Identification of Na^+, P_i -binding protein in kidney and intestinal brush-border membranes

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An Na^+, P_i -binding protein has been extracted from kidney and intestinal brush-border membranes with an organic solvent and has been purified by Kieselghur and Sephadex LH-60 chromatography. The molecular mass of this protein has been estimated to be about 155 kDa as determined by gel-filtration chromatography on Sepharose 2B. Under denaturing conditions, polyacrylamide-gel electrophoresis revealed a monomer of molecular mass about 70 kDa. The protein has high specificity and high affinity for P_i [$K_{0.5}$ (concentration at which half-maximal binding is observed) near $10 \mu\text{M}$]. Na^{2+} binding also exhibits saturation behaviour, with a $K_{0.5}$ near 7.5 mM. P_i binding is inhibited by known inhibitors of P_i transport in brush-border membrane vesicles. It appears that this protein could be involved in Na^+/P_i co-transport across the renal and intestinal brush-border membranes.

INTRODUCTION

The existence of Na^+/P_i co-transport in the kidney and intestine brush-border membrane (BBM) is a well-established phenomenon [1–3]. Recently P_i transport has been the subject of several reviews [4–6], which reflects the extent of investigative work; however, the mechanisms that regulate P_i transport are still poorly understood. This lack of information is attributable, in large part, to the fact that little is known about the molecular properties of the transporting system, which has not yet been isolated. Attempts have been made at reconstitution of the solubilized and partially purified Na^+/P_i co-transporter from renal brush-border membrane into artificial liposomes [7–9]. Electrophoretic analysis of protein incorporated into liposomes revealed a few polypeptide bands, and the question of which of them is the transporter remains open. Similarly, covalent labelling of carrier molecules by reagents reacting specifically with different amino acids were not useful in identifying the Na^+/P_i cotransporter [10]. A low-molecular-mass proteolipid named 'phosphorin', which binds P_i , has been extracted from rabbit kidney brush-border membranes [11,12]. This proteolipid can not be a complete transporter molecule, but it might be part of one.

To explore the possible role of a hydrophobic protein as a carrier in transport of P_i , in the present experiments the purification and properties of the Na^+, P_i -binding protein extracted with chloroform/methanol from kidney and intestine BBM have been examined.

MATERIAL AND METHODS

Kieselghur (Extrelute) was obtained from Merck; Sephadex LH-60, Sepharose 2B, the gel-filtration calibration kit and the electrophoresis calibration kit were from Pharmacia; acrylamide, N,N' -methylenebisacrylamide, 2,4,6-trinitrobenzenesulphonic acid and phenylglyoxal were from Serva; SDS and urea were from Bio-Rad; 4,4-di-isothiocyano-2,2-disulphonic acid was from

Calbiochem; [^{32}P] P_i (carrier-free) and $^{22}\text{NaCl}$ (carrier-free) were from Amersham International. [γ - ^{32}P]ATP was kindly given by Dr. L. Wasilewska (Institute of Biochemistry and Biophysics, Polish Academy of Science, Warsaw, Poland). It was prepared by the method of Walseth & Johnson [13]. All phospholipids standards and other reagents were from Sigma.

BBM preparation

BBM were prepared from male-rabbit (6 months old) renal cortex and intestine mucosa by the magnesium precipitation method and differential centrifugation [14]. The purity of the final vesicle preparation was monitored by assaying the activity of enzymes known to be characteristic of BBM (alkaline phosphatase), antiluminal membranes (Na^+, K^+ -ATPase), mitochondria (succinic dehydrogenase), lysosomes (acid phosphatase) and endoplasmic reticulum (glucose-6-phosphatase). The enzymes assays were carried out by previously described methods [15].

Preparation of crude extracts

The membranes were extracted by stirring for 4 h at room temperature with chloroform/methanol (2:1, v/v) at a concentration of 1 g/200 ml. The organic extract was filtered through Whatman no. 1 filter paper and washed vigorously for 2 h with 0.2 vol. of 1 M-KCl. After overnight phase separation at 4°C in a separatory funnel, the lower chloroform phase was filtered through Whatman 1 PS paper and evaporated to dryness on a rotary evaporator. The dried material was dissolved in chloroform/methanol (2:1, v/v) and kept at -20°C under N_2 .

