Ectoenzymes of the kidney microvillar membrane

Affinity purification, characterization and localization of the phospholipase C-solubilized form of renal dipeptidase

Gillian M. LITTLEWOOD, Nigel M. HOOPER and Anthony J. TURNER*

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

Renal dipeptidase (EC 3.4.13.11) was solubilized from pig kidney microvillar membranes with bacterial phosphatidylinositol-specific phospholipase C and then purified by affinity chromatography on cilastatin-Sepharose. The enzyme was apparently homogeneous on SDS/polyacrylamide-gel electrophoresis with an M_r of 47000. Immunohistochemical analysis of the distribution of the dipeptidase showed it to be concentrated in the brush-border region of the proximal tubules in close association with endopeptidase-24.11 (EC 3.4.24.11). The purified dipeptidase was shown to contain 1 mol of inositol/mol and to possess the cross-reacting determinant characteristic of the glycosyl-phosphatidylinositol membrane-anchoring domain. The glycoprotein nature of renal dipeptidase was confirmed by chemical and enzymic deglycosylation. These results establish renal dipeptidase as a glycosyl-phosphatidylinositol-anchored ectoenzyme of the microvillar membrane.

INTRODUCTION

Renal dipeptidase (dehydropeptidase-I, microsomal dipeptidase; EC 3.4.13.11) was originally described as a kidney membrane enzyme able to hydrolyse a variety of dipeptides, although more recently it has been implicated in the renal metabolism of glutathione and its conjugates, e.g. leukotriene D_4 (Campbell, 1970; Kozak & Tate, 1982). It is also responsible for hydrolysis of the β -lactam antibiotic imipenem (Kropp *et al.*, 1982). The enzyme is located in the microvillar membrane of the kidney (Welch & Campbell, 1980), but there has been some disagreement as to whether this dipeptidase is an ectoenzyme (Kim & Campbell, 1983). In particular, treatment of membranes with Triton X-100 led to the enzyme becoming associated with the detergent-insoluble (cytoskeleton) pellet, suggesting that it is not located at the luminal surface of the microvillar membrane. However, alkaline phosphatase (EC 3.1.3.1) behaves in a similar fashion, and this enzyme is now established as a glycosyl-phosphatidylinositol (glycosyl-PI)-anchored ectoenzyme (reviewed in Kenny & Turner, 1987; Low, 1987; Low & Saltiel, 1988). Furthermore, we have shown that renal dipeptidase is released from pig kidney microvillar membranes by treatment with bacterial phosphatidylinositol-specific phospholipase C (PI-PLC) (Hooper *et al.*, 1987) and that the dipeptidase gives a pattern of solubilization by a range of detergents typical of a glycosyl-PI-anchored protein (Hooper & Turner, 1988a).

A covalently attached glycosyl-PI moiety has now been demonstrated in a number of cell-surface proteins, including proteins involved in cell adhesion, trans-membrane signalling, protective surface coat, complement regulation and hydrolytic activity (reviewed in Low, 1987; Low & Saltiel, 1988). The presence of inositol

has been demonstrated in all of the glycosyl-PI-anchored proteins so far analysed (see, e.g., Ferguson *et al.*, 1985; Futerman *et al.*, 1985), and, in addition, a number of these proteins contain a common determinant in the membrane-anchoring domain, called the cross-reacting determinant (CRD) (see, e.g., Cardoso de Almeida & Turner, 1983; Bordier *et al.*, 1986).

We have now combined PLC solubilization with affinity chromatography on an immobilized inhibitor (cilastatin) to provide a rapid two-step purification of the hydrophilic form of renal dipeptidase. An antiserum was raised to the purified enzyme that was used for immunohistochemical localization of the dipeptidase in the brush border of the kidney tubules. The purified dipeptidase is shown to contain inositol and to possess the CRD characteristic of glycosyl-PI-anchored proteins. Enzymic deglycosylation cleaved the *N*-linked sugars but not the glycosyl-PI moiety from the purified dipeptidase. We also show, on the basis of inhibitor- and substrate-specificity, that renal dipeptidase is distinct from a membrane dipeptidase that hydrolyses the endogenous brain dipeptide *N*-acetyl-Asp-Glu (Robinson *et al.*, 1987).

