Proximity of Helices VIII (Ala273) and IX (Met299) in the Lactose Permease of *Escherichia coli*†

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ABSTRACT: Three double-Cys mutant pairs—Ala273→Cys/Met299→Cys, Thr266→Cys/Ile303→Cys, and Thr266→Cys/Ser306→Cys—were constructed in a functional lac permease construct devoid of Cys residues, and the excimer fluorescence or electron paramagnetic resonance (EPR) was studied with pyrene- or spin-labeled derivatives, respectively. After reconstitution into proteoliposomes, excimer fluorescence is observed with mutant Ala273→Cys/Met299→Cys, but not with the single-Cys mutants nor with mutants Thr266→Cys/Ile303→Cys or Thr266→Cys/Ser306→Cys. Furthermore, spin−spin interaction is also observed with mutant Ala273→Cys/Met299→Cys, but only after the permease is reconstituted into proteoliposomes. The results provide independent support for the conclusions that helix VIII is close to helix IX and that the transmembrane helices of the permease are more loosely packed in a detergent micelle as opposed to a phospholipid bilayer.

The lactose permease (lac permease)[1] of *Escherichia coli* is a hydrophobic polytopic membrane transport protein encoded by the *lacY* gene. The permease has been solubilized from the membrane, purified, reconstituted into proteoliposomes, and shown to be solely responsible for β-galactoside transport (reviewed in *1*) as a monomer (see *2*). All available evidence indicates that the permease is composed of 12 α-helix rods that traverse the membrane with the N and C termini on the cytoplasmic face (Figure 1) (reviewed in *3*; see *4*−*10* in addition). Furthermore, site-directed mutagenesis of wild-type permease and Cys-scanning mutagenesis of a functional mutant devoid of Cys residues (Cys-less permease) indicate that only 6 of the 417 residues in the permease are irreplaceable with respect to ligand binding and/or coupling between lactose and H^+ translocation—Glu126 (helix IV), Arg144 (helix V), Glu269 (helix VIII), Arg302 (helix IX), His322 and Glu325 (helix X) (see *11*).

By using an assortment of techniques which include second-site suppressor analysis and site-directed mutagenesis, excimer fluorescence, engineered divalent metal binding sites, chemical cleavage, electron paramagnetic resonance (EPR), thiol cross-linking, and identification of discontinuous monoclonal antibody epitopes, a helix packing model has been formulated (reviewed in *12*). Based on the proximity of the irreplaceable residues and the properties conferred by mutating these residues, a mechanism for energy coupling in the permease has been proposed (*13*). One of the important features of the mechanism is that helix VIII is sufficently close to helix IX that the guanidino side chain of Arg302 can interact with the carboxylate side chains of either Glu269 (helix VIII) or Glu325 (helix X) during turnover (*14*).

Site-directed excimer fluorescence (SDEF) and site-directed spin-labeling (SDSL) are two particularly useful techniques for investigating helix proximity in lac permease (see *12*). Since pyrene exhibits excited-state dimer (excimer) fluorescence if the two conjugated ring systems are within about 3.5 Å (see *15*), the proximity between paired pyrene-labeled Cys residues can be determined if they are in the correct orientation. Moreover, interspin distances in the range of 8−25 Å between two spin-labeled Cys residues can be measured in the frozen state (*16*−*20*). Since the proximity between helices VIII and IX is particularly important, three mutants containing paired-Cys residues in these helices were constructed, purified, and studied by SDEF and SDSL. One of the paired-Cys mutants (A273C/M299C)[2] exhibits both excimer fluorescence and spin−spin interaction, but only after reconstitution into proteoliposomes, thereby providing strong independent evidence that helices VIII and IX are closely approximated.

