Purification and Properties of Reduced Nicotinamide Adenine Dinucleotide Dehydrogenase from *Photobacterium phosphoreum*

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Reduced nicotinamide adenine dinucleotide (NADH) dehydrogenase (EC 1.6.99.3) of *Photobacterium phosphoreum* was solubilized from membrane vesicles with 2% sucrose monolaurate, and was purified almost to homogeneity by use of diethylaminoethyl (DEAE)-Sephacel, hydroxylapatite, and Butyl-Toyopearl column chromatography in the presence of 0.5% sucrose monolaurate. About 100-fold purification was achieved, and the purified enzyme is composed of a single polypeptide with a molecular weight of 49000, as determined by gel electrophoresis in the presence of sodium dodecyl sulfate. The NADH dehydrogenase contains about one mol flavin adenine dinucleotide (FAD) as a prosthetic group per mol enzyme, but does not contain acid labile sulfide or non-heme iron. This NADH dehydrogenase can use ubiquinone-1, menadione, potassium ferricyanide, and cytochrome c as electron acceptors. The enzyme barely catalyzes the oxidation of reduced nicotinamide adenine dinucleotide phosphate (NADPH) as an electron donor. The enzyme is not susceptive to rotenone, 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide, capsaicin, or *N,N'*-dicyclohexylcarbodiimide, which were reported as inhibitors of NADH dh I. On the other hand, flavone, reported as an inhibitor of NADH dh II of yeast and plant mitochondria, does inhibit the NADH dehydrogenase activity. These results indicate that the NADH dehydrogenase of *P. phosphoreum* belongs to NADH dh II.

The NADH oxidase activity of the membrane vesicles of *P. phosphoreum* is remarkably stimulated by monovalent cations such as Na^+ and K^+, but is inhibited by divalent or trivalent cations. The NADH dehydrogenase activities (especially NADH-ferricyanide and NADH-ubiquinone-1 oxidoreductase) of the purified enzyme and membrane were also enhanced by Na^+ and K^+. However, the ubiquinol-1 oxidase activity of terminal oxidase complex (cytochrome bd complex) was not enhanced largely by the monovalent cations. These results suggest that in the respiratory chain of *P. phosphoreum*, NADH dehydrogenase contributes to the enhancement of electron transfer activity by monovalent cations.

Keywords NADH dehydrogenase; respiratory chain; *Photobacterium phosphoreum*; cation enhancement

Introduction

Membrane-bound reduced nicotinamide adenine dinucleotide (NADH) dehydrogenase is located at the portal of the repiratory chain. Recent investigations suggested that NADH dehydrogenases are classified into two groups depending on certain properties.[1—4] One of the NADH dehydrogenases (NADH dh I or NDH-1) has a coupling site of the respiratory chain, and is significantly sensitive to rotenone, *N,N'*-dicyclohexylcarbodiimide (DCCD), capsaicin, 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide (HQNO) and Piericidin A.[1,3—5] The other dehydrogenase (NADH dh II or NDH-2) does not bear the coupling site, and is not susceptive to the NADH dh I inhibitors.[3] The mitochondrial NADH dh I (complex I) is composed of more than 25 unlike polypeptides,[6,7] and its bacterial counterpart has more than 10 subunits.[2,8] On the other hand, the structure of NADH dh II is much simpler. NADH dh I has several nonheme irons and flavin mononucleotide (FMN) as prosthetic groups. NADH dh II is widely present in many bacteria,[1,2,9—11] and several bacterial NADH dh II were purified.[3,4] Those are composed of only one polypeptide, and contain flavin adenine dinucleotide (FAD) as a prosthetic group, except for the enzyme of *Thermus aquaticus*, which contains FMN.[12]

Some bacterial NADH dehydrogenases have novel properties. A high concentration of monovalent cation is required for maximum enzyme activity in the purified NADH dehydrogenases of alkalophilic *Bacillus*[9] and in the partially purified enzyme of halophilic bacterium AR-I.[13] Although Imagawa and Nakamura also reported that the partially purified NADH dehydrogenase of *P.*

phosphoreum was activated with a monovalent cation,[14] they did not use the completely purified enzyme and did not examine the effect on the activity of NADH-ubiquinone oxidoreductase (ubiquinone is thought as the physiological substrate).[15] In this paper, we purified the enzyme of *P. phosphoreum* and studied its properties, including enhancement by monovalent cations.

