Purification and Characterization of a Secreted Arginine-Specific Dibasic Cleaving Enzyme from EL-4 Cells†

Eva Csuhai, Afshin Safavi, and Louis B. Hersh*

Department of Biochemistry, University of Kentucky, Lexington, Kentucky 40536

Received March 2, 1995; Revised Manuscript Received July 12, 1995®

ABSTRACT: A secreted dibasic cleaving peptidase capable of converting dynorphins into Leu-enkephalin-Arg^6 was purified from the medium of EL-4 mouse thymoma cells. The enzyme is a novel metalloendopeptidase with a neutral pH optimum (6.9) and a molecular weight of ~130 000. The dibasic cleaving enzyme was completely inhibited in the presence of 20−50 mM amine buffers, 0.1 mM EDTA, 0.5 mM 1,10-phenanthroline, 0.5 mM *N*-ethylmaleimide, and 1 mM DTNB. Unlike the Kex2 family of proteases, Ca^{2+} did not activate the endopeptidase, but high concentrations (1 mM) of metal ions such as Cu^{2+}, Ni^{2+}, Zn^{2+}, and Co^{2+} completely inhibited the enzyme. Inhibition was not seen with 0.2 mM TLCK, 1 mM DTT, and 1 mM PMSF. The enzyme will cleave Arg−Arg and Arg−Lys bonds, but not Lys−Arg or Lys−Lys bonds in identical environments, and no aminopeptidase or carboxypeptidase activity was seen. The size of the substrate does not seem to be a determining factor, since dynorphin A(1-12) is cleaved at a rate similar to prodynorphin B(228-256) containing 29 amino acids. The identity of the residues on either side of the cleavage site influences the rate of processing, as noted by different rates of cleavage for the same size peptides dynorphin A(1−13) vs dynorphin B(1−13) or dynorphin A(1−9) vs β-neoendorphin. The presence of proline in the P3′ (α-neoendorphin), P4′ (dynorphin A(1−11)), or P5′ (bovine adrenal medulla dodecapeptide) position does not prevent cleavage, but neurotensin and its (1−11) fragment containing both P2 and P2′ proline residues are not cleaved.

Many physiological peptide hormones are synthesized as inactive prohormones that are processed into smaller peptide fragments by proteolytic cleavage at specific sites. The formation of the active peptides is generally initiated by cleavage at the carboxy terminus of dibasic sequences and is finished by removal of the C-terminal basic residues by carboxypeptidases. The best known examples of enzymes responsible for cleavage at dibasic residues are the prohormone-processing enzymes, prohormone convertases 1, 2, and 4 (PC1, PC2, and PC4), furin, and PACE4 (Seidah et al., 1991, 1993; Breslin et al., 1993).

In addition to the peptidases cleaving at the carboxy side of dibasic sequences in prohormones, a group of enzymes has been reported to cleave between dibasic residues within the opioid peptides dynorphin A and dynorphin B to give Leu-enkephalin-Arg.[6] These enzymes have been described in yeast (Mizuno & Matsuo, 1984), rat testes (Chesneau et al., 1994b), human (Nyberg & Silberring, 1990; Silberring et al., 1992a) and bovine spinal cords (Silberring & Nyberg, 1989), human choroid plexus (Nyberg et al., 1991), cerebrospinal fluid (Nyberg et al., 1985), and neuroblastoma cells (Satoh et al., 1989). These enzymes, which are collectively referred to as dynorphin-converting enzymes, have widely different characteristics: among them are cysteine, serine, and metallopeptidases, with pH optima ranging from 5 to 8.8. Their substrate specificities also vary; some cleave dynorphin A at rates more than 10 times higher than dynorphin B (Silberring et al., 1992a; Chesneau et al., 1994b), while others show preference for dynorphin B (Silberring & Nyberg, 1989) or process both peptides at similar rates (Nyberg & Silberring, 1990). However, not all of the dynorphin-converting enzymes have been fully characterized in terms of their substrate specificity.

The level of dynorphin-converting enzyme activity changes during various physiological conditions: activity increased during exercise (Persson et al.; 1993), whereas a decrease in enzyme levels was observed at term pregnancy (Lyrenäs et al., 1988). Decreases in enzyme levels were reported in various pathological states (arthritis, spinal cord injury, etc.) (Silberring et al., 1992b; Sharma et al., 1992). It also has been found that enzyme activity increases as a consequence of morphine treatment of neuroblastoma cells (Vlaskovska et al., 1994). However, the physiological relevance of these enzymes *in vivo* has yet to be established. Since the dynorphins are processed and stored in intracellular granules and exert their physiological action by binding to opiate receptors located extracellularly, enzymes that act on the peptides *in vivo* should be localized at one or the other of these sites (Turner & Barnes, 1994).

We report here the identification and characterization of a new member of the dynorphin-converting enzyme family, which is secreted from EL-4 mouse thymoma cells.

MATERIALS AND METHODS

Chemicals were purchased from Sigma Chemical Co., unless otherwise indicated, and were used without further

† This research was supported in part by a grant from the National Institute on Drug Abuse (DA02243).

* Author to whom correspondence should be addressed: Department of Biochemistry, Chandler Medical Center, University of Kentucky, 800 Rose Street, Lexington KY 40536; telephone (606)-323-5549, fax (606)-323-1037.

® Abstract published in *Advance ACS Abstracts,* September 1, 1995.

0006-2960/95/0434-12411$09.00/0

purification. GEMSA[1] was obtained from Calbiochem. Peptides were purchased from the following sources: BAM12, BAM(1−9), α-neoendorphin, β-neoendorphin, dynorphin B, dynorphin A, dynorphins A(1−6),-(1−7), -(1−8),-(1−9), -(1−10), and -(1−13), [D-Pro[10]]-dynorphin A(1−11), Tyr-Gly-Gly-Phe-Met-Arg-Phe, Tyr-Gly-Gly-Phe-Met-Arg-Gly-Leu, neurotensins (1−13),-(1−11), and -(8−13), angiotensin I(1−10), somatostatin, bradykinin, and β-endorphin were from Bachem California; substance P(1−9), dynorphins A(1−11) and -(1−12), and Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Arg-Pro-Lys were from Peninsula Laboratories. Tyr-Gly-Gly-Phe-Met-Lys-Arg-Val-Gly, Tyr-Gly-Gly-Phe-Met-Arg-Lys-Val-Gly, and Tyr-Gly-Gly-Phe-Met-Lys-Lys-Val-Gly were custom synthesized at Bio-Synthesis, Inc. (Lewisville, TX). Phe-Leu-Arg-Arg-Ile and Leu-Arg-Arg-Ile were synthesized at the Macromolecular Structure Analysis Facility at the University of Kentucky. EL-4 cells were obtained from Dr. Bonnie C. Miller, University of Texas Southwestern Medical Center (Dallas, TX). Abz-Phe-Arg-Arg-Val-EDDnp was kindly provided by Dr. Eline S. Prado, Escola Paulista de Medicina (Sao Paulo, Brazil). Rabbit antiserum for dynorphin (1−6) was a generous gift of Dr. Fred Nyberg, University of Uppsala (Uppsala, Sweden).

