

# Purification and characterization of pig kidney aminopeptidase P

## A glycosyl-phosphatidylinositol-anchored ectoenzyme

Nigel M. HOOPER,\* John HRYSZKO and Anthony J. TURNER

M.R.C. Membrane Peptidase Research Group, Department of Biochemistry, University of Leeds, Leeds LS2 9JT, U.K.

Aminopeptidase P (EC 3.4.11.9) was solubilized from pig kidney membranes with bacterial phosphatidylinositol-specific phospholipase C (PI-PLC) and then purified by a combination of anion-exchange and hydrophobic-interaction chromatographies. Contaminating peptidase activities were removed by selective affinity chromatography. The purified enzyme was apparently homogeneous on SDS/PAGE with an  $M_r$  of 91000. Enzymic deglycosylation revealed that aminopeptidase P is a glycoprotein, with up to 25% by weight of the protein being due to the presence of *N*-linked sugars. The phospholipase-solubilized aminopeptidase P was recognized by an antiserum to the cross-reacting determinant (CRD) characteristic of the glycosyl-phosphatidylinositol anchor. This recognition was abolished by mild acid treatment or deamination with  $\text{HNO}_2$ , indicating that the CRD was due exclusively to the inositol 1,2-cyclic phosphate ring epitope generated by the action of PI-PLC. The activity of aminopeptidase P was inhibited by chelating agents and was stimulated by  $\text{Mn}^{2+}$  or  $\text{Co}^{2+}$  ions, confirming the metallo-enzyme nature of this peptidase. Selective inhibitors of other aminopeptidases (actinonin, amastatin, bestatin and puromycin) had little or no inhibitory effect.

## INTRODUCTION

Aminopeptidase P (EC 3.4.11.9) was first identified in pig kidney as an enzyme that could release the *N*-terminal amino acid from peptides with a penultimate prolyl residue, Gly-Pro-Hyp being used to monitor the activity (Dehm & Nordwig, 1970a). The peptidase has since been located in the microvillar-membrane fraction of pig and human kidney (Kenny *et al.*, 1977; Hooper & Turner, 1988b) and of rat intestine (Lasch *et al.*, 1986). Aminopeptidase P has been solubilized from bovine lung membranes (Orawski *et al.*, 1987), but so far the enzyme has not been extensively purified, owing, in part, to the difficulties encountered in solubilizing it from membrane components. It has been proposed that aminopeptidase P may have a role in the degradation of collagen (Dehm & Nordwig, 1970a) and in the pulmonary degradation of bradykinin (Orawski *et al.*, 1987).

Recently, we observed that aminopeptidase P can be selectively released from kidney microvillar membranes by bacterial phosphatidylinositol-specific phospholipase C (PI-PLC) or by a plasma phospholipase D, and that the peptidase gives a pattern of solubilization by a range of detergents typical of a glycosyl-phosphatidylinositol (G-PI)-anchored ectoenzyme (Hooper & Turner, 1988b, 1989b). Thus aminopeptidase P belongs to the growing family of cell-surface proteins that are anchored in the plasma membrane by a covalently attached G-PI moiety (reviewed by Low, 1987, 1989; Ferguson & Williams, 1988; Turner, 1990).

We have recently characterized the G-PI membrane anchor of another microvillar peptidase, renal dipeptidase (Hooper *et al.*, 1987a, 1990; Hooper & Turner, 1989a; Littlewood *et al.*, 1989). In the present study we have used bacterial PI-PLC to effect solubilization of aminopeptidase P from pig kidney membranes. The solubilized peptidase has been purified to virtual homogeneity by a combination of anion-exchange and hydrophobic-interaction chromatographies in conjunction with selective affinity chromatography to remove certain contaminating pep-

tidase activities. The purified peptidase has been characterized with respect to its size, extent of glycosylation and structure of its G-PI anchor. We have also examined the effect of bivalent metal ions and peptidase inhibitors on the activity of aminopeptidase P.

## EXPERIMENTAL

### Materials

PI-PLC from *Bacillus thuringiensis* was a gift from Dr. M. G. Low, Department of Physiology and Cellular Biophysics, College of Physicians and Surgeons of Columbia University, New York, NY, U.S.A. *B. cereus* phospholipase C type III was purchased from Sigma. Units of PI-PLC activity are  $\mu\text{mol}/\text{min}$ . Anti-(cross-reacting determinant) (anti-CRD) rabbit serum was a gift from Dr. M. A. J. Ferguson, Department of Biochemistry, University of Dundee, U.K. Cilastatin was attached to CNBr-activated Sepharose 4B as described by Campbell *et al.* (1984). Lisinopril was attached to Sepharose CL-4B via a 2.8 nm spacer arm as described by Hooper & Turner (1987). Proline iminopeptidase (EC 3.4.11.5) was purchased from Nacalai Tesque (Kyoto, Japan). Gly-Pro-NMec was from Bachem (Bubendorf, Switzerland). 2D-Silver Stain Kit for detection of proteins on polyacrylamide gels was purchased from Koch-Light (Haverhill, Suffolk, U.K.). Immobilon P [poly(vinylidene difluoride) (PVDF)] membranes were purchased from Millipore. *N*-glycosidase F was purchased from Boehringer Mannheim (Lewes, East Sussex, U.K.). Pig kidneys and all other materials were obtained from sources previously noted.

