The Alternating Access Transport Mechanism in LacY

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Abstract Lactose permease of *Escherichia coli* (LacY) is highly dynamic, and sugar binding causes closing of a large inward-facing cavity with opening of a wide outward-facing hydrophilic cavity. Therefore, lactose/H^+ symport via LacY very likely involves a global conformational change that allows alternating access of single sugar- and H^+-binding sites to either side of the membrane. Here, in honor of Stephan H. White's seventieth birthday, we review in camera the various biochemical/biophysical approaches that provide experimental evidence for the alternating access mechanism.

Keywords Lactose · Permease · Symport · Transport · Membrane · Membrane protein

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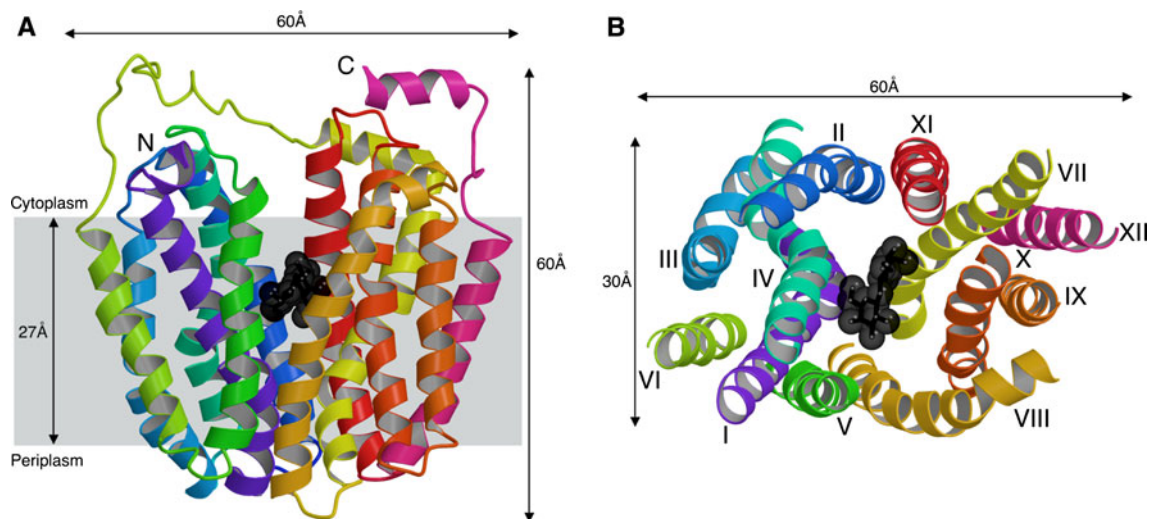e-mail: rkaback@mednet.ucla.edu

Introduction

The lactose permease of *Escherichia coli* (LacY), which catalyzes the coupled symport of a galactopyranoside and an H^+, is a paradigm for the major facilitator superfamily (MFS) of membrane transport proteins. LacY has been solubilized, purified and reconstituted into proteoliposomes in a fully functional state (reviewed in Viitanen et al. 1986). Furthermore, X-ray crystal structures of the conformationally restricted mutant Cys154→Gly have been solved in an inward-facing conformation (Abramson et al. 2003; Mirza et al. 2006), and the crystal structure of wild-type LacY exhibits the same conformation (Guan and Kaback 2006; Guan et al. 2007). Both structures have 12 transmembrane α-helices, most of which are shaped irregularly, organized into two pseudosymmetrical six-helix bundles surrounding a large interior hydrophilic cavity open to the cytoplasm only (Fig. 1). The sugar-binding site and the residues involved in H^+ translocation are at the approximate middle of the molecule at the apex of the hydrophilic cavity and distributed so that the side chains important for sugar recognition are predominantly in the N-terminal helix bundle and the side chains that form an H^+-binding site are mainly in the C-terminal bundle (Smirnova et al. 2009b). The periplasmic side of LacY is tightly packed, and the sugar-binding site is inaccessible from that side of the molecule. A similar structure has been observed for the X-ray structure of GlpT, which has little or no sequence homology with LacY and catalyzes exchange of inorganic phosphate for glycerol-3-P across the membrane (Huang et al. 2003).

Wild-type LacY is highly dynamic. H/D exchange of backbone amide protons in wild-type LacY occurs at rapid rate (le Coutre et al. 1998; Patzlaff et al. 1998; Sayeed and Baenziger 2009), and sugar binding by wild-type LacY is

Fig. 1 Overall structure of LacY. **a** Ribbon representation of LacY viewed parallel to the membrane. **b** Ribbon representation of LacY viewed along the membrane normal from the cytoplasmic side. TDG is represented as *black spheres*

mostly entropic (Nie et al. 2006), inducing widespread conformational changes (reviewed in Guan and Kaback 2006; Kaback 2005; Kaback et al. 2001). More specifically, site-directed alkylation (reviewed in Kaback et al. 2007; Nie et al. 2007, 2008; Nie and Kaback 2010), single molecule fluorescence resonance energy transfer (Majumdar et al. 2007), double electron–electron resonance (Smirnova et al. 2007), site-directed cross-linking (Zhou et al. 2008) and Trp-quenching studies (Smirnova et al. 2009a) each provide independent evidence that sugar binding increases the open probability of a wide hydrophilic cleft on the periplasmic side of LacY with closing of the cytoplasmic cavity so that the sugar- and H^+-binding sites become alternatively accessible to either side of the membrane (the alternating access model). It has also been shown that the periplasmic cleft must close, as well as open, for translocation of sugar across the membrane to occur (Liu et al. 2010; Zhou et al. 2008, 2009).

