It takes two to tango: The dance of the permease

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The lactose permease (LacY) of *Escherichia coli* is the prototype of the major facilitator superfamily, one of the largest families of membrane transport proteins. Structurally, two pseudo-symmetrical six-helix bundles surround a large internal aqueous cavity. Single binding sites for galactoside and H+ are positioned at the approximate center of LacY halfway through the membrane at the apex of the internal cavity. These features enable LacY to function by an alternating-access mechanism that can catalyze galactoside/H+ symport in either direction across the cytoplasmic membrane. The H+-binding site is fully protonated under physiological conditions, and subsequent sugar binding causes transition of the ternary complex to an occluded intermediate that can open to either side of the membrane. We review the structural and functional evidence that has provided new insight into the mechanism by which LacY achieves active transport against a concentration gradient.

**Introduction**

The lacY gene, the second structural gene in the lac operon (Müller-Hill, 1996), encodes lactose permease (LacY), the polypeptide solely responsible for β-galactoside transport in *Escherichia coli*. LacY (TCDB 2.A.1.5.1) is a member of the oligosaccharide/H+ symporter subfamily of the major facilitator superfamily (MFS), and it recognizes disaccharides or glucose. The natural substrate of LacY is D-galactopyranosyl ring and D-galactose, with no affinity for glucopyranosides or glucose. The natural substrate of LacY is lactose, a disaccharide containing a galactopyranosyl and a glucopyranosyl moieties in a β-1→4 glycosidic linkage. LacY catalyzes reversible translocation of one galactoside with one H+, and coupled translocation in the same direction is obligatory (symport).

The lacY gene is the first gene encoding a membrane transport protein to be cloned and sequenced (Büchel et al., 1980), the first symporter to be purified to homogeneity in a functional state (Newman et al., 1981) and the first symporter to have a structure determined (Abramson et al., 2003). LacY has 417 amino acid residues (65-70% of which are hydrophobic) and a molecular weight of 46,517 D, and each of the 417 residues has undergone Cys-scanning mutagenesis (reviewed in Frillingos et al., 1998) and site-directed alkylation (reviewed in Kaback et al., 2007; Nie et al., 2007) and/or other site-directed mutagenesis. Notably, only nine residues are essentially irreplaceable with regard to active lactose transport: Glu26 (helix IV), Arg144 (helix V), Thr151 (helix V), Tyr236 (helix VII), Glu269 (helix VIII), Asn272 (helix VIII), and His322 (helix X) form the galactoside-binding site (Kumar et al., 2014, 2015); and Arg302 (helix IX) and Gln325 (helix X) are essential for coupled H+ translocation (Carrasco et al., 1989; Sahin-Tóth and Kaback, 2001). All nine side chains are located in or near the aqueous cavity in the approximate middle of the LacY molecule (Fig. 1 a and Fig. 2 a).

LacY is a chemiosmotic nano-machine that performs coupled transport of a galactopyranoside with an H+ across the cytoplasmic membrane of *E. coli* (i.e., galactoside/H+ symport). Active transport (i.e., transport against a concentration gradient) is achieved by transduction of the free energy released from energetically favorable movement of H+ down the electrochemical H+ gradient (ΔμH+; interior negative and/or alkaline) as postulated by the chemiosmotic hypothesis of Mitchell (1967, 1968; Fig. 3 a). Conversely, downhill galactoside transport drives uphill H+ flux and generation of ΔμH+, the polarity of which depends upon the direction of the sugar concentration gradient (Fig. 3, b and c). Importantly, LacY also catalyzes transmembrane exchange of internal for external galactoside in a manner that is independent of ΔμH+ (Fig. 3, d and e). Thus, the primary driving force for “alternating access” (Jardetzky, 1966) of the H+– and sugar-binding sites to either side of the membrane is binding and dissociation of galactoside by protonated LacY. Without bound sugar, LacY cannot translocate H+ with or without ΔμH+, and unprotonated LacY cannot bind a galactoside because of low affinity. Therefore, binding of both sugar and H+ is required for symport.

