Transient Inactivation of the Thylakoid Photosystem II Light-harvesting Protein Kinase System and Concomitant Changes in Intramembrane Particle Size during Photoinhibition of *Chlamydomonas reinhardtii*

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Abstract. Light-dependent reduction of the plastoquinone pool regulates the activity of the thylakoid-bound protein kinase which phosphorylates the light harvesting chlorophyll *a,b*-protein complex (LHC II) and regulates energy distribution between photosystems II (PS II) and I (Staehelin, L. A., and C. J. Arntzen, 1983, *J. Cell Biol.*, 97:1327–1337). Since reduction of plastoquinone by PS II is abolished in photoinhibited thylakoids due to loss of the secondary electron acceptor Q_B protein (Kyle, D. J., I. Ohad, and C. J. Arntzen, 1984, *Proc. Natl. Acad. Sci. USA*, 81:4070–4074), it was of interest to examine the activity of the LHC II protein kinase system during photoinhibition and recovery of PS II activity. The kinase activity was assessed both in vivo and in vitro in *Chlamydomonas* cells exposed to high light intensity (photoinhibition) and recovery at low light intensity. The kinase activity was progressively reduced during photoinhibition and became undetectable after 90 min. The inactive LHC II-kinase system could not be reactivated in vitro either by light or by reduction of the plastoquinone pool following addition of reduced duroquinone ($TMQH_2$). The LHC II polypeptides were dephosphorylated in vivo when cells, prelabeled with [^{32}P]orthophosphate before exposure to high light intensity, were transferred to photoinhibiting light in the presence of [^{32}P]orthophosphate. In vivo recovery of the LHC II-kinase activity, elicited by the addition of $TMQH_2$ to the assay system, did not require restoration of Q_B-dependent electron flow or *de novo* protein synthesis, either in the cytoplasm or in the chloroplast. Mild sonication of thylakoids isolated from photoinhibited cells restored the ability of the LHC II protein kinase system to be activated in vitro by addition to $TMQH_2$. Restoration of the light-activated LHC-II kinase required recovery of Q_B-dependent electron flow.

At the structural level, photoinhibition did not affect the ratio of grana/stroma thylakoids. A reduction of ~20% of the 11–17-nm intramembrane particles and an equivalent increase in the number of 6–10.5-nm particles was observed on the E-fracture faces of stacked thylakoid membranes. Similar but smaller changes were observed also on the E-fracture faces of unstacked thylakoid membranes (more 10–14-nm and less 6–9-nm particles) and P-fracture faces of stacked thylakoid membranes (more 6–8- and less 9.5–13-nm particles). All these structural changes were reversed to normal values during recovery of PS II activity. These results suggest that alteration of PS II organization caused by photoinhibition might be related to the transient inactivation of the LHC II-kinase system that is not related to the redox state of the PQ pool. The mechanism of these changes and their physiological role are not yet understood.

After Bennett's discovery (5) of a light-activated chloroplast membrane-bound protein kinase that phosphorylates the light-harvesting chlorophyll *a,b*-protein complex of photosystem II (LHC II)[1] polypeptides, many researchers have become interested in the mechanism and the physiological significance of this reaction. Based on the results of many laboratories, it is generally agreed (1, 6, 11, 12, 30) that the activity of the thylakoid protein kinase is regulated by the redox state of the plastoquinone (PQ) pool. When PQ is reduced, the kinase is activated and the phos-

1. *Abbreviations used in this paper*: EFs; E-fracture faces of stacked thylakoid membranes; EFu; E-fracture face of unstacked thylakoid membranes; LHC II, light-harvesting chlorophyll *a,b*-protein complex of photosystem II; PFs; P-fracture faces of stacked thylakoid membranes; PFu, P-fracture face of unstacked thylakoid membranes; PQ, plastoquinone; PS I, photosystem I; PS II, photosystem II; RC II, reaction center II.

phorylation of the LHC II polypeptides causes the mobile LHC II particles to become functionally uncoupled from the photosystem II (PS II) reaction center-bound LHC II complexes. Simultaneously, these mobile antennae migrate by diffusion from the grana to the stroma regions of the thylakoids, where they can associate with the photosystem I (PS I) complex, increasing its absorptive cross-section. When the PQ pool becomes oxidized due to a decrease in electron flow from PS II and an increase in electron flow from PQH_2 to NADP via PS I, the protein kinase is inactivated. Dephosphorylation of LHC II by a permanently active membrane-bound phosphatase facilitates its reassociation with PS II. This phosphorylation-dependent reversible migration of the mobile LHC II antenna particles has been correlated with the light-induced State I-State II transition (7, 10, 17) and with partial unstacking and restacking of the grana membranes (17, 30). Because of these effects, it appears to constitute the major mechanism for regulating the distribution of excitation energy between the two photosystems under limiting light conditions.

The above scheme would predict that, at saturating light intensities, the PQ pool would be reduced, the kinase activated, and the LHC II polypeptides completely phosphorylated. Such a situation should be advantageous to the plant, since with increasing light intensity the activity of PS II is damaged and electron flow activity gradually lost, a phenomenon described as photoinhibition (15, 22, 26). To this end, phosphorylation of the mobile LHC II particles and their functional dissociation from PS II units should reduce the absorptive cross-section of this photosystem and prevent its damage. Indeed, it has been suggested that the light-activated kinase may not only regulate energy distribution under limiting light conditions, but also play a protective role under light stress conditions (13).

We have demonstrated recently (15, 22) that photoinhibition is caused by the specific loss of the 32-kD Q_B protein, the secondary electron acceptor of PS II, due to an increase in its rate of degradation beyond its rate of *de novo* synthesis. However, loss of the Q_B-dependent electron flow to PQ should result in complete oxidation of the PQ pool, inactivation of the protein kinase, dephosphorylation, migration, and functional reassociation of the mobile LHC II particles with PS II complexes. If this were the case, no protection would be offered against light stress by the LHC II-protein kinase system. To test these opposing views, we have investigated the effect of photoinhibition on the activity of the thylakoid protein kinase in vivo and in vitro, and correlated these findings with changes in the morphology of the PS II