Materials and Methods

Growth of Bacteria *P. phosphoreum* strain IAM12085 was a generous gift from the Institute of Applied Microbiology, the University of Tokyo, and was grown in the medium described previously.[16] Cells were cultured at 25 °C with vigorous aeration through shaking, and harvested in the late logarithmic phase of growth by centrifuge at $10000 \times g$. The cells were stored at -20 °C before use.

Solubilization of the NADH Dehydrogenase The preparation of sonicated membrane vesicles was described previously.[17] The frozen cells (40 g wet weight) were thawed and then suspended in 400 ml of 30 mM Tris–HCl buffer, pH 7.4, and 0.1 mM phenylmethanesulfonyl fluoride (PMSF). The suspension was sonicated with a Tomy Seiko UR-200p ultrasonic disrupter with cooling in ice water. The treated suspension was centrifuged at $7000 \times g$ for 20 min to remove undisrupted cells. The supernatant thus obtained was then centrifuged at $100000 \times g$ for 30 min. The pellet was washed twice with 10 mM Tris–HCl, pH 7.4, and 0.1 mM PMSF with centrifugation. The packed pellet (sonicated membrane) was then suspended in 10 mM Tris–HCl, pH 7.4, containing 2% sucrose monolaurate for 1 h to solubilize the NADH dehydrogenase, and the suspension was centrifuged at $100000 \times g$ for 30 min to obtain the supernatant solution. The above procedures were carried out at 4 °C.

Purification of the NADH Dehydrogenase The resultant supernatant was applied to a column of diethylaminoethyl (DEAE)-Sephacel (2.4 × 20 cm) equilibrated with 10 mM Tris–HCl, pH 7.4, containing 0.5% sucrose monolaurate. The column was washed with 10 mM Tris–HCl buffer, pH 7.4, 150 mM NaCl and 0.5% sucrose monolaurate at a flow rate of 2 ml/min. Then the NADH dehydrogenase was eluted with the same buffer containing higher concentration of NaCl (300 mM). The peak

fractions of the NADH dehydrogenase activity were collected, then diluted by adding the same volume of distilled water in order to decrease the concentration of NaCl. This was applied on a second column of DEAE-Sephacel (1.5 × 10 cm) equilibrated with 10 mM Tris–HCl buffer, pH 7.4, 150 mM NaCl and 0.5% sucrose monolaurate. The NADH dehydrogenase was eluted with 150 ml of the same buffer containing a linear gradient of 150 to 300 mM NaCl, at a flow rate of 2 ml/min. The fractions containing the majority of the NADH dehydrogenase activity were pooled. The enzyme solution was applied to a column of hydroxylapatite (1.0 × 8 cm) equilibrated with 50 mM phosphate buffer, pH 7.4, containing 0.5% sucrose monolaurate, and the column was washed with the same buffer (40 ml). Then, the NADH dehydrogenase was eluted with 150 ml of the linear gradient of 50 to 500 mM phosphate buffer, pH 7.4, containing 0.5% sucrose monolaurate, at a flow rate of 1.5 ml/min, and the peak fractions of the enzyme activity were collected. Solid ammonium sulfate was added to the enzyme fraction to 40% saturation, and the slightly cloudy suspension was applied to a Butyl-Toyopearl 650M column (1.5 × 8 cm), subjected to hydrophobic chromatography, and equilibrated with 30 mM Tris–HCl buffer, pH 7.4, containing 40% saturation of ammonium sulfate and 0.5% sucrose monolaurate. Then, the NADH dehydrogenase was eluted with 100 ml of the same buffer containing a linear gradient of 40 to 0% saturation of ammonium sulfate at a flow rate of 2 ml/min. The peak fractions of NADH dehydrogenase activity were collected and used for the experiments.

