Substrate-Related Potent Inhibitors of Brain Metalloendopeptidase†

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ABSTRACT: Rat brain metalloendopeptidase (EC 3.4.24.15) generates Leu- and Met-enkephalin from several larger opioid peptides and is capable of degrading a number of neuropeptides. Substrate-related *N*-(1-carboxy-3-phenylpropyl) peptide derivatives were synthesized and tested for enzyme inhibition. The best of these derivatives, *N*-[1(*RS*)-carboxy-3-phenylpropyl]-Ala-Ala-Tyr-*p*-aminobenzoate, inhibited the enzyme in a competitive manner with a K_i of 16 nM. The data indicate that the carboxyl group of the *N*-(1-carboxy-3-phenylpropyl) moiety coordinates with the active site zinc atom and that the remaining part of the inhibitor is necessary for interaction with the substrate recognition site of the enzyme. Replacement of the 1-carboxy-3-phenylpropyl group by a carboxymethyl group decreased the inhibitory potency by more than 3 orders of magnitude, emphasizing the importance of the hydrophobic phenyl group for inhibitor binding to a hydrophobic pocket at the S_1 subsite. Replacement of the Tyr residue by an Ala residue decreased the inhibitory potency by more than 20-fold. Changes in the structure of the residue interacting with the S_1' subsite could cause a more than 60-fold change in inhibition. The inhibitors were either ineffective or only weakly inhibitory against membrane-bound metalloendopeptidase ("enkephalinase", EC 3.4.24.11), an enzyme highly active in rabbit kidney but also present in brain. The data indicate the presence of an extended binding site in the enzyme with residues interacting with S_1, S_1', and S_3' subsites largely determining inhibitor binding. These findings are consistent with the specificity of the enzyme deduced from studies of its interaction with synthetic and natural peptides [Orlowski, M., Michaud, C., & Chu, T. G. (1983) *Eur. J. Biochem. 135*, 81–88].

Previous work in this laboratory has led to the identification and purification from rat brain of a metalloendopeptidase predominantly associated with the soluble protein fraction of brain homogenates (Orlowski et al., 1983). A membrane-bound form of the enzyme, associated with brain membrane fractions, including purified synaptosomal membranes has also been recently identified (Acker et al., 1986). The enzyme to be referred to as endopeptidase 24.15, is distinctly different from thermolysin-like membrane-bound metalloendopeptidase (EC 3.4.24.11, endopeptidase 24.11; Kerr & Kenny, 1974; Orlowski & Wilk, 1981) with respect to both specificity and molecular properties. Unlike endopeptidase 24.11, known to be identical with "enkephalinase" (Almenoff et al., 1981; Fulcher et al., 1982), an enzyme widely distributed in animal tissues, endopeptidase 24.15 is highly active in brain, pituitary, and testis with little activity in other peripheral tissues, such as liver, kidney, lung, or spleen. Also, unlike endopeptidase 24.11 which is primarily considered to have a degradative function, especially in the degradation of brain enkephalins ("enkephalinase"), endopeptidase 24.15, due to its specificity, is potentially involved in processing and formation of some bioactive peptides and degradation of others. Thus, both the soluble and membrane-bound forms of the enzyme efficiently converted several peptides, including dynorphin[1–8], α- and β-neoendorphin, and Met-enk-Arg-Gly-Leu[1] into the bioactive pentapeptides Leu-enk and Met-enk, respectively (Chu & Orlowski, 1985; Acker et al., 1986). The enzyme also showed a high affinity toward several other bioactive peptides, such as bradykinin, neurotensin, and angiotensin I, converting these into inactive products (Chu & Orlowski, 1985). These findings suggest an involvement of the enzyme in neuropeptide metabolism and thereby a role in brain function. Conclusive demonstration of the involvement of an enzyme in the metabolism of bioactive peptides requires the demonstration that its inhibition in vivo, or in intact cell preparations, leads to changes in the concentration of the affected peptides and also changes of those functions which are dependent on neuropeptide function. This requires the use of specific inhibitors as probes in such experiments. Initial studies of the specificity of endopeptidase 24.15 (Orlowski et al., 1983) indicated the presence of an extended substrate binding site accommodating at least five amino acid residues, with preference for substrates containing bulky or hydrophobic residues in the P_1 and P_3' positions.[2] On the basis of this information, we initiated work on the synthesis of specific active site directed inhibitors of the enzyme. Here we report the synthesis of a series of *N*-(1-carboxy-3-phenylpropyl) peptide inhibitors of the enzyme. Like with inhibitors of other zinc metallopeptidases (Cushman et al., 1977; Holmquist & Vallee, 1979; Kam et al., 1979; Nishino & Powers, 1979; Patchett et al., 1980), the potency of inhibition was shown to be dependent upon the presence of a sequence fulfilling the binding requirements of the substrate recognition site and the presence of a group capable of coordinating the active site zinc atom. The data underline the importance for binding and inhibition of groups capable of

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[1] Abbreviations: BOC, *tert*-butoxycarbonyl; Bz, benzoyl; Bzl, benzyl; Glt, glutaryl; HPLC, high-pressure liquid chromatography; Leu-enk, leucine-enkephalin; Met-enk, methionine-enkephalin; pAB, *p*-aminobenzoate; TLC, thin-layer chromatography.

