Journal of Bioenergetics and Biomembranes, Vol. 21, No. 6, 1989

MINI-REVIEW

Respiratory Na^+ Pump and Na^+-Dependent Energetics in *Vibrio alginolyticus*

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Received March 30, 1989

Abstract

The marine bacterium *Vibrio alginolyticus* was found to possess the respiratory Na^+ pump that generates an electrochemical potential of Na^+, which plays a central role in bioenergetics of *V. alginolyticus*, as a direct result of respiration. Mutants defective in the Na^+ pump revealed that one of the two kinds of NADH: quinone oxidoreductase requires Na^+ for activity and functions as the Na^+ pump. The Na^+ pump composed of three subunits was purified and reconstituted into liposomes. Generation of membrane potential by the reconstituted proteoliposomes required Na^+. The respiratory Na^+ pump coupled to the NADH: quinone oxidoreductase was found in wide varieties of Gram-negative marine bacteria belonging to the genera *Alcaligenes*, *Alteromonas*, and *Vibrio*, and showed a striking similarity in the mode of electron transfer and enzymic properties. Na^+ extrusion seemed to be coupled to a dismutation reaction, which leads to the formation of quinol and quinone from semiquinone radical.

Key Words: Na^+ pump; NADH: quinone oxidoreductase; marine bacteria; Na^+-circulation, respiration.

Introduction

Conservation and transduction of energy is one of the essential functions that all living organisms must retain. The chemiosmotic theory formulated by Mitchell (1961, 1973) is now widely accepted to explain the principal mode of energy coupling in membranes. According to the theory, electron transfer in the respiratory chain or photoredox chain causes the extrusion of H^+,

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0145-479X/89/1200-0693$06.00/0

leading to the generation of an electrochemical potential difference of H^+ (protonmotive force) across the membranes. It is now firmly established that the protonmotive force can drive energy-dependent reactions such as active transport of solute, ATP synthesis, or flagella motility in bacteria. These reactions are coupled to the back flow of H^+ through the respective machinery. Therefore, the circulation of H^+ plays a central role in these energy-transducing reactions. Although it has been known that some solute transport systems are dependent on Na^+ and driven by the electrochemical potential of Na^+ (sodium motive force) instead of H^+ (Tokuda and Kaback, 1977; Tsuchiya *et al.*, 1977), these systems have been thought to be rather exceptional in nonhalophilic bacteria. The generation of the sodium motive force in *Escherichia coli*, for example, is performed only by the proton motive force-driven Na^+/H^+ antiport system (Schuldiner and Fishkes, 1978). Therefore, the circulation of H^+ is still essential for Na^+-dependent transport in *E. coli* and many other nonhalophilic bacteria. In contrast, various solute transport systems in animal cells are generally driven by the electrochemical potential of Na^+ that is generated directly by a primary Na^+ pump such as Na^+/K^+ ATPase and are independent of H^+ circulation. Before the discovery of primary Na^+ pumps in bacteria, it had been thought that energetics in bacteria and animal cells differ significantly in the mode of energy coupling. The respiratory Na^+ pumps (Tokuda and Unemoto, 1981, 1982) and decarboxylation-dependent Na^+ pump (Dimroth, 1980, 1987) found in the marine bacterium *Vibrio alginolyticus* and *Klebsiella pneumoniae*, respectively, provoked fundamental questions about the energetics in bacteria. Moreover, it was revealed that the Na^+ pumps and Na^+-dependent energetics are not limited to these bacteria but rather general in salt-loving bacteria. In this short review, the energetics of the respiratory Na^+ pump and its physiological significance are discussed.

Solute Transport in *V. alginolyticus*

In contrast to nonhalophilic bacteria, most of the solute transport systems in halophilic bacteria are coupled to the sodium motive force. Lanyi (1979) has demonstrated that all amino acid transport systems in *Halobacterium halobium*, which grows optimally in the presence of saturating concentration of NaCl, are driven by the sodium motive force. In marine bacteria, the electrochemical potential of Na^+ is also a driving force for most, if not all, amino acid and sugar transport systems (Sprott *et al.*, 1975; Tokuda *et al.*, 1982; Kakinuma and Unemoto, 1985). The internal K^+ of *V. alginolyticus* can be easily replaced by Na^+ or other cations (Tokuda *et al.*, 1981; Nakamura *et al.*, 1982). The roles of K^+ and Na^+ in the active