Notably, in the conformationally restricted mutant C154G LacY, sugar binding is enthalpic and the periplasmic cleft is paralyzed in an open conformation (Majumdar et al. 2007; Nie et al. 2008; Smirnova et al. 2007). However, all X-ray structures of LacY (C154G as well as wild-type LacY) exhibit the same inward-facing conformation. Therefore, it is likely that the crystallization process selects a single conformer of LacY that is in the lowest free-energy state.

A functional LacY molecule devoid of its eight native Cys residues (C-less LacY) has been engineered by constructing a cassette *lacY* gene with unique restriction sites about every 100 bp (van Iwaarden et al. 1991). Utilizing this cassette *lacY* for Cys-scanning mutagenesis, a highly useful library of molecules with a single-Cys residue at

virtually every position of LacY has been constructed (Frillingos and Kaback 1996). Cys is average in bulk, relatively hydrophobic and amenable to highly specific modification. Therefore, Cys-scanning mutagenesis has been used in combination with biochemical and biophysical techniques to reveal membrane topology, accessibility of intramembrane positions to the aqueous or lipid phase of the membrane and spatial proximity between transmembrane domains.

Here, the experimental approaches that provide a strong case for the alternating access model are reviewed cursorily.

Site-Directed Alkylation

Site-directed alkylation (SDA) of sylfhydryl thiols by radiolabeled *N*-ethylmaleimide (NEM) or fluorescent tetramethylrhodamine-5-maleimide (TMRM), which are membrane-permeant alkylating agents, has been used to study the reactivity of single-Cys LacY mutants in the C-less background in right-side-out (RSO) membrane vesicles. The approach provides important information about the structure, function and dynamics of LacY (reviewed in Guan and Kaback 2007). The reactivity/accessibility of Cys residues depend on the surrounding environment and are limited by close contacts between transmembrane helices and/or the low dielectric of the environment. Ligand binding increases NEM reactivity of single-Cys replacements located predominantly on the periplasmic side of LacY and decreases reactivity of those located predominantly on the cytoplasmic side (Guan and Kaback 2007). The pattern suggests that during sugar transport a periplasmic pathway opens with closing of the inward-facing cavity so that the
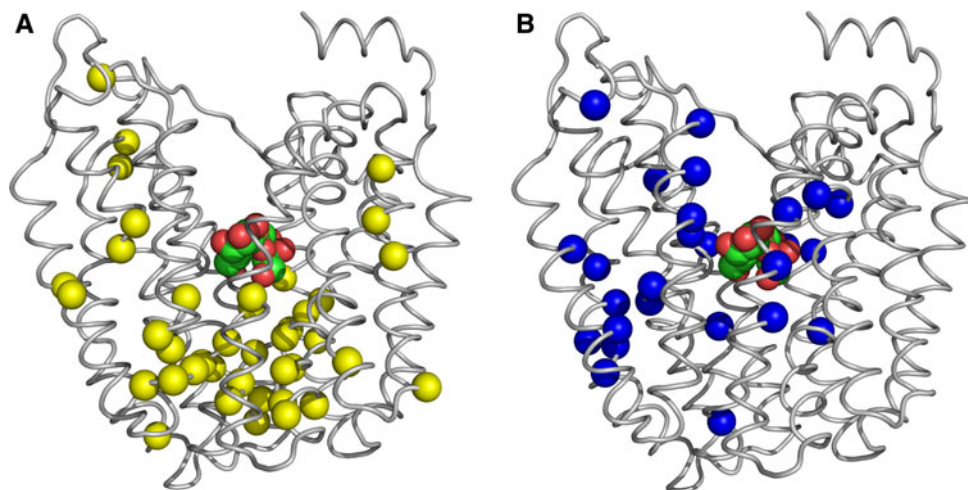
Fig. 2 Distribution of Cys replacements that exhibit changes in reactivity with NEM in the presence of TDG. **a** Positions of Cys replacements that exhibit a significant increase in reactivity with NEM (*gold spheres*) superimposed on the backbone of LacY viewed perpendicular to the plane of the membrane. TDG is shown as a CPK model at the apex of the inward-facing cavity. **b** Positions of Cys replacements that exhibit a significant decrease in reactivity with NEM (*blue spheres*) superimposed on the backbone of LacY viewed perpendicular to the plane of the membrane. The cytoplasmic surface is at the *top*, and TDG is shown as a CPK model at the apex of the inward-facing cavity (Color figure online)

sugar-binding site is alternatively accessible to either side of the membrane (Fig. 2).

