Ectoenzymes of the kidney microvillar membrane

Isolation and characterization of the amphipathic form of renal dipeptidase and hydrolysis of its glycosyl-phosphatidylinositol anchor by an activity in plasma

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Renal dipeptidase (EC 3.4.13.11) has been solubilized from pig kidney microvillar membranes with *n*-octyl- β -D-glucopyranoside and then purified by affinity chromatography on cilastatin–Sephacryl. The enzyme exists as a disulphide-linked dimer of two identical subunits of M_r 45000 each. The purified dipeptidase partitioned into the detergent-rich phase upon phase separation in Triton X-114 and reconstituted into liposomes consistent with the presence of the glycosyl-phosphatidylinositol membrane anchor. The *N*-terminal amino acid sequence of the amphipathic, detergent-solubilized, form of renal dipeptidase was identical with that of the hydrophilic, phospholipase-solubilized, form, locating the membrane anchor at the *C*-terminus of the protein. The glycosyl-phosphatidylinositol anchor of both purified and microvillar membrane renal dipeptidase was a substrate for an activity in pig plasma which displayed properties similar to those of a previously described phospholipase D. The cross-reacting determinant of the glycosyl-phosphatidylinositol anchor was generated by incubation of purified renal dipeptidase with bacterial phosphatidylinositol-specific phospholipase C, whereas the anchor-degrading activity in plasma failed to generate this determinant.

INTRODUCTION

The presence of a covalently attached glycosyl-phosphatidylinositol (G-PI) *C*-terminal anchor in a number of cell-surface proteins is now well established (reviewed in Low, 1987; Ferguson & Williams, 1988; Low & Saltiel, 1988). Renal dipeptidase (dehydropeptidase-I; microsomal dipeptidase; EC 3.4.13.11) is one of several ectoenzymes anchored via a G-PI moiety. The enzyme is located in the microvillar membrane of the kidney (Welch & Campbell, 1980), can be selectively released by bacterial phosphatidylinositol-specific phospholipase C (PI-PLC) (Hooper *et al.*, 1987a) and gives a pattern of solubilization by a range of detergents that is characteristic of a G-PI-anchored protein (Hooper & Turner, 1988). Furthermore the PI-PLC-released form of renal dipeptidase contains inositol and possesses the cross-reacting determinant (CRD) which is common to a number of G-PI-anchored proteins (Littlewood *et al.*, 1989).

The amphipathic form of renal dipeptidase, possessing the hydrophobic membrane anchor, has proved to be more resistant to detergent solubilization and purification than the hydrophilic form (Kim & Campbell, 1983; Hooper & Turner, 1988). Although the enzyme has been partially solubilized with *n*-butanol (Campbell *et al.*, 1984) and with Triton X-100 (Hitchcock *et al.*, 1987), the amphipathic nature of the isolated enzyme was not established.

We have previously shown that detergents with high critical micelle values, such as octyl glucoside and

CHAPS, are effective at solubilizing renal dipeptidase (Hooper & Turner, 1988). In the present study we have exploited this observation and used octyl glucoside to solubilize renal dipeptidase from the kidney microvillar membrane. The solubilized enzyme was then purified by affinity chromatography on cilastatin–Sephacryl. The isolated enzyme was amphipathic, as judged by phase separation in Triton X-114 and reconstitution into liposomes. The G-PI anchor of purified renal dipeptidase was hydrolysed by bacterial PI-PLC, as well as by an activity in plasma which has characteristics in common with a plasma phospholipase D (Davitz *et al.*, 1987; Cardoso de Almeida *et al.*, 1988; Low & Prasad, 1988).

EXPERIMENTAL

Materials

Cilastatin (MK 0791) was a gift from Dr. H. Kropp, Merck, Sharp and Dohme Research Laboratories (Rahway, NJ, U.S.A.). PI-PLC from *Bacillus thuringiensis* and from *Staphylococcus aureus* were gifts 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. Triton X-114 was pre-condensed before use (Bordier, 1981). Cilastatin was attached to

Abbreviations used: CRD, cross-reacting determinant; octyl glucoside, *n*-octyl β -D-glucopyranoside; G-PI, glycosyl-phosphatidylinositol; PI-PLC, phosphatidylinositol-specific phospholipase C; I_{50} , concentration of inhibitor causing 50% inhibition; E-64, *trans*-epoxysuccinyl-leucylamido-(4-guanidino)butane.

