Proteins of the kidney microvillar membrane

ASPARTATE AMINOPEPTIDASE: PURIFICATION BY IMMUNOADSORBENT CHROMATOGRAPHY AND PROPERTIES OF THE DETERGENT- AND PROTEINASE-SOLUBILIZED FORMS

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Aminopeptidase A (aspartate aminopeptidase, EC 3.4.11.7) was purified 2000-fold from pig kidney cortex. The essential step in the purification was chromatography on an immunoabsorbent column prepared from a rabbit antiserum raised against pig intestinal aminopeptidase A. Glutamyl and aspartyl substrates were attacked most rapidly and their hydrolyses were stimulated by Ca^{2+} . The 2-naphthylamide derivatives of neutral and basic amino acids were also hydrolysed by aminopeptidase A, but at rates about two orders of magnitude lower, and Ca^{2+} was inhibitory. The possibility that these atypical substrates were hydrolysed by traces of aminopeptidase M (EC 3.4.11.2) contaminating the preparation could be excluded on several grounds. Aminopeptidase A was sensitive to inhibition by chelating agents and the inactive enzyme could be reactivated by Ca^{2+} or Mn^{2+} . Atomic absorption spectrophotometry revealed 1 g-atom of Ca/143 000 g of protein. Two forms of the enzyme were purified: an amphipathic form solubilized from the membrane by Triton X-100 (detergent form) and a hydrophilic form released by incubation with trypsin (proteinase form). The detergent form exhibited charge-shift in crossed immunoelectrophoresis when anionic or cationic detergents were present. On gel filtration, mol.wts. of 350 000–400 000 and 270 000 were calculated for the detergent and proteinase forms. Electron microscopy after negative staining of the proteinase form revealed a dimeric structure. Electrophoresis of either form in the presence of sodium dodecyl sulphate revealed four polypeptides with mobilities corresponding to apparent mol.wts. of 155 000, 110 000, 90 000 and 45 000. All four bands stained positively for carbohydrate. Pig serum possesses weak aminopeptidase A activity; immunological experiments showed it to be a similar protein.

Aminopeptidase A (aspartate aminopeptidase, EC 3.4.11.7) is enriched in preparations of kidney brush border (George & Kenny, 1973) and is one of a group of microvillar enzymes present in both kidney and intestine (for review, see Kenny & Booth, 1978). The enzyme hydrolysing Glu-2-NNap was first identified histochemically in the pancreas (Glenner & Folk, 1961) and was released in a soluble form from kidney microsomal fractions by proteinase treatment (Glenner *et al.*, 1962). This

preparation, which also hydrolysed α -aspartic acid derivatives, was activated by Ca^{2+} and inhibited by EDTA. In physical properties, aminopeptidase A behaved rather similarly to the more abundant aminopeptidase M (EC 3.4.11.2) and the separation of the two proteins is the major problem in its purification (Kenny, 1977). Andria *et al.* (1976) separated the two intestinal activities on a concanavalin A–Sephacrose column. Recently, Benajiba & Maroux (1980) have purified the enzyme from pig intestine after solubilization by trypsin or detergent. The intestinal enzyme was a symmetrical dimer of two subunits of about 120 000 mol.wt., each anchored to the lipid bilayer of the membrane by a domain near the *N*-terminus.

Abbreviations used: abbreviations for amino acids and derivatives follow IUPAC–IUB recommendations; see *Biochem. J.* **126**, 773–780; -2-NNap, 2-naphthylamide; IgG, immunoglobulin G; dansyl, 5-dimethylaminonaphthalenesulphonyl.

There are several reports of a soluble enzyme that attacks the same glutamyl and aspartyl derivatives. The cytosol from dog kidneys contains an Mn^{2+} -activated enzyme preferentially hydrolysing aspartyl substrates (Cheung & Cushman, 1971). Chulkova & Orekhovich (1978) have also purified aminopeptidase A 500-fold after autolysis of beef kidney extract at pH 7.5. It hydrolysed Asp-2-NNap 10 times faster than Leu-2-NNap, was inhibited by EDTA, activated by Ca^{2+} and had a sedimentation coefficient of 3.4S, consistent with a mol.wt. of 53 000. Human serum also contains an enzyme hydrolysing glutamyl and aspartyl substrates that is activated by Ca^{2+} (Nagatsu *et al.*, 1970). However, the relationship of these relatively soluble forms of the enzyme in kidney and serum to the membrane bound forms in kidney and intestine is unclear.

In the present paper we report a novel purification that has produced a homogeneous protein, free of contamination by aminopeptidase M. This has enabled us to characterize the substrate specificity, metal content and some of the molecular properties of the detergent- and proteinase-solubilized forms.

Experimental

Tissues

Pig kidneys were generously given by ASDA Farm Stores, Lofthousegate, W. Yorks., U.K. Pig intestines, for the preparation of the antibodies to aminopeptidase A, were given by the Department of Experimental Pathology, Righshospitalet, Copenhagen, Denmark.

