Ligand-Induced Changes in Periplasmic Loops in the Lactose Permease of *Escherichia coli*

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ABSTRACT: N- and C-terminal halves of lactose permease, each with a single-Cys residue in a periplasmic loop, were coexpressed, and cross-linking was studied in the presence or absence of ligand. A Cys residue at position 36 between transmembrane helices I and II (loop I/II) forms a disulfide spontaneously with a Cys residue at position 253, 254, 255, or 256 in loop VII/VIII. Moreover, in the presence of *o*-phenanthroline−copper, a Cys residue at position 42 in loop I/II forms a disulfide with a Cys residue at position 253, 254, 257, or 258 in loop VII/VIII. Changes in the rate of cross-linking are also observed in the presence of substrate, suggesting that ligand binding induces movement between loops I/II and VII/VIII such that positions 253−256 are brought closer to position 36 or 42, while positions 257 and 258 move away from position 42.

The lactose permease (lac permease)[1] of *Escherichia coli* is a polytopic membrane transport protein encoded by the *lacY* gene and a paradigm for proteins that transduce free energy stored in electrochemical ion gradients into solute concentration gradients (reviewed in *1*, *2*). The permease has been solubilized from the membrane, purified, reconstituted, and shown to be solely responsible for the coupled stoichiometric translocation of β-galactosides and H^+ (β-galactoside/H^+ symport; reviewed in *3*) as a monomer (see *4*). All available evidence indicates that the molecule is composed of 12 transmembrane helices connected by hydrophilic loops with the N- and C-termini on the cytoplasmic face (*5*−*8*). Cys-scanning and site-directed mutagenesis of each residue in the molecule indicates that only six side chains are irreplaceable with respect to the symport mechanism (see *9*), but the permease undergoes widespread conformational changes during turnover (*10*−*20*). By using an assortment of techniques which include second-site suppressor analysis and site-directed mutagenesis, excimer fluorescence, engineered divalent metal binding sites, chemical cleavage, electron paramagnetic resonance, thiol cross-linking, and identification of discontinuous monoclonal antibody epitopes, a helix packing model has been formulated (reviewed in *21*). Based on the proximity of the irreplaceable residues and the properties conferred by mutations at these positions, a mechanism for the coupling between lactose and H^+ translocation has been proposed (*22*).

One approach for studying helix packing in lac permease in the native membrane utilizes expression of functional permease in two fragments ("split permease"; *23*−*26*), each with a single-Cys residue in a specific transmembrane helix (*27*). By this means, the proximity of the paired Cys residues is readily assessed by cross-linking the two fragments with a concomitant decrease in electrophoretic mobility. Using this methodology, the helix packing model was extended to include helices I, II, and later VI (see *21*). The technique was then utilized to estimate distances between Cys side chains in helices VII and II and to probe ligand-induced conformational changes (*15*). Most recently (*28*), site-directed thiol cross-linking was used to study the proximity between periplasmic loops in the permease. Paired Cys replacements were introduced into periplasmic loops in the N- and C-terminal halves of intact lac permease containing a cleavable factor Xa protease site in the middle cytoplasmic loop (loop VI/VII). Proximity relationships were estimated by spontaneous or copper-(1,10-phenanthroline)$_3$ (CuPh)-catalyzed disulfide formation or cross-linking by homo- or heterobifunctional reagents, followed by digestion with factor Xa protease. The findings demonstrate primarily that loop I/II is close to loops VII/VIII and XI/XII, thereby placing helix XII in close proximity to helices II and XI.

In this study, split permease with a discontinuity in the middle cytoplasmic loop (N6/C6) approach is used to study ligand-induced changes between loop I/II and loop VII/VIII or loop XI/XII. The results confirm and extend previous conclusions and demonstrate further that β,D-galactopyranosyl 1-thio-β,D-galactopyranoside (TDG) induces changes in the rate of cross-linking, thereby supporting the conclusion that the permease is a highly flexible molecule which undergoes widespread conformational changes upon ligand binding.

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[1] Abbreviations: lac permease, lactose permease; C-less permease, functional lactose permease devoid of Cys residues; CuPh, copper-(1,10-phenanthroline)$_3$; BMH, 1,6-bismaleimidohexane; NEM, *N*-ethylmaleimide; DTT, dithiothreitol; TDG, β,D-galactopyranosyl 1-thio-β,D-galactopyranoside; NaPi, sodium phosphate.

