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Membrane-Bound Kidney Neutral Metalloendopeptidase: Interaction with Synthetic Substrates, Natural Peptides, and Inhibitors†

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ABSTRACT: A neutral metalloendopeptidase with a thermolysin-like specificity was purified to apparent homogeneity, from the particulate fraction of rabbit kidney homogenates. After preparration of a deoxycholate extract, the enzyme was released from membranes by papain treatment and separated from other membrane-bound enzymes including dipeptidyl aminopeptidase IV, aminopeptidase M, and γ-glutamyl transpeptidase by chromatography on Sephadex G-200, phenyl-Sepharose, and carboxymethylcellulose columns. The isolated enzyme has a molecular weight of about 95 000 and is inhibited by thiols, metal chelators, phosphoramidon, and thiorphan. It is apparently identical with the kidney neutral metalloendopeptidase (EC 3.4.24.11) [Kerr, M. A., & Kenny, A. J. (1974) *Biochem. J. 137*, 447–488] and similar to the bovine pituitary metalloendopeptidase [Orlowski, M., & Wilk, S. (1981) *Biochemistry 20*, 4942–4950] and to an enzyme designated as "enkephalinase". Studies with a series of synthetic substrates showed that the enzyme preferentially cleaves bonds in which the amino group is provided by a hydrophobic amino acid residue. Several biologically active peptides such as methionine and leucine enkephalin, dynorphin, bradykinin, and angiotensin I are degraded by cleavage of the same type of bond. The endopeptidase acts as a dipeptidyl carboxypeptidase on peptides having a hydrophobic residue in the penultimate position. *N*-[1(*R*,*S*)-Carboxy-2-phenylethyl] derivatives of phenylalanyl- and alanyl-*p*-aminobenzoate were synthesized and tested as potential inhibitors. The two diastereomers of *N*-[1(*R*,*S*)-carboxy-2-phenylethyl]phenylalanyl-*p*-aminobenzoate were separated by high-pressure liquid chromatography; the more potent isomer had a K_I of 2.9×10^{-8} M. The inhibitory potency of the alanyl derivatives was lower by almost 2 orders of magnitude. The data indicate that, as with thermolysin, a hydrophobic residue in the P_1' position and the carboxylate group complexing with the active site zinc atom account for the inhibitory action of these derivatives.

We have previously reported the purification from bovine pituitaries of a membrane-bound metalloendopeptidase with a specificity directed toward peptide bonds in which the amino group is contributed by hydrophobic amino acid residues (Orlowski & Wilk, 1981). Subsequent studies in our laboratory have shown that this thermolysin-like endopeptidase is apparently identical with "enkephalinase", an enzyme associated with brain membrane fractions, which cleaves the Gly–Phe bond in Met-enkephalin (Tyr-Gly-Gly-Phe-Met) and in Leu-enkephalin (Tyr-Gly-Gly-Phe-Leu) (Almenoff et al., 1981). There is a growing interest in enzymes that degrade enkephalins and other biologically active peptides, since their action may control the function of these potent substances. Furthermore, inhibition of enkephalin degradation could induce morphinomimetic effects of potential pharmacological importance (Roques et al., 1980). Knowledge, therefore, of the specificity and mechanism of action of peptide metabolizing enzymes has both theoretical and practical significance.

We have previously reported that the pituitary enzyme bears resemblance to a neutral metalloendopeptidase (EC 3.4.24.11) isolated by Kerr & Kenny (1974a) from kidney brush border fractions. Recent work by these authors suggests that this enzyme, like the bovine pituitary metalloendopeptidase, is similar to brain enkephalinase (Fulcher et al., 1982).

The specific activity of the metalloendopeptidase in brain and pituitary is very low, making it difficult to isolate the enzyme from these tissues and impeding studies on its properties, specificity, and mechanism of action. This report describes a convenient procedure for isolation of the enzyme from rabbit kidney, a study of its specificity toward synthetic and natural peptides, and the effect of various inhibitors on enzyme activity. New carboxymethyl derivatives of Phe-pAB[1] were

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synthesized and shown to act as potent inhibitors with a K_I in the nanomolar range. The data suggest a similarity in the mechanism of inhibition of kidney metalloendopeptidase to that of thermolysin and angiotensin converting enzyme.

Experimental Procedures

Materials. Sodium phenylpyruvate, sodium cyanoborohydride, *tert*-butoxycarbonyl derivatives of amino acids, *N*,*N'*-dicyclohexylcarbodiimide, *N*-hydroxysuccinimide, trifluoroacetic acid, triethylamine, hippuric acid, glutathione, dithiothreitol, 2-mercaptoethanol, *p*-mercuribenzoate, iodoacetamide, iodoacetic acid, *N*-ethylmaleimide, papain, antipain, chymostatin, EDTA , *o*-phenanthroline, L-Leu-pNA, and all dipeptides were obtained from Sigma Chemical Co. (St. Louis, MO). *p*-Aminobenzoic acid was obtained from Aldrich Chemical Co. (Milwaukee, WI). DFP was obtained from Calbiochem-Behring Corp. (La Jolla, CA). Silica gel plates (13181) for thin-layer chromatography were obtained from Eastman Kodak Co. (Rochester, NY). Sephadex G-200 and phenyl-Sepharose CL-4B were obtained from Pharmacia, Inc. (Piscataway, NJ). Aminopeptidase M (EC 3.4.11.2) was obtained from Boehringer/Mannheim Inc. (Indianapolis, IN). Glutaryl-Ala-Ala-Phe-4MeO2NA was obtained from Enzyme Systems Products (Livermore, CA). Hip-Arg-Arg-Leu-2NA, Hip-Arg-Arg-Ala-2NA, Hip-Arg-Arg-Gly-2NA, and glutaryl-Ala-Ala-Phe-2NA were synthesized as previously described (Orlowski & Wilk, 1981). Thiorphan was obtained as a gift from Dr. J. C. Schwartz, Unite de Neurobiologie, 75014 Paris, France. A 0.4 mM solution was freshly prepared with 2 mM DTT and diluted several-thousand-fold for inhibition studies. At these concentrations, DTT does not inhibit the enzyme. Gly-Pro-2NA, which was used to determine dipeptidyl aminopeptidase IV (EC 3.4.14.-) was obtained from Bachem (Torrance, CA). γ-Glutamyl-pNA was synthesized and used to determine γ-glutamyl transpeptidase (EC 2.3.2.2) as described (Orlowski & Meister, 1965).

Synthetic Procedures. Peptides were synthesized by using *N*-hydroxysuccinimide esters of Boc-amino acids (Anderson et al., 1964) in the formation of the peptide bond, as described in a previous publication (Orlowski & Wilk, 1981). The purity of the compounds was determined by amino acid analysis, TLC, and HPLC.

N-Carboxymethyl derivatives of amino acid amides of pAB were prepared by reductive amination of phenylpyruvate with either Phe-pAB or Ala-pAB according to a modification of the method described by Patchett et al. (1980). ^{1}H NMR was used to confirm the structure of the diastereomers in addition to HPLC and TLC, which were used to determine purity. A Perkin-Elmer Series 2 liquid chromatograph equipped with a variable-wavelength detector was used. Emerging peaks were monitored at 210 nm. Samples were separated on a reverse-phase C_{18} column (4.5 × 250 mm, 5 μm; IBM) by elution with a linear gradient established between KH_2PO_4 (0.05 M, pH 2) and acetonitrile. The starting acetonitrile concentration was 20%, and its concentration was increased at 1%/min at a flow rate of 1 mL/min. Amino acid analysis of peptides and the products of their acid and enzymatic hydrolysis was done as previously described (Orlowski & Wilk, 1981). Ascending TLC was done on silica gel plates with fluorescent indicator in chloroform–methanol (60:40). Compounds were visualized by examination plates in a viewing box equipped with a short-wave UV light source.

