

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

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The Na<sup>+</sup>-coupled melibiose symporter MelB, which can also be coupled to H<sup>+</sup> or Li<sup>+</sup> 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<sub>St</sub>) 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<sup>+</sup> and melibiose binding to MelB<sub>St</sub>, as well as protonation of this transporter. Studies of the thermodynamic cycle for the formation of the Na<sup>+</sup>-MelB<sub>St</sub>-melibiose ternary complex at pH 7.45 reveal that the binding of Na<sup>+</sup> and melibiose is cooperative. The binding affinity for one substrate (Na<sup>+</sup> 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<sup>+</sup>-binding enthalpy at three different pH values, including the pK<sub>a</sub> value of MelB, indicate that the binding of one Na<sup>+</sup> displaces one H<sup>+</sup> per MelB<sub>St</sub> molecule. In addition, the absolute dissociation constants for Na<sup>+</sup> and H<sup>+</sup>, determined by competitive binding, show that MelB<sub>St</sub> is selective for H<sup>+</sup> over Na<sup>+</sup> by ~1,000-fold at a pK<sub>a</sub> of 6.25. Thus, the Na<sup>+</sup> coupling in MelB<sub>St</sub> is based not on ion selectivity but on ion concentrations and competitive binding because of a much higher Na<sup>+</sup> concentration under physiological conditions. Such a selectivity feature seems to be common for membrane transport proteins that can bind both H<sup>+</sup> and Na<sup>+</sup> at a common site.

## 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<sup>+</sup> electrochemical gradient for their functions, but some members are able to couple solute transport to Na<sup>+</sup> 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<sup>+</sup> but also with Li<sup>+</sup> or H<sup>+</sup> (Tsuchiya and Wilson, 1978; Niiya et al., 1980; Bassilana et al., 1987; Guan et al., 2011); however, this symporter cannot transport sugars with K<sup>+</sup>, Rb<sup>+</sup>, or Cs<sup>+</sup> (Guan et al., 2011). We have determined the high-resolution x-ray 3-D crystal structure of *Salmonella typhimurium* MelB (MelB<sub>St</sub>) at a resolution of 3.35 Å (Ethayathulla et al., 2014). This

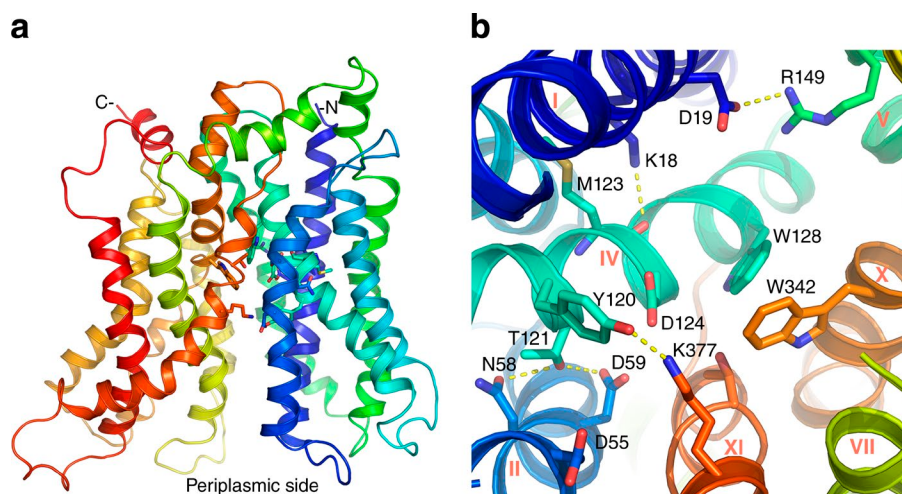
is the first high-resolution structure of a member of the MFS family that uses Na<sup>+</sup> as a coupling cation. MelB<sub>St</sub> was captured in two slightly different conformations (Ethayathulla et al., 2014). Like other MFS-fold transporters (Abramson et al., 2003; Huang et al., 2003; Guan and 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<sup>+</sup>, Li<sup>+</sup>, or H<sup>+</sup> 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<sub>Ec</sub>; 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

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Abbreviations used: ACES, N-(2-acetamido)-2-aminoethanesulfonic acid;  $\alpha$ -NPG,  $\alpha$ -nitrophenyl galactoside; D<sup>2</sup>G, 2'-(N-dansyl)aminoalkyl-1-thio- $\beta$ -D-galactopyranoside;  $\Delta$ FRET, differential FRET; FRET, fluorescence resonance energy transfer; ITC, isothermal titration calorimetry; MelB<sub>Ec</sub>, *Escherichia coli* melibiose permease; MelB<sub>St</sub>, *Salmonella typhimurium* melibiose permease; MFS, major facilitator superfamily; TMAOH, tetramethylammonium hydroxide; UDM, undecyl- $\beta$ -D-maltopyranoside.

