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The galactoside-binding site in LacY

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Abbreviations: DDM, *n*-dodecyl-β-D-maltopyranoside; FRET, Förster resonance energy transfer; IPTG, isopropyl-β-D-1-thiogalactopyranoside; KP_i, potassium phosphate; NaP_i, sodium phosphate; NEM, *N*-ethylmaleimide; NPG, *p*-Nitrophenyl-α-D-galactopyranoside; TDG, β-dgalactopyranosyl-1-thio-β-D-galactopyranoside; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Abstract. Although an x-ray crystal structure of lactose permease (LacY) has been presented with bound galactopyranoside at ~3.5 Å, residues liganding the sugar cannot be identified with precision at this resolution. Therefore additional evidence is important for identifying side chains likely to be involved in binding. Based on a clue from site-directed alkylation suggesting that Asn272, Gly268 and Val264 on one face of helix VIII might participate in galactoside binding, a molecular dynamics simulation was carried out initially. The simulations indicate that Asn272 (helix VIII) is sufficiently close to the galactopyranosyl ring of a docked lactose analogue to play an important role in binding, while the backbone at Gly268 may be involved, and Val264 does not interact with bound sugar. When the three side chains are subjected to site-directed mutagenesis, with the sole exception of mutant Asn272→Gln, various other replacements either markedly decrease affinity for substrate (i.e., high K_D) or abolish binding altogether. However, mutant Gly268→Ala exhibits a moderate eight-fold decrease in affinity, and binding by mutant Val264→Ala is affected only minimally. Thus, Asn272 and possibly Gly268 may comprise additional components of the galactoside-binding site in LacY.

The lactose permease of *E. coli* (LacY) specifically binds and transports D-galactose and disaccharides containing a D-galactopyranosyl ring with an H^+ (galactoside/H^+ symport), and does not recognize D-glucose or D-glucopyranosides, which differ in the orientation of the C4-OH only. By utilizing the free energy released from the energetically downhill movement of H^+ in response to the electrochemical H^+ gradient ($\Delta\tilde{\mu}_H+$, interior negative and/or alkaline), LacY catalyzes the uphill (active) transport of galactosides against a concentration gradient. Since coupling between sugar and H^+ translocation is obligatory, in the absence of $\Delta\tilde{\mu}_H+$, LacY can also transduce the energy released from downhill sugar transport to drive the uphill H^+ transport with the generation of $\Delta\tilde{\mu}_H+$, the polarity of which depends upon the direction of sugar gradient (reviewed in [1]).

X-ray crystal structures of WT LacY [2], the conformationally restricted mutant C154G [3, 4] and a single-Cys mutant with a covalently bound inactivator [5] have been solved in the same inward-facing conformation. In each structure, 12 transmembrane α-helices are arranged in two 6-helix pseudo-symmetrical bundles linked by a long cytoplasmic loop between helices VI and VII. The two 6-helix bundles surround a deep hydrophilic cavity that is tightly sealed on the periplasmic face and open to the cytoplasmic side only (an inward-facing conformation). Although the crystal structures suggest that LacY is rigid, a wealth of biochemical/spectroscopic data [6-14] demonstrates that the molecule can open

alternatively to either side of the membrane upon sugar binding, thereby providing evidence for an alternating-access model for symport (reviewed in [15, 16]).

The initial x-ray structure of LacY was obtained with a density at the apex of the central cavity, but at ~3.5 Å resolution, side-chain interactions with bound sugar cannot be identified clearly. However, biochemical/biophysical studies show that LacY contains a single galactoside-binding site and that the residues involved in sugar binding are located at or near the apex of the central cavity in the molecule (Fig. 1). Although the specificity of LacY is strongly directed towards the C4-OH of the D-galactopyranosyl ring, the C2-OH, C3-OH and C6-OH are also important in the following order: C4-OH >> C6-OH > C3-OH > C2-OH [17, 18]. The residues responsible for sugar binding are located on helices IV, V and VIII. Cys-scanning mutagenesis, site-directed alkylation, thiol cross-linking and direct binding assays show that Glu126 (helix IV) and Arg144 (helix V) are critical for substrate binding and probably charge paired [19-22]. Trp151 (helix V), two turns removed from Arg144, stacks aromatically with the galactopyranosyl ring [23, 24]. Glu269 (helix VIII), another irreplaceable residue [25, 26], is also essential for sugar recognition and binding and cannot even be replaced with Asp without dramatically decreasing affinity [27, 28]. Furthermore, mass spectrometry studies suggest that Glu269 may interact with the C3-OH of the galactopyranosyl ring [29].

Cys-scanning mutagenesis [30] and site-directed alkylation studies [31] suggest that Asn272, Gly268 and Val264 on the same face of helix VIII might also be

components of the sugar-binding site, and in order to gain further insight, a set of molecular dynamics (MD) simulations has been carried out. The simulations indicate that Asn272, one turn removed from critical Glu269 in helix VIII, is very likely a component of the sugar-binding site, while the backbone at Gly168 or Val164 are unlikely to be involved in binding. Consistently, binding studies on purified mutant LacY molecules show that out of a dozen replacements for Asn272 only mutant N272Q binds NPG with an affinity similar to that of the WT. All other replacements exhibit either markedly decrease affinity or no significant binding. Interestingly, mutant G168A also exhibits a significant decrease in affinity suggesting that it may be peripherally involved in binding, and mutant V164A binds sugar with good affinity demonstrating that it is not a component of the sugar-binding site.

