Location of Helix III in the Lactose Permease of *Escherichia coli* As Determined by Site-Directed Thiol Cross-Linking†

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ABSTRACT: The six N-terminal transmembrane helices (N_6) and the six C-terminal transmembrane helices (C_6) in the lactose permease of *Escherichia coli*, each containing a single Cys residue, were coexpressed, and cross-linking was studied. The proximity of paired Cys residues in helices III (position 78, 81, 84, 86, 87, 88, 90, 93, or 96) and VII (position 227, 228, 231, 232, 235, 238, 239, 241, 243, 245, or 246) was examined by using iodine or two rigid homobifunctional thiol-specific cross-linking reagents with different lengths [*N*,*N*′-*o*-phenylenedimaleimide (*o*-PDM; 6 Å) and *N*,*N*′-*p*-phenylenedimaleimide (*p*-PDM; 10 Å)]. Cys residues in the periplasmic half of helix III (position 87, 93, or 96) cross-link to Cys residues in the periplasmic half of helix VII (position 235, 238, 239, 241, or 245). In contrast, no cross-linking is evident with paired Cys residues near the cytoplasmic ends of helices III (position 78 or 81) and VII (position 227, 228, 213, 232, or 235). Therefore, the periplasmic halves of helices III and VII are in close proximity, and the helices tilt away from each other toward the cytoplasmic face of the membrane. On the basis of the findings, a modified helix packing model for the permease is presented.

Secondary active transport proteins couple the free energy stored in electrochemical ion gradients into substrate concentration gradients in membranes from *Archaea* to the mammalian central nervous system. The lactose permease of *Escherichia coli*, an important example of this class of membrane proteins, catalyzes the stoichiometric translocation of galactosides and H^+, utilizing the free energy stored in an electrochemical H^+ gradient to drive solute accumulation (*1*−*4*). This hydrophobic, polytopic cytoplasmic membrane protein was the first of its type to be solubilized, purified to homogeneity, reconstituted into proteoliposomes, and shown to be completely functional (reviewed in ref *5*) as a monomer (see ref *6*). Furthermore, all available evidence indicates that the molecule consists of 12 α-helical rods that traverse the membrane in zigzag fashion connected by relatively hydrophilic loops with the N and C termini on the cytoplasmic face (Figure 1) (reviewed in refs *7* and *8*).

Of the genomes sequenced so far, a significant percentage of the gene products are predicted to be membrane transport proteins. Many are important with respect to human disease (e.g., cystic fibrosis), and others are targets (e.g., the gastric H^+/K^+ ATPase) for widely used drugs. However, structural and dynamic information at high resolution are essential for understanding the mechanism of action, and at present, only a handful of membrane proteins have been crystallized in a form that allows structure determination at atomic resolution. In an effort to develop alternatives to crystallization that can yield both static and dynamic information at a meaningful level of resolution, a functional permease mutant devoid of native Cys residues (C-less permease)[1] has been constructed, and each residue has been replaced individually with Cys (*9*). Analysis of the mutants has led to the following developments (see refs *8*−*11*). (1) The great majority of the mutants are expressed normally in the membrane and exhibit significant activity, and only six side chains are clearly irreplaceable for active transport. (2) Helix packing, tilts, and ligand-induced conformational changes have been determined by using a battery of site-directed biochemical and biophysical techniques. (3) Positions that are accessible to solvent have been revealed. (4) Positions where the reactivity of the Cys replacement is increased or decreased by ligand binding have been identified. (5) The permease has been shown to be a highly flexible molecule. (6) A working model describing a mechanism for lactose/H^+ symport has been formulated.

A particularly powerful approach carried out in situ (*12*−*20*) involves expression of lac permease in two contiguous fragments (split permease), each with a single Cys residue on either side of the discontinuity. The proximity of the paired Cys residues is then readily assayed by disulfide formation or chemical cross-linking of the two fragments. Alternatively, permease with an engineered factor Xa protease site in the middle cytoplasmic loop has been used for the same purpose (*21*, *22*).

