Helix Packing in the Lactose Permease of *Escherichia coli* Determined by Site-Directed Thiol Cross-Linking: Helix I Is Close to Helices V and XI

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ABSTRACT: Coexpression of *lacY* gene fragments encoding the first two transmembrane domains and the remaining 10 transmembrane domains complement in the membrane and catalyze active lactose transport [Wrubel, W., Stochaj, U., et al. (1990) *J. Bacteriol. 172,* 5374−5381]. Accordingly, a plasmid encoding contiguous, nonoverlapping permease fragments with a discontinuity in the cytoplasmic loop between helices II and III (loop II/III) was constructed (N_2C_{10} permease). When Phe27 (helix I) is replaced with Cys, cross-linking is observed with two native Cys residues, Cys148 (helix V) and Cys355 (helix XI). Cross-linking of a Cys residue at position 27 to Cys148 occurs with *N*,*N*′-*o*-phenylenedimaleimide (*o*-PDM; rigid 6 Å), with *N*,*N*′-*p*-phenylenedimaleimide (*p*-PDM; rigid 10 Å), or with 1,6-bis(maleimido)-hexane (BMH; flexible 16 Å). On the other hand, with the Phe27→Cys/Cys355 pair, cross-linking is observed with *p*-PDM or BMH but not *o*-PDM. In neither case is cross-linking observed with iodine. It is suggested that a Cys residue at position 27 is within 6−10 Å from Cys148 and about 10 Å from Cys355. The results provide evidence for proximity between helix I and helices V or XI in the tertiary structure of the permease. In addition, the findings are consistent with other results [Venkatesan, P., Kaback, H. R. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 9802−9807] indicating that Glu126 (helix IV) and Arg144 (helix V) are within the membrane, rather than at the membrane−water interface on the cytoplasmic face.

The lactose (lac) permease of *Escherichia coli* is a paradigm for secondary transport proteins that couple free energy stored in an electrochemical ion gradient into a concentration gradient (*1*−*4*). This hydrophobic, polytopic membrane protein catalyzes the coupled stoichiometric translocation of β-galactosides and H^+, and it has been solubilized, purified to homogeneity, reconstituted into proteoliposomes, and shown to be solely responsible for β-galactoside transport (reviewed in ref *5*) as a monomer (see ref *6*). All available evidence indicates that the permease is composed of 12 α-helical rods that traverse the membrane in zigzag fashion connected by loops with the N and C termini on the cytoplasmic face (reviewed in refs *7* and *8*).

Site-directed and Cys-scanning mutagenesis of each amino acid residue in lac permease have demonstrated that only six amino acid residues are irreplaceable for lactose/H^+ symport—Glu126 (helix IV) and Arg144 (helix V), which are indispensable for substrate binding, and Glu269 (helix VIII), Arg302 (helix IX), and His322 and Glu325 (helix X), which are essential for H^+ translocation and coupling (reviewed in refs *8* and *9*). However, structural and dynamic information at high resolution are essential for understanding the precise role of these residues in the translocation mechanism. Since hydrophobic membrane proteins are particularly difficult to crystallize, a high-resolution structure of lac permease is not available, and alternative methods for obtaining structural information have been developed (reviewed in refs *8*−*10*). Thus, a helix packing model of the permease has been formulated based on a battery of approaches, which include second-site suppressor and site-directed mutagenesis, excimer fluorescence, spin−spin interactions, engineered divalent metal binding sites, metal−spin label interactions, thiol-specific cross-linking, and identification of discontinuous mAb epitopes.

One approach (*11*−*14*) involves expression of functional lac permease in two contiguous, nonoverlapping fragments comprising the N-terminal six transmembrane helices (N_6) and the C-terminal six transmembrane helices (C_6) (*15*, *16*), each with a single Cys residue at a defined position on either side of the discontinuity. The proximity of the paired Cys residues is then readily assayed by disulfide 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 (*17*, *18*).

To extend proximity studies on the permease by using thiol cross-linking, particularly with respect to relationships between the helices within N6, a plasmid encoding the first two transmembrane domains and the remainder of the molecule [N_2C_{10}; *22*] was constructed and used for thiol cross-linking studies. The results indicate that helix I is close to helices V and XI.

