Multiple sites of retardation of electron transfer in Photosystem II after hydrolysis of phosphatidylglycerol

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Received: 16 July 2006 / Accepted: 11 December 2006 / Published online: 19 January 2007
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Abstract Phosphatidylglycerol (PG), containing the unique fatty acid $\Delta 3$, *trans*-16:1-hexadecenoic acid, is a minor but ubiquitous lipid component of thylakoid membranes of chloroplasts and cyanobacteria. We investigated its role in electron transfers and structural organization of Photosystem II (PSII) by treating *Arabidopsis thaliana* thylakoids with phospholipase A₂ to decrease the PG content. Phospholipase A₂ treatment of thylakoids (a) inhibited electron transfer from the primary quinone acceptor Q_A to the secondary quinone acceptor Q_B, (b) retarded electron transfer from the manganese cluster to the redox-active tyrosine Z, (c) decreased the extent of flash-induced oxidation of tyrosine Z and dark-stable tyrosine D in parallel, and (d) inhibited PSII reaction centres such that electron flow to silicomolybdate in continuous light was inhibited. In addition, phospholipase A₂ treatment of thylakoids caused the partial dissociation of (a) PSII supercomplexes into PSII dimers that do not have the complete light-harvesting complex of PSII (LHCII); (b) PSII dimers into monomers; and (c) trimers of LHCII into monomers. Thus, removal of PG

by phospholipase A₂ brings about profound structural changes in PSII, leading to inhibition/retardation of electron transfer on the donor side, in the reaction centre, and on the acceptor side. Our results broaden the simple view of the predominant effect being on the Q_B-binding site.

Keywords *Arabidopsis thaliana* · Phosphatidylglycerol · Phospholipase A₂ · Photosystem II · Thylakoid lipids · Tyrosine Z

Abbreviations

BSA	Bovine serum albumin
Chl	Chlorophyll
CP43	PSII core antenna-protein encoded by <i>psbC</i>
D1 and D2	<i>psbA</i> and <i>psbD</i> gene product, respectively
DCMU	3-(3,4-Dichlorophenyl)-1,1-dimethyl urea
EDTA	Ethylenediamine tetra-acetic acid
EPR	Electron paramagnetic resonance
PG	Phosphatidylglycerol
PLA ₂	Phospholipase A ₂
PpBQ	Phenyl- <i>p</i> -benzoquinone
PSI and PSII	Photosystem I and II, respectively
P ₆₈₀ and P ₇₀₀	Photoactive Chl of the reaction centre of PSII and PSI, respectively
PQ	Plastoquinone
Q _A and Q _B	Primary and secondary plastoquinone electron acceptor of PSII
SQDG	Sulfoquinovosyldiacylglycerol
Y _Z	Redox-active tyrosine of D1 protein

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Introduction

The inner chloroplast thylakoid membranes, which perform the light reactions of photosynthesis, have a highly conserved lipid composition. Three of the four lipids are unique to thylakoids: the two major components are uncharged glycolipids, monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG), comprising between 60% and 80% of total lipids, together with the negatively charged sulfolipid, sulfoquinovosyldiacylglycerol (SQDG) (Douce and Joyard 1990). The fourth lipid, phosphatidylglycerol (PG), an anionic phospholipid which is a ubiquitous component of all other bacterial, plant and animal membranes, comprises only about 8% of total thylakoid lipids. Strangely, PG in thylakoid membranes contain the unique fatty acid, Δ^3 , *trans*-16:1-hexadecenoic acid that is absent in the PG of other eukaryotic or bacterial membranes.

Given the unusually low but uniquely acylated phospholipid content within thylakoid membranes, there has been considerable interest in the role of PG in photosynthetic function and structural organization. Photosystem II (PSII) and photosystem I (PSI) are responsible for converting light energy into electron flow. Electrons are initially generated by the photo-oxidation of primary electron donors of PSII and PSI, named P_{680} and P_{700} , respectively. On photo-excitation, P_{680} transfers an electron to the D1-bound pheophytin (Ph) molecule in a few picoseconds, forming a radical pair. The charge separation is stabilized by rapid transfer of the electron to a tightly bound plastoquinone, Q_A , and then to an exchangeable plastoquinone, Q_B . On the donor side, the tyrosine residue at position 161 on the D1 polypeptide, Y_Z , acts as an intermediate electron carrier by transferring the oxidizing equivalents from the oxidized P_{680} to a manganese cluster (Mn_4Ca cluster) (Debus et al. 1988; Metz et al. 1989). The Mn_4Ca cluster sequentially stores four oxidizing equivalents which are used to oxidize two water molecules into O_2 . The redox states of the Mn_4Ca cluster are labelled S_0 – S_4 where the index for each S state represents the number of oxidizing equivalents stored. There is another spectroscopically similar EPR signal that originates from the tyrosine residue at position 161 on the D2 polypeptide, Y_D (Vermaas et al. 1988). However, Y_D is not directly involved in the water-oxidation chemistry.

