# Ligand-Induced Movement of Helix X in the Lactose Permease from *Escherichia coli:* A Fluorescence Quenching Study

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ABSTRACT: Five single-Trp mutants were constructed by replacing Val315, Leu318, Val326, Leu329, or Val331 with Trp in transmembrane helix X of a functional lactose permease mutant devoid of Trp residues (Trp-less permease). Taking into account expression levels, each single-Trp permease except for Val331→Trp exhibits significant activity. The intrinsic fluorescence emission of each single-Trp mutant does not change significantly after addition of β-D-galactopyranosyl 1-thio-β-D-galactopyranoside (TDG), indicating that ligand induces little change in the microenvironment of the Trp residues. However, fluorescence quenching studies with the brominated detergent 7,8-dibromododecyl β,D-maltoside (BrDM) demonstrate that a Trp residue in place of Val315, Val326, or Val331 becomes less accessible to BrDM in the presence of TDG, while a Trp residue in place of Leu318 or Leu329 becomes more accessible. Acrylamide quenching studies with Leu318→Trp and Val331→Trp permeases or 2-(4-maleimidoanilino)-naphthalene-6-sulfonic acid (MIANS)-labeled Thr320→Cys and Glu325→Cys permeases indicate that positions 318 and 325 also become more accessible to a hydrophobic environment in the presence of TDG, while positions 320 and 331 become less accessible. The findings are consistent with a recently proposed mechanism for energy coupling in lactose permease [Kaback, H. R. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 5539−5543] in which substrate binding causes a conformational change resulting in movement of Glu325 to a nonpolar environment with a dramatic increase in p$K_a$.

The lactose permease (lac permease)[1] of *Escherichia coli* is a polytopic cytoplasmic membrane protein that catalyzes the coupled stoichiometric translocation of β-galactosides and $H^+$ (i.e., substrate/$H^+$ 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 as a monomer. All available evidence indicates that the permease is composed of 12 α-helix rods that traverse the membrane with the N and C termini on the cytoplasmic face (Figure 1) (*1*−*8*). Moreover, a variety of site-directed biochemical and biophysical techniques have led to a model describing helix packing in the permease (*9*).

Extensive site-directed mutagenesis with wild-type permease and Cys-scanning mutagenesis of a functional mutant devoid of Cys residues (C-less permease) indicate that as few as 4 out of over 400 residues are irreplaceable with respect to coupling substrate and $H^+$ translocation, and the residues are paired—Glu269 (helix VIII) with His322 (helix X) and Arg302 (helix IX) with Glu325 (helix X) (see *10*). Moreover, differences in the properties of the mutants indicate that Glu325 may be the only residue in the permease that is directly involved in $H^+$ translocation.

Based on helix packing (Figure 8), the interactions between the four essential residues, their proximity to the substrate translocation pathway, and the properties of site-directed mutants, a mechanism that explains the coupled translocation of substrate and $H^+$ by the permease has been proposed (*10*). Interactions between the helices are such that a ligand-induced conformational change at the interface between helices V and VIII is transmitted to the interface between helices IX and X and vice-versa. Upon ligand binding, a structural change at the interface between helices V and VIII disrupts the interaction between His322 and Glu269, Glu269 displaces Glu325 from Arg302, and Glu325 is protonated. Simultaneously, helix X is postulated to move so as to bring protonated Glu325 into contact with the hydrophobic phase of the membrane, thereby markedly increasing the p$K_a$ of the carboxylic acid. Upon return to ground state after release of substrate, the Arg302−Glu325 interaction is reestablished with release of $H^+$ from Glu325 into a water-filled crevice between helices IX and X which becomes transiently accessible to both sides of the membrane due to a change in helix tilt. One of the central ideas of the scheme is that protonated Glu325 becomes inaccessible to water due to ligand-induced movement of helix X.

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[1] Abbreviations: lac permease, lactose permease; IPTG, isopropyl 1-thio-β-D-galactopyranoside; $KP_i$, potassium phosphate; TDG, β-D-galactopyranosyl 1-thio-β-D-galactopyranoside; PCR, polymerase chain reaction; Tris, tris(hydroxymethyl)aminomethane; DM, *n*-dodecyl β,D-maltoside; BrDM, 7,8-dibromododecyl β,D-maltoside; NEM, *N*-ethylmaleimide; MIANS, 2-(4-maleimidoanilino)naphthalene-6-sulfonic acid.

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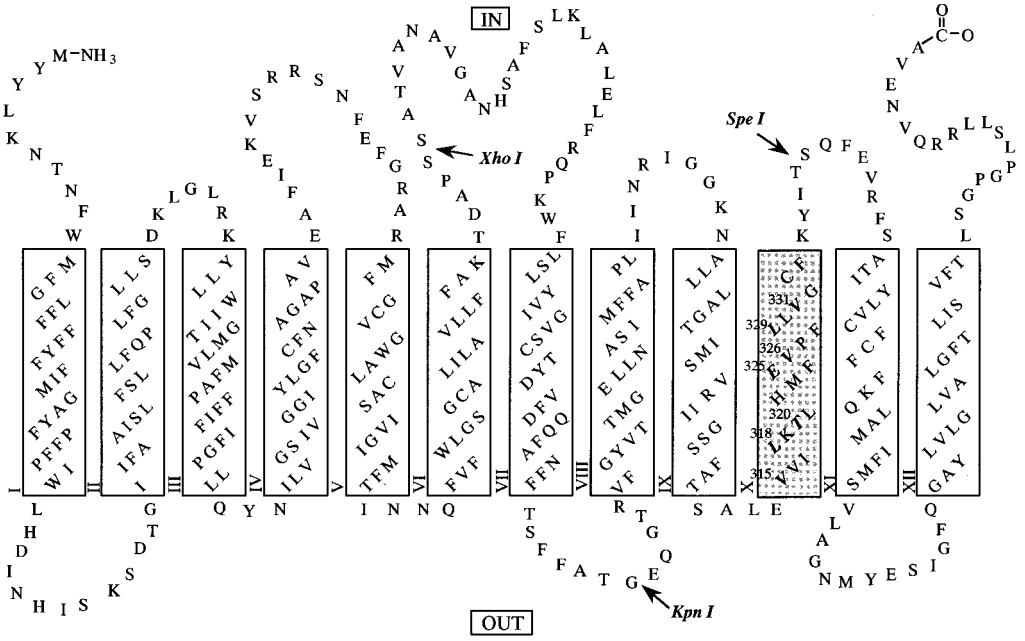


