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Studies of cyclic electron transport in bundle sheath chloroplasts and isolation of the NDH complex from chloroplasts of *Zea mays*

C. Darie, F. Drepper, B. Mutschler, S. Mauch, P. Hörth, M. Biniossek, W. Haehnel

Institut für Biologie II / Biochemie, University of Freiburg, Schänzlestr. 1, 79104 Freiburg, Germany, Fax: +49 761-203 2601, e-mail: haehnel@uni-freiburg.de

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Introduction

The eleven genes on the plastid genome encoding subunits homologue to those of the NADH dehydrogenase or complex I of mitochondria and bacteria are highly conserved in most plants. Their function as a proton-pumping NADH:plastoquinone oxidoreductase has been suggested by Friedrich et al. (1995). A structural model indicates that the plastid *ndhA – ndhG* gene products form the integral membrane subcomplex and the *ndhH – ndhK* gene products a hydrophilic connecting subcomplex probably mediating the electron transfer from NAD(P)H. Subunits homologue to the three peripheral subunits of the NADH-oxidizing domain should be nuclear encoded but have not been identified so far. NADH and not NAD(P)H or ferredoxin has been reported to function as electron donor, Sazano et al. (1998). A role in the chlororespiratory (Casano et al. (2000)) and cyclic electron transport in the dark and light, respectively, has been suggested. But several studies with deleted or inactivated NDH genes could not provide evidence for an essential function of this complex, Burrows et al. (1998), Kofer et al. (1998), Shikanai et al. (1998), Horváth et al. (2000). The C4 plants *Sorghum bicolor* and *Zea mays* have bundle sheath chloroplasts with photosystem (PS) I and very low amounts of PS II, Kubicki et al. (1996), Funk et al. (1999). In these organelles PS I has to function in cyclic electron transport to provide extra ATP needed for the Calvin cycle. The difference in redox potential between ferredoxin and plastocyanin driven by PS I is even larger than that of NADH and cytochrome (cyt) *c* in mitochondria driving proton translocation in complexes I and III (cyt *bc* complex). Cyclic electron transport involving the plastid encoded NDH complex would double the ATP synthesis if compared to a cycle with the cyt *bf* complex alone. However, it has been difficult to detect substantial turn-over rates of the NDH complex *in vivo* and *in vitro*. To contribute to the understanding of the function of this large complex in chloroplasts we have isolated the complex from *Zea mays* chloroplasts, tried to identify its subunit composition, and to measure cyclic electron transport in bundle sheath chloroplasts under well defined conditions.

Materials and methods

Antibodies. Full length cDNA was PCR-amplified and cloned into pBluescript KS. After sequencing for PCR errors and editing sites the genes were inserted into the vector pGEX-6P-2 and the GST-fusion proteins overexpressed in *E. coli.* The fusion proteins were purified by preparative SDS-PAGE, electroeluted from excised gel bands and used to raise antisera in rabbits (Charles River).

Isolation of chloroplasts and the NDH complex. Isolation of bundle sheath chloroplasts followed the procedure described by Kubicki et al. (1994). Intact bundle sheath and mesophyll chloroplasts were collected on a step gradient of 30 and 40 %, respectively, and 80 % (w/v) percoll. Isolation of the NDH complex from chloroplasts of *Zea mays* followed the initial stages of the procedure decribed by Funk et al. (1999), using the upper 2/3 of the second leaf of 14 days old maize plants. To remove most of the peripheral part of ATP-synthase the thylakoid membranes were washed either once or twice with buffered 0.75 mM EDTA, pH 8. For solubilization with 1 % (w/w) Triton X-100 the thylakoid membranes were suspended at 2 mg Chl/mL in solubilization buffer (50 mM MES buffer, pH 6, 15 mM NaCl, 5 mM $MgCl_2$). After precipitation of non-solubilized membranes by ultracentrifugation for 45 min in a Beckman Ti60 rotor at 50,000 RpM the supernatant was concentrated 3-4 times by ultrafiltration through XM300 membranes (Millipore). Aliquots of 2 ml were loaded on 24 ml of a 0.1 – 1.3 M sucrose gradient in solubilization buffer with addition of 0.2 % (w/w) Triton X-100 and centrifuged for 10 h in a Beckmann Ti60 rotor at 50,000 RpM. The bands with NDH were collected and run on blue native gels (Schägger et al., 1991). After SDS/PAGE with the Tris/Tricine buffer system in the second dimension proteins were electroblotted onto poly(vinylidene difluoride) (PVDF) membranes. Immunodetection was performed by using an ECL immunoblotting kit (Amersham) according to manufacturer instructions.

