Involvement of disulphide bonds in the renal sodium/phosphate co-transporter NaPi-2

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The rat renal brush border membrane sodium/phosphate co-transporter NaPi-2 was analysed in Western blots with polyclonal antibodies raised against its N-terminal and C-terminal segments. Under reducing conditions, proteins of 45–49 and 70–90 kDa (p45 and p70) were detected with N-terminal antibodies, and proteins of 40 and 70–90 kDa (p40 and p70) were detected with C-terminal antibodies. p40 and p45 apparently result from a post-translational cleavage of NaPi-2 but remain linked through one or more disulphide bonds. Glycosidase digestion showed that both polypeptides are glycosylated; the cleavage site could thus be located between Asn-298 and Asn-328, which have been shown to constitute the only two N-glycosylated residues in NaPi-2. In the absence of reducing agents, both N-terminal and C-terminal antibodies detected p70 and a protein of 180 kDa (p180), suggesting the presence of p70 dimers. Much higher concentrations of β -mercaptoethanol were required to produce a given effect in intact membrane vesicles than in solubilized

proteins, indicating that the affected disulphide bonds are not exposed at the surface of the co-transporter. Phosphate transport activity decreased with increasing concentrations of reducing agents [β -mercaptoethanol, dithiothreitol and tris-(2-carboxyethyl)phosphine] and was linearly correlated with the amount of p180 detected. The target sizes estimated from the radiation-induced loss of intensity of p40, p70 and p180 were all approx. 190 kDa, suggesting that NaPi-2 exists as an oligomeric protein in which the subunits are sufficiently close to one another to allow substantial energy transfer between the monomers. When protein samples were pretreated with β -mercaptoethanol [2.5% and 5% (v/v) to optimize the detection of p40 and p70] before irradiation, target sizes estimated from the radiation-induced loss of intensity of p40 and p70 were 74 and 92 kDa respectively, showing the presence of disulphide bridges in the molecular structure of NaPi-2.

INTRODUCTION

Renal reabsorption of inorganic phosphate from the glomerular filtrate takes place predominantly across the brush border membrane (BBM) of the proximal tubule [1]. This process, under hormonal and cellular control [2,3], involves specific sodium gradient-dependent transport systems [4–6]. Renal phosphate transport has been functionally well characterized and the recent molecular identification of proximal tubular Na^+/P_i co-transporters from several animal species [7–14] and renal epithelial cell lines [15,16] has broadened our understanding of their molecular structure, function and regulation. Na^+/P_i co-transporters have been classified into two families, type I and type II, on the basis of their amino acid sequences [6]. Both types differ in molecular mass, possible transmembrane domains, functional motifs and modes of regulation [6]. From their predicted molecular masses, type I co-transporters [7–9] are smaller proteins (51–52 kDa) than type II co-transporters [10–16] (69–75 kDa). Translation experiments *in vitro* have demonstrated that co-transporters of both types are N-glycosylated [7,10,17]. Both type I and type II co-transporters are found mainly in kidney cortex, but type I co-transporters are more abundant in the juxtamedullary portion of the cortex than in its superficial portion [18,19].

Type II co-transporters have been shown to be important in the adaptive response to a low- P_i diet [14,20], but type I co-

transporters do not seem to be involved [18,21]. Recently we demonstrated that phosphate deprivation not only results in higher levels of a 70 kDa protein, corresponding in size to that calculated from the amino acid sequence of the rat type II co-transporter NaPi-2 [10], but also to the overproduction of an immunologically related 40 kDa protein [22]. The present study was undertaken to further examine the possible relationship between these two proteins, which share similar immunological, chemical and physical characteristics. Earlier studies have shown that thiol groups are essential for the function of renal Na^+/P_i co-transporters, although these groups are not located at the substrate-binding sites [23–25]. The electrophoretic mobility of renal Na^+/P_i co-transporters is also known to be greatly influenced by the presence or absence of reducing agents [18,22]. Biochemical and immunochemical approaches were therefore used to study the involvement of disulphide bridges in the oligomeric structure and function of NaPi-2. Using the Western blot technique and polyclonal antibodies raised against its N-terminal and C-terminal portions, we demonstrate that this rat Na^+/P_i co-transporter is composed of two protein subunits linked together by disulphide bonds. In addition, dimers of this protein complex, also linked by disulphide bonds, were found to be present in the renal BBM. Finally, target size analysis [26–29] of the radiation-induced disappearance of NaPi-2 polypeptides revealed that this Na^+/P_i co-transporter forms a larger oligomeric

Abbreviations used: BBM, brush border membrane; DTT, dithiothreitol; TCEP, tris-(2-carboxyethyl)phosphine; TS, target size.

