Structural Changes and Lateral Redistribution of Photosystem II during Donor Side Photoinhibition of Thylakoids

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Abstract. The structural and topological stability of thylakoid components under photoinhibitory conditions (4,500 $\mu E \cdot m^{-2} \cdot s^{-1}$ white light) was studied on Mn depleted thylakoids isolated from spinach leaves. After various exposures to photoinhibitory light, the chlorophyll-protein complexes of both photosystems I and II were separated by sucrose gradient centrifugation and analysed by Western blotting, using a set of polyclonals raised against various apoproteins of the photosynthetic apparatus. A series of events occurring during donor side photoinhibition are described for photosystem II, including: (*a*) lowering of the oligomerization state of the photosystem II core; (*b*) cleavage of 32-kD protein D1 at specific sites; (*c*) dissociation of chlorophyll-protein CP43 from the photosystem II core; and (*d*) migration of damaged photosystem II components from the grana to the stroma lamellae. A tentative scheme for the succession of these events is illustrated. Some effects of photoinhibition on photosystem I are also reported involving dissociation of antenna chlorophyll-proteins LHCI from the photosystem I reaction center.

INHIBITION of photosynthesis by light, a phenomenon referred to as photoinhibition (44), is a common feature of all oxygenic organisms. At the molecular level, one main target of photoinhibition is photosystem II (PSII),[1] whose electron transport activity is severely impaired by excess illumination. Two different photoinhibition mechanisms have been suggested to be operative under different experimental conditions: an acceptor side mechanism involving over-reduction of the plastoquinone acceptors which depends on the presence of oxygen (32, 50, 52), and a donor side mechanism, involving accumulation of the strongly oxidant species Tyr_z^+ and/or P_{680}^+ and independent of the presence of oxygen (27, 54). Irrespective of the particular mechanism for electron transport damage, photoinhibition is accompanied by an increased rate of degradation of the reaction center D1-protein (6, 7, 24, 26, 51). In the light D1 turns over at a rate higher than any other polypeptide of the thylakoid membrane and this increases with light intensity (35). After this observation the loss of PSII activity under photoinhibitory conditions has been attributed to accelerated degradation, exceeding the biosynthetic rate of D1-protein (40). According to this hypothesis, recovery from photoinhibition depends on the substitution of damaged D1-protein with the newly synthesized one within the PSII structure (30). Biosynthesis of the 32-kD D1 polypeptide has been widely studied. Encoded by the plastidial *psbA* gene (13), the protein is translated on 70S ribosomes on the stroma-exposed surface of the thylakoid membrane (23), into which the protein is inserted in the form of a precursor containing an extra sequence of 9–10 amino acid residues at the COOH-terminus (33, 46). A number of posttranslational modifications such as COOH-terminal proteolytic processing to its definitive molecular size (18, 33), palmitoylation (34), and phosphorylation (37, 53) are involved in the maturation of the protein. As a result, the protein migrates from the stroma-exposed membranes to the grana compartment, where it is found assembled with other core proteins to form a functional PSII center. However, migration of the newly synthesized D1-protein does not occur as a "free" polypeptide but already associated with the other chlorophyll-proteins of the PSII complex (1). According to this scheme, photoinhibited PSII centers, initially located in the grana, migrate to the stroma lamellae where, after removal of the damaged D1-protein, they act as receptors for the newly synthesized protein (1), the cycle ending with the back migration of the repaired centers to the grana (36, 41).

We have recently shown that, after photoinhibition of isolated thylakoids, the degradation products of D1 are preferentially localized in the stroma lamellae (5), a result which confirms that damaged D1 migrates from the grana to the stroma-exposed region. However, whether migration of the D1 fragments takes place as "free polypeptides" or still integrated within the PSII core has not been investigated. This particular point is dealt with in the present study, in which changes in the topology and organization of both PSI and PSII are reported to occur as a consequence of high light illumination. A succession of events is also proposed like those which start the repair cycle by which damaged PSII centers are brought back to full activity. These involve, in order: (*a*) lowering of the oligomerization state of the PSII core (probably a transition from dimer to monomer); (*b*) cleavage of D1

1. *Abbreviations used in this paper*: CP, chlorophyll protein; LHC, light harvesting complex; OEE, oxygen evolving enhancer; PS, photosystem.

at specific sites; (*c*) dissociation of CP43 (chlorophyll-protein) from the PSII core; and (*d*) migration of CP43 and of the remaining part of the core to the stroma-exposed lamellae where the D1 fragments are released and further metabolized.

Materials and Methods

Isolation of Thylakoids and Photoinhibition Experiments

Leaves of spinach were ground in a buffer containing 0.33 M Sorbitol, 50 mM Tricine pH 7.8, 5 mM $MgCl_2$, 15 mM NaCl, and thylakoid membranes were centrifuged for few seconds at 6,000 *g*. After resuspension in 50 mM Hepes, pH 7.2, 5 mM $MgCl_2$ and 15 mM NaCl, the thylakoids were once again pelletted and resuspended in the same buffer supplemented with 0.1 M Sorbitol (Hepes/Sorbitol buffer) at a chlorophyll concentration of 0.2 mg/ml. For Tris-washing, thylakoids were resuspended in 1 M Tris-HCl, pH 8.8, and incubated in the dark for 30 min. Tris buffer was eliminated by two washing steps in Hepes/Sorbitol buffer. Photoinhibition was performed in all cases using 4,500 $\mu E \cdot m^{-2} \cdot sec^{-1}$ at 20°C. Control thylakoids were kept at the same temperature in the dark. After treatment, thylakoids were centrifuged for 10 min at 40,000 *g* and prepared for sucrose gradient centrifugation or digitonin fractionation. All preparative steps were performed in dim light.

