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Glucose Transporter Oligomeric Structure Determines the Mechanism of Glucose Transport: A Dissertation

Daniel N. Hebert

University of Massachusetts Medical School

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GLUCOSE TRANSPORTER OLIGOMERIC
STRUCTURE DETERMINES THE MECHANISM
OF GLUCOSE TRANSPORT

A Dissertation Presented

By

Daniel N. Hebert

Submitted to the faculty of the
University of Massachusetts Graduate School of Biomedical Sciences,
Worcester in the partial fulfillment of the requirements for the degree of:
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MOLECULAR BIOLOGY
December 1991
Biochemistry and Molecular Biology
GLUCOSE TRANSPORTER OLGOMERIC
STRUCTURE DETERMINES THE MECHANISM
OF GLUCOSE TRANSPORT

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Department of Biochemistry
& Molecular Biology
December 1991
ABSTRACT

The relationship between human erythrocyte glucose transporter (GLUT1) oligomeric structure and function was studied. GLUT1 was purified from human erythrocytes in the absence (GLUT1-DTT) or the presence (GLUT1+DTT) of dithiothreitol. Chemical cross-linking studies of lipid bilayer-resident purified GLUT1 and hydrodynamic studies of cholate-solubilized GLUT1 support the view that GLUT1-DTT is a homotetramer and GLUT1+DTT is a homodimer. Parallel studies on human erythrocyte, and studies employing conformation-specific antibodies (anti-GLUT1-DTT antibodies, 8-IgGs), indicate that erythrocyte-resident GLUT1 resembles GLUT1-DTT (a homotetramer). While the D-glucose binding capacities of GLUT1-DTT and GLUT1+DTT are indistinguishable, GLUT1-DTT presents at least two population of binding sites to D-glucose whereas GLUT1+DTT presents only one population of sugar binding sites. The cytochalasin B (CCB) binding capacity of GLUT1-DTT (0.4 sites/monomer) is one half of that of GLUT1+DTT. GLUT1-DTT and GLUT1+DTT contain 2 and 6 free sulfhydryls per monomer respectively. The subunits (monomers) of tetrameric and dimeric GLUT1 are not linked by disulfide bridges. Erythrocyte resident GLUT1 presents at least two binding sites to D-glucose and binds CCB with a molar stoichiometry of 0.55 sites per GLUT1 monomer. Following treatment with high pH plus dithiothreitol, the sugar binding capacity of erythrocyte membrane resident transporter is unaltered but the transporter now presents only one population of binding sites to D-glucose, binds CCB with molar stoichiometry of 1.3 sites per GLUT1 monomer and displays significantly reduced affinity for 8-IgGs. These findings demonstrate that erythrocyte resident glucose transporter is GLUT1-DTT (a GLUT1 tetramer) and that GLUT1 oligomeric structure determines GLUT1 functional properties. A model which rationalizes these findings is proposed.
ACKNOWLEDGEMENTS

I would like to express my gratitude for the support, encouragement and friendship provided to me by my thesis advisor, Dr. Anthony Carruthers. I would also like to thank the members of the Biochemistry and Molecular Biology Department with whom I have had many stimulating discussions that helped to shape this work, Dr. Reid Gilmore, Dr. Andrew Bradford, Dr. Steven Jaspers, Dr. Chee Wei Woon and Ralph Zottola. In addition, I would like to thank my parents for their unconditional support. Finally, I would like to thank Leah Kelley Hebert, who has supported and encouraged me throughout my thesis work as a friend, my fiancee and most recently my wife.
To

Leah Marie Kelley Hebert
LIST OF TABLES

TABLE I  Hydrodynamic properties of solubilized glucose transporter  37

TABLE II  Cytochalasin B binding to purified GLUT1 and GLUT1 of alkaline-treated erythrocyte membranes  66
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1</td>
<td>Topology of GLUT1</td>
<td>4</td>
</tr>
<tr>
<td>Figure 2</td>
<td>Schemes for two proposed mechanisms of glucose translocation</td>
<td>10</td>
</tr>
<tr>
<td>Figure 3</td>
<td>Relative viscosity versus $R_{avg}$ for H$_2$O and D$_2$O gradients</td>
<td>26</td>
</tr>
<tr>
<td>Figure 4</td>
<td>Sucrose gradient ultracentrifugation of purified erythrocyte glucose transporter</td>
<td>29</td>
</tr>
<tr>
<td>Figure 5</td>
<td>SEC-HPLC of ghost membrane and purified GLUT1 as assayed by ELISA</td>
<td>31</td>
</tr>
<tr>
<td>Figure 6</td>
<td>SEC-HPLC of ghost membranes and purified GLUT1 as assayed by D-glucose-inhibitable [³H]-CCB photoincorporation</td>
<td>32</td>
</tr>
<tr>
<td>Figure 7</td>
<td>[³H]-cholate binding determination</td>
<td>35</td>
</tr>
<tr>
<td>Figure 8</td>
<td>Putative locations of cysteine residues</td>
<td>38</td>
</tr>
<tr>
<td>Figure 9</td>
<td>Free sulfhydryl content of purified GLUT1</td>
<td>40</td>
</tr>
<tr>
<td>Figure 10</td>
<td>Effect of alkylation on the size of solubilized ghost GLUT1</td>
<td>41</td>
</tr>
<tr>
<td>Figure 11</td>
<td>Chemical crosslinkers</td>
<td>46</td>
</tr>
<tr>
<td>Figure 12</td>
<td>Chemical crosslinking of GLUT1 in erythrocytes</td>
<td>47</td>
</tr>
<tr>
<td>Figure 13</td>
<td>Chemical crosslinking of GLUT1 in proteoliposomes</td>
<td>49</td>
</tr>
<tr>
<td>Figure 14</td>
<td>Competition ELISA assays of erythrocyte GLUT1-DTT content</td>
<td>52</td>
</tr>
<tr>
<td>Figure 15</td>
<td>Reconstitution of GLUT1 into proteoliposomes</td>
<td>56</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>------------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>16</td>
<td>D-glucose binding to purified GLUT1</td>
<td>58</td>
</tr>
<tr>
<td>17</td>
<td>Cytochalasin B binding to purified GLUT1</td>
<td>60</td>
</tr>
<tr>
<td>18</td>
<td>Quantitation of [GLUT1] in stripped ghosts</td>
<td>62</td>
</tr>
<tr>
<td>19</td>
<td>D-glucose binding to stripped ghosts</td>
<td>65</td>
</tr>
<tr>
<td>20</td>
<td>Cytochalasin B binding to stripped ghosts</td>
<td>67</td>
</tr>
<tr>
<td>21</td>
<td>α-IgG binding to alkali-treated erythrocyte ghosts</td>
<td>68</td>
</tr>
<tr>
<td>22</td>
<td>Model of GLUT1 oligomeric structure and function</td>
<td>75</td>
</tr>
</tbody>
</table>
# TABLE OF CONTENTS

| ABSTRACT | iii |
| ACKNOWLEDGEMENTS | iv |
| LIST OF TABLES | vi |
| LIST OF FIGURES | vii |
| ABBREVIATIONS | xi |

## CHAPTER I

**INTRODUCTION: LITERATURE REVIEW**

- Superfamily
- Structure
- Heterologous associations
- Mechanisms of transport
- Strategy

## CHAPTER II

**METHODS AND MATERIALS**

- Purification of glucose transporter
- Sizing measurements
- Reconstitution into proteoliposomes
- Binding measurements

## CHAPTER III

**HYDRODYNAMICS AND CHARACTERIZATION OF SOLUBILIZED GLUT1**

- Principles
- Results and Discussion
- Conclusions

## CHAPTER IV

**OLIGOMERIC STATE OF MEMBRANE-RESIDENT GLUT1**

- Results and Discussion
- Conclusions
<table>
<thead>
<tr>
<th>CHAPTER V</th>
<th>FUNCTIONALITY AND ORIENTATION OF GLUTI SUGAR BINDING SITES</th>
<th>54</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Results and Discussion</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td>Conclusions</td>
<td>69</td>
</tr>
<tr>
<td>CHAPTER VI</td>
<td>DISCUSSION: SIGNIFICANCE &amp; FUTURE DIRECTIONS</td>
<td>71</td>
</tr>
<tr>
<td></td>
<td>Implications of this model and future directions</td>
<td>77</td>
</tr>
<tr>
<td>REFERENCES</td>
<td></td>
<td>83</td>
</tr>
</tbody>
</table>
### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-IgGs</td>
<td>antipeptide polyclonal antibodies generated against the COOH-terminus of GLUT1</td>
</tr>
<tr>
<td>CCB</td>
<td>cytochalasin B</td>
</tr>
<tr>
<td>I-β-Gs</td>
<td>polyclonal antibodies generated against holo-GLUT1-DTT</td>
</tr>
<tr>
<td>DTNB</td>
<td>5,5'-dithiobis(2-nitrobenzoic acid) Ellman’s reagent</td>
</tr>
<tr>
<td>DTPA</td>
<td>dithiobisphenylazide</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EPC</td>
<td>egg phosphatidylcholine</td>
</tr>
<tr>
<td>GA</td>
<td>glutaraldehyde</td>
</tr>
<tr>
<td>ghosts</td>
<td>erythrocyte membrane depleted of intracellular components</td>
</tr>
<tr>
<td>GLUT1</td>
<td>human erythrocyte glucose transporter</td>
</tr>
<tr>
<td>GLUT1+DTT</td>
<td>GLUT1 purified in the presence of DTT</td>
</tr>
<tr>
<td>GLUT1-DTT</td>
<td>GLUT1 purified in the absence of DTT</td>
</tr>
<tr>
<td>HPLC</td>
<td>high pressure (performance) liquid chromatography</td>
</tr>
<tr>
<td>NAG</td>
<td>N-acetylglucosamine</td>
</tr>
<tr>
<td>NTB</td>
<td>2-nitro-5-thiobenzoic acid</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecylsulfate</td>
</tr>
<tr>
<td>SEC</td>
<td>size-exclusion chromatography</td>
</tr>
<tr>
<td>SG↑pH</td>
<td>ghosts stripped at alkaline pH</td>
</tr>
<tr>
<td>SG↑pH+DTT</td>
<td>ghost stripped at alkaline pH and 10 mM DTT</td>
</tr>
<tr>
<td>SG+DTT</td>
<td>stripped ghosts treated with 10 mM DTT</td>
</tr>
<tr>
<td>stripped ghosts</td>
<td>ghosts depleted of peripheral proteins by alkaline treatment</td>
</tr>
</tbody>
</table>
CHAPTER I

INTRODUCTION

Extracellular glucose is a primary source of metabolic energy for mammalian cells. It is essential therefore that cells are able to rapidly assimilate extracellular glucose. Glucose is strongly hydrophilic therefore the plasma membrane serves as an effective barrier to movements of sugars. Most cells circumvent this problem by utilizing specific transport proteins to mediate sugar uptake. The passive translocation of glucose across the cell membrane is mediated by a family of integral membrane glycoproteins called facilitative glucose transporters. These transporters (or carriers) accelerate the translocation of hexose or pentose monosaccharides (adapting the chair or the pyranose ring configuration) down a concentration gradient into or out of the cell. Concentrative sugar transport, which is typical of epithelial cells, is mediated by a different family of transport proteins which will not be considered in this thesis. Facilitated, passive sugar transport displays saturation kinetics, is stereoselective, and is inhibited by relatively low concentrations of inhibitors such as cytochalasin B (CCB) and forskolin which bind to the transporter with high affinity.

Superfamily

Five discrete isoforms of glucose transporter have been identified by protein purification and recombinant DNA techniques. The tissue localization of these isotypes have provided valuable insight into the possible roles that each of these transporters play in glucose metabolism. These five isotypes are: GLUT1, the erythrocyte/ HepG2 type transporter which is found in almost all tissues including brain and fibroblast (Mueckler et. al. 1985), GLUT2, the liver type transporter is found in liver and β- cells of the pancreas (Thorens et.
GLUT3, is found in brain and placenta (Kayano et al. 1988), GLUT4, the so-called insulin-regulated transporter expressed in muscle and adipocytes (James et al. 1989, Kaisten et al. 1989, Birnbaum 1989, Charron et al. 1989) and GLUT5 which is found largely in the jejunum (Kayano et al. 1990).

The most extensively studied subtype is the erythrocyte/HepG2 type transporter, GLUT1. This is largely due to: 1. The ease with which a large number of isolated erythrocytes can be collected for transport studies. 2. The abundance of GLUT1 in human red cell membranes (5% of total membrane proteins). 3. The ease of purification of erythroid GLUT1 for biochemical studies.

Structure

The availability of the deduced primary structure of the five glucose transporter isotypes permits some degree of structural analysis. Hydrophathy analyses predict twelve hydrophobic, membrane spanning regions which are highly conserved among the superfamily (70 to >90% sequence homology). The overall sequence homology between isotypes is 68%.

The proposed topographical model of GLUT1 (Figure 1) has survived all immunological, molecular biological and biochemical challenges to date. Antibody binding studies have demonstrated that the N-terminus, COOH-terminus and the large loop located between hydrophobic membrane regions 6 and 7 (Davies et al. 1987 and Haspel et al. 1988) are located at the cytosolic side of the membrane. These findings have been confirmed by proteolysis studies (Cairns et al. 1984). The carrier has also been shown to be heterogenously glycosylated at asn-45 (Mueckler unpublished findings).