FIGURE 1: Secondary structure model of lac permease. The single-letter amino acid code is used, and transmembrane domains are shown in boxes. Single-Trp or Cys replacements in helix X (shaded area) are numbered and in italics. Although not shown, the single-Trp mutants are in a Trp-less background and the single-Cys mutants are in a Cys-less background. Also indicated are the restriction endonuclease sites used for construction of the mutants.

Wild-type permease contains six Trp residues, none of which is important for activity (*11*). In this report, five single-Trp mutants were constructed by introducing individual Trp residues into transmembrane helix X of a functional lactose permease mutant devoid of Trp residues (Trp-less permease; *11*). In addition, two single-Cys mutants were constructed in helix X of Cys-less permease (*12*), and the mutants were labeled with 2-(4-maleimidoanilino)-naphthalene-6-sulfonic acid (MIANS). Fluorescence quenching studies with the purified mutant permeases indicate that the face of helix X with Glu325 becomes more exposed to the hydrophobic core of the bilayer when ligand is bound, while the opposite face becomes less exposed. The results are consistent with the mechanism proposed (*10*) for energy coupling in lac permease.

## EXPERIMENTAL PROCEDURES

*Materials.* [1-$^{14}$C]Lactose and [$\alpha$-$^{35}$S]dATP were purchased from Amersham. Deoxyoligonucleotides were synthesized on an Applied Biosystems 391 DNA synthesizer. Site-directed rabbit polyclonal antiserum against a dodecapeptide corresponding to the C-terminus of lac permease (*13*) was prepared by Babco. Restriction endonuclease and T4 DNA ligase were from New England Biolabs. Taq DNA polymerase was from Promega. Sequenase was from United States Biochemicals. $\beta$,D-Galactopyranosyl 1-thio-$\beta$,D-galactopyranoside (TDG) was from Sigma. 7,8-Dibromodecyl $\beta$,D-maltoside (BrDM) was synthesized according to de Foresta et al. (*14*). MIANS was from Molecular Probes. All other materials were reagent grade obtained from commercial sources.

*Bacterial Strains. E. coli* XL1-Blue [*recA1endA1gyrA96thi-1hsdR17supE44relA1lac [F'proABlacI$^q$ZΔM15Tn10 (Tet$^r$)]*] (*15*) was used as carrier for the plasmids described. *E. coli* T184 [*lacI$^+$O$^+$Z$^-$Y$^-$(A)rpsLmet$^-$thr$^-$recAhsdM hsdR/F'lacI$^q$O$^+$Z$^{D118}$(Y$^+$A$^+$)*] (*16*) harboring plasmid pT7-5/cassette *lacY* with given mutations was used for expression of lac permease and transport studies. A cassette *lacY* gene devoid of Trp codons (*11*) under control of the *lacZ* promoter/operator was used for site-directed mutagenesis. 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 (*17*). The single-Trp residue in the biotin acceptor domain was replaced by Phe by two-stage PCR with two complementary mutagenic oligodeoxynucleotides (*18*).

*Oligonucleotide-Directed Site-Specific Mutagenesis.* Trp-replacement mutants were constructed by two-stage PCR (PCR overlap−extension; *18*) using pT7-5/cassette *lacY* encoding Trp-less permease (*11*) as template. Sequences of the synthetic mutagenic primers used are given in Table 1. The PCR products were digested with *Kpn*I and *Spe*I restriction endonucleases (see Figure 1 for location of sites) and ligated to a similarly treated pT7-5/*lacY* encoding Trp-less permease. Single-Cys mutants (T320C and E325C) were constructed using a functional lac permease mutant devoid of Cys residues (*19*).

*DNA Sequencing.* Double-stranded DNA sequencing after alkaline denaturation (*20*) was performed using dideoxy chain-termination (*21*).

*Transport Measurements.* Cells were washed with 100 mM potassium phosphate ($KP_i$, pH 7.5)/10 mM $MgSO_4$ and adjusted to an $OD_{420}$ of 10.0 (approximately 0.7 mg of protein/mL). Transport of [$^{14}$C]lactose (2.5 mCi/mmol; final concentration 0.4 mM) was assayed by rapid filtration (*22*).