Chemicals

The sources of chemicals, unless otherwise stated, were those described previously (Macnair & Kenny, 1979; Booth *et al.*, 1979). All the 2-naphthylamides of amino acids were from Bachem Feinchemikalien A.G., Bubendorf, Switzerland. CNBr-activated Sepharose and protein A-Sepharose were from Pharmacia, Uppsala, Sweden; Ultrogels AcA 22 and AcA 34 were from L.K.B. Instruments, South Croydon, Surrey CR2 8YD, U.K. Freund's incomplete adjuvant was from the Statens Serum Institut, Copenhagen, Denmark. Phosphoramidon was a gift from Professor H. Umezawa, Institute of Microbial Chemistry, Jamiosaki, Shingawa-ku, Tokyo, Japan. A low-temperature-gelling agarose was from Miles Laboratories, Stoke Poges, U.K. Agarose (type II), Trasylol, trypsin (type III) and neuraminidase (type V) were from Sigma.

Immunological methods

The programme for immunization of rabbits with pig antigens and the preparation of immunoglobulin fractions was that previously used (Danielsen *et al.*,

1977). One batch of serum was processed by using a column of protein A-Sepharose as described (Norén & Sjöström, 1980). The immunoelectrophoretic methods, including line, rocket, and crossed immunoelectrophoresis were those described by Axelsen *et al.* (1973). Crossed charge-shift immunoelectrophoresis was performed as described by Bhakdi *et al.* (1977). Immunoprecipitation of aminopeptidase A activities in serum and kidney (from pig and man) was performed as follows. Serum samples were dialysed against 10mM-Tris/HCl buffer, pH 7.5, and concentrated in the dialysis sac with solid poly(ethylene glycol): they then contained 1–3 units of aminopeptidase A activity/ml. Kidney microvilli were prepared as before (Booth & Kenny, 1974) and solubilized in 1% (w/v) Triton X-100 and diluted with 10mM-Tris/HCl buffer, pH 7.5, to give the same aminopeptidase activity/ml. Each antigen (1ml) was mixed with various amounts of IgG prepared by immunizing a rabbit to microvillar membrane by the method described by Booth *et al.* (1979). After standing for 20 h at 20°C, the mixtures were centrifuged at 18000g for 15 min and the supernatant fractions were assayed for aminopeptidase A activity. The antiserum raised to human kidney microvillar proteins was kindly given by Mr. M. T. Abbs.

Preparation of the immunoabsorbent column

Scheme 1 summarizes the preparation of the immunoabsorbent. It was first necessary to obtain a pure antigen with which to raise monospecific antibodies to aminopeptidase A. This was achieved by excising an immunoprecipitate from an agarose gel in which detergent-solubilized pig intestinal microvillar membrane was electrophoresed into antibodies raised to the same membrane proteins. These antibodies were raised in rabbits and were identical with those previously described (Danielsen *et al.*, 1977). The intestinal microvillar vesicles were obtained by a modification (Sjöström *et al.*, 1978) of the method described by Kessler *et al.* (1978). The membrane preparation was solubilized in 1% (w/v) Triton X-100 to give 3mg of protein (antigen I)/ml. Line electrophoresis with 1ml of this antigen and 12mg of IgG in 6ml of gel produced multiple precipitation lines. A pilot gel was stained for aminopeptidase A (Danielsen *et al.*, 1977); subsequent gels were not stained and the immunoprecipitate was excised (antigen II). Each rabbit immunized received antigen derived from 15 gels to produce antiserum II. Again, line electrophoresis with antigen I and antiserum II yielded an immunoprecipitate (antigen III) that was used to raise antiserum III. At the completion of the course, 40ml of blood were taken and an IgG fraction was prepared (yield 100mg/rabbit) and stored in 150mM-NaCl/15mM-Na₃N. Before coupling, the

Scheme 1. Preparation of immunoadsorbent column

IgG was dialysed against 100 mM- $\text{NaHCO}_3/\text{NaOH}$ (pH 8.3)/0.5 M- NaCl . CNBr-activated Sepharose 4B (10 ml), diluted with 10 ml of the NaHCO_3 buffer, was added to 100 mg of IgG and stirred for 20 h at 4°C. The immunoadsorbent was washed on a Buchner funnel with 200 ml of 200 mM-Tris/HCl, pH 7.0. About 90% of the IgG bound in these conditions. The column was stored in 50 mM-Tris/HCl (pH 7.5)/150 mM- NaCl /15 mM- NaN_3 . An IgG fraction from serum raised to pig kidney microvillar membrane was coupled in the same manner.

Preparation of aminopeptidase M

For comparing the properties of this enzyme with aminopeptidase A, a partially purified preparation was used. A Triton X-100-solubilized extract of pig kidney cortex was prepared and fractionated on DEAE-cellulose and Sepharose 6B as described by Macnair & Kenny (1979). The aminopeptidase M-containing fractions were further chromatographed on a column (18 ml) of concanavalin A-Sepharose 4B (Sigma) equilibrated with 10 mM-Tris/HCl (pH 7.9)/0.15% (v/v) Triton X-100/0.7 mM- CaCl_2 /0.7 mM- MnCl_2 /0.7 mM- MgCl_2 /10 mM- NaCl . It was eluted by a linear gradient (0–0.5 M) of α -methyl D-glucoside. The main peak of aminopeptidase M activity was eluted at 0.4 M- α -methyl-D-glucoside.

