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Review Letter

Proline residues in the maturation and degradation of peptide hormones and neuropeptides

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The proteases involved in the maturation of regulatory peptides like those of broader specificity normally fail to cleave peptide bonds linked to the cyclic amino acid proline. This generates several mature peptides with N-terminal X-Pro-sequences. However, in certain non-mammalian tissues repetitive pre-sequences of this type are removed by specialized dipeptidyl (amino)peptidases during maturation. In mammals, proline-specific proteases are not involved in the biosynthesis of regulatory peptides, but due to their unique specificity they could play an important role in the degradation of them. Evidence exists that dipeptidyl (amino)peptidase IV at the cell surface of endothelial cells sequesters circulating peptide hormones which are then susceptible to broader aminopeptidase attack. The cleavage of several neuropeptides by prolyl endopeptidase has been demonstrated in vitro, but its role in the brain is questionable since the precise localization of the protease is not clarified.

Neuropeptide; Peptide hormone; Proteolytic processing; Degradation; Dipeptidyl (amino)peptidase; Prolyl endopeptidase

1. INTRODUCTION

The unique cyclic and imino structure of the amino acid proline influences not only the conformation of peptide chains, but also restricts the attack of proteases. Peptide bonds involving proline residues are often resistant to the action of endo- and exopeptidases [1–4], even those with broad specificity (table 1). On the other hand, several proteases are known which specifically or selectively attack proline bonds (table 2). This report summarizes some recent findings about the influence of proline residues (i) on the proteolytic processing of neuropeptides and peptide hormones from their precursors, and (ii) on the final degradation of these regulatory peptides.

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2. DIRECTION OF THE PROTEOLYTIC MATURATION OF REGULATORY PEPTIDES BY PROLINE RESIDUES

Regulatory peptides are synthesized as part of larger precursors, namely the prepro-peptides. The N-terminal pre-sequence is cleaved by a signal peptidase in the rough endoplasmic reticulum [5–8]. Further proteolytic maturation occurs in the Golgi apparatus and in the secretory vesicles. The key proteolytic enzymes for the processing of propeptides include [7,8] an endopeptidase cleaving at paired basic residues, further a carboxypeptidase B-like enzyme and/or an aminopeptidase B-like enzyme for the removal of C- and N-terminal extended basic residues from the fragments. Other proteases [7–9] as well as further nonproteolytic modifications like glutaminyl cyclisation [10], acetylation, amidation, glycosylation [6–8] may also be necessary, depending on the complexity of

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Table 1

Peptide bonds stabilized against proteolytic attack by some common proteases by involvement of proline residues

Protease	Peptide bonds (▬), normally cleaved if Pro is replaced by other amino acids
Trypsin	– (Arg, Lys) ▬ Pro –
Chymotrypsin	– (Trp, Tyr, Phe, Leu, others) ▬ Pro –
Thermolysin	– (many) ▬ (hydrophobic) – Pro –
Staphylococcus aureus protease	– (Glu, Asp, Gln, Asn) ▬ Pro –
Aminopeptidase M[a] (= microsomal alanyl aminopeptidase)	(neutral) ▬ Pro – Pro ▬ (many) –
Leucyl aminopeptidase	(hydrophobic) ▬ Pro –
Carboxypeptidases A and B	– (many) ▬ Pro
Cathepsin B2 (= lysosomal carboxypeptidase B)	– Pro ▬ many
Angiotensin converting enzyme (= peptidyl dipeptidase A)	– (many) ▬ Pro – (many)

Data compiled from [1–3]. Reports about a different specificity of aminopeptidase M seem to be caused by contaminations with other exopeptidases, e.g. dipeptidyl peptidase IV [4]

the maturation. Fig.1 illustrates the stability of X-Pro- bonds in the precursors of some regulatory peptides to the processing proteases (further examples [9]).

