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The Periplasmic Cavity of LacY Mutant Cys154→Gly: How Open is Open?

Xiaoxu Jiang[*], **Arnold J. M. Driessen**[†], **Ben L. Feringa**[☆], and **H. Ronald Kaback**[*,¶,§,‡]

[*]Department of Physiology, University of California Los Angeles, Los Angeles, California [¶]Departments of Microbiology, Immunology & Molecular Genertics, University of California Los Angeles, Los Angeles, California [§]Molecular Biology Institute, University of California Los Angeles, Los Angeles, California [†]Molecular Microbiology, Groningen Biomolecular Sciences Institute and Biotechnology Institute and Zernicke Institute for Advanced Materials, University of Groningen, Nijenborgh 7, 9747 AG, Groningen, The Netherlands [☆]Synthetic Organic Chemistry, Stratingh Institute for Chemistry and Zernike Institute for Advanced Materials, University of Groningen, Nijenborgh 4, 9747 AG, Groningen, The Netherlands

Abstract

The lactose permease from *Escherichia coli* (LacY) is a galactoside/H^+ symporter that catalyzes the coupled stoichiometric transport of a sugar and an H^+ across the cytoplasmic membrane. x-ray crystal structures of WT LacY and the conformationally restricted mutant Cys154→Gly exhibit an inward-facing conformation with a tightly sealed periplasmic side and a deep central cleft or cavity open to the cytoplasm. Although the crystal structures may give the impression that LacY is a rigid molecule, multiple converging lines of evidence demonstrate that galactoside binding to WT LacY induces reciprocal opening and closing of periplasmic and cytoplasmic cavities, respectively. By this means, the sugar- and H^+-binding sites in the middle of the molecule are exposed alternatively to either side of the membrane. In contrast to the crystal structure, biochemical/biophysical studies with mutant Cys154→Gly show that the periplasmic side is paralyzed in an open-outward conformation. In this study, a rigid, funnel-shaped, maleimide-containing molecule was used to probe the periplasmic cavity of a pseudo WT and the Cys154→Gly mutant by site-directed alkylation. The findings provide strong support for previous observations and indicate further that the external opening of the periplasmic cleft in the mutant is patent to the extent of at least 8.5 Å in the absence of sugar or about half that of the WT cavity with bound galactoside.

Keywords

membranes; transport; permease; membrane proteins; site-directed alkylation; protein dynamics

Introduction

The lactose permease of *Escherichia coli* (LacY) is a paradigm for the Major Facilitator Superfamily (MFS) of membrane transport proteins, arguably the largest family of such proteins. As a sugar/H^+ symporter, LacY catalyzes the coupled, stoichiometric translocation of a galactopyranoside and an H^+ across the cytoplasmic membrane (1, 2). To achieve active, energetically uphill sugar transport, the free energy released by downhill transport of H^+ in response to an electrochemical H+ gradient ($\Delta\tilde{\mu}H^+$, interior negative and/or alkaline)

[‡]Corresponding author rkaback@mednet.ucla.edu; phone: (310) 206-5053, Fax: (310) 206-8623.

is utilized to drive galactoside accumulation against a gradient. Since sugar and H^+ translocation are obligatorily coupled, in the absence of $\Delta\tilde{\mu}H^+$, LacY will utilize the energy released from downhill sugar transport to drive uphill H+ transport with generation of $\Delta\tilde{\mu}H^+$, the polarity of which depends on the direction of the imposed galactoside concentration gradient.

Several x-ray crystal structures of LacY, including the conformationally restricted C154G mutant (3, 4), WT LacY (5), and the most recently an inactivator-bound single-Cys mutant (6) have been solved. All of these structures exhibit the same inward-facing conformation with two pseudo-symmetrical six-helix bundles surrounding a deep hydrophilic central cleft with a sugar-binding site at the apex in the approximate middle of the molecule (Fig. 1A). The central cavity opens to the cytoplasm only, and the sugar-binding site is inaccessible from the tightly sealed periplasmic side. However, multiple studies (reviewed in ref. 7) provide ample evidence that galactoside binding specifically induces reciprocal opening and closing of hydrophilic periplasmic and cytoplasmic cavities, respectively, which allows alternating access of the sugar- and H^+-binding sites to either side of the membrane.

Relative to WT LacY, the conformationally restricted C154G mutant, which was first to crystallize, exhibits unique properties: (i) The mutant binds sugar with about twice the affinity of the WT, but has almost negligible transport activity (8, 9). (ii) Substrate binding is almost exclusively enthalpic, as opposed to the WT where binding is mostly entropic (10). (iii) Site-directed alkylation (SDA) in the native membrane (11), single molecule fluorescence resonance energy transfer (12) and double electron–electron resonance (DEER) (13) indicate that the periplasmic side is paralyzed in an open conformation and largely insensitive to ligand binding, while the cytoplasmic side closes upon sugar binding. Furthermore, SDA and sugar-binding studies in the native membrane suggest that C154G LacY assumes an intermediate state upon sugar binding, and the conformation of mutant C154G in the membrane may differ from the crystal structure because of the detergent used for solubilization and purification (14). (iv) The transport defect in mutant C154G (helix V) is rescued dramatically by replacing Gly24 (helix I) with Cys (15). Since Cys154 and Gly24 are located at precisely the site where the two helices cross in the middle of the membrane, this finding in addition to thiol cross-linking experiments (16) suggest that sugar binding to LacY causes a local scissors-like movement between helices V and I near the point where the two helices cross.

