Novel Nuclear-encoded Subunits of the Chloroplast NAD(P)H Dehydrogenase Complex

Received for publication, July 16, 2008, and in revised form, October 28, 2008. Published, JBC Papers in Press, October 28, 2008, DOI 10.1074/jbc.M805404200

Sari Sirpiö‡, Yagut Allahverdiyeva‡, Maija Holmström‡, Anastassia Khrouchtchova§, Anna Haldrup§, Natalia Battchikova§, and Eva-Mari Arö††

From the ‡Department of Biology, Plant Physiology, and Molecular Biology, University of Turku, FI-20014 Turku, Finland and the §VKR Research Centre Pro-Active Plants, Department of Plant Biology and Biotechnology, Faculty of Life Sciences, University of Copenhagen, DK-1871 Frederiksberg C, Denmark

The NAD(P)H dehydrogenase (NDH) complex functions in photosystem I cyclic electron transfer in higher plant chloroplasts and is crucial for plant responses to environmental stress. Chloroplast NDH complex is a close relative to cyanobacterial NDH-1L complex, and all fifteen subunits so far identified in NDH-1L have homologs in the chloroplast NDH complex. Here we report on the identification of two nuclear-encoded proteins NDH48 and NDH45 in higher plant chloroplasts and show their intimate association with the NDH complex. These two membrane proteins are shown to interact with each other and with the NDH complex enriched in stroma thylakoids. Moreover, the deficiency of either the NDH45 protein or the NDH48 protein in respective mutant plants leads to severe defects in both the accumulation and the function of the NDH complex. The NDH48 and NDH45 proteins are not components of the hydrophilic domain of the NDH complex but are strongly attached to the hydrophobic membrane domain. We conclude that NDH48 and NDH45 are novel nuclear-encoded subunits of the chloroplast NDH complex and crucial both for the stable structure and function of the NDH complex.

Multisubunit protein complexes embedded in the thylakoid membrane, the photosystem (PS)I and PSII complexes, the cytochrome (cyt) b6f complex, and the ATP synthase, are fundamental prerequisites for light harvesting and solar energy conversion into chemical form. Yet there are other thylakoid multiprotein complexes that are essential for plant sustenance under changing environmental conditions. In higher plant chloroplasts, the NAD(P)H dehydrogenase (NDH) complex functions in PSI cyclic electron flow and chlororespiration (1, 2). The expression and activity of the NDH complex in plant chloroplasts are highly regulated, and this complex seems to be crucial for acclimation of the photosynthetic apparatus to environmental cues (3). Under stress conditions like heat and drought, the NDH complex diverts electrons to cyclic electron flow around PSI to avoid over-reduction of the acceptor side of the PSI complex and formation of reactive oxygen species (ROS), thus eventually protecting the photosynthetic apparatus against oxidative stress (3).

The NDH complex in chloroplasts closely resembles the cyanobacterial NDH-1L complex (4). Fifteen subunits of the NDH-1L complex have so far been identified, comprising 7 NDH subunits (NDHA-G) in the hydrophobic domain embedded in the thylakoid membrane and 8 subunits in the peripheral hydrophilic domain (NDHH-O) (5). The same subunits as in cyanobacterial NDH-1L have been found in chloroplast NDH complex (6–8), most of them being encoded by the chloroplast genome and only three (NDHM, NDHN, and NDHO) by the nuclear genome. The catalytically active subunits (homologs of NuoE, NuoF, and NuoG in Escherichia coli) that constitute the separate electron input device, if present, remain unknown both in cyanobacteria and chloroplasts.

Recently, several Arabidopsis thaliana (referred hereafter Arabidopsis) mutants with a defective NDH complex have been identified, based on chlorophyll (Chl) fluorescence characteristics typical for plants lacking NDH activity (9) and on the analysis of the epitope-tagged NDH complexes (7). By these methods, several auxiliary proteins that participate in different phases of biogenesis of the NDH complex, from gene transcription to the stability of the complex have been characterized (7, 9–14). Here we have identified the novel proteins At1g15980 and At1g64770 in Arabidopsis chloroplasts by a proteomic approach and shown their intimate association with the NDH complex and hence designated them as NDH48 and NDH45, respectively.

