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Active Site Directed *N*-Carboxymethyl Peptide Inhibitors of a Soluble Metalloendopeptidase from Rat Brain†

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ABSTRACT: A soluble metalloendopeptidase isolated from rat brain preferentially cleaves bonds in peptides having aromatic residues in the P_1 and P_2 position. An additional aromatic residue in the P_3' position greatly increases the binding affinity of the substrate, suggesting the presence of an extended substrate recognition site in the enzyme, capable of binding a minimum of five amino acid residues [Orlowski, M., Michaud, C., & Chu, T. G. (1983) *Eur. J. Biochem. 135*, 81-88]. A series of *N*-carboxymethyl peptide derivatives structurally related to model substrates and containing a carboxylate group capable of coordinating with the active site zinc atom were synthesized and tested as potential inhibitors. One of these inhibitors, *N*-[1(*RS*)-carboxy-2-phenylethyl]-Ala-Ala-Phe-*p*-aminobenzoate, was found to be a potent competitive inhibitor of the enzyme with a K_i of 1.94 μM. The two diastereomers of this inhibitor were separated by high-pressure liquid chromatography. The more potent diastereomer had a K_i of 0.81 μM. The inhibitory potency of the less active diastereomer was lower by 1 order of magnitude. Decreasing the hydrophobicity of the residue binding the S_1 subsite of the enzyme by, for example, replacement of the phenylethyl group with a methyl residue decreased the inhibitory potency by almost 2 orders of magnitude. Deletion of the carboxylate group decreased the inhibitory potency by more than 3 orders of magnitude. Shortening the inhibitor chain by a single alanine residue had a similar effect. Binding of the inhibitor to the enzyme increased its thermal stability. The present data together with previous studies with synthetic and natural peptides support the conclusion that the active site of the enzyme contains two hydrophobic pockets at the S_1 and S_3' subsites. As with other metalloendopeptidases [Holmquist, B., & Vallee, B. L. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 6216-6220], effective inhibition requires the presence in the inhibitor of a group binding to the substrate recognition site of the enzyme and a group capable of coordinating with the active site metal atom.

Metalloproteases are a distinct class of proteolytic enzymes in which a zinc atom in the active site participates in the catalytic process. Some of these enzymes are credited with important physiological functions. For example, angiotensin converting enzyme (EC 3.4.15.1), a peptidyldipeptide hydrolase, is responsible for the conversion of angiotensin I to angiotensin II, thereby helping to regulate blood pressure. It has been suggested that the degradation of the endogenous opiate-like peptides Met- and Leu-enkephalin is controlled by a membrane-bound neutral metalloenzyme (Sullivan et al., 1978; Malfroy et al., 1978) later shown to be identical with a metalloendopeptidase isolated from bovine pituitaries (Orlowski & Wilk, 1981; Almenoff et al., 1981) and also rabbit kidney (Kerr & Kenny, 1974; Almenoff & Orlowski, 1983).

Metalloproteases are inhibited by simple anions and chelating agents, such as EDTA and 1,10-phenanthroline. Data have been presented showing that metal coordinating substrate analogues, compounds that incorporate characteristics essential for binding to the substrate recognition site of the enzyme and having a group capable of coordinating with the active site zinc atom, act as highly specific inhibitors of such enzymes (Holmquist & Vallee, 1979). Indeed, peptides fulfilling the binding requirements of thermolysin, a bacterial metalloendopeptidase, and containing *N*-terminal hydroxamate, thiol, carboxyl, or phosphoramidate zinc-coordinating groups have been shown to act as potent competitive inhibitors of this enzyme (Kam et al., 1979; Nishino & Powers, 1978). Similar highly specific inhibitors of angiotensin converting enzyme (Cushman et al., 1977; Patchett et al., 1980) and of membrane-bound metalloendopeptidase have also been synthesized (Roques et al., 1980; Mumford et al., 1982; Almenoff & Orlowski, 1983).

We have recently reported on the isolation from the soluble protein fraction of rat brain homogenates of a metalloendopeptidase (M_r 67 000) optimally active at a neutral pH (Orlowski et al., 1983). Specificity studies indicate the presence of an extended substrate binding site with a preference toward peptides having aromatic or basic residues in the P_1 position,[1] or both the P_1 and P_2 positions, and an aromatic residue in the P_3' position. Several natural peptides including bradykinin, neurotensin, substance P, and LHRH were hydrolyzed at sites

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[1] The nomenclature proposed by Schechter & Berger (1967) is used to define the position (P) of amino acids in the peptides and to name the subsites (S) in the active site of the enzyme.

