Site-Directed Alkylation of Cysteine Replacements in the Lactose Permease of Escherichia coli : Helices I, III, VI, and XI †

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Site-Directed Alkylation of Cysteine Replacements in the Lactose Permease of *Escherichia coli*: Helices I, III, VI, and XI†

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ABSTRACT: To complete a study on site-directed alkylation of Cys replacements in the lactose permease of *Escherichia coli* (LacY), the reactivity of single-Cys mutants in helices I, III, VI, and XI, as well as some of the adjoining loops, with *N*-[^{14}C]ethylmaleimide (NEM) or methanethiosulfonate ethylsulfonate (MTSES) was studied in right-side-out membrane vesicles. With the exception of several positions in the middle of helix I, which either face the bilayer or are in close proximity to other helices, the remaining Cys replacements react with the membrane-permeant alkylating agent NEM. In helices III and XI, most Cys replacements are also alkylated by NEM except for positions that face the bilayer. The reactivity of Cys replacements in helix VI is noticeably lower and only 45% of the replacements label. Binding of sugar leads to significant increases in the reactivity of Cys residues that are located primarily at the same level as the sugar-binding site or in the periplasmic half of each helix. Remarkably, studies with small, impermeant MTSES show that single-Cys replacements in the cytoplasmic portions of helices I and XI, which line the inward-facing cavity, are accessible to solvent from the periplasmic surface of the membrane. Moreover, addition of ligand results in increased accessibility of Cys residues to the aqueous milieu in the periplasmic region of the helices, which may reflect structural rearrangements leading to opening of an outward-facing cavity. The findings are consistent with the X-ray structure of LacY and with the alternating access model [Abramson, J., Smirnova, I., et al. (2003) *Science 301*, 610−615].

The lactose permease of *Escherichia coli* (LacY)[1] is encoded by the *lacY* gene and catalyzes the coupled stoichiometric translocation of galactopyranosides with an H^+. As such, LacY is representative of membrane proteins from Archaea to the mammalian central nervous system that transduce free energy stored in electrochemical ion gradients into solute concentration gradients. The permease has been solubilized from the membrane, purified to homogeneity in a completely functional state (reviewed in ref *1*), and shown to act functionally and structurally as a monomer (reviewed in ref *2*). A functional LacY molecule devoid of eight native Cys residues (C-less LacY) has been constructed (*3*). Placement of single-Cys residues at almost every position of the molecule except the C-terminal 15 amino acid residues, which can be deleted with no effect (*4*−*6*), has yielded a library of mutants that represents an exceptional experimental tool for structure/function studies (see ref *7*). By using Cys, which is average in bulk, relatively hydrophobic, and amenable to highly specific modification, site-directed mutagenesis can be used in conjunction with biochemical and biophysical techniques in situ to reveal membrane topology (e.g., ref *8*) and accessibility of intramembrane residues to the aqueous or lipid phase of the membrane (e.g., refs *9*−*13*), as well as spatial proximity between transmembrane domains (e.g., refs *14*−*17*). The X-ray structure of a LacY mutant (C154G) at a nominal resolution of 3.5 Å has been solved (*18*). The mutant binds ligand as well as or better than wild-type LacY but catalyzes very little transport and is also defective with respect to the long-range conformational changes observed upon addition of ligand with the wild-type molecule (*19*).

LacY is composed of pseudosymmetrical N- and C-terminal domains, each with six transmembrane helices, many of which are highly irregular in shape. Perpendicular to the plane of the membrane, the molecule is heart shaped with a large interior hydrophilic cavity open on the cytoplasmic side, representing the inward-facing conformation. Within the cavity, a single bound sugar molecule is observed in the middle of the membrane displaced slightly toward the cytoplasmic side. 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 (reviewed in ref *7*), in conjunction with functional studies and the X-ray crystal structure has led to a postulated mechanism for lactose/H^+

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[1] Abbreviations: LacY, lactose permease; C-less permease, functional lac permease devoid of Cys residues; TDG, β-D-galactopyranosyl 1-thio-β-D-galactopyranoside; NEM, *N*-ethylmaleimide; MTSES, methanethiosulfonate ethylsulfonate; RSO, right side out; DTT, dithiothreitol; KP_i, potassium phosphate; $NaDodSO_4$−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; DDM, *n*-dodecyl β-D-maltopyranoside; BAD, biotin acceptor domain.

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symport in which the binding site is alternatively accessible to either side of the membrane (*18*, *20*, *21*).

