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Fluorescence of native single-Trp mutants in the lactose permease from *Escherichia coli*: Structural properties and evidence for a substrate-induced conformational change

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Abstract

Six single-Trp mutants were engineered by individually reintroducing each of the native Trp residues into a functional lactose permease mutant devoid of Trp (Trp-less permease; Menezes ME, Roepe PD, Kaback HR, 1990, *Proc Natl Acad Sci USA 87*:1638–1642), and fluorescent properties were studied with respect to solvent accessibility, as well as alterations produced by ligand binding. The emission of Trp 33, Trp 78, Trp 171, and Trp 233 is strongly quenched by both acrylamide and iodide, whereas Trp 151 and Trp 10 display a decrease in fluorescence in the presence of acrylamide only and no quenching by iodide. Of the six single-Trp mutants, only Trp 33 exhibits a significant change in fluorescence (ca. 30% enhancement) in the presence of the substrate analog β,D-galactopyranosyl 1-thio-β,D-galactopyranoside (TDG). This effect was further characterized by site-directed fluorescent studies with purified single-Cys W33 → C permease labeled with 2-(4′-maleimidylanilino)-naphthalene-6-sulfonic acid (MIANS). Titration of the change in the fluorescence spectrum reveals a 30% enhancement accompanied with a 5-nm blue shift in the emission maximum, and single exponential behavior with an apparent K_D of 71 μM. The effect of substrate binding on the rate of MIANS labeling of single-Cys 33 permease was measured in addition to iodide and acrylamide quenching of the MIANS-labeled protein. Complete blockade of labeling is observed in the presence of TDG, as well as a 30% decrease in accessibility to iodide with no change in acrylamide quenching. Overall, the findings are consistent with the proposal (Wu J, Frillingos S, Kaback HR, 1995a, *Biochemistry 34*:8257–8263) that ligand binding induces a conformational change at the C-terminus of helix I such that Pro 28 and Pro 31, which are on one face, become more accessible to solvent, whereas Trp 33, which is on the opposite face, becomes less accessible to the aqueous phase. The findings regarding accessibility to collisional quenchers are also consistent with the predicted topology of the six native Trp residues in the permease.

Keywords: bioenergetics; collisional quenching; Cys modification; ligand–protein interactions; membrane protein; Trp fluorescence

The lactose permease of *Escherichia coli* is a polytopic cytoplasmic membrane protein that catalyzes the coupled stoichiometric translocation of β-galactosides and H^+ (i.e., H^+/substrate symport or cotransport). The *lacY* gene, which encodes the permease, has been cloned and sequenced, and the protein has been solubilized from the membrane, purified, reconstituted into proteoliposomes, and shown to be solely responsible for β-galactoside transport (reviewed in Kaback, 1983, 1989, 1995; Poolman & Konings, 1993) as a monomer (see Sahin-Tóth et al., 1994a). Based on circular dichroic studies and hydropathy analysis of the primary amino acid sequence (Foster et al., 1983), a secondary structure was proposed in which the permease is composed of a short hydrophilic N-terminus, 12 α-helical hydrophobic domains that traverse the membrane in zig-zag fashion connected by hydrophilic loops, and a 17-residue hydrophilic C-terminal tail. Evidence supporting the general features of the model and demonstrating that both the N- and C-termini are on the cytoplasmic face of the membrane was obtained from laser

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Abbreviations: lac, lactose; NEM, *N*-ethylmaleimide; MIANS, 2-(4′-maleimidylanilino)-naphthalene-6-sulfonic acid; $\Delta\mu H^+$, proton electrochemical gradient across the membrane; DM, dodecyl-β,D-maltoside; KP_i, potassium phosphate; TDG, β,D-galactopyranosyl 1-thio-β,D-galactopyranoside; IPTG, isopropyl 1-thio-β,D-galactopyranoside; PBS, 100 mM KP_i (pH 7.5)/150 mM NaCl; TMG, thiomethylgalactoside.

Raman spectroscopy (Vogel et al., 1985), limited proteolysis (Goldkorn et al., 1983; Stochaj et al., 1986), immunological studies (Carrasco et al., 1982, 1984a, 1984b; Seckler et al., 1983, 1986; Herzlinger et al., 1984, 1985), and chemical modification studies (Page & Rosenbusch, 1988). Exclusive support for the 12-helix motif was obtained from analyses of an extensive series of lac permease–alkaline phosphatase (*lacY-phoA*) fusions (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; Kaback et al., 1993, 1994). Moreover, the helix proximity relationships of the model have been confirmed and extended by recent studies utilizing engineered metal binding sites (Jung et al., 1995; He et al., 1995a, 1995b) and site-directed chemical cleavage (Wu et al., 1995c).

