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The proton-pumping NADH:ubiquinone oxidoreductase (complex I) of *Aquifex aeolicus*

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Abstract The proton-pumping NADH:ubiquinone oxidoreductase, also called complex I, is the first energy-transducing complex of many respiratory chains. Homologues of complex I are present in the three domains of life. Here, we report the properties of complex I in membranes of the hyperthermophilic bacterium *Aquifex aeolicus*. The complex reacted with NADH but not with NADPH and $F_{420}H_2$ as electron donors. Short-chain analogues of ubiquinone like decyl-ubiquinone and ubiquinone-2 were suitable electron acceptors. The affinities towards NADH and ubiquinone-2 were comparable to the ones obtained with the *Escherichia coli* complex I. The reaction was inhibited by piericidin A at the same concentration as in *E. coli*. The complex showed an unusual pH optimum at pH 9 and a maximal rate at 80°C. We found no evidence for the presence of an alternative, single subunit NADH dehydrogenase in *A. aeolicus* membranes. The NADH:ferricyanide reductase activity of detergent extracts of *A. aeolicus* membranes sedimented as a protein with a molecular mass of approximately 550 kDa. From the data we concluded that *A. aeolicus* contains a NADH: ubiquinone oxidoreductase resembling complex I of mesophilic bacteria.

Key words: Complex I; NADH:ubiquinone oxidoreductase; NADH dehydrogenase; Extremophile; Hyperthermophile; *Aquifex aeolicus*

1. Introduction

The proton-pumping NADH:ubiquinone oxidoreductase, also called complex I, is the entry point for electrons of most respiratory chains [1,2]. It provides the proton motive force essential for energy consuming processes like the synthesis of ATP. Closely related forms of the complex are present in the respiratory chains of many bacteria and most mitochondria. Cyanobacteria and archaea contain a complex I homologue that reacts with electron donors different from NADH [3]. The archaeal complex I receives electrons from $F_{420}H_2$, while the electron donor of the cyanobacterial complex is not known yet. NADPH and ferredoxin are discussed as possible candidates [3,4]. In addition to complex I, many bacteria and mitochondria contain an alternative, non-energy converting NADH dehydrogenase [5]. This single subunit enzyme contains flavin adenine dinucleotide as single cofactor and has a 10-fold lower affinity to NADH [6]. It most likely functions as a valve for an excess of reducing equivalents [7] or is important for aerobic growth [8].

The bacterial complex I has a molecular mass of 535 kDa and consists of 14 different subunits. Seven subunits are peripheral proteins including all subunits that bear the known redox groups of complex I, namely one flavin mononucleotide and up to nine iron–sulfur (FeS) clusters [9,10]. The remaining seven subunits are most hydrophobic proteins and are predicted to fold into 54 α-helices across the membrane. Nothing is known about their function, but they are most likely involved in proton translocation [1–3]. The mitochondrial complex contains in addition to the homologues of the 14 bacterial subunits up to 28 extra proteins that add up to a molecular mass of approximately 1 MDa [1,2].

Due to its complex composition and the numerous cofactors little is known about its function. Progress in complex I research is hampered by the lack of a high resolution structure. Electron microscopy revealed that the mitochondrial as well as the bacterial complex have an unusual L-shaped overall structure [11–13]. It is made up of a peripheral arm consisting of the hydrophilic subunits and a membrane arm consisting of the hydrophobic subunits. The preparation from *Escherichia coli* has been the only preparation reported from a bacterial source so far [14,15]. Complex I preparations from a thermophilic or even hyperthermophilic organism are not yet known. However, such a preparation would be most promising in obtaining material for crystallization. From its genomic sequence the extremophile *Aquifex aeolicus* should contain a proton-pumping NADH:ubiquinone oxidoreductase [3,16]. Here, we report properties of the membrane-bound NADH dehydrogenase from *A. aeolicus*. The data obtained indicated that *A. aeolicus* possesses a complex I homologue as sole NADH dehydrogenase and that this complex resembles the *E. coli* complex I.