Fourier transform infrared (FTIR) spectroscopy (Alvarez et al. 1987) has been used to investigate the secondary structure of GLUT1. The findings of these studies are
Figure 1. Topographical map of GLUT1. A topographical map of GLUT1 generated by the Kyte-Doolittle algorithm (Mueckler et.al. 1985). Amino acid residues are denoted by their one letter codes. Residues shown by the black circles signify amino acid identity between isoforms (GLUT1 to GLUT4) of the sugar transporter superfamily. Putative membrane spanning regions are enclosed within boxes. The three darkened boxes enclose amphipathic α-helices that have been proposed to form a water-filled pore through which sugars are transported. The transporter is heterogenously glycosylated at asparagine-45.
consistent with the predominantly α-helical content assumed in the computer model. Smaller amounts of β-structure and random coils are also observable. In addition, deuterium exchange studies revealed that more than 80% of the polypeptide chain is readily accessible to solvent (Alvarez et al. 1987). This suggests that a significant proportion of intramembranous protein is accessible to bulk solvent and is not inconsistent with the notion of a water filled pore or channel for sugar translocation.

The glucose transporter can be affinity labelled by several specific reagents. The fungal metabolite, cytochalasin B (CCB), is covalently incorporated into the transporter upon ultraviolet irradiation of the GLUT1-CCB complex. This labeling is glucose-inhibitable and occurs with low efficiency levels (1-5%) (Carter-Su et al. 1982, Shanahan 1982). Forskolin (Shanahan et al., 1987) and various sugar derivatives (Holman et al., 1988) have also been employed to effectively label the transporter. Affinity labeling of the transporter with these reagents has been used extensively for quantitation of transporters, structure-function studies and as assays for transport integrity.

**Quaternary structure**

The quaternary structure of the glucose transporter has been investigated by a variety of strategies. Although milligram quantities of the purified transporter have been available since the 1970’s, the oligomeric structure of the transporter remains unknown. This is largely due to the difficulties caused by the strongly hydrophobic nature of the protein.

Radiation-inactivation can be used to study quaternary structure. These studies require only a means of assaying for protein activity (identification and purification are not necessary) and the studies take place with the protein in its native membrane bilayer. For the following experiments, CCB binding capacity or uptake of radiolabelled sugars were monitored. Conflicting results have been reported. The size of the glucose transport system of intact and peripheral protein-depleted human erythrocytes is $220 \pm 31$ kDa.
(Cuppoletti et. al. 1980, Jung et. al. 1980) or 124- kDa (Jarvis et. al. 1986). Assuming a 55-kDa monomer, these results suggest either a tetrameric or dimeric self-assembly of transporters or associations with other proteins of appropriate sizes. In addition, studies with rat adipocytes suggest that plasma membrane carrier is monomeric while intracellular pools of the carrier are dimeric or associated with an additional protein of an equivalent size (Jacobs et. al. 1987).

Freeze-fracture electron microscopy of purified glucose transporter reconstituted into proteoliposomes demonstrate that the average integral membrane particle size is 62Å in diameter (Hinkle et. al. 1979). The authors conclude that this particle size is consistent with a protein of approximately 110- kDa or a dimeric glucose transporter. However, in the absence of defined particle shape or partial specific volumes, this interpretation is qualitative at best.

Hydrodynamic studies have been performed to determine the molecular weight of the transporter system in various denaturing and non-denaturing detergents. Rampal et. al. (1986) show that octylglucoside-solubilized carrier equilibrated with 0.1% SDS is resolved (by gel-filtration) as a 43- kDa species or as a monomeric carrier. Zoccoli et. al. (1978) determined (by a combination of gel-filtration and sucrose gradient ultracentrifugation) that the triton-solubilized and dithiothreitol (DTT, a powerful reducing reagent)-treated human erythrocyte glucose transporter has a molecular mass of 225- kDa. They concluded that the transporter is either monomeric or dimeric. Masher and Lundahl (1987) observed that the glucose transporter from octylglucoside-solubilized human red cell membranes stripped of peripheral proteins and treated with DTT is resolved by gel-filtration as a 125-kDa micelle or a dimeric transporter. The extrapolation of these findings to the physiological glucose transport system is hindered by the addition of denaturing detergent (SDS) and the addition of the reducing agent, DTT.
Heterologous associations

White and Weber (1990) have reported that when fibroblasts are transformed by the src oncogene, there is a 2-5 fold increase in glucose transport and in the level of immunoprecipitated glucose transporter protein. In chicken embryo fibroblasts (CEFs) the increase in transport rate is correlated with a reduction in carrier turnover (degradation). However, in human fibroblasts no change in turnover rate was observed. When human GLUT1 is expressed in CEFs it maintains a turnover rate equivalent to the endogenous carrier. The authors speculate that this results from an heterologous association of human GLUT1 with CEF transporter. The CEF transporter is proposed to contain the necessary sequence(s) and to act in a “piggyback mechanism” to cause the heterologous transporter to behave as endogenous carrier. The possibility of other endogenous proteins associating with the carrier are not ruled out.

There is additional evidence for the association of the transporter with both hexokinase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Rist et. al. (1990) found that glucose transport and hexokinase activities are so tightly coupled that 80% of 2-deoxy-D-glucose entering the cell is phosphorylated to 2-deoxy-D-glucose 6-phosphate before entering the cytosol. Jung’s group (Lachaal et. al. 1990) have found GAPDH reversibly binds with high affinity in an ATP-dependent manner to purified GLUT1. This binding is shown to involve the large middle cytoplasmic loop of GLUT1.

There is evidence for the existence of monomeric, dimeric and tetrameric glucose carriers, and the existence of the transporter in heterocomplexes. A definitive analysis of the glucose transporter’s quaternary structure is still lacking and this significantly restricts our understanding of the physical basis of glucose transport.
Mechanisms of transport

The goal of a facilitative glucose transporter is to rapidly equilibrate intracellular and extracellular water with glucose. Two theoretical models for glucose transport have been proposed and disturbingly both are supported by the literature.

One-site carrier

Widdas (1952) proposed a one-site or alternating-conformer model (Figure 2). In this model, the transporter contains mutually exclusive, saturable glucose influx and efflux sites. At any moment, the carrier is capable of binding sugar at the efflux or influx site but not at both sites simultaneously. Once a sugar molecule is bound at a site, it is then translocated across the membrane to the opposite side. The ligand dissociates from the carrier. At this stage, the carrier can then isomerize in two directions; the actual direction being dependent upon the concentration of ligand at this side of the membrane. High concentrations of sugar will favor occupation of the newly exposed site with a sugar molecule causing a futile recycling back to the other side of the membrane (resulting in no net flux). If a lower concentration of sugar is present (a down hill concentration gradient exists), the unoccupied carrier will isomerize and restore the unoccupied sugar binding site at the original face of the membrane. This will result in a net flux of sugar. If this one-site mechanism is correct, isomerizations of unoccupied carrier in human erythrocytes occur at rates some 150 times slower than isomerizations of occupied carrier (Lowe and Wamsley 1986, Appleman and Lienhard 1989, Carruthers 1991).

Two-site transport

Widdas (1973) proposed a two-site or fixed-site model for glucose transport. The fundamental difference between the two- and one-site model is that the two-site transporter is capable of binding sugar molecules at efflux and influx sites simultaneously. Therefore,
Figure 2. Schemes for two proposed mechanisms of glucose translocation by GLUT1.  

**One-site carrier.** Schematic representation of the simple carrier. In the absence of sugar, the carrier E can exist in one or two conformations. E2 presents an influx site to extracellular sugar. Extracellular glucose binds to the influx site to form E2S2 which undergoes a conformational change to form E1S1 (the sugar is now exposed to cytosol). D-glucose now dissociates leaving E1 to undergo reisomerization to E2 for an additional round of influx. E1 and E2 are mutually exclusive. 

**Two-site carrier.** Schematic representation of the two-site carrier. In the absence of sugar, the carrier can exist in one of two conformations, E or F. Both conformations present influx and efflux sites to sugar. E and F are simply antiparallel arrangements of each other. When E isomerizes to F, the sugar binding sites are rearranged in a coupled, antiparallel fashion. The distinction between this model and the simple carrier is that the carrier can exist as a ternary complex of extracellular sugar-carrier-intracellular sugar. The two-site carrier could represent a dimer of simple carriers where sugar binding sites of each monomer of the dimer are always arranged in an antiparallel fashion and where isomerization of one monomer induces the antiparallel isomerization of the second unit.
The One-Site (Simple) Carrier

The Two-Site Carrier
The model predicts the existence of a ternary complex (Figure 2) consisting of transporter, extracellular sugar and intracellular sugar. The binding of sugar at one site and the resulting isomerization of that site causes the antiparallel isomerization of the other site regardless of occupancy. The two binding sites communicate with one another and may interact cooperatively.

Most studies that report data supporting either model cannot be used to unequivocally reject either model. This is simply a result of ambiguous experimental design. It is possible, however, to design experimental strategies that can distinguish one- and two-site carrier mechanisms and a number of such studies have been reported. The two-site model has been rejected in favor of a one-site model solely on the basis of ligand binding studies with purified GLUT1 (Sogin and Hinkle 1980, Gorga and Lienhard 1982, Appleman and Lienhard 1989). The one-site model has been rejected unequivocally in favor of a two-site model by: sugar transport studies on intact red cells and red cell ghosts (Carruthers 1991, Carruthers and Helgerson 1991), and ligand binding studies on intact red cells, red cell ghosts, alkali-extracted red cell membranes and purified GLUT1 (Carruthers 1986a, Carruthers 1986b, Helgerson and Carruthers 1987). The obvious contradiction that emerges from the studies is that ligand binding studies on purified GLUT1 support two models that are mutually incompatible. On closer examination, this discrepancy may be explained by the absence or presence of reductant during the purification process. Studies consistent with the one-site model included reductant (DTT or β-mercaptoethanol) in the purification procedure whereas those studies consistent with the two-site model omitted reductant during GLUT1 purification.

**Strategy**

It is commonly accepted that protein structure determines protein function. GLUT1 function has been demonstrated to be altered by reductant. The effect of reductant on
GLUT1 structure is unknown. Oligomeric associations are commonly generated through disulfide bond formations which are highly susceptible to cleavage by reductants. In its physiological state, GLUT1 exists in a biphasic redox environment. The cytosolic face of the carrier is exposed to a reducing environment, and the extracellular face is in an oxidative environment. This redox polarity cannot be maintained during purification. Our strategy, therefore was to purify GLUT1 in the presence and absence of reductant (DTT). We asked three simple questions: 1. What is the oligomeric structure of GLUT1? 2. Is GLUT1 oligomeric structure affected by reductant? 3. Does altered oligomeric structure explain the reported differences in GLUT1 function?
CHAPTER II

MATERIALS AND METHODS

Materials

Outdated, whole human blood was obtained from the University of Massachusetts Medical Center Blood Bank. Radiolabelled reagents were obtained from New England Nuclear. 4,4'-dithiobisphenylazide (DTPA) was purchased from Molecular Probes. Antipeptide antibodies raised against the COOH terminus of human erythrocyte glucose transporter were obtained from East Acres Biological. Peroxidase substrate kits from Bio-Rad Laboratories were used. The remaining reagents were obtained from Sigma Chemical Company.

Purification of Glucose Transporter

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 albumin as the standard. The protein content of the membrane pellet is adjusted to 4 mg/mL.
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, 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 (enzyme-linked immunosorbent assay) were performed as described by Sogin and Hinkle (1980) using rabbit antisera raised against either a synthetic carboxyl terminal peptide of the rat brain glucose transport protein (C-IgG) or against holo-transport protein purified in the absence of DTT (δ-IgG). The latter antisera has been characterized and found to react with an epitope presented on the exofacial portion of the protein (Harrison et al. 1990) whereas the COOH terminus of the protein is 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.

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 adsorption of the transporter to the microtiter plate 200 μL 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.
In addition, competition ELISA measurements of rabbit antisera (C-IgG and \( \beta \)-IgG) were performed using purified glucose carrier, red cells, red cell ghosts and alkaline treated human ghosts. Each individual ELISA plate well was coated with 50 ng of purified transporter. The antibodies were diluted in phosphate buffer and incubated for 1 hr at room temperature with varying amounts of purified carrier or red cell membranes. 200 \( \mu \)L of this solution was then applied to the wells in duplicate. The plate was then processed as above, beginning with the secondary antibody step.

**Nondenaturing Chromatography**

Size-exclusion chromatography (SEC) was performed using a Toso Haas TSK-Gel G4000 column pre-equilibrated with 150 mM NaCl, 5 mM Mops, pH 7.2 containing 20 mM cholic acid \( \pm 10 \) 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. 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.

Lectin chromatography of cholate-solubilized GLUT1 was performed using a wheat germ lectin-Sepharose 4B column (7 cm x 1 cm) equilibrated with 25 mM NaCl, 5 mM Tris-HCl, 10 mM cholic acid, pH 7.4. The 45,000 x g supernatant of cholate solubilized GLUT1 was applied to the column and samples of eluate collected at a flow rate of 20 mL/hr. GLUT1 was eluted from the column using 500 mM N-acetylglucosamine and was detected by ELISA.
**Sedimentation Velocity Studies**

GLUT1 was solubilized in 20 mM cholic acid, centrifuged at 200,000 x g for 1 hr at 4°C. The supernatant was layered on a 5 mL 5-20% sucrose gradient in 150 mM NaCl, 5 mM Mops, pH 7.2 containing 20 mM cholic acid. The H2O gradients were ultracentrifuged for 18 hr at 4°C and 40,000 rpm and the D2O gradients were ultracentrifuged for 25 hr and 40,000 rpm using a Beckman ultracentrifuge and a SW 50.1 rotor. Gradients were fractionated into 200 μL fractions using an ISCO model 185 density gradient fractionator. GLUT1 was detected by ELISA.