Early studies demonstrated that depletion of the phospholipids of spinach thylakoids by phospholipase A_2 (PLA₂) led to a marked inhibition of PSII electron transport (Rawlyer and Siegenthaler 1981; Jordan

et al. 1983). Cyanobacterial cells, in which the PG content was lowered by inactivating a gene that codes for PG phosphate synthase, suffered a partial loss of photosynthetic activity, attributable to a decrease in PSII activity (Hagio et al. 2000; Sato et al. 2000). Further studies led the authors to conclude that PG is an indispensable component of the PSII reaction centre complex in maintaining the structural integrity of the Q_B -binding site (Gombos et al. 2002). Thus, growth of the PG-deficient cyanobacterial cells in a medium that was not supplemented with exogenous PG resulted in considerably slower electron transfer from Q_A to Q_B , suggesting that PG was required for maintenance of the Q_B -binding site. Reports in the literature (Droppa et al. 1995; Sato et al. 2000; Gombos et al. 2002) favour the acceptor side of PSII, particularly the integrity of the Q_B -binding site, being the side affected by the loss of PG, resulting in the loss of PSII function.

However, thermoluminescence measurements hinted at lesions on the donor side of PSII. Droppa et al. (1995) removed the headgroup of PG molecules in pea thylakoids using phospholipase C; they observed that although Q_A^- was capable of charge recombination, the positive charges of the S_2 and S_3 states of the Mn cluster were not. Similarly, thermoluminescence results obtained after PG deprivation in cells of *Synechocystis* sp. PCC 6803 defective in the PG phosphate synthase gene suggested the accumulation of oxidized Y_Z on the donor side of PSII (Gombos et al. 2002). Other studies also suggested that the donor side of PSII could suffer inhibition. Duchene et al. (2000) showed overall differences in the distribution of PG molecules in the two monolayers of thylakoid membranes. Significantly, it was the inner PG population facing the lumen, which mainly sustained uncoupled PSII electron transport (Duchene et al. 2000), confirming an earlier conclusion from the same laboratory (Siegenthaler et al. 1987, 1989). Therefore, one would expect that hydrolysis of the inner pool of PG would affect electron transfer on the donor side of PSII. This study is a concerted investigation of the various possible sites of inhibition of electron transfer in PSII in *Arabidopsis thaliana* thylakoids following the loss of PG.

Structurally, PG plays a role in the dimerization of PSII. PLA₂ treatment of purified spinach PSII core dimers resulted in monomerization, while reconstitution with PG containing Δ^3 , *trans*-16:1-hexadecenoic acid, but not other lipid classes, induced significant dimerization of PSII monomers (Kruse et al. 2000). In *Chlamydomonas reinhardtii*, a mutation that caused PG deficiency impaired the assembly of dimeric PSII

core complexes with their associated antenna (Pineau et al. 2004). In *Synechocystis*, however, although PG plays a role in the effective dimerization and reactivation of the PSII core complex, it is not essential for dimerization since the content of PSII dimers increased on transferring the cells to low light even in the absence of PG (Sakurai et al. 2003). In this study, we have also investigated the role of PG in the assembly of higher plant PSII dimers in thylakoids, so as to test the generality of this structural role, at least in higher plants and green algae.

Materials and methods

Plant growth conditions and isolation of *Arabidopsis thaliana* thylakoids

Arabidopsis thaliana cv Columbia plants were grown in a growth cabinet with an 8-hour photoperiod and at 150 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ at 23/18°C (day/night). For preparing thylakoid membranes, leaves were homogenized in a grinding buffer containing 20 mM Tricine-KOH (pH 8.4), 300 mM sorbitol, 10 mM EDTA, 10 mM NaHCO_3 , 5 mM MgCl_2 , 10 mM KCl, 4.5 mM Na ascorbate, and 0.5% BSA. The homogenate was filtered through six layers of muslin and centrifuged at $3,000 \times g$ for 2 min at 4°C. After suspension in 20 mM Tricine-KOH (pH 7.6), 300 mM sorbitol, 5 mM MgCl_2 , and 2.5 mM EDTA, the chloroplasts were osmotically shocked in 5 mM MgCl_2 for 1 min; then an equal volume of medium was added to give a final concentration of 50 mM HEPES-KOH (pH 7.6), 330 mM sorbitol, 2.5 mM MgCl_2 , and 10 mM KCl prior to centrifugation at $3,000 \times g$ for 2 min at 4°C. Thylakoid membranes were resuspended in a small volume of supernatant and kept on ice in the dark for immediate use or stored at -80°C. Chlorophyll (Chl) concentrations were determined in 80% buffered acetone according to Porra et al. (1989).

Treatment of *Arabidopsis* thylakoids with PLA_2

Isolated thylakoid membranes were suspended in an assay buffer containing 5 mM MgCl_2 , 10 mM NaCl, 330 mM sorbitol, 20 mM Tricine (pH 7.6) and 2 mM CaCl_2 , and incubated in the dark at room temperature with PLA_2 (from bovine pancreas, Sigma) at a concentration of 10 units $(\text{mg Chl})^{-1}$.

Oxygen evolution activity of PSII

Oxygen-evolving activity of PSII: (a) from H_2O to phenyl-*p*-benzoquinone (PpBQ, 0.5 mM) or (b) from

H_2O to silicomolybdate (SiMo, 0.19 mM) plus DCMU (30 μM) was measured in the assay buffer supplemented with 5 mM NH_4Cl at 25°C using a Clark-type O_2 electrode (Hansatech, King's Lynn, UK). PpBQ and SiMo were dissolved in dimethyl sulfoxide. The Chl concentration was 10 $\mu\text{g ml}^{-1}$.

Chl fluorescence measurements

The decay of the flash-induced increase in Chl *a* fluorescence yield in a thylakoid suspension (in a cuvette of 1 mm optical path, 1 mg Chl ml^{-1}) was measured at room temperature using a pulse-modulated fluorometer (PAM101 and 103, Walz, Effeltrich, Germany). A single-turnover flash was given by an XE-STC xenon flash lamp unit (model XF-103, Walz). Weak monitoring light (650 nm) was applied at 1.6 kHz and automatically changed to 100 kHz when a single actinic flash was given. Data acquisition was achieved by home-built equipment and a computer program (Chow and Hope 2004). Twelve successive flashes were given every 15 s, and signals were averaged.

