Site-Directed Chemical Cross-Linking Demonstrates that Helix IV Is Close to Helices VII and XI in the Lactose Permease[†]

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ABSTRACT: The N-terminal six transmenbrane helices (N_6) and the C-terminal six transmembrane helices (C_6) of the lactose permease, each containing a single-Cys residue, were coexpressed, and proximity was studied. Paired Cys residues in helices IV (positions 114, 116, 119, 122, 125, or 129) and VII (227, 231, 232, 234, 235, 238, 239, 242, 243, 245, or 246) or XI (350, 353, 354, 357, 361, or 364) were tested for cross-linking in the presence of two rigid homobifunctional thiol-specific cross-linkers, *N,N′-o*-phenylenedimaleimide (*o*-PDM; 6 Å) and *N,N′-p*-phenylenedimaleimide (*p*-PDM; 10 Å). Cys residues in the middle of helix IV (position 119 or 122) cross-link to Cys residues in the middle of helix VII (position 238, 239, 242, or 243). In contrast, no cross-linking is evident with paired Cys residues at either end of helix IV (position 114, 116, 125, or 129) or helix VII (position 227, 231, 232, 234, 235, 245, or 246). On the other hand, Cys residues in the cytoplasmic half of helix IV (position 125 or 129) cross-link with Cys residues in the cytoplasmic half of helix XI (position 350, 353, or 354), while paired Cys residues at the periplasmic ends of the two helices do not cross-link. The results indicate that helices IV and VII cross in a scissors-like manner with the cytoplasmic end of helix IV tilting toward helix XI.

The lactose permease (lac permease)[1] of *Escherichia coli* is a paradigm for secondary transport proteins that couple free energy stored in electrochemical ion gradients into a solute concentration gradient (reviewed in refs *1*−*5*). 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 *6*) as a monomer (see ref *7*). All available evidence (reviewed in refs *8*−*10*) indicates that the permease is composed of 12 hydrophobic or amphipathic 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.

Site-directed mutagenesis and Cys-scanning mutagenesis have not only allowed delineation of amino acid residues in the permease that are essential for active transport and/or substrate binding, but have also yielded a unique library of mutants with a single-Cys residue at each position in the permease (reviewed in ref *11*). With the single-Cys mutant library, a variety of 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, has led to a general helix packing model of lac permease (reviewed in refs *9*, *10*, *12*) (Figure 2). Although many of the proximity relationships between helices have been documented by more than one experimental approach (see ref *10*), helical tilting is particularly interesting since it has been shown (*13*−*16*) that a solvent-filled cleft or notich is present in the permease which may represent part of the substrate translocation pathway. Recently (*17*−*19*), tilting of helices I, II, V, VII, VIII, X, and XI has been demonstrated by site-directed disulfide or chemical cross-linking of paired Cys resides in a functional permease construct expressed in two nonoverlapping contiguous fragments (N_6C_6 permease; ref *20*).

In this communication, we examine the spatial relationships between helices IV and VII or XI. The results demonstrate that helices IV and VII are in close proximity at about the middle of the two transmembrane domains, while helices IV and XI are in close proximity at the cytoplasmic ends. Binding of ligand enhances cross-linking efficiency between certain pairs of Cys residues in helices IV and VII, but not between Cys residues in helices IV and XI, suggesting that the interface between helices IV and VII may be conformationally active.

EXPERIMENTAL PROCEDURES

Materials. Protein A-conjugated horseradish peroxidase (PA-HRP) enhanced chemiluminescence (ECL) detection kits were obtained from Amersham (Arlington Heights, IL).

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[1] Abbreviations: lac, lactose; C-less permease, functional lac permease devoid of Cys residues; N_6, the N-terminal six transmembrane helices; C_6, the C-terminal six 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; $NaDodSO_4$/PAGE, sodium dodecyl sulfate/polyacrylamide gel electrophoresis; NEM, *N*-ethylmaleimide.

