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FREEZE-FRACTURE STUDIES ON BARLEY PLASTID MEMBRANES

VII. STRUCTURAL CHANGES ASSOCIATED WITH PHOSPHORYLATION OF THE LIGHT-HARVESTING COMPLEX

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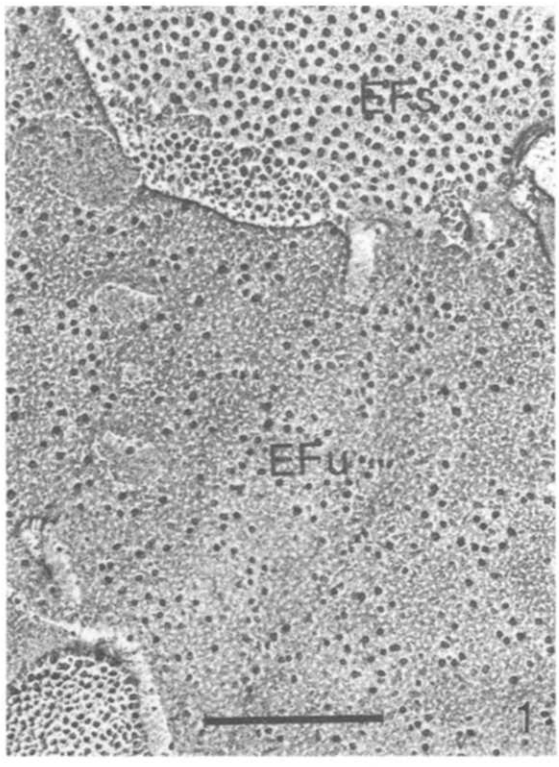
Chloroplast thylakoid membranes were isolated from barley at room temperature under redox conditions which ensured that the light-harvesting complex was either non-phosphorylated or phosphorylated. The ultrastructural appearance of these membranes was characterised by rotary shadowed, freeze-fracture electron microscopy. Upon phosphorylation, there was a slight (5%) decrease in the extent of thylakoid stacking, as evidenced by an increase in EFu face particle density. It was concluded from detailed measurements of particle density and size distribution that phosphorylation of the light-harvesting complex results in the movement of some of the Photosystem II EFs particles and some of the PFs particles containing the light-harvesting complex from grana to stroma membranes. There was also a slight increase in PFs particle size and the appearance of a population of large particles on this face, which may be due to conformational changes in the light-harvesting complex or to the movement of some Photosystem I particles from stroma to grana membranes.

