Use of Designed Metal-Binding Sites To Study Helix Proximity in the Lactose Permease of *Escherichia coli*. 1. Proximity of Helix VII (Asp237 and Asp240) with Helices X (Lys319) and XI (Lys358)

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ABSTRACT: The lactose permease of *Escherichia coli* contains two pairs of oppositely charged residues that interact functionally, Asp240 (helix VII)/Lys319 (helix X) and Asp237 (helix VII)/Lys358 (helix XI). Single- and double-His replacement mutants at these positions have been constructed and characterized with respect to transport activity and Mn^{2+} binding. The following results confirm the functional interactions between both sets of residues: (i) At pH 7.5, where the imidazole is likely to be unprotonated, the double-His mutants Asp237→His/Lys358→His and Asp240→His/Lys319→His exhibit significant transport activity while the single-His mutants Lys319→His and Lys358→His are inactive. (ii) At pH 5.5, where the imidazole is likely to be protonated, the double-His mutants Asp240→His/Lys319→His and Asp237→His/Lys358→His are inactive; however, the single-His mutant Lys319→His exhibits significant activity. (iii) The single-His mutant Asp237→His or Asp240→His is inactive at all pH values tested. In addition, a pH titration of Asp237→His/Lys358→His permease activity exhibits a midpoint at about 6.2. Finally, the purified mutant proteins Asp237→His/Lys358→His and Asp240→His/Lys319→His were assayed for Mn^{2+} binding by electron paramagnetic resonance spectroscopy. Asp237→His/Lys358→His permease binds Mn^{2+} with a stoichiometry of unity at pH 7.5, but much less binding is observed at pH 5.5, demonstrating directly that helix VII (Asp237) is in close proximity to helix XI (Lys358). In contrast, Asp240→His/Lys319→His permease does not bind Mn^{2+}, indicating that these two residues interact over a longer distance. In the following paper, the same approach is used to confirm the close proximity of helices IX (Arg302) and X (His322 and Glu325).

The lactose (lac)[1] permease of *Escherichia coli* is a hydrophobic, polytopic, plasma membrane protein that catalyzes the coupled stoichiometric translocation of β-galactosides and H^+. The permease is encoded by the *lacY* gene, which has been cloned and sequenced, and the *lacY* gene product has been solubilized from the membrane, purified to homogeneity, reconstituted into proteoliposomes, and shown to be solely responsible for β-galactoside transport [reviewed in Kaback (1983, 1988, 1992) and Poolman and Konings (1993)] as a monomer [see Sahin-Tóth et al. (1994)]. On the basis of circular dichroism and hydropathy analysis of the primary amino acid sequence, a secondary structure was proposed (Foster et al., 1983) in which the permease is composed of 12 hydrophobic α-helices that traverse the membrane in zig-zag fashion connected by hydrophilic loops with both the N and C termini on the cytosolic side (Figure 1). Evidence favoring general aspects of the model and showing that the N and C termini, as well as the loops between helices IV and V and VI and VII, are on the cytoplasmic face of the membrane has been obtained from a variety of experimental approaches [reviewed in Kaback (1992)]. Moreover, analysis of a large number of lac permease−alkaline phosphatase (*lacY−phoA*) fusions has provided unequivocal support for the 12 transmembrane−helix motif (Calamia & Manoil, 1990).

Interaction between Asp237 and Lys358 (Figure 1) was proposed initially by King et al. (1991) on the basis of second-site suppressor analysis. Permease mutants with Thr in place of Lys358 or Asn in place of Asp237 are defective in lactose transport, and second-site suppressor mutations of K358T[2] yield neutral amino acid substitutions for Asp237 (Asn, Gly, or Thr), while suppressors of D237N exhibit Gln in place of Lys358. In addition, as part of an extensive Cys-scanning mutagenesis study [reviewed in Kaback et al. (1994)] on a functional permease mutant devoid of Cys residues (C-less permease; van Iwaarden et al., 1991), putative intramembrane charged residues were systematically replaced with Cys (Sahin-Tóth et al., 1992). Individual replacement of Asp237 or Lys358 in C-less permease with Cys or Ala abolishes active lactose transport, whereas simultaneous replacement of both charged residues with Cys

