Analysis of Transmembrane Segment 8 of the Glut1 Glucose Transporter by Cysteine Scanning Mutagenesis and Substituted Cysteine Accessibility *

Running Title: Transmembrane Segment 8 of the Glut1 Glucose Transporter

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The Abbreviations used are: pCMBS, p-chloromercuribenzenesulfonate; C-less, Glut1 molecule in which all 6 native cysteine residues were changed to either glycine or serine, single-C, a Glut1 mutant constructed using the C-less parent in which a single cysteine mutation was introduced in place of one the transmembrane residues.
SUMMARY

The Glut1 glucose transporter has been proposed to form an aqueous substrate translocation pathway via the clustering of several amphipathic transmembrane helices (Mueckler, M., Caruso, C., Baldwin, S. A., Panico, M., Blench, I., Morris, H. R., Allard, W. J., Lienhard, G. E., and Lodish, H. F. (1985) Science 229, 941-945). The possible role of transmembrane helix 8 in the formation of this permeation pathway was investigated using cysteine-scanning mutagenesis and the membrane-impermeant, sulfhydryl-specific reagent, p-chloromercuribenzenesulfonate (pCMBS). Twenty-one Glut1 mutants were created from a fully functional, cysteine-less, parental Glut1 molecule by successively changing each residue along transmembrane segment 8 to a cysteine. The mutant proteins were then expressed in Xenopus oocytes and their membrane concentrations, 2-deoxyglucose uptake activities, and sensitivities to pCMBS were determined. Four positions within helix 8, alanine-309, threonine-310, serine-313, and glycine-314, were accessible to pCMBS as judged by the inhibition of transport activity. All 4 of these residues are clustered along one face of a putative α-helix. These results suggest that transmembrane segment 8 of Glut1 forms part of the sugar permeation pathway. Updated 2-dimensional models for the orientation of the 12 transmembrane helices and the conformation of the exofacial glucose-binding site of Glut1 are proposed that are consistent with existing
experimental data and homology modeling based on the crystal structures of two bacterial membrane transporters.

INTRODUCTION

Passive transport of glucose across the plasma membrane of mammalian cells is mediated by members of the Glut (SLC2a) family of membrane glycoproteins [reviewed in (1-3)]. The Glut protein family belongs to the Major Facilitator superfamily, the largest superfamily of proteins that function as membrane transporters (4). Glut1, also known as the red cell glucose transporter, is perhaps the most extensively studied of all membrane transporters (5). Kinetic analyses of glucose transport in the human red blood cell are mostly consistent with a simple alternating conformation mechanism (6). Although kinetic anomalies have been observed in human erythrocytes that appear to be inconsistent with this simple mechanism (7,8), an alternate explanation is that the anomalies may be the result of difficulties in accurately measuring steady-state kinetic properties in the red cell.

Glut1 was predicted to possess 12 transmembrane helices based on hydrophobicity analysis of the deduced amino acid sequence (9). This prediction has been confirmed by glycosylation-scanning mutagenesis (10). Several of the twelve proposed transmembrane segments are predicted to form amphipathic α-helices, an observation which led to the hypothesis that these helices form the walls of a water-filled chamber through which glucose
permeates the lipid bilayer (9). It was also proposed that hydroxyl- and amide-containing amino acid side chains within these helices form the sugar-binding site(s) of Glut1 via hydrogen bond formation with glucose hydroxyl groups, although hydrophobic interactions between aromatic amino acid side chains and the C-6 region of glucose also appear to be important (11).

Considerable experimental support for this structural model has accumulated. Cysteine-scanning mutagenesis and substituted cysteine accessibility studies implicate transmembrane segments 2 (12), 5 (13), 7 (12,14), 10 (15), and 11 (16) of Glut1 in the formation of a water-accessible cleft within the membrane. Glutamine-161 within helix 5 (17) and glutamine-282 within helix 7 (18) appear to participate in forming the exofacial substrate-binding site. Valine-165, which is positioned one helical turn distant from glutamine-161, is accessible to aqueous sulfhydryl reagents and appears to be near the exofacial substrate binding site based on mutagenesis and inhibitor studies (19). An aromatic side-chain at position 412 within helix 11 appears to be essential for transport activity (20).

Finally, hydrogen exchange studies demonstrate that 30% of peptide hydrogen atoms are exposed to water in purified, reconstituted Glut1, consistent with the presence of an aqueous cleft in the membrane (21).

In this study cysteine-scanning mutagenesis was used in conjunction with a sulfhydryl-specific chemical reagent to examine the possible role of transmembrane segment 8 in the formation the Glut1 substrate
translocation pathway. Our results suggest that transmembrane segment 8 is an amphipathic α-helix with a water-accessible face that lines the exofacial portion of the sugar permeation pathway.

