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A Stable Na^+/H^+ Antiporter of Thermophilic Bacterium PS3

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Abstract

As a first step in the isolation of a stable Na^+/H^+ antiporter, its reaction in sonicated membrane vesicles of thermophilic bacterium PS3 has been characterized. The sonicated vesicles showed quenching of quinacrine fluorescence in either NADH oxidation or ATP hydrolysis. The quenching was reversed by the addition of Na^+, Li^+, Mn^{2+}, Cd^{2+}, and Co^{2+}, but not of choline$^+$ or Ca^{2+}, regardless of their counter anions. $^{22}Na^+$ was taken up into the vesicles by NADH oxidation, and the $^{22}Na^+$ uptake was inhibited by the addition of an uncoupler. H^+ release was observed on addition of Na^+ to sonicated vesicles. The magnitude of the pH difference across the membrane induced by NADH oxidation was constant at pH 7.0 to 9.1, but the Na^+/H^+ antiport was affected by the pH of the medium (optimum pH = 8.5). The K_m's of the antiporter for Na^+ and Li^+ were 2.5 and 0.1 mM, respectively, but the V_{max} values for the two ions were the same at pH 8.0. In the presence of Li^+, no further decrease of fluorescence quenching was observed on addition of Na^+ and *vice versa*. The Na^+/H^+ antiporter activity in PS3 was stable at 70°C, and the optimum temperature for activity was 55–60°C. In contrast to mesophilic cation/H^+ antiporters, this antiporter was not inhibited by a thiol reagent.

Introduction

There are many symport systems of Na^+ and nutrients in cells [1–3]. To drive these systems, Na^+ is extruded by $Na^+ \cdot K^+$ –ATPase in eukaryotic cells

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[4], mainly via an Na^+/H^+ antiporter, which is driven by an H^+ gradient maintained by either H^+-ATPase or the respiratory chain, in prokaryotic cells [5]. The presence of an Na^+/H^+ antiporter was deduced from the observation that addition of Na^+ to *Escherichia coli* caused a decrease of the extracellular pH [6]. This Na^+/H^+ antiport reaction has been characterized in cells and membrane vesicles of various bacteria [5–11].

However, the molecular mechanism of the Na^+/H^+ antiport reaction is unknown, mainly because of the complexity of the membrane system. To study its mechanism, the Na^+/H^+ antiporter must be isolated and reconstituted into liposomes capable of this reaction. In this work we chose a thermophilic bacterium PS3 as starting material for purification of the Na^+/H^+ antiporter, since thermophilic proteins are known to be stable against various dissociation agents and detergents [12, 13]: H^+ −ATPase [12, 13], alanine carrier [14–16], cytochrome *c* [17], and cytochrome oxidase [18] purified from this thermophile were shown to be much more stable than their counterparts from mesophiles. Recently, one of the authors demonstrated that not only H^+ but also Na^+ could drive alanine transport [16]. Thus, it is of particular interest to characterize the Na^+/H^+ antiporter of this bacterium.

Materials and Methods

Preparation of Membrane Vesicles

The thermophilic bacterium PS3, isolated from Mine-hot spring, was grown as described in the previous report [19]. The membrane fraction prepared as described in that report [19] was washed twice with 10 mM Tricine-KOH (pH 8.0), suspended in the same buffer, and stored at −80°C. This preparation is called membrane vesicles. The frozen membrane vesicles were thawed at room temperature and centrifuged at 12,000 × *g* for 10 min at 4°C. The resulting precipitate was suspended in 10 mM Tricine-KOH (pH 6.5) and sonicated for 1 min using the microtip of a Branson sonifier Model 200 (output = 20 W) in an ice bath. This preparation is called sonicated membrane vesicles. ATPase activity in sonicated membranes was fully accessible from outside of the vesicles (data not shown), indicating that these membrane vesicles are everted. For pH measurements, membrane veicles were washed twice with 1 mM MOPS-TMAHO (pH 8.0) and the resulting precipitate was suspended in the same buffer. The membrane suspension was then sonicated five times for 1-min periods in the same sonicator.

