Tilting of Helix I and Ligand-Induced Changes in the Lactose Permease Determined by Site-Directed Chemical Cross-Linking in Situ†

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ABSTRACT: The N-terminal six transmenbrane helices (N_6) and the C-terminal six transmembrane helices (C_6) of lactose permease, each with a single Cys residue, were co-expressed, and cross-linking was studied. The proximity of paired Cys residues in helices I (positions 11, 14, 15, 18, 25, 28, 29, or 32) and VII (positions 227, 231, 232, 234, 235, 238, 239, 241, 242, 245, or 246) was studied by using homobifunctional thiol-specific chemical linkers of different lengths and chemical properties. The results demonstrate that Cys residues on one face of the periplasmic half of helix I (positions 32, 29, 28, or 25) cross-link to Cys residues on one face of the periplasmic half of helix VII (242 or 245). In contrast, no cross-linking is evident with paired Cys residues in the cytoplasmic halves of helices I (positions 11, 14, 15, or 18) and VII (positions 227, 230, 231, 232, 234, 235, 238, or 239). The results indicate that helices I and VII are in close proximity only at their periplasmic halves. Ligand binding decreases cross-linking efficiency of the Cys pair 28/245 or 25/242 with *N*,*N*′-*o*-phenylenedimaleimide (rigid 6 Å) and increases efficiency with *N*,*N*′-*p*-phenylenedimaleimide (rigid 10 Å) or 1,6-bismaleimidohexane (flexible 16 Å), indicating that the inter-thiol distance is about 6 Å in the absence of ligand and that ligand binding increases the distance up to 10 Å. The inter-thiol distance for Cys pairs 29/245 or 32/245 is less than 6 Å in the absence of ligand, and in the presence of ligand, distance increases to between 6 and 10 Å. Taken together, the results indicate that ligand binding induces a translational or scissors-like rigid body movement of helix I and/or VII at the periplasmic interface between the helices.

The lactose (lac)[1] permease of *Escherichia coli* is a paradigm for secondary transport proteins that couple the energy stored in an electrochemical ion gradient into a concentration gradient (*1*−*3*). This hydrophobic, polytopic membrane protein that 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 transport (*4*, *5*) as a monomer (*6*). All available evidence indicates that the permease is composed of 12 α-helical rods that traverse the membrane in zigzag fashion connected by hydrophilic loops with the N and C termini on the cytoplasmic face (reviewed in ref *7*).

Site-directed and Cys-scanning mutagenesis have allowed delineation of amino acid residues in the permease that are important for active transport and/or substrate binding (*7*−*9*). However, structural and dynamic information at high resolution are required to understand the role of these residues in the transport mechanism. 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. In this respect, a helix packing model of the permease has been formulated (*8*, *9*). Proximity relationships are based on a battery of site-directed approaches, including excimer (*10*, *11*), chemical cleavage (*12*), spin−spin interactions (*11*, *13*), engineered divalent metal binding sites (*14*−*17*), metal−spin label interactions (*18*−*20*), thiol-specific cross-linking (*21*−*24*), and identification of discontinuous mAb epitopes (*25*).

Recently, a general cross-linking approach has been used successfully to examine transmembrane helix proximity, helix tilting, inter-thiol distances, and ligand-induced conformational changes in the permease (*21*−*23*). Information regarding the tilting of individual helices with respect to each other and the plane of the membrane is particularly important for placing the essential residues in the tertiary packing structure and to localize the substrate translocation pathway. In this report, we examine proximity relationships between helices I and VII by thiol-specific chemical cross-linking of paired Cys residues at positions over the entire length of

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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; BMH, 1,6-bismaleimidohexane; Na-DodSO$_4$/PAGE, sodium dodecyl sulfate/polyacrylamide gel electrophoresis; NEM, *N*-ethylmaleimide.

