Melibiose Permease of *Escherichia coli*: Structural Organization of Cosubstrate Binding Sites As Deduced from Tryptophan Fluorescence Analyses†

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ABSTRACT: Binding of the coupling ion (Na^+ or Li^+) and sugars to the purified melibiose permease of *Escherichia coli*, reconstituted in proteoliposomes, produces selective and cooperative changes of the transporter tryptophan fluorescence. To assess the individual contribution of N- or C-terminal domains of the permease to these substrate-induced fluorescence variations, we replaced the two tryptophans located in its C-terminal half (W299 and W342) by a phenylalanine and compared the signal change in mutants and wild-type permease. None of the mutations significantly impairs transport activity. Persistence of the ion-induced signal quenching in a permease carrying only the six other tryptophans of the N-terminal domain is consistent with a previous suggestion that this domain accommodates the ion-binding site. On the other hand, the sugar-induced fluorescence increase varies from mutant to mutant in a sugar-specific fashion. While α-galactosides increase essentially the fluorescence of W299 and W342, β-galactosides enhance the signal of W299 and of one (or more) of the N-terminal tryptophans but quench that of W342. Moreover, addition of sugars produces a 10 nm blue shift of both W299 and W342 emission spectra, suggesting reduced accessibility of these residues to solvent following substrate binding. These data suggest that W299 and W342 are at or close to the sugar binding site and that this latter is lined by the C-terminal helices IX and X. Moreover, as sugars with the β-configuration also enhance the fluorescence of the N-terminal tryptophans, it is suggested that one (or more) helix of the N-terminal half may be also at or near the sugar binding site. This implies close proximity and/or tight functional linkage between some N-terminal helices and helices IX and X of the C-terminal domain of the transporter.

The structural organization of polytopic membrane proteins of the Na^+/solute symporter family is largely unknown because of the difficulties encountered in purifying and crystallizing them. The melibiose permease (Mel permease)[1] of *Escherichia coli* is one of the few proteins from the Na^+/solute symporter family (Reizer et al., 1994) that has been purified to homogeneity in an active state and in amounts allowing structural studies (Pourcher et al., 1995).

Mel permease, encoded by *melB*, 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) and methyl β-D-galactopyranoside (βMG)], by a cation/sugar symport mechanism [Wilson et al., 1982; Pourcher et al., 1990a; see Poolman et al., (1996) for a recent review]. Depending on the ionic conditions and/or sugar substrate, sugar uptake is coupled in a 1/1 ratio to either H^+ (or H_3O^+), Na^+, or Li^+ entry. The coupling ions compete for the same binding site and enhance 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; Bassilana et al., 1988). Deductions from *mel*B sequence and analysis of the N-terminal polypeptide sequence indicate that Mel permease consists of 473 amino acids with 70% of apolar residues (Yazyu et al., 1984; Pourcher et al., 1995). Hydropathy profiling of the primary amino acid sequence and analysis of a series of *mel*B−*pho*A fusions suggest that Mel permease contains 12 membrane-spanning segments with α-helical configuration (Pourcher et al., 1990b, 1996; Botfield et al., 1992).

Although Mel permease is among the few well studied Na^+/sugar cotransporters (Poolman & Konings, 1993; Poolman et al., 1996), a precise localization of the ion and sugar binding sites is still lacking. Mutagenesis of a large number of residues of the N-terminal domain of the transporter (Pourcher et al., 1991, 1993; Wilson & Wilson, 1992; Zani et al., 1993, 1994) and studies of Mel permease chimeras (Hama & Wilson, 1993) favor the hypothesis that the ion recognition site is located in the N-terminal domain of the transporter. Less information is available concerning the localization of the sugar binding site.

Recently, intrinsic fluorescence spectroscopy of purified His-tagged Mel transporter reconstituted in liposomes has been used to investigate the effects of sugar and coupling ions on the conformation of Mel permease (Mus-Veteau et al., 1995). While Na^+ and Li^+, on their own, quench Mel permease fluorescence, sugars enhance it, and the intensity of this increase is significantly potentiated by addition of the monovalent ions. These and other results led to the suggestion that this fluorescence signal increase monitors

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[1] Abbreviations: Mel permease, melibiose permease; Trp, tryptophan; N-Trp, tryptophans from the N-terminal domain; WT, wild-type; αMG, methyl α-D-galactopyranoside; TMG, methyl 1-thio-β-D-galactopyranoside; βMG, methyl β-D-galactopyranoside; αNPG, *p*-nitrophenyl α-D-6-galactopyranoside; *K*P_i, potassium phosphate.

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changes of the transporter conformation associated with binding of the substrates. The Mel permease fluorescence signal is the integrated contribution of eight different tryptophans (Trp) and is therefore too complex to identify the Trp monitoring the conformational changes and associated catalytic domains.

Studies of other soluble or membrane proteins participating in transport functions have shown that these questions can be tackled by combining Trp engineering and analysis of the resulting mutants using fluorescence spectroscopy (Martineau et al., 1990; Menezes et al., 1990). This strategy is particularly suitable for Mel permease as its Trp are distributed in two distinct regions: six of them are in its N-terminal domain (N-Trp), and the two remaining ones are on helices IX and X in its C-terminal domain (W299 and W342) (Pourcher et al., 1996). In this study, we compare the transport, binding, and fluorescence properties of wild-type permease with those of site-directed mutants carrying individual or concomitant replacement of W299 (helix IX) and W342 (helix X) by a phenylalanine. The deduced fluorescence contributions of W299, W342, and N-Trp are used to speculate on the localization of the substrate binding sites in the permease and on their possible relationships.