EXPERIMENTAL PROCEDURES

Materials— *Xenopus laevis* imported African frogs were purchased from Xenopus Express (Homosassa, FL), 3H-2-deoxyglucose and Diguanosine triphosphate (mRNA cap) were purchased from Amersham Pharmacia Biotech (Arlington Heights, IL), Megascript™ RNA synthesis kit was purchased from Ambion Inc (Austin, TX), Transformer™ Site-Directed mutagenesis kit was obtained from Clontech (Paolo Alto, CA).

General Procedures— Procedures for the site-directed mutagenesis and sequencing of human Glut1 cDNA and the in vitro transcription and purification of Glut1 mRNAs (22), isolation, microinjection, and incubation of *Xenopus* oocytes (23), preparation of purified oocyte plasma membranes and indirect immunofluorescence laser confocal microscopy (20), SDS polyacrylamide gel electrophoresis and immunoblotting with Glut1 C-terminal antibody (17), and 2-deoxyglucose uptake measurements (24), have been described in detail previously.
Treatment with pCMBS—Stage 5 Xenopus oocytes were injected with 50 ng of wild-type or mutant Glut1 mRNA. Two days after injection, groups of ~20 oocytes were incubated for 15 min in the presence or absence of the indicated concentrations of p-chloromercurisulfonate (pCMBS), in Barth’s saline at 22°C. The 100x concentrated reagent stock was prepared in 100% dimethylsulfoxide and control oocytes were treated with the appropriate concentration of vehicle alone. After a 15 min incubation period, the oocytes were washed 4x in Barth’s saline and then used for the determination of [³H]-2-deoxyglucose uptake (50 µM, 30 min at 22 °C).

Specific Activity Determinations—Plasma membranes were prepared 3 days following injection of 50 ng of mutant RNA per oocyte. Western blot analysis of each of the mutant transporters was performed on ~1 µg of total membrane protein and the intensity of the glycosylated Glut1 band was quantified by scanning densitometry using a Molecular Dynamics Phosphorimager SI. Analysis was performed using the ImageQuant NT program (Version 4.0). [³H]-2-DOG uptake (pmol/oocyte/30 minutes) of each mutant was concomitantly determined in each set of experiments. Specific activity is expressed as the 2-deoxyglucose uptake per ng of mutant Glut1 protein expressed per µg of total oocyte membrane protein. Purified human erythrocyte membranes were loaded on the same gels as the oocyte membrane samples for use as a quantitative standard.
Statistical Analysis- Uptake data were analyzed for statistical significance using the two-tailed, unpaired Student T-test.

RESULTS

We previously described the properties of a cysteine-less (C-less) human Glut1 polypeptide in which all six native cysteine residues were changed to either serine or glycine residues (19). When expressed in Xenopus oocytes the C-less transporter exhibits transport activity nearly indistinguishable from wild-type Glut1 (19,25), indicating that none of the native cysteine residues plays an essential role in transport function. C-less Glut1 cDNA was used as a template to construct single-C mutants for transmembrane segment 8. Mutant cDNAs were constructed using oligonucleotide-mediated site-directed mutagenesis in which each of the 21 residues within transmembrane segment 8 was individually changed to a cysteine residue, producing 21 mutant Glut1 molecules, each possessing only a single cysteine residue (see Table 1).

Expression of the single-C mutants in the oocyte plasma membrane was confirmed by indirect immunofluorescence laser confocal microscopy (Figure 1a) and quantitated by western blot analysis of purified oocyte membranes (Figure 1b). As we have observed for the analysis of other Glut1
helices, the single-C mutants were expressed at varying concentrations in the oocyte plasma membrane, necessitating the normalization of uptake data to expression levels in order to directly compare the catalytic activities of the mutants to the C-less parent. The expression of 20 of the 21 single-C mutants was readily detected in oocytes, but the V316C mutant was expressed at levels too low to enable further analysis.

Transport activity above the very low endogenous oocyte background level was detectable for all 20 mutants as determined by uptake of $[^3H]$-2-deoxyglucose. The absolute uptake data are shown in Figure 2a and the specific transport activities, normalized to the plasma membrane content of each mutant are presented in Figure 2b. Cysteine substitution at threonine-310, and asparagine-317, and threonine-318 significantly reduced specific transport activity relative to the C-less parent, whereas cysteine substitution at isoleucine-311 increased specific transport activity.

