Covalently bound flavin in the NqrB and NqrC subunits of Na^+-translocating NADH-quinone reductase from *Vibrio alginolyticus*

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Abstract **Na^+-translocating NADH-quinone reductase (NQR) from the marine bacterium *Vibrio alginolyticus* is composed of six subunits (NqrA to NqrF). On SDS–PAGE of the purified complex, NqrB and NqrC subunits were found to give yellow–green fluorescent bands under UV illumination. Both the NqrB and NqrC, electroeluted from the gel, had an absorption maximum at 448 nm, and the fluorescence excitation maxima at 365 and 448 nm and the emission maximum at 514 nm. The electroeluted NqrB and NqrC, respectively, were identified from their N-terminal amino acid sequences. These results clearly indicated that the NqrB and NqrC subunits have covalently bound flavins. The two subunits were digested by protease and then the fluorescent peptide fragments were separated by a reversed-phase high performance liquid chromatography. N-Terminal amino acid sequence analyses of the fluorescent peptides revealed that the flavin is linked to Thr-235 in the NqrB and Thr-223 in the NqrC subunits. This is the first example that the flavin is linked to a threonine residue. The amino acid sequence around the flavin-linked threonine was well conserved between NqrB and NqrC. Identification of the flavin group is in progress.**

Key words: Covalently bound flavin; Na^+-translocating NADH-quinone reductase; Na^+ pump; Respiratory chain; Marine bacterium; *Vibrio alginolyticus*

1. Introduction

The respiratory chain of a marine bacterium *Vibrio alginolyticus* contains an Na^+-dependent NADH-quinone reductase (NQR) that functions as an electrogenic Na^+ pump (for reviews, see [1,2]). The *nqr* operon encoding the Na^+-translocating NQR was cloned and sequenced independently by Beattie et al. [3] and by Hayashi et al. [4,5], and was found to be composed of six structural genes (*nqrA* to *nqrF*). As predicted from the sequence data, NqrA, NqrC and NqrF are relatively hydrophilic polypeptides, whereas NqrB, NqrD and NqrE are very hydrophobic polypeptides. The NQR complex was purified from the membrane fraction of *V. alginolyticus*, and the six subunits encoded by the *nqr* operon were identified in the purified NQR complex [6]. Each subunit was assigned from the N-terminal amino acid sequence.

We previously reported the presence of non-covalently bound FAD and FMN in the purified NQR complex [7,8]. FAD was localized in the purified NqrF, whereas FMN was recovered in the α fraction, which was later found to contain three hydrophobic subunits in addition to NqrA [6]. In these experiments, non-covalently bound flavins were extracted by boiling for 5 min at neutral pH and, after centrifugation, the supernatant was subjected to flavin analysis by the method of Faeder and Siegel [9]. In 1995, Pfenninger-Li and Dimroth [10] reported that the purified NQR complex from *V. alginolyticus* contains FAD but not FMN. They released flavins by treatment with trichloroacetic acid and the precipitated apoprotein was removed by centrifugation. Since our sample contained 0.2% Liponox DCH detergent and 10% (w/v) glycerol, the boiling treatment of the sample was apparently insufficient for the denaturation and removal of proteins from the supernatant fraction. The supernatant was possible to be contaminated by denatured proteins having covalently bound flavins. The purified NqrF contains non-covalently bound one FAD molecule per subunit [8]. As the amount of total flavins in the purified NQR complex could not be explained by the amount of FAD in the NqrF subunit, we reinvestigated the distribution of flavins among the constituent subunits. Very recently, Zhou et al. [11] reported the presence of a second flavin, possibly FMN, covalently attached to the NqrC subunit of the NQR complex purified from *V. harveyi*. We independently found that the NqrB and NqrC subunits from *V. alginolyticus* contain covalently bound flavins as cofactors. This paper reports the evidence for the existence of covalently bound flavins in the NqrB and NqrC subunits.

2. Materials and methods

2.1. Materials

Liponox DCH, an alkyl polyoxyethylene ether detergent [8], was kindly supplied by Lion Co., Kanagawa, Japan. The purified NQR complex and the α fraction were prepared as described in [6]. Chymotrypsin was purchased from Boehringer-Mannheim, lysyl endopeptidase from Wako Chemicals, and thermolysin from Nakarai Chemicals. Other reagents used were of analytical grade.

