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Lactose Permease and the Alternating Access Mechanism

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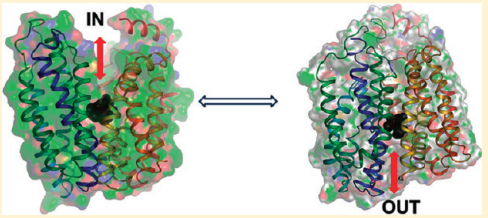
ABSTRACT: Crystal structures of the lactose permease of *Escherichia coli* (LacY) reveal 12, mostly irregular transmembrane α-helices surrounding a large cavity open to the cytoplasm and a tightly sealed periplasmic side (inward-facing conformation) with the sugar-binding site at the apex of the cavity and inaccessible from the periplasm. However, LacY is highly dynamic, and binding of a galactopyranoside causes closing of the inward-facing cavity with opening of a complementary outward-facing cavity. Therefore, the coupled, electrogenic translocation of a sugar and a proton across the cytoplasmic membrane via LacY very likely involves a global conformational change that allows alternating access of sugar- and H^+-binding sites to either side of the membrane. Here the various biochemical and biophysical approaches that provide strong support for the alternating access mechanism are reviewed. Evidence is also presented indicating that opening of the periplasmic cavity is probably the limiting step for binding and perhaps transport.



Typical of many transport proteins from organisms as widely separated evolutionarily as *Archaea* and *Homo sapiens*, the lactose permease of *Escherichia coli* (LacY), a member of the major facilitator superfamily (MFS),[1] catalyzes the coupled, stoichiometric translocation of a galactopyranoside and a proton (sugar/H^+ symport) across the cytoplasmic membrane.[2,3] Because transport is obligatorily coupled, uphill transport of sugar against a concentration gradient (i.e., active transport) is achieved by transduction of free energy released from the downhill movement of H^+ with the electrochemical H^+ gradient ($\Delta\tilde{\mu}_{H^+}$; interior negative and/or alkaline). Conversely, downhill sugar translocation by LacY drives uphill H^+ translocation with the generation of $\Delta\tilde{\mu}_{H^+}$, the polarity of which depends on the direction of the sugar concentration gradient (reviewed in refs 4−6). Thus, LacY and other ion-coupled transporters are energy transducers and not pumps,[7] which generally refer to proteins that utilize the energy released by ATP hydrolysis to generate a solute concentration gradient.

LacY functions as a monomer (reviewed in ref 5), and a single purified polypeptide is fully functional after reconstitution into proteoliposomes (reviewed in ref 8). Furthermore, X-ray crystal structures of the conformationally restricted mutant C154G (helix V) exhibit an inward-facing structure,[9,10] and a subsequent crystal structure of wild-type (WT) LacY[11] and that of a single-Cys mutant[12] have the same conformation. All structures to date have 12, mostly irregular transmembrane α-helices organized into two pseudosymmetrical six-helix bundles surrounding a large interior hydrophilic cavity open to the cytoplasm (Figure 1A,B). The sugar-binding site and the residues involved in H^+ binding are distributed at the approximate middle of the molecule at the apex of the hydrophilic cavity (Figure 1A,C). The side chains important for sugar recognition are predominantly in the N-terminal six-helix bundle, and the side chains that form a H^+-binding site(s) are in the C-terminal six-helix bundle for the most part. The amino acids involved in H^+ translocation do not form a pathway through the membrane and are organized in a tightly interconnected H-bond−salt bridge cluster, which is responsible for a remarkably high pK_a of approximately 10.5 that has been determined for the binding affinity of sugar for LacY (see refs 5, 13, and 14). The periplasmic side of LacY is tightly sealed, and the sugar-binding site is inaccessible from the external (periplasmic) side of the molecule (Figure 1A). Clearly, an alternative outward-facing conformation open to the periplasmic side is required for the transport of substrate across the membrane. An outward-facing model for LacY was proposed on the basis of thiol cross-linking studies as well as accessibility of Cys replacements for site-directed alkylation, and an alternating access mechanism of transport was proposed to describe lactose/H^+ symport catalyzed by LacY.[9,15] This mechanism, which is probably typical for other MFS transporters, has found strong support from the crystal structures of other related transporters. A similar inward-facing structure has been observed for GlpT,[16] which has little sequence homology with LacY and catalyzes exchange of inorganic phosphate for glycerol 3-phosphate across the membrane. Later, occluded intermediates [17,18] and an outward-facing[19] structure have also been observed for other MFS family members.

Molecular modeling based on existing LacY structures, as well as a wealth of biochemical data to be described below, has led to a reasonable model for the outward-facing conformation of LacY.[20] Furthermore, molecular dynamics (MD) simulations of LacY embedded in a phospholipid bilayer have reported partial closing on the cytoplasmic side and minor periplasmic conformational changes[21,22] that are much smaller than those estimated from experimental studies. Recently, a two-step

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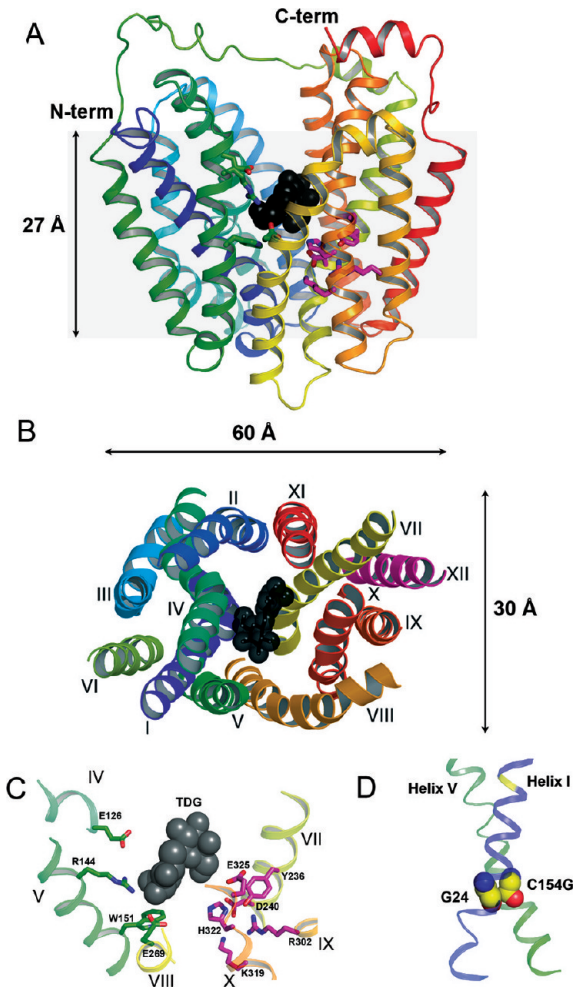