Melting points are uncorrected. Analyses for carbon, hydrogen, and nitrogen were carried out by Schwartzkopf Microanalytical Laboratory (Woodside, NY) and the Microanalytical Service of the Rockefeller University (New York, NY).

Phe-pAB. *N*-Boc-Phe *N*-hydroxysuccinimide ester (5.5 mmol), prepared according to Anderson et al. (1964), was reacted in THF with pAB (5 mmol) in the presence of 1-hydroxybenzotriazole (5 mmol). The mixture was stirred overnight at room temperature after which time the solvent was removed by flash evaporation. The remaining oil was dissolved in chloroform. The product crystallized from the solution within several hours. The crystals were collected on a sintered glass funnel, washed with chloroform, and dried. Treatment with CF_3CO_2H and ether yielded the CF_3CO_2H salt of Phe-pAB (61% yield). The salt can be converted to the free amide by dissolving it in ethanol and adding an equivalent amount of triethylamine. Phe-pAB precipitates within minutes, after which time it is washed with ethanol and dried: single peak on HPLC with a retention time of 9.2 min; mp 250 °C dec.

Hip-Phe-pAB. A total of 0.5 mmol of Phe-pAB was dissolved in THF, along with an equivalent amount of triethylamine. Hippuryl *N*-hydroxysuccinimide ester (0.525 mmol) was added and the mixture stirred for 30 min, after which time an additional 0.3 mmol of triethylamine was added. After 24 h, the THF was flash evaporated; methylene chloride was then added, and the product readily crystallized. The crystals were filtered and washed with methylene chloride and dried (81% yield): HPLC, single peak with a retention time of 25.0 min; amino acid analysis, Phe 1.09, Gly 1.0; mp 242 °C dec. Anal. Calcd for $C_{25}H_{23}N_3O_5 \cdot H_2O$: C, 64.79; H, 5.44; N, 9.07. Found: C, 65.00; H, 5.18; N, 8.81.

Glutaryl-Phe-pAB. One millimole of Phe-pAB was dissolved in THF with an equivalent amount of triethylamine. The mixture was stirred in ice, and 3 mmol of glutaric anhydride was slowly added. After 1 h, the solvent was flash evaporated. The product crystallized from chloroform: HPLC, single peak with a retention time of 19.6 min.

Acetyl-Phe-pAB. A total of 1 mmol of Phe-pAB was dissolved in THF with an equivalent amount of triethylamine. The mixture was stirred in ice, and 3 mmol of acetic anhydride was slowly added. After 1 h, the solvent was flash evaporated, and the product was obtained as an amorphous white powder. It was then washed on a filter with diethyl ether: HPLC, single peak with a retention time of 17.2 min; mp 232 °C dec.

Glutaryl-Gly-Phe-pAB. A total of 4.2 mmol of Phe-pAB was dissolved in THF and reacted with *N*-Boc-Gly *N*-hydroxysuccinimide ester (5 mmol) in the presence of 5 mmol of both 1-hydroxybenzotriazole and triethylamine. After 24 h, the solvent was evaporated, and the residue was dissolved in chloroform and washed twice with $NaHSO_4$ (0.02 M, pH 2.6) and twice with water. (The water phase was reextracted each time with a small amount of chloroform, which was then combined with the chloroform layer.) The organic phase was dried with anhydrous sodium sulfate and the solvent removed in vacuo. The residue was then treated with CF_3CO_2H and ether to yield $CF_3CO_2H \cdot$Gly-Phe-pAB, which was dried in a vacuum desiccator. A total of 0.5 mmol of the dried compound

[1] Abbreviations: Boc, *tert*-butoxycarbonyl; Hip, hippuryl; 2NA, 2-naphthylamide or 2-naphthylamine; pNA, *p*-nitroanilide or *p*-nitroaniline; 4MeO2NA, 4-methoxy-2-naphthylamide; pAB, *p*-aminobenzoic acid; DFP, diisopropyl fluorophosphate; $NaDodSO_4$, sodium dodecyl sulfate; TLC, thin-layer chromatography; HPLC, high-pressure liquid chromatography; CF_3CO_2H, trifluoroacetic acid; DMF, dimethylformamide; THF, tetrahydrofuran; DCC, *N*,*N'*-dicyclohexylcarbodiimide; EDTA, ethylenediaminetetraacetic acid; Bz, benzoyl; Tris, tris(hydroxymethyl)aminomethane; $NaDodSO_4$, sodium dodecyl sulfate.

was dissolved without further purification in THF with 1 equiv of triethylamine. The mixture was stirred in ice, and DMF was added dropwise to keep the compound in solution. An equivalent amount of glutaric anhydride was slowly added, and after 1 h, the solvent was removed. The oily residue was dissolved in chloroform from which the product crystallized: HPLC, single peak with a retention time of 15.2 min; amino acid analysis, Gly 1.1, Phe 1.0; mp 170 °C dec.

N-[1(R,S)-Carboxy-2-phenylethyl]-Phe-pAB was prepared by reductive amination of phenylpyruvate with Phe-pAB (Patchett et al., 1980). Phe-pAB (3.5 mmol) and sodium phenylpyruvate (17.6 mmol) were dissolved in 25 mL of water, and the pH was adjusted to 7.8 with 5 N NaOH. Sodium cyanoborohydride (14.8 mmol) was dissolved in 10 mL of water and added over a 5-h period with a syringe pump. After being stirred overnight at room temperature, the mixture was acidified under the hood to pH 2 with 6 N HCl. The precipitate was collected by filtration and washed with water. The dried material was then suspended in warm ethyl acetate, and the insoluble white solid was isolated by filtration. The material was dissolved in methanol after addition of triethylamine. Addition of CF_3CO_2H induced crystallization of a mixture of two diastereomeric products (49% yield): HPLC, two peaks with retention times of 21 (peak I) and 23.5 (peak II) min; TLC, two spots with R_f values 0.66 and 0.40. Anal. Calcd for $C_{25}H_{24}N_2O_5 \cdot 0.5H_2O$: C, 68.02; H, 5.71; N, 6.35. Found: C, 68.27; H, 5.89; N, 6.58.

The two diastereomers were separated by preparative HPLC on two joined C_{18} μBondapak columns (7.8 × 30 cm) with a Waters Associates HPLC apparatus, by elution with a linear gradient, estabilished between 0.28% triethylamine in water adjusted to pH 3.45 with trifluoroacetic acid and acetonitrile. The starting acetonitrile concentration was 10%, and its concentration was increased at a rate of 0.43%/min with a flow rate of 2.5 mL/min. A total of 10 mg of the diastereomeric mixture was injected per run, and emerging peaks were monitored at 285 nm. The two peaks, each of which contained a single diastereomer, were collected and evaporated under vacuum. Each compound was washed with tetrahydrofuran and ether and then dried at 100 °C in a vacuum over calcium sulfate pellets for 24 h. Peak I showed the following: TLC, one spot, with R_f 0.66; mp 257–260 °C dec; NMR (Me_2SO-d_6, δ downfield from Me_4Si) δ (AB quartet) 7.8–7.3 (J_{AB} = 8.7 Hz, 4 H, pAB), δ (aromatic) 7.2, 7.0 (10 H), δ (aliphatic) 2.9 (6 H, m). Peak II showed the following: TLC, one spot with R_f 0.40; mp 248 °C dec; NMR (Me_2SO-d_6, δ downfield from Me_4Si) δ (AB quartet) 7.9–7.6 (J_{AB} = 8.7 Hz, 4 H, pAB), δ (aromatic) 7.2, 7.1 (10 H), δ (aliphatic) 2.8 (6 H, m).

N-[1(R,S)-Carboxy-2-phenylethyl]-Ala-pAB was prepared by reductive amination of phenylpyruvate with Ala-pAB as described above. After acidification to pH 2, the filtered precipitate was suspended in ethyl acetate, placed on a sintered glass filter, and washed extensively with ethyl acetate. A white solid was obtained (51% yield): HPLC, two peaks of equal height with retention times of 9.2 and 10.4 min; TLC, two spots with R_f values of 0.52 and 0.33; mp 220 °C. Anal. Calcd for $C_{19}H_{20}N_2O_5 \cdot H_2O$: C, 60.95; H, 5.92; N, 7.48. Found: C, 61.64; H, 5.51; N, 7.46.