Subthylakoid Fractionation

Grana membranes and stroma-exposed thylakoids were isolated by the digitonin method as described by Kyle et al. (31). Briefly, digitonin was added to the thylakoid suspension (0.2 mg chlorophyll/ml) to a final concentration of 0.2%. After 2 min at room temperature, solubilization was stopped by the addition of 10 vol of Hepes/Sorbitol buffer. Unsolubilized material was removed by centrifugating the thylakoids for 2 min at 1,500 *g*. Intermediate (I), grana (G) and stroma-exposed (S) membrane fractions were isolated by differential centrifugation and obtained as pellets at 40,000, 10,000, and 144,000 *g*, respectively.

Sucrose Gradient Centrifugation

Pelletted thylakoids or membrane fractions obtained by digitonin solubilization and differential centrifugation were resuspended at the desired chlorophyll concentration in 10 mM Tricine, pH 7.8, and solubilized by the addition of *n*-dodecyl β-D-maltoside (dodecyl maltoside) to a final concentration of 1%. 1 ml of the extract was loaded onto a 0.1–1.0 M linear sucrose gradient in 10 mM Tricine, pH 7.8, and 0.03% dodecyl maltoside and spun in a Beckman SW41 rotor at 4°C at 39,000 rpm for 19 h. Gradients were fractionated into 300-μl fractions from bottom to top.

Electron Microscopy

Pellets of thylakoids were fixed overnight in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, and postfixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer, pH 7.2, for 2 h. For transmission EM the specimens were dehydrated in a graded series of ethyl alcohol and propylene oxide. Membranes were embedded in an Epon-Durcupan ACM-supplied mixture. Thin sections, cut with an ultramicrotome (Ultracut, Reichert-Jung, Vienna, Austria), were poststained with uranyl acetate and lead citrate and then examined with a transmission electron microscope (TEM 300; Hitachi, Tokyo, Japan) operating at 75 kV.

Other Methods

SDS-PAGE in the presence of 6 M Urea and immunoblotting were carried out as previously described (5). The properties of the anti-PSI (19), anti-light harvesting complex II (LHCII), anti-D1 and anti-CP43 polyclonals (5) have been described previously. Polyclonal antibodies to CP47 and D2 were raised in rabbit. CP47 was isolated as described in Dekker et al. (16). To isolate D2, reaction center complexes II obtained as described in Dekker et al. (16) were subjected to preparative SDS-PAGE and, after staining of the gel, the protein was electroeluted. Antigenic preparations were emulsified with complete Freund adjuvant and injected into rabbits. At 2-wk intervals, booster injections were given using polyA·polyU as adjuvant. Bleeding of the rabbits was according to standard procedure (28). Chlorophyll concentration was calculated according to Arnon (3). Absorption spectra at room temperature was obtained using a Beckman DU-7 spectrophotometer.

Results

Light-induced Dissociation of PSI and PSII

After Tris-washing, thylakoids were exposed to strong light irradiance. Some modifications were induced in the structural organization of both PSI and PSII. In the following experiment, two identical samples of Tris-washed thylakoids, one kept in the dark, the other illuminated with a flux of 4,500 $\mu E \cdot m^{-2} \cdot s^{-1}$ white light, were analyzed and compared. Fig. 1 *A* shows the result of sucrose gradient centrifugation of the two samples after solubilization with 1% dodecyl maltoside. For both samples, four bands were obtained with similar patterns but with significant differences in their optical absorption properties (Fig. 1 *B*) and major differences in their polypeptide composition (not shown, but see below). Thus, the red absorption maximum of bands 3 and 4 of the light-treated samples (TP) were, respectively, blue-shifted by 2–3 nm and red-shifted by 4–5 nm compared to the corresponding bands of the dark controls (TD3 and TD4). The spectrum of band 4 of the dark control sample (TD4) is typical of a PSI preparation containing CPI and LHCI (10, 38); the spectrum of band 3 (TD3) is characteristic of the PSII core devoid of chlorophyll b but containing inner antennae chlorophyll-proteins CP47 and CP43 (22, 48). The absorption spectra of bands 1 and 2 (TD1 and TD2) indicate a high level of chlorophyll b (not shown), as expected, if they contained the chlorophyll a/b antenna system

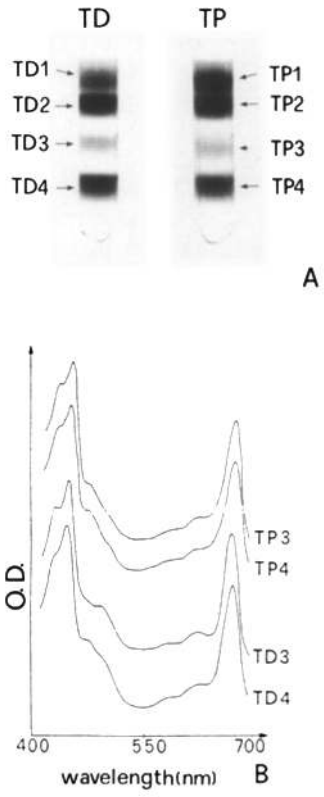


Figure 1. (*A*) Sucrose gradient centrifugation of thylakoids after 10 min solubilization in 1% dodecyl maltoside. Samples were kept in the dark (*TD*) or illuminated (*TP*) for 30 min with 4,500 $\mu E \cdot m^{-2} \cdot s^{-1}$ of white light at 20°C. Gradients were spun for 18 h at 39,000 rpm in an SW41 Beckman rotor at 4°C. (*B*) Absorption optical spectra of green bands 3 and 4 of gradients. Maxima for red absorption bands of TD3 and TD4 are at 673 and 678 nm, respectively.