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**Figure 1. X-ray crystal structure of MelB<sub>St</sub>.** See Protein Data Bank accession no. 4M64. (a) The overall fold of MelB<sub>St</sub> 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<sub>St</sub> and MelB<sub>Ec</sub>, all three Asp residues are required for Na<sup>+</sup> 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<sup>+</sup> (Granell et al., 2010). Functional studies with MelB<sub>Ec</sub> have shown that all three cations (Na<sup>+</sup>, Li<sup>+</sup>, and H<sup>+</sup>) compete for a common binding site (Lopilato et al., 1978; Damiano-Forano et al., 1986; Mus-Veteau et al., 1995). With MelB<sub>St</sub>, a common binding site for Na<sup>+</sup> or Li<sup>+</sup> 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<sup>+</sup> or Li<sup>+</sup>, 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<sup>+</sup>/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<sup>+</sup> or Li<sup>+</sup> (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<sup>+</sup> binding; most depend on binding of a sugar (e.g., Na<sup>+</sup> stimulation of [<sup>3</sup>H]α-nitrophenyl galactoside [[<sup>3</sup>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<sup>2</sup>G 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<sup>2</sup>G FRET and ITC to study Na<sup>+</sup> binding, determine the free energy for Na<sup>+</sup> and melibiose binding to MelB<sub>St</sub>, test the protonation status of MelB<sub>St</sub>, and study the competition between Na<sup>+</sup> and H<sup>+</sup> in the absence or presence of melibiose. The results show that the binding of Na<sup>+</sup> and melibiose is thermodynamically cooperative, providing insights into the coupling mechanism of this symporter. Furthermore, the binding stoichiometry of melibiose and Na<sup>+</sup> or H<sup>+</sup> was also determined, confirming the previous conclusion that MelB catalyzes stoichiometric translocation of a melibiose with a cation (Na<sup>+</sup>, Li<sup>+</sup>, or H<sup>+</sup>). Moreover, by determining the absolute dissociation constants for Na<sup>+</sup> and H<sup>+</sup>, we conclude that the use of Na<sup>+</sup> as coupling ion for sugar transport is based not on ion selectivity but on competitive binding under physiological conditions (i.e., with a Na<sup>+</sup> concentration five orders of magnitude higher than the H<sup>+</sup> 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<sup>2</sup>G, 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<sub>St</sub> expression and purification

The plasmid pK95 ΔAH/MelB<sub>St</sub>/CHis<sub>10</sub> (Pourcher et al., 1995; Guan et al., 2011) was used for the constitutive expression of the WT MelB<sub>St</sub> or MelB<sub>St</sub> mutants D55C or D59C containing a Cys in the position Asp55 or Asp59 (Ethayathulla et al., 2014), respectively. *E. coli* DW2 cells (*melA*<sup>+</sup>, *melB*, and *lacZY*) 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<sub>4</sub>)SO<sub>4</sub>, 0.5% glycerol, and 100 mg/L ampicillin. The protocols for membrane preparation and MelB<sub>St</sub> 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<sup>+</sup> stimulation constant ( $K_{0.5(\text{Na}^+)}$ ) on Trp→D<sup>2</sup>G FRET

Steady-state fluorescence measurements were performed with an AMINCO-Bowman Series 2 Spectrometer with purified MelB<sub>St</sub> or MelB<sub>St</sub> mutants D55C or D59C in a Na<sup>+</sup>-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<sup>2</sup>G, NaCl was consecutively added until no change in fluorescence intensity occurred. The Na<sup>+</sup> 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<sup>+</sup> titration to displace the bound D<sup>2</sup>G. 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 (diffFRET; the difference before and after the addition of NaCl) and corrected by the dilution effect. The diffFRET values were plotted against the Na<sup>+</sup> concentration, and the Na<sup>+</sup> activation constant (the Na<sup>+</sup> concentration for the half-maximal diffFRET [ $K_{0.5(\text{Na}^+)}$ ]) was determined by fitting a hyperbolic function to the data (OriginPro).

#### Na<sup>+</sup> 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<sub>St</sub> (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<sup>+</sup> binding to MelB<sub>St</sub>, the protein samples were dialyzed against Na<sup>+</sup>-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<sup>+</sup> 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<sub>St</sub> buffer and injected incrementally into the sample cell containing MelB<sub>St</sub>. To determine melibiose binding in the absence or presence of Na<sup>+</sup>, the melibiose solutions (10 mM or 80 mM) were buffer matched with the MelB<sub>St</sub> 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 ( $\Delta H$ ) 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<sup>+</sup> ( $K_{D(\text{Na}^+)}$ ) and H<sup>+</sup> ( $K_{D(\text{H}^+)}$ )