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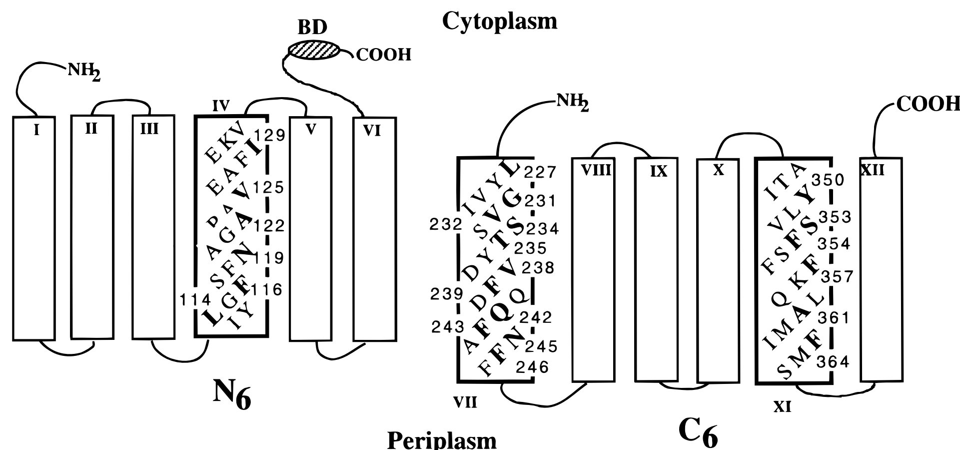

FIGURE 1: Secondary structure model of N_6/C_6 split permease. Lac permease is shown as the N-terminal six transmembrane helices (N_6) and the C-terminal six transmembrane helices (C_6). N_6 has a biotin acceptor domain (BD) at the C terminus. Single-Cys replacements in helix IV (positions 114, 116, 119, 122, 125, and 129), helix VII (positions 227, 231, 232, 234, 235, 238, 239, 242, 243, 245, and 246), and helix XI (positions 350, 353, 354, 357, 361, and 364) are numbered and highlighted.

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 N_6/C_6 Permease with Single-Cys Residues. Construction of permease mutants containing single-Cys replacements in helices IV, VII, and XI has been described (*11, 21, 22*). To each mutant with a single-Cys replacement at positions 114, 116, 119, 122, 125, or 129 in helix IV, the biotin acceptor domain from the *Klebsiella pneumoniae* oxaloacetate decarboxylase was inserted into the middle cytoplasmic loop as described (*23*). 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 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 which encodes the C-terminal six transmembrane helices of lac permease has been described (*17, 24*). To introduce a single-Cys residue in helix VII (positions 227, 231, 232, 234, 235, 238, 239, 242, 243, 245, or 246) or in helix XI (positions 350, 354, 357, 361, or 364) in C_6, the *Bst*XI-*Hind*III fragment of pC_6 was replaced with the corresponding DNA fragment from a specified single-Cys permease mutant (Figure 1). Each Cys replacement mutant in N_6 or C_6 was verified by using dideoxynucleotide termination (*25*).

Expression of N_6/C_6 Permease and Membrane Preparation. E. coli HB101 (*lacY*$^-$*Z*$^+$) was transformed with both pN_6 and pC_6, each encoding a given permease fragment containing a specified single-Cys residue. Cultures (50 mL) were grown at 37 °C in Luria-Bertani broth 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 and washed once with 20 mM Tris-HCl (pH 7.4)/2 mM ethylenediaminetetraacetate and suspended in the same buffer followed by incubation with 100 μg/mL lysozyme for 10 min in ice. Membranes were prepared by sonification, harvested by centrifugation, and suspended in 20 mM Tris-HCl (pH 7.4).

Chemical Cross-Linking and Analysis. All cross-linking experiments were carried out by adding *o*-PDM or *p*-PDM (0.5 mM final concentration) to membrane preparations at a protein concentration of 2 mg/mL. Reactions were carried out at room temperature for 30 min and terminated by adding sodium dodecyl sulfate ($NaDodSO_4$) sample buffer containing 5% (v/v) β-mercaptoethanol at given times. Iodine-catalyzed disulfide cross-linking was carried out as described (*17*). Samples were subjected to electrophoresis in $NaDodSO_4$/12% polyacrylamide gels ($NaDodSO_4$/PAGE). C_6 was detected by immunoblotting with rabbit polyclonal antibody against a C-terminal dodecapeptide corresponding to the last twelve amino acid residues of the permease (*26*). N_6 with a biotin acceptor domain at the C terminus was detected with avidin-conjugated horseradish peroxidase (*27*). Cross-linked N_6/C_6 reacts with both anti-C-terminal antibody and avidin-HRP.