**EXPERIMENTAL PROCEDURES**

Plant Material and Growth Conditions—Arabidopsis ecotype Columbia plants were used as a wild type (WT). SALK_111363 (15) line possessing a T-DNA insert within At1g64770 gene was screened by immunoblotting (for antibody, see below). The antisense ndh48 mutant plants (as-ndh48) were generated by amplifying 311 nt from the coding region of At1g15980 gene from WT cDNA by PCR using forward primer 5′-GGGGACAAGTTTGTACAAAAAAGCAGTGG-3′ and reverse primer 5′-CCCTGTTAGCTCAAGCCAGTTC-3′.
Novel NDH Subunits

GCTGGCTTCTTCATCTCATGGTACC and reverse primer 5′-GGGGACCATTTTTCAGAAGAAGGTC-AAGCTGACCGTGAACCTTTTG. The PCR product was first cloned into pDONR201 vector (Gateway® Cloning, Invitrogen), and thereafter in the antisense orientation into pK2WG7 vector under the control of the CaMV 35S promoter. The vector was transformed by electroporation into the Agrobacterium tumefaciens strain C58 (16). Arabidopsis plants were transformed by the floral dip method (17), and seeds harvested from the transformed plants were germinated on MS plates containing 2% sucrose and 50 mg liter⁻¹ kanamycin sulfate. Arabidopsis mutants lacking the NDHO protein (SALK_068922) encoded by the At1g74880 gene, and plants defective in nucleus-encoded factor, CRR2, essential for proper expression of the NDHB gene were used as controls (7, 9).

Plants were grown under a photon flux density of 150 μmol of photons m⁻² s⁻¹ in a 16-h light regime at 23 °C. Experiments were performed with 3-week-old full-size plants if not otherwise stated.

Statistical Analyses—The numerical data were subjected to statistical analysis by Student's t test with statistical significance at the p values <0.001.

Southern Blotting—Southern blotting of 2 μg of HindIII-digested WT and SALK_111363 genomic DNA was made according to the standard procedures (18). A 665-bp probe was PCR-amplified by SALK_111363 genomic DNA using T-DNA specific primers 5′-GCTTTGAAGACGTGGTGAAGC and 5′-GCACGAGTCTCATCAAGACG.

Northern Blotting—Total RNA was isolated with TRIzol (Invitrogen) according to the Tri reagent total RNA isolation protocol (Sigma), and Northern blotting was performed according to a standard procedure (18).

Isolation, Separation, and Detection of Proteins—Thylakoid membranes for immunoblotting were isolated as described in Ref. 19. Intact chloroplasts were isolated as in Ref. 20, broken up with shock buffer (50 mM Hepes-KOH pH 7.6, 5 mM sorbitol, 5 mM MgCl₂) followed by separation of the soluble stroma and thylakoids by centrifugation at 18,000 × g, 4 °C for 20 min. Thylakoid subfractionation into grana and stroma membranes was performed as in Ref. 20. Detaching of the peripheral thylakoid proteins with 2 M NaBr was performed as described in Ref. 21. The digestion of isolated thylakoid membranes with trypsin (10 μg/ml) was performed for 20 min on ice. Chl was determined according to Ref. 22 and Chl per leaf area as described in Ref. 23.

The polypeptides were separated with SDS-PAGE (15% polyacrylamide, 6 mm urea) (24), and after electrophoresis the proteins were electroblotted to a polyvinylidene difluoride membrane (Millipore, Watford, Herts, UK). Gels were loaded depending on the linearity of the antibody used (3 μg of Chl: NDH45, NDH48, NDHH, Cytb; 0.5 μg of Chl: D1, CP43, PsbO; 0.2 μg of Chl: Atpβ, PsAB, RbcL). Blue native (BN)-PAGE and two-dimensional BN/SDS-PAGE were performed as described in Ref. 25. Immunoblotting using chemiluminescence detection was performed according to standard procedures. NDH48 and NDH45 antibodies were produced by immunizing rabbits with synthetic peptides LITTERGKYTYELNKN, VYDPDDH-WPEPAEYT and ESSGWFGSEEKGPG, NTTPSKFET-IDQGR, respectively (Eurogentec, Seraing, Belgium). Quantification of different proteins was carried out with Fluor Chem™ 8000 image analyzer (Alpha Innotech Corporation, San Leandro, CA).

In Gel Trypsin Digestion and Sample Preparation for Mass Spectrometry—In gel trypsin digestion and sample preparation for mass spectrometry were performed as described in Ref. 26. MALDI-TOF (matrix-assisted laser desorption-ionization time-of-flight) analysis was performed in a reflectron mode on a Voyager-DE PRO mass spectrometer (Applied Biosystems).

