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METABOLISM OF VASOACTIVE PEPTIDES BY PLASMA AND PURIFIED RENAL AMINOPEPTIDASE M

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Abstract—Aminopeptidase M (AmM; EC 3.4.11.2) is a membrane-bound peptidase present on renal brush border and vascular plasma membrane. In the present study, AmM, purified from rabbit kidney cortex, produced a single immunoprecipitin line against AmM antisera, hydrolyzed alanyl-, leucyl- and arginyl-β-naphthylamides at rates of 5.1 ± 0.5, 3.9 ± 0.5 and 2.6 ± 0.3 μmol/min/mg, respectively, exhibited little or no α-glutamyl-, aspartyl- or glycyl-prolyl-naphthylamidase activities (≤0.14 μmol/min/mg), and was inhibited by *o*-phenanthroline, amastatin (IC_{50} = 400 nM) and bestatin (IC_{50} = 6 μM). The alanyl-naphthylamidase activity of unfractionated rabbit plasma was found to be identical to purified AmM regarding relative rates of hydrolysis of alanyl-, leucyl- and arginyl-naphthylamides (100:79:42), pH optimum, and inhibition profile. In comparative studies with the purified enzyme, immunoreactive AmM accounted for essentially all of the alanyl-2-naphthylamidase activity of rabbit plasma. N-Terminal metabolism of (Met^5)enkephalin by purified renal AmM was 3.92 ± 0.69 μmol/min/mg, followed by somatostatin (1.25 μmol/min/mg), hepta(5-11)substance P (1.14 ± 0.13 μmol/min/mg), (Asn^1)angiotensin II (1.11 ± 0.06 μmol/min/mg), angiotensin III (0.45 ± 0.04 μmol/min/mg) and des(Asp^1)-angiotensin I (0.36 ± 0.04 μmol/min/mg). In contrast, substance P, bradykinin, (Sar^1,Ala^8)angiotensin II and neurokinin analogs containing modified N-termini (e.g. Ac-Arg) were resistant to hydrolysis by AmM. Peptide degradation was optimal at neutral pH and was inhibited by amastatin (IC_{50} = 200 nM) and bestatin (IC_{50} = 5 μM). Apparent K_m values ranged from 15.7 ± 0.4 μM for angiotensin III to 102 ± 2 μM for (Met^5)enkephalin. These data support a significant role for vascular and plasma AmM in the metabolism of circulating vasoactive peptides.

Both plasma and vasculature contain enzymes that can form, convert and inactivate vasoactive peptides. These enzymes include angiotensin I converting enzyme (ACE; EC 3.4.15.1) [1], carboxypeptidase N (CPN; EC 3.4.17.3) or N-like activity [2, 3], and aminopeptidase A (AmA; EC 3.4.11.7) [4]. Previous studies have also demonstrated the presence of a vascular alanyl-naphthylamidase activity immunologically indistinguishable from renal brush border aminopeptidase M (AmM; EC 3.4.11.2) [5]. AmM is present in plasma, in cerebral microvasculature [6–8] and on the cell surface of cultured vascular endothelium and smooth muscle [9]. In addition, plasma and vascular preparations containing AmM were found to convert or degrade several members of the kinin, angiotensin, tachykinin and opioid peptide families by N-terminal metabolism [9–12]. However, although suggestive of a role of AmM in the metabolism of vasoactive peptides, no definite conclusions could be drawn since contaminating and/or co-localized peptidases in these preparations may have contributed to the observed metabolism. Further, although the hydrolysis of synthetic substrates by partially purified AmM has been examined, we are not aware of any study that has quantitatively characterized peptide metabolism by the purified enzyme.

In view of the above, the present study was conducted to examine peptide metabolism by AmM purified from rabbit kidney cortex. Purified AmM hydrolyzed a variety of biologically active peptides under conditions consistent with *in vivo* metabolism. Further, comparative analysis demonstrated that AmM accounts for the majority of such metabolism in plasma. These data support an important role for AmM in the metabolism of circulating vasoactive peptides.

MATERIALS AND METHODS

Materials. (Asn^1)angiotensin II, (Sar^1,Ala^8)angiotensin II, des(Asp^1)-angiotensin I and angiotensin III, alanyl-, leucyl- and arginyl-β-naphthylamide, alanyl-4-methoxy-2-naphthylamide (alanyl-MNA), *o*-phenanthroline, amastatin, bestatin, puromycin and phosphoramidon were obtained from the Sigma Chemical Co. (St. Louis, MO). Substance P and hepta(5-11)-substance P were obtained from Peninsula Laboratories (San Carlos, CA). The ACE inhibitor (captopril) and the CPN inhibitor (MERGETPA: D,L-mercapto-methyl-3-guanidinoethylthiopropanoic acid) were from Squibb (Princeton, NJ) and Calbiochem-Behring (San Diego, CA) respectively. Sephadex G-200, DEAE-Sephacel and Phenyl-Sepharose were from Pharmacia, Inc. (Piscataway, NJ). Neurokinin analogs were provided by Drs. Regoli and Drapeau (University of Sherbrooke, Sherbrooke, Canada). The amino acid standards, *o*-phthalaldehyde crystals and reagent solution (OPA)

