Reconstitution of the partially purified renal phosphate (P_i) transporter

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Proteins from rabbit kidney brush border membranes were solubilized with 1% Nonidet P-40 (crude membrane proteins) and fractionated according to their isoelectric points (pI) by chromatofocusing. The eluate was pooled into three fractions according to the pI of the samples (1, >6.8; 2, 6.8–5.4; 3, 5.4–4.0). The crude membrane proteins as well as the three fractions were reconstituted into liposomes and transport of P_i was measured by a rapid filtration technique in the presence of an inwardly directed K^+ or Na^+ gradient. Arsenate-inhibitable Na^+ -dependent transport of P_i was reconstituted into an osmotically active intravesicular space from both the crude membrane proteins and Fraction 1. In contrast, Fractions 2 and 3 were inactive. Treatment of the crude membrane proteins and the three fractions with the method for extracting phosphorin (a P_i -binding proteolipid found in brush border membranes) yielded Mn^{2+} -dependent binding of P_i characteristic of phosphorin only in the extracts from crude membrane proteins and Fraction 1, the same fractions in which Na^+ -dependent transport of P_i was found in the reconstituted system. When reconstituted into liposomes, phosphorin was, however, unable to yield Na^+ -dependent transport of P_i . Moreover, we cannot eliminate the possibility that Na^+ - P_i transport can occur in the absence of phosphorin, since complete recovery of Na^+ - P_i transport was not achieved. However, the present data showing localization of the recovered binding and transport systems for P_i in the same protein fraction lend support to the hypothesis that phosphorin might be a constituent of the renal P_i transport system. Whether the presence of phosphorin is necessary or accessory for Na^+ -dependent P_i transport in intact brush border membrane vesicles or in liposomes reconstituted with crude or purified membrane proteins requires further investigation.

INTRODUCTION

The brush border membrane of the renal proximal tubule reabsorbs P_i by a Na^+ -dependent transport mechanism (Dennis *et al.*, 1979; Hoffman *et al.*, 1976). Despite extensive studies of P_i transport in brush border membrane vesicles (Burckhardt *et al.*, 1981; Cheng *et al.*, 1983; Hoffman *et al.*, 1976; Sacktor & Cheng, 1981) or in isolated perfused tubules (Dennis *et al.*, 1979), our knowledge about the nature of the P_i transporter is still rudimentary. In contrast with the mitochondrial P_i transporter, which has been partially purified and characterized by different groups (Banerjee & Racker, 1979; Kolbe *et al.*, 1982; Wohlrab, 1980), there are few reported attempts to purify the renal P_i transporter (Lin *et al.*, 1984).

A proteolipid that binds P_i co-operatively and with specificity and high affinity can be extracted from renal brush border membranes. This finding led Kessler *et al.* (1982) to advance the hypothesis that this proteolipid, which was termed 'phosphorin', might be a component of the Na^+ -dependent P_i co-transport system in renal brush border membranes. An attractive feature of this hypothesis is that it could be negated if the Na^+ - P_i symporter and phosphorin were found in different fractions during the course of purification of proteins from brush border membranes. Experimental techniques required for such a test of the hypothesis include: (a) a method for extraction and assay of the ability of phosphorin to bind P_i ; (b) a method by which the Na^+ - P_i symporter could be both solubilized from renal brush

border membranes and reconstituted into an assay system *in vitro* in an active form (i.e., a form that can conduct Na^+ - P_i symport); and (c) a method for separation of proteins that have been solubilized from brush borders without elimination of either the transport or binding activities. Therefore, (i) we utilized the method of Kessler *et al.* (1982) for assay of phosphorin; (ii) we reconstituted Na^+ -dependent transport of P_i into proteoliposomes made from detergent-solubilized renal brush border membrane proteins and purified lipids by utilizing methods we recently reported for estimating the contribution of binding of P_i versus the degree of Na^+ -dependent transport of P_i (Schäli & Fanestil, 1985); and (iii) we applied the technique of chromatofocusing to the separation of detergent-solubilized renal brush border membrane proteins, since Lin *et al.* (1984) used this technique to reconstitute Na^+ -cotransport activity from calf renal brush border membranes.

In the present paper we report partial purification of the renal Na^+ - P_i symporter and of phosphorin. First, we show that only one of three protein fractions prepared by chromatofocusing contained protein that we could reconstitute in proteoliposomes that exhibit Na^+ -dependent and arsenate-inhibitable transport of P_i . This finding differs from that of Lin *et al.* (1984), who found Na^+ - P_i co-transport in all reconstituted fractions. We speculate that the lipids used in the reconstitution may account for at least some of the differences between our findings and those of Lin *et al.* (1984). Second, we find that the P_i -binding activity characteristic of phosphorin was found only in the same chromatofocusing fraction as was

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the $\text{Na}^+\text{-P}_i$ symport activity. This finding of simultaneous partial purification of both $\text{Na}^+\text{-P}_i$ symport and phosphorin fails to negate the hypothesis that phosphorin may be involved in the transport of P_i across the renal brush border membrane. However, determination of an obligatory role of phosphorin in this transport process requires further investigation.

