Evidence for a Role of Helix IV in Connecting Cation- and Sugar-Binding Sites of *Escherichia coli* Melibiose Permease†

Emmanuelle Cordat,‡ Gérard Leblanc, and Isabelle Mus-Veteau*

Laboratoire des Membranes Cellulaires (Bat. Jean Maetz), Université de Nice/Sophia-Antipolis, CNRS (ERS 1253), LRC-CEA 16V, 06238 Villefranche Sur Mer Cedex 1, France

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ABSTRACT: To improve the structural organization model of melibiose permease, we assessed the individual contributions of the N-terminal tryptophans to the transporter fluorescence variations induced by the binding of cations and β-configured sugars, by replacement of the six N-terminal tryptophans by phenylalanines and the study of the signal changes. Only two mutations, W116F located in helix IV and W128F located in the cytoplasmic loop 4−5, impair permease activity. The intrinsic fluorescence spectroscopy analysis of the other mutants suggests that W54, located in helix II, W116, and W128 are mostly responsible for the cation-induced fluorescence variations. These tryptophans, W116 and W128, would also be responsible for the β-galactoside-induced fluorescence changes observed in the N-terminal domain of the transporter. The implication of W116 and W128 in both the cation- and β-galactoside-induced fluorescence variations led us to investigate in detail the effects of their mutations on the functional properties of the permease. The results obtained suggest that the domains harboring the two tryptophans, or the residues themselves, play a critical role in the mechanism of Na^+/sugar symport. Taken together, the results presented in this paper and previous results are consistent with a fundamental role of helix IV in connecting cation- and sugar-binding sites of the melibiose permease.

The melibiose permease (MelB)[1] of *Escherichia coli*, encoded by *mel*B (*1*), belongs to the superfamily of Na^+-solute symporters (*2*). MelB catalyzes the cotransport of Na^+, Li^+, or H^+ and α- or β-galactosides (reviewed in ref *3*) and has been shown to be solely responsible for melibiose transport (*4*). Extensive investigations of the kinetic properties of MelB demonstrated that MelB function depends on the nature of the coupling cation, Na^+ and Li^+ being better activators than H^+ (reviewed in ref *5*). On the other hand, little is known about MelB structure. A topological model proposed for MelB, consisting of 12 transmembrane segments (TMS)[1] with α-helical configuration and amino-, carboxyl-terminus located in the cytoplasm, is supported by *melB-phoA* fusion analyses (*6*, *7*), anti-C-terminus polyclonal antibodies binding experiments (*8*), and proteolytic mapping (*9*) (Figure 1).

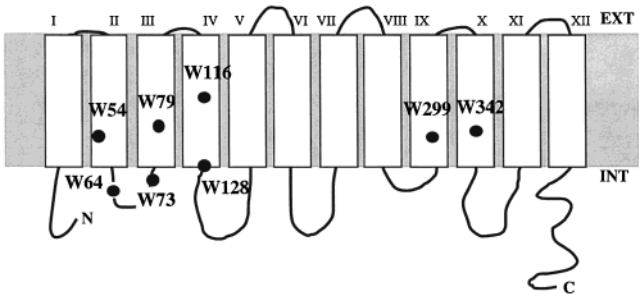

FIGURE 1: Secondary structure model of MelB. The six Trps located in the N-terminal domain are represented in boldface. The two Trps located in the C-terminal domain are shown. TMS are represented by rectangles. Topological data are derived from extensive site-directed *melB-phoA* fusion analysis (*7*).

Localization of the ion- and sugar-binding sites is still under investigation. Effects of mutagenesis of many residues of the transporter N-terminal domain and studies of MelB chimeras (*10*−*13*) favor the hypothesis that the ion recognition site is located in the N-terminal domain of MelB and constituted of four Asp residues: Asp 19, 55, 59, and 124, located in TMS I, II, and IV.

Combination of intrinsic fluorescence and fluorescence resonance energy transfer (FRET)[1] analysis and mutagenesis of MelB tryptophans (Trp)[1] proved to be valuable in gaining additional insight into the structural organization of MelB (*14*−*17*). Indeed, binding of the coupling ions and sugars to the purified MelB reconstituted in liposomes produces variations of the fluorescence corresponding to selective and cooperative conformational changes of the transporter (*14*). Of the eight MelB Trp residues, six are located between TMS I and cytoplasmic loop 4−5, and two in the TMS IX and X (Figure 1). In an initial study, the two C-terminal Trps were replaced by phenylalanines (Phe)[1] (*15*) to determine the

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* To whom correspondence should be addressed. Phone: 33-(0)4 93 76 52 13. Fax: 33-(0)4 93 76 52 19. E-mail: mus-veteau@oceane.obs-vlfr.fr.

