Two-dimensional Crystals of Photosystem I in Higher Plant Grana Margins*

Ashraf Kitmitto‡‡, Andreas Holzenburg¶, and Robert C. Ford‡‡

From the Department of Biochemistry and Applied Molecular Biology, University of Manchester Institute of Science and Technology, Manchester, M60 1QD, United Kingdom and the Department of Biochemistry and Molecular Biology, University of Leeds, Leeds, LS2 9JT, United Kingdom

In this report, we present new structural data on the size, shape, and oligomeric form of higher plant photosystem I (PSI) formed within the thylakoid grana margins. We show that PSI complexes can be assembled into ordered molecular monolayers (two-dimensional crystals) using thylakoid membranes from a variety of higher plant sources. Digital image analysis of negatively stained two-dimensional crystals (a = 26.9 nm, b = 28.0 nm, γ = 90°, p222, plane group) resulted in a projection map consisting of 4 monomers/unit cell. Higher plant PSI is slightly larger than its cyanobacterial equivalent but shows many similar features. Structural changes after urea and salt washing of the crystals supported the biochemical characterization and were mainly assigned to the stromal side of the complex where the psaC, psaD, and psaE gene products are known to be bound. Labeling with ferredoxin-colloidal gold particles enriched in the thylakoid grana margins and the two-dimensional crystals. This lateral segregation of photosynthetic complexes is important for the understanding of the kinetics of electron transfer between photosystem II and PSI in higher plants.

The thylakoid membranes of higher plants house the main constituents for the harvesting of solar energy and the light-driven electron transport of photosynthesis. The protein complexes responsible for these events in higher plants are the two photosystems I and II (PSI and PSII, respectively),1 the light harvesting chlorophyll a/b pigment-proteins I and II (LHCI and LHCII, respectively), and the cytochrome b6/f complex. PSI is believed to be asymmetrically distributed between the appressed (grana) and non-appressed (stromal lamellae) regions of the thylakoids, being predominantly located in the grana while PSI is primarily localized in the stromal lamellae (1–3).

Higher plant PSI is a membrane pigment-protein complex that catalyzes the photoinduced transfer of electrons from plastocyanin to ferredoxin. PSI contains two high molecular mass polypeptides PSI-A (83 kDa) and PSI-B (82.4 kDa), products of the psaA and psaB genes (4, 5). These two subunits bind several of the primary electron carriers and >50 chlorophyll molecules. There are also at least 13 other PSI polypeptides with molecular masses ranging between 20 and 4 kDa (6, 7) together with a light harvesting antennae (LHCl). At least three extrinsic polypeptides of PSI are exposed on its stromal surface namely PSI-C (9 kDa), PSI-D (15–18 kDa), and PSI-E (8–11 kDa), that bind the two iron-sulfur centers, Fα and Fβ (PSI-C) (8) and ferrodoxin (PSI-D and PSI-E); PSI-D and PSI-E are also thought to function as a shield for the PSI-C polypeptide (9, 10). An extrinsic 9-kDa polypeptide is thought to be located on the lumenal surface of PSI, which is suggested to be involved in binding plastocyanin (9). The exact physiological function of the other polypeptides of PSI remains unclear.

Treatment of PSI with high concentrations of urea is known to solubilize these extrinsic polypeptides (11), with milder concentrations of CaCl2 (0.4 M) and NaCl (1.0 M) shown to specifically release the luminal 9-kDa protein only (12).

It is now accepted that both the PSI and PSII populations are heterogeneous, with two subtypes identified, PSIα and PSIβ and PSIIα and PSIIβ. These sub-types are differentiated by the amounts of the respective light harvesting antennae (LHCI and LHCII) complexes associated with each photosystem core (13). PSIα and PSIIα complexes are suggested to possess a larger light harvesting antennae size (i.e. more LHCI and LHCII associated) than the β-type photosystems. Lateral segregation of these two sub-types of each photosystem has also been proposed, with PSIIα assigned to the grana core and PSIα to the grana margins. PSIβ and PSIβ are believed to be localized in the stromal lamellae (13–15).

