Proximity between Periplasmic Loops in the Lactose Permease of *Escherichia coli* As Determined by Site-Directed Spin Labeling†

Jianzhong Sun,‡,§ John Voss,‡,|| Wayne L. Hubbell,⊥ and H. Ronald Kaback*,‡

Howard Hughes Medical Institute, Departments of Physiology and Microbiology & Molecular Genetics, Molecular Biology Institute, Jules Stein Eye Institute and Department of Chemistry and Biochemistry, University of California, Los Angeles, Los Angeles, California 90095-1662

Received October 1, 1998; Revised Manuscript Received January 4, 1999

ABSTRACT: Site-directed thiol cross-linking indicates that the first periplasmic loop (loop I/II) in the lactose permease of *Escherichia* coli is in close proximity to loops VII/VIII and XI/XII [Sun, J., and Kaback, H. R. (1997) *Biochemistry 36*, 11959−11965]. To determine whether thiol cross-linking reflects proximity as opposed to differences in the reactivity and/or dynamics of the Cys residues that undergo cross-linking, single-Cys mutants in loops I/II, VII/VIII, and XI/XII and double-Cys mutants in loop I/II and VII/VIII or XI/XII were purified and labeled with a sulfhydryl-specific nitroxide spin label. The labeled mutants were then analyzed by electron paramagnetic resonance (EPR) spectroscopy, and interspin distance was estimated from the extent of line shape broadening in the double-labeled proteins. Out of six paired double-Cys mutants that exhibit thiol cross-linking, five display significant spin−spin interaction. Furthermore, there is a qualitative correlation between distances estimated by site-directed cross-linking and EPR. Taken as a whole, the results are consistent with the conclusion that site-directed thiol cross-linking is primarily a reflection of proximity.

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 galactosides and H^+ [galactoside/H^+ symport; reviewed in (*3*)] as a monomer (see *4*). The molecule is composed of 12 transmembrane helices connected by hydrophilic loops with the N and C termini on the cytoplasmic face (reviewed in *5*−*8*) (Figure 1). Cys-scanning and site-directed mutagenesis of each residue in the permease indicate that only six side chains are irreplaceable with respect to active transport but the protein undergoes widespread conformational changes during turnover. By using a battery of site-directed techniques which include second-site suppressor analysis and site-directed mutagenesis, excimer fluorescence, engineered divalent metal binding sites, chemical cleavage, electron paramagnetic resonance (EPR), thiol cross-linking, and identification of discontinuous monoclonal antibody epitopes, a helix packing model has been formulated (reviewed in *9*, *10*). Based on the proximity of the irreplaceable residues and the behavior of mutants at these positions, a mechanism for coupling between lactose and H^+ translocation has been proposed (*11*, *12*).

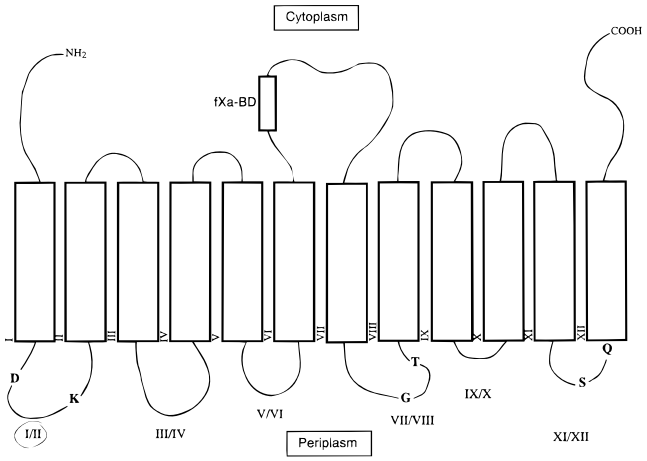

FIGURE 1: Secondary structure model of lac permease showing positions of Cys mutants in the periplasmic loops. The single-letter amino acid code is used, and the 12 hydrophobic transmembrane helices are depicted as rectangles. Residues shown were used to construct given double-Cys mutants. Loops are 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.