SDS−PAGE chromatography (Hames, 1981) and staining of the gels were carried out according to published procedures (Gottlieb & Chavko, 1987).

Iodination of Leu-Enkephalin-Arg[6]

Leu-enkephalin-Arg[6] was iodinated by the chloramine-T method, as previously reported (Christensson-Nyländer et al., 1985). The labeled peptide was purified by HPLC on a Vydac C_{18} column before use.

Enzyme Assays

Radioimmunoassay for the Detection of Dynorphin (1−6) Produced by Dibasic Cleaving Endopeptidase. The procedure utilized here is a modification of the assay described (Christensson-Nyländer et al., 1985). In 80 μL final volume of 20 mM potassium phosphate buffer (pH 7.0), 5−10 μL of enzyme solution was preincubated with 10 μM captopril, 10 μM GEMSA, 20 μM phosphoramidon, and 20 μM amastatin at 37 °C for 20 min. Dynorphin B (80 nM) was added to start the reaction, and the mixture was incubated at 37 °C for 0.5−2 h. After 5 min of boiling, 80 μL of methanol/0.2 M hydrochloric acid (1:1) was added and the samples were centrifuged for 2 min. Aliquots (25 μL) were placed into 1.5 mL centrifuge tubes, 100 μL of antibody solution (1:10000 dilution from serum) and 100 μL of ^{125}I-labeled Leu-enkephalin-Arg[6] (approximately 5000 cpm total) were added, and the vials were incubated at 4 °C for 2 h. Ice cold dextran/charcoal solution (200 μL; 0.25 and 2.5 mg/mL in water, respectively) was added, and after a 10 min incubation at 4 °C, the vials were centrifuged for 5 min. The supernatant (300 μL) was counted for γ radiation in a Beckman Gamma 8000 counter, along with standard curves of Leu-enkephalin-Arg.[6] Measurements were carried out in duplicate or triplicate. The antibody concentration was determined for each batch of iodinated peptide, such that the linear range of the standard curve was between 10 and 1000 fmol of peptide/sample.

HPLC Detection of Products from Cleavage by Dibasic Cleaving Endopeptidase. Reactions were conducted in 100 μL total volume containing 20 mM final concentration of potassium phosphate buffer (pH 7.0) and 5−50 μL of enzyme solution preincubated with 10 μM captopril, 10 μM GEMSA, 20 μM phosphoramidon, and 20 μM amastatin at 37 °C for 20 min. The appropriate peptide was added at 60 μM final concentration and the reaction was incubated at 37 °C for 10−120 min. The reaction was stopped by boiling for 3 min, and 10 μL of 5% trifluoroacetic acid was added immediately. The samples were centrifuged in an Eppendorf centrifuge for 2 min and then injected onto a Vydac C_4 column connected to a Waters HPLC chromatography system, where the peptides were eluted with a gradient of 5−75% acetonitrile containing 0.1% trifluoroacetic acid. Standards for each peptide were injected under similar conditions and used for the quantitation of reaction products and starting materials. During the purification of dibasic cleaving endopeptidase, routine enzyme assays utilized α-neoendorphin as substrate.

Lactate Dehydrogenase Assay. Lactate dehydrogenase activity was determined by measuring the decrease in fluorescence upon oxidation of NADH in the presence of pyruvate. Reaction mixtures contained 0.1 M Tris-HCl (pH 7.3), 40 mM sodium pyruvate, and 0.6 mM NADH in a total volume of 500 μL. The reaction was initiated by the addition of 20−50 μL of enzyme and followed on a Hitachi F-2000 spectrofluorometer at an excitation wavelength of 340 nm and an emission wavelength of 450 nm.

Inhibition Studies. Group-specific inhibitors (from stock solutions of 5−100 mM) were added to the enzyme solution and preincubated at 37 °C for 20 min before the substrate was added to start the reaction. PMSF, TLCK, and TPCK stock solutions were made in ethanol, which resulted in 1% and 2% ethanol concentrations, respectively, in these assays. Control experiments indicated that under these conditions ethanol did not affect peptide cleavage.

Nondenaturing Gel Electrophoresis

Native PAGE was performed in the absence of sodium dodecyl sulfate and 2-mercaptoethanol, but under otherwise identical conditions to those described for SDS−PAGE. The gel slices were gently ground with a hand-held homogenizer (Kimble) and incubated with 0.1 M potassium phosphate (pH 7) overnight at 4 °C, and the supernatant was assayed for endopeptidase activity after removal of the gel pieces by centrifugation.

[1] Abbreviations: Abz, *o*-aminobenzoyl; BAM12, bovine adrenal medulla dodecapeptide; bis-Tris propane, 1,3-bis[(tris(hydroxymethyl)-methyl)amino]propane; cFP-Ala-Ala-Phe-pAB, carboxyfluoropropyl-Ala-Ala-Phe-*p*-aminobenzoate; $C_{12}E_8$, *N*-dodecylocta(ethylene glycol) monoether; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; EDDnp, ethylenediaminedinitrophenyl; EDTA, ethylenediaminetetraacetic acid; GEMSA, guanidinoethylmercaptosuccinic acid; LHRH, luteinizing hormone-releasing hormone; γ_3-MSH, melanocyte-stimulating hormone; MES, 2-morpholinoethanesulfonic acid; NA, naphthylamide; NADH, β-nicotinamide adenine dinucleotide, reduced form; PMSF, phenylmethanesulfonyl fluoride; POMC, proopiomelanocortin; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; Suc, succinyl; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; TLCK, *N*-α-*p*-tosyl-L-lysine chloromethyl ketone; Tris, tris(hydroxymethyl)aminomethane.

Studies on the Secretion and Enzyme Activity of the Dibasic Cleaving Endopeptidase from EL-4 Cell Supernatant

EL-4 cells were subcultured at a concentration of 2.5 × 10^5 cells/mL for 24 h in RPMI 1640 medium supplemented with 5% fetal bovine serum at 37 °C and 10% CO_2. Cells were harvested by centrifugation, washed with Hank's salt solution (pH 7.4), and incubated in Hank's salt solution supplemented with 45 mM sodium phosphate buffer (pH 7.4) in the presence or absence of various reagents for 24 h at 37 °C and 10% CO_2. For secreted enzyme activity assays, cells were removed from the medium by centrifugation at 800*g* for 5 min, and the supernatant was taken as a source of enzyme. Cells were counted to assess changes in cell number during the incubation, while 20 μL of medium was added to 180 μL of 10% trypan blue and cell viability was determined by trypan blue exclusion.