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consistent with this specificity. Because of the high activity of the enzyme in brain, it became important to synthesize specific active site directed inhibitors, which could be used as probes in studies on the role of the enzyme in neuropeptide metabolism in vivo. Here we report that *N*-carboxymethyl derivatives of peptides fulfilling the specificity requirements of the enzyme act as inhibitors and that the potency of inhibition depends on the structure of the residue interacting with the S_1 subsite of the enzyme as well as on the presence of a carboxylate group coordinating with the active site zinc atom.

Materials and Methods

Hippuric acid, trifluoroacetic acid, dithiothreitol, *p*-aminobenzoic acid, glyoxylic acid, phosphoramidon, sodium phenylpyruvate, α-ketovaleric acid, sodium cyanoborohydride, chymotrypsin, and phenylacetaldehyde were obtained from Sigma Chemical Co. (St. Louis, MO). Aminopeptidase M (EC 3.4.11.2) was purified from hog kidneys according to the procedure of Pfleiderer (1970). To remove contaminating membrane-bound metalloendopeptidase, the enzyme was chromatographed on phenyl-Sepharose CL-4B as previously described (Almenoff & Orlowski, 1983). Other reagents and solvents were obtained from Fischer Scientific Co. (Pittsburgh, PA). Frozen rat brains were obtained from Pel Freeze Inc. (Rogers, AR). Dowex AG 50W-4X (100–200 mesh) was obtained from Bio-Rad (Richmond, CA). *N*-[1(*RS*)-Carboxy-2-phenylethyl]-Phe-pAB[2] and *N*-[1(*RS*)-carboxy-2-phenylethyl]-Ala-pAB were synthesized as previously described (Almenoff & Orlowski, 1983). Soluble metalloendopeptidase was prepared from frozen rat brains as previously described (Orlowski et al., 1983).

Synthesis of Inhibitors. Inhibitors were synthesized by reductive amination of aldehydes or α-keto acids with amino acid or peptide amides of pAB. Purity of these peptide derivatives was verified by HPLC, amino acid analysis, elemental analysis, and measurement of pAB content after enzymatic release of chromogen by incubation of the inhibitors with excess chymotrypsin.

Inhibitors were analyzed by HPLC on a Waters Associates liquid chromatograph equipped with a variable-wavelength detector. Emerging peaks were monitored at 210 nm. Samples were injected on a C_{18} reverse-phase μBondapak column (30 cm × 0.4 cm; 10 μm); elution was carried out with a gradient established between 0.1% phosphoric acid in water and CH_3CN. The initial concentration of CH_3CN was 10%, and its concentration was increased linearly to 40% over a 40-min period at a flow rate of 1.0 mL/min (gradient system A). Under other gradient conditions, the initial concentration of CH_3CN was 5%, and its concentration was linearly increased to either 40% over a 60-min period (gradient system B) or 35% over a 60-min period (gradient system C). Both gradient systems B and C used a flow rate of 1.0 mL/min. Inhibitors containing impurities (compounds III and V) were also purified by HPLC with the same Waters Associates apparatus except for replacement of the analytic column with a preparative C_{18} reverse-phase μBondapak column (30 cm × 0.8 cm; 10 μm). A linear gradient between 0.1% CF_3CO_2H in water and CH_3CN was used. Gradient conditions were identical with those used to analyze the inhibitors. Elemental analysis of peptide derivatives was performed by the microanalytical service of Rockefeller University (New York, NY). Melting points are uncorrected.

(*A*) *N*-[*1*(*RS*)-*Carboxy-2-phenylethyl*]-*Ala-Ala-Phe-pAB* (*I*). CF_3CO_2H–Ala-Ala-Phe-pAB (0.54 g, 1.0 mmol), synthesized as previously described (Orlowski et al., 1983), and sodium phenylpyruvate (1.86 g, 10.0 mmol) were dissolved in water (15 mL). The solution was adjusted to pH 6.5–7.0 with 1 M NaOH. A 10-mL aliquot of a 0.8 M solution of sodium cyanoborohydride was then added over a period of 3 h with a syringe pump. After being stirred for 24 h, the reaction mixture was acidified to about pH 2 with 1 M HCl; the resulting precipitate was filtered and washed with water. The remaining solid was dried and then suspended in ethyl acetate and stirred for 3 h. The insoluble white solid was filtered and washed extensively with ethyl acetate: mp 187–188 °C; HPLC, double peak with retention times of 35.8 and 37.2 min (gradient system A). Anal. Calcd for $C_{31}H_{34}O_7N_4 \cdot H_2O$: C, 62.83; H, 6.12; N, 9.45. Found: C, 62.94; H, 5.90; N, 9.26.

