A Mutation in the Lactose Permease of *Escherichia coli* That Decreases Conformational Flexibility and Increases Protein Stability†

Irina N. Smirnova and H. Ronald Kaback*

Howard Hughes Medical Institute, Departments of Physiology and Microbiology and of Immunology and Molecular Genetics, Molecular Biology Institute, University of California, Los Angeles, California 90095-1662

Received December 9, 2002; Revised Manuscript Received January 14, 2003

ABSTRACT: Lactose permease with Cys154 → Gly (helix V) binds substrate with high affinity but catalyzes little or no transport. The purified, detergent-solubilized mutant protein exhibits much greater thermal stability than the wild type and little tendency to aggregate. Stabilization is also observed in vivo with an unstable mutant that is expressed at significantly higher levels when the Cys154 → Gly mutation is introduced. In addition, ligand-induced conformational changes are markedly reduced or abolished by the Cys154 → Gly mutation: (i) Although the fluorescence of purified single Trp33 (helix I) permease is enhanced by ligand binding, introduction of the Cys154 → Gly mutation abolishes the effect. (ii) The rate of 2-(4′-maleimidylanilino)naphthalene-6-sulfonic acid (MIANS) labeling of permease with a single Cys residue in place of Val331 (helix X) is increased in the presence of ligand but reduced when the Cys154 → Gly mutation is present. (iii) Fluorescence emission intensity of MIANS-labeled single Cys331 permease is enhanced and blue shifted in the Cys154 → Gly mutant background, indicating that the latter mutation causes position 331 to become exposed to a less polar environment. The results indicate that the Cys154 → Gly mutation causes a more compact structure and decreased conformational flexibility, an alteration that specifically blocks the structural changes necessary for substrate translocation with little or no effect on ligand binding.

Typical of many transport proteins from *Archaea* to the mammalian central nervous system, the lactose permease of *Escherichia coli* (LacY)[1] transduces free energy stored in electrochemical ion gradients into a solute concentration gradient and vice versa (*1*). LacY is a polytopic cytoplasmic membrane protein that forms a 12-helix bundle with the N and C termini on the cytoplasmic face of the membrane and catalyzes the stoichiometric translocation of galactosides and H^+ (galactoside/H^+ symport) (*2*−*4*). Several lines of evidence indicate that LacY is both functionally (see ref *5*) and structurally a monomer (*6*−*8*). Application of a variety of site-directed biochemical, spectroscopic, and immunological techniques to an extensive library of mutants, particularly single Cys mutants at each position of the protein (*9*), has allowed the formulation of a tertiary structure model (*10*). Furthermore, experimental observations from structural and extensive mutational analysis have led to a postulated mechanism for lactose/H^+ symport (*11*).

Although approximately 70% of the side chains in LacY are hydrophobic, the protein appears to be in a highly dynamic state. Most remarkably, as reported recently (*12*), the N-terminal half of LacY adopts an inverted conformation relative to the C-terminal half in cells lacking phosphatidylethanolamine, and the conformation is reversed when the phospholipid is synthesized. Furthermore, as shown by attenuated total reflectance Fourier transform infrared spectroscopy (*13*), the average helix tilt in LacY is ca. 51° at a low lipid:protein ratio and decreases to about 33° at high lipid:protein ratios, and this change is reflected by an increase in transport activity. In addition, about 85% of the backbone protons exchange with deuterium in 10 min (*14*, *15*), while KcsA, the prokaryotic K^+ channel, requires 3 h to exchange only about 45% of its backbone protons for deuterium (*14*). In view of these and other properties of LacY (see ref *11*), it is not surprising that LacY, as well as some of its homologues, has resisted rigorous attempts at crystallization for over a decade.

Early site-directed mutagenesis experiments on the native Cys residues in LacY led to the isolation and characterization of a mutant with Gly in place of native Cys154 (helix V) (*16*, *17*). Remarkably, the C154G mutant binds ligand as well as or better than wild-type LacY but does little or no translocation across the membrane. Thus, the mutant appears to be locked in an outwardly facing conformation that binds ligand with high affinity but is unable to undergo the structural changes required for transport of sugar across the membrane. Consistently, the mutant does not bind IIA^{Glc}, a regulatory component of the phosphoenolpyruvate:glucose phosphotransferase system, on its cytoplasmic surface in the presence of ligand (*18*).

† This work was supported in part by NIH Grant DK51131:07 to HRK.

* To whom correspondence and reprint requests should be addressed at HHMI/UCLA, 5-748 Macdonald Research Laboratories, Box 951662, Los Angeles, CA 90095-1662. Telephone: (310) 206-5053. Telefax: (310) 206-8623. E-mail: RonaldK@HHMI.UCLA.edu.

[1] Abbreviations: LacY, lactose permease; NPG, *p*-nitrophenyl α-D-galactopyranoside; MIANS, 2-(4′-maleimidylanilino)naphthalene-6-sulfonic acid, sodium salt; TDG, β-D-galactopyranosyl-1-thio-β-D-galactopyranoside; NEM, *N*-ethylmaleimide; DDM, *n*-dodecyl β-D-maltopyranoside; DTT, dithiothreitol; KP_i, potassium phosphate; NaP_i, sodium phosphate; $NaDodSO_4$−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; HRP, horseradish peroxidase; DMSO, dimethyl sulfoxide.

10.1021/bi027329c CCC: $25.00
Published on Web 02/19/2003

In this paper, we describe the properties of the C154G mutant, both in the membrane and after solubilization and purification in dodecyl β-D-maltopyranoside (DDM). The results demonstrate clearly that this single amino acid change remarkably alters the physical properties of LacY, causing it to bind ligand with higher affinity in a manner that is resistant to inactivation by heat. In addition, the protein does not aggregate readily in the cold, unlike wild-type LacY, nor does it exhibit certain long-range conformational changes observed upon ligand binding.

