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Role of Gly117 in the cation/melibiose symport of MelB of *Salmonella typhimurium*

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Abstract

The melibiose permease of *Salmonella typhimurium* ($MelB_{St}$) catalyzes symport of melibiose with Na^+, Li^+ or H^+, and bioinformatics analysis indicates that a conserved Gly117 (helix IV) is part of the Na^+-binding site. We mutated Gly117 to Ala, Pro, Trp or Arg; the effects on melibiose transport and binding of cosubstrates depended on the physical-chemical properties of the side chain. Compared with WT $MelB_{St}$, the Gly117→Ala mutant exhibited little difference in either cosubstrate binding or stimulation of melibiose transport by Na^+ or Li^+, but all other mutations reduced melibiose active transport and efflux, and decreased the apparent affinity for Na^+. The bulky Trp at position 117 caused the greatest inhibition of melibiose binding, and Gly117→Arg yielded less than a 4-fold decrease in the apparent affinity for melibiose at saturating Na^+ or Li^+ concentration. Remarkably, the mutant Gly117→Arg catalyzed melibiose exchange in the presence of Na^+ or Li^+, but did not catalyze melibiose translocation involving net flux of the coupling cation, indicating that sugar is released prior to release of the coupling cation. Taken together, the findings are consistent with the notion that Gly117 plays an important role in cation binding and translocation.

Keywords

Na^+-coupled transporter; alternating access; ligand binding; ordered-binding model; Na^+ binding

The melibiose permease of *Salmonella typhimurium* ($MelB_{St}$) catalyzes cotransport of galactoside with Na^+, Li^+ or H^+.[1] $MelB_{St}$ belongs to the glycoside/pentoside/hexuronide:cation family[2], a subgroup of the major facilitator superfamily (MFS) of membrane transport proteins. MelB of *Escherichia coli* ($MelB_{Ec}$) is the best-studied member among all MelB orthologues.[3–14] Recently, MelB homologues in human and mouse (called the major facilitator superfamily domain-containing proteins, MFSD) have been reported.[15–17] Among them, MFSD2A protein, which is expressed in many cells, plays a role in adaptive thermogenesis;[15] it has been also identified as a human lung tumor suppressor[16] and responsible for tunicamycin sensitivity in mammalian cells.[17] MFSD2A shares ~15% identity and ~54% similarity of primary sequence with $MelB_{St}$, and the positions essential or important for melibiose transport in MelB are functionally conserved in MFSD2A.[17]

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SUPPORTING INFORMATION Conservation between $MelB_{St}$ and $MelB_{Ec}$, SDS-PAGE analysis and western blotting, as well as melibiose exchange with intact cells are available free of charge via the Internet at http://pubs.acs.org.

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MelB utilizes the free energy from the downhill translocation of one cosubstrate to drive uphill translocation of the other,[18–22] and all three cations compete for a common binding pocket.[23–25] A previous threading model,[13] basing on the crystal structures of a MFS permease, the H^+-coupled lactose permease (LacY),[26–28] predicts that $MelB_{Ec}$ is organized into two pseudo-symmetrical six-helix bundles connected by a long middle loop surrounding an internal cavity facing the cytoplasm. A similar overall fold for other MelB orthologues has also been proposed from threading analysis.[13] In contrast to LacY, the residues important for cation binding in MelB are located in the N-terminal helix bundle (Fig. 1), whereas the H^+-binding site in LacY is located primarily in the C-terminal helices.[28] Based on the location of the sugar-binding site in LacY, the melibiose-binding site is proposed to lie within the internal cavity (Fig. 1).[13] This model is consistent with numerous biochemical and biophysical results,[11, 29–36] as well as low-resolution EM structures of $MelB_{Ec}$.[37, 38] The organization of the protein into two separate helix bundles, as well as the location of cosubstrates, are consistent with the alternating-access transport model, which has been recognized as a fundamental mechanism for many other secondary transporters.[26, 39–46]

$MelB_{St}$ shares 86% identity and 96% similarity of primary sequence with $MelB_{Ec}$ (Supplemental Information, Fig. S1). All non-conserved and 82% of the conserved variations occur in the C-terminal domain and the middle loop. Bioinformatics analysis suggests that the internal cavities including two cosubstrate-binding sites are well conserved between the two MelB permeases (Fig. 1), implying similarity in function, which is supported by previous studies of melibiose transport and cation binding.[1] The docked sugar is surrounded by potential H-bonding partners and aromatic residues (Fig. 1),[13] which share common features for sugar binding.[41, 47–50] A Na^+ has been proposed to bind between helices II and IV[13]. A large body of experimental data, including those from mutagenesis, biochemistry and FTIR spectroscopy, indicates that the carboxyl groups of conserved Asp55 and Asp59 (helix II) contribute to Na^+ binding to $MelB_{Ec}$.[14, 29–34] Helix IV is in the center of a charge/H-bond network involved in the binding of the two cosubstrates (Fig. 1).[13, 30, 34, 51] Gly117 is in the middle of helix IV, and the carbonyl oxygen may participate in Na^+ coordination (Fig. 1).[13]

We know little about the role of Gly117, but it was observed that the G117D mutation rescued the function of inactive mutant D55S in $MelB_{Ec}$,[52] which supports the notion that Gly117 is in close proximity to Asp55.[13, 52] In addition, in mutant G117C $MelB_{Ec}$ the conformational equilibrium was displaced towards the outward-facing conformation.[53] In $MelB_{St}$, site-directed mutagenesis has not been used to study the binding sites for cosubstrates so far. In this study, we mutated Gly117 of $MelB_{St}$ to four different residues--Ala, Pro, Trp or Arg. The effects of the mutations on cosubstrate binding and transport depend on the physical and chemical properties of the side chain. Our studies indicate that Gly117 plays an important role in cation binding and translocation, which is consistent with our threading model.