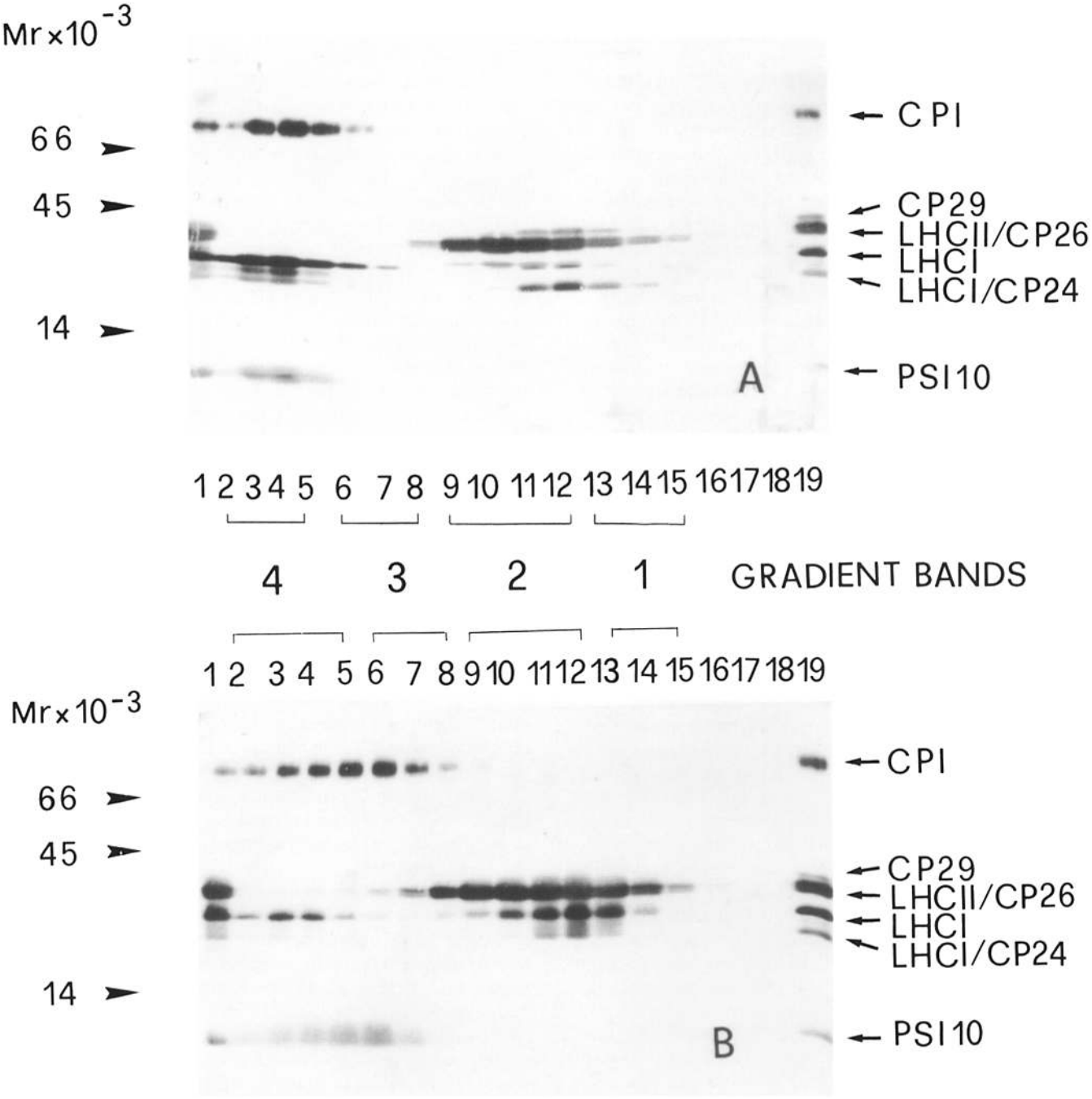


Figure 2. Immunoblots with anti-PSI 200 polyclonal of fractions obtained from (*A*) gradient TD and (*B*) gradient TP of Fig. 1. Gradients were fractionated into 300-μl aliquots from the bottom. 50 μl of every two fractions were solubilized in SDS and loaded onto gel from left to right. Approximate correspondence of fractions with gradient green bands is shown. Lanes 1 and 19 of both gels represent, respectively, photoinhibited and dark control samples of thylakoids. (*Right*) Identity of proteins recognized by polyclonal used; (*left*) relative apparent molecular mass.

of PSII (see below). The very same fractionation pattern was found for dark control thylakoids that were not subjected to Tris-washing before solubilization, indicating that inactivation of the donor side does not affect the fractionation pattern.