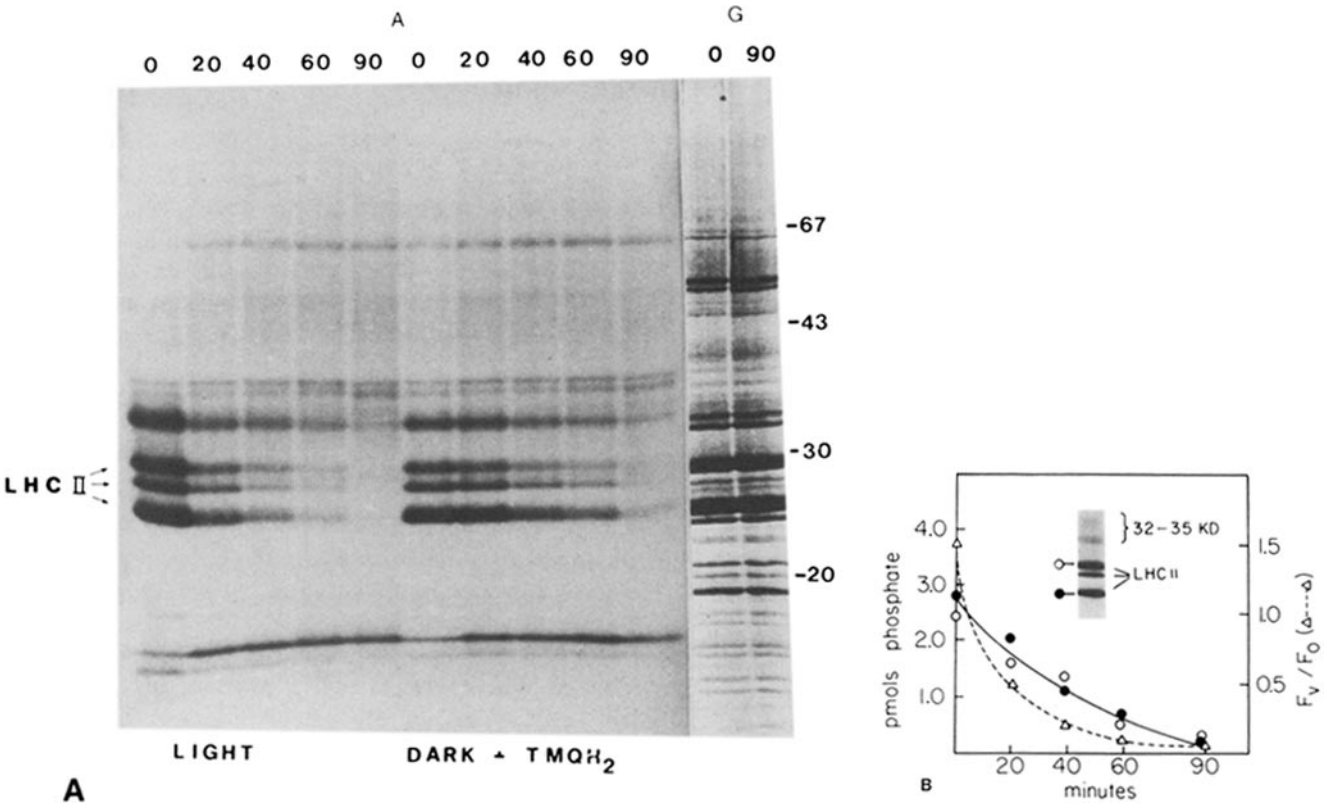


Figure 1. Time course of the loss of LHC II protein kinase activity during photoinhibition. *Chlamydomonas* cells were exposed to photoinhibitory light (2,500 W/m²) for up to 90 min and the degree of photoinhibition was assessed by measurements of variable fluorescence (the ratio of variable fluorescence to the intrinsic fluorescence; F_v/F_0) in the presence of 10 μM Diuron. Thylakoids were isolated at the times indicated and the activity of protein kinase assessed in vitro, either in the light or in the dark with addition of reduced duroquinone. No phosphorylation was obtained in the dark-incubated thylakoids in the absence of reduced duroquinone (data not shown). (*A*) Autoradiograms of thylakoid polypeptides resolved by lithium-dodecyl-sulfate-polyacrylamide gel electrophoresis. Numbers on right column, $M_r \times 10^3$. (*B*) Time course of loss of photosynthetic activity (F_v/F_0) and capacity to phosphorylate the 28-kD (○) and 24-kD (●) polypeptides of LHC II, measured by excising the stained bands and counting their radioactivity. Numbers at the top of *A*, times of photoinhibition in minutes. *A*, autoradiogram; *G*, stained gel.

complexes. The results presented here show that the LHC II polypeptides are dephosphorylated in photoinhibited membranes due to a temporary inactivation of the LHC II protein kinase that is unrelated to the redox state of the PQ pool. The dephosphorylation is, however, accompanied by a reduction in the size of the PS II particles seen on freeze-fractured membranes, suggesting that the dephosphorylated LHC II in photoinhibited membranes does not reassociate normally with the PS II complex.

Materials and Methods

Cell Growth and Preparation of Thylakoid Membranes

Chlamydomonas reinhardtii y-1 cells were grown in a semicontinuous culture apparatus in mineral medium, containing Na-acetate as a carbon source (23). Cells, at the end of the logarithmic phase of growth, were harvested by centrifugation, washed in fresh growth medium, and resuspended at a final concentration of $\sim 10^7$ cells/ml (nondividing conditions) (23). After exposure to the appropriate light conditions, the cells were pelleted by centrifugation, washed in 50 mM Tris-HCl buffer (pH 8.0), resuspended in the same buffer at 0°C at a final concentration of $\geq 10^7$ cells/ml, and broken open by passing through an ice-cold French pressure cell operated at 3,500 psi. For measurements of phosphorylation activity, the cell homogenate was layered on a sucrose solution (60% sucrose in 50 mM Tris-HCl [pH 8]) and centrifuged in a rotor (Type 40; Beckman Instruments, Inc., Fullerton, CA) at 100,000 *g* for 5 min. The membrane fraction at the buffer/sucrose interface was collected, washed by centrifugation in buffer, as above, and used immediately or stored at −80°C (28). For examination of the membrane structure by freeze-fracture, 5 mM $MgCl_2$ was added to the homogenization buffer, and the homogenate was layered over a discontinuous sucrose gradient prepared in the same buffer and containing 2, 1.75, 1.5, and 0.5 M sucrose (24). The gradients were centrifuged in a rotor (Type SW-27; Beckman Instruments, Inc.) for 30 min at 80,000 *g*, and the chloroplast membrane fraction at the interface between the 1.5 and 1.75 M sucrose collected, pelleted by centrifugation, and processed immediately for preparation of specimens for freeze-fracturing (17).

Photoinhibition, Recovery, and Measurements of Photosynthetic Activity

Cell suspensions were adjusted to a final cell concentration equivalent to 30 μg chlorophyll/ml (1.2–1.5 × 10^7 cells/ml) and exposed to white light supplied by a Tungsten-halogen lamp providing 2,500 W/m², as measured with a YSI-Kettering Model 65 radiometer (YSI Co. Inc., Yellow Spring, OH), at the surface of the cell suspension, contained in a water-jacketed double wall glass cylinder of 5 and 6.5 cm internal and external diameter, respectively. The suspension was constantly stirred by a magnetic stirrer and kept at 21 ± 1°C by water circulation. Samples of 10–20 ml were taken at timed intervals and processed for preparation of isolated thylakoids. Photosynthetic activity in whole cells was measured as the ratio of the variable fluorescence to the intrinsic fluorescence (28). A reduction of this ratio was found to correlate well with the loss of Q_B-dependent electron flow in photoinhibited *Chlamydomonas* cells (15). Actual rates of Q_B-dependent electron flow were also recorded as the reduction of 2,6-dichlorophenolindophenol by isolated thylakoids, using H_2O as an electron donor (29). For recovery from photoinhibition, the cells were washed by centrifugation and resuspended in fresh growth medium at the same concentrations (~30 μg/ml). The cells were incubated at 25°C in the same apparatus but the light intensity was reduced to 250 W/m². When used, chloramphenicol or cycloheximide (Sigma Chemical Co., St. Louis, MO) were added to a final concentration of 200 and 2 μg/ml, which inhibited 90% of chloroplast or cytoplasmic protein synthesis respectively (22).

