Volume 336, number 3, 525–529 FEBS 13474 December 1993

Mechanism of Na^+/H^+ exchange by *Escherichia coli* NhaA in reconstituted proteoliposomes

P.A. Dibrov*, D. Taglicht**

Division of Microbial and Molecular Ecology, The Silberman Institute of Life Sciences, The Hebrew University of Jerusalem, Givat Ram, 91904 Jerusalem, Israel

Received 12 November 1993

Purified NhaA, a Na^+/H^+ antiporter from *Escherichia coli*, reconstituted into proteoliposomes was used to study partial reactions catalyzed by this protein. Homologous Na^+/Na^+ exchange as well as Na^+/Li^+ exchange via NhaA were detected by monitoring the effects of external Li^+ and Na^+ ions on the ΔpH-driven sodium uptake into NH_4 Cl-loaded vesicles. Furthermore, a sodium counterflow reaction was demonstrated in proteoliposomes preloaded with non-radioactive Na^+ and placed into the experimental buffer containing low amounts of $^{22}Na^+$ under experimental conditions when both components of protonmotive force generated by the antiporter. $\Delta\psi$ and ΔpH, were dissipated by corresponding ionophores. The apparent K_m for sodium counterflow is 1.1 mM, and V_{max} is 80 μmol/min/mg of protein. External Na^+ accelerates the downhill efflux of $^{22}Na^+$ suggesting that the translocation of the Na^+-loaded form of the carrier is faster than the rest of the catalytic cycle.

Na^+/H^+ exchange; Na^+/H^+ antiporter; NhaA; Ion transport; pH regulation; *Escherichia coli*

1. INTRODUCTION

Exchange of Na^+ and H^+ is catalyzed in *Escherichia coli* by four different transport systems: NhaA, NhaB, ChaA and Kha [1]. Kha is a non-specific cation–proton exchanger which has been documented in membrane vesicles isolated from *E. coli* cells [2,3]. The affinity of this antiporter to K^+ is higher than to Na^+ suggesting that under most known physiological conditions, at which the intracellular K^+ concentration is higher than that of Na^+, its role in Na^+ extrusion is nil. ChaA is a Ca^{2+}/H^+ antiporter which has a low affinity for Na^+ but at high copy number can rather effectively transport Na^+. NhaB displays the highest affinity for Na^+ ions reported by any antiporter thus far, 40 μM [4]. Its stoichiometry is still unknown and its activity seems to be important for the cell under conditions at which NhaA is the least active, i.e. low pH and low concentration of Na^+ ions [5,6].

NhaA is an electrogenic antiporter, which exchanges $2H^+$ per each Na^+ translocated [7]. Its activity is regulated by a 'pH-sensor' in the molecule which senses intracellular pH so that when the pH increases from 7.0 to 8.5 the activity increases about 2000-fold [8,9]. A similar pH-sensor has been described for the non-erythroid Cl/HCO_3 exchanger in some cells [10] while one with an opposite polarity (activation by acid pH) has been described in detail for the eukariotic Na^+/H^+ antiporter (see [11] for a review). In all these cases, the activation by pH has been ascribed to the function of these proteins in extrusion of either base of acid from the cell in order to maintain a fixed intracellular pH.

NhaA has been purified to homogenity and reconstituted in a functional form so that it can now be studied independently from other antiporters [7,8]. It is important therefore to characterize the partial reactions catalyzed by this protein so that the mechanism of exchange can be understood in more detail. Previous studies in intact cells [12], in right side-out [13], and inside-out membrane vesicles [14] have suggested the existence of possible gating mechanisms, including lack of activity in the absence of $\Delta\bar{\mu}_{H^+}$. Moreover, failure to detect homologous sodium/sodium exchange has been pointed out [12,13] and, in addition, sodium had no detectable *trans* effect on the activity of the antiporter.

We now reevaluate these results in proteoliposomes reconstituted with purified NhaA, i.e. in a system where there are no other antiporters or leaks. In addition, the reconstituted system provides an easier way for manipulation of ion composition and gradients. We document and characterize for the first time the counterflow reaction catalyzed by NhaA. Efficient counterflow can be observed under conditions in which both components of $\Delta\bar{\mu}_{H^+}$ completely collapsed with the use of iono-

*Corresponding author. Fax: (972) (2) 666 804.

