Insights into the Inhibitory Mechanisms of the Regulatory Protein IIA$^{\text{Glc}}$ on Melibiose Permease Activity*

Received for publication, September 2, 2014, and in revised form, September 25, 2014 Published, JBC Papers in Press, October 8, 2014, DOI 10.1074/jbc.M114.609255

Parameswaran Hariharan and Lan Guan 1

From the Department of Cell Physiology and Molecular Biophysics, Center for Membrane Protein Research, Texas Tech University Health Sciences Center, Lubbock, Texas 79430

Background: The phosphotransfer protein IIA$^{\text{Glc}}$ plays a key role in the regulation of carbohydrate metabolism.

Results: ITC measurements show that IIA$^{\text{Glc}}$ binds to melibiose permease at a stoichiometry of unity and inhibits sugar binding affinity and conformational entropy.

Conclusion: IIA$^{\text{Glc}}$ inhibits MelB by restraining its conformational change.

Significance: IIA$^{\text{Glc}}$ is a useful tool for structure-function studies of its regulated permeases.

The phosphotransfer protein IIA$^{\text{Glc}}$ of the bacterial phosphoenolpyruvate:carbohydrate phosphotransferase system plays a key role in the regulation of carbohydrate metabolism. Melibiose permease (MelB) is one among several permeases subject to IIA$^{\text{Glc}}$ regulation. The regulatory mechanisms are poorly understood; in addition, thermodynamic features of IIA$^{\text{Glc}}$ binding to other proteins are also unknown. Applying isothermal titration calorimetry and amine-specific cross-linking, we show that IIA$^{\text{Glc}}$ directly binds to MelB of Salmonella typhimurium (MelB$^{\text{S}}$) and Escherichia coli MelB (MelB$^{\text{Ec}}$) at stoichiometry of unity in the absence or presence of melibiose. The dissociation constant values are 3–10 μM for MelB$^{\text{S}}$ and 25 μM for MelB$^{\text{Ec}}$. All of the binding is solely driven by favorable enthalpy forces. IIA$^{\text{Glc}}$ binding to MelB$^{\text{S}}$ in the absence or presence of melibiose yields a large negative heat capacity change; in addition, the conformational entropy is constrained upon the binding. We further found that the IIA$^{\text{Glc}}$-bound MelB$^{\text{S}}$ exhibits a decreased binding affinity for melibiose or nitrophenyl-α-galactoside. It is believed that sugar binding to the permease is involved in an induced fit mechanism, and the transport process requires conformational cycling between different states. Thus, the thermodynamic data are consistent with the interpretation that IIA$^{\text{Glc}}$ inhibits the induced fit process and restricts the conformational dynamics of MelB$^{\text{S}}$.

Sugar transport is an important process for all living organisms. To secure the energy supply, bacterial cells usually contain multiple sugar transport systems. Melibiose permease (MelB),2 which catalyzes electrogenic symport of galactoside with Na$^+$, Li$^+$, or H$^+$ (1–6), is one of the bacterial sugar transporters. MelB is encoded by the mel operon, which requires transcriptional activation induced by melibiose, as well as a global transcriptional activator (the cAMP-CAP (catabolite activator protein) complex) (7, 8). In certain bacteria, such as Escherichia and Salmonella, the phosphoenolpyruvate:carbohydrate phosphotransferase system (PTS) carries out both catalytic and regulatory functions, which promotes preferential utilization of glucose (9–11). The phosphotransfer protein IIA$^{\text{Glc}}$ plays a key role in the regulation of carbohydrate metabolism. It regulates the transcription level of various operons encoding non-PTS permeases (12), such as the mel and lac operons, by modulating the level of cAMP. Unphosphorylated IIA$^{\text{Glc}}$ regulates the activity of various non-PTS transporters (9, 11, 13–16). In addition, as a central signaling molecule, it also binds to many other proteins, such as adenylate cyclase (17) and glycerol kinase (18). Recently, it has been reported that IIA$^{\text{Glc}}$ also binds to a carbon storage regulator and affects Vibrio cholerae biofilm formation (19). It is interesting that a single rigid protein is able to bind to various families of soluble proteins as well as membrane transporters. As demonstrated by x-ray crystallography, IIA$^{\text{Glc}}$ binds to the ATPase subunit of maltose permease, preventing structural rearrangements necessary for ATP hydrolysis (16). In the H$^+$-coupled lactose permease (LuxY), it has been reported that the binding of IIA$^{\text{Glc}}$ requires the presence of sugar (13, 20, 21), but the reported stoichiometry number is not convincing.

In the Na$^+$-coupled MelB of Salmonella typhimurium (MelB$^{\text{S}}$), it was shown that mutations affect the PTS regulation (22, 23); however, there is, at present, no evidence that IIA$^{\text{Glc}}$ directly binds to MelB (11). In addition, there is no information about the energetics of IIA$^{\text{Glc}}$ binding to other proteins. Recently, we solved the three-dimensional crystal structure of MelB$^{\text{S}}$ and demonstrated that MelB (24, 25), a member of the glycoside-pentoside-hexuronide:cation symporter family (26), belongs to a subgroup of the major facilitator superfamily (MFS) permeases (5, 27–29), like LacY (30, 31). The structures were captured in an outward partially occluded and a partially outward-facing conformation, and suggest a single sugar-binding pocket within the central internal cavity (5, 32–34). The structure provides important mechanistic insights for a major

* This work was supported by National Science Foundation Grant MCB-1158085 (to L.G.) and the National Institutes of Health Grant R01 GM095538 (to L.G.).

