Proocytocin/Neurophysin Convertase from Bovine Neurohypophysis and Corpus Luteum Secretory Granules: Complete Purification, Structure–Function Relationships, and Competitive Inhibitor[†,‡]

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ABSTRACT: Structure–function relationship studies were conducted on the proocytocin/neurophysin endoprotease previously characterized in both bovine neurohypophyseal and corpus luteum granules, using as a reference substrate a synthetic peptide reproducing the entire (1–20) NH_2-terminal domain of the precursor. The [D-Arg^{12}] derivative of proocytocin/neurophysin (1–20) was found to be a good competitive inhibitor of the enzyme (K_i = 30 μM), while the [D-Lys^{11}] derivative was not. This allowed the complete purification of two isoforms of the endoprotease (M_r 58 000 and 52 000, respectively) by affinity chromatography using covalently immobilized [D-Arg^{12}] proocytocin/neurophysin (1–20) as the affinity adsorbent. The use of selectively modified or truncated forms of the reference substrate or of the [D-Arg^{12}] competitive inhibitor of the endoprotease established clearly that this basic pair specific convertase is sensitive to modification of the substrate structure either at the basic residues of the cleavage locus or at amino acids around this site (i.e., Pro^7 and Gly^9). It is concluded that longer distance interactions between amino acids situated on both the NH_2 and COOH sides of the basic doublet Lys^{11}Arg^{12} may contribute to the stabilization of a preferred substrate conformation allowing recognition by the enzyme subsites.

Limited proteolysis of polypeptide hormone precursors is a key step in the posttranslational events leading to the bioactivation of these inactive pro forms. This implies the concerted participation of both endo- and exoproteases at basic amino acid loci found in ordered and exposed regions of the precursors (for a discussion, see Cohen (1987) and Gluschankof and Cohen (1987)]. Those endoproteases, which are involved in the recognition of the dibasic signals most frequently encountered in prohormone sequences around the active domains, exhibit interesting new properties and a strict selectivity for as yet not precisely defined secondary and tertiary structures of the precursors (Rholam et al., 1986; Crêminon et al., 1988).

Ocytocin is an important nonapeptide hormone, first discovered in the hypothalamoneurohypophyseal tract, which is distributed in several tissues (Ivell et al., 1986). Originally called a "neuropeptide", it exhibits a wide spectrum of biological activities that render the study of its biosynthesis very interesting. Ocytocin appears to be synthesized in either the bovine hypothalamus or the ovarian corpus luteum as a larger molecular mass precursor (11 kDa) (Ivell & Richter, 1984; Ivell et al., 1985) coded by a 0.85-kB gene (Land et al., 1983). The nonapeptide occupies the NH_2 terminus of this pro hormone and is separated from the COOH-terminal neurophysin domain by a restriction sequence Gly^{10}Lys^{11}Arg^{12}. Synthetic peptides either mimicking (Clamagirand et al., 1986) or reproducing (Clamagirand et al., 1987a,b) the entire NH_2-terminal domain (1–20) of proocytocin/neurophysin (pro-OT/Np) were used as substrates to characterize a basic pair selective endoprotease identified in the extracts of both bovine neurohypophyseal and corpus luteum secretory granules (Clamagirand et al., 1987a,b). This enzyme cleaves the synthetic peptide corresponding to the 1–20 domain of the precursor at the Lys^{11}Arg^{12}–Ala^{13} bond, and its action is most likely associated with a carboxypeptidase B like activity (Kanmera & Chaiken, 1985) and with an enzyme involved in the amidation of the COOH terminus of peptide hormones (Bradbury et al., 1982; Eipper et al., 1983; Gomez et al., 1984). Moreover, this protease appears to be a good candidate to be involved in the first proteolytic event of the enzyme cascade leading to the release of the active peptide from its inactive precursor (Clamagirand et al., 1987a). We report herein a study of functional relationships between substrate structure and enzyme action that led to the complete purification of this endoprotease and to the identification of its competitive inhibitor. Our observations suggest that a minimal structure at the dibasic cleavage locus is required for enzyme action.

