Molecular Determinants of GLUT1: Structure and Function: A Dissertation

Ralph J. Zottola

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MOLECULAR DETERMINANTS OF GLUT1
STRUCTURE AND FUNCTION

A Dissertation Presented

By

Ralph J. Zottola

Submitted to the Faculty of the
University of Massachusetts Graduate School of Biomedical Sciences, Worcester
in partial fulfillment of the requirements for the degree of:

DOCTOR OF PHILOSOPHY

JUNE 1995

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STRUCTURE AND FUNCTION

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June 1995
ABSTRACT

Hebert and Carruthers (1992) showed that the human erythrocyte glucose transporter is an allosteric complex of four GLUT1 proteins whose structure and substrate binding properties are stabilized by reductant-sensitive noncovalent subunit interactions. The GLUT1 tetramer dissociates into dimers upon exposure to reductant but subunits are not associated via disulfide bridges. Each subunit of SDS-denatured tetrameric GLUT1 exposes only two thiols while reduced denatured GLUT1 exposes all six sulfhydryl groups. They hypothesized that glucose transporter oligomeric structure and cooperative catalytic function resulted from noncovalent subunit interactions promoted or stabilized by intramolecular disulfide bridges. These interactions give rise to an antiparallel arrangement of substrate binding sites within the transporter complex.

In the present studies, we tested aspects of this model. Specifically, we wanted 1) to understand why the native, noncovalent, homotetrameric GLUT1 complex is sensitive to reductant, 2) to determine whether the tetramer is more catalytically efficient than the dimer in situ, and 3) to test the hypothesis that it is the antiparallel arrangement of substrate binding sites between subunits that provides the transporter with its catalytic advantage. We used biochemical and molecular biological approaches to isolate specific determinants of transporter oligomeric structure and/or transport function in purified isolated transporter preparations, in intact red cells and in CHO cells. We have also examined the hypothesis that net sugar transport in the human erythrocyte is rate limited by reduced cytosolic diffusion of sugars and/or by reversible sugar association with intracellular macromolecules.

Our findings support the hypothesis that each subunit of the parental glucose transporter contains a single intramolecular disulfide bridge located between cysteine residues 347 and 421. This disulfide seems to be necessary for GLUT1 tetramerization. Our findings suggest that GLUT1 N-terminal residues 1 through 199 provide contact surfaces for subunit dimerization but are insufficient for subunit tetramerization. Our studies also show that in situ disulfide disruption by cell impermeant reductants results in
the loss of cooperative subunit interactions and a 3 to 15-fold reduction in the transport efficiency of the transporter. We further find that in situ GLUT1 is susceptible to exofacial proteolysis. Exofacial trypsin cleavage eliminates cooperativity between subunits but does not affect transporter oligomeric structure or transport activity. Thus catalytic efficiency does not derive directly from cooperative interactions between substrate binding sites on adjacent subunits. We have confirmed that 3OMG transport in human erythrocytes is a diffusion limited process. We find that steady-state sugar uptake in red cells and K562 cells measures two processes - sugar translocation and intracellular sugar binding. We propose a model for native GLUT1 structure and function.
ACKNOWLEDGEMENTS

I would like to thank my family, first and foremost, my parents, Dominick and Maria Zottola, for their unconditional support all these years. I would also like to thank my sister Elizabeth and my brother-in-law David DeJean for the stress relief they have provided over the years. A special thanks to my niece, Dominique, who every time I see her reminds me why some of us work so hard in our research. It is for our future which she represents.

I wish to express my deepest gratitude and respect for my thesis advisor, Dr. Anthony Carruthers. He provides an environment in his lab where one can truly learn without fear of mistakes. I truly felt I was a colleague while I was in his lab. I aspire to be the thinker and person that he is. I would also like to thank the members of the Department of Biochemistry and Molecular Biology with whom I have interacted over the years, especially Drs. Daniel Hebert and Chee Wai Woon.

Finally, I would like to especially thank Erin K. Cloherty. I would not have been able to complete this work without both her emotional and technical support. Whenever I think of this time, I will always say “we did it!”

“...no but ourselves can free our mind.”

Bob Marley
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ABBREVIATIONS

2DOG  2-Deoxy-D-Glucose
3OMG  3-O-Methyl-D-Glucose
6-NBDG 6-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-6-deoxyglucose
C-Ab  anti-GLUT1 Carboxyl terminal peptide antiserum
CCB  cytochalasin B
CHO  Chinese Hamster Ovary cells
∂-Ab  anti-tetrameric GLUT1 rabbit or sheep antiserum
DABCO 1,4-diazabicyclo[2.2.2]octane
DIFM  digital imaging fluorescence microscopy
DOTAP N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate
DTT  dithiothreitol
EDTA  ethylenediaminetetraacetic acid
EGTA  ethyleneglycol-bis-(β-aminoethylether) N,N,N′,N′-tetraacetic acid
ELISA  enzyme-linked immunosorbent assay
FBS  fetal bovine serum
GLUT1  erythrocyte glucose transporter
GLUT4  skeletal muscle glucose transporter
GLUT1-4C a chimeric glucose transporter consisting of GLUT1 residues 1 to 463 plus
         GLUT4 residues 480 to 509
GLUT1n-4 a chimeric glucose transporter consisting of GLUT1 residues 1 to 199 plus
         GLUT4 residues 216 to 509
GSH  glutathione
GSSG  oxidized glutathione
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<tr>
<td>Hb</td>
<td>hemoglobin</td>
</tr>
<tr>
<td>HEPES</td>
<td>(N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid])</td>
</tr>
<tr>
<td>Ht</td>
<td>hematocrit</td>
</tr>
<tr>
<td>NTB</td>
<td>2-nitro-5-thiobenzoic acid</td>
</tr>
<tr>
<td>NTSB</td>
<td>2-nitro-5-thiosulfobenzoate</td>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
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<td>SDS</td>
<td>sodium dodecyl sulfate</td>
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<td>SEC-HPLC</td>
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<td>Tris-HCl</td>
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Chapter I

Introduction: Literature Review

All cells express a variety of integral, plasma membrane proteins that catalyze transmembrane solute fluxes. These proteins are called transporters and typically catalyze cellular uptake or efflux of nucleosides, carbohydrates, amino acids, cations or anions. Solutes enter the cell via three independent mechanisms: 1) slow, bidirectional transbilayer diffusion (simple diffusion); 2) rapid, protein-mediated, bidirectional, facilitated diffusion (passive transport); and 3) rapid, active, protein-mediated transport (active transport) (Stein 1986). Since these transporters provide the cell’s metabolic machinery with its necessary substrates, it is easy to understand why transporters are essential for life. However, the mechanism of protein-mediated transmembrane solute transport remains one of the fundamental enigmas of contemporary cell biology.

We are interested in unraveling this process and have chosen the glucose transporter as our prototypical model for study. Sugars are strongly hydrophilic molecules and their diffusion into the cell is effectively blocked by the hydrophobic plasma membrane. The transport of glucose across the plasma membrane of most cells occurs via a rapid, protein-mediated, bidirectional, facilitated diffusion mechanism called uniport. The proteins responsible for this are known as glucose transporters. Net glucose transport is always from a high to low sugar concentration. This and the lack of obligatory coupling to the symport or cotransport of cations distinguishes glucose uniport from active glucose trans-
port or glucose/cation symport. The pharmacology of glucose uniport inhibition differs significantly from that of active glucose transport (Carruthers 1990). Unlike active glucose transport mechanisms, facilitated diffusion of glucose is inhibited by the fungal metabolite cytochalasin B.

Six distinct isoforms of glucose transporters have been identified by biochemical and recombinant DNA techniques and are named GLUT1 through GLUT7. GLUT1 (Mueckler et. al. 1985) is also called the erythrocyte/HEP G2 type transporter and is found in almost all tissues including the brain and fibroblasts. GLUT2, or liver type transporter (Thorens et. al. 1988) is found in the β-cells of the pancreas, hepatocytes, intestine and kidney. GLUT3 (Kayano et. al., 1988) is found mainly in the brain of most species but is more widely distributed in humans. GLUT4, the insulin-sensitive transporter (Fukumoto et. al. 1989, James et. al. 1989, Kaestner et. al 1989, Birnbaum 1989, Charron et. al. 1989), is found in muscle and adipose tissue. GLUT5 (Kayano et. al. 1990) is found largely in the jejunum and may mediate intestinal absorption of fructose rather than glucose (Burant et. al. 1992). GLUT7 (Waddell et. al. 1992), the hepatic microsomal glucose transporter, is thought to mediate glucose transport across the endoplasmic reticulum. GLUT6 is a psuedogene that was identified by a homology screening approach (Kayano et. al. 1990). It is not expressed at the protein level.

We have chosen the GLUT1 isoform for our studies on membrane transport. GLUT1 is the best studied isoform for a number of reasons of which the most important is its availability. GLUT1 is found in high abundance (5% of total membrane proteins) in human red cell membranes. Erythrocytes are easily obtainable and thus provide a good
model system for *in situ* studies. In addition, GLUT1 is easily purified from erythrocytes (yields of up to 20 mg from one unit of blood) and is thus well suited for *in vitro* studies.

GLUT1 exhibits nearly ubiquitous tissue distribution. The highest expression levels are at the blood/brain barrier but GLUT1 is abundantly expressed in all blood-tissue barriers (Gould and Holman 1993, Mueckler 1994). The regulation of GLUT1 expression has been extensively studied in cultured cells. GLUT1 expression levels are increased by: butyrate (Takano et al. 1988), cAMP (Hiraki et al. 1989), fibroblast growth factor (Hiraki et al. 1988), growth hormone (Tai et al. 1990), hypoxia (Loike et al. 1992), insulin (Tordjman et al. 1989), insulin-like growth factor I (Maher et al. 1989), oncogenes (Birnbaum et al. 1987, Flier et al. 1987), phorbol esters (Hiraki et al. 1988), platelet-derived growth factor (Rollins et al. 1988), serum (Hiraki et al. 1988), sulfonylureas (Tordjman et al. 1989), thyroid hormone (Weinstein et al. 1990), transforming growth factor-β (Kitagawa et al. 1991), tumor necrosis factor α (Stephens et al. 1992) and vanadate (Mountjoy and Flier 1990). Glucose causes a reduction in GLUT1 expression levels (Walker et al. 1988, Walker et al. 1989, Tordjman et al. 1990, Wertheimer et al. 1991). Acute hypoxia has also been shown to activate glucose transport without altering GLUT1 expression (Diamond and Carruthers 1993).

Glucose transporters are also part of a much larger superfamily. Homologous transporters have been found in algae (Sauer and Tanner 1989), cyanobacteria (Zhang et al. 1989), *Escherichia coli* (Maiden et al. 1988), plants (Sauer et al. 1990), protozoa (Cairns et al. 1989, Bringaud and Baltz 1992), yeast (Celenza et al. 1988, Szkutnicka et al. 1989) and in *Zymomonas mobilis* (Barnell et al. 1990).
There are a number of structural features which are common to the glucose transporter isoforms. The primary structures of human GLUT1-5 exhibit 39-65% sequence identity and 50-76% sequence similarity in pairwise comparison (Bell et al. 1990).

The secondary structure of GLUT1 is predicted (by hydropathy analysis of the primary structure; Mueckler et al. 1985) to contain twelve transmembrane helices (Fig. 1). Scanning hydrophobic moment analysis (Eisenberg, 1984), however, suggests only ten transmembrane domains (Carruthers, unpublished). Using anti-peptide antibodies, Fishbarg et al. (1993) suggest that GLUT1 forms a beta barrel and that the number of transmembrane domains may not be twelve. The N- and C-termini and a large middle loop are located in the cytoplasm. This has been confirmed using anti-peptide antibodies and by analysis of protease susceptibility (Cairns et al. 1987, Davies et al. 1987, Haspel et al. 1988). Spectroscopic studies suggest that purified GLUT1 is greater than 80% helical (Chin et al. 1986, Alvarez et al. 1987) and that more than 80% of the polypeptide chain is accessible to solvent (Alvarez et al. 1987). Since a larger portion of intramembranous protein is accessible to bulk solvent, the presence of a water filled pore or channel is possible. There are two conserved motifs between the N and C terminal halves of the transporters. There is an RXGRR motif between helices 2 and 3 in the N-terminus and between helices 8 and 9 in the C-terminus (Maiden et al. 1987). Another conserved motif is EXXXXXXR found between helices 4-5 and 10-11 in the N and C termini respectively (Gould and Holman 1993). Thus it is possible that the two-domain, twelve membrane spanning helical transporter structure may have arisen from gene duplication of an ancestral six transmembrane-spanning helical protein. It has been hypothesized that GLUT1 contains one or
Figure 1. Proposed 2D topology of GLUT1

A proposed topographical map of GLUT1 generated by hydropathy analysis (Mueckler et al. 1985) and optimized by the method of Jones et al. 1994. Amino acids are indicated by their one letter codes. Putative transmembrane domains are shaded. The location of the cysteine residues are denoted by dark circles. GLUT1 is heterogeneously glycosylated at asparagine-45.
more intra-molecular disulfide bonds (Hebert and Carruthers 1992).

There is no information about the tertiary structure of glucose transporters. It has proven to be extremely difficult to grow high quality protein crystals of membrane proteins for x-ray crystallographic studies. Recent data from Reithmeier’s group (Wang et al. 1994) have provided a fascinating view of the three-dimensional map of the human erythrocyte anion exchanger (Band 3). Band 3 is a membrane protein with eight to fourteen predicted transmembrane helices (Reithmeier 1993). Band 3 exists as dimers and tetramers in the membrane and anion exchange occurs by a ping pong mechanism. Band3, like GLUT1, may still be functional as a monomer in vitro. Using electron microscopy and image reconstruction, the three dimensional map of two dimensional crystals of Band 3 has been determined at 20 Å resolution. The 3D map reveals a dimer (Fig. 2) which forms a U-shaped structure. The structure is open at the top and at the sides. The monomers are in close contact at the base which is thick and is believed to span the bilayer. The exofacial domains form a canyon. Biochemical data suggest that the protrusions are dynamic. The canyon is a wide space that narrows down and converges into a depression centered between the subunits at the top of the base. The depression may form a channel.

There is a growing body of biophysical evidence concerning the quaternary structure of the glucose transporter. GLUT1 has been proposed to be a tetramer (Cuppoletti et al. 1981, Jung et al. 1980) or a dimer (Jarvis et al. 1986) by radiation inactivation studies. Freeze-fracture electron microscopy of reconstituted GLUT1 suggest it is a dimer (Hinkle et al. 1979). A number of hydrodynamic studies have suggested that the transporter is a monomer (Rampal et al. 1986), a monomer or a dimer (Zoccoli et al. 1978) and a dimer
**Figure 2. 3D map of the dimeric transport domain of Band 3 protein.**

**A.** Perspective View. The bulky basal domain is probably embedded in the lipid bilayer, indicated by the two blue planes which are separated by 35 Å. The longest dimension of the basal domain is ~110 Å. Based on the available biochemical data, it is suggested that the protrusions on the upper side of the basal domain are on the cytosolic side of the membrane and that the smooth surface on the lower side is exofacial. The two domains on the cytosolic side form two sides of a canyon that leads to a depression which is probably the entrance to a pore. Striped areas represent cuts through the map above the putative membrane surface which form protein-protein contacts in the crystals.

**B.** Top View. Shows the open canyon on the cytoplasmic side. The two sides of the canyon narrow down on the surface of the basal domain. The lower part of the protein thought to be embedded in the membrane appears blue. The bar represents 20 Å.

This figure is adapted from Wang et al. 1994.
(Masher and Lundahl (1987). These studies were all performed on purified, detergent-solubilized, reduced glucose transporter. Hebert and Carruthers (1992) showed via hydrodynamic studies and by using conformation-specific antibodies that the reduced transporter is a dimer while the non-reduced purified carrier exists as a tetramer. Furthermore, they showed that the red cell-resident carrier is a tetramer. Supporting evidence for GLUT1 oligomerization has been obtained using co-immunoprecipitation techniques in 3T3-L1 adipocytes (Pessino et al. 1991). These studies show that GLUT1 forms homo-oligomers. GLUT1 fails to oligomerize with GLUT4 in the same cell.

A number of additional features of GLUT1 have been revealed by site-directed mutagenesis studies. There are six tryptophan residues in GLUT1. All have been mutated. Trp388 and Trp412 show impaired glucose transport activity in CHO cells (Katagiri et al. 1991) and in Xenopus oocytes (Garcia et al. 1992). CCB photolabelling is also decreased (Inukai et al. 1994). Mutations at Trp48, Trp65, Trp186 and Trp363 had no effect on glucose transport. A Gln282→Leu mutation has been shown to markedly reduce binding of exofacial ligands (ATB-BMPA and ethylidene-D-glucose) with little effect on glucose transport or CCB binding (Hashiramoto et al. 1992). Mutations at Asn288 and Asn317 have no effect (Hashiramoto et al. 1992). Mutation at Asn415 decreases transport function (Ishihara et al. 1991). It has been suggested that mutagenesis of Tyr293→Leu locks the transporter in an outward facing conformation (Mori et al. 1994). Substitution of Tyr292 is without effect (Mori et al. 1994). The role of Pro385 appears to be dependant on the substituted side chain. Pro385→Ile appear to lock the transporter in an inward facing conformation whereas Pro385→Gly is without effect (Tamori et al. 1994). Pro187→His,
Pro205→Phe, Pro383→Ala/Gln, Pro385→Ala/Gln and Pro387→Ala/Gln are without effect in Xenopus oocytes (Wellner et al. 1995) but Pro196→Arg shows a reduced transport capacity. Mutagenesis of cysteines is reported to have no effect on glucose transport in the Xenopus expression system (Wellner et al. 1992, Wellner et al. 1994). Substitution of leucine or asparagine for Gln161 markedly reduced 2DOG uptake in Xenopus oocytes (Mueckler et al. 1994). Mutations at Asn100 and Gln200 were without effect (Mueckler et al. 1994).

The analysis of the transport function of the mutants is complicated by the system used to express the mutants. GLUT1 has been expressed in a number of cell types including CHO cells (Zottola et al. 1995, Harrison et al. 1990a, Asano et al. 1989), 3T3-L1 cells (Harrison et al. 1990b, Gould et al. 1989), COS cells (Schurman et al. 1992), Xenopus oocytes (Burant & Bell 1992, Gould et al. 1991, Gould & Lienhard 1989), Sf9 cells (Yi et al. 1992) and in transgenic mice (Marshall et al. 1993, Ren et al. 1993). It is difficult to study transport kinetics in systems which contain an endogenous glucose transporter since it will contribute to transport. In addition, non-mammalian systems may lack required cellular machinery for proper assembly or regulation of expressed proteins.

A large body of data is available for analysis of potential mechanisms of erythrocyte glucose transport. The facilitated diffusion of sugars across cell membranes is usually attributed (Bell et al., 1993, Mueckler, 1993) to a protein-mediated, uniport (transport) mechanism called the simple carrier. According to this model (Fig. 3), transport is a sequential (iso-uni ping pong) process in which the transporter (E) cycles between sugar import (E2) and sugar export (E1) states (Lieb & Stein, 1974, Widdas, 1952). While this
Figure 3. Proposed mechanisms of glucose translocation by GLUT1

One-site carrier. This is a schematic representation of the one-site, or simple carrier mechanism. In the absence of sugar, the carrier E can exist in one of two conformations. E2 presents a sugar influx site for extracellular sugar. Extracellular glucose binds to E2 to form E2S2 which undergoes a conformational change to form E1S1. Glucose is now exposed to the cytosol and dissociates leaving E1 which re-isomerizes to E2 for an additional round of influx. E1 and E2 are mutually exclusive. Dimeric GLUT1 is proposed to exist as two independent one-site carriers.

Two-site carrier. This is a schematic representation of the two-site (iso) carrier. In the absence of sugar, the carrier exists in one of two conformations, E or F. Both conformations present influx and efflux sites to glucose simultaneously. E and F are arranged in an antiparallel manner. When E isomerizes to F, the sugar binding sites are rearranged in a coupled, antiparallel fashion. In this model, unlike the simple carrier, the carrier can exist as a ternary complex of extracellular sugar:carrier:intracellular sugar. The two site model could represent a dimer of simple carriers in which the sugar binding sites of each monomer are always arranged in an antiparallel manner. Isomerization of one monomer forces the antiparallel isomerization of the second binding site. Tetrameric GLUT1 is proposed to exist as two two-site carriers.
Figure 3

The One-Site (Simple) Carrier

The Two-Site Carrier
model quantitatively predicts the steady-state sugar transport of some cells, it was recognized by Miller in 1968 (Miller, 1968) that key aspects of erythrocyte sugar transport are incompatible with this mechanism. This promoted systematic analyses of red cell sugar transport in which a number of groups ultimately rejected the simple carrier mechanism for erythrocyte hexose transport (Baker & Naftalin, 1979, Eilam & Stein, 1972, Ginsburg & Stein, 1975, Hankin et al., 1972, Holman, 1980, Holman et al., 1981, Lieb, 1982, Lieb & Stein, 1974, Naftalin & Holman, 1977, Stein, 1986). This conclusion stimulated unfavorable criticism and led others to re-examine earlier studies and subsequently to conclude that the simple carrier mechanism is an adequate description for erythrocyte sugar transport (Lowe & Walmsley, 1986, Wheeler, 1986, Wheeler & Whelan, 1988).

These later studies examine steady-state, unrestrained sugar transport in red cells. It had been recognized for some time, however (Baker & Widdas, 1973, Baker & Carruthers, 1981, Krupka & Devés, 1981), and later proven theoretically (Carruthers, 1991) that such analyses often lack the sensitivity to distinguish various transport mechanisms. For example, theory shows that when cellular steady-state sugar transport is consistent with the simple carrier mechanism, it is always compatible by default with a diametric transport mechanism called the simultaneous (Fig. 3) or two-site carrier (Carruthers, 1991). This carrier, unlike the simple carrier, can bind sugars at import and export sites simultaneously (Baker & Widdas, 1973). The only acceptable conclusion these later studies allow is that steady-state red cell sugar transport could be mediated by both simple- and simultaneous-carrier mechanisms.

More sensitive tests of carrier mechanisms examine sugar hetero-exchange (Miller,
1968) or inhibitions of sugar transport produced by the simultaneous presence of reversible competitive inhibitors of sugar import and export (Krupka & Devés, 1981). The results of these analyses are either consistent with simple and simultaneous-carrier models (Krupka & Devés, 1981) or support rejection of the simple carrier (Carruthers & Helgerson, 1991, Helgerson & Carruthers, 1989, Miller, 1968, Naftalin & Rist, 1994, Naftalin et al., 1985). A single analysis of pre-steady-state sugar transport by red cells has been reported (Lowe & Walmsley, 1987) and the results are incompatible with the simple carrier mechanism (Naftalin, 1988).

A plausible, physical explanation for the complex kinetic behavior of the transporter was suggested when the erythrocyte sugar transporter was found to be an allosteric, oligomeric complex of four GLUT1 proteins (Hebert & Carruthers 1991, Hebert & Carruthers 1992). This complex is stabilized by noncovalent subunit interactions but dissociates into GLUT1 dimers upon exposure to reductant. GLUT1 dimers function as prototypical simple carriers in which each subunit contributes a single transport unit which functions independently of its neighbor (Appleman & Lienhard 1989, Hebert & Carruthers 1992). The parental transporter consists of 4 such subunits whose behavior in the mature complex is hypothesized (Hebert & Carruthers, 1992) to be constrained by subunit interactions. Cooperative interactions are suggested to produce a pseudo-D2 symmetry in import and export site orientation within the transporter complex. When one subunit presents an import site, the adjacent subunit must expose an export site and vice versa. In this way, the parental transporter always exposes two sugar import and two sugar export sites although at any instant, the individual subunits contributing specific sites vary.
According to this hypothesis, subunits of the parental transporter complex can now bypass rate-limiting steps in the ping-pong transport cycle and are thus more efficient catalytically than their dimeric counterparts. This hypothesis is supported indirectly by analyses of the catalytic turnover of dimeric GLUT1 (Appleman & Lienhard, 1989) and parental transporter (Lowe & Walmsley, 1986) at 10°C which show that dimeric GLUT1 is some 10-fold more less active than the parental transporter (Hebert & Carruthers, 1992).

This hypothesis has been questioned by Burant and Bell (1992) who conclude that sugar transporters exist functionally as monomeric species in the plasma membrane. However, the conclusion of Burant and Bell was based upon two indirect observations and five untested central assumptions. The observations are: 1) Co-expression of GLUT1 and GLUT3 in Xenopus oocytes results in expression of two kinetically distinct components of sugar uptake; 2) Co-expression of wild-type GLUT3 and a mutagenized (dysfunctional) GLUT3 does not inhibit wild-type GLUT3 function. The untested assumptions were: 1) Post-translational processing of glucose transport proteins is identical in Amphibia and Mammalia; 2) Glucose transporter isoforms and mutants share identical spatial and temporal processing kinetics (i.e. the proteins can oligomerize prior to insertion at the plasma membrane) and/or, 3) Sugar transporters constantly dissociate and reoligomerize at the plasma membrane; 4) Subunits of oligomeric transporters are functionally coupled at all times; 5) Transporter isoforms can form heterocomplexes.

Recent findings invalidate the first 2 assumptions. In contrast to CHO cells (Pessino et al., 1991), GLUT1 and GLUT1/GLUT4 chimeras do not physically associate in Xenopus
oocytes where the kinetics of isoform and mutant transporter processing can differ dramatically (Hresko et al., 1994). Earlier studies from this laboratory (Hebert & Carruthers, 1991, Hebert & Carruthers, 1992) demonstrate that the third assumption is invalid in erythrocytes and CHO cells. A number of studies argue against obligate functional coupling between transporter subunits and thus refute the 4th assumption. For example, dimeric GLUT1 consists of structurally coupled but functionally independent GLUT1 proteins (Hebert & Carruthers, 1991, Hebert & Carruthers, 1992). Expression of dysfunctional GLUT1 mutants by CHO cells can be without effect on parental GLUT1 function (Tamori et al., 1994) or can inhibit parental GLUT1 functions (Mori et al., 1994) - the effect is mutant-specific. The 5th assumption is refuted by earlier studies showing that GLUT1 and GLUT4 do not form heterocomplexes (Pessino et al., 1991).