METHODS

Solubilization of the brush border membrane proteins

Brush border membrane vesicles were prepared from rabbit kidney cortex by Mg^{2+} precipitation followed by differential centrifugation according to Booth & Kenny (1974). The purity of the vesicles was checked as described elsewhere (Kessler *et al.*, 1982). Portions of membrane vesicles were stored in liquid N_2 without loss of P_i transport activity (Schäli *et al.*, 1984).

Appropriate amounts of membrane vesicles were thawed in a water bath at 25°C , diluted with KHT buffer (150 mM-KCl, 100 mM mannitol, 0.1 mM-dithiothreitol, 0.2 mM-EDTA and 20 mM-Hepes/Tris, pH 7.4) and mixed with either 1% octylglucoside (protein:detergent ratio 1:4, w/w) or 1% Nonidet P-40 (NP-40, protein:detergent ratio 1:2, w/w). The membranes were stirred for 30 min on ice and the solubilized proteins were separated from the insoluble material by centrifugation at $150\,000\text{ g}$ for 45 min. Octylglucoside was used in initial experiments in which we evaluated methods for determining the relative contributions of binding of P_i versus transport of P_i into an intravesicular space to the total uptake of P_i by the proteoliposomes (Schäli & Fanestil, 1985). The two detergents yielded similar amounts of solubilized proteins: $80.2 \pm 2.3\%$ (mean $\pm$ S.E.M, $n = 10$) and $76.0 \pm 2.1\%$ ($n = 15$) for octylglucoside and NP-40 respectively. However, transport of P_i was 15–30% of the total uptake of P_i , whereas binding accounted for 70–85% of total uptake, when octylglucoside was the detergent. On the other hand, transport of P_i was 50–60% of the total uptake when NP-40 was the detergent. Since the absolute amount of binding of P_i in the two types of proteoliposomes was nearly the same (1.09 nmol of P_i /mg of protein for octylglucoside and 0.85 nmol/mg of NP-40), intravesicular volume (calculated from the amount of P_i transported, i.e. not bound, at equilibrium) was about 5-fold greater with NP-40 ($3.34\text{ }\mu\text{l}$ /mg of protein for octylglucoside versus $17.2\text{ }\mu\text{l}$ /mg of NP-40). Thus, reconstitution with NP-40 produced a better 'signal-to-background'; therefore, all experiments presented in this paper were conducted using NP-40 as detergent, except where noted otherwise.

Chromatographic purification of the solubilized proteins

NP-40 solubilized membrane proteins were fractionated by chromatofocusing. A column (20 cm $\times$ 0.9 cm) was packed with Polybuffer exchanger 94 (Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A.) and equilibrated with start buffer (0.025 M-imidazole/HCl containing 0.5% NP-40 or 1% octylglucoside, pH 7.4) according to the instruction manual of the manufacturer. Protein (40–80 mg) was loaded on to the column and eluted with Polybuffer 74 (Pharmacia) (1:8 dilution) adjusted to pH 4.0 with 1 M-HCl in the presence or 0.5% NP-40. Fractions (1.5–2 ml) were collected and assayed for protein content and pH. The elution was continued until

the pH in the eluted sample was 4.0. The samples were then arbitrarily pooled into three fractions according to the pI of eluted proteins: Fraction 1, pI > 6.8 ; Fraction 2, pI 5.4–6.8; Fraction 3, pI 4.0–5.4. The column was regenerated with 1 M-NaCl and reused several times. Protein recovery from the column averaged $87.5 \pm 2.5\%$ ($n = 18$) of the loaded amount. In some experiments, the Polybuffer exchanger 94 was used as a normal ion-exchange resin. The column was equilibrated at pH 6.8 with 0.025 M-imidazole/HCl containing 0.5% NP-40 and the proteins were eluted with the same buffer. At the end of the first peak (Fraction 1), the elution buffer was replaced by 1 M-NaCl containing 0.5% NP-40 and a second peak was collected (Fractions 2 and 3). This modified method will be referred to as the 'batch method' throughout this paper.

