Generation of the electrochemical potential of Na^+ by the Na^+-motive NADH oxidase in inverted membrane vesicles of *Vibrio alginolyticus*

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Inverted membrane vesicles prepared from *Vibrio alginolyticus* generated a membrane potential (positive inside) and accumulated Na^+ by the oxidation of NADH. Generation of the membrane potential required Na^+ and was inhibited by 2-heptyl-4-hydroxyquinoline *N*-oxide, a specific inhibitor of the Na^+-dependent NADH oxidase. Collapse of the membrane potential by valinomycin stimulated the uptake of Na^+. In contrast, accumulation of H^+ was not detected under all the conditions tested. These results suggest that only Na^+ is translocated by the Na^+-dependent NADH oxidase of *V. alginolyticus*.

Inverted vesicle *Na^+ pump* *NADH oxidase* *Marine bacterium* *Na^+ electrochemical potential* *Respiration*

1. INTRODUCTION

The marine bacterium *Vibrio alginolyticus* retains an Na^+ pump which is coupled to respiration [1,2]. Examinations of respiratory activities in the wild-type, Na^+ pump-defective mutants, and a spontaneous revertant revealed that the Na^+ pump is coupled to Na^+-dependent NADH oxidase [3,4]. Moreover, it was shown that the Na^+-dependent NADH oxidase reconstituted into liposomes translocated Na^+ as a primary result of NADH oxidation [5]. Since NADH is impermeable to membranes, inverted membrane vesicles were expected to provide a useful system for studying the relationship between Na^+ translocation and NADH oxidation. Here, inverted membrane vesicles were prepared from *V. alginolyticus* and examined for Na^+ pump activity.

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; HQNO, 2-heptyl-4-hydroxyquinoline *N*-oxide; $\Delta\psi$, membrane potential

2. EXPERIMENTAL

2.1. *Preparation of inverted membrane vesicles*

V. alginolyticus 138-2 was grown at pH 8.5 on a complex medium [6] supplemented with 0.2% glucose and harvested at the late logarithmic phase of growth. A buffer solution containing 10 mM Hepes-KOH, pH 7.5, 0.2 M K_2SO_4, 5 mM $MgSO_4$ was used for the preparation of K^+-containing vesicles (K^+-vesicles). K^+ in this buffer solution was replaced by Na^+ for the preparation of Na^+-containing vesicles (Na^+-vesicles). The harvested cells were washed with and resuspended in the respective buffer solution at a concentration of 0.2 g wet wt/ml. The cells were ruptured by a single passage through a French pressure cell at 8000 lb/inch^2. Unbroken cells and large debris were removed by centrifugation at 30000 × *g* for 15 min. Inverted vesicles were collected by centrifuging the supernatant at 100000 × *g* for 2 h and washed once with the buffer solution. 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 −70°C. The

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intravesicular volume of inverted vesicles was determined from the difference in [^{3}H]water and [^{14}C]lactose spaces as in [7]. Protein was determined by the method in [8].

2.2. *Assays for the Na^+-motive NADH oxidase*

NADH oxidase was spectrophotometrically assayed at 30°C as in [4]. Flow dialysis [1,9] was performed at room temperature to determine $\Delta\psi$ (inside positive), ΔpH (inside acidic or alkaline) and Na^+ concentration gradient as in [5] except that 20 units of alcohol dehydrogenase (EC 1.1.1.1, Sigma) and 1% (w/v) ethanol were included in the upper chamber of the flow dialysis cell. The reaction mixture was kept under a stream of oxygen. The buffer solution pumped through the lower chamber also contained 1% ethanol and had the same salt compositions as those in the upper chamber. Radioactivities in dialysate were continuously monitored as in [1].

3. RESULTS

3.1. *NADH oxidase in inverted membrane vesicles*

When cells of *V. alginolyticus* are lysed by exposure to hypotonic medium, most populations of membranes isolated from the lysates still retain rod-shaped structure and are permeable to dextran [10]. On the other hand, rupture of the cells by passage through a French pressure cell led to the formation of inverted membrane vesicles. Na^+ seemed to be unnecessary for the preparation of inverted vesicles, since the lactose-impermeable space of K^+-vesicles (0.27 μg/mg protein) was similar to that of Na^+-vesicles (0.24 μg/mg protein). NADH oxidase in both vesicles required external Na^+ for maximum activity, whereas internal Na^+ had only a marginal effect on the activity (table 1). In the presence of 1 μM HQNO, the activity was inhibited to a similar level under all conditions examined.