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[1] Abbreviations: Cys-less permease, functional lactose permease devoid of Cys residues; DM, dodecyl β-D-maltoside; DTT, dithiothreitol; EPR, electron paramagnetic resonance; IPTG, isopropyl 1-thio-β-D-galactopyranoside; KPi, potassium phosphate; lac, lactose; NEM, *N*-ethylmaleimide; OG, octyl β-D-glucopyranoside; POPE, 1-palmitoyl-2-oleoylphosphatidylethanolamine; POPG, 1-palmitoyl-2-oleoylphosphatidylglycerol; SDEF, site-directed excimer fluorescence; SDSL, site-directed spin-labeling; TDG, β-D-galactopyranosyl 1-thio-β-D-galactopyranoside.

[2] Site-directed mutants are designated by the single-letter amino acid abbreviation for the targeted residue, followed by the sequence position of the residue in the wild-type lac permease, followed by a second letter indicating the amino acid replacement.

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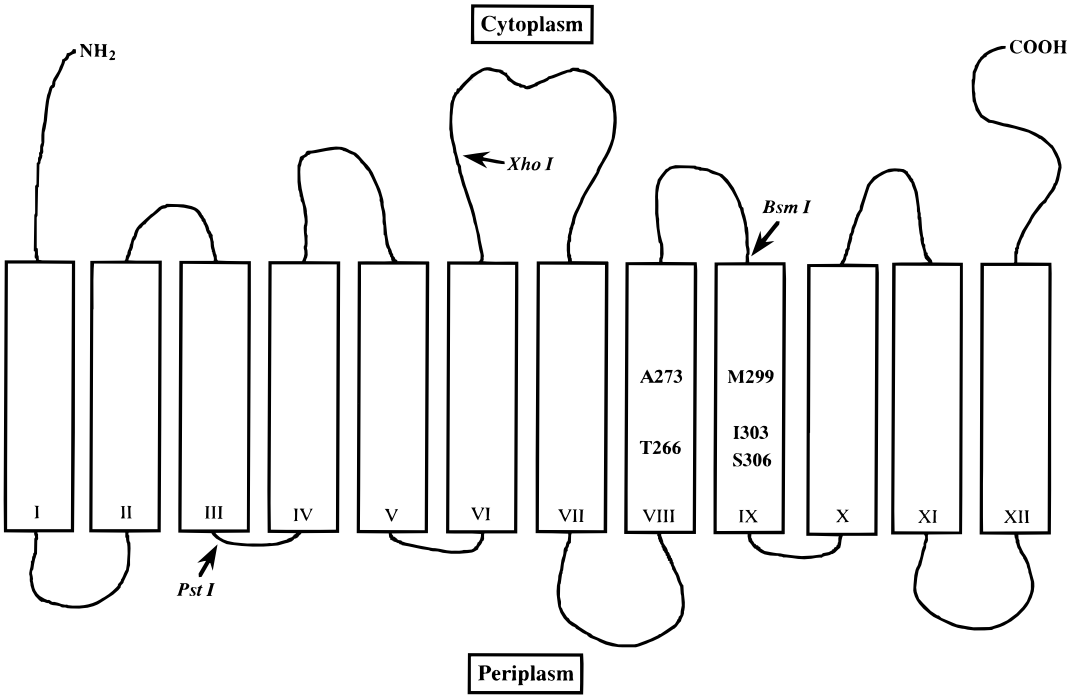

FIGURE 1: Secondary structure model of lac permease. Putative transmembrane helices are shown in boxes and numbered. The residues in helices VIII and IX that were replaced with Cys in the background of Cys-less permease are shown. Also indicated are the restriction endonuclease sites used for construction of the mutants.

MATERIALS AND METHODS

Materials. [1-^{14}C]Lactose and [α-^{35}S]dATP were purchased from Amersham, restriction endonucleases and T4 DNA ligase were from New England Biolabs, Sequenase was from United States Biochemicals, and β-D-galactopyranosyl 1-thio-β-D-galactopyranoside (TDG) was from Sigma. Proxyl methanethiosulfonate was from Reanal (Budapest, Hungary). *N*-(1-Pyrenyl)iodoacetamide was obtained from Molecular Probes, and 1-palmitoyl-2-oleoylphosphatidylethanolamine (POPE) and 1-palmitoyl-2-oleoylphosphatidylglycerol (POPG) were from Avanti Polar Lipids. All other materials were reagent grade obtained from commercial sources.

