Proximity of Periplasmic Loops in the Lactose Permease of *Escherichia coli* Determined by Site-Directed Cross-Linking

Jianzhong Sun and H. Ronald Kaback*

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

Received May 19, 1997; Revised Manuscript Received July 23, 1997[⊗]

ABSTRACT: Out of over 60 single-Cys mutants in putative periplasmic loops in lactose permease, three mutants [Tyr101 → Cys (loop III/IV), Leu313 → Cys (loop IX/X), and Ser375 → Cys (loop XI/XII)] spontaneously form disulfide-linked dimers, indicating that these loops are located on the periphery of the 12-helix bundle that comprises the permease. By using a permease construct with a factor Xa protease site in the middle cytoplasmic loop, cross-linking between paired-Cys residues in the N- and C-terminal halves of the permease was studied by spontaneous or copper−(1,10-phenanthroline)$_3$-catalyzed disulfide formation or by cross-linking with homo- or heterobifunctional reagents in which the distance between the reactive groups and the flexibility of the linker vary. The findings suggest that the longer loops are relatively flexible; however, cross-linking of residues between loops is specific, indicating that these domains are not simply flexible, hydrophilic connections between helices that interact randomly. More specifically, the findings indicate that the first periplasmic loop (loop I/II) is close to loops VII/VIII and XI/XII, placing helix XII in close proximity to helices II and XI. In addition, the observations are consistent with previous results [Wu, J., & Kaback, H. R. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 14498−502] demonstrating that helices I and II are close to helices VII and XI. Finally, evidence is presented indicating that conformational flexibility between loops I/II and XI/XII may be important for permease turnover.

The lactose (lac)[1] permease of *Escherichia coli* is a paradigm for secondary transport proteins that couple free energy stored in an electrochemcial ion gradient into a concentration gradient (Kaback, 1976, 1983, 1997; Poolman & Konings, 1993; Varela & Wilson, 1996). This hydrophobic, polytopic membrane protein which catalyzes the coupled stoichiometric translocation of β-galactosides and H^+ (i.e., symport or cotransport) has been solubilized, purified, reconstituted, and shown to be solely responsible for β-galactoside transport [reviewed in Viitanen et al. (1984)] as a monomer [see Sahin-Tóth et al. (1994)]. All available evidence 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).

With regard to helix packing (Figure 8), site-directed excimer fluorescence shows that helix VIII is close to helix X, helix IX is close to helix X, and helix X is in an α-helical conformation (Jung et al., 1993). In addition, there are two pairs of interacting Asp and Lys residues that place helix VII close to helices X and XI (King et al., 1991; Lee et al., 1992; Sahin-Tóth et al., 1992; Dunten et al., 1993; Sahin-Tóth & Kaback, 1993; Frillingos & Kaback, 1996a). Many of the spatial relationships have been confirmed by engineering divalent metal-binding sites (bis-His residues) within the permease. Permease with bis-His residues at positions 269 and 322, 302 and 325, or 237 and 358 binds Mn(II) with a stoichiometry of unity, a K_D in the micromolar range, and an apparent pK_a of about 6.3 (Jung et al., 1995; He et al., 1995a,b). Site-directed chemical cleavage confirms the positioning of helix X next to helices VII and XI and indicates further that helix V is close to helices VII and VIII (Wu et al., 1995). The relationship between helices V, VII, and VIII has been documented further by site-directed spin labeling and thiol cross-linking experiments (Wu et al., 1996). Finally, site-directed cross-linking demonstrates that helix I is close to helix VII, helix II is close to helices VII and XI (Wu & Kaback, 1996), and helix VI is close to helix VIII (J. Wu and H. R. Kaback, unpublished observations).

Monoclonal antibody 4B11 binds to an epitope comprised of the last two cytoplasmic loops in the permease (Sun et al., 1997), thereby providing independent support for the close proximity between helices VIII−XI. In addition, a portion of the helix packing model has been confirmed by distance measurements between an engineered Cu(II) binding site and spin-labeled single-Cys residues in this region of the permease (Voss et al., 1995a,b; J. Voss, W. L. Hubbell, and H. R. Kaback, submitted for publication).

In this paper, site-directed cross-linking is extended to the periplasmic loops in lac permease. Paired Cys replacements were introduced into the periplasmic loops in the N- and C-terminal halves of a lac permease construct containing a factor Xa protease site in the middle cytoplasmic loop. Proximity relationships are estimated by spontaneous or

* Author to whom correspondence should be addressed: HHMI/UCLA 5-748 MacDonald Research Labs, Box 951662, Los Angeles, CA 90095-1662. Telephone: (310) 206-5053. Fax: (310) 206-8623. E-mail: RonaldK@HHMI.UCLA.edu.

[⊗] Abstract published in *Advance ACS Abstracts,* September 15, 1997.

[1] Abbreviations: lac, lactose; Cys-less permease, functional lactose permease devoid of Cys residues; CuPh, copper−(1,10-phenanthroline)$_3$; BMH, 1,6-bis(maleimido)hexane; *o*-PDM, *N,N′-o*-phenylenedimaleimide; *p*-PDM, *N,N′-p*-phenylenedimaleimide; MBS, (*m*-maleimidobenzoyl)-*N*-hydroxysuccinimide ester; SMCC, succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate; SMPB, succinimidyl 4-(*p*-maleimidophenyl)butyrate; DTT, dithiothreitol; DM, *n*-dodecyl β-D-maltopyranoside; KP_i, potassium phosphate.

