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An Asymmetric Conformational Change in LacY

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Abstract

Galactoside/H⁺ symport by the lactose permease of *Escherichia coli* (LacY) involves reciprocal opening and closing of periplasmic and cytoplasmic cavities so that sugar- and H⁺-binding sites become alternatively accessible to either side of the membrane. After reconstitution into proteoliposomes, LacY with the periplasmic cavity sealed by cross-linking paired-Cys residues does not bind sugar from the periplasmic side. However, reduction of the S–S bond restores opening of the periplasmic cavity and galactoside binding. Furthermore, nanobodies that stabilize the double-Cys mutant in a periplasmic-open conformation and allow free access of galactoside to the binding site do so only after reduction of the S–S bond. In contrast, when cross-linked LacY is solubilized in detergent, galactoside binding is observed, indicating that the cytoplasmic cavity is patent. Sugar binding from the cytoplasmic side exhibits nonlinear stopped-flow kinetics, and analysis reveals a two-step process in which a conformational change precedes binding. Because the cytoplasmic cavity is spontaneously closing and opening in the symporter with a sealed periplasmic cavity, it is apparent that an asymmetrical conformational transition controls access of sugar to the binding site.

Graphical abstract

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Notes

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.7b00134. Materials and methods used for preparation of purified proteins, Figures S1–S5, and Table S1 (PDF)

Typical of many transport proteins from organisms as widely separated evolutionarily as *Archaea* and *Homo sapiens*, the lactose permease of *Escherichia coli* (LacY) catalyzes the coupled transport of a galactopyranoside and a H^+ across the cytoplasmic membrane (galactoside/ H^+ symport) by a recently proposed mechanism¹ and is the most intensively studied member of the major facilitator superfamily (MFS).² As broadly documented with LacY,³ MFS proteins operate by an alternating access mechanism. By this means, substrate- and H^+ -binding sites in the middle of the molecule become alternatively accessible to either side of the membrane as a result of reciprocal opening and closing of periplasmic and cytoplasmic cavities. Structures of two conformations of LacY have been determined by X-ray crystallography (Figure 1), an inward-open form with an aqueous cavity open to the cytoplasmic side and a tightly sealed periplasmic side^{4–7} and an outward-open, occluded form with bound lactose homologues^{8,9} or a nanobody (Nb)¹⁰ and a tightly sealed cytoplasmic side. However, understanding membrane transport based on alternating access requires dynamic information regarding association and dissociation of the substrate, as well as the conformational transitions of the molecule and the mechanism of coupling between sugar and H^+ transport.

Trp151, located in the galactoside-binding site in the middle of LacY, allows detection of sugar binding by Trp151 $\rightarrow$ 4-nitrophenyl α -D-galactopyranoside (NPG) FRET.¹¹ LacY reconstituted into proteoliposomes (PL) by dilution¹² is oriented with the periplasmic side facing out, as in the native bacterial membrane,^{13–17} so that the sugar-binding site can be accessed only through opening of the periplasmic cavity. The observed rate of sugar binding measured directly by stopped flow is 20–30 s^{-1} and independent of sugar concentration. Rates of conformational transition during reciprocal opening and closing of periplasmic and cytoplasmic cavities have been determined independently in real time by changes in the fluorescence of Trp or attached fluorophores due to binding or dissociation of sugar with LacY either solubilized in dodecyl β -D-maltopyranoside (DDM) or reconstituted into PL. The studies show that the opening of the periplasmic cavity limits access of sugar to the binding site from outside of the membrane.^{15,17} Moreover, the rate of opening is very similar to the turnover number of wild-type (WT) LacY in right-side-out (RSO) membrane vesicles or reconstituted PL¹⁸ and consistent with the notion that opening of the periplasmic cavity may be a limiting step in the overall transport mechanism.

In contrast to that of WT LacY, kinetic studies with certain mutants show free accessibility of the binding site for sugar, but no transport activity. With solubilized LacY mutant G46W/G262W, which is open on the periplasmic side,¹⁹ or the inward-open mutant C154G,^{4,5,15} rates of galactoside binding are linearly dependent on sugar concentration with a high

association rate constant ($k_{on} = 5 \mu\text{M}^{-1} \text{s}^{-1}$). Remarkably, however, with solubilized WT LacY under the same conditions, the sugar-binding site is considerably less accessible ($k_{on} = 0.2 \mu\text{M}^{-1} \text{s}^{-1}$),¹⁵ consistent with a dynamic molecule existing in multiple conformations.

