Supercomplexes of plant photosystem I with cytochrome b6f, light-harvesting complex II and NDH

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Abstract
Photosystem I (PSI) is a pigment-protein complex required for the light-dependent reactions of photosynthesis and participates in light-harvesting and redox-driven chloroplast metabolism. Assembly of PSI into supercomplexes with light harvesting complex (LHC) II, cytochrome b6f (Cytb6f) or NAD(P)H dehydrogenase complex (NDH) has been proposed as a means for regulating photosynthesis. However, structural details about the binding positions in plant PSI are lacking. We analyzed large data sets of electron microscopy single particle projections of supercomplexes obtained from the stroma membrane of Arabidopsis thaliana. By single particle analysis, we established the binding position of Cytb6f at the antenna side of PSI. The rectangular-shaped Cytb6f dimer binds at the side where Lhca1 is located. The complex binds with its short side rather than its long side to PSI, which may explain why these supercomplexes are difficult to purify and easily disrupted. Refined analysis of the interaction between PSI and the NDH complex indicates that in total up to 6 copies of PSI can arrange with one NDH complex. Most PSI-NDH supercomplexes appeared to have 1–3 PSI copies associated. Finally, the PSI-LHCII supercomplex was found to bind an additional LHCII trimer at two positions on the LHCI side in Arabidopsis. The organization of PSI, either in a complex with NDH or with Cytb6f, may improve regulation of electron transport by the control of binding partners and distances in small domains.

1. Introduction
Photosystem I (PSI) and photosystem II (PSII) are the major protein complexes regulating the light reactions of oxygenic photosynthesis. Light captured by PSI and PSII is ultimately used for the production of ATP and NADPH. Both PSI and PSII are universally distributed through-out prokaryotes and eukaryotes capable of oxygenic photosynthesis, including higher plants, macroalgae and cyanobacteria. PSI and PSII have the ability to form supercomplexes with other photosynthetic proteins, in particular with LHCII [1,2] and NDH [3]. Plant PSI can bind one LHCII trimer at the side of the PsaK and Psal subunits [2,4,5]. This additional antenna increases the light-harvesting capacity for PSI under state transition. The additional antenna of the PSI-LHCII complex of the model green alga Chlamydomonas reinhardtii is composed of two LHCII trimers plus CP29 [6].

Besides light-harvesting, PSI supercomplexes are also involved in regulating electron flow. Two important modes of electron flow have been described: linear electron flow (LEF) and cyclic electron flow (CEF). In LEF, PSI, Cytb6f, and PSI work in series to produce ATP and NADPH. In CEF only PSI and Cytb6f are involved as the major players, without contribution of PSII. This pathway generates extra ATP at the expense of NADPH by redirecting electron flow from PSI back to the Cytb6f. Control of LEF and CEF can thus adjust the ratio of produced ATP/NADPH in response to changing metabolic and environmental conditions. Under certain conditions, CEF appears to contribute substantially to photosynthetic electron flow, for example during induction of photosynthesis and under abiotic stress such as drought, high light and extreme temperatures [7]. Currently two cyclic pathways are known that require either the PROTON GRADIENT REGULATIONS (PGR5) and PGR5-LIKE1 (PGR5L1) complex [8] or the NADH dehydrogenase-like complex (NDH) [9,10]. Whereas the PGR5-dependent pathway is efficient in control of the ATP/NADPH ratio and
essential to induce dissipation of energy by non-photochemical quenching, the NDH-dependent pathway is considered not to affect non-photochemical quenching. Rather it alleviates over-reduction of the stromal compartment and reduces photoinhibition [11]. Although, both pathways are extremely important for photoprotection and photosynthesis, recent analysis of hcf1 (high CEF) Arabidopsis thaliana mutants, revealed a key role of the NDH in enhanced CEF and augmenting production of ATP [12]. This is consistent with previous observations that accumulation of hydrogen peroxide in barley under photoinhibitory stress was found to mediate the induction of NDH and to enhance its activity [13]. These findings posit a crucial role for the NDH-pathway of cyclic electron transport in environmental stress acclimation.