Results

MD simulations. A set of three MD simulations of wild-type, Gly268Cys and Val264Cys LacY disclose one major pose of NPG where Asn272 contributes directly to sugar binding (Fig. 2). As observed by contact analyses reporting protein-NPG interactions <3.5 Å, the sugar molecule interacts with residues thought to be involved in sugar binding (Fig. 2A). A guanidino group at position 144 (Arg), a carboxyl group at position 126 (preferably Glu), an aromatic residue at position 151 (preferably Trp) and a carboxyl group at 269 (Glu) are obligatory for sugar binding (reviewed in [1]). The nitro-group in NPG is proximal to Ala122 in all three simulations, which is consistent with previous observations [(32)]. Rather than being fixed in a single position with respect to the surrounding protein, the

NPG molecule is highly dynamic although with one clearly defined preferred binding pose. This is particularly evident in the wild-type simulation, where NPG visits the alternative locations in the wide, water-filled cytoplasmic cleft (Fig. 2A).

Residues Asn272, Glu269, and Thr 265 on helix VIII make contacts with NPG (Fig. 2A inset). In all three simulations, sugar-binding dynamics between Asn272 and the sugar interacting residue Glu269 are synchronized (Fig. 2B). However, neither the backbone at position 268 nor Val264 appear to make contact with the galactopyranosyl ring of NPG (Fig. 2A inset). In events where Asn272 and Glu269 are in close proximity to the galactopyranosyl moiety, known sugar-binding residues Glu126, Arg144, and Trp151 are also exposed (Fig. 2C).

Lactose transport. Out of the twelve Asn272 replacement mutants tested, only four--N272D, N272V, N272S and N272Q--transport lactose to a steady-state level of accumulation that is 30% or more of the steady state established by WT LacY in 30 min (in decreasing order, N272D, N272V, N272S and N272Q). Mutants N272G and N272A accumulate to about 25% and 20% of WT, respectively, and the activities of the remaining mutants are negligible (Fig. 3A & B). The initial rate of transport by mutant N272D approximates that of the WT, but the initial rates of transport observed with mutants N272V, N272Q and N272S are drastically lower than that of WT. Mutant V264A accumulates lactose as well as WT. Although the initial rate of transport by mutant G268A is similar to WT, the steady-state of accumulation is only about 40% of WT.

Western blotting with anti-His antibody reveals that each mutant is expressed at about the same levels as WT LacY (Fig. 3A, B & C, lower panels). Therefore, the differences in transport activity are not due to variations in expression of the mutants.

NPG binding. NPG is a high-affinity sugar-analogue of lactose, and previous studies [(33)] show that the distance between Trp151 in the binding site and the nitrophenyl group of NPG (~12 Å) is favorable distance for Förster resonance energy transfer (FRET). Because the analogue has a broad absorption spectrum with a maximum at 306 nm (not shown), NPG affects Trp fluorescence by two simultaneous processes: (1) by serving as a nonfluorescent FRET acceptor from Trp151 in the binding site and (2) by acting as an inner filter and absorbing irradiated excitation light at 295 nm as well as fluorescence emission of Trp. In order to discriminate between the two processes, another lactose analogue melibiose, which is not fluorescent and does not absorb light over the range of wavelengths studied, was used. Addition of saturating concentrations of melibiose in the absence of NPG causes little or no change in the emission spectrum of Trp. However, when melibiose is added after incubation with NPG, an increase in Trp fluorescence is observed due to displacement of NPG from the binding site. Thus, the increase in Trp fluorescence upon addition of melibiose represents a specific FRET effect, and the remainder of the

fluorescence change that is not restored by melibiose represents the nonspecific inner-filter effect caused by NPG in solution. The apparent affinity for NPG is estimated from the concentration dependence of the specific fluorescence change after addition of excess melibiose at various NPG concentrations (Fig. 4A-E). The calculated K_D of 19 (±1) μM obtained for WT LacY (Fig. 4F) is the same as that obtained previously [(33)].

In addition to WT LacY, significant fluorescence changes are observed with mutant N272Q (Fig. 4E) and to a lesser extent with mutant N272A (Fig. 4D), and K_Ds of 20 (±2) μM and 143 (±39) μM, respectively, are obtained (Fig. 4F; Table.1). In contrast, the fluorescence changes observed with mutants N272D, N272V, N272E, N272F and N272S were too small to allow estimates of binding constants. Significant fluoresecence changes are also observed with mutant V264A, which yield a K_D of 30 (±4) μM (Fig. 4B & Table.1). However, mutant G268A exhibits smaller changes in fluorescence and lower affinity for NPG (~8-fold) relative to WT LacY (Fig. 4C; Table.1).