Table 1

NADH oxidase in inverted membrane vesicles prepared from *V. alginolyticus*

Cation		Activity (μmol/min per mg protein)	
Internal	External	−HQNO	+HQNO
K^+	K^+	0.83	0.35
	Na^+	2.07	0.45
Na^+	K^+	1.29	0.33
	Na^+	2.23	0.43

Enzyme activity was determined in the presence of K^+ or Na^+ at pH 7.5 as described in the text using inverted vesicles containing K^+ or Na^+. The effect of 1 μM HQNO on the activity was also determined under each condition

3.2. *Generation of $\Delta\psi$ and ΔpH by inverted membrane vesicles*

$\Delta\psi$ (inside positive) was determined at pH 7.5 from the distribution of SCN^- by flow dialysis (fig.1). Since large amounts of inverted vesicles were needed for the detection of $\Delta\psi$ by flow dialysis, NADH added at 10 mM was exhausted shortly after its addition and caused only a transient accumulation of SCN^- (A). Subsequent addition of alcohol dehydrogenase in the presence of 1% ethanol led to regeneration of NADH and hence $\Delta\psi$. Therefore, the following experiments were performed in the presence of an NADH-generating system. K^+-vesicles in the presence of 0.4 M Na^+ generated 101 mV of $\Delta\psi$ which was collapsed by combined addition of HQNO and CCCP (A) or by single addition of valinomycin (B). As shown in inverted membrane vesicles of *Escherichia coli* [11], Cl^- was permeable to membranes and collapsed $\Delta\psi$ (C and E). $\Delta\psi$ generated by K^+-vesicles in the absence of Na^+ was small (76 mV) and stimulated to 99 mV by addition of 20 mM Na^+ (D). Na^+-vesicles in the presence of 0.4 M Na^+ generated $\Delta\psi$ of 148 mV at 5 min after addition of NADH (E), which was considerably larger than that generated by K^+-vesicles in 0.4 M Na^+. When inverted vesicles were treated with HQNO, generation of $\Delta\psi$ was markedly inhibited (F).

Examinations of methylamine uptake or quinacrine fluorescence quenching revealed that no ΔpH (inside acidic) was generated during oxidation of NADH by inverted vesicles. Collapse of $\Delta\psi$ by valinomycin in K^+-vesicles or by Cl^- in Na^+-vesicles did not lead to the generation of ΔpH. In contrast, Na^+-vesicles in the presence of CCCP at pH 7.5 accumulated acetate indicating the generation of inside alkaline ΔpH of 57 mV (not shown).

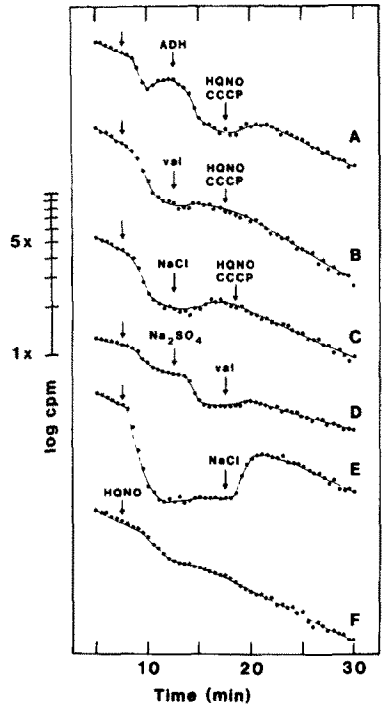

Fig.1. Generation of $\Delta\psi$ (inside positive) by inverted vesicles prepared from *V. alginolyticus*. Flow dialysis was performed as described in the text to determine $\Delta\psi$. Unless otherwise specified, assay mixture (0.4 ml) contained 50 mM Hepes-NaOH, pH 7.5, 0.2 M Na_2SO_4, 5 mM $MgSO_4$, 1% (w/v) ethanol, 20 units alcohol dehydrogenase (340 units/mg protein) and 10 mg protein of K^+-vesicles. The first arrow in each pattern indicates the addition of NADH at 10 mM. In assay A, alcohol dehydrogenase (ADH) was added after addition of NADH as indicated. Assay D was performed in a buffer containing K^+ in place of Na^+. Na^+-vesicles instead of K^+-vesicles were examined in assay E. HQNO was added together with NADH in assay F. Each assay was started by addition of $KS^{14}CN$ (58 μCi/μmol, 65 μM) at zero time. Disodium NADH was used in all assays except for assay D (dipotassium NADH). Other additions were made as indicated at following final concentrations: HQNO, 50 μM; CCCP, 5 μM; valinomycin (val), 25 μM; NaCl, 50 mM; Na_2SO_4, 10 mM.

