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Enkephalin convertase: Purification and characterization of a specific enkephalin-synthesizing carboxypeptidase localized to adrenal chromaffin granules

(endorphin/opiate receptor/carboxypeptidase assay)

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ABSTRACT **A specific carboxypeptidase that converts enkephalin precursors into enkephalin in adrenal chromaffin granules has been purified and characterized. In the adrenal this enzyme, designated enkephalin convertase, is uniquely localized to the chromaffin granules, which contain enkephalin and precursor peptides. Enkephalin convertase is markedly stimulated by $CoCl_2$ and inhibited by EDTA or 1,10-phenanthroline, unlike the lysosomal carboxypeptidase. The purified enzyme has a high affinity for the hexapeptides [Met^5]- and [Leu^5]enkephalin-Arg^6 (51 and 83 μM, respectively) and a somewhat lower affinity for the hexapeptides [Met^5]- and [Leu^5]enkephalin-Lys^6 (195 and 174 μM). Brain enkephalin convertase shows 10-fold regional variations, unlike other carboxypeptidases, which are uniformly distributed. Enkephalin convertase appears to be associated selectively and physiologically with biosynthesis of the enkephalins.**

Most biologically active peptides are synthesized from large peptide precursors by sequential actions of a trypsin-like enzyme followed by a carboxypeptidase B-like activity that removes basic amino acids from the carboxyl terminus of the trypsin fragments (1). Large enkephalin precursor peptides have been identified in which the enkephalin sequence is surrounded by dibasic amino acid pairs (2, 3). Moreover, hexapeptides comprising the enkephalin sequence with an arginine or lysine at the carboxyl terminus have been isolated from brain and adrenal (4). It is unclear whether the biosynthesis of biologically active peptides involves carboxypeptidases uniquely concerned with the formation of a single peptide or whether general carboxypeptidases serve this function.

In the adrenal, enkephalins are localized to chromaffin granules, from which they are released together with catecholamines after cholinergic stimulation (5). If enkephalin is synthesized by a unique carboxypeptidase, such an enzyme might be selectively localized to chromaffin granules and might exhibit selective affinity for enkephalin-containing hexapeptides. Conventional assays for carboxypeptidase are often nonspecific, insensitive, or, if they utilize the biologically active peptide precursor, time consuming. We have designed a novel carboxypeptidase assay using a fluorescent enkephalin analog. The assay is simple, sensitive, and specific. We now describe a unique cobalt-stimulated carboxypeptidase that, in the adrenal, is concentrated in chromaffin granules and that has selective affinity for enkephalin-containing peptides. This enzyme, designated enkephalin convertase, appears to be selectively associated with physiological synthesis of the enkephalins.

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MATERIALS AND METHODS

Synthesis of Dansylphenylalanylleucine. L-Phenylalanyl-L-leucine (100 mg) was dissolved in 20 ml of pyridine. Dansyl chloride (200 mg) was added and the mixture was stirred for 5 hr, acidified with dilute HCl (pH 1 to 2), and extracted with 1 liter of $CHCl_3$. The chloroform was dried ($MgSO_4$) and removed at reduced pressure, yielding a bright yellow oily solid. The product was purified by TLC on 1-mm silica GF (Analtech) using chloroform/methanol (4:1). Dansyl-Phe-Leu (R_F 0.5) was eluted with hot methanol, yielding 95 mg of product.

