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Use of Site-Directed Fluorescence Labeling To Study Proximity Relationships in the Lactose Permease of *Escherichia coli*†

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ABSTRACT: The lactose permease of *Escherichia coli* is a paradigm for polytopic membrane transport proteins that transduce free energy stored in an electrochemical ion gradient into work in the form of a concentration gradient. Although the permease consists of 12 hydrophobic transmembrane domains in probable α-helical conformation that traverse the membrane in zigzag fashion connected by hydrophilic "loops", little information is available regarding the folded tertiary structure of the molecule. In this paper, we describe an approach to studying proximity relationships in lactose permease that is based upon site-directed pyrene labeling of combinations of paired Cys replacements in a mutant devoid of Cys residues. Since pyrene exhibits excimer fluorescence if two molecules are within about 3.5 Å, the proximity between paired labeled residues can be determined. The results demonstrate that putative helices VIII and IX are close to helix X. Taken together with other findings indicating that helix VII is close to helices X and XI, the data lead to a model that describes the packing of helices VII–XI.

Lactose (lac)[1] permease is a hydrophobic, polytopic, plasma membrane protein that catalyzes the coupled stoichiometric translocation of β-galactosides and H^+ [reviewed in Kaback (1983, 1989, 1992)]. The permease is encoded by the *lacY* gene, the second structural gene in the *lac* operon which has been cloned (Teather et al., 1978) and sequenced (Büchel et al., 1980). Moreover, 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 (Newman et al., 1981; Viitanen et al., 1986). On the basis of circular dichroism and the hydropathy profile of the deduced amino acid sequence, a secondary-structure model was proposed (Foster et al., 1983) in which the permease is composed of a hydrophilic N-terminus followed by 12 hydrophobic segments in α-helical conformation that traverse the membrane in zigzag fashion connected by hydrophilic domains (loops) with a 17-residue C-terminal hydrophilic tail (Figure 1). Evidence favoring general aspects of the model and showing that both the N- and C-termini are on the cytoplasmic face of the membrane has been obtained from a variety of experimental approaches [see Kaback (1983, 1989, 1992)]. Moreover, analysis of a large number of lac permease–alkaline phosphatase (*lacY-phoA*) fusions has provided unequivocal support for the 12-helix motif (Calamia & Manoil, 1990).

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. In the experiments reported here, a functional permease mutant

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[1] Abbreviations: lac, lactose; C-less permease, functional lac permease devoid of Cys residues; OG, octyl β-D-glucopyranoside; KP_i, potassium phosphate; DTT, dithiothreitol; PM, *N*-(1-pyrenyl)maleimide; TDG, β-D-galactopyranosyl 1-thio-β-D-galactopyranoside; $\Delta\tilde{\mu}_{H^+}$, the H^+ electrochemical gradient.

