Purification and Properties of Membrane-Bound Aminopeptidase P from Rat Lung†

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ABSTRACT: The membrane-bound form of aminopeptidase P (aminoacylprolyl-peptide hydrolase) (EC 3.4.11.9) was purified 670-fold to apparent homogeneity from rat lung microsomes. The enzyme was solubilized from the membranes using a phosphatidylinositol-specific phospholipase C. The purification scheme also resulted in homogeneous preparations of dipeptidylpeptidase IV (EC 3.4.14.5) and membrane dipeptidase (EC 3.4.13.19). Aminopeptidase P had a subunit molecular weight of 90 000, which included at least 17% N-linked carbohydrate. The molecular weight by gel permeation chromatography varied from 220 000 to 340 000, depending on the conditions used. The amino acid composition was determined and the N-terminal sequence was found to be X^1-Gly^2-Pro^3-Glu^4-Ser^5-Leu^6-Gly^7-Arg^8-Glu^9-Asp^{10}-Val^{11}-Arg^{12}-Asp^{13}-X^{14}-Ser^{15}-Thr^{16}-Asn^{17}-Pro^{18}-Pro^{19}-Arg^{20}-Leu^{21}-X^{22}-Val^{23}-Thr^{24}-Ala^{25}-. Aminopeptidase P cleaved the Arg^1−Pro^2 bond of bradykinin with a k_{cat}/K_m of 5.7×10^5 s^{-1} M^{-1}. N-Terminal fragments of bradykinin including Arg-Pro-Pro, but not Arg-Pro, were also cleaved. The enzyme was shown to have four binding subsites (S_1, S_1', S_2', S_3'), the first three of which must be occupied for hydrolysis to occur. Neuropeptide Y and allatostatin I were hydrolyzed at the Tyr^1−Pro^2 bond and Ala^1−Pro^2 bond, respectively. The pH optimum for Arg-Pro-Pro cleavage was 6.8−7.5 in most buffers. The enzyme was most stable in the range of pH 7.0−10.5 in the presence of poly(ethylene glycol). NaCl inhibited activity completely at 2 M. Mn^{2+} had variable effects on activity, depending on its concentration and the substrate used. Various peptides having an N-terminal Pro-Pro sequence were inhibitory. The enzyme was also inhibited by EDTA, *o*-phenanthroline, 2-mercaptoethanol, dithiothreitol, *p*-(chloromercuri)benzenesulfonic acid, apstatin, and captopril. The carboxyalkyl angiotensin-converting enzyme inhibitors, ramiprilat and enalaprilat, inhibited activity in the micromolar range only in the presence of Mn^{2+}.

Aminopeptidase P (EC 3.4.11.9) removes the N-terminal amino acid from peptides that have a proline residue in the penultimate position (an aminoacylprolyl-peptide hydrolase) (Yaron & Naider, 1993). Two forms of aminopeptidase P have been purified from mammalian tissues, a soluble (cytosolic) form (Harbeck & Mentlein, 1991; Rusu & Yaron, 1992; Vanhoof et al., 1992) and a membrane-bound form (Hooper et al., 1990; Simmons & Orawski, 1992; Ryan et al., 1994a). The enzyme has also been isolated from guinea pig serum (Ryan et al., 1992, 1994a). The membrane-bound form has a much more restricted substrate specificity than the soluble form, hydrolyzing only peptides that have a small amino acid side chain in the third residue position (Yoshimoto et al., 1994). One of the best substrates for membrane-bound aminopeptidase P is bradykinin (Arg^1-Pro^2-Pro^3-Gly^4-Phe^5-Ser^6-Pro^7-Phe^8-Arg^9). Evidence is accumulating that aminopeptidase P is present on the plasma membrane of lung vascular endothelial cells and contributes to the rapid inactivation of circulating bradykinin in rat lung (Orawski et al., 1987; Ryan, 1989; Baker et al., 1991; Revann et al., 1991; Simmons & Orawski, 1992; Pesquero et al., 1992a,b; Ryan et al., 1994b; Prechel et al., 1994; Kitamura et al., 1995). Aminopeptidase P is potentially a target for the design of drugs that could elevate endogenous levels of bradykinin, a potent vasodilator and cardioprotective peptide. In this paper, we report on the purification and properties of membrane-bound aminopeptidase P from rat lung. Detailed substrate specificity data on this enzyme and preliminary results on purification have appeared elsewhere (Orawski & Simmons, 1992a,c; Yoshimoto et al., 1994).

EXPERIMENTAL PROCEDURES

Materials

Ramiprilat was a gift from Dr. Ronald J. Shebuski (The Upjohn Co., Kalamazoo, MI). Enalaprilat was supplied by Dr. Arthur A. Patchett (Merck Research Laboratories, Rahway, NJ). *N*-[1-(*R*,*S*)-carboxy-2-(phenylethyl)]-Ala-Ala-Phe-*p*-aminobenzoate and *N*-[1-(*R*,*S*)-carboxy-2-(phenylethyl)]-Phe-*p*-aminobenzoate were gifts from Dr. Marian Orlowski (Mt. Sinai School of Medicine, New York). Arg-homoPro-Pro-Ala-NH_2 was synthesized by Linda L. Maggiora (The Upjohn Co., Kalamazoo, MI). D-Phe-Pro-argininal, Boc-D-Phe-Pro-argininal, and methyl-D-Phe-Pro-argininal were obtained from Dr. Bajusz (Institute for Drug Research, Budapest, Hungary). Pro-Pro-Ala-Ala and Pro-D-Pro-Ala-NH_2 were synthesized by Research Genetics (Huntsville, AL). [2,3-*prolyl*-3,4-^{3}H]Bradykinin (62 Ci/mmol) was obtained from Dupont NEN Research Products (Boston, MA). Recombinant *N*-glycanase, cloned from *Flavobacterium meningosepticum*, was obtained from Genzyme Corp. (Cambridge, MA). Homogeneous 7.5 PhastGels, IEF 3−9 PhastGels, and isoelectric focusing calibration proteins were obtained from Pharmacia (Piscataway, NJ). Ethylene glycol, poly(ethylene glycol)-200 (and -300, -400, -600, -1450, and -8000), Gly-D-Phe, Pro-Val, arphramenine A and B, leuhistin,

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leucinethiol, actinonin, foroxymithine, Triton X-114, SDS−PAGE[1] molecular weight standards, ω-aminododecyl-agarose, and all animal venoms were obtained from Sigma (St. Louis, MO). Rat lungs were obtained from Pel-Freez (Rogers, AR). Bio-Sil SEC250 and SEC400 HPLC columns were from Bio-Rad (Hercules, CA). Poly(ethylene glycol)-20000 and D,L-dithiothreitol were from Fisher (Pittsburgh, PA). Pefabloc was the product of Boehringer Mannheim (Indianapolis, IN). Pro-Lys-Ala-4-methoxy-2-naphthylamide was from Serva (Paramus, NJ), and Pro-Pro-Pro was from Bachem Bioscience (Philadelphia, PA). Substance P (2−11) and Pro-Ala-Asn-Ile-Lys-Trp-Gly-Asp were from Bachem California (Torrance, CA). Neuropeptide (1−24)amide, human, was obtained from American Peptide Co. (Sunnyvale, VA). L-Prolinal, 2-pyrrolidinone, 3-pyrrolidino-1,2-propanediol, 1-(pyrrolidinocarbonylmethyl)piperazine, and 1-pyrrolidinecarboxaldehyde were from Aldrich (Milwaukee, WI). All other peptides, chromatography materials, and reagents were from sources indicated previously (Orawski et al., 1987; Simmons & Orawski, 1992; Yoshimoto et al., 1994).