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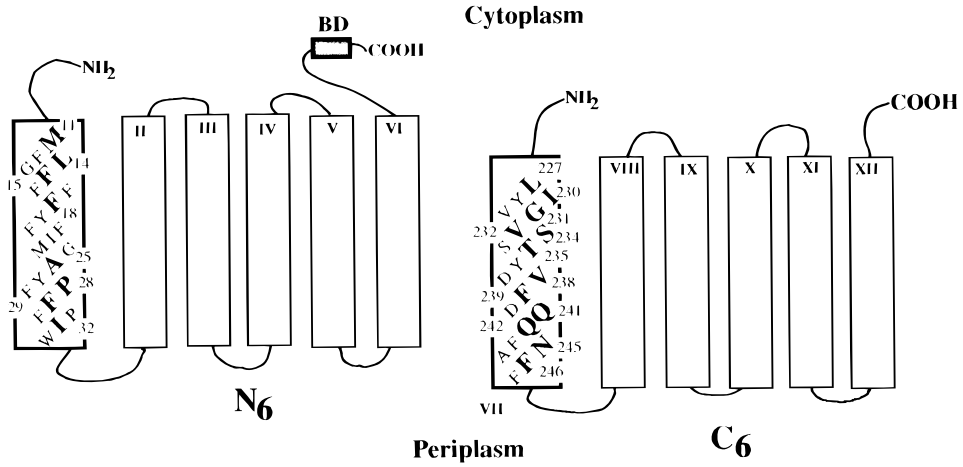

FIGURE 1: Secondary structure model of N_6/C_6 split permease. The lac permease is shown as the N-terminal six transmembrane helices (N_6) and the C-terminal six transmembrane helices (C_6). N_6 has a biotin acceptor domain (BD) at the C terminus. Single Cys replacements in helix I (positions 11, 14, 15, 18, 25, 28, 29, and 32) and helix VII (positions 227, 230, 231, 232, 234, 235, 238, 239, 241, 242, 245, and 246) are numbered and highlighted.

transmembrane domains I and VII. Paired Cys residues placed at positions in the periplasmic halves of helices I and VII are shown to be cross-linked. In contrast, no cross-linking is observed when paired Cys residues are placed in the cytoplasmic halves of the two helices. The results demonstrate that helices I and VII are in close proximity at the periplasmic ends but not at the cytoplasmic ends, indicating that the helices tilt away from each other. In addition, ligand binding alters cross-linking efficiency in a manner that is consistent with the interpretation that binding induces a translational or scissors-like displacement of helices I and/or VII.

MATERIALS AND METHODS

Materials. Protein A-conjugated horseradish peroxidase (PA-HRP) enhanced chemiluminescence (ECL) detection kits were obtained from Amersham (Arlington Heights, IL). Avidin-conjugated horseradish peroxidase (avidin-HRP) was purchased from Pierce (Rockford, IL). 1,6-Bismaleimidohexane (BMH), *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/C_6 Permease. Construction of permease mutants containing single Cys replacements in helices I and VII has been described (26, 27). To each mutant with single Cys replacement at position 11, 14, 15, 18, 25, 28, 29, or 32 in helix I, the biotin acceptor domain from the *Klebsiella pneumoniare* oxaloacetate decarboxylase was inserted into the middle cytoplasmic loop as described (*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 that encodes the N_6 fragment 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 C-terminal six transmembrane helices of lac permease has been described (*21*, *29*). Construction of pC_6 encoding C_6 with a single Cys residue in helix VII at position 227, 230, 231, 232, 234, 235, 238, 239, 241, 242, 245, or 246 has also been described (Figure 1) (*22*, *23*). Each Cys replacement mutant was verified by using dideoxynucleotide sequencing (*30*).

Expression of Split Permease and Membrane Preparation. *E. coli* HB101 (*lacY*$^-$*Z*$^+$) was transformed with both pN_6 and pC_6, each encoding a permease fragment with a given single Cys residue. Cells (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 (IPTG) for 2 h. Cells were harvested by centrifugation and washed once with 20 mM Tris-HCl (pH 7.40)/2 mM EDTA and suspended in the same buffer followed by incubation with 100 μg/mL lysozyme for 10 min in ice. Membranes were prepared by sonification and suspended in 20 mM Tris-HCl (pH 7.40).