Although the periplasmic side of mutant C154G appears to be significantly more open than in the WT, how open is open? In this study, a rigid funnel-like molecule containing a maleimide, 1-(4-trityl-phenyl) pyrrole-2,5-dione (TAM1), with a diameter of 8.5 Å (17) was employed as a probe to approximate the degree of opening of the periplasmic cavity of mutant C154G relative to the pseudo-WT (with Val in place of Cys154) in right-side-out (RSO) membrane vesicles. The results indicate that the outermost region of the periplasmic opening in C154G LacY is constitutively open to the extent of at least 8.5 Å.

Materials and Methods

Materials

TAM1 containing a maleimide was synthesized as described previously (17). Tetramethylrhodamine maleimide (TMRM; T-6027) was obtained from Molecular Probes, Invitrogen Corp. (Carlsbad, CA). ImmunoPure immobilized monomeric avidin was obtained from Pierce, Thermo Scientific Inc. (Rockford, IL). Protease inhibitor cocktail was obtained from Roche (Indianapolis, IN). Oligonuclectides were synthesized by Intergrated DNA Technologies (Coralville, CA). *p*-Nitrophenyl-α,ᴅ-galactopyranoside (NPG) was obtained

from Sigma-Aldrich (St. Louis, MO). All other materials were reagent grade and purchased from commercial sources.

Plasmid Construction

Plasmid pT7-5 with a cassette *lacY* gene encoding single-Cys replacements in C154V LacY (pseudo WT) or C154G LacY with a C-terminal biotin acceptor domain followed by a 6-His tag were constructed as described previously (18). DNA sequencing of the entire *lacY* gene verified all mutants.

Growth of Bacteria

Plasmid pT7-5 encoding a given single-Cys replacement was transformed into *E. coli* T184 [*lacI+* $O^+Z^-Y^-$*(A) rpsL* met^-thr^-*recA hsdM hsdR/F′* $lacI^q$ O^+ Z^{D118} (Y^+A^+)] (19). Transformed cells were grown in 0.5 L Luria-Bertani broth containing 0.1 mg/mL ampicillin at 37°C with shaking. Cells were induced by 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) at an OD_{600} of 0.6 and harvested by centrifugation for the preparation of right-side-out (RSO) membrane vesicles.

Preparation of RSO Membrane Vesicles

RSO membrane vesicles were prepared from 0.5 L cultures of *E. coli* T184 expressing a given single-Cys replacement by lysozyme-EDTA treatment and osmotic lysis (20, 21). The vesicles were resuspended to a total protein concentration of 10-20 mg/mL in 0.1 M potassium phosphate (KP_i; pH 7.5) containing 10 mM $MgSO_4$, flash frozen in liquid nitrogen and stored at −80°C until use.

SDA of LacY in RSO Membrane Vesicles

TAM1 and TMRM were dissolved in DMSO at stock concentrations of 10 mM and 1 mM, respectively. RSO membrane vesicles [0.1 mg total protein in 50 μL 0.1 M KPi (pH 7.5)/10 mM $MgSO_4$/protease inhibitor cocktail] containing a given single-Cys replacement were incubated with 400 μM TAM1 in the absence or presence of 1 mM NPG at room temperature. After adding DTT (20 mM, final concentration) at given times to stop the reaction, unreacted TAM1 was removed by diluting the vesicles with 1 mL of 0.1 M KP_i (pH 7.5)/10 mM $MgSO_4$ and centrifuging at 14,000 rpm for 10 min at 0°C. After washing twice, the vesicles were resuspended in 50 μL of 0.1 M KP_i (pH 7.5)/10 mM $MgSO_4$ and incubated with 5 mM NPG (except for labeling of single-Cys C148) on ice for 10 min. TMRM was then added to a final concentration of 40 μM, and the samples were further incubated on ice for 30 min. The vesicles were solubilized with 2.0% DDM and incubated with 50 μL monomeric avidin beads for 20 min at room temperature. The mixture was loaded on to a Wizard mini column (Promega, Madison, WI) and washed with 3 mL of washing buffer [50 mM NaP_i (pH 7.5), 0.1 M NaCl, 0.02% DDM]. Biotinylated LacY was eluted from the column with 40 μL of elution buffer [50 mM NaP_i (pH 7.5)/0.1 M NaCl/5 mM biotin/0.02% DDM]. Purified proteins were subjected to SDS-PAGE, and the gels were scanned by using an Amersham Typhoon 9410 Workstation (λ_{ex} = 532 nm and λ_{em} = 580 nm for TMRM) right away. The protein bands were developed subsequently by silver staining. Band intensity of fluorescent signals from TMRM labeled or silver-stained LacY bands were quantified by using ImageQuant 5.0 (Molecular Dynamics, GE Healthcare Bio-Sciences Corp). A background from the appropriate gel was subtracted. Specific TMRM labeling was calculated by dividing the fluorescent signal by the protein signal, and the specific TMRM labeling of the sample without added TAM1 was normalized to 1. Each set of data was fit by using GraFit 6 (Erithacus Software Limited) with the equation for a single exponential decay with offset:

$$y = A_0 . e^{-kt} + \text{offset}$$

Results

In this study, we chose seven positions within the tightly packed periplasmic side of LacY and one close to the sugar-binding site to test TAM1 accessibility/reactivity by SDA (Fig. 1A). The reactivity/accessibility of each single-Cys mutant was compared in Cys-less LacY with either Val (pseudo-WT) or Gly in place of Cys154. Since TAM1 is neither fluorescent like TMRM nor radioactive like N-ethylmaleimide (NEM), labeling cannot be studied directly, and the ability of TAM1 to block TMRM labeling was used as an assay.

