Structural characterization of a plant photosystem I and NAD(P)H dehydrogenase supercomplex
Kouřil, Roman; Strouhal, Ondřej; Nosek, Lukáš; Lenobel, René; Chamrád, Ivo; Boekema, Egbert J.; Šebela, Marek; Ilík, Petr

Published in:
The Plant Journal

DOI:
10.1111/tpj.12402

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2014

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the “Taverne” license. More information can be found on the University of Groningen website: https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment.

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.
Structural characterization of a plant photosystem I and NAD(P)H dehydrogenase supercomplex

Roman Kouril1,*, Ondrej Strouhal1,†, Lukáš Nosek1, René Lenobel2, Ivo Chamrăd2, Egbert J. Boekema3, Marek Šebela2 and Petr Ilík1
1Department of Biophysics, Centre of the Region Haná for Biotechnological and Agricultural Research, Faculty of Science, Palacký University, Šlechtitelu 11, 783 71 Olomouc, Czech Republic, 2Department of Protein Biochemistry and Proteomics, Centre of the Region Haná for Biotechnological and Agricultural Research, Faculty of Science, Palacký University, Šlechtitelu 11, 783 71 Olomouc, Czech Republic, and 3Electron Microscopy Group, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Nijenborgh 7, 9747 AG Groningen, The Netherlands

Received 3 October 2013; revised 21 November 2013; accepted 2 December 2013; published online 8 December 2013.
*For correspondence (e-mail roman.kouril@upol.cz).
†Both authors contributed equally to this work.

SUMMARY
Cyclic electron transport (CET) around photosystem I (PSI) plays an important role in balancing the ATP/NADPH ratio and the photoprotection of plants. The NAD(P)H dehydrogenase complex (NDH) has a key function in one of the CET pathways. Current knowledge indicates that, in order to fulfill its role in CET, the NDH complex needs to be associated with PSI; however, until now there has been no direct structural information about such a supercomplex. Here we present structural data obtained for a plant PSI–NDH supercomplex. Electron microscopy analysis revealed that in this supercomplex two copies of PSI are attached to one NDH complex. A constructed pseudo-atomic model indicates asymmetric binding of two PSI complexes to NDH and suggests that the low-abundant Lhca5 and Lhca6 subunits mediate the binding of one of the PSI complexes to NDH. On the basis of our structural data, we propose a model of electron transport in the PSI–NDH supercomplex in which the association of PSI to NDH seems to be important for efficient trapping of reduced ferredoxin by NDH.

Keywords: clear native electrophoresis, Hordeum vulgare, single particle electron microscopy, PSI-NDH supercomplex, cyclic electron transport.

INTRODUCTION
In oxygenic photosynthesis, light reactions are driven by photosystem I (PSI) and photosystem II (PSII), two multsubunit protein complexes embedded in the thylakoid membrane. The photosystems cooperatively transfer electrons released from water molecules via plastocyanine (PQ), cytochrome (cyt) b6/f complex and plastocyanin to ferredoxin (Fd). Electron transport is coupled with the translocation of protons across the thylakoid membrane, which contributes to the generation of a transmembrane ΔpH gradient utilized by ATP synthase to produce ATP. The fate of electrons transported to Fd depends on whether PSI operates in linear (LET) or cyclic (CET) electron transport. In LET, Fd reduces NADP+ via ferredoxin-NADP+ oxidoreductase (FNR) to NADPH, which is utilized in various biosynthetic pathways. In CET, electrons are driven back to the PQ pool and cyt b6/f complex and this contributes to the formation of a transmembrane ΔpH gradient and thus ATP synthesis without the net production of NADPH. Since it has been reported that LET by itself cannot meet the ATP demands of a plant even under optimal environmental conditions, CET has been generally recognized as an important electron transfer pathway in photosynthetic organisms (see Kramer et al., 2004; Munekage et al., 2004; Shikanai, 2007). Moreover, a temporary change in the relative contribution of LET and CET to overall electron transport can balance the changing ATP/NADPH demands of a plant under varying environmental conditions (for a review see Kramer et al., 2004).

