# Supporting Information

## Smirnova et al. 10.1073/pnas.1306849110

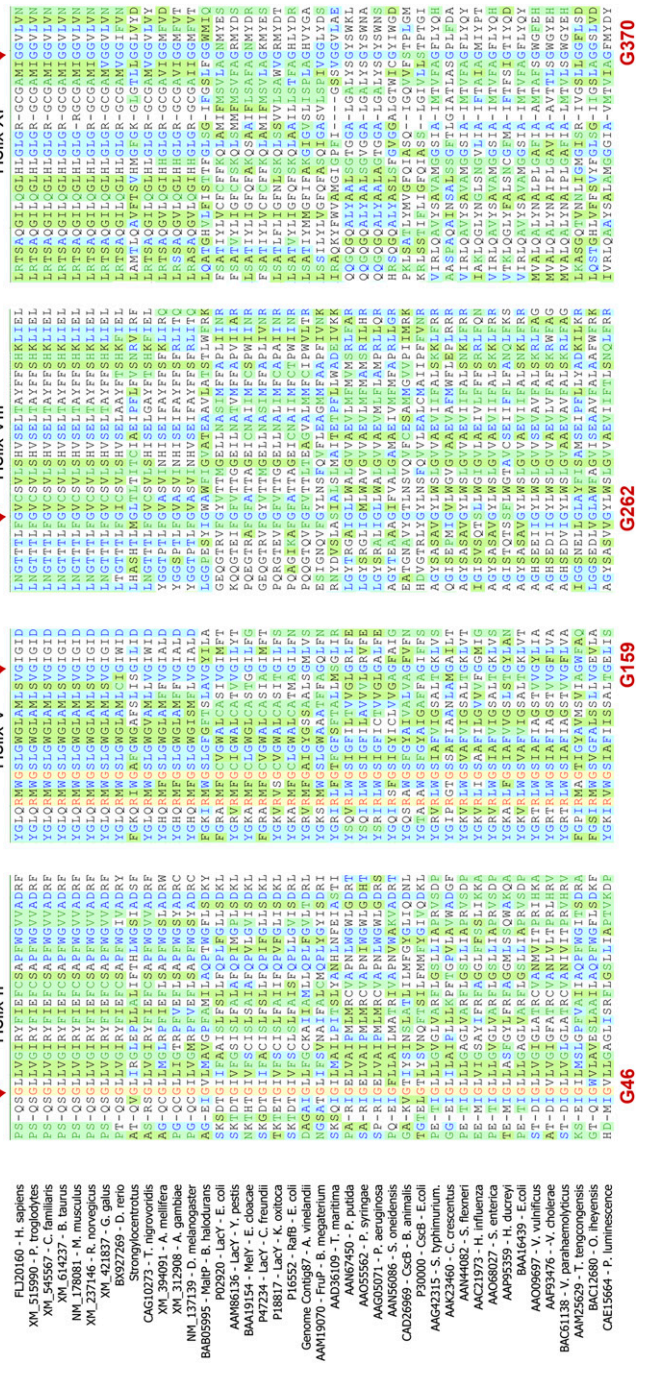


**Fig. S1.** Conservation of Gly residues at the periplasmic ends of transmembrane helices in major facilitator superfamily permeases. Sequences of eukaryotic and prokaryotic sugar transporters similar to lactose permease (LacY) (42 sequences total) were aligned over the entire length (47); only selected helices are presented. Conserved Gly residues located at the periplasmic ends of helices II (Gly46), V (Gly159), VIII (Gly262), and XI (Gly370) in LacY are indicated in red.