2.2. Measurement of flavin content in the NQR complex and the filtrate

Purified NQR complex was suspended in 100 mM sodium phosphate buffer (pH 7.7) containing 4% sodium dodecyl sulfate (SDS) at the protein concentration of 0.12 mg/ml. The mixture was incubated at room temperature for 16 min or boiled for 3 min. The denatured sample was used for the determination of total flavins. It was then filtered through a UFC3LCC Millipore filter to completely remove denatured proteins. The filtrate contains non-covalently bound flavins. The amount of flavins was calculated from the absorbance at 473 nm using the millimolar extinction coefficient of 9.2 mM^{-1} cm^{-1}, since both FAD and FMN exhibit the same extinction coefficient at this wavelength [12].

2.3. Extraction of Nqr subunits from the gel, and spectral and amino acid sequence analysis

The purified NQR complex (0.2 mg protein) was separated in a disk

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gel column (5 mm diameter) by SDS–PAGE at 9% polyacrylamide gel, and then the two fluorescent bands were separately excised from the gel. The excised gel obtained from 1.2 mg of the NQR complex was combined and the fluorescent subunit was eluted from the gel by a Max Yield AE-3590 electroelution (Atto Co., Ltd.) for 2 h at 2 W using the running buffer of SDS–PAGE as an electroelution buffer. Visible absorption spectra of the eluate were measured by a Beckman DU-640 spectrophotometer. Fluorescence excitation and emission spectra were recorded by a Shimadzu RF-1500 fluorometer.

The N-terminal amino acid sequence analysis was performed as follows. Polyvinylidene difluoride (PVDF) membrane was first immersed in methanol for a few seconds, and then equilibrated with the running buffer of SDS–PAGE. Then, the PVDF membrane was placed into the eluate and incubated at 4°C for 24 h. It was washed with water, dried and subjected to amino acid sequence analysis on a Shimadzu PPSQ-21 automated protein sequencer.

2.4. Proteolytic digestion of subunits and separation of fluorescent peptides

For identification of amino acid residues attached to flavin, fluorescent subunits were digested by a protease, and the fluorescent peptides were separated by a reversed-phase high performance liquid chromatography (HPLC). The NqrB and NqrC subunits were separated by SDS–PAGE as described in Section 2.3. The disk gel containing fluorescent NqrB or NqrC was cut into small pieces and washed with acetonitrile to remove SDS from the gel prior to proteolytic cleavage. Washed gel pieces were dried in a vacuum centrifuge, and then it was rehydrated by 70 μl of a protease reagent per disk gel. For digestion of the NqrB subunit, chymotrypsin (12.5 μg/ml) dissolved in 50 mM ammonium bicarbonate containing 5 mM $CaCl_2$ and 2 M urea was used, and the mixture was incubated for 18–20 h at 37°C. The peptides obtained from the NqrB by digestion with chymotrypsin in the presence of 2 M urea were further digested by chymotrypsin in the presence of 1.0 M guanidine hydrochloride. The dried peptide of the first digestion was dissolved in 10 μl of 5 M guanidine hydrochloride and incubated for 30 min at 50°C to denature the peptide. Then, it was diluted 5-fold with 25 mM ammonium bicarbonate containing 5 mM $CaCl_2$, and then 1 μg of chymotrypsin was added. The mixture was incubated for 5 h at 25°C. For digestion of the NqrC subunit, thermolysin (12.5 μg/ml) dissolved in 50 mM ammonium bicarbonate containing 5 mM $CaCl_2$ and 0.2% Liponox DCH was used, and the mixture was incubated for 18–20 h at 37°C. Peptide fragments were extracted from the gel as described by Jensen et al. [13]. These extracts were pooled, then dried in a vacuum centrifuge.

The dried peptides were dissolved in 0.1% TFA containing 6 M guanidine hydrochloride to prevent precipitation. It was centrifuged for 5 min at $15000\times g$ and the supernatant was applied to the reversed-phase HPLC column (Sephasil peptide C18, 4.6×250 mm, Pharmacia). Solvent A is 0.065% TFA in water, and solvent B is 0.05% TFA in 75% acetonitrile. The solvent B was increased as follows: 0–6 min, 5%; 6–60 min, 5–50%; 60–65 min, 50–100%; 65–75 min, 100%. The eluate was monitored with a Shimadzu RF-10A XL fluorescence detector. The fluorescent peaks were collected manually and then dried in a vacuum centrifuge and stored at −70°C. Each fluorescent fraction was dissolved in 30 μl of 30% acetonitrile containing 0.1% TFA, and Polybrene (sequabrene, Sigma) was employed as peptide carrier for the sequence analysis.

