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Active and monomeric human red cell glucose transporter after high performance molecular-sieve chromatography in the presence of octyl glucoside and phosphatidylserine or phosphatidylcholine

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The human red cell glucose transporter (Glut 1) was purified by ion-exchange chromatography in the presence of octyl glucoside. The state of association of the protein was studied, and the transport activity was determined after exchange of copurified membrane lipids for phosphatidylserine (PS) or phosphatidylcholine (PC). The purpose was to analyze the Glut 1 preparation for homogeneity and activity prior to attempts at crystallization. Analyses by high performance molecular-sieve chromatography showed that the Glut 1 was monomeric immediately after the ion-exchange purification: the M_r of the Glut 1 polypeptide was estimated to be 49 000 ± 6000 by TSKgel G3000SW chromatography monitored by low-angle laser light-scattering photometry, differential refractometry and UV photometry. This required determination of the absorption coefficient of the Glut 1, which was measured to be 1.13 ± 0.03 ml mg^{-1} cm^{-1} at 280 nm, referring to the polypeptide concentration. The M_r value is consistent with the cDNA-deduced M_r 54 117 of the very similar HepG2 glucose transporter polypeptide. At 2°C, pH 7 and an ionic strength of 0.06 M, the Glut 1 associated gradually during three days to form oligomers. These formed much more rapidly at room temperature or at high ionic strength. Freshly prepared Glut 1 retained high activity after separation from membrane lipids on a TSKgel G3000SW column in the presence of 40 mM octyl glucoside and 1 mM PS or PC. In contrast, most of the activity was lost when the membrane lipids were separated from the protein in the absence of eluent lipids. The presence of a phospholipid was thus essential for retention of high activity of the Glut 1 in octyl glucoside and PC was nearly as effective as PS.

Introduction

The non-ionic detergent octyl glucoside (OG) is commonly used for solubilizing and purifying integral membrane proteins, such as the D-glucose transporter from human red cells (here denoted Glut 1) [1–3], and for crystallizing membrane proteins [4,5]. OG is well suited for reconstitution experiments due to its high critical micelle concentration (CMC) of 25 mM at 25°C and 36 mM at 2°C [6]. Baldwin et al. solubilized human red cell membranes, depleted of peripheral proteins, through the use of OG. They purified the Glut 1 by ion-exchange chromatography and characterized this improved preparation [1]. Mascher and Lundahl observed low glucose transport activity after chromatography of the purified Glut 1 on Superose 6 in an eluent containing 50 mM OG and 2 mM egg-yolk phospholipids (EYP), but the Glut 1 concentration was too low for precise activity measurements [7]. The solubilization procedure was modified to increase the protein concentration, which allowed an improved molecular-sieve chromatographic study of the state of association

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and the activity of the Glut 1 in 50 mM OG after partial separation of the Glut 1 from the membrane lipids [2]. The Glut 1 retained high specific activity only where the protein and the membrane-lipid zones overlapped. The highly active Glut 1 was monomeric, as judged by elution-volume determinations and an estimate of the size of the detergent-protein complex, but most of the Glut 1 monomers had become inactivated and seemed to have formed dimers during the Superose chromatography [2]. Analyses of human red cell membrane proteins by high performance molecular-sieve chromatography in the presence of OG have been reported by Chen et al., who detected active Glut 1 after chromatography on TSKgel G3000SW [8]. The fractionations were done at room temperature (Tillotson, L.G., personal communication) and material from five runs was combined for the reconstitution. The elution position of the phospholipids was not determined [8].

The purpose of the present work was to study the preparation of monomeric (or otherwise homogeneous) and active Glut 1 for attempts at crystallization. A monodisperse preparation of high concentration is advantageous for crystallizing a protein [4,5]. We have tried to verify or disprove that the Glut 1 is monomeric when freshly prepared by ion-exchange chromatography in OG, and have studied whether it retains its activity in the presence of OG and phosphatidylserine (PS) or phosphatidylcholine (PC). Earlier analyses by chromatography on Superose 6 [2,7] or TSKgel G3000SW [8] were not conclusive in these respects. Most analyses in the present work were done by high performance molecular-sieve chromatography on TSKgel G3000SW columns in 40 mM OG. The degree of association of the Glut 1 in OG was studied by use of a measuring system that consisted of an array of a UV-photometer, a differential refractometer and a low-angle laser light scattering (LALLS) photometer which monitored the elution of the Glut 1 from the TSKgel columns with respect to molecular weight according to the method of Hayashi et al. [9]. For this purpose the absorption coefficient of the protein had to be determined. Reconstitution of the Glut 1 in liposomes and transport activity measurements were done essentially as described earlier [2].

