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ARTICLE *in* PROTEIN EXPRESSION AND PURIFICATION · AUGUST 2001

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Protein Expression and Purification 22, 337–348 (2001)
doi:10.1006/prep.2001.1440, available online at http://www.idealibrary.com on IDEAL®

Purification and Characterization of Human Erythrocyte Glucose Transporter in Decylmaltoside Detergent Solution

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Received February 1, 2001, and in revised form March 12, 2001; published online May 21, 2001

The facilitative glucose transporter from human erythrocyte membrane, Glut1, was purified by a novel method. The nonionic detergent decylmaltoside was selected for solubilization on the basis of its efficiency to extract Glut1 from the erythrocyte membrane and its ability to maintain the protein in a monodisperse state. A positive, anion-exchange chromatography protocol produced a Glut1 preparation of 95% purity with little copurified lipid. This protein preparation exhibited cytochalasin B binding in detergent solution, as measured by tryptophan fluorescence quenching. The transporter existed as a monomer in decylmaltoside, with a Stokes radius of 50 Å and a molecular mass of 147 kDa for the protein–detergent complex. We screened detergent, pH, additive, and lipid and have found conditions to maintain Glut1 monodispersity for 8 days at 25°C or over 5 weeks at 4°C. This Glut1 preparation represents the best available material for two- and three-dimensional crystallization trials of the human glucose transporter protein.

Glut1 is an integral membrane protein responsible for the facilitative transport of glucose into many cells and is a member of the mammalian facilitative glucose transporter family (44). These transporters are a group of highly related membrane proteins that share significant sequence homology and are believed to possess 12-transmembrane α-helices (2, 52). Until recently, seven members of the gene family have been described and are referred to as Glut1-7 (56). Making use of the newly available information on human genome, two new glucose transporters, Glut8 (8, 19) and Glut9 (18, 58), have recently been identified. Most of these glucose transporters are expressed in a tissue-specific manner (56). In contrast, Glut1 is expressed in all human cells and is responsible for basal level passive transport of glucose into the cell. It is particularly abundant in human erythrocytes, fibroblasts, and endothelial cells. In erythrocytes it is responsible for the uptake of glucose—the cell's major energy source (5). Its expression in human endothelial cells provides a pathway for the transport of glucose across the blood–brain barrier into the brain, which also depends on the sugar as its primary energy source (44).

Dysfunction of the Glut1 protein results in severe pathological consequences. Children born with Glut1[2] deficiency syndrome display infantile seizures, acquired microcephaly, and developmental delay (63, 68). Heterozygous mutations in the Glut1 (SLC2A1) gene causes decreased erythrocyte glucose transporter activities in the patients and reduced cerebrospinal fluid glucose concentrations. Mutations characterized to date include one large-scale deletion, five missense mutations, three deletions, three insertions, three splice site mutations, and one nonsense mutation (63, 68).

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[2] Abbreviations used: BCA, bicinchoninic acid; CB, cytochalasin B; DM, decylmaltoside; DDM, dodecylmaltoside; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Glut1, human erythrocyte glucose transporter; HPLC, high-performance liquid chromatography; OG, octylglucoside; PA, phosphatidic acid; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; PNGase F, peptide *N*-glycosidase F; PS, phosphatidylserine; SDS, sodium dodecyl sulfate; TLC, thin-layer chromatography; 2D, two-dimensional; 3D, three-dimensional.

1046-5928/01 $35.00

The mutations occur in both the transmembrane α-helices and connecting loops of Glut1.

The molecular mechanism by which Glut1 transports glucose across the membrane remains unclear largely due to the lack of detailed structural information. Two-dimensional (2D) or three-dimensional (3D) crystals of the protein are necessary for high-resolution structural analysis. Highly purified protein in detergent solution which is homogeneous and stable is needed for 2D and 3D crystallization experiments (39, 60).

The human erythrocyte is a particularly abundant source of Glut1, comprising approximately 5–10% of the total erythrocyte integral membrane protein (4). The protein consists of 492 amino acids (53) and is *N*-glycosylated on residue Asn45 in a highly heterogeneous manner (22). It has been purified using ion-exchange chromatography, producing materials for functional studies (3, 7, 32, 36, 42, 59). Following reconstitution into proteoliposomes, the protein facilitates glucose transport, as measured by transport assays, and binds the inhibitor cytochalasin B (CB), as demonstrated by tryptophan fluorescence quenching (4, 9, 15, 67). The preparation is, however, not ideal for crystallization experiments because of its high contents of copurifying lipid and short life span.

In this work, we present a novel purification method for Glut1 which is suitable for crystallization studies. The protein has less lipid copurified than previous preparations yet it binds cytochalasin B in detergent solution. We have determined the oligomeric state of the protein in detergent solution and have identified conditions that allow the protein stay in a monodisperse state for several weeks.

MATERIALS AND METHODS

Materials. Detergents were purchased from Anatrace (Maumee, OH), lipids from Avanti Polar Lipids (Alabaster, AL), peptide:*N*-glycosidase F (PNGase F) from New England BioLabs (Beverly, MA), and chromatography columns from Amersham Pharmacia (Piscataway, NJ). All other reagents were from Sigma (St. Louis, MO) and were of analytical grade or higher.

Preparation of erythrocyte membrane. Human erythrocyte "ghost" membrane was prepared by hypotonic lysis of outdated packed red cells (Tisch Hospital Bloodbank, NYU Medical Center). The membrane was stripped of cytoskeleton by 1 mM EDTA, 20 μg/ml PMSF, pH 7.5, at 37°C for 30 min. The stripped membrane was then washed with NaOH at pH 11.0 to remove other extrinsic membrane proteins (10).

Selection of detergent for extraction and solubilization. Detergents were tested for solubilization of erythrocyte membrane and extraction of Glut1 protein. For this purpose, 50 μl membrane in 50 mM Tris/HCl, pH 7.4, 0.5 mM EDTA, was solubilized with 1% micellar detergent, i.e., at a detergent concentration 1% above its critical micellar concentration. After incubation at 4°C for 30 min and removal of unsolubilized membrane by centrifugation, 25 μl of the supernatant was taken, followed by SDS–PAGE and Coomassie staining.

