Thermodynamic cooperativity of cosubstrate binding and cation selectivity of *Salmonella typhimurium* MelB

Parameswaran Hariharan and Lan Guan

Department of Cell Physiology and Molecular Biophysics, Center for Membrane Protein Research, School of Medicine, Texas Tech University Health Sciences Center, Lubbock, TX

The Na^+-coupled melibiose symporter MelB, which can also be coupled to H^+ or Li^+ transport, is a prototype for the glycoside-pentoside-hexuronide:cation symporter family. Although the 3-D x-ray crystal structure of *Salmonella typhimurium* MelB ($MelB_{St}$) has been determined, the symport mechanisms for the obligatory coupled transport are not well understood. Here, we apply isothermal titration calorimetry to determine the energetics of Na^+ and melibiose binding to $MelB_{St}$, as well as protonation of this transporter. Studies of the thermodynamic cycle for the formation of the Na^+–$MelB_{St}$–melibiose ternary complex at pH 7.45 reveal that the binding of Na^+ and melibiose is cooperative. The binding affinity for one substrate (Na^+ or melibiose) is increased by the presence of the other by about eightfold. The coupling free energies ($\Delta\Delta G$) of either substrate binding are ~5 kJ/mol, and binding of both substrates releases a free energy of ~35 kJ/mol. Measurements of the Na^+-binding enthalpy at three different pH values, including the pK_a value of MelB, indicate that the binding of one Na^+ displaces one H^+ per $MelB_{St}$ molecule. In addition, the absolute dissociation constants for Na^+ and H^+, determined by competitive binding, show that $MelB_{St}$ is selective for H^+ over Na^+ by ~1,000-fold at a pK_a of 6.25. Thus, the Na^+ coupling in $MelB_{St}$ is based not on ion selectivity but on ion concentrations and competitive binding because of a much higher Na^+ concentration under physiological conditions. Such a selectivity feature seems to be common for membrane transport proteins that can bind both H^+ and Na^+ at a common site.

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INTRODUCTION

Cation-coupled symporters use the energy stored in cation electrochemical gradients across cell membranes to translocate molecules necessary for cellular functions. Most secondary-active transporters in the major facilitator superfamily (MFS), the largest family of transporters containing over 10,000 sequenced members (Saier et al., 1999), use the H^+ electrochemical gradient for their functions, but some members are able to couple solute transport to Na^+ translocation, such as the bacterial melibiose transporter MelB (Tsuchiya and Wilson, 1978; Wilson and Ding, 2001) and the eukaryotic lysophosphatidylcholine symporter (Nguyen et al., 2014). Both proteins belong to the glycoside -pentoside-hexuronide:cation symporter family (TCDB 2.A.2; Poolman et al., 1996), a subgroup of the MFS family. MelB catalyzes galactoside symport not only with Na^+ but also with Li^+ or H^+ (Tsuchiya and Wilson, 1978; Niiya et al., 1980; Bassilana et al., 1987; Guan et al., 2011); however, this symporter cannot transport sugars with K^+, Rb^+, or Cs^+ (Guan et al., 2011). We have determined the high-resolution x-ray 3-D crystal structure of *Salmonella typhimurium* MelB ($MelB_{St}$) at a resolution of 3.35 Å (Ethayathulla et al., 2014). This is the first high-resolution structure of a member of the MFS family that uses Na^+ as a coupling cation. $MelB_{St}$ was captured in two slightly different conformations (Ethayathulla et al., 2014). Like other MFS-fold transporters (Abramson et al., 2003; Huang et al., 2003; Guan and Kaback, 2006; Guan et al., 2007; Dang et al., 2010), its N- and C-terminal six-helix bundles surround a central aqueous cavity that contains side chains important for the binding of galactoside and Na^+, Li^+, or H^+ and opens to the periplasmic side (Fig. 1, a and b). Previously, using a homology threading approach, we proposed that MelB is an MFS symporter (Yousef and Guan, 2009). The crystal structures confirmed this prediction and cleared up previous controversies about the MelB fold. The structural information is consistent with many previous biochemical and biophysical studies with *Escherichia coli* MelB ($MelB_{Ec}$; Mus-Veteau et al., 1995; Pourcher et al., 1995; Mus-Veteau and Leblanc, 1996; Maehrel et al., 1998; Ganea et al., 2001; Wilson and Ding, 2001; Meyer-Lipp et al., 2006; Granell et al., 2010). An alternating-access mechanism has been proposed to be involved in the sugar transport process (Meyer-Lipp et al., 2006; Yousef and Guan, 2009; Guan et al., 2012; Ethayathulla et al., 2014), similar to that proposed for other members of this superfamily

Correspondence to Lan Guan: lan.guan@ttuhsc.edu

Abbreviations used: ACES, *N*-(2-acetamido)-2-aminoethanesulfonic acid; α-NPG, α-nitrophenyl galactoside; D^2G, 2′-(*N*-dansyl)aminoalkyl-1-thio-β-D-galactopyranoside; $_{diff}$FRET, differential FRET; FRET, fluorescence resonance energy transfer; ITC, isothermal titration calorimetry; $MelB_{Ec}$, *Escherichia coli* melibiose permease; $MelB_{St}$, *Salmonella typhimurium* melibiose permease; MFS, major facilitator superfamily; TMAOH, tetramethylammonium hydroxide; UDM, undecyl-β-D-maltopyranoside.

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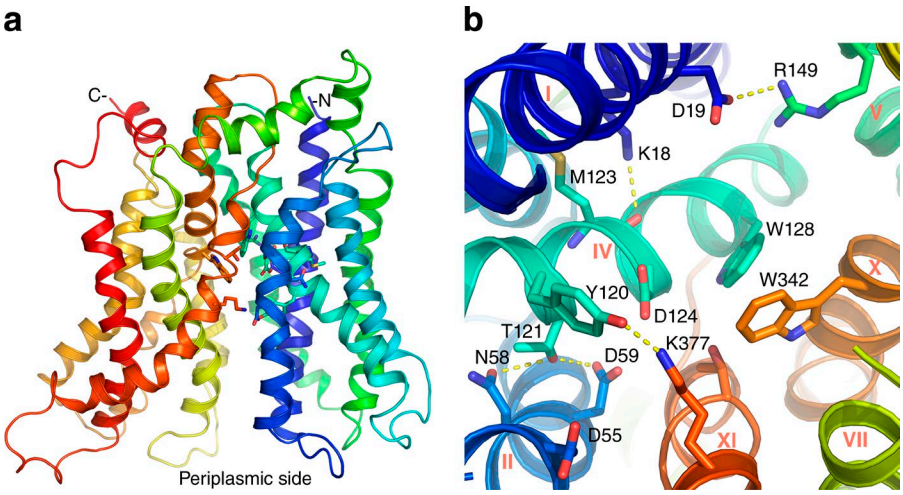

Figure 1. **X-ray crystal structure of $MelB_{St}$.** See Protein Data Bank accession no. 4M64. (a) The overall fold of $MelB_{St}$ in a periplasmic-side-open conformation. Helices in rainbow colors from blue (N terminus) to red (C terminus). (b) Cosubstrate-binding sites. The helices are labeled in roman numerals, and side chains potentially involved in the cation binding (residues D55, D59, D124, N58, and T121) or in the galactoside binding (residues D19, R149, Y120, D124, W128, W342, and K377) are highlighted as sticks. Residues Y120, D124, and K373 may be involved in both sites.

