Structural evidence for induced fit and a mechanism for sugar/H⁺ symport in LacY

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Cation-coupled active transport is an essential cellular process found ubiquitously in all living organisms. Here, we present two novel ligand-free X-ray structures of the lactose permease (LacY) of *Escherichia coli* determined at acidic and neutral pH, and propose a model for the mechanism of coupling between lactose and H⁺ translocation. No sugar-binding site is observed in the absence of ligand, and deprotonation of the key residue Glu²⁶⁹ is associated with ligand binding. Thus, substrate induces formation of the sugar-binding site, as well as the initial step in H⁺ transduction.

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Introduction

Lactose permease (LacY), a galactoside/H⁺ symporter, is a well-studied example of ion electrochemical gradient-driven symporters and antiporters. LacY carries out the coupled stoichiometric translocation of a D-galactopyranoside with an H⁺, utilizing the free energy released from downhill translocation of H⁺ to drive accumulation of galactopyranosides against a concentration gradient. Notably, in the absence of $\Delta\mu_{\text{H}^+}$, LacY also catalyzes the converse reaction, utilizing free energy released from downhill translocation of sugar to drive uphill translocation of H⁺ with generation of $\Delta\mu_{\text{H}^+}$, the polarity of which depends on the direction of the substrate concentration gradient. In the absence of substrate, LacY does not translocate H⁺; however, LacY catalyzes exchange or counterflow of sugar without translocation

of H⁺. Since substrate gradients by themselves generate a $\Delta\mu_{\text{H}^+}$ of either polarity, it is likely that the primary driving force for turnover is binding and dissociation of sugar on either side of the membrane (Kaback *et al*, 2001; Kaback, 2005).

Recently, an X-ray structure of C154G LacY, which binds ligand as well as wild-type LacY but catalyzes little transport, was solved in an inward-facing conformation (Abramson *et al*, 2003). LacY consists of 12 transmembrane helices organized in two pseudo-symmetrical α -helical bundles. The N- and C-terminal six-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 by site-directed mutagenesis in conjunction with biochemical and biophysical studies (Kaback *et al*, 2001; Guan and Kaback, 2006). Only 6 amino acid side chains are irreplaceable with respect to ligand binding [Arg¹⁴⁴ (helix V) and Glu¹²⁶ (helix IV)] and/or H⁺ translocation [Glu²⁶⁹ (helix VIII), Arg³⁰² (helix IX), His³²² and Glu³²⁵ (helix X)] (reviewed in Frillingos *et al*, 1998; Kaback *et al*, 2001; Guan and Kaback, 2005). The structure reveals that Arg¹⁴⁴ forms a bi-dentate H-bond with the O₄ and O₃ atoms of the galactopyranosyl ring, confirming the irreplaceable role of this residue in sugar binding and recognition (Venkatesan and Kaback, 1998; Sahin-Tóth *et al*, 1999; Abramson *et al*, 2003). Glu¹²⁶, another irreplaceable residue, is in close proximity to Arg¹⁴⁴ and may interact with the O₄, O₅ or O₆ atoms of the galactopyranosyl ring via water molecules. A direct interaction between Arg¹⁴⁴ and Glu¹²⁶ is not observed in the ligand-bound structure. Trp¹⁵¹ (helix V), an important residue for substrate binding (Guan *et al*, 2003; Vazquez-Ibar *et al*, 2003, 2004), stacks with the galactopyranosyl ring. Glu²⁶⁹ in the C-terminal domain, which is critical for sugar binding and H⁺ translocation, may interact with the O₃ atom of the galactopyranosyl ring (Weinglass *et al*, 2003), forms a salt bridge with Arg¹⁴⁴ and is in close proximity to Trp¹⁵¹ (Vazquez-Ibar *et al*, 2004). Therefore, it was suggested that contacts between Glu²⁶⁹ in the C-terminal half and Arg¹⁴⁴ and Trp¹⁵¹ in the N-terminal half may be key for coupling between sugar binding and H⁺ translocation.

His³²², Glu³²⁵ and Arg³⁰², as well as Tyr²³⁶ (helix VII) which is not irreplaceable with respect to activity (Roepe and Kaback, 1989), are probably directly involved in H⁺ translocation (Kaback *et al*, 2001; Guan and Kaback, 2006). His³²² may be the immediate H⁺ donor to Glu³²⁵, and Arg³⁰² may interact with Glu³²⁵ to drive deprotonation (Kaback *et al*, 2001; Sahin-Tóth and Kaback, 2001). However, in the ligand-bound conformation, there is no direct interaction between Arg³⁰², Glu³²⁵ and His³²². In addition, Glu²⁶⁹ is far removed from the H⁺ translocation pathway.

