Cysteine 148 in the Lactose Permease of *Escherichia coli* Is a Component of a Substrate Binding Site. 2. Site-Directed Fluorescence Studies

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ABSTRACT: By using site-directed fluorescence spectroscopy, we have carried out structure/function studies on lactose permease purified from *Escherichia coli* in dodecyl β,D-maltoside. Initially, permease containing a single native Cys at position 148 (helix V) was studied, since this residue is protected against alkylation by substrates of the permease. In the absence of ligand, Cys148 permease reacts rapidly with 2-(4′-maleimidylanilino)naphthalene-6-sulfonic acid (MIANS), a fluorophore whose quantum yield increases dramatically upon reaction with a thiol, indicating that this residue is readily accessible to the probe. Various ligands of the permease block the reaction, and the concentration dependence is commensurate with the affinity of each ligand for the permease (i.e., β,D-galactopyranosyl 1-thio-β,D-galactopyranoside ≪ lactose < galactose), but neither sucrose nor glucose has any effect whatsoever. Thus, the permease retains the ability to bind ligand specifically when the molecule is in dodecyl β,D-maltoside. Permease containing single Cys substitutions in the vicinity of Cys148 was also studied. Interestingly, labeling of Cys145 which is presumed to be one helical turn removed from Cys148 exhibits properties similar to those observed with Cys148 permease, but the effects of ligand are far less dramatic. On the other hand, permease with a single Cys residue at position 146 or 147 behaves in a completely different manner. Studies with iodide indicate that MIANS at positions 145 or 148 is accessible to the collisional quencher, indicating that this face of helix V is solvent exposed, while MIANS at positions 146 or 147 is not quenched by iodide in the presence or absence of ligand. Finally, iodide quenching of MIANS at position 145 is clearly diminished in the presence of ligand. Taken together with the preceding paper in this issue [Jung, H., Jung, K., & Kaback, H. R. (1994) Biochemistry (preceding paper in this issue)], the results indicate that Cys148 is a likely component of a substrate binding site in lac permease, although it does not play an essential role in transport, and that residue 145 is in close proximity.

In the preceding paper (Jung et al., 1994), site-directed mutagenesis was utilized to make multiple amino acid replacements for Cys148 in the lactose (lac)[1] permease. Evidence was presented indicating that although Cys148 is not essential for transport, it is probably located in a binding site in the permease and interacts hydrophobically with the galactosyl moiety of the substrate. Here we utilize site-directed fluorescence labeling to provide more direct evidence for the contention that Cys148 is located in a binding site in the permease. In addition, the experiments presented demonstrate that purified permease maintains the ability to bind ligand in dodecyl maltoside (DM).

EXPERIMENTAL PROCEDURES

Plasmid Construction. Using plasmids derived from pC_7S/C154V[2] (encoding C-less permease; van Iwaarden et al., 1991; EMBL X-56095) with given single-Cys replacements in putative helix V (C. Weitzman and H. R. Kaback, manuscript in preparation), the DNA sequence encoding a *Klebsiella* biotin acceptor domain was inserted into the approximate middle of each *lacY* mutant (pLacY/L6XB; Consler et al., 1993). To eliminate insertion of the biotin acceptor domain in both orientations, the *Xho*I site at the 3′ end of the biotin acceptor domain in plasmid pT7-5/lacY-L6XB was removed by PCR mutagenesis. Briefly, the DNA fragment encoding the biotin domain in pT7-5/lacY-L6XB was amplified with primers 5′-GACGCGCCCTCGAGCCTCGCGGGG-3′ (sense) which contains a *Xho*I site (underlined) and 5′-CGTGGCAGTCGACCGGCCCTCGATCAT-3′ (antisense) which contains the sequences for both a *Sal*I site (underlined) and a factor Xa protease site (emboldened). The amplified DNA fragment was digested with both *Xho*I and *Sal*I and ligated into the *Xho*I site in pC_7S/C154V encoding permease with a given single Cys residue. The resultant plasmid was termed pT7-5/Cys(residue number)-L6XB*. The new version of biotin acceptor domain was sequenced, as were each of the single-Cys mutants after insertion of the biotin acceptor domain (Sanger et al., 1977). All DNA manipulations were according to Sambrook et al. (1989).