Materials and Methods

Materials

[1-^{3}H]Melibiose was custom synthesized by PerkinElmer (Boston, MT). The 2'-(N-dansyl)aminoalkyl-1-thio-b-D-galactopyranoside (D^2G) was kindly provided by Drs. H. Ronald Kaback and Gérard Leblanc. Monensin and carbonyl cyanide m-chlorophenylhydrazone (CCCP) were purchased from *Sigma-Aldrich*. 2-(4′-maleimidylanilino)-naphthalene-6-sulfonic acid was purchased from *Invitrogen*. Oligodeoxynucleotides were synthesized by *Integrated DNA Technologies*. MacConkey agar media (lactose free) was from *Difco.* All other materials were reagent grade and obtained from commercial sources.

Bacterial strains and plasmids

E. coli DW2 strain (*mel*A$^+$, Δ*melB*, Δ*lacZY*), obtained from Dr. Gérard Leblanc, was used for the functional characterization. *E. coli* XL1 Blue cells were used for DNA manipulations. The expression plasmid pK95 ΔAH/MelB$_{St}$/CHis$_{10}$,[1, 3] which encodes the full length MelB$_{St}$ with L5→M and a His$_{10}$-tag at the C-terminus (the wild type) was used as the template. Four individual mutants with residue Ala, Pro, Trp or Arg at position Gly117 were constructed by *QuickChange*™ *Site-Directed Mutagenesis Kit* from *Stratagene*. All mutants have been confirmed by DNA sequencing.

Growth of cells and protein overexpression

E. coli DW2 cells (*mel*A$^+$, *melB,lacZY*) containing a given plasmid were grown in Luria-Bertani (LB) broth with 100 mg/L of ampicillin in a 37-°C shaker overnight. The overnight cultures were diluted by 5% to LB broth supplemented with 0.5% glycerol and 100 mg/L of ampicillin, and constitutive overexpression was obtained by shaking at 30 °C for 5 h.

Preparation of right-side-out (RSO) membrane vesicles

RSO membrane vesicles were prepared from *E. coli* DW2 cells by osmotic lysis,[1, 54, 55] extensively washed and resuspended in 100 mM KP$_i$ (pH 7.5) at a protein concentration of ~25–30 mg/ml, frozen in liquid N_2, and stored at −80 °C.

Preparation of crude membranes

The 5-hour cultures with the expressed MelB$_{St}$ were washed with 20 mM Tris-HCl (pH 7.5) once, re-suspended and adjusted with the same buffer to A_{600} ~20. Approximately 100 μL of cells from each sample were sonicated in an ice-cold water bath (Branson 2510) for 5 min, three times; and centrifuged at 20,816 g for 15 min at 4 °C. The supernatant (80 μL) was subjected to ultracentrifugation at 384,492 g (TLA-100 rotor, *Beckman Optima*™ Max Ultracentrifuge) for 20 min; the pellets were resuspended in 70 μL of 20 mM Tris-HCl (pH 7.5).

SDS-12% PAGE and Western blotting

After protein assay using *Micro BCA*™ *Protein Assay Kit* (Pierce), ~15 g of total membranes were loaded onto each well of SDS-12%PAGE. The gel was transferred onto the PVDF membrane by the *Trans-Blot Turbo* transfer system (*Bio-Rad*) at 1.3 A, 25 V for 15 min. The blocked PVDF membrane was then reacted with the penta-His horseradish peroxidase conjugate and washed according to the protocols provided in the *Penta-His HRP Conjugate Kit* (*Qiagen*). MelB$_{St}$ proteins were detected using the *SuperSignal West Pico* chemiluminescent substrate (*Thermo Scientific*) after exposing it to the X-ray film (*Kodak BioMax XAR film*).

[1-^{3}H]Melibiose transport assay

E. coli DW2 cells expressing MelB$_{St}$ in the absence of melibiose were washed with 50 ml of 100 mM KP$_i$ (pH 7.5, so-called Na^+-free buffer) twice, followed by washing with 50 ml 100 mM KP$_i$, pH 7.5, 10 mM $MgSO_4$. The cell pellets were resuspended with the latter buffer, adjusted to an A_{420} = 10 (~0.7 mg protein/ml). Melibiose transport at a final concentration of 0.4 mM and a specific activity of 10 mCi/mmol was assayed as described.[1]

Melibiose fermentation

The DW2 cells *(melA*$^+$, Δ*melB*, Δ*lacZY)* were transformed with a given plasmid, plated on MacConkey agar plates[35] containing ~100 mM Na^+ with supplement of 1–30 mM melibiose

(the sole carbohydrate source) and 100 mg/L of ampicillin, and incubated at 37 °C. After 18 h, the plates were viewed and photographed immediately.

