Substrate and Inhibitor Studies of Thermolysin-like Neutral Metalloendopeptidase from Kidney Membrane Fractions. Comparison with Bacterial Thermolysin†

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ABSTRACT: The inhibitory constants of a series of synthetic *N*-carboxymethyl peptide inhibitors and the kinetic parameters (K_m, k_{cat}, and k_{cat}/K_m) of a series of model synthetic substrates were determined for the membrane-bound kidney metalloendopeptidase isolated from rabbit kidney and compared with those of bacterial thermolysin. The two enzymes show striking similarities with respect to structural requirements for substrate binding to the hydrophobic pocket at the S_1' subsite of the active site. Both enzymes showed the highest reaction rates with substrates having leucine residues in this position while phenylalanine residues gave the lowest K_m. The two enzymes were also inhibited by the same *N*-carboxymethyl peptide inhibitors. Although the mammalian enzyme was more susceptible to inhibition than its bacterial counterpart, structural variations in the inhibitor molecules affected the inhibitory constants for both enzymes in a similar manner. The two enzymes differed significantly, however, with respect to the effect of structural changes in the P_1 and P_2' positions of the substrate on the kinetic parameters of the reaction. The mammalian enzyme showed the highest reaction rates and specificity constants with substrates having the sequence -Phe-Gly-Phe- or -Phe-Ala-Phe- in positions P_2, P_1, and P_1', respectively, while the sequence -Ala-Phe-Phe- was the most favored by the bacterial enzyme. The sequence -Gly-Gly-Phe- as found in enkephalins was not favored by either of the enzymes. Of the substrates having an aminobenzoate group in the P_2' position, the mammalian enzyme favored those with the carboxyl group in the meta position while the bacterial enzyme favored those with the carboxyl group in the para position. The data suggest that although the two enzymes have similar primary specificities apparently imposed by the hydrophobic pocket at the S_1' subsite, they differ markedly at other subsites of the active site.

Membrane-bound neutral metalloendopeptidase (EC 3.4.24.11), first identified and isolated from kidney brush border membrane fractions by Kerr and Kenny (1974a,b), exhibits a thermolysin-like specificity in that it cleaves peptide bonds on the amino side of hydrophobic amino acid residues. Studies in several laboratories (Orlowski & Wilk 1981; Almenoff et al., 1981; Altstein et al., 1981; Fulcher et al., 1982; Almenoff & Orlowski, 1983, 1984) have shown that this enzyme is identical with "enkephalinase", an activity found in brain membrane fractions that cleaves the Gly–Phe bond in Met-enkephalin (Tyr-Gly-Gly-Phe-Met) and Leu-enkephalin (Tyr-Gly-Gly-Phe-Leu). The name enkephalinase was introduced in the belief that the enzyme functions specifically in the degradation of neuronally released enkephalins (Malfroy et al., 1978; Sullivan et al., 1980; Schwartz et al., 1981). Indeed, inhibitors of the enzyme have been reported to have antinociceptive properties (Roques et al., 1980; Murthy et al., 1984), although it is not established that this effect is solely due to a slowdown of enkephalin degradation. The enzyme was shown to degrade many neuropeptides (Almenoff et al., 1981; Mumford et al., 1981), and recent studies have shown that its affinity toward many biologically active peptides is considerably higher than for the enkephalins (Matsas et al., 1984).

Unlike thermolysin whose structure, active site, and mechanism of action are rather well understood on the basis of X-ray diffraction analysis of the crystal structure of the enzyme and its complexes with substrates and inhibitors (Matthews et al., 1972; Colman et al., 1972; Kester & Matthews, 1977a; Weaver et al., 1977), the mammalian enzyme is a glycoprotein which resists crystallization. Accordingly, information about the active site of the enzyme can come only from indirect studies of its interaction with substrates and inhibitors, and from attempts to identify those residues of the active site which participate in the bond-breaking process or contribute to substrate binding. With the possible role of the enzyme in the metabolism of bioactive peptides and the potential pharmacological interest of its inhibitors, such studies acquire both practical and theoretical importance.

We report here kinetic studies on the interaction of the active site of membrane-bound neutral metalloendopeptidase and thermolysin with a series of model synthetic substrates and *N*-carboxymethyl peptide inhibitors. The data suggest that the two enzymes are remarkably similar with respect to the structural requirements for binding to the hydrophobic pockets at the S_1' [1] subsites of the active sites but differ significantly with respect to binding requirements to the other subsites (S_1 and S_2').

Experimental Procedures

Materials. Crystalline thermolysin, *N*,*N*′-dicyclohexylcarbodiimide, *N*-hydroxysuccinimide, BOC derivatives of amino acids, trifluoroacetic acid, *p*-aminobenzoic acid, glyoxylic acid, phenylpyruvate, α-ketovaleric acid, sodium phenylpyruvate, sodium cyanoborohydride, and the 2-naphthylamides of amino acids were obtained from Sigma Chemical Co. (St. Louis, MO). *o*-Aminobenzoic acid and

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[1] 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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m-aminobenzoic acid were obtained from Aldrich Chemical Co. (Milwaukee, WI). Ethyl 2-keto-4-phenylbutanoate was obtained from Chemical Dynamics Corp. (South Plainfield, NJ). Other reagents and solvents were obtained from Fischer Scientific Co. (Pittsburgh, PA). The amino acid arylamides of *p*-aminobenzoate (pAB)[2] and also the oAB and mAB derivatives were synthesized as described previously (Orlowski et al., 1983). Aminopeptidase M was purified from hog kidneys by the method of Pfleiderer (1970) and freed from the contaminating metalloendopeptidase as described previously (Almenoff & Orlowski, 1983).

Synthetic Procedures. A series of peptides containing three amino acid residues and either 2NA or one of the three isomers of aminobenzoic acid bound in amide linkage to the C-terminal amino acid were synthesized in order to map the active site of the two enzymes. The presence of the aromatic amines at the C-terminus facilitated the determination of the rate of cleavage of the peptides by a coupled enzyme assay in the presence of excess aminopeptidase M (EC 3.4.24.11) (see Determination of Enzyme Activity and Kinetic Constants). The N-terminus of the peptides was blocked by a Glt group which increased the solubility of the substrates.