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CNBr-activated Sepharose 4B as described in Campbell *et al.* (1984). Pig kidneys and all other materials were obtained from sources previously noted.

Purification of renal dipeptidase

All procedures were carried out at 4 °C. Pig kidney cortex (typically 100 g) was homogenized in 10 vol. of 10 mM-mannitol/2 mM-Tris/HCl, pH 7.1, and microvillar membranes were prepared by the method of Booth & Kenny (1974). The resultant microvillar membrane pellet was resuspended in 10 mM-Hepes, pH 7.4, and centrifuged at 31000 *g* for 90 min. The pellet was resuspended in 50 ml of 10 mM-Hepes, pH 7.4, octyl glucoside was added to a final concentration of 60 mM and the suspension was stirred for 2 h. After centrifugation at 31000 *g* for 90 min, the supernatant was diluted with an equal volume of 0.1 M-Tris/HCl/1.0 M-NaCl, pH 7.6, and applied to the cilastatin-Sepharose affinity column (20 ml bed volume) with a pre-column of unmodified Sepharose CL-4B (20 ml bed volume), equilibrated in 50 mM-Tris/HCl/0.5 M-NaCl/5 mM-CHAPS, pH 7.6, at a flow rate of 6–8 ml/h. The column was washed extensively with 50 mM-Tris/HCl/0.5 M-NaCl/5 mM-CHAPS, pH 7.6, before elution of the bound enzyme with 10 mg of cilastatin in 10 ml of the same buffer. Fractions (2 ml) were collected and those which contained material which absorbed at 280 nm were pooled and dialysed extensively against 10 mM-Tris/HCl/0.1 mM-CHAPS, pH 7.6, to remove the cilastatin. The enzyme was concentrated on a small column of DEAE-cellulose (bed volume 0.2 ml) equilibrated with 10 mM-Tris/HCl/0.1 mM-CHAPS, pH 7.6, and eluted with 10 mM-Tris/HCl/0.7 M-NaCl/5 mM-CHAPS, pH 7.6. The phospholipase-solubilized form of renal dipeptidase was purified as described previously (Littlewood *et al.*, 1989). Separate affinity columns were used for purification of the octyl glucoside- and phospholipase-solubilized forms of the enzyme. The affinity columns were washed after use as described previously (Littlewood *et al.*, 1989).

Enzyme assays

Renal dipeptidase was assayed by an h.p.l.c. method using glycyl-D-phenylalanine as substrate as described in Littlewood *et al.* (1989). Alkaline phosphatase and protein were assayed as described previously (Hooper *et al.*, 1987a).

SDS/polyacrylamide-gel electrophoresis and immunoelectrophoretic blot analysis

SDS/polyacrylamide-gel electrophoresis was per-

formed by using the system of Laemmli (1970) with a 7–17% (w/v) polyacrylamide gradient as described previously (Relton *et al.*, 1983). Immunoelectrophoretic ('Western') blot analysis was carried out as described by Towbin *et al.* (1979) and as detailed by Hooper & Turner (1987).

Triton X-114 phase separation

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 a sucrose cushion by centrifugation at 3000 *g* and assayed for enzyme activity. Activity recovered in the detergent-rich phase is expressed as percentage of the total activity.

Reconstitution of proteins into liposomes

Affinity-purified renal dipeptidase was reconstituted into liposomes by the procedure of Scotto & Zakim (1985) as detailed in Hooper *et al.* (1987b).

Solid-phase sequencing of renal dipeptidase

Samples (25–50 µg) of affinity-purified renal dipeptidase were sequenced by automated solid-phase Edman degradation using the microsequencing facility of the Sequencing Unit, Department of Biochemistry, University of Leeds, as described previously (Hooper *et al.*, 1987b).

RESULTS

Purification of renal dipeptidase after solubilization with octyl glucoside

Renal dipeptidase was solubilized from pig kidney microvillar membranes with octyl glucoside and then purified by affinity chromatography on cilastatin-Sepharose (Table 1). The purified enzyme was apparently homogeneous as assessed by SDS/polyacrylamide-gel electrophoresis (Fig. 1) and migrated with an apparent M_r of 45 000, whereas the phospholipase-solubilized form of the enzyme migrated with an apparent M_r of 47 000. Under non-reducing conditions (Fig. 1, lanes 6 and 7) both the octyl glucoside- and the phospholipase-solubilized forms migrated with an apparent M_r of approx. 80 000.

