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## Isolation and biochemical characterisation of monomeric and dimeric photosystem II complexes from spinach and their relevance to the organisation of photosystem II *in vivo*

Ben HANKAMER<sup>1</sup>, Jon NIELD<sup>1</sup>, Daniella ZHELEVA<sup>1</sup>, Egbert BOEKEMA<sup>2</sup>, Stefan JANSSON<sup>3</sup> and James BARBER<sup>1</sup>

<sup>1</sup> Wolfson Laboratories, Department of Biochemistry, Imperial College of Science, Technology and Medicine, London, UK

<sup>2</sup> Biofysische Chemie, Rijksuniversiteit, Groningen, The Netherlands

<sup>3</sup> Department of Plant Physiology, University of Umeå, Sweden

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Membranes enriched in photosystem II were isolated from spinach and further solubilised using *n*-octyl  $\beta$ -D-glucopyranoside (OctGlc) and *n*-dodecyl  $\beta$ -D-maltoside (DodGlc<sub>2</sub>). The OctGlc preparation had high rates of oxygen evolution and when subjected to size-exclusion HPLC and sucrose density gradient centrifugation, in the presence of DodGlc<sub>2</sub>, separated into dimeric (430 kDa), monomeric (236 kDa) photosystem II cores and a fraction containing photosystem II light-harvesting complex (Lhcb) proteins. The dimeric core fraction was more stable, contained higher levels of chlorophyll,  $\beta$ -carotene and plastoquinone per photosystem II reaction centre and had a higher oxygen-evolving activity than the monomeric cores. Their subunit composition was similar (CP43, CP47, D1, D2, cytochrome *b* 559 and several lower-molecular-mass components) except that the level of 33-kDa extrinsic protein was lower in the monomeric fraction. Direct solubilisation of photosystem-II-enriched membranes with DodGlc<sub>2</sub>, followed by sucrose density gradient centrifugation, yielded a super complex (700 kDa) containing the dimeric form of the photosystem II core and Lhcb proteins: Lhcb1, Lhcb2, Lhcb4 (CP29), and Lhcb5 (CP26). Like the dimeric and monomeric photosystem II core complexes, the photosystem II-LHCII complex had lost the 23-kDa and 17-kDa extrinsic proteins, but maintained the 33-kDa protein and the ability to evolve oxygen. It is suggested, with a proposed model, that the isolated photosystem II-LHCII super complex represents an *in vivo* organisation that can sometimes form a lattice in granal membranes of the type detected by freeze-etch electron microscopy [Seibert, M., DeWit, M. & Staehelin, L. A. (1987) *J. Cell Biol.* 105, 2257–2265].

**Keywords:** dimer; photosynthesis; photosystem II; spinach; structure.

Photosystem II (PSII) is a pigment-protein complex embedded in the thylakoid membrane of higher plants, algae, and cyanobacteria. By utilizing sunlight, it catalyses the splitting of water into protons, electrons, and molecular oxygen. This is the most strongly oxidizing reaction known to occur in biology. The primary photochemical process driving this highly oxidizing reaction takes place in the reaction centre of PSII which, when isolated, consists of the D1 and D2 subunits, cytochrome *b* 559 (cyt *b* 559), and the *psbI* gene product (Nanba and Satoh, 1987; Barber et al., 1987). The reaction centre proteins are closely associated with two other chlorophyll-*a*-binding proteins (CP47 and CP43), as well as the oxygen-evolving complex (OEC) composed of a four-atom cluster of manganese and the 33-kDa PsbO

extrinsic protein (Ikeuchi et al., 1985). Unlike cyanobacteria, higher plants and algae have two additional extrinsic proteins associated with the OEC which have apparent molecular masses of 23 kDa and 17 kDa (PsbP and PsbQ proteins, respectively) (Murata and Miyao, 1985). Higher plants and green algae also have chlorophyll-*a/b*-binding antenna systems that transfer excitation energy to the PSII reaction centre rather than phycobilisomes, which function in the same way in red algae and cyanobacteria (Jansson, 1994). The chlorophyll-*a/b*-binding antenna consists of LHCII (Lhcb1, b2, and b3) and the minor LHCII proteins, CP29 (Lhcb4), CP26 (Lhcb5), and CP24 (Lhcb6).

We recently presented electron micrograph images of negatively stained oxygen-evolving monomeric and dimeric PSII core complexes isolated from spinach consisting of CP47, CP43, D2, D1, cyt *b* 559 and the 33-kDa extrinsic subunit (Boekema et al., 1995). These images, which were obtained by single particle averaging, yielded structural information at a resolution of about 25 Å. The structure of a PSII-LHCII super complex isolated from spinach was characterised by the same procedure and found to have twofold symmetry with a dimeric core placed centrally. The dimeric PSII-LHCII super complex was isolated by gentle solubilisation of PSII-enriched membranes, while the highly purified dimeric and monomeric core complexes were obtained after a more rigorous detergent treatment. These results give credence to the theory that, within the granal regions, PSII

*Correspondence to* J. Barber, Biochemistry Department, Wolfson Laboratories, Imperial College of Science, Technology and Medicine, London, England SW7 2AY

**Abbreviations.** BBY, photosystem-II-enriched membranes isolated according to Berthold et al. (1981); Chl, chlorophyll; CP, chlorophyll protein; CP43, product of the *psbC* gene; CP47, product of the *psbB* gene; cyt *b* 559, cytochrome *b* 559, consisting of the  $\alpha$ - (*psbE* gene product) and  $\beta$ - (*psbF* gene product) subunits; D1, product of the *psbA* gene; D2, product of the *psbD* gene; DodGlc<sub>2</sub>, *n*-dodecyl  $\beta$ -D-maltoside; ESs, exoplasmic (luminal) surface in a stacked membrane region; OEC, oxygen-evolving complex; OctGlc, *n*-octyl  $\beta$ -D-glucopyranoside; PQ, plastoquinone; PS, photosystem; LHC, light-harvesting complex; Lhcb, light-harvesting complex of photosystem II.

exists as a dimer *in vivo* (Rögner et al., 1996). Indeed, this postulate previously emerged from electron microscopy analyses of thylakoid membranes (Seibert et al., 1987) and ordered PSII arrays (Bassi et al., 1989; Lyon et al., 1993; Santini et al., 1994), as well as from biochemical studies (Peter and Thornber, 1991; Santini et al., 1994). In this paper, we describe the isolation and biochemical characterisation of the monomeric and dimeric PSII core complexes, as well as the PSII-LHCII super complex, whose structures were reported by Boekema et al. (1995). The biochemical and structural characteristics of these complexes are compared and discussed in terms of their relevance to the organisation of PSII in the granal lamellae of thylakoid membranes.

