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The Membrane-Bound NADH:Ubiquinone Oxidoreductase in the Aerobic Respiratory Chain of Marine Bacterium *Pseudomonas nautica*

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Abstract Each oxidoreductase activity of the aerobic respiratory chain-linked NADH oxidase system in the marine bacterium *Pseudomonas nautica* was stimulated by monovalent cations including Na^+, Li^+, and K^+. In the presence of NADH or deamino-NADH as electron donors, QH_2 formation was approximately 1.3-fold higher in the presense of 0.08 M of Na^+ than K^+, whereas the other reductase activities were not significantly higher in Na^+ than K^+. The optimal pH of NADH (or deamino-NADH):ubiquinone-1 oxidoreductase was 9.0 in the presence of 0.08 M NaCl. The activity of NADH (or deamino-NADH):ubiquinone-1 oxidoreductase was inhibited by about 33% with 60 μM 2-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO). The activity of NADH (deamino-NADH): ubiquinone-1 oxidoreductase was inhibited by about 32 to 38% with 80 μM rotenone, whereas the activity was highly resistant to capsaicin. On the other hand, electron transfer from NADH or deamino-NADH to ubiquinone-1 generated a membrane potential ($\Delta\Psi$) which was larger in the presence of Na^+ than that observed in the absence of Na^+. The $\Delta\Psi$ was almost completely collapsed by 5 μM carbonylcyanide *m*-chlorophenylhydrazone (CCCP), and approximately 50% inhibited by 60 μM rotenone, or 60 μM 2-heptyl-4-hydroxyquinoline (HQNO). Also, HQNO made the $\Delta\Psi$ very unstable. The results suggest that the enzymatic and energetic properties of the NADH:ubiquinone oxidoreductase of *P. nautica* are quite different, compared with those of other marine halophilic bacteria.

Key words: Marine halophilic bacteria, *Pseudomonas nautica*, NADH (or deamino-NADH):ubiquinone oxidoreductase, H^+ pump

In the bacterial membrane, an electrochemical potential of ion is built at the expense of chemical or light energy by the respiratory chain, ATPase, or bacteriorhodopsin. This electrochemical potential of the ion can then be used by the cell to drive energy consuming reactions such as the active transport of solutes, flagella motility, and the synthesis of adenosine triphosphate (ATP). In general, H^+ is the central coupling ion in bacterial energy metabolism, but it is not the only one. For example, the halorhodopsin of *Halobacterium halobium* functions as an electrogenic chloride pump [15]. The ATPase of *Streptococcus faecalis* [7] and *Propionigenium modestum* [6, 8, 13], and the oxaloacetate decarboxylase of *Klebsiella pneumonia* [2, 3, 4, 5] and *Salmonella typhimurium* [4] are known to be a Na^+-pump. Also, the marine halophilic bacteria such as *Vibrio alginolyticus* [17, 18, 10] and *Vibrio anguillarum* [10] possess the respiratory chain-linked NADH oxidase system as a primary Na^+-pump. Thus, ions other than H^+ also can function as a coupling ion in bacterial energy metabolism.

Marine holophilic bacteria always live in Na^+-rich habitats. Thus, there is a possibility that marine halophilic bacteria may possess a primary Na^+ pump to maintain the internal Na^+ concentration at a lower level against its concentration gradient. In effect, the respiratory chain-linked NADH oxidase system, as a primary Na^+-pump, plays a central role in the energetics of marine halophilic bacteria [9, 10, 14, 17, 18, 19, 20]. The extrusion of Na^+ was revealed to be specifically coupled to the NADH:quinone oxidoreductase segment of the NADH oxidase system in *V. alginolyticus* [17, 18], *V. costicola* [20], *V. anguillarum* [10], and halotolerant bacterium Ba1 found in the Dead Sea [9]. The $\Delta\Psi$ generated by the Na^+-pump is resistant to a protonophore such as carbonylcyanide *m*-chlorophenylhydrazone (CCCP) [10, 17], but is very sensitive to a respiratory inhibitor such as 2-heptyl-4-hydroxyquinolone-*N*-oxide (HQNO) [18].

