Generalized Distribution and Common Properties of Na⁺-dependent NADH:Quinone Oxidoreductases in Gram-negative Marine Bacteria

By HAJIME TOKUDA¹* AND KAZUHIRO KOGURE²

¹*Institute of Applied Microbiology, University of Tokyo, Yayoi, Bunkyo, Tokyo 113, Japan*

²*Ocean Research Institute, University of Tokyo, Minamidai, Nakano, Tokyo 164, Japan*

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Effects of Na⁺ on respiratory NADH oxidase were studied in membranes prepared from Gram-negative marine bacteria belonging to the genera *Alcaligenes*, *Alteromonas*, *Flavobacterium* and *Vibrio*. NADH oxidases of 9 out of the 10 strains examined were found to require Na⁺ for maximum activity. Without exception, the Na⁺-dependent site was located at the level of the NADH:quinone oxidoreductase region of the respiratory chain and was highly sensitive to 2-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO). NADH dehydrogenases in the Na⁺-dependent segment were sensitive to Ag⁺ and able to oxidize reduced nicotinamide hypoxanthine dinucleotide as a substrate. Since the NADH dehydrogenase was neither dependent on Na⁺ nor sensitive to HQNO, it was suggested that reduction of quinone by the NADH:quinone oxidoreductase involves the formation of an intermediate, presumably semiquinone, and that electron transfer from the intermediate requires Na⁺ and is sensitive to HQNO. These results showed a striking similarity to those obtained with the Na⁺-dependent NADH:quinone oxidoreductase, the Na⁺ pump, of *Vibrio alginolyticus*, indicating that this enzyme is widely distributed among Gram-negative marine bacteria and shares common properties.

INTRODUCTION

The marine bacterium *Vibrio alginolyticus* was found to extrude Na⁺ as a direct result of respiration (Tokuda & Unemoto, 1981, 1982). Analysis of mutants defective in this respiratory Na⁺ pump revealed that Na⁺ was extruded by only one of the two kinds of NADH:quinone oxidoreductase found in *V. alginolyticus*, NQR1 and NQR2 (Tokuda & Unemoto, 1984). Although both NQR1 and NQR2 catalysed the reduction of ubiquinone-1 (Q₁) to ubiquinol-1 (Q₁H₂), Na⁺ extrusion was exclusively coupled to Q₁H₂ formation by the NQR1 that required Na⁺ for activity and was sensitive to 2-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO). The NQR1 has recently been purified and found to be composed of three subunits, α , β and γ (Hayashi & Unemoto, 1986). The β subunit showed an NADH dehydrogenase activity in the presence of menadione or Q₁ as an electron acceptor. However, the reaction catalysed by this subunit is the formation of ubisemiquinone radicals but not Q₁H₂. In order to form Q₁H₂, presumably by dismutation of ubisemiquinone radicals, both α and γ subunits were required. Since the reaction catalysed by the β subunit did not require Na⁺ and was resistant to HQNO, the formation of Q₁H₂ from ubisemiquinone radicals seemed to be the step that required Na⁺ and was inhibited by HQNO. On the other hand, the β subunit was highly sensitive to Ag⁺. It should be noted that the requirement for Na⁺ and the inhibition by HQNO and Ag⁺ were specific to NQR1 but not observed with NQR2.

Abbreviations: CCCP, carbonylcyanide *m*-chlorophenylhydrazone; deamino-NADH, reduced nicotinamide hypoxanthine dinucleotide; HQNO, 2-heptyl-4-hydroxyquinoline-*N*-oxide; Q₁, ubiquinone-1; Q₁H₂, ubiquinol-1.

Besides *V. alginolyticus*, Na⁺ pumps coupled to NADH oxidase have been found in *V. costicola* (Udagawa *et al.*, 1986), halotolerant bacterium Ba₁ (Ken-Dror *et al.*, 1986*a, b*) and *V. parahaemolyticus* (Tsuchiya & Shinoda, 1985). Moreover, the Na⁺ pumps in *V. costicola* and strain Ba₁ were presumed to be based on an HQNO-sensitive NADH:quinone oxidoreductase. These results suggested that the Na⁺-motive NADH:quinone oxidoreductase was a general mechanism for the generation of energy in halophilic bacteria.