Bacterial Strains and Plasmids. E. coli T184 [*lacI*$^+$*O*$^+$*Z*$^-$*Y*$^-$*(A), rpsL, met*$^-$*, thr*$^-$*, recA, hsdM, hsdR/F′,* lacI^q*O*$^+$*Z*^{D118}*(Y*$^+$*A*$^+$*)*] (*21*) was transformed with plasmid pT7-5/*lacY*-L6XB (*22*) encoding Cys-less permease with given Cys replacements and used for expression of lac permease and transport studies. To facilitate purification, the DNA sequence encoding the biotin acceptor domain from the oxaloacetate decarboxylase of *Klebsiella pneumoniae* was cloned into the *Xho*I site of the DNA encoding the middle cytoplasmic loop of each mutant.

Construction of Mutants. Construction of the single-Cys mutants by site-directed mutagenesis has been described (*23, 24*). The double mutants were constructed by restriction fragment replacement making use of the endonuclease sites shown in Figure 1. Briefly, the double mutant A273C/M299C was constructed by digesting the plasmid carrying the A273C mutation with *Pst*I and *Bsm*I and ligating the isolated insert into the vector carrying the M299C mutation which was also digested with *Pst*I and *Bsm*I. Mutants T266C/I303C and T266C/S306C were constructed in a similar fashion.

DNA Sequencing. Double-stranded DNA sequencing after alkaline denaturation (*25*) was performed using dideoxy chain-termination (*26*).

Transport Measurements. Cells were washed with 100 mM potassium phosphate (KPi; pH 7.5)/10 mM $MgSO_4$ and adjusted to an OD_{420} of 10.0 (ca. 0.7 mg of protein/mL). For measurements in the presence of pyrenyliodoacetamide, cells were further washed with 10 mM Tris·HCl (pH 7.5)/2 mM EDTA, and then incubated with 0.5 mM pyrenyliodoacetamide for 15 min at room temperature. The reaction was quenched by the addition of 10 mM DTT, and cells were assayed for transport of [^{14}C]lactose (2.5 mCi/mmol; final concentration 0.4 mM) under O_2 in the presence of 20 mM potassium ascorbate and 0.2 mM phenazine methosulfate by rapid filtration (*27*).

Purification and Spin-Labeling of A273C/M299C Permease in DM. Mutant A273C/M299C with the biotin acceptor domain in the middle cytoplasmic loop was expressed in *E. coli* T184. Twelve liters of cells was cultivated in Luria−Bertini broth and induced with 0.2 mM isopropyl thio-β-D-galactopyranoside (IPTG). Membranes were prepared as described (*28*) and solubilized with 2% DM. Solubilized biotinylated permease was purified by affinity chromatography on immobilized monovalent avidin (*22*) with the modifications descibed (*29*). In brief, the avidin resin was equilibrated with column buffer containing 50 mM KPi (pH 7.5)/150 mM NaCl/0.02% DM (w/v) (column buffer I). The DM-soluble fraction was mixed with preequilibrated avidin resin for 30 min at 4 °C with continuous rotation. The slurry was then packed into a small column, and the unbound material was removed by washing extensively with column buffer I. Bound permease was spin-labeled on the resin by incubating with 100 μM proxyl methanethiosulfonate for 1 h at 4 °C, followed by washing the column with column buffer I to remove excess spin-label. Spin-labeled permease

was finally eluted with 5 mM *d*-biotin in column buffer I and concentrated with Micro-ProDiCon Membranes (Spectrum Medical Industries).