On the other hand, Nap1, a mutant of *V. alginolyticus* defective in the Na^+-pump, can survive well in Na^+-rich environments by using only the Na^+/H^+ antiport system for Na^+ extrusion [16]. Thus, some marine halophilic bacteria

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may possess an alternative to the respiratory Na^+-pump. In the present work, in order to examine such a possibility, the enzymatic and energetic properties of the aerobic respiratory chain-linked NADH:ubiquinone oxidoreductase in the marine halophilic bacterium *Pseudomonas nautica* IAM 12929 (ATCC 27132) were investigated, and the results are reported.

Materials and Methods

Bacterial Strain and Conditions

The bacterial strain used in this work was *P. nautica* IAM 12929 (ATCC 27132). The bacterium was grown aerobically at 37°C in a liquid medium which contained 0.5% polypeptone, 0.5% yeast extract, 0.476% $MgCl_2$, and 3% NaCl in 50 mM Tris-HCl buffer (pH 7.5). A preculture grown overnight was used to inoculate the main culture to give a turbidity of approximately 0.03.

Preparation of Membranes by an Osmotic Lysis and Inside-Out Membrane Vesicles

Membranes by an osmotic lysis and inside-out membrane vesicles from *P. nautica* were prepared as described previously [1].

Measurement of Enzyme Activities

The activities of NADH (or deamino-NADH) oxidase was measured at 37°C by a decrease in A_{340}, as described previously [1]. The assay mixture of oxidoreductases contained 70 μg of membrane protein, 10 mM KCN, and a given electron acceptor in 2 ml of 20 mM Tris-HCl (pH 9.0) containing NaCl or KCl. Ubiquinone-1 (Q-1) reductase activity was measured at 340 nm with 40.5 μM Q-1, menadione reductase activity was measured at 340 nm with 0.1 mM menadione, ferricyanide reductase activity was measured at 420 nm with 1 mM ferricyanide, and dichlorophenolindophenol (DCIP) reductase activity was measured at 600 nm with 50 μM DCIP. Activity was calculated by using millimolar extinction coefficients of 6.81, 6.22, 1.0, and 16.5, respectively, for Q-1, menadione, ferricyanide, and DCIP. All reactions for reductases were started by the addition of 130 μM NADH or deamino-NADH.

Detection of $\Delta\Psi$

The generation of (inside-positive) in inside-out membrane vesicles was monitored at 37°C by following the fluorescence quenching of oxonol V as described previously [1, 12]. The fluorescence emission of oxonol V was measured at 635 nm with excitation at 580 nm by using Shimadzu spectrofluorophotometer RF 5301PC.

Protein Determination

Protein was measured by Bio-Rad protein assay, based on the Bradford method, using bovin serum albumin as a standard.

Results and Discussion

In general, the membrane-bound NADH oxidase activity of the marine halophilic bacteria, which possess a respiratory Na^+-pump, is specifically increased by Na^+, and its maximum activity is obtained at about pH 8.0–8.5 in the presence of about 0.2 M NaCl. In contrast, as described previously [1], the membrane-bound NADH oxidase of *P. nautica* IAM 12929 was stimulated by monovalent cations including Na^+, Li^+, and K^+, and the highest activation was achieved by 0.08 M Na^+. The optimal pH for NADH oxidase in the presence of 0.08 M NaCl was 9.0. Figure 1 and Table 1 show the effect of salts on each oxidoreductase activity of the NADH oxidase system. In the presence of NADH or deamino-NADH as electron donors, ubiquinone-1 reductase activity was also stimulated by monovalent cations including Na^+ and K^+, and the stimulation by Na^+ at a concentration of 0.08 M was approximately 1.3-fold higher than that by K^+. In contrast, the other reductase activities were not significantly higher in Na^+ than K^+. The optimal pH for NADH or deamino-NADH oxidation of the

