Reconstitution and characterization of a Na^+/P_i co-transporter protein from rabbit kidney brush-border membranes

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A protein with Na^+/P_i co-transporter activity has been extracted from rabbit brush-border membranes with chloroform/methanol and purified by hydroxyapatite chromatography. The protein has been incorporated by the dilution method into liposomes formed from different types and ratios of lipids. The greatest reconstitution has been achieved into liposomes prepared from cholesterol (20%), phosphatidylcholine (20%), phosphatidylethanolamine (30%) and phosphatidylserine (30%) (CH/PC/PE/PS). P_i uptake by these proteoliposomes had the following characteristics: (i) the initial rate was markedly greater in the presence of an inwardly directed Na^+ gradient (600 pmol/10 s per mg) than with a K^+ gradient (65 pmol/10 s per mg); (ii) maximal uptake was increased 8-fold above the equilibrium value ('overshoot') when a Na^+ gradient was applied; (iii) P_i was not merely bound to proteoliposomes but was transported intravesicularly; and (iv) Na^+ -dependent P_i uptake was sensitive to the known phosphate transport inhibitors. This first successful attempt of reconstitution of Na^+/P_i transport activity into proteoliposomes led us to isolate and characterize physico-chemically the protein responsible. Its isoelectric point was about 5.8, and urea/SDS gel electrophoresis revealed a broad band of molecular mass ranging from 63 to 66 kDa under both reducing and non-reducing conditions. In the native form, the molecular mass analysed by gel filtration was estimated to be 170 ± 10 kDa, suggesting that the protein is a polymer, probably stabilized by hydrophobic bonds. Endoglycosidase F treatment decreased the molecular mass to approx. 50 kDa. It is postulated that this acidic glycoprotein might represent a subunit of the intact Na^+/P_i co-transporter from rabbit kidney brush-border membranes.

INTRODUCTION

The coupled translocation of phosphate (P_i) and Na^+ by a symport mechanism across the brush-border membranes (BBM) of renal proximal tubule and intestinal epithelial cells is a well-established phenomenon [1,2]. Although Na^+ -coupled transport of P_i has been extensively studied in natural BBM vesicles (BBMV) [3–5], identification and isolation of the Na^+/P_i co-transporter from either small-intestine or kidney BBM has proven difficult for two main reasons. First, the co-transporter represents a very small percentage of total membrane proteins [6]. Secondly, we are still waiting for the generation of a specific inhibitor which binds covalently to the protein and could be used as a marker during purification. Phosphonoformic acid, a competitive inhibitor of Na^+/P_i co-transport [7], has so far appeared useless for detection of the co-transporter protein [8,9].

In the last 15 years solubilization and reconstitution experiments have been reported, but they did not result in identification of the co-transporter [10–13]. More recently, efforts to identify the Na^+/P_i co-transporter have focused on substrate-protectable covalent labelling with group-specific reagents, but these studies have not yet permitted unequivocal characterization. By using a fluorescent derivative of phenylglyoxal, a 130 kDa protein was labelled in the intestinal BBMV [14]. Other reagents such as *N*-acetyl[^3H]imidazole [15] and azido-NAD [16] identified four (31, 53, 105 and 176 kDa) and two (70 and 97 kDa) protein bands respectively as possible candidates for the Na^+/P_i co-transporter. In no instance has a single protein been unambiguously isolated or demonstrated to exhibit Na^+ -dependent P_i transport activity in a reconstituted system.

We have isolated from renal BBM and purified to homogeneity a hydrophobic protein that binds Na^+ and P_i [17]. Numerous

properties of this protein suggest that it could be involved in Na^+/P_i co-transport across BBM. The aim of the present study was to establish that this protein was actually endowed with Na^+/P_i transport activity. For this purpose, we designed a simple and fast method of purification which permitted us to demonstrate in reconstitution experiments with proteoliposomes that the Na^+/P_i -binding protein was capable of performing Na^+ -dependent P_i transport which is sensitive to phosphate transport inhibitors. This led us to characterize in more detail the physico-chemical properties of the Na^+/P_i transporter protein.

