Thermodynamic mechanism for inhibition of lactose permease by the phosphotransferase protein IIA^{Glc}

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In a variety of bacteria, the phosphotransferase protein IIA^{Glc} plays a key regulatory role in catabolite repression in addition to its role in the vectorial phosphorylation of glucose catalyzed by the phosphoenolpyruvate:carbohydrate phosphotransferase system (PTS). The lactose permease (LacY) of *Escherichia coli* catalyzes stoichiometric symport of a galactoside with an H^+, using a mechanism in which sugar- and H^+-binding sites become alternatively accessible to either side of the membrane. Both the expression (via regulation of cAMP levels) and the activity of LacY are subject to regulation by IIA^{Glc} (inducer exclusion). Here we report the thermodynamic features of the IIA^{Glc}–LacY interaction as measured by isothermal titration calorimetry (ITC). The studies show that IIA^{Glc} binds to LacY with a K_d of about 5 μM and a stoichiometry of unity and that binding is driven by solvation entropy and opposed by enthalpy. Upon IIA^{Glc} binding, the conformational entropy of LacY is restrained, which leads to a significant decrease in sugar affinity. By suppressing conformational dynamics, IIA^{Glc} blocks inducer entry into cells and favors constitutive glucose uptake and utilization. Furthermore, the studies support the notion that sugar binding involves an induced-fit mechanism that is inhibited by IIA^{Glc} binding. The precise mechanism of the inhibition of LacY by IIA^{Glc} elucidated by ITC differs from the inhibition of melibiose permease (MelB), supporting the idea that permeases can differ in their thermodynamic response to binding IIA^{Glc}.

ITC | PTS | sugar/cation symport | protein–protein interactions | protein conformation

Carbohydrate uptake in bacteria is catalyzed by a collection of sugar permeases that belong to different families of transport proteins. In *Escherichia* and *Salmonella*, the phosphoenolpyruvate:carbohydrate phosphotransferase system (PTS) carries out both catalytic and regulatory functions and plays a key role in catabolite repression resulting in preferential utilization of glucose (a constitutive process) that is transported by vectorial phosphorylation catalyzed by the PTS (1–5). The phosphotransferase protein IIA^{Glc} plays a direct role in this regulation of inducible transport systems. The *lac* operon (6) with *lacZ* encoding β-galactosidase and *lacY* encoding lactose permease (LacY) and the *mel* operon (7, 8) with *melA* encoding α-galactosidase and *melB* encoding melibiose permease (MelB) are subject to IIA^{Glc} regulation (9–13). Both LacY and MelB catalyze electrogenic symport of a galactoside with a cation (14–21). Expression of the structural genes requires the participation of both a global transcriptional activator (the cAMP–CAP complex) and a specific inducer (lactose or melibiose, respectively) (3, 22, 23). IIA^{Glc} regulates both cAMP (24) and inducer levels, and this study focuses on regulation of LacY, which influences inducer entry into the cell.

With maltose permease, an ABC permease also under PTS regulation, two molecules of IIA^{Glc} bind to the cytoplasmic ATPase subunits and consequently prevent the structural rearrangements necessary for ATP hydrolysis (25). Recently, we reported that IIA^{Glc} binds to *Escherichia coli* $MelB_{Ec}$ or *Salmonella typhimurium* $MelB_{St}$ with a stoichiometry of one and that enthalpy drives the interaction in the absence or presence of melibiose (26). Furthermore, IIA^{Glc} binding reduces sugar affinity and the conformational entropy of $MelB_{St}$, resulting in a block of melibiose entry into the cell, thus preventing induction of the *mel* operon. With LacY, IIA^{Glc} binding has been extensively studied (9, 10, 27–29). In a direct binding assay, IIA^{Glc} binding to LacY exhibits a dissociation constant (K_d) of 1 μM, and binding requires the presence of sugar substrate; however, a stoichiometry of one molecule of IIA^{Glc} to six molecules of LacY was obtained, which seems highly unusual (10).

Both X-ray crystallographic and NMR structures of IIA^{Glc} (25, 30, 31) reveal that this soluble protein is structurally rigid (Fig. 1). The crystal structures of LacY (14, 32–36) and $MelB_{St}$ (37) both exhibit N- and C-terminal domains composed of six largely irregular transmembrane helices positioned pseudosymmetrically and surrounding a deep aqueous cavity (Fig. 1). Both permeases are members of the major facilitator superfamily. WT $MelB_{St}$ was crystallized in two outward (periplasmic)-open conformations with a sealed cytoplasmic side (37). WT LacY was crystallized in an inward (cytoplasmic)-open conformation (32). Recently, a LacY conformational mutant was captured in an almost occluded, narrowly outward-open conformation with a completely liganded galactoside molecule in the middle of the protein (38). Evidence was also presented indicating that sugar binding involves induced fit, which causes LacY to transition from an open state to an occluded intermediate.

