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Physiological evidence for an interaction between helices II and XI in the melibiose carrier of *Escherichia coli*

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Abstract

The melibiose carrier from *Escherichia coli* is a cation–substrate cotransporter that catalyzes the accumulation of galactosides at the expense of H^+, Na^+, or Li^+ electrochemical gradients. Charged residues on transmembrane domains in the amino-terminal portion of this carrier play an important role in the recognition of cations, while the carboxyl portion of the protein seems to be important for sugar recognition. In the present study, we substituted Lys-377 on helix XI with Val. This mutant carrier, K377V, had reduced melibiose transport activity. We subsequently used this mutant for the isolation of functional second-site revertants. Revertant strains showed the additional substitutions of Val or Asn for Asp-59 (helix II), or Leu for Phe-20 (helix I). Isolation of revertant strains where both Lys-377 and Asp-59 are substituted with neutral residues suggested the possibility that a salt bridge exists between helix II and helix XI. To further test this idea, we constructed three additional site-directed mutants: Asp-59 → Lys (D59K), Lys-377 → Asp (K377D), and a double mutant, Asp-59 → Lys/Lys-377 → Asp (D59K/K377D), in which the position of these charges was exchanged. K377D accumulated melibiose only marginally while D59K could not accumulate. However, the D59K/K377D double mutant accumulated melibiose to a modest level although this activity was no longer stimulated by Na^+. We suggest that Asp-59 and Lys-377 interact via a salt bridge that brings helix II and helix XI close to one another in the three-dimensional structure of the carrier.

Keywords: Melibiose; Membrane transport; Mutant; *Escherichia coli*

1. Introduction

The melibiose carrier (MelB) of *Escherichia coli*, encoded by the *melB* gene [1], is a cation–substrate cotransport system located in the bacterial plasma membrane (for reviews see [2,3]). Although its physiological substrate is the α-galactoside melibiose, this carrier is able to support the uptake of a variety of sugars, including galactose, lactose, and thiomethyl-galactoside (TMG) [4,5]. Perhaps the most interesting feature of this carrier is its ability to couple the transport of melibiose to Na^+, H^+, and Li^+, with Na^+ being the most effective cosubstrate [4–7]. This flexibility for cation coupling makes the melibiose carrier unique among bacterial secondary active cotransport systems and suggests a possible evolutionary link between the H^+-coupled carriers of bacterial

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cells and the Na^+-coupled carriers of eukaryotic cells.

Based on hydropathy plots [1], *melB-phoA* reporter gene fusions [8,9], and proteolytic digestion experiments [10], the two-dimensional structure of the carrier protein is believed to consist of 12 transmembrane α-helices that traverse the membrane in a zigzag fashion connected by hydrophilic loops. Charged residues in the membrane-spanning helices have been implicated in both sugar and cation recognition. Substitution of neutral amino acids for the aspartic acid residues at positions 19 (helix I), 55 (helix II), 59 (helix II), and 124 (helix IV) leads to a loss of Na^+-stimulated sugar binding [3,11–13]. It is believed that these aspartic acid residues form part of a coordination site for cations [2,3,13,14]. While the presence of charged amino acid residues in membrane-spanning regions is thermodynamically unfavorable, Honig and Hubbel have pointed out that the transfer of an ion pair (salt bridge) from water to a region of low dielectric constant is not so energetically unfavorable [15]. Indeed, if such an ion pair made a few additional hydrogen bonds it would be extremely stable in the hydrophobic environment of a transmembrane α-helix.

There are five positively charged residues that reside on putative transmembrane domains in MelB, Lys-18 (helix I), Arg-52 (helix II), Arg-199 (helix VI), His-202 (helix VI), and Lys-377 (helix XI). It has been reported that His-202 is not important for carrier function [16] and that Arg-199 can be replaced without modifying carrier activity [10]. Recent work in our lab suggests that Arg-52 is important for cation-coupled sugar transport and that this residue participates in a salt bridge with Asp-55 [17]. In the present study, we concentrated on Lys-377 for two reasons. Isolation of substrate specificity mutants that have diminished recognition for methyl-β-D-thiogalactopyranoside (TMG) indicated that residues in loop X–XI and near the cytoplasmic border of helix XI are important for both sugar and cation recognition in the melibiose carrier [18]. In addition, although the substitution of Ser for Asp-124 causes a near complete loss of melibiose transport activity, second-site revertants that restore partial melibiose transport to this mutant (D124S) were found where Val-375 was substituted with Ala or Gly [19]. This result raised the possibility that Lys-377 could interact with negatively charged residues in the amino-terminal portion of the carrier. In this study, we wanted to determine if Lys-377 is important for sugar transport and if Lys-377 is salt-bridged to any of the Asp residues in the amino half of the carrier known to be important for cation recognition. When we substituted Val for Lys-377, transport activity was significantly reduced. This low activity allowed us to screen for functional second-site revertants on melibiose MacConkey indicator plates. Interestingly, substitution of Asp-59 with a neutral residue was the most common type of revertant. This led us to create a double mutant where the charged residues, Asp-59 and Lys-377, were exchanged. The D59K/K377D mutant regained significant transport activity over that found in either the D59K or K377D single mutants. The possibility of a salt bridge between Asp-59 and Lys-377, linking amino and carboxyl portions of the protein, is discussed.

2. Materials and methods

2.1. Reagents

Melibiose (*O*-α-D-galactopyranosyl-(1,6)-D-glucopyranose) and TMG (methyl-1-thio-β-D-galactopyranoside) were purchased from Sigma. [^{3}H]Melibiose was a generous gift from Dr. Gérard Leblanc of Départment de Biologie Commissariat à l'Energie Atomique, Villefranche-sur-mer, France. [^{14}C]TMG was purchased from Dupont-NEN. [^{35}S]Protein A was purchased from Amersham. [α-^{33}P]dATP was from Andotek. Restriction enzymes and ligase were from Pharmacia Biotech. Bacteriological media were from Difco. All other chemicals were reagent grade.