EXPERIMENTAL PROCEDURES

Materials. Deoxynucleotides were purchased from Sigma-Genosys (Woodlands, TX), restriction endonucleases and T4 DNA ligase were from New England Biolabs (Beverly, MA), and Pfu-turbo DNA polymerase was from Stratagene (La Jolla, CA). *E. coli* HB101 was purchased from Promega (Madison, WI) and XL1-Blue was from Biocrest Mfg. (Cedar Creek, TX). Penta-His antibody−HRP conjugate was obtained from Qiagen (Valencia, CA) and avidin−HRP from Pierce (Rockford, IL). *p*-Nitrophenyl α-D-[6-^{3}H]galactopyranoside (NPG) was kindly provided by Gérard Leblanc (Laboratoire J. Maetz/Commissariat a l'Energie Atomique, Ville Franche-sur-Mer, France). Yeast inorganic pyrophosphatase was from Sigma (St. Louis, MO). His-tagged biotin ligase (*19*) was expressed in *E. coli* XL1-Blue cells grown at 30 °C in Luria−Bertani broth with 100 μg/mL ampicillin and purified on Ni-NTA resin (Qiagen, Valencia, CA) according to manufacturer's instructions. Pefabloc was from Centerchem (Norwalk, CT). All other materials were of reagent grade and obtained from commercial sources.

Construction of LacY Mutants. The C154G mutation was introduced into pT7-5/cassette *lacY* (EMBL X56095) encoding LacY with single Trp33 and a biotin acceptor domain in the middle cytoplasmic loop (*20*, *21*) or a His_6 tag at the C terminus by using the QuickChange PCR method (Stratagene, La Jolla, CA) with a 30-base PCR primer (sense 5′-GCGCTGGGCGCCTCAATTGTCGGGATCATG). A unique *Mfe*I site was created in order to facilitate mutant detection. Spontaneous primer duplication during PCR was eliminated by using a lower temperature during extension (66−67 °C). The same method was used to replace Val154 with Gly in Cys-less LacY (*22*) with a C-terminal biotin acceptor domain followed by a His_6 tag. The DNA fragment from cassette *lacY* encoding single Cys331 permease was then inserted into Cys-less *lacY* or *lacY* encoding mutant C154G by restriction fragment replacement. The full length of all *lacY* genes was sequenced to confirm the mutations introduced and detect unwanted mutations. The plasmids were then transformed into *E. coli* HB101 (*lac*Z^+Y^-) for qualitative assessment of downhill lactose transport on MacConkey indicator plates containing 20 mM lactose.

Transport Measurements. Active transport of [1-^{14}C]-lactose (5 mCi/mmol) was measured in *E. coli* T184 (*lacZ*$^-$*Y*$^-$) harboring given plasmids. Cells were grown at 37 °C in Luria−Bertani broth containing 100 μg/mL ampicillin and induced with 0.3 mM isopropyl 1-thio-β-D-galactopyranoside. Cells were harvested by centrifugation after 2 h, washed with ice-cold 100 mM potassium phosphate (KP_i, pH 7.2)/10 mM $MgSO_4$, and adjusted to an OD_{120} of 10 (0.7 mg of protein/mL). Aliquots (50 μL) were equilibrated at room temperature, and [1-^{14}C]lactose was added to a final concentration of 0.4 or 4 mM, as indicated. Reactions were quenched at given times by addition of 2.0 mL of 100 mM KP_i (pH 5.5)/100 mM LiCl followed by immediate filtration (*23*). Radioactivity retained on the filters was assayed by liquid scintillation spectrometry.

Western Blots. Membrane fractions from the cells used in transport assay were prepared by osmotic lysis and sonication as described (*24*). Protein was assayed on each sample by using the MicroBCA method (Pierce, Rockford, IL) prior to electrophoresis in sodium dodecyl sulfate−12% polyacrylamide gels ($NaDodSO_4$−PAGE). Proteins were electroblotted onto polyvinylidene fluoride membrane (Immobilon-PVDF; Millipore, Badford, MA) and probed with HRP-conjugated antibody directed against the His tag or with avidin−HRP using Super Signal West Pico chemiluminescent substrate (Pierce, Rockford, IL). After exposure of the membrane to film, the density of the bands was quantitated by scanning with a Chemiimager 4400 (Alpha Innotech, San Leandro, CA).

Purification of LacY. LacY was purified from membranes from *E. coli* XL1-Blue transformed with a given plasmid essentially as described (*25*) except that extraction with DDM was carried out in 50 mM sodium phosphate (NaP_i, pH 7.2) in the absence of phospholipids. After insoluble material was removed from the DDM extract by centrifugation, affinity chromatography was carried out by either of two means: (i) LacY mutants with a His tag were purified on Ni^{2+}-NTA Sepharose (QIAGEN, Chatsworth, CA) as described (*25*). Protein eluted with 300 mM imidazole was dialyzed against 50 mM NaP_i (pH 7.5)/10% glycerol/0.008% DDM, concentrated by centrifugation with a Vivaspin 20 concentrator (30 kDa cutoff; Vivascience, Germany), and stored on ice. (ii) Mutants with a biotin acceptor domain were purified on monomeric avidin−Sepharose (Pierce, Rockford, IL) as described (*26*) after in vitro biotinylation (*27*). The membrane fraction (total volume of 15 mL from 20−25 g cells wet weight) was incubated for 30−40 min at 30 °C in 30 mM Tris-HCl (pH 8.0)/3.0 mM ATP/5.0 mM $MgSO_4$/2.0 mM *d*-biotin/10 mM β-mercaptoethanol/0.12 mg/mL Pefabloc/1 unit/mL inorganic pyrophosphatase/0.1 mg/mL biotin ligase. Biotinylated membranes were washed twice in 50 mM NaP_i (pH 7.5) to eliminate unbound biotin before solubilization with DDM. The detergent extract was slowly (0.5 mL/min) loaded onto a monomeric avidin−Sepharose column pretreated with biotin according to the manufacturer's instructions and washed extensively with column buffer [50 mM NaP_i (pH 7.5)/0.01% DDM], and LacY was eluted with 5 mM *d*-biotin in column buffer. Eluted protein was concentrated by centrifugation with a Vivaspin 20 concentrator (50−100 kDa cutoff) and stored on ice before use. The Micro BCA method (Pierce, Rockford, IL) was used to measure protein. All LacY preparations were at least 90−95% pure as judged by silver staining after $NaDodSO_4$−PAGE.

