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Sugar recognition mutants of the melibiose carrier of *Escherichia coli*: possible structural information concerning the arrangement of membrane-bound helices and sugar/cation recognition site

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Abstract

Melibiose carrier mutants, isolated by growing cells on melibiose plus the non-metabolizable competitive inhibitor thiomethyl-β-galactoside (TMG), were studied to determine sugar and cation recognition abnormalities. Most of the mutants show good transport of melibiose but have lost the recognition of TMG. In addition, most mutants show little or no transport of lactose. Cation recognition is also affected as all of these mutants have lost the ability to transport protons with melibiose. The amino acids causing these mutations were determined by sequencing the *melB* gene on the plasmid. The mutations were located on helices I, IV, VII, X and XI. We propose that these five helices are in proximity with each other and that they line the sugar/cation transport channel.

Keywords: Membrane; Transport; Melibiose; *Escherichia coli*

1. Introduction

Cation–substrate cotransport membrane carriers are found in all bacteria [1–3]. There is obligatory coupling between cation entry and substrate entry on the carrier. The energy stored in the cation gradient may be used to accumulate the substrate against a concentration gradient. The melibiose carrier of *Escherichia coli*, encoded by the *melB* gene, is one of this class of cotransport systems [4]. This carrier is unique in its ability to utilize either H^+, Na^+ or Li^+ [5–8]. Sugar binding studies with membrane vesicles have shown that the presence of Na^+ or Li^+ ions increases the carrier's affinity for galactosides [9,10].

The *melB* gene has been cloned [11] and sequenced [12]. The primary amino acid sequence deduced from the gene sequence predicts a hydrophobic protein (70% apolar) with a molecular mass of 52 kDa [12]. The results of hydropathy analysis and *melB*-phoA fusions have provided good evidence for a two-dimensional structure in which the protein forms 12 α-helical transmembrane domains connected by hydrophilic loops [11–14].

The study of a variety of mutants has given us some data on which we may make speculations concerning the organization of the helices. Neutral amino acid substitutions for Asp-19 (helix I), Asp-55 or Asp-59 (helix II), or Asp-124 (helix IV) cause the loss of Na^+-coupled sugar transport. Thus it has been

Abbreviations: TMG, thiomethyl-β-D-galactoside

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proposed [15–19] that these Asp residues provide part of the cation binding site and that helices I, II and IV are close together. Another approach to the problem of helix–helix interaction is the study of second site revertants. First, a mutation of the *melB* gene with low activity is isolated or constructed by site-directed mutagenesis. The cell containing this mutation is streaked on melibiose plates and melibiose-positive mutants can be isolated after several days incubation. One study [20] suggested that helix II is close to helices I, IV, VII and X. Another study [21] suggested that helix XI is close to helix IV.

Still another approach to determining the proximity of helices is to isolate mutants by a selection procedure. Our method [22] was to isolate mutants that would grow on melibiose in the presence of a non-metabolizable competitive inhibitor thiomethylgalactoside (TMG). Mutants were found in helices I, II, IV, X and XI. Studies of the K_m and V_{max} of TMG transport [22] indicated that each mutant showed a reduction in affinity for this inhibitor. The lithium inhibition of growth found in normal cells was greatly reduced in each of these mutants [22]. The present study is an extension of this work. In the experiments reported here, the TMG-resistant mutants were tested for sugar recognition (melibiose, TMG and lactose) and for cation recognition (H^+, Na^+ and Li^+). Most of these mutants showed a dramatic alteration in both sugar recognition and cation recognition. It is postulated that the sugar binding site must overlap with the cation binding site.

2. Materials and methods

2.1. Reagents

Melibiose and TMG were purchased from Sigma. Methyl-[^{14}C]TMG was purchased from Du Pont-New England Nuclear. Radiolabeled [glucose-1-^{14}C]lactose was from Amersham. [^{3}H]Melibiose was a generous gift from Dr. G. Leblanc. Each radioactive sugar was purified by paper chromatography on Whatman 3 mm chromatography paper using a mixed solvent of three parts 1-propanol:one part water.

