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THE ROLE OF PHOSPHOLIPIDS IN THE MOLECULAR ORGANISATION OF PEA CHLOROPLAST MEMBRANES

EFFECT OF PHOSPHOLIPID DEPLETION ON PHOTOSYNTHETIC ACTIVITIES

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Pea chloroplasts were treated with phospholipase A_2 which hydrolysed approx. 75% phosphatidylglycerol and 60% phosphatidylcholine. The major effect of the treatment was an inhibition of Photosystem (PS) II electron transport together with an (approx. 30%) increase of initial chlorophyll fluorescence (F_0) and a subsequent loss of variable fluorescence during induction, as well as an inhibition of the cation-induced rise in steady-state chlorophyll fluorescence. In contrast to the effects upon PS II activities, PS I activity was not depressed and increased slightly under certain conditions, while the coupling factor for photophosphorylation was inhibited to some extent. No significant increase in spillover was observed following the treatment with phospholipase A_2. These results are discussed in relation to the ways in which phospholipid depletion may lead to the various effects observed. It is proposed that the site of PS II inhibition after phospholipase A_2 treatment may be at the electron transfer from pheophytin to Q, the first quinone-type electron acceptor.

Introduction

It is well established that the lipid composition of thylakoid membranes differs markedly from that of other cellular membranes [1]. Because of this characteristic composition it is envisaged that these lipids have an important function in the complex organisation of the membrane and consequently in the efficiency of the photosynthetic apparatus. One potentially significant feature is that the predominant phospholipid is the anionic lipid PG rather than the more usual phospholipids, PC and phosphatidylethanolamine; this phospholipid also contains the unique fatty acid, *trans*-3-hexadecenoic acid. There is considerable speculation regarding the function of this molecule in photosynthetic activities and in the structural organisation of photosynthetic membranes [2–5]. Depletion of 82% of the total phospholipids of spinach chloroplasts by phospholipase A_2 caused a significant reduction in cyclic photophosphorylation, while retaining the major proportion of other photoactivities [2]. In contrast, Rawyler and Siegenthaler [3] observed a marked inhibition of PS II electron transport and stimulation of PS I activity in spinach using a variety of acyl hydro-

Abbreviations: Chl, chlorophyll; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCIP, 2,6-dichlorophenolindophenol; DGDG, digalactosyldiacylglycerol; Hepes, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulphonic acid; LHCP, light-harvesting Chl *a*/*b*-protein complex; MGDG, monogalactosyldiacylglycerol; PC, phosphatidylcholine; PD_{ox}, oxidised *p*-phenylenediamine; PG, phosphatidylglycerol; PS, photosystem; Q, primary electron acceptor of PS II; Tricine, *N*-tris(hydroxymethyl)methylglycine.

lases differing in substrate specificity. They concluded that the phospholipids were arranged asymmetrically across the membranes and the presence of an intact pool of PC in the inner layer is required for PS II activity. Furthermore, they considered PG to be preferentially associated with PS I electron flow and this activity required both an inner and outer pool of PG. However, using pea chloroplasts, PG depletion by phospholipase A_2 resulted in a decrease in the efficiency of light collection and in the rate of plastoquinone reduction [4]; it was suggested that PG was associated with the oligomeric forms of LHCP [6–8]. The LHCPs are principally located in the appressed regions of the granal lamellae where they transfer excitation energy predominantly to PS II [9,10] and are also thought to play a part in the formation of granal stacks. If PG is associated with LHCPs it seems likely that its absence or removal would affect the molecular organisation of the thylakoids. The role of PG in granal stacking, however, still remains an area of considerable controversy. For example, a barley mutant has been shown to have normal amounts of *trans*-3-hexadecenoic acid esterified in PG, though it is devoid of Chl *b* and granal organisation [5].

In this work we have investigated the effect of phospholipid depletion on the structural organisation of chloroplast thylakoid membranes and also upon various photosynthetic activities in an attempt to determine the exact nature of phospholipid involvement and to resolve some of the apparent contradictions.

Materials and Methods

Chloroplast preparation. Pea seedlings (*Pisum sativum* L. var. Feltham First) were grown for 2–3 weeks at 20°C; the irradiance was 22 $W \cdot m^{-2}$ with a 12 h light/12 h dark cycle. Chloroplasts were isolated from 30 g of leaf tissue by homogenising for 10–20 s in 20 mM Tricine-KOH buffer (pH 8.0) containing 0.33 M sorbitol, 0.5% bovine serum albumin, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 1% polyvinylpyrrolidone and 2 mM sodium isoascorbate. The homogenate was filtered twice through miracloth and the filtrate centrifuged at $1000 \times g$ for 1 min. The intact chloroplasts were ruptured by resuspending the pellet in a 10-fold dilution of the homogenising medium and the membranes re-isolated at $2250 \times g$ for 10 min. The supernatant was discarded and the pellet resuspended in 1–2 ml of 10 mM Tricine-KOH buffer (pH 8.0) containing 0.33 M sorbitol. Stacked or unstacked thylakoids were obtained by the addition of 5 mM $MgCl_2$ or 0.5 mM EDTA, respectively, to the resuspension buffer. Total chlorophyll concentration and Chl *a*/*b* ratios were determined by the method of Arnon [11].

