Biochimica et Biophysica Acta, 735 (1983) 314–324
Elsevier

BBA 71885

ISOLATION OF THE SODIUM-DEPENDENT D-GLUCOSE TRANSPORT PROTEIN FROM BRUSH-BORDER MEMBRANES

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(Received June 13th, 1983)

Key words: Glucose transport; Na^+ dependence; Phlorizin inhibition; Brush-border membrane; Membrane reconstitution; (Rabbit kidney, intestine)

Rabbit kidney brush-border membrane vesicles were exposed to bacterial protease which cleaves off a large number of externally oriented proteins. Na^+-dependent D-glucose transport is left intact in the protease-treated vesicles. The protease-treated membrane was solubilized with deoxycholate and the deoxycholate-extracted proteins were further resolved by passage through Con A-Sepharose columns. Sodium-dependent D-glucose activity was found to reside in a fraction containing a single protein band of $M_r \cong 165\,000$ which is apparently a dimer of $M_r \cong 85\,000$. When reconstituted and tested for transport, this protein showed Na^+-dependent, stereo-specific and phlorizin-inhibitable glucose transport. Transport activity is completely recovered and is 20-fold increased in specific activity. A similar isolate was obtained from rabbit small intestinal brush-border membranes and kidneys from several other species of animals.

Introduction

The existence of Na^+-gradient coupled solute transport in kidney and intestinal brush-border membranes is a well established phenomenon [1,2]. Although Na^+-coupled transport of D-glucose has been extensively studied in natural brush border membrane vesicles [3–8], the identification and characterization of the transport moiety has been limited both by the lack of a suitable assay system to measure transport by isolated membrane constituents and by sparse information on the nature of the chemical groups involved in the mechanism. It is fairly well established that thiol group inhibitors lead to inactivation of the transport system [9–13]. Based on this requirement for sulfhydryl groups, several investigators have attempted to identify the Na^+-dependent D-glucose transporter in small intestinal and kidney brush-border membranes. Protection by substrate, i.e. D-glucose or phlorizin, of essential sulfhydryl groups, presumed to be at the substrate binding site, from inactivation by *N*-ethylmaleimide or mercurials was the experimental approach used [14–18]. The molecular weight of the glucose binding protein assessed in these studies ranged between 30 000 and 60 000. This method has now been questioned since the sulfhydryl groups required for Na^+-dependent D-glucose transport have been demonstrated to be involved in the translocation process rather than at the substrate binding site [19]. Photoaffinity labeling with photosensitive phlorizin analogs indicate an M_r 72 000 polypeptide as the possible glucose transporter [20,21]. Using phorizin polymer as an affinity support, a protein between M_r 60 000 and 70 000 has been implied as the transporter [22]. Other studies using membrane fractionation techniques have been reported as well, with no definitive conclusions [23,24]. The identity of the

Abbreviation: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

glucose transporter from these various studies is largely inferential and no conclusive evidence has been presented.

Our attempts to identify the glucose transport protein were predicated on the unequivocal demonstration of Na^+-dependent, phlorizin-inhibited, stereospecific transport of the sugar by the isolated membrane component. With this aim and based on the methodology developed by Racker [25] we have reported that solubilized membranes can be intercalated into phospholipid bilayer vesicles and exhibit all the qualitative characteristics of the transport system observed in native membranes [26]. Using this assay system we have reported elsewhere our earlier findings on the purification of the transporter [27,28]. In this paper we report the isolation of a protein fraction from kidney and small intestinal brush border membranes of approximate M_r 165000 which when reconstituted into liposomes exhibits specific D-glucose transport which is 20-fold increased in activity when compared to crude membrane extracts similarly reconstituted.

Materials and Methods

All chemicals used were of the highest available grade. D-[U-^{14}C]Glucose (296 mCi/mmol) and sodium deoxy[*carboxyl*-^{14}C]cholate (60 mCi/mmol) were purchased from Amersham. Bacterial protease (EC 3.4.4.16, Subtilisin Carlsberg, 33.9 Anson units/g) was obtained from Calbiochem-Behring, CA. Rabbits and rats were supplied by Ace Animals, Philadelphia. Kidneys from other species were obtained fresh from a local abattoir.

Preparation of membranes

Most of these studies were done with rabbit kidney cortical brush-border membranes. Following the observations with these membranes, rabbit small intestinal and kidney brush-border membranes from other species were also used. The membranes were isolated using the methods already published [29,30] and were suspended in appropriate buffers for further processing.

Protease treatment of membranes

Isolated membranes were suspended in 0.1 M KCl buffered with 5 mM Hepes-Tris, pH 7.5 (KCl buffer) and the volume adjusted to contain about 2 mg membrane protein per ml. The suspension was held at 25°C and a freshly prepared stock solution of protease (1 mg/ml) added to give a final protease concentration of 20 μg/ml. The mixture was incubated at 25°C for 10 min, following which protease action was retarded by a 5-fold dilution with ice-cold KCl buffer. The proteolysed membranes were separated by centrifugation for 30 min at 48000 $\times$ *g*, washed once to ensure removal of the protease and suspended in suitable buffers for further testing and processing.