EXPERIMENTAL PROCEDURES

Materials. [1-^{14}C]Lactose, [α-^{35}S]dATP, L-[^{35}S]methionine, protein A-conjugated horseradish peroxidase (HRP), and enhanced chemiluminescence detection kits were purchased

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Table 1: DNA Sequences of Oligodeoxynucleotides Used for Constructing N_2C_{10} LacY Gene

Oligo name	Oligo Sequence[a]
N2C10 antisense	*CCGAGAGGGAAATAGTACATTATCTAGATCC*TAAGCT TAACTACCGAGTTTGTCAGAAAGCAG
N2C10 sense	*GGATCTAGATAATGTACTATTTCCCTCTCGG*TCTACGC AAATACCTGC

[a] Sequence of mutagenic primers are presented in 5′ → 3′ order. The termination codon (TAA), the ribosome binding sequence (AGGA), and initiation codon (ATG) are in boldface type, and the regions that are complementary are italicized.

from Amersham. Restriction endonucleases and T4 DNA ligase were from New England Biolabs. Sequenase (modified T7 DNA polymerase) and Sequenase reaction kits were from U. S. Biochemical Corp. Isopropyl 1-thio-β-D-galactopyranoside (IPTG), Taq polymerase, and associated polymerase chain reaction (PCR) reagents were obtained from Boehringer Mannheim. *N*,*N*′-*o*-phenylenedimaleimide (*o*-PDM) and *N*,*N*′-*p*-phenylenedimaleimide (*p*-PDM) were from Sigma, and 1,6-bis(maleimido)hexane (BMH) was from Pierce. Site-directed rabbit antiserum against C-terminal dodecapeptide of lac permease (*19*) was prepared by Babco (Richmond, CA). Deoxyoligonucleotides were synthesized on an Applied Biosystems 381A DNA synthesizer. All other materials were reagent grade obtained from commercial sources.

Bacterial Strains. E. coli T184 [*lacI*$^+$*O*$^+$*Z*$^-$*Y*$^-$(*A*) *rpsL met*$^-$ *thr*$^-$ *recA hsdM hsdR*/*F*′ *lacI*^{q} *O*$^+$*Z*^{D118}(*Y*$^+$*A*$^+$)] (*20*) harboring plasmid pT7−5/cassette *lacY* encoding given mutants was used for expression of lac permease and transport studies. Unless otherwise indicated, in all mutants constructed, the DNA sequence encoding the biotin acceptor domain from the oxaloacetate decarboxylase of *Klebsiella pneumoniae* was cloned into the *XhoI* site of the DNA encoding the middle cytoplasmic loop (*21*).

Construction of N_2C_{10} in C-less Permease. Two-stage PCR was used to create a *lacY* gene encoding N_2C_{10}. Two mutagenic oligonucleotides containing a complementary region, sequences encoding the termination codon (TAA), the ribosome binding sequence (AGGA), initiation codon (ATG), and sequences complementary to the insertion site of the cassette *lacY* gene were synthesized (Table 1). A few modifications were made according to Wrubel et al. (*22*) so that the N6 fragment contains residues 1−71 of wild-type permease plus an additional Ser at the C terminus, and the C6 fragment contains residues 70−417 of wild-type permease plus the sequence Met-Tyr-Tyr-Phe-Pro at the N terminus. Two independent PCR protocols were carried out using plasmid pT7-5/cassette *lacY* encoding C-less permease (*23*) as template DNA. Each mutagenic oligonucleotide was paired with a second oligonucleotide (outside primer) designed to anneal upstream or downstream depending upon the orientation of the mutagenic oligonucleotide (sense or antisense). The two PCR products were combined to form the template DNA for a second PCR amplification using the two outside primers. The resulting PCR product was then digested with *Bam*HI and *Kpn*I restriction endonucleases (see Figure 1 for location of sites) and ligated to a similarly treated pT7-5/*lacY* encoding C-less permease.

Construction of N_2C_{10} in Wild-Type Permease. N_2C_{10}/WT was constructed by replacing the *Eco*RI−*Pst*I fragment in plasmid pT7-5/cassette *lacY* encoding wild-type permease with the corresponding fragment from N_2C_{10}/C-less. The *Eco*RI site is located about 300 bp upstream of *lacY* in the plasmid (see Figure 1 for location of other sites).

