

# The respiratory chain of the thermophilic archaeon *Sulfolobus metallicus*: studies on the type-II NADH dehydrogenase

Tiago M. Bandejas<sup>a</sup>, Carlos A. Salgueiro<sup>a,b</sup>, Harald Huber<sup>c</sup>,  
Cláudio M. Gomes<sup>a,b</sup>, Miguel Teixeira<sup>a,\*</sup>

<sup>a</sup>Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Rua da Quinta Grande 6, 2780-156 Oeiras, Portugal

<sup>b</sup>Departamento de Química, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, 2825-114 Caparica, Portugal

<sup>c</sup>Lehrstuhl für Mikrobiologie der Universität Regensburg, 93053 Regensburg, Germany

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## Abstract

The membranes of the thermoacidophilic archaeon *Sulfolobus metallicus* exhibit an oxygen consumption activity of  $0.5 \text{ nmol O}_2 \text{ min}^{-1} \text{ mg}^{-1}$ , which is insensitive to rotenone, suggesting the presence of a type-II NADH dehydrogenase. Following this observation, the enzyme was purified from solubilised membranes and characterised. The pure protein is a monomer with an apparent molecular mass of 49 kDa, having a high N-terminal amino acid sequence similarity towards other prokaryotic enzymes of the same type. It contains a covalently attached flavin, which was identified as being FMN by  $^{31}\text{P}$ -NMR spectroscopy, a novelty among type-II NADH dehydrogenases. Metal analysis showed the absence of iron, indicating that no FeS clusters are present in the protein. The average reduction potential of the FMN group was determined to be +160 mV, at 25 °C and pH 6.5, by redox titrations monitored by visible spectroscopy. Catalytically, the enzyme is a NADH:quinone oxidoreductase, as it is capable of transferring electrons from NADH to several quinones, including ubiquinone-1, ubiquinone-2 and caldariella quinone. Maximal turnover rates of  $195 \text{ } \mu\text{mol NADH oxidized min}^{-1} \text{ mg}^{-1}$  at 60 °C were obtained using ubiquinone-2 as electron acceptor, after enzyme dilution and incubation with phospholipids.

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## 1. Introduction

Thermophiles constitute excellent working models for the investigation of alternative metabolic strategies operating among prokaryotes. One reason for this is the fact that these organisms branch at a deep position in the tree of life, and are thus only distantly related to the most intensively studied prokaryotes, typically proteobacteria like *Escherichia coli* or *Paracoccus denitrificans*. Furthermore, the high temperatures at which thermophiles thrive have certainly led to specific strategies to cope with these conditions, both at the level of the intrinsic protein and cellular structures stability, and at the level of distinct metabolic routes.

In the last few years, significant progress has been made towards our understanding of membrane-bound respiratory

chains from thermophiles, and as a consequence, several new features have emerged. The moderate thermophile bacterium *Rhodothermus marinus* is one good example, as it was found that this organism contains a novel protein complex which functionally substitutes the mitochondrial-like  $bc_1$  complex, having quinol:HiPIP oxidoreductase activity, which is also the first example of a physiological electron donor for this type of iron sulfur protein [1]. Further, this organism contains a proton pumping  $caa_3$  heme copper oxidase which is in fact a HiPIP:oxygen oxidoreductase [2], until then unknown catalytic activity. Among archaea, the best examples arise from the acidophilic hyperthermophiles *Acidianus (A.) ambivalens* and *Sulfolobus (S.) acidocaldarius*. The former expresses the simplest aerobic respiratory chain known so far, composed of a type-II NADH dehydrogenase [3], a succinate:quinone oxidoreductase [4,5], a pool of caldariella quinone and a single terminal quinol oxidase of the  $aa_3$  type [6,7]. Several of these proteins have unique structural features, like the

\* Corresponding author. Tel.: +351-2144-69844; fax: +351-2144-28766.

E-mail address: [miguel@itqb.unl.pt](mailto:miguel@itqb.unl.pt) (M. Teixeira).

absence of the trinuclear cluster on the succinate:caldariella quinone oxidoreductase, or alternative proton conducting pathways in the terminal oxidase [5,8,9]. On the other hand, although closely related, *S. acidocaldarius* expresses a much more intricate respiratory chain, especially on its terminal part, as it contains two distinct terminal oxidase complexes, the so-called SoxABCD and SoxM complexes [10,11]. The latter has the particular feature of congregating a cytochrome *bc*<sub>1</sub>-like putative di-heme-containing subunit [12], which together with the presence of Rieske-type proteins, suggests the fusion of structural elements from two distinct mitochondrial-like respiratory complexes into a single one.