Purification of Biotinated lac Permease with Single-Cys Residues. Permease with a given single Cys residue and the biotin acceptor domain was expressed in *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). Cultures (6–9 L) were grown at 37 °C in LB both with streptomycin (10 μg/mL) and ampicillin (100 μg/mL) and induced with 0.5

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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*-ethenyl maleimide; DM, dodecyl β,D-maltoside; KP_i, potassium phosphate; DTT, dithiothreitol; TDG, β,D-galactopyranosyl 1-thio-β,D-galactopyranoside.

[2] Single Cys mutants are designated by the single-letter amino acid abbreviation for the target residue in the C-less permease, followed by the sequence position, followed by a second letter indicating the amino acid replacement.

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mM IPTG for 4–6 h at an OD_{550} of 1.2. A membrane fraction was prepared as described (Viitanen et al., 1986) and solubilized with 2% dodecyl β,D-maltoside (DM) by incubation at 30 °C for 30 min with continuous stirring. Particulate material was removed by centrifugation at 100000g_{max}, and the supernatant was subjected to affinity chromatography on immobilized monovalent avidin (Consler et al., 1993). Monovalent avidin–Sepharose beads were washed sequentially with 100 mM NaP_i (pH 7.4) containing 150 mM NaCl (PBS), followed by 2 mM *d*-biotin in PBS, 100 mM glycine (pH 2.8), and finally PBS. The avidin resin was then equilibrated with column buffer containing 50 mM KP_i (pH 7.4)/150 mM NaCl/0.02% DM (w/v). The DM-soluble fraction was mixed with pre-equilibrated avidin resin, followed by 20-min incubation at 4 °C with continuous rotation. The slurry was then packed into a small column and unbound material was removed by washing extensively with column buffer. Bound permease was then eluted with 5 mM *d*-biotin in the column buffer and stored at 4 °C until use. The purity of each preparation was assessed by electrophoresis on 12% sodium dodecyl sulfate polyacrylamide gels (Laemmli, 1970), followed by silver staining.

Protein Determinations. Protein was assayed as described by Bradford (1976) with bovine serum albumin as standard.

Labeling of Purified Single Cys lac 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 a extinction coefficient of 17 000 M^{-1} cm^{-1} (Haugland, 1989). Fluorescence was measured at 30 °C with a SLM 8000C spectrofluorometer (SLM-Amico Instruments Inc., Urbana, IL). Emission spectra were recorded using an excitation wavelength of 330 nm and 8- and 4-nm slits for excitation and emission, respectively.

To determine the rate of MIANS reaction with Cys residues in purified single-Cys permeases, the protein (40 μg/mL) was preincubated with the 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. The reaction was initiated by addition of MIANS to a final concentration 4 μM from a 1 mM stock solution, and fluorescence was monitored continuously at an emission wavelength of 415 nm (excitation, 330 nm).

For complete modification of cysteinyl residues, reactions were carried out at 4 °C for 60 min with a 10-fold molar excess of MIANS over the protein. Unreacted MIANS was quenched with DTT and removed by dialysis against three changes of column buffer. To study the effect of ligands on the fluorescence of MIANS-modified permease, labeled protein was preincubated with a given ligand for 5 min at 30 °C followed by measurement of emission spectra.

Fluorescence Quenching Studies. Fluorescence quenching studies were carried out with freshly prepared solutions of KI (5 M). Increasing amounts of KI were added to the reaction mixtures containing MIANS labeled lac permease with a given single Cys residue. In the control assays, KCl was added at the same concentrations in order to correct for dilution and ionic strength effects. Data were analyzed by using the Stern–Volmer equation (Lakowicz, 1983):

$$F_o/F = 1 + k_q\tau_o[Q] = 1 + K_{sv}[Q]$$

where F_o and F are the fluorescence intensities in the absence and presence of quencher, respectively, k_q is the bimolar quenching constant, τ_o is the life time of fluorescence in the absence of quencher, [Q] is the concentration of quencher, and K_{sv} is $k_q\tau_o$, the Stern–Volmer quenching constant. A plot of F_o/F vs [Q] yields a slope equal to K_{sv}.

