Site-Directed Sulfhydryl Labeling of the Lactose Permease of *Escherichia coli*: Helices IV and V That Contain the Major Determinants for Substrate Binding†

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ABSTRACT: Helices IV and V in the lactose permease of *Escherichia coli* contain the major determinants for substrate binding [Glu126 (helix IV), Arg144 (helix V), and Cys148 (helix V)]. Structural and dynamic features of this region were studied by using site-directed sulfhydryl modification of 48 single-Cys replacement mutants with *N*-[^{14}C]ethylmaleimide (NEM) in the absence or presence of ligand. In right-side-out membrane vesicles, Cys residues in the cytoplasmic halves of both helices react with NEM in the absence of ligand, while Cys residues in the periplasmic halves do not. Five Cys replacement mutants at the periplasmic end of helix V and one at the cytoplasmic end of helix V label only in the presence of ligand. Interestingly, in addition to native Cys148, a known binding-site residue, labeling of mutant Ala122 → Cys, which is located in helix IV across from Cys148, is markedly attenuated by ligand. Furthermore, alkylation of the Ala122 → Cys mutant blocks transport, and protection is afforded by substrate, indicating that Ala122 is also a component of the sugar binding site. Methanethiosulfonate ethylsulfonate, an impermeant thiol reagent shown clearly in this paper to be impermeant in *E. coli* spheroplasts, was used to identify substituted Cys side chains exposed to water and accessible from the periplasmic side. Most of the Cys mutants in the cytoplasmic halves of helices IV and V, as well as two residues in the intervening loop, are accessible to the aqueous phase from the periplasmic face of the membrane. The findings indicate that the cytoplasmic halves of helices IV and V are more reactive/accessible to thiol reagents and more exposed to solvent than the periplasmic half. Furthermore, positions that exhibit ligand-induced changes are located for the most part in the vicinity of the residues directly involved in substrate binding, as well as the cytoplasmic loop between helices IV and V.

The lactose permease (LacY)[1] of *Escherichia coli* is a model for ion-gradient coupled transport proteins that convert free energy stored in electrochemical ion gradients into work in the form of solute concentration gradients (reviewed in refs *1*−*5*). Encoded by the *lacY* gene, LacY has been solubilized from the membrane, purified to homogeneity, reconstituted into proteoliposomes, and shown to be solely responsible for the stoichiometric symport of galactosides and H^+ (reviewed in ref *6*) as a monomer (see ref *7*). LacY is comprised of 12 hydrophobic, membrane-spanning, α-helical domains connected by relatively hydrophilic loops with both the N and the C termini on the cytoplasmic face of the membrane (Figure 1) (reviewed in refs *3* and *8*).

A functional LacY mutant devoid of native Cys residues has been subjected to Cys-scanning mutagenesis (reviewed in ref *9*), and use of this mutant library has led to a number of important insights (reviewed in refs *9*−*12*): (i) The great majority of the mutants are expressed normally in the membrane and exhibit significant activity, and only six side chains are clearly irreplaceable with respect to active transport [Glu126 (helix IV) and Arg144 (helix V), which are indispensable for sugar binding, and Glu269 (helix VIII), Arg302 (helix IX), and His322 and Glu325 (helix X), which are critical for H^+ translocation and coupling]. (ii) Helix packing, tilts, and ligand-induced conformational changes have been determined by using site-directed biochemical and biophysical techniques. (iii) Positions where the reactivity of the Cys replacement is increased or decreased by ligand binding have been identified (*13*−*17*). (iv) LacY is highly flexible. (v) A working model describing a mechanism for lactose/H^+ symport has been formulated (*18*).

Site-directed sulfhydryl modification of single-Cys permease mutants in situ with *N*-ethylmaleimide (NEM) is useful for studying static and dynamic features of LacY (*13*−*17*, *19*, *20*) as well as a number of other membrane proteins (e.g., see refs *21*−*32*). Alkylation with NEM is a measure of the reactivity and/or accessibility of a given Cys residue

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[1] Abbreviations: LacY, lactose permease; Cys-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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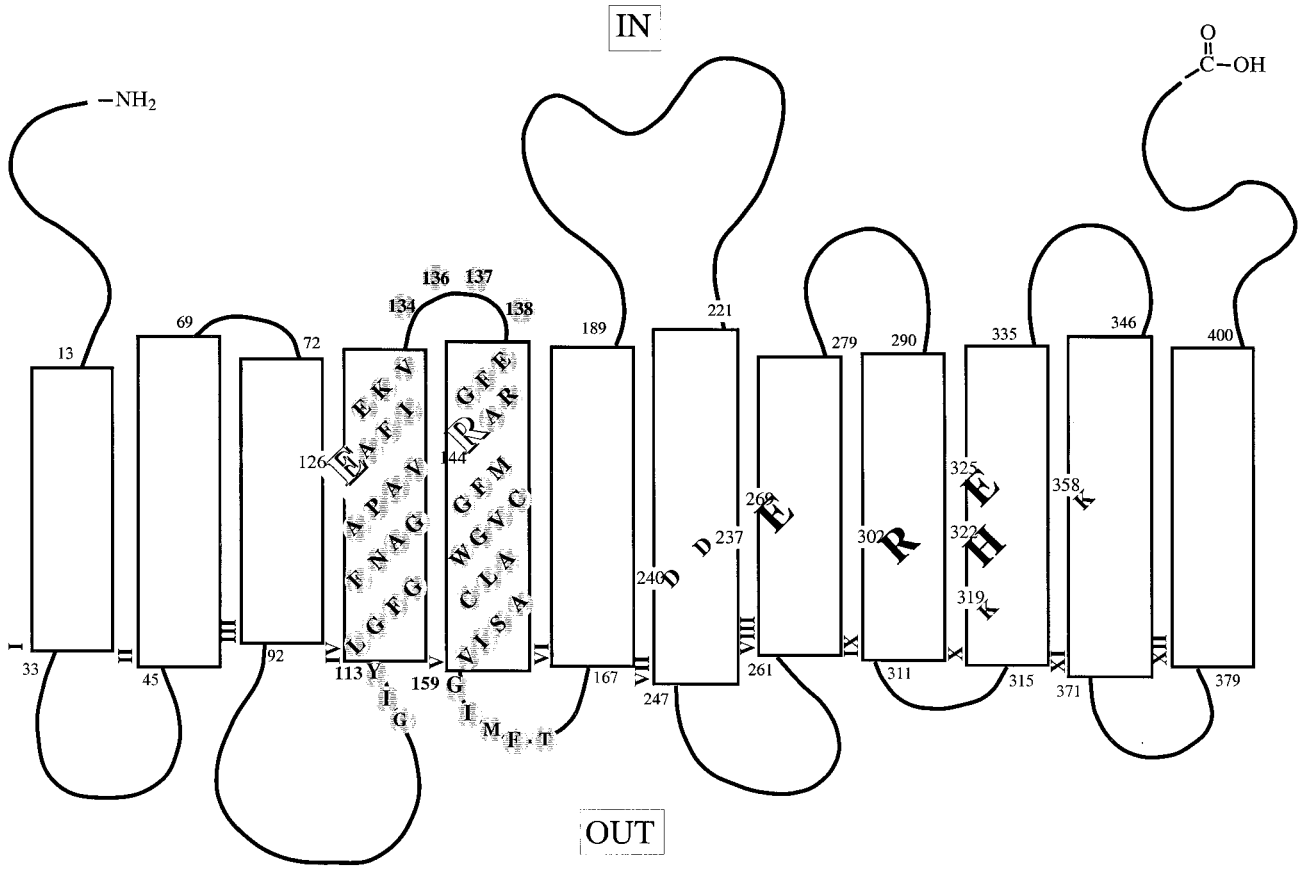