Construction of Mutants in N_2C_{10}/Wild-Type Permease. Construction of single-Cys mutants F27C, Cys154, and Cys353 in C-less permease by site-directed mutagenesis has been described (*24*−*26*). Cys replacement for Phe27 in N_2C_{10}/WT permease (F27C/N_2C_{10}/WT) was made by ligating the *Eco*RV−*Spe*I fragment from plasmid encoding N_2C_{10}/WT into plasmid encoding F27C/C-less that was digested with the same restriction enzymes. Ser replacement at positions 148 or 355 in F27C/N_2C_{10}/WT was made in several steps by using plasmids encoding C154/C-less, C353/C-less, and wild-type permease. First, mutant F27C/C117/C154/N_2C_{10}/C-less was constucted by ligating the *Bam*HI−*Nae*I fragment from plasmid encoding F27C/N_2C_{10}/WT into similarly treated plasmid encoding C154/C-less. Second, mutant F27C/C148S/N_2C_{10}/WT was constructed by ligating the *Bam*HI−*Ban*II fragment from plasmid encoding F27C/C117/C154/N_2C_{10}/C-less into plasmid encoding wild-type permease. Finally, F27C/C148S/C355S/N_2C_{10}/WT was made by replacing the *Bam*HI−*Spe*I fragment plasmid encoding C353/C-less permease with the corresponding fragment from plasmid encoding F27C/C148S/N_2C_{10}/WT permease.

Construction of Double-Cys Mutants in N_2C_{10}/C-less Permease. Single-Cys mutants C148/C-less and C355/C-less by site-directed mutagenesis have been described (*25*, *26*). Cys replacement for Phe27 in N_2C_{10}/C-less permease (F27C/N_2C_{10}/C-less) was carried out by ligating the *Eco*RV−*Spe*I fragment of N_2C_{10}/C-less into similarly treated F27C/C-less plasmid. The double-Cys mutant F27C/C148/N_2C_{10}/C-less was constructed by replacing the *Eco*RI−*Pst*I fragment of C148/C-less with the corresponding fragment from F27C/N_2C_{10}/C-less plasmid. The mutant F27C/C355/N_2C_{10}/C-less was made by replacing the *Eco*RI−*Pst*I fragment of C355/C-less with the corresponding fragment from F27C/N_2C_{10}/C-less plasmid.

DNA Sequencing. Double-stranded DNA sequencing after alkaline denaturation (*27*) was performed by dideoxy chain-termination (*28*).

Transport Measurements. Cells were washed with 100 mM potassium phosphate (KP_i; 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 [^{14}C]lactose (2.5 mCi/mmol; final concentration 0.4 mM) was assayed by rapid filtration (*29*).

In Vivo Labeling with [^{35}S]Methionine. Cloned DNA was overexpressed with the T7 RNA polymerase system (*30*−

[1] Abbreviations: N_2C_{10}, lac permease expressed in two fragments with a discontinuity in the cytoplasmic loop between helices II and III; N_2, the N-terminal two helices; N_{10}, the C-termimal 10 helices; C-less permease, functional lactose permease devoid of Cys residues; DTT, dithiothreitol; IPTG, isopropyl 1-thio-β-D-galactopyranoside; KP_i, potassium phosphate; lac, lactose; NEM, *N*-ethylmaleimide; EDTA, ethylenediaminetetraacetate; *o*-PDM, *N*,*N*′-*o*-phenylenedimaleimide; *p*-PDM, *N*,*N*′-*p*-phenylenedimaleimide; BMH, 1,6-bis(maleimido)-hexane;/WT, the mutant is constructed in the wild-type background (i.e., all the native Cys residues in lac permease are retained unless otherwise indicated);/C-less, the mutant is constructed in the C-less background.

[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.