Nap2, the Na⁺ pump-defective mutant of *V. alginolyticus*, has a single mutation in the β subunit (Asano *et al.*, 1985), whereas another mutant, Nap1, lacks three subunits of the NQR1 (Tokuda *et al.*, 1987). Moreover, it was found that Nap1 recovered the Na⁺ pump activity by conjugation with the wild-type, which contained plasmids (Tokuda *et al.*, 1987). These results suggested the intriguing possibility that a genetic determinant for the Na⁺ pump existed on a plasmid, which may be a genetic reason for the presence of similar Na⁺ pumps in different bacteria.

In this regard, we examined the Na⁺-requirement of NADH oxidase in ten Gram-negative marine bacteria and found that nine possessed Na⁺-dependent NADH:quinone oxidoreductases, which shared common properties.

METHODS

Bacterial strains and media. Bacterial strains used are listed in Table 1. Ten of these strains were generously provided by Professor Ushio Simidu, Ocean Research Institute, University of Tokyo, and are referred to as the US strains. These were grown at 30 °C in a medium containing, per litre: 30 g NaCl, 2 g MgSO₄·7H₂O, 0.8 g KCl, 0.1 g KBr, 0.026 g SrCl₂·6H₂O, 0.02 g H₃BO₃, 5 g Bactopeptone, 1 g yeast extract, 1 g glucose. *V. alginolyticus* was grown at 37 °C on a complex medium (Tokuda & Unemoto, 1983) supplemented with 0.2% glucose.

Preparation of membranes. Bacterial cells were harvested at the late exponential phase of growth. Membrane fractions of the cells were prepared by the osmotic lysis method (Tokuda, 1986). Inverted membrane vesicles were prepared from cells of strain US 16 after disruption by passage through a French pressure cell (Aminco) as described by Tokuda *et al.* (1985).

Respiratory activities of membranes. NADH and deamino-NADH (reduced nicotinamide hypoxanthine dinucleotide) oxidases were determined from the decrease in absorbance at 340 nm. Q₁H₂ oxidase was assayed by monitoring the increase in absorbance at 275 nm. Assay mixtures (1 ml) contained 20 mM-Tris/HCl, pH 7.5, and specified salt at a given concentration. The assay was started at 30 °C by the addition of NADH, deamino-NADH or Q₁H₂ at final concentrations of 0.2, 0.2 and 0.15 mM, respectively. The amounts of electron donors oxidized were calculated using values for the millimolar absorption coefficients for NADH, deamino-NADH and Q₁H₂ of 6.22, 6.22 and 12.4, respectively.

NADH dehydrogenase and NADH:quinone oxidoreductase activities were determined after solubilization of membranes in 20 mM-Tris/HCl, pH 7.5, containing 0.2 M-KCl or NaCl and 0.2% Liponox DCH, an alkyl polyoxyethylene ether detergent (Unemoto *et al.*, 1981). The activities of NADH and deamino-NADH

Table 1. *Bacterial strains used in this work*

Genus or phenotype	Source or reference
<i>Alteromonas</i> sp. US 10	U. Simidu, University of Tokyo*
<i>Alteromonas</i> sp. US 11	
<i>Alteromonas</i> sp. US 12	
<i>Flavobacterium</i> sp. US 16	
<i>Vibrio</i> sp. US 21	
<i>Vibrio</i> sp. US 22	
<i>Alcaligenes</i> sp. US 172	
<i>Alcaligenes</i> sp. US 201	
<i>Alteromonas</i> sp. Wb-30	
<i>V. parahaemolyticus</i> ATCC 17802	
<i>V. alginolyticus</i> 138-2 (wild-type)	Tokuda <i>et al.</i> (1981)
<i>V. alginolyticus</i> Nap1† (Na ⁺ pump-defective)	Tokuda (1983)
<i>V. alginolyticus</i> Nap2† (Na ⁺ pump-defective)	Tokuda (1983)

* These 10 strains are referred to as the US strains in the text.

† Nap1 lacks all three subunits of the Na⁺ pump (Tokuda *et al.*, 1987) whereas Nap2 contains inactive β subunit (Asano *et al.*, 1985).

dehydrogenases were measured at 340 nm in the presence of 0.1 mM-menadione as an electron acceptor. Assays were started by the addition of NADH or deamino-NADH at a final concentration of 0.2 mM. Q₁H₂ formation by the NADH:quinone oxidoreductase was followed by monitoring the absorbance change between 248 and 267 nm using a Shimadzu UV 3000 dual-wavelength spectrophotometer as previously described (Tokuda & Unemoto, 1984). The millimolar absorption coefficient of Q₁ was 7.8 at this wavelength pair. NADH and Q₁ were added at 0.1 mM and 15 µM, respectively. Where specified, membranes were preincubated for 1 min in the reaction mixture containing 1 µM-AgNO₃ or 2 µM-HQNO.

