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The human red cell glucose transporter in octyl glucoside. High specific activity of monomers in the presence of membrane lipids

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Human red cell membranes were stripped of peripheral proteins and partially solubilized with 50–260 mM octyl glucoside at 2–14 mg protein/ml, to find conditions that afford a high concentration of active glucose transporter after purification on DEAE-cellulose. Transporter-egg yolk phospholipid vesicles were prepared by gel filtration. The specific D-glucose equilibrium exchange activities increased with increasing dilution of the glucose transporter. At 260 mM octyl glucoside the glucose transporter became partially denaturated. At 225 mM detergent the DEAE-cellulose chromatography showed one main and one minor fraction of active glucose transporter. Nucleoside transport activity was enriched in the minor fraction. Solubilization with 75 mM octyl glucoside at 8 mg protein/ml gave a maximal concentration of purified transporter, 0.8 mg/ml, probably corresponding to complete solubilization. The phospholipids were partially retarded on the DEAE-cellulose. The specific D-glucose equilibrium exchange was high, up to 200 nmol glucose/μg transporter in two min at 50 mM glucose. High performance gel filtration in octyl glucoside indicated that the transporter formed dimers during the fractionation. These eluted at M_r 125 000, partially separated from the phospholipids, which appeared at M_r 55 000 (cf. Mascher, E. and Lundahl, P. (1987) J. Chromatogr. 397, 175–186). The D-glucose transport activity was low in the main fraction and high in the transporter-phospholipid fraction. Mixing of these fractions did not increase the activity. The glucose transporter is probably dependent on one or more specific membrane lipid(s). Presumably the transporter dimerizes and loses activity upon removal of these lipids.

Introduction

Reconstitution of active D-glucose transporter from human red cells, after solubilization with Triton X-100 or octyl glucoside, was first shown by Kasahara and Hinkle [1], who later introduced DEAE-cellulose chromatography for preparation of a transporter fraction [2]. D-Glucose transport activity after purification in the presence of Triton X-100 has been studied thoroughly by Wheeler and Hinkle [3]. Baldwin and co-workers improved the procedures using octyl glucoside and characterized the transporter preparation [4]. D-glucose transport activity after solubilization in 170 mM octyl glucoside was reported by Shelton and Langdon [5]. Other studies of the human red cell glucose transporter purified in octyl glucoside have been reported recently [6–10]. Octyl glucoside is well suited for reconstitution experiments, since it has a high critical micelle concentration (cmc): 25 mM at low ionic strength and 17 mM at 0.49 M NaCl [11] and forms relatively small micelles, M_r 8000 [12]. The use of non-ionic and bile salt

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detergents in the study of membrane proteins has recently been reviewed by Møller and co-workers [13].

Our earlier gel filtration experiments in octyl glucoside [9] indicated that the glucose transport activity depended on the presence of specific membrane lipids (or on some components co-eluting with these lipids). We have now tested a series of membrane protein and octyl glucoside concentrations to find solubilization conditions that afford a high concentration of active transporter to improve the gel filtration results. The purification procedures might be of use for crystallization experiments.

Materials and Methods

Human red cell concentrate, stored 4–5 weeks, was obtained from the Blood Bank of the University Hospital, Uppsala, Sweden. Octyl glucoside (*n*-octyl-β-D-glucopyranoside), *S*-(*p*-nitrobenzyl)-6-thioinosine, dithioerythritol and Tris ('Trizma base') were purchased from Sigma. Cholic acid was bought from Fluka, Switzerland. D-[U-^{14}C]Glucose was bought from Amersham, UK. L-[1-^{14}C]Glucose was obtained from Amersham or New England Nuclear, U.S.A. [5,6-^{3}H]Uridine was purchased from New England Nuclear. Chemicals were of analytical grade unless otherwise stated. DEAE-cellulose (DE 52) was bought from Whatman, U.K. Sephadex® G-50 M and a column prepacked with Superose® 6 were obtained from Pharmacia-LKB Biotechnology AB, Sweden. Buffers were passed through 0.2 μm Sartorius filters (SM 11107) and simultaneously degassed.

Preparation of glucose transporter. Human red cell membranes were prepared, thoroughly stripped of peripheral proteins by chromatographic and centrifugal procedures, frozen in liquid nitrogen and stored at −70°C [7].

The stripped membranes, 2–14 mg protein/ml, were stirred at 2°C for 20 min in 70 mM Tris-HCl (pH 7.0 as measured at 22°C), 1 mM dithioerythritol and 50–260 mM octyl glucoside. The amount of integral membrane proteins was 50 mg or, in two experiments including Superose 6-chromatography, 100 mg. Non-solubilized material was sedimented for 60 min at 160 000 × *g* at 2°C and the supernatant was collected.