2. Materials and methods

2.1. Growth of bacteria and preparation of membranes

A. aeolicus (VF5) was grown and cells were harvested and stored at −80°C as described previously [17]. Cells (5 g) were washed and resuspended in 15 ml 50 mM Tris–HCl, pH 7.5 containing 1 mg of DNase (Boehringer, Mannheim). The cells were disrupted by two passages through a French pressure cell (SLM Aminco) at 110 MPa. Whole cells and cell debris were removed by centrifugation for 30 min at $14\,000 \times g$, and cytoplasmic membranes were obtained by centrifugation for 90 min at $250\,000 \times g$. The membranes were resuspended in 50 mM Tris–HCl, pH 7.5 to a protein concentration of approximately 50 mg/ml.

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2.2. Enzyme assays

Respiratory activities of isolated bacterial membranes from *A. aeolicus* were measured in a Clark-type oxygen electrode at 50°C [18]. The test buffer (1.5 ml) contained 50 mM Tris–HCl, pH 7.5, 50 mM NaCl and 500 μg/ml membrane protein. The reaction was started either by addition of 10 mM NADH or 10 mM deamino (d)-NADH [6]. For titration with piericidin A, the inhibitor was added to the enzyme assay during the reaction. The samples were not pre-incubated with the inhibitor. The NADH/ubiquinone-2 reductase activity and the d-NADH/ubiquinone-2 reductase activity of the membranes were measured at 50°C with a Sigma ZWSII (Biochem) dual-wavelength spectrophotometer using the wavelengths 340 and 400 nm and ε of 6.3 mM^{-1} cm^{-1}. The assay contained 50 mM Tris–HCl, pH 7.5, 50 mM NaCl, 100 μM KCN, 5 μM antimycin A, 0.1 μM aurachin D, 0.5–20 μM ubiquinone-2 and 1–50 μM NADH or d-NADH, and 150 μg/ml membrane protein. The ferricyanide reductase activity was determined using 0.1 mM NADH, d-NADH, NADPH, and $F_{420}H_2$ as electron donors, respectively. The activity was measured at 30°C with a Sigma ZWSII (Biochem) dual-wavelength spectrophotometer using the wavelengths 410 and 500 nm and ε of 1 mM^{-1} cm^{-1}. The assay contained 50 mM Tris–HCl, pH 7.5, 50 mM NaCl, 1 mM $K_3[Fe(CN)_6]$ and 100 μg/ml membrane protein. The various pH values were adjusted using sodium citrate, MES/NaOH, Tris–HCl, bicine/NaOH, and potassium glycinate buffers, respectively. For all measurements the pH was adjusted to the appropriate temperature and the rates were corrected for the non-enzymatic reaction.

2.3. Other analytical procedures

The molecular mass of the *A. aeolicus* complex I was estimated from sucrose gradient centrifugation [14]. A suspension of cytoplasmic membranes (10 mg/ml membrane protein) was incubated with a final concentration of 5% (w/v) dodecyl maltoside for 15 min at 50°C. After centrifugation for 10 min at 20 000 × g 0.5 ml of the clear supernatant was loaded on top of a 12 ml gradient of 5–20% (w/v) sucrose with 0.2% dodecyl maltoside in 50 mM Tris–HCl, pH 7.5, 50 mM NaCl and centrifuged for 16 h at 180 000 × g. The sedimentation rate of the NADH/ferricyanide reductase activity was compared with the sedimentation rate of thyroglobin, ferritin, catalase, aldolase, γ-globulin, and the isolated *E. coli* complex I [14]. Protein concentrations were determined according to the biuret method.

3. Results

3.1. NADH oxidase activity of A. aeolicus membranes

NADH-dependent respiration was measured in Tris–HCl buffer at pH 7.5. NADH reacts with complex I as well as with the non-energy-coupled alternative NADH dehydrogenase [19]. The initial rate of the reaction at 50°C was 8.2 ± 0.7 μmol O_2/min/mg protein. With the artificial substrate d-NADH, which reacts prominently with complex I, the same rate of 8.4 ± 0.6 μmol O_2/min/mg protein was measured. More than 80% of both reactions were inhibited by 10 μM piericidin A, a specific complex I inhibitor [20]. Titration with piericidin A revealed an IC_{50}, the concentration needed for 50% inhibition of the reaction, of 1 μM (Fig. 1). These data showed that the *A. aeolicus* membranes contain only complex I but no other NADH dehydrogenases.

