J. Membrane Biol. 183, 33–38 (2001)
DOI: 10.1007/s00232-001-0053-x

The Journal of
Membrane
Biology

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# Cysteine Substitutions for Individual Residues in Helix VI of the Melibiose Carrier of *Escherichia coli*

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Received: 6 March 2001/Revised: 14 May 2001

**Abstract.** The melibiose carrier of *Escherichia coli* is a cytoplasmic membrane protein that mediates the cotransport of galactosides with $H^+$, $Na^+$, or $Li^+$. In this study we used cysteine-scanning mutagenesis to try to gain information about the position of transmembrane helix VI in the three-dimensional structure of the melibiose carrier. We constructed 23 individual cysteine substitutions in helix VI and an adjacent loop of the carrier. The resulting melibiose carriers retained 22–100% of their ability to transport melibiose. We tested the effect of the hydrophilic sulfhydryl reagent p-chloromercuribenzenesulfonic acid (PCMBS) on the cysteine-substitution mutants and we found that there was no inhibition of melibiose transport in any of the mutants. We suggest that helix VI is imbedded in phospholipid and does not face the aqueous channel through which melibiose passes.

**Key words:** Melibiose carrier — Cotransporter — Cysteine mutagenesis — Helix VI

## Introduction

The melibiose carrier of *Escherichia coli* is an integral membrane protein that can utilize a proton, sodium or lithium ion gradient to drive the accumulation of the α-galactoside, melibiose (for reviews *see* references 10 and 14). Based on the data from hydropathy plots of the amino acid sequence [23], the analysis of melB-phoA fusions [2, 16], and proteolytic digestion studies [9] the transporter is thought to have 12 transmembrane helices (*see* Fig. 1). The carboxyl terminus of the protein was shown to be on the cytoplasmic face of the cell membrane [3]. Site-directed mutagenesis of charged residues, isolation of second-site revertants that have recovered melibiose transport, and isolation of mutants with altered sugar recognition have identified amino acids in helices I, II, IV, VII and XI that are important in the carrier's function [10, 15, 20, 21, 24].

Cysteine-scanning mutagenesis has been used to study the structure of membrane proteins. Cysteine is substituted for individual amino-acid residues in a cysteine-less derivative of the normal protein. The derivative proteins that each contain a single cysteine are then tested for their susceptibility to inhibition of function by reaction with hydrophilic sulfhydryl reagents. In a number of membrane proteins patterns of cysteine residues have been found that confirm an α-helical structure and identify a hydrophilic face of the helix. These proteins include: bacteriorhodopsin [5], the tar protein [12], the maltoporin [6], the nicotinic acetylcholine receptor [1], the lactose carrier of *E. coli* [17] and the glucose-6-phosphate carrier of *E. coli* [22].

Cysteine-scanning mutagenesis has been carried out on the melibiose carrier in helix I (Ding and Wilson, 2001, Biochemistry, in press), helix II [13] and helix XI [4]. In the case of helix II and helix XI a series of cysteine mutants on one surface of the helix were susceptible to inhibition by PCMBS. We suggested that the surface of the helix where cysteine residues were sensitive to PCMBS faced the aqueous channel. On the other hand, helix I showed that many cysteine replacements around the circumference of the helix were sensitive to PCMBS. This suggested that the entire helix was surrounded by aqueous medium.

The analysis of channel size by Zeng et al. [25] suggests that six helices are needed to create a channel large enough for a disaccharide to pass through. Thus far for the melibiose carrier, the evidence cited above iden-

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tified only the three helices I, II and XI as strong candidates for lining the channel for melibiose transport. The analyses of mutations suggest that helix IV is also a possible candidate [20, 21].

In the present study, we individually replaced 23 amino-acid residues in helix VI and an adjacent loop with cysteine and checked the resulting melibiose carriers for their ability to accumulate melibiose. We then tested whether the sulfydryl reagent PCMBS has an effect on the transport in these cells. None of these cysteine substitutions showed inhibition by the water-soluble mercurial. We suggest that helix VI is imbedded in a hydrophobic region of the membrane and does not touch the aqueous channel.

## Materials and Methods

### Reagents

Melibiose (6-O-α-D-galactopyranosyl-D-glucopyranoside) was purchased from Sigma (St. Louis, MO). [$^3$H] melibiose was a generous gift from Dr. Gerard Leblanc of the Department of Biologie Cellulaire et Moleculaire du CEA, Villefranche-sur-mer, France. [α-$^{33}$P]dATP was from Andotek. Bacteriological media were from Difco (Detroit, MI). [$^{35}$S]-Protein A was purchased from Amersham (Piscataway, NJ). All other chemicals were reagent grade.

