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Role of peptide substrate structure in the selective processing of peptide prohormones at basic amino acid pairs by endoproteases

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Three putative processing enzymes, each with defined action in a prohormone system, a 'pro-ocytocin-neurophysin convertase' from bovine neurohypophysis secretory granules, a 'Leu-enkephalin Arg^6 generating enzyme' from human CSF and the endoprotease from the 'S-28 convertase' complex of rat brain cortex, were tested for their ability to hydrolyze peptides deriving from pro-ocytocin, pro-enkephalin B and pro-somatostatin, respectively at pairs of basic amino acids. The observations suggest that structural parameters specified by the peptide region around the dibasic moieties govern recognition by the enzyme and define which peptide bond is hydrolyzed.

Peptide hormone biosynthesis; Enzyme selectivity; Endopeptidase

1. INTRODUCTION

Bioactive peptides are released from biosynthetic precursors, the prohormones, in which the peptide sequences are typically flanked by pairs of basic amino acids. Endoproteases, known as processing enzymes, act at these pairs and the remaining basic NH_2- or COOH-terminal amino acid is removed by an exopeptidase [1,2]. Closer investigation of such endoproteases reveals that they exhibit, in a number of cases, a marked selectivity [3–10]. Previous work has indicated that in a family of similar peptide substrates, the same peptide bond is cleaved by a given endoprotease; i.e. either in between the dibasic pair for the pro-enkephalin B derived substrates [3–5], on the COOH-terminal side of the doublet in the case of pro-alpha-mating factor [6], pro-ocytocin/neurophysin (pro-OT/Np) [7,8] processing enzyme, or else the NH_2-terminal side of the Arg-Lys doublet in the case of somatostatin-28 [9]. Preliminary examination of the observations made in various laboratories, suggested that the peptide substrate conformation may play an important role in the specification of the cleavage loci [11,12] and that the interactions between the lateral domains of the constituting amino acids of a substrate and the appropriate subsites of the peptidase mainly govern the selection of the peptide bond involved in the hydrolytic process.

In order to test such a hypothesis, a comparative study was conducted using three highly purified putative processing enzymes and corresponding natural or synthetic substrates.

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Abbreviations: HPLC, high-performance liquid chromatography; PITC, phenyl isothiocyanate; DABSCl, 4-(dimethylamino)-azobenzene-4′-sulfonylchloride; DABITC, 4-(dimethylamino)-azobenzene-4′-isothiocyanate

2. MATERIALS AND METHODS

2.1. *Enzyme preparations*

Pro-OT/Np convertase was prepared from bovine

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neurohypophysis secretory granules as described previously [7]. The highly purified preparation was obtained after the isoelectric focusing step [8]. The endoprotease from S-28 convertase was extracted from rat brain cortices as previously described [13]. The purified fraction was recovered after ion-exchange chromatography on DEAE Sephadex [9] and further purified by HPLC on a TSK G-3000 SW column [14]. The Leu-enkephalin-Arg^6 generating protease activity was fractionated from human CSF on a tandem column system using DEAE Sepharose and phenyl-Sepharose chromatography followed by a Sephadex G-100 filtration [4].

2.2. *Peptide substrates*

Somatostatins-14 and -28 (S-14 and S-28) were purchased from Bachem (Bubendorf, Switzerland) and dynorphin B (dyn B), Leu-enkephalin, Leu-enkephalin-Arg^6 were from Peninsula (S. Carlos, CA, USA). In all cases, their purity was tested by HPLC using different buffer systems.

The following peptides were synthesized in the laboratory by the solid-phase method [12,15]. They were identified by amino acid composition, NH_2-terminal sequencing, FAB mass spectrometry, HPLC and TLC.

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                    ┌── SS ──┐
OT GlyLysArg      :Cys[1]TyrIleGlnAsnCysProLeuGlyGlyLysArg[12]
OT GlyLys         :--------------------------------Lys[11]
OT Gly            :-------------------------------Gly[10]
pro-OT/Np(1->20):-------------------------------LysArgAlaValLeu
                  AspLeuAspValArg[20]
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Scheme 1.

2.3. *Enzyme assays*

In each case, 20 μg of either pro OT/Np(1⟶20) or dynorphin B or 5 μg of hypothalamic S-28 were incubated according to the following procedures.

2.3.1. pro-OT/Np convertase

With 75 μl of enzyme preparation (2 μg of protein) for 24 h in a final volume of 100 μl in 50 mM ammonium acetate buffer, pH 7.0, at 37°C.

