Evidence for Intraprotein Charge Transfer during the Transport Activity of the Melibiose Permease from *Escherichia Coli*†

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Received June 13, 2001; Revised Manuscript Received August 24, 2001

ABSTRACT: Electrogenic activity associated with the activity of the melibiose permease (MelB) of *Escherichia coli* was investigated by using proteoliposomes containing purified MelB adsorbed onto a solid-supported membrane. Transient currents were selectively recorded by applying concentration jumps of Na^+ ions (or Li^+) and/or of different sugar substrates of MelB (melibiose, thio-methyl galactoside, raffinose) using a fast-flow solution exchange system. Characteristically, the transient current response was fast, including a single decay exponential component ($\tau \approx 15$ ms) on applying a Na^+ (or Li^+) concentration jump in the absence of sugar. On imposing a Na^+ (or Li^+) jump on proteoliposomes preincubated with the sugar, a sugar jump in a preparation preincubated with the cation, or a simultaneous jump of the cation and sugar substrates, the electrical transients were biphasic and comprised both the fast and an additional slow ($\tau \approx 350$ ms) decay components. Finally, selective inactivation of the cosubstrate translocation step by acylation of MelB cysteins with *N*-ethyl maleimide suppressed the slow response components and had no effect on the fast transient one. We suggest that the fast transient response reflects charge transfer within MelB during cosubstrate binding while the slow component is associated with charge transfer across the proteoliposome membrane. From the time course of the transient currents, we estimate a rate constant for Na^+ binding in the absence and presence of melibiose of $k > 50\ s^{-1}$ and one for melibiose binding in the absence of Na^+ of $k \approx 10\ s^{-1}$.

The melibiose permease (MelB) of *Escherichia coli* is a membrane-bound ion-coupled sugar cotransporter or symporter that uses the favorable Na^+, Li^+, or H^+ electrochemical potential gradient to drive cell accumulation of α-galactosides (melibiose, raffinose) or β-galactosides (TMG) (*1*−*8*). Detailed kinetic analysis of MelB suggests a mechanistic model with the following characteristics (*7*, *9*, *10*): (1) co-transported substrates bind to and are transported by MelB in a 1:1 ratio; (2) the coupling ion enhances the affinity of MelB for the co-transported sugar, Na^+ and Li^+ being better activators than H^+; (3) H^+, Na^+, or Li^+ compete for the same binding site; (4) the outer binding and cytoplasmic release of the substrates is best accounted for by an ordered process (outside binding, Na^+ first/sugar last; inside release, sugar first/Na^+ last); (5) Na^+ or Li^+ (but not H^+) dissociation into the cytoplasm is slow and rate-limiting for MelB cycling; (6) the membrane potential enhances active Na^+/sugar transport by increasing the rate of Na^+ dissociation in the cytoplasm.

The Mel B symporter (473 amino acids, 53 kDa) has 12 helical transmembrane domains (*11*−*15*). A recombinant transporter harboring a 6-His tag (Mel-6His permease) could be purified in large amounts, and it exhibits cation-dependent sugar-binding and transport properties comparable to those of the native permease in its natural environment when reconstituted in liposomes (*8*, *16*). Information at the molecular level has been obtained using molecular biology, biochemical, and spectroscopic approaches. Site-directed mutagenesis experiments on MelB and the construction of chimeric melibiose transporters from various microorganisms suggest an important role of a coordination network, comprising four aspartic acid residues distributed in the helices I (D19), II (D55 and D59), and IV (D124) of the N-terminal hydrophobic domains of MelB, for the cation recognition and coupling mechanism (*13*, *17*−*20*).

Biochemical and spectroscopic evidence support the earlier suggestion that some discrete steps of MelB cycling involve conformational transitions of the transporter (*21*). This includes cooperative protection against proteolysis of the highly charged cytoplasmic loop connecting helices IV and V (loop 4−5) of MelB (*14*) by MelB substrates. Cooperative changes in MelB conformation were also inferred from analysis of the intrinsic fluorescence of MelB or using fluorescence resonance energy transfer spectroscopy with a fluorescent sugar analogue (*16*, *22*).

† C. G. was the recipient of a short-term Max Planck Society fellowship and of partial financial support from the Ministry of Education, Romania.

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[1] Abbreviations: MelB, melibiose permease; SSM, solid supported membrane; DTT, dithiothreitol; NEM, *N*-ethyl maleimide; TMG, thiomethyl galactopyranoside.

10.1021/bi011223k CCC: $20.00
Published on Web 10/16/2001

Electrophysiological techniques have proven to be extremely useful tools in investigating the mechanism of ion transfer across the membrane by ion-coupled transporters in eucaryotic cells (*23*−*25*). Partial reactions that are associated with the translocation of electrical charge can be identified, which can then be interpreted in terms of, e.g., an electrogenic binding process, the translocation of ions, or a conformational transition. Although it is admitted that the bacterial counterparts of eucariotic transporters are electrogenic, the limited size of bacterial cells or derived membrane vesicles has hampered their analysis by the electrical approach. However, the progress in purification and reconstitution of MelB in liposomes opens new perspectives. Indeed, proteoliposomes adsorbed to a planar lipid membrane have been used in the past as a sensitive tool to investigate ion translocating membrane proteins (for a review, see ref *26*). Among different strategies, a novel technique, based on a rapid solution exchange combined with the high sensitivity of planar lipid membranes, is a technique that employs a solid-supported membrane (SSM) as a capacitive electrode (*27*). It was successfully used to investigate the electrogenic steps in the cycle of the Na^+,K^+-ATPase (*28*, *29*) yielding results similar to those obtained by other techniques.

Here we present the results of electrical current measurements on MelB proteoliposomes performed with the SSM technique. Activation of MelB was achieved by imposing substrate concentration jumps using a fast flow solution exchange system. The resulting electrical transient signals were recorded with a time resolution up to 10 ms. This study is the first direct electrical measurement of charge transport by a bacterial cotransporter and the first electrical characterization of a purified and reconstituted Na^+−sugar transporter.

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 salt and melibiose at various concentrations. The standard buffer solution contained a mixture of KH_2PO_4 and K_2HPO_4, prepared the following way: 100 mM KH_2PO_4 was titrated with concentrated KOH until the pH was 7. To avoid Na^+ contamination of nominally Na^+-free solutions, KH_2PO_4 (Sigma, 0.005% Na) and KOH (Merck, suprapur, 0.002% Na) were used. In addition, all solutions were prepared in plastic flasks. The Na^+ concentration in these media was determined by atomic absorption spectroscopy to be <30 μM. Melibiose and glucose were purchased from Sigma, raffinose, TMG, sucrose, and *N*-ethyl maleimide (NEM) from Fluka. All experiments using NEM-treated enzyme were performed in DTT-free buffer.

