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Biochimica et Biophysica Acta 1282 (1996) 240–248

BBA Biochimica et Biophysica Acta

Alteration of Na^+-coupled transport in site-directed mutants of the melibiose carrier of *Escherichia coli*

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Received 19 October 1995; revised 16 February 1996; accepted 22 March 1996

Abstract

Asn-58 of the *Escherichia coli* melibiose carrier was replaced by Ala, Leu, Ser, and Gln. Trp-54 was replaced by Leu and a double mutant Leu-54/Ala-58 was constructed using site-directed mutagenesis. Cation/sugar cotransport and sugar-induced cation uptake were studied for each mutant. The change of Asn-58 to Ala results in a nearly complete loss of Na^+-stimulated galactoside transport as well as sugar-stimulated Na^+ uptake. Substitutions of Leu, Gln, and Ser for Asn-58 were also defective in Na^+-stimulated sugar transport. The Trp-54 to Leu mutant shows moderate sugar accumulation with cation selectivity similar to wild-type. The double mutant Leu-54/Ala-58 shows elevated H^+-melibiose cotransport as well as reduced Na^+-stimulated melibiose cotransport. These results suggest that Asn-58 is important for Na^+ recognition.

Keywords: Melibiose carrier; Cation cotransport; Mutagenesis; *(E. coli)*

1. Introduction

The melibiose carrier of *Escherichia coli* is a cation/substrate cotransporter which catalyzes the simultaneous transport of Na^+ and melibiose across the bacterial inner membrane into the cell (for a recent review see Leblanc et al. [1]). In addition to the natural substrate the carrier is able to support the transport of a variety of sugar substrates including α-galactosides, β-galactosides, and monosaccharides [2,3]. Most bacterial secondary active transporters use H^+ as a coupling cation but some Na^+ coupled transporters have also been identified (for review see [4]). Among *E. coli* transporters the melibiose carrier has the unique capacity to exploit favorable electrochemical gradients for H^+, Na^+, or Li^+ in order to drive sugar accumulation [2,3,5,6]. Sugar binding studies in membrane vesicles show that the presence of Na^+ or Li^+ ions increase the carrier's affinity for galactosides and that these cations compete for the same binding site [7,8]. A 1:1 stoichiometry for Na^+/sugar cotransport has been determined for the carrier [8,9].

The gene encoding the *E. coli* melibiose carrier (*melB*) has been cloned [10] and sequenced [11]. The primary amino acid sequence deduced from the *melB* gene sequence predicts a highly hydrophobic protein (70% apolar) with a molecular mass of 52 kDa [11]. Hydropathy analysis has been combined with information derived from *melB-phoA* fusions to predict a topological model in which the carrier forms 12 α-helical membrane-spanning domains connected by hydrophilic loops [11,12] with the carboxyl-terminus located in the cytoplasm [13]. More recently, a refined topological model has been presented based upon information from additional *melB-phoA* fusions [14]. The melibiose carrier was predicted by Yazyu et al. [11] to consist of 469 amino acids based upon the *melB* gene sequence. However, Pourcher et al. [15] have recently purified the melibiose transport protein and subjected the amino-terminus to amino acid sequencing. They found three additional amino acids (S-I-S) preceding the N-terminal M-T-T predicted by Yazyu et al. [11]. Pourcher et al. [15] predict the true initial ATG is actually 12 bases upstream of the initial ATG postulated by Yazyu et al. [11]. Pourcher et al. [15] also suggest that the initial Met is removed by Met-specific peptidases. Consequently the mature *melB* carrier of *E. coli* is 472 amino acids in length and the N-terminal sequence is S-I-S-M-T-T.

Recent studies on the *E. coli* melibiose carrier have

Abbreviations: TMG, methyl β-D-thiogalactopyranoside; NPG, *p*-nitrophenyl α-D-galactopyranoside; Mops, 4-morpholinepropanesulfonic acid; LB, Luria-Bertani broth; KSCN, potassium thiocyanate.

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PII S0005-2736(96)00062-4

utilized site-directed mutagenesis in an attempt to identify amino acid residues involved in cation binding and transport. These studies have implicated four aspartic acid residues all of which are predicted to reside on membrane-spanning domains in the NH_2-terminal portion of the protein. When Asp-35 (helix I), Asp-55 (helix II), Asp-59 (helix II), or Asp-124 (helix IV) were individually replaced with neutral amino acids (Cys or Asn), the presence of Na^+ failed to stimulate NPG binding in all mutants except Asn-35 [16–18]. However, in the absence of Na^+ the affinity of the carrier for NPG remained comparable to wild-type for all substitutions. The replacement of Asp-55, Asp-59, or Asp-124 with Glu has the effect of dramatically reducing the carrier's affinity for Na^+ while still supporting Na^+-coupled sugar transport [1,19–21]. Taken together these data have led to the suggestion that the carboxylate groups of Asp-55, Asp-59, and Asp-124 comprise part of a coordination site for cations [1,17,21].

