Sugar Binding Induced Charge Translocation in the Melibiose Permease from *Escherichia coli*†

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ABSTRACT: Electrogenic events associated with the activity of the melibiose permease (MelB), a transporter from *Escherichia coli*, were investigated. Proteoliposomes containing purified MelB were adsorbed to a solid supported lipid membrane, activated by a substrate concentration jump, and transient currents were measured. When the transporter was preincubated with Na^+ at saturating concentrations, a charge translocation in the protein upon melibiose binding could still be observed. This result demonstrates that binding of the uncharged substrate melibiose triggers a charge displacement in the protein. Further analysis showed that the charge displacement is neither related to extra Na^+ binding to the transporter, nor to the displacement of already bound Na^+ within the transporter. The electrogenic melibiose binding process is explained by a conformational change with concomitant displacement of charged amino acid side chains and/or a reorientation of helix dipoles. A kinetic model is suggested, in which Na^+ and melibiose binding are distinct electrogenic processes associated with approximately the same charge displacement. These binding reactions are fast in the presence of the respective cosubstrate ($k > 50\ s^{-1}$).

The melibiose permease (MelB)[1] of *Escherichia coli* is a membrane bound carrier that belongs to the galactosides−pentoses−hexuronides transport family (*1*) composing the wide class of electrochemical potential driven porters (*2*, *3*). This ion-coupled sugar cotransporter uses the favorable Na^+, Li^+, or H^+ electrochemical potential gradient to drive cell accumulation of α-galactosides (melibiose, raffinose) or *β*-galactosides (methyl-1-thio-*β*-D-galactopyranoside) (*4*−*10*). Detailed functional analysis of MelB led to the proposal of a six-state mechanistic model, which accounts for many kinetic properties of the carrier (*4*, *6*, *11*, *12*).

The MelB symporter (473 amino acids, 53 kDa) has 12 α-helical transmembrane domains (*13*−*18*). A recombinant transporter harboring a 6-His tag permease can be purified in large amounts, and if reconstituted into liposomes, exhibits cation-dependent sugar-binding and transport properties comparable to those of the native permease in its natural environment (*13*, *19*). Evidence strongly suggesting that four aspartic acid residues distributed in the N-terminal helices I (D19), II (D55 and D59), and IV (D124) of MelB form a coordination network involved in cation recognition has been obtained (*1*, *20*−*23*). Moreover, several lines of evidence support the earlier suggestion that some discrete steps of MelB cycling involve conformational changes of the transporter. In particular, cooperative protection against proteolysis of the highly charged cytoplasmic loop connecting helices IV and V (loop 4−5) of MelB by its substrates has been observed (*16*). Cooperative changes in MelB conformation upon substrate binding were also inferred from the analysis of the intrinsic tryptophan fluorescence of MelB and fluorescence resonance energy transfer spectroscopy with a fluorescent sugar analogue (*19*, *24*).

Recently, analysis of transient electrical signals recorded from MelB proteoliposomes adsorbed onto a solid supported membrane (SSM) proved to be a useful strategy to investigate electrogenic events associated to partial steps of the Na^+ melibiose symport reaction (*25*). A cosubstrate concentration jump was found to give rise to a transient current corresponding to an inward movement of positive charge. Two types of transient signals were observed that differed by their characteristics of decay: imposing either a simultaneous Na^+ and sugar concentration jump or a Na^+ concentration jump to liposomes that were already equilibrated with sugar induced transient signals with biphasic decays, which could

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[1] Abbreviations: MelB, melibiose permease; SSM, solid supported membrane; NEM, *N*-ethyl maleimide; DTT, dithiothreitol; LAPAO, 3-(laurylamido)-*N*,*N*′-dimethylaminopropylamine oxide; SGLT1, Na^+/glucose cotransporter.

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be decomposed into a fast component ($\tau = 10-20$ ms) and a slower one ($\tau = 300-400$ ms). This slow component was suppressed by selective inactivation of the cosubstrates translocation with *N*-ethyl maleimide (NEM) (*5*, *25*). In contrast, a transient response including only the fast component was recorded when applying a concentration jump of Na^+ in the absence of the sugar. From all these data, it was deduced that Na^+ binding to MelB induces an intraprotein charge translocation reflected by the fast component of the transient current.

Unexpectedly, a melibiose concentration jump imposed on liposomes equilibrated with Na^+ triggered transient signals that also included a fast component (*25*). Since the sugar is an uncharged species, interpretation of the fast component is not straightforward. This electrical signal could be brought about by three different mechanisms. (1) The binding of additional Na^+ ions to the protein due to the drastically increased Na^+ affinity after addition of melibiose. (2) A melibiose induced electrogenic conformational change of the protein that displaces the cotransported H^+ or Na^+ ions. (3) A process unrelated to binding or intraprotein displacement of Na^+ but rather implying an electrogenic conformational change. To discriminate among these explanations, we studied the melibiose induced electrical signal in proteoliposomes previously equilibrated with Na^+ at different concentrations. The Na^+ concentration was raised to 100 mM, thereby ensuring that the Na^+ binding sites were completely saturated before the melibiose concentration jump took place. We present evidence that melibiose binding to MelB results in an electrogenic conformational change.

MATERIALS AND METHODS

Chemicals. Activating and nonactivating solutions contained 0.1 M KPi (pH 7) and 0.1 mM dithiothreitol (DTT, 99.5% Roth, Karlsruhe, Germany) plus salts and melibiose at concentrations as indicated next. Highest purity grade reagents (KH_2PO_4, Sigma, 0.005% Na^+ and KOH Merck, suprapur, 0.002% Na^+) were used to prepare nominally Na^+-free media (Na^+ concentration <20 μM, determined by atomic absorption spectroscopy). All other materials were obtained from commercial sources.

Purification and Preparation of the Proteoliposomes. His-tagged MelB (thereafter termed MelB) was purified from inverted membrane vesicles of *E. coli* DW2 cells (Δ*melB* Δ*lac*ZY) transformed with pK95ΔAHB plasmid that harbored the His-tagged MelB coding sequence (*13*). Inverted membrane vesicles were prepared by means of a French Press (Amercian Instruments Comp., 1200 psi), and the protein was solubilized by using 1% 3-(laurylamido)-*N*,*N*′-dimethylaminopropylamine oxide (LAPAO). A chromatography procedure combining the utilization of Ni-NTA and ion exchange (MonoQ) resins were used to prepare nearly pure MelB (generally >99%) solubilized in dodecylmaltoside (0.1%) (*13*). MelB reconstitution into liposomes (protein/lipid ratio 1/5 w/w) was performed by removing the detergent with Bio-Beads SM-2 (*26*). MelB content was assayed by a Lowry procedure including sodium dodecyl sulfate (0.2%) and using bovine serum albumin as standard (*27*).

