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On the oligomeric state of the red blood cell glucose transporter GLUT1

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Abstract

We stripped human red blood cell membranes of cytoskeleton proteins at pH 12 without reductant, partially solubilized the obtained vesicles by use of octaethylene glycol *n*-dodecyl ether and purified the glucose transporter GLUT1 by anion-exchange chromatography followed by sulfhydryl-affinity chromatography, which removed most of the nucleoside transporter (NT) and the lipids. Eighty percent of the sulfhydryl-bound GLUT1 could be eluted with sodium dodecyl sulfate (SDS) indicating that the bound protein was multimeric. Matrix-assisted laser desorption ionization-time of flight-mass spectrometry (MALDI-ToF-MS) of the trypsinized major SDS-PAGE zone of the purified material identified GLUT1 but no other membrane protein. Transmembrane helices 1 and 8 were among the detected fragments. The reconstituted purified GLUT1 showed glucose transport activity, although only approximately 0.05 high-affinity cytochalasin B (CB) binding sites were present per GLUT1 monomer. The vesicles used as starting material for the purification showed 0.4 CB sites per GLUT1 monomer, similar to vesicles prepared in the presence of dithioerythritol. The data are consistent with the coexistence of monomeric GLUT1 with high-affinity CB-binding activity and preferentially solubilized multimeric GLUT1 with no CB-binding activity in the red blood cell membrane vesicles prepared without reductant.

Keywords: Cytochalasin B; Glucose transporter GLUT1; Human red blood cell; Nitrobenzylthioinosine; Nucleoside transporter; Sulfhydryl-affinity chromatography

1. Introduction

The glucose transporters GLUT1–12 [1–5] belong to a family of facilitative glucose transporters and are distributed among different tissues and organs in mammalians. They are characterized by their 12 transmembrane helical domains. GLUT1 [2] is present in red blood cells and epithelial cells, for example, in the blood–brain barrier, and can be purified from solubilized cytoskeleton-depleted red blood cell membrane vesicles on anion exchangers, such as DEAE-cellulose [6–8], aminoethyl-Sepharose 4B [9] and HiTrap Q [10]. The nucleoside transporter (NT) is a minor component (3–5% by weight) of these GLUT1 preparations [11,12].

Binding of competitive inhibitors to membrane proteins has been analyzed to study the association state of membrane proteins. Human red blood cell membranes, membrane vesicles and proteoliposomes showed 0.4 to 1 cytochalasin B (CB) binding sites per GLUT1 monomer [7,13–19], as revealed by equilibrium dialysis [7], fluorescence quenching [16] and frontal affinity chromatography [15]. Frontal affinity chromatography at steady state on immobilized biomembranes affords accurate K_d values and numbers of binding sites [11,13,15,19–21]. Advantages of this method for studying membrane proteins have been reviewed [22,23]. K_d values of 60–100 nM were determined for CB binding to GLUT1 purified in the presence of dithioerythritol (DTE) [11,13,15]. These val-

Abbreviations: A-GLUT1, anion-exchange-purified GLUT1 prepared without reductant; A-GLUT1$_{DTE}$, anion-exchange-purified GLUT1 prepared in the presence of dithioerythritol; A-GLUT1$_{(DTE)}$, anion-exchange-purified GLUT1 prepared without reductant from membrane vesicles cytoskeleton-depleted in the presence of dithioerythritol; CaPE, calcium phosphate adsorption and ethanol precipitation; CB, cytochalasin B; $C_{12}E_8$, octaethylene glycol *n*-dodecyl ether; pCMB, *p*-chloromercurybenzoic acid; DMPC, 1,2-dimyristoyl-1-*sn*-glycerol-3-phosphocholine; DTE, dithioerythritol; EYP, egg yolk phospholipids; MALDI-ToF-MS, matrix-assisted laser desorption ionization-time of flight-mass spectrometry; NBTI, nitrobenzylthioinosine; NT, nucleoside transporter; SDS, sodium dodecyl sulfate; S-GLUT1, sulfhydryl-affinity-purified GLUT1 prepared without reductant; S-GLUT1$_{(DTE)}$, sulfhydryl-affinity-purified GLUT1 prepared without reductant from membrane vesicles cytoskeleton-depleted in the presence of dithioerythritol

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ues are similar to the K_d value of CB binding to GLUT1 in membrane vesicles [13,16] and red blood cells [19]. Values of 200–400 nM were reported for GLUT1 purified without reductant as determined by fluorescence quenching [14,18]. The competitive NT inhibitor nitrobenzylthioinosine (NBTI) shows high affinity for NT in various membranes (dissociation constant, K_d, 0.04–0.4 nM) [11,21] but binds less strongly to reconstituted GLUT1 (K_d 80 nM) [11]. Among the proposals regarding the native oligomeric state of GLUT1 [7,13,18], the most accepted one is that GLUT1 exists as tetramer with 0.5 CB binding site per GLUT1 monomer [18]. However, the reported effect of reductant to increase the number of CB binding sites per GLUT1 monomer [18] could not be confirmed [13]. The presence of reductant has also been reported to affect the association state of the purified GLUT1 [14,18].