The apparent  $K_d$  for Na<sup>+</sup> binding ( $K_{d(\text{Na}^+)}$ ) 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  $K_{d(\text{Na}^+)}$  versus H<sup>+</sup> concentration fit linearly, suggesting competition between Na<sup>+</sup> and H<sup>+</sup> for a common cation-binding site. Thus, values for the absolute  $K_{D(\text{Na}^+)}$  and absolute  $K_{D(\text{H}^+)}$  can be derived from a linear regression (Leone et al., 2015) based on the equation  $K_{d(\text{Na}^+)} = K_{D(\text{Na}^+)}\{1 + [\text{H}^+]/K_{D(\text{H}^+)}\}$ . On this  $K_{d(\text{Na}^+)}$  versus H<sup>+</sup> concentrations plot, the y intercept (i.e., H<sup>+</sup> 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  $K_{D(H+)} \cdot pK_a = -\log K_{D(H+)}$ .

#### Determination of the binding stoichiometry of $Na^+$ , $H^+$ , and MelB<sub>St</sub>

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<sub>St</sub>. 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-TMAOH, 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<sub>St</sub> 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<sup>2</sup>G 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<sup>2</sup>G bound to the galactoside-binding site of MelB (Maehrel et al., 1998; Guan et al., 2011, 2012).  $Na^+$  stimulates Trp→D<sup>2</sup>G FRET, and the differential intensity (diffFRET) induced by  $Na^+$  is concentration-dependent and saturable. The  $K_d$  for D<sup>2</sup>G binding to MelB<sub>St</sub> in membrane vesicles is ~10  $\mu$ M in the presence of  $Na^+$  and was not determined in the absence of  $Na^+$  because of poor D<sup>2</sup>G 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<sup>2</sup>G molecules bound to MelB<sub>St</sub>. This method has been widely used to determine  $Na^+$  binding in the WT MelB<sub>St</sub> and MelB<sub>Ec</sub> 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 mem-

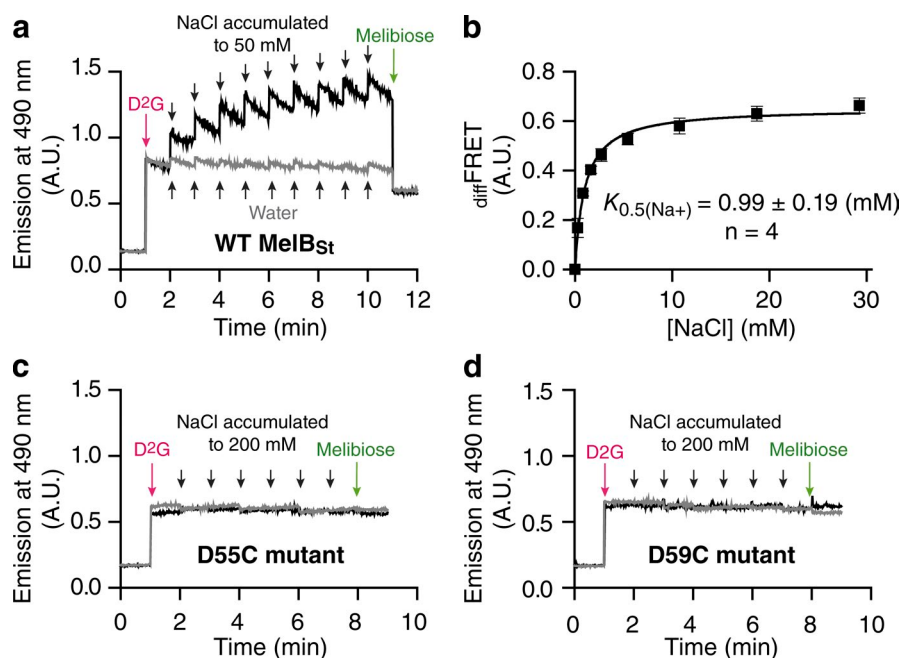
brane vesicles or reconstituted proteoliposomes (Maehrel et al., 1998; Guan et al., 2011; Jakkula and Guan, 2012). Notably, MelB<sub>Ec</sub> purified with conventional detergents, such as DDM, does not bind either D<sup>2</sup>G or melibiose, but sugar binding has been detected using these new detergents 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<sub>St</sub> in most detergent solutions binds sugar substrates, so the Trp→D<sup>2</sup>G FRET assay has been used to determine the melibiose binding to purified MelB<sub>St</sub> 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<sub>St</sub> also binds  $Na^+$ .