Methods

Enzyme Assays. Aminopeptidase P activity was determined at 37 °C in 0.1 M potassium phosphate, pH 6.8, using 0.5 mM Arg-Pro-Pro as the substrate. The extent of release of free arginine from the substrate was quantitated by measuring the amount of fluorescence produced following transfer of an aliquot of the incubation mixture to a reagent containing *o*-phthalaldehyde/2-mercaptoethanol as described previously (Simmons & Orawski, 1992). Cleavage of radiolabeled and unlabeled bradykinin and, in some cases, Arg-Pro-Pro, was determined by an HPLC technique described earlier (Orawski et al., 1987). In the case of [^{3}H]-bradykinin hydrolysis, labeled products which were separated by HPLC were detected with a Radiomatic Flo-one HS radioactive flow detector. Cleavage of Gly-Pro-Hyp by aminopeptidase P was determined by a modified ninhydrin procedure (Orawski et al., 1987). Dipeptidylpeptidase IV, aminopeptidase M, and bestatin-sensitive/amastatin-insensitive dipeptidase (Orawski & Simmons, 1992b) activities were determined with 0.5 mM Gly-Pro-2-NNap, Ala-2-NNap, and Phe-Arg, respectively, using the same fluorescent assay and buffer conditions as described above for aminopeptidase P cleavage of Arg-Pro-Pro. Membrane dipeptidase activity was determined using 0.5 mM Gly-D-Phe (Littlewood et al., 1989) in 12 mM Hepes, pH 6.5, containing 0.02 mM $ZnCl_2$, and measuring the rate of production of free amino acids by the above fluorescent assay.

Protein concentration was determined by the method of Lowry et al. (1951).

Enzyme Purification. Aminopeptidase P was purified from 158 g of Sprague-Dawley rat lung tissue by modifications and extensions of the method used to purify the enzyme from bovine lung (Simmons & Orawski, 1992). Crude microsomes were prepared, washed, and treated with phosphatidylinositol-specific phospholipase C (PI-PLC) as described previously. Following chromatography on decyl-agarose, the effluent which contained enzyme activity was concentrated using Centricon-30 ultrafiltration units and the buffer changed to 0.05 M potassium phosphate, pH 6.8, by repeated dilution and reconcentration. The enzyme in a final volume of 0.5 mL was applied to an ω-aminodecyl-agarose column equilibrated with the same buffer (rather than 0.1 M as used for the bovine enzyme). The second decyl-agarose step, the DEAE-Sephacel step, and Centricon-100 ultrafiltration step, were carried out as previously described. Two additional steps were used. The enzyme was equilibrated to 0.025 M potassium phosphate, pH 6.8, using Centricon-30 ultrafiltration units as described above and applied in a volume of 0.5 mL to a 2.5-mL prepacked ω-aminododecyl-agarose column (from the DAA-8 Affinity Chromatography Kit, Sigma) equilibrated with the same buffer. The enzyme bound to the column and was eluted with a 0−0.2 M NaCl gradient in the same buffer applied to the column stepwise in 1-mL increments of 0.005 M/mL. Fractions of 1 mL were collected. Fractions containing aminopeptidase P activity (but devoid of dipeptidylpeptidase IV activity) were pooled, and Centricon-30 units were used to concentrate the enzyme and change the buffer to 0.1 M Hepes, pH 6.5. The enzyme (0.5 mL) was then applied to a 0.2-mL DEAE-Sephacel column equilibrated with the same buffer. The column was washed with 1 mL of the equilibration buffer. Enzyme was then eluted with a 0−0.2 M NaCl gradient in equilibration buffer delivered stepwise in 1-mL increments of 0.02 M/mL. Fractions of 1 mL were collected. Active fractions devoid of membrane dipeptidase activity were pooled, concentrated with Centricon-30 units, and stored at 4 °C in 0.1 M potassium phosphate, pH 6.8.

Electrophoresis. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) was carried out in a Pharmacia PhastSystem using precast homogeneous 7.5 PhastGels according to the manufacturer's instructions. Gels were stained using the recommended silver stain. Molecular weights were determined by comparing R_f values with those of the following standards (Sigma MW-SDS-200 kit): ovalbumin (M_r 45 000), bovine serum albumin (M_r 66 000), rat muscle phosphorylase B (M_r 97 400), and *E. coli* β-galactosidase (M_r 116 000).

N-Glycanase Treatment. Purified enzyme (0.5 μg) in 10 μL of buffered 50 mM 2-mercaptoethanol (pH 7.8) was boiled for 5 min. Recombinant *N*-glycanase (1.8 units in 7 μL) was added and the mixture incubated overnight at 37 °C.

Isoelectric Focusing. Isoelectric focusing was carried out using a Pharmacia PhastSystem and precast PhastGel IEF 3−9 gels. The standard proteins used and their isoelectric points (in parentheses) were amyloglucosidase (3.50), glucose oxidase (4.15), soybean trypsin inhibitor (4.55), β-lactoglobulin A (5.20), bovine carbonic anhydrase B (5.85), and human carbonic anhydrase B (6.55).

Gel Permeation Chromatography. Molecular weight determinations were carried out by high-performance gel permeation chromatography using different columns and ionic strengths. The columns, buffer conditions, and flow rates were Superose 6 (10 × 300 mm) (0.1 M potassium phosphate, pH 6.8, containing either 0 or 1 M NaCl) (0.3 mL/min), Bio-Sil SEC400 (7.5 × 600 mm plus guard column) (0.05 M potassium phosphate, pH 6.8) (0.75 mL/

[1] Abbreviations: AP-P, aminopeptidase P; DPP IV, dipeptidylpeptidase IV; ACE, angiotensin-converting enzyme; PEG, poly(ethylene glycol); EDTA, ethylenediaminetetraacetic acid; NNap, naphthylamide; PI-PLC, phosphatidylinositol-specific phospholipase C from *Bacillus thuringiensis*; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography.

Table 1: Purification of Aminopeptidase P from Rat Lung Microsomes

step	total activity[a] (μmol/min)	total protein (mg)	specific activity (units/mg)	% rec	purif
crude microsomes	52	780	0.067	100	1.0
PI-PLC[b] treatment of washed microsomes (supernatant)	30	69	0.43	58	6.4
decyl-agarose	23	8.5	2.7	44	40
ω-aminodecyl-agarose	24	1.7	14	46	210
decyl-agarose (0.8 M NaCl)	28	1.4	20	54	300
DEAE-Sephacel	23	1.1	21	44	310
Centricon-100 ultrafiltration	18	0.71	25	35	370
ω-aminododecyl-agarose	5.7	0.20	29	11	430
DEAE-Sephacel	5.0	0.11	45	10	670

[a] The substrate was Arg-Pro-Pro. [b] PI-PLC: phosphatidylinositol-specific phospholipase C from *B. thuringiensis*.

min), and Bio-Sil SEC250 (7.8 × 300 mm) (0.1 M sodium phosphate, pH 6.8, containing either 0 or 0.15 M NaCl) (1 mL/min). The molecular weight standards used in each experiment included some of the following: equine myoglobin (M_r 17 000), chymotrypsinogen (M_r 25 000), chicken ovalbumin (M_r 45 000), bovine serum albumin (M_r 67 000), alcohol dehydrogenase (M_r 150 000), bovine γ-globulin (M_r 158 000), β-amylase (M_r 200 000), catalase (M_r 240 000), and apoferritin (M_r 443 000).

Triton X-114 Phase Separation. Triton X-114 phase separation was carried out according to Bordier (1981).

Amino Acid Analysis and Sequencing. Amino acid analysis was carried out by June M. Lull and Dr. Robert L. Heinrikson at the Upjohn Laboratories. Amino acid sequence information was determined by the automated sequential Edman procedure (Edman, 1950). The N-terminal sequence of rat aminopeptidase P was determined by Joseph W. Leone and Dr. Robert L. Heinrikson at Upjohn. Amino acid sequence data on dipeptidylpeptidase IV, membrane dipeptidase, bovine aminopeptidase P, and peptide substrate hydrolysis products were determined by Dr. Bassam T. Wakim at Loyola University's Macromolecular Analysis Facility using an Applied Biosystems 477A pulsed liquid phase protein sequencer.

Kinetic Parameters. Kinetic parameters (K_m, k_{cat}, k_{cat}/K_m) for bradykinin and its lower homologs were determined in 0.1 M potassium phosphate, pH 6.8, at 37 °C using the standard fluorescent assay (Simmons & Orawski, 1992). Values were obtained by the graphical method of Eisenthal and Cornish-Bowden (1974).