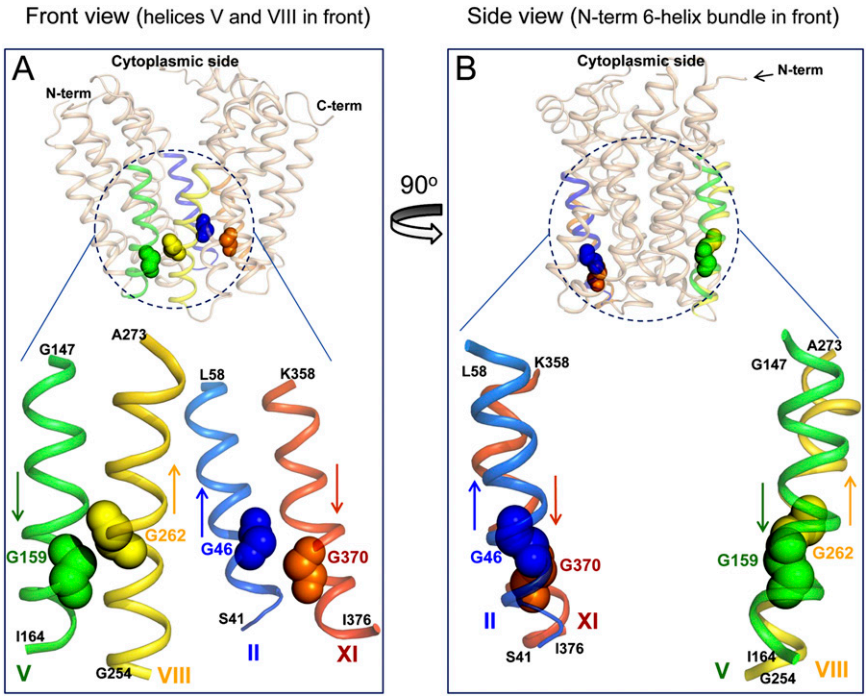


**Fig. S2.** Location of conserved Gly residues at the periplasmic ends of transmembrane helices of LacY (Protein Data Bank ID code 2CFQ). Side view of the backbone structure (*A*) is also shown at 90° rotation around an axis perpendicular to the membrane (*B*). Enlarged segments of interacting antiparallel transmembrane helices are shown in blue (helix II, from S41 to L58), orange (helix XI, from K358 to I376), green (helix V, from G147 to I164), and yellow (helix VIII from G254 to A273). Essential Gly residues are shown as spheres. Arrows indicate the directions of α-helical sequences.