Thylakoid Protein Phosphorylation In Vitro and In Vivo, and Gel Electrophoresis

Isolated thylakoids (0.2 mg chlorophyll/ml) were incubated for 5 min in 50 mM Tris-HCl buffer (pH 8.0) containing 10 mM $MgCl_2$, 0.15 Ci/mmol [γ-^{32}P]ATP, 7 μmol/ml, in the light (500 W/m²) or dark, or in the dark with 1 mM duroquinone reduced with Na-borohydride (2). Phosphorylation was stopped by rapid centrifugation in a microfuge (Beckman Instruments, Inc.), resuspension of the pellet in the electrophoresis sample buffer, and heating at 80°C for 5 min.

For measurement of thylakoid protein phosphorylation in vivo, the cells were pelleted by centrifugation, washed once, and resuspended in Tris-buffered growth medium without adding phosphate (2 × 10^7 cells/ml), and incubated in the light (250 W/m²) at 25°C for 20 min with the addition of [^{32}P]orthophosphate (0.5 Ci/mmol, 20 nmol/ml) (24). The cells were then washed and further processed, as above, to obtain isolated phosphorylated thylakoids.

Resolution of thylakoid polypeptides was carried out by denaturing polyacrylamide gel electrophoresis in the presence of lithium-dodecyl-sulfate, using the method described by Laemmli (18). The gels were stained with Coomassie Brilliant Blue R, dried, and exposed to x-ray film (29). In some experiments, the stained polypeptide bands were excised from the gel before drying, and Cerenkov radioactivity counted in a scintillation counter (Beckman Instruments, Inc.).

Chlorophyll was measured according to Arnon (4).

Freeze-fracturing and Analysis of Intramembrane Particle Size and Distribution

Membrane samples for freeze-fracturing were rapidly glycerinated (final concentration 30%, vol/vol) in the isolation medium, pelleted, and frozen in partly molten Freon 22 cooled with liquid N_2. Replicas were prepared according to standard procedures at −106°C in a 360-M freeze-etch apparatus (Balzers, Hudson, NH) (21) and were cleaned on commercial bleach overnight at room temperature. The electron micrographs were recorded and analyzed as previously described (3).

Results

Loss of Protein Kinase Activity of Isolated Thylakoids during Photoinhibition

As reported before, photosynthetic activity and variable fluorescence in vivo are rapidly lost when *Chlamydomonas* cells are exposed to high light intensity (15). When the protein kinase activity of thylakoids from cells exposed to photoinhibitory light for up to 90 min was assessed by incubation with [γ-^{32}P]ATP in the light, it was found that phosphorylation of the LHC II polypeptides was gradually lost (Fig. 1 *a*). Similarly, the time course of the inactivation of the LHC II kinase correlated well with the loss of variable fluorescence (Fig. 1 *b*). Previous studies have shown that the LHC II polypeptides (Fig. 1) and the chlorophyll content of the thylakoids remain constant during photoinhibition, while the 32-kD Q_B polypeptide is specifically degraded and Q_B-dependent electron flow to PQ is abolished (15, 22). For these reasons, inactivation of the LHC II kinase in photoinhibited membranes cannot be attributed to loss of the LHC II polypeptide substrate, but perhaps to complete oxidation of the PQ pool by PS I. Since the protein kinase can be activated in the absence of light-driven electron flow by reduction of the thylakoids with reduced duroquinone, one would expect the LHC II kinase of photoinhibited thylakoids to be reactivated by such a treatment. Fig. 1 *a*, however, demonstrates that reduced duroquinone was unable to activate the LHC II kinase of photoinhibited thylakoids under conditions that produced activation of the kinase of control membranes in the dark. It should be noted that while photoinhibition consistently blocked phosphorylation of the LHC II polypeptides, phosphorylation of polypeptides in the 32–35-kD range still occurred in some, but not all, experiments (compare Figs. 1, 3, and 4). This observation, together with the fact that phosphorylation of exogenous substrates such as casein was not abolished in photoinhibited thylakoids (data not shown), could be explained by the presence of more than

one kinase system activity (19, 20); apparently, only the kinase responsible for the phosphorylation of LHC II is consistently affected by photoinhibition. Inactivation of the LHC II kinase was also observed in experiments in which photoinhibition was carried out at 5°C (data not shown).

Loss of protein kinase during photoinhibition was also followed in vivo by pulse labeling of intact cells with [^{32}P]orthophosphate at various times of the photoinhibition process (Fig. 2). Using this assay, we found phosphorylation of the thylakoid polypeptides already drastically reduced after 30 min of photoinhibition. The phosphorylation was undetectable after 90 min of incubation in photoinhibitory light.

In a previous in vivo study of *Chlamydomonas* cells, we reported that thylakoid polypeptides are phosphorylated not only in the light, but also after incubation of the cells in the dark for up to 1 h (24). This phenomenon was explained by the finding that even in the dark the PQ pool can be reduced, presumably by reverse electron flow from reduced NADP and driven by ATP generated by respiration (chlororespiration) (8). Photoinhibited cells, however, exhibited only a residual thylakoid polypeptide phosphorylation activity in the dark after a previous exposure of 30–70 min to high light intensity (Fig. 2).

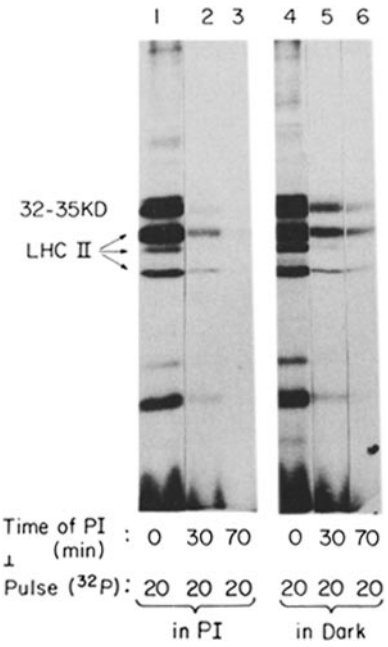


Figure 2. Decrease in the protein kinase activity in vivo during photoinhibition. *Chlamydomonas* cells were exposed to photoinhibitory light intensity for the time indicated. The cells were labeled during an additional 20 min in photoinhibitory light (lanes *1–3*) or, in the dark (lanes *4–6*) with [^{32}P]orthophosphate. Thylakoid membranes were then isolated, and the radioactivity of the various polypeptides detected by autoradiography subsequent to their separation by lithium-dodecyl-sulfate-polyacrylamide gel electrophoresis. *PI*, photoinhibition. The radioactivity at the bottom of the gel is due to ^{32}P-labeled phospholipids. Note that the phosphorylation of thylakoid polypeptides in the photoinhibited cells labeled with [^{32}P]orthophosphate in the dark (lanes *5* and *6*) is higher than that obtained in photoinhibitory light (lanes *2* and *3*). This could be due to a partial recovery of kinase activity in the dark incubated cells as compared with a continuous loss of activity in the light incubated cells, in which photoinhibition continued during the labeling period.