**Chemical crosslinking of GLUT1**

GLUT1 (5 μg GLUT1, 26 μg phospholipid) was resuspended in 1 mL of 50 mM Tris-HCl, 0.2 mM EDTA, pH 9.0 and made 0 to 13.33 mM in glutaraldehyde. This latter concentration of glutaraldehyde gave maximal crosslinking efficiency while avoiding aggregation of GLUT1 proteoliposomes as judged by phase contrast microscopy. The mixture was incubated at 20°C for 30 min then quenched by addition of 68 mM L-glycine. Samples were subjected to reducing or to non-reducing SDS gradient (3-13%) PAGE. Washed, intact red cells were suspended in 5 mL isotonic saline at a final hematocrit of 4%. The cell suspension was made 0 or 1 mM in 4,4′-dithiobisphenylazide (DTPA) and incubated for 20 min on ice. Cells were irradiated at 300 nm for 1 min using a Rayonet Photoreactor, lysed and the membranes harvested by centrifugation. Erythrocyte aggregation was not induced by this treatment. Samples were resolved by reducing and non-reducing SDS-PAGE. Immunoblot analysis using anti-GLUT1 C-terminal antibody and 125I-protein A were utilized. Autoradiograms were scanned by densitometry.
Reconstituted proteoliposomes

Detergent extracts of red blood cell ghosts membranes and of purified GLUT1 were reconstituted into egg phosphatidylcholine (EPC) by the method of cholate dialysis (Carruthers and Melchior, 1984). EPC (30 mg in hexane) was dried under N₂. The EPC lipid film was placed in vacuo for 3 hours to remove remaining solvent. The lipid was dissolved in 2 mL of 50 mM Tris-HCl, pH 7.4 containing 32 mg of cholic acid (the minimum amount found necessary to solubilize this amount of EPC). The detergent-lipid solution was centrifuged at 34,000 x g for 30 minutes and the supernatant placed on ice. GLUT1 (10 - 100 µg) or red cell membranes (500 - 5000 µg) were dissolved in 50 mM Tris-HCl, 50 mM cholic acid, pH 7.4, ±DTT by rotation at 4°C for 30 minutes. This results in a cholate : lipid molar ratio of approximately two. The sample was centrifuged at 34,000 x g for 30 min and the supernatant containing completely solubilized proteins was combined with the solubilized EPC and mixed by rotation at 4°C for 30 min. The lipid-protein mixture was dialyzed against two changes of 6 L of 50 mM Tris-HCl, pH 7.4 at 4°C for 24 hr to remove detergent. The dialyzed solution was frozen rapidly in dry ice/acetone then allowed to thaw at room temperature. This resulted in large proteoliposomes of 1 - 3 µm in diameter as judged by phase contrast microscopy. These proteoliposomes were sedimented by centrifugation at 14,000 x g for 5 min. Proteoliposomes remaining in suspension were discarded. GLUT1 concentration was quantitated by ELISA. The vesicles contain a scrambled orientation (40% right side out, 60% wrong side out) of GLUT1 as judged by C-IgG and δ-IgG binding to intact and solubilized proteoliposomes (by competition ELISA).

Sugar uptake measurements of proteoliposomes

Proteoliposomes containing GLUT1-DTT, GLUT1+DTT or erythrocyte membrane proteins were characterized for sugar translocation competency. Liposomes were incubated with 0.1 mM [14C]-3-O-methylglucose at 23°C. Uptake was stopped at the appropriate time with
ice-cold phloretin and CCB containing solutions. The liposomes were collected and washed by centrifugation. The pelleted liposomes were washed again, resuspended in 100 µL of 1% SDS and analyzed by liquid scintillation spectroscopy.

Photolabelling GLUT1 with [³H]-cytochalasin B

Red cell ghosts and purified GLUT1 were preincubated in Tris medium containing [³H]-cytochalasin B (3.7 µM, 50 µCi) plus 10 µM cytochalasin D or L-glucose (200 mM) for 20 minutes at 4°C. The suspension was irradiated at 300 nm for 0.5 to 5 min in a Rayonet Photoreactor. The photolabelled protein was then washed by resuspension and centrifugation (34,000 x g for 20 min). The cells or membranes were resuspended in 10 to 100 volumes of Tris medium and washed two more times.

[³H]-cytochalasin B binding assay

Equilibrium [³H]-cytochalasin B binding to RBCs, ghosts, alkali-treated ghosts and purified carrier were assayed. 50 µL of the carrier samples were mixed with 50 µL of [³H]-cytochalasin B solutions (5 - 5000 nM cytochalasin B) in 1.5 mL-microcentrifuge tubes and incubated for 1 hr at room temperature. Total cpms are determined by sampling 2 x 5 µL of the mixture. The tubes are then centrifuged at 4°C for 15 min in an Eppendorf bench centrifuge. Free [³H]-cytochalasin B is determined by sampling 2 x 10 µL of supernatant by liquid scintillation spectroscopy. Bound CCB is determined as (Total - Free). Millipore ultrafree-MC 10,000 molecular weight cut-off filter units were employed for purified GLUT1 due to difficulties in sedimentation caused by small vesicular size.

Quantitation of free thiols

Ellman’s reagent (5,5-dithiobis (2-nitrobenzoic acid), DTNB) was employed to determine free sulphydryl content (Rao and Scarborough 1980). GLUT1-DTT or GLUT1+DTT (each at approximately 100 µg.mL⁻¹ in 1 mL and dialyzed to remove traces of DTT) were
denatured in 0.5% SDS in 50 mM Tris-HCl, 3 mM EDTA, pH 8.0 for 30 min. The solubilized protein was collected by centrifugation and quantitated. The reaction was initiated by addition of 100 µL freshly prepared 10 mM DTNB. A reference tube lacking protein was employed. The course of the reaction at room temperature was recorded as the increase in absorbance at 412 nm. An extinction coefficient of 13,600 M⁻¹ cm⁻¹ at 412 nm for 2-nitro-5-thiobenzoic acid (NTB) was used to quantitate the reaction.

**Tryptophan fluorescence measurements**

Fluorescence was measured with a Farrand Fluorometer MK 2 at 23°C. An excitation wavelength 295 nm and an emission wavelength of 334 nm were employed. Slit widths of 2.5 to 5.0 nm were employed to minimize photolysis of protein. 25 µg of purified carrier or 200 µg of stripped ghosts suspended in 100 µL Tris medium were added to 2 mL of Tris medium. Sugars were injected into the cuvette from above while the contents of the cuvette were continually stirred with a Spectrocell Inc. cuvette stirrer. Steady-state emission levels were reached within 20s following addition of ligand. The dilution effect was corrected for by titration against L-glucose or cytochalasin D. Inherent systemic errors were addressed as described previously (Carruthers 1986).
Chapter III

HYDRODYNAMICS AND CHARACTERIZATION
OF SOLUBILIZED GLUT1

The determination of the native size of a membrane protein complex is complicated by the association of the protein with the phospholipid bilayer. Membrane proteins are commonly solubilized by detergents capable of disrupting the phospholipid matrix. This results in the formation of a protein-detergent-lipid complex or micelle. The molecular mass of the micelle can be characterized by hydrodynamic analysis (e.g. size-exclusion chromatography and ultracentrifugation). The composition of the micelle can be determined by protein and phospholipid analyses and by employing radiolabelled detergents.

The ideal detergent for these hydrodynamic studies must possess several important characteristics: 1. It must preserve the native state of solubilized protein. 2. The detergent micelle should be sufficiently small to permit facile separation from the protein-detergent complex. 3. Radiolabelled derivatives of the detergent should be available. 4. The cost of the detergent should be relatively low since large amounts will be needed to equilibrate and run columns and gradients. 5. The detergent should not contain structures (e.g. aromatic rings) that interfere with absorbance measurements. 6. The detergent should be easily removed by dialysis. The detergent which best satisfies these requirements for analysis of GLUT1 is cholate. The conservation of the native state in the cholate solubilized GLUT1 micelle cannot be determined unequivocally. However, cholate is the detergent commonly used in reconstitution studies of GLUT1. Therefore, the function of the protein is conserved, at least after removal of cholate and reinsertion into a lipid bilayer.
Principles

The molecular mass of a complex can be determined unambiguously, regardless of molecular shape, by using the Svedberg equation:

\[ M = \frac{sRT}{D(1-\nu \rho)} \]  

(3.1)

\( M \) is the molecular weight of the complex, \( s \) is its sedimentation coefficient in a solution of density \( \rho \). \( \nu \), \( D \) are the partial specific volume and diffusion coefficient respectively of the complex.

In brief, this is accomplished by determining the sedimentation coefficient and partial specific volume of the complex by sucrose gradient ultracentrifugation and by determining the diffusion coefficient of the complex by SEC-HPLC on equivalent GLUT1 samples under similar conditions.

Partial specific volume

The partial specific volume of a species can be determined by applying cholate solubilized GLUT1 samples to separate D\(_2\)O and H\(_2\)O sucrose gradients along with several marker proteins with known \( s_{20,w} \) and \( \nu \), separately. The basis of this method (Edelstein and Schachman 1967, Meunier et. al 1972) is that the sedimentation coefficient of the complex is altered by increasing the density of the solution through the use of D\(_2\)O. This provides an explicit formulation of partial specific volume with one important assumption - the protein moiety binds the same amount of detergent in H\(_2\)O and D\(_2\)O.

Marker proteins, with values of \( s_{20,w} \) and \( \nu \) used in these calculations include ribonuclease \( A \), 1.78S, 0.703 cm\(^3\)/g (Richards and Wycoff 1971); carbonic anhydrase, 2.75S, 0.731 cm\(^3\)/g (Armstrong et. al. 1966); lactate dehydrogenase, 6.95S, 0.730 cm\(^3\)/g (Schwert and
glyceraldehyde-3-phosphate dehydrogenase, 7.71S, 0.725 cm$^3$/g (Fox and Dandliker 1956) and catalase 11.30S, 0.730 cm$^3$/g (Sumner and Gralen 1938).

The velocity of travel of a macromolecule in a centrifugal field through a sucrose density gradient is constant and independent of angular acceleration (Martin and Ames 1961). Therefore, the sedimentation coefficient, $s_{T,m}$ at temperature $T$ in solvent $m$, where $r$ is the distance from the axis of rotation and $\omega$ is the angular velocity of the rotor can be expressed as:

$$s_{T,m} = \frac{V}{\omega^2 r}$$  \hspace{1cm} (3.2)

The velocity, $V$, can be obtained by measuring the distance traveled by the particle during time, $t$. $r_0$ is the distance from the top of the tube to the center of rotation and $r$ is the migration distance of the particle at time $t$ from the center of rotation.

$$V = \frac{(r-r_0)}{t}$$  \hspace{1cm} (3.3)

The half-distance of travel or $r_{avg}$ is defined as $r_{avg} = \frac{(r_0+r)}{2}$ resulting in:

$$s_{T,m,r_{avg}} = \frac{(r-r_0)}{t} \frac{1}{\omega^2 r_{avg}}$$  \hspace{1cm} (3.4)

$s_{T,m}$ can be calculated for all the resolved species and marker proteins in both D$_2$O and H$_2$O gradients.

Since the gradients used here are linear, the density at a given distance can be easily determined by a linear extrapolation of known values for 5% and 20% sucrose in H$_2$O and D$_2$O. The densities at the position $r_{avg}$ can thus be determined. However the viscosity, $\eta$, is highly temperature dependent and is not a linear function of the gradient. Therefore,
the relative viscosity at \( r_{\text{avg}} \) for each of the marker proteins in the \( \text{H}_2\text{O} \) and \( \text{D}_2\text{O} \) gradients can be calculated using the following form of the Svedberg equation:

\[
\frac{(\eta_{T,m,\text{avg}})}{(\eta_{20,w})} = \frac{(s_{20,w}(1-v(\rho_{T,\text{avg}})))}{(s_{T,m}(1-v\rho_{20,w}))}
\]  
(3.5)

The relationship between viscosity and \( r_{\text{avg}} \) in both \( \text{D}_2\text{O} \) and \( \text{H}_2\text{O} \) is then graphed separately (Figure 3) and fitted by interpolation. This allows determination of the relative viscosity at the position in the gradient where the cholate solubilized protein species travel.

