Thiol Cross-Linking of Cytoplasmic Loops in the Lactose Permease of *Escherichia coli*†

Isidore Kwaw,‡ Jianzhong Sun,§ and H. Ronald Kaback*

Howard Hughes Medical Institute, Departments of Physiology and of Microbiology & Molecular Genetics, Molecular Biology Institute, University of California, Los Angeles, California 90095-1662

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ABSTRACT: The N- and C-terminal halves of lactose permease, each with a single-Cys residue in a cytoplasmic loop, were coexpressed, and cross-linking was studied in the absence or presence of ligand. Out of the 68 paired-Cys mutants in cytoplasmic loops IV/V and VIII/IX or X/XI, three pairs in loop IV/V and X/XI, (i) Arg135 → Cys/Thr338 → Cys, (ii) Arg134 → Cys/Val343 → Cys, and (iii) Arg134 → Cys/Phe345 → Cys, form a spontaneous disulfide bond, indicating that loops IV/V and X/XI are in close proximity. In addition, specific paired-Cys residues in loop IV/V (132−138) and loop VIII/IX (282−290) or loop X/XI (335−345) cross-link with iodine and/or the homobifunctional cross-linking agents *N,N′-o*-phenylenedimaleimide, *N,N′-p*-phenylenedimaleimide, and 1,6-bis(maleimido)hexane. The results demonstrate that loop IV/V is close to both loop VIII/IX and loop X/XI. On the other hand, similar though less extensive cross-linking studies indicate that neither the N terminus nor loop II/III appear to be close to loops VIII/IX or X/XI. The findings suggest that the longer cytoplasmic loops are highly flexible and interact in a largely random fashion. However, although a Cys residue at position 134 in loop IV/V, for example, is able to cross-link with a Cys residue at each position in loop VIII/IX or loop X/XI, Cys residues at other positions in loop IV/V exhibit markedly different cross-linking patterns. Therefore, although the domains appear to be very flexible, the interactions are not completely random, suggesting that there are probably at least some structural constraints that limit the degree of flexibility. In addition, evidence is presented suggesting that ligand binding induces conformational alterations between loop IV/V and loop VIII/IX or X/XI.

The lactose permease (lac permease)[1] of *Escherichia coli*, the product of the *lacY* gene, is a paradigm for secondary active transport proteins that transduce free energy stored in electrochemical ion gradients into solute concentration gradients (*1*−*5*). This hydrophobic polytopic membrane protein catalyzes the coupled stoichiometric translocation of galactosides and H^+ (i.e., symport or cotransport) and has been solubilized, purified into 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 experimental evidence (reviewed in ref *8*) indicates that the permease is composed of 12 transmembrane helices connected by hydrophilic loops with the N and C termini on the cytoplasmic face (Figure 1).

Although lac permease forms 2D crystalline arrays (*9*), the protein has been highly resistant to crystallization in a form that diffracts to high resolution. Therefore, a battery of site-directed biochemical and biophysical approaches has been developed in an effort to gain insight into the structure and function of the permease by alternative means. In a functional mutant devoid of native Cys residues, each residue has been replaced with Cys (reviewed in ref *10*). Analysis of the mutant library by using a variety of site-directed biochemical and biophysical techniques has led to the following developments (see refs *10*−*13*): (1) The great majority of the mutants are expressed normally in the membrane and exhibit significant activity, and only 6 side chains are clearly irreplaceable for active transport: 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 critical for H^+ translocation and coupling with substrate translocation. (2) Helix packing, tilts, and ligand-induced conformational changes have been determined. (3) Positions that are accessible to solvent have been revealed. (4) Positions where the reactivity of the Cys replacement is increased or decreased by ligand binding have been identified. (5) The permease has been shown to be a highly flexible molecule. (6) A working model describing a mechanism for lactose/H^+ symport has been formulated.

A particularly powerful approach for estimating helix packing, tilts, and ligand-induced conformational changes

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* Corresponding author: Howard Hughes Medical Institute, UCLA, 5-748 MacDonald Research Laboratories, P.O. Box 951662, Los Angeles, CA 90095-1662. Phone (310) 206-5053; Fax (310) 206-8623; E-mail RonaldK@HHMI.UCLA.edu.

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§ Present address: Structural Bioinformatics Inc., Technology Place, San Diego, CA 92127.

