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Na^+-dependent NADH:quinone Oxidoreductase in the Respiratory Chain of the Marine Bacterium *Marinomonas vaga*

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The Gram-negative marine bacterium *Marinomonas vaga*, which requires 0.5 M NaCl concentration for optimal growth, is slightly halophilic. The growth of *M. vaga* was highly resistant to the proton conductor, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) under alkaline pH conditions (pH 8.5) but very sensitive to CCCP under acidic pH conditions (pH 6.5). These results suggest that the respiratory chain-linked NADH oxidase system of *M. vaga* may lead to generation of a Na^+ electrochemical gradient. In order to examine the existence of Na^+-stimulated NADH oxidase in *M. vaga*, membrane fractions were prepared by the osmotic lysis method. The membrane-bound NADH oxidase oxidized both NADH and deamino-NADH as substrates and required Na^+ for maximum activity. The maximum activity of NADH oxidase was obtained at about pH 8.5 in the presence of 0.2 M NaCl. The site of Na^+-dependent activation in the NADH oxidase system was at the NADH:quinone oxidoreductase segment. The NADH oxidase and NADH:quinone oxidoreductase were very sensitive to the respiratory chain inhibitor, 2-heptyl-4-hydroxyquinoline-N-oxide (HQNO) in the presence of 0.2 M NaCl but highly resistant to another respiratory inhibitor, rotenone. Based on these findings, we conclude that *M. vaga* possesses the Na^+-dependent NADH:quinone oxidoreductase that may function as an electrogenic Na^+ pump.

Halophilic marine bacteria can be defined as microorganisms living in sea water and requiring Na^+ for growth. They always live in Na^+-rich habitats and only a little is known about the mechanism of Na^+ circulation between their cytoplasm and the surrounding medium. In halophilic marine bacteria, the Na^+ concentration of the cytoplasm is less than that of the environment. Thus, energy is required to maintain the internal Na^+ concentration at a lower level against its concentration gradient. It has been reported that the respiratory chain-linked NADH oxidase system, as a primary Na^+ pump, plays a central role in the energetics of halophilic marine bacteria including *Vibrio alginolyticus* (18, 19), *Vibrio anguillarum* (8), *Vibrio costicola* (23), *Vibrio parahaemolyticus* (22), *Alcaligenes* strain 201 (12), and the halotolerant bacterium Ba_1 found in the Dead Sea (5, 6). The extrusion of Na^+ was revealed to be specifically coupled to the NADH:quinone oxidoreductase segment of the NADH oxidase system in *V. alginolyticus* (21), *V. anguillarum* (8), and the halotolerant bacterium Ba_1 (5, 6). The Na^+ electrochemical gradient generated by the respiratory Na^+ pump is very resistant to the proton conductor, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). This CCCP-resistant Na^+ electrochemical gradient can be used by the cell to drive energy consuming reactions, such as the active transport of sucrose (4) and amino acids (17), flagella motility (2), cell growth (14), and protein translocation across the cytoplasmic membrane (16). Although the respiratory Na^+ pump may be a general system for bacteria living in Na^+-rich environments, microbiologists have employed mainly halophilic marine *Vibrio* strains to examine the mechanism of Na^+ circulation.

Baumann *et al.* created the genus *Alteromonas* for a group of Gram-negative, aerobic, polarly flagellated, heterotrophic marine bacteria (1). Thereafter, *Alteromonas* were reclassified as the new genus *Marinomonas* by Van Landschoot and De Ley (24). Since the history of the genus *Marinomonas* is not long, little is known about the energetics of *Marinomonas* vaga.

In an attempt to understand the energetics of halophilic marine bacteria other than the marine *Vibrio* strains, we investigated the enzymatic properties of the respiratory chain-linked NADH oxidase system of *M. vaga*. Here we address the results and also demonstrate whether the respiratory chain-linked NADH oxidase of

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M. vaga is activated by Na^+ and where the primary site of Na^+-dependent activation in the NADH oxidase system is. Also, the possibility of whether the NADH:quinone oxidoreductase of *M. vaga* functions as an electrogenic Na^+ pump is discussed.