Despite a number of structures of MFS members, including 10 of LacY, mechanisms of these dynamic proteins are not completely understood. It has been demonstrated that sugar binding to highly dynamic protonated LacY triggers a global conformational change in which sugar- and H+-binding sites...
gain alternating access to either side of the membrane. Sugar binding and dissociation drive this conformational change through an induced-fit mechanism, while the proton electrochemical gradient accelerates the rate of deprotonation. LacY behaves much like an enzyme except that the transition state involves the protein rather than the substrate. X-ray structures of LacY inward- and almost occluded outward-facing conformations provide the structural basis for the alternating access mechanism. Alternating access has been documented almost unequivocally by applying pre–steady-state kinetics, as well as multiple biochemical and spectroscopic approaches, and by using kinetic data obtained in real time for several steps in the transport cycle. Thirty-one camelid nanobodies (Nbs) allow stabilization of LacY in different intermediate states that are providing an understanding of the structural changes underlying the symport mechanism.

Oligomeric state
LacY is a monomer both in detergent and in the membrane. The polypeptide reconstitutes as a monomer, as shown by fluorescence anisotropy (Vogel et al., 1985) and by freeze-fracture EM in the absence or presence of an imposed ΔμH+ (Costello et al., 1987). Initial rates of ΔμH+–driven lactose transport in proteoliposomes reconstituted at various protein-to-lipid ratios vary linearly. The linearity of the data and particularly the complete lack of sigmoidicity at very low ratios is consistent with the conclusion that LacY functions as a monomer. Furthermore, an engineered fusion protein encoded by two tandemly fused lacY open reading frames exhibits good transport activity, and negative dominance is not observed with either mutations or chemical modification in either half of the covalent dimer (Sahin-Tóth et al., 1994). Finally, cross-linking of LacY single-Cys mutants in the plane of the membrane is stochastic (Guan et al., 2002). Taken together, the findings indicate that LacY is both structurally and functionally a monomer (reviewed in Guan and Kaback, 2006).

3-D structure
LacY is organized into two six-helix bundles connected by a relatively long cytoplasmic loop between helices VI and VII, which exhibit twofold pseudo-symmetry when viewed parallel to the plane of the membrane (Fig. 1, a and b). Within each domain, there are two three-helix repeats with inverted topology. These structural features are observed in both inward- and outward-facing conformations. The protein is 86% α-helix in the x-ray structure (85% by circular dichroism; Foster et al., 1983) and contains twelve transmembrane helices traversing the membrane in zigzag fashion connected by very short hydrophilic loops with both N and C termini on the cytoplasmic side of the membrane. Most of the helices are irregular and distorted with bends and kinks. Helices III, VI, IX, and XII are largely embedded in the membrane, and the cytoplasmic regions of helices I, II, IV, and V from the N-terminal domain and helices VII, VIII, X, and XI from the C-terminal domain line a hydrophilic cavity. The inward-facing conformation may represent the resting state of the molecule (Nie and Kaback, 2010; Smirnova et al., 2011b; Kaback, 2015).
The initial structure was obtained with a conformationally constrained mutant, C154G LacY (Abramson et al., 2003), and a similar crystal structure was obtained for the WT a few years later (Guan et al., 2007). Viewed perpendicular to the membrane, both structures display heart-shaped molecules with a deep hydrophilic cavity open on the cytoplasmic side and sealed on the periplasmic side, an inward (cytoplasmic)–facing conformation (Fig. 1 a) with largest dimension of 60 × 60 Å. Viewed parallel to the plane of the membrane from the cytoplasmic side (Fig. 1 b), the molecules have a distorted oval shape that measures 30 × 60 Å. Calculation of the electrostatic surface potential reveals a positively charged ring around the cytoplasmic opening (Fig. 1, c–e; Yousef and Guan, 2009) and preferential distribution of negatively charged residues on the periplasmic side (Fig. 1 f). It is apparent that LacY conforms to the positive inside rule (von Heijne, 1992). Remarkably, LacY can be expressed in two fragments in vivo that find each other in the membrane and form a functional complex (Bibi and Kaback, 1990; Zen et al., 1994).

