A New Type-II NADH Dehydrogenase from the Archaeon *Acidianus ambivalens:* Characterization and *in vitro* Reconstitution of the Respiratory Chain[1]

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A new type-II NADH dehydrogenase (NDH-II) was isolated from the hyperthermoacidophilic archaeon *Acidianus ambivalens.* This enzyme is a monomer with an apparent molecular mass of 47 kDa, containing a covalently bound flavin, and no iron–sulfur clusters. Upon isolation, NDH-II loses activity, which can, nevertheless, be restored by incubation with phospholipids. Catalytically, it is a proficient NADH:caldariella quinone oxidoreductase (130 mmol NADH oxidized/mg protein^{-1}/min^{-1}) but it can also donate electrons to synthetic quinones, strongly suggesting its involvement in the respiratory chain. The apparent K_m for NADH was found to be ∼6 μM, both for the purified and membrane-integrated enzyme, thus showing that detergent solubilization and purification did not affect the substrate binding site. Further, it is the first example of a type-II NADH dehydrogenase that contains the flavin covalently attached, which may be related to the need to stabilize the otherwise labile cofactor in a thermophilic environment. A fully operative minimal version of *Acidianus ambivalens* respiratory system was successfully reconstituted into artificial liposomes, using three basic components isolated from the organism: the type-II NADH dehydrogenase, caldariella quinone, the organism-specific quinone, and the aa_3 type quinol oxidase. This system, which mimics the *in vivo* chain, is efficiently energized by NADH, driving oxygen consumption by means of the terminal oxidase.

KEY WORDS: NADH dehydrogenase; archaea; thermophile; respiration.

INTRODUCTION

In aerobic membrane-associated respiratory chains, there are two types of NADH:quinone oxidoreductases: complex I, or NDH-I, is a multisubunit, proton-translocating enzyme that contains one noncovalently bound FMN and 6–9 iron–sulfur clusters. The other type of NADH dehydrogenase, NDH-II, does not contain any iron–sulfur clusters and has noncovalently bound FAD as cofactor (see Kerscher, 2000; Yagi *et al.*, 1998; Finel, 1998; Ohnishi, 1998; Friedrich *et al.*, 1998; Yagi, 1993).

The latter is a simpler enzyme, which does not pump protons across the membrane. Several organisms have a combination of the two dehydrogenases (*e.g., Escherichia coli, Yarrowia lipolytica,* and *Neurospora crassa*), only NDH-I (*e.g., Paracoccus denitrificans*), or only NDH-II (*e.g., Saccharomyces cerevisiae*) (see Yagi, 1993). Little is known about the regulation of these enzymes. It has been suggested that in *E. coli,* the expression of NDH-II genes is increased at higher oxygen concentrations (Spiro *et al.*, 1989; Spiro and Guest, 1990). Recently it was proposed that in *Synechocystis,* the type-II NADH dehydrogenases might be involved in regulation, responding to the redox state of the quinone pool (Howitt *et al.*, 1999). Nevertheless, it is clear that membrane-associated NADH oxidizing activity can be solely assured by NDH-II, as shown by the cases of the organisms that exclusively contain this enzyme and by several examples of functional mutants on a complex-deficient background.

The occurrence of NADH dehydrogenases in phylogenetically distant organisms like thermophilic archaea is far less well reported. The only two reported cases of soluble NADH oxidases are the ones from *Acidianus ambivalens* (Gomes and Teixeira, 1998) and *Sulfolubus acidocaldarius* (Wakao *et al.*, 1987), although the presence of type-II NADH dehydrogenases has been previously suggested (Schuäffer, 1996; Schäffer *et al.*, 1996, 1999). The organism used in this study is *Acidianus ambivalens,* an hyperthermoacidophilic archaeon capable of growing at 82°C and pH 2.0. In recent years, we have focused on characterizing its respiratory chain, which is one of the simplest so far known (Anemüller *et al.*, 1994; Schäfer, 1996; Gomes *et al.*, 1999; Gomes, 1999). Here we report the complete physicochemical and kinetic characterization of its unique membrane-associated NADH dehydrogenase. It is a novel variant of the type-II enzymes, containing covalently bound flavin as its only redox cofactor. This enzyme was used to reconstitute *in vitro* the organism respiratory chain, using the organism-specific quinone and its only terminal oxidase (Anemüller *et al.*, 1994; Giuffrè *et al.*, 1997; Das *et al.*, 1999), thus showing not only that it is involved in respiration, but also highlighting the minimal components of an archaeal respiratory system.