Site-directed alkylation of single-Cys LacY mutants in right-side-out (RSO) vesicles with *N*-ethylmaleimide (NEM), which is membrane permeant (*22*), is a convenient biochemical tool for studying both static and dynamic features of LacY (*23*−*28*), as well as a number of other membrane proteins (e.g., refs *29*−*40*). Alkylation is a measure of the reactivity and/or accessibility of Cys residues, which depends on the environment in the vicinity of a given Cys side chain within the tertiary structure and is limited by close contacts between transmembrane helices and/or the low dielectric of the environment. In addition, the accessibility of Cys residues to the aqueous milieu can be determined by site-specific labeling with methanethiosulfonate ethylsulfonate (MTSES), a very hydrophilic, membrane-impermeant thiol reagent (*22*, *24*−*28*, *41*−*43*). When extended to each transmembrane residue, the method can be used to resolve solvent-accessible regions in LacY. Any change in labeling of a given Cys side chain upon substrate binding indicates an alteration in the local environment around the Cys residue. Although a structure of the inward-facing conformation of LacY is available and an overall mechanism for symport has been postulated, site-directed alkylation in situ is useful for identifying dynamic features induced by ligand binding and translocation (*22*−*28*, *44*).

In this study, site-directed sulfhydryl modification in right-side-out (RSO) membrane vesicles is utilized to study helices I, III, VI, and XI, thereby completing the study of most positions in LacY, particularly in the transmembrane helices. Previous evidence from single amino acid replacements, analysis of deletion mutants, and Cys-scanning mutagenesis (*8*, *45*−*49*) of helices I, III, VI, and XI indicates that although no residue per se appears to be irreplaceable for activity, structural properties are important for activity.

EXPERIMENTAL PROCEDURES

Plasmid Construction. DNA fragments encoding given single-Cys mutants were isolated from plasmids in the library containing a given mutation and inserted into either pT7-5 or pKR35 by restriction fragment replacement using appropriate pairs of restriction enzymes (e.g., *Pst*I and *Eco*RI, *Eco*RI and *Xho*I, or *Eco*RI and *Kpn*I). Plasmids encoding C-less LacY with a biotin acceptor domain (BAD) either in the central cytoplasmic loop 6−7 or at the C terminus were constructed as described (*50*).

Preparation of Right-Side-Out (RSO) Membrane Vesicles. RSO membrane vesicles were prepared from 1 L cultures of *E. coli* T184 expressing a given mutant by lysozyme/ethylenediaminetetraacetic acid treatment and osmotic lysis (*51*, *52*). Vesicles were resuspended to a protein concentration of 16−22 mg/mL in 100 mM potassium phosphate (KP_i, pH 7.3) and 10 mM $MgSO_4$, frozen in liquid N_2, and stored at −80 °C until use.

NEM Labeling. Alkylation with [^{14}C]NEM was performed essentially as described (*9*). RSO membrane vesicles [∼0.8−1.1 mg of total membrane protein in 50 μL of 100 mM KP_i (pH 7.3)/10 mM $MgSO_4$] containing a given single-Cys mutant were incubated with [^{14}C]NEM (40 mCi/mmol; final concentration 0.4 mM) in the absence or presence of 10 mM TDG at 25 °C. Labeling was terminated after 10 min by addition of 1 M dithiothreitol (DTT), and the membranes were solubilized with 2% *n*-dodecyl β-D-maltopyranoside (DDM; final concentration) for 5 min at 25 °C. The DDM extract containing solubilized membrane protein was incubated for 30 min at 4 °C with immobilized monomeric avidin−Sepharose beads previously equilibrated in 50 mM KP_i (pH 7.4)/100 mM NaCl/0.02% DDM (w/v; equilibration buffer). The resin was then extensively washed with equilibration buffer, and biotinylated permease was eluted with of 5 mM D-biotin in equilibration buffer. Sodium dodecyl sulfate−12% polyacrylamide gel electrophoresis ($NaDodSO_4$−PAGE) followed by autoradiography with a STORM 860 phosphoimager (Molecular Dynamics) was used to analyze NEM labeling.

MTSES Labeling. RSO membrane vesicles [∼0.8−1.1 mg of total membrane protein in 50 μL of 100 mM KP_i (pH 7.3)/10 mM $MgSO_4$] harboring a given single-Cys mutant were incubated with or without 10 mM TDG for 10 min at room temperature. The vesicles were then incubated without or with MTSES (200 mM final concentration) for 5 min at room temperature prior to addition of 0.4 mM [^{14}C]NEM (40 mCi/mmol). After 10 min incubation with NEM at room temperature reactions were quenched with DTT, and the samples were treated as described above. Analysis of radiolabeled bands was used to evaluate the extent of MTSES labeling, which corresponds to a decrease in the intensity of [^{14}C]NEM-labeled LacY in samples that react with MTSES relative to the untreated samples.

Immunoblots. Fractions containing affinity-purified biotinylated permease were analyzed by $NaDodSO_4$−PAGE. Protein was electroblotted onto poly(vinylidene difluoride) membranes (Immobilon-PVDF; Millipore) and probed with avidin conjugated to horseradish peroxidase (avidin−HRP).