These findings have led to new insights into basic aspects of energy transducing complexes by prokaryotes. Hence, it is challenging to further study the respiratory systems of other thermophilic organisms. *Sulfolobus* (*S.*) *metallicus* is a member of the archaeal order of the *Sulfolobales*, being a strict aerobe and an obligate chemolithoautotroph growing between 50 and 75 °C ( $T_{\text{opt}}=65$ ), at pH 1.0–4.5 ( $\text{pH}_{\text{opt}} < 4.5$ ) [13]. A spectroscopic analysis of its respiratory chain performed on intact membranes revealed the presence of a novel type of iron sulfur cluster, and indicated the presence of several respiratory enzymes [14]. In this article, we further characterise the respiratory chain of *S. metallicus*, by reporting our studies on the first respiratory protein isolated from this organism, a type-II NADH dehydrogenase with unique features: a covalently bound flavin mononucleotide, instead of the commonly encountered non-covalent flavin dinucleotide present in this enzyme family.

## 2. Experimental procedures

### 2.1. Cell growth, solubilised membrane preparation and protein isolation

Cells of *S. metallicus* were grown in 300 l fermentors (HTE, Bioengineering, Wald, Switzerland), using culture media and conditions as previously described [15]. Cells were suspended in 50 mM potassium phosphate buffer, pH 6.5 and broken at 8000 kPa in an SLM Aminco KINO20 French press. Unbroken cells were removed by centrifugation at 10,000 rpm for 20 min. The cell extract was ultracentrifuged for 15 h at  $138,000 \times g$  and 4 °C. The resulting pellet was the membrane fraction which was solubilised with dodecyl maltoside (DM), in the proportion of 1 g detergent per gram of protein, the resulting suspension being ultracentrifuged at  $138,000 \times g$  for 6 h at 4 °C. All subsequent chromatographic steps were carried out on a Pharmacia HiLoad™ system, at 4 °C, and NADH oxidase activity was monitored following the NADH-driven ferricyanide reduction (see details below). The membrane-solubilised extract was applied to a 600 ml DEAE-Sepharose Fast flow column. The fraction, which eluted at 0 mM NaCl,

was applied directly to a 90 ml HTP column, at 2 ml/min, which had been previously equilibrated with 40 mM potassium phosphate pH 6.5, 0.1% DM. An elution gradient of seven column volumes up to 1 M potassium phosphate pH 6.5, 0.1% DM was applied and the enzyme fraction eluted at around 1 M potassium phosphate. This fraction was diluted 10 times, and concentrated by ultrafiltration over a 10 kDa cut-off membrane, and once again loaded into a second HTP column, equilibrated and eluted with the same buffer system used previously. The NADH dehydrogenase fraction eluted at ~ 500 mM potassium phosphate. The purified enzyme was divided in aliquots and stored at – 70 °C. The fraction of the enzyme used for <sup>31</sup>P-NMR spectroscopy was prepared doing successive dilution and concentration steps using Tris–HCl buffer (20 mM, pH 8.0), on an Amicon Centriprep 50.

### 2.2. Enzyme assays

Oxygen consumption measurements by *S. metallicus* membranes were performed at 40 °C using a Clark-type oxygen electrode (YSI Model 5300, Yellow Springs). Assays were carried out in a micro-chamber (1300 µl) in 40 mM KPi, pH 6.5, 0.1% DM, using NADH, NADPH, succinate or decyl-quinol (3 mM final concentration) as electron donors. The Complex I inhibitor, rotenone, was tested (1 mM final concentration). NADH dehydrogenase activity was measured spectrophotometrically at 50 °C, following  $\text{K}_3\text{Fe}(\text{CN})_6$  reduction at 420 nm ( $\epsilon_{420 \text{ nm}} = 1.0 \text{ mM}^{-1} \text{ cm}^{-1}$ ). The enzyme was incubated for ~ 5 min in a reaction mixture containing  $\text{K}_3\text{Fe}(\text{CN})_6$  (1 mM) in 40 mM potassium phosphate buffer pH 6.5, 0.1% DM, and the reaction was initiated by the addition of NADH (0.2 mM). In the presence of sonicated lipids, the activity assays followed the protocol as in Refs. [3,16].

