The Na^+-translocating NADH:ubiquinone oxidoreductase from the marine bacterium *Vibrio alginolyticus* contains FAD but not FMN

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Abstract **The Na^+-translocating NADH:ubiquinone oxidoreductase from *Vibrio alginolyticus* was extracted from the bacterial membranes and purified by ion exchange chromatographic procedures. The enzyme catalyzed NADH oxidation by suitable electron acceptors, e.g. menadione, and the Na^+ and NADH-dependent reduction of ubiquinone-1. Four dominant bands and a number of minor bands were visible on SDS-PAGE that could be part of the enzyme complex. Flavin analyses indicated the presence of FAD but no FMN in the purified enzyme. FAD but no FMN were also present in *V. alginolyticus* membranes. FAD is therefore a prosthetic group of the Na^+-translocating NADH:ubiquinone oxidoreductase and FMN is not present in the enzyme. The FAD was copurified with the NADH dehydrogenase. The purified enzyme exhibited an absorption spectrum with a maximum at 450 nm that is typical for a flavoprotein. Upon incubation with NADH this absorption disappeared indicating reduction of the enzyme-bound FAD.**

Key words: FAD; FMN; NADH:ubiquinone oxidoreductase; Respiratory Na^+-pump

1. Introduction

The NADH:ubiquinone oxidoreductase from the gram-negative marine bacterium *Vibrio alginolyticus*, that is induced during growth at alkaline pH, has been reported to function as a primary sodium pump [1,2]. Within the alkaline environment the pumping of Na^+ instead of H^+ contributes in maintaining the cytoplasmic pH near neutrality. The Na^+-dependent NADH:ubiquinone oxidoreductase complex (NQR 1) is distinct from the Na^+-independent NADH:ubiquinone oxidoreductase (NQR 2) also found in *V. alginolyticus* [3], which is not coupled to a transport. NQR 1 is sensitive to inhibition by micromolar concentrations of silver ions or by 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide (HQNO), can generate a membrane potential, reduces quinone using a one-electron pathway, is sensitive to NADH preincubation in the absence of the electron acceptor, and can use the analogue deamino- NADH [4–6]. Early biochemical studies indicated that the purified NQR 1 is composed of three subunits α, β and γ in a 1:1:1 stoichiometry, with apparent molecular masses of 52, 46 and 32 kDa, respectively [7]. The FAD-containing β subunit catalyzed NADH oxidation with menadione as electron acceptor in a Na^+-independent fashion. The α-subunit was reported to contain FMN and to have no enzymic activity by itself, but to be required together with the β- and γ-subunits for the reconstitution of the Na^+-dependent NADH:ubiquinone oxidoreductase activity [8].

The Na^+-dependent NADH:ubiquinone oxidoreductase is widely distributed among marine bacteria [9] and has also been shown to exist in *Klebsiella pneumoniae* grown anaerobically on citrate [10]. Here, the enzyme catalyzes NADH formation from NAD^+ and ubiquinol. This is a reversed electron transfer which is powered by the $\Delta\mu Na^+$ generated by oxaloacetate decarboxylase [11]. Despite the abundance of the Na^+-dependent NADH:ubiquinone oxidoreductase very little is known about the catalytic mechanism of this enzyme. We therefore initiated a detailed study of its catalytic properties. A puzzling result was the presence of both FMN and FAD in the oxidoreductase. Reinvestigation of the flavin contents indicated the presence of FAD but not FMN in the purified enzyme as well as in the *V. alginolyticus* membranes.

2. Materials and methods

2.1. Preparation of membrane vesicles and enzyme purification

Cells of *Vibrio alginolyticus* were grown at 37°C aerobically in a 300 litre fermenter in a total volume of 100 litres containing 5.0 g/l polypeptone, 5.0 g/l yeast extract, 4.0 g/l K_2HPO_4, 2.0 g/l glucose and 30 g/l NaCl. The pH of the medium was adjusted to 8.5 with Tris base. Cells were harvested in the exponential phase and stored at −70°C until use without loss of NADH:ubiquinone oxidoreductase activity.

V. alginolyticus cells (10 g, wet weight) were washed twice with 50 ml of 10 mM Tris-HCl buffer, pH 7.0, containing 1.0 M NaCl followed by centrifugation at 6,500 × *g* for 10 min. Washed cells were suspended in 40 ml of 10 mM Tris/HCl buffer, pH 7.0, containing 5.0 mM $MgSO_4$, 10% (v/v) glycerol and 0.2 M Na_2SO_4 (extraction buffer). After adding 0.1 mM DFP, 1.0 mM DTT and a trace of DNase, the suspension was well homogenized with a motor-driven plunger, and passed 2–3 times through a French pressure cell at 80 MPa. The disrupted cell suspension was centrifuged at 35,000 × *g* for 30 min to remove unbroken cells, and the supernatant was ultracentrifuged at 150,000 × *g* for 60 min. The membrane vesicles contained in the pellet were washed twice with the extraction buffer and collected by centrifugation at 150,000 × *g* for 30 min.

