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CHARACTERIZATION OF THE Na^+-DEPENDENT RESPIRATORY CHAIN NADH: QUINONE OXIDOREDUCTASE OF THE MARINE BACTERIUM, *VIBRIO ALGINOLYTICUS*, IN RELATION TO THE PRIMARY Na^+ PUMP

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The Na^+-dependent respiratory chain NADH: quinone oxidoreductase of the marine bacterium, *Vibrio alginolyticus*, was extracted from membrane by a detergent, Liponox DCH, and was purified by chromatography on QAE-Sephadex and Bio-Gel HTP. The activity of NADH oxidation was separated into two fractions. The one fraction could react with several artificial electron acceptors including Q-1, but could not reduce ubiquinone and menaquinone such as Q-5 and menaquinone-4, which was called NADH dehydrogenase. The other fraction could reduce Q-5 and menaquinone-4 in addition to the NADH dehydrogenase activity, which was called quinone reductase. The purified NADH dehydrogenase consumed NADH in excess of the amount of Q-1 and the reduced Q-1 (quinol) was not produced at all due to an oxidation-reduction cycle of semiquinone radicals. The quinone reductase, however, consumed NADH with the quantitative formation of quinol on account of a dismutation reaction of semiquinone radicals. Identical to the membrane-bound NADH: quinone oxidoreductase, the quinone reductase specifically required Na^+ for the activity and was inhibited by 2-heptyl-4-hydroxyquinoline *N*-oxide. The electron transfer in the quinone reductase was formulated in a form of quinone cycle and the dismutation reaction of semiquinone radicals was assigned to be coupled to the Na^+ pump in the respiratory chain of this organism.

Introduction

The marine bacterium, *Vibrio alginolyticus*, possesses the respiratory chain-coupled primary Na^+ pump functioning at alkaline pH [1,2]. The respiratory chain of this organism specifically requires Na^+ for the maximum activity [3] and the site of Na^+-dependent activation resides in the NADH: quinone oxidoreductase segment of the respiratory chain [4]. Tokuda [5] succeeded in isolating mutants defective in the respiration-coupled Na^+ pump. Using membrane preparations from the wild-type, mutant and spontaneous revertant strains, it has been demonstrated that the Na^+-dependent NADH: quinone oxidoreductase is tightly coupled to the Na^+ pump activity [6]. To resolve the relationship between the Na^+-dependent NADH: quinone oxidoreductase and the Na^+ pump, the isolation and purification of NADH: quinone oxidoreductase segment in the respiratory chain of *V. alginolyticus* were performed.

The respiratory chain NADH: quinone oxidoreductase has been purified from *Escherichia coli* [7,8]. For the assay of quinone reductase, ubiquinone-1 (Q-1) [7] or decylbenzoquinone [8]

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Abbreviations: Q-1, ubiquinone-1; HQNO, 2-*n*-heptyl-4-hydroxy-quinoline *N*-oxide; DCIP, dichlorophenolindophenol.

has been utilized to avoid the problems involved in the use of artificial electron acceptors such as dichlorophenolindophenol (DCIP) and ferricyanide. However, since the enzyme activity was assayed from the rate of NADH consumption, the formation of reduced quinone (quinol) has not been measured. Together with conventional assay methods, we employed a direct spectrophotometric method for the determination of quinol formation from ubiquinone and menaquinone, which has been developed by us [4]. By using these assay methods, we could isolate and purify a NADH dehydrogenase and a quinone reductase from the membrane preparation of *V. alginolyticus*. The latter enzyme had the same properties as those of the membrane-bound NADH : quinone oxidoreductase and catalyzed the formation of quinol from ubiquinone and menaquinone with a long isoprenoid side chain such as Q-5 and menaquinone-4. This paper deals with the isolation and characterization of respiratory chain NADH dehydrogenase and quinone reductase of *V. alginolyticus* with special reference to the Na^+-dependent reaction.

