic enzymes can specifically interact with the cellular sorting factors. For instance, vacuolar sorting signals of yeast carboxypeptidase Y and proteinase A are within their propeptides (142, 144–146), which directly interact with receptor proteins Vps10p and, probably, Vth1p, factors of protease localization in the cell (168). pH-dependent procathepsin L association with the membrane is mediated by 9-amino acid sequence in the propeptide, which binds to the integral membrane receptor protein (151, 169). Such interaction is critical for cathepsin L delivery to lysosomes in primitive eukaryotes that lack the alternative lysosomal sorting pathway (170). Neutrophil elastase targeting to the plasma membrane depends on the C-terminal propeptide (160). The glycosylation of a specific amino acid in the prosequence of metalloproteinase ADAMT9 is essential for this enzyme secretion (161). After cathepsin E propeptide is replaced with that of cathepsin D, a related lysosomal aspartyl protease, the chimeric protein retains the capacity to form the catalytically active enzyme and to be processed; however, it cannot reach the ultimate destination in the cell (37). Thus, propeptide structural modifications in eukaryotic proteases affecting their sorting signals can alter protein transport in the cell and, consequently, alter its biological functions.

The modulation of protease functions by the propeptide can be clearly illustrated by the sorting of human cathepsin B (CB). Preprocathepsin B consists of a signal peptide, prosequence, and catalytic domain. The signal peptide cotranslationally targets the protein to the endoplasmic reticulum lumen, after which the signal peptide is removed. CB sorting to the lysosome follows the mannose 6-phosphate pathway and depends on the Asn-38 glycosylation in the propeptide (171) [although an alternative mechanism was demonstrated in some cell types (172, 173)]. However, this signal can be eliminated by alternative pre-mRNA splicing. A total of 13 exons have been identified in the CB gene and several combinations of them are possible. Most transcripts differ in the 5'- and 3'-untranslated regions; however, a transcript encoding an N-terminally truncated cathepsin B (tCB) was found in the normal and rheumatoid synovial fluids, normal and osteoarthritic cartilage tissue, and some cancers. Such transcript lacks exons 2 and 3. Because exon 3 contains the start codon in the full-length mRNA, tCB synthesis starts from an alternative codon corresponding to Met52 in exon 4. Thus, tCB lacks the signal peptide as well as 34 amino acids of the propeptide [reviewed in Ref. (174)]. This protein cannot give rise to the catalytically active form after expression in eukaryotic cells (38) and it is targeted to mitochondria (175) rather than to lysosomes (171). An amphipathic α -helix of the propeptide serves as the mitochondrial sorting signal, which becomes N-terminal and functionally active after the

removal of 51 amino acids (175). Cell culture transfection with a construct encoding tCB causes their death with nuclear fragmentation indicative of apoptosis (171, 176). It was proposed that the truncated procathepsin B provides for a physiological mechanism of cell death in tissues with slow turnover and populated by long-lived cells (174). These data illustrate another way of propeptide-mediated modulation through the cellular synthesis of enzymes with different prosequences, which cardinally change protein functioning.

A remarkable example of a principally different propeptide-mediated mechanism of protein transport control is the sorting of a part of cathepsin L (CL), a lysosomal protease, to the nucleus, where this enzyme processes nuclear proteins and, thus, mediates cell cycle regulation. CL targeting in the cell changes as a result of alternative translation giving rise to enzyme isoforms without the signal peptide, which prevents protein transport to the endoplasmic reticulum and activates an unidentified nuclear localization signal. The alternative translation is initiated at any of six (in murine CL) methionine codons in the propeptide portion of the mRNA. Thus, the propeptide structure can allow CL nuclear sorting, although the propeptide is not directly involved in it. A modification of one of several methionine codons changes the quantities of nuclear isoforms and a substitution of all of them blocks CL sorting to the nucleus (177). Hence, in this case, changes in the prosequence structure also have an impact on protease functioning in the cell.

Thus, propeptides involved in sorting can modulate functional properties of the cognate proteases. Mutations in the prosequences can substantially change cell localization of the proteins and modify their functional properties. The available examples demonstrate evolutionary emergence of systems where this propeptide property gives rise to enzymes with alternative biological activity based on the same catalytic domain.

Propeptides providing for precursor interaction with other molecules or supramolecular structures

An increasing number of publications indicate that protease propeptides can interact with other partners apart from the associated catalytic domain or components of the cell secretion system. These data are not yet abundant and the molecular details as well as the role of such interactions remain largely unclear. However, the examples discussed below suggest that the propeptide capacity to bind a wide range of molecules and supramolecular structures is highly important for the functioning of cognate proteins.