Flow Dialysis. Binding of *p*-nitrophenyl α-D-[6-^{3}H]galactopyranoside (NPG) was measured by flow dialysis (*28*). NPG bound to LacY was calculated from the flow dialysis profile as a percentage of the radioactivity released after addition of β-D-galactopyranosyl-1-thio-β-D-galactopyranoside (TDG). Dissociation constants (K_d) for NPG were calculated from the equation:

$$K_d = [P]_f[N]_f/[PN]_b$$

where $[P]_f$ is concentration of free protein, $[N]_f$ is concentration of unbound NPG, and $[PN]_b$ is the concentration of NPG bound to LacY.

Thermal Inactivation. Protein solutions in 50 mM NaP_i (pH 7.5)/150 mM NaCl/0.02% DDM were incubated at 50 °C for given times and transferred to ice for a minimum of 20−30 min. Prior to use, the samples were equilibrated to room temperature. Where indicated, precipitated protein was removed by centrifugation.

MIANS Labeling. The concentration of 2-(4′-maleimidylanilino)naphthalene-6-sulfonic acid (MIANS, sodium salt) was determined in dimethyl sulfoxide (DMSO) by absorbance at 322 nm using an extinction coefficient of 17000 (*29*). To record the time course of MIANS labeling, protein was preincubated for 10 min in 50 mM NaP_i (pH 7.5)/150 mM NaCl/0.02% DDM, and the reaction was initiated by addition of a given concentration of MIANS (0.5% DMSO final concentration). For complete modification of Cys residues in single Cys331 mutants, a 10-fold molar excess of MIANS over protein was added, and after overnight incubation at 4 °C, unbound MIANS was removed by passage through a Sephadex G-50 spin column.

Fluorescence Measurements. Fluorescence was measured at room temperature in an SLM-Aminco 8100 instrument (Urbana, IL) using 1 × 1 cm cuvettes (2 mL) with constant stirring. Emission spectra and time courses were recorded with slits of 4 and 8 mm, respectively, for excitation and emission.

RESULTS

Transport. As shown previously (*16*, *17*), replacement of native Cys154 with Gly in wild-type LacY drastically decreases transport activity to almost the same level as that of cells devoid of LacY (Figure 1A). Similar effects of the C154G mutation are observed in different mutant backgrounds. Thus, LacY with single Trp33 catalyzes lactose accumulation at high rate to a steady-state level comparable to that of wild-type LacY (*20*). However, after introduction of the C154G mutation, transport activity is almost abolished (Figure 1A). Similarly, Cys-less LacY (*22*) or single Cys331 LacY (*30*) exhibits robust transport activity (50−60% of wild type), but both mutants are practically inactive when the C154G mutation is introduced (Figure 1B). Although data are not shown, the same results are obtained when the lactose concentration is increased 10-fold.

Effect of C154G on LacY Expression. Although expression of the C154G mutant is consistently increased to only a minor extent relative to wild-type LacY (Figure 1C, compare lanes 1 and 2), introduction of the mutation into single Trp33 reproducibly results in a 2−3-fold increase in the level of expression (compare lanes 3 and 4), and a similar effect is observed when the mutant is expressed in *E. coli* HB101 or XL1-Blue (not shown). In contrast, the C154G mutation has little effect on Cys-less or single Cys331 LacY, both of which are more stable [Figure 1D; note the higher apparent MW of the biotinylated mutants (D) relative to the His-tagged mutants (C)]. Generally, the expression level of wild-type LacY or the C154G mutant is 15−20% of the total membrane protein, about 10% with Cys-less or single Cys331, and 2−4% for single Trp33.

Effect of C154G on Substrate Binding and Thermal Stability. A typical flow dialysis profile for purified wild-type LacY solubilized in DDM is shown in Figure 2A (solid symbols). At the inception of the experiment, [^{3}H]NPG is added to the upper chamber containing purified, solubilized LacY, and radioactivity in the dialysate increases rapidly to a maximum (fraction 4) and then decreases at a slow rate. When excess TDG is added to the upper chamber, bound [^{3}H]NPG is displaced, and the concentration of radioactivity in the dialysate increases. Relatively high concentrations of protein (120−140 μM) are required in order to detect NPG binding, because the affinity of wild-type LacY decreases by about an order of magnitude in DDM (from a K_d of ca. 14 μM to ca. 250 μM) (*31*, *32*). Wild-type LacY is unstable at 50 °C, and after 15 min, the protein precipitates and completely loses the ability to bind substrate. Thus, addition of excess TDG does not cause an increase in dialyzable [^{3}H]-NPG (Figure 2A, open symbols).

