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Separation and reconstitution of sodium-dependent glucose transport activity from renal brush-border membranes using gel-filtration chromatography

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Pig kidney brush-border membrane vesicles were solubilized using a final concentration of 1% Triton X-100, found optimal for quantitative reconstitution of D-glucose transport into liposomes. Using reconstituted proteoliposomes, selective permeability towards D-glucose compared to other sugars tested was shown as well as the main features of D-glucose transport in native membranes, namely sodium dependence and phlorizin inhibition of D-glucose accumulation. After removal of Triton X-100 from the detergent extract, some membrane proteins (about 40%), which are insoluble in the absence of detergent, were isolated. Among these proteins resolubilized by 1% Triton X-100, the component catalyzing the D-glucose transport was located by gel-filtration chromatography separation, using reconstitution of transport as the assay. The active fraction displayed a molecular size of 50 Å; when analyzed on SDS polyacrylamide gel electrophoresis, it contained one major protein subunit with an apparent molecular weight close to 65000. We conclude that this protein fraction is involved in D-glucose transport by renal brush borders.

Introduction

Two fundamentally different approaches are commonly used to identify the sodium-dependent D-glucose transport system in the small intestinal or kidney brush borders. The first involves different labelling of the D-glucose carrier using either radioactive [1–5] or, more recently, fluorescent [6] thiol reagents, or else radioactive D-glucose analog [7] or photoaffinity [8] and further characterization on SDS polyacrylamide gel electrophoresis. The second approach produces a disruption of the brush-border vesicles, usually by detergent action; then the polypeptide-lipid-detergent complexes obtained are separated by affinity chromatography [9,10], chromatofocusing [11] or selective precipitation [12] to isolate the D-glucose transporter. In this case, transport activity by particular polypeptides is monitored in a reassembled system, consisting of liposomes.

With one exception [10], all these techniques assign a molecular weight of less than 80000 to the D-glucose carrier. However, it is thought that the D-glucose transporter represents a larger complex, as estimated by the radiation inactivation method [13,14], chiefly on account of protein denaturation occurring during SDS-polyacrylamide electrophoresis.

The present work describes the use of gel-filtration chromatography in the separation of a protein fraction involved in sodium-dependent glucose transport as measured in reconstituted liposomes. The molecular size of this protein fraction approximates the 155000 immunoglobulin G and

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic; SDS, sodium dodecyl sulfate.

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gives a predominant subunit of M_r 65000 by electrophoretic analysis.

Materials and Methods

Methods

Solubilization of brush-border membrane vesicles. Brush-border membrane vesicles isolated from pig kidney cortex [15] were suspended in 1 mM Tris-Hepes (pH 7.4) buffer containing 100 mM KCl (KCl medium) or 100 mM NaCl (NaCl medium). The final concentration was 10 mg/ml of membrane protein. An equal volume of 2% Triton X-100 in the same medium was added to the membrane vesicles and kept for 1 h at 4°C, then centrifuged for 1 h at 180000 × *g*. The resulting clear supernatant represented the initial detergent extract.

Concentration of the initial detergent extract. The initial 1% Triton extract prepared in KCl or NaCl medium was treated with Bio-Beads SM2 (0.6 g wet weight of beads/ml) under stirring for 1 h at 4°C. After removal of the beads by filtration through fibre-glass and washing with a small volume of KCl or NaCl medium, the suspension was centrifuged for 1 h at 180000 × *g*. Soluble proteins were recovered in a clear supernatant, S_I, whereas the pellet P_I, containing insoluble proteins, was solubilized by KCl or NaCl medium supplemented with 1% Triton X-100 and 0.1% SDS at the final protein concentration of 15 mg/ml and constituted the concentrated extract.