Enzyme treatment. Stacked thylakoids were incubated in the dark at 20°C for 20 min with phospholipase A_2. Phospholipase A_2 was routinely used at a concentration of 10 Sigma U/mg Chl, although concentrations of up to 31 Sigma U/mg Chl were tested. No exogenous Ca^{2+} was added, but sufficient enzyme activity was obtained.

Lipid analysis. Total lipids were extracted by the method of Garbus et al. [12] and separated on ammonium sulphate (0.15 M) impregnated silica gel G plates in a solvent system of acetone/hexane/H_2O (91 : 30 : 8, v/v). The lipid bands were visualized by spraying with 0.05% (w/v) cresyl violet acetate in methanol. Lipids were identified by co-chromatography with known standards and differential colour reagents. Fatty acid methyl esters were prepared by transmethylation of the lipid samples at 75°C for 30 min in 14% (w/v) boron trifluoride/methanol. After extraction in petroleum ether (b.p. 40–60°C) they were separated in 15% (w/w) diethylene glycol succinate on Chromosorb W. AW (80/100 mesh) or in 15% (w/w) EGSS-x on Supelcoport (80/100 mesh) using a Pye 204 gas chromatograph. Quantitation was made with an internal standard of methyl pentadecanoate.

Chlorophyll fluorescence. Chlorophyll fluorescence measurements were made using either a Perkin Elmer Model 1000 fluorescence spectrophotometer or a combination of a red-sensitive photomultiplier, an electronic shutter and a Data Lab transient recorder DL902 at a chlorophyll concentration of 10 μg per ml of unstacking medium containing 5 mM Hepes (pH 7.6), 0.33 M sorbitol and 10 mM KCl, 33 μM DCMU being added as required. Chlorophyll fluorescence was excited with blue-green light (390–550 nm) transmitted by a Corning 4-96 filter; the emission was measured at 685 nm. Salt-induced increase of chlo-

rophyll fluorescence was observed by the addition of 5 mM $MgCl_2$.

Digitonin treatment. Stacked chloroplasts were prepared and treated with phospholipase A_2. The chloroplasts were diluted in stacking or unstacking (+0.5 mM EDTA) resuspension buffer as appropriate to give a concentration of 400 μg Chl/ml. 0.33 ml of 2% digitonin per ml chloroplast suspension was added and the samples kept on ice for 30 min. Each sample was diluted 20-fold with resuspension buffer and centrifuged at $10000 \times g$ for 30 min. Chl *a/b* ratios were determined on the pellets.

90° light scattering. Chloroplasts were added to 0.1 mM Hepes buffer (pH 7.5), 0.33 M sorbitol and 33 μM DCMU to give a final concentration of 5 μg Chl/ml. Scattered light was measured at 520 nm over a 0.1–100 mM KCl concentration range using a Perkin Elmer fluorescence spectrophotometer.

Electron transport. Oxygen evolution or consumption rates were measured at 20°C using a Clark-type O_2 electrode (Hansatech Ltd, Kings Lynn, U.K.). The media are given in the table and figure legends.

9-Aminoacridine fluorescence. Chloroplasts were incubated in the presence or absence of phospholipase A_2, in the stacked state (20 min at 20°C) They were then diluted into a medium containing 100 mM sorbitol, 1 mM KOH, 1.5 mM Hepes (pH 7.5), 10 μM DCMU, 20 μM 9-aminoacridine and 30 μM EDTA to complex divalent cations present in the mixture. The level of 9-aminoacridine fluorescence (f_{min}) was measured with a Perkin Elmer 1000 fluorescence spectrophotometer (excitation 381 nm, emission 481 nm), and was normalized with respect to the maximum fluorescence (f_{max}) observed on adding 20 mM $MgCl_2$.

Chemicals. Phospholipase A_2 from porcine pancreas and snake was purchased from Sigma Chemical. Co. (Poole, Dorset, U.K.). Lipids were purchased from Lipid Products (Redhill, Surrey, U.K.).

Results

Lipid composition of pea thylakoids and the effect of phospholipase A_2 treatment

Table I illustrates the typical lipid composition of the membranes used in these experiments. Glycolipids are the major lipid components (MGDG, DGDG and sulpholipid) with PG (9% of the total) the most abundant phospholipid present. The galactolipids (MGDG and DGDG) comprise over 70% of the total acyl lipids and contain linolenic acid as the predominant fatty acid. In contrast, PG contains far less polyunsaturated fatty acids and significantly higher levels of palmitic acid. PG is also the only lipid to contain *trans*-3-hexadecenoic acid.