Washed membrane vesicles from 10 g cells were suspended in 10 ml of solubilization buffer containing 1.0% (w/v) lauryldimethylamine oxide (LDAO), 10 mM Tris-HCl, pH 7.5, 10% (v/v) glycerol, 0.1 mM EDTA, 0.1 M NaCl and 0.1 mM DFP. After well homogenization, the suspension was stirred on ice for 20–30 min and centrifuged at 150,000 × *g* for 60 min. The supernatant usually contained 5.0–6.0 mg protein/ml. Freshly prepared 1.0% LDAO extract was immediately used for the purification of the enzyme.

All purification steps were carried out at 4°C with a FPLC apparatus (Pharmacia). 10 ml of 1.0% LDAO extract were applied directly on a column (20 ml) with the weak anion exchanger DEAE-Sephacel equilibrated with 0.1% (w/v) LDAO in 10 mM Tris-HCl buffer, pH 7.5, containing 10% (v/v) glycerol, 0.1 mM EDTA and 0.1 M NaCl (buffer A). After washing the column with 5 bed volumes buffer A, a large part of the proteins were eluted with buffer A containing 0.15 M NaCl. The active fractions were eluted with buffer A containing 0.3 M NaCl and

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Abbreviations: DFP, diisopropylfluorophosphate; DTT, dithiothreitol; LDAO, laurydimethylamine oxide; Na^+-NQR 1 (NQR 1), Na^+-translocating NADH:ubiquinone oxidoreductase.

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concentrated by ultrafiltration with a PM 10 membrane. The concentrated enzyme solution was diluted to decrease the salt concentration to 0.1 M and was loaded on a Q-Sepharose column (20 ml) equilibrated with buffer A. The active fractions were eluted with 0.4 M NaCl in buffer A and concentrated with a PM 10 membrane. Both NADH dehydrogenase and quinone reductase activities were found in this fraction. If not indicated otherwise we used the enzyme after this purification step for the analyses performed in this study. Further fractionation of the enzyme was performed where indicated by chromatography on a Hiload 16/60 Superdex 200 (Pharmacia) column which was equilibrated and developed with buffer A.

2.2. Determination of enzyme activities

The NADH dehydrogenase was assayed at 25°C with menadione, ubiquinone-1 or ferricyanide as an electron acceptor. The standard assay mixture contained in a total volume of 1.0 ml: 0.1 mM NADH or 0.1 mM deamino-NADH, 0.1 mM menadione or 15 μM ubiquinone-1, 0.2 M NaCl and 20 mM Tris-HCl, pH 8.0. The reaction was started by addition of the enzyme, and the initial velocity of decreasing absorbance at 340 nm was calculated based on the extinction coefficient $\varepsilon_{340} = 6.22\ mM^{-1} \cdot cm^{-1}$. Alternatively, 1.0 mM ferricyanide was used instead of a quinone, and the absorbance decrease was recorded at 420 nm ($\varepsilon_{420} = 1\ mM^{-1} \cdot cm^{-1}$). If membrane vesicles were used for measuring NADH dehydrogenase activity 5 mM KCN was included in the assay solution.

The quinone reductase activity was assayed at 25°C by following the formation of ubiquinol from ubiquinone. The standard assay mixture contained in a total volume of 1.0 ml: 0.1 mM NADH, 15 μM ubiquinone-1, 0.2 M NaCl, 0.01% (w/v) LDAO, 20 mM Tris-HCl, pH 8.0, and the enzyme. Changes in absorbance difference at the wavelength pair, 248–268 nm, were recorded with the Shimadzu UV-3000 dual-wavelength/double-beam recording spectrophotometer, and the rate of ubiquinol-1 formation was calculated based on the extinction coefficient $\Delta\varepsilon_{248-268} = 7.8\ mM^{-1} \cdot cm^{-1}$.

