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Properties of respiratory chain-linked Na^+-independent NADH-quinone reductase in a marine *Vibrio alginolyticus*

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Key words: Respiratory chain; NADH-quinone reductase; Sodium pump; Two-electron reduction; One-electron reduction; (*V. alginolyticus*)

The respiratory chain of a marine *Vibrio alginolyticus* contains two types of NADH-quinone reductase (NQR): one is an Na^+-dependent NQR functioning as an Na^+ pump (NQR-1) and the other is an Na^+-independent NQR (NQR-2). NQR-2 was purified about 55-fold from the membrane of mutant Nap-1 which is devoid of NQR-1, and its properties were compared with those of NQR-1. In contrast to NQR-1, the purified NQR-2 does not require any salts for activity and is not inhibited by up to 0.4 M salts. The optimum pH of NQR-2 is between 6.8 and 7.8, which is about 0.7 pH units lower than that of NQR-1. NQR-2 is insensitive to strong inhibitors of NQR-1 such as *p*-chloromercuribenzoate, Ag^+ and 2-heptyl-4-hydroxyquinoline *N*-oxide. Using inverted membrane vesicles, it was confirmed that NQR-2 has no capacity to generate a membrane potential. NQR-2 reduces menadione and ubiquinone-1 by a two-electron reduction pathway. Since the NADH-reacting FAD-containing β-subunit of NQR-1 reduces quinones by a one-electron reduction pathway, the mode of quinone reduction is closely related to energy coupling; the formation of semiquinone radicals as an intermediate is likely to be essential to functioning as an ion pump.

Introduction

The respiratory chain of marine *Vibrio alginolyticus* has two different types of NADH-quinone reductase (NQR) [1–3]: one enzyme system specifically requires Na^+ for maximum activity [4] and functions as a primary Na^+ pump [1] (designated NQR-1), and the other enzyme has no specific requirement for Na^+ (designated NQR-2). The Na^+-dependent NQR-1 was highly purified from the membrane of *V. alginolyticus*, and was shown to be composed of three subunits, α, β and γ, with apparent M_r of 52000, 46000 and 32000, respectively [5–7]. From analyses of electron transfer pathways in NQR-1, the Na^+-dependent reaction catalyzed by the FMN-containing α-subunit was assigned to be the site of the Na^+ pump [7]. Mutants defective in the respiration-coupled Na^+ pump were isolated by Tokuda [8]. These mutants were devoid of Na^+-dependent NADH oxidase and thus NQR-1, but Na^+-independent NADH oxidase activity was increased [1]. The latter activity was insensitive to 2-heptyl-4-hydroxyquinoline *N*-oxide (HQNO) and included Na^+-independent NQR (NQR-2) as a part of the respiratory chain. Both NQR-1 and NQR-2 were detected in the membrane fraction from wild type cells and about 10–20% of the total NQR activity corresponded to NQR-2. Using inverted membrane vesicles from *V. alginolyticus*, Smirnova et al. [9] and Kim et al. [10] have shown that the Na^+-independent NQR-2 has no capacity to generate membrane potential ($\Delta\Psi$), positive inside. The enzymatic properties of NQR-2, however, have not yet been described.

The presence of two types of NQR in the respiratory chain is not unique to *V. alginolyticus*. We previously reported the purification of an NADH-ferricyanide dehydrogenase from *Escherichia coli* membranes, which was found to be a part of an energy-coupled NQR [11]. This enzyme complex functions as an H^+ pump. The respiratory chain of *E. coli* also contains another NQR which has no capacity to generate $\Delta\Psi$. Since the respiratory chain of marine *V. alginolyticus* contains Na^+-transport NQR-1, it is of interest to clarify the functions of Na^+-independent NQR-2 in this organism. This paper describes the isolation and properties of NQR-2 from *V. alginolyticus*.

Abbreviations: NQR, NADH-quinone reductase; Q-1, ubiquinone-1; menadione, 2-methyl-1,4-naphthoquinone; HQNO, 2-heptyl-4-hydroxyquinoline-*N*-oxide; $C_{12}E_8$, *n*-dodecyl octaethyleneglycol monoether; oxonol VI, bis(3-propyl-5-oxoisoxazole-4-yl)pentamethine-oxonol; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; $\Delta\Psi$, membrane potential.