2.2. Bacterial strains and plasmids

Plasmid DNA was isolated using the Qiaprep Spin Miniprep kit (Qiagen) and introduced into the appropriate bacterial strains by $RbCl_2$ transformation. *E. coli* DW1 (*lacI*$^+$Δ*ZY mel*Δ*AB*) [5] and DW1/pSU-melA (*lacI*$^+$Δ*ZY mel*Δ*AB*/*melA*$^+$) [1,19] were used as host strains for plasmids expressing *E. coli melB*. The plasmid, pKKMB (ampicillin resistant) [20], that contains the gene for the melibiose carrier inserted into the vector pKK223-3 (Pharmacia Biotech) was

used for the expression of *melB*. The plasmid, pSUmelA (chloramphenicol resistant) [1,19], was used to express *melA* (α-galactosidase) which allows cells to ferment melibiose.

2.3. Site-directed mutagenesis

The Chameleon™ Double-Stranded, Site-Directed Mutagenesis kit (Stratagene) was used to substitute Lys-377 with Val. The QuikChange™ Site-Directed Mutagenesis kit (Stratagene) was used to replace Lys-377 with Asp (K377D), Asp-59 with Lys (D59K), and to create a double mutant (D59K/K377D) that swapped the residues at these positions. Mutagenic primers were 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). 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.

2.5. Selection of second-site revertant mutants

DW1/pSUmelA cells expressing the *melB* mutant K377V were used to screen for cells exhibiting a transport positive phenotype. Cells grew initially as white colonies on 0.4% melibiose MacConkey agar (Difco). After 5–8 days of incubation at 37°C, small red areas within white colonies were identified, picked, and re-streaked on the same medium to purify the colonies. Plasmid DNA isolated from red revertant colonies was used to transform DW1/pSUmelA to verify that the plasmid carrying the *melB* gene was responsible for the red phenotype on melibiose MacConkey medium.

2.6. Immunodetection of melibiose carrier in bacterial cells

The amount of melibiose carrier present in each strain was determined as previously described [21]. In summary, a known quantity of cells was lysed on nitrocellulose filters with NaOH/sodium dodecyl sulfate and then neutralized. Filters were incubated first with bovine serum albumin to block non-specific binding and then with a polyclonal antibody, anti-MBct10 [22], 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. In each experiment, values obtained for the strain DW1/pKK223-3 (*melB*$^{-}$) were used to correct for non-specific adsorption. Values for each mutant are presented as a percentage of wild-type protein levels.

2.7. Melibiose transport assays

E. coli DW1 was used as the host strain for melibiose accumulation assays [23]. Cells were grown to mid-log phase in LB medium containing ampicillin (100 μg/ml), harvested and washed twice in a buffer containing 0.1 M MOPS–Tris (pH 7.0) and 0.5 mM $MgSO_4$. The washed cells were resuspended in the same buffer to a density of approximately 3×10^9 cells/ml, and allowed to equilibrate to room temperature for 15 min. Transport was initiated by the addition of [^{3}H]melibiose (0.2 mM, 0.5 μCi/ml) or [^{14}C]TMG (0.1 mM, 0.1 μCi/ml) in the absence or presence of NaCl (10 mM), or LiCl (10 mM). A 0.2-ml aliquot was taken at various time points (0.5–15 min) and filtered rapidly through 0.65 μm pore size cellulose nitrate filters (Sartorius). To remove any remaining external sugar solution, filtered cells were washed with 5–10 ml of buffer. Filters were dissolved in 4 ml of Liquiscint (National Diagnostics) and counted. The volume of intracellular water was estimated to be 0.4 μl/6×10^8 cells [24] for calculations of sugar accumulation. Accumulation values are reported as the ratio of intracellular sugar concentration versus extracellular sugar concentration.

E. coli DW1/pSUmelA was used as the host strain for downhill melibiose transport assays. Cells were grown to mid-log phase in LB medium containing ampicillin (100 mg/ml) and chloramphenicol (30 μg/ml). Cells were prepared as described in the previous paragraph, and transport assays were initiated by the addition of [^{3}H]melibiose (0.8 mM, 0.5 μCi/ml) in the absence or presence of NaCl (10 mM), or LiCl (10 mM). Transport assays were car-

ried out as described in the preceding paragraph with time points taken between 1 and 10 min. An estimate of 0.1 mg protein/6×10^8 cells was used for calculations of melibiose transport. Transport values are reported as nmol melibiose/mg total cell protein.

2.8. Measurement of K_m and V_{max} for melibiose

The *E. coli* DW1/pSUmelA strain was used for the measurement of apparent K_m and V_{max} for downhill transport of melibiose. Downhill transport was first tested at a variety of NaCl concentrations (0–100 mM) to determine which concentration gave maximal stimulation for each MelB derivative. The Na^+ concentrations used were 10 mM for wild-type, and 75 mM for both K377V/F20L and K377V/D59N. Cells were prepared as described for the downhill transport assay and the initial rate (within 30 s) of melibiose transport was measured at six sugar concentrations (0.14–5.6 mM) to estimate the K_m for each strain. Six melibiose concentrations were chosen which bracketed the initial estimate and measurements were repeated at least two more times. Kinetic constants were determined using a Lineweaver–Burk double reciprocal plot. The values for V_{max} reported in Table 3 for revertant strains were normalized to the amount of carrier present in the wild-type strain as reported in Table 1.

2.9. Assay for melibiose-induced Na^+ transport

Sodium uptake was measured as described previously [25]. In summary, DW1 cells expressing wild-type, mutant, or revertant MelB carriers were grown to mid-log phase in LB medium containing ampicillin (100 mg/ml). Cells were harvested, washed twice and resuspended in 0.1 M MOPS–Tris (pH 7.0), 0.5 mM $MgSO_4$. An aliquot of cells was diluted to 2.5 mg protein/ml in 0.1 M Tricine–Tris (pH 8.0), and NaCl was added to a final concentration of 50 μM. Cells (6 ml) were placed in a closed plastic vial with holes in the lid to accommodate an FK1502Na Sodium Combination Ion Selective Electrode (Radiometer), a gas-tight syringe (Hamilton), and a tube for the introduction of argon. Cells were made anaerobic under argon for 30 min, followed by