The diastereomers of I were separated by HPLC; 50-μL aliquots of a 10 mM mixture of the diastereomers were injected on a C_{18} reverse-phase μBondapak column (30 × 0.4 cm; 10 μm); elution was carried out with a linear gradient established between 0.1% CF_3CO_2H in water and CH_3CN, under the same conditions as those described above for gradient system A. The emerging peaks were monitored at 210 nm and separately collected. After removal of solvent under reduced pressure, the residue from each peak was dissolved in 1.0 mL of 0.2 M Tris-HCl buffer, pH 7.0, and 0.01 mL of 1.0 M NaOH. Upon reinjection on HPLC, each dissolved fraction eluted as a single peak, indicating resolution of the diastereomers. The concentration of the inhibitor solution was determined by subjecting aliquots to chymotrypsin digestion and quantitating the amount of chromogen (pAB) released. Aliquots of 0.025 mL of an inhibitor solution, 0.175 mL of Tris-HCl buffer (0.1 M, pH 7.8), and 0.05 mL of chymotrypsin (400 units/ml; 1 unit being defined as the amount of enzyme required to hydrolyze 1 μmol of Bz-Tyr-OMe/min) were incubated for 2 h at 37 °C. After addition of 0.25 mL of 10% CCl_3CO_2H, the amount of pAB released was quantitated as described under Determination of Enzyme Activity and Inhibitory Constants. Chymotrypsin digestion resulted in total release of pAB, as followed by HPLC.

(*B*) *N*-[*1*(*RS*)-*Carboxybutyl*]-*Ala-Ala-Phe-pAB* (*II*). II was prepared by reductive amination of α-ketovaleric acid with Ala-Ala-Phe-pAB as described for I: mp 218–219 °C; HPLC, double peak with retention times of 31.4 and 31.8 min (gradient system A). Anal. Calcd for $C_{27}H_{34}O_7N_4 \cdot 0.5H_2O$: C, 60.55; H, 6.59; N, 10.46. Found: C, 60.31; H, 6.41; N, 10.26.

(*C*) *N*-[*1*(*RS*)-*Carboxyethyl*]-*Ala-Ala-Phe-pAB* (*III*). III was prepared by reductive amination of sodium pyruvate with Ala-Ala-Phe-pAB as described for I. Following completion of the reaction, the solution was acidified to pH 3 by the addition of 1 M HCl and then applied to a 100-mL column of Dowex 50 (H^+, 100–200 mesh) previously equilibrated with a 0.1 M pyridine–formate buffer, pH 3.1. Removal of starting material was accomplished by washing the column with the equilibrating buffer. The product was eluted by washing the column with 2% pyridine. A white solid was obtained upon removal of solvent under reduced pressure. The compound was suspended in 2-propanol and then filtered and washed with the same solvent: mp 290–295 °C dec; HPLC, asymmetric peak indicating the presence of two unresolved diastereomers with a retention time of 53.6 min (gradient system C). Anal.

[2] Abbreviations: pAB, *p*-aminobenzoate; Bz, N^{α}-benzoyl; CF_3CO_2H, trifluoroacetic acid; CCl_3CO_2H, trichloroacetic acid; HPLC, high-pressure liquid chromatography; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; CH_3CN, acetonitrile; LHRH, luteinizing hormone releasing hormone; Tris, tris(hydroxymethyl)aminomethane.

Calcd for $C_{25}H_{30}O_7N_4 \cdot H_2O$: C, 58.13; H, 6.24. Found: C, 58.02; H, 5.89.

(*D*) *N-(1-Carboxymethyl)-Ala-Ala-Phe-pAB (IV)*. IV was prepared by reductive amination of glyoxylic acid with Ala-Ala-Phe-pAB as described for I. The product was then isolated as for III: mp 176–178 °C; HPLC, single peak with a retention time of 27.8 min (gradient system A). Anal. Calcd for $C_{24}H_{28}O_7N_4 \cdot H_2O$: C, 57.36; H, 6.02; N, 11.15. Found: C, 57.02; H, 5.94; N, 11.09.

(*E*) *N-(1-Phenylethyl)-Ala-Ala-Phe-pAB (V)*. Ala-Ala-Phe-pAB (0.434 g, 1.0 mmol) and 20 mL of phenylacetaldehyde (1:1 in 2-propanol; 100 mmol) were dissolved in 15 mL of methanol–water (1:1). The pH was adjusted to 7.0 with 1 M NaOH. A 10-ml aliquot of a 1.0 M solution of sodium cyanoborohydride was then added over a 3-h period with a syringe pump. After being stirred for 24 h, the reaction mixture was transferred to a separatory funnel and washed twice with ethyl ether. The aqueous layer was collected, and excess ether was removed under reduced pressure. The reaction mixture was then acidifed to pH 3 with 1 M HCl; the resulting precipitate was filtered and extensively washed with water. The remaining solid was dried before suspension in ethyl acetate for 3 h. The insoluble white solid was filtered and thoroughly washed with ethyl acetate: mp 198–200 °C; HPLC, single peak with a retention time of 39.6 min (gradient system A). Anal. Calcd for $C_{30}H_{34}O_5N_4 \cdot 0.5H_2O$: C, 66.77; H, 6.54. Found: C, 66.87; H, 6.47.