A 23-ml column (29.3 × 1.0 cm) of DEAE-cellulose was equilibrated at 6°C in 70 mM Tris-HCl (pH 7.0 as measured at 22°C), containing 1 mM dithioerythritol and then with 35 ml of the above solution containing octyl glucoside of the same concentration as used for solubilization. A fresh portion of DEAE-cellulose was used in each experiment. The entire supernatant, 2.1–21 ml, was applied at 6°C onto the column at a flow rate of 12 ml/h. The material that passed the column upon isocratic elution was collected in 0.6-ml fractions (except in experiment h, Fig. 1, where the fraction volume was 0.7 ml). The three or six fractions having the highest absorbance at 280 nm were pooled, for experiment with 50 or 100 mg of protein, respectively, and analyzed for phospholipids and, following hydrolysis for 24 h with 6 M HCl, for amino acids. The amount of serine was increased by the presence of phosphatidylserine. The amounts of cysteine, tryptophan and protein serine were estimated by use of the amino acid sequence of the glucose transporter in the human hepatoma cell line HepG2 [14].

High performance gel filtration. 100 mg of integral membrane proteins were solubilized at 8 mg/ml with 75 mM octyl glucoside and fractionated on DEAE-cellulose as described above. 1 ml glucose transporter preparation, containing about 0.8 mg transporter, was made 0.5 M in NaCl and then applied at 6°C onto a 28 × 1.0 cm Superose 6-column in 50 mM Tris-HCl, pH 7.0 as measured at 22°C, 0.5 M NaCl, 1 mM dithioerythritol and 50 mM octyl glucoside. The flow rate was 9 ml/h. The column was calibrated as described earlier [9].

Egg-yolk phospholipids. 1 kg of yolks from fresh hen eggs was stirred at 22°C for 2 h with 1 l of chloroform/methanol/2-mercaptoethanol (667 : 333 : 0.5, v/v/v). The mixture was filtered on a Büchner funnel. Non-solubilized material was stirred for 1 h with 1 l of the above solution and filtered. The filtrates were combined and the phases were allowed to separate overnight. The chloroform phase was collected and the chloroform was evaporated. The lipids were dissolved in 0.4 l of diethyl ether, precipitated by dropwise addition of 2 l of acetone during stirring, washed thrice with 0.2 l of acetone and dissolved in 0.1 l of chloroform. The solvents were evaporated. The

phospholipids were stored at −70°C in 5-g aliquots in N_2-filled vials. The yield was about 65 g. The solvent evaporations were done in a vacuum rotatory evaporator at a water bath temperature of 40°C. This preparation is a modification of that of Folch and co-workers [15].

Egg-yolk phospholipid solution. The egg-yolk phospholipids (70% phosphatidylcholine, 21% phosphatidylethanolamine) were solubilized with 260 mM cholate in 200 mM NaCl, 1 mM Na_2-EDTA, 11.1 mM D-glucose, 2 mM dithioerythritol and 5 mM Tris-HCl (pH 8.4) to a final concentration of approx. 250 mM (cf. Ref. 16).

Reconstitution. 200 μl of transporter solution, diluted with detergent-free buffer (D5.5, D50 or U, below) or non-diluted, was mixed with 200 μl of the egg-yolk phospholipid solution and a 300-μl aliquot was applied at 22°C onto a 10-ml (12.7 × 1.0 cm) column of Sephadex G-50 M in 200 mM NaCl and 10 mM sodium phosphate buffer (pH 7.2) containing 5.5 or 50 mM D-glucose or 0.2 mM uridine (buffer D5.5, D50 or U, respectively). The flow rate was 3 ml/min. The column was connected to a monitor (UV-I from Pharmacia-LKB) for light absorbance at 280 nm. The front half, 1.0 ml, of the eluted protein-lipid vesicle material was collected in an Ellerman tube, frozen for 10 min in solid CO_2/ethanol and stored overnight at −25°C. The vesicles were thawed and incubated for 30 min at 25°C and homogenized for 4 s on a Vortex mixer. For calculation of the protein amount the collected 1.0-ml fraction was assumed to contain half of the applied transporter.