Solubilization of membranes

Both native and protease-treated membranes were solubilized by suspending in 5 mM Hepes-Tris buffer, pH 7.5, so as to contain 3–5 mg protein/ml, followed by the addition of 10% sodium deoxycholate in 5 mM Hepes-Tris buffer, to a final concentration of 0.4% deoxycholate. The mixture was held in an ice-bath, sonicated for 1 min using a 3/8 inch Biosonik probe and centrifuged at 192000 $\times$ *g* for 30 min. The supernatant thus obtained represents the solubilized membrane components; increasing the centrifugation time to 1 h did not alter the amount of solubilized membrane proteins. The extracted proteins were separated from the detergent by a 10-fold dilution with Hepes-Tris buffer to lower the deoxycholate concentration below its critical micelle concentration of 4–6 mM [31], addition of 0.1 M KCl and centrifugation at 192000 $\times$ *g*. The proteins aggregate and are recovered as pellets while deoxycholate stays in the supernatant.

Reconstitution of membrane extracts into liposomes

Preformed liposomes were obtained essentially as described earlier [26] except that valinomycin was added to the mixture of soybean phospholipids in acetone, prior to drying under nitrogen. The dried lipids were suspended in 0.1 M K_2SO_4 buffered with 5 mM Hepes-Tris, pH 7.5 (K_2SO_4 buffer) to a final concentration of 30 mg/ml lipids and 40 μM valinomycin. The mixture was sonicated to clarity with the 3/8 inch Biosonik probe at temperatures not exceeding 35°C and filtered through a 0.45 μm Millipore filter. The filtrate was flushed with nitrogen and stored in the cold. Solubilized and pelleted membrane components

were mixed with a suitable volume of liposomes using a tuberculin syringe fitted with a 23 g needle. This mixture was sonicated in a bath sonicator as previously described [26]. We have found it necessary to monitor for optimal reconstitution in each test since the time of sonication required to achieve this is highly variable in different preparations. The mixture was sonicated for 30 s, D-glucose transport tested as described below and the process of sonication and testing repeated. D-Glucose uptake increased with sonication and then fell off, the whole process taking anywhere between 1 and 6 min for maximal transport. The ratio of protein to liposomes was also optimized by testing serial dilutions into liposomes, of the same sample.

Assay of Na^+-dependent D-glucose transport

The transmembrane transport of solutes depending on the presence of external sodium is sensitive to the transmembrane electrochemical Na^+ gradient, $\Delta\bar{\mu}_{Na^+}$ [1]. In isolated membrane vesicles and in reconstituted liposomes the electrical and chemical potentials, $\Delta\psi$ and $\Delta\mu_{Na^+}$, respectively, can be manipulated to vary but will decay with time. In order to optimize initial rates of glucose entry, it is therefore necessary to provide a suitable electrochemical gradient of Na^+ and use short incubation periods within linear rates of uptake. Experience shows that a suitable electrochemical gradient can be achieved either with a valinomycin-induced K^+ efflux from the vesicles or with a lipophilic anion (thiocyanate) influx into the vesicles, in the presence of external Na^+. We have used the former system in the studies reported here. Membrane vesicles, native or protease-treated, were suspended in K_2SO_4 buffer and allowed to equilibrate at 0°C for 60 min in a tube containing 40 nmol valinomycin per ml of membrane suspension. Both membrane vesicles and liposomes, were tested for transport of D-glucose from a solution (200 μl final volume) containing 30 μM D-[$U^{14}C$]glucose, Hepes-Tris buffer and 0.1 M K_2SO_4 or 0.1 M Na_2SO_4, at 25°C. The reaction was initiated with 25 μl of the membranes or reconstituted liposomes and arrested by dilution into 2.0 ml of ice-cold 0.15 M KCl. The vesicles or liposomes were collected by rapid filtration on 0.22 μm Millipore filters and washed quickly with 5 ml of ice-cold 0.15 M KCl. The filters were transferred to vials and dissolved in scintillation fluid containing methyl cellosolve (25%) and toluene (75%) and counted. Routinely, uptake by native and protease-treated membranes was measured at 0.05 min and by reconstituted liposomes at 0.2 min.

Affinity chromatography on Con A-Sepharose 4B

Con A-Sepharose 4B, (Con A-Sepharose; Pharmacia) was washed with 1 M NaCl containing 1% Triton X-100 followed by washing with distilled water and stored in the cold in Hepes-Tris buffer containing 1 mM each of $CaCl_2$, $MnCl_2$ and $MgCl_2$. Columns (25 × 2 cm) of the gel were equilibrated with Hepes-Tris buffer. Deoxycholate extracts of protease-treated membranes were layered on the gel (3–4 ml/column) and eluted with equilibrating buffer. The voided fraction was collected and centrifuged in the presence of 0.1 M KCl at 192000 × *g*. Con A-Sepharose can be reused several times by washing and storage as described above.