RESULTS

Expression of Single-Cys Mutants. To test expression of the single-Cys mutants, immunoblots were carried out on membrane preparations with avidin−HRP (Figures 1−4). With the exception of Y26C, H35C, Y101C, K358C, and S366C, which are not expressed significantly, the mutants are expressed at levels comparable to C-less LacY; these five mutants were not included in this study. Mutants W171C, S174C, A177C, and L184C are expressed at 10−30% of C-less LacY and are included in the study.

Helix I. LacY mutants with single-Cys residues at positions 2−12, 15, 21, 22, 27, 31, 33, 34, 36, 38, 39, and 41−44 react well with NEM, while Cys-replacement mutants at positions 14, 17, 25, 28, and 32 react relatively poorly (Figure 1, lanes 1 and A in bottom panel). Cys residues at positions 13, 16, 19, 20, 23, 37, and 40 do not react with NEM under the same experimental conditions (data not shown). Mutants F18C, G24C, F29C, and F30C react only in the presence of β-D-galactopyranosyl 1-thio-β-D-galactopyranoside (TDG), while reactivity leads to significant increases in NEM reactivity at positions 12, 14, 15, 17, 28, 30, 31, 32, and 44. In contrast, decreased NEM reactivity is observed at positions 4, 21, 22, 27, and 34 (lanes 2 and B in bottom panel).

Studies with MTSES alone (lanes 3 and panel C in bottom panel) show that Cys residues at positions 3, 5, 7, 11, 12, 14, 15, 17, 25, and 36 are accessible in the absence or