Protein purification

Column of Kieselghur (2.6 cm $\times$ 7 cm) were prewashed with chloroform/heptane (1:1, v/v), then the sample of crude extract in 1 ml of the same solution was applied to the column. The column was eluted with following solvents: chloroform/heptane (1:1, v/v); chloroform/

methanol (20:1, v/v); chloroform/methanol (10:1, v/v); and chloroform/methanol (1:1, v/v). Five 20 ml fractions were collected and assayed for protein and P_i -binding activity. The P_i -binding fractions eluted from the Kieselghur column were delipidated on a Sephadex LH-60 column (0.9 cm $\times$ 90 cm), equilibrated, and eluted with chloroform/methanol/water (23:23:4, by vol.) and 60 mM- NH_4HCO_3 . Elution was monitored at 280 nm, and in each fraction P_i and Na^+ binding activities were tested.

Molecular-mass determination

The Na^+ , P_i -binding fractions eluted from the Sephadex LH-60 column were evaporated to dryness, dissolved in 25 mM-Hepes/Tris/0.1% Nonidet P40, pH 7.0, and loaded into a column (2.6 cm $\times$ 42 cm) of Sepharose 2B equilibrated and eluted with the same buffer. Elution was monitored at 280 nm. Molecular masses were calculated from a standard curve of $\log(\text{molecular mass})$ against K_{av} , using molecular-mass markers.

Electrophoresis was performed in a horizontal thin-layer gel on an LKB 2177 Multiphor apparatus. The acrylamide solutions contained 7.5% (w/v) acrylamide and 0.25% bisacrylamide (to exhibit proteins of molecular mass in excess of 30 kDa) or 15% (w/v) acrylamide and 0.66% bisacrylamide (to exhibit proteins of molecular mass less than 30 kDa). The acrylamide solutions also contained 6 M-urea and 0.1% SDS in 0.1 M-sodium phosphate buffer, pH 7.0. The Na^+ , P_i -binding fractions were evaporated to dryness and dissolved by incubating at 40 °C for 30 min in a solution containing (final concentrations): 1% SDS, 6 M-urea, 5 mM-dithiothreitol and 0.01 M-sodium phosphate buffer, pH 7.0. The gels were silver-stained [16].

Binding test

The Na^+ and P_i binding activities were determined by the partition test in an aqueous organic system [10]. The principle of this method is the equilibration of a water-soluble substance between a water phase and an organic

phase, in which the substance may be bound by a lipophilic compound. The tested samples were dissolved in 500 μl of chloroform/butanol/methanol (50:150:25, by vol), and 10 μl of an aqueous solution containing [^{32}P] P_i or $^{22}\text{NaCl}$ were added. The aqueous solutions were buffered with 10 mM-Hepes/Tris, pH 7.5. After vortex-mixing and leaving the mixture for 10 min at room temperature, 0.6 ml of 2 M-sucrose was added. The contents were vortex-mixed for 90 s and clean phase separation was achieved by centrifugation. The unbound P_i or Na^{2+} was localized in the lower water phase, whereas the bound P_i or Na^{2+} were present in the upper organic phase. Portions (100 μl) of the organic phase were counted for radioactivity in a liquid-scintillation system for ^{32}P or in a γ -radiation counter for ^{22}Na . All assays were made in triplicate, along with a blank containing solvent but no protein.

Other methods

Protein content was determined by the Lowry method as modified by Lees & Paxman [17]. The presence of lipids in preparations were checked by t.l.c. on silica gel, using chloroform/methanol/water (80:25:4, by vol.) as eluent. Lipids were revealed with a phosphomolybdic acid spray.

RESULTS

In the final vesicle preparation, the BBM marker, alkaline phosphatase, was enriched about 15 times over that in the homogenate. Contamination of membrane vesicles by mitochondria, as assayed by succinic dehydrogenase, was undetectable. The antiluminal membrane marker, Na^+ , K^+ -ATPase, the lysosomal marker, acid phosphatase, and the endoplasmic-reticulum marker, glucose-6-phosphatase, are enriched about 1–1.5-fold in the final vesicle preparation. The enrichment factors for kidney and intestine preparations were about the same. What is of importance is that BBM vesicle preparations did not contain mitochondria, which also have a P_i -transport system.