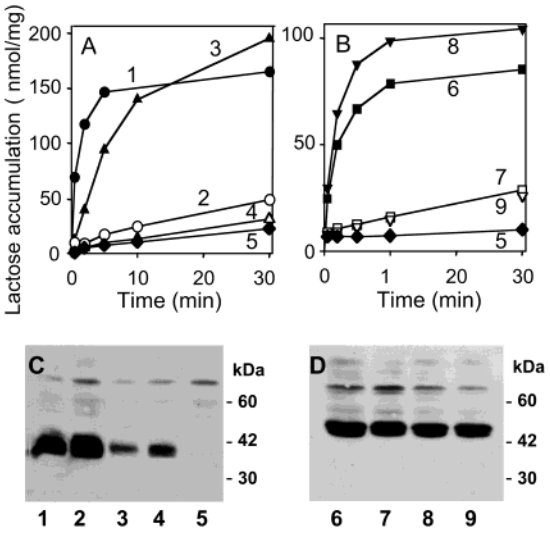

FIGURE 1: Effect of C154G mutation on transport activity and membrane expression of LacY mutants. Aliquots (50 μL) of cell suspensions containing 35 μg of total protein were assayed at 0.4 mM lactose as described in Experimental Procedures. (A) Time courses of active lactose transport by *E. coli* T184 expressing wild-type LacY (curve 1), mutant C154G (curve 2), single Trp33 (curve 3), single Trp33/C154G (curve 4), or no LacY (cells transformed with pT7-5 with no *lacY* insert; curve 5). (B) Time courses of active lactose transport by *E. coli* T184 expressing Cys-less LacY (curve 6), Cys-less/C154G (curve 7), single Cys331 (curve 8), single Cys331/C154G (curve 9), or no LacY (cells transformed with pT7-5 with no *lacY* insert; curve 5). (C and D) Expression levels of LacY in membrane fractions isolated from cells used for the transport experiments shown in panels A and B. Membrane preparations containing 40 μg of protein per sample were subjected to Na-$DodSO_4$−PAGE and Western blot analysis using a His-tagged antibody−HRP conjugate (C) or avidin−HRP (D) as described in Experimental Procedures.

In marked contrast, purified C154G LacY in DDM exhibits about 10-fold higher affinity for NPG (i.e., K_d of 30−60 μM) and much greater thermal stability. Precipitation is much slower, and even after 2 h at 50 °C, the mutant binds NPG at least half as well as the unheated control sample with an unaltered K_d (Figure 2B, Table 1). It is also noteworthy that mutant C154G can be frozen in liquid nitrogen and thawed with little or no aggregation or loss of binding activity and that the mutant is stable for at least 2 months at 0 °C (data not shown).

MIANS Labeling. Ligand protection against MIANS labeling of LacY provides a more convenient binding assay than

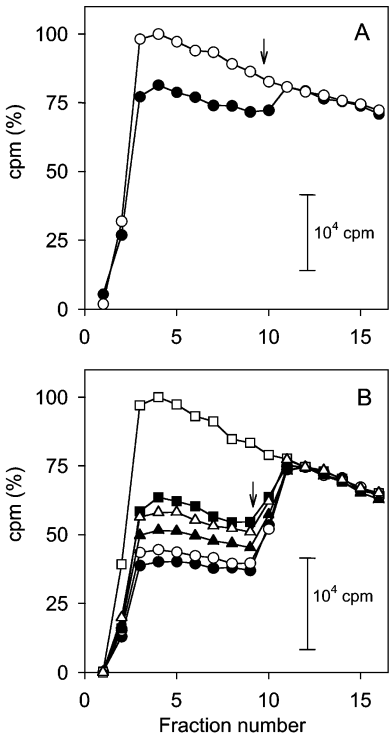

FIGURE 2: Effect of C154G mutation on substrate binding and thermal stability of purified LacY in DDM. Given samples of purified LacY were heated at 50 °C for the times indicated, and precipitated protein was removed by centrifugation at 25000*g* for 5 min. The supernatant was aspirated, and 200 μL was placed in the upper chamber of a flow dialysis apparatus; 10 μM [^{3}H]NPG (950 mCi/mmol) was added at fraction 1. As indicated by the arrow, TDG (2 μL) was added to a final concentration of 10 mM to displace bound [^{3}H]NPG. (A) Flow dialysis profiles comparing [^{3}H]-NPG binding to 140 μM wild-type LacY before (●) and after (○) 15 min incubation at 50 °C. Results are expressed as a percentage of the maximum [^{3}H]NPG level in dialysate (fraction 4) after protein thermal inactivation. (B) Flow dialysis profiles comparing [^{3}H]NPG binding to C154G LacY (57 μM) before incubation at 50 °C (●) and after 10 min (○), 30 min (▲), 60 min (△), and 125 min (■) at 50 °C. Results are expressed as a percentage of the maximum [^{3}H]-NPG level in the dialysate (fraction 4) when 10 mM TDG was added prior to [^{3}H]NPG (□). The pellets recovered by centrifugation after heating did not bind NPG to any degree whatsoever.