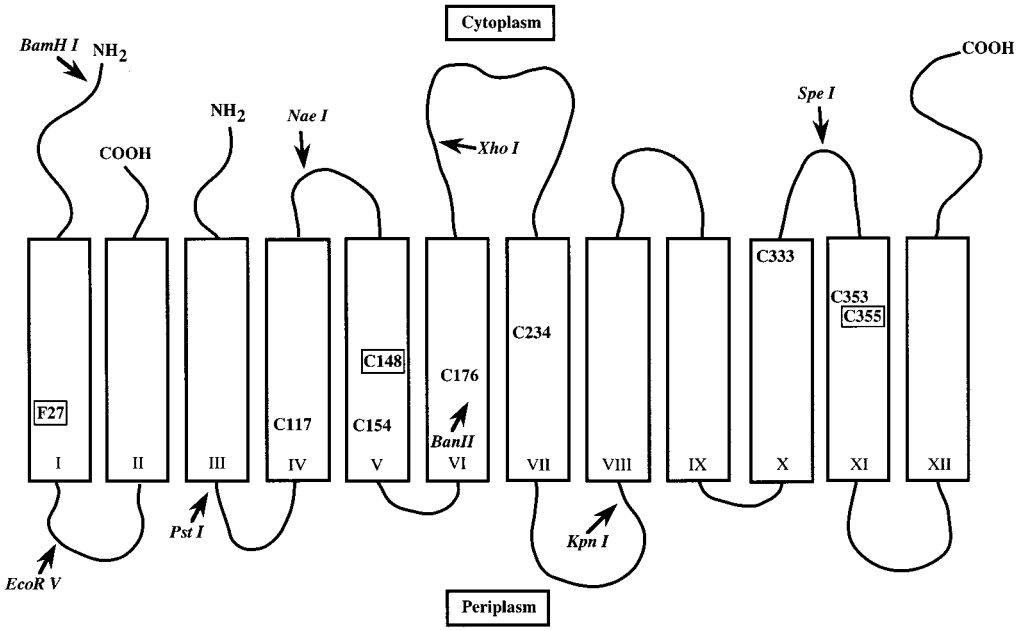

FIGURE 1: Secondary structure model of N_2C_{10} lac permease. Putative transmembrane helices are shown in boxes, and the eight native Cys residues and Phe27 are shown in boldface type. In addition, Phe27, Cys148, and Cys355 are further highlighted. Also indicated are the restriction endonuclease sites used for construction of the mutants (see text).

32). Briefly, plasmid N_2C_{10}/C-less was transformed into *E. coli* λDE3 lysogen (Novagen) bearing the T7 RNA polymerase gene and was grown at 37 °C in Luria−Bertani broth supplemented with streptomycin (10 μg/mL) and ampicilin (100 μg/mL). Overnight cultures were diluted 1:10 with fresh medium at 37 °C, and growth was continued for 3 h. The cells were washed twice in prewarmed M9 minimal medium containing 0.005% amino acids except methionine and supplemented with ampicilin (100 μg/mL). The cells were then resuspended in the minimal medium, and after growing under sulfur-starved conditions for 1 h at 37 °C, 0.2 mM isopropyl thio-β,D-galactopyranoside (IPTG) was added, and growth was continued for 12 min. Rifampicin was added to a final concentration of 0.2 mg/mL to inhibit the host cell RNA polymerase, and incubation at 37 °C was continued for an additional 40 min. Labeling was initiated by addition of [^{35}S]methionine (1000 Ci/mmol) to a final concentration of 2.5 nM. After 20 min of incubation, the cells were harvested and resuspended in 20 mM Tris·HCl (pH 7.4)/2 mM ethylenediaminetetraacetate (EDTA; potassium salt). Membranes were prepared by sonification as described (*33*) and suspended in 20 mM Tris·HCl (pH 7.4). Sodium dodecyl sulfate−polyacrylamide gel electrophoresis ($NaDodSO_4$/PAGE) and autoradiography were performed as described (*31*).

Expression of Split Permeases and Membrane Preparation. Plasmids encoding N_2C_{10} permease with given mutations were transformed into *E. coli* T184 (*lacY*$^-$Z$^-$). Cultures (25 mL) were grown at 37 °C to an OD_{600} of 1.0 and induced with 0.2 mM IPTG 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. Membranes were prepared by sonification as described (*33*) and suspended in 20 mM Tris·HCl (pH 7.4).

Chemical Cross-Linking. Chemical cross-linking was carried out at 4 °C for 20 min in the presence of a given thiol-specific homobifunctional linker at a final concentration of 0.5 mM with samples at a membrane protein concentraton of 2 mg/mL (*11*). Reactions were terminated by adding 5 mM dithiothreitol (DTT). Aliquots were mixed with Na-$DodSO_4$ sample buffer and subjected to $NaDodSO_4$/14% PAGE. Proteins were electroblotted onto poly(vinylidene difluoride) membranes (Immobilon-PVDF; Millipore) and probed with site-directed polyclonal antibody against the C-terminus of lac permease (*34*).

