Purification and Specificity of a Membrane-Bound Metalloendopeptidase from Bovine Pituitaries†

Marian Orlowski* and Sherwin Wilk

ABSTRACT: A metalloendopeptidase optimally active at a neutral pH was purified 10000-fold from particulate fractions of bovine pituitaries. The solubilized enzyme has an apparent molecular weight of about 90000, as determined by gel filtration on Sephadex G-200 and G-100 columns. The enzyme is not sensitive to inhibition by SH-blocking agents, diisopropyl fluorophosphate, leupeptin, pepstatin, antipain, and chymostatin. Thiols and metal chelators such as ethylenediaminetetraacetic acid (EDTA) and *o*-phenanthroline are inhibitory. An EDTA-treated enzyme can be reactivated by several divalent metal ions, with zinc giving reactivation at the lowest concentrations. The specificity and kinetic parameters of the enzyme were studied with a series of synthetic peptide naphthylamides. The enzyme cleaves bonds in which the amino group is provided by a hydrophobic amino acid residue (position P_1'). Replacement of this residue by small neutral amino acids decreases or virtually eliminates activity. The nature of substituents in positions P_1, P_2, P_3, and P_4 greatly influences specificity. Relatively high k_{cat} and k_{cat}/K_m ratios were obtained with substrates containing arginine residues in positions P_1 and P_2. In such cases the impression of a "trypsin-like" activity was created. High reaction rates were also observed with substrates containing small neutral amino acids in positions P_1 and P_2, provided that position P_3 was occupied by the acidic (polar) glutaryl residue. Replacement of this residue with hydrophobic substituents greatly decreased the rate of reaction. When positions P_1 and P_2, however, were occupied by arginine residues, the unfavorable effect of hydrophobic substituents in position P_3 or P_4 on catalysis was eliminated.

Pituitary proteolytic enzymes are of considerable interest because of their potential function in the generation and interconversion of peptide hormones and other biologically active peptides (Marks, 1977). A common precursor, for example, containing the amino acid sequences of ACTH, β-lipotropin, β-MSH, and β-endorphin has been demonstrated in the pituitary (Roberts & Herbert, 1977; Mains et al., 1977; Nakanishi et al., 1977, 1979). Generation of active fragments from this precursor requires a highly selective cleavage of strategic peptide bonds, a process generally referred to as "limited proteolysis" (Neurath, 1975). The responsible enzymes, however, have not been identified. Studies on pituitary proteases have greatly lagged behind structural and functional analysis of pituitary hormones.

We have recently reported on the isolation from bovine pituitaries of a neutral endopeptidase sensitive to inhibition by relatively low concentrations of monovalent cations (Wilk et al., 1979; Wilk & Orlowski, 1980). The enzyme preferentially cleaves bonds between hydrophobic and small neutral amino acids and is capable of generating the opioid peptide methionine-enkephalin from α-endorphin and leucine-enkephalin from a synthetic precursor containing the leucine-enkephalin sequence (Orlowski et al., 1980). In this paper we report the identification and purification of a metalloendopeptidase from particulate fractions of bovine pituitaries. The enzyme cleaves bonds on the amino side of hydrophobic amino acids and in this respect resembles the specificity of a group of microbial proteases, one of which is thermolysin (Matsubara et al., 1965, 1966). In addition to this primary specificity, the nature of the substituents P_1,[1] P_2, P_3, and P_4 on the amino side of the cleaved bond greatly influences activity. Substrates in which P_1 and P_2 are represented by two basic amino acids show good specificity even in the presence of unfavorable substituents in positions P_3 and P_4.

Materials and Methods

Amino acid naphthylamides and *tert*-butoxycarbonyl and benzyloxycarbonyl derivatives of amino acids were obtained from Sigma Chemical Co. (St. Louis, MO) or Bachem Inc. (Torrance, CA). *N*,*N*′-Dicyclohexylcarbodiimide, *N*-hydroxysuccinimide, trifluoroacetic acid, hippuric acid, glutathione, dithiothreitol, 2-mercaptoethanol, *p*-mercuribenzoate, iodoacetamide, iodoacetic acid, *N*-ethylmaleimide, papain, trypsin, chymotrypsin, pepstatin, antipain, chymostatin, EDTA, *o*-phenanthroline, and BOC-ε-Z-Lys-*N*-hydroxysuccinimide ester were obtained from Sigma Chemical Co. Aminopeptidase M (EC 3.4.11.2) was purchased from Boehringer/Mannheim Inc. (Indianapolis, IN). DFP was obtained from Calbiochem-Behring Corp. (La Jolla, CA). Sephadex G-100 and G-200 was obtained from Pharmacia Inc. (Piscataway, NJ). DEAE-cellulose (DE-52) and carboxymethylcellose (CM-52) were obtained from Whatman Inc. (Clifton, NJ). Plates for thin-layer chromatography were obtained from Eastman Kodak Co. (Rochester, NY). Glutaryl-Ala-Ala-Phe-4Me2NA was obtained from Enzyme Systems Products (Livermore, CA). Bz-Phe-Val-Arg-pNA, Z-Gly-Gly-Arg-2NA, Bz-DL-Arg-2NA, Z-Gly-Phe-amide, and Hip-His-Leu were obtained from Vega Biochemicals (Tucson, AZ). Palladium-on-activated charcoal (10%) was obtained

† From the Department of Pharmacology, Mount Sinai School of Medicine of the City University of New York, New York, New York 10029. *Recieved February 3, 1981.* This work was supported by Grant AM 25377 from the National Institutes of Health.

[1] The nomenclature of Schechter & Berger (1967) is used to describe the interaction between substrate and enzyme. Amino acid residues and other residues in the substrate are designated P_1, P_2, P_3, etc. in the N-terminal direction and P_1' etc. in the C-terminal direction from the bond undergoing cleavage. The corresponding subsites in the enzyme are identified with the letter S. Abbreviations used: Z, benzyloxycarbonyl; BOC, *tert*-butoxycarbonyl; Hip, hippuryl; Bz, α-*N*-benzoyl; 2NA, 2-naphthylamide or 2-naphthylamine; pNA, *p*-nitroanilide or *p*-nitroaniline; 4Me2NA, 4-methoxy-2-naphthylamide; DFP, diisopropyl fluorophosphate; $NaDodSO_4$, sodium dodecyl sulfate; TLC, thin-layer chromatography; HPLC, high-pressure liquid chromatography; CF_3CO_2H, trifluoroacetic acid or trifluoroacetate; DMF, dimethylformamide; THF, tetrahydrofuran; DCC, *N*,*N*′-dicyclohexylcarbodiimide; EDTA, ethylenediaminetetraacetic acid.

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from Ventron (Alfa Division, Danvers, MA). Z-Gly-Gly-Leu-pNA, Z-Gly-Gly-Tyr-Ala-pNA, Z-Gly-Gly-Tyr-Leu-pNA, Z-Leu-Leu-Glu-2NA, and Z-Tyr-Gly-Gly-Phe-Leu-Thr-2NA were synthesized as described previously (Wilk et al., 1979; Wilk & Orlowski, 1980; Orlowski et al., 1980). Frozen whole bovine pituitaries were obtained from Pel Freeze Inc. (Rogers, AR). Furylacryloyl-Ala-Phe-amide was obtained from the US Biochemical Corp. (Cleveland, OH). Other reagents and solvents were obtained from Fisher Scientific Co. (Pittsburgh, PA).

