Immunopurification of a subcomplex of the NAD(P)H-plastoquinone-oxidoreductase from the cyanobacterium *Synechocystis* sp. PCC6803

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An antibody against the NDH-K subunit of the NAD(P)H-dehydrogenase from the cyanobacterium *Synechocystis* sp. PCC6803 was used to isolate a subcomplex of the enzyme from Triton X-100 solubilized total membranes by immunoaffinity chromatography. The isolated subcomplex consisted of seven major polypeptides with molecular masses of 43, 27, 24, 21, 18, 14 and 7 kDa. The amino-terminal amino acid sequences of the polypeptides were determined. By comparing the sequences with the amino acid sequences deduced from DNA, three proteins were identified as NDH-H (43 kDa), NDH-K (27 kDa) and NDH-I (24 kDa). A fourth subunit (NDH-J, 21 kDa) was identified by Western blot analysis with an NDH-J antibody.

NAD(P)H-dehydrogenase; Cyanobacteria; *Synechocystis* sp. PCC6803; NDH-H; NDH-I; NDH-J; NDH-K

1. INTRODUCTION

The NADH-ubiquinone-oxidoreductase (complex I) is the first complex of the mitochondrial respiratory chain and translocates protons from the matrix to the intermembrane space. The enzyme is composed of about 30 different subunits in *Neurospora crassa* [1] and more than 40 subunits in bovine mitochondria [2]. In vertebrates, seven subunits are encoded in the mitochondrial genome [3].

Genes encoding homologues to eleven subunits have also been found in the plastid DNA of higher plants [4,5] and in cyanobacteria [6,7] and by analogy it has been concluded that here the enzyme may function as a NAD(P)H-plastoquinone-oxidoreductase [8,9]. The expression of several subunits of the cyanobacterial and plastid NAD(P)H-dehydrogenase has been demonstrated [10–13]. However, the enzyme has not yet been isolated from plants or blue-green algae and its function in chloroplast metabolism is unknown.

Another question concerns the molecular structure of the enzyme. Electron microscopy has revealed that complex I from *Neurospora crassa* has an L-shaped structure [14] and it has been shown that the enzyme can be subdivided in two parts: a peripheral arm, which protrudes into the mitochondrial matrix and a membrane arm which contains most of the mitochondrially encoded hydrophobic subunits [1]. However the exact arrangement and location of single subunits is not known.

We describe here the isolation of a subcomplex of a cyanobacterial NAD(P)H-dehydrogenase consisting of subunits NDH-H, -I, -J and -K. We propose that these polypeptides form a functional unit between the peripheral and the membrane arm.

2. MATERIALS AND METHODS

2.1. Growth of organism

Synechocystis sp. PCC6803 was grown as described in [15] in the presence of 1.25% glucose.

2.2. Isolation and solubilization of membranes

The cells from 6 l of culture were harvested by centrifugation (10 min, $3,000 \times g$), suspended in isolation buffer (100 mM Tris-HCl, pH 7.5, 400 mM sucrose, 1.3 mM $CaCl_2$ and 0.1 mM PMSF) and broken by two passages through a French pressure cell (Aminco) at 12.5 MPa. Intact cells were removed by a low-speed centrifugation (10 min, $1,500 \times g$) and the membranes were pelleted at $190,000 \times g$ for 90 min in a 45 Ti rotor (Beckman). The membranes were washed in isolation buffer and again centrifuged for 90 min at $190,000 \times g$.

The membranes were suspended in buffer (100 mM Tris/HCl, pH 7.5, 1.3 mM $CaCl_2$ and 0.1% PMSF) and adjusted to a protein concentration of 10 mg/ml. Solubilization was performed by slow addition of a 20% (w/v) Triton X-100 solution to a final concentration of 2% (w/v) and gentle stirring for 20 min on ice. The insoluble material was removed by centrifugation at $100,000 \times g$ for 60 min in a 80 Ti rotor (Beckman).