2.5. Other methods

SDS–PAGE was performed according to the method of Laemmli [14]. After electrophoresis, fluorescent gel photographs were taken and then the gel was stained with Coomassie brilliant blue G-250 as described previously [6]. Protein was determined by the method of Bradford [15] using bovine serum albumin as a standard.

3. Results and discussion

3.1. Distribution of flavins in the purified NQR complex

We previously reported that the purified NQR complex from *V. alginolyticus* contains non-covalently bound FAD and FMN [7,8]. FAD was localized in the purified NqrF and FMN was recovered in the α fraction. Later, the α fraction was found to contain NqrA, NqrB, NqrD and NqrE [6].

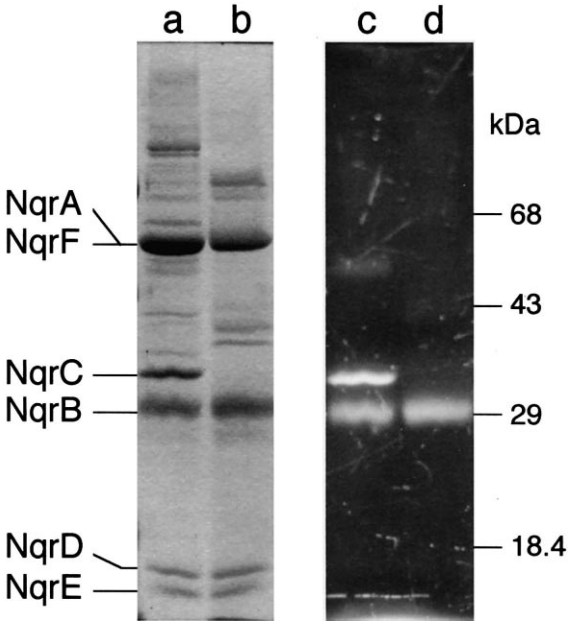

Fig. 1. Existence of covalently bound fluorescent compound in the NqrB and NqrC subunits of the NQR complex from *V. alginolyticus*. Purified NQR complex (lanes a and c, 15 μg protein) and the α fraction (lanes b and d, 10 μg protein) were separated by SDS–PAGE at 10% polyacrylamide in the presence of 6 M urea. The lanes a and b are Coomassie-stained, and the lanes c and d are a fluorescent photograph.

For the extraction of non-covalently bound flavins, sample was boiled for 5 min and then denatured proteins were removed by centrifugation. Our sample, however, contained 0.2% Liponox DCH detergent and 10% (w/v) glycerol and the removal of denatured proteins from the supernatant fraction was incomplete. Thus the supernatant was likely to be contaminated by the denatured flavin-containing proteins, leading to an erroneous conclusion on the presence of FMN. In the present study, the purified NQR complex was denatured by SDS and the sample was filtered by the Millipore filter to completely remove denatured proteins. Flavin contents in the denatured sample and the filtrate were determined spectrophotometrically as described in Section 2.2.

The purified NQR complex contained 11–12.5 nmol total flavins/mg protein. When the NQR complex was denatured by boiling for 3 min in the presence of 4% SDS, about 34% of the total flavins were extracted as non-covalently bound flavins in the filtrate. By the incubation of the NQR complex with 4% SDS for 16 min at room temperature, about 28% of the total flavins were extracted. The non-covalently bound flavin is derived from NqrF subunit that contains FAD as a cofactor [8,10]. These results indicated that there exists a non-covalently bound FAD and twice that amount of covalently bound flavins in the NQR complex.

The simplest way to separate covalently bound flavins from the non-covalently bound one is to subject the proteins to SDS–PAGE [16]. As shown in Fig. 1, two protein bands corresponding to the NqrB and NqrC subunits exhibited yellow–green fluorescence under UV illumination. To confirm this, the fluorescent bands were extracted electrically from the gel and they were subjected to amino acid sequence analysis as described in Section 2.3. The sequence obtained from the fast migrating fluorescent band (ALKKFL) coincided with the

NqrB subunit, and that obtained from the slow migrating fluorescent band (ASNNDSI) with the NqrC subunit (Fig. 1). Both the NqrB and NqrC had absorption maximum at 448 nm, and the fluorescence excitation maxima at 365 and 448 nm and the emission maximum at 514 nm. These properties clearly indicated that the NqrB and NqrC subunits have covalently bound flavins.