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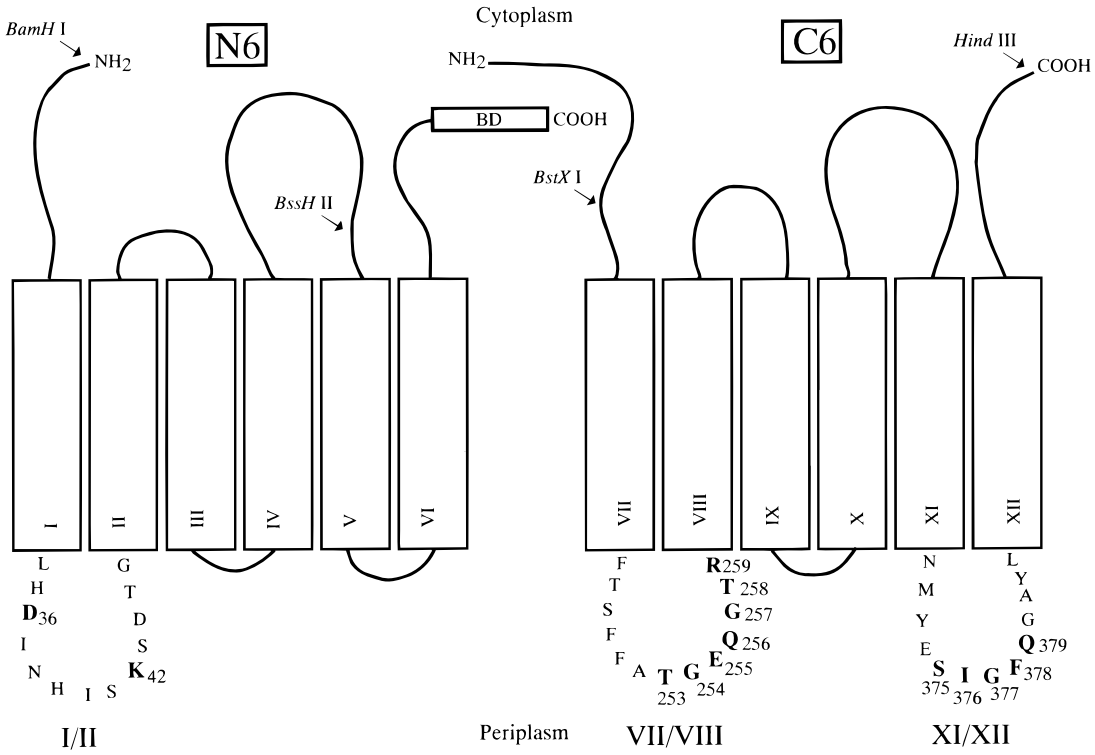

FIGURE 1: Secondary structure model of N6/C6 split permease. The lac permease is shown as the N-terminal six transmembrane helices (N6) and C-terminal six transmembrane helices (C6). Note that N6 has a biotin acceptor domain (BD) at the C-terminus. Single-Cys replacements in loop I/II (36 and 42) were constructed by *Bam*HI and *Bss*HII digestion, while mutants in loop VII/VIII (253−259) and loop XI/XII (375−379) were constructed by *Bst*XI and *Hin*dIII digestion. The positions of the mutations are emboldened and numbered.

EXPERIMENTAL PROCEDURES

Materials. All restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs, [1-^{14}C]-lactose was from Amersham, and bismaleimidohexane (BMH) was from Pierce. Rabbit polyclonal antiserum against the C-terminus of lac permease (*29*) was prepared by BAbCO. All other materials were reagent grade and obtained from commercial sources.

Construction of N6 and C6 Permease Fragments with Single-Cys Residues. Construction of plasmid pT7-5 encoding the N-terminal half of the permease followed by a biotin acceptor domain (pN6) and plasmid pACYC encoding the C-terminal half of the permease (pC6) has been described (*27*). A single-Cys replacement at position 36 or 42 was introduced into pN6 by restriction fragment replacement with *Bam*HI and *Bss*HII (Figure 1). Single-Cys replacements at positions 253−259 (loop VII/VIII) and 375−379 (loop XI/XII) were introduced into pC6 by restriction fragment replacement with *Bst*XI and *Hin*dIII. Each construct was confirmed by dideoxynucleotide sequencing (*30*) with synthetic primers after alkali denaturation (*31*).

Expression of Split Permease (N6/C6). *E. coli* T184 (*lacZ*$^-$*Y*$^-$) was transformed with both pN6 and pC6, each encoding N6 or C6 permease fragments, respectively, with a single-Cys residue at a given position. Cultures were grown aerobically at 37 °C in Luria−Bertani broth with ampicillin (100 μg/mL) and chloramphenicol (34 μg/mL). Overnight cultures were diluted 10-fold and grown for 2 h at 37 °C before induction with 0.5 mM isopropyl 1-thio-β,D-galactopyranoside. After additional growth for 2 h at 37 °C, cells were harvested by centrifugation.