The *in vivo* action of proteases is not always determined solely by their catalytic activity. They can also serve as ligands, whereby binding to receptor induces a specific cellular response. Such dual effect is probably best studied in the model of cathepsin D, a lysosomal aspartyl protease synthesized as a precursor with the N-terminal propeptide, an inhibitor of the mature enzyme and a factor of protein folding and sorting. Procathepsin D (pCD) secreted by cancer cells proved to be a mitogenic factor (178–185) and this effect of pCD did not depend on proteolytic activity (180, 183, 186, 187). Experimental data indicate that pCD binds to the sur-

face of tumor cells (188, 189), which suggests pCD interaction with an unidentified signaling receptor (190). Nonetheless, both binding and mitogenic potential of pCD is determined by the prosequence (180, 183, 188, 191). Moreover, the propeptide *per se* stimulates tumor cell growth *in vitro* and *in vivo* (180–185, 188, 192, 193). The ability to bind receptors is also observed in the propeptide of cathepsin X (CX), a lysosomal cysteine protease with a high expression level in the immune system (194–196) and cancer cells (197, 198). The propeptide of CX contains an RGD sequence unique for lysosomal cysteine proteases. This motif provides for the CX binding to integrin $\alpha_v\beta_3$, which suggests that CX in the extracellular space can modulate the migrating cell adhesion to extracellular matrix components (199). The interaction with intestinal alkaline phosphatase (IAP) and heat shock cognate protein 70 (HSC70) was demonstrated for the propeptide of cathepsin C (CC), another lysosomal cysteine protease, which is actively produced in intestinal epithelial cells. The expression of CC propeptide in Caco-2 cells that share properties with small intestinal epithelial cells proved to decrease IAP activity associated with its degradation. Because the HSC70 interaction is an essential stage in chaperone-mediated lysosomal protein degradation, CC propeptide in a complex with HSP70 and IAP was proposed to stimulate IAP sorting to the lysosome (162).

Current experimental data are supplemented by sequence analysis demonstrating that protease propeptides can contain conserved binding domains found in many other proteins apart from proteases. For instance, such domains are found in the C-terminal regions of precursors of thermolysin-like proteases (TLPs). These regions are usually missing in mature enzymes and, thus, belong to the prosequences. Not much is currently known about the function of C-terminal propeptides of TLPs. Still, the available data and comparative analysis of conserved domains in the C-terminal regions of TLPs and other enzymes suggest their involvement in the enzyme binding to insoluble proteins and polysaccharides and, probably, target the proteins to the bacterial cell surface (200).

The propeptides underlying the precursor interaction with other molecules can play a key role in protein functioning as illustrated by caspases. These intracellular cysteine proteases, substantial control factors of cell death, proliferation, and inflammation, are synthesized as precursors with N-terminal extensions (N-peptides) cleaved off in most mature caspases, which allows them to be considered as classical propeptides. The prodomains underlie the interaction of precursors of apoptotic initiator caspases and inflammatory caspases with their activation platform, a protein complex assembled in response to an apoptotic signal. This interaction leads to the formation of the active dimeric caspase and, eventually, underlies specific physiological effects. Caspase prosequences vary in structure and contain domains responsible for the specific binding to adapter molecules in the corresponding activation platform. For instance, the propeptides of apoptotic initiator caspases 8 and 10 interacting with the death inducing signaling complex contain two death effector domains (DEDs) each. The prosequences of inflam-

matory caspases 1, 4, and 5, which are activated on the inflammasomes, contain the caspase recruitment domain (CARD). [The classification and structure of caspases as well as the mechanisms of their activation and maturation have been reviewed in detail elsewhere (201, 202).] The ability of caspases to be activated in response to specific signals depends on their propeptides. A replacement of the prodomain of caspase 8 with the N-peptide of caspase 9 results in the activation of the hybrid enzyme on the apoptosome, the activation platform of caspase 9, which convincingly confirms this conclusion (203).

Thus, propeptides can have an effect on the functioning of cognate proteases through the interaction with various molecules and supramolecular complexes. In this context, there are indications that the biological properties of a precursor can substantially differ from the properties of the mature protein and, particularly, can be unrelated to catalytic activity. In this case, the mechanisms regulating the balance between the precursor and mature enzyme are not just the control mechanisms of the enzyme activation, as a balance shift towards the precursor or mature enzyme can trigger different biochemical pathways. However, it remains unclear if protease propeptides have any independent role *in vivo* after their detachment from the precursor. The available data support this possibility although it is not confirmed directly. It should be noted that different functional properties of the precursor and mature protein are not limited to proteolytic enzymes. For instance, such differences as well as the functions of autonomous prosequence-derived peptides have been demonstrated in neurotrophins [reviewed in Refs. (204, 205)].

