Energetics of Ligand-induced Conformational Flexibility in the Lactose Permease of *Escherichia coli* 

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Isothermal titration calorimetry has been applied to characterize the thermodynamics of ligand binding to wild-type lactose permease (LacY) and a mutant (C154G) that strongly favors an inward facing conformation. The affinity of wild-type or mutant LacY for ligand and the change in free energy upon binding are similar. However, with the wild type, the change in free energy upon binding is due primarily to an increase in the entropic free energy component ($\Delta S$), whereas in marked contrast, an increase in enthalpy ($\Delta H$) is responsible for $\Delta G$ in the mutant. Thus, wild-type LacY behaves as if there are multiple ligand-bound conformational states, whereas the mutant is severely restricted. The findings also indicate that the structure of the mutant represents a conformational intermediate in the overall transport cycle.

The lactose permease of *Escherichia coli* (LacY), a member of the Major Facilitator Superfamily of membrane transport proteins, couples the stoichiometric translocation of a galactoside and an H$^+$ (reviewed in Refs. 1, 2). As such, LacY utilizes free energy stored in an electrochemical H$^+$ gradient ($\Delta \mu_{H^+}$) to drive accumulation of galactosidic sugars against a concentration gradient. Conversely, in the absence of $\Delta \mu_{H^+}$, LacY utilizes free energy released from downhill translocation of galactosides to drive uphill translocation of H$^+$ with generation of H$^+$ gradients by themselves generate a $\Delta \mu_{H^+}$, the polarity of which depends on the direction of the sugar gradient. Composed of 417 amino acid residues, ~70% of which are hydrophobic, LacY has been solubilized, purified, and reconstituted into proteoliposomes in a fully functional state. Because galactoside gradients by themselves generate a $\Delta \mu_{H^+}$, and has been postulated that the primary driving force for turnover is binding and dissociation of sugar on either side of the membrane.

An x-ray structure of mutant C154G, which binds ligand as well as wild-type LacY but catalyzes very little transport and is compromised conformationally (3–6), has been solved in an inward facing conformation with bound ligand (Fig. 1) (7). Notably, wild-type LacY has the same global fold (2, 8). LacY contains 12 transmembrane helices organized in two pseudo-symmetrical $\alpha$-helical bundles. The N- and C-terminal 6-helix domains form a large internal cavity open to the cytoplasm. A single sugar-binding site is observed at the apex of the cavity near the approximate middle of the molecule. The structure confirms many previous findings obtained from site-directed biochemical and biophysical studies (1, 2).

Only a handful of side chains are essential with respect to the symport mechanism. Arg$^{144}$ (helix V) forms a bi-dentate H-bond with the O$_4$ and O$_5$ atoms of the galactopyranosyl ring (7), confirming the critical role of this residue in sugar binding and recognition (2). Glu$^{126}$ (helix IV), another important residue for binding, is in close proximity to Arg$^{144}$ and may interact with the O$_4$, O$_5$, or O$_6$ atoms of the galactopyranosyl ring via water molecules. An aromatic residue at position 151 (helix V), preferably Trp, is irreplaceable for sugar binding (9), stacking hydrophobically with the galactopyranosyl ring (10, 11). Glu$^{269}$ (helix VIII) in the C-terminal domain, which is involved in both sugar binding and H$^+$ translocation and interacts with the O$_3$ atom of the galactopyranosyl ring, forms a salt bridge with Arg$^{144}$ and is in close proximity to Trp$^{151}$ (7, 11–13). His$^{322}$ (helix X), Glu$^{325}$ (helix X), and Arg$^{302}$ (helix IX), as well as Tyr$^{236}$ (helix VII), are likely involved directly in H$^+$ translocation (2, 14). His$^{322}$ may be the immediate H$^+$ donor to Glu$^{325}$, and Arg$^{302}$ may interact with Glu$^{325}$ to drive deprotonation (14, 15).

In the ligand-free state, the essential residues for substrate binding and specificity (Arg$^{144}$, Glu$^{126}$, and Glu$^{269}$) are not in the correct configuration to bind substrate, and the following sequence of events has been suggested based upon structural changes (13). Sugar initially recognizes Trp$^{151}$ through nonspecific hydrophobic stacking between the galactopyranosyl and indole rings (9, 10). This interaction orients the galactopyranosyl moiety for recognition by Arg$^{144}$, Glu$^{126}$, and Glu$^{269}$ (i.e. induced fit) and disrupts the salt bridge between Arg$^{144}$ and Glu$^{126}$, and a bi-dentate H-bond is formed with Arg$^{144}$ through the O$_4$ and O$_5$ groups on the galactopyranosyl ring. Subsequently, protonated Glu$^{269}$ moves out of a relatively hydrophobic environment, deprotonates, and forms a salt bridge with Arg$^{144}$ and an H-bond with sugar to complete ligand binding, which triggers the global conformational change that allows accessibility of the binding site to the other side of the membrane.