Significance

Carbohydrate uptake in many bacteria is regulated by the phosphotransferase protein IIA^{Glc}, enabling cells to use glucose preferentially over other sugars. Lactose permease (LacY) is one of many sugar permeases regulated by IIA^{Glc}, but the mechanism of inducer exclusion is unclear. We now show by isothermal titration calorimetry that IIA^{Glc} binds to purified LacY with a stoichiometry of one, and that the interaction is driven by favorable solvation entropy. IIA^{Glc} binding inhibits conformational dynamics of LacY and decreases binding affinity for sugar in a manner similar to that observed for melibiose permease (MelB). However, the thermodynamic mechanism by which the inhibitory effect is expressed differs for the two permeases.

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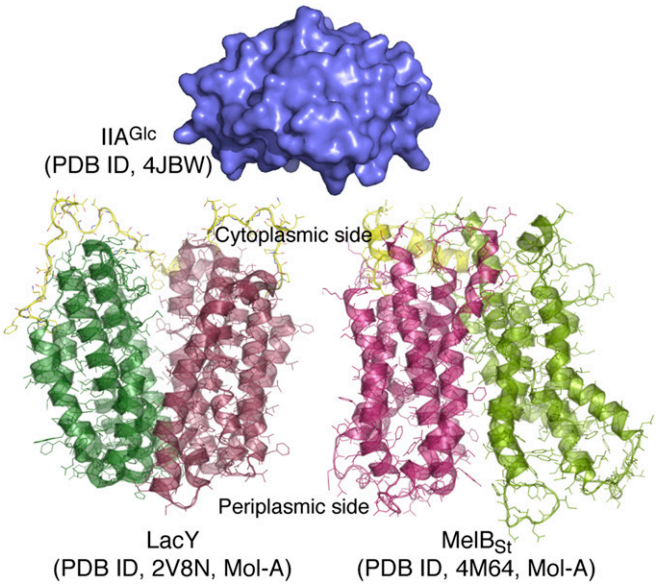

Fig. 1. Crystal structures. All of the depicted structural models are on the same scale. The surface representation of IIA^{Glc} is colored blue [Protein Data Bank (PDB) ID code 4JBW]. LacY (PDB ID code 2V8N; Mol-A) and $MelB_{St}$ (PDB ID code 4M64; Mol-A) are shown with the cytoplasmic side at the top. The N- and C-terminal domains of both permeases are shown in different colors, and their middle cytoplasmic loops are colored yellow. LacY and $MelB_{St}$ are in an inward-open and partially outward-open conformation, respectively.

It is generally believed that a crystal structure typically captures a molecule in the lowest free-energy state. With regard to LacY, abundant, independent lines of evidence demonstrate that the inward-open conformation in the absence of bound substrate represents the lowest free-energy state in both the membrane-embedded and detergent-solubilized state (39). LacY functions by exposing its substrate-binding sites (sugar and H^+) alternatively to either side of the membrane via an alternating access mechanism (36, 39, 40), and a similar model has also been proposed for MelB (37, 41, 42). With both permeases, it has been well-documented that ligand binding induces conformational changes (39, 41, 43, 44).

The ground-state conformations of protonated LacY and Na^+-bound MelB appear to have opposite orientations, LacY inward-open and $MelB_{St}$ outward-open. To compare IIA^{Glc} binding with the two symporters, we have used isothermal titration calorimetry (ITC). The results indicate that IIA^{Glc} binding inhibits conformational entropy in both transporters, but by different energetic mechanisms.

Results

IIA^{Glc} Binding to WT LacY. LacY or IIA^{Glc} samples were pre-equilibrated with melibiose (a substrate for LacY) at a saturating concentration. Titration of LacY with IIA^{Glc} yields an endothermic titration curve (Fig. 2 *A*, black and *C*). When injecting IIA^{Glc} into melibiose-containing buffer in the absence of LacY, the thermogram is flat and exothermic (Fig. 2*A*, cyan). A plot of the accumulated heat change (ΔQ) vs. the molar ratio fits a one-site independent binding model (Fig. 2*C*), which yields a K_d of 5.7 μM (SEM, 1.11; $n = 3$) and a stoichiometry of unity. The binding affinity is in a range similar to that obtained by direct binding with ^{125}I-labeled IIA^{Glc} (10), as well as the affinity obtained with $MelB_{St}$ (26). Energetically (Table 1), binding is driven by a favorable entropy change ($-T\Delta S$) and opposed by a change in enthalpy (ΔH), which is opposite to that found with IIA^{Glc} binding to $MelB_{St}$ or $MelB_{Ec}$, where binding is driven enthalpically and opposed by an entropic change (26).

