Supporting Information

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SI Methods

Materials. Oligonucleotides were synthesized by Integrated DNA Technologies, Inc. Restriction enzymes were purchased from New England Biolabs. The QuickChange II kit was purchased from Stratagene. Galactopyranosyl-β-D-galactopyranoside and octyl-α-D-galactoside were obtained from Carbosynth Limited; melibiose, *p*-nitrophenyl-α-D-galactopyranoside, and methyl-α-D-galactoside were from Sigma. Talon superflow resin was purchased from BD Clontech. Dodecyl-β-D-maltopyranoside (DDM) and n-octyl-β-D-glucoside were from Anatrace, and synthetic phospholipids [1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1'-rac-glycerol)] were from Avanti Polar Lipids, Inc. All other materials were of reagent grade obtained from commercial sources.

Construction of Mutants and Purification of Lactose Permease (LacY). Construction of mutants, expression in *Escherichia coli*, and purification of LacY were performed as described (1). All mutants contained a C-terminal 6 His-tag that was used for metal affinity purification on a Talon resin with a typical yield of approximately 0.1 mg protein/g wet cells. Purified proteins (10–15 mg/mL) in 50 mM NaP_i/0.02% DDM (pH 7.5) were frozen in liquid nitrogen and stored at −80 °C until use.

1. Smirnova I, et al. (2007) Sugar binding induces an outward facing conformation of LacY. *Proc Natl Acad Sci USA* 104:16504–16509.

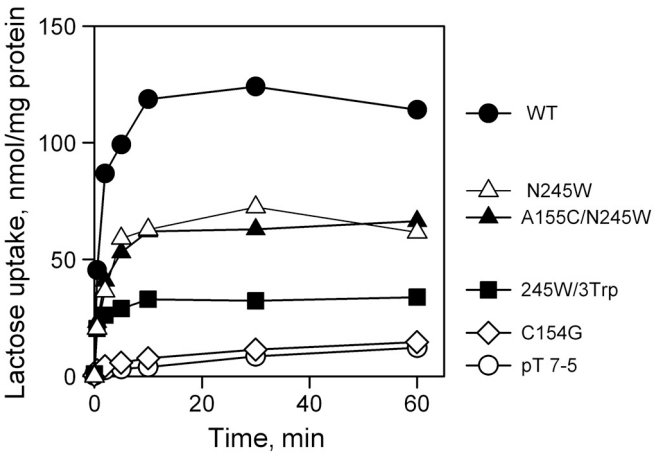

Fig. S1. Lactose transport activity of LacY mutants. Time courses of lactose accumulation by T184 cells expressing wild-type LacY (●), cells transformed with pT7-5 plasmid with no LacY insert (○), and LacY mutants C154G (◇), N245W/3Trp (■), N245W (△); N245W/A155C (▲). *E. coli* T-184 cells (*lacZ*$^-$*Y*$^-$) transformed with each plasmid were grown aerobically at 37 °C in Luria–Bertani broth containing ampicillin (100 μg/mL). Fully grown cultures of *E. coli* T-184 were diluted 10-fold and grown for another 2 h before induction with 1 mM IPTG. After 2 h, cells were harvested, washed with 100 mM KP_i/10 $MgSO_4$ (pH 7.0) and adjusted to an optical density of 25 at 420 nm (approximately 1.75 mg protein/mL). Accumulation of [^{14}C]lactose [1.0 mCi/mmol (1 Ci = 37 GBq)] at indicated time points was measured at room temperature in 50 μL aliquots (82 μg total protein) at final concentration of 0.4 mM lactose by rapid filtration method (1). Radioactivity retained on the filters was assayed by liquid scintillation spectrometry. All mutants were well expressed at levels similar to wild-type LacY as detected by Western blot analysis with anti-His antibodies.

