Site-Directed Sulfhydryl Labeling of Helix IX in the Lactose Permease of *Escherichia coli*†

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ABSTRACT: Site-directed sulfhydryl modification of transmembrane helix IX in the lactose permease of *Escherichia coli* was studied in right-side-out membrane vesicles with the thiol-specific reagents *N*-[^{14}C]-ethylmaleimide (NEM) and methanethiosulfonate ethylsulfonate (MTSES) which are permeant and impermeant, respectively. Out of ∼20 mutants with a single Cys residue at each position in the helix, only five mutants label with NEM. (i) Cys residues at positions 291, 308, and 310 label at 25 °C, and binding of substrate has no effect. (ii) Cys residues at positions 295 and 298 label only in the presence of substrate. NEM labeling at 0 °C indicates that alkylation of Cys residues at positions 295 and 308 is dependent on the thermal motion of the protein. In contrast, temperature has little effect on labeling of Cys residues at positions 291, 298, and 310. Interestingly, pretreatment with MTSES blocks NEM labeling of all the mutants. The findings demonstrate that the face of helix IX on which Arg302 is located is involved in ligand-induced conformational changes and accessible to water from the periplasmic surface of the membrane. Since Arg302 facilitates deprotonation of Glu325 (helix X) during turnover [Sahin-Tóth, M., and Kaback, H. R. (2001) *Proc. Natl. Acad. Sci. U.S.A. 98*, 6068−6073], the findings are consistent with the idea that this face of helix IX may comprise part of the H^+ translocation pathway.

The lactose permease of *Escherichia coli* (LacY)[1] is a paradigm for ion gradient-coupled transport proteins that transduce free energy stored in electrochemical ion gradients into a solute concentration gradient (*1*−*5*). Encoded by the *lacY* gene, LacY has been solubilized, purified to homogeneity, reconstituted into proteoliposomes, and shown to be completely responsible for the stoichiometric symport of galactosides and H^+ (*6*) as a monomer (see refs *7* and *8*). LacY contains 12 hydrophobic, membrane-spanning, α-helical domains of different lengths (*9*, *10*) connected by relatively hydrophilic loops with both the N and C termini on the cytoplasmic face of the membrane (*11*, *12*) (Figure 1).

A functional LacY mutant devoid of eight native Cys residues has been subjected to Cys-scanning mutagenesis, and use of this mutant library, as well as other site-directed mutants, has led to a number of important observations (reviewed in refs *13*−*16*). (i) The great majority of the mutants are expressed normally in the membrane and exhibit significant transport activity, and only six residues are irreplaceable with respect to active transport [Glu126 (helix IV) and Arg144 (helix V) which are critical for substrate binding, Glu269 (helix VIII) which may be involved in substrate binding as well as H^+ translocation, and Arg302 (helix IX) and His322 and Glu325 (helix X) which are essential for H^+ translocation and coupling]. (ii) A tertiary structure model at the level of helix packing and the relationship between the irreplaceable residues has been formulated. (iii) Positions accessible to water have been revealed. (iv) Positions where the reactivity of the Cys replacement is increased or decreased by ligand binding have been identified. (v) LacY is highly flexible. (vi) A working model describing a mechanism for lactose/H^+ symport has been formulated.

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 as well as a number of other membrane proteins (see ref *17*; *18*−*25*). Alkylation with NEM is a measure of the reactivity and/or accessibility of a given Cys residue to this small, amphipathic, membrane-permeant, thiol-specific reagent. Reactivity and 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 lipid 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. In contrast, site-specific labeling in situ with methanethiosulfonate ethylsulfonate (MTSES) (*26*, *27*), a small hydrophilic thiol-specific reagent developed to determine the water accessibility of substituted Cys residues, has been utilized to study