Materials and Methods

Chemicals. Liponox DCH, an alkyl polyoxyethylene ether detergent having average alkyl chain and polyoxyethylene chain lengths of 10 and 8, respectively, was kindly supplied by Lion Co., Tajima, Odawara-shi, Kanagawa, Japan. Ubiquinone-1 (Q-1), Q-5 and menaquinone-4 were kindly supplied by Eizai Co., Koishikawa, Bunkyo-ku, Tokyo, Japan. Horse heart cytochrome *c*, Type III, superoxide dismutase from bovine blood (3000 units/mg protein), and 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide (HQNO) were obtained from Sigma Chemical Co. Other reagents used were of analytical grade.

Bacterial growth and preparation of membrane fraction. The growth of *V. alginolyticus* 138-2 and the preparation of membrane fraction were performed as previously described [3]. The final membrane fraction was washed once with 10 mM Tris-HCl (pH 7.5). Then, it was lyophilized and stored at −40°C in a desiccator.

Enzyme assays. NADH dehydrogenase activity was assayed at 30°C from the decrease in absorbance at 340 nm with menadione as an electron acceptor. The standard assay mixture contained 0.2 mM NADH/0.1 mM menadione/0.2 M NaCl/0.1% Liponox DCH/20mM Tris-HCl (pH 7.5)/20 μM diethyldithiocarbamate and enzyme in a total volume of 1.0 ml. The reaction was started by the addition of enzyme.

Quinone reductase activity was assayed at 30°C by following the formation of reduced quinone as previously described [4]. The standard assay mixture contained 0.1 mM NADH/3 μM ubiquinone-1 (Q-1)/0.4 M NaCl/0.025% Liponox DCH/20 mM Tris-HCl (pH 7.5)/20 μM diethyldithiocarbamate and enzyme in a total volume of 2.0 ml. Changes in absorbance difference at the wavelength pair, 248–267 nm, were recorded with a Hitachi 557 two wavelengths spectrophotometer.

The rate of ubiquinone (Q-1, Q-5) and menaquinone reduction was calculated based on a millimolar absorption coefficients of 7.8 and 43, respectively [4].

One unit of activity is defined as the amount of enzyme catalyzing the oxidation of 1 μmol of NADH or the reduction of 1 μmol of quinone per min.

Detection of superoxide radicals. For the detection of superoxide radicals, the reduction of acetylated ferricytochrome *c* was measured as described by Azzi et al. [9]. The respiratory chain NADH dehydrogenase of *V. alginolyticus* weakly interacted with horse-heart cytochrome *c*, but the acetylated derivative was inactive as an electron acceptor. Thus, the latter was useful for the detection of superoxide radicals in our systems.

Horse heart cytochrome *c* was acetylated according to the method of Minakami et al. [10]. The rate of acetylated cytochrome *c* reduction was followed by the increase in absorbance at 550 nm taking a millimolar absorption coefficient of 18.4.

Extraction and purification of NADH : quinone oxidoreductase. About 1 g dry weight of lyophilized membrane was washed once with 80 ml 10 mM Tris-HCl (pH 7.0) and then suspended in 80 ml 2% (w/w) sodium cholate containing 0.2 M NaCl/0.1 mM EDTA/20 mM Tris-HCl (pH 7.0) with a Teflon-glass homogenizer. Inactive proteins extracted with sodium cholate were removed by centrifugation for 20 min at 20000 × *g*. The pellets were suspended in 100 ml of 1% (w/w) Liponox

DCH containing 10% (w/w) glycerol/0.1 mM EDTA/0.13 M NaCl/10 mM Tris-HCl (pH 7.0). The suspension was allowed to stand for 20 min at 20°C and was stored for overnight at −20°C. It was thawed at 20°C and then centrifuged for 20 min at 20000 × *g*.