Enzyme Purification

EL-4 cells were subcultured at a concentration of 0.25 × 10^6 cells/mL for 2−3 days in 40 L of RPMI 1640 medium supplemented with 5% fetal bovine serum at 37 °C and 10% CO_2. Cells were harvested by centrifugation at 1000*g* for 10 min, washed with Hank's balanced salt solution, resuspended in 8 L of Hank's balanced salt solution at a concentration of 5 × 10^6 cells/mL, and incubated for 2−3 days at 37 °C and 10% CO_2. The supernatant was harvested by centrifugation (5 min at 1000*g*) and concentrated using an Amicon stirred cell concentrator with a YM 30 membrane to ~200 mL. The sample was desalted by dilution to 500 mL with 20 mM Tris-HCl (pH 7.5), concentrated to ~100 mL, and diluted and concentrated as described earlier to a final volume of ~100 mL. The supernatant was centrifuged at 30000*g* for 20 min to remove denatured proteins and cell debris and loaded onto a 15 mL QMA Accell Plus anion exchange column previously equilibrated with 20 mM Tris-HCl buffer (pH 7.5). The column was washed with 20 mM Tris-HCl (pH 7.5) and then eluted with a NaCl gradient from 20 to 500 mM in this buffer at flow rate of 1 mL/min. Saturated ammonium sulfate was added to the pooled active fractions to reach 40% saturation, the solution was centrifuged at 30000*g* to remove all solid material, and the supernatant was applied to a 3 mL Phenyl 650S column (Tosohaas). The column was eluted with a gradient from 40% to 0% saturated ammonium sulfate in 20 mM Tris-HCl (pH 7.4) containing 5% glycerol at 0.5 mL/min flow. The active fractions were concentrated in a Centriprep 10 spin concentrator (Amicon) to <2 mL and then applied to a 1.6 × 60 cm Pharmacia Superdex 200 column. The gel filtration column was developed with 20 mM Tris-HCl buffer (pH 7.5) containing 5% glycerol at a flow rate of 0.2 mL/min. Fractions containing the dibasic cleaving activity were loaded without further concentration onto a 1 mL Mono Q column at a flow rate of 0.5 mL/min., and the proteins were eluted with a NaCl gradient from 0 to 0.7 M in the same Tris buffer as that described earlier. The active fractions were pooled, concentrated to a 1 mL final volume, and then chromatographed a second time on the Superdex 200 column as described. The desalted active fractions from the gel filtration column were applied to a 0.3 mL hydroxylapatite column (high resolution, Calbiochem, lot no. 510040), and the absorbed proteins were eluted with a gradient from 20 to 700 mM potassium phosphate buffer (pH 7.4). The active fractions were concentrated 10-fold in a Microcon 10 spin concentrator (Amicon) and stored at 4 °C in 10% glycerol. The purified enzyme retained greater than 80% activity after 4 weeks.

Release of Cytosolic Components by Digitonin

EL-4 cells were washed with 10 mL of Hank's balanced salt solution and resuspended in 0.4 mL of ice cold 0.25 mM sucrose containing 40 mM Tris-HCl (pH 7.3) at a density of 5 × 10^7 cells/mL. To this cell suspension was added 0.04 mg of purified digitonin. After 10 min on ice the cells were transferred to a 1.5 mL microcentrifuge tube and centrifuged at 14000*g* for 15 s. The supernatant was transferred to a clean tube, and the pellet was resuspended in an equal volume of 0.25 mM sucrose and 40 mM Tris-HCl (pH 7.3). To each tube was added $C_{12}E_8$ detergent to a final concentration of 0.5%. After 5 min on ice, samples were sonicated for 10 s and then centrifuged for 10 min at 14000*g*. The supernatants were removed and assayed for dibasic cleaving endopeptidase and lactate dehydrogenase activities.

RESULTS

Preliminary experiments, utilizing a sensitive radioimmunoassay (Christensson-Nyländer et al., 1985), indicated that the EL-4 mouse thymoma cell line secretes an enzyme activity that cleaves dynorphin B between the dibasic arginine pair to yield Leu-enkephalin-Arg^6 as a product. To test the characteristics of secretion and to eliminate the possibility that cell lysis causes the secretion of the dibasic cleaving enzyme activity, we compared the level of secretion of a well-known intracellular enzyme, lactate dehydrogenase, to that of the dibasic selective peptidase. Thus, cells were suspended in Hank's balanced salt solution and incubated at 37 °C with 10% CO_2 for up to 24 h. At various times, aliquots of the cells were separated from the medium by centrifugation, and the cells disrupted by sonication. Figure 1 shows the amount of dibasic cleaving enzyme being secreted during this time period compared to the amount of lactate dehydrogenase found in the medium. At 24 h, the cell medium, into which the enzyme was secreted, contained greater than 54% of the total dibasic cleaving activity. Under the same conditions, 96% of the total lactate dehydrogenase activity was detected in the cell cytosolic fraction, with only 4% present in the cell medium. The small amount of lactate dehydrogenase found in the cell medium corresponds to the observed level of approximately 5% lysed cells in these experiments. This clearly shows that the dibasic cleaving activity is indeed secreted from EL-4 cells.

We next determined whether the dibasic cleaving endopeptidase associated with EL-4 cells (at zero time in Figure 1) is a membrane bound or soluble protein. To accomplish this, cells were sonicated and separated into soluble and membrane fractions by centrifugation, and $C_{12}E_8$ detergent was added to the membrane fraction to solubilize membrane proteins. This analysis showed that more than 85% of the dibasic cleaving endopeptidase activity was localized in the soluble fraction. To determine whether the secreted dibasic cleaving endopeptidase activity was derived from the cytosol or stored and secreted through a granular pathway, EL-4 cells were treated with digitonin to release cytosolic proteins, without releasing granules and other intracellular organelles

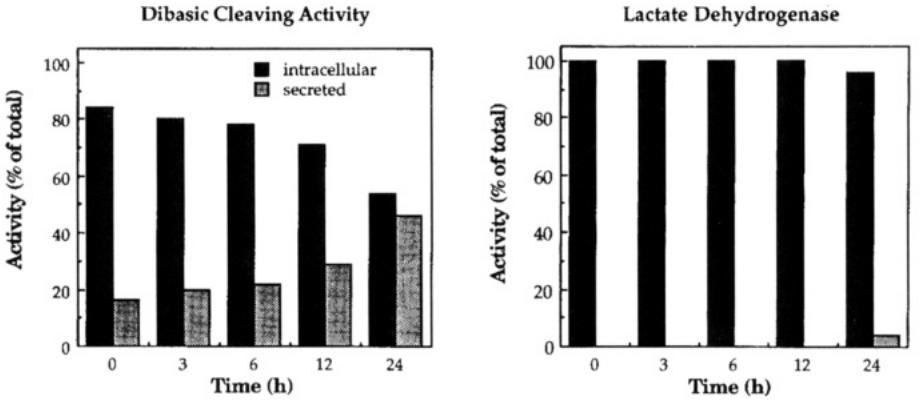

FIGURE 1: Secretion of enzymes from EL-4 cells. Cells were incubated in Hank's balanced salt solution for the indicated times at 37 °C and 10% CO_2. Supernatant and cells were separated, cells were resuspended and homogenized by sonication in the original volume of Hank's balanced salt solution, and the cytosolic fraction and the cell supernatant were assayed for lactate dehydrogenase and dibasic cleaving peptidase activity, as described in the Materials and Methods section. Activities are expressed relative to the total activity recovered in the cytosolic and secreted fractions.