Table 1: Effect of C154G Mutation on Thermal Inactivation of NPG Binding[a]

preincubation at 50 °C (min)	protein in solution (μM)	NPG bound (%)	K_d (μM)
	57	61	32
10	41	58	25
30	30	53	22
60	26	41	32
125	19	37	26

[a] Data are taken from Figure 2B.

flow dialysis, as it is more sensitive, requires less protein, and is much less dependent upon the affinity of LacY for ligand (*31*, *33*−*35*). At low MIANS concentrations (4−8 μM), the reagent covalently labels primarily one of the eight native Cys residues in LacY, Cys148, in a manner that is blocked by ligand (*36*). Since MIANS is a sulfhydryl-specific probe that is not fluorescent until the maleimide group undergoes chemical reaction (*29*, *37*), the time course of the reaction can be studied readily (Figure 3). Addition of MIANS to a reaction mixture containing purified LacY in

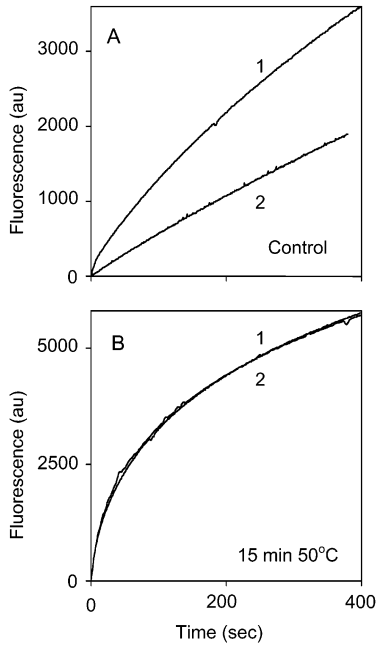

FIGURE 3: Thermal inactivation of substrate protection against MIANS labeling of wild-type LacY. Time courses of MIANS fluorescence emission were recorded at 420 nm (excitation wavelength 330 nm). The reaction was carried out in 50 mM NaP_i (pH 7.5)/150 mM NaCl/0.02% DDM containing 50 μg/mL purified wild-type LacY. Fluorescence labeling was initiated by addition of 4 μM MIANS at zero time. Traces were recorded without added TDG or with 10 mM sucrose (trace 1) or after addition of 10 mM TDG (trace 2). Panels: (A) without heating; (B) samples preincubated for 15 min at 50 °C.

DDM results in a rapid, almost linear increase in fluorescence emission intensity for over 5 min, and preincubation with TDG clearly decreases the rate of the reaction, while sucrose, which is not a ligand for LacY, has no effect (Figure 3A). On the other hand, after the sample is treated at 50 °C for 15 min, the rate of reaction increases significantly, and TDG protection is completely abrogated (Figure 3B).

When similar experiments are carried out with the C154G mutant, several differences are observed (Figure 4): (i) the rate of the reaction is slower than observed with wild-type LacY (compare Figure 3A and Figure 4A; note difference in scales); (ii) heating at 50 °C does not increase the rate of the reaction but causes a slow decrease (upper traces in Figure 4B−D); and (iii) the protective effect of TDG is only moderately affected after 30 min at 50 °C, and even after 2 h, significant protection is still observed (lower traces). Thus, the C154G mutation appears to make Cys148 less accessible to/reactive with MIANS and ligand binding more resistant to thermal inactivation.

Effect of the C154G Mutation on Ligand-Induced Conformational Changes. Although the C154G mutant binds ligand with higher affinity than wild-type LacY, it is essentially unable to transport sugar across the membrane, suggesting that the protein is locked conformationally and unable to undergo the structural changes required for translocation. Several positions in LacY that are not directly involved in ligand binding have been shown to reflect long-range conformational changes. For example, Trp33 at the periplasmic end of helix I exhibits about a 60% increase in fluorescence emission intensity upon ligand binding (Figure 5A) (*21*). Strikingly, introduction of the C154G mutation

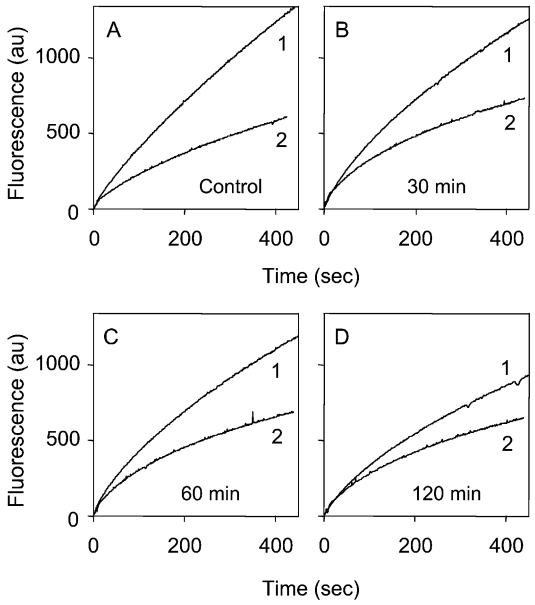

FIGURE 4: Thermal inactivation of substrate protection against MIANS labeling of mutant C154G. The experiments were carried out as described in Figure 3. (A) Mutant C154G labeled with MIANS prior to incubation at 50 °C, (B) after 30 min at 50 °C, (C) after 60 min at 50 °C, and (D) after 120 min at 50 °C. MIANS was added at zero time. Traces were recorded without TDG (trace 1) or after addition of 10 mM TDG (trace 2).

completely eliminates the effect of TDG on Trp fluorescence in the single Trp33 mutant (Figure 5B) with little or no effect on the ability of the mutant to bind ligand, as judged by TDG protection of Cys148 against MIANS labeling (Figure 5C).