## MATERIALS AND METHODS

**Isolation of PSII-enriched membranes from spinach.** The isolation procedure used to obtain PSII-enriched membranes from market spinach leaves (*Spinacea oleracea*) was modified from that reported by Berthold et al. (1981). The chlorophyll concentration was measured using the method of Arnon (1949).

**Isolation of oxygen-evolving PSII cores (OctGlc cores).** To prepare OctGlc cores, PSII-enriched membranes (15 mg Chl) were resuspended in 20 ml core buffer A (0.5 M sucrose, 40 mM Mes, pH 6.0). The sample was then centrifuged at 48 400 g for 15 min at 0°C. The washed PSII-enriched membrane pellet was carefully resuspended in 3 ml core buffer B (72 mM Mes, pH 6.0, 1.8 M sucrose, 72 mM MgCl<sub>2</sub>, 18 mM NaCl) and 2 ml 346 mM *n*-octyl  $\beta$ -D-glucopyranoside (OctGlc) purchased from Calbiochem. The sample was then thoroughly homogenised using a hand-held glass homogeniser and incubated in the dark, with constant stirring for 75 min at 4°C. The solubilised PSII core/LHCII mixture was diluted with 7.5 ml core buffer A and centrifuged at 48 400 g for 10 min at 4°C to pellet any insoluble material. The resultant supernatant was then mixed with 15.5 ml 40 mM Mes, pH 6.0, and centrifuged at 150 000 g at 4°C for 1 h to precipitate LHCII. The supernatant, which contained the oxygen-evolving PSII cores (OctGlc cores), was diluted with an equal volume of 40 mM Mes, pH 6.0, and centrifuged for 30 min at 150 000 g at 4°C. The precipitated OctGlc cores were resuspended with 0.3 M sucrose, 25 mM Mes, pH 6.5, 10 mM NaCl, 5 mM CaCl<sub>2</sub>, 10 mM NaHCO<sub>3</sub>, supplemented with 2 mM *n*-dodecyl- $\beta$ -D-maltoside (DodGlc<sub>2</sub>), before being flash frozen in liquid nitrogen and stored at -80°C.

**Isolation of monomeric and dimeric oxygen-evolving PSII cores.** To isolate PSII monomers and dimers, OctGlc cores (150  $\mu$ g Chl) were suspended in 25 mM Mes, pH 6.5, 10 mM NaCl, 5 mM CaCl<sub>2</sub>, and 10 mM NaHCO<sub>3</sub>, supplemented with DodGlc<sub>2</sub> to give a detergent concentration of 25 mM in a final volume of 300  $\mu$ l. The solubilised sample was homogenised and loaded onto a freshly prepared sucrose gradient. To prepare sucrose gradients, centrifuge tubes were filled with sucrose gradient mix solution (25 mM Mes, pH 6.5, 0.5 M sucrose, 10 mM NaCl, 5 mM CaCl<sub>2</sub>, and 0.03% DodGlc<sub>2</sub>) and frozen at -20°C. Slow thawing at 4°C resulted in the formation of a sucrose density gradient. The solubilised OctGlc cores were then loaded onto the gradients and centrifuged overnight (90 000 g) at 4°C in a Beckman SW41 swing-out rotor. The chlorophyll-rich fractions were then removed from the sucrose gradients, frozen in liquid nitrogen and stored at -80°C.

**Isolation of the PSII-LHCII super complex.** The PSII-LHCII super complex was isolated by subjecting PSII-enriched membranes (150  $\mu$ g Chl at a final concentration of 1 mg ml<sup>-1</sup> Chl) to mild solubilisation with 20 mM DodGlc<sub>2</sub> in 25 mM Mes, pH 6.5, 10 mM NaCl, 5 mM CaCl<sub>2</sub> and 10 mM NaHCO<sub>3</sub>. The solution was homogenised five times before 150  $\mu$ l were loaded

onto a sucrose gradient and centrifuged overnight at 90 000 g in a Beckman SW41 swing-out rotor at 4°C. The PSII-LHCII super complex was located in the most dense band of the sucrose gradient.

**SDS/PAGE and western blotting.** The polypeptide compositions of the isolated PSII preparations were analysed by gradient SDS/PAGE (10–17% polyacrylamide) containing 6 M urea, following the method of Laemmli (1970). The gels were stained with Coomassie brilliant blue R-250.

The protein profiles, resolved by SDS/PAGE, were transferred onto nitrocellulose (Marder et al., 1987; Towbin et al., 1979) and immunolabelled with D2-, C-terminal D1-, and Lhcb1–6-specific antibodies. The D2-specific antibody was a gift from Dr Barbato and was raised according to Barbato et al. (1992). The C-terminal D1 (Dupont 304) antibody was a gift from Dr Nixon. It was raised against a synthetic oligopeptide of the D1 molecule corresponding to the region between residue 333 and the C-terminus. Lhcb1, 2, 3, and 5 proteins were detected using the specific antibodies reported by Król et al. (1995). Lhcb4 and Lhcb6 were detected using the specific antibodies reported by Falbel and Staehelin (1992) and Høyer-Hansen et al. (1988). Biotinylated anti-rabbit IgG was used as the secondary antibody in all cases except for the Lhcb4 blots and was, in turn, labelled with ExtrAvidin-alkaline phosphatase conjugate (Sigma). The Lhcb4-specific antibody was raised in mice and on blotting was labelled with an anti-mouse IgG alkaline phosphatase conjugate. The blots were then incubated with the appropriate chromogenic substrates to facilitate band detection.

**Oxygen evolution.** Oxygen evolution measurements were made using a Clark-type oxygen electrode (Hansatech). All PSII complexes were suspended in 0.3 M sucrose, 25 mM Mes, pH 6.5, 10 mM NaCl, 5 mM CaCl<sub>2</sub>, 10 mM NaHCO<sub>3</sub>, supplemented with 1 mM 2,6-dichlorobenzoquinone and illuminated with white light. For oxygen evolution measurements, OctGlc cores, PSII monomer, and dimer samples were also supplemented with 0.03% DodGlc<sub>2</sub>.