A polyclonal antiserum raised to the phospholipase-solubilized form of renal dipeptidase (Littlewood *et al.*, 1989) recognized the octyl glucoside-solubilized form of the enzyme in a Western blot (Fig. 2). The antiserum also recognized a single polypeptide in pig kidney microvillar

Table 1. Purification of dipeptidase from pig kidney cortex

Dipeptidase was purified from 100 g of pig kidney cortex as described in the Experimental section.

Step	Protein (mg)	Total activity (µmol of D-Phe/min)	Specific activity (nmol of D-Phe/min per mg)	Recovery (%)	Enrichment (fold)
Homogenate	14200	336.0	23.7	100	1
Microvillar membrane fraction	860	96.0	111.6	29	5
Octyl glucoside-solubilized supernatant	660	58.2	88.2	17	4
Affinity chromatography	0.8	23.5	28600	7	1207

Samples were prepared and analysed as described in the Experimental section. Lane 1, pig kidney cortex homogenate (100 μg of protein); lane 2, pig kidney microvillar membrane fraction before solubilization with octyl glucoside (100 μg of protein); lane 3, supernatant fraction after solubilization of pig kidney microvillar membranes with octyl glucoside and centrifugation at 31000 g for 1.5 h (100 μg of protein); lanes 4 and 7, octyl glucoside-solubilized, affinity-purified, renal dipeptidase (4 μg of protein); lanes 5 and 6, phospholipase C-solubilized, affinity-purified, renal dipeptidase (4 μg of protein). Lanes 1–5 were electrophoresed in reducing sample buffer, lanes 6 and 7 in non-reducing sample buffer. The gel was stained with Coomassie Brilliant Blue.

Amphipathic nature of the octyl glucoside-solubilized form of renal dipeptidase

***N*-Terminal sequence analysis of renal dipeptidase**

1 5 10
Asp-Gln-Phe-Arg-Asp-Leu-Ala-Val-Arg-Ile-

Samples were prepared and analysed as described in the Experimental section. After electrophoretic transfer to nitrocellulose sheets, the tracks were blotted with anti-serum to the phospholipase C-solubilized form of pig kidney dipeptidase. Lane 1, phospholipase C-solubilized, affinity-purified, renal dipeptidase (0.1 μg of protein); lane 2, octyl glucoside-solubilized, affinity-purified, renal dipeptidase (0.1 μg of protein); lane 3, supernatant fraction after solubilization of pig kidney microvillar membranes with octyl glucoside and centrifugation at 31000 g for 1.5 h (20 μg of protein); lane 4, pig kidney microvillar membrane fraction before solubilization with octyl glucoside (20 μg of protein).

The action of PI-PLC from *B. thuringiensis*, *S. aureus* and *B. cereus* on the hydrophobic membrane anchor of purified renal dipeptidase was examined by phase separation in Triton X-114 (Fig. 4). All three bacterial PI-PLCs converted the amphipathic form of renal dipeptidase into a hydrophilic form consistent with the loss of the membrane anchor. At high concentrations of *B. thuringiensis* PI-PLC nearly all (96 %) of the membrane anchor of renal dipeptidase was hydrolysed. The *B. cereus* phospholipase activity was completely inhibited by 10 mM-ZnSO₄, but not by 2 mM-EDTA (results not shown), confirming that this activity is due to the contaminating PI-PLC in this preparation (Ikezawa & Taguchi, 1981; Fournier *et al.*, 1988).