Figure 1. Structure of LacY with transmembrane helices rainbow colored from blue (helix I) to red (helix XII) and bound TDG presented as back spheres. Residues in sugar- and proton-binding sites are shown as green and pink sticks, respectively. (A) View parallel to the membrane (Protein Data Bank entry 1PV7). The hydrophilic cavity is open to the cytoplasm. The gray area represents the approximate thickness of the membrane phospholipid bilayer. (B) Cytoplasmic view showing dimensions of the LacY molecule and spatial packing of transmembrane helices. The loop regions have been omitted for the sake of clarity. (C) Detailed view from the cytoplasm showing residues in the sugar- and H^+-binding sites. (D) Transmembrane helices I and V are tightly packed in the C154G mutant viewed parallel to the membrane (from the LacY structure of Protein Data Bank entry 2CFQ). Gly residues 24 and 154 are shown as spheres.

hybrid simulation approach led to a model that has a cavity with a pore diameter of 7 Å and exhibits sugar-induced closure on the cytoplasmic side that agrees with the alternating access mechanism based on the experimental data.[23] A novel approach based on structural repeats in the transmembrane domains of LacY suggests a model that compares very favorably with the outward-facing crystal structure determined for FucP.[24]

WT LacY is highly dynamic. Proton−deuterium exchange of virtually all of the backbone amide protons occurs at a very rapid rate with this hydrophobic protein (~75% hydrophobic side chains) embedded in either a detergent micelle[25,26] or a phospholipid membrane.[27] Moreover, sugar binding by WT LacY is mostly entropic in nature,[28] inducing widespread conformational changes (reviewed in refs 4, 5, and 15). Although an outward-open conformation of LacY has not yet been obtained crystallographically, various biochemical and biophysical approaches reviewed here provide converging evidence that binding of galactosidic sugars increases the open probability of a wide hydrophilic cleft on the periplasmic side of the molecule with closure of the cytoplasmic cavity. By this means, sugar- and H^+-binding sites in the middle of LacY become alternatively accessible to either side of the membrane as a result of reciprocal opening and closing of hydrophilic pathways on either side of the membrane. Importantly, the periplasmic cleft must open, as well as close, for translocation of sugar across the membrane to occur.[29−32]

Early site-directed mutagenesis of each Cys residue in LacY led to the isolation and characterization of a mutant with Gly in place of native Cys154 (helix V).[33,34] Remarkably, this single mutation completely changes the functional properties and physical characteristics of LacY.[35] C154G LacY binds ligand as well as or better than WT LacY but catalyzes very little translocation across the membrane. Moreover, while sugar binding is mostly entropic with WT LacY, it is enthalpic with the C154G mutant.[28] Indeed, the mutant does not exhibit the long-range conformational changes observed on the periplasmic side upon ligand binding; it is also thermostable with respect to ligand binding and aggregation[35] and arrested in a partially open outward conformation(s) in the membrane.[20,36,37] However, the X-ray structure of the mutant exhibits an open-inward conformation with a tightly sealed periplasmic side,[9,10] indicating that crystallization selects a single conformer of LacY that is not representative of the structure of the mutant in the membrane.

The X-ray crystal structures yield a clue about why the C154G mutant is conformationally restricted (Figure 1D). Helices V and I cross in the approximate middle of the membrane where the Cys154 → Gly mutation (helix V) and Gly24 (helix I) are in the proximity to each other,[9,10,38] which can lead to significantly tighter helix packing[39−42] and may partly explain the lack of conformational flexibility of the C154G mutant. Indeed, when Gly24 is replaced with Cys in the C154G mutant, the G24C/C154G double mutant exhibits a marked increase in transport activity with sugar binding and low thermostability similar to that of WT LacY.[38] Recent site-directed cross-linking studies[43] are consistent with previous observations[38] suggesting that binding of sugar to LacY causes a localized scissorslike movement between helices I and V near the point where the two helices cross in the middle of the protein. The C154G mutation may interfere with movement of helices I and V required for proper structural rearrangements during turnover.

A functional LacY molecule devoid of the eight native Cys residues (Cys-less LacY) was engineered by constructing a cassette *lacY* gene with unique restriction sites approximately every 100 bp.[44] Utilizing this cassette *lacY* gene for Cys-scanning mutagenesis, a highly useful library of mutants with a single Cys residue at virtually every position of LacY was created.[45] Cys is average in bulk, relatively hydrophobic, and amenable to highly specific modification. Therefore, Cys-scanning mutagenesis, a technique pioneered with LacY (reviewed in ref 46), was and is currently being used in conjunction with biochemical and spectroscopic techniques to reveal protein topology in the membrane, accessibility of amino acid residues to the aqueous or lipid phase [some use the acronym SCAM for the substituted cysteine accessibility method (see ref 47)], and spatial proximity between trans-

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membrane domains (see ref 48) to study LacY, as well as a wide range of other membrane proteins (see ref 49).

In this work, the experimental approaches that provide a strong case for the alternating access mechanism in LacY are reviewed.

SITE-DIRECTED ALKYLATION (SDA)

Alkylation of Cys side chains by radiolabeled *N*-ethylmaleimide (NEM) or fluorescent tetramethylrhodamine-5-maleimide (TMRM), which are membrane-permeant reagents, has been used extensively to study the reactivity and accessibility of single Cys residues introduced into Cys-less LacY. This approach, which is based on alkylation of single-Cys LacY in right-side-out (RSO) membrane vesicles containing single-Cys mutants, provides important information about the structure, function, and dynamics of LacY (reviewed in refs 50 and 51). The reactivity and accessibility of Cys residues depend on the surrounding environment and are limited by close contacts between transmembrane helices and/or the low dielectric constant of the environment. Almost every amino acid in LacY was replaced individually with Cys and tested by the SDA method for reactivity with NEM, which provided initial support for an alternating access mechanism. Engineered Cys replacements located near or within the inward-facing hydrophilic cavity react well with this alkylating agent. Binding of thiodigalactoside (TDG) increases the NEM reactivity of single-Cys replacements located predominantly on the periplasmic side of LacY (Figure 2A) and decreases the reactivity of replacements located predominantly on the cytoplasmic side (Figure 2B). Furthermore, both sets of Cys replacements in the putative cavities are located at the periplasmic (increased reactivity) and cytoplasmic (decreased reactivity) ends of the same helices and distributed in a pseudosymmetrical manner (Figure 2C,D). The pattern is consistent with a model in which the single sugar-binding site in the approximate middle of LacY is alternatively exposed to either side of the membrane because of opening and closing of cytoplasmic and periplasmic hydrophilic pathways.