2.3.2. S-28 convertase/endoprotease

With 10 μl of the purified enzyme (125 ng protein) in a final volume of 100 μl in 0.2 M Tris-HCl buffer, pH 7.2, for 10 h, at 37°C.

2.3.3. Leu-enkephalin Arg^6 generating activity

With 25 μl of endoprotease (5 μg protein) in a final volume of 200 μl in 0.2 M Tris-HCl buffer at pH 7.2 for 13 h, at 37°C.

The enzyme reaction was stopped by acidification of the medium followed by evaporation with a vacuum centrifuge. The resulting mixture was then analyzed by HPLC and the peptides generated were identified by a combination of (i) their retention time relative to standards; (ii) amino acid composition; (iii) NH_2-terminal sequencing of the four first amino acids; (iv) and in the case of both enkephalin and somatostatin related peptides, by a specific radioimmunoassay.

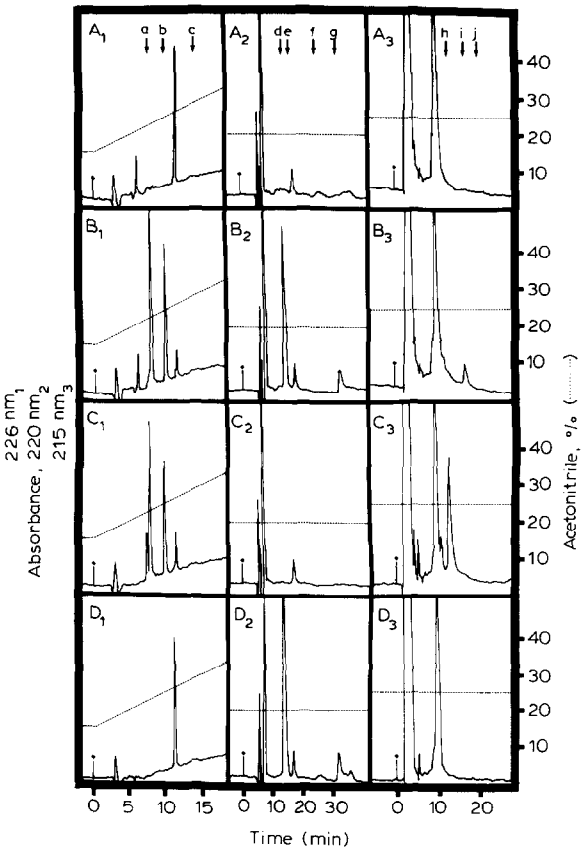

Fig.1. HPLC analysis of the endoprotease reaction mixtures. Leu-enkephalin-Arg^6 generating enzyme (B), endoprotease from S-28 convertase complex (C), and pro-OT/Np convertase (D), were incubated with: dynorphin B (1), pro-OT/Np(2⟶20) (2) and somatostatin-28 (3), as described in section 2. Line (A) represents the elution profiles of the substrates incubated in the absence of enzyme activities. The respective retention times for: dynorphin B (7⟶13) (a), Leu-enkephalin-Arg^6 (b), Leu-enkephalin (c), OTGlyLysArg (d), OTGlyLys (e), OTGly (f), pro-OT/Np(13⟶20) (g), Arg^{-2}Lys^{-1}-somatostatin-14 (h), Lys^{-1}-somatostatin-14 (i) and somatostatin-14 (j) are indicated by arrows.

2.4. *Radioimmunoassay*

Somatostatin-like material was evaluated quantitatively using RIA with anti-somatostatin antiserum 36–38 [13]. Enkephalin related peptides were evaluated using a previously described RIA [16].

2.5. *Amino acid sequencing and analysis*

Amino acid composition was established by derivatization of the hydrolyzed samples with DABSCl which were further separated by HPLC [17]. NH_2-terminal sequencing was performed using the DABITC/PITC double coupling method, and the derivatives were identified by a bi-dimensional chromatography system [18].

Table 1

Schematic representation of the peptide bond in the substrate hydrolyzed by various endopeptidases

Enzyme	Substrate		
	Dynorphin B	pro-OT/Np(1⟶20)	Somatostatin-28
Leu-enkephalin-Arg^6 generating	-ARG-ARG- ↑	-LYS-ARG- ↑	-ARG-LYS- ↑
S-28 convertase (endopeptidase)	-ARG-ARG- ↑	0	-ARG-LYS- ↑
pro-OT/Np convertase	0	-LYS-ARG- ↑	0