Under the same experimental conditions without sugar, endothermic binding is also observed (Fig. 2 *B* and *D*), but with much smaller heat changes. A best fit of the data suggests a K_d of 6.6 μM (SEM, 0.65; $n = 4$), similar to that obtained in the presence of melibiose. The finding stands in opposition to previous conclusions that IIA^{Glc} binding requires binding of substrate (10, 27, 45). It is likely that the ITC method is more sensitive than previously used methods.

IIA^{Glc} Binding to LacY Mutants. Two structurally resolved LacY mutants, C154G (33, 35) and G46W/G262W (38), bind sugar with an affinity similar to WT LacY but do not catalyze transport (32, 46, 47). In the absence of sugar, mutants C154G and G46W/G262W exhibit inward-open and outward-open conformations, respectively. A previous study reported that C154G LacY does not bind IIA^{Glc} (10). Titration of C154G LacY with IIA^{Glc} (Fig. 3, *Upper*, black curves) or with buffer (cyan) in the presence (Fig. 3*A*) or absence (Fig. 3*B*) of melibiose yields exothermic peaks with flat titration curves. The data are consistent with the conclusion that IIA^{Glc} does not bind to the C154G LacY mutant. Similar results were obtained with mutant G46W/G262W (Fig. 3, *Lower*, black curves) in the presence (Fig. 3*C*) or absence (Fig. 3*D*) of melibiose, indicating that IIA^{Glc} also does not bind to either the outward-open (47) or the almost occluded, outward-open conformation of the conformationally compromised LacY mutant.

Heat Capacity Change of IIA^{Glc} Binding to LacY. IIA^{Glc} binding to WT LacY in the presence of melibiose was tested further over a range of temperatures from 15 to 30 °C. With increasing

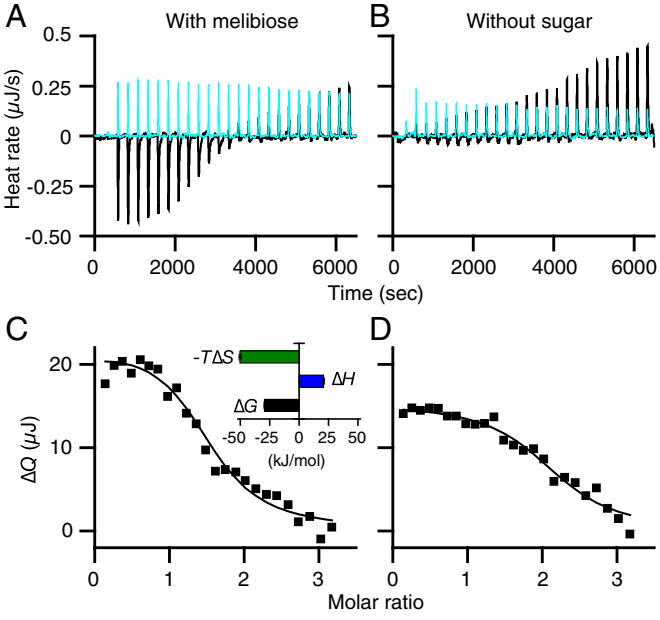

Fig. 2. ITC measurements of IIA^{Glc} binding to LacY. (*A* and *B*) Thermograms were recorded at 20 °C. Black curves, titrations of LacY (50 μM) with IIA^{Glc} (455 μM) in the presence or absence of melibiose at 10 mM as indicated. Cyan curves, titrations of buffer in the absence of LacY with IIA^{Glc} under the same conditions. (*C* and *D*) Data fitting. Accumulated heat change (ΔQ) was plotted against the IIA^{Glc}/LacY molar ratio, and the data were fitted to a one-site independent binding model as described in *Materials and Methods*. (*C*, *Inset*) Histograms showing ΔG, ΔH, and $-T\Delta S$ as described in *Materials and Methods*; the values are also presented in Table 1. Error bars denote SEM, $n = 3$.

