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Design of a membrane transport protein for fluorescence spectroscopy

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ABSTRACT **To modify the *lac* permease of *Escherichia coli* for fluorescence spectroscopy, six tryptophan residues at positions 10, 33, 78, 151, 171, and 223 were first replaced individually with phenylalanine by using oligonucleotide-directed site-specific mutagenesis. None of the tryptophan residues is critical for activity, as evidenced by the finding that the mutant permease molecules catalyze lactose/H^+ symport almost as well as wild-type permease. Subsequently, a permease molecule was designed in which all of the tryptophan residues were replaced with phenylalanine. Remarkably, the *lac* permease harboring all six mutations catalyzes active lactose transport about 75% as well as wild-type permease. The fluorescence emission spectrum of purified wild-type permease solubilized in octyl β-D-glucopyranoside and phospholipid exhibits a broad maximum centered at 350 nm, and the peak is almost completely absent from the spectrum of permease devoid of tryptophan. Furthermore, a new maximum centered at about 306 nm is apparent in the spectrum of the modified permease, suggesting that tyrosine fluorescence in the native protein is quenched by internal energy transfer to tryptophan residues. By using site-directed mutagenesis to replace specified residues in the molecule without tryptophan, it should now be possible to utilize tryptophan fluorescence spectroscopy to study static and dynamic aspects of permease structure and function.**

The *lac* permease of *Escherichia coli* is a polytopic cytoplasmic membrane protein that catalyzes the concomitant translocation of a single β-galactoside molecule with a single H^+ (i.e., β-galactoside/H^+ symport; for reviews, see refs. 1 and 2). The permease has been solubilized from the membrane, purified to homogeneity, reconstituted into proteoliposomes, and shown to be completely functional in monomeric form (3–5). Moreover, the *lacY* gene, which encodes the permease, has been cloned and sequenced, and the amino acid sequence of the polypeptide has been deduced (6). Based on hydropathy analysis of the primary sequence, a secondary structure model for the permease has been proposed (7) in which the protein is organized into 12 hydrophobic domains in α-helical conformation that traverse the membrane in zig-zag fashion connected by more hydrophilic regions with the amino and carboxyl termini on the cytoplasmic face of the membrane (see Fig. 1). The model is consistent with circular dichroic (7), laser Raman (8), and Fourier transform infrared spectroscopy (P.D.R., H.R.K., and K. J. Rothschild, unpublished information), as well as chemical modification (9), limited proteolysis (10, 11), and immunological studies (12–18). More detailed support for the topological predictions of the model has been obtained from *lacY–phoA* fusion analyses (J. Calamia and C. Manoil, personal communication).

Oligonucleotide-directed site-specific mutagenesis is useful for delineating amino acid residues in *lac* permease that are important for activity (1, 2). For example, Arg-302, His-322, and Glu-325 have been shown to be important for ligand binding and lactose-coupled H^+ translocation, and it has been postulated that the three residues may form part of a H-bond network that functions in a type of H-relay mechanism (19–22). In addition, of the eight cysteine residues in the permease, only Cys-154 is important for activity, thereby ruling out sulfhydryl–disulfide interconversion as an integral part of the symport mechanism (23). Importantly, out of more than 100 mutations characterized thus far, approximately 70% have little or no effect on activity. Therefore, it seems reasonable to conclude that individual amino acid changes do not lead to indiscriminate conformational alterations in the permease.

