FMN is covalently attached to a threonine residue in the NqrB and NqrC subunits of Na^+-translocating NADH-quinone reductase from *Vibrio alginolyticus*

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Abstract The Na^+-translocating NADH-quinone reductase (NQR) from *Vibrio alginolyticus* is composed of six subunits (NqrA to NqrF). We previously demonstrated that both NqrB and NqrC subunits contain a flavin cofactor covalently attached to a threonine residue. Fluorescent peptide fragments derived from the NqrB and NqrC subunits were applied to a matrix-assisted laser desorption ionization time-of-flight mass spectrometer, and covalently attached flavin was identified as FMN in both subunits. From post-source decay fragmentation analysis, it was concluded that FMN is attached by a phosphate group to Thr-235 in the NqrB subunit and to Thr-223 in the NqrC subunit. The phosphoester binding of FMN to a threonine residue reported here is a new type of flavin attachment to a polypeptide.

Key words: Covalently bound flavin; FMN; Na^+-translocating NADH-quinone reductase; Threonine; Matrix-assisted laser desorption ionization time-of-flight mass spectrometry; *Vibrio alginolyticus*

1. Introduction

The respiratory chain of the marine bacterium *Vibrio alginolyticus* contains the Na^+-translocating NADH-quinone reductase (NQR) [1,2]. The *nqr* operon encoding the unique redox-driven Na^+ pump was sequenced and was found to be composed of six structural genes (*nqrA* to *nqrF*) [3–5]. The NQR complex purified from the membrane fraction of *V. alginolyticus* was confirmed to be composed of the six subunits encoded by the *nqr* operon [6]. As predicted from the sequence data, NqrA, NqrC and NqrF are relatively hydrophilic subunits, whereas NqrB, NqrD and NqrE are very hydrophobic subunits.

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 NqrB, NqrD and NqrE in addition to NqrA [6]. Pfenninger-Li and Dimroth [9] reported that the purified NQR from *V. alginolyticus* contains FAD but not FMN. Therefore the distribution of flavin cofactors among the constituent subunits was reinvestigated and we came to the conclusion that in addition to the non-covalently bound FAD in NqrF, both the NqrB and NqrC subunits have a covalently bound flavin [10]. Amino acid sequence analyses of the fluorescent peptide fragments derived from NqrB and NqrC predicted that the flavin is linked to Thr-235 in NqrB and Thr-223 in NqrC [10]. The species of the flavins, however, could not be identified in the previous paper. In this paper, the fluorescent peptide fragments were applied to a matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometer to identify the flavins. The peptide sequence and the mode of flavin binding to the specified threonine residue were also determined from a post-source decay (PSD) fragmentation analysis.

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 was prepared as described in [6]. Chymotrypsin was purchased from Boehringer-Mannheim and thermolysin from Nakarai Chemicals. Other reagents used were of analytical grade.

2.2. Preparation of fluorescent peptide fragment

The fluorescent peptide fragments derived from NqrB and NqrC were prepared as described in the previous paper [10]. Briefly, the NqrB and NqrC subunits were isolated from the purified NQR complex by SDS–PAGE. The purified NqrB was digested by chymotrypsin in the presence of 2 M urea, and the purified NqrC was digested by thermolysin in the presence of 0.2% Liponox DCH. The proteolytic digest was applied to a reverse-phase high performance liquid chromatography column (Sephasil peptide C18, Pharmacia) and the eluate was monitored with a fluorescence detector.

2.3. Mass spectrometry

A MALDI-TOF mass spectrometry was performed using a Voyager-DE STR MALDI-TOF mass spectrometer (Applied Biosystems), equipped with a 377 nm N_2 UV laser. Mass spectra were obtained by co-crystallization of a 4:1 (v/v) mixture of matrix (saturated solution of α-cyano-4-hydroxycinnamic acid in 50% (v/v) acetonitrile containing 0.1% trifluoroacetic acid (TFA)) and peptide sample which was dissolved in 30% (v/v) acetonitrile with 0.1% TFA.

