Biochimica et Biophysica Acta 1817 (2012) 1817–1822

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbabio

The single NqrB and NqrC subunits in the Na^+-translocating NADH: Quinone oxidoreductase (Na^+-NQR) from *Vibrio cholerae* each carry one covalently attached FMN☆

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ARTICLE INFO

Article history:
Received 28 October 2011
Received in revised form 3 February 2012
Accepted 9 February 2012
Available online 16 February 2012

Keywords:
Covalent flavin cofactor
Protein modification
Respiratory sodium pump
NADH dehydrogenase
Na^+ transport

ABSTRACT

The Na^+-translocating NADH:quinone oxidoreductase (Na^+-NQR) is the prototype of a novel class of flavoproteins carrying a riboflavin phosphate bound to serine or threonine by a phosphodiester bond to the ribityl side chain. This membrane-bound, respiratory complex also contains one non-covalently bound FAD, one non-covalently bound riboflavin, ubiquinone-8 and a [2Fe–2S] cluster. Here, we report the quantitative analysis of the full set of flavin cofactors in the Na^+-NQR and characterize the mode of linkage of the riboflavin phosphate to the membrane-bound NqrB and NqrC subunits. Release of the flavin by β-elimination and analysis of the cofactor demonstrates that the phosphate group is attached at the 5'-position of the ribityl as in authentic FMN and that the Na^+-NQR contains approximately 1.7 mol covalently bound FMN per mol non-covalently bound FAD. Therefore, each of the single NqrB and NqrC subunits in the Na^+-NQR carries a single FMN. Elimination of the phosphodiester bond yields a dehydro-2-aminobutyrate residue, which is modified with β-mercaptoethanol by Michael addition. Proteolytic digestion followed by mass determination of peptide fragments reveals exclusive modification of threonine residues, which carry FMN in the native enzyme. The described reactions allow quantification and localization of the covalently attached FMNs in the Na^+-NQR and in related proteins belonging to the *Rhodobacter* nitrogen fixation (RNF) family of enzymes. This article is part of a Special Issue entitled: 17th European Bioenergetics Conference (EBEC 2012).

1. Introduction

Flavoproteins are a diverse class of redox proteins involved in many biological processes [1]. Typically, their flavin coenzymes are non-covalently bound, but a subset of proteins is known, which comprise a covalent linkage of the flavin moiety to an amino acid side chain [2]. Based on the type of linkage, three classes of covalent flavoproteins have been identified: (i) FAD or FMN are linked at the 8α-methyl group to a Tyr, His or Cys residue; (ii) FMN is linked at the C6 atom of the isoalloxazine ring to a Cys residue; (iii) riboflavin phosphate forms a phosphodiester with a Thr or Ser residue. The latter mode of flavin modification is found in the Na^+ -translocating NADH:quinone oxidoreductase (Na^+-NQR), a redox driven ion pump present in many pathogenic and non-pathogenic bacteria, which represents a functional equivalent of respiratory Complex I. The Na^+-NQR transports Na^+ ions upon the oxidation of NADH by ubiquinone (Q). Its six subunits (NqrA-F) are not related to subunits of complex I and harbor a different set of cofactors: a non-covalently bound FAD and a 2Fe–2S cluster in the peripheral NqrF subunit, riboflavin phosphate, which is covalently bound to the peripheral NqrC subunit, another covalently bound riboflavin phosphate plus a non-covalently bound riboflavin found in the membranous NqrB subunit, and ubiquinone-8 in the peripheral NqrA subunit [3–9].

MS analysis of proteolytic digests of Na^+-NQR from *Vibrio alginolyticus* showed attachment of riboflavin phosphate to Thr residues of subunits NqrB and NqrC *via* a phosphodiester bond to the ribityl chain [5]. In *Vibrio sp.*, the modified Thr residue is part of a conserved Thr-Gly-Ala-Thr motif. Other proteins harboring this motif were shown to contain covalently bound flavin, such as RnfG and RnfD from *Vibrio cholerae*, two subunits of the Na^+-NQR related *Rhodobacter* nitrogen fixation (RNF) complex [10].