2.3. Immunoaffinity chromatography

The preparation of the antibodies against NDH-J and NDH-K has been described in [10]. The antibody against the NDH-K subunit was coupled to protein A-Sepharose as described in [16].

The Triton X-100 solubilized membranes were adjusted to 100 mM Tris/HCl, pH 7.5, 1% (w/v) Triton X-100, 150 mM NaCl, 0.65 mM $CaCl_2$, 5 mM EDTA and 0.1 mM PMSF (incubation buffer). The final protein concentration was 2.5 mg/ml. To 9 ml membranes, 1 ml anti-

body-protein A-Sepharose conjugate (equivalent to 300 mg dry weight Sepharose) was added. The incubation was performed at 4°C overnight with constant mixing. The Sepharose beads were pelleted by centrifugation, resuspended in incubation buffer and loaded into a Pharmacia FPLC-column (HR 5/5). The column was washed with 10 vols. of incubation buffer without $CaCl_2$ and then equilibrated with 10 mM potassium phosphate buffer pH 8.0 and 0.1% β-D-dodecylmaltoside. The bound proteins were eluted with 100 nM triethanolamine pH 11.0 and 0.1% β-D-dodecylmaltoside.

2.4. Electrophoresis and immunoblot analysis

SDS-polyacrylamide gel electrophoresis was performed according to Schägger and von Jagow [17] and silver staining according to Blum et al. [18]. For immunoblot analysis, the proteins were transferred to polyvinylidene difluoride membranes (PVDF, Immobilon-P, Millipore) using the 'semi-dry' blotting protocol of [19]. Antigen-antibody complexes were detected with [125]J-labelled protein A (Amersham Buchler).

2.5. Amino acid sequence analysis

The proteins were transferred to PVDF membranes using 10 mM cyclohexylaminopropanesulfonic acid pH 11.0 and 10% methanol as transfer buffer as recommended by LeGendre and Matsudaira [20]. The bands were stained with amido black and excised from the membrane. The bound proteins were subjected to automatic Edman degradation in the pulsed-liquid-protein-sequenator 477A with the online-PTH-amino acid analyser 120A from Applied Biosystems.

3. RESULTS

Two problems arise in purifying complex I-homologous NADH-dehydrogenases from prokaryotes. Firstly, prokaryotes contain two types of NADH-dehy-

drogenases [21]. Type I enzymes are multisubunit protein complexes, contain FMN, and are homologous to mitochondrial complex I. In contrast, type II enzymes consist of only one subunit with FAD as a prosthetic group. During purification, the activity of a type II enzyme may mask the activity of the type I enzyme. Secondly, prokaryotic complex I seems to be more labile than its mitochondrial counterpart and may decay or loose subunits during purification [22]. Therefore it is necessary to monitor the purification of the enzyme by other methods than activity alone. If the DNA sequence of a subunit is known, antibodies generated via expression vectors may provide this help.

In initial experiments antibodies against the NDH-J and the NDH-K subunits were used to follow up the purification of the enzyme by ion-exchange chromatography. However the NADH-oxidizing activity and the two immunologically identifiable subunits were found in different fractions, suggesting that the enzyme had desintegrated (data not shown). To circumvent these problems, the antibody against NDH-K was covalently coupled to protein A-Sepharose. The cross-linked complex was incubated with Triton X-100 solubilized total membranes of *Synechocystis* sp. PCC6803 and the bound proteins were then eluted at high pH and analysed by SDS-polyacrylamide electrophoresis.