*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 liter of a dense culture grown in Luria−Bertini (LB) broth with streptomycin (10 $\mu$g/mL) and ampicillin (100 $\mu$g/mL) was diluted into 12 L of LB broth and grown for 2 h at 37 °C. The culture ($OD_{600}$ 0.8−1.0) was then induced with 0.2 mM isopropyl thio-$\beta$,D-galactopyranoside (IPTG) and grown for another 1−3 h at 37 °C. A crude membrane fraction was prepared as described (*23*). Membranes were solubilized

Table 1: DNA Sequence Analysis of Trp-Replacement Mutants in the Trp-less Cassette *lacY* Gene

| mutant | mutagenic oligonucleotides[a] | codon change |
|---|---|---|
| V315W sense | GCCACCTCAGCGCTGGAA**TGG**GTTATTCTGAAAACGCTGC | GTG→TGG |
| antisense | GCAGCGTTTTCAGAATAAC**CCA**TTCCAGCGCTGAGGTGGC | |
| L318W sense | GTGGTTATT**TGG**AAAACGCTGCATATGTTTGAAGTACCG | CTG→TGG |
| antisense | CGGTACTTCAAACATATGCAGCGTTTT**CCA**AATAACCAC | |
| V326W sense | ATGTTTGAA**TGG**CCGTTCCTG | GTA→TGG |
| antisense | CAGGAACGG**CCA**TTCAAACAT | |
| L329W sense | CGCTGCATATGTTTGAAGTACCGTTC**TGG**CTGGTGGGC | CTG→TGG |
| antisense | GCCCACCAG**CCA**GAACGGTACTTCAAACATATGCAGCG | |
| V331W sense | GAAGTACCGTTCCTGCTG**TGG**GGCTGC | GTG→TGG |
| antisense | GCAGCC**CCA**CAGCAGGAACGGTACTTC | |

[a] Sequences of mutagenic primers are presented in the 5′→3′ order with altered codons in boldface type.

with 2% DM (w/v) by incubating at 25 °C for 20 min with continuous stirring. Solubilized biotinylated permease was purified by affinity chromatography on immobilized monovalent avidin (*17*) with modifications (*24*). Monovalent avidin−Sepharose beads were washed sequentially with 100 mM $KP_i$ (pH 7.5)/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 preequilibrated avidin resin for 30 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 column buffer and concentrated with Micro-ProDiCon Membranes (Spectrum Medical Industries).

MIANS labeling of T320C and E325C was carried out before elution from the resin. MIANS (2.0 mM, final concentration) was mixed with the slurry described above for 15 min at 25 °C and then 30 min at 4 °C with rotation. Unreacted MIANS was removed by washing thoroughly with column buffer, and the labeled permease was eluted with *d*-biotin as described. The permease was kept at 4 °C for immediate use.

The purity of each preparation was assessed by electrophoresis on sodium dodecyl sulfate−12% polyacrylamide gels (*25*), followed by silver staining. Protein was determined as described (*26*). The quantity of MIANS covalently bound to T320C or E325C permease was determined by measuring MIANS absorption and determining the concentration by assuming an extinction coefficient of $1.4 \times 10^4$ $M^{-1}\cdot cm^{-1}$ at 313 nm. The efficiency of MIANS labeling was determined to be 80−90%.

*Immunological Analysis.* Membrane fractions were subjected to $NaDodSO_4$/PAGE; proteins were electroblotted to poly(vinylidene difluoride) membranes (Immobilon-PVDF; Millipore) and probed with site-directed polyclonal antibody against the C-terminus of lac permease (*13*).

*DM Determination.* DM concentrations were assayed by using a colorimetric method. One milliliter samples were mixed with 50 $\mu$L of phenol, and 2.5 mL of concentrated $H_2SO_4$ was added. The absorbance at 490 nm was measured after cooling the sample. A standard curve was constructed to calculate DM concentrations in given samples.

*Fluorescence Emission Spectra.* The fluorescence of single-Trp permeases or MIANS-labeled single-Cys permeases was measured at 25 °C with an SLM 8000C spectrofluorometer (SLM-Aminco Instruments Inc.). For single-Trp permeases, an excitation wavelength of 295 nm was used to avoid interference from Tyr residues; for MIANS-labeled single-Cys mutants, an excitation wavelength of 330 nm was used. Slits for excitation and emission were 16 nm and 4 nm (for single-Trp permeases) or 4 nm and 2 nm (for MIANS-labeled permeases), respectively. All steady-state emission spectra were corrected by subtracting the emission spectrum of column buffer without protein containing identical amounts of DM.

The effect of TDG on the fluorescence was determined by mixing 400 $\mu$L of a given purified permease preparation (30 $\mu$g of protein/mL) with 8 $\mu$L of a 0.5 M stock solution of TDG in 0.1 M $KP_i$ (pH 7.5). Emission spectra from 310 nm to 390 nm (for Trp fluorescence) or from 390 nm to 440 nm (for MIANS fluorescence) were recorded after 3 min incubation at 25 °C. The results were corrected for dilution effects.

*Fluorescence Quenching Measurements.* Fluorescence quenching studies were carried out with stock solutions of BrDM or acrylamide. The quencher was added to a given single-Trp or MIANS-labeled permease (30 $\mu$g of protein/mL) in 0.06% DM, in the absence or presence of 10 mM TDG or sucrose, as indicated. Fluorescence was measured as described and corrected by subtracting the emission spectrum of column buffer containing identical amounts of DM, quencher, and sugar but no protein. Effects of dilution were also corrected. The emission intensity at 333 nm (for Trp fluorescence) or 420 nm (for MIANS fluorescence) was measured, and fluorescence quenching data were analyzed according to the Stern−Volmer equation:

$$F_0/F = 1 + K_{sv}[Q] = 1 + k_q\tau_0[Q]$$

where $F_0$ and $F$ are fluorescence intensities in the absence and presence of quencher, respectively, $K_{sv}$ is the Stern−Volmer constant for collisional quenching, [Q] is the concentration of the quencher, $k_q$ is the rate constant for the quenching reaction, and $\tau_0$ is the lifetime of the fluorophore in the absence of quencher. The equation predicts a linear plot of $F_0/F$ versus [Q] for a homogeneous solution (*27*).

