Cysteine Mutagenesis of the Amino Acid Residues of Transmembrane Helix I in the Melibiose Carrier of *Escherichia coli*†

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ABSTRACT: The melibiose carrier of *Escherichia coli* is a sugar−cation cotransport system that utilizes Na^+, Li^+, or H^+. This membrane transport protein consists of 12 transmembrane helices. Starting with the cysteine-less melibiose carrier, cysteine has been substituted individually for amino acids 17−37, which includes all of the residues in membrane helix I. The carriers with cysteine substitutions were studied for their transport activity and the effect of the water soluble sulfhydryl reagent *p*-chloromercuribenzenesulfonic acid (PCMBS). Cysteine substitution caused loss of transport activity in six of the mutants (G17C, K18C, D19C, Y32C, T34C, and D35C). PCMBS caused greater than 50% inhibition in eleven mutants (F20C, A21C, I22C, G23C, I24C, V25C, Y26C, M27C, Y28C, M30C, and Y31C). We suggest that the residues whose cysteine derivatives were inhibited by PCMBS face the aqueous channel and that helix I is completely surrounded by aqueous environment. Second site revertants were isolated from K18C and Y31C. The revertants were found to have mutations in helices I, IV, and VII.

The melibiose transport carrier of *Escherichia coli* is responsible for the transport and accumulation of a variety of α-galactosides. It is a transport system that couples the downhill entry of a cation with the accumulation of the sugar. This carrier is of particular interest because of the wide variety of cations (H^+, Na^+, or Li^+) that can be utilized for cotransport (for a review see ref *1*).

This integral membrane protein has 12 transmembrane helices based on hydropathy plots (*2*), phoA fusions (*3*, *4*), and proteolytic digestion experiments (*5*). The amino and carboxy termini of the protein are on the cytoplasmic side of the membrane (*6*). These results provide good evidence for a two-dimensional structure where the protein forms 12 α-helical transmembrane domains connected by hydrophilic loops.

Cation recognition by the melibiose carrier has been investigated by site-directed mutagenesis. Studies have focused primarily on acidic residues on membrane-spanning helices in the amino-terminal portion of the carrier. Neutral amino acid substitutions for D19 (helix I), D55 and D59 (helix II), and D124 (helix IV) cause the loss of Na^+-coupled sugar transport (*7*−*10*). In these mutants, sugar binding is comparable to that of wild-type carrier in the absence of Na^+, but this binding is no longer stimulated by Na^+. These results have led to the model in which aspartic acid residues at positions 19, 55, 59, and 124 provide an important part of the network for the binding of cations by the melibiose carrier (*1*, *11*).

Very little is known about the structure of different regions of this carrier protein. One experimental approach used to study structure and function of membrane proteins has been the use of cysteine mutagenesis. Cysteine has been used to substitute for individual amino acid residues in the cysteine-less melibiose carrier in helix II (*12*) and helix XI (*13*). The importance of several residues was determined. Several cysteine residues on one side of the helix were inhibited by the organic mercurial PCMBS. These latter data suggest that both helices II and XI have one face of the helix accessible to the aqueous channel.

In the present study, cysteine was substituted individually for each amino acid residue in helix I. PCMBS, which is lipid-impermeable and reacts with cysteine residues, was added to the individual cysteine mutants and inhibition of transport occurred in a number of positions that were located around the entire perimeter of the helix. This suggests that this helix faces the aqueous environment on all surfaces.

Several cysteine substitutions led to low transport activity and appeared as white clones on melibiose MacConkey indicator plates. When these clones were incubated for several days, rare red clones appeared. These were restreaked to purify, and DNA was isolated from cells derived from a red clone. The DNA from each second site revertants was sequenced. The second site revertants derived from the mutants from helix I were D19N (helix I), M123R (helix IV), S234L and L236F (helix VII). It is inferred from these data that helix I is probably close to helices IV and VII.