(Abramson et al., 2003; Huang et al., 2003; Guan and Kaback, 2006; Meyer-Lipp et al., 2006; Kaback, 2015).

The structure shows that Asp residues at positions 55 and 59 (helix II) and 124 (IV) may form a cation-binding site (Fig. 1 b). In both $MelB_{St}$ and $MelB_{Ec}$, all three Asp residues are required for Na^+ stimulation of melibiose transport or galactoside binding (Pourcher et al., 1993; Zani et al., 1994; Granell et al., 2010; Ethayathulla et al., 2014), as well as for the specific Fourier-transform infrared signal change elicited by Na^+ (Granell et al., 2010). Functional studies with $MelB_{Ec}$ have shown that all three cations (Na^+, Li^+, and H^+) compete for a common binding site (Lopilato et al., 1978; Damiano-Forano et al., 1986; Mus-Veteau et al., 1995). With $MelB_{St}$, a common binding site for Na^+ or Li^+ has been also established (Guan et al., 2011). The crystal structure (Ethayathulla et al., 2014) suggests that the proposed cation-binding pocket could selectively coordinate a Na^+ or Li^+, but it is not known how many Asp residues among the three are protonated. A single binding site for sugar has been also suggested, and the Na^+/galactoside stoichiometric ratio has been determined to be 1:1 (Bassilana et al., 1987; Wilson and Ding, 2001; Guan et al., 2011). This galactoside-binding site, which is in close proximity to the cation site, is surrounded by residues D19 (helix I), R149 (V), Y120, D124, W128 (IV), W342 (X), and K377 (XI; Fig. 1 b). Helix IV physically hosts both cosubstrate sites, and residues Y120, D124, and K373 may contribute to the binding of both substrates, which was proposed to be the structural basis for the observed increase of the galactoside affinity by Na^+ or Li^+ (Ethayathulla et al., 2014). However, how the two sites cooperate for the binding events, the protonation status of the cation site, and the mechanism of cation selectivity are not well understood.

There are several methods to determine the affinity of MelB for Na^+ binding; most depend on binding of a sugar (e.g., Na^+ stimulation of [^{3}H]α-nitrophenyl galactoside [[^{3}H]α-NPG] binding; Damiano-Forano et al., 1986) or fluorescence resonance energy transfer (FRET) from Trp residues to the dansyl moiety of a fluorescent sugar 2′-(*N*-dansyl)aminoalkyl-1-thio-β-D-galactopyranoside (Trp→D^2G FRET; Maehrel et al., 1998; Ganea et al., 2011; Guan et al., 2011; Jakkula and Guan, 2012; Amin et al., 2014). Isothermal titration calorimetry (ITC) is a label-free technique to measure heat changes (either release or absorption) derived from molecular interactions. It can directly reveal the binding enthalpy and allow for determination of the binding association constant (Cooper, 1999; Leavitt and Freire, 2001). Therefore, the binding free energy (ΔG) can be calculated. Here, we used Trp→D^2G FRET and ITC to study Na^+ binding, determine the free energy for Na^+ and melibiose binding to $MelB_{St}$, test the protonation status of $MelB_{St}$, and study the competition between Na^+ and H^+ in the absence or presence of melibiose. The results show that the binding of Na^+ and melibiose is thermodynamically cooperative, providing insights into the coupling mechanism of this symporter. Furthermore, the binding stoichiometry of melibiose and Na^+ or H^+ was also determined, confirming the previous conclusion that MelB catalyzes stoichiometric translocation of a melibiose with a cation (Na^+, Li^+, or H^+). Moreover, by determining the absolute dissociation constants for Na^+ and H^+, we conclude that the use of Na^+ as coupling ion for sugar transport is based not on ion selectivity but on competitive binding under physiological conditions (i.e., with a Na^+ concentration five orders of magnitude higher than the H^+ concentration).

MATERIALS AND METHODS

Reagents

The detergent undecyl-β-D-maltopyranoside (UDM) was from Anatrace. 2′-(*N*-dansyl)aminoalkyl-1-thio-β-D-galactopyranoside (D^2G, dansyl-galactoside) was obtained from G. Leblanc (Institut de Biologie et Technologies-Saclay, CEA Saclay, France) and H.R. Kaback (Uni-

versity of California, Los Angeles, Los Angeles, CA). Tetramethylammonium hydroxide (TMAOH) and *N*-(2-acetamido)-2-aminoethanesulfonic acid (ACES) were from Sigma-Aldrich. 2-(*N*-morpholino)ethanesulfonic acid (MES) was obtained from Research Products International.

$MelB_{St}$ expression and purification

The plasmid pK95 ΔAH/$MelB_{St}$/$CHis_{10}$ (Pourcher et al., 1995; Guan et al., 2011) was used for the constitutive expression of the WT $MelB_{St}$ or $MelB_{St}$ mutants D55C or D59C containing a Cys in the position Asp55 or Asp59 (Ethayathulla et al., 2014), respectively. *E. coli* DW2 cells (*mel*A$^+$, *mel*B, and *lac*ZY) were used for protein overexpression (Pourcher et al., 1995). The cells were grown in Luria–Bertani broth supplemented with 50 mM KPi (pH 7.0), 45 mM $(NH_4)SO_4$, 0.5% glycerol, and 100 mg/L ampicillin. The protocols for membrane preparation and $MelB_{St}$ purification by cobalt-affinity chromatography after extracted in a detergent UDM have been described previously (Ethayathulla et al., 2014). MelB protein in 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.035% UDM, and 10% glycerol was concentrated and stored at −80°C. Protein samples were dialyzed against a specific assay buffer before a test.

Protein assay

The Micro BCA Protein Assay (Pierce Biotechnology, Inc.) was used for the protein concentration assay.

Na^+ stimulation constant ($K_{0.5(Na+)}$) on Trp→D^2G FRET

Steady-state fluorescence measurements were performed with an AMINCO-Bowman Series 2 Spectrometer with purified $MelB_{St}$ or $MelB_{St}$ mutants D55C or D59C in a Na^+-free buffer consisting of 20 mM Tris-HCl, pH 7.5, 0.035% UDM, 50 mM choline chloride (ChCl), and 10% glycerol at a protein concentration of 1 µM. The emission intensity was recorded at 490 nm using an excitation wavelength of 290 nm. After the addition of 10 µM D^2G, NaCl was consecutively added until no change in fluorescence intensity occurred. The Na^+ concentration at the end of titration was ∼50 mM for the WT and 200 mM for the mutants. Melibiose at oversaturating concentration was added after the end of the Na^+ titration to displace the bound D^2G. On a separate test, an identical volume of water instead of NaCl was used for the correction of dilution effect. For each addition, the intensities were recorded for 60 s, integrated, and averaged. Increases in fluorescence intensity are expressed as differential FRET ($_{diff}$FRET; the difference before and after the addition of NaCl) and corrected by the dilution effect. The $_{diff}$FRET values were plotted against the Na^+ concentration, and the Na^+ activation constant (the Na^+ concentration for the half-maximal $_{diff}$FRET [$K_{0.5(Na+)}$]) was determined by fitting a hyperbolic function to the data (OriginPro).