V331C LacY (cytoplasmic end of helix X) is another mutant that reflects ligand-induced conformational changes. Membrane vesicles containing a single Cys residue in place of Val331 become less accessible to/reactive with NEM in the presence of TDG and simultaneously label more efficiently with the more hydrophobic sulfhydryl reagent 7-(diethylamino)-3-(4′-maleimidylphenyl)-4-methylcoumarin (*38*). Moreover, purified single Cys331 permease reacts with MIANS, and addition of TDG elicits an increase in the rate of MIANS labeling with a decrease in the maximum level of fluorescence. The C154G mutation was introduced into single Cys331 LacY to test its effect on ligand-induced changes in MIANS labeling. Purified single Cys331 and single Cys331/C154G LacY were adjusted to the same final protein concentration, and the time course of MIANS labeling was studied (Figure 6). Single Cys331 LacY reacts at a relatively slow rate with MIANS, and in confirmation of previous results (*38*), TDG increases the rate of the reaction, but the maximum level of fluorescence is decreased (Figure 6A). Introduction of the C154G mutation causes dramatic changes: (i) The rate of MIANS labeling is significantly decreased, and the level of fluorescence achieved is slightly enhanced (compare trace 1 in panels A and B of Figure 6). (ii) In contradistinction to the effect observed with single Cys331, addition of TDG markedly decreases the rate of MIANS labeling (compare traces 2). (iii) When the data are normalized to the same final level of fluorescence for comparative purposes, it is clear that single Cys331/C154G LacY exhibits a reduced rate of MIANS labeling by a factor of 2.5 in the absence of ligand. Furthermore, the difference

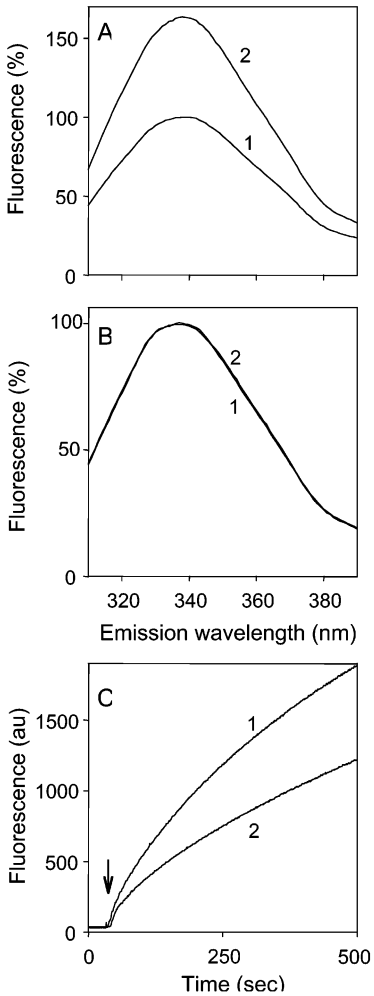

FIGURE 5: Effect of C154G mutation on substrate-induced change in single Trp33 fluorescence. Emission spectra of single Trp33 (A) or single Trp33/C154G LacY (B) were recorded with 40−60 μg/mL protein at an excitation wavelength of 295 nm before (scan 1) and after (scan 2) addition of 10 mM TDG. Addition of 10 mM sucrose had no effect in either the absence or presence of TDG. (C) TDG protection of Cys148 against alkylation by MIANS in single Trp33/C154G LacY. Fluorescence was recorded using 40 μg/mL protein and 2 μM MIANS in the absence (trace 1) or presence of 10 mM TDG (trace 2) as described in Figure 3. The arrow indicates MIANS addition.

in the rate of MIANS labeling between single Cys331 and single Cys331/C154G LacY in the presence of TDG is very marked (Figure 6C; estimated half-times are about 1 and 14 min, respectively).

The emission spectra of the MIANS-labeled mutants also demonstrate important differences (Figure 7). Clearly, the fluorescence intensity of MIANS-labeled single Cys331 LacY (curve 1) is enhanced by about 30% by introduction of the C154G mutation, and the emission maximum is blue shifted (curve 2). The findings indicate that the C154G mutation causes the fluorophor at position 331 to become accessible to an environment with the lower dielectric.

DISCUSSION

Despite an increase in high-resolution membrane protein structures that have been solved in the past few years, the number of such structures remains miniscule relative to the soluble protein structures that have been solved, which now well exceeds 12000. In addition to their hydrophobicity

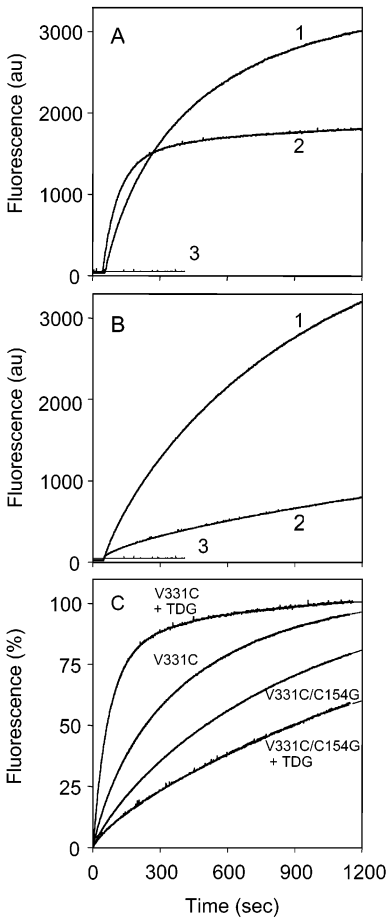

FIGURE 6: Effect of C154G mutation on the rate of MIANS labeling of single Cys331 LacY. (A) TDG effect on labeling of single Cys331 LacY. (B) TDG effect on labeling of single Cys331/C154G LacY. The experiments were carried out as described in Figure 3 with 85 μg/mL (1.5 μM) protein and 2 μM MIANS. Traces were recorded without TDG (trace 1), after addition of 10 mM TDG (trace 2), or in the absence of protein (trace 3). (C) All traces were normalized to the same maximum fluorescence intensity after subtraction of the fluorescence observed without protein and fit with an exponential equation (Sigmaplot 2001 software; SPSS Science, Chicago, IL). Estimated half-times are 1.0, 3.3, 8.5, and 14 min for V331C + TDG, V331C, V331C/C154G, and V331C/C154G + TDG, respectively.

necessitating detergent for solubilization and the frequent sparsity of hydrophilic domains which appear to be important for stabilizing a crystal lattice, many membrane proteins, ion-coupled transport proteins in particular, require a high degree of flexibility for function. LacY is a relatively small, hydrophobic, 12 transmembrane helix bundle with small exposed hydrophilic domains that is in a highly dynamic state. These properties and the strong tendency of LacY to aggregate in detergent have foiled rigorous attempts at crystallization for over a decade. This difficulty has led to the development of a battery of site-directed techniques for approximating tertiary structure (*10*) which are also useful for studying the mechanism of lactose/H^+ symport (*11*). However, a high-resolution crystal structure is sorely needed in order to verify conclusions derived from the indirect approaches to structure and function.

