Tertiary Contacts of Helix V in the Lactose Permease Determined by Site-Directed Chemical Cross-Linking in Situ†

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ABSTRACT: The six N-terminal transmembrane helices (N_6) and the six C-terminal transmembrane helices (C_6) in lactose permease, each containing a single Cys residue, were coexpressed, and cross-linking was studied. The proximity of paired Cys residues in helices V and VII, VIII, or X was studied by thiol-specific chemical cross-linking. The results demonstrate that Cys residues in the periplasmic half of helix V cross-link with Cys residues in the periplasmic half of helix VII. In contrast, no cross-linking is evident with paired Cys residues in the cytoplasmic halves of helices V and VII. Moreover, Cys residues on one entire face of helix V cross-link with Cys residues on one face of helix VIII. Finally, paired Cys residues at the cytoplasmic ends of helices V and X cross-link, but no cross-linking is observed when paired Cys residues are placed at the periplasmic ends of the two helices. Taken together, the results indicate that the periplasmic halves of helices V and VII are in close proximity and that the two helices tilt away from one another toward the cytoplasmic side of the membrane. Furthermore, helices V and VIII are in close proximity throughout their lengths and do not tilt appreciably with respect to one another, and helices V and X are in close proximity at the cytoplasmic but not at the periplasmic face of the membrane.

The lactose (lac)[1] permease of *Escherichia coli* is typical of secondary transport proteins that transduce free energy stored in an electrochemical ion gradient into a concentration gradient (reviewed in refs *1*−*3*). This hydrophobic, polytopic membrane protein which catalyzes the coupled stoichiometric translocation of β-galactosides and H^+ has been solubilized, purified to homogeneity, reconstituted into proteoliposomes, and shown to be solely responsible for β-galactoside transport (reviewed in ref *4*) as a monomer (*5*). All available evidence (reviewed in refs *6*−*8*) indicates that the permease is composed 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 (see Figure 1).

Site-directed and Cys-scanning mutagenesis have demonstrated that only six amino acid side chains in this 417-residue polypeptide are irreplaceable with respect to the mechanism of active transport: Glu126 (helix IV) and Arg144 (helix V) which are indispensable for substrate binding and Glu269 (helix VIII), Arg302 (helix IX), His322, and Glu325 (helix) which are involved in H^+ translocation and coupling (reviewed in ref *9*). However, structural and dynamic information at high resolution is required for understanding the precise role of these residues in sugar and H^+ translocation, as well as the coupling between the two processes. Since hydrophobic membrane proteins are notoriously difficult to crystallize, a high-resolution structure of lac permease is not available, and development of alternative methods for obtaining structural information is essential (*7*−*9*). By the combined use of multifaceted site-directed approaches which include excimer fluorescence, chemical cleavage, engineered divalent metal binding sites, electron paramagnetic resonance spectroscopy, thiol-specific cross-linking, and identification of discontinuous mAb epitopes, a general helix packing model of lac permease has been formulated (Figure 2). Although many of the spatial relationships between transmembrane helices have been established by more than one experimental approach (reviewed in ref *8*), determination of helix tilting is essential to placing the essential residues and to delineating the substrate and H^+ translocation pathways. In this regard, tilting of helices I, II, IV, VII, and XI has been studied by site-directed cross-linking with a functional permease construct expressed as two discontinuous, nonoverlapping fragments [N_6/C_6 permease (*10*)] containing single Cys residues in each half of the protein (*11*−*13*). The results indicate that the periplasmic halves of helices VII and I, II, or IV are in close proximity, while the cytoplasmic halves of helices XI and II or IV are close to one another.

In this report, tertiary contacts between helices V and VII, VIII, or X are examined in situ using a general cross-linking approach developed recently (*12*, *14*). Paired Cys residues were placed in two helices in N_6/C_6 permease, and proximity

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[1] Abbreviations: lac, lactose; C-less permease, functional lac permease devoid of Cys residues; N_6, six N-terminal transmembrane helices; C_6, six C-terminal transmembrane helices; TDG, β-D-galactopyranosyl 1-thio-β-D-galactopyranoside; IPTG, isopropyl 1-thio-β-D-galactopyranoside; *o*-PDM, *N*,*N*′-*o*-phenylenedimaleimide; *p*-PDM, *N*,*N*′-*p*-phenylenedimaleimide; $NaDodSO_4$, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; NEM, *N*-ethylmaleimide.

