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Enzymatic and Energetic Properties of an Aerobic Respiratory Chain-Linked NADH Oxidase System in Marine Bacterium *Vibrio natriegens*

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Abstract Membranes prepared from *Vibrio natriegens* oxidized both NADH and deamino-NADH as substrates. The maximum activity of the membrane-bound NADH oxidase was obtained at about pH 8.5 in the presence of 0.2 M NaCl, whereas that of the NADH:ubiquinone oxidoreductase was obtained at about pH 7.5 in the presence of 0.2 M NaCl. Electron transfer from NADH or deamino-NADH to ubiquinone-1 or oxygen generated a considerable membrane potential ($\Delta\psi$), which occurred even in the presence of 20 μM carbonylcyanide *m*-chlorophenylhydrazone (CCCP). However, the $\Delta\psi$ was completely collapsed by the combined addition of 10 μM CCCP and 20 μM monensin. On the other hand, the activity of the NADH oxidase and the $\Delta\psi$ generated by the NADH oxidase system were inhibited by about 90% with 10 μM HQNO, whereas the activity of the NADH:ubiquinone oxidoreductase and the $\Delta\psi$ generated at the NADH:ubiquinone oxidoreductase segment were inhibited by about 60%. Interestingly, the activity of the NADH:ubiquinone oxidoreductase and the $\Delta\psi$ generated at the NADH:ubiquinone oxidoreductase segment were resistant to the respiratory chain inhibitors such as rotenone, capsaicin, and $AgNO_3$, and the activity of the NADH oxidase and the $\Delta\psi$ generated by the NADH oxidase system were very sensitive only to $AgNO_3$. It was concluded, therefore, that *V. natriegens* cells possess a $AgNO_3$-resistant respiratory Na^+ pump that is different from the $AgNO_3$-sensitive respiratory Na^+ pump of a marine bacterium, *Vibrio alginolyticus*.

Key words: Marine halophilic bacteria, *Vibrio natriegens*, aerobic respiratory chain-linked NADH oxidase system, respiratory chain inhibitors, Na^+ pump

In the bacterial membrane, an electrochemical potential of ion is built at the expense of chemical or light energy provided 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 recognized as the central coupling ion in bacterial energy metabolism [13], but it should also be mentioned that this is not the only one [3, 5–8, 10–12, 14, 17–19].

In recent years, considerable evidence has accumulated on the importance of sodium as a coupling ion in bacterial bioenergetics [3–8, 10–12, 15–19]. Halophilic marine bacteria continually live in Na^+-rich habitats. Of them, the membrane energetics of *Vibrio alginolyticus* has attracted considerable attention, because it possesses Na^+-motive NADH: ubiquinone oxidoreductase (the Na^+ pump) [10, 15–17]. The membrane potential ($\Delta\psi$) generated by the NADH: ubiquinone oxidoreductase segment of *V. alginolyticus* is resistant to protonophores such as carbonylcyanide *m*-chlorophenylhydrazone (CCCP) [10, 16], but it has been found to be very sensitive to the respiratory chain inhibitors such as 2-heptyl-4-hydroxyquinolone-*N*-oxide (HQNO) [17] and $AgNO_3$ [1].

On the other hand, Nap1 [14], 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. Thus, some halophilic marine bacteria may possess an alternative to the respiratory Na^+ pump. In fact, the NADH:ubiquinone oxidoreductase of a halophilic marine bacterium *Pseudomonas nautica* IAM 12929 (ATCC 27132) has been shown to be a H^+-dependent one [2].

In the present paper, we report the existence of a Na^+ pump in *V. natriegens* cells that is dependent on electron transport. The site of Na^+ action is located, as in *V. alginolyticus*, at the NADH:ubiquinone oxidoreductase segment. However, the enzymatic and energetic properties of the NADH:ubiquinone oxidoreductase of *V. natriegens* are a little different from those of the NADH:ubiquinone oxidoreductase of *V. alginolyticus*. We also prove here that the enzymatic properties of the aerobic respiratory chain-

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linked NADH oxidase system of *V. natriegens* are consistent with the energetic properties