transport of α-aminoisobutyric acid (AIB), a nonmetabolizable amino acid analogue, were examined in detail using K^+-depleted Na^+-loaded cells (Tokuda *et al.*, 1982). The cells of *V. alginolyticus* growing on a synthetic medium containing 0.3 M NaCl and 14 mM K^+ retained about 0.4 M K^+ and 80 mM Na^+. On the other hand, the K^+-depleted Na^+-loaded cells contained about 0.4 M Na^+ and negligible level of K^+. The active uptake of AIB by K^+-containing cells required only Na^+, and addition of K^+ had no stimulatory effect on the uptake. The cells loaded with Cs^+ or choline$^+$ also took up AIB in the presence of Na^+ whether K^+ was present or not. In marked contrast, the Na^+-loaded cells required not only Na^+ but also K^+ for AIB uptake. It was subsequently shown that Na^+ extrusion against its electrochemical gradient by the Na^+-loaded cells requires K^+ as a counter cation. The uptake of AIB was tightly coupled to the extrusion of Na^+; AIB uptake did not occur until internal Na^+ concentration was lowered to a certain level. These results indicated that the concentration gradient of Na^+ is essential for AIB transport. Collapse of membrane potential ($\Delta\Psi$) inhibited AIB uptake, indicating that *V. alginolyticus* requires both components of the sodium motive force for AIB uptake.

It is noteworthy that the rates of K^+ uptake are significantly affected by the species of cation inside the cells. The Na^+-loaded cells quickly took up K^+ in near equimolar exchange with Na^+. On the other hand, the Cs^+-loaded cells very slowly took up K^+, and the choline$^+$-loaded cells were unable to do so. These results indicated that Na^+ and K^+ are effective counter cations that serve to maintain overall electroneutrality of ion fluxes.

Proton Conductor-Resistant $\Delta\Psi$

During the course of study on the relationship between external pH and magnitude of $\Delta\Psi$ (Tokuda *et al.*, 1981), it was found that the generation of $\Delta\Psi$ by *V. alginolyticus* at alkaline pH specifically requires Na^+ (Fig. 1). Moreover, in the presence of Na^+ at alkaline pH, $\Delta\Psi$ (negative inside) was generated even if the membrane was made permeable to H^+ by a proton conductor, carbonylcyanide *m*-chlorophenylhydrazone (CCCP). The generation of CCCP-resistant $\Delta\Psi$ was maximum at pH about 8.5 and minimum at pH 6.0 (Tokuda and Unemoto, 1981). Whenever CCCP-resistant $\Delta\Psi$ was generated, H^+ was taken up, leading to the generation of ΔpH (acidic inside). At the steady state, $\Delta\Psi$ and ΔpH generated in the presence of CCCP were similar in magnitude but opposite in polarity. As a result, the magnitude of the protonmotive force in the presence of CCCP became almost zero at any pH values examined. The generation of CCCP-resistant $\Delta\Psi$ was dependent

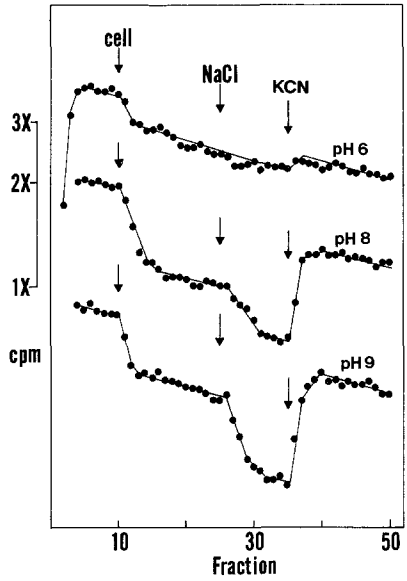

Fig. 1. Effects of Na^+ and pH on the generation of $\Delta\Psi$ by *V. alginolyticus*. Generation of $\Delta\Psi$ (inside negative) was determined in 0.1 M potassium phosphate (pH 6.0 and 8.0) containing 0.3 M KCl and 20 mM glycerol or 10 mM diethanolamine-HCl (pH 9.0) containing 0.4 M KCl and 20 mM glycerol from the distribution of [^{3}H]tetraphenylphosphonium by using flow dialysis as described (Tokuda *et al.*, 1981). The concentrated cells of *V. alginolyticus*, NaCl, and KCN were added at arrows indicated at final concentrations of 2 mg/ml, 50 mM, and 20 mM, respectively. Oxygenated buffer was pumped from the lower chamber at a flow rate of 2 ml/min. Fractions of 2 ml were collected in a Gilson fraction collector, and 0.4 ml of each fraction was mixed with 3 ml of Triton X-100/toluene scintillation liquid for the determination of radioactivity.