Glut1 purification. Four milliliters of stripped ghost membrane was solubilized by addition of decylmaltoside (DM) to a final concentration of 2 from 20% stock solution. After 30 min incubation at 4°C, the solubilized membrane was centrifuged at 10,000g for 15 min to remove unsolubilized materials and large aggregates. The sample was loaded onto a 1-ml HiTrap Q anion-exchange column (Amersham Pharmacia), preequilibrated with loading buffer (10 mM Bis–Tris, 0.5 mM EDTA, pH 6.0, 0.2% DM), at a rate of 0.5 ml/min. Following washing with 15 ml loading buffer, the column was eluted with a 15 ml linear 0–500 mM NaCl gradient at 0.25 ml/min. To better assess protein purity, Glut1 was deglycosylated with 5000 U/mg PNGase F in the presence of 1% SDS at 20°C and analyzed by Coomassie-stained SDS–PAGE. Protein concentration was measured using a BCA assay (Pierce Chemical Co., Rockford, IL). Bis–Tris buffer of 10 mM was included in the blank to minimize interference with the assay.

N-terminal sequencing. N-terminal peptide sequencing was performed by Dr. R. Beavis (Skirball Institute, NYU Medical Center) on a Perkin–Elmer sequencer using standard techniques.

Lipid analysis. The amount of phospholipids copurifying with Glut1 was measured by a phosphorus assay (14) and the types of lipids were analyzed by two-dimensional thin-layer chromatography (TLC) (37). 0.5 mg of lipids was extracted from the purified Glut1 with 10 vol of chloroform:methanol (3:1) (28). This was dried to a film with nitrogen gas and then redissolved in 100 μl chloroform:methanol. The lipid mixture was spotted at the bottom right-hand corner of a 20 $\times$ 20-cm TLC plate (250 μm, silica gel G, Fisher Scientific) and allowed to dry thoroughly. TLC plates were developed in a sealed glass chromatography tank saturated with solvent vapor, till the solvent reached $\sim$1 cm from the top of the plate. The solvent for the first dimension (basic) was chloroform:methanol:ammonia at 65:25:5, and for the second dimension (acidic) chloroform:acetone:methanol:acetic acid:water at 3:4:1:1:0.5. Plates were thoroughly dried after each development and were finally stained with molybdenum oxide (Sigma) vapor in a sealed chromatography tank. Lipids were identified on the basis of their mobility in the two solvents according to Kates (37), using synthetic lipids as standards.

Measurement of Glut1 binding to cytochalasin B. Glut1 binding to cytochalasin B was measured by tryptophan fluorescence quenching (9, 15). Purified Glut1 in 0.2% DM (10 mM Bis–Tris, 0.5 mM EDTA,

pH 6.0, 100 mM NaCl), was equilibrated for 30 min in a 250 μl cell in an Fluoromax-2 fluorimeter (J. Y. Horiba, Edison, NJ) equipped with a circulating water bath. Excitation wavelength was set at 295 nm to maximize tryptophan excitation, and emission spectra were collected between 310 and 390 nm at 25°C. To minimize UV damage to the protein, the excitation slit was set at 0.2 nm and the emission slit was set at 10 nm. Cytochalasin B was added from stock solution in ethanol, followed by mixing by inversion of the cell, and reequilibration for 5 min prior to collection of the next emission spectrum. Fluorescent emission data were corrected for dilution artifacts using control samples with the addition of cytochalasin D in ethanol.

Determination of Stokes radius and monodispersity by size-exclusion HPLC. Ten to fifty micrograms of Glut1 sample was loaded onto a Shodex KW804 analytical size-exclusion column equilibrated with 10 mM Bis–Tris, 0.5 mM EDTA, pH 6.0, 0.15% DM, 0.2 M NaCl and developed at 0.5 ml/min. The system was powered with a Waters 600S solvent delivery system controlled by the Millennium software. UV absorption spectra were collected via a photodiode array detector (Waters, Milford, MA). Chromatograms were extracted from the photodiode array data at a wavelength of 280 nm and integrated to determine the proportion of Glut1 in monodisperse and aggregated forms. Fractions were collected after separation by size-exclusion HPLC and then analyzed for protein, lipid, and detergent and later used for stability analysis using various lipids. Retention times of a set of soluble proteins of known Stokes radius (R_S) (41) were used to determine the Glut1 Stokes radius: thyroglobulin (86 Å), apoferritin (63 Å), catalase (52 Å), and aldolase (46 Å).

Determination of sedimenting coefficient by analytical ultracentrifugation. Velocity sedimentation experiments were performed using an XL-A analytical ultracentrifuge (Beckman Instruments, Palo Alto, CA) with an An60-Ti rotor. Glut1 in column buffer and approximately 100 mM NaCl was centrifuged at 40,000 rpm. The same buffer was used as a reference. Scans were collected every 5 min at 280 nm absorption wavelength and the radial distance of the sedimenting boundary r was measured. The sedimentation coefficient ($s_{20,w}$) was determined by the gradient of the plot of $\ln(r)$ vs $\omega^2 t$, where ω was the angular velocity and t was the time.

Determination of Glut1–DM complex partial specific volume. The partial specific volume of the Glut1–DM complex (v_C) was calculated, assuming volume additivity (64). The value for the amount of DM bound to Glut1 in the complex, used in the above calculation, was determined with a sugar assay (20), using Glut1 samples prepared by size-exclusion HPLC.

The protein component of the Glut1–DM complex had a molecular weight of 54,117 Da and a v of 0.755 ml/g, calculated from the sequence (53). The attached polysaccharides had an average M_W of ~6800 and a v of 0.639 ml/g (30). The partial specific volume of DM was calculated based on the value for dodecylmaltoside (DDM) (66) using the procedure of Durchslag and Zipper (21).

