ELSEVIER

Biochimica et Biophysica Acta 1188 (1994) 380–390

BBA Biochimica et Biophysica Acta

On the question of lateral migration of LHC II upon thylakoid protein phosphorylation in isolated pea chloroplasts: the stroma lamellar fraction separated from phosphorylated chloroplasts is not homogeneous

John H. Georgakopoulos, Joan H. Argyroudi-Akoyunoglou *

Institute of Biology, NCSR 'Demokritos', Aghia Paraskevi, Athens, Greece

Received 23 February 1994

Abstract

The stroma lamellar fraction (50000 × *g* supernatant) obtained by differential centrifugation from French press-disrupted chloroplasts after phosphorylation, found to be enriched in LHC II components, can be separated into an upper, LHC II-poor, and a lower, LHC II-rich, thylakoid membrane zone, by sucrose density gradient ultracentrifugation after Mg^{2+} addition. The LHC II-rich zone contains cyt *f* and peripherally bound CF1 in amount lower than the upper zone, 33 kDa PSII polypeptide in amount higher than the upper zone, and CP I in amount higher than grana membranes. The LHC II-poor zone exhibits more or less the characteristics of stroma lamellae obtained from non-phosphorylated chloroplasts. French-press disruption of isolated grana, incubated in the resuspension buffer used for phosphorylation, releases a 'light' thylakoid membrane fraction, rich in LHC II, which behaves on gradient ultracentrifugation like the LHC II-rich fragments separated from phosphorylated stroma lamellae. The results suggest that the enrichment in LHC II components of stroma lamellar fractions, obtained by differential centrifugation of French press or digitonin disrupted chloroplasts, observed after thylakoid protein phosphorylation, reflects contamination of the stroma lamellar fraction by destacked granal membranes.

Keywords: Thylakoid; Phosphorylation; Light-harvesting complex; Grana stacking/unstacking; Lateral migration

1. Introduction

The lateral heterogeneity of grana and stroma lamellae in chloroplasts is a general finding beyond doubt (Photosystem I is localized in stroma lamellae and peripheral membranes of grana; Photosystem II is localized mainly in grana appressions).

Thylakoid proteins are phosphorylated in the light by a redox regulated kinase(s), and dephosphorylated in the dark by a light-activated phosphatase [1–5]. Addition of ATP to illuminated chloroplasts results mainly in phosphorylation of the Photosystem II (PS II) proteins. This is accompanied by an enhancement of the 77 K fluorescence emission ratio F730/F685 [6,7]; on the other hand, the stroma lamellar fraction isolated by differential centrifugation from such chloroplasts, after disruption with French press or digitonin, is found to be enriched in LHC II [8]. These results are generaly considered to reflect the lateral migration of 'mobile' phospho-LHC II from grana, where it serves PS II, to stroma lamellae, where it serves PS I, upon phosphorylation-induced grana destacking, and its reversal upon dephosphorylation [9]. Such a 'lateral movement' hypothesis can explain the State transitions observed in chloroplasts under selective overexcitation of PS II or PS I [10]. Support to this hypothesis is considered to be the appearance of an 8 nm particle in stroma lamellae after phosphorylation, thought to represent the 'mobile'-phospho-LHC II [9]; recent immuno-electron microscopy studies also suggest that upon thylakoid protein phosphorylation in *Chlamydomonas*, the cyt b_6/f complex follows phospho-LHC II in this migration [11].

However, we have recently found that the stroma lamellar fraction isolated from phosphorylated pea

* Corresponding author. Fax: +30 1 6511767.

SSDI 0005-2728(94)00149-9

chloroplasts exhibits a lower F730/F685 ratio than the stroma lamellae isolated from non-phosphorylated samples; this suggested that the fluorescence changes observed in choroplasts may originate in grana, and that the excess LHC II in the stroma lamellar fraction may not be fully connected to PS I. The question therefore arose whether the excess LHC II in the light subchloroplast stroma lamellar fraction may be localized on distinct thylakoid membrane fragments [12].

In the present study we tried to assess whether the stroma lamellar fraction isolated from phosphorylated chloroplasts, after French-press disruption, indeed contains one type of membrane with randomized PS I and PS II particles, which is thus enriched in LHC II components (in favor of the lateral migration hypothesis), or if it is a mixture of membranes originating both from the PS I-rich stroma lamellae and also the LHC II-rich grana membranes. The latter was a possibility, since regions of granal membranes, destacked by the introduction of the negative repulsive charges of the phosphate groups on the threonine residue of LHC II [13,14], are expected to be extensively fragmented by the French press, and thus contaminate the light stroma lamellar fraction [15].

Our results showed that (a) the stroma lamellar fraction obtained from phosphorylated chloroplasts can be separated into LHC II-rich and LHC II-poor zones by ultracentrifugation on sucrose gradients after Mg^{2+} addition; (b) the LHC II-rich zone contains a higher amount of CP I than the grana membranes, a higher amount of 33 kDa PS II protein than the stroma lamellae, and a very low amount of cyt *f* and CF1, lower than the stroma lamellae; (c) the PS II proteins are phosphorylated in isolated grana as well; French-press disruption of isolated grana, suspended in buffers used for phosphorylation, releases a light membrane fraction, rich in LHC II, with sedimentation properties similar to those of fragments originating in stroma lamellae.

We conclude that the enrichment in LHC II of the stroma lamellar fraction, obtained from phosphorylated chloroplasts, reflects contamination of this fraction by destacked peripheral end grana membranes.

2. Materials and methods

Chloroplasts were isolated from pea plants, grown for 6 days in the dark under controlled conditions at 22°C and 80% humidity in a Conviron Phytotron, and then transferred to continuous light for a week (at 2000 × lux). Leaves (4 g fresh weight) were homogenized in 40 ml grinding buffer (0.4 M sorbitol, 50 mM Tricine-NaOH, 10 mM NaCl (pH 7.8)) in an Omni Mixer for 15 s at 35% of the line voltage, followed by 10 s at 58%. The homogenate was filtered through 6 layers of gauze; the chloroplasts, isolated at 1000 × *g* for 10 min, were washed in wash buffer (10 mM Tricine-NaOH, 10 mM NaCl, 5 mM $MgCl_2$ (pH 7.8)) recovered at 10000 × *g* for 10 min, and resuspended in resuspension buffer (0.1 M sorbitol, 30 mM Tricine-NaOH, 5 mM $MgCl_2$, 10 mM NaCl (pH 7.8)) [16,17]; their Chl was adjusted at 400 μg/ml in resuspension buffer.

For phosphorylation, to 5 ml chloroplast suspension we added 0.1 ml 0.5 M NaF, and 0.1 ml ATP (freshly prepared, 12.2 mg/ml). Illumination of samples was at 500 $\mu E/m^2$ per s (white light) in a water bath at 22°C for 20 min [16,17]. Non-phosphorylated samples contained ATP and NaF, but remained in the dark at 22°C; dephosphorylated samples contained ATP but no NaF, and following light exposure at 22°C, were incubated in the dark for 30 min.