2.2. Cells and plasmids

E. coli strain DW2 is Δ(lacZY) *melA*$^+$Δ*B* [23]. Plasmid psty91 contains both *melA* and *melB* genes in pBR322 [11]. Plasmid psty37 contains the *melB* gene in pBR322 (Tsuchiya, personal communication).

2.3. Sugar transport assays

The *E. coli* strain DW2 was used as the host strain for all transport assays. Cells were grown to late-log phase in Luria–Bertani medium containing tetracycline (1.5 μg/ml), harvested and washed twice in a buffer containing 0.1 M morpholinepropanesulfonic acid (MOPS)–Tris (pH 7.0) and 0.5 mM $MgSO_4$. The washed cells were resuspended in the same buffer to a cell density of approximately 3×10^9 cells/ml and allowed to equilibrate at room temperature for 10 min. Transport was initiated by the addition of radioactive sugar in the presence or absence of NaCl (10 mM) or LiCl (10 mM). A 0.2 ml sample was taken at a 10 min point and filtered through 0.65 μm pore size cellulose nitrate filter (Sartorius). To remove the remaining extracellular sugar solution, filtered cells were washed with 5 ml of MOPS buffer. Filters were dissolved in 4 ml of Emulsifier-safe (Packard) and counted. The volume of intracellular water was estimated to be 0.4 μl/6×10^8 cells for calculations of sugar accumulation. Accumulation values are reported as the ratio of intracellular concentration to extracellular concentration.

2.4. Isolation of mutants

Mutants were isolated on the *melB* expression plasmids psty37 (*melB*) and psty91 (*melA melB*) containing the temperature-resistant *melB* gene [22]. The plasmids were introduced into strain DW2. Cells were placed on medium 63 minimal media [24] pH 5.8 containing tetracycline with 2 mM melibiose as the sole source of carbon and 10 mM TMG as a competitive inhibitor of the melibiose carrier. Under these conditions, growth of cells containing the wild type *melB* gene was strongly inhibited. Cells with mutations that released the cells from the TMG inhibition of the normal carrier grew more rapidly and

were clearly visible as clones in 48–72 h. The plasmid was isolated and the *melB* gene sequenced.

2.5. Sugar-stimulated proton uptake

Proton uptake was measured by the method of West [25] as modified by Wilson et al. [26]. Freshly grown cells were washed twice in 120 mM KCl and resuspended in the same medium. The cell density in the assay was equivalent to 8 mg dry weight/ml. Potassium thiocyanate was added to give a final concentration of 10 mM. The cell suspension (2.5 ml) was placed in a 3 ml plastic vial and was stirred by a magnetic stirring bar. The combined pH electrode was from Radiometer Copenhagen (GK 2321-C). This electrode was inserted through a hole in the vial lid. A small vent in the lid was used for passage of argon over the fluid in the vial. A second vent served for introduction of the sugar. The assay was performed at approximately pH 6 which was the pH of the cell suspension without adjustments. After passage of argon through the vial for at least 30 min, anaerobic sugar solution (pH 6) was added to give a final concentration of 2 mM. Proton movement was recorded on a Linear Instrument recorder.

2.6. Sugar-stimulated sodium uptake

Sodium uptake was measured with a sodium electrode (Radiometer Copenhagen G502A). The cells were washed twice in 100 mM MOPS buffer (pH 7) and resuspended in 100 mM Tricine buffer pH 8 at a cell density of 8 mg dry weight/ml. The final mixture contained 50 μM NaCl. A 5 ml plastic vial (2 cm in diameter) was used with one hole in the lid for the combined sodium electrode (Radiometer Copenhagen K40) plus two additional vents for introduction of argon or substrates.

2.7. Immunodetection of the melibiose carrier

The amount of melibiose carrier present in each

Table 1
TMG-resistant mutants (in DW2 cells)