MATERIALS AND METHODS

All reagents and standard proteins for isoelectrofocusing and gel filtration were obtained from Pharmacia. Nonidet P-40 (NP-40), phosphatidylcholine Type XI E (PC), phosphatidylethanolamine Type III A from egg yolk (PE), phosphatidyl-L-serine from bovine brain (PS), cholesterol (CH), phenylglyoxal and *N*-ethylmaleimide were purchased from Sigma. Bio-Gel hydroxyapatite and all reagents and standard proteins for electrophoresis were from Bio-Rad. CHAPS was from Calbiochem, and endoglycosidase F was from Boehringer. All other chemicals used were of highest grade and obtained from Merck. [^{32}P] P_i (carrier-free) was supplied by Amersham International.

Isolation of the co-transporter protein

BBMVs were prepared from male rabbit (6 months old) renal cortex by the magnesium precipitation method and differential centrifugation [18]. The membranes were extracted with chloroform/methanol (2:1, v/v). The organic extract was washed with 1 M-KCl and, after overnight phase separation, the lower

Abbreviations used: BBM, brush-border membranes; BBMV, brush-border membrane vesicle; NP-40, Nonidet P-40; CH, cholesterol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; IEF, isoelectrofocusing.

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chloroform phase was evaporated to dryness [17]. The dried organic extract was solubilized (1 ml/100 mg of dried extract) in 5 mM-Hepes/KOH, pH 7.4, containing 0.5% NP-40, 0.1 mM-MnCl₂ and 0.1 mM-dithiothreitol. After a 1 h incubation the suspension was centrifuged for 2 h at 100 000 *g*. The supernatant (0.5 ml) was applied to a hydroxyapatite column (1 g of hydroxyapatite powder) equilibrated and eluted with detergent-free buffer. The first 3 ml eluted from the column was discarded and the next 3 ml was collected. These fractions from several columns were pooled, concentrated, and dialysed against buffer containing 100 mM-KCl, 200 mM-mannitol, 10 mM-Hepes/KOH (pH 7.4), 0.1 mM-dithiothreitol and 0.1 mM-MnCl₂ (buffer A). This sample was used for reconstitution, isoelectrofocusing (IEF), electrophoresis and gel filtration.

Preparation of liposomes and reconstitution

High-purity commercial lipids (40 mg) were dissolved under a nitrogen atmosphere in chloroform. The solvent was evaporated under reduced pressure with a rotary evaporator. The thin film of lipids was then resuspended in 1 ml of buffer A. The milky suspension was transferred into a test tube and sonicated at 0 °C under a stream of nitrogen in a bath-type sonicator until clarification.

Reconstitution was done in a final volume of 1000 µl (or multiples thereof), containing 800 µl of the concentrated hydroxyapatite eluate previously equilibrated with buffer A, and 200 µl of the sonicated lipids. After brief vortex-blending, the suspension was placed on ice for 30 min. Proteoliposomes were formed at room temperature by rapid injection of the mixture (1 ml) into 25 ml of buffer A. After mixing by five aspiration/injection cycles, the suspension was left undisturbed for 20 min. Proteoliposomes were then isolated by ultracentrifugation in the cold (4 °C, 105 000 *g*, 60 min) and resuspended in 8 ml of mannitol buffer B (300 mM-mannitol, 10 mM-Hepes/Tris, pH 7.4). The resuspended material was next briefly spun (7500 *g*, 15 min) to remove any aggregated material. Proteoliposomes were finally collected by ultracentrifugation in the cold (145 000 *g*, 60 min). The pellet was resuspended in mannitol buffer B. The proteoliposomes were equilibrated for 30 min at room temperature and for 1 h on ice before P_i uptake measurements.

