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MINI-REVIEW

Sodium-Transport NADH-Quinone Reductase of a Marine *Vibrio alginolyticus*

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Abstract

The respiratory chain of a marine bacterium, *Vibrio alginolyticus*, required Na^+ for maximum activity, and the site of Na^+-dependent activation was localized on the NADH-quinone reductase segment. The Na^+-dependent NADH-quinone reductase extruded Na^+ as a direct result of redox reaction. It was composed of three subunits, α, β, and γ, with apparent *Mr* of 52, 46, and 32 KDa, respectively. The reduction of ubiquinone-1 to ubiquinol proceeded via ubisemiquinone radicals. The former reaction was catalyzed by the FAD-containing β subunit. This reaction showed no specific requirement for Na^+. For the formation of ubiquinol, the presence of the γ subunit and the FMN-containing α subunit was essential. The latter reaction specifically required Na^+ for activity and was strongly inhibited by 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide. It was assigned to the coupling site for Na^+ transport. The mode of energy coupling of redox-driven Na^+ pump was compared with those of decarboxylase- and ATP-driven Na^+ pumps found in other bacteria.

Key Words: Na^+ transport; NADH-quinone reductase; Na^+ pump; respiratory chain; marine bacteria; moderately halophilic bacteria.

Introduction

Marine and halophilic bacteria are unique in the requirement for Na^+ for optimal growth (MacLeod, 1965; Reichelt and Baumann, 1974; Kushner, 1978). MacLeod was the first to investigate the function of Na^+ in the growth of a marine bacterium Pseudomonad B-16, which has been reclassified as *Alteromonas haloplanktis* 214. In 1957, it was reported that Na^+ and a small

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amount of K^+ are required for the oxidation of exogenous substrates by whole-cell suspensions of *A. haloplanktis* (Tomlinson and MacLeod, 1957). When cell-free extracts were used, none of the dehydrogenases that could be expected to be involved in the oxidation of substrates was found to be dependent on Na^+ for activity (MacLeod *et al.*, 1958; MacLeod and Hori, 1960). Thus, Na^+ was considered to be required for the transport of substrates into the cells. Indeed, α-aminoisobutyric acid (AIB), a nonmetabolizable amino acid analog, was accumulated by a Na^+-dependent process (Drapeau and MacLeod, 1963; Wong *et al.*, 1969). By comparing the quantitative requirements for Na^+, the Na^+ requirement for the oxidation of substrates was considered to represent a requirement for Na^+ to transport substrates into the cells (Drapeau *et al.*, 1966).

With respect to the mechanism of energy coupling to membrane transport, a direct coupling to the respiratory chain was first proposed (Thompson and MacLeod, 1974; Sprott and MacLeod, 1974), but later the Na^+-dependent transport was shown to be driven by chemiosmotic coupling (Niven and MacLeod, 1978, 1980).

We also confirmed that the active uptake of all of the amino acids by the intact cells of a marine bacterium, *Vibrio alginolyticus*, is absolutely dependent on Na^+, and other cations such as Li^+, K^+, Rb^+, and Cs^+ cannot substitute for Na^+. Intracellular cations were replaced with a desired cation under iso-osmotic conditions without damaging the function of cell membranes (Nakamura *et al.*, 1982). Detailed bioenergetic studies clearly indicated that $\Delta\tilde{\mu}_{Na^+}$ is a direct driving force for AIB uptake and that the requirement for K^+ observed with the Na^+-loaded and K^+-depleted cells is due to the necessity for K^+ to permit the generation of $\Delta\tilde{\mu}_{Na^+}$ (Tokuda *et al.*, 1982).

