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Outward-facing conformers of LacY stabilized by nanobodies

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The lactose permease of Escherichia coli (LacY), a highly dynamic polytopic membrane protein, catalyzes stoichiometric galactoside/H+ symport by an alternating access mechanism and exhibits multiple conformations, the distribution of which is altered by sugar binding. We have developed single-domain camelid nanobodies (Nbs) against a LacY mutant in an outward (periplasmic)-open conformation to stabilize this state of the WT protein. Twelve purified Nbs inhibit lactose transport in right-side-out membrane vesicles, indicating that the Nbs recognize epitopes on the periplasmic side of LacY. Stopped-flow kinetics of sugar binding by WT LacY in detergent micelles or reconstituted into proteoliposomes reveals dramatic increases in galactoside-binding rates induced by interaction with the Nbs. Thus, WT LacY in complex with the great majority of the Nbs exhibits variations in association rates in a sugar dependence of the binding site with an increase in association rate constants ($k_{on}$) of up to ~50-fold (reaching $10^7$ M$^{-1}$s$^{-1}$). In contrast, with the double-Trp mutant, which is already open on the periplasmic side, the Nbs have little effect. The findings are clearly consistent with stabilization of WT conformers with an open periplasmic cavity. Remarkably, some Nbs drastically decrease the rate of dissociation of bound sugar leading to increased affinity (greater than 200-fold for lactose).

Typical of many transport proteins, from organisms as widely separated evolutionarily as Archaea and Homo sapiens, the lactose permease of Escherichia coli (LacY), a paradigm for the Major Facilitator Superfamily (1), catalyzes the coupled, stoichiometric translocation of a galactopyranoside and an H+ (galactoside/H+ symport) across the cytoplasmic membrane (reviewed in refs. 2 and 3). Although it is now generally accepted that membrane transport proteins operate by an alternating access mechanism, this has been documented almost exclusively for LacY (reviewed in refs. 4 and 5). In contrast, with the double-Trp mutant, which is already open on the periplasmic side, the Nbs have little effect. The findings are clearly consistent with stabilization of WT conformers with an open periplasmic cavity. Remarkably, some Nbs drastically decrease the rate of dissociation of bound sugar leading to increased affinity (greater than 200-fold for lactose).

Significance

LacY, a paradigm for the major facilitator superfamily (the largest family of transport proteins) catalyzes the coupled symport of a galactoside and an H+. Although a detailed mechanism has been postulated, to test its veracity stable conformers of different intermediates would be particularly informative. Camelid single-domain nanobodies (Nbs), which stabilize specific conformers (23–27), Advantages of Nbs include small size and a unique structure that allows flexible antigen-binding loops to insert into clefts and cavities. Here we report that Nbs prepared against the outward (periplasmic)-open LacY mutant G46W/G262W effectively bind to WT LacY and inactive transport activity. However, the sugar-binding site becomes much more accessible to galactosides as a result of Nb binding, indicating stabilization of the open-outward conformations of LacY, and providing the means for detailed studies of galactoside binding to these conformers. Remarkably, several Nbs significantly increase affinity for galactosides by slowing the dissociation rate of the sugar while maintaining a high association rate. It is also apparent that the


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Nbs have the potential for crystallizing LacY trapped as otherwise unstable transient intermediates.

Results

Generation of Nbs. To generate Nbs that recognize and stabilize outward-open conformations of LacY, llamas were immunized (28) with LacY mutant G46W/G262W (20) reconstituted into PLs as the antigen. In this mutant, double-Trp replacements for Gly46 (helix II) and Gly262 (helix VIII) were introduced on the periplasmic side of LacY at positions where the two six-helix bundles come into close contact. Introduction of bulky Trp residues at these positions prevents closure of the periplasmic cavity and completely abrogates all transport activity. The double-Trp mutant reconstituted into PLs is oriented physiologically, with the periplasmic side facing the external medium (20), as demonstrated previously (18, 29). Thus, it is presumed that the llama’s immune system is presented with an antigen that has an accessible periplasmic surface of LacY with an open cavity. Selections were performed on the LacY mutant to find those nanobodies that would specifically recognize the outward-open conformation, as well as on WT LacY. Procedures used for production, selection, cloning, and purification of Nbs are provided in Methods.

Lactose Transport. Lactose/H+ symport catalyzed by WT LacY was measured in RSO membrane vesicles preincubated with each of 13 nanobodies, and the data are summarized in Table 1 and Fig. S1. Nb 9051 has no significant effect on the rate of lactose transport, but Nb 9042, Nb 9035, and Nb 9034 inhibit by 60%, 80%, and 90%, respectively, and other nine Nbs block lactose transport completely. Because it is well known that residues at these positions prevents closure of the periplasmic cavity and completely abrogates all transport activity. The double-Trp mutant reconstituted into PLs is oriented physiologically, with the periplasmic side facing the external medium (20), as demonstrated previously (18, 29). Thus, it is presumed that the llama’s immune system is presented with an antigen that has an accessible periplasmic surface of LacY with an open cavity. Selections were performed on the LacY mutant to find those nanobodies that would specifically recognize the outward-open conformation, as well as on WT LacY. Procedures used for production, selection, cloning, and purification of Nbs are provided in Methods.