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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; PMS, phenazine methosulfate; 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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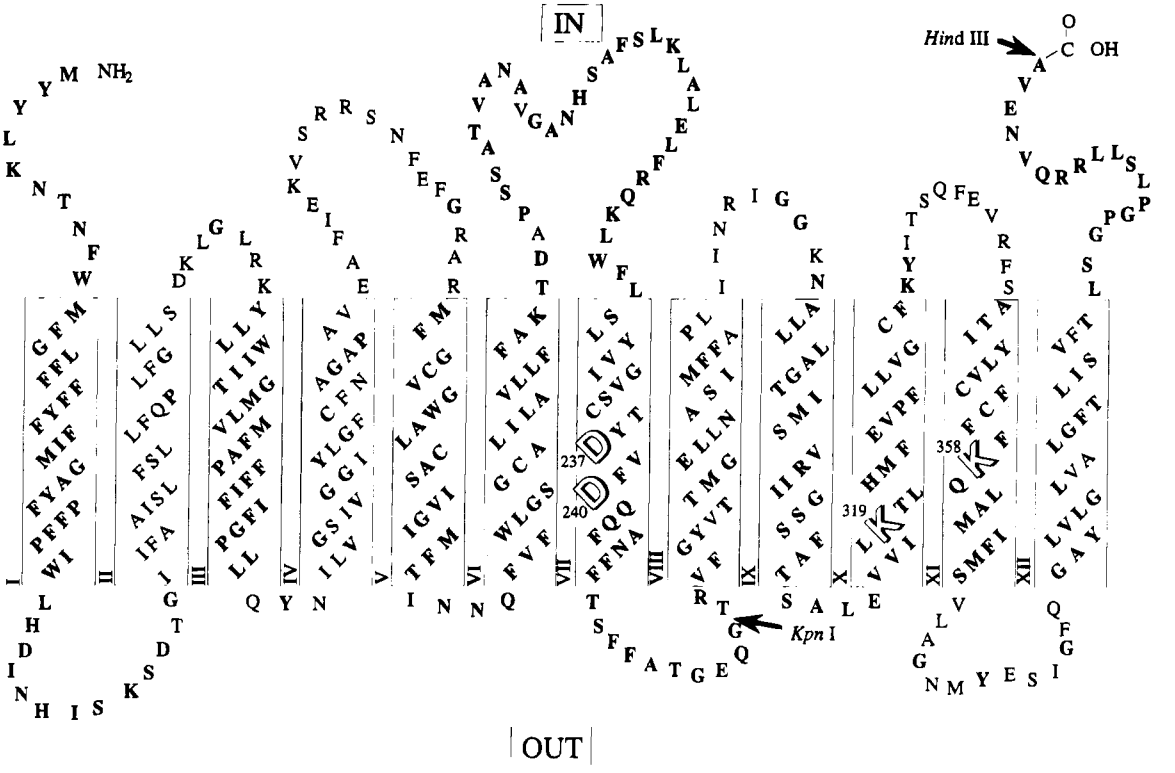

FIGURE 1: Secondary structure model of lac permease. The single-letter amino acid code is used, and Asp237, Asp240, Lys319, and Lys358 are highlighted. Hydrophobic transmembrane helices are shown in boxes. Also indicated are the restriction endonuclease sites used for construction of the mutants.