MATERIALS AND METHODS

Bacterial Strain and Culture Conditions

The bacterial strain used in this work was *Marinomonas vaga* IAM 12923 (ATCC 27119). Bacteria were grown aerobically at 30°C in liquid medium containing 0.5% polypeptone, 0.5% yeast extract, 0.4% K_2HPO_4, 3% NaCl, and 0.2% glucose.

Determination of Growth Rate

Growth of cells was determined by measuring turbidity at 600 nm using a spectrophotometer. A preculture, grown overnight, was used to inoculate the main culture to give a turbidity of approximately 0.02. The addition of CCCP, which was dissolved in dimethyl sulfoxide, was done at an A_{600} of 0.1.

Preparation of Membranes for the Determination of Respiratory Activities

Membranes from *M. vaga* were prepared by the osmotic lysis method as described previously (3), with the following modifications. Cells harvested at the late logarithmic phase of growth were washed twice in 1.0 M NaCl containing 20 mM Tris-acetate (pH 7.5) and 5 mM EDTA. Washed cells were osmotically lysed by being rapidly dispersed in hypotonic solution, 10 mM NaCl containing 20 mM Tris-acetate (pH 7.5) 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 10 mM NaCl containing 20 mM.

Tris-acetate (ph 7.5) and 0.5 mM EDTA, and then DNase, RNase, and $MgSO_4$ were added to give 5 μg/ml, 5 μg/ml, and 2 mM of final concentrations, respectively. After incubating the suspension for 20 min at room temperature, EDTA (5 mM) was added 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 10 mM NaCl containing 20 mM Tris-acetate (pH 7.5) and 0.5 M EDTA, membrane pellets were resuspended at about 20 mg protein/ml in 10 mM NaCl containing 20 mM Tris-acetate (pH 7.5) and 10% glycerol and kept frozen at -80°C.

Measurement of Enzyme Activities

NADH oxidase activity was measured at 30°C spectrophotometrically from a decrease in A_{340}. The assay mixture contained 125 μM NADH or deamino-NADH in 2 ml of 20 mM Tris-acetate (pH 7.5) containing 0.2 M KCl or NaCl. The assay was started by addition of 10 μg of membrane protein. Activity was calculated by using a milimolar extinction coefficient of 6.22. Each reductase activity of the NADH oxidase system was also measured at 30°C spectrophotometrically. The assay mixture for each reductase contained 10 μg of membrane protein, 10 mM KCN, and a given electron acceptor in 2 ml of Tris-acetate (pH 7.5) containing 0.2 M KCl or NaCl. 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, and ferricyanide reductase activity was measured at 420 nm with 1 mM ferricyanide. All reactions of reductases were started by addition of 125 μM NADH or deamino-NADH. Activity was calculated by using milimolar extinction coefficients of 6.81, 6.22, and 1.0, respectively, for Q-1, menadione, and ferricyanide.

Protein Determination

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

Chemicals

NADH (disodium salt), deamino-NADH (sodium salt), potassium ferricyanide, menadione, 2-heptyl-4-hydroxyquinoline-N-oxide (HQNO), rotenone, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), (2-[N-Morpholino]ethanesulfonic acid) (MES), (N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]) (HEPES), and N-tris[Hydroxymethyl]methylglycine (Tricine) were obtained from Sigma. Ubiqinone-1 (Q-1) was a generous gift from Hoffmann-La Roche Inc. Other chemicals used were of the highest commercial grade available.