Mutant S311C

Ser311 is located at the periplasmic end of helix X (Fig. 1A), and TMRM rapidly labels a Cys replacement at this position in a manner that is independent of the presence of galactoside (22). RSO membrane vesicles containing single-Cys311 LacY with Val or Gly at position 154 react rapidly with TAM1, as shown by inhibition of TMRM labeling. Thus, with both mutants, rapid inhibition of labeling by the fluorescent probe TMRM is observed over the first 30s, and maximum inhibition occurs by 1 min (Fig. 2).

Mutants P31C, K42C, K49C or Q242C

Position 31 is located on helix I ∼8Å away from the periplasmic end at the mouth of the periplasmic cavity (Fig. 1A). In the pseudo-WT C154V background, mutant P31C does not react with TAM1 in the absence of sugar. But in the presence of NPG, very significant reactivity is observed, and essentially complete inhibition of TMRM labeling is observed in ∼20 min (Fig. 3A). In the C154G background, TAM1 reacts with mutant P31C in the absence of sugar at a slightly lower rate than in the pseudo-WT background in the presence of sugar, and almost complete inhibition of TMRM labeling is observed in ∼30 min.

Lys42 is ∼4 Å from the periplasmic end of helix II (Fig. 1A). Like mutant P31C, in the single-Cys C154V background, mutant K42C reacts poorly if at all with TAM1 in the absence of sugar. In the presence of NPG, relatively slow reactivity is observed with ∼60% inhibition of TMRM labeling in 30-40 min. In the C154G background, TAM1 reacts with mutant K42C in the absence of sugar at about the same rate as that observed in C154V background in the presence of NPG (Fig. 3B).

Phe49 is ∼13 Å from the periplasmic end of helix II (Fig, 1A). Unlike mutants P31C and K42C, mutant F49C exhibits good reactivity with TAM1 in the absence of sugar. Complete inhibition of TMRM labeling is observed in ∼20 min (Fig. 3C). Much more rapid inhibition is observed when sugar is present, and inhibition reaches its maximum at ∼1 min. In the C154G background, TAM1 reacts with mutant F49C in the absence of sugar at a significantly faster rate than in the pseudo-WT background, but slightly slower than in the pseudo-WT background with sugar. Completion of inhibition of TMRM labeling is observed in ∼5 min.

Gln242 is ∼9Å from the periplasmic end of helix VII. In the pseudo-WT background, mutant Q242C also reacts relatively slowly with TAM1 in the absence of sugar, but by 40 min, inhibition of TMRM labeling by TAM1 is ∼40% complete (Fig. 3D). NPG binding markedly stimulates TAM1 reactivity as indicated by inhibition of labeling by the fluorescent probe, which is essentially complete in 5 min. But unlike the P31C, K42C and

F49C mutant, in the C154G and pseudo-WT backgrounds, TAM1 reactivity with Q242C in the absence of sugar is about the same.

Mutants F27C and T265C

Phe27 (helix I) and Thr265 (helix VIII) are ∼15Å and ∼14Å, respectively, from the periplasm and relatively close to the sugar-binding site. Little or no TAM1 reactivity is observed for single-Cys F27C in the C154V background in the absence of NPG, and low reactivity is induced by NPG binding. In the C154G background, mutant F27C reacts faster with TAM1 in the absence of NPG than the same mutant in the C154V background with NPG. However, only ∼50% inhibition of TMRM reactivity is observed by 40 min (Fig. 4A). Mutant Thr265 behaves in similar fashion with little or no TAM1 reactivity in the C154V in the absence or presence of NPG and relatively slow reactivity (∼60% inhibition at 40 min) in the C154G background in the absence of ligand (Fig. 4B).

Single-Cys C148

Native Cys148 is ∼25 Å from the periplasmic end of helix V in very close proximity to the sugar-binding site (Fig. 1A), and it is well documented that binding of galactosides protects Cys148 from alkylation (23, 24). In the absence of NPG, single-Cys148 in either the pseudo-WT or particularly the C154G background exhibits essentially no reactivity with TAM1 (Fig. 5).