† This work was supported in part by NIH Grant DK51131 to H.R.K.

* Corresponding author: HHMI/UCLA 5-748 MacDonald Research Labs, Box 951662, Los Angeles, CA 90095-1662. Telephone: (310) 206-5053. Telefax: (310) 206-8623. E-mail: RonaldK@HHMI.UCLA.edu.

‡ Howard Hughes Medical Institute.

§ Present address: Department of Immunology, The Scripps Research Institute, La Jolla, CA 92037.

|| Present address: Department of Biological Chemistry, University of California Davis School of Medicine, 4303 Tupper Hall, Davis, CA 95616.

⊥ Jules Stein Eye Institute and Department of Chemistry and Biochemistry.

[1] Abbreviations: lac permease, lactose permease; Cys-less permease, functional lac permease devoid of Cys residues; EPR, electron paramagnetic resonance; CuPh, copper-(1,10-phenanthroline)$_3$; TDG, β,D-galactopyranosyl 1-thio-β,D-galactopyranoside, DTT, dithiothreitol; DM, *n*-dodecyl-β,D-maltopyranoside; KP_i, potassium phosphate.

10.1021/bi982360t CCC: $18.00
Published on Web 02/13/1999

Site-directed thiol cross-linking was used initially with N_6C_6 split permease (*13*−*17*) to provide information about helix packing. Subsequently, the technique was applied to the periplasmic loops in lac permease with an engineered factor Xa protease site in the middle cytoplasmic loop (*18*). The results indicate that loop I/II is in close proximity to loops VII/VIII and XI/XII, and, more recently, the relationships were documented further by studying thiol cross-linking in N_6C_6 permease (*19*). Conclusions from these studies are based on the premise that Cys cross-linking is a measure of proximity. However, thiol cross-linking may also reflect dynamic collisions and chemical reactivity of the Cys residues, not simply proximities (*20*). That is, Cys pairs that frequently undergo collisions and are highly reactive chemically could form cross-links at a relatively rapid rate, even though they may be far apart in the average structure.

To address this problem, we have utilized site-directed spin labeling to study proximity relationships more directly (*13*, *21*−*26*). Paired double-Cys mutants with a biotin acceptor domain in the middle cytoplasmic loop were purified and labeled with a thiol-specific nitroxide spin label and subjected to EPR. The extent of the magnetic dipolar interaction between two spin-labeled Cys side chains was used to estimate the distance between the spin labels. The results are consistent qualitatively with previous cross-linking studies (*18*), thereby supporting the notion that thiol cross-linking is primarily a reflection of proximity, although other factors may also play a role.

EXPERIMENTAL PROCEDURES

Construction of Cys Mutants. Construction of single- or double-Cys mutants in plasmid pKR35 containing the cassette version of *lacY* [EMBL X-56095; (*27*)] encoding functional lac permease devoid of Cys residues (Cys-less permease) with a biotin acceptor domain and a factor Xa protease site at its N terminus in loop VI/VII (pKR35/Cys-less L6XB; Figure 1) was described previously (*18*).

Growth of Cells. E. coli T184 (*lacZ*$^-$*Y*$^-$) was transformed with pKR35/Cys-less L6XB encoding a given mutant. Cultures were grown aerobically at 37 °C in Luria−Bertani broth with ampicillin (100 μg/mL) and streptomycin (10 μg/mL). Overnight cultures were diluted 12-fold in a 12 L fermentor and grown for 2 h at 37 °C before induction with 0.3 mM isopropyl 1-thio-β,D-galactopyranoside. After additional growth for 2−3 h at 37 °C, cells were harvested by centrifugation.