EPR measurements

EPR measurements were carried out using a Bruker ESP 300E spectrometer equipped with a TM011 cavity to determine the $\text{Y}_Z^{\bullet}$ decay kinetics after each repetitive flash (Razeghifard et al. 2005). Saturating 10- μs xenon flashes from an EG&G flash lamp focused through a non-magnetic optical fibre were used to excite the sample. A non-magnetic optical fibre was used to illuminate the sample in the EPR cavity. The ESP 300E spectrometer computer controlled the EPR data acquisition and triggering. The flash lamp was triggered within a fixed delay time after the data acquisition was started. The $\text{Y}_Z^{\bullet}$ decay kinetics was measured at room temperature in the presence of 2 mM PpBQ as the electron acceptor. Thylakoid membranes at 1.5 mg Chl ml^{-1} were treated with PLA_2 and then received four sets of 1,000 flashes at different times. From all the $\text{Y}_Z^{\bullet}$ decay kinetics traces, a field-independent flash artefact signal measured at $g = 1.99$ was subtracted. The $\text{Y}_Z^{\bullet}$ decay kinetics was averaged over 4,000 events occurring at 10 Hz. The $\text{Y}_Z^{\bullet}$ decay kinetics was taken using 10 G modulation amplitude and a time constant of 20 μs . The magnetic field position was 3479 G for $\text{Y}_Z^{\bullet}$ decay kinetics. Other instrumental parameters were: modulation frequency, 100 kHz; microwave power, 100 mW; and microwave frequency, 9.7926 GHz. Instrumental parameters for acquiring $\text{Y}_D^{\bullet}$ signal were: 20 mW microwave power, 4 G modulation amplitude, 100 kHz modulation frequency, microwave

frequency 9.7920 GHz, and 82 ms time constant. Four scans were averaged for the $Y_D^{\bullet}$ signals taken at different times on PLA_2 -treated samples used for the $Y_Z^{\bullet}$ decay kinetic measurements.

Blue-native polyacrylamide gel electrophoresis (PAGE)

Blue-native PAGE was performed largely according to Suorsa et al. (2004). Thylakoids were washed with 50 mM BisTris/HCl (pH 7.0) containing 330 mM sorbitol, sedimented at $2,500 \times g$ for 2 min at 4°C, and resuspended in 25 mM BisTris/HCl (pH 7.0) and 20% (w/v) glycerol at 0.7 mg Chl ml⁻¹. An equal volume of resuspension buffer containing 1% (w/v) n-dodecyl β -D-maltoside was added to the thylakoid suspension. After incubation on ice for 5 min with occasional mixing, insoluble material was removed by centrifugation at $16,000 \times g$ for 20 min at 4°C. The supernatant was supplemented with 0.1 volume of sample buffer (100 mM BisTris/HCl, pH 7.0, 0.5 M 6-amino-*n*-caproic acid, 30% glycerol (w/v), 5% Serva blue G) and applied to a 1.5-mm-thick 5–15% acrylamide gradient gel. Electrophoresis was performed at 4°C and 10 mV for 8 h. The cathode buffer was exchanged with buffer without dye after the top half of the gel was covered with dye.

Results

Inhibition of oxygen evolution in PSII

Light-saturated, uncoupled PSII electron transport in *Arabidopsis thaliana* thylakoids was measured by the reduction of PpBQ with water as the electron donor ($H_2O \rightarrow PpBQ$). With increase in the time of incubation with PLA_2 , thylakoids lost PSII activity steadily, at 30 min, about 50% of the activity remained (Fig. 1). In contrast, control thylakoids incubated in the absence of PLA_2 suffered little or no loss of PSII activity.

Retardation of electron transfer from Q_A^- to Q_B

To test whether inhibition at the Q_B site might have contributed to the loss of PSII activity in PLA_2 -treated samples, PSII Chl *a* fluorescence yield was monitored with a very weak, modulated excitation light. In essentially dark conditions, the PSII Chl fluorescence yield was F_0 , corresponding to open PSII traps. Upon applying a single-turnover flash, the fluorescence yield F rose immediately as Q_A was reduced and converted into a non-quencher (Fig. 2, time = 0). There was an

Fig. 1 Effect of PLA_2 treatment time on PSII activity in *Arabidopsis thaliana* thylakoids. The oxygen evolution activity was measured from H_2O to PpBQ in the control (■) and PLA_2 -treated (●) thylakoid membranes. In the control, PSII activity was $208 \pm 16 \mu\text{mol O}_2 (\text{mg Chl})^{-1} \text{h}^{-1}$ at time zero

artefact signal due to the flash, resulting in the loss of data in the first 0.2 ms. After the peak was reached, F relaxed as Q_A^- was re-oxidized via various routes (Renger et al. 1995). In control thylakoids (zero incubation time), the peak was highest, and a fast phase of relaxation (~ 0.3 ms) was evident (top trace, Fig. 2), corresponding to electron transfer from Q_A^- to Q_B (Renger et al. 1995). As the time of treatment with PLA_2 increased, the peak height decreased, and the fast phase of relaxation was lost. The loss of the fast phase suggests that electron transfer from Q_A^- to Q_B was inhibited.

