Cysteine-scanning Mutagenesis and Substituted Cysteine Accessibility Analysis of Transmembrane Segment 4 of the Glut1 Glucose Transporter*

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A low resolution model has been proposed for the exofacial conformation of the Glut1 glucose transporter in which eight transmembrane segments form an inner helical bundle stabilized by four outer helices. The role of transmembrane segment 4, predicted to be an inner helix in this structural model, was investigated by cysteine-scanning mutagenesis in conjunction with the substituted cysteine accessibility method using the membrane-impermeant, sulfhydryl-specific reagent, p-chloromercuribenzenesulfonate (pCMBS). A functional, cysteine-less, parental Glut1 molecule was used to produce 21 Glut1 point mutants by individually changing each residue along transmembrane helix 4 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. In striking contrast to all of the other seven predicted inner helices, none of the 21 helix 4 single-cysteine mutants was demonstrably inhibited by pCMBS. However, cysteine substitution within helix 4 resulted in an unusually high number of severely transport-defective mutants. The low absolute transport activities of two of these mutants (G130C and G134C) were due to their extremely low levels of expression, presumably as a result of structural instability and consequent degradation in oocytes, suggesting that these two residues play an important role in maintaining the native structure of Glut1. The other two transport-defective mutants (Y143C and E146C) exhibited low specific transport activities, implying that these two residues play an important role in the transport cycle. Based on these data, we conclude that the exoplasmic end of helix 4 lies outside of the inner helical bundle in the exofacial configuration of Glut1.

Animal cells transport glucose across membranes via members of the Glut (SLC2a) family of glycoproteins (reviewed in Refs. 1–3). The Glut family belongs to the major facilitator superfamily, which includes several thousand proteins, members of which are expressed in virtually every organism examined to date. These multispanning membrane proteins are involved in the transport of numerous small molecules across membranes (4). Glut1, best known as the glucose transporter of human erythrocytes, is among the most thoroughly studied of all membrane transport systems (5).

Glut1 was predicted to possess 12 transmembrane helices based on hydrophobicity analysis of the amino acid sequence deduced from the sequence of a human cDNA clone (6). This prediction is supported by a number of experimental observations, most notably a comprehensive glycosylation-scanning mutagenesis study (7). Additionally, several of the 12 proposed transmembrane helices were predicted to be amphipathic, which led to the hypothesis that these helices form the walls of an aqueous cavity that participates in the binding and subsequent translocation of glucose across the lipid bilayer (6). 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 (6), a hypothesis consistent with earlier experimental observations (8).

The results of cysteine-scanning mutagenesis and substituted cysteine accessibility studies are generally consistent with this crude model and implicate transmembrane segments 1 (9), 2 (10), 5 (11), 7 (10, 12), 8 (13), 10 (14), and 11 (15) of Glut1 in the formation of an inner helical bundle that comprises a water-accessible cavity within the membrane. This model is also consistent with homology modeling of Glut1 based on the near atomic resolution structures recently reported for the lac permease (16) and glycerol-3-P antipporter (17), two bacterial members of the major facilitator superfamily. Homology modeling of Glut1 based on these structures 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. Our recently reported analysis of helix 3 is consistent with it being an outer helix (18).

In this study, cysteine-scanning mutagenesis was used in conjunction with a sulfhydryl-specific chemical reagent to examine the specific role of transmembrane segment 4 in the predicted structure of Glut1. In contrast to all of the other seven predicted inner helices, our results suggest that none of the 21 residues that comprise helix 4 is accessible to the external solvent. These data suggest that helix 4 lies outside of the inner helical bundle when Glut1 is its exofacial configuration and thus does not comprise a portion of the exofacial substrate-binding cavity.