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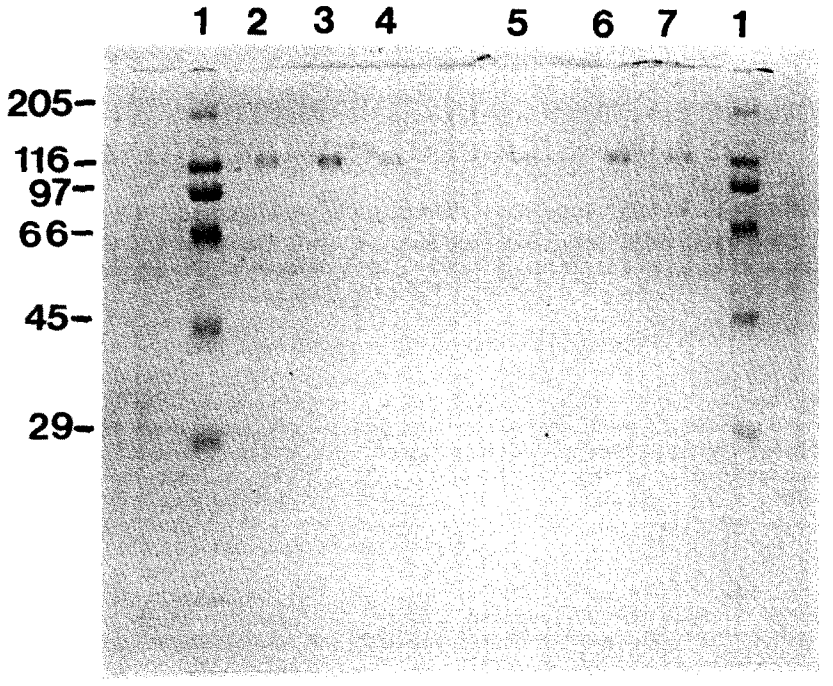

Fig. 1. SDS–PAGE of aminopeptidase M after the last purification step. Lanes 2, 3 and 4 represent 10, 20 and 10 μL, respectively, applied from fraction No. 39. Lanes 5, 6 and 7 represent 10, 20 and 10 μL, respectively, from fraction No. 42. Enzyme activity was found between fractions 30 and 50, with fraction No. 39 representing the peak of activity. Lane 1: molecular weight markers in kD.

used for HPLC were from the Pierce Chemical Co. (Rockford, IL).

Purification of aminopeptidase M from rabbit kidney cortex. Rabbit kidney cortex (75 g) was homogenized in distilled water (350 mL) and centrifuged (3000 *g*, 15 min). The supernatant fraction was adjusted to pH 3.8 with 0.1 M HCl and incubated for 24 hr (37°). The autolysate was centrifuged (18,000 *g*, 15 min) and the supernatant fraction brought to pH 7.0 with 1 M Trizma base. Solid ammonium sulfate was added to 90% saturation and the solution centrifuged (18,000 *g*, 15 min). The precipitate was resuspended in 10 mM Tris–HCl (pH 7.5) and chromatographed on a Sephadex G-200 column (5 × 100 cm) equilibrated with 10 mM potassium phosphate buffer (pH 7.5) containing 0.1 M NaCl.

Aminopeptidase M-containing fractions (assayed using leucyl-β-naphthylamide substrate as described below) were pooled and dialyzed against a 10 mM Tris–HCl buffer (pH 8.4). The dialysate was chromatographed on a DEAE-Sephacel column (2.2 × 6.5 cm) equilibrated with Tris–HCl buffer (pH 8.4) and eluted with a 400 mL linear NaCl gradient (0–400 mM) prepared in the same buffer. Aminopeptidase M-containing fractions were dialyzed against a 10 mM Tris–HCl buffer (pH 7.3) containing ammonium sulfate at 25% saturation. The dialyzed enzyme was applied to a Phenyl-Sepharose column (0.7 × 5 cm) equilibrated with the same buffer. Elution was achieved using a linear ammonium sulfate gradient prepared in the Tris–HCl buffer in which the ammonium sulfate concentration was reduced from 25 to 0% saturation. Fractions emerging with the peak tube of aminopeptidase M activity were devoid of measurable aminopeptidase A, dipeptidyl(amino)peptidase IV and neutral endopeptidase activities. Non-dissociating polyacrylamide gel electrophoresis (PAGE) [13] run on 6% gels in Tris–HCl buffer (pH 8.4) revealed a single band when stained with Coomassie blue. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was run on 10% gels by the method of Laemmli [14] and stained with Coomassie blue.