Enzyme Assay Assays of the NADH dehydrogenase activity were carried out at 25 °C in 50 mM Tris–HCl buffer, pH 7.4, using a Hitachi spectrophotometer U-3410. Ferricyanide (1 mM), menadione (100 μM), ubiquinone-1 (100 μM), horse heart cytochrome c (50 μM), or 2,6-dichlorophenolindophenol (DCIP) (50 μM) were used as electron acceptors. The routine assay medium, in a total volume of 2 ml, contained 150 μM NADH as an electron donor. NADH oxidase and ubiquinol-1 oxidase were determined polarographically with a Clark type oxygen electrode (Rank Brothers, Rank oxygen electrode) at 25 °C. One mM NADH or 120 μM ubiquinol-1 as an electron donor was added to the assay mixture (2 ml) in which 0.7 mg of protein of the sonicated membrane and 10 mM Tris–HCl buffer, pH 7.4, were contained.

Analysis of Flavin Trichloroacetic acid was added to a final concentration of 10% to the enzyme solution, and the mixture stood at room temperature for 10 min. The mixture was then centrifuged at 1000 × g for 15 min. The supernatant obtained was washed three times each with 3 volumes of diethylether to remove trichloroacetic acid. It was then concentrated in an evaporator at room temperature. The obtained sample was subjected to thin layer chromatography on a silica gel plate (Merck, 60F-254). The solvent was n-butanol : glacial acetic acid : H_2O = 4 : 1 : 5 (v/v).

Protein Determination The protein concentration was determined by the method of Lowry et al. with bovine serum albumin as a standard.[18]

Chemicals Ubiquinone-1 was generous gift from Eisai Co., Ltd. Ubiquinol-1 was prepared by the reduction of ubiquinone-1 according to the method of Kita et al.[19] DEAE-Sephacel, hydroxylapatite, and Butyl-Toyopearl 650M were purchased from Pharmacia, Nakarai Chemicals, and Tosoh, respectively. Sucrose monolaurate (SM 1200) was purchased from Mitsubishi Kasei. Other chemical reagents used were of the highest purity commercially available.

Results

Purification of NADH Dehydrogenase At first, we tried purification of the NADH dehydrogenase from P. phosphoreum using Triton X-100 or octylglucoside as the

solubilizing detergent. However, in the first DEAE-Sephacel column chromatography, the NADH dehydrogenase activity was co-eluted with many other proteins, and the purity of the dehydrogenase was not enhanced in the presence of either Triton X-100 or octylglucoside. We tried other column methods, but we could not make progress on the purification. We supposed that it would be difficult to purify the NADH dehydrogenase from the membrane fraction using these detergents. On the other hand, sucrose monolaurate, a nonionic detergent, was shown to be highly effective in the first step of purification, as indicated in Fig. 1. We used this detergent in all steps of the purification.

Table I summarizes the purification of the NADH dehydrogenase of P. phosphoreum. The enzyme was purified approximately 100-fold with an overall yield of 1.8%. The purity of NADH was analyzed by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis. As shown in Fig. 2, the purified enzyme was found to consist of a single polypeptide with a molecular weight (MW) of 49000.