Table 1. IIA^{Glc} binding to LacY

Temperature, °C (K)	K_a (/mol)	K_d (μM)	ΔG (kJ/mol)	ΔH (kJ/mol)	$-T\Delta S$ (kJ/mol)	ΔS (J/mol·K)
15 (288.15)	213,900	4.68	−29.41	28.06	−57.46	199.40
20 (293.15)	187,167 (30,739*)	5.71 (1.11)	−29.52 (0.45)	20.63 (1.02)	−50.15 (0.78)	171.1 (2.65)
25 (298.15)	173,433 (7,597)	5.79 (0.24)	−29.90 (0.11)	11.33 (0.10)	−41.23 (0.04)	138.27 (0.15)
30 (303.15)	215,400	4.64	−30.95	5.06	−36.01	118.80

Data are presented in Figs. 2 and 4. *SEM, number of tests, 2–4.

temperature, $-T\Delta S$ becomes less favorable and ΔH becomes less unfavorable (Fig. 4 and Table 1). As a result of the good enthalpy–entropy compensation, there is little change in free energy (ΔG) or K_d with temperature. The linear fit of ΔH with temperature reveals a large negative value of heat capacity change (ΔC_p) (slope) of about −1,566 J/mol·K.

The solvation entropy change (ΔS_{solv}) is associated with the change in solvation of IIA^{Glc} and LacY upon binding. Because the ΔC_p value has been determined, the ΔS_{solv} value at 25 °C can be calculated from the equation $\Delta S_{solv} = \Delta C_p \ln (298.15/385.15)$ (48, 49), yielding a value of 400.9 J/mol·K. The calculated $-T\Delta S_{solv}$ is −119.53 kJ/mol, suggesting that a very favorable solvation entropy is the major driving force for IIA^{Glc} binding to LacY (Fig. 5 and Table 2). The measured total reaction ΔS is the net value of $\Delta S_{mix} + \Delta S_{solv} + \Delta S_{conf}$ (48, 49). The mixing entropy change ΔS_{mix} can be estimated by $\Delta S_{mix} = R \ln (1/55.5)$, that is, −33 J/mol·K (48, 49). ΔS_{conf} is the conformational entropy change associated with the change in the side-chain and backbone conformation of IIA^{Glc} and LacY upon binding. Because the ΔS value at 25 °C was also determined (Table 1), ΔS_{conf} can be calculated from the equation $\Delta S_{conf} = \Delta S - \Delta S_{solv} - \Delta S_{mix}$, which yields a value of −229.63 J/mol·K. The calculated $-T\Delta S_{conf}$ is 68.46 kJ/mol with a positive sign, suggesting that the conformational entropy for the complex is largely unfavorable (Fig. 5 and Table 2).

Inhibition of Galactoside Affinity by IIA^{Glc}. Binding of nitrophenyl-α-D-galactopyranoside (α-NPG) to LacY has been determined by a number of methods including ITC (50–52). Titration of WT LacY with a solution of 1 mM α-NPG reveals exothermic peaks (Fig. 6*A*, black curve). By contrast, injection of nitrophenyl-α-D-glucoside (α-NPGlu), which does not bind to LacY, yields a flat titration curve with small exothermic peaks (Fig. 6*A*, *Inset*), strongly supporting the conclusion that the α-NPG titration curve reflects specific binding to LacY. Curve fitting using the one-site independent binding model (Fig. 6*D*, black curve) reveals a K_d value of ca 27 μM and, energetically, the interaction is driven by both favorable ΔH and $-T\Delta S$ (Fig. 6*G* and Table 3), which is consistent with previous observations (51).

Sequential injection of a 1 mM solution of α-NPG into LacY preequilibrated with IIA^{Glc} at a ratio of 3:1 (IIA^{Glc}:LacY) yields small heat changes and a flat titration, suggesting that IIA^{Glc} may significantly inhibit sugar binding (Fig. 6 *A* and *D*, orange curve or triangles). To determine the affinity and free energy for binding, a 10 mM solution of α-NPG was used, which yields a K_d value of about 1 mM, an increase of ~37-fold (Fig. 6*D*, *Inset*, violet curve). As a control, injection of α-NPGlu at the higher concentration is flat (Fig. 6*D*, *Inset*, gray triangles). Strikingly, the decrease in $-T\Delta S$ accounts for the decrease in ΔG, because ΔH does not change significantly (Fig. 6*G* and Table 3).

α-NPG binding to C154G LacY exhibits thermodynamic features similar to those described in previous studies (Fig. 6 *B*, *E*, and *H* and Table 3) (51). Titration of G46W/G262W LacY with α-NPG yields a K_d value of 6 μM (Fig. 6 *C*, *F*, and *I* and Table 3). In both of the conformationally restrained mutants, the free-energy contribution is different from the WT; ΔH drives sugar binding and is opposed by $-T\Delta S$, particularly in the G46W/G262W LacY. As further controls for the observed IIA^{Glc} inhibitory effects on sugar binding, both mutants, which do not bind IIA^{Glc}, also do not exhibit decreased α-NPG affinity in the presence of IIA^{Glc} (Fig. 6 *B* and *C*, orange curves). The results strongly support the conclusion that IIA^{Glc} inhibits the affinity of LacY for galactoside.