Enzyme assay. Amino acid–naphthylamidase activities were assayed by hydrolysis of alanyl-, leucyl- and arginyl-β-naphthylamides [9]. Specific activities are expressed as units per milligram protein where one unit equals the hydrolysis of 1 μmol substrate/min.

Qualitative assays of peptide metabolism were carried out by TLC on MN 300 cellulose plates [10, 15]. At sequential time intervals, 5-μL aliquots were spotted on the plate and immediately dried. Plates were developed in butanol : acetic acid : water (4 : 1 : 5) and the products visualized by staining with 0.4% (w/v) ninhydrin in acetone. Quantitative assays of peptide metabolism were carried out by HPLC as previously described [9–11]. Reactions (300–600 μL) were incubated at 37° in 100 mM sodium phosphate buffer (pH 7.0). At sequential time intervals, 60- to 120-μL aliquots were immersed in a boiling water bath (5 min) to terminate the reaction, cooled on ice, and centrifuged in a Brinkmann table top centrifuge (3 min); then the supernatant fraction was collected for analysis.

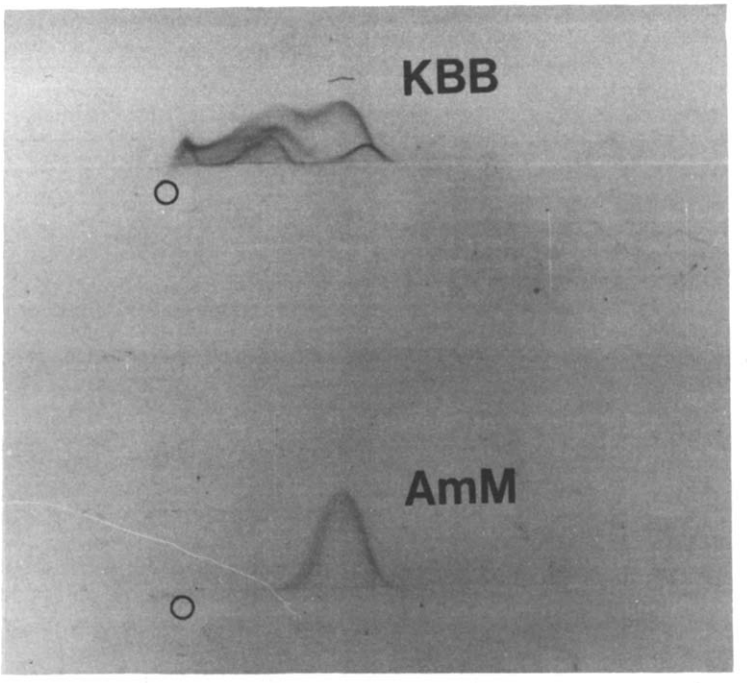

Fig. 2. Crossed-immunoelectrophoresis of solubilized kidney brush border (KBB) and purified aminopeptidase M (AmM). Horizontal electrophoresis is from the origin (O), left to right, at 15 V/cm for 1 hr, followed by vertical electrophoresis, bottom to top, at 15 V/cm for 1 hr. Vertical electrophoresis was into gel containing combined antisera to the major brush border antigens. Precipitin lines were visualized by staining for protein with Crowle's Double Stain and subsequent destaining with 0.3% acetic acid.

For determination of pH dependence, a 100 mM sodium acetate buffer was used over the pH range 4.0 to 6.0, and the above 100 mM phosphate buffer was used from pH 6.5 to 8.5. pH studies were also conducted using the Britton–Robinson universal buffer. For inhibition studies, inhibitors were preincubated with enzyme and buffer for 20 min (37°). For K_m determinations, measurements of initial velocity were made over a range of substrate concentrations. Data were plotted as $1/V$ vs $1/[S]$ and fit to the best straight line.

A Waters high performance liquid chromatograph system was employed for the HPLC analysis. Standards and unknowns (20–100 μL) were automatically derivatized with *o*-phthalaldehyde (20 μL) 3 min prior to chromatography (Pre-column Derivatization Program, Waters 710B WISP™ Autosampler) and subsequently separated on a reverse phase column (Waters, 10 m, C_{18}-Radial-PAK™, 8 mm × 10 cm) at a constant flow rate of 5.0 mL/min utilizing a linear gradient from 100% Buffer A to 40% Buffer A/60% Buffer B. Buffer A was 10 mM sodium phosphate (pH 7.0) and Buffer B was a 50/50 (v/v) mixture of Buffer A and acetonitrile. Integration of sample peak areas and quantitation of metabolites against the last-run standards were calculated automatically by the data module. Standards were run every sixth injection.

