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Dimeric and Monomeric Organization of Photosystem II

*DISTRIBUTION OF FIVE DISTINCT COMPLEXES IN THE DIFFERENT DOMAINS OF THE THYLAKOID MEMBRANE**

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Ravi Danielsson[‡1], Marjaana Suorsa[§1], Virpi Paakkarinen[§], Per-Åke Albertsson[‡], Stenbjörn Styring[¶], Eva-Mari Aro[§], and Fikret Mamedov[¶2]

From the [‡]Department of Biochemistry, Center for Chemistry and Chemical Engineering, P.O. Box 124, Lund University, S-221 00 Lund, Sweden, the [§]Department of Biology, University of Turku, FI-20014 Turku, Finland, and [¶]Molecular Biomimetics, Department of Photochemistry and Molecular Science, Uppsala University, Villavägen 6, S-752 36 Uppsala, Sweden

The supramolecular organization of photosystem II (PSII) was characterized in distinct domains of the thylakoid membrane, the grana core, the grana margins, the stroma lamellae, and the so-called Y100 fraction. PSII supercomplexes, PSII core dimers, PSII core monomers, PSII core monomers lacking the CP43 subunit, and PSII reaction centers were resolved and quantified by blue native PAGE, SDS-PAGE for the second dimension, and immunoanalysis of the D1 protein. Dimeric PSII (PSII supercomplexes and PSII core dimers) dominate in the core part of the thylakoid granum, whereas the monomeric PSII prevails in the stroma lamellae. Considerable amounts of PSII monomers lacking the CP43 protein and PSII reaction centers (D1-D2-cytochrome b_{559} complex) were found in the stroma lamellae. Our quantitative picture of the supramolecular composition of PSII, which is totally different between different domains of the thylakoid membrane, is discussed with respect to the function of PSII in each fraction. Steady state electron transfer, flash-induced fluorescence decay, and EPR analysis revealed that nearly all of the dimeric forms represent oxygen-evolving PSII centers. PSII core monomers were heterogeneous, and a large fraction did not evolve oxygen. PSII monomers without the CP43 protein and PSII reaction centers showed no oxygen-evolving activity.

The thylakoid membrane of green plant chloroplasts hosts the large membrane-bound protein and pigment-protein complexes necessary for the photosynthetic light reactions (1). The thylakoid membrane has a complex organization where several domains can be distinguished: the appressed double planed region of grana, the nonappressed single planed grana margins, single planed stroma lamellae, and the two end membranes in the grana stack (2–4). The two photosystems are segregated in the thylakoid membrane: photosystem I (PSI)[3] with LHCI located to the stroma-exposed regions and PSII with LHCII located to the stacked grana core (5). This situation is dynamic and depends on many environmental factors (sun, shade, long or short light acclimation, etc.). In our greenhouse-grown leaf, there is 4 times more PSII than PSI in the central parts of grana, whereas in some parts of the stroma lamellae, there are more than 10 PSI per PSII (6).

PSII, which contains more than 25 different proteins (7), initiates the photosynthetic electron transfer chain by using light as a driving force and water as an electron source (8, 9). To accomplish this, PSII operates at high oxidizing potentials (10, 11) and can therefore easily be damaged, especially under unfavorable environmental conditions (12). This gives rise to the photoinhibition-repair cycle, where damaged PSII centers are continuously being disassembled, repaired, and finally activated (12, 13). This continuous cycle, which at a given instance involves a large fraction of the PSII centers in the thylakoid membrane, is the main reason why PSII *in vivo* is very heterogeneous with respect to both functional and structural properties. In addition, the continuous disassembly and rebuilding involves dynamic relocation of PSII centers to different membrane domains. Therefore, PSII is very different in different parts of the membrane, but this heterogeneity is not well appreciated or understood at present (14, 15).

Recently, the crystal structure of PSII from thermophilic cyanobacteria has been solved to 3.0–3.8 Å resolution (16–19). PSII in these crystals is highly active in oxygen evolution and has been found to be in the dimeric form. The situation for PSII from higher plants is more complicated (20–29), and most evidence (although not quantitative) suggests that dimeric PSII is concentrated to the stacked, appressed regions of the grana membranes, whereas PSII monomers are found in the unstacked stroma lamellae.

Most earlier studies of PSII function have employed (i) granal preparations and utilized (ii) detergents to solubilize the thylakoid membrane. It is important to clarify whether the dimeric and monomeric forms described in these preparations represent the native organization of all PSII *in vivo* or if other PSII complexes also are abundant in the membrane. Moreover, very little is known about the supramolecular organization of PSII in other subdomains of the thylakoid membrane, and, to our knowledge, no quantitative studies exist of the distribution of PSII supramolecular complexes in the different thylakoid domains of a single chloroplast.

We have used noninvasive mechanical fragmentation and two-phase partition methods, avoiding detergents, to isolate five different fractions of the thylakoid membrane: the grana core, the grana margins, the entire grana (which contains both the grana core and the margins), the stroma lamellae, and the so-called Y100 fraction (5, 6, 30). The Y100 fraction was isolated after mild disruption of the intact membranes and, most likely, originates from stroma lamellae (31).

The approach of studying the supramolecular organization of PSII,

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[1] These two authors contributed equally to this work.

[2] To whom correspondence should be addressed. Tel.: 46-18-471-6581; Fax: 46-18-559-885; E-mail: fikret.mamedov@fotomol.uu.se.

