Melibiose Permease of *Escherichia coli*: Substrate-Induced Conformational Changes Monitored by Tryptophan Fluorescence Spectroscopy†

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ABSTRACT: Tryptophan fluorescence spectroscopy has been used to investigate the effects of sugars and coupling cations (H^+, Na^+, or Li^+) on the conformational properties of purified melibiose permease after reconstitution in liposomes. Melibiose permease emission fluorescence is selectively enhanced by sugars, which serve as substrates for the symport reaction, α-galactosides producing larger variations (13−17%) than β-galactosides (7%). Moreover, the sugar-dependent fluorescence increase is specifically potentiated by NaCl and LiCl (5−7 times), which are well-established activators of sugar binding and transport by the permease. The potentiation effect is greater in the presence of LiCl than NaCl. On their own, sodium and lithium ions produce quenching of the fluorescence signal (2%). Evidence suggesting that sugars and cations compete for their respective binding sites is also given. Both the sugar-induced fluorescence variation and the NaCl(or LiCl)-dependent potentiation effect exhibit saturation kinetics. In each ionic condition, the half-maximal fluorescence change is found at a sugar concentration corresponding to the sugar-binding constant. Also, half-maximal potentiation of the fluorescence change by sodium or lithium occurs at a concentration comparable to the activation constant of sugar binding by each ion. The sugar- and ion-dependent fluorescence variations still take place after selective inactivation of the permease substrate translocation capacity by *N*-ethylmaleimide. Taken together, the data suggest that the changes in permease fluorescence reflect conformational changes occurring upon the formation of ternary sugar/cation/permease complexes.

The melibiose permease (Mel permease)[1] of *Escherichia coli*, encoded by the *melB* gene, catalyzes the accumulation of a variety of α-galactosides (melibiose (Mel), raffinose, and methyl α-D-galactopyranoside (αMG)), as well as some β-galactoside derivatives (methyl 1-thio-β-D-galactopyranoside (TMG), methyl β-D-galactopyranoside (βMG), or to a small extent lactose), by a cation/sugar symport mechanism (Wilson et al., 1982; Pourcher et al., 1990a). Depending on the ionic conditions and/or sugar substrate, sugar uptake is coupled to H^+, Na^+, or Li^+ entry. The differences in the sugar binding and transport properties of Mel permease when functioning as a H^+-, Na^+-, or Li^+-coupled melibiose symporter (Bassilana et al., 1985) have been exploited to study the role of the cation in the coupling process. The results suggest that the coupling ion enhances the affinity of Mel permease for the cotransported sugar, Na^+ and Li^+ being better activators than H^+ (Lopilato et al., 1978; Damiano-Forano et al., 1986; Cohn & Kaback, 1980). The data also suggest that H^+, Na^+, or Li^+ ions compete for the same binding site and that the cation and sugar substrates bind to and are transported by the permease in a 1:1 ratio (Damiano-Forano et al., 1986; Bassilana et al., 1988).

From the cloning and sequencing of *melB*, it was initially suggested that the permease consists of 469 amino acids, 70% of which are apolar residues (Yazyu et al., 1984; Hanatani et al., 1984). A recent determination of the N-terminal sequence of the purified *melB* product indicates, however, that four extra amino acids have to be added to the N-terminal extremity of the predicted sequence, raising the transporter size to 473 amino acids (Pourcher et al., 1995). Secondary structure predictions based on hydropathy profiling of the primary amino acid sequence (Pourcher et al., 1990b) and analysis of a series of melB−phoA fusions (Botfield et al, 1992; Pourcher et al., unpublished experiments) suggest that Mel permease contains 12 membrane-spanning segments with α-helical configuration. Site-directed mutagenesis of the *melB* gene (Pourcher et al., 1991, 1993; Wilson & Wilson, 1992; Zani et al., 1993, 1994) and construction of chimeric melibiose transporters from various microorganisms (Hama & Wilson, 1993) recently have been used to analyze the molecular basis of the permease ionic selectivity. The results suggest that several aspartic acid residues, distributed in different helices of the N-terminal hydrophobic domains of Mel permease, form a coordination network involved in Na^+, Li^+, or H_3O^+ (rather than H^+) recognition. This suggestion will remain hypothetical, however, until additional structural information is obtained by using biophysical techniques and, ultimately, by resolving the permease 3D structure.

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[1] Abbreviations: Mel permease, melibiose permease; Mel-6His permease, Mel permease with a six-histidine tag at the C-terminus extremity; Mel, melibiose; αMG, methyl α-D-galactopyranoside; TMG, methyl 1-thio-β-D-galactopyranoside; βMG, methyl β-D-galactopyranoside; αNPG, *p*-nitrophenyl α-D-galactopyranoside; LAPAO, 3-(laurylamido)-*N*,*N*'-(dimethylamino)propylamine oxide; DM, dodecyl maltoside; KP_i, potassium phosphate; NaP_i, sodium phosphate.

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Purification of Mel permease is a prerequisite for analyzing the structural properties. This preliminary goal has been attained recently by purifying a recombinant Mel permease (Mel-6His permease) carrying an affinity tag for metallic ions (six successive histidines) at its C-terminal extremity (Pourcher et al., 1995). By using chelate affinity chromatography (Ni-NTA resin), a permease-rich fraction containing at least 90% Mel-6His permease is obtained. Ion exchange chromatography can be used to purify the permease to homogeneity (>99%). Proteoliposomes reconstituted from either the permease-rich fraction or the pure material display H^+-, Na^+-, or Li^+-dependent sugar-binding and transport properties that are comparable to those of the native permease in its natural environment. The large yield of purified Mel-6His permeases makes various approaches for analyzing the permease structure possible.

Of these, fluorescence spectroscopy of endogenous tryptophan residues presents a potentially powerful method for probing the static and dynamic aspects of protein structure–function relationships (Brandolin et al., 1985; Peerce, 1990; Martineau et al., 1990; Walmsley et al., 1993). Since Mel permease contains eight tryptophan residues, we examined the intrinsic fluorescence properties of Mel-6His permease reconstituted in liposomes from the pure or enriched cotransporter fractions and showed that the permease intrinsic fluorescence is selectively modified upon interaction of the ion and/or sugar substrates with the permease. These fluorescence changes are still observed when substrate translocation is specifically blocked by acylation of the permease with *N*-ethylmaleimide. Moreover, it was shown that the ion-dependent sugar-binding constants (sugar affinity, ion activation constants), measured by a radiolabeled sugar-binding assay, are strictly correlated with the equilibrium constants ($K_{0.5}$) describing the ion- and/or sugar-induced half-maximum change in the permease fluorescence. These and other results suggest that the substrate-induced fluorescence changes are related to conformational change(s) of the permease upon formation of the cation/sugar/permease complex.

