Acidianus ambivalens type-II NADH dehydrogenase: genetic characterisation and identification of the flavin moiety as FMN

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Abstract The thermoacidophilic archaeon *Acidianus ambivalens* contains a monomeric 47 kDa type-II NADH dehydrogenase (NDH), which contains a covalently bound flavin. In this work, by a combination of several methods, namely ^{31}P-nuclear magnetic resonance and fluorescence spectroscopies, it is proven that this enzyme contains covalent FMN, a novelty among this family of enzymes, which were so far thought to mainly have the flavin dinucleotide form. Discrimination between several possible covalent flavin linkages was achieved by spectral and fluorescence experiments, which identified an 8α-*N*(1)-histidylflavin-type of linkage. Analysis of the gene-deduced amino acid sequence of type-II NDH showed no transmembranar helices and allowed the definition of putative dinucleotide and quinone binding motifs. Further, it is suggested that membrane anchoring can be achieved via amphipatic helices.

Key words: Type II NADH dehydrogenase; Archaea; Sulfolobales; FMN; 31P NMR

1. Introduction

NADH dehydrogenases (NDHs) constitute one of the electron entry points into membrane-bound respiratory chains, oxidising NADH and generating quinol. This class of enzymes is divided into two major subfamilies, which can be discriminated on the basis of cofactor content and sensitivity towards rotenone: (i) the type-I NDH, or complex I, is a multimeric enzyme, composed of multiple subunits (up to 46 in eukarya), containing iron–sulphur centres and flavin, and (ii) the type-II NDHs (NDH-IIs), which are simple, monomeric, flavin-containing and rotenone-insensitive enzymes [1]. The latter are present in a wide range of organisms (e.g. plants, fungi and bacteria), in some cases together with complex I, and constitute an alternative pathway for donating electrons into the respiratory chain [2]. Functionally, type-II enzymes are unable to translocate protons and are typically constituted by a single ~50 kDa subunit, lacking iron–sulphur clusters and containing one non-covalent FAD cofactor [1].

We have previously demonstrated that the NDH-II from the thermoacidophilic archaeon *Acidianus* (*A.*) *ambivalens* is a functional component of the respiratory chain, having the flavin moiety covalently linked [3]. In this work we investigate and clarify the nature of the flavin and the type of covalent linkage present in this enzyme. Furthermore, the gene encoding for this enzyme was cloned and sequenced, allowing a detailed analysis of its amino acid sequence. By comparison with similar proteins, it was possible to detect conserved motifs for dinucleotide and quinone binding, as well as the probable amino acid involved in the covalent binding.

2. Materials and methods

2.1. Molecular biology procedures

A library of *A. ambivalens* DNA was constructed in the cloning vector pGemT according to the manufacturer's recommendations (Promega, Mannheim, Germany). A total of 10 000 individual clones were sequenced with standard sequencing primers from both sides. The N-terminal amino acid sequence of the pure protein determined using an Applied Biosystems Model 470A sequenator was used as a query in a TFASTA search against the *A. ambivalens* sequence database. A 1347 bp clone was retrieved from the database encoding the NADH oxidase and the promoter region.

2.2. Sequence analysis tools

Searches for similar proteins in databases were performed using the BLAST, TBLASTN, and PSI-BLAST algorithms [4] with the non-redundant GenBank databases nt and nr installed on a local server. The search for secondary and tertiary structure elements was done using the meta-search engine PredictProtein at http://cubic.bioc.columbia.edu/predictprotein/ using all available tools. The search for conserved domains was done using the CD-browser at NCBI. Fold prediction was carried out using the 3DPSSM server (http://www.bmm.icnet.uk/~3dpssm/ [5]). Multiple alignments were performed using PILEUP and CLUSTAL W version 1.6 [6].