A periplasmic-open conformation of WT LacY is stabilized by galactoside binding.^{20–22} Thus, a k_{on} of $10 \mu\text{M}^{-1} \text{s}^{-1}$ is estimated for NPG from displacement experiments with WT LacY reconstituted into PL, confirming the conclusion that bound sugar shifts the conformational distribution of LacY toward the periplasmic-open form with a readily accessible binding site. In addition, a number of camelid Nbs developed against the periplasmic-open mutant G46W/G262W LacY (Figure 1C) bind to the periplasmic aspect of WT LacY and stabilize outward-facing conformations with dramatically increased k_{on} values indicating a highly accessible galactoside-binding site.^{10,16,23}

Access to the sugar-binding site from the cytoplasmic side of LacY is more difficult to study directly. In addition to influx, LacY also catalyzes galactoside efflux, equilibrium exchange, and counterflow,^{24,25} processes that involve binding of sugar from the inner face of the membrane. The apparent affinity of galactosides for LacY in RSO or in inside-out (ISO) bacterial membrane vesicles is practically identical,²⁶ indicating that the same sugar-bound complex is formed when the galactoside enters from either side of the membrane. However, the kinetics of sugar access from cytoplasmic side of LacY solely has not been studied. With this in mind, we constructed mutants for reversibly jamming the periplasmic cavity by cross-linking paired double-Cys replacements on the periplasmic side to measure galactoside binding specifically from the cytoplasmic side.

Several X-ray structures of LacY exhibit an inward-facing conformation with a completely closed periplasmic side (Figure 1A,B). It is apparent that cavity sealing involves residues Ile32 and His35 from helix I and Asn245 from helix VII in the middle of the periplasmic side, and also two conserved pairs of Gly residues (Gly46–Gly370 and Gly159–Gly262) connecting N- and C-terminal six-helix bundles on the periphery.²⁷ Efficient cross-linking of paired-Cys replacements I32C and N245C in mutant 32C/245C with homobifunctional thiol reagents has been demonstrated in RSO membrane vesicles.²⁸ Thus, cross-linking of LacY with a short reagent (5 Å) completely blocks lactose transport that is rescued under reducing conditions. Here Cu^{2+} is used to facilitate spontaneous formation of a S–S bond between paired-Cys replacements in the 32C/245C or 35C/245C mutant, and tris(2-carboxyethyl)phosphine (TCEP) is utilized for reduction of the S–S bond, which restores conformational mobility on the periplasmic side (Figure 1B,C). Kinetic analyses of sugar binding to double-Cys mutants solubilized in DDM or reconstituted into PL show that LacY with a cross-linked periplasmic cavity remains conformationally active on the opposite side, thereby revealing asymmetric structural changes. Thus, the cytoplasmic cavity opens and closes with a locked periplasmic side, and sugar binds to the cytoplasmic-open state via a conformational selection mechanism.

MATERIALS AND METHODS

Materials used in this study, construction of mutants, purification of proteins, and reconstitution of LacY into proteoliposomes are described in the Supporting Information.

Treatment of LacY with Cu²⁺ or TCEP

Purified protein (40 μ M) was incubated with CuSO₄ (100 μ M) or TCEP (10 mM) for 10 min at room temperature in 50 mM NaP_i and 0.02% DDM (pH 7.5). Reconstituted PL were subjected to two freeze/thaw/sonication cycles in the presence of CuSO₄ (100 μ M) or TCEP (10 mM) at a protein concentration of 40 μ M in the same buffer without DDM. In stopped-flow experiments, the final concentration of TCEP or CuSO₄ was 2 mM or 1.5 μ M, respectively.

Fluorescence Measurements

Steady-state fluorescence was monitored at room temperature on a SPEX Fluorolog 3 spectrofluorometer (Horiba, Edison, NJ) in a 2.5 mL cuvette in which the sample was being constantly stirred. Time courses of labeling by 0.8 μ M 2-(4'-maleimidylanilino)naphthalene-6-sulfonate (MIANS) were recorded at excitation and emission wavelengths of 330 and 415 nm, respectively, with 0.4 μ M protein in 50 mM NaP_i and 0.02% DDM (pH 7.5) in the presence of 5 mM β -D-galactopyranosyl 1-thio- β -D-galactopyranoside (TDG) to protect highly reactive native Cys148 from alkylation and also to stabilize the outward-open conformation. LacY was preincubated at a protein concentration of 20 μ M with CuSO₄ (50 μ M) or with TCEP (100 μ M) for 30 min at room temperature and diluted 50-fold for MIANS labeling. Trp151 $\rightarrow$ NPG FRET was measured as an increase in Trp fluorescence resulting from displacement of bound NPG by excess TDG.¹¹ Trp emission spectra were recorded with 0.4 μ M LacY in the same buffer containing 1 μ M CuSO₄ and 0.2 mM NPG in the absence or presence 12 mM TDG at an excitation wavelength of 295 nm.