Kinetics of selected mutants. Kinetics of lactose transport for WT LacY and mutants N272D, N272Q. N272V, V264A and G268A were measured in order to determine whether there is a correlation between the measured K_Ds and kinetic constrants (Fig. 5; Table 2). The K_m obtained for WT LacY is ~0.8 (±0.1) mM with a V_{max} of 422 (±14) nmole/mg protein/min, in reasonable agreement with previous findings [(34)]. The K_m obtained for mutant N272D is ~5-times higher than

WT with about the same V_{max} as the WT. Replacing Asn272 with Val leads to about a 15-fold increase in K_m, with a significantly higher V_{max} than WT. Remarkably, although WT LacY and mutant N272Q have essentially the same K_D for NPG (Table 2), the K_m obtained for lactose transport is more than 40-times higher than WT, with a lower V_{max}. Finally, replacement of Val264 or Gly268 with Ala has little or no effect on either K_m or V_{max} (data not shown).

Discussion

The specificity of LacY for substrate is directed exclusively towards the galactopyranosyl ring of substrate. Thus, the monosaccharide D-galactose is the most specific sugar for LacY, although it has very low affinity [(17)]. Remarkably, substitutions on the anomeric carbon, particularly if they are hydrophobic, can lead to a marked increase in affinity (up to ~3 orders of magnitude) with no change in specificity [(35)].

Asn272 (helix VIII), positioned one turn away from Glu269 towards the cytoplasmic side, is proximal to the galactopyranosyl ring. Cys-scanning mutagenesis of helix VIII reveals that mutant N272C transports lactose only 30% as well as the WT [(30)], and site-directed alkylation demonstrates that mutants N272C, G268C and V264C are protected against alkylation by substrate, suggesting that all three positions might be in the vicinity of the sugar-binding site [(31)]. Moreover, sequence alignment data also shows that Asn272 is conserved in bacterial galactoside/H^+ symporters [(36)]. Since the interactions of LacY with

substrate are based primarily upon biochemical findings (reviewed in [1]), in order to investigate further a possible role of Asn272, Gly268 and Val264 in sugar binding, we initially utilized molecular dynamics (MD) simulations.

The simulations reveal a highly dynamic NPG substrate that adopts a similar docking position in the WT, G268C, and V264C simulations. It was possible to monitor substrate entering and leaving this docking pose by tracking interactions between residues Glu269 and Asn272 and the C4-OH on the NPG galactopyranosyl ring (Fig. 2B). It is particularly evident in the >200 ns WT simulation that the sugar visits alternative positions in the cytoplasmic cleft. In both mutant simulations, NPG remains in a binding pose that also involves interaction between the guanidino group of Arg144 and the C6-OH of the galactopyranosyl ring. While Glu126 is relatively far removed, Trp151 is stacked with the under side of the galactopyranosyl ring and the nitrophenyl group is directed towards Ala122 (Fig. 2C) [32]. It is clear that backbone positions 264 and 268, which have been implicated in sugar binding, do not interact directly with NPG. Of all atoms in these positions, the backbone carbonyl oxygen at position 268 is closest, which corroborates with an 8-fold decrease in affinity of the G268A mutant. Because the mutants G268C and V264C are relatively far from the NPG binding site, it is likely that the three simulations sample the same fundamental protein-sugar interactions.

Mutagenesis shows that except for mutant N272D, in which significant NPG

binding cannot be measured, all other replacement mutants tested exhibit significantly decreased initial rates of lactose transport. Furthermore, only mutants N272D and N272V accumulate lactose to a steady state comparable to that of the WT (Fig. 3A and B). Mutant N272Q exhibits low transport activity, but transport activity in the other mutants is essentially moribund. The transport defect in mutants N272F, N272Y and N272W may be due to the bulky aromatic side-chain at position 272, which could sterically block binding. The low activity of mutants N272G and N272A suggests that the amide group of Asn272 is probably important for binding.

Mutant N272Q with the most conservative replacement for Asn has a K_D that is essentially the same as that of the WT, and replacing the amide group with virtually any other amino acid residue except Ala essentially abolishes NPG binding. Even with Ala in place of Asn272, NPG affinity decreases by almost 8-fold. Although it is tempting to try to relate the transport data presented to the binding observations, it should be emphasized that the transport studies are carried out with lactose, which binds with very low affinity, while the binding data are obtained with the high-affinity homologue NPG. Thus, although the 45-fold increase in K_m for mutant N272Q relative to the WT may explain relatively low lactose transport activity, the K_D for NPG is the same as that of the WT. In a similar vein, there appears to be no correlation whatsoever between decreased NPG affinity, increased K_m and transport activity.

