Engineering a Metal Binding Site within a Polytopic Membrane Protein, the Lactose Permease of *Escherichia coli*[†]

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ABSTRACT: Site-directed excimer fluorescence indicates that Glu269 (helix VIII) and His322 (helix X) in the lactose permease of *Escherichia coli* lie in close proximity [Jung, K., Jung, H., Wu, J., Privé, G. G., & Kaback, H. R. (1993) *Biochemistry 32*, 12273]. In this study, Glu269 was replaced with His in wild-type permease, leading to the presence of bis-His residues between helices VIII and X. Wild-type and Glu269→His permease containing a biotin acceptor domain were purified by monomeric avidin affinity chromatography, and binding of Mn^{2+} was studied by electron paramagnetic resonance (EPR) spectroscopy. The amplitude of the Mn^{2+} EPR spectrum is reduced by the Glu269→His mutant, while no change is observed in the presence of wild-type permease. The Glu269→His mutant contains a single binding site for Mn^{2+} with a K_D of about 43 μM, and Mn^{2+} binding is pH dependent with no binding at pH 5.0, stoichiometric binding at pH 7.5, and a midpoint at about pH 6.3. The results confirm the conclusion that helices VIII and X are closely opposed in the tertiary structure of lac permease and provide a novel approach for studying helix proximity, as well as solvent accessibility, in polytopic membrane proteins.

The lactose (lac)[1] permease of *Escherichia coli* is a polytopic cytoplasmic membrane protein that catalyzes the coupled translocation of β-galactosides and H^+ with a 1:1 stoichiometry (i.e., symport or cotransport). Lac permease is encoded by the *lacY* gene, the second structural gene in the *lac* operon, which has been cloned and sequenced. The *lacY* gene product has been solubilized, purified, reconstituted into proteoliposomes, and shown to be solely responsible for β-galactoside transport [reviewed in Kaback (1983, 1989, 1992) and Poolman and Konings (1993)] as a monomer [see Sahin-Tóth et al. (1994)]. On the basis of circular dichroic studies and hydropathy analysis (Foster et al., 1983), a secondary structure was proposed in which the protein has 12 transmembrane domains in α-helical configuration that traverse the membrane in zigzag fashion connected by hydrophilic domains (loops) with the N and C termini on the cytoplasmic face of the membrane (Figure 1). Evidence favoring the general features of the model and demonstrating that the C terminus, as well as loops 4 and 6, is on the cytoplasmic face of the membrane has been obtained from laser Raman spectroscopy, limited proteolysis, and immunological studies [reviewed in Kaback (1992)]. Importantly, moreover, exclusive evidence for the topological predictions of the 12-helix motif has been obtained from analysis of a large number of lac permease–alkaline phosphatase (*lacY–phoA*) fusions (Calamia & Manoil, 1990).

By use of site-directed mutagenesis with wild-type permease or Cys-scanning mutagenesis with a functional mutant devoid of Cys residues (C-less permease), individual amino acid residues in the permease that are essential for activity have been identified [reviewed in Kaback et al. (1994)]. Over 300 of the 417 residues in C-less permease have been mutagenized, mostly by Cys-scanning mutagenesis (Dunten et al., 1993; Sahin-Tóth & Kaback, 1993; Sahin-Tóth et al., 1994a–c; Frillingos et al., 1994; Jung et al., 1995; C. Weitzman and H. R. Kaback, manuscript in preparation). Remarkably, less than a half-dozen residues have been identified thus far as being clearly essential for activity, and of the few mutants that do not catalyze active transport, most retain the ability to catalyze partial reactions or bind ligand. On the other hand, Glu269 (helix VIII) (Ujwal et al., 1994; Franco & Brooker, 1994), Arg302 (helix IX) (Menick et al., 1987; Matzke et al., 1992), His322 (helix X) (Padan et al., 1985; Püttner et al., 1986, 1989; Püttner & Kaback, 1988; King & Wilson, 1989a,b, 1990), and Glu325 (helix X) (Carrasco et al., 1986, 1989) are essential for active transport and/or binding of substrate. Although not essential for transport, Cys148 (helix V) is in the substrate binding site, interacting hydrophobically with the galactosyl moiety of the substrate, and Met145 (helix V) is on the periphery of the binding site (Jung et al., 1994; Wu & Kaback, 1994).

