Cyanobacteria contain a mitochrondrial complex I-homologous NADH-dehydrogenase

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Thylakoid and cytoplasmic membranes of the cyanobacterium *Synechocystis* sp. PCC 6803 were purified by sucrose gradient centrifugation. Both membranes oxidize NADH in a rotenone-sensitive reaction. Antibodies prepared against *psbG/ndhK* and *ndhJ* fusion proteins detect the corresponding polypeptides in both membrane preparations. This demonstrates that a NADH-dehydrogenase, homologous to the mitochondrial NADH-ubiquinone-oxidoreductase (complex I of the respiratory chain) is present in cyanobacteria. The NADH-dehydrogenase can be solubilized with the detergent β-D-dodecylmaltoside. Sedimentation analysis of the solubilized enzyme on a sucrose gradient indicates that it is a multisubunit protein complex.

1. INTRODUCTION

Respiratory and photosynthetic electron transport in cyanobacteria takes place on the thylakoid or intracytoplasmic membranes [1–3]. Recently, subunits of the cytochrome *c* oxidase and the cytochrome b_6/f complex have been identified on the thylakoid and the cytoplasmic membranes of *Anacystis nidulans* [4,5], which suggests that there is a respiratory chain also on the cytoplasmic membrane. However, the presence of a NADH-dehydrogenase complex on both membranes has not been demonstrated conclusively so far.

We have reported on the isolation of the *ndhC-psbG-ORF157* operon from *Synechocystis* sp. PCC 6803 [6]. Since the *ndhC* gene is homologous to the ND3 gene of mammalian mitochondrial DNA, encoding subunit 3 of the mitochondrial NADH-ubiquinone-oxidoreductase (complex I of the respiratory chain), this provided the first evidence for a complex I-homologous enzyme in cyanobacteria. Recently, it has been demonstrated that a homologue to *ORF157* encodes a subunit of the same complex in *Neurospora crassa* and mammals [7,8], and the gene has been named *ndhJ* [8]. The *psbG* gene product (PSII-G) was originally characterized as a subunit of photosystem II [9]. However, there is strong evidence that it specifies a component of the complex I-homolgous NADH-dehydrogenase of plastids [10]. This view is further supported by the presence of

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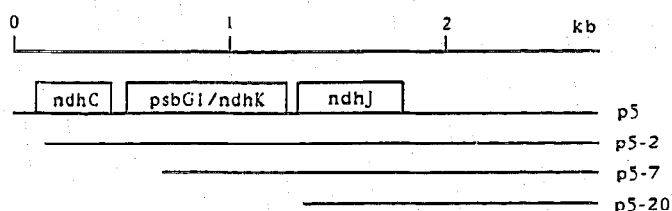
reading frames homologous to *psbG* in *E. coli* [11] and in the mitochondrial genome of *Paramaecium aurelia* [12]. Moreover, the growth of mutants of *Synechocystis* 6803, that possess a defective *psbG1* gene, is impaired in a medium containing glucose, but not under autotrophic growth conditions [13].

Here we report on the identification of the *psbG* and *ndhJ* gene products on the thylakoid and cytoplasmic membranes of *Synechocystis* 6803. We present evidence that PSII-G is a component of the cyanobacterial NADH-dehydrogenase and therefore propose to rename *psbG* into *ndhK*. Our experiments indicate that the cyanobacterial NADH-dehydrogenase is a multisubunit protein complex and related to the mitochondrial NADH-ubiquinone-oxidoreductase.

2. MATERIALS AND METHODS

2.1. Growth of Synechocystis

Synechocystis sp. PCC6803 was grown as described in Dzelzkalns and Bogorad [14] in the presence of 1.25% glucose.