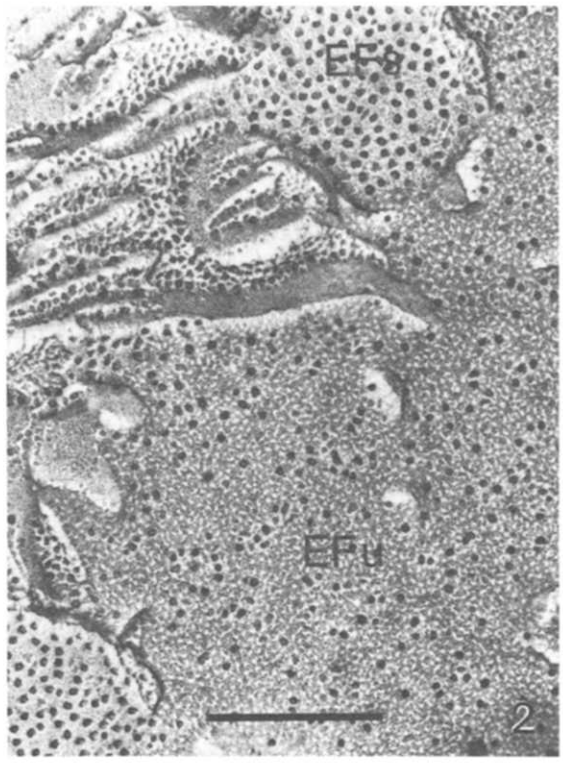
Introduction

Maximum non-cyclic photosynthetic efficiency is achieved when electron transport through the two photosystems in chloroplast membranes operates at the same rate. Photosynthetic membranes of higher plants are able to adapt to changing wavelengths of light by altering the energy distribution between the photosystems so that a greater proportion of the absorbed energy goes to the rate-limiting photosystem [1]. It is now established that the distribution of excitation energy between PS I and PS II is regulated via phosphorylation of the LHC [1,2]. The activity of thylakoid-bound kinase involved is determined by the redox state of platoquinone [2]. Illumination of plants with red light, which is mainly absorbed PS II, causes reduction of plastoquinone, activation of the kinase, phosphorylation of the LHC and redistribution of energy to PS I [2,3]. Absorption of far-red light by PS I reverses these effects. However, the kinase exhibits allosteric kinetics towards ATP and is non-competitively inhibited by ADP [4,5]. High ATP usage raises the ADP level, which decreases kinase activity, resulting in more energy going to PS II with a consequent increase in the rate of photophosphorylation. It has been proposed that this is a homeostatic mechanism to maintain ATP at physiological levels [4].

This investigation was initiated to determine the structural basis for the redistribution of light energy following phosphorylation of the LHC. It

Abbreviations: EFs, endoplasmic fracture (stacked); EFu, endoplasmic fracture (unstacked); PFs, protoplasmic fracture (stacked); PFu, protoplasmic fracture (unstacked); PSs, protoplasmic surface (stacked); LHC, light-harvesting complex; PS, photosystem; Tricine, *N*-tris(hydroxymethyl)methylglycine.


EFs
EFu
1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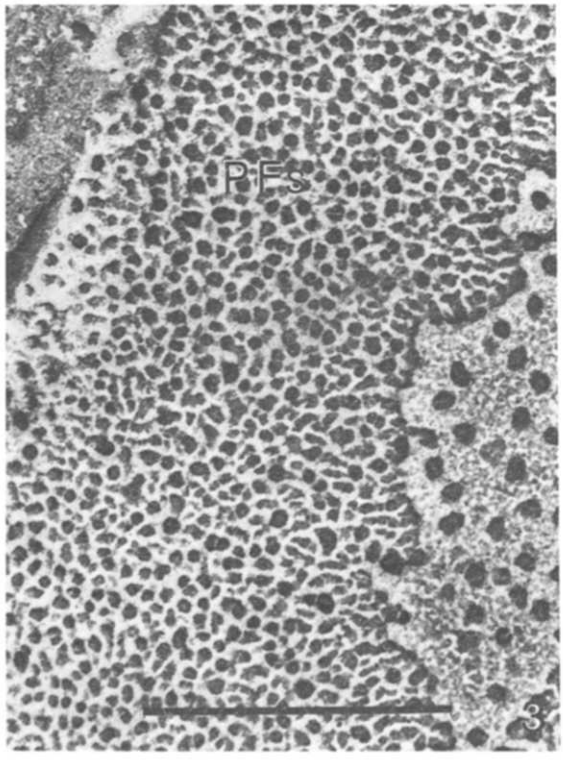
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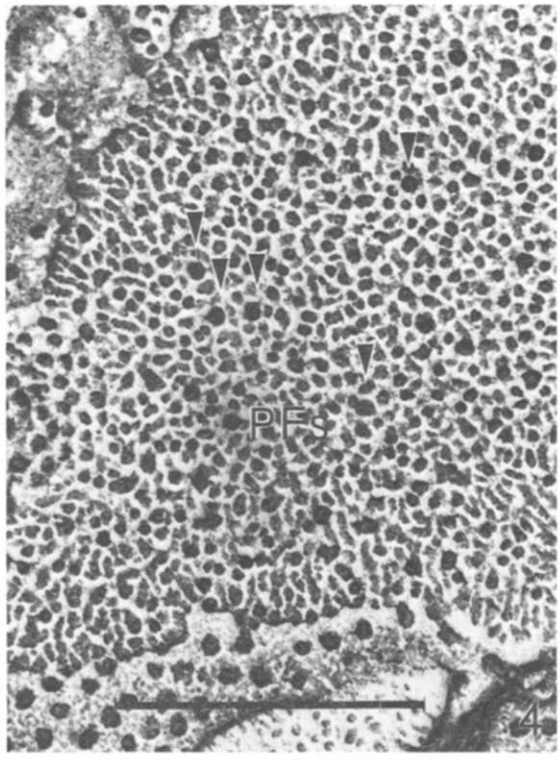
PEFs
4