1 To whom correspondence should be addressed: Dept. of Cell Physiology and Molecular Biophysics, Texas Tech University Health Sciences Center, Lubbock, Texas 79430. Tel.: 806-743-3102; E-mail: Lan.Guan@ttuhsc.edu.

2 The abbreviations used are: MelB, melibiose permease; MelB$^{\text{S}}$, MelB$^{\text{Ec}}$ of Salmonella typhimurium; MelB$^{\text{Ec}}$, MelB of Escherichia coli; LacY, lactose permease of Escherichia coli; PTS, phosphoenolpyruvate:carbohydrate phosphotransferase system; α-NPG, nitrophenyl-α-galactoside; α-NPGlu, nitrophenyl-α-glucoside; DSP, dithiobis(succinimidyl propionate); ITC, isothermal titration calorimetry.
facilitator superfamily permease that catalyzes Na\(^+\)-coupled symport (26, 35, 36). However, it was not clear where the IIA\(^{Glc}\) binding site is and how IIA\(^{Glc}\) regulates MelB activity.

It is generally believed that these IIA\(^{Glc}\) binding partners have little or no sequence or structural homology with one another (37). It is surprising that the C-terminal tail of MelBEc and MelBSt, as well as other MelB orthologues, contains the consensus region\(^ {443-459}\) that has a high sequence similarity to \(^ {129-299}\) in MalK (16, 38). Both stretches form a short helical structure (16, 25), and the underlined residues in MalK directly contact with IIA\(^{Glc}\) (16). The crystal structure determination of MelB\(_{St}\) reveals that this motif occupies two different conformations; one is closer to the membrane domain (see also Fig. 2c). The previously characterized MelB\(_{St}\) mutants (D438Y, R441S, or 1445N) (38), which are resistant to PTS inhibition, are mapped near or within this motif (see Fig. 2c). Based on this structural information, we agree with the previous postulation (38) that the C-terminal fragment of MelB could be a part of the IIA\(^{Glc}\) binding sites.

In this study, we determined the thermodynamics of the IIA\(^{Glc}\)-MelB interaction, sugar binding to MelB\(_{St}\), as well as the effect of IIA\(^{Glc}\) on the sugar-MelB interaction using isothermal titration calorimetry (ITC). We observed that IIA\(^{Glc}\) binds to MelB\(_{St}\) in the absence or presence of melibiose, and inhibits the conformational entropy and sugar affinity of the transporter.

**MATERIALS AND METHODS**

**Reagents**—The Phos-tag\(^ {TM}\) acrylamide (NARD Institute, Ltd.). Dithiobis(succinimidyl propionate) (DSP) was from Thermo Scientific. Nitrophenyl-\(\alpha\)-galactoside (\(\alpha\)-NPG) and nitrophenyl-\(\alpha\)-glucoside (\(\alpha\)-NPGlu) were from Sigma-Aldrich.

**Gene Cloning of IIA\(^{Glc}\)**—The gene encoding IIA\(^{Glc}\) was amplified from the chromosomal DNA of *Escherichia coli* DW2 strain (5) by PCR (sense primer, 5'-TATATGCTCTTCTAGT-ATGGGTTCGATAAACTAAC-3'; antisense primer, 5'-TATATGCTCTTCTACTTCTATGATCCYYT-3'), cloned into the T7-based expression vector p7XNH3 with a kanamycin resistance marker by the fragment-exchange cloning method (39). The resultant plasmid contained a 10-His tag sequence at the N terminus with a 9-residue link (MNHHHHHHHTLLLQPGS), which was verified by DNA sequencing analysis.

**Protein Expression and Purification**—The overexpression of IIA\(^{Glc}\) was performed in the *E. coli* T7 express strain (New England Biolabs). The cells were grown in LB containing 0.5% glycerol, 0.2% glucose, and 50 mg/liter kanamycin. The overnight cultures were diluted to 2% with the same medium and shaken at 30 °C. Isopropyl-1-thio-\(\beta\)-D-galactopyranoside at 0.4 mM was added at \(A\) of 0.8, and the incubation was continued for another 4 h. Cells were harvested and suspended in a buffer containing 50 mm NaPi, pH 7.5, 200 mm NaCl, 5% glycerol, and 0.1% PMSF, and broken by passage through an Emulsiflex at 10000 p.s.i. The supernatant, after ultracentrifugation at 70.409 \(\times g\) for 30 min in a Beckman rotor, type 45 Ti at 4 °C, was loaded onto a column containing Talon resin (Clontech) pre-equilibrated with 50 mm NaPi, pH 7.5, 200 mm NaCl, 5% glycerol, 5 mm imidazole for cobalt affinity chromatography. After washing with the same buffer containing 30 mm imidazole, elution was performed using the same buffer containing 200 mm imidazole; the eluate was concentrated to \(\sim 100\) mg/ml using a VIVASPIN 20 (5,000 molecular weight cut-off polysisulfone, Millipore) and dialyzed against three changes of 1 liter of dialysis buffer containing 20 mm Tris-HCl, pH 7.5, 100 mm NaCl, 10% glycerol. The protein concentration of IIA\(^{Glc}\) was measured by the Micro BCA protein assay (Pierce Biotechnology, Inc.). The protein samples were flash-frozen in liquid nitrogen and stored at \(-80 °C\). From a 1-liter culture, about 50 mg of highly pure IIA\(^{Glc}\) protein can be obtained routinely. The purified IIA\(^{Glc}\) protein was analyzed with both SDS-14% PAGE and Phos-tag SDS-12% PAGE.