Measurements of Photosynthetic Parameters—The maximal photochemical efficiency of PSII was determined as Fv/Fm using a Hansatech Plant Efficiency Analyzer (King’s Lynn, UK) after 30 min of dark incubation. The redox state of P₇₀₀ was measured with a PAM fluorometer (PAM-101/102/103, Walz) equipped with an ED-P₇₀₀-DW-E unit by monitoring absorbance changes at 810 nm and using 860 nm as a reference. Leaves were kept in the dark for 3 min prior to the measurement. The P₇₀₀ was oxidized by far-red light from a photodiode (FR-102, Walz) for 30 s, and the subsequent red-reduction of P₇₀₀⁺ in the dark was monitored. Post-illumination increase in Chl fluorescence, the Fₐ “rise”, was monitored as described in Ref. 27.

NAD(P)H Oxidation Assays—Thylakoids were isolated via intact chloroplasts (see above), and the oxidation of NAD(P)H was measured at 25 °C in the presence of either duroquinone or ferricyanide as described in Refs. 2, 28.

H₂O₂ Detection by 3,3-Diaminobenzidine (DAB) Method—H₂O₂ accumulation in leaves was detected with the DAB method as described in Ref. 29. The DAB solution was incorporated into detached 14-day-old plants overnight in darkness, and the accumulation of H₂O₂ was studied after exposure of plants to growth light conditions.

RESULTS

Novel Thylakoid Proteins Encoded by At1g15980 and At1g64770 Genes—A proteome analysis of ribosome-nascent chain complexes (associated with fragments of stroma thylakoids) from Arabidopsis chloroplasts revealed several novel proteins of unknown function (20). They may represent novel subunits of protein complexes or play a role of auxiliary proteins in the assembly and/or function of the thylakoid membrane protein complexes. Here we addressed the functional role of two nuclear-encoded, NDH-related proteins (see below), NDH48 (At1g15980) with an apparent molecular mass of 48 kDa and NDH45 (At1g64770) of 45 kDa. Both of these proteins possess the chloroplast targeting signal in the N terminus of the amino acid sequence. Homology searches (NCBI Blast) revealed that the NDH48 and NDH45 proteins are conserved among higher plants, with amino acid identities up to 62 and 52% with corresponding proteins in Oryza sativa, respectively. In contrast, cyanobacteria entirely lack the close homologs of the NDH48 and NDH45 proteins (data not shown).

Generation of the Mutant Lines—A reverse genetics approach was taken to characterize the functional roles of the NDH48 and NDH45 proteins. The SALK_111363 mutant line with a T-DNA insert within the At1g64770 gene was ordered from the SALK mutant collection. According to the immuno-
Novel NDH Subunits

FIGURE 1. Characterization of the ndh45 and as-ndh48 mutant plants. A, immunoblot of the thylakoid membrane proteins isolated from WT, ndh45, and as-ndh48 plants expressing either 10% (as-ndh48:1) or 20% (as-ndh48:2) of the NDH48 protein compared with that present in the WT plants. B, Southern blot of the HindIII-digested WT and SALK_111363 DNA probed with a radiolabeled T-DNA fragment indicating only one T-DNA insert in ndh45 plants. C, immunoblot from the soluble stroma (S) and thylakoid (T) fractions of isolated chloroplasts. Immunoblotting was performed with antibodies against NDH45, NDH48, RbcL (Rubisco large subunit), and D1 proteins. Protein gels were loaded on Chl basis, the amounts depending on the linearity of the antibody used (see “Experimental Procedures”).

TABLE 1 Characteristics of the photosynthetic apparatus in ndh45 and as-ndh48 plants

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>ndh45</th>
<th>as-ndh48</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chl/leaf area</td>
<td>24.4 ± 1.8</td>
<td>24.9 ± 1.9</td>
<td>25.3 ± 1.3</td>
</tr>
<tr>
<td>Chl a/b</td>
<td>3.7 ± 0.0</td>
<td>3.7 ± 0.1</td>
<td>3.6 ± 0.1</td>
</tr>
<tr>
<td>Photochemical efficiency of PSII (Fv/Fm)</td>
<td>0.82 ± 0.07</td>
<td>0.82 ± 0.07</td>
<td>0.83 ± 0.07</td>
</tr>
</tbody>
</table>

FIGURE 2. Interaction of NDH45 and NDH48 with the thylakoid membrane and with each other. A, immunoblot from the thylakoid membranes isolated from WT plants and washed with 2 m NaBr to detach the peripheral thylakoid membrane proteins. Antibodies against the NDH45, NDH48, NDHH, PsbO, and Atpβ were used. Thylakoid-bound proteins (T) and the proteins released by the high salt treatment (R) are shown. B, immunoblot of the NDH45, NDH48, and PsbO proteins from WT thylakoids exposed to mild trypsin digestion. C, Northern blot demonstrating the transcript levels of the NDH45 and NDH48 genes in the WT and ndh45 plants. 18S ribosomal RNA was used as a control. Protein gels were loaded on Chl basis, the amounts depending on the linearity of the antibody used (see “Experimental Procedures”).