RESULTS

Growth of *M. vaga* on Media Containing CCCP

The marine bacteria *V. alginolyticus*, *V. costicola*, *V. anguillarum*, and *Alcaligenes* strain 201 that lead to generation of a Na^+ electrochemical gradient by the respiratory Na^+ pump show a proton conductor CCCP-resistant growth under alkaline pH conditions (7, 8, 12, 20). In order to investigate the possibility of whether the cells of *M. vaga* generate a Na^+ electrochemical gradient, we employed the CCCP sensitivity test. Growth of *M. vaga* at pH 6.5 was completely stopped by addition of 2 μM CCCP (Fig. 1A, open squares), whereas growth at pH 8.5 was only slightly inhibited by 2 μM CCCP (Fig. 1B, open circles). Moreover, growth at pH 8.5 was still observed even in the presence of 20 μM CCCP (Fig. 1B, open squares). From these results, it appears that *M. vaga* possesses an electrogenic Na^+ pump and that the pump leads to generation of a Na^+ electrochemical gradient, but not that of a H^+ electrochemical gradient, under alkaline pH conditions.

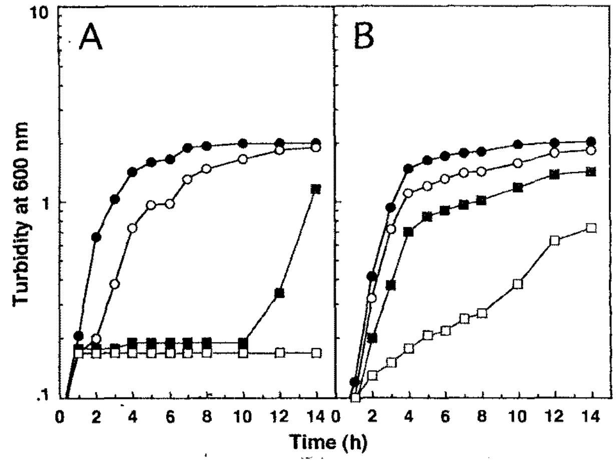

Fig. 1. Effect of CCCP on the growth of *M. vaga*.
The growth of *M. vaga* was determined by measuring the cell density at 600 nm as described under Materials and Methods. CCCP was added to give final concentrations of 0 (closed circles), 0.5 (open circles), 1 (closed squares), and 2 (open squares) μM at (A) MES-NaOH (pH 6.5) and 0 (closed circles), 2 (open circles), 10 (closed squares), and 20 (open squares) μM at (B) Tricine-NaOH (pH 8.5), respectively.

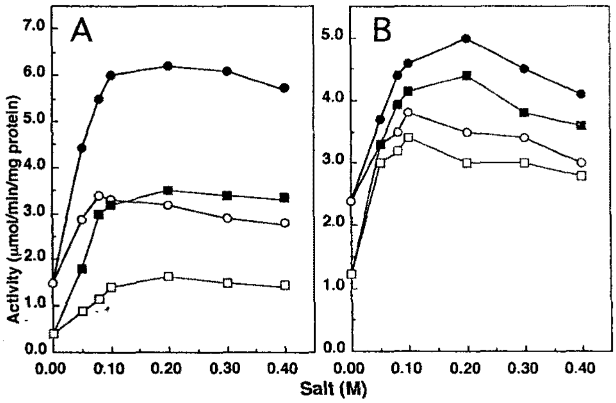

Fig. 2. Effect of salts on the respiratory chain-linked NADH oxidase and NADH:quinone oxidoreductase activities.
(A) The assay mixture of NADH oxidase (2 ml) contained 20 mM Tris-HCl (pH 7.5), 125 μM NADH (circles) or 125 μM d-NADH (squares), and various concentrations of NaCl (closed symbols) and KCl (open symbols). The assay was started by addition of 10 μg of membrane protein at 30°C. (B) The assay mixture of NADH:quinone oxidoreductase (2 ml) contained 20 mM Tris-HCl (pH 7.5), 10 μg of membrane protein, 10 mM KCN, 40.5 μM Q-1, and various concentrations of NaCl (closed symbols) and KCl. The assay was started by the addition of 125 μM NADH (circles) or 125 μM d-NADH (squares) at 30°C. The rate of NADH oxidation was determined from a decrease in A_{340}.