The supernatant (Liponox extracts) was applied to a QAE-Sephadex (A-50) column (3.2 × 16 cm) equilibrated with 10% glycerol/0.1 mM EDTA/10 mM Tris-HCl (pH 7.0)/0.2% Liponox DCH (buffer 1) containing 0.13 M NaCl. The column was washed with buffer 1 containing 0.2 M NaCl until no more protein was found in the washings. Then the column was eluted with 0.45 M NaCl in buffer 1. The enzyme activity of eluate was assayed with menadione as the electron acceptor (NADH dehydrogenase assay). The active fractions were combined, then concentrated and desalted by ultrafiltration in an Amicon apparatus fitted with a PM 10 membrane. This fraction (first QAE-Sephadex) was applied to a second QAE-Sephadex (A-25) column (2.2 × 21 cm) equilibrated with buffer 1 containing 0.05 M NaCl. The column was washed with 0.15 M NaCl in buffer 1 and then eluted with a linear gradient from 0.15 to 0.45 M NaCl in buffer 1 in a total volume of 400 ml.

The NADH dehydrogenase activity was separated into two fractions. The first fraction was eluted at about 0.22 M NaCl and the second fraction at about 0.28 M NaCl (see fractions I and II in Fig. 1). The first fraction (I), but not the second (II), could reduce Q-1 to quinol and was called quinone reductase. The quinone reductase (I) and the NADH dehydrogenase (II), respectively, were further purified by chromatography on Bio-Gel HTP.

The concentrated and desalted enzyme solution was applied to a Bio-Gel HTP column (1.5 × 2.8 cm) equilibrated with 10 mM sodium phosphate buffer (pH 6.8) containing 10% glycerol and 0.2% Liponox DCH. The column was washed with 30 mM sodium phosphate buffer (pH 6.8) and then eluted with a linear gradient from 30 to 120 mM sodium phosphate buffer containing 10% glycerol and 0.2% Liponox in a total volume of 50 ml. Active fractions were concentrated and then the suspending medium of enzyme was exchanged for buffer 1 by membrane ultrafiltration.

Other methods. SDS-Polyacrylamide gel electrophoresis on 7.5% slab gel was performed by using discontinuous buffer system of Laemmli [11]. Gels were stained and then scanned by using a Shimazu dual-wavelength chromato-scanner, CS-910, at 590-440 nm.

Kinetic constants and their standard errors were calculated according to the method of Cleland [12] as described previously [13].

Protein was determined by the method of Lowry et al. [14] with bovine albumin as a standard.

Results

Separation and purification of NADH dehydrogenase and quinone reductase

By washing the lyophilized membrane of the marine bacterium, *V. alginolyticus*, with 2% sodium cholate, about 20% of the total protein was removed without significant loss of NADH dehydrogenase activity. After that, the enzyme was extracted from the membrane by 1% Liponox DCH, an alkylpolyoxyethylene ether detergent. Details of the purification procedure were described under Materials and Methods

As shown in Fig. 1, the NADH dehydrogenase activity as measured with menadione as an electron acceptor was separated into two fractions at the second QAE-Sephadex column chromatography. In addition to the NADH dehydrogenase

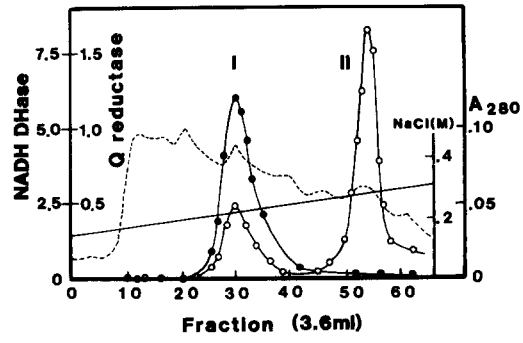


Fig. 1. The second QAE-Sephadex column chromatography. About 35 mg protein was applied to the column. The enzyme activities of eluate were measured by the NADH dehydrogenase (DHase) assay (open circles) and by the quinone (Q) reductase assay (closed circles) as described under Materials and Methods and are expressed in units/ml. The dotted line denotes the absorbance of eluate at 280 nm.