The substance P precursor is not cleaved behind the dibasic sequence -Arg-Arg-Pro- because a proline residue follows. Instead, the cleavage occurs between the two basic residues Arg-57 and Arg-58

Table 2

Proline specific mammalian endo- and exopeptidases

Enzyme	Requirements of the cleaved peptides				M_r ($\times 10^{-3}$)		Inhibitors	pH optimum	Localization
	Cleavage site ● = Pro	Other residues accepted at ●	Residues accepted at ○	Chain length (no. of residues)	Native	Subunit			
Prolyl endopeptidase	—●↓○—	–	≠Pro	4–17	70[a]	74[a]	Dip-F	7.5	S(M)
Prolyl aminopeptidase	●↓○—	–	hydrophobic	2–4	300[a]	56[a]	EDTA	8.1	S
Aminopeptidase P	○↓●—	Hyp	≠Pro	3–9			1,10-phenanthroline	7.8	M
Dipeptidyl peptidase II	⊛—●↓○—	Ala	any	3–11	110[a]	53[a]	Dip-F, cations	5.5	S(M)
Dipeptidyl peptidase IV	⊛—●↓○—	Ala, Hyp	≠Pro≠Hyp	3–200	220[b]	120[b]	Dip-F, bacitracin	8.0	M
Lysosomal prolyl carboxypeptidase	—●↓○	–	≠Pro, hydrophobic	≥3	210[c]	25[c]	Dip-F	5.2	S
Carboxypeptidase P	—●↓○	Ala, Gly	≠Pro	2–7	240[c]			7.8	M
Prolinase	●↓○	Hyp	any	2	300[c]		EDTA,Cd^{2+}-activated	8.7	S
Prolidase	○↓●	Hyp	≠Pro	2	108[c]	54[c]	EDTA, mercuribenzoate	7.5	S

The data are compiled from [2] and from work in own laboratory; for action on regulatory peptides see text. S, soluble; M, membrane-bound; molecular data for the rat brain enzymes[a], rat kidney enzymes[b] and pig kidney enzymes[c]; ⊛, any unprotected amino acid; Dip-F, diisopropyl fluorophosphate; all peptidases sensitive to chelating agents and carboxypeptidase P are activated by Mn^{2+}

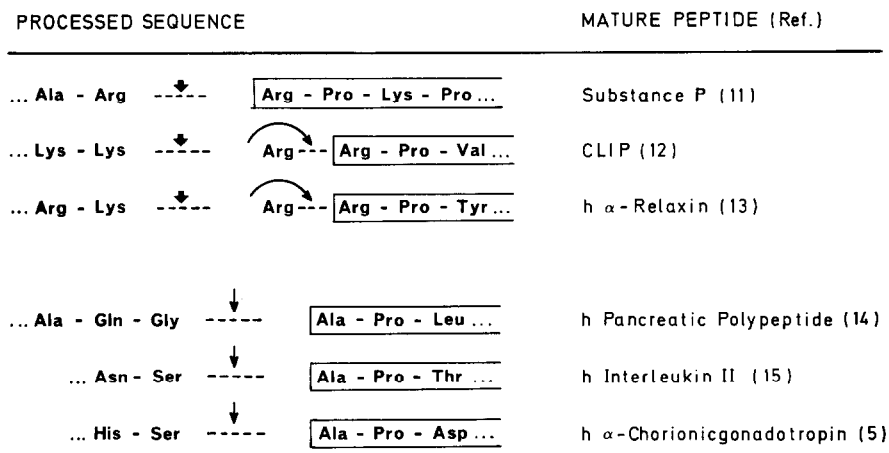

Fig.1. Influence of proline residues on the cleavage of precursors of regulatory peptides by mammalian processing proteases (see text). Observed cleavage by basic residue-specific endopeptidases —●—●↓—○— (● = Arg, Lys), signal peptidase —●—↓—○— (● = Ala and others), and aminopeptidases —●↷—○—. The N-terminal sequences of the mature peptides are boxed.

