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# Respiratory Chain-Linked Components of the Marine Bacterium *Vibrio alginolyticus* Affect Each Other

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**The aerobic respiratory chain of *Vibrio alginolyticus* possesses two different kinds of NADH oxidase systems, *i.e.*, an $Na^+$-dependent NADH oxidase system and an $Na^+$-independent NADH oxidase system. When deamino-NADH, which is the only substrate for the $Na^+$-dependent NADH oxidase system, was used as a substrate, the maximum activities of $Na^+$-dependent NADH:quinone oxidoreductase and $Na^+$-dependent NADH oxidase were obtained at about 0.06 M and 0.2 M NaCl, respectively. When NADH, which is a substrate for both $Na^+$-dependent and $Na^+$-independent NADH oxidase systems was used as a substrate, the NADH oxidase activity had a pH optimum at about 8.0. In contrast, when deamino-NADH was used as a substrate, the NADH oxidase activity had a pH optimum at about 9.0. On the other hand, inside-out membrane vesicles prepared from the wild-type bacterium generated only a very small $\Delta pH$ by the NADH oxidase system, whereas inside-out membrane vesicles prepared from Nap1, which is a mutant defective in the $Na^+$ pump, generated $\Delta pH$ to a considerable extent by the NADH oxidase system. On the basis of the results, it was concluded that the respiratory chain-linked components of *V. alginolyticus* affect each other.**

***Key words***: marine bacterium, *Vibrio alginolyticus*, respiratory chain, $Na^+$-dependent NADH oxidase, $Na^+$-independent NADH oxidase

Two kinds of respiratory chain-linked NADH oxidase systems were identified from the membrane fractions of the marine bacterium *Vibrio alginolyticus* (Tokuda, 1983; Tokuda and Unemoto, 1984a). One enzyme system requires $Na^+$ for maximum respiratory activity and functions as an electrogenic $Na^+$ pump (Tokuda and Unemoto, 1984a; Tokuda and Unemoto, 1984b). The respiratory $Na^+$ pump has a pH optimum at alkaline pH. The other enzyme system does not. The $Na^+$-dependent NADH oxidase system oxidizes both NADH and deamino-NADH as substrates and is strongly inhibited by a respiratory inhibitor, 2-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO), while the $Na^+$-independent NADH oxidase system oxidizes NADH exclusively and is highly resistant to HQNO (Tokuda, 1989). The $Na^+$-dependent site in the NADH oxidase system of *V. alginolyticus* is a type 1 NADH:quinone oxidoreductase, and this segment is also the site of HQNO inhibition. Nap1, a mutant bacterium defective in the $Na^+$ pump, lacks the function of type 1 NADH:quinone oxidoreductase but retains the activity of type 2 NADH: quinone oxidoreductase. Thus, Nap1 membranes have no capacity to generate a $Na^+$ electrochemical potential. As a consequence, the respiratory chain-linked NADH oxidase system of *V. alginolyticus* generates a $Na^+$ electrochemical potential at the type 1 NADH:quinone oxidoreductase segment and a $H^+$ electrochemical potential at the quinol oxidase segment (Tokuda, 1989; Kim *et al.*, 1991).

As mentioned above, the aerobic respiratory chain of *V. alginolyticus* consists of type 1 NADH:quinone oxidoreductase, type 2 NADH:quinone oxidoreductase, and quinol oxidase. Thus, the $Na^+$-dependent properties of the NADH oxidase system may be affected by those of $Na^+$-independent NADH:quinone oxidoreductase and quinol oxidase. In the present work, I describe how the respiratory chain-linked components of the marine bacterium *V. alginolyticus* reciprocally affect their enzyme activities and energy generation.

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## Materials and Methods

***Bacterial strain and growth conditions***

The bacterial strain used in this study was *V. alginolyticus* 138-2, which was kindly provided by Dr. H. Tokuda (University of Tokyo). The bacterium was grown aerobically at 37°C in a liquid medium containing 0.5% polypeptone,

0.5% yeast extract, 0.4% $K_2HPO_4$, 3% NaCl, and 0.2% glucose.