EXPERIMENTAL PROCEDURES

Materials. *p*-Nitrophenyl α-D-[6-^{3}H]galactopyranoside (α-NPG) was synthesized under the direction of Dr. B. Rousseau in our department. Synthesis of LAPAO ((3-laurylamido)-*N*,*N*′-(dimethylamino)propylamine oxide) was performed as described by Brandolin et al. (1980). Dodecyl maltoside (DM) was obtained from Boehringer Mannheim, and Ni-NTA resin was from Qiagen, Inc. Anion exchange resin (Macro-Prep Q support) and Bio-Beads SM-2 were from Bio-Rad Lab., Inc. Total *Escherichia coli* lipids (acetone/ether precipitated) were purchased from Avanti Polar Lipids, Inc. High purity grade chemicals (Suprapur, Merck) were used to prepare media virtually devoid of sodium salts, hereafter referred to as Na^+-free solutions. Flame photometry measurements indicated that the level of contaminating sodium salts in these solutions was generally below 20 μM. All other materials were obtained from commercial sources.

Bacterial Strain and Plasmid. *E. coli* DW2-R (Pourcher et al., 1991), a rec A^- derivative of strain DW2 (mel A^+, Δmel B, ΔlacZY) (Botfield & Wilson, 1988), was transformed with a pK31ΔAHB recombinant plasmid (Pourcher et al., 1995) derived from the pKK223-3 vector (Pharmacia LKB Biotechnology Inc.). This plasmid harbors a *melB* gene under the tac promoter that is terminated by six successive triplets encoding His residues at its 3′ extremity. The six additional histidine residues (6His tag) confer a high affinity for nickel ions and enable purification of the recombinant protein by metal-chelated affinity chromatography using Ni-NTA–agarose resin.

Protein Purification. The method of purification was essentially that described by Pourcher et al. (1995). Freshly transformed DW2-R/pK31ΔAHB cells were grown to OD_{600} = 2 at 30 °C in 200 L of culture at the Centre de Fermentation, CNRS Marseille, France. The growth medium was M9 medium supplemented with 5 g/L glycerol, 2 g/L casamino acids (Difco Co.), and 0.1 g/L ampicillin. Inverted membrane vesicles (IMV) were prepared from 100 g (wet weight) of paste cell by means of a French press (American Instrument Co., 18 000 psi). All purification steps were performed at 0–4 °C. IMV were washed once and resuspended at about 5 mg/mL in a medium containing 50 mM sodium phosphate (NaP_i) (pH 8), 10 mM Tris-HCl (pH 8), 0.6 M NaCl, 20% glycerol, 5 mM β-mercaptoethanol, and 10 mM imidazole. Solubilization was carried out in the same medium supplemented with 1% of the detergent LAPAO. After 10 min of incubation, the samples were centrifuged (280000*g*, 30 min). The supernatant was then incubated for 1 h under gentle shaking with Ni-NTA resin (25 mL of resin/g of membrane vesicle protein) preequilibrated with 50 mM NaP_i (pH 8), 10 mM Tris-HCl (pH 8), 0.6 M NaCl, 20% glycerol, 5 mM β-mercaptoethanol, 10 mM imidazole, 0.2 mg/mL *E. coli* lipids, and 0.2% LAPAO. The resin with adsorbed material was centrifuged, washed once with the same medium, and used to load an FPLC column (Waters 650). After 3 column vol washes with the same medium, the Mel permease was eluted by the same buffer containing 0.1 M imidazole. The fraction of Mel permease contained in the eluted protein solution was estimated, by scanning a Coomassie Blue-stained SDS/PAGE gel, to be around 90%. For purification of the permease to homogeneity (>99%), LAPAO was replaced by DM, and NaCl-rich buffer was replaced by a low ionic strength buffer containing 50 mM NaP_i (pH 8), 10 mM Tris-HCl (pH 8), 20% glycerol, 5 mM β-mercaptoethanol, 10 mM imidazole, 0.2 mg/mL *E. coli* lipids, and 0.2% DM during Ni-NTA chromatography. Mel permease was eluted by adding 0.1 M imidazole to the buffer. Elimination of the few contaminant proteins eluted together with the permease was achieved by applying the Ni-NTA pooled eluate to a Macro-Prep Q anion exchange support preequilibrated with the elution buffer used during Ni-NTA chromatography. The void volume containing the purified permease was collected.

Reconstitution. This was carried out by a procedure involving detergent adsorption on polystyrene beads (Rigaud et al., 1988), with all steps being performed at 0–4 °C. Pooled fractions containing enriched or pure Mel-6His permease (about 60 mL) were mixed with 1 vol of 50 mM NaP_i, 10 mM Tris-HCl buffer at pH 8, and 0.6 M NaCl. After the addition of 180 mg of *E. coli* lipids, the suspension was incubated under gentle agitation. Then, 15–20 g of BioBeads SM2 was added stepwise (four additions at 10 min intervals), and the sample was left overnight at 4 °C. The proteoliposomes thus formed were washed twice with 0.1 M potassium phosphate (KP_i) buffer (pH 7).

Equilibration of the Proteoliposomes. When required, equilibration of the internal proteoliposome space with medium containing a high concentration of sugar and/or a given salt composition was performed by submitting the proteoliposomes to repeated freezing/thawing−sonication−wash cycles.

Protein Assays. The concentration of protein was assayed according to Lowry et al. (1951) using serum bovine albumin as standard.

Binding Assays. Binding of [^{3}H]αNPG (1.6 mCi/mmol) to proteoliposomes was measured in the presence of 10 μM carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP) and 0.75 μM monensin using a flow dialysis technique at 20 °C. Apparent dissociation constants for NPG (K_D) were calculated graphically from Scatchard plots of the data. Sugar inhibitory constants (K_i) were measured by competitive displacement of bound [^{3}H]αNPG using sugar concentrations ranging from 0.1 to 20 mM. K_D values were calculated according to Rossi et al. (1980). Na^+ and Li^+ activation constants (K_{Na^+} and K_{Li^+}) were determined from plots of the apparent K_D for NPG as a function of the reciprocal concentrations of NaCl and LiCl in the range 0.2−10 mM (Damiano-Forano et al., 1986).

