Interhelical Packing Modulates Conformational Flexibility in the Lactose Permease of Escherichia coli †

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Interhelical Packing Modulates Conformational Flexibility in the Lactose Permease of *Escherichia coli*†

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ABSTRACT: A key to obtaining an X-ray structure of the lactose permease of *Escherichia coli* (LacY) (Abramson, J., Smirnova, I., Kasho, V., Verner, G., Kaback, H. R., and Iwata, S. (2003) *Science 301,* 549−716) was the use of a mutant in which Cys154 (helix V) is replaced with Gly. LacY containing this mutation strongly favors an inward-facing conformation, which binds ligand with high affinity, but catalyzes little transport and exhibits few if any of the ligand-dependent conformational changes observed with wild-type LacY. The X-ray structure demonstrates that helix V crosses helix I in the approximate middle of the membrane in such a manner that Cys154 lies close to Gly24 (helix I). Therefore, it seems likely that replacing Cys154 with Gly may lead to tighter packing between helices I and V, thereby resulting in the phenotype observed. Consistently, replacement of Gly24 with Cys in the C154G mutant rescues significant transport activity, and the mutant exhibits properties similar to wild-type LacY with respect to substrate binding and thermostability. However, the only other replacements that rescue transport to any extent whatsoever are Val and Asp, both of which are much less effective than Cys. The results suggest that, although helix packing probably plays an important role with respect to the properties of the C154G mutant, the ability of Cys at position 24 to rescue transport activity of C154G is more complicated than simple replacement of bulk between positions 24 and 154. Rather, activity is dependent on more subtle interactions between the helices, and mutations that disrupt interactions between helix IV and loop 6−7 or between helices II and IV also rescue transport in the C154G mutant.

Typical of many transport proteins from organisms as disparate as *Archaea* and mammals, the lactose permease of *Escherichia coli* (LacY)[1], a member of the Major Facilitator Superfamily (MFS) (*2*), transduces free energy stored in electrochemical ion gradients into a solute concentration gradient and vice versa (reviewed in ref *3*). LacY is a polytopic cytoplasmic membrane protein containing 12 transmembrane helices with the N- and C-termini on the cytoplasmic face of the membrane that catalyzes the stoichiometric translocation of galactosides and H^+ (galactoside/H^+ symport) (*4*−*6*). Several lines of evidence indicate that LacY is both functionally (see ref *7*) and structurally (*1, 8*−*11*) a monomer. In addition, 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 (*12*), led to the formulation of a helix-packing model (*13*). Furthermore, experimental observations from structural and extensive mutational analysis have suggested a rational mechanism for lactose/H^+ symport (*1, 14*).

The protein appears to be in a highly dynamic state. Most remarkably, 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 (*15*). Furthermore, as shown by attenuated total reflectance Fourier transform infrared spectroscopy (*16*), 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, although approximately 70% of the side chains in LacY are hydrophobic and are buried, about 85% of the backbone protons exchange with deuterium in 10 min (*17, 18*). By comparison, KcsA, the prokaryotic K^+ channel, requires 3 h to exchange only about 45% of its backbone protons for deuterium (*17*). In view of these and other properties of LacY (see ref *14*), it is not surprising that LacY, as well as some of its homologues, foiled all attempts at crystallization for many years.

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) (*19, 20*). Remarkably, this single mutation completely changes the functional properties and physical characteristics

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[1] Abbreviations: LacY, lactose permease; MIANS, 2-(4′-maleimidylanilino)naphthalene-6-sulfonic acid, sodium salt; TDG, β-D-galactopyranosyl-1-thio-β-D-galactopyranoside; NaDodSO$_4$−PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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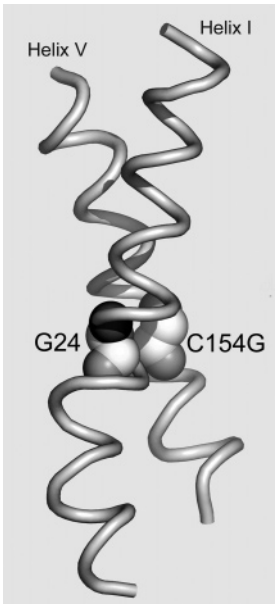

FIGURE 1: Spatial packing of transmembrane helices I and V in C154G LacY (Protein Databank entry 1PV7.pdb). Helices I and V are shown as spiral tubes. Gly residues at positions 24 and 154 are represented as spheres.

of LacY. C154G LacY binds ligand as well or better than wild-type LacY, but catalyzes very little translocation across the membrane; in addition, it does not exibit the long-range conformational changes normally observed upon ligand binding. Furthermore, C154G LacY is thermostable with respect to ligand binding and to aggregation (*21*).

The X-ray structure of the C154G mutant solved at a nominal resolution of 3.5 Å (*1*) demonstrates that the molecule is composed of pseudosymmetrical N- and C-terminal domains, each with six transmembrane helices, many of which are highly distorted. There is a large funnel-shaped hydrophilic cavity open only to the cytoplasm, which represents the inward-facing conformation of the symporter. The structure complexed with ligand reveals the sugar-binding site in the cavity, and the residues that play major roles in substrate recognition and H^+ translocation are identified. A possible mechanism for lactose/H^+ symport is proposed that is consistent with both the structure and a large body of experimental data. It has also been shown recently (*22*) that LacY contains a single binding site that exhibits the same affinity from both sides of the membrane even in the presence of a H^+ electrochemical gradient, a finding also consistent with the mechanism proposed.