Treatment of these membranes with phospholipase A_2 leads to a rapid and extensive reduction in the amounts of phospholipid present, with PG being subject to the largest loss (Table II). The preference of pancreatic phospholipase A_2 for anionic lipids as substrate may account for the

TABLE I

MAJOR ACYL LIPIDS OF PEA CHLOROPLAST MEMBRANES

Methods of analysis are given in Materials and Methods. Mean ± S.E. (data from three separate experiments and $n = 5$ unless stated otherwise in parentheses). n.d., Not detected; tr., trace (less than 0.5%). $C_{16:0}$, palmitic acid; $C_{16:1}$, *trans*-3-hexadecenoic acid; $C_{18:0}$, stearic acid; $C_{18:1}$, oleic acid; $C_{18:2}$, linoleic acid; $C_{18:3}$, linolenic acid.

Lipid	% total lipid	Fatty acid distribution (% of total)					
		$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$
MGDG	45.9 ± 1.4	2.7 ± 0.2	n.d.	0.8 ± 0.2	0.6 ± 0.2	7.0 ± 0.4	88.9 ± 0.9
DGDG	28.9 ± 2.1	8.2 ± 0.5	n.d.	3.1 ± 0.3	tr.	3.6 ± 1.7	84.8 ± 1.1
Sulpholipid	13.4 ± 0.9	22.6 ± 0.8	n.d.	5.1 ± 2.3	tr.	8.2 ± 0.7	63.7 ± 2.4
PG	8.8 ± 0.5	38.1 ± 1.4	11.5 ± 1.3	3.2 ± 1.7	4.9 ± 1.3	14.8 ± 3.6	27.4 ± 1.3
PC	3.0 ± 0.5	25.6 ± 2.4	n.d.	10.7 ± 0.7	4.1 ± 2.5	29.1 ± 5.0	30.6 ± 3.7
		(4)	(4)	(4)	(4)	(4)	(4)

TABLE II

THE EFFECT OF PHOSPHOLIPASE A_2 ON ACYL LIPIDS OF PEA THYLAKOIDS

Stacked thylakoids were incubated in the dark at 20°C for 20 min in the presence or absence of phospholipase A_2. 10 Sigma U pancreatic phospholipase A_2/mg Chl (19 Sigma U for sulpholipid data). Values represent mean ± S.E. from two separate experiments done in duplicate.

Chloroplast acyl lipid	% control
MGDG	96.2 ± 5.5
DGDG	94.8 ± 6.5
PG	23.6 ± 1.4
PC	37.5 ± 6.7
Sulpholipid	99.4 ± 6.0

different extents of hydrolysis of PG and PC, as could asymmetric phospholipid distribution. The major acyl lipid components do not appear to be hydrolysed to any extent, even at higher phospholipase A_2 concentrations (data not shown). These data are consistent with previous findings [3,4]. We conclude that the subsequent data obtained on electron transport and molecular organisation after phospholipase A_2 treatment are caused by the specific hydrolysis of phospholipids and not by glycolipid break-down or fatty acid release (in agreement with and discussed in Ref. 4).

Electron transport and photophosphorylation

The activity of a number of thylakoid membrane electron-transport systems were studied after treatment with phospholipase A_2. In nine separate experiments, electron transport was measured and the data in Table III and Fig. 1 clearly illustrate the characteristic modification of PS II and PS I activities by pancreatic phospholipase A_2. The consistent finding was the inhibition of electron transport that required PS II. In contrast, PS I activities were found to be constant or in some experiments stimulated slightly. These changes were found in both coupled and uncoupled (i.e., in the presence of 2 mM NH_4Cl) thylakoids. These data are consistent with the results of Rawyler and Siegenthaler [3,13] for spinach thylakoids treated with pancreatic phospholipase A_2, though they differ from those of Duval et al. [4] and Hirayama

TABLE III

EFFECTS OF PHOSPHOLIPASE A_2 ON ELECTRON TRANSPORT AND PHOTOPHOSPHORYLATION

Isolated chloroplasts were treated as described in the text. The basic medium for the assays contained: 330 mM sorbitol, 3 mM $MgCl_2$, 10 mM KCl, 5 mM K_2HPO_4 (pH 8.0). It was supplemented by (a) 0.1 mM methyl viologen (MV), 1 mM NaN_3, 2 mM NH_4Cl; (b) 50 μM PD_{ox}, 1 mM $K_3Fe(CN)_6$, 2 mM NH_4Cl; (c) 0.1 mM methyl viologen, 1 mM NaN_3, 50 μM DCIP, 5 μM DCMU, 1 mM sodium isoascorbate, 2 mM NH_4Cl; (i) 0.1 mM methyl viologen, 1 mM NaN_3, 1 mM ADP and (ii) 50 μM phenazine methosulphate (PMS), 1 mM ADP. Temperature 20°C, irradiance 300 $W \cdot m^{-2}$ (600–700 nm) and chlorophyll concentration approx. 20 μg/ml. The numbers in brackets indicate % of control. In item ii, Expt. Nos. 1–3, the phospholipase/Chl ratios were 9, 13 and 31 Sigma U/mg Chl, respectively. Values are expressed as μmol O_2/h per mg Chl.