Table 1: Secreted Dibasic Cleaving Endopeptidase Activity in Different Cell Growth Media[a]

no.	medium	activity (pmol/h·μL)
1	Hank's balanced salt solution	20
2	Hank's salt solution + 5% serum[b]	12
3	RPMI 1640	21
4	RPMI 1640 + 5% serum	10
5	OPTI-MEM	10
6	OPTI-MEM + 5% serum	6
7	DMEM/F12	8.8
8	DMEM/F12 + 5% serum	5
9	Waymouth	15
10	Waymouth + 10% serum	10

[a] EL-4 cells were grown to a density of 2.5×10^6 cells/mL in RPMI 1640 in the presence of 5% fetal bovine serum at 37 °C and 10% CO_2 and then resuspended at 5×10^6 cells/mL in the various growth media. After 24 h at 37 °C and 10% CO_2, 50 μL of the supernatant was assayed for the cleavage of α-neoendorphin (60 μM). Activity is expressed as the rate of formation of the cleavage product Leu-enkephalin-Arg.[6] [b] Heat-inactivated fetal bovine serum was used where the presence of serum is indicated.

(Peters et al., 1972). Digitonin-treated cells were separated from the medium by centrifugation. This treatment resulted in the release of ~70% of the total lactate dehydrogenase activity through the pores formed in the cytoplasmic membrane. In contrast, less than 45% of the dibasic cleaving endopeptidase activity was released; the rest of the endopeptidase activity remained associated with the cellular fraction, indicating that the enzyme is localized within granules or other intracellular compartments.

For optimization of the yield of enzyme, we examined different media for their effect on the amount of dibasic cleaving activity secreted in the supernatant. EL-4 cells were grown in RPMI 1640 medium to 3×10^6 cells/mL, centrifuged, and resuspended in various media at 5×10^6 cells/mL. Cells were incubated for 24 h as described in the Materials and Methods section. Table 1 shows the dibasic cleaving activity secreted in the different media tested. Cells placed in either RPMI 1640 or simply Hank's balanced salt solution secreted the same amount of enzyme, while the addition of fetal bovine serum to either medium decreased enzyme levels by 40−50%. The effect of serum was shown to be on the secretion rate, since incubation of secreted enzyme with up to 5% serum in either RPMI 1640 or Hank's salt solution for 24 h had no effect on enzyme activity. Somewhat lower levels of enzyme (70−30% of the activity observed in RPMI 1640) were obtained with cells placed in Opti-Mem, DMEM/F12, or Waymouth medium, and these samples also showed a 30−50% decrease in activity with fetal bovine serum present. Since cells suspended in RPMI 1640 medium or in Hank's buffered salt solution produced the maximum amount of activity, further experiments were conducted in these media.

Reagents commonly used to stimulate the secretion of enzymes from intracellular compartments, as shown for a monobasic specific dynorphin-converting activity secreted from AtT-20 and GH4C1 cell lines (Greco et al., 1992; Devi, 1992), were tested for their effect on the secretion of the dibasic cleaving activity. The secretagogues utilized included isoproterenol (100 nM), forskolin (10 μM), phorbol 12-myristate 13-acetate (100 nM), and KCl (50 mM). When these agents were added to EL-4 cells in Hank's balanced salt solution for 1−3 h, only small increases (117−200% of control) were seen in the secreted dibasic cleaving enzyme activity compared to the control. Attempts to use A23187, a Ca^{2+} ionophore, at 25−50 μM resulted in cell lysis, and thus this reagent could not be tested with these cells.

Purification of the Dibasic Cleaving Endopeptidase. By using the optimized growth and secretion conditions, EL-4 cells were suspended in Hank's buffered salt solution and permitted to secrete enzyme for 2−3 days. The medium containing the secreted enzyme was concentrated, desalted, and purified as described in Materials and Methods. As noted in Table 2 this scheme resulted in a 300-fold purification of the enzyme in 2.6% yield.

When analyzed by SDS−PAGE, the purified enzyme showed two bands, one with a molecular weight of 130 000 and another of 110 000 at approximately equal intensity (Figure 2). Previous enzyme preparations have shown only the 130 000 band of these two, suggesting that the 110 000 band is either a contaminant or a degradation product. When the preparation was subjected to native polyacrylamide gel electrophoresis, both bands showed endopeptidase activity. Since the ratio of the lower molecular weight band increases upon prolonged storage, and the two bands showed very similar chromatographic behavior through six purification steps, it is not unreasonable to assume that the smaller protein is a degradation product of the larger one. An analogous situation was observed with *N*-arginine dibasic convertase (NRD convertase), a similar enzyme isolated from rat testis

Table 2: Purification of Dibasic Cleaving Endopeptidase

purification step	total protein[a] (mg)	total activity (nmol/min)	specific activity (nmol/min·mg)	recovery (%)	purification-fold)
cell supernatant	596	6010	10.1	100	1
QMA Accell Plus chromatography	41.4	4512	109	75	10.8
Phenyl 650S chromatography	3.3	1152	352	19	34.8
Superdex 200 chromatography	2.0	1280	640	21	63.4
Mono Q chromatography	0.71	1164	1630	19	161
Superdex 200 chromatography	0.30	490	1601	9.1	158
hydroxylapatite chromatography	0.025	78	3120	2.6	309

[a] Protein concentrations were measured by the Coomassie Plus protein assay (PIERCE) using bovine serum albumin as standard.

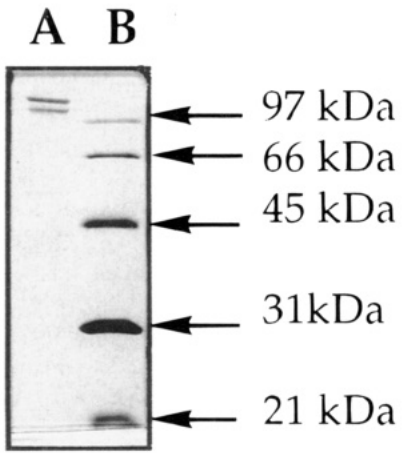

FIGURE 2: SDS−PAGE of purified dibasic cleaving endopeptidase: lane A, purified dibasic cleaving endopeptidase secreted from EL4 cells; lane B, molecular weight markers (Bio-Rad, low range). The gel was stained by the silver staining technique.

(Chesneau et al., 1994a,b). In the case of this enzyme, two bands of MW 130 000 and 110 000 were shown to be derived from the same protein. The secreted dibasic cleaving endopeptidase activity from EL-4 cells migrates on a gel filtration column as a single peak with an apparent molecular weight of ~100 000, suggesting that the enzyme is a monomer.

Characterization of the Dibasic Cleaving Enzyme. A number of group-specific agents were tested for their effect on the enzyme. As shown in Table 3, the enzyme is inactivated by the thiol reagents *N*-ethylmaleimide and DTNB, but not by iodoacetamide, indicating the presence of a reactive cysteine group on the enzyme. The metal chelators EDTA and 1,10-phenanthroline were effective in inactivating the dibasic cleaving enzyme, indicating that it is a metalloendopeptidase. Neither amastatin nor bestatin, both aminopeptidase inhibitors, GEMSA, a carboxypeptidase E inhibitor, cFP-Ala-Ala-Phe-pAB, an inhibitor of endopeptidases 24.15 and 24.16, captopril, an angiotensin-converting enzyme inhibitor, phosphoramidon, a neprilysin inhibitor, pepstatin A, a specific cathepsin D inhibitor, leupeptin, an inhibitor of papain, trypsin, and kallikrein, TPCK, a specific chymotrypsin inhibitor, or TLCK or soybean trypsin inhibitor, both specific trypsin inhibitors affected enzyme activity.