Immunoelectrophoresis. Renal brush border and antisera to the major rabbit renal brush border peptidases were obtained as previously described [5, 6, 16] and used in gel immunoelectrophoresis studies to specifically precipitate individual brush border antigens (e.g. aminopeptidase M). As previously found [5, 6, 16], the antisera to AmM do not cross-react with other brush border antigens including aminopeptidase A (EC 3.4.11.7), dipeptidyl-(amino)peptidase IV (EC 3.4.14.5), neutral endopeptidase (EC 3.4.24.11) or carboxypeptidase P (EC 3.4.17.—). Immunoelectrophoresis was performed using 1.0 mm thick 1% (w/v) agarose gel containing 1% (v/v) Triton X-100. For crossed- immunoelectrophoresis, samples (2–8 μL) were subjected to horizontal electrophoresis (15 V/cm for 1 hr) through gel followed by vertical electrophoresis into antisera-containing gel. For rocket-immunoelectrophoresis, samples were electrophoresed vertically (15 V/cm for 1 hr) directly into antisera-containing gel. After repeated dehydration/hydration of the gel to remove soluble protein, the AmM immunoprecipitin line was stained using alanyl-MNA [17, 18]. Alternatively, gels were stained for protein with Crowle's Double Stain and subsequently destained with 0.3% acetic acid [16]. Peak area measurements were determined using Sigma Scan™ (Jandel Scientific).

RESULTS

Enzyme purity. SDS–PAGE run on 10% gels revealed a single protein band for purified renal aminopeptidase M when stained with Coomassie

Table 1. Synthetic substrate hydrolysis by purified renal aminopeptidase M and plasma

Substrate	Aminopeptidase M Activity (μmol/min/mg)	Ratio	Plasma Activity (nmol/min/mL)	Ratio
Ala-Naphthylamide	5.1 ± 0.5 (7)	100	66 ± 1 (3)	100
Leu-Naphthylamide	3.9 ± 0.5 (8)	76	52 ± 2 (3)	79
Arg-Naphthylamide	2.6 ± 0.3 (5)	51	27 ± 2 (3)	42
α-Glu-Naphthylamide	0.14 ± 0.06 (3)	2.7		
Asp-Naphthylamide	0.03 ± 0.01 (3)	0.6		
Gly-Pro-Naphthylamide	0.02 ± 0.01 (3)	0.4		

Values are the means ± SE; the number of determinations is given in parentheses. Ratios are expressed as percent of alanyl-naphthylamide hydrolysis by purified renal aminopeptidase M or plasma.

blue (Fig. 1). As previously found [6, 16, 17], crossed-immunoelectrophoresis of solubilized rabbit kidney brush border against combined antisera to the major brush border peptidases revealed numerous visible precipitin lines when stained for protein with Crowle's Double Stain (Fig. 2, top). In contrast, purified renal AmM produced only a single precipitin line (Fig. 2, bottom). The AmM precipitin line could be visualized with greater sensitivity by histochemical staining (5–15 min) using alanyl-, leucyl- or arginyl-MNA (not shown), and could also be lightly visualized after prolonged incubation (12–18 hr) with glutamyl-2-MNA. Further, in contrast to the results obtained with solubilized brush border, histochemical staining of the purified renal AmM gels for aminopeptidase A (α-glutamyl-MNA) or dipeptidyl-(amino)peptidase IV (glycyl-prolyl-MNA) failed to reveal any separate contaminating precipitin lines which may have escaped detection by the less sensitive protein stain.

As shown in Table 1, purified AmM hydrolyzed alanyl-β-naphthylamide at a rate of 5.1 ± 0.5 μmol/min/mg, and the ratio of hydrolysis of alanyl-, leucyl- and arginyl-β-naphthylamides was 100:76:51. Consistent with the above immunologic studies, no significant aminopeptidase A or dipeptidyl-(amino)peptidase IV activities were present (Table 1).

Pooled rabbit plasma contained significant aminopeptidase activity (Table 1). The plasma activity hydrolyzed alanyl-, leucyl- and arginyl-β-naphthylamides in a ratio (100:79:42) comparable to that of purified AmM. Thus, subsequent studies comparatively characterized both the purified AmM and the plasma alanyl-β-naphthylamidase activity.