[2] The nomenclature of Schechter and Berger (1967) is used to describe the positions (P) of the residues in the substrate and the corresponding subsites (S) in the active site of the enzyme.

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interacting with the S_1, S_1', and S_3' subsites of the enzyme.

Materials and Methods

Materials. 2-Keto-4-phenylbutyric acid ethyl ester was obtained from Chemical Dynamics Corp. (South Plainfield, NJ). Sodium cyanoborohydride, pAB, BOC, trifluoroacetic acid, and ester derivatives of amino acids were obtained from Sigma Chemical Co. (St. Louis, MO). Dowex-50 (AG 50W-X4; H^+), 100–200 mesh, was obtained from Bio-Rad (Richmond, CA). Silica gel thin-layer plates were obtained from Macherey-Nagel (D-5160, Duren, West Germany). All other reagents and solvents were analytical grade and were obtained from Fisher Scientific Co. (Pittsburgh, PA). Brain metalloendopeptidase (EC 3.4.24.15) was prepared as described previously (Orlowski et al., 1983). Aminopeptidase M (EC 3.4.11.2) was prepared by the method of Pfleiderer (1970) and further purified as described (Almenoff & Orlowski, 1983).

Synthetic and Analytical Procedures. Inhibitors were synthesized by reductive amination of α-keto acids, with peptides containing the appropriate amino acid sequence, sodium cyanoborohydride being used as the reducing agent (Borch et al., 1971; Patchett et al., 1980; Almenoff & Orlowski, 1983; Chu & Orlowski, 1984). Purity was analyzed by HPLC, thin-layer chromatography on silica gel plates, and also elemental analysis (Galbraith Lab, Knoxville, TN). A Waters Associates liquid chromatograph equipped with a variable-wavelength detector was used for HPLC. Emerging peaks were monitored at 210 nm. Samples were dissolved in methanol and 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% phosphoric acid and acetonitrile. The initial concentration of CH_3CN was 10%, and its concentration was increased linearly to 40% over a period of 20 min, at a flow rate of 1 mL/min (solvent A). A second solvent system (solvent B) was occasionally used. This system was identical with solvent A except that the elution time of the gradient was extended to 30 min. Thin-layer chromatography was performed with a solvent system containing a mixture of chloroform–methanol and acetic acid of a composition 85:10:5 (solvent C) or 103:1:1 (solvent D).

CF_3CO_2H·Ala-Ala-Phe-pAB and related peptides were prepared as described previously (Orlowski et al., 1983). *N*-(1-Carboxymethyl)-Ala-Ala-Phe-pAB, *N*-[1(*RS*)-carboxyethyl]-Ala-Ala-Phe-pAB, *N*-[1(*RS*)-carboxybutyl]-Ala-Ala-Phe-pAB, *N*-[1(*RS*)-carboxy-2-phenylethyl]-Ala-Ala-Phe-pAB, and *N*-phenylethyl-Ala-Ala-Phe-pAB were the same preparations as described previously (Chu & Orlowski, 1984).

N-[1(RS)-Carboxy-3-phenylpropyl]-Ala-Ala-Phe-pAB (I). A total of 1.44 g (7 mmol) of ethyl 2-keto-4-phenylbutyrate was dissolved in 25 mL of methanol and saponified by the addition of 17 mL of 1 M sodium hydroxide and stirring at room temperature for 5 h; 1 mmol of CF_3CO_2H·Ala-Ala-Phe-pAB was then added, and the pH was adjusted to about 7.0–7.5 by the addition of 6 M HCl. Alternatively sodium α-ketophenylbutyrate can be used as the keto acid. Sodium cyanoborohydride (7 mmol in 10 mL of water) was added over a period of 3 h with a syringe pump. The mixture was stirred overnight and then concentrated by flash evaporation to about 20 mL. The pH was adjusted to about 1.0–2.0 by the addition of 6 M HCl, and the precipitated white solid was collected by filtration and dried. It was then suspended in warm ethyl acetate and stirred at room temperature for 2 h. The white solid was isolated by filtration, extensively washed with ethyl acetate, and dried. The yield was 300 mg (50%). The material was recrystallized from ethanol. HPLC (solvent A) gave a double peak with retention times of 24.6 and 25.4 min; TLC (solvent C) gave a single spot with an R_f value of 0.34; mp was 215–216 °C. Anal. Calcd for $C_{32}H_{36}O_7N_4$·HCl: C, 61.42; H, 5.96; N, 8.96. Found: C, 61.64, H, 6.14; N, 9.00.

N-[1(RS)-Carboxy-3-phenylpropyl]-Ala-Ala-Tyr-pAB (II). II was prepared by reductive amination of 2-keto-4-phenylbutyric acid with Ala-Ala-Tyr-pAB as described for I and crystallized from ethanol–ethyl acetate. Ala-Ala-Tyr-pAB was prepared from Tyr-pAB (Pozsgay et al., 1986) by stepwise addition of Ala residues (Orlowski et al., 1983) with the *N*-hydroxysuccinimide esters (Anderson et al., 1964) of BOC-Ala. HPLC (solvent A) gave two peaks with retention times of 20.7 and 21.6 min, respectively; TLC (solvent C) gave a single spot with an R_f value of 0.16; mp was 204–206 °C dec. Anal. Calcd for $C_{32}H_{36}O_8N_4$·HCl: C, 60.04; H, 5.83; N, 8.75. Found: C, 59.99; H, 6.03; N, 8.74.