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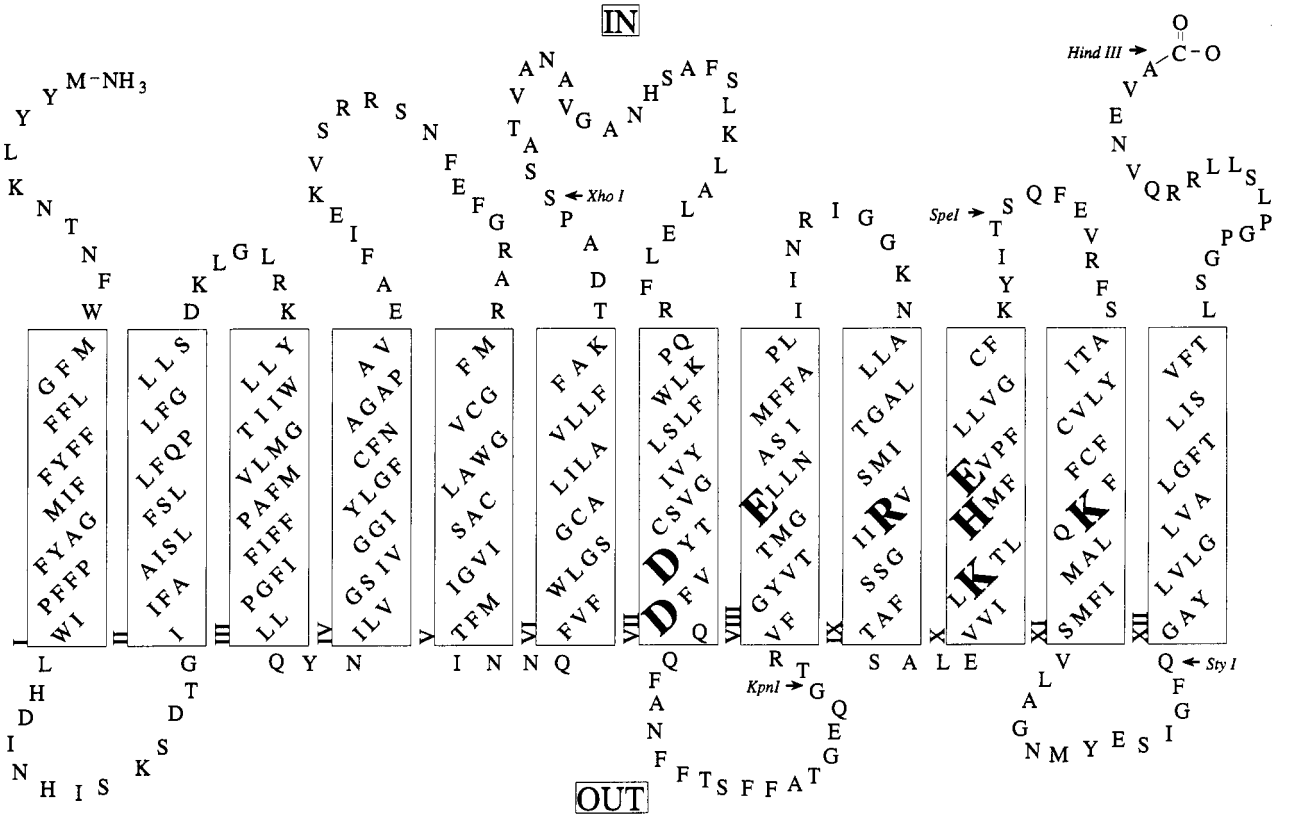

FIGURE 1: Secondary-structure model of lac permease based on hydropathy analysis (Foster et al., 1983). The single-letter amino acid code is used, and the positions of putative intramembrane charged residues (Asp237, Asp240, Glu269, Arg302, Lys319, His322, Glu325, and Lys358) are highlighted. Hydrophobic transmembrane helices are shown in boxes, and the topology of helix VII was modified according to results obtained from a series of *lacY-phoA* fusions in this region (M. L. Ujwal and H. R. Kaback, unpublished observations). Also indicated are the restriction endonuclease sites used for construction of mutants.

devoid of Cys residues (C-less permease) (van Iwaarden et al., 1991) was used, and pairs of charged amino acid residues in transmembrane domains were replaced with Cys to provide specific sites for labeling with an appropriate fluorophore. In addition, the mutants were constructed with a biotinylation domain in the middle cytoplasmic loop to facilitate purification by monovalent avidin affinity chromatography (Consler et al., 1993). As a fluorescent probe, the thiol-specific derivative *N*-(1-pyrenyl)maleimide (PM) was selected, since PM can be used to study the proximity of Cys residues (Betcher-Lange & Lehrer, 1978; Ishii & Lehrer, 1987; Lüdi & Hasselbach, 1988; Sen & Chakrabarti, 1990, Wang et al., 1992). Thus, two pyrene moieties can form an excited-state dimer (excimer) that exhibits an emission maximum at longer wavelengths (ca. 470 nm) than the monomer (ca. 380–420 nm) if the conjugated ring systems are within about 3.5 Å of each other and in the correct orientation (Kinnunen et al., 1993). By using this approach, proximity relationships between transmembrane helices in the C-terminal half of the permease are delineated.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids. *Escherichia 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) was cotransformed with (i) plasmid pT7-5/*lacY*-L6XB (Consler et al., 1993) encoding C-less permease with given Cys replacements and a biotinylation domain in the middle cytoplasmic loop and (ii) pGP1-2 (Tabor & Richardson, 1985) encoding T7 RNA polymerase under the control of a temperature-sensitive λ promotor and used for protein expression.