EXPERIMENTAL PROCEDURES

Cell Growth, Membrane Extract Preparation, and Protein Purification

Cells of *A. ambivalens* were grown as previously described (Teixeira *et al.*, 1995). Cells were broken at 6000 psi using a French press, followed by centrifugation at 18,000 rpm for 15 min to remove unbroken material. The obtained crude extract was ultracentrifuged for 6 h at 138,000 × *g*. The green pellet was the membrane fraction. This fraction was solubilized with dodecyl maltoside (DM), in the proportion of 2 g detergent/g protein; the resulting suspension was centrifuged at 138,000 × *g* for 6 h at 4°C. All subsequent chromatographic steps were carried out on a Pharmacia HiLoad system, at 4°C. The green supernatant, corresponding to the solubilized membranes, was applied to a 330-ml DEAE-Sepharose fast-flow column. After elution at 10 ml/min with 330 ml of low-ionic strength buffer (20 mM potassium phosphate pH 6.5, 0.1% DM), a gradient from 0 to 80% of high-ionic strength buffer (20 mM potassium phosphate pH 6.5, 1 M NaCl, 0.1% DM) was applied at the same flow. The NADH dehydrogenase fraction, which eluted at 20 mM NaCl, was applied directly to a 70-ml home-packed HTP Micro-beads column, at 1 ml/min, which had been previously equilibrated with 20 mM potassium phosphate pH 6.5, 0.1% DM. An elution gradient of 6 column volumes until 500 mM potassium phosphate pH 6.5, 1 M NaCl, 0.1% DM was reached was applied and the NADH dehydrogenase fraction eluted at around 40 mM potassium phosphate. This fraction was loaded into a second ionic exchanger, Q-sepharose, equilibrated, and run with the same buffer system used in the DEAE-Sepharose step, except that the pH was adjusted to 7.5. The enzyme fraction eluted at ~150 mM NaCl, was concentrated by ultrafiltration over a 10-kDa cut-off membrane, and applied to a 330-ml gel filtration Superdex S-200 column, equilibrated, and eluted with 20 mM potassium phosphate, pH 6.5, 200 mM NaCl, 0.1% DM at 1.6 ml/min. The final step consisted of a second HTP column. The purified enzyme was divided in aliquots and stored at −70°C. SDS-PAGE was used to test protein purity. *Acidianus ambivalens aa_3* quinol oxidase was purified as in Giuffrè *et al.* (1997).

Kinetic Assays

NADH dehydrogenase activity was routinely assayed at 50°C following $K_3Fe(CN)_6$ reduction at 420 nm ($\varepsilon_{420nm}=1.0$ mM^{-1}/cm^{-1}) in a medium containing $K_3Fe(CN)_6$ (1 mM) and NADH (0.2 mM) in 20 mM potassium phosphate buffer pH 6.5, 0.1% DM. After approximately 5-min incubation at the working temperature, the reaction was initiated by the addition of NADH. The activity assays performed in the presence of sonicated lipids were performed as in Björklöf *et al.* (2000).

Analytical Methods

Protein concentration was determined using the modified microbiuret method for membrane proteins and for proteins in the presence of detergent (Watters, 1978). Flavin extraction was attempted as described in Susín *et al.* (1993). Molecular mass determinations by gel permeation were performed in a 24-ml bed volume Superdex S-75 column (Pharmacia), using low-molecular mass protein standards (range 6.5–63 kDa, Pharmacia). Elution was performed with 20 mM potassium phosphate, pH 6.5, 200 mM NaCl, 0.1% DM at 0.4 ml/min. Blue dextran was used as internal standard. The protein N-terminal sequence was determined using an Applied Biosystem Model 470A sequenator. Search of protein sequences showing homology with the N-terminus was performed at the NCBI using the BLAST network service.

Spectroscopic Methods

Room temperature ultraviolet/visible spectra were recorded in a Shimadzu spectrophotometer. Redox titrations monitored by visible spectroscopy were performed in a Shimadzu diode array spectrophotometer equipped with a cell-stirring system. EPR spectra were recorded on a Bruker ESP 380 spectrometer, equipped with an ESR 900 continuous-flow helium cryostat or with a liquid nitrogen continuous-flow system from Oxford Instruments. The fluorescent photograph was recorded in a STORM 860.

Redox Titrations

Acidianus ambivalens NDH-II ($\sim$15 μM) was titrated anaerobically in 50 mM potassium phosphate pH 6.5, 0.1% DM by stepwise addition of buffered sodium dithionite. The following compounds were used as redox mediators (0.25 μM each): 1,2-naphthoquinone (E'_o = +180 mV), trimethylhydroquinone (E'_o = +90 mV), 1,4-naphthoquinone (E'_o = +60 mV), methylene blue (E'_o = 11 mV), indigo tetrasulfonate (E'_o = −30 mV), indigo trisulfonate (E'_o = −70 mV), indigo disulfate (E'_o = −182 mV), anthraquinone-2,7-disulfonate (E'_o = −182 mV), safranin (E'_o = −280 mV), neutral red (E'_o = −325 mV), benzyl viologen (E'_o = −359 mV), and methyl viologen (E'_o = −446 mV). A platinum and a silver/silver chloride electrode were used, calibrated against a saturated quinhydrone solution. The reduction potentials are quoted versus the standard hydrogen electrode. The experimental data was manipulated and analyzed using MATLAB (Mathworks, South Natick, Massachusetts) for Windows.