Determination of Enzyme Activities. Metalloendopeptidase activity was determined either with Hip-Arg-Arg-Leu-2NA or glutaryl-Ala-Ala-Phe-2NA in a coupled enzyme assay, in the presence of an excess of aminopeptidase M (EC 3.4.11.2). With glutaryl-Ala-Ala-Phe-2NA as substrate, the reaction proceeds as follows:

$$\text{glutaryl-Ala-Ala-Phe-2NA} \xrightarrow{\text{metalloendopeptidase}} \text{glutaryl-Ala-Ala} + \text{Phe-2NA} \quad (1)$$

$$\text{Phe-2NA} \xrightarrow{\text{aminopeptidase M}} \text{Phe} + \text{2NA} \quad (2)$$

In the first reaction the endopeptidase cleaves the bond on the amino side of the phenylalanine residue, releasing Phe-2NA, which is subsequently cleaved in the second reaction by the action of aminopeptidase M. The 2-naphthylamine released in this reaction was determined by a modification (Goldbarg & Rutenburg, 1958) of the diazotization procedure of Bratton & Marshall (1939) or by the procedure of Barrett (1972). The pAB released by hydrolysis of pAB containing peptides was also determined by the diazotization procedure of Bratton & Marshall (1939).

The conditions of the assay were the same as those described by Orlowski & Wilk (1981). Enzyme activity is expressed in units, one unit being defined as the amount of enzyme catalyzing the release of 1 μmol of Leu-2NA from Hip-Arg-Arg-Leu-2NA per h. Specific activity is in terms of units per milligram of protein as determined by the methods of Warburg & Christian (1942).

Commercial preparations of aminopeptidase obtained from various suppliers are usually contaminated to varying degrees with a metalloendopeptidase activity, with a specificity apparently similar to the enzyme described in the present work. It was found that this contamination could be removed by hydrophobic chromatography on a phenyl-Sepharose column. This was carried out by adding a saturated solution of ammonium sulfate in 0.035 M Tris-HCl buffer (pH 7.6) to a solution of 25 mg of aminopeptidase M (Boehringer/Mannheim) to achieve a final saturation of 30% with respect to ammonium sulfate. The solution was applied to the top of a phenyl-Sepharose CL-4B column (15 mL) that had been previously washed with 30 mL of a 30% saturated ammonium sulfate solution in 0.05 M Tris-HCl buffer (pH 7.6). The column was washed with an additional 30 mL of the same solution, and the enzyme was eluted with a linear gradient established between 150 mL of 30% saturated ammonium sulfate in 0.035 M Tris-HCl (pH 7.4) and 150 mL of 0.05 M Tris-HCl (pH 7.6). Fractions of about 3 mL were collected and tested for aminopeptidase activity with L-Leu-pNA as substrate and for metalloendopeptidase activity with Hip-Arg-Arg-Leu-2NA as described above. A virtually complete separation of aminopeptidase from the contaminating metalloendopeptidase was achieved, with the latter enzyme eluting after the aminopeptidase. The fractions containing the aminopeptidase were pooled and dialyzed against 0.05 M Tris-HCl buffer (pH 7.4). The protein concentration was adjusted to about 1 mg/mL, and the obtained solution was used in the experiments described here.

Aminopeptidase M (Pfleiderer et al., 1964) and dipeptidyl aminopeptidase IV (McDonald & Schwabe, 1977) activities were determined with Leu-pNA and Gly-Pro-2NA, respectively. Both enzymes were assayed in 0.05 M Tris-HCl (pH 7.6) at a substrate concentration of 0.4 mM. γ-Glutamyl transpeptidase activity was determined as described previously (Orlowski & Meister, 1965).

Polyacrylamide Gel Electrophoresis. Disc gel electrophoresis was performed in 8% gels under dissociating (0.1% $NaDodSO_4$) and nondissociating conditions in 0.05 M Tris-HCl (pH 8.4) according to the method of Weber & Osborn (1969). A total of 5–20 μg of protein was layered onto each gel, and a current of 5 mA/tube was applied. Gels were either stained for protein with Coomassie blue or sliced into 2-mm

Table I: Summary of Purification of Metalloendopeptidase from Rabbit Kidney[a]

purification step	vol (mL)	protein (mg/mL)	activity units/mL	activity total	sp act. (units/mg)	recovery (%)	purification (x-fold)
(1) deoxycholate extract	260	13.8[b]	138	35 880	10	100	1
(2) papain treatment and Sephadex filtration	238	0.176	60.0	14 280	341	40	34
(3) phenyl-Sepharose chromatography	67	0.170	178	11 930	1050	33	105
(4) carboxymethylcellulose chromatography	56	0.074	120	6 720	1620	19	162

[a] For details and definition of units, see Experimental Procedures. [b] Protein was determined for this step by the method of Lowry et al. (1951).

sections for determination of enzyme activity. Each slice was incubated in 0.3 mL of 0.05 M Tris-HCl (pH 7.65) overnight at 4 °C, and 0.025-mL aliquots were then assayed for metalloendopeptidase activity as described above.

Purification of Enzyme. The enzyme was purified from 100 g of frozen rabbit kidneys by a modification of the method of Orlowski & Wilk (1981). A deoxycholate extract of the particulate fraction was prepared as described, and after streptomycin precipitation, the supernatant was collected (step 1) and concentrated in an ultrafiltration cell (Amicon) to 66 mL. For each 25 mg of protein in solution, 1 mg of papain was added, followed by solid dithiothreitol to a final concentration of 5 mM. The mixture was incubated at 37 °C for 90 min, centrifuged for 10 min at 3000g, and applied to a Sephadex G-200 column (5 × 90 cm) equilibrated with 0.05 M Tris-HCl (pH 7.6). The column was eluted with the same buffer, and fractions of about 20 mL were collected. The enzyme emerged from the column in a single peak that was completely separated from dipeptidyl aminopeptidase IV (M_r 230 000), an abundant microvillus protein. Fractions containing metalloendopeptidase activity were pooled and concentrated by ultrafiltration to about 9 mL. A total of 6 mL of a saturated ammonium sulfate solution was added to the enzyme concentrate to yield 15 mL of a solution in 0.035 M Tris-HCl (pH 7.6) that was 40% saturated with respect to ammonium sulfate. This was applied to a phenyl-Sepharose column (25 mL) that was equilibrated with a 40% saturated ammonium sulfate solution in 0.035 M Tris-HCl (pH 7.6). The column was washed with 50 mL of the same buffer, and the enzyme was then eluted with a linear gradient established between 150 mL of the equilibrating buffer and 150 mL of 0.05 M Tris-HCl (pH 7.6). Fractions of 3 mL were collected and assayed for metalloendopeptidase activity (step 3). The enzyme was eluted from the column after about 200 mL of the eluting buffer passed through the column and was well separated from an aminopeptidase activity that emerged in subsequent fractions. Active fractions were pooled, concentrated to about 30 mL, and then dialyzed against two changes (4 L each) of 0.01 M sodium acetate buffer (pH 5.0). The enzyme was then applied to a carboxymethylcellulose column (CM-52, 7 mL) equilibrated with 0.01 M sodium acetate buffer (pH 5) and washed with 100 mL of this buffer. The enzyme was eluted with a gradient established between 150 mL of 0.01 M sodium acetate buffer (pH 5.0) and 150 mL of 0.01 M sodium acetate buffer containing 0.2 M NaCl. Fractions of 3 mL were collected and assayed for enzyme activity. The enzyme eluted as a single peak; active fractions were adjusted to pH 7.5 with 1 M Tris base.

