Mapping the Active Site of Meprin-A with Peptide Substrates and Inhibitors†

Russell L. Wolz,‡ Robert B. Harris,§ and Judith S. Bond*,‡

Department of Biochemistry, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061-0308, and Department of Biochemistry and Molecular Biophysics, Virginia Commonwealth University, Richmond, Virginia 23298-0614

Received April 17, 1991; Revised Manuscript Received June 12, 1991

ABSTRACT: The extended substrate-binding site of meprin-A, a tetrameric metalloendopeptidase from brush border membranes of mouse kidney proximal tubules, was mapped with a series of peptide substrates. Previous studies led to the development of the chromogenic substrate Phe^5(4-nitro)bradykinin for meprin-A. With this substrate, several biologically active peptides were screened as alternate substrate inhibitors, and, of these, bradykinin (RPPGFSPFR) was found to be the best substrate with a single cleavage site (Phe^5–Ser^6). Three types of bradykinin analogues were used for a systematic investigation of substrate specificity: (1) nonchromogenic bradykinin analogues with substitutions in the P3 to P3′ subsites were used as alternative substrate inhibitors of nitrobradykinin hydrolysis, (2) analogues of nitrobradykinin with variations in the P1′ position were tested as substrates, and (3) intramolecularly quenched fluorogenic bradykinin analogues with substitutions in the P1 to P3 sites were tested as substrates. A wide variety of substitutions in P1′ had little effect on K_M (174–339 μM) but markedly affected k_{cat} (51.5 s^{-1} = A > S > R > F > K > T > E = 0). Substitutions in P1 had a greater effect on K_M (366 μM–2.46 mM) and also strongly affected k_{cat} (98.5 s^{-1} = A > F ≫ L > E > K = 2.4 s^{-1}). The variety of allowed cleavages indicates that meprin-A does not have strict requirements for residues adjacent to the cleavage site. Substitutions farther from the scissle bond also affected binding and hydrolysis, demonstrating that multiple subsite interactions are involved in meprin-A action. It is proposed that conformational constraints at the X^6–Pro^7 bond affect bradykinin hydrolysis. A general preference for the naturally occurring bradykinin core sequence was observed, which is consistent with the possibility that bradykinin is a physiological substrate for meprin-A. A total of 15 amino acid hydroxamates were tested for inhibition of meprin-A and had K_i values ranging from 24 μM for tyrosine hydroxamate to 1.8 mM for β-aspartic acid hydroxamate. The bradykinin product analogue peptides AcRPGY and AcRPGY-NHOH were found to be very good inhibitors of meprin-A hydrolysis with K_i values of 15.6 and 3.7 μM, respectively. Both tetrapeptides inhibited via a simple noncompetitive mechanism, suggesting the possible existence of regulatory binding sites.

Meprin-A is a metalloendopeptidase in the brush border membrane of kidney proximal tubules of many mouse strains (Bond & Beynon, 1986; Craig et al., 1987). The enzyme was first identified by its hydrolytic activity at basic pH against the nonspecific protein substrate azocasein (Beynon et al., 1981). From previous studies it was found that a minimum substrate chain length of seven residues is required for peptide hydrolysis and that meprin-A has a general preference, although not a requirement, for cleavage of peptide bonds flanked by amino acids with hydrophobic side chains (Butler et al., 1987). The vasodilatory peptide bradykinin, RPPGFSPFR, is cleaved by meprin-A exclusively at the Phe^5–Ser^6 bond. Recently, a chromogenic derivative of bradykinin, Phe^5(4-nitro)bradykinin, was developed as the first peptide substrate for meprin-A that allows continuous monitoring of hydrolysis, thus providing a sensitive method to study its kinetics (Wolz & Bond, 1990).

Whereas the enzyme has been well characterized with regard to structure (Butler et al., 1987), subcellular localization (Craig et al., 1987), and genetics (Beynon & Bond, 1983; Bond et al., 1984), its physiological function(s) and substrates(s) remain unidentified. The extended substrate-binding site of meprin-A is mapped in the present work in order to gain some insight into the role of the enzyme in vivo.

To further understand its function, it is also important to develop a specific inhibitor for meprin-A. To date, only general inhibitors of metalloproteases such as the metal chelators EDTA and 1,10-phenanthroline are known to affect meprin-A activity. The most effective and most specific inhibitors are often structural analogues of substrates and/or products. Thus, to compliment the substrate studies, a series of amino acid hydroxamates, known to coordinate with the metal at the active site of metalloproteases, was tested for meprin-A inhibition. The results highlight the interactions of peptide substrates and inhibitors with meprin-A.