**Present address: Dept. Cell Biology and Anatomy, The Johns Hopkins University, School of Medicine, USA. Fax: (410) 955 4129.

Abbreviations: $\Delta\mu_{H^+}$ and $\Delta\mu_{Na^+}$, transmembrane differences in electrochemical H^+ and Na^+ potentials, respectively; $\Delta\psi$, transmembrane electric potential difference; ΔpH and ΔpNa, transmembrane H^+ and Na^+ concentration gradients; CCCP, carbonyl cyanide *m*-chlorophenylhydrazon.

phores. The kinetic properties of the counterflow reaction are presented.

2. MATERIALS AND METHODS

Methods for the overproduction and purification of NhaA, as well as preparation of NhaA containing proteoliposomes by detergent dilution were described previously [8]. Proteoliposomes were formed in buffer containing 150 mM NH_4Cl, 15 mM Tris-HCl, pH 7.5 for the ΔpH-driven sodium uptake (NH_4Cl-loaded proteoliposomes), or 100 mM potassium acetate, 10 mM NaCl, 2.5 mM $MgSO_4$, 10 mM Tris-HEPES, pH 8.6 for sodium counterflow and sodium efflux measurements (NaCl-loaded proteoliposomes).

To monitor ΔpH-driven sodium uptake, NH_4Cl-loaded proteoliposomes (4 μl) containing 200 ng of protein were diluted into 0.5 ml of medium consisting of 150 mM choline chloride, 2.5 mM $MgSO_4$, 10 mM Tris-HEPES, pH 8.6, 50 μM NaCl, and 0.5 μCi $^{22}Na^+$ (58 Ci/mmol). The reaction was stopped at different times by the rapid addition of 2.5 ml of the same ice-cold isotope-free mixture, filtered on 0.2 μ filters and washed with an additional 2.5 ml. Radioactivity on the filters was measured with a γ-counter.

Sodium counterflow reaction was initiated by dilution of NaCl-loaded proteoliposomes (4 μl) containing 114 to 200 ng of protein into 0.2 ml of medium containing 100 mM potassium acetate, 2.5 mM $MgSO_4$, 10 mM Tris-HEPES, pH 8.6, and 0.5 μCi $^{22}Na^+$. The medium contained also 0.3 mM of sodium acetate because of impurity of potassium acetate used (calculated value). To stop the reaction, samples were diluted with 2.5 ml of ice-cold acetate medium devoid of $^{22}Na^+$, filtered and washed with additional 2.5 ml. In part of the experiments, chloride was substituted for acetate in the external medium, as specified in the figure legends.

For sodium efflux experiments, NaCl-loaded proteoliposomes were equilibrated with radioactive sodium by gentle sonication in the presence of the isotope. Other conditions are given in the figure legends. *Materials:* $^{22}Na^+$ (carrier-free) was from DuPont-New England Nuclear; 0.2 μ filters were from Schleicher and Schuell. CCCP, SF6847, and nigericin were from Sigma.

3. RESULTS AND DISCUSSION

Purified NhaA reconstituted into proteoliposomes has provided an experimental system to study its activity in various modes. Thus, we have been able to show ΔpH-driven uptake of Na^+ against its electrochemical gradient [8] as well as $\Delta\bar{\mu}_{Na^+}$-driven generation of ΔpH and $\Delta\psi$ [7]. Also, the protein catalyzes downhill transport of Na^+ at maximal rates which indicates a turnover number of about 10^3–10^4 s^{-1}, one of the highest rates reported for transporters thus far [8]. We now demonstrate that NhaA also catalyzes partial reactions, i.e. Na^+/Na^+ and Na^+/Li^+ exchange.

Na^+/Na^+ and Na^+/Li^+ exchange can be demonstrated in several experimental setups. The results in Fig. 1 demonstrate an experiment in which dilution of NH_4Cl-loaded proteoliposomes generates a pH gradient across the membrane which drives Na^+ uptake against its electrochemical gradient. At this concentration of NhaA, uptake is almost linear for as long as 5 min and reaches equilibrium which is maintained for at least 75 min. These findings demonstrate the intactness of the liposomes obtained and they are consistent also with the fact that $\Delta\psi$ and ΔpH generated in these proteoliposomes are also very stable [7]. As expected, addition of higher concentrations of NhaA will bring about a proportional faster uptake (not shown, see [8]) but the equilibrium is maintained for shorter periods. This suggests that the low permeability to ions of the proteoliposomes used in Fig. 1 is due in part to the low protein concentrations used. This property allowed us to measure the effect of various agents on the levels of preaccumulated Na^+.