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structure, with considerable transfer of the absorbed energy between different subunits, as was previously shown for the type I co-transporters of rat and rabbit [30].

MATERIALS AND METHODS

Preparation of BBM vesicles

Male Sprague–Dawley rats (300–350 g; 2 months old) were purchased from the Charles River Co. (St-Constant, Que., Canada). To examine the effect of phosphate deprivation, rats were fed with either a low-phosphate (no. 86128) or a standard (no. 86129) diet from Teklad (Madison, WI, U.S.A.) containing 0.03 % (w/w) or 1 % (w/w) phosphate for 3 days. BBM vesicles were prepared from kidney cortex with a MgCl_2 precipitation method [31]. The final pellet was resuspended in 300 mM mannitol and 20 mM Hepes/Tris, pH 7.5 (buffer A) and the following protease inhibitors: chymostatin A (10 $\mu\text{g}/\text{ml}$), bacitracin (10 $\mu\text{g}/\text{ml}$), pepstatin (10 $\mu\text{g}/\text{ml}$) and aprotinin (1.85 $\mu\text{g}/\text{ml}$), or, for radiation inactivation experiments, in a cryoprotective medium composed of 150 mM KCl, 14 % (w/v) glycerol, 1.4 % (w/v) sorbitol and 5 mM Hepes/Tris, pH 7.5, and stored in liquid nitrogen until use. Protein concentration was determined by the method of Bradford [32] with BSA as standard protein. Alkaline phosphatase, a BBM marker enzyme assayed as described by Kelly and Hamilton [33], was enriched 10–15-fold in the final vesicle preparations relative to the cortex homogenate.

Immunization and antibody purification

The 15-residue peptide CPRLALPAHHNATRL, corresponding to the C-terminal portion of NaPi-2, has previously been synthesized and used for antibody production [22]. Here the 14-residue peptide MMSYSERLGGPAVS, corresponding to the N-terminal portion of NaPi-2 and synthesized with the multiple antigen peptide (MAP) system [34], was obtained from the Service de Séquence de Peptides de l'Est du Québec (Centre Hospitalier de l'Université Laval, Ste-Foy, Que., Canada) at a purity of more than 85 %. This peptide was used for antibody production with previously described immunization and antibody purification protocols [22] except that the N-terminal peptide did not require coupling to keyhole limpet haemocyanin [34].

Western blot analysis

SDS/PAGE was performed with a Mini-Protean II electrophoresis apparatus (Bio-Rad, Mississauga, Ont., Canada) and the Laemmli buffer system [35]. The separating gel contained 7.5 % (w/v) acrylamide and 0.23 % bisacrylamide before polymerization. The samples were mixed with sample buffer containing a final concentration of 20 % (w/v) glycerol, 1 % (w/v) SDS, 0.05 % Bromophenol Blue and 0.625 M Tris/HCl, pH 6.8, with the indicated concentrations of reducing agent [β -mercaptoethanol, dithiothreitol (DTT) or tris-(2-carboxyethyl)phosphine (TCEP)]. Broad-range protein standards (Bio-Rad) were used for the calibration of molecular masses. Electrophoresis was performed at 10 mA per 0.75 mm gel for approx. 1.5 h. Each well contained 12 μg of protein. After electrophoresis the proteins were transferred electrophoretically to poly(vinylidene difluoride) microporous (0.45 μm pore diameter) membranes (Millipore, Mississauga, Ont., Canada) in a Milliblot Graphite Electrobloater I apparatus (Millipore) at 1 mA/cm² for 1 h at room temperature. Non-specific binding sites on poly(vinylidene difluoride) membranes were blocked by incubation overnight in 150 mM NaCl/0.2 % (w/v) Tween-

20/50 mM Tris/HCl (pH 7.5) (buffer B), containing 5 % (w/v) powdered milk, at 4 °C. The membranes were washed three times by gentle agitation in buffer B for 10 min and incubated for 1 h with a 1:10000 dilution of either anti-peptide (N-terminal or C-terminal) antibody in buffer B. After three more washes in buffer B, the membranes were incubated for 1 h with a 1:1000 dilution of anti-rabbit IgG horseradish peroxidase-linked whole antibody from donkey (Amersham, Oakville, Ont., Canada) in buffer B. The membranes were washed a final time as described above; labelled antigens were revealed with enhanced chemiluminescence (ECL) Western blotting solutions (Amersham). The membranes were exposed to Fuji films that had been preflashed with a Sensitize flashgun unit (Amersham). The X-ray films were developed with a Mini-Med/90 X-ray film processor (AFP Imaging, Elmsford, NY, U.S.A.), scanned with a Personal Densitometer SI scanner (Molecular Dynamics) and analysed with the IPLab Gel H program (Signal Analytics Corporation).

