Dynamics of Lactose Permease of *Escherichia coli* Determined by Site-Directed Chemical Labeling and Fluorescence Spectroscopy†

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ABSTRACT: Mutants with a single Cys residue in place of Phe27, Pro28, Phe29, Phe30, or Pro31 at the periplasmic end of putative transmembrane helix I were used to study the interaction of lactose permease with ligand by site-directed chemical modification or fluorescence spectroscopy. With permease embedded in the native membrane, mutant Phe27 → Cys or Phe28 → Cys is readily labeled with [^{14}C]-*N*-ethylmaleimide (NEM), while mutant Phe29 → Cys, Phe30 → Cys, or Phe31 → Cys reacts less effectively. β,D-Galactopyranosyl 1-thio-β,D-galactopyranoside (TDG) has little or no effect on the reactivity of Phe27 → Cys, Phe29 → Cys, or Phe30 → Cys permease. Remarkably, however, Pro31 → Cys permease which is essentially unreactive in the absence of ligand becomes highly reactive in the presence of TDG. Ligand also enhances the NEM reactivity of the mutant with Cys in place of Pro28 which is presumably on the same face of helix I as position 31. The five single-Cys mutants which also contain a biotin acceptor domain in the middle cytoplasmic loop were purified by monomeric avidin-affinity chromatography in dodecyl β,D-maltoside and subjected to site-directed fluorescence spectroscopy. Mutants Phe27 → Cys, Phe29 → Cys, and Phe30 → Cys react rapidly with 2-(4′-maleimidylanilino)naphthalene-6-sulfonic acid (MIANS), and reactivity is not altered in the presence of TDG. In striking contrast, mutants Pro28 → Cys and Pro31 → Cys react extremely slowly with MIANS in the absent of ligand, and TDG dramatically enhances reactivity. Finally, when each mutant is labeled with *N*-(1-pyrenyl)maleimide and reconstituted into phospholipid vesicles, 5-doxylstearic acid is shown to quench fluorescence more effectively with pyrene-labeled Phe30 → Cys permease than with pyrene-labeled Pro28 → Cys or Pro31 → Cys permease. Taken together, the results indicate that the face of helix I with Pro28 and Pro31 is buried within the permease in the absence of ligand and becomes more reactive as the result of a ligand-induced conformational change. On the other hand, the opposing face of helix I with Phe30 is likely to be in contact with the lipid phase of the membrane and unaffected by ligand binding.

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 *lac*Y 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, 1989, 1992), Poolman and Konings (1993)] as a monomer [see Sahin-Tóth et al. (1994b)]. 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 zigzag fashion connected by hydrophilic loops with both N and C termini in the cytosolic side. 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 [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 transmembrane helix motif (Calamia & Manoil, 1990). Recently, use of second-site suppressor analysis, site-directed mutagenesis, and site-directed excimer fluorescence has led to a model describing helix packing in the C-terminal half of the permease [Jung et al., 1993; see Kaback et al. (1993, 1994)].

Site-directed mutagenesis with wild-type permease or Cys-scanning mutagenesis with a functional mutant devoid of Cys residues (C-less permease) has identified individual residues in the permease that are essential for activity [reviewed in Kaback et al. (1993, 1994)]. Over 320 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; Weitzman & Kaback, 1995). Remarkably, less than one-half dozen

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[1] Abbreviations: lac, lactose; C-less permease, functional lac permease devoid of Cys residues; MIANS, 2-(4′-maleimidylanilino)-naphthalene-6-sulfonic acid; NEM, *N*-ethylmaleimide; DM, dodecyl β,D-maltoside; pyrene maleimide, *N*-(1-pyrenyl)maleimide; KP_i, potassium phosphate; TDG, β,D-galactopyranosyl 1-thio-β,D-galactopyranoside; IPTG, isopropyl 1-thio-β,D-galactopyranoside; sodium dodecyl sulfate, $NaDodSO_4$; octyl glucoside, octyl β,D-glucopyranoside.

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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. Cys148 (helix V) is not essential for transport but is in a substrate-binding site, interacting hydrophobically with the galactosyl moiety of the substrate, and Met145 (helix V) is on the periphery (Jung, H., et al., 1994b; Wu & Kaback, 1994). Although very few amino acid residues appear to be critically involved in the transport mechanism, the activity of various active Cys replacement mutants is altered by alkylation, and these mutants appear in clusters, suggesting that surface contours within the permease may be important [Sahin-Tóth & Kaback, 1993; Dunten et al. 1993; Sahin-Tóth et al., 1994a,c; Frillingos et al., 1994; reviewed in Kaback et al. (1994)]. In brief, therefore, it appears that permease turnover involves relatively simple chemistry coupled to widespread conformational changes.