All peptide syntheses were carried out in solution by stepwise elongation from the C-terminus essentially as described previously (Orlowski & Wilk, 1981; Orlowski et al., 1983; Almenoff & Orlowski, 1983). Peptide bonds were formed by the active ester method using *N*-hydroxysuccinimide esters of BOC amino acids, prepared by the method of Anderson et al. (1981). The hydrochlorides or CF_3CO_2H salts of the amino acid arylamides were dissolved in THF or in a mixture of THF and DMF (3:1) and 1 equiv of triethylamine, and a 3% excess of the *N*-hydroxysuccinimide ester of the appropriate BOC amino acid was added. The progress of the reaction was monitored by HPLC analysis of the appearance of the product and disappearance of the starting reactants. Following completion of the reaction, the solvent was removed by flash evaporation, and the residue was dissolved in chloroform and washed twice with water and $KHSO_4$ (0.02 M, pH 2.6), and in the case of 2NA substrates with 0.5 M sodium carbonate. The chloroform layer was dried with anhydrous sodium sulfate, and the solvent was removed in vacuo. The BOC protecting group was removed by treatment with CF_3CO_2H, and the product was isolated by addition of ether as described previously (Orlowski & Wilk, 1981). Further elongation of the peptide was accomplished by repeating the coupling cycle with another *N*-hydroxysuccinimide ester of a BOC amino acid as described above. In the final step, the amino terminus of the peptide was blocked with a Glt group by reacting the peptide with a 5% excess of glutaric anhydride and triethylamine in THF. The products usually crystallized directly from THF or from one of the following solvents: chloroform, ethyl acetate, ethanol, or 2-propanol/ether, and they were then isolated by filtration.

N-[1(*R*,*S*)-Carboxy-2-phenylethyl]-Phe-pAB and its two diastereomers, *N*-[1(*R*,*S*)-carboxy-2-phenylethyl]-Ala-pAB and *N*-[1-carboxy-2-phenylethyl]-Gly-pAB, were synthesized as described previously (Almenoff & Orlowski, 1983; Murthy et al., 1984). All other inhibitors were synthesized by the same reductive amination procedure of aldehydes (glyoxylic acid) or α-keto acids with amino acid amides of pAB. For the synthesis of *N*-[1(*R*,*S*)-carboxy-2-phenylpropyl]-Phe-pAB, ethyl 2-keto-4-phenylbutanoate was converted in methanol to the sodium salt of the free acid by saponification with an equimolar amount of 1 M sodium hydroxide and then reductively aminated with Phe-pAB in a water/methanol mixture (1:2).

Table I: HPLC and Melting Point Data for Substrates and Inhibitors[a]

compound	retention time (min)	gradient	mp (°C)
Glt-Gly-Gly-Phe-2NA	23.4	A	195–197 dec
Glt-Gly-Ala-Phe-2NA	22.8	A	178–180
Glt-Gly-Phe-Phe-2NA	26.4	A	215–216
Glt-Ala-Gly-Phe-2NA	23.6	A	209–211 dec
Glt-Ala-Phe-Phe-2NA	26.0	A	262–263 dec
Glt-Phe-Gly-Phe-2NA	25.6	A	202–204
Glt-Phe-Ala-Phe-2NA	26.4	A	214–215
Glt-Phe-Phe-Phe-2NA	28.2	A	247–249 dec
Glt-Ala-Ala-Gly-2NA	18.2	A	199–201
Glt-Ala-Ala-Ala-2NA	19.6	A	259–261 dec
Glt-Ala-Ala-Phe-oAB	20.4	A	214–216
Glt-Ala-Ala-Phe-mAB	18.8	A	215–217
Glt-Phe-Ala-Phe-oAB	22.8	A	214–216
Glt-Phe-Ala-Phe-mAB	21.6	A	219–221
Glt-Phe-Ala-Phe-pAB	22.8	A	224–226
N-(1-carboxymethyl)-Phe-pAB	16.9	B	217–219
N-[1(*R*,*S*)-carboxyethyl]-Phe-pAB	17.0	B	220–221
N-(1-carboxybutyl)-Phe-pAB	20.0, 20.6	B	264–266, 224–226
N-(1-carboxy-3-phenylpropyl)-Phe-pAB	23.2, 24.4	B	262–263, 244–245
N-(1-carboxy-2-phenylethyl)-Ala-pAB	16.8, 18.4	B	220–221, 246–248
N-(1-carboxy-2-phenylethyl)-Leu-pAB	23.2, 24.8	B	228–229, 216–217
N-(1-carboxy-2-phenylethyl)-Tyr-pAB	24.4, 25.2	B	222–226
N-(1-carboxy-2-phenylethyl)-Trp-pAB	25.2, 26.2	B	234–235

[a] The two retention times and two melting points given for some of the inhibitors refer to separated *S*, *S* and *R*, *S* diastereomers, respectively. Where one value is given for melting points, it refers to the *R*, *S*; *S* diastereomeric mixture. *N*-(1-Carboxymethyl)-Phe-pAB contains only one chiral center, and the diastereomers of *N*-[1(*R*,*S*)-carboxyethyl]-Phe-pAB were not separated. The composition of gradient systems A and B is described under Synthetic Procedures.

The diastereomers of the inhibitors were separated by HPLC as described previously (Almenoff & Orlowski, 1983; Chu & Orlowski, 1984). The diastereomers with the higher inhibitory potency eluted from the column first. They were all assigned the *S*,*S* chirality since single-crystal X-ray diffraction analysis has shown that this configuration is associated with a higher inhibitory potency (Mumford et al., 1982).