In the present study, we purified GLUT1 without reductant by use of anion-exchange chromatography followed by sulfhydryl-affinity chromatography [9,24] with analysis of glucose transport and CB binding activities. Matrix-assisted laser desorption ionization-time of flight-mass spectrometry (MALDI-ToF-MS) [25] of tryptic peptide fragments was used to identify the protein. We interpreted our results to give a new view of the putative oligomeric state of GLUT1 in the red blood cell membranes.

2. Materials and methods

2.1. Materials

We purchased CNBr-activated Sepharose 4B, Superdex 75 and 200, prep grade, and 0.5 cm (i.d.) × 5 cm HR glass columns from Amersham Biosciences (Uppsala, Sweden); 1,2-dimyristoyl-1-*sn*-glycerol-3-phosphocholine (DMPC) from Avanti Polar Lipids (Alabaster, AL, USA); sodium dodecyl sulfate (SDS) from BDH (Poole, Dorset, UK); octaethylene glycol *n*-dodecyl ether ($C_{12}E_8$) from Fluka (Buchs, Switzerland); [4-^{3}H]CB, [benzyl0-^{3}H]NBTI, L-[1-^{3}H] glucose and D-[^{14}C(U)] glucose from NEN (Boston, MA, USA); 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide–HCl from Pierce (Rockford, IL, USA); trypsin solution (modified, sequence grade) from Promega (Madison, WI, USA); adenosine, CB, DTE, NBTI and *p*-chloromercurybenzoic acid (pCMB) from Sigma (St. Louis, MO, USA) and DEAE cellulose (DE-52) from Whatman (Maidstone, Kent, UK). Egg yolk phospholipids (EYP) were prepared as described in Ref. [26].

Buffer A was 70 mM Tris–HCl, pH 7.0, containing 1 mg/ml $C_{12}E_8$; buffer B was 70 mM Tris–HCl, pH 7.0, containing 1 mg/ml $C_{12}E_8$ and 1 mg/ml DMPC; eluent A was 200 mM NaCl, 50 mM D-glucose, 3 mM NaN_3 and 10 mM sodium phosphate, pH 7.2; and eluent B was 150 mM NaCl, 1 mM Na_2EDTA and 10 mM Tris–HCl, pH 7.4. The pH values were measured at 23 °C.

2.2. Phosphorus analysis, protein assays and amino acid analysis

We determined phosphorus amounts after sulfuric-acid oxidation essentially according to Bartlett [27]. Protein assays were chosen depending on the components in the sample. (a) We determined the protein amount in the GLUT1 sample prepared by anion-exchange chromatography (Section 2.3) by a micro-Bradford assay with calcium phosphate adsorption and ethanol precipitation (CaPE) [28] to eliminate the effects of endogenous lipids and $C_{12}E_8$. For this assay we solubilized proteoliposomes by mixing 30 μl of proteoliposome sample with 70-μl 10% (v/v) Triton X-100. Reagent controls with liposomes showed the same results as without liposomes. (b) For GLUT1 prepared by sulfhydryl-affinity chromatography (Section 2.4), we used the original micro-Bradford assay [29] due to the low amount of endogenous lipids and $C_{12}E_8$. (c) The protein of immobilized proteoliposomes, SDS-eluted protein from the pCMB-Sepharose 4B gel and the protein remaining bound on the gel after SDS elution were quantitated by hydrolysis with 6 M HCl for 24 h followed by automated amino acid analysis. The data were refined as in Ref. [13].

2.3. Preparation of membrane vesicle, A-GLUT1 and A-GLUT1$_{(DTE)}$

We prepared red blood cell membranes from cell concentrates (stored 5–7 weeks after blood donation) obtained from the Blood Bank at the University Hospital, Uppsala, Sweden, and stripped off the cytoskeleton at pH 10.5 and pH 12 as described in Ref. [8], except that no reductant was included in the pH-12 solution. We solubilized thawed vesicles for 20 min on ice by use of 22 mg/ml $C_{12}E_8$ in 70 mM Tris buffer, pH 7.4, at 8 mg protein/ml, centrifuged the material at 160,000 × *g* for 1 h to obtain a supernatant containing up to 65% by weight of the vesicle protein, applied the supernatant onto a DEAE-cellulose column equilibrated with buffer A (5 mg protein/ml gel bed) and collected the break-through material, the anion-exchange-purified GLUT1 (A-GLUT1).

We also purified GLUT1 from red blood cell membrane vesicles prepared as described previously with 0.2 mM DTE in the pH 12-solution for cytoskeleton stripping [8], but omitted DTE from the solubilization mixture and from the buffer for anion-exchange chromatography. This preparation was denoted A-GLUT1$_{(DTE)}$. The chromatographic purifications were performed at 5 °C.

