Biochemical Pharmacology, Vol. 33, No. 21, pp. 3471–3478, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00

HYDROLYSIS OF OPIOID HEXAPEPTIDES BY CARBOXYPEPTIDASE N

PRESENCE OF CARBOXYPEPTIDASE IN CELL MEMBRANES

RANDAL A. SKIDGEL,* ALICE R. JOHNSON and ERVIN G. ERDÖS
Departments of Pharmacology and Internal Medicine, University of Texas Health Science Center, Dallas, TX 75235, U.S.A.

(*Received* 5 *December* 1983; *accepted* 28 *February* 1984)

Abstract—Carboxypeptidase N, purified to homogeneity from human plasma, rapidly hydrolyzed Lys^6- or Arg^6-enkephalins when measured by high pressure liquid chromatography. Comparison of the kinetics of hydrolysis of the enkephalin hexapeptides and bradykinin by carboxypeptidase N revealed the following values for the K_m and k_{cat}: Arg^6-Met^5-enkephalin, 49 μM, 1024 min^{-1}; Arg^6-Leu^5-enkephalin, 57 μM, 375 min^{-1}; Lys^6-Met^5-enkephalin, 216 μM, 6204 min^{-1}; bradykinin, 19 μM, 58 min^{-1}. Thus, while bradykinin had the lowest K_m, the specificity constants (k_{cat}/K_m) for all the enkephlain hexapeptides were higher than that of bradykinin due to their high turnover numbers. Preincubation of the enzyme with 0.1 mM $CoCl_2$ increased both the k_{cat} and K_m of bradykinin and Arg^6-Met^5-enkephalin. Similar results were obtained when the above experiments were conducted with the active 48,000 dalton subunit of carboxypeptidase N. Basic carboxypeptidase activity was found in the amniotic fluid, in membrane fractions of various human and bovine tissues, and in cultured cells in the following order of decreasing specific activity: human placental microvilli, human kidney, human amniotic fluid, human lung, bovine lung, bovine pulmonary artery, human foreskin fibroblasts, human pulmonary arterial endothelial cells, and human lung fibroblasts. The membrane-bound carboxypeptidase activity had a neutral pH optimum and behaved similarly to plasma carboxypeptidase N in the presence of various inhibitors and activators. It was different from the carboxypeptidase activity in bovine adrenal chromaffin granules which had an acid pH optimum and was inhibited by sulfhydryl reagents. These studies show that human carboxypeptidase N, an enzyme found in high concentration in blood, readily hydrolyzes Arg^6- or Lys^6-enkephalins. It could thus control the levels of these peptides if they are released into the circulation from the adrenal gland. In addition, a membrane-bound carboxypeptidase N-like enzyme in various tissues may regulate the local levels of biologically active peptides containing C-terminal basic amino acids such as hexapeptide enkephalins, kinins, anaphylatoxins or fibrinopeptides.

Carboxypeptidase N (kininase I, arginine carboxypeptidase, serum carboxypeptidase B or anaphylatoxin inactivator; EC 3.4.17.3) cleaves the C-terminal basic amino acid of kinins [1, 2], anaphylatoxins [3], fibrinopeptides, and other peptide substrates [1]. The enzyme is present in plasma and is a tetramer with a mol. wt of 280,000 [4–6]. Carboxypeptidase N can be dissociated by agents which disrupt ionic and hydrophobic interactions into two active 48,000 dalton subunits and two 83,000 dalton carbohydrate-containing inactive subunits [6, 7]. The 48,000 dalton subunit is responsible for the enzymatic activity, while the 83,000 dalton subunit may stabilize the enzyme in blood [7].

Many enkephalin-containing peptides have been isolated from adrenal glands [8, 9]. These include hexapeptide enkephalins containing an additional arginine or lysine at the C-terminal end which bind to opiate receptors [8, 10] and can be converted to enkephalins by removal of the C-terminal arginine or lysine. Because of their substrate specificity, carboxypeptidases B and N are potential candidates for this converting activity *in vivo*, although carboxypeptidase B is not found in any substantial quantity in the circulation or in tissues other than the pancreas [1]. Recent studies have shown bovine adrenal chromaffin granules to contain carboxypeptidases which can convert the hexapeptide enkephalins to enkephalins [11–15]. One of these enzymes has the properties of both a metalloenzyme and a catheptic enzyme and has been called a specific "enkephalin convertase" [11, 13, 15].

We investigated the "enkephalin convertase" activity of homogeneous human plasma carboxypeptidase N and that of its active 48,000 dalton subunit. We also studied similar enzymes from bovine adrenal chromaffin granules, membrane fractions of animal and human tissues, amniotic fluid, and cultured cells.

MATERIALS AND METHODS

Outdated human plasma was obtained from the Parkland Memorial Hospital blood bank (Dallas, TX). Benzoyl-alanyl-lysine (Bz-Ala-Lys)† was

* Address all correspondence to: Dr. Randal A. Skidgel, Department of Pharmacology, Basic Sciences Research Building, The University of Texas Health Science Center, 5323 Harry Hines Blvd., Dallas, TX 75235.

† Abbreviations: Bz, benzoyl; HPLC, high pressure liquid chromatography; MGTA, DL-2-mercaptomethyl-3-guanidinoethylthiopropanoic acid; PCMS, *p*-chloromercuriphenylsulfonate; and Hepes, 4-(2-hydroxyethyl-1-piperazine-ethanesulfonic acid.

provided by Dr. Yehuda Levin of the Weizmann Institute of Science, Rehovot, Israel. L-Arginine-Sepharose was prepared from epichlorohydrin activated Sepharose 6B [16]. Lys^6-Met^5-enkephalin and Leu^5-enkephalin were purchased from Peninsula Laboratories, Belmont, CA. Met^5-enkephalin was from Boehringer Mannheim Biochemicals, Indianapolis, IN, and Arg^6-Met^5-enkephalin and Arg^6-Leu^5-enkephalin were obtained from Bachem, Torrance, CA. Bradykinin and Bz-Gly-Lys were purchased from the Sigma Chemical Co., St. Louis, MO. DL-2-Mercaptomethyl-3-guanidinoethylthiopropanoic acid (MGTA) was from Calbiochem-Behring, La Jolla, CA. HPLC-grade acetronitrile (J. T. Baker) was purchased from American Scientific Products, McGraw Park, IL. Water for HPLC was deionized, distilled, and passed through a Norganic cartridge (Millipore Corp., Bedford, MA) to remove trace organic compounds. Trifluoroacetic acid was sequanal grade from the Pierce Chemical Co., Rockford, IL.