Since membrane proteins like lac permease are inherently difficult to crystallize, development of alternative methods for obtaining static and dynamic structural information is essential. In this regard, the use of site-directed mutagenesis to engineer membrane proteins for biophysical measurements is emerging as a powerful means of examining structure/function relationships [see Kaback et al. (1993, 1994), Kaback (1994), Hubbell and Altenbach (1994), Akabas et al. (1992)]. Replacement of individual residues with Cys in C-less permease has allowed use of site-directed fluorescence as a means of studying molecular dynamics. Specifically, ligand binding or a proton electrochemical gradient ($\Delta\bar{\mu}_{H^+}$ induces a dramatic increase in the reactivity of V315C[2] permease, suggesting that ligand binding or $\Delta\bar{\mu}_{H^+}$ may cause the permease to assume a similar conformation (Sahin-Tóth & Kaback, 1993; Jung, H., et al., 1994a). Ligand-induced increases in reactivity have also been observed with E269C or H322C permease (Jung, K., et al., 1994). Furthermore, the reactivity of Cys148 or V331C permease is altered by ligand in a similar manner when the protein is in the native membrane, reconstituted into phospholipid vesicles, or solubilized in dodecyl β,D-maltoside (DM), indicating that the permease maintains close to native conformation in DM (Wu & Kaback, 1994; Wu et al., 1994). Finally, site-directed fluorescence labeling has provided more direct evidence that the permease contains more than a single substrate-binding site (Wu et al., 1994).

In this study, we focus on the periplasmic terminus of putative helix I (Figure 1). Although no residue *per se* in this region is essential for activity, structural integrity appears to be important for active lactose transport. Overath et al. (1987) isolated permease mutants defective in lactose transport which retain the ability to accumulate β,D-galactopyranosyl 1-thio-β,D-galactopyranoside (TDG) and found

[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.

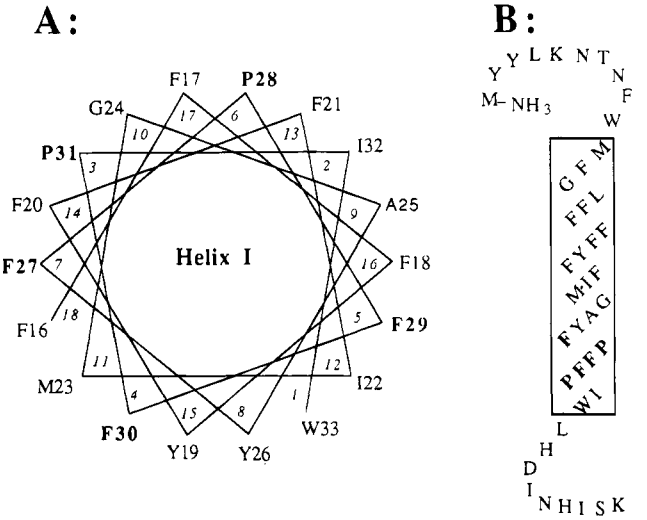

FIGURE 1: (A) Helical wheel plot of putative transmembrane helix I. Phe27, Pro28, Phe29, Phe30, and Pro31 are emboldened. Arabic numbers within the wheel represent positions in the helix from the periplasmic surface to the cytoplasmic surface. (B) Side view projection.

Ser in place of Pro28, a finding corroborated by Consler et al. (1991). Although mutants with Arg or Glu in place of Gly24 were also reported to be defective, G24C permease is fully functional (Sahin-Tóth et al., 1994a; Jung et al., 1995). Furthermore, deletion of the N terminal 22 amino acid residues of the permease does not abolish active transport, but further deletion of residues 23−38 leads to inactivation (Bibi et al., 1992). By using Cys-scanning mutagenesis, Sahin-Tóth et al. (1994a) found that although none of the N-terminal 32 amino acid residues is important for activity, the *N*-ethylmaleimide (NEM)-sensitive Cys replacement mutants cluster at the periplasmic end of helix I (i.e., residues Phe27−Trp33). For these reasons, five single-Cys replacement mutants were constructed in this region and subjected to detailed analysis. NEM labeling of permease in the native membrane or MIANS labeling of purified permease in DM shows that ligand binding dramatically enhances the reactivity of Cys residues at either position 28 or 31. Moreover, the fluorescence of permease mutants covalently labeled with *N*-(1-pyrenyl)maleimide (pyrene maleimide) at these positions is quenched less effectively by 5-doxylstearic acid relative to permease mutants labeled on the opposite face of helix I. The results provide direct evidence that the periplasmic end of helix I is involved structurally in a ligand-induced conformational change in the permease.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmid Construction. 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 pT7-5/lacY-L6XB (Consler et al., 1993) which encodes C-less permease with given Cys replacements and a biotin acceptor domain in the middle cytoplasmic loop was used for expression. Five single-Cys mutants in putative helix I (F27C, P28C, F29C, F30C, and P31C) were constructed by restriction fragment replacement (Sahin-Tóth et al., 1994a) by using a cassette version of the *lacY* gene containing unique restriction sites about every 100 bp (EMBL X-56095).