Measurement of Quinacrine Fluorescence

Quinacrine fluorescence was measured in a Hitachi fluorometer, Model 204, using an excitation wavelength of 425 nm and an emission wavelength of

500 nm at 40°C. The sonicated membrane vesicles (10–20 μl containing about 200–400 μg of protein) were placed in a cuvette containing 2.0 ml of a solution composed of 9 mM Tricine-KOH (pH 8.0), 0.9 mM $MgSO_4$, 0.2M KCl, and 1 μM quinacrine-HCl and preincubated at 40°C for 5 min. Then, K_2-NADH or K_2-ATP was added at the concentrations indicated in the legends to figures.

$^{22}Na^+$ Uptake

The reaction mixture (1 ml) containing 9 mM Tricine-KOH (pH 8.0), 0.9 mM $MgSO_4$, 0.2 KCl, 10 mM ^{22}NaCl (1 μCi/ml), and 2.5 mM K_2-NADH was preincubated at 40°C. The reaction was started by adding 50 μl of sonicated membrane vesicles (20 mg/ml) which had been warmed to 40°C. At intervals, 0.2-ml samples were withdrawn and filtered through a membrane filter (Sartorius, SM 11306). The filter was washed three times with 3 ml of 0.5 M NaCl and dried, and its radioactivity was measured in a gas-flow counter (Aloka LBC-451).

Measurement of Release of H^+ on Addition of Na^+

A mixture of 1.0 ml of sonicated membrane vesicles, 1.7 ml of 1 mM MOPS-TMAHO (pH 8.0), and 0.3 ml of 2 M KCl in the cell for pH measurement was warmed to 40°C and adjusted to pH 8.0 with KOH. The H^+ concentration was measured using a Beckman 39505 combination electrode connected to a Beckman Expandomatic SS-2 pH meter.

Protein-Concentration Determination

The method of Lowry et al. [20] was used, with bovine serum albumin as a standard.

Reagents

K_2-NADH and K_2-ATP were prepared from the sodium salts of these nucleotieds (Kyowa Hakko, Tokyo) using Dowex-50W-X4 cation-exchange resin equilibrated with 0.1 M potassium phosphate (pH 8.0). Quinacrine-HCl was purchased from Nakarai Chemicals Ltd. (Kyoto), valinomycin from P-L Biochemicals (U.S.A.), FCCP from Boehringer (Germany), and ^{22}NaCl (100 μCi/ml, carrier free) from New England Nuclear (U.S.A.). Monensin was kindly donated by Lilly Laboratories (U.S.A.). Other reagents were obtained as reported in the previous papers [15–19].

Results

Effects of Cations on the ΔpH of the Sonicated Membrane Vesicles

In assay of the Na^+/H^+ antiport, the fluorescent probe quinacrine [21] was used to monitor the pH difference across the membrane (ΔpH). Owing to accumulation of H^+ in the sonicated membrane vesicles, driven either by NADH oxidation (Fig. 1) or ATP-hydrolysis (Fig. 2), the fluorescence of quinacrine was quenched by the concentration of quinacrine in the vesicles (self-quenching) [21]. The extent of quenching reached a plateau within 1 min (Fig. 1) or 10 min (Fig. 2), and the level of quenching was maintained until the substrates had been dissipated. In both cases, the quenching was completely blocked by adding uncoupler, FCCP, to remove the ΔpH (data not shown).

As shown in Figs. 1 and 2, the addition of NaCl or LiCl to this assay system caused a rapid increase in the fluorescence, suggesting dissipation of the ΔpH as a result of the antiport reaction. Other monovalent cations, such