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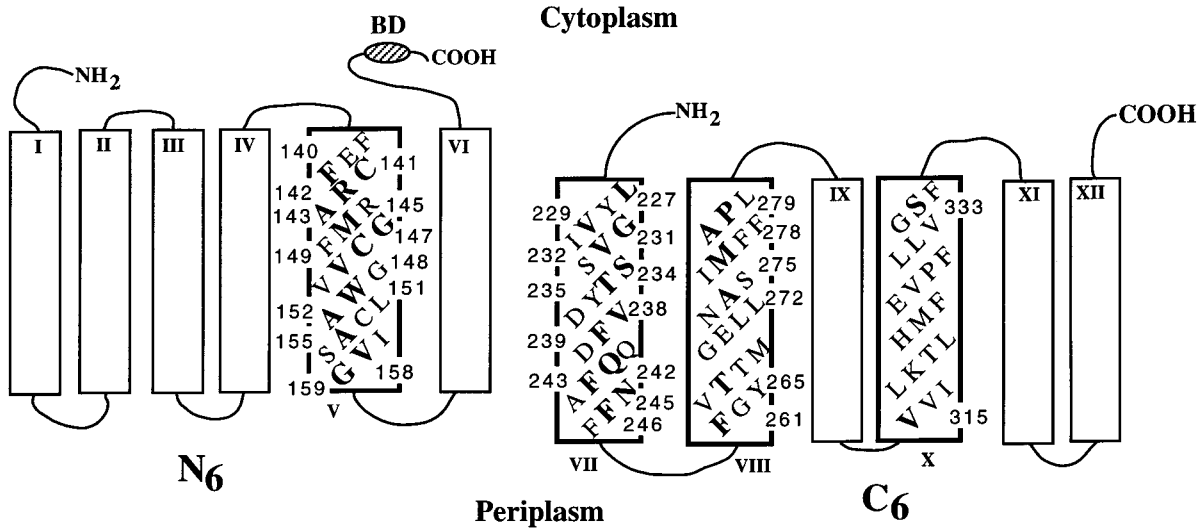

FIGURE 1: Secondary structure model of N_6/C_6 split permease. The lac permease is shown as the six N-terminal transmembrane helices (N_6) and the six C-terminal transmembrane helices (C_6). N_6 has a biotin acceptor domain (BD) at the C terminus. Single-Cys replacements in helices V (position 140, 141, 142, 143, 145, 147, 148, 149, 151, 152, 155, 158, or 159), VII (position 227, 229, 231, 232, 234, 235, 238, 239, 242, 243, 245, or 246), VIII (position 261, 265, 272, 275, 278, or 279), and X (position 315 or 333) are numbered and highlighted.

was assessed by thiol-specific chemical cross-linking in the native membrane. The results demonstrate that the periplasmic halves of helices V and VII are in close proximity, helices V and VIII are in close proximity throughout their lengths, and the cytoplasmic ends of helices V and X are in close proximity.

EXPERIMENTAL PROCEDURES

Materials. Protein A-conjugated horseradish peroxidase (PA−HRP) and enhanced chemiluminescence (ECL) detection kits were obtained from Amersham (Arlington Heights, IL). Avidin-conjugated horseradish peroxidase (avidin−HRP) was purchased from Pierce (Rockford, IL). *N*,*N*′-*o*-Phenylenedimaleimide (*o*-PDM) and *N*,*N*′-*p*-phenylenedimaleimide (*p*-PDM) were from Sigma (St. Louis, MO).