appears from biochemical [6] and ultrastructural data [7] that there is little or no PS I located in the grana membranes, which is the site of most of the PS II activity and the LHC [8]. Upon phosphorylation, there must be protein conformational changes, or a lateral redistribution of photosynthetic components, or a combination of these two events, to alter the interactions between the various pigment-protein complexes. Since the major chlorophyll-proteins have been localised to different freeze-fracture particles [7,8], it should be possible to detect ultrastructural differences and to interpret them in terms of changes in the organisation of the chlorophyll-proteins of the thylakoid.

Materials and Methods

Seeds of barley (*Hordeum vulgare* cv. Clipper) were germinated in plastic trays containing vermiculite moistened with tap water. Seedlings were harvested after growing for 7 days at 20°C in continuous white light (2500 lx, 100 $\mu E \cdot m^{-2} \cdot s^{-1}$, 400–700 nm) supplied by Sylvania Gro-lux fluorescent tubes.

Leaves were homogenised in 3 vol. of grinding medium at 20°C, using a Waring blender modified to take replaceable razor blades [9]. The basic grinding medium consisted of 0.4 M sucrose, 50 mM Tricine-NaOH, pH 7.9, and 5 mM $MgCl_2$, to which was added either 2 mM $K_3Fe(CN)_6$ or 10 mM sodium dithionite and 200 μM ATP. These conditions reproduce those used to produce non-phosphorylated and phosphorylated pea thylakoids [2]. The resulting slurry was filtered through two layers of 30 μm nylon gauze and centrifuged for 5 min at $1400 \times g$. The pellets were resuspended in the appropriate grinding medium and incubated at 20°C for 30 min. Following incubation an equal volume of 60% glycerol in 5 mM $MgCl_2$ was added and the suspension was centrifuged for 5 min at $5000 \times g$. This pellet was used for freeze-fracturing. All operations involving leaves isolated in media containing $K_3Fe(CN)_6$ were carried out under a dim green safety light, and those involving leaves isolated in the presence of dithionite and ATP were performed under room light. In both cases, the material was kept at 20°C until frozen for freeze-fracturing. The degree of thylakoid phosphorylation was monitored by recording fluorescence induction kinetics and measuring initial (F_0) and maximum (F_m) levels of fluorescence.

Rotary-shadowed freeze-fracture replicas were made as previously described [8] as were measurements of particle density and size distribution [10]. Frequency histogram plots of particle areas, assuming an elliptical cross-section, were drawn using an HP 9845B computer interfaced with an HP 9872C plotter.

Results

Isolated barley thylakoids incubated in the dark with ferricyanide had a ratio of variable fluorescence (F_v) to total fluorescence (F_m) of 0.74. Membranes incubated in the presence of dithionite and ATP showed no significant decrease in initial fluorescence (F_0), but a 28% decrease in F_m, with a consequence decrease in the F_v/F_m ratio to 0.66. This indicates that the membranes have been phosphorylated [11].

The freeze-fracture appearance of thylakoids in which the LHC is not phosphorylated is shown in Figs. 1, 3 and 5 and may be compared with corresponding faces of phosphorylated membranes

Fig. 1. Freeze-fracture appearance of the EFu and EFs faces of nonphosphorylated thylakoids. (Bar = 0.25 μm) Magnification ×92000.

Fig. 2. Freeze-fracture appearance of the EFu and EFs faces of phosphorylated thylakoids. An increase in EFu particle density is apparent, but the ultrastructure is otherwise similar to that of non-phosphorylated membranes. (Bar = 0.25 μm) Magnification ×92000.

Fig. 3. High-magnification image of the PFs face of non-phosphorylated thylakoids. (Bar = 0.2 μm). Magnification ×204000.

Fig. 4. As for Fig. 3, showing phosphorylated membranes. Note the presence of heavily shadowed particles (arrows). (Bar = 0.2 μm) Magnification ×204000.