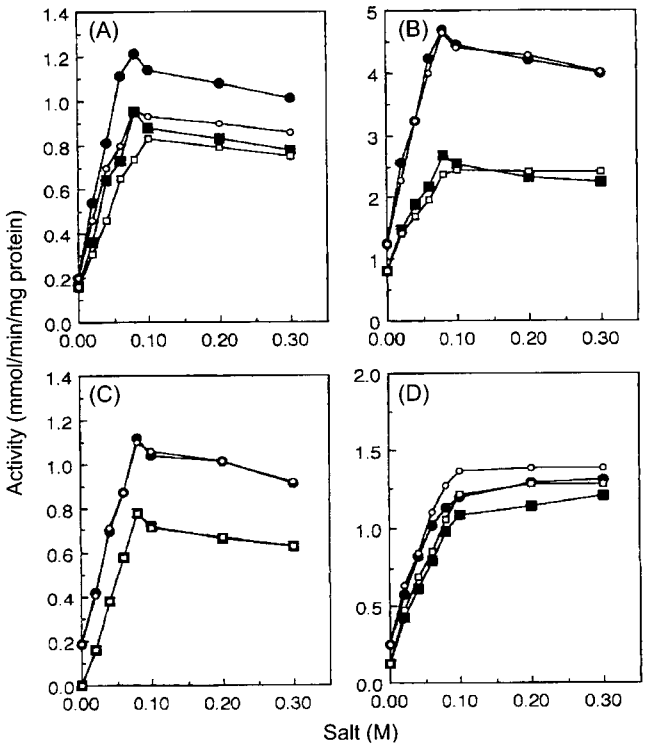

Fig. 1. Effect of salts on the activities of (A) NADH:ubiquinone-1 oxidoreductase, (B) NADH:ferricyanide oxidoreductase, (C) NADH:menadione oxidoreductase, and (D) NADH:DCIP oxidoreductase in membranes prepared from *P. nautica*.
The assay mixture of each oxidoreductase contained 70 μg of membrane protein, 10 mM KCN, and a given electron acceptor in 2 ml of 20 mM Tris-HCl (pH 9.0) containing various concentrations of NaCl (closed symbols) or KCl (open symbols). All assays were started by addition of 130 μM NADH (circles) or deamino-NADH (squares).

Table 1. Effect of salts on each oxidoreductase activity (μmol/min/mg protein) of the NADH oxidase system in membranes prepared from *P. nautica*.

Enzyme	NADH		Deamino-NADH	
	KCl	NaCl	KCl	NaCl
Oxidase	1.19	1.64	0.89	1.22
Ubiquinone-1 reductase	0.96	1.21	0.74	0.95
Ferricyanide reductase	4.65	4.69	2.47	2.56
Menadione reductase	1.12	1.12	0.78	0.78
DCIP reductase	1.27	1.13	1.05	0.98

Each enzyme activity was measured as described in the text.

ubiquinone-1 reductase in the presence of 0.08 M NaCl was 9.0 (Fig. 2).

Respiratory chain inhibitors have proved to be a useful tool for studies on the mechanism of electron transfer in the respiratory chain. The respiratory inhibitor, HQNO, which acts at the b cytochromes, is known to be also a strong specific inhibitor of Na^+-translocating NADH:ubiquinone oxidoreductase [18]. The activity of NADH (or deamino-NADH):ubiquinone-1 oxidoreductase in *P. nautica* was about 33% inhibited by 60 μM HQNO (Fig. 3A). Thus, the ubiquinone analogue HQNO in the respiratory chain of *P. nautica* did not inhibit the Na^+-activated NADH oxidation as effectively as in that of the marine halophilic bacterium *V. alginolyticus*. Interestingly, the activity of NADH (or deamino-NADH):ubiquinone-1 oxidoreductase was highly resistant to capsaicin (Fig. 3B), which is known as an inhibitor of energy-transducing NADH:ubiquinone oxidoreductase [21]. This result indicates that the energy-transducing NADH:ubiquinone oxidoreductase of *P. nautica* is different from other capsaicin-sensitive energy-transducing NADH:ubiquinone oxidoreductases. Another respiratory chain inhibitor, rotenone, which is known as the mitochondrial complex I-inhibitor, inhibited the activities of NADH:ubiquinone-1 by about 38% at a concentration of 60 μM, whereas the deamino-NADH:ubiquinone-1 oxidoreductase activity was inhibited by about 32% (Fig. 3C).