Mutations 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). Other DNA manipulations were carried out according to Sambrook et al. (1989).

[^{14}C]NEM Labeling in Native Membranes. Reactivity of single-Cys permeases was probed in crude membrane preparations by using a novel assay, which includes labeling with [^{14}C]NEM, avidin-affinity purification, and autoradiography (S. Frillingos, and H. R. Kaback, manuscript in preparation). Briefly, 50 mL cultures of *E. coli* T184 (Z^-Y^-) transformed with pT7-5/*lacY*-L6XB encoding F27C, P28C, F29C, F30C, or P31C permease were grown to midlogarithmic phase and induced with 0.5 mM isopropyl 1-thio-β,D-galactopyranoside (IPTG) for 2 h. Crude membrane was prepared as described (Dunten et al., 1993; Frillingos et al., 1994). Samples (2.5 mg of protein) in 100 mM potassium phosphate (KP_i; pH 7.5)/10 mM $MgSO_4$ were incubated with 0.5 mM [1-^{14}C]NEM (40 mCi/mmol; DuPont NEN, Boston, MA) in the absence or presence of 10 mM TDG at 25 °C. After terminating the reactions with dithiothreitol, the membranes were solubilized with 2.0% DM and biotinylated permease was purified by avidin-affinity chromatography (Consler et al., 1993). The amount of lac permease was estimated by Western blot analysis using a site-directed polyclonal antibody against the C terminus (Carrasco et al., 1984). Equal amounts of permease were then subjected to 12% sodium dodecyl sulfate ($NaDodSO_4$)−polyacrylamide gel electrophoresis (Laemmli, 1970) followed by autoradiography.

Purification of Biotinylated Permease. Permease with a given single-Cys residue and the biotin acceptor domain was expressed in *E. coli* T184 (Y^-Z^-). Cells (6−9 L) were grown at 37 °C in LB broth with streptomycin (10 μg/mL) and ampicillin (100 μg/mL), 0.5 mM IPTG was added at an OD_{600} of 1.2, and growth was then continued for 4−6 h. Cells were harvested, and crude membrane fractions were prepared (Viitanen et al., 1986) and solubilized with 2.0% DM by incubation at 30 °C for 30 min with continuous stirring. Solubilized biotinylated lac permease was purified by affinity chromatography on immobilized monovalent avidin (Consler et al., 1993; Wu & Kaback, 1994). The purity of each preparation was ascertained by electrophoresis on 12% $NaDodSO_4$ polyacrylamide gel (Laemmli, 1970) followed by silver staining. Protein was determined as described by Bradford (1976) with bovine serum albumin as standard.

Labeling of Purified Single-Cys Permease with 2-(4'-Maleimidylanilino)naphthalene-6-sulfonic acid (MIANS) and Fluorescence Measurements. MIANS (Molecular Probes, Inc.) was dissolved in methanol, and the concentration was determined by measuring absorbance at 322 nm and using an extinction coefficient of 17 000 (Haugland, 1989). Fluorescence was measured at 30 °C in an SLM 8000C spectrofluorometer (SLM-Amico Instruments Inc., Urbana, IL). Emission spectra were recorded using an excitation wavelength of 330 nm and 4 nm slits for both excitation and emission.

To determine the rate of MIANS reaction with purified single-Cys permease, protein (40 μg/mL) was preincubated with given ligands in an assay buffer containing 50 mM KP_i (pH 7.4)/60 mM NaCl/0.01% DM for 5 min at 30 °C. Reactions were initiated by addition of MIANS to a final concentration of 4 μM from a 1 mM stock solution, and fluorescence was monitored continuously at an emission wavelength of 418 nm. Where indicated, TDG or lactose was added to given final concentrations.

For complete modification of Cys residues, reactions were carried out at 4 °C for 60 min with a 10-fold molar excess of MIANS over protein. Unreacted MIANS was quenched with DTT and removed by dialysis against three changes of 50 mM KP_i (pH 7.4)/150 mM NaCl/0.02% DM. To study the effect of ligand on the fluorescence of MIANS-labeled permease, the labeled protein was preincubated with a given ligand for 5 min at 30 °C followed by measurement of emission spectra.