[3] The abbreviations used are: PSI and PSII, photosystem I and II, respectively; BN-PAGE, blue native polyacrylamide gel electrophoresis; Chl, chlorophyll; Cyt, cytochrome; DCIP, 2,6-dichlorophenolindophenol; DM, *n*-dodecyl β-D-maltoside; DPC, 2,2′-diphenylcarbonic dihydrazide; LHC, LHCI, and LHCII, light-harvesting complex, light-harvesting complex I, and light-harvesting complex II, respectively; MES, 2-(*N*-morpholino)ethanesulfonic acid; Tyr_Z, secondary electron donor in PSII, Tyr^{161} on the D1 protein; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight.

combined with detailed functional analysis, from different domains of the thylakoid membrane is important in order to understand the overall function and regulation of PSII. In this study, we separate PSII complexes with the BN-PAGE to preserve the native organization of the complexes. After this, SDS-PAGE was performed in the second dimension to enhance resolution in the subsequent immunoblot detection. This allowed us to distinguish (32, 33) and localize in the membrane a series of different forms of PSII: PSII supercomplexes (PSII core dimers together with LHCII trimers), PSII core dimers, PSII core monomers, PSII monomers without the CP43 protein, and PSII reaction centers (the D1/D2 heterodimer, Cyt b_{559}, and some low molecular mass subunits).

TABLE 1
Characterization of the different fractions of the thylakoid membrane

Fraction of thylakoid	Chl *a*/*b*	Chl/ fraction[a]	PSII/ fraction[a]	O_2 evolution
	mol/mol	*%*	*%*	*μmol of O_2 (mg of Chl)$^{-1}$ h^{-1}*
Grana core	2.2	51	71	275
Grana	2.4	64	81	240
Margins	3.1	13	10	104
Stroma lamellae	4.8	36	19	80
Y100	8.3	5	1	0
Thylakoid	2.9	100	100	120

[a] Calculated from the countercurrent distribution that provides the Chl yield in each fraction (36, 57) and from the EPR measurements for the PSII content in the different thylakoid fractions (6), S.E. is 5%.

MATERIALS AND METHODS

Preparation of Different Fractions of the Thylakoid Membrane—Spinach (*Spinacia oleracea* L.) was grown hydroponically under cool white fluorescent light at 20 °C with light-dark periods of 12 h and with the light intensity of 300 μmol of photons m^{-2} s^{-1}. Two-month-old plants were dark-adapted 24 h before harvesting to reduce the content of starch grains. All preparation procedures were made under weak green light at 4 °C, and the sample was kept on ice through the whole process. All thylakoid fractions were prepared without any detergent to preserve the membrane as intact as possible. The membranes were mechanically broken by sonication and then purified by an aqueous two-phase system to isolate the grana and the stroma lamellae fractions as described in Refs. 6, 34, and 35. The grana fraction was further purified according to Refs. 6 and 36 to isolate the grana core and the grana margin fractions. Yeda press treatment of the thylakoid membrane followed by centrifugation steps according to Refs. 6, 31, and 37 resulted in the Y100 fraction, which is considered to be a more purified stroma lamellae fraction.

All of the samples were washed once and then dissolved to a 3–4 mg/ml Chl concentration in buffer A (15 mM MES, pH 6.5, 15 mM NaCl, and 300 mM sucrose) and stored at −80 °C. The total Chl ($a + b$) concentration was determined according to Ref. 38. Steady state oxygen evolution was measured in buffer A with a Clark electrode at 20 °C using saturating white light. 2 mM ferricyanide and 0.5 mM phenyl-*p*-benzoquinone were used as electron acceptors.

BN-PAGE—BN-PAGE was performed as described in Refs. 39–41 with modifications as in Ref. 42. Thylakoid membranes and membrane fractions were resuspended in buffer B (25 mM BisTris-HCl, pH 7.0, 20% (w/v) glycerol, and 0.25 mg/ml Pefabloc (Roche Applied Science)) to a Chl concentration of 0.5 mg/ml, after which an equal volume of freshly prepared 4% DM in buffer B was added. The final concentration of DM was 2%. The samples were solubilized on ice for 2 min, followed by centrifugation at 18,000 × *g* at 4 °C for 15 min. Thylakoid membrane fractions did not produce any visible pellet after centrifugation (see also footnote 4). The supernatant was supplemented with 10% of its volume by buffer C (100 mM BisTris-HCl, pH 7.0, 0.5 M ϵ-amino-*n*-caproic acid, 30% (w/v) sucrose, 50 mg/ml Serva blue G) and loaded on BN-PAGE with a gradient of 5–12% acrylamide in the separation gel. 7 μg of Chl, corresponding on average to 80–90 μg of protein, was loaded per lane when gels were stained with silver. For D1 protein quantification by Western blotting, 50 μg of protein (corresponding on average to 4 μg of Chl) was loaded per BN lane. This amount was determined to give a linear response for different PSII complexes. Electrophoresis was carried out at 0 °C with the voltages gradually increased from 75 to 200 V for ~4 h. We have optimized our BN-PAGE protocol (short run and low temperature during the electrophoresis, short incubation time of thylakoid membranes with DM) to be as gentle as possible to prevent the dissociation of individual proteins from the complexes during electrophoresis.

SDS-PAGE—Prior to second dimension electrophoresis, the BN-PAGE gel strips were incubated in denaturing sample buffer (43) with 5% β-mercaptoethanol for 30 min at room temperature. After that, the strips were placed horizontally on a denaturing SDS-PAGE with 15% acrylamide, 6 M urea and were run in the second dimension at constant current (7 mA) overnight. After the second dimension gel electrophoresis, the proteins were visualized by silver staining or electroblotted to a polyvinylidene fluoride membrane. Protein spots from the silver-stained gels were identified with Western blotting or with MALDI-TOF mass spectrometry (44).

Western Blotting—Quantification of the different PSII complexes in each membrane fraction was based on the amount of the D1 protein. Western blotting was performed with standard techniques using a protein-specific antibody against the DE loop of the D1 protein (45). Quantitative analysis of the spots was performed with a Fluor Chem™ 8000 image analyzer (Alpha Innotech Corp.). Linearity of the immunoresponse in the range of 0.1–4.0 μg Chl was demonstrated by a series of dilutions.