blot analysis, this mutant line (ndh45) did not contain the protein produced by the At1g64770 gene (Fig. 1A). The Southern blot analysis of HindIII-digested DNA from SALK_111363 plants performed with a T-DNA specific probe confirmed the presence of the predicted 2.3-kb NDH45-T-DNA fragment (Fig. 1B) and demonstrated that the SALK_111363 line contains only one T-DNA insert. For NDH48, a T-DNA mutant was not available, and therefore an antisense mutant line was generated in our laboratory by a Gateway cloning system (Invitrogen). The majority of antisense NDH48 (as-ndh48) plants contained ~20% of the NDH48 protein compared with the amount present in WT (Fig. 1A, lane 6). However, some of the mutant plants showed more drastic down-regulation in the amount of the NDH48 protein (~10%, Fig. 1A, lane 5). To ensure adequate numbers of plants for biochemical experiments, plants containing ~20% of the NDH48 protein were used for most of the experiments.

Characterization of ndh45 and as-ndh48 Mutant Plants—No statistically significant differences in the Chl a/b ratio or in the total amount of Chl per leaf area were found in mutant plants compared with WT (Table 1). Immunoblot analysis of representative proteins from major thylakoid multiprotein complexes was taken as a first approach to analyze the impact of the deficiency of either the NDH48 or the NDH45 protein. The most dominant feature of both the ndh45 and as-ndh48 thylakoids was a distinct deficiency of the NDHH protein, representing the NDH complex (Fig. 1C). In contrast, the amounts of the PSI, PSII, and cyt b/f complexes as well as the ATP synthase, represented by PsaB, D1, Cyt f, and Atpβ proteins, respectively, remained unaltered or at a slightly elevated level in the mutant thylakoids compared with WT.

NDH48 and NDH45 Proteins Interact with Each Other on the Thylakoid Membrane—NDH48 and NDH45 were localized exclusively to the thylakoid membrane (Fig. 1C). To investigate the interaction between the NDH48 and NDH45 proteins, the thylakoids of WT plants were treated with high salt (2 M NaBr) that detaches the peripheral proteins from the thylakoid membrane (21). Such treatment indeed released the majority of the peripheral proteins, like Atpβ and NDHH, from the membrane. The PsbO protein was also partially released, indicating that the treatment was harsh enough to disrupt the thylakoid membrane in order to release PsbO from the luminal compartment (Fig. 2A). In contrast, most of the NDH48 and NDH45 proteins remained tightly bound to the thylakoid membrane after the high salt treatment (Fig. 2A). It should also be noted that those NDH48 and NDH45 proteins that released during the salt...
treatment were rapidly and efficiently degraded after release (Fig. 2A).

To determine the membrane topology of the NDH45 and NDH48 proteins more precisely, thylakoids isolated from the WT plants were subjected to mild digestion with trypsin, allowing the degradation of only the stroma-exposed parts of the thylakoid membrane proteins. Whereas the NDH48 protein was partially degraded by the protease, the NDH45 protein remained intact, similarly to the lumenal control protein, PsbO (Fig. 2B). However, the NDH45 protein seems not to locate within the lumenal compartment, because a harsh trypsin treatment that degraded the PsbO protein had hardly any effect on the NDH45 protein (data not shown).

Interestingly, the deficiency of either the NDH45 or the NDH48 protein in the two mutant plants resulted in a total loss or strong down-regulation of the other protein, respectively, from the thylakoid membrane (Fig. 1A). Thus, the accumulation of the NDH45 and NDH48 proteins seemed to be mutually co-regulated. The deficiency of the NDH48 protein from ndh48 thylakoids was more pronounced than the deficiency of NDH45 in the as-ndh48 thylakoids, which is most likely due to a small amount of NDH48 protein left in the as-ndh48 plants. To address the mutual stabilization of the NDH48 and NDH45 proteins, it was examined whether the lack of the NDH48 protein in ndh45 thylakoids results from defects in transcription of the NDH48 gene. Northern blot analysis demonstrated that the amount of the NDH48 mRNA was almost the same in WT and ndh45 plants (Fig. 2C), indicating that the loss of the NDH48 protein in ndh45 thylakoids is dependent on post-transcriptional regulation.