The overexpression and purification of MelB\(_{St}\) and MelB\(_{Ec}\) (40, 41) were carried out as described (25). MelB proteins in the MelB buffer (20 mm Tris-HCl, pH 7.5, 100 mm NaCl, 0.035% undecyl \(\beta\)-D-maltoside, and 10% glycerol) were concentrated to 20 mg/ml, flash-frozen in liquid nitrogen, and stored at \(-80 °C\).

**Isothermal Titration Calorimetry**—ITC measurements were performed in a Nano isothermal titration calorimeter (TA Instruments). Purified MelB (50 \(\mu\)M) in MelB buffer without or with 10 mm melibiose was placed into the sample cell with a reaction volume of 163 \(\mu\)l, and IIA\(^{Glc}\) (455 \(\mu\)M) in the MelB buffer without or with 10 mm melibiose, prepared by dilution from a highly concentrated protein sample, was titrated incrementally into the MelB sample. For measuring sugar binding to MelB, melibiose at 10 or 100 mm was dissolved in the MelB buffer; \(\alpha\)-NPG or \(\alpha\)-NPGlu at 1 mm was prepared by diluting 200 mm \(\alpha\)-NPG or \(\alpha\)-NPGlu in dimethyl sulfoxide into the MelB buffer. In this case, the same concentration of dimethyl sulfoxide was added into the sample placed in the sample cell. When testing the IIA\(^{Glc}\) effect, IIA\(^{Glc}\) was preincubated with MelB at a 2 to 1 ratio for 1 h.

Titrations were performed by injection of titrant with an interval of 250 or 300 s at a constant stirring rate of 250 rpm. By plotting integrated rates of heat change against the molar ratio of IIA\(^{Glc}\)/MelB or sugar/MelB, the binding stoichiometry number (n), the association constant (\(K_a\)), and the enthalpy change (\(\Delta H\)) are directly determined by fitting the data using the one-site independent binding model provided by the instrument. The dissociation constant (\(K_d\)) and the entropy change (\(\Delta S\)) are obtained by calculation using the equation of \(\Delta G = -RT \ln K_a\) and \(\Delta G = \Delta H - \Delta S\), where \(\Delta G\) is free energy change, \(R\) is the Faraday constant, and \(T\) is absolute temperature. Parameterization of \(\Delta S\) was calculated as described previously (42). Total \(\Delta S = \Delta S_{solv} + \Delta S_{mix} + \Delta S_{conf} \) Mixing entropy change \(\Delta S_{mix} = \ln(1/(55.5) = -331/mol/K\). Solvent entropy change \(\Delta S_{solv} = \Delta S_{p} \ln(298.15/385.15)\), where \(\Delta S_{p}\) is the heat capacity change. Conformational entropy change \(\Delta S_{conf} = \Delta S - \Delta S_{mix} - \Delta S_{solv}\).

**Protein Cross-linking**—The amine-reactive cross-linking reagent DSP was used for the cross-linking reaction between MelB\(_{St}\) and IIA\(^{Glc}\). Briefly, 1 \(\mu\)g of MelB\(_{St}\) (1.88 \(\mu\)M) in the absence or presence of 10 mm melibiose and IIA\(^{Glc}\) (4.8 \(\mu\)M) in 20 mm HEPES, pH 7.6, 50 mm NaCl, and 0.035% undecyl \(\beta\)-D-maltoside were preincubated for 15 min, and the cross-linking reaction was carried out by incubating with 200 \(\mu\)M DSP for 15
IIA^Glc Restrains Conformational Entropy of MelB

RESULTS

IIA^Glc Binding to MelB_{St} in the Absence or Presence of Melibiose—IIA^Glc was purified to homogeneity from the E. coli T7 express strain (Fig. 1), and the Phos-tag SDS-PAGE analysis indicates that the affinity-purified recombinant IIA^Glc protein is unphosphorylated (Fig. 1, right). By titrating IIA^Glc into a MelB_{St} sample at 25 °C, ITC measurements show exothermic binding in the absence or presence of melibiose (Fig. 2a). No detectable changes were observed when injecting IIA^Glc into the buffer (Fig. 2a, inset) or buffer to MelB_{St} (data not shown). The data fitting (Fig. 2b) suggests dissociation constant (K_d) values of 3.62 or 10.15 μM (Table 1) in the absence or presence of melibiose, respectively. The protein-protein interaction under both conditions is solely driven by favorable enthalpy change (ΔH) and opposed by negative entropy change (TΔS) (Fig. 2b, inset; Table 1). The results indicate that polar or hydrophilic interactions are the major forces governing IIA^Glc binding and that the charged or polar residues in the proposed IIA-Glc-binding site in the C-terminal tail may contribute to the enthalpy forces (Fig. 2c).