Although significant progress has been made identifying amino acid residues that are important for lactose/H^+ symport, a high-resolution structure of the permease is required to help determine the role of these residues in the mechanism. Moreover, it is equally apparent that dynamic information at high resolution is also required to solve the mechanism. Fluorescence spectroscopy of endogenous tryptophan residues represents a potentially powerful method for probing static and dynamic aspects of protein structure–function relationships at high resolution. A principal difficulty in analyzing tryptophan fluorescence in many proteins, however, is the complexity of the signal that results from the presence of multiple tryptophan residues, six in the case of *lac* permease (Fig. 1). For this reason, we have initiated studies on the permease by utilizing site-directed mutagenesis to determine which tryptophan residues can be replaced with phenylalanine without altering activity and found that none is essential. Furthermore, when all six tryptophan residues are replaced with phenylalanine in the same permease molecule (W_6F permease), about 75% of wild-type transport activity is retained. Fluorescence emission spectra of the purified proteins reveal markedly diminished tryptophan fluorescence in W_6F permease relative to the wild type, as expected, and a significant increase in fluorescence in the tyrosine region of the spectrum. In addition to demonstrating that tryptophan residues are not essential for permease activity, the study provides the groundwork for future studies in which site-directed tryptophan replacements in W_6F permease can be used to probe permease structure–function relationships.

MATERIALS AND METHODS

Materials. [1-^{14}C]Lactose was synthesized by Yu-Ying Liu under the direction of Arnold Liebman (Isotope Synthesis Group, Hoffmann–La Roche). All other materials were reagent grade and obtained from commercial sources.

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Abbreviation: W_6F, *lac* permease with all six tryptophan residues replaced with phenylalanine.

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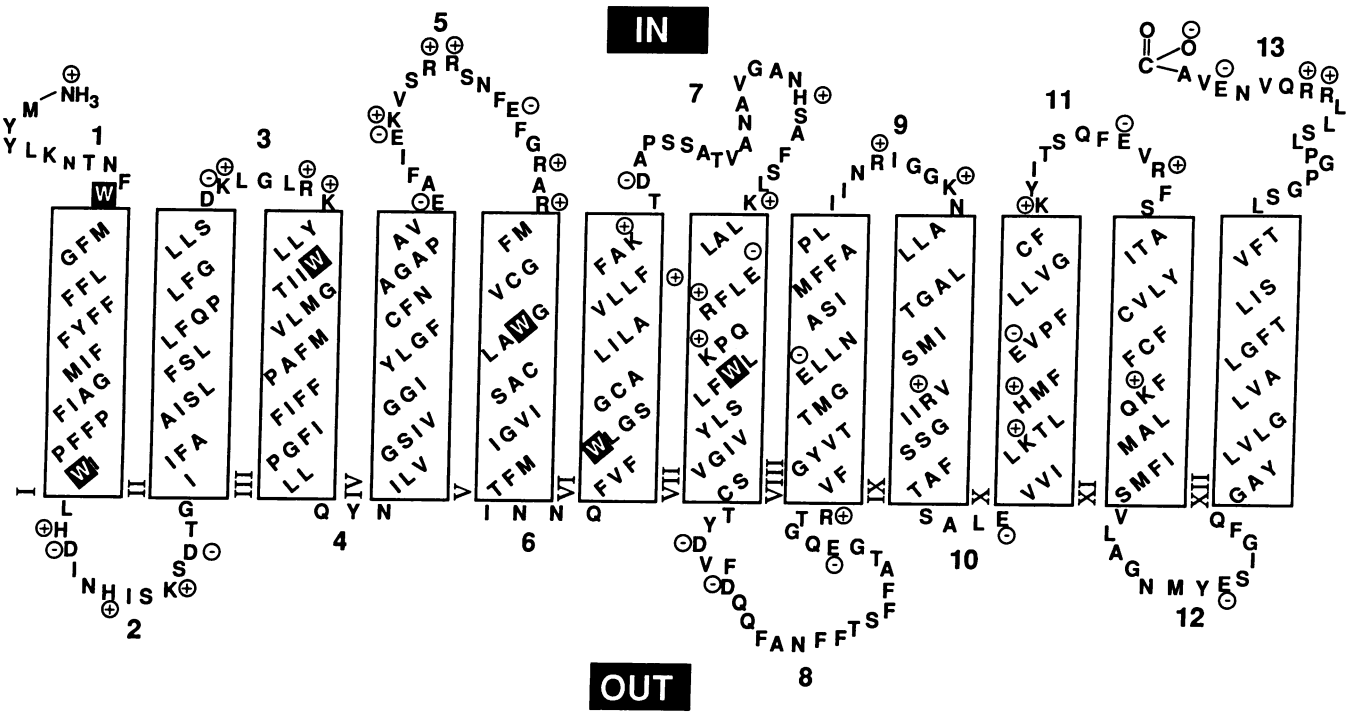

