Glucose Transporter Oligomeric Structure Determines Transporter Function

REVERSIBLE REDOX-DEPENDENT INTERCONVERSIONS OF TETRAMERIC AND DIMERIC GLUT1*

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This study investigates the relationship between human erythrocyte glucose transport protein (GLUT1) oligomeric structure and glucose transporter function. Oligomeric structure was analyzed by hydrodynamic studies of cholate-solubilized GLUT1, by chemical cross-linking studies of membrane-resident GLUT1 and by using conformation-specific antibodies. Transporter function (substrate binding) was analyzed by equilibrium cytochalasin B and D-glucose binding measurements. Erythrocyte-resident glucose transporter is a GLUT1 homotetramer, binds 1 mol of cytochalasin B/2 mol of GLUT1, and presents at least two binding sites to D-glucose. Native structure and function appear to be stabilized by intramolecular disulfide bonds and are preserved during GLUT1 purification by the omission of reductant. Native structure is independent of in vitro and in vivo membrane GLUT1 density but is transformed to dimeric GLUT1 by alkaline reduction. Dimeric GLUT1 binds 1 mol of cytochalasin B/mol of GLUT1, presents a single population of binding sites to D-glucose, and is obtained upon GLUT1 purification in the presence of reductant. Native structure and function are restored by treatment of dimeric GLUT1 with glutathione-disulfide (K_{d} glutathione disulfide = 29 μM). We propose that native structure is established prior to transporter translocation to the plasma membrane and that intrasubunit disulfide bonds promote cooperative subunit interactions that stabilize transporter structure and function.

A family of integral membrane proteins called glucose or sugar transporters mediates the facilitated diffusion of sugars across cell membranes. Neither the subunit composition of these transporters nor the mechanism by which these transporters mediate bidirectional transmembrane sugar movements is known.

Recent transport studies with human erythrocytes (cells in which the sugar transporter isoform GLUT1 mediates sugar transport) support the view that the glucose transporter can bind sugars at sugar influx and at sugar efflux sites simultaneously (1, 2). These results are consistent with a two-site mechanism for sugar transport (3, 4). Ligand binding studies of erythrocyte-resident GLUT1 substantiate this view (5, 6). However, similar studies with purified erythrocyte GLUT1 have resulted in mutually incompatible conclusions. Some studies suggest that the glucose transporter can bind two or more sugar molecules simultaneously (7), whereas others (8–10) propose that the transporter presents only a single sugar transport site (influx or efflux) at any instant. The latter result is consistent with a one-site mechanism for sugar transport (11).

Target size analyses suggest that the cytochalasin B binding and the sugar transport competent components of the human red cell sugar transporter exist as 124- or 220-kDa structures (12, 13). Hydrodynamic studies suggest that the detergent-solubilized glucose transporter is a monomeric, dimeric, or tetrameric assembly of GLUT1 proteins (14–16).

In this study we ask two questions: 1) Why does the glucose transporter exist as multiple oligomeric forms? 2) How can the ligand binding properties of purified GLUT1 be consistent with two mutually incompatible mechanisms for sugar transport? In our efforts to answer these questions we have found that the functional properties of GLUT1 are determined by GLUT1 oligomeric structure and that different laboratories have, unknowingly, studied different oligomeric forms of GLUT1.

EXPERIMENTAL PROCEDURES

Materials—[3H]cytochalasin B, [3H]cholic acid, and 125I-protein A were purchased from Du Pont-New England Nuclear. Rabbit antisera raised against a synthetic carboxyl-terminal peptide of GLUT1 (intracellular residues 480–492; C-IgGs) were obtained from East Acres Biologicals. Anti-GLUT1 antisera reacting exclusively with extracellular epitopes of GLUT1 (6-IgGs) were prepared as described previously (17). Outdated human blood was obtained from the University of Massachusetts Medical Center Blood Bank. Reagents were purchased from Sigma. Plasma membranes from Chinese hamster ovary

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1 The abbreviations used are: GLUT1, erythrocyte glucose transporter; CHO, Chinese hamster ovary; C-IgG, rabbit IgGs raised against a carboxyl-terminal peptide of GLUT1; S-IgG, rabbit IgGs raised against GLUT1-DTT; MOPS, 4-morpholino-propanesulfonic acid; DTT, dithiothreitol; ELISA, enzyme-linked immunosorbent assay; GLUT1+DTT, GLUT1 purified in the presence of DTT; GLUT1–DTT, GLUT1 purified in the absence of DTT; PAGE, polyacrylamide gel electrophoresis; SEC-HPLC, size exclusion chromatography-high performance liquid chromatography; SG, erythrocyte membranes depleted of peripheral proteins; SGpH, SG exposed to pH 12 medium; SG+DTT, SG exposed to DTT at neutral pH; SGpH+DTT, SG exposed to DTT at pH 12.
GLUT1 Oligomeric Structure Determines GLUT1 Function

(CHO) cells and from 3T3-L1 adipocytes were generously provided by Drs. A. Pessino, S. A. Harrison, and M. P. Czech.

**Solutions**—Saline consisted of 150 mM NaCl, 5 mM Tris-HCl, 0.2 mM EDTA, pH 7.4. Lysis medium consisted of 10 mM Tris-HCl, 2 mM EDTA, pH 7.4. Tris medium consisted of 50 mM Tris-HCl, 0.2 mM EDTA, pH 7.4. All alkaline wash medium contained 2 mM EDTA adjusted to pH 12.0 using NaOH. Size exclusion-HPLC column buffer and sucrose gradient medium consisted of 150 mM NaCl, 5 mM MOPS, 20 mM choline, ± 10 mM dithiothreitol, ± 0.1% SDS, pH 7.2.

**Red Cells and Red Cell Ghosts**—Red cell ghosts were prepared from washed, intact red cells as in (16). Red cell ghosts were depleted of peripheral membrane proteins by a single wash in 5 volumes of alkaline wash medium (4 °C, 20 min). Membranes were collected by centrifugation and resuspended in 10 volumes of Tris medium. These membranes were subjected to three additional wash/centrifugation cycles in Tris medium, adjusted to 4 mg of membrane protein/ml, and stored at −70 °C.

