Structural Organization of Photosynthetic Apparatus in Agranal Chloroplasts of Maize*

Received for publication, May 14, 2008, and in revised form, July 14, 2008. Published, JBC Papers in Press, July 16, 2008, DOI 10.1074/jbc.M803711200

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We investigated the organization of photosystem II (PSII) in agranal bundle sheath thylakoids from a C4 plant maize. Using blue native/SDS-PAGE and single particle analysis, we show for the first time that PSII in the bundle sheath (BS) chloroplasts exists in a dimeric form and forms light-harvesting complex II (LHCII)-PSII supercomplexes. We also demonstrate that a similar set of photosynthetic membrane complexes exists in mesophyll and agranal BS chloroplasts, including intact LHCII-PSI supercomplexes, PSI monomers, PSII core dimers, PSII monomers devoid of CP43, LHCII trimers, LHCII monomers, ATP synthase, and cytochrome b6f complex. Fluorescence functional measurements clearly indicate that BS chloroplasts contain PSI complexes that are capable of performing charge separation and are efficiently sensitized by the associated LHCII. We identified a fraction of LHCII present within BS thylakoids that is weakly energetically coupled to the PSI reaction center; however, the majority of BS LHCII is shown to be tightly connected to PSII. Overall, we demonstrate that organization of the photosynthetic apparatus in BS agranal chloroplasts of a model C4 plant is clearly distinct from that of the stroma lamellae of the C3 plants. In particular, supramolecular organization of the dimeric LHCII-PSII in the BS thylakoids strongly suggests that PSII in the BS agranal membranes may donate electrons to PSI. We propose that the residual PSI activity may supply electrons to poise cyclic electron flow around PSI and prevent PSI overoxidation, which is essential for the CO2 fixation in BS cells, and hence, may optimize ATP production within this compartment.

Oxygenic photosynthesis sustains life on Earth. It couples the formation of molecular oxygen with the biosynthesis of carbohydrates, thus providing the ultimate source of biomass, food, and fossil fuels. In the first step of photosynthesis, the solar energy is captured and converted into the energy-rich molecule ATP and the reducing equivalents (in the form of water-derived protons and electrons) used for the conversion of CO2 into carbohydrates. The light-driven charge separation is conducted by cooperative interaction of photosystem I (PSI) and photosystem II (PSII), two multimeric chlorophyll-binding protein complexes embedded in the thylakoid membranes of cyanobacteria, algae, and plants. The primary charge separation in the reaction centers of PSI and PSII triggers vectorial electron flow from PSI to PSII via the cytochrome (cyt) b6f complex, also present in the thylakoid membranes, resulting in formation of the electrochemical potential gradient across the thylakoid membrane. In this way, linear electron transport powers the activity of ATP synthase to convert ADP to ATP. Both ATP and NADPH produced in the light-driven redox reactions of photosynthesis are subsequently used for fixation and reduction of CO2 during the photosynthetic dark reactions of the Calvin-Benson cycle.

Spatial organization of the thylakoid membranes exhibits lateral distribution of the photosynthetic transport complexes. Most of the dimeric photosystem II (PSIIα) is found in the central appressed domains of the grana membranes, where it cooperates with photosystem I (PSIα) in the grana margins to conduct the linear electron flow (1). A pool of PSI, the so-called PSIIβ, also present in the stroma lamellae, donates electrons to the cyclic electron flow under oxidized conditions (1). The PSII core monomers occur predominantly in the stroma lamellae (2), although a recent study showed that some dimeric PSII is also present within this region (3). Under physiological conditions, cyt b6f is equally distributed within the thylakoid membranes, whereas ATP synthase is localized exclusively in the unstacked stroma lamellae and within the end membranes of the grana stacks (2).

The biochemical photosynthetic pathways are highly conserved among the plant species. Most green plants are C3 plants, in which the first organic product of photosynthesis is the three-carbon compound phosphoglyceric acid. A second biochemical pathway that allows efficient concentration of CO2 in leaves exists in C4 plants, which represent some of the agriculturally most productive crops. This type of plants can sustain higher rates of photosynthesis, thanks to the spatial distribution of the photosynthetic apparatus and the alteration of the leaf

