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Kinetic Properties of Na^+–H^+ Antiport in *Escherichia coli* Membrane Vesicles: Effects of Imposed Electrical Potential, Proton Gradient, and Internal pH†

Martine Bassilana, Evelyne Damiano, and Gérard Leblanc*

ABSTRACT: Modifications of the kinetic properties of the *Escherichia coli* (RA11) Na^+–H^+ antiport system by imposed pH gradients (ΔpH, interior alkaline) and membrane potential ($\Delta\psi$, interior negative) were studied by looking at the accelerating effects of ΔpH and $\Delta\psi$ on downhill Na^+ efflux from membrane vesicles incubated at different external pHs. First, variations of the Na^+ efflux rate (V_{Na}) as a function of imposed ΔpH appear to be strongly dependent on the external pH value. The individual V_{Na} vs. ΔpH relationships observed between pH 5.5 and pH 6.6 are all nonlinear and indicate the existence of a threshold ΔpH above which V_{Na} increases steeply as the ΔpH magnitude increases; threshold ΔpH values progressively decrease as the pH is raised from 5.5 to 6.6. In contrast, at or above neutrality, V_{Na} acceleration is linearly related to ΔpH amplitude. Strikingly, it is shown that the ΔpH-dependent variations in the Na^+ efflux rate measured in vesicles incubated at different external pHs can be accounted for by variations of internal pH; the observed relationship suggests that a high internal H^+ concentration inhibits the Na^+–H^+ antiport activity. This inhibition results from a drastic increase in the apparent K_m of the Na^+ efflux reaction as the internal H^+ concentration increases. On the other hand, imposed $\Delta\psi$ increases the Na^+ efflux rate linearly by a selective modification of the V_{max} value of the Na^+ efflux. Together, these data indicate that the internal H^+ concentration controls the Na^+–H^+ antiport activity and that the chemical and electrical proton gradients affect two different kinetic steps of the Na^+–H^+ exchange reaction.

It is now accepted that Na^+–H^+ exchange reactions catalyzed by the Na^+–H^+ antiporter present in bacterial membranes are involved in two important physiological functions: generation of a transmembrane Na^+ gradient (Harold & Altendorf, 1974; Lanyi, 1979) and regulation of the cytoplasmic pH (Padan et al., 1981). Operation of these cationic exchangers in the cytoplasmic membrane of bacteria was postulated by Mitchell (1968, 1970, 1973) within the frame of the chemiosmotic hypothesis, and their participation in the regulation of internal Na^+ concentration is thought to proceed in the following manner: during Na^+ extrusion, the antiporter obligatorily couples the Na^+ outflow to an inward movement of protons, the energy necessary for uphill Na^+ efflux being derived from the downhill H^+ influx along its favorable electrochemical potential gradient ($\Delta\bar{\mu}_{H^+}$)[1] created during activity of H^+-excreting devices such as the respiratory chain, H^+-ATPase, or the light-driven bacteriorhodopsin.

The existence of obligatory coupled Na^+ and H^+ movements (West & Mitchell, 1974; Zilberstein et al., 1979) as well as the dependence of the Na^+ extrusion process upon the generation of $\Delta\bar{\mu}_{H^+}$ (interior alkaline and electrically negative) has been convincingly demonstrated in many bacterial systems and derived right-side-out membrane vesicles [review in Lanyi (1979)]. Conversely, $\Delta\bar{\mu}_{H^+}$-dependent Na^+ accumulation by membrane vesicles with inverted orientation polarity has also been reported (Reenstra et al., 1980; Beck & Rosen, 1979).

Although there is general agreement that $\Delta\bar{\mu}_{H^+}$ drives the Na^+–H^+ antiport reaction, the precise mechanism underlying this activation is still unknown. In particular, the exact role of the electrical and chemical components of $\Delta\bar{\mu}_{H^+}$ remains to be elucidated. West & Mitchell (1974) first suggested that the Na^+–H^+ antiporter is solely influenced by ΔpH. Later on, Schuldiner & Fishkes (1978) proposed that $\Delta\psi$ drives the exchange reaction at alkaline external pH whereas ΔpH would specifically increase the antiporter activity at acidic pH. Finally, studies using everted membrane vesicles have shown that both ΔpH and $\Delta\psi$ can promote Na^+ accumulation in these preparations (Reenstra et al., 1980).

Recently, further insight into the mechanism of control of the antiport activity by $\Delta\psi$ and ΔpH was obtained by looking at the effects of these gradients on the kinetics of downhill Na^+ efflux catalyzed by the Na^+–H^+ exchanger in *Escherichia coli* membrane vesicles incubated at different external pHs (Bassilana et al., 1984). It was first observed that downhill Na^+ efflux is strongly enhanced in the presence of $\Delta\bar{\mu}_{H^+}$ (interior alkaline and negative). Furthermore, the results indicated that, at all external pHs tested between 5.5 and 7.5, both $\Delta\psi$ and ΔpH contribute to the acceleration of the downhill

† From the Laboratoire Jean Maetz du Département de Biologie du Commissariat à l'Energie Atomique, B.P. 68, 06230 Villefranche-sur-Mer, France. *Received February 2, 1984.*
* Address correspondence to this author. He is associated with CNRS (ER 247).

[1] Abbreviations: $\Delta\bar{\mu}_{H^+}$, electrochemical gradient of protons; ΔpH, proton gradient; $\Delta\psi$, electrical potential.

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Na^+ efflux rate. Strikingly, however, whereas at pH 6.6 the chemical and electrical gradients enhance independently the Na^+ efflux rate, the effects of the $\Delta\bar{\mu}_{H^+}$ component markedly differ at pH_{out} 5.5. Indeed, at this acidic external pH, the imposed ΔpH should exceed a threshold ΔpH value in order to give rise to an acceleration of Na^+ efflux rate; on the other hand, $\Delta\psi$, which, per se, does not promote Na^+ efflux acceleration, contributes effectively to the acceleration of the Na^+ efflux rate when a chemical proton gradient—higher than the threshold ΔpH value—is simultaneously imposed. These observations suggest that a ΔpH-sensitive step controls the Na^+–H^+ exchange reaction in acidic conditions and could be explained by assuming gating effects of the chemical proton gradient on the exchanger activity (Lanyi & Silverman, 1979). Alternatively, it can be envisaged that the existence of a threshold ΔpH value reflects an effect of the internal H^+ concentration on the Na^+–H^+ exchange reaction.

