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Dynorphin A Processing Enzyme: Tissue Distribution, Isolation, and Characterization[1]

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Limited proteolysis of the dynorphin precursor (prodynorphin) at dibasic and monobasic processing sites results in the generation of bioactive dynorphins. In the brain and neurointermediate lobe of the pituitary, prodynorphin is processed to produce α and β neo endorphins, dynorphins (Dyn) A-17 and Dyn A-8, Dyn B-13, and leucine-enkephalin. The formation of Dyn A-8 from Dyn A-17 requires a monobasic cleavage between Ile and Arg. We have identified an enzymatic activity capable of processing at this monobasic site in the rat brain and neurointermediate lobe of the bovine pituitary; this enzyme is designated "dynorphin A-17 processing enzyme." In the rat brain and neurointermediate lobe, a majority of the Dyn A processing enzyme activity is membrane-associated and can be released by treatment with 1% Triton X-100. This enzyme has been purified to apparent homogeneity from the membrane extract of the neurointermediate lobe using preparative iso-electrofocussing in a granulated gel pH 3.5 to 10, FPLC using anion exchange chromatography, and non-denaturing electrophoresis. The Dyn A processing enzyme exhibits a pI of about 5.8 and a molecular mass of about 65 kDa under reducing conditions. The Dyn A processing enzyme is a metalloprotease and has a neutral pH optimum. It exhibits substantial sensitivity to metal chelating agents and thiol agents suggesting that this enzyme is a thiol-sensitive metalloprotease. Specific inhibitors of other metallopeptidases such as enkephalinase [EC 3.4.24.11], the enkephalin generating neutral endopeptidase [EC 3.4.24.15], or NRD convertase do not inhibit the Dyn A processing enzyme activity. In contrast, specific inhibitors of angiotensin converting enzyme inhibit the activity. The purified enzyme is able to process a number of neuropeptides at both monobasic and dibasic sites. These characteristics are consistent with a role for the Dyn A processing enzyme in the processing of Dyn A-17 and other neuropeptides in the brain.

Key words: enkephalin, metalloprotease, neuropeptide, proprotein convertase.

Many peptide hormones and neuropeptides are synthesized as precursor proteins that undergo endoproteolysis at specific sites (*1-3*). These sites are usually multiple basic amino acids (*4*), although some cleavage sites are single basic ("monobasic") residues that usually fit a consensus sequence (*5, 6*).

The enzymes involved in peptide processing are important regulators of these intracellular messengers. Studies on processing endoproteases have focused mostly on the recently discovered subtilisin-like serine proteases, such as furin and prohormone convertases. These enzymes are thought to cleave peptide precursors preferentially at multibasic cleavage sites (*7*). Several enzymes have been identified that are capable of cleaving the opioid precursors or precursor fragments at single basic residues. An endoprotease designated "dynorphin converting enzyme" capable of cleaving dynorphin B-29 at monobasic sites has been purified from neurointermediate lobe of the bovine pituitary (*8*). Also a metalloprotease designated "amidorphin Gly-generating enzyme" purified from ovine chromaffin granules has been shown to process a proenkephalin fragment at a single arginine residue (*9*). A metalloprotease found in the atrial gland of *Aplysia* (*10*) cleaves Dyn A-17 at a single arginine residue resulting in the generation of Dyn A-8. Recently purified prohormone convertase 2 has been shown to process prodynorphin at monobasic sites (*11*).

Dyn A-17	Y-G-G-F-L-R-R-I[8]-R-P-K-L-K-W-D-N-Q
Dyn A-8	Y-G-G-F-L-R-R-I[8]
Dyn B-29-(9-22)	F-K-V-V-T-R-S-Q-E-D-P-N-A-Y

The ratio of the level of Dyn A-8 to the level of Dyn A-17 varies significantly among different brain regions and in various tissues (*12-14*). Multiple processing enzymes and differential regulation of these enzyme activities could account for such an effect. We used a radioimmunoassay