Membrane Preparation. Crude membranes were prepared by sonification of spheroplasts prepared by lysozyme−ethylenediaminetetraacetic acid treatment (*16*, *32*) and suspended at ca. 2.5 mg of protein/mL in 25 mM sodium phosphate (NaP_i; pH 7.5).

Cross-Linking. Cross-linking was carried out by adding 0.5 mM CuPh or 1.0 mM BMH to 20 μL samples of membrane suspensions as described (*15*, *27*, *28*). CuPh-catalyzed disulfide formation was terminated by adding freshly prepared *N*-ethylmaleimide (NEM) to a final concentration of 5.0 mM, and chemical cross-linking was terminated by adding dithiothreitol (DTT) to 10 mM. The samples were then subjected to sodium dodecyl sulfate/12% polyacrylamide gel electrophoresis ($NaDodSO_4$−PAGE). The C6 fragment was detected by chemiluminescence, after immunoblotting with the anti-C-terminal antibody followed by incubation with horseradish peroxidase (HRP)−protein A (Renaissance, New England Nuclear). For quantitation by PhosphorImaging, the blots were incubated with ^{125}I-labeled protein A (*32*).

Transport Assays. Lactose transport was determined by rapid filtration of *E. coli* T184 transformed with given plasmids (*33*).

Protein Determinations. Protein was assayed with the Micro BCA method (Pierce) with bovine serum albumin as a standard.

RESULTS

Functional Complementation of N6 and C6 Fragments with Paired-Cys Replacements. Previous experiments (*23*, *26*) show that coexpression of wild-type *lacY* fragments encoding contiguous polypeptides corresponding to the first

Table 1: Lactose Accumulation by N6/C6 Permease with Paired Cys Replacements[a]

mutant (N6/C6)	lactose transport (% C-less split permease)	mutant (N6/C6)	lactose transport (% C-less split permease)
36/253	90	42/253	40
36/254	9 (40)[b]	42/254	59
36/255	76	42/255	73
36/256	44	42/256	50
36/257	39	42/257	47
36/258	16	42/258	16
36/259	86	42/259	96
36/375	19	42/375	52
36/376	66	42/376	53
36/377	12	42/377	23
36/378	32	42/378	47
36/379	69	42/379	55

[a] Steady-state levels of lactose transport in *E. coli* T184 transformed with plasmids encoding given split permease mutants are presented. Results are expressed as a percentage of lactose accumulation exhibited by cells expressing split Cys-less permease which accumulate 44 nmol of lactose/mg of protein. All data were corrected for the transport activity of T184 cells harboring plasmid pT7-5 with no *lacY* gene.[b] In the presence of 10 mM DTT, mutant 36/254 accumulates lactose about 40% as well as the control.

and second halves of the permease leads to functional complementation. Although the ability to accumulate lactose against a significant concentration gradient is retained by most of the constructs studied here, N6/C6 permease containing a few paired-Cys mutations (e.g., 36/258, 36/375, 36/377, and 42/258; Table 1) exhibits only weak activity. Interestingly, paired-Cys mutant 36/254 accumulates lactose only 9% as well as split Cys-less permease; however, in the presence of DTT, the steady-state level of accumulation increases to 40% of control.

Cross-Linking between Position 36 (Loop I/II) and Positions 253−259 (Loop VII/VIII). When membranes containing N6/C6 with paired Cys residues at positions 36 (loop I/II) and 253, 254, 255, or 256 (loop VII/VIII) are subjected to $NaDodSO_4$−PAGE and probed with anti-C-terminal antibody, bands migrating at a molecular mass of about 52 kDa corresponding to intact permease containing the biotin acceptor domain are observed (Figure 2). However, when the membranes are treated with 5 mM DTT prior to electrophoresis, the 52 kDa bands are no longer apparent, and all of the immunoreactive material migrates at a molecular mass of about 22 kDa which corresponds to the C-terminal fragment. Thus, the 52 kDa bands represent disulfide cross-linked N6/C6 in which the disulfide bonds form spontaneously under ambient oxygen tension. Furthermore, CuPh treatment does not increase cross-linking above the levels observed spontaneously (Figure 2). No spontaneous cross-linking is observed between paired Cys residues at positions 36 and 257, 258, or 259 (loop VII/VIII) or positions 375−379 in loop XI/XII (data not shown).