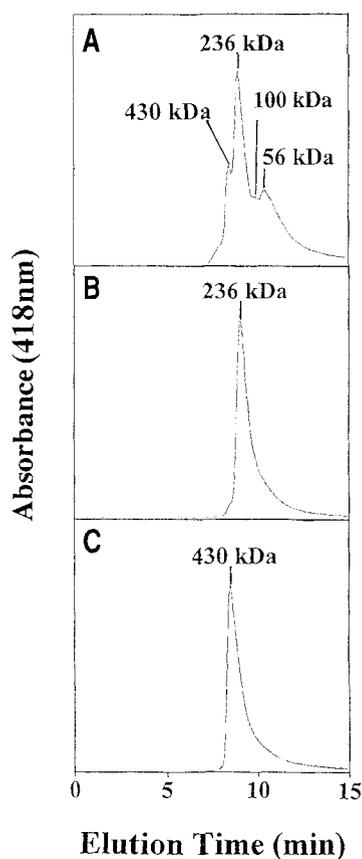
**HPLC size-exclusion analysis.** HPLC size-exclusion analysis was carried out using a Zorbax GF-450 column 9.4/250 mm (Jones Chromatography). The mobile phase consisted of 0.2 M Tris/HCl, pH 7.2, 0.05% DodGlc<sub>2</sub> and was passed through the column at a rate of 0.5 ml min<sup>-1</sup>. The elution profiles were monitored at 418 nm.

**Pigment analysis.** To determine Chl *a*:Chl *b*: $\beta$ -carotene:plastoquinone-9 (PQ-9):pheophytin ratios of isolated spinach PSII complexes, their pigments were extracted with 80% acetone at 4°C under dim light conditions, before being vortexed for 30 s and centrifuged for 1 min. The pigments were resolved by passing the supernatant through an ODS-1 Spherisorb column (Anachem) in a 68% MeOH, 30% ethyl acetate, 2% water mobile phase with a flow rate of 1 ml min<sup>-1</sup> and detected at 453 nm and 663 nm. Chl *a*, Chl *b*, and  $\beta$ -carotene peaks were calibrated using absorption coefficients 76.79 mM<sup>-1</sup> cm<sup>-1</sup> at 663.6 nm in 80% acetone for Chl *a* (Porra et al., 1989), 47.04 mM<sup>-1</sup> cm<sup>-1</sup> at 646.6 nm in 80% acetone for Chl *b* (Porra et al., 1989), and 139 mM<sup>-1</sup> cm<sup>-1</sup> at 452 nm in 100% hexane for  $\beta$ -carotene (Zechmeister and Polgar, 1943). The pheophytin standard used for calibration was produced by acidifying Chl *a* standard with 2 mM HCl. PQ-9 (obtained from Sigma) was dissolved in 100% ethanol and its concentration determined using the absorption coefficient of 15.2 M<sup>-1</sup> cm<sup>-1</sup> at 255 nm (Redfearn and Friend, 1962).

**Single particle image averaging.** Single particle image averaging was carried out using the procedures described in Boekema et al. (1995). Fig. 8A was produced using 1925 individual images of the F-type PSII-LHCII super complex. Fig. 8E was produced by placing the images of the isolated PSII core

**Table 1. Oxygen-evolution analysis of isolated PSII complexes.** The oxygen-evolution rate values are the averages of three independent determinations. The conditions used are given in the Materials and Methods section.

PSII complexes	Rate
	$\mu\text{mol O}_2 \text{ mg}^{-1} \text{ Chl}^{-1} \text{ h}^{-1}$
BBYs	$422 \pm 71$
OctGlc cores	$1246 \pm 110$
Fraction B (PSII core monomers)	$355 \pm 60$
Fraction C (PSII core dimers)	$560 \pm 56$
Fraction E (E-type PSII-LHCII super complex)	$215 \pm 113$
Fraction F (F-type PSII-LHCII super complex)	$265 \pm 61$

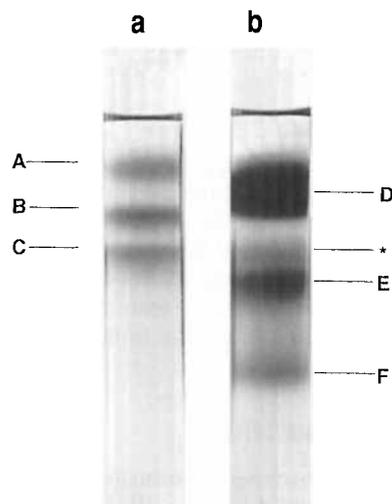


**Fig. 1. Size-exclusion HPLC elution profiles.** (A) OctGlc cores, (B) fraction B (Fig. 2a) containing PSII core monomers, and (C) fraction C (Fig. 2a) containing PSII core dimers.

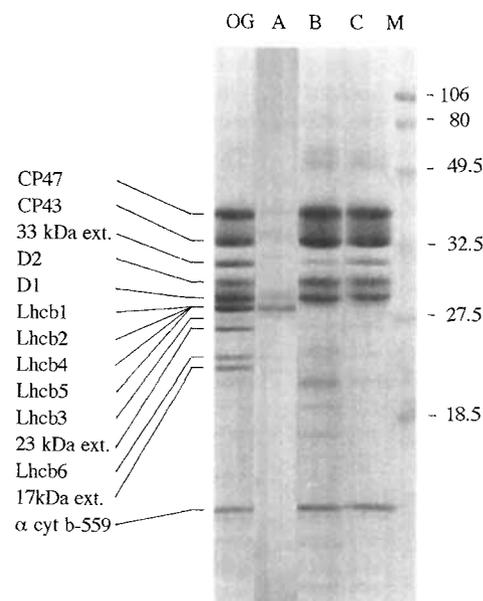
dimer (Boekema et al., 1995) at spacings identical to those of the central core dimer within the PSII-LHCII super complex in the reconstructed lattice of Fig. 8D.