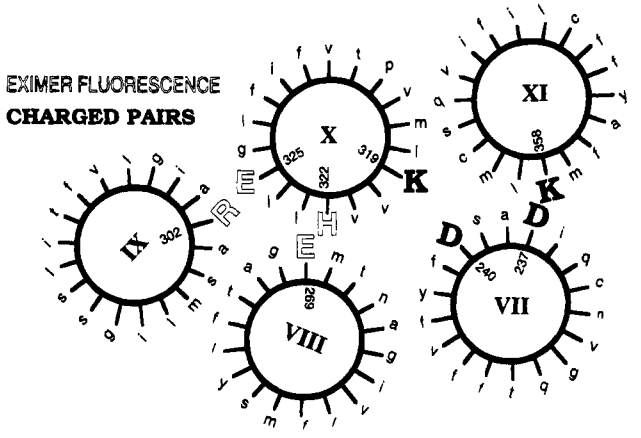

FIGURE 2: Helical wheel model of putative helices VII−XI in lac permease viewed from the periplasmic surface.

and/or Ala or reversal of the residues leads to active permease. Remarkably, mutant D237C is restored to full activity by carboxylmethylation (Dunten et al., 1993b), which recreates a negative charge at position 237, and mutant K358C is restored to full activity by treatment with ethylammonium methanethiosulfonate, which recreates a positive charge at position 358 (Sahin-Tóth & Kaback, 1993a). Taken together the findings clearly suggest that Asp237 and Lys358 neutralize each other via a salt bridge.

It has also been shown by site-directed mutagenesis (Sahin-Tóth et al., 1992; Sahin-Tóth & Kaback, 1993b) and by second-site suppressor analysis (Lee et al., 1992) that Asp240 (helix VII) interacts with Lys319 (helix X). Individual replacement of either residue in C-less permease with neutral amino acid residues inactivates the permease, but double-neutral mutants retain significant activity. In contrast to Asp237/Lys358, however, the polarity of the interaction between Asp240 and Lys319 is important, as reversal of the residues inactivates the permease. In any case, the findings indicate that helix VII (Asp237 and Asp 240) is in close proximity to helix X (Lys319) and helix XI (Lys358). In addition, site-directed pyrene excimer fluorescence has shown that (i) helix IX (Arg302) and X (Glu325) are in close proximity; (ii) helix VIII (Glu269) is in close proximity to helix X (His322); and (iii) helix X (His322 and Glu325) is probably in α-helical conformation (Jung et al., 1993; Kaback et al., 1993, 1994). On the basis of these findings, a model describing helix packing in the C-terminal half of the permease was presented (Figure 2).

Despite functional evidence indicating that Asp237 interacts with Lys358 and Asp240 interacts with Lys319, various attempts to demonstrate directly that the two sets of residues are in close proximity have not been revealing (Sahin-Tóth et al., 1995). Recently, an approach has been introduced (Jung et al., 1995) for studying helix proximity based on engineering a metal-binding site (bis-His residues) between helices thought to interact. Thus the close proximity between helices VII (Glu269) and X (His322) was confirmed by demonstrating that Mn^{2+} binds to lac permease containing a native His residue at position 322 and a His residue in place of Glu269 with a K_D of about 40 μM, a stoichiometry of unity, and a pK_a of about 6.3. In this paper, the approach is utilized to study proximity in the two functionally interacting charge pairs, Asp237/Lys358 and Asp240/Lys319. Single- and double-His replacement mutants at these positions are characterized with respect to transport activity and Mn^{2+} binding. The results confirm the functional interactions between both sets of residues and demonstrate further that D237H/K358H permease forms an Mn^{2+}-binding site. Thus, Asp237 and Lys358 must be in close physical proximity. In contrast, D240H/K319H permease does not bind Mn^{2+},

indicating that these two residues interact over a longer distance.

In the following paper (He et al., 1995), engineered metal-binding sites are used to confirm other aspects of the helix-packing model.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids. Plasmid pKR35/*lacY*-CXB was constructed by inserting a DNA fragment encoding the biotin acceptor domain from an oxaloacetate decarboxylase of *Klebsiella pneumonia* into the *Esp*I site at the 3′-end of the cassette *lacY* gene cloned into pKR35 as described (Consler et al., 1993). *E. coli* T184 (Z^-Y^-) harboring the plasmid pKR35/*lacY*-CXB encoding given mutant permeases was used for expression from the *lac* promoter/operator by induction with isopropyl 1-thio-β-D-galactopyranoside (IPTG).

