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Partial Purification and Functional Properties of an Endoprotease from Bovine Neurosecretory Granules Cleaving Proocytocin/Neurophysin Peptides at the Basic Amino Acid Doublet†

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ABSTRACT: An enriched preparation of neurosecretory granules from bovine pituitary neural lobes was used as a source of processing enzymes possibly involved in the cleavage of the proocytocin/neurophysin precursor. A synthetic eicosapeptide reproducing the entire (1–20) sequence of the NH_2-terminal domain of the bovine ocytocin/neurophysin precursor was used as a substrate to monitor an endoprotease activity cleaving at the Lys^{11}–Arg^{12} doublet. The 58-kDa endoprotease detected in the lysate of neurohypophyseal granules produced a single cleavage, after the doublet, at the Arg^{12}–Ala^{13} peptide bond. This endoprotease with pH_i 6.9 and 7.2 exhibits maximal activity at pH around neutrality (7.0) and was strongly inhibited by divalent cation chelating agents [ethylenediaminetetraacetic acid and ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,-*N*′-tetraacetic acid] and to some extent by *p*-(chloromercuri)benzoate and *p*-(chloromercuri)benzenesulfonic acid, while phenylmethanesulfonyl fluoride and pepstatin were not active. This endoprotease action was sensitive to any modification of the substrate at either basic amino acid of the doublet since replacement of either L-Lys^{11} or L-Arg^{12} by D-Lys or D-Arg and by L-Nle abolished the cleavage reaction. In contrast, reversal of the polarity of the doublet in [Arg^{11},Lys^{12}]proocytocin/neurophysin(1–20) had no effect on the mode of endoproteolytic cleavage as well as modifications of Gly^{10} (replaced by Ala^{10}). It is concluded that the selectivity of this endoprotease, which may be involved in the primary event occurring in proocytocin/neurophysin processing, is strictly dependent upon the integrity of the basic doublet but that other parameters determined by the amino acid sequence around this doublet may play an important role.

It is now well established that all known neuropeptides possess larger molecular weight biosynthetic precursors. Proteolytic cleavage of these pro forms occurs at selective loci constituted by basic amino acids often arranged as pairs, allowing the release of the corresponding active forms of the peptide hormones or neurotransmitters. In the hypothalamo–neurohypophyseal tract, as well as the ovarian corpus luteum, ocytocin (OT) is associated within secretory granules with a small (10 kDa) high disulfide containing protein called neurophysin (Np) [for reviews, see Breslow et al. (1979), Cohen et al. (1979), and Ivell et al. (1986)]. Both components derive from a common biosynthetic precursor (pro-OT/Np; 11 kDa) in which the nonapeptide ocytocin sequence, situated next to the leader peptide of the prepro form, occupies the NH_2-terminal sequence of the pro form (Land et al., 1983). It is separated from the neurophysin domain by a processing sequence Gly^{10}–Lys^{11}–Arg^{12}, which is excised during the maturation and which is probably involved in recognition by both proteolytic and amidating enzymes (Clamagirand et al., 1986; Kanmera & Chaiken, 1985). Examination of the proocytocin gene structure (Ivell & Richter, 1984a,b) indicates that the 1–21 NH_2-terminal domain of pro-OT/Np is encoded by the first (exon A) of a three exon containing 0.85 kba DNA. Prediction of the secondary structure for this domain indicates the conservation of a highly ordered structure constituted by a β-sheet/two β-turn/α-helix arrangement (Rholam et al., 1986) on which modification of amino acids at positions 2, 4, 6, 7, and 9 of the neurophysin sequence, observed in various animal species, has no influence. Therefore, we have used synthetic

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peptides reproducing the NH_2-terminal domain of the pro-OT/Np as probes to detect a basic pair specific endoprotease involved in the processing of the precursor (Clamagirand et al., 1986). The endopeptidase activity discovered in neurosecretory granule lysates was further purified, and its functional properties and mechanism of action were studied by using synthetic eicosapeptides selectively modified either at the basic amino acid residues or at Gly^{10} situated before the Lys^{11}–Arg^{12} doublet.