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[1] Abbreviations: MelB, melibiose permease; Trp, tryptophan; Mel, melibiose (6-*O*-α-D-galactopyranosyl-D-glucose); α-NPG, *p*-nitro-phenyl α-D-6-galactopyranoside; TMG, methyl-1-thio-β-D-galactopyranoside; α-MG, methyl-α-D-galactopyranoside; β-MG, methyl-β-D-galactopyranoside; FRET, fluorescence resonance energy transfer; IMV, inverted membrane vesicle; WT, wild-type; Tyr, tyrosine; Phe, phenylalanine.

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respective contributions of N- and C-terminal domains of MelB to the intrinsic fluorescence variations induced by the substrates. This study suggests that the sugar-binding site is located in the C-terminal domain of MelB close to TMS IX and X and that the N-terminal domain of MelB is required for binding β-configured sugars such as the thiomethyl-β-D-galactopyranoside (TMG).[1] On the basis of these observations, a structural organization of MelB has been proposed in which the first four TMS and the TMS IX and X would be in close proximity. This hypothetical model is supported by a FRET study of MelB carrying a mutation of each Trp, which made it possible to estimate the distances separating the sugar-site from W64 (located in the loop 2−3) and from W299 (located in the TMS IX) as approximately 20 and 15 Å respectively (*16*, *17*).

In the present paper, we further analyze the individual contribution of the N-terminal Trps to the fluorescence variations induced by cations or by β-configured sugars with the purpose of gaining additional insight into the structural organization of MelB. To this end, each of the six N-terminal Trps was independently mutated, and the corresponding permeases purified and subjected to intrinsic fluorescence spectroscopy analysis. The data suggest that the ion-induced fluorescence signal essentially results from the algebraic sum of a *quenching* effect of the W54 (in TMS II) signal and of an *enhancement* of the signal arising from W116 (in TMS IV) and/or W128 (in cytoplasmic loop 4−5). Importantly, the spectroscopic data also suggest that W116 and/or W128 are involved in the β-galactoside-induced fluorescence signal response. The information about W116 and W128 obtained from fluorescence measurements is complemented by activity information and the results are discussed in terms of a model of the structural organization of MelB in which the fourth TMS domain is postulated to play an essential role in connecting cation and sugar-binding sites.

MATERIALS AND METHODS

Materials. *p*-Nitrophenyl α-D 6-[^{3}H]galactopyranoside ([^{3}H]α-NPG) was synthesized in Dr. B. Rousseau's laboratory (Laboratoire des Molécules Marquées, CEA). Dodecyl maltoside was obtained from Boehringer Mannheim. The Ni-NTA resin was from Qiagen Inc. Bio-Beads SM-2 were from Bio-Rad Lab., Inc. Total *E. coli* lipids were purchased from Avanti Polar Lipids, Inc. All other materials were obtained from commercial sources.

Bacterial Strains and Plasmids. *E. coli* DW2-R, a recA$^-$ derivative of strain DW2 [Δmel ΔlacZY (*18*)] was transformed with pK95ΔAHB plasmids carrying either WT or a modified His-tagged-*mel*B gene with mutations W54F, W64F, W73F, W79F, W116F, or W128F as described by Cordat (*16*). Mutants W116Y, W128Y, and W54FW64F-W73FW79FW299FW342F were constructed by site-directed mutagenesis as also described by Cordat (*16*) using primers complementary to the sense strand of *mel*B except for mismatches: W116Y, ACCTACATCCTCTACGGCAT-GACTTACACC; W128Y, GATATTCCCTTCTACTCGCTG-GTTCCAACC.

Transport and Binding Activities. Freshly transformed cells were grown routinely in M9 medium at 30 °C to OD_{600} = 1.2 and then resuspended in a medium containing 0.1 M potassium phosphate (KPi) (pH 7) and 10 mM NaCl or LiCl and incubated at 23 °C for 1 min under a stream of oxygen. Transport reactions were initiated by the addition of either [^{3}H]melibiose (20 mCi/mmol) or [^{14}C]TMG (3 mCi/mmol) at final concentrations of 0.4 mM. The reactions were terminated after various times of incubation by a rapid dilution followed by immediate filtration (*19*). [^{3}H]α-NPG-binding activities were measured using a flow dialysis procedure as described by Damiano-Forano (*20*).