	Expt.	Control	Pancreatic phospholipase
Electron transport			
(a) $H_2O \rightarrow MV$	A	115.2	62.8 (54.5)
(PS I + II)	B	220	126 (57.3)
	C	93	57.4 (62.0)
(b) $H_2O \rightarrow PD_{ox}$	A	136.2	62.8 (46.1)
(PS II)	B	248	114.0 (46.0)
	C	149	64.6 (43.0)
(c) $DCIPH_2 \rightarrow MV$	A	127.8	136.2 (106.7)
(PS I)	B	209.0	211.0 (101)
	C	287.0	284 (99.0)
Photophosphorylation			
(i) MV-catalysed	1	269	94.4 (35.1)
(non-cyclic)	2	212	29.9 (14.1)
(ii) PMS-catalysed	1	437	322 (73.6)
(cyclic)	2	565	274 (48.5)
	3	310	0 (0)

and Nomotobori [2] who found little loss of electron-transport function using snake venom phospholipase A_2 on pea and spinach thylakoids, respectively. In our experiments the modification of electron transport by snake venom phospholipase A_2 was similar to that obtained with pancreatic phospholipase A_2 (data not shown). Duval et al. [4] did find some inhibition of PS II activities at low irradiances (less than 50 $W \cdot m^{-2}$) and suggested that this implied a loss of light-gathering capacity rather than an effect upon the reaction centres themselves. Fig. 2A and B shows the influence of different irradiances upon both PS II

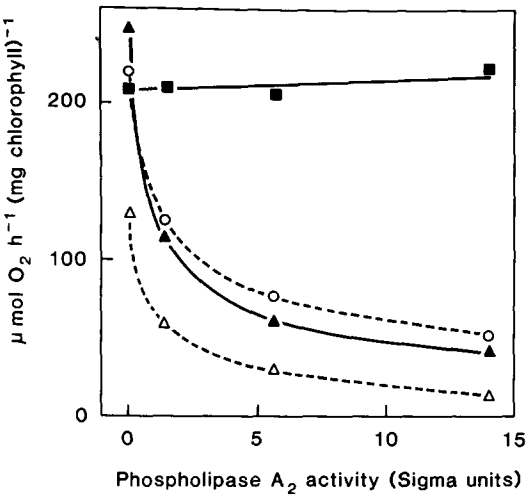

Fig. 1. The effect of phospholipase A_2 concentration on electron-transport rates. Media are the same as in Table II, except that 5 mM Hepes (pH 7.8) was also added. For the electron-transport system $H_2O \rightarrow$ DCIP, DCIP concentration was 250 μM. $H_2O \rightarrow$ methyl viologen and $DCIPH_2 \rightarrow$ methyl viologen were measured by O_2 consumption. $H_2O \rightarrow PD_{ox}$ and $H_2O \rightarrow$ DCIP were measured by O_2 evolution. Electron-transport activities measured from $H_2O \rightarrow$ methyl viologen (○), $H_2O \rightarrow PD_{ox}$ (▲), $DCIPH_2 \rightarrow$ methyl viologen (■), and $H_2O \rightarrow$ DCIP (△).

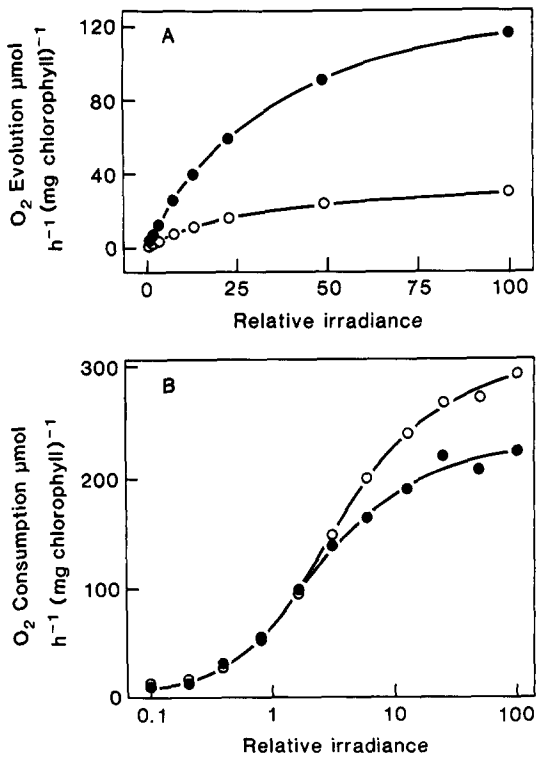

Fig. 2. The effect of phospholipase A_2 treatment on PS II (A) and PS I (B) activities at different irradiances. Control and phospholipase A_2-treated chloroplasts were suspended in media given in Table II. Maximum irradiance (blue-green light, 390–550 nm) was about 600 $W \cdot m^{-2}$. Temperature 20°C. Chlorophyll concentration 20 μg/ml. Electron-transport activities measured (A) from $H_2O \rightarrow PD_{ox}$ and (B) from $DCIPH_2 \rightarrow$ methyl viologen. Control (●) and phospholipase A_2-treated (○) membranes.