French-press disruption was done at 6000 psi in chloroplast suspensions at 400 μg Chl/ml and 22°C [18]; subchloroplast fractions were isolated from the homogenates by differential centrifugation in the cold at 1000 × *g* for 10 min (1K pellet, 1K supernatant (sup)), then from the 1K sup at 10000 × *g* for 30 min (10K pellet-grana, 10K sup), then from the 10K sup at 50000 × *g* for 30 min (50K pellet, 50K sup-stroma lamellae). The stroma lamellar fragments whenever necessary, were peletted from the 50K sup at 240000 × *g* for 30 min (240K pellet-stroma lamellae).

For separation of subgranal membrane fragments, we first isolated the 10K pellet-grana fraction as above, from chloroplasts suspended at 400 μg Chl/ml in the cold, in the absence of ATP and NaF. The grana fraction was resuspended in 50 mM Tricine NaOH (pH 7.3) for 30 min at 22°C, or in resuspension buffer, at 400 μg Chl/ml; a sample was phosphorylated as above. The granal suspensions were then French-pressed at 6000 psi; the light membrane fragments released were separated from the granal homogenate in the supernatant after centrifuging at 10000 × *g* for 30 min (new 10K sup), or in addition at 50000 × *g* for 30 min (new 50K pellet, new 50K sup).

Ultracentrifugation of chloroplast homogenates or their 50K supernatants (containing the stroma lamellar fraction), as well as of granal homogenates and their subgranal fractions (new 10K sup or new 50K sup) prior to or after addition of $MgCl_2$ at 40 mM was performed on sucrose gradients (10% to 60%, or 15% to 45% sucrose in 50 mM Tricine NaOH (pH 7.3)) at 100000 × *g* (SW 65 or SW 28 rotors, 4°C for 1 h or 2 h) (modification of the method introduced by Michel and Michel-Wolwertz [19]).

In some cases the chloroplast 50K supernatant, after separation was French-pressed at 14000 psi in the cold, prior to cation addition; this sample was then ultracentrifuged on sucrose gradients (10% to 60% sucrose, 60000 × *g* for 30 min, SW 28 rotor, 4°C).

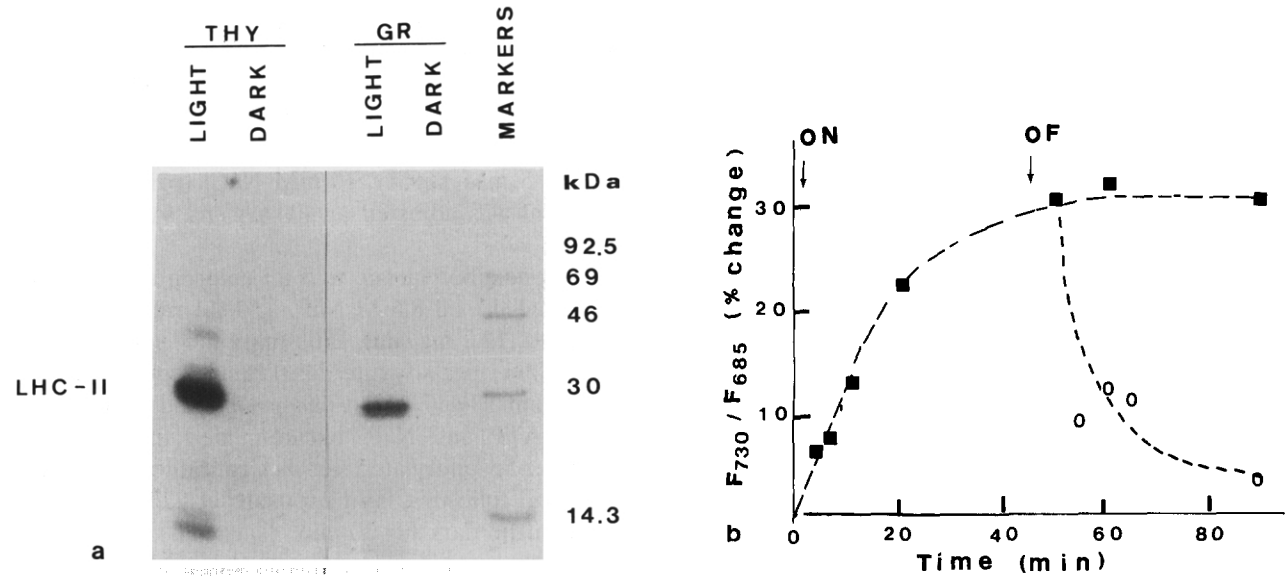

Fig. 1. (a) Autoradiogram of SDS-PAGE-resolved thylakoid (left) or granal (10K pellet) (right) polypeptides, phosphorylated in the light (500 $\mu E/m^2$ per s) or dark with [γ-^{32}P]ATP (8 μCi/ml phosphorylation assay mixture). Samples solubilized in SDS buffer containing 2 μg Chl were loaded per lane. M, ^{14}C-labelled protein markers. (b) Changes in the low temperature fluorescence emission ratio F730/F685 of chloroplasts during phosphorylation in the light (500 $\mu E/m^2$ per s) (■), and dephosphorylation in the dark (○), in the absence of NaF.

For each set of experiments, samples loaded on sucrose gradients had equal volume for reasons of comparison.

Pigment–protein complexes in thylakoid fragments, or zones, isolated by sucrose density gradient ultracentrifugation, were separated by mild SDS-PAGE at 4°C, as previously described [20]; the complexes resolved were scanned for Chl in a Joyce Loeble Chromoscan, using a cutoff 620 nm filter; quantitation was based on Chl distribution among complexes, estimated by weight of the area under each peak.

SDS-PAGE, autoradiography and immunoblotting were according to standard procedures [21–24]. Chl was determined according to MacKinney [25]. Antibod-

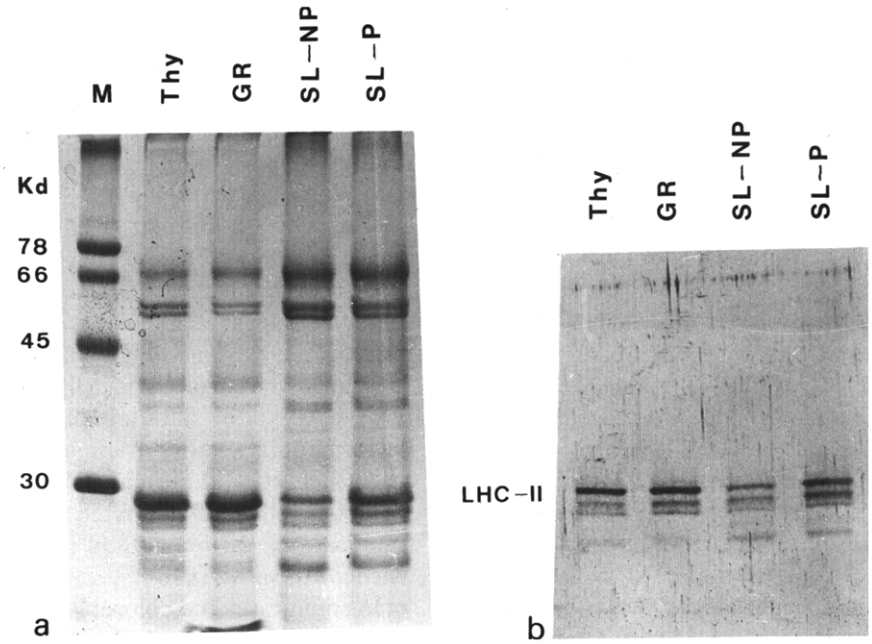

Fig. 2. SDS-PAGE polypeptide resolution pattern (a) and its immunoblot vs. anti-LHC II (b), obtained from SDS-solubilized thylakoids (Thy), isolated grana (GR), and the 240K pellet stroma lamellae fraction (SL) of phosphorylated (P) and non-phosphorylated (NP) chloroplasts. 45 μg protein loaded per lane in (a); 1 μg protein in (b). M, molecular mass protein markers.