FIG. 1. Secondary structure model of *lac* permease based on the hydropathy of the deduced amino acid sequence (7) with tryptophan residues (W) highlighted. The single-letter amino acid code is used.

Bacterial Strains. The following strains of *E. coli* K-12 were used: JM101 *supE,thi,*Δ*(lacproAB)*, [F′, *traD36,proAB, lacI*^{q}*Z*Δ*M15*] (24); T206 [*lacI*$^+$*O*$^+$,*Z*$^-$*Y*$^-$*(A),rpsL,met*$^-$,*thr*$^-$, *recA,hsdM,hsaR*/F′, *lacI*^{q}*O*$^+$*Z*^{D118}*(Y*$^+$*A*$^+$*)*] harboring plasmid pGM21 [*lac*Δ*(I)O*$^+$*P*$^+$Δ*(Z)Y*$^+$Δ*(A)tet*^{r}] (25); T184 [T206 cured of plasmid pGM21 (25)]; HB101, *hsdS20 (r*^-_B,*m*^-_B), *recA13,ara-14,proA2,lacY1,galK2,rpsL20(Sm*^{r}*),xyl-5,mtl-1, supE44*, λ$^-$/F′ (26); BMH71-18 *mutL* [Δ*(lacpro),supE,thi, proA*$^+$*B*$^+$, *lacI*^{q}*lacZ*Δ*M15/MutL*::Tn*10*] (27); CJ236, *dut, ung,thi,relA;pCJ105(Cm*^{r}*)* (Bio-Rad); MV1190, Δ*(lacproAB), thi,supE,*Δ*(srl-recA)306*::Tn*10(tet*^{r}*)* [F′:*traD36,proAB, lacI*^{q}*Z*Δ*M15*] (Bio-Rad).

Site-Directed Mutagenesis of Individual Tryptophan Residues. Oligonucleotide-directed site-specific mutagenesis using M13mp18 as the cloning vector was carried out as described (28). Deoxyoligonucleotide primers complementary to antisense *lacY* DNA with the exception of two base mismatches were synthesized on an Applied Biosystems synthesizer and purified by polyacrylamide gel electrophoresis (Table 1). In each case, phenylalanine codons (TTT) were substituted for given tryptophan codons (TGG). Oligonucleotides were annealed to single-stranded M13mp18 template DNA containing the antisense strand of *lacY*, and heteroduplex DNA was synthesized overnight at 14°C. To maximize the yield of mutant recovery, the repair-deficient strain, *E. coli* BMH71-18 *mutL* (27) was transformed with the resultant heteroduplex DNA.

Colonies were screened by hybridization with appropriate ^{32}P-labeled mutagenic primers, and the mutations were verified by dideoxynucleotide sequencing (28, 29). The replicative form of M13mp18 DNA containing specified mutations was isolated by alkaline lysis and digested with *Eco*RI, and the fragment containing *lacY* was cloned into the plasmid pACYC184. Orientation of *lacY* in the recombinant plasmid was determined by *Hin*cII restriction enzyme analysis (28). In all cases, the entire *lacY* gene was sequenced to ensure that no secondary mutations were present.