Extraction of the BBM vesicle with chloroform/methanol (2:1, v/v) resulted in the solubilization of 0.4% of the protein in the BBM and most of the lipids. This crude extract prepared from BBM appeared to be able to bind P_i and transfer it into the organic phase (Table 1). The P_i binding activity of BBM vesicle extract prepared from different segments of intestine correlated well with the level of intestinal phosphate transport, which, in the case of the rabbit, is the greatest in the duodenum [18,19]. When pure phospholipids or lecithin (crude phosphatidylcholine) were added to the organic phase, no increase in radioactivity was detected (Table 1). Because the transfer of P_i into the organic phase was mediated by hydrophobic compounds, we sought to detect possible binding of P_i to organic-solvent-soluble protein extracted from BBM. Therefore we tried to separate the P_i -binding protein from crude extract by using column chromatography. Fig. 1 shows the proteins and P_i -binding profile of the fractions eluted from the Kieselghur column. The highest binding of P_i per mg of protein was found in the second fraction eluted from the Kieselghur column with chloroform/methanol (20:1, v/v), and this fraction was used in further studies. The elution profiles of the kidney and intestine extracts had similar characteristics. The pH within the physiological

Table 1. Uptake of P_i into an organic phase containing a crude extract of rabbit BBM or different lipids

A portion of crude extract or phospholipids were dissolved in the organic phase of butanol/chloroform/methanol (6:2:1, by vol.). Binding test was performed with [^{32}P]phosphate (10 μM) under standard conditions. All assays were performed in triplicate. Values are means $\pm$ S.D.

Assay conditions	Radioactivity in the organic phase (c.p.m.)
Control chloroform alone	150 $\pm$ 9
Kidney crude extract (10 μg)	12 500 $\pm$ 105
Intestine	
Duodenal crude extract (12 μg)	10 100 $\pm$ 126
Ileal crude extract (9 μg)	1200 $\pm$ 15
Phosphatidylcholine (0.50 mg)	170 $\pm$ 10
Phosphatidylethanolamine (0.50 mg)	200 $\pm$ 12
Cardiolipin (0.25 mg)	150 $\pm$ 18
Phosphatidylserine (0.25 mg)	200 $\pm$ 14
Phosphatidylinositol (0.25 mg)	250 $\pm$ 8
Lecithin (crude phosphatidylcholine) (1.0 mg)	350 $\pm$ 10

Fig. 1. Elution profile of the crude extract from kidney brush-border membrane chromatographed on a Kieselghur column

The Kieselghur column (2.6 cm × 7 cm) was prewashed with chloroform/heptane (1:1, v/v). Five 20 ml fractions were eluted with: (1) chloroform/heptane (1:1, v/v); (2) chloroform/methanol (20:1, v/v); (3) chloroform/methanol (20:1, v/v); (4) chloroform/methanol (10:1, v/v); and (5) chloroform/methanol (1:1, v/v). The individual fractions were concentrated by evaporation on a rotary evaporator and examined for protein content (■) and P_i-binding activity (□) in the standard assay with a 10 μM-[³²P]P_i.

Fig. 2. Influence of P_i concentration on P_i binding

The second fraction was tested for P_i binding in the concentration range 1–200 μM-[³²P]P_i. Inset: reciprocal plot of upper curves. ●, Kidney; ■, intestine. Values are means ± S.D.

range was found to have slight influence on P_i binding by the kidney preparation and no effect in the case of intestine (results not shown). For this reason a pH of 7.5 was chosen for all subsequent binding studies. The P_i binding by the second fraction, measured as a function of P_i concentration over the range 1–200 μM, exhibited a saturation behaviour, indicating the participation of a carrier-mediated process. The K_{0.5} for P_i binding in the kidney and intestine fraction occurred at 10.2 μM and 12.1 μM respectively (Fig. 2).

