Identification and characterization of the major stilbene-disulphonate- and concanavalin A-binding protein of the porcine renal brush-border membrane as aminopeptidase N

Hilario SEE and Reinhart A. F. REITHMEIER

MRC Group in Membrane Biology, Department of Medicine and Department of Biochemistry, Room 7307, Medical Sciences Building, University of Toronto, Toronto, Canada, M5S 1A8

A 130 kDa glycoprotein (GP 130) was purified from porcine renal brush-border membranes by affinity chromatography using immobilized 4-acetamido-4'-isothiocyano-stilbene-2,2'-disulphonate (SITS)- and concanavalin A-Sepharose. GP 130 was the major concanavalin A-binding protein in porcine renal brush-border membranes and also bound *Ricinus communis* (castor-bean) and wheat-germ agglutinins. Endo- β -N-acetylglucosaminidase F reduced the molecular mass of GP 130 by 20 kDa as determined by SDS/PAGE, whereas endo- β -N-acetylglucosaminidase H reduced the molecular mass by 5 kDa, showing that GP 130 contained both complex and high-mannose carbohydrate structures. Western-blot analyses using an antibody raised against GP 130 showed that it was localized to the brush-border membrane fraction and was present in a membrane fraction of the pig kidney cell line LLC-PK₁. The N-terminal sequence and amino acid composition of GP 130 showed that GP 130 is similar to rat kidney zinc peptidase and human intestinal aminopeptidase N. GP 130 had aminopeptidase N enzymic activity and was inhibited by bestatin ($K_i = 36 \mu\text{M}$), 1,10-phenanthroline ($K_i 30 \mu\text{M}$), Zn^{2+} ($K_i 26 \mu\text{M}$), Cu^{2+} ($K_i 260 \mu\text{M}$), pre-incubation with EDTA and by a polyclonal antibody against GP 130. Bicarbonate and iodide blocked the binding of GP 130 to the SITS-affinity resin, showing that GP 130 has an anion-binding site. Neither these anions nor stilbene disulphonates affected the aminopeptidase N activity of GP 130.

INTRODUCTION

There is a wide range of exchangers and co-transporters present in the brush-border and anti-luminal membranes of epithelial cells in the proximal tubule of kidney that have a net effect of transcellular transport (Aronson, 1989). They are responsible for the reabsorption of anions such as chloride, sulphate, oxalate and urate and for the secretion of *p*-amino-hippurate and other organic anions. Karniski & Aronson (1985) have shown that the major pathway for chloride reabsorption in rabbit kidney is through a chloride-formate exchanger. Many of the renal anion-transport processes are inhibited by stilbene disulphonates (Karniski & Aronson, 1987; Grassl *et al.*, 1987; Aronson, 1989).

Erythrocyte Band 3 mediates the electroneutral exchange of chloride for bicarbonate, which greatly increases the bicarbonate-carrying capacity of blood (Lowe & Lambert, 1983; Passow, 1986). Stilbene disulphonates are potent inhibitors of anion-exchange activity in the erythrocyte (Knauf & Rothstein, 1971). These compounds inhibit reversibly by binding to an outward-facing site in Band 3 that may include the anion-binding site (Passow, 1986). The compound 4,4'-di-isothiocyano-stilbene-2,2'-disulphonate (DIDS) can also inhibit anion transport irreversibly by covalently reacting with one or two lysine residues in the Band 3 protein (Jennings & Passow, 1979; Ramjessingh *et al.*, 1980).

An inhibitor matrix capable of binding Band 3 consists of 4-acetamido-4'-isothiocyano-stilbene 2,2'-disulphonate (SITS) coupled to Affi-Gel 102 (Pimplikar & Reithmeier, 1986). Band 3 can be eluted from this resin by free stilbene disulphonates. Blocking the stilbene disulphonate site by covalently modifying

Band 3 with DIDS or including stilbene disulphonates in the binding solution prevents binding. This inhibitor affinity matrix was used to purify putative anion-exchangers from dog renal brush-border membranes (Pimplikar & Reithmeier, 1988). The major protein that bound to the resin and was eluted by 4-benzamido-4'-aminostilbene 2,2'-disulphonate (BADs) was a 130 kDa glycoprotein. The binding of the 130 kDa protein to the SITS-Affi-gel was blocked by free stilbene disulphonates or by covalently modifying the protein with DIDS. This showed that the 130 kDa protein has a stilbene disulphonate-binding site and this makes it a candidate for an anion-exchanger. In the present study we purified a 130 kDa glycoprotein (GP 130) from porcine renal brush-border membranes using SITS-Affi-gel and concanavalin A-Sepharose. We have characterized it as aminopeptidase N by its N-terminal sequence and enzymic activity. Moreover, GP 130 has an anion-binding site that binds bicarbonate and stilbene disulphonates. It contains both high-mannose and complex N-linked oligosaccharides and is the major concanavalin A receptor in porcine renal brush-border membranes.

MATERIALS AND METHODS

Materials

Affi-Gel 102 was purchased from Bio-Rad. SITS was obtained from U.S. Biochemical Corp., Cleveland, OH, U.S.A. SITS-Affi-Gel was synthesized as described by Pimplikar & Reithmeier (1986). BADs was synthesized as described previously (Kotaki *et al.*, 1971). n-Dodecyl octa(ethylene glycol) monoether (C_{12}E_8) was from Nikko Chemical Co., Tokyo, Japan. DIDS was

Abbreviations used: BADs, 4-benzamido-4'-aminostilbene-2,2'-disulphonate; C_{12}E_8 , n-dodecyl octa(ethylene glycol) monoether; DIDS, 4,4'-di-isothiocyano-stilbene-2,2'-disulphonate; DNDS, 4,4'-dinitrostilbene-2,2'-disulphonate; SITS, 4-acetamido-4'-isothiocyano-stilbene-2,2'-disulphonate; GP 130, 130 kDa glycoprotein.

* To whom correspondence and reprint requests should be sent.

obtained from Pierce Chemical Co. 4,4'-Dinitrostilbene-2,2'-disulphonate (DNDS) was bought from Aldrich Chemical Co. Concanavalin A-Sepharose, Sephacryl S-400 HR and DEAE-Sepharcel were from Pharmacia. Vecta Stain was bought from Vector Laboratories (Burlingame, CA, U.S.A.). Glycopeptide *N*-glycosidase F, a cocktail of endo- β -*N*-acetylglucosaminidase F (EC 3.2.1.96) and glycopeptide *N*-glycosidase F (EC 3.2.2.18) mixture, endo- β -*N*-acetylglucosaminidase H (EC 3.2.1.96), bestatin and leucine *p*-nitroanilide were obtained from Boehringer Mannheim. Biotinylated concanavalin A, tomato (*Lycopersicon esculentum*), wheat-germ and *Ricinus communis* (castor-bean) agglutinins, methyl α -D-mannopyranoside, 1,10-phenanthroline and neuraminidase were from Sigma Chemical Co. All other chemicals were reagent grade or better.