Optical Absorption Spectra and Chromophore The optical absorption spectrum of the purified, oxidized NADH dehydrogenase (Fig. 3) shows a peak at 460 nm, indicating the presence of a flavin. This is confirmed by dithionite-induced bleaching in the 460 nm region. The chromophore
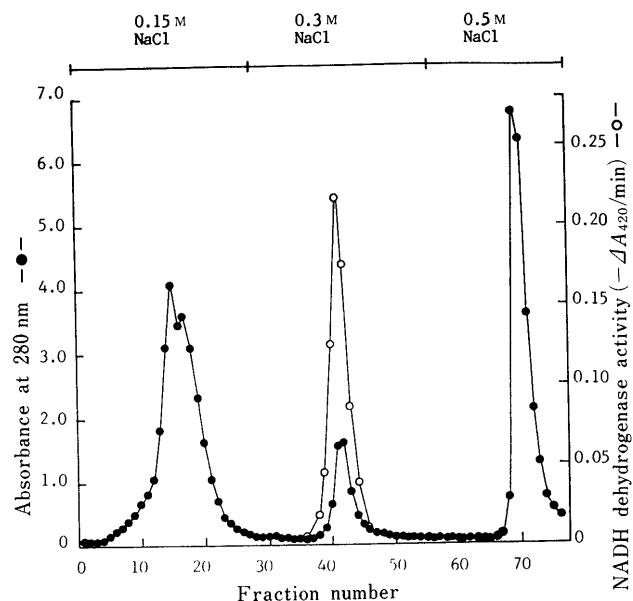

Fig. 1. Chromatogram of NADH Dehydrogenase on the First DEAE-Sephacel

The membrane protein (276 mg) solubilized by sucrose monolaurate was applied to a DEAE-Sephacel column. The running conditions were described in Materials and Methods and the volume of each fraction was 5 ml. (●) Absorbance at 280 nm; (○) NADH-ferricyanide oxidoreductase activity.

TABLE I. Purification of NADH Dehydrogenase from P. phosphoreum

	Total protein (mg)	Total activity (μmol/min)	Specific activity (μmol/min/mg)	Purification (-fold)	Yield (%)
Dodecylsucrose extract	276	997	3.6	1.0	100
1st DEAE-Sephacel	40.3	990	24.6	6.8	99.3
2nd DEAE-Sephacel	10.6	583	55.0	15.3	58.5
Hydroxylapatite	0.268	46.4	173.1	48.1	4.7
Butyl-Toyopearl	0.043	17.5	405.0	112.5	1.8

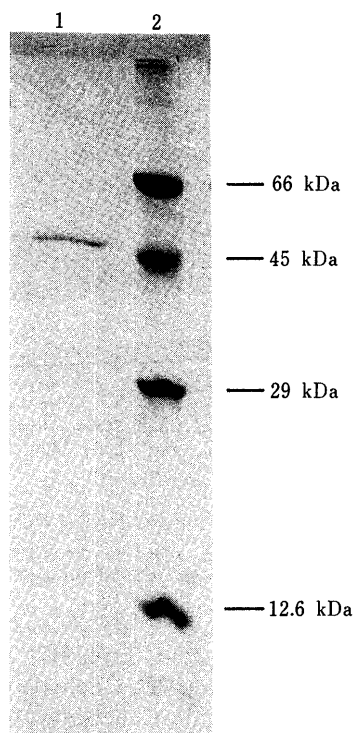The activity is measured as the NADH-ferricyanide oxidoreductase activity.

Fig. 2. SDS–Polyacrylamide Gel Electrophoresis of NADH Dehydrogenase of *P. phosphoreum*

The molecular weights of marker proteins are indicated on the right. 1, Purified enzyme; 2, markers of molecular weight.
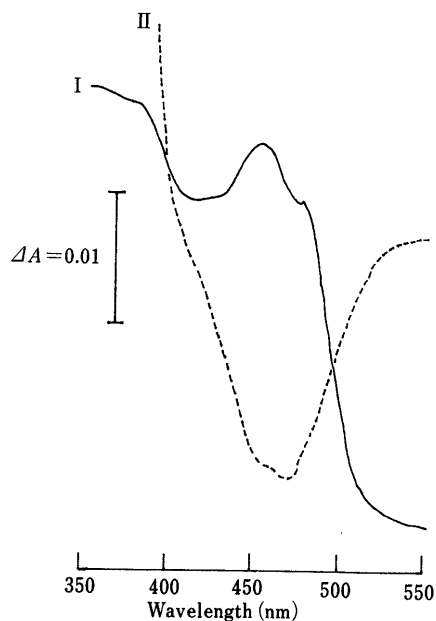

Fig. 4. pH–Activity Profile of NADH Dehydrogenase

Enzyme activity was determined by ferricyanide assay in 50 mM potassium phosphate buffer (○); 50 mM Tris–HCl buffer (●).