* The work was supported by Polish Ministry of Science and High Education Grant N303 036 31/1086. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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3 The abbreviations used are: PSI and PSII, photosystems I and II, respectively; BS, bundle sheath; BN, blue native; Chl, chlorophyll; cyt, cytochrome; DDM, β-octododecyl maltoside; LHCI and LHCII, light harvesting complex I and II, respectively; MS, mesophyll; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethyleurea.
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structure, both allowing CO₂ to be concentrated around Rubisco (4, 5). In C₃ plants, inorganic carbon is initially fixed in mesophyll (MS) cells into the four-carbon compound oxaloacetic acid. Oxaloacetate is then converted into malate or aspartate, which is transported into bundle sheath (BS) cells, where its decarboxylation provides high concentrations of CO₂ to Rubisco and where the Calvin-Benson cycle occurs (6). Chloroplasts in MS cells contain grana, whereas bundle sheath chloroplasts exhibit various degrees of granal development depending on the plant species, age, and growth conditions (4). In maize, a typical C₄ species of the NADP-malic enzyme subtype, MS chloroplasts are granal at all stages of development, whereas BS counterparts are fully agranal (7, 8).

Although the pathways for carbon assimilation in bundle sheath cells are well established, the exact supramolecular organization of the respective photosynthetic electron transport components has not been fully elucidated. Moreover, the precise biochemical role of PSII in BS chloroplasts remains controversial. Several studies suggested that PSII in maize BS chloroplasts is capable of oxygen evolution, although its activity is very low in comparison with its counterpart in MS chloroplasts (9–14). Other reports suggested that PSII is totally absent in the BS chloroplasts (15, 16) or exhibits no water-splitting activity (16, 17). Moreover, it has been debated whether all of the subunits of PSII, in particular those of the oxygen-evolving complex, are present in the agranal maize chloroplasts (13, 16, 18, 19). It appears that such contradicting reports are due to the differences in the BS isolation procedures and, as a consequence, a degree of intactness of PSII. In addition, it was demonstrated that the level of PSII activity in BS chloroplasts, as well as the presence of the oxygen-evolving complex subunits, depends on the differences in the age of the source leaf tissue, contamination with MS chloroplasts during isolation procedures, and illumination conditions (14, 20, 21). The apparent existence of the D1 degradation/repair cycle in BS chloroplasts and the identification of proteolytic enzymes responsible for these processes in these organelles confirm that PSII indeed plays an important function in the BS agranal membranes (22). Indeed, the active dimeric PSII complexes present in the stroma lamellae of C₄ chloroplasts have been shown to undergo the repair cycle (23, 24), whereby PSII with a damaged D1 reaction center subunit migrates from the grana to the stroma and subsequently undergoes disassembly, repair, and refolding (25).

The higher plant PSII core dimer and its associated light-harvesting antenna, the so-called LHClII-PSII supercomplex, form a basic highly conserved structural unit composed of two LHClII trimers and two copies of the minor Cab (chlorophyll a/b-binding) proteins CP29 and CP26, with each pair symmetrically related by the 2-fold axis of the core dimer (reviewed in Refs. 2 and 26). In C₃ plants, the LHClII-PSII supercomplexes are localized exclusively in the stacked grana regions (2, 27). More complex LHClII-PSII structures exist, in which two or three additional LHClII trimers and two copies of the minor subunit, CP24, associate with the dimeric PSII supercomplex and form complex crystalline arrays within the membranes, depending on the illumination conditions and the species analyzed (2, 3).

Although it has been widely accepted that the structure of membranes in BS chloroplasts seems analogous to the stroma lamellae of C₃ plants, limited direct evidence supporting this notion is available. In this study, we performed comprehensive biochemical and structural characterization of the membrane protein complexes of the granal and agranal maize thylakoids using mild BN-PAGE and two-dimensional SDS-PAGE combined with electron microscopy visualization of the photosynthetic complexes and single particle analyses of negatively stained particles. We demonstrate for the first time that the bundle sheath agranal thylakoid membranes differ significantly from the stroma lamellae of C₃ plants in that PSII in the BS membranes forms the fully functional dimeric supercomplex with its associated LHClII antenna. We propose that the main role of PSII exhibiting residual water splitting activity in BS chloroplasts is to donate electrons from the substrate water molecules into the cyclic electron flow around PSI.

EXPERIMENTAL PROCEDURES

Plant Material and Growth Conditions—Maize (Zea mays L. Oleňka, C₄ type NADP-malic enzyme) plants were grown in vermiculite in a growth chamber with a 14-h photoperiod and day/night regime at 24/21 °C. Photosynthetic photon flux density was 50, 350, or 800 μmol m⁻² s⁻¹ (for functional measurements). Plants were fertilized with Knop’s solution. Leaves were harvested from 3–4-week-old plants.