In any case, based primarily on the MD simulations and the NPG binding data in conjunction with the earlier site-directed alkylation experiments [30, 31], it seems highly probable that Asn272 is a component of the sugar-binding site in LacY. Previous site-directed alkylation shows that two additional mutants, V264C and G268C, are also protected by sugar against NEM labeling. In this study, Ala replacement for Val264 does not affect either NPG binding or the kinetics of lactose transport, indicating that Val264 is not a component of sugar binding site. However, mutant G268A exhibits about an 8-fold increase in K_D (i.e., a moderate decrease in affinity), but lactose transport remains unchanged. Although the results are somewhat ambiguous, they imply that the backbone at the position of Gly268 might interact with bound sugar and that the moderate decrease in affinity exhibited by mutant G268A may be due to the steric hindrance. Another possibility is that a Gly residue at position 268 provides flexibility near the binding site.

Materials and Methods

Materials

Oligonucleotides for site-directed mutagenesis were synthesized by Integrated DNA Technologies, Inc (Coralville, CA). A QuikChange II mutagenesis kit was obtained from Agilent Technologies, Inc. (Santa Clara, CA). Restriction enzymes were purchased from New England Biolabs Inc (Ipswich, MA). Lactose and *p*-nitrophenyl-α-D-galactopyranoside (NPG) were from Sigma-Aldrich (St. Louis, MO). Melibiose was obtained from Acros Organics (NJ, US). [D-glucose-^{14}C (U)]-

Lactose was from Moravek Biochemicals, Inc (Brea, CA). Penta-His antibody-horseradish peroxidase (HRP) conjugate was from Qiagen (Hilden, Germany). Supersignal West Pico Chemiluminescent substrate kits for western blotting were from Pierce (Rockford, IL). Micro BCA protein assay kits were from Thermo Scientific (Rockford, IL).

Molecular dynamics (MD) simulations

The Multipurpose Atom-Typer for CHARMM (MATCH) [37, 38] was used to obtain parameters for NPG. The crystal structure of WT, substrate-free LacY, in an inward-facing conformation, and including N-terminal Met1 to C-terminal Ala417 (Protein Data Bank ID 2V8N), was inserted into a phosphatidylethanolamine lipid bilayer by aligning the centers-of-mass of the protein TM domain and the POPE bilayer, respectively, and removing lipids to avoid protein-lipid steric clashes. The system was solvated by explicit water molecules and counter ions were added to achieve electrical neutrality. The three simulation systems were relaxed using a 10,000-step conjugate-gradient energy minimization followed by gradual heating from 0 to 310 K over 120 ps at constant temperature (310 K) and volume (NVT ensemble). Equilibrated positions of lipids, water molecules and the protein were obtained by a series of consecutive 500 ps simulations, where the harmonic restraints on these groups were successively released at constant temperature (310 K) and pressure (1 atm) (NPT ensemble). To introduce the NPG sugar molecule, WT LacY with β-D-galactopyranosyl-1-thio-β-D-galactopyranoside (TDG) coordinates (Protein Data Bank ID 1PV7) was superimposed on the 1.5 ns

NPT-equilibrated LacY structure followed by aligning NPG with TDG. Water molecules were removed to eliminate water-NPG steric clashes and the equilibration protocol was repeated, i.e. energy minimization and equilibration of water molecules and protein atoms, respectively, followed by 206 ns, 79 ns, and 89 ns of unrestrained simulation of the wild-type, Gly268Cys, and Val264Cys systems, respectively.

The MD simulations were run with the NAMD 2.7 software package [39] and the CHARMM22 and CHARMM36 force fields [40, 41] were used for protein and lipids, respectively. The TIP3P model was used for the water molecules [42]. A reversible multiple time step (MTS) algorithm [43] was used to integrate the equations of motion with time steps of 1 fs for bonded forces, 2 fs for short-range, non-bonded forces, and 4 fs for long-range, electrostatic forces. The smooth particle mesh Ewald method [44, 45] was used to calculate electrostatic interactions. The short-range interactions were cut off at 12 Å. All bond lengths involving hydrogen atoms were held fixed using the SHAKE [46] and SETTLE [47] algorithms. A Langevin dynamics scheme was used for thermostating. Nosé-Hoover-Langevin pistons were used for pressure control [48, 49]. Molecular graphics and simulation analyses were generated with the VMD 1.9.1 software package [50].

Construction of mutants

All Asn272 mutants were constructed by site-directed mutagenesis using the plasmid pT7-5 containing the cassette *lacY* gene with a C-terminal 6-His tag as template. All mutations were verified by sequencing of the entire *lacY* gene and the restriction sites.

Lactose transport

E. coli T184 [*lacI*$^+$ *O*$^+$ *Z*$^-$ *Y*$^-$ (*A*) *rpsL met*$^-$ *thr*$^-$ *recA hsdM hsdR/F' lacI*^{q} O$^+$ Z^{D118} (*Y*$^+$ *A*$^+$)] [51, 52] cells were transformed with plasmid pT7-5 encoding the WT LacY a given mutant and grew in LB broth containing 0.1 mg/mL Ampicillin at 37°C overnight. The culture was diluted into 30 mL LB broth and induced with 1 mM (final concentration) IPTG at OD_{600} of 0.6. After 1 hour induction, The cells were assayed for transport of [1-^{14}C] lactose (10 mCi/mmole, 0.4 mM final concentration) by rapid filtration [53].