EXPERIMENTAL PROCEDURES

Materials—Imported female African Xenopus laevis frogs were purchased from Xenopus Express (Hossmassa, FL). [3H]2-deoxyglucose and diguanosine triphosphate were purchased from Amersham Biosciences, Megascript RNA synthesis kits were purchased from Ambion, Inc. (Austin, TX), and Transformer site-directed mutagenesis kits were 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-PAGE and immunoblotting with Glut1 C-termi-
nal antibody (22), and 2-deoxyglucose uptake measurements (23) have been described in detail previously.

Treatment with p-Chloromercuribenzenesulfonate—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 pCMBS, in Barth’s saline at 22 °C. The concentrated reagent stock was prepared in 100% dimethyl sulfoxide, and control oocytes were treated with the appropriate concentration of vehicle alone. After a 15-min incubation period, the oocytes were washed 4 times 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—Membranes were prepared 3 days following injection of 50 ng of mutant RNA/oocyte. Western blot analysis of each of the mutant transporters was performed on 1 g of total membrane protein, and the intensity of the Glut1 band was quantified by scanning densitometry using a Molecular Dynamics phosphorimaging device SI. Analysis was performed using the ImageQuant NT program (version 4.0). [3H]2-deoxyglucose uptake (pmol/oocyte/30 min) of each mutant was concomitantly determined in each set of experiments. Specific activity is expressed as the 2-deoxyglucose uptake/ng of mutant Glut1 protein expressed/g of total oocyte membrane protein, and the data were then normalized by assigning the uptake activity of the parental 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.

2 The abbreviations used are: pCMBS, p-chloromercuribenzenesulfonate; 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 ONE

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<td>TTC → TGC</td>
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<tr>
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<td>ATC → TGC</td>
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<td>Gly → Cys</td>
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<tr>
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<td>Ser → Cys</td>
<td>TCA → TGC</td>
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</table>

### FIGURE 1.

Expression of helix 4 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/lane. Rabbit antiserum Ala-674 raised against the C-terminal 15 residues of human Glut1 was used at 1:500 dilution. Numbers above the lanes represent the quantity of human erythrocyte Glut1 loaded in each lane as quantitative standards.
FIGURE 2. 2-Deoxyglucose uptake activity of helix 4 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 ± S.E. of 5–19 independent experiments, each experiment using 15–20 oocytes/experimental group. a, raw uptake data. b, the data are normalized/ng of each mutant protein expressed/μg of total oocyte membrane protein, and the specific activity of the parental C-less transporter is arbitrarily assigned a value of 1.0. Background values observed in sham injected oocytes were subtracted prior to normalization. Stars indicate p < 0.01 for single-C mutants compared with parental C-less Glut1. ND, not determined. All of the raw update values for single-C mutants were significantly different from that or parental C-less, p < 0.01. 2-DOG, [3H]2-deoxyglucose.
Statistical Analysis—Uptake data were analyzed for statistical significance using the two-tailed, unpaired Student’s t test.

RESULTS

A Cysteine-less (C-less) human Glut1 molecule, in which all six native cysteine residues were changed to either serine or glycine residues, has been described previously (24, 25). None of the native cysteine residues is required for function, as the transport activity of C-less Glut1 is very similar to that of the wild-type transporter when expressed in Xenopus oocytes (25). C-less Glut1 was used to produce 21 mutant cDNAs in which each amino acid residue within predicted transmembrane helix 4 was individually changed to a cysteine residue (see TABLE ONE). The complete coding regions of all of the mutants were sequenced to confirm the presence of the desired mutation as well as the absence of spurious mutations.