Coreconstitution in Proteoliposomes

Caldariella quinone was purified as described in Tricone *et al.* (1989). The reconstitution was performed as described in Verkhovskaya *et al.* (1997), with the following modifications. Preformed liposomes containing caldariella quinone were prepared mixing 150 μl CQ (1 mM stock) with 16 mg azolectin and chloroform ($\sim$2 ml). The solvent was evaporated and the azolectin/CQ mix was solubilized in 200 mM HEPES-KOH, pH 7.4/55 mM octyl glucoside by brief ultrasonic treatment. Purified aa_3 quinol oxidase (40 μM) and NADH dehydrogenase (16 μM) were added to a final concentration of 1 μM each and the mixture stirred for 10 min. From here on, the procedure was as described in Verkhovskaya *et al.* (1997). The formed liposomes were then resuspended in a medium containing 100 mM KCl. Oxygen uptake experiments by the liposomes containing the reconstituted chain were performed in a Clark-type oxygen electrode at 40°C. Upon energization of this system with NADH ($\sim$4 mM final), the rate of oxygen consumption increased up to $\sim$2-fold, following addition of the ionophore gramicidin (3 μM final) plus the uncoupler carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) (16 nM final), thus indicating that the liposomes are sealed and are fairly impermeable. Potassium cyanide used on the inhibition studies was in a buffered stock solution (30 mM) at pH 8.0. Liposomes containing solely CQ and the aa_3 quinol oxidase, or only NDH-II, for control experiments were prepared identically.

RESULTS AND DISCUSSION

Evidence for the Presence of a NDH-II

The respiratory chain of *A. ambivalens* has been intensively investigated in intact membranes and cells, using spectroscopic and kinetics techniques, taking advantage of its simple composition. Thus far, only two respiratory complexes were identified: an unusual succinate dehydrogenase (Gomes *et al.*, 1999), and a aa_3-type quinol oxidase, which is the only terminal oxygen reductase (Anemüller *et al.*, 1994; Giuffrè *et al.*, 1997; Das *et al.*, 1999). However, *A. ambivalens* membranes exhibit cyanide-sensitive NADH-driven oxygen consumption, at a rate of 1 to 3 nmol O_2/min^{-1}/mg^{-1}, indicating that: (1) there is a membrane-bound enzyme that oxidizes NADH and (2) this enzyme is able to reduce caldariella quinone (CQ), the organism-specific quinone, which mediates electron transfer to the only terminal oxidase present. This respiratory rate is insensitive to classical inhibitors of type-I NADH dehydrogenase, like rotenone. Further, incubation of intact membranes with NADH does not result in any EPR-detectable resonances typical of iron–sulfur clusters, apart from those assignable to the succinate dehydrogenase complex (Gomes *et al.*, 1999). Thus, a type-II NADH dehydrogenase seems to be present in the membranes of this archaeon.

Purification of the Membrane-Bound NDH-II

Acidianus ambivalens NADH dehydrogenase was purified about 11-fold from dodecyl maltoside (DM)-solubilized membrane extracts (Table I). During purification, the fractions were routinely assayed and pooled according to its NADH-K_3Fe(CN)$_6$ oxidoreductase activity, and the final protein yield was 5 mg, obtained from $\sim$150 g cells (wet wt.) (Table I). After the last purification step, the protein practically lost activity, as assayed by the standard procedure, a typical behavior among type-II NADH dehydrogenases, which exhibit low activity rates

Table I. Purification of the *Acidianus ambivalens* NDH-II

Purification (step)	Protein (mg)	Specific activity NADH–K$_3$Fe(CN)$_6$ reductase activity (mmol NADH oxidized/min^{-1}/mg^{-1})	Enrichment (fold)
DM Extract	7000	3.7	—
Q-Sepharose	430	9.0	2.4
HTP	150	21.5	5.8
Q-Sepharose	80	25	6.7
S-200	50	28	7.5
HTP	5	40[a]	11[a]

[a] Assayed in the presence of sonicated phospholipids (see text).

once purified. Nevertheless, the activity could be restored upon assaying activity according to a recently described procedure, based on successive dilutions of the purified protein in sonicated phospholipids (Björklöf *et al.*, 2000). For *E. coli* NDH-II, this procedure resulted in a 200-fold increment of activity (Björklöf *et al.*, 2000) and, in the present case, it completely restored activity from an inactive fraction (see below). The purified protein purity was confirmed by gradient 5–20% SDS–PAGE gel electrophoresis, which showed a single band upon staining with Coomassie brilliant blue (Fig. 1).