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that allows the specific detection of Dyn-A-8 (see footnote for sequence) in the presence of a large excess of substrate, Dyn A-17, and a blocking peptide, Dyn B-29-(9-22). The latter peptide contains basic residues and is able to saturate contaminating enzyme activities thus allowing the detection of the product. This peptide has been previously used as a non-specific blocking agent for the isolation and characterization of the dynorphin converting enzyme (*8*). Dyn A-processing enzyme activity in bovine pituitary membrane extracts was examined by monitoring the formation of Dyn A-8 with a highly specific antiserum. Here we report a metalloprotease capable of processing Dyn A-17 that exhibits tissue-specific and region-specific distribution in rat and bovine brain, respectively. The purified enzyme has a pI of 5.8 and molecular mass of 65 kDa. The Dyn A-processing enzyme is a thiol-sensitive metalloprotease capable of processing regulatory peptides at mono and dibasic processing sites.

EXPERIMENTAL METHODS

Assay for Dyn A Processing Enzyme Activity—In a typical assay, the reaction mixture consisted of 50 mM Tris-Cl, pH 8.5, containing 0.1% Triton X-100, 1-2 μM Dyn A-17 (Peninsula), 100 μM Dyn B-29-(9-22), and enzyme (1-100 μg of membrane extract or 1-10 ng of purified enzyme protein) in a final volume of 100 μl. The reaction mixture was incubated for 20 min at 37°C and the reaction was terminated by boiling for 10 min. Reactions using enzyme preparations boiled before the addition of peptide were performed as a control. Boiling leads to irreversible inactivation of the enzyme activity without affecting Dyn A-8. The enzyme activity was measured in multiple dilutions of extracts and only those dilutions that give linear levels of Dyn A-8 were used for further analysis. Typically 10-20 μl of the reaction mixture was analyzed by radioimmunoassay (*15*).

Anti-Dyn A-8 antiserum was used to detect Dyn A-8. This antiserum has been previously characterized (*16*). As a highly selective and sensitive antiserum that does not recognize the C-terminal extensions or truncations of Dyn A-8; radioimmunoassay with this antiserum allows the detection of picomolar concentrations of Dyn A-8 in the presence of nanomolar concentrations of Dyn A-17.

Membrane Extraction of Dyn A-Processing Enzyme Activity—To determine the optimal detergent for extraction of the activity, the neurointermediate lobe was homogenized (Polytron) in 0.1 M NaAc buffer, pH 6.5 (buffer A), and the homogenate was centrifuged at low speed (1,500 $\times$ g for 15 min) to remove large particulate material. The supernatant was then centrifuged at high speed (100,000 $\times$ g for 1 h). Under these conditions, the majority of the peptide hormones and neuropeptides are released into the soluble supernatant. The pellet was washed twice in buffer A and resuspended in various buffers as shown in Table I, incubated for 60 min at 4°C, and centrifuged as above.

The Dyn A-processing enzyme activity in various fractions was assayed as described above. Protein estimation was carried out using BCA reagent (Pierce) with bovine serum albumin as the standard.

Distribution Of Dyn A-Processing Enzyme Activity—Tissues were removed from adult rats (Sprague-Dawley) immediately after decapitation, frozen on dry ice, and stored at −70°C until use. The bovine brain regions were dissected immediately after their transport to the laboratory from the slaughterhouse on ice, frozen on dry ice, and stored at −70°C until use. The tissues were suspended in 10 volumes of ice-cold 0.1 M sodium acetate buffer, pH 6.5, and homogenized using a Polytron (Brinkmann Scientific). The homogenate was centrifuged at low speed (1,500 $\times$ g for 15 min) to remove large particulate material and the supernatant was centrifuged at high speed (100,000 $\times$ g for 1 h). The resulting pellet was washed twice in buffer A and resuspended in buffer A containing 0.1% Triton X-100, incubated for 60 min at 4°C, and centrifuged again at high speed as above. The resulting supernatant ("membrane extract") was analyzed for Dyn A-processing enzyme activity as described above.

Purification of Dyn A-Processing Enzyme Activity—Approximately 30-50 g of the neurointermediate lobe of bovine pituitary glands (Pel-Freez) was homogenized (Polytron, Brinkmann) in 5 volumes of 50 mM sodium acetate, pH 6.5. The homogenate was centrifuged at 50,000 $\times$ g for 1 h, and the pellet was washed twice in buffer A and extracted with buffer A containing 1% Triton X-100. Following 60 min incubation at 4°C, the membranes were subjected to centrifugation as above. The resulting supernatant, representing the "Triton extract" of the membranes, was concentrated by ultrafiltration on Amicon UM 30 membranes and subjected to preparative isoelectrofocusing (IEF) at pH 3.5-10.