An extremely halophilic *Halobacterium halobium* (Lanyi, 1979) and alkalophilic bacilli (Kitada and Horikoshi, 1977; Krulwich, 1986) also employ $\Delta\tilde{\mu}_{Na^+}$ rather than $\Delta\tilde{\mu}_{H^+}$ for the active uptake of nutrients. In these organisms, a secondary Na^+/H^+ antiporter, which is driven by $\Delta\tilde{\mu}_{H^+}$, was considered to be the major mechanism for the extrusion of Na^+. In addition to $\Delta\tilde{\mu}_{H^+}$-driven Na^+/H^+ antiporter, the marine *V. alginolyticus* was found to have a respiration-driven primary Na^+ pump (Tokuda and Unemoto, 1981, 1982). Thus the $\Delta\tilde{\mu}_{Na^+}$ was generated as a direct result of respiration in the marine *V. alginolyticus*. Recently, Skulachev and his coworkers demonstrated that *V. alginolyticus* employs $\Delta\tilde{\mu}_{Na^+}$ for cell motility (Dibrov *et al.*, 1986a) and ATP synthesis (Dibrov *et al.*, 1986b). Therefore, the respiration-driven Na^+ pump plays a central role in the energetics of marine bacteria. This paper reviews the respiration-driven Na^+ pump catalyzed by a Na^+-dependent NADH-quinone reductase with special reference to the mode of energy coupling.

Na^+-Dependent Activation of Respiratory Chain

During our studies on the salt modifications of enzymes from the marine *V. alginolyticus*, periplasmic and membrane-bound phosphohydrolases were found to require salts for activity, but these enzymes were rather influenced by the species of anions, and no specific requirement for monovalent cations was detected (Unemoto *et al.*, 1974). In 1977, it was found that the membrane-bound NADH oxidase of *V. alginolyticus* is specifically activated by Na^+ (Unemoto *et al.*, 1977). Since the marine *V. alginolyticus* lyses under hypotonic conditions (Unemoto *et al.*, 1973), the membrane fraction was prepared by osmotic lysis of the cells. Electron micrographs of thin sections revealed that the lysed cells still retain their rod shape but are devoid of intracellular materials (Unemoto *et al.*, 1974). Their membrane structure had intermittent breakages and was permeable to fluorescein isothiocyanate-dextran 3 (average Mr = 3000). Since the membrane fraction prepared by the osmotic lysis exhibited full activity for the oxidation of exogenously added NADH, it was an excellent preparation for the study of NADH oxidase (Unemoto *et al.*, 1977).

As shown in Fig. 1, the membrane-bound NADH oxidase from *V. alginolyticus* required Na^+ for the maximum activity. Other cations such as Li^+, K^+, and Mg^{2+} were relatively ineffective as replacements for Na^+. The concentration of Na^+ required for a half-maximum activation ($M_{1/2}$) was estimated to be 81.8 mM. The requirement for Na^+, however, was greatly reduced in the presence of 400 mM K^+ (curve 1 in Fig. 1) or 10 mM Mg^{2+},

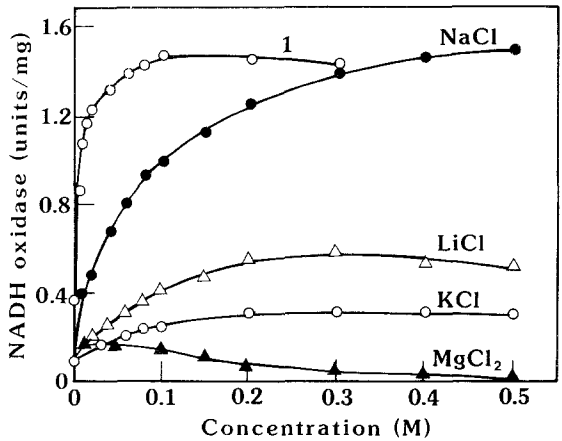

Fig. 1. Effects of cations on the NADH oxidase of the EDTA-treated membranes from *V. alginolyticus*. The NADH oxidase activity was measured in the presence of cations as indicated in the figure. In curve 1, the concentration of Na^+ was varied in the presence of 400 mM K^+. Data taken from Unemoto *et al.* (1977).

Table I. Species of Bacteria That Have Na^+-Dependent NADH Oxidase and Respiration-Driven Na^+ Pump

Bacterium	Activity*	Reference
Marine bacterium		
V. alginolyticus	*a*, *b*	Unemoto *et al.*, 1977 Tokuda and Unemoto, 1981, 1982
P. phosphoreum	*a*	Watanabe *et al.*, 1977
A. haloplanktis, *V. fisheri*, *V. natriegens*, *P. phosphoreum*	*a*	Khanna *et al.*, 1984
V. parahaemolyticus, and other marine *Vibrio*	*a*, *b*	Tsuchiya and Shinoda, 1985
Psychrophilic *Vibrio* ABE-1	*a*, *b*	Takada *et al.*, 1981, 1988
Moderately halophilic bacterium		
V. costicola	*a*, *b*	Unemoto *et al.*, 1977 Udagawa *et al.*, 1986
Halotolerant Ba_1	*a*, *b*	Ken-Dror *et al.*, 1984, 1986a, b

