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# The Succinate: Quinone Oxidoreductase of Marine Bacterium *Vibrio alginolyticus* is a $H^+$ Pump

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**Abstract** The energetics at the succinate:quinone oxidoreductase segment of *V. alginolyticus* was studied using a fluorescence quenching technique with inside-out membrane vesicles. A transient generation of the membrane potential (inside-positive) and ΔpH (inside-acidic) occurred in the presence of KCN and succinate when ubiquinone-1 (Q1) was added. The membrane potential (ΔΨ) generated by the succinate: quinone oxidoreductase segment was completely collapsed by the protonophore carbonylcyanide *m*-chlorophenylhydrazone (CCCP) and the membrane permeable anion $SCN^-$, whereas the ΔpH was completely collapsed by CCCP and $(NH_4)_2SO_4$. From these results, it was concluded that the succinate: quinone oxidoreductase segment as well as quinol oxidase [1] in the respiratory chain of *V. alginolyticus* generated $H^+$ electrochemical potential.

**Key words:** Marine bacterium, *Vibrio alginolyticus*, respiratory chain, succinate:quinone oxidoreductase, $H^+$ pump

The marine bacterium *Vibrio alginolyticus* possesses two kinds of NADH:quinone oxidoreductases (NQR) in its respiratory chain [1, 4, 5]. One enzyme system (NQR1) requires $Na^+$ for its maximum respiratory activity and functions as an electrogenic $Na^+$ pump, whereas the other enzyme system ($Na^+$-independent NQR2) has no capacity to generate an electrochemical ion gradient. However, the quinol oxidase of *V. alginolyticus* is known to be a $H^+$ pump extruding $H^+$ [1]. Accordingly, the respiratory chain of *V. alginolyticus* has extrusion systems for both $Na^+$ and $H^+$. However, the energetics at the succinate:quinone oxidoreductase segment of *V. alginolyticus* is still unknown. It has been previously reported that the succinate:quinone oxidoreductase of *Escherichia coli* generates no significant membrane potential [2]. A technique that detects the ΔΨ and ΔpH generated in the respiratory chains of bacteria by monitoring the quenching of the fluorescent probes was previously developed [1, 3]. Using this fluorescence quenching technique, the present study shows, for the first time, that the succinate:quinone oxidoreductase of *V. alginolyticus* can function as a $H^+$ pump, which generates a $H^+$ electrochemical potential.

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## MATERIALS AND METHODS

### Preparation of Inside-Out Membrane Vesicles

*V. alginolyticus* 138-2, kindly provided by Dr. H. Tokuda (University of Tokyo, Tokyo, Japan), 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. The pH of the medium was adjusted to 7.5. The cells at the mid-logarithmic growth phase were harvested by sedimentation and washed twice in a 0.4 M potassium phosphate buffer (pH 7.5) with resedimentation. The cells were then resuspended in the same buffer supplemented with 5 mM $MgSO_4$ at a 6 ml/g wet weight of cells. The cell suspension was passed once through a French pressure cell (Aminco) at 8,000 psi. The unbroken cells were then removed by centrifugation at 30,000 ×g for 10 min and a membrane pellet was obtained after centrifugation at 120,000 ×g for 2 h. The inside-out membrane vesicles were washed once in 0.4 M potassium phosphate buffer (pH 7.5) supplemented with 5 mM $MgSO_4$ and recentrifuged. The pellet was finally 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 ΔΨ and ΔpH

The generation of ΔΨ (inside-positive) and ΔpH (inside-acidic) in the membrane vesicles was monitored at 30°C by following the fluorescence quenching of oxonol V and quinacrine, respectively. Two-ml of the reaction mixture contained 0.4 M potassium phosphate buffer (pH 7.5), 5 mM $MgSO_4$, 10 mM $Na_2SO_4$, 1 μM oxonol V or

quinacrine, and 0.2 mg of membrane protein with or without 10 mM KCN. Succinate, Q1, carbonylcyanide *m*-chlorophenylhydrazone (CCCP), $(NH_4)_2SO_4$, valinomycine, and KSCN were then added at final concentrations of 10 mM, 51 μM, 5 μM, 5 mM, 25 μM, and 50 mM, respectively. The fluorescence emission of oxonol V was measured at 635 nm with an excitation at 580 nm and that of quinacrine was measured at 500 nm with an excitation at 420 nm.