Sugar Binding to Nb/LacY Complexes. Sugar binding rates were measured by Trp151→NPG FRET with WT LacY or the double-Trp mutant solubilized in n-dodecylβ-D-maltopyranoside (DDM) by using stopped-flow fluorimetry, which allows determination of association and dissociation rate constants ($k_{on}$ and $k_{off}$) of sugar binding. WT LacY exhibits a $k_{on}$ of 0.2 μM$^{-1}$s$^{-1}$, whereas $k_{on}$ for the double-Trp mutant is 5.7 μM$^{-1}$s$^{-1}$ (compare open circles in Fig. L4 with open diamonds in Fig. 1B), indicating much higher accessibility of the sugar-binding site in mutant G46W/G262W with an open periplasmic cavity. None of Nbs tested abolish sugar binding to LacY (Table 1). Two Nbs (9051 and 9035) practically do not affect sugar binding ($k_{on}$ and $k_{off}$ values are similar to those measured for WT LacY without Nbs). Interaction of Nb 9042 and Nb 9034 with WT LacY results in sugar binding with rates independent of NPG concentration ($k_{on}$s = 30 and 15 s$^{-1}$, respectively), suggesting that these two nanobodies do not alter galactoside binding. Rather, they may decrease conformational flexibility of LacY in such a manner that sugar access to the binding site is limited by a slow conformational change or slow opening of the periplasmic cavity, which could explain partial inhibition of transport.

Nine Nb/Wt LacY complexes that completely block transport, demonstrate a significant increase of NPG binding rates ($k_{on}$ increases from 5- to 50-fold) (Fig. 1A and Table 1). Dramatic increases in NPG accessibility are observed for WT LacY complexed with Nbs 9039, 9048, 9047, 9033, and 9065 to an extent comparable to that of mutant G46W/G262W (Table 1) ($k_{on}$s = 4.4, 6.8, 6.9, 7.5, and 9.3 μM$^{-1}$s$^{-1}$, respectively). Several Nbs exhibit a smaller effect on the rates of sugar binding by WT LacY, with $k_{on}$ values of 1.0, 1.2, 3.5, and 3.5 μM$^{-1}$s$^{-1}$ for Nbs 9036, 9055, 9063, and 9043, respectively (Fig. 1A and Table 1). Notably, the double-Trp mutant in complex with Nbs 9036, 9063, and 9043 is characterized by lower $k_{on}$ values than observed without Nbs, whereas all other Nbs have essentially no effect (Fig. 1B and Table 1). Kinetic parameters measured by displacement of bound NPG using a high concentration of β-D-galactopyranosyl-1-thio-β-D-galactopyranoside (TDG) show that the majority of the Nbs, which block transport, significantly increase the affinity of WT LacY for NPG ($K_{d}$s decrease up to 10 times), whereas $K_{ds}$ are mostly unaltered with the double-Trp mutant (Table 1, shaded columns). Surprisingly, similar effects of Nb 9036 are observed with both WT LacY, and mutant G46W/G262W.
The binding of Nb 9065 to reconstituted WT LacY dramatically increases NPG binding rates, but no significant change is observed with the reconstituted double-Trp mutant (Fig. 2C). Linear fits of the data yield an estimated $k_{on}$ of $\sim 20 \mu M^{-1} s^{-1}$ for both WT LacY and mutant G46W/G262W complexed with Nb 9065. Therefore, Nb 9065 binds to an epitope on reconstituted WT protein that is exposed to the external milieu, provides free access of NPG to the binding site, and blocks transport, thereby demonstrating clearly that Nb 9065 stabilizes an outward-facing conformer of WT LacY. Similar effects of Nb 9039 and 9047 on reconstituted WT LacY and of Nbs 9043, 9047 and 9065 on reconstituted mutant C154G are shown in Fig. S4.

**Nb 9036 Induces High-Affinity Galactoside Binding.** A striking effect of Nb 9036 on sugar binding is observed with both WT LacY and the double-Trp mutant. True $k_{off}$ values for NPG determined in displacement experiments decrease in the presence of Nb 9036 by about three orders-of-magnitude from 41 to 0.05 s$^{-1}$ and from 31 to 0.02 s$^{-1}$ for the WT and mutant, respectively (Table 1). With the WT LacY/Nb 9036 complex, NPG binding rates increase (Fig. S5A), demonstrating greater accessibility of the sugar-binding site ($k_{on}$ increases fivefold) (Fig. 1A and Table 1). Displacement rates are greatly decreased by Nb 9036 binding to WT LacY (Fig. S5B), resulting in a $>500$-fold increase in NPG affinity. A similar effect of Nb 9036 is observed with mutant G46W/G262W, although both $k_{on}$ and $k_{off}$ values are decreased (Table 1). Therefore, it appears that Nb 9036 binding stabilizes a specific outward-facing conformation of LacY in which the periplasmic cavity is partially open, but release of bound NPG is drastically hindered.

This effect of Nb 9036 allows characterization of the kinetic properties of lactose binding, the physiological substrate of LacY. The affinity of LacY for lactose in the absence of Nbs is extremely low with a $K_{d}$ of $\sim 10$ mM ($35, 36$). The rate of lactose displacement was measured by Trp151$\rightarrow$NPG FRET, where independent of sugar concentration, thereby indicating that binding is limited by opening of the periplasmic cavity (Fig. S3F). However, in displacement experiments with reconstituted WT LacY, opening of periplasmic cavity provides free access to binding site with a $k_{on}$ of $10 \mu M^{-1} s^{-1}$ (Fig. S3I), as shown for mutant C154G.

The accessibility of the sugar-binding site is increased by orders of magnitude, which will be discussed in detail below.

