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

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ABSTRACT: Recently we described the use of site-directed pyrene labeling of engineered lactose permease containing paired Cys residues to obtain proximity relationships between helices in the C-terminal half of the molecule [Jung, K., Jung, H., Wu, J., Privé, G. G., & Kaback, H. R. (1993) *Biochemistry 32,* 12273]. Pyrene excimer fluorescence was detected for the double Cys mutants His322→Cys/Glu325→Cys, Arg302→Cys/Glu325→Cys, and Glu269→Cys/His322→Cys, indicating that helix X (His322→Cys/Glu325→Cys) is in an α-helical conformation and that helices VIII (Glu269→Cys) and IX (Arg302→Cys) are close to helix X (His322→Cys and Glu325→Cys). In this report, these interactions are used to study dynamic aspects of the permease. Excimer fluorescence between helices VIII and X or helices IX and X is markedly diminished by sodium dodecyl sulfate, while the excimer observed within helix X is unaffected, suggesting that tertiary interactions are disrupted by the denaturant with little effect on secondary structure. Furthermore, excimer fluorescence observed between helices VIII (Glu269→Cys) and helix X (His322→Cys) is quenched by Tl^+, and the effect is markedly and specifically attenuated by ligands of the permease, suggesting that the pyrene becomes less accessible to the aqueous phase. The reactivity of single Cys residues at positions 269 or 322 was also examined by studying the rate of increase in fluorescence with *N*-(1-pyrenyl)maleimide. With both mutants, ligands of the permease cause a dramatic increase in reactivity which is consistent with the notion that these positions are transferred into a more hydrophobic environment. These and other findings are consistent with the interpretation that ligand binding causes widespread changes in the tertiary structure of the permease with little or no alteration in secondary structure.

The lactose (lac)[1] permease of *Escherichia coli* is a polytopic cytoplasmic membrane protein that catalyzes the coupled stoichiometric translocation of β-galactosides and H^+ (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 Kaback et al. (1990)]. 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 an α-helical configuration that traverse the membrane in zigzag fashion connected by hydrophilic domains (loops) (Figure 1). Evidence confirming some of the general features of the model and demonstrating that the N and C termini are on the cytoplasmic face of the membrane has been obtained from laser Raman spectroscopy, limited proteolysis, and immunological studies [see Kaback (1992)]. Furthermore, exclusive evidence for the presence of 12 transmembrane domains has been obtained from analysis of a large number of lac permease–alkaline phosphatase (*lacY–phoA*) fusions (Calamia & Manoil, 1990).

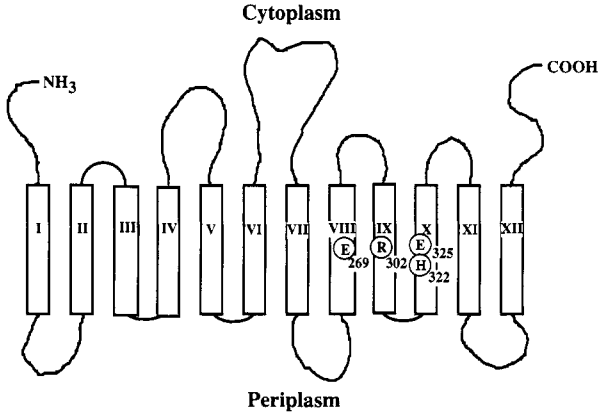

FIGURE 1: Secondary structure model of lac permease. On the basis of spectroscopic studies, hydropathy analysis (Foster et al., 1983), and a series of lac permease–alkaline phosphatase (*lacY/phoA*) fusions (Calamia & Manoil, 1990), the permease is composed of a hydrophilic N-terminus followed by 12 hydrophobic domains in α-helical configuration connected by hydrophilic loops with a 17-residue C-terminal hydrophilic tail. The positions of Glu269, Arg302, His322, and Glu325 are highlighted.

Cys-scanning mutagenesis has demonstrated that very few residues in lac permease are essential for activity (Sahin-Tóth & Kaback, 1993a; Dunten et al., 1993b; Sahin-Tóth et al., 1994); however, certain functionally important residues have been identified by site-directed mutagenesis [see Kaback (1989, 1992) and Kaback et al. (1990)]. Site-directed replacement of Arg302 (Menick et al., 1987; Matzke et al., 1992) in putative helix IX or of His322 (Padan et al., 1985; Püttner et al., 1986, 1989; King & Wilson, 1989a,b, 1990a,b;

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[1] Abbreviations: lac, lactose; C-less permease, functional lac permease devoid of Cys residues; PM, *N*-(1-pyrenyl)maleimide; TDG, β-D-galactopyranosyl 1-thio-β-D-galactopyranoside; $\Delta\bar{\mu}_{H^+}$, H^+ electrochemical gradient; SDS, sodium dodecyl sulfate; IPTG, isopropyl β-D-thiogalactopyranoside; OG, octyl β-D-glucopyranoside; KP_i, potassium phosphate; DTT, dithiothreitol; CMC, critical micelle concentration.