Materials and Methods

Granule and Enzyme Preparation. Proocytocin/neurophysin converting endoprotease was prepared starting from 15 corpora lutea (cows superovulated during the luteal phase by successive FSH and PGFα_2 injections and bred by two successive artificial inseminations 12 and 24 h after estrus, INRA, Jouy-en-Josas, France) as previously described (Clamagirand et al., 1986). The enriched granule fractions were obtained by successive fractionations as in Clamagirand et al. (1987a,b). In the case of the enzyme prepared from bovine neurohypophysis, the granule preparation was made from organs as described in detail in Clamagirand et al. (1986).

Affinity Chromatography. After lysis of the purified granules, the enzyme was prepared as described earlier (Clamagirand et al., 1986, 1987a,b) and the endoprotease was purified to the stage of isoelectric focusing (Clamagirand et al., 1987b). Further purification of the enzyme was achieved

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[‡] Dedicated to the memory of Professor C. H. Li (1913–1987).

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by affinity chromatography on peptide VIII [i.e., [D-Arg^{12}]-pro-OT/Np(1–20)] covalently conjugated to an HMD-Ultrogel AcA 34 (IBF, Villeneuve-la-Garenne, France) obtained as follows. The α and ϵ amino groups of peptide VIII were protected by citraconylation (Blake et al., 1986). Peptide VIII (11.5 mg) was dissolved in 3.35 mL of 0.4 M phosphate buffer, pH 8.0, and 7.8 μL of citraconic anhydride in 111.8 μL of dioxane was added in two portions to the peptide solution under stirring during 1 h at room temperature. NaOH (1 N) was added to maintain the pH at 7.8. At the end of the incubation, the solution was subjected to chromatography on Sephadex G-10 in 30 mM sodium bicarbonate, pH 8.0. Fractions corresponding to the peptide elution were combined and lyophilized to give 10 mg of citraconylated peptide VIII.

HMD-Ultrogel AcA 34 (0.5 mL) was aded to 4 mg of EEDQ[1] previously dissolved in 0.2 mL of ethanol. Then 10 mg of the protected peptide VIII in 0.3 mL of EtOH (50%) was added to the suspension under stirring, and the mixture was incubated for 14 h at room temperature. The gel was then filtered and washed with 50% aqueous EtOH to eliminate excess reagents. The gel was then washed three to four times with 1 volume each of 1 M NaCl and water and then rinsed with 2 mM acetic acid, pH 3.2, for about 1 h. The decitraconylated peptide conjugate was then equilibrated in a solution of 100 mM ammonium acetate at pH 7.0 and used as such for affinity chromatography.

The endoprotease fractions recovered from the isoelectric focusing step (Clamagirand et al., 1987b) were applied to the conjugate-containing column in 100 mM ammonium acetate, pH 7.0, and kept in contact for 2 h at 4 °C. The column was then washed with the indicated volumes (see Figure 2) of the equilibration buffer, and elution was performed with 0.1 M acetic acid, pH 3.0, containing 2 mM $CaCl_2$. The specific activity of starting enzyme preparation and that recovered at each step were measured as described (Clamagirand et al., 1987a,b) by using peptides II or III (see below). The protein content was evaluated by using the method of Bradford (1976) or that of Krystal (1987).

Enzyme Assay. Endoprotease and carboxypeptidase B like activities were monitored by using peptide I as the substrate and measuring the production of either peptides II and III or of peptide IV (carboxypeptidase B like). Routinely, 20 μg of either substrate was incubated with an aliquot of the fraction to be tested (containing an average of 6 μg of protein) in 100 mM ammonium acetate, pH 7.0, for 24 h at 37 °C. After acidification, the remaining substrate and the reaction products were analyzed by HPLC on a Partisil 5 ODS-3 C-18 column (Whatman) eluted isocratically with 20% acetonitrile in 0.05% trifluoroacetic acid (TFA) aqueous solution. Production of peptides II and III from peptide I was quantified by UV absorbance using a D-2000 (Merck) integrator coupled to the HPLC. Endoprotease activity was expressed as quantities (in micrograms) of either peptide II or peptide III produced.

Gel Electrophoresis. Aliquots of the endoprotease recovered after affinity chromatography were diluted in the Laemmli buffer (Laemmli, 1970) or in 1 M Tris–glycerol buffer, pH 8.0. Electrophoreses were performed by use of the Phast system (Pharmacia, Uppsala, Sweden) with an 8–25% polyacrylamide gel gradient in the presence of SDS/β-mercaptoethanol for the denaturing conditions and in 7.5% acrylamide for the nondenaturing conditions.

