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Identification of six subunits constituting Na^+-translocating NADH-quinone reductase from the marine *Vibrio alginolyticus*

Yuji Nakayama, Maki Hayashi, Tsutomu Unemoto*

Laboratory of Membrane Biochemistry, Faculty of Pharmaceutical Sciences, Chiba University, 1-33, Yayoi-cho, Inage-ku, Chiba 263, Japan

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Abstract We previously reported that the purified Na^+-translocating NADH-quinone reductase (NQR) from the marine *Vibrio alginolyticus* is composed of three major subunits, α, β and γ. NQR operon was sequenced and was found to be composed of 6 structural genes. Among these genes, *nqr1*, *nqr3* and *nqr6* were identified to code for α-, γ- and β-subunits, respectively. The protein products from *nqr2*, *nqr4* and *nqr5*, however, were not reported. The sequence data predicted that these three proteins are very hydrophobic and may be unusual in mobility and staining on SDS-PAGE. By modifying the detection method of proteins on SDS-PAGE, we could detect all six subunits encoded by NQR operon in the purified NQR complex. The open reading frame of each subunit was identified from its N-terminal amino acid sequence.

Key words: Na^+-translocating NADH-quinone reductase; Respiratory chain; Na^+ pump; Subunit structure; *Vibrio alginolyticus*

1. Introduction

The respiratory chain of a marine bacterium *Vibrio alginolyticus* contains an Na^+-dependent NADH-quinone reductase that functions as an electrogenic Na^+ pump (for reviews, see Refs. [1,2]). The Na^+-translocating NADH-quinone reductase (NQR) was purified from *V. alginolyticus*, which was reported to be composed of three major subunits, α, β and γ, with apparent molecular masses of 52, 46 and 32 kDa, respectively [3–5]. The FAD-containing β-subunit reacted with NADH and reduced 2-methyl-1,4-naphthoquinone (menadione) and ubiquinone-1 (Q-1) by a one-electron transfer pathway, which was stimulated about 5-fold by the addition of either 0.4 M Na^+ or K^+ [3]. Thus, the reaction catalyzed by the β-subunit showed no specific requirement for Na^+ and was insensitive to HQNO [3]. The αβγ-complex, however, reduced Q-1 to ubiquinol-1 with stoichiometric consumption of NADH, and the reaction stopped when all of the Q-1 was converted to ubiquinol-1 [3,6]. This reaction was specifically activated by Na^+ and strongly inhibited by HQNO. Thus the Na^+-dependent and HQNO-sensitive sites were located to the reaction catalyzed by the FMN-containing α-subunit. In 1994, Beattie et al. [7] and Hayashi et al. [8,9] independently reported the cloning and sequencing of NQR operon, and the NQR operon was found to be composed of six structural genes. Among them, *nqr1*, *nqr3* and *nqr6* were identified to code for α, γ and β-subunits, respectively [9]. Other subunits such as Nqr2, Nqr4 and Nqr5 were not reported in the purified NQR complex. Recently, Pfenninger-Li et al. [10] reported the purification and properties of NQR complex from *V. alginolyticus*. They detected four dominant polypeptides with apparent molecular masses of 50.7, 45.9, 33.3 and 31.7 kDa, respectively, in the partially purified NQR complex and the less densely stained band (33.3 kDa) located close to the γ-subunit was tentatively assigned to be Nqr2 (designated as a-subunit). Further purification of NQR complex was unsuccessful. As predicted from the sequence data, Nqr2, Nqr4 and Nqr5 were very hydrophobic polypeptides. Therefore, we reexamined the components of purified NQR complex on SDS-PAGE by modifying the detection method, and we could confirm the presence of six subunits in the complex.

2. Materials and methods

2.1. Chemicals

Liponox DCH, an alkyl polyoxyethylene ether detergent [5], was kindly supplied by Lion Co., Kanagawa, Japan. Ubiquinone-1 (Q-1) was obtained from Sigma, 2-methyl-1,4-naphthoquinone (menadione) and Coomassie Brilliant Blue G-250 from Nakarai Chemicals Ltd., and NADH from Kohjin Co., Ltd. Other reagents used were of analytical grade.