FIGURE 1: Secondary structure model of LacY. The single-letter amino acid code is used, and putative transmembrane helices are shown in boxes. The six irreplaceable residues Glu126 (helix IV), Arg144 (helix V), Glu269 (helix VIII), Arg302 (helix IX), His322 (helix X), and Glu325 (helix X) are shown as enlarged Roman letters. In addition, the charge pairs Asp237 (helix VII)/Lys358 (helix XI) and Asp240 (helix VII)/Lys319 (helix X) are highlighted. The residues in helix IV, loop IV/V, and helix V, loop V/VI, studied by site-directed NEM labeling are shown in shaded circles, and the site of the BAD is indicated.

to this small, amphipathic, membrane-permeant, thiol-specific reagent. Reactivity and/or accessibility are dependent primarily on the environment in the vicinity of a given Cys side chain and limited by close tertiary contacts between transmembrane helices and/or steric constraints of the bilayer. Hence, determination of the reactivity of Cys replacement mutants with NEM is convenient for assessing the local environment of specific positions within the tertiary structure. On the other hand, site-specific labeling in situ with methanethiosulfonate ethylsulfonate (MTSES), a small hydrophilic thiol reagent developed to determine the water accessibility of substituted Cys residues (*33*−*35*), has been utilized to study accessibility of Cys residues in LacY to the aqueous milieu (*15*−*17*, *36*). When extended to each transmembrane residue, the method should allow delineation of the solvent-accessible region(s) in LacY (*37*−*41*) that may represent all or part of the translocation pathway. Any change in labeling of a given Cys side chain upon substrate binding is indicative of an alteration of the local environment around the Cys residue or of the pathway from the bulk solution to the residue. Hence, in situ labeling is also valuable for identifying conformational alterations induced by ligand binding or other perturbants (*14*−*17*, *20*).

Glu126 (helix IV) and Arg144 (helix V) are irreplaceable with respect to all translocation reactions catalyzed by LacY (*42*, *43*). By studying site-directed NEM labeling of single-Cys148 LacY or site-directed fluorescence of single-Cys V331C[2] LacY (*20*) as well as ligand binding by flow dialysis (*43*), it is clear that a carboxylate side chain at position 126 and a guanidino side chain at position 144 are obligatory for substrate binding. Moreover, the reactivity of Cys148 with NEM is dramatically decreased when either Glu126 or Arg144 is replaced with Ala, but interchanging the residues, double-Ala replacement, or replacement of Arg144 with Lys or His does not alter reactivity, thereby indicating that Glu126 and Arg144 are charged paired (*20*).

On the basis of these and other observations, the following model for the substrate binding site has been postulated (*20*, *44*): (i) One of the guanidino NH_2 groups of Arg144 forms a H-bond with the OH group at the C-4 and/or C-3 position(s) of the galactosyl moiety of the substrate, an interaction that plays a key role in substrate specificity. (ii) The other guanidino NH_2 of Arg144 forms a salt bridge with Glu126, and the interaction holds Arg144 and Cys148 in an orientation that allows specific interaction with the galactosyl moiety. One of the oxygen atoms of the carboxylate at position 126 could also act as a H-bond acceptor from the C-6 OH of the galactosyl moiety. (iii) Cys148, which is protected by substrate against alkylation by *N*-ethylmaleimide (NEM), interacts hydrophobically with the galactosyl end of lactose and other galactosides. Although interactions with

[2] Site-directed mutants are designated by the single-letter amino acid abbreviation for the targeted residue, followed by the sequence position of the residue in the wild-type lac permease and a second letter indicating the amino acid replacement.

the nongalactosyl moiety are not clearly understood, Met145, which is on the same face of helix V as Cys148, is thought to be important in this respect for certain substrates.

One important aspect of the model is that Glu126 and Arg144 are charged paired and thus in close physical proximity. Support for this contention is provided by the demonstration that purified E126H/R144H LacY binds Mn(II) with high affinity in a pH-dependent manner (*45*). Furthermore, pyrene- or spin-labeled E126C/R144C LacY reconstituted into proteoliposomes shows eximer fluorescence or spin−spin interactions, respectively. Finally, in situ site-directed thiol cross-linking experiments (*46*) establish the proximity of positions 126 and 144 as well as neighboring amino acyl side chains. E126C/R144C LacY forms a disulfide bond spontaneously, and A122C on the same face of helix IV as Glu126 also forms a disulfide bond spontaneously with V149C on the same face of helix V as Arg144.