Propeptide variation

The above data convincingly demonstrate that propeptides to a significant extent determine protease functioning *in vivo* and that propeptide modification has a considerable impact on the biological properties of the cognate proteins. The considered examples suggest that the modulating capacity of the prosequences can underlie a specific evolutionary mechanism altering biological properties of proteins with minimal changes in their major functional domains. This is further confirmed by the comparison of precursor sequences in the enzyme with related catalytic domains.

Sequence analysis of precursors within protease families demonstrates a much higher heterogeneity of propeptides compared to the catalytic domains. For instance, mature bacterial chymotrypsin-like enzymes have similar size and 38–63% identical amino acids, whereas the propeptide lengths vary from 76 to 162 amino acids and their identity vary from 23% to 49%, which is approximately 15% lower (206). In contrast to the catalytic domains, caspase propeptides also significantly vary in size (16–219 amino acids) and contain different recruiting domains (see above) (201). Mammalian proprotein convertases are another example of this kind: in contrast to the highly conserved catalytic domains (54–70% identity to furin amino acid sequence),

their prosequences are more variable (36–51% identity to furin) (207). A lower sequence conservation of propeptides relative to mature enzymes is clearly demonstrated by our analysis of full-length TLP precursor sequences (200). All proenzymes of the family have N-terminal propeptides and one-third of them also have C-terminal propeptides. Amino acid sequence identity never drops below ~40% in mature TLPs, whereas many propeptides have no significant similarity. The N-terminal prosequences split into two non-homologous groups. The C-terminal extensions are highly variable: they are 110–670 amino acids long and contain at least 10 unrelated conserved domains (also found in diverse groups of proteins) combined in more than a dozen patterns. Thus, the rate of propeptide evolution is higher compared to the corresponding catalytic domains.

The higher variation rate relies on the propeptide tolerance to mutations. Indeed, modifications even of the most conserved amino acids or deletions of prosequence fragments often do not lead to a complete loss of the enzyme functional activity (90, 127, 131, 159, 208–210). Propeptide replacement with that of another member of the group or their partial replacement with foreign sequences can have no effect on the enzyme folding and processing (37, 210–215).

The changes in propeptide structure *in vivo* are realized in two ways. First, the prosequence can evolve together with the mature part, although at a higher rate of mutations. Second, the propeptide structure can substantially change as a result of shuffling of domains from different not necessarily related proteins. For instance, the N-terminal propeptides in TLPs accumulate mutations together with the protease domain, whereas domain shuffling seems to be the main mechanism of C-terminal prosequence modification (200). The shuffling mechanism can also underlie the structure of caspase propeptides with DED and CARD domains, which are also found in other apoptotic proteins (216).

It is of interest that protease prosequences can sometimes disconnect from the catalytic domain to become encoded by a separate gene and to acquire a separate cellular function. Protein inhibitors of proteolytic enzymes homologous to protease prosequences were found in different organisms. Five such inhibitors were identified for papain-like cysteine proteases: murine cytotoxic T-lymphocyte antigens CTLA-2 α and CTLA-2 β (217–219), *Bombyx mori* cysteine protease inhibitors BCPI α and BCPI β (220–222), and *Drosophila melanogaster* D/CTLA-2 or cer protein (223, 224). These inhibitors seem to be important regulation factors of cysteine protease activity in animals, and their effect is significant for memory functioning (225–227) and embryogenesis (228, 229). Comparative analysis of the genomic localization as well as the exon-intron structure of genes coding for cysteine protease inhibitors in mouse and *Drosophila* suggested that the CTLA-2 and D/CTLA-2 genes originated after the duplication of fragments of the *cathepsin L* (217, 230) and protease *CPI* (223) genes, respectively. Propeptide-like inhibitors of serine subtilisin-like proteases have also been found: *Pleurotus ostreatus* proteinase A inhibitor 1 (POIA1) (231) and yeast proteinase B inhibitor 2 (232). The subtilisin model was used to demonstrate that POIA1 can function as

a folding assistant (233). As no strict correlation exists between the inhibitory and folding functions of subtilase propeptides (234), their combination can point to the emergence of this inhibitor after the duplication of the cognate protease gene as in the case of CTLA-2 and cer protein.

Thus, the propeptides are relatively independent elements that allow the functional activity of proteases to be modified without significant changes in the catalytic domains. The capacity to retain their functional properties after substantial structural changes underlies high prosequence variation. Overall, the propeptides can be considered as evolutionary modules extending the functional diversity of proteins.