Stopped-flow measurements were performed at 25 °C in an SFM-300 rapid kinetic system equipped with a TC-50/10 cuvette (dead time of 1.2 ms) and an MOS-450 spectrofluorimeter (Bio Logic USA, LLC, Knoxville, TN). NPG binding was measured as Trp151 $\rightarrow$ NPG FRET at 295 nm excitation with an emission interference filter at 340 nm (Edmund Optics). Stopped-flow traces were recorded at a final LacY concentration of 0.5–1 μ M after mixing with NPG. In displacement experiments, LacY was preincubated with NPG for 5 min and then mixed with 15 mM TDG by stopped-flow. Measurements with purified protein in DDM were performed in 50 mM NaP_i and 0.02% DDM (pH 7.5). Experiments with PL were performed in 50 mM NaP_i (pH 7.5). When used, Nb9065 was added in a 1.5-fold molar excess to LacY (40 μ M) preincubated with Cu²⁺ or TCEP and kept for 20 min at room temperature. 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., Richmond, CA). Calculated standard deviations were within 10% for each presented data point. All given concentrations were final after mixing.

Stopped-flow rates of cytoplasmic cavity closing were measured using Cys-Trp mutants labeled with either (2-pyridyl)dithiobimane (PDT-bimane) or bimane-maleimide as described previously.¹⁷ Time traces of bimane fluorescence quenching by Trp were recorded at excitation 380 nm with an emission interference filter at 447 nm (Edmund Optics).

RESULTS

Cross-Linking the Periplasmic Cavity

Two double-Cys mutations, I32C/N245C or H35C/N245C, were introduced on the periplasmic side of WT LacY, as well as single replacements I32C, H35C, and N245C for controls (Figure 1). Alkylation of the Cys residues by MIANS was tested under reducing (with TCEP) or oxidizing (with Cu^{2+}) conditions to reveal formation of S–S bonds in the double-Cys mutants. An increase in MIANS fluorescence with time is observed as the maleimide moiety reacts with a free Cys residue (Figure 2A–F).^{29,30}

Purified proteins containing one Cys replacement are readily labeled with MIANS in the presence of TCEP or Cu^{2+} (Figure 2A,D, blue and red lines). In contrast, the double-Cys mutants are labeled only under reducing conditions (Figure 2B,C, blue lines), while in the presence of Cu^{2+} , both exhibit slow background labeling similar to that of WT LacY (red and gray lines, respectively, in Figure 2E,F). As expected, formation of S–S bonds in the double-Cys mutants blocks alkylation of the introduced Cys residues. However, binding of sugar to the proteins cross-linked on the periplasmic side is similar to that observed with WT LacY in steady-state displacement experiments (Figure 2G). Thus, Trp151 → NPG FRET is detected as an increase in Trp fluorescence by displacement of bound NPG with excess TDG,¹¹ and the fluorescence change in the double-Cys mutants in the presence of Cu^{2+} is in a range similar to that observed with WT LacY.

Binding of Sugar to Cross-Linked LacY

As illustrated by the cartoon at the top of Figure 3, displacement of bound NPG by a saturating concentration of TDG (Figure 2G) provides an estimate of the dissociation rate constant for sugar (k_{off}). In the presence of excess TDG, the rate of NPG dissociation is determined specifically by k_{off} and does not depend on NPG concentration.^{15,17,31–33} Stopped-flow traces recorded with 32C/245C LacY in a DDM solution exhibit an increase in Trp fluorescence due to displacement of NPG in the presence of either Cu^{2+} or TCEP (Figure 3A,B; $k_{\text{off}} = 9$ or 16 s^{-1} , respectively). However, after reconstitution into PL, the double-Cys mutant shows no sugar binding in the presence of Cu^{2+} , while the control mutant with a single Cys in place of Ile32 exhibits normal displacement (Figure 3C). This is anticipated, because LacY is incorporated into PL in the same orientation as in the bacterial membrane with the periplasmic side out,^{13–16} and access of sugar to the binding site is blocked when the periplasmic side is closed by cross-linking. In addition, binding of NPG to the reconstituted mutant is rescued when the cross-link is reduced by TCEP (Figure 3D).

Sugar binding measured directly by mixing mutant 32C/245C with NPG also demonstrates that in the presence of Cu^{2+} the binding site is accessible only when protein is solubilized in DDM, but not in reconstituted PL (compare traces 1 in Figure S1A,C), while under reducing conditions, normal sugar binding is observed in both DDM micelles and PL (traces 1 in Figure S1B,D). Therefore, it is clear that with the cross-linked mutant 32C/245C, the sugar-binding site is accessible only from the cytoplasmic side. Moreover, reduction of the cross-link allows the double-Cys mutant to regain flexibility on the periplasmic side to an extent

similar to that observed with the control single-Cys mutant I32C (Figure 3C, trace 2) or WT LacY.¹⁵ Very similar results are also obtained with the 35C/245C mutant (Figure S2).