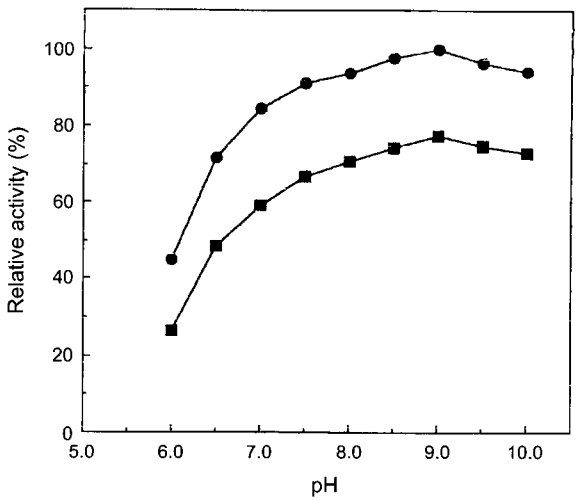

Fig. 2. Effect of pH on the activity of NADH:ubiquinone-1 oxidoreductase.

In the presence of NADH (closed circles) or deamino-NADH (closed squares) as electron donors, NADH:ubiquinone-1 oxidoreductase activity was determined at various pHs in the presence of 0.08 M NaCl. Buffers used at 20 mM were MES-KOH (pH 6 to 6.5), HEPES-KOH (pH 7 to 8), Tris-HCl (7.5 to 8.5), TRICINE-KOH (pH 8 to 8.5), and CAPSO-HCl (pH 9 to 10).

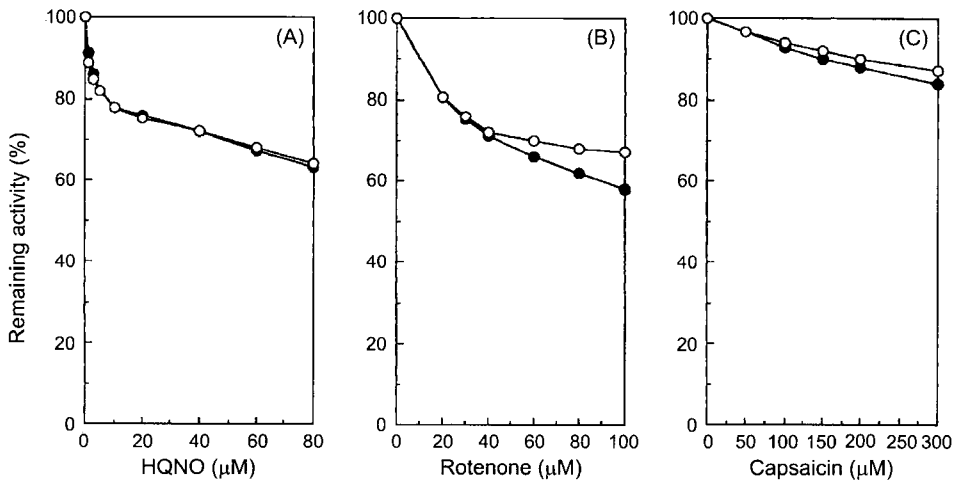

Fig. 3. Effects of respiratory chain inhibitors on the NADH:ubiquinone-1 oxidoreductase or deamino-NADH ubiquinone-1 oxidoreductase

(A) Activities of the NADH:ubiquinone-1 (closed circles) and deamino-NADH:ubiquinone-1 (open circles) oxidoreductases were measured at the various concentrations of HQNO. (B) Activities of the NADH:ubiquinone-1 (closed circles) and deamino-NADH:ubiquinone-1 (open circles) oxidoreductases were measured at the various concentrations of rotenone. (C) Activities of the NADH:ubiquinone-1 (closed circles) and deamino-NADH:ubiquinone-1 (open circles) oxidoreductases were measured at the various concentrations of capsaicin.