In order to determine which transmembrane residues are accessible to the external aqueous solvent and may therefore comprise part of the sugar permeation pathway, transport activity was measured for each of the 21 mutants after incubation in the presence of the membrane-impermeant sulphydryl-specific reagent, p-chloromercuribenzenesulfonate (pCMBS) (Figure 3). We have previously demonstrated that pCMBS can permeate the glucose permeation pathway of Glut1 and has close access to the exofacial sugar-binding site (19). Figure 3 presents the transport activities observed in
the presence of pCMBS normalized for each mutant to the activity measured in the absence of the reagent, i.e., a value of 1 indicates no effect of pCMBS, values greater than 1 indicate stimulation by pCMBS, and values less than 1 indicate inhibition by pCMBS. The activity of four single-C mutants (A309C, T310C, S313C, G314C) was significantly inhibited after incubation with pCMBS, indicating that the corresponding amino acid side chains reacted with the pCMBS and therefore must be accessible to the external aqueous solvent. V165C represents a well-characterized positive control for inhibition by pCMBS (13,19).

Discussion

Helical wheel analysis of the results of the pCMBS inhibition experiments revealed that the 4 residues accessible to pCMBS from the external aqueous solvent are clustered together along one face of a putative α-helix formed by transmembrane segment 8 (see Figure 4). These results are similar to those obtained with helices 2 (12), 5 (13), 10 (15) and 11 (16). Helix 7 appears to be unique in that it contains residues sensitive to pCMBS along its entire circumference, implying that its N-terminal half is completely immersed in solvent (14). Not unexpectedly, the 4 residues within helix 8 that are clearly reactive to pCMBS all lie within the exoplasmic half of the helix, a result similar to that observed with helices 1, 2, 5, 7, 10, and 11, all of
which possess residues close to the exoplasmic face that are accessible to pCMBS present in the external solvent.

Cysteine substitution at 3 positions (threonine-310, asparagine-317, and threonine-318) caused significant reductions in the intrinsic transport activity of Glut1. The side chains of threonine-310 and asparagine-317 are predicted to lie within the aqueous translocation pathway based on the presumed orientation of the helix according to pCMBS sensitivity, suggesting that these residues may be directly involved in hydrogen bonding to glucose lying in the exofacial binding pocket. However, pCMBS reactivity only inhibited transport at position 310, inconsistent with hydrogen bonding between glucose and the side chain of asparagine-317. Because asparagine-317 lies further towards the cytoplasmic face of the membrane than any of the amino acid positions that were sensitive to pCMBS reactivity, it is possible that helix 8 twists as it passes through the membrane such that asparagine-317 actually abuts an adjacent helix (see Fig. 5a). The serine side chain of this residue may be involved in stabilizing helical packing via hydrogen bonding to a residue in an adjacent helix (5 or 10). Likewise, cysteine substitution at threonine-318 may disrupt hydrogen bonding between the hydroxyl group of the threonine side chain and a side chain from an abutting helix. Interestingly, cysteine substitution at isoleucine-311 significantly increased specific transport activity. This residue is predicted to either be in contact with lipid or to abut helix 5 (see Fig. 5b). It is possible that the bulky
isoleucine side chain at this position slightly impairs the movement of helices 5 and 8 associated with the transporter cycle, and that this is reflected by increased activity observed upon substitution with the much more compact cysteine side chain.

A major breakthrough in our understanding of the structure of the Major Facilitator transporters was reported recently in the form of X-ray crystallographic data at near atomic resolution for the E. coli lac permease (26) and the glycerol-3-P antiporter (27). Both molecules were crystallized in their cytoplasmic-facing orientations, and in this form, the molecules exhibit a very similar basic folding pattern with a pseudo-two-fold axis of symmetry. The data for these two members of the Major Facilitator Superfamily is consistent with the 12-transmembrane helical model that was first proposed for Glut1 (9). The pseudo-symmetry also supports the notion that the 12 transmembrane transporter genes are descended from a common 6 transmembrane-encoding ancestral gene that underwent a duplication event. Based on the crystallographic data, 8 of the 12 transmembrane helices form a central cavity containing the cytoplasmic substrate-binding site. Remarkably, the structures suggest analogous mechanisms for cotransport and antiport, and, by inference, simple uniport, involving the tilting of helices such that substrate-binding sites are alternately exposed to either the cytoplasm or exoplasm. An alternating conformation mechanism of this type was originally postulated by Vidaver (28) nearly four decades ago based
purely on kinetic considerations, and inhibitor (29) and spectroscopic studies (30,31) on the red cell glucose transporter have strongly supported such a mechanism.

