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Melibiose carrier of *Escherichia coli*: use of cysteine mutagenesis to identify the amino acids on the hydrophilic face of transmembrane helix 2

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Abstract

The melibiose carrier from *Escherichia coli* is a galactoside-cation symporter. Based on both experimental evidence and hydropathy analysis, 12 transmembrane helices have been assigned to this integral membrane protein. Transmembrane helix 2 contains several charged and polar amino acids that have been shown to be essential for the cation-coupled transport of melibiose. Starting with the cysteine-less melibiose carrier, we have individually substituted cysteine for amino acids 39–66, which includes the proposed transmembrane helix 2. In the resulting derivative carriers, we measured the transport of melibiose, determined the effect of the hydrophilic sulfhydryl reagent, *p*-chloromercuribenzenesulfonic acid (PCMBS), on transport in intact cells and inside out vesicles, and examined the ability of melibiose to protect the carrier from inactivation by the sulfhydryl reagent. We found a set of seven positions in which the reaction with the sulfhydryl reagent caused partial or complete loss of carrier function measured in intact cells or inside-out vesicles. The presence of melibiose protected five of these positions from reaction with PCMBS. The reaction of two additional positions with PCMBS resulted in the partial loss of transport function only in inside-out vesicles. Melibiose protected these two positions from reaction with the reagent. Together, the PCMBS-sensitive sites and charged residues assigned to helix 2 form a cluster of amino acids that map in three rows with each row comprised of every fourth residue. This is the pattern expected of residues that are part of an α-helical structure and thus the rows are tilted at an angle of 25° to the helical axis. We suggest that these residues line the path of melibiose and its associated cation through the carrier.

Keywords: Melibiose; Helix 2; Cysteine mutagenesis; Amino acid transport; (*E. coli*)

1. Introduction

The melibiose carrier from *Escherichia coli* is an integral membrane protein of 52 000 molecular weight [1,2] that is proposed to have 12 transmembrane helices [3,4] with the amino- and carboxyl-termini of the protein on the cytoplasmic face of the cell membrane [5]. This carrier is a cationic symporter that can utilize a proton, sodium, or lithium gradient to drive the accumulation of the α-galactoside, melibiose, as well as a selection of other α- and β-galactosides (reviewed in [6]). The ability of this carrier to

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use one of several cations as the cotransported molecule provides the opportunity to study the organization of and requirements for multiple recognition sites in a single carrier.

Recently Yan and Maloney [7] have identified amino acids that line the path that glucose-6-phosphate takes through its transmembrane carrier. Similarly, Akabas et al. [8] have identified amino acids that line the channel through the cation selective acetylcholine receptor. In these studies, the site-directed replacement of an amino acid with cysteine in the studied protein allowed an analysis of the effects of hydrophilic, membrane-impermeable sulfhydryl reagents on the transport or ion conduction process. A defined set of amino acids that reacted with the sulfhydryl reagents was identified and the amino acid set was clustered as expected if it were on one face of an α-helix. The presence of the transported molecule or the receptor's ligand often altered the reactivity to the sulfhydryl reagent. Furthermore, Yan and Maloney [7] demonstrated differential reactivity of the cysteines to *p*-chloromercuribenzenesulfonic acid (PCMBS) that was dependent on which side of the membrane the reagent was located. In the lactose carrier, the systematic replacement of each amino acid with cysteine and subsequent reaction of the derivative carrier with *N*-ethylmaleimide also revealed a set of sites that exhibited an inhibition of function and a periodicity of location that was consistent with an α-helical structure (reviewed in [9]).

Several amino acids that are important for the process of galactoside/cation symport are located in the proposed transmembrane helix 2 of the melibiose carrier. Asp-55, Asn-58, and Asp-59 have been identified as participating in Na^+ recognition and/or coupling [10–13]. Arg-52 and Gly-63 (in the cytoplasmic loop adjacent to helix 2) are among the group of invariant amino acids that are found when the amino acid sequences of a number of galactoside, pentose, and hexuronide transporters are compared [14]. Transmembrane helix 2 of the melibiose carrier thus seems a good candidate to use to start the identification of the amino acids that line the path of the transported molecules through this membrane protein.