N-[1(RS)-Carboxy-3-phenylpropyl]-Ala-Ala-Tyr(O-benzyl)-pAB (III). III was prepared by reductive amination of 2-keto-4-phenylbutyric acid with Ala-Ala-Tyr(*O*-benzyl)-pAB by the method described for I. Ala-Ala-Tyr(*O*-benzyl)-pAB was prepared from Tyr(*O*-benzyl)-pAB by stepwise addition of Ala residues as described for II. The compound was further purified by crystallization from ethanol. HPLC (solvent A) gave two peaks with retention times of 31.1 and 32.1 min, respectively; TLC (solvent C) gave a single spot with an R_f value of 0.34; mp was 202–203 °C dec. Anal. Calcd for $C_{39}H_{43}O_8N_4$·HCl: C, 65.34; H, 6.14; N, 7.82. Found: C, 65.60; H, 5.95; N, 7.72.

N-[1(RS)-Carboxy-3-phenylpropyl]-Ala-Ala-Tyr(3-iodo)-pAB (IV). IV was synthesized by iodination of II in aqueous solution with 1.2 equiv of sodium iodide with chloramine T (1.2 equiv) as the oxidizing agent. Aliquots of the reaction mixture were tested by HPLC for the appearance of products (solvent A). A double peak (retention times of 32.4 and 33 min) appeared after 2–3 h. Longer reaction times led to the formation of diiodinated derivatives having longer retention times. The monoiodo derivative was isolated by HPLC. The organic solvent was removed by evaporation, and an aqueous solution of the compound was prepared by the addition of 2 equiv of base. The concentration of the inhibitor was determined by digestion with chymotrypsin and measurement of the amount of pAB released after diazotization, as described previously (Goldbarg & Rutenburg, 1958).

N-[1(RS)-Carboxy-3-phenylpropyl]-Ala-Ala-Ala-pAB (V). V was prepared by reductive amination of 2-keto-4-phenylbutyric acid with Ala-Ala-Ala-pAB as described for I. HPLC (solvent A) gave two peaks with retention times of 17.8 and 18.4 min, respectively; TLC (solvent C) gave a single spot with an R_f value of 0.18; mp was 231–232 °C. Anal. Calcd for $C_{26}H_{32}O_7N_4$·HCl: C, 56.88; H, 6.06; N, 10.2. Found: C, 56.98; H, 6.42; N, 10.57.

N-[1(RS)-Carboxy-3-phenylpropyl]-Phe-Ala-Phe-pAB (VI). VI was prepared by reductive amination of 2-keto-4-phenylbutyric acid with Phe-Ala-Phe-pAB as described for I. The crude product was dissolved in a small volume of water by addition of 1 M sodium hydroxide and then applied to the top of an AG 50W-X4 (H^+) column (2.5 × 15 cm) previously equilibrated with a 0.1 M pyridine–formate buffer (pH 3.1). The column was eluted with 150 mL of each of the following buffers: 0.1 M pyridine–formate, pH 3.1; 0.2 M pyridine–formate pH 3.1; 0.2 M pyridine–acetate, pH 4.4. Finally, it was eluted with 600 mL of 2% pyridine. The two diastereomers of IV emerged from the column in the 2% pyridine solution. Fractions containing the product were pooled and evaporated in vacuo to yield a white solid. HPLC (solvent

A) gave two peaks with retention times of 29.9 and 31.5 min; TLC (solvent C) gave a single spot with an R_f value of 0.59; mp was 206–207 °C. Anal. Calcd for $C_{38}H_{40}O_7N_4$·HCl: C, 63.46; H, 5.84. Found: C, 63.59; H, 5.87.

N-[1(RS)-Carboxy-3-phenylpropyl]-Gly-Ala-Phe-pAB (VII). VII was prepared by reductive amination of 2-keto-4-phenylbutyric acid with Gly-Ala-Phe-pAB as described for I. Gly-Ala-Phe-pAB was synthesized as described previously (Orlowski et al., 1983). HPLC (solvent B) gave two peaks with retention times of 29.5 and 30.5 min; TLC (solvent C) gave a single spot with an R_f value of 0.17; mp was 200–202 °C. Anal. Calcd for $C_{31}H_{34}O_7N_4$·HCl: C.60, 87; H, 5.77. Found: C, 61.18; H, 6.15.

Determination of Enzyme Activity and Inhibitory Constants. Enzyme activity was determined with Bz-Gly-Ala-Ala-Phe-pAB as the substrate in a two-step reaction as described previously (Orlowski et al., 1983). The following reactions form the basis of activity determination:

$$\text{Bz-Gly-Ala-Ala-Phe-pAB} \xrightarrow{\text{metalloendopeptidase}} \text{Bz-Gly} + \text{Ala-Ala-Phe-pAB}$$

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

The enzyme cleaves the Gly–Ala bond of the substrate. After completion of the reaction, the incubation mixture is heated to 100 °C for 2 min in order to inactivate the metalloendopeptidase. Addition of aminopeptidase M causes degradation of the reaction product, Ala-Ala-Phe-pAB, by the aminopeptidase with the release of *p*-aminobenzoate, which is determined after diazotization. Endopeptidase 24.11 was determined by the method previously described (Orlowski & Wilk, 1981).