Construction of Mutants. Construction of single-Cys mutants by site-directed mutagenesis has been described (Sahin-Tóth & Kaback, 1993a). The double mutants were prepared by restriction fragment replacement (Sahin-Tóth et al., 1992) using a cassette version of the *lacY* gene containing unique restriction sites about every 100 bp (EMBL X-56095). Mutant H322C/E325C was constructed by two-stage polymerase chain reaction (overlap extension) (Ho et al., 1989) using two complementary synthetic mutagenic oligonucleotide primers synthesized on an Applied Biosystems 391 DNA synthesizer. Double-stranded DNA prepared by Magic Minipreps (Promega) was sequenced by using the dideoxy chain termination method (Sanger et al., 1977) and synthetic sequencing primers after alkaline denaturation.

Expression and Purification of the Mutant Proteins. Twelve liters of cells was cultivated at 30 °C and heat-shocked for 20 min at 42 °C. Membranes were prepared as described (Viitanen et al., 1986) and extracted with 1.25% octyl β-D-glucopyranoside (OG). All permease mutants were 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 7.5)/150 mM KCl/1.25% OG (w/v)/1 mM dithiothreitol (DTT)/20 mM lactose/0.25 mg/mL acetone/ether-washed *E. coli* phospholipids (Avanti Polar Lipids) (column buffer). After application of the sample, the column was washed thoroughly with column buffer. Bound lac permease was then eluted with 2 mM biotin-*d* in column buffer without DTT. Purified samples were analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (Laemmli, 1970) and visualized by silver staining.

Labeling with PM. Fractions containing purified permease were pooled and labeled with *N*-(1-pyrenyl)maleimide (PM) (Molecular Probes) as follows: PM dissolved in *N*,*N*-dimethylformamide was added slowly to a stirred sample of

purified lac permease in column buffer until a 10-fold molar excess of reagent to protein was achieved. The reaction mixture was incubated overnight at 4 °C in the dark with stirring and centrifuged at 15000*g* for 20 min to remove undissolved reagent, and a 5-fold excess of glutathione over PM was added to quench the reaction. After being stirred for an additional 1 h, the labeled protein was reconstituted into acetone/ether-washed *E. coli* phospholipids (Avanti Polar Lipids) by detergent dilution (Viitanen et al., 1986). Proteoliposomes were washed extensively [four cycles of centrifugation (150000*g*) and resuspension in 50 mM KP_i (pH 7.5)] and resuspended in 50 mM KP_i (pH 7.5), followed by two cycles of freeze–thaw/sonication.

Fluorescence Measurements and Estimation of PM Labeling. Fluorescence emission spectra were recorded at 30 °C using an Aminco SLM spectrofluorometer 8000C equipped with a thermostated cell (excitation, 344 nm). For each preparation, the amount of PM covalently bound was determined after solubilization of the protein from the proteoliposomes with OG as follows: (i) by measuring the absorption of pyrene and determining the concentration assuming an extinction coefficient of 2.2×10^4 $M^{-1} \cdot cm^{-1}$ at 340 nm (Kouyama & Mihashi, 1981); (ii) by titrating the free thiol groups remaining in the permease with *N*-[*ethyl*-1-^{14}C]-ethylmaleimide (Amersham). Protein was determined using the amido-black method (Schaffner & Weissmann, 1973). By both methods, the double mutants contained 0.8–1.0 mol of pyrene/mol of protein and the single mutants 0.6–0.7.

RESULTS AND DISCUSSION

Attention was focused initially on residues of the permease that are important for activity and have been postulated to interact: Arg302 in putative helix IX (Menick et al., 1987) and His322 (Padan et al., 1985; Püttner et al., 1986, 1989; King & Wilson, 1989a,b, 1990a,b; Brooker, 1991) and Glu325 (Carrasco et al., 1986, 1989) in putative helix X. Moreover, Cys-scanning mutagenesis (Sahin-Tóth & Kaback, 1993a) of transmembrane domains IX and X indicates that Arg302, His322, and Glu325 are the only three residues in this region that play an important role in the transport mechanism. In order to test the proximity of the residues, the double-Cys mutants H322C/E325C,[2] R302C/H322C, and R302C/E325C, as well as the corresponding single-Cys mutants, were constructed with a biotinylation domain in the middle cytoplasmic loop, purified by avidin affinity chromatography, labeled with PM, reconstituted into proteoliposomes, and studied spectroscopically.