Na^+ binding and melibiose binding by ITC

ITC measurements were performed in a Nano Isothermal Titration Calorimeter (TA Instruments), and all data were collected at 25°C. $MelB_{St}$ (40–100 µM) was placed in the sample cell with a reaction volume of 163 µl. The titrant and titrand were prepared in the same dialysis buffers, degassed for 15 min using a TA Instruments Degassing Station model 6326. 2-µl aliquots were injected incrementally into the sample cell at an interval of 300 s with constant stirring at 250 rpm.

To determine Na^+ binding to $MelB_{St}$, the protein samples were dialyzed against Na^+-free buffer (20 mM Tris-HCl unless defined otherwise, 50 mM ChCl, 10% glycerol, and 0.035% UDM) at a given pH in the absence or presence of ∼20–50 mM melibiose. The Na^+ contamination is calculated to be lower than 5 µM. NaCl samples at concentrations of 1 to 20 mM were dissolved in a buffer matching the $MelB_{St}$ buffer and injected incrementally into the sample cell containing $MelB_{St}$. To determine melibiose binding in the absence or presence of Na^+, the melibiose solutions (10 mM or 80 mM) were buffer matched with the $MelB_{St}$ sample buffer in the absence or presence of 100 mM NaCl and placed in the syringe.

ITC data processing was performed using the one-site independent binding model in the NanoAnalyze version 3.6.0 software provided by the ITC equipment. The total heat changes were subtracted from the heat of dilution elicited by last few injections, where no further binding occurred, and the corrected heat change was normalized and plotted against the molar ratio of titrant versus titrand, as previously described (Hariharan and Guan, 2014; Hariharan et al., 2015, 2016). The association constant (K_a) and the change in enthalpy (ΔH) were determined by fitting the data with a one-site independent-binding model. The binding stoichiometry (N) was fixed to 1 because it is a known parameter, which can restrain the data fitting and achieve more accurate results (Turnbull and Daranas, 2003). $K_d = 1/K_a$; $\Delta G = -RT \ln K_a$, where R is the gas constant (8.315 J/mol·K) and T is the absolute temperature.

Determination of absolute dissociation constants for Na^+ ($K_{D(Na+)}$) and H^+ ($K_{D(H+)}$)

The apparent K_d for Na^+ binding ($K_{d(Na+)}$) in the absence or presence of 20 mM melibiose was determined in a pH range of 5.55 to 8.45 in one of the following buffers: 20 mM Tris-HCl, MES-TMAOH, potassium phosphate (KPi), Bis-Tris-HCl, or ACES-TMAOH. The apparent $K_{d(Na+)}$ versus H^+ concentration fit linearly, suggesting competition between Na^+ and H^+ for a common cation-binding site. Thus, values for the absolute $K_{D(Na+)}$ and absolute $K_{D(H+)}$ can be derived from a linear regression (Leone et al., 2015) based on the equation $K_{d(Na+)} = K_{D(Na+)}\{1+ [H^+]/K_{D(H+)}\}$. On this $K_{d(Na+)}$ versus H^+ concentrations plot, the y intercept (i.e., H^+ concentra-

tion = 0) corresponds the absolute $K_{D(Na+)}$, and the x intercept (i.e., Na^+ concentration = 0 and then $K_{d(Na+)} = 0$) corresponds to the absolute $K_{D(H+)}$. $pK_a = -\log K_{D(H+)}$.

Determination of the binding stoichiometry of Na^+, H^+, and $MelB_{St}$

ITC was used to determine the binding enthalpies for Na^+ ($\Delta H_{ITC(Na+)}$) at three pHs (6.25, 7.45, and 8.2) in five buffer systems. KPi, HEPES-TMAOH, and Tris-HCl buffers were used to test the buffer effect on $\Delta H_{ITC(Na+)}$ at pH 7.45 or 8.2. The KPi, MES-TMAOH, and ACES-TMAOH buffers were used for the test at pH 6.25, which is the pK_a value for $MelB_{St}$. The selections for these buffers are mainly based on their protonation enthalpy ($\Delta H_{(H+)}$) values and lesser Na^+ contamination. The $-\Delta H_{(H+)}$ values for phosphate buffer, MES-TMA OH, HEPES-TMAOH, ACES-TMAOH, and Tris-HCl are 3.6, 14.8, 20.4, 30.43, and 47.4 kJ/mol, respectively (Goldberg et al., 2002; Bianconi, 2003). Purified $MelB_{St}$ samples were dialyzed against a specific Na^+-free buffer system containing 50 mM ChCl, 0.035% UDM and 10% glycerol before ITC measurements. The ITC-determined $\Delta H_{ITC(Na+)}$ values were plotted against the corresponding standards $-\Delta H_{(H+)}$ from each buffer. By fitting a linear function to the data, the negative sign of the slope indicates the release of H^+ by Na^+ binding, and the slope reflects the number of H^+ replaced by Na^+.

Statistics

An unpaired *t* test was used for data analysis. P-values <0.05 were considered statistically significant.

RESULTS

Determination of Na^+ binding by Trp→D^2G FRET

Trp emission wavelength of MelB overlaps with the excitation wavelength of a dansyl group. Using an excitation wavelength of 290 nm, we detect the FRET from Trp→dansyl moiety on a fluorescent sugar analogue D^2G bound to the galactoside-binding site of MelB (Maehrel et al., 1998; Guan et al., 2011, 2012). Na^+ stimulates Trp→D^2G FRET, and the differential intensity ($_{diff}$FRET) induced by Na^+ is concentration-dependent and saturable. The K_d for D^2G binding to $MelB_{St}$ in membrane vesicles is ~10 µM in the presence of Na^+ and was not determined in the absence of Na^+ because of poor D^2G affinity (Guan et al., 2011). The increase in the FRET signal induced by Na^+ is due mainly to the increase in the number of D^2G molecules bound to $MelB_{St}$. This method has been widely used to determine Na^+ binding in the WT $MelB_{St}$ and $MelB_{Ec}$ and their mutants (Cordat et al., 1998; Maehrel et al., 1998; Meyer-Lipp et al., 2006; Ganea et al., 2011; Guan et al., 2011, 2012; Jakkula and Guan, 2012; Amin et al., 2014). Most of these reported tests were performed with membrane preparations, either right-side-out or inside-out membrane vesicles or reconstituted proteoliposomes (Maehrel et al., 1998; Guan et al., 2011; Jakkula and Guan, 2012). Notably, $MelB_{Ec}$ purified with conventional detergents, such as DDM, does not bind either D^2G or melibiose, but sugar binding has been detected using these new detergents with strong stabilizing capabilities (Amin et al., 2015; Sadaf et al., 2016; Das et al., 2017; Hussain et al., 2017). Because of greater stability, $MelB_{St}$ in most detergent solutions binds sugar substrates, so the Trp→D^2G FRET assay has been used to determine the melibiose binding to purified $MelB_{St}$ in the presence of Na^+ or Li^+ (Ethayathulla et al., 2014; Amin et al., 2015). However, there is no study showing whether the purified $MelB_{St}$ also binds Na^+.