[1] Abbreviations: lac permease, lactose permease; C-less permease, functional lactose permease devoid of Cys residues; TDG, *β*-D-galactopyranosyl 1-thio-*β*-D-galactopyranoside; DDM, *n*-dodecyl *β*-D-maltopyranoside; NEM, *N*-ethylmaleimide; *o*-PDM, *N,N′-o*-phenylenedimaleimide; *p*-PDM, *N,N′-p*-phenylenedimaleimide; BMH, 1,6-bis(maleimido)hexane; DTT, dithiothreitol; $NaDodSO_4$/PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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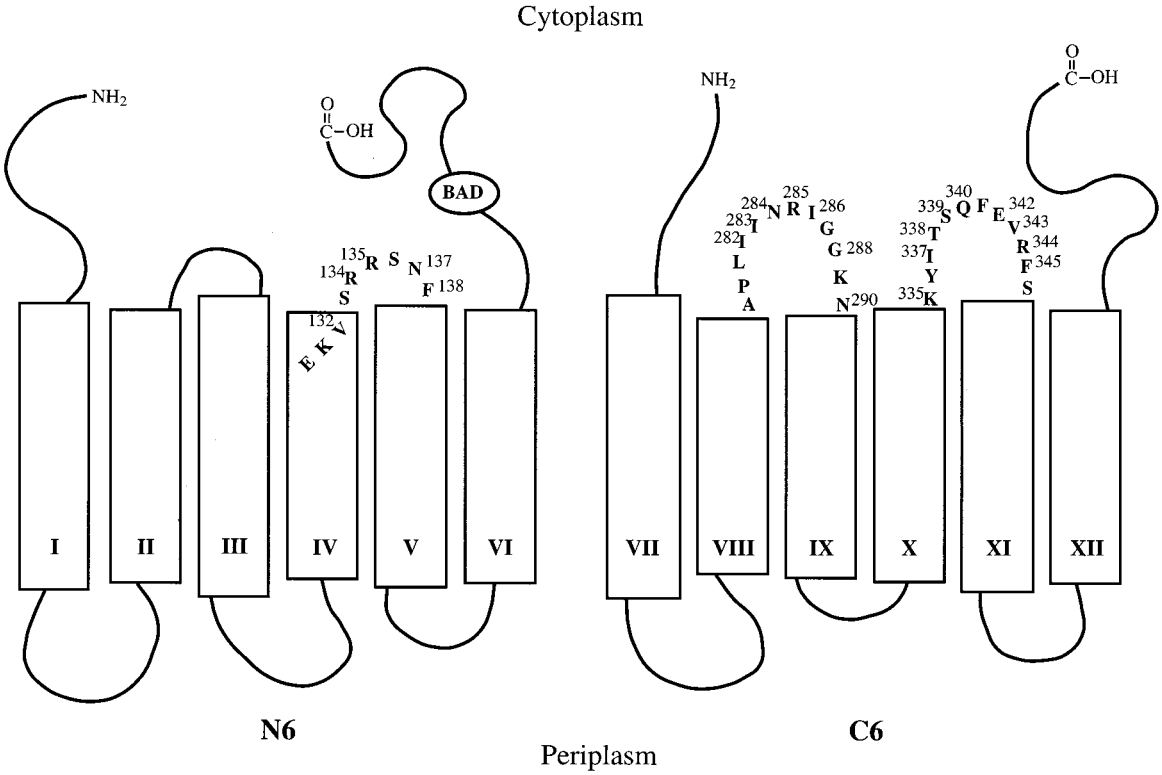

FIGURE 1: Secondary structure model of the N_6/C_6 split permease. N_6 has a factor Xa site and a biotin acceptor domain (BAD) at the C-terminus. Single-Cys replacements used in this study are shown as numbers. The 12 hydrophobic transmembrane helices are depicted as rectangles.

that is carried out in situ (reviewed in ref *13*) utilizes expression of functional lac permease in two contiguous, nonoverlapping fragments (split permease), each with a single Cys residue. Proximity of the paired-Cys residues is then assayed on Western blots by disulfide or chemical cross-linking of the fragments. Alternatively, lac permease with an engineered factor Xa protease site can be used for the same purpose (*14*−*17*).

In this communication, split permease (N6/C6) is used to determine that cytoplasmic loop IV/V is close to both loops VIII/IX and X/XI. In addition, evidence is presented that is consistent with the interpretation that ligand binding induces structural changes in these loops.

EXPERIMENTAL PROCEEDURES

Materials. All the restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA), 1,6-bis(maleimido)hexane (BMH) was from Pierce (Rockford, IL), and *N*,*N*′-*o*-phenylenedimaleimide (*o*-PDM) and *N*,*N*′-*p*-phenylenedimaleimide (*p*-PDM) were from Sigma (St. Louis, MO.). Rabbit polyclonal antiserum against the C-terminus of the permease (*18*) was prepared by Babco (Richmond, CA). *N*-[*ethyl*-1-^{14}C]ethylmaleimide was purchased from New England Nuclear (Boston, MA.). [^{125}I]-Streptavidin was from Amersham (Arlington Heights, IL), and immobilized monomeric avidin was from Pierce (Rockford, IL). All other materials were reagent grade and were obtained from commercial sources.