Gel-filtration column chromatography. The concentrated extract (1 ml) was applied to an Ultrogel ACA 34 column (1.6 × 90 cm) equilibrated at 4°C with 100 mM KCl or NaCl, 1 mM Tris-Hepes (pH 7.4), 0.5% Triton X-100 and 0.1% SDS. The eluent from the column was collected in 1 ml fractions at a flow rate of 7 ml/h. Protein content measurement of each fraction was determined by the method of Lowry et al. modified by addition of 3% SDS to prevent interference by Triton X-100 [16]. A V_e/V_0 ratio was calculated for each protein fraction; V_e is the elution volume corresponding to the peak concentration of the solute, V_0 is the void volume of the column as determined by the elution volume of blue dextran (Sigma). Four zones with V_e/V_0 ratios of 1.35, 1.42, 1.50 and 1.57, respectively, were pooled and assayed for D-glucose transport activity. The column was calibrated by selected proteins in the presence of the same Triton-SDS buffer. The V_e/V_0 ratios of the selected proteins are in linear range from 1.3 to 1.6 ratio.

Reconstitution of D-glucose transport activity. Triton extract, pellet P_I and supernatant S_I obtained by Triton removal (2 mg of protein) and fractions pooled from the gel-filtration chromatography (0.8–1 mg of protein) were added to dried egg yolk L-α-phosphatidylcholine in the ratio of one part proteins to five parts lipids. The reconstitution method was typically carried out in KCl or NaCl medium, as previously described [17]. Each pellet of proteoliposomes was suspended in the same buffer at the final concentration of 1 mg/ml of reintegrated proteins.

Transport measurements in proteoliposomes. Transport was measured by equilibrium exchange procedure as reported in a previous study [17]. Briefly, proteoliposomes (30 μl) were preincubated for 1 h at 25°C in KCl or NaCl medium containing 1.5 mM of selected and unlabelled sugar (60 μl) and the isotope exchange was initiated by addition of 15 μl of 1 mM radioactive sugar in the same medium. Transport was stopped with 2 ml of a cold stopping solution (KCl or NaCl medium supplemented with 1 mM unlabelled sugar) and the mixture was applied to presoaked membrane filter (0.45 μm, type HAMK, Millipore), then the filter was washed with 2 ml of the stopping solution. The radioactivity retained on the filter was measured using A.C.S. scintillator (10 ml) in a Packard Tricarb Spectrometer.

Materials

D-[U-^{14}C]Glucose (230 mCi/mmol), α-D-[U-^{14}C]glucose 1-phosphate (over 150 mCi/mmol) and [U-^{14}C]maltose (100–600 mCi/mmol) were obtained from Amersham International (U.K.) as the ACS scintillator. L-α-Phosphatidylcholine from egg yolk was provided by Sigma (St. Louis, MO). Ultrogel ACA 34 for gel filtration was obtained from Pharmindustrie (IBF France) and proteins markers from Boehringer (Mannheim) (F.R.G.) or Sigma. The molecular weight standards routinely used for electrophoresis were provided by Pharmacia (Uppsala, Sweden). All other chemicals were of the highest grade and obtained from Sigma or Merck (Darmstadt, F.R.G.).

Results

Previous studies on D-glucose transport by proteoliposomes obtained from Triton X-100-solubilized brush-border membrane proteins reported the low density of D-glucose transporter in liposomes, when a 0.25% detergent concentration was used [17]. To increase the specific activity of the transporter in liposomes, the Triton X-100 was added at increasing concentrations to an equal amount of purified brush-border vesicles. When comparing the rates of membrane protein solubilization and liposome insertion for detergent concentrations included between 0.25 and 2.5% (w/v), it appears that the optimal Triton concentration was close to 1%; at this concentration, 70% of membrane proteins were extracted and 32% of them were inserted into liposomes. Then, on comparing the D-glucose accumulation in proteoliposomes derived from the various Triton extracts, the equilibrium value was not modified above the 1% Triton concentration. Moreover, the percentage of D-glucose transport with regard to the equilibrium value at the different kinetic times was identical for a Triton concentration of 1% and above. It thus appears that Triton X-100 at 1% final concentration was optimal, taking into consideration protein and glucose transport reconstitution; moreover, similar equilibrium values were found for the reconstituted 1% Triton extract and purified brush-border vesicles when determined under the same conditions (see Table I): the total intravesicular space calculated from the equilibrium values was 0.4 to 0.5 μl/mg of membrane protein.