Exchange of D-glucose. The stereospecific equilibrium exchange of D-glucose at 22°C was determined at 5.5 or 50 mM D-glucose. 50 μl of D-[^{14}C]glucose in 5.5 or 50 mM D-glucose was mixed with 350 μl of freshly homogenized protein-lipid vesicles. After 1 min 50 s, 300 μl of this mixture was applied at 2 ml/min by use of two four-way valves and a 300-μl sample loop onto a 10-ml (12.7 × 1.0 cm) Sephadex G-50 column in buffer D5.5 or D50 with 0.1 mM $HgCl_2$. The incubation time was estimated at 2 min, allowing 10 s for gel filtration of vesicles into the $HgCl_2$-buffer. The vesicles were collected in two 1-ml fractions. A 1-ml aliquot of an 8-ml fraction of the external D-[^{14}C]glucose was also collected. The leakage of glucose into the vesicles was determined with L-[^{14}C]glucose exactly as above using another 350-μl aliquot of the homogenized vesicles. The leakage was always subtracted from the total uptake of D-glucose to calculate the D-glucose transport.

Exchange of uridine. The equilibrium exchange of 0.2 mM uridine was determined at 22°C as in Ref. 7. 200 μl of homogenized protein-lipid vesicles and 100 μl of buffer U were mixed with 10 μl of [5,6-^{3}H]uridine in 0.2 mM uridine. After 2 min, 100 μl of 40 μM *S*-(*p*-nitrobenzyl)-6-thioinosine in buffer U was added and 300 μl of this mixture was applied onto the Sephadex G-50 M column in buffer U with 10 μM *S*-(*p*-nitrobenzyl)-6-thioinosine. Leakage was measured as above, using another aliquot of the homogenized vesicles, except that 100 μl of 40 μM *S*-(*p*-nitrobenzyl)-6-thioinosine was added before the addition of 10 μl of [5,6-^{3}H]uridine in 0.2 mM uridine. The leakage was always subtracted.

Internal vesicle volume. Vesicles were prepared as above from 200 μl of the egg-yolk phospholipid solution mixed with 200 μl of the D5.5 or the D50 buffer. 1 ml of the vesicle suspension was mixed with 50 μl of D-[^{14}C]glucose in 5.5 or 50 mM D-glucose, frozen, thawed, incubated, homogenized and separated from external D-[^{14}C]glucose by gel filtration as above. The fraction of ^{14}C in the vesicles was assumed to be equal to the internal volume fraction.

Transport inhibition by octyl glucoside. Octyl glucoside competes with the binding of cytochalasin B to the glucose transporter from human red cells with a dissociation constant (K_d) of 5 mM [4]. Probably octyl glucoside partially inhibits the transport of D-glucose as well. This inhibition was estimated from the equilibrium GTOG ⇌ GT + OG, where GTOG is glucose transporter in complex with octyl glucoside and GT and OG represent, respectively, the free transporter and detergent. The law of mass action gives $K_d = [GT] \cdot [OG]/[GTOG]$. Since $[GT] = [GT]_t - [GTOG]$, where $[GT]_t$ is the total concentration of transporter, $[GTOG]/[GT]_t = [OG]/(K_d + [OG])$ and $[GTOG]/[GT]_t \approx [OG]_t/(K_d + [OG]_t)$ (Eqn. 1), when $[GT] \ll [OG]_t$. $[OG]_t$ is the total concentration of octyl glucoside in the vesicle suspension.

Protein immunoblotting. Immunoblotting (Western blotting) was done with the monoclonal anti-

body B315 : 32 essentially as described by Andersson and Lundahl [17]. Pre-stained molecular weight markers were purchased from Bio-Rad and were calibrated before use.

Electrophoresis. Gradient polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was done essentially as described by Lundahl and co-workers [6] except that the linear acrylamide gradient was T = 8–25% and that the membrane proteins were not reduced or heated (cf. Ref. 9). Silver-staining of the gels was done essentially as described by Andersson and Lundahl [17]. Their procedure is a modification of that described by Johansson and Skoog [18].

Phosphorus analysis. Membrane phospholipid concentrations were determined by the phosphorus assay method of Bartlett [19].