Incubation of purified renal dipeptidase with pig plasma led to a rapid concentration-dependent hydrolysis of the membrane anchor as revealed by phase

Fig. 3. Reconstitution of pig kidney dipeptidase into liposomes

Samples of pig kidney dipeptidase ($1 \mu\text{g}$ of protein) were mixed with dimyristoyl phosphatidylcholine ($200 \mu\text{g}$) as described in Hooper *et al.* (1987b) and then centrifuged through 20% (w/v) sucrose/10 mM-Tris/HCl, pH 7.5, at $100\,000 g$ for 18 h. The tubes were fractionated from the bottom (fraction 1), and the fractions assayed for enzyme activity and radioactivity. The Figure is representative of nine such experiments for the octyl glucoside-solubilized form of renal dipeptidase (a), and five such experiments for the phospholipase-solubilized form (b).

separation in Triton X-114 (Fig. 5). Also the addition of plasma to microvillar membranes caused partial conversion of both renal dipeptidase and alkaline phosphatase from amphipathic to hydrophilic forms (Fig. 5). This anchor-degrading activity in plasma was inhibited by *p*-hydroxymercuribenzenesulphonic acid, EGTA, EDTA and 1,10-phenanthroline, but not by phenylmethanesulphonyl fluoride or a range of other proteinase inhibitors (Table 2). Preincubation of the plasma for 30 min at 56°C failed to destroy the anchor-degrading activity (Table 2). In the absence of 0.1% Nonidet P-40, the plasma anchor-degrading activity was much less active on purified renal dipeptidase, but was equally as active on the dipeptidase in microvillar membranes (Table 2). A study of the divalent metal ion requirement of the anchor-degrading activity in plasma (Table 3) revealed that Co^{2+} and Zn^{2+} stimulated the activity, whereas Ni^{2+} , and to some extent Cu^{2+} , inhibited the activity. After inhibition by EGTA, the anchor-degrading activity in plasma was re-activated by Ca^{2+} , Mn^{2+} , Co^{2+} and Zn^{2+} . The anchor-degrading activity in plasma displayed a pH optimum of approx. 5.6 (Fig. 6).

Immunoreactivity of renal dipeptidase towards anti-CRD serum

Samples of the amphipathic form of renal dipeptidase were incubated with bacterial PI-PLC or plasma and then immunoblotted with an anti-CRD serum (Fig. 7). The samples of renal dipeptidase incubated with *S. aureus* or *B. cereus* PI-PLC cross-reacted with the anti-CRD serum, whereas the untreated sample or that incubated with plasma failed to cross-react. Also, binding of the anti-CRD serum to the hydrophilic form of renal dipeptidase was abolished by decyclization of the inositol 1,2-cyclic phosphate by mild acid treatment (Fig. 7). Samples of renal dipeptidase treated identically with PI-

Fig. 4. Hydrolysis of the membrane anchor of renal dipeptidase by bacterial PI-PLC

Octyl glucoside-solubilized, affinity-purified, renal dipeptidase ($1 \mu\text{g}$ of protein) was incubated with 10 mM-Hepes, pH 7.4 (50 μl total volume), for 2 h at 37°C in the absence or presence of the indicated phospholipases. Incubations with *B. cereus* phospholipase C contained 0.1 M-NaCl. After the incubation, samples were subjected to phase separation in Triton X-114 as described in the Experimental section. The results are the mean of duplicate incubations with each concentration of phospholipase. Anchor degradation of 0% is equivalent to all of the dipeptidase activity being recovered in the detergent-rich phase after phase separation in Triton X-114.

Fig. 5. Hydrolysis of the membrane anchor of renal dipeptidase by plasma

Octyl glucoside-solubilized, affinity-purified, renal dipeptidase (1 μ g of protein; 26.4 nmol of D-Phe produced/min) or pig kidney microvillar membranes (0.2 mg of protein; 27.8 nmol of D-Phe produced/min) were incubated with 0.2 M-Tris/maleate/NaOH/0.1% Nonidet P-40, pH 7.0 (50 μ l total volume) for 2 h at 37 $^{\circ}$ C in the absence or presence of the indicated amounts of pig plasma. After the incubation, samples were subjected to phase separation in Triton X-114 as described in the Experimental section. The results are the mean of duplicate incubations with each concentration of plasma. Anchor degradation of 0% is equivalent to all of the dipeptidase activity being recovered in the detergent-rich phase after phase separation in Triton X-114. No release of renal dipeptidase from the microvillar membranes was observed in the absence of plasma. $\circ$ — $\circ$, Purified renal dipeptidase; $\bullet$ — $\bullet$, microvillar membrane dipeptidase; $\circ$ --- $\circ$, microvillar membrane alkaline phospholipase.