Physicochemical Characterization

Pure *A. ambivalens* NDH-II exhibits a yellow color, suggesting the presence of a flavin cofactor. Consistently, its visible spectrum is typical of a flavoprotein, exhibit-ing bands with λ_{max} at 380 and 452 nm and a shoulder at 478 nm, suggesting that the flavin isoaloxazine ring is in a apolar environment (Fig. 2). The EPR spectra of native, NADH-reduced, and dithionite-reduced NDH-II is featureless, indicating the absence of iron–sulfur clusters (not shown).

The type of flavin cofactor could not be identified as it is covalently bound to the protein. In fact, protein incubation with trichloroacetic acid (up to 40%), even in combination with heating (up to 80°C) failed to extract the cofactor. Further, a fluorescence photograph of the SDS/PAGE gel shows that the flavin cofactor is covalently bound to the protein (Fig. 1, panel B). This is a complete novelty among type-II NADH dehydrogenases, which typically contain one noncovalently bound FAD molecule per subunit. This finding might be related to the fact that this organism thrives at extremely high temperatures and thus the covalent link may be a suitable mechanism to maintain an otherwise labile cofactor. Indeed, strong acidic treatment of intact *A. ambivalens* membranes did not result in the release of any flavin and an identical observation was made in membranes from *Sulfolobus metallicus,* another
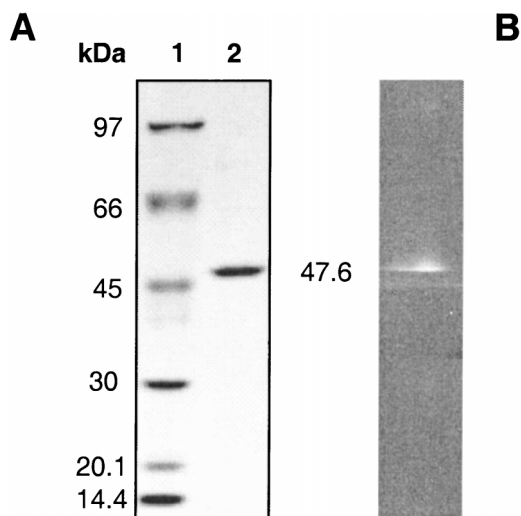
Fig. 1. SDS–Page gel electrophoresis (5–20%) and fluorescence photograph. (**A**) Lane 1, molecular mass markers; lane 2, Pure *A. ambivalens* NDH-II. (**B**) Fluorescent photograph of *A. ambivalens* NDH-II showing the existence of a covalently bound flavin.
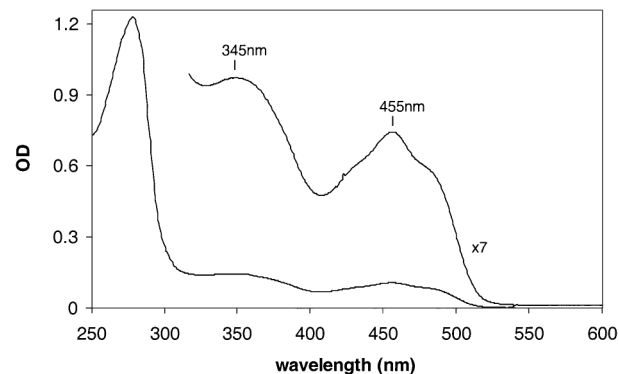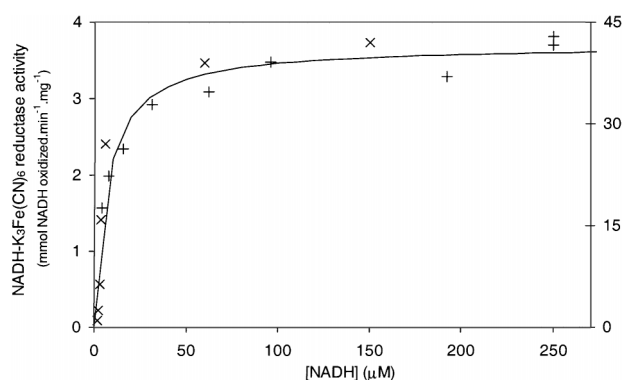

Fig. 2. UV-visible spectrum of NDH-II. Spectrum of pure NDH-II (7.5 μM), as prepared. Ratio $A_{280}/A_{345} = 8.81$, $A_{280}/A_{455} = 11.6$, and $A_{345}/A_{455} = 1.32$.

thermophilic archaeon. This further suggests that covalent linkage can be a generalized mechanism to achieve cofactor stabilization among membrane-bound flavoproteins from hyperthermophiles.