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Brooker, 1991) or Glu325 (Carrasco et al., 1986, 1989; van Iwaarden et al., 1993) in helix X indicates that these residues play a particularly important role in lactose/H^+ symport and/or substrate recognition. Moreover, Arg302, His322, and Glu325 are the only residues in this region that play an important role in the transport mechanism (Sahin-Tóth & Kaback, 1993a). In addition, Glu269 in helix VIII (Figure 1) has been shown to be a functionally important residue (Hinkle et al., 1990; Ujwal et al., 1993).

Recently, site-directed fluorescence labeling of combinations of paired Cys replacements in a functional permease mutant devoid of Cys residues (C-less permease) (van Iwaarden et al., 1991) was used to study proximity relationships in the lac permease (Jung et al., 1993). Double Cys mutant containing a biotin acceptor domain in the middle cytoplasmic loop were purified by avidin affinity chromatography, labeled with *N*-(1-pyrenyl)maleimide (PM), and reconstituted into proteoliposomes. Two pyrene moieties can form an excited-state dimer (excimer) that exhibits a unique emission maximum at approximately 470 nm if the conjugated ring systems are within 3.5 Å of each other and in the correct orientation [see Kinnunen et al. (1993)]. Excimer fluorescence was observed with PM-labeled H322C/E325C,[2] R302C/E325C, and E269C/H322C, indicating that His322 and Glu325 are located in a portion of the permease that is in an α-helical conformation and that helices VIII and IX are in close proximity to helix X. These and other findings showing that helix VII is close to helices X and XI (King et al., 1991; Sahin-Tóth et al., 1992; Lee et al., 1992; Dunten et al., 1993a; Sahin-Tóth & Kaback, 1993b) form the basis of a model describing helix packing in the C-terminal half of the permease (Jung et al., 1993; Kaback et al., 1993). Interestingly, the model is similar to that proposed by Baldwin (1993) for the eukaryotic glucose uniporters.

In this study, the paired Cys mutants that exhibit excimer fluorescence are used to study dynamic aspects of permease structure/function. Evidence is presented suggesting that denaturants such as sodium dodecyl sulfate (SDS) or ligand binding induces alterations in the tertiary structure of the permease with little or no change in secondary structure.

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) which encodes C-less permease with given Cys replacements and a biotin acceptor domain in the middle cytoplasmic loop was used for overexpression by induction with isopropyl 1-thio-β-D-galactopyranoside (IPTG). Plasmid pKR35 (Reed & Cronan, 1991) was generously provided by Dr. John Cronan, Jr. (University of Illinois).

Construction of Mutants. The construction of single Cys mutants and H322C/E325C by site-directed mutagenesis has been described (Sahin-Tóth & Kaback, 1993a; Jung et al., 1993). The other 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). 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 (Hattori & Sakaki, 1986).

Expression and Purification of Mutant Proteins. Twelve liters of cells was cultivated at 37 °C and induced with 0.3 mM IPTG. 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 8 mM *d*-biotin in column buffer without DTT. Purified samples were analyzed by SDS/polyacrylamide gel electrophoresis (Laemmli, 1970) followed by silver staining.

Reconstitution of Lac Permease into Proteoliposomes. Fractions containing purified permease were pooled, and the protein was reconstituted into acetone/ether-washed *E. coli* phospholipids (Avanti Polar Lipids) by detergent dilution (Viitanen et al., 1986). A lipid:protein ratio of 50:1 (w/w) was used. Proteoliposomes were centrifuged (150000g_{max}) and resuspended in 50 mM KP_i (pH 7.5), followed by 2 cycles of freeze–thaw/sonication.

