Cysteine Scanning Mutagenesis of Helices 2 and 7 in GLUT1 Identifies an Exofacial Cleft in Both Transmembrane Segments†

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Received September 17, 1999; Revised Manuscript Received December 6, 1999

ABSTRACT: Cysteine scanning mutagenesis in conjunction with site-directed chemical modification of sulfhydryl groups by *p*-chloromercuribenzenesulfonate (pCMBS) or *N*-ethylmaleimide (NEM) was applied to putative transmembrane segments (TM) 2 and 7 of the cysteine-less glucose transporter GLUT1. Valid for both helices, the majority of cysteine substitution mutants functioned as active glucose transporters. The residues F72, G75, G76, G79, and S80 within helix 2 and G286 and N288 within helix 7 were irreplaceable because the mutant transporters displayed transport activities that were lower than 10% of Cys-less GLUT1. The indicated cluster of glycine residues within TM 2 is located on one face of the helix and may provide space for a bulky hydrophobic counterpart interacting with another transmembrane segment or lipid side chains. Characteristic for helix 7, three glutamine residues (Q279, Q282, and Q283) played an important role in transport activity of Cys-less GLUT1 because an individual replacement with cysteine reduced their transport rates by about 80%. ParaCMBS-sensitivity scanning of both transmembrane segments detected several membrane-harbored residues to be accessible to the extracellular aqueous solvent. The pCMBS-reactive sulfhydryl groups were located exclusively in the exofacial half of the plasma membrane and, when presented in a helical model, lie along one side of the helices. Taken together, transmembrane segments 2 and 7 form clefts accessible to the extracellular aqueous solvent. The lining residues are however excluded from interaction with intracellular solutes, as justified by microinjection of pCMBS into the cytoplasm of *Xenopus* oocytes.

Members of the mammalian GLUT family (GLUT1 to GLUT5) belong to a diverse group of integral membrane proteins that facilitate the transfer of water-soluble solutes through the membrane lipid bilayer. The primary sequences of these five isoforms are very similar (i.e., 50−76% similarity at the amino acid level (*1*)), and hydrophobicity analysis performed according to Kyte and Doolitle (*2*) demonstrates almost superimposable hydropathy profiles (*3*). On the basis of the distribution of hydrophilic and hydrophobic amino acids, transmembrane segments 3, 5, 7, 8, and 11 are identified to be the most amphiphilic helices in GLUT1 (*4*). In an attempt to create a general model for the tertiary arrangement of transmembrane segments, valid for all members of the major facilitator superfamily (*5*), transmembrane segments 1, 2, 4, 5, 7, 8, 10, and 11 are postulated to possess properties that are relevant to the formation of a putative translocation pathway (*6*). Since high-resolution structure crystals are not available, which could provide unequivocal knowledge of tertiary structures for the GLUT transporter family members, several speculative membrane topology models are proposed by the use of different algorithms (*7*−*11*). With respect to membrane helix arrangements, the most recent proposal by Widdas (*11*) takes into account both the lining of the translocation pathway and the pore barrier. According to this assignment, nine transmembrane segments (i.e., transmembrane segments 1, 2, 3, 5, 7, 8, 10, 11, and 12) could surround an open cleft and only three domains (i.e., helices 1, 7, 12) would be necessary for the opening movement.

Site-directed mutagenesis of native cysteine residues (*12*), glycosylation scanning mutagenesis (*13*), and very recently cysteine scanning mutagenesis (*14*,*15*) were applied to GLUT1 providing indirect insight into secondary and tertiary structure. Cysteine scanning mutagenesis in conjunction with SH group chemical modification has proved to be a powerful strategy for investigating structure and function of integral membrane proteins such as channels or transporters. With respect to transporters, this approach has been applied successfully to *Escherichia coli* lac permease in which essentially every native amino acid residue has been substituted by cysteine. Single cysteine replacement mutants were then analyzed for SH group sensitivity toward *N*-ethylmaleimide (reviewed in ref *16*). This approach proved to be an invaluable tool for probing the secondary structure and for elucidating the packing of transmembrane spanning segments. As for the lac permease, site-directed mutagenesis of single cysteine residues in GLUT1 indicated that native cysteine residues are not essential for transport activity (*12*). We and others generated a GLUT1 construct devoid of all six native cysteine residues that encodes a functional glucose transporter (*15*, *17*, *18*). This cDNA served as a template to

† This work was supported by the Deutsche Forschungsgemeinschaft, Grant Ke 390/6-1.