*a, Na^+-dependent NADH oxidase; *b*, respiration-driven Na^+ pump.

where $M_{1/2}$ for Na^+ was calculated to be 6.4 or 13.8 mM, respectively. Thus, K^+ and Mg^{2+} cooperated with Na^+ for activation. In the absence of Na^+, any combination of cations such as Li^+, K^+, Rb^+, Cs^+, Mg^{2+}, and Ca^{2+} showed no significant activation. Thus, Na^+ was essential for the maximum activation of NADH oxidase.

A similar type of Na^+ activation was observed with the membrane-bound NADH oxidase from a moderately halophilic *V. costicola* that requires 1.0 M NaCl for optimal growth. On the other hand, the NADH oxidase of nonhalophilic *Escherichia coli* was active in the absence of salts and showed no specific requirement for Na^+ for activity (Unemoto *et al.*, 1977).

Although detailed studies on the cation dependence of NADH oxidase have not been reported in other bacteria, Table I lists the species of bacteria that have a Na^+-dependent NADH oxidase. Most of these bacteria have also been shown to have respiration-driven Na^+ pump. As early as 1977, Watanabe *et al.* reported the Na^+-dependent stimulation of NADH oxidase in a marine *Photobacterium phosphoreum*. Khanna *et al.* (1984) reported that the NADH oxidase of typical marine bacteria is activated by Na^+ and spared by K^+. The NADH oxidase of a moderately halophilic bacterium, halotolerant Ba_1, was also activated by Na^+, and Na^+ was extruded by respiration (Ken-Dror *et al.*, 1984, 1986a, b). Recently, it was confirmed that the NADH oxidase of moderately halophilic *Pseudomonas halosaccharolytica* (ATCC 29423) and an unidentified halophile (NRCC 41227) is activated by Na^+ (unpublished observations). Thus, Na^+-dependent NADH oxidase seems to be widely distributed among marine and moderately halophilic bacteria.

It is interesting to note that none of the primary Na^+ pump has been reported in extremely halophilic bacteria that grow at 4 M NaCl. Once a light-driven halorhodopsin was thought to be a primary Na^+ pump (Lanyi and Weber, 1980), but later it turned out to be a primary Cl^- pump generating inside-negative $\Delta\psi$ (Schobert and Lanyi, 1982). In extremely halophilic bacteria, light-driven H^+-pumping bacteriorhodopsin and Cl^--pumping halorhodopsin together with H^+-pumping respiratory chain seem to generate a sufficient $\Delta\tilde{\mu}_{H^+}$ to support the generation of $\Delta\tilde{\mu}_{Na^+}$ via an electrogenic Na^+/H^+ antiporter. As for extremely alkalophilic bacteria that grow optimally at pH 10–11, Skulachev (1987) posed a question on the generation of an effective Na^+ gradient (directed inward the cells) from $\Delta\tilde{\mu}_{H^+}$, and the presence of a primary Na^+ pump was suggested. Although none of the primary Na^+ pump has yet been reported in extremely alkalophilic bacteria, it is of interest that these bacteria contain unusually high cytochromes in their membranes (Lewis *et al.*, 1980).

Na^+-Dependent NADH-Quinone Reductase

The respiratory chain of *V. alginolyticus* is composed of ubiquinone (Q), menaquinone (MK), and cytochromes *b*, *c*, *d*, and *o* (Unemoto and Hayashi, 1979). As partial reactions in the respiratory chain, NADH dehydrogenase as measured with menadione or ferricyanide, and quinol oxidase as measured with duroquinol, ubiquinol-1 or tetramethyl-*p*-phenylenediamine showed no specific requirement for Na^+. On the other hand, the reduction of Q-1 to ubiquinol by NADH (NADH-Q-1 reductase) required Na^+ for maximum activity. The membrane-bound quinones were depleted from lyophilized membranes and authentic Q-10 or MK-4 were reincorporated. The reduction of incorporated quinones by NADH was found to be strictly dependent on Na^+. The cooperative effect of K^+ with Na^+ observed with the NADH oxidase was also detected in this reaction. Thus it was concluded that the site of Na^+-dependent activation in the respiratory chain of *V. alginolyticus* resides in the step of NADH-quinone reductase (Unemoto and Hayashi, 1979).