RESULTS AND DISCUSSION

Purification of Rat Lung Aminopeptidase P. A summary of the purification procedure for aminopeptidase P from rat lung microsomes is shown in Table 1. The enzyme was solubilized from microsomes by treating the membranes with phosphatidylinositol-specific phospholipase C (PI-PLC) from *Bacillus thuringiensis*. The solubilized enzyme was then purified using a combination of hydrophobic interaction and ion exchange chromatography steps and ultrafiltration. The final two steps of the purification scheme specifically removed two contaminating peptidase activities. Figure 1A shows that the ω-aminododecyl-agarose column was effective in partially separating aminopeptidase P from dipeptidylpeptidase IV (DPP IV) activity.[2] Only fractions with minimal DPP IV activity were pooled for the next step. The final DEAE-Sephacel step (Figure 1B) separated aminopeptidase P from membrane dipeptidase activity. The overall procedure resulted in an aminopeptidase P preparation that was purified 670-fold over crude microsomes in 10% yield. SDS−PAGE of the purified enzyme gave a single band following silver staining (Figure 2A). The purification procedure removed essentially all of the membrane dipeptidase and bestatin-sensitive/amastatin-insensitive dipeptidase (Orawski & Simmons, 1992b) activities, 99.8% of the DPP IV activity, and 99.9% of the aminopeptidase M activity originally present in microsomes.

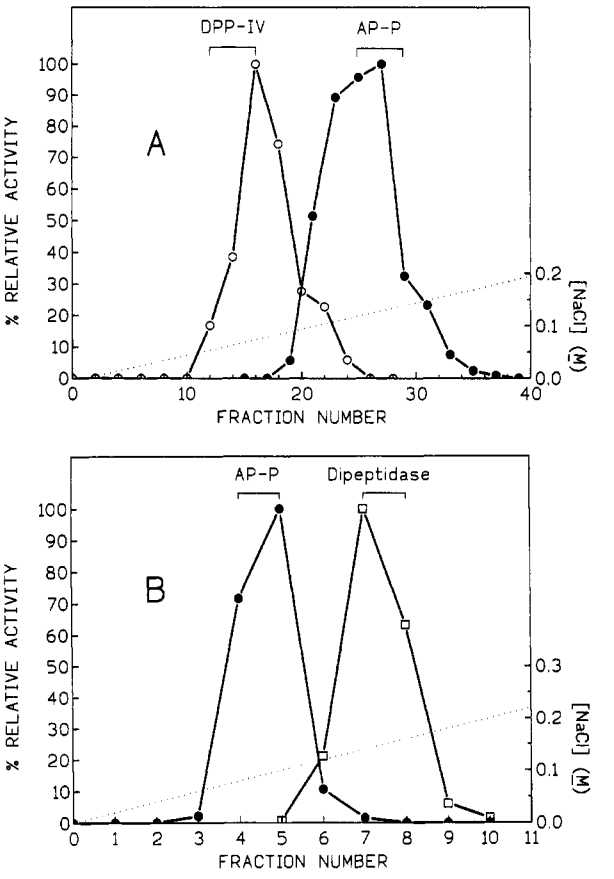

FIGURE 1: (A) Separation of aminopeptidase P and dipeptidylpeptidase IV on ω-aminododecyl-agarose. (B) Separation of aminopeptidase P and membrane dipeptidase activity in the final DEAE-Sephacel step. The ω-aminododecyl-agarose and DEAE-Sephacel columns were run as described in Methods: All fractions were 1 mL. (●) Aminopeptidase P (AP-P) activity (Arg-Pro-Pro); (○) dipeptidylpeptidase IV (DPP IV) activity (Gly-Pro-2-NNap); (□) membrane dipeptidase activity (Gly-D-Phe); (...) NaCl concentration. The brackets indicate which fractions were pooled for each enzyme.

Structural Properties. The subunit molecular weight of aminopeptidase P determined by SDS−PAGE (Figure 2A) was 90 000 ± 2100. When the purified enzyme was treated with *N*-glycanase [peptide N^4-(*N*-acetyl-β-glucosaminyl) asparagine amidase] to remove N-linked carbohydrate moieties, the subunit molecular weight decreased to 74 600 ± 1400,

[2] In contrast, during the purification of bovine lung aminopeptidase P (Simmons & Orawski, 1992), contaminating dipeptidylpeptidase IV was removed in the ω-aminodecyl-agarose step, where it eluted at a higher salt concentration than aminopeptidase P.

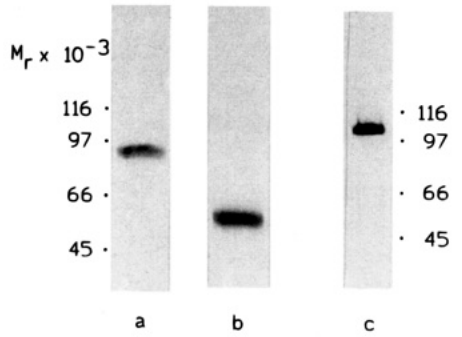

FIGURE 2: SDS−PAGE: (a) purified aminopeptidase P; (b) purified membrane dipeptidase from the second DEAE-Sephacel step; (c) purified dipeptidylpeptidase IV from the ω-aminododecyl-agarose step. Molecular weight markers were ovalbumin (M_r 45 000), bovine serum albumin (M_r 66 000), phosphorylase B (M_r 97 400), and β-galactosidase (M_r 116 000). Gels were stained with silver stain.

Table 2: Amino Acid Analysis of Rat and Bovine Aminopeptidase P[a]

amino acid	mol % rat	mol % bovine	amino acid	mol % rat	mol % bovine
Asx	9.55	9.99	Ile	4.37	3.86
Thr	6.92	7.75	Leu	9.73	7.50
Ser	7.79	8.24	Tyr	6.26	4.65
Glx	10.83	10.93	Phe	3.86	3.27
Pro	4.47	6.83	His	2.44	2.97
Gly	8.37	8.95	Lys	4.08	5.42
Ala	7.34	7.46	Trp	nd[b]	nd[b]
Val	7.17	7.32	Arg	5.12	3.82
Met	1.02	1.05			

[a] No cysteic acid appeared after hydrogen peroxide treatment of either rat or bovine aminopeptidase P. [b] nd, not determined.

suggesting that the native enzyme is composed of at least 17% carbohydrate. The glycoprotein nature of the native enzyme was also apparent from its ability to bind all lectins supplied in the Lectin-Link kit (Genzyme Corp.): concanavalin A, *Ricinus communis* agglutinin, *Datura stromonium* agglutinin, *Sambuscus nigra* agglutinin, and wheat germ agglutinin. Gel permeation chromatography in the absence of NaCl on either a Superose 6 or Bio-Sil SEC400 column gave a molecular weight of 220 000. However, values for the molecular weight of 280 000, 310 000 and 340 000 were also obtained under the following conditions: Bio-Sil SEC250 (0 M NaCl), Bio-Sil SEC250 (0.15 M NaCl), and Superose 6 (1 M NaCl), respectively. The precise role of ionic strength and enzyme concentration in subunit aggregation requires further study. The purified enzyme partitioned into the aqueous phase upon Triton X-114 phase separation, suggesting that the enzyme liberated from membranes by PI-PLC is hydrophilic. Isoelectric focusing gave a broad band between pH 4.6 and 5.1, which is presumably due to the glycoprotein nature of the enzyme.

Table 2 shows the amino acid compositions for both purified rat lung and bovine lung aminopeptidase P. No cysteine (as cysteic acid) was detected in either enzyme after hydrogen peroxide treatment. Methionine was underrepresented compared to the average protein in GenBank (Lupas et al., 1991). Tests developed by Cornish-Bowden (1980) for statistically determining the relatedness of protein sequences on the basis of amino acid composition were applied to the data in Table 2. The results indicate that the two enzymes are likely to have similar amino acid sequences

Table 3: Kinetic Parameters for Hydrolysis of Tripeptide Substrates[a]

substrate	K_m (mM)	k_{cat} (s^{-1})	$10^{-5}k_{cat}/K_m$ (s^{-1} M^{-1})
Arg-Pro-Pro	0.35	230	6.6
Arg-Pro-Pro + Mn^{2+}	0.36	180	5.0
Gly-Pro-Hyp	0.32	4.8	0.15
Gly-Pro-Hyp + Mn^{2+}	2.0	69	0.35

[a] Kinetic parameters were determined at 37 °C in 0.1 M Hepes, pH 8.0, in the presence or absence of 4 mM $MnCl_2$.