Separation and isolation of PSI and PSII have been successfully achieved by a variety of techniques (16–19). To date, most structural data for PSI have been derived from studies of PSI complexes from cyanobacteria either from crystallization of solubilized PSI complexes (20–25) or from crystallized PSI in cyanobacterial thylakoid membranes (24). The oligomeric form of cyanobacterial PSI in vivo appears to be monomeric as judged by electron microscopy (26, 27) although this has recently been disputed on the basis of mutagenesis experiments (28) where a trimeric form was postulated. In comparison, structural data for higher plant PSI are much more limited and have mainly come from freeze-fracture studies (29, 30) showing monomeric PSI particles of 10–13 nm in diameter. Also, detergent-solubilized PSI complexes from spinach have been examined (31) by electron microscopy (EM) and single particle averaging of negatively stained specimens. Without correction for attached detergent, average dimensions for three types of monomeric PSI complexes were found to be approximately 15.3 × 10.5, 16.2 × 11.1, and 20.0 × 16.0 nm. Each of the three types of PSI particle were representative of different preparation methods yielding complexes with different polypeptide and pigment compositions. The apparent lack of structural informa-

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‡ To whom correspondence should be addressed: UMIST, Dept. of Biochemistry & Applied Molecular Biology, P. O. Box 88, Manchester, M60 1QD, UK. Tel.: 44-0-161-2004186; Fax: 44-0-161-2360409.

† The abbreviations used are: PSI and PSII, photosystem I and II, LHCI and LHCII, light harvesting chlorophyll a/b pigment-proteins I and II, respectively; EM, electron microscopy; Chl, chlorophyll; MES, 4-morpholinoethanesulfonic acid; PEG, polyethylene glycol.

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tion on PSI in higher plants as compared with that in cyanobacteria is largely due to difficulties in isolating higher plant PSI suitable for two-dimensional or three-dimensional crystallization.

In this paper, we report the formation of two-dimensional crystals of PSI attached to the thylakoid grana membrane produced by partial detergent solubilization. Interpretation of data from biochemical experiments, labeling studies, and EM strongly indicate that these crystalline arrays are formed from PSI complexes. We have tested this method for crystal formation in other higher plant species and found it to yield reproducible results in terms of crystal size, quality, and unit cell dimensions. The formation of PSI crystals in the thylakoid grana margins provides direct evidence for models proposed by Albertsson et al. (13) for the organization of the two photosystems within the thylakoid membrane domains. This finding is also of importance for understanding the process of electron transfer between PSI and PSII.

**EXPERIMENTAL PROCEDURES**

Spinach (Spinacia oleracea) PSI-enriched grana membranes (BBYs) (18) were prepared as described previously (17). Chlorophyll (Chl) concentrations were determined as described by Arnon (32). Oxygen evolution was measured with a Clark-type electrode according to Ford and Evans (17). Crystal-containing PSI-enriched grana membranes were prepared by a two-step incubation procedure. Stacked thylakoid membranes (Chl = 2 mg ml⁻¹) were incubated in the dark for 20 min at room temperature (20 °C) with Triton X-100 added for a detergent/chlorophyll ratio of 13.75:1 (w/w). The sample was then centrifuged (4 °C) at 13,000 × g (Eppendorf centrifuge 5415C) for 5 min, and the pellet was resuspended in Buffer A (20 mM MES/NaOH (pH 6.3), 5 mM MgCl₂, 15 mM NaCl) at 4 °C. Resuspended membranes (2 mg ml⁻¹ Chl) were then incubated with Triton X-100 at a detergent/chlorophyll ratio of 5:1 (w/w), in the dark, at (20 °C) for 20 min. The sample was then centrifuged (4 °C) at 13,000 × g for 5 min, and the pellet was resuspended in cold Buffer A (4 °C). An aliquot was taken and diluted to a suitable concentration (20–30 μg ml⁻¹ Chl) for EM studies. No significant decline in oxygen evolution activity was measured as compared with control BBYs prepared as described previously (17).