SSM Setup and Measuring Procedure. The SSM was prepared by linking an octadecylmercaptan monolayer to a gold electrode deposited on a glass support and covering it with a lipid (diphytanoyl phosphatidylcholine, synthetic, Avanti Polar Lipids Inc., Pelham, AL) monolayer. The SSM was mounted in a flow-through cuvette, and the signal was recorded between the gold electrode and a reference Ag/AgCl electrode. After formation of the SSM, 40 μL of proteoliposomes (~15 μg of protein) were sonicated, injected into the cuvette, and allowed to adsorb to the SSM for 30−50 min. A typical solution exchange protocol for a Δmel(Na) concentration jump consisted of three phases: (1) nonactivating solution containing 100 mM KP_i, pH 7 (100 mM KH_2PO_4/KOH), 0.1 mM DDT, 20 mM glucose to minimize ionic strength and osmotic effects, and NaCl at concentrations as indicated below (1 s); (2) activating solution containing the same buffer but glucose substituted by 20 mM melibiose (1 s); and (3) nonactivating solution as described in part 1) (1 s). In the ΔNa(mel) experiment, the nonactivating solutions contained 10 mM KCl and melibiose at concentrations as indicated, and the activating solution contained the same but used 10 mM NaCl instead of KCl. In the ΔNa experiment, the nonactivating solution contained 10 mM KCl, which was substituted by 10 mM NaCl in the activating solution. The buffer was always 100 mM KPi, pH 7 as described previously. In the NEM inhibition experiment, DTT free buffers were used. Solutions were driven through the cuvette by applying pressure to the solution containers (0.6 bar). Concentration jumps at the beginning of phase 2 (on-signal) and at the beginning of phase 3 (off-signal) caused electrical signals, which were recorded via a computer. Only the on-signal will be used throughout our analysis. All experiments were carried out at room temperature (22 °C). For details of the method, see refs *25* and *28*.

Data Analysis. Parameters of the electrical signals given in the text are mean values from at least three experiments, and the standard error of the mean is given. Time constants and amplitudes were determined by fitting the decaying phase of the signal with a single- or a double-exponential function as required.

RESULTS

MelB proteoliposomes and SSM form a capacitively coupled system that enables measurements of transient currents in response to rapid concentration jumps of MelB substrates (*25*). This rapid flow technique was used in this study to examine the transient electrical signals produced by imposing melibiose or Na^+ concentration jumps on proteoliposomes equilibrated with the respective other co-substrate at various concentrations or simple Na^+ concentration jumps.

Melibiose Induced Electrical Signals. Typical sugar-induced currents recorded upon 20 mM concentration jumps of melibiose on proteoliposomes are shown in Figure 1. In the absence of Na^+ (trace 0 mM), the signal decays biexponentially (decay times 98 and 350 ms) (*25*). In the presence of 3 mM Na^+ (trace 3 mM), the resulting electrical signal was significantly bigger and faster. The indicated concentration of Na^+ was present before and during the melibiose concentration jump (i.e., sufficient time was given

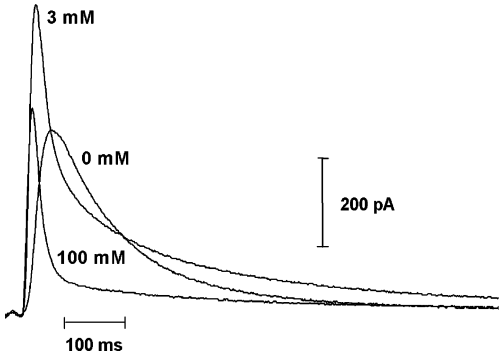

FIGURE 1: Electrical signals generated by MelB after a 20 mM melibiose concentration jump in the absence (0 mM) and presence of 3 and 100 mM Na^+ recorded on the same SSM/proteoliposome preparation. The figure shows traces from a single representative experiment.

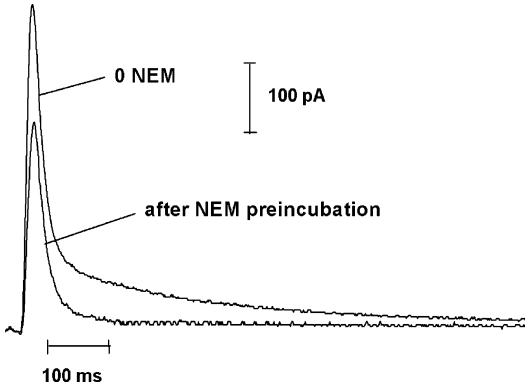

FIGURE 2: Electrical signals generated by MelB after a 20 mM melibiose concentration jump in the presence of 100 mM Na^+ with and without NEM (2 mM) preincubation. The figure shows traces from a single representative experiment.

for Na^+ to bind to the enzyme before the signal was recorded). The resulting electrical current is transient and has a biexponential decay. The fast component, which dominates the electrical signal (decay time ~15 ms), has been previously assigned to a melibiose induced charge displacement early in the first turnover, while the slowly decaying phase (decay time ~230 ms), which contributes ~40% to the total amplitude of the signal, probably corresponds to the stationary transport activity of the permease (*25*). Henceforward, the slow phase of the signal will be termed transport component. The fast component has kinetic characteristics very similar to that of the single fast component recorded on imposing a Na^+ concentration jump in the absence of melibiose (*25*). This latter has been interpreted as reflecting an electrogenic event associated to or resulting from Na^+ binding. On the other hand, melibiose carries no charge. Therefore, a possible explanation for the sugar-induced fast component recorded on liposomes equilibrated with 3 mM Na^+ medium would be the binding of additional Na^+ ions to MelB. This is a likely possibility as we have previously shown that addition of melibiose significantly increased MelB affinity for Na^+ ions. As a result of the drop of $K_{0.5}{}^{Na}$ from 2.1 mM in the absence of sugar to 0.6 mM in its presence (*25*), the occupancy of Na^+ binding sites at 3 mM Na^+ concentration should rise from 59 to 83% upon sugar addition addition (calculated from simple equilibrium binding: $[ES]/[E_{total}] = [S]/([S] + K)$, with ES = transporters with bound substrate, E_{total} = total amount of transporters, S = substrate, and K = substrate dissociation constant). If sugar-induced extra binding of Na^+ ions would exclusively account for the presence of a fast current component in the electrical response, one expects this fast component to disappear on raising the concentration of Na^+ ions in the equilibrating solution.

