Use of Designed Metal-Binding Sites To Study Helix Proximity in the Lactose Permease of *Escherichia coli*. 2. Proximity of Helix IX (Arg302) with Helix X (His322 and Glu325)

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ABSTRACT: Engineering divalent metal-binding sites into the lactose permease of *Escherichia coli* by introducing bis-His residues has been utilized to confirm the proximity of helices VIII (Glu269→His) and X (His322) [Jung, K., Voss, J., He, M., Hubbell, W. L., & Kaback, H. R. (1995) *Biochemistry 34*, 6272] and helices VII (Asp237→His) and XI (Lys358→His) [He, M. M., Voss, J., Hubbell, W. L., & Kaback, H. R. (1995) *Biochemistry 34*, 00000−00000]. In this paper, the approach is used to confirm and extend the relationship between helices IX (Arg302) and X (His322 and Glu325) [Jung, K., Jung, H., Wu, J., Prive, G. G., & Kaback, H. R. (1993) *Biochemistry 32*, 12273]. Thus, mutants Arg302→His, Glu325→His, and Arg302→His/Glu325→His were constructed, and Mn^{2+} binding was assayed by electron paramagnetic resonance. Mutant Arg302→His binds Mn^{2+} with a K_D of about 24 μM and a stoichiometry approximating unity in all likelihood because the His residue at position 302 forms a metal-binding site in conjunction with the native His residue at position 322. Mutant Arg302→His/Glu325→His also binds Mn^{2+} with a 1:1 stoichiometry, but the K_D is decreased to about 13 μM. The results suggest that Arg302 is sufficiently close to both Glu325 and His322 to form a tridentate metal-binding site in mutant Arg302→His/Glu325→His. In contrast, replacement of Glu325 with His in permease with a native His residue at position 322 does not lead to Mn^{2+} binding. The results provide strong support for the helix packing model proposed.

In the preceding paper (He et al., 1995), engineered metal-binding sites (bis-His residues) were utilized to provide the first direct physical evidence that Asp237 (helix VII) is in close proximity to Lys358 (helix XI) in the lactose (lac)[1] permease of *Escherichia coli*. Here we use the same approach to confirm and extend relationships derived from site-directed excimer fluorescence (Jung et al., 1993) which demonstrate that helix IX is in close proximity to helix X. Thus, Mn^{2+} binding sites are observed in mutants R302H[2] and R302H/E325H, and the latter mutant binds Mn^{2+} with significantly higher affinity. Taken together with previous findings (Jung et al., 1995; He et al., 1995) showing that mutants E269H and D237H/K358H contain Mn^{2+} binding sites, the data provide strong support for the helix packing model proposed by Jung et al. (1993).

EXPERIMENTAL PROCEDURES

Construction of Mutants. The His mutants were constructed by oligonucleotide-directed site-specific mutagenesis. The cassette *lacY* gene encoding wild-type permease in plasmid pKR35/*lacY*-CXB (He et al., 1995) was used as the template for mutagenesis. All site-specific mutations were directed by synthetic mutagenic oligonucleotide primers. Mutants R302H and E325H were constructed by a two-stage PCR method (overlap−extension) with two largely complementary mutagenic primers (Ho et al., 1989). The double mutant R302H/E325H was constructed by digesting plasmids encoding the single mutants with *Eco*47III and *Sty*I and ligating the isolated inserts from mutant E325H into the vector carrying the R302H mutation which was also digested with *Eco*47III and *Sty*I (Figure 1). All of the constructs contain a biotin acceptor domain at the C terminus (Consler et al., 1993).

DNA Sequencing. Double-stranded plasmid DNA was sequenced by using the dideoxynucleotide termination method and synthetic sequencing primers (Sanger et al., 1977) after alkaline denaturation (Hattori & Sakaki, 1986).

Expression and Purification of Mutant Permease. The mutant permeases were expressed and purified as described in the preceding paper (He et al., 1995). Purified R302H, E325H, or R302H/E325H permease was concentrated to 9−70 μM using a MicroDiCon (Spectrum), and the buffer was changed to 10 mM MES (treated previously with Chelex-100, Bio-Rad) at a given pH/0.01% dodecyl β-D-maltopyranoside (DM) by dialysis.