There are currently two main CET pathways that are considered to operate around PSI. One of them depends on the PROTON GRADIENT REGULATION5 (PGR5) and PGR5-LIKE1 (PGR5L1) complex (Munekage et al., 2002; DalCorso et al., 2008). The PGR5L1 complex could be the elusive Fd–PQ reductase (FQR), as has been recently proposed by
Hertle et al. (2013). The other pathway depends on NAD(P)H dehydrogenase (NDH; Burrows et al., 1998; Kofer et al., 1998; Shikanai et al., 1998). The PGR-dependent pathway has been proposed to play an essential role in the balancing of the ATP/NADPH ratio and in plant photoprotection (via energy-dependent non-photochemical quenching, qE) under naturally fluctuating environmental conditions (see Munekage et al., 2002, 2004; Kramer et al., 2004). While this function of the PGR-dependent pathway is generally accepted, the role of the NDH-dependent pathway is still a matter of debate. Studies based on mutants with impaired NDH function indicate that the NDH pathway is not essential for photosynthesis under normal conditions (e.g. Munekage et al., 2004). Nevertheless, its importance becomes more evident under stress conditions, when this pathway seems to participate in protection against oxidative stress by preventing over-reduction of the chloroplast stroma (for a review see Shikanai, 2007). Indeed, when transgenic tobacco plants with a disrupted NDH function were exposed to stresses preferentially inhibiting CO2 fixation (e.g. Horváth et al., 2000; Li et al., 2004), i.e. when these plants were experiencing conditions leading to stroma over-reduction, their photosynthetic rate or qE induction were reduced in comparison to the wild type (WT). Also a recent study with high cyclic electron flow (hcef) Arabidopsis mutants, which have constitutively impaired function of the Calvin-Benson cycle, has shown that under such conditions the NDH pathway enhanced qE and augmented production of ATP (Livingston et al., 2010). Generally, it seems that plants stressed by the factors leading to the over-reduction of chloroplast stroma utilize NDH-dependent CET for both maintenance and photoprotection of the photosynthetic function.

The discovery of the chloroplast NDH complex was based on the identification of 11 plastid genes, ndhA−ndhK, which are homologs of genes encoding subunits of mitochondrial complex I (Matsubayashi et al., 1987). Later it was shown that chloroplast NDH is even more similar to bacterial respiratory complex I (NDH-1), except for three bacterial subunits (NuoE–G) whose homologs are missing in the chloroplast NDH. These subunits form the NDH-binding domain in NDH-1 (Friedrich and Scheide, 2000) and therefore their absence indicates that the electron-binding domain in the NDH complex of photosynthetic organisms is probably different. The identity of the electron donor-binding domain in chloroplast NDH has been unknown until recently, when three specific subunits (CRR31, CRJ and CRL) were suggested to form the Fd-binding site (Yamamoto et al., 2011). This finding has prompted the reconsideration of the generally accepted idea that NAD(P)H is a direct electron donor to the chloroplast NDH complex. Except for the difference in the electron-binding domain, the photosynthetic NDH complexes have been found to contain other specific subunits which are missing in bacterial NDH-1 (see Battchikova et al., 2011; Ifuku et al., 2011; Peng et al., 2011, for recent reviews). Currently, high-resolution structural data are available only for bacterial NDH-1 (Baradaran et al., 2013). The crystal structures of its counterparts in either cyanobacteria or higher plants are still missing, but electron microscopy (EM) studies of cyanobacterial NDH-1 complexes revealed that it has an L-shaped structure similar to bacterial NDH-1 (Arteni et al., 2006).

The original idea that chloroplast NDH forms a supercomplex with PSI in the thylakoid membrane was formulated on the basis of data obtained using blue-native polyacrylamide gel electrophoresis (BN-PAGE) and the subsequent biochemical characterization (Aro et al., 2005; Peng et al., 2008). Detailed studies of the PSI–NDH supercomplex in various Arabidopsis mutants by Shikanai’s group revealed that two minor light-harvesting antenna proteins, Lhca5 and Lhca6, are required for the formation of the supercomplex and for the efficient operation of the NDH pathway (Peng et al., 2009) and that two copies of PSI can be associated with the NDH complex (Peng and Shikanai, 2011). Nevertheless, detailed information about the spatial interaction and stoichiometry of the components of the PSI–NDH supercomplex has not been available so far.

In the study presented here we provide the first structural evidence of the formation of the PSI–NDH supercomplex. We isolated PSI–NDH supercomplexes using high-resolution clear-native (CN)-PAGE under mild conditions and used single-particle EM for their structural characterization. Image analysis revealed the presence of two forms of the PSI–NDH supercomplex. The larger one consists of one NDH complex associated with two copies of the PSI complex, whereas in the smaller one only one PSI is bound to NDH. A comparison of the projection maps of electron densities of the NDH–PSI supercomplex with known atomic X-ray structures of the bacterial NDH-1 complex (Baradaran et al., 2013) and PSI complex (Amunts et al., 2010) enabled us to construct a pseudo-atomic model, which provides a structural insight into the interactions within the supercomplex and allows us to discuss their implications for the function of NDH-dependent CET.