The specific volume of the particle is determined by:

\[
v = \frac{s_H \eta_H}{s_D \eta_D} - \frac{1}{\rho_D \frac{s_H \eta_H}{s_D \eta_D} \rho_H}
\]  
(3.6)

**Sedimentation Coefficient at 20°C in \( \text{H}_2\text{O} \).**

The partial specific volume can then be used to determine the sedimentation coefficient at 20°C in \( \text{H}_2\text{O} \) (\( s_{20,w} \)) for the protein detergent micelle.

\[
s_{20,w} = \frac{s_H \eta_H}{s_D \eta_D} \frac{(1 - v_H \rho_{20,w})}{1 - v_H \rho_H}
\]  
(3.7)

**Diffusion coefficient.**

The diffusion coefficient is related to the Stokes' radius, \( S_r \), of the molecule.

\[
D = \frac{kT}{6\pi \eta S_r}
\]  
(3.8)

\( k \) is the Boltzmann constant. The Stokes' radius is determined by size-exclusion HPLC in cholate. A calibration curve is obtained using the following standards with their respective
Figure 3. Relative viscosity versus $R_{\text{avg}}$ for H$_2$O and D$_2$O gradients. The relative viscosity at the appropriate $R_{\text{avg}}$ for each protein standard was determined for H$_2$O and D$_2$O sucrose gradients. The H$_2$O plot was interpolated with a second order polynomial of: $\text{rel. viscosity} = 7.831 + 0.2176 R_{\text{avg}} - 0.00119 (R_{\text{avg}})^2$. The D$_2$O plot was interpolated by hand. This figure summarizes the results of 3 separate experiments.
Stokes' radii: ferritin, 61 Å; thyroglobulin, 85 Å; aldolase, 48.1 Å; ovalbumin, 30.5 Å and ribonuclease A, 16.4 Å.

Results and Discussion

In this section, we ask whether human erythrocyte glucose transporter exists as monomeric or as multimeric species in detergent extracts of membranes.

Sucrose gradient ultracentrifugation

$R_{avg}$ and relative viscosity at $R_{avg}$ were obtained for all standards in 5 - 20% sucrose gradients in H$_2$O and D$_2$O. The relationship between relative viscosity at $R_{avg}$ and $R_{avg}$ in D$_2$O and H$_2$O (Figure 3) is computed by using polynomial regression analysis.

Three discrete populations of GLUT1-detergent-lipid micelles are resolved upon sucrose gradient ultracentrifugation of cholate solubilized purified GLUT1 (Figure 4). The quantity of each population is dependent upon the presence or absence of reductant (DTT) in the purification process.

GLUT1+DTT solubilized in 20 mM cholate and subjected to sucrose gradient ultracentrifugation in 20 mM cholate is resolved predominantly as 3.43 ± 0.42S and 6.16 ± 0.52SS particles. A minor population of 10.54 ± 0.28S particles are also observed.

GLUT1-DTT is largely resolved as a major 10.54S particle with minor 3.43S and 6.16S populations.

The partial specific volumes of these three populations is determined by the method of Clarke (1975, see equation 3.6). These species are found to be 0.75 ± 0.08, 0.80 ± 0.02 and 0.87 ± 0.01 cm$^3$/g for 10.54S, 6.16S and 3.43S particles respectively.
Figure 4. Sucrose gradient ultracentrifugation of purified erythrocyte glucose transporter. Glucose transporter purified in the absence (GLUT1-DTT, solid lines) and in the presence of DTT (GLUT1+DTT, dashed lines) was solubilized in 20 mM cholate and applied to H2O and D2O, 5% to 20% sucrose gradients and ultracentrifuged. The following protein standards were employed: ribonuclease A (Ribo A), carbonic anhydrase (CA), lactate dehydrogenase (LDH), glyceraldehyde-3-phosphate dehydrogenase (GPDH) and catalase. This experiment is typical of seven separate experiments.
Cholate-lipid-GLUT1 micelles' Stokes' radii ($S_r$) are critically dependent upon the presence of reductant during purification as resolved by size-exclusion HPLC in 20 mM cholate. Erythocyte membranes (ghosts) solubilized in 20 mM cholate are resolved as a major peak of $S_r = 7.80 \pm 0.02$ nm (Figure 5). An appreciable shoulder at $6.01 \pm 0.21$ nm and a minor peak of $3.90 \pm 0.19$ nm are also observed. This profile is similar to that observed with GLUT-DTT although the 6.01 nm species is somewhat more prominent in GLUT-DTT. Solubilized GLUT1 from ghost membranes thus closely resembles solubilized GLUT1-DTT

GLUT1+DTT is resolved largely as a single population with a $S_r$ of 6.01 nm. Interestingly, the 6.01 nm species of GLUT1-DTT can be enriched at the expense of the 7.80 nm species by solubilization of GLUT-DTT in the presence of DTT.

These results are independent of the method of assay for GLUT1. Thus GLUT1 containing micelles detected either by direct GLUT1 immunoassay (Figure 5) or by analysis of D-glucose inhibitable [$^3$H]-cytochalasin B photoincorporation (Figure 6) into GLUT1 are indistinguishable.

Diffusion coefficients ($D_{20,w}$) are calculated for the various populations of transporter micelles resolved by SEC-HPLC (see equation 3.8). $D_{20,w}$ are $5.37 \times 10^{-7}$ cm$^2$/sec, $3.49 \times 10^{-7}$ cm$^2$/sec and $2.69 \times 10^{-7}$ cm$^2$/sec for small to large micelles respectively.
Figure 5. SEC-HPLC of ghost membrane and purified GLUT1 as assayed by ELISA. Erythrocyte ghost membranes (uppermost dashed curve), GLUT-DTT (thinsolid curve) and GLUT+DTT (lowermost dashed curve) were solubilized in 20 mM cholate and resolved by size-exclusion HPLC in 20 mM cholate. Fractions were assayed by ELISA employing anti-GLUT1 C-terminal antibodies. GLUT1-DTT was solubilized in 20 mM cholate containing 10 mM DTT (thick solid curve) and processed as described previously. Protein standards were employed to obtain a relationship between retention time (Rf) and Stokes radius (Sr): \( S_r = 21.672 - 0.4341 \text{Rf} \) (correlation coefficient = 0.965). The data sets have been displaced in the y-axis to facilitate distinction. This figure shows the results of a single experiment. This experiment was performed 25 separate occasions with indistinguishable results.
Figure 6. SEC-HPLC of ghost membranes and purified GLUT1 as assayed by D-Glucose-inhibitable [3H]-cytochalasin B photoincorporation. Elution profile of [3H]-cytochalasin B photolabelled GLUT1. Erythrocyte ghost membranes (uppermost dashed curve), GLUT-DTT (thin-solid curve) and GLUT+DTT (lowermost dashed curve) were photolabelled with [3H]-CCB in the presence of 200 mM D-glucose or L-glucose. Ordinate: cpm photoincorporated in the presence of 200 mM L-glucose minus cpm photoincorporated in the presence of 200 mM D-glucose. The photolabelled proteins were solubilized in 20 mM cholate and processed as in figure 5. The data sets shown have been displaced in the y-axis to facilitate distinction. This experiment was repeated 3 separate occasions. The results shown above are representative of 3 separate occasions.
Lipid determination

Large quantities of cholate solubilized purified GLUT1 were separated from unassociated lipid by lentil-lectin chromatography. Solubilized GLUT1 was applied to a lentil-lectin column and the flow through fraction containing unadsorbed GLUT1 and lipid collected. Bound GLUT1 was then eluted by 500 mM N-acetylglucosamine. This fraction was assayed for [GLUT1], [lipid] and [phospholipid]. Lipid acyl-chain composition and content were determined by gas chromatography (Carruthers and Melchior, 1984). Phospholipid content was determined by a modified Bartlett procedure (Carruthers and Melchior, 1984). Each solubilized glucose transporter, as assayed by Lowry protein assay, was associated with 26 ± 2 lipid molecules (Carruthers and Melchior, unpublished). A remaining problem is that each of the three populations of GLUT1 micelles may contain varying compositions of lipid. However, we are unable to cleanly isolate sufficient quantities of each population for lipid analysis at this time.

Detergent determination

The cholate content of the protein-lipid-detergent micelle is determined in a manner analogous to the method employed above for lipid determination. All cholate solutions contain [3H]-cholate at identical specific activity. The lentil-lectin column separates glycoprotein-containing micelles from cholate micelles. The N-acetylglucosamine (NAG)-eluted fractions are then assayed for radiolabelled cholate content and GLUT1 content.

The [3H]-cholate content of the lentil-lectin eluate is not systematically related to the [GLUT1] of the eluate (Figure 7A). Indeed, the [3H]-cholate content of the eluate is not significantly different from protein-free, background levels (20 mM cholate). This result is a consequence of the high critical micellar concentration of cholate and the low cholate content of GLUT1 micelles. Statistical (regression) analyses, permit computations of
Figure 7. [3H]-cholate binding determination. A) [3H]-cholate solubilized GLUT-DTT was eluted from a lentil-lectin column with N-acetyl glucosamine (NAG). GLUT1 content was determined by ELISA with anti-GLUT1 COOH peptide antibodies (thin curve). Fractions were counted for [3H]-cholate content (open circles). B) The relationship between [3H]-cholate and fraction number was analysed by the method of least squares (shown by straight line). The computed 95% confidence limits of this regression analysis are indicated by the dashed curves. The predicted [3H]-cholate contents of GLUT1 were computed using: A, the measured [GLUT1] eluted by NAG (see A); B, the known specific activity of [3H]-cholate and C, by assuming the micelles contain 50, 100, 500 or 1000 molecules of cholate per GLUT1 molecule.
confidence limits for the experimental data. Predicted profiles of $[\text{H}^3]$-cholate using values of 50, 100, 500 and 1000 cholate molecules per protein monomer per micelle can be generated (Figure 7B). Owing to the high specific activity of the background cholate, only levels greater than 50 cholate molecules per GLUT1 monomer per micelle would be resolved by this analysis. However, regression analysis indicates that the 95% confidence limits for the experimental data includes predicted cholate:GLUT1 ratios of 50:1 or lower. Therefore, cholate is present at an uppermost limit of 50 cholate molecules per GLUT1 monomer per micelle.

Unit size

The molecular mass of each population of protein-detergent-lipid micelles is determined using the Svedberg equation (see equation 3.1). Determination of lipid and maximum detergent content permits calculation of the unit size or the number of monomers per micelle. The unit sizes of the resolved micellar population are: monomer (1.21), dimer (2.21) and tetramer (3.87) (Table I). Since sodium cholate has a small aggregation number (3, Helenius and Simons 1972) the likelihood of more than one monomeric transporter existing in one micelle is remote. These calculations use 50 as the ratio of detergent to protein. If the value is smaller than 50 the unit size of the populations does not change significantly. The calculations assume that the experimental Stokes’ radii and sedimentation coefficients are directly related. This is supported by the demonstration that the 10.54S particle is resolved by SEC-HPLC as a particle with a Stokes’ radius of 7.80 nm.

Free thiol determination

These findings suggest a possible role for disulfide bonds in establishing or maintaining GLUT1 oligomeric associations in cholate. Closer examination of the putative topographical map for GLUT1 (Figure 8), indicates that four cysteine residues lie in the
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<th>Micelle</th>
<th>Micelle</th>
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<tr>
<td>Stokes Radius (nm)</td>
<td>7.80 ± 0.2 (n = 18)</td>
<td>6.01 ± 0.21 (n = 25)</td>
<td>3.90 ± 0.19 (n = 7)</td>
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<td>Phospholipid binding (mol/mol of protein)</td>
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<td>26 ± 2 (n = 3)</td>
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<td>Detergent binding (mol/mol of protein)</td>
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<td>≤50</td>
<td>≤50</td>
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<tr>
<td>Diffusion coefficient (D_{20,w}) (cm^2/sec) \times 10^7</td>
<td>2.69 ± 0.07</td>
<td>3.49 ± 0.12</td>
<td>5.37 ± 0.26</td>
</tr>
<tr>
<td>Sedimentation coefficient (s_{20,w}) (S)</td>
<td>10.54 ± 0.28 (n = 7)</td>
<td>6.16 ± 0.52 (n = 7)</td>
<td>3.43 ± 0.42 (n = 7)</td>
</tr>
<tr>
<td>Partial specific volume (cm^3/g)</td>
<td>0.75 ± 0.08</td>
<td>0.80 ± 0.02</td>
<td>0.87 ± 0.01</td>
</tr>
<tr>
<td>Molecular weight (kDa)</td>
<td>381 ± 21</td>
<td>217 ± 32</td>
<td>118 ± 17</td>
</tr>
<tr>
<td>Protein moiety (kDa)</td>
<td>210 ± 12</td>
<td>120 ± 18</td>
<td>65 ± 9</td>
</tr>
<tr>
<td>Unit size (monomers)</td>
<td>3.87 ± 0.22</td>
<td>2.21 ± 0.33</td>
<td>1.21 ± 0.17</td>
</tr>
</tbody>
</table>
Figure 8. Diagram of the putative secondary structure of GLUT1 and the topology of GLUT1 cysteine (C) residues.
lipid bilayer and one cysteine is found on each side of the membrane, immediately adjacent to the bilayer.

The free sulfhydryl content of the two purified GLUT1 preparation was determined by the use of Ellman’s reagent (Rao and Scarborough 1980). The purified protein is solubilized in SDS to unfold the protein and thereby allow uniform access of the thiol sensitive photochemical reagent (5,5’-dithiobis(2- nitrobenzoic acid), Ellman’s reagent) to the entire polypeptide. GLUT1-DTT contains 2 free sulfhydryls per GLUT1 molecule, whereas GLUT1+DTT contains 6 free sulfhydryls (Figure 9).