Light-induced kinetic measurements. Chloroplasts were suspended at a final concentration of 45 µM chlorophyll (standard condition) in 0.35 M sorbitol, 20 mM HEPES pH 8, 10 mM EDTA. 1 mM $MgCl_2$, 10 % Ficoll, 2 µM gramicidin D. Broken chloroplasts were obtained by incubation for 15 s in 0.1 M sorbitol, 20 mM HEPES pH 8, 10 mM EDTA, 1 mM $MgCl_2$ before the standard conditions were adjusted. Absorbance changes induced by laser flashes (5 ns FWHM) at 532 nm were detected with a photodiode protected by interference filters and averaged if necessary.

Results and discussion

The NDH complex was isolated from mesophyll chloroplasts of maize without anion exchange chromatography. After sucrose density gradient centrifugation of Triton X-100 solubilized thylakoid membranes the NDH complex was collected at higher density than the chlorophyll containing complexes PS I, PS II and LHCII. The fractions identified by antibodies were transferred to a blue native PAGE, shown in Fig 1, top. Three bands can be seen. The immunoblot of the 2nd dimension SDS-PAGE shows that band 1 with a mass of about 1 MDa contains all detected subunits. Band 2 originates from residual α and β subunits of ATPase (not shown). Band 3 contains a partial complex of NDH subunits without NdhJ and NdhE. The broad band of NdhE suggests some disconnection from the hydrophobic core of the complex. Band 1 has been collected and subjected also to Tricine-SDS-PAGE for high resolution of low molecular weight proteins as shown in Fig. 2. A Western blot of this type of gel (not shown) indicates that NdhH migrates with band 5 and NdhE close to

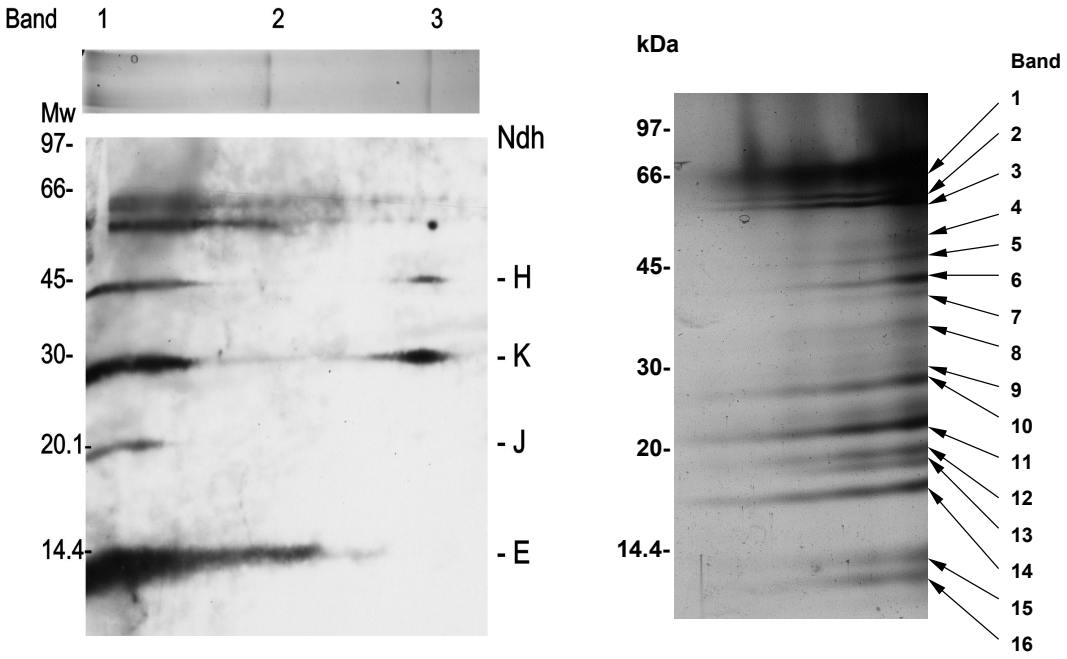

Fig. 1. (left) Horizontal lane on top: Blue native PAGE of the NDH containing fractions from a sucrose gradient, run from left to right. Below, 2nd dimension of the lane on top run on SDS-PAGE. The proteins were electroblotted onto PVDF membrane and immunodecorated with antisera against GST-fusion proteins of NdhE, NdhH, NdhJ, and NdhK labeled E, H, J, K, resp.
Fig. 2. (right) Band 1 from blue native PAGE has been collected and run on a Tricine-SDS-PAGE. The proteins are visualized by Coomassie blue stain.

band 16. Previous studies report a molecular mass of 580 kDa of the NDH complex which may indicate that we observe a dimer in Fig 1.