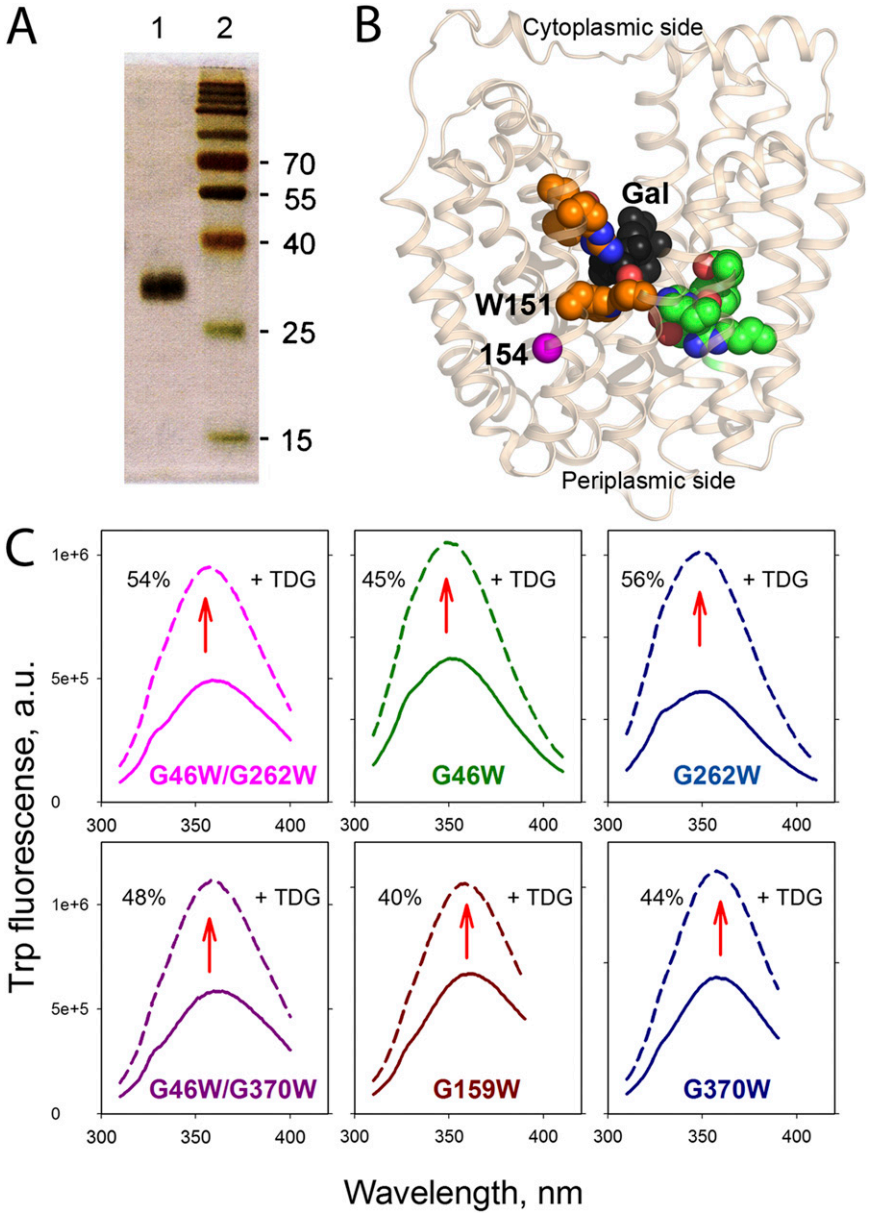


**Fig. S3.** Sugar binding to purified Gly→Trp mutants. (*A*) Silver-stained SDS/PAGE of double-mutant G46W/G262W after purification on Talon resin (lane 1, 0.4 μg of purified protein; lane 2, molecular weight markers). (*B*) Side view of LacY structure (Protein Data Bank ID code 1PV7). Bound galactoside is shown as black spheres. Amino acid residues involved in sugar (Trp151, Glu126, Arg144, Glu269) and $H^+$ binding (Asp240, Tyr236, Arg302, Lys319, His322, Glu325) are shown as orange and green spheres, respectively, with N and O atoms colored blue and red. The Cα atom at position 154 is shown as a magenta sphere. (*C*) Galactoside binding to six mutants containing indicated single or double Gly→Trp replacements. Trp→4-nitrophenyl-α-D-galactopyranoside (NPG) fluorescence resonance energy transfer (FRET) was measured with each mutant in 50 mM $NaP_i$/0.02% dodecyl-β-D-maltopyranoside (DDM) (pH 7.5) by displacement of bound NPG (0.2 mM) with excess of β-D-galactosyl-1-thio-β-D-galactopyranoside (TDG, 12 mM). Trp emission spectra were recorded at excitation wavelength 295 nm with 0.5 μM protein mixed with NPG before and after addition of TDG (solid and broken lines, respectively). The relative increase in Trp fluorescence is expressed as % of final fluorescence level at 360 nm and shown in each panel.

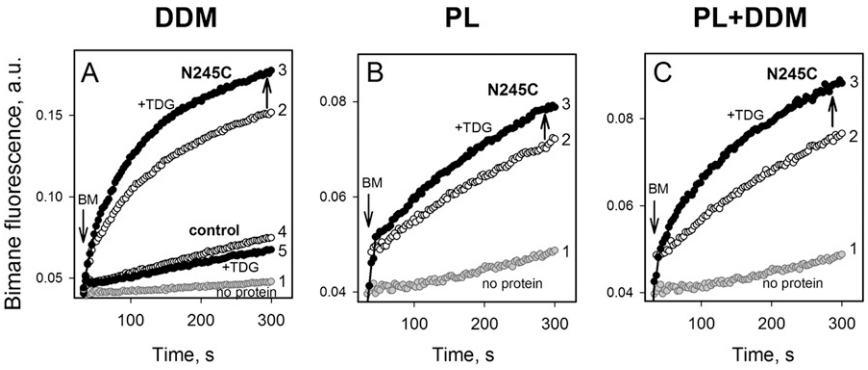


**Fig. S4.** Alkylation of a Cys residue introduced in place of Asn245 in the periplasmic pathway of mutant G370W. The highly reactive/accessible native Cys148 in the mutant was replaced with Met. Addition of 1 μM BM (at 30 s) is indicated by an inverted arrow. Traces were recorded as a function of time at excitation and emission wavelengths of 380 and 465 nm, respectively, with 0.5 μM of protein solubilized in DDM (*A*), reconstituted into proteoliposomes (*B*), or with the same proteoliposomes dissolved in DDM (*C*). In each panel, trace 1 was recorded with no protein added, and traces 2 and 3 represent labeling of Cys245 in the absence of sugar or after addition of 6 mM TDG, respectively. Labeling of a control protein with no introduced Cys (G370W LacY) is shown on left panel (traces 4 and 5 indicate labeling in the absence or presence of TDG, respectively).