Results

Solubilization

Integral red cell membrane proteins were solubilized with octyl glucoside and fractionated on DEAE-cellulose as described in Methods. The concentration of purified glucose transporter increased linearly to about 0.8 mg/ml with increasing concentration of integral membrane proteins (Fig. 1A), independent of the detergent concentration. Probably the glucose transporter was essentially completely solubilized. The glucose transporter concentration in the pooled fractions after DEAE-cellulose chromatography was up to 10.4% of the concentration of the integral membrane proteins in the solubilization mixture (Fig. 1A, hatched line), consistent with the yield 10.3% reported by Baldwin and co-workers [4]. The samples became slightly diluted in inverse relation to their volumes upon the fractionation. We estimated the amount of solubilized glucose transporter polypeptide, before fractionation, at 11.8% of the protein amount in the solubilization mixture, after correction for 0.4% nucleoside transporter (cf. Ref. 20). The concentration of membrane phospholipids in the pooled fractions increased approximately linearly with increasing concentration of octyl glucoside (Fig. 1B). Two to four phospholipid molecules were solubilized per micelle, but about 40% of the solubilized phospholipid molecules were adsorbed to the DEAE-

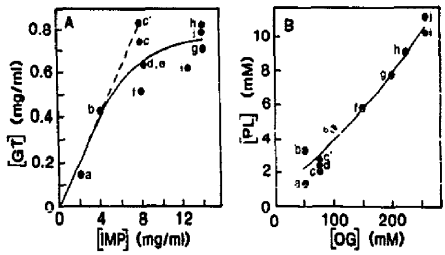

Fig. 1. Glucose transporter (GT) and phospholipid (PL) concentrations after isocratic purification of the human red cell glucose transporter on DEAE-cellulose. Integral membrane proteins (IMP) and membrane lipids were solubilized with octyl glucoside (OG) at the concentrations (a) 2 mg IMP/ml, 50 mM OG (denoted 2/50), (b) 4/50, (c, c′) 8/75, (d) 8.5/75, (e) 8.5/100, (f) 8/150, (g) 14/200, (h) 14/225, (i) 12.5/260 and (j) 14/260, as described in Methods. The amount of IMP in the solubilization mixture was 50 mg, except for experiment c′ where it was 100 mg. The points c′ represent average data from experiments 1 and 2 in Table II. The sample volumes applied to the 23-ml DEAE-cellulose columns were, in ml, (a) 20.9, (b) 11.5, (c) 4.5, (c′) 7.0, (d) 4.0, (e) 4.9, (f) 4.5, (g, h) 2.4 and (i, j) 2.1. (A) Concentration of purified glucose transporter in pooled fractions as a function of the concentration of integral membrane proteins in the solubilization mixture. (B) Concentration of membrane phospholipids in the pooled transporter fractions as a function of the concentration of octyl glucoside in the solubilization mixture.

cellulose. Gel filtration showed an apparent M_r of the mixed phospholipid-octyl glucoside micelles of 55 000 (Fig. 5 below), corresponding to micelles of approximately 7 phospholipid molecules and 170 detergent monomers (cf. Ref. 21). Solubilization of 8 mg of integral membrane protein/ml with 75 mM octyl glucoside (c and c′ in Fig. 1) was favourable. About 50% of the membrane proteins and about 46% of the membrane phospholipids were solubilized in this case. After the DEAE-cellulose chromatography the lipid concentration was relatively low, 2 mM, and the specific glucose transport activity was high (see below).

DEAE-cellulose chromatography

Typical chromatograms for purifications with 75 and 225 mM octyl glucoside are shown (Figs. 2A and 3A). These correspond to points c′ and h, respectively, in Fig. 1.

Solubilization with 75 mM octyl glucoside at 8 mg of integral membrane proteins per ml resulted in one broad glucose transporter fraction, partially

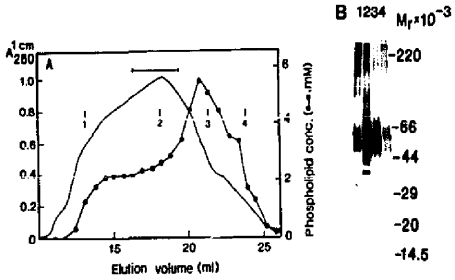

Fig. 2. (A) Isocratic DEAE-cellulose chromatography of integral membrane proteins (IMP) from human red cells, solubilized at 8 mg IMP/ml with 75 mM octyl glucoside (c′ in Fig. 1). The bar indicates the pooled fractions. (B) Electrophoretic analyses of fractions 1–4. $M_r(\times 10^3)$ values for reduced and carboxyamidomethylated calibration proteins are indicated in (B). The membrane proteins were not reduced (cf. Ref. 9).

TABLE I

SPECIFIC EQUILIBRIUM EXCHANGE OF D-GLUCOSE AND URIDINE IN THE MAJOR AND MINOR FRACTION AFTER DEAE-CELLULOSE CHROMATOGRAPHY OF INTEGRAL RED CELL MEMBRANE PROTEINS SOLUBILIZED WITH 225 mM OCTYL GLUCOSIDE

Experiment illustrated in Fig. 3. The solubilization mixture contained 14 mg of integral membrane proteins per ml (h in Fig. 1). The reconstitutions were done with nondiluted samples.