Most recently, the simple, more facile alkylation method with TMRM (Nie et al. 2007, 2008, 2009) was utilized to examine the effect of sugar binding on alkylation of single-Cys LacY mutants either in RSO membrane vesicles or with purified proteins in dodecyl-β,D-maltopyranoside (DDM) micelles (Nie and Kaback 2010). Experiments were carried out at 0°C, where thermal motion is restricted (Venkatesan and Kaback 1998; Venkatesan et al. 2000a, 2000b), and linear rates of labeling were readily obtained (Fig. 3). TMRM labeling is almost negligible, with LacY containing each of five single-Cys residues at positions on the periplasmic side of the sugar-binding site in RSO membrane vesicles or with purified protein in DDM micelles. Therefore, each of these single-Cys replacements is unreactive and/or inaccessible to the alkylating agent. The observations are consistent with the interpretation that LacY in the native bacterial membrane is in a conformation similar to that of the X-ray crystal structures in the absence of ligand. The periplasmic side is tightly closed, and an open cavity is present facing the cytoplasm (the inward-facing conformation) (Abramson et al. 2003; Guan et al. 2007; Mirza et al. 2006).

As postulated by the alternating access model, on the cytoplasmic side of the sugar-binding site, each of five single-Cys replacement mutants labels at a rapid rate in the absence of sugar both in RSO membrane vesicles and with purified protein in DDM. Moreover, the tight-binding lactose homologue β-D-galactopyranosyl 1-thio-β,D-galactopyranoside

(TDG) decreases the rate of TMRM labeling either in the membrane or with purified protein in DDM (Fig. 3; Table 1). The findings agree with a variety of other measurements (see below) showing that sugar binding induces closing of the cytoplasmic cavity and reduced reactivity/accessibility to alkylating agents.

The average increase in periplasmic TMRM labeling observed in the presence of TDG in RSO vesicles is ~ 10-fold, and the average cytoplasmic decrease in the presence of TDG is very similar (approximately ninefold) (Table 1). With purified single-Cys proteins in DDM, the comparable averages are approximately sixfold and approximately fivefold. Thus, the change in TMRM labeling induced by sugar on opposite faces of LacY appears to be about the same in RSO vesicles or with the purified single-Cys mutants in DDM. Therefore, the data provide further evidence not only that sugar binding markedly increases the open probability on the periplasmic side but that sugar binding also increases the probability of closing on the inside, the implication being that opening and closing may be reciprocal. However, reciprocity may not be obligatory as evidence has been presented showing that the periplasmic pathway is fixed in an open conformation in the C154G mutant, while the cytoplasmic cavity is able to close and open (Majumdar et al. 2007; Nie et al. 2008; Smirnova et al. 2007). It has also been demonstrated (Liu et al. 2010) that replacement of Asp68 with Glu at the cytoplasmic end of helix II blocks sugar-induced opening of the periplasmic cleft but has little or no effect on closing of the cytoplasmic cavity.
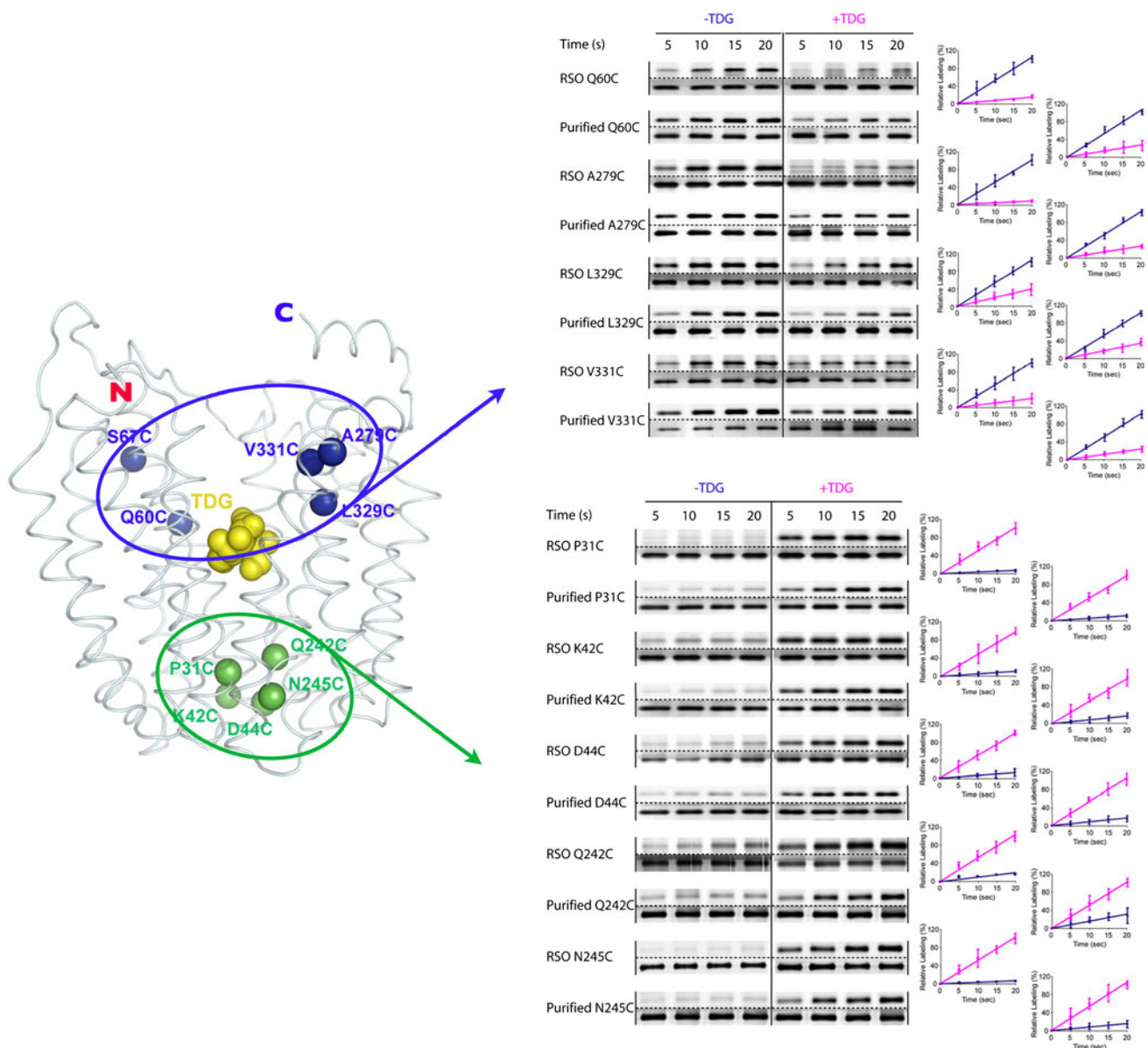
Fig. 3 TMRM labeling of cytoplasmic (*top*) or periplasmic (*bottom*) single-Cys mutants in RSO membrane vesicles or as purified proteins in DDM. Labeling of cytoplasmic single-Cys LacY mutants Q60C, S67C, A279C, L329C and V331C or periplasmic single-Cys LacY mutants Q31C, K42C, D44C, Q242C and N245C was performed with 40 μM TMRM (RSO membrane vesicles) or 4 μM TMRM (with purified proteins in DDM) for given times at 0°C in the absence of

