Engineered occluded apo-intermediate of LacY

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The lactose permease of *Escherichia coli* (LacY) utilizes an alternating access symport mechanism with multiple conformational intermediates, but only inward (cytoplasmic)- or outward (periplasmic)-open structures have been characterized by X-ray crystallography. It is demonstrated here with sugar-binding studies that cross-linking paired-Cys replacements across the closed cytoplasmic cavity stabilize an occluded conformer with an inaccessible sugar-binding site. In addition, a nanobody (Nb) that stabilizes a periplasmic-open conformer with an easily accessible sugar-binding site in WT LacY fails to cause the cytoplasmic cross-linked mutants to become accessible to galactoside, showing that the periplasmic cavity is closed. These results are consistent with tight association of the periplasmic ends in two pairs of helices containing clusters of small residues in the packing interface between N- and C-terminal six-helix bundles of the symporter. However, after reduction of the disulfide bond, the Nb markedly increases the rate of galactoside binding, indicating unrestricted access to the Nb epitope and the galactoside-binding site from the periplasm. The findings indicate that the cross-linked cytoplasmic double-Cys mutants resemble an occluded apo-intermediate in the transport cycle.

membrane transport proteins | lactose permease | fluorescence | nanobodies | stopped flow

The lactose permease of *Escherichia coli* (LacY), an intensively studied membrane transport protein, is the best understood member of the major facilitator superfamily (reviewed in ref. 1). LacY catalyzes stoichiometric lactose/H⁺ symport by a mechanism based on alternating access of single sugar- and H⁺-binding sites in the middle of the molecule to either side of the membrane, as determined experimentally by multiple biochemical/spectroscopic approaches (reviewed in ref. 2) and by X-ray crystallography (3–10). LacY consists of N- and C-terminal halves, each composed of six transmembrane helices (helices I–VI and VII–XII) connected by a relatively long loop between cytoplasmic ends of helices VI and VII. The galactoside-binding site in the middle of the molecule includes residues from helices IV, V, VII, and X. Thus, sugar binding involves side chains located in both six-helix bundles (8–10). The molecule is highly dynamic with multiple conformational intermediates in an equilibrium that is shifted toward a periplasmic-open state by binding of galactosidic sugars (11–16) or specific nanobodies (Nbs) developed against a periplasmic-open mutant G46W/G262W (LacY_{WW}) (17, 18). Different Nbs inhibit transport activity of WT LacY by trapping various outward-open conformers, which are characterized by distinct kinetic parameters of sugar binding, indicating that the Nbs recognize unique transient conformers that likely represent intermediates in the transport cycle (18, 19).

The turnover number of the overall LacY transport cycle is relatively low (~20 s⁻¹) (20), and a limiting step corresponds to the rate of opening of the periplasmic cavity (15, 16), but some conformational transitions are fast (200–300 s⁻¹), as measured by fluorescence quenching (16, 21). Kinetic parameters for galactoside binding have been determined with lactose analogs *p*-nitrophenyl- α -D-galactopyranoside (NPG), an acceptor for FRET from Trp151 (helix V) in the sugar-binding site, and β -D-galactopyranosyl-1-thio- β -D-galactopyranoside (TDG), which does not participate in FRET (22). The data show clearly that the rate of cavity opening limits sugar-binding rates on either side of

WT LacY, and that sugar has free access to the binding site when the outward- or inward-facing cavity is open (15, 21). Both outward- and inward-open conformations are characterized by a linear concentration dependence of galactoside-binding rates with high association rate constants (k_{on} = 5 to 10 μ M⁻¹s⁻¹).

Binding affinity of galactosides does not change with pH from 5.5–9.0, but decreases at alkaline pH with a midpoint at ~10.5, indicating that LacY is protonated at physiological pH (23–25). Recently, it was found that side chain of Glu325 (helix X) has a pK_a of 10.5, indicating that this functional group must be protonated for LacY to bind galactosides (26). Binding of galactoside to protonated, periplasmic-open LacY initiates an ordered series of the intermediates in the transport cycle (1). Indeed, the X-ray structure of outward-open LacY without sugar (LacY_{WW}/Nb9039, PDB ID code 5GXB) (7) exhibits a more open periplasmic cavity than observed when galactoside is bound (LacY_{WW}/TDG, PDB ID code 4OAA; LacY_{WW}/NPG, PDB ID code 4ZYR; and LacY_{WW}/NPG/Nb9047, PDB ID code 6C9W) (8–10). In the sugar-bound molecule, the periplasmic ends of helices I, II, III, and IV are shifted toward the interior of the periplasmic cavity in the direction of the C-terminal helix domain. This partial closure likely represents initial movement at the periplasmic side secondary to galactoside binding (10). Furthermore, galactosides much larger in size than lactose have unobstructed access to the binding site, and remarkably, are transported across the membrane albeit relatively slowly (27). High flexibility of transmembrane helices is necessary to accommodate the oversized substrates within the binding site let alone transport them across the membrane.