Reconstitution procedure

Unpurified proteins and the three purified protein fractions were reconstituted into liposomes. Solubilized membrane proteins (4.5 mg) were concentrated by dialysis against poly(ethylene glycol) 40000 (15% in KHT buffer). After 2–3 h, 5–10 ml of KHT buffer was added to the protein solution and the dialysis was continued until the final volume was approx. 1 ml. Purified egg yolk phosphatidylcholine (36 mg) (type V-EA; Sigma) and cholesterol (9 mg) (an amount equal to 20% of total lipids) were dissolved under a 100% N_2 atmosphere in 1 ml of chloroform in a round-bottomed flask. The solvent was evaporated under reduced pressure with a Rotavapor (Büchi; Brinkmann Instruments, Westbury, NY, U.S.A.). Complete removal of the solvent was achieved by further evaporation under high vacuum with a liquid N_2 vapour trap. The thin film of lipids was then resuspended in 1 ml of KHT buffer with a small magnetic stirring bar. The milky suspension was transferred into a test tube and sonicated at room temperature in a bath type sonicator (Bransonic 12; American Scientific Products, McGraw Park, IL, U.S.A.) until clarified. All of the above steps were conducted under an atmosphere of N_2 . The sonicated lipids (1 ml) were then frozen in liquid N_2 and thawed at room temperature prior to the addition to the dialysed proteins. After addition of 1 ml of KHT buffer, the suspension was dialysed at 4°C for 36 h against 3×1 litre of KHT buffer. After dialysis, the suspension was diluted to 8 ml with mannitol buffer (300 mM-mannitol/20 mM-Hepes/Tris, pH 7.4) and centrifuged for 10 min at 3000 g to remove any aggregated material. The resulting supernatant was centrifuged for 1 h at $150\,000\text{ g}$ to sediment the proteoliposomes. The pellet was resuspended in 1 ml of mannitol buffer and gently homogenized by aspirating the suspension into a 1 ml syringe through a 23-gauge needle (10 strokes). The liposomes were allowed to equilibrate with the mannitol for 1 h at room temperature and 1 h on ice before the uptake experiments.

Measurement of P_i uptake

Uptake of $^{32}\text{P}_i$ into reconstituted liposomes was measured by a rapid filtration method over Millipore filters (type GSWP, $0.22\text{ }\mu\text{m}$) as described (Schäli *et al.*, 1984). Liposomes ($10\text{ }\mu\text{l}$) were mixed with $20\text{ }\mu\text{l}$ of uptake buffer. Final solute concentrations in the uptake medium were: 100 mM- NaNO_3 or $-\text{KNO}_3$, 0.1 mM- K_2HPO_4 , 100 mM-mannitol, 20 mM-Hepes/Tris, pH 7.4, and $^{32}\text{P}_i$ ($25\text{ }\mu\text{Ci/ml}$).

In experiments where the osmotic sensitivity of the liposomes was measured, increasing amounts of sucrose were added to the Na^+ or K^+ uptake buffers. In some experiments, arsenate was added to the Na^+ or K^+ uptake buffers to give a final concentration of 10 mM.

SDS/polyacrylamide-gel electrophoresis

SDS/polyacrylamide-gel electrophoresis was performed by the method of Shapiro *et al.* (1967). Samples were made 5% in SDS and 2% in 2-mercaptoethanol and boiled for 5 min. After cooling on ice, the samples were made 10% in sucrose and applied onto a polyacrylamide (bisacrylamide/acrylamide, 1:30) slab gel containing 0.1 M-sodium phosphate, 6 M-urea and 0.1% SDS, pH 6.8. The running buffer contained 0.1 M-sodium phosphate and 0.1% SDS, pH 6.8. After electrophoresis the samples were silver stained according to Sammons *et al.* (1981).

Proteolipid extraction

Detergent-solubilized brush border membranes, detergent insoluble proteins (pellet of the detergent extract) and purified protein fractions obtained by the 'batch method' of chromatofocusing were dialysed to lower the salt concentration, passed over small Bio-Beads SM2 columns to remove the detergent (Momoi, 1979) and extracted with chloroform/methanol according to Kessler *et al.* (1982). The dried extracts were redissolved in a small volume of chloroform/methanol (2:1, v/v) and stored at -20°C until used for the P_i binding assay.

P_i binding assay

P_i binding to the organic solvent extracts was measured in the presence and absence of Mn^{2+} as described (Kessler *et al.*, 1982; Kessler & Vaughn, 1984). All determinations were run in duplicate along with a blank, which contained all the solvents but no extract. Each experiment was repeated three times on different extracts.

Other methods

Protein was measured by the method of Lowry *et al.* (1951) after precipitation with trichloroacetic acid, using bovine serum albumin as a standard. With extracted proteolipids, the material was first solubilized in 5% SDS in a 60°C water bath until the organic solvent had vapourized and the sample was clear. Protein samples containing Polybuffer were assayed by the method of Bradford (1976) using the BioRad protein assay (BioRad, Richmond, CA, U.S.A.) and γ -globulin as a standard.