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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.

[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 in wild-type lac permease. The sequence is followed by a second letter denoting the amino acid replacement.

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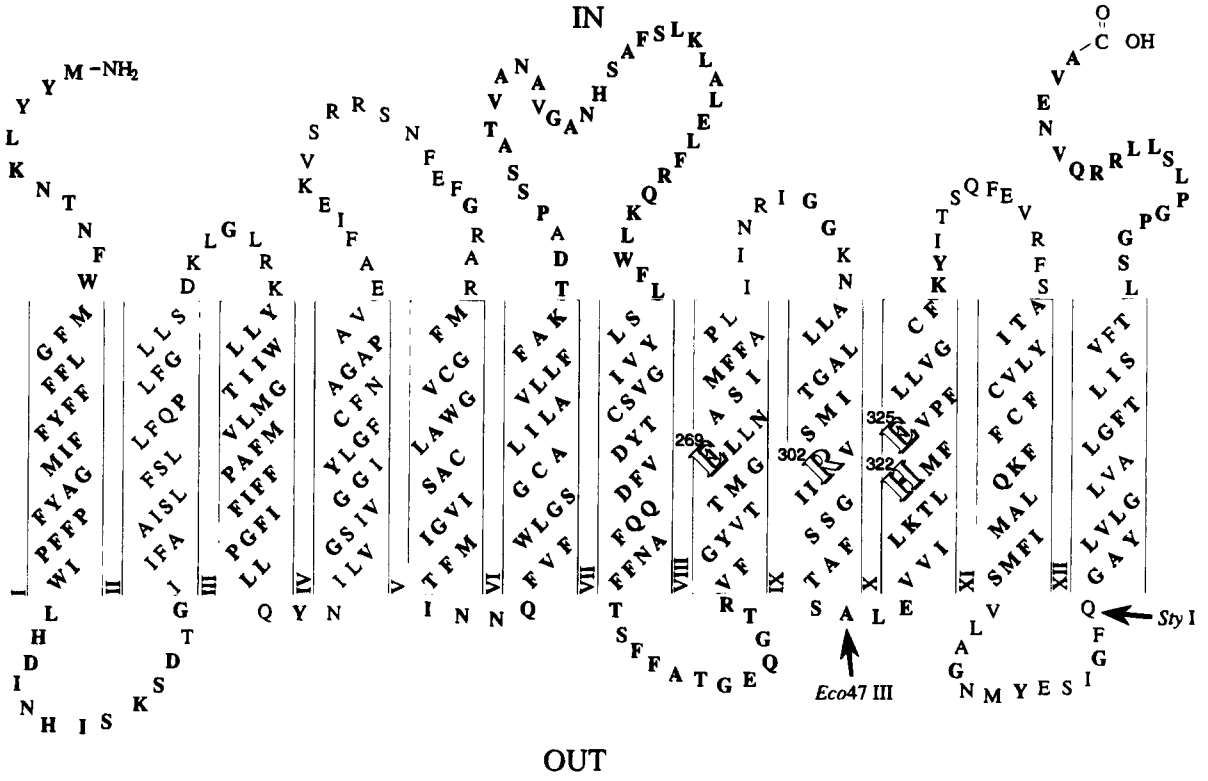

FIGURE 1: Secondary structure model of lac permease with Glu269, Arg302, His322, and Glu325 highlighted. The restriction enzyme sites used in the construction of the mutants are also shown.

Mn^{2+} Binding by EPR. Given purified, mutant permeases were incubated with freshly prepared $MnCl_2$ (Aldrich, highest available purity) 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.

Protein Determination. Protein was assayed as described (Peterson, 1977).