2.3. Spectroscopic methods

Ultraviolet/visible absorption spectra of the purified protein were recorded on a spectrophotometer (Shimadzu UV 1603) at room temperature. Fluorescence was measured on a spectrofluorimeter (SimAminco) at room temperature using an excitation wavelength of 445 nm and an emission wavelength of 520 nm. The ^{31}P-nuclear magnetic resonance (NMR) spectrum of *A. ambivalens* type-II NDH in Tris–HCl buffer (20 mM, pH 8.0) containing 10% D_2O was recorded at 202.45 MHz on a Bruker DRX500 spectrometer equipped with a quadrupole nuclei probe head. The spectrum was acquired at 25°C with the following parameters: spectral width, 20 kHz; pulse width 16 μs (70° flip angle); data size, 32K; repetition delay, 1 s; number of transients, 420K.

2.4. Analytical methods

Flavin extraction was attempted by incubating the protein with trichloroacetic acid 70%, as in [7]. A proteolytic digestion was performed on NDH-II using trypsin (Calbiochem; 0.1 mg ml^{-1} at 35°C,

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pH 7.5 for 16 h). The resulting yellowish supernatant obtained after pelleting down the undigested protein by centrifugation (15 min, 14 000 rpm, 4°C) was applied in a reverse-phase column in an attempt to isolate the flavinylated peptide(s). A Waters Delta Pak C18 column (3.9×150 mm) was equilibrated with 0.1% trifluoracetic acid in distilled water (solvent A). Applied sample was eluted with gradients up to 80% acetonitrile in solvent A. Fluorescence detection was used to monitor for flavinylated peptides (excitation: 450 nm, emission: 520 nm). Although several different gradients were tried, peptide mapping of the obtained flavin-containing bands was always unsuccessful. This may be due to a strong aggregation behaviour of the resulting peptides, a situation also observed in other flavoproteins with covalent flavins (e.g. [8]). The trypsin digestion products were used for two different strategies: (i) incubation with phosphodiesterase from *Crotalus atrox* (Sigma; 0.2 mg ml^{-1} at 37°C, pH 7.5 for 3 h) to reveal the nature of the bound flavin upon comparison of the fluorescence properties before and after the esterase reaction [8]. Controls were done in parallel using free FAD and FMN, and measuring their fluorescence differences before and after phosphodiesterase incubation; (ii) incubation in buffered solutions from pH 2.5 to pH 8.1 followed by fluorescence intensity measurements. The flavin peptides buffered at pH 3.4 were incubated for 5 min with $NaBH_4$ crystals and an aliquot of acetic acid was finally added to neutralise the pH, which was readjusted to pH 3.4 [9].

3. Results and discussion

3.1. FMN is the covalently bound flavin in A. ambivalens NDH-II

A. ambivalens NDH-II contains a covalently attached flavin group which remains bound even after treatment of the protein under strong denaturing conditions [3]. Under these circumstances the discrimination between FAD and FMN is not trivial, and in order to recognise which type of flavin is present a series of complementary experiments were performed.

A first approach consisted of evaluating the effect of phosphodiesterase on the emission spectra of a flavinylated peptide. The native protein was incubated with trypsin, the undigested protein was pelleted down by centrifugation, and a yellowish supernatant was obtained, indicating that it contained flavinylated peptides. These were used for fluorimetric identification of the flavin cofactor, a method which exploits the fact that: (i) at identical concentrations, the fluorescence of a FMN solution is 10-fold higher than that of a solution of FAD; and (ii) phosphodiesterase catalyses the conversion of FAD into FMN and adenosine monophosphate (AMP). The fluorescence intensity of the *A. ambivalens* NDH-II flavinylated peptides was measured at 530 nm upon excitation at 450 nm, before and after incubation with phosphodiesterase. No increase of fluorescence was observed, indicating that FAD is not present in the sample and that FMN is likely to be the enzyme cofactor. A positive control experiment performed in the same conditions ruled out any experimental problem. Briefly, this consisted in incubating, in identical conditions and in the same time scale, pure FAD with phosphodiesterase and observing that this led in fact to a fluorescence intensity increase, showing that in the tested conditions the enzyme was fully operational.