Synthesis of Dansylphenylalanylleucylarginine. Dansylphenylalanylleucine (85 mg) was dissolved in 6 ml of acetonitrile and the solution was cooled in ice bath. *N*-Hydroxysuccinimide (30 mg) and dicyclohexylcarbodiimide (52 mg) were added and the mixture was stirred 1 hr in an ice bath and then overnight at room temperature. The mixture was cooled and filtered, and the solvent was removed at reduced pressure, yielding 115 mg of yellow oil. The oil was dissolved in 7.5 ml of dimethylformamide, 300 mg of L-arginine·HCl in 2.5 ml of H_2O was added, and the mixture was stirred overnight. Twenty milliliters of 0.1 M HCl was added and the aqueous phase was washed with six 50-ml portions of CH_2Cl_2. The product was partially purified by cation exchange chromatography (Dowex AG-50W-X2). Then, the crude aqueous phase was loaded directly onto 2 g of the resin (prepared as directed). The column was washed with 0.1 M acetic acid and then with H_2O. Dansyl-Phe-Leu-Arg was removed by elution with 1 M NH_4OH, which was removed by lyophilization. TLC using ethyl acetate/isopropanol/water/acetic acid (40:40:19:1) showed the product to be 90% pure (ninhydrin staining shows free arginine). Dansyl-Phe-Leu-Arg was dissolved in a small volume of $CHCl_3$/MeOH (4:1) and applied to a 20-ml silica column. The column was washed with 20 ml of $CHCl_3$/MeOH (4:1) and then the product was eluted with 20 ml of $CHCl_3$/MeOH (1:1). Fractions containing product were pooled and the solvent was removed at reduced pressure, yielding 40 mg of dansyl-Phe-Leu-Arg (>99% pure). Acid hydrolysis (6 M HCl) at reduced pressure at 110°C (overnight) followed by amino acid determination (TLC) confirmed the identity of the product.

Carboxypeptidase Assay. Enzyme, substrate (40 μM dansyl-Phe-Leu-Arg), and buffer (50 mM NaOAc, pH 5.6) are incubated at 37°C for 10 min in 10 × 13 mm fluorometer test tubes (VWR Scientific). For a typical reaction volume of 0.5 ml, the reaction is quenched with 50 μl of 1 M HCl. Chloroform (1.5 ml) is then added directly to the assay tubes, which are spun on a Vortex (10 sec) and centrifuged (1 min at 2,000 × *g*). The

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amount of product formed is determined by measuring the fluorescence of the lower (chloroform) layer in an Aminco Bowman spectrofluorometer (360-nm excitation, 510-nm emission wave length). The upper (aqueous) phase is not in the path of the excitation beam and so does not interfere with the fluorometer reading. Dansyl-Phe-Leu partitions quantitatively into the organic phase, whereas the substrate is insoluble (<0.1%) in chloroform. Calibration curves using dansyl-Phe-Leu convert the relative fluorescence into picomoles of product.

Preparation of Chromaffin Granules. Chromaffin granules were prepared by a modification of the method of Smith and Winkler (6). Bovine adrenal medulles were dissected, minced, and homogenized (glass/Teflon homogenizer) in 5 vol of 0.3 M sucrose. The homogenate was centrifuged at 480 × *g* for 15 min. The supernatants were decanted and centrifuged at 12,000 × *g* for 20 min. These pellets were suspended in 0.3 M sucrose and either layered onto sucrose density gradients for subcellular localization experiments or pelleted through 1.6 M sucrose (80,000 × *g* for 1 hr). Linear sucrose density gradients of 2.2–1.0 M sucrose (30 ml) were centrifuged 120 min at 100,000 × *g*. The tubes were punctured and 0.6-ml fractions were collected (58 fractions total). Catecholamines were assayed by the method of Von Euler and Hamberg (7) using 50-μl aliquots. Fractions were then dialyzed against 10 mM NaOAc (pH 6.0). Protein was assayed by the Bradford method (8). Acid phosphate was assayed by the method of Hofstee (9). Dopamine β-hydroxylase was assayed by the modification of the method of Pisano *et al.* (10) described by Wallace *et al.* (11).

Purification of Enkephalin Convertase. The 1.6 M sucrose pellets from 10 adrenals were suspended in 5 mM NaOAc (pH 6.0) and the suspension was frozen slowly, thawed, and centrifuged at 100,000 × *g* for 60 min. The supernatant was decanted, dialyzed against 10 mM NaOAc (pH 6.0), and applied to a 3-ml agarose-Leu-Arg affinity column synthesized from Affi-Gel 10 (Bio-Rad) and L-leucyl-L-arginine (Bachem Fine Chemicals, Torrance, CA) by standard procedures. The column was washed with 10 ml of 0.1 M NaCl/10 mM NaOAc, pH 6.0. Enkephalin convertase was eluted with 6 ml of 0.1 M L-arginine·HCl/10 mM NaOAc, pH 6.0. Most of the carboxypeptidase activity (>85%) was contained in 2 ml of the eluate and it was loaded directly onto an 80-ml gel filtration column (Sephadex G-200). The column buffer was 100 mM NaCl/10 mM NaOAc, pH 6.0/1 mM 2-mercaptoethanol. Fractions (0.5 ml) were collected in tubes containing 0.5 mg of bovine serum albumin (Sigma; fraction V) unless the fraction was to be used for protein determination or gel electrophoresis.