The lipid film forming solution contained diphytanoyl phosphatidylcholine (PC, synthetic, Avanti Polar Lipids Inc., Pelham, AL) and octadecylamine (60:1 wt/wt, 98%, Riedel-de-Haen AG, Seelze-Hannover, Germany) prepared 1.5% in *n*-decane (*30*). A 1 mM octadecyl mercaptan (C_{18}-mercaptan, Aldrich, Steinheim, Germany) solution in ethanol was used for the incubation of the gold electrodes.

Purification and Preparation of the Proteoliposomes. E. coli DW2 cells (D*mel* D*lac*ZY) (*31*), transformed with p*K*95DAHB plasmid that harbors the His-tagged MelB coding sequence, were grown at 30 °C in a M9 medium supplemented with appropriate carbon sources and ampicillin (100 μg/ml) as described previously (*32*). His-tagged MelB (thereafter termed MelB) was purified from inverted membrane vesicles obtained by means of a French press (American Instrument Co.) essentially as described by (*8*). Briefly, inverted membrane vesicles were solubilized in a medium containing 50 mM Tris-HCl (pH 8), 500 mM NaCl, 1% lauryl aminopropyldiamine-*N*-oxide, and 10 mM imidazole. The supernatant was equilibrated for 1 h at 4 °C with Ni−NTA (Ni-nitriloacetic acid) resin and the mixture loaded into a column. Following extensive washout of unbound proteins with the solubilization medium, lauryl aminopropyldiamine-*N*-oxide was replaced by dodecylmaltoside (0.1%), and NaCl was reduced to 50 mM. Finally, the bound material (90% MelB and 10% contaminants) was eluted by raising the imidazole concentration to 100 mM. MelB was finally separated from the remaining contaminant protein by ion exchange chromatography using a MonoQ resin. MelB reconstitution was performed in the presence of a 5-fold excess of *E. coli* lipids (Polar Avanti), and the detergent was removed by adsorption on Bio-Beads SM-2 (*16*). To eliminate NaCl, including from the internal space, proteoliposomes were submitted to repeated freezing/thawing−sonication−wash cycles in nominally Na^+-free, 100 mM potassium phosphate buffer (pH7). As determined by flame photometry, the level of contaminating sodium salts in the proteoliposome supensions was at most 10 μM. The purity of the reconstituted MelB (generally > 99%) was assessed by silver-stained SDS/PAGE electrophoresis.

Protein Assays. Proteins contained in membrane vesicles and proteoliposomes were assayed in the presence of sodium dodecyl sulfate using serum bovine albumin as standard according to Lowry.

SSM Setup. The SSM was prepared by linking an alkanethiol (octadecyl mercaptane) monolayer to a gold electrode deposited on a glass support and covering it with a lipid (diphytanoyl phosphatidylcholine) monolayer. The planar membrane formed has an area of 1−2 mm^2. The SSM is mounted in a flow-through cuvette with an inner volume of 17 μL. The gold electrode is connected to an amplifier; the reference electrode is an Ag/AgCl electrode separated from the solution by a salt bridge. An improved setup as described in detail by ref *28* was used. In contrast to the setup described before, the solution is driven through the cuvette by applying pressure (0.6 bar) to the solution containers connected to the cuvette.

Measuring Procedure. The experiments were carried out at room temperature (22 °C). After the formation of the SSM, its capacitance and conductance were measured until they became constant after a waiting time of ∼90 min. Typical values were 300−500 nF/cm^2 for the capacitance and 50−100 nS/cm^2 for the conductance. The proteoliposomes were thawed and sonicated for ∼30 s. Then 40 μL of the suspension containing 0.37−1.4 mg/mL protein was injected into the cuvette. The proteoliposomes were allowed to adsorb to the SSM for 30−50 min. A typical solution exchange protocol consists of three phases: (1) nonactivating solution (2 s), (2) activating solution (2 s), and (3) nonactivating solution (2 s). The data recording and the solution exchange were controlled via computer as described by ref *28*.

Electrical signals are observed at the concentration jumps taking place at the beginning (on-signal) and end of phase 2 (off-signal). Only the on signal will be used throughout our analysis.

RESULTS

The SSM was prepared and the MelBproteoliposomes adsorbed onto the SSM as described in Materials and Methods. Proteoliposomes and SSM form a capacitively coupled system that enables measurement of transient currents as described previously for Na^+,K^+-ATPase (*33*). A rapid flow technique with ~10 ms time resolution (*28*) was used to impose different rapid changes in cosubstrate concentrations: (1) simultaneous concentration jumps of both cosubstrates (ΔmelΔNa); (2) concentration jumps of Na^+ in the absence of melibiose (ΔNa) or in the presence of melibiose, i.e., the proteoliposomes were pre-equilibrated with the sugar (ΔNa(mel)); (3) concentration jumps of melibiose either in the absence (Δmel) or in the presence of NaCl, i.e., the proteoliposomes were pre-equilibrated with the sodium salt (Δmel(Na)). For the sake of simplicity, the notation given in parentheses for the different concentration jump protocols will be used throughout this publication. An analogous notation is used for Li^+, TMG, and raffinose. To minimize ionic strength and osmotic effects, the nonactivating solution contained equimolar concentration of choline chloride (cholineCl) instead of NaCl and/or glucose instead of melibiose, TMG, or raffinose.

Electrical Signals Generated by Different Cosubstrate Concentration Jumps. Traces 2−6 in Figure 1 illustrate the transient electrical responses successively recorded from the same SSM/proteoliposomes system upon individual or simultaneous cosubstrate concentration jumps. The sign of the current (upward deflection) indicates the displacement of positive charge toward the SSM (or of negative charge in the opposite direction). A transient electrical response of opposite direction is observed upon removal of the added substrate(s). Preliminary experiments demonstrated that no or only a negligible artifact was observed under the following conditions: (1) ΔNaΔmel concentration jump on a SSM without liposomes or with pure *E. coli* lipid liposomes (data not shown) and (2) exchange of a glucose-containing solution to a different glucose-containing solution of the same composition (Figure 1 (control)) or a sucrose solution of the same concentration (Figure 2 (Δsucr)) using an SSM with MelB proteoliposomes.

Trace Δmel in Figure 1 shows the electrical response recorded after a melibiose concentration jump in Na^+-free medium, i.e., when MelB catalyses H^+−melibiose symport activity. The transient signal shows a rapid current rise to a maximal value (125 pA) followed by a decay toward the baseline, which could be fitted with two exponentials (τ = 98 and 350 ms). The melibiose concentration jump experiment was repeated with activating and nonactivating solutions containing 10 mM NaCl, i.e., at a condition where MelB functions as a Na^+-coupled symporter (Figure 1 (Δmel(Na))). There, the melibiose-induced signal rises faster and reaches a higher peak current (285 pA) as compared to the response in Na^+-free medium. The signal decays with a biexponential time course (τ = 17 and 385 ms).