All of the cysteine accessibility experiments on Glut1 employing pCMBS to detect solvent-accessible helical faces pertain to the exofacial conformation of the transporter, and thus the available crystallographic data for the lac permease and the glycerol-3-P antiporter may not be directly applicable to these Glut1 data. However, it is likely that Glut1 shares the same basic helical packing arrangement with these two bacterial transporters, and that this arrangement is maintained during the transport cycle, i.e., major shifts in the relative arrangement of helices in the plane of the membrane do not occur. Given this assumption, we can use homology modeling to update our crude 2-dimensional model for the exofacial substrate-binding site of Glut1.

Helices 2 (12), 5 (13), 8 (this report), 10 (15), and 11 (16) of Glut1 all have a single, discrete solvent-accessible face as defined by substituted cysteine accessibility analysis, whereas helices 1 (K. Keller, personal communication) and 7 (14) have solvent accessible residues along their entire cross-sectional perimeter (14). These experimental observations are completely consistent with the helical packing of the lac permease (26) and glycerol-3-P antiporter (27) in their cytoplasmic-facing orientations, suggesting that the transmembrane helices do not rotate substantially in the
plane of the membrane during conversion to the outward-facing form. The central solvent-accessible cavity is proposed to be formed by helices 1, 2, 4, and 5 in the N-terminal half of the molecule, and by the analogous helices, 7, 8, 10, and 11 in the C-terminal half. A ribbon diagram of the transmembrane helices as shown from the cytoplasmic face of the membrane and in the cytoplasmic-facing orientation, based on homology modeling using the lac permease structure as a template, is shown in Figure 5a. A putative model of the exofacial binding pocket is shown in Figure 5b. Residues within helices 5, 7, and 11 have been implicated in exofacial substrate binding [reviewed in (32)]. Glucose is known to be stabilized in the exofacial binding pocket via interactions between Glut1 and the hydroxyls at positions 1, 3, and 4 (11). The orientation of helices 5 and 7 in the model is consistent with hydrogen bond formation between hydroxyl groups of a dehydrated glucose molecule lying in the exofacial-binding pocket and two residues directly implicated in substrate binding, glutamine-161 (17) and glutamine-282 (18). Glutamine-282 appears to interact directly with the glucose C-1 hydroxyl group (33). The orientation of helix 5 is also consistent with the observation that valine-165 lies near the outer vestibule of the exofacial substrate binding site, although this residue itself is not directly involved in transport activity (19). Site-directed mutagenesis studies have demonstrated that side-chains bulkier than valine are not tolerated at position 165, whereas smaller side-chains are tolerated. The orientation of valine-165 in the model is consistent with bulky
substitutions at this position interfering with hydrogen bond formation
between glucose and glutamine-161. The low transport activity of the T310C
and N317 mutants along with their orientation along the solvent-exposed face
of helix 8 suggest that the side chains of these residues may be involved in
hydrogen bond formation with glucose in the exofacial substrate-binding site.
The orientation of helix 11 is consistent with a hydrophobic interaction
between the C-6 region of glucose and tryptophan-412. A hydrophobic
interaction, possibly a stacking interaction involving an aromatic ring of
Glut1 and the C-6 region of glucose, was predicted by Barnett and colleagues
(11) based on transport studies employing substituted glucose analogs. Site-
directed mutagenesis studies have shown that a tryptophan at position 412 is
critical for transport activity (20), and additional studies indicate that an
aromatic ring is essential at this position (M. Mueckler, unpublished
observations).

Determination of the structure of Glut1 in the outward-facing
configuration awaits the crystallization of the protein, a feat that has not yet
been achieved for any eukaryotic membrane protein. Although the recent
successes with the bacterial transporters are encouraging, the problems faced
with eukaryotic membrane proteins, including structural heterogeneity due to
posttranslational modifications and the lack of robust overexpression
systems, are extraordinarily daunting. In the interim, various biochemical
approaches can be used to test the applicability of homology modeling based
on the structures of the lac permease and glycerol-3-P antiporter.
Cysteine Scanning Mutagenesis of Helix 8

cDNA encoding cysteine-less human Glut1 was subjected to oligonucleotide-mediated, site-directed mutagenesis, creating a series of 21 mutant cDNAs in which each of the 21 residues within transmembrane helix 8 was individually changed to cysteine. Residue # refers to the amino acid numbering for human Glut1 given in reference (9). Amino acids are designated by the single-letter code.