Table 1
Chlorophyll distribution among subthylakoid fractions obtained by differential centrifugation from French-press-disrupted chloroplasts

Subchloroplast fraction	Chl distribution (% [a])		Ratio (Chl *a*/*b*)	
	NON-PHOS	PHOS	NON-PHOS	PHOS
1K pellet	8	8	2.5	2.6
10K pellet	48	39	2.4	2.6
50K pellet	38	44	3.1	3.0
240K pellet	6	9	4.8	3.7
10K sup	44	54	3.3	3.1

Chloroplasts phosphorylated in the light (PHOS) or dark (NON-PHOS), as in Section 2, were used.
[a] Chl distribution based on chloroplast starting material.

Table 2
Low temperature fluorescence emission ratio F730/F685 (77 K) in pea chloroplasts and subchloroplast fractions as affected by thylakoid protein phosphorylation

	NON-PHOS	PHOS	% Increase [a]
Sample	F730/F685	F730/F685	
Chloroplasts	1.1	2.1	91
Homogenate	0.73	1.4	91
1K SUP	0.73	1.38	89
10K SUP	1.1	1.9	72
50K SUP	4.1	2.9	−30

Chloroplasts prior to (NON-PHOS) or after phosphorylation (PHOS) were disrupted by French press, and the homogenates or their subchloroplast fractions (1K sup, 10K sup, 50K sup) were separated by differential centrifugation. After dilution to 30 μg Chl/ml, their 77 K fluorescence emission spectra were recorded.
[a] Increase based on respective non-phosphorylated fractions.

ies against LHC II, 33 kDa protein, cyt *f* and CF1 b were kindly offered by Professors K. Kloppstech, Hannover University, I. Ohad, Hebrew University of Jerusalem, and W. Rudiger, Munich University.

3. Results

Autoradiograms of the SDS-PAGE resolved polypeptides of thylakoids after phosphorylation in the light or dark with [γ-^{32}P]ATP showed the expected pattern earlier described (Fig. 1a). Highest label was found in the LHC II apoprotein, suggesting that in our experimental conditions, as well, the main PS II protein to be phosphorylated in the light is that of the LHC II. In addition, phosphorylation of PS II proteins altered the low temperature fluorescence ratio F730/F685, as well as the distribution of Chl between heavy and light subchloroplast fractions (Fig. 1b, Table 1). The F730/F685 ratio increased as phosphorylation proceeded in the light, and decreased upon transfer of samples to the dark. On the other hand, the Chl content in the 10K supernatant increased by about 10% in phosphorylated chloroplasts. The light membrane fractions obtained under these conditions became enriched in LHC II polypeptide and its Chl *b* component (see Fig. 2, and Table 1). The F730/F685 fluorescence emission ratio at 77 K, exhibited by the various subchloroplast fractions, is shown in Table 2. Phosphorylation resulted in enhanced F730/F685 ratio (compared to the respective non-phosphorylated samples) in chloroplasts, French-press homogenates and all subchloroplast fractions, except those of stroma lamellae (50K sup). The F730/F685 ratio changes, observed in chloroplasts upon phosphorylation, therefore, do not seem to reside in the light stroma lamellar fraction.

3.1. 'Stroma lamellae' from phosphorylated chloroplasts are not homogeneous

The poor efficiency in energy transfer by the LHC II to PS I in the stroma lamellar fraction of phosphorylated samples suggested to us that the excess LHC II in this fraction may not be connected to PS I and may belong to distinct membrane fragments. We thus attempted to analyze the sedimentation behavior of the French-press homogenates produced from phosphorylated, non-phosphorylated or de-phosphorylated chloroplasts. We used a modification of the method introduced by Michel and Michel Wolwertz [19]; by this method the French-press homogenate can be separated by ultracentrifugation at 60000 × *g* for 45 min on 12.5–50% sucrose gradients, into two heavy zones containing grana lamellae and a light zone, containing stroma lamellae. Fig. 3 shows such a separation on a 10% to 60% sucrose gradient for 1 h at 100000 × *g* of the French-press homogenates. The non-phosphorylated sample resolves 3 zones (an upper one with high Chl *a*/*b* ratio, and low Chl content, and two lower ones, with low Chl *a*/*b* ratio). The upper zone comes

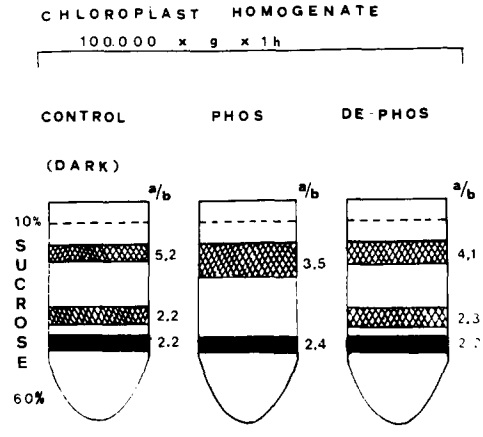

Fig. 3. Analysis by sucrose density gradient ultracentrifugation (10%–60% sucrose; 100000 × *g* for 1 h) of French-press homogenates obtained from chloroplasts phosphorylated in the light in the presence of NaF (PHOS), or phosphorylated in the light in the absence of NaF and then kept in the dark (DE-PHOS). The CONTROL sample contained ATP and NaF, but remained in the dark. a/b, Chl *a*/*b* ratio.

