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Proximity between Glu126 and Arg144 in the Lactose Permease of *Escherichia coli*†

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ABSTRACT: Evidence has been presented [Venkatesan, P., and Kaback, H. R. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 9802−9807] that Glu126 (helix IV) and Arg144 (helix V) which are critical for substrate binding in the lactose permease of *Escherichia coli* are charge paired and therefore in close proximity. To test this conclusion more directly, three different site-directed spectroscopic techniques were applied to permease mutants in which Glu126 and/or Arg144 were replaced with either His or Cys residues. (1) Glu126→His/Arg144→His permease containing a biotin acceptor domain was purified by monomeric avidin affinity chromatography, and Mn(II) binding was assessed by electron paramagnetic resonance spectroscopy. The mutant protein binds Mn(II) with a K_D of about 40 μM at pH 7.5, while no binding is observed at pH 5.5. In addition, no binding is detected with Glu126→His or Arg144→His permease. (2) Permease with Glu126→Cys/Arg144→Cys and a biotin acceptor domain was purified, labeled with a thiol-specific nitroxide spin-label, and shown to exhibit spin−spin interactions in the frozen state after reconstitution into proteoliposomes. (3) Glu126→Cys/Arg144→Cys permease with a biotin acceptor domain was purified and labeled with a thiol-specific pyrene derivative, and fluorescence spectra were obtained after reconstitution into lipid bilayers. An excimer band is observed with the reconstituted E126C/R144C mutant, but not with either single-Cys mutant or when the single-Cys mutants are mixed prior to reconstitution. The results provide strong support for the conclusion that Glu126 (helix IV) and Arg144 (helix V) are in close physical proximity.

The lactose permease (lac permease)[1] of *Escherichia coli*, a paradigm for secondary active transport proteins that transduce free energy stored in an electrochemical ion gradient into work in the form of a concentration gradient, catalyzes the stoichiometric symport of β-galactosides and H^+ (reviewed in ref *1*). The permease is encoded by the *lacY* gene of the *lac* operon (*2*) which has been cloned and sequenced. The *lacY* gene product has been solubilized, purified, reconstituted into proteoliposomes, and shown to

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[1] Abbreviations: lac permease, lactose permease; Cys-less permease, functional permease devoid of Cys residues; SDSL, site-directed spin-labeling; EPR, electron paramagnetic resonance; DM, *n*-dodecyl β-D-maltoside; KP_i, potassium phosphate.

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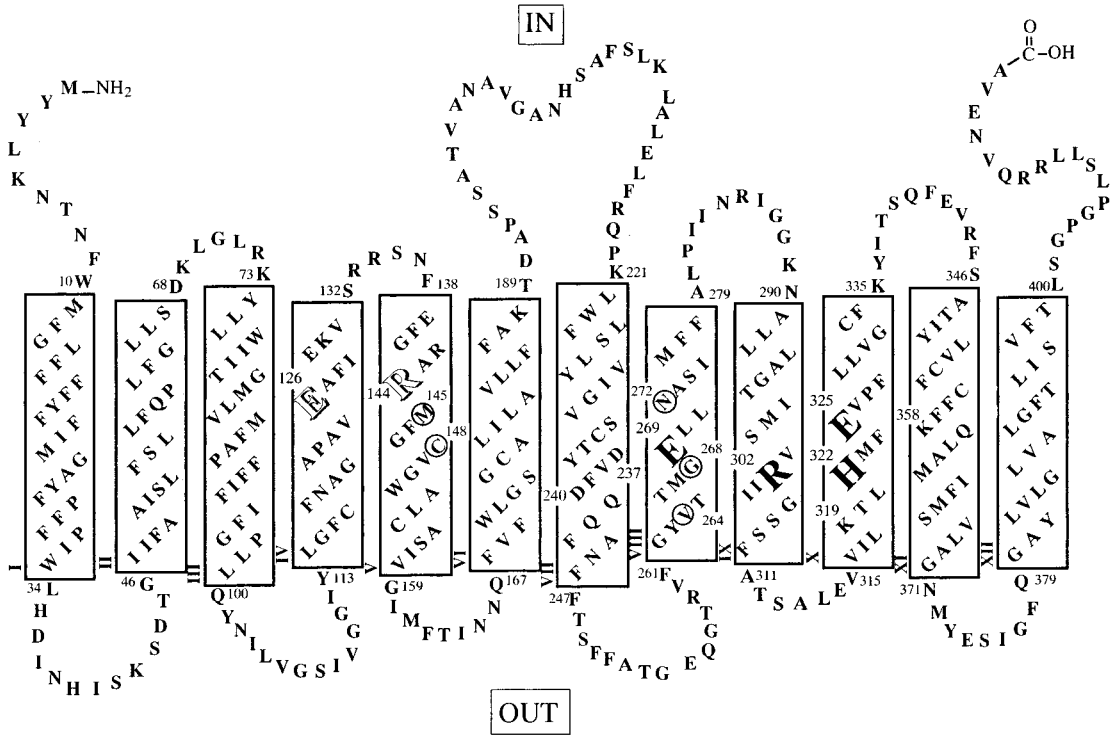