### Protein Assay

The protein concentrations were determined by Bio-Rad Protein Assay, based on the method of Bradford, using bovine serum albumin as a standard.

## RESULTS AND DISCUSSION

### ΔΨ Generation during Electron Transfer from Succinate to Oxygen or Q1 in Inside-Out Membrane Vesicles

It is already known that the succinate oxidation and coupled ΔΨ generation by the respiratory chain of the inside-out membrane vesicles of *V. alginolyticus* do not depend on $Na^+$ concentration [3]. In the present study, the energetics in the succinate:quinone oxidoreductase segment of the succinate oxidase system of *V. alginolyticus* was examined. The inside-out membrane vesicles prepared from *V. alginolyticus* quenched the fluorescence of oxonol V on addition of succinate (Fig. 1A). After the inhibition of quinol oxidase by KCN, the addition of Q1 as an electron acceptor resulted in a transient quenching of fluorescence (Fig. 1A). A second addition of Q1, after the completion of the transient quenching induced by the first addition of Q1, caused similar transient quenching (Fig. 1B). The membrane potential generated at the succinate: quinone oxidoreductase segment and by the succinate oxidase was completely collapsed by protonophore CCCP (Fig. 1C) or the membrane permeable anion $SCN^-$ (data not shown). In contrast, the membrane potential generated in the succinate:quinone oxidoreductase segment was significantly stimulated by the presence of $(NH_4)_2SO_4$, which dissipated the ΔpH (Fig. 1D). These results indicate that the fluorescence quenching induced by the addition of Q1 in the presence of KCN and succinate represents the generation of ΔΨ in the succinate:quinone oxidoreductase segment.

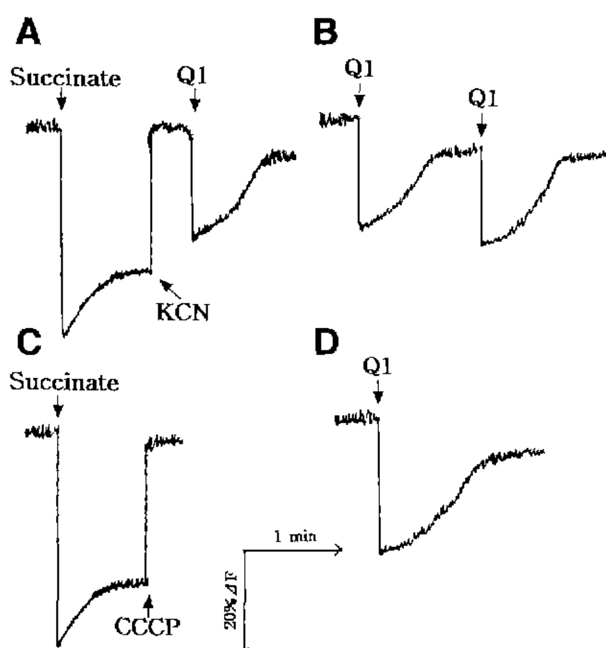


**Fig. 1.** Generation of ΔΨ at the succinate oxidase and succinate:quinone oxidoreductase segments.

Inside-out membrane vesicles prepared from *V. alginolyticus* were used to measure the generation of ΔΨ (A to D). The reaction mixture in panel B contained 0.2 mg of the membrane protein, 10 mM KCN, and 10 mM succinate The reaction mixture in panel D contained 0.2 mg of the membrane protein, 10 mM KCN, 10 mM succinate, and 5 mM $(NH_4)_2SO_4$. Succinate, Q1, CCCP, and $(NH_4)_2SO_4$ were added as specified in Materials and Methods.