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[1] Abbreviations: lac permease, lactose permease; N_6C_6, functional lac permease expressed in two nonoverlapping fragments with a discontinuity in the cytoplasmic loop between helices VI and VII; N_6, six N-terminal helices; C_6, six C-terminal helices; C-less permease, functional lactose permease devoid of Cys residues; DTT, dithiothreitol; NEM, *N*-ethylmaleimide; *o*-PDM, *N*,*N*′-*o*-phenylenedimaleimide; *p*-PDM, *N*,*N*′-*p*-phenylenedimaleimide; IPTG, isopropyl 1-thio-β-D-galactopyranoside; $NaDodSO_4$−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TDG, β-D-galactopyranosyl 1-thio-β-D-galactopyranoside.

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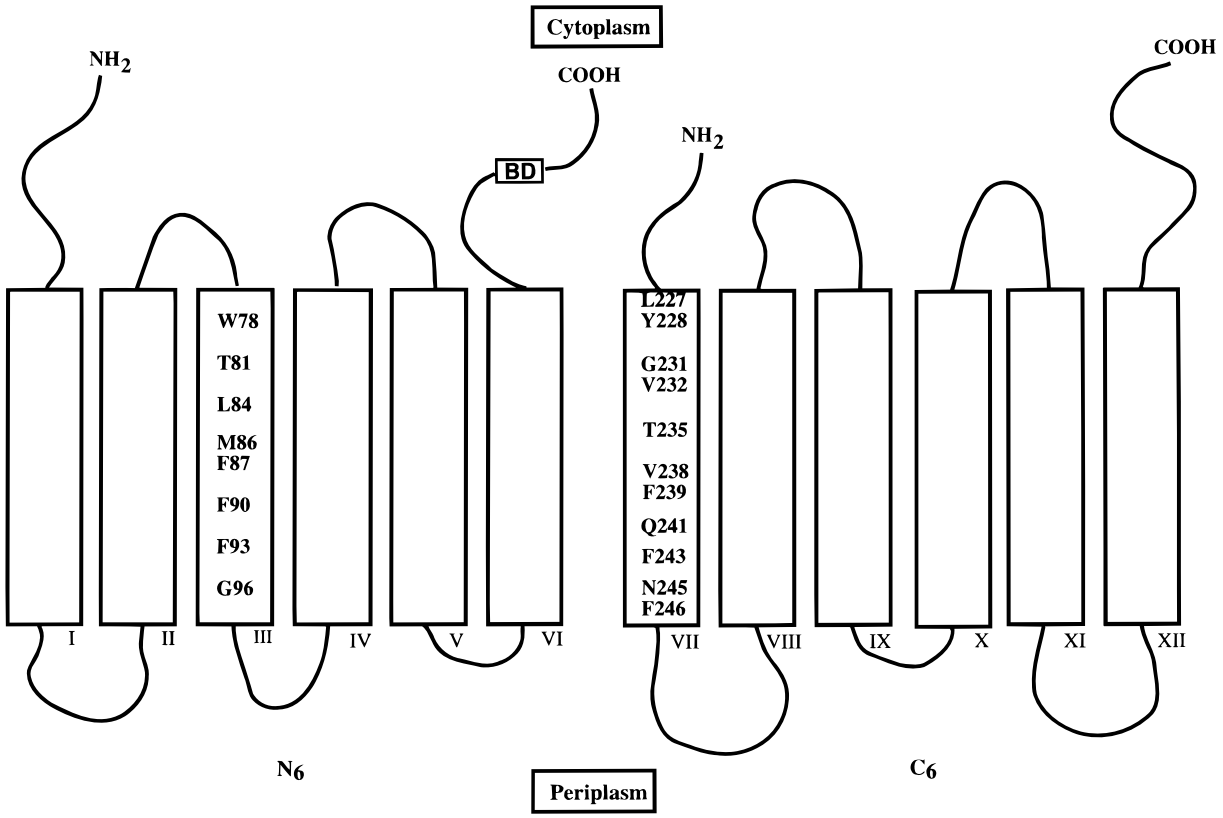

FIGURE 1: Secondary structure model of N_6C_6 lac permease. Putative transmembrane helices are shown as boxes. N_6 has a biotin acceptor domain (BD) at the C terminus. Cys replacements in helix III or VII are numbered and highlighted.