***Preparation of membrane fractions for the determination of respiratory activities and enzyme assay***

Cells of *V. alginolyticus* were osmotically lysed to prepare membrane fractions (Tokuda, 1986). Activities of NADH oxidase and NADH:quinone oxidoreductase were measured spectrophotometically at 30°C. NADH oxidase activity was measured from a decrease in absorbance at 340 nm, and the activity was calculated using the millimolar extinction coefficient of 6.22. $Q_1H_2$ formation by NADH:quinone oxidoreductase was followed by monitoring the absorbance change between 248 and 267 nm using a dual-wavelength spectrophotometer. The millimolar extinction coefficient of Q-1 was 7.8 at these wavelengths.

***Preparation of Inside-out membrane vesicles***

Cells were harvested by sedimentation at the late logarithmic growth phase, washed twice in 400 mM potassium phosphate (pH 7.5) with resedimentation. Cells were resuspended in the same buffer supplemented with 5 mM $MgSO_4$ at 5 ml/g wet weight of cells. The cell suspension was passed through a French pressure cell once at 8,000 psi. Unbroken cells were removed by sedimentation at 25,000×g for 10 min, and a membrane pellet was obtained after sedimentation at 120,000×g for 2 h. Inside-out membrane vesicles were washed once in 400 mM potassium phosphate (pH 7.5) supplemented with 5 mM $MgSO_4$ and resedimented. The pellet was resuspended in the buffer solution containing 10% glycerol to give a final concentration of about 40 mg protein/ml and kept frozen at -80°C.

***Detection of $\Delta\Psi$ and pH***

The generation of $\Delta\Psi$ (inside-positive) and $\Delta$pH (inside-acidic) in membrane vesicles was monitored at 30°C by following the fluorescence quenching of oxonol V and quinacrine, respectively. The fluorescence emission of oxonol V was measured at 635 nm with excitation at 580 nm and that of quinacrine was measured at 500 nm with excitation 420 nm.

***Protein assay***

Protein concentration was measured with a Bio-Rad protein assay kit (Bio-Rad Labs., Richmond, CA, USA) with bovine serum albumin as the standard.

## Results and Discussion

***$Na^+$ concentrations for the maximum activities of NADH oxidase and NADH:quinone oxidoreductase***

The $Na^+$-dependent NADH oxidase system of *V. alginolyticus* consists of type 1 NADH:quinone oxidoreductase and quinol oxidase, and the $Na^+$-dependent actvity of NADH oxidase system is due to the property of type 1 NADH:quinone oxidoreductase (Tokuda and Unemoto, 1984; Kim *et al.*, 1991). In order to examine whether the properties of quinol oxidase affect the $Na^+$-dependent activity of NADH oxidase system, a comparison was made

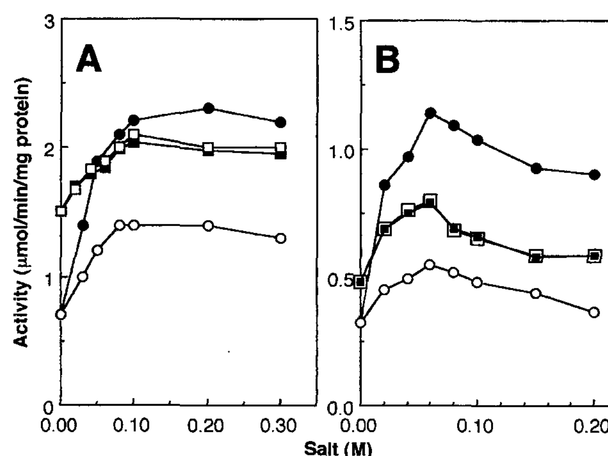


**Fig. 1.** Effect of salts on the activities of NADH oxidase and NADH:quinone oxidoreductase in the wild-type *V. alginolyticus* (circles) and Nap1 (squares) membrane fractions. (A) The assay mixture of NADH oxidase (2 ml) contained 20 mM Tris-HCl (pH 7.5), 125 μM NADH, and various concentrations of NaCl (●, ■) and KCl (○, □). The assay was started by addition of 30 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), 5 μg of membrane protein, 10 mM KCN, 55 μM NADH, and various concentrations of NaCl (●, ■) and KCl (○, □). The assay was started by addition of 10 μM Q-1 at 30°C.