Effects of Salts and pH on the Respiratory Chain-linked NADH Oxidase System of *M. vaga*

An attempt was made to investigate the requirement of Na^+ and the effect of pH on the NADH oxidase from *M. vaga* membrane fractions prepared by the osmotic lysis method. As shown in Fig. 2A, the membrane-bound NADH oxidase oxidized both NADH (circles) and deamino-NADH (squares) as substrates. The rate of NADH and deamino-NADH oxidation was specifically stimulated by Na^+ (closed circles and squares), suggesting that Na^+ is an activator of a reaction step in the respiratory chain-linked NADH oxidase system. The rate of NADH and deamino-NADH oxidation at pH 7.5 was stimulated approximately 4 and 8 fold, respectively, by 0.2 M NaCl, and this stimulation was more pronounced in the alkaline pH range than in the acidic pH range (Fig. 3). The optimal pH for NADH oxidase in the presence of 0.2 M NaCl was 8.5 (Fig. 3). In contrast, the stimulation of the rate of NADH and deamino-NADH oxidation by K^+ (open circles and squares) or other monovalent cations (data not shown) was only slight. The rate of deamino-NADH oxidation was approximately 56% of that of NADH oxidation in the presence of 0.2 M NaCl.

Primary Site of Na^+-dependent Activation in the NADH Oxidase System of *M. vaga*

The marine bacteria *V. alginolyticus* (18, 19), *V. anguillarum* (8), *V. costicola* (23), *V. parahaemolyticus* (22), *Alcaligenes* strain 201 (12), and the halotolerant bacterium Ba_1 (5, 6) are known to possess a respiratory Na^+ pump. The extrusion of Na^+ in *V. alginolyticus*, *V. anguillarum*, and the halotolerant bacterium Ba_1 was

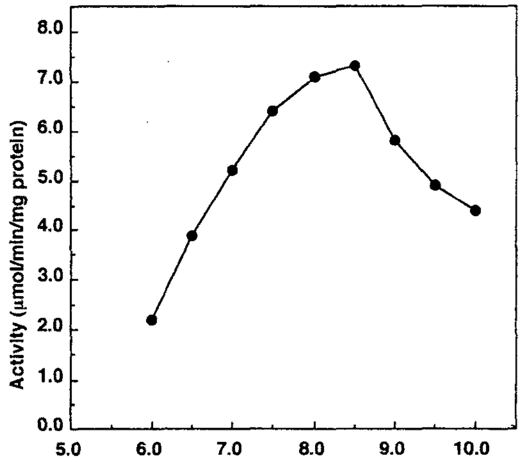

Fig. 3. Effect of pH on the respiratory chain-linked NADH oxidase activity.
NADH oxidase activity was determined with different ranges of pH in the presence of 0.2 M NaCl. Buffers used at 20 mM were MES (pH 6 to 6.5), HEPES (pH 7 to 8), Tricine (pH 8 to 8.5), and CAPSO (pH 9 to 10).

found to be specifically coupled to NADH:quinone oxidoreductase. As shown in Fig. 2B, the activity of NADH:ubiquinone-1 oxidoreductase in *M. vaga* was significantly stimulated with an increase in Na^+ concentration. To examine further whether *M. vaga* possesses a specific Na^+-stimulated NADH:quinone ox-

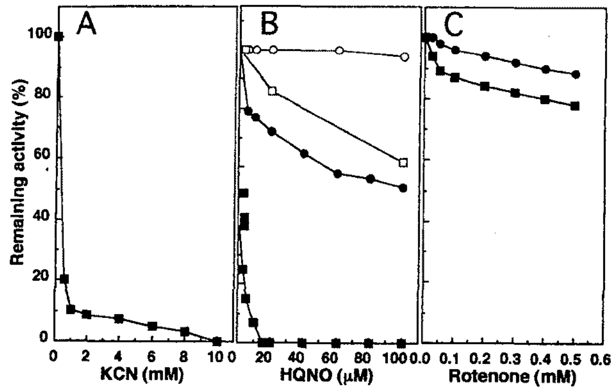

Fig. 4. Effects of respiratory chain inhibitors on the NADH oxidase and NADH:quinone oxidoreductase.