A definite confirmation that FMN is the covalently bound flavin was provided by ^{31}P-NMR spectroscopy. This method is a very powerful tool in identifying the type of flavin bound to flavoproteins, and in particular to distinguish between FMN and FAD co-enzymes. The ^{31}P-NMR spectrum of FAD-containing proteins displays two phosphorus resonances upfield-shifted, i.e. exhibiting negative shifts in relation to external 85% phosphoric acid. In contrast, the FMN co-enzyme shows a positive shift, with only one downfield resonance. The ^{31}P-NMR spectrum of *A. ambivalens* NDH-II is shown in (Fig. 1). Only one resonance at 3.2 ppm was observed, thus indicating that FMN is the covalently bound flavin to *A. ambivalens* NDH-II. This spectrum is similar to that of the *S. metallicus* NDH-II obtained in the same experimental conditions and also showing one phosphorus resonance at 3.2

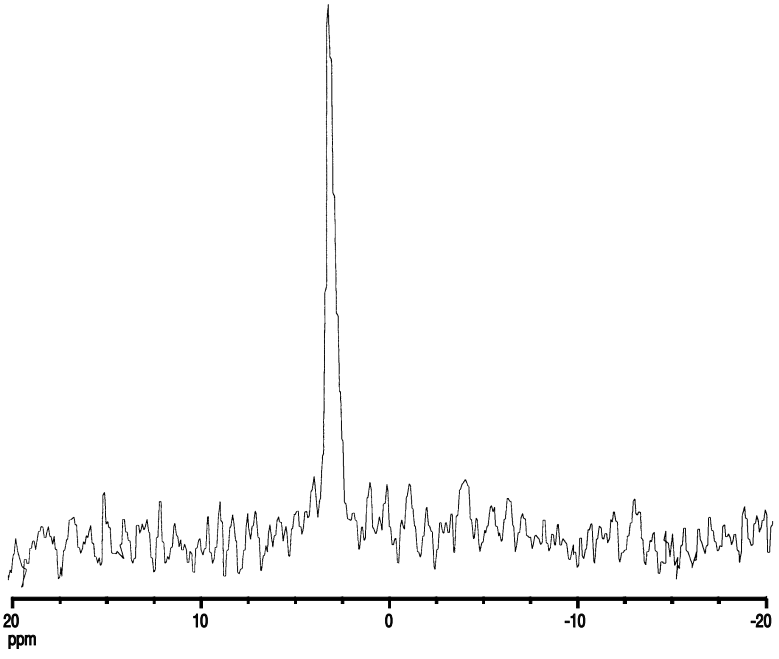
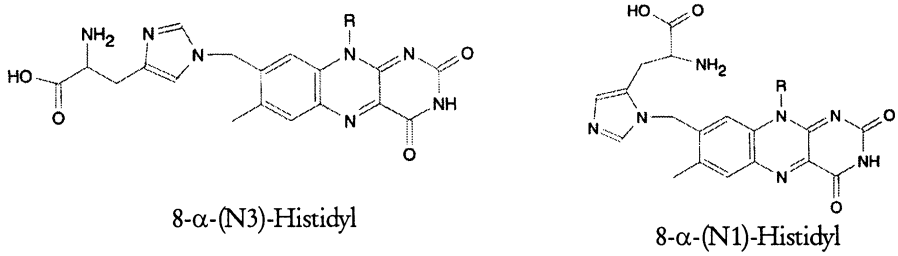


Fig. 1. ^{31}P-NMR spectrum of *A. ambivalens* NDH-II. The spectrum was recorded at pH 8.0 and 25°C and is calibrated in relation to external 85% phosphoric acid ($H_3PO_4^-$).



Scheme 1.

ppm (our own unpublished observations). Due to the similarity of both spectra it can also be concluded that the chemical environment is similar in both NDH-IIs.

