Arginine 302 (Helix IX) in the Lactose Permease of *Escherichia coli* Is in Close Proximity to Glutamate 269 (Helix VIII) as Well as Glutamate 325 (Helix X)†

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ABSTRACT: By using a variety of biochemical and biophysical approaches, a helix packing model for the lactose permease of *Escherichia coli* has been proposed in which the four residues that are irreplaceable with respect to coupling are paired—Glu269 (helix VIII) with His322 (helix X) and Arg302 (helix XI) with Glu325 (helix X). In addition, the substrate translocation pathway is located at the interface between helices V and VIII, which is in close vicinity to the four essential residues. Based on this structural information and functional studies of mutants in the four irreplaceable residues, a molecular mechanism for energy coupling in the permease has been proposed [Kaback, H. R. (1997) *Pro.c Natl. Acad. Sci. U.S.A. 94*, 5539]. The principle idea of this model is that Arg302 interacts with either Glu325 or Glu269 during turnover. Evidence that Arg302 is in close proximity with Glu325 has been presented [Jung, K., Jung, H., Wu, J., Prive, G. G., & Kaback, H. R. (1993) *Biochemistry 32*, 12273; He, M. M., Voss, J., Hubbell, W. L., & Kaback, H. R. (1995) *Biochemistry 34*, 15667]; however, the proximity of Arg302 to Glu269 has not been examined. In this report, it is shown by two methods that Arg302 is also close to Glu269: (i) permease with Glu269→His, Arg302→His, and His322→Phe binds Mn^{2+} with high affinity at pH 7.5, but not at pH 5.5; and (ii) site-directed spin-labeling of the double Cys mutant Glu269→Cys/Arg302→Cys exhibits spin−spin interaction with an interspin distance of about 14−16 Å. In addition, the spin−spin interaction is stronger and interspin distance shorter after the permease is reconstituted into proteoliposomes. Taken as a whole, the data are consistent with the idea that Arg302 may interact with either Glu325 or Glu269 during turnover.

The lactose (lac)[1] permease of *Escherichia coli* is a 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 the coupled stoichiometric translocation of β-galactosides and H^+ as a monomer (Kaback et al., 1994; Kaback, 1996). All available evidence indicates that the permease is composed of 12 α-helical rods that traverse the membrane with both N and C termini in the cytosolic side (Figure 1). Extensive site-directed mutagenesis of wild-type permease and Cys-scanning mutagenesis of a functional mutant devoid of Cys residues (C-less permease) indicate that as few as 4 out of over 400 residues are essential for the coupling between substrate and H^+ translocation (Kaback, 1997)—Glu269 (helix VIII), Arg302 (helix IV), His322 (helix X), and Glu325 (helix X). Moreover, differences in the properties of the mutants indicate that Glu325 may be the only residue in the permease that is directly involved in H^+ translocation.

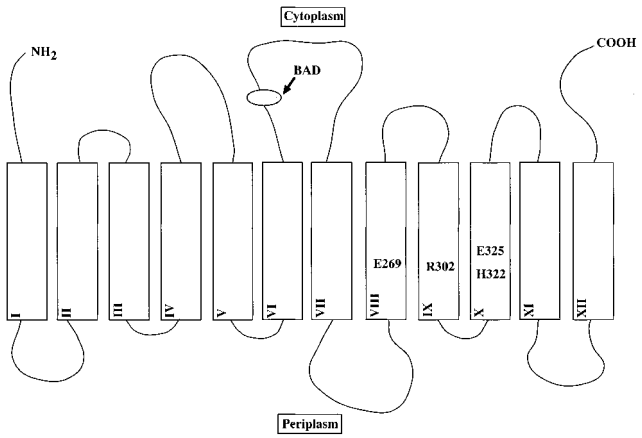

FIGURE 1: Secondary structure model of lac permease with Glu269, Arg302, His322, and Glu325 highlighted. Also indicated is the biotin acceptor domain (BAD) used for purification of lac permease by avidin affinity chromatography.