EXPERIMENTAL PROCEDURES

Materials. Melibiose (6-*O*-α-galactopyranosyl-D-glucopyranoside) and PCMBS (*p*-chloromercuribenzoicsulfonic acid) were purchased from Sigma. [^{3}H] melibiose was a generous gift from Dr. Gerald Leblanc of the Department of Biologie Cellulaire et moleculaire du CEA, Villefranche-sur-mer, France. [α-^{33}P] dATP was from Andotek. Bacteriological

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media were from Difco. [^{35}S]-Protein A was purchased from Amersham. All other chemicals were reagent grade.

Bacterial Strains and Plasmids. E. coli DW1 (LacI$^+$ ΔZY ΔmelAB) (*15*) was used as the host strain for plasmids. The gene for the cysteine-less melibiose carrier was inserted into the vector pKK223-3 (Pharmacia Biotech) as described previously (*16*). This plasmid was used as the starting material for site-directed mutagenesis.

Site-Directed Mutagenesis. The Quick Change, Site Directed Mutagenesis kit (Stratagene) was used to replace the selected amino acids with cysteine. The appropriate mutagenic primers (ranging from 40 to 45 nucleotides long) were synthesized by Dr. Charles Dahl, Harvard Medical School.

DNA Sequencing. Double-stranded plasmid DNA was isolated with the QIAprep Spin Miniprep Kit (Qiagen). Sequencing was done using (α-^{33}P)dATP with the Amplicycle Sequencing Kit (Perkin-Elmer). The melB gene was sequenced from plasmid DNA using primers that anneal at approximately 200-bp intervals.

Immunodetection and Quantitation of the Melibose Carrier. The amount of melibiose carrier present in each strain was determined as previously described by Lolkema et al. (*17*). In summary, a known quantity of cells was lysed with NaOH/SDS and neutralized on nitrocellulose filters. Filters were incubated with BSA to block nonspecific binding, followed by incubation with a polyclonal antibody, antiM-Bct10 (*6*) directed against the corboxyl-terminal 10 amino acids of the protein. [^{35}S]Protein A (Amersham) was used to label the bound antibody and the amount of the label was quantified by liquid scintillation counting. To correct for nonspecific adsorption, the value obtained for the strain DW1/pKK223-2 (melB$^-$) was used as a background control in each experiment. Values for each mutant are presented as a percentage of the wild-type protein level.

Assays for Melibiose Transport by Intact Cells. The plasmid-containing strains were grown in LB medium containing 100 μg/mL ampicillin until they reached log phase of growth. The cells were harvested and washed twice with 100 mM MOPS buffer adjusted to pH 7 with Tris base and containing and 0.5 mM $MgSO_4$.The cells were resuspended in the same buffer to a cell density corresponding to about 1 mg dry wt/mL. Cations were added to a final concentration of 10 mM. [^{3}H]Melibiose (2 μCi/mL) was added to an aliquot of the cell suspension to a final concentration of 0.l mM. After incubation for 10 min at room temperature, a 200 μL sample was filtered through a 0.65 μm cellulose nitrate filter (Sartorius). The filters were immediately washed with 4 mL of buffer and counted in Liquiscint (National Diagnostics). The effect of the sulfhydryl reagent PCMBS on transport was measured by preincubating the cell suspension with the indicated concentration of PCMBS for 10 min in the presence of 10 mM Na^+ at room temperature. The radioactive melibiose was then added and transport assayed as described.

Assay for Melibiose Transport in Inside-Out Vesicles. Forty milliters of plasmid-containing cells were grown, harvested, washed, and resuspended in 10 mL of buffer A that contained 250 mM sucrose with 10 mM NaCl. Inside-out vesicles were prepared by passing the cell suspension through a French Press (SLM-Aminco) at 8000 psi (*18*). Unbroken cells were removed by centrifugation, and the vesicle suspension was assayed for transport. An aliquot of vesicles (310 μL) was warmed to room temperature and the PCMBS was added. The suspension was incubated for 10 min at room temperature and [^{3}H]melibiose (38 μCi/mL) was added to a final concentration of 50 μM. After 60 s, the vesicle suspension was filtered through a 0.22 μm nitrocellulose filter (GSTF, Millipore), and the filter was washed with buffer containing sucrose. The filter was then counted in Liquiscint in the presence of 0.1% Triton X-100.