on respiration but not ATP (Tokuda and Unemoto, 1982). When oxygen was pulsed to the anaerobic cell suspension of *V. alginolyticus* at pH 8.5, uptake of H^+ was transiently observed in the presence of CCCP. This was consistent with the result that CCCP-resistant $\Delta\Psi$ caused the accumulation of H^+ inside the cells. On the other hand, in the absence of CCCP, the oxygen pulse induced H^+ extrusion. Although the extrusion of H^+ was relatively insensitive to 2-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO), the uptake of H^+ was highly sensitive to the respiratory inhibitor. These results clearly indicated that *V. alginolyticus* possesses a unique respiratory system for the generation of CCCP-resistant $\Delta\Psi$. This system must be different from the H^+ pump since CCCP-resistant $\Delta\Psi$, which secondarily drives H^+ accumulation, cannot be generated by the H^+ pump. The respiration-coupled Na^+ pump was, therefore, the simplest but the most astonishing conclusion drawn from these unusual results.

In order to characterize Na^+ extrusion systems in *V. alginolyticus*, the effect of inhibitors on the extrusion of Na^+ was assayed using the Na^+-loaded cells as described in the earlier section. The extrusion of Na^+ at pH 6.5 was completely inhibited by CCCP, whereas that at pH 8.5 was resistant to CCCP (Tokuda and Unemoto, 1981). CCCP-resistant Na^+ extrusion was inhibited by respiratory inhibitors such as KCN or HQNO. These results indicated that *V. alginolyticus* retains both the CCCP-sensitive Na^+/H^+ antiport system and the CCCP-resistant respiratory Na^+ pump, the latter of which functions maximally at alkaline pH.

Na^+ Pump-Defective Mutants

In order to characterize the respiratory Na^+ pump in detail, isolation of mutants lacking the Na^+ pump was attempted. It was found that collapse of the protonmotive force by CCCP completely inhibited the growth of *V. alginolyticus* at pH 6.5. In contrast, at H 8.5, where the respiratory Na^+ pump generates the sodium motive force in the presence of CCCP, the strain grew well on a medium containing CCCP (Tokuda and Unemoto, 1983). From these observations, Na^+ pump-defective mutants were expected to be unable to grow in the presence of CCCP even at pH 8.5. Among more than 1000 colonies showing defective growth on a medium containing CCCP at pH 8.5, two strains, Nap1 and Nap2, were found to be defective in O_2 pulse-induced H^+ uptake in the presence of CCCP at pH 8.5 (Tokuda, 1983). Na^+ extrusion of Nap1 and Nap2 was examined at pH 8.5. Neither strain extruded Na^+ in the presence of CCCP whereas, in its absence, both strains extruded Na^+ at a slower rate than that of the parent strain. Although both mutants did not show CCCP-dependent H^+ uptake in the presence of CCCP, O_2 pulse in the absence of CCCP caused the extrusion of H^+ in both mutants. These results indicated that both Nap1 and Nap2 were the mutants specifically defective in the Na^+ pump but retained the activity of respiration-coupled H^+ extrusion.

Both mutants were unable to generate $\Delta\Psi$ in the presence of CCCP at pH 8.5 (Tokuda *et al.*, 1988). Moreover, addition of CCCP caused the generation of inside acidic ΔpH in neither mutants. From the frequency of spontaneous Na^+ pump positive revertant, Nap2 seemed to have a single mutation in the Na^+ pump whereas no revertant was obtained from Nap1. Nap2R, a spontaneous revertant of Nap2, showed essentially the same results in the generation of $\Delta\Psi$ as the wild type. Both AIB transport and flagella motility were driven by the electrochemical potentialof Na^+ and became resistant to CCCP at pH 8.5 in the wild type and Nap2R, whereas neither of them was resistant in CCCP in the mutants.