When the Na^+ concentration was increased to 100 mM (Figure 1, trace 100 mM), an electrical signal still including a significant fast phase after a sugar concentration jump was recorded (rise time ~9 ms, decay times ~12 and 311 ms). The peak current, reflecting essentially the amplitude of the fast component, was 67 ± 2% of that recorded in the presence of 3 mM Na^+, although at 100 mM Na^+ only 2% of the cation binding sites were theoretically not occupied by Na^+ ions before the activating melibiose concentration jump was applied. Extra electrogenic Na^+ binding to the empty carrier in its first turnover can, therefore, be ruled out as mechanism for the observed electrical reaction at 100 mM Na^+. Note that the transport component of the electrical signal at 100 mM NaCl was slower, and its amplitude reduced to 20 ± 5% as compared to the signal in the presence of only 3 mM NaCl.

Inhibition by NEM. Inactivation of MelB turnover by NEM provides a means to better identify steps of the transport reaction associated with the melibiose-induced fast electrical event. As previously shown by flux measurements, acylation of MelB cysteines by this thiol reagent inhibited Na^+ and melibiose cotransport (*5*), but binding of the high-affinity melibiose analogue α-NPG was not inhibited (*12*). This has been confirmed by previous electrical measurements (*25*). A melibiose concentration jump was imposed in the presence of 100 mM NaCl with and without a 30 min preincubation with 2 mM NEM (Figure 2). The figure shows that the melibiose induced transient signal contains only a single fast component in the presence of NEM. In agreement with our previous results (*25*), the slow transport component of the signal disappeared after NEM incubation. This demonstrates that the fast electrical component is associated with melibiose binding rather than with transport of the cosubstrates. In particular, it excludes the contribution of additional electrogenic Na^+ binding during subsequent turnovers to the melibiose-induced signal. Electrogenic Na^+ binding in a second turnover can also be ruled out on grounds of the low turnover of the enzyme (~4 s^{-1} (*6*)).

Na^+ Concentration Dependence. The Na^+ concentration dependence of the normalized peak currents of the sugar-induced response is shown in Figure 3. The peak currents from five different sets of measurements are shown. The peak currents have been normalized to the respective average value obtained at Na^+ concentrations between 1 and 10 mM. The signal amplitude increased first with increasing Na^+ concentration up to ca. 1 mM. This phase is characterized by a half-saturation concentration of $K_{0.5}{}^{Na}$ = 0.25 mM, a value that agrees well with the Na^+ concentration required to produce half-maximal variation of MelB intrinsic fluorescence in proteoliposomes (~0.1 mM (*19*, *24*)). At Na^+ concentrations >1 mM, a plateau is reached, and then the fast component amplitude declines steadily being reduced by ca. 30% toward higher (100 mM) Na^+ concentrations.

At Na^+ concentrations between 10 and 100 mM, the data displayed a large variation from experiment to experiment

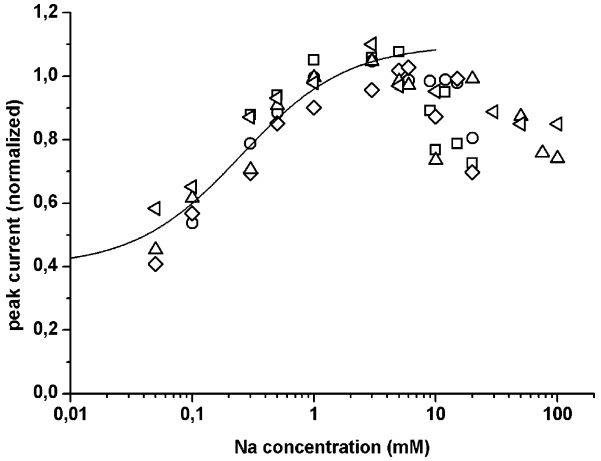

FIGURE 3: Dependence of the peak currents on the Na^+ concentration. Electrical signals were recorded after a 20 mM melibiose concentration jump in the presence of the indicated Na^+ concentration. The figure displays a compilation of five experiments (see different symbols). The solid line is a hyperbolic function: $I_p = 0.4 + 0.7c/(c + K_{0.5}^{Na})$ with I_p = peak current, c = Na^+ concentration, and $K_{0.5}^{Na} = 0.25$ mM.

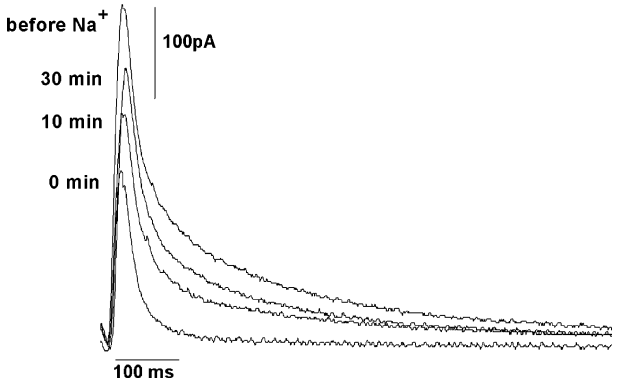

FIGURE 4: Recovery of the transport component after the incubation with 100 mM Na^+. The incubation was performed after the absorption of the proteoliposomes to the SSM. Between the recordings of the signals, the SSM was incubated with Na^+ free buffer. Electrical signals were recorded after a 20 mM melibiose concentration jump in the presence of 5 mM Na^+. The figure shows traces from a single representative experiment.