FIGURE 1: Secondary structure model of lac permease. The one-letter amino acid code is used, and putative transmembrane helices are shown in boxes. Residues irreplaceable with respect to active transport are highlighted. Glu126 (helix IV) and Arg144 (helix V) which are required for substrate binding are in white enlarged letters; Glu269 (helix VIII), Arg302 (helix IX), and His322 and Glu325 (helix X) which are irreplaceable with regard to H^+ translocation and/or coupling are in black enlarged letters. Asp237 (helix VII) and Lys358 (helix XI) and Asp240 (helix VII) and Lys319 (helix X) which are charge paired are numbered. Nonessential residues thought to be involved in substrate translocation [Met145 and Cys148 (helix V) and Val264, Gly268, and Asn272 (helix VIII)] are encircled.

be solely responsible for β-galactoside transport (reviewed in ref *3*) as a monomer (see ref *4*). All available evidence (reviewed in refs *5*−*7*) indicates that the permease consists of 12 α-helices that traverse the membrane in zigzag fashion connected by hydrophilic loops with the N- and C-termini on the cytoplasmic side of the membrane (Figure 1).

In a functional permease mutant devoid of native Cys residues, each residue has been replaced with Cys (reviewed in ref *8*). Analysis of the mutant library has led to the following developments (see refs *6*−*9*). (1) The great majority of the mutants are expressed normally in the membrane and exhibit significant activity, and only six side chains are clearly irreplaceable with respect to active transport. (2) Helix packing, tilts, and ligand-induced conformational changes have been determined by using a battery of site-directed biochemical and biophysical techniques. (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 and H^+ symport has been formulated.

Among the last residues to be subjected to mutagenesis, Glu126 (helix IV) and Arg144 (helix V) are irreplaceable with respect to all translocation reactions catalyzed by the permease (*10*). By studying the substrate binding properties of Glu126 and Arg144 mutants using site-directed *N*-ethylmaleimide labeling of Cys148 (*11*), Venkatesan and Kaback showed that replacement of either Glu126 or Arg144 with Ala markedly decreases Cys148 reactivity, while interchanging the residues, double-Ala replacement or replacement of Arg144 with Lys or His, does not alter reactivity, thereby indicating that Glu126 and Arg144 are charge paired. Furthermore, although Glu126 and Arg144 were initially placed at the membrane−water interface at the cytoplasmic ends of helices IV and V, respectively (*12*), studies with amino acid deletion mutants (C. D. Wolin and H. R. Kaback, in press), nitroxide scanning and accessibility measurements (M. Zhao, K.-C. Zen, J. Hernandez-Borrell, W. L. Hubbell, and H. R. Kaback, manuscript in preparation), and lac permease fusions with the NG domain of *FtsY* (E. Bibi, personal communication) indicate that both residues are embedded in the membrane, probably about two helical turns from the cytoplasmic ends of helices IV and V. Importantly, although alkylation of Cys148 is blocked by ligand in wild-type permease, no protection is observed with any of the Glu126 or Arg144 mutants. Further evidence for the essential role of Glu126 and Arg144 in substrate binding has been obtained by site-directed fluorescence studies (*11*) and by direct binding assays (*13*). The results indicate that Glu126 and Arg144 are charge paired and indispensable with respect to substrate binding, and a model for the binding site has been postulated (*11*).