In the present study, we investigate in detail the mechanism of control of the Na^+–H^+ antiport activity by ΔpH in membrane vesicles isolated from *E. coli* RA11 and incubated at various external pHs. Changes in the downhill Na^+ efflux rate were analyzed as a function of imposed ΔpH of varying amplitudes and artificially generated by creating outwardly directed acetate diffusion gradients (Lancaster & Hinkle, 1977; Kaczorowski et al., 1979). This analysis was completed by a determination of the effects of ΔpH on the kinetic properties of the Na^+ efflux mechanism. These effects were compared to those produced by imposition of $\Delta\psi$ of varying amplitudes on the rate of Na^+ efflux and on the kinetic parameters of the Na^+ efflux mechanism. In these later experiments, outwardly directed K^+ diffusion gradients were used to generate transmembrane electrical potentials, $\Delta\psi$ (Kaczorowski et al., 1979; Leblanc et al., 1980). The results indicate that the internal proton concentration plays a determinant role in the mechanism of stimulation of the Na^+–H^+ antiport activity by imposed chemical proton gradients. The data also suggest that the chemical and electrical components of $\Delta\bar{\mu}_{H^+}$ affect different kinetic steps of the Na^+–H^+ exchange reaction mechanism.

Materials and Methods

Materials. $^{22}NaCl$ (carrier free) was obtained from the Commissariat à l'Energie Atomique (Saclay, France). Valinomycin was from Sigma. Choline salts were prepared by titrating choline bicarbonate (Sigma) with reagent-grade acids.

E. coli RA11 cells were grown on mineral 63 medium (Cohen & Rickenberg, 1956) supplemented with 1% bactrotryptone, 10 mM melibiose, 0.5 μg/mL thiamin, and trace amounts of $FeSO_4$. Preparation of membrane vesicles was performed according to the osmotic shock procedure of Kaback (1971) as previously described (Bassilana et al., 1984).

^{22}Na Efflux Experiments. Na^+ efflux determinations were performed by following the time-dependent decrease in intravesicular $^{22}Na^+$ content of Na^+-loaded membrane vesicles after their dilution into a large volume of Na^+-free solutions. For these experiments, vesicles prepared in 100 mM potassium phosphate (pH 6.6) were thawed, resuspended in a 30-fold excess of a medium containing 100 mM potassium acetate, 10 mM sodium phosphate, and 10 mM $MgSO_4$ at the desired pH, and allowed to equilibrate at room temperature for 30 min before centrifugation (40000g × 20 min). The equilibration step was repeated twice, and the final pellet was resuspended in a minimal volume of the same solution to give a suspension as concentrated as possible (usually 30–35 mg of membrane protein/mL). Then a small aliquot of carrier-free $^{22}NaCl$ was added to the concentrated vesicle preparation (final activity 1500–2500 cpm/nmol), and the suspension was incubated in ice for about 5 h in order to allow complete equilibration of Na^+ and its isotope with the intravesicular space. At this stage, valinomycin was systematically added to give a final concentration of 1 nmol/mg of membrane protein.

Na^+ efflux determinations were initiated by diluting 2.5-μL aliquots (80–100 μg of protein) of concentrated vesicle suspension into 2 mL of appropriate solutions buffered at the same pH, quickly agitated, and placed at 21 °C. At given times, the Na^+ efflux reaction was terminated by filtering the diluted sample. The filter (Amicon, 0.45 μm) was then washed once with 2 mL of equilibration solution.

Radioactivity remaining on the filter was determined by liquid scintillation spectrometry. Nonspecific binding of $^{22}Na^+$ on the filters was estimated from the amount of radioactivity retained on the filter following filtration of 2.5-μL aliquots that had stood for 2 h or more at 21 °C and was systematically subtracted from Na^+ efflux data. Zero times were determined by diluting 2.5-μL aliquots followed by immediate filtration. Absolute values of internal Na^+ concentration at zero times were calculated by assuming 2.2 μL of intravesicular fluid/mg of membrane protein (Kaback & Barnes, 1971).

Protein Determinations. Protein was measured according to Lowry et al. (1951) using serum albumin as a standard.

Results

Effects of Imposed $\Delta\psi$ and ΔpH on Downhill Na^+ Efflux Rate. Typical modifications of the rate of downhill Na^+ efflux from Na^+-loaded vesicles provoked by imposition of artificial ΔpH (internal alkaline) or $\Delta\psi$ (internal negative) of varying amplitude are illustrated in Figure 1. In the particular experiment shown, membrane vesicles were previously equilibrated with 100 mM potassium acetate medium containing 10 mM sodium-22 phosphate (pH 6.6) and treated with valinomycin. The vesicles were then diluted in Na^+-free media at the same pH under conditions where the salt composition was changed so as to selectively create outwardly directed acetate diffusion gradients (generation of artificial ΔpH) or outwardly directed potassium diffusion gradients (generation of artificial $\Delta\psi$) during the dilution procedure. As shown in Figure 1, generation of either artificial ΔpH (left panel) or artificial $\Delta\psi$ (right panel) across the membrane vesicles enhances the Na^+ efflux rate (V_{Na}). Indeed, imposition of ΔpH or $\Delta\psi$ of theoretically 167 mV (closed diamonds) produces respectively a 5- and 10-fold acceleration of V_{Na} as compared to the rate of Na^+ efflux measured in the absence of imposed gradients (closed circles). Furthermore, as illustrated by the intermediary curves illustrated in each panel, a gradual increase in the Na^+ efflux rate is observed in response to imposed ΔpH or $\Delta\psi$ of increasing magnitude. These data not only confirm that, at an external pH of 6.6, the two components of $\Delta\bar{\mu}_{H^+}$ individually accelerate the downhill Na^+ efflux from Na^+-loaded membrane vesicles (Bassilana et al., 1984) but also show that the accelerating effects of ΔpH and $\Delta\psi$ are gradual.