In order to discriminate between passive permeability and transport, isotope-exchange experiments using D-glucose 1-phosphate, maltose and D-glucose were performed on proteoliposomes obtained from sodium or potassium 1% Triton extract and equilibrated in NaCl or KCl medium. Glucose 1-phosphate and maltose sugars were not transported by brush-border membrane vesicles (unpublished observations). The kinetic exchange rate measurements were calculated for each sugar in comparison with its equilibrium value which was reached after 2 h incubation; this equilibrium value was not modified by 6 h incubation. In this way, different permeabilities between glucose 1-phosphate and maltose substrates on the one band and D-glucose on the other were observed (Fig. 1). In the presence of sodium ions, the D-glucose isotope was accumulated faster in the intraliposomal space. Moreover, no differences were detected in kinetic exchange rates of glucose 1-phosphate and maltose, whatever sodium or potassium ions were used.

In a previous paper [17], it was demonstrated that the presence of sodium rather than potassium ions during detergent solubilization of brush-border membrane by 0.25% Triton X-100 led to a faster accumulation of the D-glucose in derived proteoliposomes. The increase of Triton concentration to 1% provided identical results (Fig.

TABLE I

EFFECT OF PHLORIZIN ON MEMBRANE VESICLES AND RECONSTITUTED PROTEOLIPOSOMES

The 1% Triton X-100 extract (2 mg) in NaCl medium was reconstituted into liposomes as described under Materials and Methods. These reconstituted proteoliposomes (30 μg) and initial brush-border membrane vesicles (30 μg) were used for each transport assay. They were incubated for 1 h at 25°C in NaCl medium containing 1 mM non-radioactive D-glucose in the absence or presence of 0.1 mM phlorizin before the addition of 1 mM radioactive D-glucose in the same medium. Each value represents the mean of three separate experiments. The errors indicated are standard deviations.

Incubation time	D-Glucose isotope exchange (pmol/mg protein)			
	Membrane vesicles		Reconstituted proteoliposomes	
	control	phlorizin-inhibited	control	phlorizin-inhibited
10 s	170 ± 35	98 ± 22	232 ± 39	95 ± 28
20 s	257 ± 79	143 ± 14	263 ± 33	149 ± 14
45 s	325 ± 56	182 ± 12	350 ± 75	205 ± 20
2 h	386 ± 14	405 ± 20	500 ± 77	429 ± 71

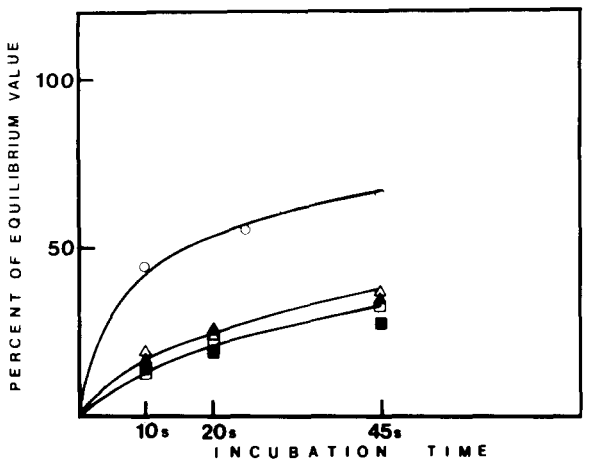


Fig. 1. Permeability of reconstituted proteoliposomes to various sugars. Transport measurements were carried out on proteoliposomes obtained from 1% Triton extract in the presence of KCl (filled symbols) or NaCl (open symbols) medium using D-glucose (○), D-glucose 1-phosphate (▲△) or maltose (■□) as detailed in Materials and Methods. The results represent the average of duplicate sample points from three experiments.

