FAD and FMN flavoproteins participate in the sodium-transport respiratory chain NADH:quinone reductase of a marine bacterium, *Vibrio alginolyticus*

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The sodium-transport respiratory chain NADH:quinone reductase of a marine bacterium, *Vibrio alginolyticus*, is composed of three protein subunits, α, β and γ. The β-subunit contains FAD as a prosthetic group and corresponds to NADH dehydrogenase, which catalyses the reduction of ubiquinone to ubisemiquinone. In addition to β, subunits α and γ are essential for the quinone reductase, which catalyses the reduction of ubiquinone to ubiquinol. The α-subunit contains FMN and the reaction catalysed by subunit α is related to the coupling site of the sodium pump in the quinone reductase.

Respiratory chain *NADH:quinone reductase* *FAD* *FMN* *Na^+ pump* *(Marine bacterium)*

1. INTRODUCTION

The respiratory chain NADH:quinone reductase of a marine bacterium, *Vibrio alginolyticus*, requires Na^+ for maximum activity [1,2], and the Na^+-dependent reaction has been shown to be tightly coupled to the extrusion of Na^+ in this bacterium [3–6]. The Na^+-dependent quinone reductase was partially purified from a membrane fraction and it was found that the reduction of ubiquinone to ubiquinol proceeds via ubisemiquinone radical [7]. The reduction of ubiquinone to ubisemiquinone was catalysed by a flavoprotein, NADH dehydrogenase. Another protein component(s) was required for the reduction of ubisemiquinone [7]. To clarify the protein component(s) catalysing ubiquinol formation, the quinone reductase was further purified by high-performance liquid column chromatography. Here, we demonstrate that, in addition to FAD-containing NADH dehydrogenase, another flavoprotein containing FMN is required for the quinone reductase activity.

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2. MATERIALS AND METHODS

2.1. *Chemicals*

Liponox DCH, an alkyl polyoxyethylene ether detergent, was described in [7]. Ubiquinone-1 was kindly supplied by Eizai, Tokyo. Other reagents used were of analytical grade.

2.2. *High-performance liquid column chromatography*

Pre-packed high-performance columns were obtained from Toyo Soda, Tokyo. Gel chromatography was performed with a TSK gel G3000SWG column (21.5 × 600 mm). The column was equilibrated and developed with a buffer solution containing 0.1 M NaCl, 5% glycerol, 0.2% Liponox DCH, 0.1 mM EDTA and 10 mM Tris-HCl (pH 7.0). For ion-exchange chromatography, TSK gel DEAE-5PW (21.5 × 150 mm) was employed. The above-described buffer solution was used except that the pH was adjusted to 7.9. The column was eluted with a linear gradient from 0.1 to 0.55 M NaCl in a total volume of 330 ml at a flow rate of 3 ml/min.

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2.3. *Enzyme assays*

NADH dehydrogenase and quinone reductase were assayed at 30°C as in [7]. Enzyme unit is defined in [7].

2.4. *Flavin analyses*

FAD and FMN were identified by thin-layer chromatography on Kieselgel (Merck, pre-coated plate) with a solvent system of *n*-butanol/acetic acid/water (4:2:2, v/v) or 5% $Na_2HPO_4 \cdot 12H_2O$. FAD and FMN contents of the enzyme preparation were determined by the method of Faeder and Siegel [8]. Flavins were extracted by boiling for 5 min at neutral pH.

2.5. *Other methods*

SDS-polyacrylamide gel electrophoresis was performed by the method of Laemmli [9]. Protein was determined by the method of Bradford [10] with bovine albumin as a standard and the dye reagent was obtained from Bio-Rad.

3. RESULTS

3.1. *Purification and protein components of quinone reductase*

High-performance liquid gel chromatography was employed to remove impurities as much as possible, and then the reductase was applied to DEAE-5PW ion-exchange column. The early stage of purification procedure is essentially similar to those described in [7]. Liponox extracts were applied to a DEAE-Sephacel column in place of QAE-Sephadex. After washing the column with 0.15 M NaCl, the reductase was eluted with 0.3 M NaCl (DEAE-Sephacel fraction). This fraction was applied to a TSK gel G3000SWG gel column as described in section 2.2. The active fraction (SWG fraction) was then applied to a TSK gel DEAE-5PW column as in section 2.2. The quinone reductase was eluted at about 0.32 M NaCl with a high specific activity of 104 units/mg protein (see table 1), which amounted to 120-fold purification from the original membrane. The NADH dehydrogenase was eluted at about 0.34 M NaCl with a specific activity of 190 units/mg protein.