Membrane preparations

Brush-border-membrane vesicles were isolated from porcine kidney outer cortex by differential centrifugation and MgCl_2 precipitation (Booth & Kenny, 1974). Anti-luminal membrane vesicles were isolated from pig kidney cortex as described by Sacktor *et al.* (1981), and ghost membranes were isolated from pig red blood cells by hypo-osmotic lysis (Dodge *et al.*, 1963). LLC-PK₁ cells were grown to confluence, washed twice with phosphate-buffered saline (5 mM-sodium phosphate/0.15 M-NaCl, pH 7.4) and then scraped off using 2 ml of buffer. The cells were collected by centrifugation at 500 *g* for 5 min at 4 °C, resuspended in 10 vol. of 10 mM-Tris/HCl (pH 7.4)/10 mM-KCl/1.5 mM-MgCl₂/2 mM-phenylmethanesulphonyl fluoride and homogenized in a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 5000 *g* for 10 min at 4 °C, and the subsequent supernatant was centrifuged at 100 000 *g* for 1 h. The pellet was resuspended in 10 mM-Tris/HCl (pH 7.4)/10 mM-KCl/1.5 mM-MgCl₂/2 mM-phenylmethanesulphonyl fluoride. All membrane fractions were stored at -20 °C until further use.

SITS-Affi-Gel affinity chromatography

Brush-border membranes (250 mg of protein) were solubilized in 228 mM-sodium citrate (pH 7.5)/1 % C₁₂E₈ for 30 min on ice at a final protein concentration of 1 mg/ml. The detergent extract was centrifuged at 100 000 *g* for 30 min at 4 °C to remove insoluble material and the supernatant was loaded on to a 1.6 cm-diameter column at a ratio of 5 mg of protein/ml of resin. The resin had been pre-washed with 5 bed vol. of 228 mM-sodium citrate (pH 7.5)/0.1 % C₁₂E₈. The resin was then washed with 10 bed vol. of 0.1 % C₁₂E₈ in 228 mM-sodium citrate, pH 7.5, and chromatography was performed at room temperature at a flow rate of 20 ml/h. The column was eluted with 4 bed vol. of 2 mM-BADS dissolved in 5 mM-sodium phosphate (pH 7.5)/0.1 % C₁₂E₈ or with 2 mM-BADS dissolved in 228 mM-sodium citrate (pH 7.5)/0.1 % C₁₂E₈ to prevent non-specific elution caused by the decrease in the ionic strength of the eluting buffer. The protein non-specifically absorbed on the resin was then eluted with 1 % (w/v) lithium dodecyl sulphate. Alternatively, the resin was removed from the column and a 100 μ l aliquot was boiled with an equal volume of Laemmli (1970) sample buffer. The column fractions (5 ml) were assayed for protein content by SDS/PAGE.

Concanavalin A-Sepharose affinity chromatography

Chromatography was done at room temperature at a flow rate of 20 ml/h, and 5 ml fractions were collected. Concanavalin A-Sepharose was washed with 10 bed vol. of Buffer 1 [10 mM-Tris/HCl (pH 7.5)/200 mM-NaCl/1 mM-MgCl₂/1 mM-MnCl₂/0.1 % C₁₂E₈]. The fractions of BADS eluant from the SITS-Affi-gel column containing the 130 kDa protein were pooled. The pooled fractions were loaded on to a 1.6 cm-diameter column at

10 ml of BADS eluant/ml of resin, and the column was washed with 10 bed vol. of Buffer 1 to remove BADS from the bound proteins. The resin was eluted stepwise, first with 5 bed vol. of 100 mM-methyl α -D-mannopyranoside in Buffer 1 and then with 1 M-methyl α -D-mannopyranoside in Buffer 1. Sometimes the 1 M-methyl α -D-mannopyranoside elution was performed at 37 °C using a batch-elution technique to increase the desorption of protein. After chromatography the resin was removed from the column and a 100 μ l aliquot was boiled with an equal volume of Laemmli sample buffer. The 1 M-methyl α -D-mannopyranoside eluant was dialysed against 10 vol. of 10 mM-Tris/HCl, pH 7.5, at 4 °C.

DEAE-Sepharcel chromatography

All steps were done at 4 °C. DEAE-Sepharcel was washed with 20 bed vol. of 10 mM-Tris/HCl (pH 7.5)/0.1 % C₁₂E₈. The dialysed 1 M-sugar-eluted fractions containing the 130 kDa protein was loaded on to a 1.0 cm-diameter column at a ratio of 2 ml/ml of resin, employing a flow rate of 30 ml/h. The resin was eluted with 300 ml of a linear 0–0.5 M-NaCl gradient in 10 mM-Tris/HCl (pH 7.5)/0.1 % C₁₂E₈ at a flow rate of 15 ml/h.

Gel filtration

All steps were done at room temperature. Sephacryl S-400 HR was packed into a 1.6 cm $\times$ 30 cm column and equilibrated with 10 mM-Tris/HCl (pH 7.5)/0.1 % C₁₂E₈. A 300 μ l portion of purified GP 130 (1 mg/ml) using SITS-Affi-Gel and concanavalin A-Sepharose was applied to the column, and 1 ml fractions were collected at a flow rate of 10 ml/h. The column was calibrated with Blue Dextran (void volume), thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa) and β -mercaptoethanol (total volume).

Antibodies

A 1 mg portion of purified pig kidney GP 130 was emulsified with 1 ml of complete Freund's adjuvant and injected subcutaneously into a New Zealand White rabbit. Booster injections containing 1 mg of protein emulsified with 1 ml of incomplete Freund's adjuvant were given at 2-week intervals for 6 weeks. Blood was collected 7 days after the final booster shot.

Lectin and antibody blots

The proteins were resolved by SDS/PAGE as described by Laemmli (1970) and blotted electrophoretically at 40 mA on to nitrocellulose membranes using a Bio-Rad Mini Transblot system, overnight at room temperature. The membranes were blocked with 10 % (v/v) ethanolamine/0.25 % gelatin/100 mM-Tris/HCl, pH 9.0, for 2 h (Olmsted, 1981) and washed with Buffer 2 [0.25 % gelatin/50 mM-Tris/HCl (pH 7.5)/5 mM-EDTA/150 mM-NaCl/0.05 % Nonidet P40] for 2 h (Olmsted, 1981). For lectin blots, the membranes were incubated overnight with biotinylated lectins, including concanavalin A (100–2000 ng/ml), wheat-germ agglutinin (5 μ g/ml) or tomato agglutinin (1 μ g/ml) in Buffer 2. The concanavalin A blot contained 10 mM-MgCl₂ during the lectin incubation. The blots were incubated with a 1:10 000 dilution of commercial Vecta stain in Buffer 2. *Ricinus communis* agglutinin (1 μ g/ml) was conjugated directly to peroxidase, so these blots did not need to be treated with avidin-biotin-peroxidase. The blots were revealed by incubation in 0.05 % (w/v) diaminobenzidine/0.1 % H₂O₂/50 mM-Tris/HCl, pH 7.5. For the antibody blot, after blocking the membranes, the blots were incubated with 1:1000 dilution of anti-(GP 130) serum in Buffer 2. The blots were then incubated with 1 μ g of biotinylated goat anti-rabbit IgG antibody/ml in Buffer 2 overnight. The membranes were incubated with 1:10 000 dilution of commercial Vecta stain and revealed as described above.