Construction of W$_6$F Permease. To construct a permease molecule harboring all six Trp → Phe mutations, single-stranded M13mp18 DNA containing *lacY* encoding the W223F mutation was transfected into *E. coli* to obtain uracil-containing template (30). Mutagenesis of tryptophan codons 151 and 171 was carried out simultaneously on this template by using two appropriate primers (see Table 1), transfecting MV1190 with the synthetic heteroduplex DNA to remove the uracil-containing wild-type strand, and then isolating single-stranded M13mp18 DNA containing the three mutations. After dideoxynucleotide sequencing to verify the presence of the mutations and the absence of additional mutations, this DNA was used in similar fashion as template for simultaneous mutagenesis of trytophan codons 10, 33, and

Table 1. DNA sequence analyses of Trp → Phe mutants in *lac* permease

Mutated tryptophan codon	Mutagenic primer	Codon change observed
10	3′-TTGTGTTTGAAAAAATACAAGCCAAAT-5′	TGG → TTT
33	3′-AAAAAGGGCTAAAAAGATGTACTGTAG-5′	TGG → TTT
78	3′-TTTATGGACGACAAAATTATTACCGGC-5′	TGG → TTT
151	3′-CCAACACAACCGAAACGCGACACA-5′	TGG → TTT
171	3′-GTCAAACAAAAGAAAGACCCGAGA-5′	TGG → TTT
223	3′-GTCGGTTTTGACAAAAAAACAGTGAC-5′	TGG → TTT

Altered codons are underscored.

78 by using given primers (see Table 1). The replicative form of M13mp18 DNA harboring all six Trp → Phe mutations was then digested and cloned into pACYC184 as described above to yield plasma pW_6F.

Qualitative Estimates of Permease Activity. *E. coli* HB101 (Z^+Y^-) was transformed with plasmids encoding mutant *lac* permeases, and the cells were grown initially on eosin/methylene blue (EMB) plates containing 25 mM lactose as a qualitative estimate of permease activity (31). For more quantitative measurements of [1-^{14}C]lactose active transport, *E. coli* T184 (Z^-Y^-) was used as host strain.

Transport Measurements. Active transport of [1-^{14}C]-lactose was measured in *E. coli* T184 harboring given plasmids (23). Cells were grown at 37°C to an OD_{420} of 0.5, induced with 0.2 mM isopropyl β-D-thiogalactopyranoside and grown for another 90 min. Cells were harvested by centrifugation, washed extensively with 50 mM potassium phosphate, pH 7.5/10 mM magnesium sulfate and resuspended in the same salt solution to an OD_{420} of 10.0 (≈1 mg of protein per ml). Aliquots (50 μl) of the cell suspensions were incubated at room temperature, and [1-^{14}C]lactose (10 mCi/mmol; 1 Ci = 37 GBq) was added to a final concentration of 0.4 mM. At given times, the reactions were terminated by rapid dilution with 3.0 ml of 100 mM potassium phosphate (pH 5.5)/100 mM lithium chloride/20 mM mercuric chloride and immediate filtration through Whatman GF/F glass fiber filters. Radioactivity retained on the filters was assayed by liquid scintillation spectrometry.

Immunoblots. Immunoblots were carried out with monoclonal antibody 4A10R and ^{125}I-labeled protein A as described (32).

Solubilization and Purification of Wild-Type and W_6F Permeases. To solubilize and purify the wild-type and W_6F permeases for fluorescence measurements, plasmids pGM21 and pW_6F were digested with *Eco*RI and *Pvu* II and *lacY* was cloned into pT7-6 (33). *E. coli* T184 harboring plasmid pGP1-2 was then transformed with the pT7-6-derived plasmids, and the permease was overexpressed by heat shock at 42°C (33). Wild-type and W_6F permeases were solubilized from the membrane with octyl β-D-galactopyranoside (octyl glucoside) in the presence of *E. coli* phospholipids and purified as described (3, 4). The preparations were stored at 4°C for no longer than 24 hr before fluorescence measurements.