Labeling of Mutant Proteins. Purified permease was labeled with 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 for 4 h at 4 °C in the dark with stirring and centrifuged at 15000g_{max} for 20 min to remove undissolved reagent, and a 5-fold excess of glutathione over PM was added to quench the reaction. After the mixture was stirred for an additional 1 h, labeled protein was reconstituted into phospholipids as described above using a lipid:protein ratio of 385:1 (w/w). Proteoliposomes were washed extensively (4 cycles of centrifugation at 150000g_{max}) with 0.1% (w/v) bovine serum albumin/50 mM KP_i (pH 7.5) in the first wash and 50 mM KP_i (pH 7.5) in subsequent washes. The preparations were then resuspended in 50 mM KP_i (pH 7.5) and subjected to 2 cycles of freeze–thaw/sonication.

Fluorescence Measurements. Fluorescence emission spectra were recorded at 25 °C using an Aminco SLM 8000C spectrofluorometer 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: (1) 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); (2) by titrating the free thiol groups remaining in the permease with *N*-[*ethyl*-1-^{14}C]maleimide (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.

In order to monitor changes in excimer fluorescence under different conditions, the ratio of the intensities at 470 nm (excimer fluorescence) and 378 nm (monomer fluorescence) (I_{470}/I_{378}) was measured.

Quenching measurements were performed using excitation at 344 nm with emission monitored at either 378 nm or 470

[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.

nm as indicated. Aliquots of a concentrated thallium(I) nitrate (Aldrich) solution were added to a suspension of proteoliposomes maintained at 25 °C with constant stirring. Values were corrected for dilution by carrying out the same experiments with sodium nitrate in place of thallium nitrate. Quenching data were analyzed by Stern–Volmer plots where $F_0/F - 1$ is plotted versus the concentration of quencher, [Q]. F is the steady-state fluorescence intensity, and F_0 denotes fluorescence in the absence of quencher. A plot of $F_0/F - 1$ versus [Q] yields a slope equal to K_{SV}, the Stern–Volmer quenching constant. K_{SV} is equal to the product of k_q, the bimolecular quenching constant, and τ_0, the lifetime of pyrene in the absence of quencher.

For time-based studies, the fluorescence emission of proteoliposomes containing the purified single Cys mutant E269C or H322C was measured continuously at 378 nm immediately after addition of proteoliposomes to a solution of 1 μM PM in 50 mM KP_i (pH 7.5). Subsequent additions were made as indicated.

RESULTS

Influence of Detergents. As reported previously (Jung et al., 1993), the purified, PM-labeled double Cys mutants H322C/E325C, R302C/E325C, and E269C/H322C exhibit excimer fluorescence with an emission maximum at around 470 nm (Figure 2, solid lines). In addition, more defined maxima characteristic of the monomer are seen at shorter wavelengths (i.e., ca. 378, 398, and 417 nm). In the presence of 0.2% SDS, the overall fluorescence with R302C/E235C or E269C/H322C is generally decreased by about 30% (Figure 2A,B, dashed lines), but the ratio of excimer to monomer fluorescence (I_{470}/I_{378}) is reduced by about 70% and 50%, respectively (Figure 3). In contrast, although 0.2% SDS also causes an overall decrease in fluorescence with PM-labeled H322C/E325C (Figure 2C, dashed line), the excimer:monomer ratio is unaffected (Figure 3). With E269C/H322C or R302C/E325C, increasing SDS concentrations cause a progressive decrease in the excimer:monomer ratio up to the critical micelle concentration (CMC) with little or no change thereafter, while PM-labeled H322C/E325C exhibits no change in I_{470}/I_{378} over the concentration range tested (Figure 3). The results suggest that the denaturing effect of SDS is due to an alteration in tertiary structure of lac permease with little effect on secondary structure. Consistently, no excimer fluorescence is observed with PM-labeled E269C/H322C or R302C/E325C in 1.25% OG (w/v), but only when the polypeptides are reconstituted into proteoliposomes (Jung et al., 1993).

Fluorescence Quenching. Fluorescence quenching has been used widely to study the relative accessibility of fluorescent groups in membrane proteins [see Peerce and Wright (1987), May et al. (1993), and Merrill et al (1993) for examples]. Thallium (Tl^+), a water-soluble collisional quencher of pyrene fluorescence (Copper & Thomas, 1977), exhibits a linear Stern–Volmer relationship with PM-labeled E269C/H322C permease (Figure 4). Strikingly, in the presence of 5 mM β-D-galactopyranosyl 1-thio-β-D-galactopyranoside (TDG), the slope of the Stern–Volmer function decreases by about 50%. Lactose at concentrations of 5 mM or particularly 50 mM also causes significant, but less marked, decreases in Tl^+ quenching, while 100 mM sucrose or glucose (not shown) has no effect. Tl^+ quenching studies were also carried out with the single Cys mutants E269C and H322C, and the Stern–Volmer quenching constants for E269C/H322C and each single mutant in the absence and presence of 5 mM TDG are tabulated in Table 1. With E269C/H322C permease, the Stern–Volmer constant is reduced from 27 to 10 M^{-1}; with H322C and E269C, from 24 to 17 M^{-1} and from 29 to 22 M^{-1}, respectively.