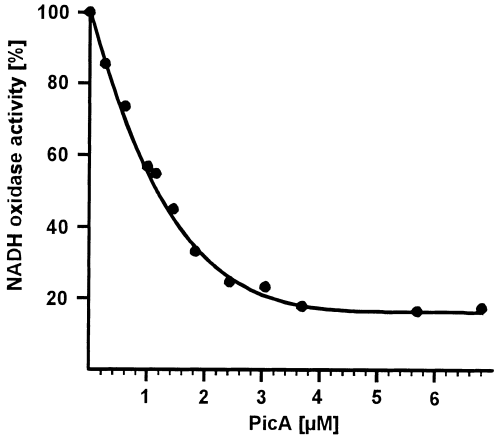

Fig. 1. Inhibition of the NADH oxidase activity of *A. aeolicus* membranes by piericidin A. Full activity corresponded to 8.2 μM O_2/min/mg protein. An identical curve was obtained using d-NADH as substrate.

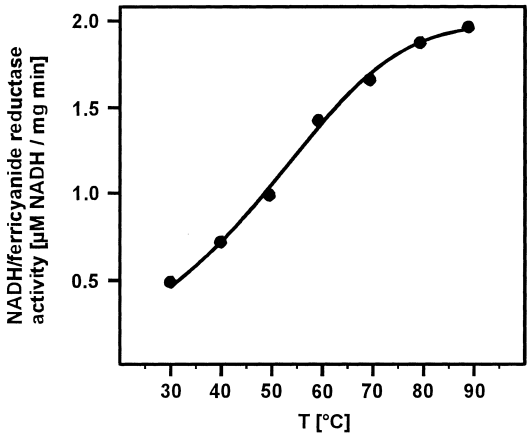

Fig. 2. Temperature dependence of the NADH/ferricyanide reductase activity of *A. aeolicus* membranes. The rates were corrected for the non-enzymatic reaction at each temperature.

The NADH as well as the d-NADH oxidase activities were 20% inhibited by KCN, an inhibitor of cytochrome *c* oxidase. The weak effect of KCN pointed to the presence of an alternative oxidase in the *A. aeolicus* membrane. From the genomic sequence [16] the presence of a *bd*-type ubiquinol oxidase is expected. Addition of 100 nM aurachin D, which has been reported to be an inhibitor of *bd*-type oxidases [21], led to 85% inhibition of the NADH oxidase activity. The IC_{50} of this reaction was 50 nM. Addition of 100 nM aurachin D and 100 μM KCN led to a complete inhibition of the NADH oxidase activity. These results indicated the presence of a cytochrome *c* oxidase as well as an *bd*-type ubiquinol oxidase in the *A. aeolicus* membrane.

3.2. NADH/ferricyanide activity of A. aeolicus membranes

In Tris–HCl pH 7.5 (d-)NADH/ferricyanide reductase activities of 0.5 ± 0.04 μmol NADH/min/mg protein and of 0.4 ± 0.02 μmol d-NADH/min/mg protein were measured at 30°C, respectively. These data were in line with the presence of complex I as the only NADH dehydrogenase in the *A. aeolicus* membranes. The hyperthermophilic bacterium *A. aeolicus* grows optimal at 85°C [16]. The optimum temperature of the enzyme complex should be close to this value. Indeed, the rate of the NADH/ferricyanide reductase activity increased with temperature (Fig. 2). The specific activity was 1.90 ± 0.2 μmol NADH/min/mg protein at 80°C and thus approximately 4-fold higher than at 30°C. Increasing the temperature to 90°C led to a slightly further increase in the reaction rate (Fig. 2).

Homologues of complex I exist in some bacteria and archaea that use either NADPH or $F_{420}H_2$ as substrate [3]. We measured the NADPH/ferricyanide and the $F_{420}H_2$/ferricyanide reductase activity to determine whether the *A. aeolicus* NADH dehydrogenase is capable to react with these electron donors. We determined the NADPH and the $F_{420}H_2$/ferricya-

nide reductase activities of the *A. aeolicus* membranes to 0.05 ± 0.005 μmol NADPH/min/mg protein and 0.08 ± 0.006 μmol $F_{420}H_2$/min/mg protein at 30°C, respectively. Consequently, the *A. aeolicus* complex I works exclusively as a NADH dehydrogenase.