Reconstitution of Pyrene-Labeled Permease and Fluorescence Quenching by Spin-Labeled Fatty Acid. Single-Cys permeases were purified in octyl β,D-glucopyranoside (octyl glucoside) as described previously (Consler et al., 1993; Jung, H., et al., 1994a). Purified permease was labeled with pyrene maleimide by incubating the protein with a 10-fold molar excess at 4 °C for 60 min. Reactions were terminated with DTT, and excess pyrene maleimide was removed by dialysis against 100 volumes of 50 mM KP_i (pH 7.4)/150 mM NaCl/1.25% octyl glucoside with four changes. Pyrene-labeled proteins were then reconstituted into liposomes prepared from 1-palmitoyl-2-oleolyl phosphatidylethanolamine and 1-palmitoyl-2-oleolylphosphatidylglycerol (3:1 mol/mol) as described (Viitanen et al., 1986), and the lipid/protein ratio was adjusted to 20:1 (w/w). Proteoliposomes were harvested by centrifugation (150000g_{max}), washed twice with 50 mM KP_i (pH 7.4), and resuspended in the same buffer followed by two cycles of freeze−thaw/sonication.

For fluorescence measurements, proteoliposomes were diluted to 0.5 mL of 50 mM KP_i (pH 7.4) to a final protein concentration of 0.1 μM. A given spin-labeled fatty acid was added from a stock ethanolic solution so that the final ethanol concentration was always less than 0.5%, and the samples were vortexed vigorously. Fluorescence measurements (excitation, 340 nm; emission, 376 nm) were made after the samples were incubated at 30 °C for 15 min, and all measurements were carried out at 30 °C. Data were analyzed by plotting F/F_o against the concentration of doxylstearic acid, where F_o and F are the fluorescence intensities in the absence and presence of a given concentration of doxylstearic acid, respectively.

RESULTS

Effect of TDG on NEM Labeling in Native Membranes. Membranes from cells expressing permease mutants with single-Cys residues and a biotin acceptor domain in the middle cytoplasmic loop were incubated with [1-^{14}C]NEM for 30 min in the presence or absence of TDG followed by solubilization and purification of the permease by monomeric avidin-affinity chromatography. The samples were then subjected to $NaDodSO_4$−polyacrylamide gel electrophoresis, and labeled permease was visualized by autoradiography (Figure 2A). In the absence of ligand, F27C or P28C permease is intensely labeled while P29C, P30C, or P31C permease is labeled only weakly. Addition of TDG during labeling has no significant effect on labeling of P27C, P29C, or P30C permease but dramatically enhances labeling of P31C permease. Although labeling of P28C is also enhanced in the presence of ligand, the effect is hardly significant when

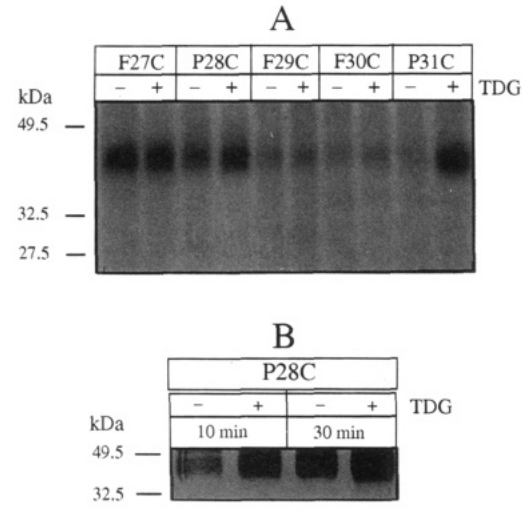

FIGURE 2: Effects of TDG on labeling of F27C, P28C, F29C, F30C, or P31C permease with [^{14}C]NEM in membranes. Membranes (2.5 mg of protein) prepared from *E. coli* strain T184 transformed with pT7-5/C-less *lacY* L6XB encoding the given single-Cys permease were labeled with 0.5 mM [1-^{14}C]NEM in the absence or presence of 10 mM TDG. The reactions were quenched by 5 mM DTT at a given time, and biotinylated permease was solubilized and purified as described under Experimental Procedures. Aliquots containing 15 μg of protein were separated on 12% NaDodSO$_4$-polyacrylamide gel electrophoresis, and the [^{14}C]NEM-labeled proteins were visualized by autoradiography. The positions of molecular weight markers ovalbumin (49.5 kDa) and carbonic anhydrase (32.5 kDa) are shown at the left. Each lane contained approximately equal amounts of lac permease as judged by Western blots (data not shown): (A) 30 min incubation and (B) 10 or 30 min incubation, as indicated.

labeling is carried out for 30 min. However, when the incubation time is shortened to 10 min, it is apparent that TDG also causes a significant increase in the reactivity of P28C permease (Figure 2B).