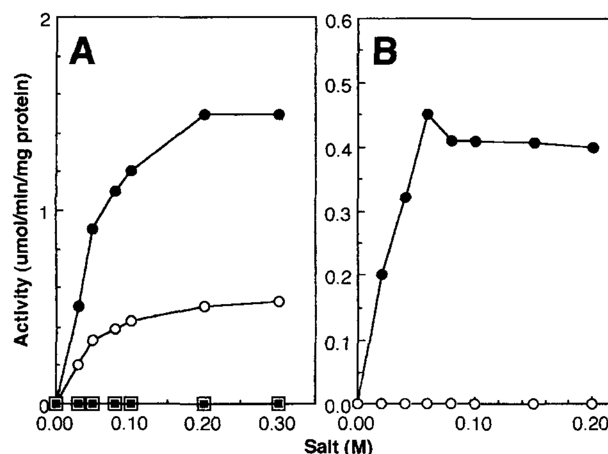


**Fig. 2.** Effect of salts on the activities of deamino-NADH oxidase and deamino-NADH:quinone oxidoreductase in the wild-type *V. alginolyticus* (circles) and Nap1 (squares) membrane fractions. (A) The assay mixture of NADH oxidase (2 ml) contained 20 mM Tris-HCl (pH 7.5), 125 μM deamino-NADH, and various concentrations of NaCl (●, ■) and KCl (○, □). The assay was started by addition of 30 μg of membrane protein at 30°C. (B) The assay mixture of NADH:quinone reductase (2 ml) contained 20 mM Tris-HCl (pH 7.5), 5 μg of membrane protein, 10 mM KCN, 55 μM deamino-NADH, and various concentrations of NaCl (●) and KCl (○). The assay was started by addition of 10 μM Q-1 at 30°C.

between the activities of $Na^+$-dependent NADH oxidase and type 1 NADH:quinone oxidoreductase. As shown in Figs. 1 and 2, membranes prepared from the wild-type bacterium, which possess the $Na^+$-independent NADH oxidase system as well as the $Na^+$-dependent NADH oxidase system oxidized both NADH and deamino-NADH as substrates. The activities of NADH oxidase and NADH:quinone oxidoreductase in the membranes of wild-type bacterium significantly increased with an increase in $Na^+$ concentration. The maximum activity of NADH oxidase was obtained at approximately 0.2 M $Na^+$, whereas the maximum activity of NADH:quinone oxidoreductase was obtained at 0.06 M $Na^+$. The stimulation by $K^+$ was only slight. On the other hand, the activities of NADH oxidase and NADH:quinone oxidoreductase in the membranes of Nap1 showed no specific requirement for $Na^+$, indicating that NADH oxidase and NADH:quinone oxidoreductase in the membranes of Nap1 are $Na^+$-independent. In order to obtain the $Na^+$-dependent activity alone except for the $Na^+$-independent activity in the membranes of wild-type bacterium, deamino-NADH as a substrate was used. Even when deamino-NADH as a substrate was used, the maximum activity of NADH oxidase was obtained at approximately 0.2 M $Na^+$, while the maximum activity of NADH:quinone oxidoreductase was obtained at 0.06 M $Na^+$. These results suggest that the $Na^+$-dependent activity of NADH oxidase system is affected by the quinol oxidase, and the type 1 NADH:quinone oxidoreductase interacts with the quinol oxidase.