### Purification of aminopeptidase P

**Solubilization with phospholipase.** All procedures were carried out at 4 °C, except for the f.p.l.c. steps, which were carried out at room temperature. Pig kidney cortex was homogenized in 10 vol. of 0.33 M-sucrose/50 mM-Hepes/NaOH, pH 7.4, and centrifuged

Abbreviations used: CRD, cross-reacting determinant; G-PI, glycosyl-phosphatidylinositol; NMec, 4-methyl-7-coumarylamide; PI-PLC, phosphatidylinositol-specific phospholipase C; PVDF, poly(vinylidene difluoride).

\* To whom correspondence and reprint requests should be addressed.

at 8000 *g* for 15 min. The supernatant was centrifuged at 26000 *g* for 2 h, and the pellet was resuspended in 10 mM-Hepes/NaOH, pH 7.4, to give a protein concentration of 10–15 mg/ml and then incubated with *B. thuringiensis* PI-PLC (0.1 unit/10 mg of protein) for 2 h at 37 °C. After centrifugation at 31000 *g* for 90 min, the supernatant was dialysed extensively against 50 mM-Tris/HCl/0.5 M-NaCl, pH 7.6.

**Affinity chromatography on cilastatin–Sepharose.** The dialysed extract was centrifuged at 31000 *g* for 90 min and then applied to a cilastatin–Sepharose affinity column (10 ml bed vol.) with a pre-column of unmodified Sepharose CL-4B (20 ml bed vol.) equilibrated in 50 mM-Tris/HCl/0.5 M-NaCl, pH 7.6. The run-through fraction was collected and dialysed extensively against 10 mM-Tris/HCl, pH 7.6.

**Anion-exchange chromatography on DEAE-cellulose.** The dialysed sample was applied to a DEAE-cellulose (DE-52) column (30 ml bed vol.) equilibrated in 10 mM-Tris/HCl, pH 7.6. Bound protein was eluted with a 200 ml linear gradient of 0–0.5 M-NaCl in 10 mM-Tris/HCl, pH 7.6. Fractions (5 ml) were collected, and those containing high aminopeptidase P activity were pooled and dialysed against 10 mM-Tris/HCl, pH 7.6.

**Anion-exchange chromatography on MonoQ.** The dialysed sample was applied to an HR5/5 MonoQ f.p.l.c. column (Pharmacia) equilibrated in 10 mM-Tris/HCl, pH 7.6. Bound protein was eluted with a non-linear gradient of 0–0.5 M-NaCl in 10 mM-Tris/HCl, pH 7.6. Fractions (1 ml) were collected, and those containing high aminopeptidase P activity were pooled and dialysed against 20 mM-sodium phosphate, pH 7.5.

**Hydrophobic interaction chromatography on alkyl-Superose.** The dialysed sample was mixed with an equal volume of 4 M-(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>/20 mM-sodium phosphate, pH 7.5, and applied to an HR5/5 alkyl-Superose f.p.l.c. column (Pharmacia) equilibrated in 2 M-(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>/20 mM-sodium phosphate, pH 7.5. Protein was eluted with a non-linear gradient of 2.0–0 M-(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> in 20 mM-sodium phosphate, pH 7.5. Fractions (1 ml) were collected and those containing high aminopeptidase P activity were pooled and dialysed against 10 mM-Tris/HCl/0.3 M-NaCl, pH 7.5.

**Affinity chromatography on cilastatin/lisinopril–Sepharose.** The dialysed sample was applied to a mixed column of cilastatin–Sepharose and lisinopril–2.8 nm–Sepharose (2 ml bed vol.) equilibrated in 10 mM-Tris/HCl/0.3 M-NaCl, pH 7.5. The run-through fraction was dialysed against 10 mM-Tris/HCl, pH 8.0.

**Anion-exchange chromatography on MonoQ.** The dialysed sample was applied to a MonoQ f.p.l.c. column equilibrated in 10 mM-Tris/HCl, pH 8.0. Bound protein was eluted with a non-linear gradient of 0–0.5 M-NaCl in 10 mM-Tris/HCl, pH 8.0. Fractions (1 ml) were collected and those containing high aminopeptidase P activity were pooled and used as purified enzyme sample.

#### Enzyme assays

Aminopeptidase P was assayed by an h.p.l.c. method with Gly-Pro-Hyp as substrate in the presence of 4 mM-MnCl<sub>2</sub> as described by Hooper & Turner (1988b). The purification was monitored by a fluorimetric assay modified from the method of Yoshimoto *et al.* (1988). Enzyme sample was preincubated in 20 mM-Tris/HCl, pH 8.0, containing 4.0 mM-MnCl<sub>2</sub> and 1 mM-di-isopropyl phosphorofluoridate (0.1 ml total volume). After 30 min at room temperature a further 0.8 ml of 20 mM-Tris/HCl,

pH 8.0, was added and the reaction started by addition of 0.1 ml of 2 mM-Gly-Pro-NMec. After incubation at 37 °C for 2 h, the reaction was terminated by boiling for 4 min. The samples were cooled on ice, and then 0.1 ml of proline iminopeptidase [1 unit (μmol/min)/ml] in 20 mM-Tris/HCl, pH 6.8, was added to release the chromogenic group. After incubation at 37 °C for 30 min, the reaction was terminated by boiling for 4 min. Water (2.0 ml) was added, and the samples were read in a fluorimeter at an excitation wavelength of 370 nm and an emission wavelength of 442 nm.

Renal dipeptidase, other peptidase activities and protein were assayed as described previously (Hooper & Turner, 1988a; Littlewood *et al.*, 1989).