Effect of Ionic Strength on Initial Transport Rates. Fresh cells were resuspended in a medium containing 0.1 M KPi (pH 7) and incubated for 1 min under a stream of oxygen. Various concentrations of salts (10 mM NaCl, 10 mM NaCl and 490 mM KCl or 500 mM NaCl) were added, and 5 s later, transport reactions were initiated by the addition of either 0.2, 0.4, 0.8, or 1.8 mM of [^{3}H]melibiose (20 mCi/mmol) or [^{14}C]TMG (3 mCi/mmol). The reactions were terminated after 15 s by rapid dilution followed by immediate filtration.

Inhibitory Constants Measurement. Fresh cells were resuspended in a 0.1 M KPi (pH 7) buffer containing 10 mM NaCl. Transport reactions were carried out for different concentrations of α-galactosides or β-galactosides added 5 s before [^{3}H]melibiose (20 or 90 mCi/mmol). After 15 s, the reactions were terminated by rapid dilution followed by immediate filtration. Melibiose initial transport rate constants were calculated for different inhibitory concentrations of α-MG (0.1, 0.5, 1, 5, and 25 mM), raffinose (0.1, 3, 10, 30, and 60 mM), and β-MG or TMG (0.1, 3, 10, 30, and 100 mM for WT and 1, 10, 30, 50, and 100 mM for W116F and W128F). The [^{3}H]melibiose concentrations used to calculate initial rates were 0.2, 0.4, 0.8, 1.8, and 3.6 mM.

MelB Purification and Reconstitution in Liposomes. This procedure was carried out as described by Cordat (*16*).

Protein Determination. Proteins contained in membrane vesicles and proteoliposomes were assayed according to Lowry (*21*) using bovine serum albumin as standard.

Fluorescence Measurements. Time-dependent fluorescence experiments were carried out with a MOS200 spectrofluorometer (BioLogic) on briefly sonicated proteoliposomes (20 μg/mL) resuspended in 20 mM Tris-HCl (pH 8), thermostated at 20 °C, and constantly stirred. Samples were excited at 297 ± 5 nm. The emission fluorescence light (F) integrated between 310 and 400 nm was recorded before and after addition of substrates, and the fluorescence variations (ΔF) were calculated as previously described by Mus-Veteau (*14*). $\Delta F/F$ values are the relative fluorescence changes calculated from at least three separate experiments and were normalized with respect to the amounts of active MelB (B_{max}, Table 2).

RESULTS

Effects of Substrates on W54F, W64F, W73F, and W79F Mutant Fluorescence Properties. To estimate the contribution of the six N-terminal Trps to the cation- or TMG-induced fluorescence variations observed in the N-terminal domain of MelB (*15*), permeases carrying individual mutations W54F, W64F, W73F, W79F, W116F, or W128F were constructed. As previously described (*16*), cells carrying MelB with mutation W54F, W64F, W73F, or W79F display transport characteristics similar to those of the wild type (WT),[1] while those carrying MelB with mutation W116F or W128F have deficient transport and binding properties.

Table 1: [^{3}H]α-NPG-Binding Characteristics of WT and Mutant Permeases after Reconstitution in Liposomes[a]

	B_{max} (nmol/mg of protein)	K_D (μM)
WT	6	0.6
W54F	6	1.5
W64F	3.5	0.9
W73F	2	1.2
W79F	6	0.9

[a] Proteoliposomes containing WT or mutant permeases (0.2−1 mg/mL) were equilibrated in 20 mM Tris-HCl (pH 8), 10 mM NaCl, 5 μM FCCP, and 0.75 μM monensin. [^{3}H]α-NPG (1.6 mCi/mmol) binding was measured as a function of free α-NPG concentration (1−250 μM) using a flow dialysis technique (*18*). The maximal number of active sites (B_{max}) and the apparent α-NPG binding constant (K_D) were calculated from Scatchard plots of the binding data.

Table 2: Effect of High Salt Concentration on the Initial Transport Rate of MelB and TMG Elicited by WT, W116F, or W128F MElB[a]

	V_{max} [nmol (mg cell protein)$^{-1}$ min^{-1}]					
	MelB			TMG		
	10 mM NaCl	0.5 M NaCl	0.5 M KCl, 10 mM NaCl	10 mM NaCl	0.5 M NaCl	0.5 M KCl, 10 mM NaCl
WT	40	40	55	85	30	50
W116F	25	45	60	11	35	35
W128F	7	45	50	4	5	10

[a] *E. coli* DW2 cells expressing WT, W116F, or W128F MelB were resuspended in a medium containing 100 mM potassium phosphate (pH 7) and incubated at 23 °C for 1 min under a stream of oxygen. Various concentration of salts (10 mM NaCl, 0.5 M NaCl, or 0.5 M KCl + 10 mM NaCl) were then added in the medium and five seconds later, transport reactions were initiated by the addition of either 0.2, 0.4, 0.8, and 1.8 mM of [^{3}H]melibiose (20 mCi/mmol) or [^{14}C]TMG (3 mCi/mmol). The reactions were terminated after 15 s by a rapid dilution followed by immediate filtration. Sugar influx is expressed in nanomoles per milligram of protein per minute.