Binding of Sugar to Double-Cys Mutants in PL under Reducing Conditions

The effect of the Cys replacements on LacY function was tested in the presence of TCEP. Kinetic parameters of binding of NPG to reconstituted double-Cys mutants were estimated from concentration dependencies of Trp fluorescence changes (Figure 4A) and observed sugar-binding rates (Figure 4B). Displacement experiments with mutant 32C/245C [Figure 4A (empty squares)] reveal high-affinity NPG binding with a K_d of $7 \pm 1 \mu\text{M}$ and a k_{off} of $24 \pm 3 \text{ s}^{-1}$. Control mutants containing only one introduced Cys replacement [I32C, H35C, or N245C (Figure S3)] bind the galactoside efficiently in the presence or absence of Cu^{2+} ($K_d = 3\text{--}17 \mu\text{M}$; $k_{\text{off}} = 33\text{--}102 \text{ s}^{-1}$). Therefore, reduction of the S–S bond in mutant 32C/245C restores opening of the periplasmic cavity and the kinetics of sugar binding to a level observed with WT LacY in PL ($K_d = 11 \mu\text{M}$; $k_{\text{off}} = 111 \text{ s}^{-1}$).¹⁵

Sugar binding rates measured directly by mixing NPG with mutant 32C/245C under reducing conditions do not depend on sugar concentration [Figure 4B (black squares)], as documented with reconstituted WT LacY,^{15,16} because access to the binding site is limited by the rate of opening of the periplasmic cavity. This rate is 10-fold slower in the TCEP-treated double-Cys mutant ($k_{\text{obs}} = 2 \text{ s}^{-1}$) than in WT LacY ($k_{\text{obs}} = 21 \text{ s}^{-1}$), which may be explained by reversible reduction and/or formation of the S–S bond between two proximal Cys residues even in the presence of TCEP, because the rates measured with control mutants {I32C, H35C, and N245C [Figure S3C,F,I (black squares)]} are in the range typical for WT LacY ($k_{\text{obs}} = 20, 33$, and 26 s^{-1} , respectively, and 21 s^{-1} for WT).

The conformational mobility of the double-Cys mutant was also tested with Nb9065. This Nb stabilizes the outward-facing conformation of LacY only when the periplasmic cavity opens and Nb-binding epitopes are exposed, as observed with highly flexible WT LacY.^{16,23} Binding of Nb9065 to mutant 32C/245C reconstituted into PL does not change the affinity for NPG or the displacement rate measured in the presence of TCEP [Figure 4A (empty triangles)] because galactoside binding itself shifts the conformational distribution of LacY toward an outward-open conformer.^{20–22} Thus, k_{on} values calculated from displacement experiments ($k_{\text{on}} = k_{\text{off}}/K_d$) in the presence and absence of Nb9065 are high (4.8 and $3.4 \mu\text{M}^{-1} \text{ s}^{-1}$, respectively), indicating that the sugar-binding site is easily accessible in both cases.

No effect of Nb9065 is detected when the periplasmic cavity is cross-linked (trace 2 in Figure S1A,C and trace 3 in Figure S2F); however, addition of Nb to TCEP-treated double-Cys mutants in the absence of sugar completely changes the kinetics of NPG binding (compare traces 1 and 2 in Figures S1B,D and S2F). A high k_{on} of $3.7 \mu\text{M}^{-1} \text{ s}^{-1}$ is estimated from the concentration dependence of binding rates measured directly [Figure 4B (green triangles)], indicating free access for sugar to the binding site in the mutant–Nb9065 complex. Therefore, the dynamic properties are restored after reduction of the disulfide bond, so that Nb9065 binds to the outward-open mutant and stabilizes conformer with an open periplasmic cavity. The data obtained with reconstituted double-Cys mutants clearly demonstrate that the Cys replacements introduced inside the periplasmic cavity do not

significantly alter the conformational transitions that govern the kinetics of sugar binding in the absence of cross-linking.

Kinetics of Binding of Sugar to Double-Cys Mutants Solubilized in DDM

Sugar binding rates measured directly in DDM micelles by mixing NPG with mutant 32C/245C under reducing or cross-linking conditions are similar [Figure 5A,B (black circles)]. However, two entirely different states of the mutant are revealed by addition of Nb9065. In the presence of TCEP, sugar binding rates are dramatically increased by Nb9065 [Figure 5A (green triangles); $k_{\text{on}} = 9.7 \mu\text{M}^{-1} \text{s}^{-1}$], indicating that the Nb stabilizes an outward-open conformer with a highly accessible sugar-binding site. In contrast, in the presence of Cu^{2+} , Nb9065 has no effect on NPG binding rates (Figure 5B and Figure S1A). Notably, the effect of Nb9065 on WT LacY is not altered by Cu^{2+} , and binding of NPG to the WT LacY–Nb9065 complex is characterized by a high k_{on} value (Figure S4). Clearly, with the double-Cys mutant cross-linked on the periplasmic side, Nb9065 is ineffective because the periplasmic cavity is locked. Therefore, in the cross-linked mutant, the sugar-binding site is accessible exclusively by opening of the cytoplasmic cavity.