(A) Activity of the NADH oxidase was measured with different concentrations of KCN. (B) Activities of the NADH oxidase (squares) and NADH:quinone oxidoreductase (circles) were measured with different concentrations of HQNO in the presence of (closed symbols) or absence (open symbols) of 0.2 M NaCl. (C) Activities of the NADH oxidase (squares) and NADH:quinone oxidoreductase (circles) were measured with different concentrations of rotenone. The activity of NADH:quinone oxidoreductase was measured in the presence of 20 mM KCN.

idoreductase, we employed artificial electron acceptors, such as those indicated in Table 1. After inhibition of the terminal oxidase with 10 mM KCN, each oxidoreductase activity of the NADH oxidase system was measured. The activity of NADH oxidase was completely inhibited by 10 mM KCN (Fig. 4A). As shown in Table 1, in the presence of NADH or deamino-NADH as electron donors, the activities of NADH (or d-NADH) oxidase and NADH (or d-NADH):ubiquinone-1 oxidoreductase were approximately 1.5-2 fold higher in Na^+ than K^+. In contrast, the other artificial electron acceptors showed no specific requirement for Na^+. These results indicate that the Na^+ requirement of the NADH oxidase system of *M. vaga* is specifically coupled to NADH-linked quinol formation.

Effects of HQNO and Rotenone on the NADH Oxidase and NADH:quinone Oxidoreductase

To determine whether the NADH:quinone oxidoreductase of *M. vaga* possesses the characteristics of the Na^+ pump of marine bacteria, a sensitivity test to respiratory chain inhibitors was employed. As shown in Fig. 4B, the activity of NADH oxidase in the presence of 0.2 M NaCl was completely inhibited by 20 μM 2-heptyl-4-hydroxyquinoline-N-oxide (HQNO), whereas its activity in the absence of 0.2 M NaCl showed far less sensitivity to HQNO.

The NADH:quinone oxidoreductase in the presence of 0.2 M NaCl was also specifically inhibited by HQNO but its activity in the absence of 0.2 M NaCl was highly resistant to HQNO, suggesting that the quinone analogue

Table 1. Effect of salts on the enzyme activities (μmol/min/mg protein) of the NADH oxidase system in membranes prepared from *M. vaga*.

Enzyme	NADH		d-NADH	
	KCl	NaCl	KCl	NaCl
Oxidase	3.2	6.2	1.6	3.5
Ubiqinone-1 oxidoreductase	3.5	5.0	3.0	4.4
Menadione oxidoreductase	4.6	4.4	4.7	4.5
Ferricyanide oxidoreductase	16.6	15.4	16.6	16.6

Each enzyme activity was measured as described under Materials and Methods.

HQNO specifically affects the Na^+-dependent NADH oxidation. On the other hand, the activities of both NADH oxidase and NADH:quinone oxidoreductase were highly resistant to another respiratory inhibitor, rotenone, which is known as the mitochondrial complex I-inhibitor. These findings indicate that the NADH:quinone oxidoreductase of *M. vaga* appears to be very similar to that of *Vibrio alginolyticus* that is known to be an electrogenic Na^+ pump.