In the body of work presented in this thesis, we test aspects of the model hypothesized by Hebert and Carruthers (1992). Specifically, we want 1) to understand why the native, noncovalent, homotetrameric GLUT1 complex is sensitive to reductant, 2) to determine whether the tetramer is more catalytically efficient than the dimer in situ, and 3) to test the hypothesis that it is the antiparallel arrangement of substrate binding sites between subunits that provides the transporter with its catalytic advantage. We used biochemical and molecular biological approaches to isolate specific determinants of transporter oligomeric structure and/or transport function in purified isolated transporter preparations, in intact red cells and in CHO cells. We have also examined the hypothesis that net sugar transport in the human erythrocyte is rate limited by reduced cytosolic diffusion of sugars and/or by reversible sugar association with intracellular
Our findings support the hypothesis that each subunit of the parental glucose transporter contains a single intramolecular disulfide bridge between cysteine residues 347 and 421. This disulfide seems to be necessary for GLUT1 tetramerization. Our findings suggest that GLUT1 N-terminal residues 1 through 199 provide contact surfaces for subunit dimerization but are insufficient for subunit tetramerization. Our studies also show that in situ disulfide disruption by cell impermeant reductants results in the loss of cooperative subunit interactions and a 3 to 15-fold reduction the intrinsic efficiency of the transporter. We also find that erythrocyte-resident GLUT1 is susceptible to exofacial proteolysis. Exofacial trypsin cleavage eliminates cooperativity between subunits but does not affect transporter oligomeric structure or transport activity. Thus catalytic efficiency does not derive directly from cooperative interactions between substrate binding sites on adjacent subunits. We have confirmed that 3OMG transport in human erythrocytes is a diffusion limited process. We find that steady-state sugar uptake in red cells and K562 cells measures two processes - sugar translocation and intracellular sugar binding. We propose a model for native GLUT1 structure and function.
Materials and Methods

Materials

[^3]H]-Cytochalasin B, [^3]H]-3-O-Methyl-D-Glucose, [^14]C]-2-Deoxy-D-glucose, [^14]C]-Iodoacetic acid and [^125]I-Protein A were purchased from New England Nuclear. Rabbit antisera raised against a synthetic carboxyl-terminal peptide of GLUT1 (intracellular residues 480-492; C-Ab) were obtained from East Acres Biologicals. Anti-GLUT1 antisera reacting exclusively with extracellular epitopes of GLUT1 (β-Ab) were prepared as described previously (Harrison et al., 1990a). Fluorescein and rhodamine-conjugated secondary antibodies were purchased from Calbiochem. Recently expired human blood was obtained from the University of Massachusetts Medical Center Blood Bank. Reagents were purchased from Sigma Chemicals. Restriction enzymes were purchased from New England Biolabs. Fetal Bovine Serum was purchased from UBI. Sculptor was purchased from Amersham Life Science Inc., and DOTAP from Boehringer Mannheim. Media, trypsin, antibiotics and G418 were purchased from Gibco. CHO-K1, K562 cells and pRSVneo are from American Type Tissue Culture. Endoproteinase glu-C (V8), endoproteinase lys-C (V9) and sequence-grade trypsin were obtained from Boehringer-Mannheim. Staphylococcus aureus α-toxin (type 616392) was purchased from Calbiochem.
Solutions

Saline consisted of 150 mM NaCl, 5 mM TrisHCl, 0.2 mM EDTA, pH 7.4. Lysis medium contained 10 mM TrisHCl, 2 mM EDTA, pH 7.4. Stop solution consisted of 150 mM NaCl, 10 μM cytochalasin B, 100 μM Phloretin and 10 μM HgCl₂ at ice temperature. Tris medium consisted of 50 mM TrisHCl, 0.2 mM EGTA, pH 7.4. Alkaline wash medium contained 2 mM EGTA adjusted to pH 12.0 using NaOH. Cytochalasin stock solutions were made in dimethylsulfoxide. Phloretin stock solutions were made in ethanol. Carrier concentrations in cytochalasin and phloretin-containing solutions were never greater than 0.1%. Size exclusion-HPLC column buffer and sucrose gradient medium consisted of 150 mM NaCl, 5 mM MOPS, 20 mM cholic acid, ± 10 mM dithiothreitol, ± 0.1% SDS, pH 7.2.

Tissue culture

CHO-K1 cells were maintained in F12 medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin in a 37°C humidified 5% CO₂ incubator. K562 cells were maintained in RPMI-1640 medium supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin and 20 mM L-glutamine in a 37°C humidified 5% CO₂ incubator. K562 cells were induced to synthesize hemoglobin by addition of 100 μM hemin (Charnay & Maniatis, 1983).

Mutagenesis of GLUT1 cDNA

A 1.7 kbp BstYI fragment of the human GLUT1 cDNA derived from pLENGT
(Harrison et al. 1990a) was subcloned into the BamH1 cloning sites of M13mp19 (M13-GT1) and pGEM3Z (pGEM3Z-GT1). Oligonucleotide-directed point mutations were introduced by the modified phosphorothioate method of Eckstein (Nakamaye & Eckstein, 1986) using the Sculptor in vitro mutagenesis system. Each of the six cysteines in GLUT1 were mutagenized individually to serine using the following primers:

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>C133S</td>
<td>5'-CATCGGTGTGTACAGCGGCCTG-3'</td>
</tr>
<tr>
<td>C201S</td>
<td>5'-CTGCTGCAGAGCATCGTGCTGCC-3'</td>
</tr>
<tr>
<td>C207S</td>
<td>5'-GCCCTTCAGCCCCGAGAG-3'</td>
</tr>
<tr>
<td>C347S</td>
<td>5'-ATGCGGGGTAAGTCCCATACCTCATG-3'</td>
</tr>
<tr>
<td>C421S</td>
<td>5'-GTGGGGCATGAGCTTCCAGTATG-3'</td>
</tr>
<tr>
<td>C429S</td>
<td>5'-GAGCAACTGTCTGCGGTCC-3'</td>
</tr>
</tbody>
</table>

where the underlined nucleotides are the codon targeted for mutagenesis. The nucleotide substitution is shown in boldface. Mutants were selected by sequencing M13 clones across the following fragments which have unique restriction sites to facilitate subcloning:

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Fragment</th>
</tr>
</thead>
<tbody>
<tr>
<td>C133S</td>
<td>244 bp Hpa I/Apa I</td>
</tr>
<tr>
<td>C201S, C207S</td>
<td>303 bp Apa I/Bst E2</td>
</tr>
<tr>
<td>C347S</td>
<td>294 bp Bst E2/Nhe I</td>
</tr>
<tr>
<td>C421S, C429S</td>
<td>461 bp Nhe I/BamH1</td>
</tr>
</tbody>
</table>
The fragment was excised from M13-GT1, subcloned into pGEM3Z-GT1 and resequenced across these restriction sites to verify the ligation region and the introduced mutation. Finally, wild-type and mutant cDNAs were subcloned into the pCMV5 expression vector (Anderson et al., 1989).

**Stable Expression of Wild Type and Mutant GLUT1 cDNAs in CHO-K1 Cells.**

Subconfluent CHO-K1 cells were co-transfected with GLUT1 cDNA and pRSVneo using the cationic lipid transfection reagent DOTAP (Leventis & Silvius, 1990). The transfected cells were grown in F12 media containing 250 μg/mL G418. G418-resistant cells were ring-cloned and expanded (Freshney, 1994). Clones expressing wild-type or mutant glucose transporters were identified by immunofluorescence and by Western blot analysis of total cellular membranes prepared as described previously (Harrison et al., 1990a) and blotted using C-Ab. CHO-cell plasma membranes and low density microsomal membranes were isolated by homogenization and sucrose-cushion centrifugation exactly as described by Harrison et al., 1990b).

**Red cells and red cell ghosts.**

Red cell ghosts were prepared from washed, intact red cells as described by Helgerson et al. (1989). Human red cells were collected from recently expired, whole human blood (University of Massachusetts Medical Center Blood Bank) by suspension in ice-cold saline (25 volumes of saline: 1 volume whole blood) followed by centrifugation at 1100 x g for 5 minutes. The supernatant fluid and buffy coat were aspirated and the red cell pellet resuspended in saline. This wash/centrifugation procedure was repeated until the superna-
tant fluid was clear and the red cell pellet lacked any discernible buffy coat (normally 3 to 4 cycles). The washed red cells were then resuspended in 100 volumes of saline and allowed to rest at room temperature for 1 hr. to deplete intracellular D-glucose. D-glucose-depleted cells were harvested by centrifugation to a final hematocrit of 80-90%.

Red cell ghosts were depleted of peripheral membrane proteins by a single wash (Carruthers, 1986a) in 5 volumes of alkaline wash medium (4°C, 20 min). Membranes were collected by centrifugation (14,000 x g for 5 min at 4°C) 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 membrane protein per mL and stored at -70°C.

When ghosts were resealed in media containing high concentrations of glycerol, the same concentration of glycerol was included in washing media to prevent subsequent hypotonic lysis.

**Glucose Transport Protein**

GLUT1 plus endogenous lipid were purified from human erythrocytes as described by Hebert and Carruthers (1992) or, in the presence of DTT as described by Cairns et al. (1984). The glucose transporter was purified from outdated human blood (Baldwin et al. 1982, Cairns et. al. 1984). All of the following operations were performed at 4°C. Red blood cells are obtained by washing one unit of blood three times with ice-cold 150 mM KCl, 0.2 mM EDTA adjusted to pH 7.4 using Tris base. The red cells are pelleted by centrifugation at 27,000 x g for 20 minutes and the buffy coat (white cells) and serum removed by aspiration. Erythrocyte membranes (ghosts) are prepared from washed red
blood cells by the method of Steck & Kant (1974). Red blood cells are lysed in 40 volumes of ice-cold lysis medium containing 10 mM Tris-HCl, 4 mM EDTA adjusted to pH 8.0 with Tris base. The membranes are pelleted by centrifugation at 27,000 x g for 20 minutes. The pellet is resuspended in lysis medium and pelleted again. This process is repeated approximately seven times to remove all the cytosolic hemoglobin or until the membranes appear white. Protein concentration of the membranes is determined by the Pierce BCA assay using bovine serum albumin as the standard. The protein content of the membrane pellet is adjusted to 4 mg/mL with 50 mM Tris-HCl adjusted to pH 7.4 with Tris base. Peripheral membrane proteins are stripped from the membranes by alkaline extraction. Five volumes of 2 mM EDTA, 15.4 mM NaOH, pH 12.0 are added to the 4 mg/mL membrane fraction and the suspension is incubated for 10 minutes without agitation. The membranes lacking peripheral proteins (stripped ghosts) are pelleted at 27,000 x g for 20 minutes. The pH of the membrane fraction is restored by washing three times with 50 mM Tris-HCl pH 7.4. The protein concentration of the membrane fraction is adjusted to 2 mg/mL with 50 mM Tris-HCl pH 7.4. Integral membrane proteins are released from the stripped membranes by solubilization in 46 mM octylglucoside while rotating for 30 minutes. The detergent solubilized membrane fraction is ultracentrifuged at 100,000 x g for 60 minutes in a Beckman Ti 50.1 rotor and the resulting pellet is discarded. The supernatant is adjusted to 25 mM NaCl. A sample (60 mL) of supernatant is applied to a column (2.5 cm x 8.0 cm) of DEAE-cellulose (Whatman DE-52) equilibrated with 34 mM octylglucoside, 25 mM NaCl, 50 mM Tris-HCl adjusted to pH 7.4 with Tris base. The flow rate was adjusted such that the supernatant is in contact with the column for 45 minutes.
The immediate flow through is collected and subsequently up to one and a half volumes (90 mL) of the added supernatant volume. The transporter is largely hydrophobic and contains very few charged residues at pH 7.4, thus is not retarded by the anion exchange column. Other integral membrane proteins liberated from the membrane by octylglucoside solubilization (anion transporter) are more extensively charged and therefore bind to the column. The flow-through material containing GLUT1 and red cell lipid is dialyzed two times against 6 liters of 50 mM Tris-HCl, 2 mM EDTA, pH 7.4 to remove more than 99% of the detergent. This results in a glucose transporter preparation in which the protein is inserted into its native lipid bilayer. The preparation is characterized by SDS-PAGE (Lamelli, 1970) and protein assay. Routinely 20 mg of 95% purified glucose transporter, as assayed by SDS-PAGE, is obtained from one unit of blood. The main contaminants observed are the nucleoside transporter (<2%) and band 7 (<4%, see Fig. 4), an Rh antigen thought to be involved in erythrocyte membrane integrity (Cherif-Zahar et al. 1990). GLUT1+DTT is prepared by adding 10 mM DTT during the alkaline treatment of ghosts and to all other steps that follow.

ELISA

ELISA and competition ELISA were performed using C- and/or δ-Ab by a modification of the method of Sogin and Hinkle as described by Hebert and Carruthers (1992). ELISA were performed using C-Ab or δAb. Rabbit and sheep δ-Ab has been characterized and found to react with an epitope presented on the exofacial portion of the protein (Harrison et al. 1990) whereas C-Ab reacts with the COOH terminus of the protein located on
the cytosolic portion of the transporter. The protein is adsorbed to a 96 well microtiter plate in 60 mM NaCO₃, pH 9.6 for 1-16 hours at 37°C. The wells are each washed three times with phosphate buffer (20 mM sodium phosphate, 145 mM NaCl, 0.05% Tween-20, pH 7.5) and then incubated with a 2,500-fold dilution of the respective antisera in phosphate buffer for 90 minutes at 37°C. The reporter molecule employed to detect rabbit IgG binding to carrier was goat anti-rabbit IgG-peroxidase conjugate (Sigma, 10,000-fold dilution). Bio-Rad peroxidase substrate kits were employed. Substrate development was arrested after the necessary time using 1% oxalic acid and product determined by detecting absorbance at 410 nm using a Dynatech MR-700 plate reader. Nonspecific IgG binding was determined by processing wells lacking glucose transport protein.

In some cases, a modified ELISA was also employed omitting the detergent (Tween-20) to avoid potential denaturation of carrier. Bovine serum albumin (1% BSA) was used in place of Tween-20 to reduce nonspecific adsorption of IgGs to wells. In addition, after absorption of the transporter to the microtiter plate 200 mL of 1% BSA in carbonate buffer is applied to the wells and incubated for 1 hour at 37°C. The plate is then processed as described above.

**Nondenaturing chromatography of GLUT1**

Size exclusion chromatography-HPLC studies of GLUT1-containing, cholate-solubilized membranes were performed using a Toso Haas TSK-Gel G4000 SWXL column as described previously (Hebert & Carruthers, 1992). Briefly, the column was pre-equilibrated with 150 mM NaCl, 5 mM Mops, pH 7.2 containing 20 mM cholic acid ±10 mM
DTT using a Waters HPLC system and developed at a flow rate of 0.3 mL/min.

The column was calibrated using Pharmacia low and high molecular weight protein gel filtration calibration kits. The calibration is: \( \log(\text{Stokes Radius in nm}) = 1.551 - 1.801 \times K_{\text{av}} \) \((R^2 = 0.9714)\).

Samples were solubilized in elution buffer containing 50 mM cholic acid \( \pm 10 \) mM DTT for 1 hr at room temperature. The 200,000 x g (1 hr) supernatant containing cholate-solubilized proteins was added to the column and fractions were collected at 1 minute intervals. Fractions were assayed by ELISA.

**Cytochalasin B binding studies**

Equilibrium, D-glucose (400 mM)-inhibitable \(^3\text{H}\)-Cytochalasin B binding to purified GLUT1 was determined as described previously (Hebert & Carruthers, 1992). Equilibrium cytochalasin B binding to red cells and red cell ghosts was measured as described in (Helgerson & Carruthers, 1987). Cytochalasin D (10 \( \mu \)M) was included in equilibrium cytochalasin B binding experiments to inhibit saturable cytochalasin B binding to non glucose transporter sites (Jung & Rampal, 1977). DTT-containing cytochalasin B solutions were made freshly for each experiment since it was observed that prolonged exposure (> 24 hr) of cytochalasin B to DTT at -70°C resulted in the irreversible loss of cytochalasin B binding to both nonreduced and reduced GLUT1.

**Determination of GLUT1 free sulphydryl and disulfide content**

Reduced and nonreduced, purified GLUT1 (each at approximately 100 \( \mu \)g/mL in 1
mL and exhaustively dialyzed to remove traces of DTT) were denatured in 0.5% SDS in 50 mM TrisHCl, 3 mM EDTA, pH 8.0 for 30 min. The solubilized protein was collected as supernatant by centrifugation (100,000 x g for 1 hr at 4°C and quantitated. The free sulfhydryl contents of reduced and nonreduced GLUT1 were determined by using Ellman’s reagent (Rao & Scarborough, 1990). The reaction was initiated by addition of 100 µl freshly prepared 10 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB). The course of the reaction at room temperature was recorded as the increase in absorbance at 412 nm. GLUT1 disulfide content was measured by the 2-nitro-5-thiosulfobenzoate (NTSB) assay in which disulfides are cleaved by sulfitolysis and the resulting free sulfhydryls quantitated (Rao & Scarborough, 1990). An extinction coefficient of 13,600 M⁻¹ cm⁻¹ at 412 nm for 2-nitro-5-thiobenzoic acid (NTB) was used to quantitate both reactions. All NTSB assays included the parallel, internal controls of porcine insulin (3 disulfides per molecule) and oxidized glutathione (1 disulfide per molecule).

The NTSB assay allows us to determine if the inaccessible cysteines in nonreduced SDS denatured GLUT1 are present as mixed disulfides (e.g. GLUT1-glutathione disulfide) or as internal disulfides. After the disulfide bonds are cleaved by sulfitolysis, the type of disulfide is identified by the following reactions:

**Internal Cysteine Disulfide**

\[
R\text{-}S\text{-}S\text{-}R + SO_3^{2-} \leftrightarrow R\text{-}S\text{-}SO_3^- + RS^- \\
RS^- + NTSB \rightarrow RSSO_3^- + NTB
\]

1 mol NTB: 2 mol SH
Mixed Disulfide

\[
R\cdot S\cdot S\cdot G + SO_3^{2-} \leftrightarrow R\cdot S\cdot SO_3^- + GS^-
\]

\[
GS^- + NTSB \rightarrow GSSO_3^- + NTB
\]

1 mol NTB: 1 mol internal S

Thus for each internal disulfide bond and for each free sulphydryl, one molecule of NTB is generated.

GLUT1 alkylation/proteolysis

The procedure of Rao and Scarborough (1990) was used to label either all cysteine residues or free cysteine residues of GLUT1 purified in the absence of DTT. Briefly, GLUT1 (1 mg/mL) was unfolded in SDS, reduced (or not reduced) and alkylated using 0.6 mM \(^{[14]C}\)-iodoacetic acid (incubation was in 0.2 M Tris-Cl, 2 mM EDTA, 1% SDS, pH 8.3 for 25 min at room temperature in the dark). Alkylation was terminated by addition of 2-mercaptoethanol (0.5%; 64 mM) and free label was removed by extensive dialysis. Labelled GLUT1 was electrophoresed on a preparative urea-acrylamide gel in the dark. This gel consisted of 9M urea, 11% acrylamide, 375 mM Tris-HCl, pH 8.8, and 0.2% SDS. Samples were solubilized in an equal volume of sample buffer and electrophoresed at 3W constant power. Gels were rinsed for 20 min in deionized water to remove urea then vacuum dried. Dried gels were exposed overnight to X-ray film to locate the radiolabelled GLUT1.
Peptide mapping of labelled GLUT1 peptides was performed either as described previously (Carruthers and Helgerson, 1989) or with the following modifications. Pieces of dried gel (5 x 8 mm) were excised from the preparative gel and inserted into wells of a stacking gel containing sample buffer and 8 µg V8 protease. The dried gel piece was allowed to soak up the buffer and running buffer was then used to cover the well. Samples were electrophoresed into the stacking gel and the power was interrupted for one hour to allow proteolytic digestion of GLUT1. Power was resumed and the peptides were allowed to separate in the 18% gel. Electrophoresis was performed in the dark. Peptides were then transferred to Immobilon-P membrane filters for immunoblot analysis or for staining with Coomassie Brilliant Blue R and subsequent sequence analysis. When peptides were isolated in this manner for N-terminal sequence analysis, acrylamide gels were pre-run and were electrophoresed in the presence of 1 mM thioglycolate. Parallel lanes were also run for visualization of alkylated peptides by autoradiography.

**Immunofluorescence microscopy of cells**

For red blood cells, circular coverslips were washed in 70% ethanol, 1% HCl for 1 hr, oven-dried, immersed in a 10% solution of polylysine for 5 minutes then air-dried overnight. Washed erythrocytes (10 µl, 50% hematocrit) were pipetted onto polylysine-coated coverslips positioned above 5 mL saline in 50 mL Falcon tubes. These tubes were then centrifuged at 1100 x g for 5 min and unattached cells were removed by addition and subsequent aspiration of saline (20 mL). Two additional saline washes were made then the erythrocyte-coated coverslips were transferred to 6 well tissue culture dishes for
immunohistochemical processing.

Antibody binding to cells was measured in cells that were either unfixed or fixed and permeabilized. Fixation was for 15 min in saline containing 4% paraformaldehyde followed by 2 washes in saline containing 1% fetal bovine serum. Fixed cells were permeabilized by 15 min incubation at room temperature in saline containing 0.05% Triton X-100 and 1% FBS followed by 4 washes in saline containing 1% FBS. Fixed and unfixed cells were then incubated for 60 min in saline containing 1% FBS plus C-Ab (1:5000 dilution of crude serum) and/or plus \( \partial \)-Ab (1:5000 dilution of crude serum). Cells were washed 3 times in FBS saline then incubated for 30 min with rhodamine-conjugated goat anti-rabbit (anti-CAb) IgGs (4 \( \mu \)g/mL) or with fluorescein-conjugated rabbit anti-sheep (anti-\( \partial \)-Ab) IgGs (4 \( \mu \)g/mL). In experiments where both C-Ab and \( \partial \)-Ab binding were measured simultaneously, cells were subsequently exposed to rhodamine-conjugated goat anti-rabbit (anti-CAb) IgGs followed by washing and exposure to fluorescein-conjugated rabbit anti-sheep (anti-\( \partial \)-Ab) IgGs. Cells were then washed 3 times in saline, the coverslips mounted on a slide with 2.5% DABCO (Sigma Chemicals), 90% glycerol and the slides sealed with clear nail polish.

Erythrocyte anion transporters were labelled exofacially using fluorescein isothiocyanate as described by (Matayoshi & Jovin, 1991, Sato et al., 1985). These cells were then attached to polylysine coated coverslips for subsequent fluorescence microscopy. In some experiments, red cells were not attached to coverslips but were processed in dilute solution and mounted later for immunofluorescence microscopy.

CHO cell lines were plated directly onto sterile coverslips in 6-well plates 48 hours
prior to processing. Cells were washed three times with PBS and fixed for 15 min at room temperature with 2\% paraformaldehyde in PBS with or without preincubation in 5mM DTT for 15 min at room temperature. Concentrations of paraformaldehyde above 2\% permeabilized the cells. Subsequent washes were done with PBS plus 1\% FBS. After fixing, cells were solubilized with 0.05\% Triton X-100 for 15 min at room temperature. Fixed, solubilized cells were then incubated for 1 hour at room temperature with PBS containing 1\% FBS, rabbit C-Ab (1:5000 dilution of crude serum), and/or sheep d-Ab (1:5000 dilution of crude serum). The cells were washed and incubated for 30 min at room temperature in PBS containing 1\% FBS, goat anti-rabbit IgG-Rhodamine (4 \text{ \mu g/mL} dilution) and then for 30 min at room temperature in PBS containing 1\% FBS, rabbit anti-sheep IgG-FITC (4 \text{ \mu g/mL}). The coverslips were washed extensively, mounted in 90\% glycerol, 2.5\% DABCO and sealed with clear nail polish.

Samples were visualized by fluorescence microscopy using a Nikon Diaphot 200 microscope with a Nikon Apo 60/1.4 oil immersion lens. Images were digitized with 14-bit resolution using a thermoelectrically cooled CCD camera (Photometrics Ltd., Tuscon, AZ) and stored on magnetic media for subsequent analysis.

Reconstitution of GLUT1.

GLUT1 was purified from human erythrocyte membranes in the absence of reductant (Hebert & Carruthers, 1992) and was reconstituted into egg phosphatidylcholine large unilamellar vesicles (diameter \(\approx 2 \mu m\) as judged by phase contrast microscopy) by cholate dialysis (Hebert & Carruthers, 1991, Zeidel et al., 1992). Equilibrium ligand (sugar or cy-
tochalasin B) binding to reconstituted GLUT1 proteoliposomes was measured by monitoring ligand-induced quenching of GLUT1 intrinsic tryptophan fluorescence (Hebert & Carruthers, 1992). GLUT1-mediated D-glucose uptake by reconstituted proteoliposomes was measured by light scattering analysis of sugar transport-induced changes in proteoliposomal volume (Carruthers & Melchior, 1983, Carruthers & Melchior, 1984a, Carruthers & Melchior, 1984b). The orientation of GLUT1 in reconstituted, sealed proteoliposomes was determined by use of antibodies that react with endofacial (C-Ab) or exofacial (d-Ab) epitopes of erythrocyte GLUT1 as described previously (Zeidel et al., 1992). Our results (63% right side out: 37% inside out) are consistent with previous demonstrations of random GLUT1 orientation upon reconstitution (Baldwin et al., 1980, Carruthers & Melchior, 1984a, Zeidel et al., 1992).

Proteolysis of GLUT1.

Packed red cells (0.5 mL) were suspended in saline to a final volume of 1.5 mL. Samples were warmed to 37°C for 10 min. and to this mixture, either saline or trypsin (final concentration ≤ 1 mg/mL) was added. Cell slurries were mixed by end over end rotation and incubated at 37°C for 30 min. Reactions were terminated by addition of 12 mL saline containing 0.2 mM PMSF. Cells were collected by centrifugation, resuspended in PMSF-saline then stored on ice until use.

Immunoblot analysis of GLUT1.

Membranes were collected by centrifugation and resuspended in 50 mM Tris-HCl,
pH 7.4. Proteins were resolved on either 10% or 15% gels as described previously (Laemmli, 1970), transferred to Immobilon P membrane filters, and subjected to Western analysis using C-Ab and \[^{125}\text{I}]\)-protein A as reporter molecules. Filters were dried and exposed to Kodak XAR-5 film at -70°C for 2 to 48 hours using a DuPont Cronex Lightning Plus intensifying screen. Resulting autoradiograms were quantitated by densitometry using a Hoefer GS300 Transmittance/Reflectance Scanning Densitometer in combination with the GS-370 Data System (Apple Macintosh Version). Peaks were integrated and expressed as a percentage of total transmittance.

**Photolabelling GLUT1 using \[^{3}\text{H}]\)-cytochalasin B.**

Red cells were preincubated in saline containing \[^{3}\text{H}]\)-cytochalasin B (3.7 \(\mu M\), 50 \(\mu \text{Ci}\)) plus 10 \(\mu M\) cytochalasin D ± D- or L-glucose (200 mM) for 20 minutes on ice. The suspension was irradiated at 300 nm for 0.5 min. in a Rayonet Photoreactor. Cells were collected by centrifugation and washed by resuspension in 10 volumes of saline. The centrifugation/wash cycle was repeated twice.

**Cytochalasin B binding to erythrocyte membranes.**

Equilibrium \[^{3}\text{H}]\)-Cytochalasin B binding measurements were as described by Helgerson and Carruthers (1987). The cytocrit of the final suspension of ghosts in \[^{3}\text{H}]\)-cytochalasin B solution was 33%. Initial studies of maltose-inhibition of cytochalasin B binding to intact cells used Sigma plant-biology grade sucrose as the osmotic substitute or
control for extracellular maltose. This source, however, was discovered to be significantly contaminated with D-glucose - a GLUT1 substrate that acts as a competitive inhibitor of exofacial maltose binding to GLUT1 and, following its transport into the cell, as a competitive inhibitor of cytochalasin B binding to GLUT1. Subsequent experiments used Sigma Sucrose ACS grade. In experiments where cytochalasin B binding was measured in the presence of DTT, solutions were made daily.

Quantitation of antibody binding to trypsinized red cell GLUT1.

Two methods were used: immunofluorescence microscopy and direct binding measurements using $^{125}$I-Protein A as the reporter molecule (Harrison et al., 1990, Diamond & Carruthers, 1993). $\partial$-Ab IgGs were also affinity purified using 500 $\mu$g purified GLUT1 that was additionally gel-purified by reducing, SDS PAGE and subsequently transferred to immobilon. That region of the blot containing intact GLUT1 ($M_r$) 45 - 68 kDa) was excised, blocked using 3% gelatin then incubated with $\partial$-Ab for 2 hr at room temperature as per Western blotting protocols (Harrison et al., 1990, Diamond & Carruthers, 1993). The membrane was washed, and IgGs eluted using 100 mM glycine (pH 2.5, 10 min incubation). The buffer was removed, neutralized using one-tenth volume 1 M Tris (pH 8.0) and dialyzed overnight against 100 volumes of saline. This was used for subsequent binding studies using $^{125}$I-protein A or ELISA. Our measurements show that 87 \pm 4\% ((208,721 \pm 9276) cpm versus (240,778 \pm 1655) cpm) of the red cell binding activity in 5 $\mu$L of $\partial$-Ab serum is recovered following $\partial$-Ab purification using denatured, reduced, intact GLUT1 as the affinity matrix. ELISA indicates superimposable $\partial$-Ab and $\partial$-Ab-IgG titration curves.
when the concentration of recovered material is adjusted for dilution during purification.