All synthesized inhibitors were resistant to degradation by isolated endopeptidase 24.15. In inhibition experiments, enzyme concentrations were 1 nM, and the incubation time was 60 min. For all inhibitors, several K_i determinations were carried out by the method of Dixon (1953; plot $1/v$ versus [I]), at three different substrate concentrations and at least six different inhibitor concentrations, with a linear regression computer program. Coefficients of determination (r^2) of better than 0.99 were obtained throughout. The type of inhibition was also determined for several inhibitors from double-reciprocal Lineweaver–Burk (Lineweaver & Burk, 1934) plots of the reaction velocity versus substrate concentration in the presence and absence of inhibitor. These plots were also used for K_i determinations from the relationship $K_m' = K_m[1 + ([I]/K_i)]$, where K_m' is the apparent K_m determined in the presence of inhibitor. For several inhibitors, K_i values were also calculated from the equation $IC_{50} = (1 + S/K_m)K_i$ in experiments in which the inhibitor concentration required for 50% inhibition (IC_{50}) was determined. A close agreement was found for the K_i values obtained with all three methods.

For tight binding inhibitors the assumption that the concentrations of free inhibitor is equal to the total inhibitor concentration ($[I] = [I]_{total}$) may not be correct, since the total inhibitor concentration is depleted by the fraction which binds to the enzyme. Goldstein (1944) has demonstrated that the ratio e/K_i, where e is equal to the total enzyme concentration, should be 0.1 or less for the Michaelis–Menten equation to be validly applied. For the most potent inhibitors (see inhibitors **2** and **3**, Table I) these values were 0.037 and 0.062, respectively. We have nevertheless applied to these inhibitors the kinetic analyses for tight binding inhibitors using the linear plots (at three substrate concentrations) as suggested by Henderson (1972). K_i values determined from the slopes of the plots were essentially identical (within 10%) with those obtained by the method of Dixon (1953).

Results

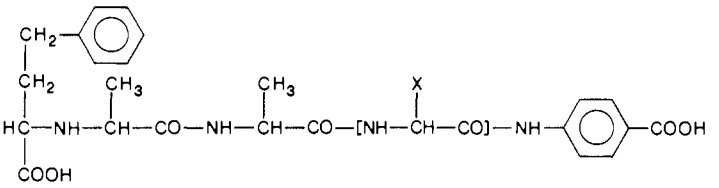
A series of inhibitors derived from reductive amination of 2-keto-4-phenylbutyrate with peptides capable of binding to the substrate recognition site of endopeptidase 24.15 were synthesized and tested for inhibition of the enzyme from rat brain. Reductive amination of the keto acid generates a new chiral center. Accordingly, two diastereomers are obtained during synthesis. All experiments described here were performed with mixtures of the *R* and *S* diastereomers in approximately equal concentrations. Experiments have shown that both diastereomers are inhibitory and that racemic mixtures give K_i values that are intermediate beween those obtained with the *R* and *S* forms, respectively. For example, a racemic mixture of *N*-[1-carboxy-2-phenylethyl]-Ala-Ala-Phe-pAB (inhibitor 2, Table III) had a K_i value of 1.94 μM. The isolated more potent diastereomer (presumably the *S* form) had a K_i of 0.81 μM while the less active diastereomer had a K_i of 7.4 μM. This ninefold difference in inhibitory potency indicates the presence of some stereospecificity of inhibitor binding (Chu & Orlowski, 1984). Similar experiments carried out with separated diastereomers of the most potent inhibitor, *N*-(1-carboxy-3-phenylpropyl)-Ala-Ala-Tyr-pAB (inhibitor 3, Table I), again showed that both the *R* and *S* diastereomers were inhibitory. The more active diastereomer had a K_i 2 times lower than that of its less active counterpart, again indicating some stereospecificity of binding, although replacement of the phenylethyl side group of the inhibitor with the phenylpropyl group led to some decrease in binding stereospecificity.

Experiments showed that all inhibitors acted in a competitive manner. Thus, inhibition was reversible and could be overcome by excess substrate. Furthermore, the same degree of inhibition was obtained in experiments in which the enzyme was preincubated with the inhibitor for 15 min, and the reaction was then initiated by addition of substrate, as in those in which inhibition was determined directly without preincubation, in mixtures containing substrate, enzyme, and inhibitor. This indicated that the dissociation of the enzyme–inhibitor complex is a rapid equilibrium process. Double-reciprocal plots obtained in the presence and absence of inhibitor gave straight lines intersecting at the *y* axis. This is consistent with the competitive nature of inhibition, with the inhibitor having no effect on the maximal velocity but causing an increase in K_m.

Previous studies of specificity with synthetic substrates (Orlowski et al., 1983) have shown that the presence of a hydrophobic or bulky group in the P_3' position favors binding and increases reaction rates. This is also evident from the effect of structural changes in this position on enzyme inhibition (Table I). The lowest K_i (16 nM) was obtained with a tyrosine residue in this position. Some decrease in inhibition was seen when the Tyr residue was replaced by a Phe. A rather pronounced decrease in inhibition was obtained by replacing the Tyr residue by an Ala residue. Indeed, this change caused a decrease in the inhibitory potency by more than 20-fold, showing the importance of this position for inhibitor binding. That there are size restrictions on the residues binding at this site is indicated by the finding that iodination of the phenol ring of tyrosine led to some increase in K_i and that a 10-fold decrease in inhibition was obtained when the phenolic hydrogen was substituted by a benzyl group.