2.2. Enzyme assays

NADH dehydrogenase (NDH) activity was assayed at 30°C from the decrease in absorbance at 340 nm with menadione as an electron acceptor. The standard assay mixture contained 0.2 mM NADH/0.1 mM menadione/0.2 M NaCl/0.1% (w/v) Liponox DCH/20 mM Tris-HCl (pH 7.8) and enzyme in a total volume of 1.0 ml.

NADH-quinone reductase (NQR) activity was assayed at 30°C by following the formation of reduced Q-1 as previously described [6]. The standard assay mixture contained 0.1 mM NADH/15 μM Q-1/0.2 M NaCl/0.015% Liponox DCH/20 mM Tris-HCl (pH 7.8) and enzyme in a total volume of 2.0 ml. Changes in absorbance difference at the wavelength pair, 242–270.5 nm, were recorded with a Hitachi 557 two wavelengths spectrophotometer. The rate of Q-1 reduction was calculated based on the millimolar absorption coefficient of 9.6 [6].

One unit of activity is defined as the amount of enzyme catalyzing the oxidation of 1 μmol NADH or the reduction of 1 μmol Q-1 in 1 min.

2.3. Enzyme purification

The Na^+-translocating NADH-quinone reductase (NQR) of *V. alginolyticus* was purified as described in [4,5] with slight modifications. Briefly, the NQR complex was extracted from the membrane fraction of *V. alginolyticus* with 1% Liponox DCH containing 10% (w/v) glycerol, 0.1 mM EDTA and 0.14 M NaCl. The Liponox extract was applied to DEAE-Sephacel column and the reductase was eluted with 225 mM NaCl. The active fraction was applied to a TSK-gel DEAE-5PW column and the column was eluted with a linear gradient from 0.2 M to 0.4 M NaCl. The active fraction was further purified by gel filtration on HiLoad Superdex 200 column (16×600 mm, Pharmacia). The NQR complex having the NDH and the NQR activities of 111 and 42 units/mg protein, respectively, was obtained. The activity ratio NDH/NQR of this sample was 2.64.

*Corresponding author. Fax: (81) (43) 290 3021.
E-mail: unemoto@p.chiba-u.ac.jp

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Table 1
Revised summary of six structural gene products of NQR operon

Subunit	Amino acid residue	Molecular mass (Da)	M_r by SDS-PAGE (kDa)	Isoelectric point (p*I*)	Membrane-spanning helix	N-Terminal sequence[a]	Comments
Nqr1	446	48 622	50	5.43	0	MITIKKGLDL	α-Subunit
Nqr2	413	45 210	30–35	8.17	10	(M)ALKKFLEDIE	ORF changed
Nqr3	255	27 571	32	4.83	1	(M)ASNNDSIKKT	γ-Subunit
Nqr4	209	22 470	20	9.15	6	(M)SSAQNVKKSI	
Nqr5	198	21 540	19	7.00	6	MEHYISLL	Blocked
Nqr6	407	45 274	46	4.55	1	MDIILGVVMF	Blocked

[a](M) denotes that methionine was not detected in the sequence analysis. Therefore methionine was omitted from the calculations in Nqr2, Nqr3 and Nqr4.

2.4. N-terminal amino acid sequence

Proteins separated by SDS-PAGE were electroblotted to Trans-blot PVDF membrane (BioRad) by a semidry blotting apparatus (Atto) using a blotting solution containing 10 mM Caps (pH 11), 0.05% SDS and 10% methanol. The blotted membrane was stained for 5 min with 0.1% Ponceau S in 2% acetic acid, and the stained protein bands were cut off and destained with 1% acetic acid. N-terminal amino acid sequence of each protein band was analyzed by a Shimadzu PPSQ-21 fully automated protein sequencer. For the sample blocked at the N-terminus, the piece of PVDF membrane was soaked with a small amount of acetonitrile and then incubated for 17–24 h at 25°C in 0.2 ml of 0.6 N HCl to remove formyl residue [11].

2.5. Other methods

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli [12]. Acrylamide gel concentrations and pre-treatments of the sample were varied as described in the text. After electrophoresis, the protein bands were fixed for 2 h with a medium containing 50% methanol and 10% acetic acid, and then it was stained for 3 h with 0.1% Coomassie Brilliant Blue G-250 in 10% acetic acid as described by Schaegger et al. [13].