Results

A summary of the purification procedure is shown in Table I. Starting with the streptomycin-treated deoxycholate extract, the enzyme was purified 160-fold with a yield of approximately 19%. Like the pituitary metalloendopeptidase, the kidney enzyme was effectively released from the membranes by treatment with deoxycholate and papain. Papain (M_r 23 000) was completely separated from the metalloendopeptidase by chromatography on Sephadex G-200 as previously described (Orlowski & Wilk, 1981). A highly purified preparation was obtained after chromatography on phenyl-Sepharose (step 3); however, the enzyme eluted in a peak that coincided with the peak of γ-glutamyl transpeptidase activity. The two enzymes were separated in step 4. In 0.01 M sodium acetate (pH 5.0), metalloendopeptidase bound to carboxymethylcellulose, while most of the transpeptidase eluted with the starting buffer. The small amount of transpeptidase that bound to CM-52 under these conditions was eluted at the beginning of the sodium chloride gradient and was thus separated from the metalloendopeptidase, which was eluted in later fractions.

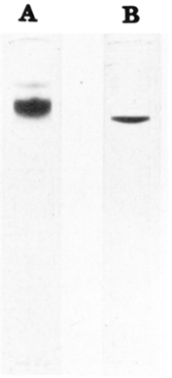


FIGURE 1: Polyacrylamide gel electrophoresis of kidney metalloendopeptidase in 8% gels: (A) nondissociating conditions (both bands have metalloendopeptidase activity); (B) dissociating conditions (0.1% $NaDodSO_4$).

Electrophoresis of the enzyme carried out under nondissociating conditions revealed one major and one minor protein band (Figure 1A). Unstained duplicate gels were sliced into 2-mm sections and assayed with Hip-Arg-Arg-Leu-2NA and glutaryl-Ala-Ala-Phe-2NA; regions corresponding to both protein bands were found to contain metalloendopeptidase activity that was proportional to the intensity of the Coomassie blue staining. Electrophoresis under dissociating conditions (0.1% $NaDodSO_4$) revealed a single sharp band (Figure 1B). Treatment of these samples with 2% and 5% 2-mercaptoethanol gave a major band with a molecular weight of about 94 000 and a faint band with a molecular weight of approximately 85 000.

Given that the enzyme was released from membranes by papain digestion, it is possible that the faint band seen under reducing conditions may represent a fragment of the main polypeptide chain, which was generated by limited proteolysis during papain treatment. Similar results have been reported for other membrane-bound enzymes prepared by proteolytic digestion (Kenny et al., 1976). The diffuse quality of both active bands seen under nondissociating conditions (Figure 1A), which in the presence of $NaDodSO_4$ migrates as a single

Table II: Effect of Inhibitors on Enzyme Activity[a]

inhibitor	final concn (mM)	inhibition (%)
leupeptin	0.023	0
antipain	0.013	0
chymostatin	0.013	0
DFP	0.11	0
p-mercuribenzoate	0.10	3
iodoacetamide	1.0	0
iodoacetic acid	1.0	0
N-ethylmaleimide	1.0	0
pepstatin	0.067	35
glutathione	1.0	76
dithiothreitol	0.4	88
	1.0	98
EDTA	1.0	70
o-phenanthroline	0.08	47
	0.2	73
2-mercaptoethanol	2.0	39

[a] Activity was determined with Hip-Arg-Arg-Leu-2NA as described under Experimental Procedures. The enzyme was not preincubated with the inhibitors with the exception of DFP and EDTA, which were preincubated with the enzyme at 37 °C for 20 min before addition of substrate. Controls in which the enzyme was preincubated in the absence of these inhibitors were included. At the concentrations tested, none of the inhibitors interfered with the aminopeptidase coupling reaction.

sharp band (Figure 2B), suggests that the enzyme is comprised of charge isomers.

The molecular weight of the enzyme was determined by gel filtration on a Sephadex G-200 column according to the method of Andrews (1965). Enzyme activity eluted as one peak with an apparent molecular weight of 98 000. These data, in conjunction with the NaDodSO$_4$ gels, suggest that the enzyme is a monomer with a molecular weight of about 95 000.

The pH optimum of the metalloendopeptidase for Hip-Arg-Arg-Leu-2NA (0.4 mM) was determined in 0.2 M Tris-HCl buffers, in the pH range between 7.0 and 8.8. A broad optimum between pH 7.5 and 8.0 was found, and enzyme activity was routinely determined at pH 7.6.

The effect of various inhibitors on enzyme activity is summarized in Table II. Leupeptin, antipain, and chymostatin, transition-state aldehyde inhibitors of several thiol and serine proteases, have no effect on activity. That neither DFP, an irreversible serine protease inhibitor, nor any of the thiol blocking agents tested affected activity further indicates that the enzyme is neither a serine nor a thiol protease. Although moderate inhibition was noted with pepstatin, the concentrations required for inhibition (35% inhibition at 0.067 mM pepstatin) are by several orders of magnitude greater than those effectively inhibiting carboxyl proteases. Thus, for example, pepsin is inhibited with a K_I of about 10^{-10} M (Kunimoto et al., 1974), and cathepsin D, another carboxyl protease, is inhibited with a similar K_I value (Knight & Barrett, 1976). We conclude, therefore, that the inhibition of the metalloendopeptidase is apparently due to nonspecific interaction of the enzyme with hydrophobic groups in the inhibitor. By contrast, the enzyme was inhibited by all thiols and metal-chelating agents, in a manner similar to the inhibition of the pituitary metalloendopeptidase by the same reagents.

The specificity of the enzyme toward various synthetic naphthylamides is summarized in Table III. The steady-state parameters K_m and k_{cat} ($=V/e$, where e = total enzyme concentration) were obtained from initial velocity measurements by a non-linear-regression program in which substrate concentration and velocity are fit to the Michaelis–Menten equation (Baing & Reid-Miller, 1980). Strict Michaelis–Menten kinetics were observed for all substrates within the concentration ranges indicated. These data are consistent with a primary specificity directed toward bonds in which the amino group is provided by a hydrophobic amino acid residue (position P_1').[2] Replacement of the hydrophobic amino acid

Table III: Kinetic Parameters for Hydrolysis of Several Peptide Naphthylamides[a]

P_3- P_2- P_1- P_1'- P_2'	[S][b] (mM)	K_m[c] (mM)	k_{cat}[d] (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
(1) Bz-Gly-Arg-Arg-Leu-2NA	0.08–0.4	0.18 ± 0.2	62.2	3.46×10^5
(2) Bz-Gly-Arg-Arg-Ala-2NA	0.10–1.2	0.97 ± 0.03	31.8	3.28×10^4
(3) Bz-Gly-Arg-Arg-Gly-2NA	0.2–0.4		negligible hydrolysis[e]	
(4) glutaryl-Ala-Ala-Phe-2NA	0.1–0.8	0.27 ± 0.02	73.4	2.72×10^5
(5) glutaryl-Ala-Ala-Phe-4MeO2NA	0.1–0.6	0.23 ± 0.02	111.0	4.83×10^5

[a] Enzyme activity was determined as described under Experimental Procedures. [b] Range of substrate concentrations used for the determination of K_m. [c] Data are mean values ±SE of four determinations. [d] Calculations based on a molecular weight of 95 000. [e] Represents less than 1% of the rate for Hip-Arg-Arg-Leu-2NA at a concentration of 0.4 mM.