### Bacterial Strains and Plasmids

*E. coli* DW1 (lacI$^+$ ΔlacZY ΔmelAB) [19] was used as the host strain for plasmids when bacteria were grown for transport assays. pSUMelA was a plasmid containing the melA gene which codes for α-galactosidase and a chloramphenicol-resistant gene [21]. In a number of experiments DW1/pSUMelA was used as host for our melB plasmid. Such a construction can grow on melibiose since the transported melibiose can be metabolized. The cysteine-less melibiose carrier gene [18] was used as a control in each experiment. The vector plasmid with no inserted gene, pKK223-3, (Pharmacia Bio Tech, Piscataway, NJ) was used in immunodetection experiments as a control for nonspecific binding.

### Site-directed Mutagenesis

The Quick Change Double-Stranded, Site Directed Mutagenesis Kit (Stratagene, La Jolla, CA) was used to replace the selected amino acids with cysteine. The appropriate phosphorylated 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 from Qiagen (Valencia, CA). Sequencing was carried out using [α-$^{33}$P] dATP with the Amplicycle Sequencing Kit (Perkin Elmer, Branchburg, NJ). The melB gene was sequenced from plasmid DNA using primers that anneal at approximately 200 bp intervals.

### Immunodetection and Quantitation of the Melibiose Carrier

The amount of melibiose carrier present in each strain was determined as previously described by Llokema et al. [11]. In summary, a known quantity of cells was placed on nitrocellulose filters and lysed with NaOH/SDS and then neutralized with Tris. The filters were incubated with BSA to block nonspecific protein binding, followed by incubation with a polyclonal antibody, anti-MBct10 [3], directed against the carboxyl-terminal ten amino acids of the protein. [$^{35}$S] protein A (Amersham) was used to label the bound antibody and the amount of label was quantified by liquid scintillation counting. To correct for nonspecific adsorption, values obtained for the strain DW1/pKK223 (ΔmelAB/pΔmelB) were used as a 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. The cells were resuspended in the same buffer to a cell density corresponding to about 1 mg dry wt/ml. In one series of experiments the cells were exposed to buffer containing either no added cation, or 10 mM NaCl or 10 mM LiCl. When required, PCMBS was added to a final concentration of 100 μM. Stock cells (200 μl) were added to 25 μl MOPS, 100 mM NaCl or 100 mM LiCl and incubated for 10 min to bring cells up to room temperature. [$^3$H]melibiose (1 mM, 25 μl) was added and incubated for another 10 min (final concentration 0.1 mM). An aliquot (200 μl) of cells was then filtered through a 0.65 μm cellulose nitrate filter (Sartorius, Hayward, CA) and immediately washed with 4 ml of MOPS buffer. The filters were placed in plastic vials and counted in the presence of Liquiscint (National Diagnostics, Atlanta, GA).

## Results

### Cysteine-Scanning Mutagenesis of Residues in Helix VI

The previously constructed plasmid containing the gene for the cysteine-less melibiose carrier [18] was used for the site-directed mutagenesis of transmembrane helix VI. The amino-acid residues from L184 to S206 in helix VI (Fig. 1) were individually converted to cysteine as described in Materials and Methods. The complete sequence of the melB gene in these mutants was determined and confirmed that there was a single amino-acid change in each case.

### Measurement of the Amount of Melibiose Carrier in the Membrane

When constructing mutations in membrane proteins it is always a possibility that the introduced amino acid will disrupt the proper membrane insertion and/or stability of the protein. The reduced protein amount will then result