Immunological studies

Female rabbits (2–3 lbs) were immunized with purified membrane protein from rat kidneys. The protein was adsorbed to bentonite [32] and suspended in phosphate-buffered saline (0.15 M NaCl + 10 mM potassium phosphate buffer, pH 7.4) to yield a suspension of 100 μg protein/ml. The rabbits were injected subcutaneously on the dorsal side (1 ml/rabbit) and a booster injection given two weeks later. Four weeks after the primary injection the animals were bled from the ear vein and the sera separated. Immune responses were monitored using an enzyme linked-immunosorbent assay (ELISA) [33] modified by Dr. M. Takahashi of this department. The assay is based on adsorption of antigen onto plastic tissue culture clusters, binding of antibody to the antigen followed by binding of anti-rabbit immunoglobulin linked to peroxidase. Peroxidase activity bound to the plates is a direct measure of antigen-directed immunoglobulins. Control sera were routinely included in all tests.

Control and immune sera were rendered free of albumin and serum proteases by passage through CM-Affigel blue obtained from BioRad Laboratories [34]. The globulins were precipitated with 45%

ammonium sulphate, dialysed against phosphate-buffered saline, centrifuged at 48000 × *g* for 30 min and frozen at −20°C in 1 ml aliquots.

Protein was measured by the method of Lowry et al. [35]. Polyacrylamide gel electrophoresis and Coomassie blue staining were carried out as previously described using 50–75 μg protein per sample [36]. For more sensitive detection of protein bands on the gel, silver nitrate was used for staining as described by Merril et al. [37]. Isoelectric focusing on a flat bed of Sephadex-G 200SF containing 1% Triton X-100 and ampholytes (pH 4–6) was performed as described by Haff et al. [38].

Results

Membrane solubilization and reconstitution

Kidney and small intestinal brush border membranes are fairly easily solubilized with neutral and anionic detergents [26]. Detergent removal is a prerequisite to liposome reconstitution by the method we use and in earlier studies using Triton X-100 this was achieved using columns of poly(vinyl styrene) beads (Biobeads SM-2) which bind Triton X-100 [39], permitting the proteins in suspension to elute out. However, in subsequent batches of the adsorbent, considerable amounts of the solubilized proteins were also retained on the beads. Attempts to circumvent this by reducing hydrophobic interactions using low ionic strength buffers (5 mm Hepes-Tris) were futile. Removal of Trition X-100 without loss of solubilized membrane components therefore presented a problem. Deoxycholate is an effective solubilizing agent and is also readily removed from solution using anion exchangers or gel filtration chromatography. It can also be removed from its association with hydrophobic proteins by dilution below its critical micelle concentration followed by centrifugal sedimentation of the aggregated proteins. Solubilization was more than 95% effective with 0.4% deoxycholate. After extraction, addition of KCl was necessary to aid aggregation of the solubilized hydrophobic membrane proteins which did not sediment readily in the absence of added salt. The amount of protein sedimented depends on the time of centrifugation (Table I). In order to isolate at least 90% of the proteins from solution, 24 h of centrifugation at 192000 × *g* was necessary. Increasing the concentration of KCl did not hasten sedimentation. However, as can be seen from Table I, the sediment after 2 h of centrifugation had almost all (97%) the glucose transport function that could be recovered from the membrane extract, although only 44% of the proteins had been centrifuged out. Continued centrifugation isolates more protein from solution with a consequent lowering of the specific activity for glucose transport. Centrifugation of the supernatant remaining after removal of the proteins sedimenting at 2 h resulted in isolation of proteins that showed little or no glucose transport activity. This would indicate that the transport function is more readily sedimented from solution that other proteins.

TABLE I

RECOVERY OF D-GLUCOSE TRANSPORT ACTIVITY FROM MEMBRANE EXTRACTS

Membranes were extracted with deoxycholate and sedimented as described in Results. The pellets were reconstituted and tested for transport as described in Methods. D-Glucose uptake was measured in the presence of 0.1 M K_2SO_4 or 0.1 M Na_2SO_4 for 0.2 min and Na^+-dependent uptake derived from the difference.

Centrifugation time (h)	Protein sedimented [a]		Na^+-dependent D-glucose transport	
	%	mg	pmol/min per mg protein	pmol/min [b]
2	44	6.5	525	3413
4	54	8.0	445	3560
6	66	9.7	365	3540
24	94	13.8	255	3519

[a] 100% protein = 14.7 mg.

[b] Recovery of Na^+-dependent D-glucose transport, represented as pmol transported per min by each fraction.

Polyacrylamide gel electrophoresis did not, however, reveal any specific separation of proteins by this procedure. Also, no enrichment of any particular membrane protein was seen in the 2-h sediment. This is perhaps due to the presence of large amounts of other membrane proteins which sediment at the same time and mask any separation that had occurred.

Purification of membrane fraction associated with D-glucose transport

The ordinarily used methods of protein fractionation such as gel-filtration, ion-exchange chromatography, hydrophobic interaction-chromatography, column and gel electrofocusing including chromato-focusing, all resulted in no resolution or inactivation of the transport system. The presence of a non-ionic detergent like Triton X-100 or Nonidet P-40, essential in most of these methods compounded the problem further since they are not easily removed from the solubilized membrane proteins.