MATERIALS AND METHODS

Bacterial Strain and Conditions

The bacterial strain used in this work was *V. natriegens* KCCM 40868 (ATCC 14048). The bacterium was grown aerobically at 37°C in a liquid medium that contained 0.5% polypeptone, 0.5% yeast extract, 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 *V. natriegens* were prepared as described previously [2, 9, 10] with the following modifications. Cells harvested at an A_{600} of 0.9 were washed twice in 20 mM Tris-HCl (pH 7.5) containing 1.0 M NaCl and 5 mM EDTA. Washed cells were osmotically lysed by being rapidly dispersed in 20 mM Tris-HCl (pH 7.5) containing 10 mM NaCl and 5 mM EDTA. The cell suspension was incubated at room temperature for 5 min and sedimented at 12,000 ×*g* for 15 min. To accomplish sufficient lysis of the cells, pellets were resuspended in the same buffer solution and resedimented. Pellets were resuspended in 20 mM Tris-HCl (pH 7.5) containing 10 mM NaCl and 0.5 mM EDTA, and then DNase, RNase, and $MgSO_4$ were added to give 10 mg/ml, 10 mg/ml, and 2 mM final concentrations, respectively. After 20 min of incubation at room temperature, EDTA was added to 5 mM and incubation was continued for an additional 5 min. Membrane fractions were obtained after sedimentation at 12,000 ×*g* for 20 min. After washing twice in 20 mM Tris-HCl (pH 7.5) containing 10 mM NaCl and 0.5 mM EDTA, membrane pellets were resuspended at about 20 mg protein/ml in 20 mM Tris-HCl (pH 7.5) containing 10 mM NaCl and 10% glycerol and kept frozen at −80°C.

Measurement of Enzyme Activities

The activities of NADH (or deamino-NADH) oxidase and NADH:quinone oxidoreductase were measured at 37°C from a decrease in A_{340} using a Varian Cary 3E spectrophotometer [2, 9, 10, 13]. The assay mixture of NADH oxidase contained 125 μM NADH or deamino-NADH in 2 ml of 20 mM Tris-HCl (pH 8.5 or 9.0) containing 0.2 M NaCl. The assay was started by addition of 40 μg of membrane protein, and activity was calculated using a millimolar extinction coefficient of 6.22. The assay mixture of NADH:ubiquinone oxidoreductase contained 30 μg of membrane protein, 30 mM KCN, and 30 μM Q-1 in 2 ml of 20 mM Tris-HCl (pH 7.5) containing 0.2 M NaCl. The reaction of oxidoreductase was started by addition of 125 μM NADH or deamino-NADH, and activity was calculated using millimolar extinction coefficients of 6.81 for Q-1.

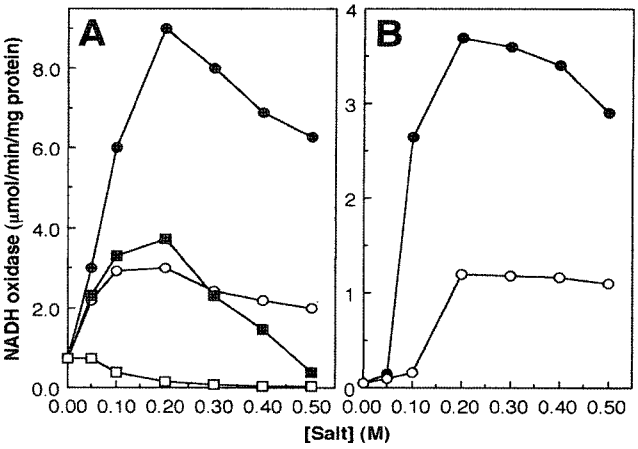

Fig. 1. Effect of salts on the activity of respiratory chain-linked NADH oxidase in the *V. natriegens* membrane fractions.
The assay mixture of NADH oxidase (2 ml) contained 125 μM NADH (A) or deamino-NADH (B), and various concentrations of NaCl (●), LiCl (■), KCl (○), or $MgCl_2$ (□) in 20 mM Tris-HCl (pH 8.5 or 9.0). The assay was started by addition of membrane suspensions containing 40 μg of protein at 37°C.