The recent cloning of the *Klebsiella pneumoniae* melibiose carrier [22] allowed a comparative physiological approach to identifying elements of the cation recognition site. Despite a 79% amino acid identity to the *E. coli* melibiose carrier the *K. pneumoniae* carrier does not use Na^+ as a coupling ion but will use H^+ or Li^+ depending on the substrate [22]. Hama and Wilson [23] constructed chimeric carriers in which amino-terminal portions of *K. pneumoniae* carrier were replaced with the equivalent *E. coli* sequence. It was found that when the first two amino-terminal transmembrane domains of the *K. pneumoniae* carrier were replaced with the equivalent *E. coli* sequence, the resulting chimera was able to couple sugar transport to Na^+. In this amino-terminal region only five residues are not conserved between the two carriers. In a related study, each of these five non-conserved amino acids of the *K. pneumoniae* carrier were individually changed to the corresponding *E. coli* residue [24]. The striking observation was that a single mutation, Ala-58 to Asn, allowed the *K. pneumoniae* carrier to use Na^+ as a coupling ion for sugar transport [24]. This result implied that Asn-58 is crucial for Na^+ recognition.

In the present study we sought to examine the role of Asn-58 (adjacent to Asp-59, helix II) of the *E. coli* melibiose carrier (see Fig. 1 for model). To this end, site-directed mutagenesis was employed to replace Asn-58 with Ala (the corresponding residue in *K. pneumoniae*), Leu, Ser, and Gln. In addition, Trp-54 (adjacent to Asp-55, helix II) was changed to Leu (the corresponding residue in *K. pneumoniae*) and a Leu-54/Ala-58 double mutant was constructed. Consequences of these mutations were assessed by analyzing changes in galactoside accumulation and sugar-induced Na^+ and H^+ uptake.

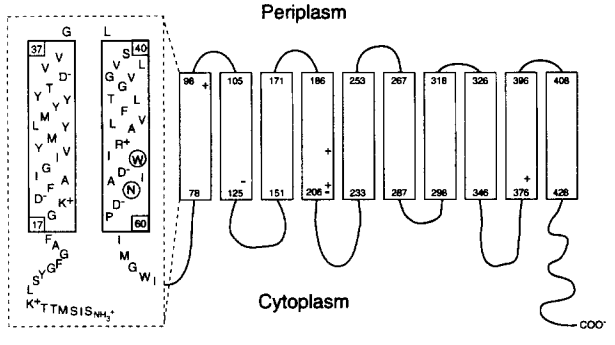

Fig. 1. Topological model of the melibiose carrier in the membrane. Rectangles represent transmembrane helices. The '+' and '−' indicate the location of charged residues within membrane helices. Numbers at the top and bottom of each rectangle indicate the first and last residue of each helix. Circled residues in helix II were subjected to site-directed mutagenesis in this study. The model is based on that of Zani et al. [14].

2. Materials and methods

2.1. Reagents

Melibiose (*O*-α-D-galactopyranosyl-(1,6)-D-glucopyranose, TMG (1-*O*-methyl-β-D-galactopyranoside) and lactose (*O*-β-D-galactopyranosyl-(1,4)-α-D-glucopyranose) were purchased from Sigma. [^{3}H]Melibiose was a generous gift from Dr. Gérard Leblanc of Département de Biologie du Commissariat à l'Energie Atomique, Villefranche-su-mer, France. [^{14}C]TMG and [^{14}C]lactose were purchased from Dupont-NEN. [α-^{35}S]ATP and ^{125}I-Protein A were purchased from Amersham. The Sequenase kit was from United States Biochemical. Restriction enzymes were from New England Biolabs. The site-directed mutagenesis kit was from Bio-Rad. All other chemicals were reagent grade.

2.2. Bacterial strains and plasmids

E. coli DW1 (*lacI*$^+$*Δ[ZY] melΔ[AB]*) [3] was used as the host strain for in vitro galactoside uphill transport assays, sugar-stimulated H^+ and Na^+ transport, and immunoblot experiments to determine the amount of carrier protein present. *E. coli* DW2 (*lacI*$^+$*Δ[ZY] melA*$^+$*ΔB*) [25] was used as a host strain for fermentation studies on melibiose MacConkey indicator plates. *E. coli* DW2R (*lacI*$^+$*Δ[ZY] melA*$^+$*ΔB* Tn10::*recA*) [26] was used as a host strain for downhill melibiose transport experiments. *E. coli* CJ236 [*dut, ung, thi, relA*; pCJ105 (*Cm*^{r})] and *E. coli* MV1190 [*D(lac-proAB), thi, supE, D(srl-recA)306*::Tn10 (*tet*^{r}) (F′: *traD36, proAB, lacI*^{q}*ZDM15*)] were from Bio-Rad.