Trace (ΔNa) in Figure 1 shows the effect of a 10 mM Na^+ concentration jump in a medium devoid of sugar substrate. A fast and large transient electric signal (peak current = 490 pA) is observed with a monoexponential decay (τ = 14 ms). Raising the concentration of glucose from 10 to 100 mM neither altered the peak current of the signal nor its decay time. A second experiment consisted in imposing a similar 10 mM Na^+ concentration jump on proteoliposomes in the presence of 50 mM melibiose (Figure 1 (ΔNa(mel))). The magnitude of the transient peak current (510 pA) was nearly the same as that in melibiose free medium (ΔNa). In contrast, the signal decay now comprised two exponential

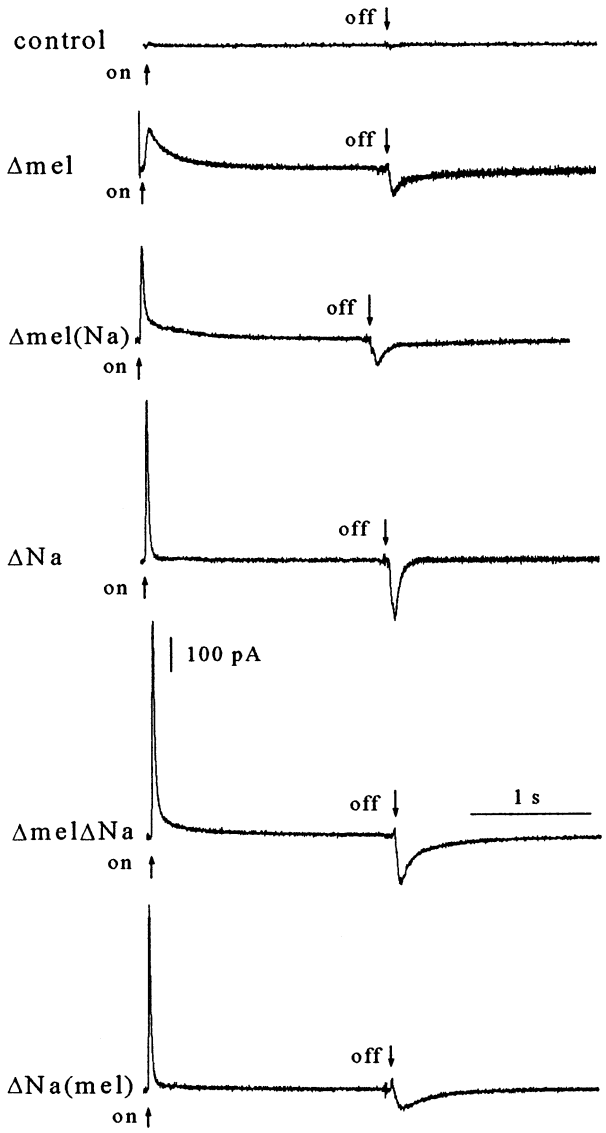

FIGURE 1: Electrical signals generated by MelB after different solution exchange protocols. In addition to the sugar and/or cation given below, the activating and the nonactivating solutions contained 100 mM KPi and 0.1 mM DTT at pH 7.0. All the data shown in the figure were recorded on the same sample. **Control:** nonactivating solution, 50 mM glucose; activating solution, 50 mM glucose. **Δmel:** nonactivating solution, 50 mM glucose; activating solution, 50 mM melibiose. **Δmel(Na):** nonactivating solution, 50 mM glucose, 10mM NaCl; activating solution, 50 mM melibiose, 10 mM NaCl. **ΔNa:** nonactivating solution, 50 mM glucose, 10 mM cholineCl; activating solution, 50 mM glucose, 10 mM NaCl. **ΔmelΔNa:** nonactivating solution, 50 mM glucose, 10 mM cholineCl; activating solution, 50 mM melibiose, 10 mM NaCl. **ΔNa(mel):** nonactivating solution, 50 mM melibiose, 10 mM cholineCl; activating solution, 50 mM melibiose, 10 mM NaCl.