RESULTS AND DISCUSSION

We aimed at a high-yield separation of PSI–NDH supercomplexes from thylakoid membranes in an intact and pure form as we were subsequently going to analyze them using mass spectrometry (MS) and single-particle EM. We decided to perform this separation using high-resolution native PAGE. Generally, native PAGE performed in gels with a gradient density starting at 4% acrylamide enables separation of large protein supercomplexes with a molecular mass even exceeding 1 MDa in the form of well distinguished and focused bands. In our separation, we preferred CN-PAGE to the more often used BN-PAGE.
because it generally works under milder conditions (Wittig and Schägger, 2005).

As the chloroplast NDH complex in plants grown under normal conditions was estimated to be only about 1–2% of PSI on a molar basis (Peng et al., 2008), it was crucial to carefully adjust the separation and isolation conditions to yield a sufficient amount of the PSI–NDH supercomplex for the subsequent analysis. In order to prepare a sample enriched in intact PSI–NDH supercomplexes from thylakoid membranes, we treated the membranes with a low concentration of n-dodecyl-β-D-maltoside (DDM). Under these conditions, the solubilization of membrane protein complexes from large grana was incomplete and the solubilized protein fraction was enriched in protein complexes from stroma thylakoids including the PSI–NDH supercomplex (Peng et al., 2008, 2009). The subsequent separation of this protein fraction by CN-PAGE showed several green bands containing PSI (Figure 1). Most of the PSI–LHCII complexes remained unsolubilized or they were retained on the top of the polyacrylamide gel.

We analyzed the separated green bands by MS and identified PSI in five green bands (bands 1–5; Figure 1). Based on the MS analysis of the bands and the comparison of our separation with similar separations using CN-PAGE, BN-PAGE (e.g. Jarvi et al., 2011) or Deriphat-PAGE (e.g. Lipová et al., 2010), we attributed the lightest PSI-containing band (band 5) to PSI monomer, which co-migrates with the dimeric PSI core. Heavier PSI-containing bands, above band 5 in Figure 1, represent the PSI/PSII supercomplex (band 4) or PSI oligomers (bands 3 and 1). In band 2 we detected a large number of NDH subunits together with PSI subunits, thus we attributed this band to the PSI–NDH supercomplex (Table 1).

Band 2 was excised and the supercomplexes were extracted from the gel by spontaneous elution and directly used for structural analysis using single-particle EM. Inspection of electron micrographs revealed the presence of two forms of PSI–NDH particle, which differed in size (Figure 2a). Image analysis of about 11 000 single-particle projections indicated that the larger particle consists of two PSI complexes attached to the sides of one NDH complex, which can be clearly recognized in the central part of the projection map due to the typically curved shape of the membrane arm and the very strong density of the hydrophilic arm (Figure 2b). The smaller particle contains only one copy of the PSI complex and one copy of NDH (Figure 2c). The assignment of the NDH complex indicates that the projection maps obtained for both the smaller and larger PSI–NDH supercomplexes represent views from the stromal side of the thylakoid membrane. Due to the fact that the PSI–NDH supercomplexes were separated by CN-PAGE as a single band (i.e. they have the same molecular mass), the smaller particles have to be considered as artificial breakdown products. Since there are hardly any free PSI complexes in the electron micrographs (Figure 2a), it is likely that the dissociation of one PSI from the supercomplex does not take place during the final preparation of the EM specimen but most probably one PSI remains in the gel during the elution of the PSI–NDH supercomplexes. Interestingly, the image analysis revealed only one form of the smaller supercomplex, where the single PSI complex is bound to the outer part of the curved membrane arm of the NDH complex (Figure 2c). This implies that the binding of PSI at this site is stronger than the binding of PSI to the inner part of the curved membrane arm of the NDH.

We constructed a pseudo-atomic model of the PSI–NDH supercomplex by fitting the EM projection map with the known X-ray structures of the plant PSI complex (Amunts et al., 2010) and the entire bacterial NDH-1 complex from Thermus thermophilus (Baradaran et al., 2013). The latter was used as an approximation of the ‘core part’ of the chloroplast NDH complex due to a high homology between 11 chloroplast NDH (NdhA-K) and bacterial NDH-1 (Nqo4–14) subunits (see Peng et al., 2011; for the nomenclature of these subunits). The validation of the model was based on the comparison of the EM projection map with the two-dimensional projection map of the PSI–NDH supercomplex generated from the pseudo-atomic model composed of the truncated X-ray structures (Figure 2e).

The pseudo-atomic model shows that the EM projection map of the chloroplast NDH complex is well fitted with the membrane subunits of NDH-1. Nevertheless, compared

![Figure 1. Separation of pigment-protein complexes by clear native-PAGE. Thylakoid membranes isolated from barley leaves were solubilized by n-dodecyl-β-D-maltoside (DDM; 6 μl of 10% (w/v) β-DDM per 100 μg of chlorophyll). PSI, photosystem I; PSII, photosystem II; NDH, NAD(P)H dehydrogenase.](Image)
et al. (2011) and Ifuku et al. (2011) and Ifuku et al. (2011). Basic protein identification statistics is related to trypsin/chymotrypsin digestion. Individual proteins are named according to Peng et al. (2011) and Ifuku et al. (2011).