Protein Assays. 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 IV and VII. Previous in situ thiol-specific cross-linking studies with N_6/C_6 permease (*17−19, 28*) demonstrate that the periplasmic halves of helices VII and I or II are in close proximity. To examine whether helices IV and VII are in close proximity, we placed paired Cys residues at various positions along one face of helix IV (position 114, 116, 119, 122, 125, or 129) in N_6 and along one face of helix VII (position 227, 231, 232, 234, 235, 238, 239, 242, 243, 245, or 246) in C_6 (Figures 1 and 2). N_6 and C_6 each with a Cys residue at a given position were coexpressed, and proximity was assessed by thiol-specific chemical cross-linking in situ. The homobifunctional thiol-specific cross-linkers *o*-PDM and *p*-PDM were chosen because of their relatively short length and hydrophobicity (*28*). Hydrophobicity is presumably important because the Cys residues are thought to be in a hydrophobic environment within the membrane. In addition, *o*-PDM and *p*-PDM are rigid cross-linking agents in which the maleimido groups are coupled to benzene rings in the *ortho* or *para* position at fixed distances of about 6 and 10 Å, respectively. C_6 which reacts with anti-C-terminal antibody migrates with an M_r of about 20 kDa (Figure 3A), N_6 with the biotin acceptor domain which reacts with avidin-