NADH : quinone Oxidoreductase as the Na^+ Pump

Alteration in respiratory activities caused by the mutation was examined in membrane fractions prepared from *V. alginolyticus* strains (Tokuda and Unemoto, 1984). Prior to the discovery of the Na^+ pump, it was known that the NADH oxidase of *V. alginolyticus* requires Na^+ for activity (Unemoto *et al.*, 1977), although the physiological meaning of this Na^+ requirement was unknown. The NADH oxidase of Nap1 and Nap2 was found to be completely independent of Na^+, whereas that of Nap2R required Na^+ (Tokuda and Unemoto, 1984). The Na^+-dependent site of NADH oxidase in the wild type resided at the segment of NADH : quinone oxidoreductase (NQR), which reduces ubiquinone-1 (Q1) to ubiquinol-1 (QH_2). In contrast, QH_2 formation by Nap1 and Nap2 membranes was independent of Na^+. Moreover, the NQR in the wild type and Nap2R was highly sensitive to HQNO, whereas that in Nap1 and Nap2 was resistant. The following properties of NQR in the mutants were also different from those in the wild type and Nap2R. Solubilization of membranes with detergent led to the inactivation of the mutant enzyme, whereas the enzyme of the wild type and Nap2R was active after solubilization. Ag^+ strongly inhibited the enzyme of the wild type and Nap2R but had no effect on the enzyme of the mutants (Asano *et al.*, 1985). Nicotinamide hypoxanthine dinucleotide (deamino-NADH) was utilized by the enzyme present in the wild type and Nap2R whereas the enzyme in the mutants did not oxidize it at all (Tokuda and Kogure, 1989). These results indicated that there are two different types of NQRs (NQR1 and NQR2) in the wild type of *V. alginolyticus* and that the NQR1, which requires Na^+, is lacking from the membranes of the mutants. In order to confirm that the Na^+-dependent NQR1 is the Na^+-motive respiratory segment, membranes prepared from the wild type were solubilized with *n*-octyl-β-D-glucopyranoside and reconstituted into liposomes prepared from soybean phospholipids (Tokuda, 1984). The reconstituted proteoliposomes generated $\Delta\Psi$ (inside positive) and took up Na^+ against its electrochemical potential when NADH and Q1 were added. Na^+ uptake by the proteoliposomes was significantly stimulated when $\Delta\Psi$ was collapsed by valinomycin in the presence of K^+. The generation of the Na^+ electrochemical potential was resistant to CCCP, indicating that the process is performed by the primary Na^+ pump. When HQNO-treated proteoliposomes were used, the electrochemical potential of Na^+ was not generated at all. Essentially the same results were obtained with inside-out membrane vesicles prepared from the wild type (Tokuda *et al.*, 1985) except that addition of Q1 was not necessary for energy generation. These results clearly established that Na^+ was translocated at the Na^+-dependent NADH : quinone oxidoreductase segment of NADH oxidase.