Chloroplast and Thylakoid Isolation—MS and BS chloroplasts were isolated according to Romanowska et al. (21). Isolation procedures were carried out at 4 °C, and 10 mM NaF was added to all buffers. The purity of the BS chloroplasts was determined as 99% (1% residual contamination with MS cells) by assaying the phosphoenolpyruvate carboxylase activity, immunoblotting with anti-phosphoenolpyruvate carboxylase antibodies, determination of Chl a/b ratio, and microscopic examination, as described in Ref. 21. For thylakoid preparation, pellets of MS and BS chloroplasts were resuspended in a washing medium containing 50 mM HEPES-NaOH (pH 8.0), 5 mM MgCl₂, 10 mM NaCl. After centrifugation at 8,000 × g for 15 min, pellets were resuspended in a washing medium supplemented with 330 mM mannitol. Thylakoid membranes prepared by this procedure were used immediately or snap-frozen in liquid nitrogen and stored at −80 °C until further use. Total chlorophyll content and Chl a/b ratio were determined in 80% acetone, according to the method of Arnon (28). PSII activity was assayed spectroscopically by measuring the photoreduction rate of the artificial electron acceptor 2,6-dichlorophenolindophenol, as described in Ref. 14. The photoreduction rates have been determined as 97–108 and 19–22 μmol of 2,6-dichlorophenolindophenol mg⁻¹ Chl h⁻¹ for MS and BS PSII in freshly isolated thylakoids, respectively. Similar values were obtained for thawed aliquots.

Blue Native-PAGE and Immunodetection—Protein solubilization and BN-PAGE were performed according to Schägger et al. (29) and Kügler et al. (30) with slight modifications. Thylakoid membranes (50 μg of Chl) were sedimented at 7,000 × g for 5 min at 4 °C and resuspended in a buffer composed of 5 mM EACA, 50 mM imidazole-HCl, pH 7, 50 mM NaCl, 0.5 mM EDTA. Membrane proteins were solubilized by the addition of
n-dodecyl β-D-maltoside (DDM) to a final concentration of 1% (w/v) (DDM/Chl ratio 20:1) for MS thylakoids and 2% (w/v) (DDM/Chl ratio 40:1) for BS thylakoids (final Chl concentration 0.5 mg/ml). Samples were incubated on ice for 5 min and centrifuged at 15,800 × g for 40 min. The supernatant was supplemented with Coomassie Brilliant Blue solution (100 mM e-aminoacaproic acid (EACA), 30% sucrose) to a final concentration of 10% and loaded directly onto the 4–10% acrylamide, 4–15% sucrose gradient gel. Electrophoresis was carried out using constant 100 V at 4 °C overnight. For separation of protein in the second dimension, the lanes of the BN gels were excised and incubated in the denaturation solution (1% β-mercaptoethanol, 1% SDS) for 15 min at room temperature, followed by 15 min at 50 °C. The strips were briefly rinsed in distilled water and layered onto SDS-polyacrylamide gels (31) with 15% (w/v) acrylamide and 6 M urea in a separating gel. Electrophoresis was performed using a constant current of 7 mA at 4 °C overnight. Proteins were visualized using Coomassie Brilliant Blue R-250 solution or electrotransferred onto a polyvinylidene difluoride membrane (Millipore) for Western immunodetection. Blots were probed with specific antibodies (anti-PSI L, −Lhca1, −Lhca4, −D2, and −Lhcb1) and visualized by chemiluminescence.

**Fluorescence Measurements of Thylakoid Membranes**—Room temperature fluorescence measurements were performed with a home-built fluorimeter, using a light source at 590 nm. The fluorescence response was detected in the near IR region. Fluorescence induction kinetics was measured in the presence of 20 μM DCMU to prevent oxidation of the primary quinone acceptor QA. Cryogenic fluorescence spectra were recorded at 77 K using a home-built spectrophotometer based on a detecting diode array (AVS-USB 200; Ocean Optics). Samples were loaded into a small metal cuvette (volume 0.8 ml Chl) were solubilized with 0.9% DDM, since it provided the optimal separation of the membrane protein complexes identified on the BN-gels (see Fig. 1, B).

**Biochemical Isolation and Characterization of Sucrose Gradient Fractions**—40 mM NaF was added to all the buffers throughout the isolation procedure to inhibit dephosphorylation of phosphoproteins. Thylakoid membranes from MS and BS chloroplasts (0.8 mg/ml Chl) were solubilized with 0.9% DDM and fractionated by centrifugation on continuous sucrose density gradients, as detailed previously (61). Protein analyses were conducted using an SDS-PAGE Tris-Tricine system described by Schägger and von Jagow (32). Protein bands were separated on 12.5% polyacrylamide gels in the presence of 6 M urea and visualized with Coomassie Brilliant Blue R-250 using standard procedures. Optical absorption spectra were obtained at room temperature using a Shimadzu UV-1601 spectrophotometer with a 2-nm slit size. Steady-state fluorescence emission spectra were measured using a PerkinElmer Life Sciences luminescence spectrometer LS 50 at 77 K and a Chl excitation wavelength of 435 nm.