**NDH48 and NDH45 Proteins Associate with the NDH Complex**—More exact location of the NDH48 and NDH45 proteins was addressed by fractionation of the thylakoid membrane into grana and stroma lamellae, followed by immunoblot analysis of the membrane fractions. Both the NDH48 and the NDH45 protein were strongly enriched in stroma thylakoids with only a minor proportion of the proteins detected in grana thylakoids (Fig. 3A). Moreover, the stoichiometry of the NDH48 and NDH45 proteins in grana and stroma thylakoids was similar (see supplemental Fig. S1 for the linearity tests of the antibodies).

**FIGURE 3. Localization of the NDH48 and NDH45 proteins in thylakoids isolated from WT Arabidopsis.**

A, immunoblot from thylakoid membranes fractionated into stroma (ST) and grana (GT) thylakoids by digitonin and subsequently separated by denaturing SDS-PAGE. Gels were loaded on Chl basis, the amounts depending on the linearity of the antibody used (see “Experimental Procedures”). Immunoblots using antibodies against NDH48, NDH45, CP43, and PsbA are shown. B, non-denaturing BN-PAGE of intact thylakoids (T) and the stroma (ST) and grana (GT) thylakoid fractions. Unstained gel (left panel) and immunoblot were performed with antibody against the NDH45 protein (right panel). C, silver-stained two-dimensional BN/SDS-PAGE of stroma thylakoids (upper panel) and pieces of corresponding immunoblots using protein-specific antibodies (lower panel). Upper panel, proteins were identified with MALDI-TOF (identified proteins are listed in Table 2). Subunits of PSI in the supercomplex of ≥1000 kDa are indicated by arrows (31).
Further study of the protein complexes from intact thylakoids as well as from isolated grana and stroma membranes by BN-PAGE, enabling the separation of the protein complexes according to their size, revealed the presence of NDH45 (Fig. 3B) and NDH48 (data not shown) proteins in a large supercomplex of ≈1000 kDa, with only minor fraction of these proteins in a smaller protein complex of ~800 kDa. For analysis of the protein composition of the ≈1000-kDa protein complex harboring the NDH45 and NDH48 proteins in the thylakoid membrane, the stroma thylakoids were subjected to two-dimensional BN/SDS-PAGE and subsequent identification of the protein subunits by immunoblotting and mass spectrometry (Fig. 3C and Table 2). Besides NDH45 and NDH48, the NDHH, NDH, and NDHK proteins were found in the same complex as well as the PPL2 protein (At2g39470), essential for accumulation and activity of the NDH complex (30). In addition, the CYP20-2 protein (At5g13120) was present in the complex, and also the PSI reaction center protein PsAB was identified by immunoblotting (Fig. 3C). Not only PsAB but also several other PSI proteins were present in the supercomplex, with identification based on our previous work (31). The composition of the ~800-kDa protein complex, in turn, remained enigmatic because according to immunoblotting, it did not contain PsAB or NDHH proteins (Fig. 3C), indicating that the PSI complex as well as the hydrophilic subunits of the NDH complex might not be present in this complex.

**ndh45 and as-ndh48 Mutants Show Impaired Function of the NDH Complex**—Functional characterization of the thylakoid membrane protein complexes did not reveal any malfunction of PSI measured as photochemical efficiency of PSI (Fv/Fm and Table 1). Functional modification, however, was recorded in the re-reduction rate of P700 + in darkness, which was slowed down both in ndh45 (t1/2 = 1.76 s) and as-ndh48 (t1/2 = 1.85 s) mutants compared with WT (t1/2 = 1.27 s) (Fig. 4A). This might be related to slower cyclic electron transfer around PSI in the mutant plants, thus indirectly suggesting a possible functional flaw in the NDH complex. To confirm the malfunction of the NDH complex in the ndh45 and as-ndh48 plants, the post-illumination rise in chlorophyll fluorescence (32), which is dependent on NDH-mediated reduction of plastocyanin pool in darkness (33), was measured. In this experiment, two earlier characterized Arabidopsis NDH mutant lines, ndho and crr2-2, were used as controls (7, 9). The reduced level of Fv/Fm “rise” after light to dark transition in as-ndh48 mutant plants and especially in ndh45 compared with WT provided direct evidence of a lack or impaired function of the NDH complex (Fig. 4B). As expected, also the ndho and crr2-2 plants showed malfunction of the NDH complex (Fig. 4B). In addition, we measured the rate of NAD(P)H oxidation using thylakoids isolated from WT, ndh45, or ndho chloroplasts. In these experiments either ferricyanide or duroquinone was used as an electron acceptor. However, no statistically significant difference was found between WT, ndh45, and ndho thylakoids in the rate of oxidation of NADH or NADPH (data not shown).