Construction of pN_6 Encoding the N-Terminal Six Transmembrane Helices (N_6) with Single-Cys Replacements. Construction of permease mutants containing single-Cys replacements at positions 8 (N terminus), 70, 71, or 72 (loop II/III) and 132, 134, 135, 137, or 138 (loop IV/V) in Cys-less permease has been described (*19*, *20*). With each single-Cys mutant, the biotin acceptor domain from a *Klebsiella pneumoniae* oxaloacetate decarboxylase (*21*) was inserted into the middle cytoplasmic loop as described (*22*, *23*). The 3′ half of the *lacY* gene in each construct was then removed by *Afl*III digestion followed by intramolecular ligation yielding 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 of the fragment (Figure 1).

Construction of pC_6 Encoding the C-Terminal Six Transmembrane Helices (C_6) with Single-Cys Replacements. Construction of plasmid pC_6 encoding C_6 has been described (*24*). To place single Cys residues at positions 282−290 (loop VIII/IX) or positions 335−345 (loop X/XI), the *Bst*XI−*Hin*dIII fragment of pC_6 was replaced with the corresponding DNA fragment from a given single-Cys mutant. All mutants were confirmed by sequencing the length of the PCR-generated or replacement segment through the ligation junctions by using dideoxynucleotide termination chain (*25*).

Expression of N_6/C_6 Permease and Membrane Preparation. E. coli T184 (*lacY*$^-$*Z*$^-$) was cotransformed with both pN_6 (conferring ampicillin resistance) and pC_6 (conferring chloramphenicol resistance), encoding N_6 and C_6, respectively, each with a given Cys residue. Cultures were grown aerobically overnight at 37 °C in Luria−Bertani broth containing ampicillin (100 μg/mL) and chloramphenicol (42 μg/mL) diluted 1:10 into fresh medium and grown for 2 h to an OD_{600} of 1.0. Isopropyl 1-thio-β-D-galactopyranoside was added to 1.0 mM final concentration, and after 2 h of induction, cells were harvested by centrifugation. Crude membranes were prepared by sonification of spheroplasts

prepared with lysozyme−ethylenediaminetetraacetate (*26*), resuspended in 100 mM potassium phosphate (pH 7.5) to about 2.0 mg of protein/mL, frozen in liquid nitrogen, and stored at −80 °C until use.

Cross-Linking. Aliquots (20 μL) of membranes from a given mutant were incubated at 25 °C for 10 min without or with β-D-galactopyranosyl 1-thio-β-D-galactopyranoside (TDG), as indicated. The samples were then incubated at 4 °C for 10 min prior to addition of a cross-linking agent as follows: (i) Iodine-catalyzed disulfide formation was carried out by adding ethanolic iodine to a final concentration of 2.5 μM, and incubations were continued for 10 min. Reactions were quenched by addition of *N*-ethylmaleimide (NEM; 9.0 mM final concentration). (ii) Chemical cross-linking with *N*,*N*′-*o*-phenylenedimaleimide (*o*-PDM; rigid, 6 Å), *N*,*N*′-*p*-phenylenedimaleimide (*p*-PDM, rigid, 10 Å), or 1,6-bis(maleimido)hexane (BMH; flexible, 16 Å) was carried out in similar fashion in the absence or presence of 10 mM TDG except that a given cross-linking agent was added to a final concentration of 1.25 mM, the samples were incubated at 4 °C for 30 min, and reactions were quenched with dithiothreitol (DTT) at a final concentration of 9.0 mM. All samples were then subjected to sodium dodecyl sulfate/12% polyacrylamide gel electrophoresis ($NaDodSO_4$/PAGE) as described (*27*). C_6 was detected by immunoblotting with rabbit anti-C-terminal antibody (*28*). Where indicated, N_6 was detected by avidin blotting.

NEM Labeling. Aliquots (50 μL) of membranes containing a given mutant were incubated at 25 °C for 10 min without or with 10 mM β-D-galactopyranosyl 1-thio-β-D-galactopyranoside (TDG), as indicated. [1-^{14}C]NEM (40 mCi/mmol; 0.4 mM final concentration) was added, and the samples were incubated at 25 °C for 10 min, at which time the reactions were quenched by adding DTT to a final concentration of 10 mM. The samples were solubilized in *n*-dodecyl β-D-maltopyranoside (DDM), and the labeled permease was purified by avidin affinity chromatography and subjected to $NaDodSO_4$/PAGE and autoradiography as described (*29*).

RESULTS

Transport Activity. Previous experiments (*30*, *31*) demonstrate that coexpression of wild-type *lacY* fragments encoding contiguous, nonoverlapping polypeptides corresponding to the first (N_6) and second (C_6) halves of the permease leads to markedly enhanced stability of the fragments and functional complementation. Although data are not shown, each of the paired Cys mutants used in this study accumulates lactose to a steady-state level of about 30−60% of C-less permease.