Effect of External pH on Na^+ Efflux Acceleration by ΔpH. Previous studies on the effect of imposed ΔpH on the Na^+ efflux rate from membrane vesicles incubated at acidic external pH (pH_{out} 5.5) suggested that imposed chemical H^+ gradients should have a magnitude higher than a critical value of about 70 mV in order to promote acceleration of the Na^+ efflux rate (Bassilana et al., 1984). This property has been examined in detail and the variations of V_{Na} as a function of the magnitude of ΔpH at pH_{out} 5.5 are illustrated in Figure 2A (closed circles). It can be observed that, below a threshold ΔpH value

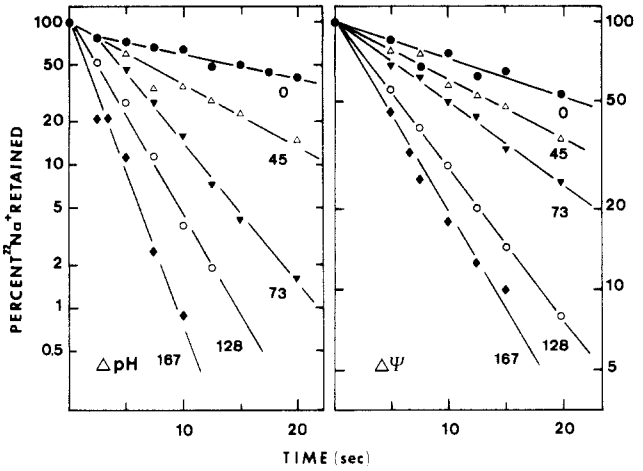

FIGURE 1: Effects of imposed $\Delta\psi$ and ΔpH of varying amplitude on the rate of downhill Na^+ efflux from Na^+-loaded membrane vesicles incubated at pH 6.6. (Left panel: ΔpH) Effect of varying ΔpH on the Na^+ efflux rate. Membrane vesicles were equilibrated with 100 mM potassium acetate, 10 mM sodium phosphate (pH 6.6), and 10 mM $MgSO_4$ and concentrated to about 30 mg of membrane protein·mL^{-1}. The vesicles were then incubated during 5 h at 5 °C with $^{22}Na^+$ (2000 cpm·$nmol^{-1}$) and finally treated with valinomycin [1 nmol·(mg of protein)$^{-1}$]. $^{22}Na^+$ efflux was measured by diluting 2.5-μL aliquots of concentrated vesicles into 2 mL of solution containing 100 mM potassium ions and various ratios of gluconate and acetate to generate the appropriate ΔpH during the dilution procedure. At the times shown, the diluted samples were filtered and the filters washed once with 2 mL of diluting solution. Radioactivity remaining on the filters, once corrected for nonspecific binding, is used to estimate the amount of Na^+ retained by the vesicles. Data are expressed as percentage of $^{22}Na^+$ retained by using zero time points for normalization. Values shown on each curve indicate the amplitude of the corresponding imposed pH gradient calculated as ΔpH (mV) = 58 log $[(acetate)_{in}/(acetate)_{out}]$. (Right panel: $\Delta\psi$) Effect of varying $\Delta\psi$ on the Na^+ efflux rate. Vesicles were prepared as described in panel A. Dilutions were performed in Na^+-free media, containing 100 mM acetate–10 mM phosphate salts (pH 6.6) and various ratios of potassium and choline. The magnitude of generated $\Delta\psi$ is given below each curve and was calculated as $\Delta\psi$ (mV) = 58 log $[(K^+)_{in}/(K^+)_{out}]$. Data are expressed as in the left panel.

of 60 mV, little or no Na^+ efflux acceleration occurs; in contrast, above this threshold ΔpH value, the Na^+ efflux rate increases almost linearly with increasing ΔpH amplitude. The resulting nonlinear relationship between V_{Na} and ΔpH is similar to the $\Delta\bar{\mu}_{H^+}$-induced gating phenomenon described for the Na^+–H^+ antiport in *Halobacterium halobium* membrane vesicles by Lanyi & Silverman (1979). At variance with these authors, we can, however, conclude that the gating effect observed here is solely related to the chemical proton gradient and not to the total protonmotive force since in our experimental conditions no $\Delta\psi$ was generated.

Since it has been previously established that ΔpH accelerates the Na^+ efflux rate at all pHs in the range of external pH between 5.5 and 7.5, it was of interest to analyze the fate of this nonlinear relationship between V_{Na} and ΔpH at higher pH. To this end, vesicles were preequilibrated in acetate buffer containing $^{22}Na^+$ at different pHs ranging from 6 to 7.5; the accelerating effect of ΔpH of increasing intensities on the Na^+ efflux rate was studied at the corresponding external pH. The results of these different experiments are shown in Figure 2. A general examination of the whole set of V_{Na} vs. ΔpH curves first indicates that the shape of the relationship between the Na^+ efflux rate and ΔpH is strongly influenced by the medium pH. Thus, from a nonlinear function at pH_{out} 5.5 (Figure 2A, closed circles), the variation of V_{Na} as a function of ΔpH is satisfactorily fitted by a linear function at pH_{out} 7.5 (Figure 2B, open diamonds). At this later pH, one can observe that