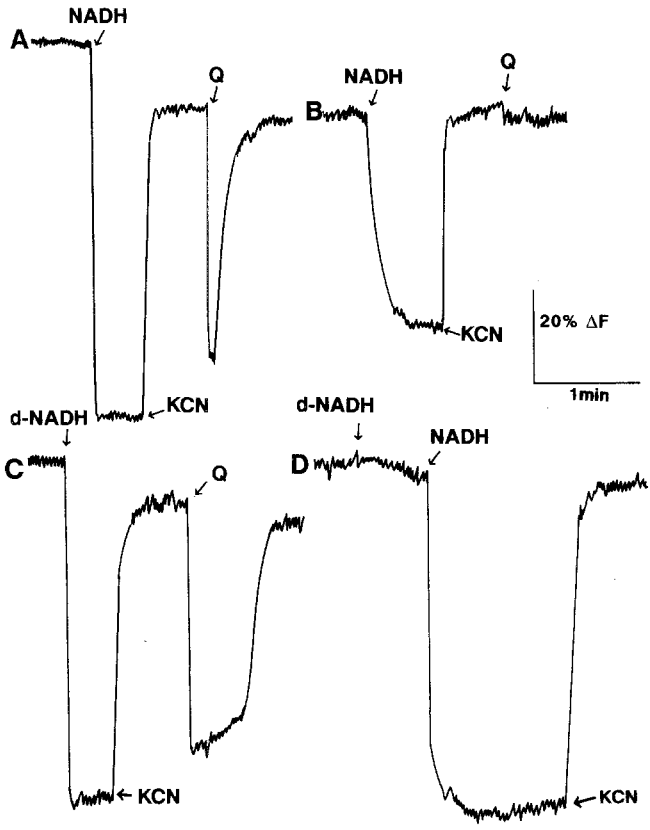

Fig. 2. Generation of $\Delta\Psi$ by inside-out membrane vesicles prepared from the wild type of *V. alginolyticus* and its Na^+ pump-deficient Nap1 mutant. $\Delta\Psi$ (inside positive) was monitored by following the fluorescence quenching of oxonol V at 635 nm with excitation at 580 nm. The membrane vesicles prepared as described (Tokuda *et al.*, 1985) from the wild type (A and C) and Nap1 (B and D) were resuspended in 0.4 M potassium phosphate, pH 7.5, containing 10 mM Na_2SO_4, 5 mM $MgSO_4$ and 1 μM oxonol V. NADH, deamino-NADH (d-NADH), KCN, and ubiquinone-1 (Q) were added as indicated at final concentrations of 1 mM, 1 mM, 10 mM, and 50 μM, respectively.

Generation of $\Delta\Psi$ can be detected by monitoring the fluorescence of oxonol V, which quenches upon the generation of inside positive $\Delta\Psi$. As shown in Fig. 2, inside-out membrane vesicles prepared from the wild type generated $\Delta\Psi$ upon the addition of NADH or deamino-NADH. Although KCN substantially collapsed the $\Delta\Psi$, addition of Q1 caused a transient quenching of fluorescence even in the presence of KCN until all added Q1 was reduced. In contrast to the wild type, addition of Q1 in the presence of NADH and KCN did not lead to the generation of $\Delta\Psi$ in the mutant membrane vesicles. Moreover, addition of deamino-NADH did not lead to

the generation of $\Delta\Psi$ in the mutants. These results indicate that NQR2 is not coupled to energy generation although it is able to catalyze the reduction of Q1 to QH_2. In *E. coli*, it is also reported that only one of two kinds of NADH dehydrogenases is coupled to energy generation (Matsushita *et al.*, 1987). It is also noteworthy that the generation of ΔpH (inside acidic) by NQR1 is not detected, indicating that Na^+ is the only cation that is translocated directly by the NQR1.

Purification of the NQR1 revealed that it was composed of three subunits, α, β, and γ (Hayashi and Unemoto, 1986; Tokuda *et al.*, 1987). The β subunit had NADH dehydrogenase activity and oxidized both NADH and deamino-NADH in the presence of menadione or Q1 as an electron acceptor. Isolated β subunit reduced Q1 to ubisemiquinone ($Q^{\cdot-}$) but not to QH_2. In order to form QH_2, both α and γ subunits were required. The β subunit did not require Na^+ for activity and was resistant to HQNO, whereas the intact NQR1 required Na^+ and was inhibited by HQNO. The purified NQR1 was reconstituted into liposomes prepared from soybean phospholipids. The reconstituted proteoliposomes generated $\Delta\Psi$ on the addition of both NADH and Q1 in the presence of Na^+ (Fig. 3A). In contrast, when assayed in the absence of Na^+, $\Delta\Psi$ was not generated at all (B). The generation of $\Delta\Psi$ was nearly completely inhibited by HQNO (C). Furthermore, replacement of Q1 with menadione, which accepts electrons at the β subunit, also failed to generate $\Delta\Psi$ (D). These results indicated that $Q^{\cdot-}$ is an intermediate of QH_2 formation and that Na^+ is required and pumped out when the α and γ subunits form QH_2 from the intermediate.

Incubation of the inactive NQR1 of Nap2 with the β subunit purified from the wild type resulted in the reconstitution of active NQR1 complex (Asano *et al.*, 1985). Therefore, Nap2 has a mutation in the β subunit but retains normal α and γ subunits. On the other hand, Nap1 membranes lacked all the subunits of NQR1. When Nap1 was conjugated with the wild type as a DNA donor, transconjugants that recovered the NQR1 were isolated. Moreover, the wild type of *V. alginolyticus* was found to retain two kinds of plasmids (Tokuda *et al.*, 1987). These results altogether suggest the intriguing possibility that the Na^+ pump of *V. alginolyticus* is encoded by one of the plasmids, which is transmissible from the wild type to Nap1 strain.