A "Na^+-free" $MelB_{St}$ sample in 20 mM Tris-HCl, pH 7.45, 50 mM ChCl, 10% glycerol, and 0.035% UDM is stable and can be used for measuring the $_{diff}$FRET stimulated by Na^+. We recorded emission at 490 nm during stepwise additions of sugar and Na^+ (Fig. 2 a). 10 µM D^2G (near the K_d value in the presence of Na^+) was added at the 1-min time point (Fig. 2, red arrows), the fluorescence intensity was recorded for 1 min, and then NaCl was incrementally added up to a final concentration of ~50 mM (Fig. 2 a, black arrows). Finally, melibiose at oversaturating concentration was added into the same solution to displace the bound D^2G, and the decrease in fluorescence intensity after the addition of melibiose reflects the magnitude of Trp→D^2G FRET (Fig. 2, green arrows). Na^+ stimulation of the Trp→D^2G FRET was observed with purified $MelB_{St}$ (Fig. 2 a). The Na^+ activation constant ($K_{0.5(Na+)}$) was determined by fitting a hyperbolic function to the data. The resulting value of 0.99 mM for $K_{0.5(Na+)}$ (Fig. 2 b) is similar to the value obtained with membrane vesicles (Guan et al., 2011; Jakkula and Guan, 2012). The Na^+-binding affinity in the absence of sugar cannot be obtained by this method, so development of a direct Na^+ binding assay was necessary.

Structural and functional studies indicate that Asp55 and Asp59 on helix II may coordinate Na^+ (Fig. 1; Pourcher et al., 1993; Zani et al., 1994; Granell et al., 2010; Ethayathulla et al., 2014). Two $MelB_{St}$ single-site mutants (D55C and D59C), in which a Cys residue replaces an Asp residue in positions 55 or 59, respectively, were purified, and the Na^+-binding assay was tested by Trp→D^2G FRET. Na^+ stimulation was absent in both mutants, even with high Na^+ concentrations (Fig. 2, c and d) or D^2G concentration (not depicted). However, the data also show that there was no melibiose displacement in both cases, which underscores the need for a direct Na^+-binding assay.

ITC measurements of Na^+ binding in the absence or presence of melibiose

A Nano ITC device was used for data collection. Positive curves denote heat release (exothermic reactions).

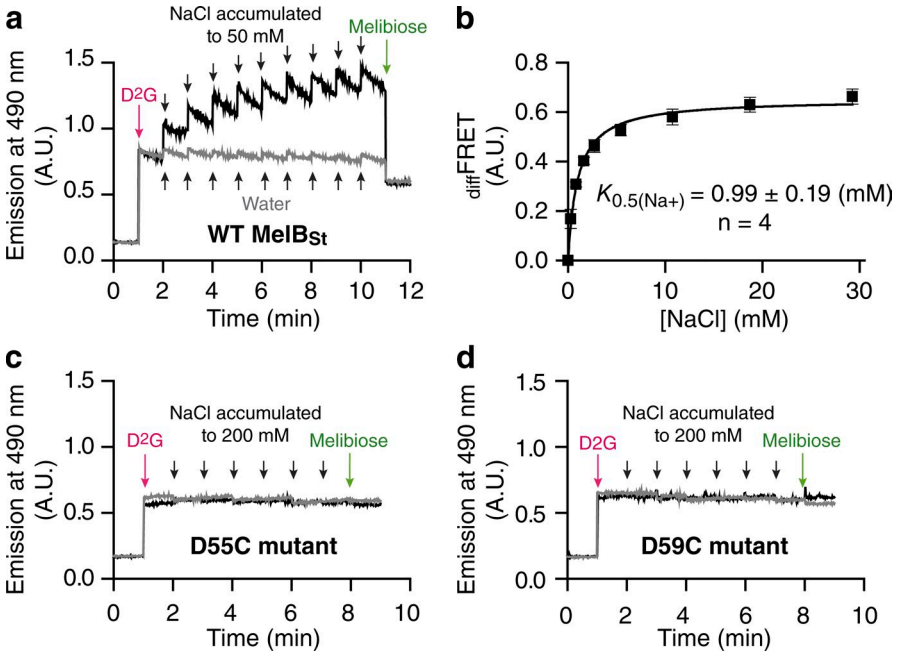

Figure 2. **Na^+ stimulation constant of the Trp→D^2G FRET ($K_{0.5(Na+)}$) with $MelB_{St}$ WT and mutants D55C and D59C.** The FRET signals from Trp residues of $MelB_{St}$ to the dansyl moiety of a fluorescent sugar (D^2G) in response to increasing Na^+ concentration were measured as described in Materials and methods. (a, c, and d) The purified WT $MelB_{St}$ (a) or $MelB_{St}$ single-site mutants D55C (c) or D59C (d) in a Na^+-free buffer containing 20 mM Tris-HCl, pH 7.5, 50 mM ChCl, 0.035% UDM, and 10% glycerol were adjusted to a protein concentration of 1 µM. D^2G in 20% DMSO was added at 1 min (red arrows) at a concentration of 10 µM (K_d value for D^2G binding to $MelB_{St}$ in the presence of Na^+). NaCl solutions of increasing concentrations were consecutively added, up to a final concentration of ~50 mM for the WT $MelB_{St}$ and ~200 mM for the mutants (black arrows). Finally, melibiose was added at an oversaturating concertation (green arrows). In control experiments, identical water volumes, instead of NaCl solution, was used (gray arrows) to control for sample dilution. Data collection and correction were as described in Materials and methods. (b) An increase in FRET intensity is expressed as $_{diff}$FRET (the difference before and after the addition of NaCl), and the value for $K_{0.5(Na+)}$ was determined by fitting a hyperbolic function to the $_{diff}$FRET versus Na^+ concentration. Error bars, standard error (SEM); the number of tests = 4.

Both NaCl solutions and purified $MelB_{St}$ samples were prepared in a Na^+-free buffer containing 20 mM Tris-HCl, pH 7.45, 50 mM ChCl, 10% glycerol, and 0.035% UDM. NaCl solutions were injected into the ITC sample cell containing $MelB_{St}$ at 25°C, and heat release and the exothermic titration thermogram were obtained (Fig. 3 a, black). As a control, parallel experiments with buffer present in the sample cell without protein yielded small exothermic peaks (Fig. 3 a, dark yellow), supporting that the thermogram is generated by Na^+ binding to $MelB_{St}$. Measurements with the two $MelB_{St}$ mutants (D55C and D59C) reveal nearly flat thermograms, and the heat releases are much smaller and indistinguishable from the buffer controls (Fig. 3, b and c), indicating that Na^+ binding in both mutants is dramatically decreased. Collectively, these experiments strongly indicate that the Na^+ titration curve shown in Fig. 3 a originates from Na^+ binding to the cation site on $MelB_{St}$.

