Cysteine-scanning Mutagenesis of Transmembrane Segment 7 of the GLUT1 Glucose Transporter*

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The human erythrocyte facilitative glucose transporter (Glut1) is predicted to contain 12 transmembrane α-helices based upon hydropathy plot analysis of the primary sequence. Five of these helices (3, 5, 7, 8, and 11) are capable of forming amphipathic structures. A model of GLUT1 tertiary structure has therefore been proposed in which the hydrophilic faces of several amphipathic helices are arranged to form a central aqueous channel through which glucose traverses the hydrophobic lipid bilayer. In order to test this model, we individually mutated each of the amino acid residues in transmembrane segment 7 to cysteine in an engineered GLUT1 molecule devoid of all native cysteines (C-less). Measurement of 2-deoxyglucose uptake in a Xenopus oocyte expression system revealed that nearly all of these mutants retain measurable transport activity. Over one-half of the cysteine mutants had significantly reduced specific activity relative to the C-less protein. The solvent accessibility and relative orientation of the residues within the helix was investigated by determining the sensitivity of the mutant transporters to inhibition by the sulfhydryl directed reagent p-chloromercuribenzenesulfonate (pCMBS). Cysteine replacement at six positions (Gln282, Gln283, Ile287, Ala289, Val290, and ribenzene sulfonate (pCMBS). Cysteine replacement at five positions (Gln282, Gln283, Ile287, Ala289, Val290, and Phe294), all near the exofacial side of the cell membrane, produced transporters that were inhibited by incubation with extracellular pCMBS. Residues predicted to be near the cytoplasmic side of the cell membrane were minimally affected by pCMBS. These data demonstrate that the exofacial portion of transmembrane segment 7 is accessible to the external solvent and provide evidence for the positioning of this α-helix within the glucose permeation pathway.

The facilitative transport of glucose across mammalian cell membranes is mediated by a family of at least four highly homologous 50–60-kDa transmembrane glycoproteins (1, 2). Glut1, the prototype member of this family, has been the most extensively studied Glut protein because of its relative abundance within the erythrocyte cell membrane. It remains the only glucose transporter to have been purified and reconstituted into lipid vesicles in a functional form (3). The kinetics of glucose transport within the erythrocyte membrane have been extensively studied (4). Several amino acid residues that likely play crucial roles in glucose binding and/or transport have been identified from affinity labeling studies and limited site-directed mutagenesis (5–7). The tertiary structure of the Glut proteins, however, remains poorly characterized.

A 12-transmembrane α-helical model for the glucose transporters was first proposed by Mueckler et al. (8) in 1985 based upon hydropathy plot analysis of the primary sequence. Fourier transform infrared spectroscopy and circular dichroism data determined that the protein is largely α-helical with the helices perpendicularly arranged relative to the lipid bilayer, in agreement with this model (9, 10). This structure has been confirmed by enzymatic (11), immunologic (12), and glycosylation-scanning mutagenesis analysis (13). Hydrogen exchange experiments have revealed that 80% of the polypeptide backbone is solvent accessible, suggesting a pore-like structure (9). Recognition that five of the putative transmembrane helices (numbers 3, 5, 7, 8, and 11) are capable of forming amphipathic helices has led to the hypothesis that these helices are arranged together to form an aqueous pathway that the glucose molecule traverses through the cell membrane (8). Molecular modeling of Glut1 has suggested that at least 5 transmembrane α-helices would be required to form an aqueous channel of sufficient size to accommodate the glucose moiety (14).

Support for the participation of TM segment 5 in forming part of this aqueous channel was recently obtained using cysteine-scanning mutagenesis. The sensitivity of Glut1 to mutations of Gln282 (5) and Tyr293 (16) in TM segment 7 suggested that this helix may also comprise part of this glucose permeation pathway. In order to investigate the functional importance, solvent accessibility, and relative orientation of helix 7 with respect to this aqueous glucose channel, we initiated a systematic study of each TM amino acid by performing cysteine-scanning mutagenesis and measured the sensitivity of each of these mutants to modification by the sulfhydryl-directed reagent pCMBS. We report here evidence that the exofacial portion of TM segment 7 in GLUT1 contains solvent accessible residues that may be positioned within or near the glucose permeation pathway.