A high-resolution structure of lac permease is not available because of the difficulty inherent in crystallizing hydrophobic membrane proteins. Therefore, development of alternative methods for obtaining structural information is important. Recently (Jung et al., 1993), *N*-(1-pyrenyl)maleimide was used to label permease mutants containing specifically engineered pairs of Cys residues for site-directed excimer

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

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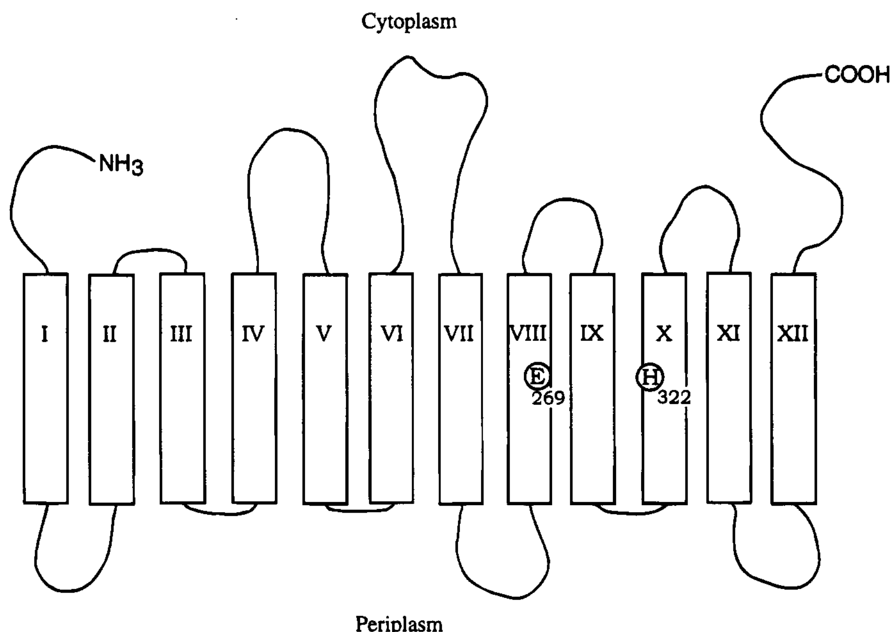


FIGURE 1: Secondary structure model of lac permease with the positions of Glu269 and His322 highlighted.

fluorescence. Attention was focused on charged residues in transmembrane domains that are essential for active transport and/or binding and had been postulated to interact. The following double-Cys mutants exhibit excimer fluorescence: H322C/E325C,[2] R302C/E325C, and E269C/H322C. The findings indicate that His322 and Glu325 are located in a portion of the permease that is in α-helical conformation (i.e., helix X), that helix IX (Arg302) is close to helix X (Glu325), and that helix VIII (Glu269) is close to helix X (His322). In addition, second-site suppressor analysis and site-directed mutagenesis provide a strong indication that helix VII (Asp237 and Asp240) is in close proximity to helices XI (Lys358) and X (Lys319) [reviewed in Kaback et al. (1993, 1994)]. The results form the basis of a model describing helix packing in the C-terminal half of the permease (Jung et al., 1993).

In the experiments reported here, a novel approach based on engineering a metal-binding site into the permease is utilized to confirm the proximity of helices VIII and X. The simplest design of a metal-binding site is based on the ability of bis-His residues to chelate divalent metal ions (Higaki et al., 1992). Thus, a lac permease mutant was constructed in which Glu269 (helix VIII) was replaced with His. If position 269 is in close approximation to His322 (helix X), as suggested by site-directed excimer fluorescence (Jung et al., 1993), we reasoned that His replacement for Glu269 would result in bis-His residues at positions 269 and 322 which might form a divalent metal-binding site. By use of electron paramagnetic resonance (EPR), it is demonstrated that E269H permease binds the transition metal Mn^{2+} with a stoichiometry of unity and a binding constant in the micromolar range, while wild-type permease exhibits no binding whatsoever. Furthermore, the mutant does not bind Mn^{2+} at pH 5.0, and the midpoint of a pH titration approximates the p*K* of imidazole.

