Evidence for an association of *ndh* B, *ndh* J gene products and ferredoxin-NADP-reductase as components of a chloroplastic NAD(P)H dehydrogenase complex

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Abstract Using non-denaturing gel electrophoresis and staining with nitro-blue tetrazolium, we reveal the presence of two NAD(P)H oxidoreductase activity bands within thylakoids membranes of *Solanum tuberosum* L. Second dimension SDS-PAGE and Western analysis show that one of the activity bands contains several polypeptides, two of them being recognized by antibodies directed against peptides corresponding to conserved domains of chloroplastic genes products NDH B and NDH J (at 32 and 18 kDa, respectively). Both activity bands also contain a polypeptide (around 36 kDa) recognized by an antibody directed against ferredoxin-NADP$^+$-reductase (FNR). We conclude from these results that both chloroplastic *ndh* B and *ndh* J gene products are components of a thylakoid NAD(P)H dehydrogenase complex. The association with FNR is suggested to allow the complex to use NADPH instead of NADH as a preferential substrate.

Key words: Chlororespiration; Cyclic electron flow; Ferredoxin-NADP$^+$ reductase; NADH dehydrogenase; *ndh* gene; Photosynthesis; *Solanum tuberosum* L.

1. Introduction

Sequencing of different chloroplastic higher plant genomes has revealed the existence of a set of genes showing significant homologies with genes coding for subunits of the mitochondrial NADH dehydrogenase complex [1,2]. These chloroplastic genes, named *ndh*, have been shown to be transcribed [3] and the polypeptide products of three of them (NDH H, NDH I and NDH K) have been found in thylakoid membranes [4,5,6]. It has been suggested that *ndh* genes encode subunits of a chloroplastic NAD(P)H dehydrogenase complex [1] which might be involved in chlororespiration, a chloroplastic respiratory process mainly studied in the unicellular green alga *Chlamydomonas* [7,8]. Alternatively, the putative NAD(P)H dehydrogenase complex might participate in cyclic electron flow around photosystem 1 (PS1) by allowing electrons to enter the plastoquinone (PQ) pool from a stromal NAD(P)H pool [9,10]. NADH dehydrogenase complexes from mitochondria [11], cyanobacteria [12] and more recently from *Escherichia coli* [13] have been isolated and characterized. Evidence for the existence of a chloroplastic NAD(P)H dehydrogenase complex has been reported in *Chlamydomonas* [14,15], but until now the existence of a NAD(P)H dehydrogenase complex in higher plants chloroplasts is still a matter of debate [16] and the participation of the *ndh* gene products to the putative NAD(P)H dehydrogenase complex remains to be proved.

In the present work, we report for the first time that two *ndh* gene products (NDH B and NDH J) are likely involved in a NAD(P)H dehydrogenase complex in thylakoids of a higher plant (*Solanum tuberosum* L.). The role of the NAD(P)H dehydrogenase complex is discussed in relation to the existence of chlororespiration and/or cyclic electron flow around PS1.

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Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; FNR, ferredoxin-NADP$^+$-reductase; FQR, ferredoxin-plastoquinone reductase; NBT, *p*-nitro blue tetrazolium; PMSF, phenylmethylsulfonyl fluoride; PQ, plastoquinone; PS, photosystem; SDS, sodium dodecyl sulfate.

2. Materials and methods

2.1. Plant material

Solanum tuberosum L. plants cv. 'Haig' were originating from *in vitro* plantlets [17] and grown on compost in a phytotron (23°C day/15°C night, 12 h photoperiod, light intensity 350 $\mu E \cdot m^{-2} \cdot s^{-1}$). Experiments were performed on leaves sampled from 1- to 2-month-old plants.