When the membranes were treated with 5 mM EDTA, the NADH oxidase activity in the absence of salts amounted to about 10% of the maximum, as shown in Fig. 1. Without EDTA treatment, the NADH oxidase activity amounting to 20–30% of the maximum was consistently observed in the absence of salts. This activity was insensitive to 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide (HQNO), whereas the Na^+-dependent activity was strongly inhibited by HQNO. Tokuda (1983) isolated Na^+ pump-deficient mutants. Detailed studies on these mutants revealed that there are two

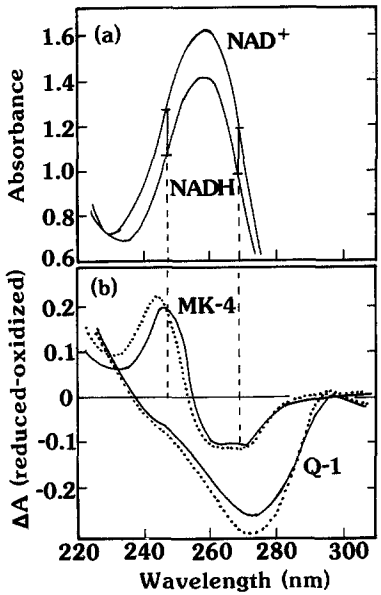

Fig. 2. Ultraviolet spectra of NADH and NAD^+ (a), and the reduced minus oxidized difference spectra of Q-1 and MK-4 (b). (a) 0.1 mM each in 25 mM Tris-HCl (pH 7.8). (b) Q-1 (22.6 μM) or MK-4 (7.2 μM) was dissolved in a solution containing 25 mM Tris-HCl (pH 7.8), 0.2 M NaCl, and 0.05% Liponox DCH, and the quinone in the sample cuvette was reduced by $NaBH_4$. The dotted lines represent the difference spectra of each quinone in ethanolic solution. Data taken from Hayashi and Unemoto (1987b).

different types of NADH-quinone reductase in the wild type cells; one is independent of Na^+ for activity and insensitive to HQNO, and the other is dependent on Na^+ and sensitive to HQNO (Tokuda and Unemoto, 1984). Therefore, all of the NADH oxidase detected in the membranes of wild type cells do not necessarily require Na^+ for activity. There is an Na^+-independent HQNO-insensitive pathway in the oxidation of NADH by the respiratory chain. The presence of similar types of NADH-quinone reductase was also reported in moderately halophilic Ba_1 (Ken-Dror *et al.*, 1986b).

Surveys of Na^+-dependent reaction in other respiratory chain-linked enzymes gave negative results. For example, when *V. alginolyticus* was aerobically grown with glycerol as a sole carbon source, respiratory chain-linked L-glycerol 3-phosphate dehydrogenase was induced (Unemoto *et al.*, 1981). Although the purified enzyme required 0.2 M salts for optimal activity, no specific requirement for Na^+ was observed. At present, the Na^+-dependent reaction in the respiratory chain was detected only in NADH-quinone reductase segment.

For the assay of NADH-quinone reductase, it was necessary to directly measure the reduction of quinones by NADH. The spectrophotometric assays were based on the difference in absorbance of the reduced and oxidized