Most of the site-directed techniques that have been developed involve replacement of specific residues with Cys in a functional LacY mutant devoid of its eight native Cys residues (*22*). Prior to the construction of functional Cys-

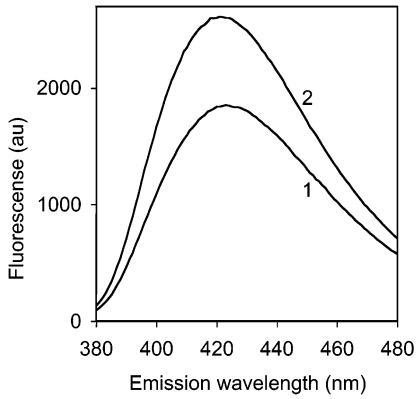

FIGURE 7: Fluorescence emission spectra of MIANS-labeled single Cys 331 (scan 1) and single Cys331/C154G LacY (scan 2). Emission spectra were recorded at an excitation wavelength of 330 nm after MIANS labeling was complete. The extent of labeling by MIANS and the final concentrations of both labeled proteins (85 μg/mL) were precisely the same as determined by UV absorption.

less LacY, each of the native Cys residues was mutated individually. Site-directed mutagenesis was used initially to replace Cys148 with Gly (*39*, *40*) or Ser (*41*, *42*). Although Cys148 is required for substrate protection against alkylation by NEM, it is not important for lactose/H^+ symport. Subsequently, it was shown (*3*, *16*) that replacement of Cys154 with Gly leads to complete loss of transport activity although the permease binds the high-affinity ligand NPG normally. In contrast, replacement of Cys154 with Ser or Val yields permease with 10% or 30%, respectively, of the wild-type rate, indicating that although Cys154 is needed for full activity, it is not mandatory. Cys176 or Cys234 (*43*) and Cys117, Cys333, or Cys353 and Cys355 (*44*) were then replaced with Ser with little or no effect on activity. Therefore, out of a total of eight Cys residues in LacY, only Cys154 appears to be important for transport, but even this residue is not essential.

Since the C154G mutant binds ligand but catalyzes little or no transport, it seems reasonable to conclude that the mutant is locked in a form that binds substrate but cannot undergo the structural changes required for translocation. In addition, in inside-out membrane vesicles, the C154G mutant does not undergo the conformational change(s) required for IIA^{Glc} binding in the presence of ligand (*18*). With this reasoning in mind, C154G was purified and characterized in the hope that it would make a better candidate for crystallization than the structurally dynamic wild-type protein.

In confirmation of previous findings, although C154G LacY catalyzes transport at an almost negligible rate, after solubilization and purification in DDM, the protein binds NPG with an affinity that is about 10 times better than that of wild-type LacY. Since the affinity of the mutant is only slightly better than that of the wild type in situ (*17*), the increased affinity observed with purified C154G LacY in DDM relative to the wild type is likely due to decreased conformational flexibility of the mutant. In any event, it is clear that C154G LacY is remarkably more stable than the wild type with respect to temperature, as judged by binding experiments using either flow dialysis or substrate protection against MIANS labeling. It is also important that mutant C154G does not aggregate after freezing and thawing and that it is stable at 4 °C for at least 2 months with respect to ligand binding.

Site-directed fluorescence studies on single Trp33 and mutant V331C demonstrate that long-range conformational changes induced by ligand binding in these mutants are either completely blocked or markedly slowed when the C154G mutation is introduced. In addition, MIANS-labeled single Cys331/C154G exhibits an increase in fluorescence and a blue shift in the emission spectrum, indicating that the cytoplasmic end of helix X is displaced to a more hydrophobic environment. Taken together, the observations provide a strong indication that replacing native Cys154 with a Gly residue dramatically changes the physical properties of LacY, locking the protein in a more compact conformation that binds ligand but cannot efficiently catalyze substrate translocation. Therefore, the C154G mutant may be a good candidate for crystallization.

ACKNOWLEDGMENT

We are indebted to Gillian Verner for providing some of the purified LacY samples and to Vladimir Kasho for many helpful discussions and suggestions. We also thank Dorothy Beckett for kindly providing a strain that overproduces biotin ligase with a His_6 tag and Gérard Leblanc for generously providing [^{3}H]NPG.