2.3. Release of enzyme-bound flavins and identification by HPLC

Flavins were identified by HPLC analysis [13,14] with the following modifications. 20–100 μg ice-cold purified enzyme or membrane vesicles were mixed with an equal volume of ice-cold 20% trichloroacetic acid in water and the mixture wsa kept on ice (to prevent hydrolysis of FAD) for 10 min in the dark and then centrifuged at 15,000 × *g* for 5 min at 4°C. The supernatant was immediately neutralized by the addition of one-fourth of its volume 4 M K_2HPO_4 [15]. The neutralized supernatant was subjected to HPLC (Bio-Rad) at 25°C on ODS Hypersil C_{18} reversed-phase (5 μm, 250 × 4 mm; Hewlett Packard) equilibrated with 5 mM ammonium acetate buffer, pH 6.0 (buffer A). Flavins were eluted with 5 mM ammonium acetate buffer, pH 6.0, containing 80% methanol (buffer B) at a flow rate of 1.0 ml/min and were detected by continuously measuring the absorbance at 265 nm. Positive controls were carried out with purified glutathione reductase from bakers yeast (contains only FAD) and glycolate oxidase from spinach (contains only FMN). Both enzymes were obtained from Sigma.

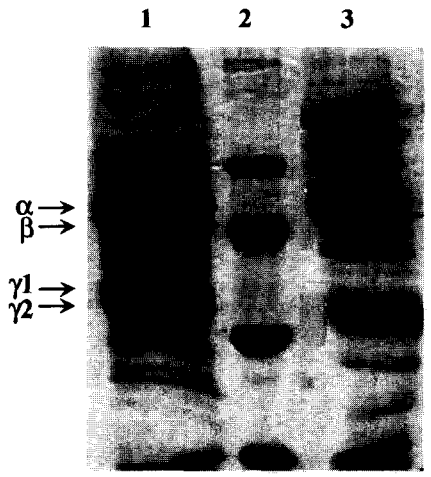

Fig. 1. Coomassie-stained SDS-PAGE of partially purified Na^+-NQR 1 from *V. alginolyticus*. 20 μg of the Q-Sepharose fraction (lane 1) and 15 μg Hiload Superdex 200 fraction (lane 3) were applied to a SDS-gel with 10% polyacrylamide [12], electrophoresed and stained with Coomassie; protein standards with molecular masses of 66, 45, 29 and 20.1 kDa, respectively (lane 2). The arrows mark the four dominant bands.

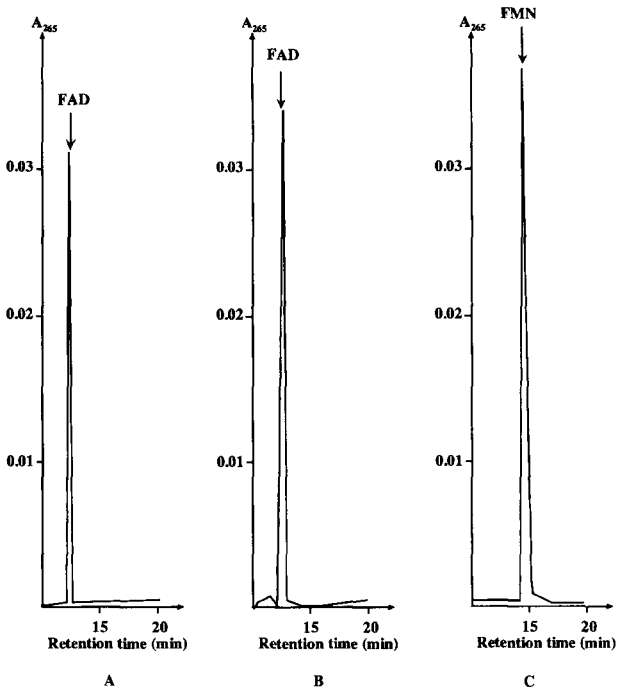

Fig. 2. The chromatograms of flavin separation. HPLC of flavins extracted from A: glutathione reductase from baker yeast (contains only FAD); B: purified NADH:ubiquinone oxidoreductase from *V. alginolyticus*; and C: glycolate oxidase from spinach (C). For details see section 2.

2.4. Quantitative determination of flavins

Commercially available FAD or FMN contains a number of impurities that must be removed before they can be used as standards for quantitative measurements. Therefore, the solid flavins were dissolved in 0.1 M potassium phosphate buffer, pH 7.7, containing 0.1 mM EDTA (standard buffer) and purified by HPLC (see above). The concentration of purified FAD and FMN was determined by measuring the absorbance at 265 nm (ε_{265}(FAD) = 38 $mM^{-1} \cdot cm^{-1}$; ε_{265}(FMN) = 31.8 $mM^{-1} \cdot cm^{-1}$).