Experimental Procedures

Neurosecretory Granule Preparations

Bovine pituitaries were collected just after sacrifice and transported on ice to the laboratory. Twenty neural lobes (roughly 8 g of fresh tissues) were dissected and homogenized in 20 mM Tris-HCl,[1] pH 7.0, and 0.25 M sucrose buffer (1 g of fresh tissue/10 mL of solution). The homogenate was then subjected to differential centrifugations to yield a granule-containing pellet (36 mg of protein). Then neurosecretory granules were obtained after two successive gradient centrifugations run in 33% Percoll/250 mM sucrose, as described previously (Masse et al., 1982; Clamagirand et al., 1986). The neurophysin-containing neurosecretory granule fractions with a refractive index ranging from 1.356 to 1.345 were found to be free of lysosomes and were pooled. Neurophysin radioimmunoassay was performed as in Masse et al. (1982), and protein content was evaluated according to the method of Bradford (1976).

Enzyme Fractionation

The endoprotease was purified from the enriched granule fractions previously obtained after lysis by osmotic shock (by 2-volume dilution in water) and by three successive cycles of thawing and unthawing.

Sephadex G-150 Filtration. The lysate prepared as described (Clamagirand et al., 1986) was obtained (7.6 mg of proteins) from granules free of lysosomal contaminant. The supernatant free of ghost membranes was then subjected to molecular sieve filtration on a Sephadex G-150 column (65 × 1 cm) equilibrated in and eluted with 50 mM ammonium acetate buffer, pH 7.0 at 4 °C. One-milliliter fractions were collected and kept frozen at −80 °C until further analysis for protein content and enzyme activities (see below).

Ion Exchange on DEAE-Trisacryl. The fractions recovered from the previous molecular sieve filtration and found entirely free of carboxypeptidase B activity (Clamagirand et al., 1986) were pooled and then applied onto an ion-exchange cationic resin (DEAE-Trisacryl column run in 50 mM ammonium acetate, pH 8.0, buffer). Elution was performed with a continuous gradient (0.05–0.4 M ammonium acetate), and each 1-mL fraction was tested both for its endoprotease activity (see below) and for its protein content. This activity represented less than 1 μg of protein (corresponding to an enrichment factor ≥40 000).

Isoelectric focusing was carried out according to the classical method (Camier et al., 1973) using an LKB (110 mL) apparatus and LKB ampholine-carried ampholytes (40% w/v; LKB, Uppsala, Sweden). The active endoprotease-containing fractions recovered either from the Sephadex G-150 filtration or from the DEAE-Trisacryl chromatography (vide supra) were applied onto the column together with the heavy sucrose solution. The pH gradient of 1.2% ampholines (w/v) was from 6.35 to 8.25 in a linear sucrose (0–47%) gradient. Power (2 W) was applied for 48 h, and then 1-mL fractions were collected and both pH and endoprotease activity were measured after elimination of sucrose and ampholines by Sep-Pak filtration.

Peptide Synthesis

All the peptides used in this work either as substrates or in order to identify products of reactions were obtained by solid-phase synthesis using a modified procedure of the Merrifield method (Merrifield, 1963) as described in detail in Nicolas et al. (1986). They were usually purified by successive fractionations on Sephadex G-25, Sephadex G-50, and CM-cellulose columns followed by preparative RP-HPLC run on a Labomat VS 200 Labomatic apparatus with an Organogen RP silica C-18 column (150 mL). Formation of the disulfide bridge was performed by air oxidation under low peptide concentration after the first Sephadex G-25 filtration. The synthetic peptides were tested and identified by a battery of successive tests: i.e., thin-layer chromatography on silica gel plates (Kieselgel 60, Merck) using the upper phase of the mixture (butanol/pyridine/0.1% acetic acid in H_2O; 50:30:110 v/v/v) and analytical RP-HPLC on an LKB 2150 apparatus using a μBondapak C-18 column (Waters Associates, Milford, MA). Amino acid composition was obtained by HPLC separation of the amino acids after derivatization by the DABSCl technique (Chang et al., 1982); Gluschankof et al., 1984). NH_2-terminal analysis was performed by using the DABITC/PITC double-coupling technique (Chang, 1983). Further characterization of each peptide compound was done by fast atom bombardment mass spectrometry on a VG 70-250 instrument (Manchester, U.K.) as described in Nicolas et al. (1986). The following peptides were synthesized and used in this work:

peptide I: Cys^1-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-Gly^{10}-Lys^{11}-Arg^{12}-Ala-Val-Leu-Asp-Leu-Asp-Val-Arg^{20} [pro-OT/Np(1–20)]

peptide II: pro-OT/Np(1–12) ($Cys^1 \rightarrow Arg^{12}$)

peptide III: pro-OT/Np(13–20) ($Ala^{13} \rightarrow Arg^{20}$)

peptide IV: pro-OT/Np(1–10) ($Cys^1 \rightarrow Gly^{10}$)

peptide V: pro-OT/Np(1–11) ($Cys^1 \rightarrow Lys^{11}$)

peptide VI: [Arg^{10},Lys^{11}]pro-OT/Np(1–20)

peptide VII: [D-Lys^{11}]pro-OT/Np(1–20)

peptide VIII: [D-Arg^{12}]pro-OT/Np(1–20)

peptide IX: [Nle^{11}]pro-OT/Np(1–20)

peptide X: [Nle^{12}]pro-OT/Np(1–20)

peptide XI: [Ala^{10}]pro-OT/Np(1–20)

Enzyme Assays

The endoprotease assay was run routinely, using an aliquot (150 μL) of enzyme (containing ≃50 μg of protein from the Sephadex G-150 filtration, 2–3 μg of protein from the IEF

[1] Abbreviations: DABITC/PITC, 4-(dimethylamino)azobenzene 4′-isothiocyanate/phenyl isothiocyanate; DABSCl, 4-(dimethylamino)azobenzene-4′-sulfonyl chloride; PCMB, *p*-(chloromercuri)benzoate; PMSF, phenylmethanesulfonyl fluoride; PCMPS, *p*-(chloromercuri)benzenesulfonic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; DEAE, diethylaminoethyl; RP-HPLC, reversed-phase high-performance liquid chromatography; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; TLC, thin-layer chromatography; IEF, isoelectric focusing; TFA, trifluoroacetic acid; kba, kilobase.

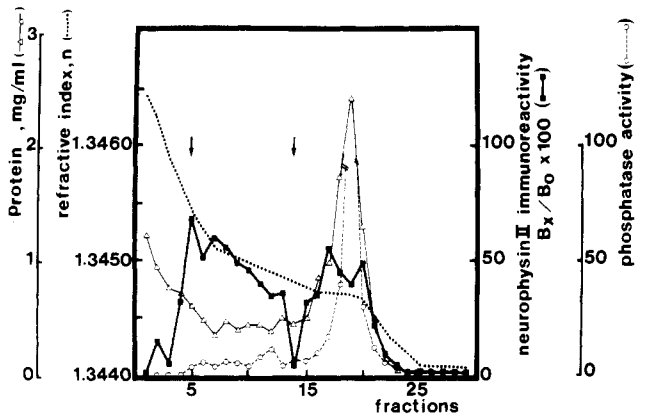

FIGURE 1: Percoll density gradient purification of neurosecretory granules. One-milliliter fractions recovered from the Percoll density gradient (run as described under Experimental Procedures) were analyzed for their refractive index (···), phosphatase activity (○, in arbitrary units), neurophysin II immunoreactivity (■) as described in Clamagirand et al. (1986), and protein content (△) (Bradford, 1976). Fractions 5–14 were pooled and subsequently used as a source of granules and processing enzymes. B_0 and B_x represent the amount of antibodies bound to iodinated neurophysin in the absence and in the presence of competitor.

preparation, or <1 μg of protein from the DEAE-Trisacryl preparation) and peptide I (20 μg) as substrate in 50 mM ammonium acetate, pH 7.0, buffer in a final volume of 200 μL during 24 h at 37 °C. All peptide analogues as well as the effects of various chemicals were tested under identical conditions. At the end of the incubation period, the solution was acidified by addition of 15 μL of 1 N HCl and applied onto the HPLC column (μBondapak C-18). Peptides II and III, generated by the endoproteolytic cleavage of peptide I, were quantified by use of an integrating system (D-2000, Merck) coupled to the HPLC apparatus.