Propeptides and protein engineering

The modulating capacity of propeptides can be used as a tool in protein engineering. The term 'prosequence engineering' was proposed for the approach to modify an enzyme functional activity by introducing mutations in the propeptide rather than in the catalytic domain (235). Several strategies were proposed within the frame of this approach. The first strategy relies on the protein memory phenomenon discussed above. It consists of producing protease conformers with altered properties resulting from point mutations in the propeptide. The second strategy consists of modifying the processing site in the prosequence to improve the autocatalytic removal of the propeptide. This can provide for the maturation or increase the yield of mature proteases synthesized in heterologous systems or of proteases with changed properties. This strategy can also be used to generate proteases with modified substrate specificity. The third strategy consists of the shuffling of prosequences or their parts from homologous proteases, which can give rise to proteins with new properties (as in the case of point mutations) and modulate the rate of propeptide degradation and, hence, the rate of catalytically active molecule production. Prosequence engineering is expected to construct proteases with altered substrate specificity, high activity, and high stability (235).

Prosequence engineering was successfully used to construct *Streptomyces griseus* protease B (SGPB) with modified substrate specificity. SGPB propeptide is removed autocatalytically, and the efficient processing requires the correspondence between the C-terminal amino acid of the prosequence at the P1 position relative to the hydrolyzed bond and the substrate specificity of the enzyme. The wild-type SGPB prefers large hydrophobic residues and has Leu at this position. No maturation takes place without this correspondence. Thus, a proper point mutation at the processing site makes it possible to select a mature active protease with modified substrate specificity from the library containing numerous enzymes with modified catalytic domain. This approach was successfully used to screen an *Escherichia coli* expression library containing nearly 30 000 SGPB mutants. In the case of Met at P1, a protease with substantially increased specificity towards substrates with Met has been selected; Val at P1 yielded a protease with wide substrate specificity; whereas P1 Phe (intact SGPB efficiently hydro-

lyzes substrates with this amino acid) gave rise to an enzyme with elevated thermostability (236).

One more achievement of prosequence engineering is the increased extracellular production of *B. subtilis* subtilisin-like thermostable protease WF146 by *E. coli* cells. After random mutagenesis of the N-terminal propeptide, two selected point mutations in the prosequence, Leu(-57)→Gln and Glu(-10)→Asp, provided a 3-fold increase in the extracellular protein production. The enzyme with the mutant propeptide demonstrated accelerated maturation relative to the wild-type protein, and no significant changes in the thermostability and catalytic properties were observed (163).

Another approach to employ the modulating properties of propeptides is to use them as specific protease inhibitors. The most attractive examples of this kind demonstrate anticancer activity of propeptides, e.g., furin propeptide. The processing of cancer-associated precursor proteins by furin is important for the acquisition of malignant phenotype and metastatic potential of tumor cells. The proregion of furin inhibits enzyme activity with a low nanomolar inhibition constant (Table 1), which suggests its anticancer activity. Indeed, the expression of furin propeptide cDNA in tumor cells or their incubation with the corresponding protein was associated with a significant reduction in tumor cell proliferation, migration, and invasion. These data advance propeptides as a new basis for anticancer drug development (237). Another demonstrative example is the development of pest-resistant plants with a transgene encoding an appropriate propeptide. The significance of extracellular proteases in the pest and pathogen interaction with the plant is well known, and their selective inhibition is considered as a strategy to control herbivorous insects, parasitic nematodes, and pathogenic microorganisms. Protease propeptides were proposed as a promising source of inhibitors in this context (95, 112, 113, 238). For instance, the expression of a prosequence of cysteine protease of soybean cyst nematode (*Heterodera glycines*) in the soybean roots has a pronounced protective effect: the development of female nematodes decelerates and the number of females (by 31%), the number of eggs per female (by 58%), and the female size decrease (239).

Discussing propeptides as a bioengineering tool, one cannot omit the modification of protein properties through the development of their artificial precursors, zymogens. No experiments of this kind on proteolytic enzymes have been published to date, although proteases largely synthesized as precursors inspire such studies (240). Apparently, ribonuclease A zymogen was the first artificial precursor. It was constructed by the insertion of a linker connecting the N- and C-termini of the enzyme, closing the active site, and containing a specific site of plasmeprin II hydrolysis. The new N- and C-termini were generated by circular permutation. The zymogen was 1000 times less active than the processing protease-treated enzyme, the catalytic properties of which were similar to those of native RNase A (240). The same approach was used to construct a promising antiviral agent, RNase A precursor activated by NS3 protease of the human hepatitis C virus (241).