The effect of structural changes in the part of the inhibitor molecule interacting with the S_1' subsite of the active site is summarized in Table II. The lowest K_i was obtained with

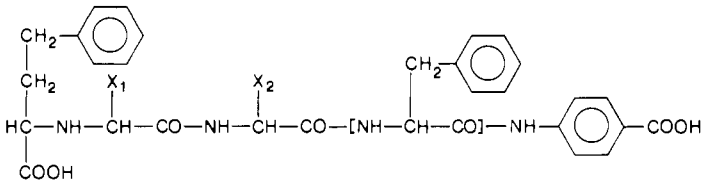
Table I: Inhibition of Rat Brain Endopeptidase 24.15 by Substrate-Related *N*-(1-Carboxy-3-phenylpropyl) Peptide Derivatives: Influence of Variations in the P_3' Position of the Inhibitor[a]



peptide moiety	X	[I] (μM)	$K_i \pm$ SE (μM)
(1) -Ala-Ala-Ala-pAB	CH_3	0.2–1.6	0.34 ± 0.38
(2) -Ala-Ala-Phe-pAB	$CH_2C_6H_5$	0.01–0.1	0.027 ± 0.003
(3) -Ala-Ala-Tyr-pAB	$CH_2C_6H_4OH$	0.007–0.053	0.016 ± 0.007
(4) -Ala-Ala-Tyr(*O*-Bzl)-pAB	$CH_2C_6H_4OCH_2C_6H_5$	0.10–0.8	0.15 ± 0.017
(5) -Ala-Ala-Tyr(3-iodo)-pAB	$CH_2C_6H_3IOH$	0.15–0.75	0.024

[a] Activity was determined with Bz-Gly-Ala-Ala-Phe-pAB as described under Materials and Methods. K_i values were obtained from Dixon plots at three substrate concentrations and at six different inhibitor concentrations. Data are mean values ± SEM from three to six determinations.

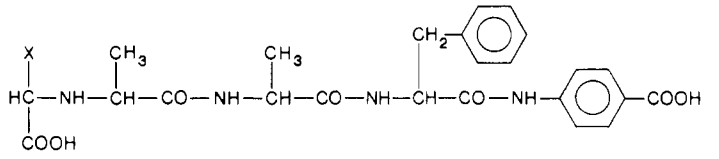
Table II: Inhibition of Rat Brain Endopeptidase 24.15 by Substrate-Related *N*-(1-Carboxy-3-phenylpropyl) Peptide Derivatives: Influence of Variations in the P_1' and P_2' Positions of the Inhibitor[a]



peptide moiety	X_1	X_2	[I] (μM)	$K_i \pm$ SE (μM)
(1) -Gly-Ala-Phe-pAB	H	CH_3	1.5–15	1.8 ± 0.07
(2) -Ala-Ala-Phe-pAB	CH_3	CH_3	0.01–0.1	0.027 ± 0.003
(3) -Phe-Ala-Phe-pAB	$CH_2C_6H_5$	CH_3	0.08–0.81	0.081 ± 0.01

[a] Experimental details are the same as those given in Table I.

Table III: Inhibition of Rat Brain Endopeptidase 24.15 by Carboxymethyl Derivatives of Ala-Ala-Phe-p-AB: Influence of Structural Variations in the *N*-(Carboxymethyl) Moiety of the Inhibitor[a]



	X	$K_i \pm$ SE (μM)
(1) carboxy-3-phenylpropyl	$CH_2CH_2C_6H_5$	0.027 ± 0.0026
(2) carboxy-2-phenylethyl	$CH_2C_6H_5$	1.94 ± 0.65[b]
(3) carboxybutyl	$CH_2CH_2CH_3$	5.17 ± 0.67[b]
(4) carboxyethyl	CH_3	20.9 ± 2.3[b]
(5) carboxymethyl	H	69.4 ± 7.7[b]
(6) *N*-(phenylethyl)[c]	$CH_2C_6H_5$	2300 ± 700

[a] Activity was determined with Bz-Gly-Ala-Ala-Phe-pAB as described under Materials and Methods. [b] Data from Chu and Orlowski (1984). [c] Compound 6 differs from 2 by the absence of the carboxyl group.

the inhibitor containing an Ala residue in the P_1' position. Replacement of the Ala residue by either a Gly or Phe residue led to an increase in K_i. Indeed, a change from an Ala residue to a Gly caused a more than 60-fold increase in K_i. Replacement of the Ala residue by a Phe also led to an increase in K_i, indicating that the nature of the residue in the P_1' position is important for the primary specificity of the enzyme.

