The Inner Interhelix Loop 4–5 of the Melibiose Permease from *Escherichia coli* Takes Part in Conformational Changes after Sugar Binding*

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Cytoplasmic loop 4–5 of the melibiose permease from *Escherichia coli* is essential for the process of Na\(^+\)-sugar translocation (Abdel-Dayem, M., Basquin, C., Pourcher, T., Cordat, E., and Leblanc, G. (2003) *J. Biol. Chem.* 278, 1518–1524). In the present report, we analyze functional consequences of mutating each of the three acidic amino acids in this loop into cysteines. Among the mutants, only the E142C substitution impairs selectivity of sugar binding. We show cooperative modification of their sugar binding sites by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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Co-transporters catalyzing specifically the transfer of organic substrates and ions across membranes are of primary importance for the life of cells. One of these co-transporters belonging to the galactosides-pentoses-hexuronides transport family (1) is the melibiose permease (MelB) of *Escherichia coli*. MelB functions as a secondary active co-transporter that links uphill solute transport (α-galactosides, such as 6-O-α-D-galactopyranosyl-D-glucose (melibiose), or β-galactosides, such as methyl-1-thio-β-galactopyranoside) to a downhill electrochemical ion gradient. It is most unusual in its ability to use either H\(^+\), Na\(^+\), or Li\(^+\) as coupling ion depending on which sugar is being transported (reviews in Refs. 1 and 2). The binding of the cation enhances the affinity of the transporter for the co-transported sugar, and vice versa melibiose enhances the affinity of the transporter for Na\(^+\) (1–5).

Consisting of 473 amino acids, this highly hydrophobic protein (70% apolar) has 12 transmembrane-spanning α-helices, organized as two asymmetric domains, each containing 6 helices, with its N and C termini facing the cytoplasm of the bacterium (6–9). While awaiting improved resolution of the MelB structure, other techniques are being used to understand better the functioning of this transporter and identify amino acids involved in substrate binding and/or translocation. For example, for MelB, Asp-19 (helix I), Asp-55 (helix II), Asp-59 (helix II), and Asp-124 (helix IV) have been shown to be essential for cation binding and Asn-58 (helix II) for cation recognition (10–14). Cysteine-scanning mutagenesis is now a widely used strategy to study systematically the structure and structure-function relationship of membrane proteins. It is currently applied to MelB using a fully functional active permease devoid of its four native cysteines (Cys-less permease) (15) as genetic background.

Extensive cysteine-scanning mutagenesis studies concentrated thus far mainly on membrane domains of MelB. However, three observations led us to focus on the role of the highly charged cytoplasmic loop 4–5 for MelB symport activity. First,
MelB substrates protect the protein cooperatively against proteolysis of loop 4–5 (7). Second, active-site-directed labeling of the protein showed that the arginyl residue 141 of this same loop is a melibiose-protected target of labeling with a photoreactive azidophenyl sugar analog (16). From the latter study, it was concluded that loop 4–5 is either directly involved in Na\(^+\) and/or sugar binding or contributes indirectly to the coupling interaction between the two binding sites. Third, among the positively charged amino acids of loop 4–5 that were individually replaced by a cysteine, only R141C, although able to bind the substrates, showed defects in the translocation process (17) suggesting that loop 4–5 is close to the sugar binding site and may participate directly in co-substrate translocation.

In the present study, we bring additional evidence for an important role of loop 4–5 for the functioning and cooperative behavior of MelB. We analyzed the effects of individual cysteine replacement of the three negatively charged residues; i.e. D137C, E140C, and E142C. Because among the mutants only E142C showed substrate translocation defects, this mutant, together with the previously characterized R141C mutant (17), was investigated in detail by combining kinetic, electrophysiological, and fluorescence spectroscopy approaches. In particular, we analyzed transient electrical currents recorded from R141C and E142C by using the solid supported membrane (SSM) technique. The SSM has already been used to monitor electrogenic events associated to partial steps of the Na\(^+\) transport, e.g. the protective effect of melibiose (5, 18). In combination with spectroscopic evidence, the results suggest that loop 4–5 contributes to the coordinated interactions between the ion and sugar melibiose binding sites and participates in conformational changes after melibiose binding.

**EXPERIMENTAL PROCEDURES**

**Materials**—Synthesis and labeling of p-nitrophenyl α-d-6-\(^{3}H\)galactopyranoside (α-[\(^{3}H\)]NPG) or 6-O-α-d-\(^{3}H\)galactopyranosyl-D-glucose ([\(^{3}H\)]melibiose), and synthesis of 2’-[(N-dansyl)aminoethyl-1-thio-β-D-galactopyranoside (DNS-S-Gal) were carried out by Dr. B. Rousseau and Y. Ambroise (Département de Biologie Joliot Curie/CEA-Saclay, France). Highest purity grade reagents (KH\(_2\)PO\(_4\), Sigma, 0.005% Na\(^+\) and KOH Merck, suprapur, 0.002% Na\(^-\)) were used to prepare nominally Na\(^+\)-free media (contaminating Na\(^+\) level of <20 μM as determined by atomic absorption spectroscopy). 3-(N-Maleimidpropionyl)biocytin (biotin maleimide, MPB), 4-acetamido-4-[\(^{3}H\)]maleimidylstilbene-2,2’-disulfonate (stilbene disulfonate), and the streptavidin-alkaline phosphatase conjugate were purchased from Molecular Probes, Inc. (Eugene, OR). The methanethiosulfonate derivatives (MTS reagents) were from Toronto Research Chemicals, Inc. (Toronto, Canada).