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10.1021/bi992160x CCC: $19.00
Published on Web 02/18/2000

reintroduce single cysteine residues into Cys-less GLUT1 replacing adjacent amino acids, one at a time. The approach will provide the opportunity to address the agreement of the various hypothetic membrane topologies with the results obtained from experiments.

Cysteine scanning mutagenesis at the boundary of exofacial loops-transmembrane segments (*14*) and of putative helix 5 (*15*), in combination with chemical modification by the sulfhydryl reagent pCMBS,[11] provided strong evidence that certain membrane-harbored amino acid residues are able to interact with this membrane-impermeant compound applied to the outer surface of the plasma membrane. To discriminate between accessibility and reactivity of sulfhydryl groups, the membrane permeable NEM was used to detect whether cysteine residues, independent of their accessibility to the extracellular aqueous solvent, are capable of influencing transport activity. Our previous investigation (*14*) provided evidence that certain residues which are predicted to be located within the plasma membrane react with the membrane-impermeant pCMBS. Targeted sulfhydryl sensitivity scanning elucidated the local structure of putative helices 2 and 7, respectively, particularly with respect to the potential formation of a sugar permeation pathway. In both helices, pCMBS-sensitive positions were found in the exofacial portions of transmembrane segments forming an exofacial cleft. They are lined up along one face in a helix model. On the opposite face of helix 2 a cluster of three glycine residues provides space for a complementary bulky or hydrophobic counterpart that may be another transmembrane helix or a hydrophobic tail of the membrane lipids.

MATERIALS AND METHODS

Mutagenesis, cRNA Preparation and Microinjection into Xenopus Oocytes. Site-directed mutagenesis and cRNA preparation were carried out according to the standard protocols described previously (*14*, *19*). A *BamH*I fragment of the human GLUT1 cDNA derived from pSPGT (*20*) was subcloned into a *Bgl*II site of the Xenopus oocyte expression vector pSP64T (*21*). All six native cysteine residues were replaced by serine or glycine to generate a Cys-less GLUT1 construct (*18*). Single cysteine residues were introduced, one at a time, to substitute residues 67 to 87 (putative helix 2) and residues 272 to 292 (putative helix 7). All mutations were confirmed by restriction fragment analysis and finally by DNA sequencing with a fluorescence DNA sequencer (ABI PRISM) connected to a Macintosh computer.

In vitro synthesis of capped cRNA of Cys-less GLUT1 or of the Cys-substitution mutants was conducted with the *Xba*I-linearized plasmid template using the in vitro transcription protocol provided by AMBION (mMESSAGE mMACHINE, Ambion, Austin, USA). The amount of transcribed RNA was calculated by counting the radioactivity of incorporated [^{35}S]thioUTP and, in addition, by calculating the 260:280 nm absorbance ratio before microinjection of cRNA into *Xenopus* oocytes was conducted.

The procedure of *Xenopus* oocyte isolation and the technique of microinjection into *Xenopus* oocytes are described elsewhere (*22*).

Transport Measurement, Transport Inhibition and Protection Assay. Determination of transport mediated by Cys-less GLUT1 or the various Cys-replacement mutants was carried out according to the standard protocol reported previously (*14*). Tritium-labeled 2-deoxy-D-glucose (50 μM, 1 μCi/0.5 mL transport assay, 30 min uptake time period) was used as the glucose analogue for the majority of uptake determinations. The membrane-impermeant sulfhydryl reagent pCMBS (2 mM, 1 h incubation) was applied to Barth's modified medium to identify those residues that are exposed to the extracellular aqueous phase (*14*). To assess accessibility of sulfhydryl groups from the cytoplasmic water, pCMBS was microinjected into *Xenopus* oocytes achieving final concentrations of 1−3 mM pCMBS (calculated on the basis of an average distribution volume of 0.5 μL per *Xenopus* oocyte (*22*)). The lipid-soluble membrane permeable NEM (10 mM, 1h incubation) was applied from outside of the membrane to detect sulfhydryl group reactivities independent of their accessibility to the aqueous solvent phase. For all transport assays containing NEM, 3-*O*-methyl-D-glucose (1 mM, 5 μCi/0.5 mL transport assay, 5 min uptake time period) was used as a substrate because NEM is known to inhibit hexokinase activity (*23*).