Here, we report a thermodynamic characterization of sugar binding with wild-type LacY and the C154G mutant by using...
isothermal titration calorimetry (ITC),\(^2\) a technique that allows measurement of changes in free energy (\(\Delta G\)), enthalpy (\(\Delta H\)), and entropic free energy component (\(T \Delta S\)), and heat capacity (\(\Delta C_p\)) (16). \(\beta\)-Galactopyranosyl 1-thio-\(\beta\)-d-galactopyranoside (TDG) or \(p\)-nitrophenyl \(\alpha\)-d-galactopyranoside (NPG) bind to wild-type LacY, and the entropic free energy component is the major component that contributes to \(\Delta G\). In striking contrast, enthalpy alone is responsible for \(\Delta G\) in the mutant. Thus, multiple ligand-bound conformational states are present in the wild-type protein, whereas the mutant is severely restricted with respect to conformational changes.

**EXPERIMENTAL PROCEDURES**

**Materials**—E. coli XL-1 Blue (recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F’ proAB lacZAM15 Tn10 (Tet’)]) was obtained from Stratagene (La Jolla, CA). TDG and NPG were purchased from Sigma. Isopropyl 1-thio-\(\beta\)-d-galactopyranoside and \(n\)-dodecyl \(\beta\)-d-maltopyranoside (DDM) were purchased from Calbiochem. TALON Superflow Metal Affinity Resin was from BD Biosciences, and Vivasin 20 concentrators (30-kDa cutoff) were purchased from Vivascience (Sartorius AG, Goettingen, Germany). Micro BCA protein assay kits were from Pierce. All other materials were of reagent grade and obtained from commercial sources.

**Protein Expression and Purification**—Plasmid pT7-5 encoding wild-type or C154G LacY with a His\(_h\) tag at the C terminus was used essentially as described (5) with minor modifications. Briefly, wild-type and C154G LacY were expressed in E. coli XL-1 Blue after induction with 0.3 mM isopropyl 1-thio-\(\beta\)-d-galactopyranoside. Membranes were prepared, washed with 5.0 M urea, and solubilized in 2% DDM. LacY was then purified by TALON Superflow metal affinity chromatography. The column was washed with 15 mM imidazole to elute weakly bound contaminants, and LacY was eluted with 150 mM imidazole, dialyzed against 50 mM NaPi (pH 7.5)/0.01% DDM, concentrated with a Vivasin 20 concentrator (30-kDa cutoff), frozen in liquid nitrogen, and stored at \(-80^\circ\text{C}\). The Micro BCA method was used to measure protein concentration. All LacY preparations were at least 90–95% pure as judged by silver staining after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

**ITC**—Heat flow resulting from binding of given ligands and associated conformational changes was measured by using a high sensitivity VP-ITC instrument (Microcal, LLC, Northampton, MA). Titration calorimetry experiments were performed as follows. Purified LacY proteins were dialyzed extensively against the target buffer (either 50 mM NaPi, (pH 7.5)/0.01% DDM or 50 mM Tris-HCl (pH 7.5)/0.01% DDM), and the sugars were dissolved in the last dialysate in order to minimize heats of dilution upon injection into the protein solution. Prior to use, solutions were degassed under vacuum to eliminate air bubbles. Each test ligand (0.25–2.0 mM) was injected into the calorimeter cell (volume 1.43 ml containing 50–100 \(\mu\)M protein) at 5-min intervals. The titration cell was stirred continuously at 300 rpm. To measure heats of dilution, ligands (at the same concentration used for test titrations) were titrated against reaction buffer without protein. Phenyl \(\alpha\)-d-glucopyranoside (0.5 mM), which does not bind to LacY (17), was used as a negative control and gives no heat change after the addition to wild-type or C154G LacY. \(N\)-ethylmaleimide (5 mM)-inactivated LacY, which does not bind ligand, also exhibits no heat change. Each measurement was repeated a minimum of three times, and the data did not vary by more than 10%.