Glycosidase treatments

All endo- β -*N*-acetylglucosaminidase F experiments employed the endo- β -*N*-acetylglucosaminidase F and glycopeptide *N*-peptidase mixture unless stated otherwise. Purified GP 130 (0.4 mg/ml) in 1% $C_{12}E_8$ was boiled in 1% SDS/1% β -mercaptoethanol for 5 min. The denatured protein was diluted 5-fold into 30 mM-sodium phosphate (pH 8.0)/4 mM-EDTA, containing 0–25 units of endo- β -*N*-acetylglucosaminidase/mg or 0–41.5 units of glycopeptide *N*-glycosidase (endo- β -*N*-acetylglucosaminidase F-free)/mg of protein for 1 h at 37 °C, or diluted 5-fold into 30 mM-sodium citrate (pH 6.0)/4 mM-EDTA, containing 0–5 units of endo- β -*N*-acetylglucosaminidase H/mg for 1 h at 37 °C. The denatured protein was diluted 5-fold into 30 mM-sodium acetate, pH 5.0, containing 0–95 units of neuraminidase/mg for 1 h at 37 °C. The reactions were terminated by adding an equal volume of Laemmli sample buffer and boiling for 5 min. The digested proteins were analysed by SDS/PAGE to determine their apparent molecular mass.

Amino acid analysis

Protein samples were hydrolysed in 5.7 M-HCl at 107 °C for 22 h and dried under vacuum. The released amino acids were quantified by using a Beckman model 121 M amino acid analyser. The contents of cysteine and tryptophan were not determined.

N-Terminal sequencing

The standard program in an Applied Biosystem model 470 A Gas-Phase sequenator was employed, and the amino acid phenylthiohydantoin derivatives were analysed using Applied Biosystem model 120 h.p.l.c. apparatus.

Enzyme assays

Aminopeptidase N enzymic activity of the brush-border membranes and GP 130 was monitored spectrophotometrically at 410 nm using leucine *p*-nitroanilide as the substrate (Booth *et al.*, 1979). The incubation mixture (2 ml) contained 1 mM-leucine *p*-nitroanilide, 100 mM-mannitol and 10 mM-Hepes/Tris, pH 7.4. In some experiments the incubation mixture was bubbled with N_2 gas for at least 15 min at 4 °C to deplete the solution of dissolved bicarbonate. Assays typically contained 200 μ g of brush-border-membrane protein or 10 μ g of GP 130 and were carried out at room temperature.

GP 130 binding to SITS-Affi-Gel

The effect of various anions on the binding of GP 130 to the SITS-affinity resin was determined. Brush-border membranes were solubilized with 1% $C_{12}E_8$ in solutions containing various anions for 30 min on ice at a final protein concentration of 1 mg/ml and centrifuged for 5 min at 12000 *g* to remove insoluble material. A 1 ml portion of solubilized membranes was added to 50 μ l of SITS-Affi-Gel prewashed with the appropriate solution containing 0.1% $C_{12}E_8$. The suspension was shaken for 30 min at room temperature and then the beads were allowed to settle and the supernatant containing the unbound fraction was removed. The resin was washed twice with 1 ml of the appropriate solution containing 0.1% $C_{12}E_8$. The resin was eluted with 2 mM-BADS dissolved in 5 mM-sodium phosphate (pH 8.0)/0.1% $C_{12}E_8$. The resin was washed twice with the appropriate solution plus 0.1% $C_{12}E_8$. A 50 μ l portion of Laemmli sample buffer was added to the resin, and the suspension was boiled for 5 min. The proteins were separated by SDS/PAGE and revealed by Coomassie Blue staining.

Protein determination

Protein concentration was assayed (Lowry *et al.*, 1951) with BSA as a standard.

PAGE

PAGE was performed as described by Laemmli (1970), and the proteins were stained with Coomassie Brilliant Blue R. Molecular-mass markers used were myosin (205 kDa), β -galactosidase (116 kDa), phosphorylase *b* (97.4 kDa), BSA (66 kDa), ovalbumin (45 kDa), and carbonic anhydrase (29 kDa). Pre-stained molecular-mass markers included phosphorylase *b* (110 kDa), BSA (84 kDa), ovalbumin (47 kDa), carbonic anhydrase (33 kDa), soybean trypsin inhibitor (24 kDa) and lysozyme (16 kDa). Biotinylated molecular-mass markers used in blots included rabbit muscle phosphorylase *b* (97.4 kDa), BSA (66.2 kDa), hen's-egg-white albumin (42.7 kDa), bovine carbonic anhydrase *b* (31 kDa), soybean trypsin inhibitor (21.5 kDa), hen's-egg-white lysozyme (14.4 kDa).

RESULTS

Purification of GP 130

Stilbene disulphonate-binding proteins were purified from solubilized porcine renal brush-border membranes by affinity chromatography employing SITS-Affi-Gel and elution from the resin by 2 mM-BADS. SITS-Affi-Gel chromatography purified two major proteins with molecular masses of 130 and 90 kDa, plus a high-molecular-mass protein and variable amounts of lower-molecular-mass proteins from the brush-border membranes (Fig. 1). No binding of the 130 kDa protein on to the SITS-Affi-Gel was observed in the presence of free stilbene disulphonate. The 130 kDa protein could be separated from contaminating proteins in the BADS eluant by lectin affinity chromatography using concanavalin A-Sepharose (results not shown). Methyl α -D-mannopyranoside (100 mM) eluted the 90 kDa protein and partially eluted the 130 kDa protein, but most of the 130 kDa protein remained bound to the resin and was

Fig. 1. Partial purification of 130 kDa protein from porcine renal brush-border membranes using SITS-Affi-Gel

SDS/10% (w/v)-polyacrylamide gel showing the protein profile of porcine renal brush-border membrane and of the purification of 130 kDa protein by SITS-Affi-Gel affinity chromatography. The gel was stained with Coomassie Blue. Lane 1, 20 μ g of brush-border-membrane proteins; lane 2, 20 μ g of solubilized brush-border-membrane proteins; lane 3, flow-through; lane 4, 2 mM-BADS eluant; lane 5, boiled resin after BADS elution; lane 6, molecular-mass markers as described in the text. The arrow on the left-hand side points to the 130 kDa protein band, and arrows on the right indicate the size of the molecular-mass markers in kDa.

Fig. 2. GP 130 is the major concanavalin A-binding protein in porcine renal brush-border membranes

Lane 1, molecular-mass markers as described in the text; lane 2, Coomassie Blue-stained gel of 1 μ g of purified GP 130; lane 3, concanavalin A (2 μ g/ml) blot of purified GP 130 corresponding to lane 2; lane 4, Coomassie Blue-stained gel of 10 μ g of porcine renal brush-border-membrane proteins; lane 5, concanavalin A (200 ng/ml) blot of porcine renal brush-border-membrane proteins corresponding to lane 4. The arrows in lanes 2 and 4 point to the 130 kDa protein band.

eluted by 1 M-methyl α -D-mannopyranoside. Occasionally a final step was employed in the purification of the 130 kDa protein using ion-exchange chromatography to remove small amounts of contaminants. The 130 kDa protein is acidic and bound to DEAE-Sephacel and was eluted at 70 mM-NaCl. A final yield of 4 mg of purified GP 130 (Fig. 2, lane 2) was obtained from 250 mg of brush-border-membrane protein.

Purified 130 kDa protein applied to a Sephacryl S-400 HR gel-filtration column eluted as a single species with a Stokes radius of 7 nm (70 Å) and an apparent molecular mass of 440 kDa in the presence of the non-ionic detergent $C_{12}E_8$. The molecular mass of the polypeptides in the detergent-protein complex cannot be calculated without determination of the sedimentation coefficient and an accurate measurement of detergent binding (Tanford *et al.*, 1974). However, the gel-filtration results are consistent with the 130 kDa protein having an oligomeric structure (dimer) and binding a micelle of detergent.