TDG (−TDG; *blue diamond*) or preincubated for 10 min with TDG prior to addition of TMRM (+TDG; *pink square*). Relative TMRM labeling rates were calculated as described (Nie and Kaback 2010); the data are plotted relative to the 20-s points in the absence (*top*) or presence (*bottom*) of TDG. For SDS/PAGE gels, *upper gel* displays the results of TMRM labeling and *bottom* is the silver-stained protein sample (Color figure online)

Although the sugar-induced changes in the global conformation of LacY are qualitatively similar in RSO membrane vesicles and with the purified mutants in DDM, it is notable that the magnitude of the effects is somewhat smaller with the purified mutant proteins (Table 1). Thus, the increases and decreases in TMRM labeling observed with the purified proteins upon addition of TDG are on average ∼60% of those observed with RSO membrane vesicles. However, this is not surprising since it is known that a lipid bilayer (le Coutre et al. 1997) as well as its

composition (Bogdanov et al. 2002) are important constraints on the structure of LacY.

Single-Molecule Fluorescence (Förster) Resonance Energy Transfer

Single-molecule fluorescence (Förster) resonance energy transfer (sm-FRET) has also been used to test the alternating access model with wild-type LacY and mutant

Table 1 TDG changes the rate of TMRM labeling of single-Cys mutants

LacY mutant	Helix	Fold change in TMRM labeling in RSO vesicles	Fold change in TMRM labeling in DDM
Periplasmic			
P31C	I	13.6	8.9
K42C	II	8.5	5.9
D44C	II	8.2	6.2
Q242C	II	5.2	3.2
N245C	VII	14.9	6.9
Cytoplasmic			
Q60C	II	−7.2	−3.8
S67C	II	−14.7	−8.3
A279C	IX	−13.1	−3.9
L329C	X	−2.6	−3.0
V331C	X	−5.1	−4.3

Rates of TMRM labeling were obtained from the time courses shown in Fig. 3 as described (Nie and Kaback 2010). For each mutant, the ratio of the estimated initial rate of TMRM labeling in the presence of TDG relative to that observed in the absence of TDG was calculated. Positive numbers indicate an increase in the relative labeling rate due to addition of TDG (periplasmic) and negative numbers indicate a decrease in the relative labeling rate due to addition of TDG (cytoplasmic)