Rate of Opening of the Cytoplasmic Cavity Limits Access to the Sugar-Binding Site

Two double-Cys mutants, 32C/245C and 35C/245C, solubilized in DDM were tested for sugar binding after cross-linking in the presence of Cu^{2+} (Figure 6). It is apparent that kinetic parameters for NPG binding to each mutant are very similar. The concentration dependencies of fluorescence changes in displacement experiments are nearly identical for both proteins (Figure 6A,B; for mutant 32C/245C or 35C/245C, $K_{\text{d}} = 78 \pm 8$ or $87 \pm 6 \mu\text{M}$ and $k_{\text{off}} = 9.1 \pm 0.9$ or $9.6 \pm 1.3 \text{s}^{-1}$, respectively). Rates of NPG binding measured directly (k_{obs}) by mixing cross-linked mutants with increasing concentrations of NPG display a nonlinear concentration dependence in both cases [Figure 6C,D (black circles)], indicating that even with periplasmic side sealed, more than one step is involved in the binding process.³⁴

Binding kinetics was analyzed by using theoretical equations derived for different mechanisms of ligand binding that include conformational transitions.³⁴ With both double-Cys mutants cross-linked on the periplasmic side, the best fits are obtained with an equation describing a two-step process involving ligand binding by conformational selection (Figure 6C,D, red lines).

According to this mechanism, the rate of binding of sugar to the cross-linked mutant is limited by the rate of opening of the cytoplasmic cavity as illustrated in Figure 6E. Thus, $\text{Trp151} \rightarrow \text{NPG FRET}$ occurs when NPG binds (black arrows), and this is preceded by conformational change on the cytoplasmic side leading to opening of the cytoplasmic cavity (red arrows). The kinetic parameters of opening and closing of the cytoplasmic cavity (k_{f} and k_{r} , respectively, in Figure 6E) were assessed by nonlinear regression analysis by substitution of k_{off} values obtained from displacement experiments (Figure 6A,B) into eq 1, describing the dependence of the observed rates (k_{obs}) on sugar concentration ($[\text{S}]$) according to the conformational selection binding mechanism.³⁴

$$k_{\text{obs}1,2} = \left[k_r + k_f + k_{\text{off}} + k_{\text{on}}[S] \pm \sqrt{(k_{\text{off}} + k_{\text{on}}[S] - k_r - k_f)^2 + 4k_r k_{\text{on}}[S]} \right] / 2 \quad (1)$$

The best fit to the data defines the kinetic constants presented in the scheme shown in Figure 6E. The first step (red arrows) is determined by forward and reverse rate constants (k_f and k_r , respectively) that characterize spontaneous movements on the cytoplasmic side of LacY in the absence of sugar and estimated as 64 ± 5 and $242 \pm 54 \text{ s}^{-1}$ for 32C/245C, respectively, or 45 ± 4 and $254 \pm 67 \text{ s}^{-1}$ for 35C/245C, respectively. These values clearly indicate that the cytoplasmic cavity closes faster than it opens.

Indeed, similar rates of cytoplasmic cavity closing in LacY are obtained from a separate experiment by site-directed quenching of bimane fluorescence with Trp (Figure 7). LacY mutants with an engineered Trp and a bimane-labeled Cys on opposite sides of the cytoplasmic cavity were used to measure the rate of cytoplasmic cavity closing upon galactoside binding as described previously.¹⁷ Rates of bimane fluorescence quenching by Trp due to closure of the cytoplasmic cavity are $160\text{--}220 \text{ s}^{-1}$, as measured with three labeled mutants and two different galactosides.

The sugar binding step (black arrows in Figure 6E) is defined by two rate constants k_{off} (measured in Figure 6A,B) and k_{on} . Data fits give estimates of the k_{on} values for binding of NPG to 32C/245C and 35C/245C mutants of 0.64 ± 0.12 and $0.70 \pm 0.16 \mu\text{M}^{-1} \text{ s}^{-1}$, respectively (Figure 6C,D, red lines). Therefore, the real K_d values for NPG binding from the cytoplasmic side are calculated as the ratio of estimated rate constants ($K_d = k_{\text{off}}/k_{\text{on}}$), which are practically identical for both mutants: 14.2 ± 2.7 and $13.7 \pm 3.1 \mu\text{M}$ for 32C/245C and 35C/245C, respectively.

DISCUSSION

To study sugar binding from the cytoplasmic side, two mutants are utilized here with paired-Cys residues placed strategically across the periplasmic cavity of WT LacY (Figure 1). When Cys residues in mutants I32C/N245C and H35C/N245C are cross-linked in the presence of Cu^{2+} , access to the binding site from the periplasmic side of the molecule is abolished. Thus, no galactoside binding is detected with cross-linked double-Cys mutants reconstituted into PL where the topological distribution of LacY molecules is periplasmic side out (Figure 3C and Figure S2D). However, sugar binding from the periplasmic side is rescued upon reduction of the S–S bond with TCEP (Figure 3D and Figure S2E). Furthermore, Nb9065, which stabilizes the periplasmic-open conformation of WT LacY,^{16,23} has no effect on the cross-linked mutant (Figure 5B and Figures S1C and S2F). Remarkably, TCEP reduction completely restores the effect of the Nb on activity, and binding of NPG to the mutant–Nb9065 complex via an open periplasmic cavity is observed with a high k_{on} value (Figures 4B and 5A) like that obtained with WT LacY upon Nb9065 binding (Figure S4F). Therefore, it is clear that Cys replacements do not change the functional properties of LacY, and both cross-linked mutants are useful for studying sugar binding kinetics from the cytoplasmic surface of the symporter.