**Bacterial Strains, Plasmids, and Site-directed Mutagenesis**—A recombinant pK95AHHB plasmid with a cassette containing the melB gene (19) encoding a permease with a His\(_6\) tag at its C-terminal end (20) and devoid of its four native cysteines (Cysless MelB (15)) was constructed by PCR and used as background for further permease engineering and as control. *E. coli* DW2-R (ΔmelB and ΔlacZ) (21) was transformed with the plasmid harboring the mutated MelB (Cys-less, D137C, R139C, E140C, R141C, or E142C, respectively). Freshly transformed cells were grown at 30 °C in M9 medium supplemented with glyceral (5 g/liter), casamino acids (0.2%), thiamine (0.5 mg/liter), and ampicillin (100 μg/ml) until an A\(_{600}\) of 1–1.2 was reached. The cells were washed and resuspended in 0.1 M potassium phosphate (KP) at pH 7.

**Preparation of Membrane Vesicles**—Right-side-out (RSO) membrane vesicles, prepared by an osmotic shock procedure (22), were concentrated to 2 mg of protein/ml and equilibrated in a medium containing 0.1 M KP\(_2\), (pH 7). Inside-out (ISO) membrane vesicles were generated using a French pressure cell as described previously (20) and concentrated to ~20 mg/ml in a medium containing 50 mM Tris-HCl (pH 8) and 50 mM NaCl (20).

**Purification and Preparation of the Proteoliposomes**—Mutated His-tagged MelB (Cys-less, D137C, R139C, R141C, or E142C) was purified from inverted membrane vesicles as described (20). A chromatographic procedure combining the utilization of nickel-nitritoliatric acid (Qiagen, Germany) and ion exchange resin (Macro-Prep-High-Q anion exchange support, Bio-Rad) were used to prepare nearly pure MelB (generally >99% (20)) solubilized in dodecylmaltoside (0.1%). MelB reconstitution into liposomes (protein/lipid ratio 1/5, w/w) (20) was performed by removing the detergent with Bio-Beads SM-2 (Bio-Rad) (23). Proteoliposomes were submitted to repeated freeze/thaw/sonication wash cycles in nominally Na\(^+\)-free, 0.1 M KP\(_2\), (pH 7) to eliminate Na\(^+\) (contaminating level <10 μM as determined by flame photometry). Purity of the reconstituted MelB was assessed by silver-stained SDS-PAGE. Protein content was measured in the presence of SDS by a Lowry assay.

**Identification of Second Site Revertants**—*E. coli* expressing E142C grew initially as pale rose colonies on 1% melibiose MacConkey agar plates. After 3–5 days of incubation at 37 °C, small red areas appeared that were picked and re-streaked for colony purification. After isolation of the plasmid DNA, mutations responsible for fermentation recovery were identified by sequencing.

**Sugar Transport in Cells**—Freshly grown cells were concentrated to 2 mg of protein/ml in 0.1 M KP\(_2\), (pH 7). The time course of transport of [\(^{3}H\)]melibiose (0.4 mM, 20 mCi/mmol) in the presence or absence of NaCl or LiCl at a concentration of 10 mM was monitored at 22 °C by a rapid filtration procedure (17). Variation of transport rate and extent never exceeded 15% from batch to batch.

**Western Blot Analysis**—Western blot analysis was applied to samples containing protein solubilized from RSO membrane vesicles (~20 μg) and probed with a mouse anti-C-terminal MelB and an anti-mouse secondary antibody (7). Results were expressed as the percentage of the signal recorded from membranes carrying the Cys-less permease.

**Binding Assays**—α-[\(^{3}H\)]NPG binding to RSO membrane vesicles (20 mg/ml) and determination of binding constants were assessed at room temperature by a flow dialysis procedure as described previously (4).
Loop 4–5 of MelB Takes Part in Conformational Changes

Entrance Counterflow Activity—Counterflow activity was assessed in RSO membrane vesicles (20 mg/ml) at room temperature as described (17).

Solid Supported Membrane Set-up and Measuring Procedure—Proteoliposomes containing Cys-less, R141C, or E142C MelB were adsorbed to the SSM (5, 24). Activating and non-activating solutions for SSM measurements contained 0.1 M KPi, (pH 7) plus NaCl/KCl and melibiose/glucose at concentrations as indicated in the figure legends. Transient currents upon addition of one or both of the co-substrates were recorded. The solution exchange protocols were as described (5). All experiments were carried out at room temperature (22 °C).

Fluorescence Assays—An LS 50 fluorometer (PerkinElmer Life Sciences) was used to measure the Na⁺-dependent fluorescence resonance energy transfer (FRET) signals (λex = 297 nm, bandpass 5 nm, λem = 410–540 nm) arising from proteoliposomes (20 µg/ml) incubated in the presence of 10 µM of the sugar fluorescent analog Dns²⁻S-Gal (25). The specific FRET signals were normalized for variation (<20%) in the amount of permeases in the different proteoliposome preparations according to a previous study (26). Intrinsic tryptophan fluorescence (λex = 297 nm, bandpass 5 nm, λem = 310–380 nm) arising from proteoliposomes (20 µg/ml) was recorded as described (27).

Orientation of the Protein in the Liposome—Liposome suspensions containing purified Cys-less or single cysteine R139C (~0.25 mg of protein/ml, in 0.1 M KPi, pH 7) were supplemented with 10 µM MPB and incubated for 5 min at room temperature. MPB was added from a stock prepared in Me₃SO. The concentration of Me₃SO in the labeling medium did not exceed 1% (v/v). Some samples were afterward subjected to three cycles of freeze-thaw sonication in the presence of MPB. Where indicated, samples were pretreated with 200 µM of the membrane-impermeable thiolstilbene disulfonate for 10 min at room temperature. Stilbene disulfonate was washed away by three cycles of centrifugation with 0.1 M KPi, pH 7. As negative control, the Cys-less transporter was incubated with varying concentrations of MPB (0–1000 µM) and subjected to three cycles of freeze-thaw sonication. The biotin maleimide-labeling reaction was stopped by the addition of 10 µM dithiothreitol to the samples. All samples were solubilized in 1% SDS and subjected to SDS-PAGE (10%) and Western Blot analysis. A streptavidin-alkaline phosphatase conjugate was used for the specific reaction with biotin maleimide and a MelB-specific monoclonal antibody (21E4, Biocytex, Marseille, France), coupled directly to an alkaline phosphatase (Davids Biotechnologie, Regensburg, Germany), for the determination of the amount of protein. Both antibodies were used in a 1:1000 dilution. The alkaline phosphatase conjugate substrate Kit (Bio-Rad) was used to visualize the bands on the membrane.