Lectin blots of porcine brush-border-membrane proteins

Since the 130 kDa protein bound tightly to concanavalin A-Sepharose, it must be a glycoprotein and likely contains one or more high-mannose oligosaccharides. To determine which lectins bind to GP 130, lectin blots were performed on purified GP 130, and porcine brush-border-membrane proteins were separated by SDS/PAGE. Purified GP 130 bound concanavalin A (Fig. 2, lane 3), as well as *Ricinus communis* and wheat-germ agglutinins (results not shown). Concanavalin A also showed strong binding to GP 130 in blots of porcine renal brush-border-membrane proteins (Fig. 2, lane 5). Blotting brush-border-membrane proteins with higher concentrations of concanavalin A resulted in more bands being revealed with apparent molecular masses of 200, 150, 110, 90, 60 and 55 kDa. Wheat-germ agglutinin, which binds to *N*-acetylglucosamine and sialic acids, showed that the major proteins among those in brush-border membranes that bound this lectin had molecular masses of 150, 130 and 90 kDa (results not shown). The *Ricinus communis* agglutinin, which binds to terminal galactose but not to oligosaccharides with terminal sialic acids, showed four major bands in blots of brush-border membrane proteins with molecular masses of 150, 130, 110 and 90 kDa (results not shown). Tomato agglutinin, which binds to *N*-acetyl- β -D-glucosamine, bound

Fig. 3. GP 130 contains both high-mannose and complex *N*-linked oligosaccharides

Purified GP 130 was deglycosylated with endo- β -*N*-acetylglucosaminidase F and H as described in the text. Lanes 1–3 are SDS/PAGE gels stained with Coomassie Blue R. Lane 1, 2 μ g of untreated GP 130; lane 2, 2 μ g of GP 130 (0.08 mg/ml) treated with 0.5 unit of endo- β -*N*-acetylglucosaminidase H/mg; lane 3, 2 μ g of GP 130 (0.08 mg/ml) treated with 2.5 units of endo- β -*N*-acetylglucosaminidase F/mg. The arrow labelled '130' points to the 130 kDa protein band. ● Indicates the partially deglycosylated 125 kDa protein. ■ Indicates the deglycosylated 110 kDa protein band, and the arrows on the right indicate the size of the molecular-mass markers in kDa.

weakly to the 130 kDa protein in the brush-border membrane, but more tightly to a 45 kDa band (results not shown).

Sensitivity of GP 130 to glycosidase treatment

Treatment of GP 130 with endo- β -*N*-acetylglucosaminidase H, which cleaves *N*-linked high-mannose oligosaccharides at 0.5 unit/mg of protein, caused a 5 kDa shift in molecular mass, as judged by SDS/PAGE (Fig. 3, lane 2). A dose-dependence assay showed that 5 kDa is the maximum shift in molecular mass observed with this enzyme. This suggests that GP 130 possesses one or more high-mannose oligosaccharide structures, confirming the concanavalin A-binding studies. Partially deglycosylated GP 130 lost most of its ability to bind to concanavalin A, but could still bind to *Ricinus communis* and wheat-germ agglutinins (results not shown). This was expected, as endo- β -*N*-acetylglucosaminidase H can only cleave the high-mannose structure, but cannot remove complex and hybrid structures.

Treatment of GP 130 with endo- β -*N*-acetylglucosaminidase F (2.5 units/mg of protein) caused a shift of 20 kDa, as determined by SDS/PAGE electrophoresis (Fig. 3, lane 3). No further reduction in molecular mass was observed with a 10-fold increase in the amount of enzyme. GP 130 had to be denatured with 1% SDS and 1% β -mercaptoethanol before glycosidase treatment to achieve the maximum shift in molecular mass. Only a small shift in molecular mass was observed without denaturation in SDS. Pure glycopeptidase F, which cleaves *N*-linked high-mannose oligosaccharides as well as biantennary, triantennary and tetra-antennary complex oligosaccharides, was also able to shift the molecular mass of GP 130 by 20 kDa (results not shown). The deglycosylated GP 130 (110 kDa) lost its affinity for concanavalin A and *Ricinus communis* and wheat-germ agglutinins (results not shown). The lack of reaction indicated that most of the *N*-linked oligosaccharide structures had been removed. Incubating GP 130 with neuraminidase (95 units/mg of

Fig. 4. Antibody localized to brush-border membrane

Western-blot analysis of porcine renal-brush-border, anti-luminal and red-cell-ghost membrane proteins using anti-(GP 130) antibody. Lanes 1–7, Coomassie Blue-stained gel. Lanes 1 and 2, 40 and 20 μ g of ghost membrane protein respectively; lanes 3 and 4, 40 and 20 μ g of anti-luminal-membrane protein respectively; lanes 5 and 6, 40 and 20 μ g of brush-border-membrane protein respectively; lane 7, molecular-mass standards; lanes 8–13, Western-blot corresponding to the gel; lanes 8 and 9, 5 and 2 μ g of ghost membrane protein respectively; lanes 10 and 11, 5 and 2 μ g of anti-luminal-membrane protein respectively; lanes 12 and 13, 5 and 2 μ g of brush-border-membrane protein respectively. The arrow labelled '130' points to the 130 kDa protein band found in the brush-border membrane of lanes 5, 6, 12 and 13. The arrow labelled '95' points to the 95 kDa Band 3 protein in ghost membranes which is the erythrocyte anion transporter.

protein) caused a slight shift in molecular mass, suggesting that GP 130 contained terminal sialic acids (results not shown).

GP 130 localized to the brush-border membrane

A rabbit polyclonal antibody was raised against purified porcine GP 130. The antibody (1:1000 dilution of serum) could detect 1 μ g of purified GP 130 on a Western blot (results not shown). The antibody reacted equally well with GP 130 that had been deglycosylated by endo- β -N-acetylglucosaminidase F, which showed that the major epitopes for the antibody likely resides in the protein moiety of GP 130.

A Western blot was performed on porcine renal-brush-border, anti-luminal- and red-cell-ghost-membrane proteins. The antibody recognized only one band in brush-border-membrane proteins, namely that with a molecular mass of 130 kDa (Fig. 4). The antibody did not cross-react with any protein from the anti-luminal or ghost membranes, including Band 3. Furthermore, the antibody did not cross-react with any proteins with a similar molecular mass to that of GP 130 from renal brush-border membranes of other species, including dog, rabbit, human, hamster and rat (results not shown). The antibody did cross-react with a 130 kDa protein in an extract of the membrane fraction of the porcine cell line LLC-PK₁ (Hull *et al.*, 1976) on a Western blot (Fig. 5). The antibody could precipitate intact brush-border-membrane vesicles. This showed that some of the epitopes for the antibody reside extracellularly, as brush-border membranes were shown to be mostly right-side-out vesicles (Booth & Kenny, 1974; Vannier *et al.*, 1976). The porcine renal brush-border-membrane vesicles were shown to be intact by their ability to concentrate tracer [³⁶Cl]chloride ion from the extracellular side into the vesicles when there was an outward-imposed chloride-ion gradient (results not shown).