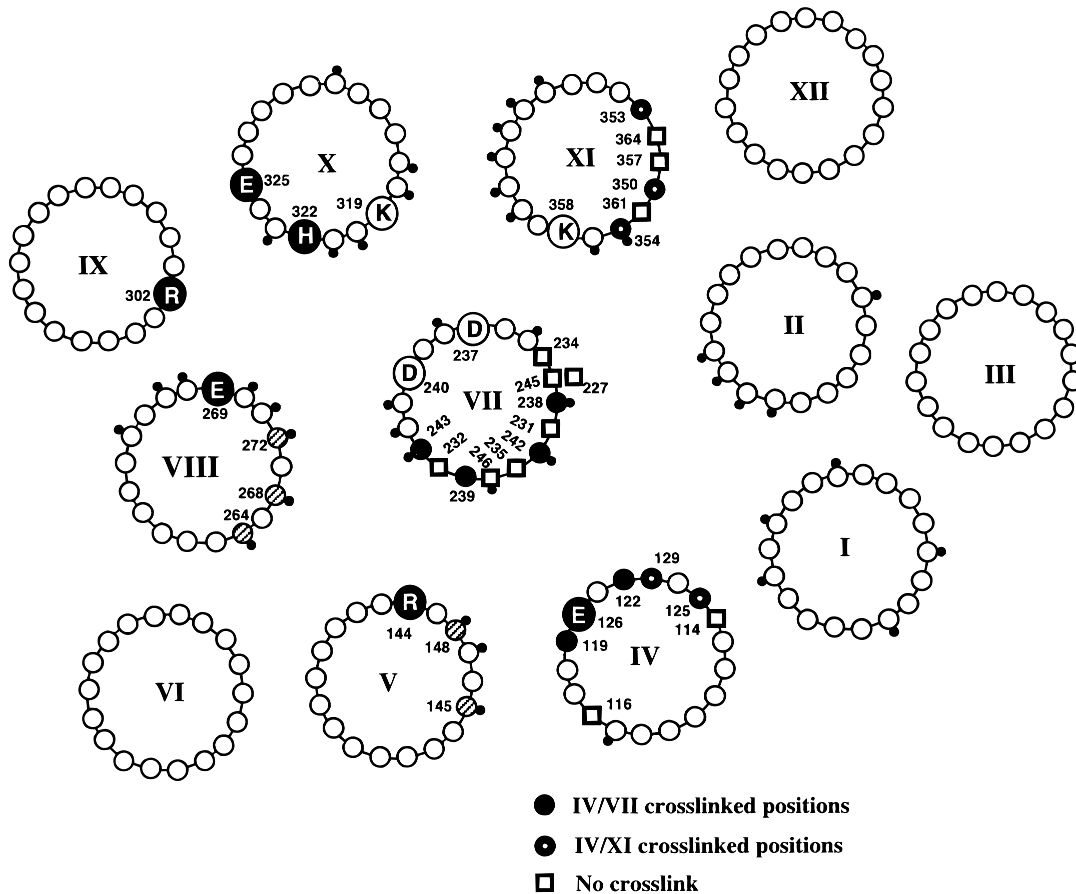

FIGURE 2: Helix packing in lac permease. The six essential residues [Glu126 (helix IV), Arg144 (helix V), Glu269 (helix VIII), Arg302 (helix IX), and His322 and Glu325 (helix X)] and two interacting pairs of Asp-Lys residues [Asp237 (helix VII)/Lys358 (helix XI) and Asp240 (helix VII)/Lys319 (helix X)] are highlighted. Positions of NEM-sensitive Cys replacements are indicated with a small black dot. Positions where single-Cys mutants are protected from alkylation by substrate (145, 148, 264, 268, and 272) are crosshatched. Cys replacement mutants in helices IV, VII, and XI tested for cross-linking in this study are shown as filled circles (positive cross-linking between helices IV and VII), filled cicles with a white dot (positive cross-linking between helices IV and XI), or unfilled squares (negative cross-linking).

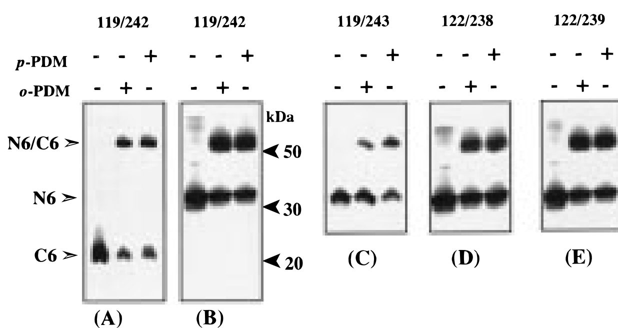

FIGURE 3: Cross-linking of N_6/C_6 permease with paired Cys residues in helices IV and VII. Membranes were prepared from cells expressing N_6 and C_6, each with a single-Cys residue at given positions as indicated. Cross-linking was carried out with 0.5 mM *o*-PDM or *p*-PDM as indicated at room temperature for 30 min. 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 blots were probed with anti-C-terminal antibody or avidin-HRP. The positions of N_6, C_6, and N_6/C_6 are indicated by arrows: (A) cross-linking of Cys pairs 119/242 probed with anti-C-terminal antibody; (B) cross-linking of Cys pairs 119/242 probed with avidin-HRP; (C) cross-linking of Cys pairs 119/243 probed with avidin-HRP; (D) cross-linking of Cys pairs 122/238 probed with avidin-HRP; and (E) cross-linking of Cys pairs 122/239 probed with avidin-HRP.

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).

When membranes containing N_6/C_6 permease with paired Cys residues at positions in the periplasmic ends of helices IV (position 114 or 116) and VII (position 245 or 246) are treated with *o*-PDM or *p*-PDM, no cross-linking is detected (Table 1). Similarly, no cross-linking is observed when paired Cys residues are placed in the cytoplasmic ends of helices IV (position 125 or 129) and VII (position 227, 231, 232, 234, or 235) (Table 1). In marked contrast, however, when paired Cys residues are placed at positions in the middle of transmembrane helices IV and VII, cross-linking is clearly evident (Figure 3; Table 1). N_6/C_6 with Cys pairs 119/238, 119/242, 119/243, 122/238, or 122/239 is readily cross-linked by *o*-PDM or *p*-PDM, indicating close proximity at the middle of the two transmembrane domains. Thus, helices IV and VII appear to cross in a scissors-like manner.

Cross-Linking N_6/C_6 Permease with Paired Cys Residues in Helices IV and XI. To study tilting of helix IV with respect to helix XI, we coexpressed N_6 with a Cys residue at various positions in helix IV with C_6 containing a Cys residue at position 350, 353, 354, 357, 361, or 364 in helix XI (Figures 1 and 2). When membranes containing N_6/C_6 with Cys pairs 114/364, 116/364, 116/361, 119/361, 119/257, 122/361, or 122/357 are exposed to *o*-PDM or *p*-PDM, no cross-linking is evident (Figure 4B and Table 1), indicating that the

Table 1: Chemical Cross-Linking of Paired Cys Residues in Helices IV/VII or IV/XI[a]