By applying site-directed mutagenesis to wild-type and Cys-scanning mutagenesis to a functional permease molecule devoid of Cys residues (C-less permease; van Iwaarden et al., 1991), individual amino acid residues that are essential for transport and/or substrate binding have been identified (Kaback, 1992, 1995; Kaback et al., 1993). Single-Cys residues have been placed at more than 350 of the 417 different positions (Dunten et al., 1993; Sahin-Tóth & Kaback, 1993; Frillingos et al., 1994; Sahin-Tóth et al., 1994b; Weitzman & Kaback, 1995) and, remarkably, less than half-dozen residues have been shown to play a critical mechanistic role thus far (reviewed in Kaback, 1992, 1995; Kaback et al., 1993). However, the activity of many active Cys replacement mutants is altered by alkylation, and these mutants appear in clusters, suggesting that surface contours within the permease are important (Dunten et al., 1993; Sahin-Tóth & Kaback, 1993; Frillingos et al., 1994; Sahin-Tóth et al., 1994b; Weitzman & Kaback, 1995). In addition, site-directed fluorescence labeling and *N*-ethylmaleimide inactivation studies demonstrate that the reactivity of various Cys residues is altered as a result of ligand binding or imposition of a H^+ electrochemical gradient (Sahin-Tóth & Kaback, 1993; Jung et al., 1994a, 1994c; Wu & Kaback, 1994; Wu et al., 1995a, 1995b). These observations suggest that ligand binding or $\Delta\bar{\mu}_{H^+}$ may cause the permease to assume the same conformation. Taken together, therefore, it appears likely that permease turnover results from relatively simple chemistry involving a small number of critical side chains, coupled to widespread conformational changes in which the transmembrane helices move relative to one another. Thus, in order to understand the transport mechanism, structural and dynamic information at high resolution is clearly required. In this regard, fluorescence spectroscopy of endogenous Trp residues represents a powerful method for probing structure–function relationships in lac permease.

Wild-type permease contains six Trp residues, none of which is important for activity (Menezes et al., 1990). In this report, each of the six native Trp residues was reintroduced individually into a completely functional permease mutant devoid of Trp (Trp-less permease), and the fluorescence properties of the purified mutant proteins in detergent were studied with respect to accessibility to polar and nonpolar collisional quenchers, as well as alterations caused by ligand binding. The results indicate that Trp 10 and Trp 151 are inaccessible from the aqueous phase, whereas Trp 33, Trp 78, Trp 171, and Trp 223 are exposed. Evidence is also presented demonstrating that ligand binding induces a conformational change that causes position 33 to become less exposed to a hydrophilic environment. The apparent K_D for TDG with respect to the phenomenon corresponds to binding at a high-affinity site. The results confirm and extend the findings of Wu et al. (1995a).

Results

Active lactose transport

Each of the six native single-Trp mutants was tested for its ability to transport lactose uphill. Cells expressing each biotinylated mutant permease exhibit active [^{14}C] lactose transport with initial rate and steady-state level of accumulation comparable to wild-type permease. Cells transformed with plasmid devoid of *lacY* transport the disaccharide to a negligible extent.

Quenching of single-Trp permease fluorescence by iodide and acrylamide

Corrected steady-state emission spectra of each native single-Trp permease in dodecyl-β,D-maltoside in the absence of quencher and in the presence of either I^- or acrylamide are shown in Figure 2. Both I^- and acrylamide exhibit similar quenching of Trp 33, Trp 78, Trp 171, or Trp 223 permease, indicating that these positions are exposed and accessible to both collisional quenchers. However, Trp 10 or Trp 151 permease are quenched by acrylamide only, which is a relatively hydrophobic quencher. Because electrostatic repulsion from neighboring acidic side-chains would result in poor I^- quenching, Trp 10 and Trp 151 were also tested for quenching by Cs^+ (not shown) with similar results (i.e., no quenching). Thus, the absence of quenching by high concentrations of both a positively and a negatively charged quencher rules out this possibility and allows us to conclude that positions Trp 10 and Trp 151 are in a more hydrophobic environment than the other Trp residues.

Effect of substrate on single-Trp permease fluorescence

Corrected steady-state emission spectra of each single-Trp mutant in the absence or in the presence of either TDG or sucrose are shown in Figure 3. Interestingly, Trp 33 permease displays a 30% enhancement of fluorescence emission in the presence of TDG, which is not observed when equimolar amounts of sucrose are added. The observation indicates that the behavior of Trp 33, like other residues at the C-terminus of helix I (Wu et al., 1995a), reflects a ligand-induced conformational change. Trp 10 and Trp 151 permeases show identical spectra in the presence and absence of sugar, indicating that the environment at these positions remains unaltered in the presence of TDG or sucrose. With Trp 78, Trp 171, or Trp 223 permease, a change is observed upon addition of sugar. The fluorescence of Trp 78 or Trp 223 permease exhibits 43% or 17% enhancement, respectively, in the presence of either TDG or sucrose. Trp 171 permease, on the other hand, shows a 25% decrease in fluorescence with both sugars. Clearly, therefore, the effects are unrelated to permease specificity because sucrose is not a substrate for lac permease. Possibly the fluorescence changes observed with these single-Trp mutants reflect exposure to the aqueous phase, which is altered by the viscous nature of the sugar solutions added.