Identity of GP 130 revealed by N-terminal sequence

The purity of GP 130 isolated by SITS-Affi-Gel followed by concanavalin A-Sepharose was demonstrated by the appearance of a single band on SDS/PAGE (Fig. 2). Purified GP 130 was subjected to automated Edman degradation and gave a single amino acid sequence. The N-terminal sequence of GP 130 is very similar to the recently published amino acid sequences of rat kidney zinc peptidase (Watt & Yip, 1989) and human intestinal aminopeptidase N (Olsen *et al.*, 1988) as deduced from their cDNA sequences (Fig. 6). Other N-terminal sequences that were

compared were the transmembrane domain of rabbit intestinal aminopeptidase N (Feracci *et al.*, 1982b) and the N-terminal sequence of the 130 kDa protein from the brush-border membrane of the proximal tubule in dog kidney (Fig. 6). The GP 130 sequence could be readily aligned with the other four sequences. In the first 40 amino acids compared, there were 35 identical residues between the rat kidney zinc peptidase, human intestinal aminopeptidase N and GP 130. The initiator methionine residue was missing from the N-terminal sequence of the 130 kDa protein from pig and dog kidney, which suggests that it was cleaved off during protein synthesis. At residue 10 there are three different amino acids (alanine in the 130 kDa protein in dog and pig kidney and rabbit intestinal aminopeptidase N, threonine in rat kidney zinc peptidase and serine in human intestinal aminopeptidase N), but these three amino acids differ from one another by a single base change in their codon. Residue 24 is a conserved small-aliphatic-amino-acid substitution, where cysteine in rat

Fig. 5. Antibody cross-reacts with a 130 kDa protein in LLC-PK₁ cells

A Western-blot analysis of a LLC-PK₁ membrane fraction is shown. Lane 1, Laemmli gel electrophoresis of LLC-PK₁ membrane fraction stained with Coomassie Blue; lane 2, immunoblot corresponding to lane 1; lane 3, molecular-mass standards. The arrow labelled '130' points to the 130 kDa protein band found in the Western blot of the LLC-PK₁ membrane fraction.

	10	20	30	40
PKAPN	AKGFYISKA	LGILGILLGV	AAVATIIALS	VVYAQEKNNK
RKAPN	MAKGFYISKT	LGILGILLGV	AAVCTIIALS	VVYAQEKNNR
HIAPN	MAKGFYISKS	LGILGILLGV	AAVCTIIALS	VVYSQEKNNK
RIAPN	YISKA	LGILGFXLG		
DKAPN	AKGFYIXKA	LGILXIXLGV	AAVXXIIAL	
	*		*	* *

Fig. 6. Comparison of the *N*-terminal sequence of porcine GP 130 with that of rat kidney zinc peptidase, human intestinal aminopeptidase N, the transmembrane domain of rabbit intestinal aminopeptidase N and the 130 kDa protein from dog renal brush-border membrane

Numbers indicate the amino acid position, starting at the initiator methionine residue, predicted from the cDNA sequence of rat kidney zinc peptidase and human intestinal aminopeptidase N. * denotes differences in amino acids. X denotes an undetermined amino acid. Abbreviations: PKAPN, porcine renal brush-border-membrane GP 130; RKAPN, rat kidney zinc peptidase (Watt & Yip, 1989); HIAPN, human intestinal aminopeptidase N (Olsen *et al.*, 1988); RIAPN, rabbit intestinal aminopeptidase N (Feracci *et al.*, 1982); DKAPN, dog renal brush-border membrane 130 kDa protein (S. W. Pimplikar & R. A. F. Reithmeier, unpublished work).

kidney zinc peptidase and human intestinal aminopeptidase N is replaced with alanine in GP 130. At residue 34, alanine in GP 130 and rat kidney zinc peptidase is replaced by a serine residue in human intestinal aminopeptidase N. Residue 39 has a conserved substitution where lysine in GP 130 and human intestinal aminopeptidase N is replaced by arginine in rat kidney zinc peptidase. The similarity in *N*-terminal sequence strongly suggests that GP 130 and aminopeptidase N are the same protein and that we have purified the porcine kidney enzyme.

Amino acid analysis

The amino acid composition of GP 130 (Table 1) is similar to that of 130 kDa protein purified from the proximal tubule of dog renal brush-border membrane (Pimplikar & Reithmeier, 1988), rat kidney zinc peptidase, and porcine and rabbit intestinal aminopeptidase N (Feracci & Maroux, 1980). This provided further evidence that GP 130 is the aminopeptidase N of porcine renal brush-border membrane.

Table 1. Amino acid composition

Abbreviations used: PKAPN, porcine renal brush-border-membrane GP 130; DKAPN, dog kidney brush-border-membrane 130 kDa protein (Pimplikar & Reithmeier, 1988); HIAPN, human intestinal aminopeptidase N (Svensson *et al.*, 1982); RKAPN, rat kidney zinc peptidase (Watt & Yip, 1989); RIAPN, rabbit intestinal aminopeptidase N (Feracci & Maroux, 1982); ND, not determined.

Amino acid	Composition (residues/100 residues)				
	PKAPN	DKAPN	HIAPN	RKAPN	RIAPN
Asp	11.1	12.7	11.9	4.6	11.8
Thr	4.8	6.8	7.0	7.0	6.4
Ser	5.8	9.0	7.4	8.3	7.7
Glu	10.6	11.2	11.6	6.4	11.0
Pro	7.3	4.4	4.7	4.6	5.4
Gly	9.7	8.2	5.5	4.6	5.2
Ala	11.6	8.1	7.8	7.4	8.0
Val	7.4	6.0	6.5	6.1	6.9
Met	0.7	0.7	2.2	1.8	1.6
Ile	3.5	4.0	5.2	5.3	5.3
Leu	8.5	9.4	10.5	9.9	11.4
Tyr	1.6	3.9	4.3	4.6	3.4
Phe	5.1	4.4	4.9	4.5	4.5
His	3.1	2.3	2.1	1.8	2.2
Lys	5.4	5.0	4.3	5.0	2.9
Arg	3.8	4.0	3.0	3.5	4.0
Cys	ND	ND	0.9	0.7	ND
Trp	ND	ND	ND	2.6	2.2
Asn	ND	ND	ND	7.3	ND
Gln	ND	ND	ND	4.2	ND

GP 130 has aminopeptidase N enzymic activity

The best way to show that GP 130 is aminopeptidase N was to demonstrate that GP 130 can hydrolyse small peptides with a neutral amino acid at the *N*-terminal side of the cleavage site. Aminopeptidase N has the highest hydrolysis rate for dipeptides with a neutral amino acid located at the *N*-terminus (Feracci *et al.*, 1981). One of the common substrates used to assay for aminopeptidase N is leucine *p*-nitroanilide. GP 130 hydrolysed leucine *p*-nitroanilide with a V_{max} of 2 $\mu\text{mol/min}$ per mg at room temperature (Table 2). Bestatin (K_i 36 μM), 1,10-phenanthroline (K_i 35 μM) and Zn^{2+} (K_i 26 μM) inhibited the aminopeptidase N enzymic activity of GP 130 (Table 2). Pre-incubation of GP 130 with 25 mM-EDTA overnight at room temperature caused 86 % inhibition of hydrolytic activity. The inhibition induced by EDTA could be reversed by adding an equivalent amount of Zn^{2+} or Cu^{2+} to saturate all the EDTA. Further addition of Zn^{2+} inhibited the enzymic activity. The inhibition caused by Zn^{2+} could also be reversed by adding an equivalent amount of EDTA. EDTA (25 mM) in the assay buffer only caused 23 % inhibition. The polyclonal antibody (1:1 dilution of serum) against GP 130 inhibited the enzymic activity by 38 %.