The fluorescence intensity of the NqrB observed on the gel is faint as compared with that of NqrC, and the detection of fluorescence by the NqrB is difficult with a small amount of NQR complex. Moreover, as reported in our previous paper [6], NqrB and NqrC co-migrate on SDS–PAGE at the gel concentration of 12%. Using 9–10% gel, the two subunits are separated and NqrB moves faster than NqrC (Fig. 1). Abnormal behavior of NqrB is apparently due to its very hydrophobic structure. It is necessary to take care of these particular properties for the detection of NqrB.

3.2. Identification of amino acid residues attached to flavin

Histidine, cysteine and tyrosine residues have been reported to bind flavins covalently [17]. To elucidate the amino acid residue attached to flavin in the NqrB and NqrC subunits, the subunits were digested by protease and the resulting peptides were subjected to the reversed-phase HPLC as described in Section 2.4.

When the NqrC subunit was digested by thermolysin in the presence of 0.2% Liponox DCH, the fluorescent peptide isolated had the sequence of LSGAXLTSNG (Fig. 2a). Addition of Liponox DCH increased the yield of the peptide. In this sequence analysis, no more identifiable residues were observed in the following cycles. The sequence corresponded to the residues 219–228 in the NqrC and Thr-223 could not be detected as shown by X. Since Thr-225 was observed as a clear peak, it was clear that Thr-223 was chemically modified. The absorption and fluorescence spectra of this peptide (Fig. 3a) are characteristic of the flavin group. These results strongly suggested that flavin must be attached to Thr-223 in the NqrC subunit.

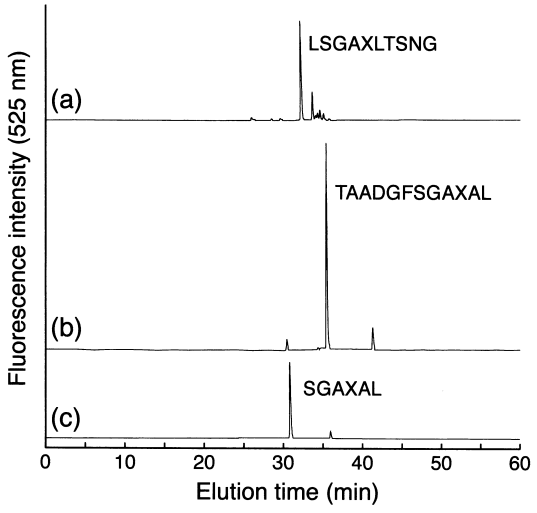

Fig. 2. Elution profiles of proteolytic digests of the NqrB and NqrC in the reversed-phase HPLC. Proteolytic digestions and the separation of fluorescent peptides were performed as described in Section 2.4. Fluorescence intensity of the eluate was monitored at 525 nm with the excitation at 450 nm, and the intensity was expressed in an arbitrary unit. The main fluorescent peak was collected and subjected to amino acid sequence analysis as in Section 2.4, and the results are shown in the figure, where X denotes a cycle with no identifiable amino acid. The NqrC subunit obtained from 1.2 mg of the NQR complex was digested by thermolysin in the presence of 0.2% Liponox DCH (a). (b) The NqrB from 2.0 mg of the NQR complex was digested by chymotrypsin with 2 M urea; (c) The NqrB from 1.0 mg of the NQR complex was first digested as in (b), and then the resulting peptide was further digested by chymotrypsin with 1 M guanidine hydrochloride as described in Section 2.4.