Cell	Helix position of mutation	melB protein expression (% of normal)	Color of colony on MacConkey plate
psty37	–	100[a]	Red
37-D19E	I	100 ± 3	Red
37-Y120H	IV	177 ± 8	Red
37-P126S	IV	148 ± 29	Red
37-A240V	VII	97 ± 8	Red
37-V349M	Loop X/XI	134 ± 9	Red
37-T373A	Loop X/XI	64 ± 11	Red
37-M374I	Loop X/XI	49 ± 5	Red
37-M374V	Loop X/XI	189 ± 18	Red
37-V376G	XI	99 ± 9	Red
37-G378S	XI	67 ± 4	Red
psty91	–	100[a]	Red
91-A21T	I	99 ± 3	Red
91-I22N	I	155 ± 3	Red
91-I65V	Loop II/III	78 ± 12	Red
91-Y120F	IV	148 ± 8	Red
91-M123V	IV	257 ± 24	Red
91-A240T	VII	90 ± 2	Red
91-V346A	X	100 ± 16	Red
91-I352V	Loop X/XI	142 ± 18	Red
91-A368V	Loop X/XI	24 ± 4	Red
91-Y369F	Loop X/XI	56 ± 10	Red
91-Q372L	Loop X/XI	186 ± 31	Red
91-V376A	XI	53 ± 8	Red

[a]The quantity of melB protein of psty37 was 1.5 times that of psty91.

Table 2
Transport of melibiose[a] by TMG-resistant mutants (in DW2 cells)

Cell	nmol of melibiose/mg cell protein
psty37[b]	125 ± 4
37-D19E	302 ± 4
37-Y120H	260 ± 11
37-P126S	362 ± 28
37-A240V	93 ± 6
37-V349M	127 ± 8
37-T373A	188 ± 6
37-M374I	178 ± 6
37-M374V	215 ± 2
37-V376G	199 ± 5
37-G378S	166 ± 8
psty91[c]	83 ± 3
91-A21T	66 ± 8
91-I22N	183 ± 10
91-I65V	226 ± 3
91-Y120F	144 ± 4
91-M123V	306 ± 14
91-A240T	49 ± 5
91-V346A	16 ± 1
91-I352V	44 ± 4
91-A368V	19 ± 0.4
91-Y369F	21 ± 2
91-Q372L	63 ± 0
91-V376A	28 ± 2

[a]Melibiose concentration was 1 mM, with 10 mM NaCl. Assay time was 10 min.
[b]Plasmid psty37 did not contain the *melA* gene (for α-galactosidase), therefore transport resulted in accumulation of the sugar.
[c]Plasmid psty91 contained *melA* gene, therefore downhill transport.

strain was determined as described by Lolkema et al. [27]. In summary, a known quantity of cells was filtered through nitrocellulose filters, then lysed with NaOH-SDS and neutralized on the nitrocellulose filters. Filters were then incubated with bovine serum albumin to block non-specific binding, followed by incubation with a polyclonal antibody, anti-MBct10 [28], directed against the carboxyl-terminal 10 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 non-specific adsorption, values obtained for the strain DW2/pKK223-3 (*melB*$^-$) were used as a background control in each experiment. Values for the mutants are presented as percentages of the wild type protein level.

3. Results

In a previous study [22], mutants were isolated which grew on agar plates on melibiose in the presence of the non-metabolizable competitive inhibitor TMG. In the studies reported here, sugar recognition and cation recognition abnormalities were investigated in these mutants.

3.1. Abnormalities in sugar recognition

The uptake of melibiose was studied under a variety of conditions. In a qualitative experiment, cells were plated on melibiose MacConkey indicator plates. All mutants were red, indicating that fermentation of melibiose was positive (Table 1). Transport of radioactive melibiose was also tested (Table 2). When exposed to 1 mM melibiose, 13 of the 22 mu-

Table 3
Transport[a] of TMG[b] and lactose[b] by TMG-resistant mutants (in DW2 cells)

Cell	In/out	
	TMG	Lactose
psty37	243 ± 5	9 ± 0.3
37-D19E	10 ± 0	0
37-Y120H	13 ± 2	0
37-P126S	25 ± 0	0.2
37-A240V	6 ± 0	0
37-V349M	86 ± 8	0.8 ± 0.1
37-T373A	3 ± 0.8	0.1
37-M374I	2 ± 0.5	0.1
37-M374V	4 ± 0.8	0.1
37-V376G	3 ± 0.5	0.5
37-G378S	23 ± 6	1 ± 0.2
psty91	228 ± 4	7 ± 0.3
91-A21T	19 ± 0.5	0
91-I22N	5 ± 0.4	0
91-I65V	5 ± 0.1	0
91-Y120F	5 ± 0.2	0
91-M123V	49 ± 2	0.5
91-A240T	12 ± 1	0
91-V346A	8 ± 0.2	0
91-I352V	29 ± 1	0.3
91-A368V	3 ± 0.7	0.1
91-Y369F	3 ± 1	0.1
91-Q372L	11 ± 0.1	0
91-V376A	27 ± 2	1