The pTZ19U phagemid (Bio-Rad) was used for the construction of site-directed mutants. pKK223-3 (Pharmacia LKB Biotechnology) was used as vector plasmid for expressing *E. coli melB* site-directed mutants. pSUBS25 [22] was used for expressing the *Klebsiella pneumoniae melB* gene. A modified form of pKKMB [13] was used for the expression of the *E. coli melB* gene. In this modified construct, pKKMB-Pst, a silent mutation has been intro-

duced at nucleotide 627 (T to A) which creates a *Pst*I restriction site without altering the amino acid sequence of the protein. In addition, the *Eco*RI and *Hin*dIII sites which flank the *melB* gene have been engineered such that the *Eco*RI site immediately precedes the start codon and the *Hin*dIII site immediately follows the stop codon for the gene as defined by Yazyu et al. [11]. This construct expresses a melibiose carrier with a deletion of the first three amino acids of the protein. However, the pKKMB-Pst construct showed steady state galactoside accumulation values comparable to the pKKMB construct which expresses a full length protein. The *melB* of pKKMB-Pst was the parental gene for all site-directed mutants.

2.3. Assays

DW1 was used as the host strain for sugar accumulation assays. DW1 bearing *E. coli* melibiose carriers were grown in LB media supplemented with 50 μg/ml ampicillin and DW1 bearing the *K. pneumoniae* melibiose carrier was grown in LB media supplemented with 15 μg/ml chloramphenicol. All strains were grown at 30°C. Cells were harvested at midlog phase and washed twice in 0.1 M Mops/0.5 mM $MgSO_4$/Tris (pH 7.0). Cells were resuspended in the same buffer to a density of approximately $3 \cdot 10^9$ cells/ml. Uphill transport experiments were carried out in the same buffer with [^{3}H]melibiose (0.2 mM, 0.4 μCi/ml), [^{14}C]TMG (0.1 mM, 0.2 μCi/ml) or [^{14}C]lactose (0.1 mM, 0.1 μCi/ml) in the absence or presence of NaCl (10 mM) or LiCl (10 mM) as indicated. At appropriate time points 200 μl aliquots were withdrawn and rapidly filtered using 0.65-μm pore size cellulose nitrate filters (Sartorius). Filters were then washed with 4–5 ml of the same buffer and dissolved in 4 ml of Liquiscint (National Diagnostics) and counted. For the experiment of Fig. 2 DW2R (*melA*$^+$*ΔB*) was used as the host strain to express wild-type or mutant *E. coli* melibiose carriers. Cells were grown at 30°C in LB media supplemented 50 μg/ml ampicillin, 25 μg/ml tetracycline and 10 mM methyl α-galactoside to induce expression of α-galactosidase. Cells were prepared as described above and the downhill transport of [^{3}H]melibiose (0.5 mM, 0.5 μCi/ml) was carried out in the presence of 0 mM, 1 mM, 5 mM, 10 mM, 20 mM or 50 mM NaCl. In the case of the wild-type carrier a measurement of transport was also carrier out with 0.5 mM NaCl. A time point of 1 minute was used to reflect initial rates of sugar uptake.

2.4. Sugar-induced H^+ transport

The measurement of sugar-induced proton uptake was done according to the method of West [27] as modified by Wilson et al. [28]. Cells were grown to midlog phase at 30°C and washed twice with 120 mM KCl. Cells were then resuspended in 120 mM KCl to a density of approximately

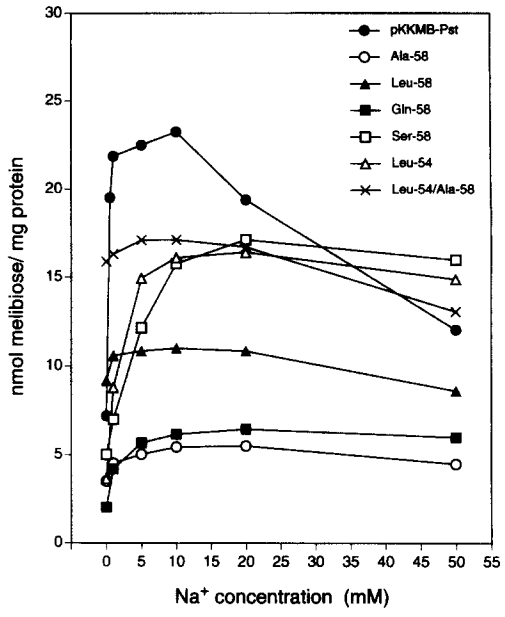

Fig. 2. Effect of Na^+ concentration on downhill melibiose transport. DW2R (*melA*$^+$*ΔB*) cells expressing pKKMB-Pst (wild-type) or mutant carriers were washed and resuspended in 100 mM Mops buffer (pH 7.0). Cells were incubated with [^{3}H]melibiose (0.5 mM, 0.5 μCi/ml) for 1 min in the presence of 0, 1, 5, 10, 20, or 50 mM NaCl. In the case of pKKMB-Pst a measurement with 0.5 mM NaCl was also included.