### ΔpH Generation during Electron Transfer from Succinate to Oxygen or Q1 in Inside-Out Membrane Vesicles

The inside-out membrane vesicles prepared from *V. alginolyticus* quenched the fluorescence of quinacrine on addition of succinate (Fig. 2A). The ΔpH generated by the succinate oxidation was completely with the inhibition of

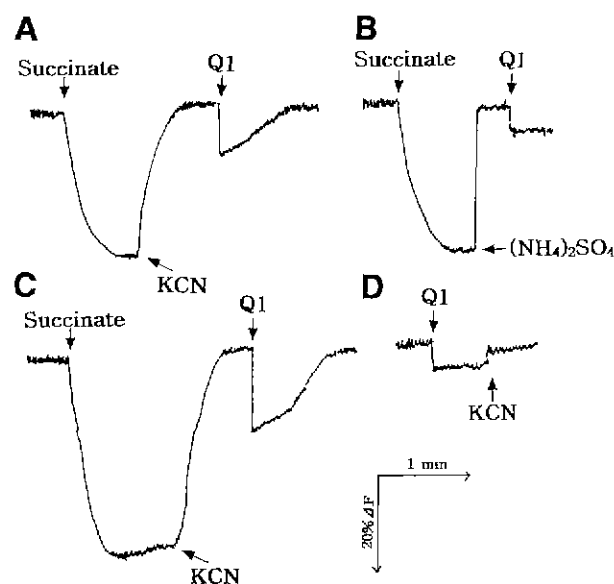


**Fig. 2.** Generation of ΔpH at the succinate oxidase and succinate:quinone oxidoreductase segments.

Inside-out membrane vesicles prepared from *V. alginolyticus* were used to measure the generation of ΔpH (A to D). Succinate, Q1, CCCP, $(NH_4)_2SO_4$, and valinomycine were added as specified in Materials and Methods.

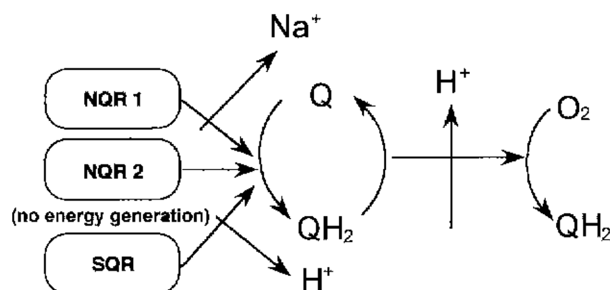


**Fig. 3.** A scheme of the respiratory chain of the marine bacterium *V. alginolyticus*. NQR and SQR refer to NADH: quinone oxidoreductase and succinate:quinone oxidoreductase, respectively.

quinol oxidase by KCN. Subsequent addition of Q1 as an electron acceptor resulted in a transient quenching of fluorescence. The ΔpH generated at the succinate:quinone oxidoreductase and succinate oxidase segments were completely collapsed by the protonophore CCCP (data not shown) or $(NH_4)_2SO_4$ (Fig. 2B). In contrast, the ΔpH generated in the succinate:quinone oxidoreductase segment was significantly stimulated by the presence of valinomycine, which dissipated the ΔΨ (Fig. 2C). Figure 2D shows quenching by Q1 in the presence of the membrane and quinacrine without the addition of succinate. Accordingly, the fluorescence quenching induced by the addition of Q1 in the presence of the membrane, KCN, and succinate represents the generation of ΔpH at the succinate:quinone oxidoreductase segment. On the basis of the above results, it was concluded that the succinate:quinone oxidoreductase of *V. alginolyticus* functions as a $H^+$ pump, which generates a $H^+$ electrochemical potential. Thus, the marine bacterium *V. alginolyticus* possesses one $Na^+$ pump and two $H^+$ pumps in its respiratory chain (Fig. 3). This data will help in understanding the $Na^+$ and $H^+$ energetics in the *V. alginolyticus* membrane.

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