Table 4: Kinetic Constants for Hydrolysis of Bradykinin and Its Lower Homologs[a]

peptide[b] P1	P1′	P2′	P3′	P4′	P5′	P6′	P7′	P8′	K_m (mM)	k_{cat} (s^{-1})	$10^{-5}k_{cat}/K_m$ (s^{-1} M^{-1})
Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg									0.021	12	5.7
Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe									0.039	13	3.3
Arg-Pro-Pro-Gly-Phe-Ser-Pro									0.051	16	3.1
Arg-Pro-Pro-Gly-Phe-Ser									0.032	11	3.4
Arg-Pro-Pro-Gly-Phe									0.048	15	3.1
Arg-Pro-Pro-Gly									0.030	17	5.7
Arg-Pro-Pro									1.0	150	1.5
Arg-Pro										0[c]	

[a] Assay conditions, 0.1 M potassium phosphate, pH 6.8. [b] Amino acid residue nomenclature is from Schechter and Berger (1967). [c] No detectable cleavage of up to 10 mM Arg-Pro.

according to the "weak" test (DI = 6.55). Neither enzyme passed the weak test for relatedness to *Escherichia coli* aminopeptidase P (Yoshimoto et al., 1989).

The N-terminal amino acid sequence of rat lung aminopeptidase P determined by Edman degradation was X^1-Gly^2-Pro^3-Glu^4-Ser^5-Leu^6-Gly^7-Arg^8-Glu^9-Asp^{10}-Val^{11}-Arg^{12}-Asp^{13}-X^{14}-Ser^{15}-Thr^{16}-Asn^{17}-Pro^{18}-Pro^{19}-Arg^{20}-Leu^{21}-X^{22}-Val^{23}-Thr^{24}-Ala^{25}-. This sequence showed no significant homology to any protein in the GenBank or NBRF-PIR data bases. The unidentified residues at positions 14 and 22 are each followed by a threonine residue two positions in the C-terminal direction. It is possible that these sites represent consensus sequences for glycosylation and that positions 14 and 22 are glycosylated asparagine residues which would not be directly detected by the Edman procedure.

Hydrolysis of Tripeptide Substrates. Table 3 shows the kinetic parameters for the hydrolysis of Arg-Pro-Pro and Gly-Pro-Hyp by purified rat lung aminopeptidase P. Arg-Pro-Pro was hydrolyzed rapidly and gave a k_{cat}/K_m of 6.6×10^5 s^{-1} M^{-1} in 0.1 M Hepes, pH 8.0. This compares with 1.5×10^5 s^{-1} M^{-1} using the standard assay conditions of 0.1 M phosphate, pH 6.8, where the K_m was 3-fold higher (Table 4). The presence of Mn^{2+} (4 mM) had little effect on the kinetic parameters for Arg-Pro-Pro cleavage under the conditions of the experiment. Gly-Pro-Hyp, a commonly used substrate for aminopeptidase P, was hydrolyzed considerably more slowly than Arg-Pro-Pro. The presence of 4 mM Mn^{2+} increased the k_{cat} by 14-fold. However, the k_{cat}/K_m was increased only 2.3-fold due to a simultaneous 6-fold increase in K_m.

Degradation of Bradykinin and Its Fragments. Rat aminopeptidase P was found to hydrolyze bradykinin at the Arg^1−Pro^2 bond using an HPLC system described previously (Simmons & Orawski, 1992) (data not shown). Table 4 shows the kinetic parameters for the degradation of bradykinin and its lower homologs at pH 6.8. For bradykinin, $K_m = 21$ μM, $k_{cat} = 12$ s^{-1}, and $k_{cat}/K_m = 5.7 \times 10^5$ s^{-1}

M^{-1}. Successively shorter homologs of bradykinin down to and including the tetrapeptide, Arg-Pro-Pro-Gly, had kinetic parameters very similar to those of bradykinin itself. However, the tripeptide, Arg-Pro-Pro, had a 50-fold higher K_m (1.0 mM), 13-fold higher k_{cat} (150 s^{-1}), and 3.8-fold lower k_{cat}/K_m (1.5×10^5 s^{-1} M^{-1}) than did bradykinin. The dipeptide, Arg-Pro, was not cleaved at all. The results suggest that rat lung aminopeptidase P has an extended binding site which can interact with four amino acid residues of a peptide substrate, P_1, P_1', P_2', and P_3' [nomenclature of Schecter and Berger (1967)]. At least three of the respective binding site subsites (S_1, S_1', S_2') must be occupied in order for hydrolysis to take place. The kinetic parameters are further modified by occupation of the fourth subsite (S_3').

Cleavage of Other Peptides. Human neuropeptide Y, which has an N-terminal sequence of Tyr-Pro-Ser-Lys-Pro-, is predicted to be a substrate for membrane-bound aminopeptidase P (Yoshimoto et al., 1994). When the neuropeptide Y (1-24)amide fragment was incubated with rat lung aminopeptidase P (pH 6.8) and the products were subjected to amino acid sequence analysis, the following PTH-amino acids were found: cycle 1, Tyr and Pro; cycle 2, Pro and Ser; cycle 3, Ser and Lys; cycle 4, Lys and Pro. The results indicate that a peotide with the N-terminal sequence of Pro-Ser-Lys-Pro- was present in the final incubation mixture along with undigested substrate, showing that aminopeptidase P cleaved the Tyr^1—Pro^2 bond. It was estimated from the relative amounts of the substrate and product PTH-amino acids that neuropeptide Y (1-24)amide was cleaved at about the same rate as bradykinin [13 μmol of bradykinin (min^{-1}) (mg of enzyme)$^{-1}$] when both peptides were assayed at 0.5 mM.

Allatostatin I, a 13-amino acid peptide with an N-terminal sequence of Ala-Pro-Ser-Gly-, was found to be cleaved at the rate of 39 μmol min^{-1} mg^{-1} using the standard fluorescence assay and 0.5 mM peptide. It was determined that cleavage occurred at the Ala^1—Pro^2 bond by amino acid sequence analysis as described above.

Human erythropoietin, a 30 400 dalton glycoprotein with an N-terminal Ala-Pro-Pro-Arg- sequence, was not hydrolyzed by aminopeptidase P in either its native state or following denaturation (but with disulfide bonds intact) (Dr. Eugene Goldwasser, University of Chicago, personal communication). The presence of a disulfide bridge seven residues from the N-terminus may have prevented hydrolysis by steric hindrance.

Aminopeptidase P has been shown to cleave peptides with a dehydroproline residue in place of proline in the penultimate position (Yoshimoto et al., 1994). To further investigate the nature of the S_1' subsite of the enzyme, a peptide with a homoproline residue in the second position was prepared. Arg-homoPro-Pro-Ala-NH_2 was found to be a good substrate with the following kinetic parameters when assayed in 0.1 M Hepes, pH 8.0: K_m = 0.15 mM; k_{cat} = 140 s^{-1}; $k_{cat}/K_m = 9.3 \times 10^5$ s^{-1} M^{-1}. The results suggest that the S_1' subsite can accommodate a six-membered piperidine ring.

pH Optimum. Figure 3 shows the activity of rat lung aminopeptidase P toward Arg-Pro-Pro at various pH values in different 0.1 M buffers. When the results from all buffer conditions are considered, activity was observed between about pH 6 and 11. The pH-dependency curves for most buffers exhibited similar acidic limbs, with activity peaking between pH 6.8 and 7.5. Activity gradually decreased in the alkaline pH range for most buffers, although activity in Tris was maintained at a peak value out to at least pH 10.

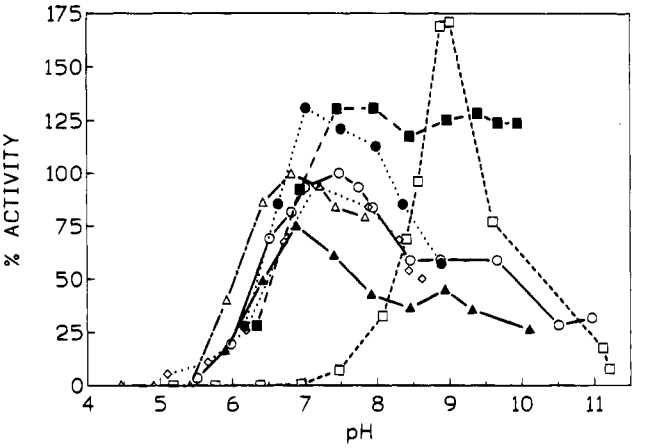

FIGURE 3: Dependence of activity on pH. Enzyme was incubated with Arg-Pro-Pro (0.5 mM) at 37 °C for 30 min in various buffers (all 0.1 M) at different pH values. Activity was determined by the fluorescence assay except for samples containing Tris, which were assayed by HPLC. Since some samples were at pH values outside the buffering range of the buffer used, the pH of each incubation mixture was measured at the end of the reaction. Activity is expressed relative to that in 0.1 M potassium phosphate, pH 7.5 (=100%): (○) potassium phosphate; (●) Hepes; (□) imidazole; (■) Tris; (△) sodium acetate; (▲) sodium borate; (◇) MES.