Construction of Single-Cys N_6 and C_6 Permease. Construction of permease mutants containing single-Cys replacements in helices V, VII, VIII, and X has been described (*15*−*18*). To each mutant with a single-Cys replacement at position 140 to 143, 145, 147 to 149, 151, 152, 155, 158, or 159 in helix V, the biotin acceptor domain from the *Klebsiella pneumonia* oxaloacetate decarboxylase was inserted into the middle cytoplasmic loop as described previously (*19*). 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 six N-terminal transmembrane helices (N_6) with a single Cys residue at a given position and the biotin acceptor domain at the C terminus (Figure 1). Construction of plasmid pC_6 encoding the six C-terminal transmembrane helices (C_6) has been described (*14*, *20*). To introduce a single Cys residue into helix VII (position 227, 229, 231, 232, 234, 235, 238, 239, 242, 243, 245, or 246), VIII (position 261, 265, 272, 275, 278, or 279), or X (position 315 or 333) in C_6, the *Bst*XI−*Hin*dIII fragment of pC_6 was replaced by the corresponding DNA fragment from an indicated single-Cys permease mutant (Figure 1). Each Cys replacement mutant in N_6 or C_6 was verified by using the dideoxynucleotide termination method (*21*).

Expression of Split Permease and Membrane Preparations. E. coli HB101 cells (*lacY*$^-$*Z*$^+$) were transformed with both pN_6 and pC_6 encoding N_6 and C_6, respectively, each with a given single Cys residue. Cultures (50 mL) were grown at 37 °C in LB medium containing 100 μg/mL ampicillin and 20 μg/mL chloramphenicol to an OD_{600} of 1.0 and induced with 1 mM isopropyl 1-thio-β-D-galactopyranoside for 2 h. Cells were harvested by centrifugation, washed once with 20 mM Tris-HCl (pH 7.4)/2.0 mM EDTA, and suspended in the same buffer followed by incubation with 100 μg/mL lysozyme for 10 min on ice. Membranes were prepared by sonification and suspended in 20 mM Tris-HCl (pH 7.4).

Chemical Cross-Linking and Analysis. All cross-linking experiments were carried out at room temperature for 30 min by adding a given thiol-specific chemical linker to a final concentration of 0.5 mM to membrane preparations at a protein concentration of 2 mg/mL. Reactions were terminated by adding sodium dodecyl sulfate ($NaDodSO_4$) sample buffer containing 5% (v/v) β-mercaptoethanol. Samples were subjected to electrophoresis in $NaDodSO_4$−12% polyacrylamide gels (PAGE). C_6 was detected by immunoblotting with rabbit polyclonal antibodies against a C-terminal dodecopeptide corresponding to the C terminus of lac permease. N_6 which contains the biotin acceptor domain at the C terminus was detected with avidin-conjugated horseradish peroxidase (avidin−HRP). Cross-linked N_6/C_6 reacts with both anti-C-terminal antibody and avidin−HRP.

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

RESULTS

Cross-Linking N_6/C_6 Permease with Paired Cys Residues in Helices V and VII. Transmembrane helix V in the lac permease was shown initially to be in close proximity to helices VII and VIII by site-directed chemical cleavage (*22*), a finding confirmed subsequently by electron paramagnetic resonance (EPR) with double nitroxide spin labeling and cross-linking studies with the 5 Å homobifunctional cross-linking agent dibromobimane (*23*). To further examine