#### SDS/PAGE and immunoelectrophoretic blot analysis

SDS/PAGE was performed with a 7–17% polyacrylamide gradient as described previously (Relton *et al.*, 1983). Protein was detected either with Coomassie Brilliant Blue or with a 2D-Silver Stain Kit. Immunoelectrophoretic ('Western') blot analysis was carried out as detailed previously (Hooper & Turner, 1987), except that Immobilon P PVDF membranes were used and a <sup>125</sup>I-labelled goat anti-(rabbit IgG) was employed as second antibody. After extensive washing of the membranes as for the first antibody, labelled protein was detected by autoradiography at –70 °C with Konica A2 film.

#### M<sub>r</sub> determination by gel-filtration chromatography

Gel-filtration chromatography was carried out on an HR10/30 Superose 12 f.p.l.c. column (Pharmacia). Duplicate samples of purified aminopeptidase P (20 μg) were chromatographed in 5 mM-Tris/HCl/0.3 M-NaCl, pH 8.0, at a flow rate of 0.2 ml/min. The marker proteins used were cytochrome *c* (M<sub>r</sub> 12400), egg albumin (M<sub>r</sub> 45000), aldolase (M<sub>r</sub> 158000), catalase (M<sub>r</sub> 232000), ferritin (M<sub>r</sub> 440000) and thyroglobulin (M<sub>r</sub> 669000).

#### Triton X-114 phase separation

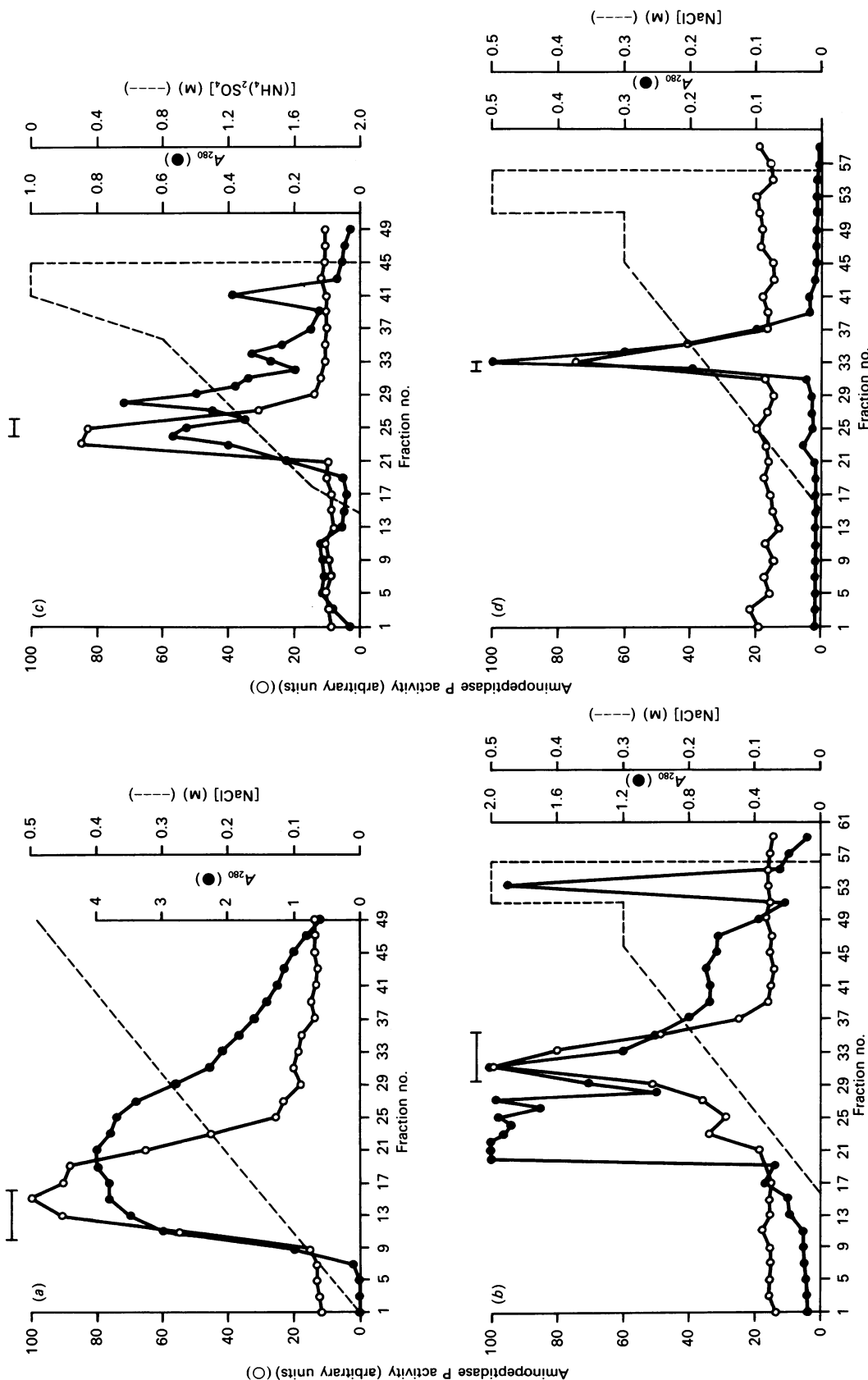
Enzyme samples were made up to 0.2 ml with 10 mM-Tris/HCl/0.15 M-NaCl/1.0% (w/v) Triton X-114, pH 7.4, and subjected to phase separation at 30 °C for 3 min as described by Bordier (1981). The detergent-rich and detergent-poor phases were separated through 0.3 ml of 6% (w/v) sucrose by centrifugation at 3000 *g* for 3 min and assayed for enzyme activity. Activity recovered in the detergent-poor phase is expressed as a percentage of the total activity.