All chemicals used were of analytical grade. PCMB, pepstatin, PCMPS, and both $CaCl_2$ and $CdCl_2$ were from Sigma (St. Louis, MO). $ZnSO_4$ was purchased from Merck (Darmstadt, FRG), Co^{2+}, Mg^{2+}, Mn^{2+}, Cu^{2+}, and EDTA were from Prolabo (Paris, France), while EGTA was from Fluka (Buchs, Switzerland).

RESULTS

The heavier granule fraction recovered from the second Percoll density gradient (Masse et al., 1982) with a refractive index ranging from 1.356 to 1.345 was found to be free of lysosomal contaminant as evaluated by the acid phosphatase test (Figure 1). It was used as a source of processing enzyme after lysis of the purified vesicles.

During enzyme fractionation, peptide I [pro-OT/Np(1–20)] (Figure 2)

Cys^1-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-
Gly^{10}-Lys^{11}-Arg^{12}-Ala-Val-Leu-Asp-Leu-Asp-Val-Arg^{20}

was used as a probe to monitor the endoproteolytic activity. An endoprotease with an apparent molecular mass of 58 kDa was detected after Sephadex G-150 filtration of the granule lysates (Figure 3). The recovered fractions, apparently free of contaminating carboxypeptidase B like activity (Figure 3; fractions 28–34), were pooled and used for subsequent fractionation either by isoelectric focusing (Figure 4, left) or by ion-exchange chromatography on a DEAE-Trisacryl (Figure 4, right) column. These last steps appeared to be efficient in removing any residual carboxypeptidase B like activity. This endoprotease activity converted peptide I into stoichiometric amounts of peptides II and III by a single proteolytic cleavage

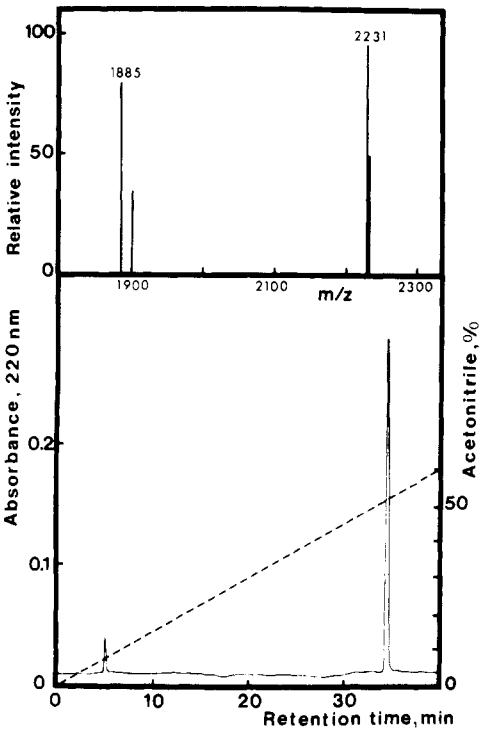

FIGURE 2: Identification of proocytocin/neurophysin(1–20) peptide by fast atom bombardment (FAB) mass spectrometry and HPLC. Upper panel: Quasi-molecular ion (high-mass) region of positive FAB mass spectrum of peptide I. (Theoretical mass calculated for M + H = 2231.) Lower panel: HPLC of purified peptide I was run on a μBondapak C-18 column eluted with a linear gradient of acetonitrile (0–60%).