RESULTS

Mn^{2+} Binding by R302H Permease. EPR difference spectra of 75 μM Mn^{2+} in the absence and presence of 9 μM R302H permease at pH 7.5 and 5.5 are shown in Figure 2. At pH 7.5, R302H permease at 9 μM causes a 12 ± 1% reduction in amplitude of the EPR spectrum of 75 μM Mn^{2+}, corresponding to a binding stoichiometry of unity (not shown). In contrast, at pH 5.5, the presence of 9 μM R302H permease decreases the EPR signal amplitude of 75 μM Mn^{2+} by only 6 ± 1%, indicating a 50% reduction of Mn^{2+} binding. Since wild-type permease does not bind Mn^{2+} to any extent whatsoever (Jung et al., 1995), the findings

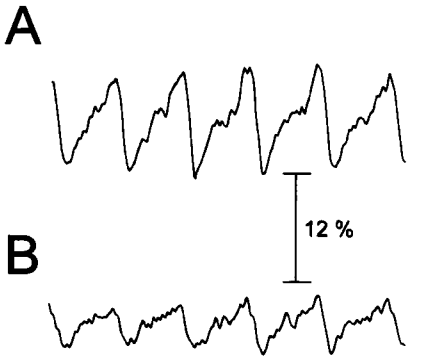

FIGURE 2: Mn^{2+} binding to R302H permease. Shown are difference spectra obtained by subtracting the spectrum of a sample containing 75 μM Mn^{2+} and 9 μM R302H permease from the spectrum of a sample containing 75 μM Mn^{2+} without protein. Spectra were acquired at room temperature in 10 mM MES containing 0.01% DM as described in Experimental Procedures: (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.

suggest that the His residue at position 302 forms a metal-binding site in conjunction with the native His residue at position 322. In order to determine the affinity of the metal-binding site introduced by the R302H substitution, metal ion binding at pH 7.5 was measured at various Mn^{2+} concentrations at a fixed permease concentration, and the data were analyzed according to the equation (Scatchard, 1949):

$$\frac{[M]_b}{[M]_f[P]_t} = \frac{1}{K_D}\left(\frac{[M]_b}{[P]_t} - n\right)$$

where $[M]_b$ is the bound Mn^{2+} concentration, $[M]_f$ is the free Mn^{2+} concentration, $[P]_t$ is the concentration of permease, K_D is the equilibrium dissociation constant, and n is the number of binding sites.

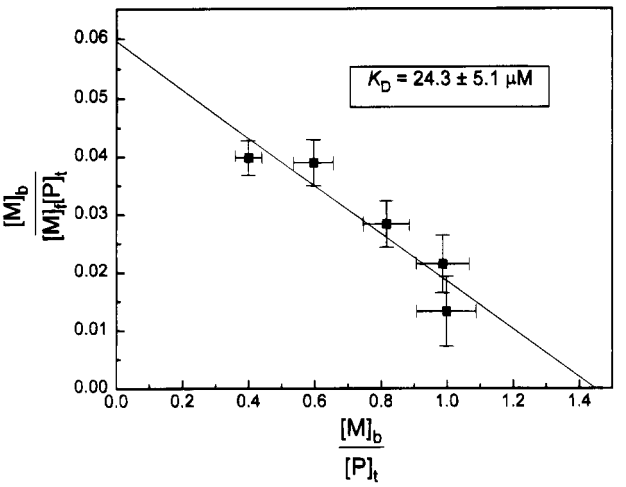

FIGURE 3: Concentration-dependent Mn^{2+} binding to R302H permease. Measurements were carried out as described in Experimental Procedures, and the data were plotted according to Scatchard (1949) as described in the text.

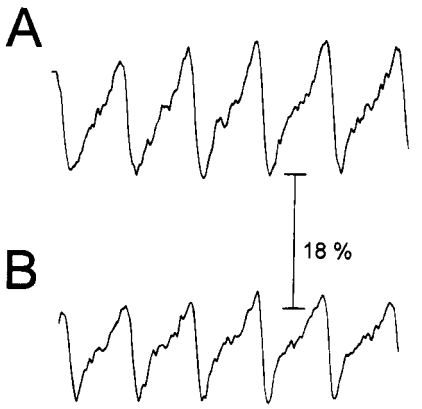

FIGURE 4: Mn^{2+} binding to R302H/E325H permease. Shown are difference spectra obtained by subtracting the spectrum of a sample containing 75 μM Mn^{2+} and 14 μM R302H/E325H permease from the spectrum of a sample containing 75 μM Mn^{2+} without protein. Spectra were acquired at room temperature in 10 mM MES containing 0.01% DM as described in Experimental Procedures: (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.