Western blotting

The cells used in the lactose transport assays were lysed by sonication. A membrane fraction was obtained by ultracentrifugation (2.2×10^5 g, 4 °C for 20 min) and suspended in 0.1 M KPi (pH 7.5)/10 mM $MgSO_4$. An aliquot of sample containing 2 μg of membrane protein was used for western blotting and probed with Penta-His antibody-HRP conjugate to indentify LacY [54].

LacY purification

E. coli XL-1 blue cells transformed with plasmid pT7-5 encoding a given mutant was grown 1L LB broth containing 0.1 mg/mL ampicillin at 37°C overnight. A 10-fold dilution of the culture was grown in fermenter and induced with 0.3 mM (final concentration) IPTG at OD_{600} of 0.6. After 3 hours induction, the cells were harvested and lysed with a French pressure cell. His-tagged LacY in the cell

lysate was purified as described previously [55]. Purified LacY was solubilzed in 50 mM NaPi (pH 7.6)/0.01%DDM, flash frozen in liquid nitrogen and stored at -80°C until use. The protein concentration was determined by Micro BCA protein assay.

NPG binding

NPG binding measurements were carried out with a SLM-Aminco 8100 spectrofluorometer (Urbana, IL) as described [33][39][33] with minor modifications. In a 1 × 1 cm cuvette, purified WT or a given Asn272 mutant was diluted in 50 mM NaP_i (pH 7.5)/0.01% DDM to a final concentration of 1 μM in a volume of 2 mL. α-NPG was added to a given concentration, and then 30 mM (final concentration) melibiose was added to displace NPG. Changes in fluorescence resulting from Trp→α-NPG FRET were recorded with constant stirring and corrected for dilution causing by addition of the ligand. K_Ds were determined by GraFit 6 (Erithacus Software, London, UK) using "1Site-Ligand Binding" equation:

$$[Bound] = \frac{Capacity.[Free]}{K_d + [Free]}$$

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Figure legends

Fig. 1. Backbone structure of LacY**.** The structure of C154G LacY (PDB ID: 1PV7) viewed from the side with the N-terminal 6-helix bundle on the left and C-terminal 6-helix bundle on the right. NPG is shown as yellow stick at the apex of the central cavity.

Figure 2. MD simulations of NPG binding. (*A*) Contact analyses of the wild-type (green), Gly268Cys (black), and Val264Cys (red) simulations reporting protein-NPG interactions < 3.5 Å. (B) The evolution of the inter-atomic distances between O4-NPG and N272-NH (black) and E269-O (blue) for the wild-type (upper panel), Gly268Cys (middle panel), and Val264Cys (lower panel) simulations. (C) NPG binding pose displayed by the final frame of the Gly268Cys simulation.

Figure 3. Lactose transport. (*A, B & C*) Transport of [^{14}C]lactose (10 mCi/mmole) of *E. coli* T184 expressing WT LacY, mutants N272D, N272E, N272K, N272Q, N272S, N272A, N272V, N272G, N272L, N272Y, N272F, N272W, V264A, G268A or no permease was measured at 0.4 mM lactose for given times as described in *Materials and Methods*. Expression of WT LacY and each mutant as determined by Western blotting.

Figure 4. NPG binding. (*A*-*E*) Trp fluorescence emission spectra at 20 mM (red),

50 mM (blue), 75 mM (pink) and 150 mM (green) NPG are shown for purified WT LacY (*A*), V264A (*B*), G268A (*C*), N272A (*D*) and N272Q (*E*). Broken lines, spectra after adding α-NPG. Solid lines, spectra after adding 30 mM melibiose. (*F*) Binding of α-NPG to purified WT LacY (●), V264A (○), G268A (△), N272A (◇) and N272Q (☆). The changes in fluorescence induced by addition of melibiose are plotted as a function of NPG concentration. Differences obtained with mutants N272D, N272E, N272F, N272S and N272V were too small for accurate measurement.

Figure 5. Transport kinetics of WT LacY and selected N272 mutants. *E. coli* T184 expressing WY LacY (●), N272D (○), N272V (△) or N272Q (□) in 0.1 M KP_i (pH 7.5)/10 mM $MgSO_4$ at an OD_{420} of 10 (50 µL) were incubated with [1-^{14}C] lactose at a given concentration at room temperature for 20 s as described in *Materials and Methods*. The samples were rapidly diluted with 3 mL stop buffer and vacuum filtered. The filters was washed once with 3 mL stop buffer and assay for radioactivity by liquid scintillation spectrometry. K_ms and V_{max}s were determined by GraFit 6 (Erithacus Software, London, UK) using the Michaelis-Menten equation (Table 1):

$$v = \frac{V_{\max}.[S]}{K_m + [S]}$$

Table 1. K_ds for NPG binding (see Fig. 4).

	App. K_d (μM)	Std. Error
WT	19.2	1.1
N272A	142.6	39.4
N272D	-	-
N272E	-	-
N272F	-	-
N272Q	20.0	1.8
N272S	-	-
N272V	-	-
V264A	30.1	4.1
G268A	154.7	57.3

-, indicates binding is too small to be determined.