Chemical Cross-Linking and Analysis. All cross-linking experiments were carried out 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 incubated at 4 °C to minimize thermal backbone motion (*22*, *31*) and terminated by adding sodium dodecyl sulfate sample buffer containing 5% (v/v) β-mercaptoethanol at given times. Samples were subjected to electrophoresis in sodium dodecyl sulfate/12% polyacrylamide gel electrophoresis ($NaDodSO_4$/PAGE). C_6 was detected by immunoblotting with rabbit polyclonal antibody raised against a dodecapeptide corresponding to the C terminus of the permease (*32*). N_6 with a biotin acceptor domain at the C-terminus was detected with avidin-conjugated horseradish peroxidase as described (33). Cross-linked N_6/C_6 product reacts with both anti-C-terminal antibody and avidin-HRP.

Protein Assay. Protein was assayed with the Micro BCA kit (Pierce, IL) using BSA as standard.

RESULTS

Cross-Linking of N_6/C_6 Permease Containing Paired Cys Residues in Helices I and VII. Previous results (*21*) show that N_6/C_6 permease containing paired Cys residues at positions 245 (helix VII) and 28 or 29 (helix I) form a disulfide bond when oxidized with iodine, indicating that the Cys residues are in close proximity. To investigate whether helices I and VII are parallel or tilted with respect to one another, Cys residues were placed in pairs at positions

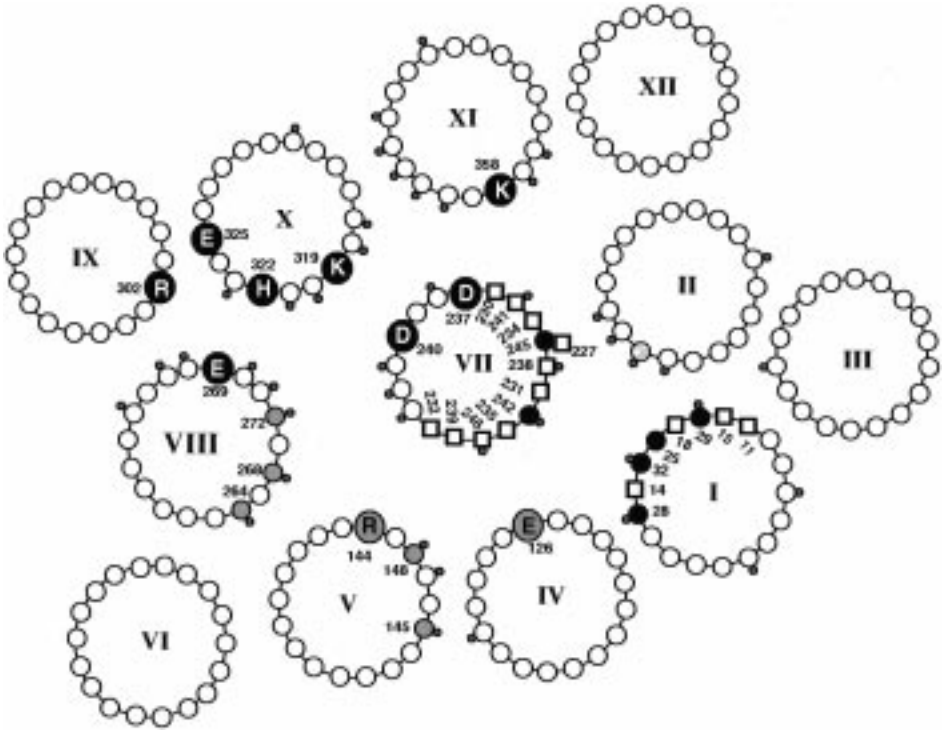

FIGURE 2: Helix packing in the lac permease. The six irreplaceable residues (Glu126, Arg144, Glu269, Arg302, His322, and Glu325) and the two interacting pairs of Asp-Lys residues [Asp237 (helix VII)/Lys358 (helix XI) and Asp240 (helix VII)/Lys319 (helix X)] are highlighted. Positions of NEM-sensitive Cys replacements are indicated with a small black dot. Substrate protectable single Cys replacement mutants (145, 148, 264, 268, and 272) are numbered. Cys replacement mutants in helices I and VII tested for chemical cross-linking in this study are highlighted and numbered. Cross-linked positions are shown as filled circles. Open squares represent positions of no-cross-linking.