In this paper, site-directed sulfhydryl labeling is applied to 48 single-Cys residues in and around helices IV and V. The results demonstrate that the cytoplasmic halves of both helices where the major binding-site determinants are located, as well as given residues in cytoplasmic loop IV/V, label with NEM and are accessible to solvent, as judged by the ability of MTSES to block NEM labeling. In addition, evidence is presented indicating that Ala122 (helix IV) may be a component of the substrate binding site. Finally, Cys residues in loop V/VI exhibit increased reactivity, suggesting that helices V and VI undergo a conformational change upon binding of substrate.

EXPERIMENTAL PROCEDURES

Materials. N-[*ethyl*-1-^{14}C]Ethylmaleimide (40 mCi/mmol) was purchased from DuPont NEN (Boston, MA). [^{125}I]-Protein A was from Amersham (Arlington Heights, IL). Immobilized monomeric avidin was from Pierce (Rockford, IL). MTSES was purchased from Toronto Research Chemicals (Toronto, Ontario, Canada). Site-directed rabbit polyclonal antiserum against a dodecapeptide corresponding to the C terminus of LacY was prepared as described (*47*). All other materials were reagent grade and obtained from commercial sources.

Plasmid Construction. Construction of the single-Cys mutants used and insertion of the DNA fragment encoding the biotin acceptor domain (BAD) from a *Klebsiella pneumoniae* oxaloacetate decarboxylase at the C terminus of the mutants by restriction-fragment replacement have been described (*48*−*50*). Each mutant was verified by using dideoxynucleotide termination (*51*).

Growth of Bacteria. E. coli T184 (*lacY*$^-$*Z*$^-$) transformed with a plasmid encoding a given mutant was grown aerobically at 37 °C in Luria−Bertani broth containing streptomycin (10 μg/mL) and ampicillin (100 μg/mL). Fully grown cultures were diluted 10-fold and grown for 2 h before induction with 0.5 mM isopropyl 1-thio-β-D-galactopyranoside. After additional growth for 2 h, cells were harvested and used for preparation of right-side-out (RSO) membrane vesicles.

Preparation of RSO Membrane Vesicles. RSO membrane vesicles were prepared from 4-L cultures of *E. coli* T184 expressing a given mutant by lysozyme−ethylenediaminetetraacetic acid treatment and osmotic lysis (*52*, *53*). The vesicles were resuspended at a protein concentration of 16−22 mg/mL in 100 mM potassium phosphate (KP_i; pH 7.5)/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 (*19*). RSO membrane vesicles [0.4 mg of protein in 50 μL of 100 mM KP_i (pH 7.5)/10 mM $MgSO_4$] harboring a given single-Cys permease mutant were incubated with [^{14}C]NEM (40 mCi/mmol; 0.4 mM final concentration) in the absence or presence of 10 mM β-D-galactopyranosyl 1-thio-β-D-galactopyranoside (TDG) at 4 or 25 °C, as indicated. Labeling was terminated after 10 min by addition of 15 mM dithiothreitol (DTT), and the membranes were solubilized with 2.0% (w/v) *n*-dodecyl β-D-maltopyranoside (DDM) for 5 min at 25 °C. The DDM extract was incubated with immobilized monomeric avidin-Sepharose, previously equilibrated in 50 mM KP_i (pH 7.5)/150 mM NaCl/0.02% DDM (w/v; equilibration buffer) for 30 min at 4 °C. The resin was then extensively washed with equilibration buffer, and biotinylated permease was eluted with 5 mM *d*-biotin in equilibration buffer. Sodium dodecyl sulfate/12% polyacrylamide gel electrophoresis ($NaDodSO_4$/PAGE) followed by autoradiography was used to analyze NEM labeling. The relative amounts of autoradiographic bands were quantitated with a STORM 860 Phosphoimager (Molecular Dynamics).

MTSES Labeling. RSO membrane vesicles [0.5−0.6 mg of protein in 50 μL of 100 mM KP_i (pH 7.5)/10 mM $MgSO_4$] harboring a given single-Cys permease mutant were incubated without or with 10 mM TDG for 10 min at 25 °C. The vesicles were then incubated with MTSES (200 μM final concentration) for 5 min at 25 °C prior to addition of 0.4 mM [^{14}C]NEM (40 mCi/mmol) for 10 min at 25 °C. Reactions were quenched with DTT, and the samples were treated as described. Analysis of the autoradiographic bands was used to evaluate the extent of MTSES labeling, which corresponds to a decrease in the intensity of ^{14}C-NEM-labeled permease in samples treated with MTSES relative to the untreated samples.

Western Blot Analysis. Fractions containing affinity-purified biotinylated permease were analyzed by $NaDodSO_4$/PAGE (*54*). Protein was electroblotted onto poly(vinylidene difluoride) membranes (Immobilon-PVDF; Millipore) and probed with a site-directed polyclonal antibody against the C terminus of LacY (*47*). The PVDF membrane was subsequently incubated with [^{125}I]protein A (30 mCi/mg; 100 μCi/mL) and autoradiographed. Quantification of the relative amounts of permease was carried out with a STORM 860 Phosphoimager (Molecular Dynamics).

Transport: *NEM Inactivation and Protection by TDG*. RSO vesicles containing A122C permease in 100 mM KP_i (pH 7.5)/10 mM $MgSO_4$ (4.0 mg protein/mL) were incubated at 4 °C with 2 mM NEM (final concentration) in the absence or presence of 10 mM TDG. At given times, DTT was added to a final concentration of 8 mM to quench the reaction. The samples were then washed 2× with a 100-fold excess of 100 mM KP_i (pH 7.5)/10 mM $MgSO_4$ and resuspended in the same solution to a concentration of 4.0 mg protein/mL. Transport of [1-^{14}C]lactose (10 mCi/mmol) was measured for 1 min in the presence of ascorbate and phenazine methosulfate under oxygen as described (*55*, *56*).