**Accessibility of the Sugar-Binding Site.** Remarkable changes in sugar-binding rates are induced by interaction of Nb 9065 with WT LacY (Fig. 2A and Table 1). As estimated from the linear concentration dependence of binding rates, $k_{on}$ increases from 0.2 to 9.3 $\mu M^{-1} s^{-1}$ (Fig. 2B), indicating free access to the sugar-binding site. Moreover, NPG binding rates are the same when the LacY/Nb binding complex is formed in the absence or presence of sugar (Fig. 2B, red triangles). WT LacY binding affinity for NPG is significantly increased by interaction with Nb 9065 (Table 1). The $K_{d}$ value measured in displacement experiments decreases from 28 to 5.3 $\mu M$ (Fig. S2 A, C, and E). Nb 9065 does not markedly alter NPG-binding kinetics with the G46W/G262W mutant (Fig. 2B, Table 1, and Fig. S2 B, D, and F).

Experiments with LacY solubilized in DDM do not specify whether NPG binding stabilizes conformers with an open periplasmic or cytoplasmic cavity. However, LacY reconstituted into PLs is oriented with the periplasmic side facing out, as in the native E. coli membrane (18, 20, 29). Therefore, a kinetic test was designed that allows discrimination between accessibility from the periplasmic or cytoplasmic sides of LacY by comparing sugar-binding rates with LacY solubilized in DDM versus reconstituted into PLs (Fig. S3). Mutants G46W/G262W or C154G with an open periplasmic or cytoplasmic cavity, respectively, are characterized by rapid sugar binding in DDM (Fig. S3 A and D) ($k_{on} = 5 \mu M^{-1} s^{-1}$). However, in PLs, sugar binding by mutant G46W/G262W is rapid and demonstrates a sharp concentration dependence of $k_{obs}$ (with $k_{on} = 14 \mu M^{-1} s^{-1}$), whereas mutant C154G exhibits a relatively slow rate of sugar binding that is independent of galactoside concentration ($k_{on} = 50 s^{-1}$) (Fig. S3 B and E). Thus, NPG has free access to the binding site from the periplasmic side in the double-Trp mutant, but limited access in mutant C154G, where the rate of opening of the periplasmic cavity is limited. However, $k_{on}$ determined by displacement with reconstituted mutant C154G in PLs (Fig. S3F) ($k_{on} = 14 \mu M^{-1} s^{-1}$) is even higher than in DDM ($k_{on} = 4.9 \mu M^{-1} s^{-1}$). Thus, when the periplasmic cavity is open, the sugar binds with a diffusion-controlled rate.

Binding of NPG by WT LacY in DDM is characterized by $k_{on} = 0.2 \mu M^{-1} s^{-1}$ and consistent with reduced access to the sugar binding site (Fig. S3G). NPG binding by WT LacY reconstituted into PLs is slow ($k_{obs} = 21 s^{-1}$), and the rate is independent of sugar concentration, thereby indicating that binding is limited by opening of the periplasmic cavity (Fig. S3F). However, in displacement experiments with reconstituted WT LacY, opening of periplasmic cavity provides free access to binding site with a $k_{on}$ of $10 \mu M^{-1} s^{-1}$ (Fig. S3I), as shown for mutant C154G.

![Fig. 1. Effect of six Nbs on kinetics of sugar binding by WT LacY (A) and mutant G46W/G262W (B) solubilized in DDM. Stopped-flow rates of NPG binding ($k_{obs}$) were measured by mixing LacY with NPG in the absence or presence of a given Nb. Stopped-flow traces of the decrease in Trp fluorescence were recorded and fitted with single-exponential equation for estimation of the sugar-binding rate ($k_{obs}$) at each NPG concentration.](https://www.pnas.org/doi/10.1073/pnas.1422265112)

![Fig. 2. Effect of Nb 9065 on accessibility of the sugar-binding site. NPG binding rates were measured directly by stopped-flow as Trp151$\rightarrow$NPG FRET with WT LacY and G46W/G262W mutant in the absence of Nbs (black lines) or after preincubation with Nb 9065 (red lines). (A) Stopped-flow traces of Trp emission decrease were recorded with WT LacY in DDM after mixing with given concentrations of NPG. (B) Concentration dependencies of sugar binding rates measured in DDM with WT LacY (open circles and red triangles) or mutant (open diamonds and red squares). WT LacY preincubated with Nb 9065 in the absence or presence of sugar (red triangles pointed down or up, respectively) exhibits the same NPG binding rates. Estimated $k_{on}$ values are presented in Table 1 in columns labeled “Binding.” With the Nb 9065 complex in gel filtration, binding rate is increased in $K_{d}$ (from 0.20 ± 0.01 to 9.3 ± 0.2 $\mu M^{-1} s^{-1}$). (C) Concentration dependencies of sugar binding rates measured with WT LacY (gray circles or red triangles) and mutant (gray diamonds and red squares) reconstituted into PLs. The red arrows indicate the change in concentration dependence of sugar binding rates after Nb 9065 binding to WT LacY.](https://www.pnas.org/doi/10.1073/pnas.1422265112)
a saturating concentration of NPG (0.2 mM) was mixed with WT LacY/Nb 9036 complex preincubated with given concentrations of lactose (Fig. 3A). The stopped-flow traces demonstrate that NPG binding occurs upon release of lactose at constant rate ($k_{off} = 1.8 \times 10^{-3}$ s$^{-1}$). As estimated from the concentration dependence of the amplitudes of the fluorescence change (Fig. 3B), the $K_d$ for lactose is 42 μM. The double-Trp mutant complexed with Nb 9036 yields a similar $K_d$ of 49 μM (Fig. 3B), suggesting that Nb 9036 stabilizes similar conformers of both proteins.