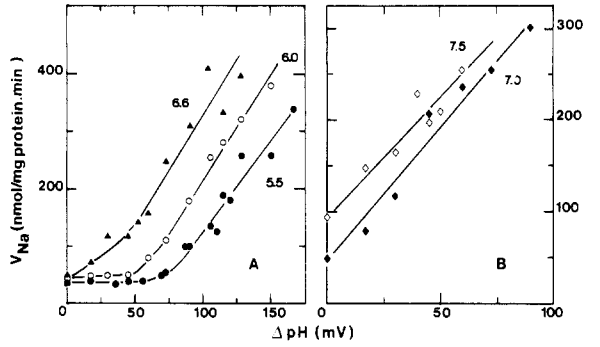

FIGURE 2: pH dependence of the relationship between Na^+ efflux rate and amplitude of imposed ΔpH. Concentrated membrane vesicles (30 mg of membrane protein·mL^{-1}) previously equilibrated with 100 mM potassium acetate media at different pHs between 5.5 and 7.5 containing 10 mM $^{22}Na^+$ ions (2000 cpm·$nmol^{-1}$) and treated with valinomycin [1 nmol·(mg of protein)$^{-1}$] were prepared as described in Figure 1. The rate of the internal $^{22}Na^+$ decrease was estimated by diluting 2.5-μL aliquots of vesicles equilibrated at a given pH into 2 mL of a medium at the same pH, devoid of Na^+ ions and containing 100 mM potassium ions and various ratios of acetate and gluconate so as to generate appropriate ΔpH during the dilution procedure. $^{22}Na^+$ efflux rates (V_{Na} in nanomoles per milligram of protein per minute) were estimated by using the rate constant for the internal $^{22}Na^+$ decrease measured graphically and the initial $^{22}Na^+$ content [20 ± 2 nmol·(mg of protein)$^{-1}$]. (A) Equilibration and dilution media were 5.5 (●), 6 (○), and 6.6 (▲). (B) Equilibration and dilution media were respectively 7 (♦) and 7.5 (◇). Magnitudes of the outwardly directed acetate diffusion gradients were calculated as ΔpH (mV) = 58 log $[(acetate)_{in}/(acetate)_{out}]$.

even reduced imposed ΔpHs significantly and linearly increase the Na^+ efflux rate. On the other hand, inspection of the dependence of V_{Na} on ΔpH at intermediary pH suggests that increasing the pH medium mainly results in a progressive translation of the curve responses toward the left of the ΔpH axis. It can also be noted that the rising phases of these curve responses are almost parallel; this is particularly striking in the range of pH values from 5.5 to 6.6 (Figure 2A). As a consequence, the amplitude of the threshold ΔpH value progressively declines as the external pH is raised. Furthermore, no ΔpH threshold is detected at pH_{out} 7 (closed diamonds in Figure 2B). Altogether, these data clearly establish that the Na^+–H^+ antiport displays a nonlinear dependence on ΔpH only in acidic conditions. Since one expects that the development of ΔpH-induced gating properties should be dependent only on the magnitude of the transmembrane chemical proton gradient and should persist at all external pHs, it is concluded that the nonlinear dependence of V_{Na} on ΔpH observed in acidic conditions is not necessarily linked to gating effects of ΔpH on the Na^+ efflux mechanism.

Importance of Internal pH Change in the Mechanism of Na^+ Efflux Stimulation by ΔpH. In looking for an alternative explanation for the strong influence of the external pH on the shape of the relationship between V_{Na} and ΔpH, it is essential to emphasize that, in the experiments described above, ΔpHs were created by shifting the internal pH (pH_i) from the equilibrium values toward more alkaline values. Since, in vesicles incubated at different external pH, the equilibration pH_i is similar to pH_{out}, it follows that the internal pH value reached during imposition of an identical ΔpH will vary greatly as pH_{out} is varied. These remarks indicate that the internal concentration of H^+ behaves as an additionnal variable. One can thus hypothesize that pH_i constitutes a critical parameter in the mechanism of stimulation of Na^+ efflux by ΔpH. Strong support for this hypothesis comes from examination of the plot of ΔpH-induced variations of the Na^+ efflux rate obtained at pH_{out} 5.5, 6, and 6.6 (taken from Figure 2A) as

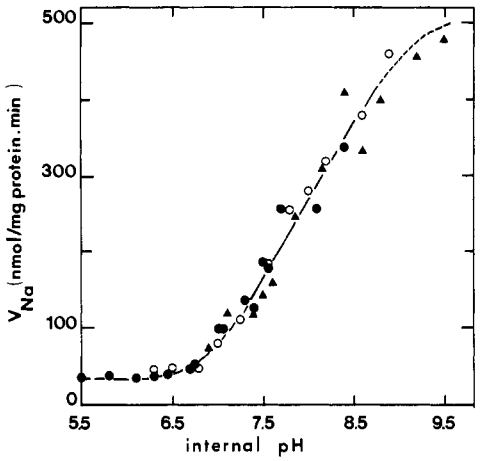

FIGURE 3: Relationship between changes in Na^+ efflux rate and variations of intravesicular pH values associated with the imposed ΔpH of varying amplitudes. Na^+ efflux rate data are taken from Figure 2A and correspond respectively to Na^+ efflux rates measured at pH_{out} 5.5 (●), 6 (○), and 6.6 (▲). Change in intravesicular pH corresponding to each Na^+ efflux data was calculated as pH_i = pH(equilibration) + 58 log $[(acetate)_{in}/(acetate)_{out}]$, where pH(equilibration) = pH_{out}.

a function of the internal pH values reached during imposition of ΔpH of increasing amplitude (Figure 3). It is remarkable to observe that a single relationship suffices to account for the totality of the Na^+ efflux variations observed at the three different external pHs. Several features of this relationship are worth mentioning. The V_{Na} vs. pH_i curve first suggests the existence of a critical internal pH_i value, around 6.5, below which no Na^+ efflux acceleration by imposed ΔpH is observed; then, above this value, V_{Na} increases as pH_i increases. Finally, there is evidence that V_{Na} saturates when pH_i reaches values above pH 9. In view that the characteristics of this relationship can be accounted for by a simple titration curve, it could be suggested that an internal chemical group with a pK_a around 7.9 is implicated in the effect of pH_i on V_{Na}. This pK_a estimation should nevertheless be considered as tentative since precipitation of phosphate salts is likely to occur at these very alkaline pHs and might interfere with the Na^+ efflux kinetics.

It should finally be mentioned that when plotted as a function of pH_i, the ΔpH-dependent variations of V_{Na} recorded at pH_{out} 7 or 7.5 (Figure 2B) can be fitted by relationships identical with that illustrated in Figure 3 but which are slightly translated toward the right of the pH_i axis (not shown). This result could be explained by assuming that at pH_{out} 6.6 or below the external H^+ concentration is saturating whereas at pH_{out} 7 or 7.5 the overall Na^+-H^+ exchange rate is limited by the external H^+ concentration, which is the substrate of the exchange mechanism.