2.2. Isolation of thylakoid and cytoplasmic membranes

Harvested cells were suspended in isolation buffer (100 mM Tris-Cl, pH 7.5, 400 mM sucrose, 1.3 mM $CaCl_2$ and 0.1 mM PMSF) and broken by passage through a French pressure cell at 12.5 MPa. Intact cells were removed by a low-speed centrifugation (10 min, 1500 × g) and the membranes were pelleted at 190000 × g for 90 min. After resuspension in isolation buffer, the thylakoid and the cytoplasma membranes were separated by sucrose gradient centrifugation for 16 h at 110000 × g in a SW28 rotor (modified after [15]). The gradient consisted of four layers: 11 ml of a 58% sucrose cushion, 9 ml membrane suspension adjusted to 40% sucrose and 9 ml of a 30%, and 8 ml of a 10% sucrose layer. Thylakoid membranes remained at the 40/58% sucrose interphase, while cytoplasmic membranes moved into the 30% sucrose layer.

0   1   2   kb

| ndhC | | psbGl/ndhK | | ndhJ |

——————————————————————————————— p5

——————————————————————————————— p5-2

————————————————————————————— p5-7

————————————————————— p5-20

Fig. 1. Map of the *ndhC-psbGl/ndhK-ndhJ* operon of *Synechocystis* 6803 and of the deletion clones that were used to construct the fusion proteins. *Synechocystis* 6803 contains two *psbG* genes; *psbG2* is a cryptic gene and not expressed in wild-type cells [13]. The fusion proteins covered the following amino acids: (i) 21–120 (*ndhC* fusion, pGX5-2); (ii) 61–248 (*psbG/ndhK* fusion, pGX5-7; and (iii) 6–157 (*ndhJ* fusion, pGX5-20).

2.3. *Sucrose gradient centrifugation of the solubilized NADH dehydrogenase*

The total membrane pellet was suspended in isolation buffer and the membranes were solubilized by addition of β-D-dodecylmaltoside to a final concentration of 1% (w/v) and gentle stirring for 30 min at 0°C. The insoluble material was removed by centrifugation at 100000 $\times g$ for 1 h and the supernatant was loaded on a linear sucrose gradient (5–32%) that contained 0.1% dodecylmaltoside. Centrifugation was for 16 h at 110000 $\times g$.

2.4. *Construction of fusion proteins*

The inserts of the deletion clones p5-2, p5-7 and p5-2 ([6] and Fig. 1) were cloned between the *Eco*RI and *Hin*dIII sites of the expression plasmids pGEMEX1 or 2 (Promega), resulting in a fusion of the reading frames of *ndhC, psbGl* and *ndhJ* to the first 260 amino acids of the bacteriophage T7 gene 10 protein. The obtained plasmids: pGX5-2 (*ndhC*-fusion), pGX5-7 (*psbGl*-fusion) and pGX5-20 (*ndhJ*-fusion) were transformed into *E. coli* JM109(DE3) and expressed according to the manufacturer's protocol. The fusion proteins were purified by preparative SDS-gel electrophoresis [16]. Antibodies were raised in rabbits using standard procedures [17].

2.5. *Miscellaneous methods*

NADH-dehydrogenase activity was measured as NADH oxidation at 340 nm. The reaction mixture consisted of 100 mM Tris-HCl, pH 8.5, 1.3 mM CaCl₂, 140 μM NADH and 100 μM DBMIB (2,5-dibromomethylisopropylbenzoquinone). The reaction was started by adding the membranes. For determination of rotenone-sensitivity, the start was with DBMIB. The chlorophyll content was measured according to Moran [18] and the protein concentration was determined after Bradford [19]. SDS-polyacrylamide electrophoresis was performed using the gel system described by Schägger and Von Jagow [20]. For immunoblot analysis, the proteins were transferred to polyvinylidene difluoride membranes (PVDF; Immobilon-P, Millipore). Antigen–antibody complexes were detected with ¹²⁵I-labeled protein A (Amersham Buchler).