IV/VII	*o*-PDM (6 Å)	*p*-PDM (10 Å)	IV/XI	*o*-PDM (6 Å)	*p*-PDM (10 Å)
129/227	−	−	129/350	+++	+++
125/231	−	−	129/353	+	++
125/232	−	−	125/353	+	++
125/234	−	−	125/354	+++	+++
125/235	−	−			
122/238	+++	+++	122/357	−	−
122/239	+++	+++	122/361	−	−
119/238	+++	+++	119/357	−	−
119/242	+++	+++	119/361	−	−
119/243	++	+			
116/245	−	−	116/361	−	−
116/246	−	−	116/364	−	−
113/245	−	−			
114/246	−	−	114/364	−	−

[a] Crosslinking experiments were carried out at 22 °C for 30 min as described for Figure 3 and probed with avidin-HRP: +++, >30% efficiency; ++, 10−30% efficiency; +, <10% efficiency; −, no detectable crosslinking.

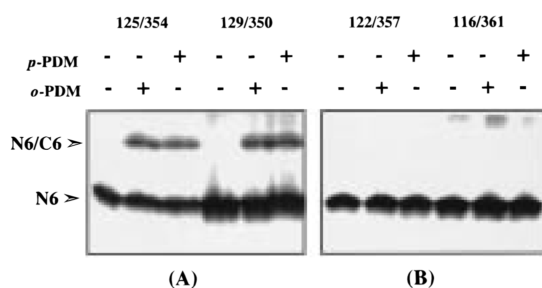

FIGURE 4: Cross-linking of N_6/C_6 permease with paired Cys residues in helices IV and XI. Membranes were prepared from cells expressing N_6 and C_6, each with a Cys residue at the position indicated. Cross-linking was carried out at room temperature by incubating membranes (2 mg of protein/mL) with 0.5 mM *o*-PDM or *p*-PDM as indicated for 30 min. Reactions were terminated by adding $NaDodSO_4$ sample buffer containing 5% (v/v) β-mercaptoethanol. Samples were analyzed as described in Figure 3 with avidin-HRP: (A) cross-linking of Cys pairs 125/354 or 129/350; and (B) cross-linking of Cys pairs 122/357 or 116/361.

periplasmic halves of helices IV and XI are not in close proximity. However, when paired Cys residues are placed at the cytoplasmic ends of the two transmembrane domains, cross-linking is readily observed. Thus, N_6/C_6 expressing Cys pairs 125/354, 129/350, 125/353, or 129/353 is clearly cross-linked by *o*-PDM or *p*-PDM (Figure 4A and Table 1). The results show that helix IV is tilted in such a manner as to cross helix VII at its approximate middle with the cytoplasmic end within 6−10 Å of the cytoplasmic end of helix XI.

Ligand-Induced Changes in Cross-Linking. Cross-linking of N_6/C_6 in situ is a sensitive probe for conformationally active interfaces between transmembrane helices (*18*, *19*, *28*). Interhelical distance changes have been observed at the periplasmic interface between helix VII and helix I or II. To test whether the interface between helices IV and VII or IV and XI is conformationally active, we carried out cross-linking of N_6/C_6 containing paired Cys residues in the absence or presence of 10 mM β,D-galactopyranosyl 1-thio-β,D-galactopyranoside (TDG). With N_6/C_6 expressing paired Cys residues at positions 119 and 238, cross-linking by *o*-PDM or *p*-PDM is weak in the absence of ligand (Figure 5A). Interestingly, cross-linking becomes stronger with both cross-linking agents in the presence of TDG (Figure 5A).

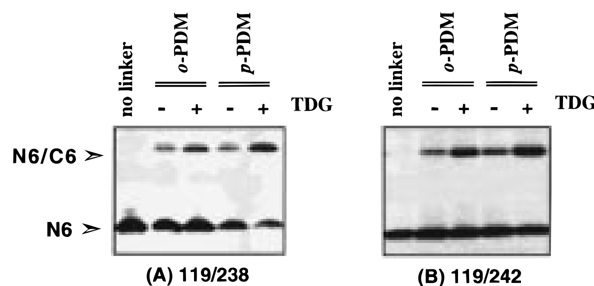

FIGURE 5: Cross-linking of N_6/C_6 permease and the effect of ligand. Membranes were prepared from cells expressing N_6 and C_6 with paired Cys residues at the positions indicated. Cross-linking was carried out at room temperature for 5 min in the absence or presence of 10 mM TDG as indicated, and samples were analyzed as described in Figure 3 with avidin-HRP: (A) Cys pairs 119/238; and (B) Cys pairs 119/242.