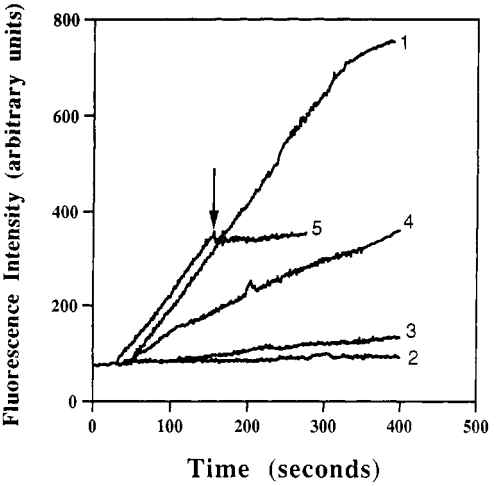

FIGURE 1: Reaction of MIANS with Cys148 in purified single-Cys148 permease. MIANS labeling was carried out in 0.5 mL of 50 mM KP_i (pH 7.4)/60 mM NaCl/0.01% DM containing 40 μg/mL purified permease. The reactions were initiated by adding MIANS to a final concentration of 4 μM, and fluorescence was recorded continuously at 415 nm as a function of time (excitation, 330 nm) as described under Experimental Procedures. Addition of ligands was as follows: (curve 1) no addition or 100 mM sucrose or glucose; (curves 2, 3, and 4, respectively) 5 mM TDG, 20 mM lactose, or 50 mM galactose was added prior to addition of MIANS; (curve 5) 5 mM TDG was added as indicated by the arrow.

RESULTS

Purification of Biotinated Single-Cys lac Permeases. Biotinated lac permeases with single Cys residues at positions 145, 146, 147, or 148 were purified by monovalent avidin affinity chromatography as described by Consler et al. (1993) with minor modifications. Thus, DM was used in place of octyl β,D-glucoside, and *E. coli* phospholipids, diththiothreitol, and lactose were excluded from the column buffer (Privé et al., 1994). Purified permeases were homogeneous as judged by silver-stained sodium dodecyl sulfate polyacrylamide gels, as well as immunoblots [see Consler et al. (1993)].

MIANS Labeling of Cys148 and the Effect of Ligands. Reactivity of Cys148 in purified single Cys148 permease was studied by labeling with MIANS, a sulfhydryl-specific probe that is not fluorescent until the maleimide group undergoes chemical reaction (Gupte & Lane, 1979; Haugland, 1989). This fluorophore has been used to probe conformational changes and ligand binding in a number of proteins, including myosin (Hiratsuka, 1992), the sarcroplasmic reticulum Ca^{2+}-ATPase (Bigelow & Inesi, 1991), the erythrocyte glucose carrier (May & Beechem, 1993), visual rhodopsin (Phillips & Cerione, 1991), and Na^+/K^+-ATPase (Gupte & Lane, 1983).

Cys148 in lac permease is readily accessible to MIANS (Figure 1, curve 1). Thus, addition of MIANS to a reaction mixture containing purified Cys148 permease in DM results in a rapid and linear increase in fluorescence emission intensity for about 5 min, after which the rate of increase in fluorescence declines. Strikingly, preincubation of Cys148 permease with 5 mM β,D-galactopyranosyl 1-thio-β,D-galactopyranoside (TDG) essentially completely abolishes the signal (curve 2). Moreover, addition of TDG during the labeling reaction also completely abrogates any further increase in fluorescence (curve 5). Although the effects are less marked, preincubation of Cys148 permease with lactose (20 mM) or galactose (50

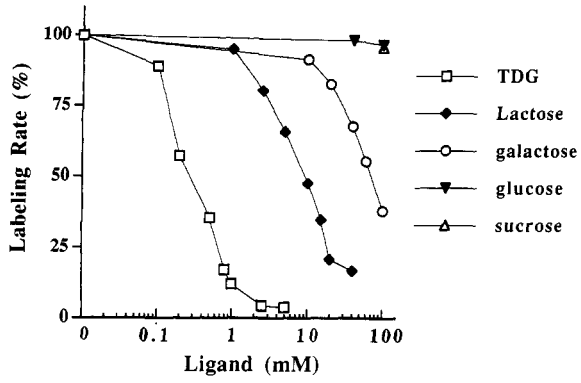

FIGURE 2: Effect of ligands on the rate of MIANS labeling of single-Cys148 permease. Purified Cys148 permease was preincubated with various concentrations of TDG (□), lactose (◆), galactose (○), glucose (▼), or sucrose (△) as described in the legend to Figure 1. The labeling rates were obtained from the initial fluorescence increase after addition of MIANS and plotted as the function of ligand concentration.