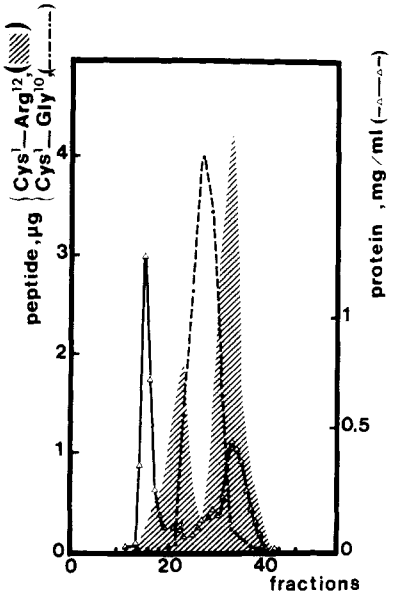

FIGURE 3: Sephadex G-150 filtration of neurosecretory granule lysate. Proteins (7.6 mg) obtained as described under Experimental Procedures were applied on the column (65 × 1 cm) equilibrated in and eluted with 50 mM ammonium acetate buffer, pH 7.0 at 4 °C. Each 1-mL fraction was analyzed. The following activities were measured: endoprotease (hatched), i.e., conversion of peptide I into peptides II (Cys^1–Arg^{12}) + III (not shown); carboxypeptidase B (●), i.e., conversion of peptide I into peptides IV (Cys^1–Gly^{10}) and III (not shown). Enzyme activities, measured on an aliquot of 50 μg of protein with 20 μg of substrate, were respectively expressed as micrograms of peptide II and peptide IV produced under the incubation period. Protein content (△) was measured by using the technique of Bradford (1976).

at the Arg^{12}–Ala^{13} peptide bond (Figure 5). Only a small shoulder in the HPLC profile was observed and represented less than 10% of the resulting fragments. Further analysis of

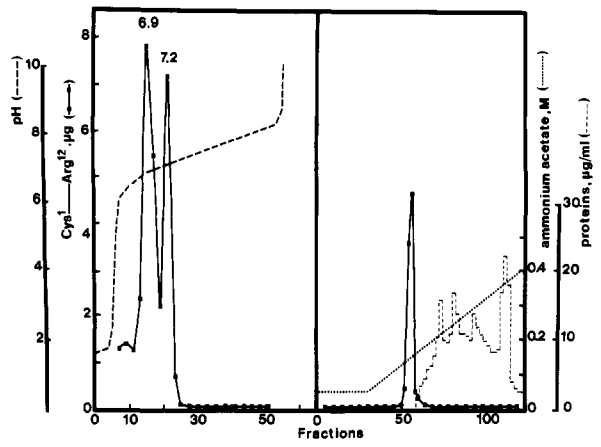

FIGURE 4: Purification of endoprotease by isoelectric focusing and ion-exchange chromatography. Left panel: Isoelectric focusing was run as described under Experimental Procedures. Enzyme activity (■) and pH (--) were measured on each fraction (1 mL). Right panel: Elution profile of the endoprotease activity after ion-exchange chromatography on a DEAE-Trisacryl column (20 × 1 cm) equilibrated in 50 mM ammonium acetate, pH 8.0, and eluted with a gradient of ammonium acetate (50–400 mM) (···). Endoprotease activity (■) was measured on each 1-mL fraction collected. Enzyme activity was expressed as micrograms of Cys^1–Arg^{12} (peptide II) produced at the end of the incubation period. Protein content (---) was measured (Bradford, 1976).

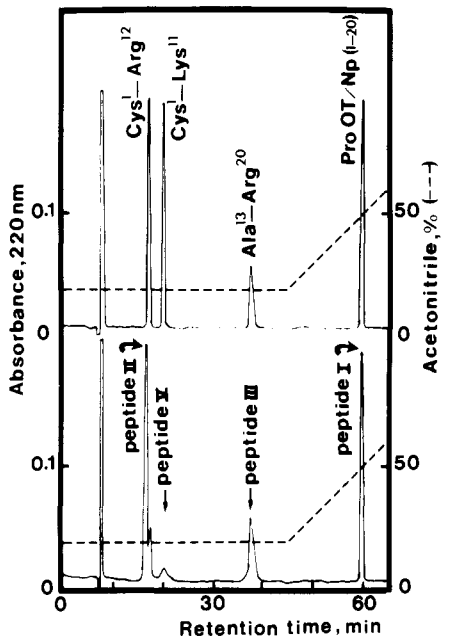

FIGURE 5: HPLC analysis of pro-OT/Np(1–20) (peptide I) conversion into peptides II and III. Upper panel: Elution of standard reference peptides II, V, III, and I on a μBondapak C-18 column using first an isocratic elution by 20% acetonitrile in H_2O containing 0.05% TFA and then a linear gradient of increasing concentration of acetonitrile in the same solution. Lower panel: The reaction mixture resulting from the incubation of peptide I with the purified endoprotease analyzed according to the same conditions as in the previous analysis (see upper panel).