1 Kaback HR (1974) Transport in isolated bacterial membrane vesicles. *Methods Enzymol* 31:698–709.

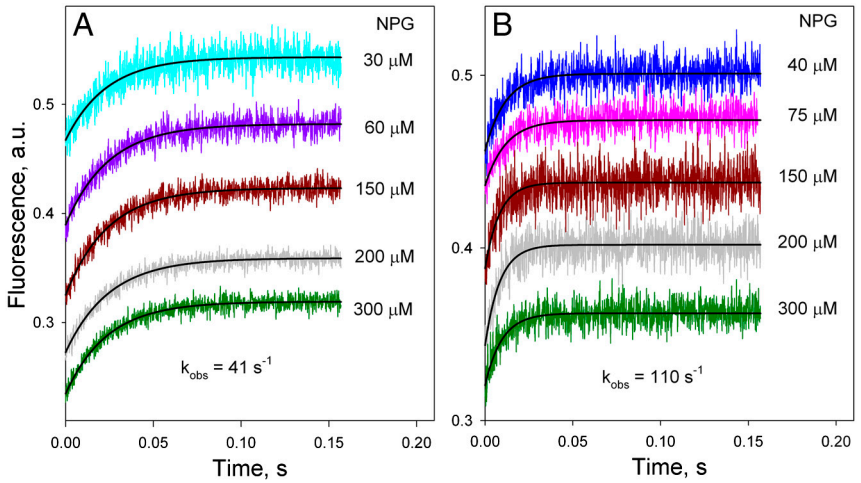

Fig. S2. Stopped-flow traces of WT LacY Trp fluorescence change [Trp151-*p*-nitrophenyl-α-D-galactopyranoside (NPG) FRET] in displacement experiments. Stopped-flow traces were recorded after mixing saturating concentration of galactopyranosyl-β-D-galactopyranoside (15 mM) with LacY preincubated with given concentrations of NPG. Preincubation of NPG with protein in DDM, as well as in reconstituted proteoliposomes, was carried out for 2–3 min prior to the stopped-flow experiments. Traces are fitted with single-exponential equation (black lines) that allows estimation of k_{obs} and the amplitude of fluorescence change at each NPG concentration (see Fig. S3*A*). Data obtained with protein in DDM solution (*A*) or reconstituted into proteoliposomes (*B*) at given NPG concentrations and displacement rates (k_{obs}) of 41 or 110 s^{-1} for DDM or proteoliposomes, respectively.

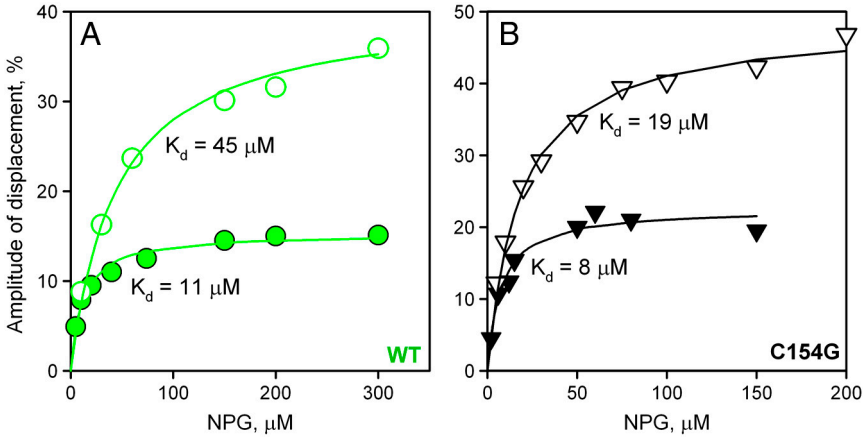

Fig. S3. NPG (*p*-nitrophenyl-α-D-galactopyranoside) binding affinity of LacY in DDM solution or reconstituted into proteoliposomes. Affinities were calculated from concentration dependences of the amplitudes of Trp151-NPG FRET in displacement experiments (stopped-flow traces for WT LacY are presented in Fig. S2). The amplitude of the fluorescence change at each NPG concentration is expressed as a percentage of the final fluorescence level in each experiment and plotted versus NPG concentration. K_d values are estimated from hyperbolic fits to the data (solid lines) and given in the figures. Data for LacY in DDM solution and in proteoliposomes are shown as open and filled symbols, respectively. (*A*) WT LacY (k_{obs} = 41 and 110 s^{-1} for DDM or proteoliposomes, respectively). (*B*) C154G mutant (k_{obs} = 75 and 105 s^{-1} for DDM or proteoliposomes, respectively).