3.2. The covalent linkage is of the histidylflavyl type

Among the known cases of flavin covalent attachment involving the amino acids histidine, cysteine, tyrosine and, as recently discovered in the *Vibrio cholerae* NqrC subunit of the NQR respiratory Na^+-pump, threonine [10], the linkage at the flavin is always made either at the C6 position of the isoalloxazine ring or at the 8α methyl group. In the case of histidynilflavins, the association to the amino acid can either be via its N(1) or N(3) position (Scheme 1).

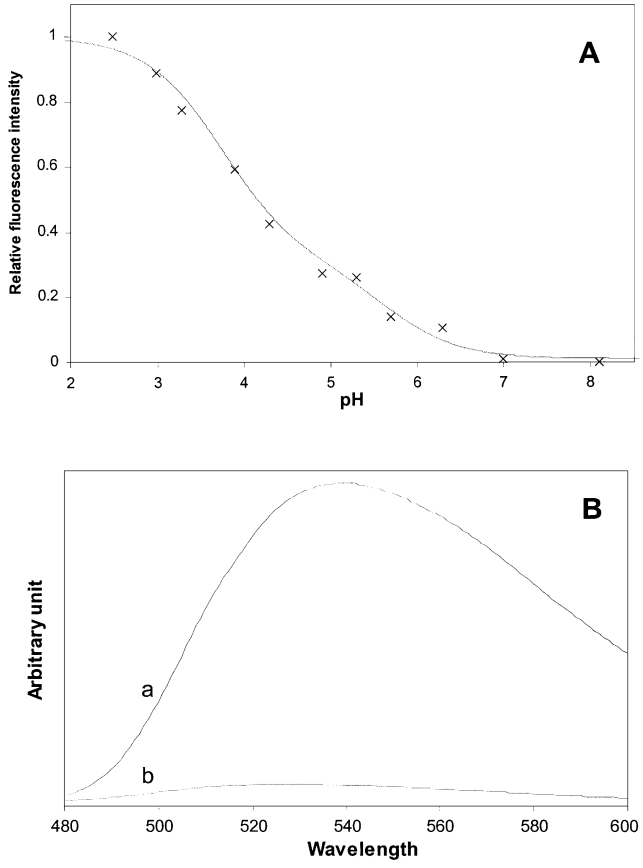


Fig. 2. Effect of pH (A) and borohydride (B) on the flavin peptide emission spectra. A: The fluorescence intensity was monitored between pH 2.5 and 8.1, and shown to increase in this range. The solid line depicts a theoretical titration curve for two protonatable species with pKa of 3.7 and 5.7, indicating the presence of at least one additional acidic amino acid in the flavinylated peptide(s). B: Fluorescence intensity of the flavinylated peptide before (trace a) and after incubation with $NaBH_4$ crystals as described in Section 2 (trace b).

The spectroscopic and chemical properties of the covalently attached flavins are not only affected by the type of linkage but are also modulated by the chemical composition of the peptide to which they are linked. Thus, the study of the spectroscopic properties of a flavinylated peptide provides some indications on the nature of the covalent link.

Unfortunately, all attempts to sequence the isolated flavinylated peptide obtained after digestion of *A. ambivalens* NDH-II with trypsin were unsuccessful, thus preventing a clear identification of the flavin-binding residue. Nevertheless, some information can be obtained from its visible spectrum, which exhibits absorption peaks at 350 and 445 nm. A comparison with the spectroscopic features of aminoacyl flavins shows that the obtained spectral bands are not compatible with the presence of a cysteinyl (maxima at 448 and 367 nm [9]) or tyrosyl flavin (maxima at 446 and 359 nm [9]). On the other hand, the bands of the isolated flavinylated peptide are identical to the ones observed in 8α-*N*-histidylflavins: the near-ultraviolet band is hypsochromically shifted with respect to the corresponding band in riboflavin, from 372 to 345–355 nm, whereas the 445 nm band remains unchanged [9]. This 10 nm shift is reported to be related to the degree of protonation at the unsubstituted *N*(1)-position of the imidazole ring, with the protonated form absorbing at 345 nm and the deprotonated one at 355 nm [9].