The molecular mass of the Glut1–DM complex (M_C) was calculated from the Stokes radius (R_S), the sedimentation coefficient ($s_{20,w}$), and the partial specific volume (v_C), using the following equation from Tanford *et al.* (65):

$$M_C = 6\pi\eta N_A \times R_S \times s_{20,w} \times (1/(1 - v_C\rho)), \quad (1)$$

where η was the solvent viscosity, N_A was the Avogadro's number, and ρ was the solvent density. The solvent viscosity and density were taken to be those of water ($\eta = 1.019 \times 10^3$ Nms^{-2}, $\rho = 1.00$ g/ml).

Determination of Glut1 monodispersity and stability. Monodispersity of Glut1 protein in various detergent solutions was measured using a modified protocol from the literature (13, 31), using size-exclusion HPLC. Other parameters investigated included temperature, time, pH, and the effects of lipids, glycerol, reducing agents, and salts. The degree of stability in various solutions and under different conditions was monitored and compared with control samples. Chromatograms extracted at 280 nm were integrated and the proportion of Glut1 in the monomer peak was calculated relative to the total Glut1. The following parameters were screened: (A) Detergent—Purified Glut1 (0.2% DM) was incubated overnight at 25°C in the presence of 1% of various detergents (added from 10% stock solutions). (B) pH—50-μl samples of purified Glut1 (10 mM Bis–Tris, pH 6.0) were titrated to the relevant pH by addition of 5 μl of 1 M buffer of the desired pH and then incubated overnight at 25°C. The following buffers were used: pH 4, acetate/acetic acid; pH 5, acetate/acetic acid; pH 6 (control), Bis–Tris/HCl; pH 7, Bis–Tris/HCl; pH 8, Tris/HCl; pH 9, Tris/HCl; pH 10, carbonate/HCl. (C) Additives—Purified Glut1 samples were prepared to the relevant concentration of additive. Glycerol was added to 20% (v/v), and cytochalasin B was taken from a 2 mM stock in ethanol (stored at −20°C), glucose from 1 M stock, ATP from 100 mg/ml stock, and DTT from 1 M stock. Samples were then incubated at 37°C overnight. (D) Lipid—Q-column purified Glut1 was delipidated by size-exclusion HPLC, followed by addition of individual lipid to a final concentration of 0.1 mg/ml. (E) Temperature—Purified Glut1 samples were incubated at 4, 25, and 37°C, and aliquots were taken at specific intervals in time.

RESULTS

Purification of Glut1

Selection of detergent for extraction of Glut1 from erythrocyte membrane. A variety of detergents were tested for extraction of Glut1 from erythrocyte membrane. They included Triton X-100, $C_{12}E_8$, Chaps, Chapso, cholate, SB-12, DM, DDM, Hecameg, nonylglucoside, and octylglucoside (OG). OG was the detergent that had often been used to solubilize and purify the Glut1 protein in previous studies (3, 4). Of all the detergents tested here, decylmaltoside and nonylglucoside were found to be most effective at solubilizing Glut1, based on Coomassie-stained SDS gel of detergent extracts from stripped erythrocyte membrane. Nonylglucoside was not further investigated due to the presence of a large amount of higher molecular weight aggregates of Glut1 at the top of the gel. These were particularly visible by Western blotting using a polyclonal antibody specific for the C-terminal region of Glut1 (data not shown). We therefore selected DM for solubilization of Glut1 and further purification.

Glut1 purification. Erythrocyte membrane was completely solubilized by 2% DM and then Glut1 was purified by anion-exchange chromatography. In this detergent, Glut1 bound to the HiTrap Q anion-exchange column, at pH 6.0 (10 mM Bis–Tris, pH 6.0, 0.5 mM EDTA) and was eluted with a linear gradient of NaCl (Fig. 1). Glut1 eluted as a sharp peak at approximately 100 mM NaCl, at a protein concentration of 0.5–1.0 mg/ml. Other erythrocyte membrane proteins, such as Band 3, did not bind significantly to the column under these conditions, and therefore the Glut1 peak dominated the chromatogram. The protein in this peak was approximately 95% pure, as determined by Coomassie-stained SDS–PAGE (Fig. 2A). Only peak fractions with A_{280} above 0.25 were collected and used for further experiments, since they contained the purest, most concentrated Glut1. The yield of the Glut1 peak was 5 ± 1% (n = 5) of total protein in the stripped erythrocyte membrane, although the entire yield of Glut1, including nonpeak fractions, was estimated to be 8–10%.

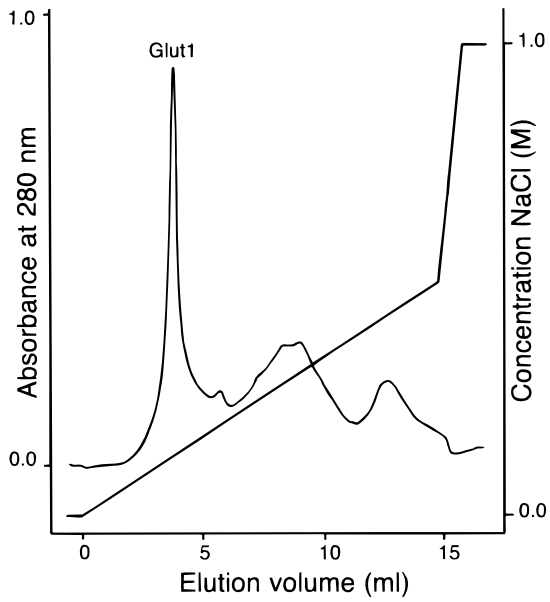

FIG. 1. Elution of decylmaltoside-solubilized erythrocyte membrane proteins from HiTrap Q anion-exchange column. Protein was monitored at 280 nm absorbance and Glut1 eluted at around 100 mM NaCl at a concentration of 0.5–1.0 mg/ml. Column buffer was 10 mM Bis–Tris, pH 6.0, 0.5 mM EDTA, 0.2% decylmaltoside.