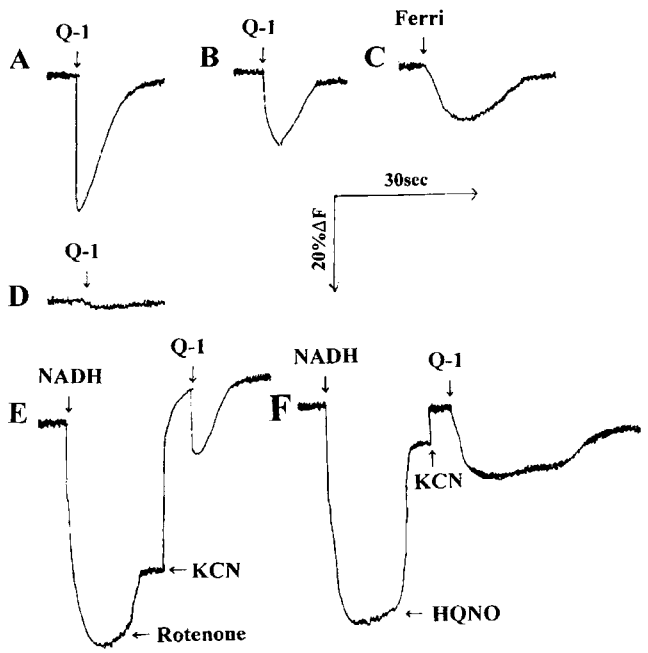

Fig. 4. Generation of membrane potential ($\Delta\Psi$) at the NADH: ubiquinone oxidoreductase segment of *P. nautica*.
The reaction mixture (2 ml) of A, B, C, and D contained 1 μM oxonol V, 10 mM KCN, inside-out membrane vesicles (0.4 mg protein), and 1 mM NADH in 50 mM Tris-acetate buffer (pH 9.0) containing 0.4 M K_2SO_4 and 5 mM $MgSO_4$. Assay D was performed in the presence of 5 μM CCCP. The reaction mixture (2 ml) of E and F contained 1 μM oxonol V, and inside-out membrane vesicles (0.4 mg protein) in 50 mM Tris-acetate buffer (pH 9.0) containing 0.4 M K_2SO_4 and 5 mM $MgSO_4$. All assays were performed at 37°C in the presence (A, C, D, E, F) or absence (B) of 0.08 M Na_2SO_4. 40.5 μM ubiquinone-1 (Q-1), 1 mM ferricyanide (ferri), 1 mM NADH, 10 mM KCN, 100 μM rotenone, and 60 μM HQNO were added where indicated.

To examine the properties of energy generated by the NADH:ubiquinone oxidoreductase segment of *P. nautica*, the fluorescence quenching technique was employed. After the inhibition of terminal cytochrome oxidase by KCN, the electron transfer from NADH (or deamino-NADH) to Q-1 in the presence of Na^+ generated a $\Delta\Psi$ which is larger than that observed in the absence of Na^+ (Figs. 4A and 4B). However, the membrane potential due to electron transfer from NADH (or deamino-NADH) to Q-1 was almost completely collapsed by 5 μM CCCP, suggesting that the H^+ is extruded by the NADH:ubiquinone oxidoreductase of *P. nautica* (Fig. 4D). In *Vibrio alginolyticus*, the $\Delta\Psi$ generated by the NADH:ubiquinone oxidoreductase segment was only 40% inhibited by 5 μM CCCP even at pH 7.5, whereas the $\Delta\Psi$ generated by the quinol oxidase segment was completely inhibited by 5 μM CCCP [10]. Thus, it is suggested that the NADH:ubiquinone oxidoreductase segment of *P. nautica* extrudes mainly a H^+. On the other hand, the activity of Na^+-specific NADH:ubiquinone oxidoreductase shows a high resistance to a respiratory inhibitor, rotenone [11], but a high sensitivity to another respiratory inhibitor, HQNO [18]. In contrast, the Na^+-stimulated NADH: ubiquinone oxidoreductase activity of *P. nautica* IAM 12929 was approximately 50% inhibited by 100 μM rotenone, and was only about 35% inhibited by 60 μM HQNO [1]. As shown in Figs. 3E and 3F, the $\Delta\Psi$ generated by the NADH: quinone oxidoreductase segment of *P. nautica* IAM 12929 was approximately 50% inhibited by 100 μM rotenone and 60 μM HQNO, and became very unstable in the presence of HQNO. Electron flow from NADH to ferricyanide also led to fluorescence quenching of oxonol V in inside-out membrane vesicles from *P. nautica* IAM 12929, but the effect was significantly smaller than that observed with Q-1 (Fig. 3C). Electron flow from NADH to menadione or DCIP did not lead to fluorescence quenching of oxonol V in inside-out membrane vesicles. On the basis of the results, it is suggested that the NADH:ubiquinone oxidoreductase segment of *P. nautica* IAM 12929 generates a H^+ electrochemical potential in place of a Na^+ electrochemical potential, and it was also concluded that the NADH:ubiquinone oxidoreductase of the marine halophilic bacterium *P. nautica* is quite different in enzymatic and energetic properties compared to that of other marine halophilic bacteria.

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