Stage 5 X. laevis oocytes were injected with mRNAs encoding each of the 21 single-C mutants. Verification that the mutants were expressed in the oocyte plasma membrane was determined by indirect immunofluorescence laser confocal microscopy using a rabbit polyclonal antibody raised against a synthetic peptide corresponding to the C-terminal 15 residues of Glut1 (Fig. 1a). 19 of the 21 single-C mutants were clearly expressed in the oocyte plasma membrane, but expression of G130C and G134C could not be detected. The expression level for each of the 21 mutants was then quantitated by Western blot analysis of purified oocyte membranes (Fig. 1b). As we have observed during our analyses of other Glut1 transmembrane helices by cysteine-scanning mutagenesis, the expression levels of the different helix 4 single-C mutants varied widely, necessitating that transport data be normalized to the membrane content of mutant protein to directly compare the catalytic activities of the mutants to that of their C-less parent. Consistent with our microscopic observations, the expression of G130C and G134C could not be detected by immunoblot analysis.

19 of the 21 single-C mutants exhibited significant transport activity above the low endogenous oocyte background level, as determined by uptake of [3H]-2-deoxyglucose. The raw uptake data are shown in Fig. 2a, and the specific transport activities, normalized to the membrane content of each mutant and with the activity of C-less assigned a value of 1.0, are presented in Fig. 2b. 10 of the 21 mutants (S148C, V147C, V144C, V140C, F139C, G138C, T137C, T136C, Y132C, and I129C) exhibited specific transport activities that were modestly reduced (by 15–78%) compared with that of the parental C-less protein. Five single-C mutants (G145C, P141C, L135C, V131C, and F127C) exhibited slight elevations in specific transport activity, and three mutants (M142C, S133C, and I128C) did not exhibit a significant difference in activity relative to that of C-less Glut1. G130C and G134C did not exhibit transport activity significantly different from that of sham injected oocytes, consistent with the lack of detectable protein expression for these mutants. Y143C and E146C exhibited normalized trans-
Transmembrane Segment 4 of the Glut1 Glucose Transporter

port activities that were reduced by 99 and 97%, respectively, compared with that of the C-less control. These latter two mutants, together with M96C within helix 3, are the most severely transport-defective Glut1 single-C mutants discovered thus far.

To determine which, if any, of the helix 4 residues are accessible to the external aqueous milieu, transport activity was measured after incubation in the presence of the sulfhydryl-specific, membrane-impermeant reagent pCMBS (Fig. 3). The transport activities of G130C, G134C, Y143C, and E146C were too low to analyze by this method. We have previously shown that pCMBS can enter the glucose permeation pathway of Glut 1 and has close access to the exofacial sugar-binding site (25). Fig. 3 shows the transport activities observed in the presence of pCMBS normalized for each single-C mutant to the activity measured in the absence of the reagent; i.e. a value of 1 indicates no effect of pCMBS, values >1 indicate stimulation by pCMBS, and values <1 indicate inhibition by pCMBS. Surprisingly, unlike all of the other seven predicted inner helices, none of the 17 helix 4 residues that could be accessed by the substituted cysteine accessibility method (SCAM) exhibited sensitivity to pCMBS. The V165C mutant represents a positive control for reaction with pCMBS (11, 25).

**DISCUSSION**

Two surprising results were observed during the analysis of helix 4 by cysteine-scanning mutagenesis and SCAM. First, the pCMBS inhibition experiments suggest that none of the helix 4 residues is accessible to the external aqueous solvent. This result is in striking contrast to the results obtained for the other seven predicted inner helices, which include helices 1 (9), 2 (10), 5 (11), 7 (10, 12), 8 (13), 10 (14), and 11 (15), all of which possess at least four pCMBS-reactive residues that are exposed to the external aqueous milieu. Most of the pCMBS-reactive residues within these seven inner helices fall within the predicted exoplasmic half of each helix, consistent with a model in which the inner helices of Glut1 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 lipid bilayer, in analogy to the cytoplasmic substrate-binding sites identified in the lac permease (16) and glycerol-3-P antiporter (17). The apparent lack of solvent accessibility of the helix 4 residues is particularly surprising in light of our previously reported results for helix 10 (14), which contains at least four solvent-accessible residues in the exoplasmic conformation. Helix 4 and helix 10 are analogous helices in the pseudo-2-fold symmetrical structures of the *Escherichia coli* lac permease and glycerol-3-P transporters in their cytoplasmic orientations. These two helices are also predicted to be analogous structures in the Glut1 model recently proposed by Salas-Burgos et al. (26). Of course, the possibility exists that one or more helix 4 residues are exposed to the external solvent in the exoplasmic conformation of Glut1, but that reaction with pCMBS at these sites does not discernibly alter transport activity. Negative data cannot be unambiguously interpreted when employing the substituted cysteine accessibility method used in this study.