3. Results and discussion

3.1. Detection of 6 subunits in the purified NADH-quinone reductase complex

On SDS-PAGE at the acrylamide gel concentration of 10%, the purified NADH-quinone reductase (NQR) complex contained 3 major proteins, α, β, and γ, together with a faintly stained band located close to the γ-subunit (Fig. 2 in Ref. [5]).

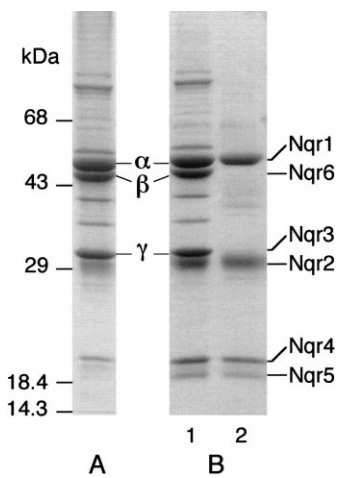

Fig. 1. Coomassie-stained SDS-PAGE of the purified NQR complex and the α-fraction. 25 μg of the purified NQR complex with NQR activity of 43 units/mg protein was applied to SDS-PAGE at gel concentration of 10% (A and B-1). The sample was pre-treated for 3 min at 100°C (A), or the heat-treatment was omitted (B-1). The α-fraction containing 16 μg protein was applied to B-2.

The NQR operon was cloned and sequenced [7–9], and was found to be composed of 6 structural genes. The α, β and γ subunits were identified to be coded by *nqr1*, *nqr6* and *nqr3* genes, respectively [9]. The unidentified Nqr2, Nqr4 and Nqr5 proteins were predicted to be very hydrophobic membrane-bound proteins from their sequence data (see Table 1). Hydrophobic proteins are likely to be faintly stained and sometimes show abnormal movements on SDS-PAGE. The heat-treatment of the sample before electrophoresis also tends to lose these protein bands. Therefore, we modified the detection method of proteins on SDS-PAGE so as to fit into hydrophobic proteins.

Fig. 1 shows the results of SDS-PAGE of the purified NQR complex.

In panel A, the sample was heated for 3 min at 100°C in the buffer containing SDS and 2-mercaptoethanol before application to the gel. The result was essentially the same as reported in [4,5], and the NQR complex was composed of 3 major subunits, α, β and γ, and several faintly stained protein bands. In panel B-1, the heat-treatment in the sample buffer was omitted and the sample was allowed to stand for 20 min at 20°C. In addition to the 3 major subunits detected in panel A, 3 other faintly stained protein bands were discernible in panel B-1. These protein bands were electroblotted to the PVDF membrane and each band was analyzed for N-terminal amino acid sequence (Table 1). From their N-terminal sequence data, each protein was assigned to correspond to the subunit of NQR complex as shown in Fig. 1.

The effect of heat-treatment of the sample for the detection of hydrophobic proteins on SDS-PAGE was examined. Although not shown here, the staining densities of hydrophobic proteins, Nqr2, Nqr4 and Nqr5, decreased with the increase in the temperature. The pre-treatment of the sample for 5 min at 100°C resulted in the disappearance of hydrophobic proteins on SDS-PAGE. Hydrophilic proteins such as Nqr1, Nqr6 and Nqr3, however, were unaffected by these treatments.

As reported in the previous paper [5], a part of the NQR complex was dissociated into subunits during purifications and these subunits were separated at the step of DEAE-5PW chromatography. Thus, 3 separate fractions, that is, the αβγ-complex having both NQR and NDH activities, the βγ-complex having only NDH activity and the α-fraction were obtained. We succeeded in the reconstitution of catalytically active NQR complex by the combination of the βγ-complex and the α-fraction. At that time, hydrophobic subunits were not detected on SDS-PAGE. Since the α-fraction contained a faintly stained minor band which appeared in front of the γ-subunit (Fig. 2c in Ref. [5]), the subunit components of the α-fraction were reexamined. As shown in panel B-2 of

Fig. 1, the α-fraction apparently contained Nqr2, Nqr4 and Nqr5 in addition to Nqr1 (α). In spite of the acidic nature of Nqr1 with p*I* 5.4 (Table 1), it was quickly eluted from DEAE-5PW. Nqr1 must be co-eluted with basic hydrophobic subunits such as Nqr2 and Nqr4. Since the α-fraction contained all 3 hydrophobic subunits, these results suggested the participation of hydrophobic subunits in the reconstitution of catalytically active NQR complex.