Dephosphorylation of Thylakoid Polypeptides In Vivo during Photoinhibition

The results presented above strongly suggest loss of activity of the thylakoid LHC II protein kinase system in photoinhibited cells. However, an alternative explanation needs to be considered, i.e., that during the initial phase of photoinhibition, the kinase is completely activated and may cause a rapid and exhaustive phosphorylation of all LHC II sites available. If the phosphatase responsible for the turnover of the esterified phosphate is inactivated in the thylakoids of photoinhibited cells, no sites will be available for additional phosphorylation by [^{32}P]orthophosphate, either during the pulse labeling experiments in vivo (Fig. 2) or in vitro when using thylakoid isolated from cells exposed to high light intensity (Fig. 1). To test this alternative possibility, intact cells were incubated for 30 min in low light and then exposed to photoinhibitory light. [^{32}P]Orthophosphate was added at the onset of illumination and was not removed during the entire incubation period. At various times, cell samples were taken, and the degree of thylakoid protein phosphorylation examined by autoradiography of lithium-dodecyl-sulfate-polyacrylamide gel electrophoresis of isolated thylakoids. The control consisted of a cell sample kept at low light intensity (250 W/m^2) for the duration of the experiment. The results (Fig. 3) show that initially the low light thylakoid polypeptides were phosphorylated in low light, and that, after exposure to photoinhibitory light, the degree of their phosphorylation gradually declined and, finally, after 90 min

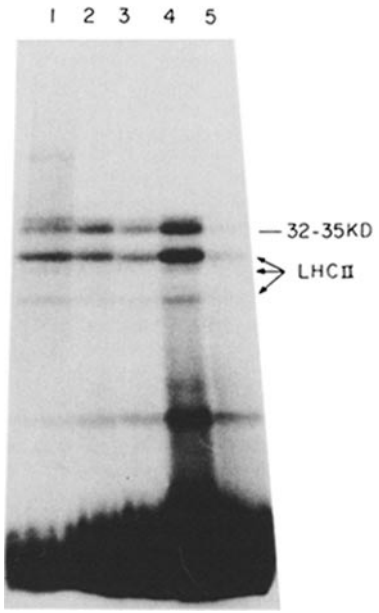


Figure 3. In vivo dephosphorylation of LHC II during photoinhibition of *Chlamydomonas* cells. *Chlamydomonas* cells were incubated in low light intensity (250 W/m^2) in the presence of [^{32}P]orthophosphate for 30 min. Part of the cell suspension was transferred to high light intensity (2,500 W/m^2) and incubation continued for 90 min. At timed intervals, samples were taken for isolation of thylakoid membranes, and the radioactivity of various polypeptides detected by autoradiography of electrophoretograms. Lanes *1*, *2*, and *4*, cells incubated in low light intensity for 30, 60, and 120 min, respectively; lanes *3* and *5*, cells incubated in low light intensity for 30 min and then subjected to high light intensity for 30 and 90 min, respectively. The high radioactivity at the bottom of the autoradiogram is presumably due to ^{32}P-labeled phospholipids.

of photoinhibition, completely disappeared. In contrast, cells kept in low light exhibited continued, strong phosphorylation of both the LHC II and the 32–35-kD polypeptides. Dephosphorylation of the thylakoid polypeptides in vivo during photoinhibition could not be ascribed to the inability of the cells to synthesize ATP, since examination of the autoradiogram in Fig. 3 shows a continuous, time-dependent increase in the amount of radioactivity of the thylakoid phospholipids, as well as an increase in the phosphorylation of the lower molecular mass polypeptides of ~13–14 kD. These results demonstrate that the thylakoids become dephosphorylated in vivo due to inactivation of the thylakoid LHC II protein kinase system.

Reactivation of the Thylakoid Protein Kinase during Recovery from Photoinhibition

Photoinhibited *Chlamydomonas* cells recover their photosynthetic PS II activity within 3–5 h when incubated in light of low intensity (22). However, recovery of the light-elicited thylakoid kinase activity, as assessed in vitro, occurred much faster than that of photosynthetic electron flow, and was already completed after 90 min (Fig. 4), when only ~20–25% of PS II electron flow activity was restored (data not shown, also reference 22). This could be explained if one considers that in the in vitro assay electron acceptors are not added, and even a low rate of light-driven electron flow will be sufficient to reduce the PQ pool and activate the kinase (22). Recovery of photosynthetic electron flow requires *de novo* protein synthesis and integration of the chloroplast encoded 32-kD Q_B protein into the preexisting PS II units (22), and is inhibited by chloramphenicol but not by cycloheximide (22). The recovery of the light-stimulated kinase activity is also inhibited by chloramphenicol but not by cycloheximide (Fig. 5, left). These results show that recovery of the light-dependent kinase activity requires restoration of PS II electron flow.

The kinase activity of normal thylakoids can be elicited also in absence of light-driven PS II activity by the addition of reduced duroquinone in the dark (Fig. 5). However, as mentioned above, reduced duroquinone does not activate the kinase of thylakoids from photoinhibited cells (Fig. 5). The question thus arises as to whether activation of the kinase in these thylakoids by reduced duroquinone requires also restoration of the potential for light-driven PS II activity and synthesis of the 32-kD Q_B protein or additional thylakoid polypeptides of chloroplast origin as yet unidentified. Fig. 5 (right) shows that reduced duroquinone activates the kinase of thylakoids from photoinhibited cells that have been incubated in light of low intensity (recovery conditions) in the presence of chloramphenicol.

Reversible Changes in the Size of Intramembrane Particles during Photoinhibition and Recovery

Photoinhibition had little effect on the ratio of stacked to unstacked thylakoid membrane regions, which remained at 1.5 throughout the experiments as determined by length

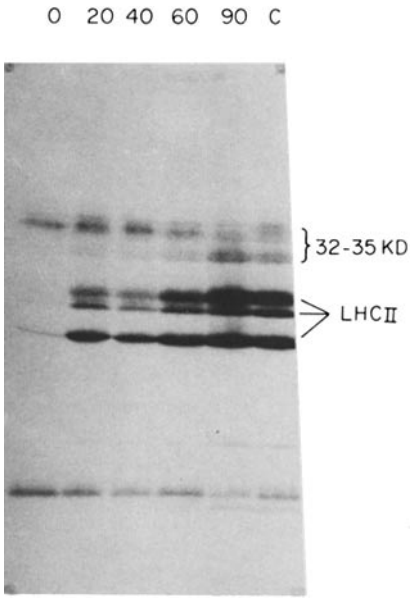


Figure 4. In vivo recovery of the light dependent LHC II protein kinase activity of photoinhibited cells. *Chlamydomonas* cells were exposed to photoinhibitory light conditions for 90 min and then allowed to recover in low light for 90 min. At the indicated times, samples were taken for isolation of thylakoid membranes and measurement of their light-dependent protein kinase activity in vitro by the addition of [γ-^{32}P]ATP and incubation in the light, as described in Materials and Methods. Only the autoradiogram of the phosphorylated thylakoid polypeptides resolved by lithium-dodecyl-sulfate-polyacrylamide gel electrophoresis is shown. Numbers on the top of the figure, times of recovery in minutes; C, thylakoid of control cells that were kept at low light intensity for 90 min.