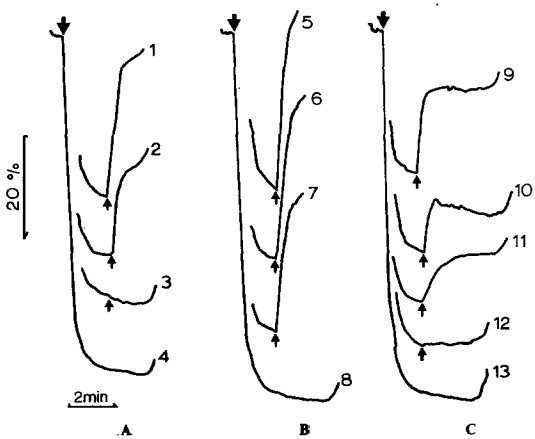

Fig. 1. Effects of various cations (A and C) and anions (B) on the NADH-driven ΔpH determined by measuring quenching of quinacrine fluorescence. The vesicles (324 μg protein) were suspended in 2.0 ml of a solution of 9 mM Tricine-KOH (pH 8.0), 0.9 mM $MgSO_4$, 0.2 M KCl, and 1 μM quinacrine. After incubation for 2 min at 40°C, 0.25 mM K_2-NADH was added (large arrow). At the indicated time (small arrow), 10 mM NaCl (curve 1), 10 mM LiCl (curve 2), 10 mM choline Cl (curve 3), 10 mM $NaNO_3$ (curve 5), 5 mM Na_2HPO_4 (curve 6), 5 mM Na_2SO_4 (curve 7), 5 mM $MnCl_2$ (curve 9), 5 mM $CdCl_2$ (curve 10), 5 mM $CoCl_2$ (curve 11), or 5 mM $CaCl_2$ (curve 12) was added. Curves 4, 8, and 13 are controls.

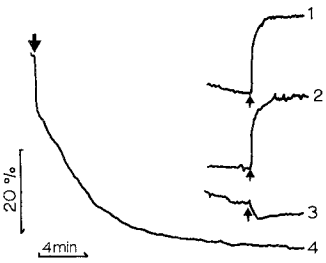

Fig. 2. Effects of various cations on the ATP-driven ΔpH determined by measuring quenching of quinacrine fluorescence. The vesicles (416 μg protein) were suspended in the same solution as for Fig. 1. After incubation for 2 min at 40°C, 1 mM K_2-ATP was added (large arrow). At the indicated time (small arrow), 20 mM NaCl (curve 1), 15 mM LiCl (curve 2), or 20 mM choline Cl (curve 3) was added. Curve 4 is the control.

as choline$^+$, did not have this effect (Figs. 1 and 2). The effects of cations was not affected by the species of anion used (Fig. 1B).

Divalent cations such as Mn^{2+}, Co^{2+}, and Cd^{2+} also reversed the quenched fluorescence, but Ca^{2+} did not affect the fluorescence (Fig. 1C). Under our assay conditions, no K^+/H^+ antiport was detectable.

Uptake of $^{22}Na^+$ Dependent on NADH Oxidation

The experiments described above indicated more or less indirectly that Na^+ ions were translocated across the membrane. Thus it was necessary to show that the uptake of $^{22}Na^+$ depended on ΔpH formation. As seen in Fig. 3, oxidation of NADH by the sonicated membrane vesicles caused uptake of $^{22}Na^+$ (Fig. 3A, curves 1 and 2), and addition of FCCP completely blocked this uptake (Fig. 3B).

Release of H^+ from the Vesicles on Addition of Na^+

In reconstitution experiments with Na^+/H^+ antiporter, it is essential to measure the extrusion on H^+ in response to influx on Na^+, because the

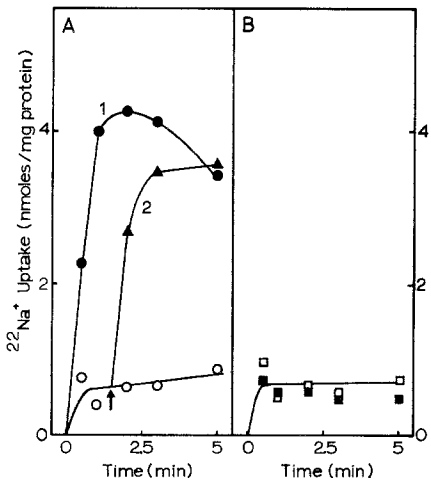

Fig. 3. Uptake of $^{22}Na^+$ into the sonicated membrane vesicles by oxidation of NADH. (A) Reactions were initiated by adding vesicles (1.04 mg protein), which had been preincubated for 5 min at 40°C in a 1.0 ml of a solution of 9 mM Tricine-KOH (pH 8.0), 0.9 mM $MgSO_4$, 10 mM NaCl, and 1 μCi $^{22}NaCl$ with (●) or without (○) 2.5 mM K_2-NADH. At the indicated times, 0.2 ml samples were filtered and washed as described in Materials and Methods. ▲: reaction started without K_2-NADH, and 1 mM K_2-NADH added at the indicated time. The incubation was carried out at 40°C. (B) Membrane vesicles were preincubated with 14 μM FCCP (final concentration) for 5 min at 40°C. Other conditions were as for (A). ■: with K_2-NADH. □: without K_2-NADH.