Table 1
Plasmids, colony phenotype and carrier expression levels

Plasmid[a]	Site of amino acid substitution(s)	Location in secondary structural model[b]	Colony phenotype on 0.5% melibiose MacConkey plates[c]	Level of protein expression[d] (% normal ± S.E.M.)
pKK223-3	melB$^-$ control	–	white	–
pKKMelB	wild-type	–	red	100
pK377V	Lys-377 → Val	helix XI	white	22 ± 7
pK377V/D59V	Lys-377 → Val	helix XI	red	33 ± 10
	Asp-59 → Val	helix II		
pK377V/D59N	Lys-377 → Val	helix XI	red	25 ± 13
	Asp-59 → Asn	helix II		
pK377V/F20L	Lys-377 → Val	helix XI	red	48 ± 10
	Phe-20 → Leu	helix I		
pD59K	Asp-59 → Lys	helix II	white	15 ± 5
pK377D	Lys-377 → Asp	helix XI	pink	10 ± 0.6
pD59K/K377D	Asp-59 → Lys	helix II	red	50 ± 16
	Lys-377 → Asp	helix XI		

[a]Plasmids expressing MelB derivatives are designated by the single letter amino acid code for the wild-type residue, followed by the position of that residue in the primary amino acid sequence of the carrier, followed by the single letter amino acid code for the mutant residue at that position.

[b]Position of the mutant or revertant amino acid residue(s) within the secondary topological model for the melibiose carrier.

[c]MacConkey agar is a rich medium lacking a fermentable carbon source. The fermentation of melibiose on this medium causes cells to form red colonies. Cells unable to ferment melibiose form white colonies. The concentration of melibiose in these plates was 15 mM (0.5%).

[d]Expression levels for melibiose carriers are reported as a percentage of the expression found for the wild-type carrier. The values are an average of at least two separate measurements ± S.E.M.

the addition of anaerobic melibiose (10 mM, final concentration). Changes in sodium levels in the extracellular medium were monitored with a chart recorder (Linear Instruments). The sensitivity of the system was tested with a known amount of anaerobic NaCl during each experiment.

2.10. Assay for melibiose-induced H^+ transport

The measurement of proton uptake was performed according to the method of West [26] as modified by Wilson et al. [27]. Briefly, DW1 cells expressing wild-type, mutant, or revertant MelB carriers were grown to mid-log phase in LB medium containing ampicillin (100 mg/ml). Cells were washed twice and resuspended to a density of approximately 3.5 mg protein/ml in unbuffered KCl (120 mM). Cells (2.5 ml) were placed in a closed plastic vial with a lid containing apertures for the introduction of argon, a PHC4406 pH electrode (Radiometer) and a gas-tight syringe (Hamilton). Potassium thiocyanate (30 mM) was added and cells were made anaerobic under argon for 30 min. Addition of anaerobic melibiose (10 mM, final concentration) was used to initiate proton uptake. Changes in the pH of the extracellular medium were monitored with a PHM64 pH meter (Radiometer) and recorded with a chart recorder (Linear Instruments) such that a 0.1-unit pH change caused a 25-cm deflection in the chart recording. Calibrations were carried out using a known amount of anaerobic HCl during each experiment.

3. Results

3.1. Site-directed mutagenesis and isolation of second-site revertants

We were interested in determining if Lys-377 is important for transport activity and if it is ion-paired with any of the functionally important aspartic acid residues. We used site-directed mutagenesis to substitute Val for Lys-377 (K377V). When K377V was expressed in DW1/pSUmelA (*melA*$^+$) and plated on 0.4% (12 mM) melibiose MacConkey indicator agar, it formed white colonies, indicating a transport defect. On this media, the wild-type strain forms dark red colonies as the result of sugar transport via MelB and subsequent fermentation. To screen for functional second-site revertants, cells of K377V were allowed to incubate on these plates at 37°C until red areas were observed (after 5–7 days) within the white colonies. These red cells were purified by re-streaking on the same type of medium until a uniform red colony phenotype was observed. Plasmid DNA was then extracted from the red colonies and used to transform the DW1/pSUmelA strain to ensure that the mutation responsible for the red phenotype was carried on the *melB* bearing plasmid. Only those samples that retained a red phenotype after transformation were saved for further analysis. DNA sequence analysis revealed that in addition to the introduced mutation at codon 377, each revertant had an additional single base substitution causing a missense mutation within the coding region of the *melB* gene. The second-site mutations identified caused the following amino acid substitutions: Asp-59 → Val or Asn and Phe-20 → Leu.

The isolation of second-site revertants neutralizing the charge at position 59 suggested that a salt bridge may exist between Lys-377 and Asp-59. To further investigate this possibility, we used site-directed mutagenesis to make the following substitutions in the wild-type carrier: Asp-59 → Lys (D59K), Lys-377 → Asp (K377D), and a double mutant Asp-59 → Lys/Lys-377 → Asp (D59K/K377D). When these three MelB derivative carriers were plated on 0.5% melibiose MacConkey plates, we observed three distinct phenotypes (Table 1). D59K formed white colonies indicating a severe defect in melibiose uptake, and the K377D strain formed pink colonies suggesting a limited amount of transport activity. In contrast, the D59K/K377D double mutant formed red colonies suggesting that transport function had been restored above that of the single mutants. The restoration of transport activity in the double mutant, where the positions of the positive and negative charges has been reversed, suggests that the formation of an ion pair between residues at these positions is important for transport activity. The phenotypic variation of these three mutants also suggests that Asp-59 is more important than Lys-377 for maintaining a transport competent carrier protein.

3.2. Measurement of the amount of melibiose carrier

When constructing mutations in membrane proteins it is always a possibility that the introduced amino acid change will disrupt proper membrane insertion and/or stability of the protein and consequently cause a loss of sugar transport. To determine the relative amount of carrier protein present in the membrane of each mutant and revertant strain, immunoblots were done using a polyclonal antibody directed against the C-terminal 10 amino acids of the carrier. The amount of melibiose carrier protein expressed in each strain is presented as a percentage of the wild-type value (Table 1). Substitution of Lys-377 with Val caused a large reduction in carrier levels down to only 22% of the wild-type value. Two of the second-site revertants that involved substitution of Asp-59, K377V/D59V and K377V/D59N, also had reduced amounts of protein with expression levels similar to that of K377V. The K377V/F20L revertant had higher expression than the other revertants, showing 48% of wild-type. The amount of carrier protein was extremely low in the D59K and K377D mutants (to 15% and 10% of wild-type, respectively). The combination of these mutants in the D59K/K377D strain showed significantly higher expression (increase to 50% of normal).