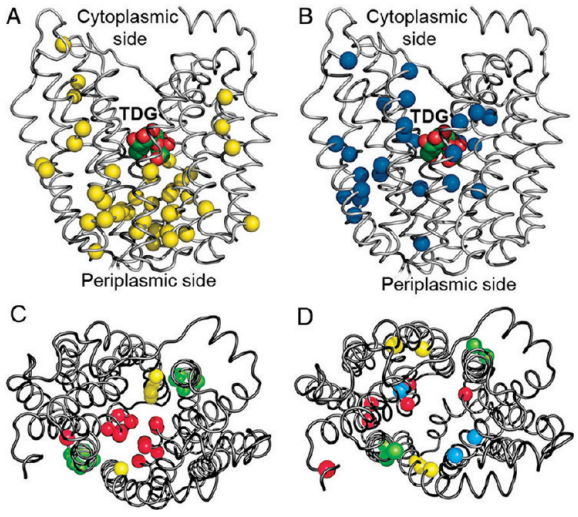

Figure 2. Distribution of Cys replacements that exhibit changes in reactivity with NEM in the presence of TDG. C_α atoms of single-Cys replacements are shown on the backbone of the LacY structure in an inward-facing conformation (Protein Data Bank entry 1PV7). (A and C) Positions of Cys residues that exhibit a significant increase in reactivity. (B and D) Positions of Cys residues that exhibit a significant decrease in reactivity. (A and B) Side view with bound TDG. (C and D) Cytoplasmic view demonstrating the pseudosymmetrical distribution of Cys replacements in putative translocation pathway. Residues located in symmetrically positioned helices are colored identically: I–VII (red), II–VIII (yellow), IV–X (blue), and V–XI (green).

More recently, a simple, more facile SDA method with TMRM[31,37,51,52] was developed to examine the effect of sugar binding on alkylation of single-Cys LacY mutants either in RSO membrane vesicles or in dodecyl β-D-maltopyranoside (DDM) micelles.[53] The experiments were conducted at 0 °C, where thermal motion is restricted,[54−56] and linear rates of labeling are readily obtained (Figure 3). TMRM labeling is almost negligible with LacY containing each of five single-Cys residues at positions on the periplasmic side in RSO membrane vesicles or with purified protein solubilized in DDM. Binding of TDG markedly increases the rate of labeling (Figure 3, bottom panel). Consistently, on the cytoplasmic side, each of four single-Cys replacement mutants is labeled at a rapid rate in the absence of sugar both in RSO membrane vesicles and with purified protein in DDM. Binding of TDG markedly decreases the rate of TMRM labeling either in the membrane or with purified protein in DDM (Figure 3, top panel).

The observations are certainly consistent with the interpretation that WT LacY in the native bacterial membrane is in a conformation similar to that of the X-ray crystal structures in the absence of ligand.[9−11] The periplasmic side is tightly closed, and an open cavity is present facing the cytoplasm (the inward-facing conformation). Sugar binding leads to closure of the cytoplasmic-facing cavity with opening of a cavity on the periplasmic side. The average increase in the level of 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 (∼9-fold) (Table 1). With purified single-Cys proteins in DDM, comparable averages are ∼6- and ∼5-fold. Therefore, the change in TMRM labeling induced by sugar on opposite faces of LacY is approximately the same in RSO vesicles or with the purified mutants in DDM.

The data obtained by SDA with TMRM provide further evidence not only that sugar binding markedly increases the open probability on the periplasmic side but also that sugar binding increases the probability of closing on the inside, the implication being that opening and closing may be reciprocal. However, reciprocity may not be obligatory. The reactivity and accessibility of periplasmic Cys replacements in C154G LacY are very high in the absence or presence of sugar, confirming that the periplasmic pathway is arrested in an open conformation in the C154G mutant, but the cytoplasmic cavity is still able to close and open.[37] It has also been demonstrated[32] that replacement of Asp68 with Glu at the cytoplasmic end of helix II blocks sugar-induced opening of the periplasmic cavity but has little or no effect on closing of the cytoplasmic cavity.

SINGLE-MOLECULE FLUORESCENCE RESONANCE ENERGY TRANSFER (SMFRET)

smFRET determined by alternating laser excitation spectroscopy was used in collaboration with D. Majumdar and S. Weiss to study ligand-induced distance changes on the cytoplasmic and periplasmic sides of LacY diffusing freely in detergent micelles.[36] The alternating access model was tested with WT LacY and the conformationally restricted mutant C154G. Pairs of Cys residues at the ends of two helices on the cytoplasmic or