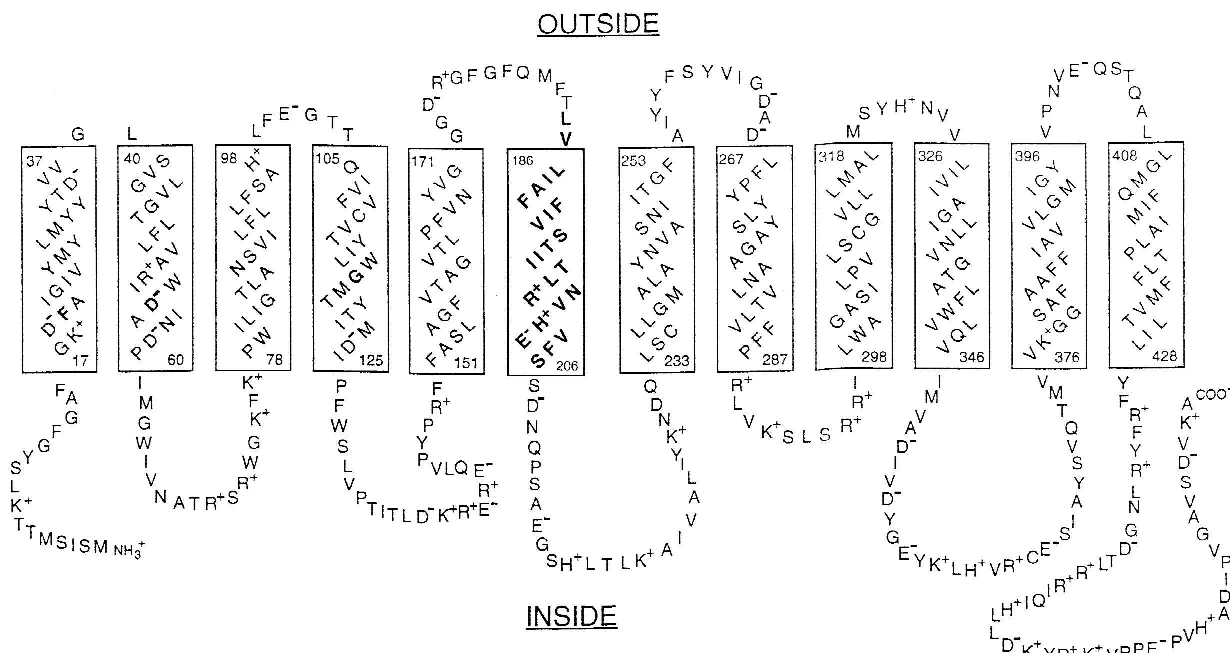


**Fig. 1.** The structure of the melibiose transport protein showing the residues in helix VI that were converted to cysteine.

in an apparently reduced function of the protein when its activity is measured. The relative amount of carrier protein present in the membrane of each strain was therefore determined by the immunoblot method using a polyclonal antibody directed against the C-terminal 10 amino acids of the carrier protein. The amount of melibiose carrier protein expressed in each strain is presented as a percentage of the wild-type value (Table 1). The expression was very good in all cases, the lowest value being 54% (V185C). Measurement of the accumulation of melibiose in the 23 mutant strains will thus not be affected by the amount of transporter that is present.

### Phenotype on Melibiose MacConkey Indicator Plates

Each of the cysteine mutant plasmids was placed in DW1 (ΔmelAB) containing a plasmid with the melA gene. These cells were each streaked on melibiose MacConkey indicator plates to determine the phenotype. On these plates melibiose enters the cell on the carrier and is then split by α-galactosidase into glucose and galactose. These sugars are fermented by the cell to acidic products which cause the pH indicator to give a red color. Under these conditions each mutant gave a red color indicating good melibiose entry in each case.

### Measurement of Cation-Coupled Accumulation of Melibiose

Transport was assayed in cells with the melB plasmid in DW1. This cell has no α-galactosidase so that the sugar that enters the cell cannot be metabolized and is accumulated within the cell as the free sugar. In a typical experiment the cell with a normal melB gene in the presence of 10 mM NaCl accumulates melibiose to a concentration 225 times higher than outside the cell (Table 2). In the presence of $Li^+$ the accumulation was slightly less (215-fold) and in the presence of only protons accumulation was much lower (11-fold). In many cases the mutants' accumulation with sodium or lithium was similar to normal. In 5 cases the accumulation was about 50% of normal (L184C, V185C, I187C, F190C, T194C). The lowest case (V201C) that showed accumulation with sodium was 22% of normal. In 10 of the mutants (T194C, I195C, I196C, T197C, N200C, V201C, H202C, E203C, V204C, and F205C), transport with proton was more than twice that of normal.

### Effect of PCMBS on Melibiose Accumulation in Intact Cells

The sulfhydryl reagent PCMBS is a hydrophilic membrane-impermeable compound and can thus be used to identify cysteines that are in a hydrophilic environment and available to the surface. When $Na^+$-coupled accumulation of melibiose in the absence or presence of PCMBS was compared (Fig. 2), no significant inhibition of transport was seen for any of the 23 cysteine mutants. Yan and Maloney [22] showed a polarity of transmembrane helix VII in the glucose phosphate carrier with respect to the accessibility to PCMBS from the two sides of the membrane. The middle section of the helix is