To test the mobility of the periplasmic ends of transmembrane helices, LacY trapped in a cytoplasmic-closed conformation was constructed by engineering paired-Cys mutants suitable for

Significance

The alternating access mechanism underlying stoichiometric symport of a galactoside and an H⁺ is mediated by LacY, a paradigm for the major facilitator superfamily, the largest family of membrane transport proteins. Although the mechanism includes multiple sequential conformational changes, only two alternative structures with a hydrophilic cavity open inward to the cytoplasm or open outward to the periplasm are available. As shown here, an empty occluded conformation of LacY with both cavities closed is stabilized by cross-linking double-Cys replacements across the cytoplasmic cavity. An occluded apo-intermediate is an obligatory component of the symport cycle, and LacY with a cross-linked cytoplasmic cavity provides insight into the nature of this conformer.

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cross-linking. Spontaneous cross-linking was documented with Cys pairs 134C-343C or 135C-338C in coexpressed N- and C-terminal six-helix halves of Cys-less LacY, demonstrating formation of S-S bonds between the cytoplasmic end of helix IV in the N-terminal half and cytoplasmic ends of helices X and XI in the C-terminal half (28). Here, the same paired Cys replacements, R134C/V343C or R135C/T338C on the cytoplasmic side of WT LacY, were introduced with the expectation that S-S bond formation would stabilize conformers in which the cytoplasmic cavity is closed but the periplasmic side remains unrestricted (Fig. 1 *A–D*).

LacY with a locked periplasmic cavity (by cross-linking I32C-N245C or H35C-I245C Cys-pairs) binds galactosides exclusively via the cytoplasmic opening, and presteady-state kinetic analysis reveals a two-step process in which opening of the cytoplasmic cavity occurs first followed by galactoside binding due to a conformational selection mechanism (21). The results indicate clearly that the cytoplasmic cavity opens and closes spontaneously in protein with a sealed periplasmic side, and the rate of opening is slower than the rate of closing. Therefore, a significant fraction of LacY molecules is closed on both sides, and an occluded conformer without bound galactoside (the apo-intermediate) is an obligatory intermediate in the transport mechanism (1).

Here we show that LacY cross-linked on the cytoplasmic side does not open on the periplasmic side, and that an occluded apo-intermediate is stabilized in which both cavities are closed with an inaccessible galactoside-binding site. This effect may be attributed to the structural differences in helix contacts between N- and C-terminal six-helix bundles in alternative conformations and to the tight contacts between the periplasmic ends of helices containing clusters of small-sized residues in the packing interface.

Fig. 1. Cross-linking of the cytoplasmic cavity. (*A–D*) Two X-ray structural models of LacY viewed from the cytoplasm (*A* and *B*) or from the side (*C* and *D*) are rainbow-colored from blue to red for helices I to XII and show conformations with open (*A* and *C*) or closed (*B* and *D*) cytoplasmic cavity (PDB ID codes 2CFQ and 5GXB, respectively). Amino acid residues used for paired Cys replacements are presented as spheres colored blue (R134 and V343), orange (R135 and T338), and red (R73 and S401). (*E* and *F*) Accessibility of Cys residues to alkylation with MANS in double-Cys mutants (R134C/V343C, blue; R135C/T338C, orange; and R73C/S401C, red) or in WT LacY (green). Arrows indicate addition of 1 μM MANS to 0.5 μM of LacY. MANS labeling is performed under reducing (*E*, with TCEP), or cross-linking (*F*, with Cu²⁺) conditions (see *Methods*).

Results

Cross-Linking the Cytoplasmic Cavity. Three double-Cys mutants were constructed—R134C/V343C, R135C/T338C, and R73C/S401C—with paired Cys replacements across the cytoplasmic cavity of WT LacY. In each pair, one Cys residue is located in the N-terminal six-helix bundle, and the other is in the C-terminal six-helix bundle (Fig. 1 *A–D*). Two pairs (134C/343C and 135C/338C) cross-link spontaneously (28), consistent with the close proximity of Arg134 (helix IV) to Val343 (loop X - XI), as well as Arg135 (helix IV) to Thr338 (helix X), in the outward-open/inward-closed conformation (Fig. 1 *B* and *D*, blue and orange spheres, respectively) (7–10). The third pair serves as a control, since Cys replacements for Arg73 (loop II - III) and Ser401 (C terminus of helix XII) are too far apart to undergo oxidative cross-linking (Fig. 1 *A–D*, red spheres).