(*F*) *N-[1(RS)-Carboxy-2-phenylethyl]-Ala-Phe-pAB (VI)*. VI was prepared by reductive amination of sodium pyruvate with Ala-Phe-pAB as described for I. The product was isolated as for I and recrystallized from ethyl acetate as the trifluoroacetate salt: mp 191–192 °C; HPLC, asymmetric peak indicating the presence of two unresolved diastereomers with a retention time of 64.0 min (gradient system B). Anal. Calcd for $C_{28}H_{29}O_6N_3 \cdot CF_3CO_2H$: C, 58.35; H, 4.9. Found: C, 58.70; H, 5.10.

Determination of Enzyme Activity and Inhibitory Constants. The activity of the soluble metalloendopeptidase was assayed with Bz-Gly-Ala-Ala-Phe-pAB as substrate in a two-stage reaction as follows:

$$\text{Bz-Gly-Ala-Ala-Phe-pAB} \xrightarrow{\text{metalloendopeptidase}} \text{Bz-Gly} + \text{Ala-Ala-Phe-pAB} \quad (1)$$

$$\text{Ala-Ala-Phe-pAB} \xrightarrow{\text{aminopeptidase}} 2\text{Ala} + \text{Phe} + \text{pAB} \quad (2)$$

In the first stage, the enzyme is incubated with substrate, resulting in hydrolysis of the Gly-Ala bond. The reaction is then terminated by placement of the incubation mixture in boiling water for 2 min. After this is cooled in ice and addition of the aminopeptidase, reaction 2 is allowed to proceed to completion at 37 °C. The amount of pAB released is determined by a diazotization procedure.

Incubation mixtures contained substrate (0.5–1.0 mM), dithiothreitol (0.25 mM), enzyme (5–25 μL), and Tris-HCl buffer (0.2 M, pH 7.0) in a final volume of 0.2 mL. Incubations were carried out at 37 °C for 15–60 min and terminated by placement of the reaction tubes in boiling water for 2 min, followed by a cooling in ice. Aminopeptidase M (10 μg) and Tris-HCl buffer (0.2 M, pH 7.0) were added to a final volume of 0.25 mL. The tubes were then incubated for 120 min at 37 °C. This time interval was sufficient to catalyze the total release of pAB. The amount of pAB released was determined by a modification (Goldbarg & Rutenberg, 1958) of the diazotization procedure described by Bratton & Marshall (1939). The absorbance of the samples was determined at 555 nm, and the amount of pAB present was calculated from a standard curve derived from known amounts of pAB. Controls in which the enzyme or substrate was separately omitted were carried through the procedure. In experiments with inhibitors, various inhibitor concentrations were included in the incubation mixture, and the reaction was initiated by addition of enzyme. In other experiments, varying amounts of inhibitor were preincubated with the enzyme for 10 min at 37 °C, and the reaction was initiated by the addition of substrate. Hydrolysis was linear with time for up to 60 min. Less than 10% of the total substrate was hydrolyzed in all experiments.

The type of inhibition for all *N*-carboxymethyl peptides was determined from double-reciprocal plots obtained in the presence and absence of inhibitor. K_i values for all inhibitors were determined by the method of Dixon (1953; plot $1/v$ vs. [I] at several different substrate concentrations).

Table I: Inhibitors of Rat Brain Soluble Metalloendopeptidase

compound	$K_i \pm$ SEM (μM)[a]
N-[1(*RS*)-carboxy-2-phenylethyl]-Ala-Ala-Phe-pAB (I)	1.94 ± 0.65
diastereomer 1	0.81 ± 0.16
diastereomer 2	7.39 ± 1.77
N-[1(*RS*)-carboxybutyl]-Ala-Ala-Phe-pAB (II)	5.17 ± 0.67
N-[1(*RS*)-carboxyethyl]-Ala-Ala-Phe-pAB (III)	20.9 ± 2.30
N-(carboxymethyl)-Ala-Ala-Phe-pAB (IV)	69.4 ± 7.72
N-(phenylethyl)-Ala-Ala-Phe-pAB (V)	2360.0 ± 700.0
N-[1(*RS*)-carboxy-2-phenylethyl]-Ala-Phe-pAB (VI)	2640.0 ± 720.0
N-[1(*RS*)-carboxy-2-phenylethyl]-Ala-pAB (VII)	no inhibition[b]
N-[1(*RS*)-carboxy-2-phenylethyl]-Phe-pAB (VIII)	no inhibition[b]
phosphoramidon (IX)	no inhibition[b]