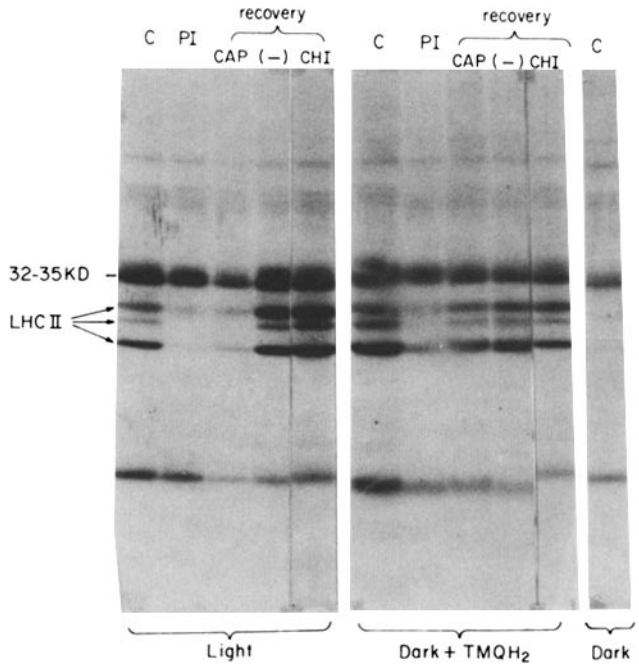


Figure 5. Effect of protein synthesis inhibitors on the in vivo recovery of the protein kinase activity of photoinhibited cells. Cells exposed to photoinhibitory light (PI) for 90 min were incubated for 3 h at 25°C in light of low intensity (250 W/m^2) in the absence (−) or presence of chloramphenicol (CAP) or cycloheximide (CHI). Thylakoids were isolated and their protein kinase activity assessed in vitro either under low light or in the dark with addition of reduced duroquinone ($TMQH_2$). C, thylakoids isolated from control, nonphotoinhibited cells. Recovery of photosynthetic activity measured by increases in the variable fluorescence of intact cells was 90% of the control value in the cells incubated with CHI and 30% in the cells incubated with CAP.

Table I. Effect of Photoinhibition on Particle Density of Chlamydomonas *Thylakoids*

Fracture face*	Particles/μm² ± SE		
	Control	Photoinhibited	Recovered
EFs	1,749 ± 45	1,359 ± 47	1,549 ± 32
EFu	596 ± 23	625 ± 42	565 ± 16
PFs	4,464 ± 162	4,176 ± 145	4,029 ± 246
PFu	3,700 ± 83	3,334 ± 164	3,285 ± 185

* Micrographs of five different chloroplasts were measured for each fracture face.

measurement of cross-sectioned thylakoids. However, our freeze-fracture micrographs revealed that photoinhibitive light conditions caused a 10–20% drop in density of particles on all fracture faces (Table I), as well as statistically significant changes in EFs, EFu, and PFs particle sizes (Figs. 6–11). All of these changes were reversed in parallel with the recovery of normal photosynthetic electron transport functions.

The most dramatic changes in particle sizes were observed for the EFs particles, where after 90 min of high light treatment, a loss of ~20% of the 11–17-nm-diam particles occurred concomitant with a quantitatively similar increase in 7- to 10.5-nm particles (Figs. 6, 7, 10, and 11). The less pronounced changes in EFu particle sizes involved a slight increase in 10–14-nm particle densities and a proportional decline in 6–9.5-nm particles (Figs. 10 and 11). The PFs particles, finally, displayed an increase in the 6–8-nm and a decrease in the 9.5–13-nm particle size ranges (Figs. 6, 9, 10, and 11). Virtually no changes were seen in PFu particle sizes (Figs. 10 and 11). Thylakoids derived from photoinhibited cells that were allowed to recover for 4 h under low light growth conditions exhibited a nearly complete reversal of all of the above listed particle size changes (Figs. 8, 10, and 11). In contrast, a significant recovery of particle densities was only observed for the EFs particles during the same time period (Table I). Longer recovery times were not assayed.

In Vitro Reactivation of the Thylakoid Protein Kinase of Photoinhibited Cells

Previous studies have correlated the 10–18-nm EFs particles with intramembrane protein complexes consisting of a reaction center II (RC II) core complex surrounded by variable amounts of LHC II antennae (3, 30). Thus, the apparent conversion of 11–17-nm EFs particles into 6–10.5-nm EFs particles during photoinhibition suggests that photoinhibition leads to destabilization of the larger RC II–LHC II complexes such that they give rise to smaller freeze-fracture particles. Such a change might be expected if photoinhibition caused dissociation of LHC II units from the RC II complex. If this were the case, changes in energy transfer from LHC II to RC II should be detected.

We have observed earlier that the rate of reduction of the primary electron acceptor of RC II, Q_A, increases during the initial stages of photoinhibition, as evidenced by a significant reduction of the variable fluorescence rise time measured in the presence of Diuron (15). We have also measured the light intensity required for 50% saturation of silicomolybdate reduction by RC II and have found no differences between control and photoinhibited thylakoids (data not shown). These results indicate that if the observed structural changes were indeed caused by dissociation of the RC II–LHC II complex, they were insufficient to disturb energy transfer from the completely dephosphorylated LHC II to RC II. However, this still leaves open the possibility that the structural changes seen in freeze-fractured membranes are related to discrete conformational changes in the organization of the protein kinase system or in its interaction with the reduced PQ activating system. The recovery process is even less well understood, but seems to involve some thermal- or energy-requiring process, since no reactivation of the protein kinase was observed within 2 h when the cells were kept at 0°C during recovery.

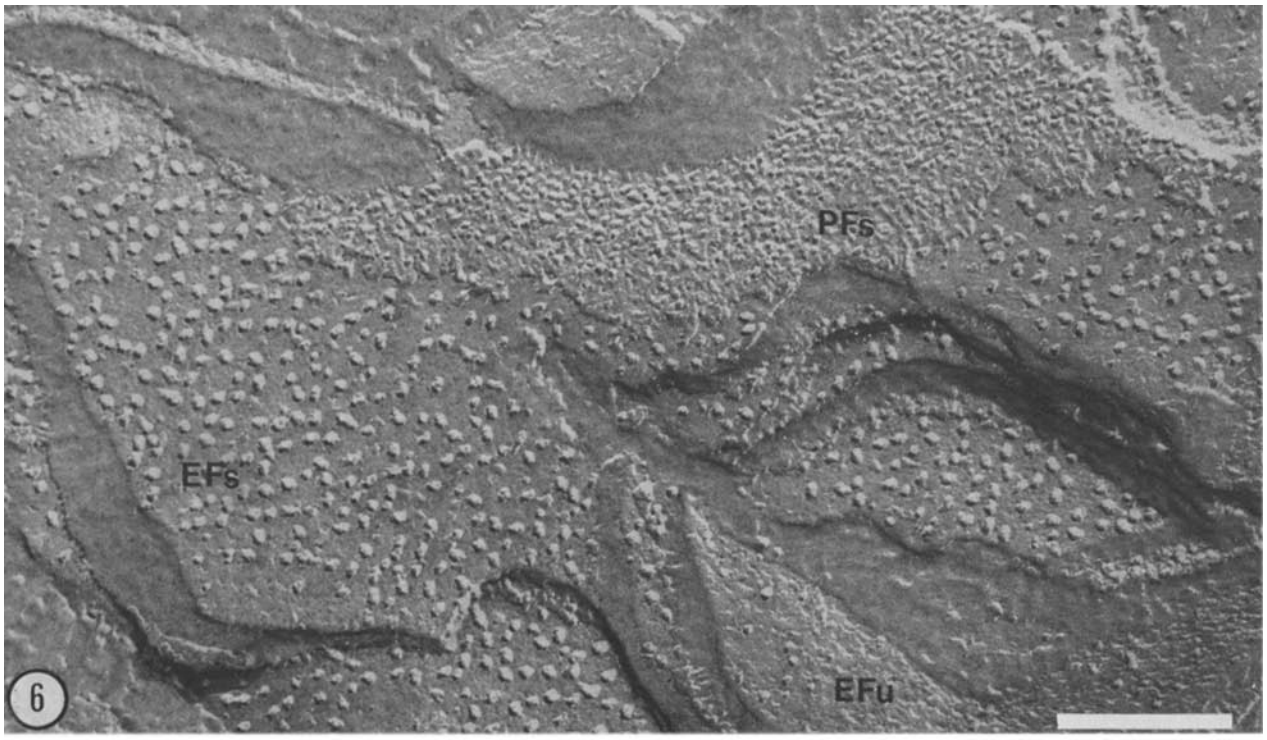
During this study we noted that in some of the experiments described above the degree of inactivation of the protein kinase was less than expected, as judged from the extent of loss of PS II activity due to photoinhibition. An examination of the protocol of these particular experiments disclosed that the isolated thylakoids were frozen at −80°C after extraction from the cells and before the phosphorylation assay in vitro. When thylakoids obtained from photoinhibited cells were tested for their kinase activity immediately after isolation or after freezing and thawing, we found that the latter procedure resulted in a partial reactivation of the protein kinase as assessed in the presence of reduced duroquinone. As Fig. 12, lanes *1–3* shows, mild sonication (30 s, 0°C) of freshly prepared thylakoids obtained from photoinhibited cells can also be used to reactivate the protein kinase. Furthermore, the same treatment exhibited no deleterious effects on the protein kinase of control thylakoids (Fig. 12, lane *4*).