In order to identify the amino acid positions that face the path of melibiose as it passes by transmembrane helix 2, we applied the cysteine replacement approach to this segment of the melibiose carrier of *E. coli*. Starting from the cysteine-less derivative of the melibiose carrier [15], we individually replaced the amino acids from Leu-39 through Val-66 with cysteine and determined the transport capability of the derivative carriers. We then measured the effect that the hydrophilic sulfhydryl reagent, PCMBS, had on the transport of melibiose in both intact cells and inside-out vesicles. Finally, we examined the ability of melibiose to protect the carrier from the inhibitory effects of the reagent.

2. Materials and methods

2.1. Reagents

Melibiose (6-*O*-α-D-galactopyranosyl-D-glucopyranoside), and PCMBS were purchased from Sigma. [^{3}H]Melibiose was a generous gift from Dr. Gerard Leblanc of the Département de Biologie Cellulaire et Moléculaire du CEA, Villefranche-sur-mer, France. [α-^{33}P]dATP was from Andotek. Bacteriological media were from Difco. The Phototope-Star Western Blot Detection Kit was from New England BioLabs and the Immun-Blot PVDF membrane was from Bio-Rad.

2.2. Bacterial strains and plasmids

E. coli DW1 *lacI*$^+$*ΔlacZY ΔmelAB* [16] was used as the host strain for the plasmids when bacteria were grown for transport assays with either intact cells or inside-out vesicles. The gene for the cysteine-less melibiose carrier was inserted in the vector, pKK223-3 (Pharmacia Biotech) as described previously [15]. This plasmid was used as the starting material for site-directed mutagenesis.

2.3. Site-directed mutagenesis

The Chameleon Double-Stranded, Site-Directed Mutagenesis Kit (Stratagene) was used to replace the selected amino acids with cysteine. The *Sca*I selection primer (Stratagene) was used along with the appropriate phosphorylated, mutagenic primer (ranging from 23 to 32 nucleotides long) that was

synthesized by Dr. Charles Dahl, Harvard Medical School.

2.4. DNA sequencing

Double-stranded plasmid DNA was isolated with the QIAprep Spin Miniprep Kit (Qiagen). The plasmid DNA was sequenced by using [α-^{33}P]dATP with the AmpliCycle Sequencing Kit (Perkin-Elmer).

2.5. Immunodetection and quantitation of the melibiose carrier in bacterial cells

Bacteria were grown, harvested, and washed as described for use in transport experiments with whole cells. The cell suspensions were stored frozen until used. Aliquots of the cell suspensions (25–75 ng of protein) were incubated with dithiothreitol containing sample buffer (as described in the brochure from the Phototope-Star Western Blot Detection Kit, New England BioLabs) for 45 min at room temperature before loading onto a 10% acrylamide denaturing gel [17]. The melibiose carrier was detected by using a polyclonal antibody prepared against the carboxyl-terminus of the protein [18]. The final chemiluminescent bands of melibiose carrier were quantitated by analyzing a digital image of the exposed X-ray film (X-Omat AR, Eastman Kodak) with the program, ImageQuant (Molecular Dynamics).

2.6. Assay of melibiose transport by intact cells

The plasmid-containing strains were grown in LB medium [19] containing 100 μg/ml ampicillin until they reached mid-log phase of growth. The cells were harvested and washed once with 100 mM MOPS buffer that was adjusted to pH 7.0 with Tris base and contained 10 mM NaCl and 0.5 mM $MgSO_4$ (buffer A). The cells were resuspended in the same buffer to a cell density corresponding to about 1 mg dry wt./ml. [^{3}H]Melibiose (2 μCi/μmol) was added to an aliquot of the cell suspension to a final concentration of 0.1 mM. After incubation for 1 min at room temperature, a 200-μl aliquot was filtered through a 0.65-μm cellulose nitrate filter (Sartorius). The filters were washed with 4 ml of buffer A and then counted in Liquiscint (National Diagnostics).

The effect of the sulfhydryl reagent, PCMBS, on transport was measured by preincubating the cell suspension with the indicated concentrations of the reagent for 10 min at room temperature. The radiolabeled melibiose was then added and the cells were processed as described above. The effect that the presence of melibiose has on the reactivity with the sulfhydryl reagent was measured by first incubating the cell suspension with 1 mM unlabeled melibiose for 1 min. The sulfhydryl reagent was then added and the incubation at room temperature was continued for 10 min. A 200-μl aliquot was then filtered, washed, and overlaid with 50 μl of buffer A that contained 0.1 mM [^{3}H]melibiose (2 μCi/μmol). After 30 s, the overlay was removed by filtration and the filter was washed again with 4 ml of buffer A. The filter was counted as described above.