Materials

Fresh kidneys were obtained from young adult New Zealand rabbits fed a standard diet. $^{32}\text{P}_i$ (carrier-free or as potassium salt) was obtained from ICN (Irvine, CA, U.S.A.). When received from the supplier, occasional preparations of carrier-free $^{32}\text{P}_i$ contained impurities that produced high background uptake. The results reported were obtained with $^{32}\text{P}_i$ that produced low uptake when assayed on the filter without liposomes. Poly(ethylene glycol) 40000 was purchased from Serva Feinbiochemica (Heidelberg, Germany); β -D-octylglucoside (n-octyl β -D-glucopyranoside) and imidazole were from Calbiochem (La Jolla, CA, U.S.A.). Egg yolk phosphatidylcholine, type V-EA, cholesterol and NP-40, as well as all other chemicals (analytical reagent grade) were obtained from Sigma.

RESULTS

Detergent solubilization of membrane proteins

Brush border membrane proteins were solubilized, at fixed protein:detergent ratios, with increasing concentrations of two detergents: octylglucoside (protein/detergent 1:4) and NP-40 (protein/detergent 1:2). The results shown in Fig. 1 indicate that at a 1% concentration both detergents solubilized similar amounts of the membrane proteins (approx. 80%). At lower concentrations, however, NP-40 was considerably more effective than octylglucoside.

Purification of solubilized membrane protein by chromatofocusing

Solubilized brush border membrane proteins were fractionated by chromatofocusing. The elution profile of a representative experiment is shown in Fig. 2. The collected samples were pooled into three fractions according to the pH limits shown in Fig. 2. In six experiments Fraction 1 contained $30.3 \pm 5.5\%$ of the loaded amount of protein, Fraction 2 $14.3 \pm 3.9\%$ and Fraction 3 $45.0 \pm 7.2\%$. Purification carried out by the batch method (see the Methods section) yielded somewhat lower amounts of proteins in Fraction 1 [$21.2 \pm 2.4\%$ ($n = 11$) of the loaded amount] as compared with normal chromatofocusing, although the total protein recovery was similar.

SDS/polyacrylamide-gel electrophoresis

Crude NP-40-solubilized proteins from brush border membrane vesicles, proteins from Fraction 1 and from Fraction 2 and 3 (obtained by the 'batch method' as described in the Methods section) were separated by SDS/polyacrylamide-gel electrophoresis. The results are shown in Fig. 3 and indicate some major differences in

Fig. 1. Solubilization of brush border membrane proteins by non-ionic detergents

Appropriate amounts of brush border membrane vesicles were solubilized (30 min at 4°C) with different concentrations, but fixed detergent:protein ratios, of octylglucoside (○) (ratio 4:1) or NP-40 (●) (ratio 2:1). Insoluble proteins were sedimented by centrifugation (45 min at 150000 g). Results are means from triplicate determinations of one experiment.

Fig. 2. Protein purification by chromatofocusing

The column (Polybuffer exchanger 94) was equilibrated with start buffer (0.025 M-imidazole/0.5% NP-40 adjusted to pH 7.4 with 1 M-HCl). Approx. 80 mg of protein was loaded on to the column and eluted with Polybuffer 74 (1:8 dilution) adjusted to pH 4.0 with 1 M-HCl. The samples were pooled into three fractions according to the pH limits shown in the Figure. Data are from a representative experiment. Fraction 1 contained 22.2%, Fraction 2 15.5% and Fraction 3 49.6% of the loaded amount of proteins. Protein recovery was 87.4%. Protein was assayed by the Bradford (1976) method (A_{595}).

the migration pattern between the three samples. Major differences in the migration pattern of proteins in the three samples were also observed in one instance when proteins were identified with Coomassie Blue instead of the silver stain. Some protein bands present in the crude solubilized proteins (lane 1) and in Fraction 2 and 3 (lane 3) are present in much smaller amounts (in the high- M_r regions) or completely absent from Fraction 1 (lane 2). On the other hand, at least four bands appear to be enriched in Fraction 1 as compared with Fraction 2 and 3. The average (two experiments) apparent M_r values of these bands were 56800, 36600, 23300 and 9300.

P_i uptake into liposomes

The three purified fractions obtained from the chromatofocusing experiments and their corresponding unpurified solubilized proteins were reconstituted into liposomes. P_i uptake was measured in the presence of a Na^+ or a K^+ gradient (out > in). The results shown in Fig. 4 indicate that only liposomes reconstituted with unpurified proteins (BBX) and those reconstituted with proteins having $pI > 6.8$, as in Fraction 1, showed a P_i uptake that was stimulated in the presence of a Na^+ gradient. Liposomes reconstituted with proteins having lower pI values, as in Fractions 2 and 3, exhibited uptake rates that were similar in the presence of Na^+ or K^+ . Note should be made of the finding that the rate at which the uptake of P_i approaches the equilibrium value in the presence of K^+ was not faster with Fractions 2 or 3 than with unpurified proteins or Fraction 1. Thus, there is no evidence that the passive permeability properties of liposomes prepared from the various protein fractions differ markedly.