3.5 mg protein/ml and KSCN was added to 30 mM. Cells (2.5 ml) were placed in a closed plastic vial with a lid containing apertures for a pH electrode, the introduction of argon, and the insertion of a needle attached to a Hamilton syringe. Cells were made anaerobic under a constant stream of water-saturated argon for at least 30 min. An anaerobic melibiose solution (25 μl of 0.5 M) was added to a final concentration of 5 mM to initiate proton uptake. Changes in the pH of the extracellular media were monitored with a Radiometer pH meter (PHM64) and electrode (GK2321-C). Changes in pH were recorded using a Linear Instruments recorder modified 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 HCl.

2.5. Sugar-induced Na^+ transport

Cells were washed twice in 0.1 M Mops/tetramethylammonium hydroxide (pH 7.0) containing 0.5 mM $MgSO_4$. Washed cells were resuspended in the same buffer to a density of approx. 25 mg protein/ml. For the assay an aliquot of cells (0.3 ml) was diluted 10-fold with 0.1 M Tricine/tetramethylammonium hydroxide (pH 8.0) containing 50 μM NaCl in a closed plastic vial. Holes were made in the lid of the vial to accommodate a G502Na Sodium Selectrode (Radiometer), a K401 Calomel Electrode (Radiometer), the insertion of a needle attached to a Hamilton syringe, and the introduction of argon. Cells were made anaerobic under a constant stream of H_2O-saturated argon. After at least 30 min under argon an

Table 1
Oligonucleotide primers used for site-directed mutagenesis

Oligonucleotide	Sequence [a]	Amino acid substitution
pN58A	5′-GGGATGCTATT(G * C * C)GATCCG-3′	Asn-58 to Ala
pN58L	5′-GGGATGCTATT(G * C * C)GATCCG-3′	Asn-58 to Leu
pN58S	5′-GGGATGCTATT(AG * T *)GATCCG-3′	Asn-58 to Ser
pN58Q	5′-GGGATGCTATT(C * A * G)GATCCG-3′	Asn-58 to Gln
pW54L	5′-GGTGGCGAGGATC(C * T * G)GATGC-3′	Trp-54 to Leu

[a] Mismatches within the *melB* gene are followed by an asterisk and the resulting codons are given in parentheses.

anaerobic solution of melibiose or TMG was added to a final concentration of 10 mM or 5 mM, respectively. Changes in the sodium levels of the extracellular media were monitored with a Linear Instruments recorder. To test electrode sensitivity calibrations were done with a known amount of anaerobic NaCl.

2.6. Site-directed mutagenesis

Site-directed mutagenesis was performed using the Bio-Rad Muta-Gene phagemid kit which employs a modified version of the method of Kunkel et al. [29]. Independent mutations were introduced using primers complementary to the antisense strand of the *melB* gene except for mismatches which altered the desired codon. Mutagenic primers are listed in Table 1. To construct the Leu-54/Ala-58 double mutant template DNA was prepared from the Ala-58 mutant and the pW54L primer (Table 1) was used to create the second mutation.

2.7. Immunoblots

Immunoblots were performed as described by Lolkema et al. [30]. The amount of melibiose carrier present in each strain was determined by using a polyclonal antibody (anti-MBct10, 1:1000 dilution) directed against the carboxyl terminal ten amino acids of the protein [13]. ^{125}I-protein A was used to label the bound antibody and the product was washed thoroughly. The amount of label was quantified with a gamma counter. To correct for non-specific absorption the number of counts obtained for the strain DW1/pKK223-3, which does not express a melibiose carrier, was subtracted from each experimental sample. Values for each mutant were then normalized to wild-type and reported as a percentage of wild-type expression.

2.8. DNA sequencing

Double-stranded plasmid DNA was isolated using a Qiagen miniprep kit. DNA was subjected to alkaline denaturation as described by Kraft et al. [31] and sequenced by the dideoxy method of Sanger et al. [32].

Table 2
Quantitation of melibiose carrier levels in wild-type and mutant strains

Strain	Melibiose carrier protein (% wild-type)
pKKMB-Pst	100
Ala-58	80
Leu-58	116
Ser-58	109
Gln-58	36
Leu-54	197
Leu-54/Ala-58	122

The level of melibiose carrier was determined by using an antibody directed against the carboxyl-terminal ten amino acids of the protein as described under Section 2.7.

3. Results

3.1. Site-directed mutagenesis

In the present study site-directed mutagenesis was employed to make substitutions at Asn-58 and/or Trp-54 of the *E. coli* melibiose carrier. Substitutions for Asn-58 included Ala, Leu, Ser, and Gln. Trp-54 was replaced by Leu and a double mutant, Leu-54/Ala-58, was constructed. In order to determine the relative amount of melibiose carrier produced for each mutant, the amount of protein was measured using an antibody directed against the carboxyl-terminal ten amino acids of the melibiose carrier. These results are given in Table 2. In general, the protein levels are comparable to wild-type with the exception of Gln-58 which showed 36% of normal.