2.7. Assay of melibiose transport in inside-out vesicles

The plasmid-containing bacteria (40 ml cultures) were grown, harvested and washed as described above and then resuspended in 10 ml of buffer A that contained 250 mM sucrose (vesicle buffer). Inside-out vesicles were prepared by passing the cell suspension through a French press (SLM-Aminco) at 8000 psi [20]. Unbroken cells were removed by centrifugation and the vesicle suspension was assayed for melibiose transport the same day. An aliquot of vesicles (312 μl) was warmed to room temperature and then the sulfhydryl reagent was added from a 40-fold concentrated stock solution that was made in vesicle buffer. The suspension was incubated for 10 min at room temperature and then [^{3}H]melibiose (25 μCi/μmol in vesicle buffer) was added to a final concentration of 50 μM. After 15 s, the vesicle suspension was filtered through a 0.22-μm nitrocellulose filter (GSTF, Millipore) and the filter was washed with vesicle buffer. The filter was counted in Liquiscint in the presence of 1% Triton X-100.

The effect of unlabeled melibiose on the reactivity with the sulfhydryl reagent was measured by preincubating the vesicles with melibiose at a final concentration of 3 mM for 1 min before adding the sulfhydryl reagent as described above. After an additional 9 min incubation, the vesicle aliquot was filtered through a 0.22-μm nitrocellulose filter and washed with 4 ml of vesicle buffer. The vesicle spot

was overlaid with 50 μl of 50 μM [^{3}H]melibiose (25 μCi/μmol in vesicle buffer) and after 1 min, the radiolabel was drawn off and the filter was washed with 3 ml of vesicle buffer. The filter was counted as described above.

2.8. Protein determination

The Bio-Rad Protein Assay (Bio-Rad) was used with bovine serum albumin as the standard.

3. Results

3.1. Site-directed mutagenesis of transmembrane helix 2

The presence of several amino acids in helix 2 that are important in the transport process suggested that the replacement of the residues of this helix with cysteine and the subsequent analysis of the transport capabilities of the derivative carriers would yield information about which residues of helix 2 face the path of melibiose through the carrier. The 21 amino acids of the proposed transmembrane helix 2 and seven amino acids in the adjacent inter-helix loops (Fig. 1) were thus individually changed to cysteine by in vitro site-directed mutagenesis that was applied to the plasmid-borne gene for the cysteine-less melibiose carrier. In all cases, only the single desired amino acid change was found after the derivative gene's complete DNA sequence was determined.

3.2. Measurement of the amount of melibiose carrier that is synthesized

The amount of melibiose carrier that was synthesized in each derivative strain relative to the amount that was synthesized by the parental, cysteine-less carrier was measured after detection of the carrier on an immunoblot by enhanced chemiluminescence that used alkaline phosphatase-linked goat anti-rabbit antibody. The exposure of the film was then quantitated as described in Section 2 and the results are presented in Table 1. In all the derivative strains except one, the amount of melibiose carrier that was synthesized was equal to at least about one-half of the amount present in the cysteine-less parental strain. Only S40C showed a substantial reduction in expression of the carrier and that was to 19% of the parental level.

3.3. Measurement of sodium-coupled melibiose accumulation

The rate at which each derivative melibiose carrier accumulated melibiose was measured. The results are presented in Table 1. Four of the substitutions,