Retardation of electron donation to $Y_Z^{\bullet}$ after PLA_2 treatment

To investigate electron transfer on the donor side of PSII, we monitored the re-reduction rate of oxidized Y_Z by time-resolved EPR spectroscopy. Oxidized Y_Z ($Y_Z^{\bullet}$) is a paramagnetic species and gives rise to a transient EPR signal in intact systems given repetitive flashes. The $Y_Z^{\bullet}$ re-reduction rates, i.e., S-state transition rates, are good indicators of the intactness of the water-oxidizing enzyme while the $Y_Z^{\bullet}$ signal intensity represents the efficiency of photo-oxidation by P_{680}^+ (Razeghifard et al. 1997b, 2005). In control thylakoids, the $Y_Z^{\bullet}$ signal intensity relaxed rapidly after the flash, but incubation with PLA_2 led to slower re-reduction of $Y_Z^{\bullet}$ (Fig. 3). During the course of

Fig. 2 Changes in Chl *a* fluorescence yield after a single-turnover flash in control and PLA₂-treated *Arabidopsis thaliana* thylakoid membranes. After PLA₂ was added to the thylakoid suspension, fluorescence was measured at different times. The traces from top to bottom refer to 0, 10, 20, 30, and 60 min treatment with PLA₂. For each experiment 12 repetitive flashes were given every 15 s at each incubation time point and traces shown here are averaged for 36 scans from three independent experiments

PLA₂ treatment (Table 1), the re-reduction kinetics can be described by the sum of initially three, but later two, exponential decays. This is because under repetitive flash measurements, the Y_Z^* decay kinetics is assumed to reflect equal contributions of all four S-state transitions. A two-component fit was then used to account for 75% and 25% contribution of earlier S-state transitions and the final S_3 to S_0 transition, respectively. This was based on earlier kinetic data showing that the Y_Z^* reduction rate for the earlier individual S-state transitions is much faster (tens of μ s) than the final S_3 – S_0 transition (hundreds of μ s) (Razeghifard et al. 1997a). A slow decaying component (15%) with a decay time constant of ~ 8 ms is also a part of Y_Z^* decay kinetics that can be attributed to inactive PSII centres. It is seen that PLA₂ treatment of thylakoids retarded electron transfer from the Mn-cluster to Y_Z^* by about three-fold for the earlier S-state transitions (characterized by τ_1) after 30 min treatment. For the $S_3 \rightarrow S_0$ transition (characterized by τ_2), however, PLA₂ treatment of thylakoids for 10 min was sufficient to cause an approximately eight-fold delay in electron transfer from the Mn cluster to Y_Z^* , the $S_3 \rightarrow S_0$ transition being a unique transition since it is closely coupled to O_2 release (Razeghifard and Pace 1999). The great

Fig. 3 Y_Z^* decay kinetics at room temperature in control and PLA₂-treated *Arabidopsis* thylakoid membranes. After PLA₂ was added to a thylakoid suspension, relaxation kinetics of the flash-induced Y_Z^* signal was measured at different times as indicated in panels (see details in Materials and Methods). Each trace is an average of 4,000 events from four independent experiments

Table 1 Analysis of decay time constant (τ_i , μ s) and amplitude (A_i , arbitrary units) of the Y_Z^* re-reduction kinetics

PLA ₂ treatment	τ_1	(A_1)	τ_2	(A_2)	τ_3	(A_3)	$A_1 + A_2 + A_3$
0 min	135	(75)	1,050	(25)	7,500	(15)	115
3 min	161	(58)	3,200	(19)	7,500	(14.5)	92
10 min	260	(62)	8,000	(36)			98
20 min	300	(54)	7,500	(31.5)			86
30 min	400	(41)	9,000	(26)			67

Kinetic fits assumed equal contributions from each S-state transition and a two-component fit model for active centres. τ_i and A_i are fit parameters for earlier transitions ($i = 1$), the $S_3 \rightarrow S_0$ transition ($i = 2$), and inactive centres ($i = 3$)

sensitivity of the $S_3 \rightarrow S_0$ transition strongly suggests that the PLA₂ treatment of thylakoids affected the water-oxidation site in such a way that even though 80% of O₂ evolution activity remained, water oxidation was kinetically retarded.

Inhibition of effective charge separation in the PSII reaction centre after PLA₂ treatment

The Y_Z^* signal intensity represents the efficiency with which P_{680}^+ photo-oxidizes Y_Z (Razeghifard et al. 1997b, 2005). From Table 1, the sum of the amplitudes of the exponential decays decreased with time of treatment of thylakoids with PLA₂. At 30 min, the amplitude was approximately halved, consistent with the 50% decrease in light-saturated PSII electron transport from water to PpBQ (Fig. 1). In the measurement of the Y_Z^* signal, the 100 ms separating two consecutive flashes was long compared with the relaxation lifetimes (0.4 ms and 8 ms for the two phases, 30 min treatment), so that Y_Z was most probably in the reduced form, available for oxidation by P_{680}^+ on the next flash; yet, the extent of oxidation of Y_Z was decreased.

Similarly, the Y_D^* signal intensity declined roughly in parallel with Y_Z^* during treatment with PLA₂. Figure 4 shows that both Y_D^* and Y_Z^* signal intensities, normalized to their respective control values, declined by about 50% after a 30 min treatment.