Fluorescence Spectroscopy. Steady-state fluorescence spectra of purified wild-type or W_6F *lac* permease (50 μg/ml in 1.25% octylglucoside/0.25 mg per ml of *E. coli* phospholipids/10 mM potassium phosphate, pH 5.8) were obtained with a Perkin–Elmer MPF-66 fluorimeter interfaced with a Perkin–Elmer 7500 data analysis terminal. Excitation was at 285 nm (bandwidth, 20 nm), and the scan rate was 1 nm/s.

RESULTS

Analysis of Mutations by DNA Sequencing. The *lacY* gene was cloned initially from pGM21 into the replicative form of M13mp18 DNA, and single-stranded phage DNA was isolated and used as template for site-directed mutagenesis. Subsequently, single-stranded phage DNA containing mutated *lacY* was isolated and sequenced (28, 29) by using appropriate primers complementary to regions of *lacY* 50–100 bases downstream from the mutations. As summarized in Table 1, the mutated *lacY* genes described contain given base changes at the sites predicted such that specified tryptophan codons (TGG) are replaced with phenylalanine codons (TTT). Furthermore, sequencing of the entire *lacY* gene of each of the mutants revealed no additional mutations, and in each case, the sequences were identical to that reported for *lacY* (6) with the exception of the base changes described. Finally, sequencing of W_6F *lacY* DNA revealed that all six tryptophan codons were replaced with phenylalanine codons, and no other mutations were detected.

Colony Morphology of Tryptophan Mutants on Lactose/EMB Plates. *E. coli* HB101 (Z^+Y^-) harboring plasmid pGM21, which expresses β-Gal from a chromosomal *lacZ* gene and wild-type *lac* permease from a plasmid-encoded *lacY* gene, grows as dark red colonies on EMB plates containing 25 mM lactose, indicating that lactose is transported and metabolized at a rapid rate. In contrast, HB101 harboring pACYC184 (the same vector with no *lacY* insert) grows as white colonies because lactose in the medium is inaccessible to β-Gal in the cytosol. *E. coli* HB101 transformed with pW10F, pW33F, pW78F, pW151F, pW171F, or pW223F grows as red colonies that are indistinguishable from HB101/pGM21. Furthermore, *E. coli* HB101 transformed with pW_6F also grows as dark red colonies. Qualitatively, therefore, replacement of each or all of the tryptophan residues in *lac* permease with phenylalanine has no apparent effect on downhill lactose translocation.

Active Lactose Transport. Assays of lactose transport in *E. coli* T184 harboring plasmids encoding wild-type permease or a single tryptophan mutation (i.e., W10F, W33F, W78F, W151F, W171F, or W223F) demonstrate that none of the tryptophan residues is essential for lactose/H^+ symport (Fig. 2). Both the initial rate of lactose transport and the steady-state level of accumulation in each of the mutants varies from 75% to 100% of that observed in *E. coli* T184/pGM21. Strikingly, moreover, the initial rate of lactose transport and the steady-state level of accumulation in *E. coli* T184 expressing W_6F permease is about 75% of that observed *E. coli* T184/pGM21 (Fig. 3). Thus, all six tryptophan residues can be replaced with phenylalanine in the same permease molecule with only a minimal effect on active transport. Although data are not shown, it is noteworthy that immunoblot analyses of membranes prepared from the wild type, each of the individual mutants, and the W_6F mutant reveal that comparable amounts of permease are present.

Fluorescence. The data presented in Fig. 4 are steady-state fluorescence emission spectra of purified wild-type and W_6F permease solubilized in octyl glucoside and *E. coli* phospholipids. With wild-type permease, a broad intense maximum centered at 350 nm was observed, and it is apparent that this band is essentially absent from the W_6F construct. As expected, therefore, fluorescence in the tryptophan region of