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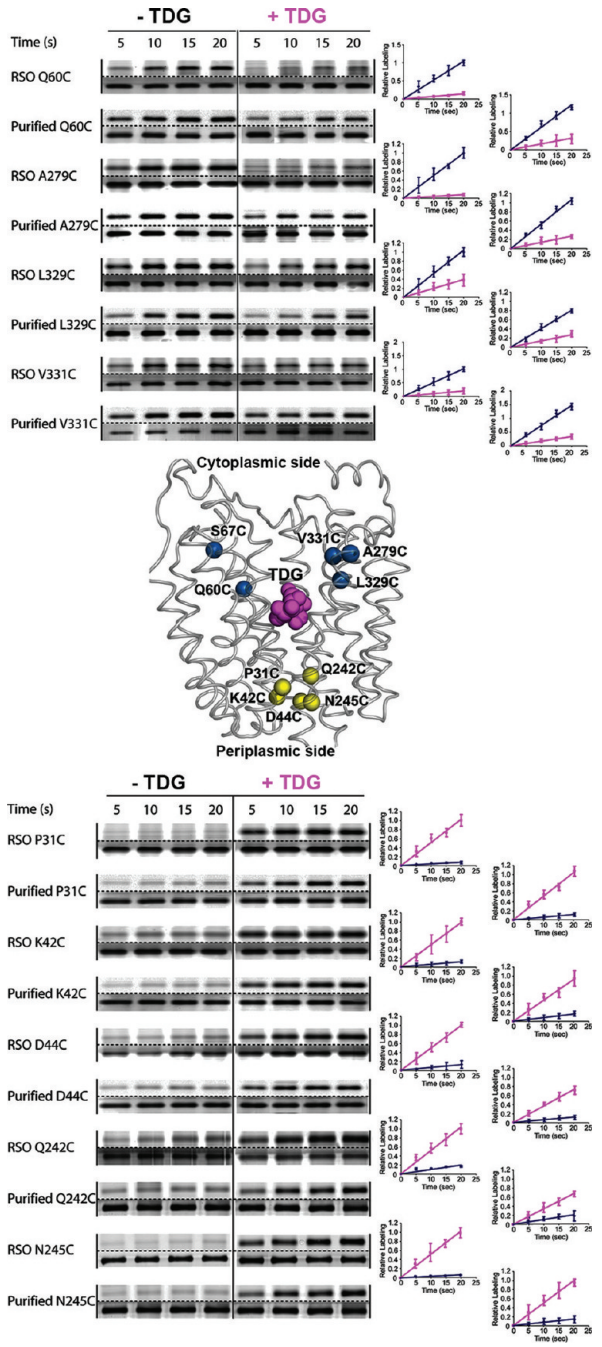

Figure 3. TMRM labeling of cytoplasmic (top) or periplasmic (bottom) single-Cys mutants in RSO membrane vesicles or as the purified proteins in DDM. Labeling of cytoplasmic single-Cys LacY mutants, Q60C, A279C, L329C, and V331C, or periplasmic single-Cys LacY mutants, Q31C, K42C, D44C, Q242C, and N245C, was performed with 40 μM TMRM (RSO membrane vesicles) or 4 μM TMRM (with purified proteins in DDM) for a given time at 0 °C in the absence of TDG (−TDG, blue plots) or preincubated for 10 min with TDG prior to addition of TMRM (+TDG, pink plots). Relative TMRM labeling rates were calculated as described in ref 53; the data are plotted relative to the 20 s points. For sodium dodecyl sulfate−polyacrylamide gel electrophoresis gels shown for each mutant, the top gel displays the results of TMRM labeling; the bottom gel is the silver-stained protein.

Table 1. Effect of TDG on the Rate of TMRM Labeling of Single-Cys Mutants[a]

	LacY mutant	helix	*x*-fold change in the rate of TMRM labeling in RSO vesicles	*x*-fold change in the rate of 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

[a]Rates of TMRM labeling were obtained from the time courses shown in Figure 3 as described previously.[53] 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 cysteines), and negative numbers indicate a decrease in the relative labeling rate due to addition of TDG (cytoplasmic cysteines).

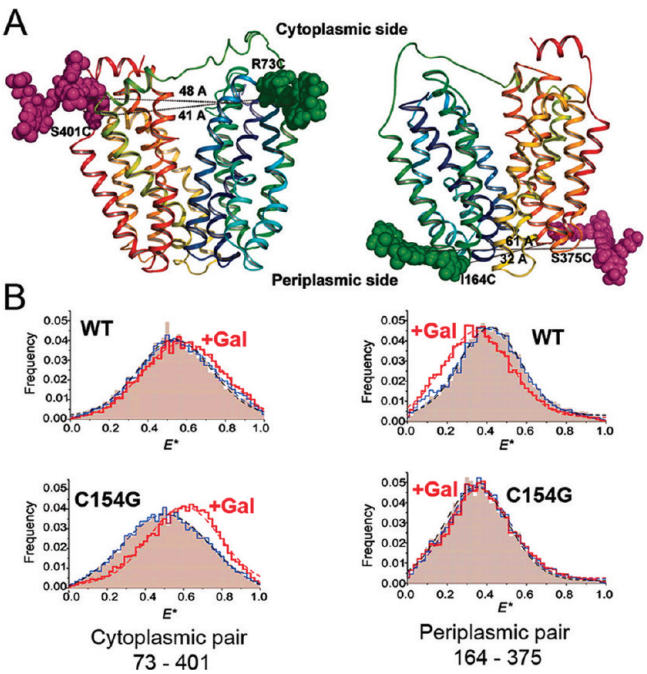

Figure 4. Interhelical distance changes on the cytoplasmic and periplasmic sides of LacY probed by smFRET. (A) LacY backbone with Alexa fluorophores attached at the ends of transmembrane helices on the cytoplasmic (left) or periplasmic (right) sides. The donor (Alexa 488) and acceptor (Alexa 647) are depicted as magenta and green space-filling models, respectively. (B) Ligand-induced effects on the smFRET efficiency distribution (E^*) measured with WT LacY (top) or the C154G mutant (bottom). A higher E^* corresponds to a shorter distance. The pink area is that without sugar. The blue line shows the addition of glucopyranoside. The red line shows the addition of galactopyranoside.

periplasmic sides were labeled with appropriate donor and acceptor fluorophores (Figure 4A); smFRET was assessed in the absence and presence of sugar, and distance changes were estimated from apparent energy transfer efficiency E^* (Figure 4B). With WT LacY, addition of a galactopyranoside, but not a glucopyranoside, results in a decrease in the distance on the cytoplasmic side and an increase in the distance and in the

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distance distribution on the periplasmic side. In contrast, with the C154G mutant, a more pronounced decrease in the distance and in the distance distribution is observed on the cytoplasmic side, but there is no change on the periplasmic side. The results are consistent with the alternating access model and indicate that the functional defect in the mutant is due to impaired ligand-induced flexibility on the periplasmic side.