The heat produced from each injection was determined by integrating the heat flow tracings. Heats of dilution in the presence of complex obtained after saturation were used to correct for heat of dilution. The heat of binding for each injection was obtained by integrating the area under the peak using ORIGIN (version 7.0; Microcal LLC). The heat evolved (\(Q\)) on addition of ligand is represented by the equation (18, 19)

\[
Q = V_h(\Delta H[M], K_a[L]/(1 + K_a[L])), \]

where \(V_h\) is the volume of the cell, \(\Delta H\) is the enthalpy of binding per mole of ligand, \([M]\) is the total macro-molecule concentration including bound and free fractions, \(K_a\) is the association constant, and \([L]\) is the free ligand concentration.

\(\Delta H\) and \(K_a\) were determined directly from the isotherm.

\(^2\)The abbreviations used are: ITC, isothermal titration calorimetry; LacY, lactose permease; TDG, \(\beta\)-d-galactopyranosyl 1-thio-\(\beta\)-d-galactopyranoside; NPG, \(p\)-nitrophenyl \(\alpha\)-d-galactopyranoside; DDM, \(n\)-dodecyl \(\beta\)-d-maltopyranoside.
Temperature, energy component) were computed from the following equation:

\[ \Delta G = -RT \ln K_a \]

and

\[ \Delta G = \Delta H - T \Delta S \]

where \( R \) is the gas constant and \( T \) is the absolute temperature in Kelvin.

Binding constants \( K_a \) of wild-type LacY or mutant C154G for TDG or NPG were calculated from experimental data by using the differential heat mode equation or integral heat mode (20). The integral heat mode calculation resulted in lower \( K_a \) values with ligand with higher affinity (24, 25), binds to wild-type or C154G LacY with a \( K_a \) of 13 \( \mu M \) (\( K_a = 7.8 \times 10^{-5} \) M\(^{-1}\)) or 5 \( \mu M \) (\( K_a = 2.1 \times 10^{-5} \) M\(^{-1}\)), respectively (Table 2), ~7-fold better affinity than TDG with either protein (Table 1). Relative to the wild type, C154G mutant LacY has 2~3 times better affinity for both ligands, as shown previously (4~6), a difference that is hardly significant energetically.

**Thermodynamic Parameters for ITC Binding**—Thermodynamic parameters, the enthalpy change (\( \Delta H \)), the free energy

\[ n_{H+} = \frac{\Delta H^\text{app}(P_i) - \Delta H^\text{app}(\text{Tris})}{\Delta H^\text{Tris} - \Delta H^\text{Tris}} \]

(Eq. 1)

where \( \Delta H^\text{Tris} \) (the enthalpy of ionization for Tris) is 11.3 kcal\( \cdot \)mol\(^{-1}\), \( \Delta H^\text{Tris} \) (the enthalpy of ionization for \( P_i \)) is 1.13 kcal\( \cdot \)mol\(^{-1}\) (22, 23), and \( n_{H+} \) is the number of protons taken up or released.

**RESULTS**

**Ligand Binding Affinity**—The heat associated with binding of lactose analogs TDG and NPG was measured by ITC (Fig. 2). Binding constants \( K_a \) of wild-type LacY or mutant C154G for TDG or NPG were calculated from experimental data and converted into dissociation constants \( K_d \) \( = \) \( 1/K_a \). Values of \( K_d \) are 83 \( \mu M \) (\( K_d = 1.2 \times 10^4 \) M\(^{-1}\)) for TDG binding to wild-type LacY and 33 \( \mu M \) (\( K_d = 3.0 \times 10^3 \) M\(^{-1}\)) for C154G LacY at 20 °C (293 K; Table 1). NPG, a
change (ΔG), and the entropic free energy component change (TΔS) accompanying binding of TDG were calculated from experimental data. Values of ΔG are similar for TDG binding to wild-type LacY (−5.4 kcal·mol⁻¹) and to C154G LacY (−6.0 kcal·mol⁻¹) (Table 1). However, the contributions from ΔH and TΔS are markedly different. For wild-type LacY, a change in the entropic free energy component (TΔS = 5.7 kcal·mol⁻¹) makes the sole contribution to ΔG, whereas a change in enthalpy (ΔH = −5.6 kcal·mol⁻¹) is the major contributor to the interaction between C154G LacY and TDG with an almost negligible change in the entropic free energy component (TΔS = 0.4 kcal·mol⁻¹) (Fig. 2, A and B, and Table 1). Furthermore, ligand binding with the C154G mutant is exothermic, and the magnitude of ΔH is much larger (−5.6 kcal·mol⁻¹) than for wild-type LacY (0.3 kcal·mol⁻¹). Thus, the interaction between TDG and C154G LacY is maintained by more or stronger interactions relative to the wild-type LacY.