Mass spectrometry. For mass spectrometry the bands of gels as that in Fig. 2 have been excised and after digestion with trypsin subjected to MALDI mass spectrometry. Fig. 3 shows a thin-layer MALDI spectrum of a trypsin digest of band 5 from a gel

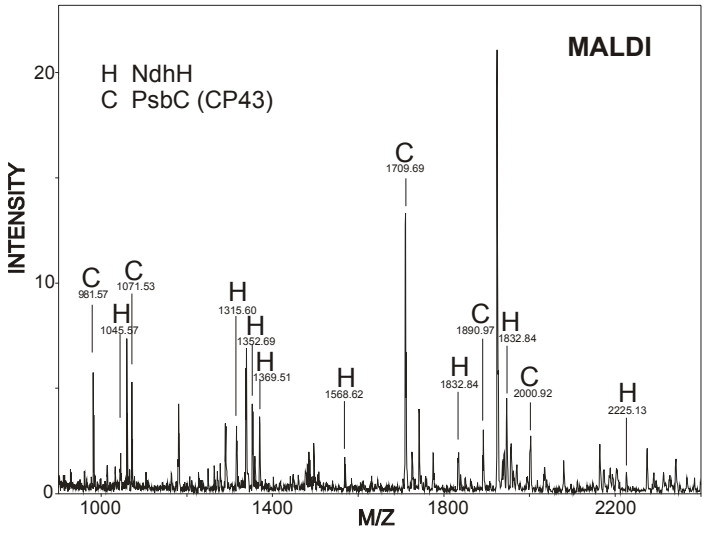

Fig. 3. MALDI mass spectra of a trypsin digest of band 5 from Tricine SDS-PAGE similar to that shown in Fig. 2. H and C label peaks attributed to NdhH and PsbC, respectively. The mass spectrometer (Reflex III, Bruker) was externally calibrated with a mixture of angiotensin I and II, substance P, bombesin, ACTH 1-17, ACTH 18-39, and somatostatin 28.

similar to that shown in Fig. 2 with a matrix of α-cyano-4-hydroxycinnamic acid mixed with nitrocellulose. More than 8 peptides could be attributed

to NdhH with an error <0.1 Da or <80 ppm. This is in agreement with the finding that the band is recognized by antibodies against NdhH. In addition we could identify PsbC (CP43), a core protein of PS II. A contamination by this subunit is surprising because our detergent concentration should not be sufficient to solubilize PS II. The high resolution of the mass spectrum made it possible to detect the two proteins in the same sample. Future work may help to identify also the other subunits even in the presence of contamination by other proteins.

Amounts of active photosystems in bundle sheath and mesophyll chloroplasts. In most of the previous studies antibodies have been used to detect the relative amounts of photosystems in bundle sheath and mesophyll chloroplasts. Light-induced absorbance changes of P700 can detect active photosystems and their interaction *via* the electron transfer chain (Haehnel, 1976). Fig. 4 A, C and B, D show light-induced absorbance changes at 820 nm observed in bundle sheath and mesophyll chloroplasts, resp. The amount of PS II connected to PS I is detected by the amount of $P700^+$ reduced after a saturating flash following far-red preillumination. For bundle sheath chloroplasts Fig. 4 A, signal SF, shows an amplitude smaller than 2 % of total P700. Total P700 is observed under reducing conditions at high time resolution, Fig. 4 C. P700 is reduced under these conditions by plastocyanin with half lives of 10 and 200 µs. A long flash of 50 ms, Fig. 4 A, LF, accumulates electrons in the plastoquinone pool until a steady state is reached. The amount of PS I being reduced by PS II increased to more than five fold as compared to the single flash. This indicates a connection of the small amount of PS II to the plastoquinone pool in bundle sheath