In two independent experiments, Mn^{2+} concentration ($[M]_t$) was varied over a range of concentrations from 60 to 180 μM, and the free Mn^{2+} concentration ($[M]_f$) was measured in the absence and presence of 14 μM R302H permease. Bound Mn^{2+} ($[M]_b$) was calculated from the difference in the spectra observed in the absence and presence of R302H permease ($[M]_t - [M]_f$). Graphic presentation of the data (Figure 3) reveals that the number of binding sites per mole of protein (n) approximates unity, and the equilibrium dissociation constant (K_D) is 24.3 ± 5.1 μM.

Mn^{2+} Binding by R302H/E325H Permease. EPR difference spectra of 75 μM Mn^{2+} in the absence and presence of 14 μM R302H/E325H permease at pH 7.5 and 5.5 are shown in Figure 4. At pH 7.5, R302H/E325H permease at 14 μM causes an 18 ± 1% reduction in amplitude of the EPR spectrum of 75 μM Mn^{2+}, suggesting a binding stoichiometry of unity. At pH 5.5, 14 μM R302H/E325H permease causes a relatively small but significant reduction of 15 ± 1% in amplitude of the EPR spectrum of 75 μM Mn^{2+}. Thus, the amount of bound Mn^{2+} is decreased by only ~15% when the pH is shifted from 7.5 to 5.5 with this mutant. In comparison to the Mn^{2+} binding properties of R302H permease at pH 7.5 and 5.5, the results suggest the presence of a metal-binding site with increased affinity in R302H/E325H permease. Therefore, metal ion binding by R302H/E325H permease at pH 7.5 was measured at various Mn^{2+} concentrations in the presence of 66 μM permease, and the data were analyzed as described above (Figure 5). Scatchard analysis yields a K_D for Mn^{2+} of 12.8 ± 1.1 μM and a stoichiometry of unity.

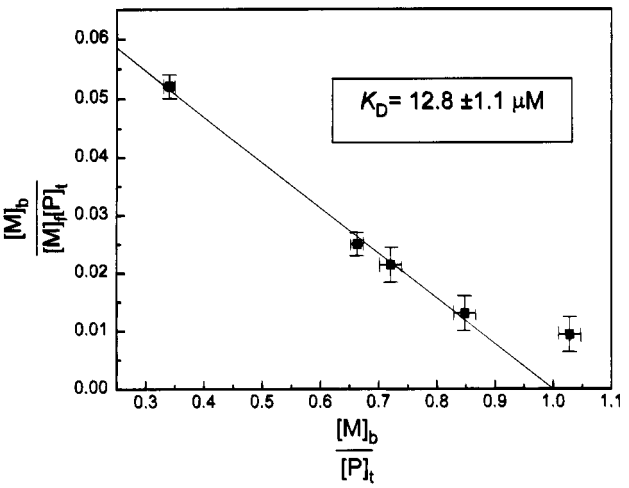

FIGURE 5: Concentration-dependent Mn^{2+} binding to R302H/E325H permease. Measurements were carried out as described in Experimental Procedures, and the data were plotted according to Scatchard (1949) as described in the text.

Mn^{2+} Binding by E325H Permease. Although data are not shown, EPR spectra of 75 μM Mn^{2+} in the absence and presence of 16 μM E325H permease at pH 7.5 and 5.5 were also measured. Since this mutant protein does not alter the spectrum to any detectable extent, it is unlikely that His residues at 322 and 325 form a metal-binding site.

DISCUSSION

The results presented here demonstrate clearly that both R302H and R302H/E325H permeases bind Mn^{2+} stoichiometrically under conditions where the imidazole group of His is expected to be unprotonated. Moreover, R302H permease binds Mn^{2+} to a reduced extent at pH 5.5, while the R302H/E325H mutant exhibits significantly more binding at the lower pH, suggesting that the latter construct contains a metal-binding site with increased affinity that is composed of three His residues at positions 302, 322, and 325.