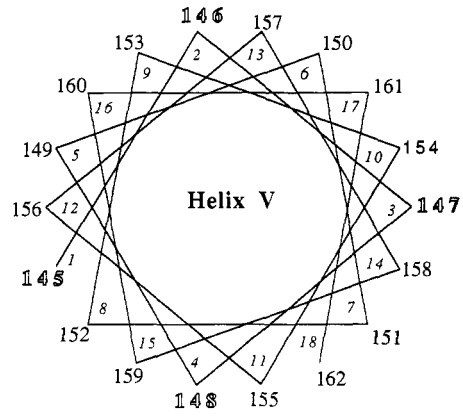

FIGURE 3: Helical wheel plot of putative helix V. Eighteen residues (positions 145–163) of putative helix V are shown on a helical wheel plot viewed from the cytoplasmic surface of the membrane. The outlined numbers represent the positions of the single-Cys replacement mutants described. The smaller, italicized numbers represent the positions in the putative transmembrane helix starting from the cytoplasmic surface.

mM), both of which are ligands of the permease with lower affinities than TDG, decreases the reactivity of Cys148 to MIANS (curves 3 and 4, respectively). In contrast, high concentrations of sucrose or glucose, sugars that are not substrates for the permease, have no effect whatsoever on the reactivity of Cys148 with MIANS (curve 1), thereby demonstrating that the effects of TDG, lactose and galactose are specific.

The effects of these sugars on the reactivity of Cys148 was then examined as a function of concentration (Figure 2). With TDG, lactose, or galactose, half-maximal protection is observed at approximately 0.3, 9.0, or 50 mM, respectively, while glucose or sucrose have no significant effect at concentrations up to 100 mM. Although the apparent affinities of the permease for TDG, lactose, and galactose are approximately an order of magnitude higher than the K_Ds and/or apparent K_ms observed for these substrates, their order is in full agreement with the apparent affinity of wild-type permease (i.e., TDG ≪ lactose < galactose).

MIANS Labeling of Other Single-Cys Mutants. As discussed in the preceding paper (Jung et al., 1994), the effect of permease ligands on the reactivity of Cys148 could be due either to a direct steric effect or to a long-range conformational change. In order to differentiate between these possibilities, single Cys substitutions were made in the vicinity of residue 148 in C-less permease where Cys148 is replaced with Ser (van Iwaarden et al., 1991). MIANS labeling of purified permease molecules with a single Cys residue at each position was then examined. A helical wheel depiction of putative helix V reveals that residue 145 is on the same helical face as 148, while residues 146 and 147 are on the opposite face (Figure 3).

Permease with a single Cys residue in place of Met145, Phe146, or Gly147 reacts rapidly with MIANS (Figure 4, curve 1 in each panel), demonstrating that the Cys residue in each molecule, like Cys148, is relatively accessible to the probe. However, the effect of ligands on MIANS labeling of the other mutants is quite different from that observed with Cys148 permease. Remarkably, reaction of MIANS with Cys145, which is presumably on the same face of helix V as 148 but one turn removed toward the cytosolic surface (Figure 3), is decreased by about 35% in the presence of a saturating concentration of TDG (Figure 4a, curve 2). Moreover, small but significant decreases in reactivity are observed with lactose or galactose (curves 3 and 4), but not with sucrose or glucose (curve 1). Thus, M145C permease behaves like Cys148 permease, but the effect of ligand is much less dramatic. With F146C permease, on the other hand, the rate of MIANS labeling is not altered in the presence of ligand (Figure 4b).

MIANS labeling of G147C permease is both different and more complex. As shown in Figure 4c, preincubation of G147C permease with TDG (5 mM) increases the reaction rate (curve 2). Furthermore, TDG induces about 30% quenching of the maximum fluorescence emission intensity observed (compare curves 1 and 2). Interestingly, the low affinity ligands lactose (20 mM) or galactose (50 mM) cause a significant decrease in the rate of MIANS labeling (curves 3 and 4, respectively) due to quenching (compare the maximum fluorescence levels in curves 3 and 4 with curve 1; see Figure 6c in addition).