## RESULTS

PSII core complexes depleted of the Lhcb proteins (OctGlc cores), but associated with the full complement of OEC subunits (33-, 23-, and 17-kDa extrinsic polypeptides), were isolated by solubilising PSII-enriched membranes with OctGlc and selectively precipitating the Lhcb components (see Materials and Methods section). OctGlc cores supported oxygen evolution rates of  $1246 \pm 110 \mu\text{mol O}_2 \text{ mg Chl}^{-1} \text{ h}^{-1}$  (Table 1). When subjected to size-exclusion HPLC in the presence of DodGlc<sub>2</sub>,



**Fig. 2. Sucrose density gradients of various PSII complexes.** Chlorophyll-containing fractions labelled A–F and \*, resolved by sucrose density gradient centrifugation of (a) DodGlc<sub>2</sub>-solubilised OctGlc cores and (b) DodGlc<sub>2</sub>-solubilised BBYs. Fractions A–C contained Lhcb proteins, PSII core monomers, and PSII core dimers, respectively. Fraction D, \*, E, and F contained Lhcb proteins, PSII core dimers, E-type PSII-LHCII super complexes, and F-type PSII-LHCII super complexes, respectively.

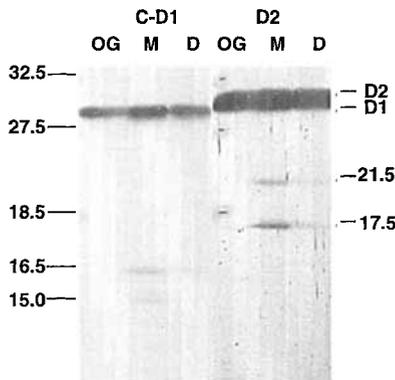
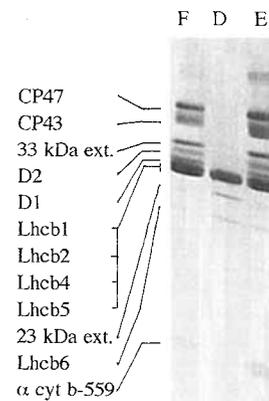


**Fig. 3. Protein content of various PSII core complexes.** SDS/PAGE gel of OctGlc cores (OG), fraction (A), fraction (B), fraction (C), and markers (M). The molecular masses of the markers are given in kDa.

OctGlc cores were partially resolved into four chlorophyll-rich components having apparent molecular masses of  $430 \pm 37$  kDa,  $236 \pm 42$  kDa, and approximately 100 kDa and  $56 \pm 3$  kDa (Fig. 1A). To purify these chlorophyll-binding complexes in bulk, DodGlc<sub>2</sub>-solubilised OctGlc cores were resolved into separate fractions using sucrose density gradients centrifugation (Fig. 2a). SDS/PAGE profiles of the three chlorophyll-rich fractions obtained (A, B, and C in Fig. 2a) are shown in Fig. 3. Fraction A consisted predominantly of Lhcb proteins. Fractions B and C both contained PSII core complexes consisting of CP47, CP43, the 33-kDa extrinsic subunit, D2, D1 and cyt b 559, but were depleted of the Lhcb proteins and the extrinsic 23-kDa and 17-kDa subunits. The efficient removal of Lhcb pro-

**Table 2. Pigment analyses of isolated PSII complexes.** The pigment analysis values are the averages of three independent determinations. The conditions used are given in the Materials and Methods section. n.d., not determined.

PSII complex	No. of molecules of				
	Chl <i>a</i>	Chl <i>b</i>	$\beta$ -carotene	PQ-9	pheophytin
OctGlc cores	50.9 $\pm$ 1.6	5.0 $\pm$ 0.2	8.6 $\pm$ 0.4	4.0 $\pm$ 0.2	2
Fraction B (PSII core monomers)	35.5 $\pm$ 1.3	0.78 $\pm$ 0.03	6.5 $\pm$ 0.5	2.3 $\pm$ 0.24	2
Fraction C (PSII core dimers)	38.5 $\pm$ 2.1	1.09 $\pm$ 0.03	8.0 $\pm$ 0.3	3.1 $\pm$ 0.2	2
Fraction E (E-type PSII-LHCII super complex)	63.6 $\pm$ 1.5	14.7 $\pm$ 0.6	12.5 $\pm$ 0.5	n.d.	2
Fraction F (F-type PSII-LHCII super complex)	78.0 $\pm$ 8.8	23 $\pm$ 3.6	12.8 $\pm$ 1.4	n.d.	2

**Fig. 4. Western blotting of D1 and D2 proteins.** Western blots of OctGlc cores (OG), PSII core monomers (M), and dimers (D), resolved by SDS/PAGE and immunolabelled with C-terminal D1 (C-D1) and D2 specific (D2) antibodies. Marker molecular masses are given in kDa.**Fig. 5. Protein content of various PSII complexes.** SDS/PAGE gel of fractions D, E, and F shown in Fig. 2b, which contained Lhcb proteins and E and F type PSII-LHCII super complexes.

teins from the B-type and C-type complexes was confirmed by the finding that both are associated with only very low levels of Chl *b* (Table 2).

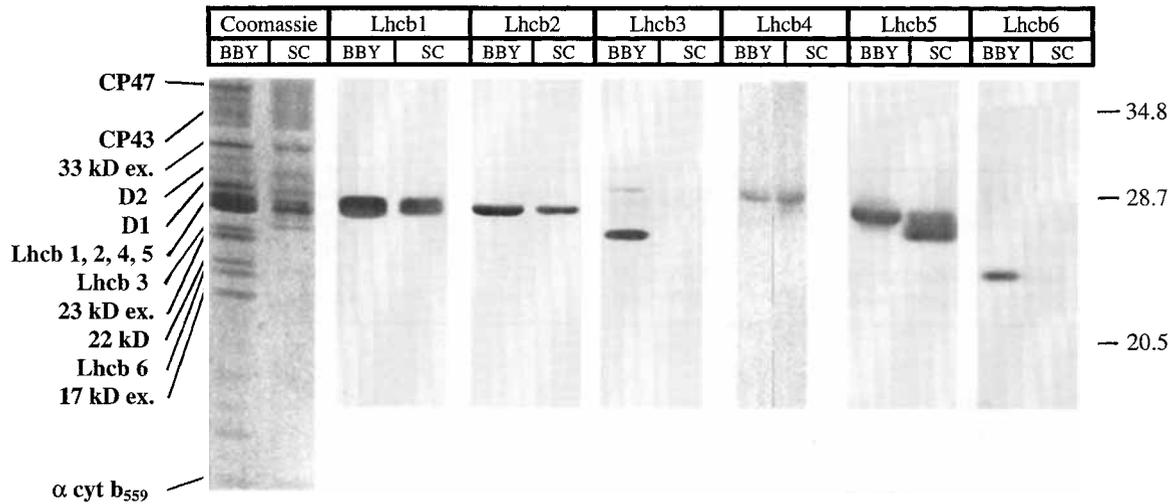
HPLC size-exclusion analysis showed that B-type and C-type PSII complexes (Fig. 1B and C) corresponded to the 236-kDa and 430-kDa components of OctGlc cores (Fig. 1A). These molecular mass data suggest that fractions B and C contain PSII monomers and dimers, respectively, since, assuming all subunits exist in a ratio of 1:1, the calculated molecular mass of a monomer would be about 250 kDa. The monomers and dimers were stable on the Zorbax GF-450 size-exclusion HPLC column and did not interconvert (Fig. 1B and C).