Comparative characterization. Both pure AmM and the plasma alanyl-β-naphthylamidase had pH optima of 7.4. As shown in Fig. 3, amastatin and bestatin were concentration-dependent inhibitors of both activities. Amastatin was the most potent inhibitor of pure AmM (IC_{50} = 400 nM) and plasma (IC_{50} = 300 nM), whereas bestatin was considerably less potent (IC_{50} = 4–6 μM). In contrast, both AmM and the plasma activity were relatively resistant to inhibition by puromycin ($IC_{50} \geq 500$ μM). Both AmM and plasma were also inhibited 100% by *o*-phenanthroline (1 mM), but were unaffected by inhibitors

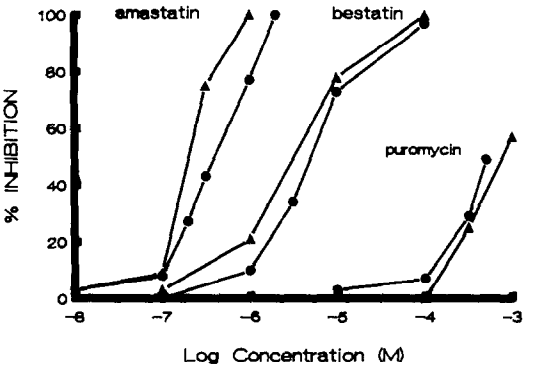

Fig. 3. Inhibition (%) of alanyl-naphthylamide hydrolysis by plasma (▲) and purified aminopeptidase M (●) by amastatin, bestatin and puromycin. Inhibitors were preincubated for 10 min (37°) with enzyme and buffer before addition of substrate. Values shown are the averages of two to four determinations. Control rates of hydrolysis were 55 nmol/min/mL and 4.6 μmol/min/mg for plasma and purified aminopeptidase M respectively.

of angiotensin converting enzyme (captopril), carboxypeptidase N (MERGETPA), neutral endopeptidase (phosphoramidon) and dipeptidyl(amino)-peptidase IV (diprotin A) (less than 12% inhibition at a final concentration of 10 μM).

Quantitative rocket immunoelectrophoresis (electroimmunoassay) [6, 9, 17] of pure renal AmM and plasma produced rocket-shaped precipitates with peak areas proportionate to the amount of sample used (Fig. 4). When peak areas [immunoreactive AmM (iAmM)] were plotted against the units of alanyl-β-naphthylamidase activity inoculated onto the gels, the relationships of iAmM to alanyl-β-naphthylamidase activity were the same for both purified AmM and rabbit plasma (Fig. 5). Thus, immunoreactive AmM accounted for essentially all of the alanyl-β-naphthylamidase activity of rabbit plasma.

Peptide metabolism by purified aminopeptidase M. In preliminary experiments, purified renal AmM was

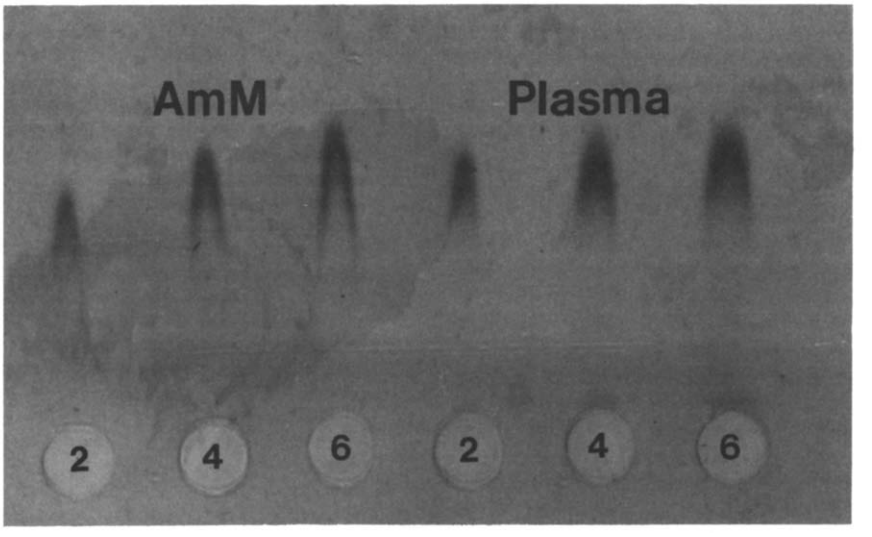

Fig. 4. Rocket immunoelectrophoresis of increasing amounts (2–6 μL) of purified renal aminopeptidase M (AmM) and rabbit plasma. Samples were electrophoresed vertically into gel containing anti-rabbit aminopeptidase M immunoglobin at 15 V/cm for 1 hr. Precipitin lines were visualized histochemically by staining for alanyl-MNA hydrolysis.

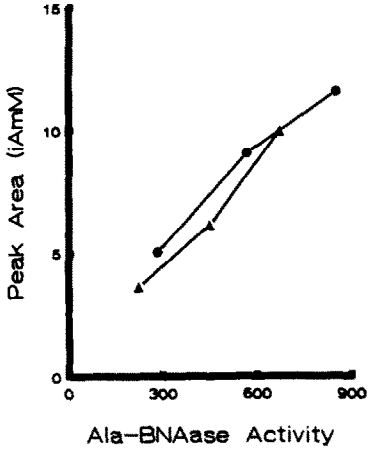

Fig. 5. Relationship of peak area (immunoreactive AmM) to the alanyl-naphthylamidase activity (pmol/min) of purified renal aminopeptidase M (●) and rabbit plasma (▲). Dilutions of each sample were electrophoresed vertically into gel containing anti-rabbit aminopeptidase M immunoglobulin at 15 V/cm for 1 hr. Precipitin lines were visualized as described in the legend of Fig. 4. Values shown are the averages of three determinations.

incubated with various peptide agonists and antagonists, and the metabolites were separated and identified by TLC. As illustrated in Fig. 6, AmM hydrolyzed the N-terminal bond of numerous peptides, whereas others were resistant to such metabolism. Further, as anticipated based on the above data regarding enzyme purity, no metabolites attributable to endo- or carboxypeptidases were detected even during extended incubations.