Melibiose efflux and exchange

RSO membrane vesicles containing $MelB_{St}$ in 100 mM KP_i, pH 7.5, 10 mM $MgSO_4$ were concentrated to 25–30 mg/ml, and pre-equilibrated overnight on ice with melibiose (20 mM, 10 mCi/mmol), 0.75 μM monensin, and 10 μM $CCCP^{22}$, in the absence or presence 20 mM NaCl or LiCl. Aliquots (2 μL) were diluted 200 fold into a given buffer in the absence (efflux) or presence (exchange) of 20 mM unlabeled melibiose, and reactions were terminated by dilution and rapid filtration at a given time.[1]

FRET (Trp→D^2G)

Fluorescence was measured with an AMINCO-Bowman Series 2 Spectrometer. Steady-state measurements were performed in a 3-mm quartz cuvette (*Starna Cells, Inc*) with RSO membrane vesicles at a protein concentration of ~0.5 mg/ml in 100 mM KP_i, pH 7.5. With excitation wavelength at 290 nm, the FRET intensity was recorded for 60 sec at 500 nm with slit widths of 2 mm for both excitation and emission.

Determination of apparent cation-stimulation constants ($K_{0.5}^{Na+}$ and $K_{0.5}^{Li+}$) of the D^2G FRET

NaCl or LiCl was consecutively added into a 3-mm quartz cuvette containing RSO vesicles after an addition of 10 μM D^2G (a K_D value for the WT). Water at identical volume was used for the control. The FRET signals were recorded for 60 sec at each condition and mean values were used for further calculation. The increase in intensity ($\Delta I^{Na+ \text{ or } Li+}$), the difference before (I_0) and after successive additions of cation solution ($I^{Na+ \text{ or } Li+}$), was expressed as the percentage of the I_0 ($\Delta I^{Na+ \text{ or } Li+}/I_0$). FRET stimulation at each cation concentration was corrected by subtracting corresponding I^{water}/I_0 value, and then plotted as a function of Na^+ or Li^+ concentration. The $K_{0.5}^{Na+ \text{ or } Li+}$ values are determined by fitting the data to a hyperbolic function.

Determination of the melibiose concentration corresponding to half-maximal displacement of bound D^2G (IC_{50})

Melibiose was added stepwise to a 3-mm quartz cuvette containing RSO vesicles after the additions with D^2G (10 μM) and NaCl or LiCl (20 mM or 200 mM) until no change occurred in fluorescence emission at 500 nm. An identical volume of water was added at each point as a negative control. FRET signals were recorded for 60 sec after each addition, and mean values were calculated. The decrease in intensity after each addition of melibiose (ΔF^{Mel}) was corrected by subtracting the emission change obtained with water (ΔF^{water}), and plotted as a function of melibiose concentration. The IC_{50} values were determined by fitting the data to a hyperbolic function.

RESULTS

Construction and protein expression of Gly117 mutants

Gly at position 117 in $MelB_{St}$ was changed by site-directed mutagenesis to four different residues: Ala (small volume); Pro (a helix destabilizer); Trp (aromatic ring); or Arg (positive charge). Membrane proteins were separated by SDS-PAGE, and the proteins were detected by both silver nitrate and Western blot using anti-His antibody (Supplemental Fig. S2). Compared to the WT, most of the mutants had similar levels of membrane expression, with the exception of G117R, which had a reduced expression.

Melibiose active transport in intact cells

In a nominally Na^+-free buffer, the WT catalyzes melibiose transport at an almost linear rate for 15 s to 80 nmol/mg protein at 2 min (Fig. 2), which was significantly stimulated by Na^+ or Li^+ as shown previously.[1, 56] Addition of the protonophore CCCP abolished transport, indicating that the primary driving force for transport is the electrochemical H^+ gradient. Furthermore, when unlabeled melibiose was added at each time point during transport, with a10-min incubation prior to the filtration, the entire internal pool of melibiose was exchanged with extracellular melibiose (Supplemental Information, Fig. S3). Thus, there is little or no hydrolysis or chemical alteration of the accumulated intracellular melibiose.

The mutant G117A catalyzed Na^+- or Li^+-dependent melibiose transport similar to the WT (Fig. 2) with somewhat lower H^+-coupled transport activity (i.e., in the absence of Na^+ or Li^+). All other mutants (P, W or R) exhibited H^+-coupled melibiose transport at a level indistinguishable from CCCP-treated WT or non-transformed DW2 cells, and showed no response to addition of Na^+ or Li^+.

Melibiose fermentation

On MacConkey agar plates containing 1–30 mM melibiose, untransformed DW-2 cells formed pale/white colonies with a translucent background, denoting little or no melibiose translocation across the membranes. At >10 mM melibiose, DW2 cells, containing the chromosome-encoded α-galactosidase and recombinantly overexpressing WT $MelB_{St}$, formed magenta colonies on a hazy background indicating melibiose fermentation and therefore downhill melibiose influx catalyzed by $MelB_{St}$ (Fig. 3). At 5 mM melibiose, few colonies were pink, whereas at 2.5 mM or lower, the colonies were pale yellow/brown. Mutant G117A exhibited similar color to WT. Mutant G117P exhibited red or pink colonies only at high concentrations of melibiose, indicating limited melibiose influx and fermentation. Mutants G117R and G117W formed colonies that were indistinguishable from untransformed cells even at 30 mM melibiose, indicating no fermentation.