3.2. Phenotype on MacConkey plates

As a qualitative assessment of melibiose uptake cells were plated on MacConkey indicator plates with melibiose (30 mM) as the sole fermentable carbon source. DW2 (*lacI*$^+$ *Δ[ZY] melA*$^+$ *ΔB*) was used for the expression of melibiose carrier mutants. The DW2 strain expresses an

active α-galactosidase but is deleted for the lactose carrier and melibiose carrier, both of which support the uptake of melibiose. When transformed with a plasmid encoding a functional melibiose carrier, these cells are able to transport and subsequently metabolize melibiose. Cells metabolizing melibiose form red colonies. Cells encoding a melibiose carrier unable to transport sugar form white colonies. All of the site-directed mutants formed red colonies indistinguishable from wild-type with the exception of the Gln-58 mutant, which formed dark pink colonies with a white halo suggesting a moderate defect in melibiose transport in this mutant.

3.3. Melibiose transport

To measure the accumulation of melibiose, wild-type and mutant plasmids were introduced into the *E. coli* strain DW1, which is deleted for the lactose and melibiose carriers as well as for α-galactosidase and β-galactosidase. These cells are unable to metabolize transported galactosides, therefore sugar transport is considered 'uphill'. The *E. coli* melibiose carrier is able to couple melibiose transport to H^+, Na^+, and Li^+. In the absence of added cations (proton cotransport) DW1/pKKMB-Pst expressing the *E. coli* wild-type protein was able to accumulate melibiose to a concentration 6-times higher than that of the external medium (i.e. 6-fold *accumulation*). In the presence of 10 mM NaCl accumulation increased to 109-fold and with 10 mM LiCl to 74-fold (i.e. 17.4-fold and 11.7-fold *stimulation*, respectively, Table 3). Hama and Wilson [22] have shown that for the *K. pneumoniae* carrier melibiose transport is coupled only to protons. In the absence of added cations DW1/pSUBS25 expressing the *K. pneumoniae* carrier showed 51-fold accumulation of melibiose (Table 3). The addition of 10 mM NaCl or LiCl failed to stimulate melibiose transport levels above this value.

In the Ala-58 mutant melibiose accumulation with protons was comparable to pKKMB-Pst but the addition of 10 mM NaCl gave only 2-fold stimulation (Table 3). Thus, it appears that changing Asn-58 to the equivalent residue in *Klebsiella* has a detrimental effect on Na^+-stimulated melibiose transport. Three additional substitutions of Leu, Ser and Gln were made for Asn-58. All showed significantly reduced Na^+-stimulated melibiose transport as compared to pKKMB-Pst. However, it was noted that the polar substitutions, Gln and Ser, remain more active than Ala and Leu. For example, in the presence of 10 mM NaCl Ser-58 and Gln-58 showed 6.1-fold and 3.6-fold stimulation respectively, while Leu-58 showed only 1.8-fold stimulation (Table 3).

In addition to Asn-58, Trp-54 of the *E. coli* carrier was implicated in cation recognition through site-directed mutagenesis studies on the *K. pneumoniae* melibiose carrier [24]. More specifically, Trp-54 is thought to be important for Li^+-stimulated melibiose transport. We replaced Trp-54 with Leu (the residue of *K. pneumoniae* at this position) and found that while melibiose transport is reduced slightly overall, near wild-type values are found for Na^+ and Li^+ stimulated transport (Table 3). In addition, a double mutant was constructed in which both Asn-58 and Trp-54 were changed to the corresponding *K. pneumoniae* residues, Ala and Leu, respectively. This double mutant showed much better melibiose accumulation with protons as compared to pKKMB-Pst (Table 3) or the two corresponding single mutants Ala-58 and Leu-54. However, the presence of 10 mM NaCl gave only 2.1-fold stimulation and 10 mM LiCl gave 2.7-fold stimulation (values similar to the Ala-58 mutant).

In an effort to ascertain the stimulatory effect of Na^+ on sugar uptake melibiose transport was measured at a variety of Na^+ concentrations. In the experiment of Fig. 2 DW2R (α-galactosidase positive) was used as a host strain for the expression of wild-type and mutant melibiose carriers. In this strain melibiose is rapidly cleaved upon

Table 3
Accumulation of melibiose in the presence of cations

Strain	Melibiose accumulation ([in]/[out])			Stimulation (uptake with cation/uptake with H^+)	
	H^+	Na^+	Li^+	Na^+	Li^+
pKKMB-Pst	6.3	109	74	17.3	11.7
pSUBS25	51	51	45	1.0	0.9
Ala-58	4.1	8.2	12	2.0	2.9
Leu-58	10	18	24	1.8	2.4
Ser-58	6.6	40	25	6.1	3.8
Gln-58	2.7	9.7	12	3.6	4.4
Leu-54	2.8	43	27	15.3	9.6
Leu-54/Ala-58	18	38	49	2.1	2.7

DW1 cells expressing wild-type or mutant *E. coli* melibiose carriers were washed and resuspended in 100 mM Mops buffer (pH 7). Cells were incubated with 0.2 mM [^{3}H]melibiose for 10 min in the absence or presence of 10 mM NaCl or 10 mM LiCl. pKKMB-Pst, wild-type *E. coli* melibiose carrier; pSUBS25, wild-type *K. pneumoniae* melibiose carrier. Mutants of the *E. coli* melibiose carrier are indented by the three later code for the mutant amino acid followed by its numerical position within the carrier's amino acid sequence.