and PS I activities of phospholipase A_2-treated samples. Inhibition of PS II was found at all irradiances used, while the effect on PS I activity was different under low and high light conditions. The lack of any phospholipase A_2 effect on PS I activity at low light intensities indicates that there was no change in spillover between the two photosystems, whereas the stimulation of PS I activity at high light levels may be explained by increased rates of diffusion or accessibility of the donors to feed electrons into PS I (Ref. 13 and references cited therein).

Table III indicates that in addition to the loss of electron-transport functions, cyclic and non-cyclic photophosphorylation activities were inhibited. Because PS I electron transport was not inhibited it is probable that the inhibition of cyclic photophosphorylation occurred as a result of the coupling factor being significantly perturbed by this treatment.

Structural organisation

The electron-transport studies show that the major deleterious effect of phospholipid depletion is associated with PS II activity. It is therefore conceivable that a specific association of phospholipid and a chlorophyll-protein complex (cf. Introduction) has been affected. To monitor changes brought about in the molecular organisation of the thylakoid by the phospholipase A_2 treatment, a number of different experimental procedures were used.

Chlorophyll fluorescence. (A) Chlorophyll fluorescence induction: The molecular organisation and energy transfer in the proximity of PS II have frequently been studied by following the chlorophyll fluorescence at room temperature; Fig. 3A illustrates the effect of phospholipase A_2 treatment on fluorescence induction. The F_0 level (Q

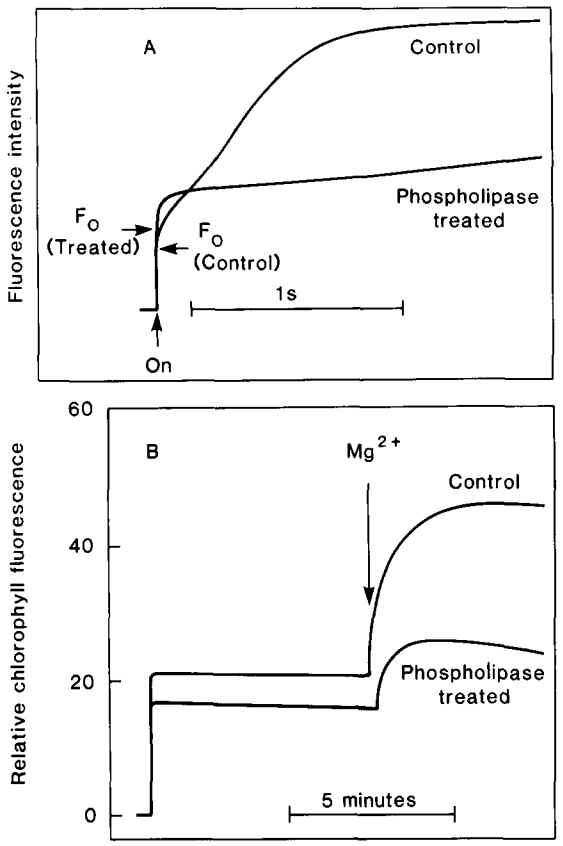

Fig. 3. (A) Chlorophyll fluorescence induction curves for control and phospholipase A_2-treated chloroplasts. Chloroplasts were suspended in a medium containing 330 mM sorbitol, 3 mM $MgCl_2$, 10 mM KCl, 5 mM K_2HPO_4, 5 mM Hepes (pH 8.0). Excitation light at 30 $W \cdot m^{-2}$ was transmitted by a Corning 4-96 blue filter and by opening an electronic shutter. Fluorescence emission selected by a Balzers B-40 686 nm interference filter was recorded with a Datalab DL 902 transient recorder. F_0 was taken to be the fluorescence level at completion of the opening of the electronic shutter (approx. 2 ms from the start of irradiation). (B) The effect of phospholipase A_2 on salt-induced chlorophyll fluorescence increase. Chloroplasts were suspended in an unstacking medium of 5 mM Hepes (pH 7.6), 330 mM sorbitol and 10 mM KCl. $MgCl_2$ was added to a final concentration of 5 mM as required. Excitation light was transmitted by a Corning 4-96 blue-green filter and fluorescence emission measured at 685 nm using a Perkin Elmer Model 1000 fluorescence spectrophotometer.

fully oxidised) shows a small increase, which was about 30% over a range of excitation light intensities. Furthermore, there was a considerable loss in variable fluorescence above the F_0 level. This result is essentially in agreement with those of Duval et al. [4], although they found no change in the F_0 value.