Polyacrylamide gel electrophoresis

Disc electrophoresis was performed with a Tris/

glycine buffer system (Davis, 1964). Electrophoresis in the presence of dodecylsulphate was performed in gradient gels [7–17% (w/v) acrylamide] by using the system described by Booth (1977). Carbohydrate was stained by basic Fuchsin after periodate oxidation as described by Glossmann & Neville (1971).

Enzyme assays

Aminopeptidases A and M were assayed fluorimetrically with Glu-2-NNap and Ala-2-NNap as substrates; neutral endopeptidase was assayed with [^{125}I]iodoinsulin B chain as substrate, both assays as previously described (Kenny, 1977). Carboxypeptidase P (proline carboxypeptidase, EC 3.4.16.2) was assayed with benzoxycarbonyl-L-prolyl-L-methionine as substrate, methionine being determined by a modification of the one-step amino acid method (Nicholson & Kim, 1975). The oxidase reagent contained (in 100 ml) 18 U of L-amino acid oxidase (type VI, Sigma, crude venom from *Crotalus atrox*), 2 mg of horseradish peroxidase (type II, Sigma), 1 ml of ethanol containing 10 mg of o-dianisidine, in a buffer comprising 108 mM- NaCl /24 mM-sodium barbitone/25 mM-sodium acetate, pH 7.75 adjusted with HCl. The incubation mixture contained 50 μl of enzyme, 50 μl of 32 mM- MnCl_2 in the barbitone buffer, 0.5 ml of a 10 mM solution of the substrate in barbitone buffer and 1 ml of oxidase reagent. After 20 min incubation at 37°C the reaction was stopped by the addition of 0.75 ml of 50% (v/v) H_2SO_4 and the absorbance at 530 nm was

determined. Trehalase and alkaline phosphatase were assayed as previously described (George & Kenny, 1973). Units for all enzyme activities are calculated as the hydrolysis of $1\mu\text{mol}$ of substrate $\cdot \text{min}^{-1} \cdot (\text{mg of protein})^{-1}$ under the conditions used.

Peptide hydrolysis

The incubation mixture (vol. $100\mu\text{l}$) contained $1\mu\text{g}$ of aminopeptidase A, 5 mM -peptide, 0.1% (v/v) Triton X-100 and 100 mM - NH_4HCO_3 , pH 7.5. After incubation at 37°C for 30 and 120 min, the mixtures were treated with dansyl chloride and the dansyl amino acids were identified by chromatography on polyamide sheets (Hartley, 1970). In the experiment with insulin or insulin B chain, the mixture contained $50\mu\text{g}$ of the peptide.

Purification of aminopeptidase A

This is summarized in Scheme 2. The steps up to the preparation of the Triton X-100 supernatant fraction were identical with those used in the preparation of dipeptidyl peptidase IV (Macnair & Kenny, 1979). The supernatant fraction (400 ml from 1 kg of kidney cortex) was stirred at 4°C for 20 h with 200 g of DEAE-cellulose (Whatman DE-52), previously equilibrated in 5 mM -Tris/HCl buffer, pH 7.5, containing 0.1% (v/v) Triton X-100. The DEAE-cellulose was washed on a Buchner funnel with 1 litre of equilibration buffer and then with 1 litre of 100 mM -NaCl and 1 litre of 200 mM -NaCl, both in the same buffer containing Triton X-100. Most of the activity was recovered in the second wash. This fraction was adjusted to bring the solution to 150 mM -NaCl and 50 mM -Tris/HCl, pH 7.5, and was pumped (10 ml/h) on to the

immunoabsorbent column (7.5 ml settled vol.) until aminopeptidase A activity was detectable in the effluent. It was then washed (20 ml/h) with 250 ml of 150 mM -NaCl/ 50 mM -Tris/HCl (pH 7.5)/ 0.1% (v/v) Triton X-100. It was eluted (10 ml/h) with 150 mM -NaCl/ 100 mM - NaHCO_3 /NaOH (pH 10.6)/ 0.1% (v/v) Triton X-100. Fractions (2 ml) were collected into tubes containing 4 ml of 200 mM -Tris/HCl (pH 7.0)/ 0.1% (v/v) Triton X-100. In some experiments the preparation was further purified by passage through a small column (2 ml settled vol.) of Sepharose 4B to which an IgG fraction of an antiserum raised to pig kidney microvillar membrane had been coupled. The fractions were stored at -20°C .

The proteinase form was purified on the same scale and in the same manner except that the microsomal suspension was treated with trypsin ($1:30$ trypsin/protein ratio) for 1 h at 37°C . Triton X-100 was omitted from the buffers.

In two experiments a small-scale purification was done in the presence of proteinase and bacterial inhibitors. A microvillar fraction was prepared from 150 g of kidney cortex. This was resuspended in 200 ml of 50 mM -Tris/HCl, pH 7.5, containing 1% (w/v) Triton X-100. In one experiment all the media used in the preparation contained Trasylol (50 k-i.u./ml). In the second, 0.1 mM -di-isopropyl phosphorofluoridate and 10 mM -NaN₃ were present.

Determination of molecular weight by gel filtration

Columns ($25\text{ mm} \times 900\text{ mm}$) of Ultrogel AcA 34 and AcA 22 were developed at 15 ml/h with 20 mM -Tris/HCl, pH 7.5, containing 0.1% Triton X-100 (omitted when the proteinase form of the enzyme was studied). The enzyme samples (1 mg in 3 ml) had been dialysed against the same buffer. The

Scheme 2. *Purification of the amphiphilic form of aminopeptidase A*

columns were calibrated with the following proteins (mol.wt. in parentheses) dipeptidyl peptidase IV (autolysis form 266 000), aminopeptidase M (papain form 280 000; detergent form approx. 400 000) and urease (480 000).