In some experiments, red cell membranes containing only integral membrane proteins were exposed to Tris medium containing GSSG/GSH buffers. In all instances, GSH was present at 2 mM. In some cases DTT (5 mM) was also present. All solutions were adjusted to pH 7.4 (20 °C). Membranes were incubated in the presence of GSSG/GSH buffers for 1 h at 37 °C. They were collected by centrifugation for 5 min at 14,000 × g and washed three times in 30 volumes of Tris medium.

**Glucose Transport Protein**—GLUT1 plus endogenous lipid were purified from human erythrocytes as described in (18). GLUT1 was also purified by omitting DTT (5 mM) from the alkali extraction step, the solubilization step, and during application of solubilized proteins to the DEAE-cellulose column (16, 19).

**Lectin Chromatography**—GLUT1 plus endogenous lipid were purified from human erythrocytes as described in (18). GLUT1 was also purified by omitting DTT (5 mM) from the alkali extraction step, the solubilization step, and during application of solubilized proteins to the DEAE-cellulose column (16, 19).

**ELISA—ELISA and competition ELISA were performed using C- and/or K IgGs as described in (17, 20).**

**Quantification of Membrane-resident GLUT1 by Immunoblot Analysis**—In some experiments it was necessary to quantitate erythrocyte membrane GLUT1 content. Membranes were collected by centrifugation and resuspended in 50 mM Tris-HCl, pH 7.4. Aliquots (5, 10, and 20 µl) were subjected to Western analysis (C-IgGs and K-IgGs A were the reporter molecules), and 0.40 µg of purified GLUT1-DTT or GLUT1+DTT was used as a standard. Blots were subjected to autoradiography and the resulting autoradiograms quantitated by scanning densitometry. The GLUT1 contents of the membranes were computed by comparison with the standard.

**Determination of GLUT1 Monomer**—Solubilized GLUT1 was purified by omitting DTT (5 mM) from the alkali extraction step, and exhaustively dialyzed to remove traces of DTT. GLUT1 was sedimented by ultracentrifugation through 50% sucrose (16, 20). After 5 min at 14,000 × g, the supernatant was removed, and GLUT1 was purified by centrifugation through 50% sucrose (16, 20).

**Characterization of GLUT1**—Two procedures were used to purify human erythrocyte GLUT1. The standard procedure includes dithiothreitol during solubilization and/or ion exchange chromatography of solubilized erythrocyte membrane proteins (18, 23). We refer to GLUT1 purified in this way as GLUT1+DTT. The second procedure omits dithiothreitol from all steps of the purification. This form of GLUT1 is termed GLUT1−DTT.

Both GLUT1+DTT and GLUT1−DTT copurify with red cell phospholipids at a lipid:protein molar ratio of approximately 350:1. This represents a 2.5-fold decrease over the endogenous, red cell phospholipid:GLUT1 ratio and may reflect preferential solubilization of GLUT1 by octyl glucoside or preferential adsorption of red cell lipids during ion exchange chromatography. Total GLUT1 yield is unaffected by the omission of dithiothreitol during purification. Both GLUT1+DTT and GLUT1−DTT are resolved as bands of broad mobility and an average molecular mass of 55 kDa upon reducing or nonreducing SDS-PAGE (see Fig. 4 below).

The accessible free thiol contents of SDS-denatured GLUT1+DTT and GLUT1−DTT differ significantly. GLUT1+DTT contains 6.2 ± 0.2 free sulfhydryls/GLUT1 monomer, whereas GLUT1−DTT contains only 2.2 ± 0.1 free sulfhydryls/GLUT1 monomer (n = 3 in each instance). The...
lower free sulfhydryl content of GLUT1–DTT seems not to result from incomplete unfolding of GLUT1–DTT since GLUT1+DTT and GLUT1–DTT share indistinguishable mobilities in and quantitative penetrations of SDS-polyacrylamide gels and the Stokes radii of SDS-denatured GLUT1+DTT and GLUT1–DTT are identical (see Fig. 4 below). As GLUT1 contains 6 cysteines/monomer (24) and both GLUT1+DTT and GLUT1–DTT are resolved as monomers by reducing and nonreducing SDS-PAGE, these findings suggest that GLUT1–DTT could contain as many as two intramolecular disulfide bonds/monomer.

The ligand binding properties of GLUT1+DTT and GLUT1–DTT also differ. Cytochalasin B, a ligand that binds very close to the sugar efflux site of GLUT1 (2), binds to GLUT1+DTT with a molar stoichiometry approaching unity (0.82 ± 0.06 mol/mol of GLUT1; n = 4; Fig. 1). This, and all other similar calculations, are based upon an assumed GLUT1 molecular mass of 55,000. The cytochalasin B binding capacity of GLUT1–DTT is one-half of that of GLUT1+DTT (0.44 ± 0.02 mol/mol of GLUT1; n = 5; Fig. 1). The $K_{d(app)}$ for cytochalasin B binding to both preparations is indistinguishable (GLUT1+DTT, $K_{d(app)} = 230 ± 31$ nM; GLUT1–DTT, $K_{d(app)} = 190 ± 24$ nM). As GLUT1+DTT was resuspended in dithiothreitol-free medium immediately prior to the assay of cytochalasin B binding, these findings cannot result from a reversible interaction of dithiothreitol with GLUT1+DTT substrate binding sites. Moreover, since dithiothreitol appears to be a competitive inhibitor of cytochalasin B binding to GLUT1+DTT (5), the expected effect of dithiothreitol on cytochalasin B binding would be to increase $K_{d(app)}$ significantly, and this is not observed. The d-glucose binding properties of each preparation were monitored by analysis of d-glucose-induced GLUT1 intrinsic fluorescence quenching. Upon d-glucose interaction with GLUT1, the intrinsic tryptophan fluorescence of the protein is diminished (9). This provides a convenient means of titrating GLUT1 d-glucose binding sites and thereby obtaining $K_{d(app)}$ for d-glucose interaction with GLUT1 (9). As reported by Gorga and Lienhard (9), equilibrium d-glucose binding to GLUT1+DTT is characterized by single site kinetics (Fig. 2). The $K_{d(app)}$ for d-glucose-induced quenching of GLUT1+DTT intrinsic fluorescence is 12.6 ± 0.9 mM, and the maximum reduction in intrinsic fluorescence ($Q_{m}$) is 9.1 ± 0.4% (n = 4).

D-glucose interaction with GLUT1–DTT is, however, characterized by at least two components of binding (Fig. 2).