Similar changes are also evident in N_6/C_6 expressing paired Cys residues at positions 119/242 (Figure 5B). Since cross-linking efficiency with the 6 or the 10 Å cross-linking agent is increased by TDG, one possibility is that binding of TDG increases the reactivity of the Cys residue at position 119. Consistently, when a Cys residue at position 122 is paired with a Cys residue at position 238 or 242, cross-linking efficiency does not change in the presence of TDG (data not shown). Moreover, TDG binding does not alter iodine-catalyzed disulfide cross-linking of Cys pairs 119/238 or 119/242 (data not shown), indicating that inter-thiol distance is not changed. Finally, binding of TDG does not alter cross-linking efficiency with N_6/C_6 containing Cys pairs 125/354 or 129/350 (data not shown), suggesting that the interface between helices IV and XI is not conformationally active.

DISCUSSION

Site-directed mutagenesis of lac permease has allowed the identification of 6 residues that are irreplaceable with respect to substrate binding and active transport: Glu126 (helix IV) and Arg144 (helix V) which are indispensable for substrate binding and recognition; and Glu269 (helix VIII), Arg302 (helix IX), His322, and Glu325 (helix X) which are essential for H^+ translocation and coupling (reviewed in refs *10*, *12*). However, structural information is a prerequisite for understanding the mechanism of the transport mechanism at the molecular level (see ref *12*). In this regard, a detailed helix packing model which includes the tilts of the helices is critical for delineation of the spatial relationship between the irreplaceable residues and to define the substrate translocation pathway. Although the permease has been impervious to crystallization attempts which precludes high-resolution structure analysis, we have utilized a wide variety of site-directed biophysical and biochemical techniques to obtain relatively high-resolution structural information regarding lac permease. In particular, use of site-directed thiol cross-linking with split permease in situ has facilitated the determination of helix proximities, tilting, and ligand-induced interhelical distance changes (*17*−*19*, *28*−*30*). Cross-linking studies with paired Cys residues in two helices across the discontinuity in N_6C_6 permease have provided proximity and tilting data between helices I and VII, II and VII, II and XI, V and VII, V and VIII, and V and X (*18, 19*). In this paper, the tertiary contacts of helix IV with helices VII and XI in the C-terminal half of the permease are documented. As

