Transmembrane Segment 3 of the Glut1 Glucose Transporter Is an Outer Helix*

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A model has been proposed for the structure of the Glut1 glucose transporter based on the results of mutagenesis studies and homology modeling in which eight transmembrane segments form an inner helical bundle surrounded by four outer helices. The role of transmembrane segment 3 in this structural model was investigated using cysteine-scanning mutagenesis in conjunction with 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 helix 3 to a cysteine. The single cysteine mutants were then expressed in Xenopus oocytes, and their expression levels, transport activities, and sensitivities to pCMBS were determined. Cysteine substitution at methionine 96 abolished transport activity, whereas substitutions at the other positions resulted in either modest reductions or no significant effect on transport activity. In striking contrast to all other helices that have been examined to date, only one of the 21 helix 3 single-cysteine mutants was inhibited by pCMBS, suggesting that only a small portion of this helix is exposed to the external solvent. This result is consistent with predictions based on our current structural model, in which helix 3 is one of four outer helices that surround the inner helical bundle that comprises the aqueous substrate-binding cavity. An updated two-dimensional model for the orientation of the 12 transmembrane helices and the conformation of the exofacial glucose-binding site of Glut1 is presented that is consistent with existing experimental data.

Facilitated diffusion of glucose across the membranes of animal cells is mediated by members of the Glut (SLC2a) family of membrane glycoproteins (reviewed in Refs. 1–3). The Glut family belongs to the major facilitator superfamily that includes several hundred proteins involved in the transport of small molecules across membranes (4). Glut1 is one of the most extensively investigated membrane transport systems (5). Kinetic analyses of glucose transport in human red blood cells and on Glut1 purified from red cells are mostly consistent with a simple alternating conformation mechanism (6).

Based on hydrophobicity analysis, Glut1 was predicted to possess 12 transmembrane helices (7). Strong support for this prediction has been provided by a number of experimental observations, including a comprehensive glycosylation-scanning mutagenesis study (8). Several of the 12 proposed transmembrane segments were predicted to form amphipathic α-helices, an observation that led to the hypothesis that these helices form the walls of a water-filled cavity involved in the binding and translocation of glucose across the lipid bilayer (7). Hydroxyl- and amide-containing amino acid side chains within these helices were predicted to form the sugar-binding site(s) of Glut1 via hydrogen bond formation with glucose hydroxyl groups (9).

Experimental support for this structural model has come from cysteine-scanning mutagenesis and substituted cysteine accessibility studies, which implicate transmembrane segments 1 (10), 2 (11), 5 (12), 7 (11, 13), 8 (14), 10 (15), and 11 (16) of Glut1 in the formation of an inner helical bundle that comprises a water-accessible cavity within the membrane. This model is also supported by homology modeling of Glut1 based on the high-resolution structures of the lactose permease (17) and glycerol-3-P antiporter (18), two bacterial members of the major facilitator superfamily. Homology modeling of Glut1 suggests that helices 1, 2, 4, 5, 7, 8, 10, and 11 comprise an inner bundle of transmembrane helices that form a water-filled substrate-binding cavity near the center of the bilayer. Helices 3, 6, 9, and 12 are predicted to surround this inner helical bundle.

In this study cysteine-scanning mutagenesis was used in conjunction with a sulfhydryl-specific chemical reagent to examine the specific role of transmembrane segment 3 in the predicted structure of Glut1. In contrast to all other helices that have been studied to date, our results suggest that only one of the 21 residues that comprise helix 3 is accessible to the external solvent. These data thus suggest that helix 3 lies outside of the inner helical bundle and does not comprise a portion of the substrate-binding cavity, a conclusion that is consistent with the predicted structure of Glut1 based on homology modeling.