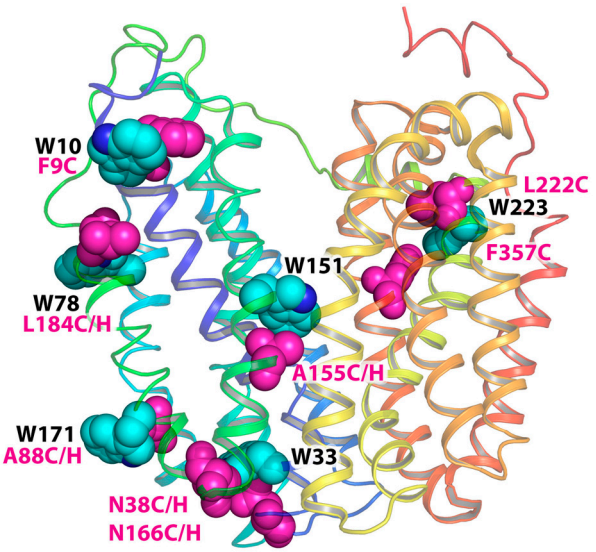

Fig. S4. Six native Trp residues in LacY. Side view of LacY in inward-facing conformation (Protein Data Bank ID 2CFQ) is shown with transmembrane helices rainbow colored from blue (helix I) to red (helix XII), and Trp residues are presented as cyan spheres. Five Trp residues are clustered in N-term six-helix bundle, which is favorable for Trp–Trp energy transfer with Förster radius up to 16 Å (1). Mutants with partial replacements of up to four Trp (W78Y, W171Y, W223Y, and W33Y) were made, and the N245W/3Trp (78, 171, and 223 replaced by Tyr) mutant was used in kinetic experiments. Inner quenchers (Cys or His) of native Trp residues were individually introduced in close proximity to each of six Trp in WT LacY. Amino acid residues replaced with inner quenchers are shown as pink spheres. The properties of individual mutants are presented in Table S1. Six out of 12 mutants tested exhibit a lower Trp emission level than WT LacY. Sugar binding is unchanged in A155C and N38C mutants only. The N245W/A155C LacY mutant was used in given kinetic studies.

1 Lakowicz JR (1999) *Principles of Fluorescence Spectroscopy* (Kluwer Academic/Plenum Publishers, New York), 2nd Ed.

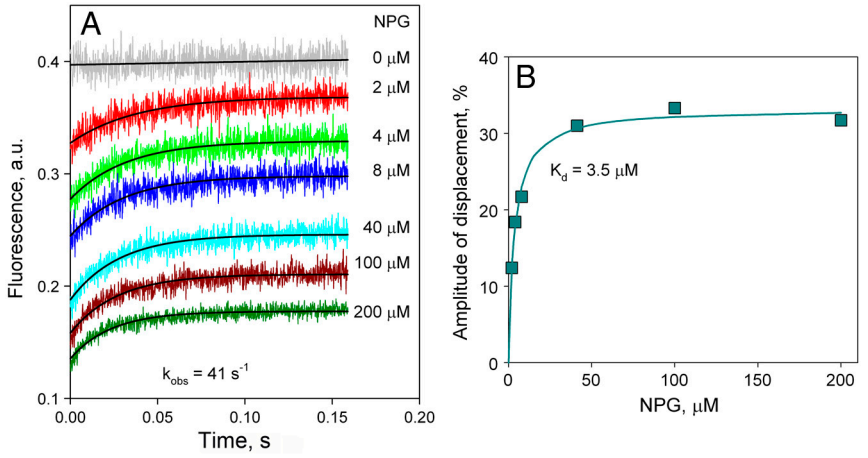

Fig. S5. Sugar binding affinity for N245W/A155C LacY reconstituted into proteoliposomes (lipid-to-protein ratio 5). (*A*) Stopped-flow traces of Trp fluorescence changes in displacement experiments. Traces are recorded after mixing a saturating concentration of galactopyranosyl-β-D-galactopyranoside (15 mM) with LacY preincubated with given concentrations of *p*-nitrophenyl-α-D-galactopyranoside (NPG). Traces are fitted with a single-exponential equation (black lines) that allows estimation of k_{obs} and the amplitude of fluorescence change at each NPG concentration. (*B*) Concentration dependences of the amplitudes of Trp-NPG FRET in displacement experiments from *A*. The amplitude of the fluorescence change at each NPG concentration is expressed as a percentage of final fluorescence level in each experiment and plotted versus NPG concentration. K_d was estimated from hyperbolic fit to the data (solid line).