In further experiments we studied whether the binding

Table 2. Exchange of bound [³²P]P_i by various anions

The second fraction eluted from the Kieselghur column was labelled with 10 μM-[³²P]P_i in the standard binding test. The bound ³²P was then displaced with sodium salts of the indicated anions at a concentration of 1 mM for 2 min before phase separation with 2 M-sucrose.

Exchanged anion	Radioactivity in the organic phase (% of control)	
	Kidney	Intestine
Control	100	100
SO ₄ ²⁻	80	96
NO ₃ ⁻	80	90
Lactate	95	95
Malate	88	92
Citrate	71	86
Cl ⁻	68	75
P _i	35	32

Table 3. Effect of different reagents on P_i binding

A portion of the second fraction eluted from the Kieselghur column and tested inhibitors were dissolved in the organic phase of butanol/chloroform/methanol (6:2:1, by vol.) and preincubated for 30 min before the addition of P_i. The samples were incubated 10 min with 10 μM-[³²P]P_i and, after addition of 2 M-sucrose for phase separation, the quantity of radioactivity transferred into the organic phase was determined. Abbreviations: DIDS, 4,4-diiodothiocyano-2,2-disulphonic acid; TNBS, 2,4,6-trinitrobenzenesulphonic acid; Pyr-S-S-Pyr, dipyrldyl disulphide.

Inhibitor	Concn. (μM)	Radioactivity in the organic phase (% of control)	
		Kidney	Intestine
DIDS	100	10	10
TNBS	100	8	10
Phenylglyoxal	100	15	18
Arsenate	25	35	40
Pyr-S-S-Pyr	100	55	60
EDTA	100	15	10

of P_i by the second fraction was specific for this anion. Therefore the exchange of the bound labelled P_i with other anions were measured. As shown in Table 2, the highest exchange was found with P_i (65%); citrate and Cl⁻ exchanged from 15 to 30%. The other tested anions, namely SO₄²⁻, NO₃⁻, lactate and malate, exchanged about 5–10% of the bound P_i.

Direct binding studies have shown that [γ-³²P]ATP was bound by the second fraction to a negligible extent.

The P_i binding capacity of the second fraction was affected by a number of compounds (Table 3). The amino-group-reactive reagents 4,4-di-iodothiocyano-2,2-disulphonic acid and 2,4,6-trinitrobenzenesulphonic acid, as well as phenylglyoxal, a specific reagent for arginine, appeared to be very effective inhibitors of the

Fig. 3. Influence of Na^+ concentration on Na^+ binding

The second fraction was tested for Na^+ binding in the concentration range of 1–40 mM- $^{22}\text{NaCl}$. Inset: reciprocal plot of upper curve. ●, Kidney; ■, intestine. Values are means $\pm$ S.D.

P_i binding activity. Phenylglyoxal is also known to be a very strong inhibitor of Na^+ -dependent P_i uptake by BBM vesicles [20]. The involvement of the amino groups of lysine and arginine in P_i binding has been described for the P_i translocator in chloroplast [21]. Similarly, in the case of cytochrome *c*, the lysine residue is located within the P_i -binding site of the molecule [22]. The arsenate analogue of P_i was an equal inhibitor of P_i transport in BBM vesicles [23] and binding of P_i . A thiol residue is the next amino acid residue involved in the functioning of the Na^+/P_i co-transport system in BBM vesicles [24]. Modification of the thiol group by dipyrindyl disulphide leads to the inhibition of P_i binding, suggesting the importance of this group in the binding process. The requirement for bivalent metal ions by the Na^+/P_i co-transport system in BBM vesicles was observed [12,25]. Similarly, binding of P_i was also inhibited by bivalent-metal-ion chelators. All these data postulate that the same groups are required both for the Na^+/P_i co-transport in BBM vesicles and for the binding of P_i .