When quantitatively examined by HPLC, N-terminal peptide metabolism was proportional to the amount of enzyme used and the time of incubation. N-terminal metabolism had a neutral pH optimum, and was inhibited by both amastatin (IC_{50} = 200 nM) and bestatin (IC_{50} = 5 μM). Puromycin was relatively ineffective (IC_{50} = 500 μM).

When the relative rates of N-terminal metabolism were determined at a standard substrate concentration (250 μM), the Tyr^1-Gly^2 bond of (Met^5)enkephalin was hydrolyzed most rapidly (3.92 ± 0.69 μmol/min/mg), followed by somatostatin, hepta(5-11)substance P, (Asn^1)angiotensin II, angiotensin III and des(Asp^1)angiotensin I (Table 2). In contrast, substance P and bradykinin (Arg^1-Pro^2 N-terminus) were resistant to AmM-mediated hydrolysis, as was (Sar^1,Ala^8)angiotensin II. Among the neurokinin analogs synthesized by Regoli *et al.* [19], the N-terminal Arg^1-Phe^2 bond of NK analog No. 1 was hydrolyzed rapidly (2.1 μmol/min/mg), whereas N-terminal modification (Ac-Arg^1-Phe^2 or β-Ala^1-Phe^2) conferred resistance (Table 2).

As shown in Table 3, the rates of AmM-mediated hydrolysis of the Tyr^1-Gly^2 bond of (Leu^5)enkephalin and C-terminally extended enkephalins decreased with increasing peptide length. N-Terminal hydrolysis of (Leu^5)enkephalin and dynorphin A(1–9) were comparable (3.31 and 3.38 μmol/min/mg) and nearly 2-fold more rapid than dynorphin A(1–10) and (1–13) (2.04 and 2.12 μmol/min/mg). In turn, dynorphin A, containing 17 amino acids, was hydrolyzed only one-sixth as fast (0.52 μmol/min/mg) as (Leu^5)enkephalin.

Despite its lower rate of N-terminal hydrolysis, dynorphin A had a higher affinity for AmM (K_m = 35.5 μM) than did (Met^5)enkephalin (K_m = 102 μM) (Table 4). Nevertheless, of all peptides tested, angiotensin III had the highest affinity (K_m = 15.7 μM).

DISCUSSION

AmM was first identified on renal and intestinal

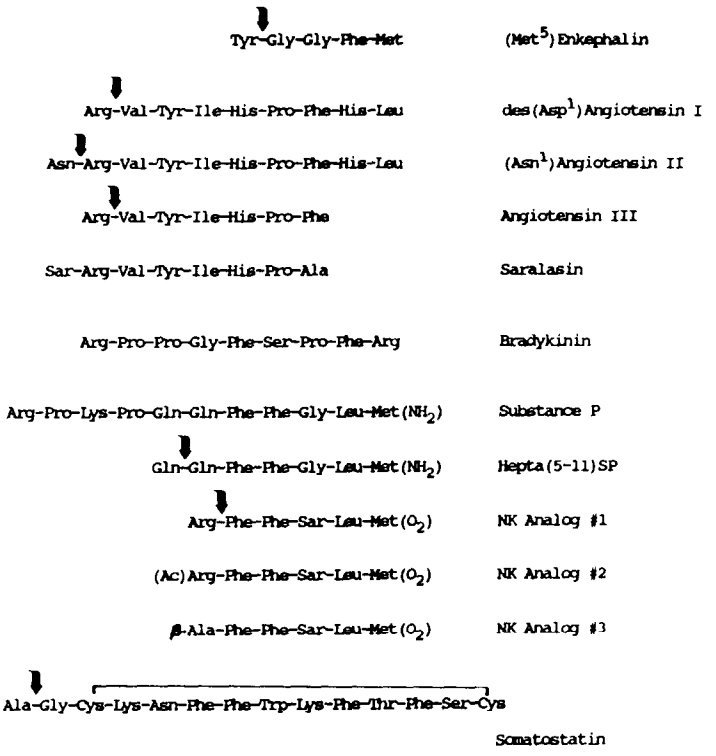

Fig. 6. Sites of N-terminal hydrolysis by purified renal aminopeptidase M as established by thin-layer chromatography and confirmed by high pressure liquid chromatography.