Table 2
Downhill melibiose transport

MelB strain	Cation concentration		
	H^+	10 mM Na^+	10 mM Li^+
	nmol [^{3}H]melibiose/mg protein ± S.E.M.		
Wild-type	35 ± 9	149 ± 40	87 ± 4
K377V	18 ± 6	20 ± 4	28 ± 2
K377V/D59V	46 ± 8	40 ± 10	38 ± 2
K377V/D59N	24 ± 10	36 ± 9	38 ± 5
K377V/F20L	37 ± 8	115 ± 21	67 ± 2
D59K	0.6 ± 0.2	0.3 ± 0.1	0.1 ± 0.1
K377D	1 ± 0.3	4 ± 0.2	1 ± 0.5
D59K/K377D	35 ± 3	33 ± 8	35 ± 6

The concentration of radiolabeled melibiose was 0.8 mM and values given represent transport at 10 min. Transport values were calculated assuming 0.1 mg of protein for every 6×10^8 cells. The data are an average of at least two separate experiments with duplicate determinations in each experiment ± S.E.M.

3.3. Downhill transport of melibiose and kinetic measurements

Colony phenotype on melibiose MacConkey plates (see Table 1) provides a qualitative measure of carrier function. To obtain a more quantitative assessment of transport activity in mutant and revertant carriers, we measured downhill melibiose transport using whole cells with an external concentration of 0.8 mM [^{3}H]melibiose under a variety of cationic conditions (Table 2). In this experiment, sugar flows into the cell via the melibiose carrier and is rapidly cleaved by α-galactosidase so that the external sugar concentration always remains higher than the intracellular concentration. In the wild-type carrier, maximal stimulation of melibiose transport has been found to require 10 mM Na^+, while higher Na^+ concentrations inhibit transport. Table 2 shows that the K377V mutant has reduced H^+-coupled melibiose transport compared to wild-type and that Na^+-stimulated transport is absent. For the second-site revertant K377V/D59V, H^+- and Na^+-coupled transport was approximately twice that found for the parental strain (K377V), but, as was the case for K377V, Na^+-stimulated transport is absent. In the K377V/D59N second-site revertant, downhill melibiose transport was twice that found for K377V in the presence of Na^+, and Na^+ stimulation of transport was less than 2-fold. Measurement of melibiose transport kinetics for K377V/D59N indicated that the apparent K_m for melibiose was 3-fold higher than normal, and the V_{max} was over 5-fold higher than normal (Table 3). The K377V/F20L revertant had near wild-type melibiose transport activity in the presence of H^+ and 10 mM Na^+ (Table 2). Although the apparent K_m of K377V/F20L for melibiose was 10-fold higher than normal, the V_{max} was also increased to over five times that found in the normal carrier (Table 3). The introduction of a positive charge in place of Asp-59 (D59K) results in a carrier that lacks downhill transport activity. Likewise, changing Lys-377 to a negatively charged Asp (K377D) almost completely disrupts melibiose transport. When these two mutants are combined (D59K/K377D), reversing the position of the charges, the H^+-coupled melibiose transport returned to wild-type levels. However, this transport activity was no longer stimulated by the addition of Na^+ or Li^+.

Table 3
Kinetic analysis of downhill melibiose transport

Strain	Mean apparent K_m (mM) ± S.E.M.	Mean apparent V_{max} (nmol of melibiose/min/mg of protein) ± S.E.M.
Wild-type	0.22 ± 0.06	71 ± 14
K377V/F20L	2.2 ± 0.6	408 ± 108
K377V/D59N	0.71 ± 0.04	400 ± 48

Transport of melibiose after 30 s was measured at six different concentrations which bracketed an initial estimate of the K_m for each strain. The values for K_m and V_{max} are the average of at least two separate experiments with triplicate data points in each experiment. Transport was done in the presence of 10 mM Na^+ for wild-type, and 75 mM Na^+ for the K377V/F20L and K377V/D59N strains. Values for V_{max} were determined by normalizing values to the amount of carrier present in the wild-type strain (see Table 1).

3.4. Measurement of galactoside accumulation

Measurement of melibiose accumulation (uphill transport) in the presence of cations allows an assessment of the coupling between cation and sugar transport. For wild-type MelB, the presence of Na^+ (or Li^+) increases the carrier's affinity for melibiose, which stimulates accumulation of sugar to concentrations many times higher than that found in the extracellular medium. For example, steady state accumulation of melibiose in wild-type MelB was 6-fold with H^+ as compared to 154-fold in the presence of 10 mM Na^+ (Table 4). The K377V mutant accumulated melibiose in the presence of Na^+ and Li^+ to 25% of wild-type levels, with Na^+ stimulating uptake approximately 4-fold over transport in the presence of protons. For second-site revertants with a substitution for Asp-59 there was elevated accumulation with protons that was either not stimulated by Na^+ (K377V/D59V), or stimulated less than 2-fold (K377V/D59N). Accumulation of melibiose in the K377V/F20L revertant was only slightly higher than, and had a cation stimulation profile similar to, accumulation in the K377V parental strain. These data clearly show that Na^+ coupling has been compromised in the mutant and revertant strains.