Amino acid analyses and HPLC, and in some cases NMR and elemental analyses, were used to determine the purity of substrates and inhibitors as described previously (Orlowski & Wilk, 1981; Orlowski et al., 1983; Almenoff & Orlowski, 1983). All peptides showed on amino acid analysis the correct ratios of amino acids. HPLC analyses were performed on a C_{18} reverse-phase μBondapak column (30 × 0.4 cm) by eluting with a linear gradient established between a 0.1% solution of phosphoric acid and acetonitrile. The initial concentration of acetonitrile was 10%, and its concentration was increased during 20 min to either 60% (gradient A) or 40% (gradient B). Emerging peaks were monitored at 210 nm. All compounds were shown to be pure on the basis of these analyses. The retention times obtained by HPLC and the melting points of the synthesized compounds are summarized in Table I.

[2] Abbreviations: pAB, *p*-aminobenzoate; oAB, *o*-aminobenzoate; mAB, *m*-aminobenzoate; 2NA, 2-naphthylamine; Glt, glutaryl; HPLC, high-pressure liquid chromatography; THF, tetrahydrofuran; CF_3CO_2H, trifluoroacetic acid; DMF, dimethylformamide; BOC, *tert*-butoxycarbonyl; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

Table II: Kinetic Parameters of Hydrolysis of Synthetic Substrates by Membrane-Bound Metalloendopeptidase and Thermolysin: Influence of Variations at the P_1' Position[a]

peptide P_3–P_2–P_1–P_1'–P_2'	[S] (mM)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} M^{-1} × 10^{-4})
		Membrane-Bound Metalloendopeptidase		
Glt-Ala-Ala-Gly-2NA	[0.6–2.0]	1.46 ± 0.08	3.51	0.24
Glt-Ala-Ala-Ala-2NA	[0.3–1.0]	0.97 ± 0.03	98.8	10.2
Glt-Ala-Ala-Leu-2NA	[0.3–1.0]	0.54 ± 0.04	482.0	89.3
Glt-Ala-Ala-Phe-2NA	[0.3–1.0]	0.2 ± 0.02	139.0	69.5
Glt-Ala-Ala-Tyr-2NA	[0.3–1.0]	0.42 ± 0.01	53.7	12.8
		Thermolysin[b]		
Z-Phe-Gly-Ala		7.4	8	1
Z-Phe-Ala-Ala		2.6	22	8
Z-Phe-Leu-Ala		0.72	416	578
Z-Phe-Phe-Ala		0.45	162	360
Z-Phe-Tyr-Ala		0.72	7	10

[a] Data are mean values ±SE from four to six determinations. Values in brackets represent the range of substrate concentrations used for K_m determinations. [b] Data of Morihara and Tsuzuki (1970).

Determination of Enzyme Activity and Kinetic Constants. Enzyme activity toward all substrates was determined in 0.05 M Tris-HCl buffer (pH 7.6) at 37 °C by a coupled enzyme assay in the presence of excess aminopeptidase M as described previously (Orlowski & Wilk, 1981). With Glt-Ala-Ala-Phe-2NA, the reaction proceeds as follows:

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

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

With both the mammalian and bacterial enzymes, there is no release of 2NA unless aminopeptidase is present in the incubation mixture. The same reaction sequence applies to all the substrates studied. The excess of aminopeptidase M assures the quantitative release of the chromogenic group (2NA or aminobenzoate) which is then determined after diazotization (Goldbarg & Rutenburg, 1958).

The steady-state parameters K_m and k_{cat} ($=V/e$, where e = total enzyme concentration) were determined from initial velocity measurements at various substrate concentrations. Strict Michaelis–Menten kinetics were obtained with all substrates. Double-reciprocal plots were obtained by a linear regression program, and correlation coefficients of 0.99 were generally obtained. In the calculation of data, the molecular weight of the enzyme was assumed to be 95 000 with one catalytic site per molecule.

K_i values for most of the inhibitors were determined by the method of Dixon (1953; plot $1/v$ vs. [I] at several substrate concentrations). A representative Dixon plot obtained for *N*-[1(*R*,*S*)-carboxyethyl]-Phe-pAB is shown in Figure 1. For inhibitors with low K_i values (inhibitors 4 and 5, Table VI), the assumption that the concentration of the free inhibitor is equal to the total inhibitor concentration ($[I] = [I]_{total}$) may not be valid. For these inhibitors, the graphical method of Dixon which is valid for potent and less potent inhibitors was used (Dixon, 1972). In all experiments, the enzyme was added to a mixture of the inhibitor, substrate, and buffer. Experiments in which the enzyme was preincubated with inhibitor and buffer (15 min at 37 °C) before addition of the substrate showed no difference in the extent of inhibition, ruling out irreversible inhibition. For all inhibitors tested, inhibition was competitive and could be overcome by high substrate concentrations.

The membrane-bound metalloendopeptidase was purified from rabbit kidneys by a modification of the procedure described previously (Almenoff & Orlowski, 1983). The procedure yielded a homogeneous enzyme preparation with a specific activity somewhat higher than that previously described. After centrifugation of the crude homogenate at 400*g* for 15 min and discarding the resulting precipitate, membrane fractions obtained by centrifugation of the supernatant at 35000*g* for 45 min were used for isolation of the enzyme as described previously (Almenoff & Orlowski, 1983).

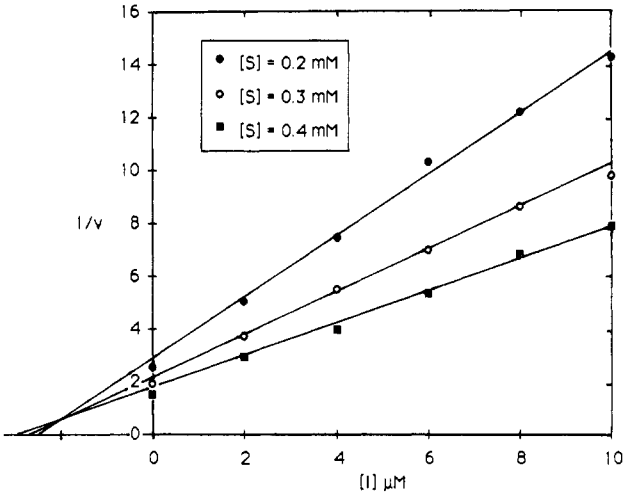


FIGURE 1: Dixon plot for the inhibition of membrane-bound metalloendopeptidase by *N*-[1(*R*,*S*)-carboxyethyl]-Phe-pAB. Substrate and inhibitor were preincubated at 37 °C in a Tris-HCl buffer (0.05 M, pH 7.6), and the reaction was initiated by addition of the enzyme.