TABLE II. Substrate Specificity of the NADH Dehydrogenase

Electron acceptor	K_m (μM) for NADH	V_{max}[a] (μmol/min/mg)
Ferricyanide	44	556
Ubiquinone-1	9	44
Menadione	15	64
Cytochrome c	12	188
DCIP	14	20

a) These values are expressed as the changes of acceptors, but expressed as that of NADH when ubiquinone or menadione is used as an acceptor.

Fig. 3. Absorbance Spectra of Purified NADH Dehydrogenase

(I) Oxidized NADH dehydrogenase. (II) Dithionite reduced minus oxidized NADH dehydrogenase.

Fig. 5. Effects of Various Cations on NADH Oxidase Activity in Membrane Vesicles

Cations were added to the assay mixture in 10 mM Tris–HCl, pH 7.4. The enzyme activities were expressed as the percentage of pH 7.4. The enzyme activities were expressed as the percentage of that measured without added salts. The 100% activity was 0.057 μmol O_2/min/mg of protein. ●, NaCl; ▲, KCl; ■, NH$_4$Cl; □, choline chloride; △, MgCl$_2$; ○, CaCl$_2$; ▽, AlCl$_3$.

was chromatographed on a silica gel thin layer plate. The Rf value of the chromophore was the same as that of authentic FAD (data not shown). A stoichiometry of 1.1 mol FAD/mol enzyme of MW 49000 could be calculated on the basis of a $\varepsilon_{460\,nm}$ of 10.3 mM^{-1} cm^{-1} for FAD in the oxidized minus reduced form.[20]

Properties of the Enzyme The effect of pH on NADH dehydrogenase activity was examined in a potassium phosphate buffer and Tris–HCl buffer of various pH (Fig.
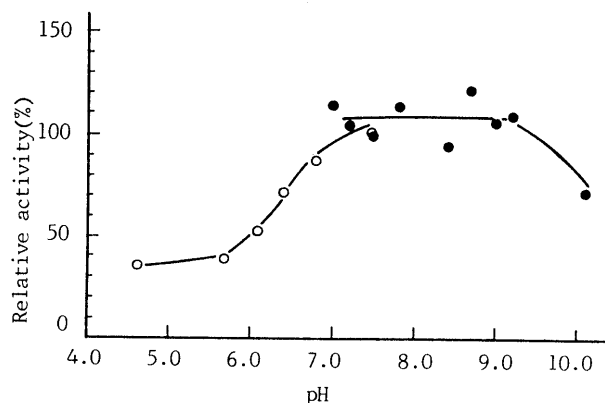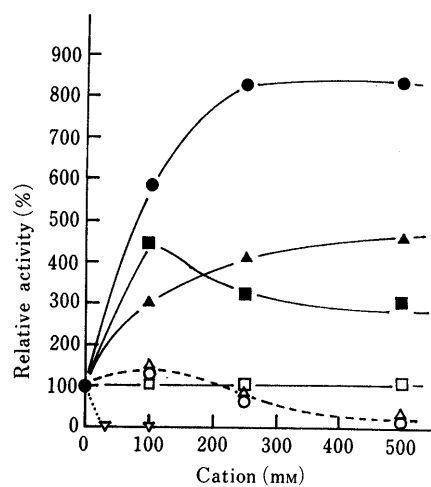
4). The optimum pH profile showed a flat maximum activity between pH 7 and 9. This tendency is similar to that of the partially purified enzyme (pH 8—10).[14]

Furthermore, we examined the substrates of the NADH dehydrogenase of *P. phosphoreum*. The enzyme can utilize NADH as an electron donor, but not NADPH effectively (data not shown). On the other hand, the enzyme can utilize ferricyanide, ubiquinone-1, menadione, cytochrome c, and DCIP as electron acceptors (Table II). The specificity of electron acceptors closely resembles that of