For each transmembrane segment, two of the most pCMBS-sensitive cysteine replacement mutants were included in an assay designed to protect against transport inhibition by pCMBS. Thus, *Xenopus* oocytes were equilibrated with 3-*O*-methyl-D-glucose (10 or 100 mM 3-OMG) for 12 h, followed by a 1 h pCMBS application, before equilibrium exchange measurements with tritium-labeled 3-*O*-methyl-D-glucose were performed.

Confocal Laser Scanning Microscopy. Cysteine replacement mutants whose basal transport activities were 20% or lower than that of Cys-less GLUT1 were included in the immunofluorescence confocal laser microscopy imaging to assess expression and membrane targeting. Water- or cRNA-injected *Xenopus* oocytes were incubated overnight in Barth's medium containing 4% paraformaldehyde. After two washings for 10 min in PBS containing 100 mM glycine and a final 15 min rinse in PBS containing 10 mM triethanolamine, the fixated oocytes were cut with a scalpel into two halves, each containing either the animal or vegetal pole. The next steps follow the protocol described previously (*24*) with slight modifications. Briefly, the oocyte halves were incubated for at least 1 h in PBS plus 2% heat-inactivated horse serum (HS) followed by three washings, two in PBS plus 0.1% Triton X-100 and the final one in PBS plus 10 mM triethanolamine. Incubation with an antipeptide antibody (AK 1462; 20 μg/mL) raised against the final 15 C-terminal amino acids of GLUT1 was for 1.5−2 h, followed by three washings as mentioned above. The FITC-conjugated goat anti-rabbit IgG second antibody (Sigma, Deisenhofen, Germany), 1:100 diluted in PBS plus 2% HS, was added to the oocyte halves in the dark for 1 h. After three washings in PBS, the oocyte halves were mounted with Fluoromount-G (Southern Biotechnology Ass., INC, Birmingham) on microscope slides covered with cover glasses and kept in the refrigerator until microscopy. Fluorescence images were obtained with a confocal laser scanning microscope (LSM 510, Carl Zeiss Jena, Jena, Germany) by scanning the fluorescence from the cut surface of each oocyte through a depth of 10 μm.

[1] [1]Abbreviations: NEM, *N*-ethylmaleimide; pCMBS, *p*-chloromercuribenzenesulfonate; 2-DOG, 2-deoxy-D-[2, 6-^{3}H] glucose; 3-OMG, 3-O-methyl-D-[1-^{3}H] glucose; TM, transmembrane segment.

Materials. The oligonucleotides for mutagenesis were from GibcoBRL (Life Technologies, Eggenstein, FRG); the radiolabeled glucose analogues (2-deoxy-[2,6-^{3}H] glucose, spec. activity 26.2 Ci/mmol and 3-*O*-methyl-D-[1-^{3}H] glucose, spec. activity 2.5 Ci/mmol), were from Amersham Buchler (Braunschweig, FRG). NEM and pCMBS were purchased from Sigma (Deisenhofen, FRG). Female *Xenopus laevis* frogs were from African Xenopus Facility C. C. (Knysna, Republic of South Africa).

RESULTS

Basal Activities of Cysteine Replacement Mutants. Successive substitutions of each single amino acid with a cysteine residue throughout the putative length of transmembrane segments 2 or 7 of Cys-less GLUT1 exerted varying effects on transport activity. Table 1 demonstrates that the majority of cysteine replacement mutants is functional. It is however obvious that the individual substitutions with a cysteine residue of F72, of the glycine residues at position 75, 76, or 79, and of S80 drastically impaired the transport activities of the indicated mutant transporters. Important to note that within a cluster of three glycines residues (G75, G76, and G79) each glycine was absolutely essential with respect to transport activity, whereas glycine at position 84 tolerated cysteine substitution. Regarding the substitution-sensitive glycine residues, no data are available that would indicate how changes in side chain shape, size, polarity, or charge affect the transport process in wild-type GLUT1. The panel of cysteine replacement mutants from helix 7 also indicates that several amino acid residues are essential for full catalytic activity of the mutant transporters. Among these, G286C and N288C retained the lowest transport activities, being less than 10% of that of Cys-less GLUT1. Those mutants from both helices that retained transport activities of 20% or lower compared to Cys-less GLUT1 were taken for confocal laser scanning imaging in order to investigate expression and plasma membrane targeting. Confocal laser scanning microscopy (Figure 1) demonstrated that these mutant transporters are expressed in the *Xenopus* oocytes, even though to various degrees, and are targeted to the plasma membrane. In TM 2, the G75C and G76C mutant transporters exhibited the lowest level of expression compared to Cys-less GLUT1, in contrast to the G79C mutant that is expressed at the normal level. By comparison, the G286C mutant of TM 7 also displayed a relatively low fluorescence intensity. On the other hand, individual substitutions of the three glutamine residues, leading to a drastic impairment of their transport activities, did not (Q279C) or only slightly (Q282C or Q283C) affected the cellular expression of these mutant transporters.