**Fig. 1.** Cytochalasin B (CCB) binding to purified GLUT1. GLUT1+DTT (●) and GLUT1–DTT (○) were resuspended in dithiothreitol-free Tris medium containing [3H]cytochalasin B (0.1–4 μM) to a final GLUT1 concentration of 10 μg/100 μl−1 (1.8 μM). Equilibrium ligand binding was measured at 20 °C. Binding data are presented in Scatchard form as mol of cytochalasin B bound per μM free cytochalasin B (ordinate) versus mol of cytochalasin B bound per mol of GLUT1 (abscissa). The straight lines drawn through the points were computed by the method of least squares. This figure pools data from seven separate experiments.

**Fig. 2.** D-Glucose binding to purified GLUT1. GLUT1+DTT (●) and GLUT1–DTT (○) were resuspended in dithiothreitol-free Tris medium to a final GLUT1 concentration of 250 μg/2.5 ml−1 (1.8 mM). The larger panel shows d-glucose-induced quenching (ordinate; fractional fluorescence quenching) versus [d-glucose] (abscissa). The curves drawn through the points were computed by nonlinear regression assuming two saturable components of glucose binding. The computed fits are: GLUT1+DTT (●), $K_{d(app)} = 12.8 ± 0.9$ mM; maximum quenching ($Q_{m1} = 0.046 ± 0.002$; $Q_{m2} = 0.045 ± 0.002$). GLUT1–DTT (○), $K_{d(app)} = 1.7 ± 0.1$ mM; maximum quenching ($Q_{m1} = 0.042 ± 0.006$; $Q_{m2} = 0.043 ± 0.005$). Since GLUT1+DTT $K_{d(app)} = K_{d(app)}$, this indicates a single functional population of sugar binding sites. The inset shows the same data presented in Scatchard form as fractional quenching/mM d-glucose (ordinate) versus fractional quenching (abscissa). The curves drawn through the points are the theoretical curves obtained from the nonlinear regression fits described above. This figure pools data from 14 experiments.

These are resolved by iterative, nonlinear regression analysis (assuming two saturable sites) as $K_{d(app)} = 1.7 ± 0.1$ mM, $Q_{m1} = 4.2 ± 0.6$% and $K_{d(app)} = 25.3 ± 0.8$ mM, $Q_{m2} = 4.8 ± 0.5$% (n = 14). The computed fits to these data are not significantly improved if three or more saturable sugar binding sites are assumed to be present. Since fluorescence quenching studies were performed using GLUT1 preparations washed three times in and finally resuspended in dithiothreitol-free medium, these findings cannot result from a reversible interaction of substrate binding sites with reductant.
two chromatographic profiles of GLUT1-DTT the time in min. Three data sets are shown and have been displaced in GLUT1+DTT curves (closed circles) show two chromatographic profiles of GLUT1 solubilized from erythrocytes following treatment of cells with or in the presence of saline (open triangles), 10 mM DTT (closed triangles), 1 mM N-ethylmaleimide (open diamonds), or 25 mM iodoacetic acid (closed diamonds). Flow rate was 0.5 ml/min. The column was calibrated using Pharmacia LKB Biotechnology Inc. high and low molecular weight gel filtration calibration kits. Stokes radius (nm) is obtained as $21.67-0.434 R_s$ (correlation coefficient $= 0.974$). The arrows indicate the retention times of 7.8, 6.0, and 3.9 nm (Stokes radius) particles. This figure includes data from nine experiments and is representative of 36 separate column runs.

The partial specific volumes of these particles is obtained by comparing their sedimentation profiles in sucrose gradients formed in $H_2O$ and $D_2O$ with those of standard proteins of known sedimentation velocities and partial specific volumes (21). Table I summarizes the results of these analyses.

Computation of the GLUT1 contents of these particles requires quantitation of the lipid and the cholic acid contents of GLUT1-containing micelles. Since SEC-HPLC of GLUT1 requires the use of small quantities of GLUT1 to achieve high resolution, it was not possible to accurately measure the lipid and cholate contents of distinct species of micelles eluting from the HPLC size exclusion column. The approach we adopted was to adsorb cholate-solubilized GLUT1 (1 mg of protein) to a lentil lectin column followed by analysis of the cholate and lipid contents of GLUT1 micelles subsequently eluted from the column by 0.5 M $N$-acetylglucosamine. These determinations of GLUT1 micelle detergent and lipid contents thus reflect an average of all particles eluted by $N$-acetylglucosamine.

The high critical micellar concentration of cholate and the low cholate content of GLUT1 micelles frustrate precise measurements of micellar cholic acid content. These measurements require subtraction of a substantial background of $[^3H]$cholate associated with the GLUT1-free column buffer. The cholic acid content of $N$-acetylglucosamine-displaced GLUT1-DTT and GLUT1-DTT micelles is $0.89 \pm 0.42$ nmol of cholic acid/µg of GLUT1 (S.E.; $n = 27$; molar ratio $= 49 \pm 231$; range $= -0.8$ to 2.9 nmol of cholic acid/µg of GLUT1). Unpaired two-tailed t tests of these data indicate that the results are significantly different from background ($t = 2.265$, $p = 0.03$, degrees freedom $= 31$). The phospholipid content of GLUT1-containing micelles is 26 ± 2 mol/mol of GLUT1 (16).

Using these data we calculate that GLUT1 purified in the presence of dithiothreitol (GLUT1-DTT) is solubilized largely as a GLUT1 dimer while GLUT1 purified in the absence of dithiothreitol (GLUT1-DTT) is solubilized largely...
as a GLUT1 tetramer (Table I).

The preceding computations of micellar cholic acid content are supported indirectly by analyses of the hydrodynamic properties of protein-free cholate/lipid micelles released upon solubilization of GLUT1. The Stokes radius, sedimentation coefficient, and partial specific volumes of these particles are 3.87 ± 0.06 nm \((n = 18)\), 1.9 ± 0.1 S \((n = 4)\), and 0.85 ± 0.07 cm\(^3\)g\(^{-1}\) \((n = 4)\), respectively. The partial specific volume of the micelle is that expected for a particle containing cholate and lipid at a molar ratio of 2:1 (25, 26). The computed mass of these micelles is 54,600 g, a value expected for a micellar cholate/lipid molar content of 62:31. These ratios are similar to those observed for cholate/lipid/GLUT1 micelles (49 ± 23:26 ± 2) and are consistent with the observation that membrane resident GLUT1 is solubilized maximally by cholic acid when the cholate:phospholipid molar ratio is 2:1 (16). It is possible, therefore, that cholate solubilization of GLUT1 results from the release of GLUT1 surrounded by a cholate-stabilized annulus of lipid bilayer. If this hypothesis is correct, the upper limit of the diameter of the membrane-embedded portion of a cylindrical, monomeric GLUT1 molecule can be estimated from Equation 1

\[
\frac{26 + 2}{\pi} \sqrt{\frac{A}{\pi}} = 33 \pm 3 \text{ Å} \tag{Eq. 1}
\]

where \(A\) is the molecular surface area of a phospholipid (50 Å\(^2\); (27)).