S0006-2960(97)01172-0 CCC: $14.00

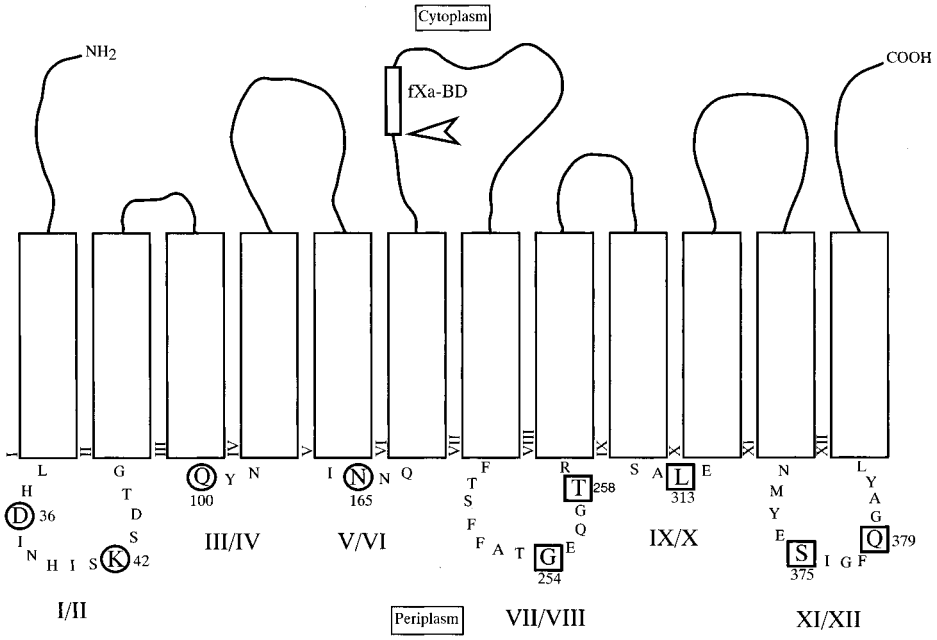

FIGURE 1: Secondary structure model of L6XB lac permease showing positions of Cys mutants in the periplasmic loops. The single-letter amino acid code is used, and the 12 hydrophobic transmembrane helices are depicted as rectangles. Residues encircled or in rectangles in the N- and C-terminal halves, respectively, were used to construct given double-Cys mutants. Loops are designated by the two connected helices separated by a slash. The fXa-BD box represents the biotin acceptor domain with a factor Xa protease site at the N terminus (arrowhead indicates the cleavable site) (Consler et al., 1993). The periplasmic ends of helices XI and XII have been modified as suggested by Cys-scanning mutagenesis and chemical modification (He et al., 1996), as well as electron paramagnetic spectroscopy (Voss et al., 1996).

copper−(1,10-phenanthroline)$_3$ (CuPh)-catalyzed disulfide formation or cross-linking by homo- and heterobifunctional reagents, followed by digestion with factor Xa protease. The results indicate clearly that the loop between helices I and II (loop I/II) is close to loop VII/VIII and loop XI/XII, thereby placing helix XII close to helices II and XI with one face in contact with the hydrophobic phase of the membrane (Voss et al., 1996).

EXPERIMENTAL PROCEDURES

Materials. All restriction endonucleases and T4 DNA ligase were from New England Biolabs. Factor Xa protease was from Boehringer Mannheim. Rabbit polyclonal antiserum against the C terminus of lac permease (Carrasco et al., 1984) was prepared by BAbCO. [1-^{14}C]Lactose was from Amersham. 1,6-Bis(maleimido)hexane (BMH), (*m*-maleimidobenzoyl)-*N*-hydroxysuccinimide ester (MBS), succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC), and succinimidyl 4-(*p*-maleimidophenyl)butyrate (SMPB) were from Pierce. *N*,*N*′-*o*-Phenylenedimaleimide (*o*-PDM) and *N*,*N*′-*p*-phenylenedimaleimide (*p*-PDM) were obtained from Sigma. All other materials were reagent grade and obtained from commercial sources.

Single-Cys Mutants. The single-Cys mutants encoded by plasmid pT7-5/cassette *lacY* have been described (Sun et al., 1996). Note that the single-Cys mutants do not contain the biotin acceptor domain.

Double-Cys Mutants. The Cys-less cassette *lacY* gene from pT7-5 (EMBL X-56095; van Iwaarden et al., 1991) was transferred into pKR35, and an oligonucleotide encoding the biotin acceptor domain from a *Klebsiella pneumoniae* oxalacetate decarboxylase with a factor Xa protease site at the N terminus was inserted to yield the pKR35/Cys-less L6XB equivalent of pLacY/L6XB (Consler et al., 1993). Given double-Cys mutants were then generated by restriction fragment replacement. Mutations were verified by restriction enzyme analysis since the Cys replacement mutations alter specific restriction endonuclease sites in the Cys-less version of the cassette *lacY* gene (van Iwaarden et al., 1991): D36C (*Eco*RV site lost), K42C (*Pst*I site gained), Q100C (*Pst*I site lost), N165C (*Ase*I site lost), G254C (*Age*I site lost), T258C (*Kpn*I site lost), L313C (*Eco*47III site lost), S375C (*Bsm*I site gained), and Q379C (*Sty*I site lost).