Solvent Accessibility of Bound MIANS. Fluorescence quenchers have been widely used to study accessibility of fluorescent groups bound to membrane proteins [for examples, see Bigelow and Inesi (1991), May and Beechem (1993), and Jung et al. (1994)], and ionic quenchers can provide information regarding the polarity and/or electrostatics of the milieu in the vicinity of the fluorophore (Eftink, 1991). Iodide, a collisional quencher (Lakowicz, 1983), was used to study the solvent accessibility of each MIANS-labeled single-Cys mutant (Figure 5). As shown, the fluorescence exhibited by MIANS-labeled M145C or Cys148 permease is significantly quenched by increasing iodide concentrations. In the absence of ligand, the Stern–Volmer constants are 1.01 M^{-1} for MIANS-labeled M145C permease and 0.52 M^{-1} for MIANS-labeled Cys148 permease. Furthermore, in the presence of TDG, MIANS-bound at position 145 is less accessible to iodide, and the Stern–Volmer constant is reduced from 1.01 to 0.65 M^{-1}. Clearly, therefore, binding of TDG decreases the solvent exposure of MIANS at position 145. On the other hand, TDG has no effect whatsoever on iodide quenching of fluorescence with Cys148 permease, a finding that is consistent with many observations demonstrating that modification of Cys148 blocks ligand binding [see Kaback (1992)]. Finally, no significant iodide quenching is observed with MIANS-labeled F146C or G147C permease either in the presence or absence of TDG.

Ligand-Induced Conformational Changes. MIANS is an environment-sensitive fluorophore whose quantum yield and emission maximum vary inversely with the polarity of the environment (Haugland, 1989; Hiratsuka, 1992; Ksenzenko

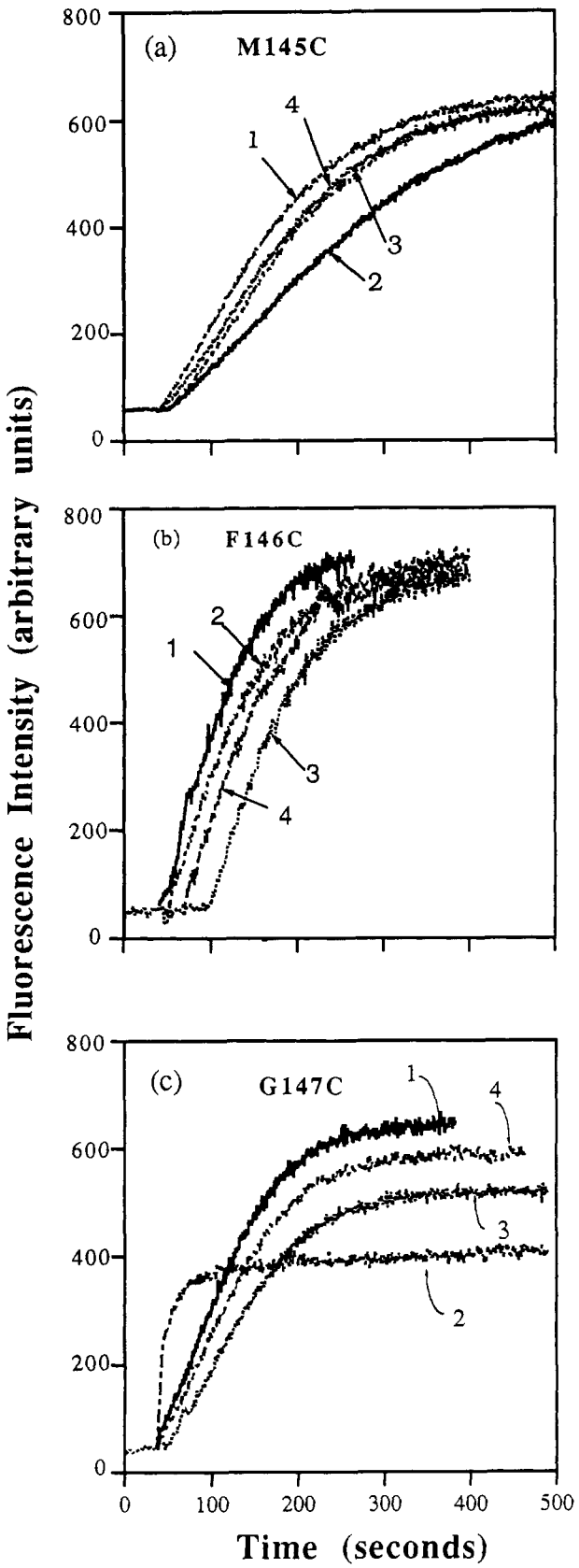

FIGURE 4: Effect of ligands on MIANS labeling of purified single-Cys permeases. Affinity purified M145C (a), F146C (b), or G147C (c) permease was preincubated with a given concentration of ligand, and reactivity with MIANS was assayed as described in Figure 1. (Curve 1) No addition, 100 mM glucose or 100 mM sucrose; (curve 2) 5 mM TDG; (curve 3) 20 mM lactose; (curve 4) 50 mM galactose.