Effects of inhibitors

Inhibitors were preincubated for 1 h at 37°C with 3 µg of enzyme in 0.2 ml of Tris/HCl (pH 7.0)/0.1% Triton X-100.

Kinetic experiments

The K_m and V_{max} values for the hydrolysis of Glu-2-NNap and Asp-2-NNap were determined from initial rates of hydrolysis for substrate concentrations in the range 0.05–0.4 mM. Appropriate corrections were made for quenching by the substrate over this concentration range. Linear regression lines were fitted to a double reciprocal plot by using a programmable calculator.

Determination of Ca, Zn and Mn

Samples of the enzyme (0.4 mg/ml) were dialysed exhaustively against double-distilled water and the metal content was determined by atomic absorption spectrophotometry with a Unicam SP.90 (series 2) or a Perkin-Elmer 303 instrument.

Treatment with neuraminidase

The procedure of Spiro (1966) was used. A sample (100 µg) of the proteinase form of the enzyme was dialysed against 100 mM-sodium acetate/acetic acid (pH 5.6)/1 mM-CaCl₂. The sample was incubated (vol. 0.2 ml) at 37°C for 6 h with 0.5 U of neuraminidase.

Electron microscopy

The methods used were those previously described (Kenny *et al.*, 1976).

Results and discussion

Purification and homogeneity

Preparation of the immunoabsorbent column. Our approach to the purification of aminopeptidase A is

one that is applicable to other enzymes, provided that the immunoprecipitate can be unequivocally identified. Three antigens of increasing purity, initially total microvillar membrane proteins, followed by two immunoprecipitates, were necessary to obtain a monospecific antiserum. Between 12 and 16 repetitions of the line electrophoresis were required to give a sufficient quantity of antigen for an immunization course. Antiserum II was not adequate for use as an immunoabsorbent column. It gave several precipitation lines against microvillar membrane proteins, one of which (corresponding to aminopeptidase A) was much intensified compared to the others. However, this precipitate was of adequate purity to produce a monospecific antiserum to aminopeptidase A. The column containing about 150 mg of the IgG fraction from this serum could bind 1.3 mg of aminopeptidase A (whether detergent or proteinase forms) of which 0.5 mg could be desorbed. Subsequent bleedings of a rabbit, following booster doses of antigen, produced IgG fractions with essentially the same binding capacity. Moreover, repeated use of the column did not diminish its capacity or specificity.

Purification of aminopeptidase A. Table 1 summarizes the purification of aminopeptidase A on a preparative scale. Triton X-100 was effective in solubilizing all the microvillar membrane peptidases (Macnair & Kenny, 1979) and in this purification about 81% of the aminopeptidase A was released from the microsomal suspension into the supernatant fraction. After solubilization by Triton X-100, only two column steps were required. Chromatography on DEAE-cellulose served to decrease by two-thirds the load of protein to be applied to the immunoabsorbent column, without much loss of enzyme. In a small-scale preparation this intermediate step could be omitted. The immunoabsorbent column achieved an enrichment of 180-fold, albeit with a loss of about 60%. The only limitation was the low capacity of this column: the preparation shown in Table 1 required ten consecutive runs. Initially, the effluent during loading was monitored to determine the capacity of the column, but once this had been established, the

Table 1. *Purification of the detergent form of aminopeptidase A*
See the Experimental section for details. The units are µmol of Glu-2-NNap hydrolysed/min.

Fraction	Protein (mg)	Activity (U)	Recovery (%)	Specific activity (U/mg)	Purification factor
Homogenate	106 500	1950	100	0.018	1
Microsomal	13 300	622	32	0.047	2.6
Triton X-100 supernatant	6000	504	26	0.0834	4.6
DEAE-cellulose	2040	413	21	0.0202	11
Immunoabsorbent chromatography	4.6	166	9	36.1	2005

quantity of enzyme for each loading could be predicted.

The proteinase form of the enzyme was also purified by the same procedure. Trypsin treatment for 1 h released 60% of the activity from the membrane.

Preparations of aminopeptidase A required for studies on the specificity of the enzyme were further purified by passage through a small immuno-adsorbent column containing an IgG fraction from an antiserum raised to kidney microvillar membrane. This column had the ability to remove trace amounts of any microvillar proteins, though since its capacity for any one protein was small, only small losses of aminopeptidase A were incurred. No measurable increase in specific activity resulted from this treatment, a finding consistent with other evidence for the homogeneity of the preparation.

Homogeneity of the purified enzyme. In polyacrylamide-gel electrophoresis of the detergent form (Fig. 1) a single Coomassie Blue-stained band was seen at a loading of 10 μ g of protein. The proteinase form (15 μ g of protein) migrated at about twice the rate as a broad band, blurred at the leading edge.

Fig. 1. Polyacrylamide-gel electrophoresis of purified aminopeptidase A

See the Experimental section for details; (a) and (b) are stained for protein with Coomassie Blue. (a) detergent form; (b) proteinase (trypsin) form; (c) as (b), but stained for enzyme activity with Glu-2-NNap (Danielsen *et al.*, 1977).