2A). In addition, when the sodium detergent extract was dialyzed against KCl prior to reconstitution, a decrease in the D-glucose exchange rate was observed, whereas no change was detected if NaCl was used during the dialysis (Fig. 2B). Therefore, it would seem that sodium rather than potassium ions preserve the functional state of the D-glucose transporter. Moreover, D-glucose exchange rates measured in proteoliposomes obtained from a Triton extract dialyzed in the presence of sodium ions or undialyzed, but kept on ice during the dialysis time, were reduced in comparison with that found for initial detergent extract, due to the prolonged exposure to the detergent. Phlorizin inhibition of the D-glucose exchange was assayed using proteoliposomes obtained from sodium detergent extract and compared to the inhibition found for brush-border vesicles in the same equilibrium exchange conditions. An identical D-glucose transport inhibition was noted in both cases with 0.1 mM phlorizin (Table I).

The subsequent step in the purification procedure of the D-glucose transport system, including chromatographic separation, involved concentration of the detergent extract. The slow dialyzability of non-ionic surfactants excluded the use of dialysis in concentration. Therefore, removal of Triton X-100 from the sodium detergent extract was performed using copolymer Bio-Beads SM_2. This procedure allowed the separation of two protein fractions, one of them pelleted by high-speed centrifugation and consisting of insoluble proteins (pellet P_I), and the other containing soluble proteins (supernatant S_I). Approx. 50–55% of detergent-extracted proteins were present in the S_I fraction, while 35–40% were recovered in pellet P_I; small amounts of proteins, estimated at less than 10%, were retained on the Bio-Beads copolymer. After insertion into liposomes, these two protein

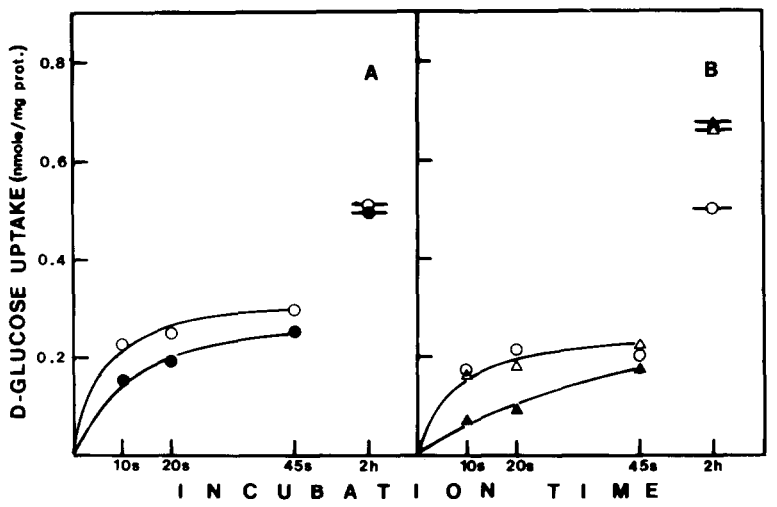


Fig. 2. Sodium-dependence of D-glucose transport by reconstituted proteoliposomes. (A) Transport measurements in liposomes associated with proteins solubilized by 1% triton X-100 in the presence of KCl (●) or NaCl (○) medium. (B) Proteoliposomes obtained from sodium 1% Triton extract kept on ice for 36 h (○) or dialyzed in the presence of 50 mM Tris-HCl (pH 6) buffer containing 100 mM KCl (▲) or 100 mM NaCl (△) during the same interval of time. All transport measurements were performed in NaCl medium. The results represent the average of duplicate sample points from six experiments.