Construction of Mutants. His mutants were constructed by oligonucleotide-directed site-specific mutagenesis. The cassette *lacY* gene encoding wild-type permease in plasmid pKR35/*lacY*-CXB was used as a template for mutagenesis. All site-specific mutations were directed with synthetic mutagenic oligonucleotide primers. Mutants D237H, D240H, K319H, and K358H were constructed by a two-stage polymerase chain reaction (PCR) method (overlap-extension) with two complementary mutagenic primers (Ho et al., 1989). Mutants containing two amino acid replacements in the same molecule were constructed by "cut and paste," making use of the restriction endonuclease sites shown in Figure 1. Briefly, the double mutants D237H/K358H and D237H/K358A were constructed by digesting the individual plasmids with *Kpn*I and *Hin*dIII and ligating the isolated inserts from mutants K358H or K358A into the vector carrying the D237H mutation which was also digested with *Kpn*I and *Hin*dIII. Mutant D240H/K319H was also made by using *Kpn*I and *Hin*dIII restriction sites.

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).

Active Transport. Active transport was measured in *E. coli* T184 (Z^-Y^-) transformed with a given plasmid. Fully grown overnight cultures of cells were diluted 10-fold and grown aerobically in Luria−Bertini broth for 2 h at 37 °C in the presence of 10 μg of streptomycin/mL and 100 μg of ampicillin/mL. To induce expression of *lacY*, 0.5 mM IPTG (final concentration) was added and the cells were grown for another 2 h before harvesting by centrifugation. Transport of [1-^{14}C]lactose (2.5 mCi/mmol; final concentration, 0.4 mM) was assayed in the presence of 20 mM potassium ascorbate and 0.2 mM phenazine methosulfate (PMS) under oxygen by rapid filtration (Konings et al., 1971). When the effect of pH was tested, cells were washed once with 5 mM ethylenediaminetetraacetate (EDTA; potassium salt) and assayed after addition of 0.2 μM nigericin (final concentration).

Expression and Purification of Mutant Permeases. For each mutant permease, 12 L of cells were 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 extracted with 2% DM. Each mutant protein was purified by affinity chromatography on immobilized monomeric avidin (Promega) (Consler et al., 1993). 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). Bound permease was then eluted with 5 mM *d*-biotin in column buffer. Purified samples were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (Laemmli, 1970) and visualized by silver staining. Purified D240H/K319H or D237H/K358H permease was concentrated to 15 μM using 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.

Mn^{2+} Binding. Purified D237H/K358H or D240H/K319H permease was 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(II) 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

Active Transport by D237H, K358H, and D237H/K358H Permeases. Time courses of [1-^{14}C]lactose transport at pH 7.5 for mutant D237H, K358H, or D237H/K358H expressed in *E. coli* T184 (Z^-Y^-), the positive control (pKR35 encoding wild-type permease) and the negative control (pT7-5 devoid of a *lacY* insert) are shown in Figure 3. While both of the single-His mutants D237H and K358H exhibit no lactose accumulation as judged by comparison to the negative control, the double-His mutant D237H/K358H has significant activity, with a rate of about 25% and steady-state level of accumulation of about 40% of wild-type permease.

Effect of pH. In order to verify that the activity of the double-His mutants is due to the neutralization of unpaired charge in the single-His mutants and also to study the pK_a of the His residues, pH titrations of the transport activity of each mutant were carried out in the presence of nigericin in order to ensure pH equilibration across the membrane (Figure 4). Wild-type permease exhibits the highest initial rate of transport at pH 7.5, and activity decreases relatively sharply at lower and higher pH values. Interestingly, while the single-His mutant K358H has no activity at any pH tested, the double-His mutant D237H/K358H displays a pH titration profile typical of a single imidazole with the highest activity at pH 7.5 and an approximate pK_a of 6.2, leading to the speculation that only the His residue at position 237 is titrated and that 358H remains unprotonated. This notion is supported by a pH titration of D237H/K358A permease. Although the activity of this mutant is lower, D237H/358A