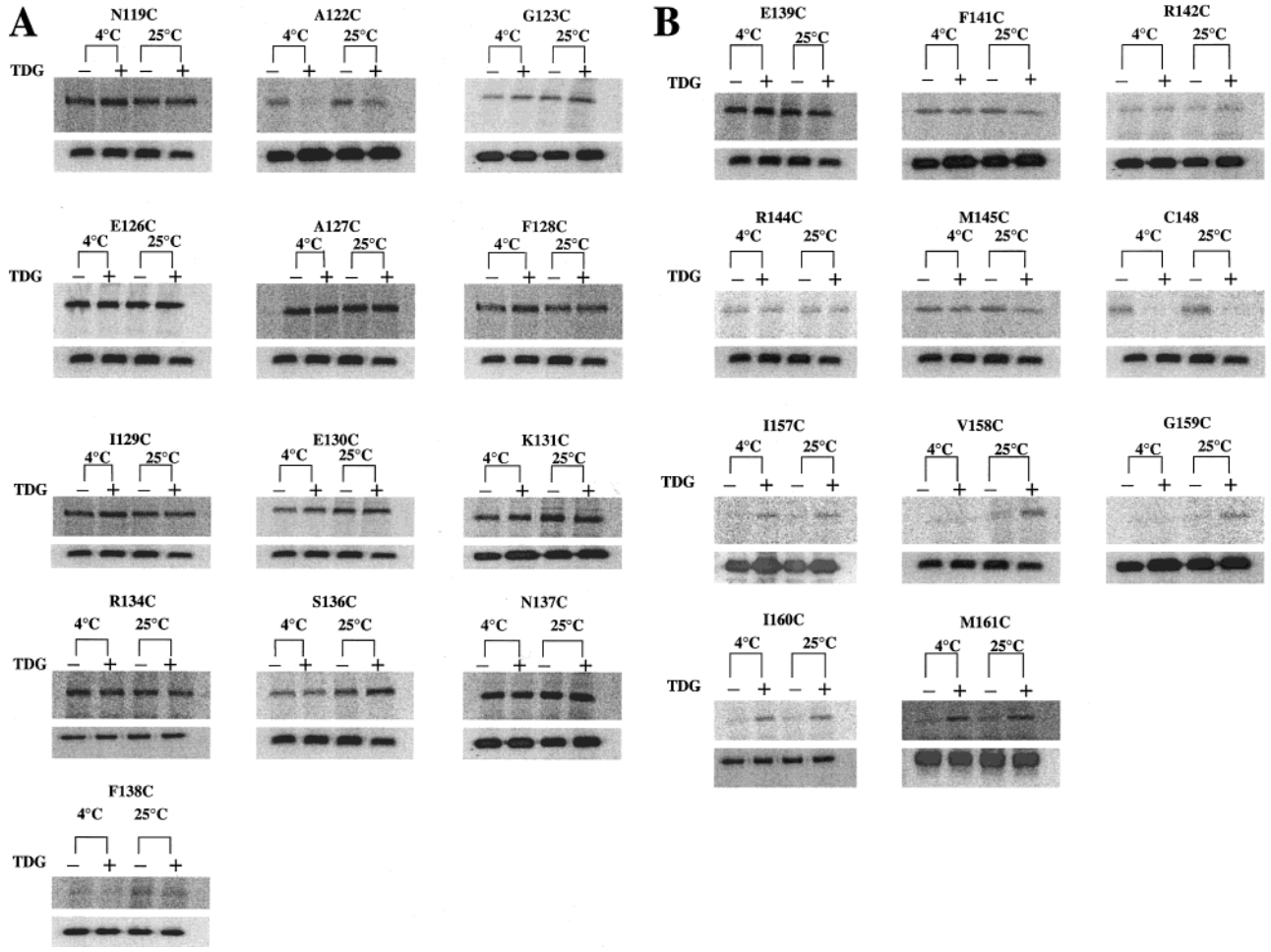

FIGURE 2: Effect of TDG and temperature on NEM labeling of given single-Cys mutants in helix IV, loop IV/V (panel A), and helix V, loop V/VI (panel B). RSO membrane vesicles [0.4 mg of protein in 50 μL of 100 mM KP_i (pH 7.5)/10 mM $MgSO_4$] prepared from *E. coli* T184 transformed with a plasmid encoding an indicated single-Cys mutant were incubated with [^{14}C]NEM (40 mCi/mmol; 0.4 mM, final concentration) for 10 min in the absence or presence of 10 mM TDG at 4 or 25 °C as shown. The reactions were terminated with DTT (15 mM, final concentration), and biotinylated permease was solubilized and purified by avidin-affinity chromatography as described in Experimental Procedures. Aliquots containing approximately 5 μg of protein were subjected to $NaDodSO_4$/12% PAGE and labeled. LacY was visualized by autoradiography (upper panels). A fraction of the protein (0.5 μg) eluted from the avidin-Sepharose beads was analyzed by Western blotting with anti-C-terminal antibody to quantify the amount of permease in each sample (lower panels).

Permeability to NEM or MTSES. Spheroplasts were prepared as described (*57*) and resuspended at 0.3 mg of protein/mL in 100 mM KP_i (pH 7.5)/0.5 M sucrose/DNase and RNase (1 mg/mL, final concentrations), and one-half of the preparation was disrupted by sonfication. To 100-μL aliquots of the intact or disrupted sample, [^{14}C]NEM (40 mCi/mmol) was added to a final concentration of 0.4 mM. Where indicated, samples were preincubated for 3 min with MTSES at a final concentration of 0.4 mM prior to addition of [^{14}C]NEM. At given times, reactions were quenched by addition of 15 mM DTT, followed by addition of 0.9 mL of cold 100 mM KP_i (pH 7.5) to lyse intact spheroplasts and centrifugation to sediment membranes and particulate debris. The supernatants were removed carefully, 400 μL of cold 10% trichloroacetic acid was added, and the samples were incubated on ice for 10 min to precipitate cytosolic proteins. The precipitates were collected by filtration through glass fiber filters and assayed by liquid scintillation spectrometry. The data were corrected for background radioactivity by adding [^{14}C]NEM to identical samples to which 15 mM DTT had been added previously.