Materials and Methods

Materials. Human red cell concentrate, stored 3–5 weeks, was obtained from the Blood Bank at the University Hospital, Uppsala. OG (1-*O*-*n*-octyl β-D-glucopyranoside) was No. RM 256 from Dojin, Kumamoto, Japan, for fractionation on TSKgel G3000SW and from Sigma, St. Louis, MO, for fractionation on Superose 6, unless otherwise stated. DEAE-cellulose (DE-52) was bought from Whatman, Maidstone, Kent, U.K. Dithioerythritol (DTE) was obtained from Sigma. Bovine brain PS (99% pure, Lot PS8–366) and PC from hen's egg yolk (99% pure, Lot EPC-236) were purchased from Avanti Polar Lipids, Alabaster, AL. D-[U-^{14}C]Glucose (10 GBq/mmol) and L-[1-^{14}C]glucose (2 GBq/mmol) were bought from Amersham, Little Chalfont, Bucks., U.K. Scintillation liquid (Quickszint 1) was bought from Zinsser Analytic, Maidenhead, Berks., U.K. For calibrating the UV, LALLS and refractometry monitors used in TSKgel G3000SW chromatography (see Introduction), chicken ovalbumin (No. A-7641) was bought from Sigma and bovine serum albumin (BSA, No. 002, 'reagent grade') from Chiba Chikusan Kogyo, Chiba, Japan. For elution volume calibrations, bovine pancreas ribonuclease A (from an LMW gel filtration calibration kit) was purchased from Pharmacia LKB Biotechnology, Uppsala, and bovine red cell carbonic anhydrase (No. C-7500) and BSA (No. A-7030) were bought from Sigma. Tris was 'Trizma base' from Sigma or was obtained from Nakarai, Kyoto, Japan. All other chemicals were of analytical grade. Solutions and samples were passed through 0.2-μm filters (SM 11107; Sartorius, Göttingen, Germany) or 0.3-μm filters (PHWP 04700; Nihon Millipore Kogyo, Yonezawa, Japan).

Preparation of Glut 1. Integral membrane proteins from human red cells (IMPs) were prepared as described earlier, by molecular-sieve chromatography of red cell hemolysate on tandem columns of Sepharose CL-4B (at pH 8) and Sepharose CL-6B (at pH 10.5) followed by centrifugation steps (at pH 10.5, 12 and 6.8) [10]. IMPs (8 mg/ml) were solubilized with 75 mM OG and the Glut 1 was purified by chromatography at 6°C on a DEAE-cellulose column essentially as described in Ref. 2. At least 0.4 ml DEAE-cellulose was used per mg IMPs. The column was equilibrated with 70 mM Tris-HCl (pH 7.0 at 22°C), 50 or 75 mM OG and 1 mM DTE. For Glut 1 purifications for TSKgel chromatography with LALLS monitoring the OG concentration was 40 mM and 3 mM NaN_3 was included in the eluent. Similar elution profiles were obtained. The central Glut 1 fraction was collected [2]. This preparation of Glut 1 (denoted DEAE-purified Glut 1) also contained membrane phospholipids [1,2] and was contaminated with about 4% of nucleoside transporter [2,11–15]. The polypeptide concentration was 0.6–0.8 mg/ml [2,17]. Unless otherwise stated, the DEAE-purified Glut 1 was prepared immediately before molecular-sieve chromatographic experiments on agarose (Superose 6) or silica (TSKgel G3000SW) gel columns.

Absorption coefficient of Glut 1. The absorption coefficient of the Glut 1 was needed for determination of the M_r and was therefore determined in a separate series of experiments as follows: NaCl was added to 300 mM concentration in a sample of DEAE-purified

Glut 1 and a 1.0-ml aliquot was applied to a Superose-6 column (28 cm × 10 mm i.d., 22.0 ml, from Pharmacia LKB Biotechnology) at 6°C in 50 mM OG, 10 mM Tris-HCl (pH 7.0 at 22°C), 300 mM NaCl and 3 mM NaN_3. The low Tris and NaCl concentrations compared to those used in other similar Superose experiments (as in Refs. 2 and 7, and as below) were chosen to reduce disturbances of amino acid analyses for determinations of protein concentrations. Still lower NaCl concentration lowered the Glut 1 yield upon the Superose chromatography. The fractionation was repeated. The two collected samples of Glut 1 (about 0.12 mg protein/ml; about 4% nucleoside transporter) had a phospholipid concentration below 10–20 μM. Eluent collected before the void volume of the column and with a stable background absorbance level was used as reference solution for the photometry. The absorbance was measured at 280 nm and 310 nm in two Shimadzu (Kyoto, Japan) UV-160A recording spectrophotometers and in a Zeiss System-Photometer PMQ3 (Carl Zeiss, Oberkochen, Germany). The samples were subjected to automated analysis of all amino acids, except cysteine and tryptophan, after 24 h and 72 h hydrolyses with 6 M HCl. The sample amounts were determined by weighing and the volumes were corrected for the density of the solution. The protein concentration for each sample was calculated for each one of the amino acids Thr, Glx, Pro, Gly, Ala, Val, Leu, Tyr, His, Lys and Arg by use of the amino acid composition (determined by cDNA sequence analysis) for the very similar or identical HepG2 glucose transporter [18,19]. The average value was then used. The average absorbance value at 280 nm was corrected by use of the average absorbance value at 310 nm, which was assumed to be due to light-scattering according to the Rayleigh rule $K = C/\lambda^4$, where K is the extinction due to scattering, C is a scattering constant and λ is the wavelength [20].