**ndh45 Plants Accumulate H2O2 in Dark-Light Transition**—NDH-mediated cyclic electron flow is important in adjusting the photosynthetic electron flow and in preventing the accumulation of ROS upon sudden changes in environmental condition. Accordingly, the production of ROS in WT, ndh45, and ndho plants was tested after transfer of 14-day-old plants from darkness to growth light conditions for 4 min and for 1 h. The DAB staining revealed a higher accumulation of H2O2 in ndh45 and ndho mutant leaves compared with WT, both immediately after the lights were turned on following the diurnal dark period and still after 1 h in light (Fig. 5), which implies higher oxidative stress in mutant plants.

---

**TABLE 2**

<table>
<thead>
<tr>
<th>Spot</th>
<th>Protein</th>
<th>AGI-code</th>
<th>MW*</th>
<th>Peptides</th>
<th>Cover</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>NDH48</td>
<td>At1g15980</td>
<td>51.3</td>
<td>12</td>
<td>27</td>
<td>151</td>
</tr>
<tr>
<td>2.</td>
<td>NDH45</td>
<td>At1g64770</td>
<td>38.3</td>
<td>9</td>
<td>22</td>
<td>141</td>
</tr>
<tr>
<td>3.</td>
<td>NADH dehydrogenase subunit H (NDHH)</td>
<td>Atcg01110</td>
<td>45.6</td>
<td>14</td>
<td>30</td>
<td>185</td>
</tr>
<tr>
<td>4.</td>
<td>NADH dehydrogenase subunit K (NDHK)</td>
<td>Atcg00430</td>
<td>25.6</td>
<td>5</td>
<td>15</td>
<td>78</td>
</tr>
<tr>
<td>5.</td>
<td>Peptidylprolyl isomerase (CYP20-2)</td>
<td>At5g13120</td>
<td>28.5</td>
<td>6</td>
<td>25</td>
<td>85</td>
</tr>
<tr>
<td>6.</td>
<td>PsbP domain protein (PPL2)</td>
<td>At2g39470</td>
<td>27.2</td>
<td>7</td>
<td>29</td>
<td>89</td>
</tr>
<tr>
<td>7.</td>
<td>PsbP domain protein (PPL2)</td>
<td>At2g39470</td>
<td>27.0</td>
<td>9</td>
<td>38</td>
<td>128</td>
</tr>
<tr>
<td>8.</td>
<td>NDH48 dehydrogenase subunit N (NDHN)</td>
<td>At5g58260</td>
<td>23.7</td>
<td>7</td>
<td>36</td>
<td>112</td>
</tr>
</tbody>
</table>

* Molecular mass is predicted from the amino acid sequence.
Accumulation and Attachment of NDH45 and NDH48 Proteins to the Thylakoid Membrane Are Distorted in crr2-2 Mutant Plants but Not in the ndho Mutant Plants—To study the role of the NDH48 and NDH45 proteins in the structure/assembly of the NDH complex, we estimated the amounts of the NDH48 and NDH45 proteins in thylakoids isolated from the ndho and from the crr2-2 mutants. ndho mutant lacks the hydrophilic subunits of the NDH connecting domain (7), whereas the accumulation of both the hydrophobic NDHB subunit and the hydrophilic NDHH is disturbed in the crr2-2 mutant plants (9). The NDH45 and NDH48 proteins were present in thylakoids of the ndho mutant in amounts similar to those in WT while the crr2-2 thylakoids contained clearly reduced amounts of the NDH45 and NDH48 proteins (Fig. 6A). This indicates that the NDH48 and NDH45 proteins do accumulate normally in the thylakoid membrane in the absence of the hydrophilic subunits of the NDH complex. Accumulation, however, is diminished when both the hydrophobic and hydrophilic subunits of the NDH complex are present in reduced amounts.

The location of the NDH45 and NDH48 proteins in thylakoid protein complexes of the ndho and crr2-2 mutant plants was studied further by BN-PAGE. In WT and in ndho thylakoids the NDH45 and NDH48 proteins were found either in the ~1000-kDa complex or in the ~800-kDa complex (Fig. 6B), of which the ~1000-kDa supercomplex represents the NDH complex together with the PSI complex (Fig. 3C). It should be noted, however, that in ndho thylakoids, the ~1000-kDa supercomplex was found to be slightly smaller because of the lack of the hydrophilic subunits of the NDH complex (Fig. 6B). In contrast, in crr2-2 thylakoids the amount of the NDH complex as well as the NDH45 and NDH48 proteins was clearly reduced compared with WT, indicating that the hydrophobic subunits of the NDH complex are required for stable attachment of NDH45 and NDH48 (Fig. 6B). Notably, some NDH45 and NDH48 proteins were found in the ~800-kDa protein complex in the crr2-2 thylakoids (Fig. 6B).