Fluorescence emission spectra for the PM-labeled permease mutants are shown in Figure 2. With the double-Cys mutant H322C/E325C, a broad band centered around 470 nm that is typical of pyrene excimer fluorescence is observed. In addition, more defined maxima characteristic of the monomer are seen at shorter wavelengths (i.e., ca. 378, 398, and 417 nm; Figure 2A). The observation is consistent with the idea (Püttner et al., 1986; Carrasco et al., 1986) that His322 and Glu325 are located in a portion of the permease that is in α-helical conformation, since the residues would be on the same face of a helix. Mutant R302C/E325C also exhibits excimer fluorescence after labeling with PM (Figure 2B). Under the same conditions, however, an excimer band is not observed with mutant R302C/H322C (Figure 2C). Taken together, the results indicate that although helix IX is close to helix X (Menick et al., 1987), Arg302 is close to Glu325, rather than His322.

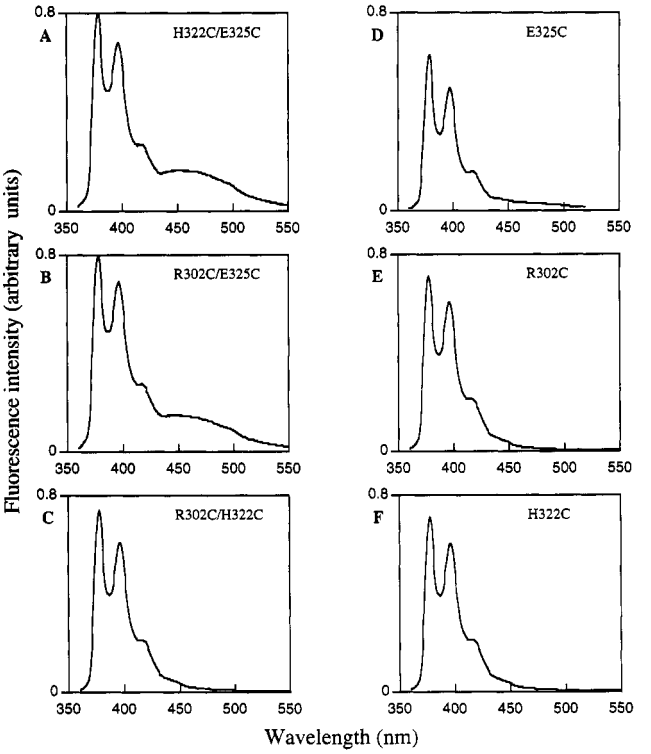

FIGURE 2: Fluorescence emission spectra of PM-labeled, purified lac permease mutants reconstituted into proteoliposomes. Spectra were obtained from samples containing lac permease (50 μg/mL) in 50 mM KP_i (pH 7.5) at 30 °C (2-mL total volume). The excitation wavelength was 344 nm.

Given the propensity of hydrophobic proteins like lac permease to aggregate, it is essential to determine whether or not the excimer fluorescence observed with the pairs H322C/E325C and R302C/E325C results from an intramolecular rather than an intermolecular interaction. Therefore, the following experiments were performed: (i) Each of the corresponding single-Cys mutants was analyzed (Figure 2D–F). Clearly, the emission spectra of the purified, labeled, reconstituted mutants R302C, H322C, and E325C exhibit characteristic monomer bands with maxima at about 378, 398, and 417 nm, but no excimer band at 470 nm is observed. (ii) Mutants containing single-Cys replacements for R302 or E325 were purified separately, labeled with PM, mixed, and reconstituted into proteoliposomes. Although not shown, the emission spectrum exhibits no excimer fluorescence at 470 nm and is identical to the spectra obtained from the unmixed single-Cys mutants (Figure 2D,E). (iii) The ratio of excimer to monomer fluorescence was measured at different lipid to protein ratios with PM-labeled R302C/H322C. If excimer fluorescence results from an intermolecular interaction, the ratio of excimer to monomer fluorescence (i.e., the 470 nm: 378 nm ratio) should be inversely related to lipid:protein ratio. Therefore, PM-labeled permease was reconstituted at lipid: protein ratios of 128:1, 385:1 (cf. Figure 2C), and 1000:1 (w/w). All three samples exhibit the same low excimer: monomer ratio. Taking (i), (ii), and (iii) together, it seems likely that the excimer fluorescence observed with PM-labeled E325C/H322C and R302C/E325C permeases results from intramolecular interactions between pyrene molecules attached to Cys residues within single molecules.