M_r determination and analyses of oligomerization. DEAE-purified Glut 1 was kept in an ice/water bath at 2°C. Aliquots of 200 μl were applied with a glass syringe prechilled to 2°C onto a TSKgel G3000SW XL column (30 cm × 7.8 mm i.d., 14.3 ml) with a TSK SW guard column (7.5 cm × 7.5 mm i.d., 3.3 ml) from Tosoh, Tokyo, at a flow rate of 0.2 ml/min. The columns were kept at 6°C by circulating liquid from a constant-temperature bath through cooling jackets and were connected to a high-speed liquid chromatograph (Type CCPD) equipped with a degasser (ERC-3522). The eluate was monitored by a UV photometer (UV-8010) and by the LALLS instrument LS-8000, which contains a light-scattering detector and a differential refractometer (RI-8011). The above pieces of equipment were obtained from Tosoh, except the degasser, which was from Erma Optical Works, Kawaguchi, Japan.

The eluent was 70 mM Tris-HCl (pH 7.0 at 22°C), 40 mM OG, 1 mM DTE and 3 mM NaN_3 (solution A, ionic strength 0.06 M). In a single series of experiments 500 mM NaCl was included. The columns were first equilibrated with the buffer solution without OG and then with 80 ml or more of the eluent (with OG), until the baselines were stable. The eluent was then recirculated except when components were being eluted. The system was calibrated with chicken ovalbumin and BSA, which were of suitable size and gave simple and reproducible elution profiles. The absorption coefficient at 280 nm is 0.733 ± 0.024 ($n = 7$, values from Refs. 16, 361, 734, 1113 and 1674–1676 in our Ref. 21) for ovalbumin and 0.665 ± 0.010 ml mg^{-1} cm^{-1} ($n = 4$, values for 279.5 and 280 nm from Refs. 11, 12, 14 and 15 in our Ref. 21) for BSA. The M_r value is 42 750 for ovalbumin according to the nucleic acid sequence [22] and 66 267 for BSA [23]. These proteins bind less than 0.1 g OG per g protein [24] and are thus suitable for this calibration [9]. Other proteins with known absorption coefficients were tested, but aggregated in the buffer/detergent solution. The M_r for the glucose transporter was estimated from a plot of

$$\frac{(\text{Output})_{LS} \times (\text{Output})_{UV}}{[(\text{Output})_{RI}]^2 \times A} \text{ versus } M_r$$

where $(\text{Output})_{LS}$ is the light scattering detector signal, $(\text{Output})_{UV}$ the UV absorbance signal, $(\text{Output})_{RI}$ the differential-refractometer signal and A the absorption coefficient. For each of these three cases, the output is the height of a peak from the baseline and is thus proportional to the difference in contribution from the solution and the solvent. The output signals were thus calculated by use of the peak heights and the detector sensitivity settings. This analysis gives the M_r of the Glut 1 polypeptide, even though the protein is glycosylated and appears in complex with OG, as explained in Ref. 9, where the applicability of the LALLS technique to determinations of the molecular weights of membrane proteins is described.

Molecular-sieve chromatography on agarose gel with activity measurements. A Superose-6 column (28 cm × 10 mm) was used at 6°C and at a flow rate of 0.15 ml/min in an FPLC-system (pump P-500, valve V-7, detector UV-1) from Pharmacia LKB Biotechnology. The column was equilibrated with 90 ml of a freshly prepared solution of 70 mM Tris-HCl (pH 7.0 at 22°C), 50 mM OG (from Sigma), 1 mM DTE and 500 mM NaCl (solution B) and then with 50 ml of solution B supplemented with 1 mM PS or 1 mM PC. These phospholipid solutions were prepared as described below. 1.0 ml of DEAE-purified Glut 1 was supplemented with NaCl to 500 mM concentration and applied onto the column. In one experiment with PC, OG from Dojin was used at a concentration of 40 mM, and

the column was kept at 2°C (Table II). Fractions were kept at 2°C before reconstitution.

Molecular-sieve chromatography on silica gel with activity measurements. A TSKgel G3000SW XL column with a TSK SW guard column (dimensions as above) were kept at 2°C by use of jackets containing ice and water and were connected to an HPLC pump (Model 510) and an LC spectrophotometer (Lambda-Max 481) both from Waters, Millipore, Milford, MA and a sample injection valve (No. 7125) from Rheodyne, Cotati, CA.

