Analysis of Transmembrane Segment 10 of the Glut1 Glucose Transporter by Cysteine-scanning Mutagenesis and Substituted Cysteine Accessibility*

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The Glut1 glucose transporter has been proposed to form an aqueous sugar translocation pathway through the lipid bilayer via the clustering of several 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 participation of transmembrane helix 10 in the formation of this putative aqueous tunnel was tested using cysteine-scanning mutagenesis in conjunction with the membrane-impermeant, sulfhydryl-specific reagent, p-chloromercuribenzenesulfonate (pCMBS). A series of 21 mutants was created from a fully functional, cysteine-less, parental Glut1 molecule by changing each residue within putative transmembrane segment 10 to cysteine. Each mutant was then expressed in Xenopus oocytes, and its plasma membrane content, 2-deoxyglucose uptake activity, and sensitivity to pCMBS were measured. Helix 10 exhibited a highly distinctive reaction profile to scanning mutagenesis whereby cysteine substitution at residues within the cytoplasmic N-terminal half of the helix tended to increase specific transport activity, whereas substitution at residues within the exoplasmic C-terminal half of the helix tended to decrease specific transport activity. Four residues within helix 10 were clearly accessible to pCMBS as judged by inhibition or stimulation of transport activity. All four of these residues were clustered along one face of a putative α-helix. These results combined with previously published data suggest that transmembrane segment 10 of Glut1 forms part of the sugar permeation pathway. Two-dimensional models for the conformation of the 12 transmembrane helices and the exofacial glucose-binding site of Glut1 are proposed that are consistent with existing experimental data.

Facilitative transport of glucose into mammalian cells is mediated by members of the Glut (SLC2a) family of membrane glycoproteins (reviewed in Refs. 1–3). Glut1, the prototype member of this family, is one of the most extensively studied of all mammalian membrane transporters (4). Kinetic studies on human red blood cell Glut1 are mostly compatible with a simple alternating conformation mechanism for sugar transport (5). However, clear kinetic anomalies have been observed in human erythrocytes that are either inconsistent with this hypothesis or suggest the presence of factors specific to the human erythrocyte that prevent accurate measurement of steady-state kinetic properties (6, 7). The human Glut1 polypeptide exhibits a molecular mass of 54,117 and contains a single N-linked oligosaccharide (8). The presence of 12 transmembrane segments was predicted based on analysis of the deduced amino acid sequence (8), and this prediction has been experimentally verified using glycosylation-scanning mutagenesis (9).

Several of the 12 putative transmembrane segments possess the potential to form amphipathic α-helices, which led to the hypothesis that these amphipathic helices cluster together in the membrane to form the walls of a water-filled tunnel through which sugar traverses the fatty acyl core of the lipid bilayer (8). It was further suggested that hydroxyl- and amide-containing amino acid side chains within these helices form the glucose binding pocket within Glut1 via the formation of hydrogen bonds with sugar hydroxyl groups, although hydrophobic interactions involving aromatic residues also appear to be important (10).

Several pieces of evidence are consistent with this general model for the structure of Glut1. First, glutamine 161 within helix 5 (11) and glutamine 282 within helix 7 (12) both appear to participate in forming the exofacial substrate-binding site. Second, valine 165, which lies near the center of helix 5 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 (13). Third, tryptophan 412 within helix 11 is essential for transport activity (14). Fourth, hydrogen exchange studies indicate that 30% of peptide hydrogen atoms are freely exposed to water in purified, reconstituted Glut1, consistent with the existence of a water-accessible permeation pathway (15). Fifth, cysteine-scanning mutagenesis and substituted cysteine accessibility studies implicate transmembrane segments 2 (16), 5 (17), 7 (18), and 11 (19) of Glut1 in the formation of an aqueous transmembrane permeation pathway.

In the present study, we used cysteine-scanning mutagenesis in conjunction with a sulfhydryl-specific chemical reagent to address the role of transmembrane segment 10 in the formation the Glut1 sugar permeation pathway. Our results suggest that transmembrane segment 10 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), [2-3H]deoxyglucose and...
TABLE I

Cysteine-scanning mutagenesis of helix 10
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 10 was individually changed to cysteine. Residue No. refers to the amino acid numbering for human Glut1 given in Muecket et al. (8).