Synthesis of Substrates. All syntheses were carried out in solution by stepwise elongation of the peptide from the C-terminal amino acid. The carboxyl group of this amino acid was bound in an amide linkage to 2NA as the chromogenic group. The presence of this group in the final peptide facilitated determination of enzyme activity by a coupled reaction in the presence of excess aminopeptidase (see Analytical Methods). *N*-Hydroxysuccinimide active esters of BOC amino acids were prepared by the method of Anderson et al. (1964) and employed in the coupling step. Removal of the BOC group was carried out by dissolving the peptide in anhydrous CF_3-COOH (Kapeller & Schwyzer, 1960) and allowing the solution to stand for 15 min at room temperature. Removal of the solvent and addition of ethyl ether yielded the CF_3COOH salt of the peptide. Benzyloxycarbonyl groups and NO_2 groups in nitroarginine were removed by catalytic hydrogenation (Bergman & Zervas, 1932) with 10% palladium-on-activated charcoal as the catalyst in a methanol–acetic acid mixture. The progress of each reaction was monitored by HPLC analysis of the disappearance of the starting reactants and the appearance of products. The purity of the synthesized peptides was determined by amino acid analysis, thin-layer chromatography on silica gel and cellulose plates, HPLC, and analysis of the 2-naphthylamine contents after enzymatic release of the chromogen by incubation of the peptide with excess trypsin and aminopeptidase M or when necessary with chymotrypsin. Peptides containing impurities (VI and VIII) were purified on a C_{18} μBondapak column (30 × 0.4 cm) using a Waters Associates HPLC apparatus and a linear gradient between 0.01 M ammonium acetate (pH 4) and methanol. The initial methanol concentration was 20% and was increased linearly to 90% during 30 min. Up to 15 mg of peptide was purified in a single run. Peaks containing the peptide were collected, and the peptide was isolated by evaporating the solvent under vacuum at 36 °C.

Substrates were analyzed for purity by ascending TLC on silica gel plates or on cellulose plates in the following solvent systems: (A) 1-butanol–acetic acid–water (60:15:25); (B) chloroform–acetic acid–methanol (80:5:15); (C) chloroform–acetic acid–methanol (50:5:45); (D) 1-butanol–pyridine–water (1:1:1). Solvents A and D were used with cellulose plates, and solvents B and C were used with silica gel plates. Location of peptides was visualized by examining the plates in a viewing box illuminated with a short-wavelength UV source and also by spraying the plates with the chlorine–starch–iodide reagent mixture of Rydon & Smith (1952) as modified by Pan & Dutcher (1956). Ninhydrin-positive compounds were visualized with a 0.1% ninhydrin solution in acetone. Peptides and products of their hydrolysis were analyzed by HPLC on a Water Associates liquid chromatograph equipped with a variable wavelength detector. Emerging peaks were monitored at 210 nm. Samples were separated on a reverse-phase C-18 μBondapak column (30 × 0.4 cm; Waters) by elution with a linear gradient established between 0.1% phosphoric acid in methanol and 0.1% phosphoric acid in water. The starting methanol concentration was 40% and its concentration was increased at the rate of 2% per min. The flow rate was 1.5 mL/min. For amino acid analysis of peptides, 0.1 μmol of each peptide was hydrolyzed in evacuated tubes in 6 M HCl at 105 °C for 24 h. Samples containing tyrosine were protected from oxidation during acid hydrolysis by addition of phenol (1 μL of liquefied phenol/mL of 6 M HCl). After completion of hydrolysis, hydrochloric acid was removed under reduced pressure, and the residue was dissolved in a sodium citrate buffer (pH 2.0; 0.1 M). The amino acid content of the hydrolysate was determined in a Technicon TSM amino acid analyzer.

For the analysis of the 2-naphthylamine content of peptides 0.1 μmol of each of the synthesized peptides was incubated with trypsin (0.2 mg), aminopeptidase M (10 μg), and Tris-HCl buffer (0.05 M; pH 7.8) in a final volume of 0.25 mL. Incubations were at 37 °C for 2 h. Under these conditions a complete release of 2-naphthylamine from arginine-containing substrates occurred. The concentration of 2-naphthylamine was determined by the method of Bratton & Marshall (1939) as modified by Goldbarg & Rutenburg (1958). Analysis of the naphthylamine content of substrates XIV and XVI was determined after incubation with crystalline chymotrypsin under the same conditions as described above.

Melting points are uncorrected. Analyses for carbon, hydrogen, and nitrogen were carried out by Schwarzkopf Microanalytical Laboratory (Woodside, NY).

Hip-Arg-Leu-2NA (I). *N*-BOC-ω-NO_2Arg-*N*-hydroxysuccinimide ester (12 mmol) prepared according to Anderson et al. (1964) was reacted in THF (40 mL) with an equimolar amount of Leu-2NA. After 24 h a small amount of insoluble material was removed by filtration, and the filtrate was flash evaporated. The residue was dissolved in 250 mL of chloroform and then washed with 100 mL of water, twice with 120 mL of $KHSO_4$ (0.02 M; pH 2.6), twice with 120 mL of water, and twice with 120 mL of 0.5 M sodium bicarbonate. The water phase was each time reextracted with 20 mL of chloroform, which was then combined with the main chloroform solution. The organic phase was dried with anhydrous sodium sulfate, and the solvent was removed in vacuo to yield a clear oil. Addition of ether yielded a white solid which was collected by filtration, washed extensively with ether, and dried (*N*-BOC-ω-NO_2Arg-Leu-2NA; 4.85 g, 73%). Treatment with CF_3CO_2H and ether gave the CF_3CO_2H salt of NO_2Arg-Leu-2NA (II) (98%). Compound II (1 mmol) was subsequently reacted in THF (5 mL) with a 10% excess of Hip-*N*-hydroxysuccinimide ester in the presence of 140 μL of triethylamine. The mixture was stirred at room temperature, and after 24 h the solvent was removed in vacuo and the product was isolated as a white solid from 2-propanol–ether. The material was hydrogenated in a mixture of methanol and acetic acid (5:25). After removal of the catalyst by filtration, the solution was evaporated under reduced pressure, and the product was obtained as a white solid from a mixture of ethanol–ether (acetate salt; 50% yield): single peak on HPLC with a retention time of 17.1 min; amino acid analysis, Arg 1.07, Gly 1.05, Leu 1.0, 2-naphthylamine 97% theoretical; R_f (A) 0.95, (B) 0.81, (C) 0.84; mp 134–136 °C. Anal. Calcd for $C_{33}H_{43}N_7O_6 \cdot H_2O$: C, 60.82; H, 6.96; N, 15.04. Found: C, 59.55; H, 6.72; N, 15.24.