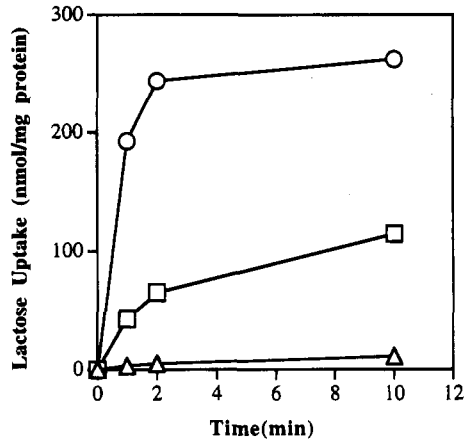

FIGURE 3: Transport of lactose by *E. coli* T184 harboring plasmids encoding wild-type permease, no permease (i.e., pT7-5 with no *lacY* insert), or permease with His replacements for Asp237 and/or Lys358. Cell suspensions were prepared as described in Experimental Procedures, and 50 μL aliquots in 100 mM KP_i (pH 7.5)/10 mM $MgSO_4$ were assayed at room temperature. Transport was initiated by addition of [1-^{14}C]lactose (2.5 mCi/mmol) to a final concentration of 0.4 mM. Reactions were quenched at the times given by addition of 5 mL of 100 mM KP_i (pH 5.5)/100 mM LiCl and rapid filtration through Whatman GF/F filters. O, Wild-type; □, D237H/K358H; △, K358H, D237H, and pT7-5 with no *lacY* insert (no significant difference is observed between cells expressing K358H permease, D237H permease, or no permease).

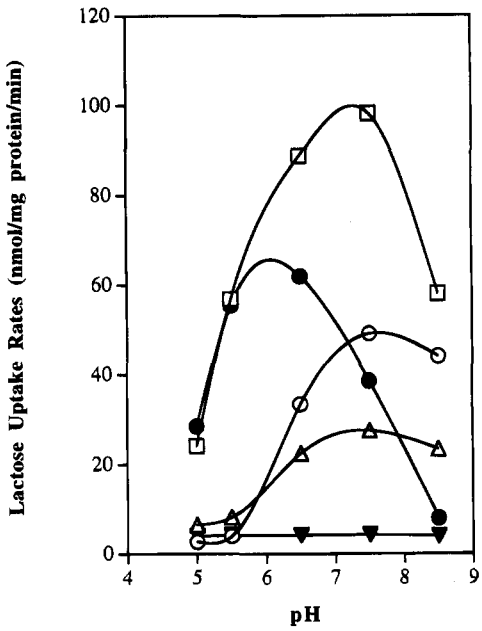

FIGURE 4: pH titration of active lactose transport by *E. coli* T184 harboring plasmid encoding wild-type (□), K358H or D237H (▼; no difference is observed between cells expressing K358H or D237H permease), D237H/K358H (O), D237C/K358C (●), or D237H/K358A (△) permease. Measurements were carried out as described in Experimental Procedures. Initial rates of [1-^{14}C]lactose transport were measured during the initial linear portion of the time course at each pH value given.

displays a pH dependence that resembles an imidazole titration. At low pH where the imidazole at position 237 is presumably charged and uncompensated by the Ala residue at position 358, activity is low. Deprotonation of the imidazole at higher pH removes the uncompensated charge, and activity is observed. Finally, the pH effects observed with the single- and double-His mutants are not due to nonspecific effects of pH on the permease. Thus, the activity