Table 2. Effect of inhibitors on the hydrolysis of the membrane anchor of renal dipeptidase by plasma

Octyl glucoside-solubilized, affinity-purified, renal dipeptidase (1 μ g of protein) and 2 μ l of pig plasma or pig kidney microvillar membranes (0.2 mg of protein) and 20 μ l of pig plasma were incubated with 0.2 M-Tris/maleate (pH 7.0) containing 0.1% Nonidet P-40 (50 μ l total volume) for 2 h at 37 $^{\circ}$ C in the absence or presence of the indicated inhibitors. The plasma and inhibitors were preincubated at 4 $^{\circ}$ C for 15 min. After the incubation, samples were subjected to phase separation in Triton X-114 as described in the Experimental section. Under these conditions the anchor degradation in the absence of inhibitors was 100% for the purified renal dipeptidase and 25% for the dipeptidase in microvillar membranes. The results are the mean of duplicate incubations which did not differ by more than 10%. Anchor degradation of 100% is equivalent to all the dipeptidase activity being recovered in the detergent-poor phase after phase separation in Triton X-114. Inhibitor cocktail A consisted of: bestatin (final concentration 0.1 mM), actinonin, amastatin, captopril, enalaprilat, kelatorphan, phosphoramidon and thiorphan (all at 10 μ M). Inhibitor cocktail B consisted of: benzamidine (final concentration 1 mM), antipain, aprotinin, chymostatin, leupeptin, pepstatin A, trypsin inhibitors type II_s and type II_o (all at 10 μ g/ml).

Inhibitor	Concentration (mM)	Degradation of dipeptidase anchor (%)	
		Purified enzyme	Microvillar membranes
None	—	100	25.0
<i>p</i> -Hydroxymercuri-benzenesulphonic acid	2.5	34.4	4.2
	0.25	79.3	—
	0.025	101.3	—
	0.0025	102.0	—
EGTA	2.5	8.5	1.8
	0.25	66.2	—
	0.025	102.0	—
EDTA	2.5	1.2	0.0
	0.25	31.0	—
	0.025	87.9	—
	0.0025	100.0	—
1,10-Phenanthroline	2.0	0.0	0.0
	0.2	17.9	—
	0.02	100.0	—
Phenylmethanesulphonyl fluoride	1.0	98.2	30.4
E-64	0.1	96.0	—
<i>p</i> -Aminobenzamidine	1.0	99.5	—
Inhibitor cocktail A	—	96.0	—
Inhibitor cocktail B	—	97.5	—
Preincubation at 56 $^{\circ}$ C, 30 min	—	100.8	—
Incubation in the absence of Nonidet P-40	—	9.2	24.1
Incubation in the presence of 0.5% Nonidet P-40	—	—	34.0

Table 3. Effect of divalent metal ions on the hydrolysis of the membrane anchor of renal dipeptidase by plasma

Incubations were performed as described in the legend to Table 2, except that 0.2 μ l of pig plasma was incubated with the dipeptidase for 1 h at 37 °C. The plasma and EGTA were preincubated for 15 min at 4 °C and then the metal ions were added at the start of the reaction. EGTA was used at a concentration of 2.5 mM. The results are the mean of duplicate incubations which did not differ by more than 10%.

Chelator/metal ion concentration	Anchor degradation (%)
None	41.3
MgCl ₂ (2.5 mM)	43.7
CaCl ₂ (2.5 mM)	45.2
MnCl ₂ (2.5 mM)	37.8
CoCl ₂ (2.5 mM)	81.4
NiCl ₂ (2.5 mM)	6.7
CuCl ₂ (2.5 mM)	20.8
ZnCl ₂ (2.5 mM)	66.5
EGTA	0.0
EGTA + MgCl ₂ (5 mM)	0.0
EGTA + CaCl ₂ (5 mM)	60.6
EGTA + MnCl ₂ (5 mM)	62.4
EGTA + CoCl ₂ (5 mM)	81.5
EGTA + NiCl ₂ (5 mM)	0.0
EGTA + CuCl ₂ (5 mM)	33.2
EGTA + ZnCl ₂ (5 mM)	85.0

Fig. 6. Effect of pH on the hydrolysis of the membrane anchor of renal dipeptidase by plasma

Octyl glucoside-solubilized, affinity-purified, renal dipeptidase (1 μ g of protein) was incubated with 0.1% Nonidet P-40 and 0.2 μ l of plasma for 1 h at 37 °C in the indicated buffer (50 μ l total volume). After the incubation, samples were subjected to phase separation in Triton X-114 as described in the Experimental section. The results are the mean of duplicate incubations at each pH. Anchor degradation of 100% is equivalent to all of the dipeptidase activity being recovered in the detergent-poor phase after phase separation in Triton X-114. Buffers used: sodium acetate/acetic acid, pH 5.0–5.6; Tris/maleate/NaOH, pH 6.0–8.0; Tris/NaOH, pH 8.0–8.9. (Below pH 5.0 renal dipeptidase was substantially inactivated, therefore determinations at lower pH were not possible.)