Hip-Arg-Arg-Leu-2NA (III). II (2.86 g, 5 mmol), triethylamine (702 μL), and *N*-BOC-NO_2Arg-*N*-hydroxysuccinimide ester (2.29 g, 5.5 mmol) were reacted in a mixture of 20 mL of THF and 5 mL of DMF. The mixture was stirred overnight, and a small amount of insoluble material was then

removed by filtration. Removal of the solvent left a solid which was washed with several portions of CH_2Cl_2, leaving a white solid of *N*-BOC-NO_2Arg-NO_2Arg-Leu-2NA (2.93 g; 77%). Treatment with CF_3CO_2H and then ether gave CF_3CO_2H·NO_2Arg-NO_2Arg-Leu-2NA (IV) (76%). IV (622 mg, 0.8 mmol) was reacted in THF (5 mL) with Hip-*N*-hydroxysuccinimide ester (10% excess) in the presence of 130 μL of triethylamine. The product which crystallized from the solution was collected by filtration and hydrogenated as described before. After removal of the solvent, the product (III) was obtained from alcohol–ether as a white solid (450 mg as the diacetate; 66%). The amorphous hygroscopic compound was stored in a desiccator over sodium hydroxide pellets: single peak on HPLC; retention time 12.8 min; amino acid analysis, Arg 2.09, Gly 1.09, Leu 1.0, 2-naphthylamine content 102% theoretical; R_f (A) 0.94, (B) 0.63, (C) 0.82; mp 145—148 °C. Anal. Calcd for $C_{41}H_{59}N_{11}O_9 \cdot H_2O$: C, 56.73; H, 7.08; N, 17.75. Found: C, 56.62; H, 7.19; N, 16.84.

Hip-Arg-Arg-Phe-2NA (*V*). This compound was obtained by reacting Phe-2NA with *N*-BOC-NO_2Arg-*N*-hydroxysuccinimide ester and removal of the protecting group with CF_3CO_2H as described for compound II. CF_3CO_2H·NO_2Arg-Phe-2NA was reacted with *N*-BOC-NO_2Arg-*N*-hydroxysuccinimide ester followed by removal of the BOC group to yield TFA·NO_2Arg-NO_2Arg-Phe-2NA by the same procedure as described for IV. The product was reacted with Hip-*N*-hydroxysuccinimide ester (10% excess) in THF in the presence of an equimolar amount of triethylamine to yield the crystalline product Hip-NO_2Arg-NO_2Arg-Phe-2NA. Catalytic hydrogenation yielded the desired compound (V) as a white solid (diacetate): single peak on HPLC; retention time 12.6 min; amino acid analysis, Arg 1.96, Gly 1.14, Phe 1.0, 2-naphthylamine 97% theoretical; R_f (A) 0.94, (C) 0.83; mp 142–145 °C.

Hip-Lys-Arg-Arg-Leu-2NA (*VI*). IV (1.97 g, 2.55 mmol), 357 μL of triethylamine, and *N*-BOC-ε-Z-Lys-*N*-hydroxysuccinimide (1.52 g, 2.55 mmol) ester (80%) were reacted in THF. After removal of the solvent in vacuo, the residue was extensively washed with ethyl acetate and then ether to yield a white solid of BOC-ε-Z-Lys-NO_2Arg-NO_2Arg-Leu-2NA, giving a single peak on HPLC. The yield was 2.14 g (82%). Removal of the protecting group yielded the CF_3CO_2H salt of ε-Z-Lys-NO_2Arg-NO_2Arg-Leu-2NA (VII); 826 mg of this product was reacted in THF (5 mL) with 276 mg of Hip-*N*-hydroxysuccinimide ester and 112 μL of triethylamine. The product crystallized from solution. It was collected by filtration, washed with THF and ethyl acetate, and dried with ether. Catalytic hydrogenation gave 360 mg of product (2-propanol–ether): after purification by preparative HPLC, single peak on HPLC with a retention time of 8.9 min; amino acid analysis, Arg 1.74, Gly 1.06, Lys 0.97, Leu 1.0, 2-naphthylamine 100%; R_f (A) 0.88, (B) 0.63, (C) 0.82; mp 132–135 °C.

Hip-Lys-Lys-Arg-Arg-Leu-2NA (*VIII*). Compound VII was reacted with *N*-BOC-ε-Z-Lys-*N*-hydroxysuccinimide ester as described for VI. The product BOC-Z-Lys-Z-Lys-NO_2Arg-NO_2Arg-Leu-2NA precipitated from the solution during the reaction. It was isolated by filtration and extensively washed with ethyl acetate and ether. The BOC protecting group was removed with CF_3CO_2H and the product reacted with Hip-*N*-hydroxysuccinimide ester (5% excess) as described for VI). After addition of ethyl acetate, Hip-Z-Lys-Z-Lys-NO_2Arg-NO_2Arg-Leu-2NA crystallized from the solution. Catalytic hydrogenation yielded VIII from 2-propanol–ether: after purification by preparative HPLC, single peak with a retention time of 5.8 min; amino acid analysis, Gly 1.02, Lys 1.87, Arg 1.86, Leu 1.0, 2-naphthylamine 98% theoretical; R_f (A) 0.81, (B) origin, (C) 0.04; mp 135–138 °C.

Succinyl-Arg-Arg-Leu-2NA (*IX*). CF_3CO_2H·NO_2Arg-NO_2Arg-Leu-2NA (IV) (0.5 mmol), triethylamine (70 μL), and succinic anhydride (0.55 mmol) were reacted in THF (3 mL). Addition of ethyl acetate produced a crystalline product which was hydrogenated in acetic acid–methanol (5:25). After evaporation of the solvent the product (IX) was obtained from 2-propanol–ether (diacetate salt, 245 mg; 73%): single peak on HPLC with a retention time of 9.2 min; amino acid analysis, Arg 2.00, Leu 1.08, 2-naphthylamine 101% of theory; R_f (A) 0.91, (B) 0.08, (C) 0.82; mp 175–178 °C.

Hip-Arg-Arg-Ala-2NA (*X*). L-Ala-2NA (3 mmol) was reacted with a 10% excess of *N*-BOC-NO_2Arg-*N*-hydroxysuccinimide ester in THF (12 mL). *N*-BOC-NO_2Arg-Ala-2NA precipitated from the solution. After addition of chloroform the white solid was collected by filtration (77% yield). The BOC protecting group was removed with CF_3CO_2H. CF_3CO_2H·NO_2Arg-Ala-2NA (1 mmol) was reacted with a 10% excess of *N*-BOC-NO_2Arg-*N*-hydroxysuccinimide ester in 5 mL of THF and 140 μL of triethylamine. The product *N*-BOC-NO_2Arg-NO_2Arg-Ala-2NA was obtained from chloroform as a white crystalline solid (85% yield). Treatment with CF_3CO_2H and ether yielded CF_3CO_2H·NO_2Arg-NO_2Arg-Ala-2NA. This product was reacted with a 10% excess of Hip-*N*-hydroxysuccinimide ester in THF (3 mL) and 70 μL of triethylamine. Addition of chloroform precipitated Hip-NO_2Arg-NO_2Arg-Ala-2NA. After hydrogenation in acetic acid–methanol (10:20) and removal of the solvent, the product (X) was crystallized from chloroform (diacetate: 73% yield): HPLC, single peak with a retention time of 7 min; amino acid analysis, Gly 1.0, Arg 2.10, Ala 0.99, 2-naphthylamine 96% of theory; R_f (A) 0.91, (B) 0.17, (C) 0.82; mp 136 °C dec.