For immunofluorescence microscopy, cells were first attached to polylysine-coated coverslips. Circular coverslips were washed in 70% ethanol, 1% HCl for 1 hr, oven-dried, immersed in a 10% solution of polylysine for 5 minutes then air-dried overnight. Washed erythrocytes were exposed to saline ± trypsin (0.5 mg/mL) for 30 min at 37°C then washed free of trypsin. These cells (10 µL, 50% Ht) were pipetted onto polylysine-coated coverslips positioned above 5 mL saline in 50 mL Falcon tubes. These tubes were then centrifuged at 1100 x g for 5 min and unattached cells were removed by addition and subsequent aspiration of saline (20 mL). Two additional saline washes were made then the erythrocyte-coated coverslips were transferred to six well tissue culture dishes for immunohistochemical processing.

Cover slips with attached control and trypsin-treated cells were exposed to saline ± 2 mM dithiothreitol (pH 7.4) for 30 min at 37°C. At this time, cells were fixed by addition of 4% paraformaldehyde (15 min) then washed twice in saline. Fixed cells were permeabilized by 15 min incubation in saline containing 0.05% Triton X-100 and 1% fetal bovine serum (FBS) followed by 4 washes in saline containing 1% FBS. Cells were incubated for 60 min in saline containing 1% FBS and either C-Ab (1:5000 dilution of crude serum) or ð-Ab (1:5000 dilution of crude serum), washed 3 times in FBS saline then incubated for 30 min with fluorescein-conjugated goat-anti rabbit IgGs (4 µg/mL). Coverslips were washed 3 times in saline, mounted on a slide with 2.5% DABCO (Sigma Chemicals), 90% glycerol and the slides sealed with clear nail polish.
Samples were visualized by fluorescence microscopy using a Nikon Diaphot 200 microscope with a Nikon Apo 60/1.4 oil immersion lens. Images were digitized with 24 bit resolution using a thermoelectrically cooled CCD camera (Photometrics Ltd., Tuscon, AZ) and stored on magnetic media for subsequent analysis.

3-O-methyl-D-glucose uptake by erythrocytes.

Zero-trans 3-O-Methyl-D-Glucose uptake and efflux was measured as described previously (Helgerson et al., 1989). Briefly, sugar-free red cells (at ice temperature) were exposed to 5 volumes of saline (ice temperature) containing variable [3OMG] and fixed [(3H]-3OMG]. Uptake was permitted to proceed for selected time points then 50 volumes (relative to cell volume) of stopper solution were added to the cell suspension. Cells were sedimented by centrifugation (14,000 x g for 15 sec at 4°C, washed twice in stopper, collected by centrifugation (14,000 x g for 15 sec at 4°C and extracted in 1 mL 3% perchloric acid. The acid extract was centrifuged (14,000 x g for 15 sec at 4°C and duplicate samples of the clear supernatant fluid were counted. Zero-time uptake points were prepared by addition of stopper to cells prior to addition of medium containing sugar and radiolabel. Cells were immediately processed. Radioactivity associated with cells at zero-time was subtracted from the activity associated with cells following the uptake period. All uptakes were normalized to equilibrium uptake where cells were exposed to sugar medium at 37°C for 30 min. prior to addition of stopper. This procedure limits the experimental 3OMG space of the cells to 10% or lower. CHO cell 2-deoxy-D-glucose uptake was measured at 22°C, in 24-well dishes using 100 μM 2-deoxy-D-glucose as described previously.
(Harrison et al., 1990a). Protein-mediated sugar uptake is defined as total sugar uptake less uptake measured in the presence of saturating levels (50 μM) of cytochalasin B, a noncompetitive inhibitor of uptake.

3-O-methyl-D-glucose exit from erythrocytes was measured as described by Helgerson and Carruthers (1989). Briefly, cells (500 μL of 30% Ht) are loaded with 0.1 mM 3-O-methyl-D-glucose for 30 minutes at 37°C. Then [3H]-3OMG (10 μCi/mL) is added to the suspension. Cells are incubated for an additional 20 minutes then sedimented by centrifugation. The cells are washed three times in 100 volumes of ice-cold saline. Then sugar exit is initiated by injecting the cells (10 μL) into 500 μL saline. At various times (0, 10, 30, 60 and 120 seconds), 1 mL of ice-cold stopper solution is added and the cells are processed as for uptake determination. Equilibrium exit is determined by allowing exit to proceed for 40 minutes at 37°C.

**Time course of net 3-O-methyl-D-glucose uptake by erythrocytes and K562 cells**

3-O-methyl-D-glucose uptake was measured as described previously (Carruthers et al., 1989). K562 cells are not adherent and may thus be manipulated like erythrocytes. Sugar-free cells (at ice temperature) were exposed to 5 volumes of saline (ice temperature) containing 50 μM 3OMG plus [3H]-3OMG. Uptake was permitted to proceed over intervals as short as 5 sec to intervals as long as 3 hr then 50 volumes (relative to cell volume) of stopper solution were added to the cell suspension. Cells were sedimented by centrifugation, washed once in stopper, collected by centrifugation and extracted in 1 mL 3% perchloric acid. The acid extract was centrifuged and duplicate samples of the clear
supernatant fluid were counted. Zero-time uptake points were prepared by addition of stopper to cells prior to addition of medium containing sugar and radiolabel. Cells were immediately processed. Radioactivity associated with cells at zero-time was subtracted from the activity associated with cells following the uptake period. All uptakes were normalized to equilibrium uptake where cells were exposed to sugar medium at 37°C for 30 min. prior to addition of stopper. Uptake assays were performed using solutions and tubes pre-equilibrated to 4°C and were carried out in a thermostatically cooled block.

In experiments where red cells were permeabilized using a-toxin, it is impossible to wash cells following sugar uptake without rapid loss of intracellular sugar. Here, sugar uptake was measured as the disappearance of sugar from the incubation medium. Cells (permeabilized or non permeabilized) were resuspended at zero-time to a final hematocrit of 50% in saline containing 50 µM [3H]-3OMG. Cells were sedimented at specific intervals by rapid centrifugation and samples (10 µl) of the supernatant counted. Parallel experiments were also made using [14C]-L-glucose (a nontransported sugar).

Equilibrium exchange 3-O-methyl-D-glucose uptake by erythrocytes

In these experiments, intracellular [3OMG] = extracellular [3OMG] and the rate of cellular equilibration with extracellular tracer radiolabelled 3OMG is monitored. The uptake assay is otherwise identical to net sugar uptake measurements. Cells were pre-equilibrated with varying [3OMG] (0 to 100 mM) by incubation in 10 volumes of equilibration medium for 1 hr at 37°C. Cells were collected by centrifugation, resuspended in 1 volume ice-cold equilibration medium and, following equilibration to
4°C, uptake was initiated by addition of labelled 3OMG. Stopper contained sucrose at a concentration identical to intracellular [3OMG].

**Calculation of net uptake rate constants**

All constants were computed by direct curve-fitting procedures using nonlinear regression of untransformed data. The software package used was KaleidaGraph 3.0 (Synergy Software, Reading PA). For radiolabelled sugar uptake, all data were fitted to the equation:

\[
\text{cpm}_t = \text{cpm}_0 + \text{cpm}_1(1 - e^{-k_1 t}) + \text{cpm}_2(1 - e^{-k_2 t})
\]

(eqn 1)

where \(\text{cpm}_t\) and \(\text{cpm}_0\) are the counts associated with the cells at times \(t\) and zero-time respectively, \(\text{cpm}_1\) and \(\text{cpm}_2\) are equilibrium counts associated with cell compartments 1 and 2 respectively and \(k_1\) and \(k_2\) are first order rate constants describing the rate of equilibration of compartments 1 and 2 respectively. Where only a single cellular compartment for sugar penetration was presented, this analysis provides unique solutions for \(\text{cpm}_1\) and \(\text{cpm}_2\) but computes statistically indistinguishable solutions to \(k_1\) and \(k_2\). In experiments where multiple cellular compartments are apparent, increasing the number of cellular compartments to 3 or greater does not statistically improve the quality of the computed fit.

For sugar exit experiments, all data were fitted to the equation:

\[
\text{cpm}_t = \text{cpm}_e + \text{cpm}_1 e^{-k_1 t} + \text{cpm}_2 e^{-k_2 t}
\]

(eqn 2)

where \(\text{cpm}_t\) and \(\text{cpm}_e\) are the counts associated with the cells at times \(t\) and equilibrium
respectively, cpm\textsubscript{1} and cpm\textsubscript{2} are equilibrium counts associated with cell compartments 1 and 2 respectively and k\textsubscript{1} and k\textsubscript{2} are first order rate constants describing the rate of loss of sugar from compartments 1 and 2 respectively. In the exit experiments reported here, only a single cellular compartment is apparent since the rate constants k\textsubscript{1} and k\textsubscript{2} are indistinguishable.

**Calculation of transport and ligand binding parameters**

\(V_{\text{max}}\) and \(K_{m(app)}\) for 3OMG uptake were computed by direct, nonlinear regression analysis of the concentration dependence of 3OMG uptake assuming Michaelis-Menten kinetics. \(K_{d(app)}\) and \(B_{\text{max}}\) for ligand (cytochalasin B and D-glucose) binding to GLUT1 were computed in a similar manner. Here, however, we modeled the data assuming two saturable components of ligand binding. When the computed \(K_{d(app)}\) for binding to the first saturable component was statistically indistinguishable from \(K_{d(app)}\) for binding to the second saturable component, we concluded that binding was described by a single saturable process. Otherwise, we concluded the assumption of multicomponent saturable binding is correct. In these instances, addition of a third or fourth saturable component to the analysis did not statistically improve the computed fit. The software package used was KaleidaGraph 3.0 (Synergy Software, Reading PA).

**Analytical Procedures**

Protein was determined by the Pierce BCA procedure (Brown et al., 1989). SDS-slab (10 or 18%) PAGE of membrane proteins and GLUT1 were as described previously
(Carruthers & Helgerson, 1989). Immuno- (Western) blotting of proteins using $\delta$- or C-Ab was as described in (Harrison et al., 1990a). Immune serum ($\delta$-Ab) specific $[^{125}I]$-Protein A binding to erythrocyte membranes was determined as described previously (Harrison et al., 1990a). Amino acid and N-terminal sequence analysis of immobilon membrane-bound peptides was performed by Dr. John Leszyk, Director of the Core Laboratory for Protein Chemistry at the Worcester Foundation for Experimental Biology, Shrewsbury, MA. Densitometric analysis of autoradiograms was carried out using either a Hoefer GS300 Transmittance/Reflectance Scanning Densitometer in combination with the GS-370 Data System (Apple Macintosh Version) or by using the software package NIH Image (v 1.5.7, National Institutes of Health) to analyze autoradiograms digitized at 8-bit resolution.
Chapter III

Molecular Determinants of GLUT1 Structure

Why is the native, noncovalent, homotetrameric GLUT1 complex sensitive to reductants?

The molecular interactions that stabilize tetrameric GLUT1 are only partly understood. Exposure to reductant causes tetrameric GLUT1 to dissociate into GLUT1 dimers but subunits of tetrameric GLUT1 are not attached via disulfide bridges (Hebert and Carruthers, 1992). Each subunit of SDS-denatured tetrameric GLUT1 exposes only 2 thiols (Hebert & Carruthers, 1992) while reduced denatured GLUT1 exposes all 6 sulfhydryl groups (Baldwin et al., 1982, Hebert & Carruthers, 1992). This suggests that glucose transporter oligomeric structure and cooperative catalytic function result from noncovalent interactions promoted or stabilized by intramolecular disulfide bridges.

We have used both biochemical and molecular biological approaches to test this hypothesis. We have confirmed the results reported by Hebert and Carruthers (1992) with Ellman’s reagent. We further show that assays of subunit disulfide bridge content suggest that two inaccessible sulfhydryl groups form an internal disulfide bridge. Differential alkylation/peptide mapping/N-terminal sequence analyses show that a GLUT1 carboxyl-terminal peptide (residues 232 to 492) contains 3 inaccessible sulfhydryl groups and that an N-terminal GLUT1 peptide (residues 147 to 261/299) contains 2 accessible thiols. The carboxyl-terminal peptide most likely contains the intramolecular disulfide bridge since
neither its yield nor electrophoretic mobility are altered by addition of reductant. Each GLUT1 cysteine was changed to serine by oligonucleotide-directed, in vitro mutagenesis. The resulting transport proteins were expressed in CHO cells and screened by immunofluorescence microscopy for their ability to expose tetrameric-GLUT1 specific epitopes. Serine substitution at cysteine residues 133, 201, 207 and 429 does not inhibit exposure of tetrameric-GLUT1 specific epitopes. Serine substitution at cysteines 347 or 421 prevents exposure of tetrameric-GLUT1 specific epitopes. Hydrodynamic analysis of GLUT1/GLUT4 chimeras expressed in and subsequently solubilized from CHO cells indicates that GLUT1 residues 1 to 199 promote chimera dimerization and permit GLUT1/chimera hetero-tetramerization. This GLUT1 N-terminal domain is insufficient for chimera tetramerization which additionally requires GLUT1 residues 200 to 463.

We conclude from these studies that each subunit of the glucose transporter contains an extracellular disulfide bridge (cys347 and cys421) that stabilizes transporter oligomeric structure. The N-terminal half of the transporter (residues 1 to 199) contains motif(s) which promote transporter dimerization.

Results

GLUT1 sulfhydryl chemistry

The purified GLUT1 preparation contains red cell lipid (Hebert and Carruthers, 1991), GLUT1 (M_r(app) = 55K; Fig. 4 lanes 2 and 3) and red cell Band 7 or RhD protein (M_r(app) = 33K, peptide a of Fig. 4, lanes 2 and 3). Amino acid composition analyses of
these proteins following electrophoretic transfer to immobilon membranes indicates that the molar ratio of GLUT1: Band 7 present in the GLUT1 preparation is 1: 0.04. This is consistent with our frequent but qualitative observation that GLUT1 is stained very poorly by Coomassie.

SDS-unfolded, nonreduced, purified GLUT1 exposes 2 thiol groups per GLUT1 monomer (Table I) and is resolved as a monomer during nonreducing SDS PAGE (Fig. 4, lanes 2 and 3). Reduced GLUT1 exposes 6 sulphydryl groups per GLUT1 monomer (Table I). Because the deduced cysteine-content of GLUT1 is 6 residues per polypeptide (Mueckler et al., 1985), this raises the possibility each GLUT1 molecule contains as many as two intramolecular disulfide bridges. We assayed nonreduced GLUT1 disulfide content by using the 2-nitro-5-thiosulfobenzoate (NTSB) assay in which disulfides are cleaved by sulfitolysis and the resulting free sulphydryls are quantitated by measuring the release of nitro-5-thiobenzoate (Rao & Scarborough, 1990). Sulfitolysis of SDS-unfolded tetrameric GLUT1 disulfides reveals an additional 3 moles of reactive sulphydryl groups per mole of GLUT1 (Table I).

Table I summarizes the results of three types of alkylation strategies in which free sulphydryls, inaccessible sulphydryls and all sulphydryl groups are labelled using $^{14}$C-iodoacetic acid. Inaccessible sulphydryl groups are revealed by first alkylating nonreduced GLUT1 with unlabeled ($^{12}$C-) iodoacetic acid then GLUT1 is reduced and alkylated using $^{14}$C-iodoacetic acid. Reduced GLUT1 incorporates almost three times more $^{14}$C-iodoacetic acid than does non-reduced GLUT1. Differential alkylation of unavailable sulf-
Figure 4. Analysis of tryptic fragments of differentially alkylated GLUT1.

Molecular weight markers are shown in Lane 1. These are (in order of decreasing molecular weight): 106.5, 80, 49.5, 32.5, 27.5 and 18.5 kDa. Coomassie-stained protein is shown in lanes 2 and 3. Peptides with intact C-termini (C-Ab-reactive peptides) are shown in lanes 4 and 5. \textsuperscript{14}C-iodoacetic acid-labelled peptides are shown in lanes 6 through 9. Lanes 8 and 9 are sections of single lane gels used to purify peptides for N-terminal sequence analysis. In lanes 2 - 5, nonreduced, tetrameric GLUT1 was subjected to reducing (DTT+) or nonreducing (DTT-) SDSPAGE. In lanes 6, 7, 8 & 9, nonreduced GLUT1 was either reduced (DTT+) or nonreduced (DTT-) prior to alkylation and reducing SDS PAGE. Peptide \textit{a} is Band 7 or RhD (see Results). The identity of peptide \textit{b} is shown in Table I.
Figure 4

<table>
<thead>
<tr>
<th>Protein</th>
<th>C-Ab blot</th>
<th>Alkylated peptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>DTT</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>-</td>
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</tbody>
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1 2 3 4 5 6 7 8 9

a b
hydryl groups in nonreduced GLUT1 results in almost 2-fold greater $[^{14}\text{C}]-i$odoacetic acid
labelling relative to $[^{14}\text{C}]-i$odoacetic acid labelling of available sites. Since these data and
quantitation of GLUT1 sulphydryls using Ellman's reagent show that GLUT1 exposes all
six potential thiols following exposure to reductant, the results of the NTSB assay are
consistent with the view that each GLUT1 molecule contains one intra-molecular disulfide
bridge and either two mixed disulfides or two free thiols that are exposed only upon
reduction of the internal disulfide bridge.

One GLUT1 cysteine residue occurs within a proposed cytosolic GLUT1 domain,
four lie in proposed transmembrane domains and the remaining residue is predicted to
contact the interstitium (Mueckler et al., 1985). To determine which GLUT1 cysteines are
inaccessible in tetrameric GLUT1, we subjected alkylated GLUT1 to proteolysis and N-
terminal sequence analysis. Reduced and nonreduced GLUT1 were alkylated, mildly
trypsinized and the resulting peptides separated by SDS-PAGE under reducing or non-
reducing conditions. Total peptides were detected by staining, alkylated peptides were
detected by autoradiography and peptides containing an intact C-terminus were detected by
immunoblot analysis using anti-GLUT1 carboxyl-terminal peptide antiserum (C-Ab).
Peptides detected by all three criteria were subjected to N-terminal sequence analysis.
Figure 4 (lanes 6, 7) shows that reduced intact GLUT1 is alkylated 4-fold more heavily than
is nonreduced intact GLUT1. Lanes 6, 7, 8 and 9 of the same figure also show that a peptide
of appMr 25 kDa (peptide b) is heavily labelled by iodoacetic acid when the transporter is
reduced prior to alkylation and proteolysis. This peptide cross-reacts with C-Ab and neither
its mobility nor yield are affected significantly by omission of reductant during
<table>
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<tr>
<th>Peptide N-terminal sequence &amp; deduced residues</th>
<th>Mol $^{[14]C}$- iodoacetic acid incorporated per mol protein or peptide</th>
<th>$^a$mol-SH per mol GLUT1</th>
<th>$^b$NTSB assay. Mol NTB per mol GLUT1</th>
</tr>
</thead>
<tbody>
<tr>
<td>TETRAMERIC GLUT1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nonreduced</td>
<td>1.8 ± 0.2</td>
<td>2.2 ± 0.1</td>
<td>4.8 ± 0.3</td>
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<tr>
<td>reduced</td>
<td>6.4 ± 0.1</td>
<td>6.7 ± 0.4</td>
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</tr>
<tr>
<td>$^{c}[^{12}C]$-alkylated then reduced</td>
<td>3.8 ± 0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$^{c}[^{12}C]$-alkylated</td>
<td>0.1 ± 0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peptide N-terminal sequence &amp; deduced residues</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>b GTADVTHDLQEMKEE (233-492)</td>
<td>nonreduced</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>b GTADVTHDLQEMKEE (233-492)</td>
<td>reduced</td>
<td>2.8</td>
<td></td>
</tr>
<tr>
<td>c VSPTALRGALGT (147-298)</td>
<td>nonreduced</td>
<td>1.7</td>
<td></td>
</tr>
<tr>
<td>d SRQMMREKKV (248-358)</td>
<td>nonreduced</td>
<td>&lt; 0.1</td>
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<tr>
<td>d KAGVQQPVYA (300-425)</td>
<td>nonreduced</td>
<td>&lt; 0.1</td>
<td></td>
</tr>
<tr>
<td>d VGPPIPWF (381-492)</td>
<td>nonreduced</td>
<td>&lt; 0.1</td>
<td></td>
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<tr>
<td>d LFSQGPRPAA (394-492)</td>
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<td>&lt; 0.1</td>
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<tr>
<td>DIMERIC GLUT1</td>
<td>reduced</td>
<td>6.2 ± 0.2</td>
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</table>
**Table I Notes:**

*GLUT1 free sulfhydryl content was assayed using Ellman’s reagent.*

*GLUT1 disulfides were cleaved sulfitolysis and the exposed thiol groups quantitated using NTSB.*

*[^12C]-alkylated protein was treated with cold (unlabeled) iodoacetic acid prior to treatment with[^14C]-iodoacetic acid. Alkylation results summarize 4 separate experiments.*

Peptides identified as b, c and d are those indicated in Figure 4. Assays of GLUT1 sulfhydryl content and disulfide bond content were made in duplicate on at least 3 separate occasions.
electrophoresis.

Quantitation of label incorporation indicates that the 25 kDa peptide incorporates 99 and 8 pmol iodoacetic acid per µg peptide when labelled after or before reduction respectively. By contrast, GLUT1 incorporates 117 and 33 pmol iodoacetic acid per µg GLUT1 when labelled after or before reduction respectively. N-terminal sequence analysis by Edman degradation indicates that the N-terminus of the 25 kDa peptide is:

\[ \text{GTADVTHDLQEMKEE} \]

Since this peptide also contains an intact C-terminus, this allows a peptide assignment of GLUT1 residues 233-(480-492). The last 13 residues are less certain since the polyclonal, GLUT1 C-terminal peptide antiserum is raised against GLUT1 residues 480-492 and may not bind to all 13 residues. Assuming all 13 C-terminal residues are present, this peptide has a deduced molecular weight of 28,705 Daltons. Iodoacetic acid incorporation into this peptide is thus 2.8 and 0.2 mol per mol reduced and nonreduced peptide respectively.

The other low molecular weight protein present in the GLUT1 preparation (peptide a of Fig 4) has a computed molecular weight of 33 kDa. This peptide does not react with C-Ab and is labelled only very weakly by iodoacetic acid. The N-terminal sequence derived for this peptide is SSKYPRSVRR which corresponds to N-terminal residues 2-11 of RhD, a component of Band 7 protein in human erythrocytes (Arce et al., 1993).

Nonreduced, alkylated GLUT1 was also subjected to urea gel electrophoresis. Intact GLUT1 was identified by immunoblot analysis, excised from the gel and subjected
to Cleland mapping using V8 protease. Peptides were transferred to immobilon and subjected to N-terminal sequence analysis. Fig. 5 (lanes 2 and 6) shows that a peptide of $M_{rapp}$ 15 kDa (peptide c) is significantly alkylated. The N-terminal sequence of this fragment is

\[ \text{147 VSPTALRGALGT}_{156}. \]

Quantitation of iodoacetic acid incorporation indicates 113 pmol reactive thiols per $\mu$g peptide. Figure 5 (lanes 2, 4 and 6) also shows that a peptide(s) of $M_{rapp}$ 11.6 kDa (peptide d) cross-reacts with C-Ab on immunoblot analysis but is not alkylated appreciably. Sequence analysis indicates that this region of the blot contains as many as 4 peptides with N-terminal sequence:

\[ \text{248} \text{SRQMMREKKV}_{257} \]
\[ \text{300} \text{KAGVQQPVYA}_{309} \]
\[ \text{381} \text{VGPGPIPWF}_{390} \]
\[ \text{394} \text{LFSQGPRPAA}_{403} \]

Each of these peptides falls within the C-terminal domain of tetrameric GLUT1 (residues 233-492) that lacks accessible thiol groups. If two of these peptides were to contain an intact GLUT1 C-terminus (i.e. GLUT1 peptides 381-492 and 394-492) they would be characterized by predicted $M_r$ of 12.3 kDa and 11 kDa respectively and could account for C-Ab binding to this region of the blot.
Figure 5. V8 peptide-mapping of urea-gel electrophoresis purified, alkylated tetrameric GLUT1.

Lanes 1 & 2 show Coomassie stained protein. Lanes 3 & 4 show peptides containing intact C-termini as judged by immunoblot analysis using anti-GLUT1 carboxyl terminal peptide antiserum (C-Ab). Lanes 5 & 6 are 2-week autoradiographic exposures of alkylated GLUT1 and show $^{14}$C-iodoacetic acid incorporation. Lane 7 shows the mobility of molecular weight markers. These are (in order of decreasing molecular weight): 139.9, 86.8, 47.8, 33.3, 26.6, 20.4, 16.9, 14.4, 6.2 kDa). Exposure to (+) or omission of (-) V8 protease (8 µg) is indicated above the lanes. Peptides c and d were subjected to N-terminal sequence analysis. Their identities are shown in Table I.
Figure 5

<table>
<thead>
<tr>
<th></th>
<th>Protein</th>
<th>C-Ab blot</th>
<th>Alkylated peptides</th>
</tr>
</thead>
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<tr>
<td>V8</td>
<td>-</td>
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<tr>
<td></td>
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</table>

- [Image of the gel with markers 1 to 7]
Mutagenesis of GLUT1 cysteines

GLUT1 cysteines were mutagenized to serines by oligonucleotide-directed *in vitro* mutagenesis. The resulting cDNAs were subcloned into pCMV5 expression vectors for CHO cell transfection. CHO cells were chosen for GLUT1 expression because sugar transport by both CHO cells and erythrocytes is inhibited by extracellular reductant (see Chapter IV). This suggests that erythrocytes and CHO cells process GLUT1 in a similar fashion.

Clonal cell lines expressing GLUT1 wild-type and cysteine mutants were screened for extent of GLUT1 overexpression by immunoblot analysis and by immunofluorescence microscopy. Two antisera were used. Ə-Ab binds to exofacial epitopes of native, tetrameric GLUT1 but fails to react with dimeric GLUT1 (Hebert & Carruthers, 1992) while C-Ab binds to the intracellular C-termini of both tetrameric and dimeric GLUT1 (Hebert & Carruthers, 1992).

Immunoblot analyses of CHO cell total membranes using C-Ab indicate that clonal cells express wild-type or mutant glucose transporter at levels 2- to 4-fold greater than levels of GLUT1 expression in parental CHO-K1 cells (Table II). CHO cell total membranes were also subfractionated into plasma membrane and low density microsomal membrane fractions. In two separate experiments where all GLUT1 cysteine mutants were examined, the ratio of plasma membrane GLUT1 content (protein detected by immunoblot analysis of 100 μg membrane total protein) to low density microsomal membrane GLUT1 content (1:0.5) was unaltered (see Fig. 6) by mutagenesis of GLUT1 cysteine residues to serines. This suggests that cellular processing of GLUT1 is unaffected by serine
Figure 6. Immunoblot Analysis of CHO Clones

Immunoblot analysis (using C-Ab as primary antiserum) of cell membranes (100 μg total protein) from CHO cell clones. The top panel shows the total membrane fraction. The middle panel shows the plasma membrane fraction. The bottom panel shows the low density microsome membrane fraction. The blots are: wild-type expresser (WT, lane 1), GLUT1 cysteine mutants C133S, C201S, C207S, C347S, C421S and C429S (lanes 2 to 7 respectively) and CHO-K1 cells (lane 8). Lane 9 contains 250 ng purified GLUT1. Lanes 10 and 11 show results of a second similar experiment using CHO-K1 cells.