A “ $Na^+$ -free” MelB<sub>St</sub> 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 diffFRET stimulated by  $Na^+$ . We recorded emission at 490 nm during stepwise additions of sugar and  $Na^+$  (Fig. 2 a). 10  $\mu$ M D<sup>2</sup>G (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<sup>2</sup>G, and the decrease in fluorescence intensity after the addition of melibiose reflects the magnitude of Trp→D<sup>2</sup>G FRET (Fig. 2, green arrows).  $Na^+$  stimulation of the Trp→D<sup>2</sup>G FRET was observed with purified MelB<sub>St</sub> (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<sub>St</sub> 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<sup>2</sup>G FRET.  $Na^+$  stimulation was absent in both mutants, even with high  $Na^+$  concentrations (Fig. 2, c and d) or D<sup>2</sup>G 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).



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  $\text{diff FRET}$  (the difference before and after the addition of NaCl), and the value for  $K_{0.5(\text{Na}^+)}$  was determined by fitting a hyperbolic function to the  $\text{diff FRET}$  versus  $\text{Na}^+$  concentration. Error bars, standard error (SEM); the number of tests = 4.

Both NaCl solutions and purified MelB<sub>st</sub> samples were prepared in a Na<sup>+</sup>-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<sub>st</sub> 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<sup>+</sup> binding to MelB<sub>st</sub>. Measurements with the two MelB<sub>st</sub> 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<sup>+</sup> binding in both mutants is dramatically decreased. Collectively, these experiments strongly indicate that the Na<sup>+</sup> titration curve shown in Fig. 3 a originates from Na<sup>+</sup> binding to the cation site on MelB<sub>st</sub>.

The normalized heat change was plotted against the molar ratio of the titrant Na<sup>+</sup> to the titrand MelB<sub>st</sub> (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<sup>+</sup> 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<sup>+</sup> binding to MelB<sub>st</sub> ( $K_{d(\text{Na}^+)}$ ) in the ab-

sence of galactoside at pH 7.45 and 25°C is ~0.64 mM (Fig. 3 a and Table 1), and  $\Delta G$  is -18.24 kJ/mol. The result is close to the  $K_{0.5(\text{Na}^+)}$  value for Na<sup>+</sup> stimulation on the Trp→D<sup>2</sup>G FRET (Fig. 2). When the Na<sup>+</sup> binding measurements were performed in the presence of 50 mM melibiose (Fig. 3 d), the apparent  $K_{d(\text{Na}^+)}$  value decreased by eightfold, from 0.64 mM to 0.08 mM, and  $\Delta G$  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<sup>+</sup> binding affinity is increased by approximately eightfold by melibiose binding to MelB<sub>st</sub>.

#### Melibiose binding in the absence or presence of Na<sup>+</sup>

Using ITC measurements, the  $K_d$  for melibiose binding to MelB<sub>st</sub> in the Na<sup>+</sup>-free buffer at pH 7.45 and 25°C is 9.28 mM and  $\Delta G$  is -11.60 kJ/mol (Fig. 3 e and Table 1). In the presence of Na<sup>+</sup> (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<sup>+</sup> binding in the presence of melibiose (Table 1 and see Fig. 6). This result indicates that melibiose binding to MelB<sub>st</sub> is increased by eightfold by Na<sup>+</sup> binding. Thus, the coupling binding free energy had similar values for both processes.

Table 1. Binding cooperativity of melibiose and Na<sup>+</sup> to MelB<sub>St</sub>

| Parameter                                               | Na <sup>+</sup> binding (NaCl in syringe) |                                | Melibiose effect on Na <sup>+</sup> binding | Melibiose binding (melibiose in syringe) |                           | Na <sup>+</sup> effect on melibiose binding |
|---------------------------------------------------------|-------------------------------------------|--------------------------------|---------------------------------------------|------------------------------------------|---------------------------|---------------------------------------------|
|                                                         | No sugar ( <i>n</i> = 4)                  | With melibiose ( <i>n</i> = 2) |                                             | No NaCl ( <i>n</i> = 3)                  | With NaCl ( <i>n</i> = 5) |                                             |
| Apparent <i>K<sub>d</sub></i> (mM) or affinity increase | 0.64 ± 0.02 <sup>a</sup>                  | 0.08 ± 0.01                    | 8-fold                                      | 9.28 ± 0.23                              | 1.09 ± 0.06               | 8.5-fold                                    |
| Δ <i>G</i> or ΔΔ <i>G</i> (kJ/mol)                      | −18.24 ± 0.08                             | −23.52 ± 0.13                  | −5.28 ( <i>P</i> < 0.05) <sup>b</sup>       | −11.60 ± 0.06                            | −16.93 ± 0.14             | −5.33 ( <i>P</i> < 0.05)                    |

ITC data were collected at pH 7.45 and 25°C with MelB<sub>St</sub> in sample cell as described in Materials and methods. Δ*G*, binding free energy; ΔΔ*G*, thermodynamics coupling free energy. *n* = number of test from a total of 5 different batches of MelB<sub>St</sub> purification.

<sup>a</sup>Standard error (SEM).