2.4. Preparation of S-GLUT1 and S-GLUT1$_{(DTE)}$

In order to synthesize pCMB-Sepharose 4B essentially as in Refs. [9,24], we suspended freeze-dried CNBr-activated Sepharose 4B gel (15 g) in 1 mM HCl, washed it on a Büchner funnel with 3 l of 1 mM HCl for 15 min, mixed the gel with a chilled solution of 19 ml of ethylenediamine

and 34 ml of 0.1 M $NaHCO_3$, adjusted the pH to 8.9 with 12 M HCl, shook the suspension gently overnight at 4 °C, washed the synthesized aminoethyl-Sepharose 4B with 4 l of chilled water on a Büchner funnel, resuspended the gel in 27 ml of 40% dimethylformamide at 23 °C, supplemented the suspension with 0.3 g of pCMB, adjusted the pH to 4.8 by addition of 5-μl aliquots of 2 M HCl during 1 h, transferred the suspension to a bottle for gentle shaking overnight, decanted the gel from pCMB particles six times and washed the gel on a Büchner funnel during 6 h with 4 l of 0.1 M $NaHCO_3$–HCl, pH 8.9, and finally with 1-l water. The addition of dimethylformamide and the subsequent procedures were repeated once. The pCMB-Sepharose 4B gel was stored at 4 °C in 0.2 mg/ml NaN_3 and bound approximately 100 mg of human hemoglobin per milliliter of sedimented gel, similarly as reported in Ref. [30]. The binding capacity was retained for at least 2 years.

We packed the pCMB-Sepharose 4B in a 0.5 cm (i.d.) × 5 cm HR glass column up to 2 mm below the end of the central part of the tube (the threaded ends were slightly wider) with the outlet of the tubing 6 cm below the buffer surface, rinsed with 70 mM Tris–HCl, pH 7.4, gently inserted the end adaptor down to the gel surface without locking the screw, and equilibrated with buffer A at a flow rate of 0.1 ml/min. The loose packing of the gel bed and the low flow rate favored the binding of protein. We applied A-GLUT1 or A-$GLUT1_{(DTE)}$, washed with buffer A or B until a stable absorbance baseline was reached and eluted the bound material with buffer A or B supplemented with 0.2% (v/v) 2-mercaptoethanol to obtain sulfhydryl-affinity-purified GLUT1; namely, S-GLUT1 or S-$GLUT1_{(DTE)}$, respectively. The chromatographic purifications were performed at 5 °C. We found 70–90 mM Tris–HCl at pH 7.4–7.8 suitable for A-GLUT1 binding and elution, whereas A-GLUT1 bound in 5 mM phosphate buffer, pH 8.0, could not be eluted by 2-mercaptoethanol in either the phosphate buffer or buffer B.

2.5. SDS-PAGE

We ran 12-μl aliquots of protein samples (10 μl) mixed with an equal volume of 2× sampling buffer containing 0.125 M Tris–HCl, pH 6.8, 20% (v/v) glycerol, 4% (w/v) SDS and 0.005% (w/v) bromophenol blue, on 4–12% Tris–glycine polyacrylamide gels (1.0-mm thick, 15-well) for 1.5 h at 125 V with a running buffer of 25 mM Tris base, 192 mM glycine and 1 mg/ml SDS. The wide-range protein standard Mark12 was applied. No DTE was added to the sample mixture. The SDS-PAGE materials were obtained from Novex (San Diego, CA, USA). The gel was fixed by an acetone solution containing formalin, sensitized by use of sodium thiosulfate, stained with silver nitrate and developed by use of sodium carbonate followed by addition of acetic acid [31].

2.6. In-gel digestion and mass spectrometry

In order to identify the GLUT1 samples, we excised the stained area of band 4.5 in the SDS-PAGE gels, which showed patterns similar to those illustrated in Fig. 1, and treated the gel piece for in-gel digestion as described [25]. Briefly, the gel pieces were washed with ammonium bicarbonate buffer in acetonitrile, dried, and a trypsin solution was allowed to be absorbed into the re-swelling gel. After overnight incubation and acidification, the generated peptides were extracted and analyzed by Peptide Mass Fingerprinting in the MALDI-ToF-MS instrument (Bruker Autoflex, Bruker Daltonics, Bremen, Germany).

Samples for MALDI were prepared by the "dried droplet" technique, using α-cyano 4-hydroxycinnamic acid as matrix. The instrument was optimized for peptide analysis and calibration was performed internally using autolytic peptides from the porcine trypsin used.

We searched for protein identity with the determined peptide masses in ProFound against a non-redundant protein sequence database (NCBInr).