Spontaneous Disulfide Formation. Out of the 68 paired-Cys mutants in loop IV/V and loop VIII/IX or X/XI, only Cys pairs 134/343, 134/345, and 135/338, all in loop IV/V and X/XI (Figure 1), exhibit spontaneous disulfide bond formation (Figure 2). The cross-linked species migrate with the same relative mobility (M_r) as intact permease with the biotin acceptor domain in the middle cytoplasmic loop (52 kDa) (*22*). In each case, the 52 kDa band disappears in the presence of DTT (Figure 2), demonstrating that the N_6 and C_6 fragments are cross-linked by a disulfide bond. Thus, loops IV/V and X/XI are within close proximity.

Proximity of Loops IV/V and VIII/IX. Representative Western blots illustrating cross-linking catalyzed by iodine or induced by homobifunctional cross-linking agents between a given Cys residue in loop IV/V of N_6 and a Cys residue in loop VIII/IX in C_6 are shown in Figure 3. Paired Cys residues at position 132 (loop IV/V) and position 282 (loop VIII/IX) form a disulfide in the presence of iodine with very low efficiency. Although significantly stronger cross-linking is observed in the presence of *o*-PDM, no cross-linking is observed with *p*-PDM or BMH, suggesting that the distance between the thiols at these two positions approximates 6 Å. In contrast, a Cys residue at position 132 exhibits high-efficiency cross-linking with a Cys residue at position 283 in the presence of iodine or each of the homobifunctional cross-linking agents tested, making distance estimates difficult to assess. With respect to position 132 and the remaining positions in loop VIII/IX (Table 1), significant cross-linking is observed with Cys pairs 132/286, 132/288, or 132/290 in the presence of *o*-PDM primarily, although the 132/288 pair is also cross-linked weakly by *p*-PDM.

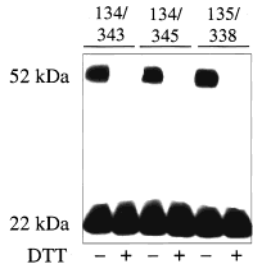

FIGURE 2: Immunoblot showing spontaneous cross-linking between Cys pairs 134/343, 134/345, and 135/338. Membranes were prepared from *E. coli* T184 expressing N_6 and C_6 with paired-Cys residues at positions given. Samples are subjected to $NaDodSO_4$/PAGE under nonreducing conditions followed by immunoblotting with anti-C-terminal antibody as described under Experimental Procedures. Lanes 1, 3, and 5 were run with untreated membranes; lanes 2, 4, and 6 were treated with DTT at a final concentration of 20 mM for 10 min prior to electrophoresis.

Cys pair 134/286 exhibits weak iodine-catalyzed disulfide formation (Figure 3). Stronger disulfide formation under the same conditions is observed with the 134/288 pair. With both pairs, cross-linking efficiency is very high with *o*-PDM, less efficient with *p*-PDM, and equally or least efficient with BMH. The same general pattern is observed when a Cys at position 134 is paired with a Cys at each of the six other positions tested in loop VIII/IX (Table 1), thereby suggesting that position 134 is an average distance of 6 Å from each position on loop VIII/IX (i.e., interaction between the loops is highly flexible).

With the exception of the 135/284 Cys pair, which exhibits no cross-linking under the conditions tested, a Cys residue at position 135 in loop IV/V exhibits weak to moderate iodine-catalyzed cross-linking with each of the positions tested in loop VIII/IX (Table 1). Although the 135/283 Cys pair (Figure 3) also cross-links better with iodine than with the chemical cross-linking agents, none of the other Cys pairs with 135 exhibit significant chemical cross-linking (Table 1).

Of the remaining Cys pairs tested for cross-linking between loops IV/V and VIII/IX, only Cys pair 137/283 cross-links weakly in the presence of iodine or BMH (Table 1). Cys pairs 137/286, 137/288, 137/289, and 137/290, as well as pairs 138/285, 138/286, 138/288, and 138/290, do not cross-link under any of the conditions tested.