P_i uptake measurements

Uptake of ³²P-labelled inorganic phosphate into reconstituted proteoliposomes was measured by a rapid filtration method over Millipore filters (0.22 µm, type GSNP). Briefly, 40 µl of proteoliposomes was mixed with 80 µl of uptake buffer. Final concentrations in the uptake buffer were 100 mM-NaCl or -KCl, 0.1 mM-KH₂PO₄, 100 mM-mannitol, 10 mM-Hepes/Tris, pH 7.4, and 25 µCi of ³²P/ml. At specific time intervals, a sample was taken from the incubation medium and diluted in 2 ml of ice-cold stop solution, which had the same composition as the incubation medium, except that the isotope was omitted and 10 mM-arsenate was added. The diluted sample was immediately mixed, applied to a 0.22 µm filter and washed twice with 5 ml of stop solution. The amount of ³²P retained on the filters was determined by scintillation counting. The results were corrected for filter blank and uptake at time 0. Each time point in a single experiment was done in triplicate. To measure the osmotic sensitivity of the proteoliposomes, increasing amounts of sucrose (0–300 mM) were added to the NaCl or KCl uptake buffers. Effects of inhibitors on the function of incorporated co-transporter were determined as follows. Proteoliposomes were preincubated for 5 min with 20 mM-phenylglyoxal, and for 30 min with 2 mM-*N*-ethylmaleimide or 2 mM-dipyridyl disulphide. Transport was then initiated by the addition of uptakes medium with final concentrations as described above.

IEF in polyacrylamide gels

IEF was performed in 5% polyacrylamide horizontal thin-layer gels (0.5 mm). The gels contained 6 M-urea, 2% (w/v) NP-40, 1% CHAPS and 2% (w/v) pharmalyte at a pH range of 3 to 10. To extract lipids before IEF, the samples were incubated with chloroform, centrifuged for 10 min at 6000 *g* and the pellet was solubilized by a 2 h incubation in a solution containing 8 M-urea, 2% (w/v) NP-40, 2% CHAPS and 2% (w/v) pharmalyte. The samples were then applied on to the gel and isoelectrofocused for 1 h at 15 W (10 °C). Thereafter the gel was fixed and silver-stained [19].

SDS/PAGE

Polyacrylamide-gel electrophoresis was performed in a minislabs gel apparatus (Bio-Rad) using the buffer system of Laemmli [20]. Two conditions were used: system 1 contained 6 M-urea, 0.1% SDS and 7.5% acrylamide, and system 2 consisted of 0.1% SDS and 5% acrylamide. Sample buffer in the first case was composed of 2% SDS and 6 M-urea with or without 5 mM-dithiothreitol, whereas in the second case it only contained 0.5% SDS. All other components were as in Laemmli sample buffer [20]. Electrophoresis was carried out for 60 min at 200 V. After fixation, the gels were silver-stained [19].

Gel-filtration chromatography

Chromatography was carried out on a Sephadex G-200 column (2.6 cm × 46 cm) equilibrated and eluted in 5 mM-Hepes/Tris and 0.1% NP-40, pH 7.2, at a flow rate of 0.8 ml/h; bed volume was 245 ml and void volume was 83 ml. Molecular masses were calculated from a standard curve of log(molecular mass) against *K_{av}*, using molecular-mass markers: ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), albumin (67 kDa) and ovalbumin (43 kDa). Markers and samples were run in triplicate.

Other methods

Enzymic deglycosylation was achieved with endo-β-*N*-acetylglucosaminidase F as suggested by the manufacturer. The purified protein was incubated with 0.2 unit of enzyme for 96 h at room temperature. The incubation buffer contained 30 mM-sodium acetate, 10 mM-EDTA, pH 6.0, 1% NP-40 and 0.1% SDS. At the end of incubation, Laemmli's sample buffer was added and the digested proteins were analysed by electrophoresis.

Protein content was determined by a modified Lowry procedure [21] after precipitation with 10% trichloroacetic acid.