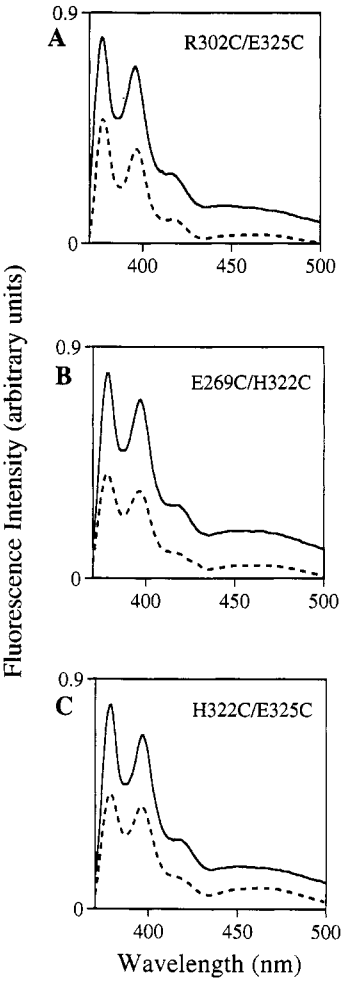

FIGURE 2: Influence of SDS on the fluorescence emission spectra of purified, PM-labeled lac permease mutants reconstituted into proteoliposomes. Spectra were obtained from proteoliposomes reconstituted with given lac permease mutants (35 μg of protein/mL) in 50 mM NaP_i (pH 7.5) at 25 °C. The excitation wavelength was 344 nm. Solid lines, spectra obtained in the absence of further additions; dashed lines, spectra obtained in the presence of 0.2% SDS (w/v).

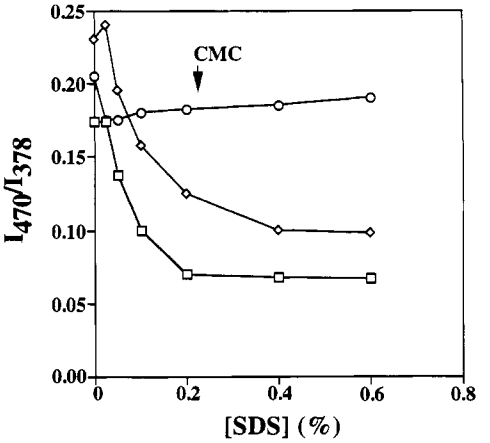

FIGURE 3: Effect of increasing concentrations of SDS on the ratio of excimer (470 nm) to monomer (378 nm) fluorescence of the labeled double Cys mutants R302C/E325C (□), E269C/H322C (◇), and H322C/E325C (○). Experiments were carried out as described under Experimental Procedures and in Figure 2.

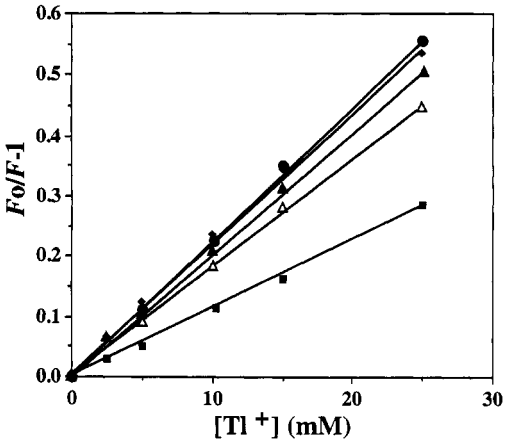

FIGURE 4: Effect of Tl^+ on the fluorescence of PM-labeled E269C/H322C permease. Fluorescence was measured at 470 nm in 50 mM KP_i (pH 7.5) at a protein concentration of 35 μg of protein/mL in the presence of given concentrations of Tl^+. The excitation wavelength was 344 nm, and the temperature was maintained at 25 °C. The data are presented as Stern–Volmer plots (see Experimental Procedures). (●) No further additions; (♦) plus 100 mM sucrose; (▲) plus 5 mM lactose; (△) plus 50 mM lactose; (■) plus 5 mM TDG.