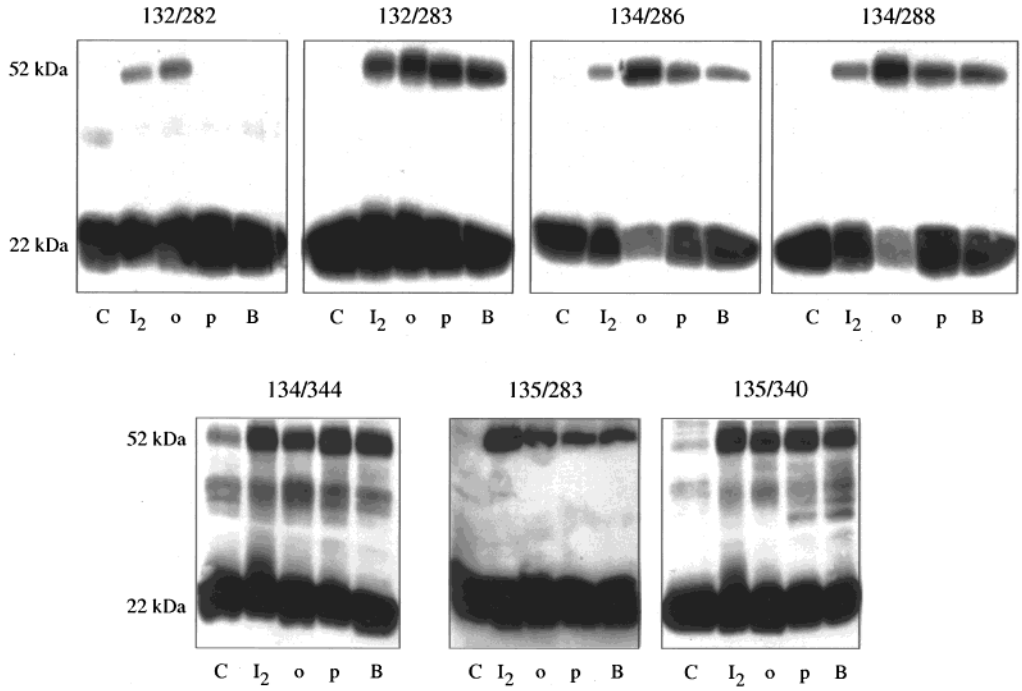

FIGURE 3: Immunoblots showing representative cross-linking experiments with N_6/C_6 paired-Cys mutants. Membranes were prepared from *E. coli* T184 expressing N_6/C_6 with paired-Cys residues at positions shown, and cross-linking was carried out with iodine or a given cross-linking agent as described under Experimental Procedures. Note that the C6 fragment migrates at about 22 kDa and the cross-linked N_6/C_6 fragment at about 52 kDa. C, control (no cross-linking agent added); I_2, iodine; o, *o*-PDM; p, *p*-PDM; B, BMH.

Table 1: Cross-Linking between N6 Residues of Loop IV/V and C6 Residues of Loop VIII/IX[a]

N6	C6	I_2	*o*-PDM (6 Å)	*p*-PDM (10 Å)	BMH (16 Å)	N6	C6	I_2	*o*-PDM (6 Å)	*p*-PDM (10 Å)	BMH (16 Å)
132	282	−/+	+	−	−	135	282	++	−	−	−
	283	++	+++	+++	+++		283	++	+	+	+
	284	−	−	−	−		284	−	−	−	−
	285	−	−	−	−		285	+	−	−	−
	286	−	++	−	+		286	++	−	−	−
	288	−	+	+	−		288	+	−	−	−
	289	−	−	−	−		290	+	−	−	−
	290	−	+	−	−	137	283	+	−	−	+
134	282	−/+	+++	+	+		286	−	−	−	−
	283	−	+++	+	+		288	−	−	−	−
	284	−	+++	+	+		289	−	−	−	−
	285	+	+++	−	+		290	−	−	−	−
	286	−/+	+++	+	+	138	285	−	−	−	−
	288	+	+++	+	+		286	−	−	−	−
	289	++	+++	−/+	+		288	−	−	−	−
	290	+	+++	+	++		290	−	−	−	-

[a] Experiments were carried out as described under Experimental Procedures, and the results shown were obtained from Western blots similar to those shown in Figures 2 and 3. (−), No cross-linking; (−/+) very minimal cross-linking; (+) minimal cross-linking; (++) moderate cross-linking; (+++) strong cross-linking.

Although it is difficult to draw quantitative conclusions, taken as a whole, the pattern observed suggests that the approximate N terminus of loop IV/V, more specifically position 134, is an average distance of 6 Å from each residue in loop VIII/IX, indicating high conformational flexibility, while position 135 may be somewhat closer.

Proximity of Loops IV/V and X/XI. Paired-Cys residues at position 132 (loop IV/V) and position 335, 337, 340, or 342 (loop X/XI) do not cross-link to a detectable extent in the presence of any of the reagents tested (Table 2). However, Cys pair 132/338 forms a disulfide in the presence of iodine with moderate efficiency, and less efficiently with *o*-PDM, *p*-PDM, or BMH. The 132/339 pair is not cross-linked by iodine but cross-links moderately well with *o*-PDM and less efficiently with *p*-PDM or BMH. Neither iodine-catalyzed disulfide formation nor cross-linking by *o*-PDM or *p*-PDM is observed with pair 132/344, and weak cross-linking is observed with BMH (Table 2). The 132/345 pair exhibits minimal cross-linking in the presence of iodine and *o*-PDM but no cross-linking with *p*-PDM or BMH (Table 2).