<table>
<thead>
<tr>
<th>Residue No.</th>
<th>Amino acid change</th>
<th>Codon change</th>
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</thead>
<tbody>
<tr>
<td>369</td>
<td>Ile → Cys</td>
<td>ATC → TGC</td>
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<tr>
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<td>Val → Cys</td>
<td>GTC → TGC</td>
</tr>
<tr>
<td>371</td>
<td>Ala → Cys</td>
<td>GCC → TGC</td>
</tr>
<tr>
<td>372</td>
<td>Ile → Cys</td>
<td>ATC → TGC</td>
</tr>
<tr>
<td>373</td>
<td>Phe → Cys</td>
<td>TTT → TGT</td>
</tr>
<tr>
<td>374</td>
<td>Gly → Cys</td>
<td>GGC → TGC</td>
</tr>
<tr>
<td>375</td>
<td>Phe → Cys</td>
<td>TTT → TGT</td>
</tr>
<tr>
<td>376</td>
<td>Val → Cys</td>
<td>GTC → TGC</td>
</tr>
<tr>
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<tr>
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<td>TGG → TGC</td>
</tr>
<tr>
<td>389</td>
<td>Phe → Cys</td>
<td>TTC → TGC</td>
</tr>
</tbody>
</table>

![Image](http://www.jbc.org/)

FIG. 1. Expression of helix 10 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 2 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. Twenty 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.

We previously constructed a mutant human Glut1 cDNA encoding a cysteine-less (C-less) Glut1 polypeptide in which all six native cysteine residues were changed to either serine or glycine residues (13). The C-less transporter expressed in Xenopus oocytes exhibits transport activity nearly indistinguishable from wild-type Glut1. We used C-less Glut1 cDNA as a template to construct single-C mutants for transmembrane segment 10. Each of the 21 residues within transmembrane segment 10 was individually changed to a cysteine residue using oligonucleotide-mediated, site-directed mutagenesis, producing a series of 21 mutant Glut1 molecules, each containing only a single cysteine residue (see Table I).

Expression of the single-C mutants in the oocyte plasma membrane was assessed visually by indirect immunofluorescence laser confocal microscopy (Fig. 1A) and quantitatively by immunoblot analysis of purified oocyte plasma membranes.

1 The abbreviations used are: pCMBS, p-chloromercuribenzenesulfonate; C-less, Glut1 molecule in which all six native cysteine residues were changed to either serine or glycine; 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.

Transmembrane Segment 10 of the Glut1 Glucose Transporter

Digenosine triphosphate (mRNA cap) were purchased from Amersham Biosciences, Inc., Megascript™ RNA synthesis kit was purchased from Ambion Inc (Austin, TX), and 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 (20), isolation, microinjection, and incubation of Xenopus oocytes (21), preparation of purified oocyte plasma membranes and indirect immunofluorescence laser confocal microscopy (14), SDS-polyacrylamide gel electrophoresis and immunoblotting with Glut1 C-terminal antibody (11), and 2-deoxyglucose uptake measurements (22) have been described in detail previously.

**Treatment with p-chloromercuribenzenesulfonate 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 pCMBS in Barth’s saline at 2°C. The 100× 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× in Barth’s saline and then used for the determination of [2-3H]deoxyglucose uptake (50 µM, 30 min at 22°C).

**Specific Activity Determinations**—Plasma membranes were prepared 3 days after injection of 50 ng of mutant RNA per oocyte. Western blot analysis of each of the mutant transporters was performed on 5–10 µg of total membrane protein, and the intensity of the upper fully 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). Protein levels were determined to the C-less Glut1 control. [2-3H]Deoxyglucose uptake (pmol/oocyte/30 min) of each mutant was concomitantly determined in each set of experiments and also normalized to C-less [2-3H]deoxyglucose uptake. Specific activity was calculated by dividing relative 2-deoxyglucose uptake by the amount of Glut1 protein (compared with the C-less transporter).

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

**RESULTS**

We previously constructed a mutant human Glut1 cDNA encoding a cysteine-less (C-less) Glut1 polypeptide in which all
Transmembrane Segment 10 of the Glut1 Glucose Transporter

FIG. 2. Relative 2-deoxyglucose uptake activity of helix 10 single-C mutants. [2-3H]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 are the mean ± S.E. or range of 2–4 independent experiments, each experiment using 15–20 oocytes per experimental group. The data are normalized to the activity of the C-less parental Glut1 protein (see “Experimental Procedures”) and, thus, represent intrinsic transport activities relative to the C-less molecule. Background values observed in sham-injected oocytes were subtracted before normalization. *, p < 0.05 for single-C mutant compared with parental C-less Glut1.