EXPERIMENTAL PROCEDURES

Materials—Xenopus laevis imported African frogs were purchased from Xenopus Express (Homosassa, FL). [3H]2-deoxyglucose was obtained from Sigma. Diguanosine triphosphate (mRNA cap) was purchased from Amersham Pharmacia Biotech, Megascript® RNA synthesis kit was purchased from Ambion Inc. (Austin, TX), Transformer® Site-directed mutagenesis kit was obtained from CLONTECH (Palo Alto, CA).

General Procedures—Mutant transporters were constructed using the Transformer® Site-directed mutagenesis kit as described previously (17). Procedures for the synthesis and purification of mutant RNA (18), isolation, microinjection, and incubation of Xenopus oocytes (19), preparation of total oocyte membranes, and laser confocal microscopy (7), SDS-polyacrylamide gel electrophoresis and immunoblotting with Glut1 C-terminal antibody (6), and 2-deoxyglucose uptake measure-
Cysteine scanning mutagenesis of helix 7

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<tr>
<th>Residue</th>
<th>Amino acid change</th>
<th>Mutagenic oligonucleotide</th>
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The treatment with p-Chloromercuribenzene sulphonate (pCMBS) at 22 °C. The 100 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 2-DOG, 2-deoxyglucose; TM, transmembrane; C-less, Glut1 in which all 6 native cysteine residues were mutated bases underlined. Mutated codons within each oligonucleotide are shown in bold with the residue refers to the amino acid numbering for human GLUT1 given in Ref. 8. Amino acid residues are designated by their three-letter code. Mutated codons within each oligonucleotide are shown in bold with the numbered bases.

Results

Construction of Cysteine Mutants and Expression in Xenopus Oocytes—Using a previously constructed Glut1 molecule devoid of all native cysteines (C-less) (20), we individually replaced each of the amino acid residues within putative transmembrane helix 7 of human Glut1 with cysteine. The specific nucleotide substitutions made and the resulting amino acid changes are shown in Table I. The C-less transporter has been previously shown to facilitate 2-deoxyglucose (2-DOG) uptake at levels comparable to the wild-type protein (21) demonstrating that none of the native cysteines are required for transport.

FIG. 1. 2-Deoxyglucose uptake activity of GLUT1 cysteine mutants. [3H]2-Deoxyglucose uptake (50 μM, 30 min at 22 °C) was measured 2 days after injection of 50 ng of C-less or mutant mRNA into stage 5 Xenopus oocytes. Results represent the mean ± S.E. of three to five independent experiments, each using 10–20 oocytes/experimental group. C-less transporter, cysteine substitution of six amino acids resulted in a greater than 75% reduction in 2-DOG uptake. Five of these mutants were found within the exofacial portion of the membrane

Estimation of Mutant Specific Activities—To investigate whether the observed differences in transport activity were due to changes in the intrinsic activity of the mutant proteins or simply a reflection of total protein expression, we first measured the amount of Glut1 protein detected by Western blot analysis of total membrane preparations (Fig. 2). In the majority of mutants, diminished 2-DOG uptake activity relative to the C-less protein correlated with decreased steady state protein expression. Since the introduced mutations could affect packaging and/or transport of these proteins to the cell membrane in addition to their effect on steady state protein levels, we investigated the subcellular localization of the mutants by laser confocal microscopy (Fig. 3).

TABLE I

The abbreviations used are: pCMBS, p-chloromercuribenzenesulfonate; C-less, Glut1 in which all 6 native cysteine residues were changed to either glycine or serine; 2-DOG, 2-deoxyglucose; TM, transmembrane; PM, plasma membrane.
Glut1 is typically seen as two distinct bands between 45 and 55 kDa (Fig. 2) when expressed in Xenopus oocytes, due to differences in glycosylation at Asn 45. Treatment of membrane preparations with \( \text{N} \)-glycanase and/or expression of Glut1 with a mutation in the glycosylation site in the exofacial loop between TM segments 1 and 2 results in the detection of a single band on Western blot analysis (22). The lower band corresponds to the core glycosylated, high mannose containing transporter present in early biosynthetic compartments. The upper band correlates with the mobility of purified PM Glut1 fractions and represents the fully processed, complex \( \text{N} \)-glycosylated protein (19). The upper band is therefore a reflection of the functional transporter within the PM. Quantification of this upper Western blot band by scanning densitometry allowed estimation of specific activities for each of the individual cysteine mutants (Fig. 4).