Growth of Cells. *E. coli* T184 cells (*lacZ*$^-$*Y*$^-$) expressing a given mutant were grown aerobically at 37 °C in Luria-Bertani broth with ampicillin (100 μg/mL) and streptomycin (10 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 the lysozyme−ethylenediaminetetraacetic acid treatment (Frillingos & Kaback, 1996b; Sun et al., 1996).

Site-Directed Cross-Linking. Membrane samples (ca. 50 μg of total protein) containing given double-Cys mutants in L6XB permease were suspended in 50 μL of 0.1 mM KP$_i$ (pH 7.5)/10 mM $MgSO_4$ buffer. Disulfide cross-linking was carried out by adding 0.4 mM CuPh for 30 min at 25 °C. The membranes were collected by centrifugation at 100000g_{max} at 4 °C and washed once with 150 mM NaCl/50 mM Tris-HCl (pH 8.0)/1.0 mM $CaCl_2$ (factor Xa digestion buffer). The membranes were dissolved in 25 μL of the same buffer containing 1% dodecyl β-D-maltopyranoside (DM) and incubated with factor Xa protease (3 μg/sample) overnight at 4 °C. Sample buffer was then added in the absence of reducing agents unless indicated, and the samples were

Table 1: Lactose Transport by Double-Cys Mutants and the Effect of DTT[a]

	initial rate (% C-less)	
double mutant	no treatment	DTT
36/258	18	33
42/258	44	33
100/258	71	77
165/258	84	100
36/313	85	80
42/313	77	85
100/313	51	53
165/313	108	99
36/375	70	69
42/375	64	58
100/375	100	106
165/375	122	118
36/254	52	53
42/254	41	52
36/379	7	36
42/379	71	65

[a] *E. coli* T184 cells harboring plasmids encoding double-Cys mutants were harvested, treated with 10 mM DTT for 30 min, and assayed as described in Experimental Procedures. Initial rates of lactose transport were measured at 1 min. The rate for Cys-less permease averaged 80 nmol min^{-1} (mg of protein)$^{-1}$. Results are expressed as a percentage of this value. All data were corrected for transport activty of T184 cells harboring pT7-5 (vector with no *lacY* gene).

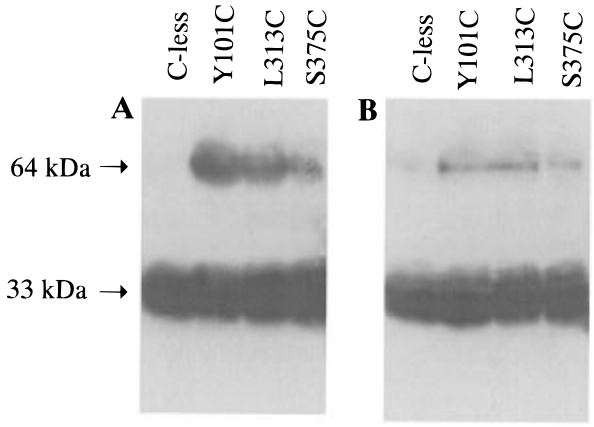

FIGURE 2: Spontaneous dimerization of single-Cys mutants in periplasmic loops: (A) nonreducing conditions and (B) reducing conditions (2% mercaptoethanol). Membranes prepared from *E. coli* T184 cells expressing given single-Cys mutants were subjected to SDS−PAGE, followed by immunoblotting with anti-C-terminal antibody. The lac permease monomer without the biotin acceptor domain migrates at 33 kDa, while the dimer migrates at 64 kDa. Note in panel B that relatively little 64 kDa material is present under reducing conditions. Although data are not shown, treatment with CuPh increases the immunoreactive material migrating at 64 kDa under nonreducing conditions, but in the presence of mercaptoethanol, most of the material migrates at 33 kDa.

subjected immediately to sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) using 12% polyacrylamide, followed by immunobloting with anti-C-terminal antibody (Carrasco et al., 1984).

Homobifunctional cross-linking was carried out in a similar manner by adding 1.0 mM BMH, *o*-PDM, or *p*-PDM (from 50 mM stock solutions in dimethylformamide), and the reactions were quenched by adding 10 mM dithiothreitol (DTT). Samples were washed once in factor Xa digestion buffer, dissolved in digestion buffer containing DM, and incubated with factor Xa protease, followed by SDS−PAGE and immunoblotting, as described.

Heterobifunctional Cross-Linking. Membrane samples containing given single-Cys mutants in L6XB permease were suspended in 50 μL of 0.1 M KP_i (pH 7.5)/10 mM $MgSO_4$ buffer. Reactions were initiated by adding 0.4 mM MBS, SMCC, or SMPB (from 20 mM stock solutions in dimethylformamide) for 30 min at 25 °C and quenched by addition of factor Xa digestion buffer. After washing once with centrifugation, the membranes were resuspended in digestion buffer with DM, incubated with factor Xa protease, and subjected to SDS−PAGE followed by immunoblotting with anti-C-terminal antibody, as described.

Transport Assays. Lactose transport was determined by rapid filtration of *E. coli* T184 cells transformed with given plasmids (Consler et al., 1991).

Protein Determinations. The protein was assayed as described (Peterson, 1977) with bovine serum albumin as the standard.