[a] K_i values were determined from Dixon plots obtained at two different substrate concentrations with Bz-Gly-Ala-Ala-Phe-pAB as substrate. At each concentration of substrate, rates were determined with five different inhibitor concentrations. Each K_i value represents the average of three to five determinations. [b] The final concentrations of compounds VII–IX were 1.25, 0.5, and 0.125 mM, respectively. These concentrations are respectively 250, 7000, and 36 000 times greater than the K_i values of these inhibitors toward the membrane-bound metalloendopeptidase.

Results

A series of *N*-carboxymethyl peptides were synthesized by reductive amination of various α-keto acids and aldehydes with Ala-Ala-Phe-pAB. This peptide was used because we have shown in previous studies (Orlowski et al., 1983) that the phenylalanyl residue is important for substrate binding by apparently interacting with a hydrophobic pocket in the S_3' subsite of the enzyme and because the two Ala residues are needed for binding to the S_1' and S_2' subsites. Furthermore, by introducing this fragment into all the inhibitors, it became possible to evaluate the importance of interactions at the S_1 subsite for inhibition and also to evaluate the contribution of the carboxylate anion as a zinc-coordinating group.

Table I summarizes the inhibition constants obtained with all the inhibitors. Reductive amination of α-ketoacids generates a new asymmetric carbon, and accordingly, two diastereomers are formed in about equimolar amounts as determined by HPLC. With the exception of *N*-[1(*RS*)-carboxy-2-phenylethyl]-Ala-Ala-Phe-pAB, which was resolved into its diastereomers by HPLC, racemic mixtures were used in studies of the other derivatives. As expected, reductive amination of glyoxylic acid (inhibitor IV; Table I) and phenylacetaldehyde (inhibitor V; Table I) yielded single products.

N-[1-Carboxy-2-phenylethyl]-Ala-Ala-Phe-pAB produced the strongest inhibition of the soluble metalloendopeptidase.

A racemic mixture of this inhibitor had a K_i of 1.94 μM. The more potent diastereomer had a K_i of 0.81 μM, while the less active diastereomer had a K_i of 7.4 μM. This almost 10-fold difference in inhibitory potency suggests considerable stereoselectivity of inhibitor binding. Decreasing the hydrophobicity of the residue binding to the S_1 subsite of the enzyme greatly decreased the inhibitory potency of the *N*-carboxymethyl peptides. For example, while the *N*-carboxybutyl derivative was only 2.5 times less potent than the *N*-carboxy-2-phenylethyl derivative, the *N*-carboxyethyl derivative was 10-fold less potent. The *N*-carboxymethyl peptide was even less potent, being 35-fold less inhibitory compared with the racemic mixture of *N*-[1(*RS*)-carboxy-2-phenylethyl]-Ala-Ala-Phe-pAB and 86-fold less inhibitory compared with the more potent isolated diastereomer.

N-(Phenylethyl)-Ala-Ala-Phe-pAB, a compound similar to *N*-[1(*RS*)-carboxy-2-phenylethyl]-Ala-Ala-Phe-pAB but lacking the Zn^{2+}-coordinating carboxylate group, was only weakly inhibitory. This derivative had a K_i almost 1200-fold higher than the corresponding carboxylate-containing compound. A similar dramatic decrease in inhibitory potency was caused by replacement of Ala-Ala-Phe-pAB with Ala-Phe-pAB. Inhibitors of membrane-bound metalloendopeptidase ("enkephalinase", EC 3.4.24.11) such as phosphoramidon, *N*-[1(*RS*)-carboxy-2-phenylethyl]-Ala-pAB, and *N*-[1-(*RS*)-carboxy-2-phenylethyl]-Phe-pAB had no effect on the soluble metalloendopeptidase activity.