The complete absence of pCMBS sensitivity in the helix 4 residues appears to be inconsistent with our previous structural model, in which this helix is one of eight inner transmembrane helices that form the exoplasmic substrate-binding site. However, the current observations on helix 4 can be reconciled with existing data. First, our helical bundle model for the exoplasmic configuration of Glut1 is based on crystal structures for the cytoplasmic configurations of the lac permease and glycerol-3-P antiporter. Given the dramatic degree of twisting and turning that several of the transmembrane helices exhibit in the cytoplasmic configuration, it is possible that the cytoplasmic end of a helix may form part of the inner helical bundle and be exposed to solvent at the cytoplasmic face of the membrane but lie outside the inner bundle at the exoplasmic face of the membrane and be shielded from the external solvent by adjacent helices. In fact, helix 4 in the crystal structures of the two bacterial transporters does exhibit a dramatic turn near the center of the bilayer, especially in the lac permease. The fourth transmembrane helices in Glut1, the lac permease, and the glycerol-3-P antiporter all contain several glycine and proline residues that disrupt helical structure and introduce kinks in the chain. A dramatic tilt in helix 4 is also predicted by the recent energy minimalization model of Glut1 proposed by Salas-Burgo et al. (26). We thus predict that in Glut1, and in perhaps all three transporters, helix 4 twists in such a manner that it comprises an outer helix in the exofacial configuration and is thus not in contact with the external solvent. The exoplasmic end of helix 4 may be shielded from the external solvent in the exofacial configuration by one or more of its adjacent helices, i.e. helices 1, 2, and 3. This hypothesis is supported by our recent cross-linking data, which showed that helices 4 and 8 are in close proximity at their cytoplasmic ends but are farther apart at their exoplasmic ends. Consistent with an important role for the conserved glycine residues in helix 4 is the observation that cysteine substitutions at Gly-130 and Gly-134 appear to cause severe disruptions in Glut1 structure, because the mutant proteins could not be detected in oocytes presumably due to rapid degradation of the improperly folded proteins.

The second surprising observation in this study was that four cysteine substitutions within helix 4 caused severe (>99%) disruptions in the function of Glut1 (see Fig. 4). This is a greater number of severe disruptions than has been observed for any of the other eight Glut1 helices analyzed by cysteine-scanning mutagenesis thus far. In addition to the presumed structural perturbations observed for G130C and G134C.
mentioned above, cysteine substitutions at Tyr-143 and Glu-146 caused severe disruptions in normalized transport activity, such that only 1 and 3%, respectively, of parental C-less activity was preserved in the mutants. Given our prediction that the exoplasmic end of helix 4 is not in contact with solvent, it is unlikely that either of these residues participates directly in exofacial substrate binding. Also, if these two residues, which lie on the same face of helix 4, were in contact with lipid, it is highly unlikely that amino acid substitutions at these positions would dramatically alter transport activity. Also, it is unlikely from an energetic standpoint that Glu-146 would be in contact with lipid. Rather, Tyr-143 and Glu-146 probably directly interact with opposing side chains in an adjacent helix and are somehow involved in helical movements that bring about conformational changes in the transporter. The almost immeasurably low residual transport activities of Y143C and E146C suggest that these two mutants may be locked in a single conformation. If so, these mutations may prove to be useful in attempts to crystallize Glut1. An updated model for the exofacial binding site of Glut1 is shown in Fig. 5.

**REFERENCES**

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