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

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Abstract Phosphatidylglycerol (PG), containing the unique fatty acid Δ3, 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 QA to the secondary quinone acceptor QB, (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 siliconolylbdate 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 Qb-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 psbC
D1 and D2 psbA and psbD gene product, respectively
DCMU 3-(3,4-Dichlorophenyl)-1,1-dimethyl urea
EDTA Ethylenediamine tetra-acetic acid
EPR Electron paramagnetic resonance
PG Phosphatidylglycerol
PLA₂ Phospholipase A₂
PpBQ Phenyl-p-benzoquinone
PSI and PSII Photosystem I and II, respectively
P₆₈₀ and P₇₀₀ Photocactive Chl of the reaction centre of PSII and PSI, respectively
PQ Plastoquinone
QA and QB Primary and secondary plastoquinone electron acceptor of PSII
SODG Sulfoquinovosyldiacylglycerol
Y₂ Redox-active tyrosine of D1 protein
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, monogalactosydialcylglycerol (MGDG) and digalactosydialcylglycerol (DGDG), comprising between 60% and 80% of total lipids, together with the negatively charged sulfolipid, sulfoquinovosydialcylglycerol (SQDG) (Douce and Joyard 1990). The fourth lipid, phosphatidylycerol (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, $\Delta_3$, 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 phophorytin (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$_D$, acts as an intermediate electron carrier by transferring the oxidizing equivalents from the oxidized P$_{680}$ to a manganese cluster (Mn$_4$Ca cluster) (Debus et al. 1988; Metz et al. 1989). The Mn$_4$Ca cluster sequentially stores four oxidizing equivalents which are used to oxidize two water molecules into O$_2$. The redox states of the Mn$_4$Ca cluster are labelled S$_7$–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$_2$ (PLA$_2$) led to a marked inhibition of PSI electron transport (Rawyler 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, theroluminomac 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 lost. Similarly, theroluminomac 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 PSI 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. PLA$_2$ treatment of purified spinach PSII core dimers resulted in monomerization, while reconstitution with PG containing $\Delta_3$, 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 reactivity 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 μmol photons m⁻² 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 NaHCO₃, 5 mM MgCl₂, 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 × g for 2 min at 4°C. After suspension in 20 mM Tricine-KOH (pH 7.6), 300 mM sorbitol, 5 mM MgCl₂, and 2.5 mM EDTA, the chloroplasts were osmotically shocked in 5 mM MgCl₂ 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 MgCl₂, and 10 mM KCl prior to centrifugation at 3,000 × 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 PLA₂

Isolated thylakoid membranes were suspended in an assay buffer containing 5 mM MgCl₂, 10 mM NaCl, 330 mM sorbitol, 20 mM Tricine (pH 7.6) and 2 mM CaCl₂, and incubated in the dark at room temperature with PLA₂ (from bovine pancreas, Sigma) at a concentration of 10 units (mg Chl)⁻¹.

Oxygen evolution activity of PSII

Oxygen-evolving activity of PSII: (a) from H₂O to phenyl-β-benzoquinone (PbBQ, 0.5 mM) or (b) from H₂O to silicomolybdate (SiMo, 0.19 mM) plus DCMU (30 μM) was measured in the assay buffer supplemented with 5 mM NH₄Cl at 25°C using a Clark-type O₂ electrode (Hansatech, King’s Lynn, UK). PbBQ and SiMo were dissolved in dimethyl sulfoxide. The Chl concentration was 10 μg 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 Y₂⁺ 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 Y₂⁺ decay kinetics was measured at room temperature in the presence of 2 mM PbBQ as the electron acceptor. Thylakoid membranes at 1.5 mg Chl ml⁻¹ were treated with PLA₂ and then received four sets of 1,000 flashes at different times. From all the Y₂⁺ decay kinetics traces, a field-independent flash artefact signal measured at g = 1.99 was subtracted. The Y₂⁺ decay kinetics was averaged over 4,000 events occurring at 10 Hz. The Y₂⁺ decay kinetics was taken using 10 G modulation amplitude and a time constant of 20 μs. The magnetic field position was 3479 G for Y₂⁺ decay kinetics. Other instrumental parameters were: modulation frequency, 100 kHz; microwave power, 100 mW; and microwave frequency, 9.7926 GHz. Instrumental parameters for acquiring Y₂⁺ 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 YZ* signals taken at different times on PLA2-treated samples used for the YZ* 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 × 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 × 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 (H₂O → PpBQ). With increase in the time of incubation with PLA2, 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 PLA2 suffered little or no loss of PSII activity.

Retardation of electron transfer from QA to QB

To test whether inhibition at the QB site might have contributed to the loss of PSII activity in PLA2-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 F₀, corresponding to open PSII traps. Upon applying a single-turnover flash, the fluorescence yield F rose immediately as QA was reduced and converted into a non-quencher (Fig. 2, time = 0). There was an 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 QA 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 QA to Qₐ (Renger et al. 1995). As the time of treatment with PLA2 increased, the peak height decreased, and the fast phase of relaxation was lost. The loss of the fast phase suggests that electron transfer from QA to QB was inhibited.