Cys pair 134/335 exhibits moderate iodine-catalyzed disulfide formation, stronger cross-linking with *o*-PDM, minimal cross-linking with *p*-PDM, and no cross-linking with BMH (Table 2). Although there are quantitative differences, Cys pair 134/337 exhibits a similar pattern with minimal iodine-catalyzed disulfide formation, moderate cross-linking by *o*-PDM, and no cross-linking by *p*-PDM or BMH. The findings suggest that position 134 in loop IV/V approximates a distance of 6 Å from positions 335 and 337 in loop X/XI. The remainder of the Cys residues in loop X/XI paired with

Table 2: Cross-Linking between N6 Residues of Loop IV/V and Loop X/XI[a]

N6	C6	I_2	*o*-PDM (6 Å)	*p*-PDM (10 Å)	BMH (16 Å)	N6	C6	I_2	*o*-PDM (6 Å)	*p*-PDM (10 Å)	BMH (16 Å)
132	335	−	−	−	−	135	335	+	+	+	+
	337	−	−	−	−		337	−	−	−	−
	338	++	+	+	−/+		338	spon			
	339	−	++	+	+		339	+	−	−	−
	340	−	−	−	−		340	+	−/+	+	+
	342	−	−	−	−		342	++	+	+	−/+
	343	++	++	+	−		343	+	++	−	−/+
	344	−	−	−	+		344	++	++	−	−/+
	345	+	+	−	−	137	339	−	−	+	+
134	335	++	+++	+	−		340	−	−	−	−
	337	+	++	−	−		342	−	−	−	−
	338	−	++	++	++		343	−	−	−	−
	339	++	++	++	−/+		344	−	−	−	−
	340	−/+	++	+	+		345	−	−	−	−
	343	spon				138	335	−	−	+	+
	344	++	++	++	++		337	−	−	−	−
	345	spon					338	−	−	+	−/+
							339	−	−	+	++
							340	−	−	−	−

[a] Experiments and notation are as described in Table 1.

a Cys residue at position 134 [positions 338, 339, 340, 343, and 344 (Figure 3) and 345 (Table 2)] exhibit significant cross-linking in the presence of each reagent tested. Therefore, although the two loops appear to be in close proximity, conformational flexibility presumably makes distance approximations untenable. Similarly, with the exception of Cys pair 135/337, which exhibits no cross-linking with any of the reagents tested, pairs 135/335, 135/338, 135/339, and 135/340 (Figure 3) and 135/342, 135/343, and 135/344 (Table 2) generally exhibit moderate to good cross-linking under each condition tested. The remaining Cys pairs tested at position 137 or 138 in loop IV/V and each position in loop X/XI exhibit no cross-linking, with a few exceptions where low efficiency cross-linking is observed with *p*-PDM and BMH (Table 2).

Although data are not shown, no cross-linking is observed under any of the conditions tested between a Cys residue at position 8 in the N terminus or between Cys residues at positions 70, 71, or 72 in loop II/III and any of the Cys replacements in either loop VIII/IX or X/XI.

Ligand-Induced Changes in Cross-Linking. To study the influence of ligand binding, cross-linking was carried out with each Cys pair in the absence or presence of the high-affinity substrate analogue TDG (Figure 4). Four of the Cys pairs tested exhibit significant changes in cross-linking. Cross-linking between Cys pairs 135/283 (helices IV/V and VIII/IX) by *o*-PDM and *p*-PDM is significantly decreased by TDG, suggesting that these two positions may move apart upon ligand binding (Figure 4). Curiously, however, a comparable decrease in cross-linking efficiency is observed with both the 6 and 10 Å cross-linkers, raising the possibility that a decrease in reactivity rather than increase in distance between the Cys residues is responsible for diminished cross-linking. To test this possibility, labeling of both Cys135 and Cys283 in N_6/C_6 permease with NEM was studied in the absence or presence of TDG, as the fragments copurify during avidin affinity chromatography (Figure 5; see avidin and immunoblots in lower panel). TDG binding induces a slight increase in NEM labeling of both Cys135 and Cys283 (Figure 5, top). Although increased reactivity is consistent