Osmotic sensitivity of the reconstituted liposomes

To determine whether the observed Na^+ -dependent P_i uptake into liposomes reconstituted with proteins from

Fraction 1 was due to Na^+ -dependent transport of P_i into an intravesicular space, we measured equilibrium P_i uptake in the presence of increasing concentrations of sucrose in the incubation medium. The results shown in Fig. 5 indicate that approximately one-half of the total uptake measured after 2 h of incubation represented transport into an osmotically sensitive intravesicular space. Almost identical binding (osmotically insensitive) values were extrapolated at infinite osmolality in the presence of Na^+ and K^+ , suggesting that the binding of P_i was not dependent upon the presence of Na^+ .

Inhibition of P_i uptake by arsenate

Uptake of P_i into liposomes reconstituted with proteins from Fraction 1 was measured in the presence of arsenate to determine whether the Na^+ -dependent uptake of P_i shown in Fig. 4 was sensitive to this inhibitor, as it is in intact brush border membrane vesicles (Hoffmann *et al.*, 1976). The results shown in Fig. 6 are consistent with the finding that part of the measured uptake is due to binding. Since arsenate is also a competitor for P_i binding, uptake of P_i at equilibrium and in the presence of excess arsenate should represent P_i accumulated into an intravesicular space (i.e., unbound P_i). Equilibrium uptake in the absence of arsenate, on the other hand, should represent bound plus unbound (i.e., transported or intravesicular) P_i . Arsenate at 10 mM inhibited control equilibrium P_i uptake by $37.4\% \pm 8.4\%$ and $39.4\% \pm 9.5\%$ ($n = 6$) in the presence of Na^+ and K^+ respectively (Fig. 6). These values, suggesting that approx. 38% of P_i is bound to the liposomes at equilibrium, are modestly lower than those determined in the osmotic equilibrium experiments (Fig. 5), but the two types of experiments demonstrate a common feature: at equilibrium, in the absence and presence of arsenate or at infinite osmolality, P_i uptake was not significantly influenced by Na^+ as compared with K^+ . Thus, neither

Fig. 3. SDS/polyacrylamide-gel electrophoresis

Lane 1, crude NP-40-solubilized membrane proteins; 2, Fraction 1; 3, Fraction 2 and 3 obtained from chromatofocusing by the 'batch method' (see the Methods section); 4, standards; 2 μ g of protein was applied to each lane of an 8.5% polyacrylamide gel. The gel was silver stained according to Sammons *et al.* (1981). At least four bands (M_r values 56800, 36600, 23300 and 9000) were enriched in Fraction 1 as compared with Fraction 2 and 3.

Fig. 5. Osmotic reactivity of liposomes reconstituted with Fraction 1

Equilibrium uptake of P_i was measured in the absence (control = 100%) or presence of increasing amounts (50–400 mM) of sucrose in the uptake medium. The results are presented as a percentage of the values observed in controls versus the reciprocal of the osmolality in the uptake buffer due to the addition of sucrose ($1/\text{osm}$). The values, obtained in the presence of a 100 mM- Na^+ or - K^+ gradient (out > in) represent means $\pm$ S.E.M. (vertical bars) for six experiments. At infinite osmolality (intersection with the y axis), extrapolated P_i binding was $50.0 \pm 8.7\%$ (in the presence of Na^+ , $\bullet$) and $48.7 \pm 9.1\%$ (in the presence of K^+ , $\circ$) of the control equilibrium uptake values.

P_i binding nor the volume of the intravesicular space appeared to be Na^+ -dependent. The rates at which P_i uptake approached equilibrium in the presence of Na^+ or K^+ in both the absence and presence of 10 mM-arsenate are also shown in Fig. 6. As shown earlier (Fig. 4), in the absence of arsenate the rate of uptake of P_i was significantly faster in the presence of Na^+ than in the

Fig. 4. Uptake of P_i into reconstituted liposomes

Fractions 1, 2 and 3 were prepared as shown in Fig. 2. These fractions and their corresponding crude solubilized membrane protein (BBX) were reconstituted into liposomes as described in the Methods section. Uptake of P_i (100 μM) was determined in the presence of ($\bullet$) 100 mM- Na^+ or of ($\circ$) 100 mM- K^+ gradients (out > in). The results shown represent average values determined on three different liposome preparations. No differences between Na^+ and K^+ equilibrium values was observed. Average values were 21.0 ± 6.3 , 6.31 ± 0.51 , 18.5 ± 5.7 and 10.7 ± 2.2 pmol of P_i /assay ($n = 3$) for BBX, and Fractions 1, 2 and 3 respectively.