Both double-Cys mutants solubilized in DDM in the presence of Cu^{2+} exhibit similar sugar binding affinities and k_{off} values in NPG displacement experiments (Figure 6A,B). Moreover, directly measured NPG binding rates increase with NPG concentration nonlinearly with k_{obs} limited to 40–50 s^{-1} at high sugar concentrations (Figure 6C,D). Therefore, general equations describing the two-step binding process involving conformational transitions³⁴ were used for data fitting and determining rate constants for conformational changes on the cytoplasmic side of LacY. The mechanism of ligand binding by conformational selection provides a satisfying data fit with kinetic parameters that are in good agreement with those measured in separate experiments. Thus, opening and closing of the cytoplasmic cavity (Figure 6E, red arrows) appear to be really fast with k_f and k_r values estimated from data fits (Figure 6C,D, red lines) as 64 and 242 s^{-1} (for 32C/245C) or 45 and 254 s^{-1} (for 32C/245C), respectively. Note that the k_r value is higher than k_f , indicating that the closing of the cytoplasmic cavity is faster than opening. Remarkably similar rates of the cytoplasmic cavity closing (160–220 s^{-1}) were measured independently with three fluorophore-labeled cytoplasmic mutants as quenching of bimane by Trp was triggered by binding of galactosides, thereby confirming the fast rate of cavity closure (Figure 7). It is also noteworthy that the same rate of conformational change ($238 \pm 12 \text{ s}^{-1}$) was measured on the cytoplasmic side of MIANS-labeled C154G/V331C LacY as the decrease in MIANS fluorescence upon sugar binding.¹¹

NPG binding, which follows the conformational transition (Figure 6E, black arrows), occurs with a k_{on} of 0.64 or 0.70 $\mu\text{M}^{-1} \text{ s}^{-1}$ and a k_{off} of 9.1 or 9.6 s^{-1} for 32C/245C or 35C/245C, respectively, resulting in practically identical true K_d values for both mutants (14.2 μM for 32C/245C and 13.7 μM for 35C/245C). These K_d values are lower than those obtained from displacement experiments (Figure 6A,B) where conformational change is involved, and apparent affinities were determined by the equation $K_d^{\text{app}} = K_d(1 + k_r/k_f)$.³⁴ When conformational equilibrium is taken into account, the true K_d values are in a good agreement with K_d values determined from the Trp151 $\rightarrow$ NPG FRET displacement experiments. The calculations result in a K_d^{app} of $14.2(1 + 242/64) = 68 \mu\text{M}$ for mutant 32C/245C and a K_d^{app} of $13.7(1 + 254/45) = 91 \mu\text{M}$ for mutant 35C/245C, which are in reasonable agreement with the measured values of 78 ± 8 and $87 \pm 6 \mu\text{M}$ (Figure 6A,B).

Theoretical considerations^{34–36} predict that when ligand binding involves conformational selection, k_{obs} increases hyperbolically with ligand concentration [Figure 6C,D (black circles)] only if $k_{\text{off}} < k_f$. In this study, the measured k_{off} (9.1 or 9.6 s^{-1}) is lower than the estimated k_f (64 or 45 s^{-1}) for both cross-linked mutants. Also, the intersection with the Y -axis (k_{obs} at $[S] = 0$) should equal k_{off} , and the limit of k_{obs} at high ligand concentrations (k_{obs} at $[S] = \infty$) should equal k_f (precisely as shown in panels C and D of Figure 6 with empty circles and red lines). Therefore, the kinetic parameters obtained with both mutants are in good agreement with the predicted mechanism (Table S1).

CONCLUSIONS

The data described here demonstrate that movements on the cytoplasmic side of LacY occur spontaneously and are independent of conformational mobility on the periplasmic side, which is incompatible with rigid-body-type movements. It is also noteworthy that a

significant fraction of LacY molecules appear to be in an occluded conformation with no bound sugar (i.e., closed cytoplasmic cavity and cross-linked periplasmic cavity), because the rate of cavity closing is higher than the rate of opening ($k_r > k_f$). These studies clearly indicate that a conformational change on the cytoplasmic side is essential for sugar binding from that side and that galactoside binding occurs by conformational selection of the cytoplasmic-open form. A similar mechanism for sugar binding was described previously for galactoside entering from the opposite (periplasmic) side of the symporter. Thus, for reconstituted LacY with the periplasmic side out, the observed binding rate is independent of sugar concentration and equal to the rate of opening of the periplasmic cavity,^{15,17} indicating that sugar binds from outside by conformational selection. Therefore, spontaneous opening of the cavity on either side of the transporter is the initial step preceding binding of the substrate. In addition, because movement on one side of the molecule is independent of dynamics on the opposite side, an occluded conformer with no sugar bound is an essential functional intermediate and may be the predominant conformer in the overall transport cycle.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