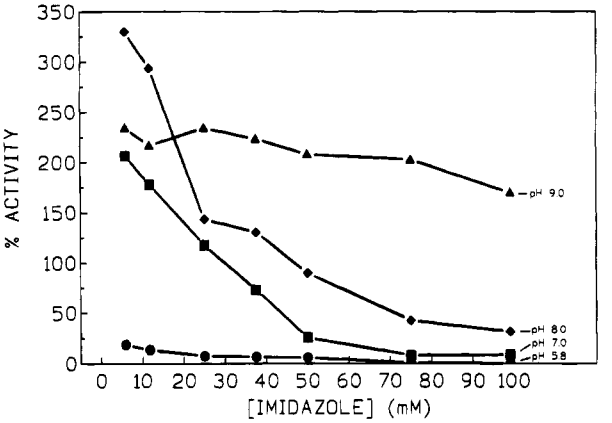

FIGURE 4: Effect of imidazole on activity. Enzyme was incubated with Arg-Pro-Pro (0.5 mM) at 37 °C for 30 min in imidazole buffers of different concentrations and pH. Activity is expressed relative to that in 0.1 M potassium phosphate, pH 7.5, to facilitate comparison with data in Figure 3: (●) pH 5.8; (■) pH 7.0; (◆) pH 8.0; (▲) pH 9.0.

Anomalous behavior was seen with imidazole, which gave little activity below pH 8.0 and had a peak at pH 9.0. As the concentration of imidazole in the assay was decreased, however, activity gradually increased in the range of pH 5.8—9.0. Figure 4 shows the activity of aminopeptidase P in different concentrations of imidazole at different pH values. At the lowest concentration of imidazole tested (6 mM), activity was highest at pH 8.0, where it was actually 2.5—9.5-fold higher than the activities observed at this pH for the other buffer conditions shown in Figure 3. As the concentration of imidazole increased, the relative activity decreased at each pH value. The extent of decrease, however, was much less at pH 9.0 than at lower pH values. As a result, at imidazole concentrations above 11 mM, the pH optimum shifted to pH 9.0. By 100 mM imidazole, there was a large difference in activity between pH 8.0 and 9.0 as

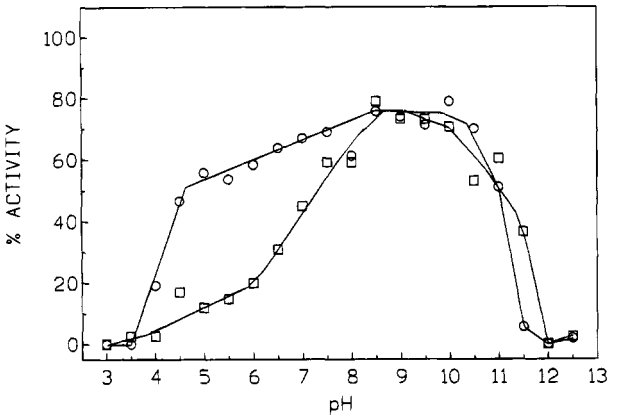

FIGURE 5: pH Stability. Enzyme was preincubated in 0.1 M potassium phosphate with (○) or without (□) 95 μg/mL PEG-20000 at different pH values at 37 °C for 2 h. Residual activity was then determined by adding buffered Arg-Pro-Pro to the preincubation mixture to give the final assay conditions of 0.2 M potassium phosphate, pH 6.8, with or without 9 μg/mL PEG-20000. Residual activity was compared to the activity of the respective control consisting of the same amount of enzyme not subjected to preincubation and assayed in the presence or absence of 9 μg/mL PEG-20000 (=100%).

shown in both Figures 3 and 4. In data not shown, the addition of increasing concentrations of imidazole to enzyme buffered in 0.1 M Hepes resulted in an increasing degree of inhibition which was more pronounced at lower pH values than at higher pH values. The results with imidazole suggest that, in addition to the effects of pH, there was an effect of imidazole itself on enzyme activity. It is proposed that the protonated imidazolium ion may be the inhibitory species in these experiments. This ionic form, which is rare at pH 9.0 (1%), increases with decreasing pH and with increasing buffer concentration.

The pH optimum for bradykinin(1−8) (0.5 mM) was 7.0 in both 0.1 M Hepes and 0.1 M potassium phosphate.

pH Stability. Figure 5 shows the stability of purified rat lung aminopeptidase P when preincubated in diluted form at different pH values for 2 h at 37 °C. In the absence of poly(ethylene glycol) (PEG-20000), activity was lost at all pH values, but was most stable in the range of pH 8.5−10. In general, enzyme stability at all pH values decreased with increasing purity and length of storage of the enzyme preparation. The enzyme was more stable when preincubated at 0 and 25 °C than at 37 °C.

When preincubated in the presence of PEG-20000, the enzyme exhibited improved stability in the range of pH 4.5−7.0 (Figure 5). However, PEG-20000 did not completely prevent loss of activity at any pH.

Temperature Stability. When preincubated without PEG-20000 in 0.1 M potassium phosphate, pH 6.8, for 30 min at various temperatures, the enzyme was relatively stable up to 55 °C and then lost activity completely by 70−75 °C.

Effect of Poly(ethylene glycol) on Activity. PEG-20000 (M_r 20 000), when present in the assay, was found to stimulate activity 32 ± 9% over a wide concentration range (0.001−2 mg/mL). A similar effect was seen with lower molecular weight poly(ethylene glycol)s. At 100 μg/mL, PEG-20000, PEG-8000, PEG-1450, and PEG-600 all stimulated activity similarly (38 ± 3%). However, at the same concentration, PEG-400, PEG-300, and PEG-200, as well as ethylene glycol, had no effect on activity. Glycerol had no effect over the range of 0.001−2 mg/mL.

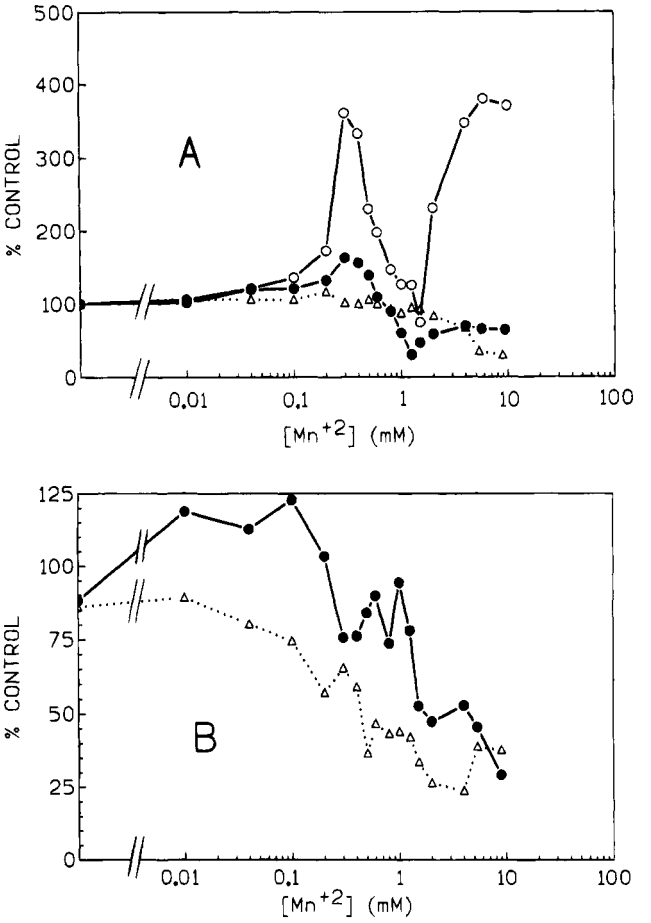

FIGURE 6: (A) Effect of Mn^{2+} concentration on activity. Activities toward Arg-Pro-Pro (0.5 mM) (●), Gly-Pro-Hyp (2 mM) (○), and [^{3}H]bradykinin (22 nM) (△) were determined in 0.1 M Hepes, pH 8.0, containing the indicated concentration of $MnCl_2$. The controls (=100%) were the respective activities in the absence of $MnCl_2$. (B) Effect of Mn^{2+} concentration on inhibition by ramiprilat. Activities toward Arg-Pro-Pro (0.5 mM) (●) and [^{3}H]bradykinin (22 nM) (△) were determined in 0.1 M Hepes, pH 8.0, containing 200 μM ramiprilat and the indicated concentration of $MnCl_2$. Each point represents the activity as a percent of its respective control activity determined in the absence of ramiprilat but in the presence of the indicated $MnCl_2$ concentration (=100%) (see panel A).