3. Results

3.1. Identification of covalently bound flavin

The fluorescent peptide isolated from the NqrB subunit had

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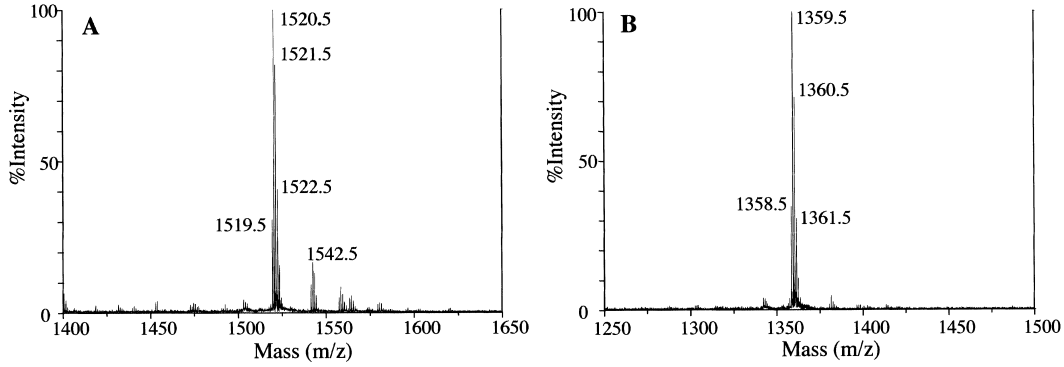

Fig. 1. MALDI-TOF mass spectra of fluorescent peptides obtained from NqrB and NqrC. A: The fluorescent peptide from NqrB with the sequence 226-TAADGFSGATAL-237, having a flavin cofactor at Thr-235. B: The fluorescent peptide from NqrC with the sequence 219-LSGATLTSNG-228, having a flavin cofactor at Thr-223.

the sequence TAADGFSGAXAL, where X could not be identified by the sequence analysis. The sequence corresponded to residues 226–237 in NqrB and X to Thr-235. Since the absorption and fluorescence spectra of the peptide were characteristic of a flavin cofactor, flavin was considered to be linked to Thr-235. The fluorescent peptide isolated from NqrC had the sequence LSGAXLTSNG, which corresponded to residues 219–228 and X to Thr-223. The flavin cofactor was considered to be linked to Thr-223. These peptides were analyzed by mass spectrometry.

Fig. 1 shows the MALDI-TOF mass spectrum of fluorescent peptide derived from NqrB (Fig. 1A) and NqrC (Fig. 1B). Assuming that FMN is covalently linked to Thr-235 via a phosphate group in NqrB, the theoretical mass of the fluorescent peptide was calculated to be 1520.62 as monoisotopic mass. This value coincided well with the observed mass of the major peak at 1520.5 (Fig. 1A). In the case of NqrC, the theoretical mass of fluorescent peptide having FMN at Thr-223 was calculated to be 1359.57. This value coincided well with the observed mass of the major peak at 1359.5 (Fig. 1B). No peaks suggesting the binding of FAD to the core peptide could be detected. These results indicate that in both NqrB and NqrC the covalently bound flavin cofactor is FMN, which is linked to the specified threonine residue by a phosphoester linkage.

3.2. Confirmation of peptide sequence and FMN-linked residue

Fig. 2 is the MALDI-TOF PSD spectrum of region *m/z* 200–1590, showing fragmentation of the fluorescent peptide from NqrB. The peptide sequence and the predicted site of FMN binding are shown in the inset. In addition to the peak of the precursor peptide (1520.9), two large peaks with masses 1063.5 and 458.5 were detected (Fig. 2). The mass difference between the precursor peptide and the mass 1063.5 is 457.4, which corresponds to the molecular mass of FMN. Thus, the observed mass 458.5 must be an ionized species of FMN, possibly with the structure of $[FMNH+H]^+$, and the observed mass 1063.5 corresponds to a core peptide devoid of FMN. From the fragmentation analysis, we noticed that when a fragment contains Thr-235 devoid of FMN, the observed mass value is always less than the theoretical mass by 18. It is highly possible that the residue Thr-235 lost water upon the removal of FMN. Thus the mass value of a Thr-235 residue without FMN would be 83.0. The theoretical mass of the core