Other methods. Q₁H₂ was prepared as described by Rieske (1967). Protein was determined by Lowry's method using bovine serum albumin as a standard.

Materials. Disodium NADH, disodium deamino-NADH and HQNO were purchased from Sigma. Q₁ and Liponox DCH were kindly supplied by Eisai (Tokyo, Japan) and Lion (Odawara, Kanagawa, Japan) respectively.

RESULTS

Phenotype of the bacteria

Growth of all US strains listed in Table 1 required Na⁺, and became substantially more resistant to the uncoupling agent carbonylcyanide *m*-chlorophenylhydrazone (CCCP) at pH 8.5 than at pH 6.5. It was shown previously that the wild-type of *V. alginolyticus* grew well in the presence of CCCP at pH 8.5 (Tokuda & Unemoto, 1983), whereas collapse of the electrochemical potential of H⁺ by CCCP was lethal to the Na⁺ pump-defective mutants (Tokuda, 1983). Since the Na⁺ pump has a pH optimum at about 8.5 and generates a CCCP-resistant electrochemical potential of Na⁺ (Tokuda, 1983; Tokuda *et al.*, 1988), it was thought that the collapse of the electrochemical potential of H⁺ was not lethal to the cells possessing the Na⁺ pump. Therefore, all US strains listed in Table 1 seemed to be good candidates for examination of the Na⁺ pump.

All US strains lysed when exposed to hypotonic medium, as did *V. alginolyticus* and *V. costicola*. Membrane fractions, isolated after lysis of cells, were examined for respiratory activities.

Effect of Na⁺ on NADH, Q₁H₂ and deamino-NADH oxidases

The activities of three oxidases were determined in the presence of 0.2 M-NaCl or KCl using membranes prepared from the various strains. Data shown in Table 2 represent the ratios of activity obtained in NaCl to those in KCl. In all the strains except US 16 (*Flavobacterium* sp.), activities of NADH oxidase were considerably higher in 0.2 M-NaCl than in 0.2 M-KCl. Other

Table 2. *Effect of salts on NADH, Q₁H₂ and deamino-NADH oxidases in US strains*

Membranes were isolated from the osmotically shocked lysates of US strains. Activity of the oxidases was determined spectrophotometrically using 0.2 mM-NADH, 0.15 mM-Q₁H₂ or 0.2 mM-deamino-NADH as electron donors. The assay mixture contained 20 mM-Tris/HCl, pH 7.5, and 0.2 M-KCl or NaCl. Activity of the oxidases was determined from duplicate assays. Values represent the ratio of activity obtained in the presence of 0.2 M-NaCl to that determined in the presence of 0.2 M-KCl.

Strain	Activity ratio of oxidases (NaCl/KCl)		
	NADH	Q ₁ H ₂	Deamino-NADH
US 10	2.10	1.04	3.85
US 11	2.51	1.04	4.60
US 12	2.31	1.06	4.30
US 16	0.80	1.33	—*
US 21	3.41	1.01	2.62
US 22	2.89	0.87	2.41
US 172	2.94	0.93	3.04
US 201	4.65	0.99	5.92
Wb-30	4.67	0.98	3.67
ATCC 17802	3.26	0.95	3.68

* Essentially no activity was found in the presence of KCl or NaCl.

Table 3. *Effect of Na⁺, Ag⁺ and HQNO on NADH dehydrogenase and NADH-linked Q₁H₂ formation in nine US strains*

Membranes of US strains were solubilized with 20 mM-Tris/HCl, pH 7.5, that contained 0.2% Liponox DCH and 0.2 M-NaCl or KCl. Effects of 1 μ M-AgNO₃ or 2 μ M-HQNO were examined with membranes solubilized in the presence of NaCl. NADH dehydrogenase and NADH-linked Q₁H₂ formation were determined as described in Methods. Values obtained in the absence of NaCl or in the presence of inhibitors were expressed as a percentage of activity obtained in the presence of NaCl without inhibitors. All the values represent the mean of duplicate determinations.