There is firm evidence that P_i forms, with Na^+ and the Na^+/P_i co-transporter, a ternary complex on the luminal surface of BBM that is translocated across the membrane into the cell interior [6,26,27]. It was of interest, therefore, to determine whether Na^+ is also bound by the P_i -binding fractions. The binding test were performed with all five fractions eluted from the Kieselghur column. As observed for P_i , the highest binding of Na^+ per mg of protein was observed in the second fraction, which was taken for kinetic studies. The binding of Na^+ was measured from 1 to 40 mM. Half-maximal binding occurred near 7.5 mM (Fig. 3). These data suggest that the second fraction is able to bind not only P_i , but Na^+ as well. Characteristics of the Na^+ binding remain to be investigated. T.l.c. analysis indicated that the second fraction was contaminated with substantial amounts of phospholipids. To separate the protein from them we chromatographed the second fraction on a Sephadex LH-60 column. The elution profile is presented in Fig. 4. P_i and Na^+ were bound by the same fractions eluted from the Sephadex LH-60 column. These fractions were significantly purified with respect to phospholipids, as detected by t.l.c. chromatography (Fig. 7a, below).

Fig. 4. LH-60 chromatography of the second fraction eluted from the Kieselghur column

The sample was dissolved in chloroform/methanol/water (23:23:4, by vol.) and 60 mM- NH_4HCO_3 and applied to a Sephadex LH-60 column (0.9 cm $\times$ 90 cm) equilibrated and eluted with the same solvent. Elution was monitored at 280 nm. The individual fractions were concentrated and examined for binding activity in the standard assay with a 10 μM - ^{32}P P_i (○) or 5 mM- $^{22}\text{NaCl}$ (●). The elution profiles for kidney and intestine samples were the same.

Fig. 5. Gel chromatography of the Na^+/P_i -binding fraction eluted from the Sephadex LH-60 column

Fractions were evaporated and dissolved in the elution buffer. Chromatography was performed on a Sepharose 2B column (2.6 cm $\times$ 42 cm). Elution was performed in 25 mM-Hepes/Tris/0.1% Nonidet P40, pH 7.0, at a flow rate of 0.8 ml/h and was monitored by absorbance at 280 nm. Arrows indicate the elution volume of molecular-mass markers: 1, thyroglobulin (669 kDa); 2, ferritin (440 kDa); 3, catalase (232 kDa); 4, aldolase (158 kDa).

The concentrated sample was then subjected to gel filtration on a Sepharose 2B column under non-denaturing conditions. Protein was eluted as a single symmetrical peak whose position was close to that of the aldolase marker (Fig. 5). By comparison with molecular-mass standards, a molecular mass of 155 kDa was

Table 4. Purification of the Na⁺,P_i-binding protein

Step	Total protein (mg)	Total binding (nmol of P _i)	Specific binding (nmol of P _i /mg)	Purification factor
Crude extract	3.0	2.5	0.83	—
Kieselghur	0.18	3.13	17.4	21
Sephadex LH-60	0.16	4.15	25.9	31

calculated. The Na⁺,P_i-binding fractions eluted from the Sephadex LH-60 column were also submitted to urea/SDS/polyacrylamide-gel electrophoresis. In this case a protein monomer with a molecular mass of about 70 kDa for both the intestine and the kidney preparation was revealed (Fig. 6a, lanes 1' and 1), suggesting that, in its native form, the Na⁺,P_i-binding protein is a dimer.

To isolate the protein components associated with the Na⁺,P_i-binding activity from brush-border membranes we decided to use chloroform/methanol extraction. A typical purification procedure for the kidney preparation is summarized in Table 4. Purification of the Na⁺,P_i-binding protein was calculated only with respect to the P_i-binding activity. This procedure solubilizes relatively little of the membrane proteins and the bulk of lipids. The application of Kieselghur column chromatography was useful in removing other proteins and polar lipids, both of which were more strongly adsorbed to the column than was the Na⁺,P_i-binding protein. In the next step of purification the Na⁺,P_i-binding protein was isolated from the bulk of phospholipids by Sephadex LH-60 chromatography. It is interesting that the total P_i-binding activity of the purified protein was consistently greater than the initial activity. This increase may be due to the removal of some unknown inhibitors. The isolation

procedure indicates that the Na⁺,P_i-binding protein is a proteolipid, i.e. a very hydrophobic protein soluble in organic solvent as defined by Folch & Lees [28].