For preparative IEF, a gel slurry was prepared by mixing 6 g of Ultrodex (Pharmacia) with 195 ml of 10% glycerol (v/v), 7.5 ml of 20% Triton X-100, 1 mM dithiothreitol, and 7.5 ml of ampholyte (Pharmacia) at pH 3.5-10. The slurry was poured onto a glass plate (24 $\times$ 11 cm) and dried with a light stream of cool air until the weight decreased by 33%. The plate was placed on the cooling unit of a Pharmacia Multifor platform (Pharmacia). Electrode strips were soaked in either 1 M phosphoric acid (anode) or 1 M NaOH (cathode) and placed at the end of the IEF plate. The plate was prefocused for 2 h at 4 watts/plate at 4°C. The concentrate containing Dyn A-processing enzyme activity was applied 3.5 cm from the cathode and focused for 8 h at 8 W/plate at 10°C. A stable pH gradient was established during this time. The resin was then sectioned into 40 fractions (0.5 cm wide) and each fraction was eluted with 10 volumes of 20 mM Tris-Cl buffer, pH 8.1, containing 10% glycerol and 0.05 mM DTT. Dyn A-processing enzyme activity was assayed in the fractions as described above. Protein estimation was carried out using BCA reagent (Pierce). The pH gradient was determined by extracting a small segment of each fraction with deionized water and then measuring the pH directly.

The Dyn A-processing enzyme extracted from the gel was concentrated on a Centriprep-30 (Amicon) concentrator, and the protein was subjected to fast protein liquid chromatography (FPLC) on a Mono Q HR, 5/5 column (Pharmacia). The column was washed for several minutes with 20 column volumes of 20 mM Tris-Cl, pH 8.1. A gradient from 0-1 M NaCl in 20 mM Tris-Cl, pH 8.1, was then applied over 75 min. Fractions of 500 μl were collected and assayed for enzyme activity.

The peak of activity was concentrated on a Centricon-30 (Amicon) column and subjected to non-denaturing gradient

gel electrophoresis in 4-15% polyacrylamide gradient gels. The gels were prerun with 0.5 mM thioglycollate to remove ammonium persulfate and other free radical reaction products generated in the gel system (*17*). The samples were not denatured by boiling; SDS and β-mercaptoethanol were excluded during sample preparation. Electrophoresis was carried out at 10°C, after which the activity in 2 mm sections was extracted with 100 mM Tris-Cl buffer, pH 8.5, containing 0.05 mM dithiothreitol and 0.2% Triton X-100. The Dyn A-processing enzyme activity in each fraction was determined as described above. The peak of Dyn A-processing enzyme activity was pooled, concentrated on Centricon-30 (Amicon), subjected to electrophoresis under denaturing conditions, and visualized by silver staining (*18*).

Characterization of the Dyn A-Processing Enzyme Activity—The Dyn A-processing enzyme in the fractions comprising the peak of activity following non-denaturing electrophoresis was characterized. Protease inhibitors (Sigma) at the concentrations indicated in Table IV were preincubated with the enzyme for 20 min at 4°C followed by addition of the substrate. Dyn A-processing enzyme activity was then measured as described above. For the determination of optimum pH for Dyn A-processing enzyme activity, the following buffers were used (at 50 mM final concentration): sodium citrate, pH 4.4-6.0; sodium phosphate, pH 6-8.5; Tris-Cl, pH 7.5-8.8. For the determination of kinetic constants, the purified Dyn A-processing enzyme was assayed with 0 to 10 μM Dyn peptides except that Dyn B-29-(9-22) was excluded from the reaction mixture. The product was measured by radioimmunoassay as described previously. To determine the site of cleavage, 1 nmol of peptide (shown in Table VI) was incubated with 10 ng of purified enzyme in 50 mM Tris-Cl, pH 8.5, containing 0.1% Triton X-100 for 1 or 18 h at 37°C. The reaction was terminated by incubation at 100°C for 10 min and the reaction mixture was subjected to MALDITOF-mass spectrometry (Dr. Ronald Beavis, Skirball Institute, NYU Medical Center, New York).