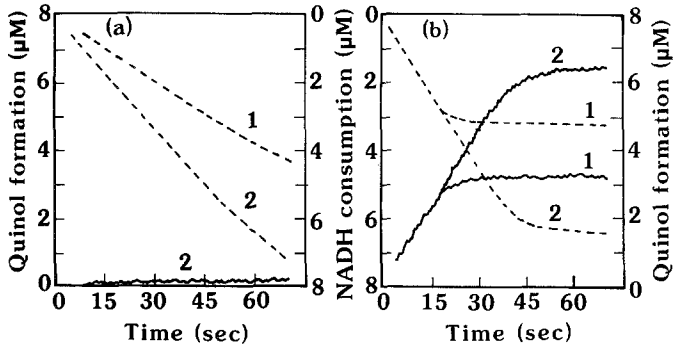

Fig. 3. Mode of Q-1 reduction by NADH dehydrogenase (a) and NADH-quinone reductase (b). The reaction mixture contained 25 mM Tris-HCl (pH 7.8), 0.1 mM NADH, 0.2 M NaCl, 0.05% Liponox DCH, enzyme, and Q-1 in a total volume of 2.0 ml. In both cases, the concentration of Q-1 is 3.4 μM in curve 1 and 6.8 μM in curve 2. The ubiquinol formation (full lines) was followed by the absorbance changes at 242–270.5 nm as described in the text. The consumption of NADH (dotted lines) was measured at 340 nm taking the millimolar absorption coefficient as 6.22 (a), and 6.81 (b). In the latter case, it was necessary to correct changes in absorbance at 340 nm due to the conversion of Q-1 to ubiquinol.

quinones. As shown in Fig. 2a, the oxidation of NADH to NAD^+ causes the increase in absorbance between 230 and 280 nm. On the other hand, the reduced minus oxidized difference spectra of Q-1 and MK-4 have characteristic absorbance changes between these wavelengths (Fig. 2b). Therefore, the absorbance difference at the wavelength pairs used for a direct assay of ubiquinone reduction (280–289 nm) (Szarkowska and Klingenberg, 1963) and menaquinone reduction (265–289 nm) (Kröger and Dadák, 1969) was inapplicable to the assay of NADH-quinone reductase. However, if the appropriate two wavelengths are selected so as to cause the same extent of increase in absorbance by the NADH oxidation, the measurement of absorbance difference between the two selected wavelengths may be unaffected by the NADH oxidation. Such a kind of wavelength pair could be successfully used for the measurement of quinol formation by NADH (Unemoto and Hayashi, 1979; Hayashi and Unemoto, 1984, 1986, 1987a). First, the absorption coefficients at the selected wavelength pair were calculated from the reduced minus oxidized difference spectra in ethanolic solution. As shown in Fig. 2b, the difference spectra were slightly shifted in a detergent-containing buffer solution. In the presence of 0.05% Liponox DCH, a nonionic detergent, the millimolar absorption coefficient at 247–268.5 nm was estimated to be 41 for MK-4 and 7.9 for Q-1. The reduction of Q-1 was also assayed at 242–270.5 nm taking the millimolar absorption coefficient as 9.6 (Hayashi and Unemoto, 1987b). This method was very useful for the analysis of reaction mechanism (see Fig. 3).

The Na^+-dependent NADH-quinone reductase of *V. alginolyticus* was extracted from the membranes by Liponox DCH and was purified by

chromatography on QAE-Sephadex (Hayashi and Unemoto, 1984). NADH dehydrogenase activity as measured with menadione as an electron acceptor was separated into two fractions at the second QAE-Sephadex column chromatography. One fraction could react with several artificial electron acceptors such as menadione, dichlorophenolindophenol, and ferricyanide, but it could not reduce quinones such as Q-5 and MK-4. It was designated as NADH dehydrogenase. In addition to the above-described activities, the other fraction could reduce Q-1, Q-5, and MK-4 to quinols. The latter fraction was designated as NADH-quinone reductase. It was found that the former is a component of the latter (Hayashi and Unemoto, 1984).

Interestingly, the NADH dehydrogenase reacted with Q-1 without producing ubiquinol. As shown in Fig. 3a, the NADH dehydrogenase consumed NADH in excess of the amount of Q-1 present, and no detectable amount of ubiquinol was produced during the reaction. On the other hand, the NADH-quinone reductase produced ubiquinol according to the consumption of NADH, and the reaction stopped when all of the Q-1 was converted to ubiquinol (Fig. 3b). Apparently, the NADH dehydrogenase reduced Q-1 by a one-electron transfer reaction and the ubisemiquinone radicals produced were auto-oxidized by molecular oxygen, forming an oxidation–reduction cycle of the radicals. Indeed, the reduction of acetylated cytochrome *c* by superoxide radicals was observed with the NADH dehydrogenase in the presence of Q-1, which was completely inhibited by the addition of superoxide dismutase. On the other hand, the NADH-quinone reductase catalyzed the stoichiometric reduction of Q-1 by NADH and no appreciable amount of superoxide radicals was detected. When menadione was used as the electron acceptor, both enzymes produced superoxide radicals and NADH was consumed in excess of the amount of menadione (Hayashi and Unemoto, 1984).