RESULTS

The three acidic residues of loop 4–5 of MelB (Asp-137, Glu-140, and Glu-142) and the positively charged residues Arg-139 or Arg-141 (17) were individually replaced by a cysteine using the Cys-less MelB sequence as genetic background. The Cys-less MelB served also as control throughout this study as its functional properties are similar to those reported for the wild-type MelB (15, 17).

Functional Properties of D137C, E140C, and E142—Initial characterization of D137C, E140C, and E142C MelB mutants included: 1) the measurement of the time course of melibiose accumulation in bacteria in the presence or absence of saturating concentrations of activating Na⁺ or Li⁺ (10 mM) and 2) the estimation of the expression level of the mutated transporter. Although initial rate and sugar accumulation at equilibrium in the presence of Na⁺ (10 min) by D137C or E140C cells were 0.9 or 0.45 times the values measured in Cys-less MelB, the level of E142C sugar accumulation was reduced by a factor of 10 (Fig. 1A). Similar to the wild-type, melibiose transport activity was stimulated by Na⁺ and Li⁺ both in the Cys-less mutant and in D137C, E140C, and E142C (Fig. 1A). Strikingly, the residual transport observed in E142C was completely abolished in the presence of 0.2 mM N-ethylmaleimide (NEM), whereas that of D137C or E140C was not sensitive to the sulfhydryl reagent (data not shown). Comparison of the expression levels of Cys-less MelB and the mutants suggests that the reduced transport activity of D137C and E140C MelB can be satisfactorily accounted for by the reduction of the expression level of the two mutants (~70 and ~60% of Cys-less, respectively, not shown). In contrast, the permease content of E142C in membranes was proportionally too high (~40%) to explain the large drop in cell transport activity. In view of these results, the E142C mutant was further characterized, and its properties were compared with those of the Cys-less and the R141C mutants.

Measurement of sugar binding affinity in RSO membrane vesicles using the high affinity radiolabeled sugar analog α-[³H]NPG (4) as a ligand revealed that E142C retained the ability to bind the sugar in a Na⁺-dependent manner. However, the affinity for the sugar analog of E142C was lowered by a factor of 3–4 as compared with that of Cys-less MelB (apparent Kd (Cys-less) = 0.6 µM, apparent Kd (E142C) = 3.4 µM, data not shown). This drop in sugar affinity was similar to that observed in R141C (17). In contrast, the Na⁺ activation constants for α-NPG binding (KNa) of E142C and Cys-less were comparable (both had a KNa of 0.32 mM, data not shown). Although NEM inhibited the residual transport in E142C, the sulfhydryl reagent added before or after addition of any substrate to the vesicles had no detectable effect on its sugar-binding properties (data not shown). On the other hand, NEM had no effect on the substrate binding or translocation properties of the Cys-less mutant (data not shown).

The sugar translocation properties of MelB can be assessed by measuring entrance counterflow in de-energized RSO membrane vesicles (17). The early transient influx of labeled substrate (overshoot) is tightly coupled to the downhill efflux of internal unlabeled sugar occurring when RSO membrane vesicles, pre-loaded with a sugar at high concentration, are diluted in a medium containing a lower sugar concentration (2, 28). The radiolabeled sugar influx is primarily associated to shunting of the loaded ternary complex (MelB-ion-sugar) across the membrane and does not include a contribution of the empty carrier. The peak of the transient uptake of [³H]melibiose observed after 1 min in Cys-less MelB was absent in E142C (Fig. 1B). Instead, the intravesicular level of radioactive melibiose in
E142C steadily increased until a plateau value was reached. This influx of labeled sugar was significantly inhibited by NEM (data not shown). Summarizing, the defect of melibiose transport function of E142C included both a small reduction of sugar affinity and an impaired sugar translocation process. As will be further emphasized in the discussion, it is striking that mutagenesis of the neighboring E142C and R141C residues (17) gave rise to similar defects of the MelB translocation mechanism.

It is finally worth mentioning that we isolated a second-site revertant of E142C with an additional mutation on I22S that is positioned on the inner half of helix I (E142C/I22S). Despite a permease expression level only slightly higher than that of E142C (~50% versus ~40%, respectively, data not shown), the revertant exhibited a Na+–stimulated melibiose transport activity five times higher than that of E142C (Fig. 1C) and a higher α-NPG affinity (apparent $K_D = 2.1 \mu M$ versus $3.4 \mu M$, data not shown). The fact that a substitution of an apolar residue (isoleucine) by a polar residue (serine) located on helix I was able to compensate the loss of the negative charge at position 142 suggests first that helix I and loop 4–5 might be close to each other. Secondly, while this negative charge is not absolutely required, the need for a local polar environment at its level is important for the transport function and is partly satisfied by introduction of the Ile to Ser mutation at position 22.

**FIGURE 1.** Functional properties of D137C, E140C, E142C, and E142C/I22S mutants. A, cellular accumulation of [3H]melibiose by E. coli DW2-R cells expressing Cys-less, D137C, E140C, or E142C permeases. Freshly grown cells were washed and resuspended at 2 mg/ml in 0.1 M KPi. According to the different transport modes of MelB, 10 mM NaCl (gray columns), 10 mM LiCl (black columns), or no added salts (white columns), respectively, were present in the medium. The uptake reaction was started by adding 0.4 mM [3H]melibiose (20 mCi/mmol) and terminated at different times using a rapid dilution/filtration procedure (17). Histograms show the amount of labeled sugars accumulated at equilibrium (±10 min). Each value is the mean of three determinations (±S.E.). B, typical entrance counterflow activity of E142C (filled squares) or Cys-less (open circles). RSO membrane vesicles were equilibrated in 0.1 M KPi containing 10 mM NaCl and 30 mM melibiose and concentrated to 20–30 mg/ml. Counterflow was assayed by diluting 200-fold the loaded vesicles into the same saline medium containing [3H]melibiose (20 mCi/mmol) at a final concentration of 0.4 mM. At the indicated time points, the reactions were terminated by rapid filtration. All data points were corrected for a nonspecific activity of 5 nmol/mg. C, [3H]melibiose accumulation in E142C/I22S cells. Measurements were performed as described in A.
Loop 4–5 of MelB Takes Part in Conformational Changes

ences in affinity and/or sizes of peak currents between the Cys-less and the WT transporter.