Table IV: Degradation of Biologically Active Peptides by Kidney Neutral Metalloendopeptidase[a]

peptide	structure	products found
Met-enkephalin	Tyr-Gly-Gly-↓Phe-Met	Tyr-Gly-Gly, Phe-Met
Met-enkephalinamide	Tyr-Gly-Gly-↓Phe-Met-NH_2	Tyr-Gly-Gly, Phe-Met-NH_2
Leu-enkephalin	Tyr-Gly-Gly-↓Phe-Leu	Tyr-Gly-Gly, Phe-Leu
dynorphin	Tyr-Gly-Gly-↓Phe-Leu-Arg-Arg-↓Ile-Arg-Pro-Lys-Leu-Lys	Tyr-Gly-Gly, Tyr-Gly-Gly-Phe-Leu-Arg-Arg, Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys, Ile-Arg-Pro-Lys-Leu-Lys, Phe-Leu-Arg-Arg
bradykinin	Arg-Pro-Pro-Gly-↓Phe-Ser-Pro-↓Phe-Arg	Arg-Pro-Pro-Gly, Phe-Arg, Phe-Ser-Pro
angiotensin I	Asp-Arg-↓Val-Tyr-↓Ile-His-Pro-↓Phe-His-Leu	Asp-Arg-Val-Tyr, Ile-His-Pro, Asp-Arg, Val-Tyr, Asp-Arg-Val-Tyr-Ile-His-Pro, Phe-His-Leu

[a] Reaction mixtures contained substrate (0.8–1.0 mM), Tris-HCl buffer (0.04 M, pH 7.6), and enzyme (1 unit) in a final volume of 0.25 mL. All incubations were for 2–4 h. Products of the reaction were separated on the Perkin-Elmer HPLC system described under Experimental Procedures and identified as previously described (Almenoff et al., 1981). In these experiments, the initial acetonitrile concentration was 10%, except for the dynorphin experiment in which the initial acetonitrile concentration was 3%.

leucine (substrate 1) with an alanine residue (substrate 2) decreased the k_{cat} by a factor of 2 and the specificity constant (k_{cat}/K_m) by a factor of 10. Introduction of glycine (substrate 3) in this position was associated with negligible hydrolysis.

High k_{cat} values and specificity ratios were also observed with substrates 4 and 5, in which the P_1 and P_2 positions are occupied by alanine residues and P_3 by a glutaryl group. Although the enzyme will hydrolyze bonds on the N-terminal side of alanyl residues (substrate 2), no such cleavages were observed for substrates 4 and 5. No free alanine was detected in incubation mixtures containing enzyme, aminopeptidase, and substrates 4 and 5. Therefore, rapid cleavage of the Ala–Phe bond renders the glutaryl-Ala-Ala peptide unsusceptible to hydrolysis.

The primary specificity of the enzyme directed toward bonds on the amino side of hydrophobic amino acid residues was also evident when its action on several biologically active peptides was studied. The results summarized in Table IV show that the enzyme has enkephalinase activity in that it cleaves both Met- and Leu-enkephalin at the Gly–Phe bond. Met-enkephalinamide is also hydrolyzed at the same bond, indicating that a free carboxyl group at the C terminus of the enkephalin molecule is not a requirement for activity. This finding is similar to that observed with the pituitary metalloendopeptidase (Almenoff et al., 1981) and indicates that the enkephalinase activity of the enzyme is that of an endopeptidase, rather than that of a dipeptidyl carboxypeptidase.

The enzyme also degrades dynorphin, a Leu-enkephalin-containing opioid peptide, by hydrolyzing bonds on the amino side of hydrophobic residues Phe^4 and Ile^8. Cleavage proceeds preferentially at the Arg^7–Ile^8 bond with the formation of an N-terminal heptapeptide and a C-terminal hexapeptide. Longer incubation results in complete hydrolysis of the Gly^3–Phe^4 bond. It is this cleavage that terminates the opioid activity of dynorphin (Chavkin & Goldstein, 1981).

Bradykinin is hydrolyzed at the Pro^7–Phe^8 and Gly^4–Phe^5 bonds, again showing that the primary specificity is directed toward bonds on the amino side of hydrophobic residues. The hydrolysis of the Pro^7–Phe^8 bond also shows that the enzyme can act as a dipeptidyl carboxypeptidase on peptides with hydrophobic residues in the penultimate position.

Angiotensin I is rapidly hydrolyzed at the Pro^7–Phe^8 bond to form Phe-His-Leu and Asp-Arg-Val-Tyr-Ile-His-Pro. The latter peptide is further degraded at the Tyr^4–Ile^5 bond, and the resulting tetrapeptide is slowly cleaved at the Arg^2–Val^3 bond. This thermolysin-like pattern of cleavage is distinctly different from the activity of the angiotensin converting enzyme, which hydrolyzes Angiotensin I at the Phe^8–His^9 bond to form angiotensin II, a potent vasoconstrictor.

Although dipeptides are not substrates, the results in Table V show the phenylalanyl, leucyl, and even alanyl dipeptides are inhibitory. By contrast, dipeptides such as Gly-Gly, Gly-Val, and Gly-Leu, with a glycine residue on the amino side, do not inhibit at concentrations as high as 2 mM. Free phenylanine was also without effect at these concentrations.

On the basis of the specificity studies described in the preceding section, the inhibitory dipeptides, all of which contain a hydrophobic residue on the amino side, can be regarded as potential products of the metalloendopeptidase-catalyzed reaction. This interpretation is based on the finding that the enzyme acts as a dipeptidyl carboxypeptidase on those peptides having a hydrophobic residue in the penultimate position. It may therefore be assumed that the inhibitory dipeptides act by binding to the S_1' and S_2' subsites of the enzyme. By contrast, the noninhibitory dipeptides having a glycine residue on the amino side lack the ability to bind to these sites. The hydrophobicity of the amino acid residue on the carboxyl side of the dipeptide, as opposed to the residue on the amino side, does not seem to be a factor in determining inhibitory potency. For example, Phe-Gly, Phe-Phe, and Phe-Leu all inhibited to a similar extent, while the inhibition obtained with Phe-Ala was much greater. A free carboxyl group in the dipeptide seems to be of importance for inhibition, since the amide of Phe-Leu showed little inhibition in comparison with Phe-Leu.

Table V: Inhibition of Metalloendopeptidase by Dipeptides and Peptide Derivatives of *p*-Aminobenzoate[a]

peptide	$IC_{50} \times 10^{-4}$ [b] (M)
(1) Gly-Gly	negligible inhibition[c]
(2) Gly-Val	negligible inhibition
(3) Gly-Phe	negligible inhibition
(4) Ala-Phe	5.9
(5) Phe-Phe	1.6
(6) Phe-Leu	4.4
(7) Phe-Leu-NH_2	negligible inhibition
(8) Phe-Ala	0.21
(9) Phe-Gly	1.6
(10) Phe	negligible inhibition
(11) Leu-Phe	0.23
(12) Leu-Trp	0.75
(13) Phe-pAB	0.19
(14) acetyl-Phe-pAB	2.5
(15) glutaryl-Phe-pAB	1.0
(16) glutaryl-Gly-Phe-pAB	1.0
(17) Bz-Gly-Phe-pAB	0.18

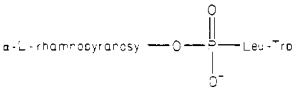
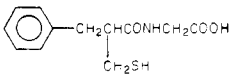
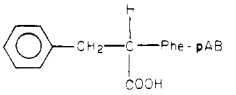
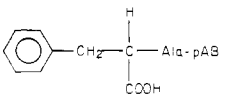
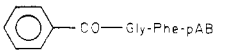
[a] Inhibition studies were done by determining activity toward 0.4 mM glutaryl-Ala-Ala-Phe-2NA in the presence of varying concentrations of inhibitor. For these determinations, the enzyme, dipeptide, and substrate were incubated, and the reaction was terminated by boiling. 2NA was then released by adding 10 μg of aminopeptidase and incubating at 37 °C for 30 min. The aminopeptidase reaction was then stopped by addition of fast garnet (Barrett, 1972). This two-stage procedure was used to prevent degradation of unblocked peptides by aminopeptidase during the course of the endopeptidase reaction. [b] IC_{50} values were obtained with a computer program (Johnson, 1979) that performs iterative curve fits to the parameters % inhibition vs. inhibitor concentration to the logistic equation. Log dose-response curves with slopes close to unity were obtained for most compounds tested. [c] No inhibition was observed at an inhibitor concentration of 2 mM.