The sucrose density gradients of Fig. 1 *A* were fractionated into 300-μl aliquots which were subjected to SDS-PAGE and immunoblotting with polyclonal antibodies raised against different thylakoid proteins. Fig. 2 shows an immunoblot with an antibody raised against a PSI 200 preparation (38). This antibody recognizes the apoprotein of CPI and some polypeptides of LHCI complex, and cross-reacts with all the components of the PSII antenna system (19). Furthermore, it recognizes a low molecular weight polypeptide associated to PSI whose identity is still unclear and which will be referred to as PSI 10. Fig. 2 *A* shows that the fractions corresponding to sucrose gradient band 4 of the dark control sample do contain the PSI-LHCI polypeptides (lanes

2–5). These fractions, corresponding to sucrose gradient bands 1 and 2, are enriched in PSII antenna components (lanes *9–15*).

In the case of light-treated thylakoids (Fig. 2 *B*), the CPI apoprotein is detected in a larger number of fractions (lanes *2–8*), indicating the presence of this polypeptide in band TP3 as well as in TP4. The maximum of immunostaining for the low molecular weight PSI protein (PSI 10) is also shifted, indicating that its association with the PSI core is maintained after illumination. This is no longer the case for LHCI apoproteins. In fact, a strong increase in the immunostaining of LHCI polypeptides is observed in the fractions corresponding to the top region of the sucrose gradient (lanes *9–15*). An immunostaining peak for these polypeptides is also found in band 4 (lanes *3* and *4*), i.e., where the CPI maximum is found in the dark control sample. This observation may be interpreted by assuming that, after over-illumination of thylakoids, the LHCI antenna complex dissociates from the PSI reaction center. As a consequence, LHCI, no longer associated with CPI, is found in the upper bands of the gradient (TP1 and TP2). The residual CPI-LHCI complex maintains the same sedimentation properties as in the dark control and is found in band 4. It is worth noticing that Tris-washing alone does not modify the structural organization of PSI, since the same fractionation pattern was obtained for normal and donor side inactivated dark control thylakoids, whereas, when thylakoids were photoinhibited with a normally active donor side, dissociation of LHCI from the reaction center was less pronounced (not shown).

When the same fractions of Fig. 2 were subjected to immunoblotting with an anti-D1 antibody, two peaks in the intensity of the immunostaining pattern were observed for the dark control sample (Fig. 3 *A*): one in the lower portion of the TD4 green band (lanes *2* and *3*), and the other, more intense, centered around sucrose density gradient band TD3 (lanes *6–8*). The relative density of the two immunostaining peaks was found to depend on solubilization time and the amount of detergent (see below); the maximum yield for the heavier band was observed with a short incubation time and a low detergent concentration. The same result was obtained with antibodies raised against other PSII proteins (anti-CP47, anti-CP43, anti-D2, and anti-*psb*H [29] polyclonals) which gave patterns characterized by the presence of two immunostaining maxima (not shown). We interpret this result as an indication that two different PSII populations are resolved by sucrose gradient centrifugation, characterized by the same polypeptide composition but with different sedimentation behavior: an oligomeric form of PSII, probably a dimer (42, 43), migrates in the lower band (TD4) together with the CPI-LHCI complex; the monomeric form of PSII migrates in the upper band (TD3). On the contrary, LHCII and oxygen evolving enhancer 2 (OEE2) polypeptides are detected by the respective polyclonals on the top bands of the gradient, indicating that they are no longer associated to the PSII core complex.

Fig. 3 *B* shows an immunoblot with an anti-D1 polyclonal antibody (light-treated thylakoids). In this case, a single maximum in the immunostaining pattern for the D1 protein is observed, indicating that the hypothesized dimeric form of the PSII core is significantly reduced after photoinhibition.

Fig. 3 *B* also shows a faint immunostained polypeptide of ~16 kD, contained in band TP3 (*arrow* in lane *7*; the corresponding band, although clearly visible in the original blot, is not easy to see in the photograph). This polypeptide, which is more clearly detected in Fig. 4 (see below), corresponds to a COOH-terminal D1 fragment, previously described and characterized (7).

To investigate further the relationship between the change in the sucrose gradient mobility of PSII and degradation of the D1-protein, we performed sucrose gradient fractionation of thylakoids after exposure to photoinhibitory light for different time periods, keeping solubilization conditions as mild as possible to avoid dark monomerization of PSII but strong enough to dissociate LHCII completely from the PSII core. This was achieved by incubating the thylakoid suspension with 1% dodecyl maltoside for 1 min instead of the 10 min used routinely. The sucrose gradient bands and their fractionation and immunoblotting analysis with anti-D1 antiserum are shown in Fig. 4. Under these conditions, sucrose gradient band 3 of the dark control is almost absent, but it appears already well defined after 2.5 min of illumination, becoming more intense after 5 and 10 min. The immunostaining pattern of the dark control (Fig. 4 *A*) reveals a single peak centered in the fractions belonging to the bottom of the gradient, as expected. However, 2.5 min of photoinhibitory illumination are sufficient to shift the immunostaining maximum in sucrose band 3, where the monomeric PSII is supposed to migrate. After longer illumination times this band becomes the one which contains most of the PSII core proteins (Fig. 4, *C* and *D*). At the same time, degradation of the D1-protein starts: the protein fragments, hardly detectable after 2.5 min of illumination, become evident after 5 min (Fig. 4, *arrow* in *C*). The main product is the 16-kD COOH-terminal fragment, previously described in Barbato et al. (7). After 10 min of illumination, a second fragment is detected, with a lower apparent mass of ~12 kD (Fig. 4, *arrows* in *D*), possibly representing a second degradation product of the 16-kD fragment.