How the functional and structural aspects of PSI supercomplexes contribute to regulation of electron flow is however not fully understood. Several years ago a supercomplex composed of PSI and Cytb6f complex was described in Chlamydomonas reinhardtii [14]. This supercomplex was proposed to play a role in CEF, but until now has not been structurally characterized. Another PSI-NDH supercomplex that appears to play a specific role in electron flow was recently described [3]. This supercomplex is composed of two PSI complexes held together by a centrally positioned NDH complex.

Despite the increasing number of tentative PSI supercomplexes, no isolation of a plant PSI-Cytb6f particle has yet been demonstrated. This stimulated us to perform a systematic search for novel supercomplexes, especially for those in which Cytb6f, NDH and LHCCI are involved. We used free-flow electrophoresis in combination with blue native electrophoresis (BN) and clear native (CN)-PAGE followed by mass spectrometric determination of the protein composition of isolated complexes. We demonstrate the isolation of PSI-Cytb6f supercomplexes from Arabidopsis chloroplast membranes. The chance of finding such complexes in single particle analysis was increased by mobility analysis of thylakoid protein complexes lacking the PPH1 phosphatase (PPH1) [15]. The Arabidopsis pph1 null mutant accumulates an increased concentration of PSI-LHCCI supercomplexes, associated with increased CEF [16]. We describe the PSI-Cytb6f supercomplex in Arabidopsis for the first time by electron microscopy (EM), and also show several novel types of PSI-LHCCI supercomplexes, where additional LHCCI trimers were determined next to the Lhca1-4 proteins of the standard PSI-LHCCI supercomplex. In addition, we describe the structural organization of novel PSI-NDH supercomplexes identified from large EM data sets. These results indicate a higher level of organization of the unstacked grana membrane than previously considered.

2. Materials & methods

2.1. Isolation of protein complexes from thylakoid membranes

Isolated of photosystem particles, thylakoid membranes were isolated from Arabidopsis thaliana plants as described and aliquots corresponding to 100 μg of Chl were frozen in liquid N2 before storage at − 80 °C [17]. Thylakoid membranes corresponding to 300 μg Chl were thawed, centrifuged for 2 min at 5000 rcf and 4 °C, and membranes were suspended in TMKS buffer (25 mM tricine/NaOH pH 7.2, 5 mM MgCl2·6H2O, 10 mM KCl, 200 mM sorbitol) at a concentration of 1 μg Chl μl−1. Membranes were solubilized in TMKS using 16 mM digitonin for 10 min at 10 °C. The sample was micro-centrifuged at 25,000 rcf for 30 min at 10 °C and the supernatant was processed by interval zone-free electrophoresis (IZE-FFE). Samples were supplemented with 0.02% (v/v) of an anionic tracking dye 2-(4-sulfophenylazo)-1,8-dihydroxy-3,6-naphthalene disulfonic acid (SPADNS) (Becton Dickenson, Franklin Lakes, NJ, USA) before application to IZE-FFE and analysis of protein complexes using 2D-BN/SDS-PAGE [17]. PSI-NDH supercomplexes were co-purified with PSII mega-complexes using clear native electrophoresis (CN-PAGE) according to [18].