3. RESULTS AND DISCUSSION

3.1. *Thylakoid and cytoplasmic membranes oxidize NADH in a rotenone-sensitive reaction*

The NADH-oxidizing activities of the soluble protein fraction and the membrane fractions of *Synechocystis* 6803 are presented in Table I. The thylakoid and cytoplasma membranes were separated by centrifugation in a sucrose step gradient. The purity of both membrane preparations was analysed by recording absorption spectra (data not shown). Thylakoid membranes contained about 169 μg chlorophyll per mg protein,

Table I

NADH-oxidizing activity of the soluble protein and the membrane fractions of *Synechocystis* 6803

	NADH-DBMIM-oxidizing activity		
	Without rotenone	With 40 mM rotenone	% inhibition
Soluble proteins (190000 $\times g$ supernatant)	246	165	33
Total membranes (190000 $\times g$ pellet)	177	122	31
Thylakoid membranes	218	109	50
Cytoplasma membranes	2678	1821	32

The activity is expressed in mU per mg protein. The values are mean values from four to six independent determinations

while cytoplasmic membranes were essentially free of chlorophyll. The NADH-oxidizing activity of cytoplasmic membranes was found to be about 12 times higher on a protein basis than for thylakoid membranes. In both cases the activity was sensitive to rotenone, though only to 32% and 50%, respectively (Table I). This might reflect the presence of an alternative, rotenone-insensitive NADH-dehydrogenase as described for plant mitochondria [21]. Alternatively, since the cyanobacterial NADH-dehydrogenase uses plastoquinone or phylloquinone as natural electron acceptors [22], rotenone as a competitive inhibitor specifically for ubiquinone-reducing enzymes may not be as effective.

3.2. *PSII-G/NDH-K and NDH-J are present in the thylakoid and the cytoplasma membrane*

The separation of cytoplasmic and thylakoid membrane proteins by SDS-gel electrophoresis, followed by silver staining, revealed differences and similarities in the protein composition of both membranes (Fig. 2A). There are proteins that are unique for each membrane type, but there are also proteins that can be found in both membranes. Because of the small contribution of the cytoplasma membrane to the overall membrane content of a cyanobacterial cell, the protein pattern of a total membrane preparation seems almost identical to that of the thylakoid membranes (Fig. 2A, lanes 2, 3).