entry into the cell such that the extracellular concentration remains higher than the intracellular concentration. Thus, transport is considered to be 'downhill'. In the case of pKKMB-Pst 0.5 mM Na^+ gave a significant stimulation of transport and a near maximal level of stimulation is achieved at a concentration of 1 mM. This result is consistent with previous measurements which have estimated the K_m for Na^+ at 0.3 mM [14,20]. At higher Na^+ concentrations (e.g. 20 mM) the stimulation of transport is less than maximal in pKKMB-Pst. Although this phenomenon has been noted previously [33,34] its explanation remains unclear at present. For the Gln-58, Ser-58, and Leu-54 mutants Fig. 2 indicates a moderate decrease in the affinity for sodium. In contrast, the Ala-58, Leu-58 and Leu-54/Ala-58 double mutant fail to show significant stimulation of melibiose uptake even at high Na^+ concentration. These results are consistent with the data for melibiose accumulation (Table 3). The substitution of Asn-58 with a non-polar residue results in a near complete disruption of sodium recognition while polar substitutions have the effect of decreasing the affinity for sodium.

3.4. TMG transport

The effect of cations on the accumulation of the β-galactoside TMG was also assayed. The pKKMB-Pst strain shows a 14-fold accumulation of TMG with protons (Table 4). The addition of 10 mM NaCl (or LiCl) yields a 10-fold stimulation of this TMG transport (approximately 140-fold accumulation). In the present study pSUBS25 supported a 10-fold accumulation of TMG with protons. The addition of 10 mM NaCl had no stimulatory effect but the addition of 10 mM LiCl elicited a 6-fold stimulation of TMG uptake (60-fold accumulation).

The mutants in this study are nearly devoid of TMG transport activity in the absence of added cations (Table 4). The best transport was seen in the Leu-54/Ala-58 double mutant with 3-fold accumulation. Interestingly, the mutants are able to catalyze H^+-melibiose cotransport. The lack of proton stimulated TMG accumulation could be explained by a reduction in affinity for TMG in the mutants. Pourcher et al. have suggested that the domain near Asp-59 is more important for the binding of sugars with a β linkage (i.e. TMG and lactose) than for those with an α-linkage [17]. When TMG uptake was assayed in the presence of 10 mM NaCl all mutants with a substitution at position 58 show severely reduced TMG accumulation levels as compared to pKKMB-Pst. The addition of Na^+ failed to stimulate TMG accumulation significantly in the Ala-58 or Leu-58 mutants. However, some Na^+-stimulated TMG transport was observed in the Gln-58 and Ser-58 mutants. The Leu-54 mutant also had considerable TMG accumulation in the presence of NaCl. While Li^+-stimulated TMG transport is defective in all position 58 mutants, this cation stimulated the highest levels of accumulation. When comparing the Ala-58 single mutant and the Leu-54/Ala-58 double mutant it is seen that the double mutant has regained a significant portion of the Li^+-stimulated TMG transport activity (Table 4). The combination of these two mutations appears to confer a cation selection profile to the *E. coli* melibiose carrier that more closely resembles that of the wild-type *K. pneumoniae* protein.

3.5. Lactose transport

The β-galactoside lactose is a relatively poor substrate for the melibiose carrier of *E. coli* and is only actively accumulated in the presence of Na^+ or Li^+. In the case of the *K. pneumoniae* protein Na^+ has no effect and in the presence of Li^+ lactose is very weakly accumulated (Table 4). The Leu-54 mutant shows a moderate reduction in activity but has an otherwise normal cation profile as compared with the wild-type *E. coli* protein. The position

Table 4
Accumulation of TMG and lactose in the presence of cations

Strain	TMG accumulation ([in]/[out])			Lactose accumulation ([in]/[out])		
	H^+	Na^+	Li^+	H^+	Na^+	Li^+
pKKMB-Pst	14	145	179	1.3	16	35
pSUBS25	11	13	64	0.6	0.6	2.8
Ala-58	1.0	1.8	8.7	1.2	0.6	0.8
Leu-58	1.8	4.2	24	0.7	0.7	2.7
Ser-58	1.0	23	30	0.5	1.4	3.3
Gln-58	1.1	6.7	19	0.4	0.8	2.0
Leu-54	1.8	89	91	0.5	8.6	19
Leu-54/Ala-58	2.9	11	61	0.5	0.8	5.6

DW1 cells expressing wild-type or mutant *E. coli* melibiose carriers were washed and resuspended in 100 mM Mops buffer (pH 7). Cells were incubated with 0.1 mM [^{14}C]TMG or 0.1 mM [^{14}C]lactose for 10 min in the absence or presence of 10 mM NaCl or 10 mM LiCl. pKKMB-Pst, wild-type *E. coli* melibiose carrier; pSUBS25, wild-type *K. pneumoniae* melibiose carrier. Mutants of the *E. coli* melibiose carrier are indicated by the three letter code for the mutant amino acid followed by its numerical position within the carrier's amino acid sequence.