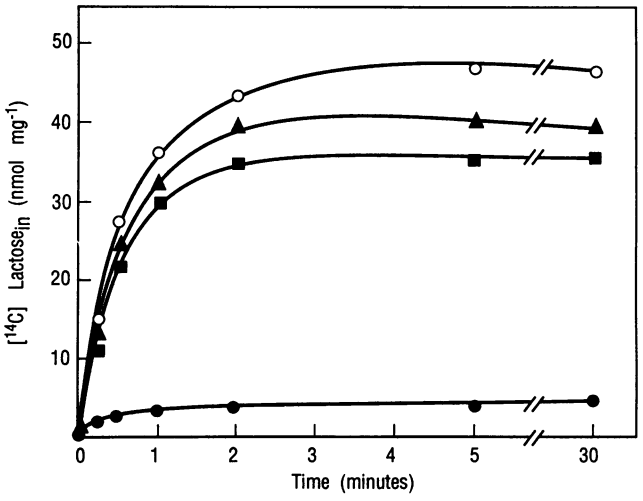

FIG. 2. Active transport of lactose in *E. coli* T184 expressing no *lac* permease (●; T184/pACYC), wild-type *lac* permease (○; T206), or *lac* permease with phenylalanine in place of Trp-33, Trp-151, or Trp-171 (○), Trp-78 (▲), Trp-10 (■), or Trp-223 (■). When no significant difference between strains was observed, the same symbol was used for purposes of clarity.

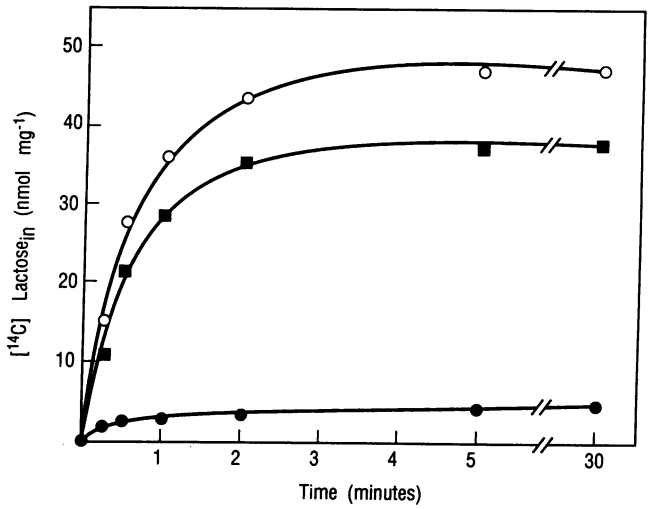

FIG. 3. Active transport of lactose in *E. coli* T184 expressing no *lac* permease (●; T184/pACYC), wild-type *lac* permease (○; T206), or W_6F permease (■).

the spectrum is largely abolished when tryptophan is replaced with phenylalanine. Interestingly, a new emission maximum centered at about 306 nm appeared in the spectrum of W_6F permease. Since tyrosine fluorescence is expected near this wavelength and the endogenous tyrosine fluorescence of many proteins is quenched by internal energy transfer to neighboring tryptophan residues (34), the new peak at 306 nm in W_6F permease can be readily explained. By removing tryptophan, the internal energy transfer acceptor, tyrosine fluorescence would be expected to increase. In any event, in addition to providing a starting point for studying the fluorescence behavior of strategically placed tryptophan residues, the W_6F permease should be useful for probing the fluorescence characteristics of the tyrosine residues in the protein.