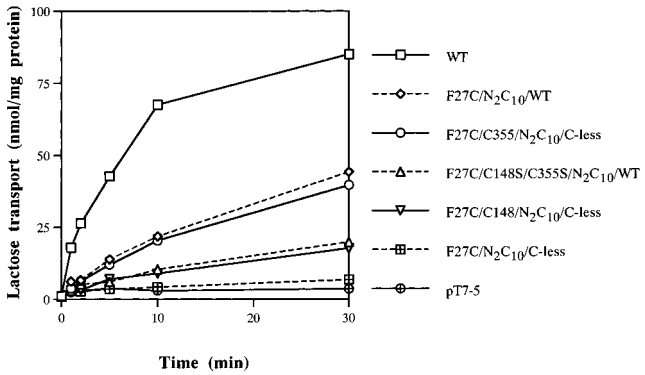

FIGURE 2: Lactose transport by N_2C_{10} permease mutants. *E. coli* T184 transformed with plasmid pT7-5/cassette *lacY* (wild type), pT7-5 (vector with no *lacY* gene), or pT7-5/cassette *lacY* encoding given N_2C_{10} permease mutants were grown at 37 °C, and aliquots of cell suspensions (50 mL containing approximately 35 mg of protein) in 100 mM KP_i (pH 7.5)/10 mM $MgSO_4$ were assayed as described in Experimental Procedures.

RESULTS

Active Transport. As reported by Wrubel et al. (*22*), *E. coli* T184 expressing F27C/N_2C_{10}/WT (Figure 2) or N_2C_{10}/WT permease (data not shown) catalyze lactose transport at about 25% the rate of wild type to a steady-state level of accumulation that is approximately 50% of wild type. However, cells expressing F27C/N_2C_{10}/C-less permease or N_2C_{10}/C-less (data not shown) are unable to accumulate lactose to a significant extent. Interestingly, by restoring the

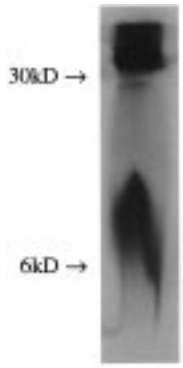

FIGURE 3: Visualization of N_2C_{10} lac permease fragments. Plasmid N_2C_{10}/C-less was transformed into *E. coli* λDE3 lysogen (from Novagen) bearing the T7 RNA polymerase gene, and the cells were grown and labeled with [^{35}S]methionine as described in Experimental Procedures. A membrane fraction was then isolated and subjected to NaDodSO$_4$/polyacrylamide gel electrophoresis, the gel was dried under vacuum, and radioautography was carried out for 24 h at −80 °C.

native Cys residue at position 355 in F27C/N_2C_{10}/C-less, which contains a Ser residue at this position (*23*), the activity of mutant F27C/C355/ N_2C_{10}/C-less becomes comparable to that of F27C/N_2C_{10}/WT permease. Thus, of the eight native Cys residues in lac permease, Cys355 appears to be responsible for the difference in the activity between F27C/N_2C_{10}/C-less and F27C/N_2C_{10}/WT. The observation is consistent with the finding (*26*) that single-Cys355 permease exhibits significantly higher activity than C-less permease. Mutants F27C/C148S/C355S/N_2C_{10}/WT and F27C/C148/N_2C_{10}/C-less transport lactose at about 10% of the wild-type rate and to a steady-state level of about 20% of wild type.

In Vivo [^{35}S]Methionine Labeling. Intact wild-type permease or C6 can be detected with anti-C-terminal antibody on immunoblots (*34*); however, the N-terminal fragment cannot. Therefore, N_2C_{10}/C-less permease was labeled with [^{35}S]methionine during expression in order to be certain that both N_6 and C_6 are expressed (Figure 3). As shown, radioactive bands corresponding to N_6 (M_r about 8000) and C_6 (M_r about 32 000) are visualized.