A summary of inhibition constants obtained as a function of variations in size and hydrophobicity of the *N*-(carboxymethyl) moiety of the inhibitor is presented in Table III. This moiety contains the zinc-coordinating carboxyl group and apparently interacts with the S_1 subsite of the active site. A progressive and great increase in K_i is seen as the size and hydrophobicity of the group are decreased. Thus, the most potent inhibitor of this series, *N*-[1(*RS*)-carboxy-3-phenylpropyl]-Ala-Ala-Phe-pAB (inhibitor 1, Table III), had a K_i more than 2500 times lower than that of the corresponding carboxymethyl derivative. It is of interest that elongation of the phenylethyl side chain by a single methylene group caused a more than 60-fold increase in inhibitory potency. These data strongly suggest the presence of a hydrophobic binding pocket at the S_1 subsite and also show the importance of this site for inhibitor binding. The presence of the zinc-coordinating carboxyl group is essential for inhibition since deletion of this group causes a dramatic decrease in inhibitory potency. Thus, *N*-(phenylethyl)-Ala-Ala-Phe-pAB (compound 6), which has the same structure as the carboxyphenylethyl derivative (compound 3) but differs by the absence of the carboxyl group, has a K_i higher by more than 3 orders of magnitude.

The inhibitors described here were also studied with respect to their effect on the activity of endopeptidase 24.11. This enzyme, having a thermolysin-like specificity, cleaves bonds on the amino side of hydrophobic amino acid residues. Accordingly, it could be expected that some of the inhibitors

Table IV: Inhibition of Membrane-Bound, Thermolysin-like Metalloendopeptidase (Endopeptidase 24.11) by Inhibitors of Endopeptidase 24.15[a]

inhibitor	[I] (μM)	K_i (μM)
(1) *N*-[1(*RS*)-carboxy-3-phenylpropyl]-Ala-Ala-Phe-pAB	10–100	17 (0.027)
(2) *N*-[1(*RS*)-carboxy-3-phenylpropyl]-Ala-Ala-Tyr-pAB	16–160	35 (0.016)
(3) *N*-[1(*RS*)-carboxy-3-phenylpropyl]-Phe-Ala-Phe-pAB	1–10	1.4 (0.081)
(4) *N*-[1(*RS*)-carboxy-3-phenylpropyl]-Gly-Ala-Phe-pAB	4–40	9 (1.8)
(5) *N*-[1(*RS*)-carboxy-3-phenylpropyl]-Ala-Ala-Ala-pAB	10–1000	>100 (0.34)

[a] Activity was determined with Glt-Ala-Ala-Phe-2NA as described previously (Orlowski & Wilk, 1981). K_i values were obtained from Dixon plots. Data are mean values from two to three determinations. Values in parentheses represent the K_i values for inhibition of endopeptidase 24.15.

studied here could be degraded by this enzyme through a cleavage of such bonds. Experiments have shown that the Phe-pAB and Tyr-pAB moieties are in fact slowly cleaved by the enzyme from the respective inhibitors. The data summarized in Table IV show that all the endopeptidase inhibitors having an aromatic residue in the P_1' or P_3' position had an inhibitory effect on the activity of endopeptidase 24.11. Replacement of the Phe residue in the P_3' position by an Ala residue led to a great increase in K_i, indicating that the Phe residue binds to the hydrophobic pocket at the S_1' subsite of the enzyme, known to be the determinant of its primary specificity (Pozsgay et al., 1986). The potency of inhibition of endopeptidase 24.11 by this group of inhibitors is, however, much lower than that of endopeptidase 24.15. Thus, for example *N*-[1(*RS*)-carboxy-3-phenylpropyl]-Ala-Ala-Tyr-pAB inhibited endopeptidase 24.11 with a K_i more than 3 orders of magnitude higher than that for endopeptidase 24.15. A similar result was obtained for the inhibitor containing a Phe residue in place of Tyr. The potency of inhibition of endopeptidase 24.11 did not parallel that of endopeptidase 24.15. Indeed, *N*-[1(*RS*)-carboxy-3-phenylpropyl]-Phe-Ala-Phe-pAB was the most effective inhibitor of endopeptidase 24.11 ($K_i = 1.4 \times 10^{-6}$ M), but one of the least effective against endopeptidase 24.15. This finding also indicates that inhibition of endopeptidase 24.11 is probably mainly related to the action of these inhibitors as substrates for the enzyme rather than to their zinc-coordinating properties. This could be rationalized on the basis of the known primary structure (Titani et al., 1972) and crystal structure of thermolysin (Matthews et al., 1972, 1974; Colman et al., 1972; Kester & Matthews, 1977; Weaver et al., 1977) and its close relationship with respect to specificity to that of endopeptidase 24.11. It can be postulated on the basis of these studies that binding of the hydrophobic residue, present in the endopeptidase 24.15 inhibitors, to the hydrophobic pocket in the substrate recognition site of endopeptidase 24.11 would place the carboxyl group of the inhibitors beyond the reach of the active site zinc atom of this enzyme. This would explain the weak inhibitory effect on endopeptidase 24.11 of this group of inhibitors.

Discussion

The inhibitor studies reported here show that substrate-related peptides containing a free *N*-(carboxymethyl) group, capable of coordinating with the active site zinc atom, act as inhibitors of endopeptidase 24.15. The potency of inhibition is largely dependent on the nature of residues at the P_1, P_1', and P_3' positions of the inhibitor. The size and hydrophobicity of the residue interacting with the S_1 subsite of the active site affect most dramatically the potency of inhibition. Thus changes in this position from an *N*-(carboxymethyl) to an *N*-(carboxyphenylpropyl) group lowered the K_i values by more than 3 orders of magnitude, indicating that residues binding to this subsite of the enzyme should be the main determinants of primary specificity. Other determinants that had a distinct effect on the potency of inhibition were related to the nature of residues interacting with S_1' and S_3' subsites of the active site. A change at S_1' from an Ala residue to a Gly caused an increase in K_i by a factor of 65, and a smaller but distinct decrease in inhibition was also observed when the Ala residue in this position was changed to a Phe. Finally, the data indicate that the nature of the residue at the S_3' subsite also has a distinct effect on K_i, with hydrophobic or aromatic residues being favored in this position.