The normalized heat change was plotted against the molar ratio of the titrant Na^+ to the titrand $MelB_{St}$ (Fig. 3 a, top/right, blue). The binding isotherm is not sigmoidal, even after exhaustive tests. To increase the fitting accuracy, the stoichiometric number of 1 for Na^+ binding to MelB was used to fit the data with a single-site independent binding model. The results are similar to those obtained without fixing the n value. The apparent K_d for Na^+ binding to $MelB_{St}$ ($K_{d(Na+)}$) in the absence of galactoside at pH 7.45 and 25°C is ~0.64 mM (Fig. 3 a and Table 1), and ΔG is −18.24 kJ/mol. The result is close to the $K_{0.5(Na+)}$ value for Na^+ stimulation on the Trp→D^2G FRET (Fig. 2). When the Na^+ binding measurements were performed in the presence of 50 mM melibiose (Fig. 3 d), the apparent $K_{d(Na+)}$ value decreased by eightfold, from 0.64 mM to 0.08 mM, and ΔG changed from −18.24 to −23.52 kJ/mol, yielding the thermodynamic coupling free energy ($\Delta\Delta G$) of −5.28 kJ/mol (Table 1). This result indicates that the Na^+ binding affinity is increased by approximately eightfold by melibiose binding to $MelB_{St}$.

Melibiose binding in the absence or presence of Na^+

Using ITC measurements, the K_d for melibiose binding to $MelB_{St}$ in the Na^+-free buffer at pH 7.45 and 25°C is 9.28 mM and ΔG is −11.60 kJ/mol (Fig. 3 e and Table 1). In the presence of Na^+ (Fig. 3 f), the melibiose-binding affinity was substantially increased; the K_d value was decreased by 8.51-fold from 9.28 mM to 1.09 mM, yielding the $\Delta\Delta G$ value of −5.33 kJ/mol, which is virtually equal to the coupling free energy determined for Na^+ binding in the presence of melibiose (Table 1 and see Fig. 6). This result indicates that melibiose binding to $MelB_{St}$ is increased by eightfold by Na^+ binding. Thus, the coupling binding free energy had similar values for both processes.

Table 1. **Binding cooperativity of melibiose and Na^+ to $MelB_{St}$**

Parameter	Na^+ binding (NaCl in syringe)		Melibiose effect on Na^+ binding	Melibiose binding (melibiose in syringe)		Na^+ effect on melibiose binding
	No sugar (n = 4)	With melibiose (n = 2)		No NaCl (n = 3)	With NaCl (n = 5)	
Apparent K_d (mM) or affinity increase	0.64 ± 0.02[a]	0.08 ± 0.01	8-fold	9.28 ± 0.23	1.09 ± 0.06	8.5-fold
ΔG or $\Delta\Delta G$ (kJ/mol)	−18.24 ± 0.08	−23.52 ± 0.13	−5.28 ($P < 0.05$)[b]	−11.60 ± 0.06	−16.93 ± 0.14	−5.33 ($P < 0.05$)

ITC data were collected at pH 7.45 and 25°C with $MelB_{St}$ in sample cell as described in Materials and methods. ΔG, binding free energy; $\Delta\Delta G$, thermodynamics coupling free energy. n = number of test from a total of 5 different batches of $MelB_{St}$ purification.
[a]Standard error (SEM).
[b]Unpaired t test.

Determination of the absolute dissociation constants for Na^+ ($K_{D(Na+)}$) and H^+ ($K_{D(H+)}$)

It has been shown that Na^+ and Li^+ compete for a common binding site on $MelB_{St}$ (Guan et al., 2011). To study the competition between Na^+ and H^+, Na^+ binding at pH values ranging from 5.5 to 8.45 was measured by ITC, in the absence of melibiose, at 25°C. The data show that the apparent $K_{d(Na+)}$ value increases (i.e., the affinity for Na^+ decreases) linearly with increasing H^+ concentration (Fig. 4, open circles), supporting the idea that Na^+ and H^+ compete for a common binding site. Thus, it is important to determine the absolute dis-