Deglycosylation

BBM proteins were solubilized in 1 % (w/v) SDS for 10 min at room temperature and diluted to a final concentration of 1 mg/ml in a buffer composed of 0.3 % (v/v) Brij-58 and 40 mM Tris/HCl, pH 7.2. Endoglycosidase F/N-glycosidase F mixture (Boehringer-Mannheim, Laval, Que., Canada) was added at 1 unit/mg of protein and the reaction continued for 17 h at 37 °C with gentle shaking. Sample buffer containing 5 % (v/v) β -mercaptoethanol was added and the samples were analysed in Western blots.

Pretreatment of BBM vesicles with reducing agents

BBM vesicles (approx. 1 $\mu\text{g}/\mu\text{l}$ protein) in buffer A were incubated at 25 °C with gentle stirring for 30 min in the presence of various concentrations of reducing agents (β -mercaptoethanol, DTT or TCEP). Buffer A was added and samples were centrifuged for 20 min at 50000 *g* at 4 °C to wash out the unreacted reagents. Two more washes were done before the pellets were finally resuspended at approx. 6 $\mu\text{g}/\mu\text{l}$ protein in buffer A. These samples were used for parallel analysis of phosphate transport activity (described below) and detection of NaPi-2-related proteins in Western blots. For Western blot analyses, samples were mixed with sample buffer without any reducing agent and loaded on an SDS/PAGE gel.

Transport measurements

Uptake of radiolabelled phosphate into BBM vesicles was measured at 25 °C with a rapid filtration method [36]. For transport assays, 5 μl of the vesicles treated with reducing agent were added to 25 μl of a solution containing 200 μM [^{32}P]KH₂PO₄/K₂HPO₄, 5 mM Hepes/Tris, pH 7.5, and 150 mM NaCl or KCl. After incubation for 9 s, the reaction was stopped with 1 ml of ice-cold stop solution containing 150 mM KCl and 5 mM Hepes/Tris, pH 7.5. The suspension was filtered immediately under vacuum through a nitrocellulose (Millipore) filter (0.45 μm pore diameter). The filter was rinsed with an additional 7 ml of stop solution and the radioactivity from the filter was measured. For the estimation of the intravesicular volume we used the method of D-glucose at equilibrium [30].

Irradiation procedure

BBM vesicles were exposed to γ -rays in a Model 220 Gammacell ⁶⁰Co irradiator (Atomic Energy of Canada) at a dose rate of approx. 0.75 Mrad/h [37]. During this procedure the samples

were kept at -78°C with crushed dry ice. The target size (TS) was calculated from the loss of intensity of the immunodetected protein bands corresponding to the phosphate co-transporter by using the following empirical equation [38], which is valid for samples irradiated at -78°C :

$$TS = 1.29 \times 10^6 / D_{37}$$

where D_{37} is the radiation dose (in Mrad) at which the intensity of the immunodetected bands has been decreased to 37% of its initial value. D_{37} values were obtained from semilogarithmic plots of the absorbance against irradiation dose by using a least-squares fit. As an internal control for the irradiation procedure we used alkaline phosphatase and 5'-nucleotidase. Both were assayed by standard procedures [39].