TABLE I. **Dyn A-processing enzyme activity in soluble and membrane extracts of the neurointermediate lobe of bovine pituitary.**

Treatment	DAP activity	
	Total activity (nmol/min)	Specific activity (nmol/min/mg)
Homogenate	110±18	0.21±0.02
Soluble	9±1	0.56±0.06
Membrane	84±9	1.05±0.09
0.1 M Sod. Ac. pH 6.5	12±2	0.56±0.05
// +1 M NaCl	34±4	0.22±0.02
// +1% Tx 100	68±8	6.24±0.03
// +1 M NaCl, 1% Tx 100	72±7	0.75±0.08
// +1% Mega 10	9±1	0.72±0.07
0. 1M Sod. Phos. pH 6.5	14±1	0.14±0.02
0.1 M Sod. Bicarb. pH 9.0	22±3	2.40±0.3
0.1 M Sod. Bicarb. pH 10.0	42±4	1.00±0.2

Sod. Ac, sodium acetate; Sod. Phos, sodium phosphate; Sod. Bicarb, sodium bicarbonate. The membranes were extracted with various treatments and assayed for Dyn A-processing enzyme activity as described in "EXPERIMENTAL METHODS." Data represent the mean±SEM from three independent determinations.

RESULTS

The production of Dyn A-8 from Dyn A-17 requires an endoproteolytic cleavage at a single arginine site. We have previously used a synthetic peptide, Dyn B-29-(9-22), to block the interference caused by a number of nonspecific peptidases; this strategy was successful in the isolation and characterization of a monobasic processing enzyme responsible for the generation of Dyn B-13 (*9*). We used a similar strategy for the identification, isolation, and characterization of the enzyme responsible for the generation of Dyn A-8. In the presence of Dyn B-29-(9-22) we find that there is a linear increase in the amount of product formed with time, suggesting that nonspecific proteases that degrade Dyn A-17 or Dyn A-8 are blocked by Dyn B-29-(9-22). This allowed the specific detection of Dyn A-8 and the characterization of the Dyn A-processing enzyme activity in the membrane extracts.

The majority of the Dyn A-processing enzyme activity was found to be associated with the membrane fraction (Table I). This activity can be released by 1 M sodium chloride in the absence or presence of Triton X-100. Also, treatment with Triton X-100 alone releases substantial amounts of activity as compared with other treatments. Although treatment with sodium chloride and Triton X-100 releases enzyme activity, this treatment also results in the release of substantial amounts of protein resulting in a lower specific activity (Table I). Treatment of the membranes with sodium bicarbonate or sodium carbonate also results in the release of enzyme activity. Since treatment with 1% Triton X-100 results in the extraction of the enzyme with high specific activity from membranes, we used the 1% Triton X-100 membrane extracts for further studies.

The levels of Dyn A-processing enzyme activity vary significantly among tissues studied (Table II). Rat brain

TABLE II. **Distribution of Dyn A-processing enzyme activity.**

	DAP activity (nmol/min/mg)
Rat tissues	
Brain	41.03±5.0
Heart	3.63±0.6
Liver	0.63±0.06
Kidney	0.31±0.06
Spleen	0.11±0.02
Ileum	0.03±0.01
Bovine brain regions	
Hypothalamus	110±15
Caudate	84±9
Occipital cortex	72±8
Singular cortex	68±7
Cerebellum	42±5
Medulla pons	34±4
Frontal cortex	22±2
Temporal cortex	14±2
Putamen	12±2
Hippocampus	9±1
White matter	9±1
Neurointermediate lobe	75±8

The assay for Dyn A-processing enzyme activity in membrane extracts was carried out as described in "EXPERIMENTAL METHODS." Data represent the mean±SEM from three independent determinations.

contains very high levels of Dyn A-processing enzyme activity. Dyn A-processing enzyme activity in the liver, kidney, and spleen is about 30-50-fold lower than the activity in brain. This distribution suggests a function for the Dyn A-processing enzyme in the processing of other regulatory peptides in addition to neuropeptides.