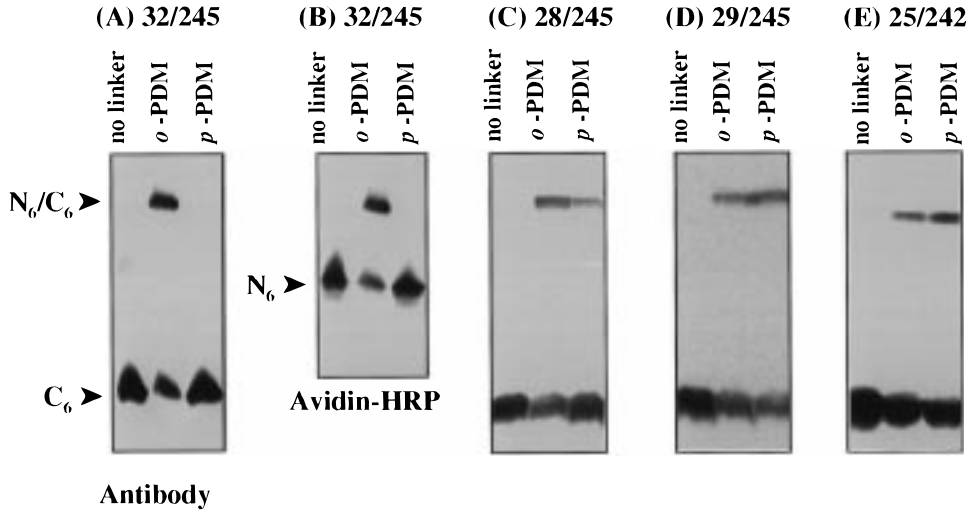

FIGURE 3: Chemical cross-linking of split N_6/C_6 permease containing paired Cys residues in helices I and VII. Membranes were prepared from cells expressing the N_6 and C_6 fragments each with a single Cys residue at a given position as indicated. Chemical cross-linking was carried out at 4 °C for 30 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 immunoblot was probed with anti-C-terminal antibody or avidin-HRP. The N_6 and C_6 fragments and the N_6/C_6 cross-linked products are indicated by the arrows. (A) Cross-linking of paired-Cys 32/245 probed with anti-C-terminal antibody. (B) Cross-linking of paired-Cys 32/245 probed with avidin-HRP. (C) Cross-linking of paired-Cys 28/245 probed with anti-C-terminal antibody. (D) Cross-linking of paired-Cys 29/245 probed with anti-C-terminal antibody. (E) Cross-linking of paired-Cys 25/242 probed with anti-C-terminal antibody.

along one face of helix I in N_6 and along one face of helix VII in C_6 (Figures 1 and 2). N_6/C_6 permease with a given pair of Cys residues was then co-expressed, and the proximity between the two Cys residues was assessed by thiol-specific chemical cross-linking in situ. Homobifunctional thiol-specific linkers *o*-PDM and *p*-PDM were chosen because of their short length and hydrophobicity. Hydrophobicity is important for cross-linking Cys residues in a hydrophobic environment such as transmembrane domains. *o*-PDM and *p*-PDM are rigid thiol-specific reagents 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. As shown in Figure 3, C_6 that reacts with anti-C-terminal antibody migrates at an M_r of about 20 kDa (Figure 3A), N_6 with the biotin acceptor domain migrates with an M_r of about 35 kDa and reacts with avidin-HRP

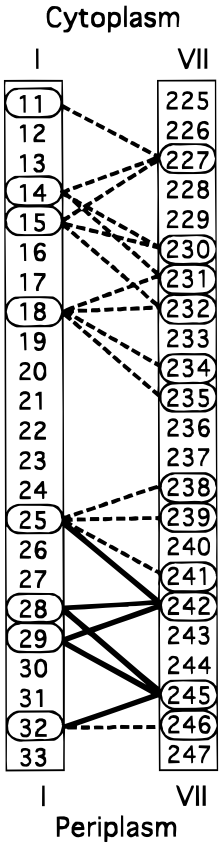

FIGURE 4: Summary of cross-linking results. Helices I and VII are depicted as boxes. Cross-linked Cys pairs are connected with solid lines. Non-cross-linked Cys pairs are shown as dashed lines.