Immunoblot analyses indicated that the permease content of IMVs harboring the mutants or WT MelB varies by less than 20% (data not shown). To identify the Trps implicated in the fluorescence variations induced by cations or TMG, proteins must possess functional properties close to WT. Therefore, only MelB carrying mutations W54F, W64F, W73F, or W79F were purified and reconstituted in *E. coli* liposomes. The affinity of these purified mutant proteins for sugar was estimated by measuring the binding of the high-affinity ligand [^{3}H]α-NPG in the presence of 10 mM NaCl in the medium (*20*). Table 1 shows that the binding constant values (K_D) for α-NPG binding on the mutants are not significantly different from that of the WT permease. The difference in content of active MelB (B_{max}) indicates that even if the mutations do not affect MelB activity in cells, they can perturb the stability of the protein after purification, as it seems to be the case for W64F and W73F mutants. As denatured MelBs do not contribute to the substrate-induced fluorescence changes, the differences in active MelB content will be used to normalize the signals during the comparison of the fluorescence properties of the mutated and WT MelB.

The effects of sodium or lithium (data not shown) and sugars on the fluorescence properties of WT and mutant permeases were assessed by recording the fluorescence variations of the Trp signal integrated between 310 and 400 nm ($\Delta F/F$%) as already described (*14*). We previously

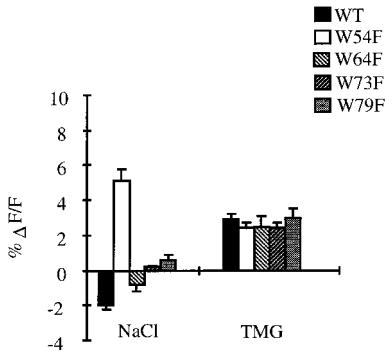

FIGURE 2: Sodium and TMG induced variations of WT and mutant MelB fluorescence. Proteoliposomes containing WT, W54F, W64F, W73F, or W79F MelB (2 mL, 20 μg/mL) were equilibrated at 20 °C in 20 mM Tris-HCl, pH 8. After excitation at 297 ± 5 nm, the emitted fluorescence signal (F) was integrated between 310 and 400 nm and recorded as a function of time. The fluorescence changes (ΔF) induced by addition of 10 mM sodium or 10 mM sodium plus 30 mM TMG were expressed as $\Delta F/F$ (%). Each of the mutant $\Delta F/F$ values was normalized with respect to the amounts of active permease (B_{max} Table 2). Values are the means of at least three experiments.

reported that addition of 10 mM NaCl or LiCl to WT MelB proteoliposomes induces a 2% *quenching* of the fluorescence signal (*14*) and also showed that this ion-induced *quenching* is due to a signal variation arising from the N-terminal Trps (*15*). For each proteoliposome preparation, the fluorescence variations recorded were normalized with respect to the variations of their active MelB contents leading to a 3- and 1.7-fold increase of the $\Delta F/F$ values for W73F and W64F, respectively. Figure 2 shows that, among the four Trp mutations introduced independently in MelB, only mutation W54F produced a major modification of the fluorescence response elicited by addition of the cation. In the absence of W54, sodium *increases* MelB fluorescence by 5%. This result suggests that the fluorescence of W54 in WT MelB is *quenched* by approximately 7% upon binding of sodium. Furthermore, since the 5% *increase* observed on W54F MelB is not due to W64, W73, or W79, it must be accounted for by the contribution of W116 and/or W128. The *quenching* of fluorescence signal results essentially from the algebraic sum of the *quenching* of the W54 (in TMS II) signal and of the *enhancement* of the W116 (in TMS IV) and/or W128 (in cytoplasmic loop 4−5). Moreover, the difference between the fluorescence spectra recorded on proteoliposomes containing WT or W54F MelB (in the same conditions of protein and lipid concentrations and of buffer) made it possible to estimate the maximal emission signal of W54 at 347 ± 5 nm (data not shown). This value indicates that W54 is located in a relatively polar environment (*22*).