RESULTS

Uptake of P_i into proteoliposomes

The fraction eluted from the hydroxyapatite column was reconstituted into liposomes, and P_i uptake was measured in the presence of a Na⁺ or a K⁺ gradient (out > in). Fig. 1(a) shows the amount of P_i taken up by reconstituted liposomes formed from different ratios and kinds of lipids. When liposomes were synthesized from CH (20%) and PC (80%), P_i uptake after reconstitution in the presence of a Na⁺ gradient was only slightly higher than in the presence of a K⁺ gradient. In this preparation, no transient accumulation of P_i above the equilibrium value (overshoot) was observed. Improvement in the reconstitution of Na⁺/P_i co-transport activity was achieved when liposomes were formed from CH (20%), PC (50%) and PE (30%). In this case, a small overshoot was observed and P_i uptake was substantially stimulated by the Na⁺ gradient. Finally, reconstitution of the Na⁺/P_i co-transporter was attempted by using the acid phospholipid PS. Liposomes formed from CH (20%), PC (20%), PE (30%) and PS (30%) showed after reconstitution the best transport properties: not only was P_i uptake stimulated by the

Fig. 1. Comparison of P_i uptake into proteoliposomes formed from different lipids

(a) Time course of uptake over a 16 h incubation period. (b) Initial rates of P_i uptake within the first 3 min of incubation. For liposome formation, the following lipid combinations were used: (▲, △) CH/PC; (■, □) CH/PC/PE; (●, ○) CH/PC/PE/PS. Closed symbols refer to P_i uptake measurements carried out in the presence of a NaCl gradient (out > in); open symbols, KCl gradient (out > in). Values are means ± S.D. of three separate proteoliposome preparations.

Table 1. Effects of liposome composition on the efficiency of protein incorporation and the activity of the Na⁺/P_i co-transporter

Rates of Na⁺-dependent P_i uptake were calculated as the difference between uptake in the presence of Na⁺ and uptake in the presence of K⁺ (gradient out > in). Values are means ± S.D. from three experiments.

Lipids present during liposome formation	Total amount of protein incorporated into liposomes (mg/ml)	Initial Na ⁺ -dependent P _i uptake rate (pmol/10 s per mg)
CH/PC	0.15 ± 0.02	150 ± 10
CH/PC/PE	0.28 ± 0.05	480 ± 42
CH/PC/PE/PS	0.40 ± 0.05	535 ± 37

presence of a Na⁺ gradient, but the maximal amount of P_i in the proteoliposomes exceeded by a factor of > 8 the value obtained at 16 h. The onset, peak value and duration of the overshoot suggested that this liposomal preparation had low permeability to both Na⁺ and P_i, thus permitting the Na⁺ gradient to persist for hours across the liposomal membranes and to serve as a driving force for P_i uptake. The equilibrium uptake of P_i was the same in all three groups, which indicated that intravesicular volume was not altered.

Fig. 1(b) depicts the initial rates of P_i uptake within the first few minutes after initiation of incubation, according to liposome composition. P_i uptake in the presence of a K⁺ gradient measured passive P_i permeabilities, in descending order: CH/PC > CH/PC/PE > CH/PC/PE/PS. In marked contrast, in the presence of Na⁺ the order was inverted and all three groups of liposomes showed a linear increase in P_i uptake over the first 3 min of the study. The linearity of P_i uptake indicated that, during this time, the Na⁺ gradient was unaltered, which allowed us to calculate initial rates of P_i uptake into the various proteoliposomes.