Purification of carboxypeptidase N and its active subunit. Carboxypeptidase N was purified to homogeneity from outdated human plasma by ion-exchange chromatography and L-arginine-Sepharose affinity chromatography as previously described [7]. The enzyme was purified 2665-fold with a 20–30% yield and had a specific activity of 144 μmoles/min/mg with Bz-Ala-Lys as substrate. Purified carboxypeptidase N was treated with 3 M guanidine–HCl to dissociate the subunits. An inactive 83,000 mol. wt subunit and an active 48,000 mol. wt subunit were obtained following separation by gel filtration on Sephadex G-75 superfine [7].

Isolation of chromaffin granules. Chromaffin granules were prepared by differential centrifugation as described [11]. Fresh bovine adrenal glands from a local slaughterhouse were kept on ice until used. The adrenal medullae were dissected, minced and homogenized in about 5 vol. of 0.3 M sucrose with a glass/Teflon homogenizer. The homogenate was centrifuged at 480 *g* for 15 min; the supernatant fraction was decanted and further centrifuged at 12,000 *g* for 20 min to sediment the granule fraction. The granule fraction was resuspended in 0.3 M sucrose and layered over 1.6 M sucrose, and the chromaffin granules were sedimented by centrifuging at 80,000 *g* for 60 min. The granules were lysed by resuspending them in 0.05 M sodium acetate, pH 6.0, followed by freeze–thawing and sonication. The lysed granules were centrifuged at 100,000 *g* for 60 min, and the supernatant fraction was used as the source of carboxypeptidase activity.

Preparation of placental microvilli. A microvillous membrane fraction of the human placental syncytiotrophoblast was prepared according to the method of Truman *et al.* [17] with modifications. Placentas were collected from mothers with uncomplicated full-term deliveries. Villous tissue was cut from the fetal surface of four to six placentas, chopped into small pieces, and washed three times with isotonic saline to remove blood. The tissue pieces (100 g) were resuspended in 300 ml of cold isotonic saline and stirred for 45 min at 4°. The suspension was filtered through gauze and centrifuged at 1200 *g* for 10 min. The resulting supernatant fraction was centrifuged for 7 min at 15,000 *g*, and the microvilli were obtained by centrifugation of the second supernatant fraction at 100,000 *g* for 60 min. The microvillous pellet was rinsed with cold, isotonic saline and resuspended in 5 ml of 10% sucrose. This suspension was centrifuged at 6000 *g* for 10 min to obtain the supernatant fraction enriched in microvilli which we call "S4".

In some experiments, the microvilli were further purified on a 25-ml sucrose step gradient consisting of 4 ml of 50%, 10 ml of 43%, 10 ml of 34%, 8 ml of 25% and 4 ml of 20% sucrose. A 2-ml portion of the microvillous-rich fraction (S4) was overlayed onto the gradient and centrifuged for 17 hr at 85,000 *g* (25,000 rpm in a Beckman SW27 swinging bucket rotor). Four fractions were obtained; Band I, at the 25%/34%-sucrose interface, Band II, at the 34%/43%-sucrose interface, Band III, from the 43%/50%-sucrose layers, and the final pellet. These fractions were diluted 2-fold with isotonic saline and pelleted at 100,000 *g* for 60 min. The pellets were resuspended in 0.1 M Tris–HCl, pH 7.5, and centrifuged again at 100,000 *g* for 60 min; the supernatant was discarded and the final pellets were suspended in 2–5 ml of 0.1 M Tris–HCl, pH 7.5.

Membrane preparations. Bovine lungs (obtained at a local slaughterhouse) or human lungs (autopsy samples from Parkland Memorial Hospital) were kept on ice until used. Pulmonary arteries and veins were dissected from lung tissue and cleaned free of connective tissue. Lung tissue was removed from the marginal regions.

Cell membranes were isolated from bovine or human lung tissue or pulmonary vessels by the method of Malathi *et al.* [18] as modified by Johnson *et al.* [19]. Tissues were washed in phosphate-buffered saline to remove blood, weighed, and then homogenized in 20 vol. of ice-cold 0.5 mM Tris–HCl (pH 7.0), 50 mM mannitol with a blender for 3 min (30-sec bursts with periodic cooling on ice). Calcium chloride was added to a final concentration of 10 mM, and the mixture was stirred at 4° for 30 min. The homogenate was strained through several layers of cheesecloth and then centrifuged at 3000 *g* for 30 min. The resulting supernatant fraction was centrifuged at 43,000 *g* for 30 min to yield a membrane pellet which was resuspended in the Tris–mannitol buffer and centrifuged again at 43,000 *g* to yield a washed membrane fraction.

Washed microsomes were prepared from blood-free cadaver kidneys as previously described [20].

Cell cultures. Endothelial cells were isolated from human pulmonary arteries by treatment with collagenase as reported [21]. All cells were grown in Medium 199 (Grand Island Biological Co.) supplemented with 10% human serum, 15% fetal calf serum and an antibiotic mixture containing penicillin, fungizone and streptomycin. Cultures were maintained at 37° in an atmosphere of 5% CO_2. Cells were washed twice with phosphate-buffered saline, detached from the flasks by scraping with a rubber spatula into about 1 ml of 0.1 M Tris–HCl, pH 7.5, and homogenized by sonication. Cell homogenates were assayed for DNA with the fluorescence method of Labarca and Paigen [22]. Cell number was cal-

culated from the DNA content using a conversion factor of 6.1 μg DNA/10^6 cells [23].