2.7. Reconstitution and immobilization

We applied a mixture of EYP solution (1.5 ml) and GLUT1 sample (2.5 ml) onto a 2 cm (i.d.) × 35 cm Superdex 75 gel bed as described in Ref. [20] in order to reconstitute the protein. For glucose transport measurements, we used eluent A as the reconstitution buffer and collected the frontal half of the proteoliposome zone, froze 200-μl aliquots of the proteoliposome suspension in polypropylene tubes at −23 °C in a temperature-controlled

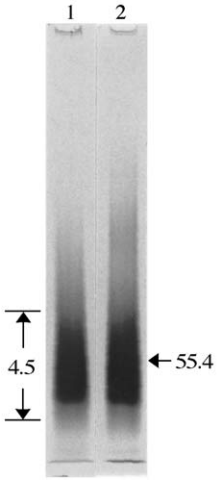

Fig. 1. SDS-PAGE of GLUT1: A-$GLUT1_{(DTE)}$ (lane 1) and S-$GLUT1_{(DTE)}$ (lane 2). A-$GLUT1_{(DTE)}$ was purified by anion-exchange chromatography and S-$GLUT1_{(DTE)}$ was prepared by sulfhydryl-affinity chromatography of A-$GLUT1_{(DTE)}$ on pCMB-Sepharose 4B (see Fig. 2). For details, see Section 2. The band 4.5 is marked, which is known to contain GLUT1 and NT [8]. The arrow indicated the position of the marker protein glutamic dehydrogenase (55.4 kDa). Approximately 1.5-μg protein was loaded to each well.

water/glycol bath, stored the material at −20 °C overnight and thawed aliquots in a water bath at 25 °C 10 min before use. For frontal affinity analysis, eluent B was the reconstitution buffer and the whole zone was collected and approximately 16-fold concentrated in a Minicon B15 cell (Millipore, Bedford, MA, USA); immobilization of proteoliposomes and vesicles in Superdex 75, packing and washing of the gel bed were essentially as described in Ref. [20].

2.8. Glucose transport measurements

For determination of the equilibrium-exchange of D-glucose, we mixed proteoliposomes (150 μl) at 22 °C with eluent A (75 μl) supplemented with D-[^{14}C]glucose and L-[^{3}H]glucose, incubated for 0, 40, 60 and 120 s, respectively, added 75 μl of eluent A with 10 mM $HgCl_2$ to stop the transport and injected the sample from a 200-μl loop into a 0.5 cm (diam.) × 18 cm gel bed of Superdex 75 for size-exclusion separation of proteoliposomes from free radioactive glucose [20]. Leakage was compensated for as described in Ref. [32]. The difference between the uptake of D- and L-glucose (mmol g^{-1} s^{-1}) in the proteoliposomes represents the D-glucose transport.

2.9. Frontal affinity chromatographic analysis of inhibitor binding

For frontal analysis of inhibitor binding to membrane proteins in immobilized proteoliposomes or vesicles in a 0.5 cm (i.d.) × 5 cm HR glass column, we connected the column to a 50-ml Superloop (Amersham Biosciences) and an on-line flow-scintillation detector (Radiomatic FLO-ONE 500TR, Packard Instruments, Meriden, CT, USA) [11,15]. Before each run we equilibrated with 10 column volumes of the sample solution without inhibitor and then applied series of large-volume samples: (a) 1 − 150 nM CB including 1 nM [^{3}H]CB, (b) 1 nM [^{3}H]CB in the presence of 0–150 mM D-glucose, (c) 0.1 − 10 nM NBTI containing 0.1–0.4 nM [^{3}H]NBTI and (d) 0.4 nM [^{3}H]NBTI in the presence of 0 − 10 mM adenosine at a flow rate of 1 ml/min. The CB binding to GLUT1 and the binding of NBTI to both NT and GLUT1 were evaluated as described in Refs. [11,15].

3. Results

3.1. Purification of the glucose transporter GLUT1

A-GLUT1$_{(DTE)}$ (Fig. 1, lane 1) and A-GLUT1 (not illustrated), obtained by anion exchange chromatography of solubilized red blood cell membranes that had been stripped of cytoskeletal proteins in the presence of 0.2 mM DTE and without reductant, respectively, both showed the broad major band 4.5 representing the heterogeneously glycosylated monomeric transporter in SDS-PAGE. A-GLUT1$_{DTE}$, obtained by anion-exchange chromatography in the presence of 1 mM DTE of solubilized red blood cell membranes depleted of their cytoskeleton at high pH in the presence of 0.2 mM DTE, showed a similar SDS-PAGE pattern [8].