Melibiose efflux and exchange

In the absence of Na^+ or Li^+, RSO membrane vesicles containing WT $MelB_{St}$ exhibited a slow rate of melibiose efflux down a concentration gradient and a slightly faster rate of equilibrium exchange (Fig. 4). Whereas the efflux rate was stimulated by Na^+ or Li^+, the exchange rate was enhanced by Na^+ but inhibited by Li^+.[1] In the presence of Na^+, 96% of the intravesicular melibiose exchanged with the extracellular unlabeled melibiose within 5 min (data not shown). Consistent with the results in intact cells, little or no melibiose hydrolysis occurred within the RSO vesicles.

The G117W mutant did not catalyze either melibiose efflux or exchange in the presence of H^+, Na^+ or Li^+. The G117P mutant catalyzed significantly reduced rates of efflux and exchange. Strikingly, mutant G117R exhibited little or no efflux but catalyzed a relatively fast rate of exchange in the presence of Na^+ or Li^+, but not H^+.

$K_{0.5}^{Na+}$ or $K_{0.5}^{Li+}$ for D^2G FRET

FRET from endogenous Trp residues to the dansyl moiety of bound D^2G has been demonstrated with $MelB_{Ec}$[4, 57] and $MelB_{St}$.[1] Thus, WT $MelB_{St}$ exhibits FRET upon the addition of D^2G, which is stimulated by addition of Na^+ or Li^+ and reversed by melibiose. The difference in intensity observed before and after melibiose addition reflects the D^2G bound specifically to $MelB_{St}$. At saturating Na^+ or Li^+, the WT $MelB_{St}$ exhibits K_d for D^2G of ~10 μM.[1] The Na^+ or Li^+ enhancement of FRET (difference in intensity before and after cation addition, $\Delta I^{Na+ \text{ or } Li+}$) results from combined effects of an increase of D^2G binding *per se* and a conformational change (distance and/or environment) induced by Na^+ or Li^+

binding.[1, 4] Mutations may affect Na^+- or Li^+-induced intensities by (a) decreasing the binding affinity for either or both cosubstrates, and/or (b) modifying the micro-environment surrounding donor Trp residue(s), and/or (c) restricting the conformational change. Thus, the same concentrations of Na^+- or Li^+-bound ternary complex from different mutants may not yield the same $\Delta I/I_0$ value.

In order to quantify cation binding to $MelB_{St}$ mutants, $\Delta I^{Na+ \text{ or } Li+}/I_0$ was measured as a function of Na^+ or Li^+ concentration (Fig. 5, upper panels). The stimulation constants ($K_{0.5}^{Na+}$ and $K_{0.5}^{Li+}$) for D^2G FRET in WT $MelB_{St}$ were 0.9 mM and 1.5 mM, respectively, and the mutant G117A yielded similar values (Table 1). $K_{0.5}^{Na+}$ and $K_{0.5}^{Li+}$ values for G117P were 25-and 6-fold higher than those for the WT, respectively. With mutant G117R, both $K_{0.5}$ values increased by 12 fold. The $K_{0.5}^{Li+}$ for G117W was only 2.5-fold higher than obtained for WT, but no Na^+ stimulation was observed even at 500 mM (data not shown). In all three mutants, the maximum stimulations by Na^+ or Li^+ were largely reduced implying that either the micro-environment and/or distance between D^2G and donors are changed, or that the number of bound D^2G molecules is reduced by lower sugar affinity. In either case, the constant ($K_{0.5}^{Na+ \text{ or } Li+}$) reflects the cation-binding affinity.

IC_{50} for melibiose displacement of bound D^2G

In order to quantify melibiose binding to $MelB_{St}$, 10 μM D^2G, Na^+ or Li^+, and then the titrate melibiose were consecutively added to RSO vesicles. IC_{50} values for WT $MelB_{St}$ of 3.8 mM or 1.7 mM were observed at 20 mM Na^+ or Li^+, respectively, and 2.2 mM or 1.8 mM at 200 mM Na^+ or Li^+, respectively (Fig. 5, bottom panels; Table 2). The IC_{50} for mutant G117A was about 2-fold higher than that of the WT at 20 mM Na^+ or Li^+. In the presence of 20 mM or 200 mM of Li^+, IC_{50} values for mutant G117W were 50 mM or 14.1 mM, respectively, and for mutant G117P were 11.3 mM or 7.5 mM, respectively. Mutant G117R exhibited IC_{50} of 5.6 mM or 4.7 mM in the presence of 200 mM Na^+ or Li^+. FRET intensities for mutants G117W or P, with Na^+ as the cosubstrate, were too weak to allow the determination of IC_{50} value.