## RESULTS

*Active Transport and Expression of Mutants. E. coli* T184 ($lacZ^-Y^+$) permease mutants L318W, V326W, or L329W transport lactose at about 20% the rate of wild type to steady-state levels of 35−70% of wild type (Figure 2). The other two mutants (V315W or V331W) exhibit low transport rates (5−10% of wild type), and mutant V315W accumulates lactose to a level of about 13% of wild type, while mutant V331W does not accumulate the dissacharide to a significant extent. As shown previously, T320C permease catalyzes active lactose transport effectively (*19*), while E325C per-

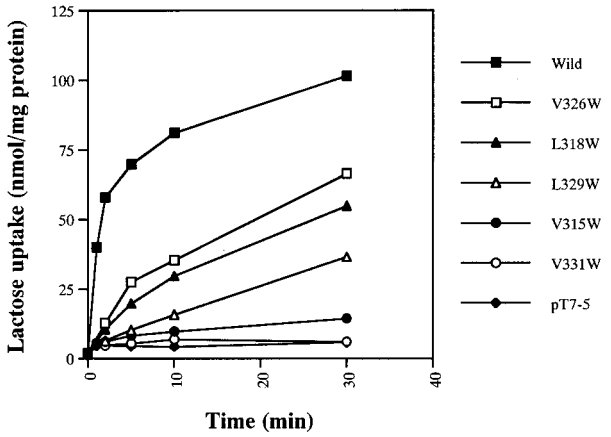


FIGURE 2: Active lactose transport by single-Trp mutants. *E. coli* T184 transformed with plasmid pT7-5/cassette *lacY* (Trp-less), pT7-5 (vector with no *lacY* gene), or pT7-5/cassette *lacY* encoding given Trp-replacement mutants in Trp-less were grown at 37 °C, and aliquots of cell suspensions (50 $\mu$L containing approximately 35 $\mu$g of protein) in 100 mM $KP_i$ (pH 7.5)/10 mM $MgSO_4$ were assayed as described under Experimental Procedures.

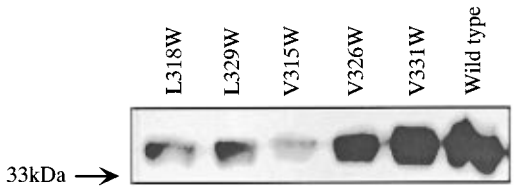


FIGURE 3: Western blots of membranes containing Trp-less lac permease or single-Trp mutants. *E. coli* transformed with pT7-5/cassette *lacY* (Trp-less) or pT7-5/cassette *lacY* encoding Trp-less with given Trp replacements were grown and induced as described under Experimental Procedures. Membranes were prepared, and samples containing approximately 50 $\mu$g of protein per sample were subject to $NaDodSO_4$/PAGE and electroblotting. The blot was incubated with antibody directed against the C-terminal dodecapeptide of lac permease, followed by horseradish peroxidase-linked protein A, and fluorescent substrate before exposure to film.

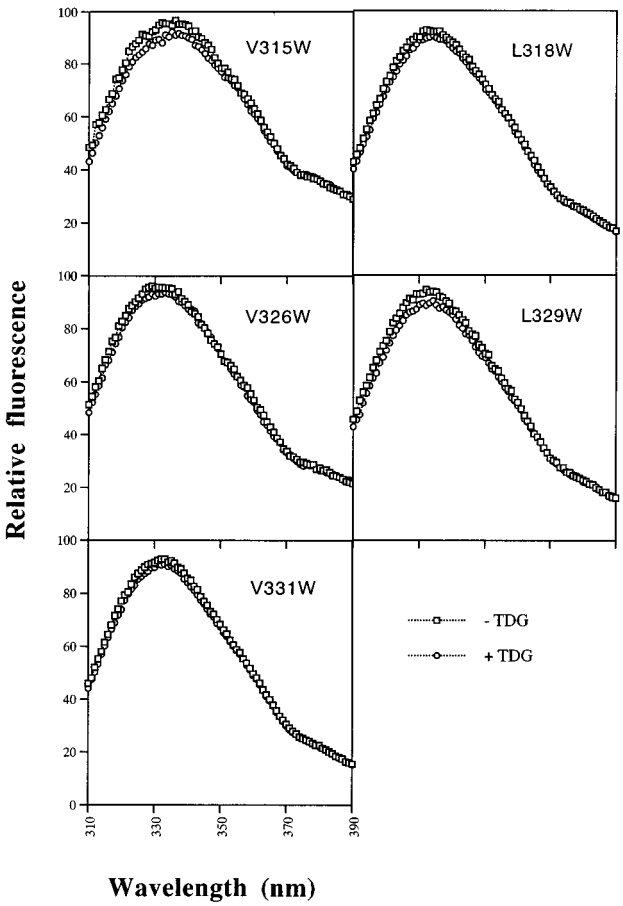


FIGURE 4: Effect of TDG on the fluorescence of single-Trp mutants. The fluorescence emission spectrum (excitation at 295 nm) of given purified single-Trp permeases (30 $\mu$g of protein/mL) was recorded as described under Experimental Procedures after 3 min incubation in the absence or presence of 10 mM TDG, as indicated.

mease is specifically defective in all translocation reactions that involve $H^+$ translocation, but catalyzes exchange and counterflow as well or better than wild-type permease (*19*, *28*).