Table 1 A summary of the identified photosystem I (PSI)–NAD(P)H dehydrogenase (NDH) complex subunits isolated from barley

<table>
<thead>
<tr>
<th>Protein</th>
<th>UniProt accession code or AGI code</th>
<th>Organism</th>
<th>Mowse score</th>
<th>Peptide counts</th>
<th>Spectral counts</th>
<th>Sequence coverage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSI complex</td>
<td>PsaA</td>
<td>spA1E9J1</td>
<td>H. vulgare</td>
<td>789/355</td>
<td>22/11</td>
<td>83/31</td>
</tr>
<tr>
<td></td>
<td>PsaD</td>
<td>triF2EJP2</td>
<td>H. vulgare</td>
<td>393/326</td>
<td>13/10</td>
<td>57/47</td>
</tr>
<tr>
<td></td>
<td>PsaE</td>
<td>triMOYUC4</td>
<td>H. vulgare</td>
<td>181/117</td>
<td>6/3</td>
<td>20/16</td>
</tr>
<tr>
<td></td>
<td>PsaF</td>
<td>triF2EWA0/triMOY678</td>
<td>H. vulgare</td>
<td>220/563</td>
<td>5/13</td>
<td>21/57</td>
</tr>
<tr>
<td></td>
<td>PsaG</td>
<td>triMOYLY3/triF2DE22</td>
<td>H. vulgare</td>
<td>84/1</td>
<td>2/3</td>
<td>6/8</td>
</tr>
<tr>
<td></td>
<td>PsaH</td>
<td>triM0WH0</td>
<td>H. vulgare</td>
<td>147/180</td>
<td>4/6</td>
<td>25/24</td>
</tr>
<tr>
<td></td>
<td>PsaK</td>
<td>triM0YA7</td>
<td>H. vulgare</td>
<td>175/75</td>
<td>5/2</td>
<td>17/9</td>
</tr>
<tr>
<td></td>
<td>PsaL</td>
<td>spP23993</td>
<td>H. vulgare</td>
<td>262/168</td>
<td>8/5</td>
<td>24/13</td>
</tr>
<tr>
<td></td>
<td>Lhca1</td>
<td>triM0V8T9</td>
<td>H. vulgare</td>
<td>342/138</td>
<td>10/5</td>
<td>38/19</td>
</tr>
<tr>
<td></td>
<td>Lhca2</td>
<td>triMOXH9</td>
<td>H. vulgare</td>
<td>240/138</td>
<td>4/3</td>
<td>15/13</td>
</tr>
<tr>
<td></td>
<td>Lhca3</td>
<td>triF2D9M7</td>
<td>H. vulgare</td>
<td>341/89</td>
<td>8/2</td>
<td>31/8</td>
</tr>
<tr>
<td></td>
<td>Lhca4</td>
<td>triM0W877/triF2CRC1</td>
<td>H. vulgare</td>
<td>312/111</td>
<td>8/2</td>
<td>34/9</td>
</tr>
<tr>
<td></td>
<td>Lhca5</td>
<td>triM0UPG7/triM0UPG6</td>
<td>H. vulgare</td>
<td>182/123</td>
<td>4/5</td>
<td>12/9</td>
</tr>
<tr>
<td></td>
<td>Lhca6</td>
<td>triM0V4V6</td>
<td>H. vulgare</td>
<td>114/127</td>
<td>4/3</td>
<td>16/10</td>
</tr>
<tr>
<td>NDH membrane subcomplex</td>
<td>NdHA</td>
<td>spP92432/ATCG01100</td>
<td>H. vulgare/A. thaliana</td>
<td>214/141</td>
<td>4/4</td>
<td>14/10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>spA1E9J6</td>
<td>H. vulgare</td>
<td>78/79</td>
<td>3/3</td>