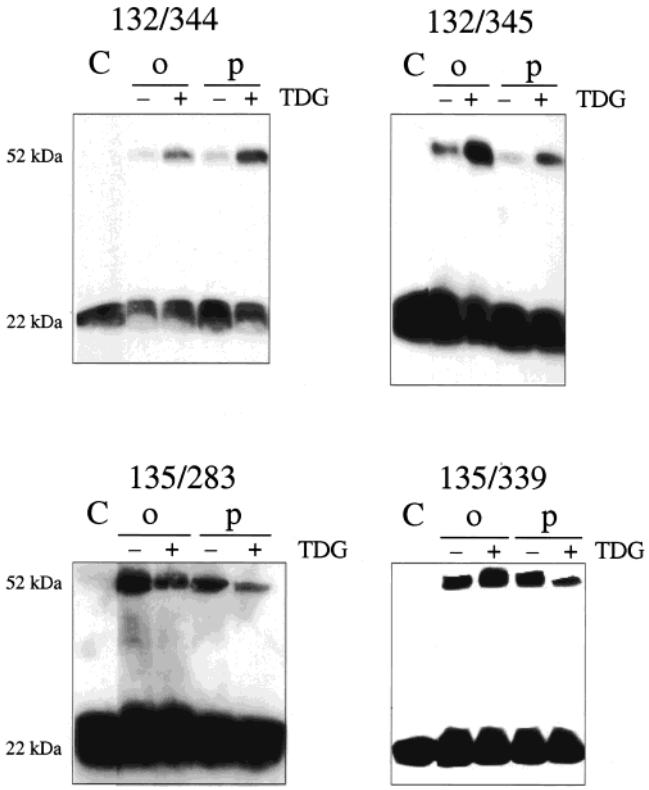

FIGURE 4: Effect of ligand binding on cross-linking. Membranes containing given paired-Cys mutants were incubated in the absence or presence of TDG, then *o*-PDM or p-PDM was added as indicated, and the samples were processed as described under Experimental Procedures. Note that the C_6 fragment migrates at 22 kDa and cross-linked N_6/C_6 at 52 kDa. C, control (no cross-linking agent); o, *o*-PDM; p, *p*-PDM.

with a ligand-induced environmental change, an increase in NEM reactivity cannot account for decreased cross-linking. Therefore, it appears that increased distance between Cys135 and Cys283 and not decreased Cys reactivity is responsible for the ligand-induced decrease in cross-linking.

In contrast to Cys pair 135/283 between loops IV/V and VIII/IX, the three Cys pairs between loops IV/V and X/XI (132/344, 132/345, and 135/339) exhibit increased or de-

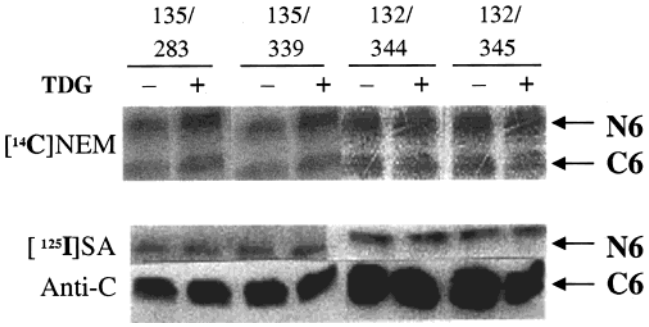

FIGURE 5: NEM labeling of Cys pairs in which cross-linking is altered in the presence of ligand. (Top panel) NEM labeling of Cys residues in paired-Cys mutants and the effect of TDG. Membranes [0.2−0.4 mg of protein in 50 μL of 100 mM potassium phosphate (pH 7.5)/10 mM $MgSO_4$] containing N_6/C_6 with paired-Cys mutants 135/283, 135/339, 132/344, and 132/345 were incubated with [1-^{14}C]NEM (40 mCi/mmol; 0.4 mM final concentration) for 10 min in the absence or presence of 10 mM TDG at 25 °C, as indicated. Reactions were terminated by addition of DTT to a final concentration of 10 mM. Biotinylated permease was solubilized and purified as described under Experimental Procedures. Aliquots containing 5 μg of protein were subjected to $NaDodSO_4$/PAGE, and the labeled protein was visualized by autoradiography. (Bottom panel) [^{125}I]Streptavidin (SA) was used to visualize N_6 (upper row), and anti-C-terminal antibody (anti-C) was used to visualize C_6 (lower row), with 0.5 μg of protein from each sample.

creased cross-linking efficiency in the presence of ligand depending on whether *o*-PDM or *p*-PDM is used as cross-linking agent (Figure 4). Thus, cross-linking efficiency between positions 132/344 and 132/345 increases dramatically with both cross-linking agents, while pair 135/339 exhibits increased cross-linking with *o*-PDM and decreased cross-linking with *p*-PDM. Labeling experiments with NEM reveal that labeling of positions 135, 132, 344, or 345 is not changed significantly in the presence of ligand, while small increases in NEM labeling are observed at positions 283 and 339 (Figure 5). Therefore, it is apparent that the changes in cross-linking efficiency observed in the presence of ligand cannot be attributed to alterations in the reactivity of the Cys residues involved.