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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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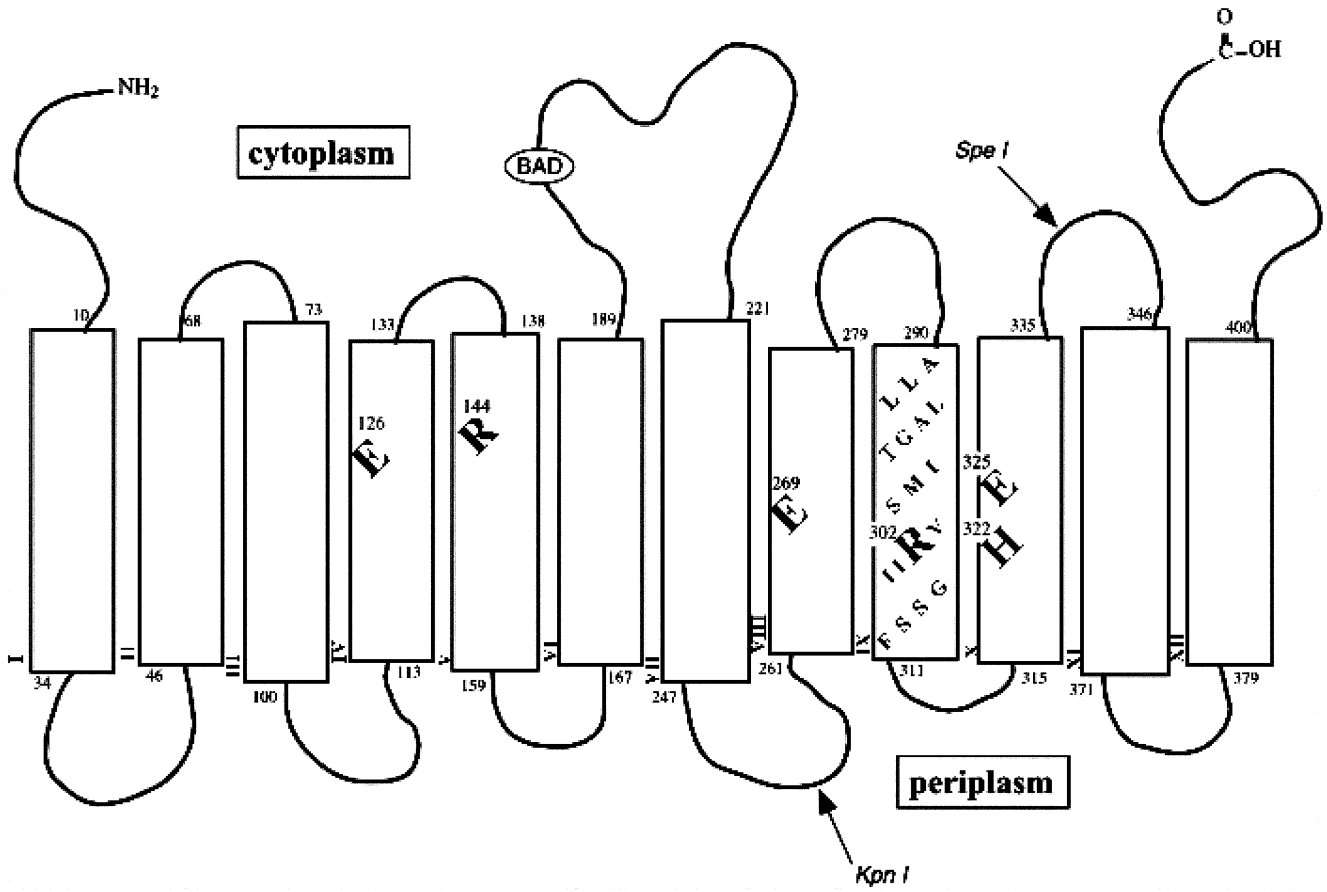

FIGURE 1: Secondary structure of lac permease. The single-letter amino acid code is used. Putative transmembrane helices are shown as rectangles. Mutants with a single Cys in place of each residue in helix IX except for Gly296 and R302 were studied. The six irreplaceable residues, Glu126, Arg144, Glu269, Arg302, His322, and Glu325, are bold; the site of the biotin acceptor domain (BAD) is indicated, and the restriction sites that were used are shown.

the accessibility of Cys residues in LacY to the aqueous milieu (*21*−*24*). Any change in labeling of a given Cys side chain upon substrate binding is indicative of an alteration in the local environment around the Cys residue. Therefore, in situ labeling is also valuable for identifying conformational alterations induced by ligand binding or other perturbants.