Na^+ Pump as a General Mechanism in Marine Bacteria

Besides *V. alginolyticus*, the respiratory Na^+ pumps were reported to be present in *V. costicola* (Udagawa *et al.*, 1986), *V. parahaemolyticus* (Tsuchiya and Shinoda, 1985) and halotolerant strain Ba_1 (Ken-Dror *et al.*, 1986a, b).

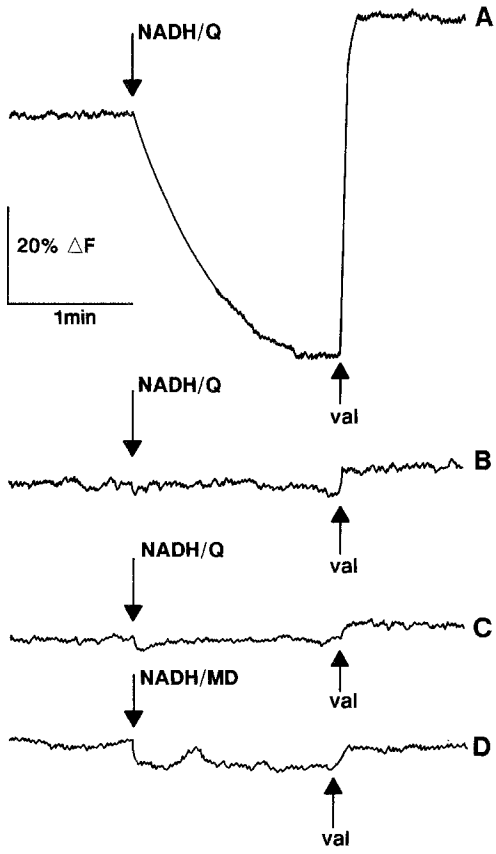

Fig. 3. Generation of $\Delta\Psi$ by proteoliposomes reconstituted with purified Na^+ pump. Reconstitution of purified NQR1 (Na^+ pump) into liposomes made from soybean phospholipids was performed as described (Tokuda, 1984). Generation of $\Delta\Psi$ (inside positive) by the reconstituted proteoliposomes having the activity to form 0.05 μmol QH_2 per min in the presence of NADH were used in each assay. Assays were performed at pH 7.5 in 50 mM potassium phosphate plus 50 mM sodium phosphate (A, C, and D) or 0.1 M potassium phosphate (B). The proteoliposomes pretreated with 1 μM HQNO were used in C. At arrows indicated, 0.2 mM NADH, 50 μM ubiquinone-1 (Q), 0.5 mM menadione (MD), and 1 μM valinomycin (val) were added.

All these bacteria retain Na^+-dependent NADH oxidases, which require Na^+ for NADH-linked QH_2 formation. The presence of the Na^+ pump was further investigated in 10 Gram-negative marine bacteria. Except for the *Flavobacterium* strain, nine strains belonging to the genera *Alcaligenes*, *Alteromonas*, and *Vibrio* were found to retain NADH oxidases that require Na^+ at the segment of NADH : quinone oxidoreductase. (Tokuda and Kogure, 1989). Moreover, the properties of the segment in these bacteria were remarkably similar to those of the NQR1 in *V. alginolyticus*. The