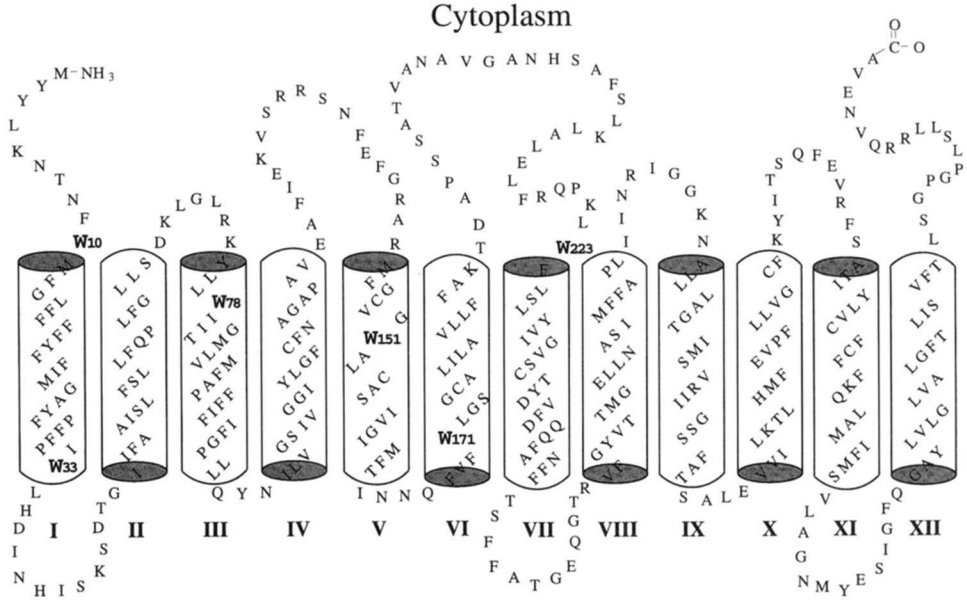

Fig. 1. Secondary structure model of *E. coli* lac permease. The single-letter amino acid code is used. Wild-type lac permease is shown with the six native Trp residues highlighted. Hydrophobic transmembrane helices are enclosed in cylinders.

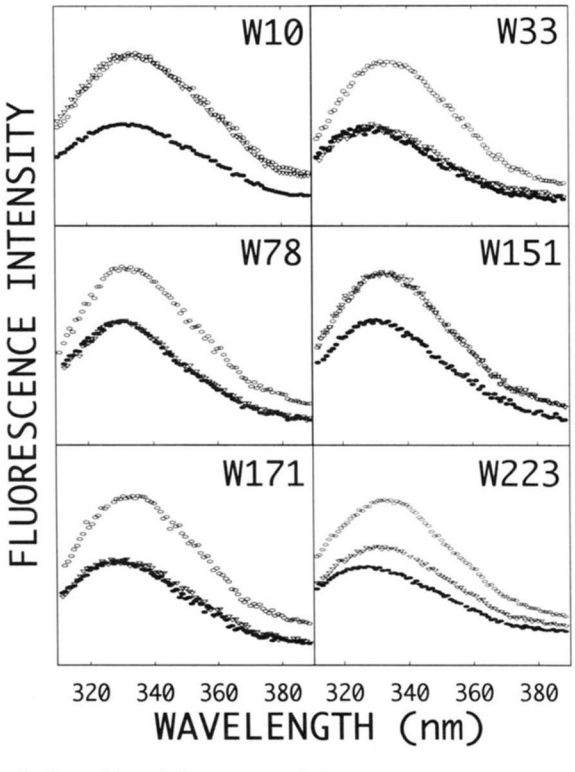

Fig. 2. Quenching of fluorescence of single-Trp mutants by acrylamide and iodide. The fluorescence emission spectra after 5 min incubation in the absence (○) and presence of 0.5 M acrylamide (●) or iodide (▽) was recorded at 30 °C with 8- and 4-nm slits for excitation and emission, respectively (excitation wavelength = 295 nm). Each spectrum represents the average of 3–7 independent measurements.