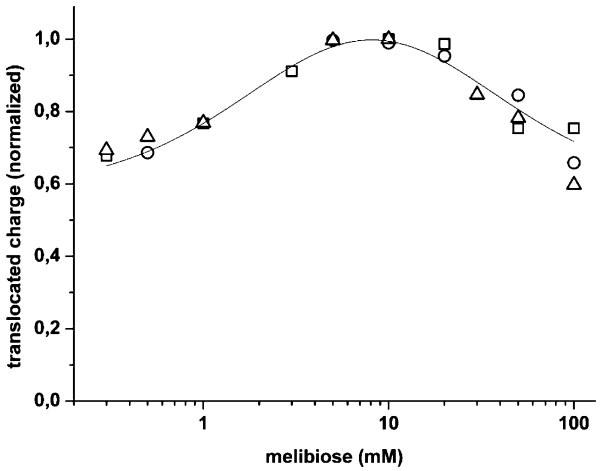

FIGURE 5: Translocated charge during a 10 mM Na^+ concentration jump at different melibiose concentrations. The translocated charge was determined from the transient currents by numerical integration in the time range 1−60 ms. The results of three experiments were normalized and are shown superimposed in the figure. The solid line is a fit to the data using the model function described in the text.

and finally at 100 mM Na^+, the transport component was significantly reduced. Furthermore, the peak currents and the transport component of the signals after a melibiose concentration jump showed the same characteristics regardless of whether the protein was preincubated with 10 mM NaCl or 10 mM NaCl and 90 mM KCl (data not shown). This suggests that no factors other than Na^+ and melibiose (e.g., ionic strength, chloride concentration, osmotic gradients) contribute to any extent to the decline of the signals observed at high Na^+ concentration. In contrast, a possible Na^+ leak into the liposomes at high Na^+ concentration may be responsible for the decrease of the transient currents. Inhibitory effects of internal Na^+ ions on MelB transport reaction are well-documented and have been ascribed to a reduction of the number of melibiose transporters facing the outer medium due to a locking of the MelB in the inwardly oriented state (*5*, *11*).

Two observations support the contention of a Na^+ leak. In a first set of experiments, we preincubated the SSM/proteoliposomes system with 100 mM Na^+ to favor Na^+ to leak into the liposomes. Directly afterward, we recorded an electrical signal using a 20 mM melibiose jump in the presence of only 5 mM Na^+. As can be seen in Figure 4 (trace 0 min), the slow transport component of the signal disappeared almost completely, and the peak current decreased. We then incubated the SSM during 10 and 30 min with Na^+-free buffer and recorded again a 20 mM melibiose jump signal in the presence of 5 mM Na^+. The peak current increased, and the slow transport component recovered with a half time of ∼10 min. In a second set of experiments, we preloaded the proteoliposomes with Na^+ prior to their adsorption to the SSM membrane by three repeated freeze−thaw sonication cycles in 100 mM Na^+ buffer. After the liposomes were adsorbed to the SSM in a medium containing 100 mM Na^+ to prevent any loss of internal Na^+, a 20 mM melibiose concentration jump in the presence of 5 mM Na^+ was applied. Such a concentration jump induced an electrical signal decaying almost monoexponentially. After 1 h of incubation with Na^+-free buffer, the peak current increased, and a significantly larger slow transport phase could be seen (data not shown). These results clearly show that the proteoliposomes are leaky and that Na^+ is able to cross the membrane within a time scale of ∼10 min via a pathway provided by the transporter or by the lipid membrane itself. Once inside the liposome, Na^+ inhibits the electrical response. From the Na^+ dependence of the transient currents at high Na^+ concentrations (see Figure 3), we estimate an affinity of the Na^+ discharge site of ∼10 mM.

Melibiose Concentration Dependence. To further analyze the mechanism of melibiose binding, concentration jumps of 10 mM Na^+ were performed on proteoliposomes preincubated with melibiose at concentrations ranging from 0.3 to 100 mM (Figure 5). The indicated concentration of melibiose was present before and during the Na^+ concentration jump was applied (i.e., melibiose was already bound to the enzyme before the Na^+ signal was recorded). The recorded transient signals had a biexponential decay. The magnitude of the peak currents increased as the melibiose concentration was raised from 0.3 to 20 mM and then progressively decreased again. The values of the peak

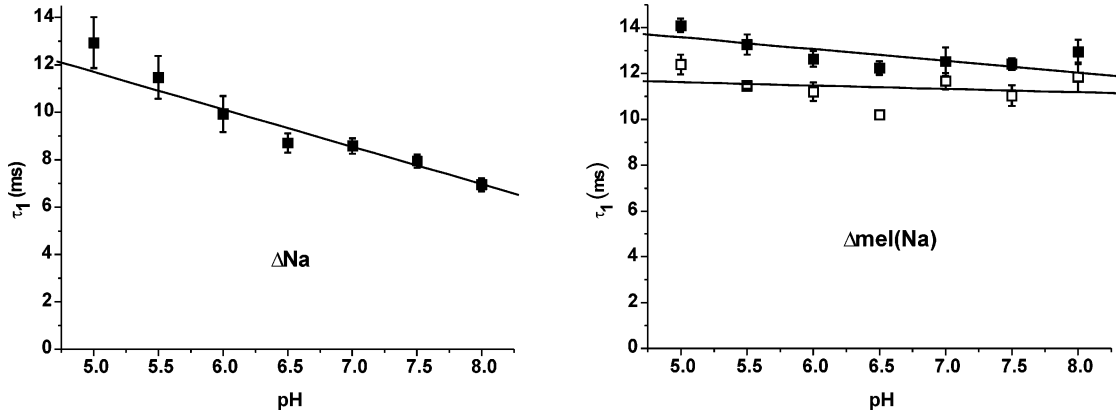

FIGURE 6: Effect of pH on the fast relaxation time constant of the decaying phase of the electrical signal. Different concentration jumps were performed, the signals recorded, and the decaying part fitted with a mono- (ΔNa and Δmel(Na), [Na^+] = 100 mM)) or biexponential (Δmel(Na), [Na^+] =10 mM) function, respectively. The resulting time constants were plotted against the respective pH. Indicated are the mean values and the standard error of three experiments. ΔNa: concentration jump of 10 mM Na^+. Δmel(Na), solid squares: concentration jump of 20 mM melibiose in the presence of 10 mM Na^+. Δmel(Na), open squares: concentration jump of 20 mM melibiose in the presence of 100 mM Na^+.