MATERIALS AND METHODS

Materials. *p*-Nitrophenyl α-D-(6-^{3}H)galactopyranoside ([^{3}H]αNPG) was synthesized in our department under the direction of Dr. B. Rousseau. Synthesis of LAPAO ((3-laurylamido)-*N*,*N*′-(dimethylamino)propylamine oxide) was performed as described by Brandolin et al. (1980). Dodecyl maltoside was obtained from Boehringer Mannheim, and Ni-NTA resin was from Qiagen. Inc. Bio-Beads SM-2 were from Bio-Rad Lab., Inc. Total *E. 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 Strains. A $RecA^-$ derivative of *E. coli* DW2 (Δ*mel* Δ*lac*ZY; Botfield & Wilson, 1988) was transformed with pK95ΔAHB plasmids carrying either the wild type or a modified *mel*B gene. pK95ΔAHB was derived from the pK31ΔAHB (Pourcher et al., 1995) in which a unique *Eco*RI site was created in the intergenic sequence between *mel*A and *mel*B, 58 bases upstream from the *mel*B gene, using site-directed mutagenesis. The *Sty*I−*Hin*dIII DNA fragment from the *mel*B gene of the modified pK31ΔAHB was replaced by the homologous *Sty*I−*Hin*dIII fragment from a previously constructed plasmid carrying all the restriction sites (pK50; Zani et al., 1994). For the *in vitro* mutagenesis experiments, a recombinant plasmid (pBS95HB) was constructed by inserting a *Sma*I−*Hin*dIII DNA fragment carrying *mel*A and *mel*B gene sequences into the BlueScript II KS^+ plasmid (Stratagene).

Site-Directed Mutagenesis. Uracil-labeled single-stranded DNA was produced by transfecting *E. coli* CJ236 cells with pBS95HB, and site-directed mutagenesis was performed following to the Bio-Rad instructions. Independent mutations (in italics) were introduced using primers complementary to the sense strand of *mel*B except for mismatches indicated by asterisks: *W299F*: 5′-C CGA CGC ATT TTA T(GT*)(GT*) GCC GGA GCA TCT-3′; *W342F*: 5′-GA ACG GCG CTT TTC T(GT*)(GT*) GTA TTA CAG GTC A-3′. After primer annealing, enzymatic filling in, and ligation, the mutated strand was selected by degrading the uracil-labeled template with uracil−DNA glycosylase and was used to transform *E. coli* DW2-R. pBS95HB DNAs carrying individually mutagenized *mel*B genes were isolated. For each mutant, a *Kpn*I−*Eco*RV DNA fragment from pBS95HB was subcloned in pK95ΔAHB. The sequence of the replaced *Kpn*I−*Eco*RV DNA fragment in pK95ΔAHB was verified by dideoxy sequencing (Sanger et al., 1977; Sanger & Coulson, 1978).

Permease Activity. The three plasmids pK95ΔAHB-(W299F), pK95ΔAHB(W342F), and pK95ΔAHB(W299F/W342F) encoding one of the two single or the double mutant, respectively, were used to transform *E. coli* DW2 grown in M9 medium supplemented with carbon sources (Mus-Veteau et al., 1995). Permease activity was assayed qualitatively on McConkey plates containing 10 mM melibiose. Transport was measured by following the time course of [^{14}C]TMG and [^{3}H]melibiose (3 mCi/mmol) accumulation in intact cells by a fast filtration assay (Kaback, 1971). Binding of [^{3}H]αNPG to RSO membrane vesicles and proteoliposomes was assayed under nonenergized conditions using a flow dialysis procedure (Damiano-Forano et al., 1986).

Protein Purification and Reconstitution in Liposomes. Freshly transformed DW2-R/pK95ΔAHB(W299F), (W342F), and (W299F/W342F) cells were grown to $OD_{600} = 1.2$ at 30 °C in 6 L of culture as described previously (Mus-Veteau et al., 1995). Preparation of inverted membrane vesicles (IMV) was carried out by means of a French press (American Instrument Co., 18 000 psi). Purification of the His-tagged permeases was carried out by affinity chromatography on a Ni-NTA column (Qiagen) as described by Pourcher et al. (1995). The eluted fraction containing about 90% Mel permease was reconstituted in *E. coli* liposomes by detergent adsorption on polystyrene beads, as described by Mus-Veteau et al. (1995). Proteoliposomes were submitted to repeated freezing/thawing−sonication−wash cycles in nominally Na^+-free, 0.1 M potassium phosphate buffer ($K P_i$) (pH 7) to eliminate NaCl from the internal space. The amounts of Mel permease contained in the wild type and the three mutant proteoliposomes were estimated by Coomassie Blue-stained SDS/PAGE gel and by immunodetection with a serum containing antibodies directed against the COOH-terminal domain of Mel permease (Botfield & Wilson, 1989).

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

Fluorescence Measurements. Emission fluorescence spectra were recorded on a LS50B Perkin Elmer spectrofluorometer using excitation light at 297 ± 5 nm to minimize the contribution of tyrosine fluorescence (Eftink & Ghiron, 1976). Each spectrum is the means of three scans using 0.5 nm step intervals with an integration rate of 1 s/nm. Time-dependent fluorescence variations were analyzed on a MOS-200 spectrofluorometer (BioLogic) as previously described by Mus-Veteau et al. (1995). Excitation was set at 297 ± 5 nm and the integrated light, emitted between 310 and 400 nm, recorded. Measurements were carried out on briefly sonicated samples (20 μg of protein/mL) resuspended in 0.1 M $K P_i$ (pH 7) containing NaCl or LiCl salts as indicated.