To address ligand binding and the mechanism of coupling between substrate and H⁺ translocation, we describe here two novel crystal structures from the same mutant (C154G LacY) at pH 5.6 and 6.5 without bound substrate.

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Table I Data collection and refinement statistics

Data collection (Beamline SLS X06SA)		
	pH 6.5	pH 5.6
Wave length (Å)	1.0775	0.9768
Resolution (Å)	40.0–2.95 (3.02–2.95)	20.0–3.3 (3.42–3.30)
Total reflections	64 905	36 939
Unique reflections	27 044	18 660
Redundancy	2.4	2.0
Completeness (%) ^a	82.5 (70.3)	83.2 (75.9)
R _{sym} (%) ^{a,b}	0.13 (0.48 ^c)	0.18 (0.59)
Space group	P4 ₃ 2 ₁ 2 (<i>a</i> = <i>b</i> = 122.47 Å, <i>c</i> = 187.78 Å)	P4 ₃ 2 ₁ 2 (<i>a</i> = <i>b</i> = 123.9 Å, <i>c</i> = 190.85 Å)
Refinement		
Resolution (Å) ^a	10.0–2.95 (3.13–2.95)	15.0–3.3 (3.5–3.3)
R factor (%) ^{a,d}	26.2 (37.5)	28.6 (38.1)
R _{free} (%) ^{a,e}	29.8 (39.5)	32.8 (38.1)
RMS deviations from ideal values		
Bond lengths (Å)	0.013	0.012
Bond angles (deg)	2.0	1.8
Dihedral angles (deg)	23.2	23.3

^aValues in parentheses are for the highest resolution shell.

^b $R_{\text{sym}} = \sum_h \sum_i |I_i(h) - \langle I(h) \rangle| / \sum_h \sum_i I_i(h)$, where $I_i(h)$ is the i th measurement.

^cThe last shell R_{sym} for the high-resolution set is rather high because of strong anisotropy.

^d $R \text{ factor} = \sum_h ||F(h)_{\text{obs}}| - |F(h)_{\text{calc}}|| / \sum_h |F(h)_{\text{obs}}|$.

^e R_{free} was calculated using more than at least 500 randomly selected reflections that were excluded from refinement.

Figure 1 Overall structure of C154G LacY in the absence of ligand. LacY consists of 12 transmembrane helices organized in two pseudo-symmetrical α -helical bundles. The N- and C-terminal 6-helix domains form an internal cavity open to the cytoplasmic side. In the ligand-free structure at pH 6.5, the residues involved in substrate binding (Glu¹²⁶ (helix IV), Arg¹⁴⁴ and Trp¹⁵¹ (helix V)) are shown in yellow, and those involved in H⁺ translocation [Tyr²³⁶ (helix VII), Arg³⁰² (helix IX), His³²² and Glu³²⁵ (helix X)] are shown in pink. The dual-function residue Glu²⁶⁹ (helix VIII) is shown in cyan. (A) Side view; (B) cytoplasmic view.

Results

The structures were obtained from a new crystal form (tetragonal), which exhibits higher-resolution limits (2.95 and 3.30 Å for the structures at pH 6.5 and 5.6, respectively) than the orthorhombic form used for the sugar-bound struc-

ture (3.6 Å). The data collection and refinement statistics are summarized in the Table I. The overall fold of the two structures is very similar to the TDG-bound complex from the orthorhombic crystals with RMSD values for the C α -atoms of 1.0 and 1.0 Å, respectively (Figure 1A and B), representing ligand-free inward-facing conformations.

Figure 2 Re-arrangement of the active site in the absence and presence of sugar. (A) Cytoplasmic stereo view of the sugar-binding site from superimposed crystal structures. The TDG-bound structure (1pv7) is shown in blue (3); ligand-free structures are shown in green (pH 5.6) and in yellow (pH 6.5). (B) Residues involved in sugar binding are shown with a 2F_o - F_c sigmaA weighted electron density map from the structure obtained at pH 6.5. (C) Stereo view of Glu²⁶⁹ in a relatively hydrophobic environment surrounded by residues Trp¹⁵¹, Cys¹⁴⁸, Asn²⁷², Ala²⁷³ and Met³²³.