2.2. Interval zone-free electrophoresis

All IZE-FFE separations were conducted in a standard free-flow electrophoresis (FFE) system (FFE Service GmbH, Germany) with a gap size of 0.2 mm and with the following buffers. As electrode buffers 100 mM formic acid and 200 mM Bis-Tris, pH 6.4 was used at the anode and 100 mM NaOH, 200 mM glycine, pH 10 at the cathode. For anodic stabilization, a buffer composed of 100 mM HCl, 50 mM formic acid and Bis-Tris, was applied through inlet 1. Anodic stabilization was increased during the experimental period by raising the pH from 4.5 (initial) to 6.4 (final). Separation buffer 1, containing 10 mM hydroxy-isobutyrinic acid (HIBA), 0.2% digitonin, and adjusted to pH 5.4 with Bis-Tris, was applied through inlets 2–4; separation buffer 2, containing 10 mM HIBA, 0.2% digitonin, and adjusted to pH 6.2 with Bis-Tris, was applied through inlets 5 and 6; separation buffer 3, containing 10 mM HIBA, 5 mM NaCl, 0.2% digitonin, and adjusted to pH 7.0 with Bis-Tris, was applied through inlet 7; and separation buffer 4, containing 10 mM HIBA, and adjusted to pH 7.0 with Bis-Tris, was applied through inlet 8. At inlet 9, cathodic stabilization buffer, composed of 150 mM HIBA, and 375 mM Imidazole pH 7.45 was applied and the separation media were neutralized at the end of the separation making use of a counter-flow solution composed of 250 mM sorbitol, 50 mM Bis-Tris, and 20 mM N-(1,1-Dimethyl-2-hydroxyethyl)-3-amino-2-hydroxypansulfonic acid (AMPSO) pH 8.04 or 250 mM sorbitol, 10 mM HIBA, 10 mM Bis-Tris, pH 6.2. Samples were applied via inlet 7, on the cathodic side of the separation chamber.

For separation of the sample, the system was operated in a preprogrammed IZE mode at a high voltage setting of 1600 V, and 86 mA, and with the buffer flow adjusted to 40 ml/h (HV). Interval conditions for sample application and separation were as follows. Sample loading was conducted using a sample pump for 70 s at 5.2 ml/h and a pump for controlling the buffer flow rate of 120 ml/h. When the sample pump was stopped, sample transport into the field chamber was completed for an additional 20 s at 120 ml/h. During electrophoresis at HV, buffer flow rate was reduced to 40 ml/h and separation conducted within 4.5 min. Elution of separated protein complexes was performed in the absence of HV at a buffer flow of 240 ml/h and samples collected in 96 well polyethylene microtiter plates (Becton Dickinson, Franklin Lakes, NJ, USA). IZE was repeated according to the amount of material required for further analysis. Samples in microtiter plates were processed for BN-PAGE [19]. Gelbands were stained using Coomassie G250 [20].

2.3. Mass spectrometry analysis

Gel slices from the native gels were excised and transferred into a 96-well PCR plate. The gel bands were cut into 1 mm² pieces, destained, reduced (DTT) and alkylated (iodoacetamide) and subjected to enzymatic digestion with trypsin overnight at 37 °C. After digestion, the supernatant was pipetted into a sample vial and loaded onto an autosampler for automated LC-MS/MS analysis.

All LC-MS/MS experiments were performed using a nanoAcquity UPLC (Waters Corp., Milford, MA) system and an LTQ Orbitrap Velos hybrid ion trap mass spectrometer (Thermo Scientific, Waltham, MA). Separation of peptides was performed by reverse-phase chromatography using a Waters reverse-phase nanoLC column (BEH C18, 75 μm i.d. × 250 mm, 1.7 μm particle size) at a flow rate of 300 nli/min. Peptides were initially loaded onto a pre-column (Waters UPLC Trap Symmetry C18, 180 μm i.d. × 20 mm, 5 μm particle size) from the nanoAcquity sample manager with 0.1% formic acid for 3 min at a flow rate of 10 μl/min. After this period, the column valve was switched to allow the elution of peptides from the pre-column onto the analytical column. Solvent A was water + 0.1% formic acid and solvent B was acetonitrile + 0.1% formic acid. The linear gradient employed was 5–35% B in 60 min. The LC eluent was sprayed into the mass spectrometer by means of a New Objective nanospray source. All m/z values of eluting ions were...
measured in the Orbitrap Velos mass analyzer, set at a resolution of 30,000. Data dependent scans (Top 20) were employed to automatically isolate and generate fragment ions by collision-induced dissociation in the linear ion trap, resulting in the generation of MS/MS spectra. Ions with charge states of 2+ and above were selected for fragmentation. Post-run, the data was processed using Protein Discoverer (version 1.2, ThermoFisher). Briefly, all MS/MS data were converted to mgf files and these files were submitted to the Mascot search algorithm (Matrix Science, London UK) and searched against the TAIR10 database, using a fixed modification of carbamidomethyl (C) and a variable modification of oxidation (M).