### 2.3. Analytical methods

Flavin extraction was attempted by treating purified NADH dehydrogenase with 70% trichloroacetic acid for 10 min at room temperature as in Ref. [17]. Protein concentration was determined using the modified micro-biuret method for membrane proteins and for proteins in the presence of detergent [18]. Determination of the protein N-terminal sequence was performed using an Applied Biosystem Model 470A sequencer, and the Blast network service at NCBI was used for searching the protein databases. Purity of the NADH dehydrogenase was determined by polyacrylamide gel electrophoresis (of 8–16%) in a BioRad instrument as described in Ref. [19]. A Superdex S-200 column (Pharmacia) was used for molecular mass determination by gel filtration. Protein elution was accomplished using 40 mM potassium phosphate, pH 6.5, 150 mM NaCl, 0.1% DM, at 0.75 ml/min. Proteins of different molecular masses were used as standards.

## 2.4. Spectral measurements

Room temperature ultraviolet–visible spectra of purified enzyme in 40 mM potassium phosphate buffer, pH 6.5 were recorded over the wavelength range 250–750 nm in a Shimadzu spectrophotometer. Redox titrations monitored by visible spectroscopy were performed in a Shimadzu diode array spectrophotometer equipped with a cell-stirring system. Protein and caldariella quinone fluorescence measurements were done on a spectrofluorimeter (Sim-Aminco) at room temperature, exciting at 345 nm for the emission spectra and monitoring at 530 nm for the excitation spectra. The samples used contained purified protein in 40 mM phosphate buffer, pH 6.5, with 0.1% DM and purified caldariella quinone solubilised in *n*-hexane.

## 2.5. Redox titrations

The anaerobic titration of *S. metallicus* purified NADH dehydrogenase ( $\sim 16.7 \mu\text{M}$ ) was carried out in an anaerobic cuvette continuously flushed with argon, containing 40 mM potassium phosphate pH 6.5, 0.1% DM, by stepwise addition of buffered sodium dithionite. The redox mediators (0.25  $\mu\text{M}$  each) used were the following: 1,2-naphthoquinone ( $E'_0 = +180 \text{ mV}$ ), trimethylhydroquinone ( $E'_0 = +90 \text{ mV}$ ), 1,4-naphthoquinone ( $E'_0 = +60 \text{ mV}$ ), indigo-tetrasulfonate ( $E'_0 = -30 \text{ mV}$ ), indigo-trisulfonate ( $E'_0 = -70 \text{ mV}$ ), indigo-disulfate ( $E'_0 = -110 \text{ mV}$ ), anthraquinone 2,7 disulfonate ( $E'_0 = -182 \text{ mV}$ ), safranin ( $E'_0 = -280 \text{ mV}$ ), neutral red ( $E'_0 = -325 \text{ mV}$ ), benzyl viologen ( $E'_0 = -359 \text{ mV}$ ), methyl viologen ( $E'_0 = -446 \text{ mV}$ ). Platinum and silver/silver chloride electrodes were used and calibrated against a saturated quinhydrone solution; the reduction potentials are expressed versus the standard hydrogen electrode. Redox titration experimental data was treated and analysed using MATLAB (Mathworks, South Natick, MA, USA) for Windows.

## 2.6. $^{31}\text{P}$ -NMR spectra

The  $^{31}\text{P}$ -NMR spectra were recorded on a Bruker DRX500 spectrometer operating at a frequency of 202.45 MHz and equipped with a quadrupole nuclei probe head. The spectra of pure *S. metallicus* NDH-2 in Tris–HCl buffer (20 mM, pH 8.0) containing 10%  $\text{D}_2\text{O}$  were measured at 25 °C with broadband proton decoupling using a spectral width of 20 kHz, a pulse width of 16  $\mu\text{sec}$  (corresponding to a 70° flip angle), and a 1 s pulse repetition rate. A total of 37,000 transients were acquired. Free induction decays were subject to a 40 Hz exponential line broadening. The spectrum of potassium phosphate 0.1% DM (40 mM, pH 8.0) buffer was acquired in the same experimental conditions but with 512 transients. The chemical shifts are in ppm and relative to external 85% phosphoric acid ( $\text{H}_3\text{PO}_4$ ).

## 3. Results and discussion

### 3.1. Respiratory activities present in *S. metallicus* intact membranes

Experiments performed on a Clark-type oxygen electrode, thermostabilised at 40 °C, showed that *S. metallicus* membranes can be efficiently energised by several respiratory substrates concomitantly with oxygen consumption: succinate (2.2  $\text{nmol O}_2 \text{ min}^{-1} \text{ mg}^{-1}$ ), NADH (0.5  $\text{nmol O}_2 \text{ min}^{-1} \text{ mg}^{-1}$ ), NADPH (0.2  $\text{nmol O}_2 \text{ min}^{-1} \text{ mg}^{-1}$ ) and decylubiquinol (8.5  $\text{nmol O}_2 \text{ min}^{-1} \text{ mg}^{-1}$ ). The effect of rotenone, a classical Complex I inhibitor was tested on the dinucleotide-driven respiratory activities. The membranes were incubated overnight at 4 °C, both in the presence and absence of the inhibitor. This procedure was necessary to assure a full binding of the inhibitor as well as to make sure that the intrinsic reducing power existing in the as-prepared membranes was fully exhausted prior to energisation. Rotenone had no effect on the measured respiratory activities, thus indicating the absence of a type-I NADH dehydrogenase.