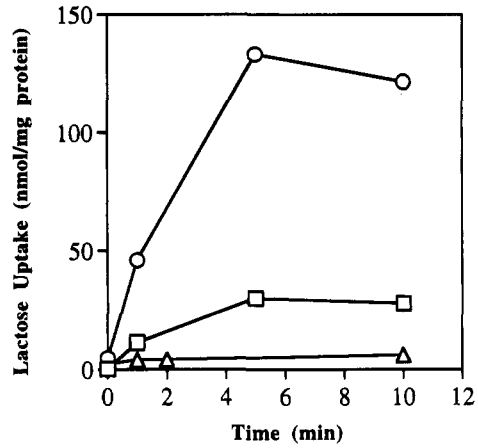

FIGURE 5: Transport of lactose by *E. coli* T184 transformed by plasmid-encoding wild-type permease, no permease (i.e., pT7-5 with no *lacY* insert), or permease with His replacements for Asp240 and/or Lys319. Measurements were carried out as described in Experimental Procedures and in Figure 3. O, wild-type; □, D240H/K319H; △, K319H, D240H, or pT7-5 with no *lacY* insert (no significant difference is observed between cells expressing either mutant permease or no permease).

of the double-Cys mutant D237C/K237C exhibits a completely different titration curve with the highest activity at pH 6.2 and sharply decreasing activity from pH 6.2 to 8.5, indicating that either one or both of the Cys residues at positions 237 and 358 has a pK_a of about 7.5.

Active Transport by D240H, K319H, or D240H/K319H Permease and Effect of pH. As shown for D237H/358H permease, at pH 7.5 the D240H/319H mutant exhibits significant, albeit somewhat lower, activity with an initial rate of about 20% and a steady-state of about 25% of wild-type permease (Figure 5). In contrast, the single-His mutants D240H and K319H retain no activity relative to the negative control, pT7-5 with no *lacY* insert. Furthermore, K319H permease has higher activity at pH 5.5, suggesting that His at position 319 is protonated at low pH and neutralizes the negative charge on Asp240 (Figure 6A). In contrast, the double-His mutant D240H/K319H has higher activity at pH 7.5 than at pH 5.5 (Figure 6B), as expected.

Mn^{2+} Binding. In order to determine whether the double-His mutants contain metal-binding sites, Mn^{2+} binding was measured with the purified mutant proteins by 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 (Reed & Markham, 1984). Since $Mn(H_2O)_6^{2+}$ EPR signals are readily observed in aqueous solution, but not when the ion is complexed with protein ligands, binding can be determined directly by measuring the decrease in amplitude of the free Mn^{2+} spectrum due to the presence of protein (Cohn, 1954). Difference spectra of 75 μM Mn^{2+} in the presence and absence of 15 μM D237H/K358H at pH 7.5 and pH 5.5 are compared in Figure 7. D237H/K358H permease at a concentration of 15 μM reduces the amplitude of the EPR spectrum by 20 ± 1% at an Mn^{2+} concentration of 75 μM which corresponds to a stoichiometry of unity (Figure 7A). Since wild-type permease does not bind Mn^{2+} (Jung et al., 1995), this finding demonstrates directly that D237H/K358H permease contains a metal-binding site with a stoichiometry of unity and a K_D of less than 60 μM (the K_D of E269H