Effect of NaCl on Activity. Aminopeptidase P was sensitive to the presence of NaCl. The following concentrations of NaCl (M) in 0.1 M potassium phosphate, pH 6.8, resulted in the indicated percent inhibition: 0.1 (20%), 0.25 (52%), 0.5 (62%), 1.0 (87%), and 2.0 (99%).

Effect of Metal Ions on Activity. The effect of different concentrations of $MnCl_2$ on activity toward Arg-Pro-Pro and Gly-Pro-Hyp was determined (Figure 6A). Mn^{2+} had little effect on either activity from 10 to 100 μM. However, Mn^{2+} stimulated both activities at 300−400 μM, reaching nearly 4-fold stimulation for Gly-Pro-Hyp and 1.6-fold for Arg-Pro-Pro. An activity minimum was then reached with both substrates at 1−2 mM Mn^{2+}, where the metal ion was actually inhibitory. Activity toward Gly-Pro-Hyp, but not Arg-Pro-Pro, was again stimulated above the control activity at higher Mn^{2+} concentrations. Figure 6A also shows that activity toward [^{3}H]bradykinin (22 nM) was not stimulated at any concentration of Mn^{2+} but was inhibited in the millimolar range. Thus, the effect of Mn^{2+} on activity depended on its concentration and on the substrate used.

Table 5: Effect of Nonsubstrate Proline-Containing Peptides on Aminopeptidase P Activity[a]

peptide[b]	rel act. (%)
none	100
Pro-Pro	99
Pro-Pro-Gly	45
Pro-Pro-Gly-Phe	27
Pro-Pro-Gly-Phe-Ser-Pro	24
Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg	11
Pro-Pro-Ala	33
Pro-Pro-Ala (0.1 mM)	(29)[c]
Pro-Pro-Ala-Ala (0.1 mM)	(12)[c]

[a] The effect of various nonsubstrate peptides (all at 0.5 mM unless otherwise indicated) were tested for their ability to inhibit cleavage of Arg-Pro-Pro (0.5 mM) in the standard assay. Incubation mixtures contained 10 μg/mL PEG-20000. Activity is expressed relative to the rate of cleavage of substrate in the absence of added peptide. [b] The following peptides caused ≤15% inhibition (relative rate ≥85%) at 0.5 mM: Pro-Val, Pro-Leu, Pro-Gly, Pro-Arg, Pro-*p*-nitroanilide, Pro-Pro-Pro, Pro-Tyr-Ala, Arg-Pro-*p*-nitroanilide, Pro-His-Ala, Ile-Pro-Ile (diprotin A), D-Ala-Pro-Phe, D-Phe-Pro-Arg-H, Boc-D-Phe-Pro-Arg-H, methyl-D-Phe-Pro-Arg-H, Pro-Arg-Gly-NH_2, Pro-Leu-Gly-NH_2, Pro-D-Pro-Ala-NH_2, Pro-Arg(Tos)-Gly-NH_2, Pro-Lys-Ala-4-methoxy-2-naphthylamide (HPLC assay), Arg-D-Pro-Asp-Val-Tyr, Tyr-D-Ala-Gly-Phe-Met, D-Pro-Pro-Arg-*p*-nitroanilide, Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2 (substance P 2−11) (0.1 mM) (HPLC assay), Pro-Ala-Asn-Ile-Lys-Trp-Gly-Asp (HPLC assay). [c] Assayed in 0.1 M Hepes, pH 8.0.

Using the same assay conditions as in Figure 6A, $ZnCl_2$ had little effect on Arg-Pro-Pro cleavage from 0.01 to 2 mM but inhibited almost completely by 10 mM. $CoCl_2$ had minimal effect on cleavage of this substrate from 0.01 to 0.1 mM, but inhibited completely by 1.5 mM. $CaCl_2$ and $MgCl_2$ had little effect on Arg-Pro-Pro hydrolysis over the concentration range of 0.01−10 mM.

Hooper et al. (1990) showed that cleavage of Gly-Pro-Hyp by pig kidney aminopeptidase P was stimulated by Mn^{2+} with a peak (about 3.5-fold) at about 1 mM and then was inhibited at concentrations between 10 and 100 mM. Guinea pig serum aminopeptidase P was only modestly stimulated (20% using Arg-Pro-Pro-[^{3}H]benzylamine as substrate) at 0.25−1.0 mM Mn^{2+} (Ryan et al., 1992). The role of Mn^{2+} in these experiments and the ones described above is unknown. Pig kidney aminopeptidase P has been shown to contain Zn^{2+} (Hooper et al., 1992). Whether added Mn^{2+} influences activity by binding at a secondary site remains to be shown. We have observed complex patterns when testing the pH dependence of the Mn^{2+} effect (data not shown). Certainly a number of variables including concentration of Mn^{2+}, type and concentration of substrate, degree of interaction of Mn^{2+} with the substrate, buffer type, pH, and whether Mn^{2+} is affecting k_{cat} or K_m could be responsible for an observed Mn^{2+} effect.

Peptides as Inhibitors of Aminopeptidase P. Table 5 shows that some nonsubstrate peptides containing proline were inhibitory to aminopeptidase P. Among these were the bradykinin fragments (2−4), (2−5), (2−7), and (2−9). The inhibitory potency of these fragments increased with increasing length. The dipeptide, Pro-Pro, had no effect on activity. Similar results with bradykinin fragments have been reported for bovine lung (Simmons & Orawski, 1992) and guinea pig serum (Ryan et al., 1992) aminopeptidase P. Pro-Pro-Ala inhibited the rat enzyme with a K_i of 65 μM (at pH 6.8) and appeared to act as a pure noncompetitive inhibitor. Pro-Pro-Ala-Ala was the most potent inhibitory peptide tested.

Table 6: Effect of Various Agents on Aminopeptidase P Activity[a]

agents[b]	concn (mM)	rel act. (%)
none		100
1,10-phenanthroline	1	0
EDTA	1	23
2-mercaptoethanol	4	12
D,L-dithiothreitol	4	7
p-(chloromercuri)benzenesulfonic acid	1	11
D,L-2-mercaptomethyl-3-guanidinoethylthiopropanoic acid	1	33
N-[1-(*R*,*S*)-carboxy-(2-phenylethyl)]-Ala-Ala-Phe-*p*-aminobenzoate	0.25	95
	1	48
apstatin	0.005	54
	0.05	11
captopril	0.01	62
	0.05	41
1-butanol	2%[c]	66
	10%[c]	5
dimethyl sulfoxide	2%[c]	104
	10%[c]	65

[a] Aminopeptidase P was assayed with 0.5 mM Arg-Pro-Pro in 0.1 M potassium phosphate, pH 6.8, in the presence of various agents at the indicated concentrations. Incubation mixtures contained 10 μg/mL PEG-20000, and the incubation time was 45 min. Activity is expressed as percent of control without added agent. [b] The following agents caused <15% inhibition (relative rate >85%): amastatin (0.5 mM), bestatin (0.21 mM), puromycin (0.3 mM), actinonin (1 mM), leuhistin (1 mM), oxidized leucinethiol (1 mM), reduced leucinethiol (0.1 mM oxidized leucinethiol plus 0.1 mM 2-mercaptoethanol), arphamenine A (1 mM), arphamenine B (1 mM), diisopropylphosphofluoridate (1.1 mM), 2-propanol (0.5%), diprotin A (1 mM), phosphoramidon (0.01 mM), thiorphan (0.1 mM), *N*-[1-(*R*,*S*)-carboxy-(2-phenylethyl)]-Phe-*p*-aminobenzoate (1 mM), cilastatin (0.1 mg/mL), phenelzine (1 mM), phenylhydrazine (1 mM), Arg-hydroxamate (1 mM), Tyr-hydroxamate (1 mM), D,L-Phe-hydroxamate (1 mM), Pefabloc (0.5 mM) (assayed in 0.1 M Hepes, pH 8.0), foroxymithine (0.5 mM), ramiprilat (1 mM), 2-pyrrolidine (1 mM), 3-pyrrolidino-1,2-propanediol (1 mM), L-prolinol (1 mM), 1-(pyrrolidinocarbonylmethyl)piperazine (1 mM), 1-pyrrolidinecarboxaldehyde (1 mM), 4-methylmorpholine (1 mM), and sodium azide (0.1%). [c] Volume/volume.