In contrast with our findings, Kessler *et al.* [11] have described the presence of a low-molecular-mass 3 kDa protein in rabbit kidney BBM that can bind P_i with high specificity and affinity. It is possible that, in BBM vesicles, there exist two distinct hydrophobic proteins able to bind P_i.

On the other hand, analysing the Na⁺,P_i-binding fraction at different stages of purification we have also noticed a low-molecular-mass band on 15%-(w/v)-acrylamide gel (Fig. 6b, lane 2), which disappeared simultaneously with delipidation of the sample (Fig. 6b, lane 1).

There are two explanations for the above results. First, Kessler *et al.*, [11] used a much more polar solvent and higher temperature of extraction than we did, and this might have solubilized different proteins. Secondly, a low-molecular-mass band revealed on polyacrylamide gel could be of lipid origin.

To answer this question we prepared a crude extract from BBM vesicles as described by Kessler *et al.* [11]. The protein was purified from that extract by Kieselghur and Sephadex LH-60 chromatography as described in the text. In the further studies the Na⁺,P_i-binding fractions were subjected to urea/SDS/polyacrylamide-gel electrophoresis and analysed by t.l.c. for the phospholipids content. As presented in Fig. 6(a), the sample investigated by electrophoresis in 7.5% acrylamide gel, always comprised the band corresponding to a molecular mass of 70 kDa. The fractions from the Kieselghur column contaminated with large amounts of phospholipids (Figs. 7a and 7b, lanes 1) had, in addition, a big smear in front of the polyacrylamide gel (Fig. 6a, lanes 2 and 4). Subsequently the same samples were electrophoresed on a 15% polyacrylamide gel. It appeared that the band in the low-molecular-mass region

Fig. 6. Urea/SDS/polyacrylamide-gel electrophoresis of the Na⁺,P_i-binding fractions at various stages of purification

(a) 7.5% Acrylamide gel; (b) 15% acrylamide gel. On both gels: lanes 1', intestine; lanes 1 and 3, kidney (Sephadex LH-60 chromatography samples); lane 2 and 4, Kieselghur-chromatography sample; lanes 5, phospholipid standards; lanes 6, phospholipids eluted from the Sephadex LH-60 column. Lanes 1', 1 and 2, crude extract preparation (as described in the text); lanes 3 and 4, crude extract prepared by the method of Kessler *et al.* [11].

Fig. 7. T.L.C. showing phospholipids associated with Na^+ , P_i -binding fractions

On both chromatograms: lanes 1, Kieselghur chromatography samples; lanes 2, Sephadex LH-60 chromatography samples. (a) Crude extract preparation (as described in the text); (b) crude extract prepared as described by Kessler *et al.* [11].

was revealed only when the samples were contaminated with large amounts of phospholipids (Fig. 6b, lanes 2 and 4). Decreasing the phospholipid content by using Sephadex LH-60 chromatography (Fig. 7) led to the disappearance of a band in the low-molecular-mass region (Fig. 6b, lanes 1 and 3) as well as of the smear visible on the gel (Fig. 6a, lanes 1 and 3). Moreover, a mixture of phospholipid standards and phospholipid fractions eluted from the Sephadex LH-60 column without P_i -binding activity also gave a smear on 7.5% acrylamide gel (Fig. 6a, lanes 5 and 6) as well as a band in the low-molecular-mass region on 15% acrylamide gel (Fig. 6b, lanes 5 and 6). Taken together, our observations suggest that the low-molecular-mass band is of lipid origin.

It is important to note that the P_i -binding sample, obtained by diethyl ether precipitation or partially purified by Sephadex LH-20 chromatography, when subjected for electrophoresis on 15% acrylamide gel by Kessler *et al.* [11] was still contaminated with phospholipids. This suggests that the low-molecular-mass protein in their samples could be attributed to the presence of phospholipids.