Purification, Labeling, and Reconstitution of Mutants A273C/M299C, T266C/I303C, T266C/S306C, A273C, or M299C. Each permease mutant with the biotin acceptor domain in the middle cytoplasmic loop was expressed in *E. coli* T184. Membranes were prepared as described above and solubilized with 1.25% octyl β-D-glucopyranoside (OG). The OG-soluble fraction was bound to monovalent avidin which had previously been equilibrated with column buffer containing 50 mM KPi (pH 7.5)/20 mM lactose/0.2 mg/mL 1-palmitoyl-2-oleoylphosphatidylethanolamine (POPE) and 1-palmitoyl-2-oleoylphosphatidylglycerol (POPG) (3:1; mol/mol)/200 mM KCl/1.25% OG (w/v) (column buffer II), and unbound material was removed by washing extensively with column buffer II. Bound permease was either spin- or pyrene-labeled on the resin by incubating with 100 μM proxyl methanethiosulfonate or 100 μM *N*-(1-pyrenyl)-iodoacetamide for 1 h at 4 °C, respectively, followed by washing the column with column buffer II to remove excess labeling reagents. Labeled permease was finally eluted with 5 mM *d*-biotin in column buffer II.

To prepare proteoliposomes, spin-labeled or pyrene-labeled permease in column buffer II was mixed with 1.35% OG (final concentration) and liposomes prepared from POPE and POPG (3:1 mol/mol); the lipid:protein ratio was adjusted to 20:1 (wt/wt). After incubation in ice for 15 min, the mixture was rapidly diluted into 45 volumes of 50 mM KPi (pH 7.5) as described (*1*). Proteoliposomes were harvested by centrifugation (150000*g*), washed twice with 50 mM KPi (pH 7.5), and resuspended in 50 mM KPi (pH 7.5) followed by two cycles of freeze−thaw/sonication. The protein concentration was adjusted to about 45 μM with spin-labeled permease and 1 μM with pyrene-labeled permease.

The purity of each preparation was assessed by electrophoresis on sodium dodecyl sulfate−12% polyacrylamide gels, followed by silver staining (*30*). Protein was determined as described (*31*). The quantity of pyrene covalently bound to the permease was determined by measuring pyrene absorption and determining the concentration by assuming an extinction coefficient of 2.2×10^4 $M^{-1}\cdot cm^{-1}$ at 340 nm. The efficiency of pyrene labeling was 70−80%.

Fluorescence Emission Spectra. The fluorescence of pyrene-labeled permeases in reconstituted proteoliposomes was measured at 25 °C with an SLM 8000C spectrofluorometer (SLM-Amico Instruments Inc.). An excitation wavelength of 340 nm was used, and emission spectra were recorded from 360 to 560 nm. Slits for excitation and emission were both 4 nm. The effect of TDG on fluorescence was determined by mixing 400 μL of a given purified permease preparation with 8 μL of a 0.5 M stock solution of TDG in 0.1 M KPi (pH 7.5). Emission spectra were recorded after 5 min incubation at 25 °C.

EPR Measurements with Spin-Labeled Permease Mutants. EPR measurements were carried out in a Varian E-104 X-band spectrometer fitted with a loop−gap resonator. Samples (10 μL) containing a final permease concentration of 100 μM in DM or 45 μM in proteoliposomes were placed in a sealed quartz capillary contained in the resonator. Room-temperature spectra were collected by signal-averaging 4−8 scans over 100 G (1 G = 0.1 mT) using a modulation amplitude of 2.5 G and a microwave power of 2 mW. To analyze dipolar interactions between spin-labels in the absence of dynamic effects, spectra were collected in the frozen state (183 K) using a microwave power of 0.05 mW and a modulation amplitude of 5 G.