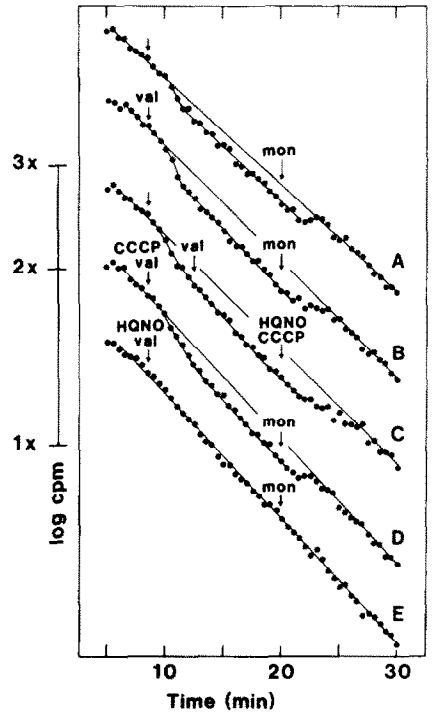

Fig.2. Na^+ accumulation by inverted vesicles. Flow dialysis was performed as described in the text and in fig.1. K^+-vesicles (10 mg protein) were examined in 0.4 ml of assay mixture containing 50 mM Hepes-KOH, pH 7.5, 0.2 M K_2SO_4, 5 mM $MgSO_4$, 1% (w/v) ethanol and 20 units alcohol dehydrogenase. $^{22}NaCl$ (1.2 μCi, carrier-free) was added at zero time to start assays. At the first arrow in each pattern, disodium NADH was added at 5 mM with or without specified reagents. Monensin (mon) was added at a final concentration of 20 μM. Other additions were as in fig.1.

3.3. *Accumulation of Na^+ by inverted vesicles*

$^{22}Na^+$ uptake by K^+-vesicles at pH 7.5 was monitored by flow dialysis (fig.2). Addition of 5 mM NADH in the presence of an NADH-generating system led to the accumulation of Na^+ by inverted vesicles (A). At about 5 min after NADH addition, the concentration gradient of Na^+ across the membrane was 8.3. Valinomycin added with NADH (B) or after addition of NADH (C) did not inhibit but rather stimulated Na^+ accumulation. $[Na^+]_{in}/[Na^+]_{out}$ of 15 was calculated at 5 min after additions of NADH and valinomycin (B). Inverted vesicles treated with CCCP and valinomycin maintained an about 12-fold concentration gradient of Na^+ throughout the assay (D). Addition of monensin or CCCP plus HQNO caused release of accumulated Na^+ under all the conditions. No accumulation of Na^+ occurred when inverted vesicles were pretreated with HQNO (E).

4. DISCUSSION

Our results confirm that the Na^+-dependent NADH oxidase of *V. alginolyticus* is an Na^+-motive redox pump. Na^+ translocation observed must be a primary event of NADH oxidation since the collapse of $\Delta\psi$ did not inhibit but stimulated Na^+ accumulation. Moreover, inverted vesicles accumulated Na^+ even in the presence of CCCP. On the other hand, H^+ uptake was not detected under all the conditions examined. These results suggest that H^+ is not translocated by the Na^+-dependent NADH oxidase. However, an oxygen pulse to anaerobic cell suspensions led to the extrusion of both H^+ and Na^+ [2]. Therefore, H^+ translocation in whole cells seemed to be ascribed to respiratory chains other than the Na^+-dependent NADH oxidase. Indeed, HQNO specifically inhibited Na^+ translocation with little effect on H^+ translocation by whole cells [4]. The effects of various energy sources on energy generation are currently under examination using inverted vesicles prepared from the wild type and Na^+ pump-defective mutants.

$\Delta\psi$ generated by Na^+-vesicles was considerably larger than that generated by K^+-vesicles although both vesicles showed similar NADH oxidase activity. Since Na^+ is more efficient than K^+ in stabilizing the membrane structure [12], these results may indicate that the presence of Na^+ during the preparation of inverted vesicles is favorable, but not essential, for the active vesicles.

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