The wild-type MelB is also able to efficiently accumulate the non-metabolizable β-galactoside TMG. A 26-fold accumulation of TMG was found without added cations (H^+ cotransport), and the addition of Na^+ or Li^+ (10 mM) stimulated this accumulation to approximately 250-fold (Table 4). In contrast, the K377V mutant accumulated TMG only 6-fold with protons. The presence of Na^+ (10 mM) did not stimulate TMG uptake in this mutant and Li^+ (10 mM) only afforded a very weak stimulation. The second-site revertants where Asp-59 has been substituted showed an interesting dichotomy. The K377V/D59V revertant was unable to accumulate TMG under any of the conditions tested. However, the K377V/D59N revertant showed significant TMG uptake. Although the stimulation of TMG transport by

Table 4
Steady state accumulation of melibiose and TMG in the presence of cations

Strain	Melibiose accumulation [IN]/[OUT] ± S.E.M.			TMG accumulation [IN]/[OUT] ± S.E.M.		
	H^+	Na^+	Li^+	H^+	Na^+	Li^+
Wild-type	6 ± 1	154 ± 21	87 ± 4	26 ± 7	250 ± 10	258 ± 9
K377V	10 ± 0.4	43 ± 10	21 ± 1	6 ± 1	4 ± 0.7	11 ± 2
K377V/D59V	18 ± 3	18 ± 6	13 ± 1	< 1	< 1	< 1
K377V/D59N	20 ± 3	35 ± 8	27 ± 3	40 ± 8	34 ± 8	43 ± 7
K377V/F20L	13 ± 3	66 ± 6	22 ± 1	9 ± 0.4	9 ± 1	16 ± 2
D59K	< 1	< 1	< 1	< 1	< 1	< 1
K377D	3 ± 1	5 ± 1	3 ± 0.06	< 1	< 1	< 1
D59K/K377D	13 ± 1	12 ± 1	11 ± 1	2 ± 0.3	2 ± 0.3	2 ± 0.3

The concentration of radiolabeled sugars in transport assays was 0.2 mM for melibiose and 0.1 mM for TMG. Values given represent transport after 15 min. Transport values were calculated assuming 0.4 μl of intracellular water for every 6×10^8 cells. The data are averages of at least two separate experiments with duplicate determinations in each experiment ± S.E.M. The concentration of Na^+ or Li^+ was 10 mM.

Na^+ and Li^+ was still defective for K377V/D59N, the H^+-coupled TMG accumulation was improved over that found for the wild-type strain. It appears that a polar amino acid is tolerated better as a substitution for Asp-59. Accumulation of TMG for the K377V/F20L revertant was only slightly better than the K377V parental strain and had a similar cation stimulation profile.

Melibiose and TMG accumulation in D59K were absent under all conditions tested. K377D had extremely low, but detectable melibiose accumulation and was unable to accumulate TMG. In contrast, the D59K/K377D double mutant had low but significant melibiose accumulation that was not stimulated by Na^+ or Li^+. Although this transport activity was weak, it was a significant improvement over that found for each of the single mutants suggesting partial reconstitution of carrier transport activity.

3.5. Measurement of melibiose-stimulated cation uptake

The transport of cations can be measured directly with pH- or Na^+-sensitive electrodes. In this experiment melibiose is added to anaerobic cells and the decrease in cation concentration (H^+ or Na^+) in the extracellular medium, due to cation–sugar cotransport, is monitored with an electrode and chart recorder. The addition of melibiose (10 mM) to wild-type cells caused a large and rapid alkalinization of the extracellular medium indicated by a downward deflection in the chart recording (Fig. 1). A modest proton influx was observed for the K377V/F20L revertant. In the second-site revertants obtained from the strains where Asp-59 was substituted, K377V/D59N had a minimal amount of melibiose driven proton uptake while K377/D59V showed significant proton influx. No proton influx was observed for the remaining mutants.

In a similar assay, Na^+ uptake was also assayed with a sodium-selective electrode. The addition of melibiose (10 mM) caused rapid Na^+ uptake in the wild-type strain indicated by an upward deflection in the chart recording (Fig. 2). A small amount of Na^+ influx was found for the K377V mutant. The K377V/F20L and K377V/D59N revertants showed a modest increase in Na^+ uptake as compared with K377V.

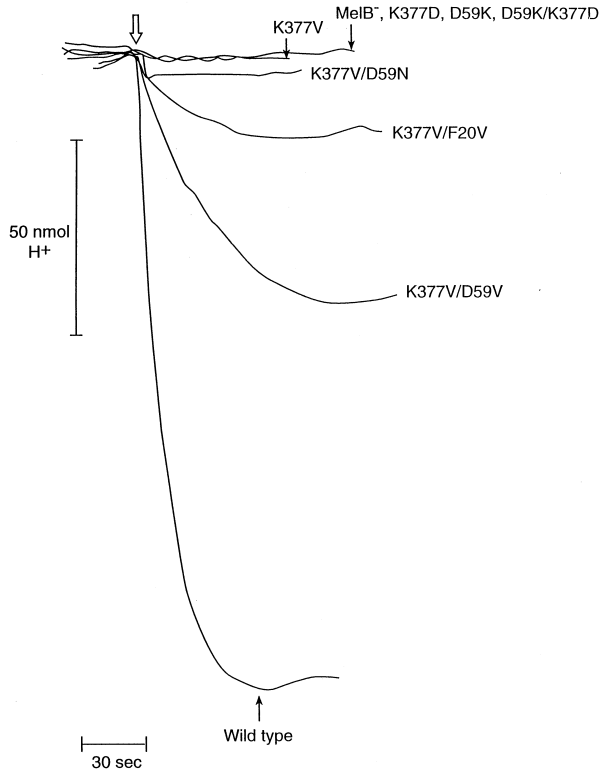

Fig. 1. Melibiose-stimulated H^+ transport. Melibiose was added to a final concentration of 10 mM and the change in extracellular pH was monitored with a pH electrode and chart recorder. A downward deflection indicates alkalinization of the extracellular media caused by sugar-stimulated proton uptake into the cell. The open arrow indicates addition of melibiose.

Melibiose-stimulated Na^+ influx was not observed in the remaining mutant or revertant strains.