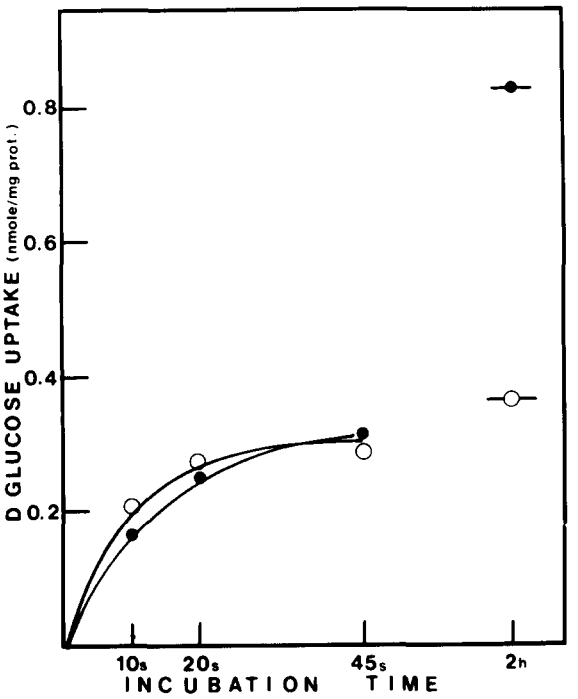


Fig. 3. Time-course of D-glucose transport by proteins fractions separated after Triton removal. The 1% Triton extract in NaCl medium was fractionated by removal of detergent in the two fractions, pellet P_I (○) and supernatant S_I (●), then reconstituted into liposomes (see Materials and Methods). Uptake measurements represents the mean of six separate experiments in duplicate sample points.

fractions were assayed for D-glucose transport; close to 50% of the proteins present in the pellet fractions were incorporated in liposomes and 15% for the supernatant; the proteoliposomes reconstituted both from the pellet, P_I, and the supernatant, S_I, displayed similar D-glucose exchange rates during the initial exchange interval (Fig. 3). Identical D-glucose isotope-exchange was found in proteoliposomes obtained for the reconstitution of the initial Triton extract in liposomes. Nevertheless, differences were noted with regard to equilibrium values measured after 2 h incubation; the D-glucose accumulation in supernatant S_I-derived proteoliposomes was twice that reached by proteoliposomes reconstituted from the pellet P_I. The differences in equilibrium values could be explained by different sizes of proteoliposomes; proteoliposomes obtained from supernatant S_I will be twice as big as proteoliposomes from pellet P_I.

Brush-border membrane proteins isolated in pellet P_I were suspended in KCl or NaCl medium containing 1% Triton X-100 and 0.1% SDS and layered on an Ultrogel ACA 34 column equilibrated in the same medium, with the exception of the Triton concentration, which was reduced to 0.5%. Fractions eluted from the gel by the same medium were measured for their protein content as shown in Fig. 4. It appears that the bulk of fractionated proteins was eluted with V_e/V_0 ratios between 1.2 and 1.6. Calibration of the same column with protein markers such as ferritin subunit (approx. 61 Å), immunoglobulin G (approx. 50 Å), alkaline phosphatase (approx. 39 Å) and serum albumin (approx. 35 Å) allowed a molecular size determination of protein fractions isolated