The columns were equilibrated with 80 ml of a freshly prepared solution of 70 mM Tris-HCl (pH 7.0 at 22°C), 40 mM OG (from Dojin), 1 mM DTE, 3 mM NaN_3, and 100 mM NaCl (solution C), unless otherwise stated. For experiments in the presence of phospholipid the columns were then equilibrated with 40 ml of solution C supplemented with 1 mM PS or 1 mM PC (see below). 0.5 ml of DEAE-purified Glut 1 was applied onto the column. Fractions were kept at 2°C before reconstitution.

Phospholipid solutions. Phospholipid solutions were prepared as follows: PS (25 mg/ml) or PC (20 mg/ml) in chloroform was added in a suction flask to solutions corresponding to solution B or C at double the concentration indicated above. Nitrogen gas was flushed onto the surface of the milky suspension, with gentle shaking, until the suspension became clear and the odor of chloroform disappeared. The flushing was then continued for 15 min and the solution was diluted to the final composition (B or C).

Phosphorus analysis. Phospholipids were determined by the 'ultramicro' phosphorus analysis method of Bartlett [25], with sample volumes of 25–50 μl.

Reconstitution of the Glut 1 and transport activity measurements. Glut 1 from fractions collected in molecular-sieve chromatographic experiments was reconstituted into EYP liposomes: 200-μl aliquots of the fractions were mixed with 200 μl of EYP solution, or 100-μl aliquots were diluted with 100 μl of 10 mM sodium phosphate buffer (pH 7.2) containing 200 mM NaCl and 50 mM D-glucose and then mixed with the EYP solution. Proteoliposomes were prepared by chromatographic removal of the detergents [2,10,26]. The stereospecific equilibrium exchange of D-glucose across the bilayer of the proteoliposomes was determined with D-[^{14}C]glucose and L-[^{14}C]glucose at 50 mM D-glucose as described in Ref. 2. The Glut 1 polypeptide concentrations in the chromatographic fractions were determined, for calculation of the specific equilibrium exchange, by analysis of all amino acids except cysteine or tryptophan following hydrolysis with 6 M HCl for 24 h. The amounts of cysteine, tryptophan and serine were estimated by use of the amino acid composition [18].

Electrophoresis. Gradient polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) and silver-staining of the gel slabs were done essentially as described in Refs. 2 and 17, respectively.

Results

Absorption coefficient of the Glut 1

The absorption coefficient at 280 nm of the purified Glut 1 was determined to be 1.13 ± 0.03 ml mg^{-1} cm^{-1}, referring to the polypeptide concentration. The final sample purification by molecular-sieve chromatography is illustrated in Fig. 1. SDS-PAGE with silver-staining showed Glut 1 monomers and some dimers and trimers, in a pattern similar to that in lane 3 of Fig. 2 in Ref. 17. The impurities seemed negligible even at high sample load (not illustrated). Two samples were analyzed. The phospholipid content of the samples was less than about 5–10 phospholipid molecules per polypeptide. The protein concentration was 122 ± 2 ($n = 2$) μg/ml as obtained by amino acid analyses with an estimated limit of error of 3%. The average $A^{1\,cm}_{280\,nm}$ value was 0.1396 ± 0.0006 ($n = 6$) and the average $A^{1\,cm}_{310\,nm}$ value was 0.0019 ± 0.0009 ($n = 6$). Details of the procedure are given in Methods. A separate experiment showed no change in $A^{1\,cm}_{280\,nm}$ upon addition of

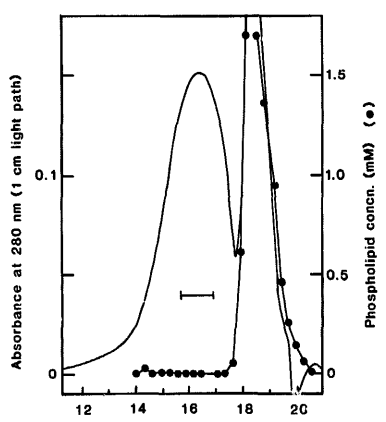

Fig. 1. Molecular-sieve chromatography of DEAE-purified Glut 1 on a Superose-6 column in the presence of OG, to obtain a Glut 1 sample solution for determination of the absorption coefficient. Phospholipids were determined by phosphorus assay. A Glut 1 fraction was collected as indicated by the bar.

sodium dodecyl sulfate (SDS) to 50 mM concentration. The denaturation of the transporter by SDS was probably only partial, due to the presence of OG.