EPR Spectroscopy—EPR measurements were performed at liquid helium temperatures with a Bruker ELEXYS E500 spectrometer equipped with the SuperX ER049X microwave bridge and an ER 4122SHQ cavity. The temperature was regulated with an Oxford-900 cryostat and ITC-4 temperature controller. Samples from the different fractions, in buffer A, were placed into calibrated EPR tubes at a Chl concentration of 1.5–4 mg/ml. The S_2 state multiline and $g = 4.1$ signals were induced by illumination at 200 K (46, 47). Full oxidation of Cyt b_{559} was achieved by illumination at 77 K (47). The time for the illumination procedures was controlled for each of the samples originating from the different parts of the thylakoid membrane in order to achieve a maximal induction of the EPR signals. Analysis of the spectra was performed using the Bruker Xepr 2.1 software.

RESULTS

Characterization of the Thylakoid Membrane Fractions—The PSII content and activity in the different thylakoid fractions is shown in Table 1. Most PSII (71%) was found in the grana core fraction. Together with the 10% of PSII present in the margin fraction, the entire grana fraction contained about 80% of PSII in the thylakoid membrane. The most active PSII was found in the grana; in the grana core fraction, the oxygen evolution was about 275 μmol of O_2/mg of Chl × h, and in the fraction containing the entire grana (the grana core and the margins together) the oxygen evolution was about 240 μmol of O_2/mg of Chl × h. The oxygen evolution was lower in the nonappressed regions. In the margins, the oxygen evolution was about 100 μmol of O_2/mg of Chl × h (comparable with the rate in the entire thylakoid

membrane). In the stroma lamellae fraction, the oxygen evolution rate was 80 μmol of O_2/mg of Chl × h, and in the Y100 fraction, we found no or very little oxygen-evolving activity. In essence, these results are in agreement with earlier reports (6, 30).

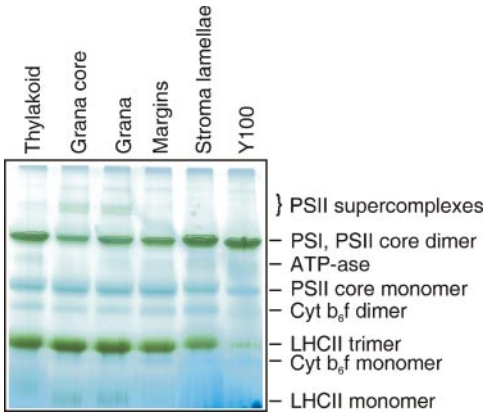

FIGURE 1. **Distribution of different protein complexes in BN-PAGE of fractions of the thylakoid membrane.** 7 μg of Chl was loaded in each lane. The electrophoresis was performed at 0 °C, with the voltage gradually increasing from 75 to 200 V during about 4 h.

Separation of the Protein Complexes by BN-PAGE—The fractions of the thylakoid membrane were analyzed by a BN-PAGE, where the different protein complexes are separated according to their size (Fig. 1). This gentle procedure preserves the native organization of the protein complexes and Chl antenna (41)[4] and has not been systematically applied to different domains of the thylakoid membrane before (44). The protein complexes separated in one-dimensional BN gel electrophoresis were identified by studying their subunit composition with the second dimension electrophoresis (see below). The individual proteins from the two-dimensional gel system have been identified by Western blotting or by MALDI-TOF mass spectrometry (42, 44). Both photosystems, different LHC complexes, the ATP synthase, and the Cyt b_6f complex were well resolved in our BN-PAGE (Fig. 1). On the upper part of the gel, there were two bands that correspond to PSII supercomplexes (*i.e.* PSII core dimers associated with LHCII trimers). They were clearly visible in the grana core and grana fractions.

[4] It should be noted that in studies with intact thylakoid membranes, the detergent (DM in our case) solubilizes mostly the stromal parts of the thylakoid membrane. Thereby, these become overrepresented on the gel and make quantitative studies impossible. In contrast, all of our fractions are vesicular and thus composed of single membranes. Therefore, all of the protein complexes are equally available for the detergent, which allows quantitative studies.