Purification and Spin Labeling of Permease. Purification and spin labeling of permease mutants were carried out essentially as described (*13*, *28*). In brief, cells were disrupted by passage through a French pressure cell, and a membrane fraction was isolated by centrifugation and resuspended in 50 mM potassium phosphate (KP_i; pH 7.5)/1 mM dithiothreitol (DTT)/5 mM $MgSO_4$/20 mM lactose/0.5 mM Pefabloc (Boehringer Mannheim). The membranes were then extracted with 2% dodecyl-β,D-maltopyranoside (DM), and after centrifugation, the supernatant was incubated with monomeric avidin−Sepharose (Pierce) for 1 h at 4 °C, followed by extensive washing with 50 mM KP_i (pH 7.5)/0.02% DM/2 mM ethylenediaminetetraacetate (potassium salt). Methanethiosulfonate spin label was added to a final concentration of 0.1 mM, the resin was incubated at 4 °C for 1 h and washed extensively with 50 mM KP_i (pH 7.5)/0.02% DM, and spin-labeled permease was eluted with 10 mM biotin in the same buffer. Fractions containing purified permease were dialyzed and concentrated by using a Micro-ProDiCon membrane (Spectrum) (*28*).

Table 1: Cross-Linking between Cys Pairs in the Periplasmic Loops of the Lactose Permease[a]

Cys pair	effective cross-linking agent
36/254	*p*-PDM (10 Å; rigid), BMH (16 Å; flexible)
36/258	CuPh, *o*-PDM (6 Å; rigid), *p*-PDM, BMH
36/379	spontaneous
42/254	CuPh, *o*-PDM, *p*-PDM, BMH
42/258	*o*-PDM, *p*-PDM, BMH
42/379	*o*-PDM, *p*-PDM, BMH

[a] See (*18*).

EPR Spectroscopy. EPR measurements were carried out in a Varian E-109 X-band spectrometer fitted with a loop-gap resonator (*29*, *30*). An aliquot of purified, spin-labeled permease (10 μL) at a final concentration of ca. 100 μM permease in 50 mM in KP_i (pH 7.5)/ 0.02% DM was placed in a sealed quartz capillary contained in the resonator. Spectra of samples at room temperature (20−22 °C) were obtained by signal averaging 3−5 scans over 100 and 200 G at a microwave power of 2 mW and a modulation amplitude optimized to the natural line width of the individual spectrum and recorded under field-frequency lock (100 G scans only) at X band. Spectra in the frozen state (183 K) were collected at a microwave power of 0.05 mW.

Data Analysis. Inhomogeneous spectral broadening was analyzed using a Voigt convolution of Gaussian and Lorentzian functions optimized by using the Levenberg−Marquardt algorithm (*31*) and the EWVoigt computer program (Scientific Software Services) for line width fitting and simulation. For a spectrum containing two dipolar interacting spin labels, the Lorentzian component was extracted by the best fit to a broadened envelope function. The envelope is a composite spectrum generated from the individually labeled samples. Prior to convolution of single labeled spectra, each spectrum was normalized to the same integrated intensity.

RESULTS

Cross-Linking in Double-Cys Mutants. As summarized in Table 1, cross-linking between a Cys residue at position 36 or 42 (loop I/II) and a Cys residue at position 254, 258 (loop VII/VIII), or 379 (loop XI/XII) varies depending upon the oxidant or homobifunctional cross-linker used. Thus, a Cys residue at position 36 spontaneously forms a disulfide with Cys at position 379, suggesting that these two positions out of the pairs tested are in the closest proximity. Although paired-Cys residues at positions 36 and 258 or 42 and 254 do not form a disulfide spontaneously, the pairs can be oxidized to form a disulfide with copper phenenthroline (CuPh) or cross-linked with *N*,*N*′-*o*-phenylenedimaleimide (*o*-PDM; 6 Å and rigid), *N*,*N*′-*p*-phenylenedimaleimide (*p*-PDM; 10 Å and rigid), or 1,6-bis(maleimido)hexane (BMH; 16 Å and flexible), thereby suggesting that the distance between the thiols can vary between 0 and at least 10 Å. In contrast, the 42/258 or 42/379 Cys pairs cross-link in the