With respect to the cation dependence and HQNO sensitivity, the purified NADH dehydrogenase was activated to the same extent by the addition of either Na^+ or K^+, and was insensitive to HQNO. The NADH-quinone reductase, however, required Na^+ for activity and was strongly inhibited by HQNO, which is a specific inhibitor of respiration-driven Na^+ pump (Tokuda and Unemoto, 1984). These results indicated that the Na^+-dependent and HQNO-sensitive site in the NADH-quinone reductase is not the reaction catalyzed by the NADH dehydrogenase but the reaction catalyzing the formation of ubiquinol from ubisemiquinone (Hayashi and Unemoto, 1984). Ken-Dror *et al.* (1986b) also arrived at the same conclusion from the data of electron-spin resonance spectra in the Na^+-dependent NADH-quinone reductase of halophilic Ba_1.

It is worth noting the pH dependence of Na^+-dependent NADH-quinone reductase. Since the pH profile of CCCP-resistant growth of *V. alginolyticus* (Tokuda and Unemoto, 1983) was similar to that of the

generation of CCCP-resistant $\Delta\psi$ (Tokuda and Unemoto, 1982), the CCCP-resistant growth at alkaline pH was concluded to be supported by the Na^+ pump functioning at alkaline pH. Indeed, the purified NADH-quinone reductase manifested its optimal activity between pH 7.5 and 8.8. At that time, the sensitivity of the growth for CCCP at acidic pH was considered to be due to the lowering of Na^+ pump activity. The purified enzyme, however, still maintained about 78 and 40% of the optimal activity at pH 7.0 and 6.5, respectively. Thus the Na^+ pump is functional even at neutral and acidic pH range. The fact that CCCP-resistant $\Delta\psi$ is generated at pH 6.5 (Tokuda and Unemoto, 1982) supports this contention. Recently, the Na^+ pump activity at pH 6.5 was also reported in a psychrophilic marine *Vibrio* ABE-1 (Takada *et al.*, 1988). Therefore, it is reasonable to consider that the growth inhibition by CCCP at acidic pH is mainly due to the collapse of ΔpH, inside alkaline. The function of the Na^+ pump in the presence of CCCP renders intracellular pH even more acidic than the external pH by the generation of CCCP-resistant $\Delta\psi$ and thus potentiates the growth inhibition at neutral and acidic pH. Thus, the pH profile of CCCP-resistant growth does not simply reflect the pH dependence of Na^+ pump. Moreover, the maintenance of CCCP-resistant growth by the Na^+ pump is feasible only at alkaline pH, where the acidification of cell interior is rather favorable for the growth of cells.

Subunit Structure and Electron Transfer Pathway

To make clear the protein component(s) that catalyze the ubiquinol formation in the NADH-quinone reductase, further purifications were performed by use of high-performance liquid chromatography (Hayashi and Unemoto, 1986, 1987a). Table II lists the subunit composition, flavin content, and enzyme activity of purified preparations. The purified NADH-quinone reductase was composed of three subunits, α, β, and γ, with apparent *Mr* of 52, 46, and 32 KDa, respectively, and contained each subunit in

Table II. Subunit Composition, Flavin Content, and Enzyme Activity of Purified Preparations from *V. alginolyticus*

Enzyme preparation	Subunit	Flavin[a]		Activity[b]	
		FAD	FMN	NQR	NDH
Quinone reductase	α, β, γ	7.2	7.0	112	181
NADH dehydrogenase	β, γ	18.5	3.0	12	255
NADH dehydrogenase	β	21.3	0	0	390
The α fraction	α	0.2	10.3	0	0

[a] nmol/mg protein.
[b] Units/mg protein; NQR, NADH-quinone reductase as measured by the reduction of Q-1 to QH_2; NDH, NADH dehydrogenase as measured with menadione.