Formation of S-S bonds was assessed by testing reactivity of thiol groups with the sulfhydryl-specific probe 2-(4'-maleimidylanilino) naphthalene-6-sulfonate (MIANS) after oxidation in the presence of Cu²⁺ or reduction with Tris(2-carboxyethyl)phosphine (TCEP). MIANS is not fluorescent until the maleimide moiety undergoes reaction with a Cys thiol group (29, 30). Purified proteins treated with TCEP or Cu²⁺ were labeled by MIANS (Fig. 1 *E* and *F*) in the presence of a saturating concentration of TDG, which protects highly reactive native Cys148 (helix V) from alkylation (31) and also stabilizes the periplasmic-open conformation (11). Under these conditions, WT LacY exhibits little labeling with MIANS (Fig. 1 *E* and *F*, green lines) indicating minimal reactivity of native Cys residues. The double-Cys mutant 73C/401C is labeled readily under either reducing or oxidizing conditions (Fig. 1 *E* and *F*, red lines). In contrast, mutants 134C/343C and 135C/338C are labeled with MIANS only after reduction with TCEP (Fig. 1 *E*, blue and orange lines), and under cross-linking conditions labeling resembles background alkylation observed with WT LacY (Fig. 1 *F*, blue and orange lines). Formation of S-S bonds prevents alkylation of Cys residues with MIANS in both 134C/343C and 135C/338C mutants. Moreover, under reducing conditions, reactivity is restored, and MIANS labeling is observed, but to a lesser extent in mutant 134C/343C than in 135C/338C due possibly to reversible reduction/formation of the S-S bond even in the presence of TCEP.

Accessibility of the Sugar-Binding Site in Double-Cys Mutants. Accessibility of the binding site to galactoside in mutants 134C/343C and 135C/338C with reversibly cross-linked cytoplasmic cavities was examined by measuring sugar-binding rates utilizing FRET from Trp151 (helix V) to bound NPG (22). As expected, under reducing conditions, stopped-flow traces exhibit a time-dependent decrease of Trp fluorescence upon mixing NPG with both double-Cys mutants (Fig. 2, trace 1). The observed rates (k_{obs}) at 50 μM NPG are $5.1 \pm 0.1 \text{ s}^{-1}$ and $32 \pm 1 \text{ s}^{-1}$ for mutants 134C/343C and 135C/338C, respectively. In contrast, after cross-linking by Cu²⁺ oxidation, no fluorescence change is observed under the same conditions with either mutant (Fig. 2, trace 3), indicating that sugar does not bind to LacY cross-linked on the cytoplasmic side. The effect of cross-linking may be explained either by blocked access to the binding site from the periplasm due to restricted conformational mobility or by structural changes in the sugar-binding site that prevent galactoside binding. Therefore, both possibilities were tested by either (*i*) using a conformation-sensitive Nb or (*ii*) cross-linking the double-Cys mutant containing bound sugar, and measuring the dissociation rate constant (k_{off}) in displacement experiments.

Locked Periplasmic Cavity in Cross-Linked Double-Cys Mutants.

Conformational mobility of the double-Cys mutants was examined with Nb9065, which recognizes the binding epitope in LacY with an open periplasmic cavity and stabilizes WT LacY in an outward-open conformation (18, 19, 21). Stopped-flow rates of NPG binding, measured with mutants 134C/343C and 135C/338C in the presence of TCEP are greatly increased by preincubation of mutants with Nb9065 (Fig. 2, compare traces 1 and 2). Binding rates measured at 50 μM NPG increase from 5.1 ± 0.1 to $500 \pm 30 \text{ s}^{-1}$ and from 32 ± 1 to $520 \pm 30 \text{ s}^{-1}$ in

Fig. 2. Galactoside binding to double-Cys mutants. Binding rates are measured directly as Trp151 $\rightarrow$ NPG FRET by mixing NPG with 134C/343C (A) or 135C/338C (B) mutants (mixing scheme shown on Top). Stopped-flow traces of Trp fluorescence change (gray lines) are recorded with LacY mutants pretreated with TCEP (traces 1 and 2) or Cu^{2+} (traces 3 and 4) as described in Methods. NPG binding rates (k_{obs}) were measured without added Nb (traces 1 and 3) or after preincubation mutants with 1.2-fold excess of Nb9065 (traces 2 and 4). Single exponential fits are shown as blue or red lines (without or with Nb9065, respectively) in the presence of TCEP and as black lines in the presence of Cu^{2+} . Under reducing conditions (+TCEP), the estimated k_{obs} values at 50 μM NPG are 5.1 ± 0.1 or $500 \pm 30 \text{ s}^{-1}$ for 134C/343C without or with Nb9065, and 32 ± 1 or $520 \pm 30 \text{ s}^{-1}$ for 135C/338C without or with Nb9065, respectively. Concentration dependencies of k_{obs} are presented on Fig. 5 (closed symbols).

mutants 134C/343C and 135C/338C, respectively (Fig. 2, blue and red lines). However, Nb9065 has no effect on double-Cys mutants oxidized in the presence of Cu^{2+} (Fig. 2, trace 4, black lines). Notably, with WT LacY, the effect of Nb9065 is not altered by Cu^{2+} (21). Thus, after oxidation of either paired-Cys mutant, the periplasmic cavity on the opposite face of LacY is not open, the epitope for Nb9065 on the periplasmic surface is not exposed, and the sugar-binding site in the middle of LacY is inaccessible.