Previous experiments on proteoliposomes containing MelB with the double mutation W299F and W342F have shown that addition of the β-galactoside sugar TMG induces an *increase* of the fluorescence of the Trps located in the N-terminal domain of MelB (*15*). Analyses of the single MelB mutants W54F, W64F, W73F, and W79F show that the fluorescence variations induced by TMG are comparable to that of WT (Figure 2). None of the four Trps mutated, 54, 64, 73, or 79, is involved in the sugar-induced fluorescence variation. From these data, we deduce that the *increase* of the fluorescence signal of N-terminal Trps elicited by TMG arises from W116 and/or W128.

In an attempt to directly confirm the effects of sodium and TMG on W116 and W128, we constructed a mutant that carries only the two Trps 116 and 128, the six other Trps (54, 64, 73, 79, 299, and 342) being replaced by phenylalanines. Although this mutant was active in *E. coli* cells and membranes, the loss of sugar-binding activity after purification and reconstitution in liposomes made any fluorescence measurements impossible.

As the data described above suggest that W116 and/or W128 participate in the cation- and TMG-induced fluorescence variations, we decided to study more precisely the kinetic properties of MelB carrying the mutations W116F or W128F.

Characterization of the Effect of Mutations W116F and W128F on MelB Activity. Mutation W128F impairs TMG accumulation by more than 90% and binding of sugar is undetectable on membrane vesicles containing W128F MelB. Transport constant values (K_t) are similar to that of WT, but mutation W128F affects initial influx rate values (V_{max}) for melibiose and TMG uptake by 80 and 95%, respectively (Table 2). To verify whether these alterations are due to an effect of mutation W128F on the ability of sodium to activate sugar transport, experiments were carried out at 0.5 M NaCl or 10 mM NaCl plus 0.5 M KCl. Table 2 shows that, in W128F mutant cells, either condition restores the V_{max} for melibiose but not for TMG, and that MelB exhibits the same activity in the presence of 10 mM and 0.5 M NaCl. The presence of 1 M NaCl or 10 mM NaCl plus 1 M KCl restores α-NPG-binding with K_D of 5μM.

Mutation W116F has less effect on MelB activity. It does not affect sugar accumulation and only slightly alters sugar binding. High ionic concentration in the medium completely restores the V_{max} for melibiose and TMG (Table 2) and also sugar binding giving a K_D of 3 μM. The data indicate that the presence of a high ionic strength in the medium stabilizes W116F and W128F MelB and that mutation W116F has no effect on the ability of sodium to activate sugar transport. Unfortunately, this high ionic strength medium did not protect against inactivation of the mutants during purification. The results do not allow any conclusion to be made concerning the effect of mutation W128F on the ability of sodium to activate sugar-transport since the TMG initial influx rate is not restored.

Melibiose being an α-galactoside disaccharide and TMG a β-galactoside monosaccharide, the possible implication of the orientation of the glycosidic linkage or of the difference in the sugar composition on sugar uptake was analyzed. The ability of different sugars to competitively inhibit melibiose uptake in the two mutants W116F and W128F was compared. The competing α-galactosides (monosaccharide α-MG[1] and trisaccharide raffinose) and β-galactosides (monosaccharides TMG, β-MG[1]) are known to be transported by MelB to some extent (*14*, *23*). Data indicated that the ability of β-galactosides to inhibit melibiose accumulation in cells was largely decreased in W116F and W128F mutant cells in comparison to WT (data not shown). Initial transport of [^{3}H]-melibiose was measured at different concentrations of α- or β-galactosides in order to estimate the apparent inhibitory constants (K_i). Table 3 shows that β-galactosides' inhibitory constants are significantly increased in W116F and W128F mutants (8−35-fold), whereas α-galactosides' K_is are only slightly affected (2−4-fold). This result suggests that mutations W116F and W128F specifically affect the transport and binding of sugars harboring a glycosydic linkage in β orientation.

Table 3: Inhibitory Constants of Melibiose Initial Transport Rates by Different α and β Galactosides[a]

	K_i (mM)			
	α galactosides		β galactosides	
	αMG	raffinose	βMG	TMG
WT	0.5 ± 0.25	3 ± 1	1 ± 0.3	0.8 ± 0.4
W116F	0.8 ± 0.2	12 ± 3	8 ± 3	18 ± 2
W128F	0.9 ± 0.3	10 ± 4	35 ± 5	27 ± 3

[a] *E. coli* containing WT or mutant permeases were grown at 30 °C in M9 medium supplemented with 5 g/L glycerol, 2 g/L casamino acids, and 0.1 g/L ampicillin, washed and resuspended in a medium containing 0.1 M KPi (pH 7) and 10 mM NaCl. Initial transport reactions were initiated by the addition of different α galactosides (α-MG or raffinose) or β galactosides (β-MG or TMG) 5 s before adding [^{3}H]melibiose (20 or 90 mCi/mmol) and terminated after 15 s by a rapid dilution. Melibiose initial transport rate constants were calculated for different inhibitory concentrations of α-MG (0.1, 0.5, 1, 5, and 25 mM), raffinose (0.1, 3, 10, 30, 60 mM), β-MG or TMG (0.1, 3, 10, 30, 100 mM for WT and 1, 10, 30, 50, 100 mM for W116F and W128F). The [^{3}H]melibiose concentrations used to calculate initial rates were 0.2, 0.4, 0.8, 1.8, and 3.6 mM. K_i values are calculated from three repeated experiments (means ± SEM).