MIANS Labeling of Purified Single-Cys Permeases and the Effect of Ligand. MIANS is a negatively charged, sulfhydryl-specific probe that is not fluorescent until the maleimide group undergoes chemical reaction (Haugland, 1989). The fluorophore has been used previously to study the reactivity of single-Cys C148 and V331C permeases (Wu & Kaback, 1994; Wu et al., 1994), as well as other membrane proteins [see Gupte and Lane (1983), Bigelow and Inesi (1991), Philips and Cerione (1991), May and Beechem (1993) for examples].

MIANS reacts readily with purified F27C, F29C, or F30C permease in DM (Figure 3a,c,d, curve 1). Addition of the fluorophore to each purified permease results in a linear increase in fluorescence emission intensity for about 2−3 min, and the reaction is essentially complete in about 5 min. Furthermore, with these mutants, TDG has no discernible effect on the labeling rate (Figure 3a,c,d, curve 2). In marked contrast, MIANS reacts almost negligibly with P28C or P31C, but ligand causes a dramatic increase in reactivity. MIANS reacts very slowly with P28C permease in the absence of ligand (Figure 3b, curve 1), and about a 12-fold increase in reaction rate is observed in the presence of 10 mM TDG (Figure 3b, curve 2). Lactose, the physiological substrate of the permease, does not significantly alter the reactivity of P28C with MIANS (not shown), an observation consistent with findings that P28S permease (Overath et al., 1987; Consler et al., 1991) or P28C permease (Sahin-Tóth et al., 1994a) transports lactose less effectively than TDG. Even more dramatically, MIANS does not appear to react with P31C permease at all in the absence of ligand over the time course shown (Figure 3e, curve 1). In the presence of 10 mM TDG, however, the probe reacts rapidly with P31C permease (Figure 3e, curve 2). Importantly, lactose also enhances MIANS reactivity with this mutant, albeit much less effectively than TDG, while sucrose or glucose has no effect whatsoever. Therefore, the effect is specific for substrates of the permease.

The effect of sugars on the reactivity of P28C or P31C permease was studied as a function of concentration (Figure 4). With both mutants, half-maximal enhancement of MIANS reactivity by TDG is observed at approximately 0.25 mM with saturation at about 1.0 mM. On the other hand, lactose enhances the reactivity of P31C permease with half-maximal enhancement and saturation at about 10 and 50 mM, respectively, and only a small effect on P28C permease reactivity is observed at concentrations up to 50 mM. Sucrose or glucose (up to 100 mM final concentration) has no effect whatsoever on the rate of MIANS labeling of either mutant (data not shown). Thus, the concentration dependence for enhanced reactivity reflects the affinity of each ligand for the permease (i.e., TDG ≫ lactose).

Fluorescence of MIANS-Labeled Single-Cys Permeases. Since the quantum yield and emission maximum of MIANS depends on the polarity of the environment (Haugland, 1989),

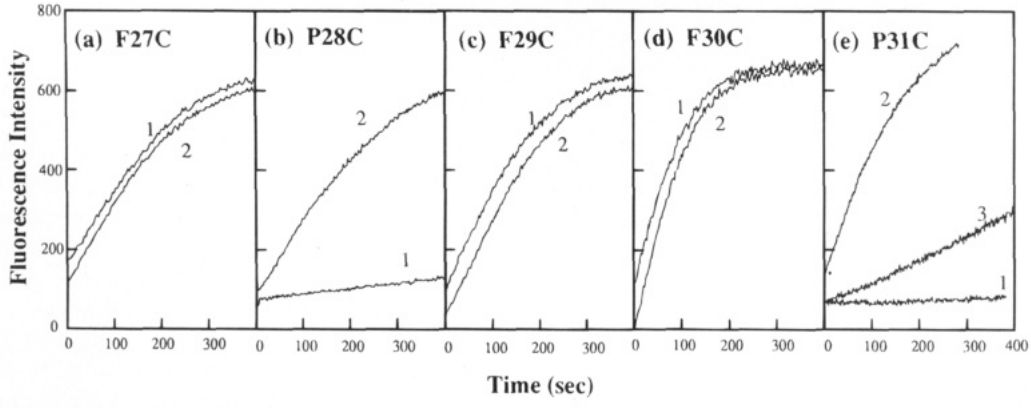

FIGURE 3: Reaction of MIANS with purified F27C (a), P28C (b), F29C (c), F30C (d), or P31C (e) permease. A given affinity purified permease (40 μg/mL) was incubated without or with a given concentration of ligand, and reaction with MIANS was carried out in 0.5 mL of 50 mM KPi (pH 7.4)/60 mM NaCl/0.01% DM. The reaction was started by adding MIANS to a final concentration of 4 μM, and fluorescence was recorded continuously at 418 nm (excitation, 330 nm) as described under Experimental Procedures: curve 1, no ligand, 50 mM sucrose or glucose; curve 2, 10 mM TDG; curve 3, 10 mM lactose.