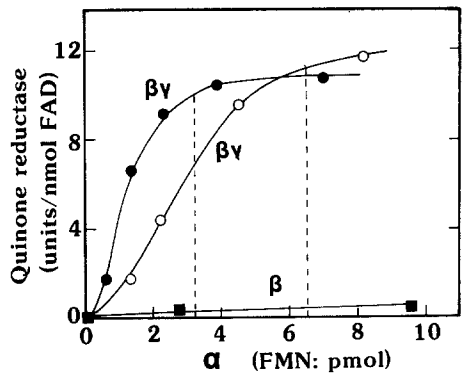

Fig. 4. Reconstitution of NADH-quinone reductase. $\beta\gamma$ corresponding to 3.25 (●) or 6.5 (○) pmol FAD, or β corresponding to 3.6 pmol FAD (■), was preincubated with various amounts of α, and the NADH-quinone reductase activity was determined. The activity is expressed in units/nmol FAD. Data taken from Hayashi and Unemoto (1987a).

equimolar quantities. Moreover, it contained FAD and FMN in equimolar quantities. The NADH dehydrogenase was first isolated as the $\beta\gamma$ complex, but further purifications revealed that the β subunit containing one FAD molecule per molecule corresponds to NADH dehydrogenase. The α subunit contained FMN as a prosthetic group, but it showed no enzyme activity by itself. As shown in Fig. 4, the Na^+-dependent NADH-quinone reductase was reconstituted from α and $\beta\gamma$, but not from α and β, indicating that three subunits are essential for the NADH-quinone reductase. Furthermore, the activity attained its maximum at the equimolar amount of FAD and FMN. The role of γ was examined with the enzyme preparations containing different ratio of β and γ, and it was found that γ increases the affinity of β for Q-1 (Hayashi and Unemoto, 1987a).

From these results, the electron transfer pathway from NADH to ubiquinol was formulated as shown in Fig. 5. The FAD-containing β subunit accepts electrons from NADH and reduces menadione or Q-1 by a one-electron transfer reaction to produce semiquinones. In the absence of α

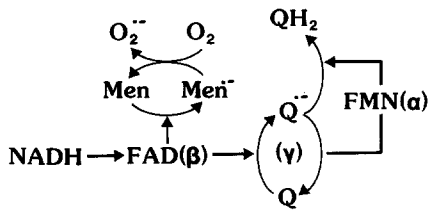

Fig. 5. Electron-transfer pathway in the Na^+-dependent NADH-quinone reductase. Curved arrows indicate chemical reactions, and straight arrows indicate the electron transfer. Men, menadione.

subunit, the semiquinones are auto-oxidized by molecular oxygen, resulting in the oxidation–reduction cycle of the radicals, which corresponds to the reaction catalyzed by the NADH dehydrogenase (β or $\beta\gamma$). This reaction is independent of Na^+ and insensitive to HQNO. In the presence of α and γ subunits, ubisemiquinones are quickly converted to ubiquinol and no significant amount of radicals is detected. At present, the γ subunit is not isolated in a pure state and the molecular nature of γ is not clear. However, γ is essential for the NADH-quinone reductase (Fig. 4). Since the presence of γ increases the affinity of β for Q-1, γ plays an important role in the interaction of Q-1 with β as well as the electron transfer from β to α. Thus γ is very likely to participate in the dismutation reaction of ubisemiquinones.

The molecular weight of purified NADH-quinone reductase complex was determined by low-angle laser light scattering combined with high-performance gel chromatography (Hayashi and Unemoto, 1987a). In the presence of 0.1% Liponox DCH, the molecular mass of the protein moiety of the active complex was calculated to be 254 KDa. This value was twice that of $\alpha + \beta + \gamma$ (130 KDa), indicating that the active complex exists as a dimer of $\alpha\beta\gamma$ or $\alpha_2\beta_2\gamma_2$ in 0.1% Liponox solution. Since no other active species are detected in the eluate of gel chromatography, it is very likely that this complex functions as a minimum active entity in the membranes also.