The X-ray structure yields a clue as to why the C154G mutant favors the inward-facing conformation and exhibits the properties described. As shown (Figure 1) (*1*), helices I and V cross in the approximate middle of the membrane where Gly24 and Cys154 are in close proximity. Two Gly residues close to each other in adjacent helices can lead to significantly tighter helix packing (*23*−*26*), which may explain the properties of the C154G mutant. To test this possibility, we replaced Gly24 with Cys in the C154G mutant. The double mutant exhibits significant rescue of transport activity and sugar binding and thermostability properties similar to wild-type LacY. Surprisingly, replacement of Gly24 with Val or Asp yields a low degree of rescue, but replacement with Ala, Ile, Met, Thr, Ser, or Glu has no significant effect. Interestingly, replacement of Cys154 with Ser or Thr yields active mutants, while the same replacements for Gly24 in the C154G mutant yield little or no activity. Moreover, a mutant with Cys at both position 24 and 154 transports like wild-type. Taken together, these results indicate that it is not merely the bulk of the side chains at the positions 24 and 154 that accounts for the properties of the C154G mutant.

This conclusion is supported by the observation that mutations which disrupt interactions between helix IV and loop 6−7 or between helices II and IV in the intracellular part of the molecule also rescue C154G LacY activity. All these positions are located at a distance from positions 24 and 154. The findings support the interpretation that highly precise, dynamic interactions between helices I and V are required for proper structural rearrangements during turnover.

EXPERIMENTAL PROCEDURES

Construction of LacY Mutants. The G24C mutation was introduced into pT7-5/cassette *lacY* encoding wild-type LacY or C154G LacY by restriction fragment replacement. A 10-histidine affinity tag (His_{10}) was placed at the C-terminus by similar means.

QuickChange PCR (Stratagene, La Jolla, CA) was used to replace Gly24 with Val, Ala, Ile, Thr, Ser, Met, Glu, or Asp in the C154G/10-His mutant with the following PCR primers, respectively: sense 5′-CTTTTTTATCATG**GTA**GCCTACTTCCCGTTTTTCCCG, 5′-CTTTTTTATCATG**GCC**GCCTACTTCCCGTTTTTCCCG, 5′-CTTTTTTATCATG**ATA**GCCTACTT-CCCGTTTTTCCCG, 5′-CTTTTTTATCATG**ACA**GCCTACTTCCCGTTTTTCCCG, 5′-CTTTTTTATCATG**AGC**GCCTACTTCCCGTTTTTCCCG, 5′-CTTTTTTATCATG**ATG**GCCTACTTCCCGTTTTTCCCG, 5′-CTTTTTTATCATG**GAG**GCCTACTTCCCGTTTTTCCCG, 5′-CTTTTTTATCATG**GAT**GCATACTTCCCGTTTTTCCCG.

Ser or Thr substitution at position 154 in wild-type-His_{10} was introduced by using the same method with sense 5′-GCGCTG**TCT**GCCTCAATTGTCGGGATCATG and 5′-GCGCTG**ACT**GCCTCAATTGTCGGGATCATG primers, respectively. The C154G mutation was introduced into pT7-5/cassette *LacY* encoding single-Cys mutants by restriction fragment replacement. All *lacY* genes were sequenced to confirm the mutations introduced and to detect unwanted mutations.

Transport Measurements. Active transport of [1-^{14}C]-lactose was measured in *E. coli* T184 [$lacI^+O^+Z^-Y^-$ (*A*) *rpsL met*$^-$ *thr*$^-$ *recA hsdM hsdR/F′ lacI*^{q} O^+Z^{D118}] (*27*) harboring given plasmids. Cells were grown overnight at 37 °C in Luria−Bertani broth containing 100 μg/mL ampicillin and induced with 0.1 mM isopropyl 1-thio-β-D-galactopyranoside. Cells were harvested by centrifugation, washed with ice-cold 100 mM potassium phosphate (KP_i; pH 7.2) and 10 mM $MgSO_4$, and adjusted to an OD_{420} of 10 (0.7 mg of protein/mL). Aliquots (50 μL) were equilibrated at room temperature, and [1-^{14}C]lactose (10 mCi/mmol) was added to a final concentration of 0.4 mM. Reactions were quenched at given times by addition of 2.0 mL of 100 mM KP_i (pH 5.5) and 100 mM LiCl followed by immediate filtration (*28*). Radioactivity retained on the filters was assayed by liquid scintillation spectrometry.

Western Blots. Membrane fractions from the same cells used for transport assays were prepared by sonification as

described (*29*). Samples (10 μL) were subjected to sodium dodecyl sulfate−polyacrylamide gel electrophoresis (12%) ($NaDodSO_4$−PAGE) and subsequent Western blot analysis with either anti-His (wild-type background) or anti-C-terminal antibody (Cys-less background) (*30*).

Purification of LacY. LacY was purified from *E. coli* XL1-Blue transformed with wild-type, C154G, or G24C/C154G plasmids essentially as described (*21*) except that Talon resin (BD Clontech, PaloAlto, CA) was used for affinity chromatography. Proteins eluted with 200 mM imidazole were dialyzed against 20 mM Tris-HCl (pH 7.4) and 0.008% dodecyl-β-D-maltopyranoside (DDM), concentrated with a Vivaspin 20 concentrator (30 kDa cutoff; Vivascience, Germany), and stored on ice before use. The Micro BCA method (Pierce, Rockford, IL) was used to measure protein concentration. All preparations were at least 90−95% pure as judged by silver staining after $NaDodSO_4$−PAGE.