Enzyme assays. Carboxypeptidase N activity was routinely measured during purification with a continuous spectrophotometric assay which monitors the hydrolysis of Bz-Ala-Lys at 254 nm in 0.1 M Hepes buffer, pH 7.0, at 37° [24–26].

Carboxypeptidase activity in tissue samples, cultured cells, membrane fractions and amniotic fluid was assayed with Bz-Gly-Lys as substrate by measurement of the Bz-Gly released spectrophotometrically. This method was adapted to measure carboxypeptidase N activity by Koheil and Forstner [27] from the original method designed to measure angiotensin I converting enzyme activity [28]. It has recently been described in detail elsewhere [25]. Briefly, enzyme samples (up to 100 μl), with or without $CoCl_2$ (20 μl of 10 mM) and inhibitor (if any), in 0.1 M Tris–HCl, pH 7.5 (final volume, 200 μl), were preincubated for 2 hr at 4°. The reaction was initiated by the addition of 50 μl Bz-Gly-Lys (25 mM), and the samples were incubated at 37°. The reaction was terminated by the addition of 250 μl of 1 N HCl. The product, Bz-Gly, was extracted by vigorously mixing the reaction sample with 1.5 ml ethyl acetate followed by centrifugation (500 *g* for 5 min) to separate the phases. A 1.0-ml aliquot of the ethyl acetate layer was transferred to a clean tube, evaporated, and reconstituted in 1.0 ml H_2O; the absorbance at 228 nm was measured in a Varian Cary 219 UV spectrophotometer. Zero time reaction blanks were run for each sample by adding 250 μl of 1 N HCl to the tube before the addition of substrate. Reactions were routinely run in the presence of $CoCl_2$ to enhance assay sensitivity since cobalt stimulates the peptidase activity of carboxypeptidases [29–31]. While a pH profile study indicated the optimum pH for the membrane carboxypeptidase with Bz-Gly-Lys was 7.0, activities between pH 7.0 and 8.0 were not tested (Fig. 1A and 1B). Separate control experiments showed that activity was still maximal at pH 7.5 and that the activity was higher in Tris buffer than in Hepes buffer at the same pH. Because Tris is a better buffer at pH 7.5 than at 7.0, pH 7.5 was chosen for routine assays of Bz-Gly-Lys hydrolysis.

Kinetic studies of the hydrolysis of Lys^6- or Arg^6-Met^5-enkephalin, Arg^6-Leu^5-enkephalin and bradykinin by intact carboxypeptidase N and its 48,000 dalton subunit were carried out by measuring product release in a high pressure liquid chromatograph. Substrate, in 0.1 M Hepes buffer, pH 7.0, and enzyme (17.5 ng carboxypeptidase N or 3.7 ng 48,000 dalton subunit) were incubated in a final volume of 100 μl at 37° for 15 min. The reactions were terminated by adding 20 μl of 5% trifluoroacetic acid. Aliquots of 40 μl were analyzed by high pressure liquid chromatography (HPLC) as stated below.

Calculations of product formation were achieved by comparing the integrated peak area of product to the peak area of a known amount of authentic standard. Standards were injected two to four times during each analysis. Kinetic constants were obtained by initial velocity measurements of product formation at seven substrate concentrations, ranging from 5 μM to 0.25 mM. Data were plotted according to Hanes ($[S]$ vs $[S]/V$) and fit to the best straight line by linear regression [32]. Correlation coefficients better than 0.97 were always obtained. The inhibition constant (K_i) for MGTA with carboxypeptidase N was determined using Arg^6-Met^5-enkephalin (50 μM) in the HPLC assay. MGTA (1–100 nM) was preincubated with enzyme and buffer for 1 hr at 4°. K_i was determined graphically by plotting the reciprocal of the velocity versus the concentration of MGTA and extrapolating the K_i from the intersection of the line with a horizontal line drawn to intercept $1/V_{max}$ (determined in the absence of MGTA) according to Dixon and Webb [33].

The conversion of Lys^6-Met^5-enkephalin to Met^5-enkephalin by placental microvilli was measured by HPLC. Placental membranes were dialyzed against 0.05 M Tris–HCl, pH 7.2. Placental microvilli were preincubated for 30 min at 4° in 0.1 M Tris–HCl (pH 7.5) containing captopril (10 μM), phosphoramidon (10 μM), puromycin (1 mM), bestatin (10 μM), aprotinin (100 units/ml) and *p*-chloromercuriphenylsulfonic acid (PCMS) (100 μM). The inhibitors were used to prevent the degradation of enkephalins by other enzymes in the microvilli. In control experiments, the hydrolysis of Lys^6-Met^5-enkephalin by purified plasma carboxypeptidase N was not decreased in the presence of these inhibitors. Lys^6-Met^5-enkephalin (0.1 mM final concentration) was added to initiate the reaction and incubations were conducted for 15 min at 37°. Reactions were terminated by the addition of 20 μl of 100% trifluoroacetic acid. Samples were diluted with 100 μl H_2O, the precipitated proteins were removed by centrifugation at 8000 *g* for 2 min, and the resulting supernatant fraction was filtered through 0.2 micron nitrocellulose microfilters (Schleicher & Schuell, Inc., Keene, NH). Aliquots of 80 μl were assayed by HPLC as stated below.

High pressure liquid chromatography. HPLC analyses were conducted in a Waters automated gradient system consisting of an M-6000A pump, an M-45 pump, a WISP 710 B automatic injector, a model 720 system controller, an M730 data module and a model 441 absorbance detector. Peptide products were separated on a Waters μ-Bondapak C-18 reverse phase column (3.9 mm × 30 cm) or a Waters Resolve C-18 reverse phase column (3.9 mm × 15 cm). Products were eluted either isocratically or with an increasing linear gradient of CH_3CN/0.05% trifluoroacetic acid (solvent A) in H_2O/0.05% trifluoroacetic acid (solvent B) at a flow rate of 1.0 ml/min. For gradient elutions, the column was equilibrated for 10 min at initial conditions before injection of sample. Peptides were detected at a wavelength of 214 nm.