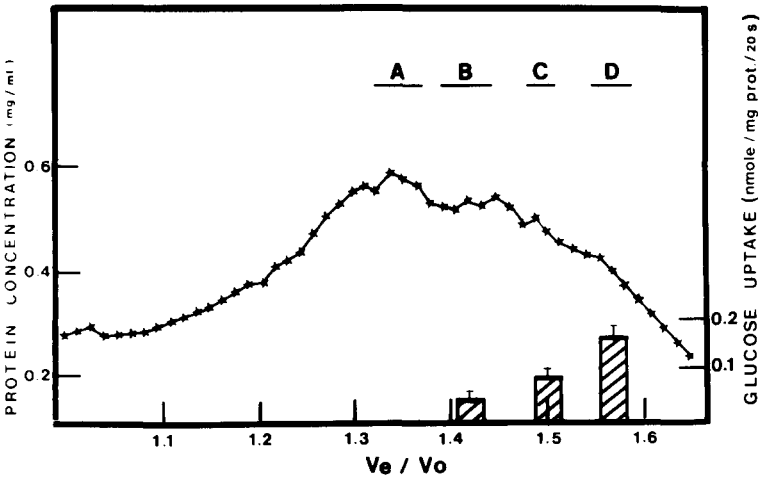
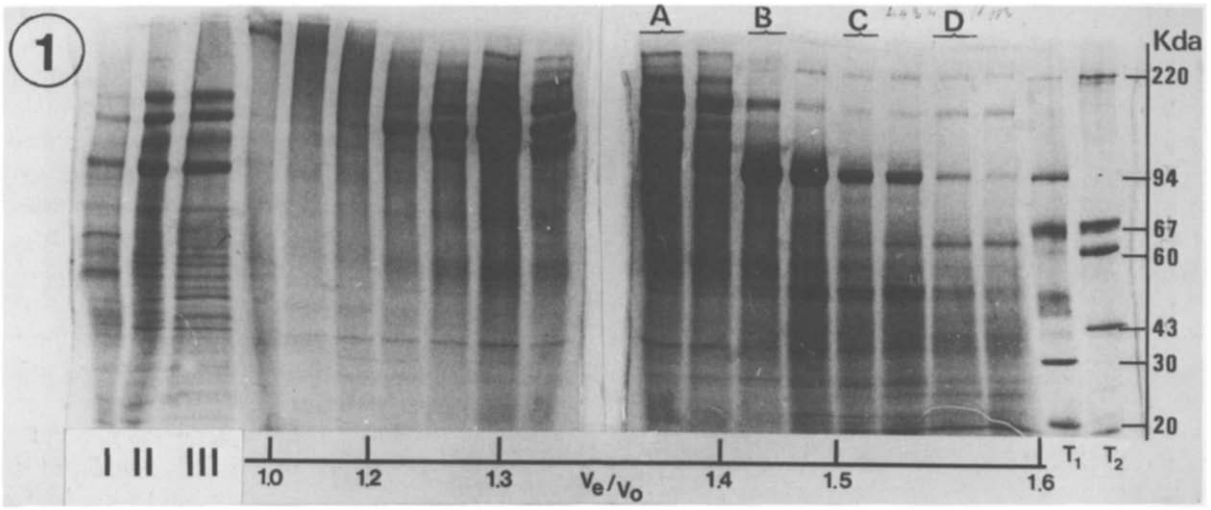


Fig. 4. Sodium-dependent D-glucose transport activity of membrane proteins eluted from gel-filtration column chromatography of pellet P_I. A sample of proteins (15 mg) was solubilized from pellet P_I by NaCl medium containing 1% Triton X-100 and 0.1% SDS and applied to an Ultrogel ACA 34 column (see Materials and Methods). The four pooled fractions A to D selected from the protein profile (★) and reconstituted into liposomes were assayed for Na^+-dependent D-glucose isotope exchange after 20 s of incubation. Na^+-independent uptake was determined by substitution of KCl for NaCl and has been subtracted. The results are the average of duplicate sample points from three experiments.

in pellet P_I between 40 and 65 Å Stokes radius.

Four pooled fractions, A, B, C and D, with V_e/V_0 ratios close to 1.35, 1.42, 1.50 and 1.57, respectively, were isolated and tested for D-glucose transport activity after incorporation into liposomes (Fig. 4). The majority of the transport activity was found associated with pooled fractions C and D; the Na^+-dependent D-glucose exchange rate, maximum in proteoliposomes obtained from fraction D, was higher than that