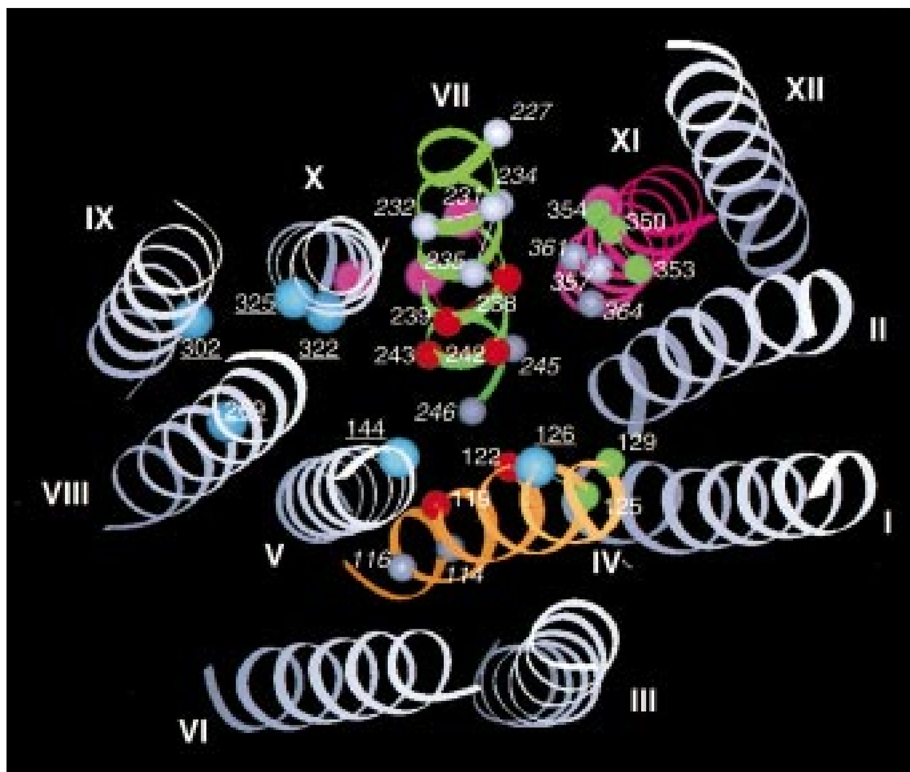

FIGURE 6: Helix packing model of lactose permease highlighting the tertiary contacts between helices IV, VII, and XI. Twelve transmembrane domains viewed from the cytoplasmic surface of the membrane are displayed as helical ribbons. The six essential residues [Glu126 (helix IV), Arg144 (helix V), Glu269 (helix VIII), Arg302 (helix IX), and His322 and Glu325 (helix X)] are shown as numbered enlarged blue balls. The two Asp-Lys charge pairs [Asp-240 (helix VII)/Lys-319 (helix X) and Asp-237 (helix VII)/Lys-358 (helix XI)] are shown as numbered enlarged purple balls. Positions that cross-link between helices IV (orange) and VII (green) are highlighted as red balls. Positions that cross-link between helices IV and XI (dark red) are highlighted as green balls. Positions that do not exhibit cross-linking are depicted as gray balls. The cytoplasmic end of helix VII tilts away from helices I, II, IV, and V. Tilting of helices I, II, V, VII, VIII, X, and XI is derived from previous studies (*18*, *19*, *28*).

shown, when paired Cys residues are placed in helices IV and VII, cross-linking is evident only with paired Cys residues in the middle of the two transmembrane domains (position 119 or 122 in helix IV; position 238, 239, 242, or 243 in helix VII). In contradistinction, no cross-linking is observed when paired Cys residues are placed in either end of the two helices. The results are consistent with the suggestion that helices IV and VII cross in scissors-like fashion (Figure 6).

To investigate the tertiary contacts between helices IV and XI, we tested cross-linking in N_6/C_6 permease containing paired Cys residues in the two transmembrane domains by using the same approach. When paired Cys residues are placed at positions near the cytoplasmic ends, high-efficiency cross-linking is observed with Cys pairs 125/354 or 129/350. In contrast, no cross-linking is evident when paired Cys residues are introduced into the periplasmic halves of helices IV (position 114, 116, 119, or 122) and XI (position 357, 361, or 364), suggesting that the two helices tilt toward each other in traversing the membrane from the periplasmic to the cytoplasmic face. Taken together, the results support the conclusion that helix IV is tilted in such a manner that the middle is close to helix VII, and the cytoplasmic end is close to helix XI (Figure 6).

Tilting of helices IV and XI (Figure 6) is consistent with previous observations. It has been shown (*18*) that helices II and VII are in close proximity at the periplasmic ends and tilt away from each other toward the cytoplasmic side of the membrane. Tilting in this manner leaves space between helices II and VII at the cytoplasmic ends. Moreover, cross-linking of paired Cys residues in helices II and XI demonstrates that the two helices are in close proximity at the cytoplasmic ends, but not the periplasmic ends. Therefore, it is reasonable to suggest that the cytoplasmic end of helix XI tilts into the cytoplasmic interface between helices II and VII (Figure 6). Finally, tilting the cytoplasmic end of helix IV toward the cytoplasmic interface between helices II and VII is consistent with the observations that the cytoplasmic ends of helices IV and XI are in close proximity and that helices IV and VII cross in such a manner that they are in close proximity in the middle of the membrane.

For the elucidation of the mechanism of sugar/H^+symport, dynamic information is clearly required in addition. In this respect, ligand-induced conformational changes have been observed in many regions of lac permease (*18, 19, 28, 30*–*35*). As shown here, the cross-linking efficiency of Cys pairs 119/238 or 119/242 is increased in the presence of ligand. Since binding of TDG increases cross-linking efficiency with both the 6 and 10 Å cross-linkers, it is unlikely that increased cross-linking reflects a decrease in interhelical distance in this instance. Consistently, iodine-catalyzed disulfide formation between the two pairs of Cys residues is not altered. Therefore, it is likely that TDG binding increases the reactivity of the Cys residue at position 119 rather than causing a decrease in distance between helices IV and VII. Nonetheless, the finding suggests that the interface between the two helices is conformationally active.

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