It is of interest that replacement of the C-terminal amino acid residues in the phenylalanyl dipeptides by *p*-aminobenzoate yielded the amino acid arylamide Phe-pAB with an inhibitory potency equal to the best of the dipeptides tested (Phe-Ala). Substitution of the amino group of Phe-pAB with an acetyl, a glutaryl, and a glutarylglycyl group diminished the inhibitory action, while a hippuryl group in the same position preserves the inhibitory potency, suggesting that a hydrophobic group in this position (P_1) favors binding.

Acylation of the amino group of Phe-pAB yields compounds that can serve as substrates of the kidney metalloendopeptidase. All four *N*-acyl derivatives of Phe-pAB listed in Table V (peptides 14–17) are hydrolyzed by the enzyme although at greatly different rates. Thus, *N*-acetyl-Phe-pAB (peptide 14) and glutaryl-Phe-pAB (peptide 15) are hydrolyzed about 1000 times slower than glutaryl-Ala-Ala-Phe-2NA (measured at a substrate concentration of 0.4 mM, as described under Experimental Procedures), and the hydrolysis of glutaryl-

[2] The nomenclature of Schechter & Berger (1967) is used to describe the interaction between substrate and enzyme. Amino acid residues and other residues are designated P_1, P_2, P_3, etc. in the N-terminal direction and P_1' etc. in the C-terminal direction from the bond undergoing hydrolysis. The corresponding subsites in the enzyme are identified with the letter S.

Table VI: Inhibition Constants of Kidney Metalloendopeptidase Inhibitors

inhibitor	K_I[a] (M)	structure
(1) phosphoramidon	$(3.4 \pm 0.31) \times 10^{-9}$	
(2) thiorphan (DL-3-mercapto-2-benzylpropanoylglycine)	8.0×10^{-8}[b]	
(3) *N*-[1(*R,S*)-carboxy-2-phenylethyl]-Phe-pAB	$(7.1 \pm 0.66) \times 10^{-8}$	
(4) *N*-(1-carboxy-2-phenylethyl)-Phe-pAB (diastereomer I)	$(2.9 \pm 0.34) \times 10^{-8}$	
(5) *N*-(1-carboxy-2-phenylethyl)-Phe-pAB (diastereomer II)	$(2.4 \pm 0.36) \times 10^{-7}$	
(6) *N*-[1(*R,S*)-carboxy-2-phenylethyl]-Ala-pAB	$(5.3 \pm 0.33) \times 10^{-6}$	
(7) Bz-Gly-Phe-pAB	$(3.0 \pm 0.48) \times 10^{-6}$	

[a] K_I values were determined by using Hip-Arg-Arg-Leu-2NA (0.3–0.7 mM) as substrate and several concentations of inhibitor, according to the method of Dixon (1972). Incubations were for 30 min. Data are mean values ±SE of three to four determinations. [b] This value represents the IC_{50}. For other details see the text.

Gly-Phe-pAB and Bz-Gly-Phe-pAB proceeds at a rate about 65 and 35 times, respectively, slower than that of glutaryl-Ala-Ala-Phe-2NA. The great differences in the rate of hydrolysis of the different peptides could be related to the length of the peptide chain, with the longer peptides being hydrolyzed more rapidly. This suggests the presence of an extended substrate binding site in the enzyme.

The specificity of the kidney metalloendopeptidase, directed toward bonds in which the amino group is contributed by hydrophobic amino acid residues, resembles that of thermolysin, a bacterial metalloendopeptidase. Thermolysin, like other metalloendopeptidases, including angiotensin converting enzyme, and also carboxypeptidases A and B, contains a zinc atom in the active site that interacts with the carbonyl oxygen of the hydrolyzed peptide bond. Many of the synthesized inhibitors of this group of enzymes contain a ligand, which coordinates with the zinc atom in the active site. The ligand is usually attached to a peptide or amino acid capable of interacting with the substrate binding site of the enzyme. Due to the similarities in the mechanism of action of zinc–metalloendopeptidases, an inhibitor of one of these enzymes can inhibit other enzymes of the same group, when modified to conform to their specificity requirements. Thus, for example, D-3-mercapto-2-methylpropanoyl-L-proline (Captopril), a potent inhibitor of angiotensin converting enzyme (Ondetti et al., 1977), was used as a model for the synthesis of thiorphan (Roques et al., 1980), an inhibitor of brain enkephalinase, having a benzylpropanoylglycine group in place of the propanoyl-L-proline group.

We have therefore examined the inhibitory effect of thiorphan and of phosphoramidon, a potent inhibitor of thermolysin, on the activity of kidney metalloendopeptidase. We also have synthesized and tested *N*-(1-carboxy-2-phenylethyl) derivatives of Phe-pAB and Ala-pAB as potential inhibitors of the kidney metalloendopeptidase. It was expected that these derivatives should inhibit the kidney enzyme, since analogous *N*-(1-carboxy-3-phenylpropyl) derivatives of several dipeptides were found to strongly inhibit the angiotensin converting enzyme (Patchett et al., 1980), and a similar derivative of Leu-Trp was shown to be a potent inhibitor of thermolysin (Maycock et al., 1981). Studies by this group on the converting enzyme inhibitors indicate that the *N*-1-carboxylate group contributes significantly to the inhibitory potency.

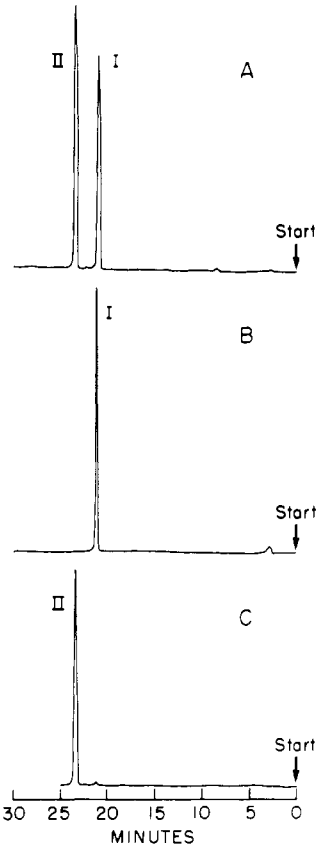


FIGURE 2: HPLC separation of diastereomers of *N*-[1(*R,S*)-carboxy-2-phenylethyl]-Phe-pAB: (A) *N*-[1(*R,S*)-carboxy-2-phenylethyl]-Phe-pAB; (B) diastereomer (peak) I; (C) diastereomer (peak) II.

The results of inhibition studies are summarized in Table VI. Reductive amination of phenylpyruvate with Phe-pAB or Ala-pAB to give the respective *N*-(1-carboxy-2-phenylethyl) derivatives generates a new asymetric carbon, and accordingly, two diastereomers are formed during synthesis. The diastereomers derived from amination of phenylpyruvate with

Phe-pAB were separated by HPLC as described under Experimental Procedures (Figure 2). Their inhibitory action was determined separately and compared with that of the mixture of the two diastereomers. The *N*-(1-carboxy-2-phenylethyl) derivatives of Ala-pAB were examined only as a mixture of the two diastereomers. Due to the low K_I values of some of the inhibitors (see Table VI), the assumption that the concentration of free inhibitor is equal to the total inhibitor concentration ($[I] = [I]_{total}$) is not correct. The graphical method of Dixon (1972), valid for both tight binding and less potent inhibitors, was therefore used for the determination of K_I values. Experiments were done by adding the enzyme to mixtures of substrate and inhibitor. Separate determinations in which the enzyme and inhibitor were preincubated for 15 min at 37 °C before addition of the substrate showed no difference in the extent of inhibition by phosphoramidon, or by any pAB derivatives, thereby ruling out irreversible inhibition and slow binding phenomena (Kam et al., 1979). Inhibition by thiorphan, however, was observed to be potentiated by preincubation. This caused difficulties in attempts to measure reliably K_I values. We report therefore, only an IC_{50} value for this inhibitor as determined in the presence of 0.4 mM substrate. For all compounds tested, inhibition was competitive in that it could be surmounted at high substrate concentrations.