of the precursor yielding a N-terminal Arg-Pro-... sequence in the mature bioactive peptide. This proteolytic conversion has earlier been classified as 'proline-directed (monobasic) arginyl cleavage' [9]. A more complex example is the conversion of ACTH to α-MSH (= acetylated and amidated ACTH 1–13, melanocyte-stimulating hormone) and CLIP (= ACTH 18–39, corticotropin-like intermediate lobe peptide) in the pituitary intermediate lobe. These bioactive fragments are linked by four basic amino acid residues. As concluded from the occurrence of intermediates [12], the endopeptidase initially cleaves ACTH(1–39) at the Lys-16–Arg-17 bond. By removal of one arginine residue, ACTH(17–39) is then processed to CLIP by the action of an aminopeptidase. However, the Arg-18 residue is stable to further aminopeptidase sequestration, because a proline residue follows. The same mechanism should occur in the processing of the human relaxin α-chain precursor. As concluded from these data, basic residue-specific endopeptidases and aminopeptidases fail to cleave -Arg-Pro- sequences in the maturation of peptide precursors. This may explain the generation of the non-processed sequence -Lys-Pro-Arg-Arg-Pro- in mature neurotensin. Also the carboxypeptidase B-like enzyme cannot readily remove Arg from -Pro-Arg peptide fragments [9].

The last residue of the signal (pre-) sequence has been found to be either Ala, Ser, Gly, Cys, Thr or Gln [5,6]. From the examples in fig.1 it appears that the removal of the pre-sequences may also be influenced by proline residues. In these examples all Ala-Pro- sequences are resistant to the signal peptidase, and instead, cleavage occurs after Ser or Gly which, of course, are also possible cleaving sites. Nevertheless, a list of 90 cleavage sites for signal peptides gives no example of X-Pro hydrolysis [5].

In conclusion, the proteases involved in the intracellular precursor processing of bioactive peptides normally are unable to cleave X–Pro- bonds. However, especially in invertebrates and lower vertebrates processing enzymes with substrate specificities to overcome this restriction must exist. The pro-sequence of honey bee promellitin consists of six X-Pro and five X-Ala repetitive dipeptidyl residues [16] which should be removed by the sequential action of a dipeptidyl peptidase of this specificity to yield the mature mellitin. The pro-sequence of the antifreeze protein from the winter flounder contains four X-Pro and seven X-Ala repetitive sequences [17], but in this case the processing to the mature peptide occurs in serum [18]. Further investigations are required to clarify whether the stepwise cleavage of dipeptides including X-Pro- sequences [19] is a particular mechanism for the intracellular maturation of propeptides also in mammals.

3. PROLINE-SELECTIVE PROTEASES IN THE DEGRADATION OF MATURE PEPTIDES

The occurrence of proline-selective proteases, which cleave peptide bonds resistant to most other proteases, suggests that these specialized enzymes could play a key role in the final degradation of mature peptide hormones or neuropeptides. Beside prolyl endopeptidase, the proline-selective dipeptidyl peptidases are the most attractive candidates for such a function since several bioactive peptides start with N-terminal X-Pro- sequences as discussed above. Like N-terminal cyclisation to pyroglutamic acid, acetylation or C-terminal amidation, these sequences with a penultimate proline could be regarded as being protected against the degradation of bioactive peptides by non-specialized exopeptidases.