DISCUSSION

An ordered-binding model for cation/melibiose symport in $MelB_{Ec}$ has been suggested.[11, 13, 14] An extended model for the melibiose efflux mechanism is proposed for $MelB_{St}$ here (Fig. 6). In this simplified scheme, melibiose efflux down a sugar concentration gradient consists of 8 steps (*black solid arrows*): [*Step-1, Na^+-bound inward-facing state, or P_I:Na^+ state*] Binding of a Na^+ to the inward-facing conformer; [*Step-2, Na^+- and melibiose-bound inward-facing state, or P_I:Na^+ :Mel state*] Binding of a melibiose from the cytoplasmic cavity; [*Step-3, occluded Na^+-and melibiose-bound state, or P:(Na^+:Mel) state*] Formation of a ternary-complex intermediate that is occluded on both sides; [*Step-4, Na^+-* and melibiose-bound outward-facing state, or P_O:Na^+ *:Mel state*] Opening of the periplasmic cavity; [*Step-5, Na^+-bound outward-facing state, or P_O:Na^+ state*] Release of the melibiose at the outer surface; [*Step-6, empty outward-facing state, or P_O:empty state*] Release of Na^+ from an outward-facing conformer; [*Step-7, occluded empty state, or P:(empty) state*] Formation of an unloaded intermediate occluded on both sides; [*Step-8, empty inward-facing state, or P_I:empty state*] Opening of the cytoplasmic cavity. The melibiose efflux involves the whole cycle, and melibiose exchange reaction involves only steps *1–5*.

Previously, we have experimentally determined[1] that the extracellular release of cation is the rate-limiting step for melibiose efflux catalyzed by $MelB_{St}$, and melibiose is released from $MelB_{St}$ extracellularly prior to the release of Na^+. These results are consistent with what observed in $MelB_{Ec}$.[22] Here, we provide further evidence to support this notion. Cells expressing mutant G117R neither ferment melibiose nor catalyze melibiose active transport

or efflux. Remarkably, however, this mutant catalyzes Na^+- or Li^+- but not H^+-coupled equilibrium exchange. Thus, in the presence of 20 mM melibiose and Na^+ or Li^+ on both sides of the membrane, the mutant can undergo the global conformational change involved in an alternating access-type mechanism but is defective in releasing Na^+ or Li^+ (Fig. 6, *step 6*), implying that Gly117 plays an important role in the cation translocation mechanism. This behavior of mutant G117R, similar to that of E325A LacY[58; 59], strongly supports the ordered-binding mechanism as postulated (Fig. 6).

The rate of exchange is believed to correlate with the binding affinity. Accordingly, at saturating Na^+ or Li^+ concentration, the IC_{50} for melibiose displacement of bound D^2G exhibits only a 2.5-fold increase (Table 2). If we assume that the mutation has a similar effect on D^2G and melibiose, then it can be estimated that the apparent binding constant (K_d) for melibiose is increased by less than 4 fold, implying that sugar binding is only slightly affected by the Arg at position 117, and therefore Gly117 may not coordinate melibiose directly.

In WT $MelB_{St}$, we have been observed previously[1] that Li^+ stimulates melibiose efflux and an outwardly directed Li^+ gradient increases exchange; however, at equal Li^+ concentrations on both sides of the membrane, the melibiose exchange rate is slower than the efflux rate. In $MelB_{Ec}$,[22,60] Li^+ also inhibits melibiose exchange, but not methyl-β-D-thiogalactoside exchange. Although the mechanism of inhibition is still not clear, it is possible that MelB/Li^+/melibiose complexes favor an occluded state (Fig. 6, *step 3*) in which there is no exchange between free and bound sugars.[1] In the presence of Li^+, the Gly117→R mutation inhibits melibiose efflux but stimulates exchange at a rate even faster than that obtained with WT $MelB_{St}$. It is likely that the mutation may decrease the stability of the MelB/Li^+/melibiose complexes at *step 3*, thus facilitating melibiose exchange at a rate similar to that obtained in the presence of Na^+.

Six of eight endogenous Trp residues are located in the N-terminal helix bundle. Trp64 (helix II) and Trp299 (IX) of $MelB_{Ec}$ are responsible for 80% of the D^2G FRET, and apparently Trp116 does not contribute significantly to the FRET.[25] Thus, it seems unlikely that the inserted Trp at position 117 could serve as an efficient FRET donor or significantly quench the Trp64 and Trp299 emission directly. Mutant G117W specifically blocked Na^+-induced D^2G FRET (ΔI), but the apparent affinity for Li^+ did not change significantly (< 3-fold increase in $K_{0.5}^{Li+}$, Table 1). Thus, lack of Na^+-induced D^2G FRET (ΔI) may be mainly due to a large decrease in Na^+ binding.

While in most Gly117 mutants there are reductions in binding of Na^+, Li^+ and melibiose, the effect on Na^+ binding is more severe than those on Li^+ and melibiose. The MelB orthologue of *Klebsiella pneumoniae* ($MelB_{Kp}$) couples melibiose transport with H^+ and Li^+ but not Na^+.[61] It has been demonstrated that Asn58, which is adjacent to Asp59, is important for Na^+ recognition, and $MelB_{Kp}$ has Ala at position 58.[31] It seems likely that selective elimination of Na^+ binding in MelB could be achieved by a minor modification around the cation-binding site. In addition, the effective inhibition of melibiose binding in mutant G117W (Table 2) may be due to a steric effect of the bulky side chain, which may also partially account for the effects of other mutations on sugar binding.