**How Do Hydrodynamic Studies Relate to Bilayer Resident GLUT1?**—The preceding demonstrate that GLUT1 released from GLUT1 proteoliposomes upon cholate solubilization exists in dimeric and tetrameric (and possibly monomeric) forms. These data do not demonstrate whether these forms represent those present in the bilayer prior to solubilization.

We approached this question by performing cross-linking studies of bilayer resident GLUT1 using the cross-linking agent glutaraldehyde. GLUT1+DTT and GLUT1–DTT proteoliposomes were treated with and without glutaraldehyde (up to 16 mM) and then resolved by SDS-PAGE and by SEC-HPLC under nondenaturing (solubilized in cholate) and denaturing (solubilized in SDS) conditions. The Stokes radii of cholate/lipid/GLUT1 micelles are unaffected by prior treatment of either GLUT1+DTT or GLUT1–DTT with glutaraldehyde (Fig. 4).

If these micelles are chromatographed under denaturing conditions (SDS-SEC-HPLC or SDS-PAGE), non-cross-linked GLUT1+DTT and GLUT1–DTT are resolved as a single species (monomer) in the presence of SDS, whereas glutaraldehyde-treated GLUT1+DTT is resolved as monomers and dimers and glutaraldehyde-treated GLUT1–DTT is resolved as monomers, dimers, and tetramers (Fig. 4). These findings suggest very strongly that nondenaturing SEC-HPLC of cholate-solubilized GLUT1 provides a snapshot of the oligomeric state of bilayer-resident GLUT1 prior to solubilization. Similar results have been reported for detergent-solubilized band 3 protein in erythrocytes (28).

We were unable to cross-link GLUT1 quantitatively using glutaraldehyde because of severe aggregation of GLUT1 at glutaraldehyde levels ≥ 16 mM.

**Which Is the Physiologic Form of GLUT1?**—The preceding demonstrate that two major forms of GLUT1 are purified by available procedures but do not indicate which of these species (if any) represents the physiologic (red cell-resident) form of GLUT1.

We approached this problem in two ways. The first was to solubilize GLUT1 from intact red cells in the presence or absence of dithiothreitol or following prior treatment of red cells with N-ethylmaleimide (1 mM) or iodoacetic acid (25 mM) to prevent promiscuous disulfide formation during or following solubilization. Fig. 3 shows that none of these treatments altered the Stokes radii (7.7 ± 0.2 nm) of cholate-released cholate/lipid/GLUT1 micelles.

A more direct approach employs conformation specific antibodies. Rabbits were immunized with GLUT1–DTT, and the resulting antisera were characterized for titer against GLUT1–DTT, GLUT1+DTT, intact erythrocytes, and leaky erythrocyte ghosts. These antisera (6-IgGs) have been previously shown to react specifically and quantitatively with extracellular epitopes of erythrocyte-resident GLUT1 and GLUT1 in parental CHO cells or in transfected CHO cells overexpressing GLUT1 (17).

Competition ELISAs using 6-IgGs show that 6-IgG-reactive epitopes are present in both GLUT1–DTT and at the surface of intact erythrocytes but are absent in GLUT1+DTT (Fig. 5). Anti-GLUT1 C-IgGs show similar titers for both GLUT1–DTT and GLUT1+DTT. C-IgGs fail to react with the intracellular carboxyl-terminal region of GLUT1 in intact

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### Table I

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<th>Parameters</th>
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<th>Medium</th>
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<td>Diffusion coefficient (d_{20,w} (\text{cm}^2/\text{s}) \times 10^{26})</td>
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* The upper limit for detergent binding.

* The diffusion coefficient of the protein-lipid-cholate complex is calculated from the Stokes radius, combining the Einstein-Sutherland equation and Stokes law.

* The molecular mass of the complex was calculated using the Svedberg equation.

* The molecular mass of the protein component of the complex was calculated as fractional protein content × mass.

* The unit size (oligomeric state) of the protein was calculated as fractional protein content × mass/55,000 assuming an average molecular mass of monomeric GLUT1 of 55 kDa.
posed to this medium at pH 12 lacking or containing 10 mM EGTA, pH 12. These membranes were subsequently reexposed to this medium at pH 12 lacking or containing 10 mM dithiothreitol or to 2 mM EGTA, pH 7.4, containing 10 mM dithiothreitol. The resulting membranes were washed three times in 30 volumes of DTT-free Tris medium and then analyzed for the following parameters: 1) d-glucose-inhibitable cytochalasin B binding capacity; 2) d-glucose binding properties; 3) GLUT1 content by immunoblot analysis and by photolabeling using [3H]cytochalasin B; 4) IgG binding capacity; 5) the Stokes radius of GLUT1-containing micelles. Fig. 6 summarizes the results of these determinations.

Treatment with dithiothreitol at alkaline pH results in the following relative to treatment with high pH alone or with dithiothreitol at neutral pH: 1) an approximate doubling of the d-glucose-inhibitable cytochalasin B binding capacity of membrane resident, C-IgG-reactive GLUT1; 2) the conversion of equilibrium d-glucose binding from multiple to single site kinetics; 3) no change in immunoreactive GLUT1 content or in the electrophoretic mobility of [3H]cytochalasin B-photolabeled proteins; 4) reduced (4-fold) IgG binding capacity; 5) conversion of GLUT1-containing micelles from 7.7-nm Stokes radius particles to 5.9-nm particles.