Although previous experiments (*22*) show that Y101C[2] permease spontanously forms disulfide-linked dimers, thereby placing helix III on the periphery of the permease, more precise evidence for the location of helix III is lacking. In this report, the proximity between helices III and VII is documented by studying cross-linking of paired Cys residues over the entire lengths of the two transmembrane domains in split permease (N_6C_6) containing the six N-terminal transmembrane helices (N_6) and the C-terminal six transmembrane helices (C_6) (*23*, *24*). The results demonstrate that helices III and VII cross-link in the periplasmic but not in the cytoplasmic halves. Thus, helices III and VII are in proximity at the periplasmic side of the membrane and tilt away from each other toward the cytoplasmic face. On the basis of the findings, the helix packing model of the permease (*11*) is modified with respect to the position of helix III.

EXPERIMENTAL PROCEDURES

Materials. [1-^{14}C]Lactose, [α-^{35}S]dATP, and protein A-conjugated horseradish peroxidase (HRP), enhanced chemiluminescence detection kits were purchased from Amersham. Restriction endonucleases and T4 DNA ligase were from New England Biolabs. Sequenase (modified T7 DNA polymerase) and Sequenase reaction kits were from United States Biochemicals. Isopropyl 1-thio-β-D-galactopyranoside (IPTG) and Taq polymerase were obtained from Boehringer Mannheim. *N*,*N*′-*o*-Phenylenedimaleimide (*o*-PDM) and *N*,*N*′-*p*-phenylenedimaleimide (*p*-PDM) were from Sigma. Site-directed rabbit antiserum against C-terminal dodecapeptide of lac permease (*25*) was prepared by Babco (Richmond, CA). Deoxyoligonucleotides were synthesized on an Applied Biosystems 381A DNA synthesizer. All other materials were reagent grade and obtained from commercial sources.

Construction of Single-Cys N_6 and C_6 Permease. Construction of permease mutants with single-Cys replacements in helices III and VII (Figure 1) has been described previously (*26*, *27*). To each mutant with a single-Cys replacement at position 78, 81, 84, 86, 87, 88, 90, 93, or 96 in helix III, the biotin acceptor domain from the oxaloacetate decarboxylase of *Klebsiella pneumoniae* was inserted into the middle cytoplasmic loop (*28*). The 3′-half of the *lacY* gene in each construct was then deleted by *Afl*II digestion followed by intramolecular ligation, resulting in plasmid pN_6, which encodes the N_6 fragment with a single Cys residue at a given position and the biotin acceptor domain at the C terminus. Construction of plasmid pC_6, which encodes the C_6 fragment, was carried out as described previously (*12*, *21*). To introduce a single Cys residue at position 227, 228, 231, 232, 235, 238, 239, 241, 243, 245, or 246 in helix VII of C_6, the *Kpn*I−*Hin*dIII restriction fragment from pC_6 was replaced with the corresponding DNA fragment from a single-Cys permease mutant. Each Cys replacement mutant was verified by using dideoxynucleotide termination (*29*) after alkali denaturation (*30*).

Transport Measurements. E. coli T184 [*lacI*$^+$*O*$^+$*Z*$^-$*Y*$^-$*(A)-rpsLmet*$^-$*thr*$^-$*recAhsdM hsdR/F′lacI*^{q} *O*$^+$*Z*^{D118}*(Y*$^+$*A*$^+$*)*] (*31*) was cotransformed with both pN_6 and pC_6, each encoding a given permease fragment with a single Cys residue. Cells

[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, followed by a second letter indicating the amino acid replacement.

were grown at 37 °C to an OD_{600} of 1.0 and induced with 0.2 mM IPTG for 2 h, harvested by centrifugation, washed with 100 mM potassium phosphate (pH 7.5)/10 mM $MgSO_4$, and adjusted to an OD_{420} of 10.0 (approximately 0.7 mg of protein/mL). Transport of [1-^{14}C]lactose (2.5 mCi/mmol) was assayed at a final concentration of 0.4 mM by rapid filtration (*32*).