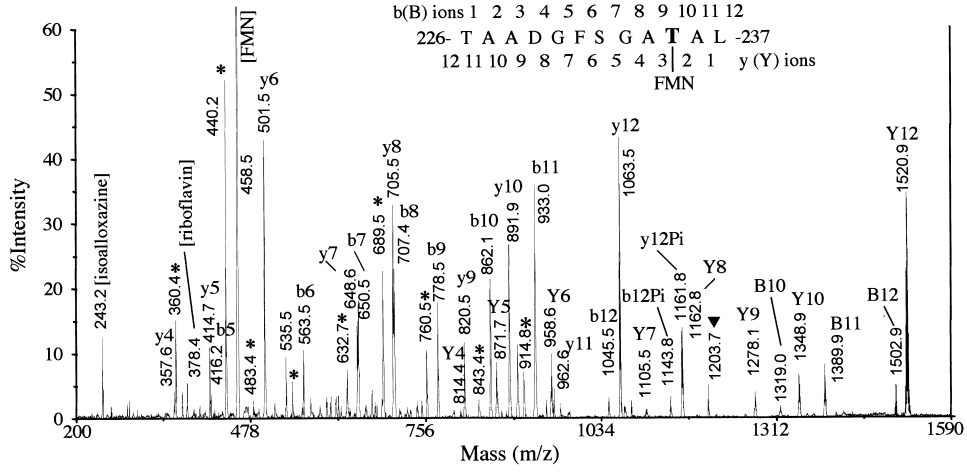

Fig. 2. MALDI-TOF PSD spectrum of region *m/z* 200–1590, showing fragmentation of the peptide mass 1520.9 from NqrB. The fragment ion mass of each ion type derived from the predicted structure of the inset was compared with the observed mass and the name of the ion type fitted for the observed mass was affixed to each peak. The fragment ions without FMN are shown in lower case letters (b, y) and the FMN-containing fragment ions by capital letters (B, Y). Inorganic phosphate attached to Thr-235 is shown by Pi. Asterisks show [minus H_2O] ions. The peak marked with a reversed triangle is an unidentified peak (see text).

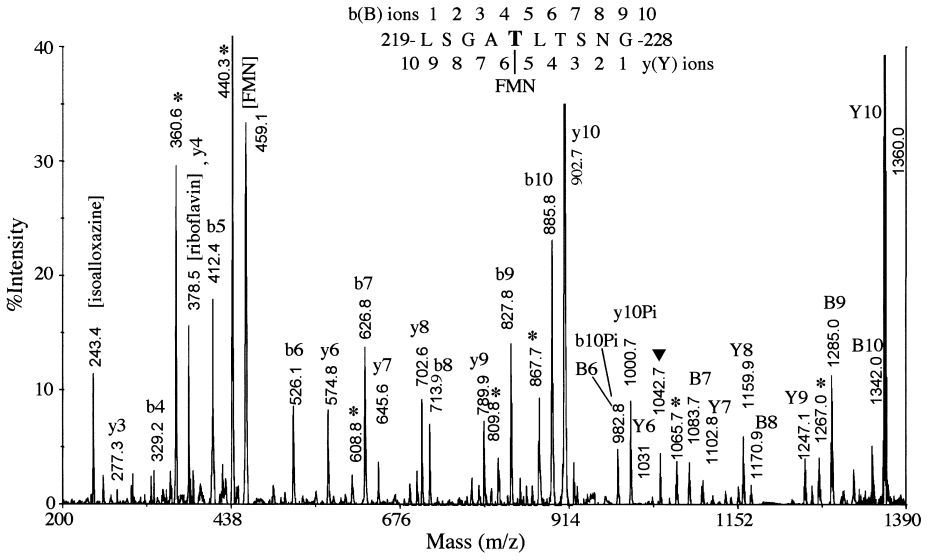

Fig. 3. MALDI-TOF PSD spectrum of region *m/z* 200–1390, showing fragmentation of the peptide mass 1360.0 from NqrC. The symbols used for the expression of the ion type are the same as described in Fig. 2, except that inorganic phosphate is attached to Thr-223 in the NqrC.

peptide without FMN is calculated to be 1063.51, which coincided with the observed mass (1063.5).

With respect to the other peaks, each observed mass was compared with the theoretical mass, which was calculated according to the ion type of the predicted structure in the inset of Fig. 2. Since the core peptide devoid of FMN was also fragmented, the fragment ions without FMN are shown in lower case letters (b, y), and the FMN-containing fragments by capital letters (B, Y). Almost all of the fragments could be assigned to those derived from the predicted structure. The fragments corresponding to b2–b12, B10–B12, y3–y12 and Y4–Y12 were detected. Of special interest, fragments containing the phosphate group at Thr-235, such as b12Pi and y12Pi, were detected. This means that the binding of FMN to the threonine residue by a hydroxyl group of ribityl residue is unlikely and that FMN is indeed attached to Thr-235 via a phosphate group. These results confirmed the peptide sequence and the site of FMN binding in the inset of Fig. 2.