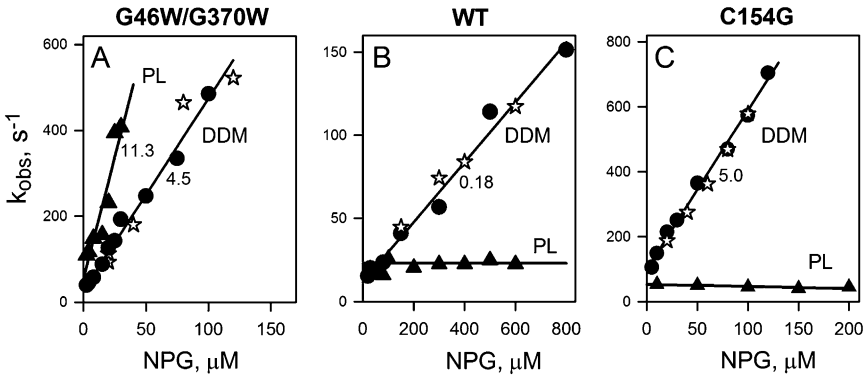


**Fig. S5.** Kinetics of sugar binding to G46W/G370W LacY. Stopped-flow data for mutant G46W/G370W (*A*) are compared with previous findings from similar experiments (1) with WT (*B*) and C154G mutant (*C*). Rates of NPG binding estimated from stopped-flow experiments (as shown in Fig. 5 *A* and *B*) are plotted vs. NPG concentration and fitted with a linear equation (as shown in Fig. 5*C*) for protein in DDM (●), reconstituted into proteoliposomes (PL; ▲), and for the same proteoliposomes dissolved in DDM (☆). Linear fits to the data are shown as black lines, with $k_{on}$ values given near the lines. Kinetic parameters for NPG binding to mutant G46W/G370W in DDM and in proteoliposomes (data in parenthesis) are: $k_{off}$ = 27 (54) $s^{-1}$, $k_{on}$ = 4.5 (11.3) $\mu M^{-1} \cdot s^{-1}$, and $K_d$ = 6.0 (4.8) μM. WT LacY and C154G mutants reconstituted into proteoliposomes bind NPG at rates that are independent of NPG concentration ($k_{obs}$ = 20 and 50 $s^{-1}$, respectively).

1. Smirnova I, Kasho V, Sugihara J, Kaback HR (2011) Opening the periplasmic cavity in lactose permease is the limiting step for sugar binding. *Proc Natl Acad Sci USA* 108(37): 15147–15151.