Table 1: Phenotype and Expression by Single Cysteine Substitution of the Cysteine-less Melibiose Carrier

cell	colony color on melibiose MacConkey plate[a]	carrier protein expression (% of cysteine-less parent)[b]
cysteine-less parent	red	100
G17C	white	81 ± 2
K18C	light pink	24 ± 2
D19C	pink	31 ± 3
F20C	red	34 ± 3
A21C	red	131 ± 13
I22C	red	151 ± 8
G23C	red	258 ± 26
I24C	red	267 ± 14
V25C	red	201 ± 19
Y26C	red	113 ± 3
M27C	pink	119 ± 6
Y28C	light pink	116 ± 5
L29C	red center	130 ± 8
M30C	red	143 ± 7
Y31C	light pink	117 ± 9
Y32C	white	45 ± 3
Y33C	pink	104 ± 14
T34C	white	84 ± 8
D35C	white	137 ± 11
V36C	light pink center	89 ± 9
V37C	red	89 ± 13

[a] Cell tested was DW2/plasmid. [b] Expression was determined by immunoblot method in DW1 cells.

RESULTS

Cysteine Mutagenesis of Residues in Helix I. Site-directed mutagenesis was used to convert 21 amino acid residues of helix I individually to cysteines. This was carried out with the cysteine-less melibiose carrier. In all cases, only the single desired amino acid change was found after the gene was completely sequenced.

The phenotype of each mutant was observed. The melB gene on a plasmid was placed into the cell DW2 which contains the melA gene (α-galactosidase). Such plasmid containing cells were streaked on melibiose MacConkey indicator plates (Table 1). The cell containing the normal melB gene took up melibiose, fermented the sugar to acid products, and produced a bright red clone on the indicator plate. Eleven of the mutants each produced a colony that was red or had a red center. Four clones (G17C, Y32C, T34C, and D35C) were white, and seven (K18C, D19C, M27C, Y28C, Y31C, Y33C, and V36C) were pink or light pink.

Quantitation of the Melibiose Carrier by Immunological Methods. The amount of melibiose carrier that was inserted into the membrane was determined by using a polyclonal antibody directed against the C-terminal 10 amino acids of the carrier protein. The amount of carrier protein was expressed as a percentage of the wild-type value (Table

Table 2: Transport[a] of Melibiose[b] by Cysteine Mutants[c] in Helix I with Three Cations[d] (In/Out)[e]

cell	H^+	Li^+	Na^+
cysteine-less parent	13 ±1	113 ± 3	161 ± 5
G17C	0.3 ± 0.1	3 ± 0.4	2 ± 0.4
K18C	0	0.4 ± 0.1	0
D19C	0	0.3 ± 0.1	0.1
F20C	17 ± 0.5	105 ± 6	113 ± 2
A21C	20 ± 3	187 ± 3	143 ± 19
I22C	24 ± 2	228 ± 3	141 ± 10
G23C	8 ± 0.4	134 ± 4	122 ± 3
I24C	2 ± 0.2	38 ± 1	21 ± 1
V25C	7 ± 0.4	96 ± 9	95 ± 5
Y26C	7 ± 0.3	111 ± 1	59 ± 3
M27C	13 ± 0.2	65 ± 2	112 ± 6
Y28C	9 ± 1	69 ± 6	88 ± 5
L29C	14 ± 1	58 ± 6	55 ± 2
M30C	13 ± 1	66 ± 1	97 ± 5
Y31C	13 ± 0.4	32 ± 1	29 ± 1
Y32C	0	0.2 ± 0.1	0.2
Y33C	9 ± 0.2	32 ± 1	39 ± 2
T34C	2 ± 0	1 ± 0.1	4 ± 0.5
D35C	0	0	0
V36C	8 ± 0	94 ± 10	116 ± 5
V37C	12 ± 1	107 ± 9	128 ± 2

[a] The standard deviation was calculated on the basis of three determinations. [b] Melibiose concentration was 0.1 mM. [c] Cells used were DW1/mutant plasmid. [d] Na^+ and Li^+ were 10 mM; H^+ was from 100 mM MOPS. [e] Melibiose concentration inside the cell/outside the cell.