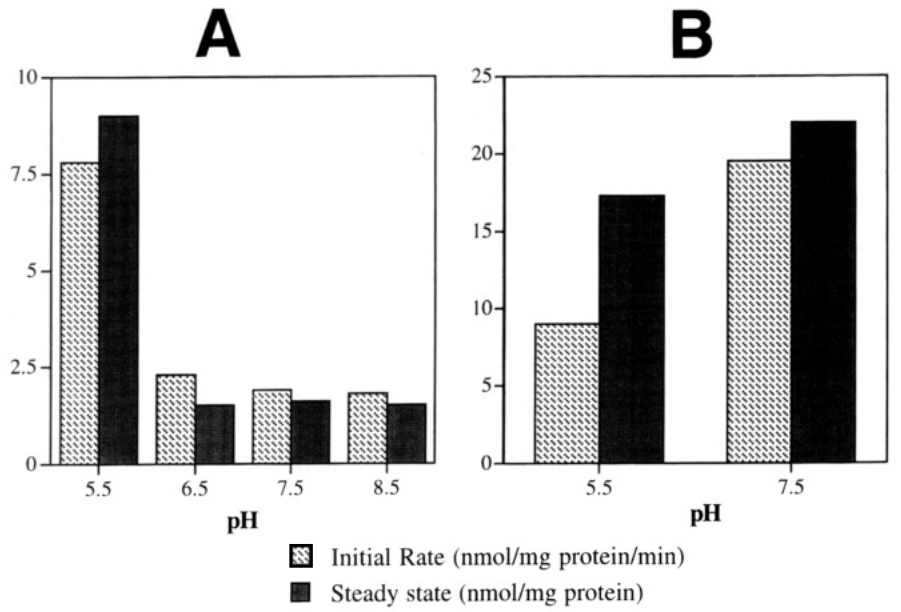

FIGURE 6: Active lactose transport at given pH values by *E. coli* T184 transformed with plasmid-encoding K319H permease (A) or D240H/K319H permease (B). Measurements were carried out as described in Experimental Procedures. For initial rates, transport was measured during the initial linear portion of the time course at each pH; steady state measurements were made at 10 min.

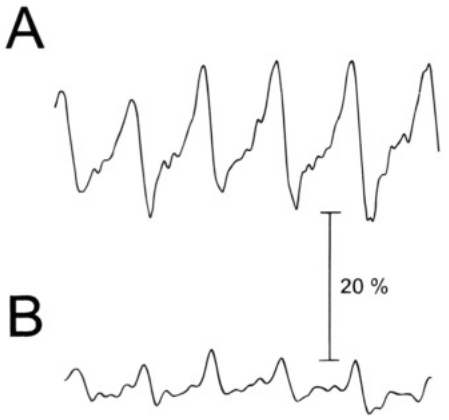

FIGURE 7: Mn^{2+} binding to D237H/K358H permease. Shown are the difference spectra obtained by subtracting the spectrum of a sample containing 75 μM Mn^{2+} and 15 μM D237H/K358H permease from the spectrum of a sample containing 75 μM Mn^{2+} without protein. Spectra were acquired at room temperature 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.

permease is about 40 μM; Jung et al., 1995). Moreover, binding of Mn^{2+} to D237H/K358H is pH dependent. At pH 5.5 the Mn^{2+} binding is reduced by approximately 70% (Figure 7B). In marked contrast, no Mn^{2+} binding whatsoever is observed with D240H/K319H permease at either pH 7.5 or 5.5 (data not shown).

DISCUSSION

Second-site suppressor analysis (King et al., 1991; Lee et al., 1992) and site-directed mutagenesis combined with chemical modification (Dunten et al., 1993a; Sahin-Tóth et al., 1992; Sahin-Tóth & Kaback, 1993a) demonstrate that Asp237 (helix VII) interacts functionally with Lys358 (helix XI) and the Asp240 (helix VII) interacts functionally with Lys319 (helix X). The results presented here confirm and extend the argument that these pairs of residues interact functionally. At pH 7.5, where the imidazole ring of His residue is likely to be unprotonated, single-His mutants D237H, D240H, K358H, and K319H contain an uncompensated charge at positions Lys358, Lys319, Asp237, and Asp240, respectively, which inactivates the permease. In contrast, His replacement of both members of the charge pair in mutant D237H/K358H or D240H/K319H permease results in significant transport activity at pH 7.5 (Figures 3 and 5). Furthermore, the activity of D237H/K358A permease is rescued partially by shifting the pH from 5.0 to 7.5. Conversely, K319H permease exhibits significant activity at pH 5.5, where the imidazole at position 319 is likely to be protonated and neutralizes the negative charge on Asp240, and inactive at pH 7.5 (Figure 6A).