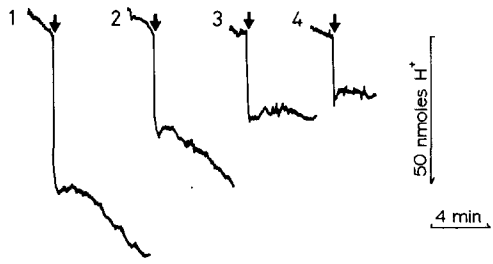

Fig. 4. H^+ release from the membranes by addition of NaCl. Assays were carried out as described in Materials and Methods. The protein concentration of the membranes in the assay mixtures was 3.6 mg. At the indicated time 10 mM NaCl was added. Curve 1: Sonicated membranes were used. Curve 2: The assay mixture contained 39.3 μM FCCP and 0.9 μM valinomycin (both added as solutions in methanol). Curve 3: Boiled membranes (100°C, 5 min) were used instead of sonicated membranes. Curve 4: Membranes were not added.

reconstituted Na^+/H^+ antiporting liposomes do not contain either the NADH oxidizing system or $H^+-ATPase$, which both pump in H^+ from the outside. As shown in Fig. 4, the extrusion of H^+ was observed in sonicated membrane vesicles (curve 1) in response to the addition of Na^+, in the absence of NADH oxidation and ATP hydrolysis. This H^+ extrusion was inhibited by the addition of valinomycin and FCCP (curve 2). Curve 4 shows the response of the pH electrode to Na^+ at this pH. The response of the pH electrode observed with boiled membranes (curve 3) was the same as curve 4. These results indicate the presence of an Na^+/H^+ antiporter in membranes of PS3.

Effects of Na^+ and Li^+ on ΔpH Dissipation

For the results shown in Fig. 5, membrane vesicles were incubated in various media. Curve 1 is that obtained in the control experiment in KCl medium. Curves 2 and 3 are in NaCl and LiCl media, respectively.The extent

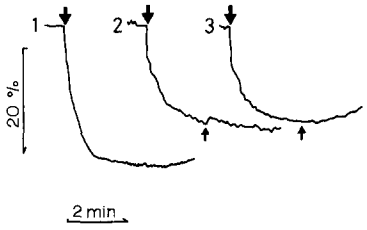

Fig. 5. Effect of Li^+ on the Na^+/H^+ antiporter. Membrane vesicles (336 μg protein) and 1.25 mM K_2-NADH were used. Other procedures were as for Fig. 1. Curve 1: Control. Curve 2: The solution contained 0.2 M LiCl instead of 0.2 M KCl before the addition of K_2-NADH, and 5 mM NaCl was added at the indicated time (small arrow). Curve 3: The solution contained 0.2 M NaCl instead of 0.2 M KCl before the addition of K_2-NADH, and 10 mM LiCl was added (small arrow).

of fluorescence quenching caused by NADH oxidation was much less when membrane vesicles were incubated in either NaCl or LiCl medium than when they were incubated in KCl medium. Furthermore, no reversal of fluorescence quenching by Na^+ or Li^+ occurred in LiCl or NaCl medium. These results indicate that Na^+ and Li^+ ions are both substrates for the Na^+/H^+ antiporter.

Characterization of the Na^+/H^+ Antiporter

Figure 6 shows the pH dependence of the Na^+/H^+ antiport activity. The extent of quenching of quinacrine fluorescence caused by NADH oxidation was almost constant between pH 7.0 and 9.1, although the initial rate of fluorescence reversal was greatly affected by the pH of the medium. The activity was almost zero at pH 7.0 and increased with increase in the pH of the medium, reaching an optimum at pH 8.5. $^{22}Na^+$ uptake driven by NADH oxidation was also optimal at this pH (data not shown).