Site-directed excimer fluorescence, site-directed mutagenesis, and second-site suppressor studies have led to a model describing the packing of helices VII−XI, where Glu269 interacts with His322 and Arg302 with Glu325 (Figure 2). In addition, there are two pairs of interacting Asp and Lys residues [Asp237 (helix VII)/Lys358 (helix XI) and Asp240 (helix VII)/Lys319 (helix X)] that are not essential for activity. The interactions have been confirmed and extended by engineering divalent metal-binding sites (bis- or tris-His

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[1] Abbreviations: lac, lactose; C-less permease, functional lac permease devoid of Cys residues; DM, dodecyl β-D-maltoside; KP_i, potassium phosphate; IPTG, isopropyl 1-thio-β-D-galactopyranoside; EPR, electron paramagnetic resonance; NEM, *N*-ethylmaleimide.

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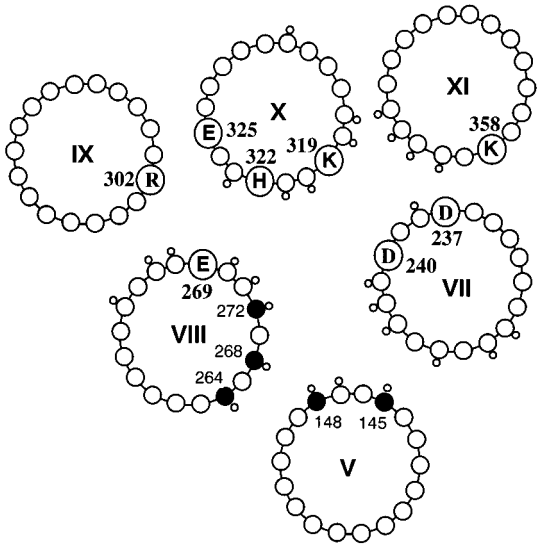

FIGURE 2: Helical wheel model of putative helices V and VII−XI in lac permease viewed from the periplasmic surface. The four essential residues and the Asp-Lys charge pairs are highlighted and indicated by larger circles. The smallest circles represent the single-Cys mutants whose activities are inhibited by NEM. The middle size black dots represent residues Val264, Gly268, Asn272, Met145, and Cys148, whose NEM inhibition of activities is blocked by addition of substrate.

residues) between helices (Jung et al., 1995; He et al., 1995a,b) and by the demonstration that monoclonal antibody 4B11 binds to the last two cytoplasmic loops (Sun et al., 1997). Furthermore, site-directed chemical cleavage (Wu et al., 1995b), thiol cross-linking, and spin-labeling studies (Wu et al., 1996) further extended the model by placing helix V at the interface of helices VII and VIII, with Cys148 (helix V) at the interface of helices VII and VIII. In addition, it has been demonstrated that helix I is close to helices V and VIII, helix II is close to helices VII and XI, and helix VI is close to helices V and VIII (Wu & Kaback, 1996, 1997; J. Wu and H. R. Kaback, unpublished information).

Site-directed mutagenesis, site-directed fluorescence spectroscopy, and site-directed chemical labeling *in situ* demonstrate that Cys148 and Met145 are located in the substrate translocation pathway (Jung et al., 1994; Wu & Kaback, 1994; Frillingos & Kaback, 1996). In addition, recent Cys-scanning mutagenesis studies on helix VIII show that the reactivity of the single-Cys mutants V264C, G268C, and N272C with *N*-ethylmaleimide (NEM) is dramatically decreased in the presence of ligand, indicating that one face of helix VIII is probably also in the substrate translocation pathway (Frillingos & Kaback, 1997; Frillingos et al., 1997). Since this face of helix VIII is predicted to be in close proximity to Cys148 and Met145 (Figure 2), it has been proposed that part of the substrate translocation pathway lies at the interface between helices V and VIII.