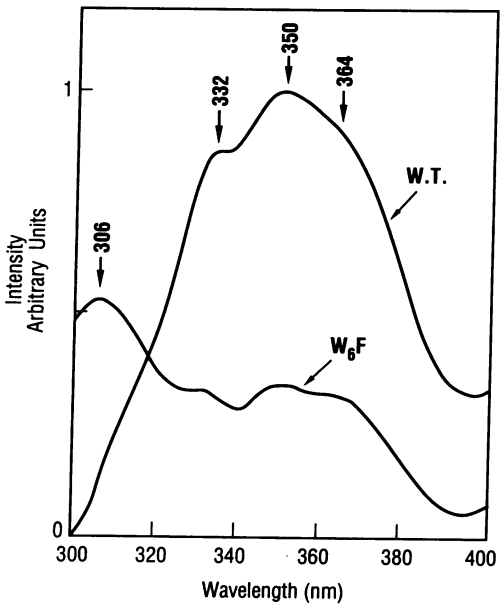

FIG. 4. Steady-state fluorescence emission spectra of purified wild-type (W.T.) or W_6F permease solubilized in octyl glucoside and *E. coli* phospholipids. Excitation was at 285 nm (bandwidth, 20 nm), and the scan rate was 1 nm/s. Since the exogenous *E. coli* phospholipid added during purification (3, 4) contains contaminating protein that is not removed completely by acetone/ether extraction, a background spectrum of column buffer (1.25% octylglucoside/0.25 mg per ml of *E. coli* phospholipid/10 mM potassium phosphate, pH 5.8) was subtracted from spectra of the purified permease samples. The correction was approximately 30% of the fluorescence intensity of the wild-type preparation.

DISCUSSION

The results presented in this paper may be summarized as follows:

(*i*) Replacement of each tryptophan residue with phenylalanine in *lac* permease does not impair the activity of the protein by more than about 25%, thereby demonstrating that none of tryptophan residues plays an essential role in lactose/H^+ symport. Since crystallographic analyses of the binding sites in certain periplasmic sugar-binding proteins from *E. coli* demonstrate that aromatic residues, including tryptophan, "sandwich" the sugar (35), mutation of the tryptophan residues in *lac* permease might have been expected to have a more adverse effect on activity. On the other hand, phenylalanine contains an aromatic side-chain that may mimic tryptophan in this respect. It should be possible to resolve this point by replacing the tryptophan residues in the permease with nonaromatic residues.

(*ii*) All six native tryptophan residues in the permease can be replaced with phenylalanine with little effect on the ability of the protein to catalyze active transport.

(*iii*) The fluorescence emission spectrum of purified wild-type permease exhibits a broad maximum centered at about 350 nm that is essentially absent from the spectrum of W_6F permease. In contrast, a new peak at about 306 nm appears with W_6F permease that is probably due to increased tyrosine fluorescence resulting from lack of internal energy transfer from tyrosine to tryptophan. Although data are not shown, both the tryptophan fluorescence of wild-type permease and the tyrosine fluorescence of W_6F permease are quenched partially upon addition of β-D-galactosyl β-D-thiogalactopyranoside. By constructing permease molecules with each of the native tryptophan residues present, it should be possible to determine which tryptophan residue(s) is responsible for this phenomenon. Furthermore, by using W_6F permease and individually mutating the four tyrosine residues shown to effect binding (36), more precise information about the role of specific tyrosine residues in substrate binding may be obtained.

In addition to the important conclusion that tryptophan residues do not play a critical role in the mechanism of lactose/H^+ symport, the results provide the basis for a general approach to the analysis of static and dynamic aspects of permease structure–function relationships. To cite a few examples, by designing tryptophan replacements for amino acid residues in the putative hydrophilic and hydrophobic domains of W_6F permease (Fig. 1), it should be possible to determine the environment of a given domain from the intensity and position of the emission maximum and from studies with water-soluble and lipid-soluble collisional quenchers. In addition, studies on the effect of the H^+ electrochemical gradient on the fluorescence properties of the modified molecules may be informative with regard to those portions of the molecule that are conformationally active. Finally, by studying energy transfer between strategically placed tryptophan residues and a variety of *N*-dansylaminoalkyl β-D-galactopyranosides (37), it might be possible to identify regions of the permease that are involved in binding. In this regard, it has been suggested that residues in putative helices IX and X are involved in binding (19–21, 38); moreover, permease with tryptophan in place of Glu-325 (putative helix X; Fig. 1) catalyzes lactose/*N*-dansylaminoalkyl β-D-galactopyranoside counterflow and binds ligand (21). Clearly, therefore, appropriate studies on W_6F permease with tryptophan in place of Glu-325 could be of immediate interest.

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