2-(4′-Maleimidylanilino)naphthalene-6-sulfonic Acid (MIANS) Labeling, Fluorescence Measurements, and Thermal Inactivation. Experiments were carried out as described previously (*21*).

Figures. All of the molecular structure representations (Figures 1, 7, and 8) were drawn with the program PyMol from crystal structure of the C154G mutant of LacY (PDB access code 1PV7).

RESULTS

C154G LacY with Replacements for Gly24 or Cys154. Expression. To test expression and membrane insertion of given LacY mutants, immunoblots were carried out on membrane preparations from *E. coli* T184 harboring plasmids encoding each mutant (Figures 2A and 3A). With the exception of G24E LacY, which is expressed relatively poorly, the mutants are expressed qualitatively at levels comparable to wild-type or C154G LacY.

Lactose Transport. The ability of each mutant to catalyze lactose transport was assayed in *E. coli* T184 (Z^-Y^-), and time courses of accumulation for mutants G24C, G24V, G24A, G24I, G24M, G24S, G24T, G24E, and G24D in the C154G background, as well as activity of wild-type and C154G LacY, are presented in Figure 2B. When rates of lactose transport are measured at 1 min or steady-state levels of accumulation at 1 h and expressed as percentage of wild-type activity, it is apparent that all mutants exhibit a low rate of transport and only one, G24C/C154G, exhibits a steady-state level of accumulation that approaches wild-type LacY (∼80%). Surprisingly, rates of transport by mutants with Val, Ala, Ile, Met, Ser, Thr, Glu, or Asp in place of Gly24 in the C154G background are all very low, but by 1 h, mutants G24V/C154G and G24D/C154G exhibit low but significant accumulation. The other mutants exhibit levels of accumulation that are only slightly higher than cells harboring pT7-5 with no *lacY* insert.

Transport activity of single mutants C154S and G24C is close to that of wild-type, while C154T exhibits ∼65% of wild-type steady state (Figure 3B). The level of accumulation by mutants G24E and G24D is 10 and 35%, respectively, of wild-type LacY.

MIANS Labeling and Thermal Inactivation. Substrate binding was tested by studying TDG protection against MIANS labeling of purified LacY mutants in DDM. When added to wild-type LacY in equimolar concentration, MIANS covalently labels Cys148, which is located near the substrate binding site, almost exclusively (*31*) and is protected from alkylation by ligand due to steric clash. MIANS is not

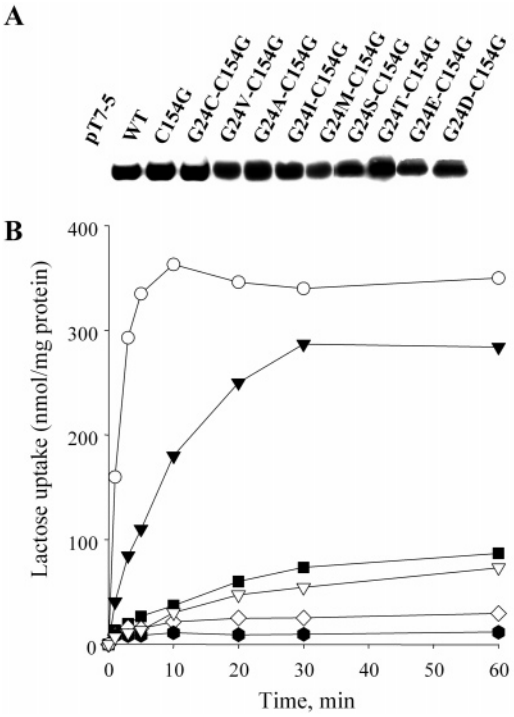

FIGURE 2: Time course of lactose transport activity by C154G LacY with various replacements for Gly24. T184 cells transformed with plasmid pT7-5/wild-type *lacY* , pT7-5 (vector with no *lacY* gene), or pT7-5 encoding given mutants were grown and assayed as described in Experimental Procedures. (A) Expression level of mutants compared to wild type. (B) Time courses of lactose uptake. ○, wild-type; ▲, G24C/C154G; ▽, G24D/C154G; ■, G24V/C154G; ◇, G24(I, A, T, M, or E)/C154G; ⬢, G24S/C154G and pT7-5 vector.

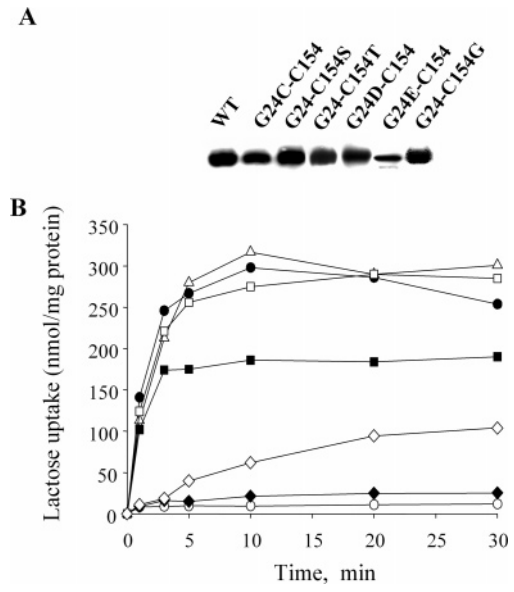

FIGURE 3: Time courses of lactose transport by 24G/C154S, 24G/C154T, G24C, G24E, and G24D single mutants of LacY. (A) Expression level of the mutants relative to wild-type. (B) The time courses of lactose uptake. ○, pT7-5 vector; ●, wild-type; △, 24G/C154S; □, G24C; ■, 24G/C154T; ◇, G24D; ◆, 24G/C154G and G24E.