(i) Proteolysis. It has been demonstrated that exposure of brush-border membrane vesicles to the action of proteolytic enzymes, results in, the solubilization of the externally oriented, enzymatically active, hydrophilic moieties of several membrane-associated enzymes leaving only their hydrophobic anchors in the membrane [40,41]. This seemed a reasonable starting point for purification of transport proteins, provided these latter are inaccessible and insensitive to the proteases used. We have published elsewhere [27], that when a partially purified membrane fraction is reconstituted into liposomes and then exposed to papain, only one major protein band of $M_r \cong 165\,000$ remained unaffected and glucose transport was fully recovered. When membrane vesicles themselves are exposed to papain, all of the larger molecular weight proteins, i.e. $> M_r$ 25 000, excepting the $M_r \cong 165\,000$ protein band, are removed. This seemed to be a simple way of eliminating extraneous proteins. However, exposure to papain, while resulting in a single large molecular weight protein, did not always result in a transport-active preparation. The other proteolytic agent that was effective was the bacterial protease, subtilisin, which yielded protease-treated membranes having only two major protein bands of $M_r \cong 200\,000$ and $M_r \cong 165\,000$ [27]. Glucose transport in these preparations is always recovered completely. The $M_r \cong 200\,000$ band is removed by subsequent treatment with low concentrations of papain but again glucose transport activity, when extracted and reconstituted into liposomes is variable. Similar problems with papain have been reported by Tannenbaum et al. [42]. The reason for this seems to be the sensitivity of the cytoplasmic side of the transporter to papain (see Discussion). It is known that when papain or trypsin is introduced to the cytoplasmic side of the membrane, phlorizin-binding activity which is also a measure of glucose transport activity [42], is lost [43]. When membrane extracts are exposed to papain, transport activity is lost presumably because the cytoplasmic side of the transporter is affected [27]. It is likely that papain treatment of membrane vesicles renders the vesicles leaky, permitting papain to penetrate and act on the cytoplasmic side of the transporter. This is supported by SDS-polyacrylamide gel electrophoresis patterns [28] and will be discussed later. Such effects do not occur, apparently, with the use of the bacterial protease, subtilisin. When membranes are treated with bacterial protease as described in Methods, 30–35% of the membrane proteins are removed. Na^+-dependent glucose transport function is completely recovered as evidenced by a corresponding increase in specific activity (3.96 nmol/mg per min for native membrane and 5.72 nmol/mg per min for protease-treated membranes). SDS-polyacrylamide gel electrophoresis of protease-treated membranes as compared to native and extracted whole membranes is shown in Fig. 1. All but two of the higher molecular weight proteins are substantially removed. An increase in the smaller molecular weight proteins, i.e. M_r 25 000 and lower, is in keeping with the observation that the small hydrophobic anchors of the proteins solubilized by the protease remain in the membrane [40]. The same electrophoretic pattern was observed when protease-treated membrane was solubilized with deoxycholate (Fig. 1). The solubilized proteins could be isolated from solution as described for whole membrane extracts. Here again the degree of protein sedimentation depended on time of centrifugation and activity was fully recovered in the 2-h centrifuge pellet which represented 40% of

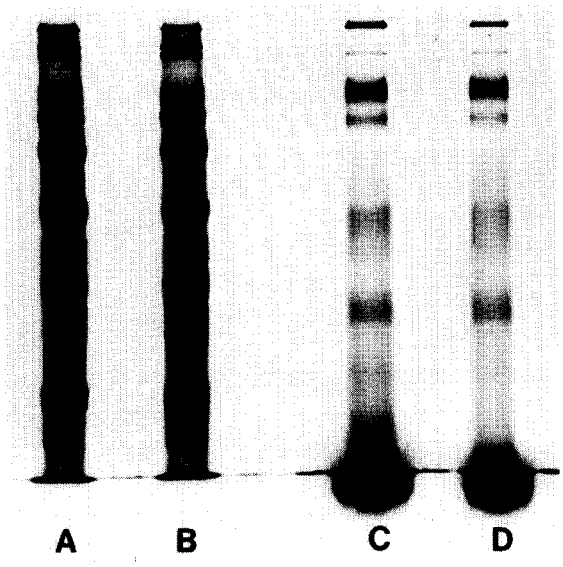

Fig. 1. Coomassie blue stained protein profiles of: (A) kidney brush-border membranes; (B) deoxycholate-solubilized kidney brush-border membranes; (C) protease-treated membranes and (D) deoxycholate-solubilized protease-treated membranes.

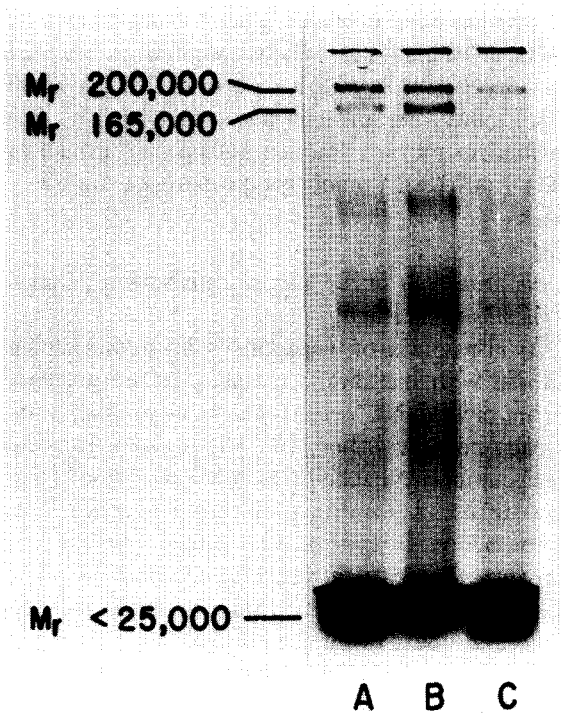

Fig. 2. Protein profiles of deoxycholate-solubilized protease-treated membranes after centrifugal sedimentation. Refer to text for details. (A) Deoxycholate extract of protease-treated membranes; (B) 2-h centrifugal sediment; (C) supernatant from (B) centrifuged for 24 h.