Protein Determinations. Protein was assayed using a Micro BCA protein determination kit (Pierce Inc., Rockford, IL).

RESULTS

Expression and Activity of Single-Cys Mutants. Expression and activity of the single-Cys mutants used in these experiments (Figure 1) have been described previously (*50*, *58*), and insertion of the BAD at the C termini does not significantly alter expression or activity (data not shown). Mutants E126C and R144C, which are completely inactive because they do not bind substrate (*20*, *43*), were included in the study, but mutants M133C and R135C were not included because of poor expression.

NEM Labeling. LacY mutants N119C, A122C, P123C, E126C to K131C, R134C, S136C, N137C, and F138C (Figure 2A; helix IV, loop IV/V) and mutants E139C, G141C, R142C, R144C, M145C, C148, and I157C to M161C (Figure 2B; helix V, loop V/VI) label significantly with [^{14}C]NEM in 10 min at 25 °C (lane 3). Labeling is unchanged in the presence of TDG (compare lanes 3 and 4 in the appropriate panels) for mutants N119C, P123C, E126C, A127C to L131C, R134C, S137C, and F138C (helix IV) as well as E139C, R142C, or R144C (helix V), implying that changes resulting from ligand binding are either not reflected by the Cys at these positions or not apparent at 25

°C (with the exception of mutants E126C and R144C, which do not bind TDG). Ligand binding completely abolishes labeling of native Cys148 (see refs *18*−*20*, *43*, *44*, and *59*) and causes marked attenuation of labeling of mutant A122C and weak protection of mutant R141C or M145C (see ref *59*). There is a significant increase in labeling of mutant S136C in the presence of TDG (lanes 3 and 4) and a much more obvious increase in labeling of mutants I157C to F161C (lanes 3 and 4). Mutants G111C to F118C, A143C, F146C, G147C, V149C to S156C, F162C, and T163C are not alkylated by NEM in the absence or presence of TDG. Furthermore, imposition of $\Delta\bar{\mu}_{H^+}$ across the membrane generated by ascorbate oxidation in the presence of phenazine methosulfate does not alter NEM labeling of any of the single-Cys mutants in helix IV or V under the conditions described (data not shown).

Alkylation was also carried out at 4 °C for 10 min (*15*−*17*). Labeling of mutants N119C, P123C, E126C to L131C, R134C, N137C, E139C, G141C, R142C, R144C, M145C, C148, I157C, V158C, G159C, I160C, or M161C at 4 °C is comparable to that observed at 25 °C (Figure 2A,B; compare lanes 1 and 3 in the appropriate panels). However, Cys residues at positions 122 and 136 or 138 do not label as strongly at 4 as at 25 °C (compare lanes 1 and 3 in the appropriate panels). Because these are single-point experiments and neither time nor concentration is varied, it is difficult to make a definitive statement on the effect of temperature on the reaction kinetics. One possibility may be that thermal motion increases the accessibility/reactivity in mutants A122C, S136C, and F138C, unlike the remaining Cys residues. No substrate-induced change in labeling is observed with mutants N119C, P123C, E126C to L131C, R134C, S136C, N137C, F138C, E139C, G141C, R142C, R144C, or M145C permease at 4 °C (compare lanes 1 and 2 in the appropriate panels). In contrast, labeling of mutant A122C, located on the same face of helix IV as Glu126, is completely abolished by TDG at 4 °C, as also observed with single-Cys148 perrmease. Cys residues at positions 157−161 at the periplasmic end of helix V show an increase in labeling with TDG at 4 and 25 °C, suggesting that ligand-induced conformational changes rather than protein thermal motion favor labeling at these positions (compare lanes 1 and 2 in the appropriate panels).

Inactivation of Transport by A122C Permease and Protection by TDG. Active lactose transport in intact cells expressing A122C permease is inactivated by only about 50% after incubation with NEM (2 mM final concentration) for 30 min (*58*). However, with RSO vesicles, NEM almost completely inactivates transport in 60 min (Figure 3). Furthermore, when incubation with NEM is carried out in the presence of TDG, complete protection against inactivation is observed. Thus, the Cys residue at position 122 behaves in much the same manner as the native Cys residue at position 148 with respect to inactivation by alkylation and protection by substrate.

Permeability of the E. coli Cytoplasmic Membrane to NEM or MTSES. NEM effectively labels the cytosolic proteins of intact spheroplasts, demonstrating that the reagent is membrane permeant (Figure 4A). Furthermore, when spheroplasts are incubated with an equimolar concentration of MTSES prior to incubation with [^{14}C]NEM, no significant effect on the rate at which the cytosolic proteins are labeled is observed. When spheroplasts are disrupted prior to incubation

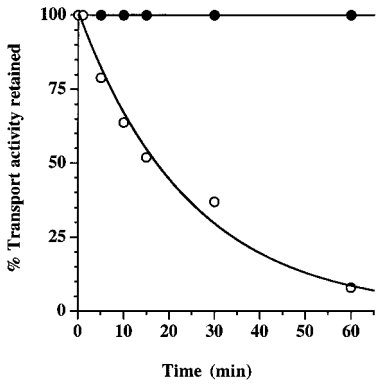

FIGURE 3: NEM inactivation and TDG protection of transport activity in RSO vesicles containing A122C permease. Aliquots (50 μL) of RSO vesicles containing A122C permease were incubated at 4 °C with NEM for the times indicated in the absence (○) or presence (●) of TDG when the reactions were quenched with DTT. Samples were then washed and assayed for transport with ascorbate and phenazine methosulfate under oxygen as described in Experimental Proceedures.