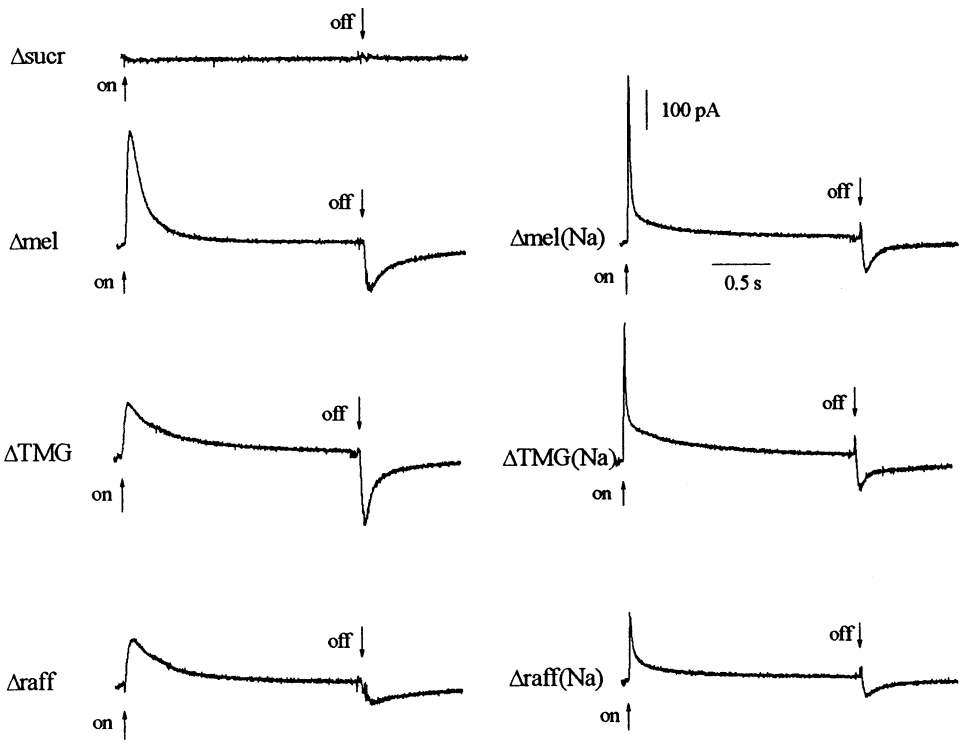

FIGURE 2: Electrical signals after a 50 mM melibiose, TMG, or raffinose concentration jump in the absence and in the presence of Na^+. In addition to the sugar and/or cation given below, the activating and the nonactivating solutions contained 100 mM KPi and 0.1 mM DTT at pH 7.0. All the data shown in the figure were recorded on the same sample. **Δsucr:** nonactivating solution, 50 mM glucose; activating solution, 50 mM sucrose. **Δmel:** nonactivating solution, 50 mM glucose; activating solution, 50 mM melibiose **ΔTMG:** nonactivating solution, 50 mM glucose; activating solution, 50 mM TMG. **Δraff:** nonactivating solution, 50 mM glucose; activating solution, 50 mM raffinose. **Δmel(Na):** nonactivating solution, 50 mM glucose, 10mM NaCl; activating solution, 50 mM melibiose, 10 mM NaCl. **ΔTMG-(Na):** nonactivating solution, 50 mM glucose, 10mM NaCl; activating solution, 50 mM TMG, 10 mM NaCl. **Δraff(Na):** nonactivating solution, 50 mM glucose, 10mM NaCl; activating solution, 50 mM raffinose, 10 mM NaCl.

components, a dominating (amplitude ≈ 97%) fast component (τ = 15 ms) and a slow component (τ = 327 ms).

Finally, Figure 1 (ΔNaΔmel) represents the electrical signal recorded upon a simultaneous concentration jump of both MelB substrates, namely, raising Na^+ to 10 mM and melibiose to 50 mM. The peak value of the transient signal (670 pA) was about 37% larger than that of the signal produced by a ΔNa concentration jump. The decay part of the electrical signal recorded in this experiment has two components, a fast one (amplitude ≈ 89%, τ = 15 ms) and a slow one (τ = 260 ms).

In summary, two situations are apparent on comparing the electrical responses. First, addition of Na^+ ions alone (ΔNa) elicits a large transient current that includes a single and fast exponential decay component. Second, the transient responses triggered by a melibiose jump in the Na^+ medium, by a Na^+ jump in the melibiose medium, or by a simultaneous Na^+ and melibiose concentration jump include both a fast and a slow exponential decay components. (Note: The slow decay component of a Na^+ jump in melibiose medium is much smaller than that in the other two cases.)

Cosubstrate Specificities. It is well established that MelB catalyses active transport of different α-galactosides (e.g., melibiose, raffinose) or β-galactosides (e.g., thio-methyl galactopyranoside, TMG) but not of sucrose and can use Na^+, Li^+, or H^+ as coupling ions (*34*, reviewed in ref *7*). The capacity of TMG, raffinose, or sucrose to trigger an electrical response was therefore compared to that of melibiose in Na^+-free or Na^+-containing media (Figure 2). A sucrose concentration jump of 50 mM generated only an insignificant artifact (Figure 2 (Δsucr)). Similar to the Δmel concentration jump, ΔTMG or Δraf also induced transient currents in Na^+-free media. The time constants of the decay were in the hundreds of milliseconds range. More specifically, the ΔTMG or Δraf peak currents were about one-half or one-third of that recorded during a Δmel concentration jump (144, 110 or 280 pA, respectively). In addition, the decay of the ΔTMG and Δraf signals could be fitted by a single-exponential component. The computed decay time constants were 405 ms (ΔTMG) and 309 ms (Δraf) in comparison to 98 ms, the fast component of the Δmel signal. The difference in peak current and/or rate of decay are reminiscent of the lower affinity or rate of H^+-coupled co-transport of these sugars by MelB (*6*). Similarly, concentration jumps of TMG or raffinose carried out in the presence of 10 mM NaCl (traces, ΔTMG(Na) or Δraf(Na)) yielded transient responses somewhat smaller than that elicited by melibiose (Δmel(Na)). The current responses were fitted by two exponentials and are dominated at shorter time by a fast and large peak current (decaying phase: τ = 15−22 ms). The slower component had a time constant of 380 ms for a melibiose jump, 507 ms for TMG, and 360 ms for raffinose. The ΔTMG(Na) or Δmel(Na) peak currents had comparable magnitudes (340 and 380 pA) and were higher than the Δraf(Na) one (190 pA). All the peak currents were significantly higher than those recorded in Na^+-free media.

Also, a Li^+ concentration jump alone (ΔLi) or in combination with a sugar concentration jump (ΔLiΔmel) gave rise

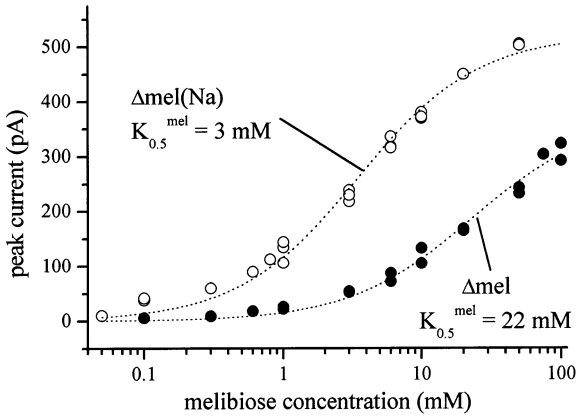

FIGURE 3: Dependence of the peak currents on the sugar concentration. In addition to the sugar and/or cation given below, the activating and the nonactivating solutions contained 100 mM KP_i and 0.1 mM DTT at pH 7.0. **Δmel** (solid circles)**:** activating solution, melibiose at different concentrations; nonactivating solution, glucose at the same concentration as melibiose. The dashed line represents a fit with a hyperbolic function; half-saturation concentration $K_{0.5}^{mel} = 22$ mM. **Δmel(Na)** (open circles): activating solution, melibiose at different concentrations, 10 mM NaCl; nonactivating solution, glucose at the same concentration as that of melibiose, 10 mM NaCl. Half-saturation concentration $K_{0.5}^{mel} = 3$ mM.