The nomenclature used to describe the membrane fractions is used to maintain consistency with previously published literature in the glucose transporter field. In contemporary cell biology literature, the total membrane fraction is called the crude membrane fraction. The low density microsomal fraction is analogous to what is now referred to as light microsomes, or golgi-enriched membrane fractions. The nomenclature for the plasma membrane fraction has not changed.
Figure 6

- TM
- PM
- LDM
Total GLUT1 expression was also quantitated by digital imaging fluorescence microscopy (DIFM) using C-Ab. Since C-Ab does not bind to intact cells (see Fig. 7), this requires that fixed cells are first permeabilized by using either nonionic detergent (e.g. 0.05% Triton X-100) or by using paraformaldehyde levels in excess of 2% (weight:vol) during fixation. Immunofluorescence quantitation was by three methods. Total, specific immunofluorescence from the field was quantitated and divided by the number of cells in the field. In the second approach, specific immunofluorescence of individual cells was quantitated and averaged over a large number of cells. These methods can suffer from depth of field phenomena (see below) so a third approach was used in which specific immunofluorescence within patches of flattened cellular extensions was measured. The results of all three approaches are in close agreement. The C-Ab immunofluorescence data of Table II are computed using the specific, cellular immunofluorescence approach and are not significantly different (2-tailed paired t-test) from the results of immunoblot analyses using C-Ab (Table II).

The depth of field phenomenon is illustrated by the fluorescence micrographs of the C347S mutant in Figure 8. For one cell in the field of view, both C-Ab and \( \partial \)-Ab staining patterns suggest an unusual, local sequestration of immunoreactive protein. The second cell in the same field does not show this behavior. In fact, this local fluorescence intensity is a cell shape phenomenon. In this hot spot, the value for the z-axis is large (reflecting cell morphology) and the intensity light collected from a given area of x y-pixels reflects the magnitude of the z-plane (Agard et al., 1989). Total cellular fluorescence is relatively
Figure 7. Immunofluorescence of CHO cells.

Parental, CHO-K1 cells were fixed using 2% paraformaldehyde (A through D), then exposed to 0 (A, B) or 0.05% Triton-X100 (C, D). Cells were stained using rabbit β-Ab (A, C) which is used to quantitate tetrameric GLUT1 or C-Ab (B, D) which quantitates total GLUT1 and developed using fluorescein-conjugated goat anti-rabbit IgGs. Cells were visualized by digital imaging microscopy.
<table>
<thead>
<tr>
<th>Tetrameric GLUT1</th>
<th>Total GLUT1</th>
<th>Tetrameric GLUT1</th>
<th>Total GLUT1</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1" alt="Intact Cells" /></td>
<td><img src="image2" alt="Permeabilized Cells" /></td>
<td><img src="image3" alt="Intact Cells" /></td>
<td><img src="image4" alt="Permeabilized Cells" /></td>
</tr>
</tbody>
</table>
Figure 8. Immunofluorescence of CHO cell clones

Cells were fixed, permeabilized and stained for δ-Ab and C-Ab reactive epitopes. Sheep δ-Ab and rabbit C-Ab were used to simultaneously stain cells for both epitopes. Fluorescein- and rhodamine-conjugated secondary antibodies were used. Results are shown for parental CHO-K1 cells (CHO), for a wild-type GLUT1 overexpresser (CWT) and for cysteine mutants 133, 201, 207, 347, 421 and 429 (C133S through C429S). For display purposes, the full range of fluorescence intensities associated with all images acquired using a given primary antiserum is compressed to a scale of 0 to 255 (8 bit resolution). This aids visual comparison of the range of staining intensities obtained with different cells but using one antiserum. For example, CWT express δ-Ab-reactive epitopes at levels 3-fold greater than do CHO-K1 parental. The actual (measured) range of fluorescence intensities in this experiment is 0 to 4753 (arbitrary units) for δ-Ab binding images and 0 to 2953 for C-Ab binding images.
Figure 8

<table>
<thead>
<tr>
<th></th>
<th>δ-Ab</th>
<th>C-Ab</th>
</tr>
</thead>
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<tr>
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</tr>
<tr>
<td>CWT</td>
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</tr>
<tr>
<td>C133S</td>
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<td></td>
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<tr>
<td>C429S</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell type</td>
<td>Immunoblot(^a)</td>
<td>(\partial)-Ab(^b)</td>
</tr>
<tr>
<td>------------</td>
<td>------------------</td>
<td>------------------</td>
</tr>
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<td>(^d)CHO-K1</td>
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<td>CWT</td>
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<td>4.8 ± 1.3</td>
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<tr>
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<td>2.4 ± 0.2</td>
<td>7.9 ± 4.5</td>
</tr>
<tr>
<td>C201S</td>
<td>2.9 ± 0.2</td>
<td>5.4 ± 1.7</td>
</tr>
<tr>
<td>C207S</td>
<td>3.3 ± 0.6</td>
<td>7.1 ± 3.5</td>
</tr>
<tr>
<td>C347S</td>
<td>2.8 ± 0.4</td>
<td>(\text{e,f}0.9) ± 0.2</td>
</tr>
<tr>
<td>C421S</td>
<td>3.1 ± 0.6</td>
<td>(\text{e,f}0.9) ± 0.2</td>
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<tr>
<td>C429S</td>
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<tr>
<td>(^b)GT3</td>
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<td>(^h)5.5 ± 1.6</td>
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<tr>
<td>(^i)GLUT1n-4</td>
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</table>
Table II Notes:

\(^a\) Intact GLUT1 was quantitated by immunoblot analysis of 100 μg CHO cell membrane protein using C-Ab as primary antiserum and \(^{125}\text{I}\)-Protein A as the reporter molecule. Resulting autoradiograms were quantitated by densitometry.

\(^b\) \(\partial\)-Ab and C-Ab binding to fixed permeabilized CHO cells was measured simultaneously by digital imaging fluorescence microscopy using fluorescein-conjugated rabbit-anti-sheep IgGs as the reporter molecule for \(\partial\)-Ab binding and rhodamine-conjugated goat anti-rabbit IgGs as the reporter molecule for C-Ab binding.

\(^c\) The ratio of cellular \(\partial\)-Ab binding (fluorescein fluorescence): C-Ab binding (rhodamine fluorescence) was computed for each cell analyzed. The numbers in parentheses indicate the range of results.

\(^d\) All results are expressed relative to those obtained with CHO-K1 parental cells within the same experiment. The following summarizes unadjusted data for CHO-K1 cells. Immunoblot analysis, \((59,701 \pm 10,121)\) arbitrary units per 100 μg membrane protein; \(\partial\)-Ab binding, \((3.2 \pm 0.37) \times 10^6\) arbitrary fluorescence units per cell; C-Ab binding, \((1.06 \pm 0.19) \times 10^6\) arbitrary fluorescence units per cell; \(\partial\): C ratio, \((3.19 \pm 0.20)\): 1.

All results are significantly greater than those obtained with control, CHO-K1 cells (\(p < 0.05\); 1 tailed paired t test) with the exception of \(^e\) in which these results are not significantly different from control and \(^g\) in which the result is significantly lower than control (\(p < 0.005\); 1 tailed paired t test).

No significant statistical differences exist across columns 1 through 3 with the exception of \(^f\) in which this result is significantly less (\(p < 0.05\); 1 tailed paired t test) than those obtained by immunoblot or immunofluorescence analysis using C-Ab and \(^h\) in which this result is significantly greater (\(p < 0.05\); 1 tailed paired t test) than that obtained by immunofluorescence analysis using C-Ab.

\(^i\) GT3 cells are CHO cell line that expresses GLUT1 at very high levels (Harrison et al., 1990a).
Immunoblot data for GLUT1-4C and GLUT1n-4 are taken from Pessino et al. (1991).

Results are shown as mean ± SEM of 3 or more separate determinations.
constant. This phenomenon was observed infrequently in all clonal cell lines and was most common when cells were characterized by a rounded morphology.

Since transfected CHO cells express GLUT1cys mutants, we next determined whether these cells also expose increased levels of tetrameric GLUT1-specific (β-Ab binding) epitopes. These data were collected from the same cells that were analyzed for GLUT1 expression levels by C-Ab fluorescence. This is possible because these cells were dual labeled for both C-Ab and β-Ab-reactive epitopes. Cells were fixed, permeabilized and exposed to sheep β-Ab and rabbit C-Ab. C-Ab binding was detected by using rhodamine-conjugated goat anti-rabbit IgGs. The cells were washed and β-Ab binding was detected by using fluorescein-conjugated rabbit anti-sheep IgGs. As with quantitation of C-Ab binding, β-Ab binding was quantitated using the specific individual cellular immunofluorescence approach. In three separate experiments, we also stained cells separately for β-Ab and for C-Ab binding and obtained results indistinguishable from those obtained by the simultaneous staining procedure.

The data we collected are expressed in two ways. Specific (β-Ab or C-Ab) cellular immunofluorescence is expressed relative to that measured in CHO-K1 parental cells. For example, Table II shows that the C429S GLUT1 mutant expresses β-Ab and C-Ab reactive sites at levels 5.3 and 3.6-fold greater respectively than do CHO-K1 cells. Figure 8 shows typical immunofluorescence micrographs where C429S GLUT1 expressing CHO cells show approximately 4-fold greater β-Ab and C-Ab staining than do CHO-K1 cells.

The ratio of β-Ab: C-Ab staining in a given cell for a given experiment is also computed and is expressed relative to this same ratio in CHO-K1 cells. This facilitates
comparison of GLUT1 cysteine mutant δ-Ab and C-Ab binding properties with those of GLUT1 in a cell (CHO-K1) where almost all GLUT1 forms a homotetrameric structure (e.g. see Hebert and Carruthers, 1992 and see Fig. 10). The ratio δ-Ab: C-Ab binding is not, however, a direct measure of the absolute ratio of cellular tetrameric GLUT1: total cellular GLUT1. The δ-Ab: C-Ab binding ratio also provides an internal control for individual experiments. Thus, while variations in levels of cellular expression of GLUT1 and GLUT1 cysteine mutants exist between experiments and are evident from standard errors computed in columns 1, 2 and 3 of Table II, the ratio of cellular δ-Ab: C-Ab binding sites (for δ-Ab binding competent GLUT1) is much less variable because this ratio is independent of GLUT1 expression level (see Fig. 9).

The results (summarized in Fig. 8 and Table II) show that serine substitution at cysteine 347 or 421 significantly reduces GLUT1 exposure of tetrameric-GLUT1-specific epitopes. Some variation between experiments is observed and ranges for δ-Ab: C-Ab binding ratios are provided in Table II.

Mapping Oligomerization Domains

It was previously shown (Pessino et al. 1991) that specific immunoprecipitation of GLUT1-GLUT4 chimeras expressed in CHO cells by using anti-GLUT4 antiserum results in co-immunoprecipitation of endogenous GLUT1 protein. The transporter chimeras used in these studies were GLUT1-4c (GLUT1 resides 1 to 463 plus the 30 C-terminal residues of GLUT4) and GLUT1n-4 (GLUT1 resides 1 to 199 plus the 294 C-terminal residues of GLUT4). Since co-immunoprecipitation of 3T3L1 adipocyte GLUT4 and GLUT1 is not
Figure 9.  $\vartheta$-Ab vs. C-Ab

CHO-K1, CWT and GT3 cells were analyzed for GLUT1 expression by immunoblot analysis and by immunofluorescence analysis of $\vartheta$-Ab binding. Results are expressed as relative $\vartheta$-Ab binding versus relative C-Ab binding. All results are expressed relative to binding observed with CHO-K1 cells. The straight line drawn through the points was computed by the method of least squares and corresponds to: relative $\vartheta$-Ab binding $= 0.7 + 0.99$ relative C-Ab binding ($R=0.944$).
Figure 9

Relative C-Ab binding

Relative C-Ab binding (immunoblot)
observed under the same conditions, Pessino et al. (1991) concluded that GLUT1-specific domains mediate GLUT1/chimera associations.

We have now determined the hydrodynamic radius of glucose transporter-containing micelles solubilized from these cells. Our analyses (Fig. 10) indicate that parental GLUT1, wtGLUT1, GLUT1-4c and 25% of expressed GLUT1n-4 co-resolve with authentic tetrameric GLUT1 upon size exclusion chromatography. The majority of GLUT1n-4, however, co-resolves with authentic dimeric GLUT1.
Figure 10. Hydrodynamic size analysis of GLUT1/GLUT4 chimeras.

Cell membranes from Parental CHO cells (■) and from CHO cells expressing Wild Type GLUT1 (○), GLUT1n-4 (□) or GLUT1-4c (●) (Pessino et al., 1991) were solubilized in cholate and applied to a size exclusion column as in (Hebert and Carruthers, 1992). Chimeras and GLUT1 were detected by ELISA using anti-GLUT4- or anti-GLUT1-C-terminal peptide antisera respectively. Authentic tetrameric (▼) and dimeric (▼) GLUT1 are also shown. Relative [GLUT1] content (ordinate) is expressed versus elution time (abscissa).
Figure 10

Tetramer   Dimer

[GLUT1] arbitrary units

GLUT1-4c
GLUT1n-4
GLUT1 wt
Parental GLUT1
Dimeric GLUT1
Tetrameric GLUT1

Rf min
Discussion

A substantial body of direct, biophysical evidence supports the hypothesis that GLUT1 exists as an oligomeric complex (Hebert & Carruthers, 1991, Hebert & Carruthers, 1992, Jarvis et al., 1986, Jung et al., 1980, Pessino et al., 1991, Sogin & Hinkle, 1978, Zoccoli et al., 1978). The present study was formulated to answer the question: Why is the quaternary structure of the glucose transporter sensitive to reductant if it is stabilized primarily through noncovalent subunit interactions? Our findings support the hypothesis that each subunit (GLUT1 protein) of the parental glucose transporter contains a single intramolecular disulfide bridge between cysteine residues 347 and 421. This disulfide seems to be necessary for GLUT1 tetramerization. Our findings suggest that GLUT1 N-terminal residues 1 through 199 provide contact surfaces for subunit dimerization but are insufficient for subunit tetramerization.

Quantitation of GLUT1 disulfide content first requires sulfitolysis of disulfides followed by quantitation of exposed thiols by the NTSB assay (Rao & Scarborough, 1990). Disulfide cleavage by sulfitolysis produces one S-sulfonated cysteine and reveals a free thiol group on the remaining cysteine. The assay for an internal disulfide thus yields 1 mol NTB per 2 mol internal cysteine while that for a mixed disulfide yields 1 mol NTB per mol internal cysteine. Our assay detects $4.8 \pm 0.3$ mol NTB which, since parental GLUT1 exposes 2 sulfhydryl groups without prior reduction, indicates that sulfitolysis reveals an additional 3 mol reactive sites. This result strongly suggests that GLUT1 contains 1 internal disulfide and either 2 mixed disulfides or 2 free sulfhydryl groups that are revealed only
when the disulfide bridge is broken. It is unlikely that the “missing” thiol is inaccessible due to covalent modification by other species since DTT reveals this group. It is unlikely that sulfitolysis is incomplete since excess (10,000-fold) sodium sulfite was used and GLUT1 (which was unfolded in SDS) is quantitatively alkylated under similar conditions.

N-terminal sequence and immunoblot analyses of $^{14}$C-iodoacetic acid labelled GLUT1 peptides indicates that a C-terminal peptide containing GLUT1 residues 233 through 492 contains 3 of the 4 inaccessible sulfhydryl groups of each parental GLUT1 subunit while a peptide containing GLUT1 residues 147 to 260/299 contains both reactive thiols. The conditions used here for GLUT1 alkylation do not favor alkylation of other functional groups (Gurd, 1972) and quantitation of presumed GLUT1 S-carboxymethylcysteine content is in agreement with GLUT1 sulfhydryl content as measured using Ellman’s reagent. It is unlikely, therefore, that these quantitative estimates are seriously flawed. The mobility of the GLUT1 C-terminal fragment is unaffected by omission of reductant during electrophoresis. If the peptide were to remain disulfide-linked to the smallest possible GLUT1 tryptic fragments containing either cysteine 133 (residues 184 to 212) or cysteines 201 and 207 (residues 127:153), its molecular weight would increase by 3308 or by 2925 Daltons respectively. No mobility shift is detected even though molecular weight markers of the same range of molecular weight but differing by only 5000 Daltons are clearly separated.

May has shown that GLUT1 cysteine 429 can be alkylated by cell impermeant maleimides (May, 1988, May et al., 1990). However, alkylation is not quantitative and
ranges between 2 to 14 mol% incorporation (May et al., 1990). This is consistent with our estimates of molar incorporation of iodoacetic acid into the carboxyl-terminal GLUT1 peptide (20 mol%). In independent studies using a Xenopus oocyte expression system, Wellner and colleagues (Wellner et al., 1992, Wellner et al., 1994) have demonstrated that GLUT1 cysteine 429 is required for exofacial pCMBS inhibition of sugar transport but suggest that mutagenesis of individual cysteine residues is without effect on sugar uptake by GLUT1 expressed in Xenopus oocytes. These studies do not permit direct comparison of the catalytic activities of the various GLUT1 cysteine mutants in Xenopus oocytes since cell surface GLUT1 content was not quantitated and transport was measured at only one or two sugar concentrations. However, if GLUT1-cysteine mutants are not inhibited, this suggests either: 1) The cysteine-hypothesis is incorrect or, 2) GLUT1 does not fold normally in oocytes. Recent experiments from this laboratory show (Fig. 11) that GLUT1 expressed in oocytes is capable of transporting sugars but neither binds $\beta$-Ab nor is inhibited by dithiothreitol. This strongly suggests that the structural and functional phenotype of oocyte-expressed GLUT1 differs fundamentally from that of human erythrocyte and CHO cell GLUT1. Oocyte-expressed GLUT1 may resemble dimeric GLUT1 - an in vitro, low affinity form of the erythrocyte sugar transporter (Hebert and Carruthers, 1992). This would explain the troubling observation that oocyte-expressed GLUT1 binds sugars with 5 to 10-fold lower affinity than does erythroid GLUT1 (Burant & Bell, 1992).

In the studies reported here, immunoblot and immunohistochemistry analyses of parental and transfected CHO cell GLUT1 content show that wild-type GLUT1 and
Figure 11. GLUT1 expression in *Xenopus* oocytes.

Capped mRNA was synthesized using the Ambion Megascript Kit. *Xenopus* oocytes were injected each with 25 ng (0.5 μg/mL in H2O) and maintained in ND96 media +2.5 mM pyruvate and gentamicin (50 μg/mL) for 3 days. 3OMG uptake at 22°C and 100 μM was measured over 1 minute. Oocytes were exposed to ND96 media ±50 μM CCB, 2 mM DTT or 2 mM GSSG for 30 minutes prior to processing for sugar influx or antibody binding. Sugar influx measurements represent SEM of 5 oocytes. Uninjected oocytes take up 3OMG at a rate of ≈22±2 fmol/oocyte/min. Oocyte immunofluorescence was performed as described for erythrocytes. Antibody binding is normalized to fluorescence intensity per sq cm oocyte surface area.
GLUT1 cysteine mutants are expressed at levels 2 to 5 fold greater than is endogenous GLUT1. We selected the highest overexpressers for analysis of GLUT1 oligomeric structure. The least ambiguous method for determining the oligomeric state of CHO cell GLUT1 would be to perform hydrodynamic size analyses on purified, solubilized GLUT1. This method requires additional quantitation of micellar lipid and detergent content and suffers from the potential for purification-associated perturbations in transporter quaternary structure (see Hebert & Carruthers, 1992). An alternative approach is to perform hydrodynamic size analyses of GLUT1 solubilized directly from CHO cell membranes. This approach, although useful, is not definitive since the protein, detergent and lipid contents of the solubilized GLUT1-containing micelles is uncertain. The strategy we adopted was to quantitate binding of an anti-tetrameric GLUT1 antiserum (d-Ab) to CHO cell GLUT1 and to contrast this binding with that of an antiserum (C-Ab) that shows no detectable sensitivity to GLUT1 quaternary structure (Hebert & Carruthers, 1992).

We have previously demonstrated that d-Ab does not cross-react with dimeric GLUT1 but binds with high affinity to tetrameric GLUT1 (Hebert & Carruthers, 1992) and to SDS-denatured, reduced GLUT1 (Harrison et al., 1990a). We have demonstrated that d-Ab binding to CHO cells is directly proportional to CHO-cell GLUT1 expression over a 20-fold range (Harrison et al., 1990a) and that d-Ab binds at an exofacial site(s) on the transporter (Harrison et al., 1990a). We have further shown that reversible dissociation of tetrameric GLUT1 into dimeric GLUT1 is associated with the reversible loss of d-Ab binding to GLUT1 (Hebert & Carruthers, 1992). We have also shown that affinity purification of d-Ab using reduced, SDS-unfolded GLUT1 results in quantitative recovery
of binding activity to intact cells (Chapter V and Coderre et al., 1995). Together these data suggest that GLUT1 contains specific epitopes that are exposed in tetrameric GLUT1 but not in the GLUT1 dimer. Thus the ability of $\partial$-Ab to bind to GLUT1 reflects the accessibility of these epitopes. It is possible that $\partial$-Ab reactive epitopes could be sequestered or exposed in the absence of any change in transporter oligomeric structure. This has not been observed experimentally (Chapter V and Coderre et al., 1995, Hebert & Carruthers, 1992).

Our results show that serine substitution at cysteines 347 and 421 significantly reduces $\partial$-Ab binding to GLUT1. The magnitude of this effect suggests that remaining $\partial$-Ab binding is accounted for by binding to parental GLUT1. While these results do not discount the possibility that serine substitution at cysteine 347 and 421 induces subtle conformational changes that mask the $\partial$-Ab epitopes, they are consistent with the view that these cysteine residues are important for GLUT1 oligomeric structure. When reviewed in the context of the peptide mapping/alkylation studies, these finding strongly suggest that the internal, GLUT1 disulfide bridge, if extant, is formed between residues 347 and 421.

Hydrodynamic analyses using GLUT1/GLUT4 chimeras indicate that GLUT1 residues 463-492 make a negligible (isoform-specific) contribution to GLUT1 oligomeric structure. This is consistent with the observation that anti-GLUT1 carboxyl terminal peptide antiserum (C-Ab) binding to GLUT1 is insensitive to GLUT1 oligomeric structure. A portion (25%) of the expressed GLUT1n-4 chimera (GLUT1 residues 1 through 199 plus GLUT4 residues 216 to 509) does resolve as a tetramer. Specific immunoprecipitation of GLUT1n-4 from CHO cells using anti-GLUT4 carboxyl terminal peptide antiserum
quantitatively precipitates parental GLUT1 (Pessino et al., 1991). Almost all parental GLUT1 from GLUT1n-4 chimera-expressing cells co-resolves with authentic tetrameric GLUT1. However, most of the chimera co-resolves with authentic dimeric GLUT1. While our studies cannot rule out the possibility that GLUT1n-4-containing micelles contain molecular species other than lipid, detergent, GLUT1n-4 and GLUT1, these data are consistent with the hypothesis that GLUT1n-4/wild-type GLUT1 complexes are heterotetramers while the remaining GLUT1n-4 forms only homodimeric structures. If correct, this would mean that wild-type GLUT1 is dominant positive over GLUT1n-4 with regard to transporter oligomeric structure.

These data further suggest that the GLUT1 N-terminal half provides dimerization motifs/contact surfaces while the carboxyl-terminal half contains the tetramerization motif (cys 347 and 421) and contact surfaces. These contact surfaces for tetramerization presumably also include domains that are well conserved in GLUT1 and GLUT4 carboxyl-terminal halves. GLUT1 residues 200 - 463 and GLUT4 residues 216 - 479 share 78% sequence homology.
Chapter IV

Effect of Reductant on \textit{in situ} GLUT1

Structure and Function

\textit{Is the GLUT1 tetramer more catalytically active than the dimer \textit{in situ}?}

In earlier studies, Hebert and Carruthers (1992) hypothesized that tetrameric GLUT1 is a more efficient transporter than its reduced, dimeric counterpart. Their hypothesis was based on indirect, \textit{in vitro} studies. Here, we test the hypothesis \textit{in situ}. We test the effect of reductants on erythrocyte and CHO cell structure and function.

Extracellular reductants (dithiothreitol, \(\beta\)-mercaptoethanol or glutathione) reduce erythrocyte 3-O-methyl-D-glucose uptake by up to 15-fold. This noncompetitive inhibition of sugar uptake is reversed by the cell-impermeant, oxidized glutathione. Reductant is without effect on sugar exit from erythrocytes. Dithiothreitol doubles the cytochalasin B binding capacity of erythrocyte-resident glucose transporter, abolishes allosteric interactions between substrate binding sites on adjacent subunits and occludes tetrameric-GLUT1 specific GLUT1 epitopes \textit{in situ}. CHO cell-resident GLUT1 structure and transport function are similarly affected by extracellular reductant.
Results

GLUT1 structure, GLUT1-mediated sugar transport and ligand binding in intact red cells

GLUT1-mediated 3-OMG uptake by human erythrocytes is inhibited 3 to 15-fold by dithiothreitol, 2-mercaptoethanol and by reduced glutathione preincubation at 37°C (Fig. 12) while oxidized glutathione stimulates red cell sugar import (Fig. 12). The inhibitory action of dithiothreitol is noncompetitive (Table III) and is half-maximal at 0.7 ± 0.1 mM (SEM of 4 experiments). This effect is exerted at the exofacial surface of the cell membrane since glutathione and oxidized glutathione are cell impermeable (Kondo et al., 1989, Srivastava & Beutler, 1969). Removal of extracellular DTT from 2 mM DTT-loaded ghosts results in reversal of transport inhibition but does not deplete ghosts of intracellular reductant as judged by quantitation of the release of DTNB-reactive species upon addition of Triton X-100. 3-OMG exit is unchanged in DTT-exposed cells shown in parallel experiments to have reduced sugar uptake (n = 3).

Cytochalasin B binding to red cells and to resealed, hemoglobin-depleted red cell ghosts is affected by DTT in two ways. 1) Sugar-inhibitable cytochalasin B binding capacity is increased 2.4-fold following red cell incubation with 2 mM DTT (Table III). 2) The kinetics of maltose-inhibition of cytochalasin B binding are converted from negative-allosteric inhibition to simple, linear competitive inhibition (Fig. 13).

Although exposure to reductant rapidly and reversibly modifies glucose transporter function and ligand binding in intact red cells, it is difficult to know whether transporter structure is affected at the same time. To address this question, we fixed red cells that were
preincubated at 37°C in the presence or absence of 2 mM DTT. These cells were attached
to polylysine coated coverslips prior to exposure to medium and fixative. The fixed cells
were permeabilized, washed free of reductant and stained separately for either tetrameric
GLUT1-specific epitopes (using \( \partial \)-Ab) or for total GLUT1 (using C-Ab) and resulting
staining intensities visualized and quantitated using digital imaging fluorescence
microscopy (Fig. 14). While C-Ab binding is unaffected by cellular exposure to reductant,
\( \partial \)-Ab binding is reduced 3.5 \( \pm \) 0.2-fold by prior cell exposure to 2 mM DTT. Similar
experiments were carried out using CHO cells but here cells were dual stained for \( \partial \)- and
C-Ab reactive epitopes. CHO cells (growing on coverslips) were exposed to saline or to
saline plus 2 mM DTT, fixed, washed free of DTT and permeabilized prior to staining. The
CHO cell \( \partial \)- and C-Ab binding images shown in Figure 14 (e.g. panels e and f) were
obtained simultaneously from the same field. DTT inhibits \( \partial \)-Ab binding to CHO cells by
76\%. As with red cells, C-Ab binding is unaffected by prior DTT exposure.