(B) Salt-induced chlorophyll fluorescence increase: Fig. 3B illustrates the effect of phospholipid depletion on the salt-induced rise of steady-state chlorophyll fluorescence. Under carefully controlled ionic concentrations there is a correlation between the increase in chlorophyll fluorescence on addition of divalent ions and the increase in granal stacking of the thylakoids [14,15]. In the control sample a typical rise in chlorophyll fluorescence was detected (routinely between 80 and 100%) on addition of Mg^{2+}. The phospholipase A_2-treated samples exhibited a steady-state fluorescence level which was lower than that of controls, and displayed a much reduced ability to increase fluorescence on addition of Mg^{2+}. Both effects may be related to the loss of variable fluorescence described above.

(C) Chlorophyll fluorescence at 77 K: At liquid nitrogen temperature, the chlorophyll fluorescence emission at 735 and 685 nm is thought to originate from PS I and PS II antennae, respectively (see Ref. 16). Therefore, the fluorescence ratio F_{735}/F_{685} should provide a measure of the relative excitation of the two photosystems. Table IV(a) shows that this ratio (uncorrected for instrument response) was similar for control and treated chloroplasts suspended in a medium containing $MgCl_2$, but differed greatly from that of unstacked chloroplasts in the absence of $MgCl_2$. This result provides evidence that there is no increase in spillover of excitation energy from PS II to PS I after the phospholipase A_2 treatment.

Digitonin treatment and packed chloroplast volume. As shown in Table IV(b), unstacked control chloroplasts ($-MgCl_2$, +0.5 mM EDTA) were readily disrupted by digitonin, resulting in a small amount of chlorophyll in the pellet with a Chl *a*/Chl *b* ratio (2.69) that was close to that of whole chloroplasts (2.82). Stacked control chloroplasts yielded a large pellet after digitonin treatment, with a Chl *a*/Chl *b* ratio of 2.26 which is characteristic of granal stacks. When phospholipase A_2-treated chloroplasts were suspended in a medium containing 3 mM $MgCl_2$, and treated with digitonin, the resultant pellet was somewhat smaller than that of control chloroplasts, but the Chl *a*/Chl *b* ratio was similar or even slightly

TABLE IV

MONITORING OF THE STRUCTURAL ORGANISATION OF CHLOROPLAST THYLAKOIDS

(a) Chlorophyll fluorescence emission spectra were obtained at 77 K using a Perkin Elmer 1000 fluorescence spectrophotometer. Excitation was by broad-band blue-green light. The ratios of uncorrected emission at 735 and 685 nm are indicated. (b) Chloroplasts were treated with digitonin at 0°C and the mixture was centrifuged, as described in the text. (c) Packed chloroplast volume was determined by centrifugation in a Beckman Microfuge B for 2 min, followed by calibration of the volume of the pellet. In a–c, control or enzyme-treated chloroplasts were monitored, in the presence of 3 mM $MgCl_2$ where indicated. (d) Normalized 9-aminoacridine fluorescence levels in suspensions of chloroplasts that had been incubated in the presence or absence of phospholipase A_2, and in either the stacked or unstacked state. Standard errors are shown, with the number of replicates indicated in brackets.

	Control $-MgCl_2$	Control $+MgCl_2$	Phospholipase A_2 $+MgCl_2$
(a) Chlorophyll fluorescence (77 K)			
F_{735}/F_{685}	8.55 ± 0.32 (3)	1.86 ± 0.04 (3)	2.42 ± 0.06 (3)
(b) Digitonin treatment			
% Chl in pellet	7.3	49.8	25.4
Chl *a*/Chl *b* in pellet	2.69	2.26	1.93
(c) Packed volume			
(μl/mg Chl)	112 ± 2 (4)	61 ± 1 (4)	65 ± 1 (4)
	Control unstacked	Control stacked	Phospholipase A_2 stacked
(d) 9-Aminoacridine fluorescence (f_{min}/f_{max})	0.619 ± 0.006 (3)	0.581 ± 0.03 (3)	0.614 ± 0.011 (3)

lower (1.93). This suggests that phospholipase A_2-treated chloroplasts, although more susceptible to digitonin attack, seem to maintain their Chl *b*-enriched granal stacks in the particular medium; there does not appear to be any extensive randomization of the supramolecular complexes (as in unstacked chloroplasts) in the plane of the membrane after phospholipid depletion. Consistent with this observation is the finding that the packed volumes of phospholipase A_2-treated and control chloroplasts in a medium containing $MgCl_2$ were similar, and were considerably smaller than that of unstacked chloroplasts ($-MgCl_2$), as shown in Table IV(c).