(Fig. 1B). As we have observed during the analysis of other Glut1 helices, roughly half of the single-C mutants were expressed in oocyte membranes at levels similar to the parental C-less construct, whereas the other half were expressed at lower levels. V370C, V371C, I372C, and G382C were expressed at particular low levels relative to the parental C-less transporter.

Transport activity above the very low endogenous oocyte background level was detectable for all 21 mutants, as determined by uptake of [2-3H]deoxyglucose. The relative intrinsic transport activities, normalized to the plasma membrane content of each mutant (see “Experimental Procedures”) are presented in Fig. 2. Consistent with our previously published findings (14), amino acid substitution at tryptophan 388 reduced intrinsic transport activity. This residue appears to be involved in the binding of the transport inhibitor, cytochalasin B (14, 23). Interestingly, cysteine substitutions at several other residues (phenylalanine 379, glutamate 380, glycine 382, proline 383, glycine 384, proline 385, isoleucine 386, and phenylalanine 389) within the C-terminal (exoplasmic) half of helix 10 significantly reduced the intrinsic transport activity, whereas cysteine substitution at several residues within the N-terminal (cytoplasmic) half of Glut1 (isoleucine 369, valine 370, isoleucine 372, and alanine 377) resulted in increased transport activity. Residues within helix 10 thus appear to be unusually sensitive to cysteine substitution relative to residues within the other transmembrane helices that have been analyzed by cysteine-scanning mutagenesis.

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, pCMBS (Fig. 3B), and compared with the activities measured in the presence of vehicle alone (Fig. 3A). We have previously demonstrated that pCMBS can permeate the glucose permeation pathway of Glut1 and has close access to the exofacial sugar-binding site (13). Fig. 3c 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. the ratio between the values in Fig. 3B and the corresponding values in Fig. 3A). The activity of three single-C mutants (V376C, F379C, E380C) was significantly inhibited after incubation with pCMBS, indicating that the corresponding amino acid side chains reacted with the pCMBS and, therefore, must be accessible from the external aqueous solvent. Additionally, the activity of P383C was stimulated by reaction of pCMBS with the cysteine side chain at this position.

DISCUSSION

Among the five transmembrane segments of Glut1 that have been analyzed thus far by cysteine-scanning mutagenesis, helix 10 exhibits a unique pattern of sensitivity wherein residues within the exoplasmic half of the helix tend to be inhibited by cysteine substitution, and residues within the cytoplasmic half of the helix are stimulated by substitution. The structural or functional implications of this unusual result, if any, are not immediately apparent. We are unaware of similar results having been reported for another membrane transporter.

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 10 (see Fig. 4). These results are similar to those obtained with helices 2 (16), 5 (17), and 11 (19). 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 (18). The 4 residues within helix 10 that are clearly reactive to pCMBS all lie in the exoplasmic half of the helix, a result similar to that observed with helices 2, 5, 7, and 11, all of which possess residues close to the exoplasmic face that are accessible to pCMBS present in the external solvent. Interestingly, however, unlike the other transmembrane helices that have been examined, none of the six residues in helix 10 that are closest to the exoplasmic face of the membrane appears to be accessible to solvent, as judged by a pCMBS-induced alteration in transport activity. There are at least two possible explanations for this result. One possibility is that one or more of these residues does, in fact, react with pCMBS but that the reaction does not result in a detectable alteration in transport function. This is a reasonable possibility considering that several of the corresponding single-C mutants exhibit low activity due to the cysteine substitution, and a further reduction due to pCMBS reactivity may not be readily detectable. A second possibility is that none of these residues is accessible to the external solvent because the solvent-exposed face of helix 10 at its extreme C terminus is in close contact with another transmembrane segment. Unfortunately, it is not possible at present to distinguish between these two interpretations.
The results of cysteine-scanning analysis and substituted cysteine accessibility studies performed on 5 of the 12 transmembrane helices of Glut1 together with other published and unpublished data allow us to propose crude two-dimensional models for the arrangement of the 12 transmembrane helices and for the helical cluster comprising the exofacial glucose-binding site. A major constraint for modeling the arrangement of the transmembrane helices in Glut1 is the short length of most of the linker domains connecting the helices, which dictates that consecutive helices in the primary structure must lie adjacent to one another in the tertiary structure, with the possible exception of helices 6 and 7. Helices 2, 5, 8, 10 (this report), and 11 (19) of Glut1 all have a single, discrete solvent-accessible face as defined by substituted cysteine accessibility analysis, whereas helix 7 has solvent-accessible residues along its entire cross-sectional perimeter (18). One possible clustering of the 12 transmembrane helices that is consistent with these constraints and experimental observations is shown in Fig. 5A. Helices 2, 3, 5, 8, 10, and 11 can be arranged to form an outer ring surrounding helix 7 that is consistent with the results of substituted cysteine accessibility analyses, site-directed mutagenesis studies, and helical wheel modeling. Residues within helices 5, 7, and 11 have been implicated in exofacial substrate binding (for review, see Ref. 24). 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 bind-
ing, glutamine 161 (11) and glutamine 282 (12). The orientation of helices 5 and 7 is also consistent with the observation that valine 165 lies at a critical region involved in transporter function, although this residue itself is not directly involved in transport activity (13). 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 between glutamine 161 and glutamine 282 is consistent with bulky substitutions at this position interfering with hydrogen bond formation between glucose and these two residues. The orientation of helix 11 is consistent with a hydrophobic interaction between the C-6 region of glucose and tryptophan 412. A hydrophobic interaction involving an aromatic ring of Glut1 and the C-6 region of glucose was predicted by Barnett et al. (10) 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 (14), and additional studies indicate that an aromatic ring is essential at this position.3