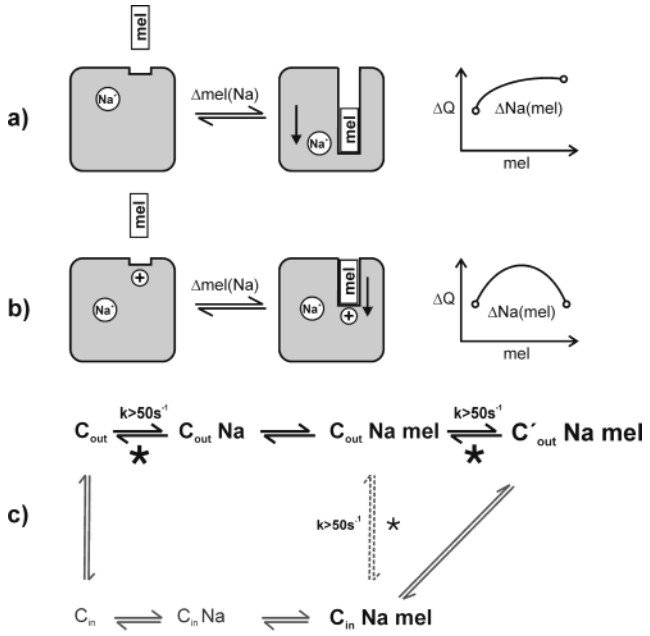

FIGURE 7: Two different mechanisms (a and b) for the charge displacement during melibiose binding in the presence of Na^+ (Δmel(Na)). For the two different mechanisms, the respective predicted melibiose concentration dependence of the translocated charge during a ΔNa(mel) experiment is shown. Panel c shows an expanded six-state model for the transport of MelB. The portion of the model relevant for our analysis is in bold. The suffixes in and out refer to the inside and outside of the liposomes. Electrogenic reactions are indicated by an asterisk. For some reaction rates, an upper limit for the rate constant could be determined ($k > 50\ s^{-1}$). In the text, two alternative transport sequences are discussed. (1) $C_{out} \rightarrow C_{out}Na \rightarrow C_{out}Na\ mel \rightarrow C'_{out}Na\ mel \rightarrow C_{in}Na\ mel$ with the melibiose induced conformational change assigned to $C_{out}Na\ mel \rightarrow C'_{out}Na\ mel$. (2) $C_{out} \rightarrow C_{out}Na \rightarrow C_{out}Na\ mel \rightarrow C_{in}Na\ mel$ with the melibiose induced conformational change assigned to $C'_{out}Na\ mel \rightarrow C_{in}Na\ mel$ indicated by dashed arrows.

currents at melibiose concentrations approaching 100 mM are comparable to those at low sugar concentrations.

As debated more extensively in the Discussion, the dependence of the Na^+ concentration jump as a function of the sugar concentration in the medium can be described by a model (see Figure 7) in which the Na^+- and sugar-induced electrical responses correspond to distinct electrogenic events taking place in parallel in the transporter. This can be quantitatively described by the following model function, which was used to fit the data points of Figure 5:

$$\Delta Q = \Delta Q_{Na} + \Delta Q_{mel}\left\{\frac{c}{c + K_{0.5}^{mel}(+Na^+)} - \frac{c}{c + K_{0.5}^{mel}(-Na^+)}\right\}$$

Here, ΔQ_{Na} and ΔQ_{mel} are the distinct charge displacements triggered by Na^+ and melibiose binding, respectively, c is the melibiose concentration, and $K_{0.5}^{mel}(+Na^+)$ and $K_{0.5}^{mel}(-Na^+)$ are the melibiose affinities of the transporter in the presence and absence of Na^+, respectively. This equation is strictly valid only at saturating Na^+ concentration, a condition that is approximately obtained with the concentration of 10 mM Na^+ in the experiment. We have chosen this relatively low Na^+ concentration because higher concentrations, although possible, would have caused larger artifacts (data not shown). For the fit, we have taken the previously determined values (*25*) $K_{0.5}^{mel}(-Na^+) = 22$ mM and $K_{0.5}^{mel}(+Na^+) = 3$ mM. ΔQ_{Na} and ΔQ_{mel} were the adjustable parameters of the fit to the normalized data. Using this model function, a good quality fit could be obtained for the experimental data (Figure 5) yielding $\Delta Q_{Na} = 0.58$ and $\Delta Q_{mel} = 0.91$. This fit, therefore, shows that Na^+ binding contributes ~40% and melibiose binding contributes ~60% of the total displaced charge during binding of both substrates.

pH Dependence of the Na^+ and Melibiose Induced Charge Displacement. It was shown in Figure 1 that the fast decaying component τ_1 of a Δmel(Na) concentration jump has a value of ~15 ms and is, therefore, very similar to that of the single fast decaying component recorded on imposing a simple Na^+ concentration jump in the absence of sugar (14 ms (*25*)). One should recall that the fast relaxation time τ_1 describes the process of filling up the binding sites of the transporter after the substrate concentration jump (*25*). Consequently, this relaxation time depends on the rate of substrate binding but may in addition be significantly modulated by the rise time of the substrate concentration at the surface of the SSM (~20 ms, unpublished results). However, since the concentration rise time is an instrumental parameter and, therefore,

constant, a change in τ_1 always reflects a change in the binding properties of the substrate. The similarity in the time constants of the signals does, however, not necessarily imply that the molecular events causing these two electrical signals are the same. To demarcate the Na^+ induced charge displacement from that induced by melibiose in the presence of saturating Na^+ concentration, we applied the two different concentration jumps at different pH values (pH 5−8). Figure 6 illustrates the variation of the fast relaxation time of the decaying phase of the electrical signals with pH. As observed in Figure 6 (graph ΔNa), a pure Na^+ jump generated a reaction that slowed at a lower pH and thereby higher H^+ concentration. A similar decrease toward lower pH was found for the integrated charge (integration time range 0−60 ms, data not shown). This phenomenon can be explained by the inhibitory effect of protons on Na^+ binding because protons and Na^+ ions compete for a common binding site (*12*).