Discussion

All crystal structures of LacY to date (3-6) exhibit indistinguishable inward-facing conformations that are tightly sealed on the periplasmic side and open to the cytoplasm. But SDA with either NEM or TMRM of single-Cys replacements in the pseudo-WT (C154V) shows that the accessibility/reactivity of Cys replacements on the periplasmic side is very low in the absence of sugar and increases dramatically upon galactoside binding. Moreover, single-Cys replacements on the cytoplasmic side of LacY behave in opposite fashion, thereby providing important additional evidence in the native bacterial membrane for the alternating access mechanism (reviewed in 7, 25). In striking contrast, with mutant C154G, the same periplasmic Cys replacements label rapidly in the absence of bound ligand, indicating that the periplasmic aspect of the C154G mutant is constitutively open, and sugar binding has little effect (11, 14). But how open is open? This is an important consideration since aqueous accessibility is required to lower the pK_a of Cys and increase reactivity.

The pK_a for the ionizable thiol group in Cys in aqueous solution is ∼9.1, and at this pH, the thiol group is ∼50% protonated statistically and relatively unreactive, and ∼50% is negatively charged and considerably more reactive. But when a Cys is buried in a hydrophobic environment, as in the tightly sealed periplasmic environment of LacY, in addition to accessibility to thiol reagents, the pK_a is elevated, causing significantly lower reactivity. Thus, the explanation for the increased labeling of single-Cys replacements on the periplasmic side of LacY upon sugar binding is likely two-fold: (i) increased physical accessibility to thiol reagents; and (ii) increased exposure to water, which decreases the pK_a with increased reactivity. Since we are unable to differentiate between the two alternatives, the term ‘accessibility/reactivity’ seems appropriate.

In this study, the maleimide-containing thiol reagent TAM1 was used to probe the potential cavity on the periplasmic side of C154G LacY. Rather than containing a planar ring system like TMRM (18) or being small like NEM (25), the three phenyl groups of TAM1 are symmetrically distributed to form a right-circular-cone shape geometry with a defined base diameter of 8.5 Å, and an overall height of ∼11 Å (Fig. 1B). Steric hindrance between the

phenyl groups provides rigidity (17). These unique structural features make TAM1 an excellent probe for our purpose, and the maleimide group exhibits relatively good reactivity with an exposed Cys replacement (Fig. 2).

Pro31 (helix I), Lys42 (helix II) and Gln242 (helix VII) occlude the mouth of the periplasmic cavity, while Phe49 (helix II), Phe27 (helix I) and Thr265 (helix VIII) are located ∼2/3 the distance to the sugar-binding site and native Cys148 is in close proximity to the binding site. Previous studies utilizing NEM or TMRM for SDA demonstrate that labeling of individual single-Cys replacements at six of these seven positions exhibit a marked increase upon galactoside binding, indicating that Cys replacements at these residues are good candidates for probing TAM1 accessibility/reactivity (11, 18, 22, 25-28). With native Cys148 (helix V), which is close to the sugar-binding site, galactoside binding blocks alkylation (23, 29).

In the C154G background, the Cys replacements at position 31 and 49 are readily labeled by TAM1 in the absence of sugar in contrast to the same Cys replacements in the pseudo-WT C154V background, which is clearly dependent upon NPG binding (Fig. 3A & C). Thus, a Cys at position 31 and 49 is much more accessible/reactive in the C154G background than in the pseudo-WT background. Since the depths of position 31 (∼8 Å) and position 49 (∼13 Å) are similar or larger than the height of the TAM1 molecule (∼11 Å), the periplasmic cavity in the C154G mutant must be open at least 8.5 Å to allow access of Cys31 and Cys49 to TAM1. Lys42 is located at the end of helix II, which is more external than position 31 (Fig. 1A). In the pseudo-WT background, mutant K42C exhibits very little labeling in the absence of sugar, and NPG binding accelerates TAM1 labeling. The labeling rate of mutant K42C in the C154G background without sugar is about the same as that in the pseudo-WT background in the presence of NPG (Fig. 3B). Again, this result shows that this position is more accessible in the C154G background than in the pseudo-WT in the absence of sugar. In TAM1, the distance from the sp^3 hybridization carbon to the double bond of maleimide is ∼8.5 Å, which is greater than the depth of position 42 (5∼6 Å). Therefore, TAM1 may label K42C without the funnel entering the cavity, and appropriately, the labeling rates are very similar.

Q242 (helix VII) is approximately 9 Å from the periplasmic end of helix VII. In the pseudo-WT background without sugar, a Cys at position 242 labels slowly with TAM1, and NPG binding markedly stimulates the rate and extent of TAM1 labeling (Fig. 3D). However, unlike mutant P31C and 49C, labeling of a Cys at position 242 in the C154G background exhibits a slow labeling rate similar to that of mutant Q242C in the pseudo-WT background in the absence of sugar. This finding differs from those observed with TMRM labeling (11, 14, 30) where the labeling rate of mutant Q242C in the C154G background is much higher than in the pseudo-WT background in the absence of sugar. Thus, it appears that position 242 is more accessible/reactive in the C154G background as shown by TMRM labeling. This difference is probably due to the physical properties of the thiol reagents; the planar, hydrophobic nature of TMRM may allow access to position 242, while the spherical, rigid structure of TAM1 may not.

Helices I and VII restrict access of sugar on the periplasmic side of LacY, and both thiol crosslinking (31) and DEER measurements (13) indicate that the maximum opening of the periplasmic cavity upon galactoside binding is 15-17 Å. Combining the TAM1 labeling results at position 242 with those obtained with mutants P31C and F49C, it is reasonable to conclude that in the native membrane, as opposed to the x-ray structure, the opening of the periplasmic cavity in the C154G mutant is fixed in a conformation that is open to the extent of at least 8.5 Å.