Expression of Split Permease and Membrane Preparation. Cultures (25 mL) were grown and induced with 0.2 mM IPTG for 2 h. Cells were harvested and washed once with 20 mM Tris-HCl (pH 7.4)/2.0 mM ethylenediaminetetraacetate/5.0 mM dithiothreitol (DTT) and suspended in the same buffer. Membranes were prepared by sonification as described previously (*27*) and suspended in 20 mM Tris-HCl (pH 7.4).

Protein Assays. Protein was assayed with the Micro BCA kit (Pierce, IL) using bovine serum albumin as the standard.

Cross-Linking. Iodine-catalyzed disulfide formation was carried out at room temperature for 30 min by adding ethanolic iodine at a final concentration of 0.5 mM to membrane preparations at a membrane protein concentration of 2 mg/mL. Reactions were terminated by adding 5 mM *N*-ethylmaleimide (NEM). Chemical cross-linking was carried out at room temperature for 30 min in the presence of *o*-PDM or *p*-PDM, as indicated, at a final concentration of 0.5 mM (*12*). Reactions were terminated by adding 5 mM DTT. Samples were mixed with sodium dodecyl sulfate ($NaDodSO_4$) sample buffer and subjected to $NaDodSO_4$−12% polyacrylamide gel electrophoresis ($NaDodSO_4$−PAGE). Proteins were electroblotted onto polyvinylidene difluoride membranes (Immobilon-PVDF, Millipore) and probed with site-directed polyclonal antibody against the C terminus of lac permease (*25*).

RESULTS

Active Transport. Coexpression of wild-type *lacY* fragments encoding contiguous polypeptides corresponding to N_6 and C_6 leads to functional complementation (*23*, *24*). Active lactose transport is observed in nearly all paired Cys N_6C_6 permease mutants used in this study. Each pair accumulates lactose to a steady state that is 30−60% of wild-type permease, except for pair 93/243 which accumulates lactose to a steady state that is only about 10% of wild-type permease (data not shown). The effect of cross-linking on permease activity was not examined since the efficiency of cross-linking (see below) is relatively low which precludes the type of study carried out previously (*13*).

Cross-Linking. Paired Cys residues were placed first at positions in the middle of helices III and VII in N_6C_6. C_6 which reacts with anti-C-terminal antibody migrates at an M_r of about 20 kDa, and cross-linked N_6C_6 migrates at about 52 kDa (Figure 2). Paired Cys mutants 87/235, 87/238, 87/239, and 87/241 are cross-linked by *o*-PDM and *p*-PDM and form a disulfide bond when oxidized with iodine, indicating that the Cys residues are in proximity (Figure 2). On the other hand, no cross-linking is observed with pairs 86/235, 86/238, 86/239, 90/238, and 90/241 in the presence of *o*-PDM, *p*-PDM, or iodine (data not shown; see Figure 4). Paired Cys mutants 84/231, 84/232, 84/235, 84/238, 90/239, and 90/243 are expressed at levels that are insufficient for studying cross-linking (data not shown; see Figure 4).