DOUBLE ELECTRON–ELECTRON RESONANCE (DEER)

Four-pulse DEER,[57,58] combined with site-directed spin labeling, is highly sensitive to distance change within the range of 20–60 Å in proteins, which is compatible with the size of LacY. The nitroxide probes are much smaller than the Alexa dyes used in smFRET and quantify distance changes more accurately. Nitroxide-labeled paired Cys replacements at the ends of transmembrane helices on the cytoplasmic or periplasmic sides of WT LacY and the C154G mutant were used for distance measurements in collaboration with C. Altenbach and W. Hubbell.[20] Six cytoplasmic and three periplasmic pairs were individually tested for distance changes in the presence of galactosidic or nongalactosidic sugars (Figure 5, top panel). Remarkably, binding of galactosidic sugars causes conformational rearrangements on both sides of WT LacY. On the cytoplasmic side, each nitroxide-labeled pair exhibits decreased interspin distances. Distance distributions shifted toward shorter distances (from 4 to 21 Å for different pairs) in the presence of galactopyranoside (compare blue and red plots for the 73–340 pair in Figure 5, middle panel). Conversely, on the periplasmic side, each of three spin-labeled pairs shows increased distances ranging from 4 to 14 Å for WT-based protein (Figure 5; blue and red plots for the 105–310 pair). Thus, in the presence of the galactopyranoside specifically, the inward-facing cytoplasmic cavity closes and a cavity opens on the tightly sealed periplasmic side. In the C154G mutant, sugar-induced closure is observed on the cytoplasmic side (Figure 5; blue and red plots for the 73–340 pair in the C154G mutant), but little or no change occurs on periplasmic side, which is partially open in the absence of sugar (Figure 5; blue and red plots for the 105–310 pair in the C154G mutant). The DEER measurements in conjunction with molecular modeling based on the X-ray structures provide strong support for the alternating access model and suggest a structure for the outward-facing conformer of LacY, which agrees remarkably well with the model proposed by Radestock and Forrest.[24] In addition, the measurements are consistent with the presence of intermediate conformation(s) in LacY (see Figure 5, bottom panel), because multiple distance distributions have been typically observed for the WT symporter with or without bound sugar.

SITE-DIRECTED CROSS-LINKING

As discussed above, the side chains essential for sugar recognition and H^+ binding are located at the apex of the cytoplasmic cavity and inaccessible from the outside (Figure 1A). On the periplasmic side, helices I and II and helix VII from the N- and C-terminal six-helix bundles, respectively, participate in sealing of the cavity from the outside. Three double-Cys mutants (I40C/N245C, T45C/N245C, and I32C/N245C) located in the interface between helices I and II and helix VII on the periplasmic side of LacY were constructed with a factor Xa protease cleavage site in the loop between helices IV and V

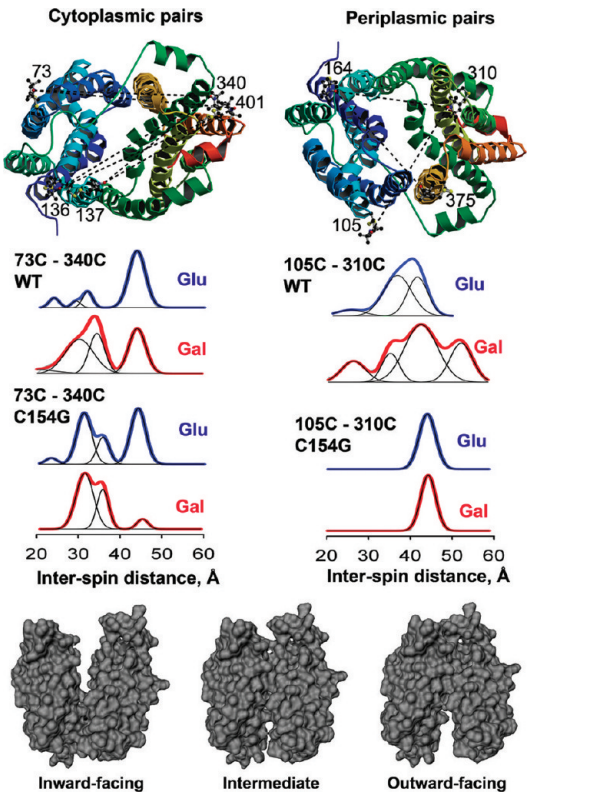

Figure 5. Effect of the binding of sugar on interhelical distances of LacY in DEER experiments. (Top) Disulfide-linked nitroxide chains are modeled on the LacY X-ray structure (Protein Data Bank entry 1PV7) viewed from the cytoplasmic (left) and periplasmic (right) sides. Nitroxides attached to LacY are shown as balls and sticks. Individual pairs used in DEER experiments are connected with dotted lines. (Middle) DEER characterization of effects of the binding of sugar on interspin distances of nitroxide-labeled double-Cys mutants located on the cytoplasmic (73–340 pair on WT or C154G background) or periplasmic (105–310 pair on WT or C154G background) side. Protein samples mixed with given sugars were frozen in liquid N_2, and measurements were taken at 50 K. Distance distributions obtained by Tikhonov regularization are shown for LacY with no sugar bound (glucosidic sugar, blue) and with bound sugar (galactosidic sugar, red). Multi-Gaussian fits (black lines) demonstrate relative distributions of conformational populations. (Bottom) Molecular modeling of major conformations of LacY based on DEER distance measurements. Space-filling representations of conformers are shown with helices II and VIII removed to illustrate openings on the cytoplasmic or periplasmic sides.

(Figure 6A) for detection of cross-linking.[29] All three pairs cross-link quantitatively and reversibly with flexible homobifunctional thiol reagents (Figure 6B,C). Strikingly, relatively short reagents (<10 Å) block lactose transport in all three mutants, whereas full or partial activity is observed when cross-linking is mediated by flexible reagents greater than ~10 Å in length (Figure 6D). The rigid cross-linking reagent naphthyl dimaleimide (NDM) practically eliminates transport, although it is sufficiently long for partial activity to occur (Figure 6). Therefore, the transport mechanism of LacY must involve closing, as well as opening, of a large periplasmic cavity. Satisfyingly, 17 Å is the minimum length of cross-linker required for maximal transport activity, a distance similar to that obtained from DEER measurements for opening of the periplasmic side in the presence of sugar.