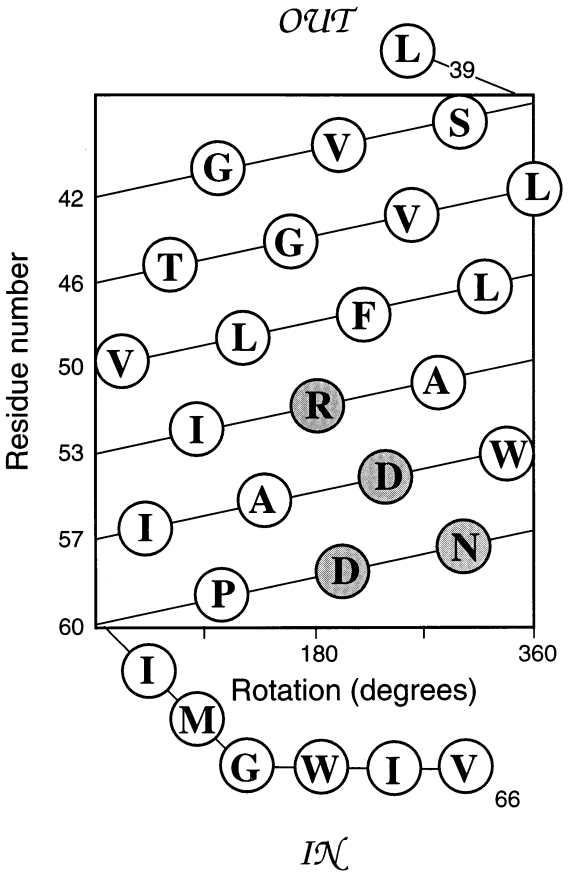

Fig. 1. A two-dimensional depiction of transmembrane helix 2 of the melibiose carrier from *E. coli*. The 28 amino acids that were individually replaced with cysteine are shown here. As proposed by Pourcher et al. [4], transmembrane helix 2 of the melibiose carrier encompasses amino acid residue 40–60. The periplasmic (OUT) and cytoplasmic (IN) ends of the helix are as indicated. Amino acid 39 and amino acids 61–66 are assigned to interhelical loops [4]. In this figure, the residue number is given on the vertical axis and the degrees of rotation about the helical axis are given on the horizontal axis. The shaded residues, R52, D55, N58, and D59, have been previously identified as residues that are important in cation-coupled transport of melibiose [10–14].

R52C, D55C, D59C, and G63C, resulted in a carrier that had essentially no measurable sodium-coupled transport of melibiose despite the fact that these strains expressed the melibiose carrier as well as the parental strain. Three of these amino acids, R52, D55, and D59, have already been shown to be important for the normal functioning of the melibiose carrier [10,11,21,22]. The fourth position, G63, represents a new site in the melibiose carrier that cannot tolerate substitution with cysteine (at least in the cysteine-less melibiose carrier). Three derivatives, N58C, W64C, and V66C, accumulated melibiose at a substantially reduced rate relative to the parental cysteine-less carrier. The reduced transport of the N58C derivative (to 27% of the parental strain) is consistent with the loss of sodium-coupled melibiose transport [13]. The remainder of the derivatives accumulated melibiose at least half as well as the cysteine-less strain and most accumulated melibiose at least 2/3 as well as the parent strain.

Table 1
Relative expression of and melibiose transport by the single cysteine replacement derivatives of the Cys-less melibiose carrier

Strain	% Cys-less expression[a]	% Cys-less transport[b]
Cys-less	100	100
L39C	73 ± 20	44 ± 3
S40C	20 ± 95	6 ± 11
V41C	160 ± 23	99 ± 18
G42C	90 ± 30	63 ± 9
L43C	68 ± 7	90 ± 8
V44C	85 ± 10	131 ± 16
G45C	76 ± 23	86 ± 6
T46C	62 ± 23	73 ± 11
L47C	64 ± 13	106 ± 19
F48C	168 ± 6	126 ± 1
L49C	126 ± 4	47 ± 6
V50C	110 ± 20	123 ± 6
A51C	68 ± 19	66 ± 6
R52C	98 ± 20	1 ± 1
I53C	71 ± 13	78 ± 20
W54C	111 ± 1	75 ± 8
D55C	104 ± 20	0
A56C	124 ± 6	120 ± 5
I57C	72 ± 28	48 ± 11
N58C	53 ± 20	27 ± 2
D59C	82 ± 4	0
P60C	48 ± 11	69 ± 19
I61C	72 ± 6	83 ± 6
M62C	50 ± 32	129 ± 1
G63C	63 ± 25	0
W64C	80 ± 11	21 ± 1
I65C	46 ± 15	104 ± 6
V66C	93 ± 11	11 ± 4

[a]Measured by quantitation of film exposure after chemiluminescent detection of melibiose carrier as described in Section 2. Mean and standard deviation are shown.
[b]Measured as sodium-coupled accumulation of melibiose in 1 min as described in Section 2. Mean and standard deviation are shown.