REFERENCES

1. Konings, W. N., Kaback, H. R., and Lolkema, J. S. (1996) *Handbook of Biological Physics: Transport Processes in Eukaryotic and Prokaryotic Organisms*, Vol. II, Elsevier, Amsterdam.
2. Kaback, H. R. (1976) *J. Cell. Physiol. 89*, 575−593.
3. Kaback, H. R. (1989) *Harvey Lect. 83*, 77−103.
4. Kaback, H. R., and Wu, J. (1997) *Q. Rev. Biophys. 30*, 333−364.
5. Sahin-Tóth, M., Lawrence, M. C., and Kaback, H. R. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 5421−5425.
6. Costello, M. J., Escaig, J., Matsushita, K., Viitanen, P. V., Menick, D. R., and Kaback, H. R. (1987) *J. Biol. Chem. 262*, 17072−17082.
7. Sun, J., and Kaback, H. R. (1997) *Biochemistry 36*, 11959−11965.
8. Guan, L., Murphy, F. D., and Kaback, H. R. (2002) *Proc. Natl. Acad. Sci. U.S.A. 99*, 3475−3480.
9. Frillingos, S., Sahin-Tóth, M., Wu, J., and Kaback, H. R. (1998) *FASEB J. 12*, 1281−1299.
10. Sorgen, P. L., Hu, Y., Guan, L., Kaback, H. R., and Girvin, M. E. (2002) *Proc. Natl. Acad. Sci. U.S.A. 99*, 14037−14040.
11. Kaback, H. R., Sahin-Tóth, M., and Weinglass, A. B. (2001) *Nat. Rev. Mol. Cell Biol. 2*, 610−622.
12. Bogdanov, M., Heacock, P. N., and Dowhan, W. (2002) *EMBO J. 21*, 2107−2116.
13. le Coutre, J., Narasimhan, L. R., Patel, C. K., and Kaback, H. R. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 10167−10171.
14. le Coutre, J., Kaback, H. R., Patel, C. K., Heginbotham, L., and Miller, C. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 6114−6117.
15. Patzlaff, J. S., Moeller, J. A., Barry, B. A., and Brooker, R. J. (1998) *Biochemistry 37*, 15363−15375.
16. Menick, D. R., Sarkar, H. K., Poonian, M. S., and Kaback, H. R. (1985) *Biochem. Biophys. Res. Commun. 132*, 162−170.
17. van Iwaarden, P. R., Driessen, A. J., Lolkema, J. S., Kaback, H. R., and Konings, W. N. (1993) *Biochemistry 32*, 5419−5424.
18. Sondej, M., Weinglass, A. B., Peterkofsky, A., and Kaback, H. R. (2002) *Biochemistry 41*, 5556−5565.
19. Beckett, D., and Matthews, B. W. (1997) *Methods Enzymol. 279*, 362−376.
20. Menezes, M. E., Roepe, P. D., and Kaback, H. R. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 1638−1642.
21. Weitzman, C., Consler, T. G., and Kaback, H. R. (1995) *Protein Sci. 4*, 2310−2318.
22. van Iwaarden, P. R., Pastore, J. C., Konings, W. N., and Kaback, H. R. (1991) *Biochemistry 30*, 9595−9600.
23. Kaback, H. R. (1974) *Methods Enzymol. 31*, 698−709.
24. Frillingos, S., Sahin-Tóth, M., Persson, B., and Kaback, H. R. (1994) *Biochemistry 33*, 8074−8081.
25. Whitelegge, J. P., le Coutre, J., Lee, J. C., Engel, C. K., Privé, G. G., Faull, K. F., and Kaback, H. R. (1999) *Proc. Natl. Acad. Sci. U.S.A. 96*, 10695−10698.
26. Consler, T. G., Persson, B. L., Jung, H., Zen, K. H., Jung, K., Prive, G. G., Verner, G. E., and Kaback, H. R. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 6934−6938.
27. Pouny, Y., Weitzman, C., and Kaback, H. R. (1998) *Biochemistry 37*, 15713−15719.
28. Rudnick, G., Schuldiner, S., and Kaback, H. R. (1976) *Biochemistry 15*, 5126−5131.
29. Haugland, R. P. (1994) *Handbook of Fluorescent Probes and Research Chemicals*, 5th ed., Molecular Probes Inc., Eugene, OR.
30. Sahin-Tóth, M., and Kaback, H. R. (1993) *Protein Sci. 2*, 1024−1033.
31. Wu, J., and Kaback, H. R. (1994) *Biochemistry 33*, 12166−12171.
32. Sahin-Tóth, M., Lawrence, M. C., Nishio, T., and Kaback, H. R. (2001) *Biochemistry 40*, 13015−13019.
33. Wu, J., Frillingos, S., Voss, J., and Kaback, H. R. (1994) *Protein Sci. 3*, 2294−2301.
34. Wu, J., Frillingos, S., and Kaback, H. R. (1995) *Biochemistry 34*, 8257−8263.
35. He, M., and Kaback, H. R. (1997) *Biochemistry 36*, 13688−13692.
36. le Coutre, J., Whitelegge, J. P., Gross, A., Turk, E., Wright, E. M., Kaback, H. R., and Faull, K. F. (2000) *Biochemistry 39*, 4237−4242.
37. Gupte, S. S., and Lane, L. K. (1979) *J. Biol. Chem. 254*, 10362−10369.
38. Wu, J., Frillingos, S., Voss, J., and Kaback, H. R. (1994) *Protein Sci. 3*, 2294−2301.
39. Trumble, W. R., Viitanen, P. V., Sarkar, H. K., Poonian, M. S., and Kaback, H. R. (1984) *Biochem. Biophys. Res. Commun. 119*, 860−867.
40. Viitanen, P. V., Menick, D. R., Sarkar, H. K., Trumble, W. R., and Kaback, H. R. (1985) *Biochemistry 24*, 7628−7635.
41. Neuhaus, J.-M., Soppa, J., Wright, J. K., Riede, I., Bocklage, H., Frank, R., and Overath, P. (1985) *FEBS Lett. 185*, 83−88.
42. Sarkar, H. K., Menick, D. R., Viitanen, P. V., Poonian, M. S., and Kaback, H. R. (1986) *J. Biol. Chem. 261*, 8914−8918.
43. Brooker, R. J., and Wilson, T. H. (1986) *J. Biol. Chem. 261*, 11765−11771.
44. Menick, D. R., Lee, J. A., Brooker, R. J., Wilson, T. H., and Kaback, H. R. (1987) *Biochemistry 26*, 1132−1136.

BI027329C