Effect of the Substitution of W116 and W128 by Tyrosine Residues. Replacement of Trp by Phe can disrupt putative hydrogen bondings with adjacent residues. To verify if the changes induced by mutations W116F and W128F are due to the absence of these putative hydrogen bondings, we replaced W116 or W128 by a tyrosine (Tyr).[1] Immunoblot experiments indicated that the permease content of IMVs harboring the mutants or WT MelB was comparable (data not shown). Accumulation of melibiose or TMG in the presence or absence of Na^+ or Li^+ (10 mM) was measured in cells expressing MelB with mutation W116Y or W128Y. Figure 3 shows that the presence of a Tyr at position 116 enhances melibiose and TMG accumulation efficiency 3-fold relative to the WT values without changing MelB ionic selectivity. In contrast, at position 128, Tyr completely inhibits TMG accumulation and decreases melibiose accumulation at 10 mM NaCl, melibiose being twice as efficiently transported in the presence of LiCl. These data indicate that replacement of Trp128 by a Tyr disrupts TMG transport but also reverse MelB ionic selectivity. Unfortunately, the W116Y and W128Y mutants were inactivated upon purification, precluding their use for subsequent spectroscopic analysis.

DISCUSSION

In the present study, we assess the individual contribution of the N-terminal Trp residues to the fluorescence changes induced by cation or β-galactoside binding as a means to analyze the role of the N-terminal domain in MelB function. To this end, each of the six Trps of the MelB N-terminal domain was replaced by a Phe by site-directed mutagenesis. Overall, the spectroscopic properties of the single Trp mutants suggest that W116 and W128, located in the fourth TMS or cytoplasmic loop 4−5, respectively, are involved in both cation- and β-galactoside-induced fluorescence variations. The information about W116 and W128 obtained from fluorescence measurements is complemented by activity

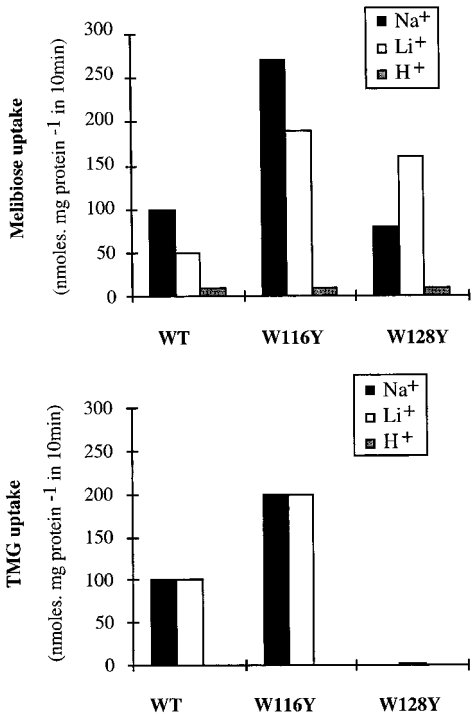

FIGURE 3: Sugar specificity and ionic selectivity of sugar transport in WT, W116Y, and W128Y MelB. *E. coli* DW2-R were transformed with pK95ΔAHB plasmids harboring the native *mel*B gene or the mutated *mel*B gene that encodes for MelB carrying a single mutation W116Y or W128Y. Cells were grown in M9 medium supplemented with 5 g/L glycerol, 2 g/L casamino acids, and 0,1 g/L ampicillin. They were washed and resuspended in a medium containing 0.1 M KPi (pH 7) and less than 20 μM of contaminating sodium salts. H^+ coupled melibiose transport was carried out in this medium. Na^+ and Li^+ coupled transport were measured in this medium supplemented with 10 mM NaCl or LiCl. Transport reactions were initiated by adding [^{3}H]melibiose (20 mCi/mmol) at a final concentration of 0.4 mM. After 10 min, a rapid dilution terminated the reaction. Each column represents the amount of sugar taken up by the cells in 10 min.

information which, together with conclusions drawn from various analyses already carried out on MelB, make apparent a fundamental role of helix IV region in connecting cation and sugar-binding sites of MelB.