The pH dependence of the NADH/ferricyanide reductase activity was investigated in various buffers (Fig. 3, see Section 2). It slowly increased from 0.5 ± 0.04 μmol NADH/min/mg protein in the range from pH 5 to 1.3 ± 0.08 μmol NADH/min/mg protein at pH 8. At pH 9 the maximum activity with 2.0 ± 0.1 μmol NADH/min/mg protein was measured which sharply decreased at pH values higher than 9.5 (Fig. 3).

3.3. NADH:ubiquinone oxidoreductase activity of A. aeolicus membranes

The NADH:ubiquinone oxidoreductase activity of the *A. aeolicus* complex I was measured at pH 7.5 in a buffer containing antimycin A, aurachin D, and KCN. Either the mixture contained 20 μM ubiquinone-2 as electron acceptor and was titrated with various amounts of NADH or it contained 50 μM NADH and was titrated with various amounts of ubiquinone-2. At 50°C an apparent V_{max} of 1.4 μmol NADH/min/mg protein with a $K_M^{app.}$ of 12 μM and 10 μM to NADH and ubiquinone-2, respectively, was determined. The membranes showed comparable activities with various short-chain ubiquinones. In the presence of 100 μM NADH an NADH:ubiquinone-2 oxidoreductase activity of 1.3 μmol NADH/min/mg protein was determined at 50°C with 20 μM ubiquinone-2 as substrate. Under the same conditions a NADH:decyl-ubiquinone oxidoreductase activity of 1.2 μmol NADH/min/mg protein and a NADH:duroquinone oxidoreductase activity of 1.5 μmol NADH/min/mg protein were determined in the presence of 20 μM electron acceptor, respectively.

3.4. Molecular mass of the A. aeolicus complex I

The *A. aeolicus* membrane proteins were extracted with dodecyl maltoside as detergent and loaded onto a 12 ml gradient of 5–20% (w/v) sucrose with 0.2% (w/v) dodecyl maltoside. The molecular mass of complex I was estimated by comparing the sedimentation rate of the NADH/ferricyanide reductase activity with the sedimentation rate of proteins with known molecular masses. The NADH/ferricyanide reductase activity sedimented approximately as a 550 kDa protein (Fig. 4). The amount of extracted complex I as judged by the NADH/ferricyanide reductase activity strongly depended on the detergent concentration, the incubation time and the incubation temperature (data not shown). At a final concentration of 5% (w/v) dodecyl maltoside and with 15 min incubation at 50°C the peak fractions of the sucrose gradient exhibited an NADH/ferricyanide reductase activity of 3.2 μmol NADH/min/ml (Fig. 4). No activity could be detected in the first quarter of the gradient, which is the typical position of the alternative NADH dehydrogenase [14]. The *A. aeolicus* complex I sedimented as a single symmetrical peak. SDS–PAGE of the fractions exhibiting NADH/ferricyanide activity revealed the presence of numerous proteins, however, it was not possible to attribute individual bands to the corresponding complex I subunits (data not shown).

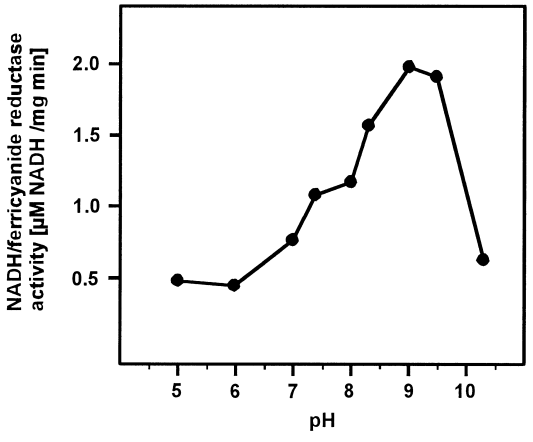

Fig. 3. Dependence of the NADH/ferricyanide reductase activity of *A. aeolicus* membranes from the pH. The rates were corrected for the non-enzymatic reaction at each pH value.