Cross-Linking. o-PDM and *p*-PDM are rigid homobifunctional reagents in which the maleimido groups are coupled to benzene rings in the *ortho* or *para* position at fixed distances of 6 or 10 Å, respectively, while BMH contains a flexible hexyl linker between the two maleimido groups, which are separated by 16 Å in the fully extended conformation. These reagents were chosen because they are hydrophobic and differ in length and flexibility. The initial purpose of constructing N_2/C_{10} permease was to study proximity between helices within N_6. However, since the N_2C_{10}/C-less permease is inactive, cross-linking was tested with F27C/N_2C_{10}/WT, which is active and contains all eight native Cys residues. Unexpectedly, although no cross-linking is observed in the presence of iodine (data not shown), *o*-PDM, *p*-PDM, and BMH effectively cross-link N_2C_{10} with increasing efficiency (Figure 4). The C_{10} fragment with a biotin acceptor domain that reacts with anti-C-terminal antibody migrates at an M_r of about 44 000, and the cross-linked N_2/C_{10} migrates at about 52 000.

To determine which of the eight native Cys residues cross-links with the Cys residue in place of Phe27, each of the eight native Cys residue was replaced individually with Val

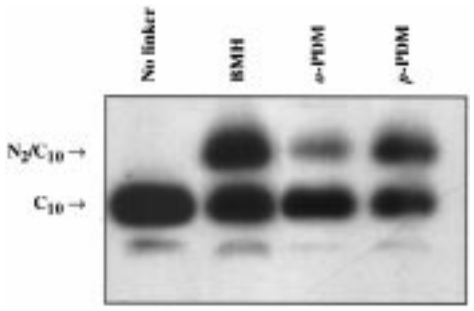

FIGURE 4: Cross-linking of F27C/N_2C_{10}/WT split permease. Membranes were prepared from *E. coli* T184 expressing F27C/N_2C_{10}/WT permease. Cross-linking was carried out by incubating membranes (2 mg of protein/mL) with 0.5 mM final concentration of a given cross-linker for 20 min at 4 °C. Cross-linking was terminated by adding DTT 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_{10} and the N_2/C_{10} cross-linked product are indicated by arrows.

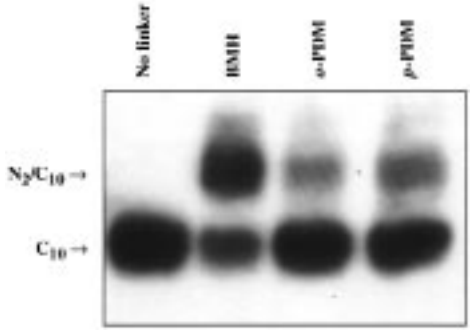

FIGURE 5: Cross-linking of F27C/C148/N_2C_{10}/C-less permease. Membranes were prepared from *E. coli* T184 expressing F27C/C148/N_2C_{10}/C-less permease. Cross-linking was carried out as indicated, and samples were analyzed as described in Figure 4.

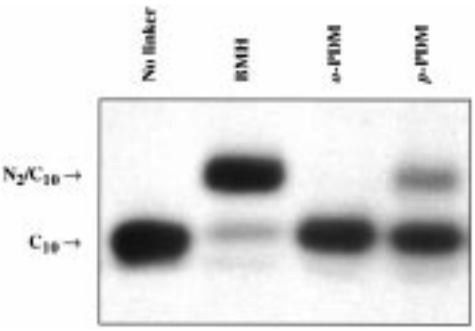

FIGURE 6: Cross-linking of F27C/C355/N_2C_{10}/C-less permease. Membranes were prepared from *E. coli* T184 expressing F27C/C355/N_2C_{10}/C-less permease. Cross-linking was carried out as indicated, and samples were analyzed as described in Figure 4.

(C154V) or Ser (each of the other native Cys residues) in F27C/N_2C_{10}/WT. Surprisingly, cross-linking was found to persist in all eight mutants (data not shown), indicating that the Cys residue at position 27 can cross-link to at least two of the eight native Cys residues.

In addition, double-Cys mutants were made in N_2C_{10}/C-less, with one Cys residue in place of Phe27 and another at each position where the eight native Cys residues are located. Significant cross-linking is observed in two of the mutants, F27C/C148/N_2C_{10}/C-less (Figure 5) and F27C/C355/N_2C_{10}/C-less (Figure 6). With mutant F27C/C148/N_2C_{10}/C-less, cross-linking is observed with all the three reagents tested,