	Specific equilibrium exchange (nmol/μg protein, in two min)	
	D-glucose [a]	Uridine [b]
Fraction I	29	0.012
Fraction Ii	7.6	0.070

[a] Specific equilibrium exchange at 50 mM D-glucose.
[b] Specific equilibrium exchange at 0.2 mM uridine.

separated from retarded membrane phospholipids (Fig. 2A). The transporter fractions 1–4 in Fig. 2A were analyzed by electrophoresis (Fig. 2B), which showed partly aggregated material in fraction 1 and minor impurities, M_r 200 000 and 36 000, in fraction 2.

At higher concentrations of detergent and protein, 225 mM and 14 mg/ml, the elution pattern showed two well separated fractions, I and II (Fig. 3A). Glucose transporter and membrane phospholipids eluted in the major one. Fraction I showed monomeric (M_r 44 000–66 000) as well as aggregated glucose transporter upon electrophoresis (Fig. 3B). In fraction II the material was mainly of apparent M_r 39 000–59 000 (Fig. 3B). Fraction II also contained a component of M_r 28 000. The C-terminal-specific monoclonal antibody B315:32 [17] reacted only with the material in fraction I (Fig. 3C). Assays with vesicles reconstituted from material from fractions I and II showed that the specific exchange for uridine was higher for fraction II than for fraction I (Table I). The specific exchange of D-glucose observed with fraction II was about one-fourth of that found with fraction I (Table 1). Part of the material in fraction II probably corresponds to an active form of the glucose

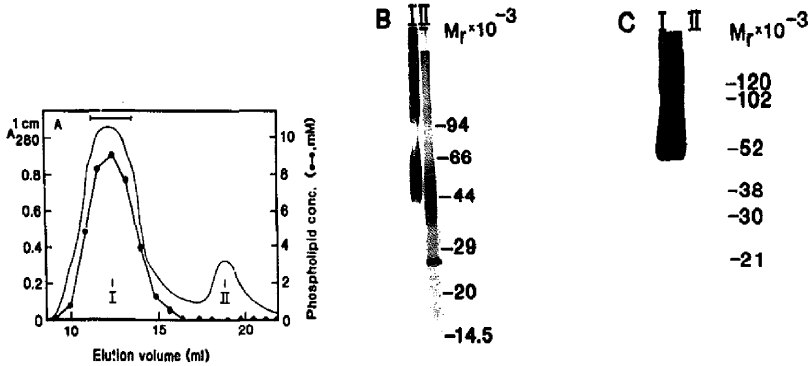

Fig. 3. (A) Isocratic DEAE-cellulose chromatography of integral membrane proteins (IMP) from human red cells, solubilized at 14 mg IMP/ml with 225 mM octyl glucoside (h in Fig. 1). The bar indicates the pooled fraction. (B) Electrophoretic analyses of fractions I and II as in Fig. 2. (C) Immunoblotting with monoclonal antibody B315:32. $M_r(\times 10^{-3})$ values for pre-stained marker proteins are indicated.

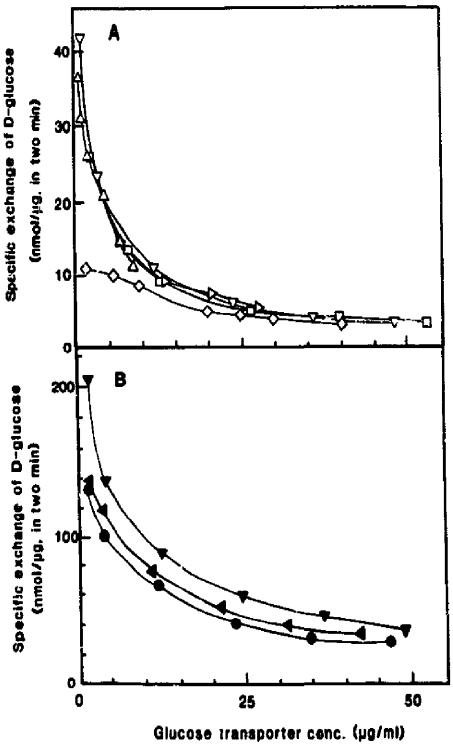

Fig. 4. The specific equilibrium exchange of D-glucose at (A) 5.5 mM and (B) 50 mM D-glucose as a function of the concentration of purified glucose transporter. The given concentrations were obtained by series of dilutions before reconstitution followed by a 7.6-fold dilution upon vesicle preparation and addition of radioactive glucose. The determinations were done with transporter purified from extracts of (A), (△) 2 mg of integral membrane protein/ml with 50 mM octyl glucoside (2/50); (▷) 4/50; (□) 14/225; (◇)12.5/260; (▽) 8/75 and (B), (▼), 8/75; (◀) 8/100 and (●) 14/200.

transporter, lacking a fragment containing a C-terminal segment.