this component (by amino acid composition or TLC separation) was unsuccessful in revealing any difference with peptide II, suggesting a very minor modification of the peptide. Alternative cleavage, if any, represented less than 5% of the total products (i.e., small peak noted as peptide V in Figure 5 results). The time course of the enzymatic conversion indicated that when very low protein enzyme quantities (microgram range), recovered from (Figure 4, left) experiment, were used in the presence of a large excess of substrate (20 μg), the rate of peptide I conversion was still linear after 24 h of incubation

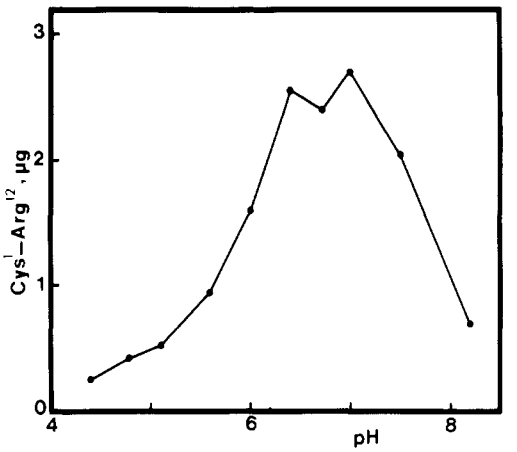

FIGURE 6: pH profile of the endoprotease activity. The enzyme activity was measured as usual at various pHs by using 50 mM ammonium acetate buffer adjusted to adequate pH.

Table I: Endoprotease Activity—Effects of Various Reagents[a]

inhibitor	activity (% of control)	inhibitor	activity (% of control)
none	100	Mg^{2+} (1 mM) ($MgCl_2$)	152
pepstatin (1.5 μM)	98	Ca^{2+} (1 mM) ($CaCl_2$)	88
PCMB (2.5 mM)	56	Mn^{2+} (1 mM) ($MnCl_2$)	97
PCMPS (2.5 mM)	33	Cu^{2+} (0.1 mM) ($CuSO_4$)	100
PMSF (1 mM)	114	Zn^{2+} (0.1 mM; 1 mM) ($ZnSO_4$)	90; 0
EDTA (2.5 mM)	0	Cd^{2+} (0.1 mM) ($CdCl_2$)	86
EGTA (2.5 mM)	5		

[a] Endoprotease activity (recovered after IEF purification) was measured using peptide I as substrate either in the absence or in the presence of various chemicals as described under Experimental Procedures. Conversion of peptide I into peptides II and III was monitored. Results are expressed as percent of the maximal conversion observed in the absence of any chemical.

(not shown). Under these conditions, more than 40–60% of the substrate was converted into fragments, allowing detection of either peptide II or III by UV absorbance (Figure 5).

This endoprotease behaves like a protein with a pH_i close to neutrality. As can be observed in Figure 4, left, two iso forms with pH_i respectively 6.9 and 7.2 were separated. By the measured catalytic properties both forms appeared to be indistinguishable except that the pH_i 7.2 species was less stable with time. Elution from the cationic resin was achieved as a sharp peak at about 150 mM ammonium ion concentration (Figure 4, right) to yield an enriched enzyme fraction corresponding to a 40 000 purification factor.

The enriched endoprotease produced no cleavage at single basic residues as could be demonstrated with another peptide substrate [see Clamagirand et al. (1986)]. The optimal activity of the convertase was found around pH 7.0 (Figure 6). This converting activity was unaffected by either PMSF or pepstatin, while PCMB, PCMPS, and EDTA (or EGTA) inhibited either partly or completely the enzyme reaction (Table I). Together these data suggest that the 58-kDa pro-OT/Np converting enzyme is neither a serine protease nor an aspartyl protease but can be classified as a metalloenzyme with possible involvement, at the catalytic site, of an active thiol group.