Preparation of Inside-Out Membrane Vesicles

For preparation of inside-out membrane vesicles, *V. natriegens* was grown aerobically at 37°C, and cells were harvested

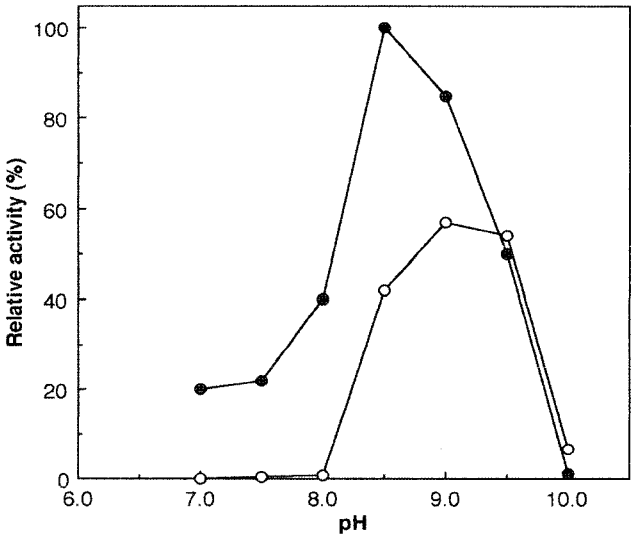

Fig. 2. Effect of pH on the activity of respiratory chain-linked NADH oxidase in the *V. natriegens* membrane fractions.
NADH (●) or deamino-NADH (○) oxidase activity was determined with different ranges of pH in the presence of 0.2 M NaCl. Buffers used at 20 mM were Tris-HCl (pH 7.1 to 8.9), Tricine-KOH (pH 7.5 to 8.5), and Capso-HCl (pH 9 to 10). The assay was started by addition of membrane suspensions containing 40 μg of protein at 37°C.

at an A_{600} of 0.9. Inside-out membrane vesicles were prepared as described previously [9, 10] with the following modifications. Cells were harvested by sedimentation, washed twice in 0.4 M potassium phosphate buffer (pH 7.5) and resedimented. The cells were resuspended in the same buffer supplemented with 5 mM $MgSO_4$ at 6 ml/g wet weight of cells, and DNase I was added to 10 μg/ml. For preparation of inside-out membrane vesicles, the cell suspension was passed through the French pressure cell twice at 8,000 psi. Unbroken cells and cell debris were removed by sedimentation at 10,000 ×*g* for 10 min, and a membrane pellet was obtained after sedimention at 120,000 ×*g* for 2 h. The pellet was finally resuspended in the buffer solution containg 10% glycerol to give a final concentration of about 40 mg protein/ml and kept frozen at −80°C.

Measurement of Enzyme Activities

The activities of NADH (or deamino-NADH) oxidase and NADH:ubiquinone oxidoreductase were measured at 37°C from a decrease in A_{340} using a Varian Cary 3E spectrophotometer [2, 9, 10, 13]. The assay mixture of NADH oxidase contained 125 μM NADH or deamino-NADH in 2 ml of 20 mM Tris-HCl (pH 8.5) containing 0.2 M NaCl. The assay was started by addition of 40 μg of membrane protein, and activity was calculated by using a millimolar extinction coefficient of 6.22. The assay mixture of NADH:ubiquinone oxidoreductase contained 30 μg of membrane protein, 30 mM KCN, and 30 μM Q-1 in 2 ml of 20 mM Tris-HCl (pH 7.5) containing 0.2 M NaCl. The reaction of oxidoreductase was started by addition of 130 μM NADH or deamino-NADH, and activity was calculated by using a millimolar extinction coefficient of 6.81 for Q-1.

Detection of ΔΨ

The generation of ΔΨ (inside-positive) in inside-out membrane vesicles was monitored at 37°C by following the fluorescence quenching of oxonol V as described previously [2, 9, 10]. The fluorescence emission of oxonol