(Figure 3B), and cross-linked N_6/C_6 migrates with an M_r of about 52 kDa that reacts with both anti-C-terminal antibody and avidin-HRP (Figure 3A,B).

When membranes containing N_6/C_6 permease with paired Cys residues at positions 32 (helix I) and 245 (helix VII) are treated with *o*-PDM or *p*-PDM, it is clear that the pair is cross-linked effectively by *o*-PDM but not by *p*-PDM (Figure 3A,B). Therefore, the inter-thiol distance between this pair of Cys residues is as great as to 6 Å but less than 10 Å. Since position 32 is in the same face of helix I as residues 28 and 29 but one helical turn removed toward the periplasmic end (Figures 1 and 2), the finding is consistent with previous observations (*21*), indicating that 245 and 28 or 29 are in close proximity. In contrast, when a Cys residue at position 32 is paired with a Cys residue at position 246, which is 108° removed from position 245, no cross-linking is evident with *o*-PDM or *p*-PDM (Figure 4). Consistently, paired Cys mutants 28/245 or 29/245 are cross-linked by *o*-PDM or *p*-PDM (Figure 3C,D), indicating that the inter-thiol distance between Cys residues at positions 245 and 28 or 29 varies between 6 and 10 Å. In addition, double Cys pairs 28/242 and 29/242 are also cross-linked effectively by *o*-PDM or *p*-PDM (data not shown).

When N_6/C_6 permease with paired Cys residues at positions 25 and 242 is treated with *o*-PDM or *p*-PDM, cross-linking is clearly evident (Figure 3E). However, Cys pairs 25/241, 25/239, or 25/238 are not cross-linked by either reagent (Figure 4). Furthermore, when paired Cys residues are placed at positions toward the cytoplasmic ends of helices I and VII, no cross-linking is detected with the following

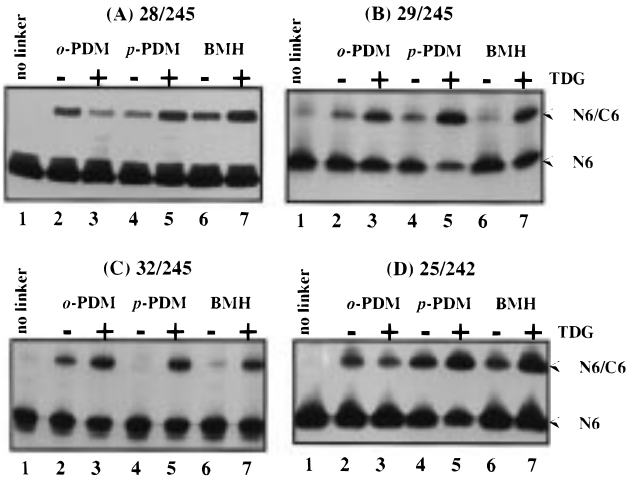

FIGURE 5: Chemical cross-linking of split permease and the effect of ligand. Membranes were prepared from cells expressing N_6 and C_6 with paired Cys residues at given positions as indicated. Cross-linking was carried out at 4 °C for 10 min in the absence or presence of 10 mM TDG as indicated, and samples were analyzed as described in Figure 3 with avidin-HRP. (A) Paired Cys residues 28/245. (B) Paired Cys residues 29/245. (C) Paired Cys residues 32/245. (D) Paired Cys residues 25/242.

pairs of Cys residues: 18/234, 18/235, 18/232, 18/231, 15/234, 15/231, 15/230, 15/227, 14/231, 14/230, 14/227, and 11/227 (Figure 4). Taken as a whole, the results indicate that helices I and VII are in close proximity at the periplasmic halves only and tilted away from each other at the cytoplasmic ends.