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Materials and Methods

Chemicals. Liponox DCH, an alkyl polyoxyethylene ether detergent [7], was kindly supplied by Lion Co., Kanagawa, Japan. *n*-Dodecyl octaethyleneglycol monoether ($C_{12}E_8$) was obtained from Nikko Chemicals, Tokyo, Japan. Ubiquinone-1 (Q-1) was kindly supplied by Eizai, Tokyo, Japan. Reduced nicotinamide hypoxanthine dinucleotide (d-NADH) and deoxyribonuclease I (type IV) were obtained from Sigma. Bis(3-propyl-5-oxoisoxazole-4-yl)pentamethineoxonol (oxonol VI) was obtained from Molecular Probes, Oregon. Other reagents used were of the highest commercial grade available.

Enzyme assays. NADH-quinone reductase activity was assayed at 30°C by following the formation of reduced quinone as previously described [2,4]. For the assay of NQR-2 activity, the standard reaction mixture contained 0.1 mM NADH/15 μM Q-1/5 mM $MgSO_4$/0.015% Liponox DCH/20 mM Tris-HCl (pH 7.0) in a total volume of 2.0 ml. For the assay of NQR-1 activity, the reaction mixture contained 0.1 mM NADH/15 μM Q-1/0.2 M NaCl/0.02% Liponox DCH/20 mM Tris-HCl (pH 7.5). Changes in absorbance difference at the wavelength pair, 242-270.5 nm, were recorded with a Hitachi 557 dual-wavelength spectrophotometer. The rate of Q-1 reduction was calculated using an absorption coefficient of 9.6 mM^{-1} cm^{-1} [2].

Alternatively, 0.2 mM menadione (2-methyl-1,4-naphthoquinone) replaced Q-1 as the electron acceptor and the activity was assayed from the decrease in absorbance at 340 nm. The formation of menadiol was measured by following changes in absorbance difference at 242-270.5 nm in the presence of 10 μM menadione. The absorption coefficient of menadiol at 242-270.5 nm was estimated to be 22.8 mM^{-1} cm^{-1}.

One unit of enzyme activity was defined as the amount of enzyme catalysing the oxidation of 1 μmol NADH, or the reduction of 1 μmol quinone or 2 μmol ferricyanide, in 1 min.

For the measurement of pH dependence, the following buffers were used at 20 mM and the pH was determined at the end of reaction: Mes (2-[*N*-morpholino]ethanesulfonic acid)-Tris (pH 5.7–6.4); Hepes (*N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid])-Tris (pH 6.8–7.9); Tricine (*N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine)-Tris (pH 7.7–8.5); Tris-Ches (2-[*N*-cyclohexylamino]ethanesulfonic acid]) (pH 8.7–9.0).

Bacterial growth and preparation of membrane fraction. V. alginolyticus 138-2 (wild-type) and Na^+-pump deficient mutant Nap-1 [8] were grown aerobically at 37°C in a medium containing 0.5% polypeptone, 0.5% yeast extract, 0.4% K_2HPO_4 and 3.0% NaCl (pH 7.2). Cells were harvested by centrifugation during the late exponential phase of growth and washed twice with 1.0 M NaCl/5 mM EDTA/20 mM Tris-HCl (pH 7.5). The membrane fraction was prepared by osmotic lysis of the cells following suspension in a lysis medium containing 10 mM NaCl/5 mM EDTA/20 mM Tris-HCl (pH 7.5) at the cell density of 40 mg wet wt./ml. After 5 min at 25°C, the suspension was centrifuged at 20000 $\times g$ for 20 min. The precipitate was resuspended in 10 mM NaCl/0.5 mM EDTA/20 mM Tris-HCl (pH 7.5) and then deoxyribonuclease I (5 μg/ml) and $MgCl_2$ (2 mM) were added to the suspension. After 10 min at 25°C, EDTA was added at 5 mM, and the suspension was incubated for 5 min before being centrifuged at 20000 $\times g$ for 20 min. The precipitate was suspended in 10 mM NaCl/10% (w/v) glycerol/10 mM Tris-HCl (pH 7.5) and stored at −75°C.