Mutants F27C (helix I) and T265C (helix VIII) are about 15 Å from the periplasmic side of LacY (Fig. 1A). No labeling is observed at these positions in the pseudo-WT background without addition of sugar. NPG binding slightly enhances labeling of mutant C154V/F27C, but has no effect on mutant C154V/265C (Fig. 4A & B). In C154G background, labeling is mildly increased for Cys replacements at both positions, indicating that the cavity is probably somewhat more open than in the pseudo-WT with bound sugar even at this depth.

Clearly, native Cys148, located in the approximate middle of LacY in close proximity to the sugar-binding site, is not labeled by TAM1 in either pseudo-WT C154V or mutant C154G background (Fig. 5). Thus, although the cavity in C154G mutant is largely open towards the periplasm, in the vicinity of Cys148, it is too narrow to allow access of TAM1. The same cannot be concluded for the pseudo-WT because in the absence of bound sugar, the entire cavity is closed. In any event, the findings presented here strongly confirm and extend the conclusion that the structure of the C154G mutant in the native bacterial membrane must be significantly open on the periplasmic face of LacY, in contradistinction to the x-ray structure, where it is tightly closed.

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Abbreviations

DDM	*n*-dodecyl-β-D-maltopyranoside
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
IPTG	isopropyl β-D-1-thiogalactopyranoside
NEM	N-ethylmaleimide
NPG	*p*-Nitrophenyl-α, D-galactopyranoside
RSO	right-side-out
SDA	site-directed alkylation
TAM1	1-(4-trityl-phenyl) pyrrole-2,5-dione
TMRM	tetramethylrhodamine maleimide