The pigment analysis data (Table 2) show that spinach PSII dimers are associated with a slightly higher number of Chl *a*, Chl *b*,  $\beta$ -carotene and PQ-9 molecules per PSII reaction centre than their monomeric counterparts. To determine whether functional differences exist between the purified PSII monomers and dimers, both were analysed for their ability to evolve oxygen. The oxygen evolution rate supported by the monomers was found to be  $355 \pm 60 \mu\text{mol O}_2 \text{ mg Chl}^{-1} \text{ h}^{-1}$ , while for the dimers the rate was  $560 \pm 56 \mu\text{mol O}_2 \text{ mg Chl}^{-1} \text{ h}^{-1}$  (Table 1). Table 2 shows that PSII dimers contain 9% more chlorophyll per PSII reaction centre than PSII monomers. As dimers support an oxygen evolution rate 57% higher than that of monomers, it is clear that each monomeric core component in a PSII dimer is more active than its isolated counterpart. In both cases, however, there is a significant drop in oxygen evolving activity compared with the OctGlc core preparation. This is almost certainly due to the loss of the 17-kDa and 23-kDa extrinsic proteins, coupled with a partial removal of the 33-kDa PsbO subunit (Fig. 3).

The difference in oxygen-evolving activity of the DodGlc<sub>2</sub> solubilised monomeric and dimeric core complexes can also be partly explained by a greater loss of the 33-kDa protein from

the former compared to the latter (Fig. 3). However, as Fig. 3 also shows, there are a number of faintly stained polypeptides running in the region between the 27.5-kDa marker and the  $\alpha$ -subunit of cyt *b* 559, particularly in the monomeric PSII complex. Western blot analyses using antibodies raised against the D2 protein and the C-terminus of the D1 protein (Fig. 4), identified these polypeptides as C-terminal D1 (15 kDa and 16.5 kDa) and D2 (17.5 kDa and 21.5 kDa) breakdown fragments. The 16.5-kDa C-terminal D1 and the 21.5-kDa and 17.5-kDa D2 fragments were also detected in dimeric PSII core preparations, but in considerably lower amounts, while no D1 and D2 breakdown fragments were detected in OctGlc cores.

The above results show that the dimeric form of PSII, solubilised in DodGlc<sub>2</sub>, is more intact and active than the monomeric form. This difference suggests that the monomeric PSII cores may be the product of dimer dissociation and that the dimers are unlikely to be the consequence of detergent-induced aggregation, a conclusion also supported by the fact that the dimer binds more chlorophyll,  $\beta$ -carotene, and PQ-9 than the monomer (Table 2). If this is the case, it could be argued that the dimeric organisation exists in the PSII-enriched membranes from which they were isolated. To test this hypothesis, PSII-enriched membranes were subjected to a single and mild DodGlc<sub>2</sub> solubilisation step and the products resolved by sucrose density gradient centrifugation. This approach yielded three major fractions labelled D, E, and F (Fig. 2b). SDS/PAGE analysis (Fig. 5) showed that fraction D consisted mainly of Lhcb proteins identified by western blotting as Lhcb1, Lhcb2, Lhcb3, Lhcb4 (CP29), Lhcb5 (CP26), and Lhcb6 (CP24) (data not shown). Fractions E and F (Fig. 5) both contained PSII core complexes consisting of CP47, CP43, the 33-kDa extrinsic subunit, D2, D1, the  $\alpha$ - and  $\beta$ -subunits of cyt *b* 559 as well as associated Lhcb

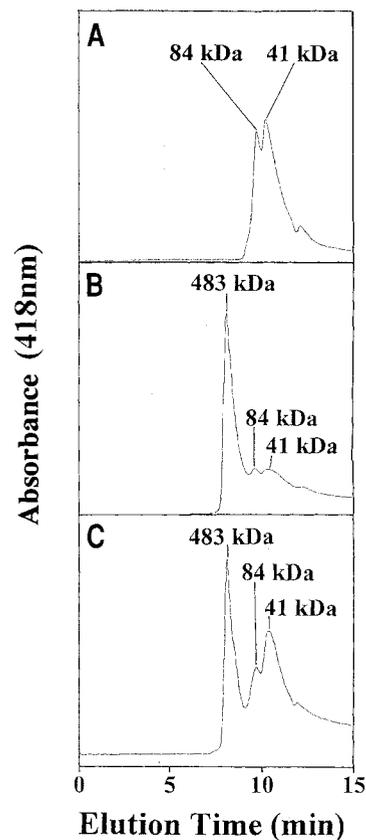


**Fig. 6. Western blotting of Lhcb proteins.** Western blots of BBY membranes (BBY) and the F-type PSII-LHCII super complexes contained in fraction F (SC), using Lhcb1–6-specific antibodies. Also shown is the Coomassie brilliant blue stain SDS/PAGE gels of BBYs and fraction F super complex.

and low-molecular-mass proteins (Fig. 5). The low-molecular-mass subunits were clearly visible in silver-stained gels (data not shown). As Fig. 6 shows, although all the Lhcb proteins were present in the PSII-enriched membranes, fraction F (Fig. 2b) contained Lhcb1, Lhcb2, Lhcb4 (CP29), and Lhcb5 (CP26), and was depleted of Lhcb3 and Lhcb6 (CP24). The reason for the change in mobility of some Lhcb5 in F compared with PSII-enriched membrane, could be due to partial proteolytic clipping.