Flavins histydil-linked at the 8α position display a characteristic higher fluorescence below pH 7, as in these circumstances the non-bonding electrons from the nitrogen atom are not available to interact with the flavin due to protonation of the nitrogen [11], and can be further distinguished on the basis of their distinct reactivity towards sodium borohydride ($NaBH_4$). The fluorescence pH dependence properties of the *A. ambivalens* flavinylated peptide showed a marked increase of fluorescence from pH 8.1 to pH 2.5 (Fig. 2, panel A, see legend for further details). Complete fluorescence quenching observed upon reaction with sodium borohydride crystals (Fig. 2, panel B) finally confirmed the hypothesis that the flavin linkage is of the 8α-*N*(1)-histidylflavin type, as this feature is exclusive for this type of association [9].

3.3. Analysis of primary sequence and putative membrane attachment

The determination of the N-terminus gave a clear amino acid sequence of 47 positions except for one position (Fig. 3) with no N-terminal methionine present. The search in a database of the randomly sequenced genomic *A. ambivalens* DNA resulted in the identification of a 1347 bp DNA fragment encoding a protein of 408 residues. The calculated molecular mass of the mature protein was 45 020 Da, which is in

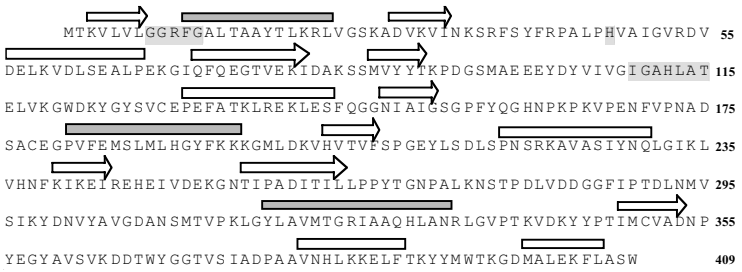


Fig. 3. Amino acid sequence of *A. ambivalens* NDH-II highlighting secondary structure. Arrows denote β sheets and boxes represent α-helices. Dark-shaded boxes show putative amphipatic helices (see Fig. 4). Grey-shaded regions represent putative dinucleotide and quinone binding regions (see text for discussion). The histidine (His-47) suggested to be involved in covalent binding is also indicated.

good agreement with the mass determined by SDS–PAGE [3]. The calculated isoelectric point was 6.8. The N-terminus matched exactly at position 2 of the deduced amino acid sequence of the ORF, showing that only the methionine is cleaved off but no signal sequence is present. The amino acid sequence (accession number: AJ489504) showed 50–80% identical residues to hypothetical proteins from the *Sulfolobus* (*S.*) *solfataricus*, *S. tokodaii*, *Thermoplasma* (*T.*) *acidophilum* and *T. volcanium* genome sequences. The most similar regions among these protein sequences comprise a βαβ fold region responsible for binding to dinucleotide molecules [12], with the glycine-containing conserved sequence ($GXGX_2G$), as well as a putative quinone binding motif LX_2HX_2T (IX_2HX_2T for *A. ambivalens* NDH-II) (Fig. 3) [13]. The fact that this enzyme catalyses the electron transfer to caldariella quinone underlines the relevance of the latter conserved region. Also present in the five sequences is a conserved histidine that naturally becomes a probable candidate for the amino acid responsible for the covalent binding to the flavin cofactor. The closest match to a protein with known function and ranking at the fifth position in the BLAST search was the sulphide quinone oxidoreductase from the cyanobacterium *Oscillatoria limetica* (36% identity). However, the pure *A. ambivalens* NDH-II did not exhibit this activity. A search for distant similarities in the structure database using the SAM T99 site [14] suggest a fold similar to that of NADH peroxidase (e.g. 1npx), dihydrolipoamide dehydrogenase (e.g. 1dxl), and flavocytochrome C sulphide dehydrogenase (1fcd). The pairwise identities of these proteins to the *A. ambivalens* NADH oxidase were below 30%.