As shown in Fig. 2, significant differences were observed between the amplitudes and/or the time courses of decay of the transient electrical currents recorded on the one hand from R141C and E142C mutants and on the other hand from Cys-less MelB. A melibiose concentration jump (Δmel), concentration jumps in the presence of the respective other co-substrate (Δmel(Na) and ΔNa(mel)), or simultaneous concentration jumps of the two co-substrates (ΔNaΔmel) resulted in signals decaying with bi-exponential time courses in wild-type (5) and Cys-less MelB (see Table 1 for an overview of the time constants of the decaying electrical currents). In contrast, when the same concentration jumps were performed on R141C and E142C proteoliposomes, the resulting electrical signals decayed only mono-exponentially showing that the electrogenic event linked to substrate binding still occurred, whereas that linked to stationary transport activity was absent or severely reduced.

We previously showed by a transport assay that reintroducing a positive charge by reacting R141C with MTSEA\(^+\) partially restores active substrate transport (17). This was now confirmed by electrical measurements. Accordingly, Fig. 3, illustrating a typical ΔNaΔmel concentration jump experiment after treatment of R141C with MTSEA\(^+\), systematically showed a partial recovery of the slow electrical transport component. This was supported by the observation that a bi-exponential function was required for fitting the time course of decay of the electrical signal and that a mono-exponential function was not sufficient (Fig. 3, inset). In contrast to this observation, any attempts to restore the transport component with the negatively charged sulfhydryl reagents MTSES\(^-\) (2-sulfonatoethyl)methanethiosulfonate) in the E142C mutants failed, perhaps due to accessibility limitation (data not shown).

Fig. 2 not only shows that the shape of the decaying part of the electrical signal is modified by the mutations but also indicates significant changes in the amplitudes of the peak currents. To better assess the mutant versus Cys-less signal differences, the relative rather than absolute values of the various peak currents were considered. To this end, we took the peak current elicited by a Na\(^+\) concentration jump for each studied mutant or wild-type as reference (100% signal in Fig. 4), because the Na\(^+\) partial reactions were not affected by the mutation, whereas the melibiose partial reactions were severely reduced (see below).

Several important observations can be made in the diagram shown in Fig. 4. First, the peak current elicited by a melibiose jump in the presence of either H\(^+\) (Δmel) or Na\(^+\) (Δmel(Na)) was much smaller in the two mutants than that of the corresponding signals in Cys-less. Second, Cys-less MelB (Fig. 4) showed a comparatively larger peak current after a ΔNaΔmel concentration jump than after a ΔNa jump. This signal represents the additive contribution of the two distinct Na\(^+\) and sugar electrogenic binding reactions (18) during a ΔNaΔmel challenge, while only the Na\(^+\) electrogenic binding makes up

**TABLE 1**
Overview of the decay time constants of the electrical signals after different solution exchange protocols

The table shows mean values of \(n\) independent experiments and the standard error (±S.E.) measured under the same conditions as described for Fig. 2. Mono- or bi-exponential functions \(g(t) = A_0 e^{-t/\tau_1} + A_2 e^{-t/\tau_2}\), \(A_2 = 0\) for mono-exponential functions), yielding the time constants \(\tau_1\) and \(\tau_2\), have been used to fit the decaying part of the electrical signals.

|          | WT       | C-less   | R141C    | R141C MTSEA | E142C    |
|----------|----------|----------|----------|-------------|----------|
| Δmel     | \(\tau_1\) (ms) | 98       | 82.5 ± 4.8 | 59.5 ± 7.9 | 60.2 ± 6.5 | 51.8 ± 6.6 |
|          | \(\tau_2\) (ms) | 350      | 258 ± 14  | 18.6 ± 1.0 | 27.5 ± 2.4 | 19.4 ± 2.3  |
| Δmel(Na) | \(\tau_1\) (ms) | 385      | 264 ± 25  | 13.0 ± 1.0 | 27.5 ± 2.4 | 19.4 ± 2.3  |
|          | \(\tau_2\) (ms) | 14       | 14.8 ± 0.7 | 11.5 ± 0.8 | 11.5 ± 0.8 | 11.5 ± 0.8  |
| ΔNa      | \(\tau_1\) (ms) | 15       | 11.5 ± 0.8 | 13.9 ± 1.3 | 12.8 ± 1.4 | 8.3 ± 0.4  |
|          | \(\tau_2\) (ms) | 327      | 172 ± 10  | 14.6 ± 0.5 | 12.1 ± 1.9 | 8.2 ± 0.3  |
| ΔNaΔmel  | \(\tau_1\) (ms) | 15       | 19.2 ± 2.0 | 14.6 ± 0.5 | 12.1 ± 1.9 | 8.2 ± 0.3  |
|          | \(\tau_2\) (ms) | 260      | 174 ± 10  | 14.6 ± 0.5 | 12.1 ± 1.9 | 8.2 ± 0.3  |
| \(n\)    |          |          | 5         | 3           | 4         |

25886 JOURNAL OF BIOLOGICAL CHEMISTRY VOLUME 281 • NUMBER 36 • SEPTEMBER 8, 2006
Loop 4–5 of MelB Takes Part in Conformational Changes

the signal during a ΔNa jump. The fact that this difference was no longer observed in the two mutants may be accounted for, at least in part, by a severe reduction or loss of sugar-induced charge transfer. Third, the melibiose-dependent increase in the peak current observed after a Na⁺ concentration jump (ΔNa(mel)) versus ΔNa) seen with Cys-less was not observed in the two mutants.