### *pH-dependent activities of $Na^+$-dependent NADH oxidase and $Na^+$-independent NADH oxidase*

It has been reported that the activity of the membrane-bound NADH oxidase of *V. alginolyticus* in the presence of 0.2 M NaCl is dependent on pH with a maximum at pH 8.0 and a minimum at pH 6.0 (Tokuda and Unemoto, 1984). The activiy in Nap1 membranes with a pH optimum at about 7.0 are less dependent on pH than those determined in $Na^+$ with the wild type (Tokuda and Unemoto, 1984). An attempt was made to investigate whether the optimum pH of $Na^+$-dependent NADH oxidase is affected by the other respiratoy components (Fig. 3A). When NADH was used as a substrate, NADH oxidase activity had a pH optimum at about 8.0. In contrast, when deamino-NADH was used as a substrate, NADH oxidase activity had a pH optimum at about 9.0. These results indicate that the true optimum pH of $Na^+$-dependent NADH oxidase is decreased by the other respiratoy components.

On the other hand, in order to test the activity of $Na^+$-dependent NADH oxidase alone at pH 6.5 and 8.5, deamino-NADH was used as a substrate. As shown in Fig. 3B, the activity of $Na^+$-dependent NADH oxidase significantly increased with an increase in $Na^+$ concentration at pH 6.5 as well as at pH 8.5. These results suggest that type 1 NADH:quinoe oxidoreductase, which is known to be a $Na^+$ pump, may extrude $Na^+$ even at acidic pH as well as at alkaline pH.

### *Generation of $\Delta\Psi$ at the $Na^+$-dependent NADH oxidase and $Na^+$-independent NADH oxidase*

Inside-out membrane vesicles prepared from the wild-type *V. alginolyticus* quenched the fluorescence of oxonol V on the addition of NADH or deamino-NADH (Figs. 4A and D). This membrane potential ($\Delta\Psi$) was highly resistant to a proton conductor, carbonylcyanide *m*-chlorophenylhydrazone (CCCP) (Figs. 4B and E), but completely collapsed by the combined addition of CCCP and monensin, an electroneutral ionophore specific to $Na^+$ and $H^+$ (Fig. 4C). In con-

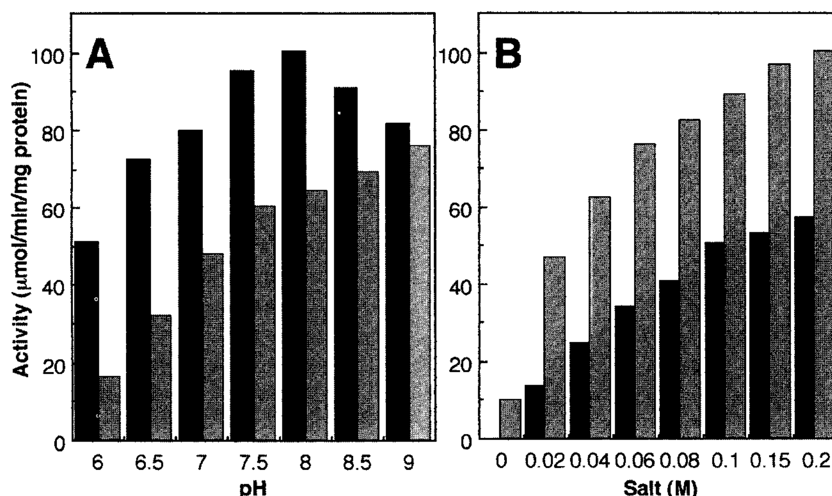


**Fig. 3.** Effect of pH on the activity of NADH oxidase. (A) NADH oxidase activity was determined with different ranges of pH in the presence of 0.2 M NaCl. Buffers used at 20 mM were MES (pH 6 to 6.5), HEPES (pH 7 to 8), and Tricine (PH 8 to 9). NADH (■) and deamino-NADH (▒) were used as substrates. (B) The effect of NaCl concentration on the activity of NADH oxidase was examined at pH 6.5 (■) and 8.5 (▒) by using deamino-NADH as a substrate.