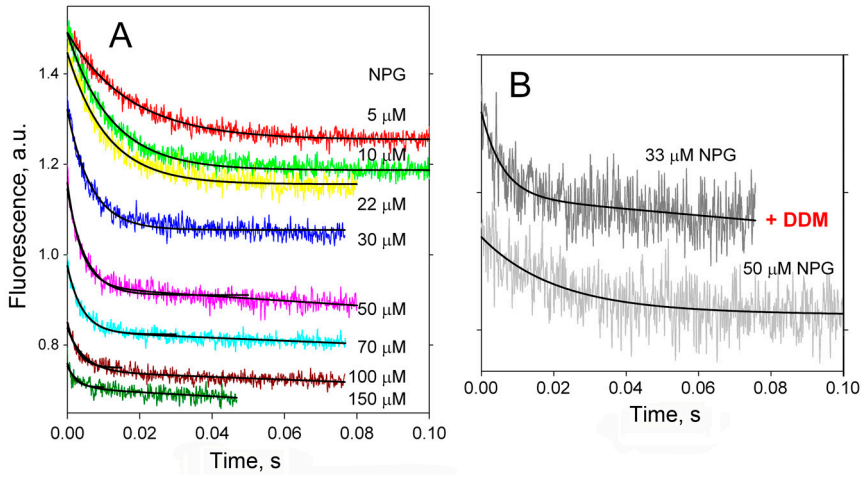

Fig. S6. Stopped-flow rates of *p*-nitrophenyl-α-D-galactopyranoside (NPG) binding to N245W/A155C LacY. (*A*) Stopped-flow traces of Trp fluorescence change were recorded after mixing protein with NPG in 50 mM NaP_i/0.02% DDM (pH 7.5). Final concentrations of sugar are shown with the traces. Single-exponential fits are shown as black lines. (*B*) Stopped-flow traces of NPG binding to the N245W/A155C mutant reconstituted into proteoliposomes (lower trace) and after dissolving the same proteoliposomes in DDM (upper trace). Single-exponential fits are shown as black lines.

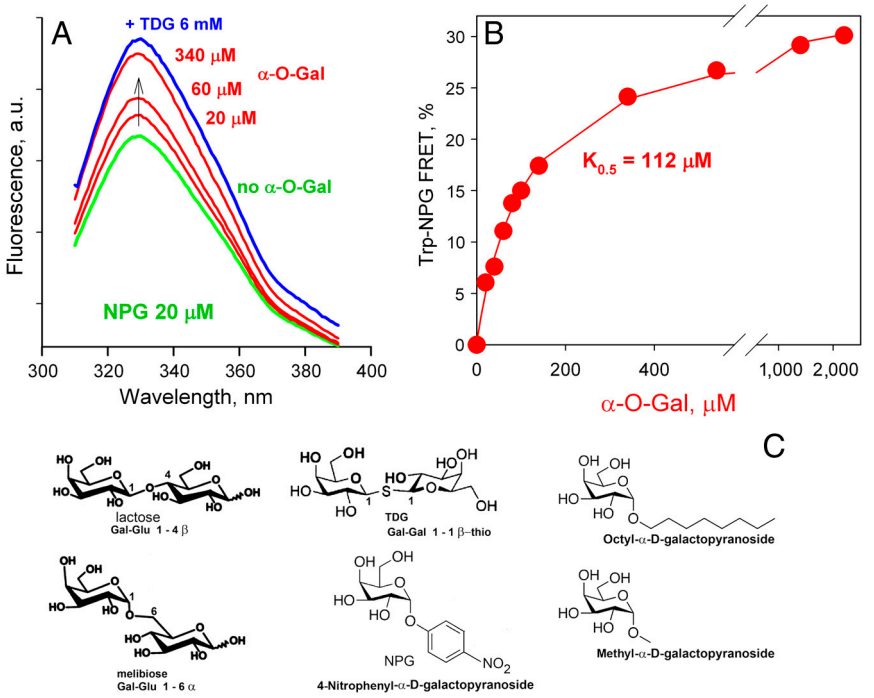

Fig. S7. Galactosidic substrates of LacY. (*A*) Substrate properties of octyl-α-D-galactoside (α-O-Gal). Displacement of bound *p*-nitrophenyl-α-D-galactopyranoside (NPG) with α-O-Gal [Trp151-NPG FRET]. Trp emission spectra of C154G LacY were recorded at 20 μM NPG (estimated K_d for NPG) without additions (green line), and after addition of different concentrations of α-O-Gal (red lines), or 6 mM galactopyranosyl-β-D-galactopyranoside (TDG) (blue line). (*B*) Binding affinity of α-O-Gal. Displacement amplitudes plotted versus α-O-Gal concentrations. The amplitude of the fluorescence increase at each α-O-Gal concentration is expressed as a percentage of the final fluorescence level in each experiment. Hyperbolic fit to the data (solid line) allows calculation of $K_{0.5}$ for displacement as 112 μM, which corresponds to $K_d = 56$ μM for α-O-Gal binding to C154G LacY. (*C*) Structures of galactosidic substrates for LacY used in this study.

Table S1. Properties of LacY mutants with introduced inner quenchers of native Trp residues

Target Trp residue in LacY	Inner quencher	Sugar binding W151-NPG FRET, % of WT	Fluorescence of 0.5 μM LacY, a.u.
W10	F9C	38	2.2
W33	N38H	89	2.9
	N38C	103	2.2
	N166H	49	2.3
	N166C	76	2.6
W78	L184C	27	1.9
W171	A88H	68	2.5
	A88C	154	2.8
W151	A155C	103	2.4
	A155H	157	2.9
W223	L222C	103	2.9
	F357C	127	2.8
WT LacY	no	100	2.8

NPG, *p*-nitrophenyl-α-D-galactopyranoside.