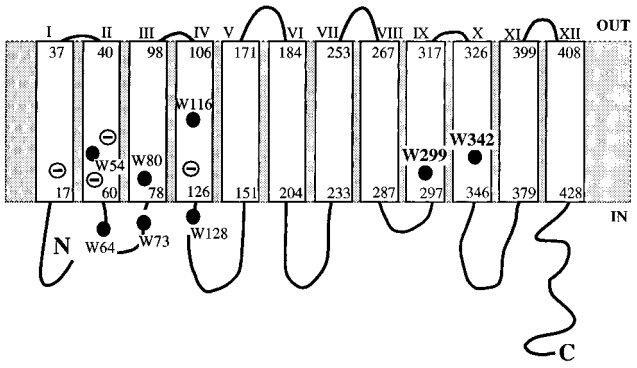

FIGURE 1: Secondary structure model of Mel permease with the location of its 8 tryptophan residues highlighted (filled circles). Topological data are derived from a site-directed *mel*B−*pho*A fusion analysis (Pourcher et al., 1996). Bottom and top numbers correspond to the first and last residues of the membrane-spanning segments numbered with Roman numerals. Circled negative charges indicate the position of aspartic acid residues putatively involved in binding of the coupling ions.

All experiments were performed with samples maintained at 20 °C and constantly stirred. To increase reproductibility, all additions were made without removing the cell from the holder. $\Delta F/F$ values are relative fluorescence changes calculated from three repeated experiments (means ± SEM), ΔF is the fluorescence variation intensity observed upon addition of the substrate, and F is the initial fluorescence intensity of the sample.

RESULTS

Construction and Activities of W299F, W342F, and W299F/W342F Mutants. The contributions of Mel permease tryptophans, W299 in helix IX and W342 in helix X (Figure 1), to the fluorescence changes induced by substrate binding to the transporter were investigated by analyzing the spectroscopic properties of permeases in which each Trp was replaced by a phenylalanine. Following *in vitro* site-directed mutagenesis of *mel*B in plasmid pB95HB, an *Eco*RI/*Kpn*I DNA fragment, carrying either single mutation or the double mutation, was inserted in place of the corresponding fragment in the wild-type (WT) *mel*B of pK95ΔAHB. The sequence of the inserts was verified by dideoxy sequencing to establish that no other modification had been introduced. The resulting single and double mutants were named W299F, W342F, and W299F/W342F, respectively.

The catalytic activities of WT and each of the mutant permeases were first compared by examining melibiose utilization by DW2-R(*mel*A$^+$, Δ*mel*B, Δ*lac*ZY) cells transformed with each plasmid. When plated on MacConkey agar supplemented with 10 mM melibiose, all transformed cells gave rise to dark red colonies, indicating that none of the mutations prevents melibiose uptake. The mutant transport properties were characterized more directly by measuring the rate and extent of cell accumulation of radiolabeled melibiose or TMG and its dependence on NaCl or LiCl. Typically, accumulation of both sugars by cells expressing WT permease was low in NaCl (or LiCl)-free media but was stimulated 5−10-fold in the presence of these salts at a final concentration of 10 mM (Bassilana et al., 1987; Table 1). In addition, Table 1 also shows that independent or concomitant mutagenesis of W299 and W342 did not significantly impair NaCl- or LiCl-dependent accumulation by Mel permease.

Table 1: Na^+, Li^+ and H^+-Dependent Melibiose (or TMG) Transport by Bacteria Expressing WT, W299F, W342F, or W299F/W342F Permease[a]

	sugar uptake [nmol/(mg of protein·min)]				
	melibiose			TMG	
Mel permease	Na^+	Li^+	H^+	Na^+	Li^+
WT	24	9	6	60	45
W299F	21	15	6	75	42
W342F	33	27	6	48	42
W299F/W342F	30	24	6	42	27

[a] *E. coli* DW2-R (*mel*A$^+$, Δ*mel*B, Δ*Lac*ZY) were transformed with pK95ΔAHB plasmids harboring the native or mutated *mel*B gene. Cells were grown in M9 medium supplemented with 5 g/L glycerol, 2 g/L casamino acids, and 0.1 g/L ampicillin at 30 °C, washed, resuspended at 1.5 mg/mL in a medium containing 0.1 M *K*P$_i$ (pH 7) and less than 20 μM of contaminating sodium salts, and incubated at 20 °C. H^+-coupled melibiose transport measurements were carried out in this medium. Na^+ or Li^+ linked sugar transport activities were measured in this medium supplemented with NaCl or LiCl added to a final concentration of 10 mM. Transport reactions were initiated by the addition of [^{3}H]melibiose (20 mCi/mmol) or [^{14}C]TMG (3 mCi/mmol) at a final concentration of 0.2 mM, terminated by dilution of the reconstitution in liposomes [a].