Hip-Arg-Arg-Gly-2NA (*XI*). Gly-2NA·HCl (3 mmol) was reacted overnight with a 10% excess of *N*-BOC-NO_2Arg-*N*-hydroxysuccinimide ester in 10 mL of THF and 420 μL of triethylamine. The solution was filtered, and the solvent was removed in vacuo. The remaining solid was crystallized from $CHCl_3$ to yield *N*-BOC-NO_2Arg-Gly-2NA (44%). The BOC protecting group was removed in the usual way and the product CF_3CO_2H·NO_2Arg-Gly-2NA (1 mmol) was reacted with a 10% excess of *N*-BOC-NO_2Arg-*N*-hydroxysuccinimide ester in 5 mL of THF and 140 μL of triethylamine. *N*-BOC-NO_2Arg-NO_2Arg-Gly-2NA was obtained from ethyl acetate (88% yield). After removal of the protecting group, CF_3CO_2H·NO_2Arg-NO_2Arg-Gly-2NA was reacted with a 10% excess of Hip-*N*-hydroxysuccinimide ester in 5 mL of THF and 120 μL of triethylamine. The product, Hip-NO_2Arg-NO_2Arg-Gly-2NA, was extensively washed with ethyl acetate and ether and hydrogenated to yield Hip-Arg-Arg-Gly-2NA from 2-propanol–ether (diacetate, 46% yield): HPLC, single peak with a retention time of 5.8 min; amino acid analysis, Arg 2.0, Gly 2.06, 2-naphthylamine 101% of theory; R_f (A) 0.87, (B) 0.08, (C) 0.8; mp 148–150 °C.

Hip-Gly-Arg-Leu-2NA (*XII*). CF_3CO_2H·NO_2Arg-Leu-2NA (II) (0.7 mmol) was reacted with a 10% excess of *N*-BOC-Gly-*N*-hydroxysuccinimide ester in 4 mL of THF and 100 μL of triethylamine. After evaporation of the solvent, the product *N*-BOC-Gly-NO_2Arg-Leu-2NA was crystallized from ethyl acetate. Removal of the BOC protecting group yielded CF_3CO_2H·Gly-NO_2Arg-Leu-2NA which was reacted with a 10% excess of Hip-*N*-hydroxysuccinimide ester. After 24 h the solvent was removed in vacuo, and Hip-Gly-NO_2Arg-Leu-2NA was crystallized from 2-propanol. Hydrogenation in acetic acid–methanol (10:20) yielded the desired product

(XII) from 2-propanol–ether (acetate salt; yield 50%): HPLC, single peak with a retention time of 15.8 min; amino acid analysis, Arg 1.0, Gly 1.87, Leu 1.0, 2-naphthylamine 97% of theory; R_f (A) 0.97, (B) 0.91, (C) 0.85; mp 134–137 °C.

Hip-Phe-Arg-Leu-2NA (XIII). CF_3CO_2H·NO_2Arg-Leu-2NA (II) (1.2 mmol) was reacted with a 10% excess of *N*-BOC-Phe-*N*-hydroxysuccinimide ester in 6 mL of THF and 170 μL of triethylamine. The solvent was removed in vacuo and the residue dissolved in chloroform. The solution was washed as described for compound I. Removal of the solvent gave a clear oil which was treated with CF_3CO_2H and ether. The white solid, CF_3CO_2H·Phe-NO_2Arg-Leu-2NA, was reacted with a 10% excess of Hip-*N*-hydroxysuccinimide ester in THF and triethylamine (170 μL). Hip-Phe-NO_2Arg-Leu-2NA was obtained from ethyl acetate and hydrogenated in a mixture of acetic acid–methanol (10:20). The product was crystallized from 2-propanol (35% yield): HPLC, single peak with a retention time of 18.5 min; amino acid analysis, Arg 1.0, Gly 0.97, Leu 1.02, Phe 1.05, 2-naphthylamine 96% of theory; R_f (A) 0.97, (B) 0.77, (C) 0.86; mp 155 °C dec.

Glutaryl-Ala-Ala-Phe-2NA (XIV). Phe-2NA (2 mmol) was reacted with *N*-BOC-Ala-*N*-hydroxysuccinimide ester (2.1 mmol) in THF (8 mL). The solvent was removed in vacuo, the residue was dissolved in $CHCl_3$, and the solution was washed as described for compound I. Removal of the solvent left a waxy solid. It was treated with CF_3CO_2H and ether to yield CF_3CO_2H·Ala-Phe-2NA (70%). This was reacted with *N*-BOC-Ala-*N*-hydroxysuccinimide ester as described above to yield a crystalline solid. Treatment with CF_3CO_2H and ether gave CF_3CO_2H·Ala-Ala-Phe-2NA (XV) which was reacted with a 5% excess of glutaric anhydride and triethylamine in THF. The crystalline product was isolated by filtration: HPLC, single peak with retention time of 15.5 min; amino acid analysis, Ala 2.05, Phe 1.0; R_f (A) 0.96, (B) 0.74, (C) 0.87; mp 178 °C dec. Anal. Calcd for $C_{30}H_{34}N_4O_6H_2O$: C, 63.82; H, 6.43; N, 9.92. Found: C, 63.15; H, 6.49; N, 9.86.

*Hip-Ala-Ala-*Phe-*2NA (XVI).* XV was treated with a 5% excess of Hip-*N*-hydroxysuccinimide ester in CF_3CO_2H. The crystalline product was collected and dried: HPLC, single peak, retention time 17.5 min; amino acid analysis, Ala 1.98, Phe 1.0; R_f (A) 0.96, (B) 0.88, (C) 0.86; mp 245 °C dec.

Determination of Enzyme Activity. Enzyme activity was determined with Hip-Arg-Arg-Leu-2NA as the substrate in a coupled enzyme assay in the presence of excess aminopeptidase M. The activity of the aminopeptidase exceeded by at least 100 times that of the metalloendopeptidase. Under these conditions this last enzyme is rate limiting, and the reaction proceeds as follows:

$$\text{Bz-Gly-Arg-Arg-Leu-2NA} \xrightarrow{\text{metalloendopeptidase}} \text{Bz-Gly-Arg-Arg} + \text{Leu-2NA}$$

$$\text{Leu-2NA} \xrightarrow{\text{aminopeptidase-M}} \text{Leu} + \text{2-naphthylamine} \quad (1)$$

There is no release of 2-naphthylamine during the reaction unless aminopeptidase M is added to the incubation mixture. That the metalloendopeptidase indeed catalyzes the hydrolysis of the Arg–Leu bond as shown in reaction 1 was demonstrated in experiments in which Bz-Gly-Arg-Arg-Leu-2NA was incubated with the enzyme in a Tris-HCl buffer (0.05 M; pH 7.8) and the products of the reaction were separated by HPLC as described previously. Two products were found in the reaction mixture. They were shown by amino acid analysis and by retention time to be identical with Bz-Gly-Arg-Arg and Leu-2NA. The site of cleavage in other substrates was studied by the same analytical procedure as described above.

Incubation mixtures for enzyme determination contained substrate (0.4 mM; 10 μL of a 10 mM aqueous solution), enzyme (10–50 μL), aminopeptidase M (10 μg), and Tris–HCl buffer (0.05 M; pH 7.6) in a final volume of 0.25 mL. Incubations were at 37 °C for 30–120 min, depending on the concentration of the enzyme. Reactions were stopped by the addition of 10% trichloroacetic acid. 2-Naphthylamine released during the reaction was determined after diazotization according to a modification (Goldbarg & Rutenburg; 1958) of the procedure described by Bratton & Marshall (1939). In some experiments determination of 2-naphthylamine was carried out by coupling the amine with fast garnet GBC (a stabilized diazonium salt) according to the procedure of Barrett (1972). Controls containing aminopeptidase alone were included in each experiment. Activity with chromogenic substrates other than Bz-Gly-Arg-Arg-Leu-2NA was determined by the same procedure as described above. Enzyme activity is defined in units as the amount of enzyme that catalyzes the release of 1.0 μmol of product/h under the conditions described above. Specific activity is expressed in terms of units/(mg of protein), as determined by the method of Lowry et al. (1951).