TABLE III. The Effects of Monovalent Cations on the Enzyme Activities of Sonicated Membrane

Enzyme	Cation	Enzyme activity	Fold
NADH oxidase[a]	0 mM	0.057	1.0
	300 mM KCl	0.262	4.6
	300 mM NaCl	0.433	7.6
Ubiquinol-1[a] oxidase	0 mM	0.547	1.0
	300 mM KCl	0.602	1.1
	300 mM NaCl	0.765	1.4
NADH-ubiquinone-1[b] oxidoreductase	0 mM	0.396	1.0
	300 mM KCl	1.27	3.2
	300 mM NaCl	1.78	4.5

a) The enzyme activity is revealed as the consumption of oxygen (μmol O$_2$/min/mg). b) The enzyme activity is revealed as the oxidation of NADH (μmol NADH/min/mg).

Fig. 7. Effects of KCl on the Purified NADH Dehydrogenase

Various electron acceptors were added to the assay mixture. ○, 100 μM ubiquinone-1; ●, 1 mM ferricyanide; ■, 50 μM cytochrome c; ▲, 100 μM menadione; □, 50 μM DCIP.

KCl or NaCl (3.2- or 4.5-fold, Table III). Furthermore, we examined the effects on NADH dehydrogenase activities of the purified enzyme and on the sonicated membrane by K$^+$ as a monovalent cation (Table III, Figs. 6, 7). The effect of KCl on the NADH-ferricyanide oxidoreductase activity of the membrane was almost the same as that of purified NADH dehydrogenase. The NADH-ubiquinone-1 oxidoreductase activity of the sonicated membrane was more effectively activated by KCl than was the purified enzyme. So, there is specificity depending on the electron acceptors. The NADH-menadione and NADH-DCIP oxidoreductases were slightly activated less than twice with the sonicated membrane. By contrast, the activity of NADH-cytochrome c oxidoreductase was inactivated with an increase in the concentration of KCl, as reported previously, on the partially purified enzyme.[14]
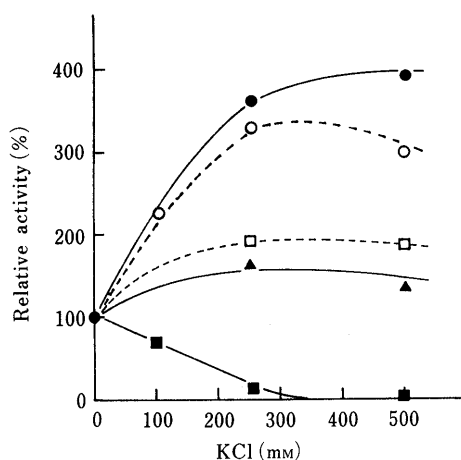

Fig. 6. Effects of KCl on NADH Dehydrogenase Activity in the Sonicated Membrane Vesicles

Various electron acceptors were added to the assay mixture. ○, 100 μM ubiquinone-1; ●, 1 mM ferricyanide; ■, 50 μM cytochrome c; ▲, 100 μM menadione; □, 50 μM DCIP.

alkalophilic *Bacillus* NADH dehydrogenase.[9]

The activity of NADH dehydrogenase was barely affected by DCCD, capsaicin, or HQNO, which were inhibitors of NADH dh I. However, flavone, which is an inhibitor of NADH dh II, inhibited the NADH-ubiquinone-1 oxidoreductase activity (50% inhibition concentration = 150 μM).