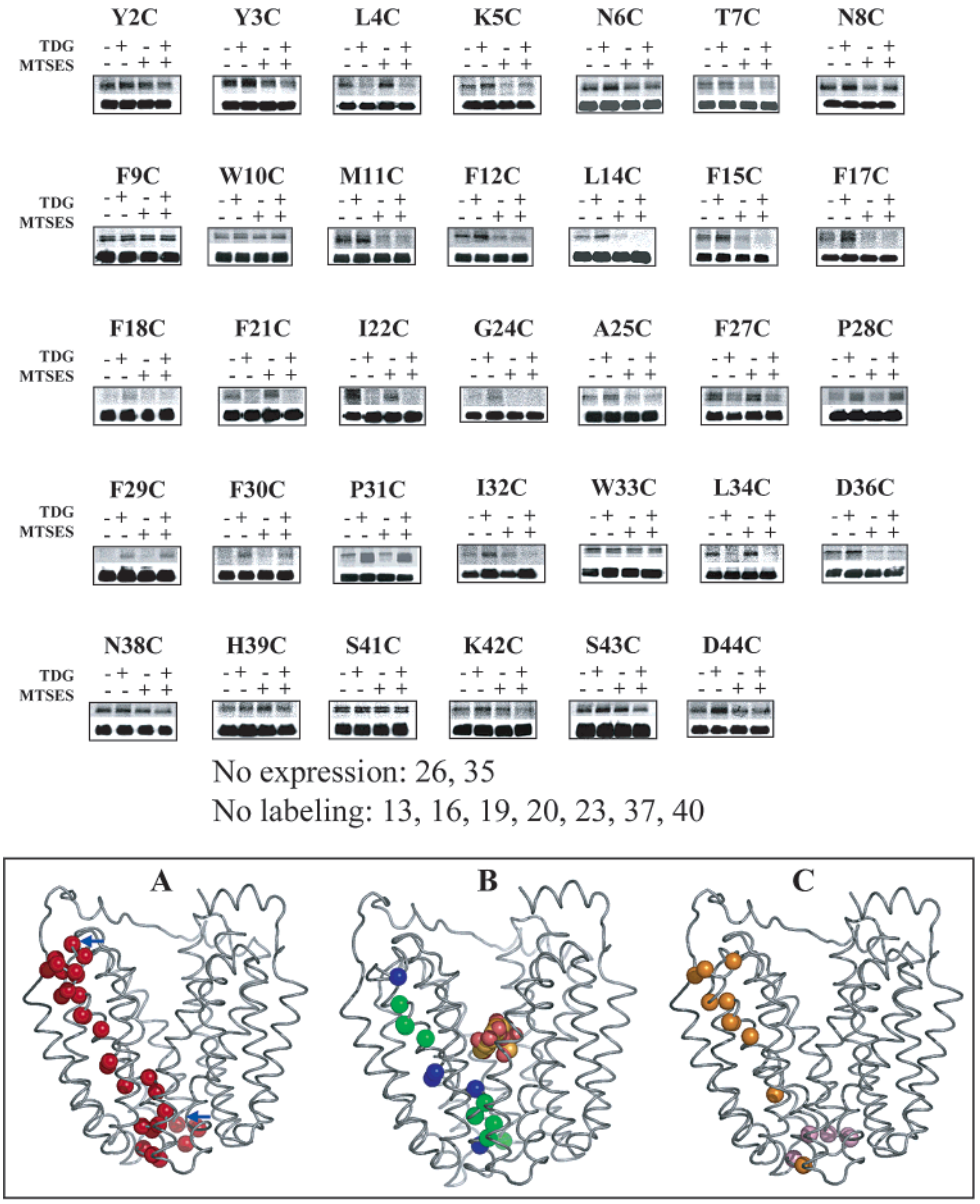


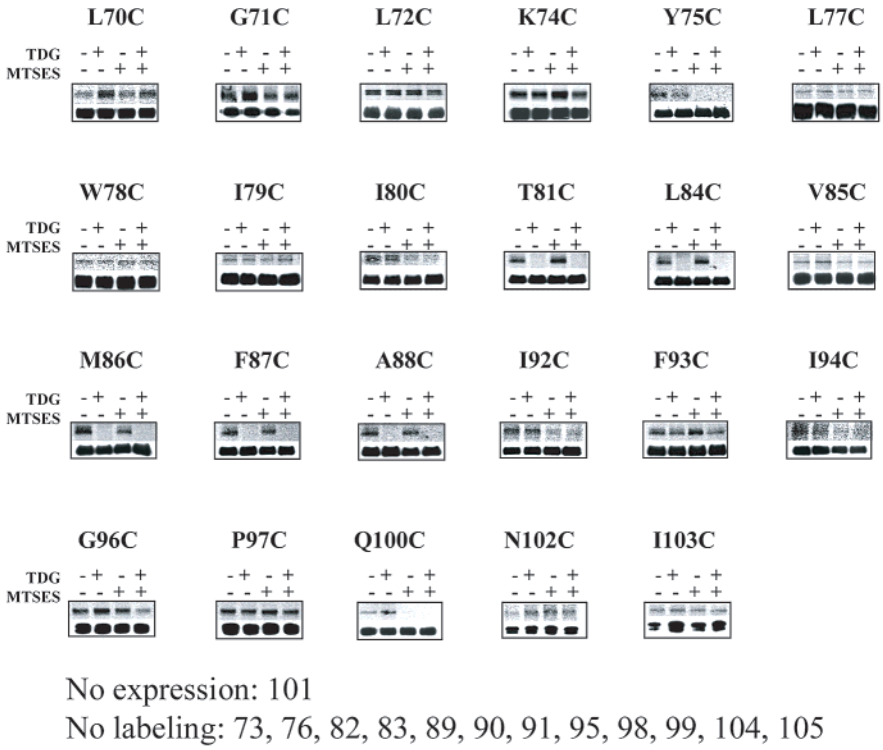
FIGURE 1: Helix I. Accessibility of given single-Cys LacY mutants to NEM and MTSES and the effect of TDG. The experiments were carried out as described in Experimental Procedures. The upper portion of each panel represents NEM labeling (autoradiography), and the bottom portion represents immunoblots of given Cys replacements in the absence or presence of TDG and/or MTSES as indicated. The panel at the bottom represents the positions of the Cys replacements within helix I (spirals) superimposed on the X-ray crystal structure of LacY (Protein Data Bank entry 1PV7). Blue arrows enclose the region studied. The C_α atoms of the positions where Cys replacements are alkylated with NEM are shown as red spheres (A). Residues that exhibit increased or decreased accessibility to NEM in the presence of substrate are shown as green and blue spheres, respectively (B). Bound TDG is shown as yellow and orange spheres in panel B. Residues that react with MTSES in the absence or presence of substrate are shown as yellow spheres. Pink spheres represent residues that are accessible to MTSES only in the presence of TDG (C).

presence of ligand. Positions 32, 38, 42, 43, and 44 are accessible to solvent from the exterior surface of the membrane only in the presence of ligand. Although weakly reactive with NEM, Cys substitutions at positions 18 and 24 are accessible to MTSES in the presence of substrate (i.e., increased reactivity in the presence of TDG is blocked by MTSES; compare lanes 2 and 4).

NEM reactivity of Cys-replacement mutants at positions 2, 6, 8, 9, 10, 33, 39, and 41 does not change significantly in the presence of substrate and/or MTSES.

Helix III. LacY mutants with single-Cys replacements at positions 71, 72, 74, 80, 81, 84, 86−88, 92, 93, 96, and 97 react well with NEM, while reactivity of the mutants with Cys replacements at positions 70, 75, 77−79, 85, 94, 100, 102, and 103 is noticeably lower (Figure 2, lanes 1 and A in bottom panel). Cys residues at positions 73, 76, 82, 83, 89, 90, 91, 95, 98, 99, 104, and 105 are essentially not reactive (data not shown). TDG binding causes significant increases in reactivity with mutants L70C, G71C, or G96C and decreased reactivity with Cys replacements at positions 81, 84, 86, 87, and 88 (compare lanes 1 and 2 and B in bottom panel).