RESULTS

Transport Activity. To assess the transport activity of the mutants, each double-Cys mutant was expressed in *E. coli* T184 cells and assayed for lactose transport (Table 1). With the exceptions of the 36/258 and 36/379 double-Cys pairs, each mutant transports lactose at 40−120% of the rate of Cys-less permease. Although the 36/379 pair transports at only 7% of the control, addition of DTT stimulates the rate to approximately 36% of that of Cys-less permease. The 36/358 pair transports at 18% of the control rate and is stimulated less than 2-fold by DTT. In contrast, the other double-Cys mutants are not significantly affected by DTT. As shown previously (Sun et al., 1996), each single-Cys mutant used exhibits highly significant transport activity.

Spontaneous Disulfide Formation. While the epitope for monoclonal antibody 4B1 was characterized (Sun et al., 1996), single-Cys mutants at each position in the putative periplasmic loops of lac permease were quantitated by immunoblotting. As a result, it was found fortuitously that, out of a total of 64 mutants, Y101C (loop III/IV), L313C (loop IX/X), and S375C (loop XI/XII) permease exhibit a significant amount of immunoreactive material that migrates at the approximate M_r of a dimer (i.e., ca. 64 kDa; Figure 2A). Hydrophobic membrane proteins like the permease frequently aggregate nonspecifically to form dimers, as well as higher-order aggregates [see Carrasco et al. (1982)], in a time- and temperature-dependent fashion in the presence of sodium dodecyl sulfate. However, the artifact can be avoided in large measure by electrophoresing samples immediately after solubilization in sample buffer at room temperature (P. Ringler and H. R. Kaback, unpublished information). In any event, the immunoreactive material at 64 kDa is dramatically reduced when the samples are electrophoresed under reducing conditions (Figure 2B). Therefore, it is apparent that the 64 kDa immunoreactive band observed with the three single-Cys mutants represents a permease dimer that is cross-linked via disulfide bonds which form spontaneously. Furthermore, treatment of these mutants with CuPh dramatically increases the amount of dimer, and the effect is reversed upon addition of DTT (data not shown).

In addition to the three single-Cys mutants, a double mutant containing Cys pairs at positions 36 (loop I/II) and 379 (loop XI/XII) also efficiently oxidizes spontaneously to form an intramolecular disulfide bond (Figure 3). Digestion of L6XB permease with factor Xa protease cleaves the

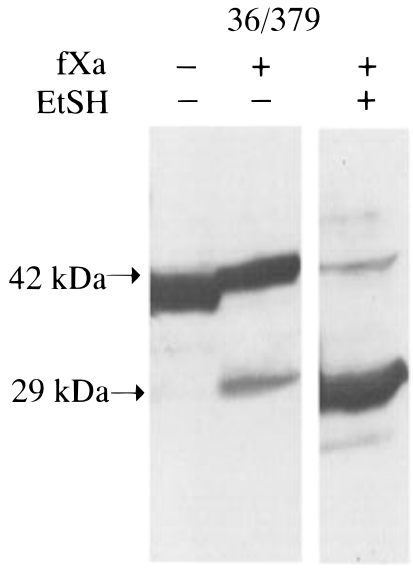

FIGURE 3: Spontaneous disulfide cross-linking between cysteinyl residues at positions 36 and 379. Membranes were prepared from *E. coli* T184 cells expressing 36/379 L6XB permease and treated with factor Xa protease as described in Experimental Procedures. Samples were subjected to SDS−PAGE under either nonreducing or reducing conditions (EtSH; 2% mercaptoethanol) as indicated, followed by immunoblotting with anti-C-terminal antibody: arrow at 29 kDa, C-terminal fragment of L6XB permease; and arrow at 42 kDa, cross-linked L6XB permease.

protein into two fragments (Consler et al., 1993; Sahin-Tóth et al., 1995; Wu et al., 1996; Zen et al., 1995), thereby allowing simple detection of cross-linking from the mobility of the C-terminal half of the permease on Western blots. The C-terminal fragment with the biotin acceptor domain migrates at about 29 kDa, while the cross-linked fragments migrate at an M_r of about 42 kDa which is identical to that of intact L6XB permease (Figure 3). Clearly, most of the L6XB permease with Cys residues at positions 36 and 379 migrates at an M_r corresponding to the intact molecule under nonreducing conditions (ca. 42 kDa) after cleavage with factor Xa protease and at ca. 29 kDa after treatment with EtSH.

CuPh-Catalyzed Disulfide Cross-Linking. Double-Cys mutant 36/258 exhibits little or no immunoreactive material at 42 kDa (Figure 4 and Table 2) but cross-links with high efficiency (ca. 40%) after treatment with CuPh. In addition,

Table 2: Disulfide and Homobifunctional Cross-Linking of Double-Cys Mutants[a]

double mutant	CuPh	*o*-PDM (6 Å)	*p*-PDM (10 Å)	BMH (16 Å)
36/258	++	+++	+++	+++
42/258	−	++	+++	+++
100/258	−			−
165/258	−			−
36/313	−			−
42/313	−			−
100/313	−			−
165/313	−			−
36/375	−	−	−	−
42/375	+	−	+	++
100/375	−			−
165/375	−			−
36/254	−	−	++	+++
42/254	+	+++	+++	+++
42/379	−	+	++	+++

[a] +++, >50% cross-linking; ++, 10−50% cross-linking; +, <10% cross-linking; −, no detectable cross-linking. Cross-linking efficiency was determined by scanning the density of 42 and 29 kDa bands using a LKB UltraScan densitometer.

the 42/254 pair cross-links significantly (ca. 10%), and a small amount of dimer is observed with the 42/375 pair, as well, after CuPh treatment. It is also noteworthy that there is a significant immunoreactive band at an M_r of about 62 kDa with all the Cys pairs in which one of the Cys residues is at position 375. Since single-Cys375 permease dimerizes spontaneously, the presence of the 62 kDa band is explained by spontaneous intermolecular cross-linking between C-terminal halves prior to digestion with factor Xa protease. Importantly, little or no cross-linking is observed with the other double-Cys mutants before or after treatment with CuPh (Table 2).