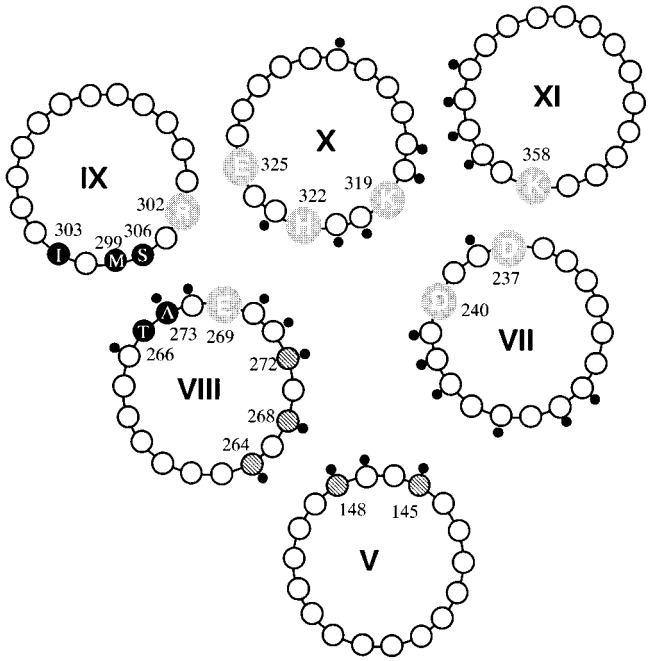

FIGURE 2: Packing of helices V and VII−XI in the permease viewed from the periplasmic surface. Four of the six irreplaceable residues (Glu269, Arg302, His322, and Glu325) and two interacting pairs of Asp-Lys residues [Asp237 (helix VII)/Lys358 (helix XI) and Asp240 (helix VII)/Lys319 (helix X)] are highlighted. Positions of NEM-sensitive Cys replacements are indicated with a small black dot. Substrate-protectable single-Cys replacement mutants (145, 148, 264, 268, and 272) are hatched. The double-Cys mutants in helices VIII and IX used in this study are shown as filled circles.

RESULTS

Active Transport. *E. coli* T184 (*lacZ*$^-$*Y*$^-$) was used to test the ability of the mutants to catalyze active lactose transport. All three double-Cys mutants transport lactose at significant rates, i.e., 70−90% of that of Cys-less permease. While the steady-state level of lactose accumulation for mutant A273C/M299C or T266C/I303C also approximates that of Cys-less permease, mutant T266C/S306C accumulates lactose to an intermediate level (30% of Cys-less) (Figure 3).

The effect of pyrenyliodoacetamide on the transport activity of each mutant was also studied. The activity of Cys-less permease is essentially unchanged by treatment with pyrenyliodoacetamide. However, mutant A273C/M299C is markedly inactivated, the initial rate by about 90% and the steady-state level of accumulation by about 80%. Mutant T266C/I303C is also inactivated significantly; the initial rate is decreased by about 75%, but the steady-state level of accumulation is inhibited by only about 10%. Although the initial rate of mutant T266C/S306C is inhibited by about 50% by pyrenylmaleimide treatment, unexpectedly, the steady-state level of accumulation is enhanced by about a factor of 2.

Fluorescence Emission Spectra of Pyrene-Labeled Permease. With purified, reconstituted double-Cys mutant

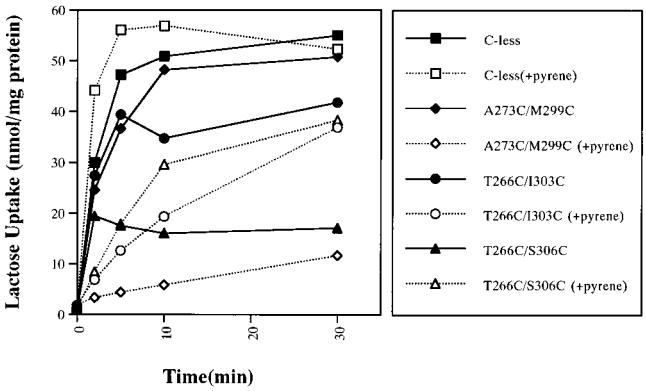

FIGURE 3: Active transport of lactose in *E. coli* T184 encoding Cys-less permease or permease with given double-Cys mutants in the absence or presence of pyrenyliodoacetamide. *E. coli* T184 transformed with plasmid pT7-5 encoding Cys-less, A273C/M299C, T266C/I303C, or T266C/S306C permease was grown and assayed as described under Materials and Methods.