Based on the properties of the four essential residues and the helical packing information described, a novel mechanism for the coupled translocation of substrate and H^+ by the permease has been proposed (Kaback, 1997). Interactions between the helices are such that a ligand-induced conformational change at the interface between helices VIII and V is transmitted to the interface between helices IX and X and vice-versa (Figure 3). Upon ligand binding, a structural change at the interface between helices V and VIII disrupts the interaction between His322 and Glu269, Glu269 displaces Glu325 from Arg302, and Glu325 is protonated. Simultaneously, helix X moves so as to bring protonated Glu325 into contact with the hydrophobic phase of the membrane which markedly increases the pK_a of the carboxylic acid. In this configuration, the permease catalyzes equilibrium exchange and counterflow. In order to return to ground state after release of substrate, the Arg302−Glu325 interaction must be reestablished which necessitates loss of H^+ from Glu325. The H^+ is released into a water-filled crevice between helices IX and X, where it is acted upon equally by either the membrane potential or the pH gradient across the membrane.

One of the principle ideas of this scheme is that Arg302 interacts with either Glu325 or Glu269 during transport. Although Arg302 is close to Glu325 (Jung et al., 1993; He et al., 1995a), the proximity between Arg302 and Glu269 has not been examined. Engineered metal-binding sites have been used to study tertiary structure in several proteins (Jung et al., 1995; Elling et al., 1995; He et al., 1995a,b). In addition, site-directed spin-label (SDSL) is a powerful approach to investigate protein structure and function (Hubbell & Altenbach, 1994). One SDSL approach uses the interaction between pairs of nitroxides measured by electron paramagnetic resonance (EPR) to demonstrate proximity, and spectra obtained in the frozen state can be used to estimate interspin distance in the range of 8−25 Å (Hyde & Rao, 1978; Anthony-Cahill et al., 1992; Farahbakhsh et al., 1995; Rabenstein & Shin, 1995; Wu et al., 1996). We now report the use of these approaches to demonstrate that Arg302 may interact with Glu269 as well as Glu325.

MATERIALS AND METHODS

Construction of Mutants. The double-His mutant E269H/R302H was constructed by restriction fragment replacement of the single-His mutants E269H and R302H (He et al., 1995a) in a cassette version of the *lacY* gene containing unique restriction sites about every 100 bp (X56095) that encodes wild-type permease. Since the single-His mutant R302H forms a metal-binding site with a native His residue at position 322, His322 was mutated to Phe by the oligonucleotide-directed two-stage polymerase chain reaction (PCR) method (Ho et al., 1989) on the background of mutant E269H/R302H. The double-Cys mutant E269C/R302C was similarly constructed by restriction fragment replacement of the single-Cys mutants E269C and R302C in a cassette version of the *lacY* gene that encodes Cys-less permease (Jung et al., 1993). Mutations in both constructs were verified by sequencing the length of the inserted restriction fragment through the ligation junctions in double-stranded plasmid DNA using dideoxynucleotide termination (Sanger et al., 1977) after alkaline denaturation (Hattori & Sakaki, 1986). In addition, a biotin acceptor domain with a factor Xa protease site at the N terminus was inserted into the C-terminal tail or in the middle cytoplasmic loop of mutants E269H/R302H/H322F or E269C/R302C, respectively, to facilitate purification by monovalent avidin affinity chromatography.

Purification of Mutant E269H/R302H/H322F. The mutant was expressed in *E. coli* T184 (*lacY*$^-$*Z*$^-$; Teather et al., 1980). Twelve liters of cells was cultivated at 37 °C in Luria−Bertini broth and induced with 0.5 mM IPTG. Membranes were prepared as described (Viitanen et al., 1986) and