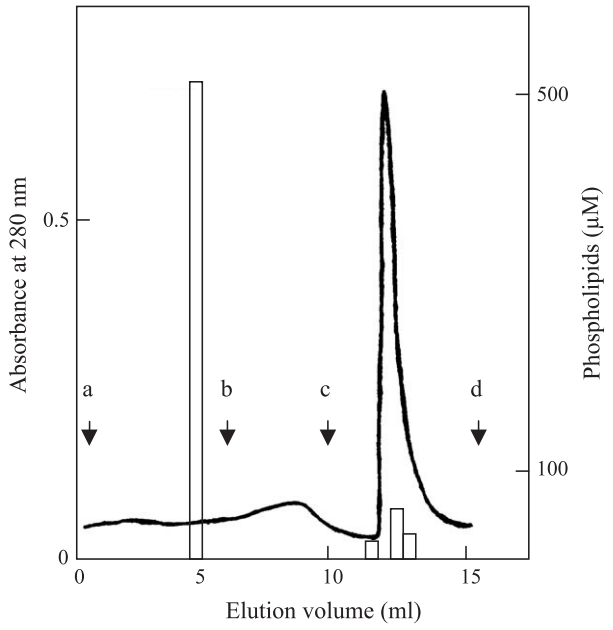

Fig. 2. Sulfhydryl-affinity chromatography of A-GLUT1$_{(DTE)}$ on pCMB-Sepharose 4B: a–b, application; b–c, washing with buffer A; c–d, elution of S-GLUT1$_{(DTE)}$ with buffer A containing 0.2% (v/v) 2-mercaptoethanol. The bars show the concentration of endogenous phospholipids. The part of the S-GLUT1$_{(DTE)}$ zone of absorbance above 0.2 was collected.

Following application of A-GLUT1$_{(DTE)}$[1] or A-GLUT1 onto a pCMB-Sepharose 4B gel bed, we washed away endogenous lipids and released S-GLUT1$_{(DTE)}$ (Fig. 2) or S-GLUT1, respectively, by use of 2-mercaptoethanol. These materials contained 7 and 30 endogenous phospholipid molecules, respectively, per GLUT1 monomer, whereas the corresponding value for A-GLUT1 was 180. The concentration of the eluted S-GLUT1$_{(DTE)}$ was up to 0.5 mg/ml. The S-GLUT1$_{(DTE)}$ (Fig. 1, lane 2) and S-GLUT1 (not illustrated) showed the same pattern on SDS-PAGE as did the anion-exchange purified materials.

MALDI-ToF-MS identified tryptic fragments of GLUT1 (Table 1). Fragments in the hydrophilic loops connecting transmembrane helices 3 and 4 and transmembrane helices 6 and 7 of both S-GLUT1 and S-GLUT1$_{(DTE)}$ were detected. Parts of the C-terminal polypeptides were also identified for both preparations. The complete transmembrane helices 1 and 8 were found for S-GLUT1 and A-GLUT1, but not for S-GLUT1$_{(DTE)}$ or A-GLUT1$_{(DTE)}$. No fragments from other

[1] The residual DTE in the vesicle suspension (approximately 5 μM after cytoskeleton-depletion in the presence of 0.2 mM DTE and washing once with Tris-HCl buffer [8]) was diluted two-fold upon anion exchange chromatography to obtain A-GLUT1$_{(DTE)}$ and did not affect the binding of A-GLUT1$_{(DTE)}$ to pCMB-Sepharose 4B significantly.

Table 1
Fragments in MALDI-ToF-MS of tryptic GLUT1[a]

S-GLUT1$_{(DTE)}$		S-GLUT1	
Residues	Position	Residues	Position
118–126	L3–4	12–38	H1
256–264	L6–7	118–126	L3–4
257–264	L6–7	233–249	L6–7
459–468	C terminus	256–264	L6–7
478–492	C terminus	301–330	H8
		459–492	C terminus

[a] The locations of the fragments in the amino acid sequence of GLUT1 are given. H1 is the transmembrane α-helical domain of GLUT1 [1], etc. L3–4 is the hydrophilic loop between the two transmembrane domains 3 and 4, etc. The C terminus is cytoplasmic.

membrane proteins than GLUT1 were identified by MALDI-ToF-MS in our GLUT1 preparations. However, amino acid analyses indicated that the A- and S-GLUT1 preparations were slightly impure. Minor contaminants are NT, hemoglobin and glyceraldehyde-3-phosphate dehydrogenase. Unknown proteins may be present in the preparations.

Eighty percent of the A-GLUT1 bound in the pCMB-Sepharose 4B gel bed could be released by 5% SDS, but not with 0.5% SDS (Fig. 3A). SDS presumably released noncovalently bound monomers in the oligomer. The material that did not elute with 5% SDS was presumably bound by -S–Hg^{2+}- bridges and could be released with 2-mercaptoethanol (Fig. 3B, lower curve). The result indicates that A-GLUT1, on the average, formed pentamers. The amount of S-GLUT1 initially bound in the pCMB-Sepharose 4B gel bed is also shown in Fig. 3B (top curve). A larger fraction of the bound protein was released by SDS at the top part of the gel bed than at the lower part, indicating that large oligomers were preferentially bound at the top of the gel bed by -S–Hg^{2+}- bridges linking a single monomer of each oligomer to the gel.