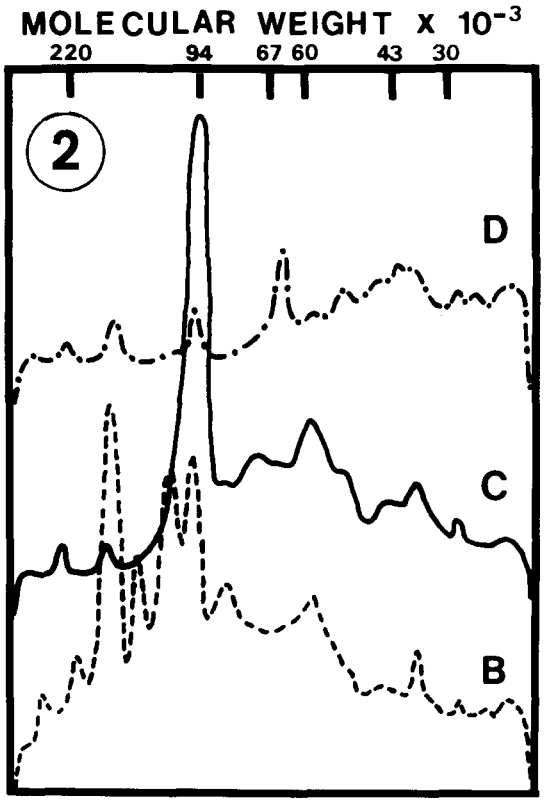


Fig. 5. SDS-polyacrylamide gels of protein fractions eluted from gel-filtration column chromatography of pellet P_I. The pellet P_I was solubilized and separated by gel-filtration chromatography as described in Materials and Methods. 5–12% SDS linear polyacrylamide gel electrophoresis prepared as previously described [17] was run with brush-border membrane (I), pellet P_I untreated (II) or treated with 10% mercaptoethanol (III) and column fractions with various V_e/V_0 ratios. All samples (60 μg of protein) were applied after boiling for 90 s in the presence of 2% SDS. The slab gels were run at 45 mA per plate for 3 h at 14°C. The protein markers used in T_1 and T_2 were ferritin (220000), phosphorylase *b* (94000), bovine serum albumin (67000), catalase subunit (60000), ovalbumin (43000), carbonic anhydrase (30000) and trypsin inhibitor (20000). In part 1 are shown protein patterns of all samples analyzed and in the part 2 the related absorbance scanning of proteins fractions B, C and D eluted from the gel-filtration column chromatography.

measured in proteoliposomes derived from the initial 1% Triton extract kept on ice during the time of chromatography (see Fig. 2B). In addition, the Na^+-independent D-glucose exchange (when KCl replaced NaCl in all experimental steps) was identical for the four fractions and reached a level of 110 ± 20 pmol/mg protein per 20 s. The equilibrium value of D-glucose isotope after 2 h incubation was the same for proteoliposomes obtained from all protein fractions and of the same magnitude as those obtained from initial 1% Triton extract. The differences in transport rate observed for protein fractions isolated after chromatographic separation were not due to the amount of proteins incorporated into liposomes, since the percentage of proteins inserted in liposomes was identical in all cases and similar to the amount found for proteoliposomes derived from initial 1% Triton extract. So the higher transport activity measured in pooled fraction D probably accounts for an enrichment of this protein fraction in the D-glucose transporter, also present in pooled fraction C. The V_e/V_0 ratio of fraction D was similar to the ratio found for immunoglobulin G in the same experimental conditions and corresponded to a Stokes radius of about 50 Å.

SDS-polyacrylamide gel electrophoresis was performed on solubilized proteins fractionated by gel filtration chromatography. As shown in Fig. 5, a decrease in molecular weight protein distribution was observed with increasing elution volume. A large concentration of proteins of molecular weight higher than 100000 was obtained in fraction A, a non-transporting fraction. The fraction B contained large amounts of the 94000 protein and decreased amounts of higher-molecular-weight proteins compared to the concentrated detergent extract. In contrast, the 65000 protein, already present in fraction C, was most concentrated in the highly transporting fraction D, while reduced amounts of the 94000 protein were observed. The minor protein bands separated by electrophoresis of transporting fractions are most likely contaminants coming from preceding or following fractions in the column. In fact, the pooled fractions with increasing Na^+-dependent D-glucose transport activity contained increasing amounts of the 65000 protein, except for the poorly transporting fraction B, where this protein was not seen.