Sulfhydryl Sensitivity Scanning using pCMBS or NEM. Table 1 documents the influence of 2 mM pCMBS or 10 mM NEM on 2-DOG (pCMBS) or 3-OMG (NEM) uptake rates when the respective sulfhydryl reagent was added to the medium 1 h before the uptake measurements were started. Among the various sulfhydryl reagents (reviewed in *25*) the organic mercury compound pCMBS, because of its strong acidic sulfonic acid group, restricts its reaction with sulfhydryl groups to those residues that are exclusively accessible to the aqueous solvent phase. Different from pCMBS, the NEM compound is a lipid-soluble and membrane-permeant sulfhydryl reagent able to access transmembrane segments.

Table 1: Effects of PCMBS or NEM on Cys-Replacement Mutants[a]

	basal uptake rate		pCMBS application		NEM application	
mutants	% activity ± S.D.	number of oocytes	% activity ± S.D.	number of oocytes	% activity ± S.D.	number of oocytes
			(a) TM 2			
L 67 C	116 ± 22	28	94 ± 27	66	86 ± 13	60
S 68 C	115 ± 26	50	98 ± 26	95	87 ± 16	88
V 69 C	115 ± 31	56	70 ± 17	71	2 ± 5	79
A 70 C	123 ± 26	15	4 ± 7	70	20 ± 10	44
I 71 C	104 ± 19	30	101 ± 29	96	78 ± 36	58
F 72 C	n.d.	90	n.d.[b]	51	n.d.	56
S 73 C	50 ± 30	88	3 ± 2	49	15 ± 9	88
V 74 C	112 ± 57	58	108 ± 30	69	78 ± 43	86
G 75 C	3 ± 5	79	19 ± 60	79	24 ± 39	60
G 76 C	3 ± 5	49	18 ± 40	74	24 ± 29	60
M 77 C	28 ± 13	38	46 ± 24	67	30 ± 18	57
I 78 C	76 ± 29	20	121 ± 49	63	62 ± 16	60
G 79 C	3 ± 6	56	12 ± 32	88	72 ± 94	57
S 80 C	7 ± 6	78	96 ± 91	159	26 ± 86	75
F 81 C	76 ± 26	67	102 ± 32	70	45 ± 23	58
S 82 C	58 ± 19	49	122 ± 44	63	57 ± 25	59
V 83 C	31 ± 9	48	119 ± 59	68	7 ± 8	60
G 84 C	98 ± 35	20	103 ± 32	69	31 ± 15	60
L 85 C	72 ± 29	40	103 ± 35	69	63 ± 21	58
F 86 C	56 ± 18	68	103 ± 28	65	42 ± 15	60
V 87 C	53 ± 20	68	97 ± 32	70	13 ± 7	60
			(b) TM 7			
I 272 C	78 ± 20	58	97 ± 33	77	34 ± 28	43
L 273 C	87 ± 26	49	92 ± 21	87	45 ± 22	67
I 274 C	88 ± 21	42	102 ± 27	65	18 ± 11	54
A 275 C	31 ± 11	41	91 ± 38	62	42 ± 24	46
V 276 C	103 ± 23	50	106 ± 28	70	58 ± 22	81
V 277 C	81 ± 16	29	106 ± 51	68	47 ± 47	84
L 278 C	66 ± 13	49	104 ± 28	74	49 ± 26	87
Q 279 C	16 ± 9	48	81 ± 28	99	7 ± 11	59
L 280 C	48 ± 14	28	93 ± 29	75	51 ± 17	90
S 281 C	83 ± 15	60	95 ± 21	87	43 ± 22	90
Q 282 C	17 ± 12	79	15 ± 8	79	73 ± 165	57
Q 283 C	20 ± 12	50	5 ± 8	79	22 ± 14	59
L 284 C	129 ± 28	59	97 ± 25	89	76 ± 21	89
S 285 C	125 ± 31	65	95 ± 22	95	90 ± 35	88
G 286 C	6 ± 11	60	388 ± 224	98	210 ± 425	73
I 287 C	82 ± 35	29	7 ± 5	70	15 ± 39	60
N 288 C	7 ± 4	55	14 ± 10	87	28 ± 37	57
A 289 C	73 ± 12	29	78 ± 23	49	81 ± 23	61
V 290 C	123 ± 19	28	44 ± 19	70	47 ± 20	63
F 291 C	104 ± 23	42	9 ± 4	77	2 ± 4	43
Y 292 C	25 ± 14	98	88 ± 29	63	49 ± 25	89