Proteins were revealed by using the simplified method (Pharmacia, Phast system) of the silver nitrate staining method (Heukeshoven & Dernick, 1985). After electrophoresis under nondenaturing conditions, the gel was cut into 2-mm slices that were eluted overnight with 0.2 mL of 0.1 M ammonium acetate, pH 7.0, at 4 °C. The enzyme activity was then assayed as described above.

Peptide Synthesis. All the peptides used in this study either as substrates or to identify reaction products were obtained by solid-phase synthesis using a modified procedure of Merrifield's method (Merrifield, 1963) as described in detail in Nicolas et al. (1986) and using a semiautomated multisynthesizer NPS 4000 (Neosystem, Strasbourg, France). 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). The disulfide bridge was formed by air oxidation, at a 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) and analytical RP-HPLC on an LKB 2150 apparatus using either a Bondapak C-18 (Waters Associates, Milford, MA) or a Partisil 5 ODS-3 C-18 column (Whatman). The amino acid composition was obtained by HPLC separation of the amino acids after derivatization by the DABSCl technique (Chang et al., 1983; Gluschankof et al., 1987). NH_2-Terminal analysis was performed by using the DABITC/PITC double-coupling technique (Chang, 1983). Each peptide compound was further characterized 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 study; they are numbered accordingly from previous papers (Clamagirand et al., 1987; Crêminon et al., 1988):

peptide I: Cys^{1}TyrIleGlnAsnCys^{6}ProLeuGly
S–S
Gly^{10}Lys^{11}Arg^{12}AlaValLeuAspLeu-AspValArg^{20}
[pro-OT/Np(1–20)]

peptide II: [pro-OT/Np(1–12)] (Cys^1 → Arg^{12})

peptide III: [pro-OT/Np(13–20)] (Ala^{13} → Arg^{20})

peptide IV: [pro-OT/Np(1–10)] (Cys^1 → Gly^{10})

peptide V: [pro-OT/Np(1–11)] (Cys^1 → Lys^{11})

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

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

peptide XII: [Ala^9]pro-OT/Np(1–20)

peptide XIII: [Val^7]pro-OT/Np(1–20)

peptide XIV: [pro-OT/Np(11–20)] (Lys^{11} → Arg^{20})

peptide XV: [D-Arg^{12}]pro-OT/Np(6–14) (Cys^6 → Val^{14})

peptide XVI: [D-Arg^{12}]pro-OT/Np(9–14) (Gly^9 → Val^{14})

peptide XVII: [pro-OT/Np(1–13)] (Cys^1 → Ala^{13})

peptide XVIII: [Phe^3,Arg^8]pro-OT/Np(1–13) [pro-Vp/Np(1–13)]

[1] Abbreviations: DABITC, 4-(dimethylamino)azobenzene 4′-isothiocyanate; PITC, 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; RP-HPLC, reverse-phase high-performance liquid chromatography; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; EEDQ *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline; TLC, thin-layer chromatography; IEF, isoelectric focusing; TFA, trifluoroacetic acid; kB kilobase.

Table I: Characterization of Endoproteases from Neurohypophysis and Corpus Luteum Secretory Granules and the Effects of Various Chemicals on Them[a]

inhibitor	enzyme activity	
	neurohypophysis	corpus luteum
none	100	100
PMSF (1 mM)	100	100
pepstatin (1.5 μM)	100	100
EDTA (2.5 mM)	0	0
EGTA (2.5 mM)	0	0
PCMPS (2.5 mM)	36	51
PCMB (2.5 mM)	53	–

	neurohypophysis	corpus luteum
optimal pH	7.0	7.0
pH_i	6.9 and 7.1	4.9 (6.9)
K_m (μM)	200	50
V_{max} (pmol·min^{-1})	50	19.6
K_i (μM)	104	30

[a] Activity was measured as described under Materials and Methods on an aliquot of enzyme (6 μg of protein) either in the absence or in the presence of the indicated quantities of various chemicals. Values were calculated as the percent of the maximal activity measured (i.e., 4 μg of peptide II produced during the incubation in the absence of inhibitor). K_m and K_i values were measured by using peptide I as the substrate in the absence or in the presence of the competitive inhibitor, peptide VIII.