2.4. Electron microscopy

For EM specimens, 4 μl of Arabidopsis fractions, purified by free flow electrophoresis, was applied onto glow-discharged carbon coated grids. The sample was absorbed for 1 min, excess sample was blotted with filter paper then stained with 2% uranyl acetate for enhancing the contrast. Images were collected on a Tecnai T20 (FEI, Eindhoven, NL) equipped with a LaB₆ tip operating at 200 kV. Images of 2048 × 2048 pixels were recorded at 133,000 x magnification using a Gatan 4000 SP 4 K slow-scan CCD camera with a pixel size of 0.224 nm. A total of 35,000 particles were manually picked from 60,000 images and single particles were analyzed with XMIPP software [21], including alignments, statistical analysis and classification and RELION software [22]. The best class members were taken for the final class sums. For three-dimension reconstruction, 4115 selected PSI-Cytb₆f projections were processed using SCIPION protocol xmlipp3-reconstruct [23].

3. Results

Free-flow electrophoresis (FFE) was employed to separate solubilized membranes into fractions of protein complexes. Separation of the protein particles by operating FFE in the IZE setup required 4.5 min and the same amount of time was used for preparation of an electron microscopy specimen. The combination of both techniques minimized the preparation time for fixation of particles and enabled examination of large fragile photosynthetic complexes and supercomplexes. Inspection of specimens by EM revealed that FFE fractions at the beginning of the separation profile (fractions 31–39, Fig. 1) contained mostly PSII complexes. Fractions 37 and 39 were highly enriched in ATP synthase, which was easily recognized due to its uniquely shaped F₀ stalk and F₁ headpiece (data not shown). Some proteins enriched in ATP synthase, which was easily recognized due to its uniquely shaped F₀ stalk and F₁ headpiece (data not shown). Some proteins...

![Fig. 1. Charge-based separation of Arabidopsis thaliana thylakoid protein complexes by IZE-FFE. Membranes corresponding to 300 μg Chl were solubilized in TMKS buffer, using digitonin (16 mM). Non-solubilized membranes were centrifuged (25,000 × g, 30 min) and the supernatant (100 μg Chl) was separated by IZE-FFE. Separation was conducted continuously in a buffer containing 10 mM HEPES, Bis-Tris pH 5.6 (fractions 24–44) and pH 6.2 (fractions 45–65). 200 mM sorbitol and 0.8 mM digitonin (blue dotted line). Thylakoid membrane protein complexes were collected in 300 μl fractions in a 96 well microtiter plate (Fraction number) and the localization of Chl-binding protein complexes was determined spectroscopically by measuring absorption at 420 nm (black curve).](image-url)
3.1. Analysis of Arabidopsis PSI-Cytb6f supercomplexes

The two protein bands in BN-PAGE that appeared to comigrate in FFE fractions 53–59 were analysed further. Mass spectrometry analysis was performed for the two protein bands at about 647 and 384 kDa (Supplemental Table 1). The higher molecular weight band was identified as PSI, since 85% of the identified peptides originate from specific PSI proteins. (Supplemental Table 2A). Identification of two peptides from PetA, and five peptides from the CF1 α and β subunits indicated however that despite the 2D separation, the 647 kDa band was not pure. The lower molecular weight band was identified as Cytb6f with 81% of the peptides originating from specific Cytb6f subunit proteins. Also here seven peptides were identified from PSI subunits PsbB, -C, and -D indicating that the 2D separation protocol provided a substantial enrichment of the PSI and Cytb6f complex, but the MS analysis indicated that bands were not pure. We therefore checked whether the comobility pattern of both protein bands in BN-PAGE was based on the destabilization of a complex composed of PSI and Cytb6f complexes by analysis of the corresponding FFE fractions by single particle analysis.