**Nbs Binding.** Homology modeling of the 3D structures of each Nbs described reveals Trp residues in the variable loops containing the complementarity determining regions (CDRs) that define the binding affinity of the Nbs (Fig. 4A). Therefore, interaction of the Nbs with LacY was studied by site-directed Trp-induced fluorescence quenching of bimane- or ATTO655-labeled LacY (19, 37, 38). WT LacY with a Cys replacement on the periplasmic side (I32C) labeled with bimane or ATTO655 exhibits a decrease in the fluorescence emission of either fluorophore upon addition of Nbs (Fig. S6). Time-courses of the fluorescence changes recorded with bimane-labeled (Fig. 4B) or ATTO655-labeled (Fig. 4C) mutant I32C LacY demonstrate various extents of fluorescence quenching after addition of Nbs 9036, 9055, and 9063, which likely reflect different distances between the Trp residues in the Nbs and the fluorophores in LacY when the Nb binds.

Stopped-flow mixing of various concentrations of Nb with 0.4 μM bimane-labeled LacY (Fig. S7) exhibits increased rates of binding with increasing Nb concentration. No change in the amplitude of the fluorescence decrease is observed even at lowest Nb concentrations (0.5–1 μM), which indicates that the affinity of Nbs for LacY is high with $K_d$ values at least in the nanomolar range. Linear concentration dependencies of Nbs binding rates (Fig. 5) yield estimated $k_{on}$ values that vary from 0.2 to 3.5 μM$^{-1}$s$^{-1}$, and extremely low $k_{off}$ values for all five Nbs. In addition, the binding rates of Nb 9036 to bimane-labeled I32C LacY are identical in the absence or presence of 5 mM TDG ($k_{on} = 0.4 \mu$M$^{-1}$s$^{-1}$), indicating that Nb recognizes the same LacY conformer with or without bound sugar.

When the Cys replacement is introduced on the cytoplasmic side of WT LacY (S401C), no significant Trp-induced fluorescence quenching is observed with bimane- or ATTO655-labeled LacY upon Nb binding (Fig. S8 A–C), although the effect of Nb 9036 on LacY binding affinity of Nb 9036, Nb 9055, or Nb 9063 was measured by steady-state titration of bimane- or ATTO655-labeled I32C LacY at low protein concentration (20 nM). Estimated $K_d$ values for all three Nbs are around 1 nM and do not depend on the structure of fluorophore attached to LacY (Fig. S9). The presence of sugar practically does not change Nb binding affinity. Measured $k_{on}$ (Fig. 5) and $K_d$ values allow calculation of $k_{off}$ as $1.2 \times 10^{-3}$, $0.4 \times 10^{-3}$, and $0.3 \times 10^{-3}$ s$^{-1}$ for dissociation of Nbs 9063, 9055, and 9036, respectively.

**Demonstration That Nb Binding Stabilizes a Conformer with an Open Periplasmic Cavity.** Trp-induced bimane unquenching allows direct demonstration of opening of periplasmic cavity in LacY (19). Thus, bimane-labeled mutant F29W/G262C exhibits unquenching of bimane fluorescence after addition of sugar, indicating opening of the periplasmic cavity and even greater unquenching is observed after addition of Nb 9036 (Fig. 6A). The increased extent of bimane fluorescence unquenching caused by Nb binding compared with effect of TDG is most likely explained by stabilization of a specific outward-open conformation of LacY, whereas sugar binding results in dynamic equilibrium of several LacY conformers including those with an open periplasmic cavity (6).

Furthermore, the rates of unquenching measured with bimane-labeled F29W/G262C at increasing concentrations of Nb 9036 exhibit a linear dependence with $k_{on} = 0.4 \mu$M$^{-1}$s$^{-1}$ (Fig. 6B). This $k_{on}$ value is identical to that measured by direct binding studies with Nb 9036 by using Trp-induced quenching of bimane-labeled I32C LacY (Fig. 5, pink circles), thereby demonstrating that binding of Nb 9036 stabilizes a conformer with an open periplasmic cavity.
the Nbs bind to epitopes on the periplasmic side of LacY. Moreover, presteady-state kinetics of Nb binding to LacY were measured by stopped-flow. The linear concentration dependencies of binding rates reveal significant variations in $k_{on}$ values for five tested Nbs (from 0.2 to 3.5 $\mu$M$^{-1}$s$^{-1}$) and exceedingly low $k_{off}$ values. Multiple $k_{on}$ values most likely correspond to interaction of the Nbs with different epitopes on periplasmic side of LacY that vary in complexity and structure. Binding affinities measured by steady-state titration are very high ($K_d$ values are around 1 nM for Nbs 9036, 9055, and 9063), which explains extremely slow dissociation rates of the Nbs. Thus, calculated $k_{off}$ values range from $3 \times 10^{-3}$ to $1.2 \times 10^{-3}$ s$^{-1}$, which is similar to published data for highly specific Nbs-antigen interactions (25).

Recognition of different epitopes in WT LacY by the Nbs results in stabilization of several conformational states of the symporter. These states may represent natural functional intermediates in overall transport cycle, as the Nbs do not interfere with sugar binding and therefore with protonation, because effective sugar binding requires the protonated form of LacY (39). Moreover, in vivo-matured Nbs do not apparently induce nonnative conformations of antigens (28). Thus, Nbs developed against the outward-open LacY mutant may be useful for crystallization of WT LacY in different conformations without the use of mutagenesis.