Four x-ray structures from LacY mutant G46W/G262W in a partially outward (periplasmic)–open conformation with a tightly sealed cytoplasmic side (Kumar et al., 2014, 2015, 2018; Jiang et al., 2016) have also been obtained (Fig. 2 a). Three of the structures (8, 9, and 27) are in an occluded conformation with a narrow periplasmic opening and a single galactoside molecule in the central sugar-binding site. The opening on the periplasmic side is too narrow to allow entrance or exit of the sugar (~3 Å at the narrowest point), and the bound galactoside is occluded. Mutant G46W/G262W is clearly able to open sufficiently to bind galactoside at rates approaching diffusion (Smirnova et al., 2013), and upon binding, the mutant tries to transit into an occluded conformation. However, it cannot do so completely because the bulky Trp residues block complete closure. Thus, the mutant binds galactoside, which initiates transition into an occluded intermediate state, but it cannot complete the operation, which accounts for why the mutant is completely unable to catalyze transport of any type. It is also clear that an occluded intermediate is part of the transport cycle.

**Galactoside binding and specificity**

There is one sugar-binding site in LacY that is selective for galactose or disaccharides containing a galactosyl moiety, and the symporter transports a single molecule per transport cycle. Two lactose analogues, β-D-galactopyranosyl 1-thio-β-D-galactopyranoside (TDG) and 4-nitrophenyl-α-D-galactopyranoside (α-NPG), as well as other homologues (Smirnova et al., 2018), bind to LacY with micromolar $K_d$. The natural substrate lactose has poor affinity with a $K_d$ of ~1 mM (Guan and Kaback, 2004), and galactose is the most specific substrate for LacY with the lowest affinity. Various adducts (particularly if they are hydrophobic) at the anomeric carbon (C1) of the galactopyranosyl ring greatly increase affinity with little or no effect on specificity (Sahin-Tóth et al., 2000). Although glucose is an epimer of galactose, neither glucose nor glucosides are recognized by LacY. The C4 OH on the galactopyranosyl ring is probably the most important determinant for specificity, with the C3, C5, and C6 OH groups playing varying roles in determining affinity/specificity, while C1 and C2 OH are of little importance (Sahin-Tóth et al., 2000, 2001).
Sugar-bound LacY in the inward-facing or partially outward-facing conformation has been resolved crystallographically (Chaptal et al., 2011; Kumar et al., 2014, 2015, 2018). Both of the inward-facing and outward-facing structures contain a single galactoside that occupies the central cavity lined by the same residues regardless of the conformation of LacY, further confirming the conclusion that there is only a single sugar-binding site (Guan and Kaback, 2004). The site is located at the apex of the internal cavity in the middle of LacY, which enables transport in either direction.

The sugar-bound, inward-facing structure is from single-Cys V122C LacY with covalently bound methanethiosulfonyl-galactoside (Chaptal et al., 2011), a suicide inactivator for the mutant (Guan et al., 2003b). Sugar-bound outward-facing structures have been obtained for the G46W/G262W mutant (Smirnova et al., 2013) with bound TDG or α-NPG (Kumar et al., 2014, 2015, 2018). The position of the galactosyl moiety in these structures is almost identical (Fig. 2, b and c), which provides strong evidence that it is indeed the galactosyl moiety that determines specificity.

Cys-scanning and site-directed mutagenesis have been used extensively to study the sugar-binding site in LacY (Frittingos et al., 1998), and the results from genetic engineering in combination with biochemical and biophysical characterization are largely confirmed crystallographically (Chaptal et al., 2011; Kumar et al., 2014, 2015, 2018). The LacY structures show that the galactopyranosyl ring of α-NPG or TDG stacks hydrophobically with Trp151 in helix V (Fig. 2, b and c), which is suggested to play important roles in orienting the galactopyranosyl ring and facilitating specific hydrogen bonding, in addition to increasing affinity (Guan et al., 2003a). Replacement of Trp151 with a Tyr or Phe residue decreases affinity for galactosides, but has little effect on lactose transport (Guan et al., 2003a; Vázquez-Ibar et al., 2003; Smirnova et al., 2009b). Glu269 in helix VIII forms two hydrogen bonds with the Cα and Cβ OH groups on the galactopyranosyl ring (Fig. 2, b and c), which explains why the Glu269 residue may be the primary determinant for sugar specificity (Weinglass et al., 2003). These important interactions are further stabilized by hydrogen bond interactions between the Cα and Cβ OH groups of the galactopyranosyl ring of TDG or α-NPG, and the Cβ OH group in another galactopyranosyl ring. As described above, the glucopyranosyl ring in lactose can be replaced by various aducts that affect binding affinity only. Glu269, in addition to stabilizing the position of Arg144, also forms multiple hydrogen bonds with the Cα and Cβ OH groups of TDG. Arg144 cannot be replaced with positively charged Lys, and Asp replacement for Glu269 also decreases affinity for galactosides significantly (Smirnova et al., 2009a).