Thermodynamic Parameters for NPG Binding—At 20 °C (293 K), the interactions of NPG with both wild-type and C154G LacY are exothermic (Figs. 2, C and D). Binding enthalpy is weakly negative for wild-type LacY (ΔH = −2.2 kcal·mol⁻¹), whereas the entropic free energy component is positive and large in magnitude (TΔS = 4.4 kcal·mol⁻¹) (Table 2). Thus, binding between NPG and wild-type LacY is characterized by both entropic and enthalpic changes, but the entropic change is clearly the dominant component. In contrast, binding of NPG to C154G LacY is characterized by a large negative enthalpy change (ΔH = −13.5 kcal·mol⁻¹), which is compensated by a large but negative change in the entropic free energy component (TΔS = −6.4 kcal·mol⁻¹) (Table 2), indicating a substantial decrease in conformational flexibility upon ligand binding.

**TABLE 1**

Thermodynamic parameters for TDG binding

| LacY | Kᵦ⁻¹ | Kᵦ⁺ | ΔH | ΔG | TΔS | ΔS |
|------|------|-----|-----|-----|-----|-----|
| Wild type | 1.2 × 10⁶ | 83 | 0.3 | −5.4 | 5.7 | 19.5 |
| C154G | 3.0 × 10⁴ | 33 | −6.6 | 0.4 | 1.3 |

* Kᵦ⁻¹ = 1/Kᵦ⁺.
* ΔG was calculated by the equation ΔG = −RT lnKᵦ⁺.
* TΔS was calculated by the equation TΔS = ΔH − ΔG.

**TABLE 2**

Thermodynamic parameters for NPG binding

| LacY | T | Kᵦ⁻¹ | Kᵦ⁺* | ΔH | ΔG | TΔS | ΔS | ΔCₐ ᵆ⁻¹ |
|------|----|------|-----|-----|-----|-----|-----|----------|
| Wild type | 279 | 8.4 × 10⁴ | 12 | −0.3 | −6.3 | 6.0 | 21.6 | −156.5 |
| | 280 | 7.5 × 10⁴ | 13 | −0.3 | −6.3 | 6.0 | 21.2 |
| | 288 | 1.0 × 10⁴ | 10 | −1.2 | −6.6 | 5.4 | 18.9 |
| | 293 | 7.8 × 10³ | 13 | −2.2 | −6.6 | 4.8 | 15.0 |
| | 298 | 7.6 × 10³ | 13 | −3.3 | −6.6 | 4.8 | 15.0 |
| C154G | 279 | 4.6 × 10³ | 2 | −7.2 | −4.4 | −7.2 | −21.8 |
| | 288 | 3.0 × 10³ | 3 | −13.4 | −7.2 | −6.2 | −21.8 |
| | 293 | 2.1 × 10³ | 5 | −13.5 | −7.1 | −6.4 | −21.8 |
| | 298 | 1.7 × 10³ | 6 | −14.1 | −7.1 | −7.0 | −23.5 |

* Kᵦ⁻¹ = 1/Kᵦ⁺.
* ΔG was calculated by the equation ΔG = −RT lnKᵦ⁺.
* TΔS was calculated by the equation TΔS = ΔH − ΔG.
* ΔCₐ ᵆ⁻¹ = ΔH/ΔT was determined from the slope of the linear fit in Fig. 4.

Over the range of temperatures tested (6–25 °C or 279–298 K), the thermodynamic parameters exhibit similar characteristics (Table 2). TΔS is the major contributor to the interaction of NPG with wild-type LacY, whereas ΔH contributes exclusively to NPG binding with C154G LacY. For all of these interactions, ΔH contributes to ΔG (Fig. 3, Table 2). However, in comparison with NPG binding to wild-type LacY, ΔH of the interaction between NPG and C154G LacY remains negative and large (Table 2, Fig. 3). Concomitantly, the entropic free energy component, TΔS, decreases due to tightening of the system, but ΔG changes little (Fig. 3, Table 2), indicating enthalpy/entropy compensation in the mutant (26).

From the change of ΔH with temperature (Fig. 4), the change in heat capacity (ΔCₐ ᵆ⁻¹) is calculated to be −156.5 and −124.5 cal·mol⁻¹·K⁻¹ for NPG interaction with wild-type and C154G LacY, respectively (Table 2). Both exhibit a similar negative ΔCₐ ᵆ⁻¹, suggesting that the binding sites in both proteins have a similar interface (7, 13) and the decrease in the entropic free energy component for NPG binding to C154G LacY is due to decreased conformational flexibility.