RESULTS

Polyclonal antibodies directed against the N-terminal and C-terminal portions of the rat renal Na⁺/P_i co-transporter NaPi-2 [10] were raised in rabbits, affinity-purified and used to identify related proteins in BBM from rats. Adaptation to chronic dietary phosphate deprivation is well known to involve an increase in Na⁺/P_i co-transport in BBM from the proximal tubule [40,41]. Under such conditions of low dietary phosphate, the initial rate of Na⁺-dependent phosphate transport into BBM vesicles is increased 3–4-fold [18]. The low dietary phosphate intake led to a 15–25-fold increase in the expression of NaPi-2-related proteins as assessed by Western blot analysis (Figure 1). Such an induction has previously been shown for p70 [22,42]. When 1 μM antigenic peptide was added during incubation with the antibodies, no proteins were detected (results not shown), demonstrating the specificity of the antibodies. Under non-reducing conditions, probing with N-terminal antibodies revealed proteins of 70–90 and 180 kDa, the first being designated the p70 group, and the latter p180. C-terminal antibodies also detected a 180 kDa protein along with a large band migrating as proteins of 70–90 kDa (also designated the p70 group). In the presence of 1% (v/v) β -mercaptoethanol or 5 mM DTT, p180 was not detected by either antibody. However, new lower-molecular-mass polypeptides became apparent. N-terminal antibodies revealed proteins of 45 and 49 kDa (together designated the p45 group), while C-terminal antibodies recognized a protein mi-

grating slightly faster, with a molecular mass of approx. 40 kDa (p40). Not only is the expression of p40 induced by a low-phosphate diet as previously shown [22], but so is the expression of p45. The amount of detected proteins was a linear function of the amount of protein from rat BBMs loaded on the gel (Figure 2).

To investigate the relationship between these NaPi-2-related proteins, slices corresponding to the regions of 70–90 and 180 kDa were excised from a first gel made without any reducing agent. Proteins from the excised gel slices were resolved on a second SDS/PAGE gel in the presence or absence of β -mercaptoethanol and analysed by Western blotting with C-terminal antibodies (Figure 3). In the presence of β -mercaptoethanol, the p70 band became much fainter and a strong band corresponding to p40 became apparent. Under the same conditions, p180 was resolved into p40 and p70, with very little p180 remaining. Reprobing of the blot with N-terminal antibodies yielded the same results, except that p45 was detected instead of p40 (results not shown).

Figure 2 Calibration of immunoblots

BBM proteins were resolved by SDS/PAGE and transferred to poly(vinylidene difluoride) membranes as described in the Materials and methods section. The Western blots were incubated with affinity-purified antibodies, followed by donkey anti-rabbit IgG horseradish peroxidase-linked antibodies. The labelled proteins were revealed with enhanced chemiluminescence Western blotting solutions. The intensities of the specifically labelled protein bands corresponding to p70 were measured by densitometric scanning of the Western blots and analysed with IPLab Gel H program.

Figure 1 Immunodetection of rat renal BBM proteins with polyclonal antibodies directed against the sodium/phosphate co-transporter NaPi-2

BBM were prepared from rats fed with a control (N) or a low-phosphate (H) diet. Proteins were solubilized in sample buffer without any reducing agent (NR) or containing 1% β -mercaptoethanol (β -EtSH) or 5 mM DTT. These protein samples were resolved by SDS/PAGE [7.5% (w/v) gel]. Immunoblots were performed as described in the Materials and methods section with antibodies directed against the N-terminal and C-terminal portions of NaPi-2.

Figure 3 Relationship between NaPi-2-related proteins

BBM proteins were resolved on a first gel under non-reducing conditions. The regions of the gel containing proteins of 70–90 kDa (p70) and 180 kDa (p180) were obtained by slicing the gel. Excised gel slices were incubated in sample buffer containing either 5% (+) or no (–) β -mercaptoethanol (β -EtSH) for 30 min at 25°C . Samples were loaded on a second gel (12.5% polyacrylamide) for Western blot analysis. BBM protein preparations (BBM) were also loaded on the second gel as controls. The blots were probed with antibodies directed against the C-terminal portions of NaPi-2 and analysed as described in the Materials and methods section.

Figure 4 Deglycosylation of renal BBM NaPi-2-related proteins

BBM proteins were solubilized in 1% SDS, followed by incubation overnight at 37 °C with endoglycosidase F/N-glycosidase F, and resolved by SDS/PAGE [7.5% (w/v) gel] under reducing conditions (lanes 2). Both N-terminal (A) and C-terminal antibodies (B) were used for the detection of NaPi-2-related proteins. Three controls were included: BBM proteins loaded directly on the gel without incubation (lanes 1), BBM proteins incubated overnight without glycosidase (lanes 3) and the enzyme mixture alone loaded on the gel without incubation (lanes 4).

Figure 5 Effect of β -mercaptoethanol concentration on the electrophoretic mobility of solubilized NaPi-2-related proteins

BBM proteins were first solubilized in sample buffer containing 1% SDS without reducing agent. β -mercaptoethanol (β -EtSH) was then added at the indicated concentrations. Membrane proteins were resolved and Western blots were probed with N-terminal anti-NaPi-2 antibodies as described in the Materials and methods section.