Extraction and purification of NQR-2. The membrane fraction was suspended in a medium containing 5 mM EDTA/5 % (w/v) glycerol/20 mM Tris-HCl (pH 8.0) at the concentration of 5 mg protein/ml and incubated for 20 min at 25°C. The suspension was centrifuged at 20000 $\times g$ for 20 min and the pellet was resuspended in 0.1 mM EDTA/5% (w/v) glycerol/20 mM Tris-HCl (pH 8.0) at 10 mg protein/ml. An equal volume of 6 mM $C_{12}E_8$ in the above buffer was added to the suspension and allowed to stand for 20 min at 25°C. The mixture was centrifuged at 20000 $\times g$ for 20 min, and the supernatant was collected and stored at −75°C ($C_{12}E_8$ extract).

The $C_{12}E_8$ extract was applied to a DEAE-Sephacel column equilibrated with 0.1% Liponox DCH/10% (w/v) glycerol/0.1 mM EDTA/20 mM NaCl/20 mM Tris-HCl (pH 8.0) (buffer A). The amount of protein applied to the column was adjusted so as to be equivalent to 5 mg/ml of the bed volume. The column was washed with buffer A containing 0.1 M NaCl until the A_{280} of the eluate had decreased to less than 0.05. Then the column was eluted with buffer A containing 0.2 M NaCl. The active fractions were combined, then concentrated and desalted by ultrafiltration with an Amicon PM 10 membrane (DEAE-Sephacel fraction).

The DEAE-Sephacel fraction corresponding to 1 mg protein/0.2 ml of the bed volume was applied to 5'-AMP affinity column equilibrated with 0.1% Liponox DCH/10% (w/v) glycerol/5 mM EDTA/20 mM Tris-HCl (pH 7.0). The column was washed with the above buffer, and then eluted with the buffer containing 1 mM NADH. The active fractions were collected, and then concentrated and NADH was removed by the ultrafiltration (5'-AMP affinity fraction).

Preparation of inverted membrane vesicles. For the preparation of inverted membrane vesicles, the cells were suspended in 0.1 M sucrose/5 mM $MgSO_4$/10 mM Hepes-Tris (pH 7.5) (buffer B) containing 1 mM dithiothreitol, then broken using a French press operating at 8000 lb/in^2. The inverted membrane vesicles

were washed with buffer B by centrifugation at 200000 $\times g$ for 1 h, then resuspended in buffer B and stored at $-75°C$.

Measurement of membrane potential. The generation of membrane potential, positive inside, was monitored from the absorbance shift of oxonol VI [11] at 625–587 nm using the dual-wavelength spectrophotometer. The reaction mixture contained 0.1 M sucrose/30 mM $MgSO_4$/50 mM Na_2SO_4/10 mM Hepes-Tris (pH 7.5)/5 μM oxonol VI/1.0 mg protein of inverted membrane vesicles in a total volume of 2.0 ml. NADH oxidase activity was initiated by the addition of 0.2 mM NADH. For the measurement of membrane potential induced by NQR reaction, 10 mM KCN was added to the reaction mixture and then 50 μM Q-1 and 0.2 mM NADH were added to start the reaction. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and monensin were used at 3 μM and 30 μM, respectively.

Other methods. SDS-polyacrylamide gel electrophoresis was performed as described by Laemmli [12]. Kinetic constants and their standard errors were calculated according to the method of Cleland [13] as described previously [14]. Protein was determined by the method of Lowry et al. [15] or Bradford [16] using bovine serum albumin as a standard.