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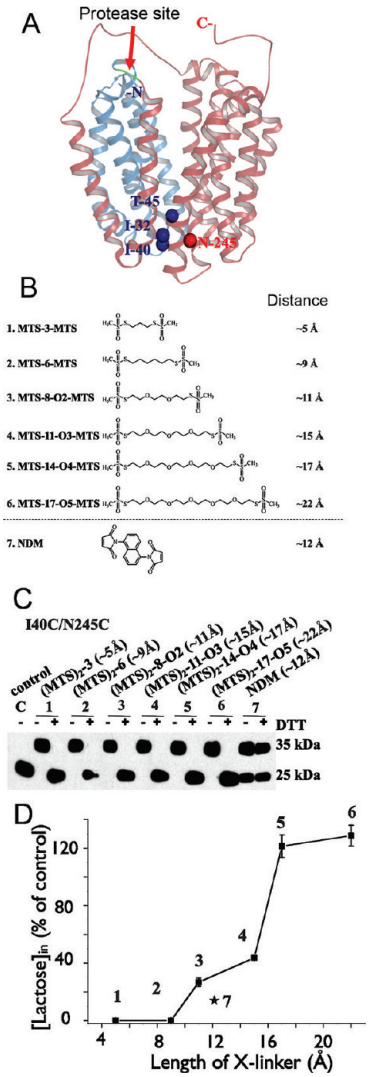

Figure 6. Effect of cross-linking at the periplasmic side of LacY on lactose transport. (A) Structural model of LacY with a closed periplasmic pathway. C_α atoms of the residues on the periplasmic side used for Cys replacements are shown as blue (helices I and II) or red (helix VII) spheres. The red arrow indicates the cleavage site for factor Xa protease located between helices IV and V. Helices I–IV and V–XII are colored blue and pink, respectively. (B) Homobifunctional cross-linking reagents with approximate S–S distances between bridging sulfur atoms in the chains. (C) Western blot analysis with the anti-C-terminal antibody in cross-linking experiments after factor Xa protease digestion: control, I40C/N245C mutant without addition of cross-linkers; lanes 1–7, results of cross-linking with indicated reagents and effect of a reducing agent (DTT). (D) Effect of cross-linking of different length reagents on lactose transport with mutant I40C/N245C. All experiments were performed with RSO vesicles.

TRP FLUORESCENCE

The fluorescence of intrinsic Trp residues in LacY provides a sensitive tool for functional studies using recently developed approaches that allow direct measurement of sugar binding, as well as global conformational changes in LacY.[59−61] As a typical membrane transporter, LacY contains multiple Trp residues located predominantly at the interface of the phospholipid bilayer that are important for insertion and stability.[62,63] The exception is Trp151, which is a component of the sugar-binding site in LacY and the only Trp residue of six in the proximity of bound galactopyranoside (Figure 7). The short distance

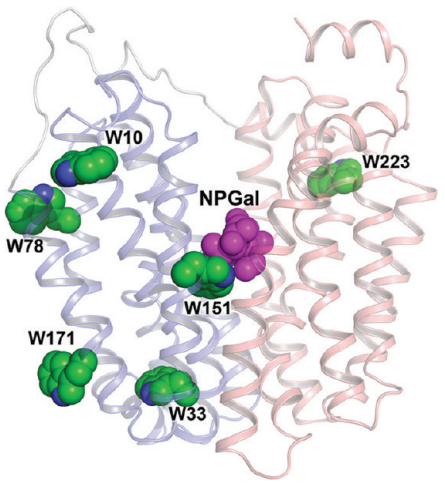

Figure 7. Location of native Trp residues in the WT LacY structure. N-Terminal and C-terminal six-helix bundles are colored blue and pink, respectively. Trp residues are presented as green spheres. NPG modeled in the sugar-binding site is shown as magenta spheres.

between Trp151 and the sugar is favorable for formation of a donor–acceptor pair between Trp and nitrophenyl or dansyl derivatives at the anomeric position of the galactosyl moiety resulting in fluorescence resonance energy transfer (FRET). Modeling of 4-nitrophenyl α-D-galactopyranoside (NPG) in the binding site of LacY places the nitrophenyl moiety ~12 Å from Trp151, a distance commensurate with the Förster distance for a Trp-nitrophenyl pair. Indeed, binding of NPG to LacY containing all six native Trp residues exhibits FRET from Trp151 (Trp151 → NPG FRET) specific for the galactopyranoside, which can be measured in steady state or stopped-flow experiments as a decrease in Trp fluorescence.[59] Importantly, binding of galactopyranosides that do not absorb UV light (e.g., lactose, TDG, and melibiose) has a negligible effect on the fluorescence of WT LacY, and replacement of Trp151 with Tyr completely abolishes FRET as a result of NPG binding.

According to the alternating access mechanism, the tightly sealed periplasmic side of LacY observed in the X-ray structures, as well as in the bacterial membrane, must open to allow access of the sugar to the binding site in the middle of the protein. Therefore, the question of whether opening of the periplasmic cavity is limiting for substrate binding and possibly transport arises. In an effort to address this question, rates of NPG binding were measured by Trp151 → NPG FRET using a stopped-flow method with purified LacY either in DDM or reconstituted into proteoliposomes.[61] A decrease in Trp fluorescence resulting from Trp151 → NPG FRET was observed with LacY in DDM as well as in proteoliposomes (Figure 8A,B), although the pre-steady state kinetics of binding of sugar to solubilized LacY is completely different from that observed with the reconstituted protein. In DDM, the rate (k_{obs}) increases linearly with an increasing substrate concentration [Figure 8C (●)]. Thus, in an inward-facing conformation—as is the case with LacY in DDM—the sugar-binding site in LacY is readily accessible from the cytoplasmic side, and binding can be described as a single-step reversible process:

$$[E] + [S] \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} [ES]$$

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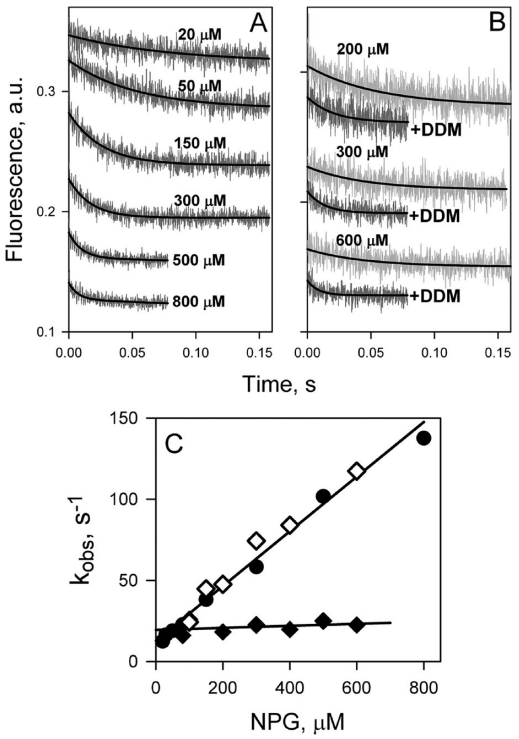