NEM labeling after preincubation with MTSES shows that positions 75, 80, 92, 94, and 100 are accessible to solvent in the absence or presence of ligand, while a Cys residue in place of Gly96 is accessible to solvent in the presence of ligand only. Single-Cys replacements at position 72, 74, 77, 78, 79, 85, 97, 102, or 103 exhibit the same level of reactivity in the absence or presence of TDG, MTSES, or both.



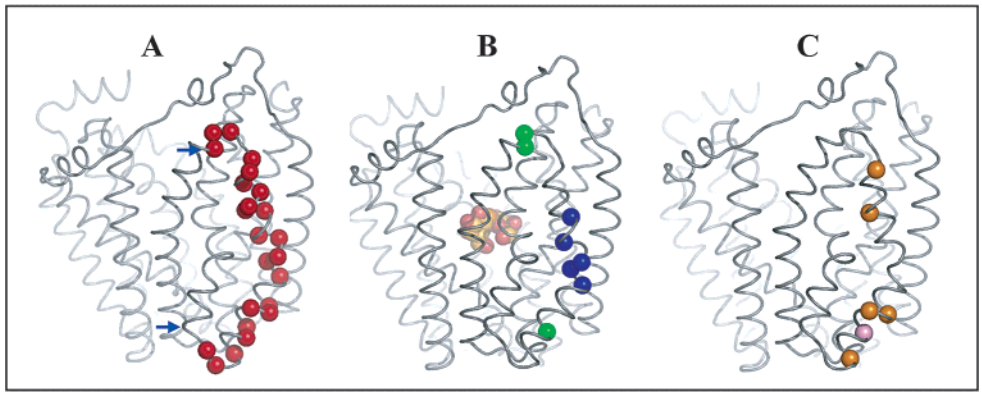


FIGURE 2: Helix III. Accessibility of given single-Cys LacY mutants to MTSES and the effect of TDG. Experiments were carried out as described in Experimental Procedures and in the legend to Figure 1.

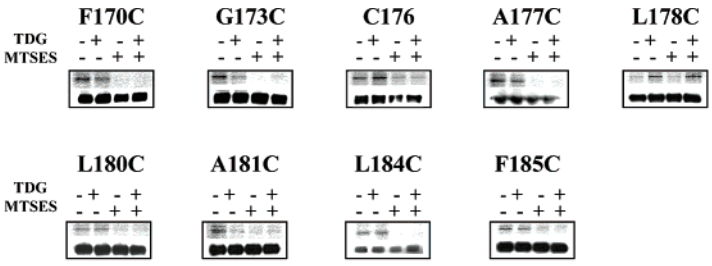
Helix VI. Permease mutants with a single Cys at positions 168−187 were labeled with NEM in the absence and presence of TDG or MTSES or both. Cys residues at positions 170, 173, 176, 177, 184, and 185 are relatively weakly reactive. Cys residues 178, 180, and 181 are even less reactive (Figure 3, lanes 1). Mutants 168, 169, 171, 172, 174, 175, 179, 182, 183, 186, and 187 do not react with NEM (not shown).

Substrate binding leads to a marked increase in reactivity of L178C LacY and a significant enhancement of labeling with native Cys176. Substrate protection from NEM labeling is observed with Cys substitutions at positions 173 and 181 (compare lanes 1 and 2 and B in bottom panel).

Labeling after incubation with MTSES shows that, with the exception of mutant L178C, the other eight replacements (170, 173, 176, 177, 180, 181, 184, and 185) are accessible to solvent in the absence or presence of ligand. Although differences in labeling of Cys residues in helix VI are observed, the reactivity of these replacements is significantly lower than Cys residues in helices I, III, or XI probably due to contact with the low dielectric of the bilayer.

Helix XI. LacY mutants with single-Cys residues at positions 347, 348, 350, 355−357, and 365 react well with NEM (Figure 4, lanes 1), while mutants with replacements at positions 351, 354, 359, and 360−364 exhibit low reactivity (Figure 4). Cys residues at positions 349, 352, and 353 are essentially unreactive (not shown).

Ligand binding causes a marked increase in reactivity of Cys residues at positions 359, 361, 362, 363, and 364. Decreased reactivity with NEM is observed with Cys replacements at positions 356 and 357 (compare lanes 1 and 2 and B in bottom panel). Studies with the impermeant reagent MTSES reveal that Cys residues at positions 347, 348, 350, 354, 355, 357, and 360−365 are accessible to solvent in the absence or presence of ligand (compare lanes 1 with 3 and lanes 2 with 4). Mutant L351C exhibits the same level of reactivity in the absence or presence of TDG, MTSES, or both.