Fig. 4 Ligand-induced effects on the FRET distribution E^* at the cytoplasmic side of LacY (R73C/S401C, helices III and XII) or at the periplasmic side of LacY (I164C/S375C, helices V and XI). *Top* LacY backbone with donor (*magenta*) and acceptor fluorophores on the cytoplasmic or periplasmic side as indicated. *Bottom* **a** Frequency vs. E^* histograms corresponding to wild-type (**a**, **c**) and C154G mutant (**b**, **d**) LacY. Measurements for each construct were obtained in the absence of sugar (*gray*) and in the presence of 1 mM (saturating) galactosidic sugar concentration (*red*) or 1 mM glucosidic sugar (*blue*) (Nie et al. 2007). High E^* indicates high smFRET (i.e., closer distance between the fluorophores); low E^* indicates low smFRET (i.e., further distance) (Color figure online)
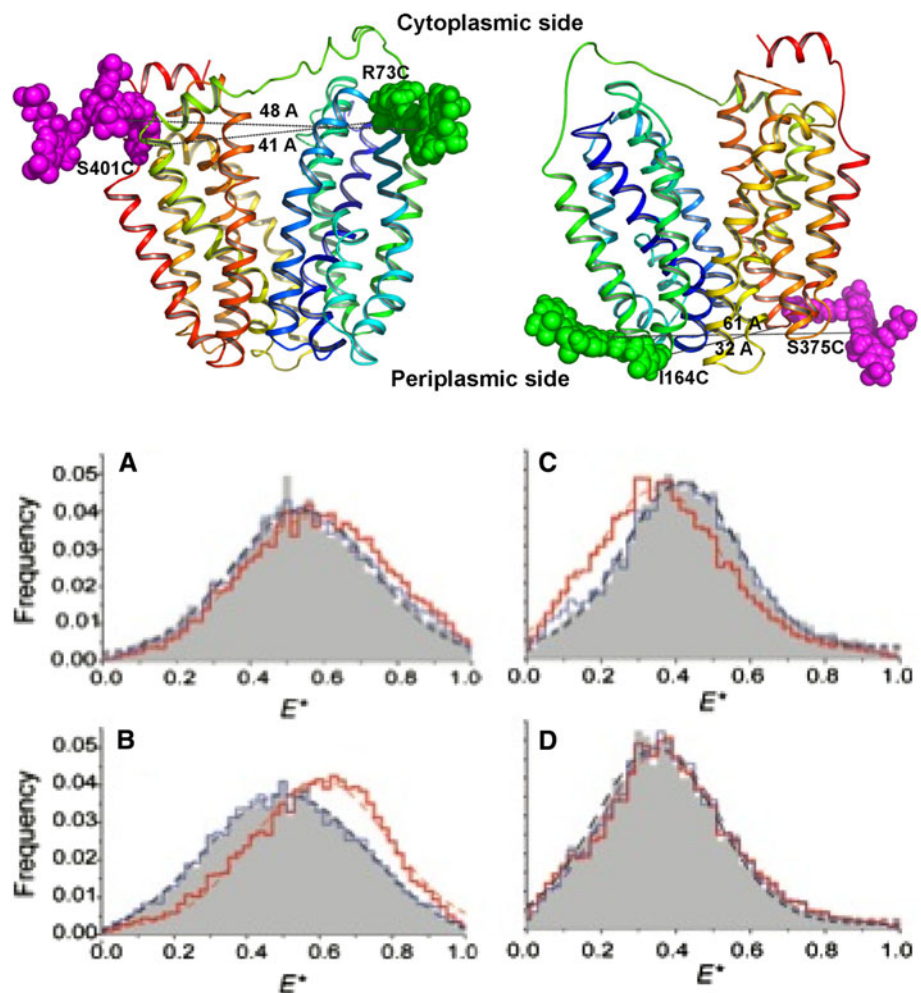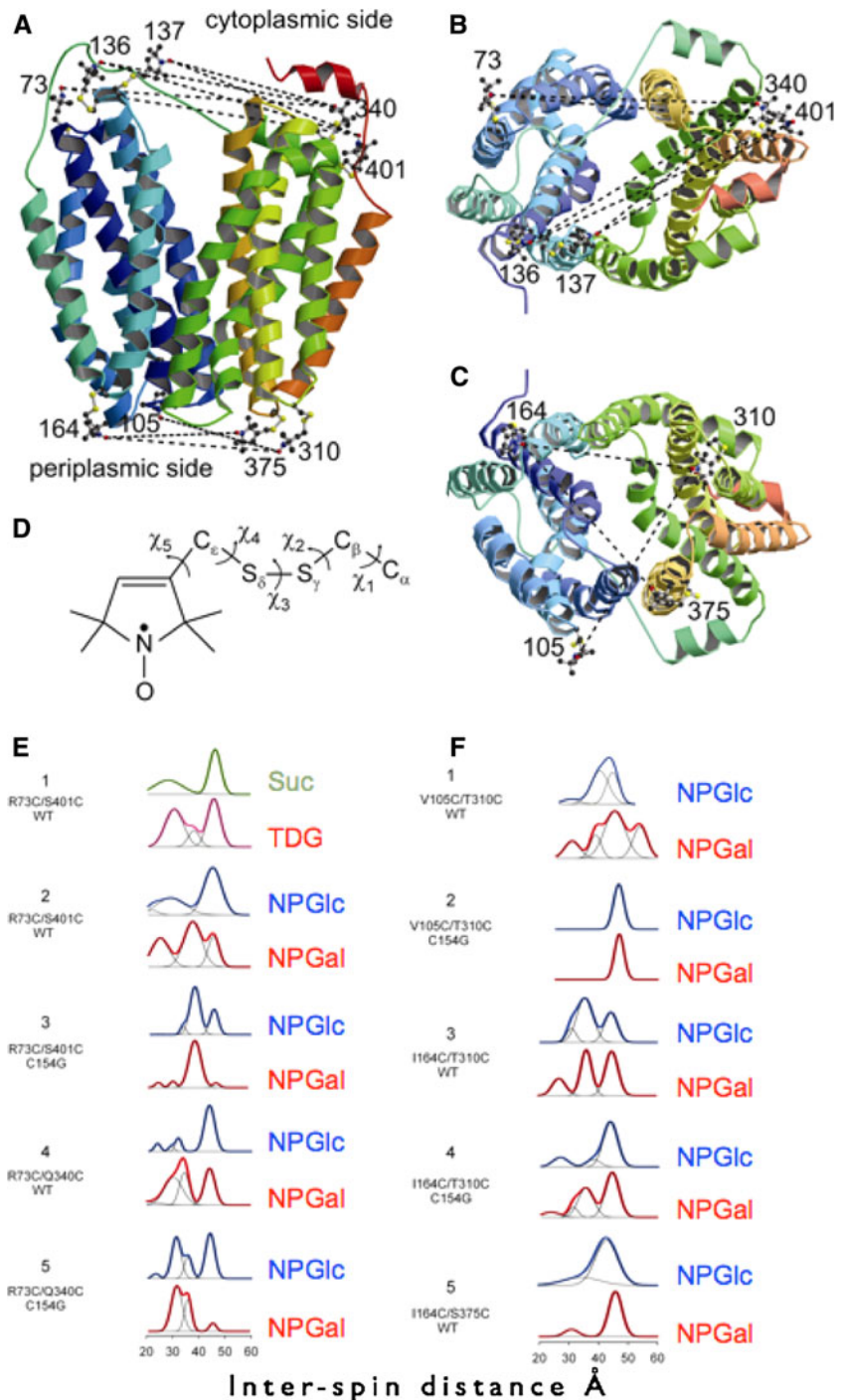
Fig. 5 *Top* Disulfide-linked nitroxide chains are modeled on the LacY X-ray structure (PDB ID 1PV6, rainbow-colored ribbons from *blue* [helix I] to *red* [helix XII]) with the cavity open to the cytoplasmic side. Nitroxides attached to the backbone of the protein are shown as *balls and sticks*. Interspin distances used for measurements are shown as *dashed lines*. Cytoplasmic pairs are viewed from the side (**a**) and from cytoplasm (**b**). Periplasmic pairs are viewed from the side (**a**) and from periplasm (**c**). Structure of the nitroxide side chain attached to the protein with indicated dihedral angles (**d**). *Bottom* DEER characterization of sugar-binding effects on interspin distances of nitroxide-labeled double-cysteine mutants located on the cytoplasmic (**e**) or the periplasmic (**f**) side of LacY. Distance distributions obtained by Tikhonov regularization are shown for protein with no sugar bound (glucosidic sugars sucrose or NP*Glc*—*green* or *blue*, respectively) and with bound sugar (galatosidic sugars TDG or NP*Gal*—*pink* or *red lines*, respectively), multi-gaussian fits (*black lines*) demonstrating relative distributions of conformational populations. See Smirnova et al. (2007) for details (Color figure online)