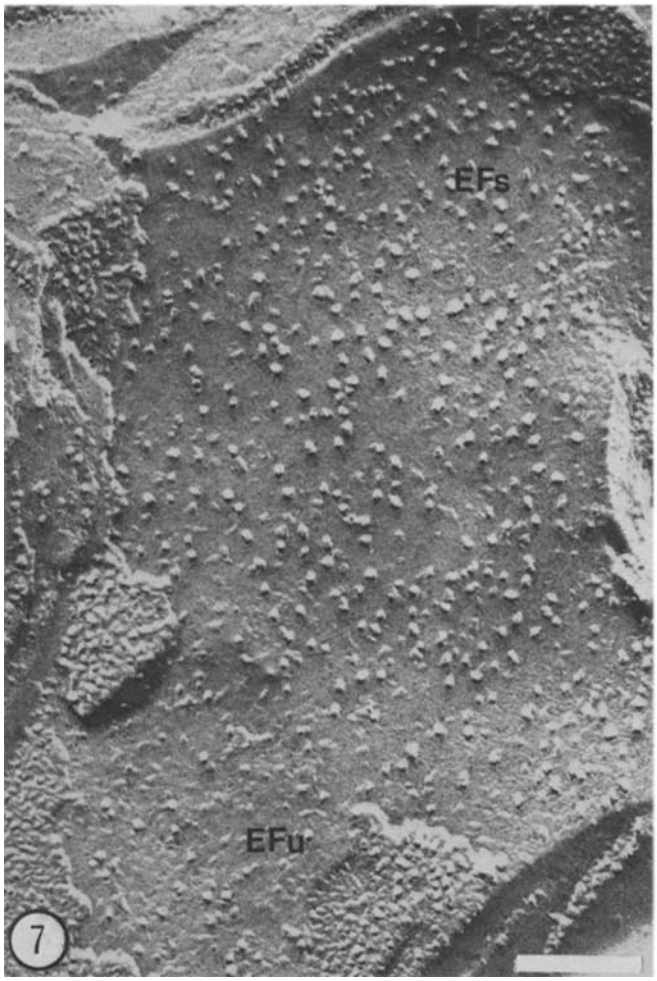
Discussion

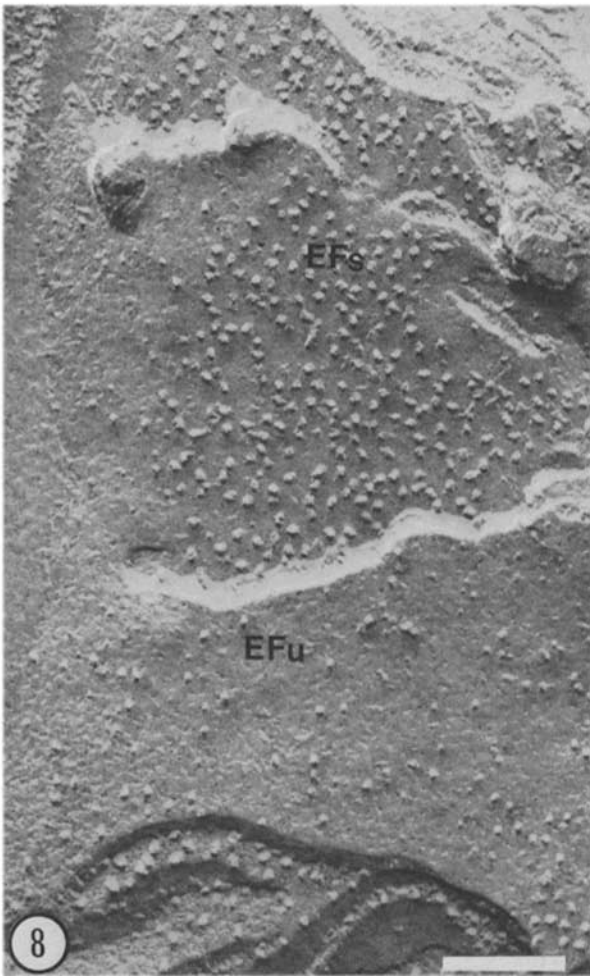
The presence of a redox controlled protein kinase which phosphorylates the mobile LHC II antenna and regulates distribution of excitation energy between the two photosystems is well established, and seems to be a general feature of higher plants and most eukaryotic algae (30), but not of prokaryotic photosynthetic microorganisms and red algae (9, 28). The question arises as to what role this regulatory system may play when the available light energy reaches levels at which photosynthesis is light-saturated and eventually causes damage to PS II by increasing the degradation rate of the 32-kD Q_B protein beyond the capacity of the chloroplast to replace it by *de novo* synthesis (15, 22).

The expected response of the LHC II protein kinase system

Figures 6–8. (Fig. 6) Freeze-fracture electron micrograph of *Chlamydomonas* thylakoid membranes isolated from control cells and depicting typical EFs, EFu, and PFs. Note the homogeneity in size of the large EFs particles. Bar, 0.2 μm. (Fig. 7) Freeze-fractured, photoinhibited thylakoid membranes of *Chlamydomonas* cells exposed to high light conditions for 90 min. The EFs particles appear both smaller and more heterogenous in size than those of control membranes (see Fig. 6). Bar, 0.2 μm. (Fig. 8) Freeze-fractured thylakoid membranes from *Chlamydomonas* cells allowed to recover from photoinhibition for 4 h. The EFs particles appear more similar in size to those of control thylakoids (Fig. 6), than of photoinhibited membranes (Fig. 7). Bar, 0.2 μm.