Previous studies have shown that addition of a saturating concentration of cation (NaCl or LiCl) or β-galactoside TMG to pure MelB reconstituted in liposomes induces, respectively, a *quenching* (−2%) or an *increase* (+5%) of the fluorescence signal emitted by Trps from the N-terminal domain of MelB (*15*). The six Trps at position 54, 64, 73, 79, 116, or 128 of MelB N-terminal domain were substituted independently by a Phe. Sugar transport and binding experiments had previously shown that only W116F and W128F mutants exhibit a reduced transport activity (*16*). Therefore, only permeases carrying mutations W54F, W64F, W73F, or W79F were studied by steady-state fluorescence spectroscopy after purification and reconstitution in liposomes. Among the four mutant permeases analyzed, W54F MelB caused a major modification of the cation-induced fluorescence response, converting the 2% *quenching* event observed in WT into a 5% *enhancement* of the fluorescence signal in the mutant. Such a change in response can be explained by assuming that the WT response is the algebraic sum of individual signal changes occurring at the level of different N-terminal Trps. Thus, upon interaction with WT permease, the cation strongly *quenches* the W54 signal (7%) and, at the same time, *increases* the fluorescence light emitted by one (or several) other N-terminal Trps, the *quenching* phenomenon being predominant. In the W54F mutant, the negative contribution of W54 no longer counterbalances the positive contribution of the N-terminal Trps, leading to a predominance of a fluorescence *enhancement* phenomenon. Moreover, as neither W64, W73, nor W79 significantly influenced the overall fluorescence signal, we deduced that W116 and/or W128 are the Trps responsible for the fluorescence *enhancement* component of the response. It is noteworthy that the Trps which participate in the overall signal change, either by a *quenching* (W54) or by an *enhancement* (W116 or W128) of their fluorescence, are located in or close to TMS harboring putative coordination ligands of the cation (*24*). W116 or W128 are either in or close to helix IV harboring Asp124. W54 is close to Asp55 and Asp59 on helix II but probably not directly in the cation-binding site since neither its mutation into a Cys residue nor subsequent modification by pCMBS impairs transport activity (*25*). Examination of the TMG-induced fluorescence increase in the different mutants does not suggest any participation of the Trps at positions 54, 64, 73, or 79 to the signal response. Thus, the signal must arise from W116 and/or W128. Unfortunately, this hypothesis cannot be experimentally verified using either the single W116F or W128F mutant or even a permease containing exclusively W116 and W128 as all these mutants are inactive after purification.

Limited information is learned from the fluorescence analysis of the Trp mutants because, due to inactivation, the behavior of the most critical Trps W116 and W128 can only be inferred indirectly. To obtain more evidence about the role of W116 and W128 in both ion and sugar binding, we investigated in further details how their mutation affects MelB activity. The first noteworthy point is that the cells expressing W116F or W128F mutant still accumulate melibiose but no or little TMG, respectively. The defect in sugar transport rate V_{max} is apparently more marked for W128F than for W116F. Thus, increasing the ionic strength rescues totally melibiose, partly TMG initial transport rates in W116F, and only initial transport rates of melibiose in W128F. No evidence was obtained for a decrease affinity of W128F for NaCl. Apparently, mutation of W116 or especially W128 preferentially impairs β-galactoside uptake. This is confirmed by the demonstration that β-galactosides are less efficient competitors of melibiose transport in the mutants than in WT MelB. This suggests that W116 and W128 play a role in sugar selectivity by stabilizing the binding and transport of sugars harboring a glycosidic linkage in the β-configuration. Remarkably, cells expressing the mutant W128Y exhibit no TMG accumulation but a higher melibiose accumulation in LiCl medium than in NaCl medium indicating a change in ionic selectivity. Taken together, the results suggest that the Trps of TMS IV region, W116 and more significantly W128, play a critical role in the sodium/sugar symport mechanism, both being involved in ionic and sugar selectivity. Moreover, it was recently reported that the loss of the sodium/sugar coupling process induced by the mutation of Arg52 in helix II was reversed by a second mutation on W116 in helix IV (*26*). Furthermore, the correlation of the 3D structural data so far collected for membrane proteins, and in particular the potassium channel,