#### Enzymic deglycosylation

Aminopeptidase P was deglycosylated with *N*-glycanase as described previously (Hooper & Turner, 1987) or with *N*-glycosidase F [0.15–1.5 units (nmol/min)] in 0.3 M-sodium phosphate, pH 7.0, containing EDTA (0.3 M) and Nonidet P-40 (7.5%, w/v) in a total volume of 40 μl. After 24 h at 37 °C the reaction was terminated by the addition of an equal volume of electrophoresis buffer and boiled for 4 min. Samples were analysed by SDS/PAGE.

#### Mild acid and HNO<sub>2</sub> treatment

The inositol 1,2-cyclic phosphate ring, formed by PI-PLC cleavage, was selectively decyclized by incubation of aminopeptidase P with 1 M-HCl for 30 min at 23 °C (Zamze *et al.*, 1988). After neutralization with NaOH, samples were subjected to Western-blot analysis. Aminopeptidase P was deaminated by treatment with 0.25 M-sodium acetate/0.25 M-NaNO<sub>2</sub>, pH 4.0, for 3 h at 23 °C (Zamze *et al.*, 1988). Control samples were treated with 0.25 M-sodium acetate/0.25 M-NaCl, pH 4.0. After neutralization, samples were acetone-precipitated and then subjected to Western-blot analysis.



**Fig. 1. Purification of pig kidney aminopeptidase P**

(a) Anion-exchange chromatography on DEAE-cellulose. The run-through fraction from the cistatin-Sepharose column was applied to a DEAE-cellulose column as detailed in the Experimental section. Bound protein was eluted with a linear gradient of 0–0.5 M-NaCl; 5.0 ml fractions were collected and assayed for aminopeptidase P activity and protein ( $A_{280}$ ). Fractions 10–16 were pooled and dialysed against 10 mM-Tris/HCl, pH 7.6. (b) Anion-exchange chromatography on MonoQ f.p.l.c. The pooled and dialysed sample from (a) was applied to an HR5/5 MonoQ column equilibrated in 10 mM-Tris/HCl, pH 7.6, as detailed in the Experimental section. Bound protein was eluted with a non-linear gradient of 0–0.5 M-NaCl; 1.0 ml fractions were collected and assayed for aminopeptidase P activity and protein ( $A_{280}$ ). Fractions 29–35 were pooled and dialysed against 20 mM-sodium phosphate, pH 7.5. (c) Hydrophobic-interaction chromatography on alkyl-Superose f.p.l.c. The pooled and dialysed sample from (b) was applied to an HR5/5 alkyl-Superose column as detailed in the Experimental section; 1.0 ml fractions were collected and assayed for aminopeptidase P activity and protein ( $A_{280}$ ). Fractions 24–26 were pooled and dialysed against 10 mM-Tris/HCl/0.3 M-NaCl, pH 7.5, before being applied to a mixed affinity column of cistatin- and lisinopril-Sepharose as detailed in the Experimental section. (d) Anion-exchange chromatography on MonoQ f.p.l.c. The run-through fraction from the cistatin- and lisinopril-Sepharose column was dialysed against 10 mM-Tris/HCl, pH 8.0, and then applied to an HR5/5 MonoQ column equilibrated in the same buffer as detailed in the Experimental section. Bound protein was eluted with a non-linear gradient of 0–0.5 M-NaCl in the same buffer; 1.0 ml fractions were collected and assayed for aminopeptidase P activity and protein ( $A_{280}$ ). Fractions 32 and 33 were pooled and used as purified aminopeptidase P. Aminopeptidase P activity was measured by the fluorimetric assay. ○, Aminopeptidase P activity; ●, protein; H, pooled fractions.

## RESULTS

### Purification of aminopeptidase P from pig kidney cortex

Aminopeptidase P was released from pig kidney cortex membranes by a purified sample of *B. thuringiensis* PI-PLC. The solubilized fraction was applied to a cilastatin-Sepharose affinity column to remove the bulk of the renal dipeptidase activity (Littlewood *et al.*, 1989). Aminopeptidase P was then purified to virtual homogeneity by a combination of anion-exchange and hydrophobic-interaction chromatographies (Fig. 1). A shoulder on the leading edge of the activity peak at the first MonoQ stage (Fig. 1*b*) constituted less than 5% of total activity and was not characterized further. Before the final chromatography on the MonoQ column, the sample was passed through a mixed column of cilastatin-Sepharose and lisinopril-2.8 nm-Sepharose, to remove any residual renal dipeptidase and angiotensin-converting enzyme respectively. The results for the purification from 400 g of pig kidney cortex are shown in Table 1. Similar results for the purification of aminopeptidase P from pig kidney were obtained when a crude sample of PI-PLC from *B. cereus* was used to release the peptidase (results not shown). After the final anion-exchange-chromatography step on the MonoQ column, the enzyme was apparently homogeneous as assessed by SDS/PAGE (Fig. 2).

This purified preparation of aminopeptidase P (specific activity 3.5  $\mu\text{mol}$  of Pro-Hyp produced/min per mg of protein) was assayed for the presence of other peptidase activities. When 100 ng of aminopeptidase P was incubated with the appropriate substrate and buffer for 2 h at 37 °C, no detectable aminopeptidase N, aminopeptidase A, aminopeptidase W, dipeptidyl peptidase IV, angiotensin-converting enzyme or endopeptidase-24.11 activities were observed. Under identical conditions, some renal dipeptidase activity (50 nmol of D-Phe produced/min per mg of protein) was detected.