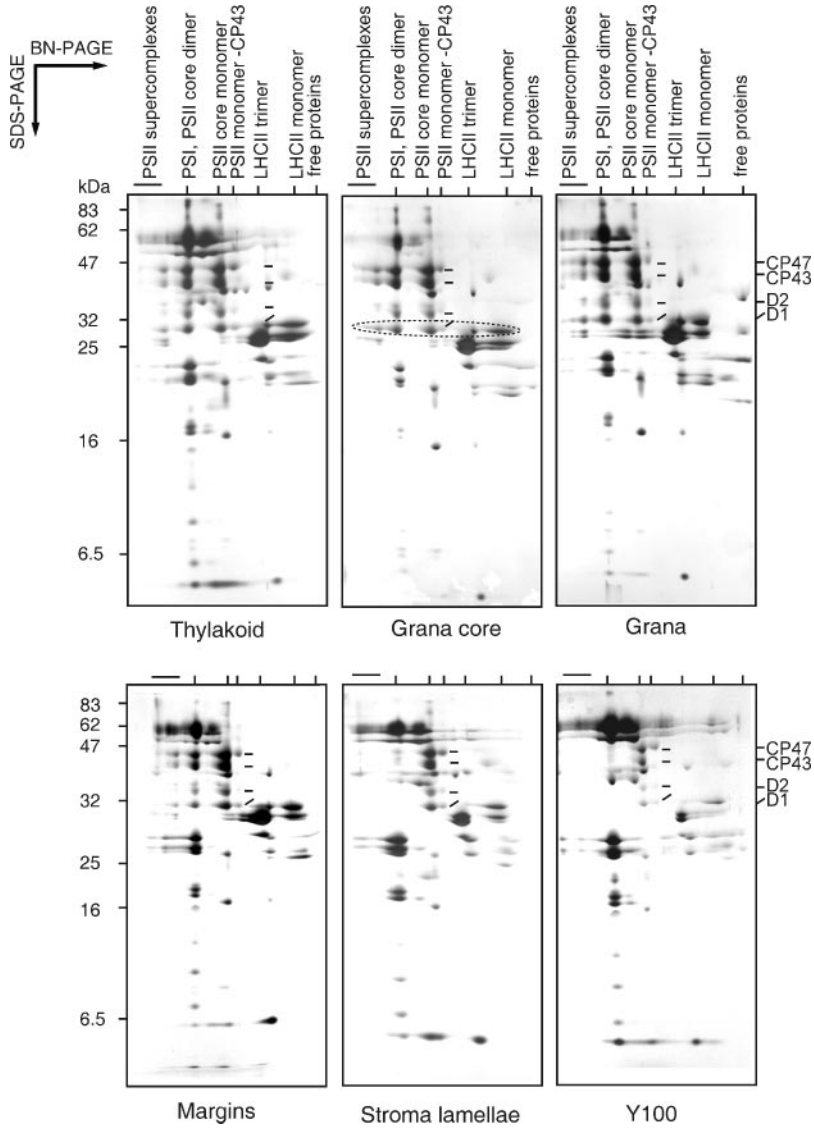

FIGURE 2. **Two-dimensional analysis of protein complexes in thylakoid membrane fractions.** The BN-PAGE strips were loaded horizontally on the SDS-PAGE and run at constant current (7 mA). 7 μg of Chl was loaded in each BN well. The silver-stained spots corresponding to the D1, D2, CP47, and CP43 subunits of PSII are indicated. The *circled dashed zone* indicates the part of the gel (for all fractions) that was used for immunoblot detection shown in Fig. 3.

Some PSII supercomplexes could also be observed in the margin fraction. In the stroma lamellae and Y100 fractions, the PSII supercomplexes were not observable (Fig. 1).

The next distinct band was mixed and is known to contain both PSI core monomers and PSII core dimers. PSII core dimers dominate the appressed membranes of the grana, whereas PSI core monomers dominate the nonappressed stroma lamellae. Therefore, this mixed band was visible and abundant in all thylakoid fractions (Fig. 1). The next band, which was faintly visible in the gel, represented PSII core monomers (Fig. 1) and was present in all parts of the thylakoid membrane. LHCII trimers, which were not associated with PSII centers, were found in the lower part of the gel. They dominated in the grana core, the grana, and the margin fractions and were clearly visible in the stroma lamellae fraction but almost absent in the Y100 fraction. Overall, the distribution of LHCII complexes seemed to follow the distribution of the PSII core dimers and monomers (Fig. 1).

Proteins Separated in the Second Dimension SDS-PAGE—Each stripe from the BN-PAGE, representing the thylakoid membrane fractions, was then analyzed by SDS-PAGE in the second dimension, enabling the separation of different protein complexes into constituting subunits (Fig. 2). The subunits retain their position in the gel according to the first dimension BN-PAGE separation (*i.e.* the position corresponding to a certain protein complex) but are separated from other subunits in the respective complex by the second dimension SDS-PAGE. The position of spots (observed after silver staining) corresponding to the D1, D2, CP47, and CP43 proteins are indicated for each fraction in Fig. 2.

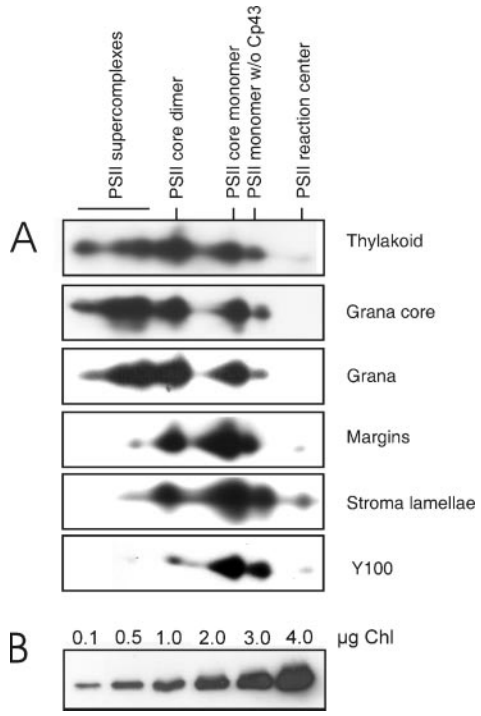

FIGURE 3. *A*, Immunoblot detection of the D1 protein in the different PSII complexes in the thylakoid membrane fractions. An antibody against the DE loop of the D1 protein was used. 50 μg of protein was loaded in each BN well (corresponding on average to 4 μg of Chl). *B*, an immunoblot demonstrating the linearity of the D1 protein DE loop immunoresponse with respect to the amount of Chl in thylakoids (proteins were separated in one-dimensional SDS-PAGE).

It is evident that the grana core, the grana, and the margin fractions show spots from the PSII core proteins originating from the lanes corresponding to PSII supercomplexes, PSII core dimers, and PSII core monomers, respectively (Fig. 2). In the stroma lamellae and the Y100 fractions, there are no traces of the PSII supercomplexes (Fig. 2). Instead, the PSII core proteins can be seen in the gel picture from the lanes corresponding to PSII core monomers and PSII monomers without the CP43 subunit. As an end point stain, silver staining provides only qualitative information about the different thylakoid membrane fractions and cannot be used for quantification of the proteins.

Quantitative Analysis of the Different PSII Forms by Immunoblotting—The last step in our analysis of the supramolecular organization of PSII was to perform immunoblot analysis of the D1 protein (Fig. 3*A*) to quantify the different types of PSII complexes. The D1 protein is an integral part of PSII and is consequently present in all different forms of PSII already separated by BN- and SDS-PAGE. For quantification purposes, it was found that the immunoresponse with the D1 antibody was linear in the range of protein concentrations present in different PSII complexes (Fig. 3*B*).

Fig. 3 and Table 2 show the results of the immunoblot analysis. In the grana core fraction, the PSII supercomplexes strongly dominated (45%). There was also 25% of PSII core dimers. We also found 25% of PSII core monomers and 5% of PSII monomers without CP43. No PSII reaction centers were found in the grana core fraction. There were more than two dimers of PSII to each monomer in this part of the thylakoid membrane (Table 3). The composition of PSII in the whole grana was quite similar to the composition in the grana core with a slight increase of the PSII core monomers and a decrease of PSII supercomplexes.