Transport activity

For measuring the exchange of D-glucose, glucose transporter-egg yolk phospholipid vesicles were prepared by rapid chromatographic removal of cholate and octyl glucoside (see Methods). Dilution of purified glucose transporter by a factor of 20–40 increased the specific exchange 4–14-fold (Fig. 4), except for purification at 260 mM octyl glucoside, which partly denatured the transporter. The condition of 8 mg of integral membrane protein/ml and 75 mM octyl glucoside gave the maximal specific exchange: 42 nmol glucose/µg transporter in two min at 5.5 mM D-glucose (Fig. 4A) and 204 nmol/µg in two min at 50 mM D-glucose (Fig. 4B). Solubilization at the above protein concentration but with 100 mM octyl glucoside gave about the same specific exchange as solubilization of 14 mg of proteins/ml with 200 mM detergent (Fig. 4B).

High performance gel filtration of purified glucose transporter

Upon fractionation on Superose 6 of the glucose transporter purified on DEAE-cellulose, the transporter eluted mainly as monomers and dimers, average apparent M_r 125 000, overlapping partially with the membrane phospholipids, which eluted at apparent M_r 55 000 (fractions c and d, respectively, in Fig. 5A). The transport activity in terms of D-[^{14}C]glucose uptake was 3.2% in the applied sample, after correction for leakage. After the fractionation on Superose 6, the highest activity, 0.4%, was found in the transporter-phospholipid fraction d, whereas the activity was very low in the main transporter fraction c. The specific D-glucose exchange was 20-times higher in fraction d than in fraction c (Table II), Expt. No. 1, and Fig. 5B). Fraction d contained 2 mM membrane phospholipid, whereas fraction c was free of phospholipids (Table II). The aggregated transporter material in the electrophoresis analyses (Fig. 5B) was probably formed upon the addition of dodecyl sulfate and was not present during the gel filtration on Superose 6, as judged from the elution volume (Fig. 5A).

To test whether the activity of fraction c could be restored or not, a fractionation similar to that shown in Fig. 5 was done and gave essentially the same result as before (Table II, Expt. No. 2). The main transporter fraction c was then mixed with the phospholipid fraction d′ in different proportions. No reactivation was obtained (Fig. 6). The uptake of D-[^{14}C]glucose did not correlate with the transporter concentration (Fig. 6A), but the specific equilibrium exchange was roughly proportional to the concentration of membrane phospholipids (Fig. 6B), i.e., to the content of the

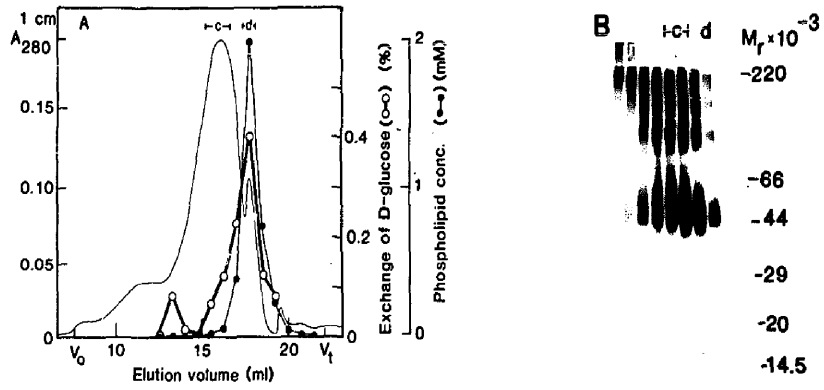

Fig. 5. (A) Chromatography on Superose® 6 in 50 mM octyl glucoside of glucose transporter purified by DEAE-cellulose chromatography of integral membrane proteins from human red cells solubilized at 8 mg/ml with 75 mM octyl glucoside (c′ in Fig. 1). Fractions c and d were analyzed for amino acid content (Expt. No. 1 in Table II). (B) Electrophoretic analysis, as in Fig. 2, of the ten 0.75 ml-fractions in A that were analyzed for D-glucose exchange (O). Fractions c and d in A are indicated. Experimental details are given in Methods. The transporter dimers in fraction c from the gel filtration experiment have partly formed monomers and partly aggregated upon electrophoresis.

fraction d′ that showed high specific activity before the mixing procedure.