Discussion

Nbs represent a unique type of single-domain antibodies with flexible antigen-binding loops containing CDR3, which is able to insert into clefts and cavities of membrane proteins and stabilize specific conformers (23–27). Therefore, Nbs were prepared against LacY mutant G46W/G262W, which is in an outward-open conformation, anticipating that such Nbs would interact with epitopes within the open periplasmic cavity to stabilize outward-facing conformers of WT LacY. As shown, 12 of the 13 Nbs characterized inhibit—and 9 totally block—lactose transport catalyzed by WT LacY in RSO membrane vesicles, indicating that they bind to periplasmic epitopes. However, sugar binding is not abolished. Rather, each of the nine Nbs significantly increases the rate of sugar binding with WT LacY solubilized in DDM, indicating that the sugar-binding site in the middle of the LacY molecule becomes much more accessible to the external medium in the presence of the Nbs. Even more impressive, WT LacY and C154G mutant reconstituted into PLs and then exposed to Nbs 9039, 9043, 9047, and 9065 exhibit virtually unrestricted sugar binding rates with high $k_{on}$ values corresponding to stabilization of conformers with an open periplasmic cavity. It is also remarkable that with few exceptions (Nbs 9036, 9063, and 9043), the Nbs have little or no effect on sugar-binding rates with the double-Trp mutant presumably because the mutant is already open on the periplasmic side.

Although Nb binding to WT LacY generally increases accessibility of the binding site to NPG, the $k_{on}$ values vary from 1 to 9 $\mu$M$^{-1}$s$^{-1}$ for different WT LacY/Nb complexes. Thus, the Nbs appear to recognize different epitopes and stabilize different outward-open conformers of LacY that may represent natural intermediates in the transport cycle.

Remarkably, three of the Nbs (9036, 9063, and 9043) significantly decrease $k_{off}$ values measured for NPG with WT LacY/Nb complexes, and dissociation of sugar is slowed nearly 1,000-fold by Nb 9036 (Table 1), resulting in markedly increased affinity for galactosides. Thus, the $K_d$ value of the Nb 9036/WT LacY complex for NPG decreases by >500-fold. This huge increase in affinity for galactoside allows determination of binding kinetics for lactose, the natural substrate of LacY where affinity increases >200-fold. Because Nb 9036 also decreases $k_{off}$ and $k_{on}$ values in complex with the double-Trp mutant to near those observed for WT LacY/Nb 9036 complex, it seems reasonable to suggest that this Nb stabilizes a conformer that approximates an occluded intermediate with fully liganded sugar.

A simple fluorescent method was developed for detection of Nb binding to LacY by using site-directed Trp-induced quenching of a fluorophore attached to the periplasmic side of LacY. Quenching of the fluorophore introduced on the periplasmic but not on cytoplasmic side of LacY also confirms that
phospholipids. The Nb-encoding ORFs were amplified from total lymphocyte
DNA and cloned into the phage display expression vector pMUSEy4. After one round of
panning, dear enrichment was seen for the LacY double-Trp mutant. Ninety-two individual colonies were randomly picked, and the Nbs were
expressed in E. coli. Purified nanobodies (2-10 mg/mL) in 100 mM potassium phosphate (pH 7.5) were frozen in liquid nitrogen and stored at –80 °C before use.

Transport Measurements. RSO vesicles for transport assay were prepared from E. coli T184 harboring plasmid pT7-5 encoding WT LacY as described in SI Methods. The effect of the Nbs on lactose transport was measured after pre-
incubation of vesicles (0.5 mg of total membrane protein) with 80 μg of each Nb (at –51 molar ratio of Nb:LacY) in 100 mM KPi/10 mM MgSO4 (pH 7.2) for 20 min. Lactose transport was assayed with 0.4 mM [14C]lactose (10 μCi/mmol) in the same buffer at room temperature (see SI Methods for details).

Fluorescence Measurements.Stopped-flow measurements were performed at 25 °C on a SFM-300 rapid kinetic system equipped with a TC-50/10 cuvette (dead-time 1.2 ms) and MOS-450 spectrophotometer (Bio-Locic). NPG binding was measured as Trp151→NPG FRET at excitation 295 nm with emission interference filters (Edmund Optics) at 340 nm. LacY/Nb complexes were formed by preincubation of purified LacY (20-30 μM) with 1.2 molar excess of each Nb in 50 mM NaPi/0.02% DDM, pH 7.5 for 10 min at room temperature. Stopped-
flow traces were recorded at final concentration 0.5–0.8 μM of LacY after mixing with NPG. In displacement experiments LacY/Nb complex was pre-
incubated with NPG and then mixed with 15 mM TDG in stopped-flow. Mea-
surements with purified protein in DDM were done in 50 mM NaPi/0.02% DDM (pH 7.5). Experiments with PLs were carried out in 50 mM NaPi (pH 7.5). To dissociate NPs from LacY/Capture Select C-tagged proteins (MW 12–15 kDa) in the periplasm of E. coli. Testing for specific binding to both the G46W/G262W mutant and WT LacY (with the fucose transporter as a negative control) resulted in 31 families with the highest signals with mutant G46W/G262W compared with WT LacY. All selections and screenings were done in the absence of sugar.

Inducible periplasmic expression of Nbs in E. coli WK6 produces milligram quantities of each nanobody using unmodified metal ion affinity chro-
tomatography (Talon resin) from the periplasmic extract of a 1-1 bacterial culture. Purified nanobodies (2–10 mg/mL) in 100 mM potassium phosphate (pH, 7.5) were frozen in liquid nitrogen and stored at –80 °C before use.