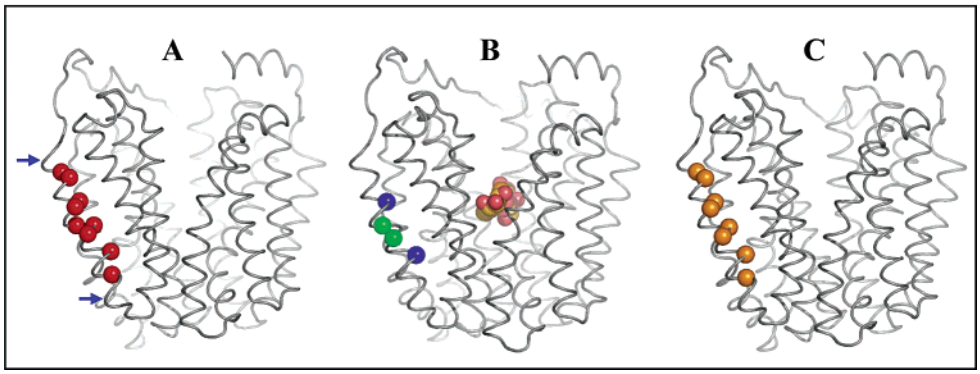


FIGURE 3: Helix VI. Accessibility of given single-Cys LacY mutants to MTSES and the effect of TDG. Experiments were carried out as described in Experimental Procedures and in the legend to Figure 1.

DISCUSSION

Helix I. Helix I lines part of the inward-facing hydrophilic cavity containing the sugar-binding site (*18*). Previous evidence (*45*−*47*) from single amino acid replacements and analysis of a series of deletion mutants in helix I led to the suggestion that this transmembrane domain may play an important role in the mechanism of action of LacY. However, results from Cys-scanning mutagenesis of helix I (*48*) demonstrate that Cys replacement for Gly24 or Tyr26 exhibits activity comparable to C-less LacY. The effect of NEM on lactose transport demonstrates that Cys-replacement mutants exhibiting significant inactivation are all located at the C terminus of helix I from F27C to W33C (*48*). Therefore, although no single residue in helix I is essential for activity, the structural integrity of the periplasmic half is important for active lactose transport (*48*). The LacY X-ray structure shows that the interface between the cytoplasmic halves of helices I and V is enriched with Gly and Phe residues that generate tight interaction between helices probably by forming multiple C_α—H···OH bonds between C_α atoms of Gly residues from helix V and backbone oxygen atoms of helix I (*53*). Moreover, the X-ray structure of LacY reveals a tightly packed cluster of residues around Gly24 and Trp151 that also includes Met23.

This study demonstrates that about 60% of the residues in the N terminus and helix I react well with NEM, indicating that the accessibility of most of the helix is not limited by close contacts with other transmembrane helices. This notion seems to be in contradiction with the previous assertion about tight contact between the cytoplasmic halves of helices I and V. However, the majority of alkylated residues is positioned on the opposite side of helix I and is not in direct contact with helix V. Moreover, disruption of tight helix−helix interactions due to a Cys residue in the place of a native Gly may disturb packing between helices I and V, resulting in increased flexibility of helix I and/or increased accessibility of a Cys side chain to NEM.

Ligand-induced alterations of labeling (Figure 1, lanes 1 and 2) suggest that substrate binding elicits a conformational change causing Cys replacements at these positions to become either more or less reactive/accessible to NEM. Most of the positions that show increased reactivity are located at the same level or on the periplasmic side as the sugar-binding site (B in bottom panel). Residues that exhibit decreased alkylation as a result of preincubation with MTSES are distributed throughout helix I and loop 1−2 (C in bottom panel). Increased reactivity/accessibility of replacements at positions on the periplasmic face of the membrane is clearly consistent with ligand-induced opening of a cavity on this face of the membrane, as postulated by the alternating accessibility model (*18*). However, the distribution of ligand-induced decreased activity of Cys residues throughout helix I suggests that this helix undergoes conformational changes that involve more than a simple translational movement.

Helix III. Helix III lies on the periphery of the N-terminal six-helix bundle in LacY (*18*). As shown previously (*49*), replacement of helix III en bloc with a putative synthetic helix containing 23 helix-forming residues [poly(Ala), poly(Leu), or poly(Phe)] abolishes active lactose transport, suggesting that helix III may contain functionally important residues. Cys-scanning mutagenesis shows that, except for mutant G96C, the single-Cys mutants exhibit little or no change in transport activity after NEM treatment. One replacement in the region, L76C, causes marked inactivation in the Cys-less background. However, the same mutation is well tolerated in wild-type LacY.