### 3.2. Isolation and properties of the membrane-bound type-II NADH dehydrogenase

Having verified that NADH is able to energise *S. metallicus* membranes and leads to oxygen consumption, the isolation of the NADH oxidising enzyme responsible for this respiratory activity was pursued. After a series of chromatographic steps, a pure protein was obtained as confirmed by gel electrophoresis, which showed a strong band upon Coomassie brilliant blue staining and a minor band probably resulting from proteolysis (Table 1, Fig. 1). The final protein yield was  $\sim 22 \text{ mg}$ , obtained from  $\sim 100 \text{ g}$  cells (wet weight). The apparent molecular mass of the purified protein was determined to be 49 kDa from a gradient 8–16% SDS-PAGE (Fig. 1). Gel filtration of the pure protein resulted in a molecular mass of 76 kDa, indicating that the protein is a monomer in solution presenting an expected increase caused by the presence of detergent. Automated sequencing of the protein N-terminus yielded a 32-amino acid-long sequence (Fig. 2), which has a high degree of similarity (12–48% identity and 28–55% similarity) towards several type-II NADH dehydrogenases,

Table 1  
Purification of *S. metallicus* NDH-2

Purification (step)	Protein (mg)	Specific activity ( $\mu\text{mol NADH oxidised min}^{-1} \text{ mg}^{-1}$ )	Enrichment (fold)
DM Extract	1646	16.7	–
Q-Sepharose	1181	18.6	1.1
Ceramic HTP (1)	306	21	1.3
Ceramic HTP (2)	22	58	3.5

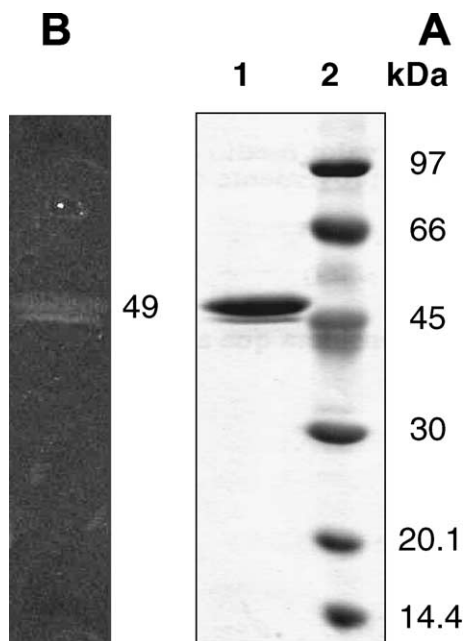


Fig. 1. SDS-PAGE electrophoresis (8–16%) and fluorescence photograph. (A) Lane 1, pure *S. metallicus* NDH-2; lane 2, molecular mass markers. (B) Fluorescent photograph of *S. metallicus* NDH-2 showing the existence of a covalently bound flavin.

namely the NDH-2 from *A. ambivalens* and *E. coli*. The N-terminal also comprises the GXG(X)<sub>2</sub>G motif that has been reported to account for binding of dinucleotide molecules. These enzymes are also referred to as alternative NADH dehydrogenases, lack iron sulfur clusters and typically contain one bound FAD molecule [20,21]. In agreement, the pure *S. metallicus* type-II NADH dehydrogenase (*Sm* NDH-2) contains no iron. However, all attempts to denature the protein and extract the flavin cofactor, by incubation with up to 70% trichloroacetic acid, did not result in cofactor release, indicating its covalent linkage to the protein. This was ultimately demonstrated by fluorescence photography performed on the SDS-PAGE gel; upon excitation at 450 nm, a fluorescent band corresponding to the

pure protein was observed (Fig. 1, panel B), indicating that the flavin remains bound to the polypeptide chain even under denaturing conditions (see also below).