<sup>b</sup>Unpaired *t* test.

### Determination of the absolute dissociation constants for Na<sup>+</sup> (*K<sub>D</sub>*(Na<sup>+</sup>)) and H<sup>+</sup> (*K<sub>D</sub>*(H<sup>+</sup>))

It has been shown that Na<sup>+</sup> and Li<sup>+</sup> compete for a common binding site on MelB<sub>St</sub> (Guan et al., 2011). To study the competition between Na<sup>+</sup> and H<sup>+</sup>, Na<sup>+</sup> 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<sub>d</sub>*(Na<sup>+</sup>) value increases (i.e., the affinity for Na<sup>+</sup> decreases) linearly with increasing H<sup>+</sup> concentration (Fig. 4, open circles), supporting the idea that Na<sup>+</sup> and H<sup>+</sup> compete for a common binding site. Thus, it is important to determine the absolute dis-

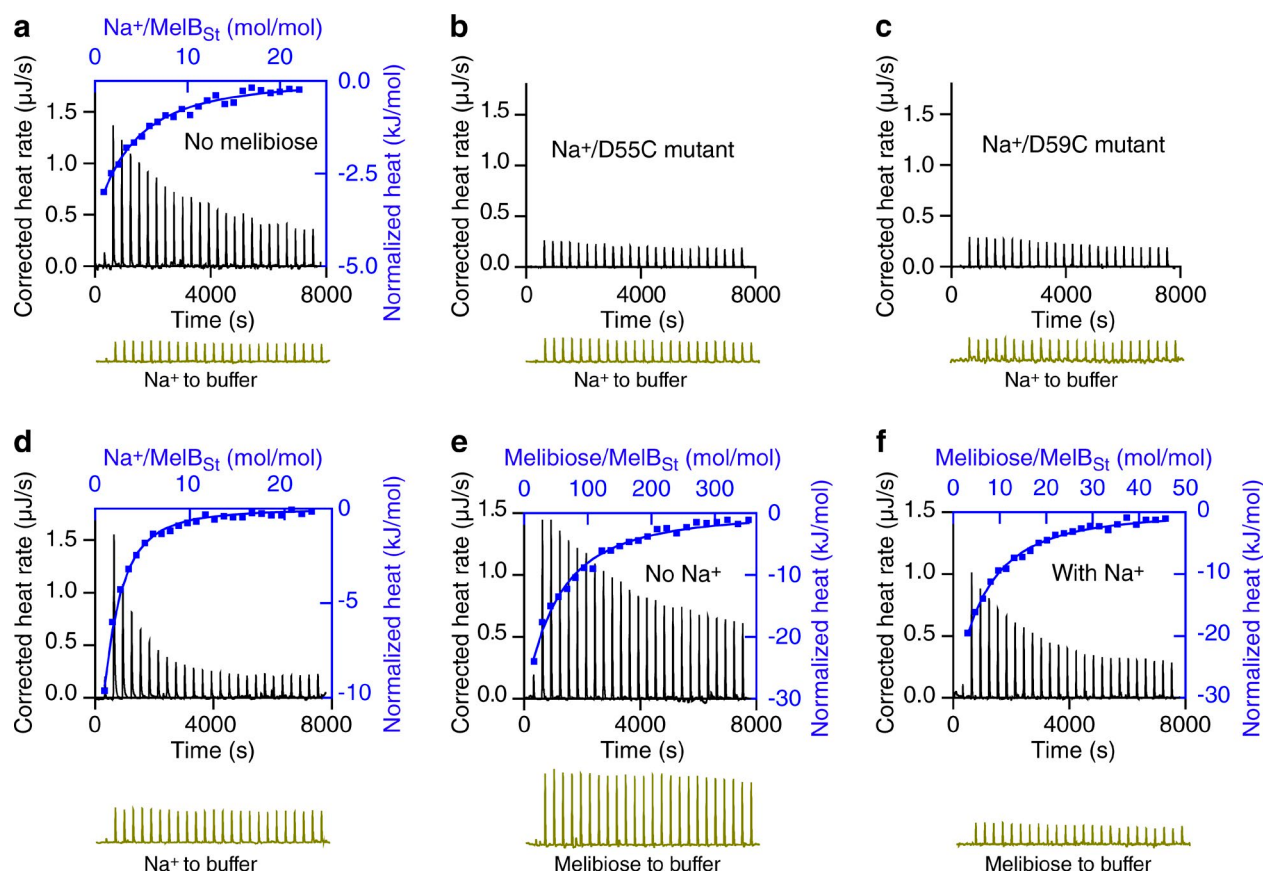


Figure 3. Cooperative binding of Na<sup>+</sup> and melibiose to MelB<sub>St</sub>. (a–f) ITC was used to determine the binding of Na<sup>+</sup> (a–d) or melibiose (e and f) to MelB<sub>St</sub> at 25°C. Data collection was performed with a Nano ITC instrument at 25°C as described in Materials and methods. Titrant and titrand samples were subjected to buffer matching and degassing before each test. The samples containing MelB<sub>St</sub> (a and d–f), or MelB<sub>St</sub> 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<sub>St</sub> 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<sub>St</sub> 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<sup>+</sup>/MelB<sub>St</sub> (a and d; top/right axis, blue curves) or melibiose/MelB<sub>St</sub> 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<sup>+</sup> or H<sup>+</sup> binding to MelB<sub>St</sub>