Consistently, no change in Trp fluorescence of 134C/343C and 135C/338C treated with Cu^{2+} is observed in displacement experiments (Fig. 3 A and B, black lines). Only in the presence of TCEP does TDG displace the previously bound FRET acceptor NPG, thereby leading to increased Trp fluorescence. (Fig. 3 A and B, blue lines). The displacement rates are $25 \pm 1 \text{ s}^{-1}$ and $32 \pm 1 \text{ s}^{-1}$ for 134C/343C and 135C/338C, respectively, and represent true k_{off} values that remain similar in the presence of Nb9065 ($27 \pm 1 \text{ s}^{-1}$ and $33 \pm 2 \text{ s}^{-1}$ for 134C/343C and 135C/338C, respectively; Fig. 3 C and D, red lines) because bound galactoside itself stabilizes the periplasmic-open conformation (11–13). Trp $\rightarrow$ NPG FRET is not detected in the presence of Cu^{2+} , and Nb9065 has no effect (Fig. 3 C and D, black lines), indicating that no opening of the periplasmic cavity occurs, and therefore, no galactoside binding is observed in mutants cross-linked on the cytoplasmic side. The single-Cys mutant R135C, as well as WT LacY, exhibit little if any effect of Cu^{2+} on galactoside binding, and k_{off} values for NPG dissociation measured for R135C LacY in the presence of Cu^{2+} ($37 \pm 3 \text{ s}^{-1}$), for WT LacY with or without Cu^{2+} (43 ± 3 or $41 \pm 1 \text{ s}^{-1}$), and for double-Cys mutants under reducing conditions (25 ± 1 and $32 \pm 1 \text{ s}^{-1}$) are comparable (Fig. 3 A and B and SI Appendix, Fig. S1).

Intact Sugar-Binding Site in Cross-Linked Double-Cys Mutants. To test for an effect of S-S bond formation on the properties of the sugar-binding site, double-Cys LacY was cross-linked in the presence of bound galactoside. Sugar binding shifts the conformational distribution toward periplasmic-open/cytoplasmic-closed form (11–13). Therefore, the double-Cys mutant with bound galactoside was expected to cross-link on the cytoplasmic side when Cu^{2+} is added

to sugar-bound protein. Mutant 135C/338C preincubated initially with TCEP to eliminate spontaneous cross-linking was mixed with galactoside (TDG or NPG), TCEP was then removed, with sugar still present and Cu^{2+} provided to catalyze S-S formation in the presence of the galactoside. MIANS labeling reveals much less reactive Cys residues after treatment with Cu^{2+} than without (Fig. 4A, black and cyan lines, respectively) indicating formation of a cross-link in the double-Cys mutant in the presence of sugar. Occupation of the binding site was then examined in displacement experiments (Fig. 4B). The stopped-flow traces clearly demonstrate an increase in Trp fluorescence due to displacement by excess TDG of NPG bound to the protein cross-linked in the presence of sugar (Fig. 4B, black line), since displacement occurs via periplasmic cavity which remains open, and addition of Nb9065 has little if any effect (Fig. 4B, red line). Estimated k_{off} values for NPG in the absence or presence of Nb9065 are $27 \pm 2 \text{ s}^{-1}$ or $33 \pm 2 \text{ s}^{-1}$, respectively. The similar k_{off} value for NPG bound to 135C/338C LacY under reducing conditions (Fig. 3 B and D) indicates that the S-S bond on the cytoplasmic surface does not alter sugar-binding site. The same conclusion was drawn when displacement rates were measured for NPG bound to the periplasmic-open double-Cys mutant in complex with Nb9065 before and after Cu^{2+} oxidation, which results in similar k_{off} values of 26 ± 1 and $30 \pm 1 \text{ s}^{-1}$, respectively (SI Appendix, Fig. S2).