Membrane preparations were assayed for PSI content by measuring P700° oxidation. Membranes ([Chl] = 40 μg ml⁻¹) were resuspended in Buffer A containing 0.2% Triton X-100 (w/v). The sample (2 ml) was equally divided between two glass cuvettes. Using a Cary 1 dual beam spectrophotometer, 5 μl of ascorbate (0.1 M) was added to one cuvette, and 5 μl of potassium ferricyanide (0.1 M) was added to the other. The absorbance difference spectrum (oxidized-reduced) was measured between 650 and 750 nm, and the Chl:P700 ratios were calculated using an extinction coefficient of 0.064 M⁻¹ cm⁻¹ for P700°. Gel electrophoresis under non-denaturing conditions was carried out as follows. Membranes ([Chl] = 2.0 mg ml⁻¹) were solubilized by incubation for 20 min on ice, in the dark, with 2% dodecyl maltoside (w/v). Insoluble material was removed by centrifugation for 5 min at 13,000 × g. A buffer containing 62 mM Tris, 10% glycerol (v/v), 2% SDS (w/v), and 0.05% bromphenol blue (w/v) was added to the solubilized protein at a Chl:SDS ratio of 1.5 (w/w) and immediately analyzed by gel electrophoresis at 4 °C (2 h at 60 V). The green bands in each lane were recorded and the gel was stained with Coomassie Brilliant Blue R250 (0.1% w/v) dissolved in 10% (v/v) methanol, 10% acetic acid (see Fig. 2). Ferredoxin-gold conjugate was prepared by incubating the 5 nm colloidal gold (Sigma) at 5 × 10¹⁵ particles/ml with a 10-fold excess of ferredoxin (Fluka), as determined by the method of Horisberger (33), overnight at 4 °C in the dark. The solution was then centrifuged (50 Ti rotor) at 4 °C for 45 min at 110,000 × g (Beckman L8-85 K ultracentrifuge). The supernatant was carefully aspirated, the wine-colored pellet was resuspended in 50 mM Tris-HCl (pH 7.5), and a 1% PEG 20,000 (w/v) solution was then added (10 parts ferredoxin-gold solution + 1 part 1% PEG). The solution was centrifuged as before. To the wine-colored pellet, an equal volume of 1% PEG was added followed by 10 volumes of the Tris buffer and again centrifuged as before. The wine-colored pellet (stabilized ferredoxin-gold conjugate) was resuspended in Tris buffer. The ferredoxin-gold conjugate at 4 × 10¹⁴ particles/ml was incubated with PSI-containing grana membranes at 50 μg/ml Chl for 20 min on ice prior to specimen preparation and examination by EM as described below.

Membranes were Tris-washed (0.8 M, pH 8.2) as described previously (34, 35) and then pelleted by centrifugation 13,000 × g for 5 min. Membranes were also treated with 5 M urea in Buffer A at a chlorophyll concentration of 250 μg ml⁻¹ for 20 min on ice. Urea-washed membranes were pelleted at 13,000 × g for 5 min. Washed pellets were resuspended in Buffer A.

Samples were mounted for examination by EM as described before (34) and examined in a Philips 301 transmission electron microscope operated at an acceleration voltage of 100 keV. Electron micrographs were recorded at a calibrated magnification of ×24,500 on Kodak electron microscope film (ESTAR Thick Base 4489). Electron micrographs were digitized (Hewlett Packard Deskscan Iic flatbed scanner) resulting in a pixel size of 0.56 nm at the specimen level. The digitized crystalline areas were selected and analyzed using the PC-based CRISP and TRIMERGE image-processing software packages (36). For each data set (control, Tris-washed, and urea-washed), amplitudes and phases were individually refined with a resolution cut-off of 2.5 nm, and individual data sets were merged for calculation of the final projection map.