Table 3: PCMBS[a] Inhibition of Melibiose Transport[b] by Cysteine Mutants in Helix I

cell[c]	% activity without PCMBS	% activity with PCMBS
cysteine-less parent	100	94 ± 3
G17C	1 ± 0.3	2 ± 0.3
K18C	0	0
D19C	0	0
F20C	82 ± 1	39 ± 1
A21C	98 ± 13	11 ± 0
I22C	97 ± 7	0
G23C	84 ± 2	0
I24C	15 ± 0.4	1 ± 0.2
V25C	59 ± 3	0
Y26C	37 ± 2	0
M27C	69 ± 4	0
Y28C	55 ± 3	19 ± 1
L29C	34 ± 1	37 ± 3
M30C	60 ± 3	3 ± 0
Y31C	24 ± 1	5 ± 1
Y32C	0	0
Y33C	33 ± 2	23 ± 0
T34C	3 ± 0	1 ± 0
D35C	0	0
V36C	63 ± 3	43 ± 2
V37C	107 ± 2	98 ± 3

[a] PCMBS concentration was 100 μM. [b] Melibiose concentration was 0.1 mM, Na^+ concentration was 10 mM, in 100 mM MOPS. [c] Cells tested were DW1/plasmid. The standard deviation was calculated on the basis of three determinations.

1). The expressions were generally good. The lowest expression was 24% for K18C. In two cases (D19C and F20C), expression was 31 and 34% respectively. An interesting observation was that in three cases (G23C, I24C, and V25C) the expressions were twice or more that of the normal value.

Measurement of Cation-Coupled Melibiose Accumulation. Membrane transport of melibiose was measured in DW1 (without melA plasmid). The melibiose entering this cell cannot be metabolized, and it accumulates inside the cell as the free sugar. In the presence of Na^+ the melibiose accumulated in the cell containing the normal melB gene was 161-fold (Table 2). In the presence of Li^+ the accumulation was 113-fold, and with H^+ it was 13-fold. In four cases (K18C, D19C, Y32C, and D35C), no accumulation was observed with any of the three cations. G17C and T34C showed extremely little accumulation. Two mutants (A21C and I22C) demonstrated abnormal cation recognition as they showed accumulation that was greater than normal with H^+ or Li^+ and accumulation with Li^+ was greater than that with Na^+ (Table 2).

Effects of PCMBS. The water-soluble mercurial PCMBS caused greater than 50% inhibition of transport in the intact cells of 11 mutants (F20C, A21C, I22C, G23C, I24C, V25C, Y26C, M27C, Y28C, M30C, and Y31C) (Table 3). In one case (K18C), the activity was zero so that inhibition could not be tested. However, the second site revertants K18C/M123R and K18C/D19N showed complete inhibition by PCMBS (Table 5). Thirteen of the cysteine mutants with good transport activities (>30% of the normal: F20C, A21C, I22C, G23C, V25C, Y26C, M27C, Y28C, L29C, M30C, Y33C, V36C, and V37C) were tested as inside-out vesicles with PCMBS (Figure 1). These mutants showed the same pattern of PCMBS inhibition with the inside-out vesicles as with the intact cells.