Glycosidase digestion of BBM proteins resulted in a significant shift in the migration of NaPi-2-related proteins (Figure 4). The apparent molecular mass of p70 was decreased to 57 kDa (Figures 4A and 4B, lanes 2) and that of p40 was decreased to 30 kDa (Figure 4B, lane 2). Reprobing the same blot with N-terminal antibodies showed that p45 was deglycosylated to a 35 kDa polypeptide (Figure 4A, lane 2). The control samples incubated overnight without endoglycosidase F/N-glycosidase F (Figures 4A and 4B, lanes 3) were unchanged. No protein band was detected when the glycosidase was loaded alone (Figures 4A and 4B, lanes 4).

To examine further the effect of reducing agents on the electrophoretic mobility of NaPi-2-related proteins, BBM vesicles from rats fed with a low-phosphate diet, which express a greater amount of NaPi-2, were either solubilized before (Figure 5) or after (Figures 6A, 7A and 8A) treatment with reducing agents. First, BBM vesicles were previously solubilized with sample buffer containing SDS, incubated with increasing concentrations of β -mercaptoethanol and analysed with N-terminal antibodies (Figure 5). The amount of p180 detected in the absence of β -mercaptoethanol was much smaller than that of p70. Between

Figure 6 Effect of β -mercaptoethanol on the electrophoretic mobility of NaPi-2-related proteins and phosphate uptake into BBM vesicles

BBM vesicles were incubated in buffer A containing the indicated concentrations of β -mercaptoethanol (β -EtSH) as described in the Materials and methods section. To remove any unreacted β -mercaptoethanol, samples were centrifuged and pellets were washed twice with buffer A. Proteins were resolved under non-reducing conditions and analysed in Western blots with N-terminal antibodies (A). Phosphate uptake into vesicles and the amount of p180 detected were plotted, with each point corresponding to a different concentration of β -mercaptoethanol (B): 100% phosphate uptake represents the phosphate transported in the absence of any reducing agent, which corresponds to 3.6×10^5 pmol of P_i /s per μ g of protein; 100% p180 detection represents the amount of p180 detected under non-reducing conditions as obtained by densitometric scanning of the band obtained on the X-ray film after Western blotting.

0.01 and 1% β -mercaptoethanol the detection of p180 was abolished. The amount of p70 detected increased as the concentration of β -mercaptoethanol was raised from 0.001 to 0.01%, and decreased rapidly as the reducing agent was brought to higher concentrations. p45 became apparent when at least 0.1% β -mercaptoethanol was added to the BBM proteins, and the amount detected increased further when the concentration of β -mercaptoethanol was raised to 1%.

Secondly, when intact membrane vesicles were pretreated with various concentrations of reducing agents and proteins were then solubilized under non-reducing conditions before analysis in Western blots, p180 was still detected along with p70 with up to 3.5–4% β -mercaptoethanol (Figure 6A), 100 mM DTT (Figure 7A) or 5 mM TCEP (Figure 8A). This result also shows that all reducing agents do not possess the same reducing power on NaPi-2 (DTT reacts at higher concentrations than β -mercaptoethanol or TCEP). At higher concentrations p180 was no longer detected, whereas the p70 signal decreased and that of p45 increased. This pattern was similar to that observed with previously SDS-solubilized BBM proteins (Figure 5), with the notable difference that much higher concentrations of β -mercaptoethanol were required to obtain the same immuno-detection band profile.

Figure 7 Effect of DTT on the electrophoretic mobility of NaPi-2-related proteins and phosphate uptake into BBM vesicles

BBM vesicles were incubated in buffer A containing the indicated amount of DTT as described in the Materials and methods section, and washed as described in the legend to Figure 6 to remove any unreacted DTT. Proteins were resolved under non-reducing conditions and analysed in Western blots with N-terminal antibodies (A). Phosphate uptake into vesicles and the amount of p180 detected were plotted, with each point corresponding to a different concentration of DTT (B); 100% phosphate uptake represents the phosphate transported in the absence of any reducing agent, which corresponds to 3.6×10^5 pmol of P_i/s per μ g of protein; 100% p180 detection represents the amount of p180 detected under non-reducing conditions as obtained by densitometric scanning of the band obtained on the X-ray film after Western blotting.