The sugar-binding site in both cross-linked double-Cys mutants is not accessible to galactosides until the S-S bond in the cytoplasmic cavity is disrupted, but even under reducing conditions, binding rates are relatively slow (Fig. 2, traces 1, blue

Fig. 3. Displacement of NPG bound to double-Cys mutants. The rates of NPG dissociation (k_{off}) are measured after mixing of saturating concentration TDG (20 mM) with double-Cys mutants preincubated with NPG (mixing scheme shown on Top). Unbinding of NPG displaced by TDG is resulted in increasing of Trp fluorescence due to Trp151 $\rightarrow$ NPG FRET. Stopped-flow traces of Trp fluorescence change (gray lines) are recorded with LacY mutants under reducing (+TCEP) or oxidizing (+ Cu^{2+}) conditions as marked, in the absence (A and B) or presence (C and D) of Nb9065 (see Methods for details). Traces are shown for 134C/343C (A and C) and 135C/338C (B and D) mutants at 0.2 mM NPG. The k_{off} values were estimated from single exponential fits under reducing conditions only (blue and red lines in the absence or presence of Nb9065, respectively). For 134C/343C mutant k_{off} is $25 \pm 1 \text{ s}^{-1}$ or $27 \pm 1 \text{ s}^{-1}$ without or with Nb9065 (A and C), and for 135C/338C mutant k_{off} is $32 \pm 1 \text{ s}^{-1}$ or $33 \pm 2 \text{ s}^{-1}$ without or with Nb9065 (B and D). Data are presented in Fig. 5 (open symbols).

Fig. 4. Cross-linking of Cys residues in R135C/T338C mutant containing bound galactoside. Double-Cys mutant was preincubated first with TCEP to eliminate spontaneous cross-link, then the galactoside (TDG or NPG) was added, TCEP removed, and, finally, Cu^{2+} was added to catalyze formation of cross-link in the presence of bound sugar (see *Methods* for details). (A) Accessibility of Cys residues to alkylation. Time courses of MIANS labeling are recorded with TDG-bound mutant cross-linked in the presence of Cu^{2+} (trace 1, black) or treated the same way but without Cu^{2+} for control (trace 2, cyan). (B) Displacement of NPG by excess TDG in the presence of Cu^{2+} . Dissociation rate constant (k_{off}) of NPG is measured by mixing of 20 mM TDG with double-Cys mutant cross-linked in the presence of 0.2 mM NPG. The stopped-flow traces are recorded without Nb (trace 1, black line) or with 1.5-fold excess of Nb9065 (trace 2, red line). The k_{off} values estimated from single exponential fits are: 27 ± 2 and $33 \pm 2 \text{ s}^{-1}$ for NPG dissociation from cross-linked LacY.

lines). The concentration dependence of NPG binding rates (Fig. 5, blue circles) fitted with a linear equation yield estimates of k_{on} for 134C/343C and 135C/338C as 0.01 and $0.23 \mu\text{M}^{-1}\text{s}^{-1}$, respectively (Fig. 5, blue lines), which is comparable to the k_{on} obtained for WT LacY (*SI Appendix*, Fig. S3, green line) (21). However, Nb9065 binding dramatically changes the NPG binding rates (Fig. 2, traces 2, red lines), and k_{on} increases by up to three orders of magnitude to 9.7 ± 0.3 or $9.8 \pm 0.2 \mu\text{M}^{-1}\text{s}^{-1}$ in 134C-343C or 135C-338C mutants, respectively (Fig. 5), values comparable to the $9.3 \pm 0.2 \mu\text{M}^{-1}\text{s}^{-1}$ observed with WT LacY/Nb9065 complex (*SI Appendix*, Fig. S3). Therefore, in each mutant, introduction of a double-Cys replacement on the cytoplasmic side by itself does not alter the dynamic mobility of LacY, and Nb9065 stabilizes a similar periplasmic-open conformer with unrestricted access to the galactoside-binding site as observed with WT LacY.

The experimental data presented in Figs. 2–5 are summarized in a cartoon explaining the effect of cross-linking of the cytoplasmic cavity on binding of sugar and interaction with Nb9065 (*SI Appendix*, Fig. S4).

Discussion

The findings presented here show that cross-linking of the cytoplasmic cavity in LacY stabilizes an occluded apo-intermediate—a predicted essential component of the transport cycle (1). Thus, Cu^{2+} -catalyzed oxidation of paired Cys replacements R134C/V343C or R135C/T338C leads to loss of MIANS labeling due to formation of an S-S bond (Fig. 1). After cross-linking the cytoplasmic cavity, the sugar-binding site becomes inaccessible to galactosides, indicating that the periplasmic side is also closed (Figs. 2 and 3 and *SI Appendix*, Fig. S4, schemes 1 and 2). Importantly, cross-linking on the cytoplasmic side does not alter the sugar-binding site per se (Fig. 4 and *SI Appendix*, Figs. S2 and S4, scheme 3), only the accessibility of the binding site.