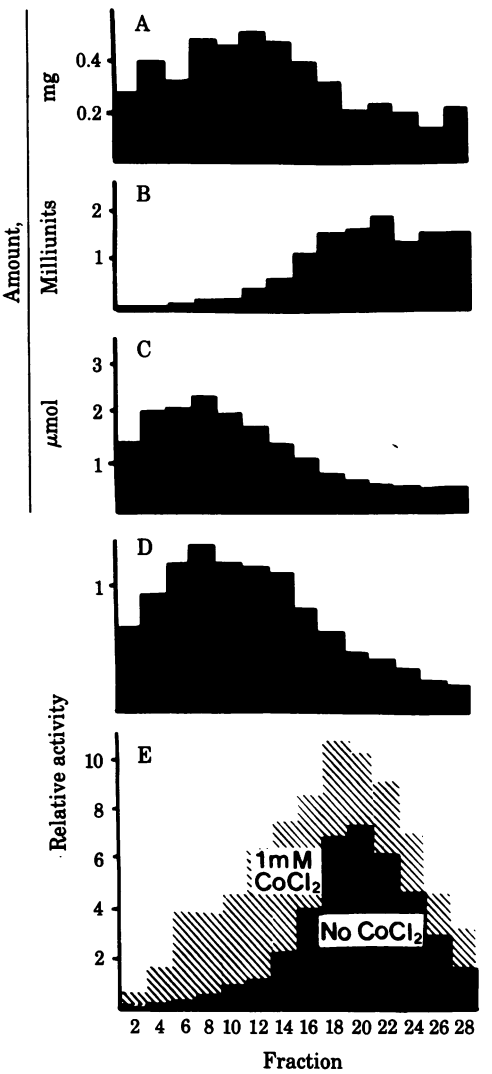

FIG. 1. Sucrose density gradient fractionation of the large granule fraction of bovine adrenal medulla. The lower half of the gradient (1.0–2.2 M sucrose) is shown. (*A*) Protein. (*B*) Acid phosphatase is a lysosomal marker. (*C* and *D*) Catecholamines and dopamine β-hydroxylase, respectively, are markers for chromaffin granules. (*E*) Carboxypeptidase activity was determined in the absence and presence of 1 mM $CoCl_2$.

RESULTS

Subcellular Localization of Enkephalin Convertase in Bovine Adrenal. Sucrose gradient fractionation can resolve lysosomes and chromaffin granules in the adrenal (12). In bovine adrenal homogenates, sucrose gradient fractionation provides a clear separation of the lysosomal enzyme acid phosphatase and the chromaffin granule constituents dopamine β-hydroxylase and catecholamines (Fig. 1). Carboxypeptidase activity in the absence of cobalt is concentrated in the lysosomal fractions of the gradients. Cobalt preferentially stimulates carboxypeptidase activity in the approximate area of the chromaffin granules. Carboxypeptidase activity in both fractions is maximally active at pH 5.5–6.0, corresponding to the intragranular pH of both chromaffin granules and lysosomes (13, 14).

The differential localization of cobalt-stimulated and unstimulated carboxypeptidase in sucrose gradients suggests that the cobalt-stimulated enzyme is contained in chromaffin granules while the non-cobalt-stimulated enzyme is localized to lysosomes and that these activities represent distinct enzymes. This conclusion is supported by the differential effect of inhibitors on carboxypeptidase activity in gradient fractions of the adrenal (Fig. 2). 1,10-Phenanthroline and cadmium selectively inhibit carboxypeptidase activity in the chromaffin granule portion of the gradient but have much less effect on the lysosomal zone of the gradient. The cobalt-stimulated chromaffin granule carboxypeptidase is designated enkephalin convertase.

Purification of Enkephalin Convertase. We have extensively purified enkephalin convertase from soluble fractions of bovine adrenal chromaffin granules. Approximately 60% of the chromaffin granule carboxypeptidase activity is soluble (100,000 × *g* for 60 min). The membrane-bound activity is similar to the soluble enzyme with respect to activation and inhibition by divalent ions and enzyme inhibitors (data not shown).