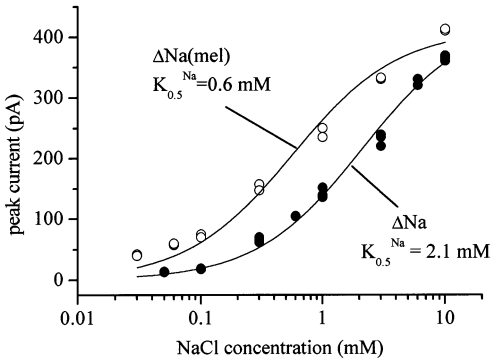

FIGURE 4: Na^+ concentration dependence of the peak currents after a jump to different Na^+ concentrations in the absence and in the presence of the active sugar. In addition to the sugar and/or cation given below, the activating and the nonactivating solutions contained 100 mM KP_i and 0.1 mM DTT at pH 7.0. **ΔNa** (solid circles): activating solution, NaCl at different concentrations and 50 mM glucose; nonactivating solution, cholineCl at the same concentration as that of NaCl and 50 mM glucose. The solid line represents a fit using a hyperbolic model function; half saturation concentration $K_{0.5}^{Na} = 2.1$ mM. **ΔNa(mel)** (open circles): activating solution, NaCl at different concentrations and 50 mM melibiose; nonactivating solution, cholineCl at the same concentration as that of NaCl and 50 mM melibiose. Half saturation concentration $K_{0.5}^{Na} = 0.6$ mM.

to signals qualitatively and quantitatively comparable to those of their Na^+-induced counterparts (data not shown).

Melibiose or Na^+ Concentration Dependence. As a first approximation, the peak current is proportional to the translocated charge (see below). We have, therefore, determined the peak current as a function of the magnitude of the concentration jump. Figure 3 depicts the variation of the peak current recorded (I_p) on imposing melibiose concentration jumps of varying amplitude. Both in the presence or absence of Na^+, the concentration dependence shown in the figure was fitted using a hyperbolic function ($I_p = I_p^{max} c/(c + K_{0.5}^{mel})$; c = melibiose concentration). The fit of the data obtained in the presence of 10 mM Na^+ yielded a half-saturation concentration for melibiose of $K_{0.5}^{mel} = 3$ mM. This $K_{0.5}^{mel}$ value estimated from the electrical measurement satisfactorily compares with the apparent affinity of MelB for melibiose determined in binding experiments (0.5−0.9 mM (*7*, *35*)) or with the concentration of sugar inducing half-maximal intrinsic fluorescence variation of MelB (1.2 mM (*16*)). In Na^+-free media, the data are best fitted by a hyperbolic function using a half-saturation concentration for melibiose of $K_{0.5}^{mel} = 22$ mM. This higher $K_{0.5}^{mel}$ value parallels the increased K_m for transport of 10 mM (*7*) or the $K_{0.5}^{mel}$ of 11 mM in fluorescence studies in corresponding Na^+-free media (*16*).

Figure 4 shows that the peak current also displays saturation kinetics as a function of Na^+ concentration alone (ΔNa). The determined $K_{0.5}^{Na}$ of 2.1 mM is close to that of 1 mM reported for the Na^+-induced change in MelB intrinsic fluorescence in proteoliposomes (*16*). When the experiment was repeated in the presence of an excess of melibiose (ΔNa(mel)), the $K_{0.5}$ for Na^+ decreased to 0.6 mM. This latter value compares well with the Na^+ activation constant of sugar binding ($K^{Na} = 0.3-0.6$ mM) estimated in right-side out membrane vesicles (*9*, *35*) or in MelB proteoliposomes (0.5 mM, (*8*)). It is also of comparable magnitude to the Na^+ activation constant for sugar transport in native *E. coli* membrane vesicles ($K^{Na} = 0.5$ mM, (*35*)). For reasons not yet well understood, the value of $K_{0.5}^{Na}$ determined in the above-mentioned and in our study is significantly higher than the Na^+ concentration required to produce half-maximal variation of MelB intrinsic fluorescence in proteoliposmes (0.1 mM, (*16*, *22*)).

Together, the electrical data show satisfactory correlation between the concentrations of melibiose and/or Na^+ producing half-maximal peak current and either the half saturation constants for melibiose transport and binding or the melibiose concentration inducing half-maximal variations of MelB intrinsic fluorescence. They also provide evidence for cooperative interactions between the MelB cosubstrates with the transporter.

Effect of NEM. Active sugar transport or all facilitated diffusion reactions (Na^+-coupled sugar influx, efflux, or exchange) catalyzed by MelB are inhibited upon acylation of one (or more) cysteine residue of MelB by NEM (*2*, *5*, *36*). In contrast, Na^+-dependent sugar binding is not impaired (*9*). To relate the transient electrical signals resulting from changes in the concentration of the transported ion and sugar substrates to given events of the transport cycle, the effect of transient concentration jumps of the substrates was analyzed in proteoliposomes incubated for 30 min with NEM at a final concentration up to 2 mM. Figure 5 compares the transient currents obtained by a ΔmelΔNa jump (20 mM melibiose and 10 mM NaCl) before and after NEM treatment at a final concentration of 1 mM. It is observed that acylation of MelB does not prevent a transient charge translocation to take place. However, while the control response includes both a fast and a slow decay components, the slow component disappears once MelB has been reacted with the SH reagent.

The inset in Figure 5 indicates that in the case of the ΔmelΔNa signal, the peak current is inhibited by about 10% after 1 mM NEM. A similar disappearance of the slow component and a small reduction of the peak current after NEM incubation was also observed for the Δmel(Na) or

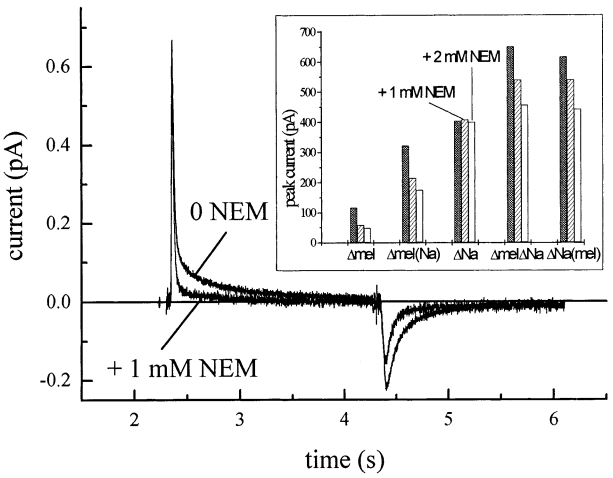

FIGURE 5: Electrical signals after a simultaneous 20 mM melibiose and 10 mM NaCl concentration jump (ΔNaΔmel) before (0 NEM) and after (1 mM NEM) addition of 1 mM NEM. In addition to the sugar and/or cation, the activating and the nonactivating solutions contained 100 mM KP_i at pH 7.0. All solutions were DTT-free. **Inset:** The peak current recorded after different concentration jump protocols before (gray) and after incubation with 1 mM NEM (hatched) and after incubation with 2 mM NEM (white). Solution exchange protocols were the same as those in Figure 1, except that 20 mM melibiose and glucose were used instead of 50 mM.