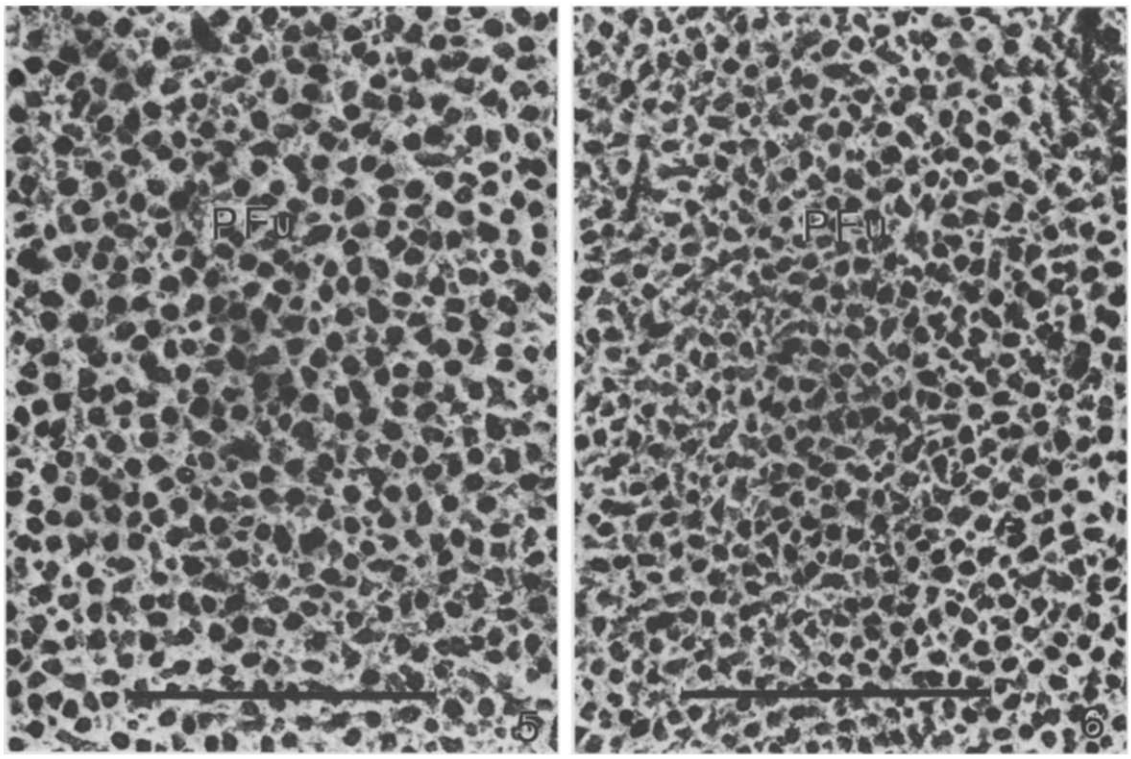

Fig. 5. High-magnification freeze-fracture image of the PFu face of non-phosphorylated thylakoids, showing large and small particles. (Bar = 0.2 μm) Magnification ×204000.

Fig. 6. The PFu face of phosphorylated thylakoids contains an increased density of small particles, with a slight decrease in large particle density. (Bar = 0.2 μm) Magnification ×204000.

in Figs. 2, 4 and 6. Under both conditions, thylakoids at 20°C showed a differentiation into stacked (grana) and unstacked (stroma) lamellae. The EF face of grana was characterised by a moderately dense population of large particles and that of stroma lamella, by a low density of somewhat smaller particles on a pitted background, precisely as has been found for thylakoids at 0°C (Figs. 1 and 2).

Similarly, the PFs face under both non-phosphorylating and phosphorylating conditions showed a high density of small particles (Figs. 3 and 4). It was possible to distinguish a slight difference in that the number of large PFs particles (arrows in Fig. 4) was greater in phosphorylated membranes (Fig. 4). There was also a subtle difference between the PF faces of unstacked membranes regarding the number of small particles, which were more numerous in phosphorylated thylakoids (Figs. 5 and 6).