Kinetic studies on the Na^+/H^+ antiport activity were carried out at different pHs (Fig. 7A). The apparent K_m values for Na^+ were 1.4 and 2.5 mM at pH 8.5 and 8.0, respectively. On the other hand, the apparent V_{max} values, expressed in arbitrary units, were the same at these pHs. Although these values were obtained in indirect measurements using a fluorescence probe, it is interesting that the K_m for Na^+ was in a similar range to that of

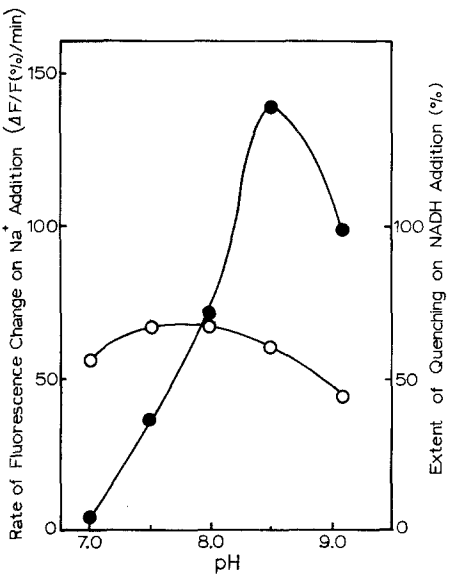

Fig. 6. Effect of pH on Na^+/H^+ antiport activity. Membrane vesicles (324 μg protein), 0.25 mM K_2-NADH, and 5 mM NaCl were used. Other procedures were as for Fig. 1. The initial rate, as the percentage change in fluorescence/min (●) was calculated from the rapid initial increase in fluorescence following addition of NaCl. The percentage quenching of fluorescence on addition of NADH (○) was calculated from the difference between the fluorescence intensities before and after addition of NADH.

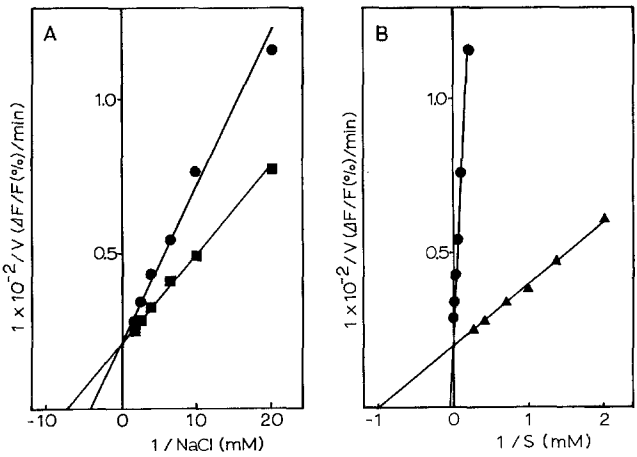

Fig. 7. Lineweaver–Burk plots of the concentration dependence of the effects of Na^+ and Li^+, and of Na^+ in media of different pHs for dissipation of ΔpH. Membrane vesicles (208 μg protein) and 0.5 mM K_2-NADH were used. Other procedures were as for Fig. 1. The initial rate of percentage change in fluorescence/min was calculated as described in the legend of Fig. 6. (A) NaCl was used as the cation for dissipation of ΔpH. Media of pH 8.0 (●) and pH 8.5 (■) were used. (B) NaCl (●) and LiCl (▲) were used as cations in medium of pH 8.0.

the *E. coli* Na^+/H^+ antiporter reported by Beck and Rosen [9]. These workers also found that Li^+ could be a substrate for *E. coli* Na^+/H^+ antiporter and that the K_m values for Na^+ and Li^+ were the same. In our system, as shown in Fig. 7B, the K_m value for Li^+ was much less (0.1 mM) than that for Na^+.

The Na^+/H^+ antiport activity of *E. coli* was inhibited by the membrane-permeable anions SCN^- [9]and NO_3^- (B. P. Rosen, personal comunication), suggesting electrogenic H^+ movement. We studied the effect of NO_3^- on the Na^+/H^+ antiport activity in sonicated membranes from PS3. In these experiments the assay medium contained K_2SO_4 instead of KCl. There was no effect of NO_3^- on the Na^+/H^+ antiport activity, suggesting that the Na^+/H^+ antiporter of PS3 transported Na^+ and H^+ by an electroneutral one-for-one exchange.