Results

Salt dependence of NQR activities in the wild type and mutant membranes

The NADH oxidase of the membrane from the wild-type cells is greatly stimulated by Na^+ [4], whereas that from mutants defective in the Na^+ pump is fully active in the absence of salts [1]. As a partial reaction of the respiratory chain, the salt dependence of Q-1 reduction by NADH was measured in the presence of 10 mM KCN; the NQR activity of the wild type membrane was strongly stimulated by Na^+ (not shown). Other cations such as K^+, Li^+ and Mg^{2+} were relatively ineffective as replacements for Na^+. Although the activity stimulated by Na^+ is sensitive to HQNO, the activity observed in the absence of salts is resistant to HQNO [1]. The activity without salts was 0.33 units/mg protein and corresponded to Na^+-independent NQR (NQR-2). With the membrane from the mutant Nap-1 cells, the activity without salts was 0.58 units/mg protein. Since this activity was not stimulated by Na^+ and was resistant to HQNO, it was concluded that the respiratory chain of Nap-1 contained exclusively NQR-2. Thus, NQR-2 was extracted from the membrane of Nap-1.

Generation of membrane potential

The generation of membrane potential ($\Delta\Psi$), positive inside, in the inverted membrane vesicles was monitored by the absorbance band shift of oxonol VI. Oxidation of NADH by oxygen (NADH oxidase) induced the oxonol response with the inverted membrane vesicles derived from both the wild-type (A) and mutant (B) cells (Fig. 1). With the wild-type membrane vesicles, the $\Delta\Psi$ was not completely collapsed by the addition of CCCP and further addition of monensin was required for complete dissipation of $\Delta\Psi$. The $\Delta\Psi$ generated by the reaction of NADH:Q-1 reductase with the wild-type membrane vesicles (C) was also resistant to CCCP. These results could be explained by the presence of Na^+-transport NQR-1 in the wild-type membrane vesicles [1,23,24]. In contrast, with the mutant membrane vesicles, the $\Delta\Psi$ generated by the NADH oxidase was completely dissipated by CCCP (B) and no detectable $\Delta\Psi$ was generated by the reaction of NADH:Q-1 reductase (not shown). Thus NQR-2 present in the mutant membrane has no capacity to generate $\Delta\Psi$. These results were consistent with the recent reports of Smirnova et al. [9] and Kim et al. [10], where the $\Delta\Psi$ was monitored by different techniques.

Extraction and purification of NQR-2

NQR-2 was purified as described in Materials and Methods (Table I). NQR activity was assayed with Q-1 and menadione as electron acceptors. Separation of the activities for Q-1 and menadione was not observed

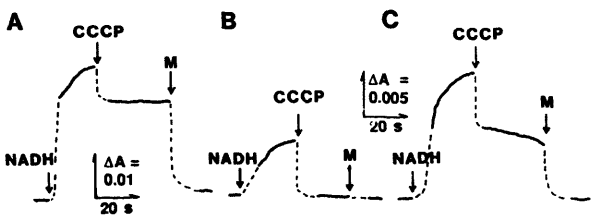

Fig. 1. Generation of membrane potential, inside positive, in the inverted membrane vesicles from the wild-type (A and C) and the mutant (B) cells. (A) and (B) oxonol response by NADH oxidase reaction; (C) oxonol response by NQR reaction in the presence of 10 mM KCN and 50 μM Q-1. Experimental conditions are described in Materials and Methods. M denotes monensin.

TABLE I

Purification of NQR-2 from the membrane of Nap-1

Fraction	Protein	Q-1 reductase		Menadione reductase	
	(mg)	units	U/mg	units	U/mg
Membrane	260	151	0.58	96	0.37
$C_{12}E_8$ extract	79	77	0.97	34	0.43
DEAE-Sephacel	24.8	56	2.26	23	0.93
5'-AMP affinity	0.3	9.5	31.7	6.2	20.7

during purifications, which was contrary to the case of NQR-1 [5–7]. As calculated on the basis of membrane protein, NQR-2 was purified about 55-fold.

SDS-polyacrylamide gel electrophoresis showed that purified NQR-2 still contained several protein bands (Fig. 2), including two major proteins with apparent M_r 44000 and 75000. It was uncertain whether or not NQR-2 was a dimer of dissimilar subunits.

Properties of NQR-2

The partially-purified NQR-2 was used for the examination of its enzymatic properties. For the comparative study, NQR-1 purified from the membrane of wild-type cells as previously described [5–7] was employed: this had a Q-1 reductase activity of 67 units/mg protein.