Disulfide cross-links between Cys residues at positions 36 and 253, 254, 255, or 256 are reduced with DTT and regenerated in the presence of CuPh (Figure 3). Time courses of CuPh-catalyzed cross-linking after reduction with DTT demonstrate that disulfide bond formation is rapid and essentially complete in 5−10 min with each pair, although cross-linking efficiency varies markedly (i.e., 36/254 ≫ 36/255 > 36/253 > 36/256). In all probability, the differences in cross-linking efficiency (i.e., the maximum level of cross-linking) are related to differences in the rate of oxidation of the cysteinyl side chains, a reaction that competes with disulfide formation (*34*).

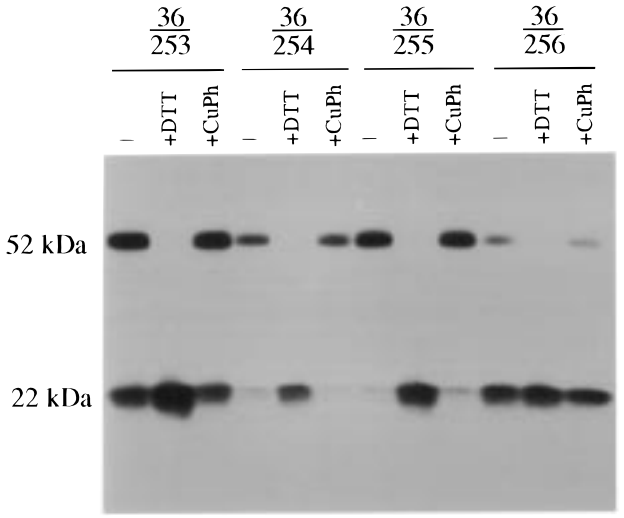

FIGURE 2: Spontaneous disulfide cross-linking of N6/C6 permease fragments containing paired Cys residues at positions 36 and 253−256. Membranes were prepared from *E. coli* T184 expressing N6 and C6, each with a single Cys residue at a given position. The samples were either subjected to SDS−PAGE directly, incubated with 5 mM DTT, or treated with 0.5 mM CuPh at 22 °C for 30 min followed by addition of NEM to a final concentration of 5 mM. Immunoblots were carried out with the anti-C-terminal antibody as described under Experimental Procedures. Note that the C6 migrates at 22 kDa, while the cross-linked N6 and C6 migrate at 52 kDa.

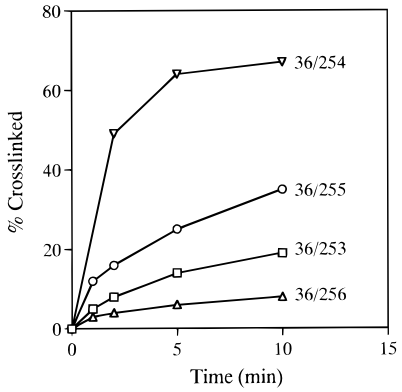

FIGURE 3: Time course of CuPh-catalyzed cross-linking between Cys residues at positions 36 and 253−256. Membranes containing split permease with given paired-Cys replacements were incubated with 5 mM DTT at 22 °C for 30 min, harvested by centrifugation, and resuspended in 20 μL of 50 mM NaP_i (pH7.5)/2 mM EDTA. Cross-linking was carried out with 0.5 mM CuPh at 22 °C for the given times, and the reactions were terminated by adding NEM to a final concentration of 5 mM. Disulfide cross-linking was quantitated from immunoblots using ^{125}I-labeled protein A by using a Model 425F phosphoImager (Molecular Dynamics). The percentage of cross-linking was calculated by dividing the intensity at 52 kDa by the sum of the intensities at 22 and 52 kDa. (□) 36/253; (▽) 36/254; (○) 36/255; (△) 36/256.

Cross-Linking between Position 42 (Loop I/II) and Positions 253−259 (Loop VII/VIII). N6 with a Cys residue at position 42 (loop I/II) was coexpressed with the C6 fragments containing a Cys residue at positions 253−259 (loop VII/VIII) or 375−379 (loop XI/XII). Although the paired Cys residues exhibit little or no immunoreactive material at 52 kDa spontaneously, CuPh catalyzes significant cross-linking