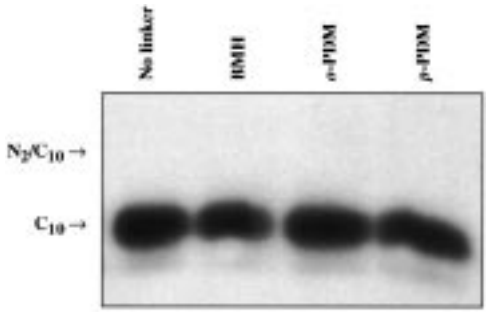

FIGURE 7: Cross-linking of F27C/C148S/C355S/N_2C_{10}/WT permease. Membranes were prepared from *E. coli* T184 expressing F27C/C148S/C355S/N_2C_{10}/WT permease. Cross-linking was carried out as indicated, and samples were analyzed as described in the legend to Figure 4.

o-PDM, *p*-PDM, and BMH. However, with mutant F27C/C355/N_2C_{10}/C-less, cross-linking is observed with *p*-PDM and BMH only. Furthermore, when both Cys148 and Cys355 are replaced with Ser and the other native Cys residues are retained, no cross-linking whatsoever is observed (Figure 7), demonstrating that Cys148 and Cys355 are the only two native Cys residues that cross-link to the Cys residue at position 27.

DISCUSSION

Based on the application of a battery of site-directed biochemical and biophysical techniques, a helix packing model of lac permease has been formulated (reviewed in refs *8*−*10*). Of the approaches utilized, *in situ* site-directed cross-linking of "split" permease molecules containing paired Cys residues has many important advantages (*11*), as it is carried out with native membranes on quantities of material that can be assayed by immunoblotting or avidin blotting. The approach is based on the observation that coexpression of lac permease in two contiguous, nonoverlapping fragments with a discontinuity in cytoplasmic or periplasmic loops leads to functional complementation (*15*, *16*, *22*, *35*). Furthermore, interthiol distances can be approximated by using iodine, copper phenanthroline, or chemical linkers of different length and rigidity (*12*). The approach has also been extended recently (*13*, *14*, *36*) to helix tilting by studying cross-linking of Cys residues placed on the faces of neighboring helices throughout the transmembrane domain.

Thus far, N_6C_6 permease only has been studied, which has the limitation that the helix proximity can be studied only between helices in the N- and C-terminal halves of the permease. Thus, functional split permease constructs with discontinuities in loops other than VI/VII would be particularly helpful. As demonstrated by Wrubel et al. (*22*) and confirmed here, N_2C_{10}/WT permease exhibits functional complementation. However, with N_2C_{10}/C-less permease, activity is nil. Therefore, an attempt was made initially to study proximity between helices I and IV in N_2C_{10}/WT, and mutant F27C/N_2C_{10}/WT was constructed. Unexpectedly, cross-linking is observed (Figure 4), and further study demonstrates that two of the eight native Cys residues in C_{10}, Cys148 and Cys355, cross-link to the Cys residue at position 27 (Figures 5 and 6).

Cross-linking between a Cys residue at position 27 and native Cys148 is observed in the presence of *o*-PDM (rigid 6 Å), *p*-PDM (rigid 10 Å), and BMH (flexible 16 Å) (Figure 4) but not iodine (data not shown), indicating the distance between the two thiol groups is between 6 and 10 Å. Thus, helix I appears to be relatively close to helix V (Figure 8). However, in the original secondary-structure model of the permease derived from hydropathy profiling (*37*), Phe27 is in the periplasmic third on helix I, while Cys148 is in the cytoplasmic third of helix V. In addition, Glu126 and Arg144, residues shown to be indispensable for substrate binding (*25*, *38*) were placed at the membrane−water interface at the cytoplasmic ends of helices IV and V. Recent studies utilizing single amino acid deletions (C. Wolin H.R.K., unpublished observations) and nitroxide-scanning and accessibility measurements (M. Zhao, J. Hernandez-Borrell, W. L. Hubbell, H.R.K., unpublished observations) indicate that loop IV/V is much smaller than indicated by hydropathy profiling, extending only from Val132 to Phe138 (Figure 1). Thus, the observation that a Cys residue at position 27 can be cross-linked to Cys148 by the 6 Å cross-linker *o*-PDM is consistent with the notion that the vertical positions 27 and 148 may be located at about the same level in the membrane and supports the proposal that Arg144 and its charge-paired partner Glu126 (*38*) are within the membrane rather than at the cytoplasmic ends of helices V and IV, respectively.