Materials and Methods

Enzyme. Meprin-A was purified from frozen ICR male mouse kidneys (Rockland Farms, Inc., Gilbertsville, PA) as previously described (Wolz & Bond, 1990).

Protein concentration was determined by the method of Lowry et al. (1951), with bovine serum albumin as a standard, or estimated by absorbance at 280 nm on the basis of $A^{0.1\%}$ = 1.0.

Peptides. The peptides listed in Tables I and II and the amino acid hydroxamates in Table V were purchased from the Sigma Chemical Co. (St. Louis, MO).

The chromogenic and fluorogenic substrates listed in Tables III and IV were synthesized by solid-phase methods on a Milligen/Bioresearch model 9600 synthesizer using diisopropylcarbodiimide as the coupling reagent and methylene chloride and dimethylformamide as the reaction solvents. All syntheses were done with 0.5 g of BOC-Arg(Tos)-substituted Merrifield resin (345–420 μmol/g; Advanced ChemTech).

† This work was supported in part by grants from the National Institutes of Health (DK 19691) and The Jeffress Memorial Trust (J-221).

* Address correspondence to this author.
‡ Virginia Polytechnic Institute and State University.
§ Virginia Commonwealth University.

0006-2960/91/0430-8488$02.50/0

Following synthesis, the peptide was cleaved from the peptide–resin in 8 mL of anhydrous HF containing 1 mL of anisole (45 min, 0 °C) and extracted with methanol, 50% (v/v) methanol in water, water, and 50% (v/v) acetic acid in water. The solvent was removed under reduced pressure, and the peptide was recovered by lyophilization from water. The peptide was purified by preparative reverse-phase HPLC on a Shimadzu LC-8A system.

Two potential inhibitor peptides, AcRPGY and AcRPGY-NHOH, were prepared by coupling Tyr-OCH_3 in solution phase to AcRPG, previously synthesized by the above solid-phase methods. For this reaction, dicyclohexylcarbodiimide was the coupling reagent, and the solvent was dimethylformamide. After completion (as judged by the extent of ninhydrin reaction), the reaction mixture was acidified, and the product, AcRPGY-OCH_3, was extracted into ethyl acetate and purified by preparative reverse-phase HPLC. At this point, half of the peptide preparation was saponified to AcRPGY in 40% (v/v) methanol/water with 1 M NaOH, and the remaining peptide was converted to the hydroxamate derivative with NH_2OH. Each of the peptides was then purified from their respective reaction mixtures by preparative reverse-phase HPLC. For the hydroxamate derivative, the presence of the hydroxamate functional group was verified by colorimetric reaction with $FeCl_3$ (Hestrin, 1949).

All peptides were obtained as acetate salts and were generally soluble in aqueous buffer to greater than 5 mM.

Assays. To follow meprin-A activity during purification and in some inhibition studies, an azocasein hydrolysis assay at pH 9.5 was used as previously described (Reckelhoff et al., 1985). Where noted, this assay was also performed in 50 mM ethanolamine/HCl buffer, pH 8.7. One unit of activity is defined as a change in absorbance at 340 nm of 0.001 per min.

Hydrolysis rates of some peptides were determined by HPLC. For these analyses, a stock solution of peptide was diluted to the desired concentration with buffer, then enzyme was added to initiate the reaction. Aliquots were withdrawn from the reaction mixture at various times, and the reaction was stopped by addition to 0.1 volume of 10% acetic acid. Reaction components were separated by reverse-phase chromatography on a Shimadzu liquid chromatography system mounted with an Axxiom octadecylsilane column (5 μm, 4.6 × 250 mm). Peptides were eluted with a linear gradient from 3.5% B to 55% B over 50 min at a flow rate of 1 mL/min. Solvent A was 0.1% trifluoroacetic acid in water, and solvent B was 0.075% trifluoroacetic acid in acetonitrile. Elution was monitored by the absorbance at 220 nm, and peak areas were determined by integration. Product concentrations were calculated by defining the peak areas measured after complete hydrolysis as equal to the initial substrate concentration. Initial rates were calculated from values obtained during the first 10% or less of substrate hydrolysis, and activity was expressed as change in substrate or product concentration per unit time.

Products were identified from amino acid compositions of the isolated peptides. A Waters HPLC amino acid analyzer employing the pico-tag method of precolumn phenylthiocarbamyl derivitization was used (Bidlingmeyer et al., 1984).