Fig. 7. 'Western' blot of pig kidney dipeptidase with anti-CRD serum

Samples were prepared and analysed as described in the Experimental section. After electrophoretic transfer to nitrocellulose sheets, the tracks were blotted with anti-serum to the CRD of the variant surface glycoprotein 221. Lanes 1–4, octyl glucoside-solubilized, affinity-purified, renal dipeptidase (6 μ g of protein) incubated in the appropriate buffer (see legends to Figs. 4 and 5) for 6 h at 37 °C: lane 1, untreated; lane 2, incubated with 0.2 unit of *S. aureus* PI-PLC/ml; lane 3, incubated with 10 units of *B. cereus* phospholipase C/ml; lane 4, incubated with 0.5 μ l of plasma. Lane 5, 0.5 μ l of plasma only. Lanes 6 and 7, phospholipase-solubilized, affinity-purified, renal dipeptidase (6 μ g of protein) incubated in the presence (lane 6) and absence (lane 7) of 1 M-HCl for 30 min at 23 °C followed by neutralization with 1 M-NaOH.

PLC, plasma or HCl all cross-reacted with the antiserum to renal dipeptidase (result not shown).

DISCUSSION

We have shown previously that octyl glucoside can solubilize efficiently G-PI-anchored proteins, with the resultant solubilized protein retaining the hydrophobic membrane anchor (Hooper & Turner, 1988). In the present study we have solubilized the G-PI-anchored renal dipeptidase from the pig kidney microvillar membrane with octyl glucoside and then purified the enzyme to homogeneity by affinity chromatography on cilastatin-Sepharose. Octyl glucoside solubilized approx. 60% of the total dipeptidase activity in the microvillar membrane fraction. The octyl glucoside-solubilized, affinity-purified form of renal dipeptidase was recognized in a Western blot by a polyclonal antiserum raised to the phospholipase-solubilized form of the enzyme and was potently inhibited by the specific inhibitor cilastatin (Kahan *et al.*, 1983), with an I_{50} value comparable to that for the phospholipase-solubilized form of the enzyme (Littlewood *et al.*, 1989).

Electrophoresis of renal dipeptidase under non-reducing conditions revealed that both the octyl glucoside-

and phospholipase-solubilized forms are disulphide-linked dimers, consistent with earlier reports for an apparent M_r of approx. 90000 on sedimentation equilibrium and gel filtration analysis of the pig kidney enzyme (Campbell, 1970; Armstrong *et al.*, 1974). Thus, renal dipeptidase is unusual amongst the pig microvillar peptidases in existing as a dimer which is covalently linked by one or more disulphide bonds, although rat endopeptidase-2 (Kenny & Ingram, 1987), mouse meprin (Beynon *et al.*, 1981) and human *N*-benzoyl-L-tyrosyl-*p*-aminobenzoic acid hydrolase (Sterchi *et al.*, 1988) are also reported to be disulphide-linked.

By the criteria of phase separation in Triton X-114 and reconstitution into liposomes, the octyl glucoside-solubilized form of renal dipeptidase was shown to be amphipathic whereas the phospholipase-solubilized form was hydrophilic. Thus the apparent difference in M_r between the amphipathic and hydrophilic forms of renal dipeptidase on SDS/polyacrylamide-gel electrophoresis is almost certainly due to the anomalous binding of SDS to the G-PI anchor as discussed previously (Littlewood *et al.*, 1989). The presence of the same *N*-terminal amino acid sequence in both the amphipathic and hydrophilic forms of renal dipeptidase confirms that the G-PI membrane anchor is located at the *C*-terminus, in common with other G-PI-anchored proteins (see Ferguson & Williams, 1988; Low & Saltiel, 1988). All three of the bacterial PI-PLCs used could release virtually 100% of the membrane anchor from purified renal dipeptidase, suggesting that the enzyme is exclusively anchored by a G-PI moiety in pig kidney microvillar membranes.