The standard solutions of FAD and FMN were diluted with standard buffer to 10 μM each (stock solutions). For the calibration curve the stock solutions were further diluted with standard buffer to 10–50 nM concentrations shortly before the assay. 2.0 ml of the standards were transferred into a fluorescence cuvette and the fluorescence emission was determined at 24 ± 1°C at 525 nm (emission slit width 25 nm) at an excitation wavelength of 450 nm (slit width 10 nm). After adding 0.2 ml of 1.0 M HCl to bring the pH to 2.6 the fluorescence was determined again. Calibration curves at both pH values were used to determine the flavin contents of the samples that were collected after HPLC and treated as the standards. The differences in fluorescence at the two different pH values was as expected for FAD or FMN, respectively [16].

3. Results

3.1. Purification and properties of NADH:ubiquinone oxidoreductase

The Na^+-dependent NADH:ubiquinone oxidoreductase from *V. alginolyticus* was partially purified by DEAE-Sephacel and Q-Sepharose chromatography as described in section 2.

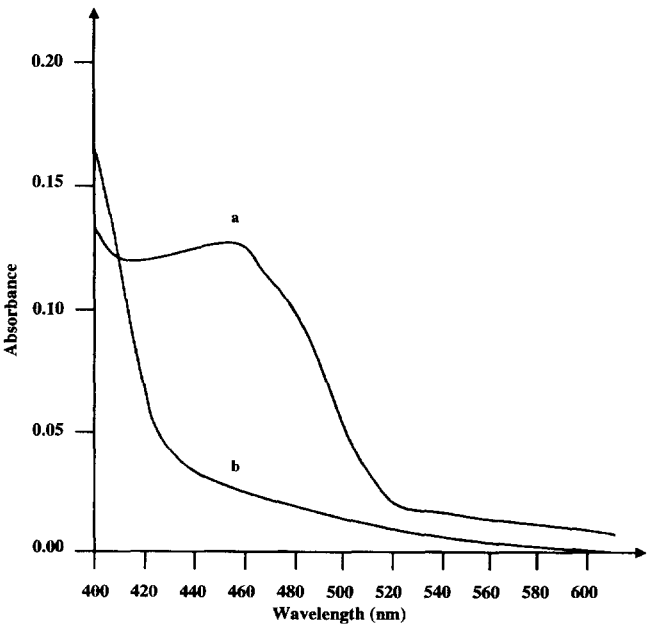

Fig. 3. Visible spectrum (420–620 nm) of purified NADH:ubiquinone oxidoreductase from *V. alginolyticus*. The cuvette (d = 1.0 cm) contained in 200 μl: 0.9 mg Q-Sepharose fraction, 50 mM Tris-HCl buffer, pH 7.5, and 0.1% LDAO (a). After additoin of 1.0 mM NADH, the spectrum of the reduced sample was recorded (b).

SDS-PAGE (Fig. 1) of the partially purified enzyme revealed after Coomassie staining 4 dominant polypeptides (α, β, γ1, γ2) with apparent molecular masses of 55, 50 34 and 32 kDa, respectively, and additional minor-stained polypeptides. The preparation contained both NADH dehydrogenase and quinone reductase activity. The quinone reductase activity was completely dependent on Na^+ ions, sensitive to silver ions and HQNO, and was inhibited by preincubation with NADH in the absence of the quinone. After reconstitution of the isolated enzyme into proteoliposomes, it catalyzed active sodium ion transport coupled to NADH oxidation by ubiquinone-1 (data not shown). All these characteristics of the preparation have indicated that the functional unit of NQR 1 was isolated. If this functional unit of NQR 1 was further purified with a Hiload Superdex 200 gel filtration, the γ2-subunit (34 kDa) was lost (Fig. 1, lane 3). This preparation still catalyzed NADH dehydrogenase activity but was devoid of the quinone reductase activity.

3.2. Determination of flavins

We used reversed phase HPLC for the separation of flavins. HPLC purified standards of FAD and FMN were separated with retention times of 13 and 15 min, respectively. Enzyme-bound flavins were released by treatment with trichloroacetic acid, and the precipitated apoprotein was removed by centrifugation. The flavin-containing supernatant was neutralized and immediately subjected to HPLC: the suitability of this procedure was controlled with glutathione reductase which contains FAD and glycolate oxidase which contains FMN (Fig. 2). In both cases only the respective prosthetic group was evident in the HPLC analysis and no hydrolysis products were detectable. On applying the same method to the isolated NADH:ubiquinone oxidoreductase of *V. alginolyticus*, FAD was identified as its prosthetic group and no trace of FMN was detectable. This result is in accord with flavin analysis of *V. alginolyticus* membranes; here too, the only flavin present was FAD and neither FMN nor riboflavin were found.