3.4. The effect of PCMBS on melibiose accumulation in intact cells

The sulfhydryl reagent, PCMBS, is a hydrophilic, membrane impermeable compound (reviewed in [23]) and can thus be used to identify cysteines that are in a hydrophilic environment. Intact cells from each single-cysteine derivative strain were incubated with 100 μM PCMBS or an equal aliquot of buffer for 10 min before melibiose transport was measured as described in Section 2. The accumulation of melibiose by the PCMBS-treated cells was compared to that of the cells that were incubated without PCMBS and the results are presented in Table 2. Seven of the cysteine-replacement derivatives showed complete or partial inhibition of melibiose accumulation after treatment with the reagent: V44C, G45C, L47C, F48C, L49C, A51C, and A56C. As seen in the helical net diagram of Fig. 1, V44C, G45C, F48C, and A51C, all lie in a cluster just above the three important charged residues of this helix, R52, D55, and D59. In addition, A56C is surrounded by the charged residues.

3.5. The effect of PCMBS on melibiose transport in inside-out vesicles

Studies on other transporters that compared the effects of impermeable sulfhydryl reagents on intact cells as opposed to inside-out or reconstituted vesicles had shown differences in the ability to inhibit the transport process [9,24] that were dependent on which side of the cytoplasmic membrane the reagent was presented. We thus examined whether any of the cysteine replacements mentioned above also reacted with PCMBS to cause inhibition of melibiose transport when the PCMBS was presented to the inner surface of the cytoplasmic membrane. The inside-out vesicles were prepared as described in Section 2 and the melibiose transported into the vesicles was

measured with or without pretreatment with PCMBS. In these vesicles, the transport process is equivalent to efflux in intact cells. The results of these experiments are presented in the right hand side of Table 2. The same set of seven cysteine replacements (V44C, G45C, L47C, F48C, L49C, A51, and A56C) that reacted with PCMBS presented from the outer surface of the cytoplasmic membrane to cause inhibition of transport also reacted with PCMBS presented from the inner surface of the membrane. Three of the sites with the greatest inhibition of transport after reaction with PCMBS are the same sites with the greatest inhibition seen in intact cells: V44C, G45C, and F48C. The two sites with partial inhibition of transport (L47C and L49C) in intact cells also show partial inhibition in inside-out vesicles. Of the remaining two sites, A51C shows less inhibition of transport after reaction with PCMBS while A56C is more sensitive to transport inhibition from the presentation of PCMBS at the inner surface of the inner membrane. Two additional sites react with PCMBS from the inner surface of the

Table 2
Effect of PCMBS on melibiose transport by intact cells and inside-out vesicles[a]

Strain	% Transport remaining	
	Intact cells	Inside-out vesicles
Cys-less	97 ± 11	98 ± 2
L39C	124 ± 23	98 ± 2
S40C	99 ± 4	90 ± 11
V41C	77 ± 8	43 ± 1
G42C	103 ± 14	88 ± 9
L43C	90 ± 5	78 ± 8
V44C	3 ± 1	2 ± 4
G45C	10 ± 2	2 ± 4
T46C	106 ± 8	74 ± 3
L47C	65 ± 5	49 ± 6
F48C	4 ± 1	4 ± 3
L49C	36 ± 6	31 ± 10
V50C	92 ± 7	70 ± 1
A51C	17 ± 4	46 ± 11
I53C	86 ± 10	12 ± 4
W54C	98 ± 2	86 ± 6
A56C	52 ± 7	4 ± 3
I57C	102 ± 5	97 ± 5
N58C	91 ± 12	93 ± 10
P60C	101 ± 5	90 ± 5
I61C	102 ± 10	94 ± 4
M62C	102 ± 6	96 ± 6
W64C	102 ± 4	94 ± 9
I65C	92 ± 10	84 ± 10
V66C	106 ± 9	80 ± 11

[a]Sodium-coupled transport was measured in intact cells or vesicles as described in Section 2. The transport remaining after incubation with 100 μM PCMBS is reported as a % of the transport in an untreated cell or vesicle sample. Mean and standard deviation are shown.