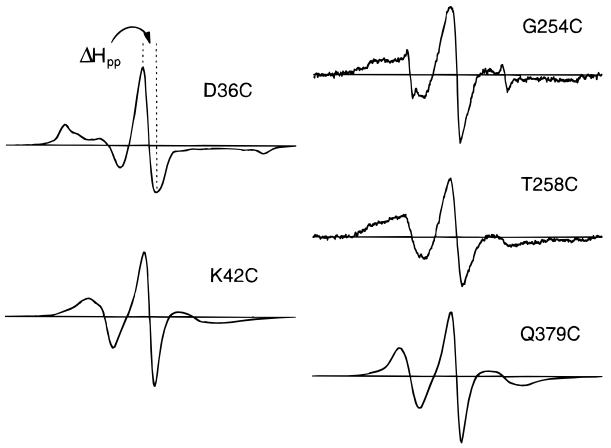

FIGURE 2: EPR spectra of spin-labeled single-Cys mutants at room temperature. Experiments were carried out as described under Experimental Procedures. The magnetic field scan is 100 G. The inverse line width of the central resonance (ΔH_{pp}^{-1}), which is a convenient experimental measure of the nitroxide mobility, is indicated. The spectrum of G254C is from (*28*).

Table 2: EPR Spectral Parameters of Spin-Labeled Single-Cys Lac Permease Mutants

single mutant	ΔH_{pp}^{-1} (room temperature) (G)	d_1/d (frozen state)
D36C	0.21	0.32
K42C	0.27	0.32
G254C	0.29	nd[a]
T258C	0.26	0.33
Q379C	0.27	0.32

[a] nd, not determined.

presence of *o*-PDM, *p*-PDM, or BMH, but not CuPh, suggesting distances of between 6 and at least 10 Å. Finally, the 36/254 pair cross-links only in the presence of *p*-PDM or BMH, indicating that the inter-thiol distance varies between 10 and 16 Å. It is noteworthy that BMH is highly flexible, and a distance of 16 Å between the maleimido groups represents the fully extended conformation. Therefore, cross-linking by BMH is probably an overestimate with respect to the pairs tested particularly when cross-linking is observed with shorter, rigid cross-linking agents.

Side Chain Dynamics of Single-Cys Mutants. The dynamics of a nitroxide side chain in a protein are reflected by the line shape of the spin-labeled Cys side chain (*22*, *32*, *33*). The inverse line width of the central resonance, ΔH_{pp}^{-1}, is a convenient experimental measure of nitroxide mobility (*28*, *34*). Nitroxides at positions along a folded protein may display a range of ΔH_{pp}^{-1} values: solvent-exposed nitroxides in interhelical loops are characterized by a high ΔH_{pp}^{-1}, whereas a low ΔH_{pp}^{-1} value is consistent with the nitroxide side chain making tertiary contact with other residues, such as a solvent-inaccessible buried site. Between these extremes are solvent-exposed residues along an ordered secondary structure, the motions of which are restricted in part by the stable backbone and in part by steric interaction with neighboring residues.

Based on the low ΔH_{pp}^{-1} (Figure 2; Table 2), the nitroxide side chain at position 36 is in a very slow motional regime, due possibly to tertiary interactions with other side chains. Conversely, position 254 displays the highest mobility, a finding consistent with previous observations suggesting a turn in the backbone near this position (*28*, *35*). The other