It is finally interesting to note that NEM added to Cys-less, R141C, or E142C proteoliposomes had no influence on the electrical signals (data not shown). Also not shown are electrical signals evoked by concentration jumps on R139C proteoliposomes, a mutant that functions similar to the wild-type (17), which had relative peak currents and time constants very similar to Cys-less MelB.

The peak current (Iₚ), plotted as a function of the respective substrate concentration, resulted in hyperbolic curves (not shown) that were fitted with a Michaelis-Menten-like function (Iₚ = Iₚmax*c/(c + Kₐ), where c = Na⁺ or melibiose concentration, respectively, Kₐ = half-saturation concentration). As shown previously, the Kₐ values of wild-type MelB correlated well with the apparent affinities for the substrates measured with other methods (5). Half-saturation concentrations determined for Cys-less MelB were similar to the wild type (Table 2). The cooperative effect of melibiose on Na⁺ binding was conserved in E142C, as the half-saturation concentration of Na⁺ decreased in the presence of melibiose (Table 2). In contrast, the Kₐ values for melibiose in the absence or presence of Na⁺. The melibiose signals were too small to yield reasonable currents at low sugar concentrations. However, we could measure stable melibiose signals with R141C in the presence of MTSEA⁺ determining a Kₐ similar to that for Cys-less but not significantly stimulated by Na⁺ (Table 2). The defect of R141C lies, thus, at least partly, on the level of the cooperative interactions between the two binding sites.

Fluorescence Analysis of R141C and E142C—Insights into co-substrate-induced structural changes of MelB can be obtained from FRET spectroscopy measurements of RSO membrane vesicles or proteoliposomes incubated in the presence of the β-galactoside fluorescent sugar analog Dns²-S-Gal (25). The fluorescence emission signal is due to a FRET phenomenon between the MelB tryptophans and the Dns²-S-Gal molecules specifically bound to the transporter (25) and is much more prominent in the presence of 10 mM NaCl than in its absence. The change in the Dns²-S-Gal microenvironment induced by Na⁺ most likely reflects a variation of MelB structure close to the sugar binding site (25). Analysis of the FRET properties of R141C and E142C proteoliposomes showed that both retained a significant Na⁺-dependent FRET signal (Fig. 5A). The emission maximum of E142C, but not of R141C, was shifted from 460 nm in Cys-less to 480 nm in E142C (Fig. 5A). This means that Na⁺ still induced a cooperative modification of the sugar binding site structure in E142C, but the vicinity of the sugar environment is more polar than in Cys-less or R141C. The specific fluorescence signal evoked by R139C proteoliposomes (not shown) was like that in the Cys-less permease.

In addition, intrinsic tryptophan fluorescence studies, reflecting essentially conformational changes of the protein after substrate binding (27), were performed on Cys-less, R139C, R141C, and E142C proteoliposomes. As for the wild-type (27), the Cys-less mutant fluorescence emission was slightly quenched by Na⁺, and subsequent melibiose addition induced a large fluorescence change (Fig. 5B). R139C showed a similar fluorescence behavior (data not shown). In contrast, melibiose and Na⁺ addition to E142C and R141C did not enhance the fluorescence signal significantly (Fig. 5B, inset). Upon addition of MTSEA⁺ to R141C, the sugar-induced fluorescence change did not return (data not shown). Also, NEM

FIGURE 3. Recovery of the stationary charge transfer in R141C proteoliposomes reacted with MTSEA⁺. R141C proteoliposomes were incubated with MTSEA⁺ (2.5 mM) for 45 min at 22 °C. Signals in response to a simultaneous concentration jump of 20 mM melibiose and 10 mM Na⁺(ΔNaΔmel) were recorded before and after the addition of MTSEA⁺. Traces: Cys-less MelB (dashed line); R141C incubated with 2.5 mM MTSEA⁺ (solid gray line); R141C unreacted with MTSEA⁺ (solid black line). For better comparison, all peak currents were normalized to the absolute peak current of the ΔmelΔNa jump of R141C after the addition of MTSEA⁺. Inset: mono- and a bi-exponential fit on a half logarithmic scale of the R141C signal after incubation with 2.5 mM MTSEA⁺.

FIGURE 4. Comparison of the relative peak currents caused by different concentration jumps. The relative peak currents of the electrical signals caused by different concentration jumps as described for Fig. 2 from at least three independent experiments were averaged. Indicated are the mean values (± S.E.). For normalization, the peak currents of the ΔNa signals were set to 100%, and the sizes of all other signals expressed in relation to it. Wild-type (WT) data are from one representative experiment and are taken from Ganea et al. (5).
TABLE 2
Kinetic constants of the fast electrical responses elicited in C-less, R141C, R141C + MTSEA*, or E142C mutants

|                  | WT (s) | Cys-less | R141C | R141C + MTSEA | E142C |
|------------------|--------|----------|--------|---------------|-------|
| \( K_{\text{Na},0.5} \) | Without melibiose (\( \Delta \text{Na} \)) | 2.1 | 2.9 ± 0.8 | 2.7 ± 0.7 | 3.0 ± 1.2 | 2.5 ± 0.1 |
|                   | In the presence of 50 mM melibiose (\( \Delta \text{Na} \text{mel}) | 0.6 | 1.0 ± 0.1 | 3.6 ± 0.6 | 1.6 ± 0.1 | 1.5 ± 0.4 |
| \( K_{\text{Na},0.5} \) | Without \( \text{Na}^+ \) (\( \Delta \text{m} \)) | 22 | 50.4 ± 1.8 | NM* | 30.6 ± 17.1 | NM |
|                   | In the presence of 10 mM \( \text{Na}^+ \) (\( \Delta \text{m} \text{Na}) | 3 | 4.5 ± 1.2 | NM | 21.5 ± 11.6 | NM |

* NM, not measurable.