The effects of treatment with dithiothreitol at high pH on cytochalasin B and IgG binding are reversed by subsequent incubation of treated membranes in GSSG-GSH buffers at pH 7.4. Fig. 7 shows that the cytochalasin B binding capacity of SGpHi-DTT membranes is halved by incubation in 0.5 mM GSSG, 2 mM GSH; similarly SGpHi-DTT binding is abolished by DTT (5 mM); and is not observed using protein-depleted membranes treated previously with dithiothreitol at pH 7.4 (SG+DTT). Similarly IgG binding to SGpHi-DTT (but not to SG+DTT) is significantly increased by prior incubation of the membranes in 1 mM GSSG, 2 mM GSH (Fig. 7).

Is GLUT1 Oligomeric State Determined by Membrane GLUT1 Content?—The human erythrocyte and purified GLUT1 preparations are characterized by a high GLUT1 content relative to membrane lipid and total protein contents. It is possible, therefore, that the oligomeric state of membrane-resident GLUT1 in these environments does not reflect that found in other cells of lower plasma membrane GLUT1 content.

Reconstitution of 100 μg of GLUT1–DTT (0.5 mg of red cell lipid) into 50 μg of egg phosphatidylcholine proteoliposomes by cholate dialysis is without effect on the amount of recoverable IgG-reactive GLUT1 (n = 2). Thus a 100-fold decrease in membrane GLUT1/lipid ratio in vitro is without effect on GLUT1 oligomeric size.

Using wild-type CHO cells and CHO cells transfected with GLUT1 cDNA, we have shown previously that cell surface GLUT1–DTT content (IgG-reactive GLUT1) and 3-O-methylglucose uptake capacity are directly proportional to CHO cell total GLUT1 content over a range of total GLUT1 levels spanning parental (CHO cells) to 23-fold greater than parental content (CHO-GT3 cells, see (17)). The major GLUT1-containing micelle released upon cholate solubilization of plasma membranes isolated from CHO and CHO-GT3 cells is resolved as a 7.8-nm particle upon cholate SEC-HPLC (Fig. 8). Because parental CHO cell surface GLUT1 density is 10–15-fold lower than that of human erythrocytes (17) and assuming that the lipid, cholate, and protein contents of these micelles are similar to those of GLUT1–DTT micelles, this finding suggests that GLUT1 oligomeric size is independent of CHO cell GLUT1 content in vivo over this range of concentrations.

**DISCUSSION**

This study characterizes two forms of purified, human erythrocyte glucose transport protein. GLUT1–DTT was pu-

**FIG. 5.** Competition ELISA assays of erythrocyte GLUT1–DTT content. A, inhibition of IgG (triangles) or C-IgG (circles) binding to 100 ng of GLUT1–DTT by GLUT1–DTT (open symbols) or GLUT1+DTT (closed symbols). Ordinate, absorbance at 414 nm (relative amount of IgG left in solution following reaction with competing GLUT1). The amount of competing GLUT1 is shown on the abscissa. B, inhibition of IgG (triangles) or C-IgG (circles) binding to 100 ng of GLUT1–DTT by intact erythrocytes (closed symbols) or unsealed erythrocyte ghosts (open symbols). The number of competing cells or ghosts is shown on the abscissa. Absorbance was normalized to that in the absence of exogenous GLUT1, cells, or ghosts. Curves through the points assume one saturable component of binding. Computed half-maximal GLUT1 binding concentrations are: β-IgG, GLUT1–DTT = 273 ± 16 ng, GLUT1+DTT > 100 μg; C-IgG, GLUT1–DTT = 155 ± 6 ng, GLUT1+DTT = 71 ± 5 ng. Computed half-maximal binding cell numbers are: β-IgG, intact cells = (0.7 ± 0.8) × 10^10 cells; C-IgG, intact cells ≥ 1 × 10^10 cells, unsealed ghosts = (0.7 ± 0.7) × 10^10 cells. IgG binding to unsealed ghosts and intact cells is indistinguishable (17). Measurements were made in duplicate. This figure pools data from and summarizes 12 separate experiments.

GLUT1 proteins are 307,029 ± 50,872/cell (using GLUT1–DTT as a standard) or > 100 × 10^6/cell (using GLUT1+DTT as a standard). Erythrocyte ghosts contain 296,651 ± 41,349 C-IgG reactive sites (using GLUT1–DTT = 10^6 cells; GLUT1–DTT = 5 ng.

**Fig. 6.** Effect of dithiothreitol on GLUT1 oligomeric size. A, effect of incubation of GLUT1–DTT or GLUT1+DTT in 1 mM GSSG, 2 mM GSH (Fig. 7).

**Fig. 7.** Summary of the effects of treatment with dithiothreitol at alkaline pH on cytochalasin B and IgG binding are reversed by subsequent incubation of treated membranes in GSSG-GSH buffers at pH 7.4. Fig. 7 shows that the cytochalasin B binding capacity of SGpHi-DTT membranes is halved by incubation in 0.5 mM GSSG, 2 mM GSH; is abolished by dithiothreitol (5 mM); and is not observed using protein-depleted membranes treated previously with dithiothreitol at pH 7.4 (SG+DTT). Similarly IgG binding to SGpHi-DTT (but not to SG+DTT) is significantly increased by prior incubation of the membranes in 1 mM GSSG, 2 mM GSH (Fig. 7).

**Fig. 8.** Photolabeling of GLUT1 with [3H]cytochalasin B using wild-type CHO cells and CHO cells transfected with GLUT1 cDNA, we have shown previously that cell surface GLUT1–DTT content (IgG-reactive GLUT1) and 3-O-methylglucose uptake capacity are directly proportional to CHO cell total GLUT1 content over a range of total GLUT1 levels spanning parental (CHO cells) to 23-fold greater than parental content (CHO-GT3 cells, see (17)). The major GLUT1-containing micelle released upon cholate solubilization of plasma membranes isolated from CHO and CHO-GT3 cells is resolved as a 7.8-nm particle upon cholate SEC-HPLC (Fig. 8). Because parental CHO cell surface GLUT1 density is 10–15-fold lower than that of human erythrocytes (17) and assuming that the lipid, cholate, and protein contents of these micelles are similar to those of GLUT1–DTT micelles, this finding suggests that GLUT1 oligomeric size is independent of CHO cell GLUT1 content in vivo over this range of concentrations.
GLUT1 Oligomeric Structure Determines GLUT1 Function