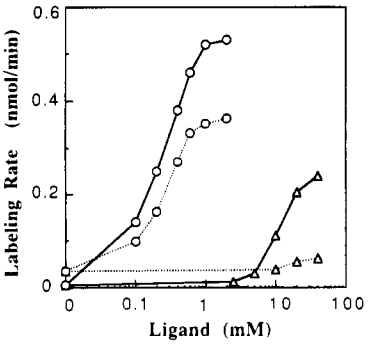

FIGURE 4: Effect of ligand on the rate of MIANS labeling of P28C or P31C permease. Purified single-Cys P28C (broken line) or P31C (solid line) was preincubated with given concentrations of TDG (○) or lactose (△), and MIANS labeling was carried out as described in the legend to Figure 3. Labeling rates were obtained from the initial fluorescence increase after addition of MIANS and plotted as the function of ligand concentration.

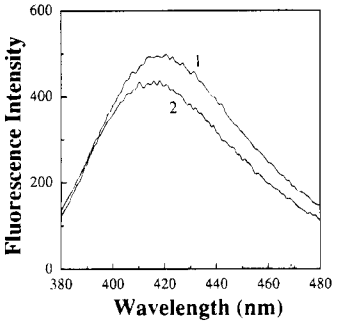

FIGURE 5: Effect of TDG on the fluorescence emission spectrum of MIANS-labeled F27C permease. Purified F27C permease was labeled with MIANS, and excess reagent was removed by dialysis as described under Experimental Procedures. Fluorescence spectra were recorded at 30 °C with MIANS-labeled F27C permease (40 μg/mL) as described in the legend to Figure 3: curve 1, no addition or 50 mM sucrose; curve 2, 10 mM TDG.

the fluorophore is useful for monitoring ligand-induced conformational changes [see Hiratruka (1992), Wu et al. (1994)]. MIANS-labeled F27C, P28C, F29C, F30C, or P31C permease exhibits fluorescence emission maxima at 414, 418, 417, 413, or 419 nm, respectively (data not shown), values that are similar to emission maxima observed for MIANS-labeled single-Cys mutants in helices V (Wu & Kaback, 1994) or X (Wu et al., 1994). Therefore, these positions are in a relatively hydrophobic environment which is consistent with the assignment of the residues to a transmembrane domain (Figure 1). No significant change in the fluorescence emission spectra of MIANS-labeled P28C, F29C, F30C, or P31C permease is observed in the presence of TDG (data not shown). However, with MIANS-labeled F27C permease, TDG induces about a 7% decrease in fluorescence and a 3 nm blue shift in the emission maximum (Figure 5).

Quenching of Pyrene-Labeled Permease Fluorescence by Spin-Labeled Fatty Acids. Fluorescence quenching by spin-labeled fatty acids (Mitra & Hammes, 1990) or phospholipids (Chattopadhyay & London, 1987) has been used to estimate the depth of fluorophores within the bilayer, as well as their accessibility to the hydrophobic phase of the membrane. In order to approximate the relationship of the periplasmic end of helix I to the surface of the membrane, purified pyrene-labeled F29C permease was reconstituted into proteoliposomes containing 5-doxylstearic acid or 12-doxylstearic acid,

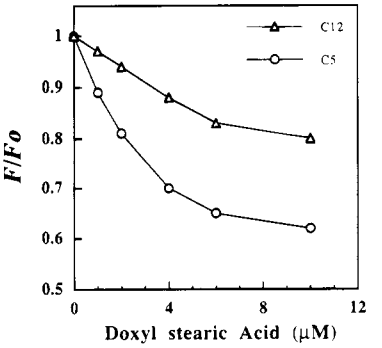

FIGURE 6: Quenching of pyrene-labeled F29C permease by 5-doxylstearic acid (○) or 12-doxylstearic acid (△). Purified F29C permease was labeled with pyrene maleimide and reconstituted into liposomes as described under Experimental Procedures. Fluorescence emission was measured at 376 nm at an excitation wavelength of 340 nm. The ratio of fluorescence intensity in the presence (F) and absence (F_o) of doxylstearic acid was plotted against the concentrations of the doxylstearic acid. Values are the average of three independent measurements.