ΔNa(mel) signals. As noted in the inset, incubation of MelB with 2 mM NEM produces a further reduction of the ΔmelΔNa signal peak current to a value comparable to that recorded after a Na^+ concentration jump (ΔNa). Importantly, neither the amplitude nor the fast decay component of the response induced by a ΔNa jump was significantly modified by NEM. Finally, a similar signal reduction by NEM was observed when a sugar concentration jump was carried out in the absence of Na^+ ions. Altogether, these observations make it likely that the fast component of the transient current response is associated to charge transfer into the transporter during cosubstrate binding while the slower component reflects charge-transfer linked to a movement of the substrates across the whole membrane.

Electrical Signal. Before establishing the possible relationship between the signal characteristics and different partial reactions of the reaction cycle, it seems worthwhile to stress specific limitation and/or constraints imposed by the solution exchange and/or the recording technique on the elicited signal. When a concentration gradient of Na^+ and melibiose is applied to the liposomes (ΔNaΔmel), the electrical signal consists of a rapid transient followed by a slowly decaying phase with a much smaller amplitude (see Figure 1). The rapid and the slow phases decay with time constants of ∼20 and ∼400 ms, respectively. These two time constants are not related to rate constants of the reaction cycle. The 20 ms time constant probably has to be assigned to the time resolution of the solution exchange. A similar limiting time constant of ∼17 ms has been found previously (*28*). Since the decay of the signal is determined by an instrumental time constant, it is approximately constant in most of the measurements. Under these conditions, the peak current is proportional to the translocated charge. Only when the electrogenic reactions become slower than ∼50 s^{-1} (= (20 ms)$^{-1}$) does the decay time increase and can the peak current no longer be considered proportional to the translocated charge, as is the case for the Δmel measurements The 400 ms time constant corresponds most probably to the so-called "system time constant" which describes charging of the liposome/SSM compound membrane. A comparable value of 330 ms has been found for liposomes containing Na^+/K^+−ATPase on a planar lipid membrane (*33*).

These arguments support the conclusion from the NEM inhibition experiments namely that the two phases in the decay of the electrical signal correspond to two distinct processes in the reaction cycle of MelB: (1) substrate binding (rapid phase) and (2) steady-state transport (slow phase). The rapid phase is over within 20 ms. Because a maximal permease turnover of about 0.2−0.3 s^{-1} can be calculated from previous kinetic experiments (*7*) and because the decay of the rapid phase is limited by the solution exchange, the underlying processes have to be much faster than 20 ms (k > 50 s^{-1}). However, at certain conditions, the partial reactions in the reaction cycle become so slow that they can be resolved (see, e.g., Δmel, Figure 1).

The slow phase (400 ms) represents steady-state turnover of the transporter. This is most clearly demonstrated by the fact that this slow component is absent after a Na^+ jump carried out in the absence of melibiose (Figure 1 (ΔNa)), a condition where no flow of charge across the membrane is expected. Because of the capacitive coupling of the liposomes via the SSM, steady-state turnover does not generate a stationary current but rather results in a transient electrical current decaying with the system time constant (*37*). We have, therefore, the ability to discriminate between electrogenic substrate binding and steady-state turnover of the exchanger.

Finally, we would like to draw attention to the fact that the experiments in this work have been performed on a liposome preparation where the protein is probably not uniformly oriented. It is known that MelB is asymmetric (*12*, *14*). Therefore, the properties determined in this study may be averages of both the inside-out and right-side-out populations. On the other hand, a large asymmetry in activity may lead to a situation where substrate (or charge) transport is dominated by a single population. Whether this is true or not is unknown. Selective side-directed inactivation of MelB with impermeant SH reagents, for example, may provide a means to better correlate the electrical events and orientation of MelB.

DISCUSSION

The experiments reported in the present study represent the first direct measurement of the electrogenic activity of a Na^+-coupled solute membrane transporter from bacteria. They were recorded at a variety of conditions that are related to different partial reactions of the transport cycle like Na^+ and/or melibiose binding to the permease and cosubstrates translocation across the membrane. This enables us to identify electrogenic steps in the reaction cycle based on a kinetic model proposed previously (*7*).

Electrical Signals Correspond to the Reconstituted MelB Transporter. The results show that transient electrical responses from MelB proteoliposomes adsorbed on SSM can be selectively triggered by independent or concerted concentration jumps of MelB substrates. The sign of the signals corresponds to the transport of positive charge toward the

interior of the liposomes in line with the anticipated influx of cations. Comparison and analysis of the different kinetics and of their modification after selective inhibition of the transporter translocation step strongly suggest that charge transfer phenomena occur not only as a result of substrate translocation across the membrane but also during binding of the substrates to the transporter.

Basically, two typical transient signal responses were recorded on performing concentration jumps of monovalent cations and/or of sugar in the medium in contact with the adsorbed MelB proteoliposomes. (1) Large and fast transient currents decaying monoexponentially are recorded upon a Na^+ or Li^+ concentration jump (ΔNa or ΔLi). (2) Transient biphasic current responses, including both a fast and a slow decay component, were observed whenever α- or β-galactosides were present, i.e., in Δmel, Δmel(Na), ΔmelΔNa, and ΔNa(mel) experiments. The fast decay component of the galactoside-induced transient response observed in the presence of Na^+ or Li^+ is replaced by a slower one in its absence, making the overall response slower.

Qualitative as well as quantitative analysis of the signals provides evidence that the associated charge transfer processes involve the reconstituted MelB transporter. First, the electrical signals are selectively triggered by well recognized cation and/or sugar substrates of MelB (Figure 1, Figure 2). This is the case for two ions known to couple sugar transport by MelB, namely Na^+ and Li^+ ions, which induce comparable fast ($\sim$15 ms) transient currents (ΔNa, ΔLi). Moreover, the Na^+- or Li^+-induced currents are converted into biphasic signals only when any of three established sugar substrates of MelB (melibiose, TMG, raffinose) are added before or together with the concentration jump. The signal consists of a fast initial current component followed by a long lasting current component with a time constant of several hundred milliseconds. Incidentally, sugar-induced responses still exhibit a fast early current component once proteoliposomes are already equilibrated with the cations. Together with the fact that currents elicited by the cation on MelB-free liposomes are of negligible amplitude (<5%), these data further establish that electrostatic interaction of the ion with the negatively charged surface of the proteoliposome lipid bilayer do not play a significant role. A direct involvement of MelB in the electrical currents is further suggested by two observations. First, the coupling ion present during the sugar challenge dictates the overall shape of the sugar induced current response, the response being much slower when the coupling ion is H^+ instead of Na^+ or Li^+. Second, the current response is modified by treatment with NEM, which is known to interfere with the transport function of MelB (Figure 5).