RESULTS

The primary specificity of membrane-bound metalloendopeptidase is directed toward peptide bonds on the amino side of hydrophobic amino acid residues. Like its bacterial counterpart, the active site of the mammalian enzyme apparently contains a hydrophobic pocket at the S_1' subsite which interacts with the side chains of hydrophobic amino acid residues. That this is indeed the case is shown in Table II, which summarizes the influence of variations in the amino acid residues in the P_1' position on substrate binding and the rate of reaction. The turnover rate constants (k_{cat}) and specificity constant (k_{cat}/K_m) increase greatly when a Gly residue is replaced by amino acid residues having hydrophobic side chains. Thus, replacement of the Gly residue by a Leu causes a 140-fold increase in the turnover rate constant and a 370-fold increase in the specificity constant. A decrease, however, in these parameters is seen when a Leu residue is replaced by a Phe residue and even more by a Tyr residue. A somewhat different progression is seen when the K_m values of the same

Table III: Kinetic Parameters of Hydrolysis of Synthetic Substrates by Membrane-Bound Metalloendopeptidase (MEP) and Thermolysin (TL): Influence of Substitutions in the P_1 and P_2 Positions[a]

peptide	MEP		TL		k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} M^{-1} × 10^{-4})		
P_3–P_2–P_1–P_1'–P_2'	[S] (mM)	K_m (mM)	[S] (mM)	K_m (mM)	MEP	TL	MEP	TL
Glt-Gly-Gly-Phe-2NA	[0.1–0.3]	0.092 ± 0.01	[0.2–0.8]	0.45 ± 0.01	2.3	0.66	2.5	0.15
Glt-Gly-Ala-Phe-2NA	[0.2–1.32]	0.49 ± 0.02	[0.2–0.8]	0.38 ± 0.01	104.0	19.8	21.2	5.2
Glt-Gly-Phe-Phe-2NA	[0.02–0.2]	0.042 ± 0.01	[0.1–0.66]	0.20 ± 0.01	2.0	88.5	4.8	44.3
Glt-Ala-Gly-Phe-2NA	[0.1–0.8]	0.074 ± 0.01	[0.15–0.5]	0.21 ● 0.01	41.6	57.6	56.2	27.4
Glt-Ala-Ala-Phe-2NA	[0.1–0.8]	0.2 ± 0.01	[0.15–0.8]	0.20 ± 0.03	139.0	267.0	69.5	133.0
Glt-Ala-Phe-Phe-2NA	[0.01–0.2]	0.036 ± 0.01	[0.08–0.33]	0.12 ± 0.06	14.1	605.0	39.2	504.0
Glt-Phe-Gly-Phe-2NA	[0.02–0.18]	0.01 ± 0.005	[0.1–0.5]	0.11 ± 0.01	42.3	65.8	423.0	59.8
Glt-Phe-Ala-Phe-2NA	[0.05–0.15]	0.058 ± 0.003	[0.04–0.5]	0.07 ± 0.01	173.0	229.0	298.0	327.0
Glt-Phe-Phe-Phe-2NA	[0.025–0.1]	0.021 ± 0.007	[0.04–0.17]	0.041 ± 0.001	2.6	58.3	12.3	142.0

[a] Data are mean values ±SE from three to four determinations. Values in brackets represent the range of substrate concentrations used for the K_m determinations.

Table IV: Kinetic Parameters of Hydrolysis of Synthetic Substrates by Membrane-Bound Metalloendopeptidase (MEP) and Thermolysin (TL): *o*-, *m*-, and *p*-Aminobenzoate-Containing Substrates[a]

peptide	MEP		TL		k_{cat} (s^{-1})		k_{cat}/K_m (s^{-1} M^{-1} × 10^{-4})	
P_3–P_2–P_1–P_1'–P_2'	[S] (mM)	K_m (mM)	[S] (mM)	K_m (mM)	MEP	TL	MEP	TL
Glt-Ala-Ala-Phe-oAB	[0.1–0.66]	0.16 ± 0.03	[0.36–2.0]	1.2 ± 0.28	7.1	0.047	4.4	0.004
Glt-Ala-Ala-Phe-mAB	[0.1–0.66]	0.25 ± 0.02	[0.72–4.0]	2.7 ± 0.15	84.4	38.9	33.8	1.4
Glt-Ala-Ala-Phe-pAB	[0.1–0.66]	0.13 ± 0.01	[0.72–4.0]	2.6 ± 0.25	12.6	132.0	9.7	5.1
Glt-Phe-Ala-Phe-oAB	[0.1–0.5]	0.07 ± 0.01	[0.36–2.0]	0.28 ± 0.01	0.99	0.55	1.4	2.0
Glt-Phe-Ala-Phe-mAB	[0.1–0.5]	0.25 ± 0.03	[0.36–2.0]	0.63 ± 0.09	99.9	56.6	39.9	9.0
Glt-Phe-Ala-Phe-pAB	[0.1–0.5]	0.19 ± 0.01	[0.36–2.0]	1.10 ± 0.2	17.0	161.0	8.9	14.6

[a] Data are mean values ±SE from three to four determinations. Values in brackets represent the range of substrate concentrations used for the K_m determinations.

substrates are considered. These values, believed to express the binding affinity of the substrate, decrease progressively in the transition from a Gly residue to a Phe residue only to increase when the Phe residue is replaced by a Tyr residue. Accordingly, the highest reaction rates were obtained with the substrate having a Leu residue in the P_1' position, but the lowest K_m was obtained with that having a Phe residue. Apparently, an increase in the size of the hydrophobic residue from Leu to Phe while improving binding causes a decrease in the catalytic efficiency of the reaction. This decrease is even more pronounced with the Tyr-containing substrate, suggesting the presence of size restrictions on residues binding to the hydrophobic pocket that are compatible with efficient catalysis.