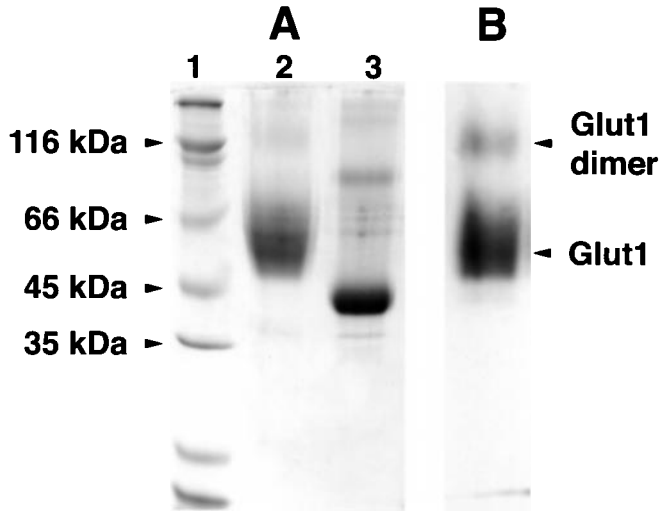

FIG. 2. SDS–PAGE showing Glut1 purification and deglycosylation. (A) Lane 1, protein molecular weight standards; lane 2, Glut 1 purified in DM detergent by HiTrap Q anion-exchange column, with a purity of approximately 95% purity. The protein was highly glycosylated. Lane 3, after PNGase F treatment in the presence of SDS, the polysaccharide was cleaved off and the Glut1 band sharpened and shifted down to 43 kDa. (B) When Glut1 was incubated with PNGase F, followed by separation from PNGase F using a size-exclusion column, and then loaded onto SDS–PAGE, the broad band of glycosylated Glut1 monomer remained intact. Minimal amounts of Glut1 dimer formed due to complete delipidation. Ten micrograms protein was loaded in each lane.

The identity of the purified protein was confirmed by N-terminal peptide sequencing. The N-terminal 12 amino acids of this Glut1 preparation were determined to be: MEPSSKXLTGRL. The seventh residue could not be detected (indicated by 'X'), and secondary sequences were detected, probably due to the poor yields during sample preparation for the sequencing experiment. This primary sequence was in agreement with the deduced N-terminal amino acid sequence of Glut1, MEPSSKKLTGRL (53). We therefore concluded that the preparation contained purified erythrocyte glucose transporter protein.

Deglycosylation of Glut1. PNGase F was added to purified Glut1 sample at 5000 units per milligram protein and subsequently loaded onto SDS–PAGE. The

Glut1 band became sharpened and shifted down to about 43 kDa, indicating complete deglycosylation of the protein (Fig. 2A). However, when Glut1 was incubated with PNGase F, followed by separation from PNGase F using a size-exclusion column, and then loaded onto SDS–PAGE, the broad band of glycosylated Glut1 remained intact (Fig. 2B). We therefore concluded that the PNGase F enzyme was only able to deglycosylate Glut1 when the protein was unfolded in the presence of SDS and was inactive against the folded protein.

Analysis of copurifying lipids. Because knowledge of the type and amount of lipids copurifying with a membrane protein is important for its crystallization, we analyzed the lipids copurifying with Glut1. Phosphorus assays were carried out on both purified protein and its lipid extraction. Measurements from purified Glut1 indicated the copurification of 75 ± 5 (n = 5) phosphates per protein molecule, equivalent to approximately 0.9:1 (w/w) lipid to protein ratio, assuming one phosphate per phospholipid. Lipid extraction using chloroform:methanol (3:1) showed that >95% of phosphate was derived from copurifying phospholipids, as opposed to from protein phosphorylation. A range of erythrocyte lipids was present in this preparation, as shown by two-dimensional TLC of the extract (Fig. 3),

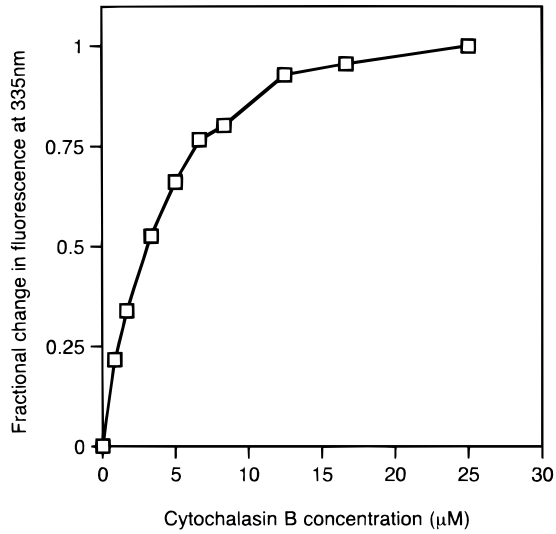

FIG. 4. Analysis of Glut1 binding to inhibitor cytochalasin B (CB) by tryptophan fluorescence quenching. The excitation wavelength was set at 295 nm on a Fluoromax-2 fluorometer. Purified Glut1 in 0.2% DM was equilibrated for 30 min and followed by emission scans between 310 and 390 nm at 25°C upon the addition of cytochalasin B. The quenching of fluorescence emission at 335 nm was corrected for dilution artifacts and the fractional changes were plotted vs CB concentration. The concentration required to give half-maximal quenching was 3.5 μM.