In addition, the lack of sugar binding to cross-linked mutants preincubated with Nb9065 indicates an absence of conformer with an open periplasmic cavity recognized by the Nb (Fig. 2, traces 4; Fig. 3 C and D, black lines; and *SI Appendix*, Fig. S4, scheme 2). Remarkably, reducing the S-S bond restores normal dynamic properties of LacY and rescues not only sugar binding

but also the effect of Nb9065 on the accessibility of the sugar-binding site to the extent reported for the WT LacY/Nb9065 complex. High k_{on} values for galactoside binding to both mutants 134C/343C and 135C/338C pretreated with Nb9065 under reducing conditions (9.7 ± 0.3 and $9.8 \pm 0.2 \mu\text{M}^{-1}\text{s}^{-1}$) indicate unrestricted access to the galactoside-binding site through an open periplasmic cavity (Fig. 5 and *SI Appendix*, Figs. S3 and S4, scheme 1). An identical k_{on} value ($9.7 \pm 0.4 \mu\text{M}^{-1}\text{s}^{-1}$) was reported for NPG binding to the complex of Nb9065 with another double-Cys mutant (I32C-N245C) on the periplasmic side of LacY under reducing conditions (21), providing additional evidence for stabilization of the same conformer. Hence, Nb9065 traps the same identifiable outward-open conformer of LacY characterized by a distinct k_{on} value for galactoside binding.

The data show that a single S-S bond on the cytoplasmic surface of LacY locks the symporter in a conformation with both cavities closed and an inaccessible sugar-binding site. However, cross-linking the periplasmic cavity does not block opening/closing of the cytoplasmic cavity as judged by accessibility of the binding site to galactoside from the cytoplasmic side (21). These observations correlate with differences in the packing of transmembrane helices (II with XI and V with VIII) between N- and C-terminal halves of the protein, which facilitate closure of cavities in alternative conformations. Crystal structures demonstrate multiple contacts of residues that participate in closing the cavities; however, the interactions between helices is different on the cytoplasmic and periplasmic sides (Fig. 6 and *SI Appendix*, Figs. S5–S7).

In the cytoplasmic-open conformation (3–6), sealing of the cavity on the periplasmic side is provided by side chains of Ile32, His35, and Asp245 clustering in the approximate middle of the closed cavity between helices I and VII, and by direct contacts of the periplasmic ends of helices II, XI, V, and VIII (*SI Appendix*, Fig. S5). The central positions in contacting portions of helices are occupied by Gly residues located in linear segments of antiparallel helices II and XI, and helices V and VIII (Fig. 6B and *SI Appendix*, Fig. S7 C and D). Both Gly pairs, G46–G370 and G159–G262, are conserved in homologous bacterial transporters of the Major Facilitator Superfamily (32). Moreover, the packing interfaces in all four helices at the periplasmic ends contain small residues spaced by one helical turn: SxxG₄₆xxxA in helix II, ASxxG₁₅₉xxxT in helix V, TxxxG₂₆₂xxxT in helix VIII, and SxxxG₃₇₀ in helix XI. The preference for small side chains on contacting faces of helices is well known (33–37), and similar

Fig. 5. Accessibility of the sugar-binding site in double-Cys mutants under reducing conditions. Concentration dependencies of NPG binding rates are shown for 134C/343C (A) and 135C/338C (B) mutants in the absence (blue) or presence (red) of Nb9065. The values of k_{obs} (filled symbols) and k_{off} (open symbols) were estimated from single-exponential fits of stopped-flow traces at various NPG concentrations (Figs. 2 and 3). The k_{on} values are calculated from the slopes of the linear fits of the concentration dependencies of binding rates ($k_{\text{obs}} = k_{\text{off}} + k_{\text{on}}[\text{NPG}]$). The estimated k_{on} for NPG binding are 0.010 ± 0.001 or $9.7 \pm 0.3 \mu\text{M}^{-1}\text{s}^{-1}$ for 134C/343C without or with Nb9065, and 0.23 ± 0.01 or $9.8 \pm 0.2 \mu\text{M}^{-1}\text{s}^{-1}$ for 135C/338C without or with Nb9065, respectively. Red arrow indicates greatly increased accessibility for sugar.

Fig. 6. Small-sized amino acid residues involved in helix packing between N- and C-terminal halves of LacY in alternative conformations. Fragments of directly contacting helices in LacY with closed either cytoplasmic or periplasmic cavities are shown with cytoplasmic side up (the entire structures are presented on *SI Appendix, Fig. S7*). Small interacting residues are presented as spheres with oxygen and nitrogen atoms colored red and blue, respectively. (A) The area of contact between helices on the cytoplasmic side of outward-open LacY_{WW} (PDB ID code 6C9W). Two pairs of contacting residues Gly64 (helix II) with Thr348 (helix XI) and Gly141 (helix V) with Pro280 (helix VIII) are shown with carbon atoms colored magenta. (B) The area of contact on the periplasmic side of inward-open WT LacY (PDB ID code 2V8N). Two pairs of connected helices (II with XI and V with VIII) contain small residues located on one face of each helix and shown with carbon atoms colored as the main chains of the helices or in magenta for conserved Gly pairs.

sequence patterns are conserved in homologous bacterial transporters threaded into the LacY structure, indicating the same principle of packing helical segments when the periplasmic cavity is closed (*SI Appendix, Fig. S8*, red underline).