et al., 1993). Although data are not presented, reaction of MIANS with 2-mercaptoethanol in aqueous solution exhibits

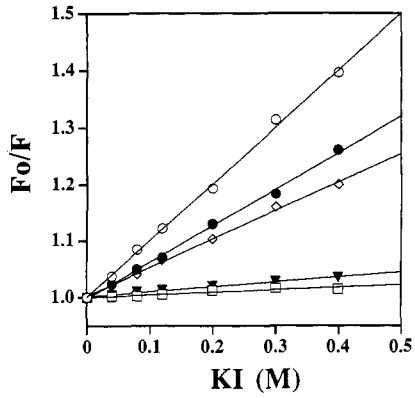

FIGURE 5: Iodide quenching of MIANS labeled single-Cys permeases. Each purified single-Cys permease was labeled with MIANS, and excess reagent was removed by dialysis as described under Experimental Procedures. Fluorescence emission maxima (see Figure 6) were then recorded in the presence of given concentrations KI (excitation 330 nm), and the spectra were corrected for identical measurements carried out at the same concentrations of KCl. The data were fit to the Stern–Volmer equation using a linear regression program as described under Experimental Procedures. (○) M145C permease; (●) M145C permease in the presence of 5 mM TDG; (□) F146C permease in the presence or absence of 5 mM TDG; (▼) G147C permease in the presence or absence of 5 mM TDG; (◇) S148C permease in the presence or absence of 5 mM TDG.

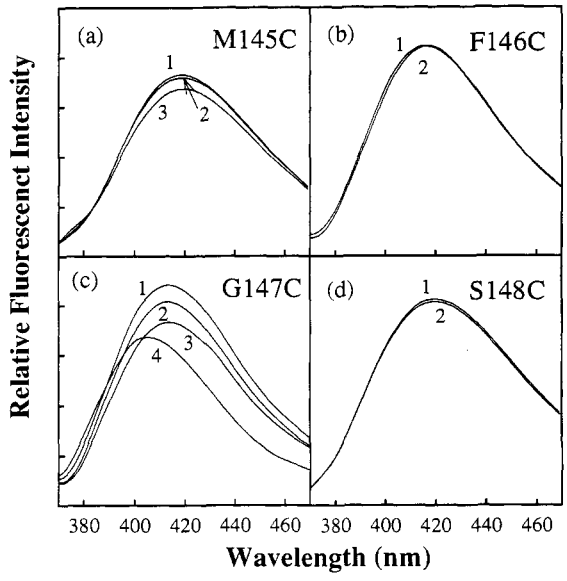

FIGURE 6: Emission spectra of MIANS-labeled single-Cys permeases and the effect of ligands. Each purified single-Cys permease was labeled with MIANS, and excess reagent was removed by dialysis as described under Experimental Procedures. The emission spectra of given single-Cys permeases were then measured (excitation 330 nm) in the absence or presence of ligand as follows: (a) MIANS-labeled M145C permease: (curve 1) no additions or 50 mM galactose; (curve 2) 20 mM lactose; (curve 3) 5 mM TDG. (b) MIANS-labeled F146C permease: (curve 1) no additions; (curve 2) 5 mM TDG, 20 mM lactose or 50 mM galactose. (c) MIANS-labeled G147C permease: (curve 1) no additions, 100 mM glucose, or 100 mM sucrose; (curve 2) 50 mM galactose; (curve 3) 20 mM lactose; (curve 4) 5 mM TDG. (d) MIANS-labeled S148C permease: (curve 1) no additions; (curve 2) 5 mM TDG, 20 mM lactose or 50 mM galactose.

an emission maximum at about 445 nm. In contrast, the emission maxima for each of the MIANS-labeled single-Cys permeases are significantly blue-shifted depending on the position labeled (Figure 6a–d). MIANS-labeled M145C, F146C, G147C, or Cys148 permease, respectively, exhibit fluorescence emission maxima at 419, 412, 413, and 418 nm. Therefore, each of these positions is in a relatively hydrophobic environment which is consistent with the topological assign-

ment of the four residues to a transmembrane domain (i.e., helix V). Furthermore, one face of helix V (i.e., positions 146 and 147) appears to be more hydrophobic than the other (i.e., positions 145 and 148; see Figure 3).