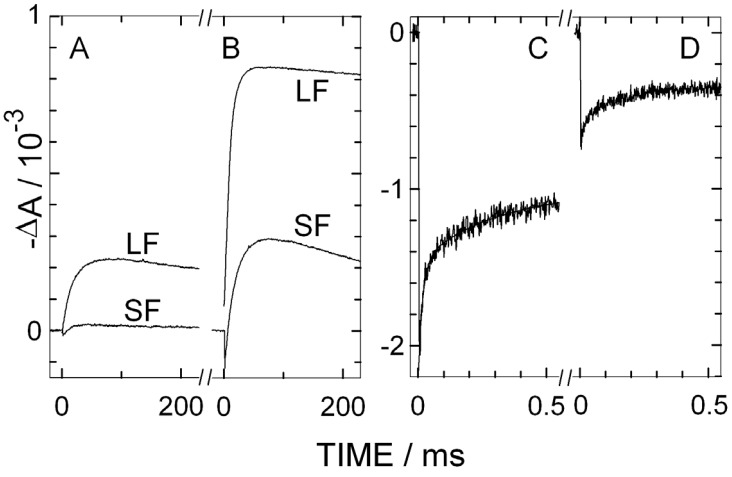

Fig. 4. Flash induced absorption changes at 820 nm in broken chloroplasts isolated from bundle sheath (A, C) and mesophyll cells (B, D). **A, B**: continuous far-red light (704 nm, FWHM 5 nm) in the presence of 0.2 mM methyl viologen. Excitation by a laser flash (SF) or a long flash of ca. 50 ms (LF) (BG39 filter, Schott) terminated at time zero. **C, D**: Time course induced by a laser flash in the presence of 15 mM sodium ascorbate.

chloroplasts. Fig. 1 B and D present the same experiments with mesophyll chloroplasts. The amplitude after a long flash is about two times larger than that after a single flash. After a long flash significant amounts of plastocyanin contribute to the amplitude at 820 nm. The amplitudes in Fig. 4 C and D indicate that bundle sheath chloroplasts contain ca. 2.5 – 3 times more PS I per chlorophyll than mesophyll chloroplasts.

Cyclic electron transfer around photosystem I. The high amount of PS I and a negligible one of PS II in bundle sheath chloroplasts present a well defined situation for a study of cyclic electron transport. Electron transfer from NADH or NADPH is only efficient in ATP production when two energy converting steps are involved both transferring protons *i.e*. the NDH and the cyt b*f* complex. Fig. 5 shows the superposition of four signals of P700 induced by groups of three laser flashes spaced at 200 ms. The dark period before the flash was long enough to ensure reduction of P700. The first two flashes of the group oxidize most of plastocyanin responsible for the fast reduction of P700 not resolved in this experiment. Traces A and B show the oxidation of P700 in the flashes and a reduction with a half-life of ca. 50 ms. DCMU has only minor effects. Inhibition of the cyt *bf* complex with DBMIB (trace C) demonstrates that the 50 ms component represents electron transfer through the cyt *bf* complex. The fast electron transfer from reduced plastocyanin is still observed. Addition of methylviologen slows down the reduction of the electron carriers to negligible rates. Methylviologen transfers the electrons from PS I to oxygen and not

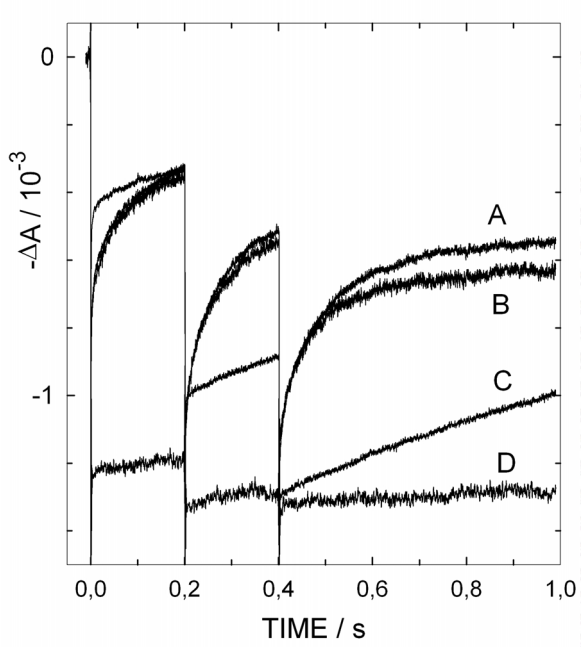

Fig. 5. Absorption changes at 820 nm induced by three laser flashes in intact bundle sheath chloroplasts after dark adaptation for 5 min. Time courses shown were measured under standard conditions (**A**), or after addition of 20 µM DCMU (**B**), 10 µM DBMIB (**C**), or 0.2 mM methyl viologen (**D**).

to $NADP^+$. The oxidation of plastoquinone at the cyt *bf* complex observed in Fig. 4 A and C has a faster halftime of 6-10 ms. This suggests a limiting turnover rate of cyclic electron transfer with a halftime of ca. 50 ms. However, this is still indirect evidence and further experiments have to show the involvement of the NDH complex.

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