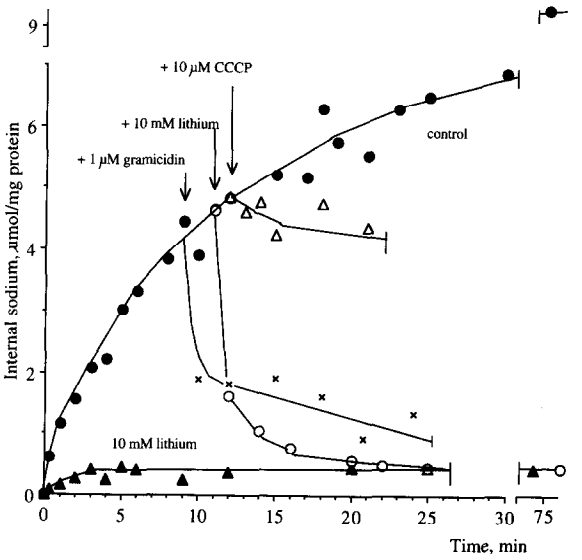

Fig. 1. ΔpH-driven sodium uptake by the NhaA proteoliposomes. NH_4Cl-loaded proteoliposomes (200 ng of NhaA) were diluted into NH_4Cl-free medium containing $^{22}Na^+$, and the isotope uptake was monitored as described in section 2. CCCP and gramicidin (final concentrations 10 and 1 μM respectively) were added to the samples as indicated. LiCl (10 mM) was added before starting the experiment (▲) or at 10 min after zero time (○).

Even though the Na^+ in the proteoliposomes can be kept in the internal milieu for long times it rapidly leaves the liposomes once treated with an ionophore such as gramicidin, which makes the membrane permeable to small monovalent cations. Addition of Li^+, a competitive inhibitor of Na^+ at the onset of the experiment inhibits Na^+ uptake almost completely. Moreover, when Li^+ is added after substantial Na^+ uptake has been achieved it induces a rapid efflux of intraliposomal Na^+, a phenomenon which indicates Na^+/Li^+ exchange. Addition of 10 mM Na^+ has an effect almost identical to that of Li^+ (data not shown).

When Na^+/Na^+ exchange is measured directly in Na^+-loaded proteoliposomes, by the addition of $^{22}Na^+$ from the outside at low concentration, no significant exchange or counterflow are detected (not shown). Under these conditions a $\Delta\bar{\mu}_{H^+}$ is generated by the antiporter acid and positive inside, which prevents any measurable antiporter catalyzed efflux [7,8]. Only when $\Delta\bar{\mu}_{H^+}$ is completely collapsed by the addition of ionophores efflux is rapid. We therefore tested the effect of ionophores on the counterflow reaction as depicted in Fig.

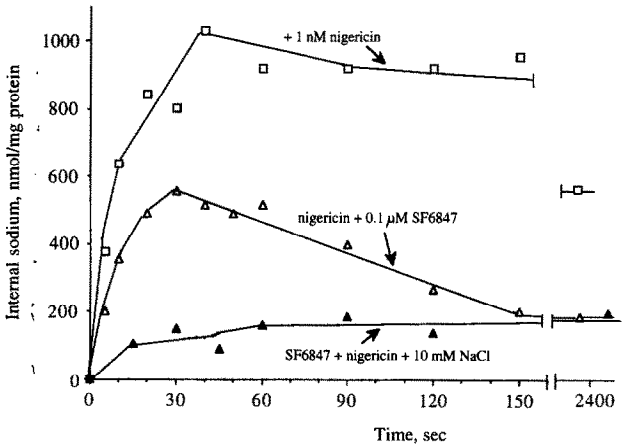

Fig. 2. Sodium counterflow in acetate medium. The process was started by the dilution of NaCl-loaded proteoliposomes (200 ng of NhaA) into acetate medium. Additions: 1 nM nigericin (□); 1 nM nigericin plus 0.1 μM SF6847 (△); SF6847 and nigericin in the presence of 10 mM NaCl (▲).