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Supporting Information

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SI Methods

Materials. Oligonucleotides were synthesized by Integrated DNA Technologies. Restriction enzymes were purchased from New England Biolabs. The QuikChange II kit was purchased from Stratagene. TDG was obtained from Carbosynth Limited. NPG, r-lactose, and ATTO 655-maleimide were from Sigma. Bimane-C3-maleimide was from Life Technologies. Talon superflow resin was purchased from BD Clontech. Octyl-galactoside (OG) and DDM was from Anaspec. Synthetic phospholipids 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPG) were from Avanti Polar Lipids. All other materials were of reagent grade obtained from commercial sources.

Preparation of Membrane Vesicles. RSO membrane vesicles were prepared by osmotic lysis from T184 Escherichia coli containing WT LacY as described previously (1, 2). The vesicles were suspended in 100 mM potassium phosphate (KP)/10 mM MgSO₄ (pH 7.2) at a protein concentration 20 mg/mL, flash-frozen in liquid nitrogen, and stored at −80 °C until use.

Transport Assay. RSO vesicles were adjusted to an OD600 of 10 (10 mg of total protein per milliliter) and 50-μL aliquots were preincubated with 80 μg of each Nb (equivalent to ∼fivefold molar excess of Nb over LacY) at room temperature for 20 min. Lactose accumulation was assayed at given times by rapid filtration after addition of 20 mM ascorbate/0.2 mM phenazine methosulfate and 0.4 mM [1-14C]lactose (10 mCi/mmol) under oxygen, as described previously (3).

Construction of Mutants, Purification of LacY, and Reconstitution into Proteoliposomes. Construction of mutants, expression in E. coli and purification of LacY were performed as described previously (4). All proteins contained a C-terminal 6-His tag that was used for metal-affinity chromatography with a Talon resin. Purified proteins (10–15 mg/mL) in 50 mM sodium phosphate (NaP)/0.02% DDM, pH 7.5 were frozen in liquid nitrogen and stored at −80 °C until use. Reconstitution into PLs was carried out with synthetic phospholipids (POPE/POPG ratio 3:1) by using the dilution method (5, 6). Briefly, purified LacY in 0.02% DDM was mixed with phospholipids dissolved in 1.2% OG maintaining a lipid:protein ratio 5:1 (wt/wt). The mixture was kept on ice for 20 min and then quickly diluted 50 times in 50 mM NaP, buffer (pH 7.5). The PLs were collected by centrifugation for 1 h at 100,000 × g, suspended in the same buffer, and subjected to two cycles of freeze-thaw/sonication before use.

Labeling of LacY with Maleimide-Based Fluorophores. Purified WT LacY with Cys residues introduced on periplasmic or cytoplasmic side [50 μM protein in 50 mM NaP/0.02% DDM (pH 7.5) containing 5 mM TDG to protect native Cys148 from labeling] was mixed with a 1.2- to 1.5-fold molar excess of bimane-maleimide or ATTO 655-maleimide and incubated for 30 min at room temperature. Unbound fluorophores and TDG were removed by buffer exchange 2-times with 50 mM NaP/0.02% DDM (pH 7.5) using an Amicon Ultra-15 centrifugal filter device with 50-kDa cutoff (EMD Millipore).