SDS/PAGE gel electrophoresis (5–20%) showed the presence of a single band with an apparent molecular mass of ~47 kDa (Fig. 1), agreeing well with those determined for NDH-II from other sources, which range from 45 (*E. coli,* Hayashi *et al.*, 1989) to 63 kDa (*B. subtilis*, Bergsma *et al.*, 1982). Determination of the molecular mass of the purified protein by gel permeation gave a value of 60 kDa, a value in agreement with the expected increase of the apparent molecular mass caused by the presence of detergent. Thus, the data suggests that the *A. ambivalens* NDH-II is a monomer.

Kinetic Characterization

As already mentioned, the pure fraction lost activity, which could only be recovered by incubation with diluted phospholipids, according to Björklöf *et al.* (2000). Other attempts involving incubation with the physiological quinone, flavins, or dithiothreitol had no effect. Thus, it seems that the enzyme needs an adequate lipidic environment to be catalytically proficient. Indeed, both membrane-integrated and purified enzyme showed identical kinetic parameters. In both cases, the affinity for the substrate NADH at 50°C was found to be very identical: 6.8 μM for the membrane-integrated enzyme and 6.2 μM for the purified enzyme (Fig. 3). The *A. ambivalens* NDH-II is reduced by NADH, but not by deamino-NADH or

Table II. Reductase Activity of *Acidianus ambivalens* NDH-II Toward Several Compounds[a]

Acceptor	Activity (%)
Ubiquinone-2 (Q_2)	100
Ubiquinone-1 (Q_1)	95
Caldariella quinone (CQ)	80
$K_3Fe(CN)_6$	25

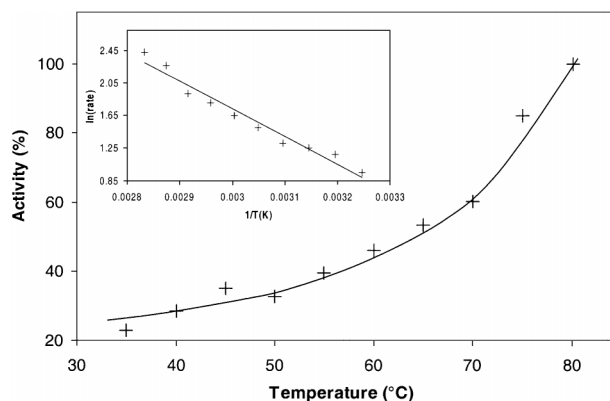[a] Assays were performed in the presence of sonicated phospholipids; maximal activity is 160 ± 40 mmol NADH oxidized/min^{-1}/mg^{-1}.

NADPH, and mediates electron transfer to several acceptors (Table II). The obtained rates are comparable to those determined for *E. coli* NDH-II activated by the same protocol (200 mmol NADH oxidized/min^{-1}mg^{-1}). Assays performed on the oxygen electrode using pure NDH-II in lipids showed that the enzyme does not catalyze the unspecific reduction of dioxygen by NADH.

The dependence of the NADH-$K_3Fe(CN)_6$ reductase activity with temperature was determined in the membrane extract. The activity increased fourfold from 35 to 80°C, the maximum possible operating temperature (Fig. 4). The Arrhenius plot of the process (Fig. 4, inset) show no discontinuities and an activation energy of 27.6 kcal/mol^{-1} was determined.

Redox Properties of NDH-II

The redox properties of the flavin cofactor of *A. ambivalens* NDH-II were investigated by redox titrations monitored by visible spectroscopy. The decrease in the

Fig. 3. Kinetic parameters for the NADH–$K_3Fe(CN)_6$ reductase activity of NDH-II. The (+) sign indicates assays performed in intact membranes (relative to the primary *y* axis), whereas (*x*) indicates experiments on the pure enzyme (relative to the secondary *y* axis). The solid line represents a fit according to the Michaelis–Menten equation, with $K_m = 6$ μM and $V_m = 40$ mmol NADH oxidized/min^{-1}/mg^{-1} for assays in the pure enzyme, and $V_m = 3.7$ mmol NADH oxidized/min^{-1}/mg^{-1} for experiments in the intact membranes.