Hydrolysis of nitrobradykinin (Wolz & Bond, 1990) and related chromogenic substrates was monitored continuously by measuring the absorbance change at 310 nm in a Shimadzu UV160-A spectrophotometer with the gain amplified 10-fold, allowing the measurement of absorbance to 10^{-4}. Typically, the reaction mixture contained substrate buffered in 50 mM ethanolamine buffer, pH 8.7, and the reaction was initiated with 1.4 μg/mL of meprin-A (16 nM enzyme at the subunit M_r of 85 000). Product [Arg-Pro-Pro-Gly-Phe(NO_2)] concentration was calculated on the basis of the measured $\Delta\epsilon_{310}$ = 750 M^{-1} cm^{-1}. Initial rates were calculated from the absorbance change during the first 10% or less of substrate hydrolysis, and activity was expressed as the change in product concentration per unit time.

The intramolecularly quenched fluorogenic substrates of the general structure 2ABz-RPGXSPFnR (Table III; Soler & Harris, 1988) were characterized in a Perkin-Elmer LS-3 fluorescence spectrophotometer interfaced with a CompuAdd 212 computer. The 2-aminobenzoyl (2ABz)[1] group attached to the N-terminal fluoresces when excited at 320 nm, and the emitted light (maximum at 420 nm) is absorbed by the nitrophenylalanine (Fn) in the C-terminal half of the peptide. Cleavage of the peptide between the groups relieves the quenching, and the increase in fluorescence at 420 nm can be monitored continuously. For the peptides tested here, the increase after complete hydrolysis was approximately 2.5-fold relative to the uncleaved peptide.

For meprin-A reactions, substrate was buffered to pH 8.7 with 50 mM ethanolamine-HCl, and the reaction was initiated by the addition of 1.4–10.6 μg/mL of meprin-A (16–125 nM at the subunit M_r of 85 000). The maximum fluorescence change for each concentration of each substrate was determined by allowing the reaction to proceed to completion as judged by a stable final fluorescence intensity. Initial velocities were calculated from the fluorescence change during the first 10% or less of substrate hydrolysis and expressed as change in concentration per unit time.

To calculate kinetic constants, initial velocity data were fitted directly to the Michaelis–Menten equation by nonlinear regression. Calculations also were performed by using the double-reciprocal linear transform method of Lineweaver–Burk. For a given experiment, values from the two fitting methods agreed to within 5%. For a given plotting method, separate experiments yielded K_M values that agreed to within 6%.

Inhibition by alternative substrates, amino acid hydroxamates, and peptide inhibitors was determined by assaying meprin-A in the presence of 5–8 concentrations of inhibitor convering a range of at least 25–75% residual activity. The concentration of 50% inhibition, ID_{50}, was determined by direct nonlinear fitting and by linear least-squares regression fitting to the Dixon linear transformation plot of inverse velocity against inhibitor concentration (Dixon, 1953).

Results

Substrates. The interaction of meprin-A with a variety of biologically active peptides was tested by two methods: (1) binding was assessed by inhibition of nitrobradykinin hydrolysis, and (2) hydrolysis rates were determined directly by HPLC (Table I). The recent development of nitrobradykinin as a chromogenic substrate for meprin-A allowed the rapid screening of many peptides by using them as inhibitors of nitrobradykinin hydrolysis. The concentration at which 50% inhibition is seen, ID_{50}, reflects the binding of the test peptide to the enzyme. If a simple competitive mechanism of inhibition is assumed, K_i can be calculated from ID_{50} by the equation

$$K_i = ID_{50}[K_{mnob}/(K_{mnob} + [NOB])] \quad (1)$$

[1] Abbreviations: 2ABz, 2-aminobenzoyl; Ac, acetyl; ACTH, adrenocorticotropic hormone; Clp, 4-chlorophenylalanine; Fn or Npa, 4-nitrophenylalanine; HPLC, high-pressure liquid chromatography; Hyp, hydroxyproline; LH-RH, luteinizing hormone releasing hormone (gonadotropin releasing hormone); Thi, thienylalanine.

Table I: Meprin-A Action on Biologically Active Peptides[a]

peptide[b]	K_i (μM)	k_{cat} (s^{-1})	k_{cat}/K_i (M^{-1} s^{-1})
α-melanocyte stimulating hormone, acetyl-Ser-Tyr-Ser↓Met-Glu-His-Phe-Arg-Trp-Gly↓Lys-Pro-Val	36	34.5[c]	9.6×10^5
neurotensin, pyroGlu-Leu-Tyr-Glu↓Asn↓Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu-NH_2	68	7.6[c]	1.1×10^5
luteinizing hormone releasing hormone, Glu-His-Trp↓Ser↓Tyr-Gly↓Leu-Arg-Pro-Gly	292	30.4[c]	1.0×10^5
angiotensin I, Asp-Arg-Val-Tyr↓Ile-His-Pro-Phe-His↓Leu	552	0.64[d]	1.2×10^3
[des Asp]angiotensin I, Arg-Val-Tyr↓Ile-His-Pro-Phe-His↓Leu	246	0.14[d]	5.6×10^2
angiotensin II, Asp-Arg-Val-Tyr↓Ile-His-Pro-Phe	740	0.15[d]	2.0×10^2
angiotensin III, Arg-Val-Tyr↓Ile-His-Pro-Phe	477	0.10[d]	2.0×10^2