Indeed, human dipeptidyl peptidase IV (DPP IV, EC 3.4.14.5) liberates X-Pro-dipeptides with high activity from several regulatory peptides of various chain lengths [20,21]: e.g. from His-Pro-NH_2 (thyroliberin-fragment generated by pyroglutaminase, of which the metabolite His-Pro [22] or the non-enzymatic formed cyclo(His-Pro) may be bioactive), β-casomorphin (opioid peptide from milk, Tyr-Pro-Phe-Pro-Gly-Pro-Ile), substance P (Arg-Pro-Lys-Pro-Gln..., M_r 1348), pancreatic polypeptide (Ala-Pro-Leu..., M_r 4240), prolactin (Thr-Pro-Val..., M_r 24000) or human chorionic gonadotropin (α-chain Ala-Pro-Asp..., M_r 48000). Only peptides with proline in the third position, e.g. bradykinin (Arg-Pro-Pro...), or glycosylation here, e.g. interleukin II (Ala-Pro-Thr(CHO)...) or α_1-microglobulin (Gly-Pro-Val-Pro-Thr-(CHO)...) are protected against DPP IV attack [21]. Since DPP IV is found in high concentrations at the vascular surface of the capillary endothelium [23] and the kidney brush border [2], circulating peptide hormones of such structure should be rapidly sequestered by this enzyme when entering the blood stream and/or during renal passage. Though no direct experiments, e.g. by use of specific DPP IV inhibitors, have yet been undertaken, some findings are consistent with this hypothesis. Sequence data for the α-subunit of chorionic gonadotropin [24], follicle-stimulating hormone [25], luteinizing hormone [26] or thyroid-stimulating hormone [27] exhibit a heterogeneity of the N-terminus with and without the X-Pro- sequence. Since this heterogeneity does not result from intracellular processing [28], the action of DPP IV on the circulating hormones may be responsible for it. However, with the exception of substance P [29] and possibly His-Pro-NH_2 [22] the loss of the terminal dipeptidyl sequences does not decisively inactivate these peptides. Rather, the DPP IV action might facilitate the further degradation, since the sequestered residue is then susceptible to subsequent aminopeptidase attack [4]. As for other neuropeptide degrading proteases, the biological function of DPP IV in brain depends on its precise localization. DPP IV is present at the surface of brain capillaries and leptomeninges [30,31], and has also been detected in synaptosomal fractions [22,32]. In the developing brain DPP IV is abundant in neuroblasts [30]. The sequestration of the N-terminal tetrapeptide Arg-Pro-Lys-Pro of substance P in neuronal cell cultures [33], which stimulates neurite outgrowth [34], shows a possible importance of DPP IV in neuronal proliferation.

A further proline-selective dipeptidyl peptidase, DPP II (EC 3.4.14.2), is a lysosomal enzyme occurring in endocrine glands, reproductive organs and neurons of the brain [2]. It liberates X-Pro- as well as X-Ala-dipeptides only from short peptides up to 11 residues long [35], even if followed by a further proline. Because of this localization and specificity it is suggested that DPP II accomplishes the final degradation of fragments from internalized or over produced regulatory peptides. Sequences involving proline bonds are stable to most lysosomal proteases. The dipeptides liberated by the action of DPP II (in contrast to tri- and oligopeptides) readily cross the lysosomal membrane, and then are metabolized by the ubiquitous cytosolic prolidase (table 2). N-terminal X-Pro- sequences may also be overcome by the sequential action of two specialized aminopeptidases, namely aminopeptidase P and prolyl aminopeptidase (or other aminopeptidases of broader specificity). Aminopeptidase P (= microsomal proline aminopeptidase, EC 3.4.11.9) is a membrane-bound enzyme with widespread tissue distribution [2,36]. In the lung it initiates the degradation of circulating bradykinin [37]. This vasoactive peptide is completely inactivated during a single passage through the rat lung. After liberation of the N-terminal Arg

by aminopeptidase P, the resulting octapeptide is easily sequestered by combined action of DPP IV and aminopeptidase M [37]. Prolyl aminopeptidase (EC 3.4.11.5) has been detected as a melanostatin (Pro-Leu-Gly-NH_2) degrading enzyme [1,2] in the kidney. It has not been investigated whether this enzyme attacks strictly this substrate or shows broader aminopeptidase properties.

Prolyl endopeptidase (= post-proline cleaving enzyme, post-proline endopeptidase, TRH-deamidase, brain-kinase B, endooligopeptidase B, EC 3.4.21.26) specifically cleaves the -Pro–X- bond (but not -Pro–Pro-) in several neuropeptides: oxytocin, vasopressin, gonadoliberin, α-MSH, substance P, neurotensin, thyroliberin, bradykinin and angiotensin II [38]. High activities of prolyl endopeptidase are found in the brain. It seems to be mainly a soluble, probably cytosolic protein, and therefore its role in the initial degradation of secreted neuropeptides is questionable. The specific function(s) of this enzyme may be clarified by the use of the selective inhibitors, Z-Pro-prolinal [39] or Z-Gly-Pro-diazomethylketone [40]. However, a prolyl endopeptidase cleaving the Pro(10)–Tyr(11) bond of neurotensin has recently been purified from rat brain synaptic membranes [41]. Further studies of the precise cellular and subcellular localization in brain are required to evaluate the role of prolyl endopeptidases in neuropeptide metabolism or other neuronal functions.