the solubilized proteins. SDS-polyacrylamide gel electrophoresis, however, revealed distinct differences in the proteins sedimented at 2 h and those remaining in the supernatant (Fig. 2). The 2-h sediment showing glucose transport had all the bands found in protease-treated membrane. When the supernatant from the 2-h centrifugation was recentrifuged for 24 h the proteins recovered as a sediment showed a marked reduction in the $M_r \cong$ 165 000 protein band and glucose transport activity was barely detectable. This is additional confirmation that the $M_r \cong 165\,000$ protein is associated with transport. In order to isolate this protein it is clear that protease-treated membrane had to be further fractionated, both to remove the $M_r \cong$ 200 000 protein and the smaller peptides resulting from proteolysis.

(ii) Affinity chromatography with Con A-Sepharose. It is fortuitous that deoxycholate was chosen to replace Triton X-100 as the solubilizing agent. Based on previous experience with Con A-Sepharose [44] further purification of deoxycholate extracts of protease-treated membrane was achieved in a one-step process which removed both deoxycholate and extraneous proteins. The void fraction from Con A-Sepharose columns contained 8–9% of the protein layered on the column and was free of deoxycholate; deoxycholate was retarded on the column and tests with deoxy-[*carboxyl*-^{14}C]cholate added to the protease-treated membrane extracts showed that deoxycholate eluted out of the column only after passage of approx. 1.5 bed volumes of eluting buffer and no radioactivity was found in the fraction eluting in the void volume. The proteins bound to the lectin matrix could not be eluted with 0.5 M α-methyl mannoside or 0.5 M α-methyl glucoside or a mixture of the two. Elution of the bound proteins was achieved only in the presence of detergents (deoxycholate or Triton X-100 at concentrations above 1%). Con A-Sepharose therefore seems in this instance to act as a hydrophobic affinity gel rather than as an immobilized lectin matrix. Similar observations have been made with human fibroblast interferon [45]. All the glucose transporting function was recovered in the fraction voided out of

the column with a considerable increase in specific activity. Extracts of whole membranes showed Na^+-dependent D-glucose uptake of 189.5 ± 20.50 (S.E.) pmol/mg protein per min ($n = 20$). The purified membrane fraction showed an uptake of 3984 ± 407 (S.E.) pmol/mg protein per min ($n = 22$).

Some properties of the glucose-transporting fraction of membrane extracts

(i) The uptake of D-glucose by liposomes reconstituted with membrane extracts, in the presence of an inward Na^+ electrochemical gradient, has already been described [26]. The sugar is transiently accumulated in the liposomes ('overshoot') and then diffuses out until equilibrium is reached. This phenomenon is qualitatively similar to the observations made with membrane vesicles [46] and is represented in Fig. 3. Uptake of D-glucose was rapid and linear in the first 0.4 min and thereafter increased to a maximum between 5 and 10 min ('overshoot') and then reached equilibrium at 5 h. In comparison, the purified fraction exhibited a similar time-dependent pattern of uptake of D-glucose (Fig. 4). Uptake was linear upto 0.4 min, but took longer to reach the 'overshoot' point and to reach equilibrium. In the absence of a Na^+-electrochemical gradient (Na^+ replaced with K^+) D-glucose entry was extremely low (therefore not

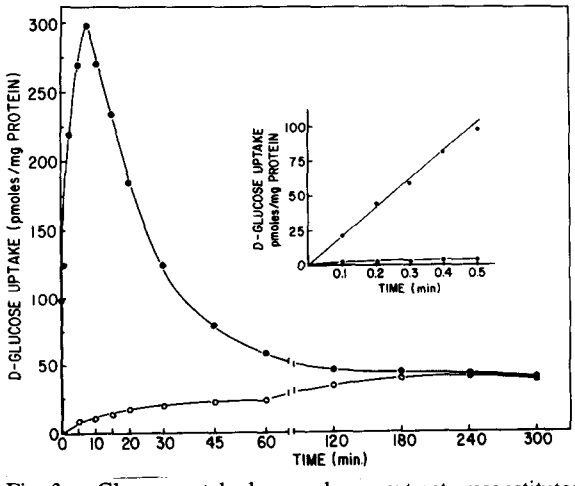

Fig. 3. D-Glucose uptake by membrane extracts reconstituted into liposomes. See Methods for details. ●——●, in the presence of 0.1 M Na_2SO_4. ○——○, in the presence of 0.1 M K_2SO_4.