Gly is known to destabilize α-helices, and the replacement with Pro, another α-helix destabilizer that may alter the tilt in helix IV, partially inhibits both efflux and exchange reactions, as well as impairs affinity for the cosubstrates to a similar extent. Thus, the data support the notion that the melibiose/cation symport mechanism requires precise positioning of helix IV.

If the carbonyl oxygen at position 117 is a part of the cation-binding site, then a small neutral replacement should not have a significant effect. Accordingly, placement of Ala at position 117 has little or no effect on Na^+- or Li^+-coupled melibiose transport (Fig. 2) or the apparent affinity for Na^+, Li^+ or melibiose (Tables 1 & 2). Taken as a whole, our findings show that the placement of Arg or Trp at position 117 abolishes melibiose uphill transport, efflux, and fermentation; G117R and P greatly decrease apparent affinities for Na^+ and Li^+. Remarkably, the mutant G117R retains significant sugar affinity and catalyzes melibiose exchange, a partial reaction, with bound Na^+ or Li^+, but does not catalyze melibiose translocation involving net flux of the coupling cation. In conclusion, Gly117 plays an important role in cation binding and translocation of $MelB_{ST}$, which is consistent with our threading structure.[13]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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ABBREVIATIONS

$MelB_{St}$	melibiose permease of *Salmonella typhimurium*
$MelB_{Ec}$	MelB of *Escherichia coli*
LacY	lactose permease of *E. coli*
***mel*A**	gene encoding α-galactosidase
LB broth	Luria-Bertani broth
D^2G	2'-(N-dansyl)aminoalkyl-1-thio-β-D-galactopyranoside
CCCP	carbonylcyanide-m-chlorophenylhydrazone
$K_{0.5}^{Na+ \text{ or } Li+}$	the Na^+ or Li^+ stimulation constant for D^2G FRET
IC_{50}	the melibiose concentration corresponding to half-maximal displacement of bound D^2G.

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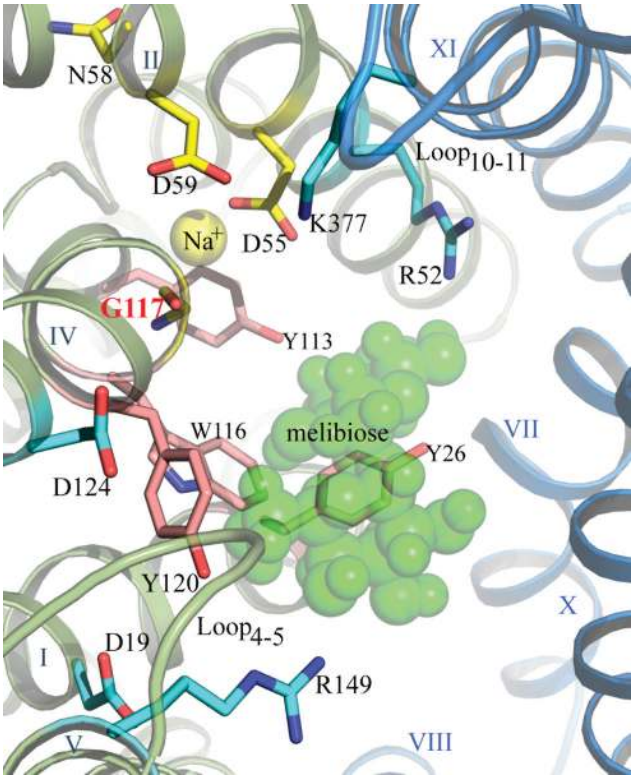

Fig. 1.

Putative cosubstrate-binding sites of MelB viewed from the cytoplasmic side. The N-and C-terminal helix bundles are shown in green and blue, respectively. The residues essential (D55 and D59) or important (N58) for Na^+ binding in $MelB_{Ec}$ are shown as yellow sticks. Residues colored in cyan (D19, D124, R52, R149, and K377) and pink (Y26, Y113, W116, and Y120) are important for melibiose binding/transport in $MelB_{Ec}$. The Gly117 is shown by backbone (yellow) and labeled in red. Important loops between helices IV–V and X–XI are labeled as $Loop_{4–5}$ and $Loop_{10–11}$, respectively. A melibiose molecule is shown in green.[13]

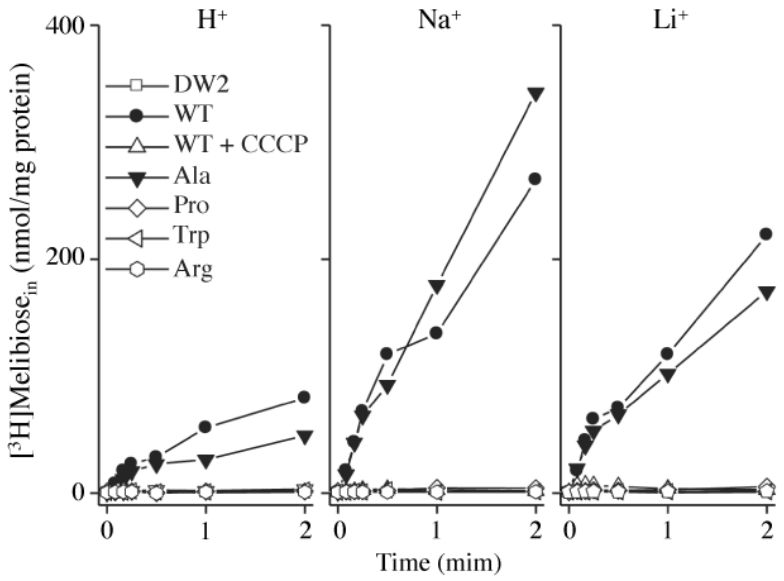

Fig. 2.