Most secondary structure prediction programmes including TMHMM and Kyte–Dolittle hydrophobicity plots did not reveal any transmembranar helices. Only the Dense Alignment Surface method ([15]) resulted in the prediction of four putative helices, of which only one had a sufficient length (positions 106–113). The identified helices were analysed with respect to the likeliness of having an amphipatic character, which could account for membrane anchoring. This feature has been observed in several other membrane-bound proteins, which also lack transmembrane helices, such as prostaglandin synthase, RGSs, CTP:phosphocoline cytidyltransferase and succinate dehydrogenase [16–20]. In fact, in *A. ambivalens*

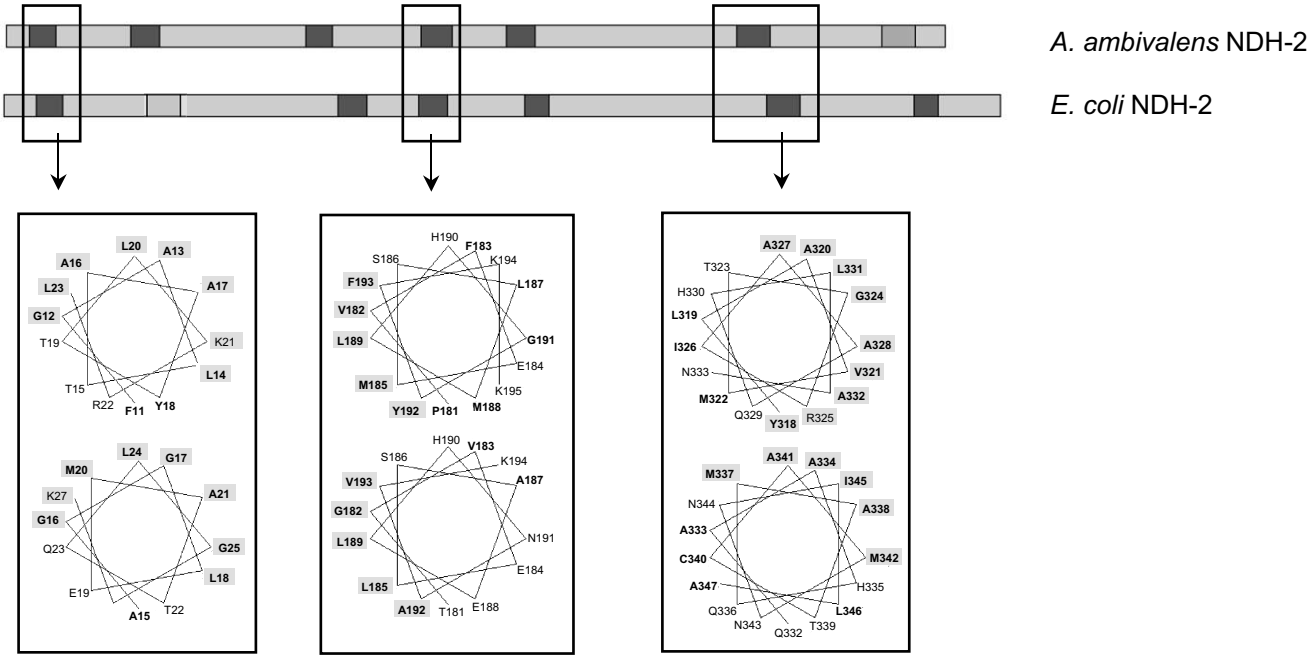


Fig. 4. Putative amphipatic helices in *A. ambivalens* and *E. coli* NDH-IIs. Grey boxes represent the region with higher density of hydrophobic residues.

NDH-II three of the putative α-helices could be amphipatic and it is noteworthy that a similar analysis performed on the *Escherichia coli* NDH-II gives identical results (Fig. 4), suggesting that membrane association via amphipatic helices may be a common feature in this family of enzymes. It is remarkable that the mode of interaction with the membranes remains undisclosed in NDH-IIs, most of which lack transmembranar helices. In this sense, the present working hypothesis constitutes a contribution for designing future experiments destined to clarify this issue.

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