To study further the interaction of the NDH45 and NDH48 proteins with the NDH complex, the thylakoids isolated from the WT, ndho, and crr2-2 plants were mildly digested with trypsin and subsequently subjected to SDS-PAGE and immunoblotting with NDH45 and NDH48 antibodies. Interestingly, while NDH48 was only partially digested by the proteases in the WT thylakoids, the protein was more susceptible to digestion in the ndho thylakoids and fully degraded in the crr2-2 thylakoids (Fig. 6C). This clearly demonstrated that the hydrophilic domain of the NDH complex protects the NDH48 protein from proteolytic degradation. On the contrary, the NDH45 protein was protected against proteolysis in WT and in both NDH mutants studied (Fig. 6C).

DISCUSSION

The structure and subunit composition of the NDH complex of plant chloroplasts is poorly known compared with other thylakoid multiprotein complexes, the PSI, PSII, and cyt b6f complexes as well as the ATP synthase (34). Contrary to these major photosynthetic protein complexes, no structural data are available from chloroplast NDH complex, and only low resolution single particle electron microscopy images of cyanobacterial NDH-1 complexes are available (35, 36). Although the NDH complex is not a component of the main linear photosynthetic electron transfer pathway, it plays a critical role in cyclic electron flow around PSI (3) together with the PGR5-dependent cyclic pathway (37). Cyclic electron transfer routes guarantee a proper production of ATP in relation to NADPH to fulfill the energy requirements of important metabolic processes. Indeed, the absence of one cyclic pathway is not lethal, but the elimination of both pathways prevents photoautotrophic growth (37). NDH-dependent cyclic electron flow has been shown to be of particular importance for plant survival under various environmental stress conditions (3).

Here we have identified two novel nuclear-encoded chloroplast proteins from Arabidopsis, NDH48 and NDH45, which are crucial for the assembly and function of the NDH complex. Both proteins locate exclusively in the thylakoid membrane.
(Fig. 1C), and the association of the NDH45 and NDH48 with the thylakoid membrane is fairly strong, because the majority of these proteins remain attached to the thylakoid membrane despite the high salt treatment (Fig. 2A). Moreover, the NDH48 protein is shown to locate on the stromal side of the thylakoid membrane, while the NDH45 protein is buried in the thylakoid membrane and not accessible to the protease treatment (Fig. 2B). This is surprising because the prediction programs (TMHMM,ARAMEMNON) expect both of the proteins to be hydrophilic. The expression of NDH45 and NDH48 is co-regulated, but it does not occur at the transcriptional level (Fig. 2C) but at the level of protein-protein interaction. Indeed, in the absence of NDH45, the NDH48 protein is largely missing from the thylakoid membrane, and vice versa (Fig. 1A), indicating that the protein-protein interaction of NDH45 and NDH48 has a stabilizing effect, and the lack of one protein does not allow stable membrane association of the other one, which instead is subjected to degradation (Fig. 2A).

Fractionation of the thylakoid membrane and separation of the multisubunit protein complexes revealed that both NDH45 and NDH48 are present in a supercomplex of ≥1000 kDa located in the stroma lamellae of the thylakoid network (Fig. 3B). Most likely, the NDH45 and NDH48 proteins are attached to the thylakoid membrane by the NDH complex, because several NDH subunits were likewise identified in the supercomplex, together with the auxiliary protein PPL2, which previously has been shown to be associated with the NDH complex (Fig. 3C and Table 2) (30). The strongest support in favor of this hypothesis, however, comes from the analysis of as-ndh48 and as-ndh45 plants, which were specifically affected in the accumulation and function of the NDH complex (Table 1, Figs. 1A and 4). Residual activity of the NDH complex in as-ndh48 plants indicates that the small amounts of NDH48, NDH45, and NDHH proteins (as well as other NDH subunits) present in these plants (Fig. 1A) are capable of sustaining small-scale activity of the complex. It is also interesting to note that the problems in NDH-mediated cyclic electron transfer in the absence of either the NDH45 or the NDHO protein are similarly reflected in the accumulation of H2O2 when the plants are suddenly exposed to light after the diurnal dark period (Fig. 5); the conditions where the carbon fixation reactions are not yet activated and cyclic electron flow is needed for dissipation of excess energy.