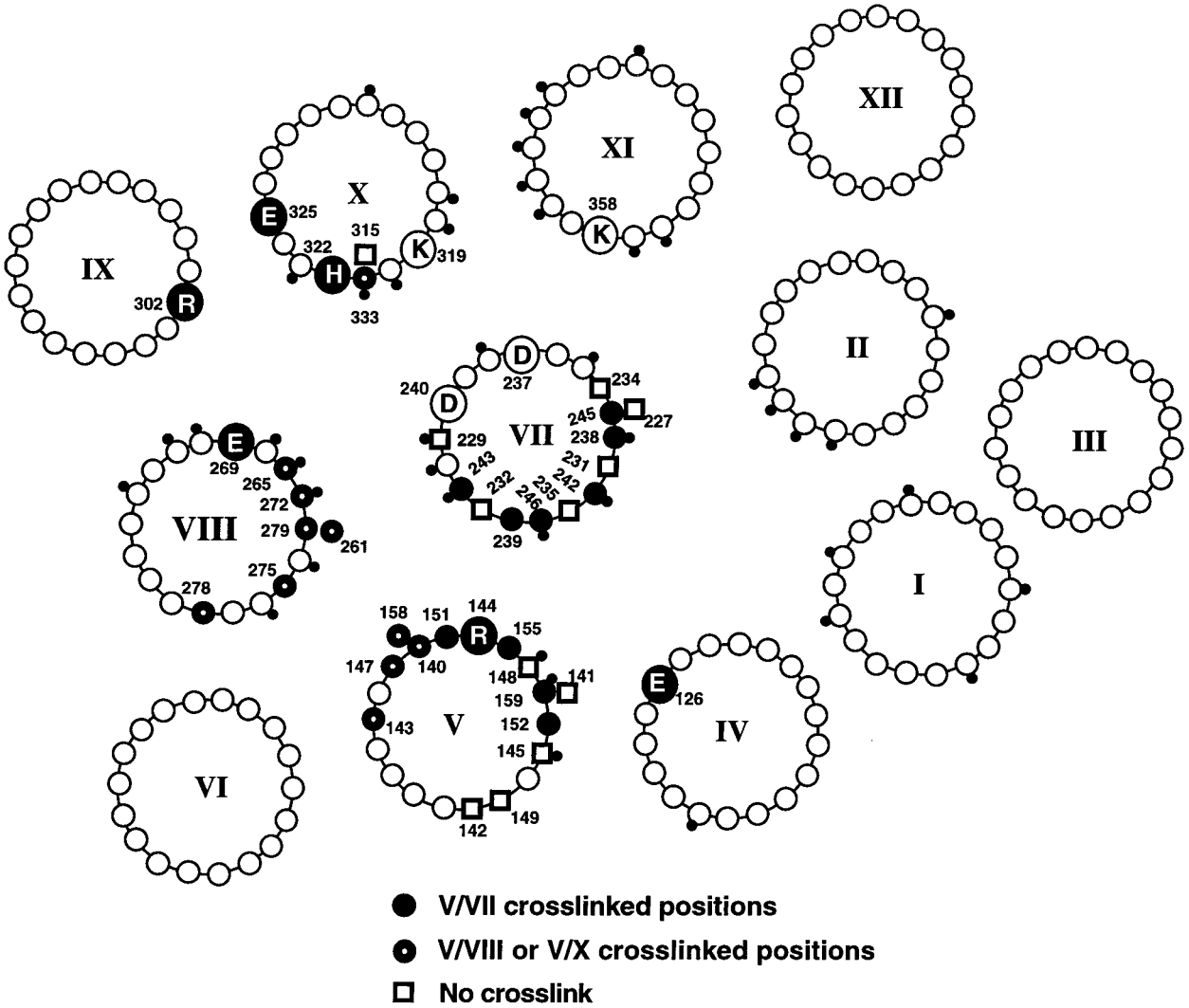

FIGURE 2: Helix packing in the lac permease viewed from the cytoplasmic face. The six essential residues [Glu126 (helix IV), Arg144 (helix V), Glu269 (helix VIII), Arg302 (helix IX), and His322 and Glu325 (helix X)] and two interacting pairs of Asp and Lys residues [Asp237 (helix VII) and Lys358 (helix XI) and Asp240 (helix VII) and Lys319 (helix X)] are highlighted. Positions of NEM-sensitive Cys replacements are indicated with a small black dot. Cys replacement mutants in helices V, VII, VIII, and X tested for cross-linking in this study are highlighted and numbered.

proximity and tilting between helices V and VII, site-directed chemical cross-linking was carried out with N_6/C_6 permease containing paired Cys residues at various positions in the two helices. As shown in Figures 1 and 2, a Cys residue was placed at given positions along the length of helix V in N_6 and along the length of helix VII in C_6. To assess the proximity between given paired Cys residues, N_6 and C_6 fragments were coexpressed and cross-linking was carried out in situ with homobifunctional chemical linkers. *o*-PDM and *p*-PDM which are thiol-specific reagents containing maleimido groups coupled to benzene rings in the ortho and para positions at fixed distances of about 6 and 10 Å, respectively, were chosen because of their relatively short length, rigidity, and hydrophobicity. Hydrophobicity is presumably important because the Cys residues are thought to be in a hydrophobic environment within the membrane.