TABLE I

SEPARATION AND PURIFICATION OF NADH DEHYDROGENASE AND QUINONE REDUCTASE FROM THE MEMBRANE OF *V. ALGINOLYTICUS*

Enzyme activities were determined by the NADH dehydrogenase assay and the quinone (Q) reductase assay as described in Materials and Methods.

Fraction	Protein (mg)	NADH dehydrogenase assay		Q reductase assay	
		(Units)	(U/mg)	(Units)	(U/mg)
1. Lyophilized membrane	500	875	1.75	288	0.58
2. Liponox extracts	289	537	1.9	248	0.86
3. First QAE-Sephadex	65	292	4.5	108	1.7
4. Second QAE-Sephadex					
(I) Q reductase	2.9	61	21	25	8.6
(II) NADH dehydrogenase	2.9	106	37	3	1.0
5. Bio-Gel HTP					
Q reductase	0.2	21	105	8.8	44
NADH dehydrogenase	0.4	85	213	0.1	0.25

activity, the first fraction (I), but not the second (II), could reduce Q-1 to quinol as revealed by the quinone reductase assay. In this paper, the activity which catalyzes the formation of quinol from quinone was regarded as quinone reductase. Thus, the first and the second fractions were called quinone reductase and NADH dehydrogenase, respectively. Both enzymes were further purified using Bio-Gel HTP column. Table I is the summary of purification. As calculated on the basis of membrane protein, the NADH dehydrogenase and quinone reductase were purified 120- and 76-fold, respectively.

As shown in Table I, the purified NADH dehydrogenase showed no appreciable quinone reductase activity. The quinone reductase, however, showed a high NADH dehydrogenase activity and the activity ratio of dehydrogenase assay to reductase assay was 2.4, which was nearly the same as those of crude enzyme fractions. Thus, most of NADH dehydrogenase seemed to be derived from the quinone reductase as a result of separation of activity catalyzing quinol formation.

On SDS-polyacrylamide gel electrophoresis, the final preparation of the NADH dehydrogenase gave a major protein band with an apparent molecular weight of 45 000 (Fig. 2). The quinone reductase, however, contained three major and several minor protein bands. One of the major bands corresponded to that of NADH dehydrogenase, suggesting the involvement of NADH dehydrogenase segment in the quinone reductase.

By rechromatography of the final preparation of quinone reductase on QAE-Sephadex under the conditions described in Fig. 1, about a half of its activity was eluted at the position of NADH dehydrogenase, which had the same properties as those

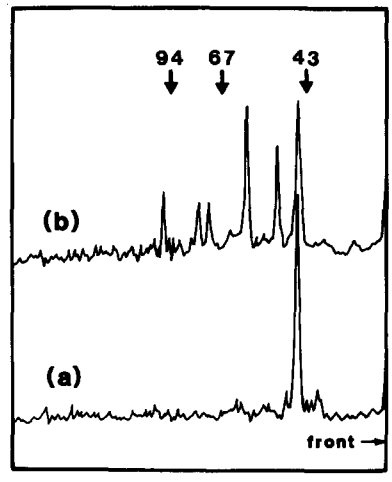


Fig. 2. SDS-Polyacrylamide gel electrophoresis of the purified NADH dehydrogenase and quinone reductase. Samples were subjected to electrophoresis, and gels were stained and scanned as described under Materials and Methods. The values for molecular mass of standard proteins are given in kilodaltons. (a) NADH dehydrogenase; (b) quinone reductase.

of the purified NADH dehydrogenase (data not shown). Thus, about a half of quinone reductase was lost by this treatment. On the other hand, the rechromatography of the purified NADH dehydrogenase gave a single protein and activity peak at the position of NADH dehydrogenase. These results also indicated that the NADH dehydrogenase was derived from the quinone reductase during treatment with QAE-Sephadex and thus the former was a component of the latter.