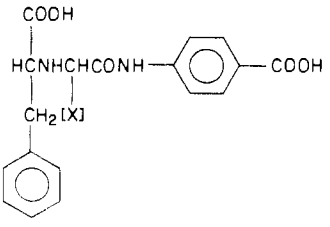
Table II also contains selected substrate data obtained by Morihara and Tsuzuki (1970) with thermolysin for a similar series of substrates. It is clear that changes in the amino acid residues in the P_1' position of the substrates have a similar effect on the kinetic parameters of both the bacterial and the mammalian enzyme.

The influence of changes of residues in the P_1 and P_2 positions of the substrate on the kinetic parameters of the mammalian enzyme and thermolysin is shown in Table III. In general, the K_m values with almost all substrates are lower for the mammalian enzyme than those for thermolysin. With the Phe residue in the P_1' position held constant, a change of a Gly residue in the P_1 position to an Ala produces a pronounced increase in the turnover rate constant and specificity constant of both enzymes. A further change to a Phe residue in this position causes a precipitous decrease in k_{cat} values for the mammalian enzyme. No such decrease is seen, however, for thermolysin. Indeed, the k_{cat} and k_{cat}/K_m ratios or thermolysin tend to increase when the Ala residue in the P_1 position is replaced by a Phe residue provided that the P_2 position is occupied by either a Gly or an Ala residue. The highest k_{cat} and k_{cat}/K_m values were obtained for thermolysin with Glt-Ala-Phe-Phe-2NA, a substrate having Phe residues in both the P_1' and P_1 positions. An additional Phe residue in the P_2 position, however, caused a decrease in the reaction rate. It is of interest that while the presence of a Phe residue in the P_1 position affected the reaction rate of the two enzymes in a divergent manner, this residue caused a marked decrease in K_m values in both enzymes, suggesting an increased binding affinity for each of the enzymes. With a Gly or Ala residue present in the P_1 position, there is a distinct tendency in both enzymes for an increase in the reaction rate with a change in the P_2 position from a Gly residue to an Ala and a Phe residue. It is notable that relatively low k_{cat} and k_{cat}/K_m ratios were obtained with substrates having Gly residues in both the P_1 and P_2 positions, a sequence seen in the enkephalins.

In an attempt to determine whether the chromogenic group in the P_2' position of the substrate affects the rate of the reaction, the uncharged 2-naphthylamide group (Table III) was replaced by the isomers of aminobenzoate. A comparison of the reaction rates for both enzymes (Table IV) indicates that the position of the carboxylate group on the phenyl ring greatly affects the rate of the reaction and that the two enzymes are affected in a quite different manner. While the membrane-bound enzyme gave the highest reaction rates and the highest specificity constant with *m*-aminobenzoate-containing substrates, the bacterial enzyme was optimally active with *p*-aminobenzoate-containing substrates. This suggests that the presence of the carboxyl group influences the alignment of the scissile bond of the substrate with the groups in the active site participating in the bond-breaking process. It is conceivable that this occurs through the interaction of the carboxylate ion with a positively charged group near the S_2' subsite of the two enzymes. Thus, differences in the kinetic parameters between the two enzymes might reflect differences in the position of this positively charged group in the active sites.

It was of interest to determine whether the substrate data presented here have predictive value in terms of structural requirements for inhibitors of the two enzymes. On the basis of the observation that Phe-pAB (Almenoff & Orlowski, 1983) inhibits the mammalian enzyme with a K_i in the micromolar range, we have synthesized a series of *N*-carboxymethyl de-

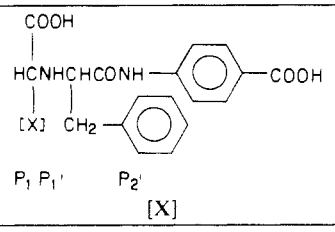
Table V: Inhibition of Membrane-Bound Metalloendopeptidase (MEP) and Thermolysin (TL) by *N*-1-Carboxy-2-phenylethyl Derivatives of Amino Acid Amides of *p*-Aminobenzoate: Influence of Substitutions in the P_1' Position on Inhibitory Constants[a]



[X]	diastereomers	inhibitory constants (M) MEP [I] (μM)	MEP K_i	TL [I] (μM)	TL K_i
(1) Gly		[40–200]	1.1×10^{-4}	[800–4000]	3.9×10^{-3}
(2) Ala	*R, S; S*	[20–100]	2.2×10^{-5}	[80–400]	3.6×10^{-4}
(3) Leu	*R, S; S*	[2.0–10]	2.6×10^{-6}	[20–100]	2.6×10^{-5}
(4) Phe	*S, S*	[0.02–0.1]	2.7×10^{-8}	[0.2–3]	2.3×10^{-6}
(4) Phe	*R, S*	[0.5–2.5]	3.9×10^{-7}	[2–30]	4.0×10^{-5}
(4) Phe	*R, S; S*	[0.02–0.1]	7.1×10^{-8}	[2–30]	7.0×10^{-6}
(5) Tyr	*R, S; S*	[10–50]	1.2×10^{-5}	[800–4000]	7.0×10^{-4}
(6) Trp	*S, S*	[20–100]	2.4×10^{-5}	[800–4000]	1.6×10^{-3}

[a] Glt-Ala-Ala-Phe-2NA (0.2–0.8 mM) was used as substrate for the determination of K_i values. Data are mean values of three to four determinations. Values in brackets represent the range of inhibitor concentrations used for the determination of K_i values.