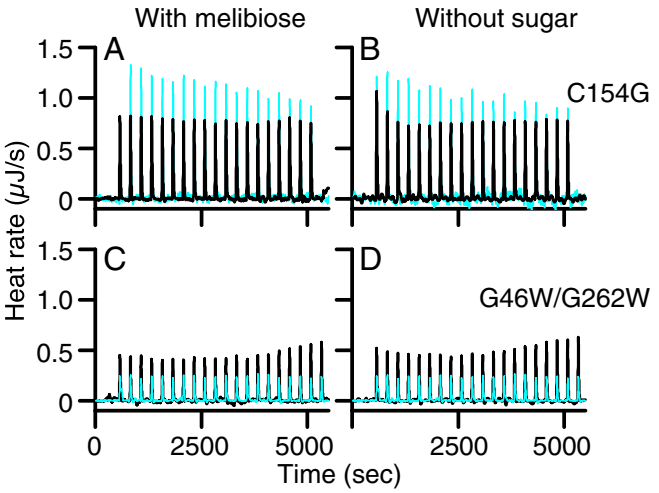

Fig. 3. Titration of LacY mutants with IIA^{Glc}. Thermograms were recorded at 25 °C in the presence or absence of melibiose at 10 mM. (*A* and *B*) C154G LacY mutant (50 μM) was titrated with IIA^{Glc} at a concentration of 780 μM. (*C* and *D*) G46W/G262W LacY mutant (50 μM) was titrated with IIA^{Glc} at a concentration of 455 μM. Black curves, injections of IIA^{Glc} into LacY mutants. Cyan curves, injections of IIA^{Glc} into buffer.

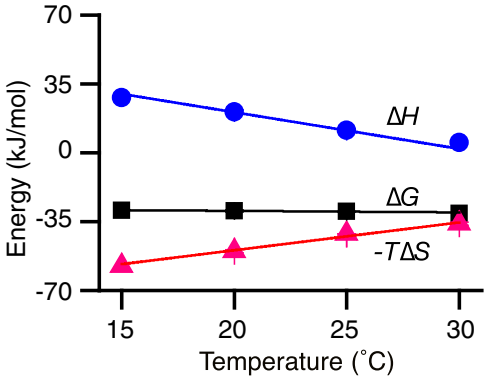

Fig. 4. Temperature effect and ΔC_p determination. Under the conditions described in the legend to Fig. 2, IIA^{Glc} binding to the WT LacY in the presence of melibiose was tested at 15, 20, 25, and 30 °C. Thermodynamic data are presented in Table 1; ΔG, ΔH, and $-T\Delta S$ values are plotted against temperature and fitted with a linear function. The slope ($\Delta H/\Delta T$) is ΔC_p, which is negative (−1,566 J/mol·K).

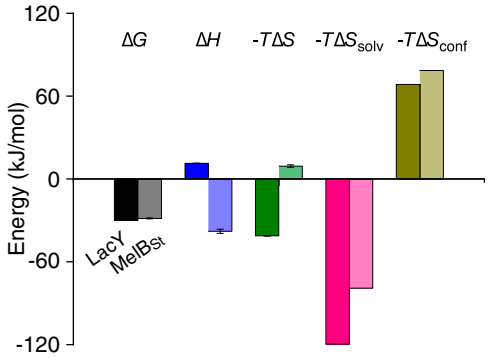

Fig. 5. Comparison of binding energy of IIA^{Glc} to LacY or MelB$_{St}$. The thermodynamic data for IIA^{Glc} binding to LacY (first bar in each pair) are from Tables 1 and 2; data for IIA^{Glc} binding to MelB$_{St}$ (second bar in each pair) are from ref. 26. Error bars denote SEM, n = 2–3.

Discussion

The ITC studies presented here show that IIA^{Glc} regulation of LacY and MelB (26) is due to direct interaction with these permeases. In both cases, IIA^{Glc} binding exhibits similar affinities and a stoichiometry of unity. With LacY, binding is endothermic and driven solely by ΔS with compensation by ΔH. In contrast, with MelB, IIA^{Glc} binding is exothermic and driven solely by enthalpic forces with entropic compensation (Fig. 5). The observation that different driving forces for IIA^{Glc} binding are observed for the two conformationally flexible permeases is notable. In either case, linear enthalpy–entropy compensation is obtained, which plays a crucial role in the thermodynamic mechanisms of the protein–protein interactions.