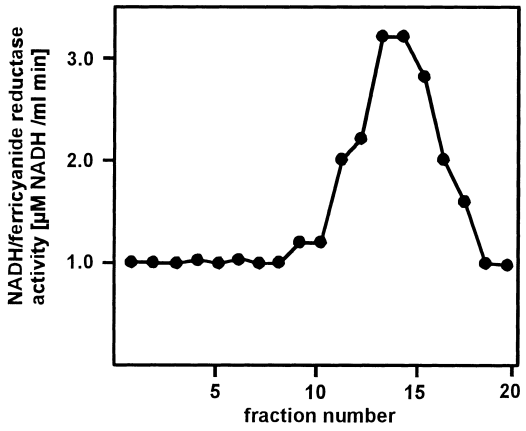

Fig. 4. Sucrose gradient centrifugation of dodecyl maltoside-solubilized cytoplasmic membranes of *A. aeolicus*. Fraction 1 refers to the top of the gradient and fraction 20 to the bottom of the gradient.

4. Discussion

A. aeolicus cytoplasmic membranes rapidly oxidized NADH at elevated temperatures indicating the presence of a membrane-bound NADH dehydrogenase in this organism. This enzyme was characterized by its NADH oxidase, NADH/ferricyanide reductase activity and NADH:ubiquinone-2 oxidoreductase activity. Within the error of the methods applied identical activities were obtained either by the use of NADH or d-NADH as substrate. These data provided evidence that *A. aeolicus* contains complex I as sole membrane-bound NADH dehydrogenase. This was in accordance with the distribution of the NADH/ferricyanide activity upon sucrose gradient centrifugation of a detergent extract of *A. aeolicus* membranes (Fig. 4). The activity sedimented like a 550 kDa protein and no activity was detected for a protein that sediments as a 50 kDa protein like the alternative NADH dehydrogenase [14]. Finally, no homologue to the *ndh* gene of the *E. coli* alternative NADH dehydrogenase is present in the *A. aeolicus* genome [16], while homologues of all 14 complex I *nuo* genes are present (see below).

Some members of the complex I family are suspected to react with other electron donors than NADH [3]. It has been shown that the complex I homologue from the methanogenic archaeon *Methanosarcina mazei* reacts with $F_{420}H_2$ [22], while it is discussed that the cyanobacterial complex I homologue as well as the complex I from *Helicobacter pylori*

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                223        *  *   *                            *    273
A. aeolicus       WLLEKGRTVCNLCPVGCEIQIEYGVGDWRSKRKVYRTKPTDELNICAKGFF
E. coli           WDMQFAPSICQQCSIGCNISPGERYGELRRIENRY-NGTVNHYFLCDRGRF
T. thermophilus   WEMEETPTTCALCPVGCGITADTRSGELLRIRARE-VPEVNEIWICDAGRF
P. denitrificans  WELTKTESIDVMDALGSSIRIDTKGREVMRILPRN-HDGVNEEWISDKTRF
R. capsulatus     WELVKTESIDVMDALGSNIRIDAKGREVKRIVPRN-HDGVNEEWLSDKGRF
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Fig. 5. Sequence comparison of the homologues of NuoG from *A. aeolicus*, *E. coli*, *T. thermophilus* [34], *Paracoccus denitrificans* [35], and *Rhodobacter capsulatus* [36]. The numbering is according to the *A. aeolicus* sequence. The conserved cysteines that presumably bind the FeS cluster N1c are marked with asterisks. Cluster N1c has not been detected in *P. denitrificans* and *R. capsulatus*.

and *Campylobacter jejuni* might react with NADPH [4,23]. However, membranes of *A. aeolicus* showed less than 10% of the reactivity with NADPH and $F_{420}H_2$ compared to NADH when assayed as ferricyanide reductase activity. The residual activities may stem from the corresponding membrane-attached NADPH or $F_{420}H_2$ dehydrogenases but not from complex I due to the low activities. In addition, the fractions of the sucrose gradient that exhibit NADH/ferricyanide reductase activity did not show any reactivity towards NADPH and $F_{420}H_2$ (data not shown). The *A. aeolicus* complex I seems to act as an NADH:ubiquinone oxidoreductase because it readily reacted with externally added short-chain ubiquinone analogues ubiquinone-2, decyl-ubiquinone, and duroquinone. The apparent affinities to the substrates NADH and ubiquinone-2 as well as to the inhibitor piericidin A were comparable with the data reported for the *E. coli* complex I [15].