Effects of Salts on the Enzyme Activity It is reported that the respiratory activity of the spheroplast of *P. phosphoreum* is enhanced by monovalent cations.[14] We re-examined the dependence of the overall reaction of the respiratory chain of the sonicated membrane on monovalent cations (Fig. 5). We used chloride as a counter anion in this experiment, and similar cation specificity was observed when a sulfate was used as an anion instead of chloride (data not shown). The additions of sodium chloride and potassium chloride resulted in activity increases of 8- and 4.5-fold, respectively (500 mM cations). On the other hand, choline did not change the activity, while di- or trivalent cation actually inactivated the oxidase activity. The terminal oxidase (ubiquinol-1 oxidase, or cytochrome bd complex) activity was slightly activated by 300 mM KCl or NaCl (1.1-fold or 1.4-fold, Table III). The NADH-ubiquinone-1 oxidoreductase activity of sonicated membrane was activated by 300 mM
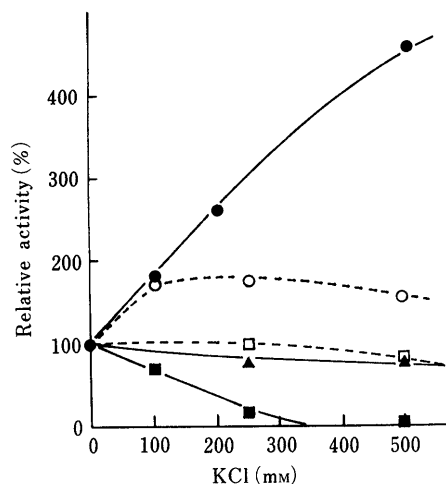
Discussion

The NADH dehydrogenase of *P. phosphoreum* had been partially purified previously by Imagawa and Nakamura.[14] They reported that the monovalent cation activated the partially purified enzyme. In this paper, we could purify almost completely the NADH dehydrogenase of *P. phosphoreum*. We then examined the properties of the enzyme, including cation enhancement.

The membrane-bound NADH dehydrogenase of *P. phosphoreum* can be effectively solubilized from the membrane with 2% sucrose monolaurate and separated from the other proteins by a combination of anion exchange chromatography, hydroxylapatite chromatography, and hydrophobic interaction chromatography in the presence of 0.5% sucrose monolaurate.

The molecular weight of the enzyme on undenatured conditions, estimated from the elution profile on high performance liquid chromatography (G2000SW column, Tosoh gel filtration), is 42000 (data not shown). That value is similar to that estimated by SDS-polyacrylamide gel electrophoresis, indicating the NADH dehydrogenase is a monomeric protein.

We confirmed that the NADH dehydrogenase of *P. phosphoreum* has almost one mol of FAD as a prosthetic

group per mol of enzyme. The NADH dh II isolated from various organisms has FAD as a prosthetic group except for *Thermus aquaticus*,[3,4,12] and the NADH dh II of alkalophilic *Bacillus* YN-1,[9] *Escherichia coli*,[10] *Thermus thermophilus* HB-8[2] and *Saccharomyces cerevisiae* mitochondria,[21] showing that the molecular stoichiometry of flavin and enzyme is approximately one. We here confirmed that the NADH dehydrogenase of *P. phosphoreum* belongs to NADH dh II because of the following criteria. 1) This enzyme is composed of one polypeptide. 2) This has one FAD per one enzyme. 3) This is not susceptive to rotenone, capsaicin, DCCD, and HQNO, which are NADH dh I inhibitors.

Both NADH oxidase activity and NADH dehydrogenase activity are drastically enhanced by monovalent cations. On the other hand, the terminal oxidase activity is barely activated by these cations. These results suggest that in the respiratory chain of *P. phosphoreum*, NADH dehydrogenase contributes to the enhancement of the electron transfer activity by monovalent cations. As ubiquinone-8 is contained in the membrane of *P. phosphoreum*,[15] it seems that the behavior of ubiquinone-1 reflects the characteristics of this bacterium. Hochstein and Dalton reported that NADH dehydrogenases of halophilic bacteria are activated by high concentrations of cations.[13] As *P. phosphoreum* is a marine bacterium, it would have some halophilic nature. It was reported that the cell contains high concentrations of cations (170 mM Na^+ and 250 mM K^+).[22] Thus, the respiratory chain and NADH dehydrogenase of this bacterium would be under the control of monovalent cations. Because the specificity of a monovalent cation is low, the activation may not be due to specific binding of the particular cation but rather to a cation-dependent structural transition.

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