Fig. 4. Temperature dependence of NADH dehydrogenase activity in intact membranes. The assays were performed in intact membranes, following NADH–$K_3Fe(CN)_6$ reductase activity after 5-min incubation at the working temperature. The maximum activity corresponds to 11 mmol NADH oxidized/min^{-1}/mg^{-1}. Inset,1Arrhenius plot.
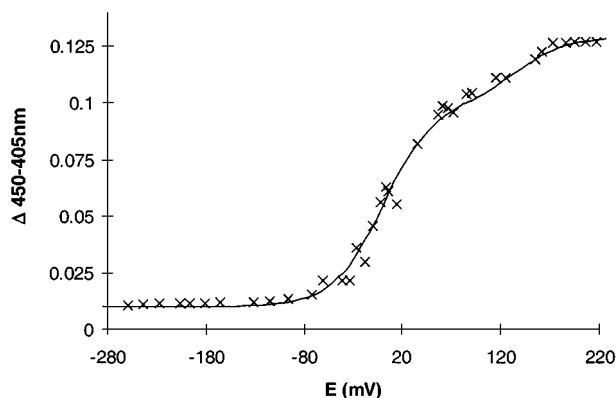
Fig. 5. Redox titration of NDH-II. The reduction of *A. ambivalens* NDH-II was followed at $450 - 405$ nm. The solid line corresponds to a sequential 2 one-step one-electron reductions, with midpoint reduction potentials of $135 \pm$ mV (Fl_{ox}/Fl_{sq}) and 0 ± 15 mV (Fl_{sq}/Fl_{red}).

intensity of the flavin absorption bands was plotted against the imposed redox potential and reduction was found to proceed through 2 one-step electron reductions, corresponding to the Fl_{ox}/Fl_{sq} and Fl_{sq}/Fl_{red} transitions at 135 ± 20 and 0 ± 30 mV, respectively (Fig. 5). Most likely, these transitions reflect coproportionation between flavin molecules in solution and do not necessarily correspond to physiological requirements, although the latter can not be excluded. The determined average reduction potential of $\sim$70 mV is perfectly adequate to mediate electron transfer between NADH ($E_{NADH/NAD} = -340$ mV) and CQ ($E_{CQ/CQH_2} = 100$ mV) (Schäffer *et al.*, 1990).

## Analysis of the N-Terminal Sequence

The N-terminus of *A. ambivalens* NDH-II was sequenced up to residue 46. A psi-BLAST search returned several sequences annotated as type-II NADH dehydrogenases, which, in the region compared, exhibit a significant similarity toward *A. ambivalens* NDH-II (22–40% identity and 42–61% similarity) (Fig. 6). This region comprises a dinucleotide binding $\beta\alpha\beta$-fold region (Wierenga *et al.*, 1996), including a partly conserved GxGxxG motif (GGxxG in a subfamily of NDH-II, which includes the *A. ambivalens* enzyme). This region has been suggested to be involved in stabilizing binding of FAD or NADH (Kerscher *et al.*, 1999). The N-terminus also comprises one of the apolar/aromatic regions that have been postulated to play a role in forming a pocket that would favor the interaction of quinone with FAD and NADH (Kerscher *et al.*, 1999). In *A. ambivalens* NDH-II, this region comprises 8 apolar/aromatic residues out of a total of 11.