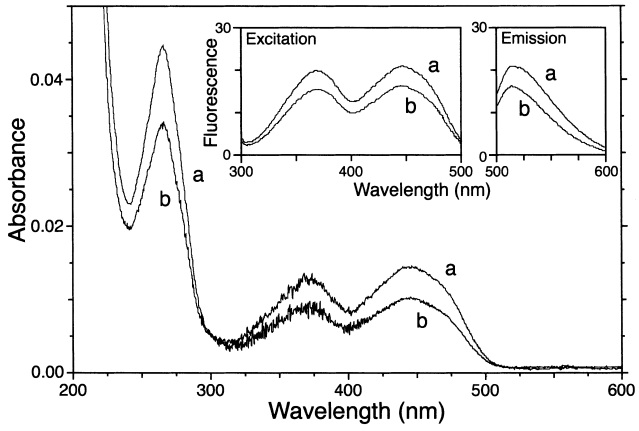

Fig. 3. Absorption and fluorescence spectra of fluorescent peptides derived from the NqrB and NqrC subunits. The fluorescent peptides from the NqrB and NqrC were prepared as described in Fig. 2c and Fig. 2a, respectively. The isolated peptide was dried and then dissolved with 20 mM sodium phosphate (pH 7.7) to record spectra. Line a, the peptide from the NqrC; line b, the peptide from the NqrB. After the measurement of absorption spectrum, the sample was diluted with 20 mM sodium phosphate (pH 7.7) to record fluorescence spectra (inset). The excitation spectrum was measured with the emission at 525 nm, and the emission spectrum was measured with the excitation at 445 nm.

Since NqrB is a very hydrophobic subunit, digestion by protease is rather difficult and the treatment by trypsin, lysyl endopeptidase and endoproteinase Glu-C gave no fluorescent peptide. Among proteases examined, only chymotrypsin gave clear fluorescent peptide. When the NqrB subunit was digested by chymotrypsin in the presence of 2 M urea, the sequence of the fluorescent peptide isolated was TAADGFSGAXAL (Fig. 2b), which corresponded to the residues 226–237 in the NqrB. In this sequence, X corresponded to Thr-235 and no more residues were detected after Leu-237. The fluorescent peptide obtained here was further digested by chymotrypsin in the presence of 1 M guanidine hydrochloride. As shown in Fig. 2c, the peptide with the sequence of SGAXAL was isolated. The sequence corresponded to the residues 232–237 in the NqrB, and X to Thr-235. The absorption and fluorescence spectra of the hexapeptide (Fig. 3b) are characteristic of the flavin group. These results strongly suggested that flavin must be attached to Thr-235 in the NqrB subunit.

Since the binding site of flavin was estimated to be a threonine residue in both the NqrB and NqrC, the sequence around the threonine residue was compared as shown in Fig. 4. Of

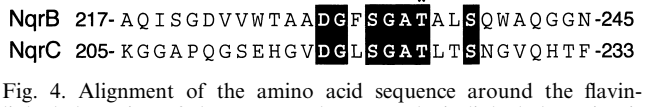

Fig. 4. Alignment of the amino acid sequence around the flavin-linked threonine of the NqrB and NqrC. Flavin-linked threonine is shown by an asterisk.

special interest, several amino acid residues around the threonine residue were conserved between NqrB and NqrC. Recently, Na^+-translocating NQR was found to be widely distributed in pathogenic bacteria [11,18]. The sequence of NqrB and NqrC around the flavin-linked residue was also well conserved among nine strains examined.

Very recently, Zhou et al. [11] reported the presence of a second flavin, possibly FMN, covalently attached to the NqrC subunit of the NQR complex from *V. harveyi*. After digestion by trypsin, fluorescent peptides were separated by HPLC and the sequence was analyzed. They reported that the flavin is linked to histidine in position 219 in the NqrC subunit. Comparing the sequence of NqrC from *V. harveyi* to that from *V. alginolyticus*, His-219 in the former corresponds to His-214 in the latter. Although not shown here, we isolated a fluorescent peptide from the NqrC that contains His-214 in the sequence. We could detect His-214 in the sequence analysis of this peptide, suggesting that His-214 is unlikely to be a binding site of the flavin in *V. alginolyticus*.

More than 20 enzymes have been reported to have covalently bound flavin [17]. But as the site of covalently bound flavin, a threonine residue has not been reported to date and this is the first report to show that a threonine residue participates in the binding of flavin. Preliminary studies with matrix-assisted laser desorption/ionization time-of-flight mass spectral analysis of the fluorescent peptide derived from the NqrB and NqrC subunits suggested that the molecular difference between the predicted and the observed values is close to that of FMN. However, detailed studies are required to finally determine the species of flavin and the mode of flavin binding to the threonine residue.

As measured from the reduced minus oxidized difference spectrum of flavins, more than 80% of the total flavins in the purified NQR complex are quickly reduced by NADH. Thus the covalently bound flavins in the NqrB and NqrC apparently participate in the redox reaction catalyzed by the NQR complex, and we need to resolve the electron transfer pathway in the complex to characterize the functions of the NqrB and NqrC subunits.

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