Ligand binding and induced fit

The sugar-binding site in both ligand-free structures exhibits a similar configuration (Figure 2A and B), which differs from the ligand-bound configuration, strongly indicating that a rearrangement occurs upon binding of sugar (Figure 2A). In the absence of ligand, Arg¹⁴⁴ (helix V) is displaced from Glu²⁶⁹ (helix VIII) and forms a salt bridge with Glu¹²⁶ (helix IV). Helix VIII is slightly removed from the cavity and the

cytoplasmic half appears to undergo a small counterclockwise rotation, consistent with biochemical finding (Frillingos and Kaback, 1997). Glu²⁶⁹ is located away from the hydrophilic cavity in a less-solvent exposed environment and H-bonded to Trp¹⁵¹, forming a platform for the initial recognition of the galactopyranosyl ring. From these movements, we conclude that the side chains interacting directly with the hydroxyl groups of the sugar are not in a configuration to

Figure 3 Stereo view of residues involved in H⁺ translocation network. (A) Residues involved in H⁺ translocation in the TDG-bound structure (blue; 1pv7) and ligand-free structure (green) at pH 5.6. (B) Side-chains involved in H⁺ translocation at pH 5.6 in the absence of ligand shown with a 2F_o–F_c sigmaA weighted electron density map.

bind galactopyranosides in the absence of the sugar. Ligand induces formation of the binding site, mainly through side-chain movements without a global conformation change.

Critical roles of Glu²⁶⁹ in ligand binding and H⁺ translocation

Glu²⁶⁹ is surrounded by Cys¹⁴⁸, Trp¹⁵¹, Asn²⁷² and Met³²³ in all structures in the absence and presence of ligand. However, in the ligand-free conformation, in addition to displacement of Arg¹⁴⁴ from Glu²⁶⁹, the carboxyl group is closer to hydrophobic Trp¹⁵¹, Cys¹⁴⁸ and Ala²⁷³; Asn²⁷² is removed from Glu²⁶⁹, suggesting that Glu²⁶⁹ is in a relatively hydrophobic environment (Figure 2C). Thus, it is likely that Glu²⁶⁹ is protonated in both ligand-free structures, which is consistent with findings from chemical modification, where Glu²⁶⁹ reacts with hydrophobic rather than hydrophilic carbo-diimides (Weinglass *et al*, 2003). Several lines of evidence indicate that substrate binds to protonated LacY. It has been also postulated that the H⁺ may be shared between Glu²⁶⁹ and His³²² (Kaback, 2005; Guan and Kaback, 2006). Here, we suggest that Glu²⁶⁹ may be the primary protonation site and that protonation precedes ligand binding.

H⁺ translocation

In the ligand-bound structure, Glu³²⁵ is in a hydrophobic environment. A similar arrangement is observed in the

ligand-free tetragonal structure at pH 6.5. However, at pH 5.6, Glu³²⁵ moves from the hydrophobic environment and becomes closer to His³²², Tyr²³⁶ and Arg³⁰², forming a salt bridge with His³²² and an H-bond with Tyr²³⁶ (Figure 3A and B). It is likely that His³²² is protonated at pH 5.6, and the positively charged imidazole ring causes Glu³²⁵ to move into a more hydrophilic environment in the deprotonated form. This H-bond/salt bridge network suggests that protonated His³²² may be able to transfer H⁺ directly to Glu³²⁵. A local acidic pH generated by Δμ_{H⁺} in the outward-facing confirmation may facilitate H⁺ translocation from His³²² to Glu³²⁵. In contrast, Glu¹²⁶, Trp¹⁵¹ and Arg³⁰² occupy nearly the same positions in each structure.

Discussion

Based on the structural observations, we propose an ordered mechanism for the initial step in galactoside/H⁺ symport. In the ligand-free conformation, the essential residues for substrate binding (Arg¹⁴⁴, Glu¹²⁶ and Glu²⁶⁹) are not in the correct configuration to bind substrate (Figure 4A). Sugar initially recognizes Trp¹⁵¹ through nonspecific hydrophobic stacking between the galactopyranosyl and indole rings (Guan *et al*, 2003; Vazquez-Ibar *et al*, 2003). This interaction orientates the galactopyranosyl moiety for recognition by Arg¹⁴⁴, Glu¹²⁶ and Glu²⁶⁹ (Figure 4B). Moreover, interaction