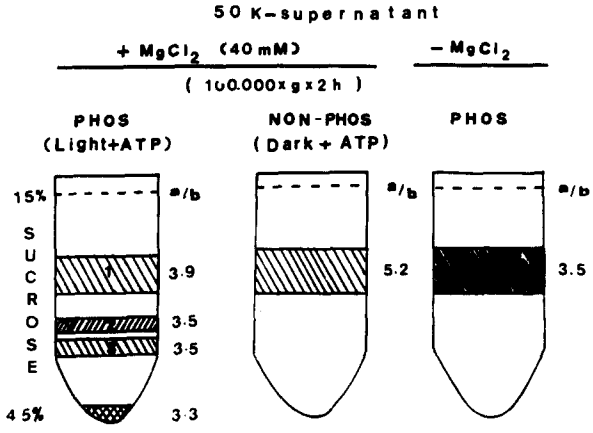

Fig. 4. Sucrose density gradient ultracentrifugation of 50K supernatants obtained by differential centrifugation from French-press-disrupted, phosphorylated (PHOS) or non-phosphorylated (NON-PHOS) chloroplasts. Ultracentrifugation was done prior to or after addition of $MgCl_2$ (40 mM).

from the stroma lamellar light fraction, obtained by differential centrifugation of French-press-disrupted chloroplasts (as judged by the behavior on similar gradients of the 50K sup-stroma lamellar fraction). On the other hand, the lower zones have low Chl *a*/*b* ratio and, as shown earlier, they originate in grana [19].

In contrast to the control sample, only two zones (the upper and lower ones) are resolved from the phosphorylated samples. The intermediate lower zone is missing, while the upper zone in this case is enhanced in Chl content and Chl *b*. The dephosphorylated sample, on the other hand, behaves like the non-phosphorylated sample, resolving the three zones, and an upper band with low Chl content and high Chl *a*/*b* ratio.

As already noted above, the 50K supernatant obtained from control non-phosphorylated chloroplasts, when analyzed on sucrose gradients by ultracentrifugation, bands in the upper part of the gradient as a distinct but unresolved band. Based on earlier work showing that $MgCl_2$ induces addhesion of LHC II containing thylakoid fragments [15,26–28], we incubated the 50K sups with $MgCl_2$, prior to ultracentrifugation. If distinct LHC II-rich fragments were present in the 50K sup, we expected them to associate forming a heavier zone on the gradient, and thus separate out of the LHC II poor stroma lamellar fragments. If, on the contrary, only one type of thylakoid fragments were present in the 50K sup, no separation was expected. The $MgCl_2$, present in the buffers (5 mM) was not adequate to induce such a separation. We thus increased its concentration to alleviate the repulsive negative forces of the phosphate groups. Fig. 4 shows the separation of the 50K sup obtained from phosphorylated and non-phosphorylated chloroplasts, prior to or after addition of $MgCl_2$ at 40 mM (15–45% sucrose gradient; 2 h at 100 000 × *g*). Similar results were obtained after ultracentrifugation for 1 h at 100 000 × *g* on a 10–60% gradient. As shown, the 50K sup prior to cation addition resolves only one zone (with sedimentation properties similar in both phosphorylated and non-phosphorylated samples). After addition of $MgCl_2$, however, and contrary to the non-phosphorylated sample, in which this zone remains unresolved, ultracentrifugation of the 50K sup from phosphorylated chloroplasts resolves an upper and a lower zone (the lower one being usually split in two).

Analysis by mild SDS-PAGE of the pigment–protein complexes in these bands showed that the 50K sup prior to separation has an LHCP/CP I ratio of 1.5 (see Fig. 5), higher than that found for stroma lamellae of

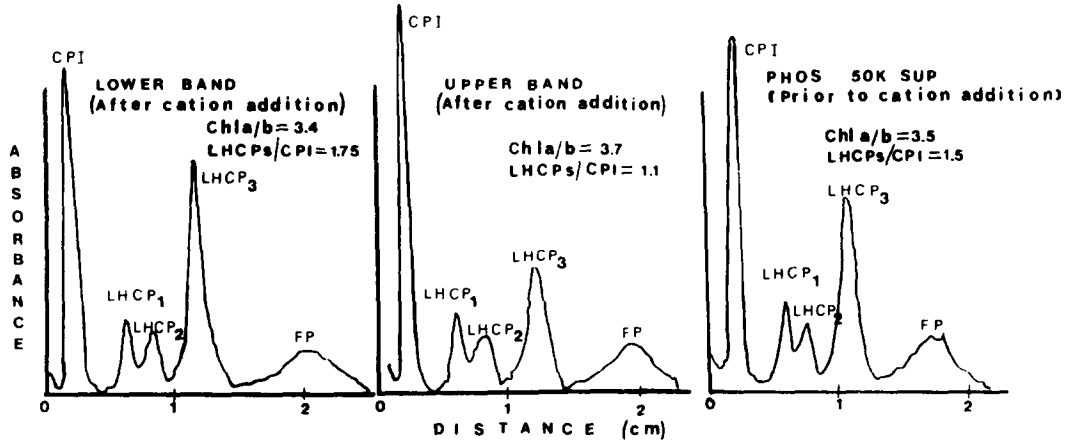

Fig. 5. Pigment–protein complex resolution by mild SDS-PAGE of SDS-solubilized thylakoid membrane fragments separated by sucrose density gradient ultracentrifugation (100 000 × *g* for 1 h; 10–60% sucrose) from the 50K sup of phosphorylated chloroplasts. The densitogram shows the Chl distribution among complexes in the original 50K sup prior to sucrose density ultracentrifugation, and in the upper and lower bands resolved after cation addition and ultracentrifugation.