Transmembrane helix IX contains an irreplaceable residue, Arg302, that plays a direct role in H^+ translocation by facilitating deprotonation of neighboring Glu325 (helix X) (*15*, *28*). Although a previous study (*29*) revealed that the transport activity of single-Cys mutants in helix IX is relatively insensitive to NEM, labeling experiments were not carried out. In this paper, structural and dynamic features of helix IX are documented by using NEM labeling of single-Cys replacement mutants in situ. The results indicate that the interface between helices IX and X is conformationally active and accessible to water, findings that are consistent with the concept that this region may comprise part of the H^+ translocation pathway.

MATERIALS AND METHODS

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, ON). Site-directed rabbit polyclonal antiserum against a dodecapeptide corresponding to the C-terminus of lac permease was prepared as described previously (*30*). All other materials were reagent-grade and obtained from commercial sources.

Plasmid Construction. Construction of the single-Cys LacY mutants in a C-less background has been described previously (*29*). To facilitate avidin affinity purification, the DNA fragment encoding a given single-Cys mutant was inserted into plasmid pT7-5/cassette Cys-less *lacY*-L6XB which encodes Cys-less permease with a biotin acceptor domain (BAD) in cytoplasmic loop VI/VII (*31*) by restriction fragment replacement using the *Kpn*I and *Spe*I restriction sites. Each mutant was verified by using the dideoxynucleotide termination method (*32*).

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 ampicillin (100 μg/mL). Fully grown cultures were diluted 10-fold and grown for 2 h before induction with 1 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG). After additional growth for 2 h, cells were harvested and used for the 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 (*33*, *34*). Vesicles were resuspended to a protein concentration of 16−22 mg/mL in 100 mM potassium phosphate (KP_i, pH 7.5) and 10 mM $MgSO_4$, frozen in liquid N_2, and stored at −80 °C until

they were used.

NEM Labeling. Alkylation with [^{14}C]NEM was performed essentially as described previously (*18*, *35*). RSO membrane vesicles [1.0 mg of protein in 50 μL of 100 mM KP_i (pH 7.5) and 10 mM $MgSO_4$] harboring a given single-Cys mutant were incubated with [^{14}C]NEM (40 mCi/mmol, final concentration of 0.4 mM) in the absence or presence of 10 mM TDG at 0 or 25 °C as indicated. Labeling was terminated after 20 min by the 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 containing solubilized membranes was incubated with immobilized monomeric avidin−Sepharose beads previously equilibrated in 50 mM KP_i (pH 7.4), 100 mM NaCl, and 0.02% DDM (w/v; equilibration buffer) for 5 min at room temperature. 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. MTSES labeling of single-Cys mutants was carried out exactly as described previously (*21*−*23*).

Western Blot Analysis. 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 a site-directed polyclonal antibody against the C-terminus of lac permease (*30*). 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).

Protein Determinations. Protein was assayed using a Micro BCA protein determination kit (Pierce).

RESULTS

NEM Labeling at 25 °C. LacY mutants with a single-Cys replacement at each position in helix IX were included in this study with the exception of G296C[2] which is not expressed and R302C which is completely inactive (Figure 1) (*29*, *36*). Mutant A291C, F308C, or T310C reacts with [^{14}C]NEM in 20 min at 25 °C, but the extent of labeling is only mildly increased (A291C or F308C) or unaltered in the presence of β-D-galactopyranosyl 1-thio-β-D-galactopyranoside (TDG) (Figure 2, compare lanes 3 and 4 in the appropriate panel). Mutants A295C and I298C react with [^{14}C]NEM only in the presence of TDG (Figure 2, compare lanes 3 and 4 in the appropriate panel), indicating that ligand binding induces a conformational change reflected by a change in the reactivity and/or accessibility of the Cys residue at these positions. In contrast, the remaining Cys replacement mutants in helix IX (L292C, L293C, L294C, T297C, M299C, S300C, V301C, I303C, I304C, G305C, S306C, S307C, and A309C) are not alkylated by NEM in the absence or presence of TDG (data not shown).