| Parameter                                                    | No sugar          | With melibiose  |
|--------------------------------------------------------------|-------------------|-----------------|
| Absolute $K_{D(Na^+)}$ (mM) <sup>a</sup>                     | $0.54 \pm 0.03^b$ | $0.09 \pm 0.02$ |
| Absolute $K_{D(H^+)}$ (μM)                                   | $0.56 \pm 0.03$   | $0.26 \pm 0.02$ |
| Cation selectivity ratio (slope = $K_{D(Na^+)}/K_{D(H^+)}$ ) | 964               | 346             |
| $pK_a$                                                       | 6.25              | 6.59            |

<sup>a</sup>Data were extracted from Fig. 4.

<sup>b</sup>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<sup>+</sup> 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<sub>St</sub> under the experimental condition is  $0.54 \pm 0.03$  mM and the absolute  $K_{D(H^+)}$  value is  $0.56 \pm 0.03$  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<sub>St</sub> 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<sub>St</sub>'s intrinsic selectivity for H<sup>+</sup> over Na<sup>+</sup> is almost 1,000-fold.

When similar measurements were performed in the presence of melibiose, the absolute  $K_{D(Na^+)}$  value was decreased from  $0.54 \pm 0.03$  mM to  $0.09 \pm 0.02$  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<sup>+</sup> 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<sup>+</sup> than with H<sup>+</sup>. This is also implied from the nonparallel shift induced by melibiose on the  $K_{d(Na^+)}$  versus H<sup>+</sup> concentration curve (Fig. 4).

#### Determination of stoichiometric ratios between Na<sup>+</sup> and H<sup>+</sup> and between H<sup>+</sup> and MelB<sub>St</sub>

The heat changes caused by Na<sup>+</sup> binding may have several origins; one of them could be protonation of the reaction buffer if MelB<sub>St</sub> undergoes deprotonation during Na<sup>+</sup> binding. If so, the ITC-measured Na<sup>+</sup>-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<sup>+</sup> were displaced by the binding of one Na<sup>+</sup>, Na<sup>+</sup> binding in five buffer systems was performed at three pH including a  $pK_a$  for MelB<sub>St</sub> via ITC measurements. Overall, the results show that, at each pH, the apparent  $K_{d(Na^+)}$  or  $\Delta G$  values are

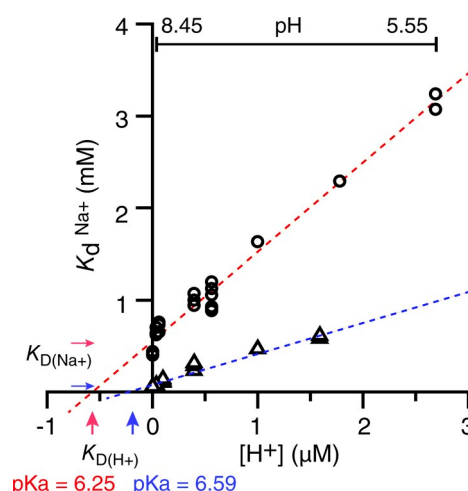


Figure 4. Determination of the absolute dissociation constants for Na<sup>+</sup> ( $K_{D(Na^+)}$ ) and H<sup>+</sup> ( $K_{D(H^+)}$ ). The apparent Na<sup>+</sup>-binding dissociation constants ( $K_{d(Na^+)}$ ) in the absence or presence of melibiose in the pH range from 5.55 to 8.45 were determined 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<sup>+</sup> 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).  $pK_a = -\log K_{D(H^+)}$ .

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<sup>+</sup> from MelB<sub>St</sub>, and the value of the slope correlates with the number of H<sup>+</sup> replaced by Na<sup>+</sup>. Because there is only one Na<sup>+</sup> binding to the MelB cation site, this result suggests that only a portion of MelB<sub>St</sub> molecules are protonated at pH 7.45.

At pH 8.2, the majority of MelB<sub>St</sub> proteins should be deprotonated because the  $pK_a$  is 6.25 and no further change in Na<sup>+</sup> 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<sub>St</sub> is unprotonated at a pH greater than 8.2.