Despite compelling evidence for functional interaction between the two sets of residues, direct evidence demonstrating close physical proximity is lacking. In this paper, an approach (Jung et al., 1995) based on engineering a metal-binding site into lac permease that confirms the proximity of helices VIII (Glu269) and X (His322) (Jung et al., 1993) is used to study the interaction between Asp237 and Lys358 as well as between Asp240 and Lys319. The results demonstrate clearly that Asp237 is in close proximity to Lys358. Thus, D237H/K358H permease chelates Mn^{2+} with the stoichiometry of unity at pH 7.5, and binding is markedly reduced at pH 5.5 (Figure 7). In contradistinction, D240H/K319H permease exhibits no Mn^{2+} binding, and furthermore [see Dunten et al. (1993b), Sahin-Tóth et al. (1992), and Sahin-Tóth and Kaback (1993b)]: (i) Unlike the Asp237/Lys358 charge pair which can be reversed with little or no loss of activity, reversal of Asp240 and Lys319 leads to inactivation. (ii) Although D237C/K358C permease accumulates lactose to essentially a normal steady state, mutant permeases with double-neutral replacements for Asp240 and Lys319 exhibit only 30%−50% of the steady state level of accumulation of the control. (iii) Double-neutral replacements for Asp240 and Lys319 are inserted into the membrane at control levels, while mutants with similar replacements for Asp237 and Lys358 are inserted poorly and are probably degraded prior to or during insertion into the membrane.

Parenthetically, similar phenomena are observed here with double-His mutants; however, the insertion defect observed with Cys and/or Ala in place of Asp237 and Lys358 is largely alleviated in the double-His replacement mutant (data not shown). When considered together, the results indicate that Asp240 and Lys319 interact over a longer distance.

Yamashita et al. (1990) have postulated that metal ions bind favorably in regions of proteins that show "high hydrophobic 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, Asp237 and Lys358 are very likely to be located in transmembrane domains of the permease about one-half to one-third of the way into the membrane from the periplasmic surface (King et al., 1991; Ujwal et al., 1995; Zen et al., 1994). Since positions 237 and 358 are on hydrophilic surfaces of amphipathic helices, the interface may be accessible to solvent. It is also possible that helices VII and XI line the solvent-filled notch in the permease, as suggested by site-directed spectroscopy (Ujwal et al., 1995). In any case, it is apparent that the metal-binding site in D237H/K358H permease, like that of E269H permease (Jung et al., 1995), is accessible to Mn^{2+} and also to the aqueous phase, as binding is reduced at pH 5.5 (i.e., when the imidazole is protonated).

Solvent accessibility of both pairs of interacting residues is also consistent with pH titrations of transport activity in the mutants. D240H/K319H permease is active at pH 7.5, but not at pH 5.5, while mutant K319H has higher activity at lower pH. Therefore, His residues at positions 319 and/or 240 undergo protonation and deprotonation as a function of pH. Moreover, the activity of D237H/K358H or D237H/K358A permease is significantly higher at relatively alkaline pH. In addition, all of the titrations display a midpoint at approximately pH 6.2, which is reasonably close to the pK of imidazole.

Finally, it is interesting that K358H permease exhibits no activity over the range of pH values tested, while the activity of the D237H/K358A mutant displays a pH titration similar to that of imidazole. Taken together with the observation that D237H/K358H permease activity also exhibits a pH profile typical of a single imidazole, it is possible that K358H remains unprotonated and is less accessible to solvent than Asp237. This suggestion is consistent with the previous studies (Dunten et al., 1993b) demonstrating that the hydrophilic thiol reagent methanethiosulfonate ethylsulfonate inactivates D237C/K358A permease more effectively than it inactivates mutant D237A/K358C. Furthermore, recent site-directed fluorescence studies (Ujwal et al., 1995) demonstrate that position 237 is more accessible to collisional quenchers than position 358.

In any case, the results presented here extend previous studies (Jung et al., 1995) indicating that introduction of bis-His sites into the transmembrane helices of polytopic membrane proteins provides a unique approach for studying helix packing. In the following paper (He et al., 1995), the general approach is used to confirm additional relationships in the helix-packing model of the C-terminal half of the permease.

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