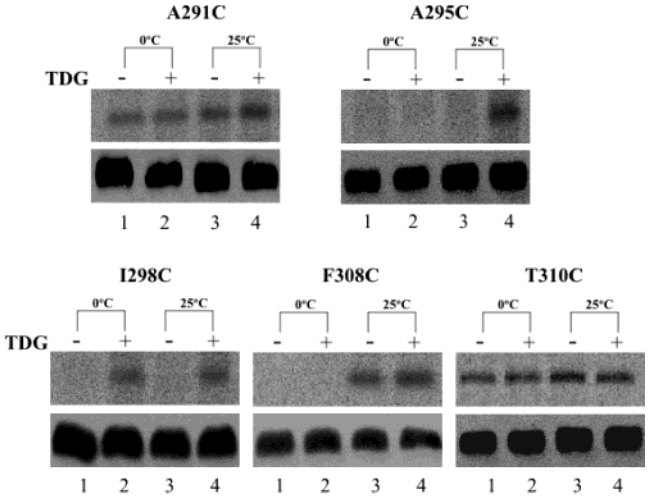

FIGURE 2: Effect of TDG and temperature on NEM labeling of single-Cys mutants in RSO membrane vesicles. RSO membrane vesicles [0.4 mg of protein in 50 μL of 100 mM KP_i (pH 7.5) and 10 mM $MgSO_4$] prepared from *E. coli* T184 transformed with a plasmid encoding an indicated single-Cys mutant were incubated with [^{14}C]NEM (40 mCi/mmol, final concentration of 0.4 mM) for 20 min in the absence or presence of 10 mM TDG at 0 or 25 °C as indicated. Reactions were terminated with DTT (final concentration of 15 mM), and biotinylated permease was solubilized with 2% DDM and purified by avidin affinity chromatography as described in Materials and Methods. Aliquots containing approximately 5 μg of protein were subjected to $NaDodSO_4$−12% PAGE, and labeled LacY was visualized by autoradiography (top 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 (bottom panels).

NEM Labeling at 0 °C. To minimize backbone thermal motion, alkylation with NEM was also carried out at 0 °C. The extent of labeling of A291C or T310C LacY is slightly lower than observed at 25 °C (Figure 2, compare appropriate lanes 1 and 3), indicating that thermal motion of the protein has little effect on the reactivity and/or accessibility of Cys to NEM at these positions. No TDG-induced change in labeling of A291C or T310C is observed at 0 °C (Figure 2, compare appropriate lanes 1 and 2). With F308C LacY, no NEM labeling is detected at 0 °C (Figure 2, F308C), suggesting that alkylation of the Cys at position 308 is highly dependent on the thermal motion of the protein. Taken together with the findings at 25 °C, it appears unlikely that ligand binding induces a conformational change that involves position 291, 308, or 310. With A295C LacY, no labeling is detected at 0 °C, even in the presence of TDG (Figure 2, appropriate lanes 1 and 2), suggesting that alkylation of the Cys at position 295 is dependent on the thermal motion of the protein even in the presence of ligand. With I298C LacY, temperature has no effect on labeling, and the mutant reacts with NEM only in the presence of TDG at either 0 or 25 °C (Figure 2, compare appropriate lanes 1 and 2) or 25 °C. Although labeling of I298C appears to be independent of the thermal motion of the protein, it should be emphasized that rates of alkylation in the absence and presence of ligand were not studied. In any event, the increase in the level of NEM labeling of Cys residues at position 295 or 298 clearly shows that these positions are involved in a ligand-induced conformational change.

[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.