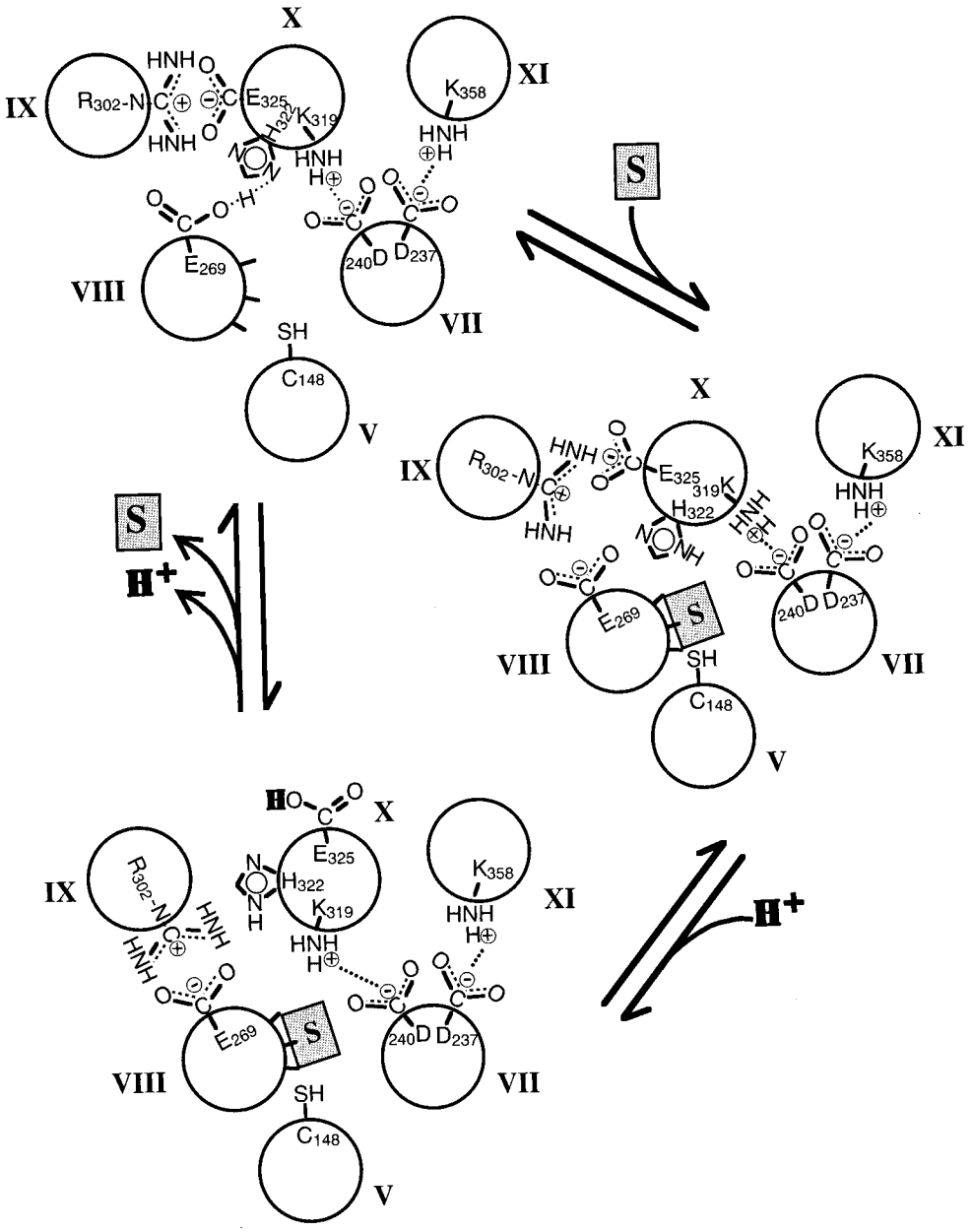

FIGURE 3: Proposed mechanism for energy coupling in lac permease. Packing of helices V and VII−XI and important side-chain interactions in the absence of substrate (upper left) are based on a variety of experimental results described in the text. Upon substrate binding (lower right), the interaction between Glu269 and His322 is disrupted, leading to the changes in side-chain interactions described in the text.

extracted with 2% dodecyl β-D-maltoside (DM). The permease was purified by affinity chromatography on immobilized monomeric avidin (Promega; Wu & Kaback, 1994). The resin was equilibrated with 50 mM potassium phosphate (KP_i, pH 7.0)/150 mM NaCl/0.02% DM (w/v). After application of the sample, the column was washed thoroughly with 10 mM MES (pH 7.0)/0.01% DM (column buffer I). Bound permease was then eluted with 5 mM biotin in column buffer I. Purified permease was analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and visualized by silver staining. Protein was then concentrated to 45 μM using a MicroProDicon (Spectrum), and the buffer was changed to 10 mM MES [treated previously with Chelex-100 (BioRad)] at a given pH/0.01% DM by dialysis. Protein concentration was determined by using a Micro BCA protein determination kit (Pierce Inc., Rockfords IL).