EXPERIMENTAL

Materials

Pig kidneys were obtained from ASDA Farm Stores (Lofthousegate, W. Yorkshire, U.K.). Cilastatin (MK0791) (monosodium *Z*-s-[6-carboxy-6-[(2,2-dimethyl-(s)-cyclopropyl)carbonyl]amino}hex-5-enyl]-L-cysteine) was a gift from Dr. H. Kropp (Merck, Sharp and Dohme Research Laboratories, Rahway, NJ, U.S.A.). PI-PLC from *Bacillus thuringiensis* was purified as described previously (Hooper *et al.*, 1987) and was a

Abbreviations used: CRD, cross-reacting determinant; PI, phosphatidylinositol; PLC, phospholipase C; PI-PLC, phosphatidylinositol-specific phospholipase C; TFMS, trifluoromethanesulphonic acid.

* To whom correspondence and requests for reprints should be addressed.

gift from Dr. M. G. Low. Rabbit anti-CRD serum and variant surface glycoprotein 117 were gifts from Dr. M. A. J. Ferguson (Department of Biochemistry, University of Oxford, Oxford, U.K.). *Bacillus cereus* PLC type III, Gly-D-Phe, *N*-acetyl-Asp-Glu and CNBr-activated Sepharose 4B were from Sigma Chemical Co. Triton X-114 was pre-condensed before use (Bordier, 1981). All other materials were from sources previously noted. The commercial preparation of broad-specificity phospholipase C from *B. cereus* is known to contain a contaminating PI-PLC activity (Ferguson *et al.*, 1985; Hooper *et al.*, 1987).

Preparation of cilastatin-Sepharose affinity resin

Cilastatin was attached to CNBr-activated Sepharose 4B as described in Campbell *et al.* (1984). Affinity resins were also made in which the cilastatin was separated from the matrix by either a 1.4 nm or a 2.8 nm spacer arm according to the procedures for lisinopril-Sepharose affinity resins [Bull *et al.* (1985) and Hooper & Turner (1987) respectively]. However, in contrast with peptidyl dipeptidase A (EC 3.4.15.1), increasing the spacer arm length decreased the apparent binding affinity of the affinity resin for renal dipeptidase, and thus all purifications were performed with the cilastatin-Sepharose matrix described by Campbell *et al.* (1984).

Purification of renal dipeptidase

All procedures were carried out at 4 °C unless otherwise stated. Pig kidney cortex (typically 100 g) was homogenized in 10 vol. of 150 mM-NaCl/10 mM-Tris/HCl buffer, pH 7.4, and centrifuged at 8000 *g* for 15 min. The supernatant was centrifuged at 26000 *g* for 2 h, and the pellet was resuspended in 200 ml of 100 mM-NaCl/10 mM-Tris/HCl buffer, pH 7.4, and centrifuged at 31000 *g* for 90 min. The pellet was resuspended in 100 mM-NaCl/10 mM-Tris/HCl buffer, pH 7.4, to give a protein concentration of 10 mg/ml and incubated with PLC (type III from *B. cereus* at 1 unit/5 ml) for 1 h at 37 °C. After centrifugation at 31000 *g* for 90 min about 90% of the dipeptidase activity was found in the supernatant. This was 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 100 mM-NaCl/10 mM-Tris/HCl buffer, pH 7.4, at a flow rate of 20 ml/h. The column was washed extensively with 100 mM-NaCl/10 mM-Tris/HCl buffer, pH 7.4, before elution of the enzyme with 10 mg of cilastatin in 10 ml of the same buffer at a flow rate of 20 ml/h. Fractions (2 ml) were collected, and those that contained material that absorbed at 280 nm were pooled and dialysed extensively against 5 mM-Tris/HCl buffer, pH 8.0, to remove the cilastatin. The enzyme was concentrated on a small column of DEAE-cellulose (bed volume 0.2 ml) equilibrated with 5 mM-Tris/HCl buffer, pH 8.0, and eluted with 0.3 M-NaCl/5 mM-Tris/HCl buffer, pH 8.0. The affinity columns were routinely washed with 50 ml of each of the following: 5 mM-Hepes buffer, pH 7.5, containing 0.5 M-NaCl, 0.5 mM-EDTA and 2% (w/v) Triton X-100; 10 mM-glycine buffer, pH 9.5, containing 0.3 M-KCl, 0.1 mM-ZnCl₂ and 1.0 M-KSCN; 5 mM-Hepes buffer, pH 7.5, containing 0.15 M-KCl, 0.1 mM-EDTA, 0.1% (w/v) Triton X-100 and 0.1% (w/v) NaN₃. Protein was determined by the method of Lowry *et al.* (1951), or by a modification of