[a] Basal uptake rates of tritium-labeled 2-DOG were assessed in *Xenopus* oocytes expressing Cys-less GLUT1 and the indicated Cys-replacement mutants of TM 2 (a) and TM 7 (b). For a particular cysteine replacement mutant, mean 2-DOG uptake rates were determined from one to three separate experiments, each containing 15−30 single *Xenopus* oocytes, and expressed as a percentage of that of the Cys-less GLUT1. For each group, equal amounts of cRNA were injected. Uptake rates of water-injected *Xenopus* oocytes were alaways subtracted. Taken from numerous independent experiments, a representative mean uptake rate of the Cys-less GLUT1 was 3.15 ± 0.37 pmol of 2-DOG oocyte^{-1} min^{-1}. The cysteine-replacement mutants were incubated with 2 mM pCMBS or 10 mM NEM at room temperature for 60 min. Thereafter, uptake rates were assessed for tritium-labeled 2-DOG in the pCMBS groups and for tritium-labeled 3-OMG in the NEM groups. For each single cysteine-replacement mutant, the retained transport activities after treatment with the sulfhydryl reagents were expressed as a percentage of uptake rates in the absence of the respective compound. For single Cys-replacement mutants, values present the mean percentage ± standard deviation of retained uptake rates obtained from two to six separate experiments, each including 20−30 single *Xenopus* oocytes. Values concerning amino acid residues 67 to 74 and 284 to 294 are mostly from ref (*14*). [b] n.d. means not detected.

With respect to interaction with water-accessible sulfhydryl groups, both reagents exerted similar inhibitory effects on