Retardation of electron donation to YZ* after PLA2 treatment

To investigate electron transfer on the donor side of PSII, we monitored the re-reduction rate of oxidized YZ by time-resolved EPR spectroscopy. Oxidized YZ (YZ*) is a paramagnetic species and gives rise to a transient EPR signal in intact systems given repetitive flashes. The YZ* re-reduction rates, i.e., S-state transition rates, are good indicators of the intactness of the water-oxidizing enzyme while the YZ* signal intensity represents the efficiency of photo-oxidation by P₆₈₀ (Razeghiparvar et al. 1997b, 2005). In control thylakoids, the YZ* signal intensity relaxed rapidly after the flash, but incubation with PLA2 led to slower re-reduction of YZ* (Fig. 3). During the course of
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 S₃ to S₀ 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 ms) than the final S₃–S₀ transition (hundreds of ms) (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 $\tau_1$) after 30 min treatment. For the S₃ → S₀ transition (characterized by $\tau_2$), 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 S₃ → S₀ transition being a unique transition since it is closely coupled to O₂ release (Razeghifard and Pace 1999). The great
Table 1 Analysis of decay time constant ($\tau$, $\mu$s) and amplitude ($A_i$, arbitrary units) of the $Y_2^*$ re-reduction kinetics

<table>
<thead>
<tr>
<th>PLA2 treatment</th>
<th>$\tau_1$</th>
<th>$A_1$</th>
<th>$\tau_2$</th>
<th>$A_2$</th>
<th>$\tau_3$</th>
<th>$A_3$</th>
<th>$A_1 + A_2 + A_3$</th>
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<tbody>
<tr>
<td>0 min</td>
<td>135</td>
<td>(75)</td>
<td>1,089</td>
<td>(25)</td>
<td>7,500</td>
<td>(15)</td>
<td>115</td>
</tr>
<tr>
<td>3 min</td>
<td>161</td>
<td>(58)</td>
<td>3,200</td>
<td>(19)</td>
<td>7,500</td>
<td>(14.5)</td>
<td>92</td>
</tr>
<tr>
<td>10 min</td>
<td>260</td>
<td>(62)</td>
<td>8,000</td>
<td>(36)</td>
<td></td>
<td></td>
<td>98</td>
</tr>
<tr>
<td>20 min</td>
<td>300</td>
<td>(54)</td>
<td>7,500</td>
<td>(31.5)</td>
<td></td>
<td></td>
<td>86</td>
</tr>
<tr>
<td>30 min</td>
<td>400</td>
<td>(41)</td>
<td>9,000</td>
<td>(26)</td>
<td></td>
<td></td>
<td>67</td>
</tr>
</tbody>
</table>

Kinetic fits assumed equal contributions from each S-state transition and a two-component fit model for active centres. $\tau_i$ and $A_i$ are fit parameters for earlier transitions ($i = 1$), the $S_3 \rightarrow S_4$ 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 $O_2$ evolution activity remained, water oxidation was kinetically retarded.

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

The $Y_2^*$ 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 $Y_2^*$ 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 $Y_2^*$ was decreased.

Similarly, the $Y_D^*$ signal intensity declined roughly in parallel with $Y_2^*$ during treatment with PLA2. Figure 4 shows that both $Y_D^*$ and $Y_2^*$ signal intensities, normalized to their respective control values, declined by about 50% after a 30 min treatment.

The parallel declines in both $Y_2^*$ 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 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 thaliana 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
the complete LHClI 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 LHClI 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\textsubscript{A} to Q\textsubscript{B}

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

Fig. 5 Effect of the PLA2 treatment on PSII activity of Arabidopsis thaliana thylakoids. The oxygen evolution activity of PSII was measured from H\textsubscript{2}O to SMO in the presence of 30 μM DCMU in the control (■) and PLA2-treated (●) thylakoid membranes. In the control, PSII activity was 103 ± 16 μmol e O\textsubscript{2} (mg Chl\textsubscript{a})\textsuperscript{-1} h\textsuperscript{-1} at time zero.

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 μg Chl was loaded per lane.

Retardation of electron donation to Y\textsubscript{Z}* after PLA2 treatment

Using time-resolved EPR kinetics of Y\textsubscript{Z}, we demonstrated that marked retardation of electron flow also occurred in the donation of electrons from the Mn cluster to oxidized tyrosine Y\textsubscript{Z}* (Fig. 3). The fast phase of reduction of Y\textsubscript{Z}* (with a time constant of ~135 μs in control thylakoids) was slowed three-fold, and the middle phase (with a time constant of ~1000 μs 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\textsubscript{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 NH\textsubscript{2}OH, and more accessible for diphenylcarbazide to act as an electron donor to Y\textsubscript{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$_2$O 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$_A$-binding site.

Jordan et al. (1983) hypothesized that PLA$_2$ treatment of thylakoids led to inhibition of the charge stabilization step: P680*'Ph*Q$_A$ $\cdot$ P680*'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$_{680}^*$ 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 µs to $\sim$1 ns (Trissl and Lavegnne 1995). That is, on closing a PSII reaction centre, charge recombination directly to the ground state would occur well before any P$_{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 Rawyler 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 LHCl (Liu et al. 2004; Standfuss et al. 2005). X-ray structure of a cyanobacterial PSI dimer has so far revealed only one PG molecule per PSI 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 PSI dimers that do not possess the complete LHCII antenna, (b) PSI dimers into monomers, and (c) LHCII trimers into monomers (Fig. 6). This is consistent with the role of PG in the formation of spinach PSI core dimers in vitro (Kruse et al. 2000), the finding in Chlamydomonas reinhardtii that PG deficiency impairs the assembly of dimeric PSI core complexes containing their associated antenna (Pineau et al. 2004), and the impairment of dimerization of PSI 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 ppg1 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 SODG and only one PG molecules, along with four DGDG and six MGDG molecules (Loll et al. 2005). The negatively charged headgroups of PG and SODG were all located at the membrane surface on the acceptor side. The protein environment of the QA, QB and the non-heme Fe(III) domain opens into a large cavity whose walls are lipophilic, thereby providing a flexible lipophilic environment for PQ/PQH2 shuttling between the QB-bonding 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 transfer at the donor and acceptor sides of PSII complex. It is probable, however, that the fragmented electron density in the large cavity at the QB-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 phospholipid 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 LHCCI 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.

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