GLUT1-DTT contains four sulfhydryl groups which are inaccessible to Ellman’s reagent. This could be due to: 1. The existence of intra-molecular disulfide bonds. 2. The existence of inter-molecular disulfide bonds between homo- or hetero-oligomers. 3. The conformation of GLUT-DTT could hinder the accessibility of the thiol sensitive reagent to the appropriate residues. 4. Mixed disulfides may exist between the protein and glutathione. Intermolecular disulfide bonds are excluded by reducing and non-reducing SDS-PAGE analysis of GLUT1±DTT (Figure 13). Intra-molecular disulfide bonds thus appear to be involved since GLUT-DTT can be converted to GLUT1+DTT by DTT-treatment of cholate solubilized GLUT1-DTT (Figure 4). These findings will be discussed in detail in chapter five.

Alkylating agents effects

To ensure that the size of the observed species is not an artifact of the protein purification procedure, the membrane permeable alkylating agent, N-ethylmaleamide (NEM), was employed to freeze the protein in its native redox state. NEM alkylates any accessible free sulfhydryl groups. Thus blocking the oxidation of free sulfhydryls (to disulfides) during
Figure 9. Free sulfhydryl content of purified GLUT1. GLUT-DTT (closed circles) or GLUT1+DTT (open circles) was denatured in 0.5% SDS for 30 minutes at room temperature. Non-solubilized GLUT1 was removed by centrifugation and the amount of GLUT1 present in the supernatant determined by protein assay. The reaction was initiated by addition of 100 μL freshly prepared 10 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB, Ellman's reagent) to the solubilized GLUT1. The course of the reaction at room temperature was recorded as the increase in absorbance at 412 nm. An extinction coefficient of 13,600 M⁻¹ cm⁻¹ at 412 nm for 2-nitro-5-thiobenzoic acid (NTB) was used to quantitate the reaction. The results of 3 separate, similar experiments indicate that GLUT1-DTT and GLUT1+DTT contain 2.2 ± 0.1 and 6.2 ± 0.2 free sulfhydryl groups per monomer, respectively.
Figure 10. Effect of alkylation on the size of solubilized erythrocyte ghost GLUT1. Erythrocyte membranes were treated with the membrane-permeable, alkylating agent, N-ethyl maleimide (NEM) and solubilized in 20 mM cholate. Solubilized proteins were resolved by SEC-HPLC. Fractions were assayed for GLUT1 content by ELISA employing anti-GLUT1 antibodies. The data sets are displaced in the y-axis to facilitate distinction. These results are representative of 3 separate experiments.
further manipulation. Prior treatment of erythrocyte ghosts with NEM has no effect on the size of GLUT1-containing micelles as resolved by size-exclusion HPLC (Figure 10).

Conclusions

1. Erythrocyte glucose transporter purified in the presence of reducing reagent (GLUT1+DTT) and solubilized in sodium cholate is resolved largely as a homo-dimer with minor tetrameric and possibly monomeric components.

2. Erythrocyte glucose transporter purified in the absence of reducing reagent (GLUT1-DTT) and solubilized in sodium cholate is resolved largely as a homo-tetramer with minor dimeric and possibly monomeric components.

3. Sodium cholate solubilized erythrocyte membranes (ghosts) resemble the GLUT1-DTT preparation and are resolved largely as tetrameric transporters.

4. GLUT1+DTT contains six free sulfhydryls upon solubilization in SDS whereas GLUT1-DTT contains two free thiol groups under equivalent conditions. The four inaccessible sulfhydryls in GLUT1-DTT form either intramolecular disulfide bonds or mixed disulfides.
Chapter IV

OLIGOMERIC STATE OF MEMBRANE-RESIDENT GLUT1

The preceding, hydrodynamic studies show that detergent-solubilized, human erythrocyte glucose transporter (GLUT1) exists as homotetramers or homodimers, in the absence or presence of reductant, respectively. These studies however, do not address the oligomeric state of GLUT1 in the native lipid bilayer- the physiological environment of GLUT1.

The study of the quaternary structure of membrane proteins has lagged behind similar studies of water soluble proteins because of complications resulting from associations of membrane proteins with the lipid matrix. Detergents disrupt this matrix enabling the solubilization of membrane proteins. However, a remaining caveat is that the structure of the protein may not be conserved during solubilization. Therefore, it is necessary to investigate the quaternary state of GLUT1 in its native lipid bilayer. In addition, hydrodynamic analyses rely on purified GLUT1. The manipulations of GLUT1 during the purification process may alter transporter oligomeric state.

Results and Discussion

Two powerful approaches for investigating membrane-resident protein oligomeric structure are chemical crosslinking and conformation-specific antibody binding.

Chemical crosslinking

The physical properties of integral membrane proteins often preclude detailed structural investigations. Biophysical techniques e.g. low angle scattering of X-rays or neutrons, or fluorescence methods can provide important information about protein structure. However, these techniques often require harsh treatment of protein in order to obtain ordered samples. Chemical crosslinking can provide important information about protein
oligomeric state without the harsh treatments required with biophysical methods (Peters and Richards 1977).

The basis of chemical crosslinking is to freeze the protein in its oligomeric structure. Crosslinking reagents contain two chemically reactive groups which are commonly symmetrical and connected by a specified linker arm available in a variety of lengths. An effective crosslink is produced by the reactive groups of a crosslinker reacting with two different subunits of a complex. Effective crosslinking is thus highly dependent upon the geometry and specificity of reactive, protein components and the length of the arm of the crosslinker. Once the protein is crosslinked in its native lipid bilayer, the protein can then be resolved by SDS-PAGE.

Crosslinking of erythrocyte GLUT1

Intact erythrocyte membrane proteins were crosslinked by using the membrane permeable, u.v. activated crosslinker, dithiobisphenyl azide (DTPA, Figure 11). DTPA crosslinking of erythrocytes at ice-cold temperature results in the formation of 55, 110 and 220 kDa GLUT1-containing complexes (figure 11). While heterocomplex formation remains a formal possibility these data are also consistent with tetrameric associations of GLUT1 in erythrocyte membranes. No red cell aggregation is observed therefore no artificial crosslinking between cells occurs.

Chemical crosslinking is not without pitfalls. Protein concentration is critical during crosslinking. A protein concentration that is too high can result in random crosslinking of adjacent, non-associated complexes. A number of considerations, however, suggest that this is not probable. The mean time between collisions of GLUT1 molecules in an erythrocyte bilayer ($t_{av}$) is (Berg et. al., 1977):

$$t_{av} = \frac{1.1a^2}{N D} \ln \frac{1.2a^2}{N s^2}$$  \hspace{1cm} (4.1)
where $a$ is the radius of the cell (assuming it has been unfolded into a circular bilayer), $s$ is the radius of GLUT1, and $N$ and $D$ are the number of GLUT1 molecules per cell and the diffusion coefficient of GLUT1 in a lipid bilayer (Krehm and Ji, 1977), respectively. The average time between GLUT1 molecular collisions in erythrocytes is thus 381 msec. The half-life of an aryl nitrene (the u.v.-activated, reactive form of N$_3$) is in the order of 1 msec (Reiser et al., 1968). The fraction of activated crosslinker that would remain at $t_{av}$ is, therefore, $2.15 \times 10^{-115}$. These calculations strongly suggest that if the observed crosslinked complexes represent dimeric and tetrameric GLUT1, they could not have arisen purely by simple, random diffusion/collision in the bilayer. Since these experiments are performed in erythrocytes, the formation of heterocomplexes cannot be excluded. Analogous calculations assuming $1 \times 10^6$ anion transporters per erythrocyte suggest that this probability is also extremely low. Furthermore, the expected size of a GLUT1-anion transporter complex would be 145 kDa. In addition, the probability of collisions between GLUT1 and lipid is much greater than that of GLUT1-protein collisions. Lipid collisions resulting in crosslinking are unlikely to have a noticeable effect on size as resolved by SDS-PAGE.

**Chemical crosslinking of purified GLUT1**

GLUT1 co-purifies with red cell lipid and integrates normally into bilayers upon detergent removal (Cairns et al. 1987). This permits the use of purified GLUT1 to examine the oligomeric structure of membrane-resident GLUT1. Glutaraldehyde reacts with primary amines. Glutaraldehyde crosslinked GLUT1-DTT is resolved as GLUT1 monomers, dimers and tetramers (Figure 12A) by SDS-PAGE. GLUT1+DTT is resolved as monomers and dimers (Figure 12B). Since glutaraldehyde is 6.6 Å in length, these findings argue strongly for homotetrameric associations of GLUT1 in GLUT1-DTT proteoliposomes and for homodimeric associations of GLUT1 in GLUT1+DTT
Figure 11 Chemical Crosslinkers
Figure 12. Chemical crosslinking of GLUT1 in erythrocytes. Intact human red cells were u.v. irradiated at ice temperature in the presence of 1 mM dithiobisphenylazide (+DTPA) or in the absence of DTPA (-DTPA). GLUT1 containing complexes were resolved by reducing (+DTT) or non-reducing (-DTT) SDS-PAGE and were detected by immunoblot assay. Ordinate: relative [GLUT1]; abscissa: electrophoretic mobility. The mobilities of molecular weight standards (kDa) are shown above the traces. The heterogenously glycosylated monomer is denoted by the bar below the traces.
Figure 13. Chemical crosslinking of GLUT1 in proteoliposomes. Purified GLUT1 (GLUT1-DTT and GLUT1+DTT) was crosslinked using glutaraldehyde (GA). GLUT1 containing complexes were resolved by reducing and non-reducing SDS-PAGE. Reducing and non-reducing electrophoresis resulted in indistinguishable traces as detected by immunoblots. Ordinate: relative [GLUT1]; abscissa: electrophoretic mobility. The mobilities of the molecular weight standards (kDa) are shown above the traces. Key: GA+, GLUT1 treated with 13 mM glutaraldehyde; GA-, GLUT1 processed in the absence of glutaraldehyde; DTT±, profiles observed in the presence or absence of DTT in the sample buffer.
205 116 66 45

GLUT1+DTT

GA+

GA-

GLUT1-DTT

Relative Mobility

Relative [GLUT1]
proteoliposomes. Moreover, the significant absence of species of >220 kDa in GLUT1-DTT and the absence of species >110 kDa in GLUT1+DTT argues strongly against non-specific complex formation. No liposomal aggregation is observed; therefore no artifactual crosslinking occurs between adjacent vesicles.

Conformation-specific antibodies

Antibody-antigen interactions are highly sensitive to antigen conformation. Certain antibodies raised against influenza hemagglutinin (HA) can bind either to HA monomers or to HA trimers but not to both (Copeland et. al., 1988). A variety of polyclonal antibodies against GLUT1 available in our laboratory were tested for their titers towards various conformations of GLUT1. Rabbit IgGs raised against GLUT1-DTT (\(\alpha\)-IgGs) react specifically with extracellular epitopes of GLUT1 in isolated red cells and in CHO cells transfected with GLUT1 cDNA and over expressing GLUT1 (Harrison et. al., 1990).

Competition ELISA assays using \(\alpha\)-IgGs show that \(\alpha\)-IgG-reactive epitopes are present in both GLUT-DTT and in intact erythrocytes but are absent in GLUT+DTT (Figure 13A). Anti-GLUT1 carboxyl-terminal peptide IgGs (C-IgGs) show similar titers for both GLUT1-DTT and GLUT1+DTT. This suggests that the C-termini of both GLUT1+DTT and GLUT1-DTT are accessible and present at similar concentrations. C-IgGs fail to react with the intracellular carboxyl-terminal region of GLUT1 in intact cells but do bind to unsealed cell ghosts (Figure 13A, see also Harrison et. al., 1990).

Competition ELISAs can be carried out using GLUT1-DTT and GLUT1+DTT as standards, to determine the number of IgG-reactive GLUT1 proteins per erythrocyte (Figure 13B). The extrapolated number of cell surface, \(\alpha\)-IgG-reactive GLUT1 proteins is 307,029 \(\pm\) 50,872 per cell using GLUT1-DTT as a standard or > 100 \(\times\) 10^6 per cell using GLUT1+DTT as a standard. Erythrocyte ghosts contain 296,651 \(\pm\) 41,349 reactive sites (using GLUT1-DTT as a standard). Since erythrocytes contain 150,000 to 300,000
Figure 14. Competition ELISA assays of erythrocyte GLUT1-DTT content. Competition ELISA assay (Carruthers and Helgerson, 1989) using rabbit anti-GLUT1-DTT IgGs (α-IgGs) were performed to determine the number of reactive, GLUT1 epitopes present in GLUT1-DTT, GLUT1+DTT, intact erythrocytes and in leaky erythrocyte ghosts. A). Prior to addition of α-IgGs (triangles) or C-IgGs (circles) to wells containing 100 ng GLUT1-DTT, the IgGs were incubated for 60 min at room temperature with GLUT1-DTT (open symbols) or GLUT1+DTT (closed symbols). The amount of GLUT1 present in the preincubation is shown on the abscissa. Absorbance was normalized to absorbance developed in the absence of exogenous GLUT1. The computed half-maximal GLUT1 binding concentrations are: α-IgGs, GLUT1-DTT = 273 ± 16 ng, GLUT1+DTT > 100 µg; C-IgGs, GLUT1-DTT = 155 ± 6 ng, GLUT1+DTT = 71 ± 5 ng. B). Prior to addition of α-IgGs (triangles) or C-IgGs (circles) to wells containing 100 ng GLUT1-DTT, the IgGs were incubated for 60 min at room temperature with intact red cells (closed symbols) or with leaky (freeze/thawed) erythrocyte ghosts (open symbols). The number of cells present in the preincubation is shown on the abscissa. The number of ghosts was calculated assuming 1 ghost contains 0.66 pg membrane protein (Carruthers, 1986). Absorbance was normalized to absorbance developed in the absence of cells or ghosts. The curves drawn through the points assume a single, saturable component of binding. The computed half-maximal binding cell numbers are: α-IgGs, intact cells = (9.7 ± 0.8) x 10⁶ cells; C-IgGs, intact cells ≥ 1 x 10¹⁰ cells, leaky ghosts = (5.7 ± 0.7) x 10⁶ cells. All measurements were made in duplicate. These results summarize 12 separate experiments.
GLUT1-specific cytochalasin B binding sites (Jung and Rampal 1977, Helgerson and Carruthers 1987), these findings suggest that erythrocyte-resident GLUT1, which closely resembles GLUT1-DTT, accounts quantitatively for erythrocyte transporter content.