Figure 8. Sugar binding rates measured as Trp151 → NPG FRET with WT LacY reconstituted into proteoliposomes or solubilized in DDM. (A) Stopped-flow traces of changes in Trp fluorescence recorded after mixing of LacY in DDM with given concentrations of NPG. (B) Stopped-flow traces showing NPG binding to LacY reconstituted into proteoliposomes (light gray traces at three sugar concentrations) and after dissolution of proteoliposomes in 0.3% DDM (dark gray traces). (C) Concentration dependence of sugar binding rates (k_{obs}) estimated from single-exponential fits shown in panels A and B. Data were obtained with LacY in DDM (●), reconstituted into proteoliposomes (◆), and after addition of DDM to proteoliposomes (◇). For protein in DDM, data were fitted with a linear equation ($k_{obs} = k_{off} + k_{on}[NPG]$) with the following estimated kinetic parameters: k_{off} = 13 s^{-1}, k_{on} = 0.2 μM^{-1} s^{-1}, and K_d = 65 μM. Reconstituted into proteoliposomes, LacY binds NPG with a k_{obs} of 21 ± 4 s^{-1}.

The k_{obs} (reciprocal relaxation time $1/\tau$) for this reaction depends linearly on ligand concentration ($k_{obs} = k_{off} + k_{on}[S]$).

In marked contrast, LacY reconstituted into proteoliposomes binds NPG at a slow rate that is independent of NPG concentration [Figure 8C (◆)]. Addition of DDM to the same proteoliposomes dissolves the membrane and restores linear concentration dependence of NPG binding rates identical to that observed with purified protein in DDM [Figure 8C (◇)]. This finding suggests that in LacY reconstituted into proteoliposomes the sugar-binding site is not readily accessible and that the binding reaction includes a limiting step that is likely represented by opening of the periplasmic cavity. LacY reconstituted into proteoliposomes is oriented with the periplasmic side facing the external medium,[64] as in the native bacterial membrane. Therefore, as shown in the X-ray structures,[9−11] as well as with RSO membrane vesicles,[53] reconstituted LacY exists predominantly in an inward-facing conformation with a sealed periplasmic cavity, because sugar binding rates do not increase even at supersaturating NPG concentrations. Evidently, the periplasmic cavity leading to the sugar-binding site must open first, and this slow opening appears to be limiting for substrate binding.

Trp fluorescence is an invaluable probe for detection of conformational changes in LacY.[60,61] Trp fluorescence in proteins is quenched by certain amino acyl side chains such as a protonated His, an amino group, or a Cys residue.[65] Measurements of quenching and unquenching of fluorescence of strategically introduced Trp residues allow differentiation between inward- and outward-facing conformations of LacY and provide strong support for the alternating access mechanism.[60] Thus, the sugar binding effect has been tested with mutants, where an additional Trp residue was introduced on either side of WT LacY far from the sugar binding site, and predicted to be in the proximity of side chains of natural quenchers in either the inward- or outward-facing conformers (Figure 9, top panel). In mutant N245W, Trp is located on the periplasmic side of LacY (helix VII) where fluorescence is quenched by native His35 (helix I) in the inward-facing conformation. The fluorescence intensity of mutant N245W increases as a result of sugar binding (Figure 9A,B) due to unquenching caused by an increase in the distance between Trp245 and quencher His35. The opposite effect is observed in mutant F140W/F334H where Trp140 (helix V) and His334 (helix X) are located across the wide open cytoplasmic cavity. Sugar binding leads to quenching of Trp fluorescence (Figure 9C,D) due to direct collision between Trp140 and His334 resulting from the closing of the cytoplasmic cavity. The pH dependence of Trp245 unquenching (Figure 9B) and Trp140 quenching (Figure 9D) exhibits a pK_a of ∼8, typical for a His side chain interacting with an aromatic group,[66] thereby confirming His as the quencher in both mutants. 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 for measuring conformational changes.

The N245W LacY mutant with Trp introduced on the periplasmic side has been used for a direct comparison between rates of opening of the periplasmic pathway and sugar binding rates with LacY reconstituted into proteoliposomes by following either unquenching of Trp fluorescence or Trp151 → NPG FRET.[61] The kinetics of binding of sugar to the N245W mutant (Figure 10) is similar to that described previously for WT LacY (Figure 8). In DDM, the rate of sugar binding exhibits a linear dependence on sugar concentration [Figure 10C (● and ◇)]. However, the mutant reconstituted into proteoliposomes binds sugar with a k_{obs} of ∼60 s^{-1}, and the rate is independent of NPG concentration [Figure 10A,C (◆)]. To measure rates of opening of the periplasmic cavity triggered by galactoside binding, four galactosidic sugars that do not change Trp fluorescence (TDG, melibiose, octyl α-D-galactoside, or methyl α-D-galactoside) were used. Rates of unquenching of Trp fluorescence in mutant N245W due to binding of these galactosides to LacY in DDM (Figure 10B) are very similar to the NPG binding rates observed in proteoliposomes (Figure 10A). Moreover, the rates of both processes are essentially independent of sugar concentration [Figure 10C (◆ vs empty symbols fitted with a dashed line)]. The similarity of the kinetics of two processes suggests that opening of the periplasmic cavity is likely the limiting step for the access of sugar to the binding site in LacY.

A turnover number of 16−20 s^{-1} was estimated for uphill lactose/H^+ symport by WT LacY in RSO membrane vesicles and with the purified protein reconstituted into proteoliposomes.[67] Very much the same rate (21 ± 4 s^{-1}) is observed for binding of sugar to WT LacY in proteoliposomes (Figure 8).