Fig. 1 shows the protein pattern of the eluted proteins, stained with Coomassie brilliant blue (Fig. 1, lanes 1 and 2) or silver (Fig. 1, lanes 3 and 4). Seven
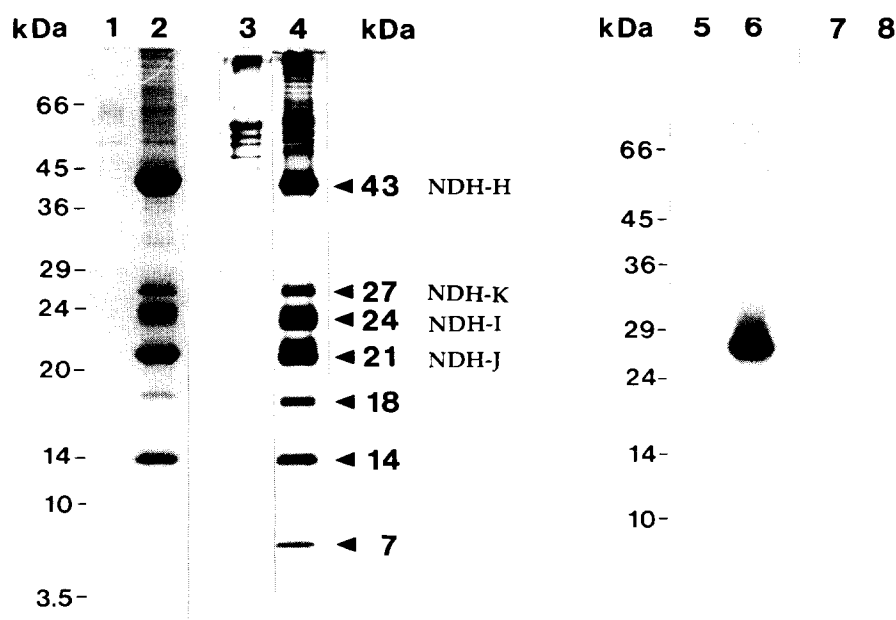

Fig. 1. SDS-gel electrophoresis (lanes 1-4) and immunoblot analysis (lanes 5-8) of the NAD(P)H-dehydrogenase subcomplex purified by immunoaffinity chromatography with an antiserum against NDH-K. In lanes 1 and 3 preimmune serum of NDH-K was used as a control. The gel was stained with Coomassie brilliant blue (lanes 1 and 2) or silver (lanes 3 and 4). The purified polypeptides were blotted onto PVDF membranes and tested with NDH-K antiserum (lane 5 and 6) and NDH-J antiserum (lanes 7 and 8). Lanes 5 and 7 correspond to the control with preimmune serum and lanes 6 and 8 to the incubation with NDH-K antiserum. Amounts of protein applied: lane 1, 3 and 5-8: 1 μg; lane 2: 10 μg, and lane 4: 3 μg.

bands with apparent molecular masses of 43, 27, 24, 21, 18, 14 and 7 kDa can be discerned. The proteins were not bound when preimmune serum was used (Fig. 1, lane 1 and lane 3). Proteins of low abundance above 45 kDa are due to unspecific binding as demonstrated by their cross-reaction with preimmune serum.

The identity of the purified proteins was investigated in two ways (Fig. 1 and Table I). An immunoblot of the polypeptides was tested with antisera against NDH-J and NDH-K. The NDH-K antibody reacted strongly with the band at 27 kDa as expected (Fig. 1, lane 6), while the NDH-J antibody recognized the 21 kDa protein (Fig. 1, lane 8). Secondly, the amino-terminal amino acid sequences of the proteins were determined and compared to the deduced amino acid sequences from the known *ndh*-genes of *Synechocystis* sp. PCC6803. The results of this comparison are shown in Table I.

The identity of the 27 kDa protein as NDH-K was confirmed; however the native protein seems to lack the first two amino acids. No amino acid sequence corresponding to NDH-J was found in the 21 kDa protein band by a search in the SWISS PROT database. Instead, the analysis revealed two amino acid sequences for which no known gene or protein could be identified.

Two other proteins could be clearly identified by the comparison of their amino acid sequences with the sequences deduced from the genes. The polypeptides at 43 kDa and at 24 kDa are NDH-H and NDH-I, respec-

tively. The native NDH-H subunit lacks the amino-terminal methionine. The sequences of the corresponding genes have been described recently [7,23].

As for the polypeptides at 21 kDa, no known homologous protein could be identified for the band at 18 kDa. However, the sequence of the 14 kDa polypeptide bears some similarity to the B13 protein of bovine heart complex I [24] as indicated in Table I.