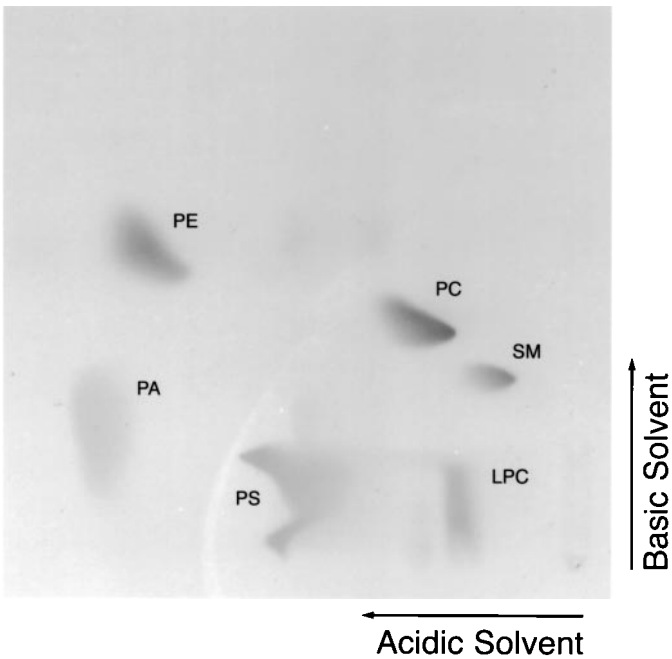

FIG. 3. Analysis of lipids copurifying with Glut1by thin-layer chromatogram. 0.5 mg lipid was spotted at the bottom right-hand corner and the plate was developed in two dimensions followed by staining with iodine vapor. Basic solvent, chloroform/methanol/ammonia 65/25/5; acidic solvent, chloroform/acetone/methanol/acetic acid/water 3/4/1/1/0.5. PC, phosphatidylcholine; PS, phosphatidylserine; PE, phosphatidylethanolamine; LPC, lysophosphatidylcholine; PA, phosphatidic acid; SM, sphingomyelin. Compared with the erythrocyte membrane, an enrichment of negatively changed PS and PA was observed.

including, phosphatidylcholine, phosphatidylserine (PS), phosphatidylethanolamine, lysophosphatidylcholine, phosphatidic acid (PA), and sphingomyelin. Compared to erythrocyte membrane (46), a relative enrichment of negatively charged lipids, such as PS and PA, was observed. The phosphorus and 2D TLC assays used were not suitable to detect nonphosphorus lipid, such as cholesterol.

Cytochalasin B Binding

To investigate the integrity of the Glut1 protein in DM detergent solution, its binding to the inhibitor cytochalasin B was measured by spectroscopic techniques. Purified Glut1 in DM displayed quenching of intrinsic tryptophan fluorescence upon successive additions of cytochalasin B (Fig. 4). The emission maximum at 335 nm shifted to a lower wavelength by approximately 2 nm and its intensity was reduced by approximately 20%, indicating that purified protein probably retained its native structure. As shown in Fig. 4, the fractional changes of Glut1 fluorescence at 335 nm displayed saturation of fluorescence quenching with increasing CB concentration. Half-maximal saturation, equivalent to dissociation constant K_{ds} was achieved at a CB concentration of 3.5 μM. Addition of cytochalasin D, an analogue of CB but not inhibitor of Glut1, did not lead to tryptophan fluorescence quenching.

Determination of Size and Oligomeric State of Glut1 Complex

Measurements of Stokes radius of Glut1–DM complex by size-exclusion HPLC. Glut1 eluted from size-exclusion HPLC, on a Shodex KW804 column, as a single major protein peak, followed by a smaller peak corresponding to solubilized lipids (Fig. 5). By comparison with the retention times of soluble protein standards, the Stokes radius of the Glut1 particle was determined to be 50 ± 5 Å (n = 5). Analysis of the eluent from this column by phosphate assay showed that Glut1 was completely separated from phospholipid, thus indicating that 50 Å was the Stokes radius of the delipidated protein–detergent complex. It also implied that the phospholipids, copurified from the HiTrap Q anion-exchange column, were not tightly associated with the Glut1 protein. Compared with the Stokes radius of 71 Å for the Band 3 membrane domain in DM, a dimeric transporter protein with a similar molecular weight (53 kDa per monomer) (13) as well as a comparable number of transmembrane α-helices (12–14 per monomer) (69), the Glut1 protein in DM was therefore likely to be a monomer.

Association state of Glut1 by analytical ultracentrifugation. The association state of Glut1 was further studied by analytical ultracentrifugation. First, sedimentation velocity of purified Glut1 showed a single sedimenting boundary, indicating a monodisperse preparation and in agreement with the size-exclusion HPLC data (Fig. 5). The sedimentation coefficient ($s_{20,w}$) was determined, from the gradient of a plot of ln(r) vs $\omega^2 t$, to be 5.5 ± 0.2 S. Second, in order to determine the Glut1–DM complex partial specific volume, fractions eluting from size-exclusion HPLC containing delipidated Glut1 were collected. They were found to contain a higher concentration of DM than eluting buffer due to its binding to the protein. A value for the amount of detergent bound to the protein of 1.45 g DM/g Glut1 protein was determined by assays of protein and detergent. The partial specific volume of the complex, v, was then calculated to be 0.786 ml/g, assuming volume additivity.

The molecular mass of the Glut1–DM complex (M_C) was calculated from the Stokes radius (R_S), the sedimentation coefficient ($s_{20,w}$), and the partial specific volume (v_C). Values for R_S of 50 Å, $s_{20,w}$ of 5.5 S, and v of 0.786 ml//g were used in Eq. (1), to obtain the molecular weight of the Glut1–DM complex of 147 kDa. Allowing for 1.45 g of bound detergent per gram of Glut1, this implied a molecular weight of the glycosylated protein of 60 kDa. The molecular weight of the polysaccharides bound to Glut1, 6.8 kDa, was then subtracted to give the molecular weight of the protein component at 53.2 kDa, in excellent agreement with the molecular weight derived from the sequence of 54,117 (53). In combination with the Stokes radius measured by size-exclusion HPLC (Fig. 5), this showed clearly that Glut1 existed as a monomer in DM solution.