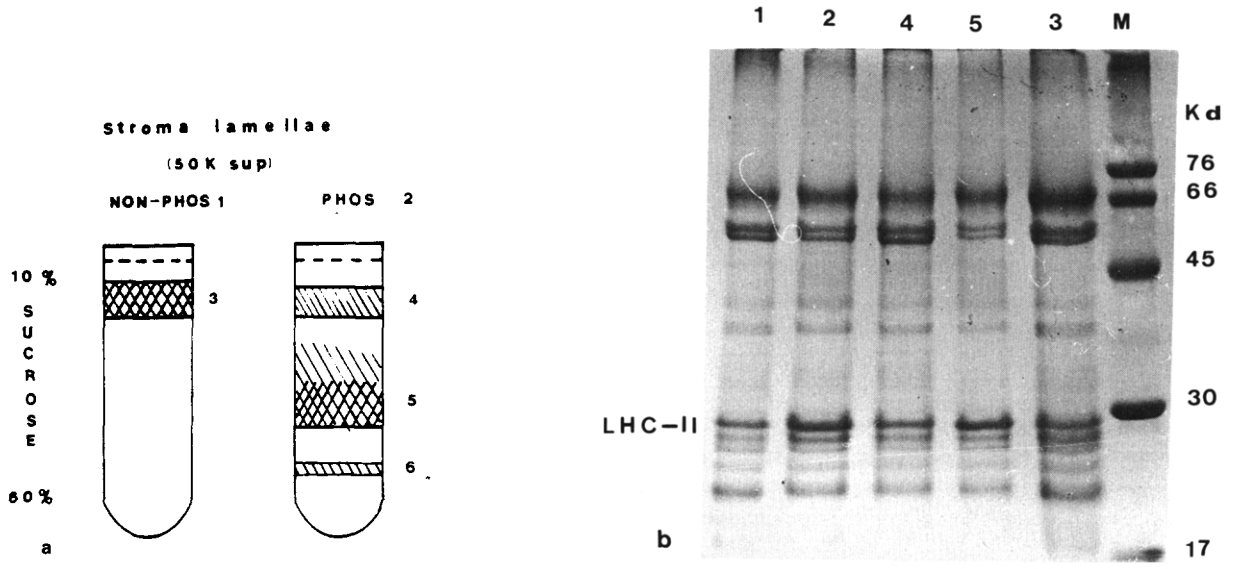

Fig. 6. Resolution by sucrose density ultracentrifugation (10%–60% sucrose; 60000 × *g* for 30 min) of extensively French-pressed stroma lamellar fractions (50K sup), from non-phosphorylated or phosphorylated chloroplasts. 50K sups, French-pressed at 14000 psi at 25 μg Chl/ml (NON-PHOS, 1), or 36 μg Chl/ml (PHOS, 2), were incubated at 0°C with $MgCl_2$ (40 mM final) for 30 min prior to centrifugation. (a) Resolution pattern; (b) SDS-PAGE analysis of the polypeptides in the 50K sups prior to centrifugation (NON-PHOS 1, PHOS 2) and in the bands resolved (3–5 as in (a)). 45 μg protein per lane. M, molecular mass protein markers. (c, d) Immunoblot analyses of thylakoids (Thy), grana (GR), stroma lamellae (240K pellet) from non-phosphorylated (SL-NP, 1) or phosphorylated (SL-P, 2) chloroplasts, and of the bands resolved as in (a) (3–5).

non-phosphorylated chloroplasts (LHCP/CP I = 0.92). After separation, this ratio in the lower band is 1.75, indicating enrichment in the granal component LHCP, while in the upper band it is reduced to 1.1, a value close to that found for stroma lamellae, which are rich in CP I, the P700-containing Chl *a*-pigment–protein

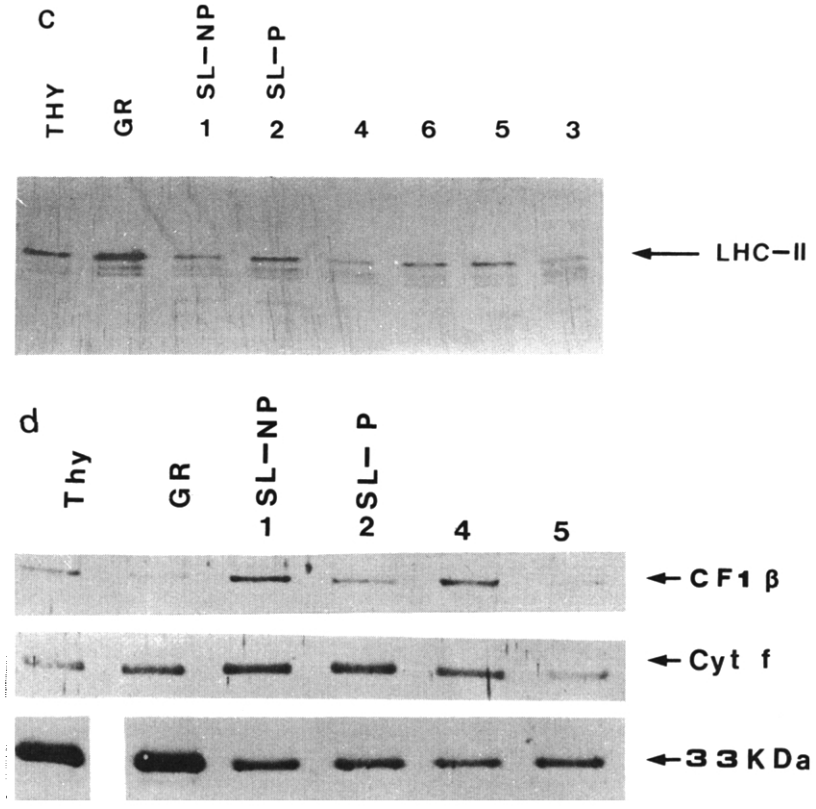

Fig. 6 (continued).

Table 3
Release of light subgranal fragments upon French-press disruption of isolated grana (French press 10K pellet)

Sample	Distribution of Chl (%)	Chl a/b ratio
(a) Tricine		
10K pellet	100	2.5
New 10K pellet	46	2.35
New 10K sup	54	2.78
(b) Sorbitol/Tricine/NaCl/$MgCl_2$		
10K pellet	100	2.5
New 10K pellet	70 (72) [a]	2.4 (2.38) [a]
New 10K sup	30 (28) [a]	2.8 (2.95) [a]

Isolated grana at 400 μg Chl/ml were incubated either in 50 mM Tricine-NaOH (pH 7.3) at 22°C for 30 min, or in 0.1 M sorbitol/30 mM Tricine-NaOH (pH 7.8), containing 10 mM NaCl and 5 mM $MgCl_2$ in the light or dark in the presence or absence of 0.44 mM ATP for 20 min at 22°C. The suspensions were French-pressed and the homogenate centrifuged at 10000 × g for 30 min.
[a] Values in parentheses are those in samples without ATP.

complex of PS I. Despite the removal of the LHC II-rich fragments from the bulk of the stroma lamellae, however, the latter still contain an appreciable amount of Chl b, as judged from the Chl a/b ratio of 3.9 in the upper band. This suggests that the upper band still contains some LHC II-rich fragments.

This separation of the lower from the upper band is more effective if the 'stroma lamellae', prior to incubation with $MgCl_2$, were disrupted for a second time by French press into smaller fragments (14000 psi at 4°C). In this case only 30 min at 60000 × g are sufficient for the separation. The upper band under these conditions separates as a very sharp zone (see Fig. 6a, band 4).

The SDS-PAGE resolution pattern of the polypeptides in the zones separated as above, are shown in Fig. 6b in comparison to the non-phosphorylated or phosphorylated stroma lamellar fraction; Western blots of these fractions vs. antisera raised against LHC II, the 33 kDa PS II polypeptide, the subunit b of ATPase and cyt f are shown in Fig. 6c and d. When compared to the upper zone, or the stroma lamellar fraction of non-phosphorylated chloroplasts (SL-NP), the membrane fragments in the lower zone are enriched in 33 kDa polypeptide and in LHC II, but contain a lower amount of cyt f and peripheral thylakoid proteins (55–57 kDa Coomassie-stained polypeptides, CF1 b).

The results suggest that the stroma lamellar fraction obtained after French-press disruption from phosphorylated chloroplasts is a mixture of membrane fragments, and that the light LHC II-rich fragments can be separated from the bulk of stroma lamellae after cation addition.

3.2. Origin of the LHC II-enriched lower band separated from the 'stroma lamellar' fraction of phosphorylated chloroplasts

To check whether the LHC II-rich thylakoid fragments in the light stroma lamellar fraction of phosphorylated chloroplasts (resolved as a lower zone after addition of $MgCl_2$), originate in destacked regions of grana, we examined the possibility that fragments of similar composition and sedimentation behavior can be released from isolated grana.