Table 1 summarizes extent of protein incorporation and initial rates (per mg of protein) of Na⁺-gradient-dependent P_i uptake into different kinds of liposomes. Although the types and amounts of protein used for incorporation were identical, the total amount of protein associated with liposomes depended

Fig. 2. Osmotic reactivity of proteoliposomes

CH/PC/PE/PS were used for liposome formation. The proteoliposomes were prepared as described in the Materials and methods section. Equilibrium uptake of [³²P]P_i was measured by a rapid filtration method after a 16 h incubation in uptake media containing NaCl (●) or KCl (○) and graded sucrose concentrations (0–300 mM). At infinite osmolarity (intersection with the y axis), the extrapolated P_i binding was 1.17 nmol/mg of protein. Data are means ± S.D. from two separate proteoliposome preparations.

on their lipid composition, and increased in the order: CH/PC < CH/PC/PE < CH/PC/PE/PS. When liposomes were synthesized with CH/PC/PE or CH/PC/PE/PS, the initial rates of Na⁺-gradient-dependent P_i uptake were 3–4-fold higher than in those composed of CH/PC, thus indicating that in the latter proteoliposomes higher inactivation of co-transporter occurred. The initial P_i uptake rates into reconstituted liposomes made up of CH/PC/PE and CH/PC/PE/PS were similar (not statistically different), and only liposomes formed from CH/PC/PE/PS were used in further studies.

When other fractions eluted from the hydroxyapatite column were similarly pooled and assayed, no Na⁺-dependent P_i uptake was detected.

Table 2. Sensitivity to inhibitors of the reconstituted Na⁺/P_i co-transporter

The proteoliposomes were preincubated with the indicated concentration of reagents. [³²P]P_i uptake was determined under Na⁺ gradient conditions. The data are corrected for uptake in the presence of KCl. Values are means ± S.D. from two experiments.

Inhibitor	Concentration (mM)	Na ⁺ -dependent P _i uptake (nmol/min per mg)	Inhibition of uptake (%)
None (control)	0	2.8 ± 0.15	0
Phenylglyoxal	20	1.4 ± 0.09	50
<i>N</i> -Ethylmaleimide	2	1.3 ± 0.10	54
Dipyridyl disulphide	2	1.2 ± 0.08	58

Osmotic sensitivity of the proteoliposomes

The observed P_i uptake into proteoliposomes could consist of two components: intravesicular transport and transport bound to proteoliposomes. To determine the amount of P_i transported intravesicularly, equilibrium uptake of P_i into reconstituted proteoliposomes was measured as a function of extravesicular osmolarity. As shown in Fig. 2, when equilibrium P_i values were plotted against the inside/outside osmolarity ratio, a straight line that intersected the *y* axis was obtained. This intersection represents P_i binding values (osmotically insensitive) at infinite osmolarity. In our preparation, this binding reached about 30 % of the total uptake value, whereas the remaining 70 % represented transport into an intravesicular space (osmotically sensitive). Equilibrium intravesicular uptake and binding values were similar whether Na⁺ or K⁺ was present in the uptake buffer.

Sensitivity to Na⁺/P_i transport inhibitors

Phosphate transport across BBM and P_i binding to the protein that we isolated from kidney cortex have been shown to be highly sensitive to inhibition by phenylglyoxal, an arginine modifier [17,22], as well as by sulphhydryl-modifying reagents [17,23–25]. Therefore we used these two classes of agents to test whether reconstituted co-transporter activity was sensitive to known inhibitors of P_i transport. Data presented in Table 2 show that Na⁺-dependent P_i uptake into proteoliposomes was decreased by phenylglyoxal, dipyridyl disulphide and *N*-ethylmaleimide. Na⁺-independent uptake and equilibrium value were not affected by this treatment (results not shown). These results indicate that,

Fig. 4. Gel chromatography of the reconstitutively active fraction eluted from the hydroxyapatite column

Chromatography was carried out on a Sephadex G-200 column. Elution was performed in 5 mM-Hepes/Tris/0.1 % NP40, pH 7.2, at a flow rate of 0.8 ml/h and was monitored by absorbance at 280 nm. Arrows indicate the elution volumes of molecular mass markers: 1, ferritin (440 kDa); 2, catalase (232 kDa); 3, aldolase (158 kDa); 4, albumin (67 kDa); 5, ovalbumin (43 kDa).

upon reconstitution, the purified co-transporter demonstrates sensitivity to inhibitors similar to that of the co-transport system in intact BBM.