### 3.3. Kinetic properties

The catalytic activity of the purified *S. metallicus* type-II NADH dehydrogenase was routinely measured using the NADH:K<sub>3</sub>Fe(CN)<sub>6</sub> oxidoreductase assay, at 50 °C. The kinetic parameters of the enzyme were determined for these conditions from Lineweaver–Burke plots: the affinity constant ( $K_m$ ) for NADH was found to be 2  $\mu$ M and the maximum velocity ( $V_{max}$ ) 58  $\mu$ mol NADH oxidized min<sup>-1</sup> mg<sup>-1</sup> at 60 °C (Fig. 3). The enzyme was observed to be inactive towards NADPH in the same conditions. Physiologically, the enzyme must operate as a NADH:quinone oxidoreductase, as suggested from the observed NADH-driven oxygen consumption in intact membranes. In fact this is the case, although the enzyme shows a reduced activity upon isolation, a recurrent observation among type-II NADH dehydrogenases [16]. It has been reported for several enzymes of this type that a higher catalytic activity can be obtained by incubation with sonicated phospholipids, which suggests that hydrophobic stabilisation from a lipidic environment is required for proper quinone docking to the protein. In these circumstances, using asolectin (1.2 mg/ml sonicated phospholipids, final concentration), a catalytic rate of 195  $\mu$ mol NADH oxidized min<sup>-1</sup> mg<sup>-1</sup> at 60 °C was obtained, when ubiquinone-2 was used as electron acceptor. This value is within the range of published values for the catalytic activity of other type-II NADH dehydrogenases towards quinones [16,22].

### 3.4. Spectroscopic analysis and identification of bound quinone

The UV–visible spectrum of the as purified type-II NADH dehydrogenase exhibits absorption bands with maxima at 335 and 450 nm (Fig. 4). While the last band is typical of flavoproteins, the characteristic 360 nm flavin band is

		% I/S
<i>S. met</i>	PKVLVLGGRFGGLSAVYTLKR---LVGNKADIKLN	: 32
<i>A. amb</i>	TKVLVLGGRFGALTAAYTLKR---LVGSKADKVIN-KSRFSYRREALPHV	: 46 48/55
<i>Ae. per</i>	VKRRIVILGGSEGGGLQAARYTLD---LLGDKADITVVNESDRIVEKFEALTYL	: 49 28/42
<i>Aq. aeo</i>	MAKHVVIGGGVGGIATAYNIRN---LMPDLKITLISDRPYFGRTAFPHL	: 48 20/32
<i>Syn</i>	ARPRICILGGGFGGLYALRLGQLSWEHGHTPPEIVLVLDQRDRFLFAZFLYEL	: 52 20/30
<i>E. coli</i>	PLKKIVIVGGGAGGLEMATQIGHK-LGRKKKAKITLVDRNHSHLWKEELLHEV	: 51 21/28
<i>Syn</i>	RRPHVIVVGGGFAGLYTAKNL-----RRSPVDITLIDKRNFHLLQELLVQV	: 46 22/30
<i>My. smeg</i>	DRHKVVIIIGSGFGGLTAAKTL-----KRADVVDVKLIARTTHHLLQELLVQV	: 46 26/36
<i>Y. lipo</i>	SKKTLVLGSGGWSVSFLKKL-----DTSNYNVIVVSPRNYFLETLLPSC	: 46 12/30
<i>S. cere</i>	DKPNVLILGSGWGAISFLKHL-----DTKKYNVSIISPRSYFLETLLPSA	: 46 16/32

Fig. 2. Amino acid sequence comparison of *S. metallicus* NDH-2 N-terminus. Comparison of *S. metallicus* NDH-2 N-terminus amino acid sequence towards other NDH-2 N-termini sequences from other organisms (NCBI accession number): *Acidianus* (*A.*) *ambivalens*; *Aeropyrum* (*Ae.*) *pernix* (hypothetical protein, APE0595); *Aquifex* (*Aq.*) *aeolicus* (sulfide quinone, 7521720); *Synechocystis* I and II (S74822, S74826); *Escherichia* (*E.*) *coli* (P00393); *Mycobacterium* (*My.*) *smegmatis* (2708705); *Yarrowia* (*Y.*) *lipolytica* (CAA07265); *Saccharomyces* (*S.*) *cerevisiae* (CAA43787). %I/S—Percentage of identity and similarity, respectively.