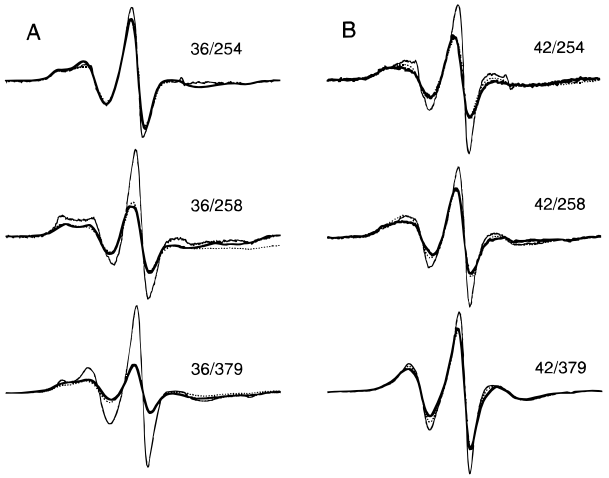

FIGURE 3: Room-temperature EPR spectra of given spin-labeled double-Cys mutants containing D36C (A) or K42C (B). The light tracing is the sum of the spectra from the two single mutants indicated. The heavy trace is the spectrum obtained with the indicated double mutant normalized to the same number of spins as the sum of spectra from the corresponding single-labeled mutants. The dotted line represents a fit to the double-labeled spectra with optimized Lorentzian broadening (see Experimental Procedures). All spectra were recorded with a 100 G scan width.

mutants exhibit intermediate mobilities (T258C < Q379C < K42C < G254C).

EPR of Double-Cys Mutants at Room Temperature. In the absence of spin−spin interaction, the EPR spectrum of a double-labeled permease mutant is expected to display a line shape comparable to that of the composite spectrum from two single-labeled samples acquired individually. Figure 3 shows EPR spectra for nitroxide-labeled permease double mutants with a Cys residue at position 36 (Figure 3A) or position 42 (Figure 3B) and position 254, 258, or 379 in comparison to the summed spectra from the corresponding single mutants. Except for the 36/254 pair, significant broadening and decreased intensity are observed with each of the other double-Cys mutants, when compared to the sum of the spectra from the corresponding single-Cys mutants. Thus, there is significant spin−spin interaction in nitroxide-labeled double-Cys mutants 36/379, 36/258, 42/254, 42/258, or 42/379.

As described under Experimental Procedures, the ΔH_d of the spectra was obtained by extracting the Lorentzian component of the envelope function (a sum of the spectra from the two individually labeled mutants) which exhibits the best fit to the double-labeled spectra (Figure 3, dotted line). The distance-dependent dipolar broadening arising from interacting spins is reflected by an increase in the spectral line width ΔH_d (see Experimental Procedures). The line widths are a steep function of interspin distance, as observed experimentally in model systems, and can be used to empirically determine whether the spins are very close (10 Å or less), close (10−15 Å), intermediate (15−20 Å), or far apart (>20 Å) (*36*).

As judged by ΔH_d measurement, the interaction between the 36/254 pair which is cross-linked only by longer cross-linkers (Table 1) is weak, while broadening is substantial with the 36/258, 42/254, 42/379, and 42/258 nitroxide-labeled paired Cys mutants (Table 3). Based on the strength of the spin−spin interaction, 36/379 is the closest pair, a conclusion

Table 3: Dipolar Broadening and the Estimated Interspin Distances of Double-Spin-Labeled lac Permease Mutants at Room Temperature

mutant	ΔH_d (G)	interspin distance range[a]
36 and 254	0.5	far
36 and 258	1.8	close
36 and 379	2.2	close
42 and 254	1.6	close
42 and 258	1.8	close
42 and 379	0.9	intermediate

[a] See text.