The parallel declines in both Y_Z^* and Y_D^* signal intensities implies that reaction-centre dysfunction, in addition to slower electron transfer from Q_A^- to Q_B and slower S-state transitions, might contribute to the decrease in light-saturated PSII activity. To investigate which was the most significant step limiting PSII electron flow, we measured electron flow from water to silicomolybdate ($H_2O \rightarrow SiMo$), which accepts electrons before Q_A (Barr et al. 1975). Figure 5 shows that the rate of electron flow to SiMo decreased by about 50% after 30 min incubation with PLA₂, to the same extent as did electron flow to PpBQ (Fig. 1). The similar extents of inhibition of electron flow to the two

Fig. 4 Effect of PLA₂ treatment of *Arabidopsis thaliana* thylakoids on Y_Z^* and Y_D^* signal intensities. After PLA₂ was added to a thylakoid suspension, the flash-induced Y_Z^* (●) and dark-stable Y_D^* (△) signals were measured at different times. The Y_Z^* signal amplitudes were taken from Fig. 3. Y_Z^* signal intensities are signal amplitudes taken from field sweeps at the 3479 G position. Intensities were normalized to the values at time zero

acceptors that intercept electrons at different sites implies that the primary “bottle-neck” for electron flow was prior to the Q_B site, probably in the reaction centre itself.

Effects on the supramolecular PSII assembly

To investigate the effects of loss of PG on the supramolecular organization of PSII, we subjected *Arabidopsis* thylakoids to blue-native PAGE following treatments with PLA₂ (Fig. 6). Control thylakoids showed a prominent band at the top of the gel (Band 1), consisting of PSII-LHCII supercomplexes. This band decreased in intensity with the time of treatment, while also migrating somewhat faster. The most intense band, consisting of a mixture of PSII dimers (without

Fig. 5 Effect of the PLA₂ treatment on PSII activity of *Arabidopsis thaliana* thylakoids. The oxygen evolution activity of PSII was measured from H₂O to SiMO in the presence of 30 μ M DCMU in the control (■) and PLA₂-treated (●) thylakoid membranes. In the control, PSII activity was $103 \pm 16 \mu\text{mol O}_2 (\text{mg Chl})^{-1} \text{h}^{-1}$ at time zero

the complete LHCII antenna) and PSI, if anything, increased in intensity following PLA₂ treatment (Band 2). PSII monomers formed a faint band, which intensified noticeably after PLA₂ treatment (Band 3). Bands 4 and 5 are LHCII trimers and monomers, respectively; they both increased slightly in intensity after PLA₂ treatment. After 60 min PLA₂ treatment, a faint band appeared (indicated by the arrow, Fig. 6), attributable to PSII monomers without CP43 (Suorsa et al. 2004). Clearly PLA₂ treatment of thylakoids had a discernible effect on the supramolecular organization of PSII.

Discussion

Inhibition of electron transfer from Q_A⁻ to Q_B

Inhibition of PSII at the Q_B site has been reported following treatment of pea thylakoids with phospholipase C (Droppa et al. 1995) or depletion of PG by growing *Synechocystis* cells that are defective in phosphatidylglycerol phosphate synthase in the absence of PG (Gombos et al. 2002). Our present results confirm the inhibition at the Q_B site following PLA₂ treatment of *Arabidopsis thaliana* thylakoids; the fast phase of re-oxidation of Q_A⁻ by Q_B was greatly diminished after 60 min PLA₂ treatment of thylakoids (Fig. 2).

Fig. 6 Structural changes of supramolecular complexes and pigment-protein complexes in blue-native gel electrophoresis of PLA₂-treated thylakoid membranes. For the control, thylakoid membranes kept in the dark for 60 min at room temperature was used. The arrow indicates CP43-less PSII monomer. Around 7 μ g Chl was loaded per lane

Retardation of electron donation to Y_Z[•] after PLA₂ treatment

Using time-resolved EPR kinetics of Y_Z, we demonstrated that marked retardation of electron flow also occurred in the donation of electrons from the Mn cluster to oxidized tyrosine Y_Z[•] (Fig. 3). The fast phase of reduction of Y_Z[•] (with a time constant of ~135 μ s in control thylakoids) was slowed three-fold, and the middle phase (with a time constant of ~1000 μ s in control thylakoids) was slowed eight-fold, after 30 min PLA₂ treatment of thylakoids (Table 1). This marked effect of PLA₂ treatment of thylakoids on the Y_Z[•] reduction kinetics, to our knowledge, has not been reported previously.

Minoda et al. (2003) observed that SQDG deficiency in a mutant of *Chlamydomonas reinhardtii* rendered the donor side of PSII unstable, and thus more susceptible to displacement of Mn from the oxygen-evolving complex by NH₂OH, and more accessible for diphenylcarbazide to act as an electron donor to Y_Z[•]. Their suggestion that this mutant could

have suffered decreased efficiency of electron donation to Y_Z^* was consistent with the increased susceptibility to photoinhibition of PSII in the same mutant (Minoda et al. 2001), the resulting longer lifetime of P_{680}^+ causing photodamage (Anderson et al. 1998). Given that SQDG and PG seem to be able to replace each other to a considerable extent (Benning et al. 1993; Güler et al. 1996; Sato et al. 2000), and given the adverse effect of SQDG deficiency on the stability of the Mn cluster (Minoda et al. 2003), one expects PLA_2 treatment to lead to the retardation of Y_Z^* reduction that we observed (Fig. 3, Table 1).