2.2. Chloroplast isolation and extraction of chloroplastic proteins

Intact chloroplasts were isolated at 4°C according to a modification of the method described by Mills and Joy [18]. Potato leaves were blended in 150 ml of medium A containing 330 mM sorbitol, 50 mM Tricine (pH 7.8), 2 mM EDTA, 1 mM $MgCl_2$, 2 mM ascorbic acid and 5 mM DTT. After filtration on three layers of 250 μm and three layers of 60 μm nylon net followed by centrifugation (2,000 × *g*, 3 min), the crude extract was layered onto a Percoll step gradient formed with two layers of medium A containing 90% and 40% Percoll, respectively. After centrifugation (3,500 × *g*, 5 min), intact chloroplasts were recovered from the 40/90% Percoll interphase, washed with medium A, pelleted at 2,000 × *g* for 5 min and resuspended in a 10 mM Tris buffer (pH 8) containing 1 mM EDTA, 15 mM NaCl and 1 mM PMSF. Purified chloroplasts were lysed by freezing and thawing and were incubated for 1 h at 4°C in a 50 mM Tris buffer (pH 7.5) containing 0.25 mM EDTA and centrifuged at 10,000 × *g* for 10 min. The pellet was then resuspended in the same buffer containing 300 mM NaCl and incubated for 90 min at 4°C. Thylakoid membranes were recovered as a pellet after centrifugation (10,000 × *g*, 10 min) and solubilized in a 50 mM Tris (pH 7.5) containing 0.25 mM EDTA and 1% (v/v) SDS. After 2 h of incubation at 4°C and centrifugation (100,000 × *g*, 1 h) to remove insoluble material, thylakoid proteins were separated by SDS/PAGE according to Laemmli [19].

Protein content was determined using a modified Lowry method, using bovine serum albumin as a standard (Sigma kit ref. B5656).

2.3. Non-denaturing gel electrophoresis

For non-denaturing gel electrophoresis, thylakoids (0.5 mg chlorophyll $\cdot$ ml^{-1}) were partially solubilized in a buffer containing 50 mM Tris (pH 7.5), 0.25 mM EDTA and 4% (v/v) Triton X-100 instead of SDS. Non-denaturing gel electrophoresis was performed at 4°C using 6% polyacrylamide gels and a migration buffer contained

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5 mM Tris/38 mM glycine and 0.1% Triton X-100. Staining of gels for NAD(P)H-dehydrogenase activity was carried out as described in [15] using 0.5 mg · ml^{-1} *p*-nitro-blue tetrazolium (NBT) as an electron acceptor and 1 mM NADH or 1 mM NADPH as electron donor in a buffer containing 50 mM Tris (pH 7.5), 0.5 mM EDTA and 0.1% Triton X-100.

Activity bands were excised from the non-denaturing gel and soaked for 30 min at 60°C in 500 μl of a buffer containing 60 mM Tris (pH 6.8), 2% SDS, 5% β-mercaptoethanol. Gel pieces were then loaded onto a 15% acrylamide gel and subjected to SDS-PAGE [19]. After electrophoresis, proteins were detected by Coomassie blue or silver staining, or electroblotted onto 0.45 μm nitrocellulose membranes using a buffer containing 25 mM Tris/193 mM glycine (pH 8.3), 20% methanol and 0.1% SDS. Membranes were incubated overnight at 4°C in a Tris 25 mM buffer (pH 8), containing 140 mM NaCl, 2.7 mM KCl and 3% BSA.

2.4. Preparation of antisera

Synthetic peptides corresponding to well-conserved and hydrophilic domains of both chloroplastic *ndh* B and *ndh* J genes were prepared by Neosystem (France). Sequence conservation was estimated by comparing gene sequences of different plant species (tobacco, rice, maize, soybean, liverwort) available in the Swiss-Prot 31 data bank and hydrophilicity was estimated according to the method of Hopp and Woods [20]. Selected sequences were Y-T-K-K-D-V-R-S-N-E-A and D-F-Q-E-R-E-S-Y-D-M-L-G-I for the *ndh* B and for the *ndh* J gene, respectively. Synthetic peptides were coupled to ovalbumin as a carrier protein. After a sole multi-intradermic immunization per rabbit, sera raised against the coupled synthetic peptide were collected (Neosystem, France).

Two different rabbit antisera raised against tobacco or spinach FNR [21] were used in this study and gave similar results. These antisera were kindly provided by Prof. G.H. Schmid (University of Bielefeld, Germany) and by Dr. K.H. Süss (Gatersleben, Germany), respectively.

For Western analysis, bound antibodies were detected using anti-rabbit Ig-G conjugated to alkaline phosphatase (Boehringer).

3. Results

Antibodies raised against synthetic peptides corresponding to well-conserved and hydrophilic domains of two chloroplastic *ndh* genes (*ndh* B and *ndh* J) were used to investigate the presence of the corresponding gene products in potato chloroplasts. The 'anti-NDH B' antibody recognized a 32 kDa polypeptide in thylakoid membranes proteins solubilized by SDS and separated by SDS-PAGE, while the 'anti-NDH J' antibody recognized a 18 kDa polypeptide (Fig. 1). When thylakoid proteins were extracted with acetone instead of SDS, an additional band at around 40 kDa was recognized by the 'anti-NDH J' serum (not shown). Estimated sizes of *ndh* B and *ndh* J gene products deduced from sequences of the corresponding chloroplastic tobacco genes (tobacco and potato are closely related plant species) are 39 and 18 kDa, respectively. None of these antisera recognized polypeptides of the stromal chloroplastic fraction and both preimmune sera did not reveal any band (not shown).