The inhibition of the soluble metalloendopeptidase by the inhibitors listed in Table I was competitive in nature since it could be surmounted by increasing substrate concentrations. Double-reciprocal plots ($1/v$ vs. $1/[S]$) in the presence and absence of inhibitor gave straight lines intersecting at the y axis. The reversibility of this inhibition was also apparent in dialysis experiments. Enzyme activity, which had been inhibited by a 6.25 μM solution of *N*-[1(*RS*)-carboxy-2-phenylethyl]-Ala-Ala-Phe-pAB, could be completely restored upon dialysis against Tris-HCl buffer (0.1 M, pH 7.6). Experiments in which the enzyme was preincubated with inhibitor for 10 min at 37 °C before the reaction was started by addition of substrate showed the same degree of inhibition as those in which the enzyme was not preincubated, and the reaction was initiated by adding the enzyme to mixtures containing substrate and buffer. This indicates that formation and dissociation of the enzyme–inhibitor complex is a rapid equilibrium process. No change in the degree of inhibition was also noted in experiments in which 0.05 mM $ZnCl_2$ was added to incubation mixtures containing enzyme and *N*-[(*RS*)-carboxy-2-phenylethyl]-Ala-Ala-Phe-pAB (2.5 μM). This indicates that inhibition was not due to an EDTA-like effect, whereby the metalloendopeptidase is stripped of its catalytically essential metal ion by the inhibitor.

The soluble metalloendopeptidase showed considerable sensitivity to heat denaturation in the absence of substrate. More than 90% of the activity was lost by heating the enzyme at 57 °C for 60 min (Figure 1). Even at 37 °C, more than 50% of the activity was lost after 60 min. Considerable protection against heat denaturation was, however, provided by addition of inhibitor. Thus, in the presence of 0.1 M *N*-[1-(*RS*)-carboxy-2-phenylethyl]-Ala-Ala-Phe-pAB only about 30% of the activity was lost by heating at 57 °C for 1 h, suggesting that inhibitor binding increases the heat stability of the enzyme.

Discussion

We have previously reported on the purification from the soluble fraction of rat brain homogenates of a metalloendopeptidase optimally active at a neutral pH (Orlowski et al., 1983). Specificity studies with model synthetic substrates and natural peptides indicated the presence of an extended substrate binding site accomodating at least five amino acid residues. Unlike, however, such metallopeptidases as carboxypeptidases A and B, thermolysin, and angiotensin converting enzyme in which specificity is determined by the structure of residues positioned in close proximity to the hydrolyzed bond, evidence was obtained suggesting that an aromatic residue (Phe, Tyr) in the P_3' position, at some distance from the hydrolyzed bond, greatly influences the rate of reaction and specificity constant of the brain enzyme. Other specificity characteristics of the enzyme include a strong preference for hydrophobic residues in the P_1 or P_1 and P_2 positions and the need for the presence of a substituent on the α-amino group in the P_1 residue of the substrate as evidenced by the failure of the enzyme to hydrolyze Met-enkephalin (Tyr-Gly-Gly-Phe-Met). On the basis of the data derived from the specificity studies, the active site of the enzyme and its interaction with the best of the model substrates can be schematically represented as shown in Figure 2A; the S_2, S_1, and S_3' subsites (probably hydrophobic pockets) accomodate the hydrophobic residues of the substrate, and the Zn^{2+} atom coordinates with the carbonyl oxygen of the hydrolyzed bond.

The high activity of the enzyme in brain prompted the present work aimed at designing specific active site directed inhibitors that could be used as probes in studies on the role of the enzyme in neuropeptide metabolism. Furthermore, it was expected that inhibitor studies would provide additional insight into the substrate recognition site of the enzyme and its mechanism of action. We proceeded, therefore, to syn-

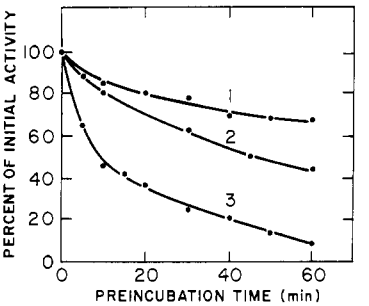

FIGURE 1: Protection of the enzyme against heat denaturation by *N*-[1(*RS*)-carboxy-2-phenylethyl]-Ala-Ala-Phe-pAB. The enzyme (after step 3 of the purification procedure; Orlowski et al., 1983) was preincubated in Tris-HCl buffer (0.2 M, pH 7.0) at 57 °C (curve 3) and 37 °C (curve 2) in the absence of inhibitor and at 57 °C (curve 1) in the presence of 0.1 M inhibitor. Aliquots were withdrawn at various times for determination of activity as described under Materials and Methods.