Table 2: Sugar-Binding Characteristics of WT and Mutant Permeases after Reconstitution in Liposomes[a]

		[^{3}H]αNPG binding		
Mel permease proteoliposomes	B_{max} (nmol/mg of protein)	$K_D^{\alpha NPG}$ (μM)	K_i^{mel} (mM)	K_i^{TMG} (mM)
WT	6	0.6	1	3
W299F	4	1	2	4
W342F	7	1.2	1	2
W299F/W342F	3	1.8	1.4	2

[a] Proteoliposomes containing WT or mutant permeases (0.2−1 mg/mL) were equilibrated in 0.1 M *K*P$_i$ (pH 7), 10 mM NaCl, 5 μM FCCP, and 0.75 μM monensin. [^{3}H]αNPG (1.6 mCi/mmol) binding was measured as a function of free αNPG concentration (1−250 μM) using a flow dialysis technique (Botfield & Wilson, 1988). The maximal number of active sites (B_{max}) and the apparent αNPG binding constant (K_D) were calculated from Scatchard plots of the binding data. The apparent inhibitory constant for αNPG binding by melibiose (K_i^{mel}) or TMG (K_i^{TMG}) was estimated from the variation in the K_D for αNPG as a function of melibiose or TMG concentration (0.1−20 mM).

The three mutant proteins were purified by affinity chromatography and reconstituted in liposomes using *E. coli* lipid extract as previously described for wild-type Mel permease (Mus-Veteau et al, 1995). In each case, analysis of the purified fractions by SDS/polyacrylamide gel electrophoresis showed a major band (90%) migrating like wild-type Mel permease (data not shown). Reconstitution was adjusted to yield similar permease contents in the different proteoliposome preparations, as judged by protein determination and immunoblot analysis with a serum containing antibodies directed against the COOH-terminal domain of Mel permease (Pourcher et al., 1992) (data not shown).

The affinities of the different mutants for the sugars were estimated by measuring the binding of a high affinity ligand [^{3}H]αNPG in the presence of 10 mM NaCl in the medium (Damiano-Forano et al., 1986). Table 2 shows that the K_D value for αNPG binding on W299F or W342F permease is only twice that of the WT permease and at most 3 times higher in the case of the double mutant. Also, progressive displacement of [^{3}H]αNPG bound to each of the three mutant proteoliposomes by increasing concentrations of melibiose or TMG indicated that the respective inhibition constants

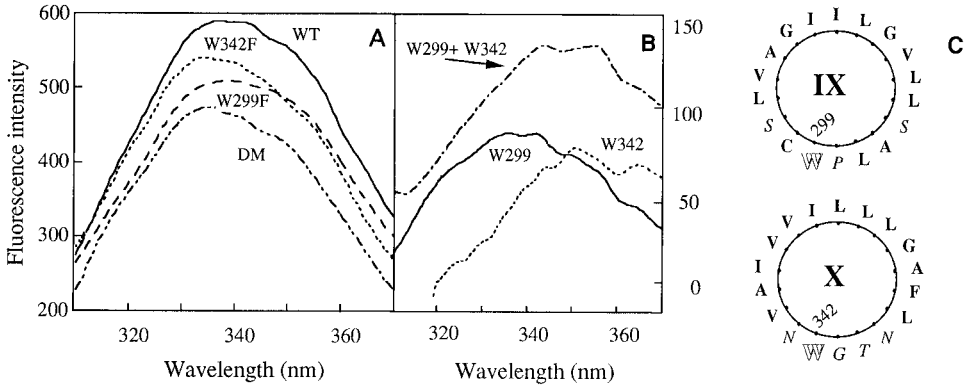

FIGURE 2: Fluorescence emission spectra of wild-type, W299F, W342F, and W299F/W342F Mel permeases and deduced spectral properties of W299 and W342. (A) Proteoliposomes containing WT, W299F, W342F, or W299F/W342F Mel permease (20 μg of protein/mL) were equilibrated in 0.1 M KP_i (pH 7) containing 10 mM NaCl and excited at 297 $\pm$ 5 nm. All fluorescence emission spectra were recorded from samples containing a constant protein concentration and also at a constant OD ($OD_{400} = 0.19$) to minimize interference on the signal due to variations of liposome concentration in the samples. Each spectrum is the means of three scans. (B) The individual contribution of W299 or W342 to the emitted fluorescence was calculated from the difference between WT and W299F or W342F spectra, respectively. The additive contribution of W299 and W342 was computed from the difference between WT and the double mutant spectra. (C) Wheel projection of helices IX (top) or X (bottom) containing W299 or W342, respectively (Pourcher et al., 1996). Trp are indicated in outlined letters. Polar and apolar residues are indicated by italics and bold letters, respectively.

K_i^{mel} and K_i^{TMG} were similar in WT or mutants. Therefore, none of the substitutions drastically impairs sugar recognition by the permease. Table 2 also indicates that the maximal number of binding sites of W342F proteoliposomes is similar to that of the WT proteoliposomes but is significantly decreased in the W299F and the double mutant proteoliposomes (66% and 50%, respectively). Since immunoblot analysis showed that the amounts of Mel permease were the same in WT and mutant proteoliposomes, the overall data suggest that replacement of W229 by a phenylanaline affects the stability of the transporter. The difference in contents of active permeases in the different proteoliposome preparations was taken into account in the comparative analysis of the fluorescence properties of the mutated transporters.