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

EXPERIMENTAL PROCEDURES

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*$^+$]) (Teather et al., 1980) harboring plasmid pKR35/*lacY*-L6XB (Consler et al., 1993) encoding wild-type or E269H permease with a biotin acceptor domain in the middle cytoplasmic loop was used for expression from the *lacZ* promoter/operator by induction with isopropyl β-D-thiogalactopyranoside (IPTG).

Construction of E269H. The cassette *lacY* gene (EMBL X-56095) encoding wild-type lac permease was used as template for mutagenesis. Mutant E269H was constructed by two-stage polymerase chain reaction (Ho et al., 1989) with two complementary mutagenic primers synthesized on an Applied Biosystems 391 DNA synthesizer. The synthetic mutagenic primers (sense and antisense) were 24 bp long, complementary to the DNA sequence of *lacY* in the region of codon 269, and encoded the replacement of Glu269 (GAA) with His (CAT). Mutations were verified by sequencing the length of the PCR-generated segment through the ligation junctions in double-stranded plasmid DNA using dideoxynucleotide termination (Sanger et al., 1977) and synthetic sequencing primers after alkaline denaturation (Hattori & Sakaki, 1986).

Expression and Purification. Twelve liters of cells were cultivated at 37 °C and induced with 0.3 mM IPTG. Membranes were prepared as described (Viitanen et al., 1986) and extracted with 2% *n*-dodecyl β-D-maltoside (DM). Lac permease was purified by affinity chromatography on immobilized monomeric avidin (Promega) (Consler et al., 1993). The resin was equilibrated with 50 mM inorganic potassium phosphate (KP_i; pH 7.0)/150 mM KCl/0.02% DM (w/v) followed by 10 mM MES (pH 7.0)/0.01% DM. After application of the sample, the column was washed thoroughly with 10 mM MES (pH 7.0)/0.01% DM. Bound permease was then eluted with 5 mM biotin-*d* in 10 mM MES (pH 7.0)/0.01% DM. Purified samples were analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (Laemmli, 1970) and visualized by silver staining. Purified wild-type

or E269H permease was concentrated to 20 μM using a microconcentrator (Filtron) or a microprodicon (Spectrum), and the buffer was changed to 10 mM MES [treated previously with Chelex-100 (Bio-Rad)] at a given pH/0.01% DM by dialysis.

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

Mn^{2+} Binding by EPR. Purified wild-type or E269H permease was incubated with freshly prepared $MnCl_2$ (Aldrich, highest available purity) at given concentrations for 1 h. 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, as indicated, with the samples in sealed quartz capillaries. Data were obtained with the following instrument settings: scan width, 800 G; scan time, 4 min; signal averaging, 10 scans; time constant, 0.032 s; microwave power, 2 mW; modulation, 4 G. The free Mn^{2+} signal, as determined from the peak-to-peak height of the fourth line of the EPR spectrum, was plotted against Mn^{2+} concentration in order to obtain a standard curve. The concentration of the free Mn^{2+} in solutions containing wild-type or mutant permease was calculated by comparison to the standard curve, and the amount bound was determined by difference. For pH dependence of Mn^{2+} binding, the free Mn^{2+} in the presence of permease was determined from the signal of a Mn^{2+} standard in the identical buffer of indicated pH.