Over one-half of the cysteine mutants had specific activities significantly less than the C-less transporter. Not unexpectedly, all of the amino acid residues that are absolutely conserved between all of the mammalian glucose transporters (Leu278, Gln282, Gln283, Gly286, Asn288, and Tyr292) had significantly diminished specific activities following mutation to cysteine. In agreement with the purported role of Gln282 in exofacial glucose binding (5), mutation of this residue to cysteine resulted in a greater than 75% reduction in 2-DOG transport activity. On the other hand, the dramatic effect of cysteine substitution at Asn288 contrasts with the negligible effect on 2-DOG transport reported following mutation to Ile (5).

**pCMBS Modification**—The solvent accessibility of the cysteine residues was investigated by measuring the effect of extracellular incubation with the sulfhydryl-directed reagent pCMBS. This compound is membrane impermeant and roughly of the same size and configuration as glucose, making it a suitable molecule to study the topology of the glucose permeation pathway (20). This reagent in conjunction with cysteine-scanning mutagenesis has been used previously to probe the structure of several transmembrane proteins including the lactose permease, which is a member of the facilitative monosaccharide transport superfamily (23). Mutant transporters that are sensitive to incubation with extracellular pCMBS must be exposed to the aqueous environment. Furthermore, pCMBS sensitivity implies that either the cysteine is positioned sufficiently close to the glucose permeation pathway to inhibit glu-

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**Fig. 2.** Expression of mutant GLUT 1 transporter proteins in Xenopus oocytes. Stage 5 Xenopus oocytes were injected with 50 ng of C-less or mutant C-less mRNAs. After incubation of the transfected oocytes in Barth’s saline at 18 °C for 2 days, total membrane fractions were prepared for immunoblot analysis. Ten \( \mu g \) of total oocyte membrane protein was loaded per lane. Rabbit antiserum A674 raised against the C-terminal 15 residues of human Glut1 was used at 1:500 dilution.

**Fig. 3.** Localization of mutant GLUT1 transporter proteins in Xenopus oocytes. Two days after injection of stage 5 Xenopus oocytes with 50 ng of C-less or mutant C-less mRNAs, oocytes were frozen, sectioned, and subjected to indirect immunofluorescence laser confocal microscopy using rabbit antiserum A674 at a dilution of 1:250. Background staining in water-injected oocytes is shown in the sham-labeled micrograph.

**Fig. 4.** Relative specific activities of GLUT1 cysteine mutants. The upper Glut1 band from total membrane Western blots of each of the cysteine mutants was quantified by scanning densitometry as described under “Experimental Procedures.” Results were normalized to the intensity of the C-less protein band. 2-DOG uptake was measured using the same batch of oocytes used for the Western blots and activity was again normalized to C-less 2-DOG uptake activity. “Relative specific activity” is the relative activity divided by relative Glut1 protein concentration. Each data point represents the mean ± S.E. of three to four independent experiments; *, \( p < .05 \) for mutant versus C-less transporters.
cose binding and/or transport or within a critical area of the protein such that modification results in a significant conformational change in the protein. Measurement of 2-deoxyglucose uptake in pCMBS-treated oocytes (Fig. 5) revealed six residues that were pCMBS-sensitive (Gln282, Gln 283, Ile 287, Ala289, Val 290, and Phe 291). All of the pCMBS-sensitive mutants were found within the exofacial half of the TM segment.

Injection of pCMBS directly into oocytes resulted in roughly a 50% reduction in C-less transport activity with no significant change in the activity of any of the mutants relative to C-less Glut1 (data not shown).

**DISCUSSION**

Helical wheel analysis of the effects of cysteine-scanning mutagenesis and pCMBS modification in TM segment 7 contrasts with the results recently reported for TM segment 5 of GLUT1 (15). Analysis of the pCMBS inhibition pattern in helix 5 revealed that all of the residues sensitive to extracellular exposure to this reagent clustered along a single face of an α-helix. In TM segment 7, pCMBS-sensitive residues were positioned over a majority of the circumference of a helical wheel plot, rather than along a single face (Fig. 6). Olsowski and colleagues (24) also reported pCMBS sensitivity of residues within the exofacial portion of TM segment 7 in their study on the boundary between TM segment 7 and extracellular loop 4. In contrast to our results, Asn288 was reported to be sensitive and Ala289 resistant to pCMBS modification. The specific activities of the cysteine mutants, however, were not determined.