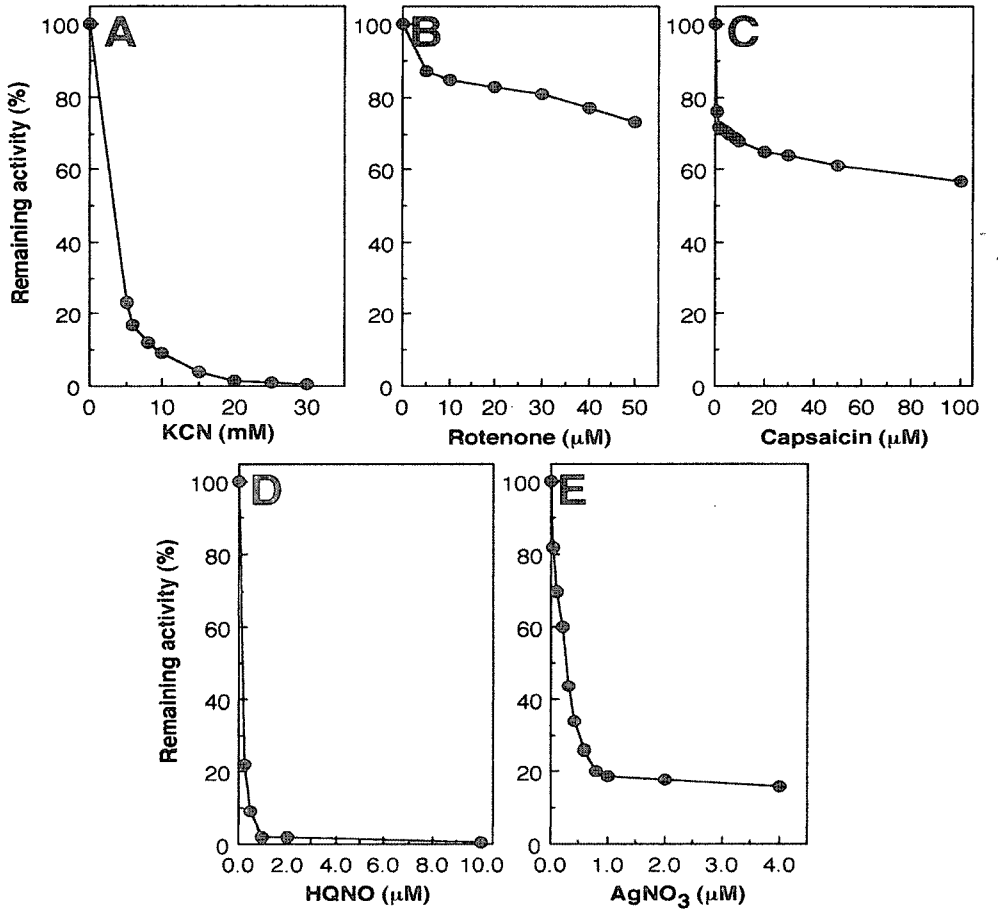

Fig. 3. Effect of respiratory chain inhibitors on the activity of respiratory chain-linked NADH oxidase.
The assay mixture of NADH oxidase (2 ml) contained 125 μM NADH, and various concentrations of (A) KCN, (B) rotenone, (C) capsaicin, (D) HQNO, or (E) $AgNO_3$ in 20 mM Tris-HCl (pH 8.5) containing 0.2 M NaCl. The assay was started by addition of membrane suspensions containing 40 μg of protein at 37°C.

V was measured at 635 nm with excitation at 580 nm by using a Shimadzu spectrofluorophotometer RF 5301PC.

Protein Determination

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

RESULTS

Effects of Salts and pH on the Activity of Membrane-Bound NADH Oxidase

The effects of salts and pH on the NADH oxidase activity were examined with *V. natriegens* membrane fractions prepared by osmotic lysis. The membrane-bound NADH oxidase oxidized both NADH (Fig. 1A) and deamino-NADH (Fig. 1B) as substrates. The rate of NADH or deamino-NADH oxidation was dramatically stimulated by Na^+, whereas that of NADH or deamino-NADH oxidation was not significantly stimulated by K^+, Li^+, and Mg^{2+}. The maximum activity of the membrane-bound NADH oxidase was obtained at about pH 8.5 in the presence of 0.2 M NaCl, whereas that of the deamino-NADH oxidase was obtained at about pH 9.0 in the presence of 0.2 M NaCl (Fig. 2).

Effect of KCN, Rotenone, Capsaicin, HQNO, and $AgNO_3$ on the Activity of NADH Oxidase

Respiratory chain inhibitors have proved to be a useful tool for studies on the mechanism of electron transfer in the respiratory chain. As seen in Fig. 3A, the NADH oxidase activity of *V. natriegens* was completely inhibited by 30 mM KCN, indicating the participation of cytochrome oxidases in electron transport. The NADH oxidase activity of *V. natriegens* was also very sensitive to 2-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO), which acts at the b cytochromes, with complete inhibition at a concentration of 10 μM (Fig. 3D) and was highly sensitive to $AgNO_3$ (Fig. 3E). On the other hand, the NADH oxidase activity of *V. natriegens* was relatively resistant to rotenone and capsaicin, which are known as the mitochondrial complex I-inhibitors; the activity was inhibited by about 20–30% with 40 μM rotenone and capsaicin (Fig. 3B and 3C).