As indicated in the introduction, evidence from genetic data point to the presence of a NADH-dehydrogenase in *Synechocystis* 6803, that is homologous to the mitochondrial complex I. To obtain antibodies against subunits of this enzyme, parts of the reading frames of *ndhC, psbGl/ndhK* and *ndhJ* were used to construct fusion proteins as described in Fig. 1. The *psbGl/ndhK* and the *ndhJ* fusion proteins were expressed at high levels in *E. coli* and were subsequently used to immunize rabbits. However, no expression was obtained with the relatively hydrophobic *ndhC* fusion protein.
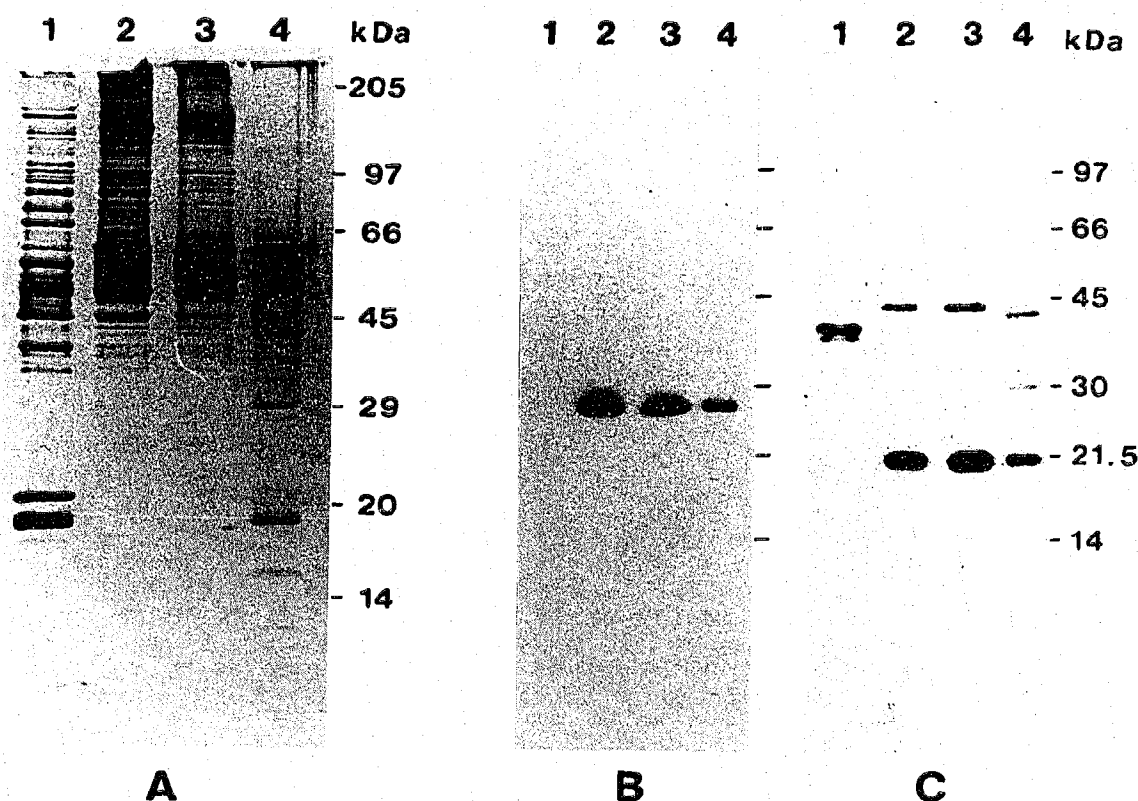
Fig. 2. SDS gel electrophoresis and immunoblot analysis of the soluble protein and the membrane fractions of *Synechocystis* 6803. The proteins were silver stained (A) or blotted onto PVDF membranes and tested with antibodies against PSII-G/NDH-K (B) and NDH-J (C). Each lane contained 4 μg (A), 8 μg (B) or 16 μg (C) protein. (Lane 1) Soluble proteins; (lane 2) total membranes; (lane 3) thylakoid membranes; and (lane 4) cytoplasma membranes. An explanation for the additional bands between 30 and 45 kDa in (C) is given in the text.

The soluble protein fraction and the membrane preparations of *Synechocystis* were tested with antibodies against the *psbG1/ndhK* and *ndhJ* fusion proteins. The calculated molecular weights for PSII-G/NDH-K and NDH-J are 27.3 kDa and 20.8 kDa, respectively. Both antibodies recognize signals of the expected size in thylakoid and cytoplasmic membrane preparations (Fig. 2B,C, lanes 3, 4). While the antibody against PSII-G/NDH-K labels a single band, the antibody against NDH-J detects one or two different bands at higher molecular weights in membrane preparations and also reacts with the 190000 × g supernatant (Fig. 2C, lane 1). The NDH-J protein is a relatively hydrophilic subunit of the so-called 'iron–protein fragment' of the NADH-dehydrogenase [8] and has probably only one membrane-spanning domain [6]. Thus, it is easily solubilized, and in its soluble form it tends to aggregate with other proteins (data not shown).