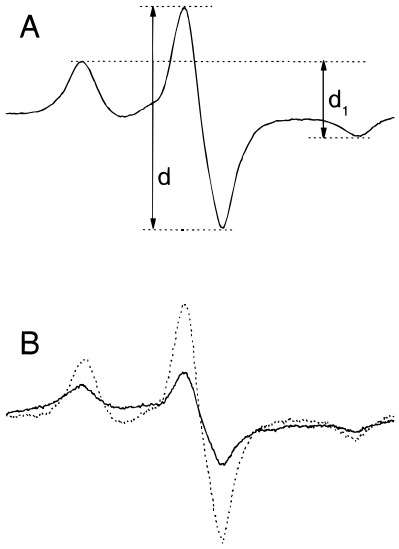

FIGURE 4: Frozen state EPR spectra of spin-labeled double-Cys mutants. (A) Spin-labeled double-Cys mutant 36/254, a pair showing no interaction; the positions of the two spectral intensities used to calculate distance, d_1 and d, are indicated. (B) Spin-labeled double-Cys mutant 36/379, a pair showing strong interaction (solid line), and reversal of the interaction by 1.0% sodium dodecyl sulfate (dotted line).

consistent with the observation that this is the only double-Cys mutant of the group that exhibits spontaneous disulfide formation. The results are consistent in general with site-directed cross-linking in which the closest pair, 36/379, cross-links spontaneously and shows the strongest spin−spin interaction, reflecting a close interspin distance (Table 3). The distances between nitroxides in the 36/258, 42/254, and 42/258 pairs are also estimated to be relatively close, while the 42/379 pair is assigned an intermediate distance due to weaker broadening. Finally, the 36/254 pair which can be cross-linked only by longer cross-linkers displays the weakest broadening, corresponding to a distance of about 20 Å or more between the nitroxides.

Spectra in the Frozen State. EPR measurements were also carried out in the absence of motion by collecting spectra in the frozen state. A measure of the extent of broadening due to dipolar interaction is obtained from the line height ratio d_1/d (Figure 4) (*37*). In the absence of dipolar interaction, a value of <0.4 is expected. Using this measure (d_1/d), values of 0.48 and 0.53 are obtained for the 36/258 and 36/379 pairs (Table 4), respectively, indicating relatively strong dipolar interaction. The d_1/d value for the other pairs is lower, in the range of 0.36−0.38, reflecting weaker interaction between nitroxides. Although weak, the values are suggestive of interaction, particularly when compared to d_1/d values of single-labeled mutants (Table 2) which are 0.32−0.33. Furthermore, addition of 1.0% sodium dodecyl sulfate to the double-labeled pairs 36/379, 42/254, or 42/258 reduces the d_1/d to values near that of the single-labeled mutants (compare Tables 2 and 4).

Table 4: Ratio of EPR Spectral Intensities (d_1/d Value) of Double-Spin-Labeled lac Permease Mutants in the Frozen State

double mutant	d_1/d	d_1/d (+SDS)[a]
36/254	0.36	nd[b]
36/258	0.48	nd
36/379	0.53	0.33
42/254	0.38	0.34
42/258	0.37	0.33
42/379	0.36	nd

[a] SDS, 1.0% sodium dodecyl sulfate. [b] nd, not determined.

DISCUSSION

Site-directed thiol cross-linking (*18*, *19*) indicates that periplasmic loop I/II is close to loops VII/VIII and XI/XII and, in addition, has provided important information regarding helix packing in lac permease (*13*−*17*). However, as emphasized by Chervitz and Falke (*20*), in particular, thiol cross-linking efficiency may depend on both dynamic collisions and the chemical reactivities of the paired Cys residues and not simply their proximities. In an attempt to examine this issue more carefully, the present study utilizing site-directed spin labeling was undertaken.

Uncertainty in the calculated values of spectral broadening depends on the quality of the alignment for convolution of single-labeled proteins, as well as the appropriateness of the double integration used to normalize the spectra. Spectra were recorded with locked-field frequency. Thus, misalignment errors that would artifactually broaden the convolution of the single-labels and diminish the magnitude of broadening due to dipolar interactions are insignificant with respect to the line width values presented here. Normalization of spectral intensities can be problematic, particularly if the signal-to-noise ratio limits the quality of the base line. Therefore, for normalization, spectra were evaluated that display a high-quality first integral with no appreciable negative region below the base line. On the other hand, possible incomplete labeling of either Cys residue must be considered as a potential source of error.