**RESULTS**

An electron micrograph of negatively stained PSI-enriched grana membranes prepared by the two-step detergent treatment described here is shown in Fig. 1a. Embedded within the central region of the grana membranes are many stain-excluding, nonordered, PSII particles typical of normal BBY preparations (37), but around the peripheral edges of the membrane sheets are several crystalline arrays of particles, as indicated by the arrows. The protein complexes forming the crystalline arrays are substantially smaller than the majority of nonor-
dered PSII particles. Virtually every membrane patch examined was found to contain these crystalline arrays at its extremities. From membrane area calculations, it was estimated that on average the crystalline areas represented about 5\% of each membrane sheet studied. The crystals were found to vary in size, and degree of order with many areas found to be mosaic, i.e. comprised of several small crystals fused together.

Yields of control PSII-enriched grana membranes prepared in this laboratory typically range between 30–40\% of the initial chlorophyll in the thylakoids, with chlorophyll $a/b$ ratios between 1.9 and 2.4. After the first incubation step of the two-stage treatment, average yields were found to be $76 \pm 15\%$ ($n = 4$), with an average chlorophyll $a/b$ ratio of $2.8 \pm 0.1$. Further detergent treatment led to a reduction in yield with an average of $50 \pm 17\%$ and a chlorophyll $a/b$ ratio of $2.5 \pm 0.1$. In general therefore, this method of preparation results in an increase in chlorophyll $a/b$ ratio and a slight increase of grana membrane yield as compared with control BBYs (17).

Non-denaturing gel electrophoresis of a crystal-containing membrane preparation and control BBYs revealed green bands in all samples. Coomassie staining of the gel did not reveal any major non-chlorophyll-containing polypeptides. There were two additional green bands (Fig. 2, A and B) in the crystal-containing sample (lane 1). Band B (molecular mass $\sim 110$ kDa) corresponds to the expected position of the reaction center (CP) complex of PSI consisting of the PSI-A and -B polypeptides and associated pigments and electron transfer components. Band A (molecular mass $\sim 150–180$ kDa) may represent CPI with some extrinsic polypeptides still attached (26). We have assigned the other green bands to PSII components: band C as LHII trimer, band D as unresolved CP47 and CP43, and band E as LHII monomer with free chlorophyll below (38). Crystal-containing membranes were found to contain PSI as determined by the spectrophotometric detection of $P700^+$ at a $P700:\text{Chl}$ ratio of $1:1470 \pm 510$ ($n = 3$). In contrast, no measurable absorbance change due to $P700^+$ could be determined for control BBY samples, giving a $P700:\text{Chl}$ ratio of $1:2800$, as judged by the expected sensitivity of the assay. The initial spinach thylakoid membranes showed a $P700:\text{Chl}$ ratio of $1:1005$, which is somewhat low, suggesting that the PSI:PSII ratio in the leaves was biased toward PSII. Attempts to separate out the crystalline areas from grana membranes containing nonordered PSII complexes by sucrose gradient ultracentrifugation were unsuccessful, implying that the crystals are physically attached to the grana.

The power spectrum of a typical crystalline area (Fig. 1b) suggests $p22_2$, symmetry. However, there are some deviations from the systematic absences expected along h,0 and 0,k for odd h and k. The presence of forbidden reflections may be attributed to partial depth staining, i.e. the stain distribution is not homogeneous along the direction perpendicular to the membrane plane (z-direction). This interpretation is not usual for negatively stained specimens, as described in detail by Harris et al. (39). Digital image analysis of twelve crystalline areas generated a projection map of the unit cell ($a = 26.9 \pm 0.5$ nm; $b = 28.0 \pm 0.6$ nm; $\gamma = 90^\circ$) as shown in Figs. 3, a and b. Crystallographic and image processing data are presented in Table I. The unit cell (Fig. 3b) is composed of two “face down” (denoted S) and two “face up” (denoted L) complexes. The S complexes have a major stain-excluding ridge running across the complex, whereas L complexes are less contrasted, with two domains (marked 1 and 2) approximately 4.4 nm apart (center to center) and separated from a third domain, labeled 3, by a small indentation. Domain 1 is also resolved from a small density labeled 4. Domains 4 and 3 are also visible in the S view of the complex.