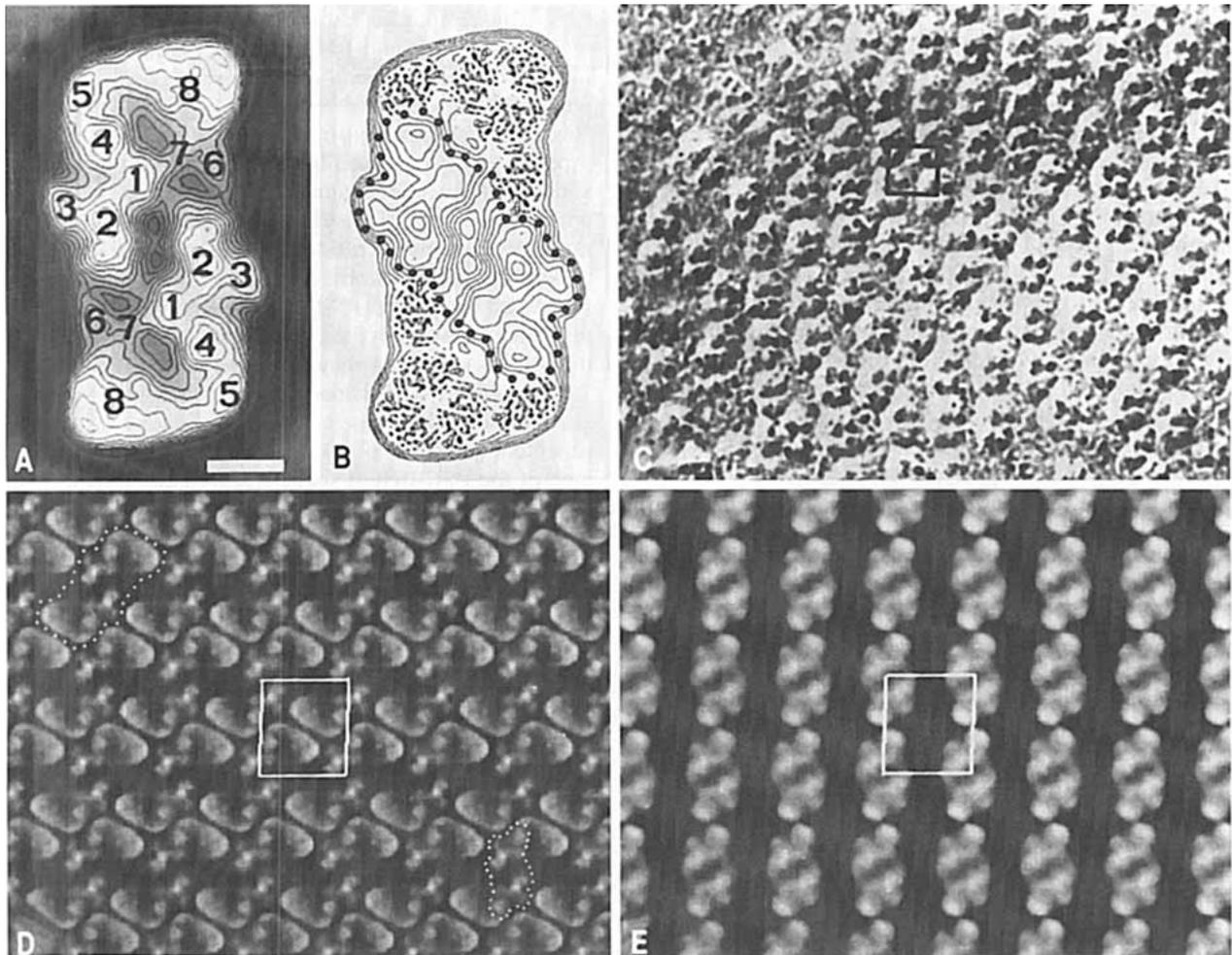
Fractions D, E, and F were subjected to size-exclusion HPLC analysis (Fig. 7). Using this approach, the Lhcb proteins contained in fraction D were resolved into two distinct populations having apparent molecular masses of  $84 \pm 7$  kDa and  $41 \pm 4$  kDa. Fig. 7B and C shows that peaks having the same apparent molecular mass were observed in the elution profiles of fractions E and F, respectively. This indicates that during Zorbax GF-450-mediated size-exclusion HPLC, the Lhcb protein components of E-type and F-type PSII-LHCII super complexes dissociated from the PSII core to which they had been bound. As the HPLC data show, higher levels of these proteins are associated with F-type (Fig. 7C) compared to E type (Fig. 7B) complexes. The Lhcb-depleted PSII cores of fractions E and F gave a 483-kDa peak similar to that observed with the DodGlc<sub>2</sub>-solubilised dimeric core preparation (Fig. 1C). Therefore, taken together with the SDS/PAGE profiles (Fig. 5), these molecular mass data confirmed that fractions E and F both contained dimeric PSII complexes, but that the latter had a larger Lhcb antenna system. When fraction F complexes were passed through a TSK-4000 column, according to the method of Dekker et al. (1988), the intact complex was calculated to have a molecular mass of  $725 \pm 14$  kDa (Boekema et al., 1995).

A closer examination of Fig. 2b shows that a faint band (marked \*), which corresponded (HPLC and SDS/PAGE data not shown) to the dimeric PSII complex (band C in Fig. 2a), was positioned just above band E. Together with the above data, this finding indicated that a large proportion of the PSII complexes isolated from DodGlc<sub>2</sub>-solubilised PSII-enriched membranes was dimeric and that they differ only in the amount of their Lhcb antenna components. We cannot, however, exclude the possibility that monomeric PSII has been isolated from the grana by this procedure but the large amount of LHCII present in fraction D (Fig. 2b) would mask its presence. The molecular mass of fraction E is unknown. However, pigment analysis sug-



**Fig. 7. Size-exclusion HPLC elution profile.** (A) Lhcb proteins (fraction D, Fig. 2b), (B) E-type PSII-LHCII super complexes (fraction E, Fig. 2b), and (C) F-type PSII-LHCII super complexes (fraction F, Fig. 2b).

gests (Table 2) that fraction E has lost 3 to 4 Lhcb polypeptides compared with fraction F, which suggests that its molecular mass is about 600 kDa. The lack of linearity in the sucrose density gradient and detergent effects probably account for this fraction running higher than expected according to the position of the core dimer (450 kDa) and PSII-LHCII super complex in fraction F (725 kDa).