In parallel experiments, 2 mM DTT inhibits 2-deoxy-D-glucose net uptake at 22°C
by 2-fold in parental CHO-K1 cells and in CHO cells overexpressing GLUT1 (CWT cells;
Table III). 2DOG phosphorylation is rate-limiting for net uptake in these experiments (\( t_{1/2} \)
for CHO cell uptake of the nonmetabolized sugar 3OMG (100 \( \mu \)M) at 4°C is 40 sec). While
we cannot rule out the possibility that DTT affects the 2DOG phosphorylation step and not
transport, this appears unlikely since the redox state of cytosol in viable cells is reducing in
the absence of exogenous reductant (Gilbert, 1982).
Figure 12. 3OMG uptake by RBCs ± reductant.

CCB-inhibitable 3OMG uptake (66 μM sugar, 4°C) by erythrocytes (RBCs) in the presence or absence of 2 mM extracellular reductants (glutathione (GSH), dithiothreitol (DTT) and β-mercaptoethanol (BME)) and by erythrocyte ghosts (ghosts) containing (e.g. DTT) or lacking 2 mM reductant and exposed to 2 mM extracellular reductants (e.g. DTT) or 2 mM oxidized glutathione (GSSG). Results represent the mean ± SEM of at least 4 separate experiments made in triplicate.
Figure 12

Ghosts
RBC + GSH
RBC + BME
RBC + DTT
RBC - DTT

Ghost + DTTi, GSSGo
Ghost + DTTi
Ghost + DDTi/o
Ghost + DTTo

CCB inhibited 3-O-methylglucose influx (µmol/L cell water/min)
Figure 13. Effect of DTT on maltose-inhibition of CCB binding in RBCs.

Effect of 2 mM DTT-treatment of red cells on maltose-inhibition of cytochalasin B binding. Ordinate: [cytochalasin B]_free/[cytochalasin B]_bound. Abscissa: [maltose]_o in mM. The curves drawn through the data points are best fit curves and were computed by nonlinear regression assuming that control cells (filled symbols) display negative, heterotropic cooperativity between maltose and cytochalasin B binding sites (Helgerson and Carruthers, 1987, also see Fig. 19) with K_o for maltose binding of 3.6 ± 0.1 mM, K_i for CCB binding of 90 ± 12 nM and a negative cooperativity factor, α, of 2.1 ± 0.1. The line drawn through the DTT data (open symbols) assumes simple competitive inhibition between cytochalasin B and maltose binding sites with K_o for maltose binding of 32.5 ± 5.4 mM and K_i for CCB binding of 90 ± 6 nM. These data summarize 7 separate experiments. Each data point represents mean ± SEM of 7 separate measurements made in quadruplicate.
Figure 13

[Graph showing the relationship between [CCB]_f/([CCB])_b and [Maltose] mM. The graph includes data points and error bars.]
Figure 14. Immunofluorescence of RBCs and CHO cells ±DTT.

Images show red cells (RBC, panels a - d) and CHO cells (panels e - h) fixed in the presence (c, d, g, h) or absence (a, b, e, f) of 2 mM DTT. Cells were then washed and stained for \( \partial \)-Ab (a, c, e, g) and/or C-Ab (b, d, f, h) reactive epitopes. Red cells were not stained simultaneously for \( \partial \)-Ab and C-Ab binding sites. Thus cells in panel a are not identical to those in panel b but are from the same population of cells and were processed in parallel to cells in a. CHO cells were dual stained for \( \partial \)-Ab (panels e, g) and C-Ab (panels f, h) reactive epitopes. Thus images shown in panels e and f show \( \partial \)-Ab and C-Ab binding associated fluorescence respectively from the same cells. Exposure to DTT prior to fixation inhibits \( \partial \)-Ab binding by as much as 4-fold. As with Figure 8 and for display purposes only, the full range of fluorescence intensities associated with all images acquired using a given primary antiserum and cell type is compressed to a scale of 0 to 255 (8 bit resolution). The actual (measured) range of fluorescence intensities in these experiments are: panels a & c, 0 to 3164 (arbitrary units); panels b & d, 0 to 4281; panels e & g, 0 to 3729; panels f & h, 0 to 1462. These results are typical of three separate experiments.
Figure 14

<table>
<thead>
<tr>
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<th>δ-Ab</th>
<th>C-Ab</th>
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<tbody>
<tr>
<td>RBC</td>
<td>a</td>
<td>b</td>
</tr>
<tr>
<td>RBC +DTT</td>
<td>c</td>
<td>d</td>
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<tr>
<td>CHO</td>
<td>e</td>
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<tr>
<td>CHO +DTT</td>
<td>g</td>
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</tr>
<tr>
<td>$^aV_{\text{max}}$</td>
<td>276 ± 28</td>
<td>138 ± 32</td>
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<tr>
<td>$^aK_{\text{m(app)}}$</td>
<td>0.11 ± 0.08</td>
<td>0.18 ± 0.05</td>
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<tr>
<td>$^bB_{\text{max}}$</td>
<td>128 ± 16</td>
<td>315 ± 22</td>
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<tr>
<td>$^bK_{\text{d(app)}}$</td>
<td>91 ± 8</td>
<td>137 ± 16</td>
</tr>
<tr>
<td>$^c$CHO-K1 cells</td>
<td>167 ± 11</td>
<td>77 ± 4</td>
</tr>
<tr>
<td>$^c$CWT cells</td>
<td>275 ± 14</td>
<td>131 ± 9</td>
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Table III: Effect of Reductant on GLUT1 Function
Table III Notes:

\(^a\) \(v_{\text{max}}\) is the maximum velocity (\(\mu\text{mol/L cell water per min}\)) and \(K_{m(\text{app})}\) the Michaelis constant (mM) for 3-\(\text{O-}\)methyl-\(\text{D-}\)glucose uptake by control and DTT (2 mM) -treated erythrocytes at 4\(^{\circ}\)C. Results represent the mean \(\pm\) SEM of 3 separate experiments made in triplicate.

\(^b\) \(B_{\text{max}}\) and \(K_{d(\text{app})}\) represent maximum binding capacity (thousands of sites per cell) and dissociation constant (nM) for equilibrium cytochalasin B binding to erythrocytes. Results represent the mean \(\pm\) SEM of 5 separate experiments made in triplicate.

\(^c\) CHO-K1 and CWT cell experiments measure cytochalasin B (20 \(\mu\text{M}\)) inhibitable 2DOG uptake (\(\mu\text{mol sugar per L cell water per minute}\)) by parental and GLUT1-transfected CHO cells at 100 \(\mu\text{M}\) sugar and 22\(^{\circ}\)C. Results are shown as mean \(\pm\) SEM of 3 separate experiments measured in quintuplicate. The water (3OMG) space of a single CHO cell is 0.98 \(\mu\text{L}\). CHO-K1 cell and CWT cell 2DOG uptake in the presence of 20 \(\mu\text{M}\) cytochalasin B are 53 and 46 \(\mu\text{M/L cell water per min}\) respectively.
Discussion

Hebert & Carruthers (1992) previously hypothesized that tetrameric GLUT1 is a more efficient transporter than its reduced, dimeric counterpart. This hypothesis is based on kinetic analyses of red cell resident (tetrameric) GLUT1 and reduced, purified GLUT1 (Appleman & Lienhard, 1989, Lowe & Walmsley, 1986) that show that the catalytic turnover of purified dimeric GLUT1 at 10°C is significantly lower than that of red cell-resident GLUT1 at the same temperature. Specifically, dimer-mediated net uptake and net exit are 2-fold and 8-fold slower respectively than that mediated by native GLUT1. While omission of a number of extrinsic environmental factors can be offered for the catalytic deficit of reduced purified GLUT1, a kinetic rationale for this behavior is provided by the observation that exchange transport (sugar uptake coupled to sugar exit) by dimeric and native GLUT1 are indistinguishable. This indicates that sugar-promoted translocation steps are largely insensitive to transporter oligomeric structure. It is the sugar-independent, transporter conformational changes (relaxation steps) inferred to be sensitive to transporter oligomeric structure. The slowest steps catalyzed by dimeric GLUT1 are relaxation steps (E1 ↔ E2) which, at 10°C occur almost 100-fold more slowly than do the sugar translocation steps (E•S1 ↔ E•S2) (Appleman & Lienhard, 1989). If the transporter could bypass relaxation steps, transport would be stimulated dramatically. In principle, this is what tetrameric GLUT1 (a simultaneous carrier) should achieve since the simultaneous carrier couples sugar import and regeneration of import sites into a single step.

This study tested this hypothesis by exposing erythrocyte-resident GLUT1 to
reductant. This reduces the intramolecular disulfide bridge of each subunit causing the transporter to dissociate into dimers. In this way the catalytic and ligand binding properties of the dimer and tetramer can be contrasted directly. Tetrameric GLUT1 dissociation into dimers should be accompanied by a loss of $\partial$-Ab binding to red cells. C-Ab binding to permeabilized cells, however, should be unchanged since this antiserum recognizes an epitope whose availability is insensitive to GLUT1 oligomeric structure. Our results show that red cell-fixation in the presence of reductant significantly reduces the availability of the $\partial$-Ab-specific epitope(s) while C-Ab binding is unchanged. Assuming the loss of $\partial$-Ab binding reflects GLUT1 dissociation into dimers, the cooperativity hypothesis predicts that the ligand binding and sugar transport properties of the cells will be modified dramatically.

Transporter conversion from a simultaneous carrier (tetramer) to a simple carrier (dimer) will impact cytochalasin B binding to GLUT1 in two ways. GLUT1 cytochalasin B binding capacity will be doubled and inhibition of cytochalasin B binding to GLUT1 by extracellular maltose will be transformed from complex allosteric inhibition to simple competitive inhibition. Increased cytochalasin B binding is expected because only half of the subunits in tetrameric GLUT1 can present an export (cytochalasin B binding) site at any instant. When tetrameric GLUT1 dissociates into dimers, subunits are released from this constraint and now isomerize independently of their neighbor. Thus each subunit now presents an export site and therefore binds cytochalasin B independently of its neighbor. This prediction was satisfied experimentally.

Inhibitions of ligand binding to tetrameric GLUT1 may be competitive or allosteric depending on the sites of action. For example, inhibition of extracellular maltose binding
by extracellular D-glucose would be competitive because both ligands compete for the import site (an E2 subunit conformation). Similarly, inhibition of cytochalasin B binding by intracellular D-glucose would be competitive since cytochalasin B binds at or close to the sugar export site (an E1 subunit conformation). Extracellular maltose-inhibition of cytochalasin B binding to tetrameric GLUT1, however, requires negative cooperative interactions between subunits. Thus maltose binding to an E2 subunit will affect cytochalasin B binding to an E1 subunit only if the occupancy state of the E2 subunit is communicated to adjacent E1 subunits and vice versa. In dimeric GLUT1 each subunit functions independently of its neighbor as an E1 or E2 conformation. Cytochalasin B binding to E1 will competitively inhibit maltose binding to E2 and vice versa. Our experiments demonstrate that dithiothreitol converts maltose inhibition of cytochalasin B binding from negative allosteric inhibition to simple competitive inhibition.

3-O-methyl-D-glucose uptake is noncompetitively inhibited following red cell exposure to reductant. The extent of import inhibition ranges from 3 to 16-fold and is rapidly reversed upon removal of extracellular reductant. Transport inhibition increases in the order dithiothreitol < β-mercaptoethanol < reduced glutathione. Transport inhibition is not observed in resealed erythrocyte ghosts that contain 2 mM DTT but are washed free of extracellular reductant. These findings indicate that the site of action is most likely extracellular and that inhibition is unrelated to the closed ring structure of oxidized dithiothreitol.

An unexpected result is the failure of reductant to inhibit erythrocyte sugar efflux. This suggests a number of possibilities: 1) The cooperativity hypothesis is incorrect; 2)
Purified reduced GLUT1 is unable to mimic faithfully the catalytic properties of erythrocyte-resident, reductant-exposed GLUT1; 3) The observed kinetics of erythrocyte sugar exit do not reflect the intrinsic catalytic properties of the transporter. Ample evidence supports the latter two possibilities. The absence of nucleotides (AMP, ADP, ATP) impacts the behavior of purified GLUT1 since the transporter is also a nucleotide binding protein (Carruthers & Helgerson, 1989, Helgerson et al., 1989) whose catalytic turnover and ligand binding properties are radically altered in the presence of nucleotide (Carruthers, 1986a, Carruthers, 1986b, Carruthers & Helgerson, 1989, Carruthers & Melchior, 1983, Helgerson et al., 1989, Jacquez, 1983). In addition, kinetic analysis suggests that intracellular sugar is distributed between free and bound compartments (Helgerson & Carruthers, 1989, Naftalin et al., 1985, Nishimura et al., 1993). The observable kinetics of transport (which assume that all intracellular sugar is free) are, therefore, unlikely to accurately reflect the intrinsic behavior of the transporter.

Other studies in our lab support rejection of the cooperativity hypothesis. We have found (Chapter V and Coderre et al. 1995) that exofacial proteolysis of GLUT1 abolishes cooperativity in ligand binding to GLUT1 but fails to impact GLUT1 oligomeric structure or sugar transport function. This suggests therefore, that rapid substrate translocation by parental (tetrameric) GLUT1 results from subunit interactions distinct from those that promote cooperative ligand binding. This will be explored in detail in the next chapter.
Chapter V

Effect of Trypsin on \textit{in situ} GLUT1

Structure and Function

\textit{How does the hypothesized antiparallel arrangement of substrate binding sites between subunits influence catalytic activity?}

In the course of these studies, we observed that human red cell-resident GLUT1 is proteolytically cleaved by extracellular trypsin. Initially, this result was unexpected since trypsin is known to inhibit GLUT1-mediated sugar transport only when present inside the red cell (Baldwin et al., 1980, Carruthers & Melchior, 1983, Masaik & LeFevre, 1977) where it acts to proteolytically cleave GLUT1 (Cairns et al., 1987, Holman & Rees, 1987, Mueckler et al., 1985). While the effect of exofacial trypsin on GLUT1-covalent structure is unreported, immunoblot analyses show that the rat adipocyte glucose transport protein GLUT4 is digested by exofacial trypsin (Czech & Buxton, 1993). GLUT1 and GLUT4 share 67\% sequence identity and similar (proposed) membrane topographies in which both transporters are suggested to expose extracellular tryptic cleavage sites (Fukumoto et al., 1989). It is not surprising, therefore, that GLUT1 is also cleaved by exofacial trypsin. The functional resistance of GLUT1 to exofacial trypsin indicates, therefore, that GLUT1-mediated sugar transport is unaffected by exofacial GLUT1 proteolysis.

Closer examination reveals that exofacial GLUT1 proteolysis uncouples coopera-
tive interactions between substrate binding sites on adjacent subunits without causing subunit dissociation or inhibition of sugar transport. Extracellular reductant, however, causes tetrameric GLUT1 dissociation, transport inhibition and the loss of cooperative ligand binding to GLUT1. This suggests that the higher catalytic turnover of tetrameric GLUT1 does not derive from functional cooperativity between ligand binding sites as originally proposed (Hebert & Carruthers, 1992) but rather, from other aspects of subunit interactions within the tetrameric transporter complex.

Results

Effects of trypsin on GLUT1 covalent structure.

Intact erythrocytes were treated with several proteases including: thrombin, endoproteinase glu-C, endoproteinase lys-C and trypsin. These proteases show high specificity for arginine, glutamic acid, lysine and arginine/lysine residues, respectively. Only extracellular trypsin (Fig. 15A, lane 1) cleaves erythrocyte-resident GLUT1. Identical results were obtained using three different batches of TPCK-trypsin and four batches of sequence-grade trypsin and the effects of trypsin are blocked by soy bean trypsin inhibitor (10:1 inhibitor to trypsin ratio by mass; n = 2). Tryptic cleavage generates a 25,100 Dalton GLUT1 C-terminal fragment as judged by immunoblot analysis using GLUT1 C-terminal peptide anti-serum (C-Ab). Since intact GLUT1 is also found under these conditions, the possibility exists that either some erythrocytes are leaky and thus allow endofacial tryptic cleavage of GLUT1, or that sub optimal conditions were used for exofacial tryptic diges-
Figure 15. Tryptic digestion of erythrocyte GLUT1

A. Western blot analysis of human erythrocyte membranes using anti-GLUT1 C-terminal antiserum. Intact erythrocytes (5 x 10^9 cells) in 1 mL saline were exposed to 200 µg trypsin (lane 1), thrombin (lane 2), V8 (lane 3) or V9 (lane 4) for 30 min. at 37°C. Cells were collected, washed, lysed and membrane proteins resolved on 15% acrylamide gels.

B. Western blot analysis of human erythrocyte membranes using anti-GLUT1 C-terminal antiserum. Intact erythrocytes (1 x 10^10 cells) were incubated in saline (lane 1), or in 2 mL saline containing 100 µg trypsin (lane 2) or trypsin and 100 µM cytochalasin B (lane 3) at 37°C for 1 hr. Cell membrane proteins resolved on 10% acrylamide gels. In lanes 4 and 5, unsealed erythrocyte ghosts (4 mg membrane protein in 2 mL saline) were incubated with (lane 5) or without (lane 4) 100 µg trypsin for 15 min.

C. Coomassie stained gel (10% acrylamide) of reconstituted GLUT1 proteoliposomes (20 µg GLUT1 in 10 mg egg phosphatidylcholine) exposed to 100 µL saline (lane 1) or to 100 µL saline containing 5 µg trypsin (lane 2) at 37°C for 30 min.

The mobility of pre-stained molecular weight markers are indicated in A, B and C.
Figure 15

A

B

C

Legend:

- 80
- 49.5
- 32.5
- 27.5
- 66
- 21
- 14.4
- 115
- 66
- 45
- 29
In contrast to its effects on GLUT1 in intact red cells, tryptic digestion of unsealed, erythrocyte membranes results in the complete loss of immuno-detectable glucose transporter (Fig. 15B, lane 5). Because peptide maps obtained from intact cells and unsealed ghosts differ significantly, these results strongly suggest that exofacial trypsin acts at an extracellular GLUT1 site in intact red cells. Maximum tryptic cleavage of erythrocyte resident GLUT1 is obtained within 30 to 40 minutes of exposure to trypsin (Fig 16, A and B). Cleavage appears to occur at least two sites. At early times, a low abundance 38 kDa C-terminal GLUT1 fragment is just detectable (≤ 8% total immunoreactive protein as judged by densitometric analysis). This fragment is lost upon longer exposure to trypsin. Maximal GLUT1 digestion is produced at a trypsin to membrane protein ratio of approximately 1:100 (3 μg trypsin per 5 x 10^8 cells; Fig. 16C). At higher trypsin concentrations, the dependence of rate of proteolysis upon trypsin level becomes nonlinear. This may result from trypsin autodigestion. Exofacial tryptic digestion of red cell-resident GLUT1 is unaffected by the presence of 2 mM DTT (pH 7.4; n = 3).

Effects of trypsin on GLUT1 oligomeric structure.

We were curious to understand whether disruption of the GLUT1 backbone affects GLUT1 oligomeric structure. GLUT1 is known to exist as a GLUT1 homotetramer in the erythrocyte membrane (Hebert & Carruthers, 1991, 1992). We examined the effects of exofacial tryptic cleavage on GLUT1 oligomeric structure by monitoring the binding of ð-Ab to intact red cells. ð-Ab is an epitope-specific antiserum that binds with high affinity to tet-
Figure 16. Kinetics of GLUT1 Digestion by Extracellular Trypsin.

A. Erythrocytes (5.5 x 10^9 cells/ml) were digested with 0.3 mg/ml trypsin in HEPES-saline for 0, 2, 5, 10, 20, 30, 45 and 60 minutes (lanes 1 through 8 respectively) at 37°C. Erythrocytes were washed and lysed. Membrane proteins were electrophoresed on 15% polyacrylamide gels, subjected to Western blot analysis using anti-GLUT1 C-terminal antiserum and immunoreactive proteins visualized by autoradiography.

B. The intensities of immunoreactive proteins revealed by autoradiography were quantitated by scanning densitometry. Ordinate: percent of total immunoreactive protein present. Abscissa: time in minutes. Three populations of immunoreactive proteins were quantitated (see arrows in panel A): intact GLUT1 (●), a 38 kDa peptide (○) and a 25 kDa peptide (△). Curves drawn through the points were computed by fourth order Runge-Kutta numerical integration assuming GLUT1 is initially proteolyzed to release a 38 kDa C-terminal peptide which is subsequently cleaved to produce a 25 kDa C-terminal peptide. The computed best fit first order rate constants for assumed first and second cleavage reactions are (0.1 and 0.75 min⁻¹ respectively).

C. Concentration dependence of GLUT1 proteolysis by exofacial trypsin. Erythrocytes were digested (as in A) for 30 minutes at varying [trypsin]. Erythrocyte membranes were collected and membrane proteins processed as in Figure 11A. The extent of GLUT1 proteolysis was quantitated as in 11B. Ordinate: rate of GLUT1 proteolysis (percent per min.). Abscissa: trypsin : erythrocyte membrane protein ratio by mass. The curve drawn through the points was drawn by eye and has no theoretical significance.
Figure 16

A

B

Percent of total immunoreactive protein

Time minutes

C

Rate of GLUT1 proteolysis (% per min)

Trypsin/protein ratio (by mass)
rameric GLUT1 but fails to bind to dimeric GLUT1 (Hebert & Carruthers, 1992). \( \partial \)-Ab binding to intact cells (212,987 ± 2166 cpm per 2 x 10^8 cells; n = 3) is unaffected by prior erythrocyte exposure to exofacial trypsin (191,789 ± 2427 cpm per 2 x 10^8 cells; n = 3). This confirms that epitopes exposed in tetrameric GLUT1 but not in dimeric GLUT1 remain accessible in the trypsin-treated transporter and strongly suggests that transporter oligomeric structure is preserved under conditions where the polypeptide backbone of each subunit is broken. Quantitative immunofluorescence digital imaging fluorescence microscopy (Fig. 17) confirms this result and further shows that exposure of red cells to 2 mM DTT for 30 min at 37°C prior to fixation reduces \( \partial \)-AB binding to control and trypsin-treated red cells by 80% and 70% respectively. C-Ab binding to permeabilized control and trypsinized cells is not inhibited by DTT.

We further examined the oligomeric structure of GLUT1 by using nondenaturing size exclusion chromatography to determine the hydrodynamic radius of GLUT1-containing micelles solubilized from erythrocyte membranes. The Stokes radius of C-Ab-reactive, protein-containing cholic acid/lipid micelles solubilized from red cell membranes (8 ± 0.2 nm; n = 3) is not affected by cellular exposure to trypsin (1 mg/mL for 40 min) prior to detergent solubilization (Fig. 18). However, red cell-exposure to DTT (2 mM for 30 min at 37°C) prior to cholate-solubilization reduces the Stokes radius of cholic acid/lipid/GLUT1 micelles from 8 to 5.1 ± 0.3 nm (n = 3). Cholic acid-solubilized, purified tetrameric and dimeric GLUT1 are characterized by Stokes radii of 7.8 ± 0.2 and 6.0 ± 0.2 nm respectively (Hebert & Carruthers, 1992).
Figure 17. Effects of Trypsin and Reductant on β-Ab and C-Ab Binding to Red Cell GLUT1

Antibody binding to permeabilized erythrocytes was quantitated by digital imaging fluorescence microscopy. Cells were preincubated for 30 min at 37°C with 0.5 mg/mL trypsin (c, d, g, h) or saline (a, b, e, f) then attached to coverslips where they were exposed to saline (a, c, e, g) or to 2 mM DTT (b, d, f, h) for 30 min at 37°C prior to fixation and permeabilization. β-Ab binding was measured in a, b, c and d. C-Ab binding was measured in e, f, g and h. The bar chart to the right of the images quantitates cellular fluorescence (relative to cells in a) in arbitrary units. Results are shown as mean ± SEM (n= 4) and are shown for β-Ab (black bars) and for C-Ab (gray bars). This experiment was repeated three times with quantitatively similar results on each occasion.
Figure 17

Relative cellular fluorescence

0  1.0

0  1.0
Figure 18. Hydrodynamic Size Analysis of In Situ GLUT1.

Erythrocytes were exposed to saline (●), trypsin (V; 0.5 mg/mL) or dithiothreitol (○; 2 mM) for 30 min at 37°C (see Fig. 17). Cells were collected by centrifugation, washed in saline, then solubilized in 150 mM NaCl, 4 mM MOPS, 80 mM cholic acid, pH 7.0. The suspension was centrifuged at 45,000 x g for 30 minutes, then 100 µl of the clear supernatant was applied to a TSK-Gel G4000 SWXL size exclusion column which was developed in the same buffer at a flow rate of 0.3 mL/min. The column was equilibrated using Pharmacia standards and is characterized by: log Stokes radius (nm) = 1.551 - 0.0649 (Rf - V), where V is the void of the column (23.03 min.) Correlation coefficient² = 0.971. The elution times of some of these standards and the Stoke’s radius of these standards (nm) are indicated by the arrows. This chart summarizes 3 analyses.
Effects of trypsin on transporter photolabelling by cytochalasin B

GLUT1 present in intact and trypsinized red cells was photolabelled using \(^{3}\text{H}\)-cytochalasin B and the labeled species resolved by SDS-PAGE. Sub-optimal conditions of proteolysis were used in order to aid comparison of ligand binding to intact and 25 kDa GLUT1 peptides within the same membrane. With trypsinized red cells, most of the incorporated label migrates with low molecular weight species that co-localize with the C-terminal peptide fragment (Fig. 19A). This region of the gel contains a greater percentage of photolabel if labeling is performed before trypsinization of red cells rather than after trypsinization (Fig. 19A). The extent of GLUT1 proteolysis is also increased by photolabelling (but not by UV irradiation \textit{per se}) prior to proteolysis (see inset of Fig. 19A). The presence of 50 \(\mu\text{M}\) cytochalasin B during red cell exposure to trypsin does not alter the extent of GLUT1 proteolysis (Fig. 15B, lanes 2 and 3). It is possible, therefore, that cytochalasin B cross-linking (but not cytochalasin binding \textit{per se}) promotes exofacial tryptic digestion of GLUT1 but that proteolysis does not prevent ligand binding to the 25 kDa tryptic fragment.

Cytochalasin B binding to the 25 kDa tryptic fragment appears to be increased relative to binding to intact GLUT1. When normalized for the amount of immunodetectable GLUT1 C-terminal peptide (Fig. 19A, inset) and corrected for the small amount of endogenous GLUT1 proteolysis detected in control cells (Fig. 19A), cytochalasin B photoincorporation into the 25 kDa peptide is 4 \(\pm\) 1.3 fold greater if labeling is performed after proteolysis rather than before trypsinization (\(n = 3\)). Label incorporation per unit 25 kDa
Figure 19. Effect of Trypsin on GLUT1-mediated Ligand Binding.

A. Erythrocytes were photolabelled using $^3$H-cytochalasin B either before (V) or after (O) exposure to 50 μg/mL trypsin for 30 min. at 37°C. Control (trypsin-free) cells are also shown (●). Erythrocytes were washed, lysed and membrane proteins were electrophoresed on 10% gels. Gels were stained with Coomassie Brilliant Blue, destained and gel lanes sliced into 2 mm aliquots. Each gel slice was digested with 10% H$_2$O$_2$ at 56°C overnight and then analyzed by liquid scintillation counting. Ordinate, percentage of total $^3$H-CCB incorporated per gel lane; abscissa, gel slice. The inset shows the densitometric quantitation of an autoradiogram of a parallel Western blot analysis of these membranes using anti-GLUT1 C-terminal antiserum. DPM $^3$H-cytochalasin B incorporated per lane are: control, 5453; labelled before trypsin, 6439; labelled after trypsin, 8683.