Mn^{2+} Binding by E269H/R302H/H322F Permease. Purified E269H/R302H/H322F permease was incubated with freshly prepared $MnCl_2$ (Aldrich, highest available purity) at a concentration of 100 μM for 1 h. 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 in the absence or presence of permease at a given pH, as indicated, with the samples in sealed quartz capillaries. Data were obtained with the following instrument settings: scan width, 600 G; scan time, 4 min; signal averaging, 10 scans; time constant, 0.032 s; microwave power, 2 mW; modulation, 4 G. The binding of hexaaquo-Mn^{2+} to the permease was determined from the mean signal change in the peak-to-peak amplitude of the four central lines corresponding to $m_I = 3/2$, 1/2, −1/2, and −3/2 with the error calculated as the standard error of the mean. As described previously (Jung et al., 1995), the amount of Mn^{2+}

bound to the permease was determined from the reduction in the free Mn^{2+} signal in the presence of permease.

Preparation of Purified Spin-Labeled Mutant E269C/R302C. Crude membrane fractions of the mutant were prepared and solubilized with 2% DM as described above. After solubilized permease was bound to monovalent avidin and unbound material was removed by washing the column with 50 mM KP_i (pH 7.4)/150 mM NaCl/0.02% DM (column buffer II), bound permease was spin-labeled by incubating with 100 μM proxylmethanethiosulfonate (Reanal, Budapest) for 2 h at 4 °C, followed by washing the column with column buffer II to remove excess spin-label. Spin-labeled permease was finally eluted with 5 mM biotin in column buffer II and concentrated to 150 μM. The purity and the concentration of the protein were determined as described above.

To prepare proteoliposomes, spin-labeled permease (150 μM) in column buffer II was mixed with 1.25% octyl glucoside (final concentration) and liposomes prepared from 1-palmitoyl-2-oleoylphosphatidylethanolamine and 1-palmitoyl-2-oleoylphosphatidylglycerol (3:1 mol/mol); the lipid:protein ratio was adjusted to 100:1 (w/w). After incubation in ice for 15 min, the mixture was rapidly diluted into 40 volume of 50 mM KP_i (pH 7.4) as described (Taglicht et al., 1991). Proteoliposomes were harvested by centrifugation (150000*g*), washed twice with 50 mM KP_i (pH 7.4), and resuspended in the same buffer followed by two cycles of freeze−thaw/sonication (Viitanen et al., 1986). The protein concentration was adjusted to about 45 μM.

EPR Measurements and Estimation of the Interspin Distance of Spin-Labeled Permease E269C/R302C. A permease sample of 10 μL at a final concentration of 150 μM in DM or 45 μM in proteoliposomes was placed in a sealed quartz capillary contained in the resonator of the same spectrometer as described above. Room temperature spectra (20−22 °C) were collected by signal-averaging 3−5 scans over 100 G 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. For an isotropic distribution of interspin vectors at distances between 8 and 25 Å, the effect is a general broadening of the resonance lines, and the degree of broadening can be used to estimate the interspin distance (Kokorin et al., 1972).

RESULTS

Mn^{2+} Binding by E269H/R302H/H322F Permease. EPR difference spectra of 100 μM Mn^{2+} in the absence and presence of 45 μM E269H/R302H/H322F permease at pH 7.5 and 5.5 are shown in Figure 4. At pH 7.5, E269H/R302H/H322F permease at 45 μM causes a 50 ± 5% reduction in amplitude of the EPR spectrum of 100 μM Mn^{2+}, corresponding to a binding stoichiometry of approximately 1:1. In contrast, at pH 5.5, the presence of 45 μM E269H/R302H/H322A permease does not significantly decrease the EPR signal amplitude of 100 μM Mn^{2+}. The reversed phase in the difference spectrum indicates that the protein-containing sample actually has more uncomplexed Mn^{2+} than the buffer control, although this difference is at the limit of experimental error. Since wild-type permease does not bind Mn^{2+} to any extent whatsoever (Jung et al., 1995), the findings demonstrate that the His residue at position 269 forms a metal binding site in conjunction with the His residue at position 302 at pH 7.5.