this method (Bensadoun & Weinstein, 1976), with bovine serum albumin as standard. Pig kidney microvillar membranes were prepared as described by Booth & Kenny (1974).

Renal dipeptidase was also purified by affinity chromatography on cilastatin-Sepharose after solubilization from the membrane with *n*-octyl β -D-glucopyranoside.

Renal dipeptidase assay

Renal dipeptidase was assayed by an h.p.l.c. method with Gly-D-Phe as substrate as described in Hooper *et al.* (1987), except that the incubation buffer was 0.1 M-Tris/HCl, pH 8.0, as the dipeptidase was found to be insensitive to Cl⁻ ions in the range 0–500 mM. The *K_m* for Gly-D-Phe degradation was 5.95 mM.

SDS/polyacrylamide-gel electrophoresis and immunoelectrophoretic blot analysis

SDS/polyacrylamide-gel electrophoresis was performed 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 of purified pig kidney dipeptidase (1 μ g of protein) were made up to 0.2 ml with 10 mM-Tris/HCl buffer, pH 7.4, containing 150 mM-NaCl and 1.0% (w/v) Triton X-114 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 in duplicate for enzyme activities. Activity recovered in the detergent-rich phase is expressed as percentage of the total activity.

Production of antiserum to renal dipeptidase

A rabbit was immunized with 50 μ g of affinity-purified renal dipeptidase in Freund's complete adjuvant (subcutaneous). The immunization was repeated 2 weeks later in Freund's incomplete adjuvant and again after a further 2 weeks. A final intravenous immunization was performed 2 weeks later. IgGs were purified from the antiserum by affinity chromatography on a column of renal dipeptidase immobilized on CNBr-activated Sepharose as described in Matsas *et al.* (1986) for endopeptidase-24.11.

Immunohistochemistry

Immunohistochemistry was performed on kidney from 2–4-week-old pigs that had been prepared by perfusion with paraformaldehyde as described in Matsas *et al.* (1986). Cryostat sections (10 μ m) were stained with a peroxidase detection system after incubation with a 1:500 dilution of the affinity-purified anti-dipeptidase IgGs or a 1:500 dilution of affinity-purified anti-endopeptidase-24.11 IgGs (Matsas *et al.*, 1986). Sections were counterstained with Harris haematoxylin stain for 10 s. Sections were mounted on microscope slides in 90% (v/v) glycerol in 0.15 M-NaCl/10 mM-sodium phosphate buffer, pH 7.4, and were viewed by a Zeiss microscope.

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. Activity was assayed with Gly-D-Phe (1.0 mM) as substrate, the product (D-Phe) being quantified by h.p.l.c.

	Protein (mg)	Total activity (μmol of D-Phe/min)	Specific activity (nmol of D-Phe/min per mg)	Recovery (%)	Enrichment (fold)
Homogenate	12285	287.8	23.4	100	1
Membrane fraction	1328	123.8	93.2	43	4
PLC-solubilized supernatant	185	115.9	626.4	40	27
Affinity chromatography	0.7	34.7	49583	24	2119

Chemical and enzymic deglycosylation of renal dipeptidase

Dipeptidase was deglycosylated either chemically with trifluoromethanesulphonic acid (TFMS) as described in Stewart *et al.* (1984) or enzymically with *N*-glycanase (glycopeptide *N*-glycosidase, EC 3.2.2.18) as described in Hooper & Turner (1987), and then analysed by SDS/polyacrylamide-gel electrophoresis. Recovery of protein after TFMS treatment was found to be highly variable.