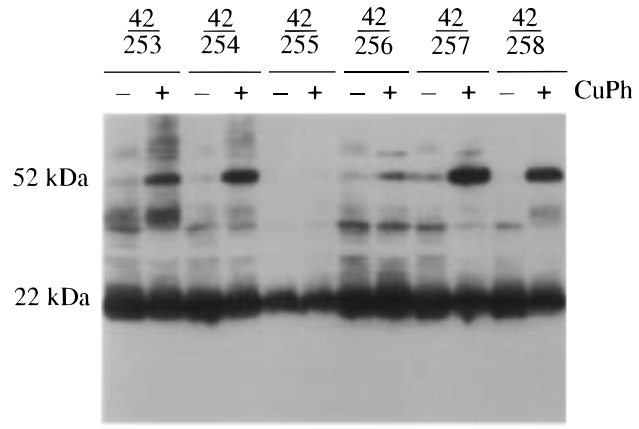

FIGURE 4: CuPh-catalyzed disulfide cross-linking of N6 and C6 permease fragments containing paired Cys residues at positions 42 and 253−258. Membranes were prepared from *E. coli* T184 expressing N6 and C6, each with a single Cys residue at a given position as indicated. Disulfide cross-linking was carried out at 22 °C by incubating membranes with 0.5 mM CuPh for 30 min. Reactions were terminated by adding NEM to a final concentration of 5 mM. Immunoblots were carried out as described under Experimental Procedures.

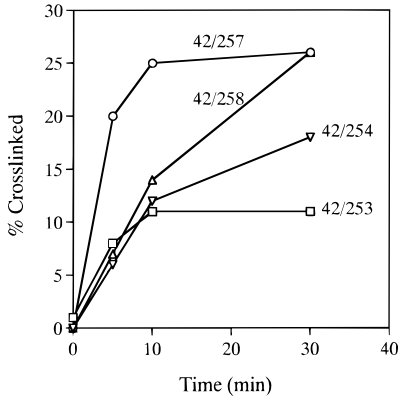

FIGURE 5: Time course of CuPh-catalyzed disulfide cross-linking between Cys residues at positions 42 and 253, 254, 257, or 258. Membranes were prepared from *E. coli* T184 expressing N6 and C6, each with a single Cys residue at a given position as indicated. Disulfide cross-linking was carried out by incubating membranes with 0.5 mM CuPh at 22 °C. Reactions were terminated by adding NEM to a final concentration of 5 mM. Immunoblots were performed as described under Experimental Procedures using ^{125}I-labeled protein A. Disulfide cross-linking was quantitated with a model 425F phosphoImager (Molecular Dynamics). The percentage of cross-linking was calculated by dividing the intensity of the signal at 52 kDa by the sum of the intensities at 22 and 52 kDa. (□) 42/253; (▽) 42/254; (○) 42/257; (△) 42/258.

between Cys residues at positions 42 and 253, 254, 257, or 258 with varying degrees of efficiency (ca. 10%, 20%, 30%, and 30%, respectively; Figure 4). In contrast, little or no cross-linking is observed with the 42/255 or 42/256 pair nor with the 42/259 pair. In addition, no significant cross-linking is observed with Cys residues at position 42 and positions 375−379 in loop XI/XII (data not shown).

Time courses of CuPh-catalyzed cross-linking exhibit different rates and maxima (Figure 5). For the 42/257 and 42/253 pairs, cross-linking is relatively rapid, reaching maxima of about 25% and 12%, respectively, within 10 min. For the 42/258 or 42/254 pair, the rate of cross-linking is slower, and increases at essentially a linear rate for 30 min, implying that the pairs may be further apart, thereby requiring greater backbone motion for cross-linking. In order to examine this possibility, cross-linking was performed at 4 °C. Although each of the pairs cross-links more slowly, pairs 42/254 and 42/258 exhibit a greater differential effect, exhibiting only about 2.0% and 1.5% cross-linked product, respectively, in 1 h (data not shown). Thus, peptide backbone motion appears to be relatively more important with respect to cross-linking the 42/258 and 42/254 pairs.

BMH-Mediated Cross-Linking. In addition to spontaneous and CuPh-catalyzed disulfide bond formation, BMH, a homobifunctional thiol cross-linking reagent with a flexible hexyl chain connecting two maleimido groups (ca. 16 Å fully extended), was also used (Figure 6). As observed with CuPh, BMH cross-links Cys residues at positions 42 and 253, 254, 257, or 258. Within 30 min, cross-linking between positions 42 and 254 is almost 100%, while BMH cross-linking between Cys residues in pairs 42/253, 42/257, or 42/258 occurs with about 10%, 70%, or 70% efficiency, respectively. In addition, BMH cross-links Cys residues at positions 42 and 378 or 379 with relatively high efficiency (ca. 50% within 30 min), but no cross-linking between Cys residues at position 42 and position 375 or 376 is observed. In contrast, BMH cross-links Cys residues at position 36 and position 257, 258, or 259 at about 50% efficiency in 30 min and cross-links Cys residues at positions 36 and 378 or 379 at 70% or 50% efficiency, respectively, but does not cross-link Cys residues at positions 36 and 375 or 376 (data not shown).