B. Effect of trypsin on cytochalasin B binding to membranes isolated from intact cells (●), intact cells exposed to 100 μg/mL trypsin for 30 min. at 37°C (O) or unsealed, erythrocyte membranes exposed to 100 μg/mL trypsin for 30 min. at 37°C (▼). Curves drawn through intact cell data (● O) were computed by nonlinear regression assuming simple saturable binding. Computed binding constants are: Control (●), $K_d(app) = 83 \pm 12$ nM, $B_{max} = 3.9$ pmol/μg membrane protein; Trypsin (O), $K_d(app) = 126 \pm 21$ nM, $B_{max} = 6.3 \pm 0.3$ pmol/μg membrane protein. Each data point represents the mean ± SEM of at least 4 separate measurements made in duplicate. The membrane protein content of intact cells (0.6 ± 0.04 pg per cell) is not significantly altered by trypsin exposure. The protein content
of unsealed erythrocyte membranes is depleted by trypsin exposure. Here (▼), binding measurements were made using an equivalent number of cell unit membranes.

C. Effect of trypsin on maltose-inhibition of cytochalasin B binding to erythrocytes.

Intact cells (●) and trypsin (1 mg/mL for 30 min at 37°C) treated cells (○) were exposed to saline containing 100 nM cytochalasin B and varying (0 to 33 mM) maltose concentrations osmotically balanced using sucrose. Cytochalasin B binding was measured at 4°C. Each data point represents the mean ±SEM of nine separate measurements. Curves drawn through the points were computed by nonlinear regression assuming binding is described by the relationship (Helgerson & Carruthers, 1987)

\[
\frac{[CCB_f]}{[CCB_b]} = \frac{[CCB_f]}{[X_t]} + \frac{K_i}{[X_t]} \left( \frac{K_o + [Maltose]}{K_o + \alpha} \right)
\]

where CCB\(_f\) and CCB\(_b\) are free and bound cytochalasin B respectively, Xt is glucose transporter, Ki and Ko are the dissociation constants for cytochalasin B and maltose (respectively) binding to Xt and α is the negative cooperativity factor. For control cells, the computed best fit (R\(^2\) = 0.972) is obtained using the following constants: Xt = 1.8 μM, K\(_i\) = 0.1 μM, K\(_o\) = 3.6 mM and α = 2.0. For trypsin-treated cells, the computed best fit (R\(^2\) = 0.992) is obtained using the following constants: Xt = 4.3 μM, K\(_i\) = 0.33 μM, K\(_o\) = 40.8 mM and α > 2 x 10\(^{14}\).
Figure 19

A

Fraction of total cpm

gel slice

B

[CCB]_{bound} mmol/µg membrane protein

[CCB]_{free} nM

C

[CCB] / [CCB]_{b} vs [Maltose] mM
peptide (fraction of total cpm/fraction of total immunodetectable protein) is $1.75 \pm 0.25$ (labeled after proteolysis; $n = 4$) versus $0.43 \pm 0.23$ (labeled before proteolysis; $n = 4$). Label incorporation per unit intact GLUT1 averages $0.39 \pm 0.13$ ($n = 5$) and is unchanged by exposure to trypsin. This latter finding substantially confirms the central assumption of this analysis (immunostaining is directly proportional to intact GLUT1 and 25 kDa GLUT1 peptide levels) since 25 kDa peptide and intact GLUT1 derived from cells that were photolabelled before proteolysis should be characterized by identical label incorporation efficiencies.

**Effects of trypsin on equilibrium cytochalasin B binding**

If increased cytochalasin B photolabelling of the 25 kDa peptide reflects increased binding of ligand (rather than increased photolabelling efficiency) this should be observable at the level of steady-state cytochalasin B binding to red cells. We therefore examined the effects of GLUT1 trypsinization on equilibrium cytochalasin B binding. Trypsin treated RBCs are characterized by significantly increased capacity to bind cytochalasin B (Table IV). Binding shows simple saturation kinetics in both control and trypsin treated cells but maximum cytochalasin B binding ($B_{\text{max}}$) and the apparent dissociation constant $K_{\text{d(app)}}$ for cytochalasin B binding are approximately doubled in trypsin-treated cells relative to control cells.

Cytochalasin B binding to intact cells is complicated by nonspecific ligand binding to intracellular hemoglobin (Jung & Rampal, 1977, Helgerson & Carruthers, 1987). We prepared erythrocyte membranes from control and trypsin-treated cells and measured bind-
ing to these nominally hemoglobin-free membranes. $B_{\text{max}}$ and $K_{d(\text{app})}$ for binding to control membranes are significantly (almost 2-fold) lower than the corresponding parameters for cytochalasin B binding to membranes prepared from trypsinized red cells (Table V; Figure 20B). These effects on $B_{\text{max}}$ and $K_{d(\text{app})}$ do not reflect trypsin-induced changes in erythrocyte membrane protein content since red cell membrane protein content (0.6±0.04 pg per erythrocyte) is unchanged following erythrocyte exposure to extracellular trypsin. Trypsinization of unsealed erythrocyte membranes results in the loss of saturable cytochalasin B binding (Figure 19B; Table IV) while trypsin treatment of GLUT1 proteoliposomes doubles $K_{d(\text{app})}$ for cytochalasin B binding to purified GLUT1 (Table IV).

Because exofacial proteolysis doubles both $B_{\text{max}}$ and $K_{d(\text{app})}$ for cytochalasin B binding to intact cells, the ratio $B_{\text{max}}/K_{d(\text{app})}$ for cytochalasin B binding to erythrocyte-resident GLUT1 is unchanged by trypsin-treatment. Since saturable cytochalasin B binding at low ligand concentrations is described by $[\text{cytochalasin B}] \cdot k$ where $k = B_{\text{max}}/K_{d(\text{app})}$, this suggests that trypsin treatment should not affect ligand binding at limitingly low ligand concentrations. This was confirmed in experiments where the effects of trypsin treatment on erythrocyte cytochalasin B binding (at low [cytochalasin B]) and 3OMG uptake were measured. Neither sugar uptake nor ligand binding are affected (Table V) in spite of extensive GLUT1 proteolysis (Fig. 15B, lane 2). In contrast, exposure of control and trypsin-treated cells to 2 mM DTT (a treatment causing transporter dissociation into dimeric GLUT1) inhibits protein-mediated sugar uptake 2-fold (Table V).
Effects of trypsin on availability of exo- and endofacial ligand binding sites

In the absence of maltose, cytochalasin B binding to control and trypsin-treated red cells are not distinguishable (Fig. 19C). However, the manner by which maltose inhibits cytochalasin B binding to GLUT1 is modified significantly following red cell exposure to trypsin. The nature of the antagonistic relationship between exofacial maltose and endofacial cytochalasin B binding sites is revealed (Helgerson & Carruthers, 1987) by expressing the ratio free [cytochalasin B]:bound [cytochalasin B] as a function of maltose concentration (Fig. 19C). If cytochalasin B and maltose binding sites cannot co-exist, the relationship is linear with positive slope. If cytochalasin B and maltose binding sites co-exist but interact with negative cooperativity, the relationship is positively curvilinear. Figure 19C shows that prior to exofacial proteolysis, exofacial maltose and endofacial cytochalasin B binding sites interact with negative cooperativity. Following proteolysis, however, cytochalasin B and maltose binding sites are mutually exclusive. The effect of trypsin on maltose-inhibition of cytochalasin B binding to red cell GLUT1 is not further modified by subsequent cellular exposure to 2 mM DTT (37°C for 30 min; n=3).

Effects of trypsin on 3-O-methyl-D-glucose transport

Neither \( V_{\text{max}} \) nor \( K_{\text{m(app)}} \) for net 3OMG uptake by erythrocytes are affected significantly by trypsin treatment (Table VI). Net 3OMG uptake by both control and trypsinized erythrocytes is inhibited significantly by 2 mM dithiothreitol (Table V). Cytochalasin B inhibition of net 3OMG uptake is unaffected in trypsin-treated cells (Table
VI). Prior trypsin treatment is without effect on subsequent tracer (60 μM) 3OMG efflux from erythrocytes at ice temperature (rate constant for exit from control and trypsin-treated cells = (0.18 ± 0.03) and (0.20 ± 0.02) min⁻¹ respectively; n=3). Basal cell membrane permeability is unchanged by trypsin treatment as 3OMG uptake in the presence of 10 μM cytochalasin B is unaffected by prior cell-exposure to trypsin (Table VI). It is unlikely, therefore, that trypsin penetrates cells to alter GLUT1 structure/function when the monosaccharide, 3OMG, is excluded by the membrane bilayer.

\(K_m(\text{app})\) for protein-mediated D-glucose transport by reconstituted GLUT1 proteoliposomes is reduced 2-fold by exofacial trypsin-treatment but \(V_{\text{max}}\) for uptake is reduced by only 20% (Fig. 20 and Table V). The fall in reconstituted transport activity (20%) upon trypsin treatment of proteoliposomes is close to the expected decline of 37%. Under the conditions of these experiments, trypsin treatment of GLUT1 proteoliposomes results in the loss of detectable protein (Figure 15C, lane 2) as judged by Coomassie staining of control and trypsin-treated proteoliposomes resolved by SDS PAGE. Protein-mediated sugar transport activity is undetectable in proteoliposomes containing GLUT1 exposed to trypsin prior to reconstitution (Fig. 20).
Figure 20. Effect of trypsin on GLUT1 mediated D-glucose uptake by reconstituted proteoliposomes.

Ordinate: rate of D-glucose uptake from saline containing 100 mM D-glucose in mmol D-glucose per L intra-proteoliposomal water per min. Abscissa: intra-proteoliposomal [D-glucose] in mM. Key: • control proteoliposomes; △ trypsin-treated proteoliposomes (see Fig 15C for details); — (straight line indicating no measurable transport), uptake by proteoliposomes exposed to 100 µM phloretin plus 50 µM cytochalasin B or by proteoliposomes formed from GLUT1 that was trypsin-treated prior to reconstitution by cholate dialysis. The rate of sugar uptake at each [D-glucose] was computed as the first order derivative of the time-course of D-glucose uptake. The curves drawn through the points were computed by nonlinear regression assuming Michaelis-Menten kinetics and have the following parameters: Control, \( V = 56 \pm 1.9, K_{m(app)} = 9.2 \pm 0.5 \); Trypsin, \( V = 47.2 \pm 1.6, K_{m(app)} = 4.6 \pm 0.2 \). \( V \) has units of mM per min. and corresponds to the rate of D-glucose uptake when intravesicular D-glucose is absent. \( K_{m(app)} \) has units of mM and corresponds to that concentration of intravesicular D-glucose that reduces sugar uptake to one-half \( V \). These data summarize 5 separate experiments.
Figure 20

D-glucose uptake (mM/min)

[D-glucose]_i mM

Control

Trypsin

Control + phloretin/CCB
Table IV: Effect of Extracellular Trypsin on Ligand Binding to Red Cell GLUT1

<table>
<thead>
<tr>
<th>CCB binding to</th>
<th>Control$^a$</th>
<th>Trypsin</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Intact Cells$^b$</strong></td>
<td>$K_d$ (app) nM 102 ± 40</td>
<td>* 324 ± 66</td>
</tr>
<tr>
<td></td>
<td>$B_{max}$ (sites/cell)$^c$ 262,000 ± 23,000</td>
<td>* 508,700 ± 48,000</td>
</tr>
<tr>
<td><strong>Membranes from intact cells$^d$</strong></td>
<td>$K_d$ (app) nM 86 ± 17</td>
<td>* 155 ± 31</td>
</tr>
<tr>
<td></td>
<td>$B_{max}$ (sites/cell) 169,200 ± 14,400</td>
<td>* 271,440 ± 32,572</td>
</tr>
<tr>
<td><strong>Membranes from leaky cells$^e$</strong></td>
<td>$K_d$ (app) nM 103 ± 32</td>
<td>Not detected</td>
</tr>
<tr>
<td></td>
<td>$B_{max}$ 120,240 ± 21,980</td>
<td>Not detected</td>
</tr>
<tr>
<td><strong>proteoliposomes$^f$</strong></td>
<td>$K_d$ (app) nM 140 ± 15</td>
<td>* 353 ± 28</td>
</tr>
<tr>
<td></td>
<td>$B_{max}$ (% quench)$^g$ 6.4 ± 0.2</td>
<td>6.0 ± 0.2</td>
</tr>
<tr>
<td><strong>D-glucose binding to proteoliposomes$^h$</strong></td>
<td>$K_d$ (low) mM 0.10 ± 0.04</td>
<td>0.07 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>$B_{max}$ (low) (% quench)$^g$ 1.37 ± 0.16</td>
<td>1.95 ± 0.64</td>
</tr>
<tr>
<td></td>
<td>$K_d$ (high) mM 17.45 ± 3.52</td>
<td>* 3.8 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>$B_{max}$ (high) (% quench)$^g$ 95.25 ± 0.27</td>
<td>4.4 ± 0.6</td>
</tr>
</tbody>
</table>
**Table IV Notes:**

*Indicates that the result is significantly different from the control measurement (p<0.05, 1 tailed t-test).

*a* All experiments are paired experiments (control versus trypsin treatment). The number of paired determination made in duplicate is 3 or greater.

*b* Cytochalasin B binding was measured in intact erythrocytes following either control or trypsin (100 µg trypsin/1 x 10^9 cells; 30 min. at 37°C) treatment.

*c* B_max for cytochalasin B binding in molecules Cytochalasin B bound per unit cell membrane (each cell membrane contains 0.6 pg protein).

*d* Cytochalasin B binding was measured in erythrocyte membranes isolated from either control or trypsin-treated erythrocytes (100 µg trypsin/1 x 10^9 cells; 30 min. at 37°C).

*e* Cytochalasin B binding was measured in erythrocyte membranes obtained following either control or trypsin-treatment (100 µg trypsin/1 x 10^9 cells; 30 min. at 37°C) of unsealed erythrocyte membranes.

*f* Cytochalasin B binding was measured in reconstituted GLUT1 proteoliposomes following either control or trypsin-treatment (5 µg trypsin/20 µg GLUT1).

*g* Binding was measured by analysis of ligand-induced quenching of GLUT1 intrinsic fluorescence. Maximum binding is represented by maximum quench (% of original fluorescence) induced by the ligand.

*h* D-glucose binding to GLUT1 is characterized by high and low affinity components. Binding was measured at 24°C following control and trypsin (5µg/20 µg GLUT1; 30 min. at
37°C) treatments.
Table V: Effect of Trypsin on Sugar Transport and Ligand Binding at Limiting Substrate Levels.

<table>
<thead>
<tr>
<th></th>
<th>Control&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Trypsin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saline</td>
<td>+DTT</td>
</tr>
<tr>
<td>b&lt;sup&gt;3&lt;/sup&gt;MG uptake</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>152.1 ± 0.4</td>
<td>75.6 ± 1.2</td>
</tr>
<tr>
<td>c plus CCB</td>
<td>5.8 ± 0.7</td>
<td>4.4 ± 0.5</td>
</tr>
<tr>
<td>d CCB binding</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>10.01 ± 0.36</td>
<td>7.43 ± 0.68</td>
</tr>
<tr>
<td>e plus phlorizin</td>
<td>1.9 ± 0.2</td>
<td>1.8 ± 0.2</td>
</tr>
</tbody>
</table>
All experiments are paired experiments (control versus 1 hr. trypsin treatment at 1 mg trypsin per 10^10 cells ± 2 mM dithiothreitol, pH 7.4). The number of paired determinations made in triplicate is 3 or more.

3-O-methylglucose uptake (at 100 μM) was measured at 4°C and is expressed as μmol sugar transported per L cell water per min.

Uptake was measured in the presence of the sugar transport inhibitor cytochalasin B (50 μM; K_i(app) for transport inhibition = 350 nM).

Cytochalasin B binding is expressed as the ratio bound [Cytochalasin B] : free [cytochalasin B]. The concentration of free cytochalasin B at equilibrium was approximately 30 nM.

Cytochalasin B binding was also measured in the presence of 100 μM phloretin - a cytochalasin B binding antagonist.

Table V Legend

- All experiments are paired experiments (control versus 1 hr. trypsin treatment at 1 mg trypsin per 10^10 cells ± 2 mM dithiothreitol, pH 7.4). The number of paired determinations made in triplicate is 3 or more.
- 3-O-methylglucose uptake (at 100 μM) was measured at 4°C and is expressed as μmol sugar transported per L cell water per min.
- Uptake was measured in the presence of the sugar transport inhibitor cytochalasin B (50 μM; K_i(app) for transport inhibition = 350 nM).
- Cytochalasin B binding is expressed as the ratio bound [Cytochalasin B] : free [cytochalasin B]. The concentration of free cytochalasin B at equilibrium was approximately 30 nM.
- Cytochalasin B binding was also measured in the presence of 100 μM phloretin - a cytochalasin B binding antagonist.
Table VI: Effect of Extracellular Trypsin on GLUT1-Mediated Sugar Transport.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Trypsin</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Proteoliposome D-Glucose uptake</strong>&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( V ) (mM/min.)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>58.8 ± 2.3</td>
<td>*47.2 ± 1.6</td>
</tr>
<tr>
<td>( K_m ) mM&lt;sup&gt;c&lt;/sup&gt;</td>
<td>8.6 ± 0.5</td>
<td>*4.6 ± 0.2</td>
</tr>
<tr>
<td><strong>Red cell 3OMG uptake</strong>&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( V_m ) (μM/min.)</td>
<td>241 ± 17</td>
<td>212 ± 10</td>
</tr>
<tr>
<td>( K_m ) mM</td>
<td>0.7 ± 0.1</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td><strong>CCB inhibition of red cell 3OMG uptake</strong>&lt;sup&gt;e&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( I_m ) (%)</td>
<td>86 ± 11</td>
<td>87 ± 16</td>
</tr>
<tr>
<td>( K_i(\text{app}) ) nM</td>
<td>419 ± 113</td>
<td>359 ± 101</td>
</tr>
</tbody>
</table>
Table VI Notes:

*Indicates that the result is significantly different from the control measurement (p<0.05, 1 tailed t-test).

a All experiments are paired experiments (control versus trypsin treatment). The number of paired determination made in duplicate is 3 or greater.

b D-Glucose (100 mM) uptake by proteoliposomes was measured at 24°C. Proteoliposomes (20 µg GLUT1) were exposed to trypsin-free saline or to saline containing 5 µg trypsin for 30 min.

c V is the computed, maximum rate of 3-O-methylglucose uptake while $K_m$ is that concentration of intra-liposomal 3-O-methylglucose that produces one-half V.

d 3-O-methylglucose uptake by erythrocytes was measured at 0-4°C. Cells ($10^9$) were exposed to saline, or to saline containing 0.3 mg.mL$^{-1}$ trypsin for 30 min. at 37°C.

e Cytochalasin B inhibition of red cell 3-O-methylglucose uptake from medium containing 2 mM 3-O-methylglucose was analyzed by nonlinear regression assuming simple saturation kinetics. $I_m$ is the maximum inhibition (%) produced by cytochalasin B while $K_{i(app)}$ is that concentration of cytochalasin B that produces one-half $I_m$. Cells ($10^9$) were exposed to saline, or to saline containing 0.3 mg.mL$^{-1}$ trypsin for 30 min. at 37°C.
Discussion

This study demonstrates that the backbone of the erythrocyte glucose transport protein can be broken at two extracellular sites without ablating protein-mediated sugar transport. In the process, however, the glucose transporter is converted from a multi-site transporter (Carruthers & Helgerson, 1991) to a simple carrier (Widdas, 1952). Previous studies have shown that trypsin cleaves GLUT1 at multiple intracellular sites and that the resulting transporter is non-functional (Baldwin et al., 1980, Cairns et al., 1987, Carruthers & Melchior, 1983, Holman & Rees, 1987, Masaik & LeFevre, 1977, Mueckler et al., 1985). These studies also concluded that GLUT1 was not cleaved at extracellular sites since transport function was unchanged by exofacial trypsin. We show here that GLUT1 is indeed cleaved at the exofacial surface, not once but twice. Proteolysis abolishes cooperative interactions between substrate binding sites but leaves transport activity and transporter oligomeric structure unchanged.

The time course of extracellular tryptic digestion of GLUT1 suggests that a rapid initial cleavage is followed by a second cleavage. The first cut releases a low abundance, C-terminal peptide of estimated $M_r$ 38,000. Within 30 minutes, this intermediate disappears yielding a 25 kDa peptide. It is possible that the initial cleavage is necessary to provide access to the second cleavage site resulting in the appearance of the 25 kDa peptide. Assuming this model is correct, our calculations suggest rate constants for the first and second cleavage reactions of 0.1 and 0.75 min$^{-1}$ respectively (see Fig. 16B). An alternative explanation is that the red cell exposes two populations of GLUT1. The smaller population
(10% of total GLUT1) is hydrolyzed slowly ($k = 0.1 \text{ min}^{-1}$) to release a 38 kDa fragment which is then rapidly degraded ($k = 0.5 \text{ min}^{-1}$). The larger population (> 90% of total GLUT1) is cleaved slowly (rate constant = 0.1 min$^{-1}$) to release the 25 kDa fragment. The available data do not allow us to distinguish between these possibilities.

The 25 kDa GLUT1 tryptic fragment contains an intact C-terminus since this peptide is detected on Western blot analysis using anti-GLUT1 C-terminal peptide antiserum. The electrophoretic mobilities of intact GLUT1 and both immuno-detectable, C-terminal GLUT1 tryptic fragments on 18% acrylamide gels are consistent with molecular weights of 64.2 ± 1.3, 38.3 ± 0.9 and 25.2 ± 1.2 kDa respectively ($n = 7$). Assuming this analysis is correct, potential tryptic cleavage sites are arginine$_{153}$ ($M_r = 37.7$ kDa) and arginine$_{264}$ ($M_r = 25.1$ kDa). However, hydropathy and glycosylation scanning mutagenesis analyses (Mueckler et al., 1985; Hresko et al., 1994a) suggest that these sites lie inside the cell and antipeptide antibody binding studies indicate that a GLUT1 region immediately N-terminal to arginine$_{264}$ (residues 218 to 232) is cytoplasmic (Andersson & Lundahl, 1988). GLUT1 and other membrane proteins that bind sodium dodecyl sulfate more avidly than do more hydrophilic proteins can show anomalous electrophoretic behavior upon SDS-PAGE. For example, intact GLUT1 and the smaller C-terminal, GLUT1 tryptic fragment are resolved as 56.4 ± 0.7 and 17.5 ± 1.8 kDa peptides in 10% acrylamide gels ($n = 4$; Figure 15B). Similarly, RhD (Band 7 protein with deduced molecular mass, 45.2 kDa) is resolved as a 33 kDa peptide upon electrophoresis in 10% acrylamide gels (Chapter III or Zottola et al, 1995). It is not possible, therefore, to make precise assignments of sites of GLUT1 proteol-
ysis based upon analysis of peptide maps alone. N-terminal sequence of the 25 kDa fragment is required. If we assume the proposed membrane topography of GLUT1 is correct, hydrolysis at exofacial residues lysine183 and lysine300 would produce peptides of $M_r$ 34.4 and 20.8 kDa respectively with theoretical relative mobilities of 0.6 : 1 (compare with experimental of ($0.65 \pm 0.05$) : 1).

GLUT1-mediated sugar transport in reconstituted GLUT1 proteoliposomes is resistant to trypsin-inhibition while reconstitution of trypsin-treated GLUT1 by detergent dialysis fails to reconstitute glucose transport. This suggests that GLUT1 function and essential, noncovalent GLUT1 structure are maintained when the proteolyzed transporter is embedded in the membrane bilayer but are lost during the reconstitution process.

Red-cell resident GLUT1 oligomeric structure was examined by two methods - size-exclusion chromatography and by analysis of β-Ab binding. The former method provides information on the hydrodynamic radius of particles released from the red cell membrane upon detergent-solubilization (Hebert and Carruthers, 1991). The latter method provides information on the surface accessibility of tetrameric GLUT1-specific epitopes (Hebert and Carruthers, 1992).

Size-exclusion analysis of cholate-solubilized red cell membranes indicates that prior cell exposure to trypsin does not change the Stokes radius (8 nm) of lipid/detergent micelles containing GLUT1 and GLUT1-carboxyl terminal peptides. While these particles may contain non-GLUT1 protein species, they also co-elute with authentic tetrameric GLUT1 (Stokes radius = 8 nm). β-Ab-reactive epitopes are lost in reductant-treated but not
in trypsin-treated cells. Our analyses demonstrate that \( \partial \)-Ab reacts with membrane-resident, tetrameric GLUT1 and with SDS-denatured, reduced GLUT1 but not with reduced, membrane-resident (dimeric) GLUT1 (see Fig. 18 and Hebert & Carruthers, 1992). The results of our current analyses, therefore, support the conclusion that exofacial, tryptic digestion of GLUT1 does not cause dissociation of the multisubunit transporter complex. Exposure to extracellular reductant, however, promotes a significant GLUT1 conformational change resulting transporter dissociation and occlusion of cell surface (\( \partial \)-Ab-reactive) epitope(s). While it is formally possible that \( \partial \)-Ab-reactive epitopes could be lost under circumstances where GLUT1 tetrameric structure is maintained or that \( \partial \)-Ab binding could be retained under conditions where GLUT1 dissociates into dimers, neither of these possibilities has been observed experimentally (see here and Hebert & Carruthers, 1992).

Steady-state cytochalasin B binding to glucose transporter present in trypsin-treated cells is almost twice that measured in untreated cells. This increased ligand binding capacity is also seen in erythrocyte ghosts prepared from trypsin-treated red cells. The increase in red cell cytochalasin B binding promoted by trypsin is not explained by a trivial loss of membrane protein because exposure to exofacial trypsin does not alter erythrocyte membrane protein content significantly. Furthermore, our analyses suggest that the 25 kDa GLUT1 C-terminal peptide is photolabelled by CCB (4 \( \pm \) 1.3)-fold more efficiently than is intact GLUT1. This analysis assumes that intact GLUT1 and the 25 kDa peptide share equal blotting affinity for C-Ab. This increased binding accounts quantitatively for the increase in steady-state ligand binding capacity of red cell membranes and confirms previous demonstrations of suppressed, erythrocyte-resident GLUT1 CCB binding potential (Hebert
The photolabelled, intact transporter is proteolyzed more efficiently than non-labeled GLUT1 or the CCB-GLUT1 equilibrium complex. Because equilibrium CCB binding does not enhance GLUT1 exofacial proteolysis, this suggests that the covalently (CCB) liganded transporter is conformationally distinct from its noncovalently (CCB) liganded and unliganded counterparts. These conclusions are consistent with those of other studies (Holman & Rees, 1987, Karim et al., 1987). While the CCB binding capacity of membranes isolated from trypsin-treated red cell is doubled, the affinity of the membranes for CCB is halved (K_d(app) is approximately doubled). This result (which is consistent with unchanged CCB binding at low ligand concentrations (see here and Baldwin et al. 1980) is equivalent to the loss of 1 van der Waal’s bond between cytochalasin B and its binding site. In contrast, K_d(app) for D-glucose binding to the sugar influx site and K_m(app) for 30MG uptake are unchanged while K_d(app) for D-glucose binding to the sugar efflux site and K_m(app) for D-glucose exit from proteoliposomes are reduced 2 to 4-fold by trypsin-treatment. Although interpretation of the latter effect is complicated by the random orientation of GLUT1 in reconstituted proteoliposomes, the evidence suggests that GLUT1 proteolysis exerts contrasting actions on the binding of two distinct ligands at the sugar efflux site.