**Electron Microscopy**—For the negative staining of protein samples, a concentration of 1–2 μg of Chl/ml was found to be optimal, prior to the addition of 2% (w/v) uranyl acetate via the droplet technique. Imaging at a magnification of ×30,000, in a low dose mode, with a FEI-Tecnai 12 transmission electron microscope, operating at 120 kV, yielded micrographs of no discernible drift or astigmatism. These were scanned using a Nikon LS9000 CoolScan densitometer at a step size of 6.35 μm, and images were transferred to a networked cluster of Linux-based PC workstations for subsequent single particle image processing.

**Image Processing**—Micrographs displayed the first minima of their Fourier power spectra in the 19–21 Å range. No correction was made for the contrast transfer function given this minimum value and the presence of negative stain. Data sets were compiled using the automatic particle selection procedures of “boxer,” a module of the EMAN software package (see Ref. 33 and references therein). All subsequent image processing was performed using the latest version of the Imagick-5 environment (Image Science GmbH, Berlin). Reference-free alignment followed by multivariate statistical analyses (34) allowed for initial two-dimensional class averages to be identified, which were then iteratively refined, resulting in the final averages shown for each preparation.

**RESULTS**

**Biochemical Characterization of Thylakoids from Mesophyll and Bundle Sheath Chloroplasts**—In order to achieve the best resolution of membrane protein complexes during one-dimensional BN-PAGE, we subjected the MS and BS membranes to several rounds of optimization of the detergent/protein ratio (data not shown). Of all of the detergent tested, we selected DDM, since it provided the optimal separation of the membrane protein complexes from both MS and BS thylakoids, especially those in the highest molecular weight range. Fig. 1 shows the BN protein profiles of the solubilized thylakoids from MS (A) and BS (B) cells. The best resolution of protein complexes on the BN-PAGE gels was achieved using detergent concentrations of 1% (w/v) (DDM/Chl ratio 20:1) and 2% (w/v) (DDM/Chl ratio 40:1) for MS and BS thylakoids, respectively. Under these conditions, 11 main protein bands corresponding to thylakoid membrane complexes from both MS and BS thylakoids were identified (Fig. 1, top panels in A and B). All of these bands were further separated in the second dimension on SDS-PAGE to determine the precise composition of the membrane protein complexes identified on the BN-gels (see Fig. 1, A and B).

Protein profiles obtained from various plant species using two-dimensional BN/SDS-PAGE are similar due to the high conservation of the subunits of the photosynthetic complexes (24, 30, 35, 36). We therefore identified the components of the BN bands from MS and BS thylakoids by comparison of the apparent molecular weight on BN/SDS-PAGE and by immunodetection using specific antibodies against the subunits of the PSII and PSI cores and the associated LHCl complex (see Fig. 1, C and D). For both MS and BS thylakoids, complexes of the highest molecular weight on the BN gels were assigned to oligomeric forms of the LHCl-PSII supercomplexes (bands 1–5 in Fig. 1, A and B), as confirmed by the identification of the PSI core subunits (PSI A/B, PSI F, PSI L, and PSI E), components of the LHCl antenna (Lhca1 and...
Lhca4), the PSII core proteins (D1, D2, CP43, and CP47), and the LHCII antenna subunits (Lhcb1). The most intense band 6 from MS thylakoids contained the PSII core dimers and the LHCI/PSI supercomplexes, as confirmed by the detection of the PSI and PSII core subunits as well as the LHCI antenna (Lhca1 and Lhca4). Band 6 from BS thylakoids contained a similar set of PSI and PSII subunits with the additional presence of the LHCII antenna (see immunodetection of Lhcb1 in Fig. 1D) and therefore was assigned to the LHCI/PSI and LHCII/PSII supercomplexes. Band 7 from both MS and BS membranes corresponded to the PSI core depleted from the LHCI antenna. Band 8 from both MS and BS thylakoids was assigned to the PSII monomer, ATP synthase, and cyt b6f. Band 9 from both types of membranes contained the PSII monomer devoid of the CP43 inner antenna subunit. Finally, bands 10 and 11 from both MS and BS thylakoids were assigned to the trimeric and monomeric forms of LHCII, respectively, as confirmed by the immunodetection of Lhcb1 in Fig. 1D).