Examination by EM of crystal-containing membranes after Tris-washing found no change to the crystal content of the sample. The final projection map from eleven crystalline areas is shown in Fig. 3, c and d. The unit cell dimensions ($a = 27.5 \pm 0.5$ nm, $b = 28.6 \pm 0.5$ nm, $\gamma = 90^\circ$) are slightly larger than those prior to washing; however, the ratio of the crystallographic $a/b$ axes (0.96) and plane group are identical. Tris-washing results in a small change in density to the central
ridge running across molecule S, perhaps suggesting the loss of a small polypeptide, the putative location of which is indicated by an arrowhead in Fig. 3c. Comparison of L in both maps (Fig. 3, a and c) suggests that Tris-washing has resulted in the loss of a density-forming part of domain 3 so that the indentation in the center of the molecule is absent, inferring the removal of an intrinsic polypeptide.

The average projection map of six urea-washed crystalline arrays (a = 25.7 ± 1.1 nm, b = 26.8 ± 1.1 nm, γ = 90°) is presented in Fig. 3f. The unit cell parameters of urea-washed crystals were smaller than control (unwashed crystals) though the ratio of the crystallographic axes remained the same, as shown in Table I. Comparison of this projection map with that of the control crystals (Fig. 3a) showed substantial structural changes to the molecule S. The central ridge running across the complex is now completely absent, revealing a small cleft separating two major domains. The shape of the L complex is similar to the corresponding complex in the Tris-washed map (Fig. 3, c and d), suggesting that urea-washing has resulted in the removal of part of domain 3 and the loss of the indentation.

The distribution of PSI in the crystal-containing membranes was confirmed by labeling PSI with ferredoxin, which binds in its oxidized form to the stromal side of the complex and accepts electrons from the (4Fe-4S) iron-sulfur centers in the PSI. The 5-nm diameter ferredoxin-gold particles appeared as electron-dense black circles in electron micrographs of negatively stained membranes (Fig. 4). The ferredoxin-gold particles were found to cluster around the periphery of the circular grana membranes (Fig. 4a) and in crystal-containing areas (Fig. 4b). In comparison, few particles were found in the central regions of the grana. These data strongly suggest that the two-dimensional crystals observed contain PSI and that the PSI complexes retain the ability to bind ferredoxin with high affinity.

**DISCUSSION**

Interpretation of electron crystallography and biochemical data leads us to conclude that these two-dimensional arrays are a crystal form of higher plant PSI. We suggest that the first detergent solubilization step partially solubilizes the stromal lamellae with the second step removing the non-appressed membranes but leaves the grana margins relatively intact. Incubation conditions at 20 °C also result in the partial solubilization of the PSI complexes from the grana membranes since the lattice plane group is p22,2 with both stromal and lumenal faces presented. There is no evidence to suggest that this configuration reflects the organization of PSI complexes in vivo. A few crystals with p1 symmetry were found in some preparations which may reflect the monomeric in vivo state of PSI in higher plant grana margins. Hefti et al. (24) previously reported small crystalline arrays formed in cyanobacterial stromal lamellae in which PSI under all conditions tested remained monomeric.