**Fig. 8. Structure and possible *in vivo* organisation of the PSII-LHCII super complex.** (A) Single particle analysis of top view projections of isolated F-type PSII-LHCII super complexes. Best average image of 1925 particles, with twofold rotational symmetry imposed (modified from Boekema et al., 1995). Stain excluding density regions have been numbered; 1–4 belong to the PSII core dimer and 5–8 to Lhc proteins. (B) An interpretation of the two-dimensional projection map of F-type PSII-LHCII super complexes. Two monomeric Lhc proteins connect the LHCII trimer to the core dimer (outlined) containing the reaction centre, CP47 and CP43 (see Boekema et al., 1995; Rögner et al., 1996). (C) Luminal surface of spinach thylakoids revealed by freeze-etching showing ES particle arrays (Seibert et al., 1987). (D) Two-dimensional lattice generated from PSII-LHCII super complexes of the type shown in A. A single PSII-LHCII super complex is outlined with a dotted line (top left hand corner). The box marked with a solid line (centre) marks the centre to centre distances of the PSII-LHCII super complexes. The central PSII core dimer is highlighted with a dotted line (bottom right corner) to aid interpretation of E. (E) The same lattice as in D is shown, but differs in that only the part occupied by the core dimer is visualized. Scale bar in A is 5 nm. The unit cell dimensions (solid line rectangles) are shown for C: 17.5 nm×20.4 nm; D and E: 19 nm×21 nm.

## DISCUSSION

In this paper, we have described the biochemical characterisation of isolated monomeric (236 kDa) and dimeric (430 kDa) PSII cores (Fig. 1), purified by sucrose density gradient centrifugation (Fig. 2a), from LHCII-depleted PSII preparations (OctGlc cores, see Fig. 1A and Fig. 3). Previously, these cores had been analysed in negative stain by single particle image averaging (Boekema et al., 1995). Both the PSII core monomers and dimers consist of CP47, CP43, the 33-kDa extrinsic subunit, D2, D1, the  $\alpha$ - and  $\beta$ -subunits of cyt *b* 559 and several low-molecular-mass polypeptides (Fig. 3). Despite the similarity in the subunit composition of the monomeric and dimeric PSII cores, pigment analyses showed the PSII dimers to be associated with a greater number of chlorophyll,  $\beta$ -carotene, and PQ-9 molecules per two pheophytin molecules (Table 2). Differences in the pigment stoichiometries of the monomers and dimers can be explained in one of two ways. Firstly, it is possible that PSII can exist as both a monomer and dimer in the granal membranes

from which they were isolated and that the monomeric form is simply more susceptible to pigment depletion, induced by the isolation procedure, than the dimer. The second is that PSII exists predominantly as a dimer *in vivo* and that the monomer is the product of detergent-induced dimer dissociation. It has been argued that PSII exists as a monomer in the grana and that these monomers aggregate on detergent solubilization of the membrane (Ford et al., 1995; de Vitry et al., 1991). Our results do not support this argument as it is hard to explain how two pigment-depleted monomers can aggregate to form a dimer having a higher chlorophyll,  $\beta$ -carotene and plastoquinone content per two pheophytin molecules, than its monomeric counterparts. A similar conclusion can be drawn from the finding that the dimers support higher rates of oxygen evolution than monomeric cores (Table 1) and that monomers contained higher levels of D1 and D2 protein breakdown fragments (Fig. 4) and lower levels of the 33-kDa subunit (Fig. 3). That both the monomers and dimers support lower rates of oxygen evolution than the OctGlc cores

from which they were isolated, is due mainly to the fact that they lack the 23-kDa and 17-kDa extrinsic proteins associated with the OEC (Fig. 3).

The above biochemical data support the view that PSII can exist as a dimer in the granal regions of higher plants as has been previously argued by Peter and Thornber (1991), Dainese and Bassi (1991), Bassi et al. (1989, 1995), Santini et al. (1994), and Lyon et al. (1993). This concept is further strengthened by the isolation of an oxygen-evolving dimeric PSII-LHCII supercomplex (fraction F) by mild detergent treatment and which has been studied previously by single particle analysis to a resolution of about 2.5 nm (Boekema et al., 1995). In Fig. 8A, a top view projection map of PSII-LHCII super complexes is shown. This map is very similar to the one presented previously, but improved, in that it is the sum of a larger data set (1925 versus 500 projections). Comparison of top views of the isolated core dimer and the PSII-LHCII supercomplex (Boekema et al., 1995) indicated that the Lhcb proteins are located in peripheral parts of the larger complex, denoted 5–8 in Fig. 8A. The density marked 8 is attributed to the Lhcb1 and Lhcb2 heterotrimer, based on the electron density map taken from Wang and Kühlbrandt (1991) and the western blot data shown in Fig. 6. Densities 5 and 6/7 are thought to be occupied by the linker proteins, Lhcb4, and Lhcb5 as suggested by Dainese et al. (1992) and by the immunoblotting data presented in Fig. 6. Since the monomeric Lhcb proteins are all similar in shape and size, the densities 5 and 6/7 are likely to be composed of single copies of Lhcb4 and Lhcb5, as depicted in Fig. 8B. There seems to be no more space available for accommodating additional Lhcb proteins, but quantitative immunoblotting would be required to confirm this stoichiometry since Dainese and Bassi (1991) have suggested a higher level of the minor Lhcb proteins per PSII. However, we determined the Chl *a* and *b* levels in the PSII-LHCII supercomplex to be 78 and 23, respectively, per reaction centre (Table 2). Subtracting the chlorophylls bound to the core (see Table 2) gives about 40 Chl *a* and 22 Chl *b* attributable to the Lhcb proteins. The precise pigment stoichiometry of Lhcb proteins is not known, but if we accept that the Lhcb *alb* trimer binds 24 and 18 Chl *a* and *b*, respectively (Kühlbrandt and Wang, 1991), this leaves 16 Chl *a* and 4 Chl *b* molecules bound to Lhcb4 and 5. Lhcb4 and Lhcb5 have been reported to bind 9 to 10 and 7–9 Chl *a*, and 3 to 4 Chl *b*, respectively (Henrysson et al., 1989; Peter and Thornber, 1991; Bassi et al., 1995). Therefore, assuming the presence of one trimer of Lhcb1/2 per reaction centre, the remaining chlorophyll level would be consistent with a single copy of Lhcb4 and Lhcb5.