A phospholipase D activity has recently been described in plasma from several species (including human, rat, rabbit, dog, bovine, chicken and horse) which hydrolyses the G-PI anchor of alkaline phosphatase, acetylcholinesterase, decay-accelerating factor, 5'-nucleotidase and the variant surface glycoprotein (Davitz *et al.*, 1987; Cardoso de Almeida *et al.*, 1988; Low & Prasad, 1988). The G-PI membrane anchor of purified renal dipeptidase is rapidly hydrolysed by an activity in pig plasma. A study of the properties of this anchor-degrading activity in plasma revealed that it is sensitive to inhibition by chelating agents and *p*-hydroxymercuribenzenesulphonic acid, but is not inhibited by a range of other proteinase inhibitors. The anchor-degrading activity in plasma displayed a pH optimum of around 5.6 and was not inactivated by heating at 56 °C for 30 min. These properties of the G-PI-anchor-degrading activity in pig plasma are similar to those described for the plasma phospholipase D (Davitz *et al.*, 1987; Cardoso de Almeida *et al.*, 1988; Low & Prasad, 1988). Although the plasma phospholipase D has been reported to be Ca^{2+} -dependent (Davitz *et al.*, 1987), the activity described here was stimulated by Co^{2+} and Zn^{2+} , and the inhibition by EGTA could be reversed by Mn^{2+} , Co^{2+} and Zn^{2+} as well as by Ca^{2+} . These results, and the inhibition observed by the transition metal ion chelator 1,10-phenanthroline (Table 2; Low & Prasad, 1988), suggest that the active site may have a requirement for divalent metal ions other than Ca^{2+} .

Renal dipeptidase is also released from pig kidney microvillar membranes upon addition of plasma and this release displays a similar inhibition profile to the activity which acts on purified renal dipeptidase. Pig kidney microvillar membranes appear to be completely free from contaminating plasma phospholipase D, as no

endogenous release of renal dipeptidase was observed in the absence of added plasma, and renal dipeptidase purified after solubilization with octyl glucoside from microvillar membranes is entirely amphipathic (see above). The plasma phospholipase D was only active on the membrane anchor of purified renal dipeptidase when detergent was present. This is in agreement with previous results where the presence of detergent was essential for the release of alkaline phosphatase from HeLa or rat osteogenic sarcoma cells by partially purified phospholipase D (Low, 1988) and the observed resistance to release of decay-accelerating factor from HeLa cells (Davitz *et al.*, 1987). Release of renal dipeptidase from microvillar membranes by the phospholipase D proceeded in the absence of added detergent, albeit at a reduced rate.

A common feature of G-PI-anchored proteins is the presence of the cross-reacting determinant (CRD) after hydrolysis of the membrane anchor by bacterial PI-PLC (Low, 1987; Ferguson & Williams, 1988). The action of *S. aureus* or *B. cereus* PI-PLC on the amphipathic form of renal dipeptidase generates the cross-reacting determinant, whereas the action of the anchor-degrading activity in plasma failed to generate this determinant. The data thus confirm that the plasma anchor-degrading activity does not have the specificity of a PI-PLC, consistent with the results of Davitz *et al.* (1987) and Cardoso de Almeida *et al.* (1988). As neither the amphipathic form of renal dipeptidase nor the hydrophilic form which had been treated with mild acid to decyclize the 1,2-cyclic phosphate cross-reacts with the anti-CRD serum, it would appear that renal dipeptidase does not contain a galactose branch in its G-PI anchor (Zamze *et al.*, 1988). Thus, the inositol 1,2-cyclic phosphate ring epitope formed by PI-PLC cleavage is essential for binding of the anti-CRD serum to renal dipeptidase.

In conclusion, in the present study we have purified the G-PI-anchored renal dipeptidase in a form which retains the hydrophobic membrane anchor after solubilization with octyl glucoside. This amphipathic form of renal dipeptidase has then been used as the substrate to characterize the properties of an anchor-degrading activity in plasma which may correspond to the plasma phospholipase D described elsewhere (Davitz *et al.*, 1987; Cardoso de Almeida *et al.*, 1988; Low & Prasad, 1988).

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