The transported substrates (D-glucose and 30MG) bind with increased affinity to proteolyzed GLUT1 while the nontransported, inhibitory ligand cytochalasin B binds with reduced affinity. These observations are consistent with the action of intra- plus extracellular trypsin on ligand binding to purified GLUT1 (Cairns et al., 1984) and support the view
that cytochalasin B and intracellular sugar binding to the transporter, although mutually exclu-

sive, are not mediated by identical chemistries (Cairns et al., 1987, Carruthers & Helg-
erson, 1991, Holman & Rees, 1987). Transported and nontransported (but reactive) ligands
have previously been shown to promote different actions at GLUT1 substrate binding sites.
Binding of nontransported species imparts negative cooperativity between import and ex-
port sites while binding of transported species does not (Helgerson & Carruthers, 1987).

How is it possible that sugar transport in control and trypsinized cells are indistin-

guishable while cytochalasin B binding to trypsinized cells is doubled? The simplest expla-
nation is that the increase in cytochalasin B binding is not associated with the glucose
transporter. Trypsinization reveals cryptic, non-GLUT1 sites with reduced affinity for
ligand (relative to GLUT1). This seems unlikely since extracellular maltose inhibits the in-
crease in binding produced by trypsin. In addition, photoaffinity labeling experiments in-
dicate that the increased binding is associated with the 25 kDa GLUT1 carboxyl-terminal
peptide.

An alternative explanation is that cooperative interactions between subunits of the
oligomeric transporter complex are relaxed upon exofacial proteolysis of GLUT1. The
erythrocyte sugar transporter is a GLUT1 tetramer (Hebert & Carruthers, 1991, Hebert &
Carruthers, 1992). Each subunit (GLUT1 protein) contributes a single sugar transport site
to the transporter complex and at any time can expose either a sugar import or a sugar ex-
port site (but not both) to available substrate. It has been proposed that cooperative interac-
tions between subunits produce an antiparallel arrangement of subunit transport sites (two
uptake and two efflux sites) at all times (Hebert & Carruthers, 1992). Thus if one subunit exposes a sugar export site, the adjacent subunit must expose a sugar import site and vice versa. Since cytochalasin B binds at or very close to the sugar efflux site and each subunit can expose only a sugar uptake or a sugar efflux site (but not both) at any instant, each GLUT1 tetramer can bind only two molecules of CCB. Tetrameric GLUT1 dissociates into GLUT1 dimers when exposed to reductant (see here and Hebert & Carruthers (1991) and Hebert & Carruthers (1992)). Cooperative interactions between subunits are relaxed in dimeric GLUT1 and the antiparallel arrangement of binding sites is lost. The CCB binding capacity of dimeric GLUT1 is thus twice that of the native, tetrameric transporter. The proteolyzed glucose transport may, therefore, functionally resemble the reduced, dimeric transporter.

This hypothesis does not require that relaxation of catalytic cooperativity between subunits is accompanied by dissociation of tetrameric GLUT1 into GLUT1 dimers (although the reverse would be required). However, the theory does predict that loss of cooperativity between subunits inhibits GLUT1-mediated sugar transport and abolishes negative cooperativity between CCB (sugar efflux) and exofacial maltose (sugar influx) binding sites (Hebert & Carruthers, 1992). Our experiments show that proteolyzed GLUT1 retains structural features unique to tetrameric GLUT1 suggesting that the transporter does not dissociate into GLUT1 dimers. Our experiments also show that \( V_{\text{max}} \) for 3OMG uptake is unaffected by trypsin-treatment of red cells. But, trypsin releases exofacial maltose and endofacial CCB binding sites from obligate, negative, cooperative interaction and ren-
ders these sites mutually exclusive. This is a methodologically independent replication of the action of reductant on ligand binding to the glucose transporter (Hebert & Carruthers, 1991, Hebert & Carruthers, 1992). In this instance, however, cooperativity between transporter subunits is lost under circumstances where structurally cohesive interactions between subunits appear to be maintained.

Red cell exposure to reductant mimics the ability of trypsin to inhibit cooperative interactions between substrate binding sites (Chapter IV or Zottola et al, 1995) but also causes inhibition of sugar import and subunit dissociation (as judged by the loss of tetramer-specific GLUT1 epitopes and the reduced Stoke’s radius of the detergent-solubilized transporter). This suggests that maintenance of tetrameric structure but not cooperativity between subunit binding sites is required for efficient catalytic turnover. Provided GLUT1 proteolysis does not introduce as yet uncharacterized compensatory changes in subunit catalytic turnover, the hypothesized antiparallel arrangement of substrate binding sites cannot contribute directly to catalytic efficiency as was originally proposed (Hebert & Carruthers, 1992).

In conclusion, our studies demonstrate that GLUT1 is vulnerable to proteolysis by trypsin in at least one extracellular site. This disruption of glucose transporter covalent structure is not accompanied by detectable changes in transporter oligomeric structure nor does it abrogate sugar transport function. Exofacial proteolysis does, however, destroy cooperative interactions between subunits allowing each subunit to behave as a simple carrier. This relaxation of obligate, antiparallel, catalytic conformations in adjacent subunits ren-
ders sugar import and sugar export sites mutually exclusive, reveals the full cytochalasin B binding potential of GLUT1 (1 mol CCB per mol GLUT1) and indicates that cooperative substrate binding is not necessary for high catalytic turnover. At this time, it is uncertain whether the loss of catalytic cooperativity results from direct proteolysis of a critical interfacial domain between subunits or indirectly from proteolysis-induced disorder in crucial interfacial domains distal to the site(s) of covalent rupture.
Chapter VI

Analysis of Net Sugar Uptake

Is human erythrocyte net sugar transport is limited by intracellular sugar binding/complexation?

Erythrocyte sugar transport is characterized by a degree of kinetic complexity that is not easily explained by models that consider the process of net transport to reflect protein-mediated transmembrane sugar movements alone (Baker & Naftalin, 1979, Carruthers, 1991, Helgerson & Carruthers, 1989, Naftalin & Holman, 1977, Naftalin & Rist, 1991, Naftalin et al., 1985). This has lead to the hypothesis that transported sugars become reversibly complexed with intracellular macromolecules (Naftalin & Holman, 1977). If correct, this means that the observable steady state kinetics of erythrocyte sugar transport describe the sum of two processes (protein mediated transport and intracellular sugar complexation) and that kinetic models for sugar transport that fail to recognize this may require significant re-evaluation.

The most striking illustrations of this problem are found in discussions of methods for sugar transport determination. When the time-course of net sugar exit from erythrocytes is analyzed using appropriate integrated Michaelis-Menten equations or by using the first derivative of the exit progress curve, relatively high $K_{m(app)}$ for exit are obtained (Baker & Naftalin, 1979, Carruthers, 1990, Carruthers & Melchior, 1983, Hankin
et al., 1972). If, however, an initial rate approach is employed to measure exit, relatively low \( K_{m(app)} \) are obtained (Lowe & Walmsley, 1986, Miller, 1971). Measurement of net sugar uptake by initial and integrated rate approaches seems not to suffer this problem (Baker & Naftalin, 1979, Ginsburg & Stein, 1975; but see Wheeler & Whelan, 1988). These approaches (initial and integrated rate analyses) exploit different expressions for the same kinetic process and should produce the same answer. Whereas initial rate analysis successfully predicts the early points of the exit progress curve but fails to predict later portions of the reaction (Carruthers, 1990, Lowe & Walmsley, 1986, Naftalin et al., 1985), integrated rate analysis successfully describes the entire course of the exit progress curve (Carruthers, 1986, Hankin et al., 1972, Miller, 1968, Sen & Widdas, 1962).

What is significant in these studies is that the integrated rate analysis follows sugar exit over prolonged intervals whereas the initial rate analysis monitors only the earliest period of the exit progress curve. If intracellular sugar binding reduces the amount of free sugar available for exit, the time-course of exit is prolonged and the measured \( K_{m(app)} \) for net exit overestimates the actual constant for protein-mediated efflux. Similarly, if sugar uptake is rate-limited by sugar binding to a complex in close proximity to the transporter and newly imported sugar first enters a compartment of restricted water content, sugar levels just below the membrane may rise extremely rapidly. This would promote significant sugar exit and thereby reduce net uptake. Considerations such as these stimulated Naftalin to propose that erythrocyte sugar transport is an intrinsically symmetric process whose operational characteristics result from factors extrinsic to the transport process per se (Baker & Naftalin, 1979, Naftalin & Holman, 1977, Naftalin et
al., 1985). Naftalin has since demonstrated that the glucose transporter of various GLUT1 expressing cells is in reversible functional and/or structural association or proximity with cytosolic hexokinase (Faik et al., 1989, Naftalin & Rist, 1990, Pedley et al., 1993) and that this association exerts a profound influence on the operational characteristics of cellular sugar transport.

In this study we ask whether human erythrocyte net sugar transport is limited by intracellular sugar binding/complexation. We surmised that if such were the case, net sugar uptake at limiting substrate levels would not be a simple exponential process. Rather, slow and rapid components of uptake may be discernible. We confirm this for uptake but not for exit. This and other findings lead us to conclude that newly transported sugar binds to an intracellular complex that is structurally associated with the sugar transporter. Sugar dissociation from this complex rate-limits further net sugar uptake or exit. Our observations substantially confirm Naftalin’s hypothesis that red cell net sugar transport is rate-limited by events extrinsic to the transport process. These observations show that steady-state red cell transport data represent two serial processes - transport and sugar binding - and thus cannot be used to model the translocation process directly. They also suggest a means for net sugar transport regulation via transporter association with sugar binding macromolecules.

Results

Is erythrocyte sugar transport diffusion-limited?

If sugar transport is a diffusion-limited process, reducing the self-diffusion
coefficient of the transported sugar by increasing the viscosity of the external or internal environments will reduce the rate of sugar transport. The strategy we adopted was that described by Blacklow et al. (1988) in which the "microviscosity" of the medium is increased by use of monomeric polyhydroxylated viscogenic species such as glycerol or sucrose. If the rate of sugar transport is limited by the frequency of collision between reactants (glucose transporter and transported sugar) and manipulation of medium viscosity exerts no additional effect on transport, relative medium viscosity and the relative second-order rate constant for transport are inversely related.

The second-order rate constant for transport \( (k_{\text{cat}}/K_m) \) is directly proportional to \( V_{\text{max}}/K_m \) \( (V_m = k_{\text{cat}} [GLUT1]) \) which is obtained as the rate constant for transport at limitingly low sugar concentrations (Carruthers, 1991). Figure 21 shows that human erythrocyte import of 50 μM 3OMG is reduced by increasing extracellular glycerol levels. This effect is also seen in resealed erythrocyte ghosts containing glycerol at levels identical to those applied externally and cannot thus be ascribed to extracellular glycerol-induced cell shrinking. The sugar analog 6-NBD glucosamine (NBDG) is transported some 3,300-fold more slowly than is 3OMG (Fig. 21) suggesting that transport of this sugar is limited by events other than sugar/transporter encounter rate. Because of this NBDG transport is expected to be less sensitive to altered medium viscosity. Cytochalasin B-inhibitable and cytochalasin B-insensitive NBDG uptakes are unaffected by addition of extracellular glycerol (Fig. 21).
Figure 21. Effect of increasing medium viscosity on erythrocyte sugar transport.

A. Effect of medium glycerol content on 3OMG uptake by red cells (●) and by red cell ghosts (○). Red cell ghosts contain glycerol at the same concentration as that in the external medium. Ordinate: rate constant for 3OMG uptake relative to that measured in the absence of glycerol. Abscissa: medium [Glycerol] (% wt:vol) and relative medium viscosity (from Blacklow et al., 1988). Data are shown as mean ± SEM of at least 3 separate determinations in duplicate or triplicate. Lines drawn through the points were computed by the method of least squares and correspond to: Red cells, relative $k = 1.0 - 0.025$ [Glycerol], $R = 0.997$; ghosts, relative $k = 0.979 - 0.024$ [Glycerol], $R = 0.994$. First order rate constants ($V_{\text{max}}/K_m$) for red cell and ghost 3OMG uptake are $2.76 ± 0.54$ and $0.30 ± 0.06$ min$^{-1}$ respectively.

B. Effect of glycerol on NBDG uptake by red cells. Rate constants (per hour) for protein-mediated (cytochalasin B inhibitable, ●) and leakage-mediated (cytochalasin B-insensitive ○) uptakes are shown relative to medium [Glycerol] and viscosity. Data are shown as mean ± SEM of at least 3 separate determinations in duplicate or triplicate. Lines drawn through the points were computed by the method of least squares.
Figure 21

A

Relative viscosity

0.8 1.2 1.6 2 2.4 2.8 3.2

k Relative

0 0.2 0.4 0.6 0.8 1

[Glycerol] %

B

Relative viscosity

0.25 0.2 0.15 0.1 0.05

NBDG uptake (per hour)

0.05 0.1 0.15 0.2 0.25

[Glycerol] %
Is erythrocyte sugar uptake a multi-component process?

We surmised that if net sugar uptake were limited in some way by diffusion across an intracellular permeability barrier (e.g. an unstirred sugar layer) or by slow complexation with an intracellular species (e.g. binding to hemoglobin), this might be revealed over a complete time course of sugar uptake as multiple kinetic components of sugar equilibration between cytosol and interstitium. Figure 22 shows that the time course of 3OMG uptake by erythrocytes at 4°C is consistent with an overall process described by a rapid filling of a small compartment plus a slow filling of a larger cellular compartment. Figure 22 also shows that when sugar uptake is inhibited using 50 μM cytochalasin B, leakage-mediated sugar import (at 37°C) is consistent with a single component of sugar uptake. This latter observation rules out the possibility (see Discussion) that multi-component sugar uptake results from sugar import into multiple cell populations of differing volumes.

Is erythrocyte sugar exit a multicomponent process?

If multicomponent sugar uptake results from sugar import by two populations of cells (e.g. old and young) with differing transporter content or catalytic turnover, then sugar exit at limiting intracellular sugar concentrations must also display the same multicomponent kinetics. The reason for this is that red cell sugar transport is passive and this requires that $V_m/K_m$ for entry = $V_m/K_m$ for exit (Krupka, 1989). Fig. 22 shows that exit of 50 μM 3OMG from human erythrocytes is consistent with a single (slow) efflux process. This suggests (see Discussion) that sugar uptake and exit occur within a uniform
Figure 22. Time course of 3OMG (50 μM) uptake and exit by red blood cells.

A. Uptake (□) and exit (▼) were measured in the absence of CCB at 4°C. Uptake was also measured in the presence of 50 μM cytochalasin B at 37°C (○). A. Raw uptake data. Ordinate: cpm [³H]-3OMG associated with cells. Abscissa: time in minutes. Curves drawn through the points were computed by nonlinear regression and have the following constants: uptake (□), k₁ = 5.4 ± 2.5 min⁻¹, C₁ = 5905 ± 987 cpm, k₂ = 0.0755 ± 0.0063 min⁻¹, C₂ = 19968 ± 1489 cpm, R = 0.96; CCB-insensitive uptake (○), k₁ = 0.13 ± 0.02 min⁻¹, C₁ = 28761 ± 3621 cpm, R = 0.99; exit (▼), k₁ = 0.114 ± 0.016 min⁻¹, C₁ = 12750 ± 428 cpm, R = 0.98. Measurements were made in duplicate at each time point.

B. The data of A replotted as log (1-fractional equilibration) versus time. The curves drawn through the points are based on the constants computed in A.
Figure 22

A

B

log(1-fractional equilibration)

time in minutes

average cpm

time in minutes
population of cells that contain two intracellular sugar compartments characterized by distinct sizes and kinetics.

Does multicomponent sugar uptake require protein-mediated import?

Cytochalasin B inhibition of sugar uptake also inhibits multi-component sugar import (Fig. 22). This result is consistent with several hypotheses which include: 1) Only sugar import via the glucose transporter conveys sugar to bulk cell water via the small, rate-limiting compartment within the cell. 2) Cytochalasin B inhibited, net uptake is no longer rate-limited by the series barrier because the rate-limiting diffusion/binding step within the cell occurs more rapidly than does transbilayer sugar diffusion. 3) The barrier is lost at higher temperatures.

If a series barrier lies between the plasma membrane and bulk cytosol, then bypassing the glucose transporter by membrane permeabilization will not bypass the series barrier. We therefore treated erythrocytes with α-toxin (20 μg/mL) and monitored 3OMG and L-glucose uptake by control and treated cells. Figure 23 shows that 3OMG and L-glucose immediately (within 5 sec) equilibrate with cell water in α-toxin-treated cells. The extracellular medium used in these experiments is K-substituted saline in which Ca^{2+} levels are buffered to < 0.1 μM using EGTA. This medium does not ablate multicomponent 3OMG uptake in the absence of α-toxin (Fig. 23). This suggests either that the series barrier between membrane and bulk cytosol is disrupted by α-toxin or that the barrier exists only between the glucose transporter and cytosol.
Figure 23. Effect of α-toxin on Sugar Transport

Effect of α-toxin on 3OMG (○, □) and L-glucose (□, ▼) uptake by red cells. Cells were treated with (○, □) or without (○, ▼) 20 μg/mL α-toxin. Uptake is shown as fractional penetration of red cell total water space versus time in seconds. Lines drawn through L-glucose (control and α-toxin-cells) and 3OMG (α-toxin cells) uptake data were computed by the method of least squares. The curve drawn through the control red cell data was computed by nonlinear regression and has the following constants: \( k_1 = 0.18 \pm 0.04 \text{ sec}^{-1} \), \( C_1 = 0.16 \pm 0.04 \), \( k_2 = 0.004 \pm 0.0001 \text{ sec}^{-1} \), \( C_2 = 0.285 \pm 0.036 \); dead space = 0.282 ± 0.029, \( R = 0.97 \). This figure summarizes 5 separate experiments made in triplicate.
Figure 23

[Graph showing sugar uptake over time in seconds]
Is the glucose transporter laterally segregated in erythrocyte membranes?

Assuming that other cell membrane structure remains unperturbed upon α-toxin treatment, the rapid cellular equilibration promoted by α-toxin suggests that the rate-limiting series barrier does not extend along the entire margin of the membrane. Rather, the barrier is associated with glucose transporter but not with bulk plasma membrane. Does this mean that the glucose transporter and the series barrier are anisotropically dispersed in the cell membrane? We examined this possibility by comparing the patterns of cell-surface staining obtained for GLUT1 (the erythroid glucose transport protein) and the anion transporter. We also compared GLUT1 staining patterns obtained with living and fixed cells using different staining reagents (primary antisera) with and without cell-attachment to polylysine coverslip. Figure 24 demonstrates that in living cells, GLUT1 is anisotropically distributed across the erythrocyte cell surface (Fig. 24d-i) while the anion transporter is more uniformly dispersed (Fig. 24j, k, l). Similar, punctate staining patterns are obtained with paraformaldehyde-fixed, detergent permeabilized cells using exofacial- (Fig. 24b) or endofacial- (Fig. 24a, c) GLUT1 epitope-reactive antisera (d-Ab and C-Ab antisera respectively). Erythrocyte pretreatment with α-toxin does not alter the pattern of GLUT1 staining (Fig. 24c). Cell attachment to polylysine-coated coverslips does not promote lateral segregation of GLUT1 because similar staining patterns are observed in free-floating cells.

What is the nature of the series barrier?

It has been suggested that transported sugars interact slowly and nonspecifically
Figure 24. Immunofluorescence micrographs.

Fluorescence micrographs of human erythrocyte GLUT1 (panels a - i, m), anion transporter (panels j, k, l) and hexokinase I (panel n) content. GLUT1 cell-surface distribution was obtained using rabbit antiserum that reacts with exofacial GLUT1 epitopes (Ab, panels b and d - h), using anti-GLUT1 C-terminal peptide antiserum (C-Ab, panels a and c) or using sheep antiserum that reacts with exofacial GLUT1 epitopes (sAb, panel m). Anion transporter was stained using FITC. Hexokinase I was detected using anti-rat hexokinase I antiserum. The secondary antibodies were fluorescein-conjugated goat anti-rabbit IgGs and rhodamine conjugated rabbit anti-sheep IgGs. Panels a, b and c were obtained using fixed, permeabilized cells. Cells in panel c were treated with α-toxin prior to fixation. Panels d, e and f were obtained using living (nonfixed) cells attached to polylysine coated coverslips. Panels g, h, i, j, k, and l were obtained using free-floating, living cells. Panels m and n are images obtained at lower magnification from erythrocyte ghosts double stained for GLUT1 (m) and hexokinase I (n).
Figure 24
with hemoglobin in red cells to form an Hb-sugar complex and that this interaction could affect the apparent kinetics of net sugar uptake by erythrocytes (Baker & Naftalin, 1979). We examined this in two ways. First we monitored the kinetics of sugar uptake by resealed, human erythrocyte ghosts. Our results show that 3OMG uptake is still characterized by multi-component uptake kinetics in substantially hemoglobin-free erythrocyte ghosts (Fig. 25). Second, we compared the kinetics of sugar uptake by control human K562 leukemic cells and by K562 cells induced to synthesize hemoglobin by prolonged exposure to hemin. Both control and Hb-synthesizing K562 cells display two component, 3OMG uptake kinetics (Fig. 25). The results obtained using erythrocyte ghosts demonstrate that if Hb impacts uptake kinetics, it must be membrane bound Hb that is responsible. The K562 studies demonstrate multicomponent sugar uptake in nucleated cells independent of cellular levels of Hb.

Even so, the erythrocyte ghost data suggest that transport (in particular the slow component of uptake) is slow relative to uptake by intact cells. Helgerson and Carruthers (1989) have shown previously that normal uptake is restored upon inclusion of Mg•ATP during ghost resealing. We confirm this in the current study (Table VII). We also examined the effects of cytoskeletal disruption of erythrocyte sugar uptake kinetics. Table VII summarizes our findings. Neither cytochalasin D nor the ionophore A 23187 plus Ca\(^{2+}\) impact multi-component nature of sugar import by erythrocytes, although treatment with A 23187 plus Ca\(^{2+}\) slows the kinetics of uptake. This may be related to the ATP-depletion promoted upon Ca-loading red cells using ionophore (Jacquez, 1983).

If multi-component sugar uptake results from a diffusion-limiting process, this
Table VII: Effect of Various Agents on Kinetics of Biphasic 3OMG Uptake.

<table>
<thead>
<tr>
<th>Condition</th>
<th>$a_k_1$</th>
<th>$bC_1$</th>
<th>$a_k_2$</th>
<th>$bC_2$</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact Red Cells</td>
<td>7.4 ± 1.7</td>
<td>29 ± 6</td>
<td>0.56 ± 0.11</td>
<td>71 ± 6</td>
<td>8</td>
</tr>
<tr>
<td>cytochalasin D</td>
<td>9.9 ± 3.4</td>
<td>29 ± 13</td>
<td>1.0 ± 0.3</td>
<td>71 ± 18</td>
<td>3</td>
</tr>
<tr>
<td>A23187 + Ca²⁺</td>
<td>0.9 ± 0.3</td>
<td>32 ± 7</td>
<td>0.054 ± 0.016</td>
<td>68 ± 14</td>
<td>3</td>
</tr>
<tr>
<td>Erythrocyte Ghosts</td>
<td>2.5 ± 1.4</td>
<td>17 ± 5</td>
<td>0.027 ± 0.013</td>
<td>83 ± 5</td>
<td>3</td>
</tr>
<tr>
<td>Ghosts + ATP</td>
<td>5.6 ± 0.8</td>
<td>25 ± 6</td>
<td>0.72 ± 0.31</td>
<td>75 ± 7</td>
<td>3</td>
</tr>
</tbody>
</table>
Table VII Notes:

a$k_1$ and $k_2$ are the pseudo first-order rate constants for 3OMG uptake at limiting $[3OMG]$ (see methods) and have units of per min.

b$C_1$ and $C_2$ are the cell volumes (% of total cell volume) equilibrated by processes 1 and 2 respectively of eqn 1 (page 40).

cCells were incubated with 50 $\mu$M cytochalasin D at 37°C for 30 min prior to uptake determinations at ice-temperature in the presence of cytochalasin D.

dCells were incubated with 5 $\mu$M A 23187 plus 100 $\mu$M Ca$^{2+}$ at 37°C for 30 min prior to uptake determinations at ice-temperature in the presence ionophore plus calcium.

eRed cells were hypotonically lysed then resealed in the absence or the fpresence of 4 mM Mg•ATP (pH 7.4) prior to washing in saline and uptake measurements.
Figure 25. Time Course of 3-O-Methyl-D-Glucose Uptake.

Time course of 3OMG uptake by control (V) and hemin induced (▲) K562 cells and by resealed human erythrocyte ghosts (○). Uptake is expressed as log(1-fractional equilibration) versus time. Curves drawn through the data points were computed by nonlinear regression as described in Fig. 22. The computed constants are: control K562 cells and ghosts (solid line), $k_1 = 6.6 \text{ min}^{-1}$, $C_1 = 0.0787$, $k_2 = 0.0567 \text{ min}^{-1}$, $C_2 = 0.893$; hemin-induced K562 cells (dashed line), $k_1 = 7.1 \text{ min}^{-1}$, $C_1 = 0.0627$, $k_2 = 0.0567 \text{ min}^{-1}$, $C_2 = 0.921$. These data summarize 3 separate experiments for each condition made in duplicate.
Figure 25

Time in minutes

log(1-fractional equilibration)
Figure 26. Is there a sugar binding complex inside the red cell?

A. Time-course of equilibrium 3OMG uptake by intact red cells. Exchange transport ([3OMG]_i = [3OMG]_o) was measured at 0.1 (△), 1 (□), 10 (■), 30 (○) and 60 (●) mM 3OMG. Uptake data are expressed as log(1-fractional equilibration) versus time (min). The straight lines drawn through the data points were computed by the method of least squares and correspond to rate constants of 0.100 ± 0.004, 0.158 ± 0.006, 0.332 ± 0.033 and 0.549 ± 0.046 min⁻¹ for exchange uptake of 60, 30, 10 and 1 mM 3OMG respectively. The curve drawn through the 0.1 mM 3OMG data was computed by nonlinear regression as in Fig. 2B and is characterized by the constants: k₁ = 6.36 ± 1.41 min⁻¹, C₁ = 0.261 ± 0.037, k₂ = 0.066 ± 0.005 min⁻¹, C₂ = 0.738 ± 0.063. This Figure summarizes 8 separate experiments made in duplicate. If the 0.1 mM 3OMG data are ignored, these data correspond to V_max and K_m(app) for exchange uptake of 7.0 ± 0.5 mmol 3OMG/L cell water/min and 12.1 ± 2.7 mM respectively (R = 0.99).

B. Measurement of 3OMG binding to cells by equilibrium 3OMG space analysis. ordinate: red cell 3OMG space in fL (●) and concentration of 3OMG binding sites in μM (○). Abscissa: extracellular [3OMG] in mM. Data represent mean ± SEM of three separate experiments made in triplicate. Curves drawn through the points were computed by nonlinear regression assuming simple Michaelis-Menten equilibrium binding kinetics and have the following constants: displaced red cell volume = 14.5 ± 2.4 fL, K_d(app) = 205 ± 130 μM 3OMG; maximum 3OMG binding = 31.1 ± 2.6 μM, K_d(app) = 446 ± 138 μM 3OMG.
Figure 26

A

B

log(1-fractional equilibrium) vs. time (min)

Cell Volume (FL), [3OMG]_bound (μM) vs. [3OMG] mM
effect should be independent of diffusant (sugar) concentration. Previous studies fail to show multi-component sugar uptake under equilibrium exchange conditions (intracellular [sugar] = extracellular [sugar]). We confirm this here (Fig. 26) for 3OMG concentrations of 1 mM or greater. However, at lower sugar concentrations, multicomponent equilibrium exchange sugar uptake is demonstrable. This suggests that the physical basis of this kinetic phenomenon derives from saturable sugar binding within the cell.