Stability of the Na^+/H^+ Antiporter at High Temperature

The temperature dependence of the Na^+/H^+ antiport activity is illustrated in Fig. 8. Although the rate of ΔpH formation induced by NADH oxidation was dependent on the temperature, the extent of fluorescence quenching was almost constant between 15 and 55°C (data not shown). The rate of dissipation of ΔpH induced by added NaCl was very low below 20°C,

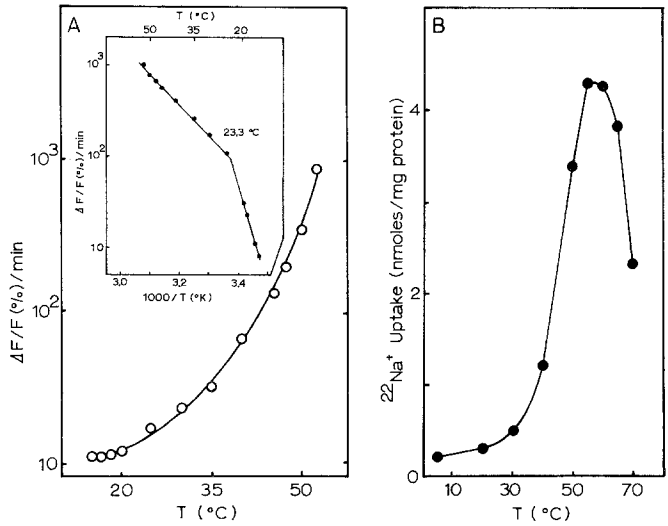

Fig. 8. Temperature dependence of Na^+/H^+ antiport activity. (A) Increase of quenching of quinacrin fluorescence dependent on NADH oxidation on addition of NaCl. Membrane vesicles (256 μg protein) were suspended in the solution described in the legend of Fig. 1 and preincubated at the indicated temperature. The mixture was incubated at the same temperature for 2 min, and then 0.75 mM K_2-NADH was added. When the quenching of fluorescence reached a maximum, 20 mM NaCl was added and the initial rate of increase of fluorescence was measured. Incubation was carried out at the indicated temperature. Na^+/H^+ antiporter activity was calculated as described in the legend of Fig. 6. The inset shows the Arrhenius plot of the reaction. (B) $^{22}Na^+$ uptake on addition of NADH. Membrane vesicles (400 μg protein) were added to 0.2 ml of a solution of 9 mM Tricine-KOH (pH 8.0), 0.9 mM $MgSO_4$, 5 mM NaCl, and 0.2 μCi $^{22}NaCl$ at the indicated temperature. The mixtures were incubated for 2 min, and then reactions were started by adding 10 μl of 50 mM NADH. After incubation for 30 sec at the same temperature, the reactions were stopped and the mixtures were assayed as described in Materials and Methods.

but increased exponentially on increase in the temperature to 55°C (Fig. 8A). The inset in Fig. 8A is an Arrhenius plot, indicating that phase transition occurred at 23.3°C. This transition temperature was close to that of respiration-dependent alanine uptake in membrane vesicles of PS3 (20°C) [22] and also to that of H^+ conductance activity by TF_0 reconstituted with PS3 phospholipids (24°C) [26].

Figure 8B also shows the temperature dependence of $^{22}Na^+$ uptake dependent on NADH oxidation. This uptake was stable at 70°C, and the optimum temperature range was 55–60°C. These values were much higher than those of alanine uptake dependent on ascorbate-phenazine methosulfate oxidation in PS3 membrane vesicles [22].

This antiport activity was not lost in 0.5 M urea solution at 40°C.

Various transport systems in mesophilic membranes are known to be inhibited by thiol reagents such as *p*-chloromercuribenzoic acid and *N*-ethylmaleimide [23, 24], and the Ca^{2+}/H^+ antiport in *E. coli* and Na^+/H^+ antiport in *Azotobacter vinelandii* were also inhibited by these reagents [11, 25]. But the Na^+/H^+ antiport activity in PS3 was not affected by incubation with 2 mM *N*-ethylmaleimide for 10 min at 40°C, like the alanine transport in PS3 membrane vesicles (unpublished data).

Discussion

In this work we demonstrated an Na^+/H^+ antiporter in sonicated membrane vesicles of thermophilic bacterium PS3. The inside acidic ΔpH formed by either hydrolysis of ATP or oxidation of NADH was dissipated when Na^+ ions were added (Figs. 1 and 2). Uptake of $^{22}Na^+$ was dependent on oxidation of NADH (Fig. 3), and H^+ extrusion in exchange for Na^+ was observed using a pH electrode (Fig. 4).