Conclusions

1. Dithiobisphenylazide (DTPA) crosslinked erythrocytes membrane-resident GLUT1 is resolved by SDS-PAGE as 55, 110 and 220 kDa species.

2. Glutaraldehyde crosslinked GLUT1-DTT is resolved as monomeric, dimeric and tetrameric species.

3. Glutaraldehyde crosslinked GLUT1+DTT is resolved as monomeric and dimeric species.

4. The use of conformation-specific antibodies demonstrates that erythrocyte-resident GLUT1 closely resembles GLUT1-DTT.

5. At the most simplistic level, the sole GLUT1 species present in GLUT1-DTT and erythrocyte membranes but absent in GLUT1+DTT is tetrameric GLUT1.
Chapter V

FUNCTIONALITY AND ORIENTATION OF GLUT1 SUGAR BINDING SITES

The preceding chapters have examined the oligomeric state of solubilized and membrane-resident GLUT1. The presence of reducing agents plays a critical role in determining the oligomeric structure of GLUT1. In this chapter, we explore the effects of reductant on the functionality and the orientation of the substrate binding sites of GLUT1.

Results and Discussion

Equivalent amounts of purified GLUT1 and GLUT1 present in erythrocyte ghost membranes were reconstituted into proteoliposomes. The functionality of the reconstituted transporter was assayed by determining the uptake of 0.1 mM [14C] 3-O-methylglucose into the GLUT1 containing proteoliposomes. Proteoliposomes containing equivalent amounts of GLUT1-DTT, GLUT1+DTT and erythrocyte ghost GLUT1 mediate cytochalasin B-inhibitable 3-O-methylglucose transport at rates which are indistinguishable at the available resolution (Figure 15).

The uptake measurements of reconstituted GLUT1 indicate that the GLUT1 preparations (GLUT1-DTT and GLUT1+DTT) contain functional species. However, recall from chapter III (Figures 4 and 5) that the preparations contain a variety of GLUT1 populations (monomer, dimers and tetramers). The fraction of these species is dependent upon the presence or absence of reductant during purification. Therefore, the formal possibility exists that a given population is dysfunctional and an additional population functions at a higher level. However, if GLUT1+DTT contains 100% dimer (as probed by SEC-HPLC) and GLUT1-DTT contains 25% dimer (Figure 4), it is difficult to envision that the 25%
Figure 15. Reconstitution of GLUT1 into proteoliposomes. Equivalent amounts of purified GLUT1 and GLUT1 from ghost membranes were reconstituted into proteoliposomes. The glucose transport activity of these GLUT1 containing proteoliposomes was assayed by monitoring the uptake of 0.1 mM [14C]-3-O-methylglucose at 25 °C in the presence and absence of cytochalasin B (CCB), a potent glucose transport inhibitor. Proteoliposomes lacking GLUT1 were also assayed to determine the leakiness of the vesicles. Uptake of [14C]-3-O-methylglucose was stopped by the addition of ice-cold medium containing 100 µM phloretin and 10 µM CCB at the appropriate time. The vesicles were sedimented by centrifugation at 4°C for 4 min at 14,000 x g, washed once more with stopper solution then solubilized with sodium dodecylsulfate. Uptake was measured at the times indicated and at up to 2 hrs. No further uptake of sugar was detectable at later time points. Uptake is expressed as 3-O-methylglucose space at time, t, divided by the equilibrated 3-O-methylglucose space of the proteoliposomes (t=2 hrs). All experiments were made in duplicate. These results summarize 3 separate experiments.
dimer of GLUT1-DTT could have equivalent transport capabilities as 100% of the dimer of GLUT1+DTT.

Ligand binding studies were employed to examine the functionality of each individual subunit of GLUT1-DTT and GLUT1+DTT in closer detail. D-glucose binding to GLUT1 can be conveniently monitored by fluorimetry since the intrinsic tryptophan fluorescence of the transporter is quenched when D-glucose binds to the carrier (Gorga and Lienhard, 1982). L-glucose, the non-transported stereoisomer of D-glucose fails to quench GLUT1 intrinsic fluorescence. Scatchard analysis of D-glucose-dependent GLUT1 intrinsic fluorescence quenching demonstrates that GLUT1-DTT contains at least 2 distinct D-glucose binding sites (high and low affinity)- a result expected of a two-site carrier (Figure 16). These sites have been shown to co-exist on a single functional complex (not different populations of GLUT1) and to correspond to the influx (high affinity) and efflux (low affinity) sites by analysis of D-glucose inhibition of cytochalasin B binding to the efflux site and of phloretin binding to the influx site (Carruthers, 1986). Furthermore, GLUT1+DTT contains a single population of sugar binding sites- a result expected of a one-site carrier (Figure 16). However, the x-intercept (number of sugar binding sites) of both GLUT1+DTT and GLUT1-DTT plots in figure 16 are indistinguishable indicating that equivalent amounts of total D-glucose binding sites are present. The two preparations therefore contain equivalent amounts of functional units as assayed by D-glucose binding capacity.

These studies show that tetrameric (but not dimeric) GLUT1 presents multiple D-glucose binding sites. However, they do not address the orientation of substrate binding sites in the transporter complex. Cytochalasin B binding studies can be employed to investigate the orientation of the binding sites of the purified transporter. Cytochalasin B binds specifically at (or very close to) the glucose transporter efflux (low affinity) site (Baldwin et. al. 1982, Carruthers and Helgerson 1991). GLUT1-DTT binds 0.42 ± 0.02 moles of
Figure 16. D-glucose binding to purified GLUT1. D-glucose binding to GLUT1 was monitored by measurements of D-glucose induced, GLUT1 intrinsic fluorescence quenching. Ordinate: D-glucose specific, GLUT1 fluorescence quenching expressed as fractional fluorescence quenching/[D-glucose] (mM D-glucose⁻¹), Abscissa: fractional fluorescence quenching. Data are shown for GLUT1+DTT (closed circles) and GLUT1-DTT (open circles). The curve through GLUT1-DTT data is the computed best fit assuming 2 saturable components of sugar binding each characterized by a binding constant (K_d(app)) and a maximum quenching parameter (Q_m). The computed parameters are: K_d(app)₁ = 1.7 mM, Q_m₁ = 0.042; K_d(app)₂ = 25.3 mM, Q_m₂ = 0.048. The straight line drawn through GLUT1+DTT data was calculated by linear regression and corresponds to a single, saturable component of binding with K_d(app) = 12.6 mM and Q_m = 0.091. This data summarizes 9 separate experiments.
cytochalasin B per mole of GLUT1 whereas GLUT1+DTT binds 0.82 ± 0.06 moles of cytochalasin B per mole of GLUT1 (Figure 17). GLUT1+DTT thus has twice the cytochalasin B binding capacity of GLUT1-DTT. Since both preparations contain the same number of total D-glucose binding sites, based upon the tryptophan quenching studies shown above, these findings are commonly interpreted as indicating that one half of the GLUT1-DTT preparation is dysfunctional with regard to cytochalasin B binding capability. However, these observations are also consistent with the following hypothesis: 1. GLUT1+DTT is a one-site transporter capable of binding one cytochalasin B or one D-glucose molecule per GLUT1 monomer. 2. GLUT1-DTT (tetramer) is a two-site transporter capable of binding one D-glucose molecule per monomer and one cytochalasin B molecule per GLUT1 dimer. Tetrameric GLUT1 must expose two sugar influx sites and two sugar efflux sites but never four sugar efflux simultaneously. This would explain both the lower cytochalasin B binding capacity and the multiple sugar binding site kinetics of GLUT1-DTT.

This model can be further tested in erythrocyte membranes depleted of peripheral proteins (stripped ghosts). This erythrocyte membrane-resident system is more representative of the physiological state of GLUT1. Based upon the above hypothesis, dithiothreitol treatment of stripped ghosts is predicted to: 1. result in the loss of multiple GLUT1 D-glucose binding sites; 2. double the cytochalasin B binding capacity of membrane-resident GLUT1 and, 3. convert tetrameric GLUT1 to dimeric GLUT1.

Erythrocyte membranes were depleted of peripheral proteins by a single alkaline wash (Carruthers, 1986). These membranes were subsequently exposed to either a second alkaline wash (SG↑pH), a second, neutral wash with dithiothreitol (SG+DTT) or to a second wash in alkaline medium containing dithiothreitol (SG↑pH+DTT).
Figure 17. Cytochalasin B binding to purified GLUT1. Purified GLUT1 preparations were assayed for cytochalasin B binding capacities. Equivalent amounts of GLUT1+DTT (closed circles) and GLUT1-DTT (open circles) (1.35 μM; 14.8 μg GLUT1 in 0.2 mL) were equilibrated with a variety of concentrations of [3H]-cytochalasin B at 25°C for 30 minutes. Samples of the total activity of [3H]-cytochalasin B were counted. Samples were spun in Millipore ultrafree-MC 10,000 molecular weight cut-off filter units for 10 minutes. The material which had spun through the filter (Free) is counted in duplicate. Bound counts are determined as: Bound = (Total - Free). Ordinate: bound [CCB], nM. Abscissa: free [CCB], nM. Curves drawn through the points were computed by nonlinear regression assuming a single, saturable component of CCB binding. The computed maximum binding capacities (B_m) are: GLUT1+DTT, B_m = 1105 ± 84 nM; GLUT1-DTT, B_m = 563 ± 60 nM. These data summarize 5 separate experiments.
Before these predictions can be tested quantitatively, it is necessary to first determine that SG↑pH, SG+DTT and SG↑pH+DTT contain equivalent amounts of GLUT1. Stripped ghost GLUT1 content was determined by first constructing a GLUT1 calibration curve by western analysis using anti-GLUT1 C-terminal antibodies and purified GLUT1 (Figure 18). Equivalent volumes of stripped ghosts were analyzed by immunoblotting to determine GLUT1 content. The resulting autoradiograms for purified GLUT1 were quantitated by densitometry and the relationship between intensity of exposure and [GLUT1] was fitted by nonlinear regression assuming a simple, saturable relationship between [GLUT1] and exposure intensity. According to this analysis the intensity of exposure, I, is related to [GLUT1] by:

\[ I = \frac{A_m [GLUT1]}{K_{0.5} + [GLUT1]} \]  

(5.1)

Thus, [GLUT1] of an unknown sample of intensity I is determined as (µg):

\[ \frac{K_{0.5}}{A_m} \cdot \frac{1}{I} - 1 \]  

(5.2)

The computed constants for purified GLUT1 are: \( A_m = 69430 \pm 3413 \) units, and \( K_{0.5} = 2.44 \pm 0.46 \) µg GLUT1 with a correlation coefficient of \( R = 0.993 \). The stripped ghost data can be analyzed in the same manner. The results are:

- **SG↑pH** \( A_m = 69445 \pm 239 \), \( K_{0.5} = 3.02 \pm 0.04 \) µL, \( R = 0.9999 \);
- **SG+DTT** \( A_m = 69365 \pm 3559 \), \( K_{0.5} = 3.58 \pm 0.69 \) µL, \( R = 0.9920 \) and,
- **SG↑pH+DTT** \( A_m = 69896 \pm 2028 \), \( K_{0.5} = 6.86 \pm 0.53 \) µL, \( R = 0.9991 \)

61
Figure 18. Quantitation of [GLUT1] in stripped ghosts. Red cell ghosts were depleted of peripheral proteins by a single alkaline wash (pH 12) as described. These stripped ghosts were then either exposed to: alkaline medium again for 5 min (SG↑pH); neutral medium plus 10 mM dithiothreitol (SG+DTT) or to alkaline medium plus DTT (SG↑pH+DTT). The membranes were collected by centrifugation and resuspended in neutral medium (50 mM Tris-HCl, pH 7.0). Aliquots of the membranes (5, 10 and 20 μL) and purified GLUT1 (0 to 40 μg) were then subjected to western analysis using anti-GLUT1 C-terminal antibodies. The resulting blots were subjected to autoradiography for 20 minutes (under exposed) and the autoradiogram quantitated by scanning densitometry. The amount of immunoreactive GLUT1 in each lane (area) is plotted as a function of applied GLUT1 (μg) or μL stripped ghosts (log scale). The curve drawn through the purified GLUT1 data was calculated by nonlinear regression assuming the relationship between area and [GLUT1] is described by simple Michaelis-Menten kinetics.
The relative amounts of GLUT1 present in these membranes can be calculated by using equation 5.1, or by simple interpolation or by dividing the $K_{0.5}$ value for purified GLUT1 by the $K_{0.5}$ value of the membrane preparation. These values are:

- $\text{SG}^{\uparrow}\text{pH}$ contains $0.79 \pm 0.04 \mu\text{g GLUT1. mL}^{-1}$;
- $\text{SG}^{\downarrow}\text{DTT}$ contains $0.67 \pm 0.04 \mu\text{g GLUT1. mL}^{-1}$ and,
- $\text{SG}^{\uparrow}\text{pH}\downarrow\text{DTT}$ contains $0.36 \pm 0.03 \mu\text{g GLUT1. mL}^{-1}$.