There is no suitable Na<sup>+</sup>-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<sup>+</sup> and Na<sup>+</sup>, as well as between H<sup>+</sup> and MelB<sub>St</sub>, 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<sub>St</sub> 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<sup>+</sup>-binding enthalpy

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

<sup>a</sup>Standards for the buffer protonation enthalpy (Goldberg et al., 2002).

<sup>b</sup> $n$  = number of tests.

<sup>c</sup>Standard error (SEM).

and Table 3). This result confirms that binding of one Na<sup>+</sup> displaces one proton, and the binding stoichiometry among H<sup>+</sup>, Na<sup>+</sup>, and MelB<sub>St</sub> is unity.

#### Melibiose binding to unprotonated MelB<sub>St</sub>

The results of Na<sup>+</sup> binding at pH 8.2 (Fig. 5 b) indicate that at pH 8.2 or above, MelB<sub>St</sub> is nearly completely unprotonated. To analyze the melibiose binding to unprotonated MelB<sub>St</sub>, the  $K_d$  values for melibiose in the absence or presence of NaCl at pH 8.45 were determined by ITC to be  $9.42 \pm 0.28$  mM (SEM,  $n = 3$ ) and  $0.95 \pm 0.06$  mM (SEM,  $n = 4$ ), respectively. Accordingly, the  $\Delta G$  values are  $-11.57$  and  $-17.26$  kJ/mol, respectively.

## DISCUSSION

It has been well documented for both MelB<sub>Ec</sub> and MelB<sub>St</sub> that Na<sup>+</sup> stimulates galactoside binding and transport (Bassilana et al., 1985; Wilson and Ding, 2001; Guan et al., 2011); however, how the Na<sup>+</sup>-binding affinity is affected by melibiose is not clear, because previous quantitative measurements of Na<sup>+</sup> binding were based

on sugar binding (e.g., the Na<sup>+</sup> stimulation of D<sup>2</sup>G FRET; Fig. 2; Maehrel et al., 1998; Guan et al., 2011) or [<sup>3</sup>H]α-NPG binding (Damiano-Forano et al., 1986). Here, we present detailed data from direct measurements of Na<sup>+</sup> and melibiose binding to MelB<sub>St</sub>, which allow us to construct thermodynamic cycles to analyze the formation of the Na<sup>+</sup>-MelB-melibiose ternary complex (Fig. 6). The thermodynamic coupling free energy ( $\Delta\Delta G$ ) values (i.e., the differences in binding free energies [ $\Delta G$ ] 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<sup>+</sup> and melibiose to MelB<sub>St</sub> is thermodynamically cooperative (Table 1 and Fig. 6). Helix IV in the N-terminal domain contains residues critical for the binding of both Na<sup>+</sup> 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.

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<sup>+</sup> and galactoside, to the symporter MelB<sub>St</sub>. The sum of  $\Delta G$  at  $-35$  kJ/mol from the path [A→B→D] (i.e., from the empty state [A] to the binary Na<sup>+</sup>-bound state [B] and then from this to the Na<sup>+</sup>- and melibiose-bound ternary complex [D]) is nearly equal to that from the path [A→C→D] (i.e., binding of melibiose before Na<sup>+</sup> binding; Fig. 6). Thus, binding of both substrates releases free energy of  $\sim 35$  kJ/mol, and it is likely that the released energy fuels the conformational changes required for transport.

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

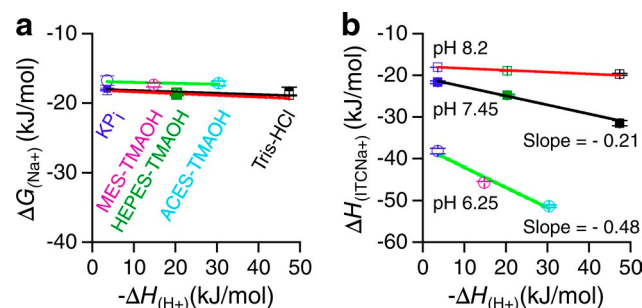
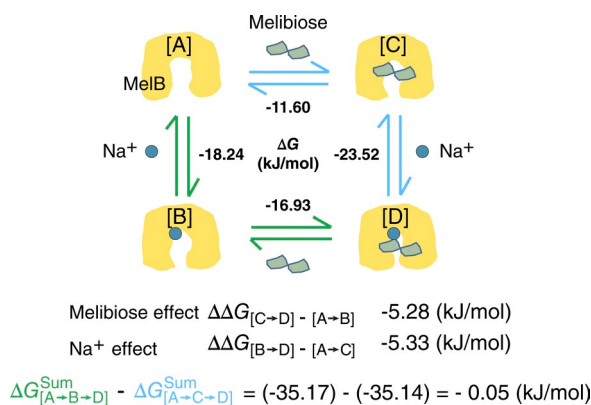


Figure 5. Buffer effects on Na<sup>+</sup> binding enthalpy ( $\Delta H_{ITC(Na^+)}$ ). ITC was used to determine Na<sup>+</sup> binding  $\Delta H_{ITC(Na^+)}$  to MelB<sub>St</sub> in a total of five buffer systems in the absence of melibiose at pH 6.25, 7.45, and 8.2 at 25°C. 5–15 mM NaCl was placed in the syringe. (a)  $\Delta G$  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.