The effects of metal ions on dibasic cleaving enzyme activity are shown in Table 4. Cu^{2+}, Zn^{2+}, and Ni^{2+} completely inactivate the enzyme at a concentration of 1 mM, while Co^{2+} was slightly less effective. At 0.1 mM concentrations these metals did not affect enzyme activity (±10% difference compared to control). These ion effects are due to the cation since varying the anion had no effect, i.e., compare $CaCl_2$ to $Ca(NO_3)_2$. The effect of cations is likely ascribed to the "soft" complex-forming characteristics of the heavy metals: Cu^{2+}, Zn^{2+}, Co^{2+}, and Ni^{2+} form stable complexes with cysteine residues, thus possibly inactivating a catalytically important cysteine.

Table 3: Functional Group Inhibition Studies on Dibasic Cleavage[a]

inhibitors	concentration (mM)	activity (% of control)
DTT	1	95
PMSF	1	100
iodoacetamide	1	100
phosphoramidon	0.02	99
pepstatin	0.002	80
leupeptin	0.004	102
soybean trypsin inhibitor	10 μg/mL	101
TPCK	0.20	94
TLCK	0.20	89
MES	32	0
bis-Tris propane	20	50
EDTA	0.01	10
	0.1	0
1,10−phenanthroline	0.10	29
	0.50	0
N-ethylmaleimide	0.10	29
	0.50	0
DTNB	0.1	43
	1.0	7
cFP-Ala-Ala-Phe-pAB	0.2	108
captopril	0.01	100
GEMSA	0.01	100
bestatin	0.1	96
amastatin	0.1	98

[a] 0.12 μg of enzyme was preincubated with the above-listed reagents for 20 min at 37 °C before the addition of α-neoendorphin as substrate. Reaction mixtures were incubated at 37 °C for 20 min to 2 h. Results are expressed as activity remaining compared to the rate of Leu-enkephalin-Arg^6 formation in the absence of added reagents taken as 100%.

Table 4: Effect of Metal IOns on Dibasic Cleaving Endopeptidase Activity[a]

metal salt	activity[b] (% of control)
$BaCl_2$	104
$CaCl_2$	72
$Ca(NO_3)$	75
$CoCl_2$	30
$CuCl_2$	0
$CuSO_4$	0
$FeCl_2$	97
$MgCl_2$	75
$Mg(OAc)_2$	91
$MnCl_2$	197
$NiCl_2$	0
$ZnCl_2$	0
$ZnSO_4$	0

[a] The procedure used in this experiment was the same as that described for Table 3. All metal salts were used at 1 mM final concentration. Cu^{2+}, Zn^{2+}, Ni^{2+} and Co^{2+} ions had no detectable effect (less than ±10% change) on enzyme activity at 0.1 mM concentration. [b] Activity is expressed as remaining rates compared to the control without added salts (100%).

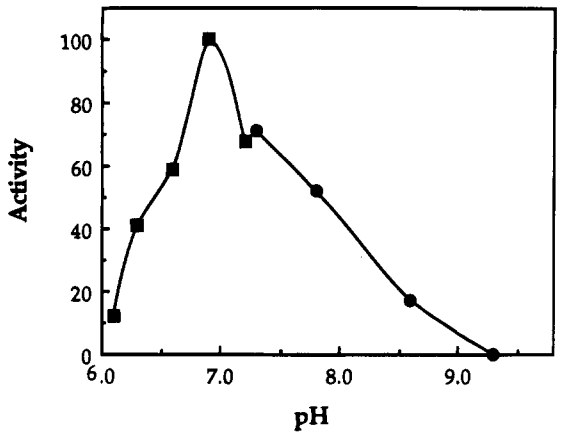

FIGURE 3: pH dependence of α-neoendorphin cleavage by EL-4 dibasic cleaving enzyme. 60 μM α-neoendorphin was incubated with 0.12 μg of purified enzyme for 20−60 min at 37 °C at different pHs. Measurements were carried out in duplicate. 100% activity was defined as the rate of cleavage at the optimum pH of 6.9. Activity is expressed as the rate of formation of Leu-enkephalin-Arg^6 from 60 μM α-neoendorphin. Buffers utilized were 20 mM potassium phosphate (■) or 20 mM glycine/NaOH (●).

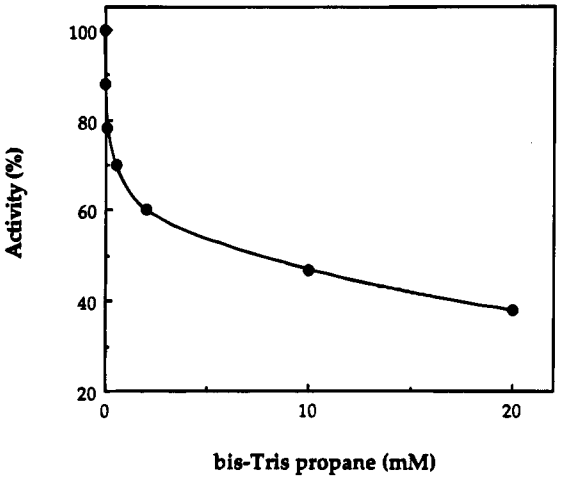

FIGURE 4: Inhibition of α-neoendorphin cleavage by bis-Tris propane. Experimental conditions are identical to those described for Figure 3, at pH 7.0. The ionic strength was kept constant during the experiment by adding NaCl at the appropriate concentration. Activity values reflect the % activity remaining compared to enzyme with no bis-Tris propane added.

The pH optimum of the dibasic cleaving enzyme using α-neoendorphin as a substrate is at pH 7, with activity extending to pH 8.5 on the alkaline side (Figure 3). Although not shown, the same pH optimum was obtained with dynorphin B as substrate using the radioimmunoassay assay method. Phosphate and glycine buffers were used in this experiment since Tris, bis-Tris propane, and MES buffers were all inhibitory (Table 3).

The inhibitory effect of amine buffers was further demonstrated by adding increasing concentrations of bis-Tris propane to the assay mixture at constant ionic strength. As shown in Figure 4, the amine buffer was a relatively potent inhibitor of the enzyme, but the maximum inhibitory effect was only approximately 60%, indicating that bis-Tris propane probably does not bind directly at the active site of the enzyme.

Specificity of the Dibasic Cleaving Endopeptidase. A number of opioid and nonopioid peptides were examined as substrates for the dibasic cleaving enzyme. Peptides were utilized at 60 μM, with cleavage being allowed to proceed to 10−20% conversion to measure initial reaction rates. Under the conditions of the experiments, 10 nmol/min·mg activity was the lower limit of detection of cleavage products. The results of these experiments are summarized in Table 5. It can be seen that a variety of opioid peptides are cleaved, including BAM12, α-neoendorphin, dynorphin A and its analogues, and prodynorphin B(228−256). In each case a single cleavage site was detected up to 70−80% conversion, yielding Leu- or Met-enkephalin-Arg^6 as determined by comparison to authentic standards. Peptides not cleaved included dynorphin A(1−7), neurotensin (1−13), angiotensin I, substance P, LHRH, somatostatin, bradykinin, β-endorphin, and the fluorogenic peptide analogues Abz-Phe-Arg-Arg-Val-EDDnp and Suc-Arg-Arg-Leu-βNA.