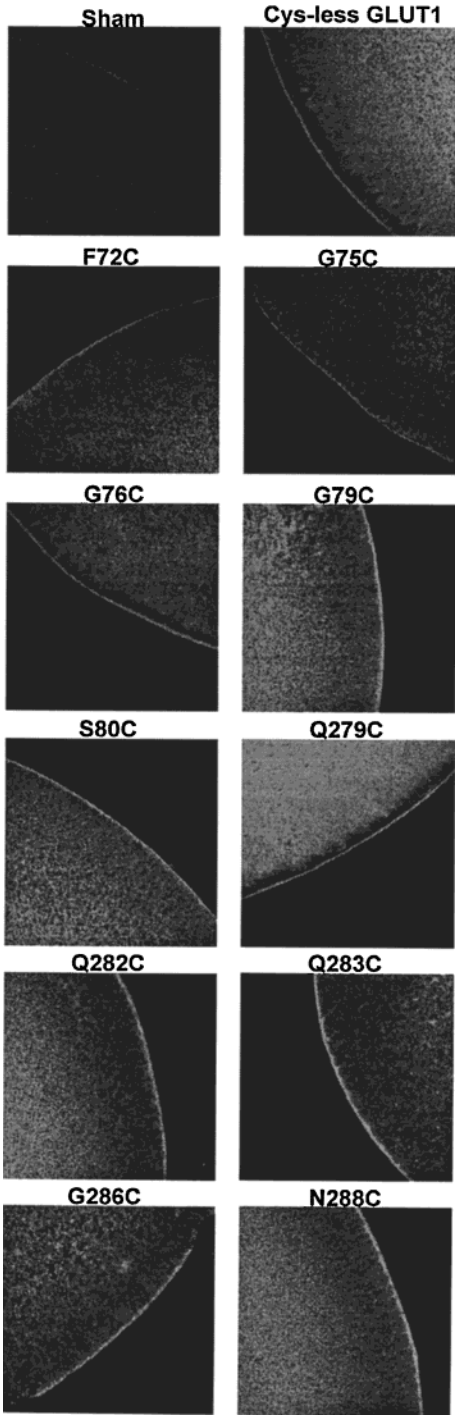

FIGURE 1: Confocal laser scanning microscopy of selected cysteine replacement mutants. A set of water- and cRNA-injected Xenopus oocytes was split into oocytes either taken for 2-DOG uptake measurements or used for microscopy. Five to six *Xenopus* oocytes per group of water-injected *Xenopus* oocytes, Cys-less GLUT1-expressing *Xenopus* oocytes, or *Xenopus* oocytes expressing mutants with basal transport activities of 20% or less than that of Cys-less GLUT1 were randomly selected for microscopy, fixated, and cut with a scalpel along the equator into two halves (for details see Materials and Methods). An anti-GLUT1 antibody raised against the final 15 C-terminal amino acids (AK 1462; 20 μg/mL) was used as the first antibody; the second antibody was a commercially available FITC-conjugated goat anti-rabbit IgG (1:100 diluted in PBS plus 2% horse serum). Images from two to three oocyte halves per group were obtained by scanning the fluorescence from the cut surface of each oocyte halve through a depth of 10 μm.

transport activity of the mutant transporters. Differences, as indicated for G79C or Q282C, may result from the negative charge introduced by pCMBS into local helical structures. In both helices, a few membrane-harbored residues reacted with the membrane impermeant pCMBS, and the positions were restricted to the exofacial portion of the transmembrane segments. With respect to the alternate conformational transporter model, it was important to show whether pCMBS from the inner side of the plasma membrane would react with one of these sulfhydryl groups. Even at an intracellular concentration of 3 mM, pCMBS did not affect 2-DOG uptake rates (data not presented). This indicates that amino acid residues in both membrane helices were not accessible from the endofacial side of the plasma membrane or that an extreme dilution of freely diffusible pCMBS occurred due to the huge number of reactive sulfhydryl groups in cytoplasmic proteins binding this compound. Taking into consideration cysteine replacement mutants with a basal activity greater than 10% of Cys-less GLUT1, pCMBS-induced transport impairment by more than 50% was observed in three mutant transporters from TM 2 (A70C, S73C, and M77C) and five mutant transporters from TM 7 (Q282C, Q283C, I287C, V290C, and F291C). Regarding all those cysteine replacement mutants that displayed extremely low basal transport activities (<10% of Cys-less GLUT1), the indicated influence of pCMBS or NEM on transport has to be interpreted with particular restraint. The reason the cysteine replacement mutant G286C exhibited transport stimulation after treatment with pCMBS or NEM remains an open question.

Locations of Substitution- and Sulfhydryl Reagent-Sensitive Residues in an α-Helical Structure. Figure 2 demonstrates convincingly that the pCMBS-sensitive sulfhydryl groups are present only in the exofacial portion of the plasma membrane and cluster on one side of the helix. Included in this assignment were only those mutants that match our threshold criteria of a basal activity higher than 10% of Cys-less GLUT1 and a pCMBS-induced transport inhibition by more than 50%. Important to note that the distribution pattern of pCMBS-sensitive residues is not consistent with an all β-strand membrane topology model, as proposed by Fischbarg et al. (*26*). Those amino acids which are extremely sensitive to a substitution with cysteine are located in the helical structure opposite to the extracellular water-accessible residues. The notion that these mutations might interfere with a transport-competent folding is consistent with their locations opposite to pCMBS-accessible positions. The cluster of three glycine residues could provide space for a complementary bulky or hydrophobic partner from another helix segment (helix−helix interaction) or a lipid tail (helix−lipid interaction). In TM 2, the NEM-sensitive positions corresponding to valine residues 69, 83, and 87 are lined up along the same helix face as the glycine residues whose replacement with cysteine led to an extremely low basal transport activity. It seems that the volume-equivalent substitution of cysteine for valine is tolerated, but addition of the bulky NEM disturbs a transport-competent folding. These results support the notion of a very tight packing at this particular portion of helix 2. Regarding TM 7, the Q279C and I274C mutants are sensitive exclusively to NEM. As a result of its membrane-burried location, Q279 is very likely excluded from the aqueous solvent phase and thus not accessible to