Several mammalian carboxypeptidases are restricted in their action by the presence of proline in the C-terminal ultimate and/or penultimate position (table 1). Proline-specific carboxypeptidases are the lysosomal prolyl carboxypeptidase (= angiotensinase C, EC 3.4.16.2) and the microsomal prolyl carboxypeptidase (= carboxypeptidase P, EC 3.4.17) which both liberate the terminal hydrophobic amino acids after the penultimate proline. The former enzyme has been considered to be important for regulating angiotensin levels in kidney [42]; however, its distribution in diverse cells such as macrophages, endothelial cells and fibroblasts suggests a broader role in intracellular protein degradation [2]. The kidney microsomal prolyl carboxypeptidase may extend the high proteolytic activity of the renal brush border membrane [43]. To define its role more precisely, additional data about its tissue localization and specificity are needed.

The proline specific dipeptidases, namely prolidase (= proline dipeptidase, EC 3.4.13.9) cleaving X-Pro and prolinase (= prolyl dipeptidase, EC 3.4.13.8) cleaving Pro-X dipeptides, are cytosolic enzymes of widespread tissue distribution [1,2]. Both are strict dipeptidases, and exhibit a complete (prolidase) or high (prolinase) specificity for proline or hydroxyproline. Pro-X dipeptides may also be hydrolysed by dipeptidases of broader specificity [1], or by prolyl aminopeptidase [2]. Due to their localization, both dipeptidases are considered to metabolize proline-containing peptide fragments produced by lysosomal or other intracellular proteases. Inherited prolidase deficiency results in a massive urinary excretion of X-Pro dipeptides, especially Gly-Pro, and mainly affects collagen metabolism with clinical signs of lathyrism [2]. This reflects the important role that proline-specific proteases also have in the degradation of structural proteins, especially collagen and elastin. But this is another topic.

4. PERSPECTIVES

Due to their unique specificity proline-specific proteases have been investigated by several enzymologists. However, the functions of these enzymes are still far from being resolved. Depending on their localization, namely extracellular (at cell surface) or intracellular (at several sites), proline selective proteases may play a role in several protein degradative systems. To clarify their role in the metabolism of regulatory peptides, future studies should be directed to examine their precise localization, especially in the brain, and to develop further specific inhibitors which could be applied to cell cultures or in vivo.

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REFERENCES

[1] Walter, R., Simmons, W.H. and Yoshimoto, T. (1980) Mol. Cell. Biochem. 30, 111–127.