In conclusion, these data strongly suggest that the internal H^+ concentration contributes to the regulation of the Na^+-H^+ antiport activity. It should be recalled, here, that a recent study using the pH-dependent fluorescence probe pyranine enclosed in membrane vesicles to monitor internal pH in *E. coli* membrane vesicles justifies calculations of pH_i changes caused by imposed outwardly directed acetate gradients using the theoretical amplitudes of the imposed pH gradients (Damiano et al., 1984).

Effects of Imposed ΔpH on Kinetic Parameters of Na^+ Efflux. The striking relationship between pH_i and V_{Na} suggests an adverse effect of internal protons on the Na^+ efflux mechanism. It was therefore of interest to investigate the modifications of the kinetic parameters of the Na^+ efflux caused by the imposition of ΔpH. The experiment was performed at pH_{out} 5.5 where the effects of pH_i changes on V_{Na} are most pronounced. ^{22}Na efflux rates from vesicles equilibrated in acetate-buffered solution (pH 5.5) containing various concentrations of Na^+ ions (from 2 to 30 mM) were measured under conditions where the intravesicular pH was fixed at different values between 6.8 and 7.7 by creating appropriate acetate diffusion gradients. Representation of the efflux data by an Eadie–Hofstee plot (Figure 4) clearly shows that the shift of pH_i toward more alkaline values results in an important decrease in the apparent affinity constant (K_m) of the Na^+ efflux mechanism without any significant effect on its maximal rate. Thus, the apparent K_m is 40 mM when pH_i is set at 6.8 and decreases down to 3.5 mM when pH_i is 7.7, and intermediary values of 20 and 7 mM are found when pH_i is 7 and 7.5, respectively. Incidentally, the K_m values of 3.5–7 mM observed when pH_i is 7.5–7.7 are comparable to that reported previously in everted membrane vesicles for the Na^+-H^+ antiport (Schuldiner & Fishkes, 1978; Beck & Rosen, 1979). Determinations of the apparent K_m values below the critical pH_i value of 6.5 were imprecise but suggest that, at pH_i 6.3, for example, the apparent K_m is higher than 100 mM. These results strongly suggest that internal protons compete with internal Na^+ ions on the internal Na^+ binding sites of the antiporter.

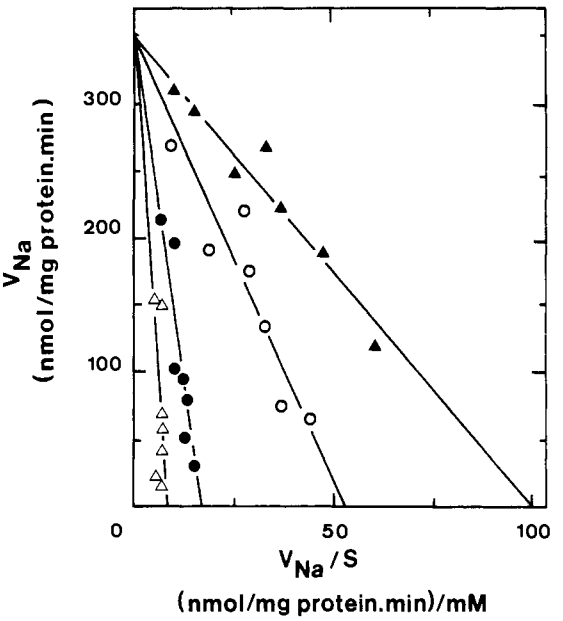

FIGURE 4: Effect of imposed ΔpH on the kinetics of Na^+ efflux in membrane vesicles incubated at pH 5.5. Concentrated membrane vesicle suspensions (30 mg of protein·mL^{-1}) were equilibrated in 100 mM acetate–10 mM phosphate media (pH 5.5) containing Na^+ ions at a final concentration ranging from 2 to 30 mM, incubated with $^{22}Na^+$ (2000 cpm·$nmol^{-1}$), and treated with valinomycin [1 nmol·(mg of protein)$^{-1}$]. The $^{22}Na^+$ efflux from these vesicles was estimated after dilution in Na^+-free media (pH 5.5) as described in Figure 1. The diluting media contained 100 mM potassium ions, valinomycin (5 μM), and the ratio of acetate and gluconate appropriate for the generation of ΔpH of 70 (△), 90 (●), 128 (○), and 150 mV (▲). Theoretically, the intravesicular pH was shifted to 6.8, 7, 7.5, and 7.7, respectively. The Na^+ efflux rate values were computed from the observed rate of intravesicular $^{22}Na^+$ decrease and corrected for the basal Na^+ efflux rate taking place in the absence of imposed gradients. The internal Na^+ concentration $(Na^+)_{int}$ was computed by using the initial $^{22}Na^+$ content and an intravesicular fluid volume of 2.2 μL·(mg of membrane protein)$^{-1}$. Kinetic parameters were determined from the Eadie–Hofstee plots shown.

Effects of Imposed $\Delta\psi$ on Na^+ Efflux at Different pHs. Figure 5 shows the effects of varying the amplitude of imposed $\Delta\psi$ (interior negative) on the rate of Na^+ efflux from vesicles loaded with 10 mM Na^+ and equilibrated in acetate media