Fig. 1. SDS/polyacrylamide-gel electrophoresis and 'Western' blot of pig kidney dipeptidase

(a) SDS/polyacrylamide-gel electrophoresis. Samples were prepared and analysed as described in the Experimental section. Lane 1, pig kidney membrane fraction (50 μg) before solubilization with PLC; lane 2, supernatant fraction (50 μg) after solubilization of pig kidney membranes with PLC and centrifugation at 31 000 g for 90 min; lane 3, affinity-purified renal dipeptidase (2 μg). The gel was stained with Coomassie Brilliant Blue. (b) 'Western' blot. 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 pig kidney dipeptidase. Lane 1, affinity-purified renal dipeptidase (0.1 μg); lane 2, pig kidney microvillar membrane fraction (20 μg).

Analysis of renal dipeptidase for inositol

Pig kidney dipeptidase that had been solubilized with phospholipase C and purified by affinity chromatography on cilastatin-Sepharose was dialysed extensively against 5 mM-sodium phosphate buffer, pH 7.5. The enzyme was then analysed by g.c.-m.s. for inositol content as described in Smith *et al.* (1987), with *scyllo*-inositol as an internal standard.

RESULTS

Solubilization and purification of renal dipeptidase

Renal dipeptidase was purified from pig kidney cortex in essentially two steps. The enzyme was released from the membrane by PI-PLC, which gave nearly a 7-fold purification, and was then applied to a cilastatin-Sepharose affinity column, which gave a final purification of over 2000-fold. Typical results for purification from 100 g of pig kidney cortex are shown in Table 1. Similar results for purification of the dipeptidase from pig kidney were obtained when a purified sample of

Fig. 2. Immunoprecipitation of pig kidney dipeptidase

Pig kidney dipeptidase was incubated at 4 °C for 18 h with increasing amounts of a polyclonal antiserum raised to pig kidney dipeptidase. After precipitation, enzyme was assayed as described in the Experimental section. ○, Dipeptidase with immune serum ($n = 3$); ●, dipeptidase with pre-immune serum ($n = 2$).

PI-PLC from *B. thuringiensis* was used to release the enzyme (results not shown). After affinity chromatography, the enzyme was apparently homogeneous as assessed by SDS/polyacrylamide-gel electrophoresis (Fig. 1a) and migrated with an apparent M_r of 47000. The purified pig kidney dipeptidase, when analysed by phase separation in Triton X-114, partitioned predominantly ($97.1 \pm 0.4\%$, $n = 3$) into the detergent-poor phase, indicative of a hydrophilic nature.

A polyclonal antiserum to pig kidney dipeptidase recognized the purified enzyme in 'Western' blots (Fig. 1b), as well as a single polypeptide in pig kidney microvillar membranes with an apparent M_r of 45000. The polyclonal antiserum was also able to immunoprecipitate purified pig kidney dipeptidase (Fig. 2). No precipitation was observed with pre-immune serum.

Localization of renal dipeptidase

Immunohistochemical analysis with the polyclonal antiserum raised to pig kidney dipeptidase and a peroxidase detection system showed renal dipeptidase to

be present in pig kidney cortex but not in medulla. The anti-dipeptidase antibody was seen to be concentrated in the lumen of the proximal tubules (Fig. 3a), with a distribution similar to the anti-endopeptidase-24.11 antibody (Fig. 3c). The enzyme was not present in the glomeruli (Fig. 3b), and no immuno-staining was observed when the antibody was replaced with pre-immune serum (Fig. 3d).