[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.

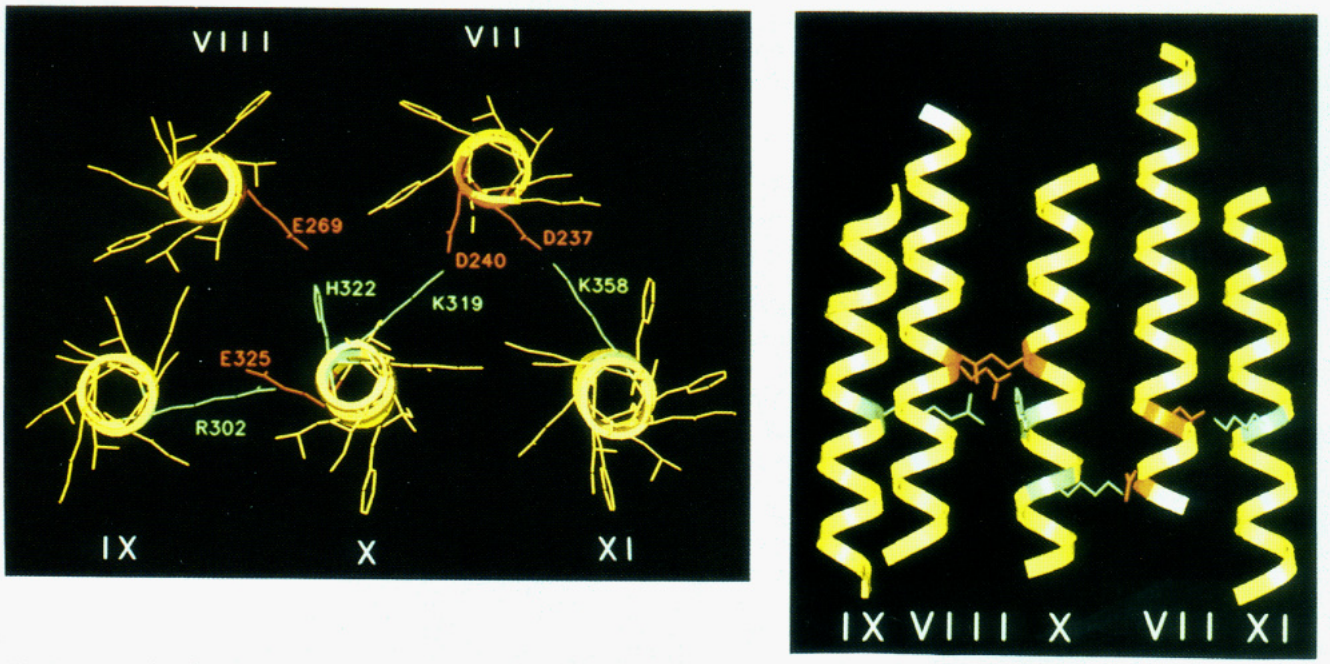

FIGURE 3: Model of transmembrane helices VII–XI of lac permease. (A, left) View from the periplasm. Ideal α-helices for the five segments were arranged with the proper polarity and the nonpolar regions aligned to place each helix at its proper "depth" within the membrane. The helices were then moved laterally so as to maximize the deduced interactions: D237/K358, D240/K319, E269/H322, and R302/E325. For clarity, the distances between the helices are greater than the normal separation between packed, parallel α-helices. (B, right) Side view. Helices VII and VIII are meant to be in front of helices IX, X, and XI. Red side chains indicate acidic residues, and green side chains are basic.