2. SF6847 at 0.1 μM electrogenically transports H^+ ions across the membrane and it collapses the $\Delta\psi$ generated by the antiporter activity [7]. Under these counterflow conditions, addition of SF6847 induces a small and transient accumulation of Na^+ which reaches equilibrium within 30 s (not shown). On the other hand, addition of nigericin, at a concentration at which it specifically exchanges K^+ for H^+ ions, leads to the collapse of ΔpH but increases $\Delta\psi$ [7]. Under these conditions, a dramatic and rapid accumulation of $^{22}Na^+$ against its electrochemical gradient occurs (Fig. 2). The accumulation of $^{22}Na^+$ into the vesicles plateaus at about 30 s and remains relatively stable for a further 120 s, then slowly returning to the equilibrium value. We have previously shown that the activity of NhaA generates under these conditions a stable membrane potential suggesting that the $^{22}Na^+$ accumulates as a result of a combination of counterflow and $\Delta\psi$-driven uptCake. In order to analyze independently each component we have tested the effect of an agent that collapses $\Delta\psi$, SF6847. When both nigericin and SF6847 are added, conditions at which $\Delta\bar{\mu}_{H^+}$ is equal to zero [7], the uptake detected is smaller and more transient, reflecting solely the actual counterflow activity of NhaA. The penetrating weak base, methylamine of 20 mM, can replace nigericin as a ΔpH-dissipating agent in the sodium counterflow (data not shown). Valinomycin may also be used to discharge $\Delta\psi$ instead of SF6847 (not shown). From the results presented in Fig. 2 we estimate that during counterflow a transient gradient of at least three-fold ($[Na^+]_{in}/[Na^+]_{out}$) is generated. A membrane potential drives a larger accumulation of about 6 to 7-fold.

The activity of the electrogenic antiporter carrying Na^+ downhill from the proteoliposome to the medium generates almost instantaneously a $\Delta\bar{\mu}_{H^+}$ composed of $\Delta\psi$ and ΔpH [7] and the acidic intraliposomal pH (pH_i) prevents NhaA from any massive uptake of $^{22}Na^+$. Collapsing the pH gradient with 1 nM nigericin allows for the establishment of a slightly larger membrane potential and, since pH_i rises, it allows for the accumulation of $^{22}Na^+$. Only upon collapse of both $\Delta\psi$ and ΔpH we detected real counterflow. Data concerning the Na^+ efflux (which drives the counterflow reaction) measured in parallel under the same conditions are in agreement with this scheme. Indeed, collapse of the membrane potential generated by the antiporter activity greatly accelerated efflux [7,8].

The experiments described thus far were performed in media containing high concentrations of acetate salts on both sides of the membrane. The protonated acetic acid rapidly permeates the membrane and is capable of equilibrating a proton gradient across the membrane. Its efficiency, however, is limited by the total flux that crosses the membrane relative to the rate of the divice generating the gradient. At pH 8.6, the pH at which these experiments were performed the concentration of the protonated acid is very low. From the results shown in Fig. 2 (see also [7]) only in the presence of 1 nM nigericin we see a complete collapse of the pH gradient, demonstrating again the high capacity of NhaA and its ability to change and regulate pH.

Acetate salts of K^+ contain contamination of Na^+ equivalent to a concentration of 0.3 mM in 100 mM solution. In order to determine the kinetic parameters of the counterflow reaction, we measured it in a medium where acetate has been replaced by chloride (Fig. 3). The results shown in Fig. 3 demonstrate counterflow in the presence of both nigericin and SF6847 at 50 μM $^{22}Na^+$ in the medium. If the vesicles are loaded only with 30 μM Na^+ inside, no counterflow is observed, as ex-