Remarkably, the inhibitory pH effect no longer took place in proteoliposomes preincubated with 10 mM Na^+ (Figure 6, graph Δmel(Na), solid squares). In this case, no or little dependency of the relaxation time from the pH value was observed. The same effect was observed when the concentration of Na^+ was raised to 100 mM (Figure 6, graph Δmel-(Na), open squares) leading to a 98% occupancy of the Na^+ binding sites ($K_{0.5}^{Na} = 2.1$ mM). A similar observation has been made previously (*12*), namely, that increasing H^+ concentrations inhibited the Na^+-dependent sugar binding and that the inhibitory effect of protons disappeared as the Na^+ concentration increased. These data strongly suggest that the molecular basis of the fast electrical events generated during a melibiose jump in proteoliposomes equilibrated with Na^+ is different from that triggered by a pure Na^+ jump.

DISCUSSION

The electrical signal following the binding reaction of melibiose to MelB was investigated by means of the SSM technique. It was of special interest to notice that a neutral substrate, namely, the sugar melibiose, can trigger an electrical signal upon binding to the MelB transporter (*25*). In this study, we have considered different scenarios to explain this finding: first, due to the cooperative nature of transport by MelB, the increase of the cation affinity upon melibiose binding could induce Na^+ filling of the unoccupied cationic binding sites, which would lead to a charge displacement via an electrogenic cation binding mechanism. Alternatively, the already bound coupling ion (Na^+ or H^+) could be displaced within the core of the protein by this conformational change**.** Finally, binding of melibiose could initiate a conformational change that displaces charged residues of the protein or reorients the electrical dipoles of α-helices. In this study, we have addressed these questions and found evidence that Na^+ is not involved in the melibiose-induced charge translocation neither via cooperative binding of additional Na^+ ions nor by displacement of bound Na^+. In contrast, our data support a mechanism, in which melibiose binding triggers a conformational change that displaces charged intra-protein amino acid side chains or reorients electrical dipoles.

Electrogenic Conformational Change rather than Extra Na^+ Binding Is Responsible for the Melibiose-Induced Electrical Signal. Our data demonstrate that a sugar-induced fast electrical signal is still observed at conditions, in which Na^+ is bound to all MelB transporters (a Δmel(Na) jump at saturating 100 mM Na^+, Figure 1). In this situation, additional Na^+ binding after a melibiose concentration jump is excluded as cause for the electrical signal. Furthermore, the fast sugar-induced transient current persists after inactivation of substrate transport by NEM treatment, indicating that it is not linked to steady state cosubstrate transport (Figure 2). Consequently, the underlying molecular event must correspond to a movement of charges within MelB, most likely dependent upon conformational changes triggered by melibiose binding. Cosubstrate-triggered conformational variation has been documented by analysis of the intrinsic MelB fluorescence properties (*19*, *29*), by fluorescence resonance energy transfer spectroscopy with a fluorescence sugar analogue (*24*), or finally, by Fourier transform infrared spectroscopy (*17*, *30*). A conformational change upon sugar binding at close to saturating Na^+ has also been reported for the human Na^+/glucose cotransporter (hSGLT1) (*31*). This conformational change was also associated with charge movement. For hSGLT1, it was postulated that the observed reaction is the final step of sugar binding preceding the sugar translocation step. This is a likely interpretation also for MelB.

Molecular Events Underlying Electrogenic Na^+ and Melibiose Binding Are Different. The fast relaxation time constant (τ_1) of the decaying part of the transient signal was used to demarcate Na^+ binding from melibiose binding (Figure 6). τ_1 depends strongly on the pH in the case of ΔNa but not in the case of Δmel(Na) experiments. This suggests that the molecular processes underlying the electrical responses observed in these two experiments are different. In the ΔNa solution exchange, Na^+ binding is the current-generating process and accounts for the pH dependence. If the electrical signal of the Δmel(Na) concentration jump was kinetically controlled by binding of additional Na^+ ions, a similar dependency would have been observed. This was, however, not the case. Together with the persistence of a fast electrical signal in transporters fully occupied by Na^+ ions, these experiments, therefore, support the notion that the electrical signal observed during melibiose binding in the presence of Na^+ is not caused by extra Na^+ binding.

Charge Movement Observed during Melibiose Binding Is not Linked to an Intraprotein Displacement of Already Bound Na^+ Ions. According to the arguments given previously, melibiose binding results in an electrogenic conformational change. We can now ask the question: what are the charges displaced during this structural rearrangement of the protein? Two different schemes can be envisioned for melibiose binding in the presence of Na^+ (see Figure 7, left part). In mechanism a, the charge displaced during binding of melibiose corresponds to the cosubstrate Na^+ that is moved deeper into the protein. In mechanism b, melibiose binding induces a charge movement not related to further displacement of bound Na^+. From the polarity of the transient currents, we infer that either positive charges are moved inward (into the liposomes) or negative charges are moved outward during this process. For simplicity, we arbitrarily have represented this in Figure 7 as positive charge moving into the liposome with melibiose. Fortunately, the two schemes predict distinct dependencies of the amount of

displaced charge induced by a given Na^+ concentration jump at increasing melibiose concentrations.

In mechanism a in Figure 7, the hypothesis of a deeper migration of filled Na^+ binding sites in the presence of melibiose predicts that for a Na^+ jump in the presence of melibiose the displaced charge during Na^+ binding should increase monotonically until saturation with increasing melibiose concentration (Figure 7a, ΔNa(mel) curve on the right). In mechanism b, the Na^+ binding site remains at the same position whether or not melibiose is bound to MelB. If a Na^+ concentration jump is performed in the absence of melibiose, the resulting charge translocation will result only from Na^+ binding to MelB. If melibiose is present at saturating concentration, a Na^+ concentration jump will yield a charge translocation coming alike only from Na^+ binding. Consequently, the Na^+-induced translocated charge is the same at zero and at saturating melibiose concentration. At intermediate melibiose concentrations, not all of the melibiose binding sites are saturated with the sugar when the Na^+ concentration jump is applied. Therefore, a Na^+ concentration jump causes additional melibiose binding to the protein due to the cooperative interaction of the two binding sites. Since melibiose binding was found to be electrogenic, this results in an extra charge translocation. Therefore, at rising melibiose concentration, the displaced charge rises to a maximum at intermediate sugar concentration (Figure 7b, ΔNa(mel)). This behavior as predicted by mechanism b is indeed experimentally observed (Figure 5). Consequently, our data demonstrate that upon binding, melibiose induces a charge translocation that does not represent the displacement of the cosubstrate Na^+. This charge displacement has to be assigned to a movement of charged amino acid residues and/or electrical dipoles of the protein itself (*32*).