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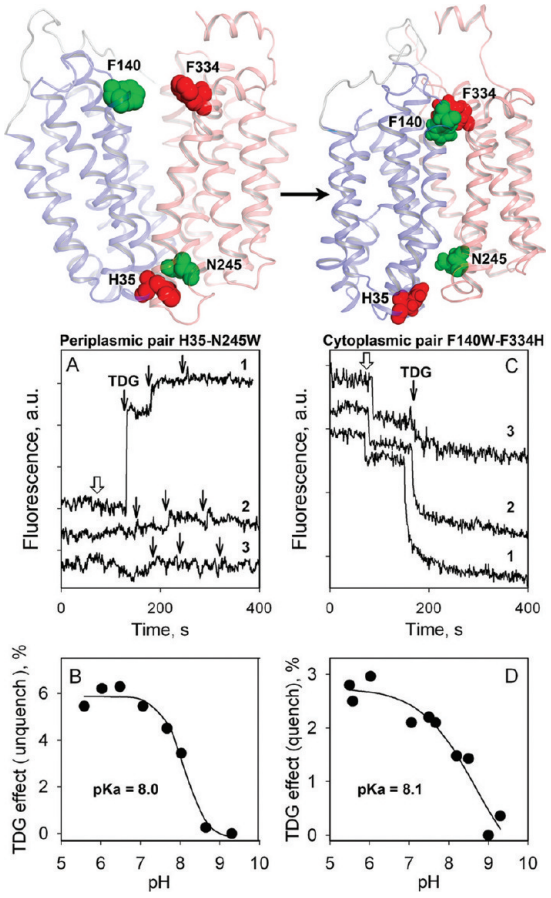

Figure 9. Alternating access mechanism probed by quenching and unquenching of Trp fluorescence. (Top) Backbone of the inward-facing LacY structure (left) and outward-facing model (right) with N-terminal and C-terminal six-helix bundles colored blue and pink, respectively. The arrow indicates the conformational change resulting from sugar binding. Residues used for Trp substitutions are shown as green spheres. Residues used as the quenchers of Trp fluorescence are presented as red spheres. (Bottom) Effect of a conformational change in LacY triggered by sugar binding on Trp fluorescence. Excitation and emission wavelength were 295 and 330 nm, respectively. (A and B) Unquenching of Trp fluorescence in mutant N245W after addition of TDG. (C and D) Quenching of Trp fluorescence in mutant F140W/F334H after addition of TDG. (A) Trp fluorescence change after addition of sucrose (empty arrow) or TDG (filled arrows) to the N245W mutant at pH 6 (trace 1) and pH 9 (trace 2) or to control LacY without Trp substitution at position 245 at pH 6 (trace 3). (B) Dependence of the fluorescence change on pH for the N245W mutant. (C) Trp fluorescence change after addition of sucrose (empty arrow) or TDG (filled arrow) to the F140W/F334W mutant at pH 5.5 (trace 1), pH 8.5 (trace 2), or pH 9.0 (trace 3). (D) Dependence of the fluorescence change on pH for the F140W/F334W mutant.

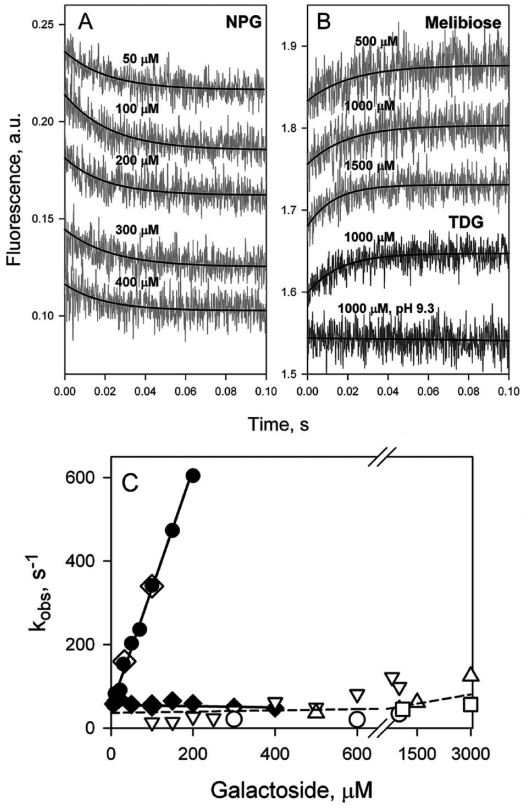

Figure 10. Comparison of the rates of binding of galactoside to the N245W mutant reconstituted into proteoliposomes with the rates of opening of the periplasmic cavity. (A) Binding of NPG to protein reconstituted into proteoliposomes measured by Trp151 → NPG FRET. Stopped-flow traces are shown for five sugar concentrations. Single-exponential fits are shown as black lines. (B) Unquenching of Trp245 fluorescence resulting from opening of the periplasmic cavity upon sugar binding. Stopped-flow traces are recorded with the mutant in DDM after mixing with melibiose (top three traces) or TDG (bottom two traces) at pH 6.0, except for the trace at pH 9.3. (C) Concentration dependence of sugar binding rates and rates of periplasmic pathway opening. Binding rates (k_{obs}) estimated for the purified mutant in DDM (●), reconstituted in proteoliposomes (◆), and after proteoliposomes had been dissolved in DDM (◇). The reconstituted mutant binds NPG with a k_{obs} of 56 ± 7 s^{-1}. Rates of unquenching of Trp245 fluorescence resulting from opening of the periplasmic cavity after sugar binding were measured in a DDM solution and are presented: TDG (▽), melibiose (△), octyl α-D-galactoside (○), and methyl α-D-galactoside (□). The rate of opening of the periplasmic cavity in DDM is 50−100 s^{-1} at the saturating concentrations of all four galactosides tested.

Therefore, opening of the periplasmic pathway may also be the rate-limiting step for the overall symport mechanism catalyzed by WT LacY.

In view of this scenario, how does transport of sugar across the membrane occur? On the basis of the available biochemical and structural information, a probable description of the overall symport cycle of LacY can be formulated. In the absence of an external galactopyranoside, opening of LacY on the periplasmic side occurs spontaneously, but with low frequency. Thus, at any given time, only a small population of molecules in the membrane is open on the periplasmic side statistically. However, binding of a galactopyranoside from the periplasm results in formation of a high-energy intermediate in which both cavities are closed and sugar and H^+ are occluded in the middle of the molecule. Depletion of the sugar-free, open-outward form of LacY shifts the equilibrium from the inward-facing conformation to the high-energy intermediate, which is metastable and has a much higher probability of opening the periplasmic cavity. To complete the transport process, the intermediate with a bound sugar opens to the cytoplasm, and sugar dissociates followed by dissociation of an H^+. In the absence of $\Delta\tilde{\mu}_{H^+}$, the limiting step of the transport is deprotonation, and in the presence of a driving force on the H^+, dissociation of sugar or a conformational change becomes limiting.

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