Inhibitor-sensitivity of renal dipeptidase

The inhibitor-sensitivity of pig kidney dipeptidase with a variety of enzyme inhibitors was examined (Table 2). The metalloenzyme inhibitors EDTA and 1,10-phenanthroline, as well as dithiothreitol, caused substantial inhibition of the dipeptidase. Other selective peptidase inhibitors, including inhibitors of aminopeptidases, peptidyl dipeptidase A and endopeptidase-24.11 (Kenny & Turner, 1987), had no significant inhibitory effect on the pig kidney dipeptidase activity, except for amastatin and bestatin, both of which partially inhibited the enzyme. The specific inhibitor of renal

Fig. 3. Immunohistochemical localization of dipeptidase in pig kidney

Sections of pig kidney cortex were prepared and stained as described in the Experimental section. Arrows show the position of tubules. (a) Immunostained for renal dipeptidase with nuclei counterstained with Harris haematoxylin; (b) as (a) with arrow marked 'g' showing glomerulus; (c) immunostained for endopeptidase-24.11; (d) incubated with pre-immune serum.

dipeptidase, cilastatin (Kahan *et al.*, 1983), potently inhibited the purified pig kidney dipeptidase activity (Table 2).

Analysis of renal dipeptidase for inositol

Inositol analysis revealed the presence of 0.93 mol of *myo*-inositol/mol of dipeptidase 47000- M_r subunit, protein concentration being determined by the modified Lowry assay method of Bensadoun & Weinstein (1976).

Immunoreactivity of renal dipeptidase towards anti-CRD serum

Samples of purified pig kidney dipeptidase were immunoblotted with an anti-CRD serum (Fig. 4). The hydrophilic PLC-solubilized form of the enzyme cross-reacted with the anti-CRD serum, whereas no cross-reactivity was observed with an amphipathic detergent-solubilized form.

Deglycosylation of renal dipeptidase

Samples of pig kidney dipeptidase were deglycosylated both by enzymic (*N*-glycanase) and chemical (TFMS) methods and the products analysed by SDS/polyacrylamide-gel electrophoresis (Fig. 5a). *N*-Glycanase treatment produced two polypeptide bands with apparent M_r 44000 and 40500, both of which were recognized by the anti-dipeptidase serum (results not shown). Even with incubation times of up to 48 h at 37 °C, complete conversion of the 44000- M_r polypeptide into the 40500- M_r polypeptide was not observed (results not shown).

Table 2. Sensitivity to selected inhibitors of renal dipeptidase

Purified pig kidney dipeptidase (50 ng) was incubated with Gly-D-Phe for 20 min at 37 °C in the absence and in the presence of inhibitors. Amastatin, dithiothreitol, EDTA and 1,10-phenanthroline were preincubated with enzyme for 15 min at room temperature. In the absence of any inhibitors, approx. 10% substrate breakdown was observed. The I_{50} value is the concentration of inhibitor giving 50% inhibition of enzyme activity. The results are the means $\pm$ S.E.M. for three determinations.

Inhibitor	Relative activity (%)
None	100
1.0 mM-1,10-Phenanthroline	54.4 $\pm$ 2.9
10 mM-EDTA	50.6 $\pm$ 1.2
1.0 mM-Dithiothreitol	6.8 $\pm$ 1.4
10 μ M-Actinonin	102.4 $\pm$ 6.5
10 μ M-Amastatin	72.9 $\pm$ 2.4
0.1 mM-Bestatin	63.1 $\pm$ 2.5
10 μ M-Captopril	98.3 $\pm$ 0.8
10 μ M-Enalaprilat	101.9 $\pm$ 2.2
10 μ M-Kelatorphan	98.8 $\pm$ 4.1
10 μ M-Lisinopril	101.5 $\pm$ 9.7
10 μ M-Phosphoramidon	106.4 $\pm$ 5.4
0.1 mM-Quisqualate	106.9 $\pm$ 5.1
10 μ M-Thiorphan	102.0 $\pm$ 6.7
	I_{50} (μ M)
Cilastatin	0.65 $\pm$ 0.04

Fig. 4. '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 antiserum to the CRD of the variant surface glycoprotein 221. Lane 1, PLC-solubilized affinity-purified renal dipeptidase (10 μ g); lane 2, variant surface glycoprotein 117 whose only site of cross-reactivity with the anti-CRD serum is the CRD itself (2 μ g); lane 3, detergent-solubilized affinity-purified renal dipeptidase (12 μ g).