3.2. Affinity of and binding sites for inhibitors on red blood cell membrane vesicles, A-GLUT1 and S-GLUT1

Immobilized S-GLUT1 and A-GLUT1 showed indistinguishable dissociation constants of inhibitors and substrates, similar to those of A-GLUT1$_{DTE}$, except for the K_d for adenosine binding on GLUT1 (Table 2). However, the number of CB-binding sites per A-GLUT1 or S-GLUT1 monomer was dramatically lower than that for vesicles or A-GLUT1$_{DTE}$ (the latter according to data from Ref. [11]) (Table 3). S-GLUT1$_{(DTE)}$ purified from the partially solubilized membrane vesicles stripped with alkaline solution containing 0.2 mM DTE showed no significant CB-binding activities upon analysis by frontal affinity chromatography. The molar ratio of NBTI-binding sites on the minor impurity, NT, over the total amount of S-GLUT1 protein (assuming a molecular weight of 54,117) was 1/7 of the corresponding ratio for A-GLUT1 (Table 3) and 1/36 of that for A-GLUT1$_{DTE}$ (the latter according to data from Ref. [11]). The A- and S-GLUT1 showed a number of NBTI binding sites per GLUT1 monomer that was 50% and 30%, respectively, of the number of NBTI binding sites per A-

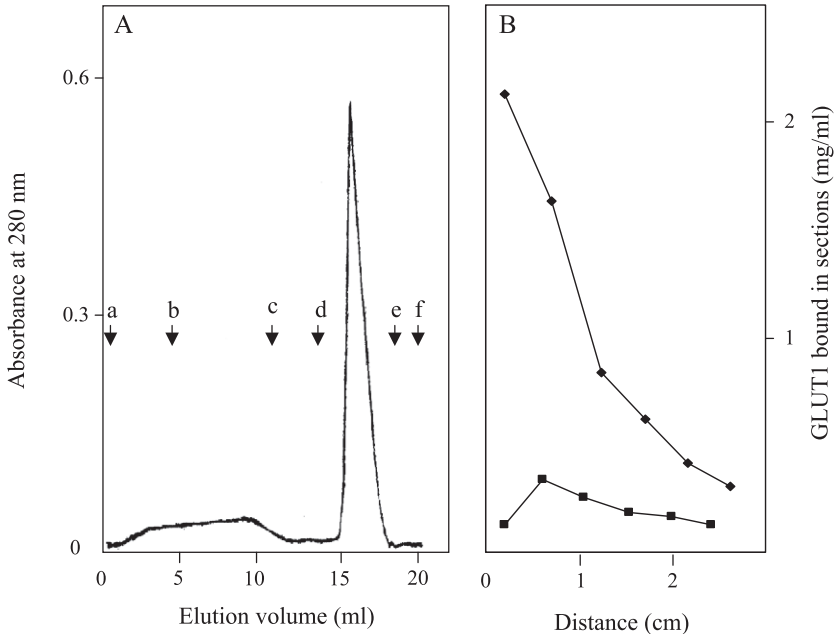

Fig. 3. Partial elution by SDS washing of A-GLUT1 bound on pCMB-Sepharose 4B. (A) a–b, application; b–c, washing with buffer B; c–d, washing with buffer B containing 0.5% SDS; d–e, elution with buffer B containing 5% SDS warmed to 30 °C to keep the SDS in solution; e–f, washing with buffer A. (B) Quantitation of the amount of A-GLUT1 bound on pCMB-Sepharose 4B and of the amount remaining bound after SDS washing. We applied A-GLUT1 until the protein began to elute, washed the column either with buffer A alone (♦) or with buffer B containing 5% SDS followed by buffer A (■) as in Fig. 3A, sectioned the gel beds by Pasteur pipetting and released the bound GLUT1 by 2% 2-mercaptoethanol in buffer A. The protein amounts were quantitated by the original micro-Bradford assay [29]. The *x*-axis shows the distance in the column from the top of the gel bed.

Table 2
Dissociation constants of CB, glucose (Glc), NBTI and adenosine (Ad) interacting with GLUT1 or NT in red blood cell membrane vesicles and reconstituted GLUT1 preparations (proteoliposomes), all immobilized in Superdex 200 and analyzed by frontal affinity chromatography

Preparation	CB-GLUT1 (nM)	Glc-GLUT1 (mM)	NBTI-NT (nM)	Ad-NT (nM)	NBTI-GLUT1 (nM)	Ad-GLUT1 (mM)
A-GLUT1	38	20	0.35	24	43	4.6
S-GLUT1	35	30	0.31	23	55	8.2
A-GLUT1$_{DTE}$[a]	61	41	0.38	21	79	0.2
V[b]	29	26	0.15	Nd[c]	–[d]	–[d]
V_{DTE}[e]	59	18	0.16	Nd[c]	–[d]	–[d]

Estimated relative error limit: ± 15%.
[a] A-GLUT1$_{DTE}$, data from Ref. [11].
[b] V, red blood cell membrane vesicles prepared without reductant.
[c] Nd, not determined.
[d] –, no measurable interaction.
[e] V_{DTE}, red blood cell membrane vesicles prepared with reductant, data from Refs. [13,21].