The both enzymes had fluorescence peaks of excitation and emission at 450 and 526 nm, respectively, indicating the presence of flavin as a cofactor. Further details of the molecular properties of these enzymes will be described elsewhere.

Although the quinone reductase still contained contaminated proteins, it was used in the following experiments without further purifications.

Electron acceptor specificities of NADH dehydrogenase and quinone reductase

Table II shows the electron acceptor specificities of purified NADH dehydrogenase and quinone reductase. The activity was measured from the rate of NADH consumption except for DCIP and ferricyanide. Both enzymes reacted with artificial electron acceptors such as menadione, DCIP and ferricyanide. The quinone reductase reacted with ubiquinone and menaquinone having a long isoprenoid side chain such as Q-5 and menaquinone-4, whereas the NADH dehydrogenase was practically inactive with these quinones.

TABLE II

ELECTRON ACCEPTOR SPECIFICITIES OF NADH DEHYDROGENASE AND QUINONE REDUCTASE

The reaction was carried out under the conditions of quinone reductase assay and the activities for menadione, Q-1, Q-5 and menaquinone-4 were measured from the rate of NADH consumption and those for DCIP and ferricyanide from the changes in absorbance at 600 and 420 nm, respectively.

Electron acceptor	Concn. (μM)	NADH dehydrogenase (Units/mg)	Quinone reductase (Units/mg)
Menadione	100	223	109
	10	134	61
DCIP	100	192	45
$K_3Fe(CN)_6$	1000	417	152
Q-1	13	49	51
	4	18	49
Q-5	30	<1	14
Menaquinone-4	10	<1	11

TABLE III

KINETIC PARAMETERS OF NADH DEHYDROGENASE AND QUINONE REDUCTASE FOR MENADIONE, Q-1 AND Q-5

The reaction was carried out under the conditions of quinone reductase assay except that concentrations of electron acceptor were varied in the presence of 0.1 mM NADH. The activities of quinone reductase for Q-1 and Q-5 were measured from the quinol formation and other activities were measured from NADH consumption.

Electron acceptor	NADH dehydrogenase		Quinone reductase	
	K_m (μM)	V (U/mg)	K_m (μM)	V (U/mg)
Menadione	10.8±1.1	243 ±6.5	9.5±1.0	120 ±4.1
Q-1	15.0±2.6	83.9±8.5	<1.0	41
Q-5	-	-	26.1±5.5	19.5±2.4

The NADH dehydrogenase, however, reacted with Q-1 when measured by the NADH consumption: A remarkable difference was observed between the two enzymes in the concentration dependence. Therefore, kinetic parameters of the both enzymes were compared as shown in Table III. Apparent K_m value for menadione was identical with the both enzymes. On the other hand, the K_m value for Q-1 of the NADH dehydrogenase was about 15 μM, which was considerably larger than that of quinone reductase. The affinity of latter enzyme for Q-1 was very high and the K_m was estimated to be less than 1 μM. The K_m value for Q-5, however, was 26 μM with the maximum velocity of about a half of that for Q-1. In this experiment, the K_m values of quinone reductase for Q-1 and Q-5 were determined from the rate of quinol formation, but essentially the same values were obtained when the initial velocity was measured from the rate of NADH consumption.