Complementary and more quantitative evidence for an implication of MelB in the generation of the transient responses is brought about by the good correlation existing between the kinetic constants determined from the cosubstrate dependence of the peak currents and the kinetic constants previously obtained in sugar binding or transport studies (*7*, *35*) or MelB intrinsic fluorescence measurements (*16*, *22*). The half saturation constants determined from the electrical measurements are in general about 2 times higher than those estimated from the binding, transport, or biophysical studies measured at steady state. Part of the discrepancy may result from unstirred layer effects at the proteoliposomes/SSM surface that restrict complete equilibration of the added substrate at a time when the current reaches its maximum value (*28*). Also, a fraction of MelB transporters with inside-out orientation may partially increase the constants as they expose their low affinity internal binding sites outside (*36*).

It is finally remarkable that $K_{0.5}^{Na}$ obtained from the electrical measurements is reduced by the presence of sugar (2.1 mM vs 0.6 mM) and that $K_{0.5}^{mel}$ decreases in the presence of Na^+ ions (22 mM vs 3 mM). These data are consistent with cooperative interactions between the substrates on MelB. The cooperative effect of Na^+ on sugar binding and transport by MelB is already well documented (*7*, *9*, *16*, *22*, *36*). The present data provide the first evidence for a cooperative influence of the sugar substrate on MelB affinity for the coupling cation.

Correlation of the Electrical Signal with Partial Reactions of MelB. Components of the observed currents or associated charge transfers can be assigned to distinct steps of the transport mechanism by examining the ΔNa and ΔNaΔmel transient currents in the light of basic principles of the mechanism of ion-linked solute cotransport and/or a 6-state ordered kinetic model as well as more specific properties of MelB (review in (*7*)). Upon addition of the coupling ion in the absence of sugar substrate (ΔNa), one expects Na^+ binding to MelB but not its translocation across the membrane into the inner compartment. Accordingly, the fast and monophasic transient response recorded in ΔNa (or ΔLi) experiments must be linked to an ion-induced charge transfer within the transporter and, more precisely, into a MelB domain that is electrically insulated from the external proteoliposome aqueous phase. Since the time constant of this current component ($\tau = 15$ ms) is limited by the time constant of the solution exchange (see above), the Na^+-induced charge transfer within MelB has a time constant of 15 ms or shorter, corresponding to a rate constant $k \geq 50\ s^{-1}$.

A second process, associated with cosubstrate translocation across the membrane, can be identified from the slowly decaying component (350−450 ms) of the biphasic responses induced by the ΔmelΔNa concentration jump. There, the simultaneously added Na^+ and melibiose bind to MelB and are translocated downhill as an electrogenic Na^+-coupled melibiose inflow across the membrane. Such an inward Na^+−melibiose translocation gives rise to a stationary current which is converted into a slow transient current decaying with a time constant of $\sim$350 ms by the capacitive recording system (*37*). Clear independent support for this interpretation is provided by the observation that the slow current component of the ΔmelΔNa response is suppressed upon selective inactivation of the translocation capacity of MelB by NEM (Figure 5).

Combination of the two distinct charge-transfer processes can account for the overall shape of the two additional current responses observed either on adding melibiose to proteoliposomes incubated in Na^+ (Δmel(Na)) or on adding Na^+ to proteoliposomes incubated in melibiose (ΔNa(mel)). The persistence of a fast component of smaller amplitude in the Δmel(Na) response from proteoliposomes already equilibrated with 10 mM Na^+ may represent filling of unoccupied Na^+ binding sites induced by the cooperative effect of melibiose on MelB affinity for Na^+ (see Figure 4) or an

independent electrogenic melibiose binding process. Further experiments are needed to evaluate these possibilities.

In addition, the coexistence of a large fast and a small slow components in response to a Na^+ jump in the presence of melibiose (ΔNa(mel)) suggests a fast Na^+ binding reaction followed by a rate limiting step, probably Na^+ release into the trans compartment (*7*, *36*).

Finally, the current response to a melibiose jump in Na^+-free medium (Δmel) includes a component decaying with $\tau \approx 100$ ms in place of the fast current component observed in Na^+-containing solution (15 ms). This may be assigned to cooperative H^+ binding triggered by slow melibiose binding. Alternatively, the cation binding sites could be already filled with H^+ (or H_30^+), the 100 ms phase representing a distinct electrogenic melibiose binding reaction. In both cases, it can be concluded that the melibiose binding reaction in the absence of Na^+ takes place with a rate constant of $\sim 10\ s^{-1}$.

Pioneered by Wright and colleagues (*38*, *39*) on the eucaryote Na^+−glucose transporter (SGLT1) and then extended to a large variety of Na^+ transporters (review in ref *24*), electrical studies of presteady-state currents have revealed the existence of rapid charge transfer within the transporters in response to membrane voltage change. In closer relation to the present study, refs *25* and *40* reported transient currents in the GABA transporter (GAT1) induced by Na^+-concentration jumps. These studies demonstrated charge transfer due to a voltage and Na^+-dependent readjustement of the distribution of the empty and loaded forms of the carriers. In particular, Na^+-binding/dissociation and the reorientation of the negatively charged empty transporter were proposed to be electrogenic (*24*). Interestingly, the eucaryote and bacterial MelB transporters share very similar six-state ordered kinetic models for Na^+-coupled solute transport (compare for example refs *7* and *39*). Moreover, conformational changes appear to play a determining role during cosubstrate binding and/or transport by both classes of transporters (*16*, *22*, *41*). However, the difference in the dependence on the membrane potential of MelB (*5*, *7*) and SGLT1 (*42*) functioning as Na^+-, Li^+-, or H^+-coupled transporters seems to suggest differences in the rates of some of the partial reactions of their transport cycles.

The overall appearance of similarity of the eucariotic and the procariotic Na^+/solute transporters is further supported by the electrical measurements presented here. In MelB, we have demonstrated a Na^+-induced charge tranfer process. In agreement with the kinetic model for SGLT1, this can be explained by electrogenic Na^+ binding and possibly a contribution from the preceding electrogenic reorientation of the unloaded carrier. In addition, the slow phase of the electrical signal can be explained on the basis of the existing kinetic model, namely, in terms of the coordinated action of all electrogenic steps which are rate limited by the slow Na^+ dissociation reaction.

Finally, experimental evidence supports the notion that several aspartic residues located near the cytoplasmic extremity of N-terminal helices of MelB could organize in a coordination network for the coupling ion and be responsible for MelB ionic selectivity. Neutralization of the negatively charged ionic site by Na^+ or Li^+ ions may change electrostatic interactions within the protein and trigger a conformational change. In fact, conformational changes upon substrate binding are a likely requirement for the direct or indirect cooperative interaction of cation and sugar. Since conformational transitions may lead to rapid intraprotein charge transfers, we have to consider the possibility that only a part of the charge translocation observed upon Na^+ binding is due to the displacement of Na^+. Similarly, electrogenic sugar binding is an option that has to be kept in mind. Experiments aiming at the investigation of this mechanism are currently underway by examining the electrical properties of MelB mutated on these aspartic acids or exhibiting impaired cosubstrate translocation.