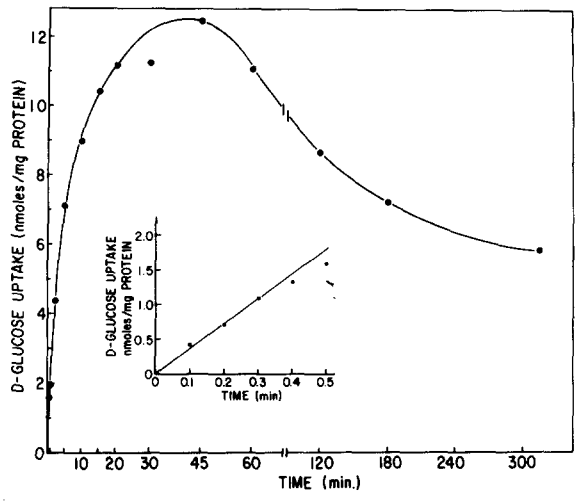

Fig. 4. D-Glucose uptake by the membrane fraction purified from protease-treated membranes and reconstituted into liposomes. ●——●, in the presence of 0.1 M Na_2SO_4.

shown in figure) and the sugar was not equilibrated even after 5 h of incubation; the values at 0.1 min were 8 and 414 pmol/mg protein in K^+ and Na^+ media, respectively; at 'overshoot' the corresponding values were 42 and 12426. Equilibrium was reached only after 10 h. The equilibrium values are comparable in both membrane extract and purified preparations and it can therefore be concluded that there are no gross differences in the intravesicular volumes of the two preparations. L-Glucose does not exhibit these uptake characteristics in the presence of Na^+ observed with D-glucose and no differences were seen in the uptake of L-glucose in the presence of K^+ or Na^+. Phlorizin (1 mM) inhibits D-glucose uptake in the presence of Na^+, inhibition being more than 95%.

(ii) SDS-polyacrylamide gel electrophoresis of the purified fraction revealed the presence of a single protein band of $M_r \cong 165\,000$ (Fig. 5A). As reported earlier, when this protein fraction in SDS is held at the temperature of boiling water a breakdown to a protein band of $M_r \cong 85\,000$ occurs (Fig. 5B). A similar resolution occurs when the isolated protein is subjected to electrofocusing or to low pH, between 4 and 4.3 (Fig. 5D). The $M_r \cong 165\,000$ protein reappears upon titration to neutral pH; however, transport activity is not reconstitutable after exposure to low pH even after titration to neutral pH.

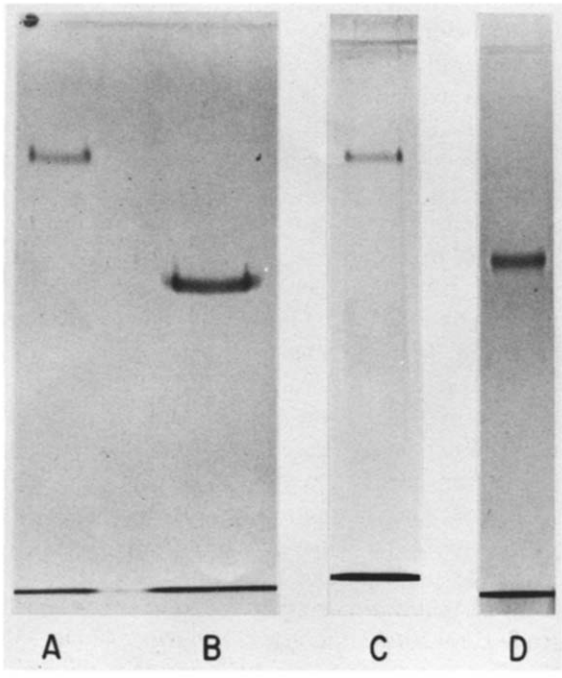

Fig. 5. Coomassie blue stained slab gel protein patterns. (A) and (C) Con A-Sepharose eluates of protease-treated membrane extracts. (B) Sample (A) held in boiling water for 3 min before electrophoresis. (D) Sample (C) isoelectrofocused on Sephadex G200SF flat beds.

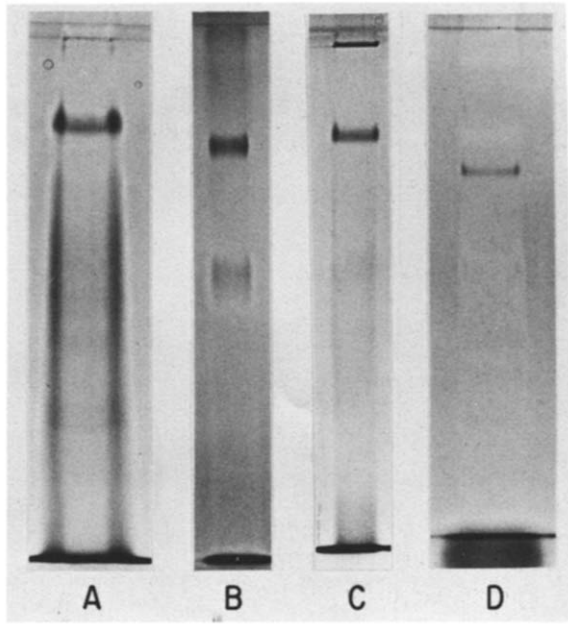

Fig. 6. Purified membrane fraction patterns from brush-border membranes. (A) Rabbit small intestine; (B) calf kidney cortex; (C) rat kidney cortex; (D) goat kidney cortex.