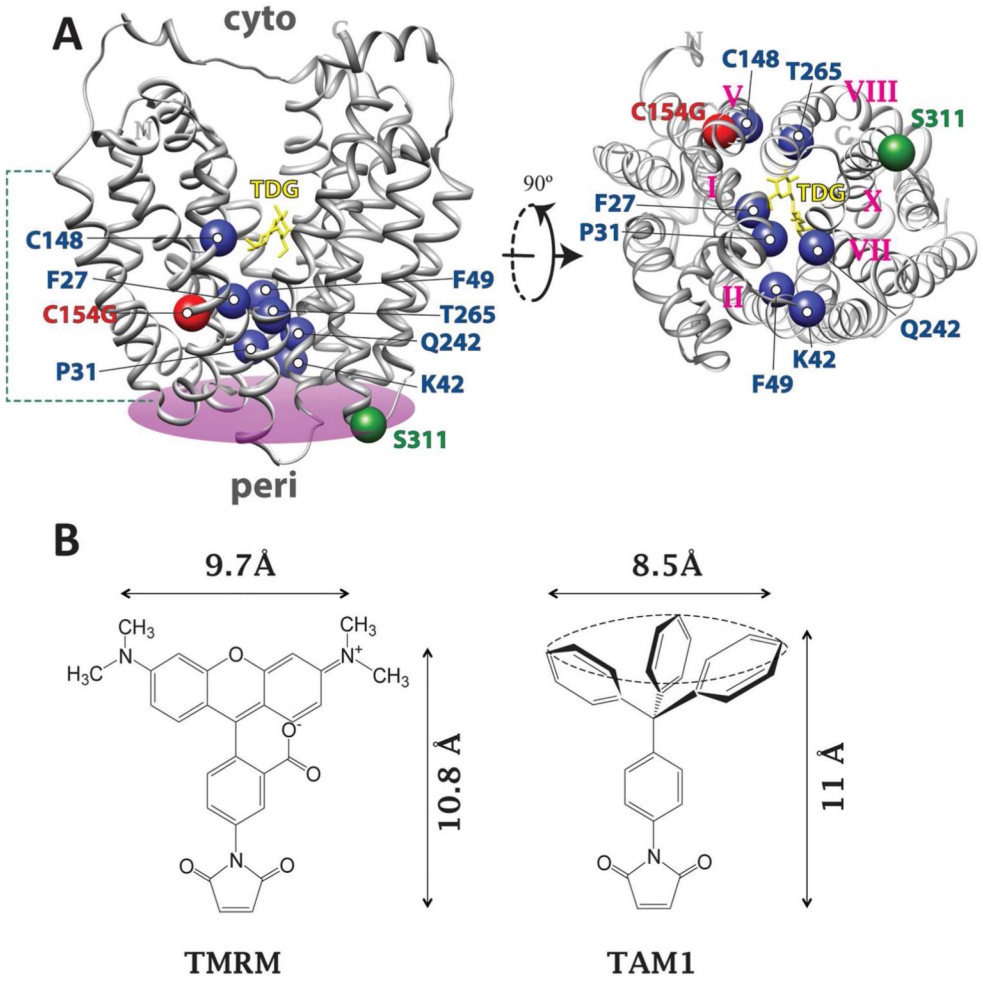

Fig. 1. Single-Cys replacements tested in this study

(A) Left, 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. TDG is shown as yellow stick at the apex of the central cavity. The C_α carbons of F27 (helix I), P31 (helix I), K42 (helix II), F49 (helix II), C148 (helix V), Q242 (helix VII) and T265 (helix VIII) are superimposed on the backbone of C154G LacY as blue spheres. A plane was defined (colored in purple) based on the position of five helix-terminal residues (D36, Q100, Q256, T310 and E374) on the periplasmic side of LacY to define the border of the coherent structural domain. The distance of each Cα carbon to the periplsmic side of LacY is measured as the distance from each C_α carbon to the defined plane on the periplamic side of LacY: 27, 15 Å; 31, 8 Å; 42, 4 Å; 49, 13 Å; 148, 25 Å, 242, 9 Å; 265: 14 Å. The C_α carbon of G154 (helix V) and S311 (helix X) are presented as red and green spheres, respectively. The green dashed lines indicate the dimension of the cytoplasmic membrane. Right, the image of LacY on the left side is turned 90° and viewed from the periplasmic side. Roman number labels the helices where the single-Cys replacements are located. (B) The chemical structures of TMRM and TAM1.

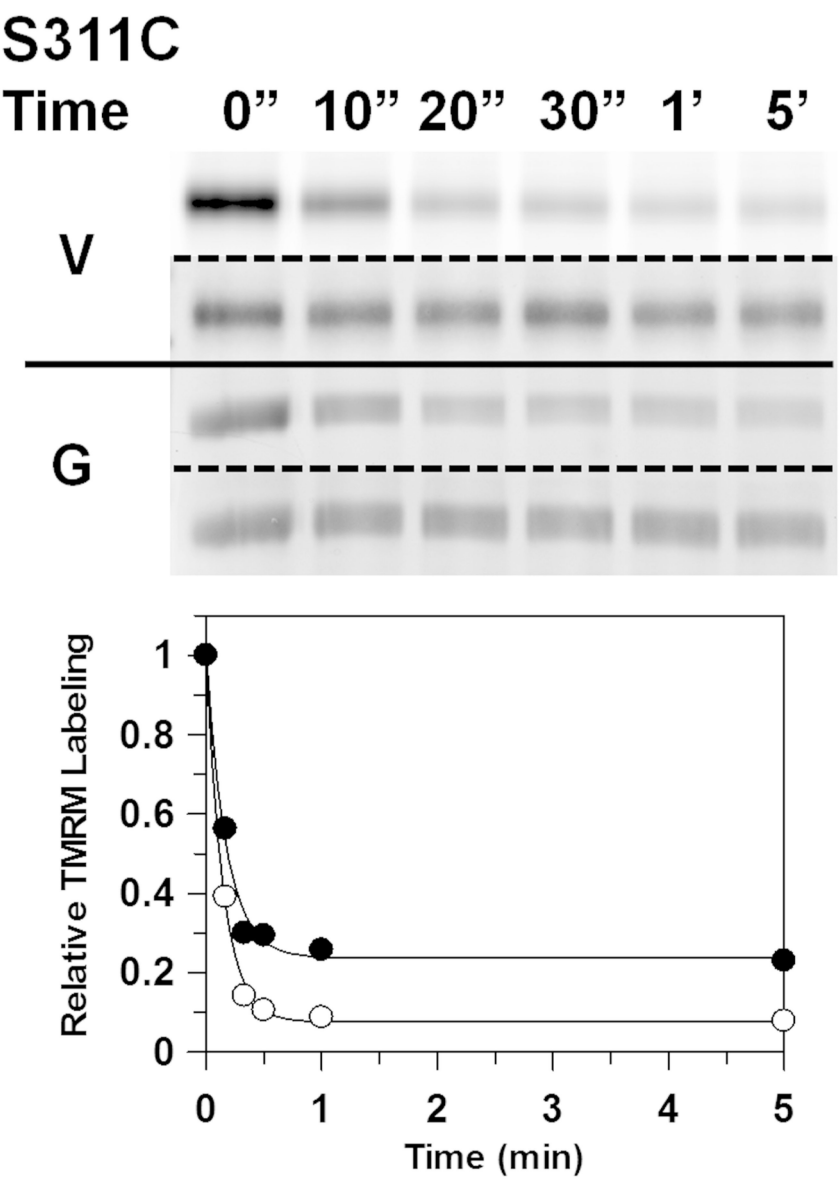

Fig. 2. SDA of single-Cys S311C

RSO membrane vesicles containing single-Cys C154V/S311C (○) or single-Cys C154G/S311C (●) were incubated with 400 μM TAM1 for the given times at room temperature in the absence of sugar. After terminating the reaction and removing the remaining TAM1, the vesicles were labeled with 40 μM TMRM at 0°C in the presence of 5 μM NPG for 30 min. LacY was solubilized and purified in DDM by monomeric avidin chromatography and subjected to SDS-PAGE. The gel was scanned for TMRM fluorescence (bands above the dotted lines) and silver stained for protein (bands below the dotted lines). Relative TMRM labeling was calculated semi quantitatively as described in *Materials and Methods*. Relative TMRM labeling of the 0-sec TAM1 labeling point was set to 1. V, C154V background; G, C154G background.