4. Discussion

The study of the relationship between structure and function in membrane transport proteins has shown that charged residues play an important role in carrier function. Of particular interest are charged residues that reside on transmembrane helices. Given the energetically unfavorable positioning in the hydrophobic environment of the lipid bilayer, these residues often are targets for functional studies. For example, the importance of such residues has been demonstrated in lactose carrier of *E. coli* where Glu-325 has been shown to be critical for efficient proton coupling [28,29], and Glu-126 and Arg-144 are required for substrate binding [30,31]. Charged resi-

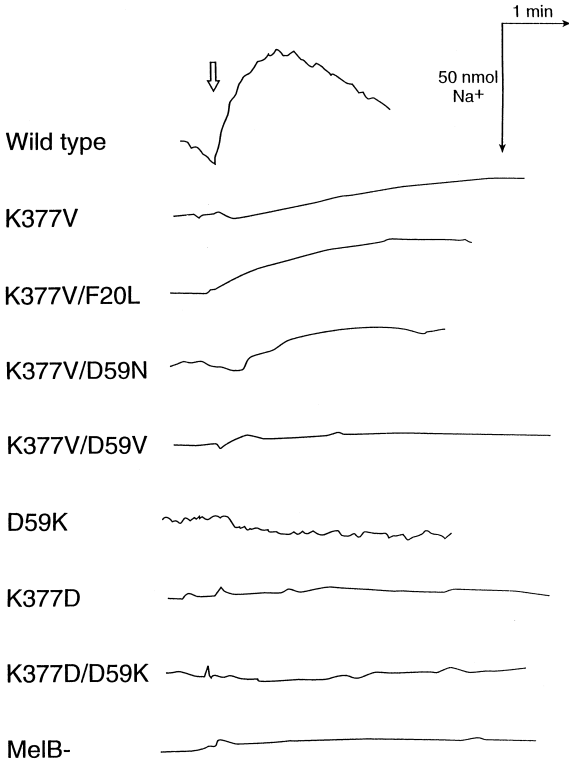

Fig. 2. Melibiose-stimulated Na^+ transport. Melibiose was added to a final concentration of 10 mM and the change in extracellular Na^+ was monitored with a sodium-selective electrode and chart recorder. An upward deflection in the chart recording indicates sugar-stimulated Na^+ uptake into the cell. The open arrow indicates addition of melibiose.

dues located on transmembrane helices have also been found to participate in interhelical ion pairs or salt bridges. Evidence for a salt bridge has been presented for bacteriorhodopsin [32], the voltage-gated Na^+ channel [33], CFTR [34], the H^+ ATPase of *Saccharomyces cerevisiae* [35], the lactose carrier of *E. coli* [36–39], and a vesicular monoamine transporter [40]. The identification of this type of salt bridge interaction provides information concerning the three-dimensional helical arrangement for these proteins.

In the current study, we wanted to determine if Lys-377 is important for sugar transport. We replaced this residue with Val and analyzed the transport properties of the mutant carrier. The K377V strain showed reduced melibiose and TMG transport activity. The reduced level of Na^+-stimulated sugar transport suggested that Lys-377 is important for efficient cation coupling. The defect in transport for the K377V mutant could be attributed to different problems caused by the removal of a positive charge at position 377. For example, Lys-377 could participate directly in sugar and/or cation binding. In support of this notion a previous study found that mutations that disrupt TMG recognition map to a cluster of residues between Ala-368 and Gly-378 [18]. In addition, for sugar binding proteins that have been crystallized (e.g. the arabinose binding protein [41]), lysine residues are found to make hydrogen-bonding contacts with sugar substrates. An alternative explanation is that Lys-377 interacts with aspartate residue(s) in the amino-terminal half of the carrier, regulating the pK_a of residues that are important for cation coordination. Because sodium increases the affinity of the melibiose carrier for sugar substrates, disruption of the coordination site for cations could, therefore, cause a decrease in sugar transport.

The low level of downhill melibiose transport activity for K377V (white phenotype on melibiose MacConkey indicator plates) allowed us to screen for functional second-site revertants. The goal of this screen was to determine if Lys-377 participates in a salt bridge with important negatively charged residues (e.g. Asp-19, Asp-55, Asp-59, or Asp-124). A second-site revertant in which a negatively charged residue was neutralized would provide evidence for such an interaction. Two types of revertants were isolated that involved residues at two distinct locations in the amino-terminal portion of the carrier.

The first type of revertant found was in helix I, where Phe-20 was substituted with Leu. The K377V/F20L revertant recovered near wild-type downhill transport activity and significant melibiose accumulation. Melibiose-stimulated H^+ and Na^+ transport was also observed. Although it is difficult to ascertain the mechanism by which this revertant allows recovery of transport activity, it is interesting to note that Phe-20 is adjacent to Asp-19 in helix I. Asp-19 is believed to participate in cation coordination and to be salt-bridged to Arg-52 in helix II [3,17]. Perhaps the substitution of Phe-20 with Leu changes the position of Asp-19 (helix I), altering the structure of the cation coordination site and allowing the K377V/F20L revertant to regain transport activity. Alternatively, Phe-20 might be located at or near the sugar binding site and substitution with Leu al-

lows more efficient sugar binding and/or transport. The V_{max} for melibiose transport in this revertant was found to be increased 5.7-fold over wild-type. In either case, it is interesting that this revertant substitution is far removed from the original mutation in helix XI, suggesting a possible functional interaction between the two halves of the carrier.

The second type of second-site revertant substituted the negatively charged Asp-59 (helix II) with the neutral residues Asn or Val. The gain of transport function in these revertants suggested that the loss of the negative charge at Asp-59 was compensating for the loss of the positive charge at Lys-377, indicating that these two residues interact via a charge neutralizing salt bridge that is important for carrier function. Consistent with the red phenotype on melibiose MacConkey indicator plates, each of these revertants had improved downhill transport as compared with the K377V parental strain. However, analysis of the transport characteristics for position 59 second-site revertants showed interesting differences between the two. The K377V/D59V revertant recovered significant downhill melibiose transport with protons as the cotransported cation and was able to drive melibiose-stimulated H^+ influx. However, Na^+ failed to stimulate melibiose transport above levels found with H^+, and no melibiose-stimulated Na^+ influx was observed. Although melibiose accumulation was better than the K377V parental strain with H^+, this activity was also not stimulated by Na^+. In addition, the β-galactoside, TMG, could not be accumulated.