The generation of an artificial zymogen of adenosine diphosphate ribosyl transferase is another example of this

kind. This *B. cereus* enzyme also known as the vegetative insecticidal protein 2 (Vip2) in combination with another *B. cereus* protein Vip1 is toxic towards the larvae of the western corn rootworm (WCR), a major pest of corn in the United States. Vip2 is an intracellular toxin that modifies actin, which suppressed its polymerization and microfilament network formation. Vip1 provides for Vip2 entry into the eukaryotic cell through the interaction with the cell surface (242). The Vip1-Vip2 system looks very promising for WCR control. However, Vip2 introduced into the plant causes severe developmental pathology and substantial phenotype changes. This problem was solved by the generation of an artificial Vip2 precursor inactive in maize cells but activated in the digestive system of WCR larvae. Random propeptide library was introduced to the C-terminus of Vip2, and the variants with low cytotoxic activity were selected in yeast. The expression of the resulting proVip2 (with a 49 amino acid propeptide) had no effect on maize development and phenotype. Nonetheless, the prosequence was efficiently removed in the WCR digestive system, which activated the enzyme. Feeding the larvae with proVip2 and Vip1 caused 100% death after 72 h (243).

Thus, propeptides are an attractive protein engineering tool that allows protein properties to be modified without affecting the structure of the key functional domains. Moreover, an artificial prosequence can be inserted *de novo*, which can impart absolutely new properties to the protein. In a way, prosequence engineering reproduces the natural evolutionary process. However, propeptide-mediated modification of protein functional activity is not widely used at present, which can be attributed to insufficient knowledge of the mechanisms of propeptide functioning.

Expert opinion

The majority of proteolytic enzymes are synthesized as precursors containing propeptides. The function and mechanisms of action of protease prosequences were actively studied in past two decades and the results are summarized in several reviews (2, 3, 5, 41–43, 206, 244, 245). However, analysis of propeptides is usually limited to two main issues: their involvement in protein folding and cell protection from undesirable protease activity through the rigid control of the time and location of protease activation.

In the present review, we considered all known functions of propeptides and attempted to demonstrate that propeptides modulate protease functional activity irrespective of the specific mechanism of their action. Propeptide modifications, sometimes minor, can substantially alter protein functioning in living organisms. This modulating activity coupled with high variation allows us to consider propeptides as specific evolutionary modules underlying modifications in protease biological properties without significant changes in the highly conserved catalytic domains. Although it remains beyond the scope of this review, it should be stressed that prosequence-mediated modulation of protein function is not limited to proteases or even enzymes [as is clearly exemplified

by neurotrophins mentioned above (204)], suggesting that it is a universal phenomenon of the living world.

Outlook

It is beyond question that the data on the structure and functions of propeptides (in proteases as well as other proteins) will continue to accumulate. New data on the three-dimensional structure of the precursors, functional properties of propeptides of individual proteins *in vivo* and *in vitro*, as well as on protein partners of propeptides will inevitably uncover new specific biochemical mechanisms. However, the experimental data available to date make it clearer that the mechanisms of action and biological functions of propeptides are extremely diverse, and there is no clear boundary between propeptides and constituent protein domains.

In this context, the concept proposed in this review considering propeptides as evolutionary modules and transient protein domains can be fruitful for the identification of future research trends. The propeptide capacity to modulate the biological functions of proteins is the primary concern here. Knowledge of the function of individual prosequences and their interaction with cognate protein domains is not sufficient to understand the mechanisms of propeptide-mediated evolution. Disembodied studies on individual molecules should be replaced with systemic studies considering both physicochemical and biological aspects in groups of related proteins in taxonomically close organisms (or even the same organism) but with different propeptide structure.

Highlights

- Most proteases and many other proteins are synthesized as precursors containing propeptides.
- The main propeptide functions include: assistance in cognate protein folding, inhibitor/activation peptide function, sorting, and interaction with other molecules or supra-molecular structures.
- The same propeptide can have several functions mentioned above.
- Irrespective of the propeptide specific function and mechanism of action, its structure alterations can modulate functional properties of the protein.
- The propeptide structure is much less conserved compared to the cognate catalytic domains.
- The combination of modulatory activity and high variation makes propeptides specific evolutionary factors changing biological properties of proteins without significant modifications on the highly conserved functional (e.g., catalytic) domains.
- Propeptide engineering based on their modulatory activity can be used to generate artificial proteins with desired properties.
- Further studies of prosequences can focus on groups of related proteins functioning in taxonomically close organ-

isms (or even in the same organism) but with different propeptide structure.

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