### Structural properties of aminopeptidase P

On SDS/PAGE under reducing conditions, purified aminopeptidase P migrated with an apparent  $M_r$  of 91 000 (Fig. 2, lane 9). Under non-reducing conditions the enzyme migrated with an apparent  $M_r$  of 95 500 (Fig. 2, lane 10). When aminopeptidase P was subjected to gel-filtration chromatography on a Superose 12 column, the enzyme displayed an apparent  $M_r$  of 280 000 (mean of duplicate runs). The purified pig kidney aminopeptidase P, when analysed by phase separation in Triton X-114, partitioned predominantly ( $96.9 \pm 1.3\%$ ,  $n = 3$ )

into the detergent-poor phase, indicative of a hydrophilic nature.

Samples of pig kidney aminopeptidase P were enzymically deglycosylated with *N*-glycosidase F and the products analysed by SDS/PAGE (Fig. 2, lane 11). Two polypeptide bands were

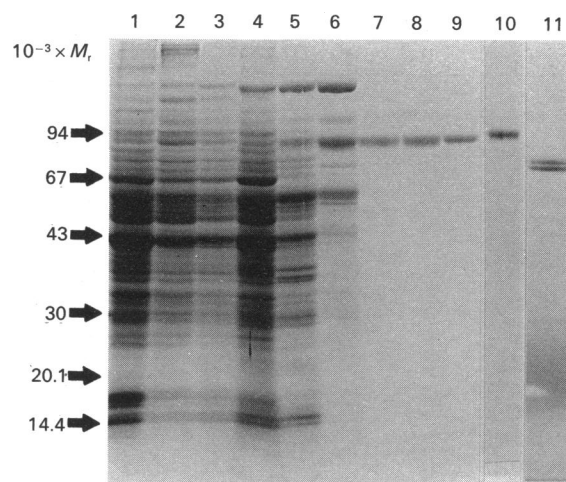


Fig. 2. Purification and enzymic deglycosylation of pig kidney aminopeptidase P

Samples were prepared and analysed by SDS/PAGE as described in the Experimental section. Lane 1, pig kidney-cortex homogenate (0.1 mg of protein); lane 2, microsomal membrane fraction before solubilization with *B. thuringiensis* PI-PLC (0.1 mg of protein); lane 3, supernatant fraction after solubilization of microsomal membranes with *B. thuringiensis* PI-PLC and centrifugation at 31 000  $g$  for 1.5 h (0.1 mg of protein); lane 4, sample from lane 3 after chromatography on cilastatin-Sepharose (0.1 mg of protein); lane 5, sample from lane 4 after chromatography on DEAE-cellulose (50  $\mu\text{g}$  of protein); lane 6, sample from lane 5 after chromatography on MonoQ by f.p.l.c. (26  $\mu\text{g}$  of protein); lane 7, sample from lane 6 after chromatography on alkyl-Superose by f.p.l.c. (10  $\mu\text{g}$  of protein); lane 8, sample from lane 7 after chromatography on cilastatin-Sepharose and lisinopril-2.8 nm-Sepharose (10  $\mu\text{g}$  of protein); lane 9, sample from lane 8 after chromatography on MonoQ by f.p.l.c., i.e. purified aminopeptidase P (5  $\mu\text{g}$  of protein); lane 10, purified aminopeptidase P in non-reducing sample buffer (5  $\mu\text{g}$  of protein); lane 11, purified aminopeptidase P (10  $\mu\text{g}$  of protein) incubated with 0.5 units of *N*-glycosidase F for 24 h at 37 °C. Lanes 1–10 were stained with Coomassie Brilliant Blue; lane 11 was silver-stained.

Table 1. Purification of aminopeptidase P from pig kidney cortex

Aminopeptidase P was purified from 400 g of pig kidney cortex as described in the Experimental section. Activity was assayed with Gly-Pro-Hyp as substrate.

	Protein (mg)	Total activity ( $\mu\text{mol}$ of Pro-Hyp/min)	Specific activity (nmol of Pro-Hyp/min per mg)	Recovery (%)	Enrichment (fold)
Homogenate	64400	464.4	7.2	100	1
Microsomal membranes	9240	207.3	22.4	45	3
PI-PLC-solubilized supernatant	3180	145.0	45.6	31	6
After cilastatin-Sepharose	1540	128.4	83.4	28	12
After DEAE-cellulose	110	43.2	392.7	9	55
After MonoQ	20	17.3	866.7	4	120
After alkyl-Superose	5.4	14.4	2673.3	3	371
After cilastatin- and lisinopril-Sepharose	4.2	12.9	3066.7	3	426
After MonoQ	1.2	4.2	3534.0	1	491

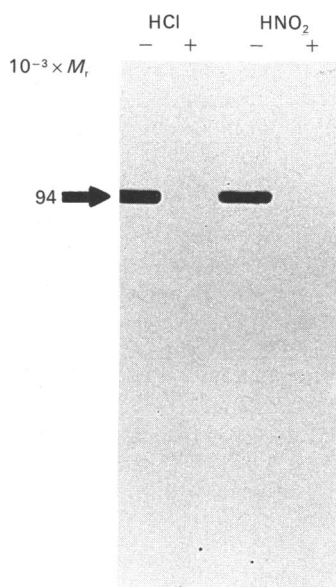


Fig. 3. Western blot of aminopeptidase P with anti-CRD antiserum

Samples of phospholipase-solubilized purified pig kidney aminopeptidase P ( $5 \mu\text{g}$  of protein per track) were prepared and analysed as described in the Experimental section. After electrophoretic transfer to PVDF membranes, the tracks were blotted with antiserum to the CRD of the variant surface glycoprotein 221. Bound antiserum was detected with a  $^{125}\text{I}$ -labelled second antibody, followed by autoradiography.

produced, with apparent  $M_r$  71 500 and 68 000. The intensity and apparent  $M_r$  of the two polypeptide bands were the same when aminopeptidase P was incubated with three times the amount of *N*-glycosidase F (1.5 units) or with *N*-glycanase (0.4 unit) for 24 h at  $37^\circ\text{C}$  (results not shown).