The relative molecular mass (M_r) of each subunit was estimated from SDS-PAGE (Table 1). As compared with the M_r values deduced from the sequence data, Nqr1, Nqr3 and Nqr6, respectively, gave an expected value. Hydrophobic Nqr4 and Nqr5 moved a little faster than was expected from their M_r. On the other hand, Nqr2 with M_r 45.3 kDa moved faster than expected and its mobility was altered by the gel concentrations. Thus, the estimated M_r of Nqr2 from SDS-PAGE changed from 30 to 35 kDa at gel concentrations from 10 to 14%. Indeed, at the gel concentration of 12%, Nqr2 and Nqr3 moved at the same rate and overlapped with each other on SDS-PAGE. These properties apparently interfered with the detection of Nqr2 on SDS-PAGE.

3.2. Identification and properties of NQR complex and each subunit

Table 1 summarizes properties of six subunits of NQR operon. Pfenninger-Li et al. [10] reported that Nqr2, which was tentatively assigned as the a-subunit, had blocked N-terminus. However, the N-terminal amino acid sequence of Nqr2 could be analyzed without any pre-treatment. From its amino acid sequence, the open reading frame of Nqr2 was altered to start from the next methionine and the amino acid residues were shortened from 426 (predicted by Beattie et al. [7]) to 413 with the calculated M_r 45 210 Da (Table 1). The N-terminal amino acid sequences of Nqr5 and Nqr6 could not be directly analyzed due to the blocking of terminal amino group. When the sample was pre-treated overnight at 25°C with 0.6 N HCl, the N-terminal sequence could be analyzed as shown in Table 1. From these sequence data, the open reading frame of each subunit in the NQR operon was finally identified.

During our studies on the constructions of plasmids containing a definite region of NQR operon, nucleotide sequences derived from the NQR operon have been repeatedly analyzed. These sequence data indicated that several nucleotides were different from that reported by Beattie et al. [7] in the region from *nqr1* to *nqr3*. For example, 3- and 5-nucleotide residues were different in *nqr1* and *nqr2*, respectively. As a result, some predicted amino acid residues were also altered. This information and the revised nucleotide sequence data of NQR operon have been submitted under accession number AB008030. The data presented in Table 1 were calculated based on the revised sequences.

We previously estimated the molecular mass of protein moiety of the complex to be 254 kDa [5]. Since this value was close to twice that of (α+β+γ), the catalytic NQR complex was predicted to be a dimer of αβγ in 0.1% Liponox DCH solution without considering the participation of hydrophobic subunits. In this paper, the active NQR complex was found to contain all 6 subunits. Since the sum total of six subunits amounts to 211 kDa, the NQR complex is more likely to be composed of equimolar quantities of six subunits.

Contrary to our previous results, Pfenninger-Li and Dimroth [14] reported that the NQR complex from *V. alginolyticus* contains FAD but not FMN. Therefore, we analyzed the purified enzyme again and came to the conclusion that the NQR complex contains both FAD and FMN as reported in our previous paper [4,5]. Details will be reported elsewhere. It is not so easy to precisely decide the purity of NQR complex. As a simple method, the calculation of the activity ratio NDH/NQR is helpful. If the sample contains excess Nqr6, or the complex loses Nqr1, the NDH/NQR ratio increases. The NQR activity of the complex used for flavin analysis was reported to be 14.8 units/mg protein [14]. Since the assay was performed at 25°C, the specific activity is not directly comparable with our preparation. The ratio NDH/NQR was calculated to be about 6.1 [14], which was significantly higher than our purified NQR complex with the ratio of 2.6. As pointed out in our previous paper [3,5], Nqr1 is likely to be released from the NQR complex during storage or by treatment with ion-exchange chromatography. Recently, we constructed several plasmids that allow the overproduction of required subunits in *E. coli*, which must be useful for the elucidation of the role of each subunit in the NQR complex.

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