Fig. 6. Inhibition of P_i uptake by arsenate

P_i uptake into liposomes reconstituted with Fraction 1 was measured in the presence of an Na^+ (●, ▲) or a K^+ (○, △) gradient, and in the presence of (▲, △) or absence (○, ●) of 10 mM-arsenate in the uptake medium. Results represent means $\pm$ S.E.M. for six experiments run in triplicate. Equilibrium values were: ●, 2.90 ± 0.58 ; ○, 2.90 ± 0.57 ; ▲, 1.98 ± 0.56 and △, 1.95 ± 0.50 nmol of P_i /mg of protein.

presence of K^+ . For example, the uptake at 10 s in the presence of Na^+ was 0.904 ± 0.232 nmol of P_i /mg of protein, while in the presence of K^+ the value was 0.614 ± 0.232 nmol of P_i /mg of protein. Despite the overlapping S.E.M. values (Fig. 6), the paired difference of 0.290 ± 0.097 nmol/mg of protein was statistically significant. The same also applies to the 1, 2 and 5 min time points, but not to the equilibrium value. In the presence of 10 mM-arsenate, however, the P_i uptake was no longer influenced by the presence of Na^+ in the uptake medium. The paired difference at 10 s was 0.003 ± 0.050 nmol of P_i /mg of protein ($P > 0.05$).

P_i binding to chloroform/methanol extracts

Chloroform/methanol extracts of (1) solubilized proteins from brush border membranes, (2) detergent-insoluble proteins and (3) the purified fractions, were assayed for their ability to bind P_i in the presence and absence of Mn^{2+} . The results were expressed as pmol of P_i bound/mg of protein from which the proteolipids were extracted (start protein) to avoid possible bias resulting from variations in the yield of extracted material. Our data presented in Fig. 7 show: (i) that the organic solvent extracts of detergent-solubilized protein (BBX) exhibited P_i -binding activity, suggesting that phosphorin was solubilized by the detergent, (ii) that the solubilization of phosphorin was nearly complete, since the extract of detergent-insoluble proteins (pellet BBX) only exhibited a small P_i -binding activity as compared with BBX, (iii) that P_i binding to organic solvent extracts of Fractions 2 and 3 was negligible and (iv) that the chloroform/methanol extract of Fraction 1 showed an average 2.9-fold increase in binding activity as compared with BBX in the absence ($2.6 \times$) and presence ($3.2 \times$) of Mn^{2+} . Thus, only one of the purified fractions (Fraction 1) showed increased P_i binding activity. This is the same fraction from which the apparent Na^+ -dependent P_i transport system was reconstituted into liposomes (Fig. 4).

Fig. 7. P_i binding to chloroform/methanol extracts

P_i binding was measured in chloroform/methanol extracts of different starting material (from left to right): (i) membrane proteins solubilized from renal brush borders with detergent (BBX); (ii) detergent-insoluble proteins (pellet BBX); (iii) Fraction 1 and Fractions 2 and 3 obtained by the 'batch method' (see the Methods section). P_i binding ($10 \mu M$) was measured in the presence (▨) and absence (□) of $10 \mu M$ - Mn^{2+} . Results are means $\pm$ S.E.M. for three different experiments, expressed as pmol of P_i bound/mg of protein from which the proteolipids were extracted.

Uptake of P_i into liposomes reconstituted with phosphorin

The fact that the Na^+ -phosphate transporter was found in the same protein fraction as that containing phosphorin might suggest that phosphorin is somehow involved in phosphate transport. To test this hypothesis we reconstituted phosphorin (obtained from intact brush border membrane vesicles) into liposomes and measured uptake of P_i in the presence of Na^+ or K^+ . However, the results in Fig. 8 show that, in contrast with liposomes reconstituted with the partially purified P_i transporter, Na^+ was unable to accelerate the influx of P_i as compared with K^+ in liposomes containing phosphorin even though 54% of the equilibrium uptake of P_i was into an osmotically sensitive space (results not shown).

DISCUSSION

Proteins solubilized from renal brush border membranes with NP-40, when reconstituted into proteoliposomes, transport P_i into an intravesicular space in a Na^+ -dependent fashion. However, the recovered activity of the Na^+ - P_i transport process represented only about 20% of that found in intact brush border membrane vesicles (Table 1). Numerous causes of this loss of apparent activity are possible. For example, random orientation of a vectorial carrier in the proteoliposomes would automatically cause an apparent loss of 50% of activity. Other possible causes include inefficient reassembly of a multi-subunit carrier, reconstitution of only one of two or more Na^+ - P_i carrier systems, differences in the rate of 'turnover' of the carrier due to a change in the lipid environment surrounding the carrier, and degradation of the carrier. Our data do not allow us to select or reject any of these or other possible explanations. We do

Fig. 8. Uptake of P_i into liposomes reconstituted with phosphorin

Phosphorin was extracted from brush border membrane vesicles as described (Kessler *et al.*, 1982). Phosphorin was solubilized with 2% octylglucoside, after evaporation of the organic solvent, and reconstituted into liposomes at a protein:lipid ratio of 1:50. Uptake of P_i was determined in the presence of a 100 mM- Na^+ (●) or $-K^+$ (○) gradient (out > in). Results are means $\pm$ S.E.M. for three experiments. Equilibrium P_i uptake results were 6.95 ± 2.48 and 7.06 ± 1.73 pmol of P_i /assay in the presence of Na^+ and K^+ respectively.

emphasize two points, however. First, in the partial purification of another renal brush border carrier, the Na^+ -glucose carrier, Koepsell *et al.* (1983) recovered only about 4% of the initial activity. Second, that lipids can play a crucial role is exemplified by the finding of Connolly *et al.* (1985) that as little as a 2.5% change in bilayer cholesterol could result in as much as a 100-fold change in maximal transport rate of the erythrocyte glucose carrier.