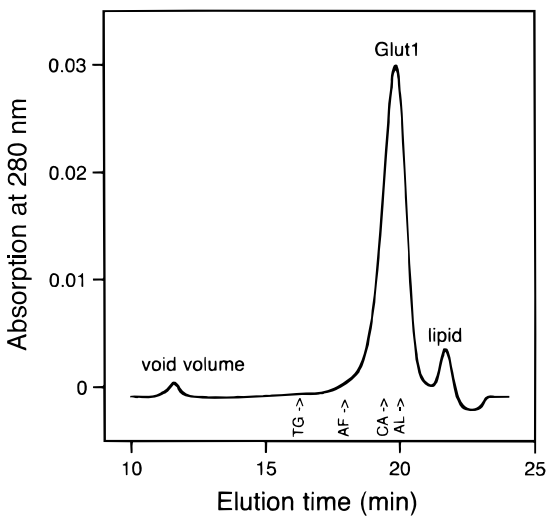

FIG. 5. Size-exclusion HPLC chromatogram of Glut1 in DM solution. The sample was run on a Shodex KW-804 column, equilibrated with 10 mM Bis–Tris/HCl, pH 6.0, 0.5 mM EDTA, 0.2 M NaCl, and 0.15% DM. Peaks were identified by their retention time, by their spectrum from photodiode array data, and by protein and lipid analysis of collected fractions. The Glut1–DM complex had a Stokes radius of 50 Å, corresponding to a Glut1 monomer. Elution times of standard proteins of known Stokes radius are indicated; TG, thyroglobulin (86 Å); AF, apoferritin (63 Å); CA, catalase (52 Å); AL, aldolase (46 Å).

Monodispersity and Stability of Glut1

To maintain Glut1 in a monodisperse state for crystallization, various factors were screened for their ability to influence the stability of the protein. They included detergent, pH, additive, lipid, and temperature. Conditions were eventually found to maintain the protein monodisperse for weeks.

Effect of detergent. Several detergents were assayed for their effect on the stability of Glut1 in solution (Table 1a). Only a limited amount of information can be deduced from this study because the addition of a high concentration (1%) detergent tended to destabilize Glut1, regardless of the detergent type, leading to high amounts of aggregation. Nevertheless, various degrees of protein stability have been observed for different detergents, suggesting possible alternative detergents suitable for crystallization experiments. Maltoside detergents generally retained Glut1 monodispersity, with decylthiomaltoside being the best. This and dodecylmaltoside were slightly better than decylmaltoside. Octylglucoside, which was used in many established protocols for Glut1 purification (4), resulting in large amounts of aggregation relative to the maltoside detergents, as did $C_{12}E_8$, which had been used in a recent protocol (30).

TABLE 1

Stability of Purified Glut 1 Protein under Various Conditions

Reagent	Conditions	Proportion of monomer after treatment	Effect on monodispercity
(a) Detergents			
Decylmaltoside	25°C overnight without glycerol	0.22	Control
Nonylmaltoside		0.22	−
Undecylmaltoside		0.22	+/−
Dodecylmaltoside		0.25	+
Decylthiomaltoside		0.26	+
Cymal-3		0.19	−
Octylglucoside		0.05	—
Nonylglucoside		0.04	—
Decylglucoside		0.13	—
$C_{12}E_8$		0.06	—
$C_{10}E_8$		0.04	—
MEGA-10		0.07	—
(b) pH			
4	25°C overnight	0.10	−
5		0.70	++
6		0.95	+++
7		0.90	+++
8		0.65	++
9		0.15	−
(c) Additives			
None	37°C overnight	0.24	Control
20% glycerol		0.59	++++
10 μM cytochalasin B		0.42	+++
100 mM glucose		0.36	++
1 mM ATP		0.19	—
5 mM DTT		0.24	−/+
(d) Lipids			
None	4°C overnight	0.61	Control
Phosphatidylcholine		0.75	+
Phosphatidylethanolamine		0.56	−
Sphingomyelin		0.63	+/−
Cholesterol		0.59	−
Phosphatidylinositol		0.79	++
Phosphatidylserine		0.93	+++
Total erythrocyte lipids		0.81	++

Note. Effect of (a) detergents, (b) pH, (c) additives, and (d) lipids on the stability of Glut 1. Concentrations of reagents were as follows: 0.1 mg/ml lipid (added to delipidated Glut 1), 1% detergent. The proportion of monomeric Glut 1 was 100% before treatment. Each parameter was assayed separately, and a combination of the positive factors identified in such a way used to preserve Glut 1 monodispersity for an extensive period of time.

Effect of pH. As expected, pH had strong effects upon the stability of Glut1 (Table 1b). A range of pH of 6–7 was found to be optimal for stabilizing the protein at 25°C. Glut1, having an isoelectric point of 8.4 (30), was positively charged at this pH range, thus maximizing charge repulsion between solubilized protein molecules (62).

Effect of additive. Several other reagents were assayed for their ability to prevent the protein from aggregating and thus increased its effective lifetime (see Table 1c). Twenty percent glycerol was found to be the most effective additive, increasing the stability of the protein significantly. The inhibitor, cytochalasin B, also had a positive effect, as did glucose. Surprisingly, the reducing agent DTT, which was commonly included in earlier Glut1 preparations, slightly decreased Glut1 stability.

Effect of lipid. The effect of lipid upon the monodispersity of delipidated Glut1 was also investigated (Table 1d). Completely delipidated Glut1 tended to aggregate, even at 4°C overnight. Different lipids displayed various degrees of capabilities of keeping Glut1 monodisperse. The most marked stabilization effect was observed with the negatively charged lipid, phosphatidylserine, although other lipids, such as phosphatidylinositol and phosphatidylcholine, showed some beneficial effects.

Effect of temperature. Combination of the stabilizing factor identified above resulted in conditions to

maintain Glut1 monodispersity over a wide temperature range. At 4°C the Glut1 complex undergoes minimal amounts of aggregation over time. Moreover, in the presence of 20% glycerol, Glut1 could be stored for at least 6 months at −20°C without any apparent aggregation. Assuming a monoexponential decay, the half-life of the monodisperse Glut1 in DM at pH 6 (with 50% monomer retained) was calculated to be 13 h, 8 days, and 5 weeks, respectively, at 37, 25, and 4°C. The Glut1 protein prepared in DM at pH 6.0 in the presence of 20% glycerol was therefore particularly suitable for two-dimensional crystallization.

DISCUSSION

There is currently very limited structural information available about Glut1 owing to the lack of any crystals of this protein. Either 2D or 3D crystals of membrane protein are most readily grown from protein preparations which are pure, homogeneous, active, and stable (40, 60). Protein purity >95% and monodispersity, i.e., having a single oligomeric state in solution, often are the most critical criteria. The presence of as little as possible of copurifying lipid is also important while keeping the protein stable and active.