When the substrate dependence for the rate of the reaction of dynorphin B with the enzyme was measured, substrate inhibition was observed (Figure 5). It can be estimated from this data that the dynorphin B K_M is ~14 μM with a V_{max} of approximately 2.7 μmol/min·mg. In contrast, the substrate dependence for the rate of cleavage of α-neoendorphin and bovine adrenal medulla dodecapeptide did not exhibit substrate inhibition, yielding K_M values of 30 and 20 μM, respectively, with respective V_{max} values of 3.5 and 3.4 μmol/min·mg.

DISCUSSION

These studies have led to the identification of a novel peptidase secreted from EL-4 cells. This enzyme, which is a monomeric protein with a molecular weight of ~130 000, appears to be a metallopeptidase exhibiting maximal activity at neutral pH. The enzyme shows sensitivity to sulfhydryl reagents, indicating the presence of a reactive cysteine.

The substrate specificity of this endopeptidase appears to be unique. It clearly falls into the classification of a dynorphin-converting enzyme, on the basis of its ability to cleave dynorphin B between the Arg^6-Arg^7 bond, but it can also be classified as a dibasic cleaving endopeptidase since it cleaves peptides only between paired dibasic residues. The enzyme requires an arginine residue in the P1 position and either an arginine or a lysine residue in the P1′ position. This is evident from its ability to cleave between the Arg-Arg and Arg-Lys dibasic sequences in dynorphins, α-neoendorphin, and bovine adrenal medulla (BAM12) peptides, but its inability to cleave Lys^6-BAM12(1−9) or Lys^6,Lys^7-BAM12(1−9), whose sequences are otherwise analogous to that of BAM12(1-9), one of the best substrates of the enzyme. This is further supported by the lack of cleavage with mastoparan, which contains paired lysines, although in a different environment than opioid peptides. The enzyme does not cleave at monobasic residues, as evidenced by its inability to cleave angiotensin I, somatostatin (16−28), LHRH, bradykinin, and substance P, all of which contain single arginine or lysine residues, or derivatives of bovine adrenal medulla peptide, where the second arginine was substituted by other amino acids (e.g., glycine or phenylalanine).

Other requirements for cleavage are the presence of P3, P2, and P2′ residues, as shown by the ability to cleave dynorphin A(1−8), which contains a P2′ Ile, but the inability to cleave dynorphin A(1−7), which does not contain a P2′ residue. Similarly, Arg-Lys-Glu-Val-Tyr and Arg-Arg-Pro-

Table 5: Cleavage of Neuropeptides and Other Substrates by Dibasic Cleaving Endopeptidase[a]

Name	Sequence[b]	Rate of Cleavage (μmol/min.mg)
BAM12	Tyr-Gly-Gly-Phe-Met-**Arg-Arg**-Val-Gly-Arg-Pro-Glu	2.12
BAM12 (1-9)	Tyr-Gly-Gly-Phe-Met-**Arg-Arg**-Val-Gly	4.71
adrenorphin, free acid	Tyr-Gly-Gly-Phe-Met-**Arg-Arg**-Val	3.60
α-neoendorphin	Tyr-Gly-Gly-Phe-Leu-**Arg-Lys**-Tyr-Pro-Lys	3.12
β-neoendorphin	Tyr-Gly-Gly-Phe-Leu-**Arg-Lys**-Tyr-Pro	0.20
dynorphin B (1-9)	Tyr-Gly-Gly-Phe-Leu-**Arg-Arg**-Gln-Phe	1.12
dynorphin B	Tyr-Gly-Gly-Phe-Leu-**Arg-Arg**-Gln-Phe-Lys-Val-Val-Thr	1.35
dynorphin A (1-7)	Tyr-Gly-Gly-Phe-Leu-**Arg-Arg**	[c]NC
dynorphin A (5-8)	Leu-**Arg-Arg**-Ile	NC
dynorphin A (4-8)	Phe-Leu-**Arg-Arg**-Ile	0.54
dynorphin A (3-8)	Gly-Phe-Leu-**Arg-Arg**-Ile	1.94
dynorphin A (1-8)	Tyr-Gly-Gly-Phe-Leu-**Arg-Arg**-Ile	2.66
dynorphin A (1-9)	Tyr-Gly-Gly-Phe-Leu-**Arg-Arg**-Ile-Arg	2.23
dynorphin A (1-10)	Tyr-Gly-Gly-Phe-Leu-**Arg-Arg**-Ile-Arg-Pro	3.60
dynorphin A (1-11)	Tyr-Gly-Gly-Phe-Leu-**Arg-Arg**-Ile-Arg-Pro-Lys	1.24
dynorphin A (1-12)	Tyr-Gly-Gly-Phe-Leu-**Arg-Arg**-Ile-Arg-Pro-Lys-Leu	0.83
dynorphin A (3-13)	Gly-Phe-Leu-**Arg-Arg**-Ile-Arg-Pro-Lys-Leu-Lys	0.26
dynorphin A (1-13)	Tyr-Gly-Gly-Phe-Leu-**Arg-Arg**-Ile-Arg-Pro-Lys-Leu-Lys	0.37
dynorphin A (1-17)	Tyr-Gly-Gly-Phe-Leu-**Arg-Arg**-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln	<0.01
prodynorphin B (228-256)	Tyr-Gly-Gly-Phe-Leu-**Arg-Arg**-Gln-Phe-Lys-Val-Val-**Thr**-Arg-Ser-Gln-Glu-Asp-Pro-Asn-Ala-Tyr-Tyr-Glu-Glu-Leu-Phe-Asp-Val	0.85
(D-Pro[10])-dynorphin A(1-11)	Tyr-Gly-Gly-Phe-Leu-**Arg-Arg**-Ile-Arg-D-Pro-Lys	NC
dynorphin (1-6)	Tyr-Gly-Gly-Phe-Leu-**Arg**	NC
	Tyr-Gly-Gly-Phe-Met-**Lys-Arg**-Val-Gly	NC
	Tyr-Gly-Gly-Phe-Met-**Lys-Lys**-Val-Gly	NC
	Abz-Phe-**Arg-Arg**-Val-EDDnp	NC
	Suc-**Arg-Arg**-Leu-βNA	NC
	Arg-Lys-Glu-Val-Tyr	NC
	Tyr-Gly-Gly-Phe-Leu-**Arg-Lys**-Tyr-Arg-Pro-Lys	NC
	Tyr-Gly-Gly-Phe-Met-**Arg**-Gly-Leu	NC
	Tyr-Gly-Gly-Phe-Met-**Arg**-Phe	NC
neurotensin (1-13)	pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-**Arg-Arg**-Pro-Tyr-Ile-Leu	<0.05
neurotensin (1-11)	pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-**Arg-Arg**-Pro-Tyr	NC
neurotensin (8-13)	**Arg-Arg**-Pro-Tyr-Ile-Leu	NC
mastoparan	Ile-Asn-Leu-Lys-Ala-Leu-Ala-Ala-Leu-Ala-**Lys-Lys**-Ile-Leu	NC
somatostatin 28	Ser-Ala-Asn-Ser-Asn-Pro-Ala-Met-Ala-Pro-Arg-Glu-**Arg-Lys**-Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys	NC
somatostatin 28 (1-12)	Ser-Ala-Asn-Ser-Asn-Pro-Ala-Met-Ala-Pro-Arg-Glu	NC
angiotensin I (1-10)	Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu	NC
substance P (1-9)	Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly	NC
LHRH	pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly	NC
somatostatin (16-28)	Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Dys	NC
bradykinin	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg	NC
β-endorphin	Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ile-Ile-Lys-Asn-Ala-Tyr-**Lys-Lys**-Gly-Glu	<0.01