However, histochemical staining showed that the entire band possessed enzyme activity. Treatment of a sample with neuraminidase gave a preparation that stained as a more compact band of activity in a slightly retarded position (result not shown). Crossed immunoelectrophoresis against an antiserum raised to all microvillar membrane proteins gave only one precipitate (Fig. 2a). At the concentration of IgG used, this method is exquisitely sensitive in detecting contamination with other microvillar proteins.

The preparation was assayed for other pig kidney microvillar enzymes (Table 2) with a variety of substrates. Two substrates in this group (Glu-2-NNap and Asp-2-NNap) were hydrolysed at high rates. However Arg-2-NNap, Lys-2-NNap, Ala-2-NNap and Leu-2-NNap were also attacked at significant rates. The hydrolysis of other substrates was at the limit of detection. Ala-2-NNap is the preferred assay substrate for aminopeptidase M; hence the possibility that this enzyme contaminated the preparation needed further investigation.

Table 3 compares the relative activities of aminopeptidases A and M on four substrates in the presence and absence of 1 mM- Ca^{2+} . The preparation of aminopeptidase M was partially pure (specific activity, 7.2 U/mg of protein for Leu-2-NNap) but was essentially free of aminopeptidase A (sp.act., 0.07 U/mg of protein for Glu-2-NNap). It is clear that the patterns of substrate specificity are different for the two enzymes. Furthermore, the addition of Ca^{2+} was strongly inhibitory of aminopeptidase A, but only weakly so for aminopeptidase M. When aminopeptidase A was heated to 60°C the loss of activity towards Glu-2-NNap and Ala-2-NNap was similar, reaching 50% loss at 20 min. Aminopeptidase M activity was unaffected when Triton X-100-solubilized microvillar membrane was similarly treated. Rocket immunoelectrophoresis showed that the same immunoprecipitated material hydrolysed Lys-2-NNap, Arg-2-NNap, Ala-2-NNap and Leu-2-NNap (Fig. 2b). For these reasons we conclude that the hydrolyses of some basic and neutral amino acid 2-naphthylamides can be attributed to aminopeptidase A and that our preparation was enzymically, immunologically and electrophoretically homogeneous.

Enzymic properties of the detergent form of aminopeptidase A

pH-activity curve. Maximum activity was observed at pH 6.5–7.0. The curve was symmetrical and sharp: little activity was measurable outside the range pH 4.5–8.5.

Effect of metal ions. Ca^{2+} , at 0.1 mM and above, activated the hydrolysis of Glu-2-NNap about 4-fold (Fig. 3); Ba^{2+} was slightly less effective, whereas

Fig. 2. Immuno-electrophoresis of the detergent form of aminopeptidase A

See the Experimental section for details. (a) Crossed charge-shift immuno-electrophoresis (stained for protein): T, gel containing Triton X-100 only; C, with addition of cetyltrimethylammonium bromide; D, with addition of sodium deoxycholate. (b) Rocket immuno-electrophoresis. Each gel is identical in loading of enzyme and IgG. The histochemical stain for aminopeptidase activity with Lys-2-NNap, Arg-2-NNap, Leu-2-NNap and Ala-2-NNap as substrates (Danielsen *et al.*, 1977) is shown.

Table 2. Substrate specificity of aminopeptidase A

The detergent form of the enzyme was used. All the naphthylamide substrates were present at 0.2 mM. The specific activity for each substrate is given as mU (i.e. nmol of substrate hydrolysed · min⁻¹)/mg of protein. The hydrolysis of Gly-Pro-2-NNap, γ-Glu-2-NNap, *p*-nitrophenylphosphate, and trehalose was, in each case, at the limit of detection even when the enzyme concentration was increased 10-fold.

Substrate	Specific activity (mU/mg of protein)	Relative activity
Glu-2-NNap	36 100	100
Asp-2-NNap	8460	23
Arg-2-NNap	1496	4.1
Lys-2-NNap	640	1.8
Ala-2-NNap	525	1.4
Leu-2-NNap	137	0.38
Cbz-Pro-Met	6	0.017
[¹²⁵ I]iodoinsulin B chain	0.28	0.0008

Table 3. *Effect of Ca^{2+} on the hydrolysis of various 2-naphthylamides by aminopeptidases A and M*
The conditions were similar to those given in Table 2.

Substrate	[Ca^{2+}] (mM)	Relative activities	
		Aminopeptidase A	Aminopeptidase M
Ala-2-NNap	0	100	100
	1	33	91
Leu-2-NNap	0	26	76
	1	8	67
Arg-2-NNap	0	285	53
	1	54	43
Lys-2-NNap	0	122	26
	1	37	20

Fig. 3. *Effect of metal ions on aminopeptidase A*
●, CaCl_2 ; ○, BaCl_2 ; ■, MgCl_2 ; □, MnSO_4 ; ▲, CuCl_2 ; △, ZnCl_2 . The salts were not preincubated with the enzyme; the concentrations shown are those in the assay medium. The enzyme activities are shown relative to that determined in the absence of metal ions.

Mg^{2+} and Mn^{2+} had little influence. Cu^{2+} and Zn^{2+} were strongly inhibitory.