The results in Table VI show that phosphoramidon, a thermolysin inhibitor of bacterial origin, is the most potent of the inhibitors tested and that thiorphan is also highly inhibitory. These results further support the conclusion that the kidney metalloendopeptidase, like the analogous bovine pituitary enzyme, has thermolysin-like properties and that it is also similar to brain enkephalinase. The *N*-carboxymethyl derivatives of Phe-pAB, all show high inhibitory potency. The most active diastereomer (diastereomer I, Table VI) of *N*-(1-carboxy-2-phenylethyl)-Phe-pAB has a K_I of 2.9×10^{-8} M. This K_I is 2 orders of magnitude lower than that of Bz-Gly-Phe-pAB, a compound with an inhibitory potency similar to that of Phe-pAB (see Table V). This indicates that N-alkylation of Phe-pAB by the carboxyphenylethyl group increases the inhibitory potency of Phe-pAB by almost 2 orders of magnitude. It is of interest that diastereomer II was less inhibitory by a factor of 10 and that the mixture of diastereomers had an intermediate potency, suggesting some stereoselectivity of inhibitor binding. A significant finding is that replacement of a phenylalanine residue in the inhibitor by an alanine residue decreased the inhibitory potency by a factor of 100. This indicates that the interaction between the aromatic ring of phenylalanine and a hydrophobic pocket in the S_1' subsite of the enzyme contributes significantly to binding, a conclusion consistent with the results of specificity studies described in a preceding section. A common feature of the potent inhibitors listed in Table VI is the presence of a group capable of interacting with the Zn^{2+} in the active site of the enzyme. This property is shared by the thiol group in thiorphan, the phosphoryl group in phosphoramidon, and the carboxyl group in the *N*-carboxymethyl derivatives of Phe-pAB and Ala-pAB.

Discussion

The characteristics of the kidney metalloendopeptidase described in this paper indicate that this enzyme is apparently identical with that purified by Kerr & Kenny (1974a), from rabbit kidney brush border preparations, and shown to cleave the B chain of insulin, by hydrolyzing bonds involving the amino group of hydrophobic residues. The enzyme also appears to have properties similar to those of a neutral metalloendopeptidase isolated from bovine pituitaries (Orlowski & Wilk, 1981) and also detected in pancreatic membrane preparations (Mumford et al., 1980), in rat brain membrane fractions (Almenoff et al., 1981) and in porcine kidney (Mumford et al., 1981). All of these enzymes are membrane-bound endopeptidases exhibiting a thermolysin-like specificity. All share the common property of being sensitive to inhibition by thiols and by metal chelators and being resistant to inhibition by thiol blocking agents, DFP, and several thiol, serine, and carboxyl protease inhibitors of bacterial origin, such as leupeptin, chymostatin, antipain, and pepstatin. Furthermore, the pituitary and the kidney enzymes have the same molecular weight of about 90 000–95 000 (Kerr & Kenny, 1974b) and seem to be composed of a single polypeptide chain.

Kerr & Kenny (1974a) have reported that the kidney enzyme was firmly bound to the microvillous membranes and resisted solubilization by treatment with papain, Triton X-100, various concentrations of $NaDodSO_4$, and several proteolytic enzymes. Successful solubilization was only obtained after treatment with toluene and a rather prolonged subsequent exposure to trypsin. We have found that the enzyme can be efficiently solubilized after preparation of a deoxycholate extract, removal of excess of deoxycholate with streptomycin sulfate, and a subsequent short (90-min) treatment with papain. The solubilized enzyme can be readily separated from other membrane-bound kidney peptidases on the basis of molecular size, hydrophobic interactions, and charge differences. Thus, chromatography on Sephadex G-200 columns removed dipeptidyl aminopeptidase IV, an enzyme having a molecular weight more than twice that of the metalloendopeptidase (McDonald & Schwabe, 1977); hydrophobic chromatography on phenyl-Sepharose eliminated the contamination by aminopeptidase M; and chromatography on carboxymethylcellulose eliminated the accompanying γ-glutamyl transpeptidase, an enzyme closely associated with kidney brush border membranes (Albert et al., 1961; Orlowski & Meister, 1965).

The specificity of the rabbit kidney metalloendopeptidase studied with peptide naphthylamide substrates synthesized in our laboratory shows close resemblance to the specificity of the enzymes purified from membrane fractions of bovine pituitary and rabbit brain (Orlowski & Wilk, 1981; Almenoff et al., 1981). A hydrophobic residue in the P_1' position is required for activity with all of these enzymes, and replacement of this residue by small neutral amino acids such as alanine or glycine greatly diminishes or virtually eliminates activity. It is of interest that high (k_{cat}/K_m) ratios are obtained with those substrates having either arginine or alanine residues in positions P_1 and P_2. Apparently, the S_1 and S_2 subsites of these enzymes accomodate well either small neutral amino acids or basic amino acid residues. Thus, synthetic chromogenic substrates having a hydrophobic residue in the P_1' position and either small neutral or basic residues in positions P_1 and P_2 can be used for the convenient and sensitive determination of enzyme activity in a coupled enzyme assay with aminopeptidase M, as described in the present paper. Such substrates are particularly useful for kinetic studies and should also find application for the determination of activity of bacterial metalloendopeptidases including thermolysin.

Kidney metalloendopeptidase also showed the same specificity as the pituitary enzyme with respect to hydrolysis of naturally occurring peptides. Both enzymes hydrolyzed the Gly–Phe bond in Met- and Leu-enkephalin and in Met-enkephalinamide and also readily hydrolyzed bonds on the amino

side of hydrophobic residues in several other biologically active peptides. This activity poses the question of a possible role of the enzyme in the metabolism of these peptides. While it is not known what role, if any, such an activity would have in kidney function, it could be anticipated that hydrolysis of neuropeptides could profoundly affect the function of the central nervous system and also of the pituitary. The activity of the enzyme hydrolyzing the Gly–Phe bond in enkephalins is of particular interest, because it is identical with an activity associated with brain membrane fractions and designated as enkephalinase (Sullivan et al., 1978; Roques et al., 1980). It was proposed that brain enkephalinase functions as a specific brain enkephalin inactivator in a manner similar to the inactivation of acetylcholine by acetylcholinesterase. The main arguments advanced in support of this hypothesis were that the distribution of the enzyme in brain parallels the distribution of opiate receptors and that thiorphan, DL-3-mercapto-2-benzylpropanoylglycine, a synthetic inhibitor of enkephalinase, potentiated the morphinomimetic effect of an enkephalin analogue, D-Ala^2-Met-enkephalin, and had itself some antinociceptive activity (Roques et al., 1980).

We have presented several lines of evidence suggesting that the pituitary metalloendopeptidase (Orlowski & Wilk, 1981) and a similar enzyme associated with brain membrane fractions are identical with enkephalinase (Almenoff et al., 1981). It is now clear that enkephalinase is not an enzyme specific for the brain but a metalloendopeptidase widely distributed in animal tissues and particularly abundant in the kidney. The identity of the kidney metalloendopeptidase with enkephalinase and the membrane-bound pituitary metalloendopeptidase is indicated by an identical specificity toward synthetic and natural peptides, the same pH optima, an identical sensitivity to inhibition by metal chelators and thiols, and resistance to inhibition by several thiol, serine, and carboxyl protease inhibitors. We have shown in the present study that the kidney metalloendopeptidase is strongly inhibited by thiorphan and phosphoramidon, both compounds known to strongly inhibit brain enkephalinase (Roques et al., 1980; Hudgin et al., 1981; Fulcher et al., 1982).