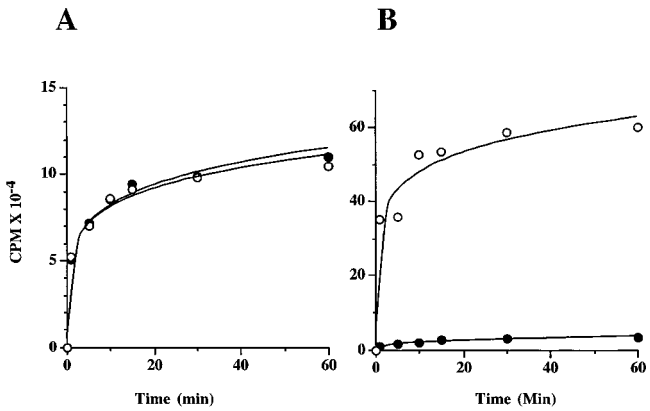

FIGURE 4: Permeability of spheroplasts to NEM or MTSES. *E. coli* T184 was grown aerobically overnight at 37 °C in Luria−Bertani broth with streptomycin (10 μm/mL); spheroplasts were prepared and treated as described in Experimental Procedures. (A) Intact spheroplasts were incubated with [^{14}C]NEM (40 mCi/mmol; 0.4 mM, final concentration) for given times in the absence (○) or presence (●) of 0.4 mM MTSES (final concentration). (B) Disrupted spheroplasts were treated identically in the absence (○) or presence (●) of 0.4 mM MTSES.

with [^{14}C]NEM (Figure 4B), the rate and extent of labeling are markedly increased, and dramatically, preincubation with equimolar MTSES almost completely blocks labeling. Therefore, it is clear that, while NEM is membrane permeant, MTSES is not.

Accessibility to MTSES. Pretreatment of mutants N119C, P123C, E126C, E129C, K131C, S136C, and N137C (Figure 5A; helix IV, loop IV/V) and mutants G141C, R142C, M145C, I157C, V158C, G159C, L160C, and M161C (Figure 5B; helix V, loop V/VI) with MTSES has no effect on [^{14}C]-NEM labeling, indicating that the Cys side chain at these positions is not accessible to MTSES, which further indicates that there is no water-filled pathway from bulk solution to these residues, at least not one wide enough for MTSES to pass (compare lanes 1 and 3). In contrast, NEM labeling of mutants A122C, A127C, F128C, E130C, R134C, F138C, E139C, R144C, or single-Cys148 permease is blocked by MTSES, demonstrating that the Cys side chain at these positions is accessible to solvent from the external surface of the membrane (compare lanes 1 and 3). Considering the