In this report, three site-directed techniques are used to determine the proximity between Glu126 and Arg144. Glu126 and Arg144 were replaced with His residues, and the mutant permease was purified and shown to bind Mn(II) at pH 7.5 with a K_D in the micromolar range. Glu126 and Arg144 were replaced with Cys residues, and site-directed spin-labeling (SDSL) was employed to demonstrate that the paired nitroxides exhibit spin−spin interactions. The double-Cys mutant was labeled with pyrene and shown to

exhibit excimer fluorescence. The results demonstrate that positions 126 and 144 in helices IV and V, respectively, are in close proximity in the tertiary structure of the permease.

EXPERIMENTAL PROCEDURES

Materials. Deoxyoligonucleotides were synthesized on an Applied Biosystems 391 DNA synthesizer. Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA). *Taq* DNA polymerase was purchased from Promega (Madison, WI), immobilized monomeric avidin from Pierce (Rockford, IL), and $MnCl_2$ (highest purity) from Aldrich (Milwaukee, WI). The proxyl methanethiosulfonate spin-label was obtained from Reanal (Budapest, Hungary). All other materials were reagent grade and were obtained from commercial sources.

Plasmid Construction. E126H,[2] R144H, and E126H/R144H permease mutants were constructed by oligodeoxynucleotide-directed, site-specific mutagenesis in plasmid pT7-5/cassette *lacY* encoding wild-type permease by using either one- or two-step PCR. The PCR products were digested with restriction endonucleases *Pst*I, *Bst*BI, and *Xho*I and ligated to similarly treated plasmid pKR35/cassette *lacY*-CXB (encoding wild-type permease containing a biotin acceptor domain at the C-terminus) (*14*). Mutant E126C/R144C was constructed by restriction fragment replacement of DNA encoding E126C/R144C in pKR35/Cys-less *lacY*-CXB (encoding Cys-less permease containing a biotin acceptor domain at the C-terminus) (*11*).

Growth of Cells. E. coli T184 (*lacZ*$^-$*Y*$^-$) cells transformed with a given plasmid were grown aerobically at 37 °C in 1 L of Luria-Bertani broth containing streptomycin (10 μg/mL) and ampicillin (100 μg/mL). Overnight cultures were diluted into 12 L and grown aerobically for 2 h before induction with 0.3 mM isopropyl 1-thio-β-D-galactopyranoside. After additional growth for 2 h, cells were harvested and used for membrane preparation.

Purification and Labeling. Membranes were prepared as described previously (*15*) and solubilized with 2% *n*-dodecyl β-D-maltoside (DM). Solubilized biotinylated permease was purified by affinity chromatography on immobilized monovalent avidin (*14*) with the modifications described in ref *16*. In brief, the avidin resin was equilibrated with column buffer containing 50 mM potassium phosphate (KP_i; pH 7.5)/150 mM NaCl/0.02% DM (w/v) (column buffer I). The DM-soluble fraction was mixed with pre-equilibrated 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. Where indicated, bound permease was either spin-labeled or fluorescently labeled on the resin by incubating with 100 μM proxyl methanethiosulfonate or 100 μM *N*-(1-pyrenyl)-iodoacetamide, respectively, for 1 h at 4 °C, followed by washing the column with column buffer I to remove unreacted label. Labeled permease was finally eluted with 5 mM D-biotin in column buffer I and concentrated with Micro-ProDiCon Membranes (Spectrum Medical Industries, Houston, TX). The buffer was dialyzed to adjust the pH to 7.5 or 5.5 in 10 mM MES [treated with Chelex-100 (Bio-Rad)]/0.01% DM, as indicated. The purified protein was analyzed by sodium dodecyl sulfate−12% polyacrylamide gel electrophoresis (*17*) and visualized by silver or Coomassie blue staining. The protein was assayed using a Micro BCA kit (Pierce).

Mn(II) Binding. Purified E126H, R144H, or E126H/R144H permease (120 μM final concentration) was incubated with freshly prepared $MnCl_2$ (final concentration ranging from 40 to 400 μM) for 30 min. Electron paramagnetic resonance (EPR) spectra were then acquired on a Varian E-104 X-band spectrometer fitted with a loop-gap resonator at room temperature with the samples sealed in quartz capillaries. Each spectrum was the average of three scans over 1000 G (1 G = 0.1 mT) using a 0.032 s time constant, a 2 mW microwave power, and a 10 G modulation. Free Mn(II) was assessed using the average peak-to-peak heights of the middle four lines of the EPR spectrum, and calibrated against a standard curve determined at various Mn(II) concentrations at a given pH.