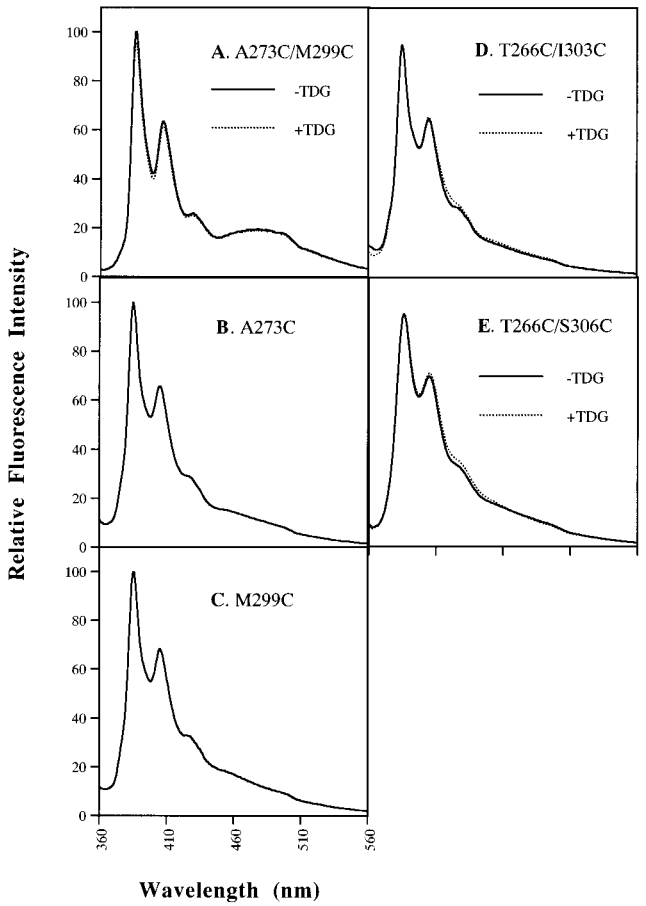

FIGURE 4: Fluorescence emission spectra of pyrene-labeled lac permease mutants reconstituted into proteoliposomes. Spectra were obtained with samples containing lac permease (1 μM, final concentration) in 50 mM KPi (pH 7.5) at room temperature. TDG (10 mM, final concentration) was added as indicated. The excitation wavelength was 340 nm, and data were collected as described under Materials and Methods.

A273C/M299C, a broad band centered around 470 nm which is typical of pyrene excimer fluorescence is clearly observed (Figure 4A). In addition, more defined maxima characteristic of the monomer are present at shorter wavelengths (i.e., ca. 378, 398, and 417 nm). In contrast, no excimer band is observed with mutant T266C/I303C or T266C/S306C (Figure 4D,E). Furthermore, no significant changes in the spectra

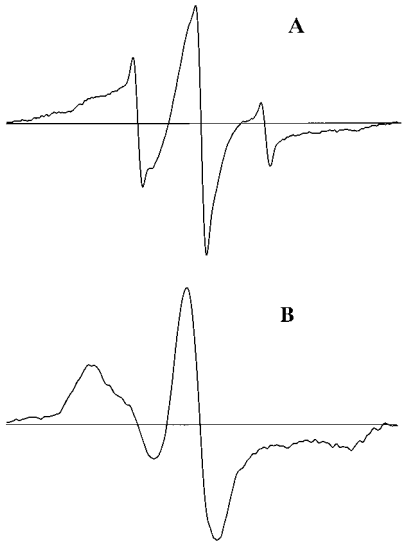

FIGURE 5: EPR spectra of spin-labeled A273C/M299C mutant permease at room temperature. (A) Permease in DM; (B) permease reconstituted into proteoliposomes. Experiments were carried out as described under Materials and Methods.

are observed after the addition of the substrate analogue β-D-galactopyranosyl 1-thio-β-D-galactopyranoside (TDG) with all three mutants.