When melibiose is preincubated with MelB_{St} (Fig. 2a, Table 1), the ΔS becomes less unfavorable. The measured stoichiometry number (n) without melibiose is 0.98. In the presence of...
melibiose, the \( n \) number is about 0.78; however, at 20 °C, it is 1.1 (Table 2). It is noteworthy that asymmetric peaks appear at the beginning of the titration in the absence of sugar (Fig. 2a).

The interaction of \( \text{IIA}^{\text{Glc}} \) with \( \text{MelB}_{\text{St}} \) was further tested by amine-specific cross-linking studies. In the absence or presence of melibiose, a band with \( M_r \approx 62,000 \) was obtained only in the presence of cross-linking reagents, which corresponds to the cross-linked product containing one \( \text{MelB}_{\text{St}} \) and one \( \text{IIA}^{\text{Glc}} \) (Fig. 2d). These data are consistent with the results from the ITC measurements and support the conclusion that the stoichiometry of \( \text{IIA}^{\text{Glc}} \) to \( \text{MelB}_{\text{St}} \) is unity in the absence or presence of melibiose.

\( \text{IIA}^{\text{Glc}} \) binding to \( \text{E. coli} \) MelB (MelBEc) was also examined by ITC. The data reveal thermodynamic features similar to that observed when injecting \( \text{IIA}^{\text{Glc}} \) into \( \text{MelB}_{\text{St}} \), except for the higher \( K_d \) value of 25 \( \mu \text{M} \) (Fig. 3, Table 2); furthermore, there is no difference in the absence or presence of melibiose. The following studies focused only on \( \text{IIA}^{\text{Glc}} \) binding to \( \text{MelB}_{\text{St}} \).

**Table 1**

| \( K_a \) (mol \(^{-1} \)) | \( K_d \) (\( \mu \text{M} \)) | \( \Delta G \) (kJ/mol) | \( \Delta H \) (kJ/mol) | \( T \Delta S \) (kJ/mol) | \( \Delta S \) (kJ/mol) | \( n^a \) |
|-----------------|-----------------|-----------------|-----------------|--------------------|--------------------|------|
| No sugar        | 290,433 (436,09) | 3.62 (0.59)      | -31.13 (0.39)   | -48.74 (2.73)      | -17.61 (2.42)      | -59.10 (8.12)    | 0.98 (0.10) |
| Melibiose\(^c\) | 104,415 (24,785) | 10.15 (0.80)     | -28.56 (0.60)   | -37.91 (1.52)      | -9.33 (0.92)       | -31.32 (3.09)    | 0.78 (0.06) |

\( a \) Stoichiometry of \( \text{IIA}^{\text{Glc}} \) versus \( \text{MelB}_{\text{St}} \).
\( b \) S.E., number of tests = 2–3.
\( c \) Melibiose at 10 \( \mu \text{M} \) was pre-equilibrated with both proteins.

**Table 2**

| \( K_a \) (mol \(^{-1} \)) | \( K_d \) (\( \mu \text{M} \)) | \( \Delta G \) (kJ/mol) | \( \Delta H \) (kJ/mol) | \( T \Delta S \) (kJ/mol) | \( \Delta S \) (kJ/mol) | \( n^a \) |
|-----------------|-----------------|-----------------|-----------------|--------------------|--------------------|------|
| No sugar        | 39,195 (5355)   | 25.76 (3.33)    | -37.49 (0.25)   | -11.73 (0.08)      | -40.01 (0.28)      | 1.22 (0.07) |
| Melibiose\(^c\) | 39,640 (3220)   | 25.4 (2.10)     | -39.23 (0.89)   | -13.44 (1.10)      | -45.84 (3.75)      | 1.27 (0.11) |

\( a \) Stoichiometry of \( \text{IIA}^{\text{Glc}} \) versus \( \text{MelB}_{\text{St}} \).
\( b \) S.E., number of tests = 2–3.
\( c \) Melibiose at 10 \( \mu \text{M} \) was pre-equilibrated with both proteins.
IIA\textsubscript{Glc} Restrains Conformational Entropy of MelB

in $\Delta G$ and $K_d$ with temperature. A linear fitting of $\Delta H$ versus temperature reveals the heat capacity change ($\Delta C_p$, the slope) of $-1.17$ and $-1.04$ kJ/mol/K in absence or presence of melibiose, respectively. There is no significant difference at either condition.

Because the $\Delta C_p$ value was determined, the $\Delta S$ can be parameterized (42) (Table 4; Fig. 4b), which reveals a large increase in the solvent-entropy change ($\Delta S_{solv}$) and a large unfavorable conformational entropy change ($\Delta S_{conf}$). Without sugar, the binding yields a more favorable change in $\Delta S_{solv}$ and a more unfavorable change in $\Delta S_{conf}$.