[a] K_i values were determined from inhibition of nitrobradykinin hydrolysis. Initial velocities for peptide hydrolysis were determined by HPLC, and k_{cat} was calculated by substituting K_i for K_m in the Michaelis–Menten equation. [b] (↓) Cleavage site. [c] Rate of substrate disappearance. [d] Rate of product appearance.

Table II: Meprin-A Inhibition by and Hydrolysis of Bradykinin Analogues[a]

peptide[b]	K_i (μM)	k_{cat} (s^{-1})	k_{cat}/K_i (M^{-1} s^{-1})
Arg-Pro-Pro-Gly-Phe↓Ser-Pro-Phe-Arg	520	22.0	4.2×10^4
Ac-Arg-Pro-Pro-Gly-Phe↓Ser-Pro-Phe-Arg	593	54.3	9.2×10^4
Arg-Pro-Gly-Phe↓Ser-Pro-Phe-Arg	383	63.7	1.7×10^5
Arg-Pro-Pro-Gly-**Clp**↓Ser-Pro-**Clp**-Arg	282	26.6	9.4×10^4
Arg-Pro-**Hyp**-Gly-Phe↓Ser-Pro-Phe-Arg	134	2.4	1.8×10^4
Arg-Pro-Pro-Gly-Phe↓**Thr**-Pro-Phe-Arg	127	0.4	3.1×10^3
D**Arg**-Arg-Pro-**Hyp**-Gly-**Thi**↓Ser-D**Phe**-**Thi**-Arg	422	0.2	5.3×10^3
Arg-Pro-Pro-Gly-Phe-Ser-D**Phe**-Phe-Arg	410	0	0

[a] K_i values were determined from inhibition of nitrobradykinin hydrolysis. Initial velocities for peptide hydrolysis were determined by HPLC, and k_{cat} was calculated by substituting K_i for K_M in the Michaelis–Menten equation. [b] Abbreviations: Clp, 4-chlorophenylalanine; Thi, thienylalanine; Hyp, hydroxyproline; ↓, cleavage site.

Table III: Effect of Variation of P1′ Position on Meprin-A Hydrolysis of Nitrobradykinin[a]

peptide[b]	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})
Arg-Pro-Pro-Gly-Npa↓**Ser**-Pro-Phe-Arg	290	40.9	1.41×10^5
Arg-Pro-Pro-Gly-Npa↓**Ala**-Pro-Phe-Arg	331	51.5	1.56×10^5
Arg-Pro-Pro-Gly-Npa↓**Arg**-Pro-Phe-Arg	174	19.6	1.13×10^5
Arg-Pro-Pro-Gly-Npa↓**Phe**-Pro-Phe-Arg	226	7.6	3.34×10^4
Arg-Pro-Pro-Gly-Npa↓**Lys**-Pro-Phe-Arg	182	5.0	2.75×10^4
Arg-Pro-Pro-Gly-Npa-**Glu**-Pro-Phe-Arg	339[c]	0	0

[a] Hydrolysis was monitored spectrophotometrically and values for K_M and k_{cat} were determined from initial velocities at varying substrate concentrations. [b] Abbreviations: Npa, 4-nitrophenylalanine; ↓, cleavage site. [c] K_i value determined from inhibition of nitrobradykinin hydrolysis.

Table IV: Effect of Variation in P Positions on Meprin-A Hydrolysis of Fluorogenic Bradykinin Analogues[a]

peptide[b]	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})
2ABz-Arg-Pro-Gly-Phe↓Ser-Pro-Npa-Arg	366	81.2	2.22×10^5
2ABz-Arg-Pro-**Ile**-Phe↓Ser-Pro-Npa-Arg	296	132.7	4.48×10^5
2ABz-Arg-**Hyp**-Gly-Phe↓Ser-Pro-Npa-Arg	183	26.7	1.46×10^5
2ABz-Arg-**Gly**-**Pro**-Phe↓Ser-Pro-Npa-Arg	220	1.1	4.81×10^3
2ABz-Arg-Pro-Gly-**Ala**↓Ser-Pro-Npa-Arg	1380	98.5	6.92×10^4
2ABz-Arg-Pro-Gly-**Glu**↓Ser-Pro-Npa-Arg	1220	4.9	4.05×10^3
2ABz-Arg-Pro-Gly[3]↓**Lys**[1]↓Ser-Pro-Npa-Arg	402	2.4[c]	6.07×10^3
2ABz-Arg-Pro-Gly[1]↓**Leu**[5]↓Ser-Pro-Npa-Arg	2460	12.0[c]	4.87×10^3