C154G in collaboration with Devdoot Majumdar and Shimon Weiss (Majumdar et al. 2007). Pairs of Cys residues at the ends of two helices on the cytoplasmic or periplasmic side of wild-type LacY and the mutant were labeled with appropriate donor and acceptor fluorophores, sm-FRET was determined in the absence and presence of sugar and distance changes were calculated. With wild-type LacY, binding of a galactopyranoside, but not a glucopyranoside, results in a decrease in distance on the cytoplasmic side and an increase in distance and in distance distribution on the periplasmic side (Fig. 4). In contrast, with the mutant, more pronounced decreases in distance and in distance distribution are observed on the cytoplasmic side but there is no change on the periplasmic side (Fig. 4). The results are consistent with the alternating access model and indicate that the translocation defect in the mutant is due to paralysis in the outward-facing conformation.

Double Electron–Electron Resonance

Double electron–electron resonance (DEER), a site-directed spin labeling technique, was applied to measure interhelical distance changes induced by sugar binding in collaboration with Christian Altenbach and Wayne Hubbell (Smirnova et al. 2007). Nitroxide-labeled paired-Cys replacements were constructed at the ends of transmembrane helices on the cytoplasmic or periplasmic side of LacY and in the conformationally restricted mutant C154G (Fig. 5a–d). Distances were then determined in the presence of galactosidic or nongalactosidic sugars (Fig. 5e, f). Strikingly, specific binding causes conformational rearrangements on both sides of the molecule. On the cytoplasmic side, each of six nitroxide-labeled pairs exhibits decreased interspin distances, ranging 4–21 Å. Conversely, on the periplasmic side, each of three spin-labeled pairs shows increased distances, ranging 4–14 Å. Thus, the inward-facing cytoplasmic cavity closes and a cavity opens on the tightly packed periplasmic side. In the C154G mutant, sugar-induced closing is observed on the cytoplasmic face but little or no change occurs on periplasmic side. DEER measurements in conjunction with molecular modeling based on the X-ray structure provide strong support for the alternative access model and suggest a structure for the outward-facing conformation of LacY.