Inhibition of Melibiose Affinity by IIA\textsubscript{Glc}—We further analyzed the effect of IIA\textsubscript{Glc} binding on sugar affinity. As we previously showed (25), the binding of melibiose to the Na\textsuperscript{+}-bound MelB\textsubscript{ec} was detected by ITC measurement at 25 °C; the binding is exothermic with a $K_d$ value of 950 $\mu$M (Fig. 5a). The binding is driven by both favorable $\Delta H$ and favorable $\Delta S$ (Table 5). Strikingly, when injecting the melibiose solution to the MelB\textsubscript{ec}-IIA\textsubscript{Glc} complex at 25 °C (Fig. 5b) or 35 °C (data not shown), the heat changes are smaller than the control. By injecting 10-fold higher melibiose (100 mM), a titration curve after correction for the buffer control shows an endothermic effect (Fig. 5c, inset). As a control, injection of 100 mM sucrose (a non-substrate) does not produce detectable binding signals (Fig. 5d). Although the weak signals were not amenable for fitting, the affinity is apparently inhibited.

![Figure 5](image-url)

TABLE 4 Parameterization of $\Delta S$

|                     | No sugar | 10 mM melibiose |
|---------------------|----------|-----------------|
| $\Delta C_p$ (J/mol/K) | $-1167$       | $-1036$         |
| $\Delta S^b$ (J/mol/K) | $-39.10$      | $-31.32$        |
| $\Delta S_{conf}$ (J/mol/K) | $-33.00$      | $-30.00$        |
| $\Delta S_{solv}$ (J/mol/K) | $298.75$       | $265.21$        |
| $\Delta S_{total}$ (J/mol/K) | $-324.85$     | $-263.54$       |

$^a$ $\Delta C_p = \Delta H/\Delta T$, the slope from the linear fitting (Fig. 4a).

$^b$ Total $\Delta S = \Delta S_{mix} + \Delta S_{solv} + \Delta S_{conf}$.

$^c$ Mixing entropy change $\Delta S_{mix} = R \ln (1/55.5) = -33$ (J/mol/K).

$^d$ Solvent entropy change $\Delta S_{solv} = \Delta C_p \ln (298.15/385.15)$.

$^e$ Conformational entropy change $\Delta S_{conf} = \Delta S - \Delta S_{mix} - \Delta S_{solv}$.

![Figure 5](image-url)

**TABLE 5**

Energetics of sugar binding to MelB\textsubscript{ec} at 25 °C

*The binding data are presented in Figs. 5–6.*

| Sugars Proteins | $K_d$ (µM) | $\Delta H$ (kJ/mol) | $\Delta S$ (kJ/mol K) |
|-----------------|------------|--------------------|----------------------|
| Melibiose MelB\textsubscript{ec} | 950 (12) \textsuperscript{a} | -17.28 (3.09) | -9.27 (0.05) |
| $\alpha$-NPG MelB\textsubscript{ec} | 1545 (0.35) | -27.46 (0.05) | -43.25 (0.44) |
| MelB\textsubscript{ec}-IIA\textsubscript{Glc} | 76.13 (4.52) | -23.51 (0.15) | 21.28 (5.03) |

$^a$ S.E., number of tests = 2.
**DISCUSSION**

Previous studies reported that the binding of IIAGlc to LacY requires the presence of sugar substrate (13, 20, 21); in contrast, we show here that IIAGlc binds to MelBEc or MelBSt in the absence or presence of the sugar. The binding to MelBSt has higher affinity than that to MelBEc. Without or with melibiose, the titration curves of IIAGlc to MelBEc are similar. When injecting IIAGlc into MelBSt without sugar, the heat change peaks in the beginning of the thermogram are asymmetric, which indicates that IIAGlc binding is relatively slow. It is likely that MelBSt occupies several conformational states; not all the conformers can be recognized by IIAGlc, which causes the slow binding. The results suggest that the conformational transition is slow in the absence of melibiose. It is possible that the lower number of MelBSt molecules available for the IIAGlc binding may explain this behavior. Sugar binding facilitates the conformational dynamics associated with IIAGlc binding.

Although the IIAGlc-bound state of MelB and the details of the interaction are not known, two conformations, an outward partially occluded conformation and a partially outward-facing conformation, were determined by crystallography (25); both structures show a close surface at the cytoplasmic side, reflecting its low energy state. Accordingly, it seems reasonable to postulate that the cytosolic IIAGlc binds between the C-terminal tail and a closed face on the cytoplasmic side of MelB (Fig. 2c).

IIAGlc binding to MelB is involved in a large favorable \( \Delta S_{\text{solv}} \) with compensation of a large unfavorable \( \Delta S_{\text{conf}} \). Because IIAGlc is structurally rigid (16, 45), it is likely that the large change in \( \Delta S_{\text{solv}} \) may mainly result from the conformationally flexible MelBSt, as well as the binding interface. The inhibition of conformational entropy upon the binding implies that IIAGlc bind-
IIA\textsubscript{Glc} Restrains Conformational Entropy of MelB

...ing prevents the conformational changes of MelB, similar to that proposed by the structural approach in maltose permease (16). The less restrained $\Delta S_{\text{conf}}$ is observed in the presence of melibiose, which is probably due to the idea that the conformational entropy was restrained to a certain extent by the sugar binding prior to the IIA\textsubscript{Glc} binding.