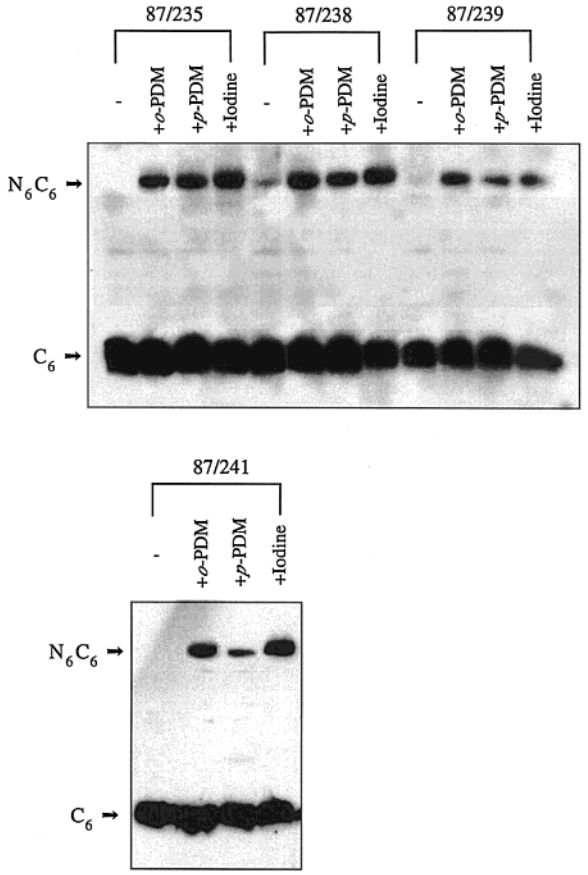

FIGURE 2: Cross-linking of N_6C_6 split permease containing paired Cys residues in helices III and VII (87/235, 87/238, 87/239, and 87/241). Membranes were prepared from cells expressing the N_6 and C_6 fragments each with a single Cys residue at a given position as indicated. Cross-linking was carried out by incubating membranes (2 mg of protein/mL) at a final cross-linker or iodine concentration of 0.5 mM for 30 min at 20 °C. Cross-linking was terminated by adding DTT (for *o*-PDM or *p*-PDM) or NEM (for iodine) to a final concentration of 5 mM. Samples containing approximately 50 μg of protein were subjected to $NaDodSO_4$−PAGE and electroblotted. The blots were probed with anti-C-terminal antibody. C_6 and the N_6C_6 cross-linked products are denoted with arrows.

With paired Cys residues near the periplasmic ends of helices III and VII, highly efficient iodine-catalyzed disulfide formation is observed with mutants 93/245 and 96/246, less efficient cross-linking is observed in the presence of *o*-PDM (6 Å), and the least efficient cross-linking is observed in the presence of *p*-PDM (10 Å) (Figure 3), suggesting that the distance between pair 93/245 or 96/245 is less than 6 Å. No cross-linking is observed with pair 93/243 (data not shown; see Figure 4), and pairs 93/246, 96/243, and 96/246 are not expressed adequately for study (data not shown; see Figure 4). With paired Cys residues near the cytoplasmic ends of helices III and VII (pairs 78/227, 78/228, 78/231, 81/231, and 81/232), no cross-linking whatsoever is observed under any of the conditions that were tested (data not shown; see Figure 4).

DISCUSSION

Although a high-resolution structure of lac permease is not available (see ref *33*), application of a battery of site-directed biochemical and biophysical techniques that include second-site suppresser analysis and site-directed mutagenesis, excimer fluorescence, spin−spin interactions, engineered di-

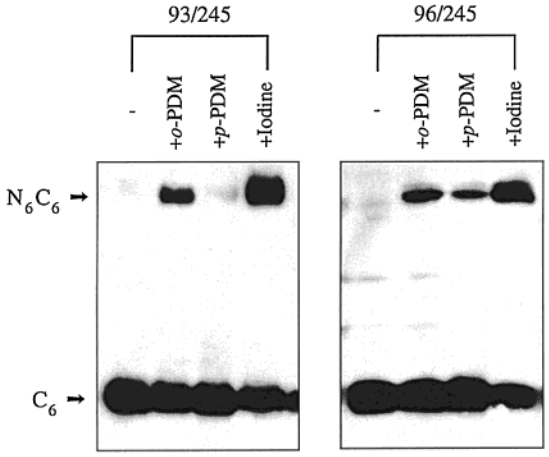

FIGURE 3: Cross-linking of N_6C_6 split permease containing paired Cys residues in helices III and VII (93/245 and 96/245). Membranes were prepared from cells expressing the N_6 and C_6 fragments each with a single Cys residue at a given position as indicated. Cross-linking was carried out, and samples were analyzed as described in the legend of Figure 2. C_6 and the N_6C_6 cross-linked products are denoted with arrows.