Second-Site Revertants. The cysteine mutant K18C showed very low transport activity and the cell containing this mutant (DW1/pmelA/pK18C) was very light pink on melibiose MacConkey indicator plates. When plates containing these cells were incubated for several days at 37° C a rare bright red spot was observed. This red area was restreaked to purify, and the plasmid DNA was isolated from one of the purified red clones. This DNA was then used to transform DW1/pmelA to verify that the plasmid carrying the melB gene was responsible for the red phenotype on melibiose MacConkey plates. The DNA was then sequenced. Two second-site revertants were isolated from K18C (Table 4). In one case (K18C/M123R), a neutral amino acid in helix IV was changed to an arginine. In a second case (K18C/D19N), the neighboring aspartic acid was changed to a neutral residue. In both of these second site revertants, the transport activity was much greater than the parent (K18C) but still much less than normal melB (Table 5). Another cysteine mutant (Y31C) also had very little activity and was almost white on melibiose MacConkey indicator plates. After several days of incubation, a few bright red clones appeared. These were purified, and DNA isolated and sequenced. Two second-site revertants were isolated: Y31C/S234L and Y31C/L236F. The second site mutations were located in helix VII. Both of these revertants showed good transport activities. The Y31C/L236F revertant showed about normal transport with Na^+ or Li^+ and twice normal activity with H^+ (Table 5).

DISCUSSION

Very little is known about the three-dimensional structure of the melibiose transport protein. Information is available on the two-dimensional structure in relation to the phospholipid of the membrane. Hydropathy plots (*2*), melB-phoA

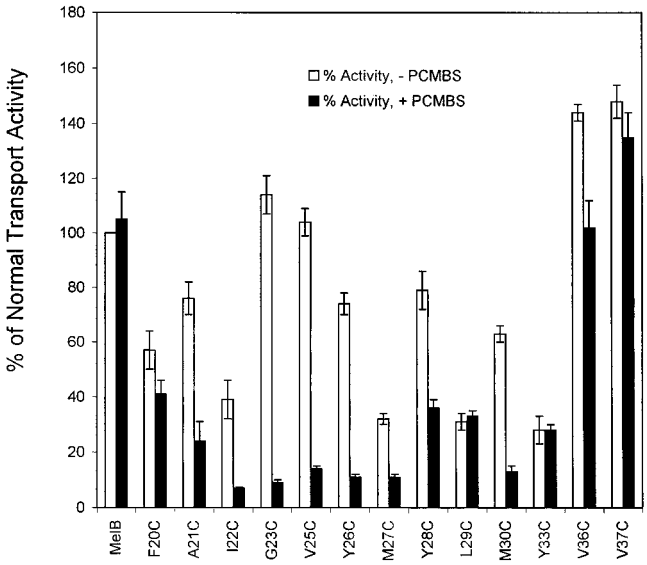

FIGURE 1: The effect of PCMBS on the transport of melibiose by inside-out vesicles. Melibiose concentration was 50 μM, PCMBS concentration was 100 μM. All tests were done in the presence of 10 mM Na^+ at room temperature. Melibiose was incubated with the membrane vesicles for 1 min before sampling.

Table 4: Phenotype and Expression of Second-Site Revertants of Cysteine Mutants

cell	color of colony on melibiose MacConkey plate[a]	helix position of second-site	expression level[b]
C-less parent	red		100
K18C	light pink		24 ± 2
K18C/M123R	red	IV	43 ± 5
K18C/D19N	red	I	44 ± 9
Y31C	light pink		117 ± 9
Y31C/S234L	red	VII	83 ± 3
Y31C/L236F	red	VII	60 ± 9

[a] Cell was DW1 with melA plasmid. [b] Expression was measured by the immunoblot method using DW1 cells.

Table 5: Transport of Melibiose[a] by Second-Site Revertants[b] in the Presence of Cations[c] and PCMBS[d]

	in/out			
cell	H^+	Li^+	Na^+	PCMBS/Na^+
C-less parent	13 ± 1	113 ± 3	161 ± 5	156 ± 1
K18C	0	0.4 ± 0.1	0	0
K18C/M123R	2 ± 0.4	29 ± 1	21 ± 1	0
K18C/D19N	2 ± 0.1	62 ± 5	46 ± 1	0
Y31C	13 ± 0.4	32 ± 1	29 ± 1	6 ± 0
Y31C/S234L	15 ± 1	71 ± 3	64 ± 4	27 ± 2
Y31C/L236F	23 ± 0	147 ± 5	143 ± 4	62 ± 2

[a] Melibiose concentration was 0.1 mM. [b] All in DW1 cells. [c] H^+ was from 100 mM MOPS; Li^+ and Na^+ were 10 mM. [d] PCMBS concentration was 100 μM (in the presence of 10 mM Na^+).

fusions (*3*, *4*), and proteolytic digestion (*5*) provide evidence for 12 transmembrane α-helices which traverse the phospholipid bilayer. However, little is known about which helices line the aqueous channel through which the sugar and cation are transported.