Determination of Inter-Thiol Distance and Ligand-Induced Distance Changes. Chemical cross-linking of paired Cys N_6/C_6 permease in situ has proven to be a sensitive method for estimating inter-thiol distances as well as a means to probe ligand-induced distance changes by using linkers of different length and rigidity (*22*, *23*). As shown in Figure 5A, at 4 °C in the absence of ligand, *o*-PDM (rigid 6 Å) cross-links paired Cys residues at positions 28 and 245 effectively (Figure 5A, lane 2), BMH (flexible; 16 Å) cross-links with comparable efficiency to *o*-PDM (Figure 5A, lane 6), and *p*-PDM (rigid 10 Å) cross-links least effectively (Figure 5A, lane 4). Cross-linking by BMH and particularly *p*-PDM is much more effective in the presence of TDG (Figure 5A, lanes 5 and 7). In contrast, cross-linking by *o*-PDM decreases (Figure 5A, lane 3). The results suggest that the distance between Cys residues at positions 28 and 245 varies up to 6 Å in the absence of ligand and that binding of TDG increases distance to a maximum of 10 Å. The effect of ligand on cross-linking of paired Cys residues at positions 25 and 242 is similar to that observed with the 28/245 pair. Thus, in the absence of ligand, N_6/C_6 permease containing 25/242 paired Cys residues is cross-linked weakly by *o*-PDM or BMH (Figure 5D, lanes 2 and 6) relative to *p*-PDM (lane 4). Binding of TDG increases cross-linking efficiency with *p*-PDM or BMH significantly (lanes 5 and 7), and cross-linking by *o*-PDM is slightly decreased (lane 2). The results indicate that the inter-thiol distance of this pair of Cys residues also varies between 6 and 10 Å in the absence of ligand and that binding of TDG increases the distance to around 10 Å.

The effect of ligand is also evident in N_6/C_6 permease containing paired Cys residues at positions 245 and 29, which

is presumably 108° removed from position 28 and about 1.5 Å displaced toward the periplasm (Figures 1 and 2). In the absence of ligand, this pair of Cys residues is cross-linked relatively weakly by *o*-PDM, *p*-PDM, and particularly BMH (Figure 5B, lanes 2, 4 and 6). In the presence of TDG, however, cross-linking efficiency is significantly increased for all three linkers (Figure 5B, lanes 3, 5 and 7). One possibility is that inter-thiol distance is shorter than 6 Å in the absence of ligand and that binding of TDG increases distance to between 6 and 10 Å. Alternatively, since no reciprocal changes are evident between the shorter (6 Å) and longer (10 and 16 Å) linkers, the results could be interpreted to indicate that the inter-thiol distance with this pair of Cys residues varies up to 10 Å and that binding of TDG does not alter distance but changes the reactivity of one or both of the Cys residues.

A ligand-induced effect on N_6/C_6 permease containing paired Cys residues 32/245 is also clearly evident. As shown in Figure 5C, in the absence of ligand, *o*-PDM cross-links much more effectively than BMH (compare lane 2 with lane 6), and *p*-PDM does not cross-link significantly. In the presence of TDG, cross-linking efficiency with *o*-PDM is significantly increased (compare lane 3 with lane 2), and cross-linking efficiency with *p*-PDM or BMH increases dramatically (lanes 5 and 7). The results indicate that the inter-thiol distance is shorter than 6 Å in the absence of ligand and that binding of TDG increases the distance to between 6 and 10 Å.

DISCUSSION

Site-directed disulfide cross-linking of N_6/C_6 permease demonstrates that a Cys residue at position 245 in helix VII is close to a Cys residue at position 28 or 29 in helix I (*21*). In this paper, we extend this approach to investigate transmembrane helix tilting by placing Cys residues pairwise down the length of the two helices. As shown, paired Cys residues in the periplasmic halves of helices I (positions 32, 29, 28, and 25) and VII (positions 245 and 242) are in close proximity. Thus, paired Cys residues at positions 32/245, 29/245, 28/245, or 25/242 are cross-linked by the 6 Å *o*-PDM. The data are consistent with previous observations showing that a Cys residue at 245 forms a disulfide with a Cys residue at positions 28 or 29 in the presence of iodine (*21*). Furthermore, the results demonstrate that the close proximity between helices I and VII extends from the periplasmic end to near the middle of each transmembrane domain. In marked contrast, when paired Cys residues were placed at positions in the cytoplasmic halves of helices I and VII, no cross-linking is detected, suggesting that helices I and VII are tilted away from each other at their cytoplasmic ends (Figure 4). These data are also consistent with previous observations (*26*, *34*−*36*) indicating that the periplasmic end of helix I is conformationally active while the cytoplasmic half is not. Thus, no single amino acid residue in helix I is essential for transport activity (*26*), but the Cys replacement mutants that are inactivated by alkylation cluster at the periplasmic end (i.e., F27C−W33C). Moreover, deletion of the N-terminal 22 residues of the permease does not abolish active transport, but deletion of 38 residues inactivates (*34*). Finally, the face of helix I with residues 28, 31, and 33 undergoes a conformational change when the ligand is bound (*35*, *36*). Together with results presented here, it is likely that the periplasmic end of helix I makes tertiary contact with a conformationally active face of helix VII (*37*), while the cytoplasmic half of helix I tilts away from this conformationally active domain of the protein and most likely makes contact with the membrane bilayer.