The specificity of the enzyme toward its substrate was analyzed by using selectively modified eicosapeptides (see Table II). The striking feature of this study was that any modification of one of the basic amino acids of the Lys–Arg pair was lethal for the convertase action. Indeed, replacement of either L-Lys or L-Arg by its D-Arg or D-Lys isomer abolished the cleavage reaction (Table II). Moreover, if the basic side

Table II: Relationships between Pro-OT/Np(1–20) Structure and Endoprotease Action[a]

			% cleavage of control
Peptide I		Cys[1]-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-Gly[10]-Lys[11]-Arg[12]-Ala[13]-Val-Leu-Asp-Leu-Asp-Val-Arg[20] (S—S)	100
"	XI	Arg[11]-Lys[12]	100
"	VII	DLys[11]-Arg[12]	0
"	VIII	Lys[11]-DArg[12]	0
"	IX	NLe[11]-Arg[12]	0
"	X	Lys[11]-NLe[12]	0
"	XI	Ala[10]-Lys[11]-Arg[12]	100

[a] Effects of various modifications of peptide I were tested on an aliquot (20 μg) of each of the modified peptides. This was incubated in the presence of an aliquot of the purified enzyme (1–2 μg recovered from IEF purification) under the same experimental conditions (see Experimental Procedures), and the result of the reaction was analyzed by HPLC as usual (see Figure 5 legend). Under these conditions 20 μg of peptide I was converted into 6–7 μg of peptide II and 4–5 μg of peptide III (100% basis). Results obtained with each analogue are referred to this basis. No trace of peptide III was detected in the case where peptides VII, VIII, IX, and X were used as substrates and recovered unmodified at the end of the enzymatic test.

chain of either residue 11 or 12 was replaced by a neutral, hydrophobic one (L-Arg or L-Lys by Nle[11] or Nle[12]), no cleavage of the modified peptides was observed. These observations reinforce strongly the conclusion that the endoprotease is strictly selective for basic L-amino acids arranged in a doublet. But reversal of the polarity of this pair did not appear to affect the locus of the cleavage reaction since in [Arg[11],Lys[12]]pro-OT/Np(1–20) hydrolysis was observed at the Lys[12]–Ala[13] bond (Table II). In order to evaluate the possible contribution of Gly[10], a residue involved in the amidation of the carboxyl terminus (Bradbury et al., 1982; Eipper et al., 1983; Gomez et al., 1984), this was replaced by Ala in peptide XI without measurable effect in the cleavage reaction (see Table II).

Discussion

The characterization of proteolytic systems possibly participating in the posttranslational processing of prohormones raises several questions relative to their biological relevance as well as about their mechanism of action. The model of the common precursor to ocytocin and neurophysin is particularly well suited for such an analysis since the structure of this prohormone and of its corresponding fragments is relatively simple and, now, well established. The endoprotease here described represents a good candidate to be involved in the in vivo processing of the corresponding precursor. Its endoproteolytic action, completed by the removal of the extra Lys–Arg residues by a carboxypeptidase B like enzyme (Kanmera & Chaiken, 1985; Clamagirand et al., 1986) and by a peptidylglycine amino monooxygenase involved in COOH-terminal amidation of OT-Gly[10] into OT(NH_2) (Kanmera & Chaiken, 1985), could account for the entire in vivo process [see Clamagirand et al. (1986)]. It is likely that these enzyme systems are colocalized in membrane-limited vesicles originating from the hypothalamus (Kanmera & Chaiken, 1985; Clamagirand et al., 1986). Moreover, a similar endoprotease, by its catalytic properties, selectivity toward the substrate, molecular size, and spectrum of sensitivity to various chemicals, was characterized in secretory granules from bovine corpus luteum (Clamagirand et al., 1987), a site of high ocytocin production at days 7–13 after ovulation (Ivell et al., 1985). Indeed, the detection, in extracts made from secretory granules of these corpora lutea, of COOH-terminally extended OT, i.e., OT-Gly, OT-Gly–Lys, and OT-Gly–Lys–Arg, gives indirect support to such a mechanism (Clamagirand et al., 1987). Interestingly, the endoprotease previously described as a proopiomelanocortin (POMC) converting enzyme was more recently reported to be also able in vitro to generate Vp-Gly[10]–Lys[11]–Arg[12] from a crude preparation of provasopressin/neurophysin (pro-Vp/Np) by cleavage on the carboxyl side of the Arg[12] residue (Parish et al., 1986). Except for the nature of the peptide bond that was cleaved, the two enzymes differ quite significantly in both their size and functional properties. In particular, the POMC convertase was reportedly completely inhibited by pepstatin and unaffected by EDTA.