When membranes containing N_6/C_6 permease with paired Cys residues at positions in the periplasmic halves of helices V (position 151, 152, 155, 158, or 159) and VII (position 238, 239, 242, 243, 245, or 246) are treated with *o*-PDM or *p*-PDM, cross-linking of the N_6 and C_6 fragments is clearly evident (Figures 3 and 6). C_6 which reacts with anti-C-terminal antibody migrates at an M_r of about 20 kDa (Figure 3A); N_6 with the biotin acceptor domain which reacts with avidin−HRP migrates with an M_r of about 35 kDa (Figure 3B), and cross-linked N_6/C_6 which reacts with both anti-C-terminal antibody and avidin−HRP migrates with an M_r of about 52 kDa (Figure 3A,B). *o*-PDM with 6 Å separating the maleimido groups readily cross-links paired Cys residues 159 and 246, 158 and 246, 155 and 243, 155 and 242, 152 and 239, 151 and 239, and 151 and 238 (Figures 3 and 6). Similar results are obtained by using the 10 Å cross-linking agent *p*-PDM (data not shown). In marked contrast, when paired Cys residues are placed at positions in the cytoplasmic halves of helix V (position 140, 141, 142, 143, 145, 147, 148, or 149) and helix VII (position 227, 229, 231, 232, 234, or 235), no cross-linking is observed (Figure 6). The results indicate that helices V and VII are in close proximity within the periplasmic side of the membrane and tilt away from each other toward the cytoplasmic side.

Cross-Linking N_6/C_6 Permease with Paired Cys Residues in Helices V and VIII. To examine proximity and tilting between helix V and helix VIII, N_6 containing a Cys residue at given positions in helix V was coexpressed with C_6 with

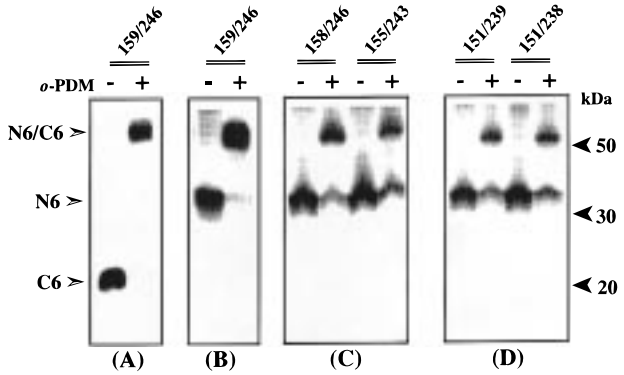

FIGURE 3: Chemical cross-linking of split N_6/C_6 permease containing paired Cys residues in helices V and VII. Membranes (2 mg of protein/mL) were prepared from cells expressing N_6 and C_6 fragments, with each containing a single Cys residue at given positions as indicated. Chemical cross-linking was carried out at room temperature for 30 min by adding *o*-PDM to a final concentration of 0.5 mM. Reactions were terminated by adding $NaDodSO_4$ sample buffer containing 5% (v/v) β-mercaptoethanol. Samples containing approximately 20 μg of membrane protein were subjected to $NaDodSO_4$−PAGE and electroblotted. The immunoblot was probed with anti-C-terminal antibody or avidin−HRP. N_6 and C_6 fragments and the N_6/C_6 cross-linked product are denoted by arrows: (A) cross-linking of paired Cys residues 159 and 246 probed with anti-C-terminal antibody, (B) cross-linking of paired Cys residues 159 and 246 probed with avidin−HRP, (C) cross-linking of paired Cys residues 158 and 246 and 155 and 243 probed with avidin−HRP, and (D) cross-linking of paired Cys residues 151 and 239 and 151 and 238 probed with avidin−HRP.