**FIGURE 5.** Na+-dependent FRET signal and tryptophan fluorescence emission spectra of Cys-less, R141C, and E142C MelB proteoliposomes. A, difference FRET spectra (with or without Na+) recorded from Cys-less MelB, R141C, or E142C proteoliposomes (20 \( \mu \)g/ml) incubated in the presence of the fluorescent sugar analog Dns\(^-\)S-Gal (10 \( \mu \)M) and excited at 297 nm (bandpass, 5 nm) (25). The FRET signal (average of three scans) was recorded (410–540 nm, bandpass, 5 nm) before and after the addition of 20 mM NaCl. B, tryptophan fluorescence spectra for Cys-less MelB. Samples (20 \( \mu \)g) in 0.1 M KPi, pH 7, medium, were excited at 297 nm (bandpass, 5 nm). Emission spectra (310–380 nm, bandpass, 5 nm) were recorded before any addition (lane 1) or after successive addition of 10 mM Na\(^+\) (lane 2), and then 30 mM melibiose (lane 3). Inset: relative fluorescence change after addition of Na\(^+\) or Na\(^+\) plus melibiose for Cys-less, R141C, and E142C proteoliposomes. Measurements were the same as described in B. The emitted fluorescence light was integrated between 310 and 380 nm and the difference after addition of the substrates calculated. Values are the mean of three experiments (± S.E.).

**FIGURE 6.** Orientation of MelB in the proteoliposomes. A, labeling was carried out in the presence of MPB (10 \( \mu \)M), and stilbene disulfonate was used at concentration of 200 \( \mu \)M for protection against labeling. Labeling conditions were as follows: 1, labeling of all transporters: proteoliposomes incubated with MPB were submitted to freeze-thaw sonication to allow MPB access to both sides of the membrane (lane ALL). 2, labeling of inside-out oriented transporters: proteoliposomes incubated with MPB without subsequent freeze-thaw sonication (lane ISO). 3, labeling of right-side-out oriented transporters: preincubation of the proteoliposomes with the membrane-impermeable stilbene disulfonate to block all cysteines accessible from the outside followed by addition of MPB and freeze-thaw sonication (lane RSO). 4, no labeling: preincubation with stilbene disulfonate before incubation with MPB (NO lane). Signals were evaluated by Western blot analysis using streptavidin-alkaline phosphatase conjugate for MPB labeling (streptavidin lane), and an anti-C-terminal antibody for MelB detection (protein lane). B, proteoliposomes containing Cys-less MelB were incubated in the presence of varying MPB concentrations (0–1000 \( \mu \)M), subjected to three freeze-thaw sonication cycles, and incubated for 5 min at room temperature. Detection of labeled Cys-less MelB as in B.

loop 4–5 of MelB Takes Part in Conformational Changes

did not change the fluorescence properties of E142C (data not shown). In conclusion, the results of the tryptophan emission studies suggest that conformational changes following melibi-
Loop 4–5 of MelB Takes Part in Conformational Changes

(Fig. 6A, lane NO), but when freeze-thaw sonication cycles were applied to the MPB labeling reaction after the incubation with stilbene disulfonate, a faint band was visible (Fig. 6A, RSO) indicating that a small portion of the protein is RSO-oriented. The negative control, Cys-less transporter, showed nonspecific reactions of the maleimide at concentrations higher than 10 μM MPB (Fig. 6B), but these weak diffusive bands were clearly distinguishable from the very strong bands detected in R139C. Western blot analysis ensured equal amounts of protein in all samples (see Protein lanes in Fig. 6). Similar results were obtained for the non-transporting mutant R141C (data not shown) suggesting that functional as well as non-functional MelBs orient mainly in the ISO configuration in the liposomes. Consequently, the kinetic properties determined in this study by SSM measurements refer to the backward running carrier.

Finally, proteolysis experiments with trypsin (7) showed that the full-length MelB in proteoliposomes disappeared very rapidly (60% after 10 min of incubation) with the concomitant appearance of the cleavage fragments (data not shown). Because only cytoplasmic domains of MelB are processed by the protease (7), this result reinforces the conclusion of a predominant ISO orientation of MelB in proteoliposomes.

DISCUSSION

This study provides evidence that loop 4–5 connecting helices IV and V of MelB, and especially the adjacent charged residues Arg-141 and Glu-142 within it, play a key role in MelB symport activity. Together with the previous characterization of the R141C mutant (17), the kinetic, electrophysiological, and spectroscopic data reported above suggest that both Arg-141 and Glu-142 participate in conformational changes involved in the mechanism of Na⁺-sugar symport.

MelB Is Uniformly Oriented Inside-Out in the Liposomes—Using the impermeable thiol-specific compound MPB to assess MelB orientation in the proteoliposomes we have demonstrated that the transporters are uniformly ISO-oriented (Fig. 6). The observed extensive rate of cleavage of MelB cytoplasmic domains in those proteoliposomes by trypsin supports this conclusion (see results and also Ref. 7).

The finding of the unidirectional configuration was unexpected, because the reconstitution method that was used, i.e. detergent removal and subsequent freeze-thaw sonication of the samples, is generally believed to lead to scrambled rather than homogenous protein orientation (29). However, a slow rate of detergent removal, in particular during progressive addition of SM-2 Biobeads, gives rise to a mixture of small detergent-saturated liposomes, lipid-protein-detergent micelles, and protein-detergent micelles, and the remaining detergent participates in the process of protein insertion (29). This situation mimics to some extent the one in which the use of pre-formed, detergent-saturated liposomes has been shown to favor unidirectional ISO insertion of proteins with their large and/or charged hydrophilic domains exposed outside (e.g. for bacteriorhodopsin (29), the MelB homologue LacS (30), DtpT (31), and PutP (32)). According to topological and biochemical studies, also MelB has bulky and charged hydrophilic cytoplasmic domains, which should favor ISO insertion (7, 9).