Fluorescence Studies. All experiments were carried out at 20 °C. After excitation at 297 ± 5 nm, fluorescence spectra were recorded on a PTI spectrofluorometer, and time-dependent changes in fluorescence were analyzed on a MOS-200 spectrofluorometer (BioLogic). In the latter case, fluorescence emission intensity was detected with a photomultiplier unit PMS-200 between 310 and 400 nm, using a combination of WG320 and black glass filters, and a 2 s integration time. Measurements were carried out on samples containing 20 μg of protein/mL that were previously sonicated for 30 s and placed in 1 × 1 cm methacrylate UV/visible cuvettes (CML). A special component on top of the cuvette holder allows efficient mixing with a propeller and injection of the reagents through two holes with Hamilton syringes. Additions were made directly to the cell without removing it from the cell holder to increase reproducibility. When needed, uncoupling reagents (2 μM valinomycin, 0.75 μM monensin, and 0.75 μM nigericin) were added to the proteoliposome suspension. Data were fitted with the analysis program Sigma plot (Jandel Scientific) using the equation $\Delta F/F = \Delta F/F_{max}[L]/K_{0.5} + [L]$, where ΔF is the fluorescence variation intensity observed upon addition of the ligands, F is the initial fluorescence intensity of the sample, [L] is the ligand concentration, $\Delta F/F_{max}$ is the maximum fluorescence change induced by the ligand, and $K_{0.5}$ is the apparent equilibrium constant describing the half-maximum change of the permease fluorescence. Each experiment was performed in triplicate. The standard deviation of the $K_{0.5}$ determinations was in all cases lower than 10% of the mean values.

RESULTS

Intrinsic Tryptophan Fluorescence Spectrum of Mel Permease. Figure 1 shows the emission fluorescence spectrum recorded upon illumination (297 ± 5 nm) of a proteoliposome suspension prepared from the Mel-6His permease-rich fraction (90% permease) and also illustrates the typical spectral changes elicited by successive addition of NaCl- and melibiose. The proteoliposome spectra were corrected for the NaCl or melibiose-insensitive fluorescence signal recorded from pure liposomes. In the absence of substrates, the proteoliposome fluorescence spectrum (spectrum 1) is characterized by a maximum emission wavelength centered around 337 nm. NaCl was then added at a final concentration (10 mM) known to produce maximal activation of the sugar-binding and transport activities of the permease (Lopilato et al., 1978; Damiano-Forano et al., 1986). This addition induces general and limited quenching of the fluorescence spectrum (spectrum 2) with no concomitant shift of the maximum emission wavelength, the quenching effect being more pronounced in the region around 345 nm. Melibiose was then added at a concentration (10 mM) at which binding and transport activities are maximal. This addition produces a broad increase in fluorescence emission between 320 and 350 nm (spectrum 3). Although the maximum emission wavelength is not shifted significantly, the contribution of the light emitted in the region around 327 nm becomes proportionally more important. The spectral characteristics of Mel permease and the substrate-induced changes were observed repeatedly.

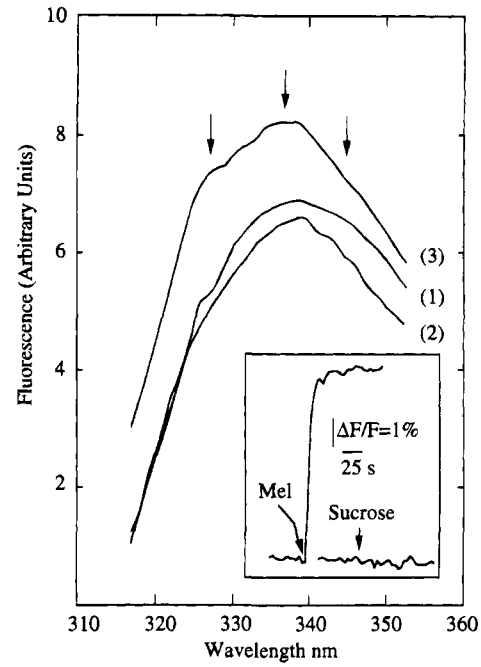

FIGURE 1: Effects of sodium and melibiose on the intrinsic fluorescence of proteoliposomes containing Mel-6His permease. A permease-rich fraction containing 90% Mel-6His permease was reconstituted into liposomes using total *E. coli* lipids as described in Experimental Procedures. Samples (2 mL, 20 μg of protein/mL) equilibrated in 0.1 M KP_i (pH 7) and 0.5 M KCl were illuminated at 297 ± 5 nm, and the fluorescence emission spectrum was recorded between 320 and 350 nm (spectrum 1). The maximum emission wavelength (337 nm) and the regions at 327 and 345 nm are indicated by arrows. NaCl and melibiose were successively added to a final concentration of 10 mM, and the effects on the fluorescence spectra were recorded (spectra 2 and 3, respectively). All spectra were corrected for inner filter effects using the signal measured from a pure liposome preparation with identical absorbancy. Inset: Effect of the addition of sugar on the integrated fluorescence signal recorded from proteoliposome samples (20 μg of protein) equilibrated in 50 mM NaP_i, 10 mM Tris-HCl (pH 8), and 10 mM NaCl. After excitation at 297 ± 5 nm, the emitted fluorescence light (F) was integrated between 310 and 400 nm and recorded as a function of time. The sugar-induced change (ΔF) in signal was expressed as $\Delta F/F$ (in %). Arrow: addition of the indicated sugar at a final concentration of 10 mM. All experiments were carried out at 20 °C.

The results of several experiments, not illustrated here, support the contention that the NaCl- and melibiose-induced changes in fluorescence spectra are specifically related to interaction of the two substrates with Mel-6His permease. Firstly, no effect on the fluorescence spectrum was observed upon adding either substrate to proteoliposomes carrying only contaminant proteins copurified with Mel-6His permease (Pourcher et al., 1995). Secondly, proteoliposomes prepared from the fraction containing pure Mel-6His permease exhibited substrate-induced fluorescence changes similar to those described for proteoliposomes containing the permease-rich fraction. It is also relevant that the substrate-induced changes in fluorescence persist in the presence of an uncoupling mixture containing valinomycin (2 μM), nigericin (0.75 μM), and monensin (0.75 μM), suggesting that the ion and/or electrical gradients that may be generated across the proteoliposome membrane upon addition of the substrates are not responsible for the substrate-induced fluorescence changes. Finally, the fluorescence signal from Mel-6His permease solubilized in the presence of LAPAO and NaCl is enhanced by the addition of melibiose. This sugar-induced fluorescence variation of the LAPAO-solubilized permease is, however, significantly lower than that induced in proteoliposomes and subsequently disappears. This probably reflects an instability of the transporter solubilized in detergents. In any case, these data indicate that the substrate-induced variations in the fluorescence signal of the permease in the proteoliposomes are not linked to osmotic artifacts. Since the substrate-induced changes in intrinsic fluorescence of Mel-6His permease can be recorded on proteoliposomes reconstituted from the permease-rich fraction obtained after only one chromatographic step, these preparations were used for subsequent studies.