RESULTS

Endoprotease Purification. The endoprotease activity was highly enriched by the use of conventional gel filtration and isoelectric focusing (Clamagirand et al., 1987a). To achieve complete purification, an affinity chromatography step was added. It was previously observed that modification of pro-OT/Np(1–20) substrate affecting the configuration of either basic residue Lys[11] or Arg[12] of the doublet abolished the endoprotease cleavage at the Arg[12]–Ala[13] bond (Clamagirand et al., 1987b). In particular, neither the [D-Lys[11]] nor [D-Arg[12]] derivative of the eicosapeptide substrate was cleaved by the enzyme. When the capacity of either peptide VII or peptide VIII to inhibit pro-OT/Np(1–20) cleavage by the enzyme was tested, only the [D-Arg[12]] compound (peptide VIII) exhibited inhibitory properties (Table II). Lineweaver–Burk representation of the inhibition effects of this derivative demonstrated that it behaves as a strong competitive inhibitor, and a K_i of 30 μM was measured (Figure 1). This affinity of the inhibitor for the enzyme makes it adequate for use as a ligand for endoprotease purification by affinity chromatography.

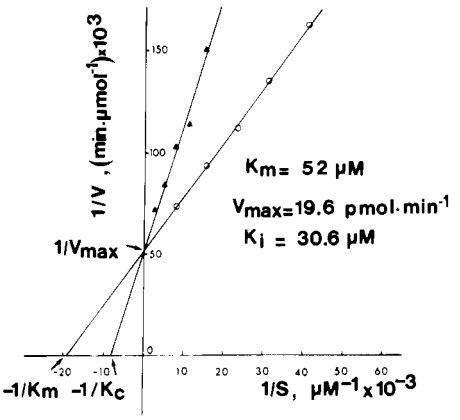

FIGURE 1: Determination of K_m, V_{max}, and K_i of the proocytocin/neurophysin convertase. The indicated amount of peptide I was incubated in a mixture containing 50 μL (average 3 μg of protein) of pooled fractions from the isoelectric focusing, 100 mM in ammonium acetate, pH 7.0 (see Enzyme Assay), either in the absence or in the presence of 43 μM of the modified peptide [D-Arg[12]] pro-OT/Np-(1–20). The resulting peptide mixture was analyzed by HPLC as described under Materials and Methods. Data are expressed as micromoles of peptide II produced during the 24-h incubation period.

Therefore, a conjugate of peptide VIII covalently bound to a derivatized Ultrogel was prepared. To allow coupling of the inhibitor to the NH_2 groups of the matrix by its free carboxylates (Asp[16], Asp[18], and Arg[20]), both α and ε amino groups were previously protected by citraconylation (Materials and Methods).

After deprotection of the NH_2 groups, the conjugate was used as an affinity adsorbent to achieve final purification of the endoprotease preparation highly enriched by isoelectric focusing (Clamagirand et al., 1987a). Figure 2 indicates that additional contaminating inactive proteins were removed by

Table II: Relationships between Substrate Structure and Enzyme Action[a]

Peptide			Cleavage % of control	% Inhibition
	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	–
"	XIV	Lys[11]-Arg[12]	0	12
"	XVII	Lys[11]-Arg[12]-Ala[13]	0	nd
"	XII	Ala[9]	0	nd
"	XIII	Val[7]	0	nd
"	VII	DLys[11]-Arg[12]	0	0
"	VIII	Lys[11]-DArg[12]	0	40
"	XV	Cys[6]—Lys[11]-DArg[12]-Ala-Val[14]	0	0
"	XVI	Gly[9]-Gly-Lys[11]-DArg[12]-Ala-Val[14]	0	0
"	XVIII	Phe[3]—Arg[8]—Lys[11]-Arg[12]-Ala[13]	0	nd