Inhibition of effective charge separation in the PSII reaction centre

The total flash-oxidizable Y_Z^* was less after PLA_2 treatment of thylakoids (Table 1). The 50% loss in Y_Z^* signal intensity after treatment of thylakoids with PLA_2 can be explained by two possibilities. In the first, PLA_2 treatment prevented electron transfer from the Mn cluster to Y_Z^* . In this case, one would expect that Y_Z remains oxidized in 50% of inactive PSII centres during the course of measurement and therefore would not be detected as a transient after a flash. In these inactive PSII centres P_{680}^+ could undergo charge recombination or oxidize another species like Y_D . We therefore measured the Y_D^* signal intensity in PLA_2 -treated thylakoids. The Y_D^* signal intensity was also decreased by about 50% after 30 min treatment, suggesting that Y_D did not act as an alternative electron donor to P_{680}^+ in these inactive centres. Therefore, we favour a second scenario as an explanation for the approximately parallel loss of signal intensities of both Y_Z^* and Y_D^* (Fig. 4), namely, inhibition of effective charge separation and/or of charge stabilization in PSII. Our finding that electron transport from H_2O to SiMo declined by about 50% after 30 min treatment strengthens the idea that the main limitation might have been in the reaction centre rather than the Q_B -binding site.

Jordan et al. (1983) hypothesized that PLA_2 treatment of thylakoids led to inhibition of the charge stabilization step: $P_{680}^+Ph^-Q_A \rightarrow P_{680}^+PhQ_A^-$, where Ph is the primary electron acceptor. This hypothesis is consistent with our observations that (a) electron transport to SiMo was inhibited following the treatment (Fig. 5), and (b) fewer electrons flowed to P_{700}^+ on each flash (data not shown). If the charge-stabilization step were inhibited, and the electron remained on Ph following charge separation, the PSII reaction centre would be effectively closed, particularly if some electrons were to leak through to Q_A and no further. When a PSII

reaction centre goes from an open to a closed state, the time constant for non-radiative loss of the radical pair $P_{680}^+Ph^-$ (i.e., charge recombination directly to the ground state) changes from $\sim 1 \mu s$ to $\sim 1 ns$ (Trissl and Lavergne 1995). That is, on closing a PSII reaction centre, charge recombination directly to the ground state would occur well before any P_{680}^+ could oxidize Y_Z in the sub- μs scale or Y_D in a slower time scale; the amplitude of both Y_Z^* and Y_D^* signal intensities, therefore, would be decreased, as observed (Table 1, Fig. 4). Further, fast charge recombination directly to the ground state would serve to quickly dissipate excitation energy, thereby quenching the variable fluorescence, as observed (Fig. 2). Inhibition of the PSII reaction centre after PLA_2 treatment of thylakoids is also consistent with the finding of Rawlyer and Siegenthaler (1981) that using the electron donor diphenylcarbazide instead of water did not restore electron flow to 2,6-dichlorophenylindophenol.

The relation of PG to the structural organization of PSII

It has been established by high resolution X-ray crystallography that cyanobacterial PSI contains at least three PG molecules and one MGDG molecule located at the outer membrane surface (Jordan et al. 2001), and that PG is required for trimerization of PSI (Domonkos et al. 2004). Further, PG is intimately involved in the trimerization of plant LHCII (Liu et al. 2004; Standfuss et al. 2005). X-ray structure of a cyanobacterial PSII dimer has so far revealed only one PG molecule per PSII reaction centre (Loll et al. 2005).

Our study confirms that removal of PG by PLA_2 treatment of thylakoids led to the partial dissociation of (a) PSII supercomplexes into PSII dimers that do not possess the complete LHCII antenna, (b) PSII dimers into monomers, and (c) LHCII trimers into monomers (Fig. 6). This is consistent with the role of PG in the formation of spinach PSII core dimers in vitro (Kruse et al. 2000), the finding in *Chlamydomonas reinhardtii* that PG deficiency impairs the assembly of dimeric PSII core complexes containing their associated antenna (Pineau et al. 2004), and the impairment of dimerization of PSII core monomers in PG-deprived mutant cyanobacterial cells (Sakurai et al. 2003). Probably, the requirement of PG for both the dimerization of PSII and the trimerization of LHCII is of such importance that in a *pgp1* mutant of *Arabidopsis thaliana* deficient in PG, the chloroplasts each contain only a few swollen thylakoid membranes without a granum structure (Hagio et al. 2002).

The most recent crystal structure of PSII reveals the first visualization of fourteen integrally bound lipids in a cyanobacterial PSII including four anionic lipids, three SQDG and only one PG molecules, along with four DGDG and six MGDG molecules (Loll et al. 2005). The negatively charged headgroups of PG and SQDG were all located at the membrane surface on the acceptor side. The protein environment of the Q_A , Q_B , and the non-heme Fe^{2+} domain opens into a large cavity whose walls are lipophilic, thereby providing a flexible lipophilic environment for PQ/PQH₂ shuttling between the Q_B -binding site and the PQ pool in the bilayer (Loll et al. 2005). The presence of only one PG per cyanobacterial PSII (Loll et al. 2005) raises the question of how the PLA₂ treatment reported here could have such widespread effects on electron transfers at the donor and acceptor sides of PSII complex. It is probable, however, that the fragmented electron density in the large cavity at the Q_B -binding site “is not interpretable and could be due to two or three disordered lipophilic molecules” (Loll et al. 2005). Removing such PG molecules from the acceptor side could somehow affect the acceptor side of PSII.