Ligand-Induced Changes in Cross-Linking. In order to study the influence of ligand binding, CuPh-catalyzed cross-linking was compared in the absence or presence of saturating concentrations of TDG. As shown (Figure 3), paired Cys residues at positions 36 and 253−256 cross-link spontaneously in a manner that is reversed by DTT and reestablished by treatment with CuPh. The initial rate of cross-linking of the 36/253, 36/255, and 36/256 pairs is significantly enhanced by TDG (Figure 7). In contrast, the rate of cross-linking of the 36/254 Cys pair is very rapid, and no TDG effect is detected at room temperature (data not shown). For this reason, cross-linking was performed at 4 °C with this pair in order to slow the reaction. However, even at low temperature, no TDG effect is observed.

TDG also alters the rate of cross-linking between paired Cys residues at positions 42 and 253, 254, 257, or 258 (Figure 8). The homolog increases CuPh-catalyzed cross-linking with the 42/253 and 42/254 pairs by about 2-fold. In contrast, ligand markedly decreases the rate of cross-linking with the 42/258 pair, and decreases the rate of cross-linking with the 42/257 pair as well, but to a much less significant extent. Thus, in the presence of TDG, positions 253 and 254 may move closer to position 42, while positions 257 and 258 may move further away.

DISCUSSION

In the experiments reported here, site-directed cross-linking between paired-Cys residues in periplasmic loops in the N- and C-terminal halves of lac permease (*28*) is further documented in the absence and presence of ligand by using split permease constructs (*15*, *27*) rather than an engineered factor Xa protease site in the middle cytoplasmic loop (*28*). Although relatively subtle, it is apparent that there are

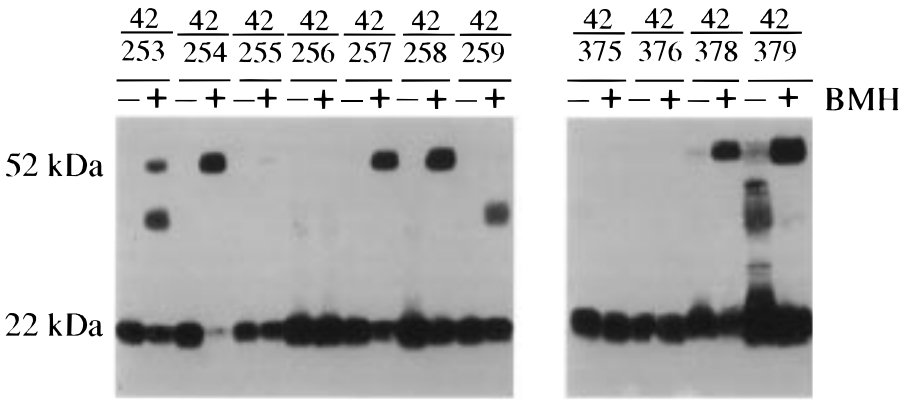

FIGURE 6: BMH-mediated cross-linking of N6/C6 permease. Conditions for cross-linking and immunoblotting are described under Experimental Procedures. The band migrating at 22 kDa represents the C-terminal half-fragment; the band at 52 kDa, cross-linked N6/C6 permease. The band observed at about 44 kDa for the 42/253 and 42/259 pairs probably represents cross-linked C-terminal fragments.