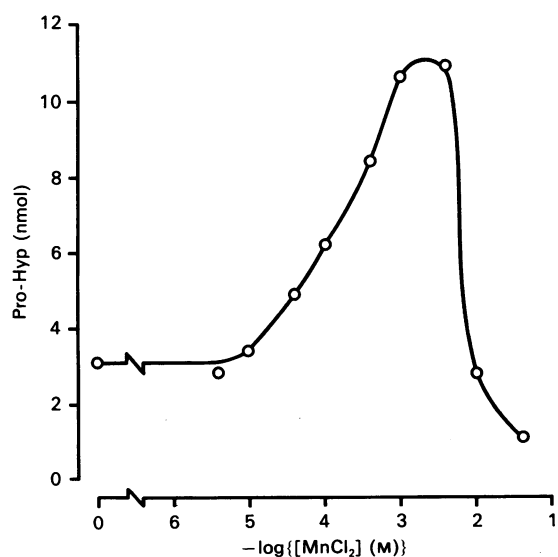


Fig. 4. Effect of  $\text{Mn}^{2+}$  concentration on the activity of aminopeptidase P

Purified aminopeptidase P (100 ng) was incubated with Gly-Pro-Hyp for 1.5 h at  $37^\circ\text{C}$  in the presence of various concentrations of  $\text{MnCl}_2$ . The  $\text{MnCl}_2$  was preincubated with enzyme for 15 min at  $4^\circ\text{C}$ . The results are the mean of duplicate determinations for each concentration of  $\text{MnCl}_2$ , which did not differ by more than 10%.

Table 2. Effect of bivalent cations on the activity of aminopeptidase P

Purified aminopeptidase P (100 ng) was incubated with Gly-Pro-Hyp for 1.5 h at  $37^\circ\text{C}$  in the presence of various bivalent cations (chloride salts). The cations were preincubated with enzyme for 15 min at  $4^\circ\text{C}$ . The results are the mean of duplicate determinations for each concentration of cation, which did not differ by more than 8%.

Bivalent cation	Concn. (mM)	Amount of Pro-Hyp (nmol)	Relative activity (%)
None	—	4.8	100
$\text{Mn}^{2+}$	4	21.7	452
	0.4	12.6	263
	0.04	7.7	160
$\text{Co}^{2+}$	4	17.7	369
	0.4	12.1	252
	0.04	9.4	196
$\text{Ca}^{2+}$	4	11.3	235
	0.4	6.7	140
	0.04	4.5	94
$\text{Mg}^{2+}$	4	8.1	169
	0.4	5.7	119
	0.04	4.1	85
$\text{Ni}^{2+}$	4	0	0
	0.4	0.6	13
	0.04	2.0	42
$\text{Cu}^{2+}$	4	0	0
	0.4	0.2	24
	0.04	1.3	27
$\text{Zn}^{2+}$	4	0	0
	0.4	0.6	13
	0.04	0.9	19

#### Characterization of the CRD on aminopeptidase P

The presence of the CRD on pig kidney aminopeptidase P was examined by using an anti-CRD antiserum in Western-blot analysis (Fig. 3). Purified aminopeptidase P was recognized by the anti-CRD antiserum. This recognition was abolished by prior treatment of the peptidase either with 1 M-HCl or with  $\text{HNO}_2$ .

#### Effect of bivalent metal ions on the activity of aminopeptidase P

The effect of increasing concentration of  $\text{Mn}^{2+}$  ions on the activity of purified aminopeptidase P was examined (Fig. 4). Optimal activity was observed at 4 mM- $\text{MnCl}_2$ , with inhibition of enzyme activity at higher  $\text{Mn}^{2+}$  concentrations. The effect of other bivalent cations on the activity of purified aminopeptidase P was also examined (Table 2).  $\text{CoCl}_2$  stimulated the activity of aminopeptidase P to a similar extent as  $\text{MnCl}_2$ .  $\text{CaCl}_2$  and  $\text{MgCl}_2$  stimulated the enzyme activity to a lesser extent, whereas  $\text{NiCl}_2$ ,  $\text{CuCl}_2$  and  $\text{ZnCl}_2$  were all inhibitory over the range (0.04–4.0 mM) examined.

#### Inhibitor sensitivity of aminopeptidase P

The inhibitor sensitivity of purified pig kidney aminopeptidase P with a variety of enzyme inhibitors was examined (Table 3). Dithiothreitol, 2-mercaptoethanol, *p*-hydroxymercuribenzoate and *p*-hydroxymercuribenzenesulphonate caused substantial (> 68%) inhibition of aminopeptidase P. The chelating agents EDTA, 1,10-phenanthroline, and to a lesser extent EGTA, also inhibited the peptidase. A variety of other general proteinase inhibitors were without effect on the activity of aminopeptidase

**Table 3. Sensitivity to inhibitors of aminopeptidase P**

Purified aminopeptidase P (100 ng) was incubated with Gly-Pro-Hyp and 4 mM-MnCl<sub>2</sub> for 1.5 h at 37 °C in the absence and in the presence of inhibitors. All inhibitors were preincubated with enzyme for 15 min at 4 °C. \*These incubations were carried out in the absence of MnCl<sub>2</sub> for 3 h at 37 °C, and the values are relative to a control with no MnCl<sub>2</sub> included. In the absence of any inhibitors, approx. 10% substrate breakdown was observed. The results are means of duplicate determinations which did not differ by more than 10%. Abbreviation: E-64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane.