<td>16/9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>spI03060</td>
<td>H. vulgare</td>
<td>168/160</td>
<td>4/6</td>
<td>12/11</td>
</tr>
<tr>
<td>NDH subcomplex A</td>
<td>NdHh</td>
<td>spA098691</td>
<td>H. vulgare</td>
<td>416/290</td>
<td>11/13</td>
<td>54/24</td>
</tr>
<tr>
<td></td>
<td>Ndhl</td>
<td>ATCG01090</td>
<td>A. thaliana</td>
<td>136/39</td>
<td>3/3</td>
<td>13/13</td>
</tr>
<tr>
<td></td>
<td>NdHj</td>
<td>triMOVEF6</td>
<td>H. vulgare</td>
<td>105/132</td>
<td>3/3</td>
<td>13/15</td>
</tr>
<tr>
<td></td>
<td>NdHk</td>
<td>spQ85X3</td>
<td>H. vulgare</td>
<td>228/213</td>
<td>6/7</td>
<td>25/22</td>
</tr>
<tr>
<td></td>
<td>NdHl</td>
<td>triF2CUR7</td>
<td>H. vulgare</td>
<td>69/1</td>
<td>3/3</td>
<td>7/4</td>
</tr>
<tr>
<td></td>
<td>NdHm</td>
<td>triMOVHJ2</td>
<td>H. vulgare</td>
<td>235/55</td>
<td>7/5</td>
<td>22/5</td>
</tr>
<tr>
<td></td>
<td>NdHn</td>
<td>triMOXNR3</td>
<td>H. vulgare</td>
<td>397/99</td>
<td>12/2</td>
<td>46/8</td>
</tr>
<tr>
<td></td>
<td>NdHo</td>
<td>triMOUSN8</td>
<td>H. vulgare</td>
<td>163/169</td>
<td>4/5</td>
<td>17/25</td>
</tr>
<tr>
<td>NDH subcomplex B</td>
<td>NDF1</td>
<td>triF2D3G1</td>
<td>H. vulgare</td>
<td>605/401</td>
<td>14/14</td>
<td>60/34</td>
</tr>
<tr>
<td></td>
<td>NDF2</td>
<td>triF2D932</td>
<td>H. vulgare</td>
<td>720/308</td>
<td>18/8</td>
<td>70/32</td>
</tr>
<tr>
<td></td>
<td>NDF6</td>
<td>triF2CPQ4</td>
<td>H. vulgare</td>
<td>239/81</td>
<td>7/5</td>
<td>16/6</td>
</tr>
<tr>
<td></td>
<td>NDH18</td>
<td>triF2DWH9</td>
<td>H. vulgare</td>
<td>78/7</td>
<td>3/3</td>
<td>5/5</td>
</tr>
<tr>
<td></td>
<td>POL</td>
<td>triM0XTT4</td>
<td>H. vulgare</td>
<td>79/49</td>
<td>3/2</td>
<td>9/5</td>
</tr>
<tr>
<td>Lumen subcomplex</td>
<td>PPL2</td>
<td>triMOXDE0</td>
<td>H. vulgare</td>
<td>231/185</td>
<td>6/6</td>
<td>31/15</td>
</tr>
<tr>
<td></td>
<td>CYP20-2</td>
<td>triF2CTD7</td>
<td>H. vulgare</td>
<td>134/112</td>
<td>6/6</td>
<td>13/9</td>
</tr>
<tr>
<td></td>
<td>FKB1P16-2</td>
<td>triF2DIC1</td>
<td>H. vulgare</td>
<td>182/57</td>
<td>6/2</td>
<td>19/7</td>
</tr>
<tr>
<td></td>
<td>POL</td>
<td>triF2CTW7</td>
<td>H. vulgare</td>
<td>147/127</td>
<td>3/4</td>
<td>22/10</td>
</tr>
<tr>
<td>NDH electron donor-binding</td>
<td>NdHU</td>
<td>triF2DYa4/triF2DEX3</td>
<td>H. vulgare</td>
<td>168/102</td>
<td>4/4</td>
<td>15/9</td>
</tr>
</tbody>
</table>