Fig.1 shows the results obtained from SDS-polyacrylamide gel electrophoresis. Three protein components, designated as α, β and γ, were concentrated in the quinone reductase (lane 3),

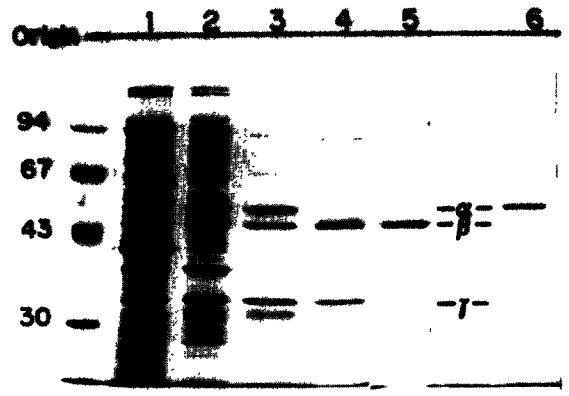

Fig.1. SDS-polyacrylamide gel electrophoresis of each enzyme preparation. Numbers in parentheses are the amounts of protein applied to electrophoresis. (1) DEAE-Sephacel fraction (35 μg), (2) SWG fraction (18 μg), (3) quinone reductase from DEAE-5PW (3 μg), (4) NADH dehydrogenase from DEAE-5PW (2.3 μg), (5) NADH dehydrogenase further purified by Bio-Gel HTP (1.2 μg), (6) the α fraction from DEAE-5PW (1.5 μg). Migrations of standard proteins and their molecular masses (in kDa) are indicated to the left of lane 1.

whereas the NADH dehydrogenase contained β and γ (lane 4). The M_r values of α, β and γ were estimated to be approx. 52000, 46000 and 32000, respectively. The NADH dehydrogenase was further purified by Bio-Gel HTP column as described in [7], which contained a single protein band β (lane 5). We could isolate a protein fraction containing α (lane 6) from fractions not adsorbed to the DEAE-5PW under the present conditions. This fraction contained a flavin as judged from its absorption and fluorescence spectra.

3.2. *Flavin contents of the enzyme preparation*

Flavins were identified as in section 2.4. The quinone reductase contained both FAD and FMN, whereas the NADH dehydrogenase contained only FAD. Table 1 lists the flavin contents of each enzyme preparation together with its enzyme activity. The quinone reductase contained FAD and FMN in equal quantities. From table 1, it is apparent that subunit β is a FAD-containing and subunit α a FMN-containing flavoprotein.

3.3. *Reconstitution of quinone reductase*

The α fraction showed no enzyme activities by

Table 1

Flavin content and activity of enzyme preparations with varied subunit components

Enzyme preparation	Subunit	Flavin (nmol/mg)		Activity (units/mg)	
		FAD	FMN	QR	NDH
Quinone reductase (lane 3)	$\alpha\beta\gamma$	6.7	6.6	104	232
NADH dehydrogenase (lane 4)	$\beta\gamma$	9.4	1.1	7	190
NADH dehydrogenase (lane 5)	β	21.3	0	0	390
The α fraction (lane 6)	α	0.6	6.1	0	0

Enzyme preparations are those used in fig.1 and the lane number is indicated for each preparation. QR, quinone reductase; NDH, NADH dehydrogenase

itself, but the combination of α with $\beta\gamma$ manifested quinone reductase activity. Since the quinone reductase was not reconstituted from α and β, subunit γ was also an essential component of this enzyme. The K_m value for ubiquinone-1 of $\beta\gamma$ was lower than that of β, suggesting that γ was related to the interaction of β with ubiquinone.

4. DISCUSSION

The purified quinone reductase is composed of three protein subunits, α, β and γ (fig.1), β and α containing FAD and FMN, respectively, as prosthetic groups (table 1). Thus two flavoproteins participate in the quinone reductase. The FAD-containing β with an apparent M_r of 46000 corresponds to NADH dehydrogenase and catalyses the reduction of ubiquinone to ubisemiquinone. On the other hand, the FMN-containing α participates in the reduction of ubisemiquinone to ubiquinol. Because the latter reaction requires Na^+ and is very sensitive to 2-heptyl-4-hydroxyquinoline *N*-oxide [7], the reaction catalysed by α is considered to be coupled to the extrusion of Na^+ in the quinone reductase.

Several dehydrogenases containing both FAD and FMN as prosthetic groups have been reported [11–13]. These enzymes contain both flavins in a single protein. The quinone reductase, however, contains FAD and FMN in a separate subunit. This is the first report of respiratory chain-linked NADH:quinone reductase that is composed of subunits containing both FAD and FMN flavoproteins.

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