Spectral Properties of W299F and W342F Mutants in the Absence of Substrate. The intrinsic fluorescence properties of WT and mutant permeases were first compared in the absence of ligands (Figure 2A). At a constant protein concentration, the overall fluorescence signals recorded from each of the single mutants or from the double mutant are about 10% or 20% lower than that of WT, respectively. The light emitted by W299F proteoliposomes has a broad maximum around 335−350 nm while that of W342F proteoliposomes is shifted toward lower wavelengths, with a maximum centered around 333 nm. The individual contribution of either W299 or W342 to the fluorescence signal of Mel permease can be deduced from the difference between the spectrum of WT and that of the corresponding single mutant, assuming that the fluorescence of Mel permease is entirely due to emission from Trp and that there is no energy transfer between them (see Martineau et al., 1990). As expected, the extracted spectra differences appear as normal Trp spectra with maxima between 300 and 360 nm (Lehrer & Fasman, 1967; Burstein et al., 1973) (Figure 2B). The fluorescence maximum of W299 is at about 335 nm (spectrum 1) and that of W342 is at around 350 nm (spectrum 2), suggesting that W342 is in a more polar environment than W299. Essentially similar conclusions were drawn by analyzing the differences between the double and single mutant spectra. These data are consistent with the proposed environment of these two Trp on their respective membrane-spanning segments. Indeed, the wheel projection of helices IX and X suggests that W299 is surrounded by more apolar residues than W342. Finally, the maximum emission of the double mutant around 333 nm (Figure 2A) suggests that many of the six N-Trp are in an apolar environment.

Effect of Substrates on the Mutant Fluorescence Properties. We reported previously that addition of 10 mM NaCl or LiCl induces a 2% quenching of the fluorescence signal from proteoliposomes containing WT permease (Mus-Veteau et al., 1995). Similar limited signal quenching is observed with all three mutant permeases constructed in this study (data not shown), indicating that the N-Trp but not W299 or W342 participate in the ion-induced fluorescence variations.

The effect of sugars on the fluorescence properties of WT and mutant proteoliposomes incubated in the presence of 10 mM NaCl was assessed qualitatively by recording the spectra of the different permeases before and after addition of a saturating concentration of either melibiose or TMG (Figure 3). Figure 3 indicates that melibiose produces a much larger increase of the fluorescence signal in WT proteoliposomes (3A) than in any of the three mutant proteoliposomes (3B, 3C, and 3D). The relative variations of the fluorescence signal integrated between 310 and 400 nm ($\Delta F/F$, %) were used to quantify these changes (Mus-Veteau et al., 1995). When normalized with respect to the variations of Trp number and active permease contents in each proteoliposome sample, the melibiose-dependent $\Delta F/F$ change observed with either of the single mutants is about 40% of the WT signal change. In the double mutant, it is only 11% that of the WT signal (Table 3). These data indicate additive contributions of W299, W342, and N-Trp to the overall fluorescence variation induced by melibiose, the contribution of the former Trp being the largest. The absolute magnitude of the individual contributions was however much more difficult to ascertain. This is illustrated by examining the melibiose-induced fluorescence variations of the different permeases in the presence of NaCl (Table 3, 1st column). The calculation of the contribution of W299 and W342 would be overestimated or underestimated depending on the reference used (WT or double mutant) due to the errors ac-

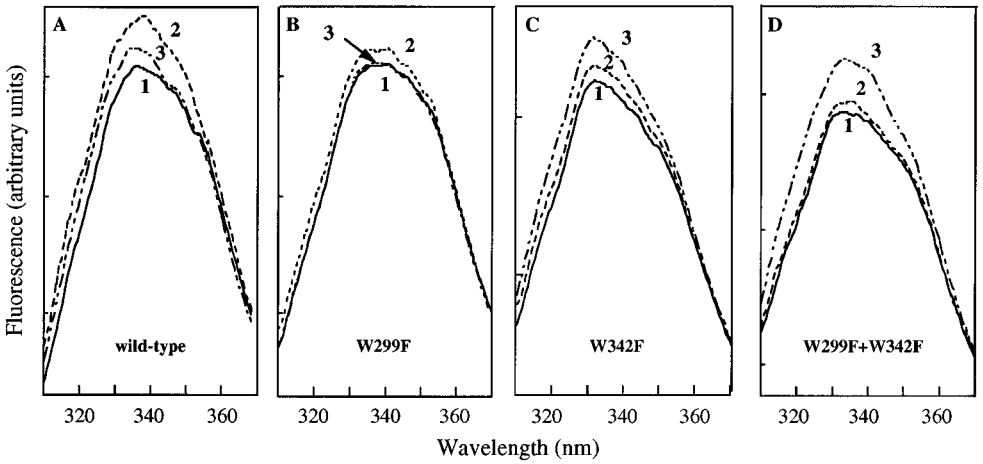

FIGURE 3: Effects of melibiose or TMG on the fluorescence spectrum of proteoliposomes containing WT (A), W299F (B), W342F (C), and W299F/W342F (D) permeases. Proteoliposomes containing wild-type or mutant permeases (20 μg/mL) were equilibrated at 20 °C in 0.1 M *K*P$_i$ (pH 7) and 10 mM NaCl. After excitation at 297 ± 5 nm, the emission spectra were recorded between 310 and 370 nm, in the absence of sugar (1), in the presence of 20 mM melibiose (2), or in the presence of 20 mM TMG (3). Spectra were not corrected for the dilution effect produced on adding the sugars (1%). Each spectrum is the means of three scans.