For kinetic studies with carboxypeptidase N, its 48,000 dalton subunit and Arg^6-Met^5-enkephalin, Arg^6-Leu^5-enkephalin or Lys^6-Met^5-enkephalin, products were separated isocratically on a Waters Resolve C-18 column using a mixture of 50% A and 50% B. Under these conditions the following retention times were observed: Arg^6-Met^5-enkephalin, 8.5 min; Arg^6-Leu^5-enkephalin, 9.4 min; Lys^6-Met^5-enkephalin, 8.6 min; Met^5-enkephalin, 3.0 min; and Leu^5-enkephalin, 3.4 min.

The products of hydrolysis of Lys^6-Met^5-enkephalin by placental microvilli were separated on

a Waters μ-Bondapak C-18 column using a linear gradient of 20% A to 40% A in 15 min. The retention times were: Lys[6]-Met[5]-enkephalin, 8.0 min; Met[5]-enkephalin, 11.8 min.

Kinetic studies of bradykinin hydrolysis were carried out using a Waters μ-Bondapak C-18 column with a linear gradient of 25% A to 35% A in 10 min. Under these conditions, bradykinin eluted in 6.5 min and des-Arg[9]-bradykinin in 10.2 min.

Protein determinations. Protein concentrations of purified plasma carboxypeptidase N and its 48,000 dalton subunit were determined by quantitative amino acid analysis. Protein values for cultured cells and tissue preparations were determined according to the method of Bradford [34].

RESULTS

Kinetics of the conversion of enkephalin hexapeptides. Table 1 summarizes the kinetic parameters of the hydrolysis of Arg[6]- and Lys[6]-enkephalins by homogeneous human plasma carboxypeptidase N and its 48,000 dalton active subunit. Although carboxypeptidase N has long been known to hydrolyze the Phe[8]-Arg[9] bond of bradykinin [2], kinetic constants have never been determined for this nonapeptide with the pure enzyme. For the sake of comparison, data obtained with bradykinin are also included in Table 1. Turnover numbers are expressed as molecules of substrate hydrolyzed per min per active site assuming two active sites per intact 280,000 dalton molecule and one active site per 48,000 dalton molecule of active subunit [7].

In agreement with the known properties of carboxypeptidase N [1, 5, 31], C-terminal lysine was hydrolyzed six times faster than arginine. The turnover number of Lys[6]-Met[5]-enkephalin was the highest of any substrate tested (k_{cat} = 6204 min^{-1}). Arg[6]-Met[5]- and Arg[6]-Leu[5]-enkephalin were hydrolyzed slower (k_{cat} = 1024 min^{-1} and 375 min^{-1}).

To show that the activity was due to carboxypeptidase N and not a trace contaminant in the purified preparation, the K_i for MGTA (a potent, specific inhibitor of basic carboxypeptidases) was determined using Arg[6]-Met[5]-enkephalin as substrate as described in Materials and Methods. MGTA inhibited the hydrolysis of Arg[6]-Met[5]-enkephalin with a K_i of 5 nM.

The hydrolysis of Arg[6]-Met[5]-enkephalin was also studied in the presence of 0.1 mM $CoCl_2$ which optimally accelerates the cleavage of peptide substrates by carboxypeptidase N [31]. Addition of $CoCl_2$ increased the turnover number of this peptide about 3-fold but it also raised the K_m. Thus, the specificity constant, k_{cat}/K_m, did not change.

Surprisingly, bradykinin was cleaved slower (k_{cat} = 58 min^{-1}) than the hexapeptide enkephalins although the K_m (19 μM) was the lowest of all substrates tested. As with Arg[6]-Met[5]-enkephalin, $CoCl_2$ greatly accelerated the rate of hydrolysis (k_{cat} = 296 min^{-1}) and doubled the K_m to 45 μM. The specificity constant also increased about 3-fold in the presence of 0.1 mM $CoCl_2$ but was still lower than that of Arg[6]- or Lys[6]-Met[5]-enkephalin. Although carboxypeptidase N was originally named "kininase I" for its kininase activity [1, 2], under the *in vitro* conditions in this study the hexapeptide enkephalins were the preferred substrates of this enzyme.

Similar results were obtained when the 48,000 dalton active subunit of carboxypeptidase N was tested with the hexapeptide enkephalins (Table 1). Again, Lys[6]-Met[5]-enkephalin was cleaved fastest with a k_{cat} of 4213 min^{-1}, yielding the same specificity constant as with the intact enzyme. Addition of 0.1 mM $CoCl_2$ to the reaction mixture increased both the K_m and k_{cat} of Arg[6]-Met[5]-enkephalin hydrolysis without changing the k_{cat}/K_m significantly. In general, the K_m for the hexapeptide enkephalins was lower with the 48,000 dalton subunit than with the intact enzyme, and the k_{cat} was also lower with the isolated active subunit. Thus, the specificity constants (k_{cat}/K_m) of both carboxypeptidase N and its active subunit were similar for all of the substrates tested.

As with intact 280,000 dalton enzyme, the 48,000 dalton subunit cleaved bradykinin slower than the hexapeptide enkephalins. Addition of $CoCl_2$ only doubled the k_{cat} of the subunit with bradykinin in contrast to the 5-fold increase seen with the intact enzyme. However, the K_m decreased slightly in the presence of $CoCl_2$ (35 μM to 25 μM) and, consequently, the specificity constant was the same as with the intact enzyme.