Enkephalin convertase activity binds to an affinity column consisting of L-leucyl-L-arginine linked to agarose. This substrate is both positively and negatively charged at pH 6, so a large amount of protein also adsorbs to the affinity column.

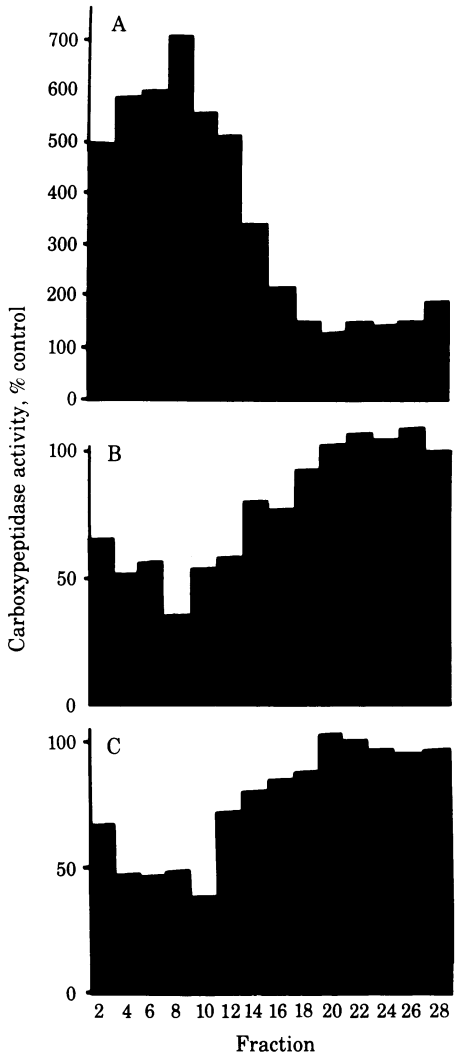

FIG. 2. Differential effect of activators and inhibitors on sucrose density gradient fractions. Activators and inhibitors were incubated with the gradient fraction for 2 hr at 4°C before assay. (*A*) $CoCl_2$. (*B*) $CoCl_2/CdCl_2$. (*C*) *o*-Phenanthroline.

Elution with high salt buffer removes most of this nonspecifically bound protein and also the contaminating lysosomal carboxypeptidase. Enkephalin convertase is then eluted with 0.1 M arginine, providing a fraction that has <0.05% of the original protein but ≈30% of the original cobalt-stimulated carboxypeptidase activity. The eluate from the affinity column is applied to a Sephadex G-200 column, which further purifies the enkephalin convertase and removes arginine from the enzyme preparation.

Properties of Purified Enkephalin Convertase. HPLC shows that purified enkephalin convertase converts the [Met]enkephalin and [Leu]enkephalin hexapeptides containing carboxyl-terminal arginine or lysine residues to [Met]enkephalin and [Leu]enkephalin with no further degradation of enkephalin. The results with [Met]enkephalin-Arg, [Met]enkephalin-Lys, and [Leu]enkephalin-Lys (data not shown) are essentially the same as for [Leu]enkephalin-Arg (Fig. 3). HPLC also shows that the heptapeptide [Met]enkephalin-Arg-Arg is sequentially degraded to [Met]enkephalin-Arg and then to [Met]enkephalin (data not shown).

Peptidase inhibitors and peptides differentiate enkephalin convertase and the lysosomal carboxypeptidase activity by their