Table 2. Kinetics of selected mutants (see Fig. 5).

	K_m (mM)	Std. Error	V_{max} (nmole/mg protein/min)	Std. Error
WT	0.8	0.1	422	14
N272D	3.7	0.8	450	40
N272Q	36	28	336	139
N272V	12	2	547	47

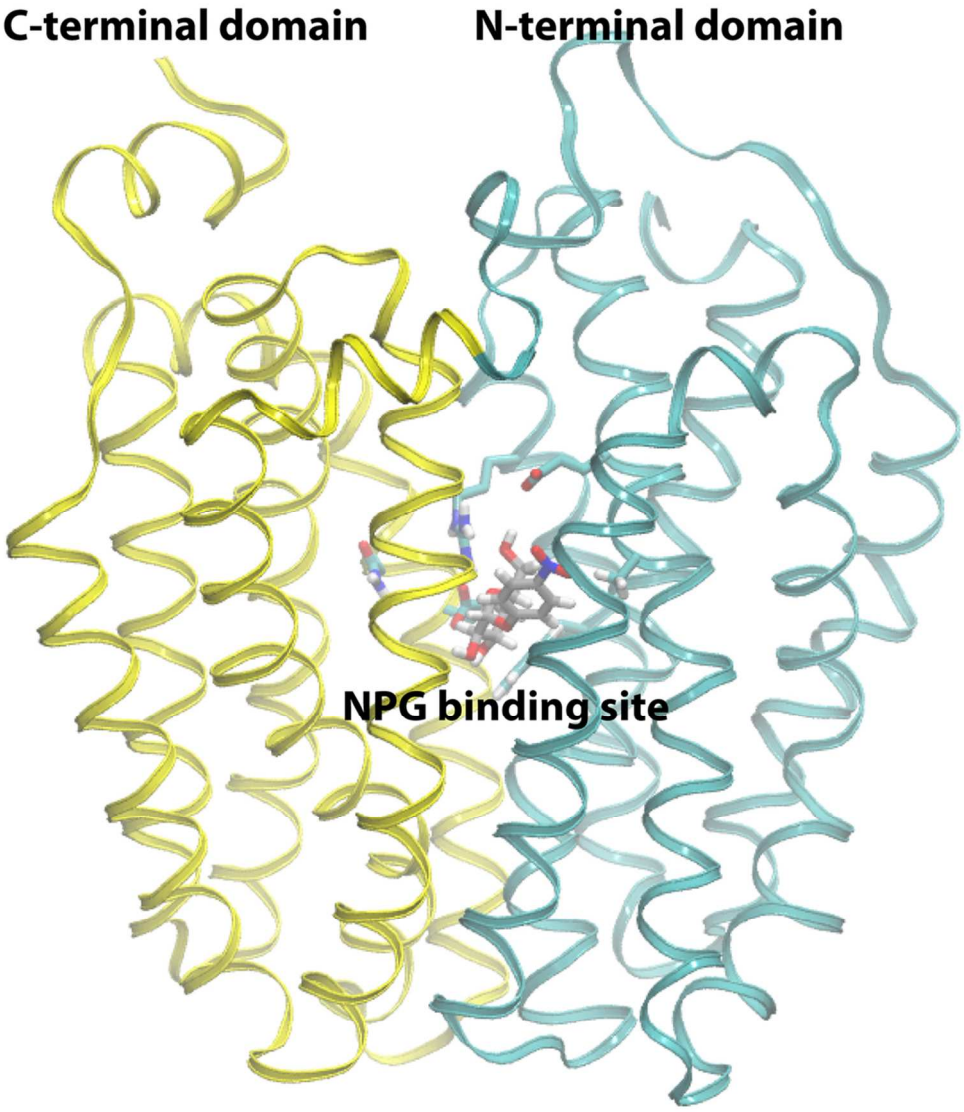

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Figure 1