Table 1. Hydrolysis of hexapeptide enkephalins and bradykinin by carboxypeptidase N (280K) and its active subunit (48K)*

Substrate	$CoCl_2$†	K_m (μM)		k_{cat} (min^{-1})		k_{cat}/K_m (μM^{-1} min^{-1})	
		280K	48K	280K	48K	280K	48K
Arg[6]-Met[5]-Enk	−	49	15	1024	596	20.9	39.7
Arg[6]-Met[5]-Enk	+	135	72	2871	2590	21.3	36.0
Arg[6]-Leu[5]-Enk	−	57	44	375	370	6.6	8.4
Lys[6]-Met[5]-Enk	−	216	149	6204	4213	28.7	28.3
Bradykinin	−	19	35	58	70	3.0	2.0
Bradykinin	+	45	25	296	159	6.6	6.4

* Kinetic constants were determined as described in Materials and Methods. The results shown are the average values from two reactions. The variation between reactions averaged 11% for the K_m and 4% for the k_{cat}. Enk = enkephalin.

† Enzyme was preincubated for 2 hr on ice in the presence (+) or absence (−) of 0.1 mM $CoCl_2$.

Table 2. Carboxypeptidase activity in tissues and cultured cells

Source	N*	Preparation†	Activity‡ nmoles/min/mg	Activity‡ nmoles/min/10^6 cells
Human placenta	5	Microvilli, Band I	65.2 ± 8.5	
Human placenta	6	Microvilli, S4	34.8 ± 6.4	
Human kidney	3	Membrane fraction	18.1 ± 2.1	
Human plasma	5		16.3 ± 4.8	
Human lung	3	Membrane fraction	7.8 ± 0.3	
Bovine lung	2	Membrane fraction	3.0 (3.6, 2.4)	
Bovine pulmonary artery	2	Membrane fraction	2.3 (2.3, 2.4)	
Human foreskin fibroblasts	2	Homogenate	2.0 (2.1, 1.9)	0.65 (0.62, 0.67)
Human pulmonary arterial endothelial cells	3	Homogenate	1.9 ± 0.1	0.43 ± 0.08
Human lung fibroblasts	2	Homogenate	1.1 (1.3, 1.0)	0.34 (0.39, 0.28)

* N = the number of different preparations tested.
† Tissues and cells were homogenized and fractionated as described in Materials and Methods.
‡ Activity was determined at pH 7.5 using Bz-Gly-Lys as substrate and is given ± S.E.M. for N ≥ 3. For N = 2, the range of activities is given in parentheses.

Carboxypeptidase in tissues and cells. Carboxypeptidase activity was detected in membrane fractions from various bovine and human tissues including lung, pulmonary artery, kidney and placenta (Table 2). Activity was also found in various human cells in culture (Table 2). The enzymatic activity was assayed in subcellular fractions of the various tissues, and the highest specific activity was in the washed membrane fractions that sediment at 43,000 *g*.

The richest source of carboxypeptidase activity was a microvillous fraction isolated from human placenta. This preparation had a 4-fold higher activity than plasma on a per mg protein basis (Table 2). High specific activities were also found in human kidney and lung (Table 2).

Because bovine adrenal chromaffin granules contain carboxypeptidase B-like enzyme activity [11–15], we tested the granule lysate with Bz-Gly-Lys as substrate. At pH 7.5, the activity was 8.9 nmoles/min/mg, while at pH 6 the activity increased 8-fold, indicating an acid pH optimum.

Of the cultured human cells tested, foreskin fibroblasts had the highest activity on a per cell basis followed by pulmonary artery endothelial cells and lung fibroblasts (Table 2). When calculated on a per milligram protein basis, the specific activity in cultured cells was lower than that in the tissue preparations (Table 2).

To distinguish the membrane-bound carboxypeptidase activity from other tissue carboxypeptidases (such as catheptic carboxypeptidase), the hydrolysis of Bz-Gly-Lys was studied at different pH values. While lysosomal or catheptic carboxypeptidases have an acid pH optimum [35, 36], the activity of the membrane-bound enzyme from human placenta or lung was maximal at pH 7, the same as plasma carboxypeptidase N (Fig. 1A). The carboxypeptidase activity in the membrane fraction of human kidney was also maximal at pH 7 (data not shown). In contrast, the enzyme from bovine adrenal chromaffin granules was optimally active at pH 6.

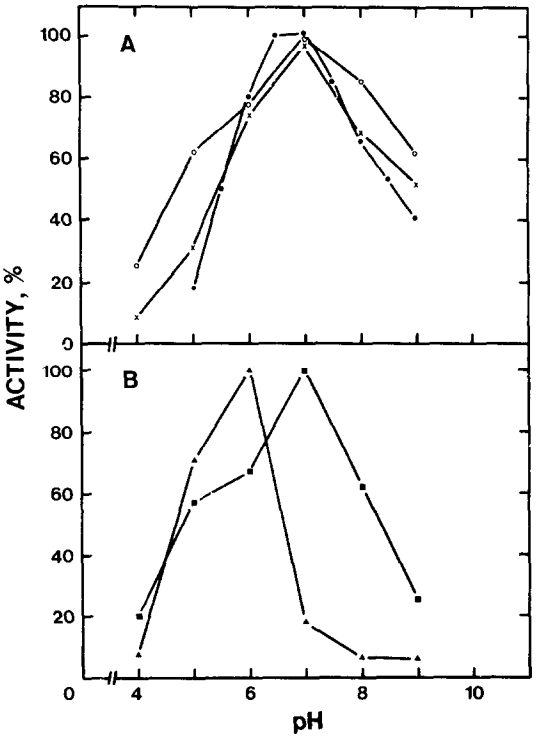

Fig. 1. Effect of pH on carboxypeptidase activity. Tissue samples and homogeneous human plasma carboxypeptidase N were prepared as described in Materials and Methods. In all cases, carboxypeptidase activity was assayed in the presence of either 0.1 M sodium acetate buffer (pH 4–6) or 0.1 M Tris–HCl buffer (pH 7–9). Tissue samples were assayed with Bz-Gly-Lys and purified human plasma carboxypeptidase N with Bz-Ala-Lys as substrate. For further details, see Materials and Methods. (A) Human: placental microvilli (○), lung membrane fragments (×), and purified plasma carboxypeptidase N (●). (B) Bovine: lung membrane fragments (■), and adrenal chromaffin granule lysate (▲).

with only 20% of the activity remaining at pH 7 (Fig. 1B). This was not due to a species difference as the activity from bovine lung showed the same pH profile as that from human lung (Fig. 1 A and B).