**Sugar binding and induced fit**

Methanethiosulfonyl-galactose is a unique suicide substrate for a LacY mutant with Cys in place of Ala122 (Guan et al., 2003b). The x-ray structure (Chaptal et al., 2011) reveals that a molecule of methanethiosulfonyl-galactose is covalently bound to a Cys engineered at position 122 near the sugar-binding site, and the overall conformation of LacY remains in an inward-open conformation, although typically galactoside binding shifts LacY to the outward-open state (reviewed in Smirnova et al., 2011b). However, many side-chain interactions with the galactopyranoside ring are perturbed; for example, Asn272, His322, and Arg144 are no longer sufficiently close to interact with the galactopyranosyl ring. Therefore, although the binding site is insufficient to drive transition into the occluded state.

**H+ binding**

Unexpectedly, LacY exhibits a perturbed apparent pK of ~10.5 with respect to galactoside-binding affinity, and the pH-dependent apparent pK is abrogated when Glu325 (helix X) is replaced with a neutral side chain (Smirnova et al., 2008; Fig. 4). Mutant E325A binds sugar with an affinity similar to WT, but affinity is independent of pH up to at least pH 11. Even more
Figure 4.  \( pK_a \) of Glu325 in LacY. The pH dependence of \( \Delta-I_R \) intensity change at 1,742 cm\(^{-1} \) was measured with the LacY G46W/G262W mutant in the absence or presence of \( \alpha \)-NPG (filled red circles) or E325A LacY (open red circles). The \( K_a \) values for \( \alpha \)-NPG binding to WT LacY (filled green circles), E325A LacY (open green circles), or LacY G46W/G262W mutant (filled cyan circles) were calculated as the ratio of rate constants \( (k_{off}/k_{on}) \) measured by stopped-flow fluorescence.

Remarkably, Glu325 also exhibits a \( pK_a \) of 10.5 by direct measurement with surface enhanced infrared absorption spectroscopy (SEIRAS; Grytsyk et al., 2017; Fig. 5). The perturbed \( pK_a \) is due to location in a hydrophobic pocket between helices IX and X (Figs. 5 and 6). Therefore, Glu325 is clearly the primary (and possibly the only) functional H\(^+\)-binding site in LacY. A \( pK_a \) value of 10.5 translates to a \( K_a \) for H\(^+\) of less than ~30 pM, so Glu325 is completely protonated over a very broad pH range. The overall conclusion is that the galactoside-binding site in LacY is remarkably stable to alkaline pH, but in order to elicit this property, Glu325 must be replaced with a neutral side chain.

Of potential importance, Glu325 is in close proximity to an electrostatic/hydrogen-bond network involving His322 and Lys319 on helix X, Tyr236 and Asp240 on helix VII, and Arg302 on helix IX (Fig. 6). Glu269 and particularly His322, initially thought to be involved in H\(^+\) translocation, are both ligands for the galactopyranosyl moiety in the sugar-binding site (Fig. 2, b and c). Mutation of Glu269, His322, or Tyr236 causes a marked decrease in sugar-binding affinity (Smirnova et al., 2008). Another salt bridge (Asp237 in helix VII and Lys358 in helix XI) also lies in the cavity, but does not make a direct contact with either site and is not involved in sugar or H\(^+\) binding. However, this salt bridge is essential for the insertion of LacY into the membrane and for maintaining protein stability (Kaback et al., 2001).