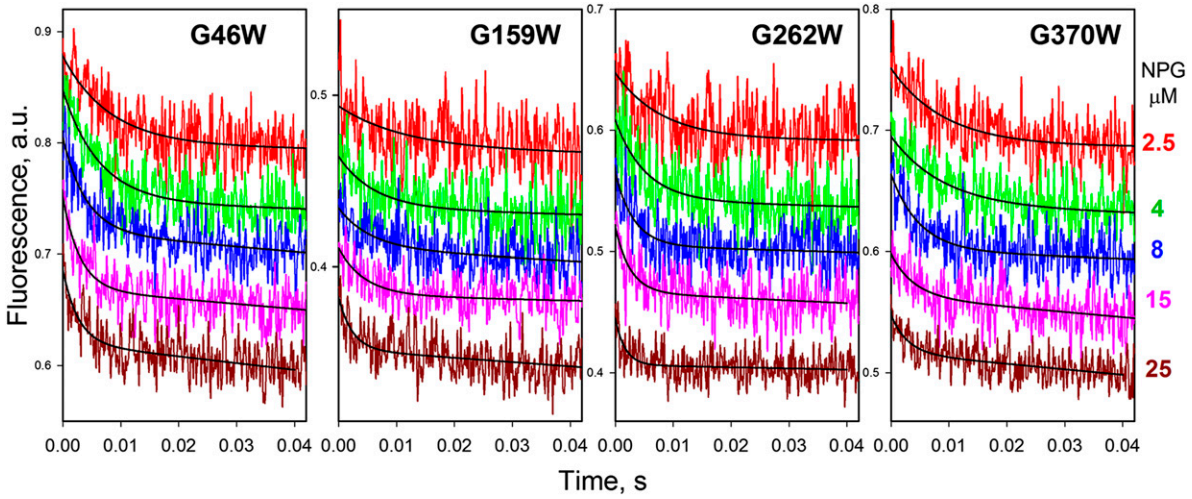


**Fig. S6.** NPG binding to reconstituted LacY mutants with single G→W replacements. Stopped-flow traces of Trp fluorescence change (excitation and emission wavelengths, 295 and 340 nm, respectively) were recorded after mixing of NPG (at final concentrations indicated), with each purified mutant (0.5 μM) reconstituted into proteoliposomes. Sugar binding rates ($k_{obs}$) were estimated from single exponential fits (black lines) and plotted vs. NPG concentrations (shown as triangles in Fig. 6).

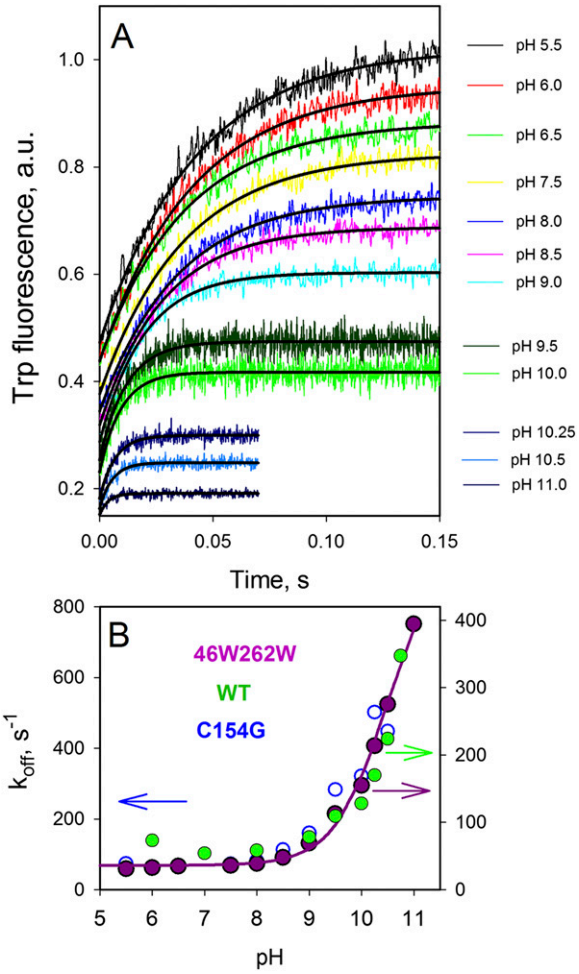


**Fig. S7.** Effect of pH on NPG dissociation rate ($k_{off}$) for mutant G46W/G262W. (*A*) Stopped-flow traces showing the increase in Trp fluorescence after displacement of bound NPG by excess TDG (Trp→NPG FRET) at given pH values. Protein preincubated with NPG was mixed with TDG, and the fluorescence change was recorded at excitation and emission wavelengths 295 and 340, respectively. Final concentrations were protein, 0.8–1.6 μM; NPG, 0.2–0.4 mM; and TDG, 30–60 mM. Buffers (50 mM) containing 0.02% DDM were citrate/$NaP_i$ (pH 5.5–6.5); $NaP_i$ (pH 7.5–8.0); *N*-(1,1-dimethyl-2-hydroxyethyl)-3-amino-2-hydroxypropanesulfonic acid/NaOH (pH 8.0–9.5); and 3-(cyclohexylamino)-1-propanesulfonic acid/NaOH (pH 9.5–11.0). Single exponential fits are shown as solid black lines. Amplitudes of the fluorescence changes (expressed as percentage of final level) decreased from 54% at pH 5.5 to 20% at pH 11.0. Displacement rates ($k_{off}$) estimated from (*A*) are plotted as a function of pH (*B*) in comparison with previous data for WT LacY (green circles) and mutant C154G (open circles) (1). Vertical axis are as follows (arrows): right axis, G46W/G262W, purple, and WT LacY, green; left axis, C154G, blue. The solid line represents a sigmoidal fit (SigmaPlot 10.0) with an estimated $pK_a$ of ~10.5.

1. Smirnova IN, Kasho VN, Sugihara J, Choe JY, Kaback HR (2009) Residues in the $H^+$ translocation site define the $pK_a$ for sugar binding to LacY. *Biochemistry* 48(37):8852–8860.