The shift of the immunostaining maximum from sucrose band 4 to band 3, which is assigned to the monomerization of a dimeric PSII core, was also evident when polyclonals against other PSII proteins, such as anti-CP47 and anti-D2, were used (not shown). However, when an anti-CP43 antibody was used, an unexpected result was obtained. In fact, as shown in Fig. 5 *B*, this protein is also found in fractions corresponding to the top region of the gradient (lanes *5–8*), where D1, as shown in *B* of Fig. 3 (lanes *15–18*) is not present. Immunodetection of CP43 in this region of the gradient was not observed in the dark control thylakoids (Fig. 5 *A*), suggesting that one of the effects of photoinhibition is dissociation of this inner antenna from the core, possibly as a consequence of the degradation of the D1-protein.

Fractionation of Thylakoids into Grana Membranes and Stroma-exposed Lamellae

Apart from the dissociation of LHCI from the PSI core, the structural changes of the thylakoid membrane after donor side photoinhibition reported above, namely, monomerization of the PSII core, dissociation of CP43, and cleavage of D1 within the PSII core, may well represent some of the steps involved in the repair cycle of damaged PSII centers. According to current models (8, 36, 45), one would expect that the

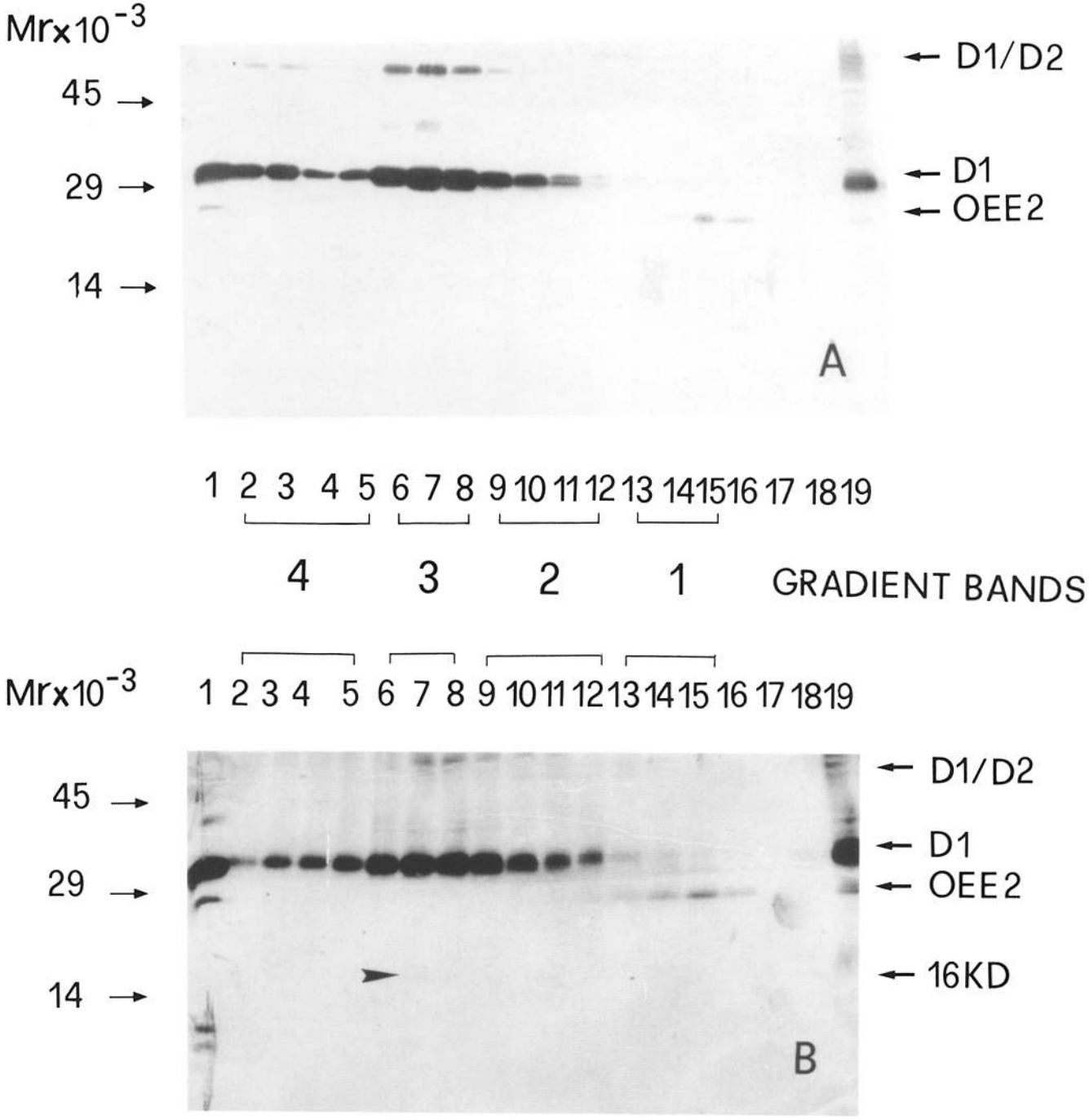


Figure 3. Immunoblot with anti-D1 and anti-OEE2 polyclonals of same fractions shown in Fig. 2.