There is a growing interest in inhibitors of zinc metallopeptidases, because of their potential pharmacological effects, which are related to the function of these enzymes in vivo. For example, captopril (D-3-mercapto-2-methylpropanoyl-L-proline), an inhibitor of angiotensin converting enzyme, has found application in the treatment of hypertension (Antonaccio & Cushman, 1981); and *N*-carboxymethyl dipeptides, a new class of converting enzyme inhibitors, may find the same application (Patchett et al., 1980). With the potential role of the metalloendopeptidase described in this paper in the degradation of enkephalins and other neuropeptides, inhibitors of this enzyme are of interest because of their potential pharmacological effects. Indeed, study of such effects may provide clues to the physiological function of this enzyme in vivo. There are clear similarities in the basic mechanism of action of the angiotensin converting enzyme, the metalloendopeptidase described here, thermolysin, and also carboxypeptidases A and B. All of these enzymes have a zinc atom in the active site that participates in catalysis by polarizing the carbonyl group of the bond undergoing hydrolysis. Differences between the enzymes relate to substrate specificity and binding. Accordingly, principles of design of potent inhibitors of these enzymes are based on the need for the presence of a group capable of interacting with the zinc atom in the active site, in analogy to the interaction of the carbonyl oxygen of the hydrolyzed bond with this zinc atom, and in the presence of a group with optimal binding

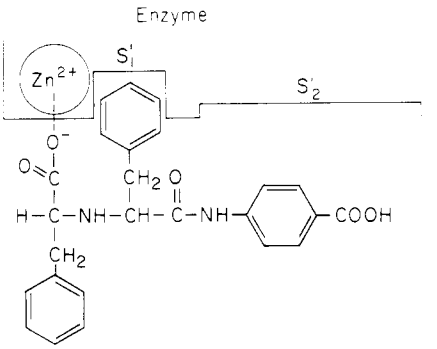


FIGURE 3: Schematic drawing of mode of binding of *N*-(1-carboxy-2-phenylethyl)-Phe-pAB to kidney metalloendopeptidase.

characteristics to the substrate binding site of the enzyme.

Thermolysin and the kidney metalloendopeptidase are closely related with respect to specificity in that a hydrophobic residue in the P_1' position is needed for effective binding. Although both of these enzymes can act as dipeptidyl carboxypeptidases on peptides having a hydrophobic residue in the penultimate position, unlike the angiotensin converting enzyme, a free carboxyl group is not required for activity. Several potent synthetic thermolysin inhibitors have been described (Kam et al., 1979; Nishino & Powers, 1979; Maycock et al., 1981). Each of these inhibitors contains a peptide capable of interacting with the substrate binding site of the enzyme and a group such as a hydroxamic acid, phosphoramidate, thiol, or carboxyl capable of coordinating with the zinc atom of the active site. It could be anticipated that similar compounds would be effective inhibitors of the kidney metalloendopeptidase. Indeed, inhibition of the enzyme by phosphoramidon, a naturally occurring analogue of a series of phosphoramidates synthesized by Kam et al. (1979), supports this conclusion. The use of phosphoramidates in in vivo experiments presents difficulties due to their susceptibility to enzymatic degradation. Similarly, thiol-containing inhibitors such as thiorphan may present problems related to the facile oxidation of the sulfhydryl group.

We have therefore proceeded to synthesize *N*-carboxymethyl derivatives of amino acid amides containing *p*-aminobenzoate in amide linkage, as potential inhibitors of the enzyme. This approach was based on the finding that the enzyme is inhibited by dipeptides having a phenylalanyl residue on the amino side and that Phe-pAB itself is a potent inhibitor with a K_I in the micromolar range. It could be expected that addition to Phe-pAB of a zinc-binding group should greatly increase its inhibitory potency. Indeed, *N*-(1-carboxy-2-phenylethyl) derivatives of Phe-pAB have proved to be potent inhibitors of the enzyme, with the most active diastereomer having a K_I of 2.4×10^{-8} M. As expected, the alanyl derivative of the same inhibitor was less potent by almost 2 orders of magnitude, reflecting the lower binding affinity of alanine to the hydrophobic pocket of the enzyme, as compared with the phenylalanine-containing derivative. This finding also shows the large contribution of the phenylalanyl residue to the binding energy of the inhibitor to the enzyme. On the basis of the available information, the mode of binding of the inhibitor to the enzyme can be presented schematically as shown in Figure 3. This simplified scheme does not consider the contributions to binding provided by the carbonyl group of the Phe–pAB bond and by the carboxylate group of *p*-aminobenzoate. That these groups contribute to binding is suggested by the finding that Phe-pAB is strongly inhibitory while free phenylalanine is not. This binding mode presented in Figure 3 is similar to that presented for several inhibitors of thermolysin (Kam et al.,

1979; Nishino & Powers, 1979; Holmes & Matthews, 1981). Another factor thought to contribute to the inhibtory potency of the *N*-carboxymethyl derivatives is their resemblance to the tetrahedral transition state involved in the hydrolysis of peptide substrates (Patchett et al., 1980).

N-(1-Carboxy-2-phenylethyl)-Phe-pAB should be an excellent tool with which to probe the in vivo function of the enzyme. It is resistant to enzymatic degradation by metalloendopeptidase, aminopeptidase, and dipeptidyl carboxypeptidase and should therefore be useful in the elucidation of the function of the metalloendopeptidase in both brain and peripheral tissues.

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Registry No. Phe-pAB, 68036-08-8; *N*-Boc-Phe *N*-hydroxysuccinimide ester, 3674-06-4; pAB, 150-13-0; Phe-pAB·CF_3CO_2H, 84041-47-4; Hip-Phe-pAB, 84041-48-5; hippuryl *N*-hydroxysuccinimide ester, 66134-74-5; glutaryl-Phe-pAB, 84041-49-6; glutaric anhydride, 108-55-4; acetyl-Phe-pAB, 68036-07-7; acetic anhydride, 108-24-7; glutaryl-Gly-Phe-pAB, 84041-50-9; *N*-Boc-Gly *N*-hydroxysuccinimide ester, 3392-07-2; Gly-Phe-pAB·CF_3CO_2H, 84041-52-1; *N*-[1(*R*,*S*)-carboxy-2-phenylethyl]-Phe-pAB, 84041-53-2; sodium phenylpyruvate, 114-76-1; *N*-[1(*R*)-carboxy-2-phenylethyl]-Phe-pAB, 84041-54-3; *N*-[1(*S*)-carboxy-2-phenylethyl]-Phe-pAB, 84041-55-4; *N*-[1(*R*,*S*)-carboxy-2-phenylethyl]-Ala-pAB, 84041-56-5; pepstatin, 39324-30-6; glutathione, 70-18-8; dithiothreitol, 3483-12-3; EDTA, 60-00-4; *o*-phenanthroline, 66-71-7; 2-mercaptoethanol, 60-24-2; Bz-Gly-Arg-Arg-Leu-2NA, 78496-77-2; Bz-Gly-Arg-Arg-Ala-2NA, 78496-79-4; Bz-Gly-Arg-Arg-Gly-2NA, 78496-80-7; glutaryl-Ala-Ala-Phe-2NA, 84041-57-6; glutaryl-Ala-Ala-Phe-4MeO2NA, 84041-58-7; Met-enkephalin, 58569-55-4; Met-enkephalinamide, 60117-17-1; Leu-enkephalin, 58822-25-6; dynorphin(1–13), 72957-38-1; bradykinin, 58-82-2; angiotensin I, 484-42-4; Ala-Phe, 3061-90-3; Phe-Phe, 2577-40-4; Phe-Leu, 3303-55-7; Phe-Ala, 3918-87-4; Phe-Gly, 721-90-4; Leu-Phe, 3063-05-6; Leu-Trp, 5156-22-9; phosphoramidon, 36357-77-4; thiorphan, 76721-89-6; kidney neutral metalloendopeptidase, 82707-54-8; enkephalin dipeptidyl carboxypeptidase, 70025-49-9; thermolysin, 9073-78-3.

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