LacY	lactose permease of <i>E. coli</i>
MFS	major facilitator superfamily
Nb	nanobody
NPG	4-nitrophenyl α -D-galactopyranoside
FRET	Förster resonance energy transfer
PL	proteoliposomes
RSO	right-side-out membrane vesicles
ISO	inside-out membrane vesicles
DDM	dodecyl β -D-maltopyranoside
TCEP	tris(2-carboxyethyl)phosphine

MIANS	2-(4'-maleimidylanilino)naphthalene 6-sulfonate
TDG	β -D-galactopyranosyl 1-thio- β -D-galactopyranoside
PDT-bimane	(2-pyridyl)dithiobimane
PDB	Protein Data Bank

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Figure 1.

Cys replacements of amino acid residues for cross-linking of the periplasmic cavity. LacY structural models viewed from the side (A) or from the periplasm (B and C) with rainbow-colored helices (from blue to red from helix I to helix XII, respectively) are shown in inward-open (A and B, PDB entry 2CFQ) and outward-open (C, PDB entry 4OAA) conformations. Residues Ile32, His35, and Asn245 located in the middle of the periplasmic cavity were used for Cys replacements and are shown as yellow spheres. Double-Cys mutants I32C/N245C and H35C/N245C were made for cross-linking. Two Gly pairs on the periphery of the cavity (G46–G370 and G159–G262) are shown as gray spheres. Note Gly → Trp replacements in mutant G46W/G262W (C).

Figure 2.

Binding of sugar to cross-linked mutants solubilized in DDM. (A–F) Accessibility of introduced Cys residues to alkylation. Time traces of the MIANS fluorescence change after addition of 0.8 μ M fluorophore (marked by black arrows) to the 50 mM NaP_i /0.02% DDM buffer (pH 7.5) with no protein (trace 1) or to the same buffer containing 0.4 μ M WT LacY (trace 2) or a given Cys mutant (trace 3) in the presence of TCEP (A–C) or Cu^{2+} (D–F). MIANS labeling of Cys mutants [I32C (A and D), I32C/N245C (B and E), and H35C/N245C (C and F)] performed under reducing (with TCEP) or cross-linking (with Cu^{2+}) conditions is indicated by blue or red lines, respectively. (G) Binding of sugar to WT LacY and the double-Cys mutants measured under cross-linking conditions. Displacement of protein-bound NPG (200 μ M) by excess TDG (12 mM) was detected as the relative fluorescence change in the Trp emission spectra (Trp151 $\rightarrow$ NPG FRET) recorded before and after the addition of TDG (solid and broken lines, respectively). The increase in fluorescence intensity at the spectral maxima (red arrows) is presented as a percentage of the final level of fluorescence after the addition of TDG (F/F).

Figure 3.

Displacement of NPG bound to 32C/245C LacY in DDM micelles (A and B) or reconstituted into PL (C and D). Schematic representation of the displacement of bound sugar (S) by an excess of TDG (T) in the LacY mutant with a cross-linked periplasmic cavity (yellow bar) and an open cytoplasmic side shown at the top. Rates of NPG displacement (k_{off}) were measured with protein preincubated with Cu^{2+} (A and C) or TCEP (B and D). The results of the experiment with the control mutant containing only one additional Cys (I32C LacY) are shown in panel C (trace 2). Stopped-flow traces (gray lines) show the Trp fluorescence change with time as a result of Trp151 $\rightarrow$ NPG FRET. Single-exponential fits to the data are presented as red or blue lines in the presence of Cu^{2+} or TCEP, respectively. Traces were recorded at NPG concentrations of 100 μ M (A and B) or 400 μ M (C and D).

Figure 4.

Effect of Nb9065 on binding of NPG to mutant 32C/245C reconstituted into PL under reducing conditions. (A) NPG binding affinity was measured by displacement as described for Figure 3D at given sugar concentrations. Data obtained in the absence or presence of Nb9065 are shown as empty black squares or green triangles, respectively. The amplitude of the Trp fluorescence increase is expressed as a percentage of the final fluorescence level of the individual traces (F/F). K_d values estimated from hyperbolic fits of concentration dependencies are 7 ± 1 and $6 \pm 2 \mu\text{M}$ for the mutant alone and the mutant in complex with Nb9065, respectively. Average values of k_{off} calculated from individual traces in the absence and presence of Nb9065 are 24 ± 3 and $29 \pm 9 \text{ s}^{-1}$, respectively. (B) Rates of NPG binding measured directly as in Figure S1D at given NPG concentrations. Data obtained in the absence or presence of Nb9065 are shown as filled black squares or green triangles, respectively. Empty symbols represent displacement rates (k_{off}) estimated from experiments described in panel A. Reconstituted mutant 32C/245C with no added Nb9065 binds sugar with a k_{obs} of $2.2 \pm 0.4 \text{ s}^{-1}$ that is independent of NPG concentration (solid black line). The mutant–Nb9065 complex exhibits NPG binding with a k_{on} of $3.7 \pm 0.2 \mu\text{M}^{-1} \text{ s}^{-1}$ (solid green line). The green arrow indicates the change in NPG binding rates induced by Nb9065.