Bestatin (K_i 36 μM), Zn^{2+} (K_i 26 μM) and Cu^{2+} (K_i 260 μM) inhibited the aminopeptidase N enzymic activity of porcine renal brush-border membranes, as summarized in Table 2. Bestatin and Zn^{2+} inhibited the aminopeptidase N enzymic activity in brush-border membranes and GP 130 with the same K_i . This suggests that the protein responsible for aminopeptidase N enzymic activity in porcine brush-border membrane is GP 130. Pre-treatment of membranes with 25 mM-EDTA overnight at room temperature caused 86 % inhibition. This inhibition could be reversed by adding equivalent amount of Zn^{2+} . Excess addition of Zn^{2+} again, however, inhibited the enzymic activity. Other bivalent cations, such as Ca^{2+} , Mn^{2+} and Mg^{2+} , were much weaker inhibitors. The polyclonal antibody did not inhibit the enzymic activity in brush-border-membrane vesicles. Intact brush-border membranes incubated with 1 % C_{12}E_8 for up to 12 h at 4 °C suffered only a slight loss in activity compared with intact brush-border membranes. The non-ionic detergent (C_{12}E_8) therefore had little effect on enzymic activity; however, when 1 % C_{12}E_8 was added directly to the assay buffer, there was a reduction in activity (Table 2). This may be an artefact due to substrate binding by the detergent micelles.

GP 130 has an anion-binding site

GP 130 binds to the SITS-affinity resin and can be specifically eluted by BADS, suggesting that it likely contains an anion-binding site. The binding of GP 130 to SITS-Affi-Gel is dependent on ionic strength (Table 3). Increasing the concentration of citrate from 2.3 to 228 mM greatly increases the binding. This is

Table 2. Aminopeptidase N activity of GP 130

Treatment	Relative activity	
	GP 130*	BBM†
Control	100	100
Inhibitors		
10 μ M-Bestatin	64	54
100 μ M-Bestatin	17	16
10 μ M-1,10-Phenanthroline	88	ND‡
100 μ M-1,10-Phenanthroline	18	86
Bivalent cations and chelator		
5 mM-EDTA	109	107
25 mM-EDTA	67	66
10 μ M-Zn ²⁺	84	89
100 μ M-Zn ²⁺	10	10
10 μ M-Cu ²⁺	121	ND‡
100 μ M-Cu ²⁺	124	84
500 μ M-Cu ²⁺	32	13
1 mM-Cu ²⁺	3	0
1 mM-Mg ²⁺	101	98
1 mM-Mn ²⁺	55	60
1 mM-Ca ²⁺	68	62
Anion and stilbene disulphonate		
10 mM-Bicarbonate	80	94
100 mM-Bicarbonate	31	42
150 mM-Chloride	83	87
275 mM-Chloride	80	65
1 mM-Oxalate	95	93
9 mM-Oxalate	74	79
1 mM-Phosphate	99	108
9 mM-Phosphate	111	94
1 mM-Sulphate	96	97
9 mM-Sulphate	89	80
100 mM-SITS	105	105
100 μ M-DNDS	104	97
100 μ M-DIDS	95	103
Detergents		
0.01 % C ₁₂ E ₈	99	87
0.1 % C ₁₂ E ₈	72	80
1 % C ₁₂ E ₈	42	37
0.01 % Deoxycholate	97	100
0.1 % Deoxycholate	44	27
1 % Deoxycholate	16	12
0.01 % SDS	119	74
0.1 % SDS	74	12
1 % SDS	0	0

* Activity of purified GP 130 at room temperature with 1 mM-leucine-*p*-nitroanilide was 2 μ mol/min per mg.

† Activity of porcine renal brush-border-membrane vesicles at room temperature with 1 mM-leucine *p*-nitroanilide was 500 nmol/min per mg.

‡ ND, not determined.

consistent with the finding of Pimplikar & Reithmeier (1988), who showed that the binding of the 130 kDa protein from dog renal brush border membrane on to SITS-Affi-Gel also increased with higher ionic strength.

Different anions should also be able to increase the binding of GP 130 at high ionic strength unless the anion competes with SITS for the anion-binding site in GP 130. Anions that caused increased binding with increasing ionic strength were phosphate, chloride, formate and sulphate (Table 3). Interestingly, the potassium salts compared with sodium salts for chloride and phosphate gave higher binding of GP 130. On the other hand, higher amounts of iodide inhibited binding. This suggests that iodide competes with SITS for the anion-binding site on GP 130. Bicarbonate blocked GP 130 binding at 10 mM, and increasing the concentration to 100 mM-bicarbonate did not promote binding.

Table 3. Effect of anions on GP 130 binding to SITS-Affi-Gel

Solubilized porcine brush-border membranes in the different solutions listed above were incubated with SITS-Affi-Gel. The bound 130 kDa protein was eluted by BADS and subjected to SDS/PAGE. The gel was stained with Coomassie Blue and quantified by scanning with a densitometer. The intensity of the 130 kDa band in 228 mM-sodium citrate solution was used as the control and arbitrarily assigned as 1.00, and the intensity of the 130 kDa band in other solutions were calculated relative to the control. All the solutions included 10 mM-Tris/HCl, pH 7.5, except for NaHCO₃, which had a pH 8.0.

Solution	Intensity of 130 kDa band
228 mM-Sodium citrate	1.00
23 mM-Sodium citrate	0.68
2.3 mM-Sodium citrate	0.16
10 mM-Tris chloride	0
10 mM-NaCl	0.19
100 mM-NaCl	0.19
10 mM-KCl	0.22
100 mM-KCl	0.31
10 mM-NaHCO ₃	0
100 mM-NaHCO ₃	0
10 mM-Sodium formate	0.33
100 mM-Sodium formate	0.56
10 mM-KI	0.20
100 mM-KI	0
10 mM-Na ₃ PO ₄	0.42
100 mM-Na ₃ PO ₄	0.43
10 mM-K ₃ PO ₄	0.50
100 mM-K ₃ PO ₄	1.19
10 mM-Sodium oxalate	0.22
10 mM-Na ₂ SO ₄	0.37
100 mM-Na ₂ SO ₄	0.66

Effect of anions and stilbene disulphonates on GP 130 aminopeptidase N enzymic activity

Since GP 130 has an anion-binding site, anions might play a role in its catalytic activity. The enzymic assay was performed in nominally bicarbonate-free assay solution. Addition of 10 mM-bicarbonate did not stimulate the activity of the aminopeptidase N in brush-border membranes. However, 250 mM-bicarbonate inhibited the aminopeptidase N activity of brush-border membrane by 89%. Bicarbonate inhibited 50% of the activity at 75 mM. Since NaHCO₃ had a pH of 8, the addition of a large excess of bicarbonate to the assay media would increase the pH of the assay media to 8. As a control, an assay was done in the absence of bicarbonate, where the medium had a pH of 8. At pH 8 there was 31% inhibition of activity. This shows that the inhibition noticed between 250 mM-bicarbonate, pH 8 (89% inhibition) and an assay solution with pH 8 (31% inhibition) has to be attributed to some factor other than an increase in pH. Chloride (150 mM) inhibited the enzymic activity by 12%, and 275 mM-chloride inhibited 35% of the activity. Other anions tested, such as sulphate, phosphate and oxalate up to 9 mM, had little effect on the activity (Table 2).