Table 3: Melibiose- and TMG-Induced Variations of WT and Mutant Permease Fluorescence[a]

Mel permease proteoliposomes	fluorescence variations depending on the substrate added ($\Delta F/F$, %)			
	Mel/Na^+	Mel/Li^+	TMG/Na^+	TMG/Li^+
WT	6.8 ± 0.8	8.1 ± 1	2.9 ± 0.3	4.7 ± 0.8
W299F	3 ± 0.4	3.8 ± 0.8	1.7 ± 0.4	2.3 ± 0.5
W342F	2.5 ± 0.2	3.6 ± 0.5	5.8 ± 0.4	7.1 ± 0.6
W299F/W342F	0.8 ± 0.1	1.8 ± 0.2	5.1 ± 0.6	7 ± 0.7

[a] Proteoliposomes containing WT, W299F, W342F, and W299F/W342F mutant permeases (2 mL, 20 μg/mL) were equilibrated at 20 °C in 0.1 M *K*P$_i$ (pH 7) and 10 mM NaCl or LiCl. 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 fluorescence changes (ΔF) induced by addition of sugar (melibiose or TMG) at a final concentration of 20 mM were expressed as $\Delta F/F$ (in %). Each of the mutant $\Delta F/F$ values were corrected for variation of the number of Trp and of proteoliposomes active permease contents. Values are the means of three experiments.

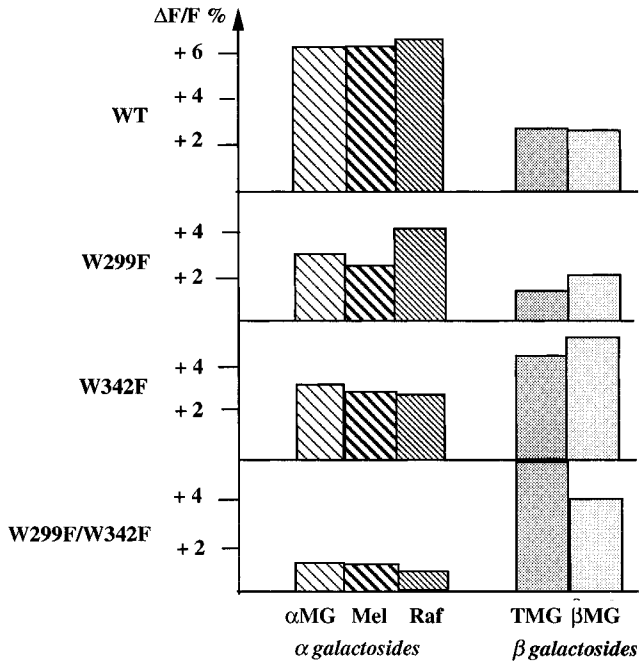

FIGURE 4: Effect of α- and β-galactosides on the intrinsic fluorescence of proteoliposomes containing WT, W299F, W342F, and W299F/W342F permeases. Proteoliposomes (20 μg/mL) were equilibrated at 20 °C in 0.1 M *K*P$_i$ (pH 7) and 10 mM NaCl. After excitation at 297 ± 5 nm, the emitted fluorescence light (F) was integrated between 310 and 400 nm and recorded as a function of time. The fluorescence change (ΔF) induced by addition of the different sugars at a final concentration of 15 mM for α-galactosides (α-MG, melibiose, and raffinose) or 35 mM for β-galactosides (β-MG and TMG) was expressed as $\Delta F/F$ (in %). Each of the mutant $\Delta F/F$ values were corrected for variation of the number of Trp and of proteoliposome active permease contents. Values are the means of three experiments.

companying the corrections applied to the mutant signals (protein concentration and active permease contents). Irrespective of the mode of calculation, however, it should be noticed that the contribution of the C-terminal Trp (W299 *plus* W342) is always proportionally higher than that of the N-Trp, a fact that agrees with the conclusion that melibiose perturbs preferentially the C-terminal Trp, W299, and W342 (Figure 4).

On its own, TMG increases WT permease fluorescence to a lesser extent than does melibiose (Figure 3A, Table 3). The signal enhancement produced in the W299F mutant is 50% lower than that observed in WT permease (Figure 3B, Table 3), indicating that W299 contributes to the fluorescence increase produced by TMG. Unexpectedly, the signal increase produced by TMG in the W342F mutant (Figure 3C) or the double mutant (Figure 3D) is twice as large as that elicited by this sugar in WT permease. Taking into account that, in both the W342F and the double mutant, W342 is replaced by a phenylalanine, these effects as well as the sequence of $\Delta F/F$ variations produced by TMG (W342F > double mutant ≫ WT > W299F) are best explained by assuming that the W342 fluorescence is quenched by TMG (Figure 4). This observation suggests that W342 fluorescence is affected in a different fashion upon interaction of melibiose or TMG with the permease. In addition, it is observed that TMG produces a much larger variation than melibiose in the double mutant which carries only Trp from the N-terminal domain of the permease (Figure 4). This finding suggests that pertubation of the N-Trp fluorescence is also sugar-specific. In this context, it is important to note that other sugars which carry a glycosidic linkage in the α-configuration (αMG or raffinose) behave like melibiose, whereas βMG, a methyl analog harboring a

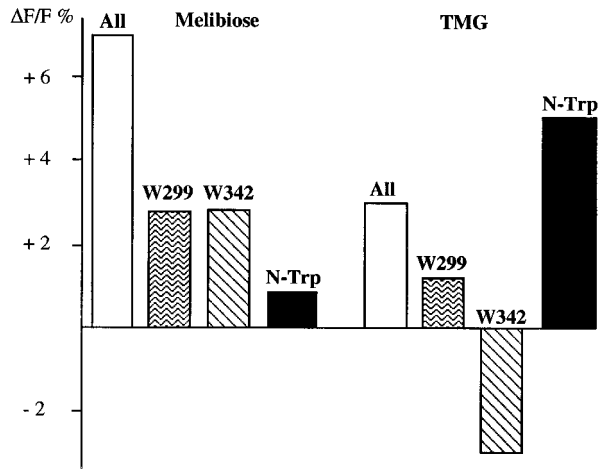

FIGURE 5: Deduced contributions of W299, W342, and the cluster of six Trp of the N-terminal domain to the Mel permease fluorescence changes induced by melibiose or TMG. Contributions of W299 and W342 were calculated from the data given in Table 3 and using the signal change observed in either WT or double mutant permease as a reference. The magnitude of W299 or W342 contribution given in this figure is the mean value of the individual contributions calculated by using the two different references (see text). The contributions of all Trp (all) and of the N-terminal Trp (N-Trp) of the permease correspond to the $\Delta F/F$ given in Table 3 for WT and double mutant permeases, respectively.