Western blots of membrane fractions expressing the Trp-replacement mutants (Figure 3) demonstrate that mutant V326W or V331W is present in the membrane at a level comparable to wild type, while mutant L318W or L329W is expressed at a reduced but significant level. V315W permease is expressed at the lowest level which is consistent with the low activity of this mutant (Figure 2). Thus, with the exception of mutant V331W, the alterations in activity of the other mutants correlate generally with the amount of permease present in the membrane.

*Fluorescence Emission Spectra.* Corrected steady-state emission spectra of each single-Trp permease in DM in the absence or presence of TDG are shown in Figure 4. Each mutant exhibits a similar spectrum in the absence or presence of sugar, indicating that the environment of the Trp residues remains essentially unchanged upon addition of TDG. The slight decrease in fluorescence after addition of TDG may be due to an osmotic effect, as similar changes are observed with sucrose. It is noteworthy that the emission maximum of V326W permease is the most blue-shifted of the five mutants (V315W, 332 nm; L318W, 332 nm; V326W, 331 nm; L329W, 333 nm; V331W, 333 nm), which is consistent with the localization of position 326 in the approximate middle of helix X. Fluorescence emission spectra of MIANS-labeled T320C and MIANS-labeled E325C permeases also exhibit no significant changes in the absence or presence of TDG, indicating that the microenvironment of MIANS at these positions experiences little change upon ligand binding (Figure 5).

*Quenching of Single-Trp Permease Fluorescence by BrDM.* Figure 6 shows Stern−Volmer plots of fluorescence quenching of the five single-Trp permeases by BrDM in the absence or presence of TDG or sucrose. When TDG is added, Trp residues at positions 315, 326, and 331 become less accessible to BrDM (i.e., decreased quenching is observed), while those at positions 318 and 329 become more accessible (i.e., increased quenching is observed). In contrast, addition of sucrose which is not a substrate of lac permease has no significant effect. Because there is an equilibrium between the aqueous and micellar phases with respect to BrDM, the local concentration of BrDM in the micelles (i.e., the true quencher concentration) is expected to differ from the overall concentration of BrDM in solution, and the mechanism of quenching could be partially static in addition to collisional which may explain the downward curvature of the Stern−Volmer plots. In addition, the differences in downward curvature observed for the mutants may reflect the position of a given Trp residue in helix X relative to the bromine moiety in BrDM.

*Acrylamide Quenching of L318W, V331W, MIANS-Labeled T320C, or MIANS-Labeled E325C Permeases.* Stern−Volmer plots of fluorescence quenching of L318W,

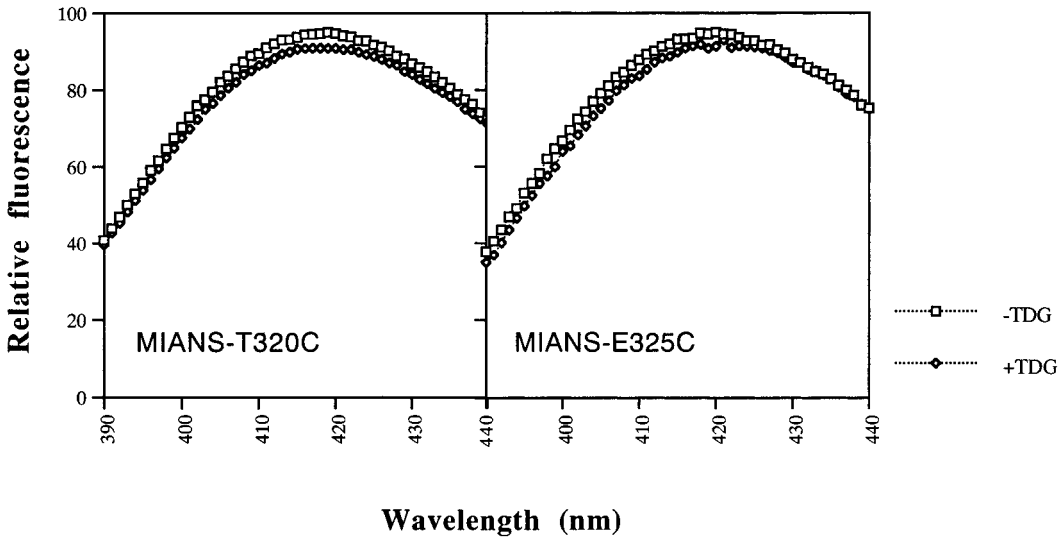


FIGURE 5: Effect of TDG on the fluorescence of MIANS-labeled single-Cys mutants. The fluorescence emission spectrum (excitation at 330 nm) of MIANS-labeled T320C or MIANS-labeled E325C permease (30 $\mu$g of protein/mL) was recorded as described under Experimental Procedures after 3 min incubation in the absence and presence of 10 mM TDG, as indicated.

V331W, MIANS-labeled T320C, and MIANS-labeled E325C permeases by acrylamide are shown in Figure 7, and the calculated constants are given in Table 2. With acrylamide as a collisional quencher, the Stern−Volmer plots are linear, as expected (see *29*). More importantly, however, in the presence of TDG, fluorophore at positions 318 and 325 is quenched more effectively, while that at positions 320 and 331 is quenched less effectively. In contrast, sucrose has no significant effect.