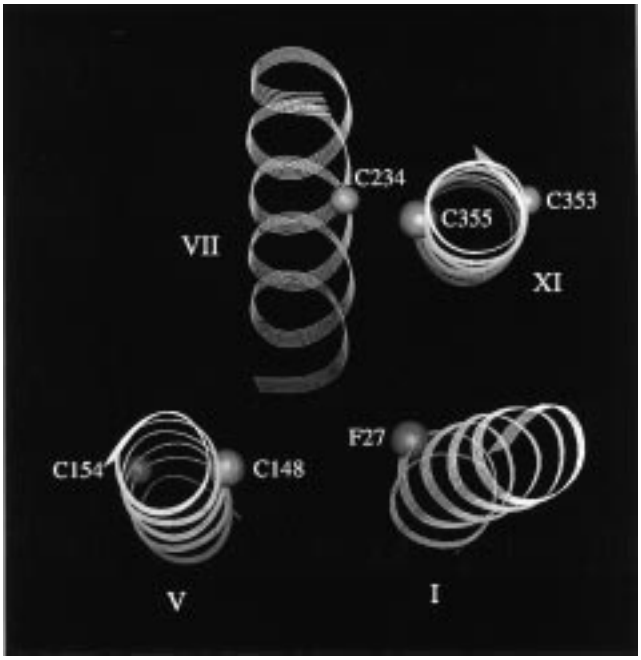

FIGURE 8: Packing of helices I, V, VII, and XI in the lac permease viewed from the cytoplasmic surface of the membrane. Positions found to cross-link (Phe27, Cys148, and Cys355) are depicted as enlarged balls. Three native Cys residues in this region of the permease that do not cross-link with a Cys residue at position 27 (Cys154, Cys234, and Cys353) are shown as smaller balls.

Similarly, cross-linking between a Cys residue at position 27 and native Cys355 provides the first evidence for proximity between helices I and XI (Figure 8). However, cross-linking of this pair is observed in the presence of *p*-PDM or BMH only, but not *o*-PDM or iodine (data not shown), suggesting that the distance between the two thiol groups is relatively long (i.e., a minimum of 10 Å). The result also suggests that the two positions are at about the same vertical level in the membrane, although the current secondary structure model (Figure 1) indicates that the two positions

are located at different depths in the membrane. On the other hand, *p*-PDM and BMH are 10 Å and up to 16 Å in length, respectively, and may cross-link two Cys residues at somewhat different depths in the membrane. Since Asp237 (helix VII) and Lys358 (helix XI) are ion-paired (*39*−*44*) and Asp237 and Asp240 approximate the middle of the membrane (*45*, *46*), the position of Lys358 and therefore Cys355 with respect to the membrane appear to be relatively well established. While further studies are in progress in an effort to address this question, the following points are noteworthy. Caution should be taken in interpreting results from cross-linking studies, since cross-linking may trap a conformation that is removed from the equilibrium structure of the protein. Possibly, there are both rotational and vertical motions of the helices in the permease, and cross-linking may trap relatively rare states resulting from structural fluctuations in the molecule. Since mutant F27C/C355/N_2C_{10}/C-less is active and cross-links to nearly completion with BMH (Figure 6), experiments were carried out to test the effect of the cross-linking on active transport. The mutant is inactivated completely by treatment with BMH (data not shown), thereby indicating that conformational flexibility between helices I and XI is important for active transport and that cross-linking traps the permease in an inactive form. Despite the caveats, it seems reasonable to conclude that position 27 is in relatively close proximity to both positions 148 and 355.

No cross-linking is observed between Phe27 → Cys and the other six native Cys residues in the permease. It is evident from the current helix packing model (reviewed in refs *8*−*10*) either that Cys117 (helix IV), Cys176 (helix VI), and Cys333 (helix X) are distant from Phe27 or that there is a steric barrier that blocks cross-linking. Helix VII tilts away from helices I, II, IV, and V toward the cytoplasmic surface of the membrane (*13*, *14*, *36*) in such a manner that a Cys residues at position 27 (helix I) cross-links to Cys355 but not to Cys234 (helix VII) (Figure 8). Cys154 is two turns below Cys148 in helix V and on the opposite face. Thus, it is not surprising that Cys residues at positions 27 and 154 do not cross-link. Although a Cys residue at position 27 does not cross-link to Cys353 in N_2C_{10} permease, cross-linking between the two positions is observed in N_6C_6 permease (unpublished data). Further experiments are in progress to document further the relationship between helices I and XI.

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