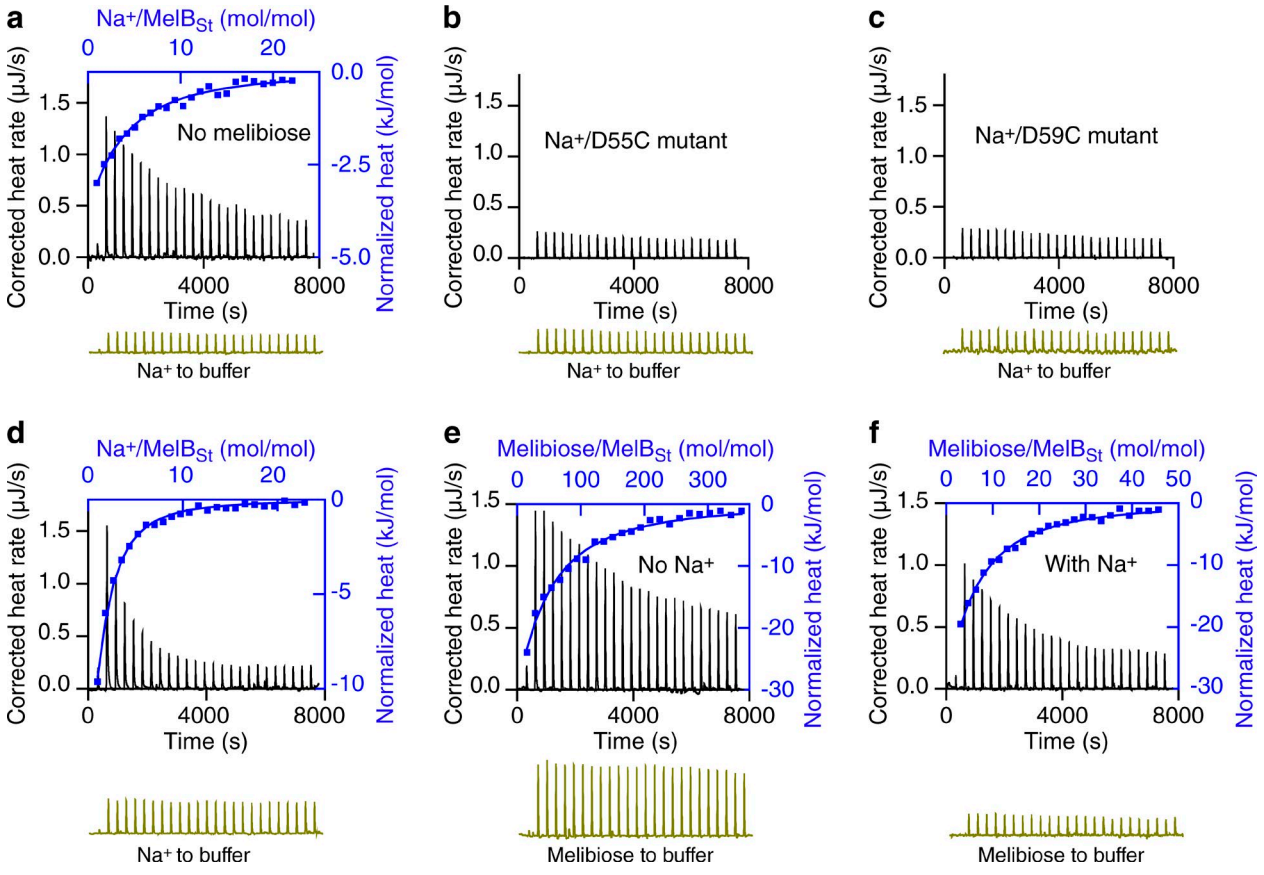

Figure 3. **Cooperative binding of Na^+ and melibiose to $MelB_{St}$.** (a–f) ITC was used to determine the binding of Na^+ (a–d) or melibiose (e and f) to $MelB_{St}$ at 25°C. Data collection was performed with a Nano ITC instrument at 25°C as described in Materials and methods. Titrant and titrand samples were subjected to buffer matching and degassing before each test. The samples containing $MelB_{St}$ (a and d–f), or $MelB_{St}$ mutants D55C (b) or D59C (c), at a protein concentration of 80 μM were placed in the sample cell. NaCl samples (5 mM) in the absence or presence of 50 mM melibiose placed in the syringe were injected into the $MelB_{St}$ samples in the absence or presence of 50 mM melibiose or corresponding buffers without protein (controls, bottom of each panel, dark yellow). The melibiose binding to $MelB_{St}$ was measured in the absence or presence of 100 mM NaCl by placing melibiose solutions (10–80 mM) in the syringe. The normalized heat changes (kJ/mol) were plotted against the $Na^+/MelB_{St}$ (a and d; top/right axis, blue curves) or melibiose/$MelB_{St}$ molar ratio (e and f; top/right axis, blue curves), and fitted with a one-site independent-binding model with fixed stoichiometry (n = 1).

Table 2. **Absolute dissociation constants for Na^+ or H^+ binding to $MelB_{St}$**

Parameter	No sugar	With melibiose
Absolute $K_{D(Na+)}$ (mM)[a]	0.54 ± 0.03[b]	0.09 ± 0.02
Absolute $K_{D(H+)}$ (μM)	0.56 ± 0.03	0.26 ± 0.02
Cation selectivity ratio (slope = $K_{D(Na+)}/K_{D(H+)}$)	964	346
pK_a	6.25	6.59

[a]Data were extracted from Fig. 4.
[b]Standard error from curve fitting.

sociation constants for both cations $K_{D(Na+)}$ and $K_{D(H+)}$. As discussed in Materials and methods, the absolute $K_{D(Na+)}$ value corresponds to the y-intercept gained from the extrapolation of the linear fit where the H^+ concentration is zero, and the absolute $K_{D(H+)}$ corresponds to the x-intercept (Fig. 4; Leone et al., 2015). The results show that the absolute $K_{D(Na+)}$ value for $MelB_{St}$ under the experimental condition is 0.54 ± 0.03 mM and the absolute $K_{D(H+)}$ value is 0.56 ± 0.03 μM (Table 2). Calculated from the $K_{D(H+)}$ value by the equation pK_a = −log $K_{D(H+)}$, the pK_a of the cation-binding site in $MelB_{St}$ is 6.25 in the absence of melibiose. Therefore, the cation selectivity ratio ($K_{D(Na+)}/K_{D(H+)}$) = slope = 540/0.56 = 964 (Fig. 4 and Table 2), indicating that $MelB_{St}$'s intrinsic selectivity for H^+ over Na^+ is almost 1,000-fold.

When similar measurements were performed in the presence of melibiose, the absolute $K_{D(Na+)}$ value was decreased from 0.54 ± 0.03 mM to 0.09 ± 0.02 mM (Fig. 4 and Table 2). Interestingly, the absolute $K_{D(H+)}$ decreased from 0.56 μM to 0.26 μM, only 2.1-fold increase for the H^+ affinity, and the pK_a changed from pH 6.25 to 6.59, an ∼0.3-unit increase (Table 2). Accordingly, the cation selectivity ratio was reduced from 964 to 346. These data clearly show that melibiose has a stronger cooperativity with Na^+ than with H^+. This is also implied from the nonparallel shift induced by melibiose on the $K_{d(Na+)}$ versus H^+ concentration curve (Fig. 4).

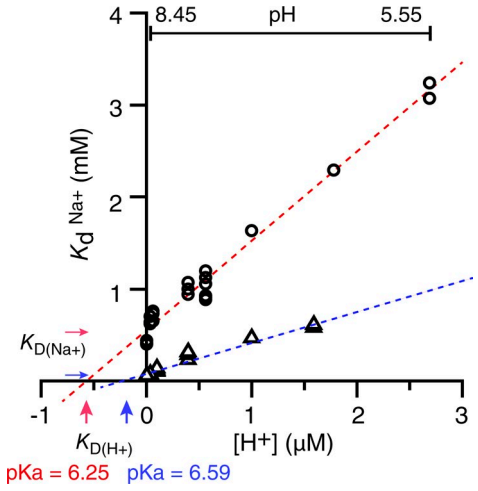

Figure 4. **Determination of the absolute dissociation constants for Na^+ ($K_{D(Na+)}$) and H^+ ($K_{D(H+)}$).** The apparent Na^+-binding dissociation constants ($K_{d(Na+)}$) in the absence or presence of melibiose in the pH range from 5.55 to 8.45 were determined by ITC at 25°C. 1–20 mM NaCl was placed in the syringe. All apparent $K_{d(Na+)}$ data were plotted against the H^+ concentration without averaging and fitted by a linear function; the absolute $K_{D(Na+)}$ value corresponds to the y-axis intercept, and the absolute $K_{D(H+)}$ value corresponds to the x-axis intercept (see Materials and methods). pK_a = −log $K_{D(H+)}$.

Determination of stoichiometric ratios between Na^+ and H^+ and between H^+ and $MelB_{St}$

The heat changes caused by Na^+ binding may have several origins; one of them could be protonation of the reaction buffer if $MelB_{St}$ undergoes deprotonation during Na^+ binding. If so, the ITC-measured Na^+-binding enthalpy ($\Delta H_{ITC(Na+)}$) should be buffer dependent with an unchanged $\Delta G_{(Na+)}$. This is because the intrinsic protonation enthalpies ($\Delta H_{(H+)}$) of various buffer systems differ (i.e., the $\Delta H_{(H+)}$ values for phosphate and Tris-HCl buffer are −3.6 kJ/mol and −47.4 kJ/mol, respectively; Table 3; Goldberg et al., 2002; Bianconi, 2003). To determine how many H^+ were displaced by the binding of one Na^+, Na^+ binding in five buffer systems was performed at three pH including a pK_a for $MelB_{St}$ via ITC measurements. Overall, the results show that, at each pH, the apparent $K_{d(Na+)}$ or ΔG values are similar in different buffers, but the binding enthalpy $\Delta H_{ITC(Na+)}$ values vary (Fig. 5, a and b; and Table 3). The determined $\Delta H_{ITC(Na+)}$ values plotted against the standard protonation enthalpies of each buffer ($-\Delta H_{(H+)}$) yield linear relationships (Fig. 5 b).