The numbers of tryptophan ($N_{Trp} = 6$) and tyrosine ($M_{Tyr} = 13$) residues per Glut 1 according to the sequence of the HepG2 Glut 1 [18] were used to calculate a theoretical absorption coefficient value (A) for comparison with the experimental value. The equation from Ref. 27,

$$\epsilon_{280} = N_{Trp}5690 + M_{Tyr}1280$$

where ϵ_{280} is the molar absorption coefficient, gave $A = 0.94$ ml mg^{-1} cm^{-1} which is lower than our experimental value, 1.13 ± 0.03. However, our measurements were done in OG, whereas Edelhoch's calculated values in Ref. 27 were found to agree with experimental values for proteins in guanidinium chloride. Furthermore, the red cell Glut 1 may contain more tyrosine or tryptophan residues than the HepG2 Glut 1, although these transporters are very similar [18], and it may contain one or two disulfides, the contribution of which to the absorption has not been taken into account [27]. Finally, the content of about 4% of nucleoside transporter may increase the absorption.

Estimated M_r of the Glut 1 after molecular-sieve chromatography

Chromatography of the Glut 1 on Superose 6 at 6°C in the presence of 50 mM OG gave elution volumes corresponding to an M_r of 100000–125000 for the complex between the glycosylated Glut 1 and OG (data not shown). This is similar to the result described in Ref. 2 and indicates partial formation of dimers during the fractionation. The retention time for the main protein peak was about 110 min at the flow rate 0.15 ml/min.

The Glut 1 separated well from the membrane phospholipids (similarly as in Fig. 3 below) upon fractionation on TSKgel G3000SW at 2°C in the presence of 40 mM OG. The M_r for the main OG-Glut 1 complex (corresponding to peak 'M' in Fig. 3) was 70000–90000, as estimated from the elution volume in the gel chromatography by the of the calibration line (Fig. 2) (data not given). This is consistent with elution of the Glut 1 as a monomer (see Ref. 2), in agreement with the results below. The retention time for the monomer peak was about 20 min at the flow rate 0.4 ml/min. Upon chromatography at 25°C, most of the Glut 1 associated to form oligomers (not shown).

M_r and association state of the Glut 1 after molecular-sieve chromatography

As described in Methods, the M_r of the Glut 1 polypeptide could be determined after chromatography

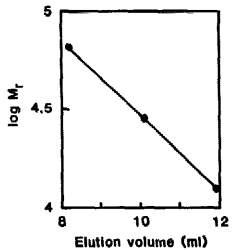

Fig. 2. Log M_r versus elution volume for calibration proteins upon high performance molecular-sieve chromatography in the presence of OG, on a TSKgel G3000SW column with a TSK SW guard column. The calibration line was used to estimate the M_r of the OG-Glut 1 complex. The calibration proteins were BSA (M_r 66267), bovine carbonic anhydrase (approx. M_r 29000) and bovine pancreas ribonuclease A (approx. M_r 13700).

on a TSKgel G3000SW column with three detectors (Fig. 3). The M_r value for the main Glut 1 peak was 49000 ± 6000 as illustrated in Fig. 4. (The limit of error includes the limit of error of the absorption coefficient). This shows that the Glut 1 in this peak

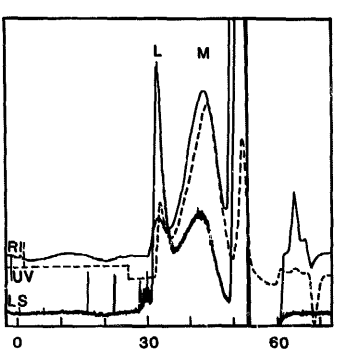

Fig. 3. High performance molecular-sieve chromatography of DEAE-purified Glut 1 on a TSKgel G3000SW column with a TSK SW guard column, in the presence of OG, for analysis of the M_r of the Glut 1 and its state of association. The DEAE-purification of Glut 1 was finished about 4 h after the start of the solubilization of the IMPs. The run shown here was done at 5.6 h (arrow in Fig. 5). Three detectors were used, for light-scattering (LS), differential refractometric (RI) and absorbance (UV) measurements. The peaks marked 'L' correspond to Glut 1 oligomers; the ones marked 'M' correspond to Glut 1 monomers.

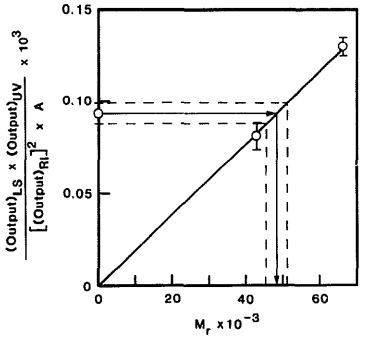

Fig. 4. Calibration line and data for the Glut 1 from high performance molecular-sieve chromatographic experiments on a TSKgel G3000SW column with a TSK SW guard column, in the presence of OG. Averages for five runs with ovalbumin (M_r 42750), six runs with BSA (M_r 66267) and (open circle on the vertical axis) 16 runs with DEAE-purified Glut 1. The light-scattering signal $[(Output)_{LS}]$ times the protein concentration $[(Output)_{UV}/A]$, over the square of the differential refractometric signal $[(Output)_{RI}]$ is plotted versus the molecular weight (M_r). The polypeptide M_r of the Glut 1 was determined as indicated by the arrows.