In the margins, the PSII core monomers started to outnumber the dimers (Table 3). Margins had 13% of PSII supercomplexes, 28% of PSII core dimers, and as much as 41% of PSII core monomers. There were also 14% of PSII monomers without the CP43 subunit. An interesting observation was that we found a small amount of PSII reaction centers in this fraction (4%) (Table 2).

In the stroma lamellae, we found no PSII supercomplexes and only 15% of PSII core dimers. Here PSII core monomers (>50%) and PSII monomers without the CP43 subunit (28%) dominated (Table 2). Also, about 6% of PSII was present in the reaction center form. The distribution of PSII complexes in the Y100 fraction was similar to that in the stroma lamellae fraction. There were 11% PSII core dimers, 48% PSII core monomers, and 29% PSII monomers without the CP43 subunit. The amount of PSII reaction centers increased to 12% in this part of the thylakoid membrane (Table 2).

EPR Spectroscopy on the Thylakoid Membrane Fractions—Steady state oxygen evolution (Table 1) is indicative of PSII activity. However, it

TABLE 2

Distribution of different PSII complexes in the thylakoid membrane, in percentage of the total PSII content in each fraction

Quantification was performed by quantitative immunoblotting of the D1 protein. ND, not detected.

Fraction of thylakoid	PSII supercomplex	PSII core dimer	PSII core monomer	PSII monomer without CP43	PSII reaction center
	%	%	%	%	%
Grana core	45 ± 8	25 ± 4	25 ± 3	5 ± 1[a]	ND
Grana	27 ± 2	35 ± 2	33 ± 2	5 ± 1[a]	ND
Margins	13 ± 1	28 ± 2	41 ± 4	14 ± 3	4 ± 2[a]
Stroma lamellae	ND	15 ± 5	51 ± 3	28 ± 6	6 ± 2[a]
Y100	ND	11 ± 3	48 ± 12	29 ± 1	12 ± 5

[a] Note that the quantification is less precise at very low protein abundance.

TABLE 3

Comparison of the fraction of oxygen-evolving centers and oxidized Cyt b_{559} with dimeric and monomeric forms of PSII (percentage of total) in the different fractions of the thylakoid membrane

Fraction of thylakoid	Fraction of O_2-evolving centers[a]	F_v recombination to the Mn_4 cluster, % of total F_v[b]	PSII dimers + core monomers[c]	Total PSII dimers[d]	Total PSII monomers[e]	Oxidized Cyt b_{559}, % of total[f]
	%	%	%	%	%	%
Grana core	91	77	95	70	30	21
Grana	84	84	95	62	38	35
Margins	66	66	82	41	55	54
Stroma lamellae	43	≥39	66	15	79	75
Y100	0	0	59	11	77	87

[a] Estimated from DCIP reduction by PSII measured in the absence (water to DCIP, oxygen-evolving centers) and presence of DPC (water and DPC to DCIP, total amount of PSII centers) from Ref. 30.
[b] Fraction of oxygen-evolving centers estimated from the flash-induced variable fluorescence decay kinetics, measured in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea from Ref. 30.
[c] Sum of PSII supercomplexes, PSII core dimers, and PSII core monomers (columns 2, 3, and 4 in Table 2).
[d] Sum of PSII supercomplexes and PSII core dimers (columns 2 and 3 in Table 2).
[e] Sum of PSII core monomers and PSII monomers without the CP43 subunit (columns 4 and 5 in Table 2).
[f] Total amount of Cyt b_{559} was estimated after saturating illumination at 77 K.

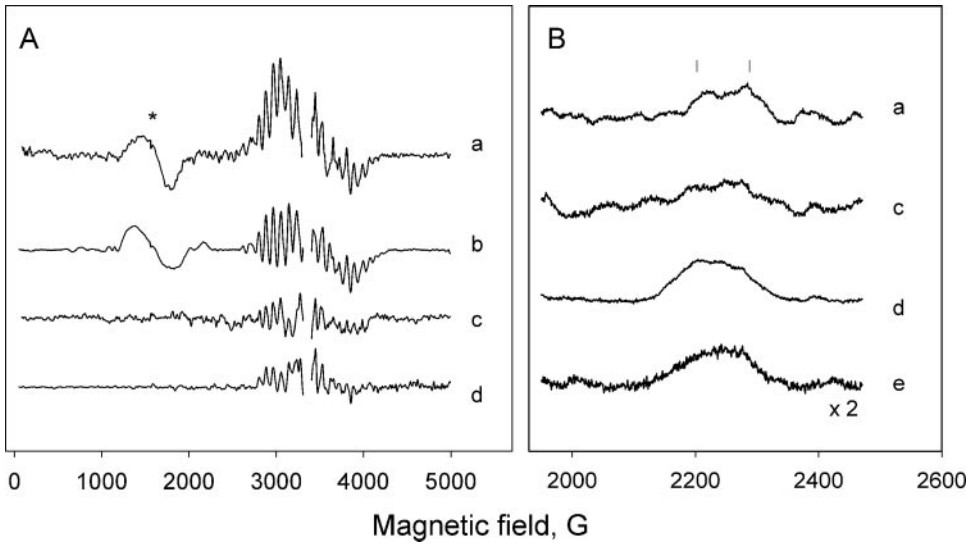

FIGURE 4. *A*, the light minus dark difference spectra showing the S_2 state $g = 4.1$ (*) and multiline signals from the oxygen-evolving complex in PSII from the different fractions of the thylakoid membrane: the grana core (*a*), the grana (*b*), the margins (*c*), and the stroma lamellae (*d*). The spectra were normalized to the same Chl concentration. The S_2 state EPR signals were induced in the absence of an electron acceptor by illumination at 200 K. Experimental conditions were as follows: microwave frequency, 9.41 GHz, microwave power, 10 milliwatts; modulation amplitude, 15 G; temperature, 7 K. *B*, the EPR spectra from the g_z region of oxidized Cyt b_{559} from different fractions of the thylakoid membrane: the grana core (*a*), the margins (*c*), the stroma lamellae (*d*), the Y100 (*e*). The *bars* indicate the position for the high potential form (*left*, $g = 3.06$) and the low potential form (*right*, $g = 2.96$) of Cyt b_{559} (53). The spectra were normalized to the same Chl concentration (*spectrum e* was multiplied 2 times). Experimental conditions were as follows: microwave frequency, 9.41 GHz; microwave power, 5 milliwatts; modulation amplitude, 15 G; temperature, 15 K.