A total of 35,000 particles were picked from micrographs of FFE fractions containing PSI and Cytb6f (fractions number 53–59) and was submitted to single particle analysis including classification. Many classes showed PSI particles in a highly tilted position but in about half of all projections PSI was more or less in a top-view position, as in the membrane plane. Out of 20,000 particles in top-view projection, 4500 show a PSI-Cytb6f supercomplex in which Cytb6f is present as a dimer (classes of Fig. 3A-H). In 9500 particles, the attached Cytb6f is monomeric (Fig. 3I-K), and in the rest of the particles the Cytb6f was loosely bound (Fig. 3M-O) or completely detached from PSI upon staining (Fig. 3P). Single Cytb6f dimers were analyzed as well and a map is depicted (Fig. 3L) in a position as in the non-tilted dimer classes (Fig. 3A,B). The dimer is rectangular and the overall size and shape is comparable to the high-resolution structures determined by X-ray crystallography [25,26]. The dimer also has strong stain-excluding “bright” features, in comparison to the deeper stain-embedded PSI complex. This is related to the larger dimension of Cytb6f vertical to the membrane.

The structure of Cytb6f dimers detached from PSI (Fig. 3L) is very similar to those bound to PSI (Fig. 3A–D). However, the edges of bound dimers are rounded up as a result of a low overall resolution in these maps. A similar loss of detail is visible in a 20 Å resolution EM map of the Cytb6f complex, compared to a map at 8 Å resolution [27]. The low resolution is caused by a limited number of summed images in each class. All classes show the same supercomplex, but seen from many different angles (Fig. 3A–H). To deduce where the Cytb6f dimer is attached to PSI we calculated a low-resolution 3D model from 4115 2D projection from classes of Fig. 3A–H, see Fig. 4A, B. The model was shifted into a position compatible to the regular PSI (Fig. 4C) and the PSI-LHCII (Fig. 4D), of which we know by comparison to the high-resolution 3D model that they show PSI from the stromal side. The model can be correlated to these 2D maps because according to the high-resolution structure, the shape of Cytb6f is highly asymmetrical in respect to the membrane [25,26]. It protrudes strongly on its luminal side and minimally on the stromal side, in contrast to PSI with its rather flat luminal surface. This difference is reflected in the model, where the Cytb6f part of the PSI-Cytb6f supercomplex is flat on the stromal side (Fig. 4A), but substantially protruding from the luminal side (Fig. 4B). The surface profile of PSI further confirms the position of the 3D model with respect to the 2D maps. The stromal-exposed bump on PSI, made by the Psac, -D and -E subunits [28,29], is on the upper half of PSI (Fig. 4A), whereas the lower thinner side of the PSI model is occupied by the flat LHCII antenna proteins. Thus the model shows that in the PSI-Cytb6f supercomplex Cytb6f is bound to the left side of the light-harvesting part of PSI, where the Lhc1 subunit is located [28,29], as also depicted in a model (Fig. 7A).

3.2. Analysis of Arabidopsis PSI-LHCII supercomplexes

A set of 15,000 potential PSI-LHCII supercomplexes were selected and processed by single particle image analysis. The standard PSI-
LHCII supercomplex was the most common particle in the FFE fractions 48–49 (Fig. 5A). Interestingly, particles of double size were present in low frequency, and image analysis showed indeed that PSI-LHCII supercomplexes can dimerize (Fig. 5B). FFE fractions also contained particles resembling the standard PSI-LHCII particle, but with additional extensions. The map of one of these particles, comprised of over 600 projections indicated that the additional density has the size and shape of a LHCII trimer (Fig. 5C) and modelling of the atomic models of PSI and LHCII into this PSI-LHCII$_2$ supercomplex map indicates that the second LHCII trimer was attached to Lhca2 and Lhca4 antenna subunits (Fig. 7D). In another supercomplex, similar to PSI-LHCII$_2$ (Fig. 5C), the second LHCII trimer attached to the Lhca2 and Lhca3 antenna subunits (Fig. 5D). Two similar maps may represent the same particle in a slightly tilted situation (Fig. 5E, F).