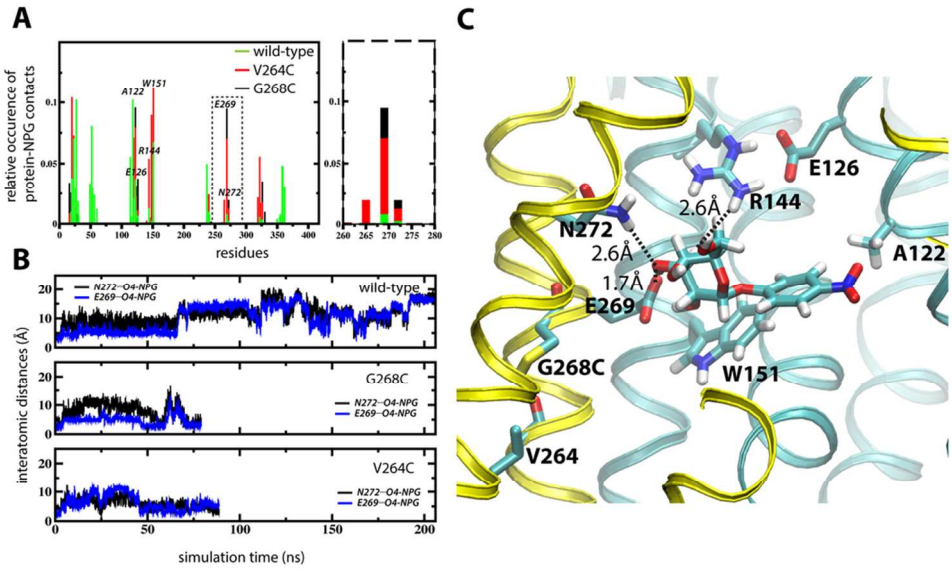

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Figure 2

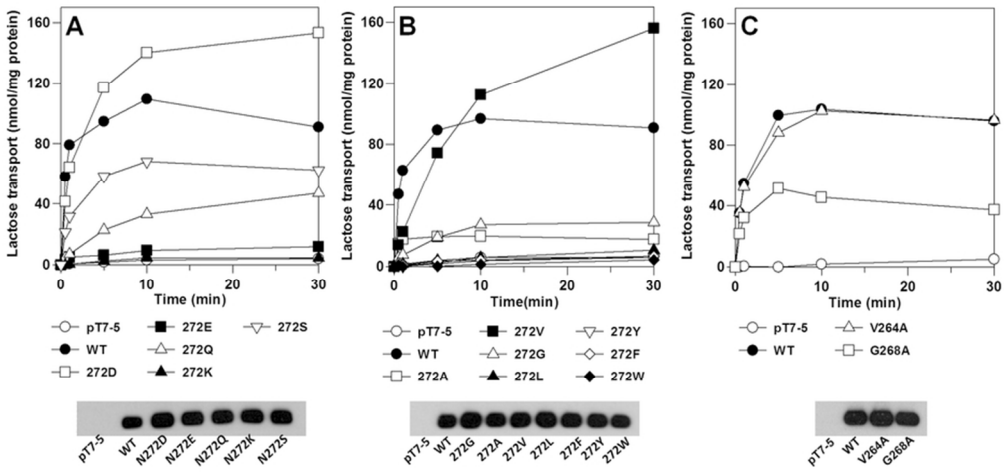

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Figure 3

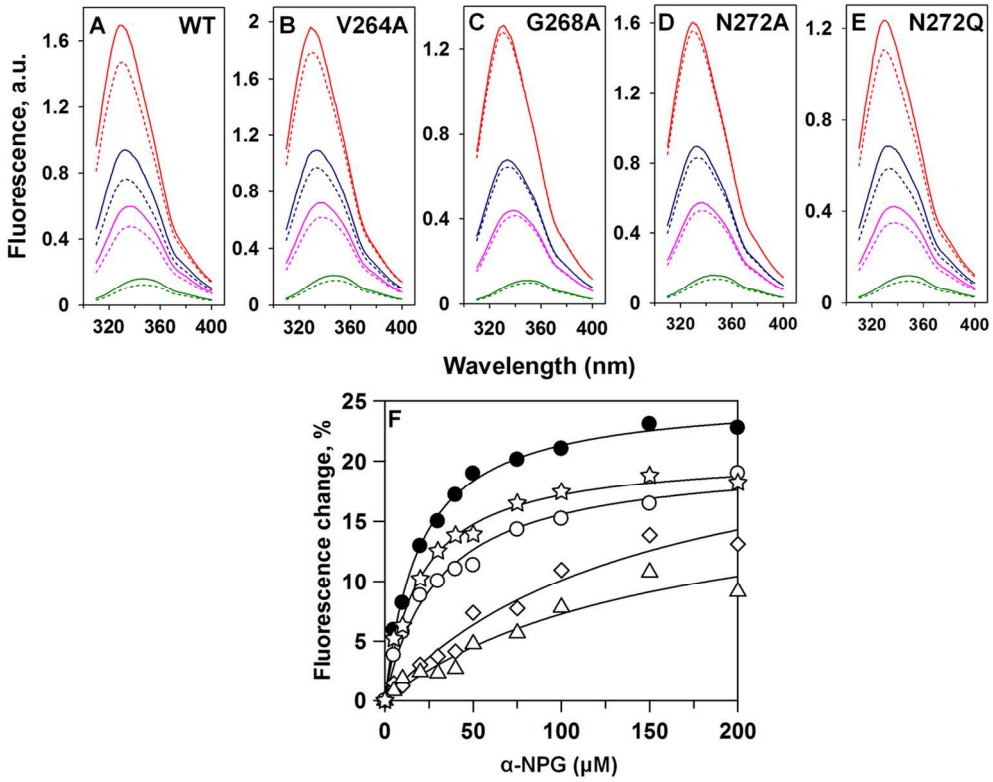