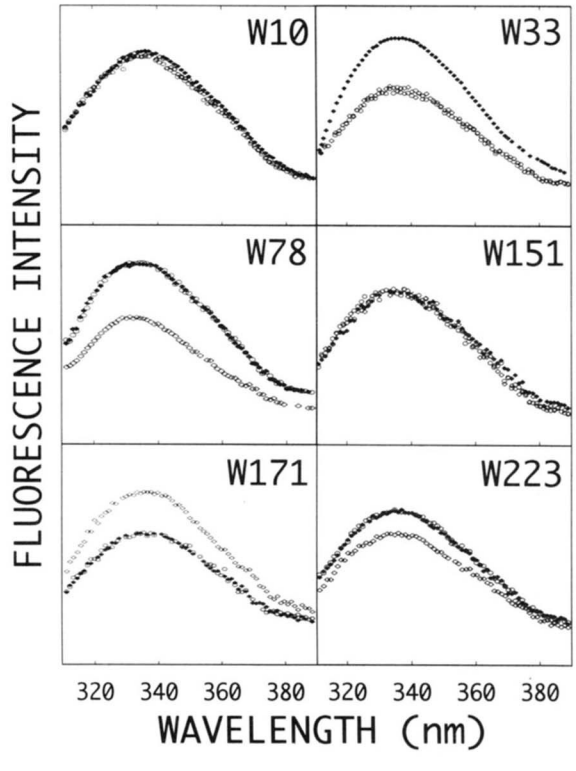

Fig. 3. Effect of ligand on fluorescence of single-Trp mutants. The fluorescence emission spectrum (excitation wavelength = 295 nm) of each pure single-Trp permease (20–40 μg/mL) was recorded as described in the Materials and methods after 5 min incubation in the absence (◇) and in the presence of 10 mM TDG (◆) or 10 mM sucrose (○). Each spectrum represents the average of 3–5 independent measurements.

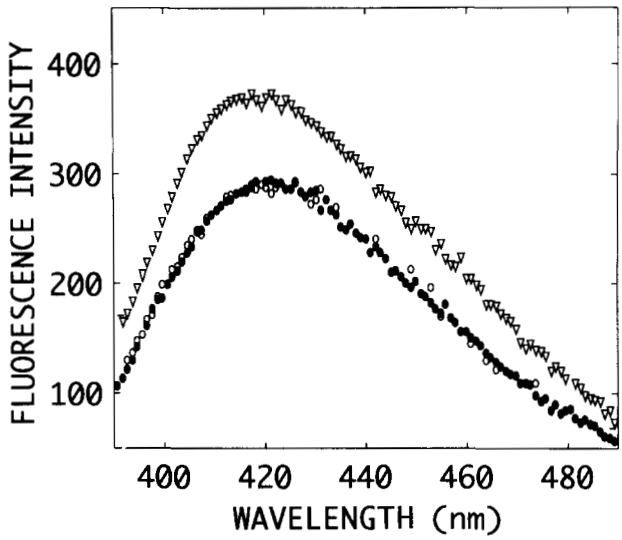

Fig. 4. Effect of ligand on the fluorescence emission spectrum of MIANS-labeled W33C permease. Complete modification of the single Cys residue was carried out at 4 °C as described in the Materials and methods, and the fluorescence of MIANS-modified permease was measured (excitation 330 nm), after 5 min incubation with and without ligand. ●, no addition; ○, 10 mM sucrose; ▽, 10 mM TDG. Each spectrum represents the average of 4–6 independent measurements.

Effect of substrate on MIANS-labeled W33C permease

In order to further investigate the properties of position 33, studies were carried out with single-Cys W33C[2] permease. When MIANS-labeled W33C permease is exposed to TDG, a high-affinity ligand, a 30% enhancement of MIANS fluorescence and a 5-nm blue shift in the emission maximum are observed (Fig. 4). Importantly, no effect whatsoever is observed in the presence of sucrose, which is not a substrate of the permease. When the effect of TDG is examined quantitatively (Fig. 5), a single exponential increase in fluorescence is observed over a range of TDG concentrations from 0 to 10 mM. Moreover, Scatchard analysis is consistent with a single binding site exhibiting an apparent K_D of 71 μM (Fig. 5, inset).

Effect of substrate on the reactivity of W33C permease with MIANS

MIANS is a sulfhydryl reagent that reacts specifically and covalently with thiol groups (Gupte & Lane, 1979), and it is not fluorescent until the maleimide group reacts (Haugland, 1994). The probe has been used extensively to study the reactivity of various single-Cys permease mutants (Wu & Kaback, 1994; Wu et al., 1995b). As shown in Figure 6, W33C permease reacts with MIANS, as evidenced by the linear increase in fluorescence for about 10 min after exposure to the probe. Dramatically, addition of TDG completely blocks reactivity when added during the reaction or prior to addition of MIANS (not shown), whereas sucrose has no effect whatsoever. Although not shown, it is important that 10 or 20 mM lactose decreases the rate of MIANS labeling by 50% or 100%, respectively. Clearly, the observations with W33C permease complement those made with single-Trp 33 permease, and the findings taken together indicate strongly that ligand binding causes position 33 to become less accessible from the aqueous phase.

Effect of substrate on acrylamide and iodide quenching of MIANS fluorescence

Table 1 summarizes the values obtained for acrylamide or I^- quenching of MIANS-labeled W33C fluorescence in the presence of either TDG or sucrose. Addition of TDG significantly decreases the quenching efficiency of iodide with no change in acrylamide quenching efficiency, whereas sucrose has no effect on quenching by either compound. This result confirms the notion that position 33 becomes occluded from the aqueous phase.