events described so far would be paralleled by a redistribution of PSII proteins between the different domains of the thylakoid membranes, i.e., grana membranes and stroma-exposed lamellae. To investigate this topic, thylakoids were fractionated into grana (G), intermediate (I) and stroma-exposed (S) membranes by using the digitonin method described in Kyle et al. (31). Fig. 6 shows electron micrographs of Tris-washed dark control and light-treated thylakoids: although changes in the general pattern of the membrane and considerable swelling are observed, differentiation into grana and stroma lamellae is largely preserved after photoinhibitory treatment. It has recently been demonstrated that, during photoinhibition, cyclic electron flow via photosystem I may be activated, causing thylakoid swelling without dissociation of the grana domain (55). This allows a meaningful analysis of the effect of light exposure on the polypeptide composition of the different membrane regions. Immunoblots of the membrane fractions with anti-D1 and anti-CP43 polyclonals are shown in Fig. 7. In Fig. 7 *A* we notice that the 16-kD fragment is present in each of the three fractions. Although saturation of the blots prevents quantitative evaluation of the relative amounts of the fragments in the different membrane compartments, this finding suggests that, after cleavage in the grana region, the D1-protein migrates to the

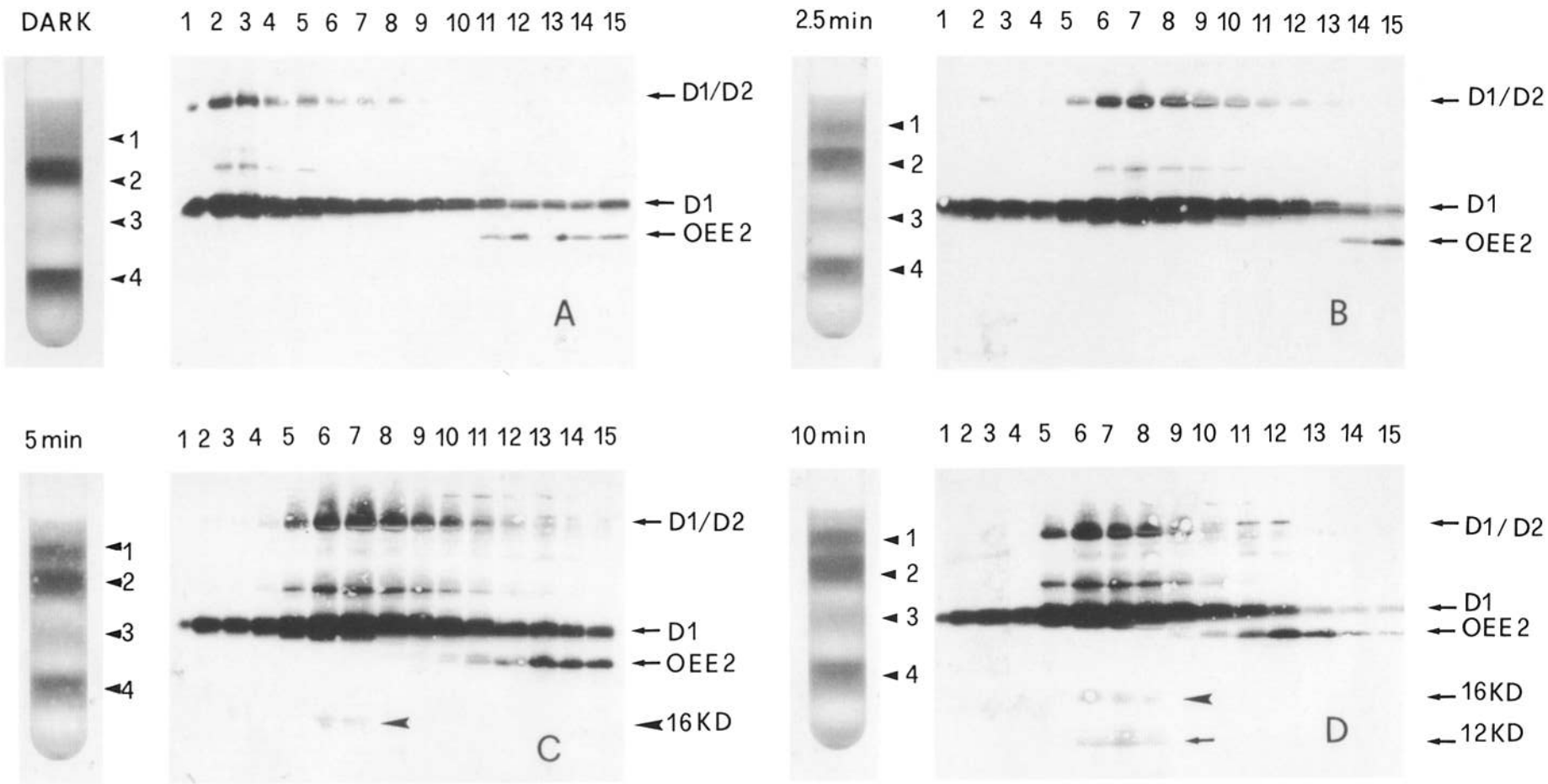


Figure 4. Sucrose gradient fractionation and immunoblot analysis with anti-D1 and anti-OEE2 polyclonals of 300-μl fractions of each gradient (see also legend of Fig. 2). Tris-washed thylakoids were illuminated with white light of 4,500 μE $m^{-2}s^{-1}$ at 20°C for (*A*) 0; (*B*) 2.5; (*C*) 5; and (*D*) 10 min. (*C* and *D*) Spots corresponding to 16- and 12-kD fragments are marked by arrows.