[a] 60 μM each peptide was incubated with 0.12 μg of purified enzyme for 10−60 min at 37 °C. After boiling and the addition of trifluoroacetic acid, the reaction mixtures were analyzed on a reverse phase HPLC column. Measurements were done in triplicate, and the error was generally less than 10% between separate determinations. The lower limit of detection was approximately 0.01 μmol/min.mg enzyme. [b] Possible cleavage sites are indicated in bold type. [c] NC, not cleaved.

Tyr-Ile-Leu, which lack a P2 residue, are not cleaved, just as dynorphin A(5−8) lacking a P3 residue is not cleaved. However, cleavage is detectable in the case of Phe-Leu-Arg-Arg-Ile (dynorphin A(4−8)), which appears to represent the minimal substrate (Table 5).

The nature of the amino acids in the P2 and/or P2′ positions is important, as seen by the lack of cleavage for somatostatin 28 with glutamate and alanine in these positions and for neurotensin (1−13) or (1−11), which contain proline residues in these positions. Proline has previously been indicated as a factor in preventing cleavage at dibasic residues if it is located next to a putative cleavage site (e.g., Nilsson & Heijne, 1992). There are other, more complex effects of sequence as demonstrated by the poor cleavage of β-neoendorphin, dynorphin A(1−17), and [D-Pro[10]]-dynorphin A(1−11).

Chain length, however, does not appear to be a major factor since prodynorphin B, which is 29 amino acids in length, is cleaved more efficiently than the shorter 13- and 17-amino acid-containing peptides dynorphin A(1−13) and dynorphin A(1−17).

The specificity of the dibasic cleaving enzyme described here distinguishes it from the prohormone-processing enzymes PC1, PC2, furin, and PACE4, which predominantly

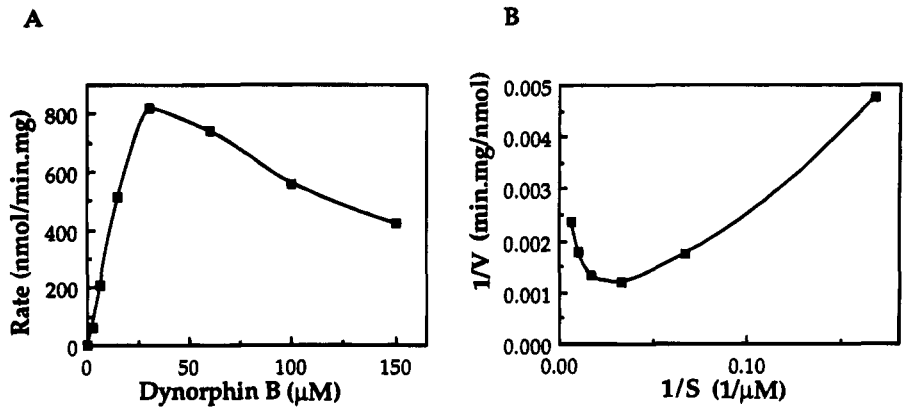

FIGURE 5: Substrate inhibition of the cleavage of dynorphin B. Varying concentrations of dynorphin B were incubated with 0.12 μg of purified dibasic cleaving enzyme for 10−20 min at 37 °C. Measurements were carried out in duplicate, with a maximum difference of approximately 10% between determinations. (A) Rate of Leu-enkephalin-Arg^6 formation vs dynorphin B concentration. (B) Double-reciprocal plot of the data from panel A.

cleave at the carboxy terminus of dibasic sequences (Seidah et al., 1991, 1993; Breslin et al., 1993) or after monobasic sequences (Dupuy et al., 1994), although cleavage between two basic residues has also been reported (Zhou et al., 1993).

The dibasic cleaving enzyme purified from EL-4 cells is also clearly distinguished from other "dynorphin-processing enzymes" on the basis of its profile of inhibition by metal chelators and group-specific reagents. For example, the yeast enzyme, the human cerebrospinal fluid enzyme, and one of the human spinal cord enzymes are inhibited by PMSF at 0.20 mM concentration (Mizuno & Matsuo, 1984; Nyberg et al., 1985; Nyberg & Silberring, 1990). The neuroblastoma enzyme containing an active cysteine residue was not capable of cleaving α-neoendorphin and showed preference for full size dynorphin A over its (1−13) fragment (Satoh et al., 1989). The bovine spinal cord dibasic specific peptidase is not inhibited by EDTA up to 1 mM concentration (Silberring & Nyberg, 1989), and neither is the human spinal cord enzyme that also showed preference for dynorphin A over dynorphin B (Silberring et al., 1992a).

The enzyme that is closest in its properties to the EL4 dibasic cleaving endopeptidase is a cytosolic dibasic specific metalloendopeptidase named *N*-arginine dibasic convertase (NRD convertase) with a molecular weight of 133 000 Da that has recently been purified from rat testis (Chesneau et al., 1994b). Both enzymes are inhibited by metal chelators and *N*-ethylmaleimide at relatively low concentrations, but not by iodoacetamide or DTT at 1 mM concentration. However, the rat testis enzyme was completely inhibited by amastatin and bestatin at 20 μM concentration, which can be contrasted to the insensitivity of the dibasic cleaving endopeptidase described in these studies to either of these reagents at up to 100 μM. Furthermore, TPCK did not inhibit the purified EL-4 enzyme up to 0.20 mM, while TPCK inhibited the rat testis metalloendopeptidase by 50% at 0.25 mM. The pH optimum observed for the rat testis enzyme was around pH 8.8, as opposed to neutral pH for the EL-4 enzyme. The reactivity toward different opioid peptides is also fundamentally different between these enzymes. For example, α-neoendorphin is hydrolyzed 2 and 300 times faster by the EL-4 enzyme at identical concentrations than dynorphin B and dynorphin A, respectively, while the former peptide is the poorest substrate for the rat testis enzyme. NRD convertase cleaves somatostatin 28 at the N-terminal side of the first arginine of the dibasic pair. The rate of cleavage of this peptide by NRD convertase is similar to those of dynorphin B and α-neoendorphin. In contrast, the secreted dibasic cleaving endopeptidase described in this study did not cleave somatostatin 28 at a detectable rate. On the basis of the limits of detection of the assay, α-neoendorphin is cleaved at a rate at least 600 times faster than that of somatostatin 28.