Table 2. Peptide hydrolysis by purified renal aminopeptidase M

Peptide	N-Terminal bond	Activity (μmol/min/mg)	Ratio
(Met^5)Enkephalin	Tyr-Gly	3.92 ± 0.69 (5)	100
Somatostatin	Ala-Gly	1.25 (1.4/1.1)	32
Hepta(5-11)Substance P	Gln-Gln	1.14 ± 0.13 (3)	29
(Asn^1)Ang II	Asn-Arg	1.11 ± 0.06 (6)	28
Angiotensin III	Arg-Val	0.45 ± 0.04 (9)	11
des(Asp^1)Ang I	Arg-Val	0.36 ± 0.04 (5)	9
Substance P	Arg-Pro	0	0
Bradykinin	Arg-Pro	0	0
(Sar^1,Ala^8)Ang II	Sar-Arg	0	0
Neurokinin analogs:			
NK No. 1	Arg-Phe	2.1 (1.9/2.2)	53
NK No. 2	(Ac)Arg-Phe	0	0
NK No. 3	β-Ala-Phe	0.1	2

Assays were performed as described in Materials and Methods at a final substrate concentration of 250 μM. Values are means ± SE (with the number of determinations in parentheses) or averages of two determinations.

brush border as an enzyme hydrolyzing N-terminal neutral and basic amino acids from naphthylamide and peptide substrates [20]. However, although several studies have examined quantitatively the hydrolysis of synthetic substrates by partially purified preparations of AmM [20], we are not aware of any previous study that has characterized quantitatively N-terminal peptide metabolism by the purified enzyme.

Based on both immunologic and enzymatic criteria, the AmM preparation obtained in the present study was free of contamination by other renal brush border peptidases including aminopeptidase A and dipeptidyl(amino)peptidase IV.

Table 3. Metabolism of opioid peptides by purified renal aminopeptidase M

Peptide	Activity (μmol/min/mg)	Ratio
(Leu^5)Enkephalin	3.31 ± 0.44 (7)	100
Dynorphin A		
Sequence (1–9)	3.38 ± 0.53 (5)	102
Sequence (1–10)	2.04 ± 0.22 (6)	61
Sequence (1–13)	2.12 ± 0.26 (6)	64
Dynorphin A	0.52 ± 0.11 (6)	16

Assays were performed as described in Materials and Methods at a final substrate concentration of 100 μM. Values are means ± SE; the number of determinations is given in parentheses. Dynophin A = (Leu^5)enkephalin-Arg^6-Arg^7-Ile^8-Arg^9-Pro^{10}-Lys^{11}-Leu^{12}-Lys^{13}-Trp^{14}-Asp^{15}-Asn^{16}-Gln^{17}.

Table 4. Kinetics of peptide hydrolysis by purified renal aminopeptidase M

Peptide	K_m (μM)	V_{max} (μmol/min/mg)
(Met^5)Enkephalin	102 ± 2	4.3 ± 1.0 (3)
Dynorphin A	35.5 ± 3.8	0.9 ± 0.1 (3)
Hepta(5-11)Substance P	48.5 ± 7.2	1.0 ± 0.2 (4)
Angiotensin III	15.7 ± 0.4	0.4 ± 0.1 (5)
des(Asp^1)Ang I	22.8 ± 3.3	0.4 ± 0.1 (4)

Assays were performed over a range of substrate concentrations and plotted as $1/V$ vs $1/[S]$. Values are means ± SE; the number of determinations is given in parentheses. Dynorphin A = (Leu^5)enkephalin-Arg^6-Arg^7-Ile^8-Arg^9-Pro^{10}-Lys^{11}-Leu^{12}-Lys^{13}-Trp^{14}-Asp^{15}-Asn^{16}-Gln^{17}.

Renal AmM hydrolyzed alanyl-β-napththylamide 1.3- and 2-fold more rapidly than leucyl- and arginyl-naphthylamide, respectively, and displayed little or no α-glutamyl-, aspartyl- or glycyl-prolyl-naphthylamidase activities. AmM was 20-fold more sensitive to inhibition by amastatin than bestatin and was relatively resistant to the effects of puromycin. Collectively, these characteristics differentiate AmM from other neutral/basic amino acid-specific aminopeptidases. Leucine aminopeptidase (EC 3.4.11.1) hydrolyzes leucyl-naphthylamide more rapidly than alanyl- or arginyl-naphthylamides, and aminopeptidase B (EC 3.4.11.6) is relatively insensitive to inhibition by amastatin [20]. Two membrane-bound aminopeptidases identified within the CNS (MI and MII) can also be differentiated from AmM since MI displays little or no alanyl- or leucyl-naphthylamidase activity, and MII is sensitive to inhibition by puromycin [21].