The membrane-bound carboxypeptidase was further distinguished from catheptic carboxypeptidase by the use of inhibitors and activators. While the catheptic enzyme requires a thiol reagent for maximal activity and is inhibited by sulfhydryl reagents and heavy metals [36], the membrane carboxypeptidases from human kidney, placental microvilli and bovine lung were not inhibited by $HgCl_2$ or PCMS and were not stimulated with dithiothreitol (Table 3). Similarly, sulfhydryl reagents had no effect on the plasma enzyme (Table 3). The plasma and membrane-bound carboxypeptidases were all inhibited by cadmium acetate, *o*-phenanthroline and MGTA, and their activities were stimulated markedly by added $CoCl_2$ (Table 3). The enzymatic activity from bovine adrenal chromaffin granules, however, resembles both carboxypeptidase N and catheptic carboxypeptidase. For instance, while its activity was enhanced by $CoCl_2$ and inhibited by MGTA, cadmium acetate and *o*-phenanthroline, it was also activated by dithiothreitol and inhibited by $HgCl_2$ and PCMS (Table 3).

Because of the high carboxypeptidase activity found in placental microvillous membranes, we investigated the carboxypeptidase activity of human amniotic fluid. The activity with Bz-Gly-Lys as substrate was 11.3 nmoles/min/mg, about 70% the specific activity found for carboxypeptidase N in plasma. The inhibition pattern and pH profile were similar to those found for plasma carboxypeptidase N and the membrane carboxypeptidases (not shown).

Placental microvillous membranes contain kappa opiate receptors [37], and Lys^6- or Arg^6-enkephalins bind to kappa opiate receptors [10]. It was therefore of interest to determine whether these membranes were capable of converting hexapeptide enkephalins to enkephalins. Placental microvillous membrane fragments (20 μg) were incubated (37°, pH 7.5) with 0.1 mM Lys^6-Met^5-enkephalin as described in Materials and Methods. Under these conditions, the placental membranes hydrolyzed 10% of the substrate in 15 min, giving a rate of 3.7 nmoles/min/mg protein. Thus, the placental microvilli readily convert hexapeptide enkephalins to enkephalins at physiological pH.

DISCUSSION

The major findings of this study are 2-fold. First, carboxypeptidase N, an enzyme found in high concentration in blood, readily converted hexapeptide enkephalins containing a C-terminal arginine or lysine to enkephalins. Second, membrane fractions of various human and animal tissues contained a similar enzyme capable of hydrolyzing C-terminal basic amino acids from peptide substrates at neutral pH.

Investigation of the kinetics of the hydrolysis of bradykinin by homogeneous human plasma carboxypeptidase N yielded a K_m value of 19 μM. This is in contrast to an earlier study which reported the K_m to be 0.4 μM [38]. The reason for this difference is not clear, however; several factors could be involved. The earlier study followed the hydrolysis of ^{14}C-labeled bradykinin by a preparation of carboxypeptidase N partially purified by gel filtration and ion-exchange chromatography [38]. Because of a limited supply of [^{14}C]bradykinin, which had to be purified by high voltage electrophoresis, the highest substrate concentration which could be used in the previous study was 7×10^{-8} M which is about 6-fold lower than the reported K_m and 270-fold lower than the K_m we found. Use of such low substrate concentrations could have influenced the value of the

Table 3. Activation and inhibition of carboxypeptidase activity

		Activity† (%)				
		Human			Bovine	
Addition	Concn* (mM)	Kidney microsomes	Placental microvilli	Plasma	Lung membrane fraction	Adrenal chromaffin granule lysate
CdOAc	0.1	74	42	65	76	16
PCMS	0.1	104	99	105	120	1
$HgCl_2$	0.001	89	99	99	133	43
MGTA	0.01	15	12	4	56	8
o-Phenanthroline	1	15	11	7	32	0
$CoCl_2$	1	200	332	491	380	836
Dithiothreitol	5		64	55		163

* Activators or inhibitors were preincubated with sample for 2 hr on ice at the concentration given. Addition of substrate to start the reaction decreased the final concentration by 20%.

† Tissue fractions were prepared as described in Materials and Methods. Activity was determined using Bz-Gly-Lys as substrate in 0.1 M Tris, pH 7.5, with the exception of the bovine adrenal chromaffin granule lysate which was assayed in 0.1 M sodium acetate at pH 6.0. Results are expressed as percent of control activity in the absence of inhibitors or activators. Control activities were (nmoles/min): human renal microsomes, 0.15; human placental microvilli, 0.59; human plasma, 0.49; bovine lung membrane fraction, 0.37; and bovine adrenal chromaffin granule lysate, 0.49.

K_m as it is preferable to use substrate concentrations near the K_m for the most accurate determination [39]. Another factor which may be involved is the use, in the previous study, of partially purified carboxypeptidase N which was prepared without the use of proteolytic inhibitors [38]. It is now known that carboxypeptidase N is very sensitive to proteolytic degradation during purification and that proteolysis leads to an increase in activity and a decrease in stability [6, 7]. Finally, in the previous study, the partially purified preparation was stored in the presence of an unspecified concentration of $CoCl_2$ which would have affected the activity.

While bradykinin had the lowest K_m value of all the peptide substrates tested, the specificity constants of all the enkephalin hexapeptides were higher because of the 6- to 107-fold higher turnover rates. Similar results were obtained when the isolated 48,000 dalton subunit of carboxypeptidase N was used. In agreement with the known properties of carboxypeptidase N, enkephalins with C-terminal lysine had the highest turnover numbers. If alanine precedes arginine, then the C-terminal arginine is released very rapidly [5] as in the anaphylatoxin C3a [40], hence the name "anaphylatoxin inactivator" [3]. However, when tested at a 60 μM substrate concentration, the hexapeptide enkephalins were cleaved by the enzyme two to twelve times faster than anaphylatoxin C3a.* Thus, of the physiologically active substrates tested so far, the hexapeptide enkephalins were cleaved the fastest, illustrating the futility of naming an enzyme after a single substrate only, such as "kininase", "anaphylatoxin inactivator" or "enkephalin convertase".