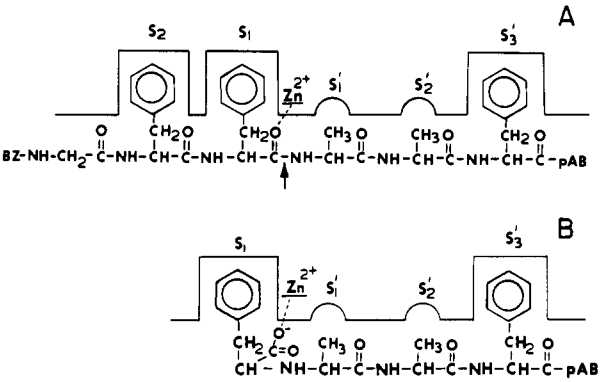

FIGURE 2: Schematic representation of the active site of the enzyme and its binding of the substrate Bz-Gly-Phe-Phe-Ala-Ala-Phe-pAB (A) and inhibitor *N*-[1-carboxy-2-phenylethyl]-Ala-Ala-Phe-pAB (B).

thesize a series of *N*-carboxymethyl derivatives containing that part of the substrate which was postulated to bind to the S_1'-S_3' subsites of the enzyme (Figure 2A). These derivatives were selected because *N*-carboxymethyl derivatives of peptides or peptide analogues fulfilling the binding requirements of the substrate recognition site have been previously shown to act as potent inhibitors of several Zn^{2+} metallopeptidases. Thus, Patchett et al. (1980) have shown that *N*-carboxymethyl derivatives of proline-containing dipeptides act as potent inhibitors of angiotensin converting enzyme. Subsequently, *N*-carboxymethyl derivatives of peptides binding to the active site of thermolysin and mammalian membrane-bound neutral metalloendopeptidase were shown to strongly inhibit these two enzymes (Maycock et al., 1981; Fournie-Zaluski et al., 1982) Mumford et al., 1982; Almenoff & Orlowski, 1983). Indeed, as expected the synthesized derivatives (Table I) act as strong inhibitors of the soluble metalloendopeptidase.

The assumption that the interaction of a hydrophobic residue with a hydrophobic pocket in the S_1 subsite of the enzyme is important for binding was tested by determining the inhibitory potency of a series of *N*-carboxymethyl derivatives with varying degrees of hydrophobicity in the P_1 position. The finding that the inhibitory constant of *N*-[1(*RS*)-carboxy-2-phenylethyl]-Ala-Ala-Phe-pAB (Table I) is 35 times lower than that of the corresponding *N*-carboxymethyl derivative (K_i of 1.94 μM vs. 69.4 μM) indeed supports this assumption.

The importance for inhibition of the carboxylate group as a moiety capable of coordinating with the active site zinc atom is shown by the finding that *N*-(phenylethyl)-Ala-Ala-Phe-pAB, a compound not having this group, is by 3 orders of magnitude less inhibitory than the analogous *N*-[1(*RS*)-carboxy-2-phenylethyl]-Ala-Ala-Phe-pAB. In addition to coordinating with the zinc atom, this group may also impose some conformational restrictions on the hydrophobic side chain of the inhibitor, thereby favoring its interaction with the S_1 subsite of the enzyme. In the absence of this group, the benzyl group of the phenylethyl derivative is free to assume many additional conformations that might not lead to interaction with the hydrophobic S_1 subsite.

The importance of interactions at the active site zinc and at both the S_1 and S_3' subsites of the enzyme for inhibition is also indicated by the finding that *N*-[1(*RS*)-carboxy-2-phenylethyl]-Ala-Phe-pAB, an analogue of the most potent inhibitor missing a single Ala residue, is only weakly inhibitory (K_i of 2640 μM vs. 1.94 μM; Table I). One can suggest that the binding of this inhibitor to the S_1 subsite and to the zinc atom prevents interaction between the Phe residue and the S_3' subsite and, vice versa, binding to the S_3' subsite prevents interactions with the zinc atom and the S_1 subsite. None of the membrane-bound metalloendopeptidase inhibitors (compounds VII–IX, Table I) inhibited the soluble brain enzyme.

Our data indicate that inhibition of the soluble metalloendopeptidase requires the presence of a group fulfilling the binding requirements of the active site and the presence of a zinc-coordinating group, as postulated by Holmquist & Vallee (1979) for metallopeptidases in general. In addition, a tetrahedral geometry is present in these inhibitors at the scissile CO–NH locus, through the R, H, COOH, and NH groups, probably mimicking the transition state for peptide hydrolysis. As suggested by Wolfenden (1972) and Leinhard (1973), compounds mimicking the transition state of a substrate would be expected to bind tightly to the active site of an enzyme and therefore act as potent enzyme inhibitors. The proposed mechanism by which metalloproteases catalyze the hydrolysis of peptide bonds is not totally resolved; however, there are some indications that substrates for these enzymes undergo a transient change into a tetrahedral geometry (Hartsuck & Lipscomb, 1971). Therefore, the inhibition exhibited by the *N*-carboxymethyl derivatives might be partially contributed by their resemblence to the transition state for peptide hydrolysis.