Thus far, the conformation of the IIA^{Glc}-bound state has not been revealed with either MelB or LacY. MelB$_{St}$ has a closed cytoplasmic surface, and IIA^{Glc} binding is enthalpy-driven. LacY is open on the cytoplasmic side, and IIA^{Glc} binding is entropy-driven, specifically by solvation entropy. A favorable solvation entropy change ($-T\Delta S_{solv}$ with negative value) usually indicates that water molecules, which are localized on the surfaces of the binding molecules, gain degrees of freedom by being released to the bulk solvent upon binding (53). This suggests that LacY must undergo a structural rearrangement(s) to form a surface that binds IIA^{Glc}, accompanied by a large conformational change that involves release of water molecules. Consistently, the two conformationally constrained LacY mutants (C154G in an inside-open conformation and G46W/G262W in an inside-closed configuration) do not bind IIA^{Glc}. Probably, both proteins are unable to form a IIA^{Glc}-binding surface because of restricted conformational flexibility.

Regardless of the different thermodynamic mechanisms responsible for IIA^{Glc} binding to LacY and MelB, a common feature is observed. The observed large unfavorable ΔS_{conf} indicates that the IIA^{Glc}-bound LacY or MelB loses degrees of freedom with respect to conformational changes. This is consistent with the functionality of IIA^{Glc}. On the other hand, sugar binding to LacY involves induced fit (38) in which there is a conformational transition from an open conformer to an occluded state that can open to either side of the membrane. We have shown that IIA^{Glc} decreases α-NPG affinity by more than 30-fold with LacY (Table 3) and by 5-fold with MelB$_{St}$ (26). IIA^{Glc} binding prevents both permeases from interacting optimally with sugars and thereby inhibits the induced-fit process with both regulated permeases. This conclusion is also supported by the energetic change observed with respect to the driving force for sugar binding. With the LacY–IIA^{Glc} complex, the large decrease in sugar-binding affinity is due solely to a decrease in favorable entropy change (Fig. 6). It appears that the initial contacts involving the specificity of sugar binding are enthalpically driven, probably through polar or hydrophilic interactions. Taken together, the thermodynamic insight into the mechanism of IIA^{Glc} regulation of disaccharide uptake by LacY and MelB involves blocking conformational transitions and trapping both permeases in a low-affinity state.

When any PTS sugar is being transported, IIA^{Glc} is predominantly in the dephosphorylated form, which can bind to and inhibit the activity of non-PTS permeases. In the absence of PTS sugars, IIA^{Glc} is mainly in the phosphorylated form, which does not bind to or inhibit permeases (3, 5). Thus, the global mechanism for regulation is clearly dependent on the functionality of the PTS. The regulatory protein IIA^{Glc} blocks inducer entry into the cell, which prevents activation of a specific operon. As a consequence, cells preferentially use glucose by the PTS via a mechanism defined previously as inducer exclusion (54).

A comparison (Fig. 5) of the current study on LacY with the previous (26) study on MelB indicates that the objective of permease inhibition by IIA^{Glc} can be accomplished by a variety of thermodynamic mechanisms.

Materials and Methods

Reagents. Nitrophenyl-α-galactoside and nitrophenyl-α-glucoside were purchased from Sigma-Aldrich. The detergents undecyl-β-D-maltopyranoside and dodecyl-β-D-maltopyranoside were from Anatrace.

Plasmids and Strains. The T7-based expression plasmid p7XNH3/IIA-NH10 encoding *E. coli* IIA^{Glc} with a 10-His tag and a 9-residue linker (MHHHHHH-HHHHLEVLFQGPS) at the N terminus was constructed as described (26). Construction of plasmids pT7-5 LacY (32), pT7-5/C154G LacY (33), and pT7-5/G46W/G262W LacY (38), each with a His tag at the C terminus to allow metal-affinity purification, has been described. Overexpression of IIA^{Glc} or LacY was performed in the *E. coli* T7 express strain (NEB) or XL1 Blue (Stratagene), respectively.

IIA^{Glc} Expression and Purification. The expression and purification of IIA^{Glc} were carried out as described (26). Briefly, cells were grown in LB containing 5 mM glucose and induced with isopropyl β-D-1-thiogalactopyranoside. IIA^{Glc} was purified by cobalt-affinity chromatography and concentrated to ~100 mg/mL using a Vivaspin 20 (5,000 MWCO PES; Millipore) in a buffer containing 20 mM Tris·HCl (pH 7.5), 100 mM NaCl, and 10% (vol/vol) glycerol. Protein was measured by the Micro BCA Protein Assay (Pierce Biotechnology). Purified IIA^{Glc} was in the unphosphorylated form as analyzed with both SDS/PAGE and Phos-tag SDS/PAGE (26).