The hydrolysis of dipeptides and tripeptides was tested in incubation mixtures containing 0.1 unit of enzyme, 0.5 μmol of each of the peptides, and buffer (Tris-HCl, 0.05 M; pH 7.6) in a final volume of 0.25 mL. The formation of products was tested by TLC on cellulose plates with solvent systems A and D. The presence of hydrolytic products was tested after 6- and 24-h incubation with the ninhydrin stain and by the method of Pan & Dutcher (1956).

Polyacrylamide Gel Electrophoresis. Electrophoresis was performed under nondissociating conditions in 0.05 M Tris–acetate buffer (0.05 M; pH 8.3) in 6% gels. Samples containing 10–20 μg of protein were layered on top of the gel, and a current of 4 mA was applied per tube until the tracking bromphenol blue dye reached the bottom of the gel (Weber & Osborn, 1969). Staining of proteins was carried out with a 0.25% solution of Coomassie brilliant blue in 12.5% trichloroacetic acid. Enzyme activity was located in gels by incubating them at 37 °C in a solution containing glutaryl-Ala-Ala-Phe-2NA or succinyl-Arg-Arg-2NA (0.8 mM), Tris-HCl buffer (0.05 mM; pH 7.0), and fast garnet GBC (0.5 mg/mL) in the presence of aminopeptidase M (40 μg/mL). The location of the enzyme becomes visible during the incubation as a brown-reddish band.

Kinetic Studies with Metalloendopeptidase. Kinetic studies were performed with a series of synthetic substrates (Table III) containing 2-naphthylamine or 4-nitroaniline as the chromogenic group. Enzyme activity was determined as described above. The steady-state parameters K_m and k_{cat} ($=V/e$, where e = total enzyme concentration) were calculated from initial velocity measurements plotted vs. various substrate concentrations. Double reciprocal plots (Lineweaver–Burk, 1934) were obtained by a linear regression program. Correlation coefficients better than 0.99 were obtained throughout. In the calculations the molecular weight of the enzyme was taken as 90 000. One active site per enzyme molecule was assumed, and the maximal specific activity of the isolated enzyme was taken as equal to 160 units/(mg of protein).

Purification of the Enzyme. All steps were carried out at 4 °C; 185 g of frozen bovine pituitaries were homogenized in a Waring blender for 3 min with 750 mL of 0.05 M Tris-HCl buffer (pH 7.8). The homogenate was centrifuged at 34000*g* for 15 min, and the supernatant was discarded. The precipitate was washed with 450 mL of buffer, and the suspension was centrifuged as above. The supernatant was discarded and the

Table I: Summary of Purification of Metalloendopeptidase from Bovine Pituitaries[a]

purification step	volume (mL)	protein (mg/mL)	activity units/mL	activity total	sp act. (units/mg)	recovery (%)	purification (x-fold)
(1) homogenate	880	20.0	0.22	194	0.011	100	1
(2) deoxycholate extract	420	4.75	0.69	289	0.145	149	13
(3) papain treatment and Sephadex filtration	136	0.13	1.25	170	9.6	88	870
(4) DEAE-cellulose chromatography	107	0.065	0.97	104	14.9	54	1350
(5) carboxymethylcellulose chromatography	16	0.03	3.3	54	110	28	10000

[a] For details and definition of units, see Materials and Methods.

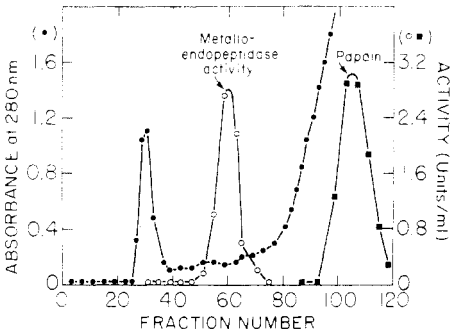

FIGURE 1: Sephadex G-200 gel filtration of the papain-treated metalloendopeptidase. The activity of both enzymes was determined with Hip-Arg-Arg-Leu-2NA as the substrate as described under Determination of Enzyme activity.

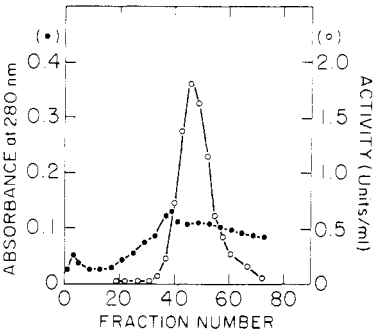

FIGURE 2: Chromatography of the enzyme on a DEAE-cellulose column (DE-52). For details see text.

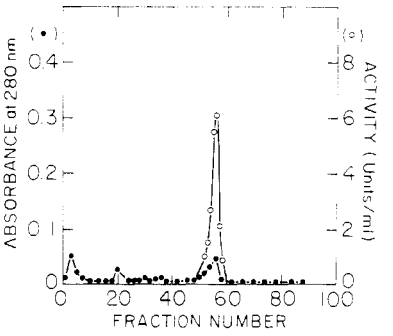

FIGURE 3: Chromatography of the enzyme on carboxymethylcellulose (CM-52).

precipitate was again thoroughly washed with the same buffer and centrifuged as above. The precipitate was then suspended in 450 mL of a 1% deoxycholate solution in 0.05 M Tris–HCl buffer (pH 7.8) and stirred overnight. The suspension was then centrifuged at 34000*g* for 15 min, and the supernatant was treated with a 20% solution of streptomycin sulfate and centrifuged as before (step 2). The supernatant was collected and concentrated in an ultrafilration cell (Aminco) to about 40 mL. For each 25 mg of protein in solution, 1 mg of papain was added, followed by solid dithiothreitol to a final concentration of 0.01 M. The mixture was incubated for 90 min at 37 °C and then applied to the top of a Sephadex G-200 column (5 × 48 cm) equilibrated with a 0.05 M Tris-HCl buffer (pH 8.0) containing 0.1 M sodium chloride. The column was eluted with the same buffer, and fractions of about 10 mL were collected (step 3). The enzyme emerged from the column after a peak of high molecular weight protein and was completely separated from papain, which emerged together with the low molecular weight material (Figure 1). The active fractions were pooled and concentrated by ultrafiltration to about 50 mL. The solution was dialyzed against 4 L of 0.01 M Tris-HCl buffer (pH 8.0). The solution was then applied to the top of a DE-52 column (1.2 × 12 cm) previously equilibrated with the same buffer. Elution was carried out with a linear gradient established between 150 mL of 0.01 M Tris-HCl (pH 8.0) and 150 mL of the same buffer containing 0.2 M sodium chloride. Fractions of about 4 mL were collected and tested for activity. Protein concentrations were estimated from absorbance at 280 nm. The enzyme emerged from the column in the middle of the gradient (step 4; Figure 2) and was closely followed by a peak of activity which hydrolyzed Leu-2NA. Active fractions were pooled, concentrated by ultrafiltration to about 40 mL, and dialyzed against two changes (2 L each) of 0.01 M sodium acetate buffer (pH 5.6). The enzyme was then applied to the top of a CM-52 column (8.0 × 0.9 cm) equilibrated with the same buffer. The enzyme did not bind to the carboxymethylcellulose and emerged with the eluting buffer. A considerable amount, however, of inactive protein was removed by this procedure. The active fractions were collected and dialyzed against 4 L of 0.01 M sodium acetate buffer (pH 5.0). The solution was then applied to the top of a CM-52 column (6 × 0.9 cm) equilibrated with the same buffer, and elution was started with a linear gradient established between 100 mL of 0.01 M sodium acetate buffer (pH 5.0) and 100 mL of the same buffer containing 0.2 M sodium chloride. Fractions of about 2.5 mL were collected and tested for activity and protein by measuring the absorbance at 280 nm. The enzyme was eluted from the column as a sharp peak of activity coinciding with a distinct protein peak (step 5; Figure 3). The aminopeptidase contaminating the enzyme obtained after DEAE-cellulose chromatography did not bind to the carboxymethylcellulose column and was completely removed by the initial washing of the column with the starting buffer. Active fractions were collected and adjusted to about pH 6.0 by addition of 1.0 M Tris base. The enzyme was stored in ice, and under such conditions lost about 20% of activity during 6 weeks.