### 3.3. Analysis of Arabidopsis PSI-NDH supercomplexes

The discovery of novel PSI-LHCII supercomplexes raised the question about the existence of supercomplexes larger than the ones composed of NDH and 1–2 copies of PSI, as found in a previous study in barley [3]. Indeed, a former biochemical analysis indicated that NDH might associate with up to three copies of PSI [30]. FFE fractions enriched in PSI lacked substantial numbers of PSI-NDH supercomplexes necessary for a refined analysis by single particle EM. Nevertheless, our recent search for large molecular assemblies in mildly solubilized thylakoid membranes from Arabidopsis thaliana using CN-PAGE revealed, in addition to large PSII megacomplexes [18], the presence of large variable associations between NDH complex and multiple copies of PSI. A batch of PSI-NDH supercomplexes with a total number of 35,000 particles was analyzed. As a result, a wealth of novel information was obtained. Besides the previously found supercomplexes with one or two PSI...
complexes flanking NDH (Fig. 6A,B), three other types of PSI-NDH particles that can bind additional copies of PSI on one side were identified (Fig. 6C–F). If all possible maps are combined, NDH can, hypothetically, associate with 5 PSI complexes on one side, as schematically outlined (Fig. 6G). Indeed, a PSI cluster containing 5 copies, but without NDH, was found (Fig. 6H). In this cluster, the PSI complexes are proposed to be organized at positions according to the model (Fig. 6G).

Another intriguing aspect is that the NDH complex from Arabidopsis, although strongly related to NDH-1 from cyanobacteria, appeared to be similar to mitochondrial Complex I, which has a much higher number of additional subunits than NDH-1. In tilted views or side view positions, the protrusions of NDH, attached to the membrane-bound arm, are visible in a non-overlap position (Fig. 6L,K, red arrowheads). These protrusions, as seen in the side view map (Fig. 6L) are remarkably similar to those of plant Complex I (Fig. 6M). It is tempting to see this as a mistaking-exchange, but it should be noted that the protrusions are also visible if PSI still connected (Fig. 6L) and that on the other side plant Complex I, as studied by Peters and coworkers, was partially connected to Complex III, an unmistakable component of a mitochondrial supercomplex [31].

4. Discussion

In this study, supercomplexes of PSI were isolated that may be discussed as a structural basis for the regulation of CEF in the non-stacked thylakoid membrane. The structures of PSI [28,29], the Cytb6f complex [25,26,32], and NDH complex [33] have been characterized, and chemical steps in energy trapping [34] and electron transfer via iron-sulfur centers are known in detail. However, less is known about the structural arrangement of the complexes in the non-stacked chloroplast membrane and the functional role of supercomplexes composed of these components in regulation of CEF. Clear evidence for structural arrangement of two PSI with NDH in a supercomplex has been shown [3]. Despite the claims for the elusive PSI-Cytb6f supercomplex in the green alga Chlamydomonas reinhardtii [14], no progress has been published regarding purification and subsequent structural characterization of its counterpart in green plants. Several types of particles were characterized in an extensive survey of PSI supercomplexes in non-stacked chloroplast membranes, but none of them had Cytb6f attached [35]. Apparently the interaction between PSI and Cytb6f is very loose and can be easily disrupted by detergents during purification.

In this work we have pursued the structural characterization of elusive PSI supercomplexes in two ways: (1) we pushed the collection of single particle projections to the limit by recording about 100,000 EM

Fig. 5. Projection maps of PSI-LHCII supercomplexes, obtained from single particle image processing. (A) PSI-LHCII supercomplex. (B) Dimeric PSI-LHCII supercomplex, with the lower part in a position as in frame A. (C) PSI-LHCII2 supercomplex with a second LHCII trimer attached to the Lhca2 and Lhca4 antenna subunits. (D–F) Similar maps of a PSI-LHCII supercomplex with the second LHCII trimer attached to the Lhca2 and Lhca3 antenna subunits. The particles may differ mostly in a tilt out of the membrane plane. The scale bar is 100 Å.