The effect of ligand on the fluorescence emission spectra of each MIANS-labeled single-Cys permease was also examined. As shown in Figure 6b,d, when MIANS is bound at Cys146 or Cys148, no change in the emission spectrum is observed on addition of ligand. In contrast, the fluorescence of MIANS-labeled M145C permease is quenched by about 5% in the presence of TDG without a detectable shift in the emission maximum, while little or no effect is observed with lactose or galactose or with glucose or sucrose (Figure 6a). Strikingly, substantial ligand-induced fluorescence quenching is observed with MAINS-labeled G147C (Figure 5c). With TDG (curve 4), lactose (curve 3), or galactose (curve 2), respectively, about 25%, 16%, or 9% quenching is observed, but importantly, no quenching is observed with glucose or sucrose (curve 1). Furthermore, it is apparent that the emission maximum of MIANS bound at position 147 is blue-shifted by about 9 nm (from 413 to 404 nm) in the presence of TDG (curve 4), indicating that TDG binding causes the environment around the fluorophore to become more hydrophobic. No spectral shift is evident in the presence of lactose or galactose (curves 2 and 3).

DISCUSSION

Fox and Kennedy (1965) demonstrated that lac permease is inactivated by *N*-ethylmaleimide (NEM) and that protection is afforded by substrates such as TDG leading to the hypothesis that a Cys residue in the permease is in or near the substrate binding site. Sixteen years later, Beyreuther et al. (1981) showed that Cys148 is the substrate-protectable residue. Shortly thereafter, Trumble et al. (1984) utilized site-directed mutagenesis to replace Cys148 with Gly with relatively little effect on active lactose transport and concluded that Cys148 is not essential for permease activity. This conclusion was subsequently confirmed by a number of additional observations (Viitanen et al., 1985; Neuhaus et al., 1985; Sarkar et al., 1986; Jung et al., 1994), and, parenthetically, it was also demonstrated that none of the other seven Cys residues is essential for function (Menick et al., 1985, 1987; Brooker & Wilson, 1986; van Iwaarden et al., 1991). In view of these findings and observations demonstrating that mutants containing single-Cys residues at various positions in the permease undergo marked increases in reactivity in the presence of ligand (Sahin-Tóth & Kaback, 1993; Jung et al., 1994a,b), the possibility arises that Cys148 is neither at nor near a substrate binding site, but becomes inaccessible to thiol reagents due to a ligand-induced conformational change.

In the preceding paper (Jung et al., 1994), site-directed mutagenesis was utilized to replace Cys148 in lac permease with different hydrophobic, hydrophilic, or charged residues. In confirmation of previous observations (Trumble et al., 1984; Viitanen et al., 1985; Neuhaus et al., 1985; Sarkar et al., 1986), Cys148 is not essential for transport. However, the size and polarity of the side chain modifies activity and specificity in subtle ways. Small, hydrophobic amino acid residues (e.g., Ala) cause a relatively small increase in apparent affinity for substrate, whereas hydrophilic substitutions (e.g., Ser) cause the opposite effect. Therefore, weak hydrophobic interactions appear to contribute to substrate binding. The weak hydrophobic contribution is of minor importance with disaccharides, but removal of the non-galactosyl moiety of the substrate reveals the functional importance of these interactions probably by decreasing nonspecific interactions between the non-galactosyl portion of the substrate with the permease. Thus, hydrophilic side chains at position 148 markedly decrease the transport of monosaccharides (galactose and methyl-1-thio-β,D-galactopyranoside) relative to transport of disaccharides (lactose, melibiose, and TDG). The results suggest that Cys148 may be located in a sugar binding site in lac permease interacting weakly and hydrophobically with the galactosyl moiety of the substrate. Although the findings are clearly consistent with this conclusion, the evidence is indirect. For this reason, the experiments presented here were undertaken.