The proteins we solubilized with NP-40 were separable by chromatofocusing into three fractions. Importantly, Na^+ - P_i symport activity was reconstitutable only from that fraction (Fraction 1) of proteins having $pI > 6.8$. Since an average of 82% of the total reconstitutable Na^+ - P_i symport activity present in total NP-40 extract of brush border membranes was recovered in Fraction 1 (Table 1), this Na^+ - P_i symport activity represents the major part of this transport activity in the NP-40 extract. The specific activity of the Na^+ - P_i symport system increased by 2.7-fold, from 0.159 nmol/10 s per mg of protein in the total NP-40 extract to 0.431 nmol/10 s per mg in Fraction 1 (Table 1).

Proteins solubilized from renal brush border membranes with NP-40, when extracted and assayed, bound P_i in a Mn^{2+} -augmented fashion that is characteristic of the binding of P_i to the proteolipid phosphorin (Kessler & Vaughn, 1984). Recovery of P_i -binding activity was approx. 90% (Table 2), suggesting that little degradation of phosphorin occurred in the solubilization process. Upon separation of the solubilized proteins by chromatofocusing, we found that 72% of the P_i -binding activity in the detergent extract was recovered in Fraction 1 (Table 2), the same fraction in which Na^+ - P_i transport

was recovered. The P_i -binding activity in Fraction 1 represents the major part of this activity in the NP-40 extract. The specific activity of the P_i -binding system increased by 3-fold, from 47 pmol/mg of protein in the total NP-40 extract to 146 pmol/mg of protein in Fraction 1 (Table 2).

The Na^+ -dependent P_i uptake activity found only in liposomes reconstituted with the proteins in chromatofocusing Fraction 1 has a number of features that should be discussed. First, initial P_i uptake measured after 10 s, 1, 2 and 5 min was significantly faster in the presence of a Na^+ gradient than in its absence (K^+ gradient) (Fig. 4). Second, transport occurred into an intravesicular space, as inferred from two lines of evidence. (i) An increase in the external osmolality by addition of sucrose decreased the equilibrium P_i uptake measured after 2 h (Fig. 5). Extrapolated uptake values for infinite osmolality suggested that approx. 50% of the equilibrium uptake in the absence of sucrose represented unbound P_i accumulated into an osmotically sensitive intra-vesicular volume or, in other words, that the remaining 50% of the equilibrium uptake represented binding of P_i to the liposomes (Fig. 5). (ii) The equilibrium uptake of P_i was also depressed by arsenate. Arsenate displaces P_i from several biological binding sites for P_i and, in particular, it inhibits P_i binding by phosphorin (Kessler *et al.*, 1982). Equilibrium P_i uptake was inhibited by about 38% by 10 mM-arsenate, suggesting that this portion of accumulated P_i was due to binding of P_i (Fig. 6) or that about 60% of the uptake was into the intravesicular space. Third, equilibrium uptake in the absence or presence of arsenate (Fig. 6), as well as in the absence or presence of sucrose (Fig. 5), was not dependent on the presence of Na^+ in the incubation media. Thus, binding of P_i to the liposomes appears to be independent of the presence of Na^+ . Fourth, the Na^+ -dependent uptake of P_i observed over the first 5 min of incubation was completely abolished by arsenate. This is consistent with the interpretation that the non-mediated uptake of P_i ('leak') measured in the presence of arsenate was also not Na^+ -dependent. Therefore, the Na^+ -dependent uptake of P_i that is reconstituted into liposomes by the proteins in Fraction 1 has several of the characteristics expected of the Na^+ - P_i symport activity from renal brush border membranes.