Melibiose transport in intact cells. *E. coli* DW2 cells were washed and resuspended with 100 mM KP_i, pH 7.5, 10 mM $MgSO_4$, and adjusted to 0.7 mg/ml of protein. Transport was initiated by adding melibiose (0.4 mM, 10 mCi/mmol) in the absence or presence of 20 mM NaCl or LiCl. Intracellular melibiose is plotted as a function of time. The cells expressing WT $MelB_{St}$ were treated with 10 μM CCCP prior to the transport assay.

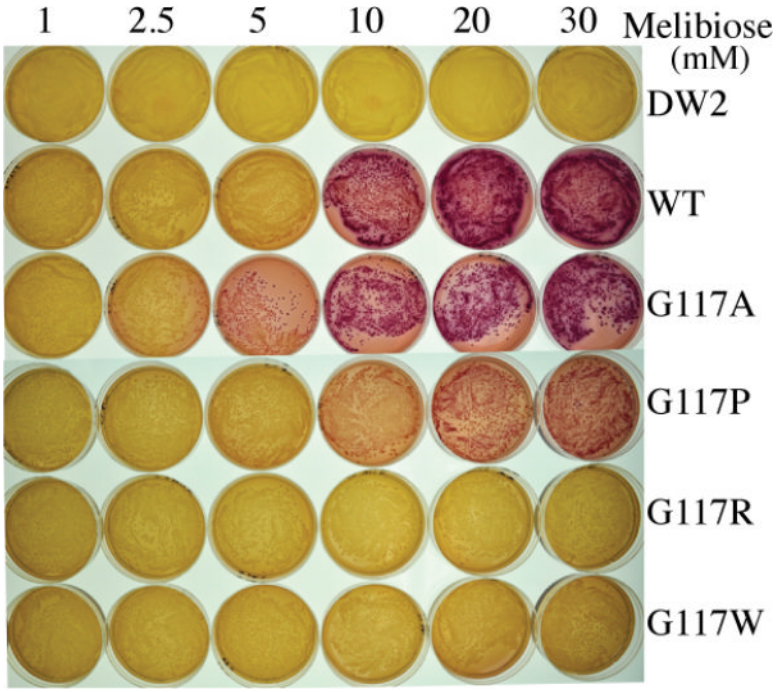

Fig. 3.

Melibiose fermentation. *E. coli* DW2 (*lacY*$^{-}$*Z*$^{-}$*melA*$^{+}$*B*$^{-}$) cells were transformed with a plasmid encoding the WT or a $MelB_{St}$ mutant, and plated on MacConkey agar (lactose free) containing concentrations of melibiose ranging from 1 mM to 30 mM, and incubated at 37 °C for 18 h.

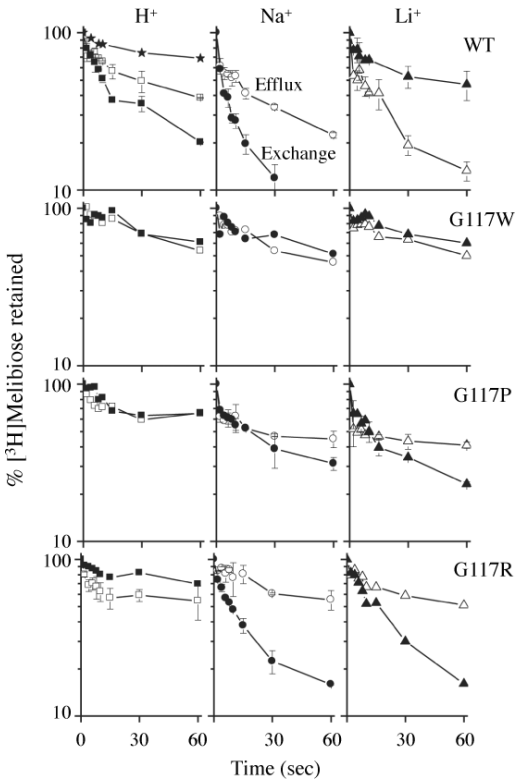

Fig. 4.

Melibiose efflux and exchange. Melibiose efflux (open symbols) was measured by diluting the preloaded vesicles without (*squares*) or with 20 mM NaCl (*circles*) or LiCl (*triangles*) in a buffer containing 100 mM KP_i, pH 7.5, without or with 20 mM NaCl or LiCl, respectively. Melibiose exchange (*filled symbols*) was assessed in the same way except for the presence of 20 mM unlabeled melibiose in the dilution buffer. For a negative control, RSO vesicles containing WT $MelB_{St}$ were treated with 3.6 mM 2-(4'-maleimidylanilino)-naphthalene-6-sulfonic acid for 5 hr at 23 °C (*stars*), and diluted into 100 mM KP_i, pH 7.5 in the absence of melibiose.[1] Intracellular melibiose was expressed as a percentage of the zero-time point. Error bars indicate S.D.