Helix III is in close proximity to helix IV at the periplasmic end (*18*) and has a similar distribution of Cys replacements that exhibit decreased labeling in the presence of TDG (*22*). Thus, replacements in helix IV in the immediate vicinity of

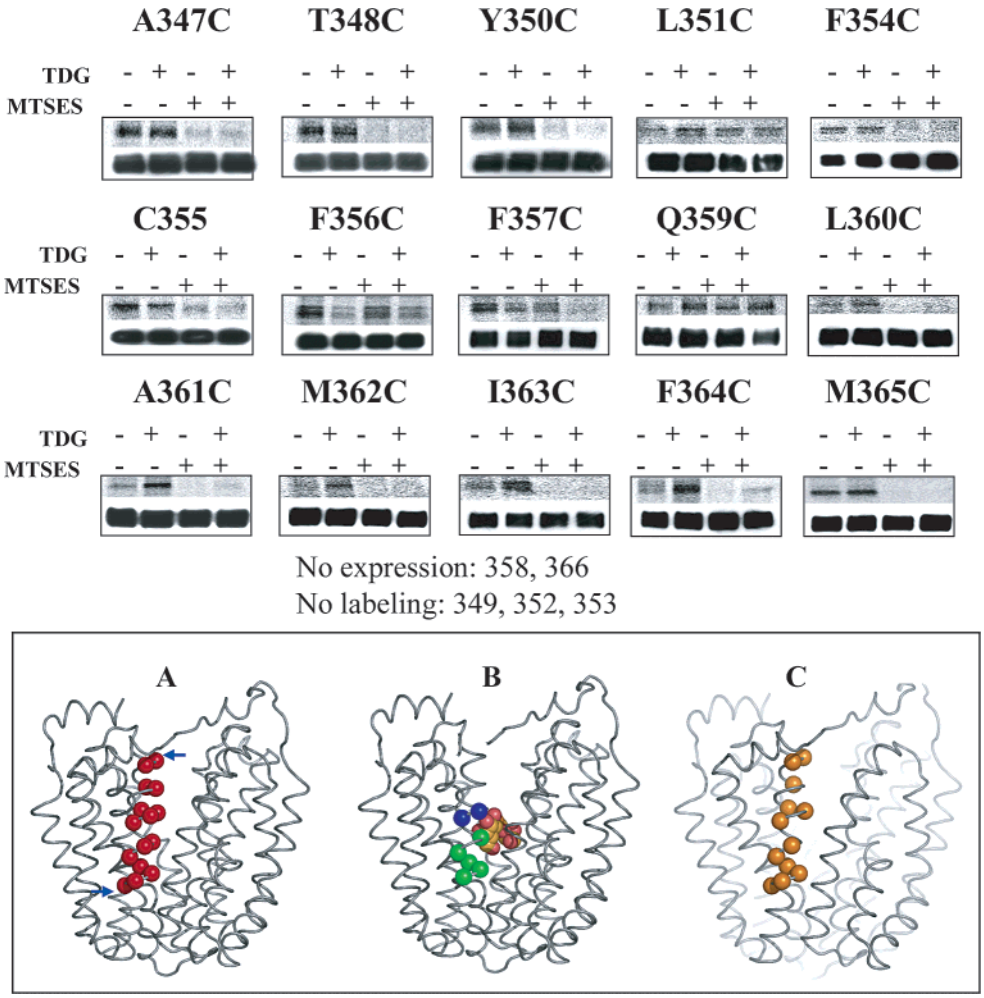


FIGURE 4: Helix XI. Accessibility of given single-Cys LacY mutants to MTSES and the effect of TDG. Experiments were carried out as described in Experimental Procedures and in the legend to Figure 1.

the sugar-binding site and in helix III, where replacements with like behavior are at the same level as the binding site, exhibit decreased labeling in the presence of TDG. Furthermore, in helix IV, these residues are located between two kinks. Perhaps ligand binding induces a conformational change(s) in helix IV that results in tighter packing with helix III, thereby decreasing reactivity/accessibility of Cys residues at the same level as the binding site. Mutants G111C to F118C, some of which appear to contact helix III, are not alkylated in the absence or presence of TDG (*22*), indicating that accessibility of this region to NEM is restricted by close contacts with neighboring helices.

Three replacements at positions close to the N and C termini of helix III (70, 71, and 96) exhibit increased labeling with NEM upon substrate binding. The structure reveals that L70C is in tight contact with Leu66 and Leu76, which interact with Phe63 to form a dense hydrophobic patch that may prevent NEM and MTSES labeling. The conclusion is consistent with experimental data showing little or no labeling of mutants F63C, L66C (*26*), and L76C with NEM. Substrate binding alters interaction such that a Cys residue at position 70 at the C terminus reacts better with NEM, while the environment surrounding other residues more deeply embedded in the membrane is unaffected by ligand binding.