Purification of Glut1

A protocol for purification of Glut1, first appearing in the literature in 1977 (36), has been modified several times (3, 7, 32, 42, 59). Fundamentally, the method consists of passing detergent-solubilized erythrocyte membrane proteins down a DEAE anion-exchange column at pH 7.4 and collecting Glut1 in the flowthrough fraction (4). Although this method produces relatively pure protein with high activity, it is unsuitable for crystallization studies for a number of reasons. First, because this is a negative purification, i.e., the protein is collected in the flowthrough and never binds to the column, a large amount of erythrocyte lipid is copurified (lipid:protein ratio ~ 3:1 w/w) which severely complicates 2D and 3D crystallization trials. Second, even in the presence of a large amount of native membrane lipid, this preparation of Glut1 is highly unstable in detergent solution and must immediately be reconstituted to form proteoliposomes for activity to be retained (3). Third, the rapid denaturation of this preparation results in the formation of heterogeneous aggregates which are likely to hinder any attempts to form ordered crystals of the protein, while most crystallization trials require the protein to be stable in detergent solution for several days or longer (27, 60).

We have now developed a completely novel method for the purification of Glut1 with the specific aim of producing monodisperse protein for crystallization trials. This has involved selection for a more suitable detergent for solubilization, development of a positive purification protocol by anion-exchange chromatography, and search for conditions to keep the protein monodisperse and stable in detergent solution for an extensive period of time after purification.

A positive purification procedure is required in order to reduce the amounts of lipids copurifying with the protein. We have tested the binding of DM-solubilized Glut1 to both anion- and cation-exchange resins at various pHs. The Glut1 protein is found to bind to HiTrap Q anion-exchange column, yielding a positive purification protocol. The binding of Glut1 to an anion-exchange matrix at such a pH, however, is unexpected, given the high isoelectric point of Glut1 of around 8.4 (23–25, 30). At this pH, Glut1 should be positively charged (cationic) and should not bind to an anion-exchange column (62). We propose that lipids are involved in the binding of the transporter protein to the column. The copurifying lipids contain a substantial proportion of negatively charged lipids, such as PA and PS. These lipids could bridge the positively charged protein and the positively charged column matrix, resulting in the binding of Glut1 to the anion-exchange resin. This theory is supported by our observations that the Q column purified Glut1 will not bind to the same anion-exchange column again, but will bind weakly to a cation-exchange column instead (data not shown), suggesting that the localized negative charges holding Glut1 to the first Q column are dispersed upon release from the column matrix. There have been reports of a lower isoelectric point for Glut1 (49); however, given the high isoelectric point predicted from the amino acid sequence of 8.8 (24), such a low pH seems unlikely.

The amount of lipid copurifying with Glut1 in this preparation at 0.9/1 lipid-to-protein ratio, approximately 75 lipid molecules per protein monomer, is still relatively high, although still much lower than in previous preparations. This amount of lipid seems to stabilize Glut1, since their removal results in more rapid aggregation of the protein. In addition, it has been noted for many membrane proteins that small amounts of membrane lipids are required to maintain structure and function in detergent solutions (55, 70). Stabilization of Glut1 by the lipids phosphatidylcholine and phosphatidylserine has been reported (43, 48). Our observations have revealed similar effects when these lipids are added back to the delipidated Glut1. Two-dimensional crystals of membrane proteins often form at a lipid-to-protein ratio of (0.5–1)/1 (w/w) (39). Extra lipids in the reconstituted membrane can be removed enzymatically by phospholipase A2 treatment to induce crystal packing (47). Our preparation is therefore particularly suitable for 2D crystallization experiments.

Deglycosylation of Glut1

Plasma membrane proteins from mammalian cells are normally glycosylated and the polysaccharide is often critical for the folding and targeting of the proteins (50, 57). It has been shown that glycosylation is necessary for efficient Glut1 intracellular targeting (1) but not for glucose transport (16). Enzymes like peptide:*N*-glycosidase F are routinely employed to remove the sugars from similar membrane proteins (11). We have found that, in contrary to expectation, PNGase F is unable to deglycosylate Glut1 in decylmaltoside solution, unless the transporter protein is unfolded, implying a buried Asn45 residue of the protein. A similar situation has been observed in the case of the Na,K-ATPase, where the glycosylation on its β-chain is insensitive to PNGase F in the absence of SDS (Dr. D. Martin, personal communication). Interestingly, the deglycosylation reaction of Glut1 took place within a short period of time, probably only in the first few minutes in the SDS–PAGE before the transporter protein and the enzyme became separated. A large amount of PNGase F, 5000 U/mg Glut1, was therefore required for deglycosylation (Fig. 2A). For comparison, only 5 U PNGase F per milligram is needed for deglycosylation of Band 3, which completes overnight at 20°C (11). Another noticeable point is that SDS actually inhibits PNGase F activity (45, 54). The PNGase F that we used (New England BioLabs), however, contains 10% NP-40 detergent, which revives the deglycosylation activity of the enzyme (45, 54).

Glut1 Binding to Cytochalasin B

Direct measurement of the transport activity of facilitative membrane transport proteins, such as Glut1, is not possible in detergent solution because of the vectorial nature of the transport process and the anisotropy of the solubilized protein. Either the protein must be reconstituted into proteoliposomes and the transport of labeled substrate may subsequently be measured or the binding of a ligand to the solubilized protein may be studied in detergent solution. In the case of Glut1, there is a well-documented quenching of the fluorescence of tryptophan residues of the protein upon binding of many ligands, particularly inhibitor cytochalasin B (9, 15). This method has been used as a convenient measure of the integrity and functionality of the protein. The previous binding studies of purified Glut1 have however been conducted in reconstituted membrane (9, 15). Now we have produced a preparation stable enough for the fluorescence quenching experiment in detergent solution.