[a] Effects of various modifications on the cleavage of peptide I into peptides II and III were tested on an aliquot (20 μg) of each of the modified peptides. This mixture was incubated in the presence of an aliquot of the purified enzyme (1–2 μg recovered after IEF purification) under the same experimental conditions (see Materials and Methods), and the results of the reaction were analyzed by HPLC as usual. Under these conditions 20 μg of peptide I were converted into 6–7 μg of peptide II and 4.5 μg of peptide III (100% basis). No trace of peptide III was detected when peptides VII, VIII, XII, XIII, and XIV were used as substrates; the latter were recovered unmodified at the end of the enzyme tests. No trace of peptide II was detected when peptide XVII was used as the substrate. Peptides XV, XVI, and XVIII were recovered unchanged at the end of the incubation period. Inhibition is calculated as the percent of the maximal activity measured (i.e., 6–7 μg of peptide II produced during the incubation in the absence of each of the modified peptides) under these conditions. Each of the modified peptides was used at a double concentration of peptide I.

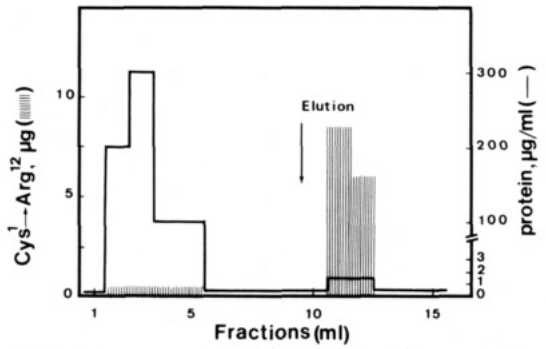

FIGURE 2: Endoprotease purification by affinity chromatography. The endoprotease fractions recovered from the isoelectric focusing step were applied to the affinity column in 100 mM ammonium acetate, pH 7.0, and kept in contact for 2 h at 4 °C. The column was washed with the indicated volumes of the equilibration buffer to remove contaminating inactive proteins, and 0.1 M acetic acid, pH 3.0, containing 2 mM $CaCl_2$, was used to elute the enzyme. activities were measured as described under Materials and Methods and are expressed as micrograms of $Cys^1 \rightarrow Arg^{12}$ (peptide II) produced during the incubation period. Protein content was determined by using either the method of Bradford (1976) or that of Krystal (1987).

adsorption and washing of the gel with diluted ammonium acetate, pH 7.0. Elution was performed by using the substrate as the eluent (not shown) or by changing the pH and ionic conditions (to pH 3.0 and 2 mM $CaCl_2$) and led to the recovery of high activity with a very low protein concentration (Figure 2). The total recovery following affinity chromatography was approximately 70%, and this step further enriched the endoprotease 150-fold (Figure 2). Electrophoretic analysis of the purified protease under either nondenaturing conditions or in the presence of SDS/β-mercaptoethanol revealed that the enzyme preparation was homogeneous. In both cases, two populations of activities, with slight differences in electrophoretic mobility, were observed (Figures 3 and 4). This is in keeping with previous observations that two closely related forms of the protease were recovered after isoelectric focusing (Clamagirand et al., 1986, 1987a). The apparent M_r of these two isoforms was approximately 58 000 and 52 000, respectively (Figure 3).

Comparison of Endoproteases from Bovine Corpus Luteum and Neurohypophysis. Comparison of the properties of inhibitors on the purified endoproteases from either type of granule (corpus luteum or neurohypophysis) indicated clearly that both enzyme activities are indistinguishable. In either case, serine protease or aspartylprotease inhibitors had no effect (Table I), but divalent ions chelators such as EDTA and EGTA were totally effective against both forms.

Thermodynamic and kinetic parameters determined for the purified fractions of the enzymes indicated similar properties for the neurohypophyseal and corpus luteum preparations. Both enzymes were inhibited competitively by [D-Arg^{12}]pro-OT/Np(1–20) and had K_i of values 104 and 30 μM, respectively, and V_{max} values of 50 and 19.6 pmol·min^{-1}. Both enzyme activities were an optimal value around pH 7.0. However, some slight differences in K_m were observed between the two enzymes (200 and 50 μM, respectively, using peptide I as the substrate), and the corpus luteum enzyme showed a pH_i of either 4.9 or 6.9 depending upon the preparation. Why these differences exist is not as yet clear, but they may be due to the extraction conditions. Overall, these data confirm that both endoproteases possess similar properties.