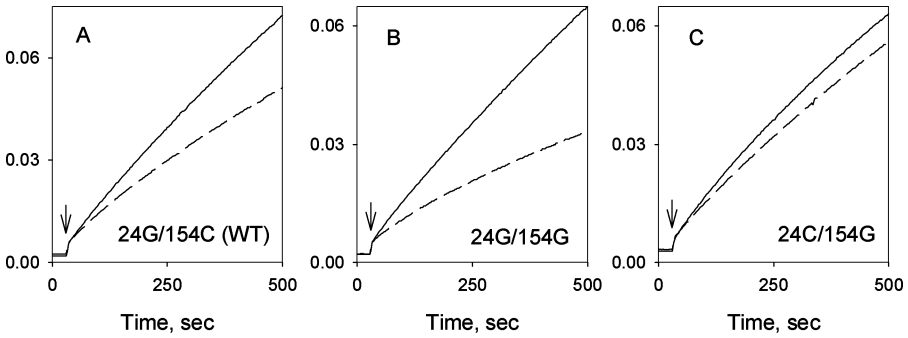

FIGURE 4: Effect of Cys or Gly substitutions at positions 24 and 154 on substrate protection against MIANS labeling of purified LacY mutants. (A) G24/C154/LacY (wild-type); (B) G24/C154G; (C) G24C/C154G. An amount of 0.5 μM of protein solution in 50 mM NaP_i (pH 7.5), 150 mM NaCl, and 0.02% DDM was mixed with 1 μM of MIANS at the time indicated by an arrow. Fluorescence emission was recorded at 415 nm (excitation wavelength 330 nm) without added TDG (solid lines) or after addition of 10 mM TDG (dashed lines).

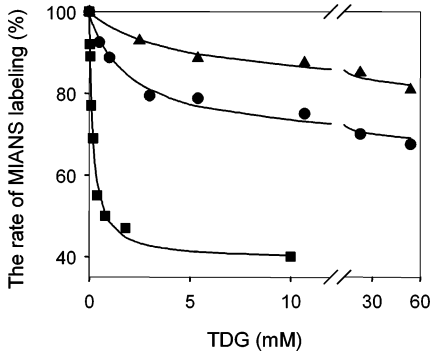

FIGURE 5: Substrate affinity of purified LacY mutants with Cys or Gly substitutions at positions 24 and 154. The rates of MIANS labeling at given TDG concentrations were measured at the conditions described in Figure 4 for wild-type LacY (●), G24/C154G (■) and G24C/C154G (▲). Solid lines represent hyperbolic equation fit to the data (SigmaPlot 2001 software, SPSS Science, Chicago, IL). Estimated K_d values are 2.2 ± 0.5 mM for G24/C154 LacY, 0.17 ± 0.03 mM for G24/C154G, and 4.8 ± 1.5 mM for G24C/C154G.

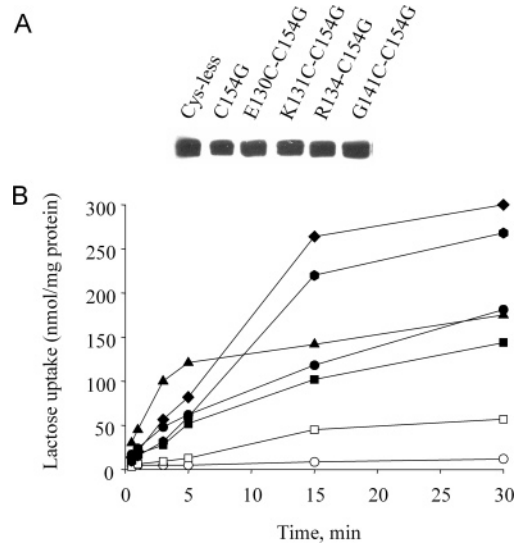

FIGURE 6: Recovery of transport activity of C154G mutant (Cys-less background) by Cys replacements in helices IV and V. T184 cells transformed with plasmid pT7-5/Cys-less *lacY*, pT7-5, or pT7-5 encoding given single-Cys mutants were grown and assayed as described in Experimental Procedures. Expression level of the mutants compared to Cys-less LacY are shown in A. (B) Time courses of lactose uptake. ○, pT7−5; ●, Cys-less (C154V); □, C154G; ■, E130C/C154G; ▲, K131C/C154G; ◆, R134C/C154G, ⬢, G141C/C154G.

fluorescent until it reacts with Cys and becomes fluorescent in a time-dependent fashion (*21*, *32*−*37*).