Pretreatment of intact brush-border membranes with 1 mM-DIDS for 1 h at 37 °C at pH 9.5 caused DIDS to covalently attach to the proteins; however, this treatment did not inhibit enzymic activity. The aminopeptidase activity of GP 130 was not inhibited by DNDS and SITS up to 100 μ M (Table 2).

DISCUSSION

The present results demonstrate that the stilbene disulphonate-binding protein, GP 130, is not a transport protein but rather a

membrane-anchored proteolytic enzyme, aminopeptidase N. The strongest evidence for the identity of GP 130 as aminopeptidase N was its *N*-terminal sequence, which showed a high degree of identity with the predicted amino acid sequences of rat kidney zinc peptidase and human intestinal aminopeptidase N. The membrane-anchoring domain of rabbit intestinal aminopeptidase N (Feracci *et al.*, 1982b) and the 130 kDa protein purified from dog renal brush-border membranes have been partially sequenced and also showed a high degree of identity with the *N*-terminal portion of porcine GP 130 when aligned for optimum correlation. The amino acid compositions of GP 130, the 130 kDa protein in dog renal brush-border membranes, rat kidney zinc peptidase, porcine and rabbit intestinal aminopeptidase N are all similar.

The type of oligosaccharide chains on GP 130 was inferred partially by its affinity for lectins and sensitivity to glycosidases. GP 130 bound strongly to concanavalin A, wheat-germ and *Ricinus communis* agglutinins and weakly to tomato agglutinins, showing that GP 130 has both high-mannose and *N*-linked complex or hybrid oligosaccharide chains. GP 130 was the major concanavalin A-binding protein in the renal brush-border membrane. Endo- β -*N*-acetylglucosaminidase F reduced the electrophoretic mobility (in molecular-mass terms) of GP 130 by 20 kDa. In a study of the biosynthesis of the porcine renal aminopeptidase N, Stewart & Kenny (1984) showed that the immature protein could be deglycosylated by endo- β -*N*-acetylglucosaminidase H to 115 kDa when the oligosaccharides were not yet fully processed. Danielsen *et al.* (1982) used cell-free translation to show that the unglycosylated porcine intestinal aminopeptidase N has a molecular mass of 115 kDa. In addition, our study of the enzymic deglycosylation of the native protein showed that the molecular mass could be reduced from 130 to 110 kDa by glycopeptidase *N*-peptidase F. The molecular mass calculated from the deduced amino acid sequences of human intestinal aminopeptidase N (Olsen *et al.*, 1988) and rat kidney zinc peptidase (Watt & Yip, 1989) (110 kDa) is in excellent agreement with the deglycosylation results.

The sensitivity of GP 130 to endo- β -*N*-acetylglucosaminidase H showed that 5 kDa out of the 20 kDa are *N*-linked high-mannose structures, whereas the rest could be *N*-linked complex or hybrid structures. The average size of an *N*-linked carbohydrate structure is 2.5 kDa; therefore we can speculate there are approximately eight oligosaccharide chains on the porcine 130 kDa protein, two of which may be of a high-mannose type. This is in reasonable agreement with the results of Yamashita *et al.* (1988), who have found that the aminopeptidase N in rat kidney brush-border membrane contains on average 5.5 *N*-linked oligosaccharide structures per 130 kDa subunit of aminopeptidase N. The oligosaccharide structures were comprised of a mixture of five types of high-mannose sugar chains and 32 types of mono-antennary, biantennary, triantennary, and tetra-antennary complex sugar chains. There are nine potential *N*-linked glycosylation sites [Asn-Xaa-Ser(Thr)] available in rat kidney zinc peptidase (Watt & Yip, 1989) and ten potential *N*-linked glycosylation sites in human intestinal aminopeptidase N (Olsen *et al.*, 1988), values that are in good agreement with our predictions of eight *N*-linked oligosaccharide chains for the porcine renal enzyme.

GP 130 was shown to be localized to the brush-border membrane by subcellular fractionation and immunoblotting. This is in agreement with other methods, such as immunofluorescence (Feracci *et al.*, 1982a), which localized the aminopeptidase N to brush-border membrane in rabbit and pig enterocytes. The antibody recognized the deglycosylated protein, but did not cross-react with any protein from other species' brush-border membranes. The lack of cross-reactivity leads us to speculate that there are differences in the amino acid sequences of the different species' aminopeptidase N, even though the first

40 amino acids of human intestinal and rat and porcine renal aminopeptidase N are very similar. The antibody cross-reacted with a protein in LLC-PK₁ with a molecular mass of 130 kDa, suggesting that this pig kidney-cell line contains aminopeptidase N.

The active site of aminopeptidase N is located in the large extracellular domain (Maroux *et al.*, 1973), as proteinase treatment of intact brush-border membrane released a functional and soluble aminopeptidase N (Feracci *et al.*, 1981). The antibody did not inhibit the aminopeptidase N enzymic activity in porcine renal brush-border membrane, even though some of the antibody may bind to the functional domain. The antibody did inhibit the aminopeptidase N enzymic activity in purified GP 130. Access of the antibody to the inhibitory epitope may be restricted in the brush-border membranes.

Porcine renal aminopeptidase N was shown to contain two Zn²⁺ ions per mol of protein (Wacker *et al.*, 1971) or one Zn²⁺ per subunit, since pig renal aminopeptidase N is dimeric (Lehky *et al.*, 1973). Overnight incubation of GP 130 with 25 mM-EDTA almost completely inhibited the aminopeptidase N activity, which is probably due to the removal of the Zn²⁺ ions from aminopeptidase N. However, its activity could be fully restored by adding back equivalent amounts of Zn²⁺ and Cu²⁺, but not by adding Mg²⁺, Mn²⁺ and Ca²⁺. This is in good agreement with results of other investigators (Lehky *et al.*, 1973), who showed that Zn²⁺ and Cu²⁺, but not Mg²⁺, Mn²⁺ and Ca²⁺, could restore the activity of apo-aminopeptidase N whose bivalent cations have been chelated away.

Excess Zn²⁺ inhibited the aminopeptidase N enzymic activity of GP 130 and the porcine renal brush-border membranes. Excess Zn²⁺ inhibits pig kidney aminopeptidase N (Lehky *et al.*, 1973). The inhibition of aminopeptidase enzymic activity by excess Zn²⁺ could be a similar mechanism to Zn²⁺ inhibition of carboxypeptidase A (Hirose *et al.*, 1987), which is also a metalloenzyme. This suggests that there might be another bivalent-cation-binding site present in GP 130 which caused inhibition of activity upon binding of a bivalent cation.

Bestatin and 1,10-phenanthroline also inhibited the aminopeptidase N enzymic activity of GP 130 and the porcine renal brush-border membranes. These two inhibitors are also known to inhibit rabbit intestinal aminopeptidase N enzymic activity (Hirota *et al.*, 1985), suggesting that the protein in brush-border membrane with aminopeptidase N activity is indeed aminopeptidase N.