Mode of Q-1 reduction by NADH dehydrogenase and quinone reductase

Although the NADH dehydrogenase consumed NADH in the presence of Q-1, no significant activity was measured by the quinone reductase assay. To compare the mode of Q-1 reduction

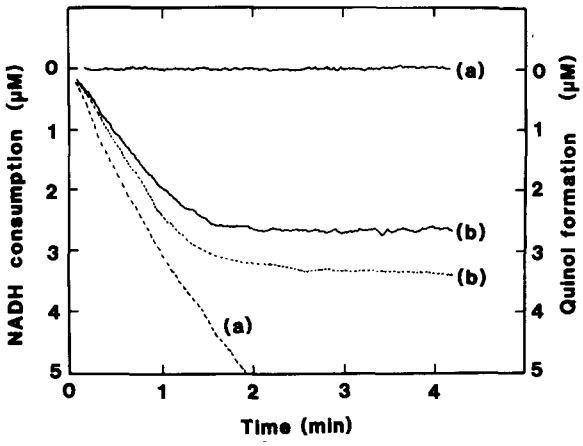


Fig. 3. Mode of Q-1 reduction by the NADH dehydrogenase and quinone reductase. The reaction was carried out under the standard conditions of quinone reductase assay, and the NADH consumption (dotted lines) or the quinol formation (full lines) was monitored. (a) NADH dehydrogenase; (b) quinone reductase.

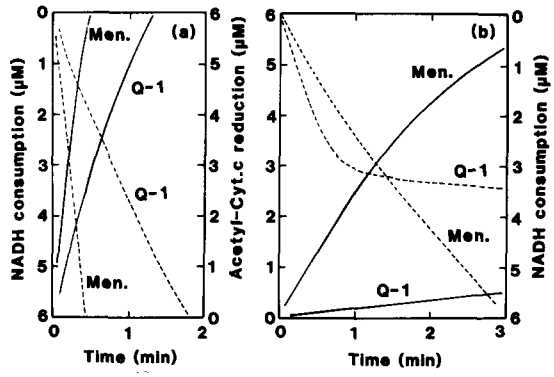


Fig. 4. Reduction of acetylated cytochrome *c* by the NADH dehydrogenase (a) and quinone reductase (b). The reaction was carried out under the conditions of quinone reductase assay in the presence of 10 μM acetylated cytochrome *c* and 3 μM each of menadione (Men.) or Q-1 as indicated in the figure. The NADH consumption (dotted lines) or the reduction of acetylated cytochrome *c* (full lines) was monitored under the same conditions.

between the two enzymes, the NADH consumption and quinol formation were simultaneously measured in the presence of a limiting amount of Q-1 (3 μM). As shown in Fig. 3, the NADH dehydrogenase consumed NADH in excess of 3 μM and no detectable amount of quinol was produced during the reaction. On the other hand, the quinone reductase consumed NADH with a concomitant formation of quinol and the reaction stopped when almost all of Q-1 was converted to quinol. These results indicated that, similar to mitochondrial NADH dehydrogenase [15] and microsomal NADPH : cytochrome P-450 reductase [16], the NADH dehydrogenase reduced Q-1 by one-electron transfer reaction and semiquinone radicals produced were autooxidized by molecular oxygen, forming an oxidation-reduction cycle of the radicals.

Since the reduction of molecular oxygen by semiquinone radicals lead to the formation of superoxide radicals, the latter was detected by use of acetylated cytochrome *c*. As shown in Fig. 4, when menadione was used as the electron acceptor, the both enzymes reduced acetylated cytochrome *c* following to the NADH consumption. In this case, the NADH consumption proceeded in excess of the amount of menadione (3 μM). Furthermore, it was ascertained that menadiol was not produced at all with the both enzymes (data not shown). These results indicated that, in contrast to DT-diaphorase [15,16], menadione was reduced by one-electron transfer reaction with the both enzymes.

When Q-1 was used as the electron acceptor, the reduction of acetylated cytochrome *c* was observed with the NADH dehydrogenase. On the contrary, the quinone reductase showed no appreciable reduction of acetylated cytochrome *c* and the NADH consumption stopped at about 3 μM.

The reduction of acetylated cytochrome *c* observed here was highly sensitive to superoxide dismutase and about 95% of the activity was inhibited by the addition of 10 μg of superoxide dismutase per ml. Apparently, the NADH dehydrogenase generated superoxide radicals during the reaction with menadione and Q-1. On the other hand, no significant amount of superoxide radicals was detected when the quinone reductase was reacted with Q-1, where quinol was produced with a stoichiometric oxidation of NADH (see Fig. 3).