The room-temperature spectra reflect dynamics of the intermediate motional regime which are attributed to local segmental dynamics (*36*). How such dynamics modulate the interaction of two nitroxides is difficult to predict, and no suitable method for quantitatively describing the interaction currently exists. Since the scope of this study is to identify the proximity of domains within the lactose permease, the proximity of nitroxide pairs is described qualitatively according to the extent of line broadening in the double-labeled protein. Hustedt et al. (*38*) have introduced a powerful method for determining both the interspin distance and the individual nitroxide geometries for situations in which both probes are fixed and adopt unique orientations with respect to one another. Due to the observed mobility in solution of the spin labeled sites examined here, the nitroxide pairs in the frozen state likely contain a range of distances and orientations. Analysis of the static dipolar interactions

according to a Pake pattern has been presented (*21*, *39*). In these studies, a quantitative evaluation of interspin distances was carried out by assuming a random distribution in the orientations of spin label 1 relative to spin label 2. It is highly unlikely that such an isotropic distribution of angles exists in the system studied here, and the orientational dependence of observed dipolar splittings in the absence of motion has been demonstrated by simulation (*40*). We have limited our evaluation of static (frozen) pairs to a qualitative estimate of nitroxide proximities by using the d_1/d broadening parameter (*37*). However, as shown here, the use of a Guassian and Lorentzian convolution to evaluate the room temperature data clearly offers more sensitivity in the analysis of dipolar interactions. Analysis of nitroxide pairs under a range of orientational and dynamic circumstances is currently an intense area of study. It should be noted, however, that qualitative analysis is often sufficient to answer specific questions regarding the tertiary or quarternary structure of a given protein (*36*, *40*, *41*).

Dynamics of the protein backbone and of the two side chains relative to the backbone may generate a substantial distribution in the apparent distances separating the reactive or the spectroscopic moieties. While EPR measurements report the average distance separating two spins, thiol cross-linking measurements may be biased toward shorter distances, since even relatively infrequent collisions between reactive pairs may accumulate over time to a significant population of cross-linked product (*20*). In addition, where sterics allow, such as with the surface-exposed sites studied here, spin labeled side chains may adopt conformations that minimize contact. This consideration may also explain the weaker than expected interactions between the nitroxides when compared to the lengths of the reagents used to cross-link the same pairs.

As reported, EPR spectra of nitroxide-labeled double-Cys mutants 36/258 and 36/379 exhibit substantial spin−spin interaction at room temperature, while the spectrum of the 36/254 mutant does not, a conclusion confirmed by spectra obtained in the frozen state. In addition, room-temperature spectra of the spin-labeled 42/254, 42/258, and 42/379 double-Cys pairs exhibit line width broadening which is consistent with the notion that these pairs are in close proximity, although data obtained in the frozen state provide only marginal support for close proximity.

In summary, thiol cross-linking occurs between six double-Cys pairs located in two periplasmic domains of lac permease (*18*, *19*), and proximity relationships in five of the six pairs are now confirmed by EPR spectroscopy (36/254 displays little if any dipolar interaction). However, as reported previously for positions 148 (helix V) and 228 (helix VII) (*13*) and shown here, distances estimated from dipolar interaction are consistently greater than those estimated from thiol cross-linking. On the other hand, relative distances between pairs are reasonably comparable, indicating that the relationship between the pairs in the folded protein is similar as determined by either approach. In other words, the two methods are complementary although they do not yield the same results on a quantitative basis. Taking the observations as a whole, the data are consistent with the interpretation that thiol cross-linking is primarily a reflection of proximity, although other factors such as dyamics and reactivity may be of secondary importance.

ACKNOWLEDGMENT

We thank Cathy Lawrence for the excellent technical assistance in protein purification.

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BI982360T