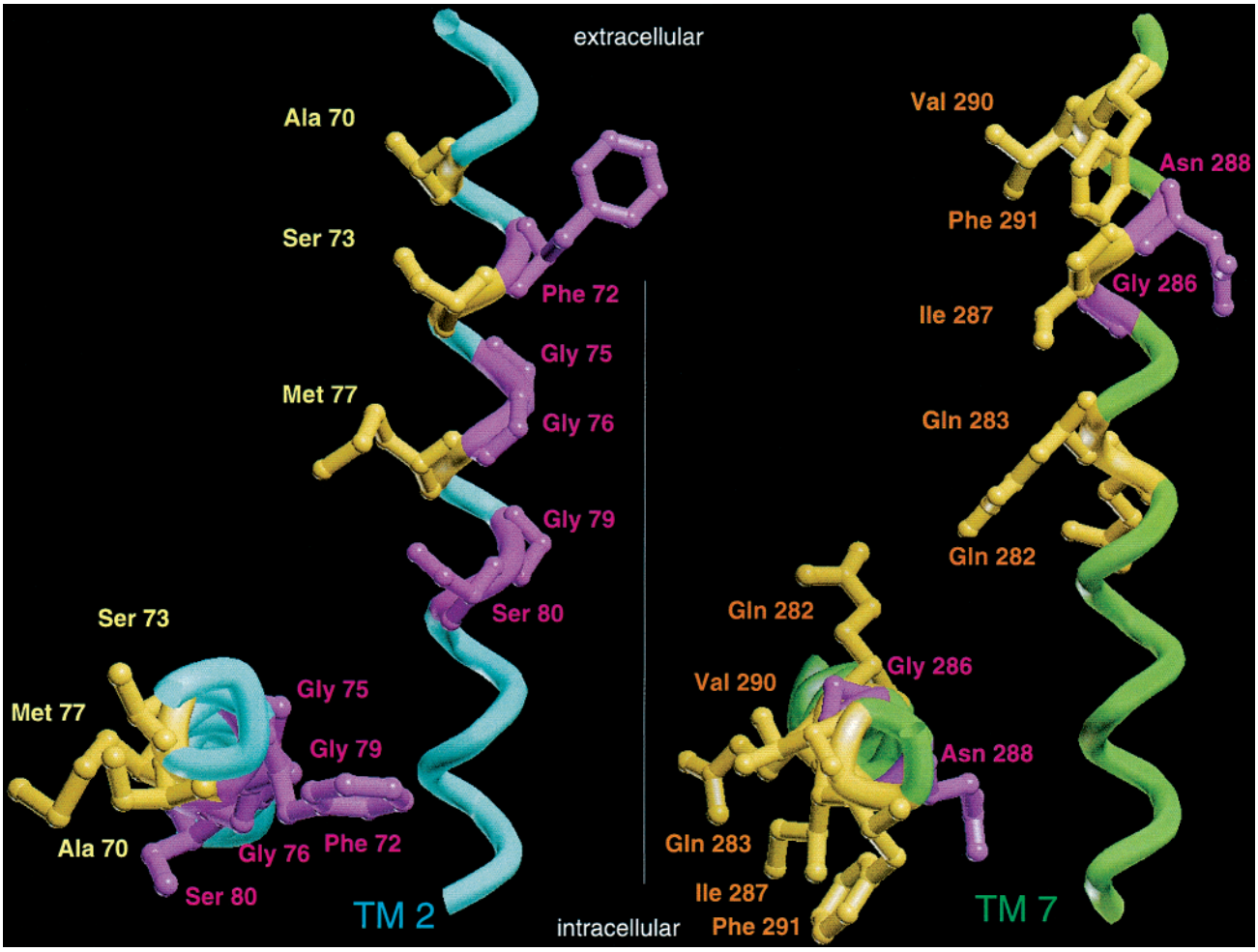

FIGURE 2: Computer models of helix 2 and helix 7 presented at extracellular and transmembrane views. Residues in magenta represent cysteine insertions that reduced the basal catalytic activities of the respective mutants to less than 10% of Cys-less GLUT1. Residues in yellow indicate those sulfhydryl groups within the Cys-replacement mutants that fulfill both criteria, i.e., having basal activities greater than 10% of Cys-less GLUT1 and exhibiting transport inhibition by at least 50% after pCMBS treatment. Visualization and sequence homology searches of the 3D-protein structure database (PDB) were done with the program package Sybyl 6.4, TRIPOS Inc., St. Louis.

pCMBS. Isoleucine-274 is localized to the opposite face of the helix like N288. Addition of NEM at position 274 may disturb the tight hydrophobic packing at the endofacial half of TM 7.