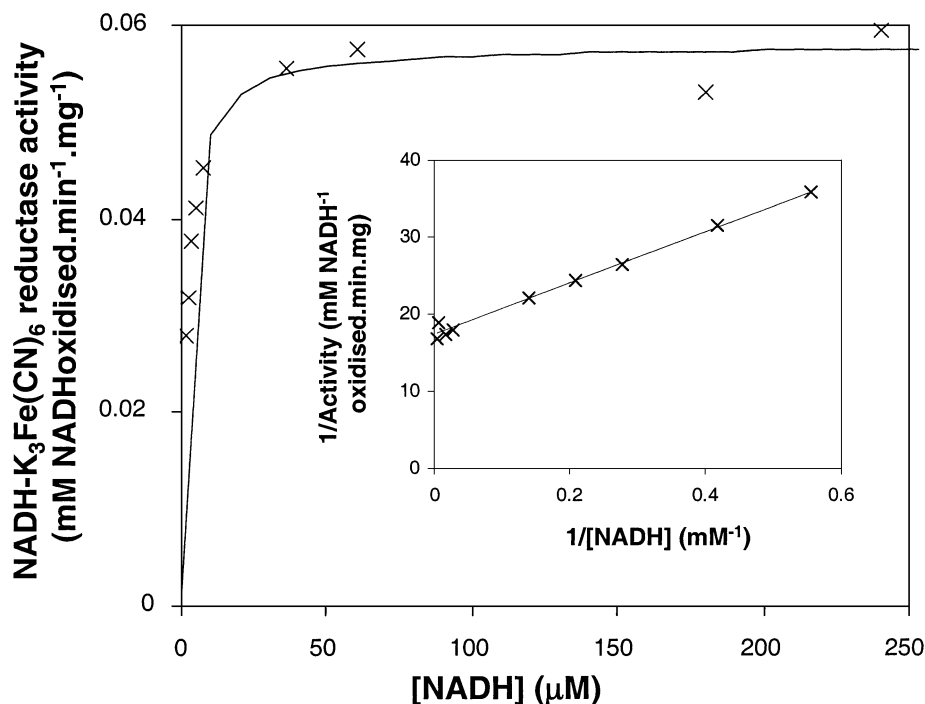


Fig. 3. Kinetic parameters for the NADH:K<sub>3</sub>Fe(CN)<sub>6</sub> reductase activity of NADH dehydrogenase. The (×) sign indicates assays performed on the pure enzyme. The solid line represents a fit according to the Michaelis–Menten equation, with  $K_m = 2 \mu\text{M}$  and  $V_m = 58 \mu\text{mol NADH oxidized min}^{-1} \text{mg}^{-1}$  for the experiments on the pure enzyme.

masked by a more intense feature at  $\sim 335 \text{ nm}$ . This band resembles the one observed on the spectrum of caldariella quinone (CQ), the physiological quinone of the *Sulfolobales*, the archaeal order to which *S. metallicus* belongs. In order to determine if the purified *S. metallicus* NDH-2 contains a bound caldariella quinone molecule, the fluorescence emission spectra of pure *S. metallicus* NDH-2 and CQ were recorded after excitation at  $345 \text{ nm}$  (Fig. 5). The similarity between the two spectra is remarkable, despite the observation of a shift of  $\sim 20 \text{ nm}$  between bands, likely to be due to the fact that pure CQ is in *n*-hexane, an environment distinct

from that of the purified protein. Conclusions on the protein:quinone stoichiometry are impaired by the presence of different solvents on the samples preparation; also, at this stage, we cannot eliminate the possibility that caldariella quinone co-purified with NDH-2.

### 3.5. Redox properties

The redox properties of the flavin cofactor of *S. metallicus* NDH-2 were determined from redox titrations monitored by visible spectroscopy. The decrease in the absorp-

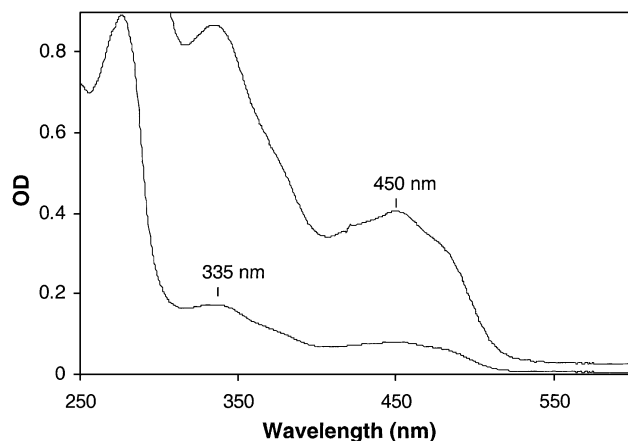


Fig. 4. UV–visible spectrum of *S. metallicus* NDH-2. Spectrum of pure *S. metallicus* NDH-2 ( $7 \mu\text{M}$ ), as prepared. Upper spectrum is magnified five times.