A linear correlation was observed between phosphate transport activity and the amount of p180 detected in BBM vesicles pretreated with different concentrations of β -mercaptoethanol (Figure 6B), DTT (Figure 7B) or TCEP (Figure 8B). Such a correlation was observed only for p180; in fact, as the concentration of β -mercaptoethanol was brought from 0 to 3%, the intensity of p45 and p70 increased, whereas the transport activity decreased markedly (Figure 6A). When vesicles were pretreated with any of these three reducing agents and then washed (i.e. uptake and detection were determined under non-reducing conditions), their intravesicular volume remained unchanged, showing that the vesicles kept their integrity (results not shown). The uptake was linear for at least 10 s of incubation; the transport measurements used, which were done at 9 s, were thus within the linear portion of the curve (Figure 9).

Exposure of BBM vesicles to ionizing radiation resulted in a dose-dependent decrease in the intensity of the NaPi-2-related proteins (Figure 10A). The radiation-induced disappearance of p40, p70 and p180, each detected with C-terminal antibodies, was a simple exponential function of the radiation dose (Figure 10B). The target sizes calculated from the data shown in Figure 10(B) were 178 ± 20 kDa (mean $\pm$ S.D.) for p40, 179 ± 18 kDa for p70 and 199 ± 22 kDa for p180. When the vesicles were pretreated with 2.5% or 5% β -mercaptoethanol [to obtain an optimal amount of p70 or p40 respectively (Figure 11A)] before ir-

Figure 8 Effect of TCEP on the electrophoretic mobility of NaPi-2-related proteins and phosphate uptake into BBM vesicles

BBM vesicles were incubated in buffer A containing the indicated amount of TCEP as described in the Materials and methods section, and washed as described in the legend to Figure 6 to remove any unreacted TCEP. Proteins were resolved under non-reducing conditions and analysed in Western blots with N-terminal antibodies (A). Phosphate uptake into vesicles and the amount of p180 detected were plotted, with each point corresponding to a different concentration of TCEP (B); 100% phosphate uptake represents the phosphate transported in the absence of any reducing agent, which corresponds to 3.6×10^5 pmol of P_i/s per μ g of protein; 100% p180 detection represents the amount of p180 detected under non-reducing conditions as obtained by densitometric scanning of the band obtained on the X-ray film after Western blotting.

Figure 9 Initial rate of phosphate uptake by BBM vesicles

BBM vesicles were isolated as described in the Materials and methods section. Phosphate uptake was measured in the presence of 150 mM NaCl as described in the text. Each value represents the mean $\pm$ S.D. for three experiments in which each assay was performed in quadruplicate.

radiation, the *TS* values obtained from the radiation-induced disappearance of p40 and p70 (Figure 11B) were approx. 74 and 92 kDa respectively (from Figure 11C). As previously shown, the intravesicular volume was not affected by the radiation dose [30].

Figure 10 Determination of the target size of the rat renal sodium/phosphate co-transporter NaPi-2

BBM vesicles were irradiated and analysed in Western blots with C-terminal antibodies as described in the Materials and methods section. (A) Effect of radiation dose on the structural integrity of NaPi-2-related proteins. For the analysis of p180 and p70, electrophoresis was performed in the absence of reducing agents. Samples used for the analysis of p40 were solubilized in sample buffer containing 5% β -mercaptoethanol. (B) Disappearance of the immunodetected proteins was measured by laser densitometry. The results are expressed as the logarithm of the percentage area remaining under each peak relative to the unirradiated control for p180 ($\circ$), p70 ($\bullet$) and p40 ($\blacksquare$). Each value represents the mean $\pm$ S.D. for four independent experiments.

To establish the validity of the irradiation method in our system, the inactivation of two intrinsic enzyme markers was investigated. For alkaline phosphatase the inactivation curve gave a molecular mass of 104800 ± 3500 Da (Figure 12A). This value is similar to that reported for calf kidney alkaline phosphatase (115 kDa) estimated by the same method [43] and for isolated alkaline phosphatase [44] purified by SDS/PAGE (120 kDa). The estimated molecular mass of 5'-nucleotidase was 89400 ± 1800 Da (Figure 12B). This is similar to the value estimated in fibroblasts (80 kDa) [45], but somewhat higher than that found in rabbit kidney (58 kDa) [46].