Salt dependence. NQR-2 was highly active in the absence of salts, was unaffected at the concentrations up to 0.4 M NaCl or KCl, and was progressively inhibited at higher concentrations (Fig. 3). In contrast, NQR-1 strictly required salts for activity (Na^+ was the most effective) and maximum activity was attained at 0.3–0.6 M NaCl. These results clearly indicated that, in contrast to NQR-1, NQR-2 does not require any salts for maximum activity.

pH dependence. As compared with NQR-1, the pH optimum of NQR-2 was about 0.7 pH unit lower (Fig. 4). Thus the optimum pH for NQR-2 was between 6.8 and 7.8. The pH-NQR activity curves observed with the wild-type and Nap-1 membranes were similar to those of NQR-1 and NQR-2, respectively, and the NQR activity of wild-type membranes was apparently highest at alkaline pH.

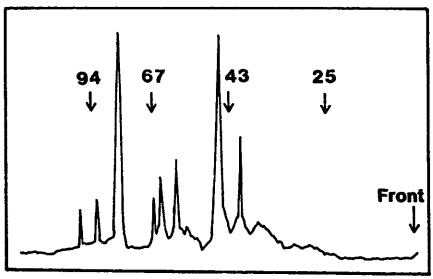

Fig. 2. Densitometer traces of SDS-polyacrylamide gel electrophoresis of 5'-AMP affinity fraction. The molecular weights of standard proteins are indicated in thousands.

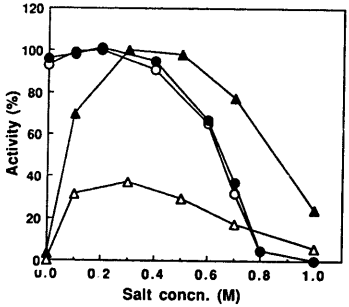

Fig. 3. Effects of NaCl (closed symbols) and KCl (open symbols) on the activities of NQR-2 (circles) and NQR-1 (triangles). A 100% activity corresponds to 30 units/mg protein for NQR-2 and 67 units/mg protein for NQR-1.

Substrate specificity. Table II shows the electron acceptor specificities of NQR-2 and NQR-1. NQR-2 was the most active toward Q-1. As compared with NQR-1, the activities of NQR-2 for artificial electron acceptors such as menadione, 2,6-dichlorophenolindophenol (DCIP) and ferricyanide were relatively low. Similar to NQR-1, the β-subunit showed a high reactivity toward artificial electron acceptors.

The energy-coupled NQR of *E. coli* that functions as an H^+ pump reacts with d-NADH as an electron donor [17]. The Na^+-transport NQR-1 of *V. alginolyticus* also reacts with d-NADH [18]. NQR-2, however, could not react with d-NADH at all, and was therefore

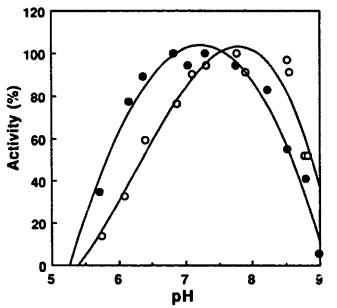

Fig. 4. Effects of pH on the activities of NQR-2 (closed circles) and NQR-1 (open circles). Buffers used were described in Materials and Methods.

TABLE II

Electron acceptor specificities of NQR-2 and NQR-1

Electron acceptor	Concn. (μM)	Relative activity		
		NQR-2	NQR-1 [a]	β-subunit [a]
Q-1	15	100 [b]	100 [b]	100 [b]
Menadione	10	18	120	273
	100	57	214	455
	200	62	–	–
DCIP [c]	40	11	88	392
Ferricyanide	1000	20	149	426

[a] Data from Ref. 5.

[b] 100% activities of NQR-2, NQR-1 and β-subunit for Q-1 correspond to 30, 51 and 49 units/mg protein, respectively.

[c] 2,6-Dichlorophenolindophenol.

TABLE III

Effects of inhibitors on the activities of NQR-2 and NQR-1

Inhibitor	Half-inhibitory concn. [a]	
	NQR-2	NQR-1
p-CMB [b]	nil [c]	0.01
Ag^+	nil [d]	0.02
HQNO	6.0	0.1
Zn^{2+}	3.5	1.0
Capsaicin	> 250	175
5'-AMP (mM)	1.5	> 10

[a] The half-inhibitory concentration of each inhibitor is expressed in μM except for 5'-AMP, which is expressed in mM.