Since transmembrane exchange does not involve \( \Delta \psi \), it is apparent that alternating access is driven by binding and dissociation of sugar, and does not involve turnover of H\(^+\) (Kaback, 2015). Because the driving force for accumulation against a gradient is \( \Delta \psi \), and E. coli has a stable internal pH of 7.6 (Padan et al., 1976; Ramos et al., 1976; Zilberstein et al., 1979), a \( pK_a \) of 10.5 for LacY (Smirnova et al., 2008; Grytsyk et al., 2017) implies that decreasing the p\( K_a \) by three orders of magnitude to cytoplasmic pH would release only ~50% of the transported lactose from LacY. How to increase the rate of deprotonation without decreasing the H\(^+\) concentration of the E. coli cytoplasm? Arg302 is relatively close to Glu325 (~6 Å), and mutants R302A or R302S cannot perform active transport, but catalyze transmembrane exchange (Sahin-Töth et al., 2001; Weinglass et al., 2002). A recent SEIRAS study (Grytsyk et al., 2019) on the effect of mutating residues in the immediate vicinity of Glu325 shows that only mutation R302K alters the Glu325 \( pK_a \), causing over a 2-pH U acid shift. Overall, the findings support the idea that H\(^+\) is extracted from Glu325 by spatial fluctuations in the position of Arg302, expelling H\(^+\) by moving near Glu325 (Weinglass et al., 2001) or vice versa.

Although nothing more is known at present, neutral replacements for specific carboxyl residues in the E. coli melibiose (Ethayathulla et al., 2014), fucose (Dang et al., 2010), and xylose permeases (Sun et al., 2012), as well as the Staphylococcus epi- dermidis glucose permease (Iancu et al., 2013), behave functionally in a manner similar to neutral replacement mutants for Glu325 in LacY. Thus, each of these sugar permease mutants is defective with respect to active transport (i.e., H\(^+\)/sugar symport), but still able to catalyze transmembrane exchange reactions. At the least, the findings indicate that these MFS family members likely have an ordered kinetic mechanism similar to that found with LacY.

**Alternating access and the symport mechanism**

An alternating-access type of mechanism is widely accepted to explain membrane transport; thus, H\(^+\) symporters expose both H\(^+\)- and cargo-binding sites alternatively to either side of the membrane through conformational changes. In LacY, both the sugar- and H\(^+\)-binding sites are located at the approximate middle of the molecule at the apex of the cavity, providing a structural basis for such an alternating-access mechanism.

The periplasmic- and cytoplasmic-open conformations with bound galactoside and H\(^+\) reflect static states. However, LacY must undergo dynamic conformational changes to expose both the sugar- and H\(^+\)-binding sites to either side of the membrane. Multiple independent experimental methods (Smirnova et al., 2011a), e.g., site-directed alkylation of single-Cys mutants; thiol cross-linking; single molecule FRET; double electron-electron resonance spectroscopy; and time-resolved FRET techniques to detect conformational change, sugar-binding kinetics, and nanobodies, have been used to examine sugar-induced conformational changes which provide independent converging evidence that periplasmic and cytoplasmic cavities open...
reciprocally and that opening of the periplasmic cavity controls closing of the cytoplasmic cavity (Kaback et al., 2011; Smirnova et al., 2017). In the absence of a galactoside, LacY may favor an inward-facing conformation, the resting state, as indicated by the crystal structure (Fig. 1), but galactoside binding causes LacY to populate intermediate occluded and outward-open conformations.

The LacY transport cycle includes alternating-access and several other steps that are explained by a simplified eight-step kinetic scheme (Fig. 7). Based upon an ordered-binding model (Kaczorowski et al., 1979), different transport modes are explained in detail below.

**Active transport**

Active transport of lactose against a lactose concentration gradient is driven by $\Delta \tilde{\mu}_{H^+}$ (interior negative and/or alkaline). LacY is completely protonated in the outward-open state, which primes it for sugar binding with relatively high affinity (1). Binding of galactoside from the periplasm (2) to protonated LacY induces the formation of an occluded intermediate (3), in which sugar and $H^+$ are bound and both solvent accessible pathways from either side of protein are closed. The intermediate with a bound sugar and $H^+$ then opens to the cytoplasm (4), sugar dissociates (5) followed by deprotonation of Glu325 (6) to the cytoplasm, unloaded LacY returns to the outward-facing conformation by steps (7 and 8), and Glu325 is protonated immediately (1), ready for the next transport cycle.