Table 3 shows the distribution of Chl among heavy and light subgranal fragments produced by French-press disruption from isolated 10K grana fractions (Chl a/b = 2.5), preincubated for 30 min at 25°C in 50 mM Tricine-NaOH (pH 7.3), or phosphorylated in the light or dark in the resuspension buffers used for phosphorylation. As shown, in the case of Tricine (a low-salt medium), about 50% of the Chl in grana ends up in the

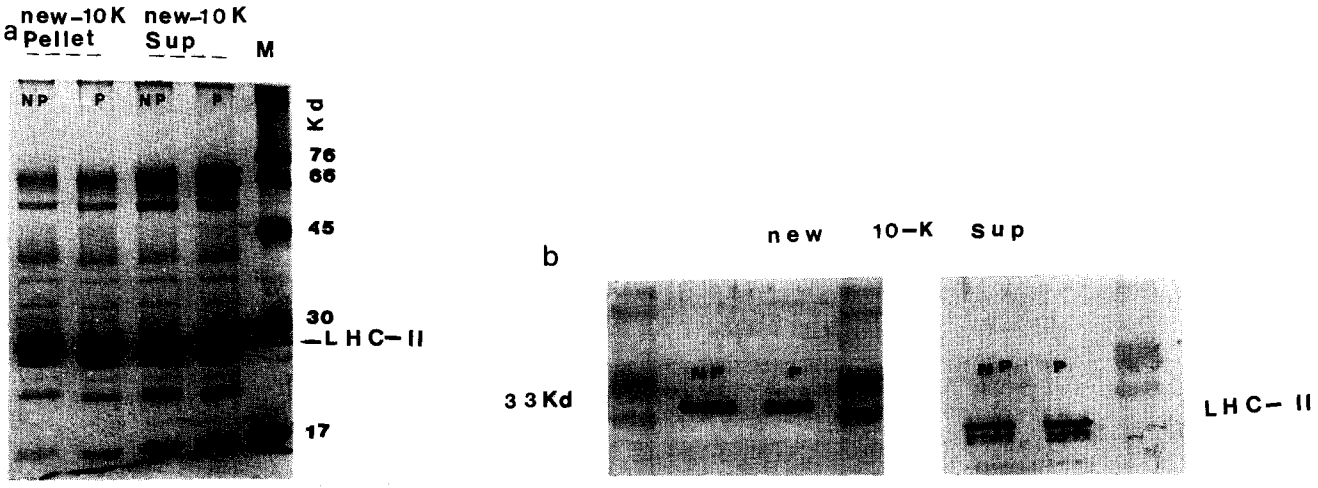

Fig. 7. (a) SDS-PAGE of polypeptides in new 10K pellet or new 10K sup obtained by differential centrifugation from French-press-disrupted phosphorylated (P) or non-phosphorylated (NP) grana (10K pellet). Samples containing 2 μg Chl loaded per lane. M, molecular mass markers. (b) Immunoblot analysis of the new 10K sup obtained as above, vs. anti LHC II and anti 33 kDa protein.

light fraction (new 10K sup); this value in the samples incubated in the resuspension buffer used for phosphorylation (containing 10 mM NaCl and 5 mM $MgCl_2$) is lower (27–29%).

Phosphorylation of the 10K pellet grana fraction in the light also leads to phosphorylation of LHC II (see autoradiogram in Fig. 1). The distribution of Chl among heavy and light subthylakoid fractions, under these conditions, changes slightly in favor of the light fractions. In all cases, the subgranal fragments released as light fragments by French press (new 10K sup, new 50K sup), have a lower Chl *a*/*b* ratio and a higher LHC II and 33 kDa protein content than stroma lamellae (see the SDS-PAGE polypeptide resolution pattern and the immunoblots in Fig. 7). When compared to the remaining grana pellet in the heavy fraction (new 10K pellet), however, they have slightly higher content of CP I and 55–57 kDa peripheral proteins.

These light subgranal fragments (new 50K sup), when loaded on sucrose gradients and centrifuged at 100 000 × *g* for 1 h, resolve only one band, which bands at a position similar to that of the 50K sup obtained from French-press-disrupted chloroplasts (Fig. 8; compare with Fig. 4). $MgCl_2$ addition to the light subgranal fragments (new 10K sup) followed by ultracentrifugation at 100 000 × *g* for 2 h (15–45% sucrose gradient) results in the resolution of a heavy double lower zone, with sedimentation properties similar to those of the LHC II-rich band, separated out of the 'stroma lamellar' fraction of phosphorylated chloroplasts (see Fig. 8 in comparison to Fig. 4). This suggests that the LHC II-rich fragments contaminating the stroma lamellar fraction of phosphorylated chloroplasts may indeed originate in destacked membranes of grana.

Finally, to further check whether the separation of the various types of membrane fragments in the phosphorylated stroma lamellae can be also achieved by differential centrifugation, we phosphorylated chloroplasts in the presence or absence of NaF and French-pressed them immediately; the homogenates were incubated in the dark at room temperature for 30 min to dephosphorylate; dephosphorylation was expected to alleviate the repulsive negative forces on the phosphate groups, and allow reassociation of LHC II-containing fragments in the 5 mM $MgCl_2$ resuspension buffer [15,26–28]. Table 4 shows the distribution of Chl and the Chl *a*/*b* ratio found in the light 240K fraction, obtained after differential centrifugation of the homogenates. In all cases prolonged incubation in resuspension buffer leads to increased Chl yield in heavy

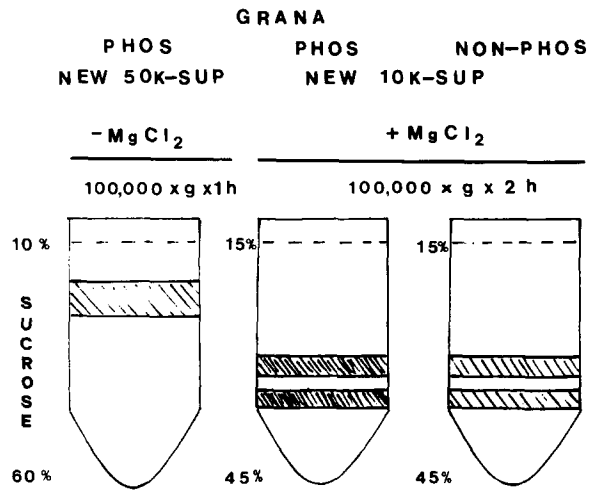

Fig. 8. Resolution by sucrose gradient ultracentrifugation of subgranal fragments produced by French-press disruption of phosphorylated (PHOS) or non-phosphorylated (NON-PHOS) grana. Left: new 50K supernatant obtained from phosphorylated grana (10%–60% sucrose gradient; 100 000 × *g* for 1 h). Right: new 10K supernatant from phosphorylated or non-phosphorylated grana after $MgCl_2$ addition (15%–45% sucrose gradient; 100 000 × *g* for 2 h).