RESULTS

In order to determine whether or not wild-type permease or the E269H mutant contains a metal-binding site, Mn^{2+} binding was measured with purified proteins by using EPR spectroscopy. Mn^{2+} is a high-spin, paramagnetic ion with a sufficiently long relaxation time that spectra can be obtained at room temperature. Six lines arising from the nuclear spin ($I = ^5/_2$) are characteristic for this transition metal (Figure 2). Since Mn^{2+} EPR signals are observed readily when the metal is in aqueous solution, but not when the ion is complexed with protein ligands, binding can be determined directly by measuring the free Mn^{2+} concentration in the absence and presence of protein (Cohn & Townsend, 1954). EPR spectra of Mn^{2+} at a concentration of 100 μM in the absence and presence of 20 μM wild-type or E269H permease are shown in Figure 2. Clearly, wild-type permease does not alter the Mn^{2+} EPR spectrum, while E269H permease causes reduction in amplitude. The observations demonstrate that the free Mn^{2+} concentration is reduced in the presence of E269H permease but not in the presence of wild-type permease.

Metal ion binding at pH 7.0 was then measured at various Mn^{2+} concentrations using equal amounts of E269H permease, 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. In two independent experiments, the Mn^{2+} concentration ($[M]_t$) was varied over a range of concentrations from 10 to 400 μM, and the free Mn^{2+} concentration ($[M]_f$) was measured in the absence and presence of E269H permease at a final concentration of 10 μM (Table 1). Bound Mn^{2+} ($[M]_b$) was calculated from the difference in the spectra in the absence and presence of E269H 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 about 43 μM. Mn^{2+} binding to wild-type permease is undetectable over the same range of metal concentrations.

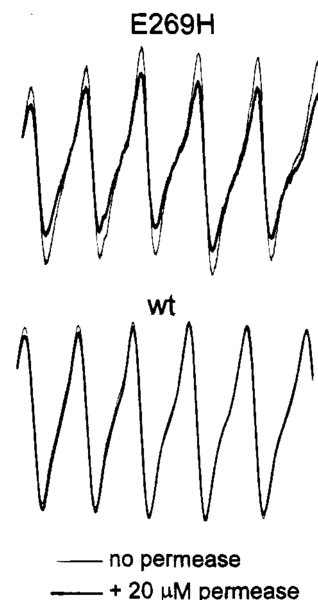


FIGURE 2: Mn^{2+} binding as determined by EPR for wild-type and E269H permease. EPR spectra were obtained at room temperature with 100 μM $MnCl_2$ in 10 mM MES (pH 7.0)/0.01% DM in the absence (thin line) or presence of wild-type (thick line; bottom) or E269H permease (thick line; top) at 20 μM, final concentration, as described in Experimental Procedures.

Table 1: Concentrations of Free Mn^{2+} As Determined by EPR[a]

$[Mn^{2+}]$ in buffer (μM)	$[Mn^{2+}]$ + E269H (μM)	Mn^{2+} bound (mol/mol of protein)
14.56	10.02	0.23
42.72	33.55	0.46
87.77	74.51	0.66
179.00	156.00	1.15
398.78	377.63	1.06

[a] Free Mn^{2+} was measured by EPR in 10 mM MES (pH 7.0)/0.01% DM in the absence or presence of E269H permease (20 μM, final concentration) as described in Experimental Procedures. The values presented represent average data points obtained from two independent experiments. Although data are not shown, the free Mn^{2+} concentration observed in the presence of wild-type permease was identical to that observed with buffer alone in the absence of protein.

Binding of Mn^{2+} to E269H permease is pH dependent (Figure 4). At pH 5.0, no binding whatsoever is observed, while at pH 7.5, E269H permease binds 1 mol of Mn^{2+}/mol of protein. Moreover, the midpoint of the titration approximates pH 6.3, which is reasonably close to the pK of the imidazole group of His.

DISCUSSION

The results presented here demonstrate clearly that E269H permease binds one Mn^{2+} ion with relatively high affinity