Fig. 3 is the PSD spectrum of region *m/z* 200–1390, showing fragmentation of the fluorescent peptide from NqrC. The mass difference between the two large peaks, 1360 and 902.7, corresponds to the molecular mass of FMN. As described above, the mass value of Thr-223 devoid of FMN is 83.0. Almost all of the fragments could be assigned as those derived from the predicted structure in the inset of Fig. 3. Fragments corresponding to b4–b10, B5–B10, y3, y4, y6–y10 and Y6–Y10 were detected, and fragments containing the phosphate group at Thr-223, such as b10Pi and y10Pi, were also detected. These results confirmed the peptide sequence and the site of FMN binding in the inset of Fig. 3.

As expected, FMN and its fragments, such as the ionized species of FMN, FMN–H_2O, riboflavin, riboflavin–H_2O and isoalloxazine, could be detected in both Figs. 2 and 3. These results further support both NqrB and NqrC having FMN as a flavin cofactor.

With respect to the peaks with masses 1203.7 in Fig. 2 and 1042.7 in Fig. 3, the mass difference from each precursor molecule were both found to have the same value of 317. This means that the unidentified peaks in Figs. 2 and 3 were derived from the precursor molecules by the loss of the same size of 317. The common structure in the precursor molecules from NqrB and NqrC is FMN. If FMN were cleaved between the 3′ and 4′ carbons of the ribityl residue, the precursor molecule would lose mass 315. Thus, the unidentified peaks in Figs. 2 and 3 may be fragments containing part of ribityl residue and the phosphate group of the precursor molecule.

4. Discussion

The results of MALDI-TOF mass spectrometry of the fluorescent peptides derived from NqrB and NqrC clearly dem-

Table 1
Homology search of amino acid residue around the FMN-linked threonine of the NqrB and NqrC subunits

Amino acid residue from *V. alginolyticus* NqrB:	D	G	F	S	G	A	T[a]	A	L	S
Number of species having identical residue	14	14	10	13	14	14	14	10	14	11
Number of species having different residue	0	0	4(Y)	1(A)	0	0	0	3(P), 1(S)	0	2(A), 1(G)

Amino acid residue from *V. alginolyticus* NqrC:	D	G	L	S	G	A	T[a]	L	T	S
Number of species having identical residue	14	9	12	13	14	12	12	14	14	12
Number of species having different residue	0	5(A)	2(I)	1(A)	0	1(S), 1(G)	2(S)	0	0	2(G)

Amino acid residues around the FMN-linked threonine of NqrB and NqrC were compared among 14 species of bacteria known to have an *nqr* operon. Symbols in the parentheses denote amino acid residues. Sequence data were obtained from the Institute for Genomic Research Website (http://www.tiger.org).
[a]FMN-linked threonine.

onstrate that the flavin cofactor linked to both subunits is FMN (Fig. 1). The PSD fragmentation analysis also confirms the peptide sequence and localizes the extra FMN mass units to the specified threonine residue, that is, Thr-235 in NqrB and Thr-223 in NqrC. Of special interest, the FMN is attached to these threonine residues by phosphoester bonds. This type of flavin binding to a peptide has not been previously reported, and is therefore a new kind of flavinylation.

The amino acid sequence around the FMN-linked threonine residue is well conserved between NqrB and NqrC. Recently, Na^+-translocating NQR's were found to be widely distributed in pathogenic bacteria [11]; therefore, the amino acid residues around the FMN-linked threonine among 14 species of bacteria known to have *nqr* operons were compared. As shown in Table 1, the sequence around the FMN-linked threonine in NqrB and NqrC was well conserved throughout the 14 species examined. Thus, the conserved sequence is likely to be a motif surrounding the FMN binding site. The FMN-linked threonine residue in NqrC is replaced by a serine residue in two species, *Neisseria gonorrhoeae* and *Neisseria meningitidis*. These species are possible to use serine residue as a binding site of FMN in the NqrC.

The Na^+-translocating NQR complex reduces ubiquinone to ubiquinol by two successive reactions [7,8]. The first reaction is catalyzed by NqrF, which contains non-covalently bound FAD and reduces ubiquinone by a one-electron transfer pathway to produce ubisemiquinone radical. This reaction does not require Na^+ for activity. The second reaction is catalyzed by the other subunits (NqrA to NqrE) to produce ubiquinol. This reaction is tightly coupled to the Na^+ pumping activity and specifically requires Na^+ for activity [8]. The bound FMN in NqrB and NqrC probably participate in both the electron transfer reactions and the Na^+ pumping activity catalyzed by the NQR complex. Elucidating the electron transfer pathway between the two FMN cofactors may help understand the latter activity.

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