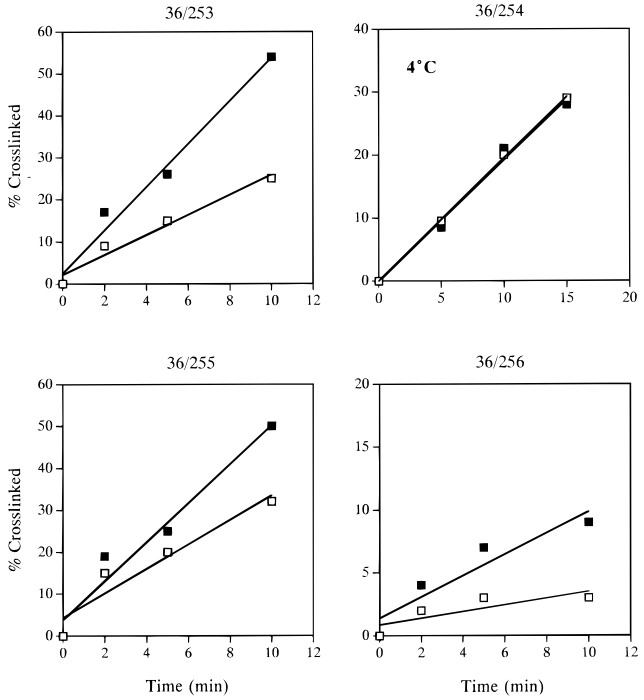

FIGURE 7: Effect of TDG on CuPh-catalyzed cross-linking of Cys residues at positions 36 and 253−256. Membranes were incubated with 5 mM DTT at 22 °C for 30 min, harvested by centrifugation, and resuspended in 20 μL of 50 mM NaP_i (pH 7.5)/2 mM EDTA. TDG was added to a final concentration of 10 mM prior to the incubation with 0.5 mM CuPh at 22 °C, except that with the 36/254 mutant incubation was at 4 °C. Reactions were quenched at given times with 5 mM NEM, and immunoblotting was carried out as described under Experimental Procedures using ^{125}I-labeled protein A. The intensity of the radioactive bands was quantitated with a Model 425F phosphoImager (Molecular Dynamics). Percentage cross-linking was calculated by dividing the intensity of the signal at 52 kDa by the sum of the intensities at 22 and 52 kDa. (□) No addition; (■) in the presence of 10 mM TDG.

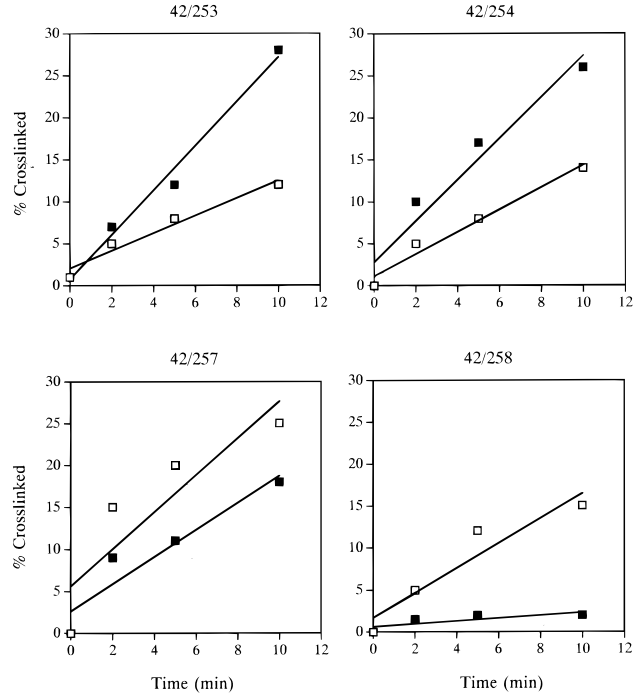

FIGURE 8: Effect of TDG on CuPh-catalyzed cross-linking of Cys residues at positions 42 and 253, 254, 257, or 258. The experiments were carried out as described in Figure 7 without DTT pretreatment, and the immunoblots were quantitated with a Model 425F phosphoImager (Molecular Dynamics). Percentage cross-linking was calculated by dividing the intensity of the signal at 52 kDa by the sum of the intensities at 22 and 52 kDa. (□) No addition; (■) in the presence of 10 mM TDG.

differences between intact permease with a factor Xa protease site (*28*) and N6/C6 permease. Thus, spontaneous cross-linking is observed between Cys residues at positions 36 and 379 in intact permease, but not in N6/C6; however, the pairs are cross-linked with BMH in N6/C6. Alternatively, spontaneous cross-linking between Cys residues at position 36 and positions 253−256 is observed in N6/C6, while Cys residues at positions 36 and 254 cross-link only in the presence of cross-linking agents that are 10 Å or greater with intact permease. In addition, although Cys pairs 42/254 exhibit CuPh-catalyzed cross-linking in either N6/C6 or intact permease, the 42/258 pair cross-links in the presence of CuPh in N6/C6, but not with intact permease, while the 36/258 pair exhibits the opposite behavior. Finally, all pairs that exhibit cross-linking with BMH in intact permease do so with N6/C6 as well. In summary, therefore, it appears that loops I/II and XI/XII may be further apart in N6/C6 relative to intact permease, although activity is altered to a relatively minor extent. That is, although N6/C6 exhibits lower activity than intact permease, the split construct catalyzes highly significant accumulation.

A Cys residue at position 36 spontaneously forms disulfide bonds with Cys residues at positions 253 through 256, indicating that they are probably in close proximity. Cys pairs at positions 36/253 and 36/256 cross-link with 60%

and 10% efficiency, respectively, in a manner that is not increased by CuPh. As discussed (*34*), Cys residues may be oxidized by trace metal ions or endogenous oxidants, thereby preventing disulfide formation. Moreover, Cys residues at positions 36 and 257−259 are also cross-linked by BMH. It has been proposed that there is a turn at Gly254 (*32*, *35*), and it is possible that the side chains at positions 253−256 are oriented in the same direction facing position 36.