Table 2. Parameterization of reaction entropy change ΔS

	ΔC_p*	$\Delta S^{\dagger}$	ΔS_{mix}	ΔS_{solv}	ΔS_{conf}	$-T\Delta S^{\ddagger}$	$-T\Delta S_{mix}$	$-T\Delta S_{solv}$	$-T\Delta S_{conf}$
Permease	(J/mol·K)					(kJ/mol)			
LacY	−1,566	138.27	−33	400.90	−229.63	−41.23	9.84	−119.53	68.46
MelB[§]	−1,036	−31.32	−33	265.21	−263.54	9.34	9.84	−79.07	78.58

*$\Delta C_p = \Delta H/\Delta T$, obtained from linear fitting (Fig. 4).
[†]ΔS determined at 25 °C.
[‡]$T\Delta S = 298.15 \times \Delta S$.
[§]Data are from ref. 26.

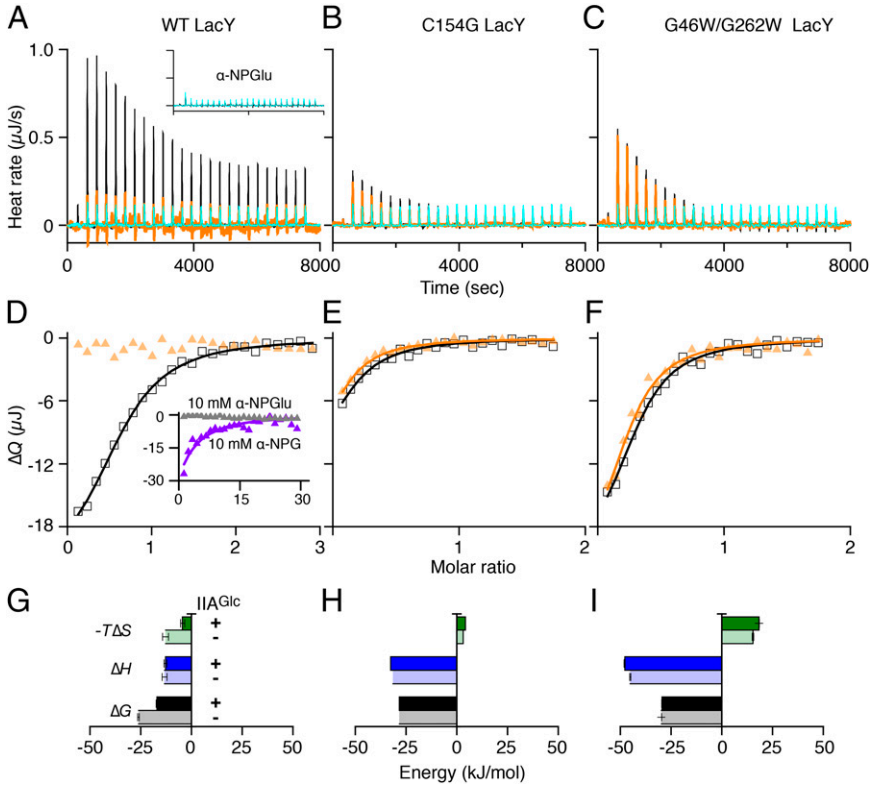

Fig. 6. Sugar-binding energetics and IIA^{Glc} effect. (*A–C*) Equilibrated to 25 °C, α-NPG at 1 mM was injected into the cell containing WT LacY (120 μM), or α-NPG at 250 μM was titrated into the C154G mutant or G46W/G262W LacY (50 μM), without (black curves) or preequilibrated with IIA^{Glc} at a concentration threefold higher than the LacY concentration (orange curves). DMSO (0.5%) was present in both titrand and titrant to guarantee α-NPG solubility. Cyan curves, injections of α-NPG at 1 mM (*A*) or 250 μM (*B* and *C*) solution into buffers under the same conditions. (*A*, *Inset*) Thermograms were recorded from the injection of 1 mM α-NPGlu solution into 120 μM LacY (black curve) or buffer (cyan curve). (*D–F*) ΔQ was plotted against the α-NPG/LacY molar ratio and fitted to a one-site independent binding model. Empty squares, titration of LacY with α-NPG at 1 mM solution in the absence of IIA^{Glc}; filled yellow triangles, titration of LacY–IIA^{Glc} complex with α-NPG at 1 mM solution. (*D*, *Inset*) Data derived from injecting a solution of 10 mM α-NPG (violet curve) or α-NPGlu (gray triangles) into 120 μM LacY/360 μM IIA^{Glc} complex. (*G–I*) Sugar-binding energy. ΔG, ΔH, and $-T\Delta S$ values in the absence (histograms filled with lighter colors) and presence of IIA^{Glc} (histograms filled with darker colors) that are from Table 3. Error bars denote SEM, $n = 2$.