Equilibrium uptake experiments can be used to quantitate 3OMG “binding sites” within the erythrocyte. Cells were equilibrated with various levels (0 to 50 mM) of unlabeled 3OMG then tracer quantities of labelled 3OMG were added to the equilibrated suspension. If 3OMG is bound by intracellular components in a saturable manner, the labelled 3OMG space of the cell will fall with increasing unlabeled 3OMG. Table VIII summarizes the results of 3 experiments which demonstrate saturable 3OMG binding with $K_{d(app)} \approx 450 \mu M$ and $B_{\text{max}} = 31 \mu \text{mol per L cell water (}1.2 \times 10^6 \text{ sites per cell)}$.

What cellular components form the sugar transport/binding complex?

Glyceraldehyde phosphate dehydrogenase and phosphoglycerate kinase are reported to associate with the red cell membrane (Kliman & Steck, 1980, Lachaal et al., 1990, Mercer & Dunham, 1981). Immunoblot analysis of intact erythrocytes and of red cell membranes indicates that $28.8 \pm 1.7\% \ (n = 3)$ of total cellular hexokinase I is retained by erythrocyte ghosts. While 3OMG is not expected to interact with hexokinase, this enzyme could form one component of a glycolytic particle that interacts with glucose following sugar transport into the cell. We therefore stained erythrocytes for GLUT1 and
hexokinase I to determine whether both cellular components co-localize. Hexokinase staining patterns also reveal an anisotropic distribution within the cell (Fig. 24n) however, this distribution does not co-localize with that obtained simultaneously for GLUT1 within the same cell (Fig. 24m).

**Discussion**

The results of this chapter demonstrate that 3OMG uptake by human erythrocytes is a diffusion controlled process and that net sugar uptake and exit are limited by saturable sugar binding to a small, intracellular compartment that lies between the glucose transporter and the bulk cell sol.

A general criterion for assessing the catalytic efficiency of an enzyme is the $k_{cat}/K_m$ ratio which estimates the second-order rate constant for an enzyme-catalyzed reaction. Theoretical approaches predict that the second-order rate constant for a diffusion-limited, enzyme-mediated reaction is in the range of $10^8$-$10^{10}$ M$^{-1}$ s$^{-1}$ (Blacklow et al., 1988, Solc & Stockmayer, 1973). However, very few of the fastest enzyme-mediated reactions ever achieve this range of $k_{cat}/K_m$ (Hammes & Schimmel, 1970). Several reasons have been suggested for this apparent discrepancy. Unlike the diffusion of two small unsolvated species in solution, the binding of a substrate to the active site of an enzyme involves more than a simple encounter. Binding may involve changes in ion pairing, in hydrogen bonding partners and in local solvent structure both for substrate and the active site. These exchanges will produce an enthalpic contribution to what for a small unsolvated species is normally considered to be a purely entropic diffusion phenomenon. An enthalpic
contribution to activation energy of the docking process will reduce the rate of association.
Lower values of second-order rate constants for enzyme-substrate association are also
expected if the substrate binds only to a rare conformational or solvation state of the active
site.

If an enzyme-catalyzed reaction is limited by the rate of substrate enzyme
association, then reducing the rate of substrate diffusion in solution is expected to reduce
the frequency of substrate-enzyme encounters. For small molecules such as glucose, this
requires use of monomeric polyhydroxylated species such as sucrose or glycerol
(Blacklow et al., 1988). Polymeric species such as poly (ethylene glycol) or
polyacrylamide while effective in reducing the self diffusion of larger molecules such as
proteins, have little effect on the diffusion of smaller molecules (Stokes & Weeks, 1964).
Since most enzymes are larger than the substrates upon which they act, the overall
encounter rate between enzyme and substrate will be governed by the rate at which
substrate diffuses.

Blacklow et al. (1988) have developed simple criteria for determining whether the
rate of an enzyme-mediated reaction is affected directly by altered rates of substrate
diffusion. First, the rate of the reaction should be reduced by increased solution
(micro)viscosity. Second, if a “good substrate” reacts at or near the diffusion limit and this
reaction is sensitive to solution viscosity, the use of a “poor substrate” (for which some
other non-diffusive step is rate-limiting) provides an internal check for whether the
viscogenic agent exerts additional (nonspecific) effects on the reaction. Third, where
possible a “poor enzyme” (a mutant form of the enzyme with reduced catalytic efficiency)
should be used to determine whether the visco genic agent affects the enzyme directly.

Glucose and 3OMG transport by human red cells are characterized by $k_{cat}/K_m$ of $10^5 M^{-1} s^{-1}$ at both ice and room temperatures (Carruthers, 1990). At 37°C, this value approaches $10^6 M^{-1} s^{-1}$ (Lowe & Walmsley, 1986). While these second order rate constants are significantly lower than those expected of diffusion-limited enzymes, protein-mediated erythrocyte sugar transport is reduced by increasing medium viscosity. For an $n$-fold increase in solution viscosity, the second-order rate constant for 3OMG transport by cells and erythrocyte ghosts falls $n$-fold. Transport of the poorly transported sugar 6-NBD glucosamine (which occurs 3,300-fold more slowly than does transport of 3OMG) is unaffected by medium viscosity. Transbilayer diffusion of sugar (a process limited by the mobility of sugar within the hydrocarbon core of the bilayer (Lieb & Stein, 1986)) is also unaffected by aqueous solvent viscosity. These findings suggest very strongly that GLUT1-mediated sugar transport is a diffusion-limited process. This supports the hypothesis of Naftalin and co-workers (Baker & Naftalin, 1979, Naftalin & Holman, 1977, Naftalin & Rist, 1991, Naftalin et al., 1985) that the observed kinetics of erythrocyte sugar transport - being influenced by environmental factors - do not reflect the intrinsic properties of the transporter.

The observations presented in the current study, however, suggest that an intracellular sugar binding compartment complicates accurate determination of sugar transport rates. This study describes sugar uptake by human erythrocytes and by human K562 leukemic cells at low sugar concentrations as a bi-exponential process. In red cells, the first process rapidly fills a small cellular compartment ($k = 7.4 \pm 1.7 \text{ min}^{-1}$; vol = 29 ±
6%) while the second process slowly fills the bulk of the cytosol \((k = 0.56 \pm 0.11 \text{ min}^{-1}; \text{vol} = 71 \pm 6\%\). The transported sugar (3OMG) is nonmetabolizable thus subsequent metabolic transformation cannot account for the phenomenon. Several explanations could account for bi-exponential sugar uptake.

Were the transporter to contain a high affinity, negative-feedback sugar binding site within a cytosolic GLUT1 domain, sugar uptake would be rapid during initial phases but would slow as intracellular sugar rises and the regulatory site becomes saturated. We modelled this possibility in two ways. The first model assumes that occupancy of the regulatory site locks the transporter in a dysfunctional state (the competitive model). The second assumes that occupancy of the site does not prevent sugar binding at catalytic sites but rather slows translocational steps significantly (the allosteric model). Both models predict that sugar uptake shows biphasic kinetics. However, both models also predict that exit is accelerated as the regulatory site desaturates \((K_{m(app)}\text{ for exit falls with falling intracellular sugar})\) and this results in a hysteresis in the exit progress curve that is not observed experimentally. We therefore reject the kinetic explanation for biphasic sugar uptake.

A more simple explanation describes uptake of sugar into two parallel compartments (i.e. two populations of cell sizes are present in the cell suspension). This seems unlikely, however, since it can be shown that the first-order rate constant for sugar uptake (protein-mediated or leakage) into a sphere of volume \(v\), is given by:

\[
k = \text{constant} \cdot \text{vol}^{\frac{1}{3}}
\]
Thus for particles of volume $v$ and $v/2.5$, $k$ would vary by only 1.3-fold. The observed
difference is 13-fold. Moreover, it can also be shown that for any body, the ratio of
GLUT1-mediated sugar uptake (transport) to transbilayer diffusion (leakage) is
independent of volume and is given by:

\[
\frac{[\text{GLUT1}] k_{\text{cat}}}{P \cdot N \cdot K_m}
\]

where [GLUT1] is surface glucose transporter density, $P$ is the permeability coefficient of
the membrane bilayer to sugar, $K_m$ is the Michaelis constant for GLUT1-mediated sugar
uptake and $N$ is Avagadro’s constant. Thus, if two populations of cell sizes give rise to fast
and slow components of protein-mediated sugar uptake, sugar leakage should also show
fast and slow components. This was not observed and we thereby reject this hypothesis.

However, were a second population of cells to exist with lower volume and higher
sugar transporter cell-surface density, this population would transport sugars more rapidly
than the larger cells. Our calculations show that such a population of spherical cells would
be characterized by a diameter of 75% of that of the larger cells and would present cell-
surface GLUT1 at a density 10-fold higher than that of the larger cells. More importantly,
this population of cells would also display elevated rates of sugar exit relative to the larger
cells at limiting sugar concentrations and this was not observed. We therefore reject the
hypothesis of multiple cell populations of differing GLUT1 content.

If small and large cellular compartments do not exist in parallel, they must be
present in series. Since the smaller compartment fills more rapidly than the larger, we
conclude that this compartment lies between the glucose transport and the bulk cell
cytosol. The questions we now address are: 1) Does the compartment extend uniformly across the cytoplasmic surface of the erythrocyte membrane? 2) Why is sugar exit a single exponential process?

We approached the first question by permeabilizing the red cell membrane using α-toxin of *Staphylococcus aureus*. Our rationale was to bypass the glucose transporter as the major portal for sugar import but in a way as to enable uptake rates that would still be limited by a slower intracellular process. α-Toxin monomers interact at the cell surface to form hexameric pores of 2 to 3 nm diameter. These pores limit the passage of macromolecules but allow smaller molecular species (<1000 daltons) to move freely across the membrane (Ahnert-Hilger et al., 1985, Bader et al., 1986, Fussle et al., 1981, Hildebrand et al., 1991). The large mass of the α-toxin monomer (>28 kDa) thus limits its distribution to the cell surface.

Our studies show that permeabilized erythrocytes fill within 5 seconds of exposure to extracellular sugar. This suggests that the series barrier does not extend uniformly across the endofacial surface of the membrane. Rather, it must be limited (structurally or functionally) to locations coincident with glucose transporter-mediated sugar entry. While our studies cannot eliminate the possibility that toxin-treated red cells lose the series barrier below the cell membrane, these same studies demonstrate that the characteristically anisotropic GLUT1 cell surface distribution is maintained suggesting that at least major structural elements remain in position in permeabilized erythrocytes. When examined in other cell types, α-toxin permeabilization neither disrupts membrane cycling processes nor depletes cellular enzyme activities (Baldini et al., 1991, Bauldry et
indicating that fundamental cellular structure and function are retained by permeabilized cells. If the series barrier were an unstirred sugar layer acting to reduce the rate of cytosolic sugar diffusion, bypassing the glucose transporter by cell membrane permeabilization is not expected to impact this phenomenon.

Because of the very high glucose transporter content of human red cells (6% of total erythrocyte surface area is occupied by GLUT1 (Hebert & Carruthers, 1992)), we expected to observe uniform surface staining when erythrocytes were stained for GLUT1. Contrary to expectations, the GLUT1 staining patterns obtained suggest that GLUT1 distribution is restricted to surface domains of very high local transporter density. This contrasts with the uniform cell-surface distribution of the anion transporter. It is probable, therefore, that the series barrier is also restricted to these locations of high GLUT1 density. Occasionally, GLUT1 staining intensity coincides with red cell crenations. However, this is an unusual rather than a normal occurrence. If GLUT1 were restricted to crenations resulting from echinocytosis (Lin et al., 1994), the sugar binding complex must also co-localize within these structures.

The series barrier is not formed from macromolecules that are lost upon cellular lysis because multicomponent sugar uptake is still observed in erythrocyte ghosts which lack more than 95% cellular hemoglobin. Human K562 leukemic cells (a pre-erythroid cell line) also shows multi-component sugar uptake which is unaffected by subsequent cell-induction to synthesize hemoglobin. This suggests that sugar binding to hemoglobin (whether free in bulk cytosol or anchored to the membrane) is not responsible for this phenomenon. While hypotonically lysed and washed erythrocytes retain a
disproportionate amount of Hexokinase I suggesting a membrane association (28% based upon immunoblot analysis), the distribution of this enzyme does not coincide with that of GLUT1. Other enzymes of glycolysis are suggested to associate strongly with the erythrocyte membrane. These include glyceraldehyde phosphate dehydrogenase and phosphoglycerate kinase (Kliman & Steck, 1980, Mercer & Dunham, 1981). Indeed, GAPDH has been shown to associate in a nucleotide-dependent fashion with erythrocyte GLUT1 in vitro (Lachaal et al., 1990). At this time, the molecules that associate with erythrocyte GLUT1 remain uncertain. Equilibrium 3OMG space analysis indicates that erythrocytes contain some $1.2 \times 10^6$ 3OMG binding sites per cell (30 μM; GLUT1:binding site molar ratio = 1:4) with $K_{d(app)}$ for 3OMG binding at 4°C of 450 μM. This apparent binding constant is close to $K_{m(app)}$ for 3OMG net uptake at this temperature (Helgerson et al., 1989). It is interesting that ATP-depletion (e.g. ghost-formation) reversibly slows the rapid (presumed translocation) step somewhat and the slower (presumed sugar desorption) step considerably. If transported sugar were to bind to a hypothetical glycolytic complex via a substrate tunneling-like phenomenon, slower sugar dissociation from the complex may favor more efficient metabolic processing.

We conclude that net sugar transport by human erythrocytes and by human K562 cells is the sum of two sequential processes - sugar translocation and reversible sugar binding to a complex in very close association with the glucose transporter. Because of this, steady-state sugar transport measurements report both translocation and binding steps and cannot be used directly to model the translocation process in isolation. This process also illustrates the potential for control of net sugar transport by reversible recruitment of
sugar binding complexes to the transporter - a process known to occur in thymocytes, CHO cells and in macrophages (Faik et al., 1989, Naftalin & Rist, 1989, Naftalin & Rist, 1990, Pedley et al., 1993).
Chapter VII

Significance and Future Directions

In the body of work presented in this thesis, we tested aspects of the model hypothesized by Hebert and Carruthers (1992). Specifically, we wanted 1) to understand why the native, noncovalent, homotetrameric GLUT1 complex is sensitive to reductant, 2) to determine whether the tetramer is more catalytically efficient than the dimer in vivo, and 3) to test the hypothesis that it is the antiparallel arrangement of substrate binding sites between subunits that provides the transporter with its catalytic advantage. We used biochemical and molecular biological approaches to isolate specific determinants of transporter oligomeric structure and/or transport function in purified isolated transporter preparations, in intact red cells and in CHO cells. We also examined the hypothesis that net sugar transport in the human erythrocyte is rate limited by reduced cytosolic diffusion of sugars and/or by reversible sugar association with intracellular macromolecules.

We have shown that serine substitution at cysteines 347 and 421 significantly reduces ß-Ab binding to GLUT1. ß-Ab binds to epitopes presented by the GLUT1 tetramer. The magnitude of this effect suggests that remaining ß-Ab binding is accounted for by binding to parental GLUT1. While these results do not discount the possibility that serine substitution at cysteine 347 and 421 induces subtle conformational changes that mask the ß-Ab epitopes, they are consistent with the view that these cysteine residues are important for GLUT1 oligomeric structure. When reviewed in the context of the peptide mapping/
alkylation studies, these finding strongly suggest that the internal, GLUT1 disulfide bridge is formed between cysteine residues 347 and 421.

Hydrodynamic analyses using GLUT1/GLUT4 chimeras indicate that GLUT1 residues 463-492 make a negligible (isoform-specific) contribution to GLUT1 oligomeric structure. This is consistent with the observation that anti-GLUT1 carboxyl terminal peptide antiserum (C-Ab) binding to GLUT1 is insensitive to GLUT1 oligomeric structure. A portion (25%) of the expressed GLUT1n-4 chimera (GLUT1 residues 1 through 199 plus GLUT4 residues 216 to 509) does resolve as a tetramer. Specific immunoprecipitation of GLUT1n-4 from CHO cells using anti-GLUT4 carboxyl terminal peptide antiserum quantitatively precipitates parental GLUT1 (Pessino et al., 1991). Almost all parental GLUT1 from GLUT1n-4 chimera-expressing cells co-resolves with authentic tetrameric GLUT1. However, most of the chimera co-resolves with authentic dimeric GLUT1. While our studies cannot rule out the possibility that GLUT1n-4-containing micelles contain molecular species other than lipid, detergent, GLUT1n-4 and GLUT1, these data are consistent with the hypothesis that GLUT1n-4/wild-type GLUT1 complexes are heterotetramers while the remaining GLUT1n-4 forms only homodimeric structures. If correct, this would mean that wild-type GLUT1 is dominant positive over GLUT1n-4 with regard to transporter oligomeric structure.

These data further suggest that the GLUT1 N-terminal half provides dimerization motifs/contact surfaces while the carboxyl-terminal half contains the tetramerization motif (cys 347 and 421) and contact surfaces (Fig. 27). These contact surfaces for tetramerization presumably also include domains that are well conserved in GLUT1 and GLUT4 carboxyl-
Figure 27. 2D Structure of GLUT1.

This is our modified model for the 2D structure of GLUT1. The location of the cysteines is denoted by the dark circles. We propose that the N-terminal half (residues 1-199) provides the dimerization domain or contact surfaces for dimerization. The structure is stabilized by an intramolecular disulfide bridge between cysteine residues 347 and 421. The C-terminal half of the protein has been hypothesized to contain the catalytic domain. Thus the location of the disulfide bond is well suited to impact transport function.
Figure 27
terminal halves. GLUT1 residues 200 - 463 and GLUT4 residues 216 - 479 share 78% sequence homology.

Our studies show that in situ disulfide disruption by cell impermeant reductants results in the loss of cooperative subunit interactions and a 3 to 15-fold reduction the intrinsic efficiency of the transporter. 3-O-methyl-D-glucose uptake is noncompetitively inhibited following red cell exposure to reductant. The import inhibition is rapidly reversed upon removal of extracellular reductant. Transport inhibition increases in the order dithiothreitol < β-mercaptoethanol < reduced glutathione. Transport inhibition is not observed in resealed erythrocyte ghosts that contain 2 mM DTT but are washed free of extracellular reductant. These findings indicate that the site of action is most likely extracellular and that inhibition is unrelated to the closed ring structure of oxidized dithiothreitol.

We are currently initiating studies to determine which regions of GLUT1 mediate oligomerization. Our plan is to utilize a co-immunoprecipitation strategy using GLUT1/GLUT4 chimeric transporters to broadly map the dimerization and tetramerization domains. This will be done by swapping transmembrane domains between GLUT1 and GLUT4 and assessing the effect on oligomerization. In this way, we can begin to understand the role of defined subsequences in GLUT1 oligomerization. GLUT1 does contain two motifs which may form contact regions between subunits in the transporter complex. A leucine zipper motif (L-X₆-I-X₆-I-X₆-L; residues 64 - 85) is present in all glucose transporters. The role of leucine zipper motifs in membrane protein oligomerization is without precedent. GLUT1 also contains the glycophorin A
dimerization motif (L-X₃-G-X₃-G; residues 159 - 167). The role of the glycophorin A
dimerization motif in glycophorin A oligomerization is well established but this protein
spans the bilayer only once and thus may not serve as a useful paradigm for understanding
membrane proteins the bilayer many times. It will be interesting to see if these domains
have any role in GLUT1 oligomerization. We may also be able to exploit chimeric
transporters to map GLUT1 domains required for rapid transport function and/or
cooperative ligand binding domains. Such an approach may assist in determining whether
domains that establish transporter catalytic properties are distinct from or inseparable from
domains that establish oligomeric structure.

We have shown that GLUT1 is vulnerable to proteolysis by trypsin at least one extracellular site. This disruption of glucose transporter covalent structure is not accompanied by detectable changes in transporter oligomeric structure nor does it abrogate sugar transport function. Exofacial proteolysis does, however, destroy cooperative interactions between subunits allowing each subunit to behave as a simple carrier. This relaxation of obligate, antiparallel, catalytic conformations in adjacent subunits renders sugar import and sugar export sites mutually exclusive, reveals the full cytochalasin B binding potential of GLUT1 (1 mol CCB per mol GLUT1) and indicates that cooperative substrate binding is not necessary for high catalytic turnover. At this time, it is uncertain whether the loss of catalytic cooperativity results from direct proteolysis of a critical interfacial domain between subunits or indirectly from proteolysis-induced disorder in crucial interfacial domains distal to the site(s) of covalent rupture. It is our hope that more detailed studies of
cooperative ligand binding and transport function in chimeric transporters will allow us to define domains which regulate catalytic activity independently of oligomeric structure and vice versa.

We have shown that net sugar transport by human erythrocytes and by human K562 cells is the sum of two sequential processes - sugar translocation and reversible sugar binding to a complex in very close association with the glucose transporter. Because of this, steady-state sugar transport measurements report both translocation and binding steps and cannot be used directly to model the translocation process in isolation. This process also illustrates the potential for control of net sugar transport by reversible recruitment of sugar binding complexes to the transporter.

We are currently initiating experiments to determine the nature of the barrier. These experiments may involve nondenaturing chromatographic separation of membrane constituents in conjunction with sugar binding assays to determine whether sugar binding complexes have been isolated.

Based on the results presented in this thesis, we propose a model (Fig. 28) for the native transporter is a GLUT1 tetramer. This structure is stabilized by a single, extracellular disulfide bridge within each subunit. Contacts between subunits are of two types. Dimerization surfaces are provided by the N-terminal 199 residues of GLUT1. Tetramerization and cooperativity contacts are provided by GLUT1 carboxyl-terminal residues 200 - 463. The disulfide bridge between cysteine residues 347 and 421 spans the proposed ligand binding/catalytic domain of GLUT1 (Clark & Holman, 1990, Deziel et al., 1984, Holman et al., 1988, Holman & Rees, 1987, Karim et al., 1987, Oka et al., 1991) and
Figure 28. Proposed Model for GLUT1 Structure.

A hypothetical glucose transporter is shown looking into the plane of the bilayer. The section is across two of the four subunits that form the transporter. The complex forms a water-filled cavity that almost extends across the entire bilayer. Dimensions are based in part upon measured hydrodynamic radii and detergent/lipid/protein ratios of GLUT1 micelles (Hebert and Carruthers, 1992). The catalytic domain is at the center of the water-filled cavity. Each subunit contributes 1 transport site to the complex (these domains and their conformational changes are shown in expanded-view at the bottom of the figure). Subunit 1 is shown in an $e_1$ state (exposing an intracellular sugar binding site and shaded for clarity) while subunit 2 is in an $e_2$ state (binding extracellular sugar). When subunit 2 translocates its bound sugar across the membrane (an $e_2\leftrightarrow e_1$ conformational change), subunit 1 must undergo the antiparallel ($e_1\leftrightarrow e_2$) conformational change and vice versa. In this way, the transporter complex always exposes two sugar influx sites and two sugar efflux sites. The structure of each subunit is stabilized by an extracellular, internal disulfide between cysteine residues 347 and 421. Reduction of this bridge results in tetramer dissociation into dimers. Reduced subunits adopt an altered conformation that causes the loss of functional coupling between adjacent catalytic sites. Each site can now isomerize between $e_1$ and $e_2$ states without affecting (and independently of) the state of its neighbor.
Figure 28

120 Å

Glucose outside

Subunit 1

Subunit 2

Catalytic domain

Inside

e1 e2

e1 e2
is thus well positioned to impact the ligand binding and catalytic properties of each subunit. Like the potassium-channel of the endoplasmic reticulum (Miller, 1982) and the erythrocyte water channel (Smith & Agre, 1991), we suggest that the catalytic domain of GLUT1 does not span the entire membrane but, rather, is formed from a limited structure that bridges a larger, water-filled cavity. This is consistent with the very high (> 80%) solvent accessibility of the GLUT1 polypeptide backbone (Alvarez et al., 1987, Jung et al., 1986) and the amphipathic nature of a number of proposed membrane spanning domains (Mueckler et al., 1985). This hypothesis is also consistent with the observation that GLUT1-mediated sugar transport is a diffusion-limited process and allows for disulfide bridge formation within protein domains hypothesized previously to be buried within the lipid bilayer and thus inaccessible to extracellular solvent.

The hypothesis of Hebert and Carruthers (1992) cannot explain our observation that GLUT1 cytochalasin B binding capacity is doubled but sugar transport capacity remains unchanged following exofacial trypsin-treatment of in situ GLUT1. We are now considering the possibility that each subunit of the glucose transporter is indeed a simple carrier (i.e. contains a single catalytic site at any instant) but additionally contains endo- and exofacial ligand binding sites that serve as noncatalytic, regulatory allosteric sites. The cytochalasin B binding site of the transporter may be the endofacial allosteric site which when occupied by its ligand blocks access to the catalytic E1 (exit) site. This would explain why cytochalasin B competitively inhibits sugar exit. Thus each subunit contains one catalytic site (E1 or E2) but two allosteric (1 endo- and 1 exofacial) sites that interact cooperatively to modify E1 <-> E2 transition rates. Subunit packing in the tetramer may
occlude 1 of every 2 endofacial allosteric sites and thereby halve the cytochalasin B binding capacity of tetrameric GLUT1. Exofacial proteolysis or reduction of the tetramer may subsequently disocclude these sites. This model allows for the observed multi-site sugar binding and sugar transport kinetics yet may permit apparent uncoupling of sugar binding and sugar transport function. Future studies must address this possibility.

We also propose a model for net erythrocyte sugar transport as a three compartment process (Fig. 29). Sugar transporters are laterally segregated into domains of very high local transporter density. Below these domains lie the sugar binding species which form a complex with cytoplasmic domains of GLUT1 in order that sugar released at the exit site is rapidly bound by the complex. Sugar association with the complex and sugar transport are more rapid than sugar dissociation from the complex. Some leakage is permitted between transporter and bulk cytosol. When sugar levels are low, net sugar uptake is rate-limited by sugar dissociation from the complex into cytosol. At higher sugar levels, binding sites are saturated and leakage between bulk cytosolic and peri-transporter water spaces dominates. Sugar exit is rate limited by sugar dissociation from the complex into the sugar exit cleft. Exit is slow and monoexponential because the amount of free sugar in the transporter/sugar binding particle complex is extremely low (the free water content of the complex is \(<<\) cell water) and slow desorption from the complex limits hexose availability to the transporter. Uptake appears bi-exponential because the locally high concentration of binding sites on the sugar binding particle must fill (rapidly) before slow dissociation into cytosol can proceed.
Figure 29. A model for net sugar transport by human erythrocytes.

The upper diagram shows clusters of transporter delivering extracellular sugar (S_o) to a sub-membranous compartment of limited free water content. This sugar (S_m) can exit rapidly via transport, can leak slowly into bulk cytosol to form S_l or can bind rapidly to a sugar binding complex to form bound sugar (S_b). S_b then dissociates slowly back to S_m or to bulk cytosolic sugar S_l. The lower diagram is a schematic representation of this model.
Figure 29

Cytosol

Outside

membrane
memb. compartment
binding complex
bulk cytosol

$S_o \xrightleftharpoons[k_{-1}]{k_1} S_m \xrightleftharpoons[k_{-2}]{k_2} S_b$
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