The Glut1 protein in our preparation is in an active form, as determined by its binding of cytochalasin B. The total change in fluorescence emission of around 20% is significantly higher than that reported for previous preparations, possibly implying that more of the protein remain active in this preparation. The affinity of Glut1 for cytochalasin B may be crudely estimated, from the point of half-saturation of the protein, to be about 3.5 μM, which is approximately 10–20 times lower affinity than that measured in previous studies performed on protein reconstituted into liposomes (9, 15). This may be due either to a minor change in the conformation of the protein resulting from detergent solubilization or it may be due to the inhibition of the cytochalasin B binding by the maltose head group of the maltoside detergent. Maltose is an inhibitor of Glut1 transport activity (38) and also inhibits cytochalasin B binding to the protein.

Oligomeric State of Glut1 in DM

The association state of Glut1 in solution in decylmaltoside has been determined to be monomeric by size-exclusion HPLC and analytical ultracentrifugation. The Glut1–DM complex is found to have a Stokes radius of 50 Å, with a mass of 147 kDa. This result on oligomeric state is in contrast to some determinations in the native membrane (34, 35), and in solution in cholate (32, 33), which indicate dimers and/or tetramers. However, some recent studies on membrane (6) and in detergent solutions (OG and $C_{12}E_8$) (30, 43) show a monomeric association state. Although the association state of membrane proteins in solution in nonionic detergents has frequently been shown to mirror that in the native membrane (12), it is possible that in the case of Glut1, solubilization disrupts its oligomeric state or drives the oligomeric equilibrium to the monomeric form (71). However, for the pursuit of crystallization studies, in which the protein is generally crystallized from the soluble state, knowledge of the oligomeric state in solution is more relevant than that in the native membrane. More importantly, we observe only one oligomeric state for Glut1 in DM, and such a high degree of monodispersity is vitally important for crystallization experiments (27, 29, 60).

Stabilization of Glut1

Stability of a protein is a concept with many implications, depending on the context. For protein crystallization experiments, it means a basic requirement for its presence in a monodisperse state over a certain period of time, often several days to weeks at 4 or 20°C (27, 60). For membrane protein, this is often rather challenging. Due to the duality of their surfaces—hydrophilic, where they are exposed to aqueous solutions at their polar caps, and hydrophobic, where they interact with the nonpolar lipid core of the membrane—they tend to aggregate and precipitate even in detergent solution, thus aborting crystallization attempts. This is particularly

true for transporter proteins with multiple transmembrane spans and hence larger hydrophobic surface areas, such as Glut1. Careful selections of detergent to protect the protein molecules as well as other conditions, like pH and additive, will help to maintain the protein in soluble form.

In order to search for conditions in which Glut1 protein would stay monodisperse for days, we have developed a assay to screen detergent and other conditions efficiently. We have reasoned that if the protein could be kept stable at 37°C for a few hours it should stable monodisperse for days at 20°C. The effects of chemical and physical factors can be tested by incubating the protein sample in the presence of the factor at 37°C for a few hours, followed by assessment of the stability of the protein by analytical size-exclusion chromatography on HLPC. Each parameter is assayed separately, and combination of the positive factors identified in such a way will be able to preserve Glut1 monodispersity for an extensive period of time.

The analytical size-exclusion chromatography on HLPC is particularly adequate for assessing the protein monodispersity (13, 31). Incubation the samples at 25 or 37°C significantly speeded up the process of searching for optimal conditions for retaining protein monodispersity. A small amount of a protein sample, as little as 30 μg of Glut1, is enough to produce a signal with a high signal-to-noise ratio, and only a period of 30 min is needed to finish one experiment on the HPLC. A Shodex SW804 column can easily separate proteins differing by 50 kDa in molecular weight. These make the incubation/SE-HPLC assay more attractive than many other techniques for measuring membrane protein monodispersity, including native electrophoresis, analytical ultracentrifugation, and quasi-elastic light scattering (27, 60).

Glut1 has been observed to aggregate and denature irreversibly in nonionic detergents such as octylglucoside (7, 43). However, with careful selection of the solubilizing detergent and other conditions, this problem can usually be overcome (13, 51). Maltoside detergents, because of their mild properties, have been used in many membrane protein preparations. The erythrocyte anion exchanger Band 3, another membrane protein from the erythrocyte membrane, is most stable in dodecylmaltoside detergent (61). However, the 10-carbon chain length decylmaltoside is more efficient for extraction of Glut1 and maintained the protein in a stable state. In addition, the detergents selected by the SE-HPLC assay can later be used for detergent exchange on a preparative chromatography column for crystallization experiments.

Apart from maltoside detergents, other factors which stabilized Glut1 were, a pH range between 6 and 7, 20% glycerol, lipid, and specific ligands. A combination of these factors is required for optimizing the protein's stability. A slightly basic pH stabilizes Glut1 by giving the protein a net positive charge, thus maximizing ionic repulsion between protein molecules and maintaining the monodisperse state. It is also possible that the positive charges on the protein play a role in attracting stabilizing negatively charged phospholipid. Glycerol has been shown to stabilize many purified proteins, probably by reducing the activity of the water molecules and thus minimizing denaturation (17). The role of specific ligands in stabilizing Glut1 probably results from reduction in the conformational flexibility of the protein and also the formation of favorable bonds between residues in the active site and the ligand. D-Glucose, which increases the thermostability of Glut1 by shifting its denaturation transition to higher temperature (26), also helps to maintain its monodispersity.

The stability of this preparation is superior to previously published preparations. For crystallization experiments, temperatures between 20 and 40°C have proved most successful for integral membrane proteins (39). It is therefore essential that minimal aggregation occurs over the time course of experiments (~1–10 days) at these temperatures. This preparation stays monodisperse over 5 weeks at 4°C and 8 days at 25°C, and it represents the best material available for crystallization trials for the human erythrocyte glucose transporter protein.

ACKNOWLEDGMENTS

We thank Dr. Ronald Beavis for protein sequencing, Dr. Reinhart Reithmeier for anti-Glut1 antibodies, Dr. Dwight Martin for communicating unpublished results, Dr. Xiao-Dan Li and Joanne Lemieux for helpful discussions, Heather Griffith for critical reading of the manuscript, and Jonathan Weider and Anthony Villa for assistance with graphics and imaging. The research was financially supported by NIH (DK-53973).

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