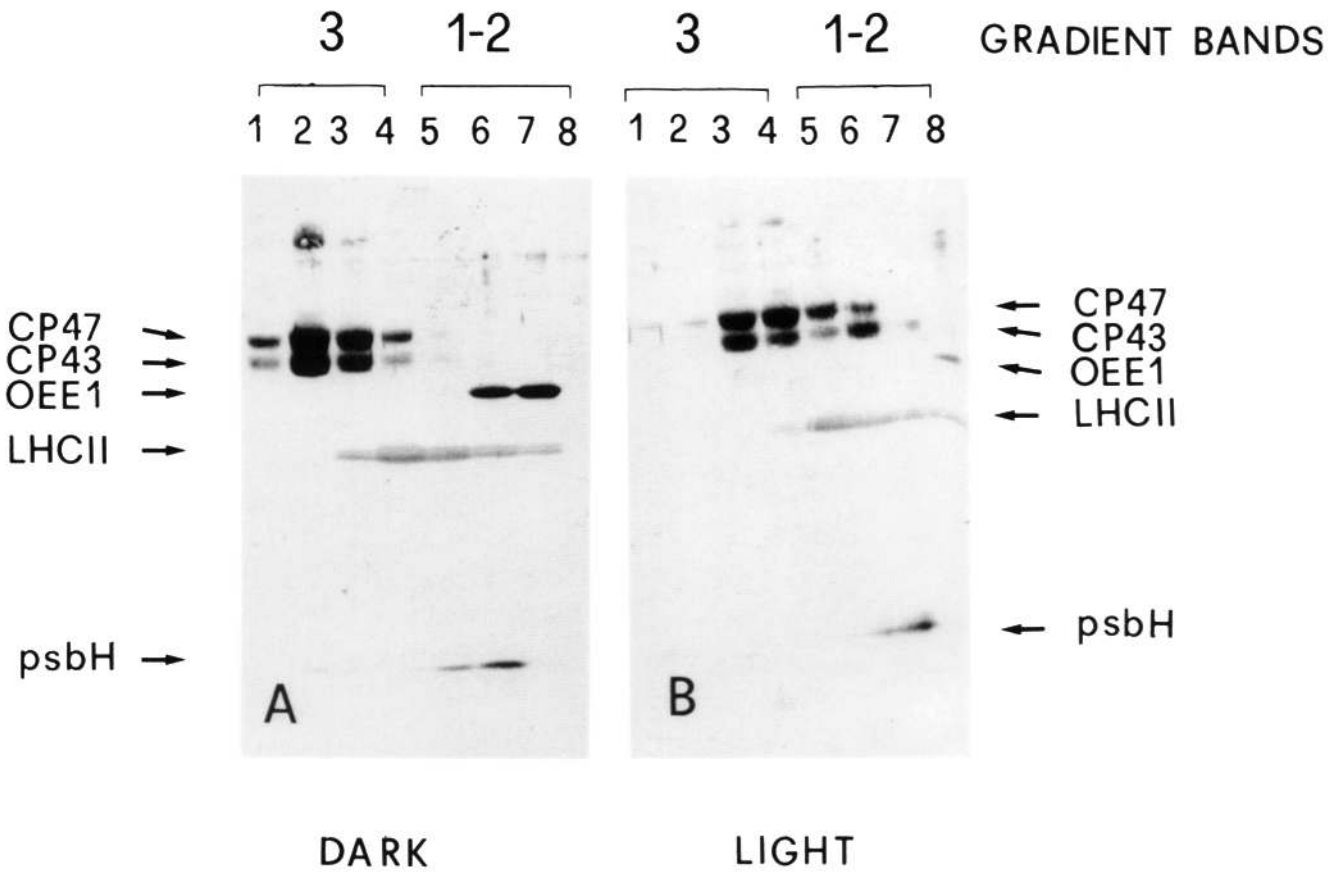


Figure 5. Immunodetection of CP43 in upper portion of sucrose gradients reported in Fig. 1. Lanes 1–4 correspond to green band 3 and lanes 5–8 to bands 2 and 1. Nitrocellulose filters were reacted with a mixture of polyclonals including anti-CP47, -CP43, -OEE1, -LHCII, and -psbH gene product.

stroma lamellae, in agreement with previous reports under different conditions (for examples, see ref. 5). At the same time, a remarkable increase in CP43 in the stroma lamellae is observed after photoinhibition (Fig. 7 *B*).

The presence of the OEE1 polypeptide in the intermediate and grana fractions of dark control Tris-washed thylakoids indicates rebinding of this protein from the lumen space when the low salt condition is restored. However, when the thylakoids are exposed to the light, no trace of OEE1 is found after fractionation into grana and stroma membrane compartments.