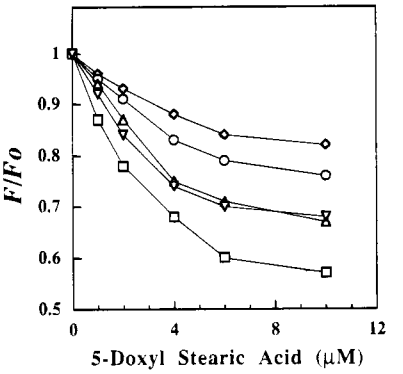

FIGURE 7: Quenching of pyrene-labeled single-Cys permease fluorescence by 5-doxylstearic acid. Purified single-Cys permease F27C (▽), P28C (○), F29C (△), F30C (□), or P31C (◇) was labeled with pyrene maleimide and reconstituted into liposomes as described under Experimental Procedures. Given concentrations of 5-doxylstearic acid were added, and fluorescence was measured at 376 nm with an excitation wavelength of 340 nm. Data were analyzed as described in the legend to Figure 6. Values are the average of three independent measurements.

and the decrease in fluorescence intensity at 376 nm (excitation, 340 nm) was monitored as a function of spin-labeled fatty acid concentration (Figure 6). Clearly, 5-doxylstearic acid quenches more effectively than the 12-doxyl homolog, indicating that pyrene at position 29 is relatively close to the surface of the membrane.

The effect of 5-doxylstearic acid was then tested on purified, pyrene-labeled, reconstituted F27C, P28C, F29C, F30C, or P31C permease (Figure 7). As shown, the fluorescence of pyrene-labeled P30C permease is quenched most effectively by 5-doxylstearic acid, while the fluorescence of labeled P28C or P31C permease is quenched much less effectively, and F27C and F29C exhibit intermediate behavior.

DISCUSSION

The results presented in this communication are consistent with previous observations (Overath et al., 1987; Consler et al, 1991; Bibi et al., 1992; Sahin-Tóth et al., 1994a) indicating that the periplasmic terminus of helix I of lac permease plays a structural role in a conformational change

resulting from ligand binding and provide more direct evidence for this contention. Thus, although no single residue among the N terminal 32 amino acid residues in the permease is essential for transport activity, the Cys replacement mutants that are inactivated by alkylation cluster at the periplasmic end of putative helix I, and as shown here, two of these mutants (P28C and P31C) which lie on the same face of the helix undergo dramatic increases in reactivity as a result of ligand binding.

It is particularly noteworthy that the ligand-induced increases in reactivity observed with either P28C or P31C permease have been documented with native membranes containing the mutant permeases, as well as with purified permease solubilized in DM. Therefore, as suggested previously (Wu & Kaback, 1994; Wu et al., 1994), it is likely that lac permease maintains close to native conformation in DM. On the other hand, the permease is probably not completely native, as the excimer fluorescence observed between pyrene-labeled Cys residues in opposing helices is not observed in octyl glucoside but only after the protein is reconstituted into proteoliposomes (Jung et al., 1993). However, since a proximity of 3.5 Å and proper orientation are required for excimer fluorescence, this may reflect minor differences in structure. In any event, ligand binding *per se* and conformational changes observed as a result of ligand binding are clearly retained when the protein is solubilized in DM.

The reactivity of the five mutants in the absence of ligand clearly differs depending upon the position of the Cys replacements. Thus, with membrane-embedded permease, Cys residues at positions 29 and 30 react relatively poorly with NEM in 30 min while Cys residues at positions 27 and 28 are significantly more reactive, although it is apparent that P28C permease labels more slowly in the absence of ligand (Figure 2B). Since these positions represent approximately one helical turn and Phe27 and Pro28 are on opposite faces from Phe29 and Phe30 (Figure 1), it is likely that the two faces of helix I at the C terminus are exposed to different environments. Pro31 which is on the same face of the helix as Phe27, but one turn removed toward the C terminus, behaves anomalously with respect to NEM labeling, as it is relatively unreactive in the absence of ligand. With purified permease solubilized in DM, Cys residues at 27, 29, and 30 react rapidly with MIANS, a negatively charged probe, while Cys residues at positions 28 and 31 react slowly (Figure 3). Even though the solubility properties of NEM and MIANS differ considerably, both sets of results are consistent with the interpretation that the face of helix I with Pro28 and Pro31 is in a different environment from the opposite face.