## DISCUSSION

Fluorescence quenching is used widely to study the relative accessibility of fluorescent groups in membrane proteins (*30*, *31*). In this regard, brominated detergents, such as BrDM, should provide powerful tools to determine the environment of transmembrane helices containing fluorophores, as well as the dynamics of the reporter groups (*14*). With respect to the general use of collisional quenchers with membrane proteins, BrDM differs from acrylamide or ionic quenchers ($I^-$, $Cs^+$, etc.) in that most of the BrDM molecules are in micellar form with DM and the solubilized protein (in this case, lac permease) which restricts the movement of BrDM and places the quencher in close contact with the protein. Furthermore, a number of studies (see *1*) demonstrate that lac permease maintains close to native conformation in DM.

As postulated recently (*10*), Glu325 (helix X) may be the only functional group in the permease that is directly involved in $H^+$ translocation. In the ground state, Glu325 interacts electrostatically with Arg302 (helix IX), and in this configuration, the p$K_a$ of the carboxylate is infinitely low. Upon binding of substrate at the interface between helices V and VIII, a conformational change occurs that disrupts the interaction between His 322 (helix X) and Glu269 (helix VIII), and Glu269 displaces Glu325 from Arg302. As a result, Glu325 is protonated and becomes inaccessible to solvent due to movement of helix X, thereby increasing the p$K_a$ of the carboxylic acid dramatically. Subsequently, when substrate is released, the protein returns to ground state with loss of $H^+$ from Glu325, an event that results from reestablishing the Arg302−Glu325 interaction. In brief, therefore, in one conformation, Glu325 interacts with Arg302 as an unprotonated carboxylate, and in another conformation, Glu325 is embedded in the nonpolar hydrophobic phase of the membrane as a carboxylic acid with an extremely high p$K_a$ (see *32*). In an effort to test the postulate that ligand binding induces such a change in helix X, five single-Trp and two single-Cys replacement mutants were constructed in helix X, in functional mutants devoid of Trp or Cys, respectively.

The five single-Trp mutants vary in transport activity, from essentially none (V331W) to relatively high activity (Figure 2). When expression levels are taken into account (Figure 3), it appears that four of the five single-Trp mutants retain reasonable activity. In contrast, Cys replacement at the same positions (i.e., V315C, L318C, V326C, L329C, and V331C) yields permease with high activity and normal expression levels (*19*). Therefore, none of the five native residues plays a direct role in the transport mechanism, and the effects of the Trp replacements on activity relative to Cys are probably related to the bulk of the respective side chains. In a similar vein, Thr320 can be replaced with Cys with little or no effect on activity or expression (*19*), while E325C permease is specifically defective in all reactions that involve net $H^+$ translocation, but catalyzes exchange and counterflow at least as well as wild-type permease (*28*, *33*).

Since little change in the fluorescence emission maxima of the five single-Trp mutants or the two MIANS-labeled single-Cys mutants is observed in the presence of TDG, ligand binding does not induce a gross change in the microenvironment of helix X. However, as revealed by BrDM quenching studies, upon addition of TDG, Trp residues at positions 315, 326, or 331 in the permease become less accessible to BrDM, while Trp residues at positions 318 or 329 become more accessible (Figure 6). Acrylamide, a neutral collisional quencher, which has accessibility to indoles within detergent micelles (*34*) was also used. Although acrylamide quenching was not studied with each mutant, results similar to those observed with BrDM are observed with L318W and V331W permeases [i.e., Trp at position 318 becomes more accessible to acrylamide in the presence of TDG, while Trp at position 331 becomes less accessible (Figure 7)]. Moreover, acrylamide quenching studies with MIANS-labeled T320C permease indicate that position 320 becomes less accessible to the quencher in the