DISCUSSION

The experiments presented in this paper utilize in situ cross-linking of N6/C6 split permease with a single Cys residue in each fragment to study proximities between cytoplasmic loops in lac permease, an approach similar to that used previously to study distance relationships and conformational dynamics between transmembrane helices, as well as periplasmic loops (reviewed in ref *13*). The results demonstrate that loop IV/V, which bridges the two helices containing the major determinants for substrate binding in the permease [Glu126 (helix IV) and Arg144 (helix V)], is close to loops VIII/IX and X/XI, which bridge helices containing the four residues essential for H^+ translocation and coupling [Glu269 (helix VIII), Arg302 (helix IX), and His322 and Glu325 (helix X)] (see ref *13*). Furthermore, the findings indicate that ligand binding at the interface between helices IV and V induces a change in conformation reflected by alterations in the proximity between certain positions in loop IV/V and loop VIII/IX or X/XI.

The approach used is based on the premise that Cys cross-links are a measure of proximity. However, it is noteworthy that cross-link formation may detect dynamic collisions and chemical reactions between residues, not simply their proximities. For instance, Cys pairs that frequently undergo collisions and are highly 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 (*32*−*34*). Therefore, proximities between loops are estimated by spontaneous disulfide formation between engineered cysteinyl side chains, iodine-catalyzed disulfide formation, or cross-linking by homobifunctional cross-linking agents in which the distance between the reactive groups and the flexibility of the linker between the functional groups varies. In addition, many of the experiments were carried out at 4 °C in order to decrease thermal motion of the polypeptide backbone and thereby limit long-range collisions. Finally, it is noteworthy that experiments (*35*) in which rates of cross-linking of paired Cys residues in periplasmic loops were compared with distances estimated from spin−spin interactions between the nitroxide-labeled pairs are consistent with the conclusion that site-directed thiol cross-linking is primarily a reflection of proximity.

The observations that Cys pairs 134/343, 134/345, and 135/338 spontaneously form disulfide bonds provides an indication that loop IV/V may be closer to loop X/XI than to loop VIII/IX. In addition, qualitative perusal of the data presented in Tables 1 and 2 suggests that there is significantly more efficient cross-linking between Cys pairs in loops IV/V and X/XI than those in loops IV/V and VIII/IX. On the other hand, there seems to be more conformational flexibility between loops IV/V and X/XI than between loops IV/V and VIII/IX. Thus, there is relatively little difference in cross-linking efficiency with the various cross-linking agents with Cys pairs in loops IV/V and X/XI, in contrast to loops IV/V and VIII/IX, where the 6 Å rigid *o*-PDM cross-links position 134 (loop IV/V) most efficiently to each Cys replacement tested in loop VIII/IX (Table 2). However, even in this case, there must be considerable conformational mobility even at 4 °C for *o*-PDM to cross-link Cys134 to each position in loop VIII/IX with equal efficiency. Because of the presumed flexibility of the loops, it is difficult to draw quantitative conclusions with respect to distances. On the other hand, as discussed previously (*17*), the cross-linking patterns observed are specific, which is consistent with the notion that the loops are not merely flexible, hydrophilic connections between transmembrane helices that interact in a completely random fashion, a conclusion that receives further support from the absence of cross-linking between Cys residues in the N terminus or loop II/III with Cys residues in loops VIII/IX or X/XI.

Site-directed fluorescence studies and labeling experiments with NEM demonstrate that ligand binding results in widespread conformational changes (reviewed in refs *12* and *13*). Therefore, it was suggested that rigid-body movements of the helices initiated at the interface between helices IV and V are transmitted cooperatively throughout the molecule in order for turnover to occur. Cross-linking of paired Cys residues at positions 135 and 283 is decreased in the presence of TDG; which is consistent with the interpretation that the two positions move further apart in the presence of ligand. In contrast, for the most part, ligand binding increases cross-linking of Cys pairs 132/344, 132/345, and 135/339, sug-

gesting that the pairs move closer together. Although it is difficult to envisage the precise nature of the structural change, it seems reasonable to suggest that a conformational change induced by ligand binding may cause certain positions in loop IV/V to move away from certain positions in loop VIII/IX and closer to other positions in loop X/XI.

Finally, it should be emphasized that the cross-linking studies presented here, as well as those dealing with the transmembrane helices and the periplasmic loops (reviewed in ref *13*), provide strong support for the secondary structure model of lac permease. If the permease did not consist of 12 transmembrane helices that traverse the membrane in zigzag fashion with the N and C termini on the cytoplasmic face of the membrane and the loops in the positions indicated (see ref *36*), markedly different results would be expected from the cross-linking studies that have been described.

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