In line with a model for the organization of the photosynthetic complexes within the various thylakoid domains (13), these PSI complexes forming the two-dimensional arrays would be expected to correspond to PSI molecules formed from an outer PSI-rich area surrounding the grana core enriched in PSII. Previous reports have indicated that the grana membranes contain PSI complexes (15). Earlier immunolabeling studies of BBYs indicated that a domain exclusive to PSI particles existed around the grana core (40), and we have confirmed these studies using a ferredoxin-gold complex to specifically label PSI complexes in the grana margins. The formation of PSI crystals by the method described here provides additional evidence for such a PSI-enriched grana region. The identification of an enriched PSI population in the grana margins is of significance for understanding how linear electron transport occurs between granal PSII and PSI (13). Crystals with identical unit cell parameters and plane group were also identified in grana membranes prepared, as described here, for both Vicia faba (broad bean) and Pisum sativum (pea) (data not shown).

Comparison of the average projection maps in Fig. 3 with cyano bacterial PSI structures leads us to assign the view denoted S as that corresponding more closely to the stromal surface of PSI and L to the lumenal surface, with the differentiation facilitated by the partial-depth negative staining of the crystal. This assignment is reinforced by the structural changes observed after urea washing, which is expected to remove stromally located extrinsic polypeptides (11). There is a striking similarity between the lumenal and stromal views...
reported here for higher plants and that from solubilized PSI from the thermophilic cyanobacterium *Synechococcus* sp. (20, 23). Bottcher et al. (23) report two-dimensional crystals with \( p12_2 \) symmetry showing preferential staining for both the stromal and luminal surfaces with a unit cell of \( 16.0 \times 15.0 \) nm containing two monomers. The size of the cyanobacterial PSI monomer is smaller (13 \( \times \) 10 nm) than the higher plant PSI reported here (16 \( \times \) 11 nm), which compares closely to the dimensions reported for single particles of PSI-100 (16 \( \times \) 11 nm) (31). However, the principle protein domains are clearly comparable between higher plant and cyanobacterial systems, a finding that was absent from previous structural studies of higher plant PSI. Karrasch et al. (20) more recently report upon the formation of highly ordered two-dimensional crystals of cyanobacterial PSI with \( p12_2 \) plane group but with slightly smaller dimensions than those of Bottcher et al. (23) and than higher plant PSI described here. However, structural features are again strikingly similar to the higher plant crystals.

Substantial structural changes to the stromal side of PSI are found by urea-washing, revealing two large domains separated by a small cavity. We tentatively propose that these two underlying protein domains correspond to PSI-A and PSI-B exposed by the removal of the extrinsic polypeptides, PSI-C, PSI-D, and PSI-E. A similar projection map for a cyanobacteria P700.FX preparation has been calculated (41). However, it may be that urea-washing also has additional effects upon the luminal surface of the higher plant PSI complex, implying that there are several polypeptides that are removed by high concentrations of urea-washing.

Tris-washing was undertaken to assess whether these crystals could have been core complex PSIII since Tris-washing of PSI is known to remove three extrinsic polypeptides bound to the luminal surface of the molecule (33, 34), and thus, if these crystals were PSIII or a subcomplex of PSIII, then large structural changes to one surface of the molecules would be expected. However, it is clear from comparison of the average projection maps before and after Tris-washing that there are only minor density changes to both sides of the map, which would be incongruous for a PSIII assignment. Bassi et al. (42) also report the formation of two-dimensional crystals in grana membranes by detergent treatment at room temperature. However, the conditions employed here are significantly different to those of Bassi et al. (42), with much lower detergent to chlorophyll ratios employed in this method. We interpret the density change on S after Tris-washing as possibly being due to the removal of the PSI-E polypeptide (molecular mass of 8–11 kDa) from the stromal surface, but this remains to be confirmed. The loss of density to the luminal side of PSI may be due to the removal of a 9-kDa extrinsic protein (12) that is known to be removed by high salt concentrations.

In summary, these data provide new and important structural information for higher plant PSI, describing a complex with many similarities to cyanobacterial PSI but indicating it to be larger. The novel method described here presents a new approach to studying higher plant PSI structure and function with future work aimed at optimization of the crystallization procedure and the refinement of a three-dimensional structure.

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