Basic protein identification statistics is related to trypsin/chymotrypsin digestion. Individual proteins are named according to Peng et al. (2011) and Ifuku et al. (2011).

with the generated projection map, in the EM projection map we observed a stronger density variation in the membrane arm of the NDH. Our MS analysis (Table 1) suggests that this difference can be attributed to the presence of additional subunits, specific to the chloroplast NDH complex (see Ifuku et al., 2011; Peng et al., 2011). An unassigned strong density of the hydrophilic arm (blue asterisk, Figure 2d) may also represent subunits specific to the chloroplast NDH complex. We might speculate that the additional density comes from the recently discovered CRR31, CRRJ and CRRL subunits, which form the Fd-binding domain in the chloroplast NDH (Yamamoto et al., 2011). One of these subunits, CRRL (NdHU), was indeed identified in the PSI–NDH supercomplex by the MS analysis (Table 1).

Fitting of the PSI complexes was facilitated by resolved strong densities of the PSI core complex at the periphery of the supercomplex, which indicates that the PSI complexes interact with the membrane domain of the NDH through their light-harvesting antenna proteins. Inspection of the pseudo-atomic model shows that the PSI complex at the outer part of the curved membrane domain binds to NdHd and NdHf subunits through Lhca2/4 proteins. Involvement of the NdHd and NdHf subunits in association with PSI and NDH is in agreement with earlier biochemical data (Peng et al., 2009). The other PSI complex, which is attached to the inner part of the curved membrane domain, interacts with NdHB/F subunits mainly through Lhca2/4, where Lhca4 is also in the vicinity of NdHE and NdG. The pseudo-atomic model indicates that Lhca1 is facing the area of strong density corresponding to the hydrophilic arm, which overlaps the tip of the NDH membrane arm.
The fact that PSI binds to NDH in two different ways is quite unusual. To facilitate their binding, PSI complexes possibly need 'help' from additional proteins. There is an unassigned protein density between the Lhca2/3 of the left PSI complex and the NdhD/B subunits (Figure 2d). The area is large enough to accommodate additional light-harvesting proteins or another Ndh subunit, specific to the chloroplast NDH complex. We propose that the unassigned area contains minor light-harvesting proteins, Lhca5 and/or Lhca6, which were found to play a critical role in supercomplex formation (Peng et al., 2009). This is supported by our MS analysis, which identified both Lhca5 and Lhca6 in the PSI–NDH supercomplex (Table 1). The proposed location of the Lhca5/6 proteins is in agreement with the cross-linking studies, which indicate that Lhca5 associates with PSI complex at the Lhca2/3 site (Lucinski et al., 2006). Nevertheless, a complete absence of association of PSI to NDH complex was observed in the Arabidopsis lhca5–lhca6 double mutant (Peng and Shikanai, 2011). In the light of this finding, one would expect that Lhca5 and Lhca6 proteins mediate binding of PSI complexes to both sides of the NDH complex. The same conclusion can also be drawn from a former study of individual Arabidopsis lhca5 and lhca6 single mutants, where only a smaller form of the PSI–NDH supercomplex (one PSI complex/NDH) was detected (Peng et al., 2009). However, our data imply that, at least in barley, both Lhca5 and Lhca6 participate in binding of only one of the PSI complexes and support its strong association with the NDH complex (Figure 2b–d). The binding of the other PSI complex to NDH is weak, which is evidenced by the fact that it can dissociate from the intact PSI–NDH supercomplex during its elution from the gel (see discussion above). Based on our current data obtained for barley we can only speculate whether the

Figure 2. Structural characterization of the photosystem I (PSI)–NAD(P)H dehydrogenase (NDH) supercomplex by single-particle electron microscopy.

(a) Examples of two raw electron micrographs of a negatively stained specimen with two forms of the PSI–NDH supercomplex. A more abundant larger form is highlighted in the blue box, a smaller form in the yellow box.
(b) Averaged projection map of the larger PSI–NDH supercomplex (sum of 4808 particles) consisting of the NDH complex and two copies of the PSI complex.
(c) Averaged projection map of the smaller PSI–NDH supercomplex (sum of 1031 particles) containing single copies of both NDH and PSI complex.
(d) Structural assignment of the larger PSI–NDH supercomplex based on fitting with the X-ray structures of the PSI complex (Amunts et al., 2010) and the respiratory complex I (Baradaran et al., 2013; the Protein Data Bank accession numbers 3LW5 and 4HEA, respectively). The PSI complex is shown in green with highlighted Lhca proteins (Lhca1 in red, Lhca4 in yellow, Lhca2 in cyan, Lhca3 in magenta). Subunits of the membrane part of the respiratory complex are shown in orange (NdhF), green (NdhD), salmon (NdhB), warm pink (NdhE), pale cyan (NdhG) and blue (NdhA and NdhC). The hydrophilic arm is in light-orange. Nqo1–3 subunits, which are missing in the chloroplast NDH complex, were omitted in the model. The blue asterisk indicates an unassigned density area of the hydrophilic arm.
(e) Projection map of the PSI–NDH supercomplex generated from the proposed pseudo-atomic model at 20 Å resolution.
smaller PSI–NDH supercomplex formed in Arabidopsis lhca5 and lhca6 single mutants represents the complex with the strongly or the weakly bound PSI. Nevertheless, a complete absence of PSI–NDH supercomplexes in the Arabidopsis lhca5–lhca6 double mutant indicates that (i) the strongly bound PSI requires these minor antenna proteins for its stable association with the NDH complex and (ii) the interaction between the strongly bound PSI and NDH is required for the association of the weakly bound PSI to the NDH complex. However, further structural analysis of individual Arabidopsis mutants is needed to fully clarify this issue.