There is another class of enzymes referred to as "dynorphin-converting enzymes", which cleave prodynorphin at monobasic residues: these enzymes are clearly distinguished from dibasic specific dynorphin-converting enzymes by their cleavage site specificity (Petanceska et al., 1993; Devi, 1992; Greco et al., 1992).

The dibasic specific endopeptidase described in this paper is a novel metalloendopeptidase with unique inhibition characteristics and is the first example of a secreted dibasic cleaving enzyme. Whether this enzyme acts as a prohormone-processing enzyme, an extracellular convertase, or an extracellular regulator of peptide levels available for receptor occupancy remains to be determined. This enzyme is unlikely to be a prohormone-processing enzyme for prodynorphin and proenkephalin, since in these molecules (except for a single Arg-Lys site in proenkephalin) the sites for processing into physiologically active dynorphins and neoendorphins all contain Lys-Lys and Lys-Arg sequences, which are not cleaved by the EL-4 enzyme. On the other hand, the sequence of proopiomelanocortin (POMC) contains two dibasic sites with an N-terminal arginine. Cleavage at these sites (Arg-Lys and Arg-Arg) would yield γ_3-melanocyte-stimulating hormone (γ_3-MSH). By processing at these sites, the enzyme could be involved in the generation of γ_3-MSH from proopiomelanocortin. POMC also contains a Lys-Lys-Arg-Arg sequence, the processing of this sequence by the EL-4 dibasic cleaving enzyme has yet to be established.

The high affinity of this enzyme for dynorphin B and α-neoendorphin makes it a likely candidate for being the neuropeptidase that is at least partly responsible for the regulation of the levels of these peptides extracellularly. The conversion of dynorphin B to Leu-enkephalin-Arg^6 after cleavage at the dibasic sequence (possibly coupled with carboxypeptidase action to give Leu-enkephalin) changes the specificity of this peptide from κ-opioid receptors to δ-opioid receptors (Pleuvry, 1991). This in turn would cause a change in the analgesic properties of these substances *in vivo*

(Dickenson, 1991). Since different effects for opioid peptides of differing receptor specificity have been described in cells of the immune system (e.g., enhancement of lymphocyte proliferation by δ-receptor agonists or enhancement of natural killer activity by μ- and κ-receptor agonists, etc.) (Carr, 1991), the conversion of dynorphin B to Leu-enkephalin-Arg^6 could also be involved in the regulation of opioid action in immune cells.

ACKNOWLEDGMENT

We thank Dr. Joseph McGillis (Department of Microbiology and Immunology, University of Kentucky) for his help and the use of his laboratory facilities for the iodinations. The technical assistance of Mr. John Yordy and Mr. Brent Mayer is greatly appreciated.

REFERENCES

Breslin, M. B., Lindberg, I., Benjannet, S., Mathis, J. P., Lazure, C., & Seidah, N. G. (1993) *J. Biol. Chem. 268*, 27084−27093.
Carr, D. J. J. (1991) *Proc. Soc. Exp. Biol. Med. 198*, 710−720.
Chesneau, V., Pierotti, A. R., Prat, A., Gaudoux, F., Foulon, T., & Cohen, P. (1994a) *Biochimie 76*, 234−240.
Chesneau, V., Pierotti, A. R., Barré, N., Créminon, C., Tougard, C., & Cohen, P. (1994b) *J. Biol. Chem. 269*, 2056−2061.
Christensson-Nyländer, I., Nyberg, F., Ragnarsson, U., & Terenius, L. (1985) *Regul. Pept. 11*, 65−76.
Devi, L. (1992) *Endocrinology 131*, 1930−1935.
Dickenson, A. H. (1991) *Br. Med. Bull. 47*, 690−702.
Dupuy, A., Lindberg, I., Zhou, Y., Akil, H., Lazure, C., Chrétien, M., Seidah, N. G., & Day, R. (1994) *FEBS Lett. 337*, 60−65.
Gottlieb, M., & Chavko, M. (1987) *Anal. Biochem. 165*, 33−37.
Greco, L., Daly, L., Kim, S., & Devi, L. (1992) *Neuroendocrinology 55*, 351−356.
Hames, B. D. (1981) in *Gel Electrophoresis of Proteins: a Practical Approach* (Hames, B. D., & Rickwood, D., Eds.) pp 1−86, IRL Press, London.
Lyrenäs, S., Nyberg, F., Lindberg, B., & Terenius, L. (1988) *Obstetrics Gynecol. 72*, 54−58.
Mizuno, K., & Matsuo, H. (1984) *Nature 309*, 558−560.
Nilsson, I., & Heijne, G. (1992) *FEBS Lett. 299*, 243−246.
Nyberg, F., & Silberring, J. (1990) *Prog. Clin. Biol. Res. 328*, 261−265.
Nyberg, F., Nordström, K., & Terenius, L. (1985) *Biochem. Biophys. Res. Commun. 131*, 1069−1074.
Nyberg, F., Kankaanranta, S., Brostedt, P., & Silberring, J. (1991) *Brain Res. 552*, 129−135.
Persson, S., Jónsdóttir, I., Thorén, P., Post, C., Nyberg, F., & Hoffmann, P. (1993) *Life Sci. 53*, 643−652.
Petanceska, S., Zikherman, J., Fricker, L. D., & Devi, L. (1993) *Mol. Cell. Endocrinol. 94*, 37−45.
Peters, T. J., Muller, M., & deDuve, C. (1972) *J. Exp. Med. 136*, 1117−1139.
Pierotti, A. R., Prat, A., Chesneau, V., Gaudoux, F., Leseney, A.-M., Foulon, T., & Cohen, P. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 6078−6082.
Pleuvry, B. J. (1991) *Br. J. Anaesthesia 66*, 370−380.
Rawlings, N. D., & Barrett, A. J. (1993) *Biochem. J. 290*, 205−218.
Satoh, M., Yokosawa, H., & Ishii, S.-I. (1989) *J. Neurochem. 52*, 61−68.
Seidah, N. G., Day, R., Marcinkiewicz, M., Benjannet, S., & Chrétien, M. (1991) *Enzyme 45*, 271−284.
Seidah, N. G., Day, R. & Chrétien, M. (1993) *Biochem. Soc. Trans. 21*, 685−691.
Sharma, H. S., Nyberg, F., & Olsson, Y. (1992) *Neurosci. Res. 14*, 195−203.
Silberring, J., & Nyberg, F. (1989) *J. Biol. Chem. 264*, 11082−11086.
Silberring, J., Castello, M. E., & Nyberg, F. (1992a) *J. Biol. Chem. 267*, 21324−21328.
Silberring, J., Sakurada, T., & Nyberg, F. (1992b) *Life Sci. 50*, 839−847.
Turner, A. J., & Barnes, K. (1994) *Biochem. Soc. Trans. 22*, 122−127.
Vlaskovska, M., Nyländer, I., Schramm, M., & Terenius, L. (1994) *25th International Narcotics Research Conference*, North Falmouth, MA, T27.
Zhou, A., Bloomquist, B. T., & Mains, R. E. (1993) *J. Biol. Chem. 268*, 1763−1769.

BI950483X