However, Duchene et al. (2000) have demonstrated with spinach right-side-out and inside-out vesicles that electron flow through PSII depends in part on the integrity of an inner PG pool facing the lumen, a result consistent with an earlier conclusion arising from work on thylakoids in the same laboratory (Siegenthaler et al. 1987, 1989). Even earlier Rawyler and Siegenthaler (1981) had demonstrated that an easily-accessible pool of PG (about 67%) was hydrolyzed by short (5 min) treatment of thylakoids with PLA₂ at 20°C, the hydrolyzed phospholipids almost certainly coming from the outer monolayer. The loss of water-oxidation activity in PSII was only about 25% after 5 min PLA₂ treatment of thylakoids at 20°C (Rawyler and Siegenthaler 1981). The further loss of the bulk of the water-oxidation activity in PSII occurred beyond 5 min PLA₂-treatment, presumably due to the transbilayer movement of PG to the outer monolayer from the inner monolayer where PG normally confers PSII activity. In this way, PLA₂ accessing PG only on the outer monolayer could bring about an effect on the donor side of PSII.

Quite apart from the loss of PG from both monolayers and its effects on both sides of PSII, the monomerization of PSII supercomplexes and of LHClI trimers was accompanied by the loss, from some PSII monomers (indicated by an arrow in Fig. 6), of CP43, a Chl *a*-protein antenna complex in the PSII core. Such disruption of the supramolecular organization of PSII by PLA₂ must have been severe enough to bring about

the widespread effects observed here in electron transfers in PSII.

In conclusion, removal of PG by PLA₂ treatment of *Arabidopsis thaliana* thylakoids brings about profound structural changes in PSII, leading to inhibition of electron transfer at the donor side, the reaction centre, and the acceptor side of PSII. Our results, therefore, broaden the simple view of the predominant effect being on the acceptor side.

Acknowledgments An ANU/RSBS PhD Scholarship supported E-HK. We thank Dr Ron Pace and Dr Paul Smith for constructive comments on the manuscript.

References

- Anderson JM, Park Y-I, Chow WS (1998) Unifying model for the photoinactivation of photosystem II in vivo under steady-state photosynthesis. *Photosynth Res* 56:1–13
- Barr R, Crane FL, Giaquinta RT (1975) Dichlorophenylurea-insensitive reduction of silicomolybdic acid by chloroplast photosystem II. *Plant Physiol* 55:460–462
- Benning C, Beatty JT, Prince RC, Somerville CR (1993) The sulfolipid sulfoquinovosyldiacylglycerol is not required for photosynthetic electron transport in *Rhodobacter sphaeroides* but enhances growth under phosphate limitation. *Proc Natl Acad Sci USA* 90:1561–1565
- Chow WS, Hope AB (2004) Electron fluxes through photosystem I in cucumber leaf discs probed by far-red light. *Photosynth Res* 81:77–89
- Debus RJ, Barry BA, Sithole I, Babcock GT, McIntosh L (1988) Direct mutagenesis indicates that the donor to P_{680}^+ in photosystem II is tyrosine-161 of the D1 polypeptide. *Biochemistry* 27:9071–9074
- Domonkos I, Malec P, Sallai A, Kovács L, Itoh K, Shen G, Ughy B, Bogos B, Sakurai I, Kis M, Strzalka K, Wada H, Itoh S, Farkas T, Gombos Z (2004) Phosphatidylglycerol is essential for oligomerization of photosystem I reaction centre. *Plant Physiol* 134:1471–1478
- Douce R, Joyard J (1990) Biochemistry and function of the plastid envelope. *Annu Rev Cell Biol* 6:173–216
- Droppa M, Horváth G, Hideg E, Farkas T (1995) The role of phospholipids in regulating photosynthetic electron transport activities: treatment of thylakoids with phospholipase C. *Photosynth Res* 46:287–293
- Duchene S, Smutny J, Siegenthaler P-A (2000) The topology of phosphatidylglycerol populations is essential for sustaining photosynthetic electron flow activities in thylakoid membranes. *Biophys Biochim Acta* 1463:115–120
- Gombos Z, Várkonyi Z, Hagio M, Iwaki M, Kovács L, Masamoto K, Itoh S, Wada H (2002) Phosphatidylglycerol requirement for the function of electron acceptor plastocyanin Q_B in the photosystem II reaction centre. *Biochemistry* 41:3796–3802
- Güler S, Seeliger A, Härtel H, Renger G, Benning C (1996) A null mutant of *Synechococcus* sp. PCC7942 deficient in the sulfolipid sulfoquinovosyl diacylglycerol. *J Biol Chem* 271:7501–7507
- Hagio M, Gombos Z, Várkonyi Z, Masamoto K, Sato N, Tsuzuki M, Wada H (2000) Direct evidence for requirement of phosphatidylglycerol in photosystem II of photosynthesis. *Plant Physiol* 124:795–804