Time courses of MIANS labeling of wild-type LacY (G24/154C), G24/C154G, and G24C/C154G mutants are shown in Figure 4. The rate of MIANS labeling in all three instances decreases after preincubation of solubilized, purified proteins with β-D-galactopyranosyl-1-thio-β-D-galactopyranoside (TDG). The protective effect of 10 mM TDG is significantly greater for G24/C154G LacY than for wild-type (G24/154C) (*21*) or mutant G24C/C154G. To test differences in affinity of the mutants for substrate, the rate of MIANS labeling at increasing TDG concentrations was also measured (Figure 5). Estimated K_d values are 2.2 mM for wild-type and 4.8 mM for G24C/C154G LacY and 0.17 mM for G24/C154G LacY. The maximum extent of TDG protection under the same conditions is higher for the G24/C154G mutant (60%) than for proteins with a Gly/Cys pair (20−30%) (Figure 5). The conformational stability of proteins was tested by thermal inactivation of substrate protection against MIANS labeling as described (*21*). Samples treated at 50 °C for 15 min demonstrate visible precipitation with both Gly/Cys pairs, but no precipitation of G24/C154G LacY. Protection by TDG against MIANS labeling is observed with only the G24/C154G mutant after 15 min incubation at 50 °C (data not shown). Thus, the apparent correlation between increased TDG protection against MIANS labeling and substrate affinity of the mutants (G24/C154G > G24/C154 > G24C/C154G) may reflect a more tightly packed and less-flexible conformation of proteins with the Gly/Gly pair compared to the Gly/Cys and Cys/Gly pairs and therefore its overall stability.

Rescue of C154G LacY Transport Activity by Single-Cys Replacements in Helices IV and V. Expression of Single-Cys Mutants. Expression levels of single-Cys mutants in given backgrounds were tested by Western blot analysis with anti-C-terminal antibody, and levels of expression are comparable to that of Cys-less LacY (Figure 6A).

Active Lactose Transport. The transport activity of single-Cys mutants with the C154G mutation relative to Cys-less LacY and C154G are shown in Figure 6B. Cys-less LacY displays about 50−60% of the activity of wild-type LacY, as shown previously (*38*). Introduction of the 154G mutation causes a large decrease in transport activity of Cys-less LacY (*21*). However, single-Cys substitutions at positions 130, 131, 134, or 141 lead to significant recovery of the steady-state level of accumulation by 154G LacY (80%, 95%, 165%, or

150% of Cys-less, respectively; Figure 6B). Mutation F140C results in ~10−15% recovery of transport activity (data not shown). Other single-Cys mutations in helices IV and V (E126C, S133C, and R144C) do not influence C154G LacY transport activity (data not shown).

DISCUSSION

Interaction between Helices I and V. Previous evidence from single amino acid replacements and analysis of a series of deletion mutants indicate that certain residues in the region of helix I may be important for active lactose transport. Thus, Bibi et al. (*39*) found that the region between residues 23 and 38 contains elements essential for both active transport and membrane insertion. Moreover, Overath et al. (*40*) identified LacY mutants defective in lactose transport with Arg and Glu in place of Gly24 or Ser in place of Pro28. It was also shown that permease with Y26F is devoid of transport activity due to low-substrate affinity (*41*).

However, results of the further experiments utilizing Cys-scanning mutagenesis of the helix I (*42*) demonstrate that Cys replacement for Gly24 or Tyr26 exhibits activity comparable to Cys-less permease, which led to the conclusion that no residue in the N-terminus or in the first transmembrane helix is irreplaceable for active lactose transport. It was concluded that the important elements in helix I are more related to general physicochemical properties of the amino acid residues (e.g., bulk, hydrophobicity) than to more specific properties such as shape or ability to form H-bonds (*42*). The effect of NEM on lactose transport demonstrates that the Cys-replacement mutants in helix I, which are significantly inactivated, are all located at the C-terminus of the first helix from F27C to W33C (*42*). Apparently addition of a relatively bulky hydrophobic moiety to a cysteinyl side chain in this region may interfere with packing. The observation that NEM labeling does not influence the transport activity of the G24C mutant (Cys-less background) may be explained by inaccessibility of this residue to NEM due to tight contacts between helix I and helix V at this point. This suggestion is confirmed by results of NEM labeling of single-Cys mutant C154 (*43*) which does not react with the alkylating agent. Remarkably, NEM labeling of mutant G24C exhibits reactivity only in the presence of substrate (TDG), while in the absence of TDG, G24C is unreactive (N.V.E., and H.R.K., manuscript in preparation), thereby indicating that the helix interface may be very tightly packed in this region.