Given the propensity of hydrophobic proteins such as lac permease to aggregate, it is essential to determine whether the excimer fluorescence observed with the A273C/M299C mutant results from an intramolecular rather than an intermolecular interaction. Therefore, each of the corresponding single-Cys mutants was analyzed (Figure 4B,C). Clearly, the emission spectra of the purified, pyrene-labeled, reconstituted A273C and M299C mutants exhibit characteristic monomer bands with maxima at 378, 398, and 417 nm, but no excimer band at 470 nm is observed. Therefore, it seems likely that the excimer fluorescence observed with A273C/M299C permease results from intramolecular interaction between pyrene molecules attached to Cys residues within a single molecule.

EPR of Spin-Labeled A273C/M299C Permease. The line shape of the EPR spectrum at room temperature with purified, spin-labeled A273C/M299C permease in reconstituted proteoliposomes indicates that the nitroxides are immobilized (Figure 5B). In comparison, when the permease is solubilized in DM, the nitroxides are more mobile, as indicated by the line shape of the low-field peak of the spectrum (Figure 5A). This observation is consistent with previous findings describing looser packing of the helices in detergent micelles relative to that in a phospholipid bilayer (*14*, *32*, *33*). The notion is further confirmed by the observation that the high-field line shape of the spectrum with the proteoliposomes is depressed (Figure 5B), due probably to a population of dipolar interacting spins in close proximity. The spectrum displays broadened features consistent with a distance of <20 Å between the two probes (*34*). In contrast, the spectrum of the sample in DM displays little or no dipolar broadening, indicating little if any interaction between the two nitroxides. Although spectra are not shown, with spin-labeled mutant T266C/I303C or T266C/S306C reconstituted into proteoliposomes, no dipolar broadening is observed.

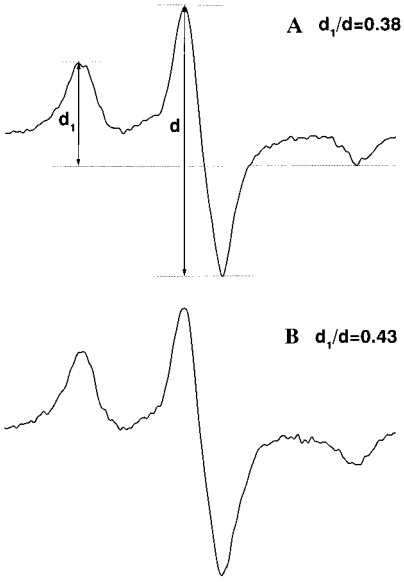

FIGURE 6: EPR spectra of spin-labeled A273C/M299C mutant permease in the frozen state. (A) Permease in DM; (B) permease reconstituted into proteoliposomes. Experiments and data analysis were carried out as described under Materials and Methods.

To evaluate broadening due to static dipolar interaction only, quantitative analysis of the interspin distance was carried out in the absence of motion by collecting spectra in the frozen state (Figure 6). An estimate of the extent of broadening due to dipolar interaction is obtained from the line height ratio d_1/d (*17*). In the absence of interaction, a value of <0.4 is expected. The d_1/d value of the A273C/M299C mutant in DM (Figure 6A) or in reconstituted proteoliposomes (Figure 6B) is 0.38 or 0.43, respectively. This is consistent with the room-temperature findings indicating that the nitroxide pair is in close proximity following reconstitution but not close enough to cause dipolar broadening when the permease is solubilized in DM. Empirical estimation of the d_1/d values yields an approximate distance of 14−16 Å for the sample in proteoliposomes and >20 Å for the sample in DM. The d_1/d value for spin-labeled T266C/I303C or T266C/S306C permease in proteoliposomes is 0.37 and 0.33, respectively, indicating no spin−spin interaction between the two probes in either of these mutants (data not shown).