Fig. 6. Analysis of PSI-NDH supercomplexes from Arabidopsis. (A) Map of the PSI1-NDH supercomplex. (B) Map of the PSI1-NDH supercomplex. (C) Map of the PSI1-NDH supercomplex. (D) Map of the PSI1-NDH supercomplex in a slightly tilted position, in which the upper left PSI complex becomes larger in projection and the right PSI complex smaller. (E) Map of a PSI1-NDH supercomplex. (F) Map of another PSI1-NDH supercomplex. (G) Schematic model for the largest possible PSI6-NDH supercomplex with 5 copies of PSI attached at the left and one at the right. (H) Particle consisting of 5 PSI complexes, in positions as in the model. (I) Map of NDH-PSI, in a tilted position, protruding densities of the NDH part are indicated by red arrowheads. (J) NDH complex in a tilted position, protrusions are not visible, the hydrophilic arm is partially in a non-overlap position with the membrane domain. (K) NDH in side-view position, with protrusions as in frame I marked by red arrowheads. (L) Same map as in frame J, shifted in a horizontal position. (M) Map of mitochondrial Complex I (reproduced from [31]. Orange arrowhead point to the attached Carbonic anhydrase. Purple arrowheads in frames L and M point to a characteristic density present in both chloroplast NDH and mitochondrial Complex I, and named P2 in the latter. Scale bar is 100 Å.
micrographs of potential PSI-Cytb6f supercomplexes and other supercomplexes. Large data sets of single particle projections, obtained by manual picking and semi-automated extractions from these images, were submitted to single particle averaging, including automated classification. (2) We used free-flow electrophoresis for separation of the complexes. The approach avoids typical problems associated with gel-electrophoresis like size filtration, artificial charge shift, and high internal concentration of proteins especially at the liquid gel junction. Protein complexes are separated based on the pH dependent native charge of the complexes and resolution is achieved by mobility differences in defined interval pH zones (Fig. 1). A concentration of complexes in bands is achieved at pH steps during the separation process. A typical separation of all solubilized membrane protein complexes is achieved in parallel within about 4–6 min. Potential fractions of PSI-Cytb6f and other supercomplexes were selected in the isolation buffer after spectroscopic screening and additional biochemical examinations of the FFE fractions. In this combined way, new supercomplexes were found, which are discussed below.

4.1. PSI-LHCII in Arabidopsis

In a previous study the PSI-LHCII supercomplex was purified by sucrose gradient fractionation and only one particle was found, consisting of a PSI complex and one LHCII trimmer [5]. No larger particles were present. The use of free-flow electrophoresis provides an access to the solubilized complexes within minutes. (Labile) supercomplexes could therefore be analyzed much faster, which appears beneficial to characterization. On the other side, the lower resolution of the one-step purification strategy results in some heterogeneity in the obtained fractions. This was evident from the detection of low amounts of proteins originating from complexes with similar molecular weight by MS analysis after native BN-PAGE and was also evident during inspection by EM. However, in the structural characterization the problem of heterogeneity can be circumvented to a large extent. Extensive sorting of projections by statistical analysis has been applied during the structural analysis by single particle EM. In this way we could separate the projection maps belonging to different types of particles.

In the free-flow fractions several types of larger PSI-LHCII supercomplexes were found and homogeneous 2D projection maps were obtained by image processing of large numbers of EM projections (Fig. 5). For the first time dimers of PSI-LHCII particles were detected, which we propose to name PSI$_2$-LHCII$_2$ (Fig. 5B). Further, PSI-LHCII particles which contain an additional LHCII trimmer in two slightly different positions were revealed (Fig. 5C and D). The position indicates that the additional LHCII trimmer is connected to the PSI core part either via the peripheral antenna subunits Lhca2/Lhca3 or Lhca4/Lhca2 (Figs. 5C and D, 7D). In the first case, the luminal PsaN subunit, located next to Lhca3 [28], might also be involved in the binding. The finding of PSI complexes with two LHCII trimers, which we propose to name PSI-LHCII$_2$, is not totally unexpected. It was recently suggested from biochemical experiments that PSI binds more than one LHCII trimer [36-37]. In conclusion, the novel PSI-LHCII$_2$ supercomplexes suggest that the interaction with the peripheral LHCII antenna is more complex than previously considered. Therefore, the current view that part of the mobile fraction of LHCII moves towards PSI and binds at only one specific site under state transition [5], should be revised.