Substrate Structure and Endoprotease Activity. Previous studies using selectively modified eicosapeptides derived from the reference peptide I, proocytocin/neurophysin(1–20), had shown that the endoprotease activity is sensitive to modifications of the substrates at the level of either amino acid of the basic doublet (Clamagirand et al., 1987b). Further use of such synthetic derivatives indicated that the inhibitor properties of peptide VIII depend upon the presence of a [D-Arg] moiety in position 12 as well as upon the length of the peptide (Table II). The derivatives of peptide VIII, i.e., peptides XV and XVI,

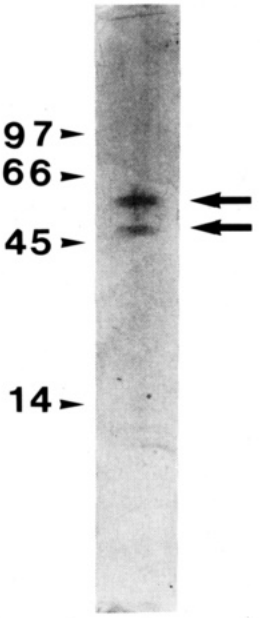

FIGURE 3: SDS/β-mercaptoethanol polyacrylamide gel electrophoresis of purified proocytocin/neurophysin convertases. Fractions 11 and 12 obtained from the affinity chromatography column were pooled. An aliquot (200 ng of protein) was run on an 8–25% polyacrylamide gradient gel in the presence of SDS/β-mercaptoethanol by use of the Phast system (Pharmacia, Uppsala, Sweden). The gel was silver-stained to visualize the proteins. The mobilities of different molecular mass markers (bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; phosphorylase *b*, 97 kDa; lysosyme, 14 kDa) are indicated. The revelation of two bands with slightly different electrophoretic mobilities demonstrates that the enzyme preparation was homogeneous. The apparent M_r of these two isoforms was approximately 58 000 and 52 000, respectively.

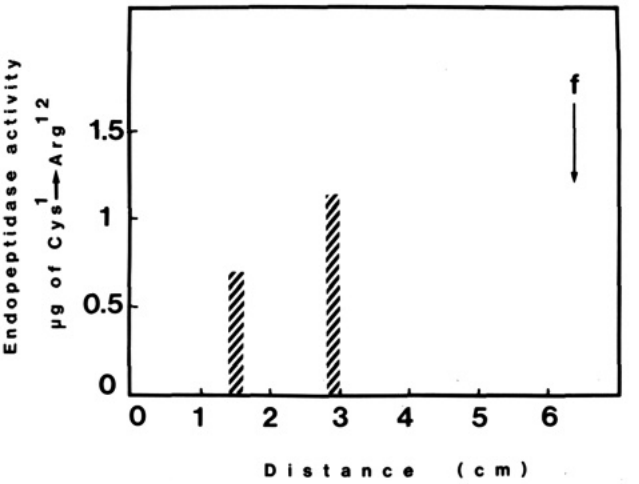

FIGURE 4: Gel electrophoresis of purified proocytocin/neurophysin convertases. Fractions 11 and 12 obtained from the affinity chromatography column were pooled. An aliquot (850 ng of protein) was run on a 7.5% acrylamide gel by use of a Tris–glycine buffer system. The gel was cut, and each 2-mm slice was eluted overnight at 4 °C with 0.2 mL of 0.1 M ammonium acetate buffer, pH 7.0. Each eluate was assayed for the presence of the endoprotease by incubation at 37 °C with 20 μg of peptide I for 24 h. Two populations of activities were observed, in keeping with previous observations that two closely related forms of the protease were recovered after isoelectric focusing (f, migration of bromophenol blue).

did not exhibit measurable inhibitory effects (Table II). This observation was further corroborated by the fact that both the NH_2- and COOH-terminally truncated forms of the reference substrate, still bearing the $Lys^{11}Arg^{12}$ doublet, remained uncleaved by the endoprotease.

Peptides XIV, XVII, and XVIII were not cleaved and showed very weak inhibitory properties (Table II). Furthermore, selective modification at amino acids thought to play a key role in the specification of a particular conformation at the cleavage site was tested for enzyme action. Table II data indicate that either replacement of Pro^7 by Val^7 or of Gly^9 by Ala^9 was lethal for enzyme action.