DISCUSSION

The results of the present study clearly demonstrate a strong effect of reducing agents on the structure and function of the rat renal Na^+/P_i co-transporter NaPi-2. Under non-reducing conditions the major proteins detected with antibodies directed against the N-terminal or C-terminal portions of NaPi-2 had a molecular mass of 70–90 kDa (p70). Because NaPi-2 is glycosylated [10,17], this size is in good agreement with that calculated from its amino acid sequence (68.7 kDa) [10]. Previous studies with polyclonal antibodies have demonstrated that NaPi-2 is present as proteins of 80–90 kDa in the rat renal BBM [17,47] and 70–110 kDa in cRNA-injected *Xenopus laevis* oocytes [17].

Figure 11 Determination of the target size of the rat renal sodium/phosphate co-transporter NaPi-2 after pretreatment with β -mercaptoethanol

BBM vesicles were pretreated with 0%, 2.5% and 5% β -mercaptoethanol (β -EtSH), then irradiated and analysed in Western blots with C-terminal antibodies as described in the Materials and methods section. (A) Immunodetection of NaPi-2-related proteins pretreated with 0%, 2.5% and 5% β -mercaptoethanol. (B) Effect of radiation dose on the structural integrity of NaPi-2-related proteins pretreated with 0%, 2.5% (for optimal p70 detection) or 5% β -mercaptoethanol (for optimal p40 detection). ECL detection was done with Bio-Rad's Molecular Imager System. (C) Disappearance of the immunodetected proteins was measured with Bio-Rad's Molecular Imager System and the IPLab Gel software from Molecular Dynamics. The results are expressed as the logarithm of the percentage area remaining under each peak relative to the unirradiated control for p70 ($\bullet$) and p40 ($\circ$). Each value represents the mean $\pm$ S.D. for two independent experiments.

Under reducing conditions, however, additional proteins of 45–49 kDa (p45) and 40 kDa (p40) were detected with N-terminal and C-terminal antibodies respectively. Recent studies have shown the presence of a 51 kDa protein in OK cells by using antibodies against NaPi-4 [48,49], and a NaPi-2-related 37 kDa protein in mouse BBM [50]. The authors assume these lower bands are degradation products. In contrast, our results clearly show that p45 and p40 are not degradation proteins (Figure 3). Furthermore the addition of protease inhibitors during BBM purification or the incubation of purified BBM with protease inhibitors were without any effect on the detection of p70, p45 and p40, indicating that p45 and p40 are not degradation artifacts created during membrane isolation. Using C-terminal

Figure 12 Radiation-inactivation curves for alkaline phosphatase (A) and 5'-nucleotidase in membrane vesicles

BBM vesicles were irradiated at the indicated doses, and phosphatase (A) and 5'-nucleotidase (B) activities were measured with *p*-nitrophenyl phosphate or AMP as substrate respectively [37] ($n = 4$).

antibodies, we have recently shown by tryptic proteolysis experiments that p40 is closely related to p70 [22]. In the present study we demonstrate that an increase in the amount of p40 or p45 detected, owing to the presence of β -mercaptoethanol, DTT or TCEP, is accompanied by a loss of p70. Each of these three reducing agents possesses a different strength of reduction on NaPi-2 (e.g. DTT reacts at much higher concentrations than β -mercaptoethanol or TCEP). These results strongly suggest that p70 is composed of two protein fragments held together by one or more disulphide bonds and resulting from a specific post-translational proteolytic cleavage of the NaPi-2 polypeptide. Both of these fragments, p40 and p45, were shown to be glycosylated. Because NaPi-2 has previously been shown, with site-directed mutagenesis experiments, to be glycosylated at only two of its four potential N-glycosylation sites [17], the proteolytic cleavage site could be located between Asn-298 and Asn-328. Our proposed model is reinforced by a recent report suggesting that NaPi-II is a co-transporter that could originate from two ancient independent genes [51]. The first would represent a protein with three transmembrane regions; the second would represent a protein with four transmembrane regions. These proteins could represent p45 and p40 respectively.

Glycosidase digestion significantly decreased the apparent molecular masses of p40, p45 and p70. After deglycosylation, p70 migrated as a protein of 57 kDa, a value somewhat smaller than that calculated from the amino acid sequence of NaPi-2 (68.7 kDa) [10]. This difference, however, might be due to the presence of disulphide bonds in p70, which could affect its electrophoretic mobility. In contrast, once deglycosylated, p40 and p45 had apparent molecular masses of 30 and 35 kDa respectively. The sum of these values is in remarkably good

agreement with the predicted size of NaPi-2, suggesting that little or no further proteolysis occurs after the initial cleavage of the native polypeptide.