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Figure 4

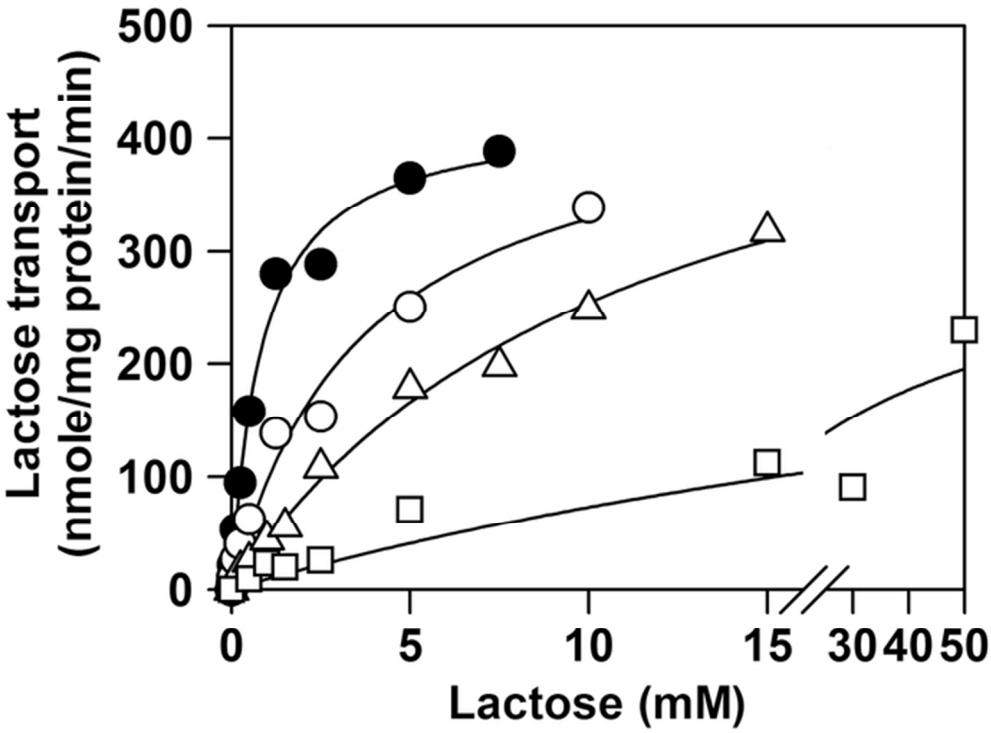

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Figure 5

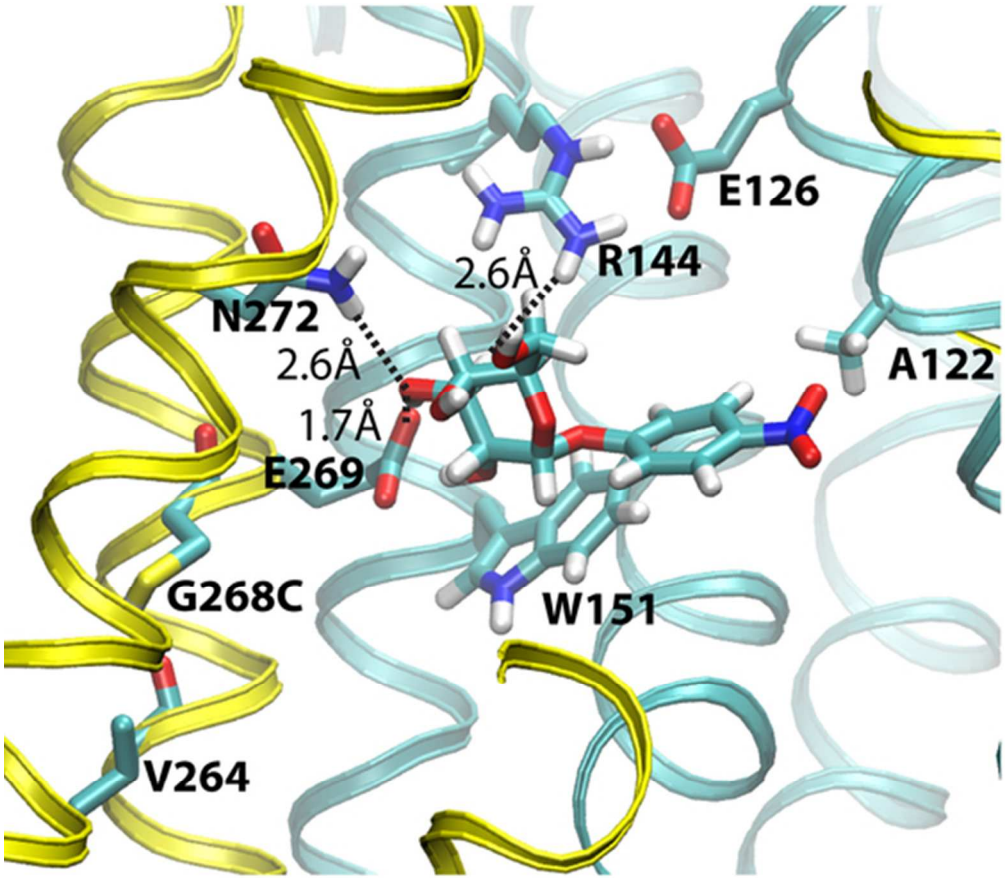

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Figure 6