4. DISCUSSION

It has been shown that antibodies against a single subunit of a multisubunit protein complex can precipitate the whole complex if the interaction of the subunits is strong enough to prevent decay [25,26]. Here, this approach was used to purify a subcomplex of the NAD(P)H-dehydrogenase of the cyanobacterium *Synechocystis* sp. PCC6803. The complex consisted of a minimum of seven subunits, four of which were identified as NDH-H, -I, -J and -K.

The sequences of several genes encoding subunits of the NAD(P)H-plastoquinone-oxidoreductase of *Synechocystis* sp. PCC6803 are known [6,7,23,27,28]. Among these genes are seven subunits (*ndhA-G*) of which homologue (ND1-ND4L-ND6) are encoded in the mitochondrial genome of vertebrates [3]. The *ndhF* gene has also been isolated and partially sequenced (Ellersiek and Steinmüller, in preparation). However, no corresponding proteins to these genes were found in the

Table I

Protein sequences of the subunits of the purified subcomplex

Apparent molecular mass (kDa)	Protein sequence (a) According to amino acid analysis (b) Deduced from DNA sequence	Subunit
43	(a)　　TKIETRTEPMVLNMGPH (b)　　MTKIETRTEPMVLNMGPH	NDH-H
27	(a)　　PNPANPT (b) MSPNPANPT	NDH-K
24	(a)　　MFNNILKQVGDYAKESLQAAKYIGQ (b)　　MFNNILKQVGDYAKESLQAAKYIGQ	NDH-I
21	(a1)　AEEVNSPNEAVNLQEETAI (a2)　TQRLKTLYQ-TI	? ?
	(b)　　VGPVSTWLTTNGFEHQSL	NDH-J
18	(a)　　SKTVKVVLNETINKLGF	?
14	(a)　　MLVKSTTRHVRIFSAEVQG (c) MAGLLKKTTGLVGLAVCETPH (　)　　 *　*　**　*　　　*	B13 ?

The sequences from the amino-terminal amino acid analysis are compared to the sequences deduced from the DNA sequence of the corresponding genes published in [6,7,23]. From the 21 kDa band two sequences were obtained: a dominant sequence (a1) and a side sequence (a2). For the 14 kDa polypeptide the sequence of the B13 protein from bovine mitochondria is listed under (c) and identical amino acids are marked by asterisks.

purified subcomplex. This is a further indication for the decay of the enzyme during purification as mentioned earlier.

Interestingly, the cyanobacterial subcomplex contains four subunits (NDH-H, -I, -J and -K) of which homologous proteins are encoded in the plastid genome, while in *Neurospora crassa* and in vertebrates the corresponding genes are found in the nucleus [1,2]. At least four more proteins for which no known homologous proteins are known could be identified in the subcomplex. Two of them have a similar apparent molecular mass and run together during SDS-gel electrophoresis at 21 kDa (Table I). Also at 21 kDa runs the NDH-J subunit as was demonstrated by immunoblot analysis. The amino-terminus of NDH-J may be blocked, so that no sequence could be obtained. Therefore it is possible that the band at 21 kDa comprises three different polypeptides. The two other proteins which could not be identified have molecular masses of 18 and 7 kDa. For the 14 kDa polypeptide, some similarity to the B13 protein of bovine complex I was observed (Table I). However, whether or not these proteins are truly related can only be decided when the complete sequence is known.

Thus, adding the total number of polypeptides identified in the subcomplex [4] to the number of polypeptides encoded by already identified genes *ndhA-F* and *ndhL* [8] the NAD(P)H-dehydrogenase of *Synechocystis* sp. PCC6803 contains at least 12 subunits. It should be noted that subunits homologous to the 51, 78 and 24 kDa subunits which have important functions in the mitochondrial enzyme (NADH- and FMN-binding, carriers of iron-sulfur clusters) have not yet been identified in cyanobacteria. These subunits are present in the prokaryotic enzymes from *Paracoccus denitrificans* and *E. coli* [21,29].