Abbreviations: Na^+-NQR, Na^+-translocating NADH:quinone oxidoreductase; RNF complex, *Rhodobacter* nitrogen fixation complex; Q, ubiquinone; DDM, *n*-dodecyl-β-D-maltoside; SVPD, snake venom phosphodiesterase; β-ME, β-mercaptoethanol

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0005-2728/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbabio.2012.02.012

Notably, in subunit RnfD, which is related to NqrB, the Thr residue within the conserved sequence stretch (T-G-A-T) was not modified [10], indicating that the attachment site for FMN in NQR and its homologs cannot be predicted from this amino acid sequence. Here we show that each of the single NqrB and NqrC subunit of the Na^+-NQR from *V. cholerae* carry one covalently attached riboflavin phosphate bound via a phosphodiester between Thr and the 5'-position of the ribityl side chain as in authentic FMN. The experimental strategies described herein are applicable for all members of the growing families of the NQR and the related RNF complexes, which play a prominent role in energy conservation in many bacteria.

2. Material and methods

2.1. Analytical methods

NADH, FAD, FMN and FMN were purchased from Sigma-Aldrich. Riboflavin was obtained from Fluka. FMN (4 mM in 50 mM Na^+-acetate buffer, pH 5.0) was further purified by HPLC on an EC 250/4 Nucleosil 120–5 C18 column [3,11]. Fractions collected from 27.8 to 29.0 min contained FMN. Fractions collected from 21.4 to 27.6 min contained FMN (riboflavin 5'-monophosphate) and riboflavin 4'-monophosphate at a ratio of approximately 1:1. The structures of the flavins are shown in Fig. 1. Protein was determined by the bicinchoninic acid method using the reagent from Pierce [12]. BSA (fraction V; from Applichem) served as standard.

2.2. Purification of Na^+-NQR

Na^+-NQR from *V. cholerae* was produced and purified as a recombinant protein containing a His_6-tag at the N-terminus of subunit NqrA [3]. Briefly, His_6-tagged Na^+-NQR was expressed in *V. cholerae*, solubilized with *n*-dodecyl-β-D-maltoside (DDM) and purified via nickel affinity chromatography. The enzyme was concentrated to 1–4 mg ml^{-1} in buffer containing 50 mM sodium phosphate, pH 8.0, 5% glycerol, 300 mM NaCl and 0.05% DDM and further purified by gel filtration [3].

2.3. Cleavage of FMN with snake venom phosphatase

Snake venom phosphodiesterase I (SVPD) type IV from *Crotalus atrox* venom was purchased from Sigma-Aldrich. Fifty microliters of Na^+-NQR (2.5 mg ml^{-1}) were mixed with 0–0.8% SDS (final concentration) using a 10% (w/v) SDS solution and incubated in the dark for 30 min under gentle shaking at 25 °C. By adding 2 ml buffer A (10 mM HEPES•NaOH, pH 8.0, 300 mM NaCl, 0.05% DDM, 5% glycerol) the concentration of SDS was reduced to less than 0.02% to maintain the enzymatic activity of SVPD [13]. Na^+-NQR was concentrated again to 0.1 ml using centrifugal filter devices (Ultrafree-MC, 5 kDa cut-off, Millipore). Two aliquots of 40 µl were withdrawn. One aliquot was mixed with 2 µl SVPD solution (3 units ml^{-1}), the other served as control. Both aliquots were incubated for 10 min at 25 °C, followed by analysis on SDS-PAGE. SDS-PAGE was performed with 10% polyacrylamide gels in the presence of 6 M urea [14,15]. The Na^+-NQR was allowed to react with sample buffer (50 mM Tris•HCl, pH 6.8, 5% SDS, 5.8% glycerol, and 0.1 M β-mercaptoethanol) for 30 min at room temperature to prevent precipitation of the hydrophobic NqrB, NqrD and NqrE subunits [15]. Thirty micrograms of protein were loaded per lane. Gels were analyzed for flavins with a fluorescence scanner (excitation, 457 nm; emission, 526 nm; Typhoon 9400 scanner, Molecular Dynamics). Proteins were stained with Coomassie brilliant blue G-250.