On the basis of an analysis of second-site suppressor mutations in lac permease, King et al. (1991) proposed that Asp237 (helix VII) interacts with Lys358 (helix XI) to form a salt bridge. This conclusion has received strong support from experiments with C-less permease (Sahin-Tóth et al., 1992; Dunten et al., 1992; Sahin-Tóth & Kaback, 1993b). Individual replacement of Asp237 or Lys358 (Figure 1) with Cys or Ala abolishes active lactose transport, while simultaneous replacement of both charged residues with Cys and/or Ala or reversal of the residues leads to fully active permease. Remarkably, moreover, mutant D237C is restored to full activity by carboxymethylation 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 (Dunten et al., 1993; Sahin-Tóth & Kaback, 1993a). In addition to this interaction, evidence has been presented (Sahin-Tóth et al., 1992; Lee et al., 1992) that Asp240 (helix VII) and Lys319 (helix X) also interact. Individual replacement of either Asp240 or Lys319 in C-less permease with neutral amino acid residues inactivates the permease, but double neutral substitutions exhibit significant transport activity. In contrast to Asp237–Lys358, however, the polarity of the interaction between Asp240 and Lys319 is important, since reversal of the residues inactivates the permease (Sahin-Tóth et al., 1992). In any event, the studies indicate that helix VII (Asp237 and Asp240) is in close proximity to helices X (Lys319) and XI (Lys358).

Combining results obtained from pyrene fluorescence with mutagenesis studies leads to the model shown in Figure 3A, where helices VII, IX, X, and XI are shown to interact via ion pairs between R302 and E325, K319 and D240, and K358 and D237. A side-view projection of the helices reveals that these residues are at approximately the same level with respect to depth (Figure 3B).

In addition to neutral substitutions for Lys319, a second-site suppressor mutant for Asp240 has been described (Lee et al., 1992) with Val in place of Gly268. Moreover, recent

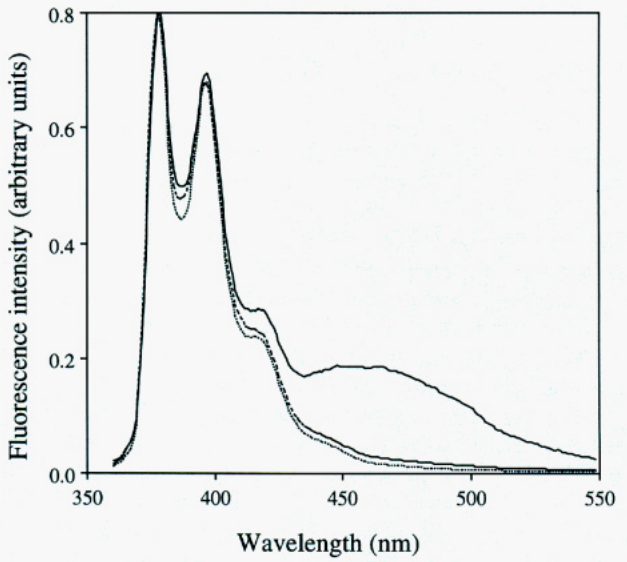

FIGURE 4: Emission spectra of the PM-labeled, purified lac permease mutant E269C/H322C (solid line) and the corresponding single-Cys mutants E269C (dotted line) and H322C (broken line) reconstituted into proteoliposomes. Spectra were obtained under the same conditions as described in Figure 2. Although not shown, when the single-Cys mutants were purified, labeled with PM, mixed, and reconstituted, spectra identical to those observed with E269C or H322C were obtained.

experiments (J. Wu and H. R. Kaback, unpublished information) demonstrate that Ser substitution for Gly262 suppresses the phenotype of E325C. These observations led to the notion that putative helix VIII may interact with helices VII and X, thereby bringing Glu269 into proximity with His322 (Figure 3).

Regarding Glu269, Hinkle et al. (1990) utilized cassette mutagenesis of *lacY* DNA encoding putative helix VIII to identify mutants in this region of the permease that retain the ability to catalyze lactose accumulation. A stripe of residues, largely on one side of helix VIII opposite Glu269, was identified that tolerates mutations with relatively little effect on activity,

suggesting that this mutable strip of low-information content is probably in contact with the membrane phospholipids. Since no active mutants in Glu269 were identified, this residue was subjected to site-directed mutagenesis, and permease with Cys or Gln in place of Glu269 is completely inactive, while Asp269 permease is completely defective with respect to lactose transport but catalyzes significant accumulation of β-D-galactopyranosyl 1-thio-β-D-galactopyranoside (TDG) (Ujwal et al., 1993). It is also noteworthy that paired double mutants containing E269C and Cys replacements for each of the other charged residues in transmembrane domains are inactive (Sahin-Tóth et al., 1992). In brief, therefore, Glu269 plays an important role in the transport mechanism.