Interpretation of the changes in the fluorescence spectrum of Mel-6His permease induced by the substrates is complex. The carrier intrinsic fluorescence properties were therefore analyzed by measuring the relative variation in the fluorescence signal ($\Delta F/F$) integrated between 310 and 400 nm. Figure 1 (inset) shows that, in the presence of a saturating concentration of sodium (10 mM), the fluorescence signal increases by 17% upon addition of the physiological substrate melibiose, while it decreases only to an extent corresponding to the dilution effect (0.25%) when a nontransported sugar such as sucrose is added. This experiment establishes that the fluorescence variation is sugar-selective.

The Permease Intrinsic Fluorescence Variations Are Sugar-Specific. Mel permease recognizes a broad range of α- and β-galactoside derivatives and, to a lesser extent, galactose (Wilson & Wilson, 1987). To compare the effects of various sugar substrates on Mel permease fluorescence properties, proteoliposomes were equilibrated in the presence of a saturating concentration of sodium (10 mM), and the concentration of each sugar giving a maximal fluorescence change ($\Delta F/F_{max}$) was determined. Figure 2 shows that saturating concentrations (10−15 mM) of α-galactosides such as the monosaccharide αMG, the dissaccharide melibiose, and the trisaccharide raffinose produced a large $\Delta F/F_{max}$ (13−17%), whereas the maximal $\Delta F/F$ produced by β-galactosides such as the monosaccharide β-MG, the monosaccharide with a thiol in β-position TMG, and the disaccharide lactose was at best only 7% and necessitated a higher sugar concentration (>35 mM). It was finally shown

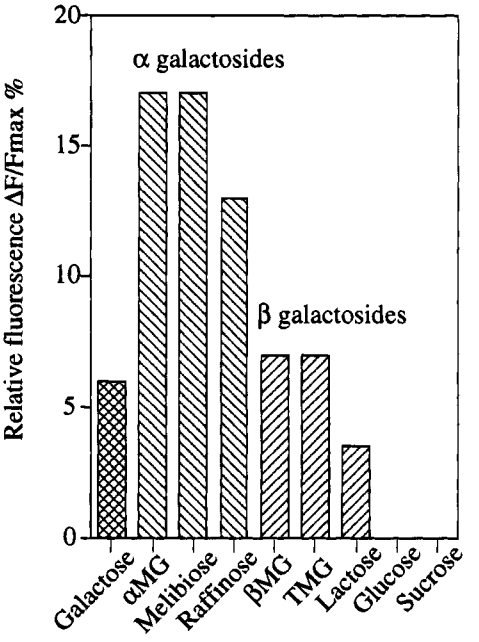

FIGURE 2: Effect of different galactosides on the tryptophan fluorescence of Mel-6His permease. Proteoliposomes prepared from the permease-rich fraction (90% Mel-6His permease) were equilibrated in 50 mM NaP_i, 10 mM Tris-HCl (pH 8), and 10 mM NaCl. Samples (2 mL, 20 μg of protein/mL) were incubated at 20 °C and illuminated at 297 ± 5 nm, and the relative variation in fluorescent light emitted between 310 and 400 nm ($\Delta F/F$) was recorded. Each sugar was added at a final concentration known to induce maximal variation in the fluorescence signal. The following additions were made: melibiose (10 mM), raffinose and methyl α-D-galactopyranoside (αMG) (15 mM), methyl β-D-thiogalactopyranoside (TMG), methyl β-D-galactopyranoside (βMG), galactose, lactose, and sucrose (35 mM).

that galactose produces a $\Delta F/F_{max}$ of 6%, while glucose or sucrose has no effect. These data indicate that the change in the transporter intrinsic fluorescence is primarily dependent on the galactopyranosyl moiety and the α-configuration of the glycosidic linkage of the sugar substrate. Finally, measurement of the sugar-binding constants indicated that the Mel permease affinity for the three α-galactosides tested was about an order of magnitude higher than that for the β-derivatives or galactose. The respective inhibition constants (K_i) are 0.5−0.7 mM for melibiose, αMG, and raffinose, 2−4 mM for TMG, 6 mM for β-MG, and 7 mM for galactose. These data indicate a correlation between the amplitude of fluorescence changes produced by a given sugar and the permease affinity for this sugar.

The difference in amplitude of $\Delta F/F_{max}$ elicited by α- and β-galactosides was exploited to assess the competitive interaction between these two classes of sugar substrate (Figure 3). The fluorescence variation (about 7%) induced by the addition of 35 mM TMG (about 10 times its K_i) is approximately doubled when a large excess of melibiose (25 mM, i.e., 50 times its K_i) is added after the TMG (Figure 3B). The magnitude of the $\Delta F/F$ change produced by the addition of the two sugars is not the algebraic sum of their individual effects (Figure 3A). Moreover, when a maximal $\Delta F/F$ is first induced by 5 mM melibiose (i.e., 10 times its binding constant), subsequent addition of 50 mM TMG (about 15 times its K_i) reverses the fluorescence signal (Figure 3C). Since the maximal number of sugar-binding sites is constant, these data must indicate that the fluorescence changes are not additive and they suggest that TMG and

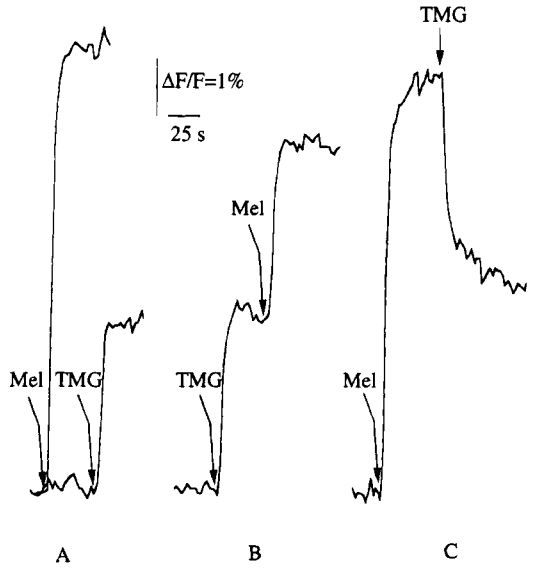

FIGURE 3: Competition effects of α- and β-galactosides on the tryptophan fluorescence of Mel-6His permease. Proteoliposomes were prepared from the permease-rich fraction (90% Mel-6His permease) and equilibrated at 20 °C in 50 mM NaP_i, 10 mM Tris-HCl (pH 8), and 10 mM NaCl. Samples (2 mL, 20 μg of protein/mL) were illuminated at 297 ± 5 nm, and the integrated fluorescence signal was measured as described in Figure 1 (inset). The traces show the $\Delta F/F$ produced by the addition of given sugars at final concentrations indicated in brackets. (A) Addition of TMG (35 mM) or melibiose (10 mM); (B) successive addition of TMG (35 mM) and melibiose (25 mM); (C) successive addition of melibiose (5 mM) and TMG (50 mM).