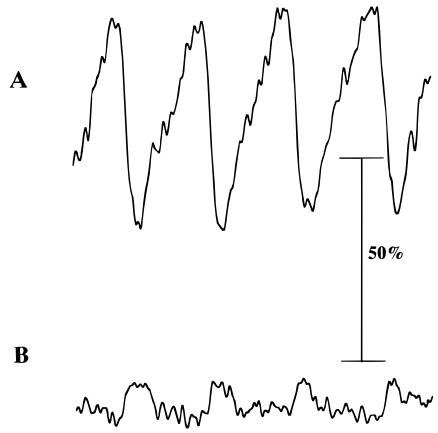

FIGURE 4: Mn^{2+} binding to E269H/R302H/H322F permease. Shown are difference spectra obtained by subtracting the spectrum of a sample containing 100 μM Mn^{2+} and 45 μM mutant permease from the spectrum of a sample containing 100 μM Mn^{2+} without protein. Spectra were obtained at room temperature in 10 mM MES containing 0.01% DM as described under Materials and Methods: (A) pH 7.5; (B) pH 5.5. The bar represents the scale for the percentage reduction of the Mn^{2+} spectrum due to metal binding at pH 7.5.

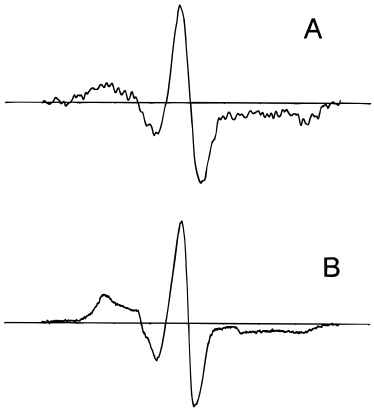

FIGURE 5: EPR spectra of spin-labeled E269C/R302C mutant permease at room temperature. (A) Permease reconstituted in proteoliposomes; (B) permease in detergent DM. Experiments and data analysis were carried out as described.

SDSL of E269C/R302C Permease at Room Temperature. In order to approximate the distance between residues Glu269 and Arg302, SDSL of mutant E269C/R302C was carried out both in reconstituted proteoliposomes (Figure 5A) and in detergent DM (Figure 5B). If the two spin-labels are within a distance of 20 Å, it is possible to detect spin−spin interactions, as reflected by broadening of the line shape in the EPR spectrum (Likhtenshtein, 1993). The composite spectra of spin-labeled E269C/R302C permease are shown in Figure 5. In each case, the spectra indicate that the nitroxide-labeled side chains are immobilized, although the sharper line shape of the sample in DM suggests that a population of spins in this sample are more mobile relative to reconstituted permease. This observation is consistent with previous findings describing a looser structure for the protein in DM (Jung et al., 1993; Wu et al., 1996). In addition, the broadened features of the reconstituted permease are indicative of a population of dipolar-interacting spins in close proximity. This observation is consistent with the notion that the permease is in a less relaxed conformation

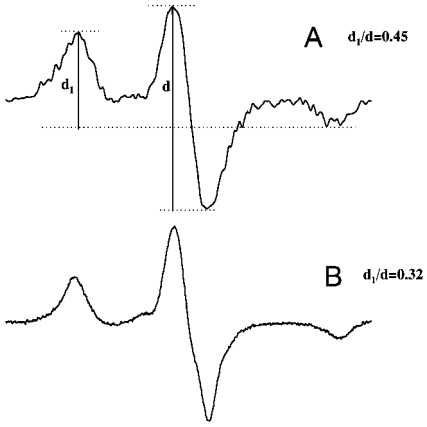

FIGURE 6: EPR spectra of spin-labeled E269C/R302C mutant permease in the frozen state. (A) Permease reconstituted in proteoliposomes; (B) permease in detergent DM. Experiments and data analysis were carried out as described.

in a lipid bilayer relative to DM, since the interaction is stronger in the reconstituted sample, indicating a closer average distance separating the nitroxides at positions 269 and 302.