In order to test the notion that Glu269 interacts with His322, the double-Cys mutant E269C/H322C was constructed. The mutant and the corresponding single-Cys mutants containing biotinylation domains in the middle cytoplasmic loop were purified, labeled with PM, and reconstituted into proteoliposomes. Strikingly, PM-labeled E269C/H322C permease exhibits a clear excimer emission band at 470 nm (Figure 4). Importantly, no excimer fluorescence is observed with PM-labeled E269C or H322C nor when the single-Cys mutants are mixed prior to reconstitution. The results demonstrate that Glu269 interacts with His322 and imply strongly that helix VIII is in close proximity to helix X (Figure 3).

Preliminary experiments indicate that the excimer fluorescence observed between transmembrane domains can be used to study certain dynamic aspects of permease folding. Thus, the excimer observed with reconstituted PM-labeled R302C/E325C or E269C/H322C permease is markedly diminished by increasing concentrations of sodium dodecyl sulfate (up to 0.6%), while the excimer band with the H322C/E325C mutant is unaffected (data not shown). The results suggest that the detergent disrupts tertiary interactions within the permease with little effect on secondary structure. Consistently, the double mutants E269C/H322C and R302C/E325C do not exhibit excimer fluorescence after labeling with PM in octyl β-D-glucopyranoside, but only after reconstitution into proteoliposomes.

Although individual replacement of each of the charged residues in transmembrane domains inactivates active lactose transport (Padan et al., 1985; Püttner et al., 1986, 1989; Carrasco et al., 1986, 1989; Püttner & Kaback, 1988; Lee et al., 1989; King & Wilson, 1989a,b, 1990a,b; Sahin-Tóth et al., 1992; Ujwal et al., 1993), preliminary experiments indicate that some of the constructs described here exhibit ligand-induced conformational alterations. Excimer fluorescence in proteoliposomes containing PM-labeled E269C/H322C permease is quenched by Tl^+, and the effect is markedly attenuated by 5 mM TDG (i.e., Stern–Volmer plots reveal an decrease in the quenching constant for Tl^+ from 27 to 10 M^{-1} in the presence of 5 mM TDG). In contrast, Tl^+ quenching of the excimer band observed with PM-labeled H322C/E325C permease is unaffected by TDG. Interestingly, it has also been demonstrated recently (Sahin-Tóth & Kaback, 1993a) that C-less permease with a single Cys residue in place of Val315 (presumably the N-terminal residue in helix X; Figure 1) is inactivated by *N*-ethylmaleimide much more rapidly in the presence of TDG or a proton electrochemical gradient ($\Delta\bar{\mu}_{H^+}$). The findings imply that ligand binding or $\Delta\bar{\mu}_{H^+}$ induces a change in tertiary structure without effecting secondary structure.

Finally, it should be emphasized that the use of site-directed mutagenesis to place Cys residues at specific positions has been used successfully in cross-linking studies of the tar receptor (Falke & Koshland, 1987; Pakula & Simon, 1992) and for site-directed spin-labeling of bacteriorhodopsin (Altenbach et al., 1990), for example. The approach described here utilizes functional lac permease devoid of Cys residues with a biotinylation domain in the middle cytoplasmic loop to facilitate purification. Pairs of charged residues in the construct were replaced with Cys, and after purification, the proteins were labeled with PM as reporter group because of the unique property of the probe to exhibit excimer fluorescence if two molecules of the fluorophore are within about 3.5 Å. Findings obtained using this approach are consistent with the following conclusions: (i) transmembrane domain X is probably in α-helical conformation; (ii) Arg302 (helix IX) and Glu325 (helix X) are in close proximity; (iii) His322 (helix X) is in close proximity to Glu269 (helix VIII). Taken together with other experimental results indicating that helix VII is close to helices X and XI, the observations provide a model that describes the packing of helices VII–XI.

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