glycosidic linkage in the β−configuration, has TMG-like effects (Figure 5). Taken together with the other results, the contrasted effects of the anomers αMG and βMG conclusively demonstrate that the orientation of the glycosidic linkage is a determining factor in the fluorescence response. As previously described for WT Mel permease (Mus-Veteauet al., 1995), the addition of a nontransported sugar such as sucrose has no effect on the fluorescence signals of the three mutant proteins, showing that the fluorescence variations observed are sugar-selective (data not shown).

The following two observations provide further characterization of the fluorescence response of WT and mutant permeases to the addition of sugars. In a first series of experiments, the spectra of WT and mutant proteoliposomes resuspended in Na^+ medium were recorded before and after addition of sugars (see Figure 2B). The difference was used to assess the resulting modification of the spectral properties of the Trp at positions 299 or 342 or in the N-terminal domain. The data indicate that interaction of melibiose or TMG with the permease induces about the same blue shift (10 nm) of the emission spectra of W299 and W342, the maximum of the W299 spectrum being shifted from 336 to 325 nm and that of W342 from 351 to 341 nm (data not shown). Although the fluorescence variations are less marked in the absence Na^+, similar sugar-induced spectral shifts were observed with H^+ acting as coupling ion. These observations are consistent with a decreased accessibility to the solvent of W299 and W342 upon sugar binding to either H^+ or Na^+ coupled Mel permease. On the other hand, no significant wavelength shift is associated with the increase of N-Trp fluorescence in the double mutant. In a second set of experiments, we observed that the sugar-induced variations of fluorescence recorded in every mutant are systematically higher by about 20−40% in the presence of LiCl than in the presence of NaCl (Table 3, see also Mus-Veteau et al., 1995). Moreover, all mutants display melibiose- or TMG- induced fluorescence changes that saturate as a function of the NaCl or LiCl concentration, the half-maximum effect being reached at a salt concentration similar to that previously reported for wild-type permease (0.1−0.2 mM). These findings suggest that none of the mutations significantly modifies activation of sugar binding to the permease by the monovalent ions.

DISCUSSION

The experiments reported above detail the relative contributions of Trp located either in the C-terminal domain of *E. coli* Mel permease (W299 and W342) or in the N-terminal one (N-Trp) to the changes in fluorescence properties of the transporter occurring upon binding of the coupling ion and sugar substrate to the permease (Mus-Veteau et al., 1995). Comparison of the fluorescence properties of Mel permease carrying either a Trp or a Phe at positions 299 and 342 provides information on the localization of its ion and sugar binding domains and on their possible relationships.

Characterization of the activities of the single mutants W299F and W342F and of the double mutant indicates that the Na^+- or Li^+-activated sugar binding and transport activities of Mel permease are not significantly modified upon mutagenesis of these two Trp (Tables 1 and 2). These data indicate that neither W299 nor W342 is directly involved in catalysis and, in particular, sugar binding, as is the case for several sugar-binding proteins in bacterial members of the ABC transporter family (Vyas et al., 1982; Quiocho et al., 1987; Martineau et al., 1990). Consequently, the change in the fluorescence properties induced by the substrates must reflect local, or more extensive, structural reorganization of the transporter during substrate binding. In addition, examination of the fluorescence emission spectra of W299 and W342 (Figure 2B) suggests that W299 is in a more apolar environment than is W342. These data are in good agreement with predictions from the secondary structure model of Mel permease (Pourcher et al., 1996). Wheel projection of the two putative helical membrane-spanning segments IX and X carrying W299 and W342, respectively, indeed suggests that W299 is on a more apolar helical sector than W342 (Figure 2C).

The similarity of the quenching of the fluorescence signal induced by Na^+ and Li^+ in WT and mutant permeases (about 2%) suggests that this effect is restricted to perturbation of one (or more) Trp of the N-terminal domain. This suggestion is consistent with the hypothesis that the N-terminal domain of Mel permease accommodates at least part of the cationic binding site (Pourcher et al., 1993; Zani et al., 1993; Wilson & Wilson, 1994). Whether quenching results from direct interaction of the coupling ion with one or more N-Trp and/or from an ion-dependent conformational readjustment of the N-terminal domain of Mel permease remains to be determined. The report that replacing W54 by an alanine had no effect on the ion selectivity and transport properties of Mel permease excludes direct participation of W54 in the ion binding process (Hama & Wilson, 1994). Mutagenesis of the other N-Trp and characterization of the activities and spectral properties of the corresponding mutants will be useful for evaluating their catalytic importance as well as their participation in the quenching of the fluorescence signal.