Figure 4 Proposed model for the initial step in coupling between sugar and H⁺ translocation. H-bonds are represented by broken lines; charge pairs by solid lines. The proton is represented by a white sphere and the water by a turquoise sphere. (A) Glu²⁶⁹ is protonated and close to Trp¹⁵¹ to form a platform in the absence of substrate. Arg¹⁴⁴ forms a salt bridge with Glu²⁶⁹. (B) The Glu²⁶⁹/Trp¹⁵¹ platform orients the galactopyranosyl ring to allow specific interactions with Arg¹⁴⁴, Glu¹²⁶ and Glu²⁶⁹. (C) Arg¹⁴⁴ moves towards the galactopyranoside ring to form a bi-dentate H-bond with the O₄ and O₃ positions. Glu²⁶⁹ moves into a hydrophilic environment, deprotonates and interacts with the O₃ position of the sugar, as well as a salt bridge with Arg¹⁴⁴. (D) The H⁺ is transferred to His³²² via water.

of sugar with Trp¹⁵¹ disrupts the salt-bridge between Arg¹⁴⁴ and Glu¹²⁶, and a bi-dentate H-bond is formed with Arg¹⁴⁴ through the O₄ and O₃ groups on the galactopyranosyl ring (Figure 4C). Subsequently, protonated Glu²⁶⁹ moves out of a relatively hydrophobic environment, deprotonates, forms a salt bridge with Arg¹⁴⁴ and an H-bond with sugar to complete ligand binding (Figure 4C). In other words, sugar binding drives the deprotonation of Glu²⁶⁹, the initial step in H⁺ translocation. In the ligand-bound complex, His³²² is far from Glu²⁶⁹, and the H⁺ released from Glu²⁶⁹ may be delivered to His³²² through a tightly bound water molecule(s) either on the O₃ group of the galactopyranosyl moiety or bound to His³²² (Figure 4D). Although bound water cannot be observed at this resolution, it is common in high-resolution structures of carbohydrates (Mirza *et al*, 2001) and membrane proteins (Pebay-Peyroula *et al*, 1997).

The postulated scheme for lactose influx based on an alternating access model (Abramson *et al*, 2003; Guan and Kaback, 2004, 2006) is updated in the following manner (Figure 5). Initially, in the absence of ligand, LacY in the outward-facing conformation is unstable (Figure 5A), and Glu²⁶⁹ is rapidly protonated (Figure 5B). In this conformation, the side chains involved in direct interaction with sugar are not in a configuration to bind galactopyranosides. However, Trp¹⁵¹ and Glu²⁶⁹ are located in the middle of

helices V and VIII in the closely opposed interface between the N- and C-terminal helical bundles at the apex of the water-filled cavity. Therefore, the recognition platform formed by Trp¹⁵¹ and protonated Glu²⁶⁹ may also be present in the outward-facing conformation (Figure 5B). Initial recognition of galactopyranoside by Trp¹⁵¹ orientates Glu²⁶⁹ and Arg¹⁴⁴ in the correct configuration to bind specifically and induces deprotonation of Glu²⁶⁹ as well (Figure 5C). As a consequence, the salt bridge between Arg¹⁴⁴ and Glu¹²⁶ is disrupted and a new salt-bridge between Arg¹⁴⁴ and Glu²⁶⁹ is formed. This switch is induced by substrate to complete formation of binding site. The H⁺ released from Glu²⁶⁹ is transferred to His³²², and rapid transition to the inward-facing conformation occurs (Figure 5D). His³²² may be the immediate H⁺ donor to Glu³²⁵ through the local H-bond/charge-pair network. Substrate is then released into the cavity facing the cytoplasm (Figure 5E), and the salt bridge is broken between Arg¹⁴⁴ and Glu²⁶⁹ and re-established between Arg¹⁴⁴ and Glu¹²⁶. The H⁺ is then released into the same cavity (Figure 5F) due possibly to a decrease in the pK_a of Glu³²⁵ caused by either approximation to Arg³⁰² (Kaback *et al*, 2001; Sahin-Tóth and Kaback, 2001) or exposure to solvent in the aqueous cavity (cytoplasmic pH is constant at 7.6) or both. After releasing the H⁺, transition back to the outward-facing conformation occurs.

Figure 5 An updated influx mechanism for lactose/H⁺ symport. See main text for details. The proton (H⁺) and the substrate (S) are shown with red and green, respectively. Charge pairs are indicated by solid lines.