Functional Analysis of Thylakoids from Mesophyll and Bundle Sheath Chloroplasts—Room temperature fluorescence emission measurements of MS and BS thylakoids showed a significantly decreased fluorescence yield in BS thylakoids (Fv/Fm = 0.33) compared with MS membranes (Fv/Fm = 0.65) (see Fig. 2). Several
possibilities can account for this observation, including a decreased PSII/PSI ratio. PSII is responsible for variable fluorescence ($F_v$) emission at room temperature, whereas PSI contributes only to the constant $F_o$ emission (37). Therefore, a large decrease in the PSII/PSI ratio, as expected in the case of BS chloroplasts, may lead to a decreased $F_v/F_o$ ratio. Alternatively, a weak energetic coupling between LHClI and PSII may decrease the $F_v/F_o$ ratio by increasing the $F_o$ emission component. Indeed, the relatively large pool of LHClI present in BS chloroplasts (14) may not be completely connected to the reduced number of PSI reaction centers. Finally, the occurrence of sustained energy spillover from PSII to PSI may also decrease the fluorescence yield of PSII and thus the $F_v/F_o$ ratio.

In order to distinguish among these hypotheses, we first assessed the actual PSI/PSII ratio in BS and MS chloroplasts by monitoring the electrochromic shift signal, a technique previously used to evaluate the PSI/PSII ratio in freshwater green algae (38). The electrochromic shift signal is triggered by the light-induced electric field that develops across the thylakoid membrane upon charge separation within the reaction centers of the two photosystems (reviewed in Ref. 39). Although the relative area of the two major PSII bands (685 and 695 nm) did not change between the BS and MS chloroplasts, there was a significant increase in the 680 nm peak, which represented ~30% of the total PSII emission in BS chloroplast and ~10% in the MS membranes. This suggests that a fraction of the LHClI complexes present within BS thylakoids is weakly coupled to the PSI reaction center.

The weak energetic coupling of the specific fraction of the LHClI complexes was confirmed by measuring fluorescence emission spectra at room temperature. In these conditions, sustained emission in the 675 nm region was seen in BS thylakoids, as expected if they contain some poorly coupled LHClI (data not shown). Nevertheless, it clearly appears that the majority of the LHClI complexes are connected to PSII in BS thylakoids. This is further confirmed by the observation that the rise of fluorescence observed in BS chloroplasts was ~40% faster compared with MS membranes (upon normalization of $F_o$; see Fig. 3B). This indicates that the optical section of the PSI reaction centers was larger in BS compared with MS thylakoids, as expected if most of the LHClI present in the BS membranes was connected to the remaining reaction centers.

An additional conclusion that can be derived from Fig. 3B is the lack of sustained spillover from PSII to PSI in BS thylakoids (42). This is indicated by the shape of the fluorescence rise measured in the presence of the PSI inhibitor DCMU, which is clearly sigmoidal. This feature reflects the progressive increase in the light harvesting capacity of a PSI photochemical unit as its PSII neighbors become photochemically inactive due to the reduction of the quinone acceptor $Q_a$ (43, 44). This feature is therefore indicative of an efficient energy transfer between closed and open PSII traps, without any significant competition by energy quenching through PSI photochemistry.

**Isolation and Biochemical Characterization of Mesophyll and Bundle Sheath Photosynthetic Complexes—**Sucrose gradient fractionation of thylakoid membranes solubilized with 0.9% DDM was performed in order to isolate photosynthetic complexes from MS and BS chloroplasts. Three main Chl-containing bands were obtained for both types of thylakoids (F1, F2, and F3, for MS and BS, respectively). SDS-PAGE of sucrose density fractions isolated from BS thylakoids (see Fig. 4) showed the presence of free LHC proteins in F1, a range of proteins indicative of a mixture of PSII and PSI core subunits in F2 (see identification of PSI A/B, LHCI subunits, CP43, and cyt$b_{559}$), and the enrichment of the PSI core subunits (PSI A/B) and the
partially depleted from the LHCl antenna (see Fig. 5, C and D). The fluorescence emission spectrum of fraction 3 from BS thylakoids (BS-F3) peaked at 735 nm, indicating the enrichment of LHCl-PSI within this fraction (see Fig. 5D). A small peak at 685 nm (less than 10% of the PSI peak) indicated a small proportion of PSI present in the BS-F3 fraction (Fig. 5D). In contrast, the densest fraction from MS thylakoids (MS-F3) showed two major fluorescence peaks at 685 and 735 nm, implying the presence of a mixture of PSI and PSI complexes in this fraction (see Fig. 5C).