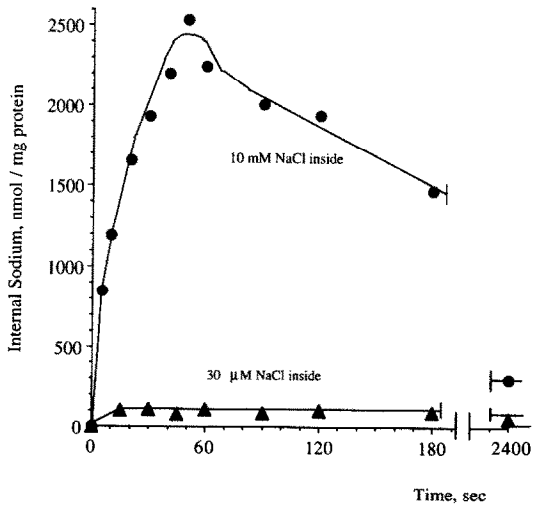

Fig. 3. Sodium counterflow in chloride medium. 4 μl of proteoliposomes (114 ng of protein) preloaded with 10 mM NaCl and treated with 1 nM nigericin and 0.1 μM SF6847 were diluted into 0.2 ml of chloride medium containing 0.2 mM Na^+ (●). For control experiment, the proteoliposomes were prepared 'without' NaCl (30 μM; ▲).

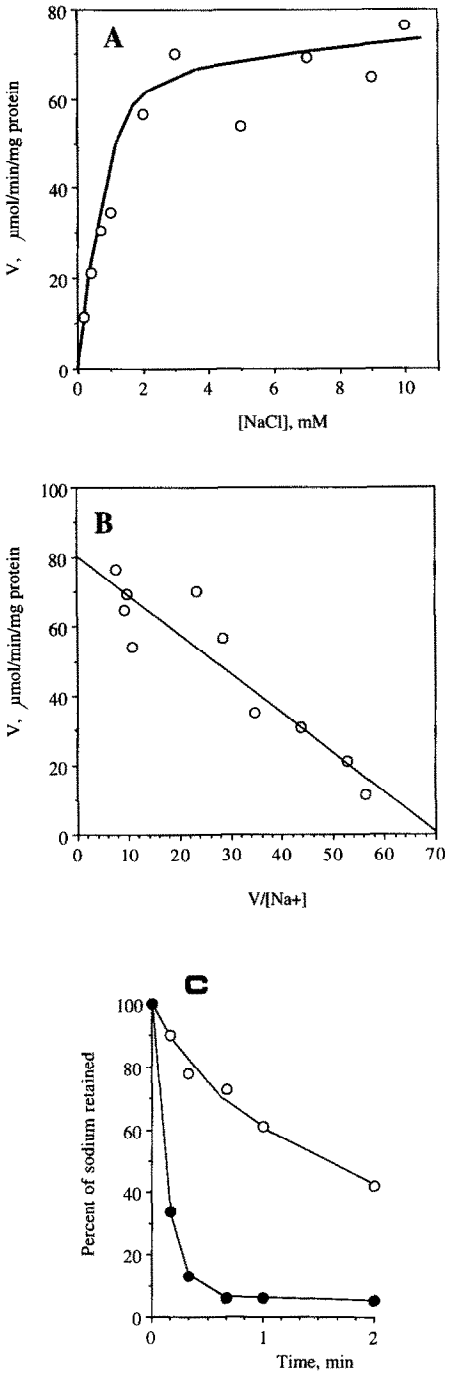

Fig. 4. Determination of the kinetic parameters of sodium counterflow. (A) The initial rate of sodium accumulation into NaCl-loaded proteoliposomes treated with a combination of nigericin and SF6847 for 10 s was determined at different NaCl concentrations (0.2 to 10 mM). Other experimental conditions were as in Fig. 3. (B) Linear regression of an Eadie-Hoffstee plot. (C) *Trans*-acceleration of sodium efflux. Sodium efflux from proteoliposomes (114 ng of NhaA) loaded with 10 mM NaCl and equilibrated with $^{22}Na^+$ (30 μCi/ml) was initiated by dilution of 4 μl of vesicles into 0.4 ml of the isotope-free chloride medium, pH 8.6 (○) or into 0.4 ml of the same medium supplemented with 10 mM NaCl (●). Nigericin and SF6847 were added to the vesicles as in Fig. 2.