LacY drives accumulation of lactose against a 50- to 100-fold concentration gradient (Robertson et al., 1980) with a turnover number of $\sim 20 \text{ s}^{-1}$. Both $\Delta Y$ (interior negative) and $\Delta pH$ (interior alkaline) have quantitatively the same effect on transport, with a 50- to 100-fold decrease in $K_m$ and the same thermodynamic equilibrium. In opposition to time-honored conjecture, the $K_d$ for galactosides on either side of the membrane is essentially the same in the absence or presence of $\Delta \tilde{\mu}_{H^+}$ (Guan and Kaback, 2004). In the absence of $\Delta \tilde{\mu}_{H^+}$, there is three- to fourfold inhibition of lactose transport in the presence of deuterium oxide (D$_2$O), indicating that deprotonation is rate limiting (Viitanen et al., 1983; García-Celma et al., 2009, 2010; Gaiko et al., 2013). However, when there is a driving force on the H$^+$ in the presence of $\Delta \tilde{\mu}_{H^+}$, deprotonation is no longer rate-limiting, and the transport rate is unaffected by D$_2$O. It is particularly noteworthy that a number of experimental findings indicate that $\Delta \tilde{\mu}_{H^+}$ functions kinetically as a driving force on the H$^+$, but alternating access itself is driven by galactoside binding and dissociation independent of $\Delta \tilde{\mu}_{H^+}$ (Guan and Kaback, 2006).

**Transport down a concentration gradient**

Inwardly directed flux (influx) or outwardly directed flux (efflux) down lactose concentration gradients (Fig. 3, b and c) occurs in a similar fashion, starting at step 1 for influx and step 6 for efflux and proceeding around the circle. Both transport modes generate $\Delta \tilde{\mu}_{H^+}$, the polarity of which depends on the direction of the flux, and the limiting step involves deprotonation of Glu325. Both reactions exhibit deuterium isotope effects; i.e., influx and efflux are inhibited by D$_2$O.

**Transmembrane exchange**

Equilibrium exchange and counterflow represent alternating access of the galactoside-binding site to alternative sides of the membrane. In either reaction, LacY binds a sugar molecule on
the external side of the membrane (unlabeled if equilibrium exchange; labeled if counterflow) and exchanges that sugar molecule for its opposite member on internal side of membrane. With equilibrium exchange, the concentrations are the same on both sides of the membrane; with counterflow, the internal concentration of unlabeled galactoside is much higher than the external concentration of labeled sugar. Under both conditions, the H\textsuperscript+ does not dissociate from LacY, and both reactions are unaffected by ambient pH or Δ\textmu\textsubscript{H\textsuperscript{+}} and do not exhibit a deuterium isotope effect. Only steps 2 through 5 in Fig. 7 are engaged. For counterflow, the initial 1/1 exchange of internal unlabeled substrate for external labeled galactoside leads to a rapid increase in internal radioactivity until all of the internal unlabeled sugar inside is exchanged for external labeled galactoside. At this point, the increase in radioactivity stops, and the internal radioactivity decreases with time as the concentrations equilibrate and the specific activity of the external labeled substrate decreases, thereby causing the typical “overshoot” profile that is observed.

Ordered binding and release of H\textsuperscript+ and galactoside are strongly supported by the behavior of neutral replacement mutants in Glu325 that do not catalyze any reactions involving net H\textsuperscript+ translocation (i.e., electrogenic transport), but catalyze transmembrane exchange (i.e., electroneutral transport) at the same rate as WT LacY (Carrasco et al., 1989).