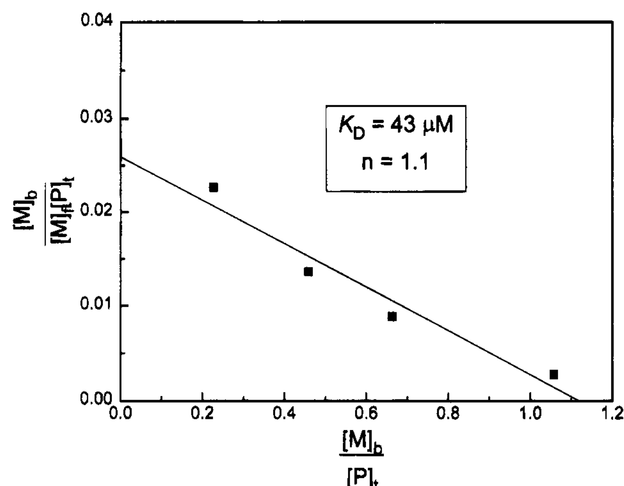


FIGURE 3: Concentration-dependent Mn^{2+} binding to E269H permease. The data tabulated in Table 1 were plotted according to Scatchard (1949) as described.

under conditions where the imidazole group of His is expected to be unprotonated. Conversely, binding is undetectable with wild-type permease under any condition studied or with E269H permease at pH 5.0. On the basis of these observations, it seems likely that introduction of a His residue at position 269 in place of Glu results in the formation of a divalent metal binding site due to the presence of bis-His residues in the interface between helices VIII (E269H) and X (H322) (Figure 5). As such, the results provide novel support for site-directed excimer fluorescence studies (Jung et al., 1993) which led to the same conclusion and provided the impetus for these experiments.

The K_D of about 43 μM for Mn^{2+} determined for E269H permease compares favorably with the K_D values observed for natural metalloproteins or other proteins containing engineered metal-binding sites. For example, the metal-binding site in the bioluminescent protein aequorin exhibits a K_D of about 1 μM for Mn^{2+} (Kemple et al., 1990), L-lactate dehydrogenase has a K_D of 260 μM (Mayr et al., 1982), and a number of proteins containing engineered His-X_3-His sites in exposed α-helices have K_Ds that vary from 2 to 200 μM (Arnold & Haymore, 1991). The relatively high affinity for Mn^{2+} of the bis-His site within a hydrophobic region of the E269H permease mutant is consistent with the notion of Yamashita et al. (1990) that metal ions bind favorably in regions of proteins that show "high hydrophobicity contrast". That is, metal-binding sites are usually centered in a shell of hydrophilic liganding groups surrounded by more hydrophobic side chains. Thus, the transmembrane helices of lac permease which are generally hydrophobic may provide an environment conducive to the construction of metal-binding sites. On the other hand, the site must be accessible to the metal ion, and in this instance with E269H permease, the bis-His residues are presumably located within the transmembrane portion of the protein about one-third of the way into the membrane from the cytoplasmic surface (Figure 5). Since positions 269 and 322 are on hydrophilic surfaces of amphipathic helices, it is possible that solvent is accessible to the interface. Alternatively, helices VIII and X may line the solvent-filled notch in the permease (Costello et al., 1984, 1987; Li & Tooth, 1987). In any case, it is apparent that the metal-binding site in E269 permease is accessible to Mn^{2+}