Figure 5.

Effect of Nb9065 on the accessibility of the sugar-binding site of mutant 32C/245C in a DDM solution under reducing and cross-linking conditions. Concentration dependencies of NPG binding rates were measured directly as described in Figure S1A,B in the absence or presence of Nb9065 (filled black circles or filled green triangles, respectively). (A) Data obtained under reducing conditions (with TCEP). (B) Data obtained under cross-linking conditions (with Cu^{2+}). The mutant–Nb9065 complex exhibits NPG binding with a k_{on} of $9.7 \pm 0.4 \mu M^{-1} s^{-1}$ (A, green line), and the green arrow indicates the change in the concentration dependence of sugar binding rates caused by Nb9065 binding.

Figure 6.

Kinetics of binding of sugar from the cytoplasmic side to LacY with the cross-linked periplasmic cavity. (A and B) Concentration dependencies of the Trp fluorescence change measured in displacement experiments with 32C/245C and 35C/245C, respectively, in a DDM solution as described for Figure 3A and Figure S2A. Hyperbolic fits to the data are shown as solid black lines with estimated kinetic parameters for sugar binding: for 32C/245C, $K_d = 78 \pm 8 \mu\text{M}$ and $k_{\text{off}} = 9.1 \pm 0.9 \text{ s}^{-1}$, and for 35C/245C, $K_d = 87 \pm 6 \mu\text{M}$ and $k_{\text{off}} = 9.6 \pm 1.3 \text{ s}^{-1}$. (C and D) Concentration dependencies of NPG binding rates (black circles) measured directly with 32C/245C and 35C/245C, respectively, as described for Figure S1A (trace 1) and Figure S2C (trace 2). Displacement rates (k_{off}) are shown as empty circles. Red solid lines are the best fits to the data using the equation for the two-step binding mechanism with conformational selection. The values of the rate constants were estimated as follows: for 32C/245C, $k_f = 64 \pm 5 \text{ s}^{-1}$, $k_r = 242 \pm 54 \text{ s}^{-1}$, and $k_{\text{on}} = 0.64 \pm 0.12 \mu\text{M}^{-1} \text{ s}^{-1}$, and for 35C/245C, $k_f = 45 \pm 4 \text{ s}^{-1}$, $k_r = 254 \pm 67 \text{ s}^{-1}$, $k_{\text{on}} = 0.70 \pm 0.16 \mu\text{M}^{-1} \text{ s}^{-1}$ using k_{off} values obtained from corresponding displacement experiments (A and B). (E) Schematic representation of the binding mechanism that involves the conformational change preceding binding of sugar to LacY with the cross-linked periplasmic cavity (yellow bar). The conformational transitions are shown as red arrows (k_f and k_r are forward and reverse rate constants, respectively), and sugar binding and release are shown as black arrows (k_{on} and k_{off} are association and dissociation rate constants, respectively).

Figure 7. Rate of closing of the cytoplasmic cavity measured by site-directed Trp-induced quenching of bimane fluorescence. Three LacY mutants with Trp and Cys residues introduced on opposite sides of the cytoplasmic cavity were labeled with bimane and used for direct measurement of the rate of cytoplasmic cavity closing in a DDM solution upon galactoside binding (see Materials and Methods). The cartoon at the top shows the location of the Ca atoms of inserted Trp (pink spheres) and bimane-labeled Cys (green spheres) residues in the inward-facing structure of LacY (gray-colored backbone) with indicated N- and C-terminal six-helix bundles. Stopped-flow traces of the bimane fluorescence decrease were recorded after rapid mixing of protein (0.5–1 μ M) with saturating concentrations of galactosides (blue and gray traces for NPG and TDG, respectively). The observed decrease in fluorescence is the result of closing of the cavity and quenching of bimane by Trp upon sugar binding. Rates of fluorescence change were estimated from single-exponential fits (black lines) as 160–220 s^{-1} for the three mutants with two sugars. (A) NPG (0.2 mM) mixed with PDT-bimane-labeled mutants F140W/V343C (trace 1) and F140W/V331C (trace 2). (B) TDG (3 mM)

mixed with two mutants: PDT-bimane-labeled F140W/V343C (trace 1) and bimanemaleimide-labeled S67C/V343W (trace 2).

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