Electron Microscopy—Direct visualization of the protein complexes, in two-dimensional projection, was made possible through the single particle analysis of transmission electron micrographs of negatively stained particles, from the BS sucrose gradient fractions F2 (12 micrographs) and F3 (13 micrographs). After the application of multivariate statistics and subsequent classification of the two data sets, it became apparent that fraction BS-F2 contained two main subpopulations. These were assigned as PSI monomeric core (594 particles), devoid of the LHCl antenna, having dimensions of 150 and 100 Å (see Fig. 6B) and a PSI core monomer (330 particles) with dimensions of 145 and 104 Å (Fig. 6C). In fraction BS-F3, the most intact populations we assigned to a typical PSI core dimer (502 particles) having dimensions of 220 × 150 Å (see Fig. 6A) and the LHCl-PSI supercomplex (103 particles), the latter characterized by a crescent of four LHca antenna subunits localized asymmetrically on one side of the complex (see Fig. 6D). An approximate outline contour of the recent x-ray crystal structure of the pea LHCl-PSI supercomplex (Protein Data Bank code 2o01) (45) was overlaid, to scale, onto the electron microscopy-derived two-dimensional projection maps of the maize PSI core monomers (Fig. 6B; PSI monomeric region only) and LHCl-PSI supercomplexes (Fig. 6D) to aid orientation of the structural domains within these particles. It is of note that in the crystal structure, the PSI L and PSI H subunits form a characteristic bulge at the tip of the PSI monomer, which is also present along the lower edge of the averaged particles shown in Fig. 6B. Another characteristic feature of the pea LHCl-PSI supercomplex x-ray structure is a crescent of four distinct domains corresponding to the LHca antenna subunits, flanking the side opposite to PSI L/H. Similar features in the projection maps of the maize LHCl-PSI supercomplex and PSI monomer were therefore assigned to PSI L/H and LHca subunits and used for the approximate alignment and confirmation of the stromal orientation of the projection maps. Notably, neither in fractions BS-F2 nor BS-F3 did we observe larger particles indicative of the putative larger LHCl-PSI supercomplex that might have retained bound mobile LHClII antenna.

DISCUSSION

Features of BS Thylakoids—The spatial organization of thylakoid membranes in BS chloroplasts has been suggested to be analogous to that of the stroma lamellae of C₃ plants. Surprisingly, limited data concerning the exact composition of the photosynthetic apparatus in the BS thylakoids are available. Our study presents a comprehensive analysis of the membrane protein complexes of the granal and agranal thylakoids from the typical C₃ plant maize. Using blue native PAGE we identified intact LHCl-PSI and LHClII-PSII supercomplexes, PSI
monomers, PSII core dimers, PSII monomers devoid of CP43, LHCII trimers, LHCII monomers, ATP synthase, and cyt b6f complex in the MS and BS thylakoid membranes (Fig. 1). As expected, our results of BN- and BN/SDS-PAGE confirm that the protein composition of the C4 MS membranes is similar to that of the C3 thylakoids, where all of the complexes listed above are present (24, 30, 35, 36). Our BN/SDS-PAGE identification of the LHCII/PSII supercomplexes in the BS agranal membranes (Fig. 1, B and D) demonstrates that the BS thylakoids differ in their protein composition from the stroma lamellae of the C3 plants, in which no dimeric LHCII/PSII supercomplex has been reported to date (3). In addition, the structure of the BS chloroplasts was suggested not to be influenced by the light intensity and was similar in plants grown in full tropical sunlight or under low irradiance (46). In contrast, our recent results have shown that various light growth conditions may alter polypeptide composition of the BS thylakoids of maize (14).

BN-PAGE has been proven a useful approach to investigate the photosynthetic apparatus of higher plants, in particular for the identification of the intact photosynthetic complexes (30). In the present study, we achieved the finest electrophoretic resolution of the thylakoid membrane complexes from the MS and BS chloroplasts of maize by optimization of the procedures described by Kügler et al. (30) and Schägger et al. (29), in particular by optimizing various detergent concentrations used to solubilize the two types of membranes. In this way, we achieved the best resolution of the protein bands within the highest molecular weight range, including the components of the PSII and PSI reaction centers (see Fig. 1). In agreement with Refs. 24, 30, 35, and 36, we did not observe the oxygen-evolving complex polypeptides, since they are easily detached from PSII during solubilization procedures. Moreover, a pool of PSII monomer lacking CP43 was identified in our study in both types of maize thylakoids (see Fig. 1). A similar complex was reported recently during BN/SDS-PAGE separation of the stroma lamellae of spinach (3). In vivo detachment of CP43 occurs during the PSII repair cycle, whereby CP43 undergoes dephosphorylation, which in turn triggers its detachment from the PSII core (47). Interestingly, DDM caused more intensive detachment of CP43 compared with digitonin (data not shown), in agreement with Heinemeyer et al. (35). It cannot be excluded that a subpool of the PSII devoid of CP43 may be a product of the PSII repair cycle, since this pathway has been shown to exist in the BS agranal membranes (22). Additional experiments are required to test this possibility.