The high complexity of the NADH-dehydrogenases from mitochondria has led to a search for ways to break up the enzyme in defined subfractions. In the first studies the complex was desintegrated with chaotropic anions. This fractionation yielded three subcomplexes; the flavoprotein fragment with three subunits, one of them containing FMN and the NADH-binding site; the iron-protein fragment which comprises six subunits, some of them containing iron-sulfur clusters and a hydrophobic fraction, consisting mainly of membrane proteins of unknown function [30,31].

Our preparation has many similarities in common with the iron-protein fragment. The polypeptide composition of this fragment was initially thought to consist of six proteins with molecular masses of 75, 49, 30, 18, 15 and 13 kDa [30,31]. The 49 kDa protein is homologous to NDH-H [32] and the 30 kDa protein to NDH-J [33,34]. Recently, Masui et al. [35] have suggested that two more subunits belong to this fragment: a protein of 20 kDa (in the bovine enzyme) which is homologous to NDH-K [35,36] and the B13 protein [24,35]. Thus at
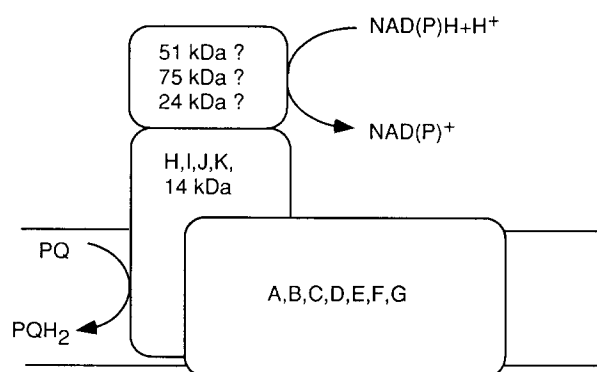

Fig. 2. Schematic representation of the cyanobacterial and plastidial (?) NAD(P)H-dehydrogenase. The drawing is in analogy to the mitochondrial complex I as depicted in [1,2]. Subunits A–G are shown as components of the membrane arm. Subunits H–K and the 14 kDa protein (B13 homologue) are part of the isolated subcomplex. No proteins homologous to the 51, 78 and 24 kDa subunits of the mitochondrial complex have yet been identified in cyanobacteria.

least three, or if the 14 kDa polypeptide (B13 homologue) is included, maybe four polypeptides, are the same in the iron-protein fragment and our preparation. It may be that further proteins can be added when the complete sequences of the two polypeptides at 21 kDa and the 18 and the 7 kDa proteins are known.

One remaining problem is the localization of NDH-I. The homologous mitochondrial subunit, the 23 kDa protein (or TYKY as characterized by its first four amino acids according to Walker [2]) is a likely candidate for the iron-sulfur cluster N-2 [37], the cluster donating electrons to ubiquinone [1,38]. This subunit is found in the hydrophobic fraction [31] and in the membrane arm of the enzyme from *Neurospora crassa* [38]. However, recently Finel et al. [39] described a fractionation procedure of complex I from bovine heart by the detergent lauryldimethylamine oxide (LDAO), which splits the enzyme into two subcomplexes, $I\alpha$ and $I\beta$. $I\alpha$ contains FMN, the NADH-binding site and mainly hydrophilic subunits and thus corresponds in many features to the peripheral arm of the enzyme from *Neurospora crassa*, while $I\beta$ is composed of 13 polypeptides, nine of which contain one or more hydrophobic domains, and therefore resembles the membrane arm of the fungal enzyme. Interestingly, in this preparation NDH-I is found in the subcomplex $I\alpha$.

Thus, it seems that the subcomplex that we have isolated from *Synechocystis* sp. PCC6803, represents a connecting part between $I\alpha$ (or the peripheral arm) and $I\beta$ (or the membrane arm). A schematic representation of the cyanobacterial NAD(P)H-dehydrogenase is presented in Fig. 2. Further research will be necessary to identify the NAD(P)H-oxidizing part of the enzyme.

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