Fig. S1. Effect of Nbs on lactose transport by WT LacY. Experiments were carried out as described in SI Methods. (A) Inhibition of lactose transport by increasing concentrations of Nb 9063. Accumulation of lactose (0.5 min) was measured after preincubation of RSO vesicles (500 μg total membrane protein) containing WT LacY with the indicated amount of Nb 9063 (circles). In control experiments (triangles), uptake of [3H]fucose (0.4 mM) by RSO vesicles containing FucP was measured after preincubation of vesicles with the same concentrations of Nb 9063. (B) Time courses of lactose accumulation by RSO vesicles containing WT LacY after preincubation with the indicated Nbs (300 μg Nb/mg membrane protein, black symbols) or without addition of Nb (blue circles). Control RSO vesicles did not contain LacY (red circles). (C) Transport of lactose measured with RSO vesicles containing WT LacY preincubated with indicated Nbs (300 μg Nb/mg membrane protein). Two time points (30 and 60 s) for transport are shown for each experiment. Data are expressed as percentage of lactose transport in the absence of Nb, averaged, and presented in Table 1.
Fig. S2. Effect of Nb 9065 on kinetic parameters of NPG binding to WT LacY and mutant G46W/G262W. Affinity for NPG ($K_d$) and $k_{off}$ values were determined using Trp151—NPG FRET in displacement experiments with WT LacY (Left) and mutant G46W/G262W (Right) by mixing a saturating concentration TDG (15 mM) with a protein solution containing indicated concentrations of NPG. Stopped-flow traces of Trp fluorescence increase were recorded in the absence of Nb (A and B) or with LacY preincubated with a 1.2 molar excess of Nb 9065 (C and D). Single exponential fits to the data without Nb (black lines) yield estimated $k_{off}$ values of 41 and 31 s$^{-1}$ for the WT and mutant, respectively. Single exponential fits to the data in the presence of Nb (red lines) yield estimated $k_{off}$ values 40 and 58 s$^{-1}$ for the WT and mutant, respectively. NPG binding affinity was determined from hyperbolic fits of concentration dependence of the fluorescence changes at each NPG concentration in the stopped-flow traces recorded in the absence (black lines) or presence (red lines) of Nb 9065 (E and F). The fluorescence change at each NPG concentration (amplitude of displacement) is expressed as percentage of final fluorescence level in the stopped-flow trace. The affinity of WT LacY for NPG increases in the presence of Nb 9065 ($K_d$ decreases from 28 ± 2 to 5.3 ± 0.3 μM), whereas the $K_d$ for mutant G46W/G262W in complex with Nb 9065 changes only slightly (from 6.1 ± 0.4 to 9.1 ± 0.5 μM). The values of $k_{on}$ calculated from displacement experiments ($k_{on} = k_{off}/K_d$) in the presence of Nb are 7.5 and 6.4 μM$^{-1}$s$^{-1}$ for WT and mutant, respectively.
Fig. S3. Accessibility of the sugar-binding site in LacY from periplasmic or cytoplasmic side. NPG binding was measured by Trp151→NPG FRET with mutants G46W/G262W (A–C), C154G (D–F), or WT LacY (G–I) either solubilized in DDM or reconstituted into PLs. Stopped-flow rates were measured by mixing indicated concentrations of NPG with 0.5–0.8 μM proteins in DDM solution (A, D, and G, blue circles), in reconstituted PLs (B, E, and H, brown squares), or after dissolving the PLs in DDM (A, D, and G, stars). The linear concentration dependencies of the NPG binding rates \( k_{\text{obs}} = k_{\text{off}} + k_{\text{on}}[\text{NPG}] \) measured in DDM (A, D, and G, blue circles) give estimates of \( k_{\text{on}} \) values (slope of the solid blue line) for G46W/G262W (5.7 μM\(^{-1}\)s\(^{-1}\)), C154G (5.0 μM\(^{-1}\)s\(^{-1}\)), and WT LacY (0.2 μM\(^{-1}\)s\(^{-1}\)). Mutant G46W/G262W reconstituted into PLs exhibits a linear concentration dependence of binding rates (B) \( k_{\text{on}} = 5.0 \text{ μM}^{-1}s^{-1} \), whereas C154G and WT LacY bind sugar with constant rates independent of NPG concentration (E, \( k_{\text{obs}} = 50 \) and H, \( 21 \text{ s}^{-1} \), respectively). Dissolving the PLs in DDM results in NPG binding rates similar to those observed in DDM before reconstitution (A, D, and G, stars). Displacement experiments (as described in Fig. S2) were carried out with the same proteins in DDM or reconstituted into PLs. The concentration dependencies of the amplitudes of the fluorescence changes (C, F, and I) allow estimation of \( K_d \) values in DDM (open circles) or in PLs (open squares). The rate of displacement is a true \( k_{\text{off}} \) and does not depend on NPG concentration. Therefore, \( k_{\text{on}} \) values were calculated using measured \( K_d \) and displacement rates \( k_{\text{on}} = k_{\text{off}}/K_d \). Kinetic parameters of NPG binding obtained in displacement experiments are shown in blue or brown for data obtained in DDM and PLs, respectively. NPG binding rates with \( k_{\text{on}} \) values calculated from displacement are presented as linear concentration dependencies for comparison (broken lines in panels A, B, D, E, G, and H). The sharp slope of the linear concentration dependence of observed rates with high \( k_{\text{on}} \) value (5–14 μM\(^{-1}\)s\(^{-1}\)) indicates free access for sugar to the binding site of LacY.