The X-ray structure of C154G LacY (*1*) provides a clue to how residues in this region may be involved in transport and why certain mutations result in the loss of activity. It was shown earlier (*19*−*21*) that, although the C154G mutant binds ligand as well as or better than wild-type LacY, transport activity is drastically decreased to almost the same level as for cells or vesicles devoid of LacY, suggesting that the protein strongly favors one conformation and is unable to translocate substrate. This loss of conformational flexibility may also be the reason for high stability. 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 and does not exhibit certain long-range conformational changes observed upon substrate binding (*21*). This set of data combined with analysis of the C154G mutant crystal structure led to the suggestion that tighter packing of helices I and V causes the mutant to favor one particular conformation by decreasing the distance between helix I and helix V at the point of intersection. It was suggested that the loss of conformational flexibility is due to Gly residues at positions 24 and 154, which serve to allow tighter helix−helix interaction (see Figure 1). This postulate is based on packing analysis of membrane proteins with known X-ray structures, which strongly suggests that small, polar residues enhance helix−helix interactions. Gly is one of the most abundant residues found at tightly packed helical interfaces, exhibiting the highest overall packing value in membrane proteins, followed by Pro and Ala (*44*, *45*). In polytopic membrane proteins, Gly residues also facilitate helix packing either by forming favorable van der Waals surfaces for hydrophobic packing or by allowing closer dipolar interactions of the polar backbone atoms (*23*−*26*). Gly may not disrupt the secondary structure of helical transmembrane segments, but rather functions as a molecular notch to facilitate helix packing (*26*). Parallel right-handed helix−helix interactions appear to favor C_α—H···O H-bond formation between helices with the structural motif GxxxG (*46*). In addition, because of the absence of a side chain, Gly residues tend to form interhelical nonpolar contacts with bulky aromatic residues (*47*).

In accordance with the structure of C154G LacY, Gly residues at positions 24 and 154 are located in close proximity to each other, providing tight interaction between helices I and V at the point of intersection. Wild-type LacY contains Gly and Cys at positions 24 and 154, correspondingly. Replacement of Cys154 with Ser or Val in Cys-less LacY yields a protein with 10% or 50−60%, respectively, of wild-type activity (*38*). These data indicate that, although Cys154 is important for transport, it is not irreplaceable. However, the decreased activity observed with these substitutions for Cys154 suggests that a Gly−Cys pair at the point where helices I and V intersect provides optimal conditions for helix packing and the flexibility of the protein necessary for the translocation of the substrate. As shown in Figure 2, mutation G24C rescues transport in the C154G mutant to a highly significant level. MIANS labeling of purified LacY mutants and thermal inactivation studies are in full agreement with the transport results and demonstrate remarkable differences between proteins with Gly/Cys or Gly/Gly pairs at positions 24 and 154. Mutant G24C/C154G and wild-type (G24/C154) have lower substrate affinity relative to the G24/C154G mutant (Figures 4 and 5) and relatively low stability.

Analysis of the X-ray structure of C154G LacY (1pv7.pdb) shows that several amino acid residues are located in the vicinity of Gly24 and Gly154 in close proximity to residues directly involved in substrate binding. Trp151 and Arg144 (helix V) are direct ligands for substrate; Met23 is close to the C6 atom of the galactopyranosyl ring, and Phe20 may participate in positioning Trp151. Possibly, small distance changes between helices I and V may modify binding of substrate and/or subsequent conformational changes that lead to the dissociation of substrate. As shown in Figure 7A,B, the interface between parallel regions of helices I and V is enriched with Gly residues (G13 and G24 in helix I and G141, G147, G150, and G154 in helix V) and Phe residues (F16, F17, F20, and F21 in helix I and F146 in helix V) that

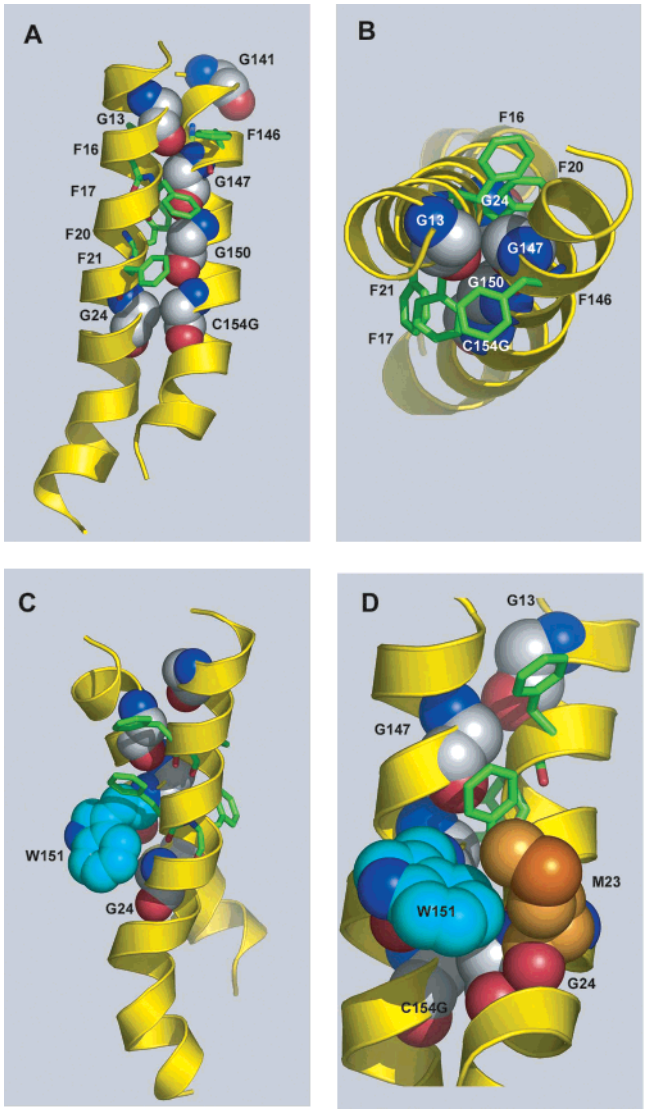

FIGURE 7: Side-chain interactions in transmembrane helices I and V drawn from X-ray structure of C154G LacY (Protein Databank entry 1PV7.pdb). Helices are shown as ribbons. (A) Side view of helices I and V. Gly residues at positions 13, 24, 140, 147, 150, and 154 are shown as spheres. Phenylalanine residues at positions 16, 17, 20, 21, and 146 are represented as green sticks. (B) Cytoplasmic view of helices I and V. Residues are labeled as in A. (C) Side view of helices I and V with Trp151 shown as blue spheres, Gly as gray spheres, and Phe as green sticks. (D) Side view of the helices I and V with Met23 represented as yellow spheres.