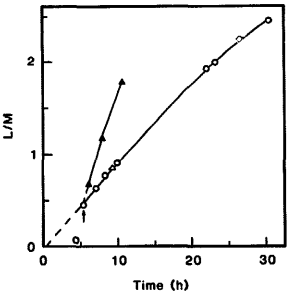

Fig. 5. High performance molecular-sieve chromatographic analyses of the association of Glut 1 monomers to form oligomers in 40 mM OG at 2°C, pH 7, and an ionic strength of 0.06 M (solution A). DEAE-purified Glut 1 (batches a and b) was kept at 2°C and aliquots were applied onto a TSKgel G3000SW column with a TSK SW guard column (○, batch a, first series of experiments; △ and ▲, batch b, second series of experiments). The ratios between the UV signal L corresponding to the Glut 1 oligomer peak ('L' in Fig. 3) and the UV signal M corresponding to the Glut 1 monomer peak ('M' in Fig. 3) were plotted as a function of the time elapsed from the start of the solubilization of the IMPs from which the Glut 1 was purified, to the start of the molecular-sieve chromatographic experiment. ▲, In the second series of analyses, most of the batch b of DEAE-purified Glut 1 was supplemented with NaCl to a concentration of 500 mM at the time 5.5 h (arrow); an aliquot of the original batch b (without NaCl) was analyzed at time 9.5 h (△).

('M' in Fig. 3) is monomeric, since the cDNA-deduced M_r value for the HepG2 Glut 1 polypeptide is 54117, and since a very similar value is expected for the red cell Glut 1 [18].

However, the monomers associated slowly to form oligomers, as shown by repeated chromatographic experiments with aliquots of DEAE-purified Glut 1 kept at 2°C in a solution of 40 mM OG (pH 7) and ionic strength 0.06 M (solution A) (Fig. 5). The oligomers were eluted near the void volume of the column (for example, peak 'L' in Fig. 3, which was calculated to correspond to complexes of, on the average, 20 Glut 1 monomers). The Glut 1 associated rapidly in the presence of 500 mM NaCl (in the sample and in the eluent) (Fig. 5, ▲) and even more rapidly at 26°C in the absence of NaCl (not illustrated).

TABLE I

Specific equilibrium exchange of D-glucose mediated by the Glut 1 from the main protein zone after chromatography of DEAE-purified Glut 1 on a Superose-6 column in the presence of OG and PS or PC

The eluent contained 500 mM NaCl and 1.0–1.2 mM PS or PC. The column temperature was 6°C in the experiments with 50 mM OG and 2°C in the experiment with 40 mM OG

Lipid in the eluent	OG concn. in the eluent (mM)	Specific equilibrium exchange of D-glucose after reconstitution [a] (nmol/μg per 2 min)	Glut 1 concn. [a] (μg/ml)	Phospholipid concn. above baseline [a] (μM)
PS	50	25±10	110±6 [b]	≈0 [c]
PC	50	9± 3	116±7 [b]	≈0 [c]
PC	40	92±17	43±1 [d]	60±30

[a] Average values for two main Glut 1 fractions (corresponding to the bar in Fig. 1).
[b] The concentration was 15-times lower in the transport assay incubation mixture.
[c] Uneven baseline.
[d] Aliquots were diluted 1:1 before mixing with lipids for reconstitution to make the Glut 1 concentration 30-times lower in the transport assay incubation mixture.

Specific glucose transport activity after molecular-sieve chromatography on agarose gel

Fractionation of DEAE-purified Glut 1 on Superose 6 with 50 mM OG and 1 mM PS or 1 mM PC in the eluent resulted in low specific equilibrium exchange of D-glucose after reconstitution of the Glut 1 (Table I). This is consistent with the low activity levels obtained earlier on Superose in the presence of 50 mM OG and 2 mM EYP (at most about 0.25% stereospecific uptake of D-[^{14}C]glucose in the equilibrium exchange assay [7], compared to up to 1.6–2.1% in the experiments illustrated in Fig. 6). The chromatograms were similar to that in Fig. 5 of Ref. 2. An experiment was also done on Superose 6 in the presence of 40 mM OG and 1 mM PC. A higher specific equilibrium exchange of D-glucose was observed under these conditions. This indicates that the OG concentration is important. The value 92 ± 17 nmol D-glucose per μg protein, in two min, (Table I) is nevertheless only about half of the corresponding value, 198 ± 9, obtained after chromatography on TSKgel at 40 mM OG (see Table II and Fig. 6C). The above TSKgel column experiments were 5.5-times faster than the Superose experiments (Table I) and did not lead to formation of significant amounts of Glut 1 dimers.