**DISCUSSION**

Wild-type LacY is a highly dynamic membrane transport protein in which ligand binding induces widespread conformational changes (1, 2, 13). In contrast, C154G LacY binds galactosidic sugars with relatively high affinity but is conformationally constrained and catalyzes very little translocation across the membrane (3–6).
In addition, C154G LacY exhibits higher thermostability and has less tendency to aggregate than the wild type (5), which led to its crystallization and x-ray structure determination (7). Because the C154G mutation in helix V abuts Gly24 in helix I where the two helices cross at the approximate middle of the membrane and replacement of Gly24 with Cys rescues activity (6), it was suggested that tighter packing between helices V and I may be responsible for the properties of the C154G mutant. As shown previously (4, 5), the C154G mutation results in a small increase in affinity for TDG or NPG relative to wild-type LacY at all temperatures tested (Tables 1 and 2). The effect may result from the tighter interactions between ligand and specific residues in the binding site. By allowing tighter packing between helices I and V, the C154G mutation may directly inhibit conformational flexibility near the binding site and indirectly alter positioning of residues that make direct contact with the sugar (e.g. Glu269, Trp151, Arg144, Glu126, Met23, and/or Asp237/Lys358) (6, 7, 13). In this study, the thermodynamics of binding between wild-type or C154G LacY and two galactosidic ligands are analyzed calorimetrically.

Remarkably, although the change in free energy of binding (ΔG) is similar for both proteins as expected, in wild-type LacY the change is due primarily to an increase in the entropic free energy component (TΔS), whereas in the C154G mutant an increase in enthalpy (ΔH) markedly predominates. Thus, ΔH for TDG binding is shifted from a small positive value in wild-type LacY to a relatively large negative value in C154G LacY (Table 1). The large negative value observed with the mutant suggests tighter packing upon ligand binding, which extends the conclusion that the C154G mutation causes decreased conformational flexibility (5, 6) and that the mutant favors a particular conformation(s). Thus, a specific conformer of C154G LacY is selected from the dynamic ensemble of conformations upon ligand binding.

Wild-type LacY is in a highly dynamic state (14, 28), and widespread conformational changes accompany ligand binding (2, 13). Therefore, the number of conformers for ligand-bound wild type is very likely greater than that for the unliganded protein, resulting in an increase in the entropic free energy component upon ligand binding. However, TΔS is reversed from positive in wild-type LacY to negative in the C154G mutant (Fig. 3 and Table 2). A negative change in the entropic free energy component is observed with H-bond formation, a decrease in the number of isoenergetic conformations, or a decrease in soft internal vibrational modes (29–31). There-

**TABLE 3**

Thermodynamic parameters for NPG binding in NaPi, or Tris

| Buffer | ΔH<sup>app</sup> (kcal mol<sup>−1</sup>) | ΔG<sup>app</sup> (kcal mol<sup>−1</sup>) | TΔS<sup>app</sup> (kcal mol<sup>−1</sup> K<sup>−1</sup>) | ΔS (cal mol<sup>−1</sup> K<sup>−1</sup>) | n<sub>1</sub> | n<sub>2</sub> |
|--------|-------------------------------|------------------|------------------------|-----------------|---------|---------|
| Wild   | −2.2                          | −6.6             | 4.4                    | 15.0            | 0.05    |         |
| Tris   | −1.7                          | −6.8             | 5.1                    | 17.4            |         | −0.04   |
| C154G  | −13.5                         | −7.1             | −6.4                   | −21.8           |         |         |
| Tris   | −13.9                         | −6.6             | −7.3                   | −25.0           |         |         |

<sup>ΔG was calculated from the equation ΔG = −RTlnK<sub>p</sub>.</sup>
<sup>TΔS were calculated from the equation TΔS = ΔH − ΔG.</sup>
<sup>n<sub>1</sub> = [ΔH<sup>app</sup>(Pi) − ΔH<sup>app</sup>(Tris)]/[ΔS<sup>1</sup> − ΔS<sup>2</sup>]</sup>
C154G LacY is compromised by the entropic free energy component, which restricts the number of conformers that can be occupied (32). With this restriction, the mutant is able to undergo the induced-fit phenomenon associated with ligand binding (13) but can hardly overcome the energy barrier to achieve the outward facing conformation and catalyzes translocation extremely poorly (Fig. 5). Finally, as observed by using P$_i$ or Tris, buffers with very different enthalpies of protonation, no significant change in different enthalpies of protonation, no significant change in

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