SDSL of E269C/R302C Permease in the Frozen State. To evaluate broadening due to static dipolar interactions only, quantitative analysis of the interspin distance was carried out in the absence of motion by collecting spectra in the frozen state (183 K). An estimation of the extent of broadening due to dipolar interaction is obtained from the line height ratio d_1/d (Figure 6A; Likhtenshtein, 1993). In the absence of interaction, a value of <0.4 is expected. The d_1/d value of the mutant in reconstituted proteoliposomes is 0.45, consistent with the room temperature findings of the close proximity for this pair following reconstitution. The value of the mutant in detergent DM is only 0.32 (Figure 6B), indicating that in the frozen state few, if any, spin pairs of the DM-solubilized permease are sufficiently close to cause dipolar broadening. Empirical estimation of the d_1/d values yields approximate distances of 14−16 Å for the sample in proteoliposomes and >22 Å for the sample in DM.

DISCUSSION

The purpose of these studies is to test one aspect of a mechanistic model for energy coupling in lac permease (Kaback, 1997) by focusing on the possibility that Arg302 (helix IX) can interact with Glu269 (helix VIII), as well as Glu325 (helix X). The close proximity of Arg302 with Glu325 has been demonstrated by site-directed excimer fluorescence and by the demonstration that R302H/E325H forms a divalent metal-binding site (Jung et al., 1993; He et al., 1995a). In the experiments reported here, an engineered divalent metal-binding site and site-directed spin-labeling are used to demonstrate that Arg302 is able to interact with Glu269, as well as Glu325, as predicted by the model.

Permease E269H/R302H/H322F binds Mn^{2+} at pH 7.5 but not at pH 5.5, consistent with protonation of the imidazole at pH 5.5. This finding is in agreement with previous results showing that the pairs Glu269 and Arg302, as well as Asp237 and Lys358, are probably located in water-filled crevices (Jung et al., 1995; He et al., 1995a,b). The result also shows that the mutant permease at a concentration of 45 μM causes a 50% reduction in amplitude of the EPR spectrum of 100 μM Mn^{2+}, suggesting that the binding stoichiometry may slightly exceed unity. Previous studies show that Glu residues are able to participate in metal chelation (Witmer et al., 1994; He et al., 1995a). Since Glu325 is in close proximity to E269H and R302H, and all three residues are at about the same depth into the membrane, it is possible that Glu325 is responsible for this effect. In any case, the excess is small, and the data clearly show that position 302 is sufficiently close to position 269 to form a metal-binding site with His residues at both positions.

Spin−spin interactions provide more definitive evidence for the proximity of Glu269 with Arg302. Room temperature spectra of spin-labeled mutant E269C/R302C have features consistent with a dipole−dipole interaction between the two spins. More importantly, EPR spectra in the frozen state yield a interspin distance of about 14−16 Å. This distance is relatively close as determined by spin−spin interaction, and the distance may be even closer in the native membrane. For example, spin−spin measurements in mutant Cys148/Y228C revealed a distance of about 13−15 Å in DM and ca. 11−13 Å in reconstituted proteoliposomes. However, the two Cys residues are cross-linked in native membranes by dibromobimane, which is only about 5 Å in length (Wu et al., 1996). In addition to possible differences in conformation in different environments, this discrepancy may also result from differences in the orientation of the two spin-labels in the tertiary structure which may act to increase the interspin distance.

It is noteworthy that the spin−spin interaction in mutant E269C/R302C is stronger when the permease is reconstituted into proteoliposomes. This observation is consistent with the interpretation that there may be small conformational differences between the permease in DM micelles vs a phospholipid bilayer (Jung et al., 1993; Wu et al., 1996), although many observations demonstrate that the permease retains near-native structure in detergent DM (Jung et al., 1993, 1995; Wu & Kaback, 1994; He et al., 1995a,b; Wu et al., 1995a; Frillingos & Kaback, 1996). It is also important to note that the experiments described here involve mutations in two of the four irreplaceable residues in the permease. In future experiments, mutations will be introduced at positions one turn up or down from the essential residues on the same helical face so that interactions between irreplaceable residues are likely to be maintained.

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