However, at this stage we could not eliminate the possibility that a low-molecular-mass protein with a high affinity for P_i was present in the purified (by Kieselghur chromatography) P_i -binding sample, but was removed together with most of the phospholipids on the Sephadex LH-60 column. To resolve the above mentioned problem, the following experiments were undertaken. The standard binding test was carried out with samples isolated by Kieselghur chromatography. The [^{32}P] P_i -labelled organic phases were then taken and chromatographed on silica-gel plates with chloroform/methanol/water (85:25:4, by vol.) as developing solvent. After spraying with Molybdenum Blue the plates were submitted to auto-

radiography. It was found that the radioactivity applied to the plates remained at the origin in both tested samples. No Molybdenum Blue-staining material was detected at the origin. About 95% of radioactivity applied to the plates were eluted with methanol/water (1:1, v/v). After evaporation to dryness the material was dissolved in SDS buffer and subjected to urea/SDS/polyacrylamide-gel electrophoresis; only a single protein band, with a molecular mass of about 70 kDa, was revealed. From these observations it can be concluded that the radioactivity transferred into the organic phase was associated with the 70 kDa protein. Also, as previously, we were not able to detect any low-molecular-mass protein in the P_i -binding samples isolated from crude extracts prepared by either method.

DISCUSSION

The foregoing studies provide the first evidence that, in kidney and intestine BBM, there is present a hydrophobic protein that binds Na^+ and P_i . The initial step in lumen-to-blood P_i transport involves secondary active carrier-mediated Na^+ / P_i co-transport across the luminal BBM. This is a major regulatory site in trans-epithelial P_i transport and it is subjected to modulation by many hormonal and metabolic factors. The molecular basis of BBM co-transport remains unknown at present. Studies on the definition of the transporter are therefore important if we are to understand better the absorption process of P_i under normal physiological conditions and in certain diseases.

The physiological significance and involvement in P_i transport of the Na^+ , P_i -binding protein relates to its origin and properties. First of all the protein has been isolated from BBM that are known to conduct Na^+ / P_i co-transport. The distribution of the P_i -binding activities in the small intestine (Table 1) corresponds to that of the P_i transport, which is greatest in the duodenum and decreases in the distal segments.

The further observation that the protein can bind both Na^+ and P_i provides some basic argument in support of assumption that this protein is involved in Na^+ / P_i co-transport in BBM. All Na^+ /solute co-transport models are based on the premise that there exists a carrier with specific binding sites for each solute and Na^+ [29]. It is of interest also that the apparent affinities of the Na^+ / P_i co-transport system in BBM vesicles for P_i (K_{m,P_i} 30–200 μM) is two to three orders of magnitude lower than for Na^+ (K_{m,Na^+} ~ 70 mM) [26,30–32]. It reflects the differences in the affinities for P_i and Na^+ binding ($K_{0.5,\text{P}_i}$ ~ 10 μM ; $K_{0.5,\text{Na}^+}$ 7.5 mM). Similarly the observation that the binding of P_i is inhibited by all inhibitors of P_i transport (Table 3) supports the suggestion that this protein might be related to P_i transport.

The next property of importance is the strong hydrophobicity of the protein. The Na^+ / P_i co-transporter is exposed on both the luminal and the cytoplasmic side of the BBM [5]; thus the transporter molecule has to contain a hydrophobic membrane-spanning polypeptide domain.

Another interesting finding that favours the suggestion that the Na^+ , P_i -binding protein may be a co-transporter is its molecular mass. The electrophoretic mobility of the protein (Fig. 6a) in urea/SDS/polyacrylamide gels is identical with that of one of the proteins identified in the

sample incorporated into liposomes conducting Na⁺/P_i co-transport [8].

In many respects, the Na⁺/P_i and Na⁺/D-glucose co-transporters of BBM share some similarities, in this light also the occurrence of the Na⁺,P_i-binding protein as a dimer would be in accordance with Na⁺/D-glucose co-transporter which was found to consist of two subunits in their active form [33].

Evidence presented here suggests that the Na⁺,P_i-binding protein may be a strong candidate for the co-transporter responsible for transport of P_i across the BBM membrane.