**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 Ambion Inc. (Austin, TX), and the Transformer™ site-directed mutagenesis kit was obtained from Clontech (Palo 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 (19), isolation, microinjection, and incubation of Xenopus oocytes (20), preparation of purified oocyte plasma membranes and indirect immunofluorescence laser confocal microscopy (21), SDS-polyacrylamide gel electrophoresis and immunoblotting with Glut1 C-terminal antibody (22), and 2-deoxyglucose uptake measurements (23) have been described in detail previously.

**Specific Activity Determinations**—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 oocyte membrane protein, and the intensity of the Glut1 band was quantified by scanning densitometry using a Amersham Biosciences PhosphorImager SI. Analysis was performed using the ImageQuant NT program (version 4.0). [3H]2-Deoxyglucose uptake (picomole/oocyte/30 min) of each mutant was concomitantly determined in each set of experiments. Specific activity is expressed as the 2-deoxyglucose uptake per nanogram of mutant Glut1 protein expressed per microgram of total oocyte membrane protein, and the data were then normalized by assigning the uptake activity of the parental cysteine-less (C-less) protein a value of 1.0. Purified human erythrocyte membranes were loaded on the same gels as the oocyte membrane samples for use as quantitative standards.

**Statistical Analysis**—Uptake data were analyzed for statistical significance using the two-tailed, unpaired Student t test.

1 The abbreviations used are: pCMBS, p-chloromercuribenzensulfonate; C-less, Glut1 molecule in which all six 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.

**Table I**

| Residue no. | Amino acid change | Codon change |
|-------------|------------------|--------------|
| 96          | Met → Cys        | AGT → TGT    |
| 97          | Leu → Cys        | CTG → TGT    |
| 98          | Met → Cys        | ATG → TGT    |
| 99          | Met → Cys        | ATG → TGT    |
| 100         | Asn → Cys        | ATG → TGT    |
| 101         | Leu → Cys        | CTG → TGT    |
| 102         | Leu → Cys        | CTG → TGT    |
| 103         | Ala → Cys        | GCC → TGC    |
| 104         | Phe → Cys        | TTC → TGC    |
| 105         | Val → Cys        | GTG → TGT    |
| 106         | Ser → Cys        | TCC → TGC    |
| 107         | Ala → Cys        | GCC → TGC    |
| 108         | Val → Cys        | GTG → TGT    |
| 109         | Leu → Cys        | CTC → TGC    |
| 110         | Met → Cys        | ATG → TGT    |
| 111         | Gly → Cys        | GGC → TGC    |
| 112         | Phe → Cys        | TTC → TGC    |
| 113         | Ser → Cys        | TCG → TGT    |
| 114         | Lys → Cys        | AAA → TGT    |
| 115         | Leu → Cys        | CTG → TGT    |
| 116         | Gly → Cys        | GGC → TGC    |

**Fig. 1. Expression of helix 3 single-C mutant transporters in Xenopus oocytes.** Stage 5 Xenopus oocytes were injected with 50 ng of wild-type, C-less, or mutant C-less mRNAs, and 2 days later frozen sections were prepared and analyzed by indirect immunofluorescence laser confocal microscopy, or oocytes were used to prepare purified 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 quantitative standards.
RESULTS

The creation of a functional C-less human Glut1, in which all six native cysteine residues were changed to either serine or glycine residues, has been described previously (24). The transport activity of C-less Glut1 is nearly indistinguishable from that of the wild-type transporter when expressed in Xenopus oocytes (24, 25), indicating that none of the native cysteine residues is required for function. Oligonucleotide-mediated site-directed mutagenesis was used to produce 21 mutant cDNAs in which each amino acid residue within the predicted transmembrane helix 3 of C-less Glut1 was individually changed to a cysteine residue (see Table I).

The single-C mutants were expressed in stage 5 X. laevis oocytes after injection of the corresponding mRNAs. Verification that the mutants were expressed in the oocyte plasma membrane was required for function. Oligonucleotide-mediated site-directed mutagenesis was used to produce 21 mutant cDNAs in which each amino acid residue within the predicted transmembrane helix 3 of C-less Glut1 was individually changed to a cysteine residue (see Table I).