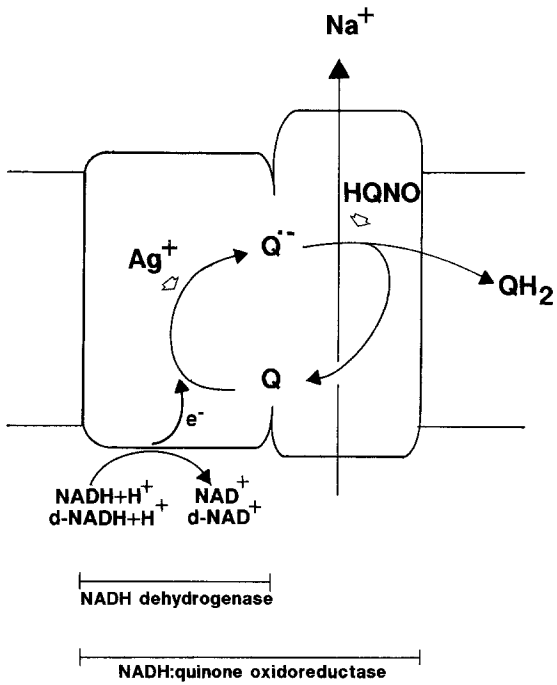

Fig. 4. Common properties of Na^+-dependent (Na^+-motive) NADH : quinone oxidoreductase in marine bacteria. The Na^+-dependent NQR is composed of two functional units; one has an NADH dehydrogenase activity and another catalyses QH_2 formation from semiquinone radical ($Q^{\cdot-}$). Open box arrows indicate the site of inhibition. For more detail, see the text.

characteristic properties of Na^+-dependent (Na^+-motive) NQRs are depicted in Fig. 4. Na^+-dependent NQRs contain NADH dehydrogenases that are able to oxidize deamino-NADH and are highly sensitive to Ag^+. The NADH dehydrogenases in all the bacteria do not require Na^+ and are resistant to HQNO. On the other hand, QH_2 formation requires Na^+ and is inhibited by HQNO. These results suggest that QH_2 formation by all Na^+-dependent NQRs takes place *via* semiquinone radical as an intermediate and that Na^+ is extruded when QH_2 is formed from the semiquinone radical by dismutation reaction. It is of great interest whether these similarities reflect any homology at a molecular level.

Physiological Roles of the Respiratory Na^+ Pump

It is certain that the respiratory Na^+ pump is a general energy-yielding mechanism in Gram-negative marine bacteria, which usually require Na^+ for

growth. The bacteria so far examined also require Na^+ for active transport (Sprott *et al.*, 1975; Tokuda *et al.*, 1982; Kakinuma and Unemoto, 1985) and flagella motility (Chernyak *et al.*, 1983; Tokuda *et al.*, 1988). Therefore, the role of the respiratory Na^+ pump seems to be the generation of the Na^+ electrochemical potential as a direct result of respiration, especially in weak alkaline marine water. However, it is not completely clear why these bacteria must possess the primary Na^+ pump in addition to the secondary Na^+/H^+ antiport system. It was shown that growth of Nap1 and Nap2 becomes defective in alkaline medium compared to that of the wild type and Nap2R (Tokuda *et al.*, 1988). It thus seems likely that the presence of the primary Na^+ pump is advantageous to cells living under alkaline Na^+-rich environments. The respiratory Na^+ pump directly converts redox energy to the sodium motive force. In contrast, for conversion of the protonmotive force to the sodium motive force by the Na^+/H^+ antiport system, redox energy needs to be converted to the protonmotive force by respiratory H^+ pump. As the number of systems involving energy coupling increases, loss of energy during the coupling also increases. Moreover, since the protonmotive force is composed of $\Delta\Psi$ alone at alkaline pH, the Na^+/H^+ antiport system must be electrogenic and requires the influx of more than 1 mol H^+ for the extrusion of 1 mol Na^+. Therefore, concerning energy economy for the generation of the sodium motive force, the respiratory Na^+ pump seems to be superior to the Na^+/H^+ antiport system especially under marine environments (Tokuda and Unemoto, 1985).

So far reported, the cytoplasmic pH in bacteria is regulated near neutral and becomes even more acidic than the external pH under alkaline conditions (Booth, 1985). Generation of $\Delta\Psi$ by the H^+ pump inevitably causes alkalization of cytoplasm, which must be neutralized by influx of H^+ in exchange with Na^+ (Padan *et al.*, 1981; Krulwich, 1986) or K^+ (Plack and Rosen, 1980; Nakamura *et al.*, 1984). On the other hand, generation of $\Delta\Psi$ by the Na^+ pump without alkalization of cytoplasm is possible. This may be another advantage in having the Na^+ pump at alkaline pH.

Acknowledgment

This work was supported in part by a Grant-in-Aid for Scientific Research on Priority Areas of "Bioenergetics" from the Ministry of Education, Science and Culture of Japan.

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