Enkephalin-containing peptides have been detected in blood and their levels are elevated during stress, presumably due to release from the adrenal glands [41]. These opioid peptides may be involved as mediators of stress-induced analgesia [42]. Enkephalins have also been implicated as mediators released in shock [43, 44]. The present study showed that Lys^6- or Arg^6-enkephalins are excellent substrates of carboxypeptidase N. Thus, hexapeptide enkephalins (and possibly other enkephalin-containing peptides with C-terminal arginine or lysine) released into the blood would be converted rapidly to their des-arginine or des-lysine forms. Carboxypeptidase N in blood, along with circulating aminopeptidases, may therefore regulate the levels of these opioid peptides in the circulation.

Carboxypeptidases at tissue sites could also be involved in the activation or inactivation of opioid peptides. Recent publications report the presence of carboxypeptidase B-like activity in bovine adrenal chromaffin granules which may be involved in this process [11–15]. The methods used for purification of a carboxypeptidase from bovine adrenal chromaffin granules were similar to those employed with plasma carboxypeptidase N [11]. This enzyme was named "enkephalin convertase" [11, 13, 15] although these authors state that the enzyme may be involved in cleaving other biologically active peptides since it is also found in the brain and pituitary gland [13, 15]. It has many properties in common with plasma carboxypeptidase N. For instance, its activity is enhanced in the presence of $CoCl_2$, and it is inhibited by *o*-phenanthroline, EDTA and cadmium [11, 13, 15]. While some of the similarities are striking, there are also many differences between the two enzymes. In agreement with previous studies [11–15], we found that the bovine adrenal carboxypeptidase had an acid pH optimum, was stimulated by dithiothreitol, and was inhibited by $HgCl_2$ or PCMS, similar to catheptic carboxypeptidases previously described [36]. In contrast, plasma carboxypeptidase N had a neutral pH optimum, was not stimulated by dithiothreitol, and was not inhibited by $HgCl_2$ or PCMS. In addition, a recent study showed quantitative differences in the inhibition of bovine plasma carboxypeptidase N and bovine adrenal carboxypeptidase by derivatives of guanidinomercaptosuccinic acid and guanidinopropylsuccinic acid [45].

It was of considerable interest that cultured cells such as human fibroblasts or endothelial cells and membrane fractions of various organs contained a basic carboxypeptidase. Although the specific activity in membrane fractions from either lung or kidney was relatively high, a microvillous membrane fraction from human placenta had the highest value of any tissue tested. Human amniotic fluid also contained a basic carboxypeptidase with about 70% of the specific activity of carboxypeptidase N in plasma. While this enzyme could be released from the placenta, it may also come from other sources (e.g. fetal urine, amnion, etc.).

The pH profile and inhibition pattern of the membrane-bound carboxypeptidase are similar to those of plasma carboxypeptidase N and distinct from lysosomal carboxypeptidases and the carboxypeptidase activity in bovine adrenal chromaffin granules. However, in contrast to the soluble plasma enzyme, it is tightly bound to the membrane as treatment with 0.1% Triton X-100 failed to solubilize the activity.†

Such a membrane-bound carboxypeptidase could play an important role in the regulation of the action of kinins, enkephalins, anaphylatoxins and other active peptides at tissue sites. For example, a recent paper showed kappa opiate receptors to be in the placental microvilli [37], where we found the highest specific activity of the carboxypeptidase. A study of the relative binding of enkephalin peptides at mu, delta and kappa binding sites in guinea pig brain showed pentapeptide enkephalins to be relatively selective for the delta binding site with virtually no affinity for the kappa binding site [10]. The addition of Arg or Lys in position 6 caused a marked loss of activity at the mu and delta binding sites but led to an increase in affinity for the kappa binding site [10]. Thus, a membrane carboxypeptidase N-like enzyme, by cleaving the Arg^6- or Lys^6- from the hexapeptide enkephalins, may regulate the relative affinity of enkephalin peptides towards kappa opiate receptors in the placenta.

In addition to enkephalin peptides, this enzyme in the placenta, lung, kidney and other tissues could play an important role in the inactivation and/or modulation of the activity of other biologically active

* R. A. Skidgel, M. S. Kawahara and T. E. Hugli, manuscript in preparation.

† R. A. Skidgel and E. G. Erdös, unpublished experiments.

peptides containing C-terminal basic amino acids (e.g. kinins, anaphylatoxins, fibrinopeptides). For instance, while removal of the C-terminal arginine on bradykinin abolishes its activity in most tissues, the activity of des-Arg^9-bradykinin assayed on the rabbit aorta is 11-fold higher than that of native bradykinin [46]. These data suggest that there are at least two types of bradykinin receptor, one which interacts with bradykinin and one which binds preferentially des-Arg^9-bradykinin [46]. Similarly, anaphylatoxin C5a, the most potent anaphylatoxin, is both spasmogenic and chemotactic [40]. Removal of the C-terminal arginine from C5a causes a loss of spasmogenic and histamine-releasing activity, while its chemotactic effectiveness is retained [40]. Thus, a membrane carboxypeptidase, capable of removing the C-terminal arginine from these potent peptides, may be a modulator as well as an inactivator of peptide hormone action.

Acknowledgements—We would like to thank Dr. H. Y. T. Yang of the National Institute of Mental Health for her advice and initial collaborations on this study. We also thank Richard M. Davis and Youngsook Kim for their expert assistance and Brenda Moore for help in preparation of the manuscript. We are grateful to the Department of Obstetrics, Parkland Memorial Hospital, Dallas, TX, and the Dallas County Medical Examiners for providing the human tissues used in this study. This work was supported by Grants HL 16320, HL 28813 and HL 18826 from the National Institutes of Health (N.H.L.B.I.) and by a Grant-in-Aid (82782) from the American Heart Association.