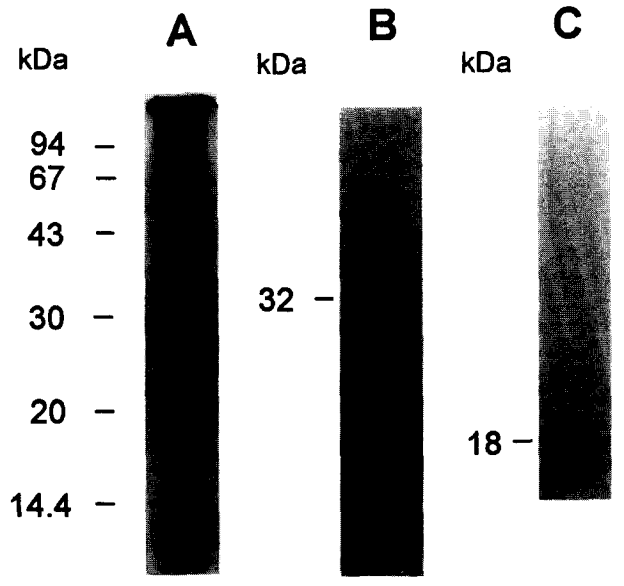

Fig. 1. Western blot analysis of thylakoid proteins from *Solanum tuberosum* chloroplasts solubilized with 1% (v/v) SDS and separated by SDS-PAGE. (A) thylakoid membrane proteins stained by Coomassie blue. (B) Western analysis using an 'anti-NDH B' antibody. (C) Western analysis using an 'anti-NDH J' antibody. 15 μg protein/lane.

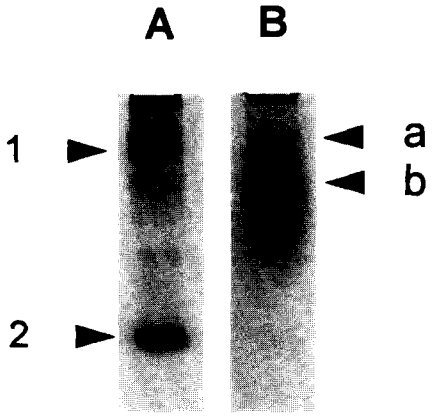

Fig. 2. Non-denaturing gel electrophoresis of thylakoid proteins of *Solanum tuberosum* partially solubilized using 4% Triton X-100. NAD(P)H oxidoreductase activity was revealed by following nitro-blue-tetrazolium reduction in the presence of 1 mM NADPH (A) or 1 mM NADH (B) as electron donors. Activity bands (blue color) are shown by arrows 1 and 2 (A). Chlorophyll protein complexes present in both lanes (green color) are shown by arrows a and b (B). 50 μg protein/lane.

In order to investigate whether *ndh* gene products are organized in a thylakoid protein complex, we carried out non-denaturing gel electrophoresis on thylakoid membranes partially solubilized using Triton X-100. NAD(P)H oxidoreductase activity bands were stained using NBT as an electron acceptor and either NADH or NADPH as electron donor. Two green bands corresponding to chlorophyll protein complexes were observed before starting the staining procedure in both lanes (Fig. 2A,B – bands a, b). When NADPH was used as a substrate, two activity bands showing a blue color (Fig. 2A – bands 1,2) were revealed in a few minutes. On the other hand, when NADH was used, no substantial activity could be detected. The two activity bands observed with NADPH could be observed when pursuing the staining in the presence of NADH for a longer period (more than 1 hour). Activity bands were excised, subjected to second dimension SDS-PAGE, transferred onto a nitrocellulose membrane and used for Western analysis. Two polypeptides of 32 and 18 kDa were recognized by the 'anti-NDH B' and by the 'anti-NDH J', respectively, among polypeptides originating from the slow migrating activity band (Fig. 3B,C). Note that an intense band at around 40 kDa, also observed in one dimension SDS/PAGE when thylakoid proteins were extracted by acetone, was also recognized by the 'anti-NDH J' antibody. The nature of this band, which might