The Dyn A-processing enzyme activity was examined in various regions of bovine brain (Table II). The highest levels of activity are observed in the hypothalamus; substantial amounts of activity are also detected in the caudate, occipital cortex, and singular cortex. Cerebellum and medulla pons exhibit two times lower levels of activity, whereas the frontal cortex, temporal cortex, putamen, hippocampus, and white matter contain even lower levels of activity. The neurointermediate pituitary contains substantial Dyn A-processing enzyme activity, at levels comparable to the levels in the hypothalamus and caudate.

We used the selective assay that allows the specific detection of the Dyn A-8 for the isolation of the Dyn A-processing enzyme. We choose bovine pituitary as the source since pituitary contains relatively high levels of neuropeptides including Dyn A-8 and thus should contain their processing enzymes. Accordingly, we found a high level of this activity in the pituitary. Triton X-100 extraction of the membranes resulted in a 10-fold purification of the enzyme with about 80% yield. Preparative IEF on a granuated gel gave another 110-fold purification with about a 10–20% yield (Table III). The Dyn A-processing enzyme exhibits a pI of about 5.8. Ion-exchange chromatography on Mono Q yielded an additional 6-fold purification. At this point Dyn A-processing enzyme appeared as a major protein band along with a few minor protein bands (Fig. 1). Non-denaturing gradient gel electrophoresis resulted in an additional 2-3-fold purification. Under non-denaturing and denaturing conditions the enzyme migrated as a 65 kDa protein (Fig. 1).

The purified bovine pituitary enzyme is inhibited by metal-chelating agents (Table IV), and the activity is completely inhibited by 1 mM EDTA, EGTA, or 1,10 phenanthroline. The enzyme is thiol-sensitive as seen by its inhibition by 100 μM *p*-chloromercuriphenylsulfonic acid. Inhibitors of cysteine proteases, aspartyl proteases, aminopeptidases, serine proteases, or lysosomal proteases do not significantly inhibit the Dyn A-processing enzyme activity (Table IV). *N*-Cpp-Ala-Ala-Phe-pAB, a specific inhibitor of soluble endopeptidases [EC 3.4.24.15] (*19*) and [EC 3.4.24.16] (*20*), does not inhibit the activity. Also, neither *N*-Cpp-Phe-pAB nor phosphoramidon, inhibitors of enkephalinase (*21*), inhibit the activity. Additionally, bestatin, a potent inhibitor of NRD convertase (*22*) does not substantially inhibit the activity, suggesting that the Dyn A-processing enzyme has properties distinct from these metalloproteases (*22*). Interestingly, captopril and lisinopril, specific inhibitors of angiotensin converting enzyme-like enzymes (*23*) significantly inhibit the Dyn A-processing enzyme (Table IV). Furthermore, we found that $ZnCl_2$ at neutral pH inhibits the enzyme activity (Table IV). This and the fact that the Dyn A-processing enzyme shows a pH optimum between 8 and 8.5, but is inhibited at pH 5.5 in

TABLE III. **Purification of Dyn A-processing activity from bovine pituitary membranes.**

Step	Activity (nmol/min)	Specific activity (nmol/mn/mg)	Fold purification	Yield (%)
Homogenate	260	0.008	1	100.0
Membrane extract	200	0.075	10	77.0
Isoelectrofocusing	40	9.0	1,125	15.0
FPLC Mono Q	10	53.0	6,625	4.0
Non-denaturing PAGE	2	130.0	16,250	0.8

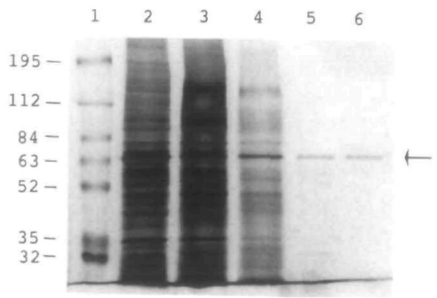

Fig. 1. **Silver-stained gel following denaturing electrophoresis.** Fractions containing Dyn A-processing enzyme activity were subjected to SDS-polyacrylamide gel electrophoresis under denaturing conditions in a 7.5% gel. The numbers at the top correspond to the various purification steps: 1, molecular weight markers (Sigma); 2, homogenate; 3, Triton X-100 membrane extract; 4, peak activity following isoelectrofocusing; 5, fraction representing peak activity following FPLC on Mono Q; 6, fraction representing peak activity following non-denaturing gel electrophoresis. Arrow points to the Dyn A-processing enzyme seen as a protein band at about 65 kDa. The numbers on the left represent molecular mass in kDa.