Replacement of the Gly residues with bulky Trp disrupts contacts between the N- and C-terminal six-helix bundles and stabilizes the periplasmic-open conformation (17). All four X-ray structures of the double-Trp mutant LacY_{WW} with or without bound galactoside (7–10) exhibit the same arrangement of residues closing the cytoplasmic cavity, where tight helix packing between the N- and C-terminal halves is mediated by two pairs of residues Gly64–Thr348 and Gly141–Pro280, between helices II and XI, and helices V and VIII, respectively. Also, multiple side-chain interactions seal the cavity in the middle (*SI Appendix, Fig. S6*). However, no Gly–Gly contacts are present on the cytoplasmic side of LacY with a closed cytoplasmic cavity, where Gly–Thr and Gly–Pro residues are found in the packing interface between N- and C-terminal helix bundles (Fig. 6A and *SI Appendix, Fig. S7 A and B*). These two pairs of residues are conserved in homologous transporters, but sequences with runs of small residues are not observed on one face at the cytoplasmic ends of contacting helices (*SI Appendix, Fig. S8*), suggesting that interaction between N- and C-terminal helix bundles is weaker on the cytoplasmic than on the periplasmic side.

As shown by Cys-scanning mutagenesis of each residue in LacY (38), although small residues can generally be replaced with retention of activity, those involved in packing interfaces on both sides of LacY cannot be replaced without compromising transport activity. The transport cycle requires alternating opening and closing of cavities, and LacY activity is significantly decreased by bulky replacements of residues in areas of direct helix-helix contacts between N- and C-terminal halves on both sides of the molecule. For example, lactose transport is essentially abolished by Cys mutations of Gly64, Gly141, Pro280, or Thr348 on the cytoplasmic side (38), and by replacement of any side chain larger than Ala or Ser for Gly residues in the contacting Gly–Gly pairs on the periplasmic side of LacY (17, 39).

However, Gly residues in contacting periplasmic ends of helices in LacY are not a part of the Gly-containing sequence motifs GxxxG or GxxxGxxxG found frequently in packing interfaces with strong helix-helix connections that are important for stability and oligomerization of membrane proteins (33–37). Therefore, connections between N- and C-terminal halves of LacY on the periplasmic side formed by Gly pairs surrounded by small residues are sufficient enough to provide tight packing of helices, but allow opening of the periplasmic cavity and other conformational transitions necessary for transport. Structural studies suggest that sequences similar to GxxxG but with small residues in place of Gly may be important for membrane protein folding (36, 37). In LacY, association of elongated segments in pairs of helices on the periplasmic side could be a driving force for the formation and stabilization of active 12-helix molecules when N- and C-terminal domains are coexpressed as individual fragments in the membrane (40, 41). Although WT LacY does not contain GxxxG motifs in any areas of helix-helix contacts, mutation C154G creates a G₁₅₀xxxG₁₅₄ sequence that strengthens interaction between helices I and V resulting in markedly decreased conformational mobility and increased thermostability that led to the first X-ray structure (3, 42, 43).

Notably, tighter packing with stronger interactions between helices on the periplasmic side of LacY relative to the cytoplasmic side (helix II with XI and helix V with VIII) is consistent with a slower rate of conformational change on the periplasmic side. The rate of opening of the periplasmic cavity ($21 \pm 1 \text{ s}^{-1}$) is slower than opening of the cytoplasmic cavity ($55 \pm 5 \text{ s}^{-1}$), as estimated from galactoside binding and fluorescence quenching studies (15, 21). Clearly, structural features determine functional properties, and specific packing interactions between helices in alternative conformations play a critical role in the conformational transitions.

Importantly, closing of the cavity on either side of LacY is faster than opening. Thus, rates of cavity closing are estimated as $240 \pm 60 \text{ s}^{-1}$ and $300\text{--}400 \text{ s}^{-1}$, respectively, for cytoplasmic and periplasmic sides, compared with opening rates of $55 \pm 5 \text{ s}^{-1}$ and $21 \pm 1 \text{ s}^{-1}$ (15, 21). Because closing rates are faster than opening rates, the occluded conformer is present as a significant population. The conformation with both cytoplasmic and periplasmic cavities tightly closed requires flexibility within the two six-helix bundles to maintain the occluded form and is inconsistent with a strictly rigid body conformational change. Moreover, the finding that LacY transports oversized galactosides implies significant changes in the shape of the transmembrane helices to form a sugar-binding cavity large enough to accommodate these substrates (27).