Although as few as six residues are irreplaceable with respect to H^+-coupled sugar translocation, which suggests that relatively few chemical interactions drive the mechanism (*7*, *9*, *38*, *39*), ligand-induced conformational changes are widespread (*9*, *22*, *23*, *35*, *36*, *40*−*43*). As shown here, the interface between the periplasmic halves of helices I and VII is clearly conformationally active (Figure 5). The conformational changes are documented by ligand-induced changes in inter-thiol distances between paired Cys residues placed at the interface between helices I and VII. Thus, in the absence of ligand, the inter-thiol distance is close to 6 Å for paired Cys residues 28/245 or 32/245 since the shorter reagent *o*-PDM (6 Å) cross-links more effectively than the longer ones (*p*-PDM or BMH). On the other hand, the distance for paired Cys residues 25/242 is close to 10 Å in the absence of ligand because the longer rigid linker *p*-PDM (10 Å) cross-links more effectively than *o*-PDM. With paired Cys residues 29/245, *o*-PDM or *p*-PDM cross-links weakly in the absence of ligand, indicating that the distance is between 6 and 10 Å. Interestingly, binding of TDG alters cross-linking efficiency for all four paired Cys residues. The changes for paired Cys residues 28/245 or 25/242 are most readily interpreted as the result of a ligand-induced increase of inter-thiol distance because efficiency is decreased for the shorter *o*-PDM and increased for the longer reagents *p*-PDM or BMH in the presence of TDG. For the 32/245 or 29/245 pairs, binding of TDG increases cross-linking efficiency for all three linkers. One possibility is that the inter-thiol distance for Cys pair 32/245 or 29/245 is less than 6 Å in the absence of ligand, which could account for low cross-linking efficiency with each linker, and the TDG-induced increase in cross-linking efficiency for all linkers results from an increase in distance to between 6 and 10 Å. Alternatively, it is possible that binding of ligand does not alter the inter-thiol distance significantly but increases the reactivity of one or both Cys residue(s) in the pair. The former interpretation is favored because Cys pairs 32/245 and 29/245 are on the same interface as the 28/245 and 25/242 pairs where TDG binding clearly increases inter-thiol distance. Therefore, it is likely that binding of TDG causes a translational or scissors-like movement of helix I and/or helix VII. If rotational displacement were the case, reciprocal distance changes between the pairs would be expected in the presence of ligand. This interpretation is consistent with previous site-directed fluorescent labeling and NEM modification studies in situ with single Cys replacement mutants at the periplasmic end of helix I (positions 27−31), which indicate that the helical face with residues 28 and 31 undergoes a rigid body movement when the permease binds ligand (*35*). Since both helices I and II are in close proximity to helix VII at their periplasmic ends (*21*−*23*), movement of helix VII may change its relationship with helix II as well as helix I. In this respect, it has been shown recently (*22*, *23*) that binding of TDG induces a scissors-like movement at the periplasmic interface between helices II and VII that increases inter-helical distance at the periplasmic ends and decreases distance at the approximate middle of the two transmembrane

domains. Finally, it is also noteworthy that the face of helix VII with residues Tyr228 and Gln242 is in close proximity to helices IV and V (*12*,*13*; J.W., D.H., and H.R.K., manuscript in preparation), both of which contain residues that are essential for substrate binding and recognition (*44*−*47*).

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