Fig. 6. Effects of alkaline reduction on GLUT1 oligomeric size and ligand and α-IgG binding properties. Erythrocyte membranes were depleted of peripheral membrane proteins by a single alkaline wash. The membranes were then exposed to 2 mM EGTA solutions at pH 12 (SGpH; open circles), pH 12 plus 10 mM DTT (SGpH+DTT; closed circles), or pH 7.4 plus 10 mM DTT (SG+DTT; open triangles) at 4 °C for 30 min prior to resuspension in Tris medium. A, cytochalasin B (CCB) binding SGpH, SGpH+DTT, and SG+DTT. Ordinate, mol of cytochalasin B bound per mol of immunodetectable GLUT1 per μmol of free cytochalasin B. Abscissa, mol of cytochalasin B bound per mol of immunodetectable GLUT1. The straight lines drawn through the points were computed by least squares and correspond to the following binding parameters: SGpH, Bmax = 0.61 ± 0.03, Kd(app) = 230 ± 11 nM; SG+DTT, Bmax = 0.63 ± 0.04, Kd(app) = 233 ± 8 nM; SGpH+DTT, Bmax = 1.31 ± 0.07, Kd(app) = 283 ± 9 nM where Bmax has units of mol of cytochalasin B binding sites per mol of immunodetectable GLUT1. This figure pools data from four separate experiments. B, D-glucose binding to SGpH and to SGpH+DTT measured as in Fig. 2. Binding is shown in Scatchard form as fractional specific fluorescence quenching per mM D-glucose versus fractional specific fluorescence quenching. The curve drawn through the SGpH data was computed assuming two saturable components of binding. Computed maximum quenching and dissociation constants are: Qm1 = 0.029 ± 0.003, Qm2 = 0.047 ± 0.006, Kd(app)1 = 1.3 ± 0.2 mM and Kd(app)2 = 31 ± 2.6 mM. The line through the SGpH+DTT was computed assuming a single saturable component of binding with Kd(app) of 16.5 ± 0.7 mM and Qm = 0.071 ± 0.005. Similar results were found with SG+DTT membranes (not shown). These data summarize four separate experiments. C, SEC-HPLC (in 20 mM cholate) elution profiles of cholate-solubilized GLUT1 from SGpH, SG+DTT, and SGpH+DTT. Electrophoretic mobility of [3H]cytochalasin B-photolabeled GLUT1 from SGpH, SGpH+DTT, and SG+DTT. Ordinate, [3H]cytochalasin B photoincorporation (dpm × 10^-4/slice); abscissa, gel slice number. Each lane of the polyacrylamide gel was loaded with erythrocyte membranes containing 5 μg of immunodetectable GLUT1. Membranes were irradiated at 300 nm in the presence of 250 nM cytochalasin B. The positions of molecular weight markers are indicated. D, α-IgG binding to GLUT1 present in SG membranes. Ordinate, membrane type (SGpH+DTT, SG+DTT, and SGpH); abscissa, cpm (× 10^-4) of 125I-protein A bound per nmol of immunodetectable GLUT1. Data are corrected for nonspecific binding (427 ± 57 cpm/nmol of GLUT1) that occurs in the absence of immune serum by using preimmune serum as a control. This chart summarizes three experiments. Data are shown as mean ± S.E.

rified from red cells in the absence of dithiothreitol, and GLUT1+DTT was purified in the presence of 5 mM dithiothreitol.

The results of our analyses demonstrate that glucose transporter substrate binding properties are determined by transporter oligomeric structure. The erythrocyte glucose transporter is a GLUT1 homotetramer in which subunits (GLUT1 monomers) interact cooperatively. GLUT1–DTT most closely resembles the physiologic transporter. Transporter structure and subunit interactions appear to be stabilized and promoted by intramolecular disulfides which, in the erythrocyte membrane, are resistant to reduction at physiologic pH. Following reduction, tetrameric GLUT1 dissociates into GLUT1 dimers in which subunit interactions are lost. GLUT1+DTT resembles this reduced form of glucose transporter. Physiologic structure and subunit interactions are restored by treatment of GLUT1 dimers with glutathione-disulfide. We speculate that in vivo GLUT1 oligomeric struc-
FIG. 7. Effects of GSSG on cytochalasin B and 6-IgG binding to GLUT1 present in SG+DTT (A) and in SGpH+DTT (B). A, SG+DTT and SGpH+DTT were incubated in the presence of 2 mM GSH plus varying [GSSG] ± DTT (5 mM) for 1 h at 37 °C. Membranes were harvested and then assayed for [3H]cytochalasin B binding capacity. The computed B_{max} (pmol of cytochalasin B binding sites) for binding is expressed on the ordinate. The GSSG:GSH ratio is shown on the abscissa. The curve drawn through the SGpH+DTT data was computed by weighted (S.E.) nonlinear regression assuming simple saturation kinetics. GSSG reduces B_{max} for cytochalasin B binding to SGpH+DTT by a factor of 2.05 ± 0.08 and is half-maximally effective at 29 ± 7 μM. Closed triangles and circles indicate the effects of GSSG + 5 mM DTT on cytochalasin B binding to SGpH+DTT and SG+DTT respectively. Each data point represents the mean ± S.E. of four separate determinations. B, 6-IgG binding to SG+DTT and SGpH+DTT. These membranes were treated as in A. Ordinate, GSSG:GSH ratio (at 2 mM GSH). Abscissa, cpm of 125I-protein A bound. Nonspecific binding was subtracted. Binding to SGpH+DTT is shown by the open bars. Binding to SG+DTT is shown by the dark bars. Data are shown as the mean ± S.E. of six separate measurements.

FIG. 8. Size exclusion chromatography of cholate-solubilized GLUT1 from plasma membranes isolated from CHO cells (C) and from CHO-GT4 cells (D). Elution profile of GLUT1 detected by ELISA using C-IgGs. Ordinate, [GLUT1] in arbitrary units. Abscissa, retention time in min. The column was calibrated as in Fig. 3. The elution times of 7.8 and 6.0 nm (Stokes radius) particles are indicated by the arrows.