A high resolution model for the structure of Glut1 has recently been proposed by Zuniga et al. (25). Their model is based primarily on the proposed helical bundle arrangement of the Lac permease and was refined using an energy minimization algorithm. Despite having distinct derivations, our crude two-dimensional model and the high resolution model of Zuniga et al. (25) share some similarities. Both models place helix 7 within a water-filled channel formed by the clustering of either 7 or 9 of the other transmembrane helices, with either two or four transmembrane helices lying outside of the central helical bundle. The first seven helices are similarly disposed in the two models, but the precise arrangement of the C-terminal five helices varies between the two models. Both models suggest the presence of two independent channels or pathways formed by the transporter, with the centrally positioned helix 7 playing a key structural role in defining the two pathways.

The two models also differ in some respects. Whereas our model is based entirely on experimental observations and is thus necessarily of relatively low resolution, the model of Zuniga et al. (25) is consistent with some, but not all, of the available data. For example, it is difficult to conceive of a key role for glutamine 161 in the context of the model of Zuniga et al. because this residue is predicted to lie a large distance from the proposed translocation pathways and from the proposed substrate-binding site. As noted above, there is experimental evidence that glutamine 161 is directly involved in exofacial substrate binding, and even highly conserved amino acid substitutions at this site dramatically affect transport activity and the apparent exofacial affinity of Glut1 for a non-transported glucose analog (11). Zuniga et al. (25) classify a single-C mutant at this site as being insensitive to pCMBS inhibition. However, the data suggest that a single-C mutant at glutamine 161 is sensitive to pCMBS inhibition but that the inhibition did not reach statistical significance because of the extremely low transport activity of the sin-

3 M. Mueckler, unpublished observations.
gle-C substitution at this critical site (17). This contention is consistent with the fact that a single-C mutant at valine 165, a residue that lies one helical turn distant from glutamine 161 and should therefore lie on the same face of the helix, is clearly sensitive to pCMBS inhibition (17). In fact, the valine 165 single-C mutant is routinely used as a positive control in our pCMBS inhibition studies. The results of a comprehensive study strongly suggest that valine 165 lies within the glucose permeation pathway, close to the exofacial substrate-binding site (13). The disposition of valine 165 in the model of Zuniga et al. (25) appears to be inconsistent with these observations. Curiously, valine 165 is not discussed by Zuniga et al. (25) and is missing from Table V (see Ref. 25). This table lists mutants that have been analyzed by site-directed mutagenesis and have been found to be sensitive to pCMBS inhibition. Regardless of these inconsistencies, the model is a useful starting point and presumably can be modified to more fully account for the existing data and to accommodate new data as they accumulate.

Dwyer (26) proposes a structural model for the Glut3 glucose transporter based on homology modeling using the MsL protein, a mechano-sensitive ion channel. This model is difficult to evaluate given the lack of experimental data regarding the structure of Glut3. It would also seem that a homology model for a membrane transporter based on an ion channel is of uncertain significance given the fundamental mechanistic differences between ion channels and transporters (27). Unfortunately, no crystallographic data exists for any member of the major facilitator superfamily of membrane transporters.

The crude model we propose is extremely tentative and will undoubtedly require extensive modification, but it provides a useful framework for further structural analysis of Glut1. The power of mutagenesis and biochemical approaches to the study of transporter structure/function is amply demonstrated by the work of Kaback et al. (28) on the Escherichia coli Lac permease (28).

REFERENCES

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