is not sensitive to small structural changes in the vicinity of the Mn_4 cluster. In the S_2 state, the Mn_4 cluster exhibits a much studied multiline EPR signal (8, 47, 48) and the so-called $g = 4.1$ EPR signal (46, 49–51). Observation of either or both of these signals depends on the temperature and illumination conditions to induce the $S_1 \rightarrow S_2$ state transition (49, 50, 52) but also on the chemical environment of PSII (see Ref. 51 and references therein). Therefore, it was of interest to investigate whether the multiline and/or the $g = 4.1$ EPR signals are sensitive to differences in the supramolecular state of the PSII complex.

Fig. 4*A* shows light minus dark difference EPR spectra, which have been recorded in the different fractions of the thylakoid membrane after illumination at 200 K. It is clearly visible that the S_2 state multiline signal was inducible in all fractions that showed steady state oxygen evolution. We were not able to induce any multiline signal in the Y100 fraction (not shown). Interestingly, the granal fractions (the grana core and the grana fractions) showed, in addition to the multiline signal, also the $g = 4.1$ EPR signal (Fig. 4*A*, *spectra a* and *b*) but not in any of the other fractions.

Cyt b_{559} is an important component in PSII and participates in the electron transfer reactions, although it is not a part of the linear electron flow (53). Cyt b_{559} can exist in several potential forms, and its redox state depends on the functional status of the PSII complex (15, 53–55). We therefore characterized the redox state of Cyt b_{559} in the different fractions of the thylakoid membrane, and the results are presented in Fig. 4*B* and Table 3. In the grana core fraction, the oxidized form involved 21% of the total Cyt b_{559} (Fig. 4*B*, *spectrum a*). This proportion gradually increased in fractions from the appressed regions of the grana to the nonappressed regions of the stroma lamellae (Fig. 4*B*, *spectra a–d*), and finally in the Y100 fraction almost 90% of Cyt b_{559} was found to be oxidized (Fig. 4*B*, *spectrum e*, Table 3).

DISCUSSION

Supramolecular Differentiation of PSII in the Thylakoid Membrane—In analysis of the supramolecular organization of PSII in different domains of the thylakoid membrane, we applied methods that do not disturb the original, native organization of the thylakoid membrane fractions and their protein complexes. Mechanical fragmentation and a two-phase separation technique were therefore used to prepare differ-

TABLE 4

Functional and supramolecular properties and membrane localization of different PSII complexes studied in this work

The supramolecular motif of the PSII dimer association with LHCII and small peripheral antennae proteins (PSII supercomplexes) was taken from electron microscopy and single particle analysis studies as in Ref. 29.

Type of PSII complex	Possible structural arrangement [1]	Membrane localization and functional properties
PSII supercomplex		- Only found in the appressed regions of the grana core; - Active O_2 evolution and efficient electron transport
PSII core dimer		- Distributed in all regions of the thylakoid membrane, mostly in the grana; - Nearly all the centers are active in O_2 evolution
PSII core monomer		- Distributed in all regions of the thylakoid membrane, mostly in the non appressed regions of grana margins and stroma lamellae; - Functional heterogeneity in O_2 evolution and electron transport, oxidized Cyt b_{559}
PSII monomer without CP43		- Found mostly in the stroma lamellae and Y100; - No O_2 evolution, oxidized Cyt b_{559}
Reaction center		- Only found in the non appressed regions of grana margins and stroma lamellae, mostly in Y100; - No O_2 evolution, electron transport blocked on the acceptor side, oxidized Cyt b_{559}

[1] Blue central part corresponds to the PSII dimeric or monomeric complexes; the red link corresponds to the minor antennae proteins CP24, -26, and -29, and the green external part corresponds to the LHCII trimers.

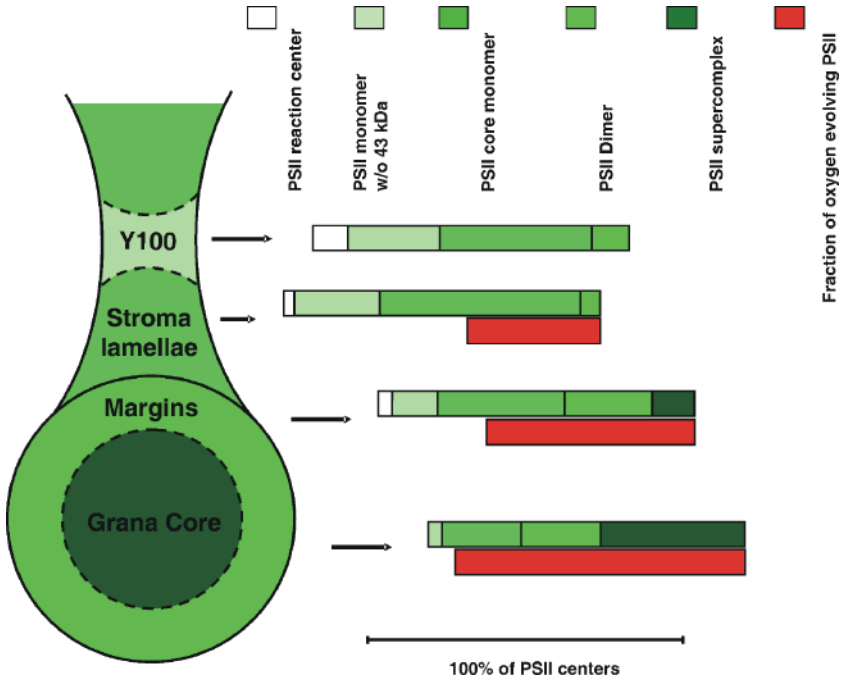

FIGURE 5. **Quantitative distribution of the various PSII complexes in the different domains of the thylakoid membrane.**

ent fractions of the thylakoid membrane (3, 56, 57).[5] Conditions were chosen to ensure that the grana were stacked during fragmentation. In fact, due to the presence of the polymers, the environment of the thylakoids during fragmentation and separation is likely to resemble the natural environment in the intact chloroplast (58). The obtained fractions of the thylakoid membrane were then subjected to BN-PAGE for separation of different PSII complexes. This electrophoretic strategy separates large, multisubunit protein complexes, maintaining their native structural organization, and has been proved to be the most efficient method available for separation of different PSII complexes (32, 33, 41, 42, 59–61).