[a] Hydrolysis was monitored fluorometrically and values for K_M and k_{cat} were determined from initial velocities at varying substrate concentrations. [b] Abbreviations: 2ABz, 2-aminobenzoyl; Hyp, hydroxyproline; Npa, 4-nitrophenylalanine; ↓, cleavage site. [c] Two cleavage sites; $k_{cat} = -d[S]/dt$. The numbers preceding the arrows indicate the proportion of cleavage.

where K_{mnob} is the Michaelis–Menten constant for the chromogenic substrate nitrobradykinin (290 μM) and [NOB] is the initial concentration of this substrate (usually 100 μM).

Peptides (with their respective K_i values) that were screened as inhibitors included renin inhibitor (140 μM), substance-P (240 μM), vasopressin (0.7 mM), Kemptide (1.4 mM), Leu-enkephalin (2.2 mM), ACTH fragment 34–39 (1.5 mM), ACTH fragment 5–10 (3.6 mM), neurotensin fragment 1–8 (>5 mM), and neurotensin fragment 8–13 (>5 mM).

Reaction mixtures of meprin-A with the peptides listed in Table I were also analyzed by HPLC to observe hydrolysis and determine initial velocities. The K_i values determined from the inhibition experiments can be assumed to be equivalent to K_{mi}, the Michaelis–Menten constant for the competing alternative substrate. Hence, the catalytic rate constant, k_{cat}, can be estimated from a single initial velocity, v_o (determined by HPLC for meprin-A and peptide alone), by substituting K_{mi} (determined from inhibition of nitrobradykinin hydrolysis) into the rearranged Michaelis–Menten equation

$$k_{cat} = [v_o(K_{mi} + S_o)]/(E_oS_o) \quad (2)$$

where S_o and E_o are, respectively, the concentration of peptide and meprin-A in the HPLC reaction.

The results in Table I reveal a wide range of binding and catalytic constants. Of these, α-melanocyte stimulating hormone had the highest k_{cat}/K_i ratio (equivalent to the specificity ratio) of 10^6 M^{-1} s^{-1}, and neurotensin and luteinizing hormone releasing hormone (LH-RH) also were good substrates for meprin-A. All three of these peptides, however, were cleaved at multiple sites and thus were not suitable for use in an active site mapping study. Bradykinin was found to have the highest specificity ratio of peptides tested with a single cleavage site.

To assess the effects of variations within the bradykinin sequence, several commercially available bradykinin analogues were tested by this combination of methods. The results listed in Table II show that a variety of substitutions in several positions had relatively little effect on binding (127 μM < K_i < 593 μM). The effects on hydrolysis were, however, quite marked. Notably, the subtle change from Pro[3] to Hyp[3] in the P3 position[2] slowed hydrolysis by a factor of 10. Substitution of D-Phe[7] for Pro[7] (position P2′) prevented hydrolysis.

To more systematically map single-position substitutions, a series of variations of nitrobradykinin were synthesized with different residues in the P1′ position (Table III). The kinetic parameters for these derivatives were determined spectrophotometrically by measuring initial velocities at various substrate concentrations. For all substrates of this type, simple Michaelis–Menten-type kinetics were observed, indicating a homogeneous population of active sites. Variations in this P1′ position had little effect on the binding with the K_M values all falling in the narrow range between 174 and 339 μM. Catalytic rate constants were, however, markedly affected and ranged from 51.5 s^{-1} for the Ala derivative to 0 for the unhydrolyzed Glu derivative.

The bradykinin analogue studies (Table II) showed that deletion of Pro[2] (position P4) lowered K_M and raised k_{cat}

[2] The nomenclature of Schecter and Berger (1967) is used.