The K_D of about 13 μM for Mn^{2+} observed for R302H/E325H permease is one-half to one-third of the K_Ds determined for R302H permease (Figure 3) and E269H permease (Jung et al., 1995), respectively. This finding suggests strongly that the Mn^{2+} binding site in mutant R302H/E325H probably contains three His ligands that coordinate to the metal, rather than two, which leads to a higher affinity for Mn^{2+} (Arnold & Haymore, 1991). All three His residues in mutant R302H/E325H are located at about the same depth into the membrane, and they are likely to be close enough and in the correct orientation to form a tridentate metal-binding site (Figure 6). Alternatively, it is possible that introduction of a third His residue at position 325 leads to increased affinity for Mn^{2+} by perturbing the pK_a of the imidizoles at positions 302 and 322. As shown, Glu269 may also contribute to the interaction of the mutant with divalent metal.

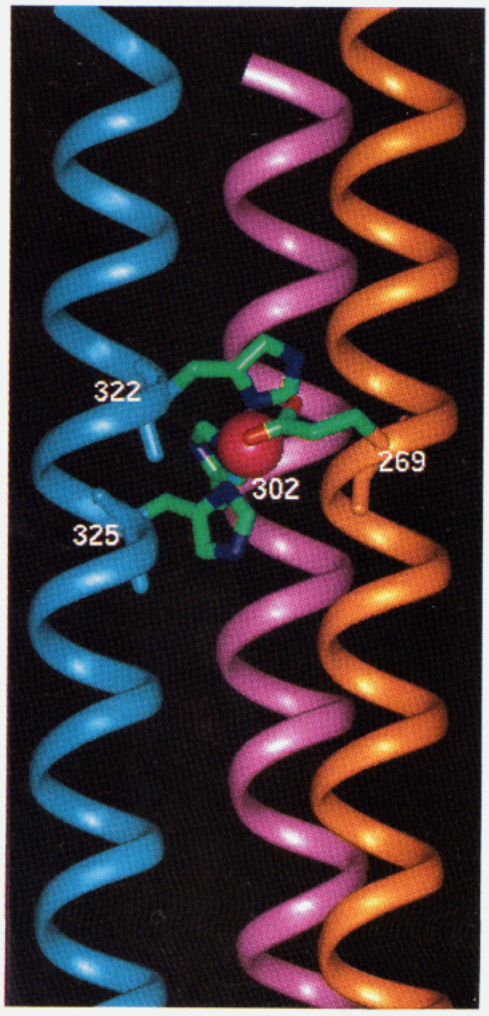

FIGURE 6: Model of transmembrane helices VIII (gold), IX (purple), and X (blue) showing a tridentate divalent metal ion binding site. Helices are shown with His residues at positions 302 (helix IX), 322, and 325 (helix X) chelating Mn^{2+} (magenta). Also shown is Glu269, which forms a metal-binding site with His322 when replaced with His. For clarity, the distances between the helices are greater than the normal separation between packed, parallel α-helices.

It is of particular interest that site-directed excimer fluorescence studies (Jung et al., 1993) indicate that position 302 (helix IX) interacts with position 325 (helix X), but not with position 322 (helix X), while the studies presented here suggest that all three residues may interact. This observation may be of importance with regard to the postulated role of these residues in lactose-coupled H^+ translocation [reviewed in Kaback (1987)].

Finally, as discussed by Arnold and Haymore (1991), the geometric requirements for metal chelation by coordination with His residues are highly specific. While two His residues separated by three other amino acid residues in an undistorted α-helix (His-X_3-His) form a divalent metal ion binding site, His-X_2-His or His-X_4-His sites in α-helical conformation do not chelate divalent metals. On the other hand, in a reverse β-turn configuration, a His-X_2-His motif forms a divalent metal-binding site. Therefore, the finding that mutant E325H, which contains His322-X_2-His325 in a putative transmembrane α-helix, does not form a Mn^{2+} binding site is consistent with previous findings (Jung et al., 1993) indicating that this region of the permease is indeed in α-helical conformation.

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