Site-Directed Cross-Linking

As discussed, the residues essential for sugar recognition and H^+ translocation are located at the apex of the cavity and are inaccessible from the outside. On the periplasmic side, helices I/II and VII from the N and C six-helix bundles, respectively, participate in sealing the cavity from the outside. Three paired double-Cys mutants—Ile40 → Cys/Asn245 → Cys, Thr45 → Cys/Asn245 → Cys and Ile32 → Cys/Asn245 → Cys—located in the interface between helices I/II and VII on the periplasmic side of LacY were constructed with tandem factor Xa protease
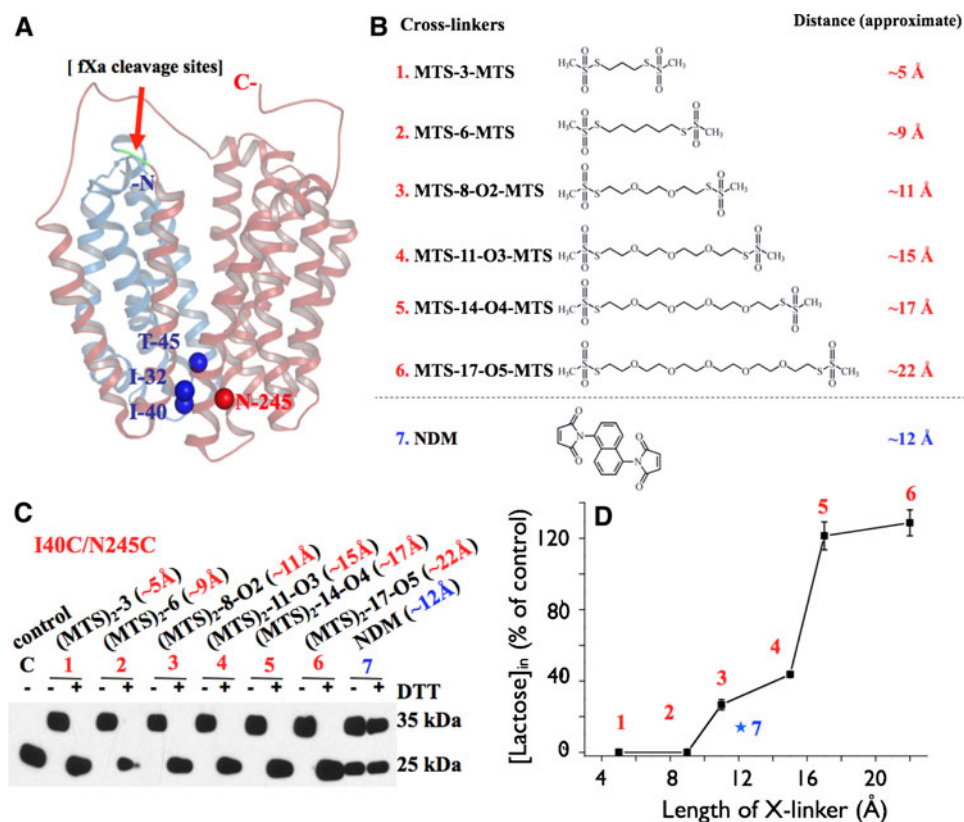

Fig. 6 a Structure model of LacY (PDB ID 2V8 N). Residues Ile32 (helix I), Ile40 (loop I/II) and Thr45 (helix II) are presented as *dark blue spheres*, and residue N245 (helix VII) is presented as a *red sphere*. The N-terminal four helices (N_4) and the C-terminal helices (C_8) are shown in *blue* and *red*, respectively, and separated by tandem factor Xa protease sites (*left side, green*), as indicated by the *red arrow*. b Homobifunctional cross-linking reagents. For MTS reagents, the approximate S–S distances between bridging sulfur atoms in the chains are as given by the manufacturer. The distance for NDM is from Green et al. (2001). Cross-linking (c) and lactose transport (d) with mutant I40C/N245C. All experiments were performed with RSO vesicles. 1, MTS-3-MTS; 2, MTS-6-MTS; 3, MTS-8-O2-MTS; 4, MTS-11-O3-MTS; 5, MTS-14-O4-MTS; 6, MTS-17-O5-MTS; 7, NDM (Color figure online)

sites between the two Cys replacements (Fig. 6a) (Zhou et al. 2008). After quantitative cross-linking with flexible homo-bifunctional reagents less than about 15 Å in length, all three mutants lose the ability to catalyze lactose transport (Fig. 6b–d). Strikingly, however, full or partial activity is observed when cross-linking is mediated by flexible reagents greater than about 15 Å in length. Moreover, 17 Å is the minimum required for maximum activity, a distance very similar to that obtained from DEER (Smirnova et al. 2007). The results provide further support for the argument that transport via LacY involves opening and closing of a large periplasmic cavity.