Discussion

Earlier we have solubilized the human red cell glucose transporter with octyl glucoside at an integral membrane protein concentration of 4 mg/ml [7,9,10,17,22]. For attempts at transporter crystallization and for determination of transport activity after partial removal of membrane lipids we have now further increased the transporter concentration in the solubilization mixture. As shown above, purification on DEAE-cellulose

TABLE II

SPECIFIC EXCHANGE OF D-GLUCOSE IN THE MAIN TRANSPORTER FRACTION AND IN THE TRANSPORTER-PHOSPHOLIPID FRACTION AFTER HIGH-PERFORMANCE GEL FILTRATION OF TRANSPORTER PURIFIED ON DEAE-CELLULOSE

Expt. No.	1 [a]		2 [b]	
Gel filtration fraction [c]	c	d	c	d′
Glucose transporter concn. (μg/ml)	197	43	196	69
Membrane phospholipid concn. (mM)	0.0	2.0	0.0	3.0
Exchange of D-glucose (%, in two min) [d]	0.09	0.40	0.30	0.48
Specific exchange of D-glucose (nmol/μg protein, in two min) [d]	3.4	70	11	48

[a] The gel filtration on Superose 6 is illustrated in Fig. 5.

[b] Gel filtration as that illustrated in Fig. 5.

[c] Fraction volumes: c, d′ 1.5 ml, d 0.75 ml. Fraction d′ corresponds to fraction d combined with the fraction between c and d in Fig. 5.

[d] Determined at 50 mM D-glucose.

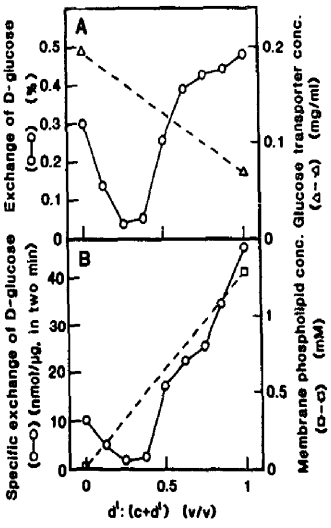

Fig. 6. (A) Equilibrium exchange and (B) specific equilibrium exchange of D-glucose in vesicles reconstituted with mixtures of the main glucose transporter fraction (c) and the transporter phospholipid fraction (d') after chromatography on Superose 6 in octyl glucoside of DEAE-cellulose-purified material (Expt. No. 2 in Table II). 200 μl of the mixture of aliquots of fractions c and d' was supplemented with 200 μl of the egg yolk phospholipid solution for vesicle preparation and assay. The concentrations of purified glucose transporter and membrane phospholipids are indicated (broken lines).

showed glucose transporter concentrations up to 0.8 mg/ml. By diluting the samples of purified transporter we were able to demonstrate a high specific equilibrium exchange activity with egg-yolk phospholipid vesicles, similar to that in experiments by Wheeler and Hinkle [3], in which Triton X-100-solubilized material and soy-bean phospholipids were used. Our maximal specific activity for the equilibrium exchange at 50 mM glucose is about 204 nmol/μg in two min at 22°C, equivalent to at least 102 nmol/μg in one min, since the time dependence is non-linear even though we used a low protein concentration. The initial rate would be still higher. The specific exchange for red cell membranes at 50 mM glucose is 14–21 nmol/μg per min at 20°C, as calculated by use of Michaelis-Menten's equation with $K_m = 38$ mM and $V_{max} = 260$ nmol/cell unit per min [23] or $K_m = 32$ mM and $V_{max} = 357$ nmol/cell unit per min [24], $1.6 \cdot 10^{13}$ cells/cell unit [25] and 0.66 pg of protein/ghost [26]. We estimate that 50% of the ghost protein is integral membrane protein (cf. Ref. 27) and that about 12% of the integral membrane proteins (see Results), i.e., 0.048 pg of the ghost protein, is glucose transporter polypeptides. For a transporter polypeptide of M_r 54 117 (cf. Ref. 14) this corresponds to $5.3 \cdot 10^5$ monomers/cell, which is much higher than the number of cytochalasin B-binding sites/cell reported earlier, $1.2 \cdot 10^5$ [28] and $2.5 \cdot 10^5$ [29]. The specific exchange for the reconstituted transport system with purified glucose transporter, at 50 mM glucose, should be a factor of 0.66 pg ghost protein/0.048 pg glucose transporter, i.e. 14-times higher than in ghosts, or 190–290 nmol glucose/μg transporter per min. Our highest specific exchange (Fig. 4), at least 102 nmol/μg · min, corresponds to 35–55% of these values. This is reasonably high, since probably only part of the glucose transporters became incorporated into the lipid bilayers of the vesicles: Wheeler and Hinkle [3] have reported that the specific activity increased by a factor of 2.5 when non-incorporated protein was removed. Furthermore, the freeze-thawed vesicles are partly multilamellar (cf. Ref. 30).