Table 1 shows the FAD content of the membranes and the isolated enzyme. Also shown are the NADH dehydrogenase activities and the protein content of these preparations. The specific NADH dehydrogenase activity of the purified enzyme was 12 times higher than that of the membranes and the specific FAD content increased about 10-fold. This indicates that the NADH dehydrogenase and FAD were purified together, as to be expected if this is the prosthetic group of the enzyme. The FAD content in the purified enzyme is equal to about 1 mol per 440,000 g protein. The participation of the FAD prosthetic group in the electron transfer from NADH to ubiquinone was shown by measuring the absorption spectrum in the range from 400–620 nm. A band with an absorption maximum at 450 nm was observed (Fig. 3) that is characteristic for a flavoprotein with the flavin at its oxidized state. Upon incubation of the enzyme with NADH, this absorbance around 450 nm was bleached which is in accord with the reduction of the enzyme-bound FAD by NADH.

4. Discussion

We show here that the Na^+-translocating NADH:ubiquinone oxidoreductase from *Vibrio alginolyticus* contains FAD as a prosthetic group. It is therefore distinct from its H^+-translocating counterpart which contains FMN. The amount of FAD in the partially purified enzyme was about 1 mol per 440,000 g protein. We consider this as the maximum molecular weight of Na^+-NQR because the enzyme employed for the flavin analyses was not pure (see Fig. 1). Nevertheless FAD was copurified with the NADH dehydrogenase (Table 1) and the flavin absorbance was bleached upon incubation of the enzyme with NADH (Fig. 3). These observations clearly indicating that the FAD is a prosthetic group of NQR-1 and that it is involved in the electron transfer from NADH to ubiquinone. SDS-PAGE of partially purified enzyme indicates four dominant bands after staining with Coomassie with apparent molecular masses of 55, 50, 34 and 32 kDa. Additional polypeptides apparent on the gel may or may not be part of the complex, the correct composition of which can not be derived from this type of analysis. Loss of the γ1 subunit on further fractionating the

Table 1
FAD content in the membrane vesicles and the purified Na^+-NQR 1 from *V. alginolyticus*

Sample	Protein (mg)	NDH* specific activity (U/mg)	Yield (%)	FAD (mol)	FAD (nmol/mg)	Yield (%)
Membrane vesicles	200	1.2	100	48×10^{-9}	0.24	100
Q-Sepharose fraction	0.88	14.8	5.4	2.0×10^{-9}	2.27	4.2

*NDH = NADH dehydrogenase was measured with 15 μM ubiquinone-1 as electron acceptor.

protein on a gel filtration column (Fig. 1) did not affect the NADH dehydrogenase activity but abolished the quinone reductase activity. The $\gamma 1$ subunit is therefore probably essential for the later function.

Hayashi and Unemoto have previously reported the purification of Na^+-NQR 1 from *V. alginolyticus* [7]. Based on Coomassie-stained SDS-PAGE, the composition of only three subunits α, β, γ with molecular masses of 55, 50 and 32 kDa was proposed. The α-subunit was reported to contain FMN and the β-subunit was reported to contain FAD. In our studies we found FMN neither in the purified Na^+-NQR 1 nor in the membranes of *V. alginolyticus* and conclude therefore that the previous analyses are erroneous. It is possible that the FMN found in the previous analyses was formed by partial hydrolysis of the pyrophosphate bound of FAD. Inspection of the published data indicates that the isolated β-subunit contained FAD in the expected amounts but that the FMN content of the α, β, γ complex was only 32% of that expected from the proposed 1:1:1 stoichiometry of these subunits [19]. The lack of FMN from the α-subunit is further in accord with recent DNA sequencing data which do not indicate a FMN binding motif within the deduced protein sequence of the α-subunit [17].

Based on N-terminal amino acid sequences for the α and $\gamma 2$ subunits that are identical to analyses performed in our laboratory (unpublished data), the genes for these subunits have been identified and have been shown to be part of a lager operon that has been termed nqr operon. The DNA sequences of four additional open reading frames of the putative operon have been determined by Beattie et al. [17] and Hayashi et al. [18], but none of these sequences could be correlated yet to the protein sequence of a Na^+-NQR 1 subunit. The gene nqr 6 has been proposed to encode the β-subunit and to contain a structural motif for NADH or FAD binding. Thus, the Na^+-NQR 1 is more complex than anticipated: it consists of more than the three subunits recognized previously [7] and contains FAD but not FMN as prosthetic group. Additionally, we have evidence from EPR spectroscopy that Na^+-NQR 1 contains iron-sulfur cluster(s) that are essential for quinone reductase activity. The function of the isolated complex as a primary Na^+ pump in a reconstituted system and the essence of the prosthetic groups for this function will be described elsewhere.

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