Specific glucose transport activity after molecular-sieve chromatography on silica gel

The specific equilibrium exchange of D-glucose mediated by the reconstituted Glut 1 at 50 mM D-glucose was 160–180 nmol D-glucose per μg protein, in two min, after fractionation of DEAE-purified Glut 1 on TSKgel G3000SW in the presence of 40 mM OG, 1 mM PS or PC and 100 mM NaCl (Fig. 6A'; Table II), but much lower after fractionation in the absence of eluent lipids. In the latter case, the specific equilibrium

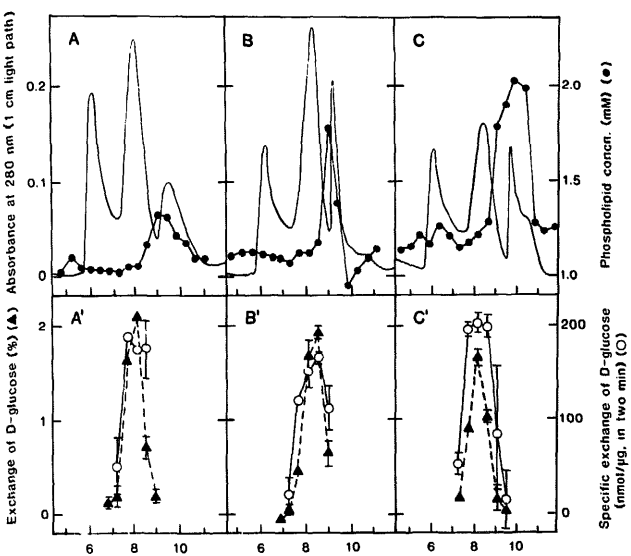

Fig. 6. High performance molecular-sieve chromatography of the Glut 1 on a TSKgel G3000SW column with a TSK SW guard column, in 40 mM OG. DEAE-purified Glut 1 was applied and 0.4-ml fractions were collected for phosphorus analyses, reconstitutions, transport activity measurements and amino acid analyses. The columns had been equilibrated in buffer solution (see Methods) containing: (A, A'), 1 mM PS and 100 mM NaCl; (B, B') 1 mM PC and 100 mM NaCl; and (C, C') 1 mM PC and 500 mM NaCl. Panel A' illustrates the same run as in panel A, etc. The absorbance peaks at about 8.2 ml represent the monomeric Glut 1. Limits of error are indicated in panels A'–C'. In several cases they are covered by the symbols. Data illustrated here are summarized in Table II.

exchange was only 27 nmol/μg, in two min, in the main lipid-free Glut 1 fraction (Table II). In the TSKgel fractionations illustrated in Figs. 6A,A' and B,B' the NaCl concentration in the eluent was 100 mM, whereas it was 500 mM, as in the Superose experiments, in the experiment illustrated in Fig. 6C,C'. The specific activity seemed to increase slightly with the NaCl concentration. In separate reconstitutions of non-purified IMPs, the NaCl concentration in the samples was adjusted to four values in the range 150–350 mM before reconstitution. This corresponds to the range of the final NaCl concentrations before reconstitution in the experiments summarized in Tables I and II. The D-[^{14}C]glucose uptake in the corresponding equilibrium exchange assays increased slightly with increasing NaCl concentration and was, on the average, 3.20 $\pm$ 0.06% for duplicate assays of the four samples (not illustrated). A comparison between the data in Table I, last line, and those in Table II, last line, indicates that the lower activity after a run on Superose 6 than after a corresponding run on TSKgel G3000SW was caused by the 5.5-fold longer retention time for the main Glut 1 fraction in the former case and was related to dimerization of the Glut 1 in the Superose runs. However, additional effects related to the different structures of the agarose and silica gels cannot be excluded.

The specific equilibrium exchange at 50 mM D-glucose was, on the average, about 180 $\pm$ 10 nmol/μg, in two min, after reconstitution of DEAE-purified Glut 1 that had been run on TSKgel in the presence of lipids and 100 or 500 mM NaCl. The Glut 1 concentration in the incubation mixture for the equilibrium exchange assay was 4.7 $\pm$ 1.0 μg/ml in these experiments (Table II). The above specific exchange value, after chromatographic separations in the presence of exogenous phospholipids, was equal to or exceeded those obtained earlier [2,26] for Glut 1 that had been only DEAE-purified, for example 135–180 nmol/μg, in two min at 3–6 μg protein/ml (Fig. 4 in Ref. 2). The purity of the active, monomeric Glut 1 was only slightly improved by molecular-sieve chromatography on the TSKgel column. The activity thus seemed to be retained during the TSKgel chromatography.