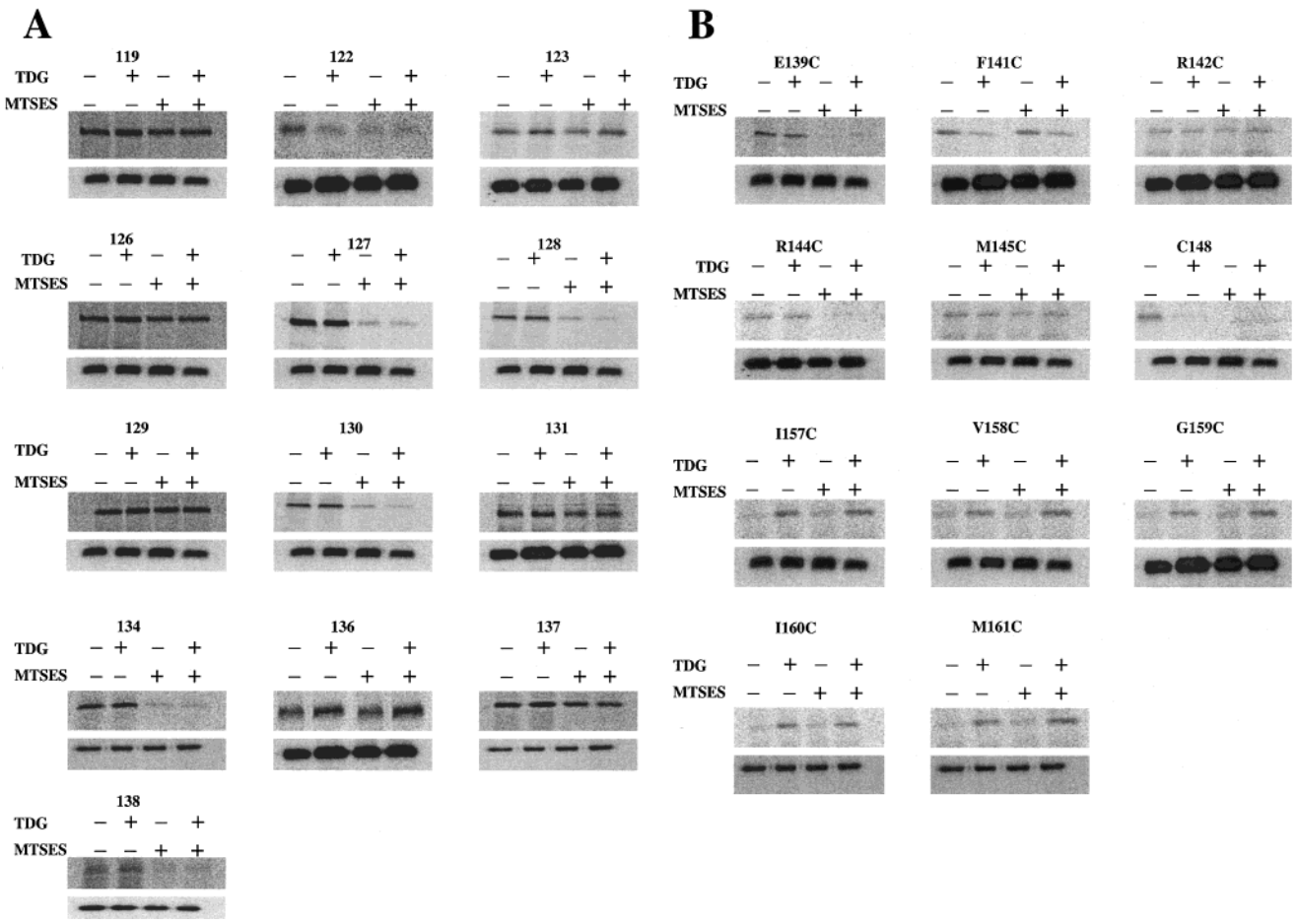

FIGURE 5: Accessibility of given single-Cys permease mutants in helix IV, loop IV/V (panel A) and helix V, loop V/VI (panel B), to MTSES and the effect of TDG. RSO membrane vesicles [0.5−0.6 mg of protein in 0.5 mL of 100 mM KP_i (pH 7.5)/10 mM $MgSO_4$] prepared from *E. coli* T184 transformed with plasmid encoding the indicated single-Cys mutant were incubated without or with MTSES (200 μM, final concentration) for 5 min at 25 °C in the absence or presence of TDG. The vesicles were washed 2× with ice-cold buffer and resuspended in 50 μL of the same buffer, and TDG (10 mM, final concentration) was added back to the samples initially treated with TDG. The samples were then treated with [^{14}C]NEM (40 mCi/mmol; 0.4 mM, final concentration) for 30 min at 25 °C. Reactions were quenched with DTT, and biotinylated permease was solubilized and purified as described in Experimental Procedures. Aliquots containing approximately 5 μg of protein were subjected to $NaDodSO_4$/12% PAGE, and labeled LacY was visualized by autoradiography (upper panels). A fraction of the protein (0.5 μg) eluted from the avidin-Sepharose beads was analyzed by Western blotting with anti-C-terminal antibody to quantify the amount of permease in each sample (lower panels).

limitations of this methodology (i.e., only changes greater than 2-fold are considered to be significant), it appears that none of the mutants exhibit a significant change in NEM labeling when pretreated with TDG in the absence or presence of MTSES (compare lanes 1 and 2 with lanes 3 and 4), indicating that ligand binding has small if any effect on the accessibility of a given Cys side chain to solvent.

DISCUSSION

Site-specific alkylation of 48 LacY mutants containing single-Cys replacements in and around helices IV and V was used to study structural and dynamic features of the region of LacY that contains the major determinants for substrate binding, which is the likely trigger for turnover (*18*). The cytoplasmic halves of both helices IV and V clearly label with NEM more readily than the periplasmic halves (Figure 6), as shown previously for helix X (*16*). With the exception of mutants I157C, V158C, G159C, I160C, and M161C at the periplasmic end of helix V, which label significantly only in the presence of TDG, none of the Cys replacements at positions 111−118 or positions 143, 146, 147, and 149−163 in the periplasmic halves of helices IV and V label with NEM. Most of the unreactive Cys replacements may lie either at the presumed interface between helices IV or V and the interior of the bilayer or in regions abutting other helices. Tertiary contacts within the protein or between the permease and the lipid bilayer may sterically and/or electronically disfavor alkylation of the thiol group in these mutants. Furthermore, poor labeling of Cys residues in the periplasmic halves of helices IV and V may suggest tighter tertiary packing between the two helices and the neighboring helices in the periplasmic half relative to the cytoplasmic halves of helices IV and V, as suggested previously for helix X (*17*). In any case, NEM labeling studies with intact cells containing A122C or Y113C LacY (data not shown) exhibit the same difference in labeling as observed with RSO vesicles (i.e., A122C LacY labels readily with NEM, while Y113C LacY is unreactive). Therefore, it is unlikely that the results can be attributed to the use of RSO vesicles rather than intact cells.

Ligand-induced changes in LacY are reflected by altered labeling of Cys residues at positions 122, 136, 141, 145, 148, and 157−161 in the presence of TDG (Figure 6). Labeling of Cys148 permease is sterically blocked in the presence of

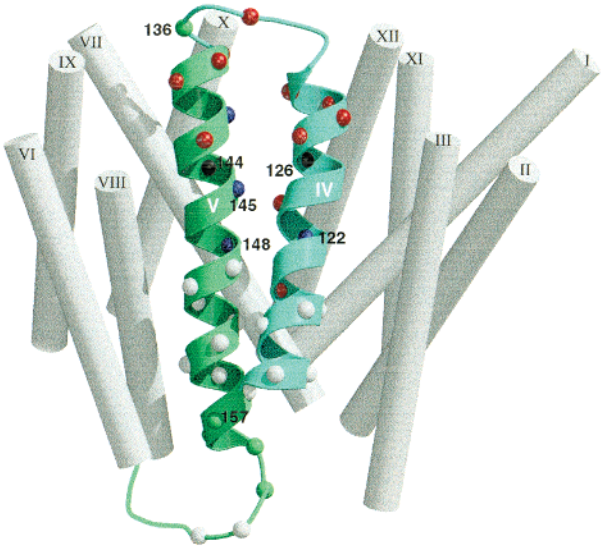

FIGURE 6: NEM labeling and the effect of ligand on single-Cys replacements in helix IV, loop IV/V, and helix V, loop V/VI. The results from Figures 2 and 3 are shown in a 3D representation of the helices and adjoining loops as follows. Positions that are labeled with NEM are presented as colored spheres: red, labels with NEM with no change in the presence of TDG; green, increased labeling in the presence of TDG; blue, decreased labeling in the presence of TDG. Positions that are not labeled are shown as white spheres. Residues Glu126 and Arg144 are shown as black spheres. Helices other than helices IV and V are shown as rods, and their positions are derived from modeling studies (*65*) using approximately 100 constraints (see ref *12*).