Table VI: Inhibition of Membrane-Bound Metalloendopeptidase (MEP) and Thermolysin (TL) by *N*-Carboxymethyl Derivatives of Phe-*p*-aminobenzoate: Influence of Substitutions in the P_1 Position on Inhibitory Constants[a]



[X]	diastereomers	inhibitory constants (M) MEP [I] (μM)	MEP K_i	TL [I]	TL K_i
(1) –H	*S*	[2–10]	5.1×10^{-6}	[20–100]	5.4×10^{-5}
(2) $-CH_3$	*R, S; S*	[2–10]	2.0×10^{-6}	[5–25]	2.3×10^{-5}
(3) $-CH_2CH_2CH_3$	*S, S*	[0.5–2.5]	2.7×10^{-7}	[1–5]	2.4×10^{-6}
	R, S	[1–5]	1.8×10^{-6}	[16–80]	4.0×10^{-5}
	R, S; S	[0.5–2.5]	3.8×10^{-7}	[1–5]	4.4×10^{-6}
(4) $-CH_2C_6H_5$	*S, S*	[0.02–0.1]	2.7×10^{-8}	[0.8–4.0]	2.3×10^{-6}
	R, S	[0.5–2.5]	3.9×10^{-7}	[20–100]	4.0×10^{-5}
	R, S; S	[0.02–0.1]	7.1×10^{-8}	[2–10]	7.0×10^{-6}
(5) $-CH_2CH_2C_6H_5$	*S, S*	[0.02–0.1]	2.1×10^{-8}	[0.2–0.1]	2.6×10^{-7}
	R, S	[0.5–2.5]	3.7×10^{-7}	[1–10]	2.2×10^{-6}
	R, S; S	[0.02–0.1]	3.8×10^{-8}	[0.4–2]	8.5×10^{-7}

[a] Glt-Ala-Ala-Phe-2NA (0.2–0.8 mM) was used as substrate for the determination of K_i values. Data are mean values of three to four determinations. Values in brackets represent the range of inhibitor concentrations used for the determination of K_i values.

rivatives of amino acid-*p*-aminobenzoates and tested their inhibitory potency toward both the membrane-bound metalloendopeptidase and thermolysin. Table V summarizes the inhibitory constants of *N*-1-carboxy-2-phenylethyl derivatives of amino acid amides of *p*-aminobenzoate. The pAB and the phenylethyl group in these inhibitors are presumed to bind to the S_2' and S_1 subsites, respectively, while the carboxylate group coordinates with the active-site zinc atom. It can be seen that the inhibitory potency for both enzymes increases progressively with the replacement of a Gly residue in the P_1' position with residues having greater hydrophobic side chains, reaching a maximum with the inhibitor containing a Phe residue. Replacement, however, of a Phe by a Tyr residue causes a pronounced decrease in the inhibitory potency which is even more marked with the introduction of a Trp residue. This effect is clearly visible with both enzymes. Apparently, there are similar size restrictions on residues capable of binding to the hydrophobic pocket in the S_1' subsite of both enzymes.

With both enzymes, the inhibitory potency of the *S, S* diastereomers is about 1 order of magnitude higher than that of the corresponding *R, S* diastereomers, whereas the potency of the *R, S; S* diastereomers is intermediate. Although the pattern of inhibition associated with changes in residues in the P_1' position of the inhibitors was the same for both enzymes, the K_i values for thermolysin were higher by 1–2 orders of magnitude than those toward the mammalian enzyme.

The effect of structural variations in that part of the inhibitor molecule expected to bind to the S_1 subsite of the enzyme on the inhibitory potency is summarized in Table VI. The potency of inhibition increases with the increase in size and hydrophobicity of the residue binding to the S_1 subsite. Thus, the K_i values decrease with the change in the side chain of the inhibitor from the methyl through the ethyl, butyl, and phenylethyl to the phenylpropyl group. The highest inhibitory potency was obtained with inhibitors having a phenylethyl or phenylpropyl side chain. This is of interest since such groups can be considered as related to a Phe residue in the corresponding position (P_1) of substrates. As was discussed previously (Table III), such groups did not favor catalysis in the case of the mammalian enzyme but gave high turnover rate constants with thermolysin. With both enzymes, however, substrates with a Phe residue in the P_1 position tended to have lower K_m values than those with either Gly or Ala residues in the same position.

Discussion

The membrane-bound metalloendopeptidase and bacterial thermolysin share a common primary specificity in that both enzymes cleave peptide bonds on the amino side of hydrophobic amino acid residues. Elucidation of the amino acid sequence of thermolysin (Titani et al., 1972) and X-ray diffraction analysis of its crystal structure and its complexes with inhibitors (Matthews et al., 1972, 1974; Colman et al., 1972; Kester & Matthews, 1977a; Weaver et al., 1977) have led to a good understanding of the topography of its active site. This work also became the basis for a proposed mechanism of peptide bond hydrolysis catalyzed by the enzyme. Some extensions of this mechanism were recently proposed on the basis of an interactive computer graphics analysis of peptide bond cleavage catalyzed by the enzyme (Hangauer et al., 1984). This study also led to rationalization of the kinetic parameters obtained during earlier specificity studies of the enzyme with model peptide substrates (Morihara & Tsuzuki, 1970). Although no comparable data are available for the mammalian enzyme, it could be assumed that a detailed kinetic analysis of its interaction with substrates and inhibitors could provide some information about the topography of the active site of the enzyme with the data for thermolysin serving as a comparison.

Like other proteases, the membrane-bound enzyme has an extended substrate binding site accommodating at least four amino acid residues. Analysis of the data obtained with substrates in which the residues binding to the S_1', S_2', S_1, and S_2 subsites were systematically varied showed some similarities, as well as distinct differences, between the two enzymes.