TABLE IV. **Effect of protease inhibitors on Dyn A-processing enzyme activity.**

Inhibitor	Concentration	% Inhibition
EDTA	1.0 mM	100±12
EGTA	1.0 mM	100±9
1,10-Phenanthroline	1.0 mM	100±11
4,7-Phenanthroline	1.0 mM	4±1
PCMPS	0.1 mM	91±9
E-64	1.0 mM	20±5
Calpain inhibitor: ALLN	0.1 mM	4±1
Pepstatin A	1.0 mM	6±2
Leupeptin	0.1 mM	4±2
TPCK	0.1 mM	12±3
PMSF	1.0 mM	6±3
TLCK	0.1 mM	8±4
SBTI	0.1 mg/ml	17±1
CETI	0.1 mg/ml	15±2
N-Cpp-Ala-Ala-Phe-pAB	0.1 mM	9±3
N-Cpp-Phe-pAB	0.1 mM	4±3
Phosphoramidon	0.1 mM	12±5
Bestatin	0.1 mM	25±7
Captopril	3 nM	41±6
	30 nM	77±8
Lisinopril	3 nM	88±7
	30 nM	100±11
$ZnCl_2$	10 μM	37±4
	100 μM	100±7

The abbreviations used are: PCMPS, *p*-chloromercuriphenylsulfonic acid; E-64, *trans*-epoxysuccinly-L-leucinamido-(4-guanidino)butane; ALLN, *N*-Ac-Leu-Leu-norleucinal; TPCK, L-1-tosyl-amido-2-phenylethyl chloromethyl ketone; PMSF, phenyl methyl sulfonyl fluoride; TLCK, 1-choloro3-tosylamido-7-amino-2-heptanone; SBTI, soybean trypsin inhibitor; CETI, chicken egg white trypsin inhibitor; Cpp, *N*-[1(*R,S*)-carboxy-3-phenylpropyl]-; pAB, *p*-aminobenzoate.

sodium acetate buffer (not shown) suggest that the Dyn A-processing enzyme is a thiol-sensitive neutral metalloprotease with properties similar to the angiotensin converting enzyme.

The Dyn A-processing enzyme exhibits typical Michaelis-Menten kinetics with an apparent K_m for Dyn A-17 of 0.4 μM and a V_{max} of 0.15 mmol/min/mg protein. The apparent K_m value with Dyn A-(2-17) is 1 μM and the V_{max} is about 75 nmol/min/mg protein; these values are lower than the values for Dyn A-17 suggesting the importance of the N-terminal tyrosine in recognition by the enzyme. The apparent K_m for Dyn A-13 of 1 μM and V_{max} of 35 nmol/min/mg protein are much lower, suggesting that C-terminal amino acids also play a role in substrate recognition.

To test the specificity of the Dyn A-processing enzyme, the conversion of Dyn A-17 was measured in the presence of high concentrations of a number of dynorphin-related peptides containing single or paired basic residues (Table V). Substantial inhibition is displayed by the orphanin FQ peptide (Table V); Dyn B-29 and BAM18 also inhibit the Dyn A-processing enzyme activity, although the K_i is substantially higher than the K_i for the orphanin FQ peptide. Interestingly, neither Dyn A-(2-13) nor Dyn B-29-(9-22), which contain cleavage site residues, inhibit the activity, suggesting that the presence of the cleavage site residue alone is not sufficient and that the regions around the cleavage site are necessary for recognition of the peptide substrate by the Dyn A-processing enzyme.