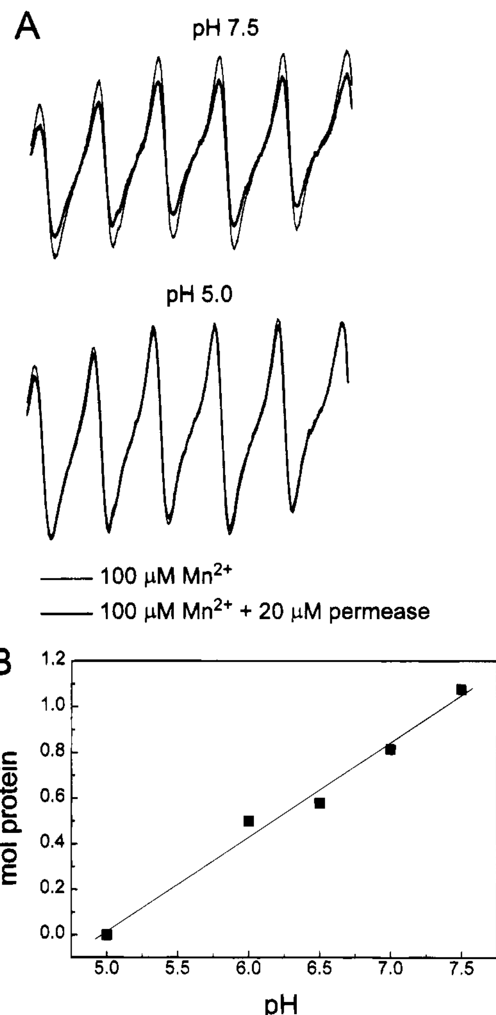
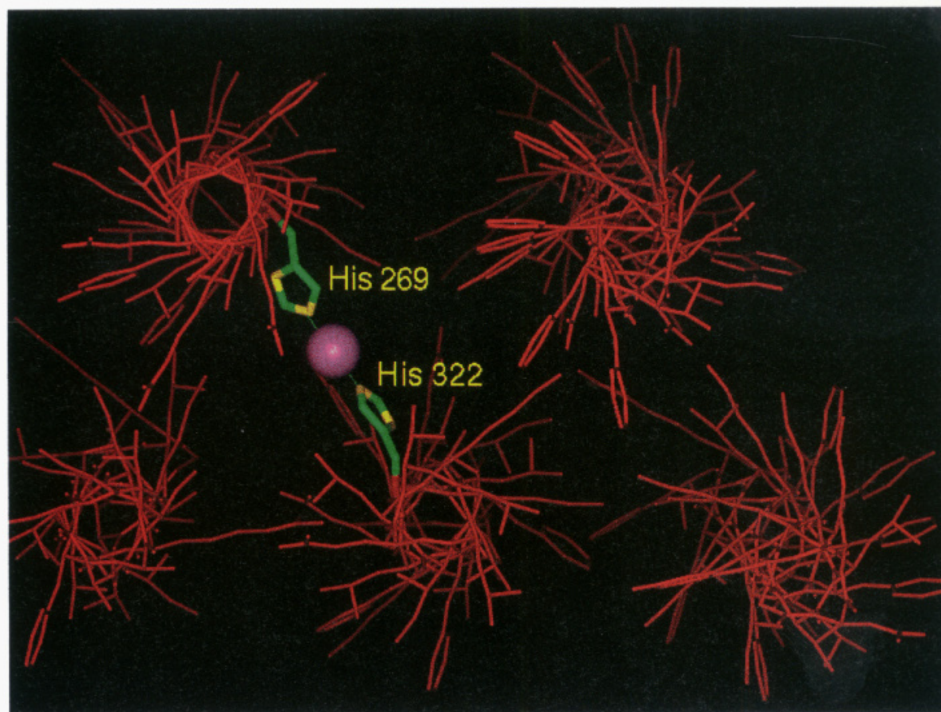


FIGURE 4: pH dependence of Mn^{2+} binding to E269H permease. (A) Spectra obtained at pH 7.5 and 5.0. Representative EPR spectra at pH 7.5 or 5.0 were measured in 10 mM MES at a given pH containing 0.01% DM in the absence (thin line) or presence of 20 μM E269H permease (heavy line). (B) pH titration. Binding (mol of Mn^{2+} bound/mol of protein) was determined from the difference in the free Mn^{2+} concentration calculated from EPR spectra obtained in the absence or presence of 20 μM E269H permease at given pH values as described in Experimental Procedures.

and also to the aqueous phase, as the midpoint of the pH titration approximates the pK of imidazole.