GLUT1$_{DTE}$ monomer [11] according to the data in Table 3. No NBTI-binding sites on GLUT1 in red blood cell membrane vesicles stripped without reductant could be detected, similarly as reported for vesicles stripped with reductant [33] and red blood cell ghosts [34,35]. For the above calculations, GLUT1 was assumed to represent 12% of the total protein amount in vesicles [13].

3.3. Glucose transport activity

The S-GLUT1 showed glucose transport activity after reconstitution (Fig. 4). The equilibrium exchange for S-GLUT1 eluted without added lipids at 50 mM glucose was 0.80 ± 0.02 mmol g^{-1} s^{-1} for 1-min incubation, similar to the reported values of 1.1 mmol g^{-1} s^{-1} for GLUT1 with a 20-s incubation [20] and 0.35 ± 0.04 and 0.43 ± 0.03 mmol g^{-1} s^{-1} without and with lipids, respectively, with a 2-min incubation [17].

Table 3
The number of binding sites of CB and NBTI per GLUT1 monomer in membrane vesicles and GLUT1 proteoliposomes

Preparation	NT-T[a]	CB-T[b]	NBTI-T[c]
A-GLUT1	0.0035	0.066	0.31
S-GLUT1	0.0005	0.028	0.19
A-GLUT1$_{DTE}$[d]	0.018	0.40	0.59
V[d]	0.018	0.41	–[e]
V_{DTE}[d]	0.029	0.51	–[e]

Estimated relative error limit: ± 15%.
[a] NT–T, the molar ratio of NBTI sites on NT over the total amount of protein molecules in the column when the molecular weight of all the protein was taken as 54117 (GLUT1).
[b] CB–T, the ratio of CB sites on GLUT1 over the total amount of protein molecules in the column when the molecular weight of all the protein was taken as 54117.
[c] NBTI–T, the ratio of NBTI sites on GLUT1 over the total amount of protein molecules in the column when the molecular weight of all the protein was taken as 54117.
[d] As described in Table 2.
[e] –, no measurable interaction.

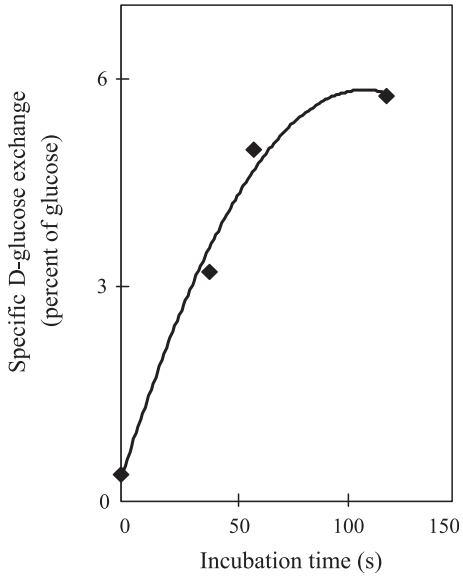

Fig. 4. Specific D-glucose equilibrium exchange (percent of 50 mM D-glucose) by S-GLUT1 reconstituted with EYP. The exchange of L-glucose has been subtracted. The protein concentration in the proteoliposomes was 46 μg/ml.

4. Discussion

In our study, no GLUT1 oligomers were observed on SDS-PAGE for GLUT1 in vesicles (not shown). However, treatment of membrane vesicles with reductant can convert the multiple oligomeric bands of the oxidized anion transporter, the major component of red blood cell membrane vesicles, into a single monomeric band on SDS-PAGE [36]. The partial solubilization without reductant of membrane vesicles stripped without or with reductant may be related to the cross-linking of anion transporters in the vesicles. If a protein appears simultaneously in different states in the vesicles, incomplete and selective solubilization of the vesicles would critically affect the characterization of the membrane protein.

Different association states of GLUT1 in the red blood cell membranes have been indicated in earlier studies [14,37–39]. The immunofluorescence staining of human red blood cells showed an uneven distribution of GLUT1 over the red blood cell membranes [37]. GLUT1 in red blood cell vesicles stripped with reductant was solubilized mostly as monomers by *n*-octyl *β*-D-glucopyranoside or $C_{12}E_8$ [17,40] and GLUT1 in red blood cell vesicles stripped without reductant was solubilized mostly as tetramers by cholate [14,18]. Multimers up to octamers with glucose transport activity have been observed by cholate solubilization of non-stripped human red blood cell ghosts [38]. Alkaline reduction caused conversion from oligomeric