(iii) Intestinal brush-border membranes from rabbits, when similarly processed yielded a membrane isolate very similar to the fraction isolated from kidneys (Fig. 6A). D-Glucose transport by this isolate when reconstituted was 20–24-fold greater than that in reconstituted whole membrane extracts. In one representative experiment Na^+-dependent D-glucose uptake by reconstituted whole membrane extracts was 90 pmol/min per mg protein compared to 2360 pmol/min per mg protein by the purified fraction. Processing of kidney cortices from other species of animals which included goat, rats and calves (bovine), all resulted in the purification of one major protein band of M_r approx. 160000–165000 (Figs. 6B–D). All these preparations show Na^+-dependent, phlorizin-inhibitable D-glucose transport when reconstituted. The recovery of activity has not been established in these various species but the final preparation is at least as active as the one obtained from rabbits. For example, rat kidney membrane extracts show D-glucose uptake of 445 pmol/min per mg protein compared to the purified fraction which shows 7930 pmol/min per mg protein.

(iv) When rabbits were challenged with subcutaneous injections of the purified protein fraction obtained from rat kidneys, the immune response was positive and high titers of antibodies were detected; 1×4000 dilution of serum produced a very positive ELISA response against 4 μg/ml of pure antigen or whole membranes. The immunoglobulins were isolated from both control and immunized rabbits. When the antigen was exposed to the globulins isolated from immunized rabbits a visible precipitation reaction occurred. After overnight incubation at 5–10°C in the refrigerator the reaction mixtures were centrifuged down, washed with KCl buffer and tested for transport. The results were unanticipated. The activity in the complex of antigen and specific antibody was increased at least 2-fold while no such effect was observed using the control globulins. Fig. 7 shows the SDS-polyacrylamide gel electrophoresis pattern of the preparations. Although several bands of extraneous proteins derived from

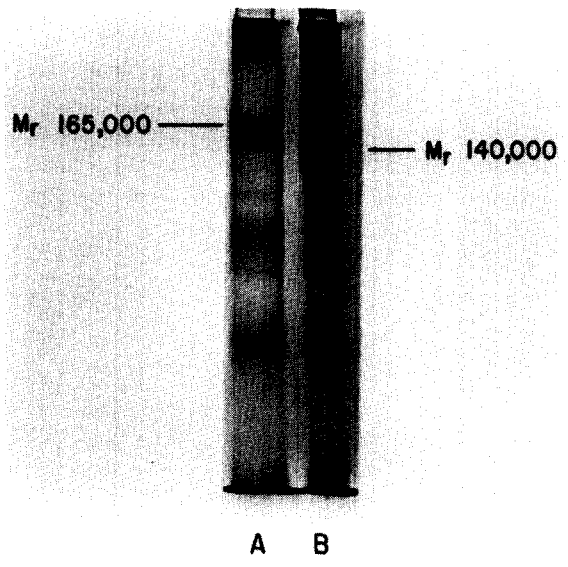

Fig. 7. Con A-Sepharose eluates from rat kidney brush-border protease-treated membrane preincubated with (A) control serum globulins and (B) immune serum globulins from rabbits.

the globulins used are seen, quite clearly the antigenic component at $M_r \cong 165\,000$ is not visible in panel (B) which represents antigen incubated with its specific immunoglobulins. The large band of protein at 140000 in panel (B) represents IgG. In order to determine whether or not the IgG class of globulins were involved, binding of antigen-antibody complex to immobilized protein A (Protein A-Sepharose) was tested since protein A specifically binds the IgG group of globulins [47]. In preliminary tests, glucose transport activity was not removed by such a treatment. This observation coupled with the fact that the antigenic protein band is lost from its position on SDS-polyacrylamide gel electrophoresis inspite of large amounts of bound IgG after exposure to antibody has prompted us to isolate other globulin classes like IgM in order to establish the cause of band loss or displacement. This work is currently in progress.