Reconstitution of a Minimal Respiratory Chain Using NDH-II

In order to investigate the involvement of the purified NDH-II in respiration, a minimal respiratory chain of *A. ambivalens* was reconstituted into artificial liposomes. Uncoupled liposomes containing NDH-II, caldariella quinone, and the terminal oxidase (Fig. 7,A) exhibit NADH-driven, cyanide-sensitive oxygen consumption (Fig. 7,B). NADH can energize the terminal
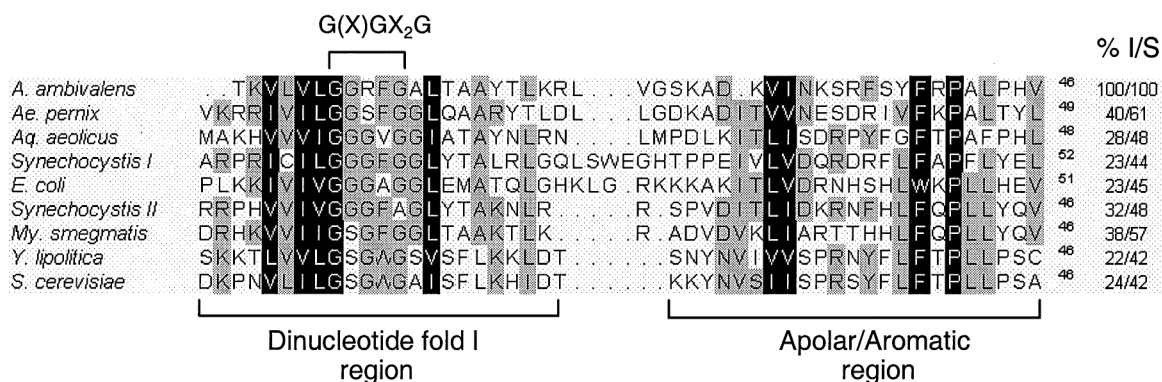
Fig. 6. Amino acid sequence comparison of *A. ambivalens* NDH-II N-terminus. The N-terminal sequence of *A. ambivalens* is compared toward other type-II NADH dehydrogenases (accession numbers are given between parenthesis): *Aeropyrum* (*Ae.*) *pernix* (E72645), *Aquifex* (*Aq.*) *aeolicus* (7521720), *Synechocystis* I (S74822) and II (S74826), *E. coli* (P00393), *Mycobacterium* (*My*) *smegmatis* (2708705), *Yarrowia* (Y.) *lipolytica* (AJ006852), and *Sacharomyces* (*S.*) *cerevisiae* (CAA43787). The two conserved regions are highlighted, as well as the GXGXXG motif. The percentage of identity and similarity (% I/S) in respect to the *A. ambivalens* NDH-II is given in the far left column.
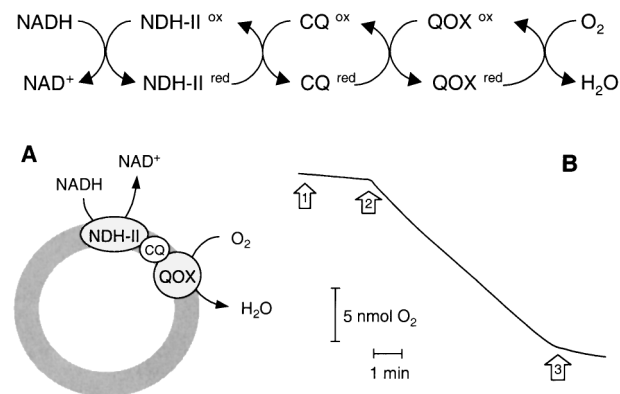
Fig. 7. Reconstitution of the minimal *A. ambivalens* respiratory chain. (Top) scheme showing the reconstituted electron transfer chain linking NADH oxidation to oxygen reduction. Three components are involved: the NADH dehydrogenase (NDH-II), caldariella quinone (CQ), and the terminal quinol oxidase (Q$_{OX}$). (Bottom) (**A**) representation of the liposome-integrated components, simulating the organism respiratory chain; (**B**) oxygen consumption traces at 40°C. Arrow 1, addition of liposomes prepared as described in the materials section (*cf.* A) in 100 mM KCl containing 3 μM gramicidin (ionophore) and 16 nM FCCP (uncoupler). Arrow 2, addition of 20 mM NADH. Arrow 3, addition of 0.5 mM KCN.

reductase at a rate of 20 ± 2 min^{-1}, in a cyanide-sensitive manner, thus confirming the involvement of the NDH-II in respiration (Fig. 7, top). The presence of the ionophore gramicidin and of the uncoupler FCCP assures that the reaction rate is not limited by the proton availability inside the liposomes. Oxygen consumption is strictly associated with the operation of the terminal oxidase. A control experiment using liposomes containing, exclusively, the NDH-II showed that upon energization with NADH, no oxygen consumption is observed. Catalase also has no effect on the measured oxygen consumption rates, indicating that, as expected, oxygen is being completely reduced to water. Thus, these experiments exclude the presence of unspecific oxygen reduction reactions occurring in the liposomes. Another type of control experiment was performed, in which uncoupled liposomes containing only the terminal oxidase and caldariella quinone were used. It showed that upon energization this system uptakes oxygen at a rate of $\sim$17 min^{-1}. This rate is comparable to the one determined for the complete system (Fig. 7), indicating that the membrane-bound NDH-II is an efficient electron entry point into the respiratory chain.

CONCLUSION

A new kind of type-II NADH dehydrogenase was isolated and thoroughly characterized from the membranes of the archaeon *A. ambivalens*. This is the first unequivocal example for a type-II enzyme in archaea, whose presence has been postulated for a long time (Schäfer *et al.*, 1996). Indeed, recent studies on the halophilic archaeon *Halobacterium halobium* have suggested the presence of a type-II NADH dehydrogenase in this organism, but the protein was not isolated (Sreeramulu *et al.*, 1998). The *A. ambivalens* NDH-II protein was found to be a 47-kDa monomer, lacking iron–sulfur clusters, but containing a covalently bound flavin cofactor with an E_m of 70 mV. Sequence analysis showed that it is a member of the NDH-II family, containing typical conserved regions with possible structural relevance on its N-terminal regions. Kinetically, it is an appropriate NADH:quinone oxidoreductase. A minimal archaeal respiratory pathway was reconstituted in liposomes, using three components from the *A. ambivalens* chain: an aa_3-type quinol oxidase, caldariella quinone, and the NDH-II. This artificial system allowed not only the demonstration of the direct involvement of the new NDH-II in respiration, but the *in vitro* assembly of the minimal functional unit of an archaeal respiratory chain.