Table V: Selected Substrate Data for Endopeptidase 24.15[a]

peptide	K_m (mM)	k_{cat}/K_m (s^{-1} M^{-1})
-P_1-P_1'-P_2'-P_3'		
BOC-Phe-Ala-Ala-Phe-pAB	0.22	9.77×10^4
Bz-Gly-Ala-Ala-Phe-pAB	0.49	9.73×10^3
Bz-Gly-Ala-Ala-Leu-pAB	2.5	1.24×10^3
Bz-Gly-Ala-Ala-Gly-pAB	3.10	3.70×10^2
Bz-Gly-Gly-Ala-Phe-pAB	2.00	7.30×10^2
-Gly-Phe-Ser-Pro-Phe- (bradykinin)	0.051	3.0×10^5
-Phe-Met-Arg-Arg-Val- (BAM-12P)	0.39	4.6×10^4
-Phe-Leu-Arg-Lys-Tyr- (β-neoendorphin)	0.038	2.4×10^5

[a] Data from Orlowski et al. (1983) and Chu and Orlowski (1985). The amino acid sequences of the natural peptides represent partial sequences at the site of cleavage. BAM = bovine adrenal medulla dodecapeptide.

Previous studies on the specificity of brain endopeptidase 24.15, both with synthetic and with natural peptides, have shown that the enzyme has an extended substrate recognition site capable of binding at least five amino acid residues. Selected substrate data on the interaction of the enzyme with synthetic and natural peptides are presented in Table V. These data show a pattern consistent with the conclusion that the potency of inhibition is related both to the K_m and to the k_{cat}/K_m ratio of the corresponding substrates. Thus a change from a Phe to a Gly residue at the P_1 position of the substrate causes both a decrease in binding affinity (increase in K_m) and a decrease in the specificity constants (k_{cat}/K_m). Also consistent with the inhibitor data is the finding that a decrease in size and hydrophobicity at the P_3' position of the substrate and a change from an Ala to a Gly residue at P_1' had a similar effect. The importance of hydrophobic residues in the P_3' position for activity is also evident from studies with natural peptides, since those peptides having a Phe or Tyr residue in this position (bradykinin and β-neoendorphin) show a lower K_m and a higher k_{cat}/K_m ratio than those having other residues in this position (Val).

On the basis of the substrate and inhibitor data it can be concluded that the primary specificity of the enzyme is directed toward substrates having hydrophobic residues at the P_1 position and that a hydrophobic residue is also favored in the P_3' position. This specificity is apparently imposed by the presence of hydrophobic binding pockets at both these sites. A scheme of the active site of the enzyme and its interaction with the best of the inhibitors is shown in Figure 1.

It is of interest to note that this specificity is quite different from that of endopeptidase 24.11 ("enkephalinase"), a related zinc metalloendopeptidase widely distributed in animal tissues. This enzyme, highly concentrated in the kidney but only weakly active in brain, exhibits a thermolysin-like specificity,

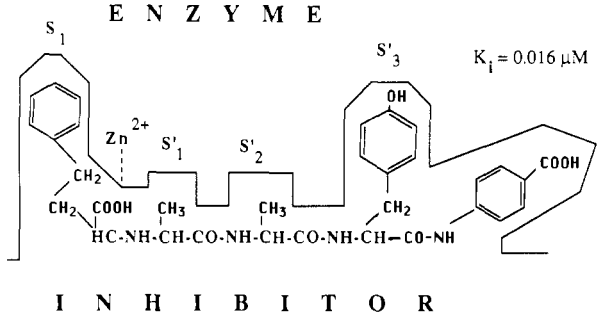


FIGURE 1: Scheme of interaction of the best of the inhibitors with the inactive site of endopeptidase 24.15.

in that it cleaves bonds on the amino site of hydrophobic residues. Studies on the interaction of this enzyme with substrates and inhibitors (Pozsgay et al., 1986) indicate that this specificity is imposed by the presence of a hydrophobic binding pocket at the S_1' subsite of the enzyme, quite similar to that present in thermolysin. The differences in the specificity of the two enzymes is also underlined by the fact that inhibitors of endopeptidase 24.11 have no effect on endopeptidase 24.15 activity and that inhibitors of this latter enzyme are only weakly inhibitory toward endopeptidase 24.11.

Inhibitors of zinc metalloendopeptidases are of interest because of their potential pharmacological properties related to the function of these enzymes. Thus, inhibitors of angiotensin converting enzyme (Antonaccio & Cushman, 1981; Patchet et al., 1980) have found application in the treatment of hypertension and congestive heart failure, and inhibitors of endopeptidase 24.11 seem to have analgesic properties (Roques et al., 1980; Murthy et al., 1984; Fournie-Zaluski et al., 1985). The physiological function of endopeptidase 24.15 in brain is not sufficiently explored. The finding, however, that the enzyme is the major factor in degradation of neurotensin by synaptic membranes (Checkler et al., 1985) and that both the soluble and membrane-bound forms of the enzyme rapidly convert larger precursor peptides into Leu- and Met-enkephalin (Chu & Orlowski, 1985; Acker et al., 1986) indicates the potential use of inhibitors of endopeptidase 24.15 in studies on the pharmacological effects associated with the inhibition of this enzyme.