AmM is co-localized on renal and intestinal brush border with a number of other peptidases including aminopeptidase A, dipeptidyl(amino)peptidase IV, neutral endopeptidase, carboxypeptidase P and angiotensin I converting enzyme. In concert with these enzymes, intestinal AmM is involved in protein/peptide digestion, and renal AmM is thought to play a role in the degradation of peptides filtered into the renal proximal tubule [16, 20].

In more recent studies, we and others have found that AmM is also present within the vascular compartment. AmM had been identified on the cell surface of porcine endothelium and smooth muscle [9], and in porcine, rabbit and rat microvasculature [6–8]. Further, the results of the present study demonstrate that the majority (if not all) of the neutral/basic amino acid-naphthylamidase activity in plasma can be attributed to AmM. These data, which are consistent with the results of Tokioka-Terao *et al.* [22] in human plasma, suggest that, like plasma and vascular angiotensin converting enzyme, plasma and vascular AmM may play a significant role in the metabolism of circulating vasoactive peptides.

In general, the characteristics of peptide metabolism by purified AmM found in the present study are consistent with those peviously reported for metabolism by microvascular and vascular membrane fractions containing immunoreactive AmM [5, 6, 9]. Purified AmM hydrolyzed the N-terminal bonds of (Met^3)- and (Leu^5)-enkephalin, somatostatin, hepta(5-11)substance P, (Asn^1)angiotensin II, angiotensin III and des(Asp^1)angiotensin I. N-Terminal metabolism was optimal at neutral pH, and the K_m values obtained (15–100 μM) are comparable to those reported for angiotensin I with angiotensin I converting enzyme (K_m = 30 μM) [23]. In contrast, both substance P and bradykinin were resistant to AmM-mediated hydrolysis, as was (Sar^1,Ala^8)-angiotensin II and several neurokinin analogs with modified N-terminal residues.

(Met^5)- and (Leu^5)-enkephalin were degraded more rapidly by AmM than any other peptide tested. Consistent with a physiologic role for AmM-mediated metabolism of enkephalins, previous studies have shown that N-terminal metabolism is the principal route of degradation of the pentapeptides in plasma [24], and in cerebral microvessel, endothelium and smooth muscle membrane preparations containing AmM [6, 9, 11]. As the pentapeptides were extended at their C-termini, the rates of degradation by purified AmM decreased. These data are consistent with our previous studies with vascular membrane preparations containing immunoreactive AmM [6, 11] and suggest that the physiologic significance of plasma and vascular AmM may be more relevant to the degradation of lower, rather than higher, molecular weight opioid peptides.

Due to the presence of N-terminal sequences containing alternating proline residues, neither bradykinin (Arg^1-Pro^2) nor substance P (Arg^1-Pro^2-Lys^3-Pro^4) is susceptible to degradation by AmM. However, both post-proline cleaving enzyme [15] and dipeptidyl(amino)peptidase IV [25] can convert substance P to the hepta(5-11)substance P metabolite. Thus, if substance P is normally metabolized by either plasma or vascular post-proline cleaving enzyme or dipeptidyl(amino)peptidase IV, AmM may play a role in the subsequent degradation of this biologically active metabolite [26]. Further, since AmM can rapidly degrade some neurokinin analogs (e.g. NK analog No. 1; Arg^1-Phe^2), N-terminal modifications (e.g. NK analog No. 2; Ac-Arg^1-Phe^2) which prevent AmM-mediated degradation may

result in more potent receptor agonists and antagonists.

Plasma and vascular angiotensin I converting enzyme converts angiotensin I to angiotensin II [1, 27], and aminopeptidase A converts angiotensin I and II to des(Asp^1)angiotensin I and angiotensin III respectively [4, 17]. Despite their slower rates of hydrolysis, these des(Asp^1)angiotensins had the highest affinity for AmM. This high affinity suggests preferential metabolism at low (physiologic) substrate concentrations, and is consistent with studies demonstrating that the degradation of des(Asp^1)-angiotensins *in vivo* is primarily due to N-terminal hydrolysis [28]. Direct support for a role for AmM in such metabolism has been obtained in recent studies in our laboratory demonstrating that *in vivo* inhibition of rat plasma and vascular AmM alanyl-naphthylamidase by amastatin potentiates the pressor effects of both angiotensin III and des(Asp^1)angiotensin I [29].

In summary, the results of the present study demonstrate that purified AmM, free of contamination by other amino-, endo- and carboxypeptidases, is capable of metabolizing a wide variety of biologically active peptides. Kinetic data were consistent with metabolism under physiologic conditions, and the majority of such metabolism in plasma was due to AmM. These data, in conjunction with previous data demonstrating the presence of AmM in vasculature and microvasculature [6–9], support an important role for AmM in the metabolism of circulating vasoactive peptides.

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