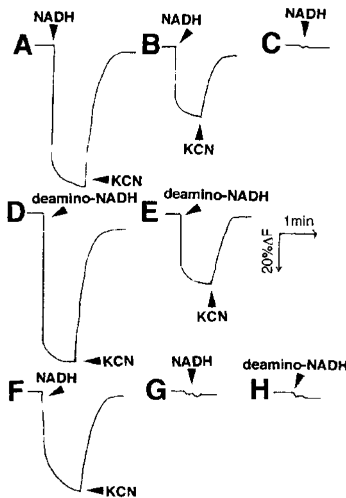


**Fig. 4.** Generation of ΔΨ during electron transfer from NADH or deamino-NADH to oxygen in inside-out membrane vesicles. Inside-out membrane vesicles prepared from the wild-type (0.2 mg membrane protein, A to E) or Nap1 (0.4 mg membrane protein, F to H) were used to measure the generation of ΔΨ. Assays B, E, and G were performed in the presence of 5 μM CCCP, and assay C was performed in the presence of both 5 μM CCCP and 30 μM monensin at 30°C. NADH, deamino-NADH, and KCN were added at final concentrations of 1 mM, 1 mM, and 10 mM, respectively.

trast, the inside-out membrane vesicles prepared from Nap1 lacking type 1 NADH : quinone oxidoreductase generated the CCCP-sensitive ΔΨ (Fig. 4G), and did not oxidize deamino-NADH (Fig. 4H). These results indicate that CCCP-resistant ΔΨ generated by the wild-type strain is a ΔΨ due to the type 1 NADH:quinone oxidoreductase, and the CCCP-sensitive ΔΨ generated by the Nap1 strain is a ΔΨ due to the quinol oxidase segment.

#### *Generation of ΔpH at the $Na^+$-dependent NADH oxidase and $Na^+$-independent NADH oxidase*

It has been reported that the NADH oxidase in inside-out membrane vesicles prepared from wild-type *V. alginolyticus* appears to generate little ΔpH, suggesting that no $H^+$ is extruded by the NADH oxidase of *V. alginolyticus* (Tokuda *et al.*, 1985). In order to ascertain whether the NADH oxidase of *V. alginolyticus* extrudes $H^+$ or not, a fluorescence quenching technique was employed. Inside-out membrane vesicles prepared from the wild-type bacterium, which type 1 NADH:quinone oxidoreductase generates a $Na^+$ electrochemical potential, generated a very small ΔpH on the addition of NADH (Fig. 5A). This ΔpH was considerably stimulated when a membrane permeable anion, $SCN^-$, was added to the reaction mixture to collapse ΔΨ (Fig. 5C). In contrast, when the inside-out membrane vesicles prepared from Nap1 that possess only type 2 NADH:quinone oxidoreductase lacking the energy coupling site were used, the generation of ΔpH by the NADH oxidase was more significant (Fig. 5B). This ΔpH was

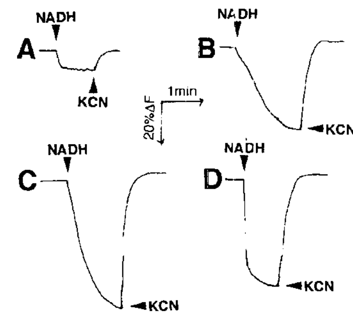


**Fig. 5.** Generation of ΔpH during electron transfer from NADH to oxygen in inside-out membrane vesicles. Inside-out membrane vesicles prepared from the wild-type (0.2 mg membrane protein, A and C) or Nap1 (0.4 mg membrane protein, B and D) were used to measure the generation of ΔpH. Assays C and D were performed in the presence of 50 mM KSCN at 30°C. NADH and KCN were added at final concentrations of 1 mM and 10 mM, respectively.

also stimulated when a membrane permeable anion, $SCN^-$, was added to the reaction mixture to collapse ΔΨ (Fig. 5D). These results suggest that the $Na^+$ chemical gradient ($\Delta pNa^+$) generated by type 1 NADH:quinone oxidoreductase disturbs the generation of ΔpH by the quinol oxidase segment.

From the above-described results, it was concluded that the respiratory chain-linked components of *V. alginolyticus* affect each other.

## Acknowledgment

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