generate tight interaction between helices probably by forming multiple C_α—H···O H-bonds between C_α atoms of Gly residues from helix V and backbone oxygen atoms of helix I. It is possible that the C_α of Gly150 forms an H-bond with the backbone oxygen of Phe20 and the C_α of Gly147 forms an H-bond with the backbone oxygen of Phe16 (Figure 7A). Previous Cys-scanning mutagenesis data support this assumption (*42*, *48*, *49*). Cys-replacement of Gly141, -147, -150, or -159 results in loss of transport activity while Phe16, -17, -20, -21, or -146 can be replaced with Cys without loss of function. Furthermore, according to sequence alignment with other MFS members, Gly141, Gly147, Gly150, and Gly159 are highly conserved. Insertion of an additional Gly at position 154 strengthens the preexisting association of helices I and V and does not allow flexibility, which is necessary for turnover. In addition, Gly24 is located in close proximity to Trp151 (Figure 7C) and may form an interhelical nonpolar contact with this bulky aromatic residue. For the G/W pair, the C and C_α atoms of Gly are often found in contact with the Trp aromatic ring in transmembrane helices of known structures (*47*). The X-ray structure of LacY reveals a tightly packed cluster of residues around Gly24 and Trp151 that also includes Met23, which is close to the C-6 position of substrate (Figure 7D). The unique position of Gly24 close to Trp151, which is essential for transport, may explain why any substitutions other than Cys at position 24 do not rescue the transport activity of the C154G mutant (Figures 2B and 3B). This exclusive Cys substitution is in agreement with current structural and biochemical data indicating that Cys residues have the lowest frequency of amino acyl side chains found in H-bonds of transmembrane helices (Ser > Tyr > Thr > His > Arg > Trp > Glu > Asn > Asp > Gln > Lys > Cys) (*50*). Comparison with the transport activity of mutants with Cys, Asp, Gly, Ileu, Ala, Thr, Met, Glu, and Ser at position 24 shows that the transport activity of the mutants decreases in an order opposite to the ability of the amino acyl side chains to form H-bonds (Cys > Asp > Gly (Ile, Ala, Thr, Met, Glu) > Ser). Thus, residues other than Cys may H-bond with another residue in the vicinity, thereby disturbing correct positioning of residue 24 and surrounding residues. Most likely, this may be also true with mutants G24C, G24D, and G24E in the wild type-background (C154). The transport activity of these mutants decreases in the following order Cys > Asp > Glu (Figure 3B). Taken together, the results provide an indication that proper helix packing in C154G LacY tolerates a Cys side chain only at position 24 because of specific physicochemical properties other than simply bulk. On the other hand, Cys154 can be replaced with Ser or Thr without significant loss of activity (Figure 3B), indicating that this position has less specific physicochemical requirements. Most likely, optimal bulk of the side chain is necessary to constrain Cys154 without disturbing flexibility. Other residues in the vicinity of Cys154 suggest that the Cys154 side chain may not be pointed directly toward Gly24 in wild-type LacY. This notion is supported by experiments with G24C/C154 LacY that do not exhibit any difference in transport activity after treatment with 50 μM $CuSO_4$ (data not shown), suggesting that both Cys residues are positioned in a way that may preclude cross-linking.

Cys Replacements in the Cytoplasmic Portions of Helices IV and V Rescue C154G Cys-Less LacY. Single- or double-Cys replacements in Cys-less LacY are used extensively in structure−function studies (reviewed in ref *12*). Cys-less LacY, which has a Val residue at positon 154 and Ser residues in place of the remaining seven Cys residues, displays about 60% of the activity of wild-type with a similar K_m (ca. 0.3 mM) (*38*). Insertion of the C154G mutation into Cys-less LacY causes about an 80% decrease in the steady-state level of lactose accumulation (*21*). Replacement of several amino acid residues in the cytoplasmic portions of helices IV and V with Cys restores activity to levels approximating that of Cys-less LacY (Figure 6). Interestingly, these replacements are located at a distance from the intersection of helices I and V, where positions 24 and 154 are in close proximity. A possible explanation for this effect is that optimal orientation of helices I, IV, and V during