In order to examine if these peptides serve as substrates for the Dyn A-processing enzyme and to examine the sites of cleavage, the peptides generated were subjected to MALDITOF-mass spectrometry. Dyn A-17 was found to be cleaved at Ile^8, directly generating Dyn A-8 (Table V). In addition, small amounts of cleavage at Arg^6 and Arg^7 were also seen upon prolonged incubation, suggesting that the Dyn A-processing enzyme is capable of cleaving at both monobasic and dibasic sites in Dyn A-17. Dyn B-29 is also cleaved at a monobasic site, Arg^{14}, releasing Dyn B-14, and at a dibasic site, Arg^6-Arg^7, releasing the C-terminally Arg-extended Leu-Enk. Interestingly, although BAM18 contains both monobasic and dibasic sites, cleavage is seen only at the dibasic Arg^6-Arg^7 site. Also, orphanin FQ is processed at dibasic Arg^{12}-Lys^{13} and Arg^8-Lys^9 sites (Table V). These results suggest that the Dyn A-processing enzyme is able to process a variety of sites in a number of peptides, and that the cleavage site recognition depends not only on the residue at the cleavage site but also on the residues surrounding the cleavage site.

TABLE V. **Specificity of the Dyn A-processing enzyme.**

Peptide	K_i (μM)
Orphanin FQ	3.3
Dyn B-29	7.0
BAM 18	29.0
Dyn A-17-(2-13)	>100
Dyn B-29-(9-22)	>100
GLP-1	>100

Purified Dyn A-processing enzyme (1 ng) was incubated with 1-100 μM peptide and 1 μM Dyn A-17 in 50 mM Tris-Cl buffer, pH 8.5, containing 0.1% Triton X-100 for 20 min at 37°C. The reaction was terminated by boiling for 10 min. The Dyn A-8 generated was measured by RIA as described in "EXPERIMENTAL METHODS."

DISCUSSION

We used an assay that permits the specific detection of Dyn A-8 to identify and examine the distribution of enzyme activities in various brain regions and tissues. In the brain, the Dyn A-processing enzyme activity is detected in all regions, although the levels of activity vary significantly. The distribution of the Dyn A-processing enzyme activity generally matches the distribution of prodynorphin mRNA and immunoreactive Dyn A-8 in rat and bovine brain (*13, 14*). The Dyn A-processing enzyme activity is high in the hypothalamus and caudate and low in the cortex. A parallel distribution of Dyn A-8, high levels in the hypothalamus, moderate levels in the caudate, and low levels in the cortex, has been reported (*13, 14*). The Dyn A-processing enzyme activity is high in cerebellum; this does not correlate with the extremely low levels of Dyn A-8 in this region, suggesting that the Dyn A-processing enzyme might be involved in the processing of other neuropeptides. Other peptide processing enzymes, such as carboxypeptidase D, also show high levels of activity in the cerebellum (*24*). In addition, the distribution of carboxypeptidase D activity generally matches the level of Dyn A-processing enzyme activity: highest in the hypothalamus and pituitary, moderate in the striatum, and lowest in the cortex (*24*), suggesting that like carboxypeptidase D, the Dyn A-processing enzyme may be involved in the processing of a number of neuropeptides.

The specific activity of the Dyn A-processing enzyme is highest in the brain and pituitary; these tissues also show the highest levels of dynorphin and other neuropeptides (*14*). In these tissues, the Dyn A-processing enzyme may be responsible for the processing of neuropeptides. In other tissues, such as heart, that have no detectable dynorphins, the Dyn A-processing enzyme may be responsible for the processing of other regulatory peptides such as atrial natriuretic factor, a product of monobasic processing. The presence of the Dyn A-processing enzyme activity in tissues that contain bioactive peptides suggests that the Dyn A-processing enzyme may be involved in the processing of a number of peptide hormones and neuropeptides. The neuroendocrine distribution of the Dyn A-processing enzyme activity supports such a notion.

The Dyn A-processing enzyme activity has been purified to homogeneity using preparative isoelectrofocusing, hydrophobic chromatography, FPLC on an anion-exchange column, and non-denaturing gradient gel electrophoresis. The enzyme activity exhibits a pI of 5.8 and a molecular mass of 65 kDa under both non-denaturing and denaturing conditions. The Dyn A-processing enzyme is a thiol-sensitive metalloprotease as evidenced by its inhibitory profile (Table IV). The enzyme activity is not inhibited by specific inhibitors of metallopeptidases [EC 3.4.24.15] (*19*), [EC 3.4.24.16] (*20*), [EC 3.4.24.11] (*21*), or NRD convertase (*22*). These results suggest that the Dyn A-processing enzyme is distinct from these neutral metalloproteases and other endoproteases that process regulatory peptides at mono or dibasic sites (*25-32*).