The kinetic parameters obtained with substrates in which the residues binding to the hydrophobic pocket at the S_1' subsite of the mammalian enzyme were varied showed that a Leu residue gave the highest k_{cat} whereas Phe residues gave the lowest K_m. Replacement of the Phe residue by a Tyr led to a marked increase in K_m and to a decrease in the turnover rate constant. These results are very similar to those obtained with thermolysin by Morihara and Tsuzuki (1970). Crystallographic studies of inhibitor binding to thermolysin led to the conclusion that there are size restrictions on residues binding to the hydrophobic pocket of the enzyme and that the phenolic group of Tyr is likely to generate bad contacts with the bottom of the hydrophobic cleft which may result in a misalignment between the scissile bond and the bond-breaking residues of the enzyme, leading to a decrease in k_{cat} (Kester & Matthews, 1977a; Hangauer et al., 1984). Our substrate data obtained with the membrane-bound metalloendopeptidase (Table II) and also with the corresponding inhibitors (Table V) suggest the presence of similar size restrictions in the mammalian enzyme. Thus, the two enzymes seem to be quite similar with respect to the dimensions of the hydrophobic pocket at the S_1' subsite. Indeed, there is a strong correlation ($r = 0.94$) between the potency of inhibitors of the two enzymes as a function of changes of residues in the P_1' position. One can speculate that these similarities might be the result of a convergent evolutionary process, or alternately point to a high degree of preservation of this part of the enzyme structure that forms the hydrophobic pocket should the two enzymes be derived from a common ancestral gene. Kester and Matthews (1977b) considered this possibility with respect to thermolysin and carboxypeptidase A because of similarities in the positioning of about 22 atoms in the binding pockets of the two enzymes, although the three-dimensional organization of the structural elements beyond these residues is quite different (Liebman & Weinstein, 1984). While there is some correspondence in the spatial arrangement in the two enzymes of crucial residues participating in the catalytic process, there is an absence of the degree of structural homology found in most serine proteases of both mammalian and bacterial origin (Subtilisin being an exception). Accordingly, Kester and Matthews (1977b) concluded that the similarities between the two enzymes are the result of a "convergent rather than divergent evolution". The specificity, however, of thermolysin resembles much more closely the specificity of the membrane-bound metalloendopeptidase than that of carboxypeptidase A. It is therefore possible that elucidation of the primary structure of the enzyme could reveal a degree of homology between the two endopeptidases not found for thermolysin and carboxypeptidase A.

In contrast with the restrictions on the size of residues capable of binding to the S_1' subsite of thermolysin, no such restrictions seem to be imposed on residues binding to the S_1 subsite (Kester & Matthews, 1977a). Indeed, in agreement with this conclusion, the data show that the highest k_{cat} and k_{cat}/K_m ratios were obtained with substrates having Phe residues in this position, providing that the residue in the P_2 position was represented by either an Ala or Gly residue. The presence of a Phe residue in the P_1 position, however, had a distinctly different effect on the kinetic parameters of the mammalian metalloendopeptidase. This residue caused a pronounced decrease in the turnover rate constant that was independent of the kind of residue present in the P_2 position, suggesting the presence of definite size restrictions on residues binding at the S_1 subsite. Indeed, the highest reaction rates were obtained with substrates having an Ala residue in this position. It is of interest, however, that with both enzymes the lowest K_m values were obtained with a Phe residue in the P_1 position, suggesting that this residue gives the highest binding affinity.

Consistent with the data reported by Morihara and Tsuzuki (1970) for thermolysin, in a set of substrates in which the amino acid residue in the P_2 position was represented by a Gly, Ala, or Phe residue the highest reaction rates were obtained with those having an Ala residue, although the lowest K_m values were obtained for Phe residues. Similar low K_m values were obtained for the mammalian enzyme when the P_2 position was represented by a Phe residue. Also, substrates with this residue gave both high reaction rates and low K_m values when the P_1 position was represented by either an Ala or Gly residue. Accordingly, the highest specificity constant for thermolysin was obtained with a substrate having the sequence -Ala-Phe-Phe- in positions P_1, P_2, and P_1', respectively, while the corresponding sequence for the membrane-bound metalloendopeptidase was -Phe-Gly-Phe-. It is interesting to note that recent work by Matsas et al. (1984) has shown that substance P, which has a similar sequence (-Phe-Gly-Leu-) at the site of hydrolysis, was the most rapidly hydrolyzed, among a series of neuropeptides, and that this peptide also had the lowest K_m. By contrast, the sequence -Gly-Gly-Phe- found in the enkephalins was not favored. These results suggest that our substrate data obtained with model synthetic substrates are relevant for the prediction of the specificity of the enzyme with natural peptide substrates.

It is of interest that replacement of the 2NA group in the P_2' position by the isomers of aminobenzoate had a pronounced effect on the kinetic parameters of the reaction and that the position of the carboxyl group giving optimal reaction rates was different for the two enzymes. Thus, while the mammalian enzyme gave the highest reaction rates with the mAB-containing substrates, the bacterial enzyme showed the highest activity with the pAB-containing substrates. It is likely that this effect is caused by an interaction between the charged carboxyl group on the aromatic ring and a positively charged

group near the S_2' subsite of the mammalian enzyme. The presence of such a group was also suggested on the basis of studies showing that inhibitors having a β-alanine or a γ-aminobutyrate residue in the P_2' position had lower K_i values than those having a Gly residue in the same position (Mumford et al., 1982).

Inhibitors of zinc metallopeptidases are of interest because of their pharmacological effects which are related to the in vivo function of these enzymes. Thus, inhibitors of angiotensin converting enzyme have found application in the treatment of hypertension and congestive heart failure (Antonaccio & Cushman, 1981), and inhibitors of the membrane-bound metalloendopeptidase discussed here show analgesic properties (Roques et al., 1980; Murthy et al., 1984). Zinc metallopeptidases show marked similarities in their basic mechanism of action, differences between these enzymes being mainly related to substrate specificity and structural requirements for efficient binding to the active site. Accordingly, principles of design of metallopeptidase inhibitors are based on the need for the presence of a group fulfilling the binding requirements of the substrate recognition site and the presence of a group capable of coordinating with the active-site zinc atom (Holmquist & Vallee, 1979).