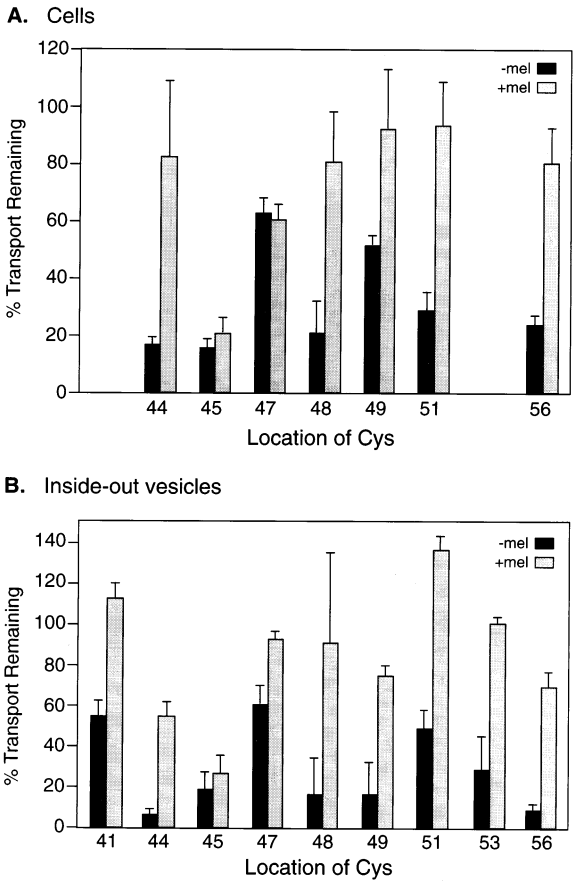

Fig. 2. The effect of the presence of melibiose on the inhibition of the melibiose carrier by PCMBS. Sodium-coupled melibiose transport was measured in intact cells (A) or inside-out vesicles (B) as described in Section 2. The percent of transport (the mean value) that remains after incubation with the thiol reagent is given on the vertical axis. The dark bars represent the percent activity remaining after incubation with PCMBS alone. The shaded bars represent the percent activity remaining after first adding melibiose and then adding PCMBS. The standard deviation for each mean is indicated on its bar. The horizontal axis gives the residue number at which the cysteine substitution was made for each of the melibiose carriers that were tested. The concentration of PCMBS used for each strain is as follows. (A) Intact cells: V44C, 15 μM; G45C, 0.6 μM; L47C, 100 μM; F48C, 1 μM; L49C, 2 μM; A51C, 100 μM; and A56C, 200 μM. (B) Inside-out vesicles: V41C, 50 μM; V44C, 60 μM; G45C, 40 μM; L47C, 60 μM; F48C, 30 μM; L49C, 50 μM; A51C, 100 μM; I53C, 50 μM; and A56C, 50 μM.

inner membrane, V41C and I53C. V41C shows only partial inhibition which reaches a maximum of about 60%. However, the reaction of inside-out vesicles from the I53C derivative with PCMBS results in nearly complete inhibition of transport.

3.6. The effect of melibiose on transport inhibition by PCMBS

The ability of the carrier's substrate to protect a cysteine replacement from reaction with a hydrophilic sulfhydryl reagent has been used as corroborating evidence for the presence of the reactive residue in the path of the transported compound [7,25,26]. The effect of melibiose on the inhibitory action of PCMBS was tested in intact cells from the seven derivatives with reactive cysteine replacements or in inside-out vesicles from the nine derivatives with reactive cysteine replacements. The concentrations of PCMBS that were chosen for this experiment gave about 80% of maximal inhibition in the respective derivative strain (see legend to Fig. 2). Of the seven sites that were inhibited by PCMBS in intact cells, five were significantly protected against inhibition by PCMBS if the cells were preincubated with melibiose (Fig. 2A). Only two sites, G45C and L47C, were not protected against inhibition by PCMBS after preincubation with melibiose. When these same seven sites were examined in inside-out vesicles, the same pattern of protection by melibiose was seen (Fig. 2B). The two additional sites, V41C and I53C, that were inhibited by reaction with PCMBS only in inside-out vesicles were both protected from inhibition by melibiose (Fig. 2B).

4. Discussion

In this study, we individually changed each amino acid from L39 to V66 in the cysteine-less melibiose carrier to a cysteine. We found that all of the derivative melibiose carriers except one were present in amounts similar to the starting parent strain. However, substitution with cysteine at four out of the 28 positions, resulted in a carrier with little or no ability to accumulate melibiose. We found a set of seven cysteine replacements that reacted with PCMBS to cause partial or complete loss of ability to transport melibiose. In this set of seven, it did not matter whether the PCMBS was presented from the periplasmic side or the cytoplasmic side of the inner membrane. For a subset of five of these cysteine replacements, the reaction with PCMBS was prevented by the presence of melibiose. Two additional cysteine replacements resulted in a carrier that was inhibited by PCMBS only when inside-out vesicles were examined and these two replacements could be protected from reaction with the sulfhydryl reagent by the presence of melibiose.