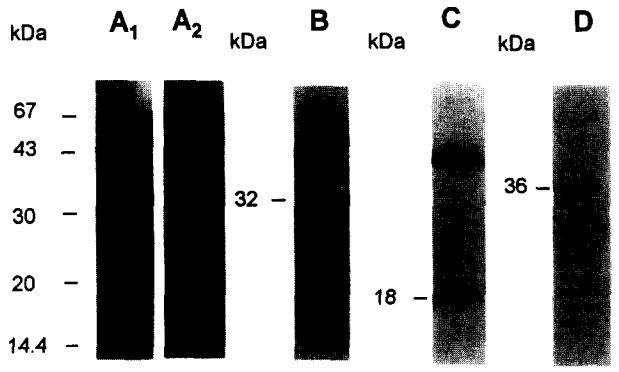

Fig. 3. Second-dimension SDS/PAGE and Western analysis of the NAD(P)H dehydrogenase activity band shown on Fig. 2 (band 1). After excision, the activity band was subjected to second dimension SDS/PAGE. (A_1): Coomassie blue staining. (A_2): silver staining. Western analysis was performed using an 'anti-NDH B' antibody (B), an 'anti-NDH J' antibody (C) and an 'anti-FNR' antibody (D). Lane (A_2) was obtained from an activity band excised when carrying out the first dimension with 50 μg of solubilized proteins and lanes (A_1, B, C, D) starting with 300 μg of proteins.

be due either to a dimeric form of the *ndh* J gene product or to its association with another polypeptide, remains however undetermined. On the other hand, denatured polypeptides contained in the fast migrating band (Fig. 2A, band 2) were recognized neither by the 'anti-NDH B' nor by the 'anti-NDH J' antibody (not shown). Since FNR is able to catalyze the oxidation of NADPH through diaphorase activity, we investigated the presence of FNR in the different activity bands. Antisera raised against FNR recognized a single 36 kDa polypeptide in potato thylakoid membranes (not shown). The 'anti-FNR' antibody also recognized a 36 kDa polypeptide among polypeptides originating from both the slow (Fig. 3D) and fast migrating (not shown) activity bands. The fast migrating activity band, recognized by the 'anti-FNR' antibody but not by 'anti-NDH' antibodies was presumably attributed to the diaphorase activity of FNR. A possible contamination of the slow migrating activity band with soluble FNR was unlikely since membranes were washed using 0.3 M NaCl to remove extrinsic proteins. Moreover, Matthijs et al. [22] previously reported that part of the FNR was associated to a 17.5 kDa polypeptide and that this association could be removed by washing thylakoid membranes with 10 mM CHAPS. When washing potato thylakoids according to this procedure [22], most of the FNR associated to the complex was removed, and the activity of the slow migrating activity band disappeared concomitantly (data not shown). Silver or Coomassie blue staining of the polypeptides contained in the slow migrating activity band showed the presence of at least 14 polypeptides ranging from 16 to 67 kDa (Fig. 3A_1,A_2). Polypeptides of 36, 32 and 18 kDa likely correspond to FNR, NDH B and NDH J, respectively. We conclude from these experiments that the slow migrating activity band likely corresponds to the NAD(P)H dehydrogenase complex which is composed of different polypeptide subunits such as NDH B, NDH J and FNR. From migration on a non-denaturing polyacrylamide gel gradient (4–15%), the size of the chloroplastic NAD(P)H dehydrogenase complex was estimated to approximately 670 kDa (data not shown)

4. Discussion

Previous studies showed that chloroplastic *ndh* genes are transcribed [3]. However, although the polypeptide products of three *ndh* genes (NDH H, NDH I, NDH K) have been found in thylakoid membranes of higher plants [4,5,6] it was not known whether *ndh* gene products are involved in a NAD(P)H dehydrogenase complex as it is the case in bacteria, cyanobacteria or mitochondria. Further, the occurrence of a NAD(P)H dehydrogenase complex in higher plant chloroplasts was recently seriously questioned [16]. Results presented in this paper supply evidence for a participation of both chloroplastic *ndh* B and *ndh* J gene products to a membrane thylakoid complex supporting NAD(P)H dehydrogenase activity.