Table 1: Stern–Volmer Quenching Constants for Tl^+ and the Influence of Ligand Binding[a]

	Stern–Volmer constants, K_{SV} (M^{-1})	
mutant	–TDG	+TDG
E269C/H322C	27	10
H322C	24	17
E269C	29	22

[a] Protein concentration: 35 μg/mL. Values are each the average of three determinations calculated from Stern–Volmer plots such as those shown in Figure 4.

Although data are not presented, concentrations of TDG ranging from 0.1 to 30 mM were tested with each mutant. TDG concentrations below 1 mM are ineffective, and concentrations above 10 mM do not lead to further reduction of Tl^+ quenching. Since the K_D for TDG approximates 53 μM with wild-type permease (Wright et al., 1983), but mutations in H322 (Püttner et al., 1989) or E269 (Ujwal et al., 1993) cause a markedly reduced affinity for ligand, it is not surprising that TDG is effective in only the millimolar range with these mutants. In any event, the results described are specific for substrates of the permease. The findings indicate that the PM-labeled mutants are able to bind substrate, albeit with low affinity, and that ligand binding causes a conformational change leading to decreased accessibility of the fluorescence label at positions 322 and 269 to the aqueous phase.

Ligand Binding Influences the Reactivity of E269C or H322C Permease. In another series of experiments, the single Cys mutants E269C and H322C were purified and reconstituted into proteoliposomes prior to labeling with PM. Since PM is nonfluorescent until it reacts with a thiol, the reactivity of Cys residues can be studied fluorometrically (Figure 5). In the experiments shown, proteoliposomes containing either E269C or H322C permease were added to a cuvette containing 1 μM PM in 50 KP_i (pH 7.5) to initiate the reaction. As shown, fluorescence increases very slowly with E269C permease (Figure 5A) or H322C permease (Figure 5B). Dramatically, when 10 mM TDG is added to either mutant, marked stimulation in the rate of increase of fluorescence is observed, but no effect whatsoever is observed with 100 mM sucrose. Consistent with the results of Tl^+ quenching, TDG

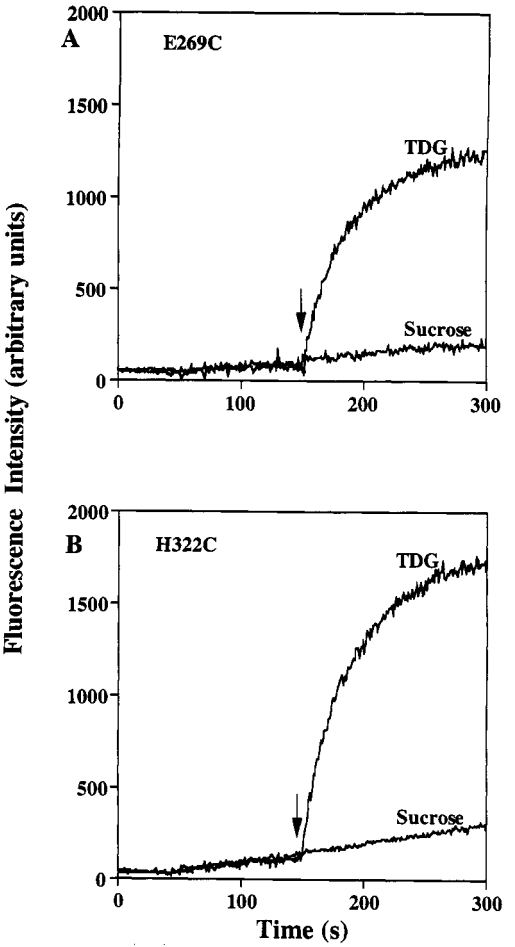

FIGURE 5: Time course of labeling of E269C (*A*) or H322C (*B*) permease with PM. Two milliliters of 50 mM KP_i (pH 7.5) containing a final concentration of 1 μM PM was placed in a cuvette, and fluorescence was recorded (excitation wavelength, 344 nm; emission wavelength, 378 nm) with continuous stirring at 25 °C. After 60 s, 30 μL of proteoliposomes containing purified E269C or H322C (35 μg of protein) was added, and the measurements were continued. As indicated by the arrow, either TDG or sucrose was added to final concentrations of 10 or 100 mM, respectively. With TDG, the rate of labeling increases markedly; with sucrose, no change is observed. The data have been corrected for controls carried out with proteoliposomes reconstituted with C-less permease or liposomes without protein. With either of these preparations, a low rate of increase in fluorescence is observed, and no change is observed when TDG is added. The correction did not exceed 20% of the labeling rate observed prior to addition of TDG.

concentrations below 1 mM are ineffective, and concentrations above 10 mM have no further effect (data not shown). In control experiments with proteoliposomes containing C-less permease or liposomes without protein, the slow increase in fluorescence remains constant when TDG is added. The results provide strong additional evidence for the argument that ligand binding leads to a conformational change in the permease which involves helices VIII (E269) and X (H322).