Cross-Linking by Homobifunctional Reagents. BMH is a homobifunctional thiol cross-linking reagent with a flexible hexyl chain connecting two maleimido groups (ca. 16 Å fully extended). The 36/258 and 36/254 double-Cys pairs exhibit relatively little or no cross-linked product without BMH treatment, but a marked increase in the intensity of the 42 kDa band correlated with a decrease in the 29 kDa band is observed after incubation with the cross-linking agent (Figure 5 and Table 2). A Cys residue at position 42 readily cross-links with a Cys residue at position 258, 254, or 379 in the presence of BMH, and a relatively small but significant amount of cross-linking is also observed with the 42/375

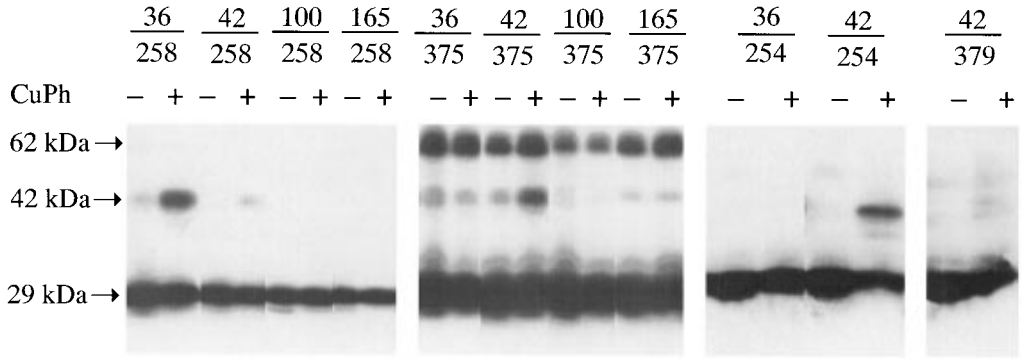

FIGURE 4: CuPh-catalyzed disulfide cross-linking of double-Cys mutants. Membranes prepared from *E. coli* T184 cells expressing the indicated double-Cys mutants were treated with 0.4 mM CuPh, followed by factor Xa protease as described in Experimental Procedures. Samples were subjected to SDS−PAGE, followed by immunoblotting with anti-C-terminal antibody: arrow at 29 kDa, C-terminal fragment of L6XB permease; and arrow at 42 kDa, cross-linked L6XB permease. The higher-molecular mass bands (62 kDa) observed with the double-Cys mutants containing a cysteinyl residue at position 375 are due to intermolecular disulfide formation prior to factor Xa protease treatment.

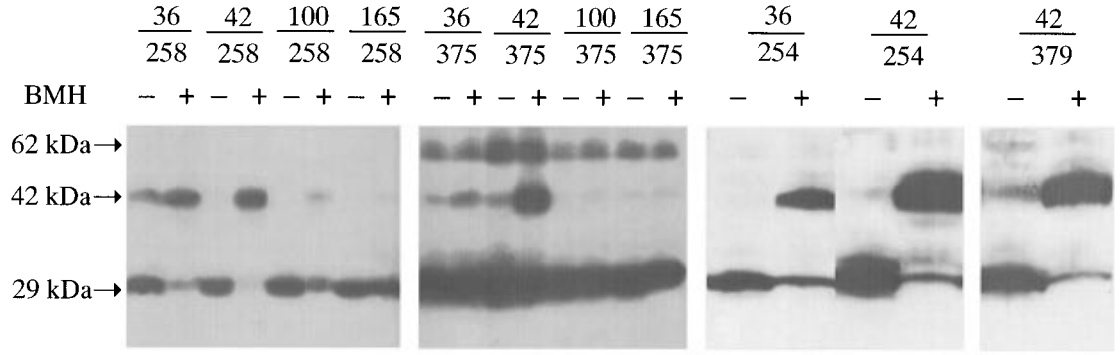

FIGURE 5: BMH cross-linking of double-Cys mutants. Conditions for cross-linking and immunoblotting are described in Experimental Procedures: arrow at 29 kDa, C-terminal fragment of L6XB permease; and arrow at 42 kDa, cross-linked L6XB permease. The higher-molecular mass bands (62 kDa) observed with the double-Cys mutants containing a cysteinyl residue at position 375 are due to intermolecular disulfide formation prior to factor Xa protease treatment.