Discussion

Efforts to identify and isolate the Na^+-dependent D-glucose transporter from brush-border membranes have largely focused on the labeling of the binding site for D-glucose or phlorizin [14–18,20,21]. The conclusions from these studies have implicated proteins of molecular weights ranging from M_r 30000 to 72000. In no instance has a definitive protein been identified or demonstrated to exhibit specific D-glucose transport. In our studies, using Na^+-dependent D-glucose transport as the test of function, the first indication that a membrane protein of $M_r \cong 165\,000$ was associated with D-glucose transport came from papain proteolysis of membrane extracts purified by passage through Con A-Sepharose and incorporated into liposomes [27,44]. Only this protein remained unaffected in the treated liposomes and specific transport activity was increased 10–12-fold. It is now possible to isolate this protein from membranes. When reconstituted into liposomes, specific glucose transport is increased at least 20-fold with full recovery as compared to the total extracts from membranes. This still represents 4–5% of membrane proteins and arbitrary estimates have indicated that not more than 1% of the membrane proteins constitute the glucose carrier [42,48–55]. In preliminary tests to establish whether this protein fraction represents more than one Na^+-dependent transport system we measured L-alanine transport with ambiguous results; low levels of Na^+-dependent transport were present in some experiments and none in others. On the one hand, this indicates that the isolated protein fraction may represent an array of Na^+-dependent transport systems which probably are inactivated in the extraction procedure or are not properly assembled in liposomes; Na^+-dependent citrate and α-ketoglutarate transport is inactivated when extracted from membranes and reconstituted [27]. On the other hand, while SDS-polyacrylamide gel electrophoresis reveals only one band after Coomassie blue staining or even the more sensitive silver staining, other co-purifying transport systems like those for L-alanine may represent occasional contaminants that are not sensed by gel electrophoresis and staining but can be detected by activity. The apparent discrepancy in the molecular weight estimates between our laboratory and others who suggest an molecular weight between 60000 and 72000 stems from the differences in methodologies routinely employed. In most laboratories, samples to be electrophoresed are dissolved in SDS, mercaptoethanol added, and the mixture held in boiling water. We do not use

the heat-treatment procedure. However, when our isolate is heat-treated and then electrophoresed, a single protein band at $M_r \cong 85\,000$ results. When the isolate was electrofocused on flat bed gels, a single band at approximate $M_r \cong 85\,000$ resulted at a pH of 4.3 which showed no transport activity when reconstituted. When the $M_r \cong 165\,000$ band is exposed to buffers at low pH, a similar breakdown occurs. Interestingly enough, reassociation to $M_r \cong 165\,000$ occurs when the pH is raised to 7.4 but activity is irreversibly lost. The reasons for this are not clear at the moment. The heating process also revealed why papain treatment led to loss of activity while the $M_r \cong 165\,000$ band was seemingly unaffected. As published elsewhere [27] papain does alter the $M_r \cong 165\,000$ band because when held in boiling water, this band now was converted to one of approximate $M_r \cong 40\,000$. Thus, papain cleaves essential linkages in a dimer of $M_r \cong 165\,000$ breaking it down to a tetramer which remains in the associated state. On the other hand, the bacterial protease, subtilisin, does not seem to affect the $M_r \cong 165\,000$ protein. That this protein band is not an artifect of subtilisin proteolysis is supported by our earlier observations with unproteolysed membrane extracts. Con A-Sepharase removes 80% of the extraneous proteins from these extracts, yielding a purified fraction of proteins that clearly contained the $M_r \cong 165\,000$ protein [27]. This band is not distinctly seen in SDS-polyacrylamide gel electrophoresis of whole membranes due to the excessive presence of other proteins which mask it. That bacterial protease does not in any perceptible way alter this protein is also borne out by the fact that when whole membrane extracts in the solubilized state are exposed to it, both glucose transport and the $M_r \cong 165\,000$ protein are recovered. Therefore, unlike papain, bacterial protease does not affect this protein even when exposed to its cytoplasmic domain.

The reconstituted system is quantitatively different from natural membrane vesicles. Transport activity in reconstituted membrane extracts is 20-fold reduced as compared to the natural vesicles (240 pmol/min per mg protein by membrane extracts and 4000 pmol/min per mg protein by membrane vesicles, in one representative experiment). The reason for this is not clear. It is likely that during incorporation only a limited number of transport proteins are assembled with the right orientation into the liposomes. We intend to measure phlorizin binding [47] as a measure of the sidedness of the preparation, compared to native membranes. Also in preliminary binding studies with D-glucose carried out by F. Dorando of our laboratory the reconstituted carrier has a turnover number which is half that in membrane vesicles [28].

Isolates from rabbit intestinal brush border membranes and kidney brush border membranes from other species of animals gave surprisingly similar results with this purification procedure. The isolated proteins showed reconstitutable Na^+-dependent, phlorizin-inhibited, stereospecific glucose transport and the molecular weights were very comparable. Heat treatment or low pH also gave results similar to that discussed above.

Although we have compelling evidence that the isolated protein is not an artifact of proteolysis, we have yet to establish whether the membrane-bound native form is the same. Recently, for example, Meyer et al. [56] have demonstrated that vectorial translocation of nascent proteins through the rough endoplasmic reticulum, mediated by a proteolytically derived polypeptide of M_r 60 000, is in fact M_r 72 000 in the native state. Antibodies raised against the M_r 60 000 fragment react with a protein of M_r 72 000 in native unproteolysed membranes. Based on the same rationale, we have raised antibodies in rabbits using isolate from rat kidneys as antigen. The antibody reacts not only with the protein used as the antigen but equally well with native membranes. Binding of antibody to antigen results in a 2-fold increase in activity. It is too early to speculate on what conformational changes cause this effect. We are in the process of identifying and isolating the globulin fraction responsible for antibody activity. This should facilitate isolation and characterization of the transporter from native membranes using immobilized antibody supports.

Acknowledgement

We are grateful to Professor R.K. Crane for the many useful suggestions and discussions during the course of this work and the preparation of this manuscript. We thank Mrs. M. Burd for her excel-

lent technical assistance. This study was supported by a grant from USPHS AM-10696-17.

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