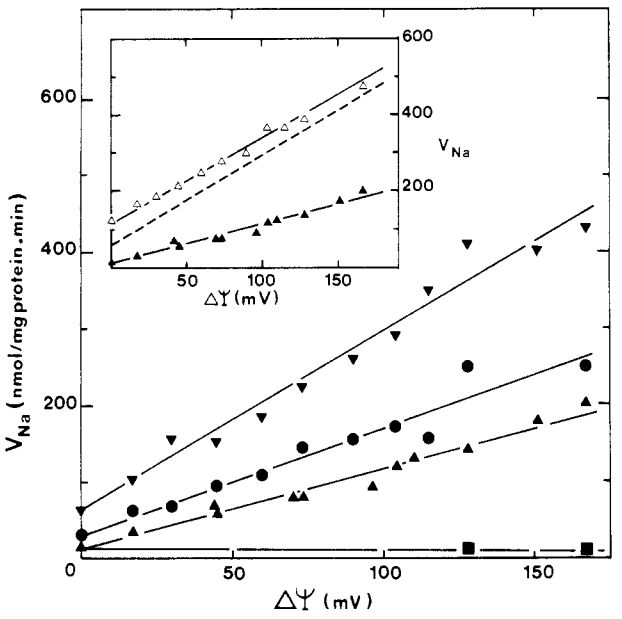

FIGURE 5: pH dependence of the relationship between the Na^+ efflux rate and the amplitude of imposed $\Delta\psi$. Concentrated membrane vesicle suspensions (30 mg of protein·mL^{-1}) equilibrated in 100 mM acetate–10 mM phosphate media at different pHs between 5.5 and 7.5, containing 10 mM Na^+ ions, were incubated in the presence of $^{22}Na^+$ (2000 cpm·$nmol^{-1}$) and treated with valinomycin [1 nmol·(mg of protein)$^{-1}$]. Na^+ efflux rates were estimated by measuring the rate of internal $^{22}Na^+$ decrease after diluting 2.5-μL aliquots of concentrated membrane vesicles into 2 mL of Na^+-free media at the corresponding pH. The dilution media were composed of 100 mM acetate, 10 mM phosphate, and various ratios of potassium and choline appropriate for the generation of artificial $\Delta\psi$ ranging from 20 to 167 mV and valinomycin (5 μM). The pH of equilibration and dilution media was 5.5 (■), 6.6 (▲), 7 (●), and 7.5 (▼). (Inset) Dependence of the relationship between the Na^+ efflux rate and $\Delta\psi$ on the intravesicular pH. Membrane vesicles were loaded with 10 mM $^{22}Na^+$ and equilibrated in acetate–phosphate buffer (pH 6.6) as described above. (▲) 2.5-μL aliquots were diluted in 100 mM acetate media (pH 6.6) containing varying ratios of potassium and choline ($\Delta\psi$ variable; ΔpH = 0, i.e., $pH_i = pH_{out} = 6.6$). (△) 2.5-μL aliquots were diluted in media containing acetate and gluconate in a ratio appropriate for the generation of ΔpH = 0.9 unit, i.e., $pH_i = 7.5$ and $pH_{out} = 6.6$, and varying ratios of potassium and choline ($\Delta\psi$ variable). Dashed line: relationship taken from the main figure and observed when $pH_i = pH_{out} = 7.5$. All diluting media contained 10 mM phosphate salts, 10 mM $MgSO_4$, and valinomycin (5 μM).

buffered at different pHs ranging from 5.5 to 7.5. In agreement with previous observations (Bassilana et al., 1984), no significant modifications of the Na^+ efflux rate by imposed $\Delta\psi$ are observed in vesicles incubated at pH_{out} 5.5 (closed squares). In contrast, at external pH equal or higher than 6.6, the Na^+ efflux rate increases as a linear function of imposed $\Delta\psi$ (Figure 5), and no indication of a threshold $\Delta\psi$ value can be noted at pH_{out} 6.6 (closed triangles). Moreover, it is apparent that the slope of these relationships becomes steeper as the medium pH is raised. The variation in the extent of V_{Na} activation by a given $\Delta\psi$ at different pHs cannot be explained by assuming variations in the magnitude of $\Delta\psi$ actually generated at different pHs since it has been previously shown that outwardly directed potassium diffusion gradient generates transient $\Delta\psi$s of comparable amplitude in the pH range between 5.5 and 7.5 (Leblanc et al., 1980). It appears more reasonable to suggest that these differences are related to variations of the medium pH. However, it cannot be concluded whether the internal or external pH is the determinant parameter since in our experiments pH_i and pH_{out} are modified simultaneously. The experiment illustrated in the inset of Figure 5 suggests that pH_i rather than pH_{out} modulates the accelerating effect of $\Delta\psi$. In this experiment, the variations of V_{Na} as a function of $\Delta\psi$ were studied in conditions where (a) $pH_i = pH_{out} = 6.6$ (closed triangles) and (b) pH_{out} was 6.6 while pH_i was set at pH 7.5 by concomitantly generating an appropriate acetate gradient (open triangles). The inset shows that the slope of the relation between V_{Na} and $\Delta\psi$ is higher when pH_i is shifted to 7.5 than when pH_i is 6.6. Interestingly, the slopes of the V_{Na} vs. $\Delta\psi$ relationships are identical when vesicles incubated at pH 6.6 (open triangles) or pH 7.5 (dashed line) have the same internal pH value (7.5).

Modifications of Kinetic Constants of Na^+ Efflux by $\Delta\psi$. Since, in vesicles incubated at pH 6.6 or higher, $\Delta\psi$ (interior negative) independently accelerates the Na^+ efflux rate, it is of interest to compare the effects of a transmembrane electrical field on the kinetics of Na^+ efflux to those described above for ΔpH. The experiment was conducted using vesicles loaded with Na^+ concentrations varying from 5 to 30 mM and equilibrated at pH 7; acceleration of the Na^+ efflux in response to imposed $\Delta\psi$ ranging from 45 to 128 mV was analyzed. Examination of the Na^+ efflux data by a Lineweaver–Burk plot (not shown) indicates that the imposition of $\Delta\psi$ of increasing amplitude modifies exclusively the maximal Na^+ efflux rate and has no effect on the apparent K_m of the Na^+ efflux reaction (about 10 mM). This result is in agreement with the observation that dissipation of $\Delta\psi$ in respiring inverted *E. coli* vesicles specifically decreases the V_{max} of the antiport reaction (Beck & Rosen, 1979). A 4-fold increase in V_{max} is observed when $\Delta\psi$ is increased from 45 to 128 mV (77–360 nmol·(mg of protein)$^{-1}$·min^{-1}). Compared to the selective modification of K_m produced by imposed ΔpH, the specific effect of $\Delta\psi$ on V_{max} suggests that the mechanisms of activation of the Na^+–H^+ antiport by imposed $\Delta\psi$ and ΔpH are different.

Discussion

The results presented in this paper provide strong support for the concept that the intracellular concentration of H^+ contributes to the regulation of the Na^+–H^+ antiport activity of *E. coli* RA11 membrane vesicles. Furthermore, the distinct kinetic effects of imposed $\Delta\psi$ and ΔpH on the Na^+ efflux mechanism suggest that the chemical and electrical components of $\Delta\bar{\mu}_{H^+}$ affect two different kinetic steps of the Na^+–H^+ exchange reaction.