Mechanism for Substrate Translocation in MelB. As noted before, fast-decaying transient electrical currents are recorded on imposing either a Na^+ concentration jump or a melibiose concentration jump in the presence of Na^+ (*25*). Remarkably, these signals share similar properties: they have comparable amplitudes, they display fast decaying signals with time constants of ∼15 ms, and they are insensitive to treatment with NEM. This demonstrates that the underlying processes are fast ($k > 50\ s^{-1}$) and not related to MelB turnover (see also ref *25*). We have, therefore, assigned these signals to electrogenic Na^+ and melibiose binding, respectively. By electrogenic binding, we understand a binding process that is electrogenic by itself or is electroneutral and immediately followed by a charge displacement in the protein.

It is noteworthy that integration of the current responses after a Na^+ concentration jump or a melibiose concentration jump in the presence of Na^+ in the time range of 0−150 ms yielded a total displaced charge of equal amount (12−13 pC), while a simultaneous concentration jump of Na^+ and melibiose gave approximately twice that value (24 pC, using the signals of Figure 1 of ref *25*). This shows on one hand that each substrate displaces approximately an equal amount of charge upon binding, which is in agreement with the quantitative analysis of the experiment shown in Figure 5 yielding 60% charge displacement after binding of melibiose and 40% after Na^+ binding. On the other hand, this suggests that the final position of the binding site of a substrate is the same whether the respective cosubstrate is present or not and that cooperativity only comes in at the level of the speed or affinity of the binding process.

Different models for the mechanism of cotransport have been suggested in the past. Intriguing for its simplicity is a single site alternate accessibility mechanism that requires only a single site for each substrate, which is alternatively made accessible to the cytoplasmic or to the periplasmic side of the membrane. This model essentially agrees with Mitchell's concept of a "mobile osmotic barrier" (*33*) and has recently found support by structure determination of LacY and GlpT (*34*, *35*). In this mechanism, the substrates reach their binding sites through a large hydrophilic cavity. When both substrates are bound, the general reorientation of the carrier takes place opening up the binding sites to the opposite side of the membrane.

Our data indicate a more complex mechanism in MelB. The single-site alternate accessibility mechanism as described previously predicts no or little electrogenicity for the individual binding reactions of the two substrates and a major electrogenic event when both are present simultaneously and the reorientation takes place. This concept is, however, not compatible with our data since we find major electrogenic binding reactions for the individual substrates. We, therefore, propose a transport model for MelB (Figure 7c) that is not based on a single general reorientation reaction that displaces both substrates simultaneously across the membrane. Our electrical study suggests that the Na^+ ion traverses a part (or all) of the protein dielectric already during binding (see Figure 7c: $C_{out} \rightarrow C_{out}Na$) in a rapid reaction ($k > 50\ s^{-1}$). This could possibly take place in a deep ion well (*32*), which explains electrogenicity of Na^+ binding. Subsequent melibiose binding then initiates a fast ($k > 50\ s^{-1}$) conformational change that displaces charged amino acid residues and/or electrical dipoles of the protein. This conformational change could be a separate step ($C_{out}Na$ mel $\rightarrow$ $C_{out'}Na$ mel) following melibiose binding and preceding the general reorientation ($C_{out'}Na$ mel $\rightarrow$ $C_{in}Na$ mel) as suggested for the Na^+ glucose cotransporter SGLT1 (31) and may reflect accommodation of the bulky sugar in the protein. In this case, we would have to assume that $C_{out'}Na$ mel $\rightarrow$ $C_{in}Na$ mel is slow and/or electroneutral. Alternatively, the electrogenic conformational change could be the reorientation reaction itself ($C_{out}Na$ mel $\rightarrow$ $C_{in}Na$ mel). Our analysis demonstrates once more that Na^+ coupled cotransport is a complex process. By assigning molecular processes to individual partial reactions of the transport cycle, electrical measurements can help to unravel details of its mechanism.

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REFERENCES

1. Poolman, B., Knol, J., Vanderdoes, C., Henderson, P. J. F., Liang, W. J., Leblanc, G., Pourcher, T., and Mus-Veteau, I. (1996) Cation and sugar slectivity determinants in a novel family of transport proteins, *Mol. Microbiol. 19*, 911−922.
2. Saier, M. H., Jr. (2000) A functional-phylogenetic classification system for transmembrane solute transporters, *Microbiol. Mol. Biol. Rev. 64*, 354−411.
3. Reizer, J., Reizer, A., Saier, M. H., Jr. (1994) A functional superfamily of sodium/solute symporters, *Biochim. Biophys. Acta 1197*, 133−166.