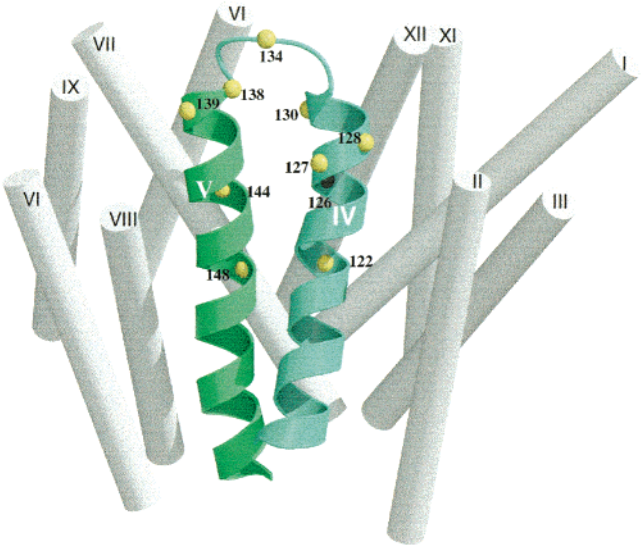

FIGURE 7: Solvent accessibility of single-Cys replacements in helix IV, loop IV/V, and helix V, loop V/VI, as judged by MTSES blockade of NEM labeling. The results from Figures 4 and 5 are shown in a 3D representation. Positions where NEM labeling is significantly blocked by MTSES are shown as yellow spheres. Helices other than helices IV and V are shown as rods, and their positions are derived from modeling studies (*65*) using approximately 100 constraints (*12*). Residues Glu126 and Arg144 are shown as black spheres.

ligand, and labeling of A122C permease is also markedly attenuated in the presence of ligand (Figure 2). In contrast, Cys residues at positions 157−161 undergo labeling only in the presence of TDG, demonstrating clearly that the ligand-induced changes in labeling at these positions is conformational. Thus, substrate binding enhances labeling at the periplasmic end of helix V and decreases labeling in the vicinity of the Glu126/Arg144 charge pair (positions 122, 141, 145, and 148). Similar results have been observed with single-Cys replacement mutants in helix II (*15*) and helix X (*17*). In any case, the finding that native-Cys148 labeling is completely attenuated by TDG and M145C is protected slightly is consistent with arguments that Cys148 interacts directly with the galactosyl moiety of the substrate (see refs *20*, *59*, and *60*).

A Cys residue at position 122 in helix IV, which cross-links with a Cys residue at position 149 (*46*) and is likely therefore to be within a single helical turn of Glu126/Arg144 charge pair and in close proximity to Cys148, also shows marked attenuation of labeling in the presence of ligand (Figures 2 and 6). Furthermore, active transport catalyzed by the A122C mutant is essentially completely inactivated by NEM, and complete protection is observed when alkylation is carried out in the presence of ligand (Figure 3). Thus, the A122C mutant exhibits properties similar to those of Cys148. It is also noteworthy that replacement of Cys148 with Ala has little effect on activity (*60*). Finally, the A122C mutant is the only Cys replacement mutant in helix IV that exhibits decreased labeling in the presence of TDG, an observation that argues against a ligand-induced conformational change being responsible for protection. Therefore, on the basis of the observations as a whole, it seems likely that attenuation of NEM labeling of mutant A122C in the presence of TDG reflects a direct interaction of Ala122 with substrate, possibly via a hydrophobic interaction with the β face of the galactosyl moiety of substrate, as is the case with Cys148 (*20*, *44*, *60*).

Thermal motion markedly influences labeling of several Cys residues, and labeling at 4 °C frequently reveals substrate-induced structural alterations that are not observed at 25 °C (*15*−*17*). Also, comparison of labeling at 25 vs 4 °C gives a qualitative indication of the contribution of protein dynamics to the reactivity/accessibility of a given Cys residue. Unlike Cys148, labeling of A122C permease is increased at 25 °C relative to 4 °C, but attenuation by ligand is observed at both temperatures, as is the case for Cys148. Thus, thermal motion enhances NEM labeling, but substrate protection is still observed, a finding that also favors the notion that substrate protection of mutant A122C is due primarily to steric rather than conformational effects. Mutant S136C or F138C (loop IV/V) also exhibits increased labeling at 25 °C relative to 4 °C. F138C permease labels poorly at 25 °C, but hardly labels at all at 4 °C, while mutants G141C, M145C, Cys-148 V158C, G159C, I160C, and M161C do not display significant thermally induced changes in labeling.

As observed with helices II and X (*15*, *17*), studies with MTSES indicate that the solvent accessible positions in helices IV and V (122, 128, 130, 134, 138, 139, and C-148) may be located near or on the cytoplasmic face of the membrane, while none of the Cys residues at the periplasmic ends are accessible to solvent (Figure 7). Moreover, binding of substrate does not appear to alter solvent accessibility in the cytoplasmic halves of helices IV and V. Because only certain positions within the binding site and in the immediate vicinity appear to be exposed to solvent, it is possible that the MTSES-reactive positions are part of the solvent-filled cleft in the permease that may represent all or part of the translocation pathway through LacY (*37*−*41*).

It is demonstrated clearly in this paper that NEM is membrane permeant, while MTSES is impermeant (Figure 4). Labeling of cytosolic proteins in intact spheroplasts with NEM is unchanged in the absence or presence of equimolar MTSES. However, when the membrane is disrupted, NEM labeling is completely blocked by MTSES. Therefore, it is clear that the accessibility of positions thought to be deeply buried in LacY or even in cytoplasmic loops to MTSES cannot be due to labeling from the cytosol. Although a possible explanation for the MTSES accessibility of these positions is that they are present in so-called P-loops, this suggestion is incompatible with observations demonstrating that Cys replacements in loop IV/V cross-link with Cys replacements in loops VIII/IX or X/XI (*61*). In addition, deletion (*62*) or insertional mutagenesis (*63*) of loop IV/V does not compromise activity. In view of these observations, the high rate of backbone H/D exchange (*64*) and the high degree of conformational flexibility exhibited by LacY (reviewed in ref *12*), it seems likely that, in addition to the solvent-filled cleft in LacY, there may be water-filled crevices between the helices that open and close transiently, thereby allowing access to small impermeant reagents such as MTSES.

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