Inhibitor	Relative activity (%)
None	100
5 mM-Dithiothreitol	29.9
5 mM-2-Mercaptoethanol	31.3
1 mM- <i>p</i> -Hydroxymercuribenzoate	23.6
1 mM- <i>p</i> -Hydroxymercuribenzenesulphonate	18.5
0.1 mM-E-64	96.6
1 mM <i>N</i> -Ethylmaleimide	93.2
1 mM-Iodoacetamide	100.4
1 mM-Phenylmethanesulphonyl fluoride	107.2
0.1 mM-Di-isopropyl phosphorofluoridate	101.3
1 mM-Benzamide	104.1
Leupeptin (10 µg/ml)	92.1
1 mM-EDTA*	28.7
1 mM-EGTA*	72.7
1 mM-1,10-Phenanthroline*	35.7
10 µM-Actinonin	100.4
10 µM-Amastatin	102.3
0.1 mM-Bestatin	79.4
0.1 mM-Puromycin	109.0
0.1 mM-Cilastatin	100.4

P. A range of selective aminopeptidase inhibitors had no significant inhibitory effect on pig kidney aminopeptidase P activity, except for partial inhibition by bestatin. The renal dipeptidase inhibitor cilastatin had no effect on the activity of aminopeptidase P.

## DISCUSSION

We have previously shown that aminopeptidase P is one of several proteins that can be selectively released from the renal microvillar membrane by bacterial PI-PLC (Hooper & Turner, 1988b). In the present study we have purified aminopeptidase P to apparent homogeneity after solubilization by bacterial PI-PLC by using a combination of anion-exchange and hydrophobic-interaction chromatography. Selective affinity-chromatography procedures were also employed to remove contaminating peptidase activities. At each step of the purification, fractions were pooled to maximize specific activity at the expense of yield.

The solubilization by bacterial PI-PLC provides a useful initial purification step, as most other kidney microvillar peptidases, in particular aminopeptidases N, A and W, are not released by this treatment (Hooper & Turner, 1988a,b; N. M. Hooper & A. J. Turner, unpublished work). The only other G-PI-anchored peptidase, renal dipeptidase, was selectively removed by affinity chromatography on cilastatin-Sepharose (Littlewood *et al.*, 1989). Contaminating angiotensin-converting enzyme, released from the membrane by an endogenous hydrolase (Hooper *et al.*, 1987b), was selectively removed by chromatography on lisinopril-2.8 nm-Sepharose (Hooper & Turner, 1987).

On SDS/PAGE purified pig kidney aminopeptidase P exhibited an  $M_r$  of 91 000 under reducing conditions and an  $M_r$

of 95 500 under non-reducing conditions and, unlike renal dipeptidase, is therefore not disulphide-linked. On gel filtration, aminopeptidase P exhibited an  $M_r$  of approx. 280 000. The PI-PLC-solubilized form of aminopeptidase P was hydrophilic, as analysed by phase separation in Triton X-114, consistent with removal of the hydrophobic lipid anchor. Deglycosylation of the purified pig kidney aminopeptidase P with *N*-glycosidase F resulted in two polypeptide bands, of  $M_r$  71 500 and 68 000 on SDS/PAGE. We have previously observed that enzymic deglycosylation of the G-PI-anchored renal dipeptidase of both pig and human also generated two polypeptide products which differed in  $M_r$  by 3500 (Littlewood *et al.*, 1989; Hooper *et al.*, 1990). For pig renal dipeptidase, the presence of two polypeptide bands, both of which possessed the CRD epitope and hence the G-PI anchor, suggested that there were two distinct populations of *N*-linked sugars which differed in their susceptibility to enzymic cleavage (Littlewood *et al.*, 1989); a similar phenomenon occurs with aminopeptidase P. The result with *N*-glycosidase F indicates that up to 25% by weight of the  $M_r$ -91 000 polypeptide of aminopeptidase P consists of *N*-linked sugars.

The purified phospholipase-solubilized form of aminopeptidase P was recognized by an anti-CRD antiserum. The epitope recognized by this antiserum is known to be cryptic in the membrane-bound form of G-PI-anchored proteins and only exposed after treatment with bacterial PI-PLC (Cardoso de Almeida & Turner, 1983; Low, 1987). This recognition of aminopeptidase P was abolished by prior treatment of the enzyme with mild acid, which decyclizes the inositol 1,2-cyclic phosphate ring epitope formed on PI-PLC cleavage (Zamze *et al.*, 1988), or with HNO<sub>2</sub>, which deaminates the glucosamine residue in the G-PI anchor, releasing the inositol 1,2-cyclic phosphate group (Ferguson *et al.*, 1985).

When aminopeptidase P was first identified, and partially purified from pig kidney, it was observed that Mn<sup>2+</sup> ions were required for optimal activity (Dehm & Nordwig, 1970b). The effect of MnCl<sub>2</sub> on the activity of purified aminopeptidase P was therefore examined. Mn<sup>2+</sup> ions enhanced the activity of aminopeptidase P, with a maximum at 4 mM-MnCl<sub>2</sub>, in agreement with earlier observations (Dehm & Nordwig, 1970b; Holtzman *et al.*, 1987; Orawski *et al.*, 1987; Lasch *et al.*, 1988). The effect of other bivalent cations on the activity of aminopeptidase P was also examined. Of the other transition-metal ions, only Co<sup>2+</sup> stimulated the enzyme activity to a similar extent to Mn<sup>2+</sup> ions, whereas Cu<sup>2+</sup>, Ni<sup>2+</sup> and Zn<sup>2+</sup> were all inhibitory.