[b] *p*-Chloromercuribenzoate.

[c,d] No inhibitory effect at 0.1 (c) and 0.5 μM (d).

very similar to the energy-uncoupled NQR-2 from *E. coli* [11,17].

Kinetic studies of NQR-2. Double-reciprocal plots of initial velocity versus concentration of NADH at various fixed levels of Q-1 yielded parallel lines characteristic of a ping-pong mechanism (Fig. 5). Secondary plots of intercepts against the reciprocal of Q-1 concentration gave linear plots. From these data, the K_m values for NADH and Q-1 were calculated to be 75 $\pm$ 15 μM and 15 $\pm$ 3 μM, respectively.

Inhibitor studies. The effects of several potential inhibitors of NQR-2 and NQR-1 were investigated (Table III). NQR-1 was more sensitive to all of these inhibitors except 5'-AMP. *p*-Chloromercuribenzoate and Ag^+ were strong inhibitors to NQR-1, but NQR-2 was not inhibited at all even in the presence of 10-fold amount of each half-inhibitory concentration for NQR-1. The effect of Ag^+ on the β-subunit of NQR-1 (NADH dehydrogenase) has been reported previously [19]. Using the membranes from wild-type and mutant cells, HQNO has been shown to be a specific inhibitor

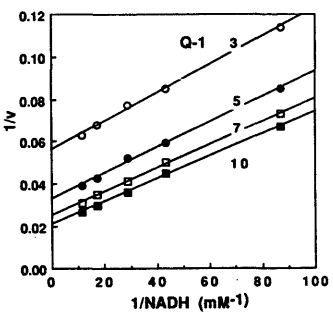

Fig. 5. Double-reciprocal plots of initial velocity versus NADH concentration at various fixed levels of Q-1. The concentration of Q-1 in the reaction mixture was indicated in the figure in μM. The initial velocity (v) is expressed in units/mg protein.

of NQR-1 and Na^+ pump activities without any effect on NQR-2 activity [1]. The partially-purified NQR-2, however, was significantly inhibited by HQNO, although the half-inhibitory concentration was still 60-fold nigher than that for NQR-1. The sensitivity toward Zn^{2+} was different between NQR-1 and NQR-2, which were completely inhibited in the presence of 3 and 10 μM Zn^{2+}, respectively. The energy-coupled NQR of *E. coli* was reported to be specifically inhibited by capsaicin [20]. This compound was relatively ineffective against both NQR-1 and NQR-2 from *V. alginolyticus*. Among inhibitors examined, only 5'-AMP inhibited NQR-2 more than NQR-1, but very high concentrations were required.

Mode of quinone reduction

Quinones are reduced by either one-electron or two-electron transfer reaction depending on flavin enzymes [21,22]. If quinones are reduced by a one-electron reduction pathway, semiquinone radicals produced are autoxidized by molecular oxygen, forming an oxidation-reduction cycle of the radicals. This reaction leads to a continuous consumption of NADH in excess of the amount of quinones present in the reaction mixture. Therefore, the rates of NADH consumption and quinol formation were measured spectrophotometrically, and NQR-2 was shown to consume NADH with the stoichiometric formation of ubiquinol-1 or menadiol (Fig. 6). It was therefore concluded that NQR-2 reduced Q-1 and menadione by the two-electron reduction pathway. In contrast, the β-subunit of NQR-1 has been shown to reduce Q-1 and menadione by the one-electron reduction pathway in our previous paper [5].