Table V: Inhibition of Meprin-A by Amino Acid Hydroxamates

hydroxamate	ID_{50} (μM) azocasein	nitrobradykinin
L-tyrosine	71	24
L-tryptophan	160	24
L-histidine	360	180
DL-methionine	220	390
L-cystinedihydroxamate	490	300
L-arginine	620	960
L-lysine	610	320
L-isoleucine	720	160
glycine	1640	390
DL-valine	1810	460
DL-threonine	2500	720
DL-serine	2620	750
DL-alanine	2870	670
L-γ-glutamic acid	2340	1660
DL-β-aspartic acid	4260	1840

relative to the native nonapeptide. Thus, intramolecularly quenched fluorogenic substrates of the general sequence 2ABz-RPGXSPFnR were developed to study changes in the P1–P3 positions (Soler & Harris, 1988). Kinetic parameters for this set of substrates were determined by measuring initial velocities at varying substrate concentrations (Table IV), and all exhibited simple Michaelis–Menten kinetics. A greater effect on K_M was produced by variations in this (P1) position than in other positions with values ranging from 366 μM to 2.46 mM. A wide range of k_{cat} values also was observed from 2.4 s^{-1} for the Lys peptide to 98.5 s^{-1} for the Ala peptide. Two substitutions, Lys and Leu, introduced a second cleavage site into the peptide, allowing hydrolysis on either the N- or C-terminal sides of these residues. Note that, for these latter peptides, the measured k_{cat} is the sum of the two product appearance rate constants and equals the rate constant of substrate disappearance.

Peptides of the fluorogenic type also were synthesized to assess effects at the P2 position. When Gly was replaced by an Ile residue, K_M was lowered somewhat and k_{cat} increased 1.6-fold, resulting in the highest specificity ratio found of 4.48 × 10^5 M^{-1} s^{-1}. A reversal of the P2 and P3 residues (RPGFSPFnR → RGPFSPFnR) lowered k_{cat}/K_M by a factor of 50.

Inhibitors. To further map the active site of meprin-A and to begin development of a specific inhibitor, a series of amino acid hydroxamates was tested for inhibition of meprin-A hydrolysis of azocasein and nitrobradykinin. Table V lists the hydroxamates in order of potency according to the ID_{50} values. The values against both substrates cover about a 100-fold range, and, generally, the ID_{50} for nitrobradykinin hydrolysis is about 2–3-fold lower than the corresponding ID_{50} for azocasein hydrolysis. On the basis of the observed inhibition constants, the side chains fall into groups according to their chemical nature. Tyrosine hydroxamate is a quite good inhibitor with an ID_{50} of 24 μM against nitrobradykinin and 71 μM against azocasein. The other aromatic hydroxamates are nearly as good, followed by those with side chains containing sulfur, basic groups, aliphatic groups, and finally acidic groups.

Previous studies (Wolz & Bond, 1990) indicated that the products of meprin-A hydrolysis of bradykinin (RPPGF and SPFR) were weak inhibitors (ID_{50} > 1 mM). Combining this information with the knowledge gained here from the substrate and hydroxamate inhibitor experiments, the peptides AcRPGY and AcRPGY-NHOH were synthesized as inhibitors of meprin-A. The peptide with the free carboxy-terminal was a suprisingly good inhibitor of nitrobradykinin hydrolysis with an ID_{50} of only 15.6 μM. The hydroxamate derivative showed about a 4-fold improvement with an ID_{50} of 3.7 μM.

These tetrapeptides also were tested for inhibition of meprin-A hydrolysis of azocasein at pH 8.7. The ID_{50} values were 45.9 and 4.9 μM for the peptide with the free carboxy-terminal and the hydroxamate derivative, respectively.

The mechanism of inhibition of nitrobradykinin hydrolysis by both peptides was elucidated by determining ID_{50} in the presence of two different substrate concentrations (100 and 300 μM). For both inhibitors, Dixon plots (not shown) at the two substrate concentrations were linear, indicating no partial activity in the enzyme–inhibitor complex, and the lines intersected on the horizontal (inhibitor concentration) axis. Additionally, the K_M of nitrobradykinin was determined in the absence and presence of each inhibitor near the respective ID_{50} concentrations. Lineweaver–Burk plots (not shown) for both inhibitors were linear, and the lines intersected on the horizontal (reciprocal substrate concentration) axis. Thus, for both the free carboxyl peptide and the hydroxamate derivative, substrate concentration had no effect on ID_{50} and the presence of inhibitor had no effect on K_M. Both determinations indicated that both peptides inhibited meprin-A hydrolysis of nitrobradykinin by the simple, purely noncompetitive mechanism in which K_i is equal to ID_{50}.

Discussion

Of a variety of biologically active peptides tested, bradykinin was found to be the best substrate with a single cleavage site. Hence, its sequence was chosen for systematic substitutions to map the extended substrate-binding site of meprin-A. A wide variety of substitutions in the P1′ position (Tables I and II) had little effect on substrate binding whereas variations in the P1 position (Table III) yielded K_M values spanning almost an order of magnitude. Catalytic rate constants were markedly sensitive to changes in either P1′ or P1. In order of decreasing rate constants, preferred P1′ substitutions were A > S > R > F > K > T > E and for P1 substitutions A > F ≫ L > E > K. The variety of allowed cleavages indicates that meprin-A does not have strict requirements for residues adjacent to the cleavage site.