Purified aminopeptidase P was inhibited by metallo-enzyme inhibitors, consistent with previous observations that it is a metallo-peptidase (Holtzman *et al.*, 1987; Orawski *et al.*, 1987; Lasch *et al.*, 1988). The activity of aminopeptidase P was also substantially inhibited by dithiothreitol and 2-mercaptoethanol, as well as by *p*-hydroxymercuribenzoate and *p*-hydroxymercuri-phenylsulphonate, again in agreement with studies on partially purified preparations (Holtzman *et al.*, 1987; Orawski *et al.*, 1987; Lasch *et al.*, 1988). None of the other general proteinase inhibitors examined had any significant inhibitory effect. Of the selective aminopeptidase inhibitors, only bestatin partially inhibited the purified aminopeptidase P. Thus the inhibitory profile of aminopeptidase P is distinct from the other microvillar aminopeptidases (Kenny *et al.*, 1987; Kenny & Hooper, 1990).

In summary, we have purified pig kidney microvillar aminopeptidase P to near homogeneity after its release from the membrane by bacterial PI-PLC. The purified peptidase is a glycoprotein of  $M_r$  91 000 and possesses the CRD epitope characteristic of G-PI-anchored proteins. The availability of purified enzyme will allow more detailed studies on its structure and specificity to be carried out.

We thank the Wellcome Trust, the Medical Research Council and the Royal Society for financial support. N. M. H. is a Mr. and Mrs. John Jaffé Donation Research Fellow of the Royal Society.

## REFERENCES

- Bordier, C. (1981) *J. Biol. Chem.* **256**, 1604–1607
- Campbell, B. J., Forrester, L. J., Zahler, W. L. & Burks, M. (1984) *J. Biol. Chem.* **259**, 14586–14590
- Cardoso de Almeida, M. L. & Turner, M. J. (1983) *Nature (London)* **302**, 349–352
- Dehm, P. & Nordwig, A. (1970a) *Eur. J. Biochem.* **17**, 364–371
- Dehm, P. & Nordwig, A. (1970b) *FEBS Lett.* **9**, 225–228
- Ferguson, M. A. J. & Williams, A. F. (1988) *Annu. Rev. Biochem.* **57**, 285–320
- Ferguson, M. A. J., Low, M. G. & Cross, G. A. M. (1985) *J. Biol. Chem.* **260**, 14547–14555
- Holtzman, E. J., Pillay, G., Rosenthal, T. & Yaron, A. (1987) *Anal. Biochem.* **162**, 476–484
- Hooper, N. M. & Turner, A. J. (1987) *Biochem. J.* **241**, 625–633
- Hooper, N. M. & Turner, A. J. (1988a) *Biochem. J.* **250**, 865–869
- Hooper, N. M. & Turner, A. J. (1988b) *FEBS Lett.* **229**, 340–344
- Hooper, N. M. & Turner, A. J. (1989a) *Biochem. J.* **261**, 811–818
- Hooper, N. M. & Turner, A. J. (1989b) *Biochem. Soc. Trans.* **17**, 885–886
- Hooper, N. M., Low, M. G. & Turner, A. J. (1987a) *Biochem. J.* **244**, 465–469
- Hooper, N. M., Keen, J. N., Pappin, D. J. C. & Turner, A. J. (1987b) *Biochem. J.* **247**, 85–93
- Hooper, N. M., Keen, J. N. & Turner, A. J. (1990) *Biochem. J.* **265**, 429–433
- Kenny, A. J. & Hooper, N. M. (1990) in *Degradation of Bioactive Substances: Physiology and Pathophysiology* (Henricksen, J. H., ed.), CRC Press, Boca Raton, FL, in the press
- Kenny, A. J., Booth, A. G. & Macnair, R. D. C. (1977) *Acta Biol. Med. Germ.* **36**, 1575–1585
- Kenny, A. J., Stephenson, S. L. & Turner, A. J. (1987) *Mammalian Ectoenzymes: Res. Monogr. Cell Tissue Physiol.* **14**, 169–210
- Lasch, J., Koelsch, R., Ladhoff, A.-M. & Hartrodt, B. (1986) *Biomed. Biochim. Acta* **45**, 833–843
- Lasch, J., Koelsch, R., Steinmetzer, T., Neumann, U. & Demuth, H.-U. (1988) *FEBS Lett.* **277**, 171–174
- Littlewood, G. M., Hooper, N. M. & Turner, A. J. (1989) *Biochem. J.* **257**, 361–367
- Low, M. G. (1987) *Biochem. J.* **244**, 1–13
- Low, M. G. (1989) *FASEB J.* **3**, 1600–1608
- Orawski, A. T., Susz, J. P. & Simmons, W. H. (1987) *Mol. Cell. Biochem.* **75**, 123–132
- Relton, J. M., Gee, N. S., Matsas, R., Turner, A. J. & Kenny, A. J. (1983) *Biochem. J.* **215**, 519–523
- Turner, A. J. (ed.) (1990) *Molecular and Cell Biology of Membrane Proteins: The Glycolipid Anchors of Cell-Surface Proteins*, Ellis Horwood, Chichester, in the press
- Yoshimoto, T., Murayama, N. & Tsuru, D. (1988) *Agric. Biol. Chem.* **52**, 1957–1963
- Zamze, S. E., Ferguson, M. A. J., Collins, R., Dwek, R. A. & Rademacher, T. W. (1988) *Eur. J. Biochem.* **176**, 527–534

---

Received 25 September 1989/3 November 1989; accepted 1 December 1989