Discussion

By the isolation and purification of NQR-2 from the membrane of *V. alginolyticus*, it became quite clear that, in contrast to NQR-1, NQR-2 does not require

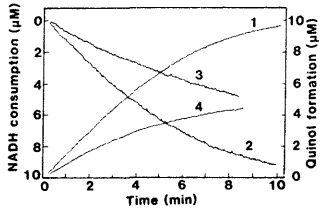

Fig. 6. Mode of quinone reduction by NQR-2. The reaction was carried out in the presence of 10 μM Q-1 (curves 1 and 2) or 10 μM menadione (curves 3 and 4). The consumption of NADH (curves 2 and 3), or the formation of ubiquinol-1 (curve 1) or menadiol (curve 4) was monitored as described in Materials and Methods.

any salts for activity and is not inhibited by up to 0.4 M salts. The substrate specificity and inhibitor sensitivity of NQR-2 is also dissimilar to that of NQR-1. The most remarkable difference between NQR-2 and NQR-1 is in their capacities to generate $\Delta\Psi$. As also shown by other investigators [9,10], the respiratory chain of *V. alginolyticus* apparently has extrusion systems for Na^+ and H^+. The Na^+-dependent NQR-1 functions as Na^+ pump and the Na^+-independent NQR-2 has no capacity to generate $\Delta\Psi$. Since a CCCP-sensitive $\Delta\Psi$ is generated by the NADH oxidase in the mutant membrane vesicles, the quinol oxidase must function as an H^+ pump. Thus, the electron flow from NADH to oxygen via NQR-2 generates only a protonmotive force. In this connection, it is interesting to note that the optimal pH of NQR-2 is more acid than that of NQR-1. For the regulation of cytoplasmic pH in *V. alginolyticus*, it is necessary to alkalinize the cell interior by the H^+-pumping respiratory chain [25,26]. Therefore, it is reasonable to consider that when the cytoplasmic pH falls, electron flow via NQR-2 may increase, resulting in the extrusion of H^+ from the cytoplasm without concomitant generation of $\Delta\Psi$ by the Na^+ pump. This may be of advantage to the alkalinization of cell interior and thus the regulation of cytoplasmic pH.

Recently, Yagi [27] reviewed the bacterial NADH-quinone reductases and pointed out the presence of two types of NQR in the respiratory chains of several species of bacteria. Yagi classified type-1 NQR (designated NDH-1) as those having an energy coupling site and type-2 NQR (designated NDH-2) as those having no energy coupling site. The two types of NQR found in the respiratory chain of marine *V. alginolyticus* coincide with the above classification except that the energy-coupled NQR-1 functions as the Na^+ pump, not as the H^+ pump. As shown in our previous papers [5–7], NQR-1 is composed of three subunits, α, β, γ, and the FAD-containing β-subunit accepts electrons from NADH and reduces Q-1 or menadione by a one-electron transfer reaction to produce semiquinone radicals. This reaction is independent of Na^+ and insensitive to HQNO. On the other hand, the reaction catalyzed by the FMN-containing α-subunit is dependent on Na^+ and sensitive to HQNO, and is directly coupled to the translocation of Na^+. As a whole complex, NQR-1 catalyzes the reduction of Q-1 to ubiquinol-1, but menadione is not reduced to menadiol due to the direct interaction of menadione with the β-subunit. NQR-2, however, reduced both Q-1 and menadione by the two-electron transfer pathway. Therefore, it is likely that the energy-coupled NQR-1 reduces ubiquinones by one-electron reduction, whereas the energy-uncoupled NQR-2 catalyses two-electron reduction. The respiratory chain of *E. coli* also has two types of NQR [11]. The reduction of quinones by the purified enzymes from *E. coli* were examined. Although not shown here, FMN-containing NADH-ferricyanide dehydrogenase, which is the NADH-reacting site of energy-coupled NQR-1, reduced menadione by one-electron reduction pathway, whereas FAD-containing energy-uncoupled NQR-2 reduced Q-1 and menadione by two-electron reduction pathway. Although NQR-1 of *E. coli* functions as the H^+ pump, the generation of semiquinone radicals as an intermediate of quinone reduction must be a common factor for NQR-1 to function as an ion pump. Indeed, a semiquinone EPR signal sensitive to HQNO has been detected in *Thermus thermophilus* HB-8 membrane, which contains H^+-pumping type-1 NQR composed of ten unlike polypeptides, FMN and three iron-sulfur clusters [28,29]. Further studies are required to make clear the mode of coupling between the redox reaction of semiquinone radicals and the translocation of ions.

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