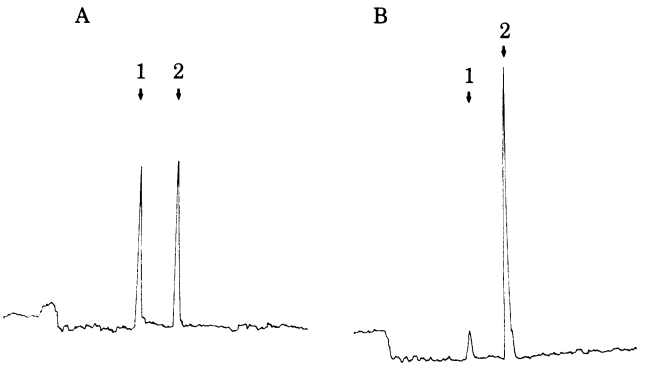

FIG. 3. HPLC of the conversion of [Leu^5]enkephalin-Arg^6 to [Leu^5]enkephalin by enkephalin convertase. Purified enzyme was incubated at 37°C for 1 hr (*A*) or for 3 hr (*B*) with 0.4 mM [Leu^5]enkephalin-Arg^6 in 10 mM NaOAc, pH 6.0/1 mM $CoCl_2$. Aliquots (25 μl) were injected onto the HPLC column (Waters) and elution was with a linear gradient of 10% acetonitrile/90% H_3PO_4 (0.1%) to 50% acetonitrile/50% H_3PO_4 (0.1%) over 30 min. A reverse-phase C_{18} column (μBondapak) was used with a flow rate of 2 ml/min. Absorption at 280 nm was monitored. No change in peak height or retention time was detected after 7 hr of incubation at 37°C (not shown). Peaks: 1, [Leu^5]enkephalin-Arg^6; 2, [Leu^5]enkephalin.

relative inhibitory potencies (Table 1). At 1 mM, EDTA and 1,10-phenanthroline markedly inhibit enkephalin convertase activity while having negligible effects on the lysosomal car-

Table 1. Activators and inhibitors: Enkephalin convertase vs. lysosomal carboxypeptidase B

Addition	Concentration, mM	% control activity: Enkephalin convertase	% control activity: Lysosomal carboxypeptidase B
EDTA	1	15	99
1,10-Phenanthroline	1	7	86
$CdCl_2$	0.1	12	79
$CuCl_2$	0.1	9	3
$CoCl_2$	1	1,090	95
$NiCl_2$	1	200	78
$NiCl_2/CoCl_2$	1/1	780	—
$ZnCl_2$	0.1	106	79
$ZnCl_2/CoCl_2$	0.1/1	180	—
p-Chloromercurophenyl-sulfonate	0.01	76	10
$HgCl_2$	0.001	81	14
[Met^5]Enkephalin-Arg^6	0.5	13	68
[Met^5]Enkephalin-Lys^6	0.5	42	68
[Leu^5]Enkephalin-Arg^6	0.5	20	44
[Leu^5]Enkephalin-Lys^6	0.5	27	49

Lysosomal carboxypeptidase was purified from sucrose gradient fractions 19–27 by passing the soluble fraction (50,000 × *g*, 60 min) through the agarose-Leu-Arg affinity column. Approximately 80% of the carboxypeptidase activity was not retained and was used as the lysosomal carboxypeptidase fraction. Ions and inhibitors were incubated 2 hr at 0°C with enzyme, and then substrate (dansyl-Phe-Leu-Arg) was added to a final concentration of 40 μM. The peptide inhibitors were not incubated with enzyme before addition of substrate and were assayed in the presence of 1 mM $CoCl_2$. The following had no effect on enkephalin convertase: 1 mM $SrCl_2$, 1 mM $MgCl_2$, 1 mM $BaCl_2$, 1 mM $CaCl_2$, 1 mM $MnCl_2$, 1 mM iodoacetamide, leupeptin at 10 μg/ml, bacitracin at 100 μg/ml, soybean trypsin inhibitor at 10 μg/ml, pepstatin A at 10 μg/ml, 0.1 mM tosylphenyl chloromethyl ketone, 0.1 mM tosyllysyl chloromethyl ketone, 1 mM succinic acid, 1 mM captopril, chymostatin at 10 μg/ml, and 1 mM phenylmethylsulfonyl fluoride.

boxypeptidase. Among the divalent cations, 0.1 mM cadmium differentiates the two enzymes, markedly inhibiting enkephalin convertase while having a minimal effect on lysosomal carboxypeptidase, whereas copper (0.1 mM) inhibits both enzymes and strontium, magnesium, barium, and calcium inhibit neither.

In contrast to the inhibitory effects of some divalent cations, cobalt stimulates enkephalin convertase activity 11-fold while having no influence on lysosomal carboxypeptidase. The cobalt stimulation of enkephalin convertase is blocked by zinc which, however, does not affect enkephalin convertase activity in the absence of cobalt. Nickel can partially stimulate enkephalin convertase but also partially blocks cobalt stimulation.