LacY Expression and Purification. Cell growth, overexpression, and purification of LacY proteins were carried out as described for LacY (32) with a few modifications. The buffer used for the WT LacY contained 20 mM Tris·HCl (pH 7.5), 100 mM NaCl, 0.035% undecyl-β-D-maltopyranoside, and 10% glycerol. The buffer used for the LacY mutants was the same except that dodecyl-β-D-maltopyranoside (0.01%) was used instead of undecyl-β-D-maltopyranoside. Proteins were concentrated to 15–20 mg/mL, aliquoted, flash-frozen in liquid nitrogen, and stored at −80 °C.

ITC. ITC measurements were performed on a Nano Isothermal Titration Calorimeter (TA Instruments). Purified LacY was placed in the sample cell with a reaction volume of 163 μL. IIA^{Glc} was prepared in the same buffer by dilution from a highly concentrated sample, and then 2- or 2.5-μL aliquots were injected incrementally into the sample cell at an interval of 250 or 300 s with constant stirring at 250 rpm.

For sugar binding to LacY or the LacY–IIA^{Glc} complex, α-NPG or α-NPGlu was dissolved in dimethyl sulfoxide (DMSO) and diluted with assay buffer to 1 or 10 mM containing 0.5% DMSO. DMSO was added to the protein samples for buffer matching. When testing for the IIA^{Glc} effect, IIA^{Glc} was incubated with LacY at a 3:1 ratio for 3 h before injection of sugar.

The raw thermogram was applied for a baseline correction. A blank correction for the heat of dilution was made by subtracting the integrated peak area from a constant estimated from the convergence value of the final injections. The corrected heat change (ΔQ) was plotted against the molar ratio of IIA^{Glc}/LacY or sugar/LacY. Binding stoichiometry, the association constant (K_a), and ΔH values were directly determined by fitting the data using the one-site independent binding model (55) provided by NanoAnalyze version 2.3.6 software (TA Instruments). The dissociation constant is $1/K_a$. ΔS values were obtained by calculation using the equation $\Delta G = -RT \ln K_a$ and $T\Delta S = \Delta H - \Delta G$. R, gas constant, 8.315 J/mol·K; T, absolute

Table 3. IIA^{Glc} inhibition of α-NPG binding

Protein	K_d (μM)	ΔG (kJ/mol)	ΔH (kJ/mol)	$-T\Delta S$ (kJ/mol)
WT LacY	27.50 (4.40*)	−26.06 (0.40)	−13.25 (1.15)	−12.80 (1.55)
WT/IIA^{Glc}	1,013 (114)	−17.11 (0.27)	−12.74 (0.75)	−4.38 (1.02)
C154G mutant	10.80	−28.33	−31.56	3.23
C154G/IIA^{Glc}	10.70	−28.38	−32.66	4.28
G46W/G262W mutant	6.16 (0.28)	−29.75 (0.11)	−45.10 (0.33)	15.35 (0.44)
G46W/G262W/IIA^{Glc}	7.05 (0.69)	−29.42 (0.24)	−47.84 (0.31)	18.42 (0.07)

ITC measurements were studied at 25 °C; data are presented in Fig. 6. *SEM, number of tests, 2.

temperature. Parameterization of ΔS was calculated as described previously (49, 53). Total $\Delta S = \Delta S_{mix} + \Delta S_{solv} + \Delta S_{conf}$. ΔS_{mix} was calculated as reported (48), which reflects the mixing of solute and solvent molecules, and entropy change due to changes in translational/rotational degrees of freedom. Based on the bimolecular binding reaction using a 1 M standard state, $\Delta S_{mix} = R \ln (1/55.5) = -33$ J/mol·K. The entropy of polar and apolar solvation is close to zero at a temperature of near 385 K, and then solvent entropy change at 25 °C is given as $\Delta S_{solv} = \Delta C_p \ln (298.15/385.15)$ (48). ΔC_p of the binding was obtained by fitting ΔH versus temperature. $\Delta S_{conf} = \Delta S - \Delta S_{mix} - \Delta S_{solv}$.

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