Reconstitution. To prepare proteoliposomes, 150 μM spin- or pyrene-labeled permease was mixed with octyl glucoside (OG) to a final concentration of 1.25% before addition of liposomes prepared from 1-palmitoyl-2-oleoylphosphatidylethanolamine and 1-palmitoyl-2-oleoylphosphatidylglycerol (3:1 molar ratio). The lipid:protein ratio was adjusted to 50:1 (w/w). After incubation on ice for 15 min, the mixture was rapidly diluted into a 240-fold volume of 50 mM KP_i (pH 7.5), and the proteoliposomes were harvested by ultracentrifugation. The pellets were resuspended in the same buffer followed by two cycles of freezing−thawing and sonication, and the protein concentration was adjusted to 50 μM.

EPR Spectra of the Spin-Labeled E126C/R144C Permease. EPR spectra were obtained with 10 μL of a permease sample at a final protein concentration of 150 μM in DM or 50 μM in proteoliposomes in sealed quartz capillaries. Room-temperature spectra (295 K) are the average of six scans over 100 G using a 0.032 s time constant, a 2.5 G modulation, and a 2 mW microwave power. To analyze dipolar interactions in the absence of side chain motion, frozen-state (243 K) spectra were collected, and the average of six scans over 200 G using a 0.032 s time constant, a 5 G modulation, and a 0.5 mW microwave power is presented.

RESULTS

Mn(II) Binding by E126H/R144H Permease. To determine whether E126H/R144H permease binds divalent metal, the EPR spectrum of Mn(II) was measured in the absence or presence of purified protein. The paramagnetic ion Mn(II) has a high spin with a sufficiently long relaxation time so that spectra can be obtained at room temperature. Six lines arising from the electron−nuclear spin coupling ($I = {}^5/_2$) are characteristic for this transition metal (Figure 2). Because Mn(II) EPR signals are readily observed when the metal is free in solution, but not when the ion is complexed with protein, the extent of binding can be determined directly by measuring the free Mn(II) concentration in the absence and presence of protein (*18*). Clearly, E126H/R144H permease causes a significant reduction in Mn(II) signal amplitude, while E126H or R144H permease does not alter the Mn(II) EPR spectrum.

Metal ion binding at pH 7.5 was then titrated at various Mn(II) concentrations using a constant amount of E126H/

[2] Site-directed mutants are designated as follows. The one-letter amino acid code is used followed by a number indicating the position of the residue which is followed by the desired mutation at the position.

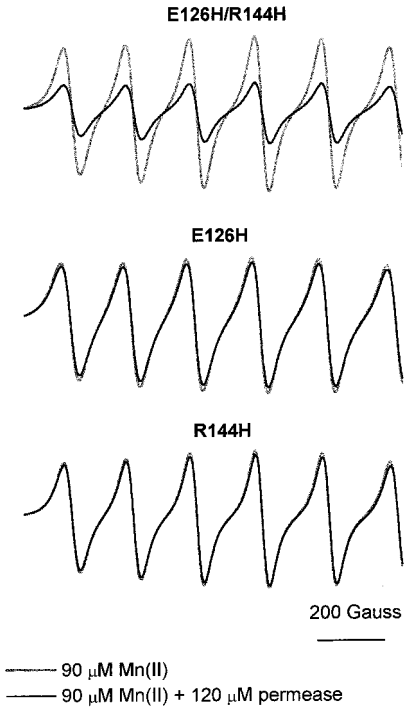

FIGURE 2: Mn(II) binding as assessed by EPR for E126H/R144H, E126H, or R144H permease. EPR spectra were obtained at room temperature with 90 μM $MnCl_2$ in 10 mM MES (pH 7.5)/0.01% DM in the absence (thick gray lines) or presence of E126H/R144H, E126H, or R144H permease (thin black lines) at a final concentration of 120 μM, as described in Experimental Procedures.

Table 1: Concentrations of Free Mn(II) As Determined by EPR[a]

[Mn(II)] in buffer (μM)	[Mn(II)] with E126H/R144H (μM)	Mn(II) bound (mol/mol of protein)
44.8	14.0	0.256
89.6	35.5	0.451
134.4	57.6	0.689
179.2	95.9	0.694
268.8	177.5	0.760
358.4	251.4	0.892

[a] The concentration of free Mn(II) was determined by EPR in 10 mM MES (pH 7.5)/0.01% DM in the absence or presence of E126H/R144H permease (120 μM final concentration) as described in Experimental Procedures. The values represent averages obtained from two independent experiments.