When objective measurements were made of particle densities, differences were detected (Table I). Phosphorylated membranes were found to have a slightly higher particle density on all four fracture faces. In the case of the PFu face, this was due to a greater number of small particles, there being the same number of large particles. For comparison, the particle densities for thylakoids isolated at 0°C are included with the particle densities for thylakoids isolated at 20°C in the presence of redox compounds.

The average dimensions of the particles on each face are given in Table II for non-phosphorylated and phosphorylated thylakoids, again with values from thylakoids isolated at 0°C for comparison. The differences between non-phosphorylated and

TABLE I

FREEZE-FRACTURE PARTICLE DENSITIES (PARTICLES PER μm^2) OF CHLOROPLAST THYLAKOIDS

Values are ± S.E.

Face	Temperature			Significance level [a]
	0°C	20°C		
		Non-phosphorylated	Phosphorylated	
EFu	358 ± 11	370 ± 6	429 ± 10	0.027
PFu	4729 ± 27	4005 ± 21	4479 ± 26	0.001
EFs	1622 ± 12	1448 ± 12	1478 ± 9	0.050
PFs	6525 ± 30	6190 ± 29	6334 ± 33	0.061
EFu (holes)	3262 ± 25	3250 ± 24	3312 ± 20	–
PFu (large)	3290 ± 27	3253 ± 24	3219 ± 22	0.363
PFu (small)	1439	752	1260	–

[a] Comparison of non-phosphorylated and phosphorylated particle density by Student's *t*-test.

phosphorylated membranes are small, except for the PFs face particles, which are larger on average in phosphorylated thylakoids. Statistical analysis by the χ-squared test on the size distributions, revealed a significant difference only for the PFs face particles (Table III). These comparisons can be made graphically by referring to Fig. 7 in which frequency plots of particle area are shown for non-phosphorylated versus phosphorylated thylakoids.

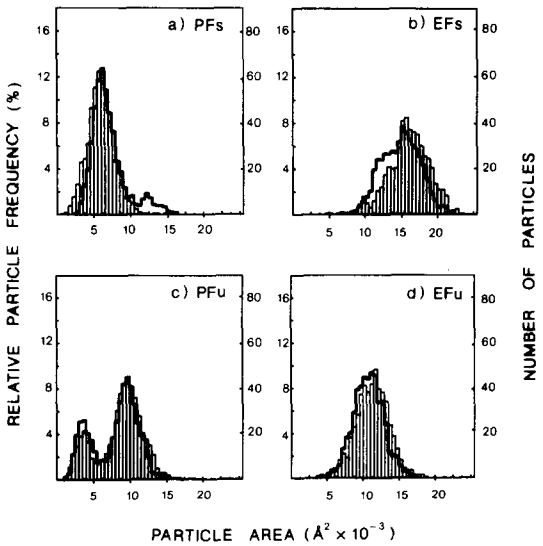

Fig. 7. Frequency histogram of particle size distribution for the four freeze-fracture faces of non-phosphorylated ($K_3Fe(CN)_6$) (thin lines) and phosphorylated ($Na_2S_2O_4$ + ATP) (thick lines) thylakoids prepared at room temperature.

TABLE II

FREEZE-FRACTURE PARTICLE SIZES OF CHLOROPLAST THYLAKOIDS

Values are in Å.

Face	Temperature		
	0°C	20°C	
		Non-phosphorylated	Phosphorylated
EFu	99 × 118	113 × 130	111 × 125
PFu	94 × 115	95 × 114	92 × 110
EFs	115 × 151	129 × 161	123 × 156
PFs	80 × 94	80 × 96	88 × 105

Calculations of the percentage of stacked membranes and of EFu particles (as a percent of total EF particles) are given in Table IV. These figures are based on the particle density values in Table I

TABLE III

STATISTICAL ANALYSIS OF PARTICLE SIZE DATA: NON-PHOSPHORYLATED VERSUS PHOSPHORYLATED THYLAKOIDS

df, degrees of freedom.