Effects of Salts and pH on the Activity of NADH:Ubiquinone Oxidoreductase

The activity of NADH (or deamino-NADH):ubiquinone oxidoreductase was markedly stimulated by Na^+, similar to the NADH oxidase. The optimal pH of NADH:ubiquinone oxidoreductase was found at about 7.5 in the presence of 0.2 M NaCl, whereas that of the deamino-NADH:ubiquinone oxidoreductase was obtained at about 8.0 in the presence of 0.2 M NaCl (Fig. 4).

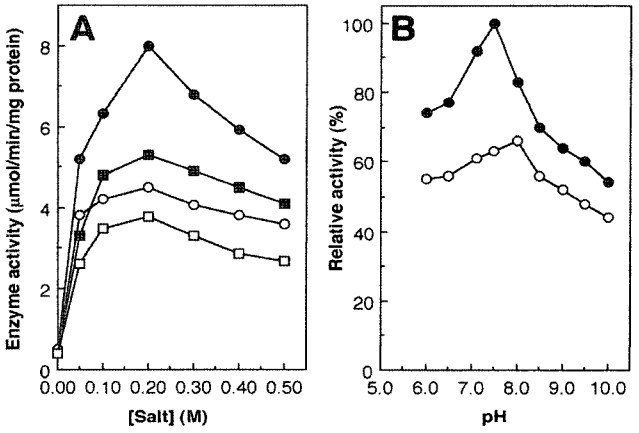

Fig. 4. Effects of salts and pH on the activity of NADH:quinone oxidoreductase.

(A) The assay mixture of NADH:quinone oxidoreductase (2 ml) contained 30 μg of membrane protein, 30 mM KCN, and 30 μM Q-1 in 2 ml of 20 mM Tris-HCl (pH 7.5 or 8.0) containing various concentrations of NaCl (●, ■) or KCl (○, □). All assays were started by addition of 125 μM NADH (●, ○) or deamino-NADH (■, □) at 37°C. (B) In the presence of NADH (●) or deamino-NADH (○) as electron donors, NADH:quinone oxidoreductase activity was determined at various pHs in the presence of 0.2 M NaCl. Buffers used at 20 mM were MES (pH 6 to 6.5), Tris-HCl (pH 7.1 to 8.9), Tricine-KOH (pH 7.5 to 8.5), and Capso-HCl (pH 9 to 10). All assays were started by addition of 125 μM NADH or deamino-NADH at 37°C.

Effect of Rotenone, Capsaicin, HQNO, and $AgNO_3$ on the Activity of NADH:Quinone Oxidoreductase

Like NADH oxidase, the NADH:quinone oxidoreductase activity of *V. natriegens* was relatively resistant to rotenone and capsaicin; the activity was inhibited by about 26–28% with 40 μM rotenone and capsaicin (Figs. 5A and 5B). The respiratory chain inhibitor, 2-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO), which acts at the b cytochromes, is also known to be a strong specific inhibitor of Na^+-translocating NADH:ubiquinone oxidoreductase [17]. As shown in Fig. 5C, the NADH:ubiquinone oxidoreductase activity of *V. natriegens* was inhibited by about 60% with HQNO. $AgNO_3$ is known to be a strong inhibitor of the Na^+-translocating NADH:quinone oxidoreductase in *V. alginolyticus* [1]. However, the NADH:quinone oxidoreductase activity of *V. natriegens* was very resistant to $AgNO_3$ (Fig. 5D).