Strain	Relative activity (%)					
	NADH dehydrogenase			Q ₁ H ₂ formation		
	-Na ⁺	+Ag ⁺	+HQNO	-Na ⁺	+Ag ⁺	+HQNO
US 10	90	6	105	6	1	26
US 11	94	3	143	4	1	16
US 12	88	42	122	2	25	50
US 21	106	6	102	7	2	33
US 22	100	5	99	11	6	42
US 172	101	7	121	16	4	20
US 201	98	5	166	7	1	51
Wb-30	98	9	105	9	3	31
ATCC 17802	86	16	159	7	7	29

monovalent cations such as Li⁺, Rb⁺, Cs⁺ or choline⁺ were ineffective in replacing the stimulatory effect of Na⁺ (results not shown). In contrast, activities of Q₁H₂ oxidase in the presence of KCl or NaCl were essentially the same in membranes from all the strains. These results suggested that NADH oxidases from nine of the bacterial species specifically required Na⁺ at the NADH : quinone oxidoreductase segment of the respiratory chain. Although results are not shown, inverted membrane vesicles prepared from strain US 16 also had no Na⁺-dependent NADH oxidase, indicating that failure to detect the Na⁺-requirement in this strain was not dependent on the method of membrane preparation.

It was recently reported that *Escherichia coli* membranes retain two kinds of NADH dehydrogenase, which differ in their abilities to generate energy and oxidize deamino-NADH (Matsushita *et al.*, 1987). Oxidation of deamino-NADH was observed only with NADH dehydrogenase coupled to the extrusion of H⁺. Since *V. alginolyticus* also retains two kinds of NADH dehydrogenase, the ability to oxidize deamino-NADH was examined in membranes isolated from the wild-type, Nap1 and Nap2. Although results are not shown, membranes from the wild-type showed both deamino-NADH dehydrogenase and oxidase activities. Moreover, the oxidase activity determined in the presence of 0.2 M-NaCl was 3.5-fold higher than that in 0.2 M-KCl. On the other hand, essentially no activity was detected in membranes from Nap1 or Nap2. These results clearly indicated that the ability to oxidize deamino-NADH was specific to the NQR1, which required Na⁺ for activity.

Deamino-NADH oxidase was next examined with membranes prepared from the US strains (Table 2). Membranes of nine US strains having Na⁺-dependent NADH oxidase were found to possess Na⁺-dependent deamino-NADH oxidase activity. In contrast, the ability to oxidize deamino-NADH was not detected with membranes of strain US 16, which retained an Na⁺-independent NADH oxidase. Na⁺-dependence of oxidase activity in some strains was appreciably enhanced when deamino-NADH was used instead of NADH.

Effects of Na⁺, Ag⁺ and HQNO on NADH dehydrogenase and NADH-linked Q₁H₂ formation

Although the NQR2 of *V. alginolyticus* was inactivated by solubilization of membranes, the NQR1 retained full activity in the presence of the non-ionic detergent Liponox DCH, and reduced Q₁ to Q₁H₂ in the presence of NADH. Solubilized membranes prepared from nine US strains also retained activities of NADH dehydrogenase and NADH-linked Q₁H₂ formation. Effects of salt on both activities were assayed in the presence of 0.2% Liponox DCH. The

activity determined in the presence of 0.2 M-KCl expressed as a percentage of that determined in the presence of 0.2 M-NaCl is shown in Table 3. It was clear that the NADH-linked Q₁H₂ formation by all the membranes substantially decreased when NaCl was omitted. On the other hand, the activity of NADH dehydrogenase was essentially the same in KCl and NaCl. These results indicated that NADH:quinone oxidoreductases of nine US strains required Na⁺ for electron transfer after NADH dehydrogenase.

NADH dehydrogenase in solubilized membranes of nine US strains was strongly inhibited by Ag⁺, which presumably acts as a thiol reagent, whereas HQNO had no effect on the enzyme activity (Table 3). When NADH-linked Q₁H₂ formation was examined, however, both Ag⁺ and HQNO caused marked inhibition. These results indicated that Ag⁺ and HQNO inhibited different sites of Na⁺-dependent NADH:quinone oxidoreductase. HQNO specifically inhibited the site where Na⁺ was required. It is remarkable that the Na⁺-dependent site and the Ag⁺- or HQNO-sensitive sites were common in all the NADH:quinone oxidoreductases examined.