Discussion

Fluorescence quenching has been used widely to study the relative accessibility of fluorescent groups in membrane proteins (see Bigelow & Inesi, 1991; Jung et al., 1994c; Wu & Kaback, 1994; Wu et al., 1995a for examples). Ionic quenchers, such as iodide, provide information regarding the polarity of the milieu in the vicinity of the fluorophore, and nonpolar quenchers, such as acrylamide, provide information regarding steric resistance (Eftink et al., 1991). The data presented here are in agreement with the proposed location of native Trp residues in the lac permease with respect to their degree of exposure to the aqueous environment outside the bilayer, and provide the basis for further studies in which the environment of other positions can be examined. Reintroduction of each of the native Trp residues into Trp-less permease has no effect on active transport (data not shown). Therefore, it can be assumed that the conformation of the single-Trp mutants is virtually identical to wild-type. Two distinct categories of mutants are apparent from quenching studies on purified, detergent-solubilized protein: (1) mutants that display quenching only in the presence of acrylamide and no

Table 1. *Effect of substrate on acrylamide and iodide quenching of fluorescence of MIANS-labeled W33C permease*[a]

	% Quenching[b]	
Quencher	TDG	Sucrose
Iodide	69	97
Acrylamide	100	98

[a] Quenching of fluorescence of purified MIANS-labeled W33C permease either in the presence of 10 mM TDG or 10 mM sucrose by either 0.5 M iodide or 0.5 M acrylamide is shown as a percentage of the quenching observed in the absence of ligand. Each value corresponds to an average of 2–4 independent measurements.

[b] Percent of quenching by either TDG or sucrose, measured in KP_i buffer.

[2] Site-directed mutants are designated by the single-letter amino acid abbreviation for the targeted residue, followed by the sequence position of the residue in wild-type lac permease, followed by a second letter indicating the amino acid replacement.

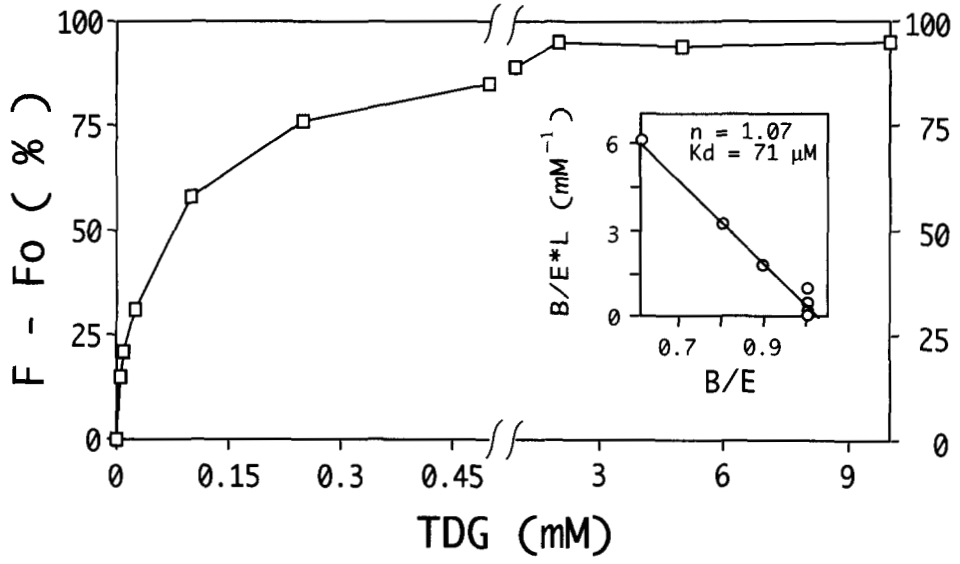

Fig. 5. Titration of MIANS-labeled W33C fluorescence with TDG. Purified W33C permease was labeled with MIANS as described in the Materials and methods and the fluorescence intensity of MIANS-labeled W33C permease (30 μg/mL) was measured at 415 nm in the absence and presence of TDG at given concentrations. The curve represents the average of two independent measurements. **Inset:** Scatchard plot of the data presented in the body of the figure. Data were analyzed as described in the Materials and methods.

quenching by I^- (or Cs^+); and (2) mutants that display similar quenching by both acrylamide and iodide. The first category corresponds to Trp residues that are not solvent-exposed, and the second category includes Trp residues exposed to the aqueous phase. Based on the proposed topology model of lac permease (Fig. 1), Trp 10 (near surface in helix I), Trp 33 (near surface in helix I), Trp 78 (near surface in helix III), and Trp 223 (near surface in helix VI) are predicted to fall in the second category,