To determine which, if any, of the transmembrane residues within helix 3 are accessible to the external aqueous solvent, transport activity was measured after incubation in the presence of the membrane-impermeant sulfhydryl-specific reagent, pCMBS (Fig. 3). The activity of M96C was too low to permit this analysis. We previously demonstrated that pCMBS can at least partially enter the glucose permeation pathway of Glut1 and has close access to the exofacial sugar-binding site (24).
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 only one single-C mutant, G111C, was significantly inhibited after incubation with pCMBS, and the observed inhibition was modest in magnitude (20%). The V165C mutant represents a well characterized positive control for reaction with and inhibition by pCMBS (12, 24). The results of the pCMBS inhibition experiments suggest that only one of the helix 3 residues is accessible to the external aqueous solvent (see Fig. 4). This result is in striking contrast to the results obtained for helices 1 (10), 2 (11), 5 (12), 7 (11, 13), 8 (14), 10 (15), and 11 (16), all of which possess four or more pCMBS-reactive residues that lie in direct contact with the external aqueous solvent. All of the pCMBS-reactive residues fall within the predicted exoplasmic half of each helix, consistent with the notion that the eight inner helices of Glu1 tilt in the plane of the membrane in such a way as to form a cavity containing the exoplasmic substrate-binding site near the center of the bilayer analogous to the cytoplasmic substrate-binding sites identified in the lactose permease (17) and glycerol-3-P antiporter (18). The relative lack of pCMBS sensitivity in

**DISCUSSION**

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**FIG. 4. Helical wheel representation of helix 3.** Transmembrane helix 3 of Glu1 as viewed from the exoplasmic surface of the plasma membrane. Amino acids are represented by the single letter code. The black arrow points to the single residue that was accessible to pCMBS from the external solvent, and the red arrow points to a methionine residue critical for transport activity.
the helix 3 residues is consistent with our current structural model, in which this helix is one of four transmembrane helices that surround the inner cavity-forming bundle of eight helices (see Fig. 5). The single residue within helix 3 that was observed to react with pCMBS, glycine 111, is predicted to lie a distance of roughly one-third the length of the helix from the exoplasmic face of the membrane. The accessibility of this residue to a membrane-impermeant reagent indicates that at least a small portion of this outer helix is exposed to the aqueous solvent in the exofacial orientation of the transporter. Presumably a small crevice exists between inner helices 2 and 4 or helices 1 and 4 that permits access of solvent to glycine 111. Based on homology modeling of the cytoplasmic-facing orientation of the transporter, such a crevice is predicted to exist between helices 2 and 4 (see Fig. 5a in Ref. 14). The solvent accessibility of this single residue in helix 3 allows us to roughly orient this helix in the membrane (see Fig. 5).

Cysteine substitution at methionine 96 resulted in a ~98% reduction in transport activity. This is one of the largest effects observed on normalized transport activity of any Glut1 point mutant analyzed to date, suggesting that this residue plays a critical role in the structure and function of the molecule. Methionine 96 is predicted to lie very close to the cytoplasmic face of the membrane, and given the predicted orientation of helix 3 as an outer helix, this residue is not likely to play a direct role in substrate binding in either conformation of the transporter. Because methionine has a low probability of participating in reverse turns, it is also unlikely that this residue plays a role in forming the hinge region between helices 2 and 3. Furthermore, if methionine 96 were to lie in direct contact with lipid, it is unlikely that a cysteine substitution at this position would have a dramatic impact on transport activity. Therefore, it is most likely that methionine 96 lies in direct apposition to an adjacent inner helix, where it may interact with an apposing residue or residues and is perhaps involved in helical movements involved in the transport cycle. This hypothesis is supported by the observation that methionine 96 lies on the same face of the helix as glycine 111, which must face the aqueous cavity and thus also lies directly opposite to an adjacent inner helical face. An updated version of a model for the exoplasmic substrate-binding site of Glut1 based on the current data is presented in Fig. 5.

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