REFERENCES

Acker, G. R., Molineaux, C., & Orlowski, M. (1986) *J. Neurochem. 48*, 284–292.

Almenoff, J., & Orlowski, M. (1983) *Biochemistry 22*, 590–599.

Almenoff, J., Wilk, S., & Orlowski, M. (1981) *Biochem. Biophys. Res. Commun. 102*, 206–214.

Anderson, G. W., Zimmerman, J. E., & Callahan, F. M. (1964) *J. Am. Chem. Soc. 86*, 1839–1842.

Antonaccio, M. J., & Cushman, D. W. (1981) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 40*, 2275–2284.

Borch, F. B., Bernstein, M. D., & Durst, H. D. (1971) *J. Am. Chem. Soc. 93*, 2897–2904.

Checler, F., Vincent, J. P., & Kitabgi, P. (1985) *J. Neurochem. 45*, 1509–1513.

Chu, T. G., & Orlowski, M. (1984) *Biochemistry 23*, 3598–3603.

Chu, T. G., & Orlowski, M. (1985) *Endocrinology (Baltimore) 116*, 1418–1425.

Colman, P. M., Jansonius, J. N., & Matthews, B. W. (1972) *J. Mol. Biol. 70*, 701–724.

Cushman, D. W., Cheung, H. S., Sabo, E. F., & Ondetti, M. A. (1977) *Biochemistry 16*, 5484–5491.

Dixon, M. (1972) *Biochem. J. 129*, 197–202.

Fournie-Zaluski, M. C., Coulaud, A., Bouboutou, R., Chaillet, P., Devin, J., Waksman, G., Costentin, J., & Roques, B. P. (1985) *J. Med. Chem. 28*, 1158–1169.

Fulcher, I. S., Matsas, R., Turner, A. J., & Kenny, A. J. (1982) *Biochem. J. 203*, 519–522.

Goldbarg, J. A., & Rutenburg, A. M. (1958) *Cancer 11*, 283–291.

Goldstein, A. (1944) *J. Gen. Physiol. 27*, 529–580.

Henderson, P. J. F. (1972) *Biochem. J. 127*, 321–333.

Holmquist, B., & Vallee, B. L. (1979) *Proc. Natl. Acad Sci. U.S.A. 76*, 6216–6220.

Kam, C. M., Nishino, N., & Powers, J. C. (1979) *Biochemistry 18*, 3032–3038.

Kerr, M. A., & Kenny, A. J. (1974) *Biochem. J. 137*, 447–488.

Kester, W. R., & Matthews, B. W. (1977) *Biochemistry 16*, 2506–2516.

Lineweaver, H., & Burk, D. (1934) *J. Am. Chem. Soc. 56*, 658–666.

Matthews, B. W., Colman, P. M., Jansonius, J. N., Titani, K., Walsh, K. A., & Neurath, H. (1972) *Nature (London) 238*, 41–43.

Matthews, B. W., Weaver, L. H., & Kester, W. R. (1974) *J. Biol. Chem. 249*, 8039–9044.

Murthy, L. R., Glick, S. D., Almenoff, J., Wilk, S., & Orlowski, M. (1984) *Eur. J. Pharmacol. 102*, 305–313.

Nishino, N., & Powers, J. C. (1978) *Biochemistry 17*, 2846–2850.

Orlowski, M., & Wilk, S. (1981) *Biochemistry 20*, 4942–4950.

Orlowski, M., Michaud, C., & Chu, T. G. (1983) *Eur. J. Biochem. 135*, 81–88.

Patchett, A. A., Harris, E., Tristram, E. W., Wyvratt, M. J., Wu, M. T., Taub, D., Petyerson, E. R., Ikeler, T. J., ten-Broeke, J., Payne, L. G., Ondeyka, D. L., Thorsett, E. D., Greenlee, W. J., Lohr, N. S., Hoffsommer, R. D., Joshua, H., Ruyle, W. V., Rothrock, J. W., Aster, S. D., Maycock, A. L., Robinson, F. M., Hirschmann, R., Sweet, C. S., Ulm, E. H., Gross, D. M., Vassil, T. C., & Stone, C. A. (1980) *Nature (London) 288*, 286–288.

Pfleiderer, G. (1970) *Methods Enzymol. 19*, 514–521.

Pozsgay, M., Michaud, C., Liebman, M., & Orlowski, M. (1986) *Biochemistry 25*, 1292–1299.

Roques, B. P., Fournie-Zaluski, M. C., Soroca, E., Lecomte, J. M., Malfroy, B., Llorens, C., & Schwartz, J. C. (1980) *Nature (London) 288*, 286–288.

Titani, K., Hermodson, M. A., Ericsson, L. H., Walsh, K. A., & Neurath, H. (1972) *Nature (London) 238*, 35–37.

Weaver, L. H., Kester, W. R., & Matthews, B. W. (1977) *J. Mol. Biol. 114*, 119–132.