Although alkylation of mutant G96C increases to only a small extent in the presence of TDG (Figure 2), it is apparent that reaction with MTSES is enhanced in the presence of TDG. Taken together, the observations indicate that although no residue per se appears to be essential, helix III undergoes important structural rearrangements upon substrate binding.

Helix VI. Like helix III, helix VI also lies on the periphery of the N-terminal six-helix bundle in LacY (*18*). Cys-scanning mutagenesis of helix VI and the flanking hydrophilic domains (positions 164−211) reveals that three mutants (F185C, A187C, and F208C) exhibit lower but significant levels of accumulation (*9*). Cys replacement for Ala177 or Leu184 causes more drastic inactivation, but the same mutations are tolerated well in the wild-type background. Transport activity of the great majority of the mutants is unaffected by NEM. Relatively modest but significant inactivation is observed with mutants F170C, G173C, and A187C, which cluster on the same face of helix VI as Ala177 and Leu184. Consistent with these observations, the majority of Cys replacements in helix VI do not react with NEM, and the eight residues that react do so relatively weakly. However, mutants F170C and G173C exhibit inhibition of transport activity after NEM treatment.

Mutations G173C, C176, L178C, and A181C, in the same plane as the sugar-binding site, exhibit clear TDG-dependent changes in labeling (Figure 3, B in bottom panel). Furthermore, Cys residues at positions 173 and 181 on a face of helix VI that is oriented toward the interior of the N-terminal six-helix bundle label better with NEM in the presence of

TDG. In contrast, native Cys176 and mutant L178C, located on the opposite face, label worse. Native Cys176 is accessible to MTSES with or without ligand, while L178C is inaccessible under either condition. The results are consistent with previous cross-linking studies between helix VI (position 170) and helices V (position 158 or 161) and VIII (position 264 or 265) at the periplasmic ends (*54*), suggesting that sugar binding results in significant rotation of helix VI.

Helix XI. Helix XI in the C-terminal six-helix bundle comprises part of the inward-facing cavity. Furthermore, Lys358 forms a salt bridge with Asp237 (helix VII), makes direct contact with one end of TDG to increase affinity, and is involved primarily with insertion of LacY into the membrane (see refs *20* and *55*). Cys-scanning mutagenesis (*8*) reveals that most of the mutants exhibit very significant transport activity, and Cys replacements for Thr348, Tyr350, and Lys358 result in inactive permease. In addition, single-Cys mutants that are inhibited by NEM exhibit a periodicity consistent with α-helical structure. It was proposed that the strongly inhibited positions (Gln359, Ser366, Cys355, and Met362) directly adjoin helix X. Interestingly, sulfhydryl modification stimulates transport activity of mutations F357C and I363C which are located on opposite sides of the helix, suggesting that these residues are also accessible to NEM and modification restores some of the bulk of the original residues, thereby improving transport.

About 80% of the Cys residues tested in helix XI are alkylated by NEM, and there is a good qualitative correlation between inactivation of transport (*8*) and NEM labeling (Figure 4). However, the labeling pattern differs significantly with respect to location of the Cys replacements in helix XI. Residues that are accessible to NEM and MTSES independent of substrate line the hydrophilic cavity. Residues F356C and F357C exhibit TDG protection against alkylation and are located at the level of the sugar-binding site. Cys replacements at positions 359, 361, 362, 363, and 364, which are on the periplasmic side of the binding site, exhibit increased labeling in the presence of TDG. This may reflect conformational changes that lead to opening of a cavity on the periplasmic face of the protein. The notion is consistent with the results of MTSES labeling, indicating increased accessibility of most of these residues to the aqueous milieu. Furthermore, mutants T45C, F49C, and S53C at the periplasmic surface of helix II, which faces helix XI, show similar increases in NEM and MTSES reactivity/accessibility (*26*).

In summary, site-directed Cys modification is utilized in situ to analyze helices I, III, VI, and XI. Combined with previous findings (*7*, *9*, *10*, *22*, *24*−*27*, *48*, *56*−*59*), the study now covers the great majority of the positions in the transmembrane helices of LacY, as well as portions of many loops. Although the combined data are not shown here, a group of residues in the periplasmic region exhibits increased labeling upon ligand binding, as well as accessibility to MTSES. Furthermore, there is a corresponding group of positions lining the inward-facing cytoplasmic cavity that exhibits decreased alkylation in the presence of ligand. Clearly, the observations are consistent with the alternating access model for transport (*18*). Remarkably, Cys replacements at positions lining the inward-facing cavity are accessible to MTSES, a very impermeant reagent (*22*). Notably, the X-ray crystal structure of LacY does not have re-entrance loops. The observations highlight conclusions drawn from Cys accessibility studies with presumably impermeant thiol reagents. So how does MTSES gain access to positions in the cytoplasmic cavity? Possibly, the reagent gains access via the pathway by which the sugar reaches the binding site, an idea that is also consistent with the alternating access model.

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