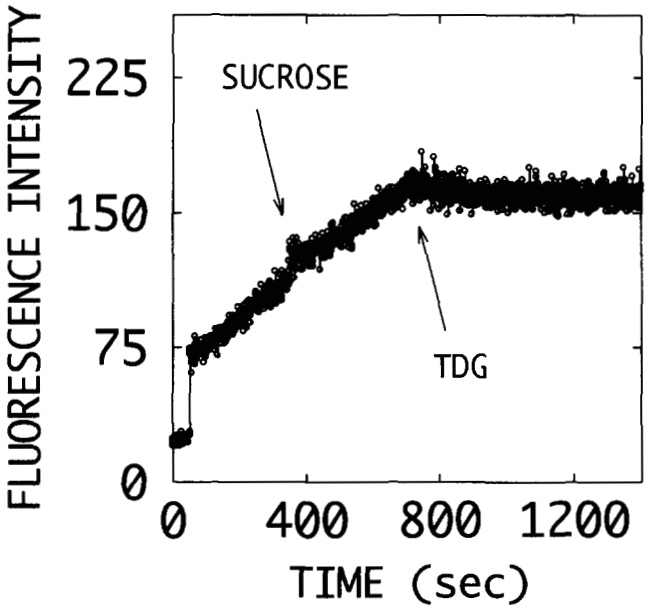

Fig. 6. Effect of ligand on the reaction of MIANS with purified W33C permease. MIANS labeling was carried out with 0.4 mL pure protein (30 μg/mL) and reactions were initiated by adding MIANS to a final concentration of 4 μM. Fluorescence was recorded continuously at 415 nm (excitation 330 nm) as described in the Materials and methods. Addition of 10 mM sucrose and 10 mM TDG is indicated.

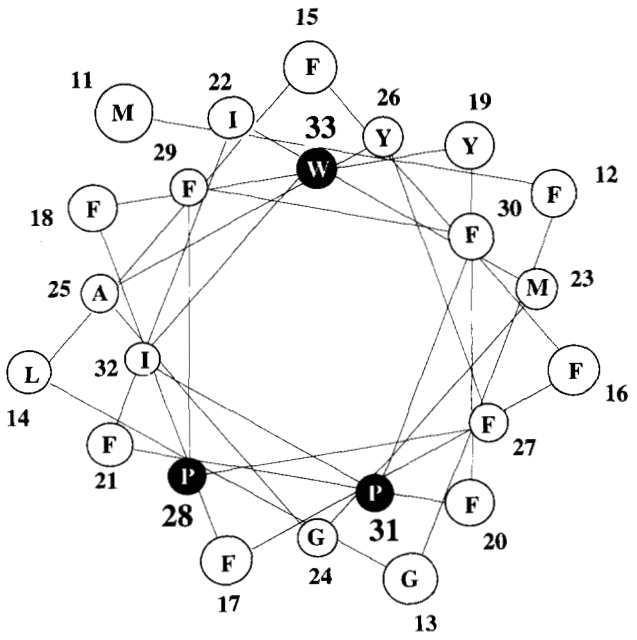

Fig. 7. Helical-wheel representation of residues in putative helix I of *E. coli* lac permease. Positions corresponding to single-Cys mutants that exhibit enhanced reactivity with MIANS in the presence of TDG (Wu et al., 1995a) and Trp 33 are highlighted.

and Trp 151 (middle of helix V) is predicted to fall in the first category. The data presented are consistent with the predictions for all the Trp residues but Trp 10 (Fig. 2). The inconsistency may be related to observations indicating that the N-terminus either does not protrude sufficiently from the membrane or is buried within the tertiary structure of the protein (Carrasco et al., 1982; Zen et al., 1995). Analysis of single-Trp fluorescence, as demonstrated here, is a powerful approach to monitoring conformational changes in lac permease induced by ligand binding. The following observations are consistent with previous results (Overath et al., 1987; Consler et al., 1991; Bibi et al., 1992; Sahin-Tóth et al., 1994b; Wu et al., 1995a) indicating that the periplasmic terminus of helix I of lac permease undergoes ligand-induced conformational change. (1) Thirty percent enhancement of single-Trp 33 fluorescence is observed in the presence of TDG, but not in the presence of sucrose (Fig. 3). (2) Binding of TDG but not sucrose causes a 30% enhancement of MIANS-labeled W33C permease fluorescence and a 5-nm blue shift in the emission maximum (Fig. 4). (3) Upon addition of saturating concentrations of TDG or lactose, but not of sucrose, the reactivity of W33C permease with MIANS is blocked (Fig. 6). (4) Iodide quenching of MIANS-labeled W33C permease fluorescence decreases by 30% in the presence of TDG and sucrose has no effect (Table 1). These changes are consistent with a hydrophobic shift in the polarity of the environment at position 33 (Lakowicz, 1983). When viewed on a helical wheel plot (Fig. 7), these results are in complete agreement with site-directed spectroscopic studies on single-Cys mutants F27C, P28C, F29C, F30C, and P31C in helix I, which indicate that the two faces of the C-terminus of this helix are exposed to different environments (Wu et al., 1995a). The face containing positions 27, 29, 30, and 33 is more accessible from the lipid bilayer, suggesting that it may be in direct contact with the hydrophobic phase of the membrane. In contrast, the face with positions 28 and 31 is less accessible from the bilayer, suggesting that it may interact with another part of the permease (Wu et al., 1995a). In addition, the reactivity of P28C and P31C with MIANS increases upon TDG binding (Wu et al., 1995a), indicating that ligand binding causes that face of helix I to become more accessible to the probe. Consistently, as shown here, position 33, which is on the opposite face of helix I from positions 28 and 31, becomes less accessible to MIANS (Fig. 6).