IIA\textsubscript{Glc} effects on MelB\textsubscript{St} affinity for sugars were examined with the lower affinity melibiose and the higher affinity ligand $\alpha$-NPG. The ITC measurements show that IIA\textsubscript{Glc} binding affects the sugar binding significantly. It inhibits the sugar binding affinity; on the other hand, it alters the thermodynamic features of sugar binding from an exothermic to an endothermic reaction. Because the affinity of MelB\textsubscript{St} for melibiose is at the lower sensitivity boundary of the Nano ITC equipment, the decreased sugar binding affinity by IIA\textsubscript{Glc} is out of the detectable range of this method. We confirmed the IIA\textsubscript{Glc} inhibition of sugar binding with $\alpha$-NPG. The ITC measurements reveal that $\alpha$-NPG binds to MelB\textsubscript{St} with $>$60-fold higher affinity than does melibiose. Different from the entropy-driven melibiose binding, the $\alpha$-NPG binding is solely driven by $\Delta H$, suggesting that the increased affinity mainly results from polar or hydrophilic interactions between $\alpha$-NPG and MelB\textsubscript{St}. Furthermore, $\alpha$-NPG binding to MelB\textsubscript{St} in the IIA\textsubscript{Glc}-bound complex becomes solely driven by $\Delta S$ with a 5-fold higher $K_a$ value. Because the sugar binding to the permease is likely involved in an induced fit process as demonstrated in LacY (46, 47), the results could be explained by the observed unfavorable $\Delta S_{\text{conf}}$ of the IIA\textsubscript{Glc}-MelB\textsubscript{St} complex. Thus, IIA\textsubscript{Glc} inhibits the induced fit process for sugar binding by restricting the conformational entropy.

Overall, the observed direct interaction of IIA\textsubscript{Glc} with MelB\textsubscript{St} inhibits the sugar affinity and conformational dynamics of the transporter protein. It is likely that by such a mechanism, unphosphorylated IIA\textsubscript{Glc} blocks entry of melibiose, the inducer of the mel operon, so that the cells utilize glucose via the PTS transport system.

Acknowledgments—We thank Colette Quinn and Abdul S. Ethayathulla for helpful discussions, Raimund Dutzler and Eric R. Geertsma for the fragment-exchange cloning vector, Gerard Leblanc for the E. coli DW2 strain and MelB expression vector, and Tomofusa Tsuchiya for the S. typhimurium LT2 strain. We also thank Alan Peterkofsky and Luis Reuss for critical reading of the manuscript, and Ron Kaback for stimulating discussions.

REFERENCES

1. Wilson, D. M., and Wilson, T. H. (1987) Cation specificity for sugar substrates of the melibiose carrier in Escherichia coli. Biochim. Biophys. Acta 904, 191–200
2. Tokuda, H., and Kaback, H. R. (1977) Sodium-dependent methyl 1-thio-$\beta$-D-galactopyranoside transport in membrane vesicles isolated from Salmonella typhimurium. Biochemistry 16, 2130–2136
3. Tsuchiya, T., Raven, J., and Wilson, T. H. (1977) Co-transport of Na\textsuperscript+ and methyl-$\beta$-D-thiogalactopyranoside mediated by the melibiose transport system of Escherichia coli. Biochem. Biophys. Res. Commun. 76, 26–31
4. Bassilana, M., Pourcher, T., and Leblanc, G. (1987) Facilitated diffusion properties of melibiose permease in Escherichia coli membrane vesicles: release of co-substrates is rate limiting for permease cycling. J. Biol. Chem. 262, 16865–16870
5. Guan, L., Nurva, S., and Ankeshwarapu, S. P. (2011) Mechanism of melibiose/cation symport of the melibiose permease of Salmonella typhimu-