DISCUSSION

While a number of studies have been presented documenting the energetics of cells for the generation of a H^+ electrochemical gradient, few studies have been done concerning the energetics of marine bacteria in the generation of a Na^+ electrochemical gradient. Moreover, the studies concerning energetics of halophilic marine bacteria have concentrated on marine *Vibrio* strains. With this work, we have demonstrated that the respiratory chain linked-NADH oxidase system of the marine bacterium *Marinomonas vaga*, like marine *Vibrio* strains, also shows Na^+-dependent activity. The Na^+-dependent site of NADH oxidation was revealed to be the reduction of quinone to quinol, and this step was also shown to be the site for HQNO inhibition. This NADH:quinone oxidoreductase oxidized both NADH and deamino-NADH as substrates. On the other hand, the activities of both NADH oxidase and NADH:quinone oxidoreductase were highly resistant to another respiratory inhibitor, rotenone, which is known as the mitochondrial complex I-inhibitor.

The marine bacteria *V. alginolyticus*, *V. anguillarum*, *V. costicola*, *V. parahaemolyticus*, *Alcaligenes* strain 201, and the halotolerant bacterium Ba_1 are known to possess a respiratory Na^+ pump. The NADH:oxidoreductase segment of the NADH oxidase system in *V. alginolyticus*, *V. anguillarum*, and the halotolerant bacterium Ba_1 was identified as an electrogenic Na^+ pump. Extrusion of Na^+ by the Na^+ pump leads to generation of a Na^+ electrochemical gradient , which is highly resistant to the proton conductor, CCCP and inhibited by the respiratory inhibitor, HQNO but not by rotenone. When growing under alkaline pH conditions, where a Na^+ electrochemical potential instead of a H^+ electrochemical potential across the membrane exists, they exhibit highly resistant growth to CCCP. This property was used by Tokuda to select for CCCP-sensitive mutants that were detected to lack the Na^+ pump activity (15). In the present work, we have employed the CCCP sensitivity test so as to examine the possibility of whether *M. vaga* possesses the electrogenic Na^+ pump. In our experiments, *M. vaga* also showed CCCP-resistant growth at alkaline pH (pH 8.5). Moreover, the Na^+-dependent activity of the NADH oxidase of *M. vaga* was greater at alkaline pH values than at acidic pH values. The pH optimum for NADH oxidation in the presence of 0.2 M NaCl was 8.5.

It has been reported that there are two types of NADH: quinone oxidoreductase in bacteria (11, 13, 21, 25, 26). Type-1 NADH:quinone oxidoreductase reacts with d-NADH as well as with NADH, shows high affinities for NADH (5 to 15 μM), possesses an energy coupling site, and is inhibited by the respiratory inhibitors rotenone and capsaisin, whereas type-2 NADH:quinone oxidoreductase reacts exclusively with NADH without any energy coupling site, shows low affinities for NADH (50 to 60 μM), and is only slightly inhibited by rotenone or capsaicin. Additionally, the type-1 NADH:quinone oxidoreductase of the marine bacterium *Vibrio alginolyticus* shows slightly different properties. Type-1 NADH:quinone oxidoreductase of *Vibrio alginolyticus* requires Na^+ for maximum respiratory activity and functions as an electrogenic Na^+ pump. Moreover, the NADH:quinone oxidoreductase is resistent to rotenone, but highly sensitive to HQNO. HQNO appears to be a specific inhibitor of the Na^+-dependent step. From the above results, *E. coli* (13), *V. alginolyticus* (21), and Thermus thermophilus HB-8 (25) are known to possess both type-1 and type-2 NADH:quinone oxidoreductases. However, *Zymomonas mobilis,* interestingly, has been revealed to possess only type-2 NADH:quinone oxidoreductase lacking the energy coupling site (11).

On the basis of the results presented above, the properties of Na^+-dependent NADH:quinone oxidoreductase existing in *M. vaga* appear to be quite similar to those of *V. alginolyticus,* which is known to be an electrogenic Na^+ pump. Accordingly, we propose the possibility that the type-1 NADH:quinone oxidoreductase of *M. vaga* functions as an electrogenic Na^+ pump. In order to elucidate in more detail whether the NADH:quinone oxidoreductase of *M. vaga* functions as an electrogenic Na^+ pump, we are trying to prepare inside-out membrane vesicles and right side-out membrane vesicles using Kim's methods (8-10) with modification.

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