Structural studies

The reconstitutively active fraction eluted from the hydroxyapatite column was further analysed by IEF and urea/SDS/PAGE. The isoelectric point of the isolated protein was calculated to be ~ 5.8 (Fig. 3a). Urea/SDS electrophoresis followed by silver staining revealed a broad diffuse band with a molecular mass ranging from ~ 63 to 66 kDa under both reducing and non-reducing conditions (Fig. 3b). The presence of carbohydrate in the co-transporter was assessed by treatment with specific enzymes. The purified protein that had been enzymically deglycosylated with endoglycosidase F gave a band with a molecular mass of ~ 50 kDa (Fig. 3b). These results establish that the co-transporter is an acidic glycoprotein.

The molecular mass of the isolated protein was also assessed by Sephadex G-200 gel-filtration chromatography under non-

Fig. 3. Electrophoretic analysis of the reconstitutively active fraction eluted from the hydroxyapatite column

The protein was isolated as described in the Materials and methods section and the samples were then submitted to one of the following treatments. (a) IEF: lane 1, standard protein markers; lane 2, isolated protein. (b) Urea/SDS/PAGE in a 7.5 % polyacrylamide gel with heat treatment of the sample. Lane 1, standard protein markers; lane 2, isolated protein (reduced with dithiothreitol); lane 3, isolated protein (unreduced); lane 4, isolated protein digested for 96 h with endoglycosidase F. (c) SDS/PAGE under mild conditions in a 5 % polyacrylamide gel without heat treatment of the sample: lane 1, isolated protein; lane 2, standard protein markers.

denaturing conditions. A single peak was eluted with an estimated molecular mass of 170 ± 10 kDa (Fig. 4). This indicates that, in the native form, the co-transporter protein may exist as a polymer in which single peptide molecules are not associated with each other by disulphide bonds. The protein probably has a strong propensity to form multiunit complexes stabilized by hydrophobic bonds. This assumption was supported by SDS/PAGE performed under mild conditions without urea and sample heating. In this case, a high-molecular-mass protein band migrating in the 170–180 kDa area was detected (Fig. 3c).

DISCUSSION

The object of the present work was to demonstrate that the Na⁺/P_i-binding protein that we had previously isolated [17] could function as a Na⁺/P_i co-transporter. This led us to set up a fast purification procedure to avoid denaturation of the protein during isolation. Purification was achieved by extraction of BBM with chloroform/methanol, followed by hydroxyapatite chromatography. Previous attempts to restore Na⁺-P_i co-transporter activity into artificial liposomes were not successful, and only poor Na⁺-dependent P_i uptake was measured after reconstitution of the separated protein fractions [12,13]. We believe that generation of functional proteoliposomes was possible for three reasons.

First, lipids present at the time of protein solubilization seem to play a critical role in the maintenance of functional activity. Restoration of activity of solubilized membrane protein by phospholipid addition during removal of detergents has been observed in a number of different systems, including P_i-linked antiporters [26,27]. With this in mind, we selected a purification method that allowed us to isolate protein in association with naturally occurring phospholipids. After analysis by t.l.c. of our protein fraction eluted from hydroxyapatite, it was indeed found that samples contained large amount of phospholipids (results not shown).

Second, the nature of the lipid components used for liposome formation has been shown to be another important factor involved in proper incorporation of the protein [28,29]. The role of acidic lipids in binding proteins to liposomes or monolayers was established by several investigators [30]. Therefore, to select the appropriate conditions for reconstitution, different ratios and types of lipids were used for liposome formation. The best efficiency of protein incorporation was achieved into liposomes formed from CH/PC/PE/PS which were also relatively impermeable to both Na⁺ and P_i, thus preserving gradients over several hours (Fig. 1 and Table 1).