Discussion

The reconstitution method reported here has been used for the insertion of the band 3 protein from human erythrocyte membrane [16] and used successfully by our team for the reconstitution of sodium-dependent D-glucose transport; the starting material used in that study consisted of 0.25% Triton X-100 solubilized brush border proteins [17]. The increase in the Triton X-100 concentration to 1% to improve the extraction of D-glucose transporter did not affect the selective permeability of the D-glucose substrate, and the sodium dependence of the transport. Moreover, an identical phlorizin inhibition level of D-glucose transport was found both in native membranes and reconstituted proteoliposomes. Thus the main features of D-glucose transport on the luminal side of the proximal renal tubule were recovered after detergent extraction and reconstitution.

Chromatographic methods were used to separate membrane proteins from human erythrocytes by ion-exchange chromatography [18–20] or from adipocyte cells by gel filtration [21]. Until now, kidney brush-border glucose transport activity was separated by affinity chromatography [9,10] or chromatofocusing [11] and reconstituted in liposomes, but these chromatographic methods did not allow a direct estimation of the molecular size of the glucose transporter. Previous attempts to isolate the kidney brush-border D-glucose transporter by gel-filtration chromatography were unsuccessful [22,23], no Na^+-dependent D-glucose uptake was detected after reconstitution of the separated protein fractions. It is possible that the removal of detergent from fractionated proteins before reconstitution leads in this case to the instability of the D-glucose transporter; our less drastic procedure, replacing detergent by phospholipids in one reconstitution step, eliminates this risk. This hypothesis concords with the observations of Carter-Su et al. [21], who showed higher sodium-independent glucose transport activity of adipocyte solubilized and fractionated proteins, when phospholipids were added during detergent removal and prior to sonication. When applied to 1% Triton X-100 solubilized pig kidney brush-border proteins, the gel-filtration chromatography on Ultrogel ACA 34 permitted the

isolation of Na^+-dependent D-glucose transport activity; the higher level of transport was obtained for an elution volume corresponding to a molecular size of 50 Å. This particle size is in the range one would predict for a protein that spanned the membrane [21]. When compared with the total protein of initial membrane vesicles, the bulk of proteins associated with Na^+-dependent glucose transport did not exceed 3%; this approximates the figure of 2% estimated for the M_r 75000 intestinal brush-border transporter [6].

Specific activity comparison of glucose transporter between proteins fractionated by chromatography and native membranes was impossible, on account of different percentages of transport and non-transport proteins in both samples, even though the intravesicular and intraliposomal spaces seem to be comparable.

Although no clear purification of transport protein was obtained, the SDS-polyacrylamide electrophoretic pattern of fractionated proteins associated with glucose transport activity showed few protein bands; one of these, with an apparent molecular weight of 65000, was enriched in transporting fractions and absent from the other chromatographically separated fractions. This 65000 protein band probably represents a part of kidney brush-border glucose transporter, previously found as a specific D-glucose transport component by double labelling with *N*-ethylmaleimide [4] and partially purified by affinity chromatography on phlorizin polymer [10]. Nevertheless, the minor high-molecular-weight bands, also present in the electrophoretic pattern, could be possible candidates for the glucose transporter, though they represent probably contamination of preceding column fractions.

On the other hand, the involvement of the M_r 55000 Na^+-independent passive glucose transporter in the glucose transport activity measured in fractionated proteins seems to be ruled out by the absence of cytochalasin B inhibition of glucose transport in proteoliposomes reconstituted from the initial Triton extract (unpublished data).

Finally, proteins associated with glucose transport activity exhibit a Stokes radius identical to that of the 155000 molecular weight immunoglobulin G, close to the value found for the kidney brush-border glucose transporter by Malathi et al. [9]. Thus, the 65000 protein band found after SDS polyacrylamide electrophoresis could derive from a large protein complex, which results from the association of a 65000 polypeptide with another identical or different polypeptide.

Further experiments will be required to identify the glucose transporter among the polypeptides recovered in fraction D, but chromatographic separation followed by reconstitution under mild conditions reported above represents some progress in the path of its identification under non-denaturing conditions.

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