DISCUSSION

As demonstrated here, pyrene labeling of engineered Cys residues in C-less permease is a powerful approach to monitoring conformational changes in lac permease induced by detergents or, more importantly, by ligand binding. Previous studies (Jung et al., 1993) with PM-labeled lac permease mutants containing double Cys replacements for H322 and E325, two residues predicted to be on the same face of helix X, exhibit excimer fluorescence. Similarly, PM-labeled R302C (helix IX)/E325C (helix X) or E269C (helix

VIII)/H322C (helix X) also exhibits excimer fluorescence. The findings indicate that helix X is probably α-helical and that helices VIII and IX are close to helix X. By using these interactions, we now show that the denaturant SDS reduces the excimer fluorescence observed *between* helices (IX and X or VIII and IX) but has little or no effect on the interaction observed *within* helix X. Presumably, therefore, SDS has a strong influence on the tertiary structure of lac permease with relatively little effect on the secondary structure, at least in the region tested. The observations are consistent with circular dichroic measurements demonstrating that the permease remains about 70% helical in 10% SDS (D. M. Foster, M. Boublik, and H. R. Kaback, unpublished information) and with the finding that the excimer fluorescence observed with mutants R302C/E325C or E269C/H322C is observed only after the proteins are reconstituted from OG into proteoliposomes.

The fluorescence of the PM-labeled double mutant E269C/H322C or the single mutants E269C and H322C is quenched by the hydrophilic collisional quencer Tl^+, indicating that these regions of the permease are accessible to the aqueous phase. Since Glu269 and His322 are presumably located in the middle of hydrophobic transmembrane domains VIII and X, respectively, these putative amphipathic helices may line the walls of a solvent-filled notch or cleft in the molecule (Costellow et al., 1984, 1987; Li & Tooth, 1987). In any event, ligands of the permease (particularly TDG which has a relatively high affinity, and lactose at higher concentrations) cause a marked decrease in the accessibility of the residues to Tl^+ and a decrease in the Stern–Volmer quenching constant. Thus, ligand binding apparently results in a conformational change that causes positions 269 and 322 to become less accessible to the aqueous phase. Taken together with the previous observations, the findings are consistent with the interpretation that ligand binding induces alterations in the tertiary structure of the permease.

The reaction rates of the single Cys mutants E269C or H322C with PM are markedly stimulated by ligands with specificity for the permease. Since PM is hydrophobic and partitions into the proteoliposomes, it is likely that reaction with Cys takes place from within the hydrophobic phase rather than from the aqueous phase. Thus, the increased reactivity with PM observed on addition of TDG is entirely consistent with decreased accessibility to Tl^+ and supports the notion that the site-directed pyrene moieties in the permease are being transferred into a more hydrophobic environment upon ligand binding. Although the studies reported here concentrate on E269 and H322 in helices VIII and X, respectively, similar experiments demonstrate that single Cys replacements in a number of other helices also exhibit dramatic changes in reactivity in the presence of ligand. For example, permease molecules with V315C (helix X) (Sahin-Tóth & Kaback, 1993a; H. Jung and H. R. Kaback, unpublished results), P31C (helix I), or F162C (helix V) also exhibit increased reactivity in the presence of TDG, while the reactivity of C148 (helix V) is essentially abolished on addition of the analog (J. Wu and H. R. Kaback, unpublished results). Therefore, the overall results indicate that ligand binding probably induces widespread conformational alterations in the permease. It is also of interest that enhanced reactivity of V315C permease with *N*-ethylmaleimide is observed upon generation of $\Delta\bar{\mu}_{H^+}$ (Sahin-Tóth & Kaback, 1993a), suggesting that ligand binding or $\Delta\bar{\mu}_{H^+}$ may induce the same conformational state in the molecule. In this context, it will be informative to compare the effects of TDG and $\Delta\bar{\mu}_{H^+}$ on the reactivity of these and other single Cys replacements.