PFs
EFs
EFu
6


EFs
EFu
7


EFs
EFu
8

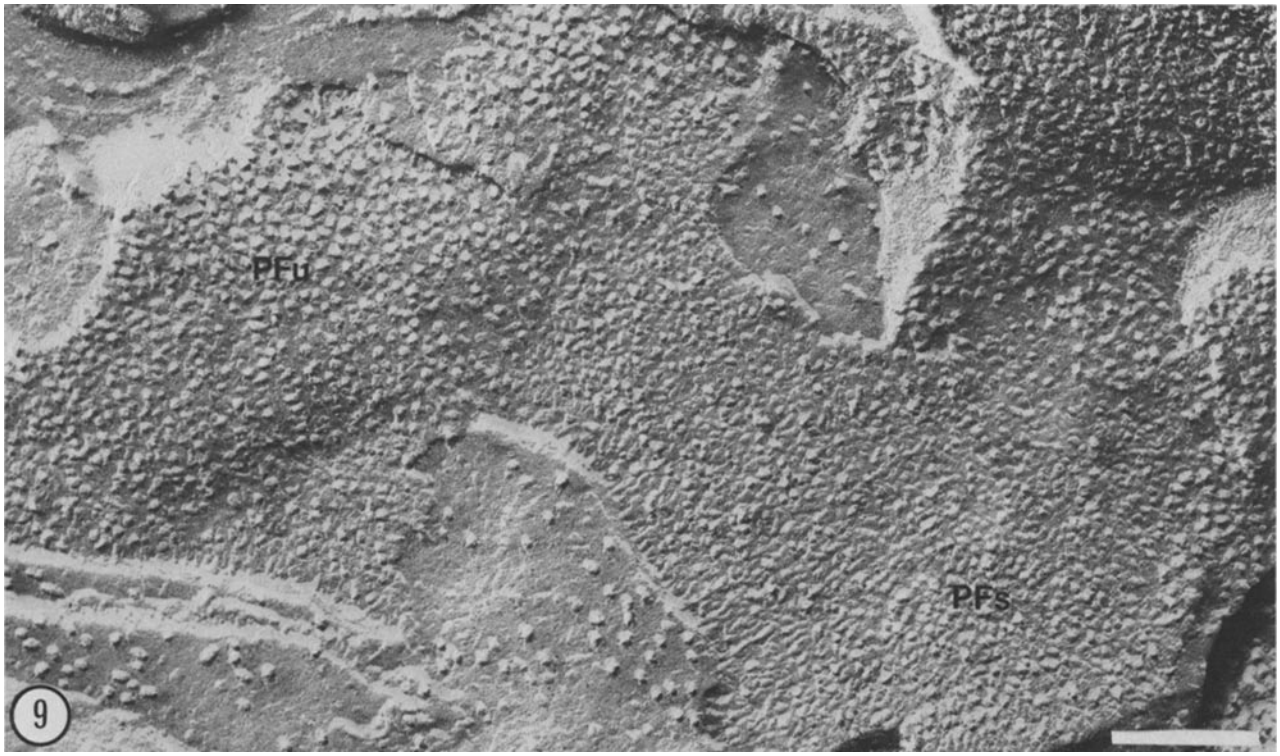


Figure 9. Freeze-fracture micrograph illustrating typical PFs and PFus of photoinhibited thylakoids. Bar, 0.2 μm.

to high light intensity, which should result in reduction of the plastoquinone pool, would be a complete phosphorylation of the LHC II polypeptides and maximal dissociation of the mobile LHC II antennae from the PS II complex, to reduce its absorptive cross-section. With increasing photo damage and loss of the Q_B-dependent electron flow, one would anticipate a gradual decrease in the ratio PQ^{-}/PQ^{++}, which eventually would reach a level at which the kinase is no

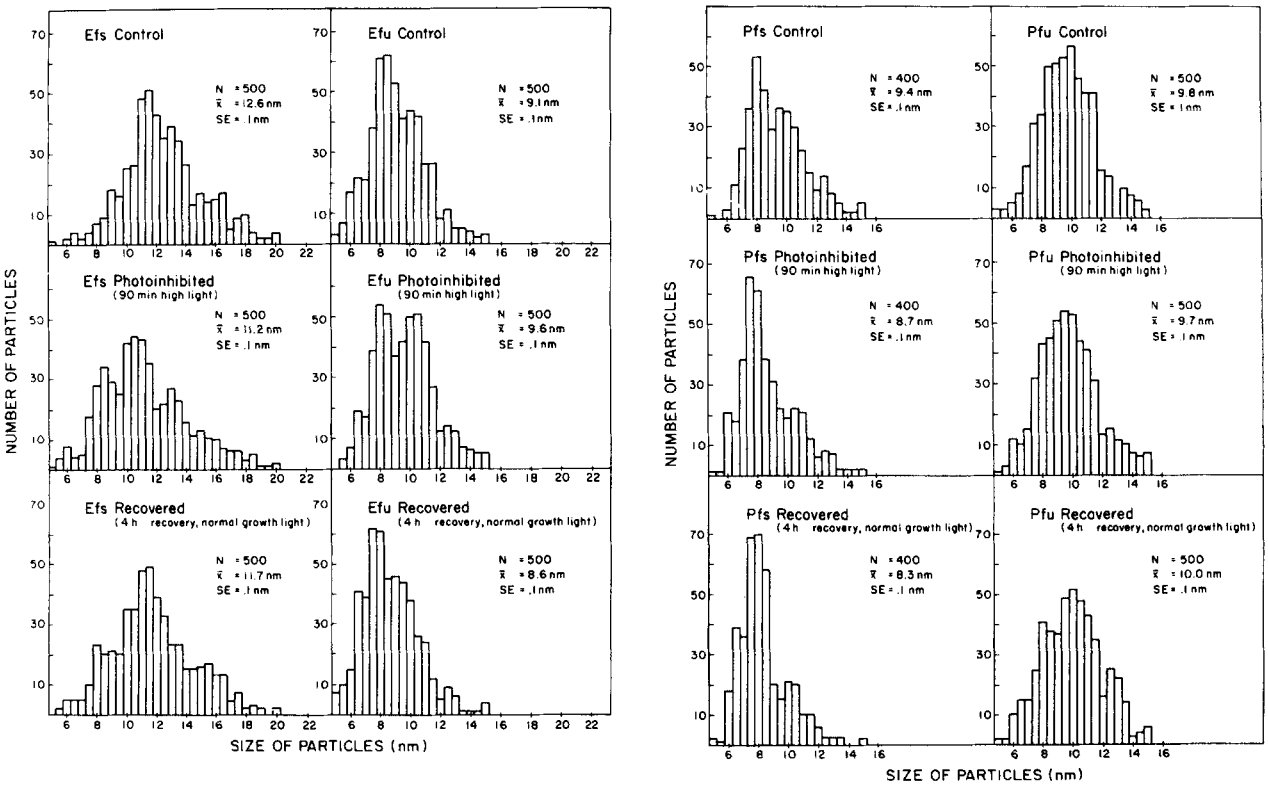


Figure 10. Histograms of intramembrane particle sizes on EFs, EFu, PFs, and PFu of thylakoids obtained from control, photoinhibited, and recovered *Chlamydomonas* cells.