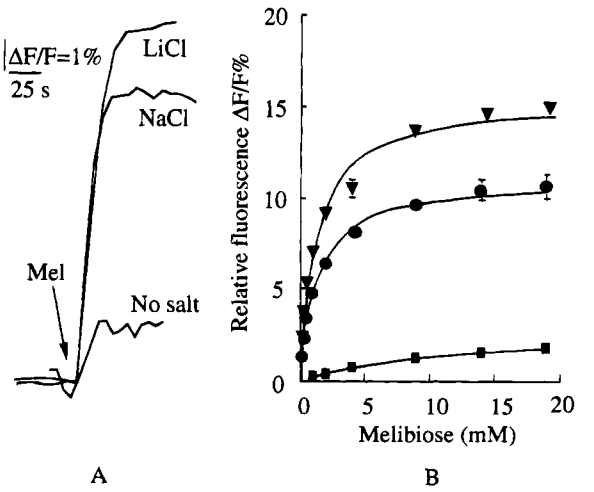

FIGURE 4: Effects of melibiose on Mel-6His permease fluorescence in the presence or absence of NaCl (or LiCl). Proteoliposomes were prepared from the permease-rich fraction (90% Mel-6His permease) and equilibrated at 20 °C in 0.1 M KP_i (pH 7) and 0.5 M KCl buffer. Samples (2 mL, 20 μg of protein/mL) were illuminated at 297 ± 5 nm, and the integrated fluorescence signal was measured as described in Figure 1 (inset). (A) $\Delta F/F$ induced by the addition of melibiose (10 mM) to proteoliposomes resuspended in a medium lacking NaCl or LiCl (lower trace) or a medium containing 10 mM NaCl (middle trace) or 10 mM LiCl (upper trace). (B) Dose–response curve of the melibiose-induced increase in fluorescence in proteoliposomes incubated in the absence of salt (■) or in the presence of 10 mM NaCl (●) or 10 mM LiCl (▼). Each point is the mean of four experiments (±SE). Plots gave $K_{0.5}$ for melibiose of 10.6 ± 0.8 mM in the absence of salt, 1.2 ± 0.1 mM in the presence of Na^+, and 1.3 ± 0.2 mM in the presence of Li^+.

melibiose compete for the same sugar-binding site. Similar competitive behavior was observed with various combinations of different α- and β-galactosides (data not shown).

Ionic Dependence of the Melibiose-Induced Fluorescence Change. This was investigated in proteoliposomes incubated in the absence or presence of NaCl or LiCl (10 mM) in the medium at pH 7. In the absence of these salts, i.e., when H^+ is the coupling ion, the addition of melibiose (10 mM) leads to a $\Delta F/F$ of only 2.2%. In the presence of NaCl or LiCl, however, a similar addition of melibiose induces a much higher $\Delta F/F$ of 10 or 14%, respectively (Figure 4A). The difference (30–40%) between the NaCl- and LiCl-induced $\Delta F/F$ variations is significant and has been observed repeatedly. Furthermore, the fluorescence variation produced by melibiose is concentration-dependent (Figure 4B), and the data can be fitted by Michaelis–Menten equations in all ionic conditions tested. The melibiose concentration producing a half-maximum effect ($K_{0.5}$) is 11 mM in the absence of NaCl or LiCl and 1.2–1.3 mM in the presence of these salts. As shown in Table 1, the $K_{0.5}$ value calculated in a given ionic condition is on the same order of magnitude as the apparent melibiose-binding dissociation constant determined in that condition. These results also indicate that the change in fluorescence is produced by the interaction of a single sugar molecule with the permease. Finally, we observed that the characteristics of the melibiose-induced fluorescence change recorded in medium devoid of NaCl or LiCl are pH-dependent. Thus, the $K_{0.5}$ value is 4 times higher at pH 8 than at pH 7 (45 and 11 mM, respectively) and remains constant upon further acidification of the medium ($K_{0.5}$ is 9 mM at pH 6); in contrast, the maximal change in fluorescence recorded is pH-independent. These data suggest that H^+ acts as an activator during the melibiose-induced

Table 1: Effect of Ionic Conditions on the Melibiose Concentration Producing Half-Maximal Fluorescence Variations and on the Binding Constants[a]

coupling cation	fluorescence $K_{0.5}^{Mel}$ (mM)[b]	[^{3}H]αNPG binding K_D^{Mel} (mM)[c]
10 mM NaCl	1.3	1
10 mM LiCl	1.2	1
H^+	11	4

[a] All experiments were performed in 0.5 M KCl and 0.1 M KP_i (pH 7), using proteoliposomes containing 20 μg/mL protein for fluorescence measurements and 0.5 mg/mL protein for [^{3}H]αNPG binding measurements. [b] Fluorescence was determined as described in Figure 4. The $K_{0.5}$ values are the melibiose concentrations producing half-maximal fluorescence changes in the absence (H^+) or presence of 10 mM NaCl or LiCl. They were calculated from curve fits for increasing concentrations of melibiose. [c] [^{3}H]αNPG (1.6 mCi/mmol) binding to proteoliposomes incubated in a medium lacking or containing 10 mM NaCl or LiCl with carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (5 μM) and monensin (0.75 μM) was assayed by flow dialysis. K_D^{Mel} was calculated as described by Damiano et al. (1986).

fluorescence change. Moreover, since the $K_{0.5}$ value measured at pH 7 in the absence of NaCl or LiCl is 10 times higher than that in their presence, it is concluded that Na^+ or Li^+ ions are better activators than H^+. Overall, the observed ionic dependency of the melibiose-induced change in permease fluorescence is reminiscent of that previously described for sugar binding to or transport by the transporter (Damiano-Forano et al., 1986; Bassilana et al., 1988).