Effect of chelating agents and the role of Ca^{2+} . The hydrolysis of Glu-2-NNap was inhibited by EDTA and EGTA (Fig. 4a), each with I_{50} values in the range 2–5 mM, EDTA being slightly more potent. The hydrolysis of Ala-2-NNap was inhibited by EDTA to the same extent as that of Glu-2-NNap. When the enzyme was fully inhibited by treatment with 100 mM-EDTA, it could, after dialysis, be

reactivated with either Mn^{2+} or Ca^{2+} to about 90% of the activity exhibited by the untreated enzyme assayed in the absence of Ca^{2+} . Mn^{2+} was the more effective (Fig. 4b). Other divalent metal ions (Mg^{2+} , Co^{2+} , Ni^{2+} , Ba^{2+} , Fe^{2+} and Cu^{2+}) achieved no more than 10% reactivation when tested in the concentration range 0.1 μM –10 mM.

The activation by Ca^{2+} of the hydrolysis of Glu-2-NNap has been examined in a kinetic experiment (Fig. 5). The K_m value for Glu-2-NNap was decreased by the presence of Ca^{2+} without any significant effect on V_{max} . (47.3 ± 2.2 units/mg of protein). At 0.15 mM- Ca^{2+} the K_m value was 75 μM compared with 350 μM in the absence of Ca^{2+} . In the presence of 1.25 mM- Ca^{2+} the K_m values were 62 μM (Glu-2-NNap) and 190 μM (Asp-2-NNap).

Stability of the purified enzyme. At -20°C the detergent form of the enzyme was stable over a period of several months. At 4°C it slowly lost activity and failed to respond effectively to Ca^{2+} with Glu-2-NNap as substrate.

Hydrolysis of peptides and peptide derivatives. The results are shown in Table 4. Dipeptides and tripeptides with aspartic or glutamic acids as *N*-terminal residues were readily hydrolysed, provided the α -amino group was unsubstituted. Substrates with other *N*-terminal amino acid residues (leucine, alanine, histidine, valine) were hydrolysed less readily, and the Ala-Pro bond resisted attack, as did glycine-containing peptides. No significant hydrolysis of insulin B chain or insulin could be demonstrated.

Inhibitors. Thiol compounds inhibited aminopeptidase A: 10 mM-2-mercaptoethanol and 10 mM-dithiothreitol inhibited by 71 and 97%. No inhibition was demonstrated with iodoacetamide (1–10 mM), 4-chloromercuribenzoate (1–10 mM), di-isopropyl phosphorofluoridate (0.1–1 mM) and phosphoramidon (0.1–1 mM).

Molecular properties of aminopeptidase A

Molecular weight determinations. Both the detergent and proteinase forms of the enzyme were

EXPLANATION OF PLATE 1

Electron micrograph of aminopeptidase A (detergent form)

The preparation was negatively stained with uranyl acetate. The horizontal bar represents 20nm. Most of the structures appear to be dimers, each monomer being about 6.5 nm in diameter.

Fig. 4. Inhibition by EDTA and EGTA and reactivation by Ca²⁺ and Mn²⁺

Enzyme activities are shown relative to that of the control determined in the absence of Ca²⁺. (a) ●, EDTA; ○, EGTA. (b) A sample of enzyme previously treated for 1 h with 10 mM-EDTA was exhaustively dialysed against water. No residual activity could be determined. Aminopeptidase A activity was assayed in the presence of CaCl₂ (●) or MnCl₂ (○) at the concentrations shown.

Fig. 5. Effect of Ca²⁺ on K_m and V_{max} values for hydrolysis of Glu-2-NNap

See the Experimental section for details. ●, No Ca²⁺; ○, 0.015 mM-CaCl₂; ■, 0.050 mM-CaCl₂; □, 0.15 mM-CaCl₂. The concentrations shown are those in the assay mixture.

subjected to gel filtration on columns of Ultrogel AcA 22 and Ultrogel AcA 34. The proteinase form was eluted in the same volume as the proteinase forms of dipeptidylpeptidase IV and aminopeptidase M, corresponding to a mol.wt. of about 270 000. The detergent form was significantly larger, with a mol.wt. from three experiments in the range 350 000–400 000. The two peptidases used as reference proteins are known to be dimeric in pig kidney. Electron microscopy of the proteinase form of aminopeptidase A (Plate 1) showed it to be

predominantly dimeric in appearance after negative staining, rather similar to dipeptidylpeptidase IV (Macnair & Kenny, 1979). Each monomer was about 6.5 nm in diameter.

Electrophoresis of both forms of aminopeptidase A in the presence of dodecylsulphate consistently showed four bands (Fig. 6). The apparent mol.wts. for the polypeptides in the detergent form were 155 000, 110 000, 90 000 and 45 000, the last of which appeared as a broad, rather diffuse band. The stained bands observed with the proteinase form were generally similar, but the two higher molecular-weight peptides migrated very slightly in advance of those derived from the detergent form. The multiplicity of bands strongly suggested that fragmentation by proteinases may have occurred during the preparation. However, the same pattern was seen in preparations purified in the presence of Trasylol or di-isopropyl phosphorofluoridate and NaN₃.