The replacement of the amino acids of transmembrane helix 2 and the adjacent inter-helix loops with cysteine resulted in only four cases where there was loss of ability to transport melibiose in the derivative strain even though the amount of melibiose carrier present in these strains was between 63 and 104% of the parental, cysteine-less strain. Three of these were the charged residues, R52, D55, and D59, that have been shown to be important in cation recognition and selectivity. [10,11,21,22]. The D55C and D59C substitutions in a wild-type carrier have previously been shown to result in loss of sodium-coupled transport of melibiose [10]. The fourth case, G63, is the highly conserved glycine found in the galactose–pentose–hexuronide family of bacterial carriers [14]. Curiously, the major facilitator superfamily [27,28] has a similarly located and conserved glycine in transmembrane helix 2. Jessen-Marshall et al. [29] and Frillingos et al. [30] found that a cysteine replacement at this glycine in either the wild-type or the cysteine-less lactose carrier resulted in an inactive carrier. Our findings have corroborated and extended the previously published studies that identify the importance of position R52, D55, D59, and G63 in the effective functioning of the melibiose carrier.

A set of seven cysteine replacements, V44C, G45C, L47C, F48C, L49C, A51C, and A56C, resulted in a melibiose carrier that was partially or completely inactivated by reaction with the hydrophilic sulfhydryl reagent, PCMBS. The inactivation occurred whether the reagent was present on the periplasmic or cytoplasmic side of the cell membrane. An additional two cysteine replacements, V41C and I53C, were partially or completely inactivated only when the reagent was present on the cytoplasmic face of the cell membrane. This pattern of reactivity is in contrast to

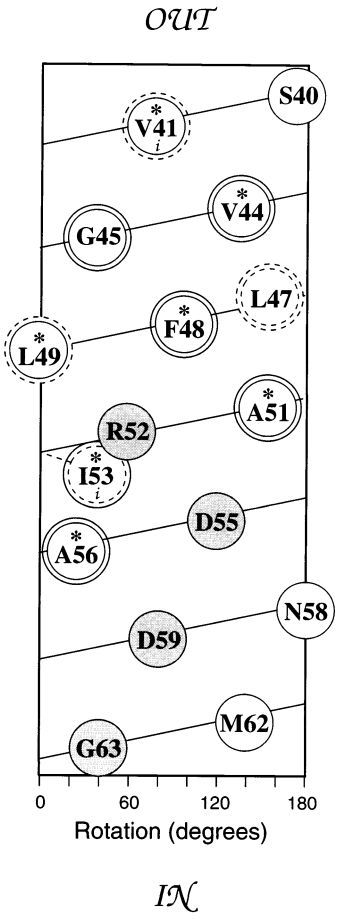

Fig. 3. A two-dimensional depiction of the hydrophilic face of transmembrane helix 2 from the melibiose carrier. The half of helix 2 is shown that includes the positions where substitution of the wild-type amino acid by cysteine results in sensitivity to PCMBS. The cysteine substitutions that resulted in greater than 80% inhibition of transport after reaction with PCMBS are indicated by a second solid line around the residue name. The positions that are protected from inhibition by melibiose are indicated by an asterisk (*) above the residue name. The substitutions that were partially inhibited by PCMBS (less than 50%) are indicated by a second dashed line around the residue name. The cysteine substitutions that are at the two positions marked by '*i*' below the residue name showed inhibition by PCMBS only when the reagent was introduced from the cytoplasmic face of the membrane. The two residues (L47 and I53) that are found on the opposite half of the helix have an inner dotted line. The cysteine substitutions at the four positions (R52, D55, D59, and G63) that resulted in a carrier with no transport activity are indicated by shading. The periplasmic (OUT) and cytoplasmic (IN) ends of the helix are indicated. The position of each residue on this side of the helix is given as the rotation in degrees about the helical axis.

that found by Yan and Maloney [7] for the glucose-6-phosphate/phosphate antiporter. They found three classes of sulfhydryl reactive cysteine replacements: accessible only from the cell exterior, accessible only from the cell interior, and accessible from both sides. They proposed that this pattern suggested a 'mobile barrier' that provides specificity to the uhpT carrier. In the melibiose carrier, the positions that are accessible only from the outside are missing and the positions accessible from the inside show an unusual pattern. Whether or not the differences in accessibility reflect mechanistic differences between the uhpT antiporter and the melB symporter awaits further study.