9-Aminoacridine fluorescence quenching. The mechanisms that govern the differentiation of thylakoid lamellae into the granal and the stromal phases have been extensively investigated [17–22]. It has been proposed that the aggregation of thylakoids into grana is initiated by the segregation of components in the plane of the membranes, leading to a lateral heterogeneous distribution [23,24] so that stacking occurs at regions of low Coulombic repulsion and high Van der Waals attraction. If the phospholipase A_2 treatment exposes any additional charged groups on the membrane components to the aqueous phase, changes in membrane charge density and Coulombic repulsion would be expected. The quenching of 9-aminoacridine fluorescence by negatively charged surfaces has been used to monitor their surface charge density [25,26] and it can be seen that thylakoids previously incubated with phospholipase A_2 showed a slight increase in the 9-aminoacridine fluorescence, corresponding to less quenching (Table IV(d)). This suggests that the surface charge per unit area of membrane was, if at all, decreased slightly after the treatment.

90° light scattering. The intensity of 90° light scattering has been used as an indicator of grana formation or membrane appression [27–29]. Fig. 4 shows the relative light scattering of control and phospholipase A_2-treated samples as a function of monovalent cation concentration. At low concentrations where stacked chloroplasts would remain stacked [14,30–32], the signal was diminished in the treated samples. This may reflect a redistribution of membrane components during

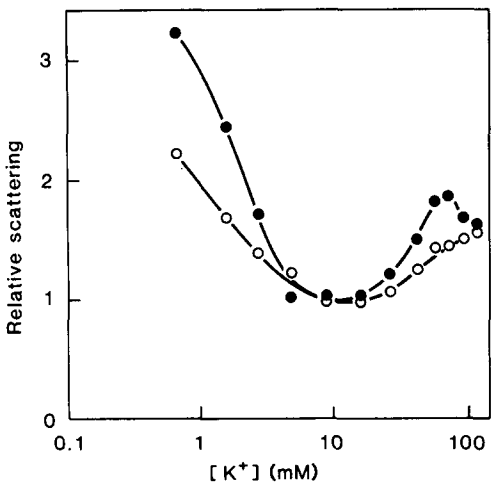

Fig. 4. The intensity of light scattering at 520 nm for control and phospholipase A_2-treated chloroplasts as a function of K^+ concentration. Incident light was transmitted by a Corning 4-96 filter (about 4 $W \cdot m^{-2}$). Scattered light was measured at 520 nm. The medium contained 33 μM DCMU, 0.1 mM Hepes (pH 7.5), 25 μM carried-over $MgCl_2$, and KCl and sorbitol to maintain the total osmolarity at 0.33 osM and to give the required K^+ concentration. Control (●) and phospholipase A_2-treated (○) membranes, the chlorophyll concentration being 5 μg/ml in both cases.

the incubation with phospholipase A_2 or it could related to a swelling of the membranes. At a monovalent ion concentration (about 10 mM) that is known to bring about unstacking [14,30–32], treated and control samples fell to the same value. As the concentration was raised above 10 mM the normal response, indicative of restacking, was somewhat diminished in the treated samples.

Discussion

The results of treating chloroplasts with phospholipase A_2 under our conditions suggest the following conclusions: (i) there is a specific release of fatty acids from a large percentage of the phospholipids, but not from the glycolipids; (ii) PS II is inhibited in agreement with Ref. 3 and the inhibition takes place at all irradiances in contrast to Ref. 4. There is also an associated loss of variable fluorescence and an inhibition of the cation-induced rise in chlorophyll fluorescence; (iii) there is no significant increase in spillover of excitation energy from PS II to PS I, whether measured by chlorophyll fluorescence spectra at 77 K or by the rate of PS I electron transport under light-limiting conditions; (iv) no unstacking occurs in such a way that the photosystems are randomized in the plane of the thylakoid membrane, or that the packed chloroplast volume is substantially increased, although the treated chloroplasts exhibited different light-scattering properties and lower resistance to disruption by digitonin, properties which are usually reminiscent of unstacked membranes; (v) the data suggest that the coupling factor for ATP formation is affected.

It has been suggested that there is a tight coupling between *trans*-3-hexadecenoic acid-esterified PG and oligomeric LHCP, and that this may be correlated with a better efficiency of light collection and energy distribution between the two photosystems in illuminated seedlings of *Picea abies* [8]. An association between *trans*-3-hexadecenoic acid-esterified PG and oligomeric LHCP seems to be substantiated by the findings that (i) treatment of thylakoids with phospholipase A_2 at 22°C for 2 h led to the disappearance of oligomeric LHCP and (ii) oligomeric LHCP could be reconstituted from the monomer with PG or, to a lesser extent, with other thylakoid lipids [7]. Therefore it is surprising that, in the present study, phospholipase A_2 treatment (20 min at 20°C) did not lead to any significant change in spillover. Possibly, the treatment adopted was not severe enough to dissociate oligomeric LHCP, although it effectively led to PS II inhibition. Certainly, no dissociation of LHCP oligomers could be detected under the conditions of our experimental procedure (Burton, K.S., personal communication). Consequently, it does not appear that a specific phospholipid association with LHCPs contributes to the results of this study.