The arrow indicates the peptide bond hydrolyzed. 0 means no reaction (i.e. unmodified substrate was recovered). Dynorphin B is: Tyr^{1}GlyGlyPheLeuArg-ArgGlnPheLysValValThr^{13}. Somatostatin-28 is: Ser^{1}AlaAsnSerAsnProAla-MetAlaProArgGluArgLysAlaGlyCysLysAsnPheTrpLysThrPheThrSer-Cys^{28}. pro-OT/Np(1⟶20) is: Cys^{1}TyrIleGlnAsnCysProLeuGlyGlyLysArg-AlaValLeuAspLeuAspValArg^{20}

2.6. *High-pressure liquid chromatography*

Somatostatin-like material was eluted by an isocratic gradient made of 25% acetonitrile in 0.1 M triethylamine phosphate, pH 3.0, from a μ-Bondapack C-18 column. The OT/Np material was eluted by an isocratic system of 20% acetonitrile in 0.05% trifluoroacetic acid from the same column. The pro-enkephalin B derived peptides were analyzed on a column TSK ODS-120T (LKB, Uppsala, Sweden) with a linear gradient from 15% to 45% acetonitrile containing 0.04% trifluoroacetic acid, in 30 min.

3. RESULTS

The highly purified enzyme preparations were incubated with three different substrates: S-28, dyn-B and pro-OT/Np(1⟶20). The peptides generated by the reaction with the endoproteases pro-OT/Np convertase, Leu-enkephalin-Arg^6 generating activity, and the one implicated in S-28 convertase respectively were analyzed by HPLC (fig.1). Analysis of the results led to the following observations: (i) the 'Leu-enkephalin-Arg^6 generating activity' cleaves the three substrates; (ii) the endopeptidase involved in the 'S-28 convertase' complex cleaves S-28 and dyn B; and (iii) the 'pro-OT/Np convertase' cleaves only its own substrate, i.e. pro-OT/Np(1⟶20). In all cases the peptide generated after enzyme incubation is the product of hydrolysis of a unique peptide bond around the cleavage site (i.e. either, before, in between or after the pair of basic amino acids). These results indicate that in the case where enzymes recognize only pairs of basic amino acids, their specificity for the substrate differs from one enzyme to the other. Interestingly, the endoproteases 'Leu-enkephalin-Arg^6 converting activity' and the one implicated in 'S-28 convertase' hydrolyze different peptide bonds in the various substrates.

Indeed, the endoprotease from 'S-28 convertase' which was demonstrated to cleave S-28 before the ArgLys doublet, generating the intermediate [Arg^{-2}Lys^{-1}]somatostatin-14, produces from dyn B the peptide Leu-enkephalin-Arg^6 by cleaving in between the ArgArg doublet. The 'Leu-enkephalin-Arg^6 generating activity' which hydrolyzes the peptide bond situated in between the ArgArg doublet, generates the product OTGlyLysArg from pro-OT/Np(1⟶20) by hydrolysis of the peptide bond situated after the cleavage site. In all cases, no secondary cleavage reaction was detected and in the case where no cleavage was observed (table 1) the original peptide substrate was recovered unchanged after the reaction. Table 1 summarizes these results in a schematic way.

4. DISCUSSION

It is now clear that two different types of proteases have to be involved in the excision of peptide hormones from their precursors. Indeed, in order to account for the selective release of bioactive

fragments from their pro-forms, both an endoprotease and exopeptidase(s) must participate in the removal of the remaining basic amino acids on either the NH_2- or COOH-termini. There is also evidence for the presence of these enzyme species [19,20] in the secretory organelles. At this time their relevance to the in vivo process has not, as yet, been unequivocally established except for KEX_2, KEX_1 and STE_{13} gene products in yeast [21]. Although in the case of these two gene products the enzyme material was alleged to be segregated in distinct secretory compartments (Fuller, R.S. et al., unpublished), other examples suggest co-localization of these components [19,20].

It is known that polyenzyme complexes may favor regulatory processes, and that in the case of proneuropeptide maturation, obviously, the endoprotease specificity and mode of peptide bond cleavage both define the nature of exopeptidase(s) necessarily involved in the subsequent steps (see Discussion in [1,2]). The reported data strongly suggest that endoprotease selectivity is both specified by the basic doublet and by the nature of the subsites surrounding the catalytic site. The known specificity for basic amino acids of these proteases shows that binding sites involved in the recognition of both Lys and/or Arg side chain are necessary. The nature of the peptide bond to be cleaved should be determined, therefore by structural contacts with enzyme subsites situated either on the NH_2- or COOH-side of the basic pair of the substrate (Sn and Sn′ subsites, respectively). These are not as yet fully characterized. The use of selectively modified substrates may prove useful in a more precise definition of the conformational parameters underlying these interactions [12].

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