REFERENCES

Anemüller, S., Schmidt, C. L., Pacheco, I., Schäfer, G., and Teixeira, M. (1994). *FEMS Microbiol. Lett.* **117**, 275–280.

Bergsma, J., Van-Dongen, M. B., and Konings, W. N. (1982). *Eur. J. Biochem.* **128**, 151–157.

Björklöf, K., Zickermann, V., and Finel, M. (2000). *FEBS Lett.* **467**, 105–110.

Das, T. K., Gomes, C. M., Teixeira, M., and Rousseau, D. L. (1999). *Proc. Natl. Acad. Sci. USA* **96**, 9591–596.

Finel, M. (1998). *Biochim. Biophys. Acta* **1364**, 112–124.

Friedrich, T., Abelmann, A., Brors, B., Guénebaut, V., Kintscher, L., Leonard, K., Rasmussen, T., Scheide, D., Schlitt, A., Schulte, U., and Weiss, H. (1998). *Biochim. Biophys. Acta* **1365**, 215–219.

Giuffrè, A., Gomes, C., Antonini, G., D'Itri, E., Teixeira, M., and Brunori, M. (1997). *Eur. J. Biochem.* **250**, 383–388.

Gomes, C. M. (1999). Ph.D. dissertation, Instituto Tecnologia Química e Biológica, Universidade Nova de Lisboa.

Gomes, C. M., and Teixeira, M. (1998). *Biochem. Biophys. Res. Commun.* **243**, 412–415.

Gomes, C. M., Lemos, R. S., Teixeira, M., Kletzin, A., Huber, H., Stetter, K. O., Schäfer, G., and Anemüller, S. (1999). *Biochim. Biophys. Acta* **1411**, 134–141.

Hayashi, M., Miyoshi, T., Takashina, S., and Unemoto, T. (1989). *Biochim. Biophys. Acta* **977**, 62–69.

Howitt, C. A., Udall, P. K., and Vermaas, W. F. J. (1999). *J. Bacteriol.* **181**, 3994–4003.

Kerscher, S., Okun, J. G., and Brandt, U. (1999). *J. Cell Sci.* **112**, 2347–2354.

Kerscher, S. J. (2000). *Biochim. Biophys. Acta*, **1459**, 274–283.

Ohnishi (1998). *Biochim. Biophys. Acta* **1364**, 186–206.

Schäfer, G. (1996). *Biochim. Biophys. Acta* **1277**, 163–200.

Schäfer, G., Anemüller, S., Moll, R., Meyer, W., and Lübben, M. (1990). *FEMS Microbiol. Rev.* **75**, 335–348.

Schäfer, G., Purschke, W., and Schmidt, C. L. (1996). *FEMS Microbiol. Rev.* **18**, 173–188.

Schäfer, G., Engelhard, M., and Müller, V. (1999). *Microbiol. Mol. Biol. Rev.* **63**, 570–620.

Spiro, S. and Guest, J. R. (1990). *FEMS Microbiol. Rev.* **75**, 399–428.

Spiro, S., Roberts, R. E., and Guest, J. R. (1989). *Mol. Microbiol.* **3**, 601–608.

Sreeramulu, K., Schmidt, C. L., Schafer, G., and Anemuller, S. (1998). *J. Bioenerg. Biomembr.* **30**, 443–453.

Susín, S., Abián, J., Sánchez-Baeza, F., Peleato, M. L., Abadía, A., Gelpí, E., and Abadía, J. (1993). *J. Biol. Chem.* **268**, 20958–20965.

Teixeira, M., Batista, R., Campos, A. P., Gomes, C., Mendes, J., Pacheco, I., Anemüller, S., and Hagen, W. R. (1995). *Eur. J. Biochem.* **227**, 322–327.

Tricone, A., Lanzotti, V., Nicolaus, B., Zillig, W., De Rosa, M., and Gambacorta, A. (1989). *J. Gen. Microbiol.* **135**, 2751–2757.

Verkhovskaya, M. L., García-Horsman, J. A., Puustinen, A., Rigaud, J. L., Morgan, J. E., Verkhovsky, M. I., and Wikström, M. (1997). *Proc. Natl. Acad. Sci. USA* **94**, 10128–10131.

Wakao, H., Wakagi, T., and Oshima, T. (1987). *J. Biochem (Tokyo)* **102**, 255–262.

Watters, C. (1978). *Anal. Biochem.* **88**, 695–698.

Wierenga, R. K., Terpstra, P., and Hol, W. G. J. (1986). *J. Mol. Biol.* **187**, 101–107.

Yagi, T. (1993). *Biochim. Biophys. Acta* **1141**, 1–17.

Yagi, T., Yano, T., Di Bernardo, S., and Matsuno-Yagi, A. (1998). *Biochim. Biophys. Acta* **1364,** 125–133.