**Table 1.** Phenotype and expression by single cysteine substitution of cysteine-less carrier in helix VI

| Cell | Colony color on melibiose MacConkey plate[a] | Carrier protein expression (% of cysteine-less parent)[b] |
|---|---|---|
| Cysteine-less parent | Red | 100 |
| L184C | Red | 67 ± 7 |
| V185C | Red | 54 ± 3 |
| L186C | Red | 76 ± 11 |
| I187C | Red | 86 ± 8 |
| A188C | Red | 97 ± 7 |
| F189C | Red | 95 ± 12 |
| F190C | Red | 110 ± 5 |
| I191C | Red | 121 ± 11 |
| V192C | Red | 104 ± 21 |
| S193C | Red | 63 ± 5 |
| T194C | Red | 89 ± 10 |
| I195C | Red | 110 ± 9 |
| I196C | Red | 122 ± 23 |
| T197C | Red | 106 ± 13 |
| L198C | Red | 109 ± 14 |
| R199C | Red | 92 ± 18 |
| N200C | Red | 106 ± 14 |
| V201C | Red | 102 ± 18 |
| H202C | Red | 114 ± 21 |
| E203C | Red | 118 ± 13 |
| V204C | Red | 126 ± 16 |
| F205C | Red | 95 ± 5 |
| S206C | Red | 106 ± 18 |

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

**Table 2.** Transport of melibiose[a] by cysteine mutants[b] in helix VI with three cations[c] (In/Out)[d]

| Cell | $H^+$ | $Li^+$ | $Na^+$ |
|---|---|---|---|
| Cysteine-less parent | 11 ± 0.6 | 215 ± 3 | 225 ± 13 |
| L184C | 11 ± 0.6 | 118 ± 1 | 115 ± 1 |
| V185C | 11 ± 1 | 111 ± 4 | 124 ± 1 |
| L186C | 10 ± 0 | 139 ± 5 | 158 ± 5 |
| I187C | 13 ± 4 | 131 ± 4 | 124 ± 1 |
| A188C | 9 ± 0 | 159 ± 3 | 186 ± 5 |
| F189C | 11 ± 0.5 | 196 ± 14 | 196 ± 1 |
| F190C | 0.6 | 96 ± 5 | 105 ± 1 |
| I191C | 13 ± 0 | 226 ± 1 | 255 ± 11 |
| V192C | 14 ± 0 | 218 ± 6 | 218 ± 20 |
| S193C | 10 ± 0.4 | 140 ± 6 | 128 ± 13 |
| T194C | 26 ± 0.6 | 123 ± 5 | 106 ± 3 |
| I195C | 30 ± 0.6 | 200 ± 6 | 194 ± 3 |
| I196C | 28 ± 1 | 158 ± 3 | 139 ± 3 |
| T197C | 26 ± 2 | 234 ± 2 | 218 ± 2 |
| L198C | 16 ± 0 | 188 ± 2 | 149 ± 5 |
| R199C | 11 ± 0 | 139 ± 5 | 150 ± 3 |
| N200C | 39 ± 0.6 | 190 ± 14 | 168 ± 6 |
| V201C | 23 ± 1 | 59 ± 3 | 49 ± 3 |
| H202C | 39 ± 1 | 229 ± 3 | 233 ± 3 |
| E203C | 25 ± 3 | 173 ± 5 | 128 ± 4 |
| V204C | 25 ± 0.6 | 170 ± 5 | 150 ± 3 |
| F205C | 29 ± 1 | 84 ± 3 | 69 ± 4 |
| S206C | 15 ± 0.6 | 123 ± 0.6 | 131 ± 11 |

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

open to the reagent from both sides of the membrane, whereas the cytoplasmic and periplasmic sections were only accessible to their respective sides.

## Discussion

Identifying which transmembrane helices surround the channel through which melibiose and the cotransported ion pass is an important step in understanding how the melibiose carrier functions and in elucidating the three-dimensional structure of the carrier. Characterization of mutants has already identified five transmembrane helices as likely candidates for this role. Acidic and basic residues in helices I, II, IV and in the junctions of helices VII, XI with their cytoplasmic loops have been identified as important in sugar recognition, cation binding and formation of interhelical charge pairs [7, 8, 10, 15, 21]. Cysteine-scanning mutagenesis combined with studies of inhibition of function by reaction with the hydrophilic sulfhydryl reagent PCMBS has provided corroborating evidence for an α-helical structure and identified a hydrophilic face on three of these helices: I (Ding and Wilson, 2001, Biochemistry, in press), which is completely in the aqueous channel, and II [13] and XI [4], each having one face in the aqueous channel.