FIGURE 5. Optical absorbance and fluorescence properties of sucrose gradient fractions obtained from MS (A and C) and BS (B and D) thylakoids. Spectra corresponding to F1 (LHCs), F2 (PSI/PSII), and F3 (LHCII-PSII) fractions are presented as dashed, solid, and dotted-dashed lines, respectively. A and B, room temperature (RT) absorbance spectra. C and D, fluorescence spectra were recorded at 77 K using excitation at 435 nm. Peaks have been normalized to aid their comparison. a.u., absorbance units.
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Role of PSII and LHCII in BS Thylakoids—Our functional analysis clearly indicates that BS chloroplasts contain a significant fraction of PSII complexes (about one-third of the PSI centers), which are capable of performing charge separation and are efficiently sensitized by LHCII. Although some of the LHCII complexes present in the BS thylakoids are weakly energetically coupled to the PSII reaction centers, a significant fraction of this light-harvesting complex remains excitonically connected with PSI. Although it is possible that the weakly connected LHCII complexes may form aggregates in the membranes, where absorbed energy is largely dissipated nonphotochemically, our fluorescence analysis at room temperature suggests that at least a fraction of LHCII is neither in a quenched state nor connected to the PSII reaction center.

An alternative hypothesis for the functional arrangement of PSII and LHCII in BS chloroplasts is that LHCII remains bound to PSI and may form arrays in the thylakoid membranes facilitating energy transfer between PSII and PSI. This hypothesis is not consistent with the fluorescence kinetic measurements, which clearly indicate lack of sustained spillover and a major association of LHCII to PSII. What is then the function of the LHCII-PSII complexes in the bundle sheath cells? In C₃ plants, photosynthetic protein complexes show heterogeneous lateral distribution along thylakoid membranes. PSII is mostly located in the appressed grana stacks, whereas PSI is concentrated in the stroma lamellae, the grana margins, and the end membranes. In contrast, the cytochrome b₅f complex is homogeneously distributed (e.g. reviewed in Ref. 1). Because of the restricted diffusion of the soluble electron carriers (see review in Ref. 48), it has been proposed that PSII may be able to fuel only those cyt b₅f com-
plexes that are located in the grana. This would elicit an electron flow to the LHCI-PSI supercomplexes that are found at the periphery of the grana and then to ferredoxin. Ultimately, NADPH would be generated at the grana margins and/or grana ends and then consumed, together with ATP, for driving the Benson-Calvin cycle. Although PSI in the stroma lamellae would also reduce ferredoxin, the latter would only access cytochrome complexes, leading to cyclic electron flow (reviewed in Ref. 49).

In C₄ plants, a similar segregation of photosynthetic complexes is observed, which, however, involves a significant decrease of the amount of PSII in a specific compartment (i.e. the BS chloroplast). Thus, the segregation between linear and cyclic flow does not occur within the same chloroplast but rather between two separate leaf compartments. The BS chloroplast is essentially involved in the cyclic electron flow leading to the production of ATP that is required, together with the reducing power provided by the mesophyll chloroplasts via the malate shuttle, for CO₂ assimilation (5).

The reduced amount of PSII, which is still present and fully functional in the bundle sheath (i.e. in the “cyclic compartment” of maize leaves) may therefore be required to provide reducants as needed for the redox poise control of cyclic flow. Indeed, although in a “perfect” cyclic electron flow system electrons are indefinitely recycled around PSI, when the electron carriers in the stroma become overoxidized, electrons can leak from the cyclic pathway, ultimately leading to a complete inhibition of this process (50). In this sense, the role of PSII would be complementary to that of the NADH-plastoquinone reductase (NDH), which is enriched in the BS chloroplasts (51) and is supposed to inject electrons into the plastoquinone pool, depending on the redox status of the stroma. Alternatively, it has been proposed that ascorbate and malate can cause reduction of stromal donors, which establishes conditions for activation of cyclic electron flow around PSI (52).