The most marked variation of WT permease fluorescence is the increase occurring upon addition of sugars (Mus-Veteau et al., 1995). The magnitude of the change is sugar-specific (melibiose >TMG) and is potentiated by the

monovalent cations ($Li^+ > Na^+$). Comparison of the relative fluorescence variations ($\Delta F/F$) observed for WT and for the three mutant permeases enables the deduction of the relative contributions of W299, W342, and N-Trp to the fluorescence variation occurring in WT permease (Figure 4). This analysis shows that (1) W299 and W342 contribute most to the melibiose-induced fluorescence change elicited in WT permease, and (2) TMG binding also perturbs W299 and W342, although in a different manner from melibiose: it increases the W299 fluorescence while quenching that of W342. The opposite effect of melibiose and TMG on W342 fluorescence is related to the α to β change of the glycosidic linkage configuration as it is also observed when using the two sugar analogs (αMG and βMG) which differ only in the configuration of the glycosidic linkage. In addition to these findings, it was also observed that the maximum of the emission spectrum of both W299 and W342 is shifted by 10 nm toward lower wavelengths upon interaction of either melibiose or TMG with the permease, suggesting that interaction of either α- or β-galactosides with the permease reduces the accessibility of these two Trp to the solvent. All these data can be simply explained by assuming that W299 and W342, although not involved in catalysis, are at or close to the sugar binding site. This suggests that the sugar binding site may be lined by the two C-terminal helices IX and X. Studies of other transporters suggest that their C-terminal domains may also be involved in sugar recognition. For example, Chin et al. (1992) interpreted the reduction of accessibility to water of Trp in helices X and XI of the human erythrocyte glucose transporter upon D-glucose binding as an indication of their proximity to the sugar binding site. Also, several studies of Lac permease of *E. coli* suggest that the C-terminal domain of the transporter contains most if not all essential structural components associated with high affinity ligand binding [see Wu et al., (1996) and references therein].

Analyses of the double mutant properties established that the fluorescence emission of one or more N-Trp also increases in a fashion dependent on the configuration of the sugar glycosidic linkage. Thus TMG causes a far greater increase of the fluorescence emission of one or more of these N-Trp than does melibiose (Figure 4). This observation suggests that one (or more) helix of the N-terminal domain is also at or close to the sugar binding site. If this is the case, this would imply close proximity of some N-terminal helices to helices IX and X of the C-terminal domain of Mel permease. Lines of evidence consistent with a proximity of the N- and C-domains in the Lac permease have been recently discussed by Kaback and co-workers (Frillingos & Kaback, 1996). Such a structural organization of the sugar binding site in Mel permease would readily explain that a simple change of orientation of the oxygen (or sulfur) atom of the glycosidic linkage from an α- to a β-configuration could reduce or even reverse the fluorescence contribution of C-terminal W342, located in a given area of the sugar binding pocket, and, at the same time, recruiting that of one (or more) Trp located in the N-terminal domain. These observations could mean that part of the N-terminal domain could also line the sugar binding site, which is in keeping with the identification of two major clusters of residues critical for sugar specificity located in or near the cytoplasmic extremity of the four N-terminal helices and in the cytoplasmic loop flanking helix X, respectively (Botfield et al., 1988; Zani et al., 1994; review in Poolman et al.,1996). However, the fact that β-analog binding leads to a reduced accessibility of the C-terminal residues W299 and W342, but not that of the N-Trp, suggests that the N-terminal domain does not really participate in the sugar binding site. It should be stressed that the data presented here were not discussed in terms of a kinetic transport model containing two different and similtaneously accessible sugar binding sites as envisioned for Lac permease (Loklema & Walz, 1990; Wu & Kaback, 1994; Wu et al., 1996; Frillingros & Kaback, 1996). At the present time, no experimental evidence supports such a mechanistic model for Mel permease.

It has been previously suggested that the N-terminal domain accommodates at least part of the ion recognition site (Pourcher et al., 1993; Zani et al., 1994; Hama & Wilson, 1993). The hypothesis that some N-terminal domains may be at or close to the sugar recognition site raises the possibility of an overlap between the ion and sugar binding sites. Such an overlap would account for the high frequency of single mutations producing simultaneous alteration of the sugar and ionic recognition properties of Mel permease, a phenomenon probably reflecting cooperative interactions between cation and sugar binding processes (Botfield & Wilson, 1989; Zani et al., 1994; Mus-Veteau et al., 1995). In this context, it is worth recalling that the magnitude of the relative contribution to the fluorescence variations induced by sugars depends on the ion activating strength, whereas the reduction of accessibility to solvent of W299 and W342 induced by sugars does not depend on this activating strength, as is observed with permeases using both H^+ or Na^+. Furthermore, these two events can happen in sequence, as one can first record the fluorescence shift upon addition of melibiose to proteoliposomes in a Na^+-free medium and then measure the fluorescence increase after addition of NaCl. These data are consistent with the notion that both the sugar and the activating ions participate in the structural reorganization of the sugar binding site that takes place during the substrate binding process.

In conclusion, the data presented here are consistent with a structural model in which the two C-terminal helices IX and X and possibly domains of the N-terminal half are at or close to the sugar binding site of Mel permease, implying that they are close to each other. However, additional or alternative contributions of long range effect on the conformation of Mel permease cannot be excluded. Transient kinetic analysis of the fluorescence changes in these mutants will provide valuable complementary information regarding the conformational events monitored by the N- and C-termini fluorescent reporters. In addition, mutagenesis of Trp from the N-terminal domain of Mel permease will help to identify the Trp(s) involved in the ion-induced fluorescence quenching and the Trp(s) responsible for the β-galactoside-dependent signal increase as well as the related structural events.

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