[a]Transport data are presented as in/out ratio in 10 min.
[b]TMG and lactose concentrations were each 0.1 mM, both with 10 mM NaCl in 100 mM MOPS buffer.

tants showed greater transport than normal. Five mutants showed more than 50% of normal and four showed less than 50% activity. Of this latter group, three of the four mutants showed expression level about 50% or lower (Table 1). Thus, most of the mutants show good activity with 1 mM melibiose.

All of the mutants showed a very severe defect in the transport of TMG. The transport was less than 15% of normal in 20 out of 22 mutants (Table 3). The remaining two gave 21% (91-M123V) and 35% (37-V349M) of normal transport. Similarly, the transport of lactose was zero or extremely low in all of the mutants (Table 3).

Table 4
Transport of melibiose[a] by TMG-resistant mutants with different ions[b]

Cell	nmol of melibiose/mg cell protein		
	H^+	Li^+	Na^+
psty37[c]	9 ± 0.4	59 ± 2	64 ± 4
37-D19E	10 ± 0.1	96 ± 2	75 ± 6
37-Y120H	14 ± 1	71 ± 3	68 ± 4
37-P126S	1 ± 0.2	58 ± 1	136 ± 3
37-A240V	6 ± 0.3	40 ± 1	24 ± 1
37-V349M	10 ± 1	139 ± 4	134 ± 3
37-T373A	1 ± 0	40 ± 5	86 ± 4
37-M374I	1 ± 0	36 ± 2	75 ± 5
37-M374V	2 ± 0.1	90 ± 5	73 ± 2
37-V376G	5 ± 0.1	53 ± 4	53 ± 2
37-G378S	15 ± 1	121 ± 1	130 ± 4
psty91[d]	15 ± 0.3	19 ± 1	27 ± 1
91-A21T	7 ± 0.4	31 ± 1	31 ± 1
91-I22N	3 ± 0.3	60 ± 4	54 ± 1
91-I65V	2 ± 0.2	71 ± 5	60 ± 1
91-Y120F	1 ± 0	38 ± 2	37 ± 2
91-M123V	15 ± 0.4	97 ± 7	109 ± 5
91-A240T	15 ± 1	31 ± 1	27 ± 1
91-V346A	4 ± 0.3	14 ± 1	15 ± 0.2
91-I352V	17 ± 1	48 ± 2	40 ± 3
91-A368V	6 ± 0.1	10 ± 0.2	8 ± 0.6
91-Y369F	2 ± 0	8 ± 0.2	6 ± 0.1
91-Q372L	20 ± 0.4	42 ± 1	37 ± 2
91-V376A	12 ± 0.3	31 ± 0.3	36 ± 2

[a]Melibiose concentration was 0.2 mM. Time was 10 min.
[b]H^+ (100 mM MOPS buffer), Li^+ and Na^+ concentrations were both 10 mM.
[c]Plasmid psty37 did not contain *melA* gene (for α-galactosidase), therefore transport resulted in accumulation.
[d]Plasmid psty91 contained the *melA* gene so that entering melibiose was metabolized and therefore transport was 'downhill'.

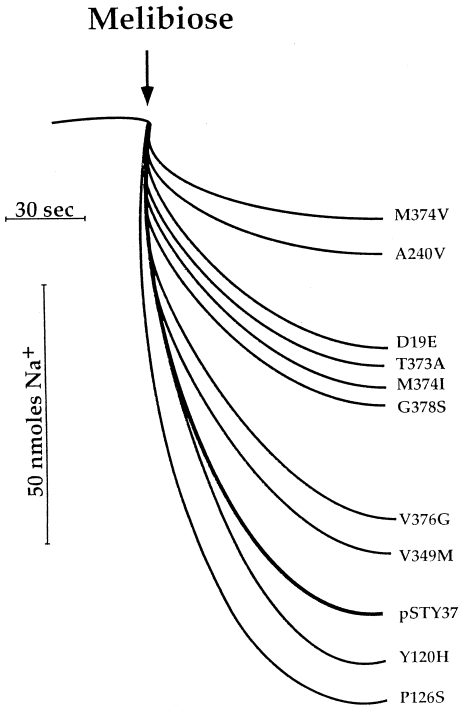

Fig. 1. Melibiose-stimulated sodium ion uptake by psty37 and its mutants.