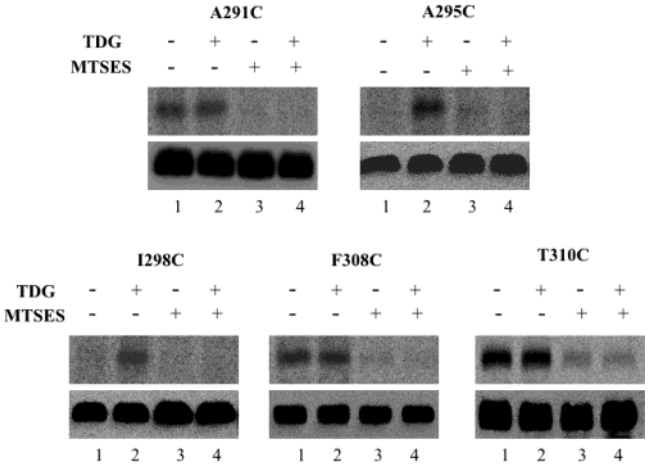

FIGURE 3: Accessibility of given single-Cys permease mutants in helix IX to MTSES and the effect of TDG. RSO membrane vesicles [1.0 mg of protein in 50 μL of 100 mM KP_i (pH 7.5) and 10 mM $MgSO_4$] prepared from *E. coli* T184 transformed with a plasmid encoding the indicated single-Cys mutant were incubated without or with MTSES (final concentration of 200 μM) for 5 min at 25 °C in the absence or presence of TDG. The vesicles were washed twice with ice-cold buffer and resuspended in 50 μL of the same buffer, and TDG (final concentration of 10 mM) was added back to the samples initially treated with TDG. The samples were then treated with [^{14}C]NEM (40 mCi/mmol, final concentration of 0.4 mM) for 20 min at 25 °C. Reactions were quenched with DTT, and biotinylated permease was solubilized with 2% DDM and purified as described in Materials and Methods. Aliquots containing approximately 5 μg of protein were subjected to $NaDodSO_4$−12% PAGE, and labeled LacY was visualized by autoradiography (top panels) (*1*−*5*). 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 (bottom panels).

MTSES Accessibility. The LacY mutants that label with NEM were tested for reactivity with methanethiosulfonate ethylsulfonate (MTSES), a hydrophilic sulfhydryl reagent shown to be membrane impermeant (*24*). Pretreatment of RSO membrane vesicle with MTSES in the presence or absence of TDG uniformly blocks NEM labeling in mutants A291C, A295C, I298C, F308C, and T310C (Figure 3, appropriate panels, lanes 3 and 4). Thus, Cys residues at positions 291, 295, and 298 which are located in the cytoplasmic half of helix IX, as well as Cys residues at positions 308 and 310 which are near the periplasmic end, are exposed to solvent from the periplasmic surface of the membrane. Furthermore, TDG-induced conformational changes do not appear to alter the accessibility of Cys residues at any of these positions to MTSES.

DISCUSSION

Previous studies (*29*) indicate that transport activity of single-Cys mutants in helix IX is relatively unaffected by NEM in RSO membrane vesicles. In this paper, single-Cys replacement mutants for each native residue in helix IX were studied by site-directed NEM labeling. Among 19 single-Cys mutants in helix IX, only five react with [^{14}C]NEM. Cys residues at positions 308 and 310 near the periplasmic end of helix IX and a Cys residue at position 291 in the cytoplasmic half react well with NEM in a manner that is unaffected by ligand binding. In contrast, Cys residues at positions 295 and 298 in the cytoplasmic half do not label unless ligand is bound. The other single-Cys replacement