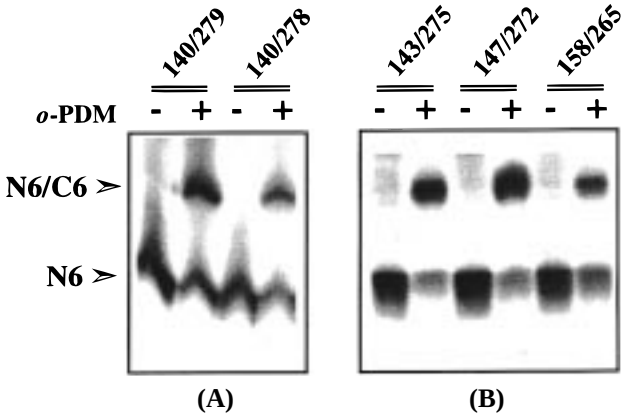

FIGURE 4: Chemical cross-linking of N_6/C_6 permease containing paired Cys residues in helices V and VIII. Membranes were prepared from cells expressing N_6 and the C_6 fragments, each with a Cys residue at given positions as indicated. Chemical cross-linking was carried out at room temperature by incubating membranes (2 mg of protein/mL) with 0.5 mM *o*-PDM for 30 min. Reactions were terminated by adding $NaDodSO_4$ sample buffer containing 5% (v/v) β-mercaptoethanol. Samples were analyzed as described in the legend of Figure 3 with avidin−HRP: (A) paired Cys residues 140 and 279 and 140 and 278 and (B) paired Cys residues 143 and 275, 147 and 272, and 158 and 265.

a Cys residue at given positions in helix VIII (Figures 1 and 2). When membranes containing N_6/C_6 with paired Cys residues 140 and 279, 140 and 278, 143 and 278, 143 and 275, 147 and 275, 147 and 272, 158 and 265, or 158 and 261 are treated with *o*-PDM (Figures 4 and 6) or *p*-PDM (not shown), cross-linking is clearly observed. The results indicate that helices V and VIII are in close proximity throughout the entire transmembrane domain. In addition, since no cross-linking is evident in N_6/C_6 containing paired

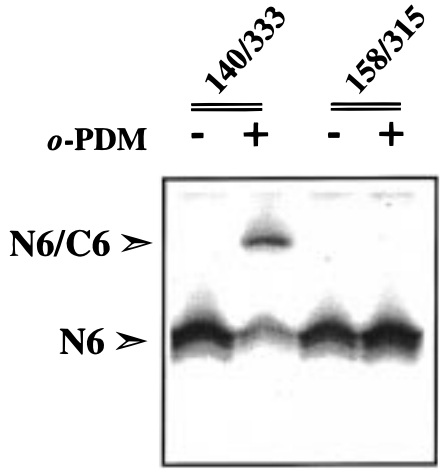

FIGURE 5: Chemical cross-linking of split N_6/C_6 permease containing paired Cys residues in helices V and X. Membranes were prepared from cells expressing N_6 and C_6 with paired Cys residues at given positions as indicated. Cross-linking was carried out at room temperature for 30 min, and samples were analyzed as described in the legend of Figure 3 with avidin−HRP.

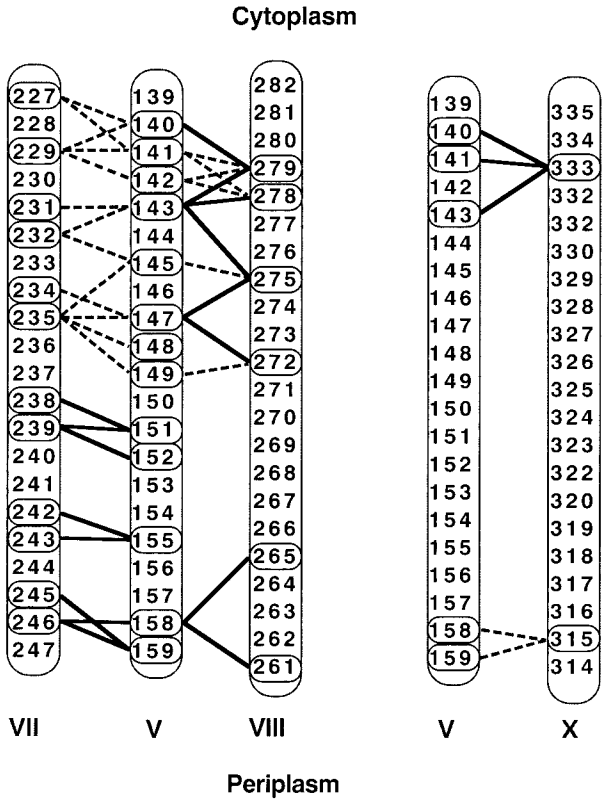

FIGURE 6: Summary of data for cross-linking between helices V and VII, V and VIII, and V and X in N_6/C_6 permease. The Cys replacement mutants used in this study are encircled. Paired Cys residues that exhibit significant cross-linking by *o*-PDM and *p*-PDM are connected with a solid line. Pairs which exhibit no cross-linking are connected with a dashed line.