3.2. Effect of cations on melibiose transport

One of the striking observations was the low transport activity (less than 50% of normal) in the absence of Na^+ or Li^+ in 11 mutants, some values being extremely low (Table 4). Thus, cotransport with protons is defective in many of the mutants. Another cation abnormality was that 11 mutants showed higher transport with Li^+ than with Na^+. Mutant 91-M123V showed a very striking abnormality: its expression was 2.6 times normal. Melibiose transport with Na^+ was three times higher than normal and transport with Li^+ five times higher than normal. Similar results were obtained with 37-V349M, 37-P126S and 37-G378S. In each case, melibiose uptake in the presence of Na^+ was twice normal and in two cases (37-V349M and 37-G378S), uptake in the presence of Li^+ was also twice normal. Thus, the effect of cations on transport is altered in most of the mutants.

3.3. Sodium ion entry in response to melibiose addition

When melibiose (2 mM) was added to normal cells

suspended in a low concentration of sodium ion, sugar and cation entered the cell. In such an experiment, one measures the extracellular sodium ion with a sensitive sodium electrode. On the addition of melibiose, the external concentration of sodium ion falls and reaches the lowest level in 30 s to 1 min. Fig. 1 shows the results with psty37 and its 10 mutants. In all of the mutants, sodium uptake was observed. In two cases (37-Y120H and 37-P126S), there was distinctly more sodium uptake than the normal. At 0.2 mM melibiose concentration, 37-P126S showed twice as much melibiose uptake as the parental cell psty37, whereas 37-Y120H showed about the same melibiose transport as normal. But at 1 mM melibiose concentration, 37-Y120H showed twice as much melibiose transport as the normal (Table 3). It is likely that 37-Y120H has higher K_m and V_{max}. Fig. 2 shows the results with psty91 and its 12 mutants. Again there is sodium uptake in each mutant. In three cases (91-V376A, 91-A21T and 91-M123V), there is distinctly more sodium uptake than the normal. In one of these cases (91-M123V), there was much more melibiose uptake than the parent, psty91.

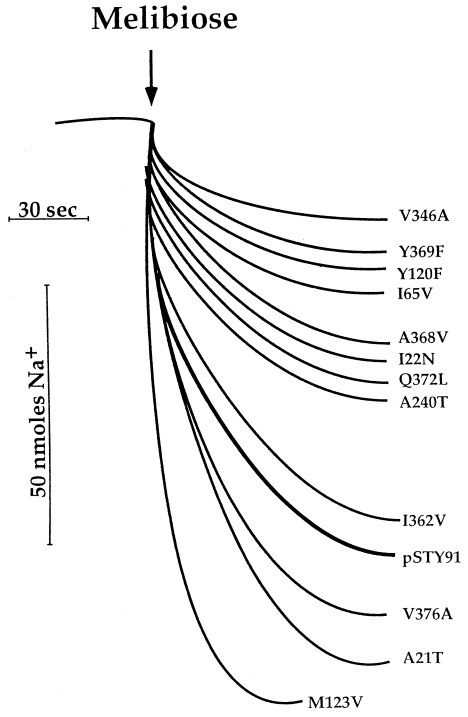

Fig. 2. Melibiose-stimulated sodium ion uptake by psty91 and its mutants.