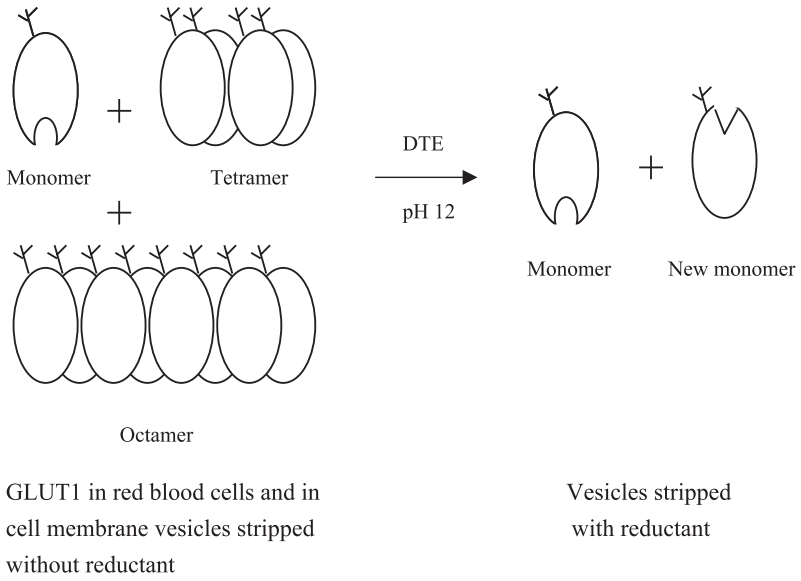

Fig. 5. Schematic illustration of hypothetical GLUT1 association states in red blood cells and red blood cell membrane vesicles stripped without reductant and in membrane vesicles stripped in the presence of reductant. The native monomeric GLUT1 represents 40% of the total GLUT1 molecules and the ratio of tetramers to octamers is 3:1. The monomers formed from the oligomeric GLUT1 do not bind CB significantly compared to the native GLUT1 monomers.

GLUT1 to dimeric [14,18] or monomeric GLUT1 [17,40]. In our study, the competitive inhibitor CB bound similarly, at a ratio of 0.4–0.5 sites per GLUT1 monomer, to GLUT1 in the vesicles stripped without and with reductant (Table 3). However, GLUT1 partially solubilized and purified without reductant from vesicles stripped with or stripped without reductant did not attain the CB-binding ratio of 0.4 reported previously for GLUT1 purified in the presence of reductant [13]. The ratio was found to be much lower for the $C_{12}E_8$-solubilized GLUT1 prepared without reductant (Table 3), which showed on the average pentamers to judge by SDS washing of GLUT1 bound on the pCMB gel bed (Fig. 3). The few but similarly active CB-binding sites in A-GLUT1 and S-GLUT1 proteoliposomes compared to A-$GLUT1_{DTE}$ proteoliposomes (Table 2) implied that there are different GLUT1 association states and that the partial solubilization was selective, i.e., GLUT1 in a conformation which did not favor CB binding was preferentially solubilized from vesicles without reductant and these GLUT1 molecules were natively oligomeric, averaged as pentamers according to our analysis. We further speculate that CB-binding GLUT1 monomers represented approximately 40% of the GLUT1 in the vesicles as judged by the CB binding of GLUT1 in the vesicles. Furthermore, it was harder to release this GLUT1 from the vesicles by the detergent compared to the oligomeric GLUT1. The oligomeric GLUT1 converts to monomers by alkaline reduction, but this monomeric GLUT1 does not necessarily show CB-binding activity, since vesicles stripped without reductant did not differ significantly from the vesicles stripped with reductant concerning the CB binding site/GLUT1 monomer ratio [13]. MALDI-ToF-MS revealed differences among the detected tryptic fragments obtained from S-$GLUT1_{(DTE)}$, which may consist of newly produced monomeric GLUT1, and from S-GLUT1, which probably consists of native oligomeric GLUT1 (Table 1). The number of phospholipids per S-GLUT1 monomer of the oligomeric material was 30, similar to the value 26 reported in Ref. [14] for GLUT1 oligomers, i.e., more than the value 7 for the S–$GLUT1_{(DTE)}$.

We propose a scheme for the oligomeric states of GLUT1 in red blood cells or vesicles stripped without reductant and in vesicles stripped with reductant (Fig. 5). The native GLUT1 state might be a mixture of monomers, tetramers, octamers and possibly other forms, in which the monomers constitute approximately 40% of the total amount of GLUT1. In this scheme, we combine the previously observed monomeric and oligomeric states. The discrepancy between this scheme and the proposition that GLUT1 in the membranes is tetrameric [18] may be explained by the selective partial solubilization of the vesicles, i.e., mainly GLUT1 tetramers together with small amounts of monomers and other forms were solubilized. The biological importance of the putative multiple oligomeric states of GLUT1 is uncertain. They might be related to the age of the protein and/or provide a way to adjust the glucose transport activity. Such a multiplicity of GLUT1 oligomeric states not only complicates the characterization of GLUT1, but also hinders both three-dimensional and two-dimensional crystallization.

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