Presumably, the cytoplasmic cavity opens more frequently than the periplasmic cavity in the apo-occluded conformation, and the symporter exhibits a statistical distribution of multiple conformers with a larger fraction in the cytoplasmic-open than the periplasmic-open form at any time. Therefore, the inward-facing conformation is readily detected in the absence of sugar by site-directed thiol labeling (11), single-molecule fluorescence (12), double electron-electron resonance (DEER) distance measurements (13), or by fluorescence quenching (14), although it is not the predominant conformation. The outward-open form is miniscule but exists and is detected with Nbs developed against periplasmic-open LacY (18, 19). The stable occluded conformation of LacY described here likely resembles the apo-intermediate of the transport cycle identified in DEER studies by interspin distance measurements (13, 44).

Methods

The materials used in this study, construction of mutants and purification of LacY, are described in *SI Appendix, SI Methods*.

Treatment of LacY with Cu²⁺ or TCEP. Purified double-Cys mutants (25–40 μM) in 50 mM NaP₀/0.02% dodecyl- β -D-glucopyranoside (DDM; pH 7.5) were incubated with 100 μM CuSO₄ or 10 mM TCEP for 20 min at room temperature to catalyze formation or reduction, respectively, of S–S bonds between Cys residues. Samples diluted 40–60 fold were used for MIANS labeling or for measurements of sugar-binding rates. To maintain reducing conditions in stopped-low experiments, TCEP concentration was 2 mM.

For cross-linking of galactoside-bound protein, purified LacY (40 μ M) was initially incubated with TCEP (10 mM) to reduce spontaneously formed S-S bonds, then a given galactoside (10 mM TDG or 0.2 mM NPG) was added and TCEP was removed by passage through a spin-column (60 μ L solution loaded on 1 mL Sephadex-G25 equilibrated with the same galactoside). Galactoside-bound LacY (20–30 μ M) in 50 mM NaP_i/0.02% DDM (pH 7.5) buffer containing 10 mM TDG or 0.2 mM NPG was then mixed with either CuSO₄ (70–100 μ M) or TCEP (10 mM) for 20 min at room temperature and used for MIANS labeling or measurements of sugar-binding rates.

MIANS Labeling. Steady-state fluorescence was monitored at room temperature on a SPEX Fluorolog 3 spectrofluorometer (Horiba) in a 2.5-mL cuvette with constant stirring. Time courses of labeling with MIANS were recorded at excitation and emission wavelengths of 330 nm and 415 nm, respectively, with 0.5 μ M LacY in 50 mM NaP_i/0.02% DDM (pH 7.5) in the presence of 10 mM TDG to protect highly reactive native Cys148 from alkylation and to stabilize the outward-open conformation of LacY. Reaction was started by addition of 5 μ L of 0.5 mM MIANS solution in DMSO. Residual concentrations of TCEP or CuSO₄ were 0.2 mM or 2 μ M, respectively. Background level of MIANS fluorescence without protein was insignificant and subtracted from the traces.

Sugar Binding. Galactoside binding rates (k_{obs}) were measured directly by mixing LacY with given concentration of NPG as the decrease of Trp fluorescence due to FRET from Trp151 of LacY to bound NPG. In displacement experiments, LacY was preincubated with NPG for 10 min and then mixed with excess TDG (20 mM final concentration). TDG is not a FRET acceptor, and Trp151 fluorescence increases due to dissociation of bound NPG and its

displacement by TDG, that allows determination of a dissociation rate constant, k_{off} , which does not depend on NPG concentration (22, 45, 46).

Stopped-Flow Measurements. Fluorescence of Trp was measured at 25 °C in a SFM-300 rapid kinetic system equipped with a TC-50/10 cuvette (dead-time 1.2 ms), and MOS-450 spectrofluorimeter (Bio-Logic USA, LLC) at excitation wavelength 295 nm with collection of emitted light using interference filter IF340 nm with a bandwidth of 26 nm (Edmund Optics). Stopped-flow traces were recorded at final concentration 0.5–1 μ M of LacY or LacY/Nb complex in 50 mM NaP_i/0.02% DDM (pH 7.5). Nb9065 was added at a 1.2–1.5-fold molar excess to LacY treated with Cu²⁺ or TCEP, and incubated at room temperature for 20 min before stopped-flow mixing with a given galactoside. Measurements under cross-linking conditions were done at 1.2–2.5 μ M CuSO₄, and under reducing conditions at 2 mM of TCEP. Typically, 10–30 traces were recorded for each data point, averaged and fitted with an exponential equation using the built-in Bio-Kine32 software package or by using Sigmaplot 10 (Systat Software Inc.). A linear fit of concentration dependence of observed rates ($k_{\text{obs}} = k_{\text{off}} + k_{\text{on}}[\text{ligand}]$) was used to estimate the association rate constant, k_{on} . Calculated SDs were within 10% for each data point presented. All given concentrations were final after mixing.

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