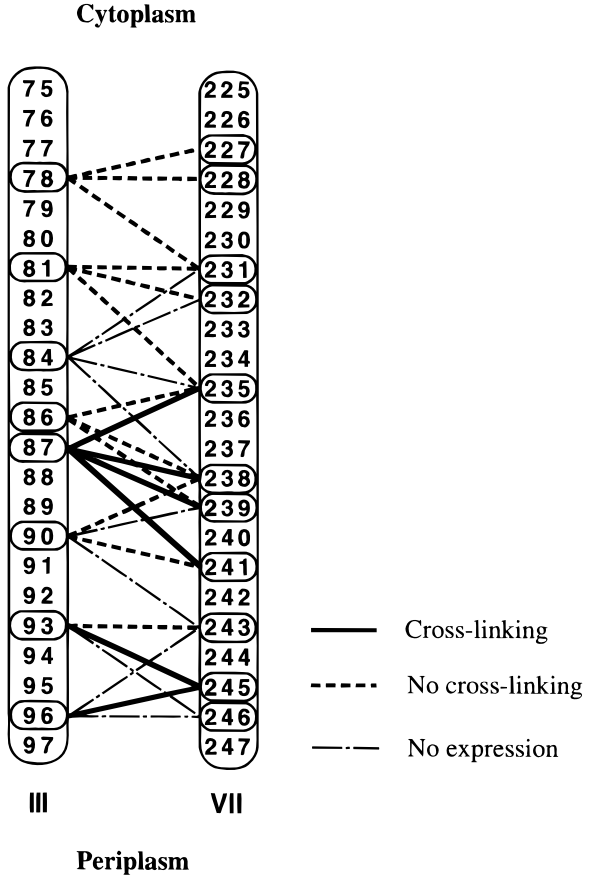

FIGURE 4: Summary of cross-linking data. Helices III and VII are depicted as boxes. Cross-linked Cys pairs in N_6C_6 permease are connected by solid lines. Cys pairs that do not cross-link are connected by broken lines. Cys pairs that do not exhibit an expression level sufficient for cross-linking study are connected by broken lines with alternating long and short breaks.

valent metal binding sites, metal−spin-label interactions, thiol-specific cross-linking, and identification of discontinuous mAb epitopes has led to the formulation of a helix packing model (reviewed in refs *8*−*11*). Of the approaches that were utilized, in situ site-directed cross-linking of split permease molecules containing paired Cys residues has many advantages (*12*). The technique is based on the observation that coexpression of lac permease in two contiguous, non-overlapping fragments with a discontinuity in cytoplasmic or periplasmic loops leads to functional complementation (*23*, *24*, *34*, *35*) and is carried out with native membranes on quantities of material that can be assayed by immunoblotting or avidin blotting. Furthermore, inter-thiol distances can be approximated by using appropriate oxidants or chemical linkers with different properties (*13*). The approach has also been used to study ligand-induced conformational changes (*13*−*15*), as well as helix tilts (*14*−*16*, *20*), by examining cross-linking of Cys residues on the faces of neighboring helices throughout transmembrane domains.

The approach that was used is based on the premise that Cys cross-links are a measure of proximity. However, it is noteworthy that cross-link formation may detect dynamic collisions and chemical reactions between residues, not simply their proximities. For example, Cys pairs that frequently undergo collisons and are highly chemically reactive could form cross-links at a relatively rapid rate, even though they may be distant in the average structure. On the other hand, a strong correlation is expected between collision rates and proximity (*36*). Therefore, proximities between loops are estimated by spontaneous disulfide formation between engineered cysteinyl side chains, iodine-catalyzed disulfide formation, or cross-linking by homobifunctional cross-linking agents in which the distance between the reactive groups and the flexibility of the linker between the functional groups vary. In addition, many of the experiments are carried out at 4 °C to decrease the thermal motion of the polypeptide backbone and thereby limit long-range collisions. Finally, it is noteworthy that experiments (*37*) in which rates of cross-linking of paired Cys residues in periplasmic loops were compared with distances estimated from spin−spin interactions between the nitroxide-labeled pairs are consistent with the conclusion that site-directed thiol cross-linking is primarily a reflection of proximity.

Although evidence has been presented indicating that helices III and VI are probably on the periphery of the 12-helix bundle that comprises the permease (*22*), data regarding a precise location of helices III and VI in the helix packing model of the molecule are lacking (*11*). In this report, the position of helix III in the tertiary structure of the permease is documented by studying site-directed thiol cross-linking of helices III and VII in N_6C_6 permease. The results are summarized in Figure 4, and a modified helix packing model is proposed in Figure 5.