Our findings with chromatofocusing and reconstitution of the Na^+ - P_i symport system differ from those recently reported by Lin *et al.* (1984). We found reconstitutable Na^+ - P_i symport only in Fraction 1 ($pI > 6.8$), whereas Lin *et al.* (1984) found it in all four of their fractions ($pI > 6.6$, 6.6–5.8, 5.8–5.3 and 5.3–4.6). We note a number of methodological differences between the study by Lin *et al.* (1984) and the present work. (i) The species from which the kidneys were obtained differs (calf versus rabbit). (ii) The detergent used for solubilization differs (octylglucoside versus NP-40). That the detergent used may considerably influence experimental results is suggested by our finding that proteoliposomes reconstituted from NP-40 extracts, when compared with octylglucoside extracts, have a greater total uptake of P_i and greater intravesicular volume, while the absolute amount of P_i bound per mg of protein was similar (results not shown). (iii) The method for formation of liposomes differed (centrifugation versus dialysis). (iv) The recovery of protein from the chromatofocusing column differed (45–70% versus 87.5%). (v) The type of lipid

Table 1. Recovery of Na⁺-dependent P_i transport activity

Proteins from total NP-40 extract and Fractions 1, 2 and 3 were reconstituted into liposomes and Na⁺-dependent P_i transport was measured as described in the Methods section. The rate of transport was calculated as the difference between uptake in the presence of Na⁺ and uptake in the presence of K⁺ (100 mM gradient, out > in). In Fractions 2 and 3 the values were not appreciably different from zero. For comparison, Na⁺-dependent P_i transport was also measured in intact brush border membrane vesicles. Results are means ± S.E.M. for *n* experiments.

Protein fraction	Protein (% of total)	Rate of transport (nmol/10s per mg of protein)	<i>n</i>	Activity (% of total)
Brush border membrane vesicles	100	0.630 ± 0.057	4	100
NP-40 extract	80	0.159 ± 0.052	6	20.2
Fraction 1	24.2	0.431 ± 0.084	6	16.6
Fraction 2	11.4	—	—	—
Fraction 3	36.0	—	—	—

Table 2. Recovery of P_i-binding activity

Proteins of total NP-40 extract, Fraction 1, and Fractions 2 and 3 were extracted with chloroform/methanol and P_i binding (10 μM-P_i) was measured in the presence and absence of 10 μM-Mn²⁺ as described in the Methods section. The results shown, which are the means ± S.E.M. of *n* experiments, represent the amount of binding of P_i/mg of protein originally extracted with chloroform/methanol.

Protein fraction	Protein (% of total)	P _i binding (pmol/mg of start protein)		<i>n</i>	Activity (% of total)
		— Mn ²⁺	+ Mn ²⁺		
Brush border vesicles	100	—	50*	—	100
NP-40 extract	80	15.6 ± 0.49	46.8 ± 1.87	3	~90
Fraction 1	20	41.2 ± 9.8	146 ± 22	3	~64
Fraction 2 and 3	60	0.35 ± 0.09	2.6 ± 0.8	3	~3

* Calculated from data in Kessler & Vaughn (1984)

used for the liposomes differed (asolectin versus purified egg yolk phosphatidylcholine plus cholesterol). Our choice of purified lipids was based on initial observations with an asolectin preparation we obtained. We observed that the asolectin itself (i.e., without added protein) could produce an apparent Na⁺-dependent transport of P_i (but not of glucose). For example, the uptake of P_i after 30 s was nearly five times greater in the presence of a 100 mM-NaCl gradient than in the presence of a 100 mM-choline chloride gradient (3.57 pmol/assay versus 0.73 pmol/assay; no brush border protein added). Other workers have also deduced that some commercially available lipids that are derived from biological sources contain proteins that may mimic transport activities (Gögelein & Koepsell, 1984). Our inability to reconstitute Na⁺-P_i transport activity in chromatofocusing Fractions 2 and 3 cannot be attributed to generalized leakiness or non-selective 'pores' of the vesicles prepared from these fractions, since the uptake of P_i was no faster than with the vesicles prepared from Fraction 1 (Fig. 4). Since we recovered only 20% of total Na⁺-P_i transport activity (Table 1), we cannot eliminate the possibility that there are other Na⁺-P_i transport systems in intact brush border membranes which might be located in an inactive form in Fractions 2 and 3.

We recovered 20% of the Na⁺-P_i transport activity and 90% of the P_i-binding activity characteristic of phosphorin

in a total detergent extract of renal brush border membrane vesicles. About three-fourths of both transport and binding activities that were recovered were partially purified into chromatofocusing Fraction 1. The failure of phosphorin alone, when incorporated into liposomes, to facilitate transport of P_i into an osmotically sensitive space in the presence of a Na⁺ gradient appears to indicate that phosphorin by itself is not sufficient for the Na⁺-dependency of the P_i uptake observed in Fraction 1. Moreover, we cannot eliminate the possibility that we failed to recover a Na⁺-P_i symport activity without phosphorin in Fractions 2 and 3. On the other hand, the initial nearly quantitative purification of the recovered Na⁺-P_i symport activity and of the recovered phosphorin in Fraction 1 fails to negate the hypothesis that phosphorin is involved in the Na⁺-P_i symport activity we assayed. Whether the presence of phosphorin is necessary, accessory or non-essential for the activity of the Na⁺-dependent P_i transport process we reconstitute from crude or purified renal brush border membrane proteins requires further detailed investigation.

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