| Residue # | Amino Acid Change | Codon Change |
|-----------|-------------------|--------------|
| 307       | Val → Cys         | GTG → TGC    |
| 308       | Tyr → Cys         | TAT → TGT    |
| 309       | Ala → Cys         | GCC → TGC    |
| 310       | Thr → Cys         | ACC → TGC    |
| 311       | Ile → Cys         | ATT → TGT    |
| 312       | Gly → Cys         | GGC → TGC    |
| 313       | Ser → Cys         | TCC → TGC    |
| 314       | Gly → Cys         | GGT → TGT    |
| 315       | Ile → Cys         | ATC → TGC    |
| 316       | Val → Cys         | GTC → TGC    |
| 317       | Asn → Cys         | AAC → TGC    |
| 318       | Thr → Cys         | ACG → TGC    |
| 319       | Ala → Cys         | GCC → TGC    |
| 320       | Phe → Cys         | TTC → TGC    |
| 321       | Thr → Cys         | ACT → TGC    |
| 322       | Val → Cys         | GTC → TGC    |
| 323       | Val → Cys         | GTG → TGC    |
| 324       | Ser → Cys         | TCG → TGC    |
| 325       | Leu → Cys         | CTG → TGC    |
| 326       | Phe → Cys         | TTT → TGT    |
| 327       | Val → Cys         | GTG → TGC    |
FIGURE LEGENDS

FIG. 1. **Expression of helix 8 single-C Glut1 transporter proteins in Xenopus oocytes.** Stage 5 *Xenopus* oocytes were injected with 50 ng of wild-type, C-less, or mutant C-less mRNAs and two days later frozen sections were prepared and analyzed by indirect immunofluorescence laser confocal microscopy or oocytes were used to prepare purified plasma membrane fractions for immunoblot analysis. a) Confocal Micrographs of oocytes expressing each of the 21 single-C mutants; b) Immunoblot: 10 µg of total oocyte membrane protein were loaded per lane. Rabbit antiserum A674 raised against the C-terminal 15 residues of human Glut1 was used at 1:500 dilution. Numbers above the lanes on the right represent the quantity of human erythrocyte Glut1 loaded in each lane as a quantitative standard.

FIG. 2. **2-Deoxyglucose uptake activity of helix 8 single-C mutants.** [³H]-2-deoxyglucose uptake (50 µM, 30 min at 22 °C) and the plasma membrane content of each single-C mutant were quantitated 2 days after injection of mRNAs. Results represent the mean ± SE of 5-10 independent experiments, each experiment using 15-20 oocytes per experimental group. a) raw uptake data; b) the data are normalized per ng of each mutant protein expressed per µg of total oocyte membrane protein. Background values observed in sham-
injected oocytes were subtracted prior to normalization. *, p < 0.05 for single-C mutant compared to parental C-less Glut1. ND, not determined.

FIG. 3. Effect of pCMBS on transport activity of helix 8 single-C mutants. Three days after injection of mRNAs, groups of 15-20 oocytes were incubated in the presence or absence of 0.5 mM pCMBS in Barth’s saline at 22 °C for 15 min. Oocytes were washed 4x in Barth’s saline and then subjected to 2-deoxyglucose uptake measurements under the conditions described in the legend to Fig. 2. Results represent the mean ± SE of 5-10 independent experiments, each experiment using 15-20 oocytes per experimental group. Data are expressed as relative uptake activity, i.e., uptake observed in the presence of pCMBS divided by the uptake observed in the absence of pCMBS. C-less represents the parental cysteine-less Glut1 construct. V165C is a well-characterized positive control whose activity is inhibited by pCMBS (19). *, p < 0.01 for 2-deoxyglucose uptake in the presence versus absence of pCMBS. ND, not determined.

FIG. 4. Helical wheel representation of helix 8. Transmembrane helix 8 of Glut1 as viewed from the exoplasmic surface of the plasma membrane. Amino acids are represented by the single letter code. Arrows point to residues that are accessible to pCMBS from the external solvent.
FIG. 5. 2-Dimensional models for the arrangement of the 12 transmembrane helices of Glut1 and for the exofacial sugar-binding site.  a) Theoretical arrangement of the 12 putative transmembrane helices of Glut1 in the cytoplasmic-facing conformation based on homology modeling using the E. coli lac permease as the template (26).  b) Proposed low-resolution model of the exofacial glucose-binding site. The model is consistent with numerous experimental observations (see text). Glucose is not drawn to scale. The dotted lines represent possible hydrogen bonds formed between glucose hydroxyl groups and various side chains on Glut1. Numbered residues are accessible to pCMBS from the external solvent. See text for detailed discussion.
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Figure 1a
Figure 1b
Figure 2a
Specific Activity (pMoles/oocyte/30 min./ng protein)

Figure 2b
Figure 3
Figure 4
Figure 5b
Analysis of transmembrane segment 8 of the glut1 glucose transporter by cysteine scanning mutagenesis and substituted cysteine accessibility
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