When interpreting the results below, we will frequently refer to a previously proposed extended six-state kinetic model for the Na⁺-melibiose co-transport reaction (3, 4, 18, 33). In light of the evidence presented under “Results,” which suggests a uniform ISO orientation of MelB in proteoliposomes, we have adapted the original kinetic model to the situation of the transporter functioning in the reverse direction (see below and Fig. 7).

Substrate Binding in R141C and E142C Is Intact, but Substrate Translocation of the Loaded Carrier Is Impaired—Cysteine-scanning mutagenesis of the charged residues

| TABLE 3 |
| --- |
| Partial activities of C-less, NEM-inhibited WT, and R141C and E142C MelB |
| Binding | Conformational transition | Stationary activity |
| α[^H]NPG binding | ΔNa(Dm2S-Gal) | Trp fluorescence Δmel(Na) | Transient current Δmel(Na) |[^H]mel accumulation |[^H]mel entrance counterflow | Stationary current Δmel(Na) |
| C-less | + (17) | + (17)⁵ | a | a | + (17) | + (17) | + (17) |
| WT + NEM | + (4) | + (35) | + (27) | a | - (53) | - (33) | - (5) |
| R141C | + (17) | + (17)⁵ | + (5) | a | - (17) | - (17) | - (5) |
| E142C | + a | a | a | a | - (17) | - (17) | - (5) |

⁵ Results from the present study.

The _ sign indicates that the respective activity is completely or to a large extent inhibited.
Loop 4–5 of MelB Takes Part in Conformational Changes

of loop 4–5 indicates that only mutagenesis of the two adjacent residues Arg-141 and Glu-142 strongly impairs MelB functioning (Fig. 1 and Ref. 17). MelB transport activity is either completely (R141C) or largely (E142C) suppressed. Despite defective transport activity, both mutants retain the capacity to bind sugars, including the high affinity sugar-α-[3H]NPG (see “Results” and Ref. 17) or the fluorescent sugar analog Dns²-S-Gal (Fig. 5A and Ref. 17). Because previous data showed that melibiose can displace bound α-[3H]NPG or Dns²-S-Gal irrespective of whether the transporters are outward facing (in RSO vesicles) or inward facing (in ISO vesicles or proteoliposomes) (6, 25), we infer that melibiose still efficiently binds to R141C and E142C. Other findings suggest also that the cation interaction with the two mutated transporters is comparable to that in wild-type or Cys-less MelB. Indeed, Na⁺ still activates sugar binding (α-[3H]NPG, see “Results” and Ref. 17), enhances Dns²-S-Gal fluorescence emission (Fig. 5A and Ref. 17), and induces fast transient currents in R141C and E142C proteoliposomes (Fig. 2). Furthermore, the corresponding Na⁺ activation constant for α-NPG binding (KₐNa) and the Na⁺ concentration (K₀Na), at which the fast electrical responses (∆Na) in the mutants are half-maximal, are equal to those of wild type or Cys-less MelB (see “Results” and Table 2).

Partial activities of wild-type and R141C and E142C variants of MelB are summarized in Table 3. As a support for the following discussion this table also includes results from previous work. From Table 3 it is obvious that substrate binding in R141C and E142C MelB still occurs (columns “Binding” in Table 3), whereas the stationary activities (columns “Stationary Activity” in Table 3) are inhibited. In particular, the total loss (R141C (17)) or almost complete reduction (E142C, Fig. 1B) of melibiose counterflow of the two mutants is interesting, because counterflow occurs without Na⁺ transmembrane flow and involves only transitions 2 → 3 ↔ 4 ↔ 5 (28). The inhibition in R141C and E142C MelB must, therefore, be confined to the substrate translocation or release steps of the carrier (transitions 3 ↔ 4 ↔ 5 ↔ 6 in Fig. 7). In addition, formation of the Na⁺-melibiose-MelB ternary complex is still observed in RSO membrane vesicles as demonstrated by the observation of Na⁺-dependent α-[3H]NPG binding and Dns²-S-Gal FRET (4, 25). This excludes periplasmic substrate binding or release as a candidate for inhibition in the mutants and leads to the conclusion that only substrate translocation is inhibited in R141C and E142C MelB (3 ↔ 4 in Fig. 7).

Conformational Changes after Melibiose Binding Are Disturbed in R141C and E142C—Other electrical properties and spectroscopic features of R141C and E142C provide a means to tentatively relate the mutant translocation defects to modified conformational changes during MelB cycling (column “conformational transition” in Table 3). Both R141C and E142C lose the capacity to generate fast transient electrical responses after melibiose concentration jumps in the absence (∆mel) or presence of Na⁺ (∆mel(Na⁺)) even when the final sugar concentration was saturating (50 μM melibiose). A detailed characterization of these sugar-induced fast transient electrical signals in wild-type or Cys-less MelB has led to the conclusion that they are linked to conformational changes moving yet unidentified charged amino acids or helical dipoles (18). According to this explanation, these sugar-induced electrogenic conformational changes would be missing in R141C and E142C.

Further evidence for a lack of sugar-induced conformational changes in the two mutants came from Trp fluorescence experiments. The typical Na⁺-dependent increase in the Trp fluorescence signal recorded in the native or Cys-less transporter after sugar binding was no longer observed in R141C or in E142C (Fig. 5B). This Trp fluorescence signal rise is due to a light emission increase from Trp-299 and Trp-342 in the C-terminal helices IX and X of MelB, respectively (19, 27) and has been attributed to cooperative conformational changes either associated to or following binding of melibiose. It must be recalled here that helices X and XI are among the different helices putatively lining the sugar binding site and/or the sugar pathway (16, 19, 26, 34, 35).