Trp Quenching

Since Trp fluorescence is quenched by certain amino acyl side chains such as a protonated His or amino group, Trp residues were placed on either side of LacY where they are predicted to be in close proximity to the imidazole side chains of His in either the inward- or outward-facing conformation (Fig. 7, top) (Smirnova et al. 2009a). In the inward-facing conformation, LacY is tightly packed on the periplasmic side and Trp residues placed at position 245 (helix VII) or 378 (helix XII) are in close contact with His35 (helix I) or Lys42 (helix II), respectively. Sugar binding leads to *unquenching* of Trp fluorescence in both mutants, a finding clearly consistent with opening of the periplasmic cavity (Fig. 7a). The pH dependence of Trp245 unquenching exhibits a pKa of ~ 8, typical for a His side chain interacting with an aromatic group. On the cytoplasmic side, Phe140 (helix V) and Phe334 (helix X) are located on opposite sides of a wide open hydrophilic cavity. In precisely the opposite fashion from the periplasmic side, mutant Phe140 → Trp/Phe334 → His exhibits sugar-induced Trp *quenching* (Fig. 7b). Again,
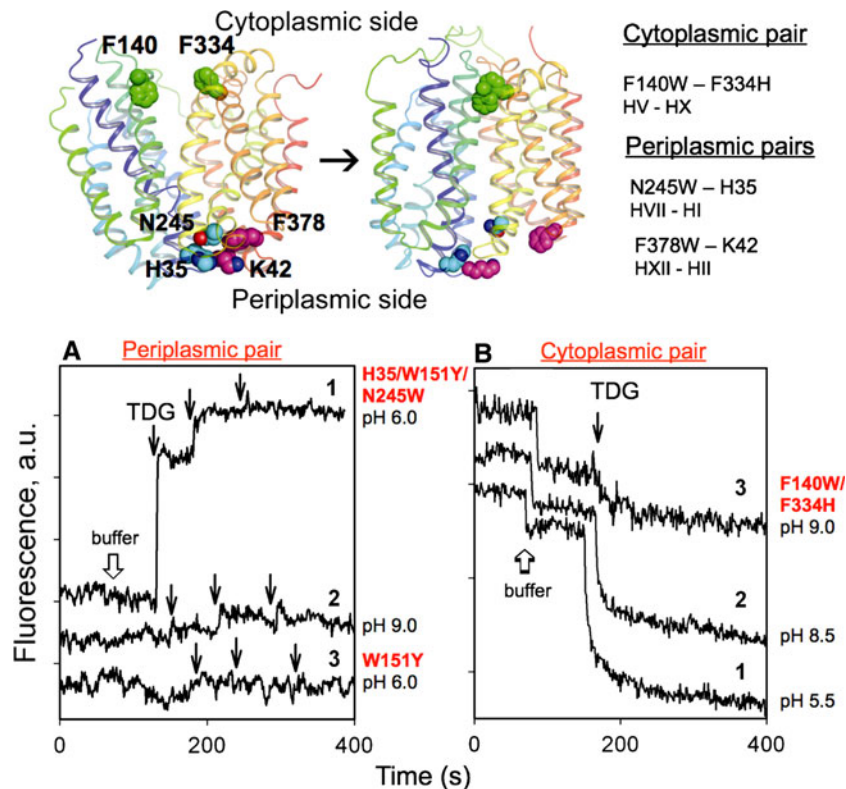

Fig. 7 *Top* Pairs of amino acid residues selected for Trp substitutions are shown on the backbone structure of LacY. The F140–F334 pair (helices V and X) on the cytoplasmic side is shown as *green spheres*. The H35–N245 (helices I and VII) and K42–F378 (helices II and XII) pairs on the periplasmic side are shown as *cyan* and *pink spheres*, respectively. A side view of the overall structure in the inward-facing conformation (PDB ID 2CFQ) is shown on the *left*, and the structure in the outward-facing conformation modeled (Smirnova et al. 2007) is shown on the *right*. Transmembrane helices are rainbow-colored from *blue* (helix I) to *red* (helix XII). *Arrow* indicates the conformational change resulting from sugar binding. The LacY structure is presented using Pymol 0.97 (DeLano Scientific, San Carlos, CA). **a** TDG effect on fluorescence of Trp introduced on the periplasmic side. Sequential additions of 6 μl of buffer (*open arrow*) and 6-μl aliquots of 1.8 M TDG (*black arrows*) to the mutant W151Y/N245 W at pH 6.0 (*trace 1*) or at pH 9.0 (*trace 2*) and to the control mutant W151Y at pH 6.0 (*trace 3*). **b** TDG effect on Trp fluorescence of cytoplasmic pair F140 W–F334H at pH 5.5 (*trace 1*), pH 8.5 (*trace 2*) and pH 9.0 (*trace 3*). Additions of 20 μl of buffer (*open arrows*) or 20 μl of 1.5 M TDG (*black arrows*) were made into 2 ml of protein solutions (Color figure online)

quenching is pH-dependent with a pKa of ~ 8. The results provide yet another strong, independent line of evidence for the alternating access mechanism and demonstrate that the methodology described provides a sensitive probe to measure conformational changes in membrane transport proteins.

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