At pH 7.45 in KPi, HEPES-TMAOH, or Tris-HCl buffer, a negative slope of ∼0.21 is obtained (Fig. 5 b). The negative sign of the slope suggests the release of H^+ from $MelB_{St}$, and the value of the slope correlates with the number of H^+ replaced by Na^+. Because there is only one Na^+ binding to the MelB cation site, this result suggests that only a portion of $MelB_{St}$ molecules are protonated at pH 7.45.

At pH 8.2, the majority of $MelB_{St}$ proteins should be deprotonated because the pK_a is 6.25 and no further change in Na^+ affinity was observed (Fig. 4); i.e., the $\Delta H_{ITC(Na+)}$ values measured with the three buffers at this pH should be similar, and this is in fact the case (Table 3), generating a nearly flat curve (Fig. 5 b). These data support the conclusion that the cation-binding site on $MelB_{St}$ is unprotonated at a pH greater than 8.2.

There is no suitable Na^+-free buffer system at an acidic pH where all MelB molecules are protonated. Taking advantage of the determined pK_a value of 6.25, the stoichiometry between H^+ and Na^+, as well as between H^+ and $MelB_{St}$, can be established by determining the slope at this specific pH. Using KPi, MES-TMAOH, and ACES-TMAOH buffers adjusted to pH 6.25, where the protonated and unprotonated $MelB_{St}$ levels are equal, the obtained slope is 0.48, which is very close to 0.5 (Fig. 5 b

Table 3. **Buffer effects of Na^+-binding enthalpy**

Buffer	Buffer pH	$-\Delta H_{(H+)}$[a]	$K_{d(Na+)}$ (mM)	$\Delta G_{(Na+)}$	$\Delta H_{ITC(Na+)}$	Slope
		kJ/mol	*mM*	*kJ/mol*	*kJ/mol*	
KPi	6.45 (n[b] = 2)	3.6	1.16 ± 0.04[c]	−16.76 ± 0.08	−38.22 ± 0.67	
MES-TMAOH	6.25 (n = 3)	14.8	0.90 ± 0.01	−17.38 ± 0.02	−45.70 ± 0.28	−0.48 ± 0.09
ACES-TMAOH	6.25 (n = 2)	30.43	0.99 ± 0.06	−17.16 ± 0.16	−51.37 ± 0.32	
KPi	7.45 (n = 3)	3.6	0.65 ± 0.02	−18.19 ± 0.09	−21.67 ± 0.34	
HEPES-TMAOH	7.45 (n = 3)	20.3	0.52 ± 0.03	−18.75 ± 0.12	−24.79 ± 0.23	−0.21 ± 0.03
Tris-HCl	7.45 (n = 4)	47.4	0.64 ± 0.02	−18.24 ± 0.08	−31.50 ± 0.63	
KPi	8.2 (n = 1)	3.6	0.72	−17.94	-18.06	
HEPES-TMAOH	8.2 (n = 2)	20.3	0.56 ± 0.01	−18.55 ± 0.11	−18.93 ± 0.12	−0.04 ± 0.01
Tris-HCl	8.2 (n = 2)	47.4	0.50 ± 0.01	−18.90 ± 0.05	−19.76 ± 0.26	

[a]Standards for the buffer protonation enthalpy (Goldberg et al., 2002).
[b]n = number of tests.
[c]Standard error (SEM).

and Table 3). This result confirms that binding of one Na^+ displaces one proton, and the binding stoichiometry among H^+, Na^+, and $MelB_{St}$ is unity.

Melibiose binding to unprotonated $MelB_{St}$

The results of Na^+ binding at pH 8.2 (Fig. 5 b) indicate that at pH 8.2 or above, $MelB_{St}$ is nearly completely unprotonated. To analyze the melibiose binding to unprotonated $MelB_{St}$, the K_d values for melibiose in the absence or presence of NaCl at pH 8.45 were determined by ITC to be 9.42 ± 0.28 mM (SEM, n = 3) and 0.95 ± 0.06 mM (SEM, n = 4), respectively. Accordingly, the ΔG values are −11.57 and −17.26 kJ/mol, respectively.

DISCUSSION

It has been well documented for both $MelB_{Ec}$ and $MelB_{St}$ that Na^+ stimulates galactoside binding and transport (Bassilana et al., 1985; Wilson and Ding, 2001; Guan et al., 2011); however, how the Na^+-binding affinity is affected by melibiose is not clear, because previous quantitative measurements of Na^+ binding were based on sugar binding (e.g., the Na^+ stimulation of D^2G FRET; Fig. 2; Maehrel et al., 1998; Guan et al., 2011) or [^{3}H]α-NPG binding (Damiano-Forano et al., 1986). Here, we present detailed data from direct measurements of Na^+ and melibiose binding to $MelB_{St}$, which allow us to construct thermodynamic cycles to analyze the formation of the Na^+–MelB–melibiose ternary complex (Fig. 6). The thermodynamic coupling free energy ($\Delta\Delta G$) values (i.e., the differences in binding free energies [ΔG]) obtained from binding of one component in the absence or presence of the other are quite similar (approximately −5 kJ/mol or eightfold increases in affinity); therefore, the binding of Na^+ and melibiose to $MelB_{St}$ is thermodynamically cooperative (Table 1 and Fig. 6). Helix IV in the N-terminal domain contains residues critical for the binding of both Na^+ and galactoside, and both binding sites are physically connected (Fig. 1 b; Ethayathulla et al., 2014), which may be the structural basis for the positive cooperativity of galactoside and cation binding in MelB.

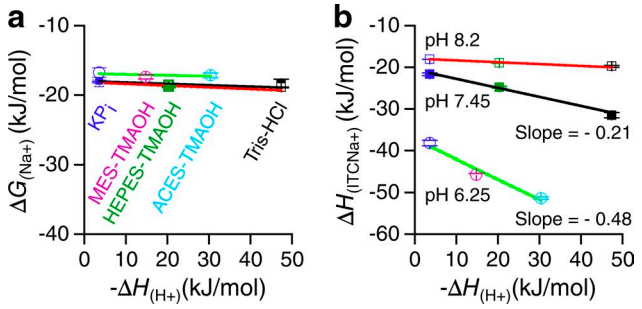

Figure 5. **Buffer effects on Na^+ binding enthalpy ($\Delta H_{ITC(Na+)}$).** ITC was used to determine Na^+ binding $\Delta H_{ITC(Na+)}$ to $MelB_{St}$ in a total of five buffer systems in the absence of melibiose at pH 6.25, 7.45, and 8.2 at 25°C. 5–15 mM NaCl was placed in the syringe. (a) ΔG versus standard buffer $-\Delta H_{(H+)}$ plot. (b) $\Delta H_{ITC(Na+)}$ determined by ITC versus buffer standard $-\Delta H_{(H+)}$ plot, with linear fits to the data.