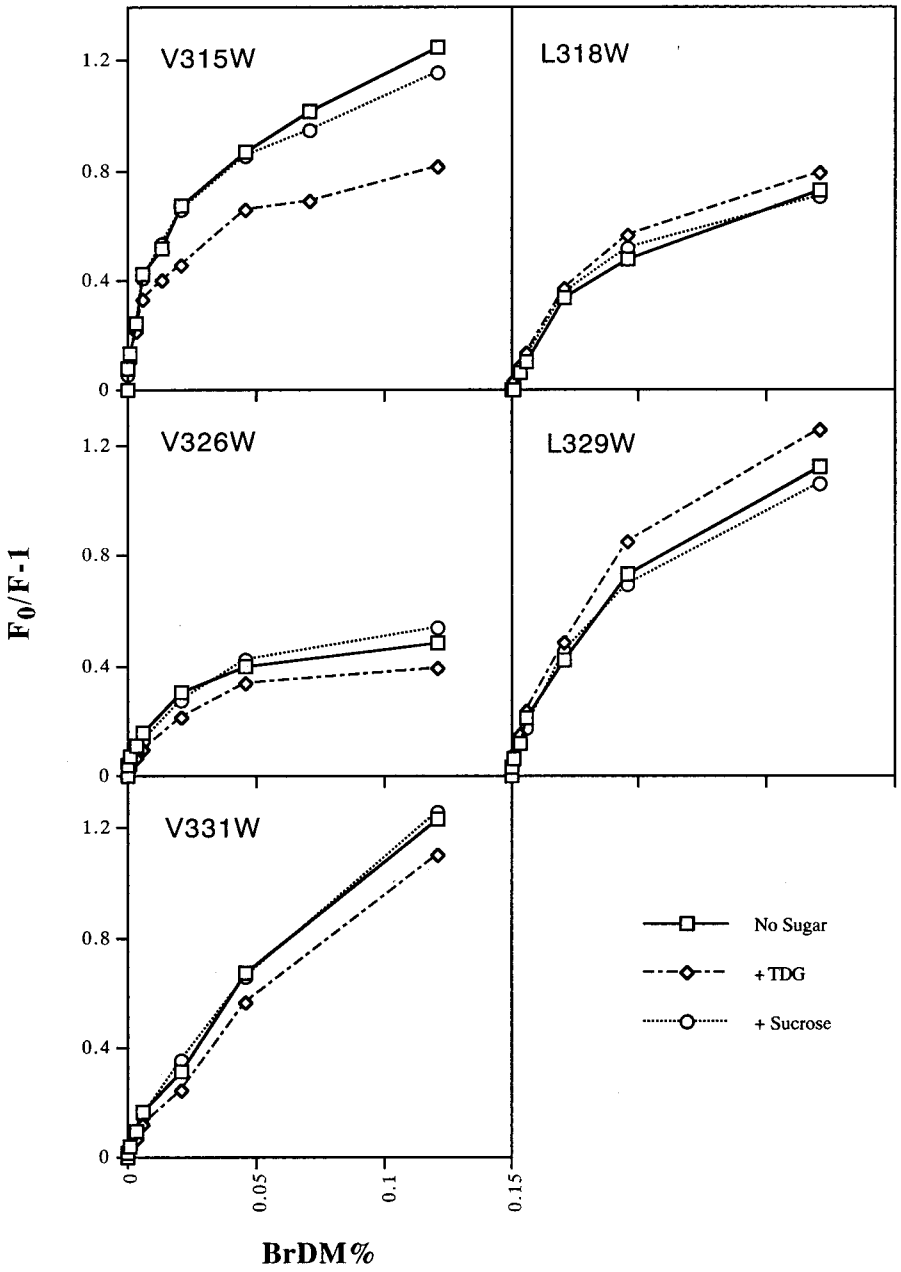


FIGURE 6: Quenching of the fluorescence of single-Trp mutants by BrDM. Samples (30 $\mu$g of protein/mL) of purified given single-Trp permeases in 0.06% DM were incubated in the absence or presence of 10 mM TDG or 10 mM sucrose, as indicated. BrDM was then added from a concentrated stock solution to a given final concentration. Fluorescence was measured at an excitation wavelength of 295 nm and an emission wavelength of 333 nm, and Stern−Volmer plots were constructed as described under Experimental Procedures.

presence of ligand. It is also highly noteworthy that previous studies with V315C (*19*, *35*, *36*) or V331C permease (*37*) demonstrate that ligand binding induces marked changes in the reactivity and/or the fluorescent properties of both mutants, observations which support the argument that ligand binding induces a conformational change in helix X. Taken as a whole, the findings are consistent with the notion that ligand binding induces clockwise rotation of helix X such that the face with positions 318 and 329 comes into closer contact with the hydrophobic phase of the membrane, while positions 315, 326, or 331 move toward the interior of the protein (Figure 8). In apparent contradiction, MIANS-labeled single-Cys325 permease exhibits only a small increase in accessibility to acrylamide in the presence of TDG (E325W permease cannot be expressed in quantities sufficient for purification). However, given the mechanism of coupling proposed (*10*), it is possible that position 325 in the E325C mutant is already in contact with the hydrophobic phase of the membrane prior to addition of ligand. That is, when Glu325 is replaced with Cys, Arg302 may displace Glu269 from His322 so that E325C permease assumes the same conformation as wild-type permease in the presence

Table 2: Stern−Volmer Constants for Acrylamide Quenching of L318W and V331W Permeases or MIANS-Labeled T320C and E325C Permeases in the Absence or Presence of TDG or Sucrose[a]

| | $K_{sv}$ ($M^{-1}$) | | |
|---|---|---|---|
| mutant | no sugar | +TDG | +sucrose |
| L318W | 2.70 | 3.07 | 2.73 |
| V331W | 2.80 | 2.04 | 2.93 |
| MIANS-T320C | 2.62 | 2.14 | 2.49 |
| MIANS-E325C | 2.17 | 2.37 | 2.14 |

[a] Experiments were carried out as described under Experimental Procedures. $K_{sv}$ is the Stern−Volmer quenching constant determined from the slopes of the lines for the plots of $F_0/F - 1$ versus [Q]; slopes were determined by linear regression analysis of the fluorescence quenching data using the least-squares method.