Carbohydrate. All four of the bands resolved by electrophoresis in the presence of sodium dodecyl sulphate stained positively for carbohydrate by the periodic acid/Schiff reagent (Fig. 6).

Metal content. Samples of the detergent form of the enzyme were assayed by atomic absorption spectrophotometry for Zn, Mn and Ca. The first two metals were not detectable, but Ca was identified to the extent of 1 g-mol/143 000 g of protein, a result consistent with each dimer containing two non-diffusible calcium ions.

Amphipathic properties of the detergent form of aminopeptidase A. Crossed immunoelectrophoresis (Fig. 2) in which the antigen was electrophoresed into a gel containing antibodies raised to all

Table 4. *Hydrolysis of various peptides by aminopeptidase A*

See the Experimental section for details. Parentheses indicate that the dansyl amino acid was at the limit of detection.

Substrate	Products identified after incubation for:	
	0.5 h	2.0 h
α -Glu-Ser	Glu,Ser	Glu,Ser
α -Asp-Ser	Asp,Ser	Asp,Ser
Cbz- α -Glu-Tyr	Not hydrolysed	Not hydrolysed
Gly-Glu	Not hydrolysed	Not hydrolysed
Leu-Leu	(Leu)	Leu
Ala-Ala-2-NNap	(Ala)	Ala
His-Ser-2-NNap	(His)	His
Glu-Thr-Tyr	Glu,(Thr)(Tyr)	Glu,Thr,Tyr
Ala-Pro-Gly	Not hydrolysed	Not hydrolysed
Ala ₄	Ala	Ala
N-Acetyl-Ala ₄	Not hydrolysed	(Ala)
Val-Ala-Ala-Phe	(Val)	Val,(Ala)
Gly ₅	Not hydrolysed	(Gly)
Insulin B-chain	Not hydrolysed	Not hydrolysed
Insulin	Not hydrolysed	Not hydrolysed

Fig. 6. Polyacrylamide-gel electrophoresis in the presence of dodecylsulphate

See the Experimental section for details. An acrylamide gradient [7–17% (w/v)] was used. Mv, microvillar membrane (100 μ g of protein). (a) Detergent form; (b) proteinase form; (c) detergent form (all 10 μ g loading). Mv, (a) and (b) were stained with Coomassie Blue; (c) is stained for carbohydrate and is from a different gel.

microvillar proteins produced a single precipitation peak, indicative of the immunological homogeneity of the preparation. When the experiment was repeated with the same (Triton X-100) detergent form of the enzyme, but in the presence of either deoxycholate or cetyltrimethylammonium bromide, the mobility in the first dimension was clearly

changed: decreased to 50% by the cationic detergent and doubled by the anionic detergent (Fig. 2). These results indicate the presence of a hydrophobic domain to which substantial amounts of detergent are bound. The proteinase form did not show charge-shift in these experiments.

Immunological properties

The antiserum used for the preparation of the immunoabsorbent column was raised to an antigen present in pig intestinal microvilli. It follows that the kidney and intestinal enzymes possess extensive cross reactivity, though we have not attempted to quantify the number of common antigenic determinants. This cross-reactivity is not unexpected for microvillar enzymes. Most, though not all, of the determinants have been shown to be common to the renal and intestinal forms of aminopeptidase M (Vannier *et al.*, 1976). A similar relationship holds for dipeptidylpeptidase IV and γ -glutamyl-transferase (Norén *et al.*, 1979).

The presence of an enzyme in human serum with similar properties to that in kidney (Nagatsu *et al.*, 1970) posed questions concerning the identity of the two enzymes. Pig serum also contained detectable aminopeptidase A activity (0.8 mU/ml). An IgG fraction from rabbit antiserum raised to pig kidney microvillar membrane proteins reacted with the enzyme in serum (Fig. 7). Both the kidney and serum activities were precipitated and/or inhibited by the immunoglobulin fraction, whereas a similar fraction prepared from the same rabbit before immunization (i.e. pre-immune serum) had no effect. In a second experiment, the antiserum was absorbed by either kidney or serum aminopeptidase A and the absorbed sera now failed to react with the serum or

Fig. 7. Immunological cross-reactivity between aminopeptidase A in pig plasma and pig kidney

See the Experimental section for details. Aminopeptidase activity is shown relative to that in samples not treated with antiserum after centrifugation at 18000 *g* for 15 min. ●, Pig kidney microvillar activity; ▲, pig serum activity; ○ and △, kidney and serum activity with IgG from control (i.e. pre-immune) serum; ■, pig serum activity, antiserum absorbed with purified aminopeptidase A; □, pig serum activity, antiserum absorbed with serum antigen. The absorbed sera were prepared by adding an excess of each antigen. After precipitation overnight the immunoprecipitate was removed by centrifugation and any remaining antigen was removed by the addition of the immunoadsorbent [anti-(aminopeptidase A)–Sepharose 4B].

kidney activities. The serum enzyme also bound to the immunoadsorbent column and could be desorbed by the same method. It was also found that an antiserum raised against human kidney microvillar membrane was capable of precipitating and/or inhibiting the aminopeptidase A activity in both human kidney and human serum. It thus appears that the serum and kidney enzymes are related proteins.