3.3. *The cyanobacterial NADH-dehydrogenase is a multisubunit protein complex*

The mitochondrial NADH-ubiquinone-oxidoreductases of *Neurospora crassa* and mammalians consist of about 25 non-identical subunits and contain FMN and iron-sulfur clusters [23,24]. In order to investigate the complexity of the NADH-dehydrogenase from *Synechocystis* 6803, total membranes were solubilized with the detergent β-D-dodecylmaltoside and separated on a linear sucrose gradient. The highest NADH-oxidizing activity was detected in the middle of the gradient, just above the chlorophyll-containing photosystems (Fig. 3A). An immunoblot analysis demonstrates that the distribution of PSII-G/NDH-K and NDH-J follows the activity profile (Fig. 3B,C). This reinforces the view, that PSII-G is a component of the NADH-dehydrogenase and therefore we propose to rename *psbG* into *ndhK*.

The migration of the NADH-dehydrogenase in the sucrose gradient indicates that the enzyme is a protein complex, which consists of multiple subunits, like the mitochondrial counterpart. This conclusion is further supported by the isolation and sequencing of genes encoding *ndhH, -A, -I, -G, -E, -D* and *-F* from *Synechocystis* 6803 (Steinmüller et al., in preparation).

In procaryotes, there appear to be at least two different classes of NADH-dehydrogenases [25]. Enzymes of the first class use FAD as a prosthetic group and are usually composed of a single polypeptide. Enzymes of this type have been described for *Escherichia coli* [26], *Bacillus subtilis* [27], or *Rhodopseudomonas capsulata* [28]. The second class consists of enzymes, that are
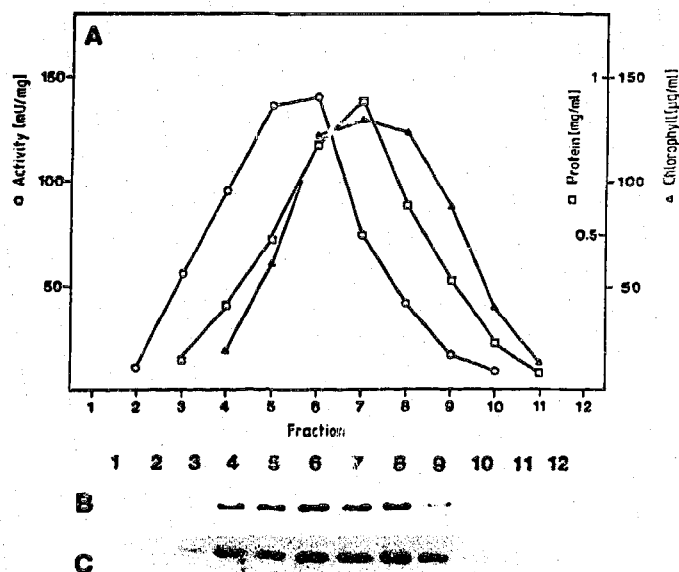
Fig. 3. Sedimentation analysis of the β-D-dodecylmaltoside solubilized membranes of *Synechocystis* 6803 on a sucrose gradient. The NADH-oxidizing activity, the protein and the chlorophyll concentrations in (A) were determined as described in section 2. Aliquots of each fraction, containing 2.4 μg protein, were separated by SDS gel electrophoresis, blotted onto a PVDF membrane and tested with antibodies against PSII-G/NDH-K (B) and NDH-K (C).

composed of about 10 different subunits and that contain FMN and iron–sulfur clusters. Examples are the enzymes of *Paracoccus denitrificans* [29] or *Thermus thermophilus* [25]. The characteristics of the latter enzymes point to a relation to the NADH-ubiquinone-reductase of mitochondria. However, this has yet to be confirmed by a search for homologous subunits. The NADH-dehydrogenases isolated so far from cyanobacteria [30,31] belong to the first class, since they contain FAD and are composed of only one subunit. The results presented in this paper clearly indicate that cyanobacteria also possess an enzyme, that is related to the mitochondrial NADH-ubiquinone-oxidoreductase. The location of this enzyme on the cytoplasma and the thylakoid membrane confirms earlier suggestions that each membrane system carries a complete respiratory chain [1] and raises interesting questions as to the interaction and regulation of photosynthetic and respiratory electron transport at the molecular level in cyanobacteria.

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