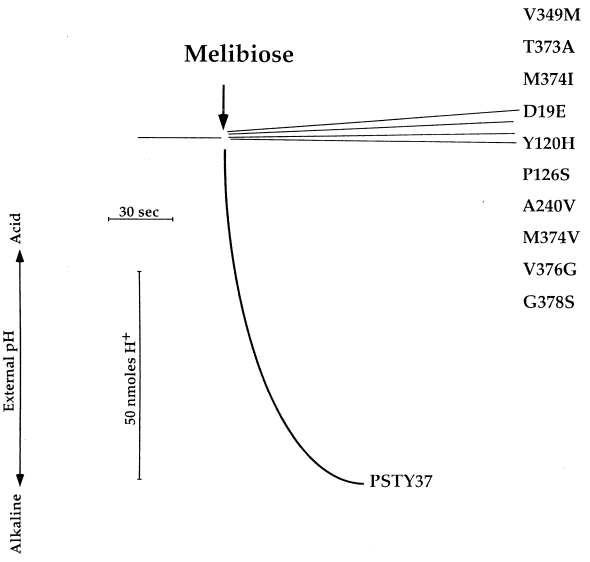

Fig. 3. Melibiose-stimulated proton uptake by psty37 and its mutants.

3.4. Proton uptake in response to melibiose addition

In a second set of experiments, the proton uptake by cells exposed to 2 mM melibiose was measured (Figs. 3 and 4). Cells were incubated in an unbuffered solution and the external pH measured. The addition of sugar to normal cells resulted in the uptake of protons with the sugar and a rise of pH in the external medium. The very striking result of these experiments was that none of the mutants showed a significant amount of proton uptake on the addition of melibiose. Eleven of the mutants showed little or no sugar uptake with protons (Table 3) but many of them showed considerable uptake under these conditions. In these latter mutants, the uptake of melibiose in the absence of sodium or lithium ions without associated proton uptake suggests sugar uptake without cation. This represents an uncoupling between the normal obligatory coupling between sugar and cation entry on the carrier. The mechanism of transport in these mutants is not understood at the present time.

4. Discussion

The mutants described in this communication were isolated as mutants that grew on 2 mM melibiose plus 10 mM TMG [22]. Under these conditions,

growth of the cells containing wild type *melB* gene (psty37 and psty91) was strongly inhibited. Cells released from this TMG inhibition via mutation of the *melB* gene grew more rapidly and were clearly visible on the agar plates after 48 h. The *melB* gene in the plasmid was sequenced. These mutants showed a poor affinity (high K_m) for the inhibitor, TMG.

The studies reported in this communication indicated that most of the mutants transport melibiose (1 mM) quite well. Thirteen out of 22 mutants showed melibiose transport better than the wild type. Most of the mutants showed extremely poor transport of TMG and all of the mutants showed little or no transport of lactose. Thus, there was a marked alteration in sugar recognition by the mutants. Cation recognition abnormalities were also observed. While melibiose-stimulated sodium ion uptake was observed in all mutants, sugar-stimulated proton uptake was completely lost in all mutants. Thus each mutant showed abnormalities in both sugar recognition and cation recognition.

The possibility that the sugar might contribute to the binding sites for cations is suggested by several experiments. α-Galactosides are cotransported with H^+, Na^+ or Li^+. D-Galactose shows uptake with H^+, Na^+ and Li^+ while D-fucose and L-arabinose show cotransport with Na^+ and Li^+ but not H^+ [8]. In the present experiments, it was found that every mutant with altered TMG and lactose recognition has completely lost melibiose-stimulated proton uptake. By virtue of its two free pairs of electrons, the ring oxygen of simple sugars is available to act as a coordination atom and participate in complexing with the cation. The finding that different cations interact with the coupling site of the melibiose carrier has important implications. Boyer [29] pointed out that if a carrier can use both H^+ and Na^+ (or Li^+), the protein–ion interactions are likely to be similar in each case. For this reason, he suggested that the proton is probably taken in the form of its hydrate (H_3O^+) rather than H^+.