Regarding the mechanism of excision of the Lys–Arg pair, it may, at least in theory, result from three different hydrolytic processes [for a discussion, see Cohen (1987) and Gluschankof and Cohen (1987)]. Those may involve a primary cleavage generated by an endoprotease either (i) before the doublet, (ii) in between the Lys–Arg pair, or (iii) on the carboxyl side of the Arg residue. Evidence illustrating any of these mechanisms is now available [for a review, see Gluschankof and Cohen (1987)]. Evidently, the pro-OT/Np converting endoprotease here described belongs to the category of protease acting according to the third mechanism. Evidence for such a cleaving mechanism was reported in some cases (Cromlish et al., 1986; Fuller et al., 1986; Loh et al., 1985), but this enzyme appears to be quite different from the other reported systems [for a discussion, see Gluschankof and Cohen (1987)] except for some functional analogies with the yeast pro-α-mating factor converting enzyme (Julius et al., 1984; Fuller et al., 1986). In particular, it cannot be classified as a serine protease, but its strict selectivity toward basic amino acids in tandem is noteworthy, a property demonstrated only for a few of the presently reported processing systems (Gluschankof et al., 1984; Mizuno et al., 1985; Clamagirand et al., 1986). Moreover, its catalytic action appears to be quite sensitive to either conformational or charge modifications at the basic doublet, suggesting that sterically accurate contacts between the basic amino acid side chains in tandem and the active site of the endoprotease may govern recognition of the cleavage loci. Although the latter were foreseen to belong in most cases to both exposed regions and highly ordered domains of the substrate molecules, i.e., β-turns flanked by either β-sheets and/or α-helix (Rholam et al., 1986), it is difficult to envision which structural parameters may specify the mode of endoproteolytic cleavage of those basic moieties. Both kinetic and thermodynamic measurements on various peptide analogues

should help solve these problems.

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Conformation of the ATP Binding Peptide in Actin Revealed by Proton NMR Spectroscopy†

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ABSTRACT: The actin peptide 106–124 exists in a completely conserved region of the sequence and binds strongly to both ATP and tripolyphosphate. Binding particularly affects residues 116 and 118 and generally affects the two segments 115–118 and 121–124 [Barden, J. A., & Kemp, B. E. (1987) *Biochemistry 26*, 1471–1478]. One-dimensional nuclear Overhauser enhancement difference spectroscopy was used to detect molecular interactions between both adjacent and nonadjacent residues. The N-terminal segment 106–112 was found to be largely extended. A sharp bend was detected between Pro-112 and Lys-113. The triphosphate moiety binds to the strongly hydrophilic central segment of the peptide. Evidence was obtained for a reverse turn involving residues 121–124. Amide proton temperature coefficients and coupling constants provide evidence for a type I β-turn. A model of the ATP binding site is proposed together with its relationship to other parts of the actin structure and to the phalloidin binding site.

Actin filaments are assembled, disassembled, and reorganized in cells. These processes provide a basis for the several behavioral, contractile, and structural events occurring in the cells (Korn, 1982).

The tertiary structure of actin has been determined at a resolution of 0.45 nm (Kabsch et al., 1985). Although this resolution is not yet sufficient to enable the backbone of the peptide chain to be followed, the electron density map reveals that there are two major domains. These are separated by a pronounced cleft, at the base of which is the triphosphate moiety of the bound ATP. Actin monomers denature in the absence of the bound nucleotide (Asakura, 1961; Waechter & Engel, 1977). Thus, the binding of ATP appears to be needed to stabilize the structure of the cleft region. Moreover, the nucleotide is dephosphorylated following the incorporation

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