There are two more similarities between the *A. aeolicus* and the *E. coli* complex I. First, 14 genes called *nuoA* to *nuoN* encode the subunits of bacterial complex I [10]. In *E. coli* *nuoC* and *nuoD* are fused to one gene *nuoCD* [24,25]. The same holds true for the *A. aeolicus* complex I: the gene *Aq1314* [16] is homologous to the fused *E. coli nuoCD*. Beside *E. coli* and *A. aeolicus*, this has also been demonstrated to be the case in *Buchnera aphidicola*, an endosymbiont of aphids and a close relative to *E. coli* [26]. Second, the subunit NuoG of *E. coli* contains an additional binding motif for an FeS cluster [14]. The cluster bound by this motif has been attributed to the EPR detectable FeS cluster N1c [14,24]. The motif is also present in the homologous genes from *Salmonella typhimurium*, *Thermus thermophilus*, and a few more bacteria. It is also present in *Aq437*, the *A. aeolicus* homologue of NuoG (Fig. 5). It has been proposed that the occurrence of the FeS cluster N1c may correlate with the ability to use menaquinone as a substrate in addition to ubiquinone [27]. Genes involved in ubiquinone biosynthesis are present in the *A. aeolicus* genome, while no homologues of *menA*, *B*, *D*, *F* and *G* are detectable ([28,29]; http://www.ncbi.nlm.gov/COG/). Thus, it is very likely that *A. aeolicus* complex I reacts exclusively with ubiquinone as substrate and that the presence of the FeS cluster N1c does not correlate with the ability of complex I to react with menaquinone.

In most prokaryotes, the structural genes coding complex I subunits cluster together in one operon or gene cluster. The gene order within this *nuo* cluster is conserved [10]. As one exception of this rule *A. aeolicus* contains several gene clusters containing homologues of complex I genes [16]. There is one locus containing the homologues of *nuoE* and *F* (*Aq573* and *574*) and another one containing *nuoG* (*Aq437*). These genes encode subunits that comprise the electron input part of complex I, namely the NADH dehydrogenase module [3]. This conserved module is present in many different enzymes and it is discussed that it has been acquired by complex I late in evolution [3,10,30]. The presence of these genes at two separate loci in *A. aeolicus* supports this idea. The residual complex I genes cluster in one large locus (*Aq1310* to *1322*) leading to the presence of three different *nuo* gene clusters in *A. aeolicus*. There are three more loci in the *A. aeolicus* genome containing *nuo* homologues ([16]; *Aq551*, *Aq866* and *Aq1373* to *1385*). These genes encode part of the complex I but their function is not yet clear. They may encode an isoform of complex I as it has been reported for cyanobacteria [4]. They do not contain a homologue of NuoB which is part of all members of a family of membrane-bound multi-subunit hydrogenases, which is closely related to complex I [3,31]. Therefore, the three additional *nuo* loci most likely do not encode such a hydrogenase. It is unlikely as well that these genes encode a member of the family of alkali/H^+ antiporters [30,32], which is related with complex I, because they contain a homologue of NuoI, which is not present in this type of antiporters.

As expected for a hyperthermophilic organism the enzyme activity raised with the temperature and exhibited an optimum around the natural growth temperature of 85°C. At these temperatures, the *E. coli* complex I is denatured. This cannot be envisaged by means of simple sequence comparison as phylogenetic calculations revealed that the homologous complex I genes of *A. aeolicus* and *E. coli* cluster fairly close together ([3]; http://www.ncbi.nlm.gov/COG/). The molecular basis of the hyperthermophily, especially for this huge membrane-bound protein complex, is still unknown [33] and may only be revealed by comparison of high resolution structures. The complex I from *A. aeolicus* seems to be a good starting material to obtain this highly desired structure.

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