DISCUSSION

Helix packing is an important aspect of the mechanistic model for energy coupling proposed for lac permease (*13*). Thus, placement and orientation of helix VIII are essential not only because helix VIII contains Glu269, an essential residue proposed to interact with either His322 (helix X) or Arg302 (helix IX) during turnover (*14*), but also because helix VIII contains residues that are thought to comprise part of the substrate translocation pathway (*23*, *35*).

Placement of helix VIII in the helix packing model of the permease (Figure 2) is based upon the following previous observations: (i) Pyrene-labeled E269C/H322C permease exhibits excimer fluorescence (*15*), and mutant E269H which contains His residues at positions 269 and 322 binds Mn(II) with high affinity (*36*). Thus, helix VIII is in close proximity to helix X. (ii) Site-directed chemical cleavage (*37*) and SDSL (*20*) place helix VIII in close proximity to helix V. (iii) The epitope for monoclonal antibody 4B11 is composed of cytoplasmic loops VIII/IX and X/XI (*7*), thereby placing helices VIII to XI in close proximity. (iv) Most recently, it has been shown by engineering a Mn(II) binding site and by SDSL that Arg302 (helix IX) is sufficiently close to helix VIII to interact with Glu269 (*14*) in addition to Glu325 (helix X) (*15*, *38*).

For this study, the double-Cys mutant A273C/M299C was constructed in which Cys replacements are present at positions one turn up from the irreplaceable residues Glu269 and Arg302 in helices VIII and IX, respectively, and it is demonstrated by SDEF that position 273 (helix VIII) is in close proximity to position 299 (helix IX). Thus, pyrene-labeled A273C/M299C permease reconstituted into proteoliposomes exhibits excimer fluorescence, indicating that the two pyrene rings are within about 3.5 Å. Since no excimer band is observed with either of the single-Cys mutants A273C or M299C, the excimer observed with A273C/M299C permease cannot be due to aggregation and must be due to an interaction between the two pyrene moieties in the same molecule. Further evidence for close proximity between Ala273 and Met299 is provided by SDSL. Room-temperature spectra of spin-labeled mutant A273C/M299C are suggestive of possible dipole−dipole interaction between the two spins, and more definitively, EPR spectra in the frozen state yield an interspin distance of about 14−16 Å. Possibly, the discrepancy in the distance measurements between SDEF and SDSL results from differences in the orientation of the two sets of probes in the tertiary structure. In a similar vein, spin−spin measurements in mutant Cys148/Y228C yield a distance of about 11−13 Å in reconstituted proteoliposomes, but the two Cys residues are cross-linked in native membranes by dibromobimane which is only about 5 Å in length (*20*).

It is noteworthy that the spin−spin interaction in mutant A273C/M299C is stronger when the permease is reconstituted into proteoliposomes. The observation is consistent with the notion that the helices in the permease may be more loosely packed in detergent micelles versus a phospholipid bilayer (*14*, *15*, *33*). On the other hand, many observations provide a strong indication that lac permease retains near-native structure in DM (*5*−*7*, *15*, *29*, *36*, *38*−*41*).

SDEF and SDSL between helices VIII and IX was also used to probe ligand-induced conformational changes at the interface between the two helices. However, no significant changes in the fluorescence or EPR spectra are observed in the presence of the high-affinity analogue TDG. This is disappointing, but not surprising because the permease is inactivated by modification with pyrenyliodoacetamide (Figure 3). Although measurements were not made, it is presumed that the spin-label has a similar inactivating effect, as the single-Cys mutant A273C is inactivated by more than 75% with *N*-ethylmaleimide (*23*).

In a continuous effort to study dynamic aspects of lac permease, two other mutants, T266C/I303C and T266C/S306C, were constructed and studied, since the single-Cys mutant T266C is not inactivated by NEM (*23*) and the two pairs are also expected to be in close proximity (Figure 2). However, no excimer fluorescence or spin−spin interaction is observed with the two mutants. Although disappointing, this is not surprising because the orientation of the side chains and the attached probes may vary greatly and/or the helices

may be tilted so that the residues in question are not as close at the A273C/M299C pair.

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