In contrast to K377V/D59V, a weak stimulation of both downhill melibiose transport and melibiose accumulation (>2-fold) was found for the K377V/D59N revertant in the presence of Na^+. Consistent with this weak stimulation, a small amount of melibiose-stimulated Na^+ influx was found. However, only marginal melibiose-stimulated H^+ uptake was observed. In stark contrast to the K377V/D59V revertant, K377V/D59N was able to efficiently accumulate TMG, however Na^+ did not stimulate this transport activity. The observation that the K377V/D59N revertant can accumulate TMG whereas the K377V/D59V revertant cannot suggests that the architecture of the side-chain at position 59 influences sugar binding directly. The structurally conservative substitution of Asn for Asp allows the carrier to continue to recognize TMG while a Val substitution disrupts binding of this sugar. It has previously been shown that Asp-59 is required for efficient cation coupling and, in particular, Na^+-stimulated sugar binding (for review see [3]). Our results reiterate the importance of Asp-59 for Na^+ recognition and also suggest that the cation and sugar binding sites overlap at this position.

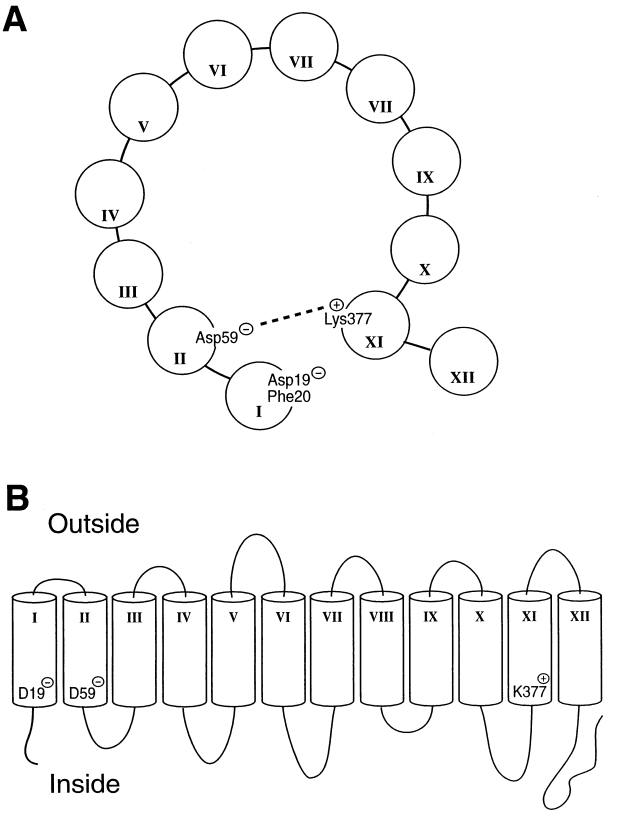

Fig. 3. Salt bridge between Lys-377 and Asp-59. Cartoon illustrating the proposed salt bridge between Lys-377 and Asp-59 that brings helix XI in close proximity to helix II.

The isolation of second-site revertants of K377V which neutralize the negative charge at Asp-59 strongly indicates that these residues form a salt bridge that would require helices II and XI to be close to one another in the three-dimensional structure of the carrier. A salt bridge between these residues seems plausible as the topological model for the carrier predicts that both residues are found at similar positions in their respective helices, near the cytoplasmic face of the membrane (Fig. 3B). To further investigate this possibility, we created three additional mutants: Asp-59 was substituted with Lys

(D59K), Lys-377 was substituted with Asp (K377D), and a double mutant was created that combined these substitutions (D59K/K377D) and reversed the position of the charges as compared to the wild-type carrier. If Asp-59 and Lys-377 form a salt bridge, reversing these charges should result in a carrier that retains melibiose transport activity.

The single mutants K377D and D59K lacked significant melibiose transport activity. In contrast, the D59K/K377D double mutant behaves as a carrier that has lost the Na^+-stimulated transport characteristics of the wild-type protein. In both melibiose downhill and accumulation assays this double mutant showed transport similar to wild-type when H^+ was the coupling cation. But this transport was clearly not stimulated by Na^+ or Li^+. The accumulation of melibiose with protons suggests that this mutant was able to couple sugar transport to the proton electrochemical gradient. Unfortunately, we were unable to demonstrate melibiose-stimulated H^+ transport in this strain. However, this may be due to the sensitivity of the assay itself rather than a lack of proton uptake. It has been shown that for H^+-coupled melibiose transport the K_m for melibiose increases 10-fold when the membrane potential is disrupted [42]. The decrease in affinity for melibiose under anaerobic conditions coupled with a low initial rate of sugar transport for the double mutant could lower the proton uptake response below measurable levels in our assay. The retention of melibiose transport in the D59K/K377D double mutant, but loss of Na^+ and Li^+ coupling to that transport, suggests that it is not just the charge pairing of Asp-59 and Lys-377 that is important for carrier function. Rather, it appears that a combination of the presence of the salt bridge, and the precise positioning of Asp-59 within the cation coordination site is required for efficient sugar transport and cation coupling.

In summary, our data suggest that Lys-377 and Asp-59 form a charge pair in the wild-type melibiose carrier which brings helices II and XI near one another in the three-dimensional structure of the protein (Fig. 3A). This conclusion is supported by both the isolation of functional revertants for the K377V mutant where Asp-59 is substituted with neutral residues, and by the transport activity found for the D59K/K377D double mutant. Although reversal of the charges at these two positions results in a transport competent carrier, the coupling of Na^+ and Li^+ to sugar transport is lost. Thus, the presence of a salt bridge between residues at position 59 and 377 (e.g. D59K/K377D) is not sufficient to produce wild-type carrier activity. Rather, an aspartate residue is required at position 59 to properly construct the cation coordination site. This result agrees with several previous studies that have shown the importance of Asp-59 for Na^+ recognition (for review see [3]). In other complex membrane proteins the proximity of amino-terminal and carboxyl-terminal helices has been suggested. For example, two independent models of helix-packing in the lactose carrier of *E. coli* place helices II and XI close to one another [30,43]. In addition, a salt bridge between residues Lys-139 (transmembrane domain II) and Asp-427 (transmembrane domain XI) of VMAT 2, a vesicular monoamine transporter, has been reported to promote high affinity substrate recognition [40]. Information from the present study will be used to target specific areas of the melibiose carrier for biochemical studies. For example, chemical cross-linking of double cysteine mutants in a recently constructed C-less carrier could be used to verify the proximity of specific helices.