DISCUSSION

Substitution-Sensitive Residues. Successive substitution of adjacent native residues in TM 2 or TM 7 with a cysteine residue, one at a time, revealed that the majority of cysteine replacement mutants is functional. Five mutants in helix 2, however, had basal transport activities lower than 10% of Cys-less GLUT1 and the same number of mutants in helix 7 displayed uptake rates lower than 20% of the parental construct. In helix 2, three glycine residues out of a total number of four were irreplaceable (<10% retained activity of Cys-less GLUT1). It is important to note that the replacement of glycine residues at the positions 75, 76, and 286 resulted in lower cellular expression of mutant transporters compared with Cys-less GLUT1, whereas expression for the G79C mutant was unimpaired. The reasons for the varying transporter levels after substitution of glycine for cysteine at different positions are not known but could reflect variation in cRNA half-lives. The arrangement of three glycine residues on a helix face opposite to the pCMBS-accessible residues could form a grove to give space to a lipid side chain. This structural detail is reminiscent of a similar detail in the recently presented heterotrimeric bacteriorhodopsin crystal structure (*27*). The presence of several glycine residues facilitates an interaction with another transmembrane helix or lipid side chains of the plasma membrane. By analogy, a cluster of three glycine residues lines up on one side in helix D of bacteriorhodopsin allowing local lipid−protein interaction (*27*, *28*).

Among those amino acid residues in helix 7 that responded to cysteine substitution with a decline in transport activity by about 80% or greater are Q282 and Q283. Both residues were accessible to extracellular pCMBS and hypothetically may form hydrogen bonds with sugar moieties. Glutamine at position 279 was also sensitive to a substitution by cysteine (16% remaining activity). The Q279C mutant, located in a deeper portion within the helix, exhibited only marginal changes in transport activity after pCMBS treatment, but was extremely sensitive to NEM. The helical conformation of TM 7 with an arrangement of pCMBS-accessible residues at the same face of the exofacial portion of this helix is supported by a 3D-sequence homology consideration at the 3D

protein structure database (PDB). The portion (LQLSQQLSG) of the TM 7 sequence was found with a high homology in helix α1 (L**Q**LSQS*L*K*G*) at the crystal structure of HFE, a nonclassical major histocompatibility complex class I protein (*29*). It is interesting to note that the crystal structure contains a homodimer and that the glutamine residue, which corresponds to G282 in TM 7 of GLUT1, mainly participates in forming this homodimer by mutual glutamine interaction (glutamine finger). Even if there is no functional relationship between both proteins, possible dimerization of GLUT1 via a "glutamine phase" adds a novel point to the discussion on oligomerization of the GLUT1 glucose transporter. The indicated structure at least demonstrates that the "glutamine phase" of TM 7 has the potential of hydrophilic and helix−helix interaction.

Sufhydryl Group Sensitivity Scanning. Almost all models of helical arrangement in GLUT1 (*7*−*11*) propose that helix 7 takes part in the lining of a sugar translocation pathway. Conclusion drawn from an analysis of 65 members of the major facilitator superfamily (*6*) suggests that TM 2 could be a suitable candidate for a lining of a solute transfer pathway. This notion is supported very recently by Widdas' proposal of a helix arrangement (*11*) that takes into account the lining of the translocation pathway and the pore barrier. Together with eight additional helices TM 2 is predicted to surround an open cleft in close proximity to helix 3, 4, and 11. In a different model containing a helix bundle of five transmembrane segments, helix 2 is also predicted to line the transmembrane channel for glucose (*10*). The sulfhydryl sensitivity scanning data suggest that helices 2 and 7 each form an exofacial cleft in the plasma membrane, as has been shown for helix 5 (*15*). The indicated three regions (i.e., TM 2, TM 5, and TM 7) have in common that residues within their endofacial membrane portions were not accessible to the extracellular aqueous solvent. Moreover, pCMBS had no access to intramembranous residues if present on the inner surface of the plasma membrane after its microinjection into the cytoplasm of *Xenopus* oocytes. The difference between helix 5 and the here investigated transmembrane segments 2 and 7 refers to the fact that a particular portion in TM 5 may participate in forming a glucose binding site, as justified by the positive protection assay (*30*). It is important to note that a sugar translocation pathway may not be the only permeation pathway in GLUT1. Facilitated permeation of water (*31*, *32*) or dehydroascorbic acid (*33*) has been detected in GLUT1 so that distinct helices may be involved in different pore-forming structures.

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BI992160X