Cys residues 141 and 278, 141 and 279, 142 and 278, 142 and 279, 145 and 275, or 149 and 272 (Figure 6), it is likely that the face of helix V with Phe140, Ala143, Gly147, and Val158 is close to the face of helix VIII with Pro279, Ala278, Met275, Ala272, Thr265, and Phe261 (Figures 2 and 7).

Cross-Linking N_6/C_6 Permease with Paired Cys Residues in Helices V and X. To examine the proximity and tilting between helices V and X, cross-linking of N_6/C_6 permease

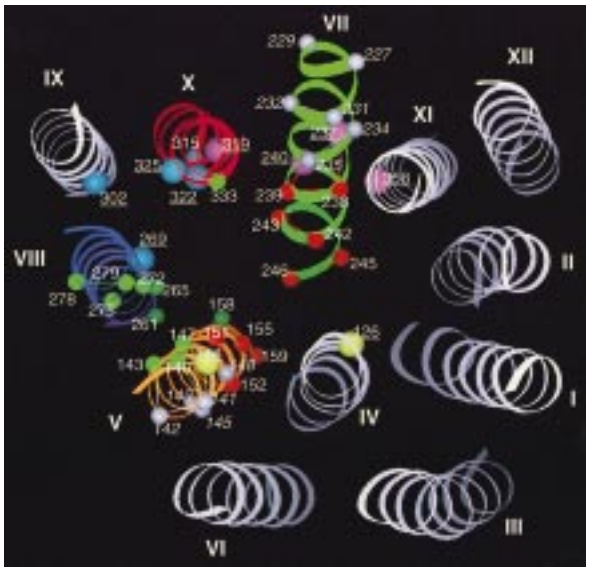

FIGURE 7: Helix packing and tilting of lactose permease viewed from the cytoplasmic side. Twelve transmembrane domains are displayed as helical ribbons. The six irreplaceable residues [Glu126 (helix IV), Arg144 (helix V), Glu269 (helix VIII), Arg302 (helix IX), and His322 and Glu325 (helix X)] are shown as enlarged, numbered balls. The two Asp-Lys charge pairs [Asp240 (helix VII) and Lys319 (helix X) and Asp237 (helix VII) and Lys358 (helix XI)] are shown as enlarged, numbered purple balls. Helices V, VII, VIII, and X are shown as colored ribbons. Cross-linked positions between helices V and VII are highlighted as red balls. Positions in helix VII shown as gray balls (227, 229, 231, 232, 234, and 235) do not cross-link with Cys residues in helix V. Positions that exhibit Cys cross-linking between helices V and VIII are shown as green balls. Positions in helix V shown as gray balls (141, 142, 145, 148, and 149) exhibit no cross-linkings with Cys residues in helix VIII. The cytoplasmic end of helix X (residue 333) tilts toward helix V (residue 140). The cytoplasmic end of helix VII (residue 227) tilts away from helices I, II, IV, and V, since no cross-linking is observed with Cys pairs between helix VII and any of the other helices. Tilting of helices I, II, IV, VII, and XI has been documented previously (*11*−*13*, *40*).

containing paired Cys residues in the two helices at positions in the cytoplasmic or periplasmic ends was carried out. As shown (Figures 5 and 6), paired Cys residues at positions 333 (helix X) and 140, 141, or 143 (helix V) are cross-linked by *o*-PDM (Figures 5 and 6) or *p*-PDM (not shown), indicating that the two helices are in close proximity in the cytoplasmic side of the membrane. In contrast, when paired Cys residues are placed at the periplasmic ends of the two helices at positions 315 (helix X) and 158 or 159 (helix V), no cross-linking is evident by either *o*-PDM (Figures 5 and 6) or *p*-PDM (not shown), suggesting that the two helices tilt away from each other toward the periplasmic side of the membrane (Figure 7).