The question is, what benefit is brought by the organization of NDH and PSI in such a peculiarly shaped supercomplex? Firstly, the binding of two PSI complexes along the membrane arm of the NDH maximizes the interaction interface between these complexes, which clarifies the stabilization of the NDH complex observed when NDH is part of the supercomplex (Peng and Shikanai, 2011). Secondly, the formation of the supercomplex guarantees short distances between the hydrophilic arm of the NDH, which most probably accommodates the Fd-binding domain of the NDH, and two PSI–Fd-binding sites (approximately 11 and 22 nm). Further, the 2:1 stoichiometry between PSI and NDH in the supercomplex can locally increase the population of reduced Fd. Taken together, these facts suggest that supercomplex formation can make the trapping of the reduced Fd by the NDH complex more efficient. This interpretation coincides with the recent results of Yamamoto et al. (2011), who showed the importance of Fd in the function of the chloroplast NDH complex. These authors showed that the NDH complex accepts electrons from Fd rather than from NAD(P)H and thus they proposed that the chloroplast NDH complex may function in a similar way to the PGRL1 complex, i.e. as the FQR (Hertle et al., 2013). From this point of view, NDH-dependent CET might be much more similar to PGR-dependent CET than previously assumed.

Despite this similarity, we have to bear in mind that in the case of NDH-dependent CET, NAD(P)H is widely accepted as the main electron donor and therefore we can ask what the exact role of Fd in this pathway is. We hypothesize that within the PSI–NDH supercomplex, Fd can be reduced at the expense of NAD(P)H via the reverse reaction of FNR. The Fd then reduces the plastoquinone (PQ) molecule, which is bound at the back side of the membrane arm of the NDH complex (continuous violet arrow). Then the cyclic pathway is completed by a transfer of the electron from the PQ molecule back to the PSI complex via the cyt b/f complex and plastocyanine. Alternatively, Fd can be reduced by NAD(P)H through the reverse reaction of FNR, which is attached to the NDH complex. In this way, the alternative reduction of Fd can alleviate the stromal over-reduction. The NDH complex may also function as a proton pump. LHCI, light-harvesting complex I.

**Figure 3.** Structure–functional model of the photosystem I (PSI)–NAD(P)H dehydrogenase (NDH) supercomplex in the thylakoid membrane. Ferredoxin (Fd) binds to the hydrophilic arm of the NDH complex (continuous red arrows) upon its reduction at the acceptor side of the PSI complex (dotted blue arrow). Then Fd reduces the plastoquinone (PQ) molecule, which is bound at the back side of the membrane arm of the NDH complex (continuous violet arrow), via intermediate NDH electron carriers (dotted red arrow). Then the cyclic pathway is completed by a transfer of the electron from the PQ molecule back to the PSI complex via the cyt b/f complex and plastocyanine. Alternatively, Fd can be reduced by NAD(P)H through the reverse reaction of FNR, which is attached to the NDH complex. In this way, the alternative reduction of Fd can alleviate the stromal over-reduction. The NDH complex may also function as a proton pump. LHCI, light-harvesting complex I.
CONCLUSION

The first evidence of the existence of the PSI–NDH supercomplex was based on extensive biochemical studies (Peng et al., 2008, 2009) and the absence of structural data can be ascribed mainly to the low abundance of the NDH complex in the thylakoid membrane. The fact that the NDH content is generally as low as 1–2% of the total PSI content (Peng et al., 2008) makes any structural analysis of the supercomplex rather challenging. An optimized mild separation of solubilized thylakoid membrane using CN-PAGE, followed by MS of separated bands, allowed us to isolate an intact PSI–NDH supercomplex with a yield sufficient for single-particle structural analysis. The structural analysis shows asymmetric binding of two PSI complexes to an NDH complex and indicates the position of the Lhca5 and Lhca6 antenna proteins of PSI, which are present in the thylakoid at enigmatically low substoichiometric levels (Ganeteg et al., 2004). These antenna proteins are probably needed for the attachment of one of the two PSI complexes to the low-abundant NDH.

The formation of the PSI–NDH supercomplex is another factor supporting the hypothesis that the key reactions in photosynthesis and in the mitochondrial respiratory chain require a high level of organization. Besides the increase in the stability of individual components, supercomplex formation has important functional implications, which result from possibly enhanced electron transfer rates and substrate channeling (Dudkina et al., 2010). It has been reported that in *Chlamydomonas reinhardtii*, CET depends on the formation of a PSI–LHCI–LHCII–FNR–cytb~5/f–PGRL1 supercomplex (Iwai et al., 2010). Here, we provide a structural evidence for PSI–NDH supercomplex formation in higher plants. As the formation of the PSI–NDH supercomplex was found to be necessary for the efficient operation of the NDH complex (Peng et al., 2008), it is essential for proper function of the NDH-dependent pathway of CET. The formation and dissociation of supercomplexes can function as a switch between CET and CET. Different triggers have been proposed to induce the formation of supercomplexes participating in CET, including a low ATP content (Joliot and Joliot, 2002), a high NADPH concentration (Joliot and Johnson, 2011) or protein phosphorylation (Iwai et al., 2010). Nevertheless, further studies are necessary to understand the exact mechanism governing the reversible formation of these supercomplexes.