In addition to introduction of metal-binding sites for protein purification by metal chelate chromatography [see Hochuli et al. (1987), Loddenkötter et al. (1993), and Berhe et al. (1995) for examples], this operation is important in the development of a new technique which utilizes paramagnetic metal–spin label interactions to measure distance within proteins [Voss et al., 1995; see Hubbell and Altenbach (1994) in addition]. By this means, a metal-binding site is incorporated into a protein containing a single Cys residue which is purified and labeled with a thiol-specific nitroxide spin label. Binding of paramagnetic metals such as Cu^{2+} or Mn^{2+} reduces the intensity of the nitroxide signal in a distance-dependent manner. In this context it is noteworthy that two or six contiguous His residues can be inserted into most of the hydrophilic loops of the permease with little or no effect on activity (McKenna et al., 1992) and that permease with six contiguous His residues in the cytoplasmic loop between helices III and IV binds Mn^{2+} or Ni^{2+} in the micromolar range (J. Voss, W. L. Hubbell, and H. R. Kaback,



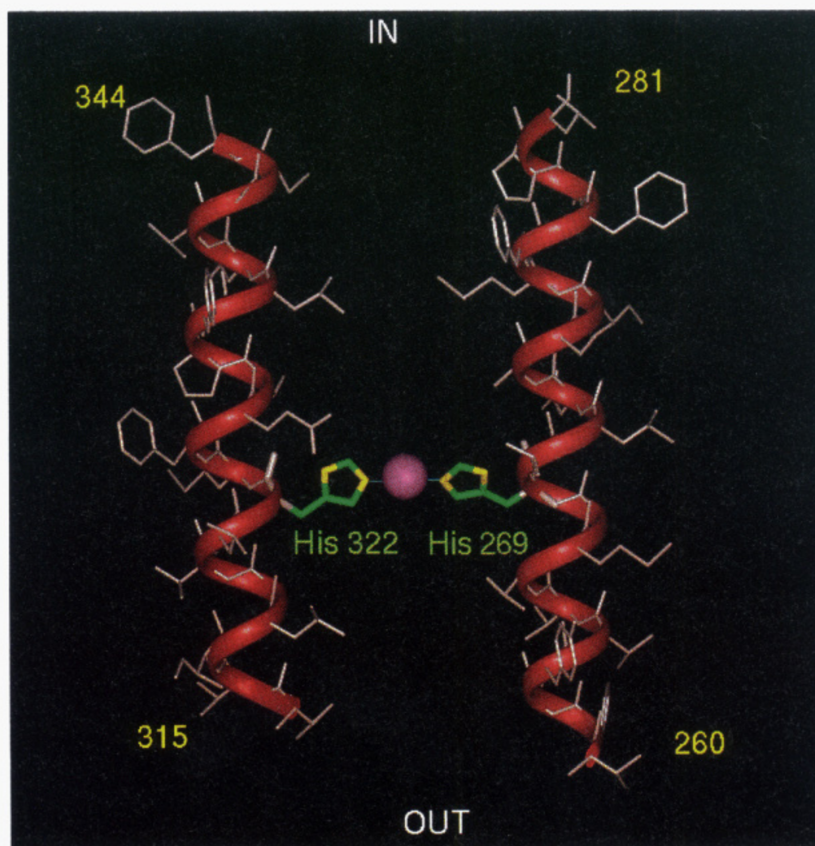


FIGURE 5: Model of transmembrane helices VII–XI of E269H permease. (A, top) View from the periplasm. Ideal α-helices for the five segments were arranged with the proper polarity and the nonpolar regions aligned to place each helix at its proper "depth" within the membrane. The helices were then moved laterally so as to maximize the deduced interaction between bis-His residues at positions 269 (helix VIII) and 322 (helix X) and Mn^{2+} (magenta). For clarity, the distances between the helices are greater than the normal separation between packed, parallel α-helices [see Jung et al. (1993) in addition]. (B, bottom) Side view. Helices VIII and X are shown with bis-His residues at positions 269 (helix VIII) and 322 (helix X) chelating Mn^{2+} (magenta).

unpublished information). Moreover, permease containing His residues in place of Asp237 (helix VII) and Lys358 (helix XI), two residues thought to be salt bridged [see Kaback et al. (1993, 1994)], binds Mn^{2+} stoichiometrically in a manner similar to that described for E269H permease (M. He, J. Voss, W. L. Hubbell, and H. R. Kaback, manuscript in preparation). Finally, permease with His residues in place of Met145 and Val149 on the same face of helix V also binds Mn^{2+} with high affiniy (J. Wu, J. Voss, and H. R. Kaback, unpublished information).

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