Although the affinity of the permease for ligand is markedly diminished by mutations in Arg302 (Menick et al., 1987), His322 (Püttner et al., 1986, 1989), or Glu269 (Ujwal et al., 1993), it is apparent from the findings described here that Cys replacement at these positions, followed by reaction with pyrene, does not completely abolish ligand binding. Thus, TDG or lactose decreases Tl^+ quenching with PM-labeled E269C/H322C permease, and the effect appears to be related qualitatively at least to the affinity of the permease for these ligands. Thus, TDG is effective at concentrations ranging from 1 to 10 mM, while lactose concentrations of 50 mM are needed to observe clear effects on Tl^+ quenching. Importantly, sucrose or glucose, which have little or no affinity for the permease, have no effect on either phenomenon, demonstrating that the effects are specific. The residual low apparent affinities may be due to modification either of a single high-affinity site or of a second, regulatory site (Lolkema & Walz, 1990; Lolkema et al., 1991; van Iwaarden et al., 1993). In any case, it should be noted that it is difficult to accurately quantitate the effective concentrations of ligand with respect to Cys reactivity, as the hydrophobic nature of PM makes it impossible to saturate the velocity of its reaction with Cys residues in the permease.

In view of the putative similarity in helix packing in the C-terminal halves of lac permease (Jung et al., 1993) and the eukaryotic glucose uniporters (Baldwin, 1993), it is interesting that effects similar to those shown here have been observed with respect to the fluorescence of Trp residues in the C-terminal half of the human erythrocyte glucose transporter (Pawagi & Deber, 1990; Chin et al., 1992).

REFERENCES

Baldwin, S. A. (1993) *Biochim. Biophys. Acta 1154*, 17.

Bhattacharyya, T., & Roy, D. (1993) *Biochemistry 32*, 9268.

Brooker, R. J. (1991) *J. Biol. Chem. 266*, 4131.

Calamia, J., & Manoil, C. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 4937.

Carrasco, N., Antes, L. M., Poonian, M. S., & Kaback, H. R. (1986) *Biochemistry 25*, 4486.

Carrasco, N., Püttner, I. B., Antes, L. M., Lee, J. A., Larigan, J. D., Lolkema, J. S., Roepe, P. D., & Kaback, H. R. (1989) *Biochemistry 28*, 2533.

Chin, J. J., Jhun, B. H., & Jung, C. Y. (1992) *Biochemistry 31*, 1945.

Consler, T. G., Persson, B. L., Jung, H., Zen, K. H., Jung, K., Privé, G. G., Verner, G. E., & Kaback, H. R. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 6934.

Copper, M., & Thomas, J. K. (1977) *Radiat. Res. 70*, 312.

Costello, M. J., Viitanen, P. V., Carrasco, N., Foster, D. L., & Kaback, H. R. (1984) *J. Biol. Chem. 259*, 15570.

Costello, M. J., Escaig, J., Matsushita, K., Viitanen, P. V., Menick, D. R., & Kaback, H. R. (1987) *J. Biol. Chem. 262*, 17072.

Dunten, R. L., Sahin-Tóth, M., & Kaback, H. R. (1993a) *Biochemistry 32*, 3139.

Dunten, R. L., Sahin-Tóth, M., & Kaback, H. R. (1993b) *Biochemistry 32*, 12644.

Foster, D. L., Boublik, M., & Kaback, H. R. (1983) *J. Biol. Chem. 258*, 31.

Fox, C. F., & Kennedy, E. P. (1965) *Proc. Natl. Acad. Sci. U.S.A. 51*, 891.

Hattori, M., & Sakaki, Y. (1986) *Anal. Biochem. 152*, 232.

Hinkle, P. C., Hinkle, P. V., & Kaback, H. R. (1990) *Biochemistry 29*, 10989.

Jung, K., Jung, H., Wu, J., Privé, G. G., & Kaback, H. R. (1993) *Biochemistry 32*, 12273.

Kaback, H. R. (1983) *J. Membr. Biol. 76*, 95.

Kaback, H. R. (1989) *Harvey Lect. 83*, 77.