Third, we used a newly described technique for reconstitution of membrane proteins [31]. Proteoliposomes were formed as a result of the removal of detergent by the dilution method from mixed micelles containing phospholipid, protein and detergent. This procedure is mild, possibly resulting in less damage to protein during the incorporation phase. By contrast, in preliminary experiments using the freeze-thaw sonication method, only a minor fraction of the active co-transporter could be incorporated into proteoliposomes.

The conclusion that the protein that we have purified is, in fact, the Na⁺/P_i co-transporter relies on the following properties demonstrated after incorporation into liposomes: (i) Na⁺-gradient-dependent uptake of P_i into an intravesicular space; (ii) 'overshoot' transient Na⁺-gradient-dependent accumulation of P_i above equilibrium value; and (iii) sensitivity to classical P_i transport inhibitors.

The purified BBM preparation reconstituted in active form in proteoliposomes and exhibiting the main features of the BBM Na⁺/P_i co-transporter seems to contain a single acidic glyco-

protein with the following characteristics: (i) a pI of ~ 5.8 , (ii) a molecular mass under non-denaturing conditions of 170–180 kDa, (iii) a molecular mass under denaturing conditions of 63–66 kDa, and (iv) a molecular mass of the deglycosylated form of ~ 50 kDa. In the first paper describing this protein [17], we found a molecular mass of about 70 kDa under denaturing conditions and of 155 kDa by gel filtration on Sepharose 4B. These discrepancies are explained by the different conditions under which molecular masses were estimated.

It has been noted that most membrane transport proteins are oligomeric and that oligomerization may be required for transport activity [32,33]. Similarly, it is very likely that in its native form the Na⁺/P_i co-transporter is a multimer made up of subunits linked by hydrophobic bonds. This hypothesis is supported by our recent data as well as by those obtained by Beliveau *et al.* [34] using an irradiation-inactivation method. These authors suggested that the functional state of the Na⁺/P_i co-transporter in the membrane should have a molecular mass of ~ 200 kDa. If this estimate is correct, the co-transporter may exist in a trimeric aggregate form in BBM. This also assumes that the molecular mass of monomers is ~ 66 kDa. The exact molecular mass may, however, be somewhat lower and the co-transporter could be a tetramer. Indeed, the molecular mass of glycoproteins is difficult to determine precisely by using SDS/PAGE and gel filtration, for two reasons. First, since the protein is glycosylated, decreased migration in SDS gels and consequently overestimation of molecular mass may be caused by decreased SDS binding by the glycoprotein [35]. Second, molecular masses of glycoproteins may not be appropriately deduced from the calibration curves established for globular proteins by the calibration kit.

Using a completely different approach based on expression and cloning of the Na⁺/P_i co-transporter, it has been suggested that a membrane protein of ~ 50 kDa could be involved in Na⁺/P_i co-transport activity [36,38]. This estimation was made before post-translational processing of the protein. Interestingly, the molecular mass calculated from the amino acid sequence deduced from the cDNA is very similar to that of the deglycosylated form of the protein identified in our laboratory as the Na⁺/P_i co-transporter.

Na⁺-P_i co-transport in BBM is a rate-limiting step in P_i absorption, as well as a major target of regulation by a multitude of hormonal, metabolic and dietary factors. A number of regulatory mechanisms have been described [1,3,4,5,37,38], but further advances are limited by the lack of knowledge of the structure, spatial conformation, precise subcellular distribution and trafficking of the transporter molecule. Purification and characterization of a reconstitutively active form of the Na⁺/P_i co-transporter from rabbit kidney BBM represent an important step towards better understanding of the molecular events involved in the regulation of P_i transfer across the membrane. Accordingly, we have raised a panel of monoclonal antibodies against the purified co-transporter protein [39], and these antibodies can be used for subcellular localization of the antigen and identification of crucial functional epitopes.

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