Several types of thermolysin inhibitors have been synthesized. These contain a group capable of binding to the substrate recognition site of the enzyme and one of several groups such as thiol, phosphoramidate, phosphonamidate, carboxyl, or hydroxamic acid capable of coordinating with the active-site zinc atom (Kam et al., 1979; Nishino & Powers, 1979; Maycock et al., 1981; Bartlett & Marlowe, 1983). Because of the similarity in the primary specificity of thermolysin and the mammalian membrane-bound metalloendopeptidase, inhibitors of one enzyme could be expected to exert an inhibitory effect also on the other. The data of Mumford et al. (1982) and also those reported here (Table V) show that this is indeed the case. Marked differences, however, can be observed between the inhibitory potency of the same inhibitors toward the two enzymes. Thus, the data in Table V clearly show that the potency of thermolysin inhibition was lower than that of the mammalian enzyme by 1–2 orders of magnitude. The reasons for this are not clear, but it is notable that the K_m values obtained for almost all the model substrates were uniformly lower for the mammalian enzyme than for thermolysin (see Tables III and IV). This suggests that the potency of the inhibitors may be to a great extent related to the binding affinity of the corresponding substrates. That this is indeed the case is shown by the finding that replacement of a Gly residue in the P_1' position by a Phe residue increased the potency of inhibitors by a factor of 4000 for the mammalian enzyme and a factor of 1600 for thermolysin (Table V). This conclusion is also consistent with the decrease in K_m of the corresponding substrates induced by replacement of a Gly residue by a Phe. That the potency of inhibitors is related to the binding affinity of the corresponding substrates is also indicated by the finding that the lowest K_i values were obtained with inhibitors having phenylethyl or phenylpropyl groups in the P_1 positions. This is in agreement with the finding of the lowest K_m values for substrates having a Phe in this position.

Bartlett and Marlowe (1983) have shown for a series of phosphonamidate inhibitors of thermolysin the presence of a strong correlation between the inhibitory potency and the k_{cat}/K_m values of corresponding substrates, suggesting that these inhibitors are transition-state analogues. Monzingo and Matthews (1984) determined by X-ray crystallography the mode of binding to thermolysin of *N*-(1-carboxy-3-phenylpropyl)-L-leucyl-L-tryptophan, a potent inhibitor of this enzyme (Maycock et al., 1981), and concluded that the mode of inhibitor binding has much in common with the presumed transition state in peptide hydrolysis. Our substrate and inhibitor data show a strong correlation ($r = 0.96$) between the K_i of thermolysin inhibitors and the k_{cat}/K_m ratios of the corresponding substrates, a finding consistent with such a suggestion.

While the kinetic analysis presented here has used the nomenclature of Schechter and Berger (1967) to describe the positions of substrate residues and subsites in the enzyme, other potentially significant factors need to be considered. Peptides of the size used in this study can exhibit characteristic conformational preferences similar to those assumed by natural peptides, as shown by the analysis of amphiphilicity of peptide helices (Blanc & Kaiser, 1984). A tetrapeptide, for example, could form an amphiphilic helix presenting a tightly wound conformation. Alternately, the same peptide may assume an extended conformation. The resulting surfaces would present different pairs of amino acids to the active groove of the enzyme. An additional factor related to peptide conformation is the surface accessibility of the peptide bond to be cleaved. It can be noted, although not generalized, that the tetrapeptides (Table II) expected to have the greatest tendency to attain a helical conformation (Chou & Fasman, 1978) are the ones which tend to give high k_{cat} values. Other factors that should be considered in probing the multidimensional aspects capable of influencing K_m and k_{cat} include the potential of dipolar interactions in the binding site (Warshel, 1981), the relative stability of conformations vs. their ease of interconversions (Hangauer et al., 1984), and the complementation of the electrostatic potential surfaces and van der Waals surfaces of both the peptide and the residues in the active cleft. Examination of these factors could further contribute to the understanding of the interactions between enzyme and substrate in the active site of the endopeptidase.

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Kinetic Studies of L-Aspartase from *Escherichia coli*: Substrate Activation†

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ABSTRACT: The enzyme L-aspartase from *Escherichia coli* was observed to have a time lag during the production of aspartic acid from fumarate and ammonia. This time lag is pH dependent, with little lag observed below pH 7.0 and a very extensive lag observed above pH 8.0. This time lag was also found to be dependent on both substrate and divalent metal ion concentrations and on the degree of proteolysis of L-aspartase. The observed lag, in the reaction examined in the amination direction, has been found to be correlated with the nonlinear kinetics seen at higher pH in the deamination direction. Both phenomena are consistent with a model in which there is a separate activator site for the substrate, L-aspartic acid, that is distinct from the enzyme active site. Occupation of this site by the substrate, or by various substrate analogues, eliminates both the nonlinearity and the time lag. The D isomer of aspartic acid, which does not bind at the active site, can bind at this newly identified activator site.

The enzyme L-aspartase (L-aspartate ammonia-lyase, EC 4.3.1.1) catalyzes the reversible deamination of L-aspartic acid to yield fumaric acid and ammonia. The enzyme from *Escherichia coli* is composed of four apparently identical subunits with a molecular weight of about 48 000. Limited proteolysis of the enzyme with trypsin (Mizuta & Tokushige, 1975), as well as several other proteases (Yumoto et al., 1982), results in a severalfold increase in L-aspartase activity measured at pH 7.0. The divalent metal ion requirements and the circular dichroism and UV spectra of the enzyme are also changed on trypsinolysis (Mizuta & Tokushige, 1976). These changes appear to be concomitant with the loss of one or several small peptides, seven to eight amino acids in length, from the carboxyl-terminal end of the polypeptide chain (Yumoto et al., 1980, 1982).

L-Aspartase displays complex kinetics at higher pH, with positive cooperativity observed at pH 7.5 and above (Williams and Lartigue, 1967; Rudolph & Fromm, 1971). Williams & Scott (1968) have reported that certain nucleotide effectors either activate (AMP, IMP) or inhibit (GTP, UTP) L-as-

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