After phospholipase A_2 treatment of chloroplasts and subsequent disruption by digitonin, the resultant pellet was enriched in Chl *b*, indicating the absence of randomization of PS II (with Chl *b*-rich LHCP) and PS I. This is consistent with there being no increase in spillover, for randomization of PS II and PS I in the plane of the membrane (as in unstacked chloroplasts) would give rise to a smaller average separation between them and hence better excitation energy transfer to PS I [15,24]. The present results argue against any

phospholipase A_2-induced randomization of the photosystems. Indeed, the finding that the quenching of 9-aminoacridine fluorescence [26] was not increased by phospholipase A_2 (Table IV(d)) also rules out any possibility that unstacking could have resulted from the exposure of negative charges on the thylakoid membranes. The different light-scattering properties of the treated chloroplasts and the lower resistance to disruption by digitonin, although reminiscent of unstacked membranes, were probably associated with some localized swelling of membranes, or instability of the treated membranes; it is possible that phospholipid depletion renders the membranes less stable, particularly if depletion occurs predominantly at one monolayer.

The major effect of phospholipase A_2 treatment of chloroplasts in this study was the inhibition of PS II, in agreement with Ref. 3, together with a loss of variable chlorophyll fluorescence. It has been suggested that the site of PS II inhibition is located somewhere between the electron entry point from 1,5-diphenylcarbazide and the plastoquinone pool [3]. We propose that phospholipase A_2 treatment inhibits the electron-transfer step:

$$P^+\text{-}680Ph^-Q \rightarrow P^+\text{-}680PhQ^-$$

where P-680 is the PS II reaction centre Chl *a*, Ph (pheophytin) the intermediary electron acceptor, and Q the first quinone-type PS II acceptor. Inhibition of electron transfer from pheophytin$^-$ to Q would favour charge recombination between P^+-680 and pheophytin$^-$ (see Ref. 33), resulting in enhanced chlorophyll fluorescence emission; the higher initial level of fluorescence in the induction curves of Fig. 3A for the treated chloroplasts may be a result of enhanced charge recombination after inhibition of forward electron transfer. In the subsequent phase of the induction curves, it is expected that P^+-680 would be reduced by (slower) electron donation from H_2O to yield the state P-680Ph$^-$Q, or P*-680Ph$^-$Q when P-680 is excited. A state with pheophytin$^-$ would quench chlorophyll fluorescence by energy transfer from excited chlorophyll molecules to pheophytin$^-$ [34] and this may explain the loss of variable fluorescence (Fig. 3).

It may be of interest to speculate on the mechanism by which phospholipid depletion could lead to inhibition of electron transfer from pheophytin$^-$ to Q. From an exchange interaction between pheophytin$^-$ and Q^-, it is inferred that the distance between pheophytin and Q is very short [35]; a short distance is probably essential for the rapid localization of the electron at Q (with a mid-point potential 0.5 V higher than that of pheophytin) after charge separation, so as to stabilize the separated charges against recombination. It is conceivable that removal of phospholipid could have significantly altered the relative distance/orientation between pheophytin and Q, thus affecting the rate of forward electron transfer.

Recent experiments have shown that PS II centres are almost exclusively located in the appressed membranes of granal stacks [36]. In view of our finding that the major effect of phospholipase A_2 treatment is on PS II, it follows that the enzyme has ready access into the 'partition gap' of appressed membranes. Whilst chymotrypsin with a molecular weight of 25 300 does not readily penetrate the partition gap [37], pancreatic phospholipase A_2 with a molecular weight of approx. 14 000 may be sufficiently small to do so. The three dimensional structure of pancreatic phospholipase A_2 is that of a flattened cylinder ($2.2 \times 3.0 \times 4.2$ nm), held tightly together by a high content of disulphide linkages [38]. These dimensions would allow passage of the molecule between appressed thylakoid membranes separated by an estimated 4.0 nm [39]. Because PS II is primarily located in the granal regions of the thylakoid and as PS II is most strongly inhibited by phospholipid depletion, the implication is that an important phospholipid pool resides in this region of the membrane. Our data do not rule out the possibility that PC could be more important than PG in this context [3,13], although the small percentage of PC present suggests PG depletion is the more likely cause of the deleterious effects on PS II-associated processes (electron transport and chlorophyll fluorescence). We are unable to comment on the asymmetrical arrangement of the phospholipids across the thylakoid bilayer as has been described recently [3,40]. However, the inhibition of the coupling factor, which is thought to be located primarily in non-appressed membranes, certainly implies other important functions for phospholipids in thylakoid organisation.

Finally, it was noted that phospholipase A_2 treatment of chloroplasts sometimes enhanced PS I activity, in agreement with Refs. 3 and 13. This enhancement occurred only at a relatively high irradiance (Fig. 2B), and is consistent with the suggestion [13] that the accessibility of electron donation could be a limiting step at high irradiances.

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