Discussion

The presence of an endoprotease selective for the cleavage of the pro-OT/Np(1–20) Arg^{12}–Ala^{13} bond, in granules purified from two organs in which production of both ocytocin and neurophysin is well established, can be taken as a strong argument in favor of its involvement in the processing of this hormone precursor. The specificity of this enzyme for hydrolysis of this particular peptide bond on the carboxyl side of the $Lys^{11}Arg^{12}$ doublet is corroborated by the characterization, in bovine corpus luteum, of COOH-terminally extended forms of OT, i.e., OT-Gly, OT-Gly-Lys, and OT-Gly-Lys-Arg (Clamagirand et al., 1987a). While the finding by others, using similar techniques, of incompletely processed forms of the pro-OT/Np precursor during postnatal development in the rat (Alstein & Gainer, 1988) supports this conclusion, these observations cannot, as yet, definitively establish the involvement of this enzyme in a particular processing pathway. Another candidate for this function was also reported to cleave either proopiomelanocortin or pro-OT/Np and pro-Vp/Np (Parish et al., 1986), and alternative hydrolysis in between or after the doublet was observed. In its optimal pH (4.6), size, and inhibitor properties, this enzyme does not resemble the presently described endoprotease.

It is noteworthy that purification of the enzyme led to two closely related forms of the protease. It can be hypothesized that we are dealing with isoforms possibly derived from a larger native protein. It is interesting in this respect that elucidation of the primary structure of carboxypeptidase E (Fricker et al., unpublished data) and of the enzyme involved in the post-translational COOH-terminal amidation of neuropeptides (Mains et al., unpublished data) indicated that the soluble forms of these enzymes were indeed derived from a larger protein containing a transmembrane domain that was lost, possibly during extraction.

Striking features of pro-OT/Np convertase are its high degree of selectivity for the substrate and its sensitivity to modifications of the amino acid sequence around, or at, the cleavage locus. From the data presented herein and in previous papers (Clamagirand et al., 1987b; Crêminon et al., 1988) it can be inferred that (i) this enzyme strictly requires a basic doublet (LysArg or ArgLys) to hydrolyze the peptide bond on the COOH-terminal side, (ii) this enzyme will not cleave any monobasic site either on the relevant substrate or on a model peptide, and (iii) although the presence of the dibasic moiety is a necessary condition, it is not sufficient per se. It is clear that both the cleavage reaction and the inhibitory action of the D-Arg derivatives showed a size-dependent relationship. This is particularly obvious from the observation that the pro-OT/Np convertases failed to hydrolyze NH_2- or COOH-terminal fragments of the reference substrate still bearing the basic $Lys^{11}Arg^{12}$ doublet, as in peptides XIV, XVII, and XVIII. Therefore, it can be concluded that the length of the peptide contributes to a stabilization of the key structure and that a given conformation of the substrate at the cleavage locus is required for enzyme action (Rholam et al., 1986; Crêminon et al., 1988). While these secondary and tertiary structures cannot as yet be defined precisely, it is noteworthy that modification of amino acids on the NH_2 side of $Lys^{11}Arg^{12}$, which are assumed to play a key role in the formation of β-turns, was devastating for enzyme action. This is true in particular for the Pro^7 and Gly^9 residues replaced by amino acids, such as Val and Ala, respectively. Physicochemical studies on these and an extended series of model peptides may help to establish the existence of such structures in solution.

Acknowledgments

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Registry No. Peptide I, 107945-26-6; peptide II, 90685-16-8; peptide III, 106905-38-8; peptide IV, 98791-57-2; peptide V, 98823-76-8; peptide VII, 110414-83-0; peptide VIII, 110351-95-6; peptide XII, 115945-10-3; peptide XIII, 115921-56-7; peptide XIV, 118658-13-2; peptide XV, 118658-14-3; peptide XVI, 118658-15-4; peptide XVII, 103185-69-9; peptide XVIII, 118658-16-5; proocytocin/neurophysin endoproteinase, 110353-43-0.