A Cys residue at position 42 also cross-links with Cys residues at positions 253, 254, 257, or 258 in the presence of CuPh with varying efficiency, indicating that these positions are in close proximity to position 42, as well as position 36. Time courses of CuPh-catalyzed cross-linking demonstrate that Cys at position 42 cross-links with Cys residues at positions 253 and 257 at a relatively rapid rate, implying that they are in closer proximity than position 42 and positions 254 and 258. Cross-linking at 4 °C demonstrates that the 42/254 and 42/258 pairs cross-link at a very slow rate, indicating that a relatively greater degree of backbone motion may be needed for these two pairs to interact. BMH also cross-links paired Cys residues at 42/253, 42/254, 42/257, and 42/258, while cross-linking of the 42/255, 42/256, and 42/259 Cys pairs is not detected, presumably because of structural hindrance.

Regarding loops I/II and XI/XII, BMH cross-links Cys residues at either position 36 or position 42 with a Cys residue at position 378 or 379, indicating that the two loops are in close proximity. Permease with Cys at position 377 is poorly expressed which precludes cross-linking studies. However, a Cys residue at position 375 or 376 does not exhibit cross-linking with a Cys residue at either position 36 or position 42 even in the presence of BMH. Thus, positions 375 and 376 are either relatively far from loop I/II, or there is a structural constraint that prevents cross-linking.

It has been demonstrated by site-directed fluorescence and labeling experiments with radioactive NEM that ligand binding results in widespread conformational changes. Cross-linking of Cys residues at position 52 or 53 (helices II) with position 245 (helix VII) indicates that ligand binding induces a scissors-like movement which increases the distance between the pairs by 3−4 Å (*15*). Therefore, it was suggested that rigid body movements of the helices initiated at the interface between helices V and VIII are transmitted cooperatively throughout the molecule in order for turnover to occur. CuPh-catalyzed cross-linking of paired Cys residues at positions 42 and 253, 254, 257, or 258 is altered in the presence of TDG. Ligand binding increases cross-linking of Cys pairs 42/253 and 42/254, but decreases cross-linking of the 42/257 and 42/258 pairs. Although it is difficult to envisage the structural change precisely, it seems reasonable to suggest that ligand binding brings positions 42 and 253 or 254 closer and moves positions 42 and 257 or 258 further apart by some type of torsional movement in loop I/II and/or loop VII/VIII. On the other hand, TDG binding appears to cause position 36 to come into closer proximity to positions 253, 255, and 256 (Figure 9).

The conclusions in this study are based on the premise that Cys cross-links are a measure of proximity. However, it should be emphasized that cross-link formation detects dynamic collisions and chemical reactions between residues, not simply their proximities. For example, Cys pairs that frequently undergo collisions and are highly chemically reactive could form cross-links at a relatively rapid rate, even though they may be distant in the average structure. On the other hand, a strong correlation is expected between collision rates and proximity (*36*). Therefore, it is likely that proximities between loops are estimated by spontaneous disulfide formation between engineered cysteinyl side chains, CuPh-catalyzed disulfide formation, or cross-linking by BMH. Further support for this contention will be presented subsequently (J. Sun, J. Voss, W. L. Hubbell, and H. R. Kaback, manuscript in preparation) by documenting spin−spin interactions between certain Cys pairs, as well as ligand-induced changes in the interactions.

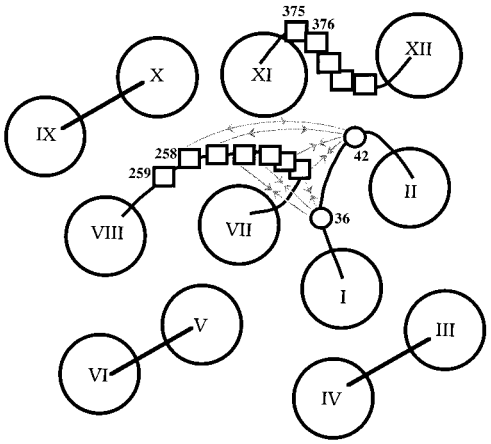

FIGURE 9: Schematic representation of the spatial arrangement of periplasmic loops in lac permease (*28*). Transmembrane helices are depicted as large circles with Roman numerals from I to XII. Positions of the Cys mutants in the N- and C-terminal halves of N6/C6 permease are indicated as circles and rectangles, respectively. The TDG effect on cross-linking of the pairs is represented by arrowheads: < > indicates that the residues move further apart; > < indicates that the residues move closer together.

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