Some pronounced changes in k_{cat} were brought about by relatively minor changes in the P1′ side chain. A comparison of the kinetic constants for the residues Ala and Ser in P1′ showed little difference. The addition of a methyl group to Ser to give Thr, however, slowed catalysis by a factor of 100 while lowering K_M 3-fold. This could be due to different torsional angles for Thr and Ser, which may give rise to different side-chain orientations within the S1′ subsite of the enzyme.

In this study, a few substitutions farther from the scissle bond also were tested. In the fluorogenic substrate 2ABz-RPGFSPFnR, replacement of Gly (position P2) with the bulkier more hydrophobic Ile residue doubled k_{cat}/K_M to yield the highest specificity ratio found (4.48 × 10^5 M^{-1} s^{-1}). This was the only bradykinin analogue tested that had a significantly higher specificity ratio than that with the naturally occurring bradykinin core sequence. Preference for the naturally occurring bradykinin sequence is consistent with the possibility that bradykinin is a physiological substrate of meprin-A.

The Pro in P4 has a small negative effect on binding and catalysis. Its deletion (RPGFSPFR) resulted in a 4-fold increase in k_{cat}/K_i compared to native bradykinin (Table II). The adjacent Pro in P3 can be quite important to catalysis. The subtle change to hydroxyproline in the complete nonapeptide (RPHypGFSPFR) lowered K_M 4-fold but slowed catalysis by a factor of 10 (Table II). In the fluorogenic octapeptide (2ABz-RHypGFSPFnR) the same substitution

had the same effects, but to a lesser degree (Table IV). Reversal of G and P in the sequence 2ABz-RPGFSPFnR to give 2ABz-RGPFSPFnR had almost no effect on K_M but caused a dramatic 80-fold lowering of k_{cat}.

The effects of replacement of Pro in position P2′ were assessed by a novel method described elsewhere (Birket et al., 1991). Briefly, a mixture of peptides was synthesized that included approximately equimolar amounts of 13 peptides with different residues in the X position of AcRPGFAXFR. The mixture was allowed to react with meprin-A, and the product mixture was subjected to automated Edman degradation. Since the N-termini of the substrate peptides were acylated, only the newly produced C-terminal portions were sequenceable. The quantities of the various amino acids in the sequenceable products reflected the degree of hydrolysis of the corresponding substrates.

By this method, it was found that peptides containing Glu, Lys, or Leu in the P2′ position were not hydrolyzed significantly. All other substitutions (D, S, T, G, A, Y, R, V, I) except the native-like Pro-containing peptide shifted the cleavage site from the expected F–A bond to the A–X bond or allowed cleavage of both the F–A and A–X bonds. These data indicate that Pro in the P2′ position is apparently important in directing the cleavage to a given site.

In reviewing the cleavages of the other unrelated peptides in Table I, it can be seen that, in each of the peptides, a cleavage site was found with a Pro in either P2′ or P3′. Thus, a proline in one of the P′ sites close to the scissle bond seems to be a significant factor for hydrolysis by meprin-A.

Because of its unique imide bond, replacement or relocation of proline can affect peptide conformation. The conformation of native bradykinin in aqueous solution has been studied in detail by NMR (Denys et al., 1982). It was found that, except for the imide bonds, the peptide is flexible and in rapid equilibrium among multiple conformations. Each of the X–Pro bonds in native bradykinin exists predominantly (>90%) as the trans isomer (London et al., 1979). Thus, the prolines do confer some local conformational constraints. Side-chain variations near to a proline can affect this isomerization. ^{13}C and ^{19}F NMR studies demonstrated that, in bradykinin, replacement of Ser^6 (the P1′ position for meprin-A cleavage) with Gly^6 significantly increased the proportion of the cis conformer at the Pro^7 bond from 10% to 35% (Denys et al., 1982; London et al., 1979). Kinetic differences observed here could be due to such effects of residue replacement on conformation.

The NMR studies showed additionally that when a low concentration of sodium dodecyl sulfate (5.2 mM) was included in the solution of Gly^6-bradykinin (0.69 mM), a detergent–peptide complex was formed in which the proportion of the cis-Pro^7 conformer is further increased from 35% to 42% (Cann et al., 1990). This conformational change in bradykinin upon binding to a molecule of mixed hydrophilic/hydrophobic character provides a model for bradykinin–protein interaction. A conformational change in bradykinin to cis-Pro^7 when bound to meprin-A could add strain on the adjacent scissile bond, thereby accelerating hydrolysis. The return to the energetically favored trans conformation in the C-terminal product upon release also could be a driving force in the reaction. Although substrate binding to the enzyme is little affected by P1′ substitutions, side chains in this position that lower product binding and thereby enhance product release could increase the catalytic rate constant.