DISCUSSION

Since an Na⁺-dependent NADH oxidase activity was found in 9 out of the 10 halophilic strains examined, there is no doubt that this is a general mechanism present in a wide variety of Gram-negative marine bacteria. Moreover, the Na⁺-dependent site of the NADH oxidases was without exception at the NADH:quinone oxidoreductase segment of the respiratory chain. The Na⁺-dependent NADH:quinone oxidoreductases have been identified and characterized as Na⁺ pumps in *V. alginolyticus* (Tokuda & Unemoto, 1984), *V. costicola* (Udagawa *et al.*, 1986) and halotolerant bacterium Ba₁ (Ken-Dror *et al.*, 1986*a, b*). Although results are not presented, we found that inverted membrane vesicles prepared from strain US 201 possessed the respiratory Na⁺ pump, which was energized by NADH. From these data, it is quite likely that Na⁺-dependent NADH:quinone oxidoreductases in nine of the US strains also function as the respiratory Na⁺ pump.

In addition to the unique property of Na⁺-requirement, marked sensitivity to HQNO was a characteristic property of the Na⁺-dependent NADH:quinone oxidoreductases. Furthermore, the mode of electron transfer by these enzymes was similar. In *V. alginolyticus*, reduction of Q₁ to Q₁H₂ by the NQR1 took place via a semiquinone radical (Hayashi & Unemoto, 1984). Formation of Q₁H₂ from the semiquinone radical was shown to require Na⁺ whereas formation of the semiquinone radical was independent of Na⁺ and catalysed by NADH dehydrogenase, which was sensitive to Ag⁺ (Asano *et al.*, 1985). Remarkably, NADH dehydrogenases and Na⁺-dependent NADH:quinone oxidoreductases in nine of the US strains were similarly inhibited by Ag⁺ and HQNO, respectively. It is likely that these enzymes of US strains also form a semiquinone radical as an intermediate of Q₁H₂ formation. Moreover, all Na⁺-dependent NADH:quinone oxidoreductases in *V. alginolyticus* and US strains oxidized deamino-NADH while the Na⁺-independent NQR2 of *V. alginolyticus* was unable to do so. These results indicate that Na⁺-dependent NADH:quinone oxidoreductases in taxonomically different bacteria have common properties, which are summarized schematically in Fig. 1. Since the NQR1 of *V. alginolyticus* seemed to be encoded by a self-transmissible plasmid (Tokuda *et al.*, 1987), it is of great interest to examine whether these enzymes have any homology at the molecular level.

It may be noteworthy that no strain possessed Na⁺-dependent Q₁H₂ oxidase. Although the number of bacterial strains examined is still limited, these results suggest that the presence of a Q₁H₂ oxidase that requires Na⁺ is rare even in marine halophiles. Bacterial membranes contain various dehydrogenases and are able to reduce quinone by utilization of a number of substrates (Ingledew & Poole, 1984). Little is known about energy coupling of quinol formation in bacteria. In *E. coli* and *V. alginolyticus*, however, generation of energy seems to be exclusive to NADH-linked Q₁H₂ formation (unpublished observation). Since Na⁺-dependent NADH:quinone oxidoreductases presumably require Na⁺ as a coupling ion for energy generation, requirement for Na⁺ may be limited to the pathway of NADH-linked quinol formation. At present, however, it is not completely excluded that a Na⁺ pump coupled to other respiratory segments exists in

Fig. 1. A scheme depicting common properties of Na^+ -dependent (Na^+ -motive) NADH:quinone oxidoreductase in Gram-negative marine bacteria. The Na^+ -dependent NADH:quinone oxidoreductase in *V. alginolyticus* is composed of three subunits. Subunit compositions of the enzyme in other strains described in this paper are not known. Q_1H_2 formation by the enzyme proceeds via the ubisemiquinone radical ($\text{Q}_1^{\cdot-}$) as an intermediate. Ag^+ and HQNO inhibit reactions before and after the intermediate, respectively. Na^+ is required and, presumably, translocated at the site after the intermediate. NADH dehydrogenase is able to utilize deamino-NADH as a substrate. For more details, see the text.

micro-organisms. In this connection, the phenotype of strain US 16 seems of interest. Growth of this strain was substantially more resistant to CCCP at pH 8.5 than at pH 6.5, whereas membranes prepared from this strain had no Na^+ -dependent NADH oxidase. Further investigations are necessary to clarify whether or not strain US 16 has a different type of ion pump.

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