Effect of monovalent cations and HQNO on the activities of NADH dehydrogenase and quinone reductase

In previous studies [4,6], we have shown that the membrane-bound NADH : quinone oxidoreductase which catalyzes the quinol formation from

TABLE IV

EFFECT OF SALTS AND HQNO ON THE ACTIVITIES OF NADH DEHYDROGENASE AND QUINONE REDUCTASE

The reaction was carried out under the conditions of quinone reductase assay except that the NADH dehydrogenase was assayed from the NADH consumption in the presence of 13 μM Q-1 and that the quinone reductase was assayed from the quinol formation in the presence of 4 μM Q-1. The concentration of HQNO was 2.5 μM and the reaction was started by the addition of enzyme. The activity is expressed in units/mg protein.

Salt addition	NADH dehydrogenase		Quinone reductase	
	−HQNO	+HQNO	−HQNO	+HQNO
0.4 M NaCl	52	54	41	6
0.4 M KCl	53	55	8	1
None	11	12	2	±

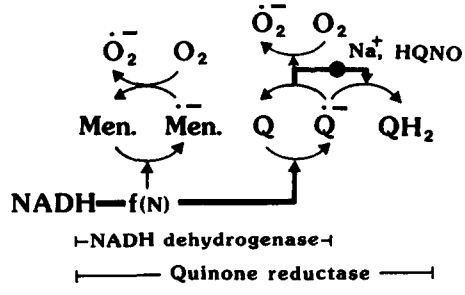
ubiquinone and menaquinone specifically requires Na^+ for the activity and is very sensitive to HQNO, whereas the membrane-bound NADH dehydrogenase measured with menadione as the electron acceptor shows no specific requirement for Na^+. Therefore, the effect of monovalent cations and HQNO on the activities of the both enzymes were examined. In this experiment, Q-1 was used as the electron acceptor. As shown in Table IV, the NADH dehydrogenase was stimulated to the same extent by the addition of either NaCl or KCl, indicating no specific requirement for Na^+. Furthermore, the activity was not inhibited by HQNO, which is a specific inhibitor of Na^+ pump [6]. On the other hand, the quinone reductase specifically required Na^+ for the activity and was strongly inhibited by HQNO. Thus, the quinone reductase had the same properties as those observed with the membrane-bound NADH : quinone oxidoreductase [4,6]. These results also indicated that the Na^+-dependent and HQNO-sensitive site in the quinone reductase was directly related to the step of quinol formation.

When Q-1 was used as the electron acceptor, the half-maximal inhibition of HQNO for the quinone reductase was estimated to be about 0.4 μM. Thus, in *V. alginolyticus* the quinone reductase is very sensitive to HQNO unlike its mitochondrial analogue. However, it should be mentioned that HQNO inhibits Na^+-dependent, but not Na^+-independent, quinone reductase of *V. alginolyticus* [6].

Discussion

The oxidation of NADH by the respiratory chain of the marine bacterium, *V. alginolyticus*, is unique in the specific requirement for Na^+ [3,4] and the site of Na^+-dependent reaction has been localized at the step of quinone reduction [4,6]. In the present work, the quinone reductase catalyzing the formation of quinol from quinone by the NADH oxidation was isolated and partially purified from the membrane of this organism. From the results of Na^+ dependence and the sensitivity to HQNO (Table IV), this enzyme can be identified as the Na^+-dependent respiratory chain NADH : quinone oxidoreductase. Since the NADH dehydrogenase is considered to be a component of quinone reductase, the former may be best characterized as a flavoprotein part of the latter.