Results

A summary of the purification procedure is given in Table I. A 10000-fold purification with a yield of about 30% was

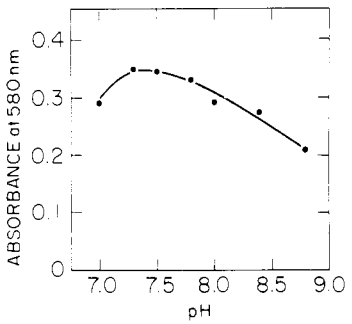

FIGURE 4: Dependence of activity on pH. The activity was determined with Hip-Arg-Arg-Leu-2NA as the substrate in 0.2 M Tris-HCl buffers. Other conditions were the same as those given under Materials and Methods.

obtained. All of the enzyme activity was initially associated with the particulate fraction of pituitary homogenates. Treatment with deoxycholate solubilized the enzyme; however, the activity was still associated with high molecular weight membrane fractions eluting predominantly in the void volume during Sephadex G-200 gel filtration. A short treatment with papain in the presence of dithiothreitol was sufficient to release the enzyme from these fragments, as a molecular species eluting as a single peak of activity which was significantly retarded during passage through a Sephadex G-200 column. Papain (M_r 23 000) was completely separated from the metalloendopeptidase and was eluted with the low molecular weight fraction. Although both enzymes cleaved the substrate, Hip-Arg-Arg-Leu-2NA, they could be distinguished by the effect of dithiothreitol on activity. This thiol strongly inhibited the metalloendopeptidase but was necessary for the expression of papain activity. A consistent increase in total activity was observed in step 2 of the purification procedure, which involved deoxycholate extraction of the particulate fraction and treatment with streptomycin sulfate, intended to remove excess deoxycholate and ribonucleoproteins Orlowski & Meister, 1965). This activity increase is probably due to the removal of inhibitors present in the crude homogenate. It is noteworthy that a single peak of enzymatic activity was observed during each of the chromatographic steps (Figures 1–3).

The pH optimum of the enzyme was determined in 0.2 M Tris-HCl buffers, in the pH range between 7.0 and 8.8. A broad optimum between pH 7.3 and 7.8 was found (Figure 4). Enzyme activity was therefore determined routinely at pH 7.6.

Electrophoresis of the enzyme was carried out in polyacrylamide gels at pH 8.3 and 7.8. After electrophoresis the gels were incubated with glutaryl-Ala-Ala-Phe-2NA or succinyl-Arg-Arg-Leu-2NA under the conditions described under Materials and Methods. A protein band was found corresponding with enzymatic activity. An inactive protein component which could not be removed by ion-exchange chromatography was also present.

The molecular weight of the enzyme was determined by gel filtration on Sephadex G-100 and G-200 columns according to the method of Andrews (1964, 1965). Apparent molecular weights of 88 000 and 92 000 were found with the two procedures. An average molecular weight of about 90 000 is therefore assumed.

The effect of various inhibitors on enzyme activity is summarized in Table II. Leupeptin, pepstatin, antipain, and chymostatin, inhibitors of several thiol, serine, and carboxyl proteases, did not influence activity. DFP inhibited only at high concentrations (1.1 mM) and was without effect at lower concentrations. Since DFP has been reported to react

Table II: Effect of Inhibitors on Enzyme Activity[a]

inhibitor	final concn (mM)	inhibition (%)
leupeptin	10 μg/mL	0
pepstatin	0.2	14
antipain	8 μg/mL	0
chymostatin	8 μg/mL	0
DFP	1.1	57
	0.11	0
p-Mercuribenzoate	0.1	0
iodoacetamide	1.0	0
iodoacetic acid	1.0	0
N-ethylmaleimide	1.0	0
GSH	2.0	57
2-mercaptoethanol	2.0	38
dithiothreitol	2.0	93
L-cysteine	2.0	22
EDTA	1.0	37
o-phenanthroline	1.0	100
phosphate	4.0	20

[a] Activity was determined with Hip-Arg-Arg-Leu-2NA as described under Materials and Methods. The enzyme was not preincubated with the inhibitors with the exception of DFP which was preincubated with the enzyme at 37 °C for 30 min before addition of the substrate. Controls in which the enzyme was preincubated in the absence of DFP were included in the experiment.

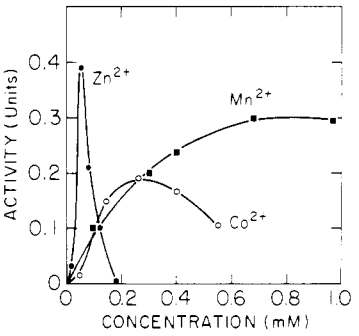

FIGURE 5: Reactivation of pituitary metalloendopeptidase after dialysis against EDTA. The enzyme was dialyzed for 24 h against several changes of 0.01 M EDTA (pH 7.8) and then dialyzed against deionized water. Activity was determined as described under Materials and Methods. No activity was detected unless metal ions were present.

occasionally with tyrosine residues (Maylie et al. 1969), determination of whether phosphorylation of a tyrosine residue near or at the active center is a cause of this inhibition would seem to be indicated. None of several thiol-blocking agents had any effect on activity. These results suggest that the enzyme does not belong to the group of serine or thiol proteases. By contrast, all of the thiols tested were inhibitory, with dithiothreitol showing the strongest inhibition. It is assumed that the inhibitory effect of thiols is due to their metal-chelating properties, although the exact mechanism of this inhibition has not been studied in detail. That the enzyme is indeed a metalloendopeptidase is suggested by the inhibition of activity observed with metal-chelating agents such as EDTA and *o*-phenanthroline and also by the inhibitory effect of phosphate ions. This is also supported by experiments which showed that dialysis of the enzyme against EDTA (0.01 M; pH 7.8) followed by removal of the chelating agent by dialysis against deionized water caused complete inactivation of the enzyme. The enzyme could be reactivated by the addition of several divalent cations. The reactivation profiles obtained with various metal ions differed in shape in a concentration-dependent manner (Figure 5). A sharp reactivation maximum within a narrow concentration range was observed with zinc. At optimal zinc concentration 55–70% of the activity was

Table III: Kinetic Parameters of Metalloendopeptidase-Catalyzed Hydrolysis of Peptide Naphthylamides