Although certain paired Cys mutants (84/231, 84/232, 84/235, 84/238, 90/239, 90/243, 93/246, 96/243, and 96/246) are expressed in insufficient quantity for study (Figure 4), other paired Cys mutants provide clear-cut cross-linking results indicative of proximity between helices III and VII within the periplasmic half of the membrane. Thus, pairs 87/235, 87/238, 87/239, and 87/241 (Figure 2) exhibit cross-linking with iodine, *o*-PDM, or *p*-PDM, and pairs 93/245 and 96/245 (Figure 3) cross-link with iodine or *o*-PDM, but weakly with *p*-PDM. Since the rigid longer reagent *p*-PDM (10 Å) cross-links paired Cys residues in the middle of the two helices (87/235, 87/238, 87/239, and 87/241) significantly more effectively than those near the periplasmic ends

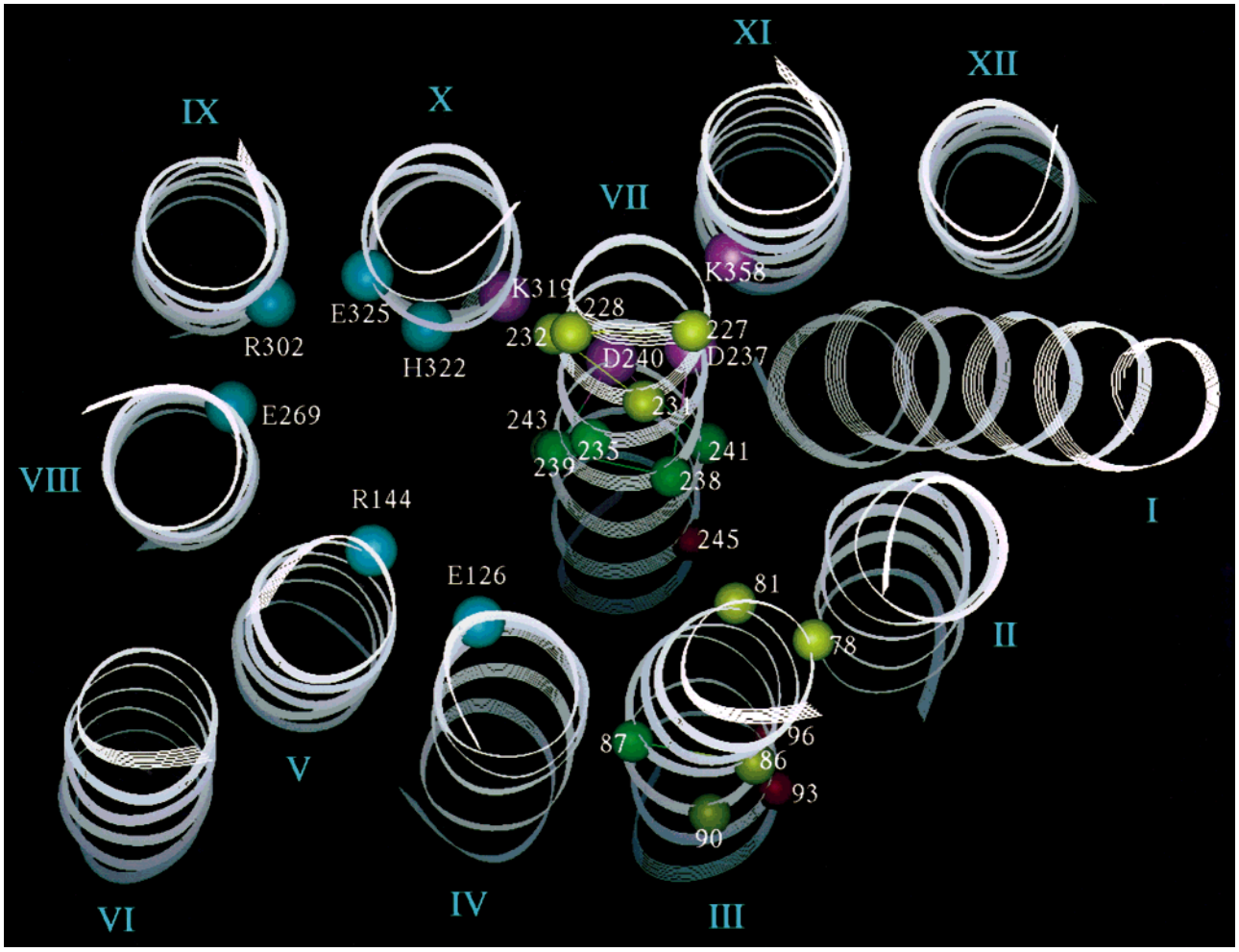

FIGURE 5: Helix packing in the lac permease viewed from the cytoplasmic surface of the membrane. The six irreplaceable residues (Glu126, Arg144, Glu269, Arg302, His322, and Glu325) are shown as cyan balls. The two interacting pairs of Asp and Lys residues [Asp237 (helix VII)/Lys358 (helix XI) and Asp240 (helix VII)/Lys319 (helix X)] are shown as pink balls. Positions in helices III and VII tested for cross-linking in this study are highlighted and numbered. Cross-linked positions are shown as red (periplasmic third) or dark green (middle third) balls. Light green balls represent positions that do not exhibit cross-linking under any of the conditions that were tested. The red ball denoting residue 96 is difficult to visualize in this orientation of helix III. Preliminary results (A. B. Weinglass and H. R. Kaback, unpublished information) suggest that helix VI may cross-link with helix V.

(93/245 and 96/245), it appears that the periplasmic ends of the two helices may be closer than the middle. These observations in addition to the finding that no cross-linking is evident when paired Cys residues are placed in the cytoplasmic ends of the two helices (Figure 4) are consistent with the interpretation that the two helices tilt away from each other toward the cytoplasmic face of the membrane. Furthermore, pairs 86/235, 86/238, 86/239, 90/238, and 90/241 do not cross-link under any of the conditions that were tested, suggesting that positions 86 and 90 (Met and Phe, respectively, in the wild type) are located on a face of helix III that is directed away from helix VII. The suggestion is consistent with the previous finding that Tyr101 is located on the periphery of the permease (*22*). In addition, previous results show (*26*) that G96C is the only