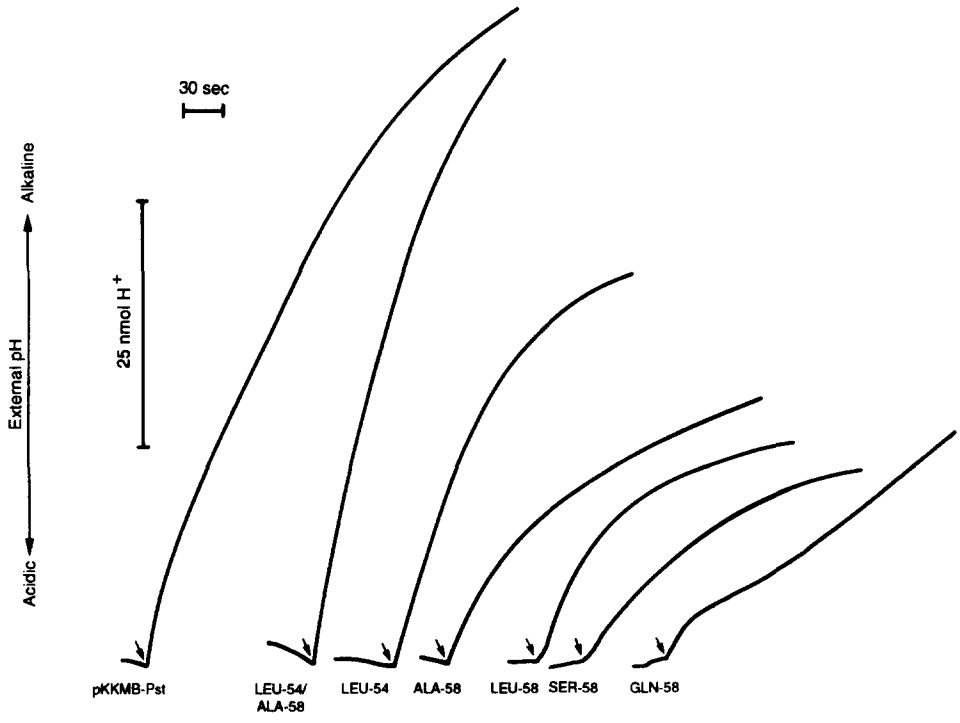

Fig. 3. Melibiose-induced proton uptake. DW1 expressing pKKMB-Pst or mutant carriers were washed and resuspended in 120 mM KCl (unbuffered) and 30 mM KSCN. Cells were made anaerobic under argon for 30 min. Arrows indicate the addition of 25 μl of an anaerobic melibiose solution (0.5 M) giving a final concentration of 5 mM.

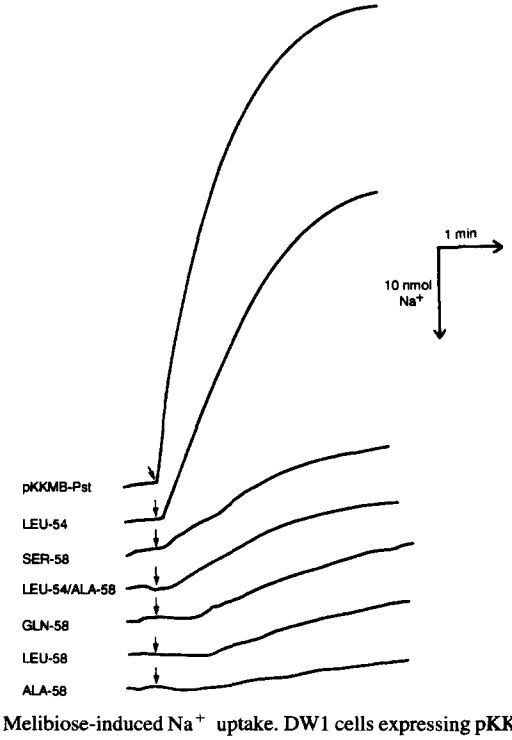

Fig. 4. Melibiose-induced Na^+ uptake. DW1 cells expressing pKKMB-Pst and mutants carriers were resuspended in 100 mM Tricine buffer (pH 8) with 30 mM KSCN and 50 μM NaCl in the presence of a sodium-selective electrode. Cells were made anaerobic under argon for 30 min. Arrows indicate the addition of 30 μl an anaerobic melibiose solution (1 M) giving a final concentration of 10 mM.

58 mutants showed little or no ability to accumulate lactose in the presence of 10 mM Na^+. With regard to Li^+-stimulated lactose transport the Leu-58, Gln-58, and Ser-58 mutants exhibited a marginal level of accumulation, while Ala-58 was devoid of activity. Again, the Leu-54/Ala-58 double mutant shows a cationic profile similar to the *K. pneumoniae* carrier.