rion. J. Biol. Chem. 286, 6367–6374
6. Guan, L., Jakula, S. V., Hodkoff, A. A., and Su, Y. (2012) Role of Gly117 in the cation/melibiose symport of MelB of Salmonella typhimurium. Biochemistry 51, 2950–2957
7. Kahramanoglou, C., Webster, C. L., El-Robh, M. S., Belyaeva, T. A., and Busby, S. I. (2006) Mutational analysis of the Escherichia coli melR gene suggests a two-state concerted model to explain transcriptional activation and repression in the melibiose operon. J. Bacteriol. 188, 3199–3207
8. Grainger, D. C., Belyaeva, T. A., Lee, D. J., Hyde, E. I., and Busby, S. J. (2003) Binding of the Escherichia coli MelR protein to the melAB promoter: orientation of MelR subunits and investigation of MelR-DNA contacts. Mol. Microbiol. 48, 335–348
9. Postma, P. W., Lengeler, J. W., and Jacobson, G. R. (1993) Phosphoenolpyruvate:carbonate-phosphotransferase systems of bacteria. Microbiol. Rev. 57, 543–594
10. Saier, M. H., Jr. (1989) Protein phosphorylation and allosteric control of inducer exclusion and catabolite repression by the bacterial phosphoenolpyruvate:sugar phosphotransferase system. Microbiol. Rev. 53, 109–120
11. Deutscher, J., Aké, F. M., Derkaoui, M., Zébré, A. C., Cao, T. N., Bouraoui, H., Kentache, T., Mokhtari, A., Milohanic, E., and Joyet, P. (2014) The bacterial phosphoenolpyruvate:carbonate-phosphotransferase system: regulation by protein phosphorylation and phosphorylation-dependent protein-protein interactions. Microbiol. Mol. Biol. Rev. 78, 231–256
12. Guan, L., and Kaback, H. R. (2013) Glucose/Sugar Transport in Bacteria. In Encyclopedia of Biological Chemistry (Lennarz, W. J., and Lane, M. D., eds), pp 387–390. Second Ed., Elsevier Science Publishers, Oxford
13. Osumi, T., and Saier, M. H., Jr. (1982) Mechanism of regulation of the lactose permease by the phosphotransferase system in Escherichia coli: evidence for protein-protein interaction. Ann. Microbiol. (Paris) 133, 269–273
14. Peterkofsky, A., Wang, G., Garrett, D. S., Lee, B. R., Seok, Y. J., and Clore, G. M. (2001) Three-dimensional structures of protein-protein complexes in the E. coli PTS. J. Mol. Microbiol. Biotechnol. 3, 347–354
15. Meadow, N. D., and Roseman, S. (1982) Sugar transport by the bacterial phosphotransferase system: isolation and characterization of a glucose-specific phosphocarrier protein (III\textsuperscript{Glc}) from Salmonella typhimurium. J. Biol. Chem. 257, 14526–14537
16. Chen, S., Oldham, M. L., Davidson, A. L., and Chen, J. (2013) Carbon catabolite repression of the melibiose transporter revealed by x-ray crystallography. Nature 499, 364–368
17. Lengeler, J. W., and Jahreis, K. (2009) Bacterial PEP-dependent carbohydrate-phosphotransferase systems couple sensing and global control mechanisms. Contrib. Microbiol. 16, 65–87
18. Hurley, J. H., Faber, H. R., Worthylake, D., Meadow, N. D., Roseman, S., Pettigrew, D. W., and Remington, S. I. (1993) Structure of the regulatory complex of Escherichia coli III\textsuperscript{Glc} with glycerol kinase. Science 259, 673–677
19. Pickering, B. S., Smith, D. R., and Watanick, P. I. (2012) Glucose-specific enzyme IIA has unique binding partners in the Vibrio cholerae biofilm. mBio 3, e00228–00212
20. Sondej, M., Weinglass, A. B., Peterkofsky, A., and Kaback, H. R. (2002) Binding of enzyme IIA\textsuperscript{Glc}, a component of the phosphoenolpyruvate:sugar phosphotransferase system, to the Escherichia coli lactose permease. Biochemistry 41, 5556–5565
21. Sondej, M., Vázquez-Ibar, J. L., Farshidi, A., Peterkofsky, A., and Kaback, H. R. (2003) Characterization of a lactose permease mutant that binds IIA\textsuperscript{Glc} in the absence of lactose. Biochemistry 42, 9153–9159
22. Kuroda, M., Wilson, T. H., and Tsuchiya, T. (2001) Regulation of galactose-side transport by the PTS. J. Mol. Microbiol. Biotechnol. 3, 381–384
23. Kuroda, M., Osaki, N., Tsuda, M., and Tsuchiya, T. (1992) Preferential utilization of glucose over melibiose, and vice versa, in a pts mutant of Salmonella typhimurium. Chem. Pharm. Bull. (Tokyo) 40, 1637–1640
24. Yousef, M. S., and Guan, L. (2009) A 3D structure model of the melibiose permease of Escherichia coli represents a distinctive fold for Na\textsuperscript{+} symporters. Proc. Natl. Acad. Sci. U.S.A. 106, 15291–15296
25. Ethayathulla, A. S., Yousef, M. S., Amin, A., Leblanc, G., Kaback, H. R., and Guan, L. (2014) Structure-based mechanism for Na\textsuperscript{+}/melibiose symport by MelB. Nat. Commun. 5, 3009
IIA^Glucose Restrains Conformational Entropy of MelB

26. Poolman, B., Knol, J., van der Does, C., Henderson, P. J., Liang, W. J., Leblanc, G., Pourcher, T., and Mus-Veteau, I. (1996) Cation and sugar selectivity determinants in a novel family of transport proteins. Mol. Microbiol. 19, 911–922

27. Saier, M. H., Jr., Beatty, J. T., Goffeau, A., Harley, K. T., Heijne, W. H., Huang, S. C., Jack, D. L., Jaehn, P. S., Lew, K., Liu, J., Pao, S. S., Paulsen, I. T., Tseng, T. T., and Virk, P. S. (1999) The major facilitator superfamily. J. Mol. Microbiol. Biotechnol. 1, 257–279

28. Wilson, T. H., and Ding, P. Z. (2001) Sodium-substrate cotransport in bacteria. Biochim. Biophys. Acta 1518–1524

29. Saier, M. H., Jr., Beatty, J. T., Goffeau, A., Harley, K. T., Heijne, W. H., Huang, S. C., Jack, D. L., Jaehn, P. S., Lew, K., Liu, J., Pao, S. S., Paulsen, I. T., Tseng, T. T., and Virk, P. S. (1999) The major facilitator superfamily. J. Mol. Microbiol. Biotechnol. 1, 257–279