4. Bassilana, M., Damiano-Forano, E., and Leblanc, G. (1985) Effect of membrane potential on the kinetic parameters of the Na^+ or H^+ melibiose symport in *Escherichia coli* membrane vesicles, *Biochem. Biophys. Res. Commun. 129*, 626−631.
5. Lopilato, J., Tsuchiya, T., and Wilson, T. H. (1978) Role of Na^+ and Li^+ in thiomethylgalactoside transport by the melibiose transport system of *Escherichia coli*, *J. Bacteriol. 134*, 147−156.
6. Pourcher, T., Bassilana, M., Sarkar, H. K., Kaback, H. R., and Leblanc, G. (1990) The melibiose/Na^+ symporter of *Escherichia coli*: kinetic and molecular properties, *Philos. Trans. R. Soc. London, Ser. B. 326*, 411−423.
7. Tsuchiya, T., and Wilson, T. H. (1978) Cation-sugar cotransport in the melibiose transport system of *Escherichia coli*, *Membr. Biochem. 2*, 63−79.
8. Tsuchiya, T., Oho, M., and Shiota-Niiya, S. (1983) Lithium ion-sugar cotransport via the melibiose transport system in *Escherichia coli*. Measurement of Li^+ transport and specificity, *J. Biol. Chem. 258*, 12765−12767.
9. Wilson, T. H., Ottina, K., and Wilson, D. M. (1982) Melibiose transport in bacteria, in *Membranes and transport* (Martonosi, A. N., Ed.) pp 33−39, Plenum Publishing Corporation, New York.
10. Wilson, D. M., and Wilson, T. H. (1987) Cation specificity for sugar substrates of the melibiose carrier in *Escherichia coli*, *Biochim. Biophys. Acta 904*, 191−200.
11. Bassilana, M., Pourcher, T., and Leblanc, G. (1988) Melibiose permease of *Escherichia coli*. Characteristics of cosubstrates release during facilitated diffusion reactions, *J. Biol. Chem. 263*, 9663−9667.
12. Damiano-Forano, E., Bassilana, M., and Leblanc, G. (1986) Sugar binding properties of the melibiose permease in *Escherichia coli* membrane vesicles. Effects of Na^+ and H^+ concentrations, *J. Biol. Chem. 261*, 6893−6899.
13. Pourcher, T., Leclercq, S., Brandolin, G., and Leblanc, G. (1995) Melibiose Permease of *Escherichia coli*—Large Scale Purification and Evidence That H^+, Na^+, and Li^+ Sugar Symport Is Catalyzed By a Single Polypeptide, *Biochemistry 34*, 4412−4420.
14. Botfield, M. C., Naguchi, K., Tsuchiya, T., and Wilson, T. H. (1992) Membrane topology of the melibiose carrier of *Escherichia coli*, *J. Biol. Chem. 267*, 1818−1822.
15. Pourcher, T., Bibi, E., Kaback, H. R., and Leblanc, G. (1996) Membrane topology of the melibiose permease of *Escherichia coli* studied by melB-phoA fusion analysis, *Biochemistry 35*, 4161−4168.
16. Gwizdek, C., Leblanc, G., and Bassilana, M. (1997) Proteolytic mapping and substrate protection of the *Escherichia coli* melibiose permease, *Biochemistry 36*, 8522−8529.
17. Dave, N., Troullier, A., Mus-Veteau, I., Dunach, M., Leblanc, G., and Padros, E. (2000) Secondary structure components and properties of the melibiose permease from *Escherichia coli*: a Fourier transform infrared spectroscopy analysis, *Biophys. J. 79*, 747−755.
18. Hacksell, I., Rigaud, J. L., Purhonen, P., Pourcher, T., Hebert, H., and Leblanc, G. (2002) Projection structure at 8 Å resolution of the melibiose permease, an Na-sugar cotransporter from *Escherichia coli*, *EMBO J. 21*, 3569−3574.
19. Mus-Veteau, I., Pourcher, T., and Leblanc, G. (1995) Melibiose permease of *Escherichia coli*: substrate-induced conformational changes monitored by tryptophan fluorescence spectroscopy, *Biochemistry 34*, 6775−6783.
20. Hama, H., and Wilson, T. H. (1993) Cation-coupling in chimeric melibiose carriers derived from *Escherichia coli* and *Klebsiella pneumoniae*. The amino-terminal portion is crucial for Na^+ recognition in melibiose transport, *J. Biol. Chem. 268*, 10060−10065.
21. Wilson, D. M., and Wilson, T. H. (1992) Asp-51 and Asp-120 are important for the transport function of the *Escherichia coli* melibiose carrier, *J. Bacteriol. 174*, 3083−3086.
22. Pourcher, T., Zani, M. L., and Leblanc, G. (1993) Mutagenesis of acidic residues in putative membrane-spanning segments of the melibiose permease of *Escherichia coli*. I. Effect on $Na(^+)$-dependent transport and binding properties, *J. Biol. Chem. 268*, 3209−3215.
23. Zani, M. L., Pourcher, T., and Leblanc, G. (1993) Mutagenesis of acidic residues in putative membrane-spanning segments of the melibiose permease of *Escherichia coli*. II. Effect on cationic selectivity and coupling properties, *J. Biol. Chem. 268*, 3216−3221.
24. Maehrel, C., Cordat, E., Mus-Veteau, I., and Leblanc, G. (1998) Structural studies of the melibiose permease of *Escherichia coli* by fluorescence resonance energy transfer—I. Evidence for ion-induced conformational change, *J. Biol. Chem. 273*, 33192−33197.
25. Ganea, C., Pourcher, T., Leblanc, G., and Fendler, K. (2001) Evidence for intraprotein charge transfer during the transport activity of the melibiose permease from *Escherichia coli*, *Biochemistry 40*, 13744−13752.
26. Rigaud, J. L., Paternostre, M. T., and Bluzat, A. (1988) Mechanisms of membrane protein insertion into liposomes during reconstitution procedures involving the use of detergents. 2. Incorporation of the light-driven proton pump bacteriorhodopsin, *Biochemistry 27*, 2677−2688.
27. Lowry, O. (1951) Protein measurement with the folin phenol reagent, *J. Biol. Chem. 193*, 265−275.
28. Pintschovius, J., and Fendler, K. (1999) Charge translocation by the Na^+/ K^+-ATPase investigated on solid supported membranes: Rapid solution exchange with a new technique, *Biophys. J. 76*, 814−826.
29. Mus-Veteau, I., and Leblanc, G. (1996) Melibiose permease of *Escherichia coli*: structural organization of cosubstrate binding sites as deduced from tryptophan fluorescence analyses, *Biochemistry 35*, 12053−12060.
30. Dave, N., Lorenz-Fonfria, V. A., Villaverde, J., Lemonnier, R., Leblanc, G., and Padros, E. (2002) Study of amide-proton exchange of *Escherichia coli* melibiose permease by attenuated total reflection-Fourier transform infrared spectroscopy—Evidence of structure modulation by substrate binding, *J. Biol. Chem. 277*, 3380−3387.
31. Meinild, A. K., Hirayama, B. A., Wright, E. M., and Loo, D. D. (2002) Fluorescence studies of ligand-induced conformational changes of the $Na(^+)$/glucose cotransporter, *Biochemistry 41*, 1250−1258.
32. Läuger, P. (1991) *Electrogenic Ion Pumps*, Sinauer Associates, New York.
33. Mitchell, P. (1990) Osmochemistry of solute translocation, *Res. Microbiol. 141*, 268−278.
34. Abramson, J., Smirnova, I., Kasho, V., Verner, G., Kaback, H. R., and Iwata, S. (2003) Structure and mechanism of the lactose permease of *Escherichia coli*, *Science 301*, 610−615.
35. Huang, Y., Lemieux, M. J., Song, J., Auer, M., and Wang, D. N. (2003) Structure and mechanism of the glycerol-3-phosphate transporter from *Escherichia coli*, *Science 301*, 616−620.

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