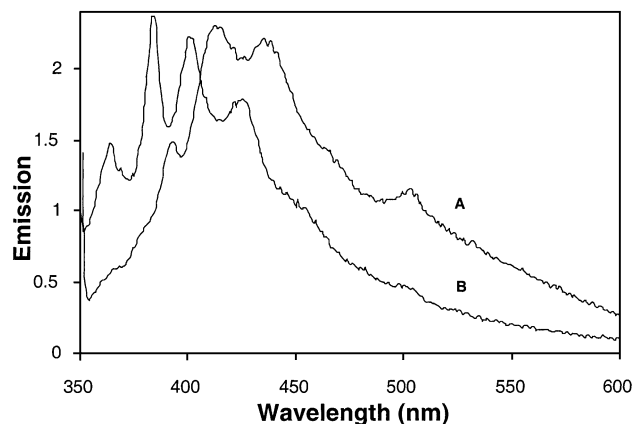


Fig. 5. Fluorescence emission spectra (excitation at  $345 \text{ nm}$ ) of the *S. metallicus* NDH-II (A) and caldariella quinone (B). NDH-2 was in  $40 \text{ mM KPi}$ ,  $\text{pH } 6.5$ , with  $0.1\% \text{ DM}$  and caldariella quinone was in *n*-hexane.

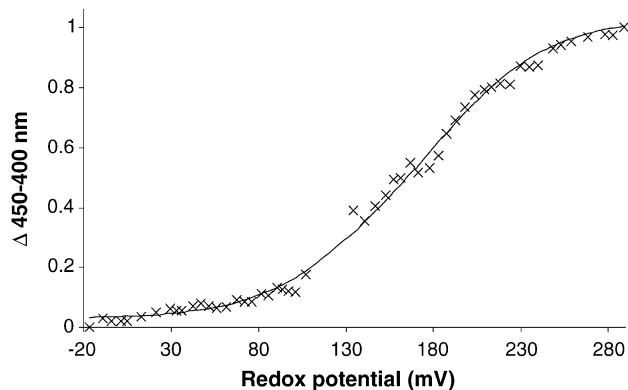


Fig. 6. Redox titration of NADH dehydrogenase. The reduction of *S. metallicus* NDH-2 was followed at 450–400 nm. The solid line corresponds to sequential two-step one-electron reduction, with midpoint reduction potentials of  $195 \pm 15$  mV ( $Fl_{ox}/Fl_{sq}$ ) and  $125 \pm 15$  mV ( $Fl_{sq}/Fl_{red}$ ).

tion intensities at 450–400 nm was plotted against the imposed redox potential (Fig. 6). The reduction process observed in the redox curve occurs via two sequential one-electron reduction steps with reduction potentials of 195 and 125 mV, corresponding to an average reduction potential of 160 mV. This observation does not imply that physiologically the reaction follows the same process, since both the different environment in the archaeal membranes and the interaction with the physiological substrate can significantly change the redox behaviour of the flavin moiety. In particular, although in vitro the reduction potential of the flavin in

*S. metallicus* is higher than that determined, also in vitro, for caldariella quinone, in vivo either reduction potential can be slightly different. Furthermore, the flux of electrons will also depend on the relative concentrations of  $NAD^+/NADH$  and  $CQH_2/CQ$ , which will dictate the actual driving force for the overall electron transfer process. The determined redox potential for the *S. metallicus* NDH-2 is the highest observed for type-II NADH dehydrogenases:  $-370$  mV for *Saccharomyces cerevisiae* NDH-2 [23] and  $\sim 70$  mV for *A. ambivalens* NDH-2 [3], also containing covalently bound flavin. These data suggest a modulation of the redox potential by the flavin covalent bound. In fact, high reduction potentials are common among covalent flavoproteins, which may rise from the chemical modifications of the flavin moiety. For example, by mutating the amino acid bound to the flavin in vanillyl-alcohol oxidase, which did not cause structural changes [24], the reduction potential decreases 120 mV. Nevertheless, the wide range of covalent flavoprotein redox potentials (from  $-101$  to  $84$  mV) [24–27] indicates that the protein environment surrounding the flavin is also strongly influencing its reduction potential, as observed for any other redox group in proteins.

### 3.6. Identification of the flavin type by $^{31}P$ -NMR spectroscopy

The type of flavin bound to *S. metallicus* NDH-2 was identified by  $^{31}P$ -NMR spectroscopy. This technique can be successfully applied in the identification of the type of flavin