There are several possible explanations of how PCMBS causes inhibition of transport when it reacts with cysteines at the nine positions in the melibiose carrier (Fig. 3). The presence of PCMBS in the transported molecule's path through the melibiose carrier could act as a physical barrier preventing entry of melibiose or preventing effective interaction of melibiose with its binding site. The reaction of PCMBS with the carrier at these cysteine-substituted positions could also alter the position of the transmembrane helices in such a way as to exclude melibiose from its binding site. Seven of the positions in question (all except L47 and I53) lie in a defined cluster of three rows on one half of the α-helix (Fig. 3). These three rows include three charged residues (R52, D55, and D59) known to be involved in cation-coupled transport. It thus seems most likely that the reaction with PCMBS has produced a physical barrier that prevents melibiose from entering or effectively interacting with amino acids on its path through the membrane. Melibiose was shown to protect all but two of the replacement cysteines from reaction with PCMBS. In the same way that PCMBS acts as a barrier blocking melibiose entry, melibiose could act as a barrier blocking PCMBS entry. Interestingly, the two positions with little or no protection by melibiose, G45C and L47C, lie at the fringe of the group of residues depicted in Fig. 3. In addition, these two sites are on the periplasmic end of the helix. Perhaps the entrance of the path through the carrier is a little wider than the path further into the carrier. Thus the presence of PCMBS linked to a cysteine at one of these two positions results in only a partial blockage of the passageway.

In inside-out vesicles, the treatment of two deriv-

atives, V41C and I53C, with PCMBS resulted in inhibition of transport. These two positions could represent sites that were only accessible to PCMBS when PCMBS was presented from the cytoplasmic face of the cell membrane or they could represent sites that, when reacted with PCMBS, only affect the efflux portion of the transport process and not entry of melibiose as was measured in intact cells. Further experiments that examine whether or not these two cysteines react with the sulfhydryl reagent when the reagent was presented from the outside are needed to clear up this point. Whether the results show sidedness of accessibility and/or sidedness of effect, the results would provide interesting information about the difference between the periplasmic face of the melibiose carrier and entry of melibiose into the cell as opposed to the cytoplasmic face of the carrier and exit of melibiose from the cell. Pourcher et al. [31] have already shown that influx and efflux differ kinetically.

In the helical net depiction of one-half of transmembrane helix 2 (Fig. 3), the pattern of PCMBS-sensitive positions is seen to form three rows with each row comprised of every fourth residue in the α-helix. Chothia (reviewed in [32]) has described a common packing arrangement of α-helices that utilizes just this arrangement of every fourth amino acid side chain. If an α-helix is viewed on a axis that is tilted 25° from the axis of the peptide backbone, the rows of every fourth residue are now perpendicular to the plane of viewing. The studies of Le Coutre et al. [33] on the lactose carrier and three-dimensional structures of membrane proteins (e.g. [34]) have demonstrated that the transmembrane helices are not necessarily oriented perpendicular to the membrane bilayer, but rather may be tilted at a an angle of 25–33° relative to the cell membrane. A tilt of the helix axis 25° away from perpendicular to the cell membrane would make all the PCMBS reactive residues in the melibiose carrier line up in a path that is now perpendicular to the cell membrane. A tilted helix, however, requires that amino acids beyond the 21 assigned for a perpendicular orientation [4] be included in the helix in order that it span the minimum width of the cell's membrane (30 Å). For a tilt of 25°, an additional two residues would be required to span 30 Å and a tilt of 33° would require an additional four residues in the α-helix to span the distance. The α-helix of Fig. 3 has three additional residues beyond the proposed structure of Pourcher et al. [4]. The inclusion of I61 through G63 allows G63 to become a part of the proposed path for symport. Interestingly, N58, shown to be necessary for efficient sodium-coupled transport [13], lies on this half of the helix but just outside the three rows.

In this study, we have found a group of amino acid positions that, when these amino acids are replaced with cysteine, exhibit inhibition of transport of melibiose by the carrier after reaction with the hydrophilic, membrane-impermeable sulfhydryl reagent, PCMBS. It seems highly likely that these amino acids line a portion of the passageway of melibiose through the cytoplasmic membrane. The application of this analysis to other transmembrane helices of the melibiose carrier that have residues known to be important in transport in the transport process (for example, helices 1, 4, and 11 as reviewed in [14]) could potentially result in the description of the entire lining of the passageway.

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