The role of the Na^+/H^+ antiporter has been postulated to be to convert $\Delta\bar{\mu}_{H^+}$ to $\Delta\bar{\mu}_{Na^+}$ [6], and the $\Delta\bar{\mu}_{Na^+}$ is utilized for Na^+/nutrient symport systems in microorganisms [5]. In the thermophilic bacterium PS3, the alanine carrier is driven by both $\Delta\bar{\mu}_{H^+}$ and $\Delta\bar{\mu}_{Na^+}$ [16]. K^+/H^+ antiport activity has been postulated to be responsible for regulation of intracellular pH in *E. coli* [27]. However, in the Gram-positive organism *Bacillus alcalophilus,* Na^+/H^+ exchange may carry out this function [28]. Since PS3 is Gram-positive, the Na^+/H^+ antiporter of this organism may play a similar role. The Na^+ concentration in the water of the hot springs from which this bacterium was obtained is 31 mM (Cl^- = 29.8 mM), which is about 3×10^5 times higher than the H^+ concentration (pH 7.1) [29].

The kinetics of the Na^+/H^+ antiport activity of PS3 membranes differed from that of the activity in *E. coli* membranes: with the latter, the K_m values for Na^+ and Li^+ were equal [9], but the K_m for Li^+ was about one-twentieth of that for Na^+ with the Na^+/H^+ antiport of PS3. On the other hand, in PS3 the V_{max} for Na^+ and Li^+ were the same. These results show that the transmembrane movements of Na^+ and Li^+ were similar, and may depend on the extent of the ΔpH in PS3. The K_m of the Na^+/H^+ antiporter for Na^+ was dependent on the pH of the medium, whereas the V_{max} was not (Fig. 7A). Thus the Na^+/H^+ antiporter in PS3 may be regulated by the affinity for Na^+ ions rather than H^+ ions.

The Na^+/H^+ antiport activity was temperature dependent with a definite transition temperature of 23.3°C (Fig. 8A), suggesting that the Na^+/H^+ antiporter is influenced by the state of its environmental phospholipids, and may not be a simple channel-like molecule.

There are other cation/H^+ antiporters besides the Na^+/H^+ antiporter in microorganisms; the Ca^{2+}/H^+ antiporter [8, 11, 30] and K^+/H^+ antiporter [27] are well known. Moreover PS3 may have cation/H^+ antiporter(s) for CO^{2+}, Cd^{2+}, and Mn^{2+} in addition to the Na^+/H^+ antiporter (Fig. 1).

The Na^+/H^+ antiporter has been characterized using membrane vesicles, but the molecular mechanism of exchange of Na^+ and H^+ has not been studied. To understand this mechanism, it is necessary to isolate the Na^+/H^+ antiporter from membranes, and reconstitute it into functional vesicles. For this purpose, we used a thermophilic bacterium because its proteins, such as H^+–ATPase [12, 13], alanine carrier [14–16], and cytochrome oxidase [18] are stable against high temperature and high concentrations of detergents and other protein-dissociating reagents [13]. The Na^+/H^+ antiporter activity in PS3 was also stable at high temperature (Fig. 8B). The great stability of these proteins depends on their high content of salt bridges and also on their very low content of thiol residues [19]. Since its activity is insensitive to thiol reagent, the Na^+/H^+ antiporter in PS3 probably does not contain thiol residues. Considering the homology of the amino acid sequence of an H^+ channel protein of PS3 with those of mesophilic species [31], the results obtained with the Na^+/H^+ antiporter of PS3 may be essentially applicable to other Na^+/H^+ antiporters. The solubilization and purification of this stable Na^+/H^+ antiporter of PS3 are now in progress.

Acknowledgments

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Because the question of the electrogenicity of cation/H^+ antiport systems is controversial, we have performed similar experiments using membrane vesicles from PS3, *E. coli,* and a mutant of *E. coli* [32] lacking the K^+/H^+ antiporter together with Dr. B. P. Rosen of the University of Maryland during his recent visit to our laboratory.

Abbreviations

Tricine, *N*-tris(hydroxymethyl)methylglycine; MOPS, morpholinopropane sulfonic acid; TMAHO, tetramethylammonium hydroxide; DCCD, *N,N'*-dicyclohexylcarbodiimide; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; H^+–ATPase, proton-translocating adenosine triphospha-

tase; $\Delta\bar{\mu}_{H^+}$, electrochemical proton gradient across membrane; $\Delta\bar{\mu}_{Na^+}$, electrochemical Na^+ gradient across membrane; ΔpH, pH difference across membrane.

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