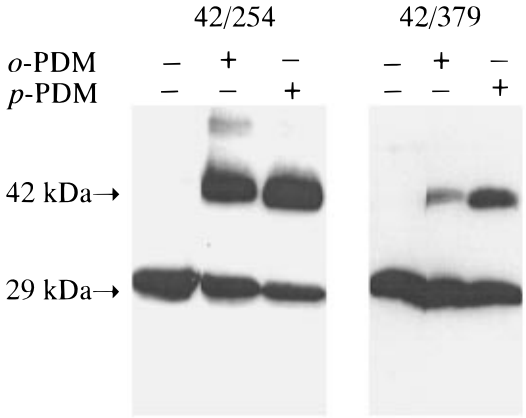

FIGURE 6: *o*-PDM and *p*-PDM cross-linking of double-Cys mutants. Conditions for cross-linking and immunoblotting are described in Experimental Procedures: arrow at 29 kDa, C-terminal fragment of L6XB permease; and arrow at 42 kDa, cross-linked L6XB permease.

pair. In contrast, no significant BMH-induced cross-linking is observed with pair 100/258 or 165/258 (Figure 5) or any of the other pairs tested (Table 2).

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 about 6 or 10 Å, respectively. The double-Cys pair 42/254 cross-links effectively with both *o*-PDM and *p*-PDM, while the 42/379 pair cross-links weakly with *o*-PDM and more effectively with *p*-PDM (Figure 6). Although the immunoblots are not shown (see Table 2), the 42/258 pair also cross-links with both reagents, but more efficiently with *p*-PDM. In addition, the double-Cys pair 36/258 cross-links strongly with *p*-PDM and *o*-PDM, 36/254 cross-links moderately well with *p*-PDM, but not with the shorter reagent, and 42/375 cross-links weakly with *p*-PDM only.

Heterobifunctional Cross-Linking. Since there is only one Lys residue in the periplasmic loops of lac permease (Figure 1), heterobifunctional cross-linking between the primary amine of Lys42 in loop I/II and Cys residues in the C-terminal half was also investigated (Figure 7). SMPB (ca. 15 Å in length), SMCC (ca. 12 Å in length), or MBS (ca. 10 Å in length) each has an *N*-hydroxysuccinimide group that reacts with primary amines and a maleimide group that reacts with Cys residues. The reagents cross-link Lys42 with a Cys residue at position 254, 258, or 379 with varying efficiencies. However, significant cross-linking between Lys42 and Cys375 is not detected (see Table 3 in addition).

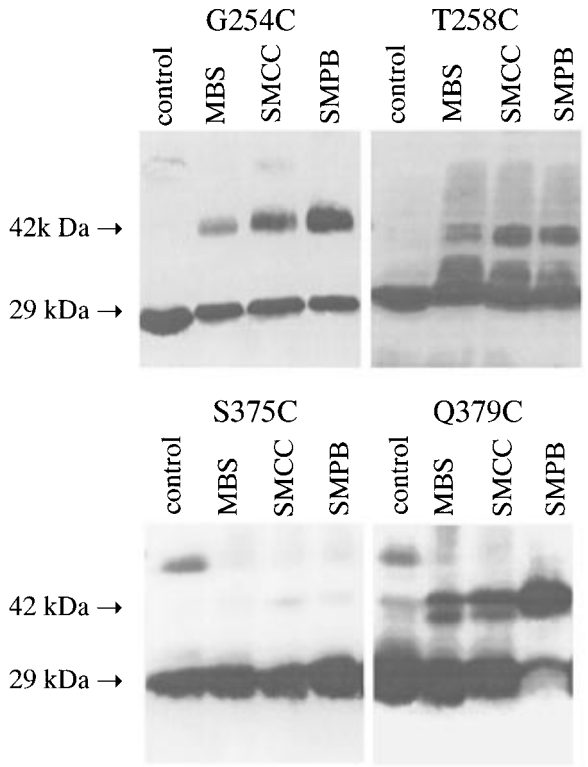

FIGURE 7: Heterobifunctional cross-linking of thiol groups with Lys42 (loop I/II) in single-Cys mutants G254C, T258C, S375C, or Q379C. Conditions for cross-linking and immunoblotting are described in Experimental Procedures: arrow at 29 kDa, C-terminal fragment of L6XB permease; and arrow at 42 kDa, cross-linked L6XB permease.

Table 3: Heterobifunctional Cross-Linking of Cys Residues with Lys42 in Single-Cys Mutants[a]

single mutant	MBS (10 Å)	SMCC (12 Å)	SMPB (15 Å)
G254C	+	++	+++
T258C	+	++	++
S375C	−	−	−
Q379C	+	+	+++

[a] +++, >50% cross-linking; ++, 10−50% cross-linking; +, <10% cross-linking; −, no detectable cross-linking. Cross-linking efficiency was determined by scanning the density of 42 and 29 kDa bands using a LKB UltraScan densitometer.

DISCUSSION

Spontaneous Dimerization by Single-Cys Mutants. Single-Cys replacement mutants Y101C (loop III/IV), L313C (loop