Effect of Other Agents on Activity. Table 6 shows the effect of various chemicals including a variety of peptidase inhibitors on aminopeptidase P activity. The enzyme was inhibited by metal chelating agents such as 1,10-phenanthroline and EDTA, indicating that the enzyme is a metallopeptidase. Aminopeptidase P was also inhibited by the thiol-containing compounds 2-mercaptoethanol and dithiothreitol. D,L-2-Mercaptomethyl-3-guanidinoethylthiopropanoic acid (a carboxypeptidase N inhibitor) (Plummer & Ryan, 1981) and *N*-[1-(*R*,*S*)-carboxy-(2-phenylethyl)]-Ala-Ala-Phe-*p*-aminobenzoate (an endopeptidase 24.15 inhibitor) (Orlowski et al., 1988) were weak inhibitors of the enzyme. A new compound developed in this laboratory called apstatin [(2*S*,3*R*)-3-amino-2-hydroxy-4-phenylbutanoyl-L-prolyl-L-prolyl-L-alaninamide] (Prechel et al., 1994) inhibited aminopeptidase P in the micromolar range. Footnote *b* in Table 6 indicates that a large number of other agents including a variety of aminopeptidase inhibitors such as amastatin, bestatin, puromycin, actinonin, leuhistin, reduced and oxidized leucinethiol, arphamenines A and B, and amino acid hydroxamates had no significant effect on activity at the concentrations and under the conditions used. It can be noted that bestatin, at 0.2 mM, had different effects on the enzyme depending on the substrate and assay conditions used. These included no effect (e.g., conditions in Table 6), partial inhibition (e.g., 48% inhibition of the hydrolysis of 1 mM

Gly-Pro-Hyp in 0.1 M Hepes, pH 8.0, containing 3.6 mM $MnCl_2$), and even stimulation (e.g., up to 2-fold using various substrates assayed in 0.1 M imidazole, pH 8.0). Inhibition by bestatin under specific conditions has been observed for membrane-bound aminopeptidase P from bovine lung (unpublished), rat intestine (Lasch et al., 1988), and pig kidney (Hooper et al., 1990, 1992), as well as for cytosolic forms of the enzyme (Harbeck & Mentlein, 1991; Vanhoof et al., 1992). In all cases, inhibition by bestatin has only been seen at or near millimolar concentrations.

A number of animal venoms were tested for the presence of a natural aminopeptidase P inhibitor. Venoms from the following species had no effect on activity at 5 mg/mL: *Apis mellifera, Condylactis gigantea, Agkistrodon halys blomhoffii, Dendroaspis angusticeps, Bothrops jararaca, Bufo marinus, and Polistes apachus* (1.2 mg/mL).

Angiotensin Converting Enzyme Inhibitors. Table 6 indicates that captopril, an angiotensin-converting enzyme (ACE) inhibitor, is also an inhibitor of aminopeptidase P in agreement with observations by others (Hooper et al., 1992; Ryan et al., 1992). Captopril exhibited a mixed-type (β = 0) (α = 4) inhibition with a K_i = 9 μM (assayed in Hepes, pH 8.0). In contrast to studies using pig renal aminopeptidase P (Hooper et al., 1992), we were unable to detect inhibition of rat lung aminopeptidase P in our standard assay by ramiprilat, a carboxyalkyl inhibitor of ACE. In the published studies on the pig enzyme, activity had been assayed with Gly-Pro-Hyp as substrate in the presence of 4 mM $Mn.^{2+}$ We therefore investigated the possible requirement of Mn^{2+} for the inhibitory activity of ramiprilat. Figure 7A shows that ramiprilat was much more inhibitory in the presence of 4 mM Mn^{2+} than in its absence regardless of whether Gly-Pro-Hyp or Arg-Pro-Pro was used as the substrate. The IC_{50} for ramiprilat in the presence of Mn^{2+} was 20 μM for Gly-Pro-Hyp and 140 μM for Arg-Pro-Pro. Figure 6B shows the dependence of inhibition by a single concentration (200 μM) of ramiprilat on the Mn^{2+} concentration. Investigation of other metal ions in the same concentration range (0.01−10 mM) showed that $ZnCl_2$, $CoCl_2$, $MgCl_2$, and $CaCl_2$ could not substitute for $MnCl_2$ in producing significant inhibition by ramiprilat.

Inhibition by enalaprilat, another carboxyalkyl ACE inhibitor, was also enhanced by 4 mM Mn^{2+} (Figure 7B). Some inhibition by enalaprilat was seen in the absence of Mn^{2+} (IC_{50} = 380 μM with Gly-Pro-Hyp), but inhibition was much greater in the presence of Mn^{2+} (IC_{50} = 2.6 and 17 μM for Gly-Pro-Hyp and Arg-Pro-Pro, respectively). Lisinopril, a carboxyalkyl ACE inhibitor in which a lysyl residue replaces the alanyl residue in enalaprilat, did not inhibit aminopeptidase P up to 4 mM either in the presence or absence of 4 mM Mn^{2+}. Inhibition by a carboxyalkyl inhibitor of endopeptidase 24.15, *N*-[1-(*R,S*)-carboxy-(2-phenylethyl)]-Ala-Ala-Phe-*p*-aminobenzoate, was also enhanced by Mn^{2+}. In contrast to the results at pH 6.8 (see Table 6), this compound at 1 mM had no effect on Arg-Pro-Pro cleavage at pH 8.0 (Hepes), but inhibited 72% at this higher pH in the presence of 4 mM $MnCl_2$.

The role of Mn^{2+} in enhancing the inhibitory potential of the carboxyalkyl-type ACE and endopeptidase 24.15 inhibitors is unknown. Perhaps Mn^{2+} acts as a bridge between the negatively charged carboxylate of the inhibitor and a putative carboxylate or other Lewis base in the active site normally involved in binding the substrate N-terminal amino group.