The experiments presented in this current study demonstrate that the major physiologic form of human erythrocyte GLUT1 is tetrameric GLUT1 (GLUT1–DTT). The ligand binding properties of erythrocyte-resident GLUT1 (see here and Refs. 2, 5, and 6) most closely resemble those of GLUT1–DTT. The use of conformation-specific antibodies (δ-IgGs) supports this view. δ-IgGs display undetectable affinity or titer for GLUT1–DTT but bind to GLUT1–DTT and intact erythrocytes with high titer. Erythrocytes contain 3.1 × 10^{6} δ-IgG-reactive sites. Since human erythrocytes contain 1.5 × 10^{7} δ-glucose-inhibitable cytochalasin B binding sites and 2.9 × 10^{5} anti-GLUT1 carboxyl-terminal peptide IgG-reactive sites, this suggests that human erythrocyte-resident

fluence is established prior to transporter translocation to the plasma membrane.

Previous hydrodynamic studies from this laboratory are consistent with the view that the erythrocyte glucose transporter is an oligomeric complex of GLUT1 proteins (16). We have also shown that endogenous CHO cell GLUT1 is quantitatively and specifically communoprecipitated by anti-GLUT4 carboxyl-terminal antibodies when CHO cells express a chimeric glucose transporter in which the 29 carboxy-terminal amino acids of GLUT1 are replaced by the 30 carboxy-terminal amino acids of GLUT4 (29). Our current hydrodynamic studies demonstrate that GLUT1–DTT and erythrocyte-resident GLUT1 are solubilized as GLUT1 tetramers, whereas GLUT1+DTT is solubilized as GLUT1 dimers. These conclusions are supported by the results of chemical cross-linking studies.

We demonstrate in this present study that the ligand binding properties of GLUT1 are determined by GLUT1 oligomeric structure. Tetrameric GLUT1 presents multiple δ-glucose binding sites to available substrate, whereas dimeric GLUT1 presents only a single population of δ-glucose binding sites. The δ-glucose binding capacities of tetrameric and dimeric GLUT1 are, however, indistinguishable. Tetrameric GLUT1 binds cytochalasin B (a ligand that binds at or very close to the sugar efflux site) with a molar stoichiometry of 1:2 (mol of cytochalasin B/mol of monomeric GLUT1). The cytochalasin B binding capacity of dimeric GLUT1 is double that of tetrameric GLUT1. Conversion of tetrameric GLUT1 to dimeric GLUT1 by reductant results in the loss of multiple δ-glucose binding sites and doubles the cytochalasin B binding capacity of GLUT1. Zoccoli et al. (14) also report that the cytochalasin B binding capacity of GLUT1 solubilized from red cell membranes by Triton X-100 is doubled upon inclusion of dithiothreitol.

We demonstrated previously (16) that the cytochalasin B binding properties of GLUT1–DTT and GLUT1+DTT reconstituted into egg phosphatidylcholine liposomes by detergent dialysis were indistinguishable. These analyses were in part based upon quantitation of GLUT1 by using δ-IgGs. We now know that δ-IgGs do not bind to dimeric GLUT1 and that prolonged exposure to detergent can result in GLUT1 aggregation. It is possible, therefore, that the contribution of dimeric GLUT1 to the total cytochalasin B binding capacity of each preparation was overlooked and/or that some conversion of dimeric GLUT1 to tetrameric GLUT1 occurred during reconstitution. It is also possible that the functional properties of dimeric or tetrameric GLUT1 reconstituted into egg phosphatidylcholine bilayers differ from those of GLUT1 in red cell membranes, a possibility supported by the results of reconstitution studies (19). Future reconstitution studies aimed at comparing the catalytic properties of dimeric and tetrameric GLUT1 must address these possibilities.

The experiments presented in this current study demonstrate that the major physiologic form of human erythrocyte GLUT1 is tetrameric GLUT1 (GLUT1–DTT). The ligand binding properties of erythrocyte-resident GLUT1 (see here and Refs. 2, 5, and 6) most closely resemble those of GLUT1–DTT. The use of conformation-specific antibodies (δ-IgGs) supports this view. δ-IgGs display undetectable affinity or titer for GLUT1–DTT but bind to GLUT1–DTT and intact erythrocytes with high titer. Erythrocytes contain 3.1 × 10^{6} δ-IgG-reactive sites. Since human erythrocytes contain 1.5 × 10^{7} δ-glucose-inhibitable cytochalasin B binding sites and 2.9 × 10^{5} anti-GLUT1 carboxyl-terminal peptide IgG-reactive sites, this suggests that human erythrocyte-resident
GLUT1 most closely resembles and is quantitatively accounted for by GLUT1–DTT. Erythrocyte GLUT1 oligomeric structure and ligand binding characteristics are unaffected by cell lysis and resealing (ghost formation). The ligand binding properties of erythrocyte- and erythrocyte-ghost-resident GLUT1 are indistinguishable (6), and IgG binding to the exterior of erythrocytes is unaffected by cell lysis and resealing (17).

Both GLUT1–DTT and erythrocyte-resident GLUT1 (tetrameric GLUT1) are converted to GLUT1+DTT (dimeric GLUT1) with its associated ligand binding and hydrodynamic properties upon treatment with dithiothreitol. The available evidence suggests that this results from reduction of GLUT1 intramolecular disulfides which, in the erythrocyte membrane, are either stabilized by salt bridges or are reduced only at alkaline pH. Reduction of GLUT1–DTT and the concomitant doubling of GLUT1 cytochalasin B binding capacity are reversed by glutathione disulfide–reduced glutathione buffers. This effect is half-maximal at 30 μM GSSG, a level similar to that found in the erythrocyte (30).

Although further characterization is necessary, our findings suggest that glucose transporter subunit composition is independent of membrane GLUT1 density. It is possible, therefore, that transporter oligomeric structure is established prior to transporter translocation to the plasma membrane. Because native, tetrameric GLUT1 is reduced only at alkaline pH, this structure is expected to be resistant to reduction at physiologic pH in vivo.

These findings are consistent with a model for transporter function shown in Fig. 9. GLUT1 can exist in two forms: as dimeric GLUT1 (obtained upon purification in the presence of dithiothreitol) and as tetrameric GLUT1 (erythrocyte-resident transporter and that form purified in the absence of DTT). Each monomer (subunit) is functional in both structures and exposes a sugar binding site (shown as a cleft) to cytosol or to extracellular medium but never to both environments simultaneously. Each monomer is thus a prototypic one-site transporter (11). Tetrameric and dimeric GLUT1 differ in the manner in which their subunits interact.