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REFERENCES

- Murer, H., Stern, H., Burckhardt, G., Storelli & Kinne, R. (1980) *Adv. Exp. Med. Biol.* **128**, 11–23
- Cheng, L. & Sactor, B. (1981) *J. Biol. Chem.* **256**, 1556–1564
- Berner, W., Kinne, R. & Murer, H. (1976) *Biochem. J.* **160**, 467–474
- Murer, H. & Burckhardt, G. (1983) *Rev. Physiol. Biochem. Pharmacol.* **96**, 1–53
- Mizgala, C. L. & Quamme, G. Q. (1985) *Physiol. Rev.* **65**, 431–466
- Gmaj, P. & Murer, H. (1986) *Physiol. Rev.* **66**, 36–70
- Kinne, R. & Faust, R. G. (1977) *Biochem. J.* **168**, 311–314
- Lin, J. T., Schwarc, K. & Stroh, A. (1984) *Biochim. Biophys. Acta* **774**, 254–260
- Schali, C. & Fanestil, D. D. (1985) *Biochim. Biophys. Acta* **769**, 277–283
- Strevey, J., Brunette, M. G. & Beliveau, R. (1984) *Biochem. J.* **223**, 793–802
- Kessler, R. J., Vaughn, D. A. & Fanestil, D. D. (1982) *J. Biol. Chem.* **257**, 14311–14317
- Kessler, R. J. & Vaughn, D. A. (1984) *J. Biol. Chem.* **259**, 9059–9063
- Walseth, T. F. & Johnson, R. A. (1979) *Biochim. Biophys. Acta* **562**, 1131–1149
- Biber, J., Stieger, B., Haase, W. & Murer, H. (1981) *Biochim. Biophys. Acta* **647**, 169–176
- Turner, R. J. & Moran, A. (1982) *Am. J. Physiol.* **242**, 406–414
- Görg, A., Postel, W., Weser, J., Schivara, H. W. & Boesken, W. H. (1985) *LKB Instrum. J.* **32**, 5–9
- Lees, M. B. & Paxman, S. (1972) *Anal. Biochem.* **47**, 184–192
- Danisi, G. & Straub, R. W. (1980) *Pfluegers Arch.* **385**, 117–122
- Hildman, B., Storelli, C., Danisi, G. & Murer, H. (1982) *Am. J. Physiol.* **242**, 533–539
- Beliveau, R. & Stervey, J. (1987) *Kidney Int.* **31**, 160
- Fluge, U. J. & Heldt, H. W. (1987) *Biochem. Biophys. Res. Commun.* **84**, 37–44
- Taborsky, G. & McCollum, K. (1979) *J. Biol. Chem.* **254**, 7069–7075
- Hoffman, N., Thees, M. & Kinne, R. (1976) *Pfluegers Arch.* **362**, 147–156
- Dębiec, H. & Lorenc, R. (1984) in *Epithelial Calcium and Phosphate Transport: Molecular and Cellular Aspects* (Bronner, F. & Peterlik, M., eds), pp. 349–353, Alan R. Liss, New York
- Rouse, D. & Suki, W. N. (1985) *J. Clin. Invest.* **76**, 630–636
- Szczepańska-Konkel, M., Yusufi, A. N. K. & Dousa, T. P. (1987) *J. Biol. Chem.* **262**, 8000–8010
- Amsutz, M., Mahrman, M., Gmaj, P. & Murer, H. (1985) *Am. J. Physiol.* **248**, F705–F710
- Folch, J. & Lees, M. (1951) *J. Biol. Chem.* **191**, 807–817
- Alvarado, F. & Lherminier, M. (1982) *J. Physiol. (Paris)* **78**, 131–145
- Cheng, L. & Sactor, B. (1981) *J. Biol. Chem.* **256**, 8080–8084
- Kempson, S. A., Berndt, T. J., Turner, S. T., Zimmerman, D., Knox, F. & Dousa, T. P. (1983) *Am. J. Physiol.* **244**, R216–R223
- Levine, B. S., Ho, K., Kraut, J. A., Coburn, J. W. & Kurokawa, K. (1983) *Biochim. Biophys. Acta* **727**, 7–12
- Malathi, P. & Preiser, H. (1983) *Biochim. Biophys. Acta* **735**, 314–324

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