Fig. 5. Deglycosylation of pig kidney dipeptidase

(a) SDS/polyacrylamide-gel electrophoresis. Samples were prepared and analysed as described in the Experimental section. Affinity-purified renal dipeptidase (2 μ g) was incubated with *N*-glycanase (10 units/ml) at 37 °C for: lane 1, 0 h; lane 2, 2 h; lane 3, 6 h; lane 4, 24 h. Lane 5, renal dipeptidase (10 μ g) treated with TFMS for 6 h. The gel was silver-stained. (b) 'Western' blot. Samples were prepared and analysed as described in the Experimental section. After electrophoretic transfer to nitrocellulose sheets, the tracks were blotted with anti-CRD serum. Lane 1, renal dipeptidase (2 μ g) incubated for 24 h at 37 °C; lane 2, renal dipeptidase (2 μ g) incubated with *N*-glycanase (10 units/ml) for 24 h at 37 °C.

Deglycosylation of renal dipeptidase with TFMS produced only one polypeptide, which co-migrated with the 40 500- M_r polypeptide produced by *N*-glycanase treatment. The *N*-glycanase deglycosylation products were immunoblotted with the anti-CRD serum (Fig. 5b). Both the 44 000- M_r and the 40 500- M_r polypeptides cross-reacted with the anti-CRD serum in a similar manner to the native 47 000- M_r polypeptide.

DISCUSSION

We have previously shown that renal dipeptidase is one of a group of proteins that can be selectively released from the microvillar membrane by bacterial PI-PLC (Hooper *et al.*, 1987), a useful indication of the presence of a glycosyl-PI membrane anchor (Low, 1987; Low & Saltiel, 1988). In the present study we have utilized this ability of bacterial PI-PLC to release renal dipeptidase selectively, in order to solubilize the enzyme before purification. The released enzyme was then purified to apparent homogeneity by affinity chromatography on cilastatin-Sepharose (Table 1 and Fig. 1). The solubilization with bacterial PI-PLC provides a useful initial purification step, as most other kidney microvillar peptidases are not released by this treatment (Hooper & Turner, 1988a,b). On SDS/polyacrylamide-gel electrophoresis the purified pig kidney dipeptidase exhibited an M_r of 47 000 (Fig. 1), consistent with earlier reports (René & Campbell, 1969; Hitchcock *et al.*, 1987).

A polyclonal antiserum raised to affinity-purified pig kidney dipeptidase recognized the purified enzyme (Figs. 1b and 2). In a pig kidney microvillar membrane fraction the antiserum revealed a single polypeptide band of M_r 45 000 (Fig. 1b). As shown by phase separation in Triton X-114, the PLC-solubilized affinity-purified form of renal dipeptidase is hydrophilic, whereas the form of the enzyme in kidney microvillar membranes is an amphipathic (Hooper & Turner, 1988a). Thus this difference in apparent M_r on SDS/polyacrylamide-gel electrophoresis may be due to anomalous binding of SDS to the hydrophobic glycosyl-PI anchor. A similar phenomenon has been observed for the variant surface glycoprotein (Cardoso de Almeida & Turner, 1983) and the scrapie prion protein (Stahl *et al.*, 1987). The polyclonal antiserum was also used to localize the dipeptidase in sections of pig kidney (Fig. 3). The distribution of renal dipeptidase was comparable with that of another well-characterized microvillar ectoenzyme, endopeptidase-24.11. The enzyme was concentrated on the brush border of the kidney proximal tubules in a similar manner to dipeptidase in rat kidney (Hirota *et al.*, 1987), but, unlike the rat enzyme, did not appear on the basolateral membrane.