Reacting with Q-1, the purified NADH dehydrogenase gives rise to labile semiquinone radicals which are readily autooxidized by molecular oxygen as revealed by the nonstoichiometric NADH oxidation (Fig. 3) and by the generation of superoxide radicals (Fig. 4). On the contrary, the quinone reductase produces quinol by the stoichiometric NADH oxidation (Fig. 3) without generating significant amount of superoxide radicals (Fig. 4). Although there is no direct evidence for the



Scheme I. Proposed scheme of electron transfer from NADH to quinol. Curved arrows indicate chemical reactions, and straight and right-angled arrows the direction of electron transfer. The thick arrows indicate the electron transfer in the quinone reductase. The Na^+-dependent and HQNO-sensitive site is shown by the closed circle. Men., menadione; f(N), flavoprotein component.

dismutation reaction of semiquinone radicals, the electron transfer from NADH to quinol can be formulated as shown in Scheme I. In this scheme, the quinone reductase first reduces Q-1 by one-electron transfer reaction to produce semiquinone radicals and then the latter are further converted to Q-1 and quinol due to the presence of activity catalyzing a dismutation reaction. In the absence of latter activity, the semiquinone radicals may be autooxidized by molecular oxygen resulting in the oxidation-reduction cycle of the radicals, which corresponds to the reaction catalyzed by the NADH dehydrogenase. The latter enzyme is independent of Na^+ and insensitive to HQNO (Table IV). Thus, the Na^+-dependent and HQNO-sensitive site, that is the coupling site of Na^+ pump [6], can be localized at the dismutation reaction of semiquinone radicals. As shown in Scheme I, menadione is likely to interact at the flavoprotein component, generating superoxide radicals.

The corresponding mitochondrial enzyme system, known as complex I, is the most complicated of the respiratory chain enzymes (see Ref. 17 for a review). Suzuki and King [18] reported that the complex I isolated from bovine-heart mitochondria produces stable ubisemiquinone radicals upon reduction by NADH in a rotenone-sensitive manner. They suggested the presence of an ubiquinone-binding protein called QP-N for the stabilization of radicals. Such a protein is verly likely to be present in the quinone reductase also, since no appreciable amount of superoxide radicals is generated during the reaction. They proposed the operation of protonmotive quinone cycle in this region. This cycle has been originated by Mitchell [19] as a direct-coupling redox loop model of H^+ extrusion in the bc_1 complex. On the other hand, Wikström and Krab [20] proposed an indirect-coupling redox pump *b* cycle model. In either case, ubisemiquinone radicals play an essential role. It is very interesting to note that the same type of dismutation reaction of semiquinone radicals as suggested in the bc_1 complex is possible to occur in the quinone reductase reaction also. Furthermore, the coupling site of Na^+ pump to the electron transfer is localized at this reaction step from the Na^+-dependence and HQNO-sensitivity [6]. Therefore, the quinone cycle seems to function as a mechanism of energy coupling between the cation extrusion and the electron transfer in the quinone reductase segment. Although the nature of component(s) catalyzing the dismutation reaction in the quinone reductase is not clear at present, further studies on the bacterial enzyme system will give a new insight into the mechanism of energy conservation associated with the coupling site I.

The respiratory chain NADH dehydrogenase has been purified to homogeneity from *E. coli* membrane by Jaworowski et al. [7] and by Thompson and Shapiro [8]. The highly purified enzyme consisting of a single polypeptide species of molecular weight 47000 was confirmed to be a component of respiratory chain [21]. In these reports, Q-1 [7] or decylbenzoquinone [8] was used as the electron acceptor for the assay of quinone reductase, but the quinol formation has not been measured. In this regard, it is noteworthy that the NADH dehydrogenase isolated from *V. alginolyticus* reacts with Q-1 without producing quinol. Moreover, the enzyme is incapable of reacting with Q-5 and menaquinone-4. It is apparent that in addition to NADH dehydrogenase another protein factor(s) is required for the quinol formation. Thus, the direct measurement of quinol formation employed in this work may be helpful as an effective means for the characterization of quinone reductase and related enzymes.

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