A Different Phenotype of Inhibition Is Observed in R141C and E142C as Compared with NEM-inhibited Wild-type MelB—For comparison, the effect of NEM on the partial activities of wild-type MelB has been included in Table 3. At first glance the phenotypes of R141C and E142C MelB seem to be the same as that of the NEM-inhibited WT protein: substrate binding is still intact while turnover is inhibited. Similar to the mutants, NEM-inhibited WT MelB can still form a ternary complex in RSO membrane vesicles as demonstrated by the observation of Na⁺-dependent α-[3H]NPG binding and Dns²-S-Gal FRET as well as a Na⁺-induced Trp fluorescence signal in proteoliposomes (4, 25, 27). Therefore, in the NEM-inhibited WT enzyme substrate translocation is inhibited similar to R141C and E142C MelB.

However, an important distinction shows up in the activities related to a conformational transition following melibiose binding (columns “conformational transition” in Table 3). The conformational transition was still observed in NEM-inhibited WT MelB, whereas it was absent in the mutants, suggesting that the partial reactions inhibited by NEM and by the mutations at position 141 and 142 are different. This could fit a model in which substrate translocation in MelB is a two-step process consisting of an electrogenic conformational transition inhibited in R141C and E142C MelB (3 ↔ 4 in Fig. 7) followed by a second NEM inhibitable reaction (3’ ↔ 4 in Fig. 7).

An attractive hypothesis arises in light of the recent proposal that the shuttling of co-transporters from one side of the membrane to the other may involve more intermediates than initially deduced from the simple one-step alternate access model (36, 37). In particular, voltage-jump experiments on hSGLT1 demonstrated different conformational states occurring during the reorientation of the empty carrier from the inward to the outward facing conformation (38). An extra- and hypothetical intra-membrane kinetic intermediate (C’ Na mel) has also been included in the MelB kinetic model recently on grounds of electrical measurements (18). The hypothetical step was assigned to an electrogenic conformational transition after melibiose binding (18). Also, combining cryo-electron microscopy data of OxlT at 6.5 Å with a model of the “open” state of the oxalate transporter deduced from the atomic structures of related transporters like the lactose permease from E. coli (39) hypothesized the existence of an oxalate-bound “closed” or “occluded” OxlT state. It is, therefore, tempting to associate the occluded state in MelB with the extra state C’ Na mel (state 3’ in Fig. 7) between the electrogenic conformational transition inhibited...
in the R141C and E142C variants and the subsequent NEM inhibitable reaction.

Mutations of R141 and E142 Induce Changes in Structural and Cooperative Properties of MelB—FRET studies indicate that the Na\(^{+}\)-induced spectroscopic variations of Dns\(^{2-}\)-S-Gal bound to the sugar site differ in the two mutants. Whereas the emission in R141C is comparable to that from the Cys-less mutant (17), there is clearly a red-shift in E142C emission (Fig. 5A) indicating that the sugar binding site environment after binding of Na\(^{+}\) becomes proportionally less apolar as compared with Cys-less MelB (25). Thus, both mutations have not a strictly comparable impact on the structural properties of MelB. In addition, we noted that the \(K_{\text{m}}\) is dependent on melibiose in Cys-less and E142C but not in R141C. This latter finding is pointing out to an alteration of the cooperative interaction between the sugar and cation binding sites in R141C.

Analysis of site-directed mutants, second-site revertants, or cysteine-screen scanning in several predicted helices combined with sulfhydryl-reagent accessibility, suggest that several membrane domains from the N- or C-terminal half of MelB (I, II, IV, X, and XI) are lining the substrate sites and/or the translocation pathway (16, 19, 26, 34, 35, 40–42). Consistently, the projection map or three-dimensional model, derived from two-dimensional crystals of MelB, at 8- or 10-Å resolution, respectively (6, 43), suggests that a central curve-shaped depression, tentatively assigned to the substrate pathway and/or substrate binding sites, is surrounded by about seven densities putatively interpreted as transmembrane helices. The derived three-dimensional structure of MelB (43) bears similitude to that emerging from the recently published structures at high resolution of LacY (44, 45), GlpT (46), or OxlT (47–49). All these transporters are supposed to operate according to an alternating-access mechanism where conformational changes are associated to binding and translocation of the substrates. In this context, recent Fourier transform infrared and differential attenuated total reflectance-Fourier transform infrared studies (50–52) support the existence of such structural changes by indicating sugar-induced increases in MelB compactness and substrate-induced changes of the transporter absorbance tentatively assigned to secondary structure components. Finally, several observations favor the notion that the N-terminal domains of MelB are essential for recognition of the coupling Na\(^{+}\), whereas other domains in the C-terminal half preferentially participate in the interactions with the sugar substrate. Coordinated interactions between some of the domains lining the two substrate binding sites are likely to be crucial for the cooperative and coupling properties of H\(^{+}\) or cation-coupled transporters.

On the basis of this and previous studies, our current working hypothesis is that concerted interactions between several putative helices lining the central hydrophilic pathway (and/or the substrate-binding sites) are essential for substrate binding and translocation in MelB. Loop 4–5 may, thereby, be essential for the stabilization of the structure after binding of the substrates and/or for the translocation process. The set of interactions initially promoted by substrate binding (giving rise to the ternary MelB complex, C\(_{\text{in}}\) Na mel) may be distinct from that linked to translocation (C\(^{+}\) Na mel). This working hypothesis has the merit to account for the observed dissociation of the binding and translocation events in the mutants. At the same time, it provides a simple explanation for the diversity of changes of cooperative and structural properties introduced by neighboring mutations located in a domain that cannot be strictly classified as a transmembrane region because of its accessibility to proteases (7).

Conclusions—The data presented here suggest that the local domain of loop 4–5 harboring the neighboring Arg-141 and Glu-142 residues plays an important role in the mechanism of Na\(^{+}\)-sugar symport by MelB due to an interaction with the substrate binding sites. Loop 4–5 might contribute to the coordinated interactions between the ion and sugar binding sites and participates in a conformational change after melibiose binding that is essential for the subsequent obligatory coupled translocation of substrates. It is also proposed that substrate translocation proceeds as a two-step reaction: in the backward running carrier the first step is inhibited in R141C or E142C MelB, whereas the second is inhibited by NEM.

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**Loop 4–5 of MelB Takes Part in Conformational Changes**

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