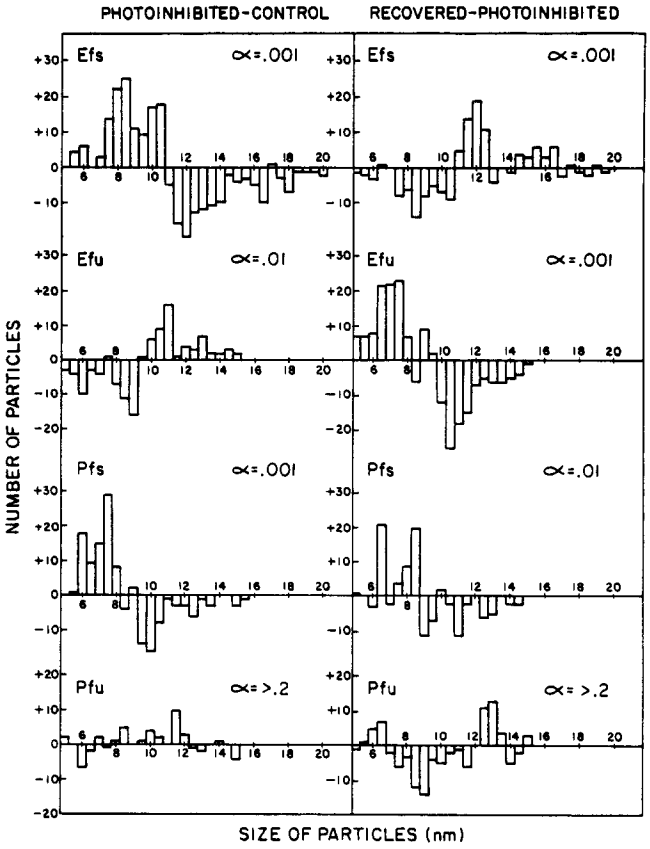


Figure 11. Differential histograms showing differences in intramembrane particle sizes between control, photoinhibited, and recovered *Chlamydomonas* thylakoids. The α-values indicate levels of significance of the differences between the particle size histograms as determined by the Kolmogorov-Smirnov (27) test.

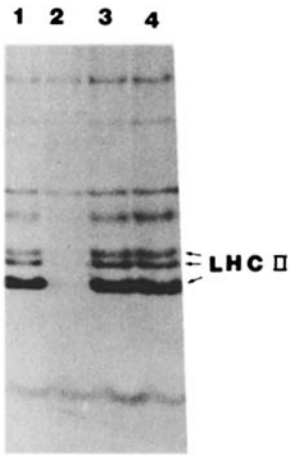


Figure 12. In vitro reactivation of the thylakoid protein kinase of photoinhibited cells by mild sonication. *Chlamydomonas* cells were incubated under photoinhibitory light for 90 min; the thylakoids were then isolated and the activity of protein kinase assessed in vitro in the dark with addition of reduced duroquinone. Lane *1*, control thylakoids isolated from non-photoinhibited cells; lane *2*, thylakoids obtained from photoinhibited cells; lane *3*, thylakoids as in lane *2* were sonicated at 0°C in a Ladd Research Industries Inc. (Burlington, VT) sonicator for 30 s; lane *4*, thylakoids as in lane *1*, sonicated as in lane *3*.

longer activated and LHC II would become dephosphorylated. At this stage, the dephosphorylated LHC II should reassociate with the PS II complex and, by increasing its absorptive cross-section, further accelerate its light-induced inactivation.

Based on the present knowledge of the regulatory mechanism governing the LHC II-protein kinase interaction (1, 5–7, 10–12, 17, 30) and the fact that the PS II-associated 32-kD Q_B protein is present in only one copy per electron transfer chain (14, 16, 31), while the PQ pool contains 8–10 molecules/chain and is free to diffuse rapidly and mix throughout the bilayer plane of the membrane, the following testable predictions can be made: (*a*) During photoinhibition, the loss of PS II activity and Q_B-dependent electron flow should be directly proportional to the loss of the 32-kD Q_B protein. Indeed, this prediction is in agreement with experimental results (15). (*b*) The PQ pool should be able to accept electrons from any residual, active Q_B site. Thus, at photoinhibitory light intensity when electron flow is light saturated, generation of reduced PQ and phosphorylation of LHC II should continue even when a significant fraction of the electron transfer chains have lost their active Q_B sites. Hence, a lag in the dephosphorylation of LHC II in vivo and inactivation of the light dependent protein kinase in vitro would be expected relative to the time course of the loss of PS II activity. (*c*) The LHC II kinase system should lose its light-dependent activity in thylakoids depleted of Q_B sites, since no PQ reduction should occur in the light. However, the kinase should still be activated if the PQ pool is artificially reduced by the addition of appropriate reducing agents.

The experimental results presented here, however, show no major lag in the dephosphorylation of LHC II in vivo or the inactivation of the protein kinase assessed in vitro. Both seem to follow the same time course as that of photoinhibition, within the resolution of the time points used. Experiments in which the activity of the LHC II kinase system was assessed immediately upon the onset of exposure to high light intensity and at close time intervals thereafter, showed no initial increase in the phosphorylation activity nor a lag in its loss (data not shown). Furthermore, the LHC II protein kinase system could not be activated by reduced duroquinone in isolated photoinhibited membranes. These results are not compatible with the kinase activation mechanism discussed above, which appears to operate only at low light intensities. We conclude that the lack of phosphorylation of the LHC II polypeptides by the protein kinase at high light intensity is not related to the redox state of the PQ pool. A possible explanation for the reversible inactivation of the LHC II kinase system in membranes exposed to high light intensity is that degradation products of the 32-kD Q_B protein, or other membrane components, may interfere with the LHC II-kinase interaction. Reactivation of the LHC II-kinase might then require the removal of such inhibitors, either due to metabolic activity in vivo or the in vitro mild sonication procedure as described here.

Our conclusion is supported by all the experimental data presented here, including (*a*) inability of the protein kinase system to respond to reducing agents in photoinhibited membranes, (*b*) time- and temperature-dependent recovery in vivo of enzyme activity that can be elicited by reducing agents independent of *de novo* protein, synthesis and of reactivation of electron flow by *de novo* synthesis with integration of nascent 32-kD Q_B protein, and (*c*) in vitro recovery of the LHC II kinase activity by physical disturbance of the membrane by freezing and thawing or mild sonication. Based on these observations, we suggest that at high light intensity, and possibly because of increased 32-kD Q_B turnover and degradation, structural changes are induced in the organization of the PS II complex resulting in a transient inactivation of the LHC II protein kinase system. These changes are presumably also related to the changes in EFs

particle sizes documented in Figs. 7, 8, 10, and 11. However, we do not know whether the conversion of the larger EFs particles into smaller ones during photoinhibition is directly related to the destruction and/or removal of the 32-kD Q_B protein from the PS II complex, or to the inactivation of the LHC II protein kinase system. Alteration of the PS II complex structure brought about by high light intensity is also manifested by loss of variable fluorescence and reduction of the fluorescence quantum yield (15) as well as alteration of the accessibility of RC II-Q_A to various electron acceptors (Siderer, Y., and I. Ohad, unpublished observations).

It has been suggested that phosphorylation of PS II complex polypeptides, including LHC II reaction center polypeptides of 44–47 kD and the 32–35-kD polypeptides (24), might play a role in the assembly of the PS II complex (25). Hence, one should consider that complete dephosphorylation of thylakoid polypeptides induced by photoinhibition in vivo, as reported here, might be part of the underlying mechanism responsible for the observed structural changes and the inactivation of the LHC II protein kinase system. It is attractive to consider that these changes might alter the pattern of deexcitation of LHC II and eventually help to dissipate the light and heat energy absorbed at high photon fluency by radiationless transition. The mechanism of these changes and their possible physiological significance remain to be studied.

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