Na^+- or Li^+-Induced Fluorescence Change in Proteoliposomes Incubated in Excess Sugar. Experiments were carried out on proteoliposomes equilibrated in the presence of a saturating concentration of melibiose (0.1 M), and the fluorescence changes produced by the addition of NaCl or

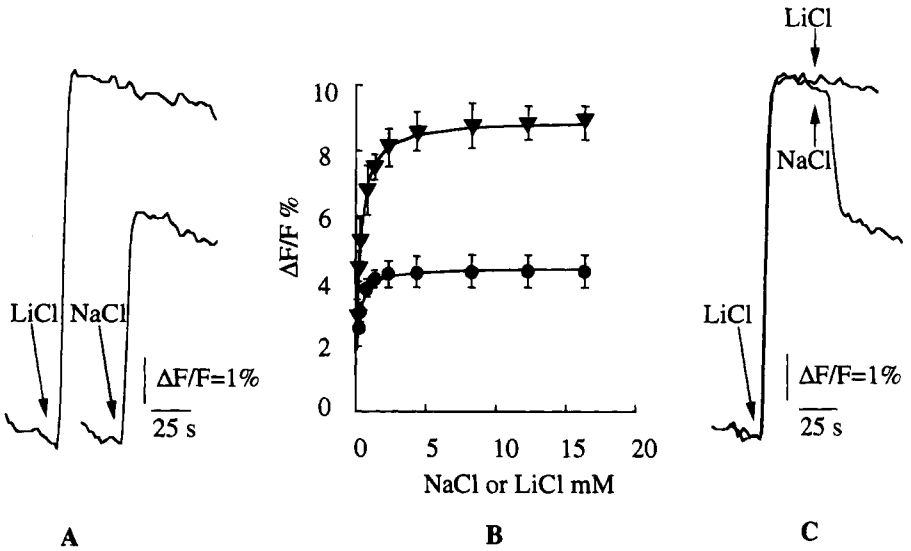

FIGURE 5: Potentiation of the melibiose-induced fluorescence changes by NaCl or LiCl. Proteoliposomes were prepared from the permease-rich fraction (90% Mel-6His permease) and equilibrated at 20 °C in 0.1 M KP_i (pH 7) buffer containing 0.5 M KCl and 0.1 M melibiose. Samples (2 mL, 20 μg of protein/mL) were illuminated at 297 ± 5 nm, and the variation in the fluorescence signal was measured as described in Figure 1 (inset). The following additions were made: (A) 20 mM NaCl or 20 mM LiCl; (B) variable NaCl (●) or LiCl (▼) concentrations were added and the $\Delta F/F$ was measured. Each point is the mean of four separate experiments (±SE). Plots gave a $K_{0.5}$ for the Na^+ activating effect of 0.13 ± 0.005 mM and a $K_{0.5}$ for the Li^+ activating effect of 0.22 ± 0.008 mM. (C) Addition of LiCl (4 mM) followed by the addition of NaCl (20 mM) (lower trace) or LiCl (20 mM) (upper trace).

Table 2: Correlation between NaCl or LiCl Concentrations Producing Half-Maximal Potentiation of the Melibiose-Induced Change in Fluorescence

activating cation	fluorescence $K_{0.5}^{Na^+}$ or $K_{0.5}^{Li^+}$ (mM)[a]	[^{3}H]αNPG binding K_{Na^+} or K_{Li^+} (mM)[b]
Na^+	0.1	0.3
Li^+	0.2	0.5

[a] Fluorescence measurements were carried out on proteoliposomes (20 μg/mL) equilibrated in 0.1 M melibiose in a medium containing 0.5 M KCl and 0.1 M KP_i (pH 7), and the effects of increasing concentrations of NaCl or LiCl were measured as described in Figure 5. The NaCl or LiCl concentration producing half-maximal changes in the fluorescence signal ($K_{0.5}^{Na^+}$ or $K_{0.5}^{Li^+}$) was computed from data presented in Figure 5B. [b] [^{3}H]αNPG binding was measured as a function of NaCl or LiCl concentration using samples containing 0.5 mg of protein/mL. Na^+ or Li^+ (K_{Na^+} or K_{Li^+}) was calculated as described by Damiano et al. (1986).

LiCl were analyzed. In the experiment illustrated in Figure 5A, the addition of 20 mM NaCl or LiCl induced a maximal increase effect of 5.2 or 8.4%, respectively, in the tryptophan fluorescence. Moreover, the fluorescence change saturates as a function of NaCl or LiCl concentration and can again be fitted by simple Michaelis–Menten equations (Figure 5B). A half-maximum change in the fluorescence signal ($K_{0.5}$) occurs at concentrations of Na^+ and Li^+ of 0.1 and 0.2 mM, respectively. Table 2 shows that these apparent potentiation constants are comparable to the activation constants (K_{Na^+} or K_{Li^+}) of NaCl or LiCl on sugar binding determined on the same samples. These data suggest that the $\Delta F/F$ change is produced by the interaction of only one ion with the transporter.

The fact that NaCl and LiCl elicit different $\Delta F/F$ values provides a means for analyzing the competitive interaction between the two activating cations. Figure 5C shows that the fluorescence change initially induced by 4 mM LiCl (20 times the Li^+ activation constant) can be partially reversed (35%) by adding a 7-fold excess of NaCl (20 mM, i.e., 200 times the Na^+ activation constant). On the other hand, addition of 20 mM LiCl has no effect. Moreover, the maximal fluorescence change reached upon adding 4 mM NaCl is increased by adding 20 mM LiCl, the final level being similar to that produced by the addition of LiCl alone (not shown). These data indicate that the NaCl- and LiCl-induced changes in fluorescence are not additive. Similar results were obtained with proteoliposomes incubated in excess TMG (not shown). These results suggest that the two cations compete for a common binding site on the permease and that they are exchangeable.