Besides *ndh* B and *ndh* J gene products, FNR appears as another component of the NAD(P)H dehydrogenase complex. Initially, chloroplastic *ndh* sequences were identified on the basis of their homology with genes coding for subunits of the mitochondrial NADH dehydrogenase which uses NADH as a substrate within mitochondria. However, NADPH is the main cofactor present within chloroplasts [23]. We suggest that association between FNR and the *ndh* gene products allows the NAD(P)H dehydrogenase complex to use NADPH instead of NADH as a preferential substrate (Fig. 4). This would explain the higher activity observed when NADPH instead of NADH was supplied to the complex (Fig. 2). Interestingly, Friedrich et al. [24], noticing that the cyanobacterial complex was lacking the three subunits involved in the binding of NADH, and suggesting that it might be the same for the chloroplastic complex, also proposed that association to a FNR subunit could allow these complexes to use NADPH as a substrate. Association of FNR with an unidentified membrane component of 17.5 kDa was previously reported [22]. Presumably, this unidentified polypeptide might be a *ndh* (for instance NDH J) gene product.

Several NADH dehydrogenase complexes originating from different organisms have been purified to date. The NADH dehydrogenase complex of bovine mitochondria is an assembly of at least 41 different polypeptides [11]. Bacterial enzymes show a much simpler organization. For instance, Berger et al. [12] showed that the cyanobacterial complex of *Synechocystis* contains at least 12 subunits and the recently purified *Escheri-*

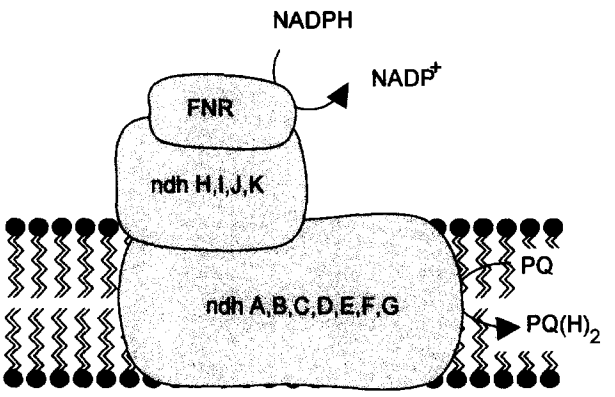

Fig. 4. Hypothetical scheme of the chloroplastic NAD(P)H dehydrogenase subunit organization. This scheme is based on the published organizations of the cyanobacterial [12] and bacterial [13] NADH dehydrogenase complexes as well as on the results presented in this paper. We suggest that the association of a FNR subunit allows the complex to use NADPH instead of NADH as a preferential substrate.

chia coli complex was shown to contain 16 subunits [13]. The estimated size of the chloroplastic complex (about 670 kDa) is quite higher when compared to the value (about 450 kDa) obtained by adding the size of the gene products of the eleven chloroplastic *ndh* genes discovered to this date. This suggests that the NAD(P)H complex might contain other yet undiscovered subunits.

As already suggested, the NAD(P)H dehydrogenase complex may allow the entry of electrons from a soluble stromal pool of NAD(P)H to the plastoquinone (PQ) pool. Bendall and Manasse in a recent review [16] concluded that FNR associated to FQR (ferredoxin-PQ reductase) rather than a NAD(P)H dehydrogenase would be involved in that reaction. It appears from this work that the NAD(P)H dehydrogenase complex formed by the association of *ndh* gene products with FNR, rather than the still unidentified FQR, is most likely the functional complex involved in the reduction of the PQ pool.

One of the most intriguing questions concerns the physiological significance of the thylakoid NAD(P)H dehydrogenase complex. During illumination, this complex might participate to a cyclic electron pathway around PS 1 [10,16]. This is well-supported by the fact that high amounts of *ndh* gene products (NDH H, NDH K) are present within chloroplasts of bundle sheath cells of C_4 plants, which lack PS2 and produce ATP required for CO_2 fixation through cyclic photophosphorylations [25]. Alternatively, the NAD(P)H dehydrogenase complex may be involved in chlororespiration [8,26]. However, if chlororespiration has been extensively studied in unicellular green algae [7,8,27], its occurrence in higher plants chloroplasts is still controversial [27,28].

In conclusion, we have identified for the first time a higher plant thylakoidal NAD(P)H dehydrogenase complex which likely catalyzes the entry of electrons from a soluble stromal pool of NADPH to the PQ pool. By being able to carry out either $NADP^+$ reduction from ferredoxin when loosely bound to the membrane or PQ reduction from NADPH when bound to the NAD(P)H dehydrogenase complex, FNR would regulate the partition between cyclic and non-cyclic pathways. Whether phosphorylation of FNR which has recently been demonstrated [29] is involved in such a regulation will need further experiments to be elucidated.

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