In this study transmembrane helix VI was studied as a possible channel-facing helix because of the presence of three charged amino acids (Glu203, Arg199 and His202). Each of the 23 residues on helix VI was converted individually to cysteine. This was followed by testing the effect of the hydrophilic sulfhydryl reagent PCMBS on melibiose transport of each mutant. PCMBS failed to significantly inhibit any of the mutants with cysteine substitutions. One possibility is that the helix is out of the membrane and lies on the surface. Although the PCMBS would react with the cysteines, this would probably not interfere with the transport. We suggest that helix VI is in the membrane and completely surrounded by phospholipid so that the water-soluble inhibitor is unable to reach the cysteines. Also the fact that we have never been able to isolate second-site mutations in helix VI that recover transport is consistent with its being outside the aqueous channel.

One unexpected finding was that cysteine substitutions of Arg199, His202, and Glu203 led to very little loss of activity. When charged residues in helix I, II, IV

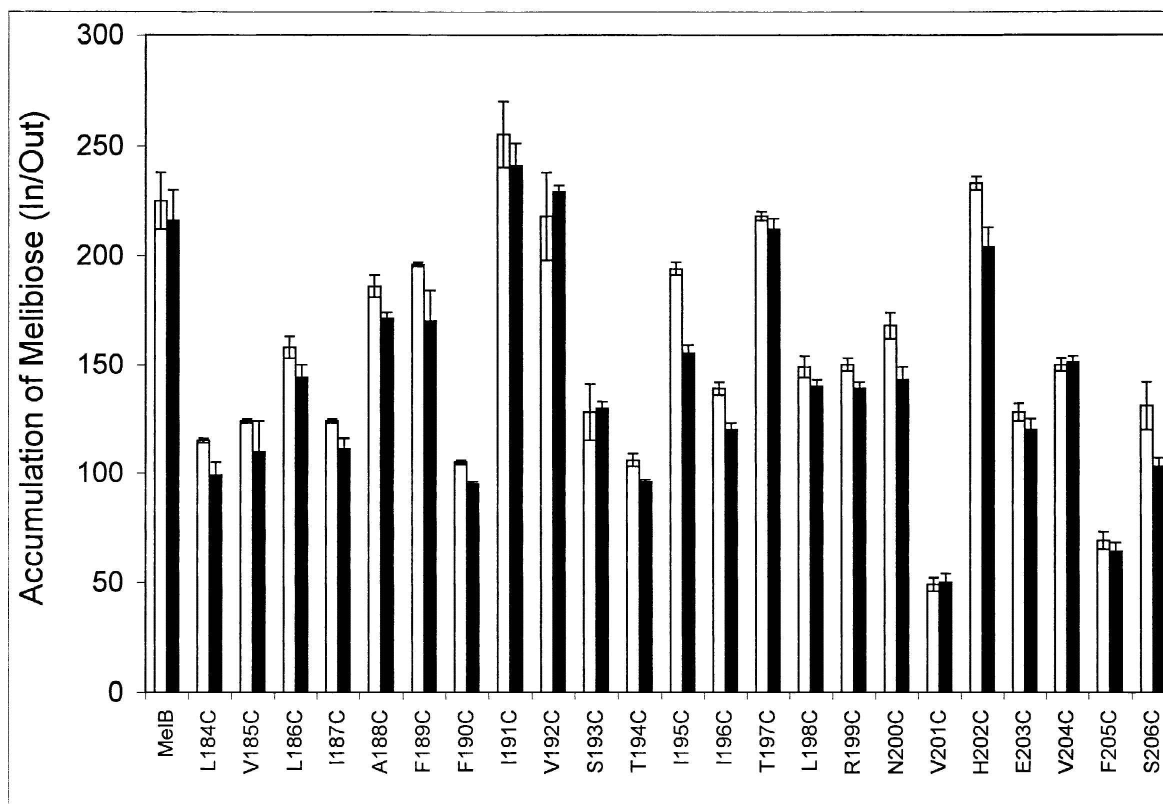


**Fig. 2.** The effect of PCMBS on the melibiose (0.1 mM) transport in the presence of 10 mM NaCl with each cysteine mutant of helix VI. Sugar accumulation in the absence of inhibitor is indicated by a white bar and in the presence of 100 μM PCMBS is indicated by a black bar.

and XI were changed to cysteine they all resulted in total loss of activity of the carrier. One of the possible explanations for the failure of cysteine substitutions to reduce activity of the three charged residues in helix VI is that these residues are not located in the lipid region of the helix but in a cytoplasmic loop. Charged residues in loops are much less sensitive to mutational changes than such residues in the transmembrane region of the helix. A segment of the loop V/VI containing G178 to V185 could be included in the helix VI, leaving the charged residues in the loop VI/VII.

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