Acknowledgements

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References

[1] H. Yazyu, S. Shiota-Niiya, T. Shimamoto, H. Kanazawa, M. Futai, T. Tsuchiya, J. Biol. Chem. 259 (1984) 4320–4326.

[2] G. Leblanc, T. Pourcher, M.-L. Zani, The melibiose permease of *Escherichia coli*: importance of the NH_2-terminal domains for cation recognition by the Na^+/sugar cotransporter, in: L. Reuss, J.M. Russell, M.L. Jennings (Eds.), Molecular Biology and Function of Carrier Proteins, The Rockefeller University Press, New York, 1993, pp. 213–227.

[3] B. Poolman, J. Knol, C. vanderDoes, P.J. Henderson, W.J. Liang, G. Leblanc, T. Pourcher, I. Mus-Veteau, Mol. Microbiol. 19 (1996) 911–922.

[4] T. Tsuchiya, J. Raven, T.H. Wilson, Biochem. Biophys. Res. Commun. 76 (1977) 26–31.
[5] D.M. Wilson, T.H. Wilson, Biochim. Biophys. Acta 904 (1987) 191–200.
[6] T. Tsuchiya, T.H. Wilson, Membr. Biochem. 2 (1978) 63–79.
[7] T. Tsuchiya, M. Oho, S. Shiota-Niiya, J. Biol. Chem. 258 (1983) 12765–12767.
[8] M.C. Botfield, K. Naguchi, T. Tsuchiya, T.H. Wilson, J. Biol. Chem. 267 (1992) 1818–1822.
[9] T. Pourcher, E. Bibi, H.R. Kaback, G. Leblanc, Biochemistry 35 (1996) 4161–4168.
[10] C. Gwizdek, G. Leblanc, M. Bassilana, Biochemistry 36 (1997) 8522–8529.
[11] M.L. Zani, T. Pourcher, G. Leblanc, J. Biol. Chem. 268 (1993) 3216–3221.
[12] T. Pourcher, M. Deckert, M. Bassilana, G. Leblanc, Biochem. Biophys. Res. Commun. 178 (1991) 1176–1181.
[13] T. Pourcher, M.L. Zani, G. Leblanc, J. Biol. Chem. 268 (1993) 3209–3215.
[14] M.L. Zani, T. Pourcher, G. Leblanc, J. Biol. Chem. 269 (1994) 24883–24889.
[15] B.H. Honig, W.L. Hubbell, Proc. Natl. Acad. Sci. USA 81 (1984) 5412–5416.
[16] T. Pourcher, H.K. Sarkar, M. Bassilana, H.R. Kaback, G. Leblanc, Proc. Natl. Acad. Sci. USA 87 (1990) 468–472.
[17] P.J. Franco, T.H. Wilson, J. Bacteriol. 181 (1999) 6377–6386.
[18] M.C. Botfield, T.H. Wilson, J. Biol. Chem. 263 (1988) 12909–12915.
[19] T.H. Wilson, D.M. Wilson, Biochim. Biophys. Acta 1374 (1998) 77–82.
[20] M.C. Botfield, T.H. Wilson, J. Biol. Chem. 264 (1989) 11643–11648.
[21] J.S. Lolkema, I.B. Puttner, H.R. Kaback, Biochemistry 27 (1988) 8307–8310.
[22] M.C. Botfield, T.H. Wilson, J. Biol. Chem. 264 (1989) 11649–11652.
[23] H. Hama, T.H. Wilson, J. Biol. Chem. 268 (1993) 10060–10065.
[24] S. Shiota, Y. Yamane, M. Futai, T. Tsuchiya, J. Bacteriol. 162 (1985) 106–109.
[25] P.J. Franco, T.H. Wilson, Biochim. Biophys. Acta 1282 (1996) 240–248.
[26] I.C. West, Biochem. Biophys. Res. Commun. 41 (1970) 655–661.
[27] D.M. Wilson, T. Tsuchiya, T.H. Wilson, Methods Enzymol. 125 (1986) 377–387.
[28] P.J. Franco, R.J. Brooker, J. Biol. Chem. 269 (1994) 7379–7386.
[29] S. Frillingos, A. Gonzalez, H.R. Kaback, Biochemistry 36 (1997) 14284–14290.
[30] S. Frillingos, M. Sahintoth, J.H. Wu, H.R. Kaback, FASEB J. 12 (1998) 1281–1299.
[31] M. Sahin-Toth, J. leCoutre, D. Kharabi, G. leMaire, J.C. Lee, H.R. Kaback, Biochemistry 38 (1999) 813–819.
[32] L.J. Stern, H.G. Khorana, J. Biol. Chem. 264 (1989) 14202–14208.
[33] W.A. Catterall, Science 242 (1988) 50–61.
[34] J.F. Cotten, M.J. Welsh, J. Biol. Chem. 274 (1999) 5429–5435.
[35] S. SenGupta, N.D. DeWitt, K.E. Allen, C.W. Slayman, J. Biol. Chem. 273 (1998) 34328–34334.
[36] S.C. King, C.L. Hansen, T.H. Wilson, Biochim. Biophys. Acta 1062 (1991) 177–186.
[37] J.I. Lee, P.P. Hwang, T.H. Wilson, J. Biol. Chem. 268 (1993) 20007–20015.
[38] J.I. Lee, M.F. Varela, T.H. Wilson, Biochim. Biophys. Acta 1278 (1996) 111–118.
[39] M. Sahin-Toth, R.L. Dunten, A. Gonzalez, H.R. Kaback, Proc. Natl. Acad. Sci. USA 89 (1992) 10547–10551.
[40] A. Merickel, H.R. Kaback, R.H. Edwards, J. Biol. Chem. 272 (1997) 5403–5408.
[41] F.A. Quiocho, Ann. Rev. Biochem. 55 (1986) 287–315.
[42] T. Pourcher, M. Bassilana, H.K. Sarkar, H.R. Kaback, G. Leblanc, Philos. Trans. R. Soc. Lond. B Biol. Sci. 326 (1990) 411–423.
[43] V.C. Goswitz, R.J. Brooker, Protein Sci. 4 (1995) 534–537.