P_6-P_5-P_4-P_3-P_2-P_1-P_1'	[S][a] (mM)	K_m[b] (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} mM^{-1})
Bz-Gly-Arg-Arg-Leu-2NA	0.049–0.66	0.18 ± 0.02	240	1330
Bz-Gly-Arg-Arg-Phe-2NA	0.04–0.22	0.11 ± 0.01	120	1090
Bz-Gly-Arg-Arg-Ala-2NA	0.04–0.6	0.30 ± 0.02	8.8	29
Bz-Gly-Arg-Arg-Gly-2NA	0.05–0.7		negligible hydrolysis	
Bz-Gly-Arg-Leu-2NA	0.05–0.7	0.19 ± 0.02	2.9	15
Bz-Gly-Lys-Arg-Arg-Leu-2NA	0.05–0.27	0.08 ± 0.01	109	1360
Bz-Gly-Lys-Lys-Arg-Arg-Leu-2NA	0.05–0.27	0.03 ± 0.007	23	767
Bz-Gly-Gly-Arg-Leu-2NA	0.05–0.27	0.11 ± 0.01	3.0	27
Bz-Gly-Phe-Arg-Leu-2NA	0.05–0.7		negligible hydrolysis	
succinyl-Arg-Arg-Leu-2NA	0.07–0.74	0.45 ± 0.03	379	842
glutaryl-Ala-Ala-Phe-4Me2NA	0.12–0.8	0.42 ± 0.05	317	755
glutaryl-Ala-Ala-Phe-2NA	0.17–1.0	0.59 ± 0.03	592	1000
Bz-Gly-Ala-Ala-Phe-2NA	0.12–0.32		negligible hydrolysis	
Z-Gly-Gly-Leu-pNA	0.06–0.8	0.13 ± 0.01	6.3	48

[a] Range of substrate concentrations used for the determination of K_m. [b] Data are mean values ± SE of four to six determinations.

recovered. At concentrations higher than 0.2 mM this metal was strongly inhibitory when added to incubation mixtures containing the native enzyme (not dialyzed against EDTA). A broader and lower reactivation maximum was seen with Co^{2+}. The reactivation with Mn^{2+} plateaued at concentrations higher than 0.8 mM. Ca^{2+} activated only at higher concentrations (1–5 mM), and the maximal activity reached with Ca^{2+} was only about 30% of that obtained with Mn^{2+} (not shown). No activity was noticed in the presence of Mg^{2+} at concentrations up to 5 mM.

The specificity of the enzyme toward a variety of synthetic substrates was studied as described under Materials and Methods. The results summarized in Table III allow certain conclusions concerning both the "primary specificity" of the enzyme as determined by the two amino acids which form the bond undergoing hydrolysis and the "secondary specificity" which is a consequence of interaction of other amino acids in the substrate with the enzyme (Fruton, 1975). The site of cleavage of the substrates as determined by the identification of the products of enzymatic cleavage is indicated by the arrow (Table III). Strict Michaelis–Menten kinetics were observed with all the substrates within the indicated concentration ranges. The data clearly indicate that the primary specificity of the enzyme is directed toward bonds in which the amino group of residue P_1' is provided by a hydrophobic amino acid (Leu and Phe). When this amino acid was replaced by an alanine residue (substrate 3), there was a great decrease in both the rate of reaction (k_{cat}) and the specificity constant (k_{cat}/K_m). Introduction of a glycine residue in this position virtually eliminated activity. In addition to this primary specificity, the nature and the number of substituents in position P_1, P_2, P_3, P_4, and possibly also P_5 and P_6 greatly influenced the specificity. Thus, relatively high k_{cat} and k_{cat}/K_m ratios were obtained for substrates in which positions P_1 and P_2 were occupied by two arginine residues. Deletion of one arginine residue (substrate 5) greatly decreased both the maximal rate of reaction and the specificity constant. It is of interest, however, that an increase in the number of basic residues to 3 and 4 (substrates 6 and 7), while causing a decrease in k_{cat}, did not affect greatly the specificity constant. This resulted from a simultaneous decrease in the K_m values for the two substrates, so that the k_{cat}/K_m ratios remained high. If K_m expresses the "binding specificity" of the substrate to the enzyme, an increase in binding does not result in this case in a better catalysis. One can speculate that the presence of additional basic residues in position P_3 and P_4 makes possible the binding of these residues to subsites S_1 and S_2 of the enzyme, normally occupied by the residues P_1 and P_2 of the substrate. The consequence of such binding is the positioning of the scissile bond beyond the proper alignment with the bond-breaking catalytic site of the enzyme, slowing thereby the catalytic process.

It is of interest that replacement of an arginine residue in position P_2 with either a glycine or phenylalanine residue greatly decreased the rate of the reaction and the specificity constant. That the presence, however, of basic residues in positions P_1 and P_2 is not a prerequisite for good catalysis is shown by the high rate of hydrolysis of substrates in which positions P_1 and P_2 are represented by the small neutral amino acid alanine, provided that position P_3 is occupied by the relatively polar (or acidic) glutaryl residue (substrates 11 and 12). It is, however, noteworthy that replacement of the glutaryl residue by a hippuryl group (Bz-Gly; positions P_3 and P_4) almost completely eliminated activity. Similarly, a low rate of reaction and low specificity was observed with Z-Gly-Gly-Leu-pNA (substrate 14) in which position P_3 is occupied by the hydrophobic benzyloxycarbonyl group. Apparently the presence of hydrophobic groups in either position P_3 or P_4 greatly decreases the rate of reaction. In contrast, however, to alanine residues, the presence of two arginine residues in positions P_1 and P_2 eliminates this unfavorable effect of the hydrophobic hippuryl group.

Additional considerations should be discussed with respect to the specificity data presented in Table III. Since the enzyme favors a hydrophobic amino acid residue in position P_1' and also accommodates an alanine residue in this position (substrate 3), peptides containing two hydrophobic residues (substrates 9, 11, 12, and 13) may present to the enzyme a second position for binding, one that would not result in catalysis (nonproductive binding). Such substrates would also act as inhibitors (substrates 9 and 13). The presence of a glutaryl group such as in substrate 11 and 12 might make it more difficult for the substrate to bind at this second position, giving a single productive binding mode and a good catalysis rate. With these substrates, binding of the Ala residue at position P_1' would position the anionic glutaryl group in position P_1 or P_2, normally occupied by either a neutral or cationic group. The absence of activity with Bz-Gly-Ala-Ala-Phe-2NA could therefore be explained by binding of Ala at P_1' and Ala or Gly at P_1, resulting in a nonproductive binding. A similar explanation could be applied to the absence of activity with substrate 9, where binding of the Phe residue in P_1' provides a nonproductive binding. On the other hand the inability to bind a glycine residue in position P_1' would prevent such nonproductive binding with Z-Gly-Gly-Leu-pNA, explaining cleavage of this substrate by the enzyme.