REFERENCES

1. E. G. Erdös, in *Handbook of Experimental Pharmacology* (Ed. E. G. Erdös), Vol. 25, Suppl., p. 428. Springer, Heidelberg (1979).
2. E. G. Erdös and E. M. Sloane, *Biochem. Pharmac.* **11**, 585 (1962).
3. V. A. Bokisch and H. J. Müller-Eberhard, *J. clin. Invest.* **49**, 2427 (1970).
4. G. Oshima, J. Kato and E. G. Erdös, *Biochim. biophys. Acta* **365**, 344 (1974).
5. G. Oshima, J. Kato and E. G. Erdös, *Archs Biochem. Biophys.* **170**, 132 (1975).
6. T. H. Plummer and M. Y. Hurwitz, *J. biol. Chem.* **253**, 3907 (1978).
7. Y. Levin, R. A. Skidgel and E. G. Erdös, *Proc. natn. Acad. Sci. U.S.A.* **79**, 4618 (1982).
8. A. S. Stern, R. V. Lewis, S. Kimura, J. Rossier, S. Stein and S. Udenfriend, *Archs Biochem. Biophys* **205**, 606 (1980).
9. H-Y. T. Yang, T. Hexum and E. Costa, *Life Sci.* **27**, 1119 (1980).
10. J. Magnan, S. Paterson and H. W. Kosterlitz, *Life Sci.* **31**, 1359 (1982).
11. L. D. Fricker and S. H. Snyder, *Proc. natn. Acad. Sci. U.S.A.* **79**, 3886 (1982).
12. V. Y. H. Hook, L. E. Eiden and M. J. Brownstein, *Nature, Lond.* **295**, 341 (1982).
13. L. D. Fricker, S. Supattapone and S. H. Snyder, *Life Sci.* **31**, 1841 (1982).
14. C. M. Troy and J. M. Musacchio, *Life Sci.* **31**, 1717 (1982).
15. L. D. Fricker and S. H. Snyder, *J. biol. Chem.* **258**, 10950 (1983).
16. J. Porath and N. Fornstadt, *J. Chromat.* **51**, 479 (1970).
17. P. Truman, J. S. J. Wakefield and H. C. Ford, *Biochem. J.* **196**, 121 (1981).
18. P. Malathi, H. Preiser, P. Fairclough, P. Mallett and R. K. Crane, *Biochim. biophys. Acta* **554**, 259 (1979).
19. A. R. Johnson, M. John and E. G. Erdös, *Ann. N.Y. Acad. Sci.* **384**, 72 (1982).
20. K. Nishimura, F. Alhenc-Gelas and E. G. Erdös, *Proc. natn. Acad. Sci. U.S.A.* **77**, 4975 (1980).
21. A. R. Johnson, *J. clin. Invest.* **65**, 841 (1980).
22. C. Labarca and K. Paigen, *Analyt. Biochem.* **102**, 344 (1980).
23. A. R. Johnson and E. G. Erdös, *J. clin. Invest.* **59**, 684 (1977).
24. T. H. Plummer and E. G. Erdös, *Meth. Enzym.* **80**, 442 (1981).
25. R. A. Skidgel and E. G. Erdös, in *Methods of Enzymatic Analysis* (Ed. H. U. Bergmeyer), Vol. 4, in press. Verlag Chemie, Weinheim (1984).
26. E. C. Wolff, E. W. Schirmer and J. E. Folk, *J. biol. Chem.* **237**, 3094 (1962).
27. A. Koheil and G. Forstner, *Biochim. biophys. Acta* **524**, 156 (1978).
28. D. W. Cushman and H. S. Cheung, *Biochem. Pharmac.* **20**, 1637 (1971).
29. J. E. Folk and J. A. Gladner, *J. biol. Chem.* **235**, 60 (1960).
30. J. E. Folk and J. A. Gladner, *Biochim. biophys. Acta* **48**, 139 (1961).
31. E. G. Erdös, E. M. Sloane and I. M. Wohler, *Biochem. Pharmac.* **13**, 893 (1964).
32. M. Dixon and E. C. Webb, *Enzymes*, 3rd Edn, pp. 60–62. Academic Press, New York (1979).
33. M. Dixon and E. C. Webb, *Enzymes*, 3rd Edn, pp. 345–52. Academic Press, New York (1979).
34. M. M. Bradford, *Analyt. Biochem.* **72**, 248 (1976).
35. L. M. Greenbaum and R. Sherman, *J. biol. Chem.* **237**, 1082 (1962).
36. J. K. McDonald and C. Schwabe, in *Proteinases in Mammalian Cells and Tissues* (Ed. A. J. Barrett), Vol. 2, p. 335. North-Holland, New York (1977).
37. G. Porthé, A. Valette, A. Moisand, M. Tafani and J. Cros, *Life Sci.* **31**, 2647 (1982).
38. R. Zacest, S. Oparil and R. C. Talamo, *Aust. J. exp. Biol. med. Sci.* **52**, 601 (1974).
39. I. H. Segel, *Enzyme Kinetics*, p. 46. John Wiley, New York (1975).
40. T. E. Hugli, in *Critical Reviews in Immunology* (Ed. M. Z. Atassi), Vol. 1, p. 321. Chemical Rubber Co., Boca Raton, FL (1980).
41. M. R. Boarder, E. Erdelyi and J. D. Barchas, *J. clin. Endocr. Metab.* **54**, 715 (1982).
42. J. W. Lewis, M. G. Tordoff, J. E. Sherman and J. C. Liebeskind, *Science* **217**, 557 (1982).
43. J. W. Holaday and A. I. Faden, *Nature, Lond.* **275**, 450 (1978).
44. N. J. Gurll, D. G. Reynolds, T. Vargish and R. Lechner, *J. Pharmac. exp. Ther.* **220**, 621 (1982).
45. L. D. Fricker, T. H. Plummer and S. H. Snyder, *Biochem. biophys. Res. Commun.* **111**, 994 (1983).
46. D. Regoli and J. Barabe, *Pharmac. Rev.* **32**, 1 (1980).