In agreement with our results, a recent study with maize bundle sheath chloroplasts, rich in the NDH complex, showed that the NDH48 and NDH45 homologs co-migrate in BN-PAGE with the NDH complex (38). Co-expression analysis also links the NDH48 protein to the same regulon with PPL2, NDHN, and NDHL (30). Besides NDH45, NDH48, and PPL2 proteins, yet another protein, CYP20-2 was identified in the same supercomplex by the two-dimensional BN/SDS-PAGE (Fig. 3C). The presence of CYP20-2 in the NDH supercomplex is surprising because this protein, thylakoid lumen cyclophilin of 20 kDa, has been reported to function as an auxiliary protein assisting the insertion of the light harvesting complex II (LHCII) to PSII and thus functioning in the formation of the PSII-LHCII supercomplexes (39). Our result, however, suggests that CYP20-2 protein probably has an alternative role with respect to the NDH complex and this interpretation is further supported by the co-migration of the homolog of CYP20-2 (TLP20) with the maize NDH complex in BN-PAGE analysis (38). Moreover, in the supercomplex of ≥1000 kDa, several subunits of the PSI complex were identified, which is in line with a recent report showing that the chloroplast NDH complex interacts with the PSI complex in Arabidopsis (40). The intimate co-operation of the NDH with PSI would certainly favor the cyclic electron flow around PSI and possibly explains why the genes encoding the activity subunits of the NDH complex are missing from plant and cyanobacterial genomes. In this context it is worth noting that when NAD(P)H was used as a substrate for the NDH complex no difference in activity could be recorded between WT and any of the ndh mutant thylakoids (including ndho which completely lacks the NDH complex) giving support to recent speculations about electron donors other than NAD(P)H for the chloroplast and cyanobacterial NDH complex.

Besides the major ≥1000-kDa complex, NDH45 and NDH48 were also present in a smaller supercomplex of ~800 kDa, which seems not to contain the subunits of the PSI complex or hydrophilic NDHH subunit of the NDH complex (Fig. 3C). The composition and role of this protein complex remains to be resolved but it might represent a subassembly step of the NDH supercomplex.

Although the final answer to the question whether the NDH45 and NDH48 proteins are intrinsic components of the NDH complex or whether they play an auxiliary function in the biogenesis, assembly and function of NDH is waiting for the high-resolution structural analysis of the NDH complex, the results presented here strongly support the first assumption. We show that the NDH45 and NDH48 proteins are not components of the hydrophilic connecting domain of the NDH complex but instead are attached to the hydrophobic membrane domain (Fig. 7). This interpretation is based on the fact that NDH45 and NDH48 form a complex and are stable in the absence of the NDHO protein, which is crucial for the assembly of the hydrophilic connecting domain of the NDH complex, whereas in the crr2-2 mutant both the hydrophobic membrane arm and also the NDH45 and NDH48 proteins are in shortage
Novel NDH Subunits

(Fig. 6, A and B). On the other hand, the NDH45 and NDH48 proteins are crucial for accumulation of NDHH (Fig. 1A) and therefore probably also for the stable attachment of the hydrophilic connecting domain to the hydrophobic membrane domain of the NDH complex. The hydrophilic arm of the NDH complex seems to surround the NDH48 protein thus partially protecting it from digestion by proteases (Fig. 6C) while the NDH45 protein is likely to be buried inside the thylakoid membrane below the NDH48 protein thus being protected from digestion by proteases when NDH45 is present.

It is interesting to note that the NDH45 and NDH48 proteins do not have homologs in cyanobacterial genomes and it may turn out that the chloroplastic NDH complex has acquired more nuclear-encoded subunits than have been envisaged so far (7, 38), and thus the chloroplastic NDH complex is likely to have more structural complexity than the cyanobacterial one.

Acknowledgments—We thank Salk Institute Genomic Analysis Laboratory for providing the sequence-indexed Arabidopsis T-DNA insertion mutants, Prof. Toshiharu Shikanai for providing the crr2-2 Arabidopsis mutant line, and the proteomics unit in the Turku Center for Biotechnology for maintenance of the mass spectrometry facility.

REFERENCES

Novel Nuclear-encoded Subunits of the Chloroplast NAD(P)H Dehydrogenase Complex
Sari Sirpiö, Yagut Allahverdiyeva, Maija Holmström, Anastassia Khrouchchova, Anna Haldrup, Natalia Battchikova and Eva-Mari Aro

doi: 10.1074/jbc.M805404200 originally published online October 28, 2008

Access the most updated version of this article at doi: 10.1074/jbc.M805404200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2008/10/29/M805404200.DC1

This article cites 39 references, 18 of which can be accessed free at http://www.jbc.org/content/284/2/905.full.html#ref-list-1