Fractionation in the presence of PS resulted in a slightly higher specific activity (at similar protein concentration) than did fractionation in the presence of PC both on Superose (Table I) and on TSKgel (Table II; Figs. 6A' and 6B'). The small difference can depend on the lengths of the fatty acid chains of the PS and PC and the position of the double-bonds in these chains, although the type of the polar head group may be the most important factor, as in reconstituted systems. The activity of the Glut 1 in dimyristoylPS liposomes is much higher than in dimyristoylPC liposomes [28].

Discussion

The transport activity of the Glut 1 was measured by its ability to mediate D-glucose equilibrium exchange after reconstitution into liposomes. The specific transport activity was considerably better retained when the final purification of the OG-Glut 1 complex was done by molecular-sieve chromatography in the presence of PS or PC than in the absence of these lipids, especially when the run time was short as on the TSKgel columns. However, the hypothesis that PS is specifically needed [2,30] is not supported by the present experiments. PC was nearly as effective as PS in preserving the specific

TABLE II

Specific equilibrium exchange of D-glucose mediated by the Glut 1 from the main protein zone after chromatography of DEAE-purified Glut 1 on a TSKgel G3000SW column in the presence of 40 mM OG and PS or PC

The PS or PC concentration was 1.0–1.2 mM. The column temperature was 2 °C. The values given with limits of error are averages for two main Glut 1 fractions. The corresponding specific equilibrium exchange values are averages of duplicate values for aliquots diluted 1:1 before mixing with lipids for reconstitution. Aliquots of the other fractions were not diluted

Lipid in the eluent	NaCl concn. in the eluent (mM)	Specific equilibrium exchange of D-glucose after reconstitution (nmol/μg per 2 min)	Glut 1 concn. [a] (μg/ml)	Phospholipid concn. above baseline [a] (μM)
–	0	27	84	0
PS	0	146	83	60
PS [b]	100	181 $\pm$ 7	158 $\pm$ 26	25 $\pm$ 0
PC [c]	100	158 $\pm$ 12	173 $\pm$ 5	45 $\pm$ 35
PC [d]	500	198 $\pm$ 9	98 $\pm$ 27	25 $\pm$ 15

[a] In the Glut 1 fraction(s).
[b] Experiment illustrated in Fig. 6A, A'.
[c] Experiment illustrated in Fig. 6B, B'.
[d] Experiment illustrated in Fig. 6C, C'.

activity of the Glut 1 in OG, as judged by high performance molecular-sieve chromatographic experiments on TSKgel G3000SW at an ionic strength of about 0.16 M (solution B). This ionic strength was chosen to resemble that in the red cell. The non-specific effect of PS and PC is probably partly related to a lowering of the OG monomer concentration (the effective CMC) in the presence of these lipids.

Requirements for essential lipids have been reported for the *Escherichia coli* lactose transporter and it was suggested that the specificity may depend on the polar head of such lipids in that case [29]. The present results support a model for the freshly prepared, native Glut 1 in OG as monomeric, in agreement with an earlier estimate and hypothesis [2], and associated with a small number of phospholipid molecules (cf. Ref. 30). The Glut 1 retained its activity in the presence of eluent phospholipids when the OG concentration did not greatly exceed the CMC. Accurate data on the micellar properties of OG in aqueous solutions [6,31] are important for the choice of a suitable OG concentration.

The oligomeric state of canine renal Na^+/K^+-ATPase solubilized by octaethylene glycol *n*-dodecyl ether ($C_{12}E_8$) has been studied similarly by LALLS photometry coupled with high performance molecular-sieve chromatography [32]. The $\alpha\beta$-protomer of this protein showed up to 79% of the specific activity of the membrane-bound enzyme after chromatography in the presence of $C_{12}E_8$ supplemented with PS of a sufficient concentration. The specific activity became much lower in the absence of PS. The $\alpha\beta$-protomer appeared in equilibrium with the $(\alpha\beta)_2$-diprotomer.

The tendency of Glut 1 monomers to associate in, for example, OG, cholate [33] and SDS [1,2,34] may earlier have contributed to difficulties in identifying this protein [33,35]. The present results show that monodisperse active Glut 1 can be obtained in OG for attempts at crystallization. It appears difficult, but not impossible, to prepare a stable monodisperse Glut 1 preparation of high concentration. Further experiments are being planned in Uppsala with the purpose of increasing the concentration and improving the stability of the preparation. Preliminary results obtained by high performance molecular-sieve chromatographic analyses indicate that the concentration of the monomeric Glut 1 can be increased considerably compared to the concentration of 0.6–0.8 mg/ml of the DEAE-purified Glut 1 [2,17].

Note added in proof (Received 12 July 1991)

A paper published on May 14, 1991 (Hebert, D.N. and Carruthers, A. (1991) Biochemistry 30, 4654–4658) on Glut 1 dimers and tetramers in the presence of sodium cholate, confirms and extends the results by Acevedo et al. [33] on active glucose transporter complexes of heterogeneous size in cholate, and is an interesting counterpart to the present article on Glut 1 monomers in octyl glycoside.

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