Purified renal dipeptidase was inhibited by metallo-enzyme inhibitors and by dithiothreitol (Table 2) to a similar extent as the partially purified enzyme (Hooper *et al.*, 1987). The specific inhibitor of renal dipeptidase, cilastatin, potently inhibited the purified enzyme with an I_{50} value (Table 2) comparable with other reports (Kahan *et al.*, 1983; Hooper *et al.*, 1987). Selective inhibitors of endopeptidase-24.11, peptidyl dipeptidase A and aminopeptidases failed to inhibit significantly the activity of renal dipeptidase (Table 2). However, some inhibition was observed with amastatin and bestatin, 27% and 37% respectively, presumably as a result of non-specific interactions of these low- M_r peptides (Rich *et al.*, 1984) with the active site of dipeptidase. These

inhibitory effects are probably not due to the presence of contaminating aminopeptidases in the purified preparation of renal dipeptidase, as the substrate Gly-D-Phe is resistant to hydrolysis by aminopeptidases, and the only aminopeptidase that is released by bacterial PI-PLC, aminopeptidase P (EC 3.4.11.9), has a strict requirement for a proline as the penultimate residue (Kenny & Turner, 1987; Hooper & Turner, 1988a,b).

A metallo-dipeptidase activity has been described in rat brain and kidney membranes that cleaves the endogenous brain dipeptide *N*-acetyl-Asp-Glu and that is potently inhibited by L-quisqualate (Robinson *et al.*, 1987). There are several similarities between this dipeptidase and renal dipeptidase; however, the two enzymes do not appear to be identical, as quisqualate failed to inhibit pig renal dipeptidase (Table 2), and *N*-acetyl-Asp-Glu was not hydrolysed by purified renal dipeptidase [negligible (< 6%) decrease in the amount of *N*-acetyl-Asp-Glu was observed when incubated in the presence of 500 ng of purified renal dipeptidase for 24 h at 37 °C; results not shown].

Analysis of the PLC-solubilized affinity-purified pig kidney dipeptidase revealed the presence of 1 mol of inositol/mol of enzyme, a result consistent with the presence of a glycosyl-PI anchor (Ferguson *et al.*, 1985; Futerman *et al.*, 1985; Low & Saltiel, 1988). The purified hydrophilic form of dipeptidase was also recognized by an anti-CRD serum (Fig. 4). However, the detergent-solubilized affinity-purified form of renal dipeptidase, which displays amphipathic properties and contains the hydrophobic membrane anchor (N. M. Hooper & A. J. Turner, unpublished work), did not cross-react with the anti-CRD serum (Fig. 4). This result is consistent with the CRD epitope being cryptic in the membrane-bound form of glycosyl-PI-anchored proteins and only exposed when the protein is released by the action of PI-PLC (Cardoso de Almeida & Turner, 1983; Low, 1987).

Deglycosylation of the purified kidney dipeptidase with TFMS resulted in a single polypeptide band of M_r 40 500 on SDS/polyacrylamide-gel electrophoresis, whereas *N*-glycanase produced two polypeptide bands of M_r 44 000 and M_r 40 500 (Fig. 5a). The presence of two polypeptide bands upon deglycosylation with *N*-glycanase, both of which possess the CRD epitope (Fig. 5b) and hence the glycan region of the glycosyl-PI anchor, would suggest that there are two distinct populations of *N*-linked sugars that differ in their susceptibility to cleavage by *N*-glycanase. In contrast, TFMS does not distinguish between these two populations and efficiently removes all the sugars. Renal dipeptidase is known to contain carbohydrate (Kim & Campbell, 1983; Campbell *et al.*, 1984), and the similar results obtained in the present study with *N*-glycanase and TFMS suggest that the bulk of the carbohydrate is present as *N*-linked sugars.

In conclusion, as well as being released from the membrane by bacterial PI-PLC (Hooper *et al.*, 1987) and giving a pattern of solubilization by a range of detergents typical of a glycosyl-PI-anchored protein (Hooper & Turner, 1988a), the present study has shown that renal dipeptidase also contains inositol and *N*-linked sugars and possesses the CRD epitope. These results are consistent with this microvillar ectoenzyme belonging to the group of proteins that are anchored in the plasma membrane by covalent attachment to a glycosyl-PI moiety.

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