EXPERIMENTAL PROCEDURES

Plant material and sample preparation

Barley (*Hordeum vulgare* L. cv. Akcent) seedlings were grown in a growth chamber with a 16-h/8-h light/dark photoperiod at 25°C. During the light period, the seedlings were illuminated with a white fluorescent lamp at an intensity of about 100 μmol photons m~−2 s~−1~ (400–700 nm). Thylakoid membranes were isolated as described in Dau et al. (1995) and a quantity of thylakoids corresponding to 100 μg of chlorophyll was solubilized by the addition of 6 μl of 10% (w/v) DDM. The mixture was supplemented with solubilizing buffer (50 mM HEPES, 400 mM sucrose, 15 mM NaCl, 5 mM MgCl₂, pH 7.2) containing 10% (v/v) glycerol to a final volume of 50 μl. After solubilization, membrane fragments were removed by centrifugation (22 000 g, 4°C, 10 min). Then CN-PAGE was performed with a 4–8% gradient resolving gel and 4% stacking gel (Wittig et al., 2007). The separation started at a constant current of 7 mA for 15 min and continued at a constant current of 15 mA for an additional 2 h. The chlorophyll content was determined by extraction and assay in 80% acetone (Lichtenthaler and Buschmann, 2001).

Sample preparation and liquid chromatography (LC)-MS/MS-based analysis of the PSI–NDH supercomplex

The PSI–NDH supercomplex separated by CN-PAGE was excised from the gel slab and in-gel digested either with trypsin or chymotrypsin as described elsewhere (Shevchenko et al., 2006). The resultant peptides were desalted on C18 microcolumns (Rappsilber et al., 2007) and analyzed using a nanoLC-electrospray ionization-ultrahigh resolution-quantitative time-of-flight LC-MS system (Proxeon, Denmark, www.proxeon.com; Bruker Daltonik, Germany, www.bruker.com). Protein identification was achieved by searching the acquired data against a custom protein database with the Mascot search algorithm (Perkins et al., 1999). For detailed information on the digestion procedure, peptide desalting, LC-MS/MS analysis and data processing see the Supporting Information (Methods S1–S3, Tables S1 and S2, Data S1 and S2).

Electron microscopy and image processing

The gel stripe containing PSI–NDH supercomplexes was excised from the CN-PAGE gel, chopped up and placed in an Eppendorf tube with 50 μl of buffer (50 mM HEPES, 15 mM NaCl, 5 mM MgCl₂, pH 7.2) for 2 h at 4°C to achieve spontaneous elution of the supercomplexes. The solution was then directly used for EM specimen preparation by negative staining with 2% uranyl acetate on a glow-discharged carbon-coated copper grid. Electron microscopy was performed on a Philips CM120 electron microscope (www.ﬁe.com) equipped with a LaB₆ filament operating at 120 kV. Images were recorded with a Gatan 4000 SP (www.gatan.com) 4K slow-scan charge-coupled device camera at 130 000 × magnification with a pixel size of 0.23 nm at the specimen level after binning the images to 2048 × 2048 pixels. GRACE software was used for semi-automated data acquisition (Oostergetel et al., 1998). Single-particle analysis was performed using GRIP software including multi-reference and non-reference alignments, multivariate statistical analysis and classification. A pseudo-atomic model of the PSI–NDH supercomplex was created using Pymol (DeLano, 2002). Truncated versions and a two-dimensional projection map of the generated model for the PSI–NDH supercomplex at 20Å resolution were generated using routines from the IMAG package (Ludtke et al., 1999). A schematic model of the PSI–NDH supercomplex was created using a free version of Google Sketchup software.

ACKNOWLEDGEMENTS

This work was supported by the Ministry of Education, Youth and Sports of the Czech Republic (ED0007/01/01 to Centre of the Region Haná for Biotechnological and Agricultural Research and CZ.1.07/2.3.00/20.0057), the Grant Agency of the Czech Republic (project 13-28093S/P501 to RK) and by a Marie Curie Career Integration Grant call FP7-PEOPLE-2012-CIG (322139 to RK). We thank Dr Iva Iličová for editing the manuscript.

© 2013 The Authors

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Table S1. Complete list of identified proteins for trypsin digestion.

Table S2. Complete list of identified proteins for chymotrypsin digestion.

Data S1. Complete list of identified proteins for trypsin digestion.

Data S2. Complete list of identified proteins for chymotrypsin digestion.

Methods S1. Protein digestion and peptide desalting.

Methods S2. Liquid chromatography-MS/MS analysis.

Methods S3. Protein data analysis.

REFERENCES