Both antibodies also detected a protein of 180 kDa (p180) that disappeared gradually as the concentration of reducing agent was increased, with a concomitant increase in the detection of the lower-molecular-mass NaPi-2-related proteins. p180 thus seems to correspond to an oligomeric form of NaPi-2 composed of p70 subunits linked through disulphide bonds. These disulphide bridges could be involved in stabilizing the functional oligomeric structure of the Na⁺/P_i co-transporter molecule, as was demonstrated for the type-C natriuretic peptide receptor [52] and the GLUT1 glucose transporter [53]. A striking correlation was demonstrated between the transport activity and amount of p180 detected. When p180 was reduced by the addition of any of the three reducing agents, detection of p70 was increased but p180 disappeared in correlation with a diminution of phosphate uptake into the vesicles. This, as well as the target sizes obtained for NaPi-2-related proteins under non-reducing conditions, strongly suggests that the protein responsible for the transport of phosphate into vesicles is an oligomer rather than a monomer.

At a given concentration the reducing agents had a much weaker effect on the electrophoretic mobility of NaPi-2-related proteins when added to intact BBM vesicles than when added to solubilized proteins. The inter-polypeptide disulphide bonds thus seem to be protected in the intact membrane and become readily exposed only once the membrane proteins have been solubilized and unfolded by SDS.

Our results are at variance with those of Suzuki et al. [54], who reported a slight but significant stimulation of phosphate transport in rabbit renal BBM vesicles preincubated with 5 mM DTT. Phosphate transport was also increased in vesicles reconstituted from bovine renal BBM when 1 mM DTT was included in the buffer used to solubilize the proteins [55]. The different effects of reducing agents observed in these studies could, however, be due to the use of different animal species and experimental conditions. For example, in the present study, the vesicles were incubated at least twice as long and, for most experiments, with higher concentrations of the reducing agents.

Similar target sizes, ranging from 178 to 199 kDa, were estimated from the radiation-induced disappearance of p40, p70 and p180 protein bands in Western blots probed with C-terminal antibodies. These values, which are not significantly different from one another, strongly suggest that all three polypeptide species are part of the same oligomeric structure. Because the oligosaccharide moiety of glycoproteins does not contribute to the molecular sizes determined with the radiation inactivation method [26–29], these TS values correspond to almost three times the size of a p70 subunit. Target sizes corresponding to three times the molecular mass of the constituent monomer have also been measured for the type I Na⁺/P_i co-transporter [30]. In both types of phosphate co-transporter, the different polypeptides thus seem to be closely associated with one another because, after a single radiation hit, the absorbed energy causes the fragmentation of three subunits. Disulphide bonds between p70 subunits might contribute to this energy transfer. The results obtained from vesicles pretreated with β -mercaptoethanol before irradiation demonstrate the effect of reducing agents on the state of oligomerization of NaPi-2. Under these reducing conditions, NaPi-2 exists as a monomer, not an oligomer.

The size of the functional rat renal Na⁺/P_i co-transporter complex, estimated from the radiation-induced loss of phosphate transport activity in BBM vesicles, has been previously shown to be 234 kDa [56]. Similarly, large radiation inactivation sizes have also been reported for the Na⁺/P_i co-transporters of rabbit [30],

mouse [57] and cow [58]. Phosphate transport across the rat renal BBM is, however, probably not performed only by NaPi-2 but also by a type I phosphate co-transporter. Although no type I co-transporter has yet been cloned from rat, both types of phosphate co-transporter have been cloned from rabbit [7,14], human [8,10] and mouse [9,12,13] kidney cortex. In addition, a protein of 65 kDa is detected specifically in Western blots of rat BBM proteins with antibodies raised against the C-terminal portion of the rabbit type I co-transporter NaPi-1, a region in which NaPi-1 and NaPi-2 share little similarity [18,30]. Because type I co-transporters are smaller than type II co-transporters, the size of the functional NaPi-2 oligomer is thus probably larger than 234 kDa. NaPi-2 would consist of a tetramer composed of four p70 subunits each composed of a p40 and a p45 polypeptide. Disulphide bonds would play an important role in the structure and function of this Na^+/P_i co-transporter by holding p40 and p45 polypeptides together within each subunit and forming bridges between some of the p70 subunits.

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