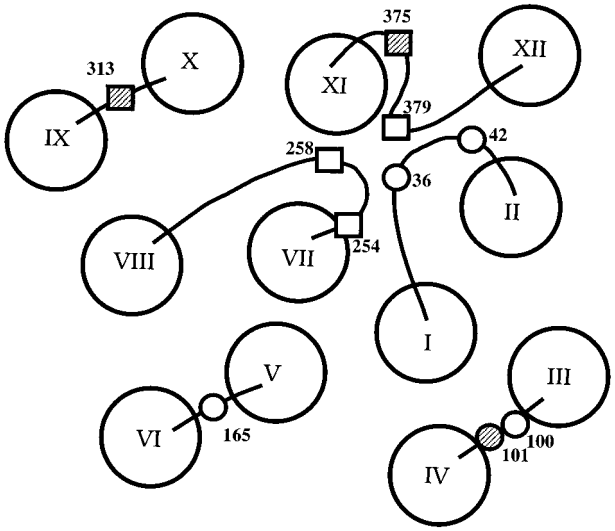

FIGURE 8: Schematic representation of the spatial arrangement of periplasmic loops in lac permease. The transmembrane helices are depicted as circles with Roman numerals from I to XII. Positions of the Cys mutants in the N- and C-terminal halves of the permease are indicated as small circles and rectangles, respectively. The arrangement of helices VII−XI has been established [reviewed in Kaback et al. (1995)]. The placement of helices V, I, and II has also been described (Wu et al., 1995, 1996; Wu & Kaback, 1996). Placement of helix XII is based on data presented in this paper demonstrating that loop I/II is close to loops VII/VIII and XI/XII. The positioning of helices III and IV is based on intermolecular cross-linking of Y101C, L313C, or S375C (crosshatched) permease and on the estimated length of the loops between helices II and III and III and IV (Figure 1).

IX/X), and S375C (loop XI/XII) spontaneously dimerize in a manner that is accelerated under oxidizing conditions (i.e., in the presence of CuPh) and reversed under reducing conditions (i.e., treatment with DTT). Furthermore, dimerization is not observed with permease devoid of Cys residues or with 60 other single-Cys mutants in periplasmic loops. The results suggest that periplasmic loops III/IV, IX/X, and XI/XII are on the periphery of the 12-helix bundle that comprises the permease (Figure 8). In addition, the findings are consistent with the notion that dimerization is a stochastic process resulting from random collisions between monomers, a conclusion consistent with previous observations [reviewed in Sahin-Tóth et al. (1994)] indicating that the permease is a monomer.

Intramolecular Cross-Linking. In most of the experiments reported here, site-directed cross-linking is documented between paired Cys mutants in the N- and C-terminal halves of a permease construct containing a factor Xa protease site in the middle cytoplasmic loop which allows ready detection of cross-linking *in situ* by SDS−PAGE, followed by Western blotting with anti-C-terminal antibody. The technique is an alternative to that of Wu and Kaback (1996, 1997) which utilizes "split permease" constructs rather than an engineered protease site.

The approach used is 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 collisons 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. Therefore, proximities between loops are estimated by spontaneous disulfide formation between engineered cysteinyl side chains, CuPh-catalyzed disulfide formation, or cross-linking by homo- or heterobifunctional cross-linking agents in which the distance between the reactive groups and the flexibility of the linker between the functional groups vary.

The findings are consistent with the interpretation that the first periplasmic loop (positions 36 and 42) is close to loops VII/VIII (positions 254 and 258) and XI/XII (positions 375 and 379) which places helix XII close to helices II and XI with one face exposed to the membrane bilayer (Figure 8), as indicated by site-directed electron paramagnetic spectroscopy studies (He et al., 1996; Voss et al., 1996). The results are also consistent with previous studies (Wu & Kaback, 1996) showing that helices I and II are in close proximity to helices VII and XI. Since cross-linking is observed between Cys residues in the longer loops only (I/II in the N-terminal half with VII/VIII and XI/XII in the C-terminal half) and not with the shorter loops (III/IV, V/VI, or IX/X), the findings support general aspects of the secondary structure model (Figure 1) and suggest further that the longer loops are relatively flexible. On the other hand, the cross-linking patterns observed are clearly specific which is consistent with the notion that the loops are not merely flexible, hydrophilic connections between transmembrane helices that interact randomly.

The data are consistent with the model shown in Figure 8. Cys residues at positions 36 and 379 cross-link spontaneously. Therefore, it is reasonable to conclude that these two positions are the closest of the pairs tested. Position 36 does not spontaneously cross-link with position 258, but it is sufficiently close that CuPh catalyzes disulfide formation. Furthermore, this pair is cross-linked more efficiently by the three homobifunctional reagents, suggesting that proximity can vary up to at least 6 Å, a conclusion consistent with the notion of flexibility. The 36/254 pair is cross-linked effectively only by *p*-PDM and BMH and not by CuPh or *o*-PDM, suggesting a minimum distance of 10 Å. A Cys residue at position 42 does not cross-link spontaneously with a Cys residue at position 254 or 258; however, weak disulfide formation is observed between positions 42 and 254 with CuPh, and the longer cross-linking agents *o*-PDM, *p*-PDM and BMH cross-link 42 and 254 or 258 effectively. Position 42 also appears to be closer to position 379 than to 375 because the 42/379 pair is cross-linked effectively by *p*-PDM and BMH only, while the 42/375 pair is cross-linked with modest efficiency by BMH and weakly by *p*-PDM. Consistently, the findings with the heterobifunctional cross-linking agents suggest that position 42 is close to positions 254, 258, and 379 but not to position 375.

Finally, addition of DTT to the double-Cys mutant 36/379 stimulates transport activity about 5-fold, indicating that reduction of the spontaneously formed disulfide bond in this mutant releases the permease from an inhibited state. Cys-scanning mutagenesis of the permease [see Kaback (1996)] reveals that many mutants inactivated by alkylation cluster on putative helical faces, particularly I, II, VII, VIII, and XI, indicating that surface interactions in these regions of the protein are important for activity. In addition, second-site suppressor analysis of D68T or D68S mutants (loop II/III; Jessen-Marshall & Brooker, 1996), as well as the demonstration that disulfide cross-linking between helices

II and VII inhibits transport (Wu & Kaback, 1997), provides a strong indication that conformational flexibility between helices II and VII is important for turnover. The results presented for the 36/379 double-Cys mutant provide a preliminary indication that conformational flexibility of certain loops may also be important.