Indications that the internal H^+ concentration is a major parameter involved in the regulation of the Na^+–H^+ antiport activity come from the detailed study of the accelerating effect of imposed ΔpH on the rate of downhill Na^+ efflux from membrane vesicles incubated at different external pHs (Figure 2). This analysis first established that, in vesicles incubated at acidic pH, V_{Na} is nonlinearly related to the magnitude of the imposed ΔpH (Figure 2A). In analogy with a previous claim of Lanyi & Silverman (1979), who demonstrated a nonlinear relationship between the Na^+–H^+ antiport activity and $\Delta\bar{\mu}_{H^+}$ in *H. halobium*, the peculiar dependence of V_{Na} on the ΔpH magnitude might indicate gating effects of the chemical gradient on the Na^+–H^+ antiport activity in *E. coli* membranes. In the present study, however, we have shown that these gating properties are not displayed at all external pHs (Figure 2B). Indeed, at neutral or alkaline external pH, the rate of Na^+ efflux increases linearly with ΔpH, even in the presence of ΔpH of reduced amplitude (Figure 2B). This latter observation argues against the existence of gating effects of the chemical proton gradient itself. Indeed, one expects the development of ΔpH-induced gating effects depends primarily on the magnitude of the transmembrane H^+ gradient rather than on the value of the external pH per se. It thus appears that other factors, besides gating, are involved in the mecha-

nism of activation of the Na^+–H^+ antiport by imposed chemical gradients.

The observation that a unique relationship fits the ΔpH-dependent variations of V_{Na} and concomitant changes in pH_i in vesicles incubated at different external pHs (Figure 3) clearly points to an important role of the internal concentration of H^+ in the activation mechanism of Na^+ efflux associated with the imposition of ΔpH. This relationship, which apparently follows a simple titration curve, calls for two comments. First, it is apparent that the accelerating effects of imposed ΔpH are more closely related to the change in internal pH than to the magnitude of the imposed chemical H^+ gradients. One can thus observe that identical V_{Na} accelerations are recorded in response to imposed ΔpH of different amplitudes across the membrane of vesicles incubated at different external pHs as long as pH_i is identical. In the second place, at all external pH values in the range 5.5–6.6, the acceleration of Na^+ efflux by ΔpH is only observed when pH_i is shifted to values near or above neutrality, i.e., when the internal concentration of H^+ is lowered; in contrast, no accelerating effect of ΔpH occurs when pH_i remains at values below 6.5. These findings indicate that high internal proton concentrations have adverse effects on the Na^+ efflux mechanism.

Finally, direct support for the above contention is provided by the analysis of the modifications of the kinetic constants of the Na^+ efflux mechanism during activation by imposed ΔpH. Lowering the internal H^+ concentration by imposition of ΔpH leads to a strong decrease in the apparent K_m value of the Na^+ efflux reaction with no significant effect on its maximal rate (Figure 4). These data are characteristics of pure competitive inhibition and can be readily interpreted by assuming competition between internal protons and Na^+ ions for common intravesicular Na^+ binding sites on the antiporter.

The second important result provided by this study comes from the analysis of the kinetic effects of imposed ΔpH (interior alkaline) and $\Delta\psi$ (interior negative) on the Na^+ efflux mechanism, which clearly established that the two components of $\Delta\bar{\mu}_{H^+}$ lead to distinctly separate modifications of the kinetic parameters of the Na^+–H^+ antiport. It is thus evident that imposed ΔpH selectively changes the apparent affinity constant of the Na^+–H^+ exchange reaction (Figure 4) whereas $\Delta\psi$ increases specifically the maximal Na^+ efflux rate. These findings provide primarily a straightforward explanation for the observation that in vesicles incubated at acidic pH, $\Delta\psi$ raises V_{Na} only when imposed simultaneously with ΔpH (Bassilana et al., 1984). Indeed, when the individual effect of $\Delta\psi$ is analyzed at any pH and in particular at pH 5.5, the internal pH has the same value as the external pH. According to the data presented in Figure 4, the apparent affinity constant of the antiporter for internal Na^+ ions at pH_i 5.5 is probably higher than 100 mM; consequently, the rate of Na^+ efflux from vesicles loaded with only 10 mM Na^+ ions is low and an accelerating effect of $\Delta\psi$ can thus barely be detected experimentally. On the other hand, when a chemical proton gradient (interior alkaline) is imposed simultaneously with $\Delta\psi$, the apparent K_m is shifted to values near or below the internal Na^+ concentration used to load the vesicles and the $\Delta\psi$-dependent accelerating effect can then be observed. These data indicate that the step involving binding of Na^+ ions on the antiporter becomes rate limiting for the overall Na^+–H^+ exchange mechanism at acidic pH_i. On the other hand, according to the same reasoning, one can expect that the fractional increase in V_{Na} as a function of $\Delta\psi$, i.e., the slope of the V_{Na} vs. $\Delta\psi$ curves shown in Figure 5, could increase as pH_{out} (and more importantly pH_i) is shifted toward alkaline values. Indeed, the electrical field would exert its accelerating effect on a Na^+–H^+ exchange reaction having enhanced affinity at more alkaline pH_i. This hypothesis is further substantiated by the finding that the slope of the V_{Na} vs. $\Delta\psi$ relationship is increased when vesicles incubated at the same outer pH have more alkaline pH_i. Importantly, this latter observation excludes the possibility that the variations in slope of the V_{Na} vs. $\Delta\psi$ curves be the result of a change in the stoichiometry of the Na^+–H^+ antiport as a function of pH_{out}.