Generation of Membrane Potential ($\Delta\Psi$) During Electron Transfer from NADH, or Deamino-NADH (d-NADH), to Q-1 or Oxygen in Inside-Out Membrane Vesicles

Generation of $\Delta\Psi$ (inside-positive) in the NADH oxidase system of *V. natriegens* was examined by measuring the fluorescence quenching of oxonol V with inside-out membrane vesicles. Inside-out membrane vesicles prepared from *V. natriegens* quenched the fluorescence of oxonol V upon the addition of NADH or deamino-NADH (Fig. 6A and 6B), indicating the generation of $\Delta\Psi$. This membrane potential due to electron transfer from NADH or deamino-

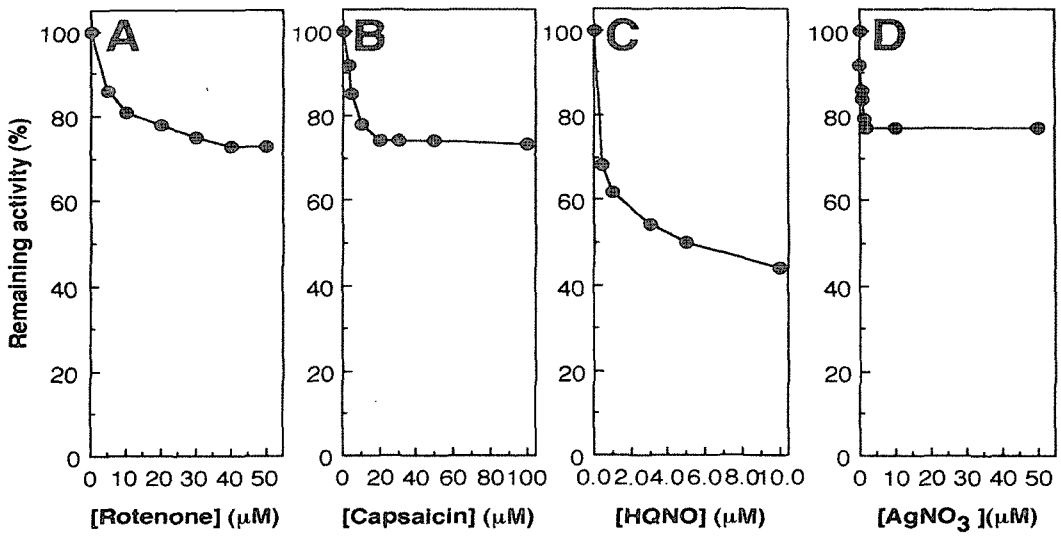

Fig. 5. Effect of respiratory chain inhibitors on the activity of NADH:quinone oxidoreductase.
The assay mixture of NADH:quinone oxidoreductase (2 ml) contained 30 μg of membrane protein, 30 mM KCN, and 30 μM Q-1 in 2 ml of 20 mM Tris-HCl (pH 7.5) containing 0.2 M NaCl. Activity of the NADH:quinone oxidoreductase was measured with different concentrations of (A) rotenone, (B) capsaicin, (C) HQNO, or (D) $AgNO_3$. All assays were started by addition of 125 μM NADH at 37°C.

NADH to Q-1 or oxygen was resistant to carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (Fig. 6C). However, the $\Delta\psi$ was completely collapsed by the combined addition of 10 μM CCCP and 20 μM monensin, an electroneutral ionophore specific to Na^+ and H^+ (Fig. 6D), although monensin added alone had little effect on the $\Delta\psi$ (data not shown). The $\Delta\psi$ generated by the NADH oxidase system was collapsed by about 90% with 10 μM HQNO, whereas the $\Delta\psi$ generated at the NADH:ubiquinone oxidoreductase segment was collapsed by about 60% (Fig. 7C). These results suggest that Na^+ is extruded at the NADH:ubiquinone oxidoreductase segment of *V. natriegens*. The $\Delta\psi$ generated at the NADH:ubiquinone oxidoreductase segment was highly resistant to rotenone, capsaicin, and $AgNO_3$ (Figs. 7A, 7B, and 7D). On the other hand, although the level of Na^+ contamination was significant in the reaction mixture, externally added Na^+ considerably stimulated the generation of $\Delta\psi$ (data not shown).

DISCUSSION

The results presented in this paper demonstrate that *V. natriegens*, a halophilic marine bacterium, possesses a $AgNO_3$-resistant respiratory Na^+ pump that extrudes Na^+ as an immediate result of respiration, and proves that the enzymatic properties of the aerobic respiratory chain-linked NADH oxidase system of *V. natriegens* are consistent with the energetic properties. The membrane-bound NADH oxidase of *V. natriegens*, which oxidizes both NADH and deamino-NADH as substrates, required 0.2 M Na^+ concentration for maximum activity. The optimal pH for NADH oxidase in the presence of 0.2 M NaCl was 8.5, whereas that for deamino-NADH oxidase was 9.0.