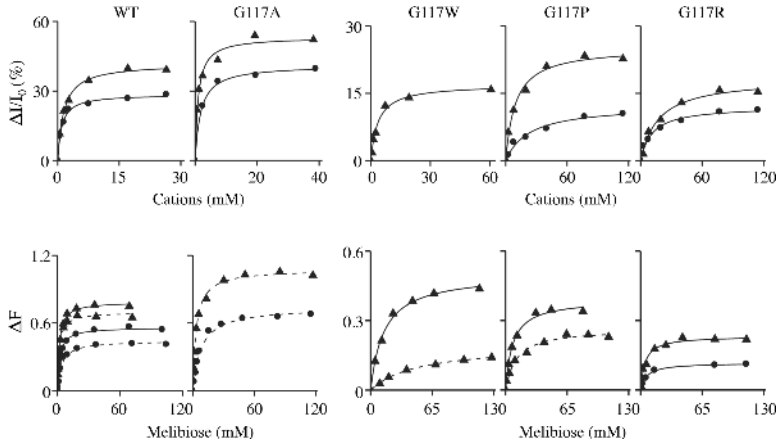

Fig. 5.

Apparent affinity. RSO vesicles (0.5 mg/ml, 100 mM KP_i, pH 7.5) containing WT or a $MelB_{St}$ mutant were used for D^2G FRET assay at excitation and emission wavelengths of 290 and 500 nm, respectivley. (*Top panels)*: Determination of $K_{0.5}^{Na+}$ and $K_{0.5}^{Li+}$ for the D^2 0.5 G FRET. With 10 μM D^2G, Na^+ or Li^+ was added at increasing concentrations. The normalized emissions were plotted as a function of Na^+ (circles) or Li^+ (triangles) concentration. $K_{0.5}^{Na+}$ or $K_{0.5}^{Li+}$ value was determined by fitting a hyperbolic function to the data. (*Bottom panels)*: Determination of IC_{50} for melibiose displacement of bound D^2G. With D^2G (10 μM) and Na^+ or Li^+at a given concentration, melibiose were added stepwise until the displacement of the bound D^2G is complete. After correction for the water dilution effect, the change in intensity (ΔF) was plotted as a function of melibiose concentration. The IC_{50} value was determined by fitting a hyperbolic function to the data obtained in the presence of 20 mM Na^+ (circles with dashed lines), 200 mM Na^+ (circles with solid lines), 20 mM Li^+ (triangles with dashed lines), or 200 mM Li^+ (triangles with solid lines).

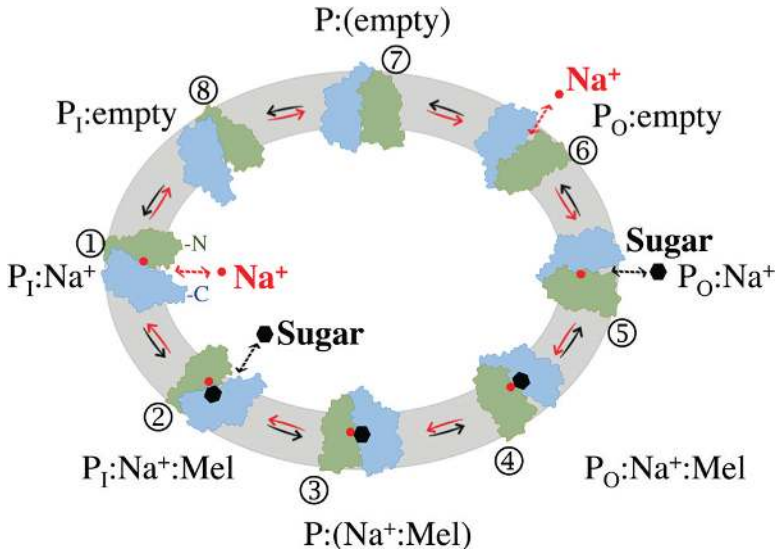

Fig. 6.

An ordered-binding model for melibiose/Na^+ efflux catalyzed by $MelB_{St}$ (see text). The N- and C-terminal domains of $MelB_{St}$ molecule are colored in green and blue, respectively. Sugar and Na^+ are shown as black hexagons and red spheres, respectively. P_I or P_O represents the permease at an inward-facing or outward-facing conformation, respectively. P:(Na^+:Mel) or P:(empty), cosubstrates-bound or unloaded permease at the occluded state, respectively.

Table 1

Cation stimulation constants for D^2G FRET ($K_{0.5}^{Na+ \text{ or } Li+}$, mM)

	WT	G117A	G117W	G117P	G117R
Na^+	0.9	1.7	/[a]	23.2	11.2
Li^+	1.5	0.97	3.6	9.5	17.2

[a] not determined due to weak signals.

Table 2

IC_{50} for melibiose displacement of bound D^2G (mM)

		WT	G117A	G117W	G117P	G117R
Na^+	20 mM	3.8	8.1	/[a]	/	/
	200 mM	2.2	-[b]	/	/	5.6
Li^+	20 mM	1.7	3.4	50.0	11.3	/
	200 mM	1.8	-	14.1	7.5	4.7

[a] not determined due to weak signals.

[b] not determined