In order to differentiate between a direct steric effect of ligand versus a long-range conformational change secondary to ligand binding, we have utilized site-directed fluorescence spectroscopy, an approach developed recently to study both static (Jung et al., 1993) and dynamic aspects (Jung et al., 1994a,b) of permease structure/function. Initially, permease containing a single native Cys residue at position 148 and the biotin acceptor domain from a *Klebsiella* carboxylase in the middle cytoplasmic loop was purified by avidin affinity chromatography in DM and tested for reactivity with MIANS, a fluorophore that is nonfluorescent until it undergoes chemical reaction. The results demonstrate that Cys148 is readily accessible to MIANS and reacts completely within about 5 min. Strikingly, TDG essentially completely blocks the reaction whether it is added before or after reaction with MIANS is initiated. Moreover, lactose and galactose also block reaction of Cys148 with MIANS, but sucrose and particularly glucose have no effect whatsoever. Thus, the specificity of the permease for substrate appears to be exclusively related to the asymmetry at the fourth carbon of the galactosyl portion of the substrate. In addition, the concentrations of TDG, lactose, and galactose required to block the rate of MIANS reactivity by 50% (0.3, 9.0, and 50 mM, respectively) reflect the relative apparent affinities of the permease for these ligands. On the other hand, the apparent K_Ds are about an order of magnitude higher than the K_Ds and/or apparent K_ms found for these substrates with wild-type permease in the membrane. Possibly the discrepancies are due to the fact that the measurements here were made with single-Cys permeases in DM in the absence of exogenous phospholipid. In any event, it is important that the permease retains the ability to bind ligand specifically under these conditions.

Since the experiments with MIANS labeling of Cys148 permease merely reproduce the findings of Fox and Kennedy (Fox & Kennedy, 1965; Kennedy et al., 1974), they shed little light on the question at issue in-and-of themselves. Therefore, three additional single-Cys replacement mutants were constructed encompassing one full turn of putative helix V from Cys148 toward the cytosolic surface. The only position in the permease that mimics Cys148 is position 145 where TDG, lactose, and galactose also inhibit MIANS labeling, but far less dramatically than observed with Cys148. Residues 146 and 147 behave in a completely different manner. Although both are accessible to MIANS, labeling of Cys146 is completely unaffected by ligand, while labeling of Cys147 is altered by ligand in a complex fashion (see below). Since three of the four single-Cys constructs studied exhibit ligand-induced changes in MIANS labeling, it is still difficult to differentiate between a direct steric effect versus a long-range conformational alteration. However, the iodide quenching experiments presented in Figure 5 provide stronger support for a direct interaction between TDG and residues 148 and

145 on the same face of helix V. Thus, the fluorescence of MIANS-labeled permease with a single-Cys residue at position 145 is quenched by iodide, and accessibility to the collisional quencher is attenuated in the presence of TDG. Furthermore, TDG quenches the fluorescence of MIANS-labeled Cys145 permease (Figure 6a). Although MIANS bound at position 148 is also quenched by iodide, ligand has no effect; however, permease modified at position 148 no longer binds ligand. Finally, residues 146 and 147 which are on the opposite face of helix V are inaccessible to iodide in the presence or absence of ligand.

It is apparent that the properties of G147C permease are not straightforward. This mutant is accessible to MIANS, but its behavior with respect to ligand is complex (Figure 4c). TDG, a high-affinity ligand, accelerates the rate of labeling much like other mutants with single-Cys residues at positions 315 (Sahin-Tóth & Kaback, 1993; Jung et al., 1994b), 322, and 269 (Jung et al., 1994a). However, TDG also quenches the fluorescence observed with Cys147 permease and causes a blue-shift in the fluorescence emission spectrum (Figures 4 and 6). In contrast, lactose or galactose which bind with lower apparent affinity cause quenching but no blue shift, and sucrose or glucose have no effect even at higher concentrations. Although data are not shown (J. Wu and H. R. Kaback, unpublished observations), it is also important that fluorescence quenching with TDG is observed over a relatively low concentration range of ligand, while the blue-shift is observed over a significantly higher range of TDG concentrations. At face value, the results are consistent with other observations (Lolkema & Walz, 1990; Lolkema et al., 1991; van Iwaarden et al., 1993) indicating that the permease may contain two binding sites, a high-affinity site responsible for quenching and a low-affinity site responsible for the blue-shift. Similar results are obtained with a single-Cys permease containing Cys in place of Val331 (J. Wu and H. R. Kaback, unpublished observations).

In conclusion, the results presented in this and the preceding paper (Jung et al., 1994) indicate that Cys148 is in a binding site of lac permease and that Met145 is in close proximity.

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