- Hagio M, Sakurai I, Sato S, Kato T, Tabata S, Wada H (2002) Phosphatidylglycerol is essential for the development of thylakoid membranes in *Arabidopsis thaliana*. *Plant Cell Physiol* 43:1456–1464
- Jordan BR, Chow WS, Baker AJ (1983) The role of phospholipids in the molecular organization of pea chloroplast membranes. *Biochim Biophys Acta* 725:77–86
- Jordan P, Fromme P, Witt HT, Klukas O, Saenger W, Krauss N (2001) Three-dimensional structure of cyanobacterial photosystem I at 2.5 angstroms resolution. *Nature* 411:909–917
- Kruse O, Hankamer B, Konczak C, Gerle C, Morris E, Radunz A, Schmid GH, Barber J (2000) Phosphatidylglycerol is involved in the dimerization of photosystem II. *J Biol Chem* 275:6509–6514
- Liu Z, Yan H, Wang K, Kuang T, Zhang J, Gui L, An X, Chang W (2004) Crystal structure of spinach major light-harvesting complex at 2.72 Å resolution. *Nature* 428:287–292
- Loll B, Kern J, Saenger W, Zouni A, Biesiadka J (2005) Towards complete cofactor arrangement in the 3.0 Å resolution structure of photosystem II. *Nature* 438:1040–1044
- Metz JG, Nixon PJ, Rögner M, Brudvig GW, Diner A (1989) Directed alternation of the D1 polypeptide of photosystem II: evidence that tyrosine-161 is the redox component, Z, connecting the oxygen-evolving complex to the primary electron donor, P₆₈₀. *Biochemistry* 28:6960–6969
- Minoda A, Sonoike K, Okada K, Sato N, Tsuzuki M (2003) Decrease in the efficiency of the electron donation to tyrosine Z of photosystem II in an SQDG-deficient mutant of *Chlamydomonas*. *FEBS Lett* 553:109–112
- Minoda A, Sonoike K, Okada K, Sato N, Tsuzuki M (2001) Contribution of SQDG in photosystem II of *Chlamydomonas reinhardtii*. In: PS2001Proceedings: 12th International Congress on Photosynthesis, S5–039, CSIRO Publishing, Melbourne (CD-ROM)
- Pineau B, Girard-Bascou J, Eberhard S, Choquet Y, Trémolières A, Gérard-Hirne C, Bennardo-Connan A, Decottignies P, Gillet S, Wollman F-A (2004) A single mutation that causes phosphatidyl deficiency impairs synthesis of photosystem II cores in *Chlamydomonas reinhardtii*. *Eur J Biochem* 271:329–338
- Porra RJ, Thompson WA, Kriedemann PE (1989) Determination of accurate extinction coefficients and simultaneous equations for assaying Chls *a* and *b* extracted with four different solvents: verification of the concentration of Chl standards by atomic absorption spectroscopy. *Biochim Biophys Acta* 975:384–394
- Rawyler A, Siegenthaler P-A (1981) Transmembrane distribution of phospholipids and their involvement in electron transport, as revealed by phospholipase A₂ treatment of spinach thylakoids. *Biochim Biophys Acta* 635:348–358
- Razeghifard MR, Pace RJ (1999) EPR kinetic studies of oxygen release in thylakoids and PSII membranes: a kinetic intermediate in the S3 to S0 transition. *Biochemistry* 38:1252–1257
- Razeghifard MR, Chen M, Hughes JL, Freeman J, Krausz E, Wydrzynski T (2005) Spectroscopic studies of photosystem II in Chl *d*-containing *Acaryochloris marina*. *Biochemistry* 44:1178–1187
- Razeghifard MR, Klughammer C, Pace RJ (1997a) Electron paramagnetic resonance kinetic studies of the S States in spinach thylakoids. *Biochemistry* 36:86–92
- Razeghifard MR, Wydrzynski T, Pace RJ, Burnap RL (1997b) Y_z reduction kinetics in the absence of the Mn-stabilizing protein of photosystem II. *Biochemistry* 36:14474–14478
- Renger G, Eckert H-J, Bergmann A, Bernarding J, Liu B, Napiwotzki A, Reifarth F, Eichler HJ (1995) Fluorescence and spectroscopic studies of exciton trapping and electron transfer in photosystem II of higher plants. *Aust J Plant Physiol* 22:167–181
- Sakurai I, Hagio M, Gombos Z, Tyystjärvi T, Paakkari V, Aro E-M, Wada H (2003) Requirement of phosphatidylglycerol for maintenance of photosynthetic machinery. *Plant Physiol* 133:1376–1384
- Sato N, Hagio M, Wada H, Tsuzuki M (2000) Requirement of phosphatidylglycerol for photosynthetic function in thylakoid membranes. *Proc Natl Acad Sci USA* 97:10655–10660
- Siegenthaler P-A, Rawyler A, Smutny J (1989) The phospholipid population which sustains the uncoupled non-cyclic electron flow activity is localized in the inner monolayer of the thylakoid membrane. *Biochim Biophys Acta* 975:104–111
- Siegenthaler P-A, Smutny J, Rawyler A (1987) Involvement of distinct populations of phosphatidylglycerol and phosphatidylcholine molecules in photosynthetic electron-flow activities. *Biochim Biophys Acta* 891:85–93
- Standfuss J, Terwisscha van Scheltinga AC, Lamborghini M, Kuhlbrandt W (2005) Mechanism of photoprotection and nonphotochemical quenching in pea light-harvesting complex at 2.5 Å. *EMBO J* 24:919–928
- Suorsa M, Regel RE, Paakkari V, Battchikova N, Herrmann RG, Aro E-M (2004) Protein assembly of photosystem II and accumulation of subcomplexes in the absence of low molecular mass subunits PsbL and PsbJ. *Eur J Biochem* 271:96–107
- Trissl H-W, Lavergne J (1995) Fluorescence induction from Photosystem II: analytical equations for the yield of photochemistry and fluorescence derived from analysis of a model including exciton-radical pair equilibrium and restricted energy transfer between photosynthetic units. *Aust J Plant Physiol* 22:183–193
- Vermaas WJF, Rutherford AW, Hansson O (1988) Site-directed mutagenesis in photosystem II of the cyanobacterium *Synechocystis* sp. PCC 6803: Donor D is a tyrosine residue in the D2 protein. *Proc Natl Acad Sci USA* 85:8477–8481