**Outward-facing conformers stabilized by Nbs**

Single-domain camelsid Nbs raised against a LacY mutant immobilized in an outward-open conformation bind to LacY completely inhibiting transport and stabilizing the open-outward conformers of the WT (Smirnova et al., 2014). Using site-directed, distance-dependent Trp quenching/unquenching of fluorescent probes on opposite surfaces of LacY, conformational states of LacY complexed with each of eight unique, high-affinity Nbs that bind exclusively to the periplasmic side and block transport, but increase accessibility of the sugar-binding site, were examined (Smirnova et al., 2015). Each Nb induces quenching with three pairs of cytoplasmic Trp/bimane probes, indicating closure of the cytoplasmic cavity. In reciprocal fashion, the same Nbs induce unquenching of fluorescence in three pairs of periplasmic probes due to opening of the periplasmic cavity. Since the extent of fluorescence change with various Nbs differs and the differences correlate with changes in the stopped-flow rate of sugar binding, it is also concluded that the Nbs stabilize several different outward-open conformations of LacY. Thus, Nb binding clearly involves conformational selection of LacY molecules, which has been confirmed by thermodynamic studies with isothermal titration calorimetry (Hariharan et al., 2016). X-ray crystal structures of three Nb/LacY complexes, one apo structure (Jiang et al., 2016) with no bound sugar substrate, and two with bound lactose analogues (Kumar et al., 2015, 2018), confirm the biochemical data indicating that the Nbs bind stoichiometrically to the periplasmic face of the C-terminal six-helix bundle with nanomolar affinities.

**IIA\textsuperscript{Glc} regulation of lactose utilization by catabolite repression**

Transcription of the lacY gene and LacY transport activity are down-regulated by the phosphoenolpyruvate-glucoside phosphotransferase system (PTS; Postma et al., 1993; Park et al., 2006). A phosphotransfer protein IIA\textsuperscript{Glc} of the PTS, the most abundant cytosolic protein in E. coli, plays a key role in the regulating carbohydrate metabolism by binding to various families of sugar transporters and many other soluble proteins, modulating their activities (Deutscher et al., 2014; Hariharan and Guan, 2014; Vogt et al., 2014; Hariharan et al., 2015). Binding measurements (Hariharan et al., 2015) show that unphosphorylated IIA\textsuperscript{Glc} binds to LacY in the absence or presence of galactoside. Glucose transport by PTS-mediated vectorial phosphorylation (Kaback, 1968) leads to preponderance of unphosphorylated IIA\textsuperscript{Glc}, which binds to LacY and inhibits galactoside binding and subsequent inducer formation. By this means, synthesis of inducer is prevented so that the cell preferentially utilizes glucose via the PTS. The phenomenon is known as “inducer exclusion.”

**Summary and future prospects**

Studies with LacY have provided new insights into the mechanism of H\textsuperscript+/galactoside symport as follows. (1) High-resolution structural information of at least two important conformations of LacY in apo and substrate-bound forms have been obtained. (2) Detailed understanding of galactoside and H\textsuperscript+ binding and symport, much of which was unanticipated, has been obtained. (3) Although chemiosmosis is the driving force for the overall process by which lactose is accumulated against a concentration gradient, the basic alternating access conformational change itself is independent of Δ\textmu\textsubscript{H\textsuperscript{+}}. (4) Active transport does not involve a change in affinity for galactoside on either side of the membrane. Rather, there is a marked change in affinity for H\textsuperscript+ driven by Δ\textmu\textsubscript{H\textsuperscript{+}}. And perhaps most surprisingly, (5) The mechanism of coupling between H\textsuperscript+ and galactoside binding is relatively simple—in order to bind/transport galactoside, Glu325 must be protonated.

Despite these advances, several fundamental questions remain unsolved, some of which are the following: How generalized are the findings with LacY with respect to other permeases? Why does a negative charge on Glu325 block galactoside binding? Is Glu325 the only side chain in LacY directly involved in coupled H\textsuperscript+ translocation? Although there is no change in K\textsubscript{m} for galactosides, why kinetically is there a decrease in K\textsubscript{m} in the presence of Δ\textmuH. What structural changes resulting from release of sugar during the transport cycle cause LacY deprotonation? Is an increase in water accessibility involved with deprotonation of Glu325? Does the transport mechanism in other symporters involve a carboxyl side chain with a perturbed pK\textsubscript{a} that can be detected by SEIRAS? By using Nbs, can cryo-EM be applied successfully to a protein the size of LacY?

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