Kaback, H. R. (1992) *Int. Rev. Cytol. 137A*, 97–125.
Kaback, H. R., Bibi, E., & Roepe, P. D. (1990) *Trends Biochem. Sci. (Pers. Ed.) 15*, 309.
Kaback, H. R., Jung, K., Jung, H., Wu, J., Privé, G. G., & Zen, K. H. (1993) *J. Bioenerg. Biomembr. 25*, 627.
King, S. C., & Wilson, T. H. (1989a) *J. Biol. Chem. 264*, 7390.
King, S. C., & Wilson, T. H. (1989b) *Biochim. Biophys. Acta 982*, 253.
King, S. C., & Wilson, T. H. (1990a) *J. Biol. Chem. 265*, 3153.
King, S. C., & Wilson, T. H. (1990b) *Biochim. Biophys. Acta 1022*, 373.
King, S. C., Hansen, C. L., & Wilson, T. H. (1991) *Biochim. Biophys. Acta 1062*, 177.
Kinnunen, P. K. J., Koiv, A., & Mustonen, P. (1993) in *Fluorescence Spectroscopy* (Wolfbeis, O. S., Ed.) pp 159–171, Springer Verlag, Berlin, Heidelberg, and New York.
Kouyama, T., & Mihashi, K. (1981) *Eur. J. Biochem. 114*, 33.
Laemmli, U. K. (1970) *Nature (London) 227*, 680.
Lee, J.-I., Hwang, P. P., Hansen, C., & Wilson, T. H. (1992) *J. Biol. Chem. 267*, 20758.
Li, J., & Tooth, P. (1987) *Biochemistry 26*, 4816.
Lolkema, J. S., & Walz, D. (1990) *Biochemistry 29*, 11180.
Lolkema, J. S., Carrasco, N., & Kaback, H. R. (1991) *Biochemistry 30*, 1284.
Matzke, E. A., Stephenson, L. J., & Brooker, R. J. (1992) *J. Biol. Chem. 267*, 19095.
May, J. M., Zhio-chao, Q., & Beecham, J. M. (1993) *Biochemistry 32*, 9524.
Menick, D. R., Carrasco, N., Antes, L. M., Patel, L., & Kaback, H. K. (1987) *Biochemistry 26*, 6638.
Merrill, A. R., Palmer, L. R., & Szabo, A. G. (1993) *Biochemistry 32*, 6974.
Padan, E., Sarkar, H. K. Viitanen, P. V., Poonian, M. S., & Kaback, H. R. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 6765.
Pawagi, A. B., & Deber, C. M. (1990) *Biochemistry 29*, 950.
Peerce, B. E., & Wright, E. M. (1987) *Biochemistry 26*, 4272.
Püttner, I. B., Sarkar, H. K., Poonian, M. S., & Kaback, H. R. (1986) *Biochemistry 25*, 4483.
Püttner, I. B., Sarkar, H. K., Padan, E., Lolkema, J. S., & Kaback, H. R. (1989) *Biochemistry 28*, 2525.
Reed, K. E., & Cronan, J. E., Jr. (1991) *J. Biol. Chem. 266*, 11425.
Sahin-Tóth, M., & Kaback, H. R. (1993a) *Protein Sci. 2*, 1024.
Sahin-Tóth, M., & Kaback, H. R. (1993b) *Biochemistry 32*, 10027.
Sahin-Tóth, M., Dunten, R. L., Gonzalez, A., & Kaback, H. R. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 10547.
Sahin-Tóth, M., Persson, B., Schwieger, J., Cohan, M., & Kaback, H. R. (1994) *Protein Sci.* (in press).
Sanger, F., Nicklen, S., & Coulsen, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463.
Schaffner, W., & Weissmann, C. (1973) *Anal. Biochem. 56*, 502.
Teather, R. M., Bramhall, J., Riede, I., Wright, J. K., Fürst, M., Aichele, G., Wilhelm, V., & Overath, P. (1980) *Eur. J. Biochem. 108*, 223.
Ujwal, M. L., Sahin-Tóth, M., Persson, B., & Kaback, H. R. (1994) *Membr. Mol. Biol. 1*, 9.
van Iwaarden, P. R., Pastore, J. C., Konings, W. N., & Kaback, H. R. (1991) *Biochemistry 30*, 9595.
van Iwaarden, P. R., Driessen, A. J. M., Lolkema, J. S., Kaback, H. R., & Konings, W. N. (1993) *Biochemistry 32*, 5419.
Viitanen, P. V., Newman, M. J., Foster, D. L., Wilson, T. H., & Kaback, H. R. (1986) *Methods Enzymol. 125*, 429.
Wright, J. K., Teather, R. M., & Overath, P. (1983) *Methods Enzymol. 97*, 158.