DISCUSSION

On the basis of previous studies in which site-directed chemical cleavage (*22*), site-directed spin labeling, and cross-linking between Cys residues (*23*) were used, helix V was placed in the vicinity of helices VII and VIII (Figure 2). To further investigate tertiary contacts between helix V and the C-terminal half of lac permease, site-directed thiol-specific cross-linking was carried out with N_6/C_6 split permease containing paired Cys residues at various positions in helices V (N_6) and VII, VIII, or X (C_6). As shown previously (*11*−*14*), cross-linking of paired Cys residues in N_6/C_6 split permease has provided proximity and tilting information for helices I, II, IV, VII, XI, and XII in the lac permease, and the technique has also provided important information regarding other other membrane proteins (*24*−*30*).

With paired Cys residues in helices V and VII, cross-linking is observed only when the two Cys residues are in the periplasmic halves of the helices (positions 151−159 in helix V and 238−246 in helix VII), while no cross-linking whatsoever between helices V and VII is evident with paired Cys residues in the cytoplasmic halves of these helices. The results indicate that the two helices are in close proximity within the periplasmic side of the membrane and tilt away from each other toward the cytoplasmic side of the membrane. In addition, since no cross-linking is observed with paired Cys residues at positions in the cytoplasmic halves of helices VII and I, II, or IV (*12*, *13*), it is likely that the cytoplasmic end of helix VII tilts away from the helices in the N-terminal half of the permease (Figure 7).

Since cross-linking is evident with paired Cys residues at positions extending over the entire length of the transmembrane domains of helices V and VIII, it is likely that these two helices are in close proximity as they traverse the membrane without major tilting with respect to one another. Furthermore, no cross-linking is evident between helices V and VIII when Cys residues are placed at positions 141, 142, 145, or 149 in helix V and positions 279, 278, 275, or 272 in helix VII. Thus, it is likely that the face of helix V with Phe140, Ala143, Gly147, and Val158 is directed toward the face of helix VIII with Pro279, Ala278, Met275, Ala272, Thr265, and Phe261 (Figures 2 and 7).

On the basis of previous studies (*8*, *12*, *13*) and the findings presented here, a tertiary helix packing model is presented (Figure 7). This model is consistent with the following observations. (i) Close proximity between the periplasmic halves of helices VII and I, II, IV, or V has been documented by thiol cross-linking (*11*−*14*). (ii) Helix VII tilts away from the N-terminal half of the lac permease toward the cytoplasmic face of the membrane, since no cross-linking is observed with paired Cys residues at various positions in the cytoplasmic halves of helices VII and I, II, IV, or V (*11*−*14*). (iii) Tilting of the cytoplasmic end of helix XI toward helices II and IV is consistent with cross-linking between paired Cys residues at positions in the cytoplasmic halves of helices XI and II or IV, but not the periplasmic halves (*12*). (iv) Helices V and VIII are in close proximity without major tilting with respect to one another, since cross-linking is observed with paired Cys residues on one face of each of these helices throughout their lengths. (v) The cytoplasmic end of helix X tilts toward helix V, since paired Cys residues at the cytoplasmic ends of the two helices cross-link effectively. (vi) The periplasmic end of helix I tilts toward helix VII (*13*). (vii) Close proximity between helices IV and V has been documented recently by site-directed cross-linking of pairs of Cys residues placed in the two helices (C. Wolin and H. R. Kaback, unpublished results).

Electron microscopic studies (*31*−*35*) reveal a notch or cleft in the permease that is likely to result from helical tilts and may be related to the substrate translocation pathway. As shown in Figure 7, the tilting of helices I, II, IV, V, VII, VIII, X, and XI results in a cleft in the tertiary structure which is composed of one face of helices II, V, and VIII, the cytoplasmic halves of helices IV, X, and XI, and the

periplasmic halves of helices I and VII. Consistent with the notion that the cleft may be related to the sugar translocation pathway, residues thought to be directly involved in substrate binding [Glu126 (helix IV) and Arg144, Cys148, and Met145 (helix V)] (*36*−*38*) are clearly accessible within the cleft. Moreover, substrate protectable single-Cys replacements at positions 264, 268, and 272 (*39*) on one face of helix VIII lie in the vicinity of helix V and line the cleft. Finally, NEM-sensitive single-Cys replacements in helices I, II, V, VII, VIII, X, and XI cluster on helical faces that also line the cleft (*8*, *9*), and Cys residues at these positions are accessible to solvent (P. Venkatesan and H. R. Kaback, manuscript in preparation).

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