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Identification and Purification of Truncated Insulin-like Growth Factor I from Porcine Uterus. Evidence for High Biological Potency†

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ABSTRACT: We report the completion of the purification of uterine-derived growth factors (UDGF) described previously by this laboratory [Ikeda, T., & Sirbasku, D. A. (1984) *J. Biol. Chem. 259*, 4049-4064]. During isolation, the mitogenic activity was monitored by using the human MCF-7 breast cancer cells in serum-free Ham's F12 and Dulbecco's modified Eagle's medium (1:1, v/v) containing 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.2), 200 μg/mL bovine serum albumin, and 10 μg/mL human transferrin. This medium sustained growth for several days in response to a single addition of growth factor. The isolation of UDGF began with acetic acid extraction followed by sulfopropyl-Sephadex chromatography, Bio-Gel P-10 molecular sieve fractionation, and a series of reverse-phase high-pressure liquid chromatography separations. Purifications [[(1.0-8.5) × 10^6]-fold] of three mitogens (5-20 ng each) were achieved. The mitogens were shown by protein microsequencing to be DES 1→3 to DES 1→6 forms of insulin-like growth factor I (truncated IGF-I). An M_r estimated by ^{125}I labeling, urea–sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and autoradiography was consistent with a DES 1→3(4) N^{α} truncation. Immunoadsorption and radioimmunoassay confirmed immunological properties equivalent to IGF-I. Radioreceptor assays showed truncated IGF-I was functionally equivalent to recombinant IGF-I. The ED_{50} values of DES 1→3 IGF-I and recombinant IGF-I for MCF-7 cell growth were 0.8-6.0 and 30-150 pg/mL, respectively. With Balb/c 3T3 mouse fibroblasts, the ED_{50} of DES 1→3 IGF-I was 100 times lower than that of IGF-I. We conclude that the major acid-stable low-M_r mitogenic activities isolated from uterus are very potent forms of truncated IGF-I capable of stimulating growth of epithelial and mesenchymal cells.

Previously, this laboratory has shown that extracts of uterus contained estrogen-inducible growth factor activities for breast cancer cells in culture (Sirbasku, 1978; Sirbasku et al., 1981; Ikeda et al., 1982). When extracts were prepared at neutral pH, one of the activities identified was an M_r 70000 protein (Sirbasku et al., 1981) later purified to homogeneity and identified as transferrin (Riss et al., 1986; Riss & Sirbasku, 1987a). In contrast, acid extraction of uterus yielded lower M_r activities that were heat stable and readily assayed with breast, pituitary, or uterine cancer cells (Ikeda et al., 1984b).

The purification of an acid-stable low M_r mitogenic activity from uterus had been described by Ikeda and Sirbasku (1984). This material, designated UDGF,[1] was obtained in milligram quantities per kilogram of lyophilized powder of pregnant sheep uteri after a 150-fold purification. The M_r of UDGF was estimated between 3700 and 7200. The ED_{50} was measured in nanomolar concentrations. Although moderatively active, the ED_{50} of this preparation was from 10 to 1000 times greater than those of other well-characterized mitogens (Barnes & Sirbasku, 1987a,b; Ogasawara & Sirbasku, 1988; Karey & Sirbasku, 1988).

Analytical data obtained by a variety of methods initially indicated UDGF had been purified to near homogeneity (Ikeda & Sirbasku, 1984). Nevertheless, additional evaluation by protein chemistry methods confirmed this preparation was not

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[1] Abbreviations: UDGF, uterine-derived growth factor; IGF-I, insulin-like growth factor I; IGF-II, insulin-like growth factor II; EGF, epidermal growth factor; bFGF, basic fibroblast growth factor; TGFα, transforming growth factor α; SP-Sephadex, sulfopropyl-Sephadex; RP-HPLC, reverse-phase high-pressure liquid chromatography; F12/DME, a 1:1 (v/v) mixture of Ham's F12 nutrient medium and Dulbecco's modified Eagle's medium (high glucose concentration formulation) containing 2.2 g/L sodium bicarbonate; PBS, Dulbecco's phosphate-buffered saline, pH 7.2; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.2; TFA, trifluoroacetic acid; FBS, fetal bovine serum; RIA, radioimmunoassay; Tf, human transferrin; BSA, bovine serum albumin, fraction V; PMSF, phenylmethanesulfonyl fluoride; cpm, counts per minute; cpd, cell population doublings; ELISA, enzyme-linked immunosorbent assay; RRA, radioreceptor assay; SFM-3T3, serum-free defined medium for 3T3 cell growth.

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