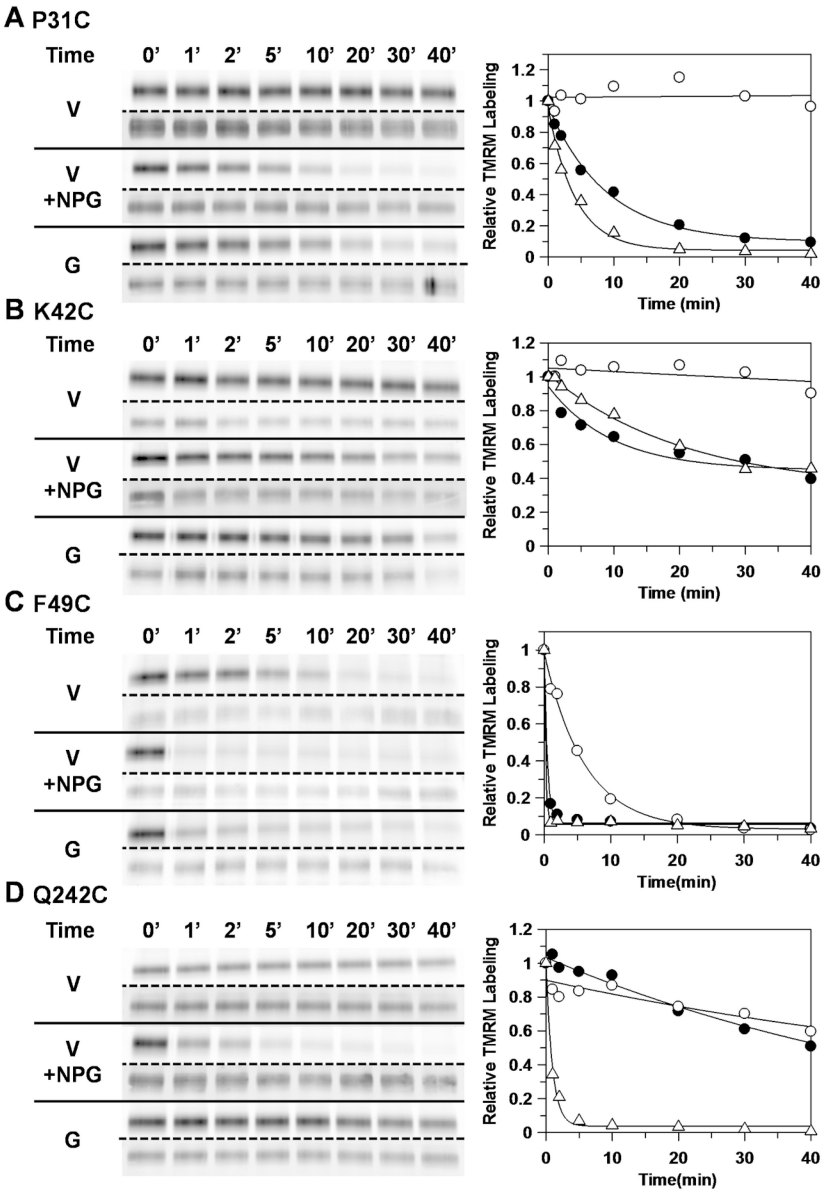

Fig. 3. SDA of single-Cys mutants P31C (A), K42C (B), F49C (C), and Q242C (D)

RSO membrane vesicles containing a designated single-Cys mutant in the C154V background were incubated with TAM1 in the absence (○) or presence (△) of 1 mM NPG. Vesicles containing the same single-Cys mutants in the C154G background was incubated with TAM1 in the absence of sugar (●). Experiments and data analysis were carried out as described in Fig. 2.