pected. Upon dilution of liposomes loaded with 10 mM Na^+ a rapid accumulation is observed which peaks at around 50 s and slowly decreases to almost equilibrium values after 40 min. These experimental conditions were then used for determination of the kinetic parameters of the sodium counterflow reaction (Fig. 4). The initial rate of Na^+ uptake in the presence of SF6847 and nigericin was titrated by external NaCl in the concentration range of 0.2 to 10 mM (Fig. 4). An Eadie–Hoffstee plot gave the following values: apparent K_m for sodium 1.1 mM, and V_{max} 80 μmol/min/mg of protein (Fig. 4B). This apparent K_m is about tenfold higher than the app. K_m for ΔpH-driven $^{22}Na^+$ uptake [8]. The V_{max} of the counterflow reaction is also more than 20-fold higher than the respective V_{max} for the ΔpH-driven uptake. On the other hand, the V_{max} for downhill $^{22}Na^+$ efflux at this pH is about 20-fold higher.

NhaA displays a complex behaviour and its activity is tightly regulated by pH. The difference in the V_{max} at the various catalytic modes may reflect different rate-limiting steps. An attempt to identify such a step is shown in Fig. 4C. When the downhill efflux of $^{22}Na^+$ is measured in the presence of nigericin and SF6847, addition of Na^+ (10 mM) to the reaction medium accelerates it severalfold: $t_{1/2}$ is less than 10 s in the presence of $^{22}Na^+$ as compared to about 120 s in the absence. These findings suggest that the Na^+-loaded form of the transporter operates faster than the protonated form. In the latter statement we include of course not only the step responsible for the movement of the two H^+ ions but also their binding and release during the catalytic cycle. We cannot thus far dissociate between the various steps mainly due to the steep pH dependence of NhaA activity [8].

The data reported here represent the first demonstration of partial reactions catalyzed by NhaA and provide tools for a more detailed analysis which is required for understanding how NhaA performs its functions i.e. acidification of the cytoplasm to the homeostatic value [7–9,15].

Acknowledgements: We thank Prof. E. Padan, Prof. S. Schuldiner and Prof. V.P. Skulachev for fruitful discussions and critical reading of the manuscript. This work was supported by grants from the Israel-USA Binational Science Foundation (to E.P. and S.S.) and from the Israel Ministry of Science and Technology (to P.D. and S.S.)

REFERENCES

[1] Schuldiner, S. and Padan, E. (1993) Int. Rev. Cytol. 137C, 229–266.
[2] Brey, R., Beck, J. and Rosen, B. (1978) Biochem. Biophys. Res. Commun. 83, 1588–1594.
[3] Brey, R., Rosen, B. and Sorensen, E. (1980) J. Biol. Chem. 255, 39–44.
[4] Pinner, E., Padan, E. and Schuldiner, S. (1992) J. Biol. Chem. 267, 11064–11068.
[5] Karpel, R., Alon, T., Glaser, G., Schuldiner, S. and Padan, E. (1991) J. Biol. Chem. 266, 21753–21759.
[6] Pinner, E., Kotler, Y., Padan, E. and Schuldiner, S. (1993) J. Biol. Chem. 268, 1729–1734.

[7] Taglicht, D., Padan, E. and Schuldiner, S. (1993) J. Biol. Chem. 268, 5382–5387.
[8] Taglicht, D., Padan, E. and Schuldiner, S. (1991) J. Biol. Chem. 266, 11289–11294.
[9] Gerchman, Y., Olami, Y., Rimon, A., Taglicht, D., Schuldiner, S. and Padan, E. (1993) Proc. Natl. Acad. Sci. USA 90, 1212–1216.
[10] Jennnings, M. (1992) in: The Kidney: Physiology and Pathophysiology, 2nd Edn. (Seldin, D. and Giebish, G., Eds.) pp. 503–535, Raven Press, New York.
[11] Aronson, P. (1985) Annu. Rev. Physiol. 47, 545–560.
[12] Borbolla, M. and Rosen, B. (1984) Arch. Biochem. Biophys. 229, 98–103.
[13] Bassilana, M., Damiano, E. and Leblanc, G. (1984) Biochemistry, 23, 5288–5294.
[14] Nakamura, T., Hsu, Ch. and Rosen, B. (1986) J. Biol. Chem. 261, 678–683.
[15] Schuldiner, S. and Padan, E. (1993) in: Alkali Cation Transport Systems in Procaryotes (E.P. Bakker, Ed.) pp. 25–51, CRC Press, Boca Raton.