Table 4: Sugar Uptake and Initial Transport Rate of Melibiose and TMG Elicited by WT, Y120F, D124E, or P126S MelB[a]

	melibiose			TMG		
	accumulation at 10 min [nmol (mg protein)$^{-1}$]	K_t (mM)	V_{max} (%)	accumulation at 10 min [nmol (mg protein)$^{-1}$]	K_t (mM)	V_{max} (%)
WT	100	0.1	100	100	0.2	100
Y120F (ref *24*)	30	2.5	275	3	ND	ND
D124E (ref *24*)	55	1.7	325	1	ND	ND
P126S (Zani, unpublished results)	150	0.7	210	45	ND	ND

[a] *E. coli* DW2 cells expressing WT or mutant MelB were resuspended in a medium containing 100 mM potassium phosphate (pH 7) and 10 mM NaCl, and incubated at 23 °C for 1 min under a stream of oxygen. Transport reactions were initiated by the addition of either [^{3}H]melibiose (20 mCi/mmol) or [^{14}C]TMG (3 mCi/mmol) at final concentration of 0.4 mM for sugar uptake and 0.2, 0.4, 0.8, or 1.8 mM for initial transport rates. The reactions were terminated by a rapid dilution after 15 s of incubation for initial transport rates, or 10 min for accumulation, and followed by immediate filtration. ND, not determinable.

shows that Trps are often situated at the end of membrane-spanning helices as to anchor them into the membrane (*27*, *28*). W128, being localized at the helix IV extremity region, might be involved in the structural stabilization of helix IV into the membrane.

Several mutations carried out on other residues of MelB TMS IV, i.e., Tyr120, Asp124 (*12*, *24*), Met123 (*18*), and Pro126 (*18*, *29*), have been shown to induce similar pleiotropic defects as those described for W116F, W128F, or W128Y mutation (loss of TMG accumulation, reduction of the affinity for sugars, alteration of the ionic selectivity) (see Table 4). The concomitant disruption of both ion and sugar recognition in all these mutants is best explained by assuming an overlap of the substrate-binding sites (*12*, *24*). Since six different single point mutations located in the cytoplasmic region of TMS IV were shown to disrupt both ion and sugar recognition and transport properties considerably, this region must be crucial for MelB activity. The higher variability in the primary sequence observed between the four Na^+ and H^+ dependent melibiose permeases so far isolated (*30*) is localized in TMS IV. This observation argues in favor of an important role of TMS IV region for the cation and sugar-coupling process. Moreover, the increase of resistance of loop 4−5 to protease cleavage observed in the presence of the cation alone or the cation plus melibiose (*9*) and the observation from covalent photolabeling that Arg141 in the same loop may (directly or not) participate to sugar binding (*31*) provide independent evidence that the helix IV region of MelB (helix IV and loop 4−5) is involved in the binding of the two cosubstrates. On the basis of these various results, we propose that the cytoplasmic region of helix IV could play a role in connecting cation- and sugar-binding sites.

Accordingly, an hypothetical model of the structural organization of MelB is proposed in Figure 4. This model takes into account the present results and those reported in different studies carried out on the *E. coli* melibiose transporter which suggest that (i) the cation-binding site is located between helices I, II and IV (*11*−*13*, *24*, *32*−*34*), (ii) the sugar-binding site is located close to helix IX (*15*, *35*), and (iii) there is a proximity between the cation- and sugar-binding sites with a distance of around 20 Å between the loop 2−3 and the sugar-binding site (*16*). The merit of the helix arrangement in the model, together with the position of helix IV as a hinge between the two substrate-binding sites, is to provide a rational background to explain that residues conferring lithium and/or TMG resistance are

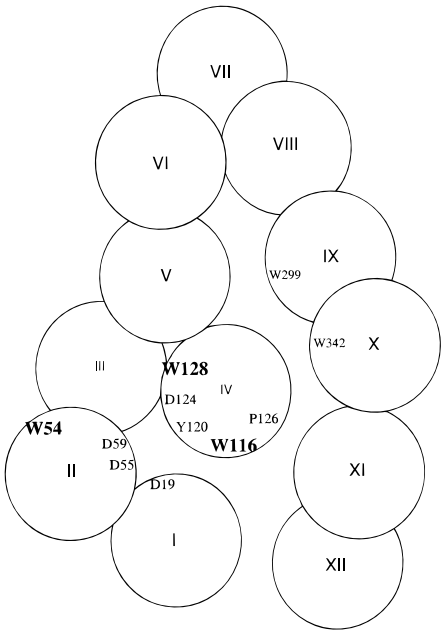

FIGURE 4: Model showing the role of helix IV in connecting the sugar- and cation-binding sites.

clustered in both halves of the permease (*18*, *36*, *29*). The localization of helix IV in connecting cation and sugar-binding sites could account for the cooperative interaction of cation and sugar in the transport process.

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