General conclusions

One unexpected finding in these studies was the broad specificity of an enzyme originally considered (and hence so named) to be specific for peptides with *N*-terminal acidic amino acid residues. Its ability to hydrolyse basic and neutral substrates, albeit at much slower rates, could not be attributed to contamination by other aminopeptidases. The evidence shows that this extended specificity is a property of aminopeptidase A. In hydrolytic function, the enzyme neatly complements that of other exopeptidases in the renal microvillar membrane so as to provide a comprehensive system for the hydrolysis of almost any oligopeptide (Kenny & Booth, 1978). There are some common features among this group of peptidases. All those so far fully

characterized (with the exception of the serine peptidase, dipeptidylpeptidase IV) are metallo-proteins. Aminopeptidase M, peptidyl dipeptidase and neutral endopeptidase are Zn^{2+} -enzymes. Aminopeptidase A is unusual in containing Ca^{2+} . There are also some general similarities in structure. They are intrinsic membrane proteins of substantial size (monomeric mol.wts. in the range 90000–160000). All are glycoproteins with the bulk of the polypeptide chain exposed at the external surface of the microvillus, with only a relatively small hydrophobic domain anchored in, and traversing, the lipid bilayer (Booth & Kenny, 1980). In common with most of the microvillar peptidases (neutral endopeptidase being the exception) the bulky hydrophilic portion of aminopeptidase A can be cleaved from the anchor peptide by trypsin or papain. Aminopeptidase A, in either the detergent or proteinase form, is dimeric when purified, with an appearance in electron micrographs indistinguishable from dipeptidylpeptidase IV.

One finding that might suggest a structural difference compared with other microvillar peptidases was the consistent demonstration of four stained bands on dodecylsulphate electrophoresis. Both forms of the enzyme exhibited essentially similar patterns and the use of reagents to limit proteolysis during the preparation did not affect the pattern. Aminopeptidase M from intestine is invariably multibanded on gel electrophoresis in the presence of dodecyl sulphate (Maroux *et al.*, 1973). So, too, is renal aminopeptidase M after treatment with papain or trypsin (Wacker *et al.*, 1976; Booth & Kenny, 1976). However, the detergent form of the kidney enzyme and that from the intestine, provided pancreatic enzymes are excluded by prior duct ligation (Sjöström *et al.*, 1978), are symmetrical dimers composed of only two polypeptide chains. While it is possible that the four polypeptides found in aminopeptidase A represent subunits existing in the membrane, it is more likely that they arose by proteolysis. The mol.wts. of the four components (155000, 110000, 90000, 45000) do not summate, in any simple ratio, to a dimeric mol.wt. of about 270000. Moreover, Benajiba & Maroux (1980) have found that both the detergent and proteinase forms of pig intestinal aminopeptidase A, prepared by a different procedure, are single banded in sodium dodecyl sulphate electrophoresis. So, too, is a preparation of intestinal aminopeptidase A purified in this laboratory by the method described here for the kidney enzyme (S. Sivakami, E. M. Danielsen & A. J. Kenny, unpublished work). This is a surprising result, since the risk of proteinase attack might be expected to be greater in intestine than kidney. However, the inclusion of inhibitors in the media used for preparing the kidney enzyme did not alter the pattern. Nor could we demonstrate any intrinsic

Fig. 8. *Speculative model showing possible role of Ca^{2+} in hydrolysis of L-glutamylglycine*

The binding of Glu-Gly is assumed to require two electrostatic interactions, one involving the γ -carboxylate group and Ca^{2+} , the second with the α -amino group. The nondiffusible Ca^{2+} is shown coordinating with the carboxyl group of the peptide bond.

proteinase activity by the immunoabsorbent by using a sensitive assay with [^{125}I]iodoinsulin B chain. The origin of the four bands remains unclear and the question deserves further investigation.

The role of Ca^{2+} is two-fold. The tightly bound Ca^{2+} is essential for catalytic activity and probably for stabilizing the enzyme. Chelating agents inhibit the hydrolysis of all types of substrate and reactivation of the inhibited enzyme never achieves the maximum activity of untreated samples. The second activating effect of Ca^{2+} is limited to the hydrolysis of acidic substrates, Glu-2-NNap and Asp-2-NNap. Here it promotes binding of the side chain carboxylate ion and reduces the K_m value substantially. Basic substrates may bind at this same site without the mediation of Ca^{2+} , and indeed the presence of Ca^{2+} is strongly inhibitory to the hydrolysis of both these and neutral substrates. A speculative view of the active site binding L-glutamylglycine is shown in Fig. 8.

Aminopeptidase A purified from kidney microvilli may well be identical in most structural respects with the enzyme in the intestinal brush border. More

surprisingly, the rather weak activity demonstrable in serum is also a related protein, binding to the same immunoabsorbent column and reacting with aminopeptidase A antibodies in an antiserum raised to kidney microvillar proteins. The enzyme purified about 500-fold from bovine kidneys by Chulkova & Orekhovich (1978) probably still contained aminopeptidase M, since it hydrolysed Leu-2-NNap at a substantial rate. The mol.wt. was reported to be 53000, a value surprisingly low in comparison with microvillar peptidases in other species. In catalytic properties it resembles the pig kidney enzyme. However, the cytosolic aspartate aminopeptidase reported in dog kidney (Cheung & Cushman, 1971) is a distinct enzyme differing in specificity and metal ion requirement from the microvillar enzyme.

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