R144H permease, and the data were analyzed according to Scatchard (*19*). In two independent experiments, the total Mn(II) concentration was varied over a range of concentrations from 40 to 400 μM, and the free Mn(II) concentration was measured in the absence and presence of E126H/R144H permease at a final concentration of 120 μM (Table 1). Bound Mn(II) was calculated from the difference in the spectra in the absence and presence of E126H/R144H permease. A Scatchard plot of the data (Figure 3) reveals that the number of binding sites per protein molecule (n) approaches unity, and the equilibrium dissociation constant (K_D) is 42 μM. At pH 7.5, E126H/R144H permease binds 1 mol of Mn(II) per mole of protein, while at pH 5.5, no significant binding is observed (data not shown; see refs *21*−*23*).

SDSL of E126C/R144C Permease. To approximate the distance between positions 126 and 144, SDSL of E126C/R144C permease was carried out both in reconstituted proteoliposomes and in DM. As reflected by the broadening

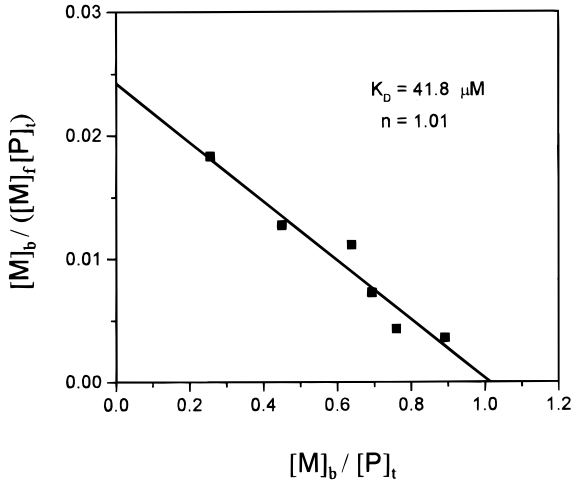

FIGURE 3: Concentration dependence of Mn(II) binding to E126H/R144H permease. The data in Table 1 were plotted according to Scatchard (*19*). $[M]_f$ is the free Mn(II) concentration; $[M]_b$ is the bound Mn(II) concentration, and $[P]_t$ is the total protein concentration.

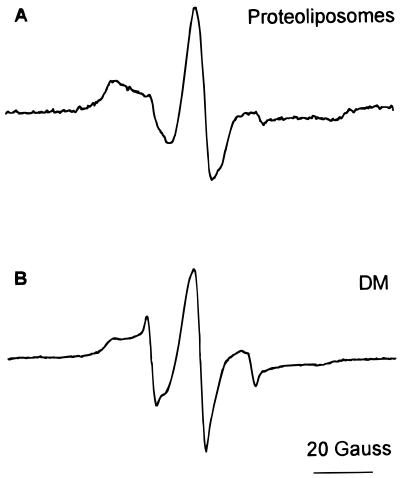

FIGURE 4: EPR spectra of spin-labeled E126C/R144C mutant permease at room temperature: (A) permease reconstituted in proteoliposomes and (B) permease in detergent DM. Experiments and data analysis were carried out as described in Experimental Procedures.

of the line shape in the EPR spectrum (*20*), spin−spin interactions can be detected in reconstituted proteoliposomes (Figure 4A), but not in DM (Figure 4B). In addition, the line shapes of both spectra suggest that the nitroxide side chains are immobilized, although the sharper lines of the sample in DM indicate more motional freedom of spin-labels relative to that of proteoliposomes. The observation is consistent with the notion that the permease is in a more tightly packed conformation in the lipid bilayer than in DM micelles, resulting in a shorter average distance between the nitroxides at positions 126 and 144 in the reconstituted sample. Moreover, the strength of the nuclear−electron spin coupling observed with E126C/R144C permease indicates that the spin-labels are located in a relatively hydrophilic environment, with the sample in lipid bilayers being slightly more hydrophilic than in DM.