![Fig. 9. Model for GLUT1 oligomeric structure and function.](image)

In dimeric GLUT1, conformationally active regions of each subunit are unconstrained, allowing each monomer to isomerize independently of the other between influx (E2) and efflux (E1) states. Since cytochalasin B binds at the efflux site, each monomer can bind one molecule of cytochalasin B.

Tetrameric GLUT1 is comprised of two dimers of GLUT1 (a pseudo-D2 symmetry). These dimers differ from those of dimeric GLUT1. Conformationally active regions of each subunit are now constrained, resulting in coupled isomerizations and an antiparallel arrangement of sugar binding sites. Thus each dimer of the tetramer presents one influx and one efflux site and, when one subunit isomerizes from an efflux to an influx conformation, the second must isomerize from an influx to an efflux conformation and vice versa. Each dimer thus exists in one of two states, E or F, each presenting only one efflux and therefore one cytochalasin B binding site.

This model is consistent with data demonstrating that erythrocyte sugar transporter and GLUT1–DTT behave as multisite, allosteric transporters (1, 2, 5–7), whereas GLUT1+DTT functions as a simple, one-site transporter (8–10). This model also shares some similarities with the proposed quaternary structure of the Rho hexamer (31). Although each Rho monomer contains a single ATP binding domain and a single RNA binding domain, the native hexamer appears to bind only three ATP molecules strongly (31, 32) and three RNA oligonucleotides (33). Thus native Rho is comprised of three dimers in which the functional sites of each monomer are arranged in an antiparallel fashion.

Alternative explanations for the multisite, D-glucose binding kinetics and reduced cytochalasin B binding capacity of GLUT1–DTT appear less likely. Consider, for example, that GLUT1–DTT consists of two, dysfunctional forms of one-site transporter (GLUT1+DTT). Here, one-half of the transporter may be trapped in a form exposing a sugar influx site while the other half is trapped as a form exposing a sugar efflux (and cytochalasin B binding) site. This hypothesis seems untenable since GLUT1–DTT is at least as catalytically active as GLUT1+DTT (16). If true, this observation also permits rejection of the possibility that GLUT1–DTT...
can predict the transport properties of erythrocytes containing GLUT1+DTT at this temperature (1). The results are shown in Table II and are contrasted with the known sugar transport properties of the red cell at this temperature (34).

These comparisons strongly suggest that dimeric GLUT1 is 2-8-fold catalytically less efficient than native, tetrameric GLUT1 at 10°C. The reason for this is that the tetramer avoids wait times in which the unoccupied (nontranslocating) carrier must reisomerize to a translocation competent form. When GLUT1+DTT can circumvent these wait times (during exchange transport when sugar is present at both sides of the membrane), its catalytic efficiency approaches that of GLUT1–DTT.

These considerations indicate the potential for transport acceleration upon tetramerization of GLUT1 and may explain why the kcat of reconstituted GLUT1+DTT approaches a value 20-fold lower than that of erythrocyte-resident GLUT1 (35), whereas the kcat of reconstituted GLUT1–DTT can approach that of erythrocyte-resident GLUT1 (15, 36, 37). It remains to be established whether alterations in GLUT1 oligomeric structure play a role in the regulation of GLUT1 catalytic efficiency.

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References


Table II

<table>
<thead>
<tr>
<th>Parameter <em>a</em></th>
<th>Tetrameric GLUT1</th>
<th>Dimeric GLUT1</th>
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<td>Km(net efflux)</td>
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<tr>
<td>Km(exchange transport)</td>
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<td>11.6</td>
<td>1.1</td>
</tr>
</tbody>
</table>

*a* Vmax parameters refer to maximum velocities of transport and have units of mmol-liter of cell water min⁻¹. Km parameters refer to apparent Michaelis constants and have units of mmol liter⁻¹ min⁻¹.

The d-glucose transport properties of intact erythrocytes at 10°C were taken from Ref. 34 and are assumed to reflect those of the native, tetrameric carrier.

The transport properties of the dimeric carrier at 10°C were predicted from the studies of Appleman and Lienhard (10) in which the catalytic turnover of GLUT1+DTT was analyzed.

\[ \frac{V_{\text{max}}}{K_{\text{m}}(\text{app})} \]

\[ \text{Refer to apparent Michaelis constants and have units of mmol liter^{-1} min^{-1}.} \]

\[ V_{\text{max}}, \text{ or } K_{\text{m}}(\text{app}) \]

\[ \text{Parameter for dimer-mediated transport.} \]

\[ \text{D-Glucose influx when intracellular d-glucose is absent.} \]

\[ \text{D-Glucose efflux when extracellular d-glucose is absent.} \]

\[ \text{Equilibrium exchange D-glucose uptake or efflux when intracellular and extracellular [d-glucose] are identical.} \]

\[ \text{Kcat(OT) exchange transportY 12.4 11.6 1.1} \]

\[ \text{Kcat(OT) exchange transportY 12.4 11.6 1.1} \]

\[ \text{Ratio of Vmax, or Kcat(OT) parameter for dimer-mediated transport to Vmax, or Kcat(OT) parameter for dimer-mediated transport.} \]

\[ \text{D-Glucose efflux when intracellular d-glucose is absent.} \]

\[ \text{D-Glucose influx when extracellular d-glucose is absent.} \]

\[ \text{Equilibrium exchange D-glucose uptake or efflux when intracellular and extracellular [d-glucose] are identical.} \]

\[ \text{Vmax parameters refer to maximum velocities of transport and have units of mmol-liter of cell water min^{-1}.} \]

\[ K_{\text{m}}(\text{pp}) \]

\[ \text{Refer to apparent Michaelis constants and have units of mmol liter^{-1} min^{-1}.} \]

\[ V_{\text{max}} \text{ parameters refer to maximum velocities of transport and have units of mmo} \text{l-liter of cell water min^{-1}.} \]

\[ K_{\text{m}}(\text{pp}) \]

\[ \text{Refer to apparent Michaelis constants and have units of mmol liter^{-1} min^{-1}.} \]

\[ V_{\text{max}}, \text{ or } K_{\text{m}}(\text{app}) \]

\[ \text{Parameter for dimer-mediated transport.} \]

\[ \text{D-Glucose influx when intracellular d-glucose is absent.} \]

\[ \text{D-Glucose efflux when extracellular d-glucose is absent.} \]

\[ \text{Equilibrium exchange D-glucose uptake or efflux when intracellular and extracellular [d-glucose] are identical.} \]