Hydrolysis rates several hundred times slower than with Bz-Gly-Arg-Arg-Leu-2NA were also obtained with the following substrates (measured at a concentration of 0.4 mM under the conditions described under Materials and Methods): Z-Gly-Gly-Tyr-Ala-pNA, Z-Gly-Gly-Tyr-Leu-pNA, and Z-Tyr-Gly-Gly-Phe-Leu-Thr-2NA. This observation lends further support to our finding that the presence of hydrophobic groups in positions P_3 and P_4 does not favor catalysis. Large amounts of enzyme (0.2 unit) and prolonged incubation periods (up to 24 h) were also required to detect a trace of hydrolysis in the substrates Z-Gly-Phe-NH_2 and furylacryloyl-Ala-Phe-NH_2. No hydrolysis was observed of the peptides Z-Leu-Leu, Z-Phe-Leu, Z-Leu-Leu-Glu-2NA, and the angiotensin-converting enzyme substrate Hip-His-Leu (Cushman & Cheung, 1971). No hydrolysis was also observed of the trypsin substrates Bz-DL-Arg-2NA, Z-Gly-Gly-Arg-2NA, and Bz-Phe-Val-Arg-pNA.

Discussion

The peptide naphthylamide substrates synthesized in the course of the present work provided a convenient tool for both determination of enzyme activity and the investigation of its primary and secondary specificity. The sensitivity of measuring naphthylamine as the final product of the enzymatic reaction facilitated the study of the kinetic parameters of the enzyme. Because of the advantages offered by substrates containing in an amide linkage chromogenic groups such as naphthylamine, *p*-nitroaniline, and related aromatic amines, such substrates are increasingly used not only for detection of specific peptide bond cleaving enzymes (Erlanger et al., 1961; Orlowski & Szewczuk, 1962; Orlowski & Meister, 1963, 1965) but also for specificity and kinetic studies of proteases (Yoshida et al., 1980).

The final preparation obtained by our purification procedure had a 10000 times higher specific activity than the crude pituitary homogenate. The isolated enzyme, however, was not completely homogeneous, and the presence of an inactive protein component was detected by polyacrylamide electrophoresis. Because of the rather low initial activity in crude pituitary homogenates, the isolation of sufficient amounts of enzyme for more detailed physicochemical studies would require the processing of large quantities of pituitaries and application of affinity techniques after the enzyme had been solubilized. Affinity chromatography was successfully used by Pangburn et al. (1973) for the purification of thermolysin, an enzyme similar in certain respects to the pituitary metalloendopeptidase. It is likely, therefore, that similar techniques might facilitate the isolation of the pituitary enzyme. Because of the presence of the inactive protein contaminant, our reported values of k_{cat} should not be regarded as representing the maximal turnover rate constant of the enzyme.

The primary specificity of the enzyme is clearly directed toward peptide bonds in which the amino group is provided by a hydrophobic amino acid. This specificity is similar to the specificity of a group of microbial proteases represented among others by thermolysin (Matsubara et al., 1965, 1966). Like the bacterial enzyme, the pituitary metalloendopeptidase is inhibited by metal chelators, and it is likely that it is a zinc-containing protease. There are, however, differences between the two enzymes. Thus, the mammalian enzyme is membrane bound and has a molecular weight of about 90000. By contrast, the bacterial enzymes are generally soluble proteins, found in filtrates of bacterial cultures, and their molecular weight is about 35000–40000. Differences in the secondary specificity of the enzymes seem also significant. Thermolysin is relatively efficient in the hydrolysis of Z-Gly-Phe-NH_2 and substrates of the general structure Z-Gly-Gly-Leu-X (Morihara & Tsuzuki, 1970), while the pituitary enzyme attacks such substrates very slowly, apparently being inhibited by the presence of hydrophobic groups in positions P_3 and P_4 of the substrate. Similarly the pituitary enzyme showed almost no activity toward furylacryloyl-Gly-Phe-NH_2 (Feder, 1968), a substrate frequently used for the determination of thermolysin activity. To our knowledge, no studies have been carried out on the specificity of thermolysin toward arginine-containing substrates, and potential differences between the two enzymes in this respect would require a separate evaluation.

The pituitary enzyme shows some resemblance to the neutral endopeptidase purified from rabbit kidney brush borders by Kerr & Kenny (1974). This enzyme is a Zn^{2+} metalloendopeptidase cleaving the B chain of insulin on the amino side of hydrophobic amino acids. It is of interest that native insulin is relatively resistant to the action of this enzyme despite the presence of a relatively great proportion of hydrophobic amino acids. The presence of a zinc metalloendopeptidase, hydrolyzing the Ala–Phe bond in the fluorogenic substrate succinyl-Ala-Ala-Phe-7-amino-4-methylcoumarin, has been detected in crude pancreatic membrane extracts by Mumford et al. (1980). This enzyme has not been purified, and its specificity has not been studied in detail. The kidney enzyme is tightly bound to the microvillar membrane, and treatment with several proteolytic enzymes, including trypsin, papain, chymotrypsin, and others, failed to solubilize the enzyme. The only effective solubilization procedure involved treatment of the membranes with toluene followed by incubation with relatively large amounts of trypsin. In contrast, the pituitary enzyme was easily released from membrane fractions by treatment with deoxycholate and short exposure to relatively small amounts of papain. While the kidney enzyme was resistant to prolonged incubation with trypsin (up to 16 h), the pituitary enzyme lost activity under such conditions. Other differences between the two enzymes concern the reactivation profiles after dialysis against EDTA. The kidney enzyme was efficiently reactivated with Ca^{2+} and showed also activity with Mg^{2+}. In contrast, the pituitary enzyme showed little activity with Ca^{2+} and was inactive with magnesium ions. The specificity of the kidney enzyme with respect to model synthetic peptides has not been studied, and therefore no comparison in this respect can be made with the pituitary enzyme. It is possible that species differences or the presence of various forms of the metalloendopeptidase in different organs could account for the dissimilarities of the two enzymes.

It is presently established that many peptide hormones are generated from larger precursors or prohormones by limited proteolysis [for a review see, for example, Steiner et al. (1980)]. A common precursor containing the amino acid sequences of ACTH, α-MSH, β-lipotropin, β-endorphin, and β-MSH has been demonstrated in the pituitary. Active fragments in this precursor are separated by pairs of basic amino acids, and cleavage at these sites by a "trypsin-like" enzyme is believed to be involved in the release of the fragments. A similar type of cleavage is necessary in the generation of several other hormones, for example, insulin, glucagon, and parathyroid hormone. These findings stimulated the search for enzymes capable of catalyzing this type of cleavage. Our arginine-containing substrates were synthesized for the purpose of screening tissues and particularly the pituitary for the presence of such enzymes. The use of these substrates led to the detection of the metalloendopeptidase described in this work. Preliminary studies with fractions obtained from the pituitary

by differential centrifugation showed that the enzyme has the highest specific activity in the microsomal fraction, where the processing of hormones is believed to occur. Although the primary specificity of the metalloendopeptidase is directed toward the hydrophobic amino acids which provide the amino group of the attacked bond, the presence of a pair of basic amino acids adjacent to this site greatly favors catalysis. This creates the impression of a "trypsin-like" activity, although the mechanism and specificity of the enzyme have no resemblance to trypsin. Knowledge of the specificity of an enzyme toward model synthetic peptides, furthermore, is not a sufficient basis for predicting its action on large peptides and proteins, where the conformation of the peptide chain may limit the accessibility of the sensitive bond to enzyme action. In addition, many of the amino acids which follow the pair of basic amino acids in prohormones are represented by small neutral and acidic amino acid residues, eliminating them as a target for the metalloendopeptidase. In many cases, however, a pair of basic amino acids is followed by a hydrophobic residue. For example, the release of β-endorphin from β-lipotropin requires cleavage of a bond between a pair of basic amino acids and tyrosine. The possibility that this and other similar bonds are cleaved by the metalloendopeptidase is worth exploration.

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