On the other hand, the optimal pH for NADH:ubiquinone oxidoreductase in the presence of 0.2 M NaCl was 7.5, whereas that for deamino-NADH:ubiquinone oxidoreductase was 8.5. These results imply that the aerobic respiratory chain-linked NADH oxidase system of marine bacterium *V. natriegens* is of two different types [9, 10, 13, 17].

Generally, the membrane-bound NADH oxidase activity of the marine halophilic bacteria, which possess a respiratory Na^+ pump, was specifically increased by Na^+, and its maximum activity was obtained at pH around 8.0–8.5 in the presence of about 0.2 M NaCl. The membrane-bound NADH oxidase of *V. natriegens* was also activated by Na^+, and its maximum activity was obtained at around pH 8.5 in the presence of about 0.2 M NaCl.

Respiratory chain inhibitors have been widely used to probe the mechanism of electron transfer in the respiratory chain. Generally, the H^+-translocating NADH: ubiquinone oxidoreductases are inhibited by the respiratory inhibitors rotenone and capsaicin, whereas the NADH: ubiquinone oxidoreductases that lacks an energy coupling site are only slightly inhibited by these inhibitors [9, 20]. Interestingly, the NADH:ubiquinone oxidoreductase of *V. natriegens* was resistant to rotenone and capsaicin.

On the other hand, the respiratory chain inhibitors such as 2-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO) and $AgNO_3$ are known as potent inhibitors of the Na^+-translocating NADH:ubiquinone oxidoreductase of marine bacterium *V. alginolyticus* [1, 17]. In the present study, the NADH: ubiquinone oxidoreductase activity of *V. natriegens* was

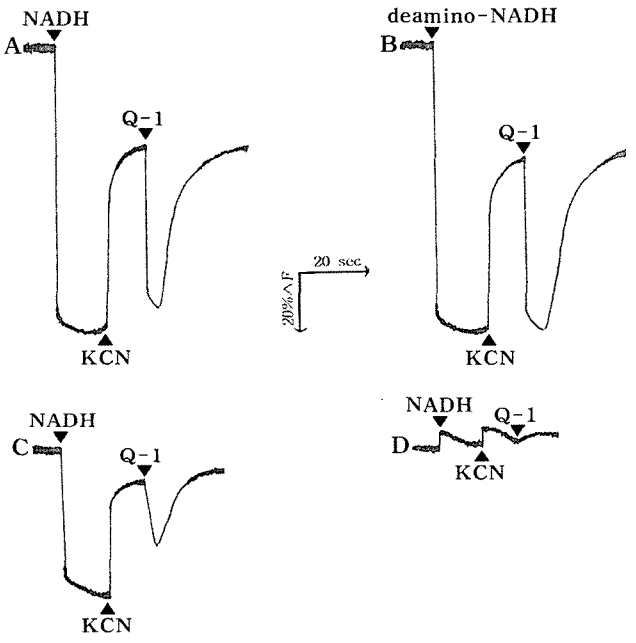

Fig. 6. Generation of $\Delta\psi$ during electron transfer from NADH or deamino-NADH to ubiquinone (Q-1) or oxygen in inside-out membrane vesicles.

The reaction mixture (2 ml) of A, B, C, and D contained 1 μM oxonol V and inside-out membrane vesicles (0.2 mg protein) in 0.4 M potassium phosphate (pH 7.5) containing 10 mM Na_2SO_4 and 5 mM $MgSO_4$. Assay C was performed in the presence of 10 μM CCCP, and assay D was performed in the presence of both 10 μM CCCP and 20 μM monensin. NADH, deamino-NADH, KCN, and ubiquinone-1 (Q-1) were added at final concentrations of 1 mM, 1 mM, 30 mM, and 40.5 μM, respectively. All assays were performed at 37°C.