The Dyn A-processing enzyme is distinct from the prohormone-processing serine proteases that have the ability to cleave precursors at monobasic sites (*4*). These enzymes differ in their physio-chemical properties (molecular mass and pI), distribution, substrate specificity, and

inhibitory profiles (*11, 30-32*). Other metalloendoproteases that process peptide hormones at monobasic sites include the dynorphin converting enzyme (*8*) and the adrenorphin Gly-generating enzyme (*9*). The Dyn A-processing enzyme has some properties that are similar and some properties that are distinct from these two enzymes (*8, 9*). All three enzymes cleave peptide substrates at monobasic sites, exhibit neutral pH optima, and are thiol-sensitive metalloproteases. However, the three enzymes differ in their pI and molecular masses; the dynorphin converting enzyme has a pI of 5.1 and a molecular mass of 58 kDa, the adrenorphin Gly-generating enzyme has a pI of 5.2 and a molecular mass of 45 kDa, whereas the Dyn A-processing enzyme has a pI of 5.8 and a molecular mass of about 65 kDa. Additionally, the adrenorphin Gly-generating enzyme is substantially inactivated by phosphate buffer whereas the Dyn A-processing enzyme and dynorphin converting enzyme are not (*8*). These results suggest that the Dyn A-processing enzyme is distinct from these metalloproteases.

The extracellular processing of neuropeptides appears to be mediated largely by metalloproteases. This is in contrast to the intracellular processing of neuropeptides, which is mostly mediated by subtilisin-like serine proteases (*4*). Many members of the metalloprotease family have been implicated in the extracellular processing of neuropeptides. Endopeptidase 24.15 has been shown to cleave a variety of opioid peptides generating a bioactive pentapeptide (*19*). In contrast, enkephalinase and angiotensin converting enzyme have been shown to degrade primarily opioids and other neuropeptides (*33, 34*). In this study, we show that the Dyn A-processing enzyme shares some properties with these enzymes in that it can generate bioactive peptides such as Leu-Enk, Met-Enk, and Dyn A-8 from larger opioid peptides. In addition, we have found that this enzyme can also inactivate biologically active peptides such as orphanin FQ since C-terminally processed orphanin FQ (1-11) has been shown to lack biological activity (*35*). These results suggest an important role for the Dyn A-processing enzyme in regulating the expression of bioactive opioids and other peptides in the brain.

The Dyn A-processing enzyme shares some properties with the angiotensin converting enzyme. Captopril, a widely used inhibitor of the angiotensin converting enzyme and lisinopril, a more selective and potent inhibitor of the angiotensin converting enzyme, inhibits the Dyn A-processing enzyme with relatively high potency, suggesting that these two enzymes share similar functional properties. However, the enzymes differ in their molecular masses and tissue distribution. The Dyn A-processing enzyme migrates as a 65 kDa protein under both non-denaturing and denaturing electrophoretic conditions (Fig. 1), whereas the angiotensin converting enzyme migrates as a 120-170 kDa protein under denaturing conditions and as a 330 kDa protein under non-denaturing conditions (*36, 37*). The Dyn A-processing enzyme is expressed at high levels in many regions of the brain whereas the angiotensin converting enzyme is expressed in only a few regions (*37*). It is possible that the Dyn A-processing enzyme and the angiotensin converting enzyme are related members of a family of metalloendoproteases that process peptide hormones and neuropeptides.

In summary, we have purified a Dyn A-processing enzyme that generates Dyn-8 from Dyn A-17. This thiol-sensitive metalloprotease is a membrane-associated enzyme with some properties similar to the angiotensin converting enzyme. The ability of the Dyn A-processing enzyme to recognize a number of peptide precursors with high selectivity and its wide neuroendocrine distribution suggest a broad role for this enzyme in the processing of a number of peptide precursors in addition to the generation of Dyn A-8.

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