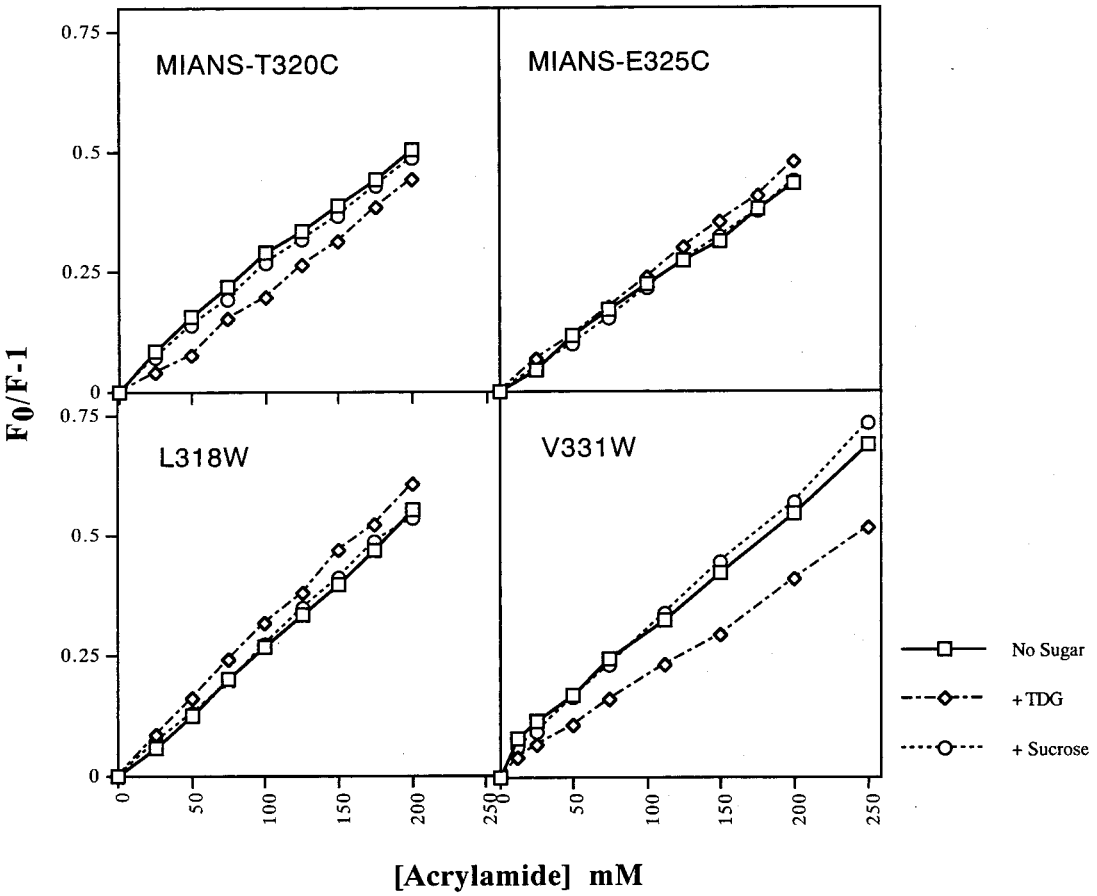


FIGURE 7: Acrylamide quenching of the Trp fluorescence of L318W and V331W permeases or the MIANS fluorescence of MIANS-labeled T320C and E325C permeases. Samples (30 $\mu$g of protein/mL) of a given purified permease in 0.06% DM were incubated in the absence or presence of 10 mM TDG or 10 mM sucrose, as indicated. Acrylamide was then added from a concentrated stock solution to a given final concentration. Fluorescence was measured at an excitation wavelength of 295 nm (for Trp fluorescence) or 330 nm (for MIANS fluorescence) and an emission wavelength of 333 nm (for Trp fluorescence) or 420 nm (for MIANS fluorescence), and Stern−Volmer plots were constructed as described under Experimental Procedures.

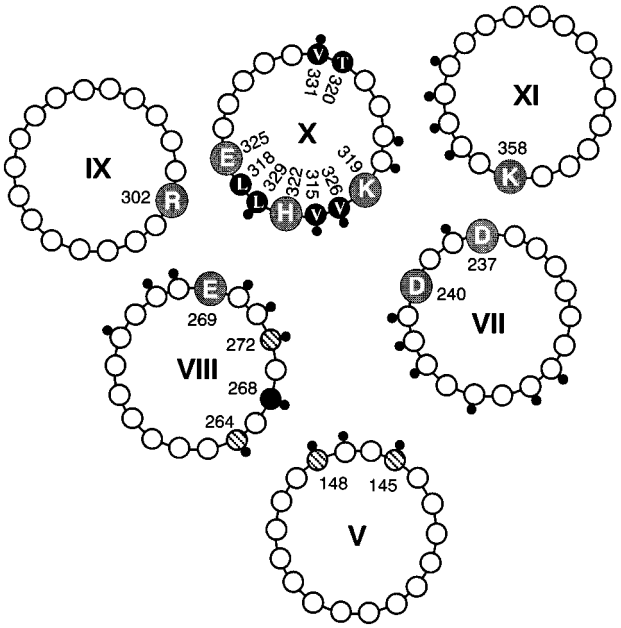


FIGURE 8: Packing of helices V and VII−XI in the lac permease viewed from the periplasmic surface. The four essential residues (Glu269, Arg302, His322, and Glu325) and two interacting pairs of Asp−Lys residues [Asp237 (helix VII)/Lys358 (helix XI) and Asp240 (helix VII)/Lys319 (helix X)] are highlighted. Positions of *N*-ethylmaleimide-sensitive Cys replacements are indicated with a small black dot. Substrate-protectable single-Cys replacement mutants (145, 148, 264, 268, and 272) are hatched. The single-Trp or Cys mutants in helix X which were used in this study are shown as filled circles.

of substrate with position 325 in contact with the hydrophobic phase of the membrane. Under these circumstances, ligand would be expected to produce little or no change in the accessibility of position 325 to the hydrophobic phase of the membrane.

Finally, it should be emphasized that the postulated ligand-induced conformational change in helix X may involve a change in helix tilt, as well as rotational movement. In addition, the magnitude of the structural changes involved need not be particularly large, as it is possible that relatively small changes in protein conformation coupled with diffusion of phospholipids (or detergent molecules) may result in relatively large changes in the accessibility of a given side chain to the hydrophobic phase of the membrane (or micelle).

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