When these data are back-calculated for membrane yield and quantitated loss of peripheral proteins, the estimated numbers of GLUT1 per erythrocyte (assuming 1 erythrocyte contains 0.66 pg membrane protein) are:

- $\text{SG}^{\uparrow}\text{pH}$ contains $343,373 \pm 17,386 \text{ cell}^{-1}$;
- $\text{SG}^{\downarrow}\text{DTT}$ contains $316,265 + 23,602 \text{ cell}^{-1}$ and,
- $\text{SG}^{\uparrow}\text{pH}\downarrow\text{DTT}$ contains $299,184 \pm 24,932 \text{ cell}^{-1}$.

The stripped ghosts were assayed for D-glucose binding sites by tryptophan fluorescence quenching. D-glucose interaction with GLUT1 in $\text{SG}^{\uparrow}\text{pH}$ is characterized by at least two components of binding (Figure 19). Following exposure to a second alkaline wash containing dithiothreitol ($\text{SG}^{\uparrow}\text{pH}\downarrow\text{DTT}$), membrane D-glucose binding displays single site kinetics (Figure 19). D-glucose binding was unaffected by treatment with dithiothreitol alone ($\text{SG}^{\downarrow}\text{DTT}$).

The cytochalasin B binding capacity of these stripped ghost preparations was also determined. Dithiothreitol treatment in alkaline medium ($\text{SG}^{\uparrow}\text{pH}\downarrow\text{DTT}$) but not in neutral
Figure 19. D-glucose binding to stripped ghosts. D-glucose binding to GLUT1 in alkali-treated erythrocyte membranes was monitored by D-glucose induced, GLUT1 intrinsic fluorescence quenching measurements. 200 μg of membrane protein was resuspended into 2.5 mL Tris-medium. Fluorescence was sampled at a rate of 10 readings per second and signal averaged over two seconds. A. Changes in fluorescence of SG↑pH and SG↑pH+DTT as a function of time was monitored. Time scale and extent of fluorescence is shown by the bars. Starting fluorescence for SG↑pH = 489 mV and SG↑pH+DTT = 487 mV. The arrows indicate successive additions from a stock solution of 1.25 M D-(lower trace) or L-Glucose (upper trace). These additions were 1, 1, 1, 1, 6, 10, 10, 10, 20, 40, 50, 50, μL of D- or L-glucose. B. Ordinate: D-glucose-specific, GLUT1 fluorescence quenching expressed as fractional fluorescence quenching/[D-glucose] (mM D-glucose⁻¹). Abscissa: fractional fluorescence quenching. Data are shown for membranes exposed to alkaline medium containing (closed circles) or lacking (open circles) dithiothreitol. The curve drawn through the stripped ghost -DTT data is the computed best fit assuming 2 saturable components of sugar binding. The computed parameters are: K_d(app)₁ = 1.3 mM, Q_m₁ = 0.039; K_d(app)₂ = 31 mM, Q_m₂ = 0.031. The line drawn through the stripped ghost +DTT data was calculated by linear regression and corresponds to a single saturable component of binding of K_d(app) of 16.5 mM and Q_m of 0.07. This figure summarizes three separate experiments.
Fractional Quenching
Table II: Cytochalasin B binding to purified GLUT1 and to membrane-resident GLUT1.

<table>
<thead>
<tr>
<th>GLUT1 species</th>
<th>CCB binding (moles CCB/mol GLUT1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLUT1-DTT</td>
<td>0.44 ± 0.02</td>
</tr>
<tr>
<td>GLUT1+DTT</td>
<td>0.82 ± 0.06</td>
</tr>
<tr>
<td>SG↑pH</td>
<td>0.61 ± 0.01</td>
</tr>
<tr>
<td>SG↑pH+DTT</td>
<td>1.31 ± 0.02</td>
</tr>
<tr>
<td>SG+DTT</td>
<td>0.63 ± 0.01</td>
</tr>
</tbody>
</table>
Figure 20. Cytochalasin B binding to stripped ghosts. Cytochalasin B binding to stripped ghosts was determined by incubating the alkaline-treated erythrocyte membranes with varying concentrations of $[^{3}$H]-CCB for 30 min at 25°C. Total cpm samples were taken. The samples were spun for 10 min in a table-top Eppendorf centrifuge. The supernatant was sampled and counted. Bound CCB was determined as: (Total - Free).

Ordinate: mol CCB bound per mol GLUT1. Abscissa: [CCB]$_{free}$ in nM (note log scale).

The curves were calculated by nonlinear regression. The parameters calculated are:
SG+high pH+DTT, $B_{max} = 1.310 \pm 0.017$, $K_d = 282.36 \pm 9.03$ nM, $R = 0.9996$;SG+DTT, $B_{max} = 0.635 \pm 0.0087$, $K_d = 233.49 \pm 7.88$ nM, $R = 0.9996$ and, SG+high pH, $B_{max} = 0.605 \pm 0.012$, $K_d = 230.09 + 11.04$ nM, $R = 0.9992$. This figure summarizes 3 separate experiments.
Figure 21. \( \delta \)-IgG binding to alkali-treated erythrocyte ghosts. \( \delta \)-IgG binding to stripped ghosts was determined by incubating erythrocyte stripped ghosts with \( \delta \)-IgG for 30 min at 25°C. Membranes were washed by ultracentrifugation. \( ^{125} \)I-protein A was incubated with membranes for 30 min at 25°C. Membranes were washed by ultracentrifugation and the pellets resuspended and counted. Data are corrected for non-specific binding (quantitated using pre-immune serum), and are expressed as cpm \( ^{125} \)I-Protein A bound per nmol immunodetectable GLUT1. Key: SG\( \uparrow \)pH, additional treatment with high pH media; SG+DTT, additional treatment with 10 mM DTT, and SG\( \uparrow \)pH+DTT, additional treatment with high pH and 10 mM DTT. This figure summarizes 3 separate experiments. Measurements were made in triplicate.
medium (SG+DTT) doubles the cytochalasin B binding capacity of membrane-resident GLUT1 (Figure 20, Table II).

The final prediction can be tested by measuring conformation specific \( \alpha \)-IgG binding to depleted membranes. \( \alpha \)-IgGs bind to GLUT1-DTT (tetramer) with high affinity but have undetectable affinity for GLUT1+DTT (dimeric GLUT1, as shown in Figure 14). \( \alpha \)-IgG-specific protein A binding to depleted membranes was reduced 4.3 \( \pm \) 0.9-fold following exposure to a second alkaline wash containing dithiothreitol (SG\( \uparrow \)pH+DTT) (Figure 21). Binding was unaffected by treatment with dithiothreitol alone (SG+DTT).

Thus each prediction of this hypothesis was satisfied experimentally. However, it was necessary to simultaneously expose membrane-resident GLUT1 to high pH and dithiothreitol, in order to achieve these results. Exposure to high pH depletes membranes of peripheral proteins through disruption of salt bridges. The simplest explanation for these findings is that erythrocyte membrane-resident, tetrameric GLUT1 intramolecular disulfides are masked by intra- or intermolecular salt bridges. The disruption of these salt bridges permits access of dithiothreitol to the disulfide(s) resulting in conversion of tetrameric GLUT1 to dimeric GLUT1.

Conclusions

1. GLUT1-DTT, GLUT1+DTT and erythrocyte membrane-resident GLUT1 reconstituted into egg phosphatidylcholine bilayers, transport 3-O-methylglucose at equivalent rates.

2. GLUT1-DTT possesses two discrete D-glucose binding sites (high and low affinity). GLUT1+DTT contains a single population of D-glucose binding sites.
3. GLUT1-DTT binds 0.42 moles of cytochalasin B per mole of GLUT1 whereas GLUT1+DTT binds 0.82 moles of cytochalasin B per mole of GLUT1.

4. Dithiothreitol treatment of peripheral protein depleted erythrocyte membranes results in the loss of multiple D-glucose binding site kinetics but leaves the total number of glucose binding sites unchanged.

5. Dithiothreitol treatment of stripped erythrocyte ghosts doubles the cytochalasin B binding capacity of membrane-resident GLUT1.

6. Dithiothreitol treatment of stripped erythrocyte ghosts decreases the binding of ð-IgG, a conformation (tetramer)-specific antibody.
CHAPTER VI

DISCUSSION: SIGNIFICANCE & FUTURE DIRECTIONS

This study examines the relationship between human erythrocyte glucose transporter (GLUT1) oligomeric structure and function. A controversy presently exists in the literature concerning the mechanism of glucose translocation by the glucose transporter. This study resolves this controversy by demonstrating that the mechanism of sugar transport is determined by transporter oligomeric structure. Different laboratories have, unknowingly, studied different oligomeric forms of GLUT1. The differences in GLUT1 oligomeric structure result from the omission or inclusion of reductant (dithiothreitol) during GLUT1 purification.

GLUT1 was purified from human erythrocytes in the absence (GLUT1-DTT) or presence (GLUT1+DTT) of the reducing reagent, dithiothreitol (DTT). Hydrodynamic (size-exclusion HPLC and sucrose gradient ultracentrifugation) analysis of cholate-solubilized GLUT1±DTT show that GLUT1-DTT is largely a homotetramer and GLUT1+DTT is largely a homodimer. Chemical crosslinking studies of lipid bilayer-resident GLUT1 support these conclusions. Sodium dodecylsulfate-solubilized GLUT1-DTT and GLUT1+DTT contain 2 and 6 free sulfhydryls per monomer of GLUT1, respectively. However, the subunits are not linked by intermolecular disulfide bridges.

Similar studies with intact and cytosol-depleted erythrocytes suggest that membrane-resident GLUT1 resembles GLUT1-DTT, a homotetrameric protein. Studies using conformation-specific antibodies support this view. Antibodies raised against GLUT1-DTT (\(\partial\)-IgGs) fail to react with GLUT1+DTT but do react with GLUT1-DTT and bind at 307,000 sites on the surface of human erythrocytes. \(\partial\)-IgGs react with CHO cells.
overexpressing GLUT1 and increased $\beta$-IgG binding is directly related to total GLUT1 concentration and expressed transport activity (Harrison et. al. 1990). Anti-GLUT1-C-terminal antibodies (C-IgGs) show similar titers for both GLUT1-DTT and GLUT1+DTT and react with 296,700 sites in leaky erythrocyte ghosts. These results support the view that erythrocyte-resident GLUT1 is quantitatively accounted for by GLUT1-DTT.

Since protein function is determined by protein structure, the functional properties of GLUT1-DTT and GLUT1+DTT were analyzed. The sugar transporter serves to bind then translocate sugars across the cell membrane. Translocation can be studied in reconstituted proteoliposomes but a number of practical considerations reduce the utility of studying sugar translocation as a means of detailed analysis of transporter function. These include: heterogeneities in reconstituted protein numbers per proteoliposome; heterogeneities in proteoliposomal size (Carruthers and Melchior, 1984); random orientation of reconstituted proteins (Carruthers and Melchior, 1984); the small sizes and relatively high basal sugar permeabilities of proteoliposomes (Carruthers and Melchior, 1984). Ligand binding studies on purified GLUT1 are, however, experimentally facile and do provide information on numbers and affinities of sugar binding sites present in the transporter complex. For these reasons, the D-glucose and cytochalasin B (CCB, a potent transport inhibitor) binding characteristics of purified GLUT1 were characterized. While the D-glucose binding capacities of GLUT1-DTT and GLUT1+DTT are indistinguishable, GLUT1-DTT presents at least two populations of binding sites to D-glucose and GLUT1+DTT presents only one population of sugar binding sites. The orientation of the binding sites can be investigated by using cytochalasin B, a ligand that binds only at (or very close to) the sugar efflux site. The cytochalasin B binding capacity of GLUT1-DTT (0.4 sites per GLUT1 monomer) is one half of GLUT1+DTT. Multiple D-glucose binding sites and one CCB binding site per dimer are properties consistent with a two-site transporter in which the binding sites of each monomer of the dimer are organized in an antiparallel fashion. In contrast, a single
D-glucose binding site and a cytochalasin B binding capacity of one mol of CCB per mol of GLUT1 is characteristic of a one-site transporter in which each monomer of the dimer is unrestrained and capable of independent isomerizations.

On closer examination, laboratories whose findings support the view that GLUT1 functions as a one-site transporter routinely purify GLUT1 in the presence of 10 mM DTT (Sogin and Hinkle 1980, Gorga and Lienhard 1982, Appleman and Lienhard 1989). Laboratories whose work supports the two-site transport mechanism, isolate GLUT1 in the absence of DTT (Carthers 1986a, Helgerson and Carthers 1987). The source of this controversy over transport mechanism thus appears to be related to the use of dithiothreitol during purification and, consequently the oligomeric structure of GLUT1.