In order to study the environments of the two conformationally distinct faces of helix I, quenching of reconstituted, pyrene-labeled single-Cys permease by spin-labeled stearic acid was tested. Initially, F29C permease was used to approximate the spatial relationship between the C-terminal end of helix I and the surface of the membrane. Clearly, 5-doxylstearic acid is a significantly more effective fluorescence quencher than 12-doxylstearic acid (Figure 6). Since the C5 and C12 positions of stearic acid are about 6 and 15 Å, respectively, from the carboxyl headgroup (Voges et al., 1987), the results are consistent with the placement of Phe29 at about 1.5 helical turns from the surface of the membrane. Quenching of pyrene-labeled single-Cys permeases was then studied as a function of 5-doxylstearic acid concentration (Figure 7), and the results indicate that the face with residues 27, 29, and 30 is more accessible from the lipid bilayer, suggesting that it may interact directly with the hydrophobic phase of the membrane. On the other hand, pyrene-labeled P28C and P31C permeases are less accessible to 5-doxylstearic acid which is consistent with the notion that this face of helix I may interact with another part of the permease.

Strikingly, with either membrane-embedded permease and NEM or purified permease and MIANS, marked increases in reactivity are observed with single-Cys replacements at two positions only, 28 and 31, and in both sets of experiments, no alteration in reactivity is observed with Cys residues at the other positions (Figures 2 and 3). Therefore, it seems reasonable to conclude that ligand binding induces a conformational change in the permease that increases the accessibility of this face of helix I to the probes. Similar ligand-induced increases in reactivity have been observed with MIANS and purified permease containing a single Cys residue at position 315 (Sahin-Tóth & Kaback, 1993; Jung, H., et al., 1994a; Frillingos and Kaback, unpublished observations) or 331 (Wu et al., 1994) in helix X and with pyrene maleimide and purified permeases containing a single Cys residue at position 269 in helix VIII or 322 in helix X (Jung, K., et al., 1994).

Interestingly, the reactivity of P31C, but not of P28C, permease is enhanced by lactose, the physiological substrate of lac permease. This finding is consistent with previous observations (Overath et al., 1987; Consler et al., 1991) showing that replacement of Pro28 with Ser abolishes lactose transport but leaves significant TDG transport activity. Since P28S exhibits a higher affinity for *p*-nitrophenyl α,D-galactopyranoside than wild-type permease (Overath et al., 1987; Consler et al., 1991), it was suggested that Pro28 may be involved in sugar binding. In this context, Huang et al. (1992) have postulated that Trp33 which is on the opposite face of helix I is also involved in substrate recognition. In light of the observations presented here demonstrating that the reactivity of P28C or P31C permease is increased in the presence of TDG, it seems unlikely that this portion of helix I is directly involved in binding substrate [see Wu and Kaback (1994)].

Evidence has been presented (Lolkema & Walz, 1990; Lolkema et al., 1991; van Iwaarden et al., 1993) indicating that lac permease has at least two substrate-bindings sites with differing affinities. Recent site-directed fluorescence studies with V331C permease (Wu et al., 1994) provide more direct evidence for the postulate and suggest that the sites may be occupied simultaneously. Since half-maximal enhancement of the MIANS reactivity of P28C or P31C permease is observed at approximately 0.25 mM TDG (Figure 4), increased reactivity at both positions apparently reflects occupation of a relatively high-affinity binding site. Moreover, TDG blockade of Cys148 reactivity, a phenomenon due in all likelihood to steric interaction between ligand and a binding site residue (Jung, H., et al., 1994b; Wu & Kaback, 1994), occurs over the same range of TDG concentrations. Taken together, the data are consistent with the interpretation that occupation of a relatively high-affinity site containing Cys148 induces a conformational change in the permease leading to increased accessibility of the face of helix I on which Pro28 and Pro31 are situated. The finding that TDG induces quenching and a blue shift of

MIANS-labeled P27C permease is also consistent with this interpretation (Figure 5). Although no ligand-induced fluorescence change is detected with MIANS-labeled P28C or P31C permease, it is likely that modification of these mutants results in loss of ability to bind ligand.

In conclusion, on the basis of the data as a whole, we suggest that ligand binding to lac permease induces a widespread conformational change in which helix I undergoes a rigid-body movement much like that proposed recently for the C helix of visual rhodopsin during photoexcitation (Z. T. Farahbakhsh, K. D. Ridge, H. G. Khorana, and W. L. Hubbell, submitted for publication). Alternatively, however, it is possible that helix I does not move but that neighboring membrane-spanning domains move relative to helix I, thereby exposing Pro28 and Pro31.

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