Enkephalin convertase seems to be less dependent on sulfhydryl groups than lysosomal carboxypeptidase; concentrations of *p*-chloromercurophenylsulfonate and mercuric chloride that inhibit 90% of lysosomal carboxypeptidase activity produce only 20% inhibition of enkephalin convertase activity.

The selectivity of enkephalin convertase for enkephalin precursors is apparent in the effect of the enkephalin hexapeptides, which are more potent in inhibiting enkephalin convertase than lysosomal carboxypeptidase. Interestingly, arginyl[Met]enkephalin and -[Leu]enkephalin hexapeptides are more potent inhibitors of enkephalin convertase than the corresponding lysyl hexapeptides. By contrast, the arginyl and lysyl hexapeptides inhibit lysosomal carboxypeptidase to a similar extent.

Enkephalin convertase has considerable specificity for peptides having carboxyl-terminal basic amino acids (Table 2). A large number of di- and tripeptides lacking carboxyl-terminal basic amino acids fail to influence enkephalin convertase activity at 1 mM. By contrast, several peptides having carboxyl-terminal basic amino acids are potent at micromolar levels. While arginine alone is a weak inhibitor, dipeptides containing arginine are often as potent as hexapeptides having carboxyl-terminal arginine residues. The preferential affinity of the enzyme for carboxyl-terminal arginine residues is apparent in the 2- to 4-fold greater affinity of the arginyl hexapeptides of [Leu]enkephalin and [Met]enkephalin than of their respective lysyl hexapeptides. [Met]enkephalin-Arg-Arg has only one-fourth the affinity of [Met]enkephalin-Arg.

Table 2. Peptide inhibitors of enkephalin convertase

Peptide	K_i, μM
Benzyloxycarbonyl-Gly-Arg	180
Hippuryl-Arg	180
Gly-Gly-Arg	1,170
Gly-Arg	53
Benzyloxycarbonyl-Ala-Arg	196
Ala-Arg	94
Ile-Arg	64
Leu-Arg	61
Phe-Arg	133
Tyr-Arg	125
Arg-Arg	266
Lys-Arg	297
[Leu^5]Enkephalin-Lys^6	174
[Leu^5]Enkephalin-Arg^6	83
[Met^5]Enkephalin-Lys^6	195
[Met^5]Enkephalin-Arg^6	51
[Met^5]Enkephalin-Arg^6-Arg^7	205

The following peptides and amino acids did not significantly (>15%) inhibit enkephalin convertase at 1 mM (all L-form unless specified): Gly-Gly, Gly-Gly-Glu, Gly-Gly-Trp, Gly-Leu-Gly, Gly-Gly-Pro, Gly-Gly-Tyr, Gly-Gly-Leu, benzyl-Gly-His-Leu, Gly-Tyr-Gly, Try-Gly-Gly, Nle-Ala, Phe-Met, Phe-Ile, Gly-Thr, Gly-Nva, Gly-Asn, Gly-Gly-Nle, Arg-Glu, [Leu^5]enkephalin, arginine, D-arginine, homoarginine, arginosuccinate, Arg-OMe, and Arg-Lys. The K_i values of the di- and tripeptides were obtained from displacement curve IC_{50} values as described in *Materials and Methods*, except the substrate concentration was 10 μM and 1 mM $CoCl_2$ was included. The K_i values of the hexa- and heptapeptides were determined from Lineweaver–Burk plots of enkephalin convertase activity in the absence and presence of 100 μM peptide. Aliquots (200 μl) were removed every 8 min from tubes containing enzyme, 1 mM $CoCl_2$, 50 mM NaOAc (pH 5.6) and dansyl-Phe-Leu-Arg (200, 100, 50, 25, and 12.5 μM) and the reaction was quenched with 50 μl of 1 M HCl. Chloroform (1.5 ml) was added, the tubes were spun on a Vortex and centrifuged, and the fluorescence in the $CHCl_3$ layer was determined. All peptides showed competitive type inhibition of the substrate dansyl-Phe-Leu-Arg (K_m, 53 μM).