The distinct effects of ΔpH and $\Delta\psi$ on the kinetic constants of the Na^+ efflux mechanism suggest that the two components of $\Delta\bar{\mu}_{H^+}$ act on different steps of the Na^+–H^+ exchange reaction. Thus, generation of ΔpH (interior alkaline), by reducing the internal concentration of protons that inhibit competitively the binding of Na^+ ions on internal sites of the antiporter, would increase the affinity of the antiporter for Na^+ ions to be extruded. On the other hand, $\Delta\psi$ (interior negative) would affect a step involved in the translocation of one of the two exchanged species. The latter could be H^+ if one assumes unequal transfer of H^+ and Na^+ ions during each cycle of the exchange reaction (2 H^+/1 Na^+) as previously suggested (Schuldiner & Fishkes, 1978; Beck & Rosen, 1979). Alternatively, $\Delta\psi$ might increase the number of functional Na^+–H^+ antiport units. Experimental determination of the H^+/Na^+ stoichiometry of the antiporter in *E. coli* membrane would be helpful for a distinction between these two possibilities. Finally, it is reasonable to question to what extent the results presented, obtained under conditions of downhill Na^+ transport, are relevant for the in vivo situation, where sodium transport may be uphill rather than downhill. As shown in our previous paper (Bassilana et al., 1984), the Na^+ efflux properties are not modified by the presence of external Na ions; this suggests that no major change in the kinetic scheme of the Na^+–H^+ antiport occurs when the exchange reaction pumps Na^+ ions down or against their chemical gradient.

The effect of internal pH on the Na^+–H^+ antiport activity reported here adds to previous evidence (Komor et al., 1983; Bakker & Mangerich, 1983) or suggestions (Booth, 1981) that the cytoplasmic pH participates in the control of H^+-linked or H^+-independent transport activities in prokaryote membranes. It is, nevertheless, worth stressing that the effect of internal H^+ is likely to vary according to the transport system under study. Thus, Bakker & Mangerich (1983) have shown that a high internal cytoplasmic concentration of H^+ acts on the rate of K^+ transport by the trk system of *E. coli* by reducing the maximal velocity of the transport system.

Finally, recent evidence indicates that the Na^+–H^+ antiport, in addition to its participation in the establishment plus maintenance of a transmembrane Na^+ gradient, contributes also to the homeostasis of the cytoplasmic pH (Padan et al., 1981). The regulatory role played by the internal H^+ concentration on the Na^+–H^+ antiport activity reported in this paper might be considered as part of this physiological mechanism. Indeed, reduction of the Na^+–H^+ exchange reaction in cells whose cytoplasmic pH becomes acidic would tend to decrease the net influx of protons catalyzed by the antiporter and thus to reduce its contribution to further cell acidification. It would be of interest to analyze if similar feed-back mechanisms regulate the activities of other cation-exchange systems involved in internal pH regulation such as the K^+–H^+ exchange in prokaryote systems (Brey et al., 1978, 1980).

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Photolabeling on β-Subunit of the Nucleotide Site Related to Hysteretic Inhibition of Mitochondrial F_1-ATPase†

Gilles Fellous, Catherine Godinot,* Hélène Baubichon, Attilio Di Pietro, and Danièle C. Gautheron

ABSTRACT: While F_1-ATPase can hydrolyze about any nucleoside triphosphate, it can undergo a hysteretic inhibition only in the presence of nucleotides or analogues bearing an adenine ring [Baubichon, H., Godinot, C., Di Pietro, A., & Gautheron, D. C. (1981) *Biochem. Biophys. Res. Commun. 100*, 1032–1038]. This difference in specificity has been used to identify the location of the regulatory site in F_1-ATPase. 3′-*O*-[3-[*N*-(4-Azido-2-nitrophenyl)amino]propionyl]adenosine 5′-diphosphate (NAP_3-ADP) behaves as ADP to induce the hysteretic inhibition of F_1-ATPase. The radioactive analogue also binds to F_1-ATPase with the same stoichiometry and the same concentration dependence as ADP. It is therefore an excellent photoaffinity label to localize the regulatory site. Catalytic sites being occupied by guanosine 5′-(β,γ-imidotriphosphate), the photoirradiation-induced covalent binding of NAP_3-ADP to the β-subunit of F_1-ATPase can be directly related to the hysteretic inhibition. On the contrary, there is no correlation between the inhibition of ATPase activity and the limited binding of NAP_3-ADP to the α-subunit. It is therefore concluded that the regulatory site must be located on the β-subunit of the mitochondrial F_1-ATPase.

Previous studies from our laboratory (Di Pietro et al., 1980) have shown that preincubation of pig heart mitochondrial F_1-ATPase with ADP[1] leads to binding of ADP, which induces a progressive hysteretic inhibition of MgATP hydrolysis. Since this binding was Mg dependent and since it was reversed by ammonium sulfate precipitation, this ADP was not "tightly bound" as defined by Harris et al. (1978). This binding occurs at regulatory site(s) specific to adenine nucleotides (Baubichon et al., 1981; Di Pietro et al., 1981). On the contrary, all nucleoside triphosphates can be hydrolyzed at the catalytic site(s) (Schuster et al., 1975; Pedersen, 1975; Harris et al., 1978). The aim of this study is to use this difference in specificity of catalytic and regulatory sites to localize the

† From the Laboratoire de Biologie et Technologie des Membranes du CNRS, Université Claude Bernard de Lyon, F-69622 Villeurbanne, France. *Received January 6, 1984; revised manuscript received May 9, 1984.* This work was supported by the CNRS (LP 5421, ATP 8154). This work will constitute part of the Doctorat de Spécialité in Biochemistry of G.F., who is supported by a grant from Laboratoires Hoechst, France.

[1] Abbreviations: ATPase, adenosine-5′-triphosphatase; F_1-ATPase, pig heart mitochondrial F_1-ATPase purified according to the procedure of Penin et al. (1979), omitting the last step (gel filtration in the presence of 50% glycerol); ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; GMP-P(NH)P, guanosine 5′-(β,γ-imidotriphosphate); NADH, reduced nicotinamide adenine dinucleotide; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; EDTA, ethylenediaminetetraacetic acid; NAP_3, [*N*-(4-azido-2-nitrophenyl)amino]propionyl; NAP_3-ADP or arylazido-β-alanyl-ADP, 3′-*O*-[3-[*N*-(4-azido-2-nitrophenyl)amino]-propionyl]adenosine 5′-diphosphate; TDAB, tetradecyltrimethylammonium bromide; FSBA, [*p*-(fluorosulfonyl)benzoyl]adenosine.

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