**Validity of the F-type PSII-LHCII super complex as a model of an *in vivo* complex.** The question arises as to whether the detergent-solubilised PSII-LHCII super complex isolated from granal membranes is indeed an *in vivo* form of PSII. A closer examination of the luminal surface of the granal membranes, as studied by freeze-etch analysis (Seibert et al., 1987), shows that some ESs particles attributed to PSII are arranged into small two-dimensional arrays. Fig. 8C shows one of these ESs particle arrays (kindly provided by Andrew Staehelin and taken from Seibert et al., 1987). In this image, the arrays have an almost rectangular lattice with spacings of 17.5 nm×20.4 nm. Other investigations of green plant thylakoid membranes have shown similar ESs-type particle lattices, but with slightly different dimensions. For example, Miller and Jacob (1991) found repeating lattices of 18 nm×22.5 nm and 18 nm×24 nm in wild-type barley thylakoids while Simpson, using the same material, reported centre to centre distances of 17.5 nm×24.7 nm and 15.2 nm×23.7 nm (Simpson, 1978, 1979). Tsvetkova et al. (1995) studied crystalline arrays in *Arabidopsis thaliana*; two

fatty acid desaturase mutants showed lattices with dimensions of 19 nm×23 nm and 18 nm×23 nm, respectively. To relate the top view projection map of the F-type PSII-LHCII super complex (Fig. 8A) with the array of ESs particles observed *in vivo* (Fig. 8C), we have constructed the lattice shown in Fig. 8D. To produce this lattice, we placed the super complex at an angle of about 45 degrees in an almost rectangular array with dimensions of 19 nm×21 nm (Fig. 8D). These dimensions fall within the range of the above mentioned published values and suggest that the crystalline arrays observed in thylakoid membranes could be composed of F-type PSII-LHCII super complexes. Moreover, this analysis supports the conclusions of Seibert et al. (1987) that the ESs particles they observed were PSII-LHCII dimers.

The above suggestion is further strengthened by the similarity between the luminal surface topology of the PSII-LHCII super complexes and ESs particles. The side view projection map of the PSII-LHCII super complex has shown that the Lhcb proteins (densities 5–8 in Fig. 8A) have a thickness of about 6.0 nm (Boekema et al., 1995). This is consistent with the height of LHCII as determined from high-resolution electron crystallography (Kühlbrandt et al., 1994) and is close to the thickness of the thylakoid membrane. Together these results indicate that the Lhcb proteins of the PSII-LHCII super complex would not protrude much above either the stromal or luminal thylakoid membrane surfaces. In contrast, the extrinsic proteins of the OEC (Murata and Miyao, 1985) and the large extrinsic loops of both CP47 and CP43 (Bricker, 1990) extend into the thylakoid lumen. This information was used to model the luminal surface topology of a F-type PSII-LHCII super complex array to allow a more direct comparison with the shadowing data of Fig. 8C. For this reason, the structural detail of the PSII core dimer (densities 1–4 in Fig. 8A) is maintained in the reconstructed lattice (Fig. 8E), while the stained regions corresponding to the Lhcb proteins were deliberately omitted. Note that the spacing of the central dimeric PSII cores (Fig. 8E) is the same as those in Fig. 8D. The modified view of the PSII-LHCII super complex lattice shown in Fig. 8E compares well with the ESs particle array reported by Seibert et al. (1987) in that it reproduces a similar striation pattern due to its surface topology. This analysis therefore further supports the hypothesis that the ESs particles could be PSII-LHCII super complexes of the type depicted in Fig. 8A.

One argument that could be raised against the proposal that the ESs particle arrays observed in freeze-etch images of granal membranes of dimeric PSII being closely packed F-type PSII-LHCII super complexes is that, on average, PSII reaction centres in the granal membranes are associated with about 250 Chl molecules (Lam et al., 1983). Thus, a dimeric complex of PSII would be expected to bind 500 Chl molecules as opposed to the 200 Chl (per two reaction centres) we have measured for the F-type PSII-LHCII super complex. It is clear that there is insufficient space within the unit cell of the constructed crystalline ESs particle arrays (Fig. 8C) to accommodate the extra LHCII subunits required to bind the additional 300 chlorophylls. However, it should be stressed that the value of 250 Chl:reaction centre obtained for granal membranes, is an average value. The freeze-etch images of Seibert et al. (1987) clearly show that the ESs particles in a large proportion of the granal membranes are not in a crystalline array but are more widely spaced. It is quite possible that these non-ordered particles are associated with a much larger antenna system so that on average there are about 250 Chl per reaction centre. This heterogeneity could also account for reported stoichiometric differences for the Lhcb proteins in our supercore complex and in other types of PSII preparations (Dainese and Bassi, 1991).

Studies with PSII-enriched membranes delipidated with Triton X-100 often form two-dimensional arrays that have been analysed by electron crystallography to be PSII core dimers (Bassi et al., 1989; Santini et al., 1994; Lyon et al., 1993; Miller and Jacob, 1991), with a single exception, where a monomeric model was argued (Holzenberg et al., 1993). In the latter case, the unit cell was assumed to contain a full complement of Lhcb proteins. However, Lyon and colleagues (Lyon et al., 1993) showed that similar crystals contained little or no LHCI proteins (low Chl *b* level) but did have Lhcb4, 5, and 6 (Marr et al., 1996). The detection of these linker-type proteins suggests their close association with the core in line with their position in Fig. 8A and B. We did not, however, detect Lhcb6 in the F-type PSII-LHCII.

**Regulatory implications of arrayed and non-arrayed PSII complexes.** A more detailed analysis of the reconstructed crystal consisting of the F-type PSII-LHCII super complex (Fig. 8D) shows that each LHCI protein antenna set (densities 5–8 in Fig. 8A), is in close contact with the PSII core of a neighbouring PSII-LHCII super complex. It is therefore reasonable to expect that excitation energy transfer can occur not only within the PSII super core, but between PSII-LHCII super complexes within such a crystalline array. The non-arrayed ESs particles are more widely spaced and presumably associated with larger antenna systems, more suitable for exciton trapping under low light conditions. These results are also in agreement with those of Spangfort and Andersson (1989) who suggested that Lhcb1 and 2 form part of a mobile LHCI pool. The apparent heterogeneity of PSII/LHCI organisation in the granal membranes may thus reflect a regulatory mechanism used by plants to adapt to changing levels of illumination. Biochemical fractionation studies could help to address how this highly dynamic system adapts to changing environmental conditions and in this context the work of Albertsson (1995) is relevant in that it emphasises the heterogeneity of PSII within the grana, particularly in terms of differing antenna sizes.

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