The reduced specific activity observed in over half of the helix 7 mutants suggests an important structural role for this TM segment. However, it does not appear that any of the helix 7 amino acids are essential for sugar transport since nearly all of the mutants retain some residual activity. Although the mutation of Asn288 to cysteine resulted in a greater than 10-fold reduction in specific activity, mutation of this residue to Ile has been previously shown to have little effect on transport (5). While pCMBS sensitivity clearly demonstrates that a portion of helix 7 is exposed to aqueous solvent and suggests positioning close to the glucose permeation pathway, helices 5 and 7 must have different orientations relative to the aqueous channel. The data indicate that the outer third of helix 7 is highly accessible to solvent, suggesting that it lies completely within the putative aqueous tunnel.

Several authors have speculated on the packing arrangement of the 12 transmembrane α-helices in Glut1. Adapted from Kaback’s (23) model for helix packing within the *E. coli* lactose permease.

**FIG.5.** Effect of extracellular pCMBS on 2-deoxyglucose transport activity. Groups of 10–20 oocytes were incubated in Barth’s saline at 22 °C in the presence or absence of 0.5 mM p-chloromercuri-ribenzenesulfonate (dissolved in 100% dimethyl sulfoxide) for 15 min. Oocytes were washed 4× in Barth’s saline and then subjected to 2-deoxyglucose uptake measurements under the same conditions described in the legend to Fig. 1. Results represent a percentage of the pCMBS-treated oocytes compared with vehicle alone within the same batch of oocytes. Each data point represents the mean ± S.E. of three to five independent experiments; ▲, p < .05 and ★, p < .01 for mutant versus C-less control. DMSO, dimethyl sulfoxide.

**FIG.6.** Helical wheel representation of transmembrane helix 7. Transmembrane helix 7 of Glut1 as viewed from the cytoplasmic 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. ★, designates residues with significantly reduced specific activity relative to C-less Glut1.
Zeng’s second model is compatible with the positioning of helices 5 and 7 within the glucose permeation pathway. This model also predicts that helices 2, 8, and 11 participate in forming the aqueous channel. The observation of pCMBS-sensitive residues on both sides of helix 7, however, is difficult to reconcile with this specific helix packing arrangement.

The most extensive data supporting the tertiary structure of the monosaccharide transport proteins has been obtained for the lactose permease from *E. coli*. The packing of the 12 transmembrane α-helices within this protein has been well characterized through a number of experimental approaches including electron spin resonance labeling, fluorescence labeling, cysteine-scanning mutagenesis, and chemical cross-linking (23). Helix 7 appears centrally located within the lactose permease with proximity to helices 1, 2, 5, 10, and 11. Given the high degree of similarity in membrane topology between members of the monosaccharide transporter superfamily, it is reasonable to speculate that the glucose transporters share a common helix packing arrangement with the lactose permease. The positioning of helix 7 in Glut1 within the center of the helix bundle (Fig. 7) is entirely consistent with pCMBS-sensitive residues on opposing faces of the α-helix. It is possible that TM segment 7 has a high degree of conformational flexibility, allowing alternating sides of the α-helix to be solvent accessible during glucose flux. Disruption of this helix movement by pCMBS modification on either side of the helix could impair glucose transport.

The failure to observe any pCMBS-sensitive residues within the cytoplasmic half of TM segment 7 is similar to the results obtained from cysteine-scanning mutagenesis of TM segment 5 (15). The inhibition pattern indicates that either pCMBS is not accessible to the cytoplasmic portion of the α-helix from the external solvent or that chemical modification is not sufficient to inhibit transport activity. The former explanation is consistent with the simple alternating conformational model for the mechanism of glucose transport whereby the cytoplasmic and exofacial glucose-binding sites are not accessible to solvent from the opposing side of the lipid membrane. These results are also consistent with the accessibility of the helix 7 TM amino acids in the glucose-6-P antipporter of *E. coli* (26).

Although labor intensive, cysteine-scanning mutagenesis is proving to be a powerful tool in the elucidation of the tertiary structure of polytopic membrane proteins. With a complete set of cysteine mutants for two of the 12 transmembrane-spanning domains in Glut1 now available, it will be possible to begin more detailed measurements of the distances between these α-helices by performing chemical cross-linking experiments with bifunctional cysteine-directed reagents. Extension of our current study to the remaining TM α-helices will also likely provide further insights into the mechanism of facilitative glucose transport. This approach will allow direct testing of the current models for Glut1 tertiary structure.

REFERENCES

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