30. Guan, L., and Kaback, H. R. (2006) Lessons from lactose permease. Annu. Rev. Biophys. Biomol. Struct. 35, 67–91

31. Guan, L., Mirza, O., Verner, G., IWata, S., and Kaback, H. R. (2007) Structural determination of wild-type lactose permease. Proc. Natl. Acad. Sci. U.S.A. 104, 15294–15298

32. Abdel-Dayem, M., Basquin, C., Pourcher, T., Cordat, E., and Leblanc, G. (2003) Cytoplasmic loop connecting helices IV and V of the melibiose permease from *Escherichia coli* is involved in the process of Na^+^-coupled sugar translocation. J. Biol. Chem. 278, 1518–1524

33. Zani, M. L., Pourcher, T., and Leblanc, G. (1994) Mutation of polar and charged residues in the hydrophobic NH2-terminal domains of the melibiose permease of *Escherichia coli*. J. Biol. Chem. 269, 24883–24889

34. Granell, M., Leon, X., Leblanc, G., Padrós, E., and López-Fonfría, V. A. (2010) Structural insights into the activation mechanism of melibiose permease by sodium binding. Proc. Natl. Acad. Sci. U.S.A. 107, 22078–22083

35. Nguyen, L. N., Ma, D., Shui, G., Wong, P., Cazenave-Gassiot, A., Zhang, X., Wenk, M. R., Goh, E. L., and Silver, D. L. (2014) MfsD2a is a transporter for the essential omega-3 fatty acid docosahexaenoic acid. Nature 509, 503–506

36. Berger, J. H., Charron, M. J., and Silver, D. L. (2012) Major facilitator superfamily domain-containing protein 2a (MFSD2A) has roles in body growth, motor function, and lipid metabolism. PLoS One 7, e50629

37. Feese, M. D., Comolli, L., Meadow, N. D., Roseman, S., and Remington, S. J. (1997) Structural studies of the *Escherichia coli* signal transducing protein IIA^Glucose^; implications for target recognition. Biochemistry 36, 16087–16096

38. Kuroda, M., de Waard, S., Mizushima, K., Tsuda, M., Postma, P., and Tsuchiya, T. (1992) Resistance of the melibiose carrier to inhibition by the phosphotransferase system due to substitutions of amino acid residues in the carrier of *Salmonella typhimurium*. J. Biol. Chem. 267, 18336–18341

39. Geertsema, E. R., and Dutzler, R. (2011) A versatile and efficient high-throughput cloning tool for structural biology. Biochemistry 50, 3272–3278

40. Pourcher, T., Leclercq, S., Brandolin, G., and Leblanc, G. (1995) Melibiose permease of *Escherichia coli*: large scale purification and evidence that H^+^, Na^+^, and Li^+^ sugar sympol is catalyzed by a single polypeptide. Biochemistry 34, 4412–4420

41. Chae, P. S., Rasmussen, S. G., Rana, R. R., Gotfryd, K., Chandra, R., Goren, M. A., Kruse, A. C., Nuruva, S., Loland, C. J., Pierre, Y., Drew, D., Popot, J. L., Picot, D., Fox, B. G., Guan, L., Gether, U., Byrne, B., Kobilka, B., and Gellman, S. H. (2010) Maltose-neopentyl glycol (MNG) amphiphiles for solubilization, stabilization and crystallization of membrane proteins. Nat. Methods 7, 1003–1008

42. Zakariassen, H., and Soreide, M. (2007) Heat capacity changes in heme protein-ligand interactions. Thermochimica Acta 464, 24–28

43. Pourcher, T., Deckert, M., Bassilana, M., and Leblanc, G. (1991) Melibiose permease of *Escherichia coli*: mutation of aspartic acid 55 in putative helix II abolishes activation of sugar binding by Na^+^ ions. Biochem. Biophys. Res. Commun. 178, 1176–1181

44. Nie, Y., Smirnova, I., Kasho, V., and Kaback, H. R. (2006) Energetics of ligand-induced conformational flexibility in the lactose permease of *Escherichia coli*. J. Biol. Chem. 281, 35779–35784

45. Cai, M., Williams, D. C., Jr., Wang, G., Lee, B. R., Peterkofsky, A., and Clore, G. M. (2003) Solution structure of the phosphoryl transfer complex between the signal-transducing protein IIA^Glucose^ and the cytoplasmic domain of the glucose transporter IICB^Glucose^ of the *Escherichia coli* glucose-phosphotransferase system. J. Biol. Chem. 278, 25191–25206

46. Kumar, H., Kasho, V., Smirnova, I., Finer-Moore, J. S., Kaback, H. R., and Stoud, R. M. (2014) Structure of sugar-bound LacY. Proc. Natl. Acad. Sci. U.S.A. 111, 1784–1788

47. Chaptal, V., Kwon, S., Sawaya, M. R., Guan, L., Kaback, H. R., and Abramson, J. (2011) Crystal structure of lactose permease in complex with an affinity inactivator yields unique insight into sugar recognition. Proc. Natl. Acad. Sci. U.S.A. 108, 9361–9366