Face	χ^2 value	df	Signficance level
EFu	28.4	34	0.69
PFu	20.7	38	0.99
EFs	40.7	37	0.31
PFs	60.2	29	0.0006

TABLE IV

DISTRIBUTION OF EF PARTICLES

Treatment	% stacked [a]	% EFu particles
0°C, control	63.7	10.2
20°C, $K_3Fe(CN)_6$	72.4	8.9
20°C, $Na_2S_2O_4$ + ATP	68.8	11.7

[a] Calculated from EFs and EFu particle densities in Table I using the formula:

$x[\text{EFs}]+(1-x)[\text{EFu}]=1151$

where x is the fraction of stacked membranes.

and a destacked EF face density of 1151 particles per μm^2. It can be seen that there was about a 5% decrease in the extent of stacking after phosphorylation. It should be noted that incubation of thylakoids in the presence of dithionite alone had no significant affect on particle size or distribution.

Discussion

The conditions for isolation of the thylakoids for ultrastructural examination were designed to be as close as possible to the in vivo state. For this reason all operations prior to rapid freezing of the final pellet were carried out at room temperature, in the presence of either potassium ferricyanide, or sodium dithionite and ATP. It should be noted that there are differences in both particle density and particle sizes for all fracture faces in thylakoids prepared at 20°C compared with the more usual temperature of 0°C (Tables I and II). The ultrastructural appearance is not greatly altered, as, for example, occurs with membranes that undergo lipid phase transition, and can probably be attributed to vertical displacement of the particles with respect to the plane of the lipid bilayer [12,13].

Incubation of isolated chloroplast thylakoids in the presence of ATP and dithionite [2] or light [1] results in the phosphorylation of surface-exposed parts of the LHC. This causes a redistribution of excitation energy between the two photosystems so that a greater proportion is absorbed by PS I. The work of Haworth et al. [11,14,15] has shown that barley thylakoids are extensively phosphorylated and that, unlike peas, most of the phosphate is incorporated into LHC alone with perhaps 80% of this protein being phosphorylated. Upon phosphorylation the proportion of energy initially absorbed by PS I almost doubles, and there are similar increases in the amount of energy transferred from PS II to PS I. There is a decrease in the connectivity between PS II and LHC and between individual PS II centres as a result of phosphorylation. In other words, energy absorbed by LHC is less likely to be transferred to PS II centres and more likely to go to PS I. There is a distinct difference, however, in the changes induced by membrane phosphorylation, and those caused by cation-induced destacking. Haworth et al [14] state that LHC phosphorylation does not affect the degree of membrane stacking as defined by light scattering, in contrast to cation-depleted thylakoids.

It has been proposed that phosphorylation of LHC at the membrane surface (PSs) causes an increased net electrical charge and a partial destacking [16]. The freeze-fracture results show only a 5% decrease in the degree of stacking after phosphorylation (Table IV), as evidenced by changes in EFu particle density ($p = 0.027$). There is also an increase in the number of small PFu particles, from 752 to 1260 per μm^2 (Table I). The size of the particles is consistent with that of the PFs particle containing the light-harvesting [8] complex, again indicating movement of some grana particles into the stroma lamellae.

Approx. 10% of the particles on the PFs face of phosphorylated thylakoids are large and heavily shadowed. They have an average size of 111 × 131 Å, which is much greater than the smaller PFs particles (Table II), but similar to that of the large PFu particles (103 × 125 Å) [7]. Even excluding these large particles, there is a slight increase in the average size of PFs particles (84 × 100 Å vs. 80 × 96 Å).

Studies on the mechanism of restacking of cation-depleted thylakoids have shown that it occurs in several steps. Partial neutralisation of the negative charge on LHC allows initial membrane contact and the lateral aggregation of the small PF particles containing this protein [17]. Under these conditions EF particles are excluded from the areas of adhesion, but with increasing cation con-

centration, the EFs particle density increases due to entropic ordering. The important feature of this process, as far as particle movements are concerned, is the lateral aggregation of the LHC particle population, with the exclusion of the PS I particles, which remain in the unstacked region. Random lateral movements of the EF particles cause them to move into regions of membrane adhesion where they remain due to their affinity with the LHC particles. By analogy, an increase in the charge on LHC due to phosphorylation may allow penetration of stacked regions by some PS I PFu particles.