ACKNOWLEDGMENT

The authors would like to acknowledge Raymonde Lemonnier and Eva Grabsch for excellent technical assistance. For his interest and support of this work, Ernst Bamberg is thanked. We thank Ernest Wright for the valuable discussion about our kinetic interpretation.

REFERENCES

1. Tsuchiya, T., and Wilson, T. H. (1978) *Membr. Biochem. 2*, 63−79.
2. Lopilato, J., Tsuchiya, T., and Wilson, T. H. (1978) *J. Bacteriol. 134*, 147−56.
3. Wilson, T. H., Ottina, K., and Wilson, D. M. (1982) in *Membranes and Transport* (Martonosi, A. N., Ed.) pp 33−39, Plenum Publishing Corporation, New York.
4. Tsuchiya, T., Oho, M., and Shiota-Niiya, S. (1983) *J. Biol. Chem. 258*, 12765−7.
5. Bassilana, M., Damiano-Forano, E., and Leblanc, G. (1985) *Biochem. Biophys. Res. Commun. 129*, 626−31.
6. Wilson, D. M., and Wilson, T. H. (1987) *Biochim.Biophys. Acta 904*, 191−200.
7. Pourcher, T., Bassilana, M., Sarkar, H. K., Kaback, H. R., and Leblanc, G. (1990) *Philos. Trans. R. Soc. London, Ser. B: Biol. Sci. 326*, 411−23.
8. Pourcher, T., Leclercq, S., Brandolin, G., and Leblanc, G. (1995) *Biochemistry 34*, 4412−4420.
9. Damiano-Forano, E., Bassilana, M., and Leblanc, G. (1986) *J. Biol. Chem. 261*, 6893−9.
10. Bassilana, M., Pourcher, T., and Leblanc, G. (1988) *J. Biol. Chem. 263*, 9663−7.
11. Botfield, M. C., Naguchi, K., Tsuchiya, T., and Wilson, T. H. (1992) *J. Biol. Chem. 267*, 1818−22.
12. Pourcher, T., Bibi, E., Kaback, H. R., and Leblanc, G. (1996) *Biochemistry 35*, 4161−8.
13. Poolman, B., Knol, J., Vanderdoes, C., Henderson, P. J. F., Liang, W. J., Leblanc, G., Pourcher, T., and Mus-Veteau, I. (1996) *Mol. Microbiol. 19*, 911−922.
14. Gwizdek, C., Leblanc, G., and Bassilana, M. (1997) *Biochemistry 36*, 8522−9.
15. Dave, N., Troullier, A., Mus-Veteau, I., Duñach, M., Leblanc, G., and Padros, E. (2000) *Biophys. J. 79*, 747−55.
16. Mus-Veteau, I., Pourcher, T., and Leblanc, G. (1995) *Biochemistry 34*, 6775−83.
17. Wilson, D. M., and Wilson, T. H. (1992) *J. Bacteriol. 174*, 3083−6.
18. Pourcher, T., Zani, M. L., and Leblanc, G. (1993) *J. Biol. Chem. 268*, 3209−15.
19. ama, H., and Wilson, T. H. (1993) *J. Biol. Chem. 268*, 10060−5.
20. Zani, M. L., Pourcher, T., and Leblanc, G. (1993) *J. Biol. Chem. 268*, 3216−21.
21. Leblanc, G., Pourcher, T., and Bassilana, M. (1989) *Biochimie 71*, 969−79.
22. Maehrel, C., Cordat, E., Mus-Veteau, I., and Leblanc, G. (1998) *J. Biol. Chem. 273*, 33192−33197.
23. Kappl, M., and Hartung, K. (1996) *Biophys. J. 71*, 2473−2485.

24. Hazama, A., Loo, D. D. F., and Wright, E. M. (1997) *J. Membr. Biol. 155*, 175−186.
25. Mager, S., Naeve, J., Quick, M., Labarca, C., Davidson, N., and Lester, H. A. (1993) *Neuron 10*, 177−188.
26. Bamberg, E., Butt, H. J., Eisenrauch, A., and Fendler, K. (1993) *Q. Rev. Biophys. 26*, 1−25.
27. Seifert, K., Fendler, K., and Bamberg, E. (1993) *Biophys. J. 64*, 384−91.
28. Pintschovius, J., and Fendler, K. (1999) *Biophys. J. 76*, 814−826.
29. Pintschovius, J., Fendler, K., and Bamberg, E. (1999) *Biophys. J. 76*, 827−36.
30. Bamberg, E., Apell, H.-J., Dencher, N. A., Sperling, W., Stieve, H., and Läuger, P. (1979) *Biophys. Struct. Mech. 5*, 277−92.
31. Botfield, M. C., and Wilson, T. H. (1988) *J. Biol. Chem. 263*, 12909−15.
32. Mus-Veteau, I., and Leblanc, G. (1996) *Biochemistry 35*, 12053−60.
33. Gropp, T., Cornelius, F., and Fendler, K. (1998) *Biochim. Biophys. Acta 1368*, 184−200.
34. Tsuchiya, T., Takeda, K., and Wilson, T. H. (1980) *Membr. Biochem. 3*, 131−46.
35. Zani, M. L., Pourcher, T., and Leblanc, G. (1994) *J. Biol. Chem. 269*, 24883−9.
36. Bassilana, M., Pourcher, T., and Leblanc, G. (1987) *J. Biol. Chem. 262*, 16865−70.
37. Fendler, K., Jaruschewski, S., Hobbs, A., Albers, W., and Froehlich, J. P. (1993) *J. Gen. Physiol. 102*, 631−66.
38. Parent, L., Supplisson, S., Loo, D. D. F., and Wright, E. M. (1992) *J. Membr. Biol. 125*, 49−62.
39. Parent, L., Supplisson, S., Loo, D. D. F., and Wright, E. M. (1992) *J. Membr. Biol. 125*, 63−79.
40. Mager, S., Kleinberger-Doron, N., Keshet, G. I., Davidson, N., Kanner, B. I., and Lester, H. A. (1996) *J. Neurosci. 16*, 5405−5414.
41. Loo, D. D. F., Hirayama, B. A., Gallardo, E. M., Lam, J. T., Turk, E., and Wright, E. M. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 7789−7794.
42. Hirayama, B. A., Loo, D. D. F., and Wright, E. M. (1997) *J. Biol. Chem. 272*, 2110−2115.