Direct Effect of Na^+ and Li^+ on the Mel Permease Intrinsic Fluorescence. The direct effects of sodium and lithium ions on the fluorescence properties of Mel-6His permease were studied in proteoliposomes equilibrated in [Na^+]- and [K^+]-free choline phosphate buffer. Free movement of Na^+ or K^+ across the membrane was ensured by adding valinomycin (2 μM), nigericin (0.75 μM), and monensin (0.75 μM). In the experiment shown in Figure 6A, the addition of 10 mM NaCl or LiCl produces limited but significant quenching of the fluorescence signal (2.3 or 1.6%, respectively). In contrast, no quenching above a dilution effect was observed upon adding 10 mM KCl (Figure 6A). Control experiments carried out on pure liposomes or proteoliposomes containing only the copurified contaminant proteins indicate that NaCl, LiCl, or KCl produces a quenching of fluorescence (0.25%) that has the magnitude of the dilution effect. These data suggest that the NaCl- or LiCl-induced fluorescence quenching is specifically an interaction of Na^+ or Li^+ with the permease and is not related to nonspecific effects on the contaminant proteins or membrane lipids (Akutsu & Seelig, 1981). When analyzed as a function of the concentration of NaCl or LiCl, the quenching phenomenon saturates and is best described by a Michaelis–Menten equation. The $K_{0.5}$ constants calculated from these curves are 1 mM for Na^+ and 3 mM for Li^+.

Effect of Substrates on the Mel Permease Intrinsic Fluorescence on Inhibition of the Transport Activity. It has been reported that the acylation of Mel permease in the

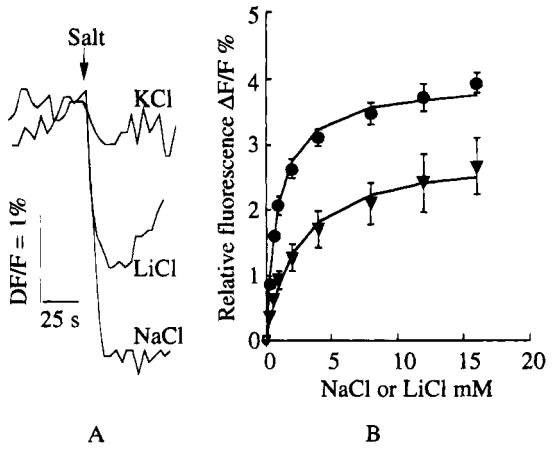

FIGURE 6: Direct effects of Na^+ or Li^+ cations on the Mel-6His permease intrinsic fluorescence. Proteoliposomes were prepared from the permease-rich fraction (90% Mel-6His permease) and equilibrated at 20 °C in 0.1 M choline phosphate buffer at pH 7. Samples (2 mL, 20 μg of protein/mL) were illuminated at 297 ± 5 nm, and the variation in the fluorescence signal was measured as described in Figure 1 (inset). (A) $\Delta F/F$ recorded upon the addition of KCl, LiCl or NaCl at a final concentration of 10 mM. (B) Dose–response curve of the NaCl (●) or LiCl (▼) induced $\Delta F/F$ change. Each point is the mean of four experiments (±SE). Plots gave $K_{0.5}$ of 1 ± 0.2 mM for NaCl and 2.9 ± 0.5 mM for LiCl.

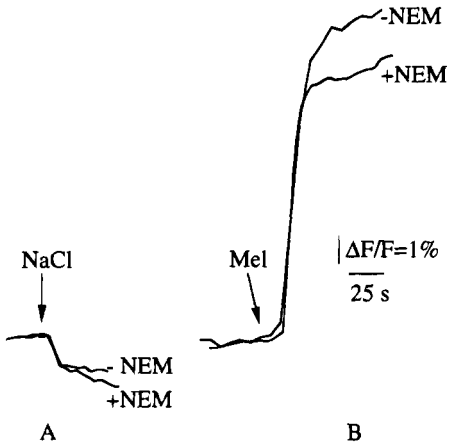

FIGURE 7: Effects of substrates on tryptophan fluorescence of proteoliposomes treated with *N*-ethylmaleimide. Proteoliposomes were prepared from the permease-rich fraction (90% Mel-6His permease) and equilibrated at 20 °C in 0.1 M choline phosphate buffer at pH 7 lacking NaCl or LiCl (A) or in 0.1 M KP_i 0.5 M KCl (pH 7) containing 10 mM NaCl (B). Half of the proteoliposomes in each condition was incubated for 15 min in the presence of 0.5 mM NEM at room temperature and the other half was used as controls. Samples (2 mL, 20 μg of protein/mL) were illuminated at 297 ± 5 nm, and the integrated fluorescence signal was measured as described in Figure 1 (inset). Traces show the $\Delta F/F$ recorded from NEM-treated or control proteoliposomes upon adding NaCl (10 mM) to proteoliposomes resuspended in 0.1 M choline phosphate buffer at pH 7 (A) or upon adding melibiose (10 mM) to proteoliposomes resuspended in a medium containing 0.1 M KP_i, 0.5 M KCl (pH 7), and 10 mM NaCl (B).

presence of *N*-ethylmaleimide (NEM) almost completely inactivated translocation of the substrates (>90%), while the Na^+-dependent sugar-binding capacity was reduced by only 10% (Lopilato et al., 1978; Damiano-Forano et al., 1986). A similar inactivation by NEM of energy-dependent melibiose transport was observed in proteoliposomes (not shown). As seen in Figure 7, the quenching of fluorescence produced by the addition of NaCl (Figure 7A) and the increase in fluorescence elicited by melibiose in the presence of NaCl (Figure 7B) or LiCl (not shown) are essentially similar before and after NEM treatment. The small reduction in fluorescence change may be attributed to a slight diminution of the total number of active sites during the NEM treatment. These experiments suggest that inhibition of substrate translocation by Mel permease has no effect on the fluorescence properties of the permease.

DISCUSSION

The present work shows that the tryptophan residues of Mel-6His permease constitute a powerful internal probe to monitor the interaction of the transporter with its cation and sugar substrates. The intrinsic fluorescence properties of the purified Mel permease reconstituted in proteoliposomes are selectively modified by the sugar and cation substrates of the transport reaction. Detailed examination of these fluorescence variations suggests that they may reflect conformational changes appearing upon the formation of the ternary (cation/sugar/protein) complex. Because Mel-6His and native Mel permeases have similar catalytic activities (Pourcher et al., 1995), we implicitly assume that their fluorescence properties are also similar.

The ability of given sugars and/or cations to elicit a change in Mel permease fluorescence closely correlates with their capacity to serve as substrates for the symport reaction or to activate it (Lopilato et al., 1978; Bassilana et al., 1985; Damiano-Forano et al., 1986). Only well-recognized sugar substrates of the permease such as the α-galactoside melibiose, raffinose, α-MG, or the β-derivative TMG produce significant changes in fluorescence. Analysis of these changes as a function of the structure of the different sugars tested suggests that the galactopyranosyl moiety and the configuration of the glycosidic linkage of melibiose are the molecular elements of the disaccharide structure that govern the magnitude of the fluorescence change. Finally, the differences in amplitude of the fluorescence change produced by these sugars seem to correlate better with differences in the permease affinity for these sugars than with differences in the rate at which they are transported. Indeed, Mel permease shows a greater affinity for binding α-derivatives (melibiose, raffinose, and α-MG) than it does for galactose or β-derivatives (TMG or β-MG). In contrast, the V_{max} of transport of the various sugars follows a different order (raffinose < melibiose ≤ TMG) (Bassilana et al., 1985; Wilson & Wilson, 1987).