Scatchard analysis of TDG enhancement of MIANS-labeled W33C permease fluorescence reveals a single binding site with an apparent K_D of 71 μM, which coincides with the high-affinity site described previously (Lolkema & Walz, 1990; Lolkema et al., 1991; van Iwaarden et al., 1993; Wu et al., 1995b). Therefore, it appears that occupancy of a high-affinity site in lactose permease causes position 33 to move into a more hydrophobic environment that is less accessible to MIANS, and positions 28 and 31 to move into a more hydrophilic environment that is more accessible to the probe.

It has been postulated that Trp 33 could be directly or indirectly involved in sugar recognition, based on the finding that substitution of Trp 33 by Ser, Gln, Tyr, Ala, Gly, and Phe leads to higher rates of thiomethylgalactoside transport relative to lactose or melibiose (Huang et al., 1992).

Taken as a whole, the spectroscopic findings presented here and by Wu et al. (1995a) in conjunction with the observations that replacement of Trp 33 with Phe (Menezes et al., 1990) or Cys (Sahin-Tóth et al., 1994b) has essentially no effect on permease activity make it highly unlikely that this residue is directly involved in sugar recognition.

Materials and methods

Materials

Bacterial strains included T206 *lacI*$^+$*O*$^+$*Z*$^-$*Y*$^-$(*A*$^+$), *rpsL*, *met*, *thr*, *recA*, *hsdM*, *hsdR* [*F'*: *lacI*^{q}*O*$^+$*Z*^{D118}(*Y*$^+$*A*$^+$)] harboring plasmid pGM21 [*lacD*(*I*)*O*$^+$*P*$^+$*D*(*Z*)*Y*$^+$*D*(*A*)*tet*^{r}] (Teather et al., 1980); T184 [T206 cured of plasmid pGM21] (Teather et al., 1980).

All restriction endonucleases, T4 DNA ligase, and VentR DNA polymerase were purchased from New England Biolabs (Beverly, Massachusetts). Sequenase was purchased from United States Biochemicals (Cleveland, Ohio). Mutagenic deoxyoligonucleotide primers were synthesized on an Applied Biosystems DNA synthesizer and used without further purification. Kits for carrying out site-directed mutagenesis according to the methods of Kunkel (1985) were obtained from BioRad (Richmond, California). All other materials were reagent grade and obtained from commercial sources.

Methods

Site-directed mutagenesis

Single-Trp permeases W78 and W223 were engineered by ligation of wild-type lacY *Acc* I/*Bss*H II and *Xho* I/*Kpn* I fragments, respectively, with *Acc* I/*Bss*H II- and *Xho* I/*Kpn* I-digested Trp-less gene, respectively. Single-Trp permeases W10 and W33 were assembled from *Eco*R I/*Acc* I fragment of partial clones A and B into *Eco*R I/*Acc* I-digested Trp-less (Fig. 8). Single-Trp permeases W151 and W171 were assembled from *Bss*H II/*Xho* I fragment of partial clones C and D into *Bss*H II/*Xho* I-treated Trp-less. Partial clones A, B, C, and D were

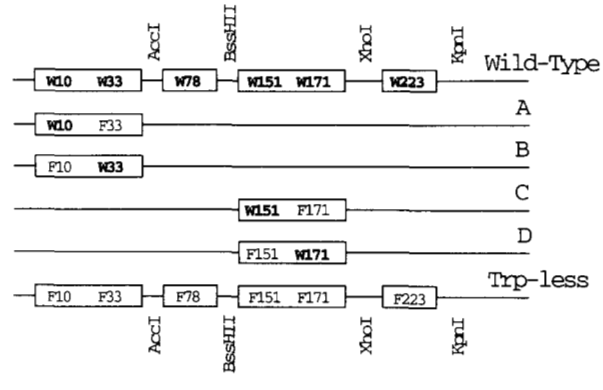

Fig. 8. Schematic representation of the *lacY* cassette genes used in the construction of single-Trp mutants. W78 and W223 result from ligation of the *Acc* I/*Bss*H II and *Xho* I/*Kpn* I fragments, respectively, from wild-type lacY, with the Trp-less gene digested with the corresponding restriction enzymes. W10 and W33 were assembled from the *Eco*R I/*Acc* I fragment from partial clones A and B into the corresponding fragments from Trp-less. W151 and W171 were assembled from the *Bss*H II/*Xho* I fragment from partial clones C and D into the corresponding fragments from Trp-less.

obtained from a single round of M13 mutagenesis as described (Kunkel, 1985), using the wild-type *lacY* cassette as template and six deoxyoligonucleotides encoding Phe in place of each of the native Trp codons. In order to facilitate purification, the DNA sequence encoding the biotin acceptor domain from the oxaloacetate decarboxylase of *Klebsiella pneumoniae* was cloned into the *Xho* I site of the DNA encoding the middle cytoplasmic loop of each single-Trp mutant (Consler et al., 1993). The single-Trp residue in the biotin acceptor domain was replaced with Phe by two-stage PCR with two complementary mutagenic oligodeoxynucleotides (Ho et al., 1989).