Structure of PSII and PSI Complexes in BS Thylakoids—To gain a deeper insight into the structure of maize photosynthetic complexes from the BS agranal membranes, we applied electron microscopy of negatively stained particles, followed by single-particle averaging of the two-dimensional projections. As expected, we confirmed that maize LHCI-PSI supercomplex exists as a monomer with four Lhca antenna subunits associated asymmetrically with the PSI core (Fig. 6D). This is in agreement with the recent 3.4 Å x-ray structure of the pea LHCI-PSI supercomplex (45) in which four Lhca subunits of the LHCI complex form a crescent of two dimers that bind asymmetrically to the PSI core. The higher molecular weight oligomeric forms of LHCI-PSI supercomplex identified by our BN-PAGE analysis (Fig. 1) represent artifacts caused by detergent solubilization, as reported previously (53, 54). A relatively high ratio of LHCI/PSII in maize bundle sheath chloroplasts (14, 56) and the significant increase of the functional antenna of PSI within these membranes prompted suggestions that LHCI may act as the antenna for PSI in this compartment (19, 57, 58). We therefore examined whether a fraction of LHCI may transiently associate with the LHCI-PSI supercomplex, leading to an observed increase of the PSI absorption cross-section (data not shown). A similar phenomenon occurs during the process of

![FIGURE 6. Single particle analysis of PSI and PSII complexes obtained from bundle sheath thylakoids. Shown are top view class average projections of the PSII dimer (A), PSI core (B), PSII monomer (C), and PSI-LHCI supercomplex (D) overlaid with an outline contour of a 3.4 Å pea three-dimensional x-ray structure (Protein Data Bank code 2o01) (45). Scale bar, 50 Å.](image-url)
state transitions when redistribution of reversibly phosphorylated LHCII between PSII and PSI maintains optimal excitation balance of both photosystems (55, 59). However, we did not detect any projections over and above the classic LHCI-PSI supercomplex, suggesting that in maize, LHCCI remains associated with the PSII core. It cannot be excluded, however, that a subfraction of mobile LHCCI may transiently associate with PSI but it is displaced following the detergent treatment, as previously reported (60, 61). To explore this possibility, further investigation is required using digitonin solubilization, an approach that has successfully been used to identify a labile LHCCI-LHCl-PSI supercomplex in the membranes of a C₄ plant, Arabidopsis thaliana (60). Alternatively, maize LHCl-PSI may migrate toward PSII to ensure equal excitation energy distribution between both photosystems without the involvement of mobile phospho-LHCCI, as recently suggested by Tikkanen et al. (62), who reported such a phenomenon during state transitions in the spinach grana margins.

Importantly, our single particle analysis revealed for the first time that PSII forms dimers in addition to monomers in maize BS agranal membranes (Fig. 6A), in contrast to the earlier report postulating an exclusively monomeric aggregation state of PSII in maize BS thylakoids (19). The maximum diagonal length of the maize PSII dimer is 200 Å, including a 15-Å detergent shell around this particle. This dimension matches the size of the two-dimensional projection of the spinach PSII core dimer (63). Our single particle analysis did not reveal any LHCCI-PSII supercomplexes within sucrose gradient fractions, in contrast to the BN-PAGE results. This apparent discrepancy can be explained by the experimental approach of the sucrose gradient fractionation, which was aimed primarily at the isolation of the intact LHCl-PSI supercomplexes. In this approach, alkaline pH, which is optimal for LHCl-PSI isolation, has been shown to dissociate LHCCI from the PSII core (63).

In conclusion, this report demonstrates that the organization of photosynthetic apparatus in BS agranal chloroplasts of a C₄ plant species is clearly distinct from that of the stroma lamellae of the C₃ plants. The dimeric character of PSII and formation of the LHCCI-PSII supercomplex in maize BS agranal thylakoids identified in this study support the idea that PSII plays an important function in this type of membranes, in contrast to the current dogma suggesting functional redundancy of bundle sheath PSI. In particular, the supramolecular organization of the dimeric LHCCI-PSII (Figs. 1 and 6) and the existence of the PSII repair cycle (22) in the BS thylakoids strongly suggest that PSII in the BS agranal membranes may donate electrons from the substrate water molecules to a cyclic electron transport around PSI. In C₃ plants, ATP is used to drive the C₄ cycle, and the requirement for ATP is cell-specific and varies between MS and BS cells. In bundle sheath chloroplast, the cyclic electron flow plays a role in supplying the surplus ATP needed for C₄ photosynthesis, and as a consequence, the ATP/NADPH ratio is higher in this type of chloroplasts compared with mesophyll counterparts. This type of electron flow is very sensitive to redox poise and requires activation by a reductant. Residual PSII activity can therefore supply electrons to poise cyclic electron flow, essential for the CO₂ fixation in BS cells, and, hence, may optimize ATP production within this compartment.

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Acknowledgments—We thank Professors E.-M. Aro, P. E. Jensen, and S. Jansson for providing the antibodies used in this study. We thank Dr. S. Santabarbara for help with spectra deconvolution.

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