A 10% partial destacking, resulting from membrane phosphorylation, has been deduced from changes in the linear dichroism signal in the red chlorophyll absorption band resulting from changes in selective polarised light scattering [18]. This sort of change is consistent with the ultrastructural results reported in this paper. Haworth et al. [19] report particle movements upon phosphorylation of pea thylakoids. They find no movement of EFs particles out of grana partitions, with only LHC-containing PFs particles moving into destacked regions. In contrast to earlier work [14], they find a 23% decrease in grana stacking as measured by changes in 180° light scattering. This is significantly greater than found by measuring linear dichroism of pea thylakoids, and freeze-fracture EF particle distribution of barley thylakoids. No change in PFs particles was found, but the use of rotary shadowed freeze-fracture replicas in the present study allows a more sensitive measurement of small changes in particle size and shape. The large PFs particles are readily detected because they are taller than their neighbours, so they receive more platinum during shadowing and stand out due to their greater electron density.

The nature of these large PFs particles remains to be elucidated. Their appearance in the grana of phosphorylated thylakoids is the most significant change in particle size distribution to occur upon phosphorylation (Table III). There have been suggestions that there is a structural heterogeneity of PS I units in terms of Mg^{2+} effects due to their environment within the membrane [20], i.e., in stroma versus grana lamellae. On the other hand, analysis of grana partition membranes [6,21] and PS I mutants [7] indicates that the amount of PS I in grana, under normal isolation conditions, is very small, possibly zero. But, if PS I particles migrate into grana only as a result of phosphorylation of the LHC, this would only occur in thylakoids isolated in the presence of ATP. The movement of such fluorescent quenching centres into the grana may more readily account for the magnitude of the fluorescence decrease upon phosphorylation [11,22] than the movement of a number of LHC particles out of the grana.

To summarise, phosphorylation of the LHC results in a partial (5%) destacking of chloroplast thylakoid membranes, as evidenced by the movement of some of the PS II EFs particles and some of the PFs particles containing the LHC from grana to stroma lamella. Conformational changes concomitant with phosphorylation may be reflectd in a slight decrease in average EFs particle size and a slight increase in PFs particle size, possibly related to the decrease in connectivity between PS II and LHC. The appearance of a population of large PFs particles following phosphorylation may be a further manifestation of these conformational changes, or the result of a movement of PS I particles from stroma lamellae to the grana.

Note added in proof (Received September 7th, 1983)

A freeze-fracture investigation of the effect of phosphorylation on thylakoid ultrastructure was reported recently by Kyle et al. [23]. Isolated thylakoids in 200 μM ATP were phosphorylated by illumination with 500 $\mu E \cdot m^{-2} \cdot s^{-1}$ of white light for 20 min. Slight decreases in packing density of EFu and PFs particles and a decrease in PFu particle size were found. Phosphorylation was accompanied by a decrease in the extent of stacking, measured from cross-fractured thylakoids. These authors interpreted the data as indicating a movement of the light-harvesting PFs particles out of stacked regions, while the PS II-containing EFs particles remain in the stacked regions. Calculation of total numbers of the different types of particles from their data, however, shows that phosphorylation has apparently led to a loss of EFs particles (from 850 to 699), with no change in the number of EFu particles, and a loss of PFs particles (from 3748 to 2752) with only about half

this number appearing on the PFu face (from 1271 to 1785). Dephosphorylation led to the reappearance of the missing EFs and PFs particles, but a loss of EFu particles (from 221 to 159). It is difficult to see how the proposed structural changes can explain the increase in energy spillover directly from PS II to PS I to the extent observed under phosphorylating conditions. The high light intensity used (500 $\mu E \cdot m^{-2} \cdot s^{-1}$) would have caused considerable photoinhibition in isolated thylakoids, as confirmed by the incomplete reversibility of fluorescence changes. The effect of photoinhibition on thylakoid ultrastructure is not known, but it does cause fluorescence changes similar to those characteristic of membrane phosphorylation, namely, a decrease in F_{max} and a decrease in F_{685} relative to F_{735} [24].

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