**Figure 6. Thermodynamic cycle for the formation of the melibiose- $\text{Na}^+$ -MelB<sub>st</sub> ternary complex at pH 7.5.** Symbols for MelB<sub>st</sub>, melibiose or  $\text{Na}^+$  are indicated. State [A], cartoon for MelB<sub>st</sub> from the crystal structure (Protein Data Bank accession no. 4M64) represents a periplasmic side-open apo state. States [B] and [C] represent a  $\text{Na}^+$ -bound or melibiose-bound binary complex, respectively. State [D] represents a  $\text{Na}^+$ -melibiose-MelB<sub>st</sub> ternary complex.  $\Delta G$  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.  $\Delta G^{\text{sum}}$ , the sums of free energies for the path [A→B→D] or [A→C→D]. The difference in the  $\Delta G$  from paths [A→B→D] and [A→C→D] is close to zero.

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

ITC is also a useful tool for the study of protein protonation and stoichiometry between  $\text{H}^+$  and its competitive cation. The observed negative linear relationships between the  $\text{Na}^+$  binding enthalpy and the buffer intrinsic protonation enthalpy at pH 6.25 and pH 7.45 suggest that buffer protonation occurs upon  $\text{Na}^+$  binding (i.e., MelB<sub>st</sub> releases  $\text{H}^+$  when it binds  $\text{Na}^+$ ). The demonstration of competitive binding between  $\text{Na}^+$  and  $\text{H}^+$  (Fig. 4), as well as between  $\text{Na}^+$  and  $\text{Li}^+$  (Guan et al., 2011), indicates that all three cations ( $\text{Na}^+$ ,  $\text{H}^+$ , and  $\text{Li}^+$ ) compete for a common binding site in MelB<sub>st</sub>. A similar conclusion was previously drawn in MelB<sub>Ec</sub> (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  $\text{pK}_a$  value, suggesting that the binding of one  $\text{Na}^+$  displaces one  $\text{H}^+$  from the cation binding site per MelB<sub>st</sub>, 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  $\sim 20\%$

MelB<sub>st</sub> 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  $\text{Na}^+$  or  $\text{Li}^+$ . The initial rate and the steady-state level of  $\text{H}^+$ -coupled melibiose transport were  $\sim 30\%$  of  $\text{Na}^+$ -coupled melibiose transport (Guan et al., 2011). With an empty cation site, MelB<sub>st</sub> still binds melibiose, but with very low affinity. Thus, at pH 7.5, only a small portion of MelB<sub>st</sub> 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  $\text{Na}^+$  and  $\text{H}^+$  yields an absolute  $K_{D(\text{Na}^+)}$  of 0.54 mM and  $K_{D(\text{H}^+)}$  of 0.56  $\mu\text{M}$ ; thus, the  $\text{Na}^+$  affinity is  $\sim 1,000$ -fold lower than that for  $\text{H}^+$  in the absence of melibiose, and the MelB<sub>st</sub> cation site is intrinsically selective for  $\text{H}^+$  over  $\text{Na}^+$ . Even in the presence of melibiose, the  $\text{H}^+$  selectivity persists, with a greater than 300-fold higher affinity. In MelB<sub>Ec</sub>, previous studies yielded an  $K_{D(\text{Na}^+)}$  of 0.3 mM and a  $\text{pK}_a$  of 6.3, also suggesting that the cation site in MelB<sub>Ec</sub> is intrinsically selective for  $\text{H}^+$  (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  $K_{D(\text{Na}^+)}$  (0.29 mM) and  $\text{pK}_a$  (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  $\text{Na}^+$  and  $\text{H}^+$ . Although intrinsically selective for  $\text{H}^+$ , the availability of  $\text{H}^+$  in physiological environments (pH 7.5,  $[\text{H}^+] = 32 \text{ nM}$ ) is very low, and the availability of  $\text{Na}^+$  is often high enough to ensure that  $\text{Na}^+$  can effectively compete for the cation site. Such a cation site thus appears to have evolved for the effective use of the metal cation  $\text{Na}^+$  under physiological conditions. Because the living environments for bacteria are not always  $\text{Na}^+$  rich, an elevated  $\text{pK}_a$  value for Asp residues in the cation site allows the bacteria to use  $\text{H}^+$  as the coupling cation for melibiose transport, albeit with less efficiency. This elegant mechanism secures MelB's important biological function.

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