Free-energy changes in a thermodynamic cycle are state functions independent of their paths because of thermodynamic equilibrium. This is the case for the binding of the two cotransporting substrates, Na^+ and galactoside, to the symporter $MelB_{St}$. The sum of ΔG at −35 kJ/mol from the path [A→B→D] (i.e., from the empty state [A] to the binary Na^+-bound state [B] and then from this to the Na^+- and melibiose-bound ternary complex [D]) is nearly equal to that from the path [A→C→D] (i.e., binding of melibiose before Na^+ binding; Fig. 6). Thus, binding of both substrates releases free energy of ∼35 kJ/mol, and it is likely that the released energy fuels the conformational changes required for transport.

MelB also catalyzes H^+-coupled melibiose transport, so it is important to determine its affinity to H^+ and protonation status. Analysis of the competitive binding between H^+ and Na^+ by measuring the apparent $K_{d(Na+)}$ at a range of pH values indicates that the pK_a for $MelB_{St}$ cation site is 6.25, and the absolute $K_{D(H+)}$ is 0.56 µM.

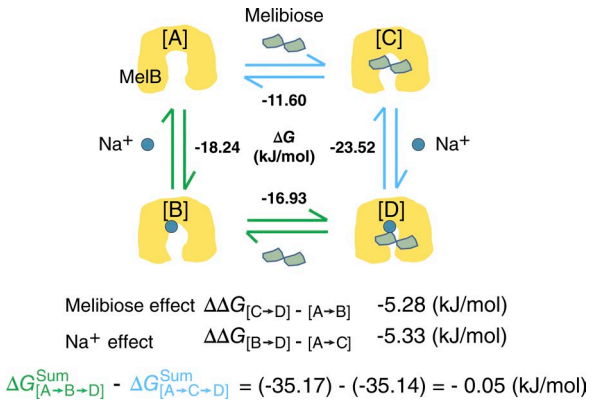

Figure 6. **Thermodynamic cycle for the formation of the melibiose–Na^+–$MelB_{St}$ ternary complex at pH 7.5.** Symbols for $MelB_{St}$, melibiose or Na^+ are indicated. State [A], cartoon for $MelB_{St}$ from the crystal structure (Protein Data Bank accession no. 4M64) represents a periplasmic side-open apo state. States [B] and [C] represent a Na^+-bound or melibiose-bound binary complex, respectively. State [D] represents a Na^+–melibiose–$MelB_{St}$ ternary complex. ΔG values are listed for each binding step. The thermodynamic coupling free energy ($\Delta\Delta G$) reflects the effect of one substrate on the binding of the other. ΔG^{sum}, the sums of free energies for the path [A→B→D] or [A→C→D]. The difference in the ΔG from paths [A→B→D] and [A→C→D] is close to zero.

This result is in agreement with data previously obtained from $MelB_{Ec}$ by an indirect assay (i.e., Na^+ stimulation of [^{3}H]α-NPG binding; Damiano-Forano et al., 1986). Melibiose and H^+ are also cooperative; however, the galactoside effect on MelB affinity for H^+ is smaller (i.e., the pK_a increases by only ∼0.3 units, which is equivalent to a twofold decrease in $K_{D(H+)}$; Table 2). Compared with the much greater effect on Na^+ binding, it is clear that melibiose cooperates with Na^+ stronger than with H^+, although the protein is intrinsically selective for H^+.

ITC is also a useful tool for the study of protein protonation and stoichiometry between H^+ and its competitive cation. The observed negative linear relationships between the Na^+ binding enthalpy and the buffer intrinsic protonation enthalpy at pH 6.25 and pH 7.45 suggest that buffer protonation occurs upon Na^+ binding (i.e., $MelB_{St}$ releases H^+ when it binds Na^+). The demonstration of competitive binding between Na^+ and H^+ (Fig. 4), as well as between Na^+ and Li^+ (Guan et al., 2011), indicates that all three cations (Na^+, H^+, and Li^+) compete for a common binding site in $MelB_{St}$. A similar conclusion was previously drawn in $MelB_{Ec}$ (Bassilana et al., 1987). Furthermore, the slope of −0.48 is obtained from the linear fit when the tests were done at a buffer pH equal to the pK_a value, suggesting that the binding of one Na^+ displaces one H^+ from the cation binding site per $MelB_{St}$, because at this pH, only half of the MelB molecules are protonated. At pH 7.45, the slope value of −0.21 is obtained, which suggests that only ∼20% $MelB_{St}$ proteins are protonated at this pH. These data may explain the results on lower transport rates and the lower melibiose binding at pH 7.5 in the absence of Na^+ or Li^+. The initial rate and the steady-state level of H^+-coupled melibiose transport were ∼30% of Na^+-coupled melibiose transport (Guan et al., 2011). With an empty cation site, $MelB_{St}$ still binds melibiose, but with very low affinity. Thus, at pH 7.5, only a small portion of $MelB_{St}$ molecules are able to perform the symport function, which is consistent with the notion that concurrent binding of both substrates is required for the symport process (Yousef and Guan, 2009).

The study of competitive binding between Na^+ and H^+ yields an absolute $K_{D(Na+)}$ of 0.54 mM and $K_{D(H+)}$ of 0.56 µM; thus, the Na^+ affinity is ∼1,000-fold lower than that for H^+ in the absence of melibiose, and the $MelB_{St}$ cation site is intrinsically selective for H^+ over Na^+. Even in the presence of melibiose, the H^+ selectivity persists, with a greater than 300-fold higher affinity. In $MelB_{Ec}$, previous studies yielded an $K_{D(Na)}$ of 0.3 mM and a pK_a of 6.3, also suggesting that the cation site in $MelB_{Ec}$ is intrinsically selective for H^+ (Damiano-Forano et al., 1986). Such a selectivity feature has been recognized in several ATP synthases (Krah et al., 2010; Schlegel et al., 2012; Leone et al., 2015), such as in the F-type ATP synthase of *Ilyobacter tartaricus*, where the ion-driven membrane rotor exhibits very similar value of $K_{D(Na+)}$ (0.29 mM) and pK_a (6.5; Leone et al., 2015). Collectively, these studies from radically different membrane transporters reveal a common principle of cation selectivity in membrane proteins with a common cation site used by both Na^+ and H^+. Although intrinsically selective for H^+, the availability of H^+ in physiological environments (pH 7.5, [H^+] = 32 nM) is very low, and the availability of Na^+ is often high enough to ensure that Na^+ can effectively compete for the cation site. Such a cation site thus appears to have evolved for the effective use of the metal cation Na^+ under physiological conditions. Because the living environments for bacteria are not always Na^+ rich, an elevated pK_a value for Asp residues in the cation site allows the bacteria to use H^+ as the coupling cation for melibiose transport, albeit with less efficiency. This elegant mechanism secures MelB's important biological function.

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Author contributions: L. Guan conceived and directed this research. P. Hariharan performed all experiments and data process-

ing. L. Guan and P. Hariharan analyzed the results, and L. Guan wrote the manuscript.

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