3.6. Melibiose-stimulated H^+ transport

The ability of the melibiose carrier to couple proton influx to melibiose influx can be measured directly using a pH electrode. In such an experiment cells are made anaerobic under argon to halt proton extrusion via the respiratory chain. If an anaerobic sugar solution is added under these conditions the coupled flow of protons and sugar into the cell via the melibiose carrier will cause the extracellular medium to become more alkaline. In the experiment of Fig. 3 melibiose (5 mM) was used to stimulate proton uptake in the wild-type and mutant strains. Although the rate of H^+ uptake is variable, all of the mutants were found to catalyze significant H^+ influx upon the introduction of melibiose. Interestingly, the rate of H^+ influx for Leu-54/Ala-58 is faster than the rate for the Leu-54 or Ala-58 single mutants and was actually faster than pKKMB-Pst. This result agrees with the in vitro transport data which showed an enhanced level of H^+/melibiose cotransport for the Leu-54/Ala-58 double mutant.

3.7. Sugar-stimulated Na^+ transport

The coupled transport of Na^+ to sugar was also assayed. In this experiment cells are made anaerobic and extracellular Na^+ levels are monitored with a sodium sensitive electrode. In Fig. 4 melibiose was added to a final concentration of 10 mM. The wild-type and Leu-54 strains exhibit a rapid and extensive Na^+ influx as indicated by the upward deflection in the chart recording. In contrast, strains carrying a position 58 substitution show severely reduced Na^+ influx with melibiose. Of the position 58 substitutions Ser-58 shows the best Na^+ uptake and the Ala-58 is worst. In a similar experiment when TMG was added to a final concentration of 5 mM only the wild-type and Leu-54 strains were seen to catalyze Na^+ influx (data not shown). Thus, substitution for Asn-58 appears to disrupt Na^+-coupled sugar transport.

4. Discussion

The melibiose carrier of *E. coli* can use H^+, Na^+, or Li^+ for cotransport while a similar carrier in *Klebsiella pneumoniae* uses only H^+ or Li^+. Since the amino acid sequences of the two proteins are extremely similar (79% identical) it was believed that the residues responsible for Na^+ recognition might be identified. It was subsequently shown that the substitution of a single non-conserved amino acid, Ala-58 to Asn, in the *Klebsiella* carrier permitted that carrier to utilize Na^+ for cotransport [24]. This result suggested a prominent role for Asn-58 of the *E. coli* protein in Na^+ recognition. The Asn-58 residue resides in a region of the protein already identified to be important for cation recognition as the nearby residues Asp-55 and Asp-59 have both been shown to be critical for Na^+-stimulated sugar transport [16–18,20].

The most striking observation was that Ala-58 and Leu-58 showed severe defects in Na^+-stimulated melibiose transport although proton coupled transport was similar to normal. Melibiose-stimulated Na^+ uptake was also extremely low for these two mutants (Fig. 4). In the case of TMG and lactose there was little or no Na^+ stimulation of accumulation for either sugar. There was also no TMG stimulated Na^+ uptake. Thus, Na^+ coupling was almost completely lost in these two mutants. In contrast, the polar substitutions of Gln and Ser for Asn-58 had the effect of dramatically reducing steady-state accumulation levels while retaining a limited portion of the Na^+-stimulated transport activity. Melibiose-stimulated Na^+ uptake was slightly better for the Gln-58 and Ser-58 mutants but was also very defective. No TMG-stimulated Na^+ uptake was observed for either of these mutants. The study of the effect of Na^+ concentration on melibiose transport (Fig. 2) indicated that the affinity of all position 58 mutants for Na^+ was poor.

When Trp-54 was replaced with Leu (the residue found at the equivalent position in the *Klebsiella* carrier) accumulation levels for melibiose, TMG and lactose were moderately reduced as compared with the wild-type. However, 10 mM Na^+ or Li^+ was able to stimulate transport of these sugars. This mutant also showed melibiose- and TMG-stimulated Na^+ transport that was only moderately reduced. Other than a slight reduction in activity the transport characteristics of the melibiose carrier are not altered in the Leu-54 mutant.

In the Leu-54/Ala-58 double mutant Na^+ stimulation of transport was found to be similar to that in the Ala-58 single mutant. However, an interesting characteristic not seen with the Leu-54 or Ala-58 single mutants was found: a marked increase in proton coupled melibiose transport as compared with the normal *E. coli* carrier. This property of very active proton coupling is characteristic of the *Klebsiella* melibiose carrier which utilizes only H^+ for melibiose cotransport. Although this double mutant weakly couples Na^+ to sugar transport it has a poor affinity for Na^+ (Fig. 2).

It is concluded that Asn-58 is required for the efficient coupling of Na^+ to sugar transport by the melibiose carrier of *E. coli*. Asn-58 may participate directly in the coordination of Na^+ or may influence the position of nearby aspartates (Asp-55 and Asp-59) that have been shown to be critical for Na^+ recognition. Although the Trp-54 to Leu mutant did not have a dramatic effect on the transport properties of the carrier the combination of the Ala-58 with this mutant conferred a *Klebsiella*-like cation profile to sugar transport.

Acknowledgements

This work was supported by NIH grant DK05736.

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