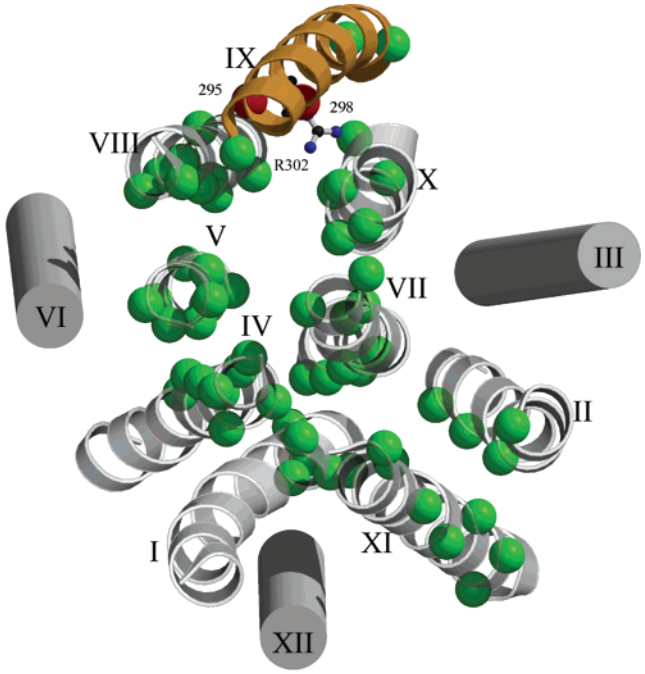

FIGURE 4: Helix packing of LacY viewed from the cytoplasmic surface showing positions where Cys replacement mutants are alkylated by NEM. The packing of the helices is taken from Sorgen et al. (*16*), and helix IX is highlighted. Positions where Cys replacements are alkylated by NEM are depicted as green balls. The two red balls shown in helix IX represent Cys replacements at positions 295 and 298 which react with NEM only in the presence of TDG; in addition, Arg302 is shown. Helices III, VI, and XII are shown as rods to indicate that none of the single-Cys mutants in these helices are inactivated by NEM, although NEM labeling studies have not been carried out on these helices. The face of helix XI with the NEM-reactive Cys replacements appears to face away from the middle of the 12-helix bundle, but only one constraint was used to model this helix (*16*), the salt bridge between Lys358 (helix XI) and Asp237 (helix VII). Therefore, it is possible that helix XI may be rotated counterclockwise by ≥45° which would bring the face with the NEM-reactive Cys replacements toward the interior of the protein.

mutants in helix IX are not alkylated by NEM over the 20 min time course tested in these experiments. The NEM reactive Cys mutants clearly cluster on the face of helix IX that contains Arg302, one of the six irreplaceable residues in LacY which is thought to be required for deprotonation of Glu325 in neighboring helix X (Figure 4). Furthermore, positions 298 and 295 which are alkylated by NEM in the presence of ligand only are one and two turns of helix IX removed from Arg302, respectively, toward the cytoplasmic end. In contrast, most of the unreactive Cys replacement mutants lie on the face helix IX that makes contact with the interior of the bilayer. The findings are consistent with the interpretation that tertiary contacts within the protein or between LacY and the low dielectric of the membrane may sterically and/or electronically disfavor alkylation of the thiol group (*8*, *21*−*23*).

Comparison of labeling at 25 and 0 °C provides a qualitative indication of the contribution of backbone dynamics to the reactivity and accessibility of a given Cys residue. The observation that the reactivity of mutants A295C and F308C is essentially nil at 25 °C implies that backbone motion is primarily responsible for the reactivity of Cys residues at these positions. In contrast, labeling of A291C, I298C, and T310C exhibits little or no difference at the two

temperatures, indicating that the reactivity and/or accessibility of the thiol groups at these positions are not altered by backbone motion.

Many observations demonstrate that ligand-induced conformational changes within LacY are reflected by altered NEM reactivity of single-Cys mutants induced by ligand binding (*18*, *21*−*24*, *37*). In this study, TDG binding is shown to enhance NEM labeling of Cys residues at positions 295 and 298. The two positions are on the same face of helix IX as Arg302 which was postulated to be charge-paired with Glu325 in helix X in the ground-state conformation and to be responsible for deprotonation of Glu325 when LacY returns to the ground state during turnover (*15*, *28*). Interestingly, NEM labeling of Cys residues at positions 315, 326, and 329 in helix X is also altered in the presence of TDG (*21*). Since these positions are on the same face of helix X as the irreplaceable residues Glu325 and His322 and appear to be close to the interface with helix IX (Figure 4), the results support the contention that the interface between helices IX and X is important for H^+ translocation.

Thiol modification studies with the hydrophilic reagent MTSES indicate that all five NEM-labeled single-Cys residues mentioned above are accessible to the aqueous phase from the periplasmic face of the membrane, and TDG has no effect on accessibility. LacY is highly dynamic conformationally, and most of the protein backbone is accessible to water (*38*, *39*). From these and previous studies (*18*, *21*−*24*, *37*), it appears that many of the residues in transmembrane helices that are accessible to bulk solvent form part of a hydrophilic cleft (*16*, *40*−*43*). These solvent accessible positions also reflect ligand-induced conformational changes, supporting the notion that these residues may provide pathways for substrate or H^+.

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