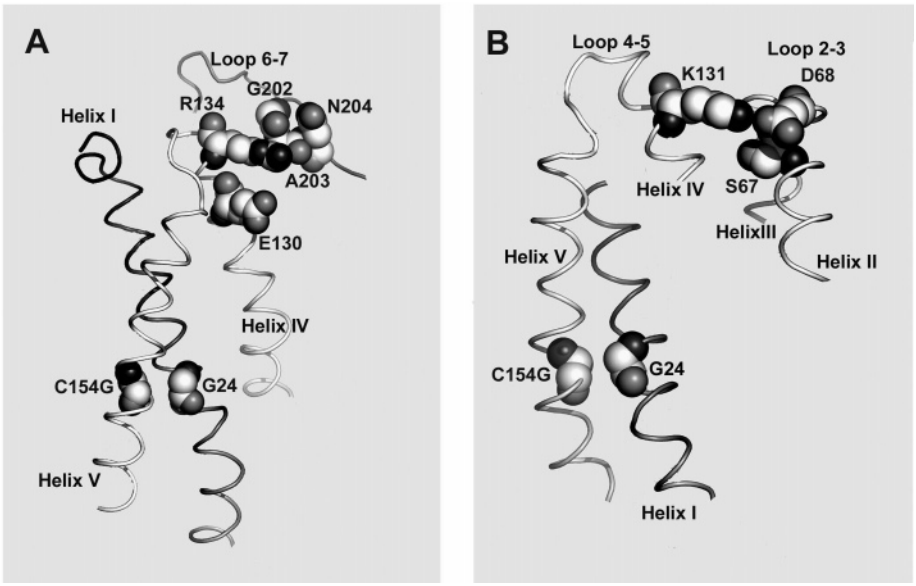

FIGURE 8: Structural interactions in N-terminal 6-helix bundle of C154G LacY (Protein Databank entry 1PV7.pdb). Helices are shown as spiral tubes and interacting amino acyl side chains as spheres. Panel A shows close contacts between Arg134 and Glu130 at the cytoplasmic end of helix IV with Gly202, Ala203, and Asn204 from loop 6−7. Panel B shows contacts between Lys131 at the cytoplasmic end of helix IV with Ser67, Asp68 at the cytoplasmic end of helix II.

substrate translocation requires coordinated movement of these helices which is largely lost with the C154G mutation and rescued by replacement of Glu130, Lys131, Arg134, or Gly141 with Cys. Possibly, Cys replacement of Glu130, Lys131, or Arg134 alters interactions in the vicinity of these residues, leading to recovery of flexibility between the helices and partial rescue of activity. Cross-linking studies are consistent with movement of the cytoplasmic regions of helices IV and V upon substrate binding (*51*).

Arg134 is in close contact with Gly202, Ala203, and Asn204 in loop 6−7 and forms a salt bridge with Glu130 (helix IV) (Figure 8A). These interactions appear to fix the cytoplasmic ends of helices IV and V, a conclusion supported by in-frame deletion analyses of the central cytoplasmic loop (*52*). Deletion of residues 206−210 (Δ5) results in properties very similar to those of wild-type LacY, while deletion of residues 199−210 (Δ12) causes a significant decrease in activity with little reduction in LacY expression. Cys-scanning mutagenesis shows that transport activity is not altered by replacement of Gly202, Ala203, or Asn204 (*53*). Thus, the region containing positions 202−204 does not play a direct role in the transport mechanism, although it may participate in stabilizing tertiary contacts. Replacement of Arg134 or Glu130 with Cys may disturb contact between helix IV and loop 6−7 (Figure 8A), which presumably relaxes the structure and results in loss of transport function. In contrast, with C154G LacY, where helices I and V may be more tightly packed than in wild-type LacY at the point of intersection, relaxation of the structure may act to rescue the activity of C154G LacY. Cys replacement of Arg134 also partially rescues the activity of the C154G mutation in the wild-type background (I.N.S. and H.R.K., unpublished data).

Another interesting residue is Lys131 at the end of helix IV (Figure 8B), which interacts with both Asp68 and Ser67, thereby potentially altering structural rearrangements in C154G LacY. Site-directed and Cys-scanning mutagenesis confirm the importance of Asp68 and, to a lesser extent, Ser67 with respect to transport activity (*54*, *55*). Although the S67C mutant exhibits significant activity, transport activity is inhibited by ca. 60% after alkylation with *N*-ethylmaleimide (*55*). These findings and others suggest that one face of helix II participates in structural changes induced by ligand binding and is important for the conformational changes that occur during turnover (*55*−*58*). In contrast to S67C, single-Cys D68C does not react with *N*-ethylmaleimide, possibly reflecting close tertiary contact between helix II and neighboring helices. Moreover, all other substitutions for Asp68 (Glu, Ala, Ser, Thr, Tyr, Asn, and His) in the wild-type background cause inactivation (*54*). Therefore, it appears likely that structural contacts between residues Lys131, Ser67, and Asp68 (Figure 8B) result in a relatively fixed structure at the cytoplasmic end of helix IV. Introducing a Cys for Lys131 loosens this interacting cluster and results in the loss of function in the wild-type, but partially rescues activity in C154G LacY by causing local relaxation which may be transmitted to the intersection between helices IV and V.

Rescue of C154G activity by replacing Gly141 with Cys may be explained by disruption of the helix structure in the vicinity of position 141 (Figure 7A). Gly141 is the N-terminal residue in helix V. Thus, replacement with Cys may disorder the helix at its beginning or change the pitch of the helix and lead to relaxation of the interaction between helix V and I, which allows rescue of C154G LacY.

The data presented here show that the conformational changes that occur during LacY turnover are not restricted to movements of the N- and C-terminal 6-helix bundles as rigid bodies (*1*). There also appear to be changes in the positions of the transmembrane helices within C-terminal 6-helix bundle, and conditions that restrict these changes may compromise activity (*59*). Additional structural information regarding different proposed conformations of LacY are clearly necessary to delineate a detailed mechanism for LacY turnover.

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