The dimeric *versus* monomeric organization of PSII is important in several aspects, and dimerization is reported to contribute to the stability of PSII centers (21, 27). There are different dimeric forms of PSII described in the literature. They mainly differ by the number of LHC trimers and their binding strength to the core dimer of PSII (29, 32). One studied dimeric form contains a large, tightly bound outer antenna with

[5] It should be stressed that the isolated vesicles are not "pure" preparations of the different thylakoid membrane domains. Instead, the obtained fractions should rather be considered as preparations enriched in fragments from the respective domains.

2–4 attached LHC trimers and is known as the PSII supercomplex (32), the nomenclature also used here. They are found in the grana, where they are organized in special structures in the granal membranes (62, 63). From our results, it is also clear (Tables 2 and 3) that there are no PSII supercomplexes in the stroma lamellae.

The grana itself is not homogenous with respect to PSII (Tables 2 and 3). Indeed, there is a gradient of the antennae composition from being very large in the central part of the grana to a smaller antenna in the margins. It is important to note that the much studied so-called BBY preparation (64, 65) is dominated by the central part of the grana. Consequently, it can be expected to be dominated by PSII supercomplexes (Tables 2 and 4, Fig. 5). In the appressed grana regions, we find substantial amounts of free LHCII trimers and monomers (Figs. 1 and 2). This reflects that about half of the LHCII trimers are loosely associated with PSII (6, 66, 67).

We also find a fraction of PSII dimers with a smaller antenna, the PSII core dimers. These PSII dimers are more spread over the thylakoid membrane and are found also in the stroma lamellae fractions. In the grana, they are differently distributed as compared with the PSII supercomplexes and are more frequent in the margins than in the grana core. Even stroma lamellae and the Y100 fraction have more than 10% of their PSII in a dimeric form.

The PSII core monomers were found to be heterogeneous and also differentially distributed in the membrane fractions. Our protein analysis distinguished three forms of monomeric PSII (note that our activity analysis indicates an ever higher heterogeneity; see below): (i) PSII core monomers with most of the PSII subunits present, (ii) PSII monomers lacking the CP43 protein (see also Refs. 32 and 68), and (iii) PSII reaction centers. The dominating form of the PSII monomers is the "entire" core monomer, whereas the reaction center form only amounts to ~2% of total PSII (data not shown). CP43-less PSII monomers were previously suggested to result from DM solubilization of the thylakoid membrane and were thus artifacts of the solubilization procedure (32). We have, however, carefully optimized the DM solubilization conditions and previously showed for pumpkin thylakoids that the grana are devoid of CP43-less PSII monomers, whereas they are quite prominent in the stroma thylakoids (44). Moreover, the amounts of CP43-less PSII monomers increase in stroma thylakoids upon increasing light intensity and enhanced damage to PSII.[6] In line with a hypothesis that CP43-less monomers are part of the PSII repair cycle (44), we show here that the proportion of CP43-less PSII monomers logically differs between the thylakoid membrane fractions. The monomeric PSII is not evenly spread, and there is a gradient with fewer monomers in the grana core and with a larger relative fraction in the stroma lamellae. Interestingly, the Y100 fraction is characterized by the very high abundance of PSII monomers lacking CP43 (29%) and by PSII reaction centers (12%) (Tables 2 and 4, Fig. 5). Earlier, the monomeric forms of PSII were reported to mainly originate from the nonappressed regions of the stroma lamellae (21, 24, 25, 69), and our analysis extends this model to a higher resolution.

Interestingly, we found quite substantial amounts of the PSII reaction center, composed of the D1, D2, and Cyt b_{559} subunits, in the margin and stromal fractions. These reaction centers constitute the "minimal PSII unit" and represent less than 2% of total PSII complexes in the thylakoid membrane. Consequently, we know very little about these reaction centers. Compositionally, they resemble the so-called D1-D2-Cyt b_{559} preparation that is purified by rather harsh detergent treatment from grana fractions of the thylakoid (70). This D1-D2-Cyt b_{559} preparation has been extensively studied by spectroscopy, and much is known about its photochemistry and how light affects the photoreactions (71–73). The PSII reaction center we find here is not prepared by detergent and originates from totally different membrane locations in the margins, stroma lamellae, and the Y100 fraction. Thus, we propose that the presence of these reaction centers reflects the PSII repair cycle. From work done on the D1 protein degradation, it seems that once the D1 protein is damaged and PSII has migrated to the stroma lamellae, the D1 protein is rather quickly degraded (13). Thus, since we find as much as 12% of PSII in the Y100 fraction to be in the reaction center form, we propose that they originate from the assembly part and that Y100 is the location where the first steps during PSII assembly and repair take place (Tables 2 and 4, Fig. 5). Attempts to study these PSII reaction centers directly in the membrane are in progress.