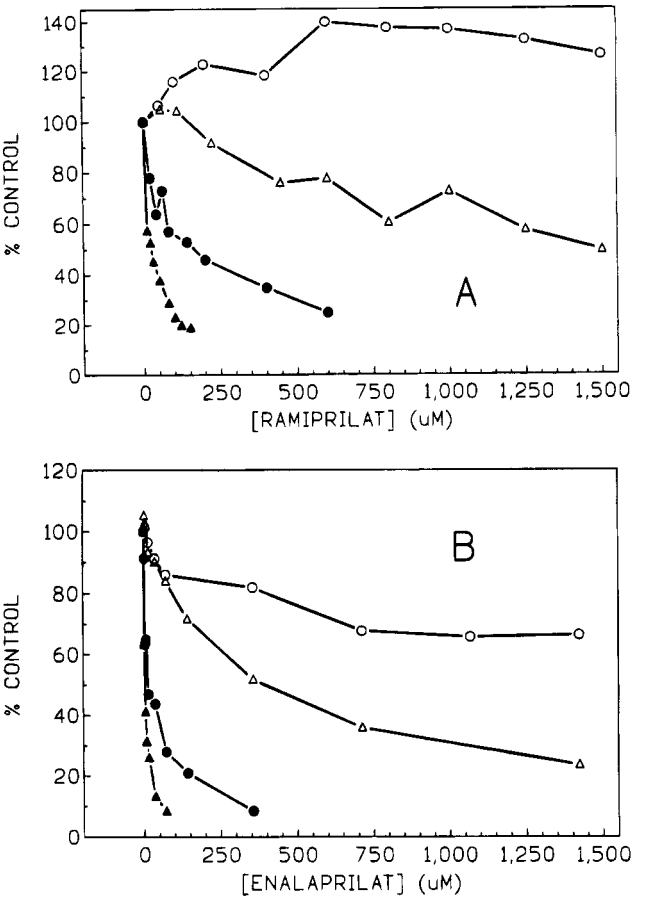

FIGURE 7: Effect of carboxyalkyl angiotensin-converting enzyme inhibitors on aminopeptidase P activity. Aminopeptidase P activity toward either Arg-Pro-Pro (1 mM) or Gly-Pro-Hyp (1 mM) was determined alone and in the presence of increasing concentrations of either (A) ramiprilat or (B) enalaprilat. Assays were carried out at 37 °C in either 0.1 M Hepes, pH 8.0, or in the same buffer containing 4 mM $MnCl_2$: (○) Arg-Pro-Pro, no Mn^{2+}; (●) Arg-Pro-Pro + Mn^{2+}; (△) Gly-Pro-Hyp, no Mn^{2+}, (▲) Gly-Pro-Hyp + Mn^{2+}. The data are expressed as percent control, where 100% is the activity of the respective substrate−Mn^{2+} condition in the absence of inhibitor.

In contrast to the carboxyalkyl-type inhibitors, inhibition of aminopeptidase P by the sulfhydryl ACE inhibitor captopril was actually reduced by the presence of Mn^{2+}. The inhibition of cleavage of Gly-Pro-Hyp (1 mM) by 0.32 mM captopril (in 0.1 M Hepes, pH 8.0) was reduced from 93% to only 4% in the presence of 4 mM $MnCl_2$.

Comparison with Purified Bovine Lung Aminopeptidase P. During the course of the studies on rat lung aminopeptidase P, additional parallel data were obtained on purified bovine lung membrane-bound aminopeptidase P (Simmons & Orawski, 1992). The subunit molecular weight of the bovine enzyme as determined by SDS−PAGE decreased from 94 500 ± 2800 to 76 400 ± 2000 upon treatment with *N*-glycanase, indicating that the enzyme is composed of at least 19% N-linked carbohydrate. The amino acid composition of bovine aminopeptidase P is shown in Table 2 and was discussed above. The N-terminus of the bovine enzyme was apparently blocked as no sequence could be obtained by the Edman procedure (Simmons & Orawski, 1992). However, a cyanogen bromide cleavage fragment was isolated and found to have the following sequence:[3] Val-

Thr-Lys-Ala-Val-Lys-Asn-Trp-Lys-Glu-Gln-Thr-Leu-Leu-Arg-Ala-X-His-Val-Arg-Asp-Ala-Val-Ala. Of the peptides listed in Table 5, Pro-Pro-Ala-Ala was also the most potent inhibitor of bovine aminopeptidase P, causing 90% inhibition at 0.1 mM and pH 8.0.

Copurification of Dipeptidylpeptidase IV and Membrane Dipeptidase. The ω-aminododecyl-agarose step used in the purification of rat lung aminopeptidase P (Figure 1A) yielded fractions that contained pure dipeptidylpeptidase IV (DPP IV) (EC 3.4.14.5) as shown by SDS−PAGE (Figure 2C). When the purified DPP IV was subjected to Triton X-114 phase separation (Bordier, 1981), the enzyme was found in the aqueous phase, suggesting that the DPP IV was devoid of its transmembrane domain and therefore presumably was formed by autolysis during the PI-PLC treatment step. However, the N-terminus was inexplicably blocked as no sequence information could be obtained on the intact protein by the Edman procedure. Cyanogen bromide cleavage, however, resulted in a peptide fragment that had the sequence His-Ala-Ile-Asn-Lys-Arg-Leu-Gly-Thr-Leu-Glu-Val-Glu-, which is identical to amino acids 593−605 of rat liver DPP IV (Ogata et al., 1989). The purified enzyme had a subunit molecular weight of 104 000 ± 3600 (Figure 2C), which was reduced to 92 500 ± 1600 after removal of carbohydrate moieties by *N*-glycanase treatment.

The final DEAE-Sephacel step separated aminopeptidase P from membrane dipeptidase activity (EC 3.4.13.19) as assayed with the substrate Gly-D-Phe (Figure 1B). The indicated fractions in Figure 1B contained pure membrane dipeptidase as shown by SDS−PAGE (Figure 2B). The N-terminal amino acid sequence was X-X-Phe-Arg-Asn-Gln-Ala-Glu-Asn-Ile-Met-Arg-Thr-Thr-Pro-Val-Ile-Asp-Gly-, which agrees with the N-terminal sequence of rat renal membrane dipeptidase deduced from the cDNA sequence (Adachi et al., 1992). The subunit molecular weight of 56 300 ± 900 (Figure 2B) was reduced to 38 600 ± 2100 by *N*-glycanase treatment. Removal of carbohydrate moieties was probably complete in this experiment since the protein molecular weight determined from the cDNA sequence is about 41 000. This indicates that the native enzyme contains about 30% N-linked carbohydrate.

Concluding Remarks. Rat lung membrane-bound aminopeptidase P, like the bovine lung and pig kidney enzymes (Simmons & Orawski, 1992; Hooper et al., 1990), appears to be attached to membranes by a glycosylphosphatidylinositol anchor since the enzyme was solubilized by a phosphatidylinositol-specific phospholipase C in the first step in the purification. In contrast to the purification scheme devised for the bovine lung enzyme, rat lung aminopeptidase P required additional steps to remove two peptidases, dipeptidylpeptidase IV and membrane dipeptidase. However, these steps led to the simultaneous purification to homogeneity of all three enzymes.

Rat lung aminopeptidase P hydrolyzed bradykinin at the Arg^1-Pro^2 bond with a k_{cat}/K_m of 5.7×10^5 s^{-1} M^{-1}. This is consistent with the proposal that membrane-bound aminopeptidase P is involved in the pulmonary inactivation of bradykinin (Orawski et al., 1987; Ryan, 1989; Baker et al., 1991; Revann et al., 1991; Simmons & Orawski, 1992; Pesquero et al., 1992a,b; Ryan et al., 1994b; Prechel et al., 1994; Kitamura et al., 1995). An inhibitor of aminopeptidase P, apstatin, has been shown to reduce the degradation of bradykinin in the isolated perfused rat lung (Prechel et al., 1994) and to potentiate the blood pressure lowering response to bradykinin in the anesthetized rat (Kitamura et al., 1995).

Neuropeptide Y (Tyr-Pro-Ser-...) was also predicted to be a substrate for aminopeptidase P because of the penultimate proline residue and an amino acid with a small side chain in the third position (Yoshimoto et al., 1994). We found that neuropeptide Y (1−24)amide was hydrolyzed by purified aminopeptidase P with the release of the N-terminal tyrosine. The rate of cleavage was similar to that of bradykinin (when both peptides were at 0.5 mM). Whether aminopeptidase P has a role in hydrolyzing circulating neuropeptide Y is unknown. However, unlike bradykinin, neuropeptide Y is also a substrate for dipeptidylpeptidase IV (Mentlein et al., 1993; Medeiros & Turner, 1994), which releases the N-terminal Tyr-Pro dipeptide very rapidly ($k_{cat}/K_m = 12 \times 10^6$ s^{-1} M^{-1}). In experiments with human serum and human cultured endothelial cells, DPP IV activity predominated over aminopeptidase P in cleaving neuropeptide Y (Mentlein et al., 1993). It seems likely that aminopeptidase P has a more important role to play in the degradation of bradykinin than of neuropeptide Y, at least at sites where the two enzymes are colocalized.

[3] Interestingly, this sequence is most homologous with a portion of the gag polyprotein of human and simian immunodeficiency viruses (at least 56% identity over an 18-amino acid overlap and 82% identity over an 11-amino acid overlap with HIV-2. Although aminopeptidase P has not yet been shown on the surface of $CD4^+$ T cells, we offer the possibility that the above sequence is involved in enzyme subunit interaction and that the virus may have mimicked this sequence to bind to the membrane-bound protein as a part of its life cycle.

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