In conclusion, the data presented here suggest a simple mechanism for the initial step in coupling. However, it is still not clear how sugar binding correlates with the global conformational change that presumably occurs between the inward- and outward-facing conformations or how the H⁺ is released from the molecule. Furthermore, it is notable (Guan and Kaback, 2006) that the residues involved in H⁺ translocation are aligned parallel to the plane of the membrane at a level similar to the sugar-binding site (Figure 1) and may be exposed to a water-filled cavity in both the inward- and outward-facing conformations. Therefore, the H⁺, like the sugar, can be released directly into either cavity. In any event, it is apparent that H⁺ translocation through LacY cannot involve a pathway through the molecule as observed in bacteriorhodopsin (Pebay-Peyroula *et al*, 1997; Lanyi, 2004) or cytochrome *c* oxidase (Iwata *et al*, 1995; Tsukihara *et al*, 1996). These structural features may also help explain how lactose-driven H⁺ translocation can occur in both directions across the membrane via the same residues.

Materials and methods

Purification and crystallization

The expression and purification were carried out as described (Abramson *et al*, 2003). The solubilization step was modified by increasing the detergent:protein ratio to 2.5:1.0 (wt/wt; 10 mg protein/ml) (Guan *et al*, 2006). Both tetragonal crystals were obtained at the late equilibrium stage under conditions used for the orthorhombic crystals (Abramson *et al*, 2003), which yielded a pH 6.5. To decrease the pH to 5.6, crystals were soaked in buffer (100 mM MES (pH 5.6)/36% PEG 400/200 mM CaCl₂/3% 1,6 hexanediol and 8 mM CHAPS) for 2 min before frozen.

Diffraction and data collection

Diffraction data were collected at 100 K from single crystals on beam-line X06SA (data from pH 6.5) at the Swiss Light Source (Villigen). Diffraction was anisotropic with both crystals. The crystals at pH 5.6 or 6.5 diffracted to 2.95 or 2.6 Å in one and 4.5 or 3.5 Å in the other dimension, respectively. Due to the relative

radiation sensitivity of the crystals, a maximum of 10° was collected at any single position of the crystal. Data processing was performed using Denzo and Scalepack (Otwinowski and Minor, 1997). The crystals belong to the space group P4₃2₁2. Due to anisotropic diffraction, a sigma cutoff of -1 was used during the Scalepack run, preventing merging of very weak or nonexistent reflections with relatively strong reflections.

Structure determination

The structure at pH 6.5 was solved at a resolution of 2.95 Å by molecular replacement with the C154G mutant monomer by using 1PV6 as template (Table I) (Abramson *et al*, 2003). The structure at pH 5.6 was solved at a resolution of 3.3 Å by isomorphous molecular replacement using the structure at pH 6.5 (Table I). Subsequent refinement of both structures was carried out by using CNS (Brünger *et al*, 1998) initially with rigid body minimization with three separate rigid bodies (residues 1–190, 191–210 and 211–417) followed by simulated annealing, individual restrained *B*-factor refinement and minimization. Approximately 2.5% of the data were set aside for calculation of *R*-free values. Manual rebuilding was done by using O (Jones and Kennedy, 1969) with sigma A-weighted 2F_o–F_c and F_o–F_c electron density maps (Read, 1986). The stereochemistry of the model was evaluated using Procheck (Laskowski *et al*, 1996). Due to significant anisotropic diffraction with both crystals, local scaling was applied to the data at the later steps of refinement, calculating individual scale factors for 64 different zones of the reciprocal space. This significantly improved the quality of the maps, and an approximate decrease of 1.5% in *R*-factors was observed. All refinement statistics are given in Table I. Five mercury ions were included in the refinement. For the structure at pH 6.5, none of the residues were found in the disallowed, 1.4% in the generously allowed, 28.1% in the additionally allowed, and 70.5% in the most favored regions; for the pH 5.6 structure, the corresponding values are none, 0.8, 32.2 and 66.9%, respectively.

The electron densities throughout the structure were coherent, and most of the amino-acyl side chains were easily assigned with the exceptions of the central cytoplasmic loop (residues 191–205) and the C-terminal helix (residues 404–417). All residues forming the sugar-binding site show clear electron densities, and their positions were determined unambiguously. There is an incoherent electron density in the cavity, stacking on Trp¹⁵¹, which could not be modeled. The coordinates for the pH 6.5 and 5.6 structures have been deposited in the Protein Data Bank (entries 2cfq and 2cfp, respectively).

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