GP 130 has an anion-binding site, as judged by the ability of certain anions, such as bicarbonate, to block specifically the binding of GP 130 to SITS-Affi-Gel. Bicarbonate had been suggested to take part in the activity of the metalloproteins lactoferrin and transferrin (Baker *et al.*, 1987). These two proteins require Fe³⁺ as a cofactor. Since Fe³⁺ has three positive charges, it will have to be stabilized by negative charges in the binding site or by the negative charge of an anion like bicarbonate. GP 130 might have a similar metal-binding site, because GP 130 is a metalloenzyme and has an anion-binding site. Addition of bicarbonate did not stimulate aminopeptidase activity, even though the assay buffer was bubbled with N₂ gas to deplete bicarbonate. High concentrations of bicarbonate inhibited enzyme activity, but this may be due to an ionic-strength effect. Stilbene disulphonates did not alter the enzymic activity. This suggests that GP 130 contains an anion-binding site, but that the presence or absence of anions does not interfere with its aminopeptidase enzymic activity. GP 130 might have two binding sites, where one binding site has a tightly bound anion so that even depleting the assay media of bicarbonate or the addition of stilbene disulphonates was not sufficient to displace the tightly bound anion from the aminopeptidase N. The other anion-

binding site may be where the stilbene disulphonates bind, but the presence or absence of stilbene disulphonate does not affect enzymic activity, suggesting that this anion-binding site does not affect the catalytic activity.

In conclusion, GP 130 is identified as the aminopeptidase N in porcine renal brush-border membranes by its *N*-terminal sequence and enzymic activity. It contains approx. 20 kDa of *N*-linked high-mannose and complex carbohydrate. GP 130 is the major concanavalin-A binding protein in the porcine renal brush-border membrane and it is present in LLC-PK₁ cells. It possesses one or more anion-binding sites that also bind stilbene disulphonates. Aminopeptidase N is unlike other aminopeptidases which are activated by anions, like the chloride-activated aminopeptidase A in human brain (McDermott *et al.*, 1988) or the anion-activated aminopeptidase B of human skeletal muscle (Ishiura *et al.*, 1987).

This work was funded by a generous grant from the Medical Research Council of Canada. H.S. was supported by a University of Toronto Open Scholarship. Dr. M. Blum is acknowledged for performing the amino acid analysis and protein sequencing. Dr. J. H. M. Charuk provided help and advice concerning the lectin binding and endoglycosidase experiments. We thank Dr. T. Salerno and Dr. K. L. Skorecki for providing the pig kidneys and LLC-PK₁ cells respectively, and Dr. D. B. Williams and D. M. Lieberman for critical readings of the manuscript.

REFERENCES

- Aronson, P. S., (1989) *Annu. Rev. Physiol.* **51**, 419–441
- Baker, E. N., Rumball, S. V. & Anderson, B. F. (1987) *Trends. Biochem. Sci.* **12**, 350–353
- Booth, A. G. & Kenny, A. J. (1974) *Biochem. J.* **142**, 575–581
- Booth, A. G., Hubbard, L. M. L. & Kenny, A. J. (1979) *Biochem. J.* **179**, 397–405
- Danielsen, E. M., Norén, O. & Sjöström, H. (1982) *Biochem. J.* **204**, 323–327
- Dodge, J. T., Mitchell, C. & Hanahan, D. J. (1963) *Arch. Biochem. Biophys.* **100**, 119–130
- Feracci, H. & Maroux, S. (1980) *Biochim. Biophys. Acta* **599**, 448–463
- Feracci, H., Benajiba, A., Gorvel, J. P., Doumeng, Ch. & Maroux, S. (1981) *Biochim. Biophys. Acta* **658**, 148–157
- Feracci, H., Bernadac, A., Gorvel, J. P. & Maroux, S. (1982a) *Gastroenterology* **82**, 317–324
- Feracci, H., Maroux, S., Bonicel, J. & Desnuelle, P. (1982b) *Biochim. Biophys. Acta* **684**, 133–136
- Grassl, S. M., Holahan, P. D. & Ross, C. R. (1987) *J. Biol. Chem.* **262**, 2682–2687
- Hirose, J., Ando, S. & Kidani, Y. (1987) *Biochemistry* **26**, 6561–6565
- Hirota, T., Nishikawa, Y., Takahagi, H., Igarashi, T. & Kitagawa, H. (1985) *Res. Commun. Chem. Pathol. Pharmacol.* **49**, 435–455
- Hull, R. N., Cherry, W. R. & Weaver, G. W. (1976) *In Vitro* **12**, 670–677
- Ishiura, S., Yamamoto, T., Yamamoto, M., Nojima, M., Aoyagi, T. & Sugita, H. (1987) *J. Biochem. (Tokyo)* **102**, 1023–1031
- Jennings, M. L. & Passow, H. (1979) *Biochim. Biophys. Acta* **554**, 498–519
- Karniski, L. P. & Aronson, P. S. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 6362–6365
- Karniski, L. P. & Aronson, P. S. (1987) *Am. J. Physiol.* **253**, F513–F521
- Knauf, P. A. & Rothstein, A. (1971) *J. Gen. Physiol.* **58**, 190–210
- Kotaki, A., Naoi, M. & Yagi, K. (1971) *Biochim. Biophys. Acta* **229**, 547–556
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Lehky, P., Lisowski, J., Wolf, D. P., Wacker, H. & Stein, E. A. (1973) *Biochim. Biophys. Acta* **321**, 274–281
- Lowe, A. G. & Lambert, A. (1983) *Biochim. Biophys. Acta* **694**, 353–374
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Maroux, S., Louvard, D. & Baratti, J. (1973) *Biochim. Biophys. Acta* **321**, 282–295
- McDermott, J. R., Mantle, D., Lauffart, B., Gibson, A. M. & Biggins, J. A. (1988) *J. Neurochem.* **50**, 176–182
- Olmsted, J. B. (1981) *J. Biol. Chem.* **256**, 11955–11957
- Olsen, J., Cowell, G. M., Königshofer, E., Danielsen, E. M., Möller, J., Lausten, L., Hansen, O. C., Welinder, K. G., Engberg, J., Hunziker, W., Spiess, M., Sjöström, H. & Norén, O. (1988) *FEBS Lett.* **238**, 307–314
- Passow, H. (1986) *Rev. Physiol. Biochem. Pharmacol.* **103**, 62–223
- Pimplikar, S. W. & Reithmeier, R. A. F. (1986) *J. Biol. Chem.* **261**, 9770–9778
- Pimplikar, S. W. & Reithmeier, R. A. F. (1988) *J. Biol. Chem.* **263**, 4485–4493
- Ramjessingh, M., Grinstein, S. & Rothstein, A. (1980) *J. Membr. Biol.* **57**, 95–102
- Sacktor, B., Rosenbloom, I. L., Liang, C. T. & Cheng, L. (1981) *J. Membr. Biol.* **60**, 63–71
- Stewart, J. R. & Kenny, A. J. (1984) *Biochem. J.* **224**, 549–558
- Svensson, B., Sjöström, H. & Norén, O. (1982) *Eur. J. Biochem.* **126**, 481–488
- Tanford, C., Nozaki, Y., Reynolds, J. A. & Makino, S. (1974) *Biochemistry* **13**, 2369–2376
- Vannier, Ch., Louvard, D., Maroux, S. & Desnuelle, P. (1976) *Biochim. Biophys. Acta* **455**, 185–199
- Wacker, H., Lehky, P., Fischer, E. H. & Stein, E. A. (1971) *Helv. Chim. Acta* **54**, 473–485
- Watt, V. M. & Yip, C. C. (1989) *J. Biol. Chem.* **264**, 5480–5487
- Yamashita, K., Tachibana, Y., Matsuda, Y., Katunuma, N., Kochibe, N. & Kobata, A. (1988) *Biochemistry* **27**, 5565–5573

Received 14 December 1989/9 April 1990; accepted 24 April 1990