Regional Distribution of Enkephalin Convertase Activity in Rat Brain. We have partially purified from soluble rat brain extracts a cobalt-stimulated carboxypeptidase whose properties are essentially the same as those of adrenal enkephalin convertase (data not shown). Carboxypeptidase activity not stimulated by cobalt in soluble brain extracts does not display any regional variation (Table 3). By contrast, apparent enkephalin convertase activity of soluble extracts shows considerable regional variations. Enzyme activity is highest in the thalamus-hypothalamus, corpus striatum, hippocampus, and midbrain, while activity in the cerebellum and brain stem is <1/10th these levels. Cerebral cortical levels of enzyme activity are intermediate.

DISCUSSION

The major finding of the present study is that a cobalt-stimulated carboxypeptidase designated enkephalin convertase appears to be selectively associated with biosynthesis of the enkephalins. Evidence for this is as follows. (*i*) In the adrenal, enkephalin convertase activity is localized to chromaffin granules, in contrast to the carboxypeptidase not stimulated by cobalt, which is concentrated in the lysosomes. (*ii*) The arginyl and lysyl hexapeptides of the enkephalins have micromolar level affinities for enkephalin convertase and lesser affinity for the lysosomal carboxypeptidase. (*iii*) The regional distribution in the brain of apparent enkephalin convertase activity is heterogeneous with similarities to the distribution of enkephalin and opiate receptors. By contrast, the carboxypeptidase activity that is not stimulated by cobalt is similar in all regions examined.

Recently, Hook *et al.* (15) described a carboxypeptidase activity in adrenal homogenates that converted [Met]enkephalin-Arg to [Met]enkephalin, which appears to reflect lysosomal carboxypeptidase and not enkephalin convertase. Thus, EDTA and 1,10-phenanthroline (1 mM) are potent inhibitors of enkephalin convertase but, at 1 mM concentration, fail to inhibit lysosomal carboxypeptidase or the enzymatic activity of Hook *et al.* Moreover, mercuric chloride and *p*-chloromercurophenylsulfonate

Table 3. Regional distribution of cobalt-stimulated and not cobalt-stimulated carboxypeptidase in rat brain

Region	Specific activity, nmol·min^{-1}·mg^{-1}	
	Co^{2+} stimulated	Not Co^{2+} stimulated
Cerebral cortex	1.6	5.3
Cerebellum	0.22	6.8
Brainstem	0.42	5.4
Midbrain	2.0	4.9
Thalamus/hypothalamus	2.6	5.6
Striatum	1.9	4.9
Hippocampus	3.0	5.1

Rat brains were dissected and homogenized (Polytron–Brinkman) in 100 vol of 10 mM NaOAc (pH 6.0). Homogenates were centrifuged at 50,000 × *g* for 60 min and supernatants were decanted and assayed. Cobalt-stimulated carboxypeptidase activity was determined by subtracting the activity in the presence of 1 mM $CoCl_2$ from the activity in the absence of cobalt. Protein was determined by the Bradford method (8).

potently inhibit the enzyme activity of Hook *et al.* and lysosomal carboxypeptidase but are much weaker at inhibiting enkephalin convertase. Though Hook *et al.* used an adrenal chromaffin granule preparation for their enzymatic studies, this preparation contains a substantial amount of lysosomes (6, 12, 16, 17).

Extensive characterization of enkephalin convertase was made feasible by a simple, sensitive, and specific assay using a dansylated enkephalin analog as substrate. Conventional assays monitoring the conversion of enkephalin-Arg^6 to enkephalin by HPLC require about 30 min per sample. In contrast, with the dansylated substrate, we can assay 100–200 samples in an hour. Moreover, the fluorescent assay is $\approx$100 times more sensitive, readily detecting the formation of 10 pmol of product. While the use of radiolabeled enkephalin hexapeptide substrates provides sensitivity, the assays are substantially more time consuming than the fluorescence procedure.

The data presented here suggest that enkephalin convertase is a specific carboxypeptidase that is physiologically involved in the biosynthesis of the enkephalins. Our partial characterization of the enzyme from brain indicates that the same enzymes are involved in the formation of brain and adrenal enkephalins. The selective association of this enzyme with enkephalin synthesis does not rule out the possibility that it may serve other functions. Thus, DOPA decarboxylase converts dihydroxyphenylalanine into dopamine as well as forming serotonin from 5-hydroxytryptophan. Nonetheless, the existence of a specific enzyme selectively associated with a step of enkephalin biosynthesis suggests that similar selective enzymes might be involved in the biosynthesis of other biologically active peptides.

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