The endopeptidase purified in our laboratory exhibits all the characteristics indicative of a metalloenzyme. Since, however, only minute amounts of enzyme could be isolated from rat brain, we have not been able to determine its zinc content by metal analysis. The demonstration that the presence of a carboxylate group capable of interacting with the active site metal is required for inhibition in the series of *N*-carboxymethyl peptides described here represents additional proof that this enzyme is indeed a metalloendopeptidase. Furthermore, our inhibitor studies fully confirm the conclusions related to the topography of the active site as derived from studies with model synthetic substrates. A schematic representation of the interaction of the inhibitor with the active site is given in Figure 2B. The phenylethyl group and the Phe residue of the inhibitor fit into the hydrophobic pockets of the active site in a similar fashion as the Phe residues of the substrate (Figure 2A). Also, the zinc ion that polarizes the carbonyl group of the scissle bond of the substrate is coordinated by the carboxylate group of the inhibitor. While these studies verify the preference of the enzyme for hydrophobic residues in the P_1 position, information concerning the binding requirements of the S_1' and S_2' subsites and the importance of hydrogen bonding between groups of the enzyme and the carbonyl oxygens of the substrate is still lacking. Such information could provide a basis for the synthesis of even more potent inhibitors than those described here. Nevertheless, these inhibitors are sufficiently potent to be useful as probes in studies on the role of this enzyme in vivo.

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Primary Structure of Human Preangiotensinogen Deduced from the Cloned cDNA Sequence†

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ABSTRACT: Cloned cDNA sequences for human preangiotensinogen have been isolated from a human liver cDNA library by hybridization with a restriction fragment derived from a previously cloned cDNA for rat preangiotensinogen. Analyses by nucleotide sequence determination, S1 nuclease mapping, and RNA blot hybridization indicate that human preangiotensinogen is encoded by two mRNAs that differ only in the length of the 3′-untranslated region. The deduced amino acid sequence shows that the mature angiotensinogen consists of 452 amino acid residues with the angiotensin sequence at its amino-terminal portion. Two potential initiation sites have been discussed. These are the methionine codon located at the position exactly corresponding to the initiation site of rat preangiotensinogen mRNA and an additional methionine codon positioned nearest the 5′ end of the mRNA. The amino acid sequences starting at either of the initiation sites and preceding the angiotensin sequence constitute a large number of hydrophobic amino acid residues, thus representing the signal peptide characteristic of the secretory proteins. Human and rat preangiotensinogens show that 63.6% of the amino acid positions of the two proteins are identical. However, the amino-terminal portions directly distal to angiotensin I diverge markedly between the two proteins and differ in their possible glycosylation sites. These structural differences may contribute to the known species specificity exhibited by renin.

The renin–angiotensin system plays an important role in the regulation of blood pressure and hydromineral balance [see reviews from Reid et al. (1978) and Skeggs et al. (1980)]. Activation of this system is initiated by the release of angiotensin I from its precursor angiotensinogen by processing with the enzyme renin (EC 3.4.99.19). Angiotensin-converting enzyme (dipeptidyl carboxypeptidase, peptidyldipeptide hydrolase, EC 3.4.15.1) then cleaves a dipeptide from angiotensin I to form the octapeptide angiotensin II. Angiotensin II is the principal biologically active peptide that causes arteriolar vasoconstriction and stimulates aldosterone secretion. Several lines of evidence indicate that angiotensinogen is as important as renin in determining the rate of formation of angiotensin and therefore the activity of the renin–angiotensin system (Reid et al., 1978).

A number of inhibitors of angiotensin-converting enzyme have recently been developed, and their wide applicability for treating hypertensive patients suggests that the renin–angiotensin system may involve the pathogenesis of various forms of human hypertension including essential hypertension [see review from Ondetti & Cushman (1982)]. Human angiotensinogen has been purified to homogeneity in several laboratories [see review from Tewksbury (1983)], and its amino-terminal amino acid sequence up to the 25 amino acid residues has been reported (Tewksbury et al., 1981). However, the primary structure of a large carboxyl-terminal portion of human angiotensinogen remains to be determined. Furthermore, little is known about the regulatory mechanism responsible for the biosynthesis of human angiotensinogen, although it has been reported that the concentration of human plasma angiotensinogen varies under various physiological and pathological conditions (Reid et al., 1978).

We have previously reported the whole primary structure of rat preangiotensinogen by determining the nucleotide sequence of cloned DNA complementary to its mRNA (Ohkubo et al., 1983). In the present study, the construction and sequence analysis of cDNAs for human preangiotensinogen have been undertaken as an initial approach to investigate the regulatory mechanism involved in the biosynthesis of human angiotensinogen and to study the possible involvement of the renin–angiotensin system in the pathogenesis of human hypertension. We here report the entire amino acid sequence of human preangiotensinogen deduced from the nucleotide

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