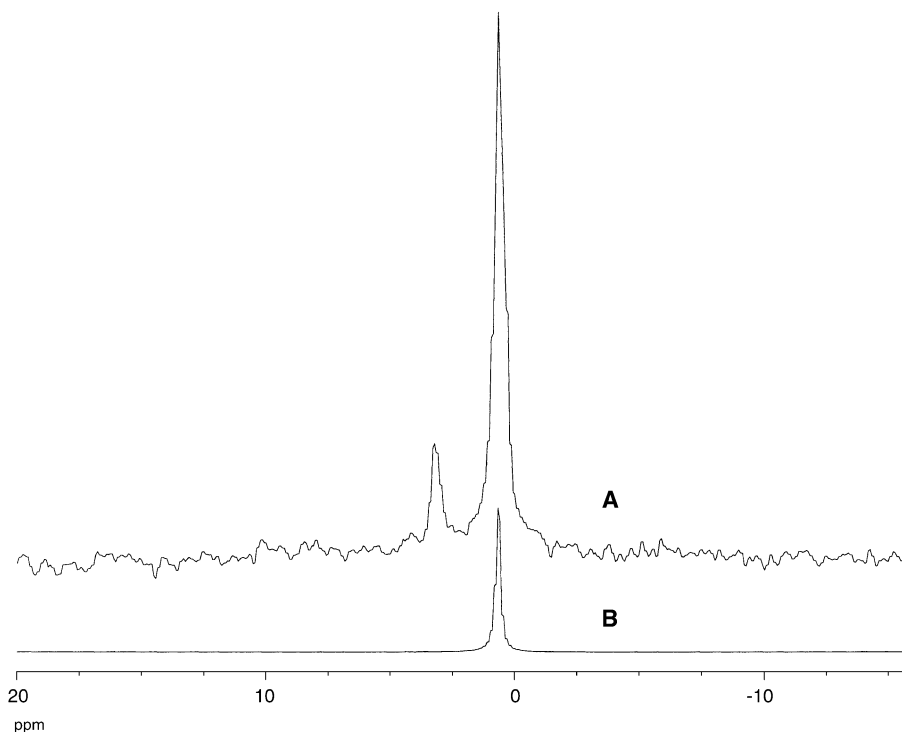


Fig. 7. *S. metallicus* NDH-2 (A) and potassium phosphate buffer (B)  $^{31}P$ -NMR spectra. The peak at 0.62 ppm originates from the phosphate buffer which did not interfere with the *S. metallicus* NDH-2 spectrum.

bound to flavoproteins, either FMN or FAD, since the  $^{31}\text{P}$  chemical shifts and the spectral type of each flavin are quite different. The FMN-containing flavoproteins described in the literature show a single resonance for the dianionic monoesters between 3.7 and 8.8 ppm [28–31], whereas the FAD-containing proteins exhibit two phosphorous resonances in the high field region of the  $^{31}\text{P}$  spectrum with chemical shifts ranging between  $-7.0$  and  $-13.5$  ppm ([28,29] and references therein). These patterns remain unaltered either in the case of covalently or non-covalently bounded flavins. The *S. metallicus* NDH-2  $^{31}\text{P}$ -NMR spectrum shows two resonances: one at 3.2 ppm and another at 0.62 ppm (Fig. 7A). The resonance at 0.62 ppm was assigned to traces of phosphate buffer, by comparison with its spectrum, which shows a single peak at 0.62 ppm (Fig. 7B). The result obtained for *S. metallicus* NDH-2 excludes the possibility of FAD being the bound flavin, and consequently, the signal at 3.2 ppm was attributed to the covalently bound FMN phosphate. The sarcosine oxidase isolated from *Corynebacterium* sp. P-1 [29] was the only flavoprotein with covalently bound FMN so far studied by  $^{31}\text{P}$ -NMR. In the native enzyme, the resonance of the FMN phosphate group appears at 5.7 ppm, and at 2.8 ppm upon complete hydrolysis of the protein. This change in the chemical shift of the FMN moiety may result from the different interactions involving the phosphate group upon denaturation of the protein. Similarly, the difference observed for the FMN signal in *S. metallicus* NDH-2 (3.2 ppm) and that in sarcosine oxidase (5.7 ppm) may originate from different protein binding environments surrounding the FMN phosphate group in both proteins. Although, to our knowledge, there are no evidences for the existence of different flavin cofactors in *Sulfolobales*, this possibility cannot be excluded. Further structural studies will allow to clarify this point.

#### 4. Concluding remarks

In conclusion, the type-II NADH dehydrogenase from the thermoacidophilic archaeon *S. metallicus*, which appears to be the major NADH dehydrogenase in the membranes, has unique features among this family of enzymes: it is the second example in which the flavin group is covalently bound, as previously shown for the homologous enzyme from *A. ambivalens*, and, also a novelty in these enzymes, it contains a flavin mononucleotide instead of a flavin dinucleotide, as clearly proven by  $^{31}\text{P}$ -NMR. Furthermore, the enzyme was purified with one bound quinone, the physiological electron acceptor caldariella quinone.

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