In an effort to assess the distance between paired spin-labels more quantitatively, spectra were collected in the frozen state (243 K). The ratio d_1/d (Figure 5A; *20*) can be used to estimate empirically the strength of static spin−spin

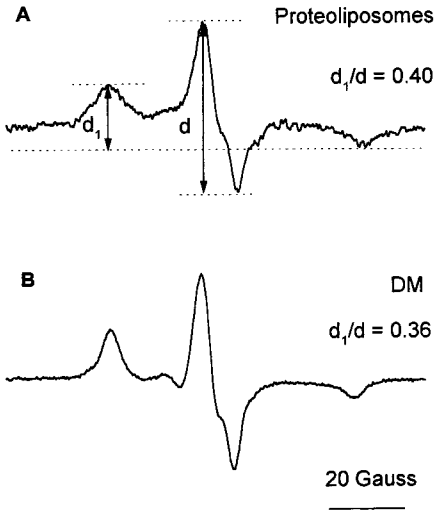

FIGURE 5: EPR spectra of spin-labeled E126C/R144C mutant permease in the frozen state: (A) permease reconstituted in proteoliposomes and (B) permease in detergent DM. Experiments and data analysis were carried out as described in Experimental Procedures.

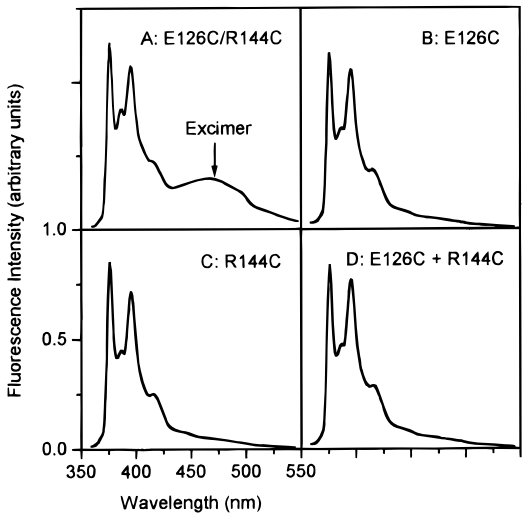

FIGURE 6: Fluorescence emission spectra of pyrene-labeled, purified lac permease mutants reconstituted in proteoliposomes. Spectra were acquired from samples containing lac permease (50 μM final concentation) in 50 mM KP_i (pH 7.5) at 22 °C. The excitation wavelength was 344 nm. For E126C + R144C (panel D), the purified single-Cys mutants were mixed at equimolar concentrations prior to reconstitution into proteoliposomes as described in Experimental Procedures.

interactions, and hence the distance separating the pair of interacting spin-labels. In proteoliposomes, the frozen-state spectrum of the E126C/R144C permease yields a d_1/d ratio of 0.40 (Figure 5A), while in frozen DM micelles, a value of only 0.36 is obtained (Figure 5B), suggesting that there are static spin−spin interactions in proteoliposomes, but not in DM micelles.

Site-Directed Excimer Fluorescence of E126C/R144C Permease. With purified, reconstituted double-Cys mutant E126C/R144C labeled with pyrene, a broad band centered around 470 nm typical of pyrene excimer fluorescence is clearly observed (Figure 6A). In addition, more defined maxima characteristic of the monomer are present at shorter wavelengths (i.e., ca. 378, 398, and 417 nm). Given the propensity of hydrophobic proteins such as lac permease to aggregate, it is essential to determine whether the excimer fluorescence observed with the E126C/R144C mutant results from an intramolecular rather than from an intermolecular interaction. Thus, each of the corresponding single-Cys mutants was analyzed. Clearly, the emission spectra of the purified, pyrene-labeled, reconstituted E126C (Figure 6B) and R144C (Figure 6C) mutants exhibit characteristic monomer bands with maxima at 378, 398, and 417 nm, but no excimer band at 470 nm is observed. Furthermore, when the purified pyrene-labeled single-Cys mutants are mixed and then reconstituted into proteoliposomes, no excimer band is observed (Figure 6D). Therefore, the excimer fluorescence observed with E126C/R144C permease results from intramolecular interaction between pyrene molecules attached to Cys residues within a single molecule.