DNA sequencing

Double-stranded DNA sequencing after alkaline denaturation (Hattori & Sakaki, 1986) was performed using the dideoxy chain-termination method (Sanger et al., 1977).

Transport measurements

Active [^{14}C]lactose transport activity was determined in *E. coli* T184 harboring given plasmids as described (Consler et al., 1991).

Purification of mutant lac permeases

Each mutant permease with the biotin acceptor domain in the middle cytoplasmic loop was expressed in *E. coli* T184. One L of a dense culture grown in Luria-Bertini (LB) broth with streptomycin (10 μg/mL) and ampicillin (100 μg/mL) was diluted into 12 L and grown for 2 h at 37 °C in LB broth. The culture (OD_{600} of 0.8–1.0) was then induced with 0.2 mM isopropyl 1-thio-β,D-galactopyranoside and grown for another 4 h at 37 °C. A crude membrane fraction was prepared as described (Viitanen et al., 1984). Membrane proteins were solubilized with 2% DM by incubation at 30 °C for 30 min with continuous stirring. Solubilized biotinylated permease was purified by affinity chromatography on immobilized monovalent avidin (Consler et al., 1993) with modification as described (Wu & Kaback, 1994). Monovalent avidin sepharose beads were washed sequentially with 100 mM KP_i, pH 7.5, 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 7.5/150 mM NaCl/0.02% DM (w/v). The DM-soluble fraction was mixed with pre-equilibrated avidin resin for 20 min at 4 °C with continuous rotation. The slurry was then packed in a small column and the 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 kept at 4 °C for immediate use. The purity of each preparation was assessed by electrophoresis on 12% SDS gels (Newman et al., 1981), followed by silver staining.

Protein determinations

Protein concentrations were determined by the Bradford method (Bradford, 1976).

Fluorescence measurements

Trp fluorescence was measured at 30 °C with a SLM 8000C spectrofluorometer (SLM-Amico Instruments Inc., Urbana, Illinois). For Trp fluorescence, emission spectra were recorded using an excitation wavelength of 295 nm and 8- and 4-nm slits for excitation and emission, respectively. All steady-state emission spectra were corrected by subtracting the emission spectrum of column buffer containing identical amounts of dodecyl-β,D-maltoside as the pure permease solutions.

The effect of ligand on the fluorescence of single-Trp mutants was determined by mixing 400 μL of a given purified permease (20–40 μg/mL) with 20 μL of a 0.2-M stock solution of a given sugar in 0.1 M KP_i, pH 7.5. Emission spectra from 310 to 390 nm were recorded after 5 min incubation at 30 °C. Dilution effects were corrected by subtracting a spectrum obtained after addition of 0.1 M KP_i, pH 7.5.

The rate of MIANS reaction with purified W33C permease was determined by mixing 400 μL of the protein (40 μg/mL) with 2 μL of MIANS 1 mM stock solution (prepared in methanol using an extinction coefficient of 17,000 at 322 nm [Haugland, 1994]). Fluorescence emission was monitored continuously at 415 nm, with fixed excitation at 330 nm.

For complete modification of W33C permease, the reaction was carried out at 4 °C for 45 min with a 10-fold molar excess of MIANS over protein. Unreacted MIANS was quenched with dithiothreitol and removed by dialysis against three changes of column buffer. To titrate the effect of TDG on the fluorescence of MIANS-modified permease, labeled protein was incubated with a given concentration of ligand for 5 min at 30 °C followed by measurement of the emission spectra from 385 to 500 nm.

Ligand binding

The dissociation constant (K_D) and number of binding sites (n) in MIANS-labeled W33C permease were determined by Scatchard analysis as described previously (Wu et al., 1995b). The fluorescence of MIANS-labeled W33C permease (0.75 μM) was measured as described above, and bound TDG was calculated from the fluorescence increase measured at given TDG concentrations. It was assumed that at saturation one mol of TDG is bound per mol of permease (Lolkema & Walz, 1990).

Fluorescence quenching

Fluorescence quenching studies were carried out with freshly prepared solutions of 5 M potassium iodide and 5 M acrylamide in 0.1 M KP_i, pH 7.5. The quenchers were added to the reaction mixtures containing single-Trp permease or MIANS-labeled W33C permease to a final concentration of 0.5 M. Fluorescence (20–40 μg/mL of permease) was measured as described above. To correct for dilution and ionic strength effects, control assays were carried out with addition of potassium chloride at the same concentration.

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