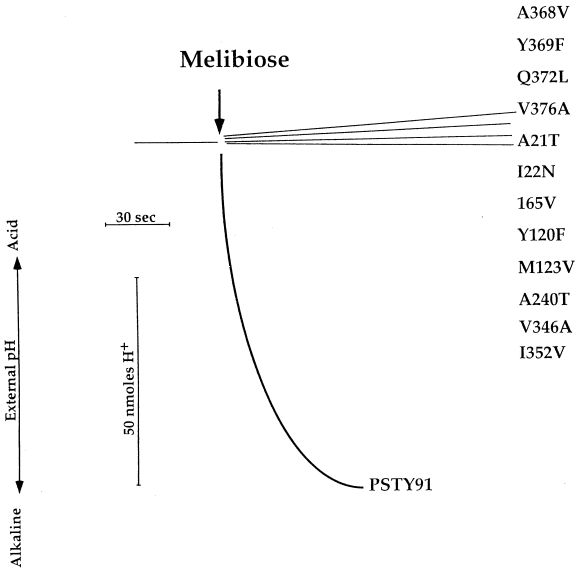

Fig. 4. Melibiose-stimulated proton uptake by psty91 and its mutants.

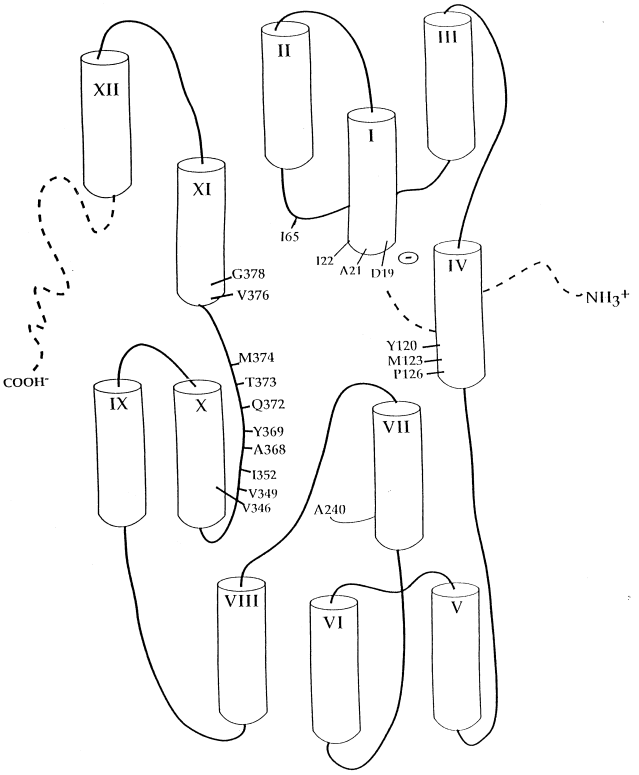

Fig. 5. A possible model of the arrangement of the helices of the melibiose carrier protein showing a central channel into which the mutated residues face.

Previous experiments have provided evidence for the close interaction between several transmembrane helices. Several of these experiments involved the isolation of second site revertants. First, a mutant was constructed which showed little or no activity. This mutant was white on melibiose MacConkey indicator plates. When such cells were incubated for several days, rare red mutants appeared on the plates. When melibiose-positive mutants were sequenced, they frequently contained the original mutation in

addition to a second site mutation. In one such study [20], Arg-52 (in helix II) was converted to serine, resulting in extremely low activity. Second site mutations were found in helices I, IV, VII and X. This suggested that these four helices are close to helix II. This agrees very well with the data given in Fig. 5. In another study [21], Asp-120 (helix IV) was converted to serine which resulted in low activity. Second site revertants were found in V375 (helix XI). This suggested the close proximity of helix IV and helix XI.

Another approach to this problem is to isolate mutants selected for growth under conditions which alter the sugar recognition. The experiments reported in this communication represent an example of this type. Cells were grown on melibiose in the presence of a high concentration of a non-metabolizable competitive inhibitor (TMG). Mutations were found in helices I, IV, VII, X and XI (Fig. 5). In addition, several mutations were located in the cytoplasmic loop between helix X and helix XI. It is of course possible that the mutations alter some distant region of the carrier that is involved in sugar recognition. Alternatively, the mutations are themselves in or near the sugar recognition site itself. In this case, the results agree with those obtained with second site revertants since both types of mutants are located in similar regions of the carrier. It is interesting to note that a lithium-resistant mutant isolated by Yazyu et al. [30] proved to be P126S which is identical to one of our mutants (37-P126S). In Fig. 5, one can see that the five helices which are close together might form a binding site for sugar and cation as well as a channel through which these substrates could pass during the transport event. Therefore, we suggest that these helices are likely to be adjacent to one another.

Acknowledgements

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