Substrate and product binding were factors considered for inhibitor design. The product analogue AcRPGY-NHOH had a K_i of 3.7 μM and should lead to the development of even more specific inhibitors. It was unexpected that, despite their structural homology to the N-terminal of the substrate, the tetrapeptides nevertheless inhibited nitrobradykinin hydrolysis by a simple noncompetitive mechanism. Inhibition of this type, in which substrate and inhibitor binding are random and independent, is usually interpreted to imply that the substrate and inhibitor bind at different sites. In some enzymes, it has been proposed that these can be different subsites within the same active site (Wolz et al., 1990). Even though meprin-A has an extended substrate-binding site, it seems unlikely that a tetrapeptide could bind to a given active site at the same time as a nonapeptide substrate.

The hydroxamate peptide was designed with the metal-binding moiety for the purpose of interaction with the active-site metal. Usually (for single amino acids), hydroxamate derivatization enhances binding by a factor of at least 100–1000 compared to the underivatized form (Wolz et al., 1990). The fact that only a 4-fold increase was seen for this peptide may indicate that metal–hydroxamate interaction is not a major contributor to inhibitor binding. Hence, the inhibitor-binding site is not necessarily the active site.

Since meprin-A exists as a tetramer in its native form, there are potentially four active sites (Butler et al., 1987). All bradykinin type substrates tested here exhibited simple Michaelis–Menten kinetics, indicating a homogeneous population of independent active sites (and thus the k_{cat} values herein were calculated on the basis of one active site per subunit). It could be, however, that the enzyme exhibits complete negative cooperativity (sometimes referred to as "half of the sites reactivity") such that if substrate or product is bound to the active site of one subunit, another subunit(s) is completely inactivated. Alternatively, all of the subunits might not be catalytic and the noncompetitive inhibitor-binding site could be on a different, putatively regulatory, subunit(s). Future investigations into the activity of separated subunits and the use of specific meprin inhibitors with the membrane bound form of the enzyme should contribute to further understanding its function.

References

Beynon, R. J., & Bond, J. S. (1983) *Science 219*, 1351–1353.

Beynon, R. J., Shannon, J. D., & Bond, J. S. (1981) *Biochem. J. 199*, 591–598.

Bidlingmeyer, B. A., Cohen, S. A., & Tarvin, T. L. (1984) *J. Chromatogr. 336*, 93–104.

Birket, A. J., Soler, J. F., Wolz, R. L., Bond, J. S., Wiseman, J., Berman, J., & Harris, R. B. (1991) *Anal. Biochem. 196*, 137–143.

Bond, J. S., & Beynon, R. J. (1986) *Curr. Top. Cell. Regul. 28*, 263–290.

Bond, J. S., Beynon, R. J., Reckelhoff, J. F., & David, C. S. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 5542–5545.

Butler, P. E., McKay, M. J., & Bond, J. S. (1987) *Biochem. J. 241*, 229–235.

Cann, J. R., Vavrek, R. J., Stewart, J. M., & Mueller, D. D. (1990) *J. Am. Chem. Soc. 112*, 1357–1364.

Craig, S. S., Reckelhoff, J. F., & Bond, J. S. (1987) *Am. J. Physiol. 253*, C535–C540.

Denys, L., Bothner-By, A. A., Fisher, G. H., & Ryan, J. W. (1982) *Biochemistry 21*, 6531–6536.

Dixon, M. (1953) *Biochem. J. 55*, 170–171.

Hestrin, S. (1949) *J. Biol. Chem. 180*, 249–253.

London, R. E., Stewart, J. M., Williams, R., Cann, J. R., & Matwiyoff, N. A. (1979) *J. Am. Chem. Soc. 101*, 2455–2462.

Lowry, O. H., Rosenbrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Reckelhoff, J. F., Bond, J. S., Beynon, R. J., Savarirayan, S., & David, C. S. (1985) *Immunogenetics 22*, 617–623.
Schechter, I., & Berger, A. (1967) *Biochem. Biophys. Res. Commun. 27*, 157–162.
Soler, J. F., & Harris, R. B. (1988) *Int. J. Pept. Protein Res. 32*, 35–40.
Wolz, R. L., & Bond, J. S. (1990) *Anal. Biochem. 191*, 314–320.
Wolz, R. L., Zeggaf, C., Stocker, W., & Zwilling, R. (1990) *Arch. Biochem. Biophys. 281*, 275–281.