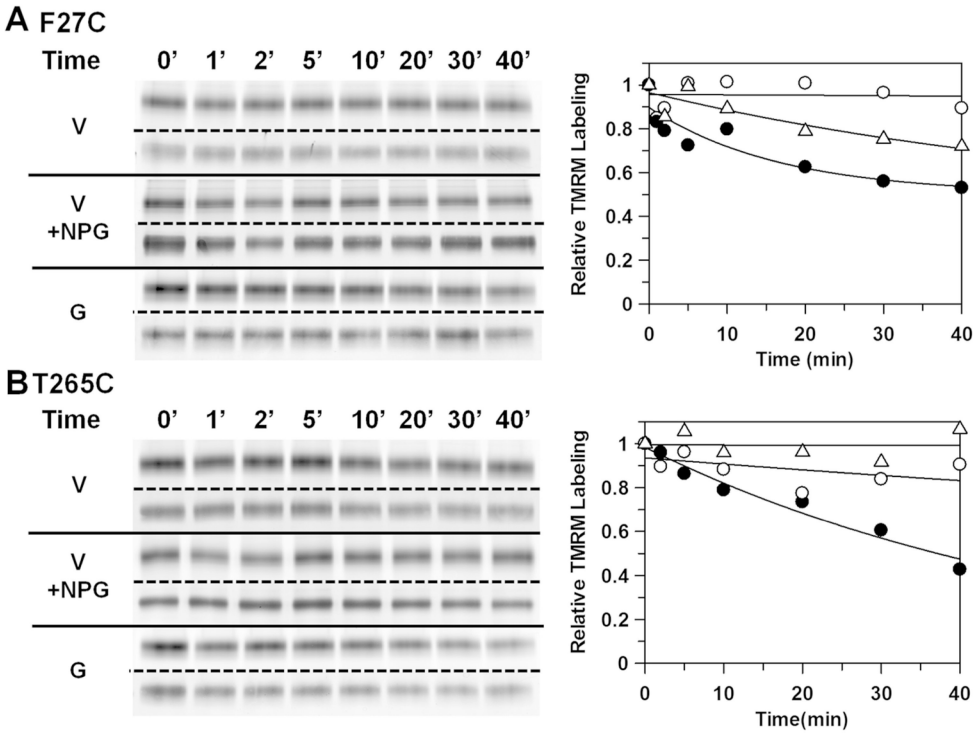

Fig. 4. SDA of single-Cys F27C (A) or T265C (B)

RSO membrane vesicles containing the designated single-Cys mutant in the C154V background were incubated with TAM1 in the absence (○) or presence (△) of 1 mM NPG. Vesicles containing the same single-Cys mutants in the C154G background were incubated with TAM1 in the absence of sugar (●). Experiments and data analysis were carried out as described in Fig. 2.

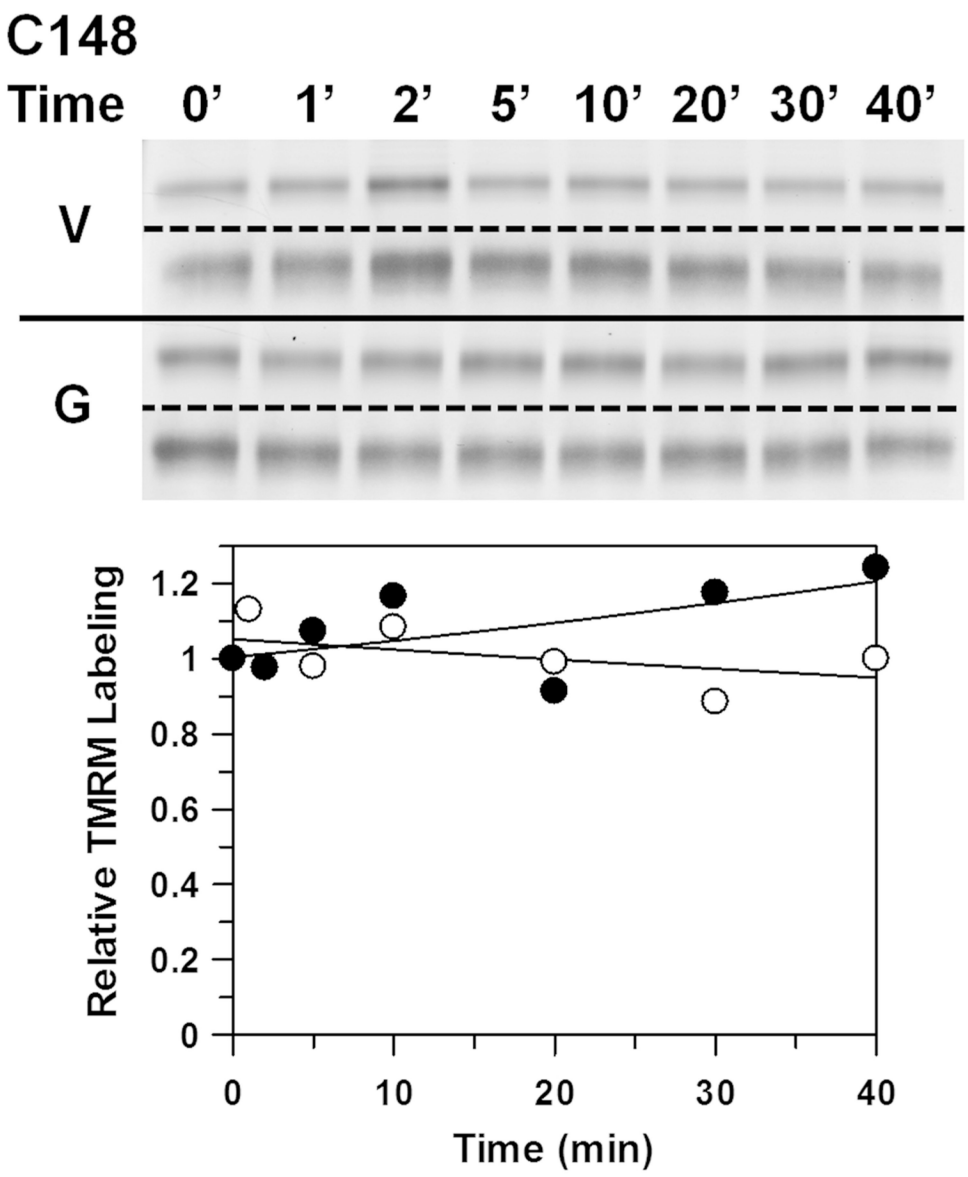

Fig. 5. SDA of single-Cys C148

RSO membrane vesicles containing single-Cys mutant C148 in the C154V background (○) or the C154G background (●) were incubated with TAM1 in the absence of sugar. Experiments and data analysis were carried out as described in Fig. 2.