4.2. PSI-Cytb6f in Arabidopsis

The comparison of the 3D model of Arabidopsis PSI-Cytb6f supercomplex with existing 2D maps of known orientation clearly indicates one unique binding site of Cytb6f to PSI, next to the peripheral antenna subunit Lhca1 (Fig. 7). The Cytb6f complex binds as a monomer or dimer, although only the dimer is considered to be the active form [34]. There is no clear evidence that any other additional protein is binding to the PSI-Cytb6f supercomplex, but given the low resolution of the maps and model, which is around 30 Å, association of other small proteins cannot be ruled out. There is no indication in Arabidopsis for the elusive supercomplex between PSI, Cytb6f, LHCII, FNR and PGRL1, proposed some years ago for Chlamydomonas [14]. But in principle, the binding of Cytb6f to Lhca1 would not prevent binding of a LHCII trimer to Lhca2–4. Interestingly, the Cytb6f dimer associates with its short side rather than its long side to PSI. This way of binding could explain why PSI-Cytb6f particles are prone to dissociation and are present in low abundance, even if analysis is performed about an hour after their solubilization, as in this study. The small protein plastocyanin functions as an electron transporter between the cytochrome f subunit of Cytb6f and PSI. In the way that Cytb6f and PSI associate in the supercomplex, this distance is relatively small (black circles, Fig. 7A). This could be a reason why the PSI-Cytb6f supercomplex has this particular shape, as further discussed in Section 4.4.

4.3. PSI-NDH in Arabidopsis

The extended analysis of PSI-NDH complexes revealed that NDH can bind up to 6 PSI complexes. This number is higher than the 1–2 PSI complexes found earlier in barley [3,33]. This likely means that NDH is able to make specific clusters with PSI. We identified low amounts of these clusters, but without NDH (Fig. 6H). Clusters of PSI are novel, because earlier attempts to extract PSI multimers ended up in artificial detergent-induced aggregates in which the particles were oriented both upside-up and upside-down [4]. It is possible that the low-abundant Lhca5 and 6 antenna subunits function in pasting the PSI complexes to NDH, as discussed earlier [3]. The EM maps do not have enough detail to see such proteins, but according to the modeling there is enough open space available to put in Lhca5 and 6 (green asterisks, Fig. 7C). Of the two subunits, Lhca5 was found to be present in the NDH band by MS analysis (not shown).
There is one intriguing aspect about the analysis of supercomplexes, although not directly related to the main issue of this work about PSI interactions. We can now compare NDH, as bound to PSI, with mitochondrial Complex I, as a single entity or as bound to Complex III in supercomplexes [31]. The chloroplast NDH complex strongly resembles the mitochondrial complex (Fig. 6L, M), although they are not closely related from a sequence perspective. It is striking that NDH from Arabidopsis contains a central protrusion, just as in Complex I (orange arrowhead in Fig. 6M). This protrusion was determined in plant mitochondria as the carbonic anhydrase domain, functional in carbon metabolism [38]. Chloroplast NDH is composed of about 30 subunits [39,40] and mitochondrial Complex I has over 40 subunits [41], of which many have an unknown function. This may explain the similarity. The striking resemblance of chloroplast NDH and mitochondrial Complex I needs further attention, the more because the origin of the particles studied is inconceivable. PSI and Complex III are specific for chloroplasts and mitochondria, respectively, and each complex has a unique, and easily recognizable shape.

4.4. Function of spatial separation of PSI, Cytb6f and NDH

The structural organization of the thylakoid membrane has been often studied, but investigations mostly deal with stacked grana membranes, where PSI often forms semi-crystalline arrays [1,42]. It also the non-stacked grana membrane is largely composed of PSI, Cytb6f, and ATP synthase, and besides these proteins it is considered to accommodate monomeric PSI complexes and single trimers of LHCII [43]. It is not known how all these components interact. The last years have shown that PSI is undoubtedly a key protein in the regulation of electron transport via plastocyanin and ferredoxin is diffusion limited. This may explain the similarity. The striking resemblance of chloroplast NDH and mitochondrial Complex I needs further attention, the more because the origin of the particles studied is inconceivable. PSI and Complex III are specific for chloroplasts and mitochondria, respectively, and each complex has a unique, and easily recognizable shape.

Transparency document

The Transparency document associated with this article can be found, in online version.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.bbabio.2016.10.006.

References