Preliminary data on the arrangement of helices came from previous studies of second site revertants of R52S and R52V (*19*). These two mutants failed to ferment melibiose on MacConkey indicator plates (white in color). From the white clones, arose rare red melibiose positive revertants containing a second-site mutation in addition to the original mutation. The second-site revertants were found to have mutations in helices I, II, IV, VII, and X. It is inferred from these studies that the five helices are close together. A similar study was carried out with K377C and L391C which failed to show transport activity (*20*). From these two inactive mutants second-site revertants were found that contained mutant sites in helices I, II, and V.

A second approach to the study of structure has been the use of the water-soluble and lipid-impermeable mercurial PCMBS to test inhibition of the transport of individual cysteine mutants in a helix. If PCMBS fails to inhibit a carrier containing a cysteine residue, it is probably because the cysteine faces the phospholipid which the water-soluble inhibitor cannot enter, even though it is not impossible that the cysteine residue is located in an aqueous position which the PCMBS cannot have access to. On the other hand, inhibition indicates that the cysteine is in an aqueous channel and that the inhibitor containing benzene sulfonic acid obstructs the movement of sugar through this channel. We have found (in unpublished observations) that PCMBS has no inhibition of transport of any of the cysteine mutants of helix VI. Each residue in helix II (in the cysteine-less carrier) was individually changed to cysteine and the effect of PCMBS tested on each (*12*). The pattern of inhibition indicated that the residues sensitive to inhibition were clustered along a single face of the helix suggesting that this side of the helix faced the aqueous channel while the other side faced the phopholipid. Similar results were obtained in the study with residues of helix XI (*13*). We deduce from these experiments that PCMBS inhibition is a good criterion for predicting which region of the helix faces the aqueous environment.

The present study is a continuation of the study of PCMBS inhibition of cysteine mutants using helix I as the test object. In 11 out of 21 cysteine mutants of helix I, PCMBS caused inhibition of transport in intact cells. The same pattern was observed for inside-out vesicles (Figure 1). This suggests that PCMBS has the same ease of access from both the cytoplasmic and the periplasmic sides to the interior of the channel. There are cases in which a carrier protein in the membrane has "sidedness" on a helix. From the periplasmic side, PCMBS cannot reach the residues on the cytoplasmic side and vice versa, but a certain region in the interior is accessible to PCMBS from both sides (*14*). It is posssible that the conversion of a hydrophobic residue such as tyrosine to cysteine creates an artificial hydrophilic region of the protein with which PCMBS can interact and therefore not reflect the nonmodified parental structure. However, a variety of different amino acids were substituted by cysteine with 11 cysteines inhibited by PCMBS being distributed all around the circumference of helix I (Figure 2). Thus, we speculate that helix I is probably located in the center of the aqueous channel and is surrounded by aqueous medium (Figure 3). Similar observations were made by Hruz and Mueckler (*21*) on helix VII of the GLUT1 glucose transporter. In that study, PCMBS was added to each cysteine mutant of helix VII and PCMBS sensitive residues were positioned over a majority of the circumference of the helical wheel plot. Thus, they concluded that helix VII was completely surrounded by aqueous medium.