Therefore, the following hypothesis can be proposed: GLUT1-DTT exists largely as a tetramer exhibiting two-site transport. Dithiothreitol treatment of GLUT1 converts tetrameric GLUT1 to dimeric GLUT1 which is characterized by one-site transport.

This hypothesis was further tested in erythrocyte membranes depleted of peripheral proteins. This hypothesis predicts that subsequent dithiothreitol treatment should: 1) double the cytochalasin B binding capacity of membrane-resident GLUT1; 2) result in the loss of multiple GLUT1 D-glucose binding sites and, 3) convert tetrameric GLUT1 to dimeric GLUT1.

The ligand binding predictions of this hypothesis were tested and satisfied, but only if dithiothreitol-treatment was carried out at high pH. The prediction of the conversion of tetrameric GLUT1 to dimeric GLUT1 was not tested by hydrodynamic or crosslinking studies. Rather, the specificity of ß-IgGs for tetrameric GLUT1 was exploited. ß-IgGs-specific protein A binding to depleted membranes was reduced (4.3 ± 0.9-fold) following membrane exposure to a second alkaline wash containing dithiothreitol. Binding was unaffected by treatment with dithiothreitol alone. Thus all three predictions are satisfied
but require dithiothreitol treatment in the presence of high pH. Since alkali-treatment of proteins disrupts salt bridges, these findings suggest that erythrocyte membrane-resident, tetrameric GLUT1 intramolecular disulfides are masked by intra- or intermolecular salt bridges.

In a collaborative effort, Pessino et. al. (1991) find that endogenous CHO cell GLUT1 is quantitatively co-immunoprecipitated by anti-GLUT4 C-terminal antibodies when CHO cells express a chimeric glucose transporter in which the 29 C-terminal amino acids of GLUT1 are replaced by the 30 C-terminal amino acids of GLUT4. These findings are consistent with GLUT1 existing as a homomultimer.

These findings are consistent with the model shown in Figure 22. GLUT1 can exist in two forms: as *Dimeric GLUT1* (obtained upon GLUT1 purification in the presence of dithiothreitol) and as *Tetrameric GLUT1* (erythrocyte-resident transporter and that form purified in the absence of dithiothreitol). Each monomer (subunit) is functional in both structures, exposing a single sugar binding site (shown as a cleft) to cytosol or to extracellular medium but never to both environments simultaneously.

*Dimeric GLUT1* contains unconstrained subunits. Each monomer is capable of independently isomerizing between influx (E2) and efflux (E1) states. Since cytochalasin B binds at the efflux site, each monomer can bind one molecule of cytochalasin B (binding of CCB will eventually arrest isomerization and freeze all GLUT1 monomers in the E1 state).

*Tetrameric GLUT1* is comprised of two dimers of GLUT1. These dimers differ from those of *dimeric GLUT1*. Conformationally active regions of each subunit are now constrained resulting in coupled isomerizations and an antiparallel arrangement of sugar binding sites. Thus each dimer of the tetramer presents one influx and one efflux site and, when one subunit isomerizes from an efflux to an influx conformation, the second must
Figure 22. Model of GLUT1 oligomeric structure and function. Proposed structures for dimeric (GLUT1+DTT) and tetrameric (GLUT1+DTT) GLUT1 are shown in schematic. Isomerizations of the minimal functional units of both structures are shown. Cytochalasin B binding results are modeled.
Sugar binding sites

Isomers of Monomeric GLUT1

Cytochalasin B (CCB)

Key

CCB is present when complexes formed

Isomerizations of units and functional structure

Transporter GLUT1

Dimeric GLUT1

Tetrameric GLUT1

J-Isomers of Monomeric GLUT1

Sugar binding sites

Functional units and isomerizations

Complexes

Cytochalasin B (CCB)
isomerize from an influx to an efflux conformation and vice versa. Each dimer thus exists in one of two possible states - E or F - each presenting one efflux and therefore one cytochalasin B binding site.

This model is consistent with data demonstrating that erythrocyte sugar transporter and GLUT1-DTT behave as two-site, allosteric transporters (Carruthers 1986a and 1986b, Helgerson and Carruthers 1987, Carruthers 1991, Carruthers and Helgerson 1991) while GLUT1+DTT functions as a simple, one-site transporter (Sogin and Hinkle 1980, Gorga and Lienhard 1982, Appleman and Lienhard 1989).

*Implications of this model and future directions*

The goal of the facilitative glucose transporter is to equilibrate the intracellular water with extracellular glucose. It is presently unknown whether tetrameric GLUT1 could accomplish this goal more efficiently than dimeric GLUT1. However, upon closer examination of one- and two-site transport mechanisms, some degree of informed speculation is possible. A one-site carrier sugar uptake cycle in the absence of intracellular sugar can be represented as:

\[
E_2 + S_o \xrightarrow{k_{-1}} E_2S_o \xrightarrow{k_0} E_1S_i \xrightarrow{k_{-1}} E_1 + S
\]

where E1 and E2 correspond to the components in Figure 22 and S_o and S_i refer to extracellular and intracellular sugars respectively. There are two rate-limiting steps in this mechanism: sugar translocation (\(k_{-1}\)) and the E1→E2 isomerization (\(k_0\); Widdas 1952, Appleman and Lienhard 1989, Carruthers 1991).
Sugar uptake cycles in the absence of intracellular sugar for the two-site carrier can be represented as:

\[ F + S_o \xrightarrow{k_{-1}} FS_o \xrightarrow{k_1} ES_i \xrightarrow{k_2} E + S_i \]

and

\[ E + S_o \xrightarrow{k_{-2}} ES_o \xrightarrow{k_2} S_iF \xrightarrow{k_1} F + S \]

where \( E \) and \( F \) correspond to the components in Figure 22. Only the sugar translocation steps (\( k_{-1} \) or \( k_2 \), Carruthers 1991) are rate-limiting in two-site transport. Both extra- and intracellular sugar are capable of binding to \( E \) and \( F \). For example, \( F \) binds sugar and translocates to the interior of the cell. This step is coupled to the immediate isomerization of \( E \rightarrow F \), a repriming of the transporter. Thus, the transporter is immediately ready for another round of uptake. The rate-limiting step of isomerization (\( k_0 \)) is thereby avoided.

The predicted transport properties of GLUT1+DTT at 10°C can be calculated from the studies of Appleman and Lienhard (1989) in which the catalytic turnover of GLUT1+DTT was examined. The duration of a single round of dimeric GLUT1-mediated sugar uptake (assuming no oxidation of dimeric to tetrameric occurred in the experiment) is 194 msec. \( k_0 \) or the unoccupied carrier isomerization step (\( E_1 \rightarrow E_2 \)) accounts for 192 msec of this time. Similarly for sugar efflux, the cycle is 114 msec and the isomerization step (\( E_2 \rightarrow E_1 \)) accounts for 111 msec of this time. Since tetrameric GLUT1 avoids the isomerization step, these results strongly suggest that dimeric GLUT1 is catalytically less efficient than erythrocyte-resident, tetrameric GLUT1 when sugar is absent at the opposite side of the membrane. This conclusion is supported by the studies of Lowe and Walmsley (1986) in which red cell (GLUT1-DTT)-mediated sugar transport at 10°C occurs at rates 10-fold greater than those predicted from the studies of GLUT1+DTT (Appleman and Lienhard, 1989). To further these studies, more extensive analyses of
reconstituted GLUT1±DDT are needed. The reconstitution experiment of Figure 15 lack sufficient resolution to provide suitable information.

Does the conversion of dimeric to tetrameric GLUT1 occur physiologically? The calculations above suggest that this conversion could produce an activated GLUT1 and possibly be a mode of regulation. Recall from Figure 8, the putative location of the 6 cysteine residues. The cysteine residues are located within the proposed transmembrane region or adjacent to it. Small conformational changes in GLUT1 could readily change the topographical location and redox environment of a cysteine residue. For example, exposure of a free sulphydryl (normally present in the membrane) to the extracellular, oxidizing environment may cause tetramer production, or *vice versa*.

In addition, the intracellular reducing environment is largely controlled by a redox buffer of reduced glutathione (GSH) and oxidized glutathione (GSSG). The redox potential of this environment is controlled in part by a glutathione transporter in erythrocytes (Srivastava and Beutler, 1969) which transports oxidized glutathione out of the cell in an ATP-dependent fashion (Kondo et. al., 1980). ATP inhibits glucose transporter in erythrocytes (Hebert and Carruthers, 1986), by direct association with GLUT1 (Carruthers and Helgerson, 1988). ATP may also inhibit glucose transport by formation of dimeric transporters- an effect resulting from an increased intracellular reducing environment. Of course, this mechanism is highly speculative. However, it could be investigated by GLUT1 reconstitution studies with varying intra- and extracellular redox buffers. In addition, an *in vivo* oligomeric structure assay could be developed employing complementary fluorescent probes in fluorescent resonance energy transfer studies (FRET). Fluorescence would be monitored in the presence of varying ATP levels and redox buffers.
Hurtley and Helenius (1989) define an oligomeric membrane protein by its ability to fulfill one or more of four operational criteria: “(a) the main function and/or stability critically depends on the quaternary structure, (b) intracellular transport requires oligomerization, (c) isolation using various methods routinely yields an oligomeric complex, and (d) the subunits are permanently associated, and are handled and degraded by the cell as a unit.” It is apparent by the finding of this study that both criteria (a) and (c) are satisfied. However, criteria (b) and (d) focus on an area yet to be investigated for GLUT1.

Plasmal membrane proteins fold and are assembled into their oligomeric structure in the lumen of the endoplasmic reticulum (Hurtley and Helenius, 1989). Correct assembly is required for transport from the endoplasmic reticulum to the Golgi. A “quality control” network is responsible for retaining incorrectly folded and/or assembled proteins (Hurley and Helenius, 1989) in the endoplasmic reticulum.

This area has strong potential for assisting in our understanding of glucose transport and its role in disease. Large, complex and hydrophobic proteins such as GLUT1 are excellent candidates for proteins which fold in a protein assisted manner (Rothman, 1989). As hydrophobic proteins are translated and translocated into the lumen of the endoplasmic reticulum (ER), co-translational folding occurs. However, it is becoming increasingly apparent that these newly exposed hydrophobic sequences associate with folding proteins. This disrupts the premature occurrence of nonspecific hydrophobic interactions, before the portion of the protein needed for correct folding can emerge. In addition, N-terminal glycosylations are proposed to play a role in these disruptions (Rose and Doms, 1988). GLUT1 is glycosylated at asparagine-45. The correctly folded protein assembles into oligomers and is transported.

Preliminary observations with glucose transporter isotypes support the importance of correct ER folding and assembly. GLUT1 is commonly overexpressed in cell lines
possessing endogenous GLUT1 (CHO and 3T3L1 fibroblasts, Harrison et al., 1991). However, overexpression of GLUT4 in the same cell lines (CHO and 3T3L1 fibroblasts) which lack endogenous GLUT4 is unsuccessful (Woon and Czech, unpublished). The protein appears to be translated and retained in a perinuclear membrane believed to be the ER (Mueckler, unpublished). Interestingly, chimeric glucose transporters possessing the N-terminal 199 residues of GLUT1 and the remaining COOH-terminal residues of GLUT4 are overexpressed 2 to 6-fold in these cell lines (Pessino et al., 1991). These results are consistent with the requirement of isotype-specific folding or assembly proteins which associate with the N-terminal sequences. An emerging powerful technique to study in vivo protein folding and assembly is to combine a pulse-chase with radiolabeled amino acids with immunoprecipitations of proteins using conformation-specific monoclonal antibodies (antigenic towards folding and assembly intermediates).

The mode and topology of interactions which are involved in forming and maintaining oligomeric associations in GLUT1 remain to be determined. Evidence exists for the occurrence of interactions in all three topological domains of membrane proteins: ectodomain for influenza hemagglutinin (Boulay et al., 1988), transmembrane region for chicken asialoglycoprotein receptors (Loeb and Drickamer, 1987), and cytosolic domains for formation and stability of LDL receptor dimers (van Driel et al., 1987).

The dithiothreitol sensitivity of GLUT1 oligomeric structure suggest that the disulfide bonds of cysteine residues play a critical role in oligomeric associations. To determine the extent of this role, the six cysteine residues can be mutated separately and in various combinations. The resulting oligomeric structures can then be determined as discussed earlier. Leucine/isoleucine zippers have been found recently to be involved in oligomeric association (Landschulz et al., 1988). Interestingly, leucine/isoleucine zipper motifs are found in transmembrane regions 2 and 3 (White and Weber, 1989). The importance of these motifs to GLUT1 structure/function remains to be determined.
The topology of interactions could be investigated by proteolysis studies. GLUT1 would be photolabelled and subjected to various proteases at both sides of the membrane, separately and together. Nondenaturing size-exclusion chromatography would be employed to determine the effects of these treatments on oligomeric structure.

The fundamental problem of GLUT1 oligomeric structure was investigated in this thesis. The elucidation of the oligomeric structure has revealed new insight into the relationship between quaternary structure and function. The oligomeric structure of GLUT1 controls the mechanism of glucose transporter and possibly the catalytic efficiency of the protein. It is hoped that this new understanding of GLUT1 oligomeric structure and its relationship to GLUT1 function will aid in understanding the physiology of the glucose transporter.
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