Table 4
Reassociation of light granal membrane fragments, released by French-press disruption from phosphorylated chloroplasts, upon dephosphorylation

Sample	Chl Distribution (%) [a]			Chl *a*/*b*
	1K + 10K-p	10K-sup	240K-p	240K-p
(1) Non-dephosphorylated (PHOS (+NaF) --Fp--+ Dark)	75	25	2.5	3.5
(2) Dephosphorylated (PHOS (-NaF) --Fp--+ Dark)	85	15	1.3	4.9
(3) Non-dephosphorylated (PHOS (-NaF) --Fp--+ NaF + Dark)	76	24	2.0	4.3
(4) Non-phosphorylated (NON-PHOS (+NaF) --Fp--+ Dark)	82	18	1.0	5.7

Reassociation is monitored by the distribution of Chl in the heavy membrane fraction (1K + 10K pellet) isolated by differential centrifugation from the French-press homogenates.

[a] Chl distribution based on starting chloroplast amount. (1), (2): phosphorylated in the presence or absence of NaF, respectively, French-pressed and kept in the dark for 30 min. (3): Sample (2) to which NaF was added after French press. (4): phosphorylated in the dark, in the presence of NaF, then French-pressed and incubated in the dark for 30 min. NaF was used to inhibit dephosphorylation prior to or after French-press disruption. p, pellet.

fragments (compare with Table 1); dephosphorylation further enhances the yield of heavy fractions (sample 2) allowing the separation of a light fraction with higher Chl *a*/*b* ratio than that in the phosphorylated sample (compare sample 2 with 1). This suggests that dephosphorylation of the French-press homogenate allows the reassociation of LHC II containing fragments in the presence of 5 mM $MgCl_2$; when these are collected in the heavy fraction, stroma lamellae with high Chl *a*/*b* ratio are separated.

4. Discussion

The results of this study suggest that the stroma lamellar fraction obtained by differential centrifugation of French-press-disrupted phosphorylated chloroplasts is a mixture of stroma lamellar and destacked granal membrane fragments. This became evident from the successful resolution of the LHC II-rich fragments present in the light subthylakoid fraction (50K supernatant) from the bulk of stroma lamellae. The enrichment in LHC II components of this fraction on phosphorylation, therefore, cannot be considered as evidence for lateral migration of LHC II. If this were due to lateral migration of LHC II from grana to stroma lamellae, one would expect this fraction to be composed of one type of light thylakoid fragments with similar composition and sedimentation properties.

Most of the work done with stroma lamellar fractions considers as stroma lamellae any subthylakoid fraction sedimenting above $40\,000 \times g$. Our results suggest that subthylakoid fractions cannot be considered as stroma lamellae merely on the grounds of their sedimentation properties. On the other hand our results also show that the fraction isolated as 'grana' in the 10K pellet separated by differential centrifugation from homogenates of French-pressed chloroplasts, suspended in the buffers used for phosphorylation (0.1 M sorbitol, 0.03 M Tricine, 5 mM $MgCl_2$, 10 mM NaCl (pH 7.8)) [16,17] can release a light fraction (representing almost 30% of the granal Chl) upon additional passage through the French press. These fragments, when compared to the granal core, are as rich in LHC II, but they contain a higher amount of the 62 kDa PS I polypeptide and of the 55–57 kDa peripheral proteins. This suggests that they originate in the peripheral grana membranes, known to contain PS I particles; we cannot exclude the possibility, however, that some stroma lamellae may be entrapped in the 'granal' fraction, obtained from chloroplasts suspended in such cationic media.

How this destacking of isolated grana occurs is not known. It is possible that following the first French-press disruption, grana ends are loosened, so that they destack mechanically. Chow and Barber [29], using grana isolated from digitonin-disrupted pea chloroplasts, have also found that about 35% of their Chl is released in the 10K sup by a second digitonin disruption. This value is not very different from what we have found for French-press disruption of the French-press 10K pellet. However, in our case, grana destacking can be further facilitated by incubation in Tricine ('low-salt' conditions, see Table 3), in contrast to the findings of Chow and Barber [29] who reported no change in destacking at KCl concentrations ranging from 1 to 100 mM.

The similar composition and sedimentation properties of these light subgranal fragments with the LHC II-rich zone separated from the bulk of stroma lamellae of phosphorylated samples strongly suggests that the latter originates from destacked membranes of grana.

Our study also showed that in contrast to chloroplasts and their grana fraction, which acquire a higher F730/F685 ratio as a result of protein phosphorylation, the stroma lamellar fraction of phosphorylated chloroplasts exhibits a lower ratio than that of non-phosphorylated chloroplasts. This is explained by the finding that the excess LHC II in this fraction belongs to distinct membrane fragments, it is not connected to PS I, and cannot serve this unit, increasing its absorption cross section.

In agreement to our study are recent results on the incorporation in thylakoids of in vitro synthesized LHC II protein, after its import into isolated intact pea chloroplasts; it was found that independent of light conditions or mutations directed to the threonines at position 5 and 6, involved in phosphorylation, the protein accumulates in grana [30].

Quite a controversy exists in the experimental findings concerning the absorption cross section of PS I upon phosphorylation, as judged by fluorescence emission studies or the kinetics of photochemical reduction in isolated chloroplasts. In some cases its size was found to increase, in others it remained unaffected by phosphorylation; on the other hand, the results concerning the absorption cross-section of PS II, more or less agree that thylakoid protein phosphorylation leads to the decrease of its effective absorption cross-section [31–37]. Our results suggest that such changes may be the result of granal destacking induced by phosphorylation; repulsive forces during destacking may remove some LHC II from its tight association with the PS II core in grana appressions, and lead to its association with the PS I units, present in the outer granal end membranes. Variations in destacking might lead to varying degree of association, and to variations in the absorption cross-section.

PS I particles (PS I_a) of larger functional antenna size than those in stroma lamellae (PS I_b) have been actually separated from grana [38]; these particles con-

tain LHC II, and seem to be located in the periphery of the grana [38]. A recent study in our laboratory has also shown that digitonin action on phosphorylated pea chloroplasts releases a light membrane fraction which is enriched in LHC II, contains PS I components and has a characteristic high F730/F685 fluorescence emission ratio at 77 K; these fragments are beleived to originate in destacked granal regions [39].

It has been proposed that the physiological role of PS II protein phosphorylation is the regulation of excitation energy distribution between the photosystems, underlying the State transitions: overexcitation of PS II leads to phosphorylation of PS II proteins by the redox regulated kinase, and to migration of phospho LHC II from grana to stroma lamellae, where it serves PS I (State II, low Chl *a* fluorescence yield at room temperature, high F730/F685 at 77 K), while overexcitation of PS I leads to dephosphorylation of phospho LHC II by the phosphatase, and its return to granal PS II (State I, high Chl *a* fluorescence yield at room temperature, low F730/F685 at 77 K). This hypothesis explains the increase in the rate of PS I electron transfer as a result of protein phosphorylation, and the modulation by light quality of the overall oxygen yield in the early experiments of Bonaventura and Myers [40].