Mode of Energy Coupling

Proteoliposomes containing the purified NADH-quinone reductase were prepared by a cholate-deoxycholate dialysis procedure, and the generation of $\Delta\psi$, positive inside, was monitored by the absorbance band shift of oxonol VI (Hayashi and Unemoto, 1987a). By the addition of NADH in the presence of Q-1, the $\Delta\psi$ was generated concomitant with the formation of ubiquinol, which was completely dependent on Na^+ and was inhibited by HQNO. When menadione, which accepts electrons from FAD-containing β subunit, was used as the electron acceptor, no significant $\Delta\psi$ was generated in spite of the oxidation of NADH. Thus, the Na^+-dependent and HQNO-sensitive reaction catalyzed by the FMN-containing α subunit directly participated in the generation of $\Delta\psi$, indicating that this reaction step is the coupling site of Na^+ transport.

The mitochondrial NADH-quinone reductase, known as Complex I, contains FMN as a sole flavin and at least six iron–sulfur clusters, and translocates H^+ coupled to the reduction of ubiquinone by NADH (Ragan, 1987). Although the electron-transfer pathway through these components and the site of energy coupling remain to be established, Ragan (1987) proposed that the flavosemiquinone, ubisemiquinone, or both radicals

mediate the redox-linked H^+ translocation. Since the Na^+-dependent NADH-quinone reductase translocates Na^+ instead of H^+, it is unlikely that these semiquinone radicals directly function as a carrier of Na^+. Thus, an indirect coupling redox pump mechanism is plausible for this enzyme system.

The arrangement of electron flow in Fig. 5 is analogous to the "b cycle" model of Wikström and Krab (1980) proposed for cytochrome bc_1 complex. In the "b cycle" model, ubisemiquinone is produced by the oxidation of ubiquinol and its dismutation reaction catalyzed by two *b* cytochromes is intimately involved in H^+ translocation. In the case of Na^+-transport NADH-quinone reductase, ubisemiquinone is produced by one-electron reduction of ubiquinone, and the dismutation reaction catalyzed by the FMN-containing α subunit is the site of Na^+ translocation. Interestingly, a highly reactive ubisemiquinone functions as a direct energy donor for cation transport in both cases.

Primary Na^+ pumps coupled to chemical reactions other than respiratory chain have been reported in anaerobic bacteria (Dimroth, 1987). Among them, Na^+ transport oxaloacetate decarboxylase from *Klebsiella aerogenes* (Dimroth and Thomer, 1983) and Na^+ transport ATPase from *Propionigenium modestum* (Laubinger and Dimroth, 1987) were investigated in detail. The Na^+ transport oxaloacetate decarboxylase was composed of three subunits, α, β, and γ, and α subunit contained 1 mol covalently bound biotin. The transfer of the carboxyl group from the substrate to the biotin prosthetic group catalyzed by α was completely independent of Na^+, whereas the decarboxylation of the carboxybiotin enzyme catalyzed by β or $\beta\gamma$ was dependent on Na^+. Thus, the arrangement of the subunits in the membranes was assumed to be similar to that of H^+-translocating F_1F_0-ATPase, in which α corresponded to F_1 and $\beta\gamma$ to F_0 (Dimroth and Thomer, 1983). In this model, the Na^+-dependent process and thus the site of Na^+ transport was presumed to be catalyzed by membrane-bound β. As for the Na^+-transport ATPase of *P. modestum*, the catalytic part of ATPase (F_1) extracted by a low ionic-strength treatment was not stimulated by Na^+, but the intact ATPase complex (F_1F_0) extracted with Triton X-100 or the reconstituted ATPase from purified F_1-ATPase and F_1-depleted membranes was activated by Na^+ (Laubinger and Dimroth, 1987). Thus, the site of Na^+ action was localized on the membrane-bound F_0 part of the enzyme complex. Therefore, in spite of the complete difference in the chemical reactions coupled to Na^+ transport, the proposed reaction schemes for Na^+ pumps driven by respiration, decarboxylation, and ATP hydrolysis are very similar to one another. In all cases, the first Na^+-independent reaction provides a highly reactive intermediate, which is then utilized by the Na^+-dependent process to drive the translocation of Na^+. For example, the intermediate in the Na^+-transport NADH-quinone reductase is ubisemiquinone radicals and that in the

Na^+-transport decarboxylase is the enzyme-bound carboxybiotin. Although molecular mechanisms for Na^+ transport still remain unsolved, elucidation of the coupling mechanism in these Na^+ pumps is urgent for the understanding of ion-translocating systems.

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