REFERENCES

Carrasco, N., Tahara, S. M., Patel, L., Goldkorn, T., & Kaback, H. R. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 6894−8.

Carrasco, N., Herzlinger, D., Mitchell, R., DeChiara, S., Danho, W., Gabriel, T. F., & Kaback, H. R. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 4672−6.

Consler, T. G., Tsolas, O., & Kaback, H. R. (1991) *Biochemistry 30*, 1291−8.

Consler, T. G., Persson, B. L., Jung, H., Zen, K. H., Jung, K., Prive, G. G., Verner, G. E., & Kaback, H. R. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 6934−8.

Dunten, R. L., Sahin-Toth, M., & Kaback, H. R. (1993) *Biochemistry 32*, 3139−45.

Frillingos, S., & Kaback, H. R. (1996a) *Biochemistry 35*, 13363−7.

Frillingos, S., & Kaback, H. R. (1996b) *Biochemistry 35*, 3950−6.

He, M. M., Voss, J., Hubbell, W. L., & Kaback, H. R. (1995a) *Biochemistry 34*, 15667−70.

He, M. M., Voss, J., Hubbell, W. L., & Kaback, H. R. (1995b) *Biochemistry 34*, 15661−6.

He, M. M., Sun, J., & Kaback, H. R. (1996) *Biochemistry 35*, 12909−14.

Jessen-Marshall, A. E., & Brooker, R. J. (1996) *J. Biol. Chem. 271*, 1400−4.

Jung, K., Jung, H., Wu, J., Privé, G. G., & Kaback, H. R. (1993) *Biochemistry 32*, 12273−8.

Jung, K., Voss, J., He, M., Hubbell, W. L., & Kaback, H. R. (1995) *Biochemistry 34*, 6272−7.

Kaback, H. R. (1976) *J. Cell. Physiol. 89*, 575−93.

Kaback, H. R. (1983) *J. Membr. Biol. 76*, 95−112.

Kaback, H. R. (1996) in *Handbook of Biological Physics: Transport Processes in Eukaryotic and Prokaryotic Organisms* (Konings, W. N., Kaback, H. R., & Lolkema, J. S., Eds.) pp 203−27, Elsevier, Amsterdam.

Kaback, H. R. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 5539−43.

Kaback, H. R., Jung, K., Jung, H., Wu, J., Privé, G. G., & Zen, K. (1995) in *Advances in Cell and Molecular Biology of Membranes and Organelles* (Dalbey, W., Ed.) Vol. 4, pp 129−44.

King, S. C., Hansen, C. L., & Wilson, T. H. (1991) *Biochim. Biophys. Acta 1062*, 177−86.

Lee, J. L., Hwang, P. P., Hansen, C., & Wilson, T. H. (1992) *J. Biol. Chem. 267*, 20758−64.

Peterson, G. L. (1977) *Anal. Biochem. 83*, 346−56.

Poolman, B., & Konings, W. N. (1993) *Biochim. Biophys. Acta 1183*, 5−39.

Sahin-Tóth, M., & Kaback, H. R. (1993) *Biochemistry 32*, 10027−35.

Sahin-Tóth, M., Dunten, R. L., Gonzalez, A., & Kaback, H. R. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 10547−51.

Sahin-Tóth, M., Lawrence, M. C., & Kaback, H. R. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 5421−5.

Sahin-Tóth, M., Dunten, R. L., & Kaback, H. R. (1995) *Biochemistry 34*, 1107−12.

Sun, J., Wu, J., Carrasco, N., & Kaback, H. R. (1996) *Biochemistry 35*, 990−8.

Sun, J., Li, J., Carrasco, N., & Kaback, H. R. (1997) *Biochemistry 36*, 274−80.

van Iwaarden, P. R., Pastore, J. C., Konings, W. N., & Kaback, H. R. (1991) *Biochemistry 30*, 9595−600.

Varela, M. F., & Wilson, T. H. (1996) *Biochim. Biophys. Acta 1276*, 21−34.

Viitanen, P., Garcia, M. L., & Kaback, H. R. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 1629−33.

Voss, J., Hubbell, W. L., & Kaback, H. R. (1995a) *Proc. Natl. Acad. Sci. U.S.A. 92*, 12300−3.

Voss, J., Salwinski, L., Kaback, H. R., & Hubbell, W. L. (1995b) *Proc. Natl. Acad. Sci. U.S.A. 92*, 12295−9.

Voss, J., He, M., Hubbell, W., & Kaback, H. R. (1996) *Biochemistry 35*, 12915−8.

Wu, J., & Kaback, H. R. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 14498−502.

Wu, J., & Kaback, H. R. (1997) *J. Mol. Biol. 270*, 285−93.

Wu, J., Perrin, D., Sigman, D., & Kaback, H. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 9186−90.

Wu, J., Voss, J., Hubbell, W. L., & Kaback, H. R. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 10123−7.

Zen, K., Consler, T. G., & Kaback, H. R. (1995) *Biochemistry 34*, 3430−7.

BI971172K