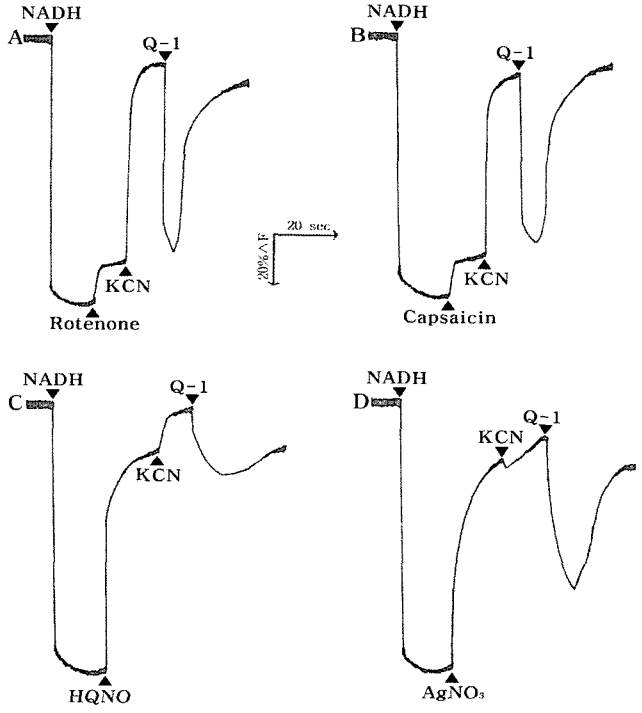

Fig. 7. Effect of respiratory chain inhibitors upon respiration-linked $\Delta\psi$.

The reaction mixture (2 ml) of A, B, C, and D contained 1 μM oxonol V and inside-out membrane vesicles (0.2 mg protein) in 0.4 M potassium phosphate (pH 7.5) containing 10 mM Na_2SO_4 and 5 mM $MgSO_4$. NADH, KCN, ubiquinone-1 (Q-1), rotenone, capsaicin, HQNO, and $AgNO_3$ were added at final concentrations of 1 mM, 30 mM, 40.5 μM, 40 μM, 40 μM, 10 μM, and 1 μM, respectively. All assays were performed at 37°C.

inhibited by about 60% with HQNO (Fig. 5C), whereas the NADH:ubiquinone oxidoreductase activity of *V. natriegens* was very resistant to $AgNO_3$ (Fig. 5D). To examine the properties of energy generated by the Na^+-activated NADH: quinone oxidoreductase segment of *V. natriegens*, we employed the fluorescence quenching technique. The membrane potential ($\Delta\Psi$) due to electron transfer from NADH or deamino-NADH to Q-1 or oxygen was resistant to carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (Fig. 6C). However, the $\Delta\psi$ was completely collapsed by the combined addition of 10 μM CCCP and 20 μM monensin (Fig. 6D), suggesting that Na^+ is extruded by the NADH oxidase system of *V. natriegens*. The $\Delta\psi$ generated at the NADH:quinone oxidoreductase segment was sensitive to HQNO (Fig. 7E). Thus, it is suggested that the extrusion of Na^+ in the aerobic respiratory chain of *V. natriegens* is coupled to the NADH:ubiquinone oxidoreductase segment. On the other hand, the $\Delta\psi$ generated at the NADH: ubiquinone oxidoreductase segment was very resistant to rotenone, capsaicin, and $AgNO_3$ (Figs. 7A, 7B, and 7D). The NADH oxidase was enzymatically and energetically very sensitive to $AgNO_3$, whereas the NADH:ubiquinone oxidoreductase was very resistant to $AgNO_3$, suggesting that $AgNO_3$ inhibits the quinol oxidase in *V. natriegens*, but not the NADH:ubiquinone oxidoreductase.

In conclusion, first, the aerobic respiratory chain-linked NADH oxidase system of *V. natriegens* possesses a CCCP-resistant and HQNO-sensitive Na^+ pump. The extrusion of Na^+ is specifically coupled to the NADH:ubiquinone oxidoreductase segment. Second, interestingly, the NADH: ubiquinone oxidoreductase activity of *V. alginolyticus* is very sensitive to $AgNO_3$, whereas that of *V. natriegens* is very resistant to $AgNO_3$. In *V. natriegens*, $AgNO_3$ inhibits the quinol oxidase segment. On the other hand, the activity of the NADH:ubiquinone oxidoreductase and the $\Delta\psi$ generated at the NADH:ubiquinone oxidoreductase segment were very resistant to rotenone and capsaicin. Third, this paper proved the enzymatic results by using the energetic results. That is, the enzymatic results from *V. natriegens* were consistent with the energetic results.

Acknowledgment

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