Comparison of Functional and Structural Properties of PSII in the Thylakoid Membrane Domains—It is also interesting to discuss the PSII pool present in the different parts of the thylakoid membrane with respect to the fraction of oxygen-evolving PSII centers (Tables 3 and 4), which we first reported and described in detail in Ref. 30. In the grana core, the situation is quite clear. Here, 80–90% of PSII was active in oxygen evolution, as revealed from electron transport and fluorescence decay measurements (Table 3). Since PSII mainly was present as supercomplexes (45%), PSII core dimers (25%), and PSII core monomers (25%, Table 2), it can be concluded that all of those complexes were oxygen-evolving. In addition, it is safe to assume that the PSII monomer without the CP43 subunit (Table 3, columns 2 and 4) was not oxygen-evolving. In the margins, the situation is quite different, and here we find 66% of the centers to be active in oxygen evolution (Table 3). ~40% of PSII is present in different dimeric forms, which probably are all active in oxygen evolution. There are also >50% monomers. If all of these were oxygen-evolving, there would be a larger fraction of oxygen-evolving centers than we find. Instead, our data indicate that the monomeric fraction is heterogeneous, with a little more than half of the monomers present in the margins being inactive in oxygen evolution.

In the stroma lamellae, a very similar picture emerges (Tables 3 and 4). Only 43% of the PSII centers were oxygen-evolving. Also about 40% of the PSII centers showed flash-induced fluorescence decay kinetics in the 1–3-s time scale, reflecting oxidizable manganese. The kinetics, which were too fast (44 ms, recombination to Tyr_Z^{ox}) or too slow (>10 s, nonrecombining PSII centers) reflect PSII centers without a manganese cluster (30). Here, about 30% of PSII was in the monomer form without the CP43 protein or in the reaction center form. We can safely conclude that these were inactive in oxygen evolution. The rest of PSII were either in the dimeric form (15%) or the monomeric form (51%). Such a large fraction clearly exceeds the fraction of oxygen-evolving centers (~40%). It is likely that the dimeric form is active in oxygen evolution. Again, we must consequently conclude that the monomeric form is not homogenous. Similar to in the margins, it contains both oxygen-evolving centers and centers inactive in oxygen evolution. In our preparation of the stroma lamellae, a little more than half of the monomeric centers are active in oxygen evolution, whereas the rest are inactive in oxygen evolution. We also conclude that both the oxygen-inactive monomers and the monomers without the CP43 protein are active on the acceptor side and active in photooxidation of Tyr_Z (which is the donation site for DPC). It is highly likely that these forms of PSII are found on the repair and assembly pathway, where we earlier have identified PSII intermediates active, but slow, on the acceptor side but inactive in oxygen evolution (54, 74).

[6] R. Danielsson, M. Suorsa, V. Paakkarinen, P.-Å. Albertsson, S. Styring, E.-M. Aro, and F. Mamedov, unpublished results.

We found no oxygen-evolving centers in the Y100 fraction (Tables 3 and 4). Flash-induced fluorescence decay kinetics in this fraction could be solely attributed to recombination between Q_A^- and Tyr_Z^{ox} (30). Therefore, it was surprising that about 10% of the PSII in this part of the thylakoid membrane were PSII core dimers. However, the amount of PSII centers in this fraction is very small as compared with other fractions and constitute about 1% of total PSII (Table 1). Thus, the estimated amount of these PSII core dimers then constitutes about 0.1% of the total PSII in the thylakoid membrane.

In addition to our functional analysis with respect to the fraction of the oxygen-evolving centers, our EPR measurements brought more information about PSII behavior in the different fractions of the thylakoid membrane. It can be concluded that the induction of the $g = 4.1$ signal was possible only in the granal fractions with a substantial amount of PSII supercomplexes (Fig. 4*A* and Table 2). It is known that the induction of the $g = 4.1$ signal is sensitive to treatments that can affect the overall structure of PSII (51). It is therefore likely that the special arrangement of PSII in the large supercomplexes somehow affects the magnetic properties of the Mn_4 cluster, inducing the conditions where the $g = 4.1$ signal can be observed. This is unlikely to involve the direct interaction between the two Mn_4 clusters present in dimeric PSII complex. Instead, minor changes in the close environment of the oxygen-evolving complex must be induced by supercomplex formation. It is also indicative that only the fully active PSII centers in the supercomplexes are able to induce the $g = 4.1$ signal by our illumination protocol.

The determined oxidation state of Cyt b_{559} also reflects the composition of PSII in the different fractions. Fully active PSII centers usually have most of Cyt b_{559} in the reduced form in the dark-adapted state (53). Analysis of the data in Table 3 reveals that the fraction of oxidized Cyt b_{559} closely follows the total amount of PSII monomers in each fraction. Thus, monomeric PSII comes with preoxidized Cyt b_{559} in the dark. Interestingly, this oxidized fraction contains both the low and high potential forms of Cyt b_{559}, as revealed from analysis of the g values in the EPR spectra (Fig. 4) (53). Oxidation of Cyt b_{559}, which is not a part of linear electron transfer events leading to water splitting, indicates that there was a necessity for electron donation in PSII (15, 54). Since PSII monomers are functionally heterogeneous, this implies that even oxygen-evolving monomers require alternative electron donation. This phenomenon could refer to incomplete maturation of PSII in monomers and possibly has a significant impact on electron transfer events during PSII turnover.

To conclude, our analysis reveals that all dimeric forms of PSII found here are active in oxygen evolution (Table 4 and Fig. 5). The PSII reaction center and the PSII monomer without CP43 both are inactive in oxygen evolution. The seemingly more intact PSII core monomeric fraction must in contrast be divided into at least two different forms. One of these is active in oxygen evolution, and we find this in both grana cores (very little), margins (about half of the monomers), and stroma lamellae. The other form of the monomeric PSII complexes is inactive in oxygen evolution. These are dominating the Y100 fraction, where 48% of PSII are core monomers without any oxygen evolution. In both stroma lamellae and margins, this PSII monomeric form is abundant and amounts to almost half of the monomers. Thus, among monomeric PSII, there seems to be a gradient from the Y100, where monomers are inactive in oxygen evolution and a large fraction is incomplete with respect to subunit composition, via the stroma lamellae, where a large fraction is active in oxygen evolution and the incomplete centers are less frequent, to the margins, where dimers begin to be frequent and the PSII core monomers are more active in oxygen evolution and less incomplete in their subunit composition (Table 4 and Fig. 5). Finally, in the grana core, PSII core dimers dominate, supercomplexes are very abundant, and nearly all dimers are active in oxygen evolution.

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