2.4. Quantification of FMN released from denatured Na^+-NQR under alkaline conditions

To determine the content of covalently bound FMN in the Na^+-NQR, it was necessary to remove the non-covalently bound flavins. Briefly, protein from a concentrated solution (1–4 mg ml^{-1}) was precipitated with 6.25% TCA and removed by centrifugation [3]. The supernatant containing the non-covalently bound flavins was neutralized by adding 0.8 M K_2HPO_4 and passed through PVDF filter (0.22 µm; Millipore) prior to analysis by HPLC. The protein pellet obtained by the initial TCA precipitation was further used to extract covalently bound FMN. The pellet was washed repeatedly with 6.25% TCA (0 °C), until the supernatants were free of non-covalently bound flavins as confirmed by HPLC.

To release the FMN, ice-cold LiOH (0.5 M, 0.1 ml) was added to the pellet, which was resuspended by vortexing and stored on ice for 24 h. At this step, protection of flavins from light was crucial to prevent their photolysis. Fifty microliters 30% TCA (0 °C) were added, shifting the pH to the acidic range and precipitating proteins. After an incubation of 5 min at 0 °C, the suspension was cleared by centrifugation (5 min, 15,800 × *g*). The supernatant containing the flavins released from subunits NqrB and NqrC was neutralized by adding 0.8 M K_2HPO_4 and passed through a PVDF filter (0.22 µm; Millipore) prior to analysis by HPLC [3]. Standard solutions for quantification and identification of flavins by HPLC contained 120 pmol FAD, 37 pmol FMN and 56 pmol riboflavin. Flavin concentrations were determined photometrically using the following extinction coefficients at 450 nm:

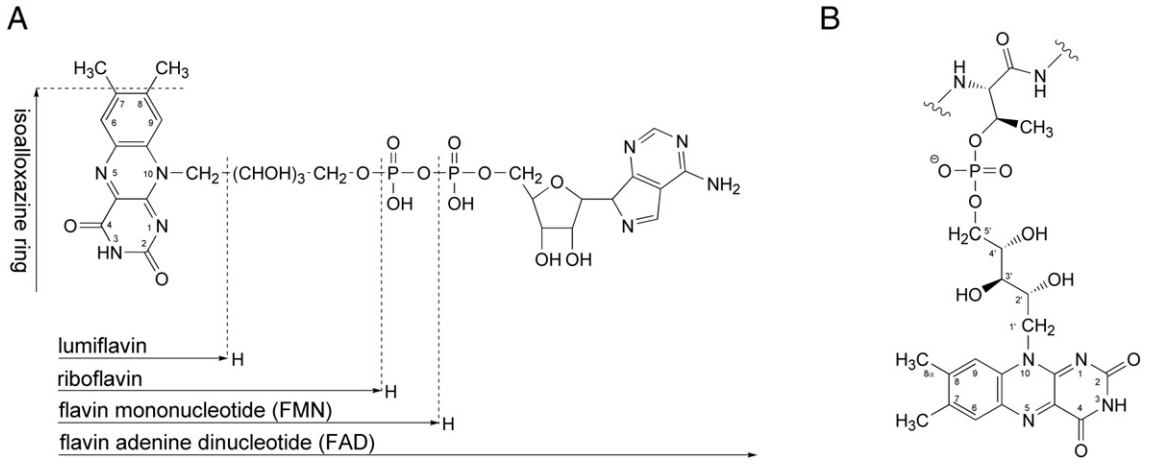

Fig. 1. Structure of common flavins and phosphodiester-linked FMN. Nomenclature of flavins (A) and structure of the phosphodiester-bound FMN (B), which is linked between the 5'-hydroxyl group of the ribityl chain and the hydroxyl group of a Thr side chain to NqrB and NqrC subunits of the Na^+-NQR.