DISCUSSION

One important aspect of the model for the substrate binding site in lac permease (*11*) is that Glu126 (helix IV) and Arg144 (helix V) are charge paired and thus in close physical proximity in the tertiary structure. In the studies presented here, this conclusion receives strong support from the demonstration that E126H/R144H has the ability to bind the divalent metal Mn(II) with a high affinity in a pH-dependent fashion, from site-directed spin-labeling of E126C/R144C permease, and from site-directed excimer fluorescence with the double-Cys mutant.

E126H/R144H permease binds Mn(II) stoichiometrically with a high affinity at pH 7.5 where the imidazole group of His is expected to be unprotonated. Conversely, binding is undetectable with E126H/R144H permease at pH 5.5 where the imidazole group is expected to be protonated or with the single mutants E126H and R144H which do not contain bis-His residues. The observation that neither E126H nor R144H permease binds Mn(II) is important in that it demonstrates that neither position is sufficiently close to native His322 (helix X) to form a divalent metal binding site (see ref *9*). In addition, as shown previously (*21*), wild-type permease does not bind Mn(II). The K_D of 42 μM observed with E126H/R144H permease is comparable to the K_D values observed for other permease mutants containing engineered metal-binding sites (*21*−*24*). The relatively high affinity for Mn(II) of the engineered bis-His residues in lac permease is consistent with the notion of "high hydrophobicity contrast" (*25*), suggesting that metal-binding sites are usually centered in a shell of hydrophilic ligands surrounded by more hydrophobic side chains. Thus, the high-contrast environment provides a site conducive to binding of metal ions. In this context, the bis-His residues at positions 126 and 144 are probably located within the membrane about two helical turns from the cytoplasmic surface (Figure 1; C. D. Wolin and H. R. Kaback, in press; M. Zhao, K.-C. Zen, J. Hernandez-Borrell, W. L. Hubbell, and H. R. Kaback, in preparation). Since positions 126 and 144 are readily accessible to Mn(II) and bind the metal at pH 7.5 but not at pH 5.5, it is likely that the site is accessible to the aqueous phase. This conclusion is consistent with observations demonstrating that Cys148 which is on the same face of helix V as Arg144, but one turn removed, is clearly accessible to solvent from both sides of the membrane (*26*).

SDSL provides further evidence for the proximity of Glu126 and Arg144. Room-temperature spectra of spin-labeled E126C/R144C permease reconstituted into proteoliposomes demonstrate features, albeit weak, of dipolar interactions between the paired spin-labels. Further evidence of spin−spin interaction is derived from frozen-state EPR spectroscopy. A d_1/d ratio of 0.40 for permease reconstituted in proteoliposomes is consistent with spin−spin interactions, while a d_1/d ratio of 0.36 for the protein in DM indicates little or no interaction. Furthermore, the downfield peak in the frozen spectrum of the reconstituted permease (d_1 in Figure 5A) appears to contain a sharp component (noninteracting) superimposed on a broad component (interacting), suggesting that the d_1/d ratio that is obtained may be underestimated. In any case, it is important that pyrene-labeled E126C/R144C permease exhibits a clear excimer band, while the single-Cys mutants E126C and R144C do not, even when they are mixed prior to reconstitution. Furthermore, bis-Cys residues at positions 126 and 144 cross-link spontaneously in a manner that is reversed by dithiothreitol (C. D. Wolin and H. R. Kaback, manuscript in preparation). Therefore, the spin−spin interactions observed by SDSL probably underestimate the proximity between positions 126 and 144, as documented previously for other paired residues in the permease (*27*). In addition, paired Cys replacements for other residues in helices IV and V (e.g., A127C/A143C permease) exhibit stronger spin−spin interactions as determined by SDSL (K.-C. Zen, M. Zhao, W. L. Hubbell, and H. R. Kaback, unpublished observations), suggesting that removal of the charge pair Glu126−Arg144 may alter the proximity between helices IV and V.

It is noteworthy that the dipolar interactions observed with E126C/R144C permease are stronger in reconstituted proteoliposomes than in DM micelles. The observation is consistent with other findings (*21*−*23*, *26*, *28*−*30*) indicating that although the permease may be in a more relaxed conformation in DM micelles relative to a phospholipid bilayer, the protein is maintained in near-native conformation in the detergent. Such differences, albeit relatively small, may act to reduce the strength of the dipolar interactions by altering the orientation between the two spin-labels. In any event, taken together, the results presented in this communication provide strong support for the contention that Glu126 and Arg144 are in close proximity in the tertiary structure of lac permease.

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