The concentration of octyl glucoside remaining in the vesicle preparation was calculated (Eqn. 1 in Methods), assuming that octyl glucoside was removed to the same extent, 94%, as cholate (unpublished data) during the vesicle preparation procedure. Results of Mimms and co-workers [31] indicate that only 0.1% octyl glucoside may remain in similar vesicle preparations. About 30% inhibition by octyl glucoside was estimated for non-diluted samples of high detergent concentration, 75 mM octyl glucoside gave at most 10% inhibition. For 20–40-fold diluted transporter samples the inhibition was negligible. For the material solubilized at 8 mg/ml with 75 mM octyl glucoside, the specific exchange was 12-times higher at 50 mM than 5.5 mM D-glucose for non-diluted sample, whereas it was 5-times higher for the most diluted sample (Fig. 4). The latter value agrees with calculations by use of Michaelis-Menten's equation, using the above K_m and V_{max} values. Thus the inhibition for non-diluted transporter samples was negligible at 50 mM D-glucose but not at 5.5 mM.

The internal vesicle volumes were $9.4 \pm 0.2\%$ and $7.0 \pm 0.1\%$ of the suspension volume at 5.5 and 50 mM D-glucose, respectively. The non-linearity of the plots in Fig. 4B is mainly due to this limited internal volume of the transporter-lipid vesicles.

The glucose transporter becomes partially denatured in the presence of high concentrations of cholate [32,33] or octyl glucoside [4]: the higher the detergent concentration the more rapid the denaturation. In the present work up to 225 mM octyl glucoside was used with retention of activity, whereas 260 mM octyl glucoside concentration resulted in partial loss of activity. Probably denaturation of the red cell glucose transporter by these detergents are related to displacement of one or more loosely bound essential lipid(s), as suggested earlier [4,8,9,33–35]. The removal of one or more essential lipid(s) from the transporter monomer may lead to dimerization and denaturation. The observations that the transporter eluted at M_r 125 000 upon gel filtration (cf. Fig. 5A), between the M_r values expected for monomers and dimers (cf. Ref. 9) and that the dimer fraction showed low activity, whereas the activity in the monomer-phospholipid fraction was high (Fig. 5, Table II), support our hypothesis. Similarly, Rampal and co-workers have suggested that cytochalasin B-binding to the glucose transporter decreases upon dimerization of the transporter in octyl glucoside [36]. A binding site for an essential lipid could be involved in the dimerization. An increase in detergent concentration probably favours the release of lipids.

There are indications that phosphatidylserine may interact with the transporter. The immunoblotting of Fraction I (Fig. 3C) showed a sharp, but weakly stained, zone with an apparent M_r of about 30 000. A similar zone was found with another monoclonal antibody against the human red cell glucose transporter [7]. The M_r-30 000 zone may correspond either to a transporter fragments or to the band 7-protein, which is known to co-purify with the glucose transporter on DEAE-cellulose in some cases (cf. Ref. 4). According to results of Connor and Schroit [37], the M_r 30 000 zone may represent a phosphatidylserine transporter or translocator, which could be identical with the band 7 protein. The monoclonal antibody B315 : 32 used in our immunoblotting is directed against a C-terminal segment of the glucose transporter, residues 478–492, and possibly also against another segment, residues 218–231, exposed at the inside of the membrane (Ref. 17, residue denotation according to Ref. 14). Since this antibody reacted not only with the glucose transporter but also with the M_r 30 000-component it is possible that the antibody reacts with a binding site for phosphatidylserine in the phosphatidylserine transporter as well as in the glucose transporter. The negatively charged phosphatidylserine molecules, which are confined to the inner leaflet of the membrane lipid bilayer [38,39], may interact with the positively charged transporter segment 218–231 in a phosphatidylserine-binding site.

Requirement of essential lipids have also been reported for the lactose transporter in *Escherichia coli* [40]. Recently, binding of long-chain phosphatidylcholine and -serine to rhodopsin has been reported [41]. The specificity of lipid association with membrane proteins may reside in the polar head(s) of the lipid(s) (cf. Ref. 42).

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