In addition to displaying the same sugar selectivity pattern, the fluorescence and substrate-binding properties also exhibit the same ionic dependency. Although NaCl and LiCl, on their own, quench Mel permease fluorescence in a selective maner, they potentiate the fluorescence changes induced by sugars. The concentration of melibiose producing a half-maximal change in permease fluorescence ($K_{0.5}$) is nearly 10 times higher in the absence of NaCl and LiCl than in their presence (11 and 1.2 mM, respectively). The relationship of these $K_{0.5}$ values is comparable to that of the melibiose-binding constants measured in the corresponding medium (Table 1). Also, the Na^+ or Li^+ activation constants for melibiose binding to the permease (K_{Na^+} or K_{Li^+}) are similar to the $K_{0.5}$ values of the Na^+ and Li^+ potentiating effects on the sugar-dependent fluorescence change (Table 2). It should also be noted that NaCl and LiCl induce saturable quenching of Mel permease fluorescence with half-

maximal effects at a concentration 10 times higher than that of their potentiating effect on the sugar-dependent fluorescence change. This discrepency may be explained by assuming that sugar and cation interact with the permease in a cooperative fashion. Measurement of the binding characteristics of $^{22}Na^+$ on purified permease in proteoliposomes may help to advocate this interpretation rather than that of a nonspecific effect of the ions on Mel permease fluorescence. All of the observations discussed in this and the preceding paragraphs indicate a strong correlation between the substrate effects on the intrinsic fluorescence and the substrate-binding properties. Further support for this correlation is given by the observation that NEM-treated permeases, which retain 90% of the original substrate-binding activity but have an impaired substrate translocation (Damiano-Forano et al., 1986; Bassilana et al., 1987), exhibit fluorescence properties similar to those of unmodified permeases. The simplest explanation to account for all of these observations is that the sugar-induced fluorescence variations and the associated ionic dependency reflect events occurring during the initial step of the transport cycle, i.e., upon formation of the ternary cation/sugar/permease complex.

Three observations strongly support the hypothesis that the fluorescence increase monitors variations in transporter conformation induced by the coupling ion in the presence of the sugar: (1) NaCl and LiCl, on their own, quench Mel permease fluorescence; (2) in the absence of these cations, the sugar-induced fluorescence increase has a limited amplitude; (3) these ions potentiate the sugar-induced fluorescence increase. Moreover, the final level of fluorescence emission of the ternary complex recorded at a saturating concentration of sugar varies according to the coupling ionic species and increases in the order $H^+ < Na^+ < Li^+$. This observation is consistent with the view that the ternary complex adopts a different conformation when either H^+, Na^+, or Li^+ is a partner of the complex. This conclusion is in line with a previous suggestion derived from kinetic analysis of Mel permease (Pourcher et al., 1990a). In the same manner, the higher level of fluorescence emission recorded in the presence of α-derivatives relative to β ones, in either NaCl or LiCl medium, may signify that the conformation of the ternary complex is also dependent on the sugar structure. The existence of different conformational sugar/transporter complexes in the presence of different sugars has already been suggested for the human erythrocyte hexose transporter (Pawagi & Deber, 1990). Finally, the observations suggesting competition of the sugar and cation substrates for their respective binding sites (Figures 3 and 5) also suggest that, in the complex, sugar and cation are exchangeable rather than completely occluded. Taking together the interpretations of the Mel permease fluorescence changes given in this paper and a previous indication of Na^+-induced conformational change in the intestinal Na^+/sugar cotransporter (Peerce & Wright, 1987), one is tempted to propose that an ion-induced change in carrier conformation is a general phenomenon during ion-linked solute cotransport.

In the preceding discussion of the fluorescence properties of Mel permease, we have only considered the information provided by recording an integrated fluorescence signal and its variations at equilibrium. Analysis of the Mel permease fluorescence spectrum and spectral changes elicited by substrates may first give further insight into the transporter structure–function relationship. The peak emission fluorescence is centered at 337 nm, suggesting that many of the tryptophan residues of Mel permease are located in domains of reduced polarity (Burstein et al., 1973). This agrees with the putative location of five of the eight tryptophans in predicted transmembrane domains, three being distributed in the N-terminal half of the transporter and two in the C-terminal half (Botfield et al., 1992; Pourcher et al., unpublished experiments). Interaction of the cation with the permease would appear to attenuate preferentially the light emitted around 345 nm, whereas subsequent addition of the sugar preferentially enhances the light emitted at a lower wavelength. As previously proposed for D-glucose binding to the human erythrocyte hexose transport protein (Pawagi & Deber, 1990) and to the intestinal brush border Na^+/glucose cotransporter (Peerce, 1990), this suggests that one (or more) of the tryptophan residues of Mel permease may be shifted to a region less water-accessible upon substrate binding. Interpretation of the spectrum variations, however, is complicated by the presence of more than one tryptophan residue in the transporter. We intend to combine site-directed mutagenesis and fluorescence spectroscopy to identify the tryptophan residues that monitor the putative conformational changes elicited by the substrates.

Previous analyses of the intrinsic fluorescence properties of the lactose permease (Page, 1992), the L-arabinose-H^+, or the galactose-H^+ symporters of *E. coli* (Walmsley et al., 1993, 1994; Henderson et al., 1994) indicate that the study of the time course of substrate-induced variations in the fluorescence signal using stopped-flow fluorescence spectroscopy provides a means to identify catalytic intermediates of the transport cycle, as well as their rates of interconversion. It will be of interest to undertake similar transient kinetic studies to further dissect the ion-dependent sugar-induced fluorescence response exhibited by Mel permease to characterize the ionic selectivity properties of the transporter and the role of the ion in the coupling mechanism. In addition, this approach may also be useful to understand further the cation recognition defect produced upon mutating several aspartic residues of the N-terminal hydrophobic domains of Mel permease (Pourcher et al., 1991, 1993; Zani et al., 1993).

Finally, the intrinsic fluorescence characteristics of Mel permease are currently used as a tool to monitor the activity and stability of the detergent-solubilized permease used in crystallization assays.

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