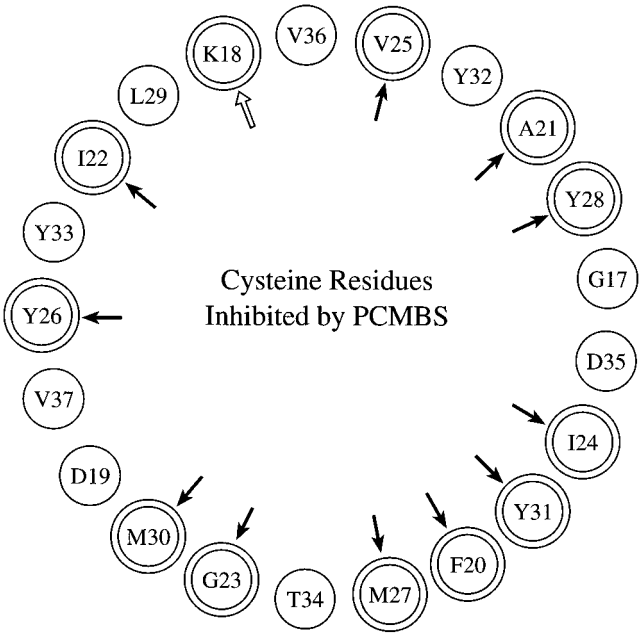

FIGURE 2: Cysteine residues inhibited by PCMBS. A helical wheel plot of residues in helix I. Residues inhibited by PCMBS are indicated with a black arrow. The open arrow (K18C) has no transport activity but K18C/D19N and K18C/M123R have activity and are inhibited 100% by PCMBS.

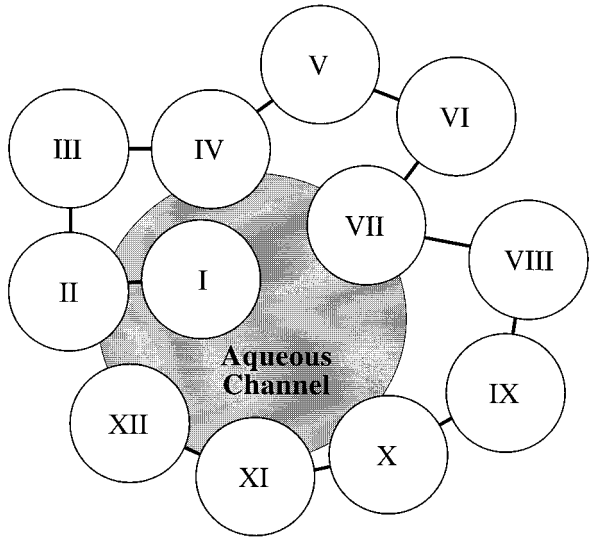

FIGURE 3: A proposed arrangement of helices showing that helix I is surrounded by aqueous medium.

The considerable number of hydrophobic residues (F20, I22, I24, V25, Y26, Y28, L29, Y31, Y32, Y33, V36, and V37) outnumbers the hydrophilic residues (K18, D19, T34, and D35), and therefore one would anticipate that helix I would have hydrophobic binding sites in the phospholipid or in the hydrophobic regions of other helices. On the other hand, the evidence for the helix being in the aqueous environment is the large number of PCMBS sensitive positions distributed around the entire helix. Although there is a remote possibility that this helix may be lying on the surface of the membrane facing the aqueous environment, this is unlikely because in that case it would not have extensive inhibition sites to the transport.

All three of the charged residues (K18, D19, and D35) are sensitive to mutagenesis. Conversion of each cysteine resulted in the complete loss of activity. K18C gave rise to the second-site mutation D19N. This suggests a salt bridge between Lys18 and Asp19. An additional second-site revertant from K18C was found to be M123R, which suggests that the positive charge is important for function of the carrier and the charge normally at position 18 can be substituted by a similar charge at position 123 to regain partial activity. Both of these positions are located near the cytoplasmic ends of their respective helices. Thus, if helix I and helix IV are close together positions 18 and 123 would be immediately adjacent. Y31C gave rise to two revertants, S234L and L236F (helix VII). This suggests that helix I is close to helix VII. Figure 3 sumarizes the current hypothesis concerning the three-dimensional arrangement of the helices.

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