Fig. S4. Effect of Nbs on accessibility of sugar-binding site in reconstituted LacY. Sugar binding rates were measured directly as Trp151→NPG FRET as described in Fig. 2C, by mixing NPG with reconstituted WT LacY (A) or mutant C154G (B) in the absence of Nbs (gray symbols) or after preincubation with a 1.2-molar excess of the indicated Nb. The red arrows indicate the change in concentration dependence of sugar binding rates due to Nb binding. (A) For WT LacY/Nb complexes, \( k_{\text{on}} \) values were estimated as 9.7 and 22 μM\(^{-1}\)s\(^{-1}\) for Nbs 9039 and 9047, respectively. Sugar binding in the absence of Nb is limited by opening of periplasmic cavity \( k_{\text{obs}} = 21 \text{ s}^{-1} \). (B) For C154G LacY/Nbs complexes, \( k_{\text{on}} \) values were estimated as 2.5, 5.6, and 7.1 μM\(^{-1}\)s\(^{-1}\) for Nbs 9047, 9043, and 9065, respectively. Sugar binding in the absence of Nb \( (k_{\text{obs}}) \) is 50 s\(^{-1}\). Measured by displacement, true \( k_{\text{off}} \) values are shown as open symbols on y axis. Broken lines represent the concentration dependence of rates of NPG binding to the open periplasmic cavity \( (k_{\text{obs}} = 10 \text{ and } 14 \text{ μM}^{-1}s^{-1} \text{ for WT LacY and C154G, respectively}) \) calculated from displacement experiments with reconstituted LacY. See also Fig. S3 E, F, H, and I.
Fig. S5. Effect of Nb 9036 on sugar binding to WT LacY. NPG binding rates measured as Trp151–NPG FRET in direct binding experiments or by TDG displacement of NPG bound to LacY without Nbs or preincubated with 1.2-excess of Nb 9036. (A) Stopped-flow traces of Trp fluorescence decrease after mixing indicated concentrations of NPG with 0.4 μM WT LacY. The concentration dependencies of the observed rates are shown in Fig. 1 A (black and pink lines) with estimated $k_{on} =$ 0.2 and 1.0 μM$^{-1}$ s$^{-1}$ for WT LacY and WT LacY/Nb 9036 complex, respectively (Table 1). (B) Stopped-flow traces of Trp fluorescence increase in displacement experiments after mixing 15 mM TDG with WT LacY (0.2 μM) preincubated with indicated concentrations of NPG. Estimated $k_{off}$ values are 41 and 0.05 s$^{-1}$ for WT LacY and WT LacY/Nb 9036 complex, respectively. The $K_d$ value for NPG binding calculated from $k_{off}/k_{on}$ is 0.05 μM for the LacY/Nb 9036 complex compared with 28 μM measured for WT LacY without Nb (Table 1).
Fig. 56. Detection of Nb binding to LacY by using Trp-induced fluorescence quenching. Homology modeled structures of Nbs 9063, 9065, and 9036 are presented at the top with Trp residues shown as green spheres. Structures of bimane-maleimide and ATTO-655-maleimide fluorophores are shown on right. Fluorescence emission spectra of fluorophore-labeled mutant I32C LacY were recorded in 50 mM NaPi/0.02% DDM (pH 7.5) before and after addition of a twofold molar excess of the indicated Nb. (A–C) Bimane emission spectra were recorded at excitation wavelength 380 nm with 0.3 μM bimane-maleimide labeled LacY. (D–F) ATTO655 emission spectra were recorded at excitation wavelength 650 nm with 0.2 μM ATTO655-maleimide labeled LacY. Arrows indicate Trp-induced fluorescence quenching in the presence of the Nb.
Fig. 5.7. Presteady-state rates of Nb binding to LacY measured as Trp-induced fluorescence quenching. Stopped-flow traces are recorded in 50 mM NaPi/0.02% DDM (pH 7.5) after mixing bimane-labeled mutant I32C LacY (0.4 μM) with given concentrations of Nb 9034 (A), Nb 9063 (B), Nb 9036 (C), and Nb 9055 (D). Single exponential fits are shown as colored lines with estimated rates and amplitudes of the fluorescence changes given on the right. The concentration dependencies of the Nb binding rates are shown in Fig. 5. Homology models of the Nbs are shown on the left with Trp residues presented as green spheres.
Fig. S8. Confirmation that Nb binds to the periplasmic side of WT LacY. (A–C) Cys residues introduced on periplasmic (I32C) or cytoplasmic (S401C) side of WT LacY were labeled with bimane- or ATTO655-maleimides, and the time courses of the fluorescence emission changes were recorded in 50 mM NaPi/0.02% DDM (pH 7.5) at excitation/emission wavelengths 380/465 nm and 660/677 nm for bimane and ATTO655, respectively. The fluorescence of LacY labeled on the cytoplasmic or periplasmic sides are shown as blue or red lines, respectively. Black arrows indicate addition of 0.6 μM of a given Nb to 0.3 μM LacY labeled with bimane-maleimide (A) or ATTO-655-maleimide (B and C). The inward-open conformer of LacY (PDB ID code 2CFQ) with Cys replacements is shown on right. (D) Effect of Nb 9036 on kinetic parameters of bimane-labeled S401C mutant. The concentration dependencies of NPG binding rates were measured in the absence of Nb (filled red circles, labeled mutant S401C; open circles, WT LacY) or with Nb 9036/LacY complex (red diamonds, labeled S401C; red triangles, WT LacY). As estimated from linear fits, $k_{on}$ values increase from 0.2 to 1.0 μM$^{-1}$·s$^{-1}$ for both the WT and bimane-labeled S401C LacY complexes with Nb 9036 (see also Fig. 1). The effect of Nb 9036 on the NPG displacement rate by TDG measured with bimane-labeled S401C LacY is the same as that observed with WT LacY ($k_{off}$ decreases from 41 to 0.05 s$^{-1}$, black circle and open diamond, respectively) as presented on Fig. S5B.
Fig. S9. Nb binding affinity. Fluorophore-labeled I32C LacY (20 nM in 2.5 mL of 50 mM NaPi/0.02% DDM pH 7.5) was titrated with Nbs 9063, 9055 or 9036. (A and B) Nbs binding to bimane-labeled LacY. (C and D) Nbs binding to ATTO655-labeled LacY. Measurements were done as described in Fig. 4. (A and C) Time courses of fluorescence quenching after sequential additions (1–3 μL) of increasing concentrations of Nb 9063 (total amount of added Nb is shown). Titrations were done at 0.1 mM NPG or without added sugar (green and cyan lines, respectively). Note slow binding rates because of low concentrations of LacY and Nb. (B and D) Concentration dependencies of the fluorescence change because of binding of the indicated Nb to labeled LacY. Fluorescence changes are expressed as percent of total fluorescence quenching in each experiment: 100(F0 − Fn)/F0, where F0 is fluorescence level without Nb; Fn is fluorescence level at indicated Nb concentration; F0 is final level of fluorescence at saturating Nb concentration. Data are fitted with an equation derived from equilibrium $K_d = [Nb]_{free} [P]_{free}/[Nb\cdot P]$, where [Nb$\cdot$P] is a concentration of Nb/LacY complex, and [Nb]$_{free}$ and [P]$_{free}$ are concentrations of unbound Nb and LacY. Final equation used for fitting is: $f = 100((Nb + P + K_d)/2 - ((Nb + P + K_d)^2 - (4Nb\cdot P))^{0.5}/2)/P$, where concentrations of Nb and LacY are total (in micromolars). Estimated $K_d$ values are: 0.9 ± 0.2 nM for binding Nb 9063 and Nb 9036 to bimane-labeled LacY (B, green line); 0.9 ± 0.2 nM and 0.6 ± 0.3 nM for binding Nb 9063 and Nb 9036 to ATTO-labeled LacY (D, green and pink lines, respectively); and 1.6 ± 0.3 nM and 2.8 ± 0.7 nM for binding Nb 9055 to ATTO-labeled LacY in the absence or presence of sugar (D, broken and solid blue lines, respectively).