In view of our results, the migration of phospho LHC II from grana to stroma lamellae is questioned. The question is raised, therefore, as to what the State transitions may reflect, and how can they be explained. Two types of mechanisms may be proposed. The first might involve the destacking-induced detachment of LHC II from PS II in grana appressions and its association to the PS I in the peripheral granal membranes, discussed above. The second might involve the organization of the photosynthetic unit assemblies themselves. Thylakoid protein phosphorylation may affect, for example, the organization of the PS I unit itself, in a way that the association of the PS I core with its own LHC-I may be enhanced, just as in the case of chloroplasts suspended in low-salt [41–43]. Such an organization has been shown to enhance the F730/F685 ratio, at least in low-salt chloroplasts [15,41–43]. The organization of the oligomeric supramolecular pigment–protein complexes and of the CP I-LHC I (CP Ia) PS I complex was indeed found to be affected by thylakoid protein phosphorylation [12]. This may show that phosphorylation renders the photosynthetic unit assemblies less succeptible to degradation. Indeed, a recent report has shown that phosphorylation of the LHC II, which is newly incorporated into pea thylakoids, inhibits its degradation [30]; we have also found that addition to thylakoids of benzamidine, the proteinase inhibitor, prior to SDS-solubilization, favors the separation of oligomeric pigment–protein complexes (unpublished results). Such changes, therefore, in the organization of complexes (association of granal PS II-LHC II to granal PS I; association of PS I core with LHC-I) may lead to the increase of the PS I effective absorption cross-section, or to a more effective energy transfer between PS II and PS I assemblies, reflected by the F730/F685 ratio increase.

References

[1] Bennett, J. (1977) Nature 269, 344–346.
[2] Bennett, J. (1993) Annu. Rev. Plant Physiol. Plant Mol. Biol. 42, 281–311.
[3] Allen, J.F. (1992) Biochim. Biophys. Acta 1098, 275–335.
[4] Gal, A., Schuster, G., Frid, D., Canaani, O., Schwieger, H.-G. and Ohad, I. (1988) J. Biol. Chem. 263, 7785–7791.
[5] Silverstein, T., Cheng, L. and Allen, J.F. (1993) Biochim. Biophys. Acta 1183, 215–220.
[6] Bennett, J., Steinback, K.E.. and Arntzen, C.J. (1980) Proc. Natl. Acad. Sci. USA 77, 5253–5257
[7] Krause, G.H. and Behrend, U. (1983) Biochim. Biophys. Acta, 723, 176–181.
[8] Kyle, D.J., Kuang, T.Y., Watson, J.L. and Arntzen, C.J. (1984) Biochim. Biophys. Acta 765, 89–96.
[9] Kyle, D.J., Staehelin, L.A. and Arntzen, C.J. (1983) Arch. Biochem. Biophys. 222, 527–541.
[10] Barber, J. (1983) Photobiochem. Photobiophys. 5, 181–190.
[11] Vallon, O., Bulte, L., Dainese, P., Olive, J., Bassi, R. and Wollman, F.-A. (1991) Proc. Natl. Acad. Sci. USA 88, 8262–8266.
[12] Georgakopoulos, J. and Argyroudi-Akoyunoglou, J.H. (1991) in Regulation of Chloroplast Biogenesis (Argyroudi-Akoyunoglou, J.H., ed.), NATO ASI Vol A226, pp. 539–544, Plenum, New York.
[13] Mullet, J.E. (1983) J. Biol. Chem. 268, 9941–9948.
[14] Miller, K. and Lyon, M.K. (1985) Trends Biochem. Sci. 10, 219–222.
[15] Argyroudi-Akoyunoglou, J.H. and Akoyunoglou, G. (1986) in Regulation of Chloroplast Differentiation (Akoyunoglou, G. and Senger, H., eds.), pp. 329–338, Alan R. Liss, New York.
[16] Kyle, D.J., Haworth, P. and Arntzen, C.J. (1982) Biochim. Biophys. Acta 680, 336–342.
[17] Steinback, K.E., Bose, S. and Kyle, D.J. (1982) Arch. Biochem. Biophys. 216, 356–361.
[18] Sane, P.V., Goodchild, D.J. and Park, R.B. (1970) Biochim. Biophys. Acta 216, 162–178.
[19] Michel, J.-M. and Michel-Wolwertz, M.-R. (1967) in Carnegie Institution Year Book 67, pp. 508–514.
[20] Anderson, J.M., Waldron, W.C. and Thorne, S.W. (1978) FEBS Lett. 92, 227–233.
[21] Laemmli, U.K. (1970) Nature 277, 680–685.
[22] Bonner, W.J. and Laskey, R.A. (1974) Eur. J. Biochem. 46, 83–88.
[23] Towbin, H. and Gordon, J. (1984) J. Immunol. Methods 79, 313–340.
[24] Blake, M.S., Jhonston, K.H., Russel, G.J. and Gotschlich, E.C. (1984) Anal. Biochem. 136, 175–186.
[25] MacKinney, G. (1941) J. Biol. Chem. 140, 315–322.
[26] Argyroudi-Akoyunoglou, J.H. (1976) Arch. Biochem. Biophys. 176, 267–274.
[27] Argyroudi-Akoyunoglou, J.H. and Akoyunoglou, G. (1977) Arch. Biochem. Biophys. 179, 370–377.
[28] Arntzen, C.J. and Ditto, C.L. (1976) Biochim. Biophys. Acta 449, 259–274.
[29] Chow, W.S. and Barber, J. (1980) Biochim. Biophys. Acta 593, 149–157
[30] Kohorn, B.D. and Yakir, D. (1990) J. Biol. Chem. 265, 2118–2123.

[31] Forti, G. and Fusi, P. (1990) Biochim. Biophys. Acta 1020, 247–252.
[32] Torti, F., Gerola, P.D. and Jennings, R.C. (1984) Biochim. Biophys. Acta 767, 321–325.
[33] Deng, X. and Melis, T. (1986) Photobiochem. Photobiophys. 13, 41–52.
[34] Haworth, P. and Melis, A. (1983) FEBS Lett. 160, 277–280.
[35] Horton, P. and Black, M.T. (1981) FEBS Lett. 132, 75–77.
[36] Telfer, A., Bottin, H., Barber, J. and Mathis, P. (1984) Biochim. Biophys. Acta 764, 324–330.
[37] Bassi, R., Giacometti, G., M. and Simpson, D.J. (1988) Biochim. Biophys. Acta 935, 152–165.
[38] Andreasson, E. and Albertsson, P.-A. (1993) Biochim. Biophys. Acta, 1141, 175–182.
[39] Georgakopoulos, J.H. and Argyroudi-Akoyunoglou, J.H. (1994) J. Photochem. Photobiol. B: Biol. 26, 57–65.
[40] Bonaventura, E. and Myers, J. (1969) Biochim. Biophys. Acta 189, 366–383.
[41] Argyroudi-Akoyunoglou, J.H. (1991) Arch. Biochem. Biophys. 286, 524–532.
[42] Argyroudi-Akoyunoglou, J.H. (1984) FEBS Lett. 171, 47–53.
[43] Kuang, T.Y., Argyroudi-Akoyunoglou, J.H., Nakatani, H., Watson, J. and Arntzen, C.J. (1984) Arch. Biochem. Biophys. 235, 618–627.