single-Cys mutant in helix III inactivated by NEM, and the rate of inactivation is slightly enhanced by β-D-galactopyranosyl 1-thio-β-D-galactopyranoside (TDG). Both properties are also consistent with the proposed orientation of helix III; i.e., position 96 (Gly in the wild type) is located at the interface between helices III and II (Figure 5) where it may be important for helix packing, ligand-induced conformational changes, or both.

On the basis of the results of this study, the modified helix packing model shown in Figure 5 is proposed. As shown, helix III is placed between helices II and IV, and it is tilted in such a manner that the periplasmic half is close to helix VII and the cytoplasmic half tilts away toward the cytoplasmic face of the membrane. In the model that is shown, helix VII appears to be located near the center of the permease, with helices I (*15*), II (*14*), III and IV (*16*), and V (*17*) in proximity toward the periplasmic face of the membrane, but tilting away toward the cytoplasmic face. Strong evidence has been presented (*38*−*44*) showing that Asp237 and Asp240 (helix VII) are ion-paired with Lys358 (helix XI) and Lys319 (helix X), respectively. Therefore, in the approximate middle of the membrane, helix VII must be close to helices X and XI, and given the proposed packing model, helices X and XI are likely to be close to helix VII at the cytoplasmic face of the membrane, a hypothesis that can be tested with N_7C_5 permease (*24*).

Cross-linking between helices III and VII was also used to probe ligand-induced conformational changes at the interface between the two helices. Cross-linking experiments were carried out on ice for 30 min in the absence or presence of high-affinity substrate analogue TDG (10 mM). However,

no significant changes in cross-linking efficiency were observed (data not shown). The findings suggest that there are little or no distance changes between the paired Cys residues shown to cross-link in this study. The observation that the rate of NEM inactivation of transport by the G96C mutant is enhanced only 2-fold in the presence of TDG at ambient temperature (*26*) may not be reflected by thiol cross-linking studies at low temperatures. Furthermore, with the sole exception of the G96C mutant, the single-Cys mutants exhibit no significant change in transport activity upon alkylation with NEM (*26*). Therefore, the data as a whole indicate that there is little or no ligand-induced change in conformation between helices III and VII.

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