[2] McDonald, J.K. and Barett, A.J. (1986) Mammalian Proteases: A Glossary and Bibliography, vol.2. Exopeptidases, Academic Press, London.
[3] Croft, L.R. (1980) Handbook of Protein Sequence Analysis, 2nd edn, p.10, Wiley, Chichester.
[4] Heymann, E. and Mentlein, R. (1986) J. Dairy Res. 53, 229–236.
[5] Von Heijne, G. (1983) Eur. J. Biochem. 133, 17–21.
[6] Richter, D., Ivell, R., Schmale, H., Nahke, P. and Krisch, B. (1985) in: Neurobiochemistry – 36. Colloquium Mosbach 1985, pp.33–42, Springer, Berlin.
[7] Gainer, H., Russel, J.T. and Loh, Y.P. (1985) Neuroendocrinology 40, 171–184.
[8] Loh, Y.P. and Parish, D.C. (1987) in: Neuropeptides and Their Peptidases (Turner, A.J. ed.) pp.65–84, VCH Verlagsgesellschaft, Weinheim.
[9] Schwartz, T.W. (1986) FEBS Lett. 200, 1–10.
[10] Fischer, W.H. and Spiess, J. (1987) Proc. Natl. Acad. Sci. USA 84, 3628–3632.
[11] Nawa, H., Hirose, T., Takashima, H., Inayama, S. and Nakanishi, S. (1983) Nature 306, 32–36.
[12] Fenger, M. (1986) Biochem. J. 235, 715–722.
[13] Hudson, P., Haley, J., John, M., Cronk, M., Crawford, R., Haralambidis, J., Tregear, G., Shine, J. and Niall, H. (1983) Nature 301, 628–631.
[14] Takeuchi, T. and Yamada, T. (1985) Proc. Natl. Acad. Sci. USA 82, 1536–1539.
[15] Taniguchi, T., Matsui, H., Fujita, T., Takaoka, C., Kashima, N., Yoshimoto, R. and Hamuro, J. (1983) Nature 302, 305–309.
[16] Lane, C.D., Champion, J., Haiml, L. and Kreil, G. (1981) Eur. J. Biochem. 113, 273–281.
[17] Davies, P.L., Roach, A.H. and Hew, C.-L. (1982) Proc. Natl. Acad. Sci. USA 79, 335–339.
[18] Hew, C.L., Wang, N.C., Yan, S., Cai, H., Sclater, A. and Fletcher, G.L. (1986) Eur. J. Biochem. 160, 267–272.
[19] Mollay, C., Vilas, U., Hutticher, A. and Kreil, G. (1986) Eur. J. Biochem. 160, 31–35.
[20] Püschel, G., Mentlein, R. and Heymann, E. (1982) Eur. J. Biochem. 126, 359–365.
[21] Nausch, I. (1987) Thesis, Frankfurt.
[22] Coggins, P.J., McDermott, J.R., Snell, C.R. and Gibson, A.M. (1987) Neuropeptides 10, 147–156.
[23] Palmieri, F.E. and Ward, P.E. (1983) Biochim. Biophys. Acta 755, 522–525.
[24] Bellisario, R., Carlsen, R.B. and Bahl, O.P. (1973) J. Biol. Chem. 248, 6796–6809.
[25] Rathman, P. and Saxena, B.B. (1975) J. Biol. Chem. 250, 6735–6746.
[26] Liu, W.-K., Nahm, H.S., Sweeny, C.M., Lamkin, W.N., Baker, H.N. and Ward, D.N. (1972) J. Biol. Chem. 247, 4351–4364.
[27] Liao, T.H. and Pierce, J.G. (1971) J. Biol. Chem. 246, 850–865.
[28] Birken, S., Fetherston, J., Desmond, J., Canfield, R. and Boime, J. (1978) Biochem. Biophys. Res. Commun. 85, 1247–1253.
[29] Blumberg, S. and Teichberg, V.I. (1982) in: Regulatory Peptides: From Molecular Biology to Function (Costa, E. and Trabucchi, M. eds) pp.445–452, Raven, New York.
[30] Bernstein, H.-G., Schön, E., Ansorge, S., Röse, I. and Dorn, E. (1987) Int. J. Dev. Neuroscience 5, 237–242.
[31] Mitro, A. and Lojda, Z. (1988) Histochemistry 88, 645–646.
[32] O'Connor, B. and O'Cuinn, G. (1986) Eur. J. Biochem. 154, 329–335.
[33] Horsthemke, B., Schulz, M. and Bauer, K. (1984) Biochem. Biophys. Res. Commun. 125, 728–733.
[34] Narumi, S. and Maki, Y. (1978) J. Neurochem. 30, 1321–1326.
[35] Mentlein, R. and Struckhoff, G. (1988) Eur. J. Biochem., in press.
[36] Holtzman, E.J., Pillay, G., Rosenthal, T. and Yaron, A. (1987) Anal. Biochem. 162, 476–484.
[37] Orowski, A.T., Susz, J.P. and Simmons, W.H. (1987) Mol. Cell. Biochem. 75, 123–132.
[38] Wilk, S. (1983) Life Sci. 33, 2149–2157.
[39] Chu, T.G. and Orlowski, M. (1984) Biochemistry 23, 3598–3603.
[40] Knitsatschek, H. and Bauer, K. (1986) Biochem. Biophys. Res. Commun. 134, 888–894.
[41] Checler, F., Vincent, J.-P. and Kitabgi, P. (1986) J. Biol. Chem. 261, 11274–11281.
[42] Oyada, C.E., Marinkovic, D.V., Hammon, K.J., Steward, T.A. and Erdös, E. (1978) J. Biol. Chem. 253, 5927–5931.
[43] Kenny, J. (1986) Trends Biochem. Sci. 11, 40–42.