Sucrose Gradient Fractionation of Stroma-exposed Membranes

In a previous paper we showed that photoinhibition of donor side inactivated PSII cores produces only a 16-kD fragment of the D1-protein when CP43 is associated to the PSII core complex and also a 24-kD fragment when CP43 is dissociated (7). The appearance of free CP43 in the gradient of photoinhibited thylakoids, together with that of the 16-kD fragment, suggests that dissociation of CP43 from the PSII core takes place after the cleavage of D1. CP43 increases in the stroma lamellae after photoinhibition; what is left to establish is whether this complex migrates to the stroma region mainly as a free protein or still associated to the PSII core.

To verify this, sucrose gradient fractionation of stroma membrane isolated both from dark controls and photoinhibited thylakoids was performed and the results were analyzed. Fig. 8 shows the sedimentation pattern of stroma membranes from a dark control sample, with an intense green band (STD4) containing the PSI-LHCI complex and only a faint trace of PSII core (STD3). In the light-treated samples a strong increase in band 3 (STP3) is observed, corresponding to some PSI after dissociation of LHCI (see above) and some PSII core including D1 fragments (Fig. 8 *B*, lanes *10* and *11*). However, most of the CP43 complex is not associated with this band, but is found in the upper fractions of the gradient (STP1/STP2) where free antennae are also found (Fig. 8 *B*, lanes *14* and *15*).

This finding indicates that dissociation of CP43 after D1 cleavage probably takes place in the grana region and is followed by its migration as a dissociated subunit to the stroma lamellae.

Discussion

Photoinhibition of isolated thylakoids whose donor side has been inactivated by Tris-washing brings about a number of events involving the molecular structure and composition of the two photosystems. So far little attention has been given to the effects of over-illumination on PSI. Besides a decrease in low temperature fluorescence emission at 730 nm (14), a decrease in photochemical activity has been reported, possibly due to impairment of iron-sulfur center(s) (see 45). Al-

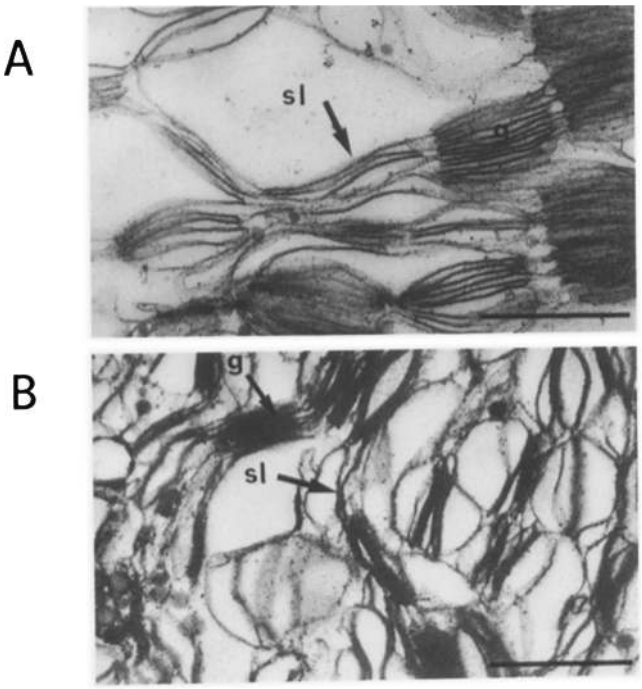


Figure 6. Electron micrographs of (*A*) dark control and (*B*) light-treated (30 min) thylakoids. *sl*, stroma lamellae; *g* grana. Bar, 1 μm.

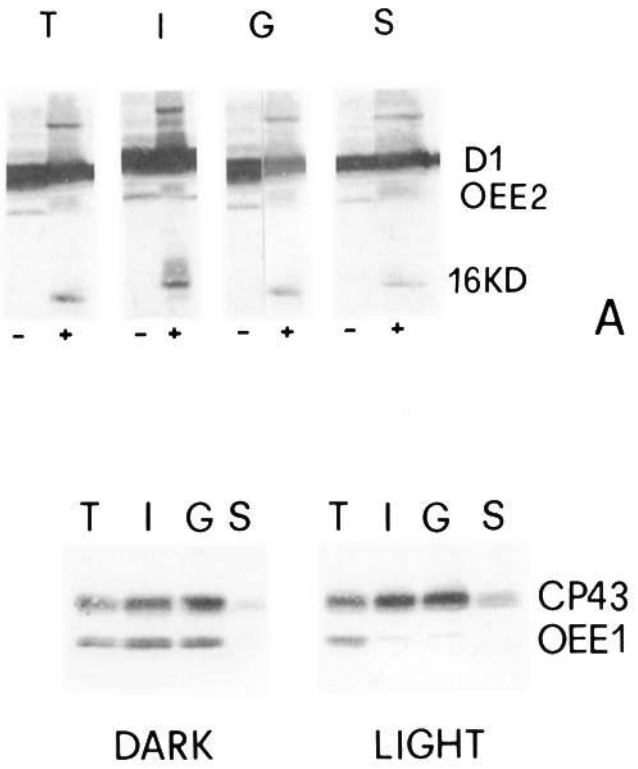


Figure 7. Immunoblots with anti-D1 plus anti-OEE2 (*A*) and anti-CP43 plus anti-OEE1 (*B*) of whole thylakoid membrane (*T*), intermediate (*I*), grana (*G*) and stroma (*S*) regions before and after photoinhibitory illumination. (*A*) Dark control (−) and light-treated (+) samples are compared for each membrane region. (*B*) Three regions and whole thylakoids are compared for dark control (*left*) and light-treated samples (*right*).

though it has not been further investigated, the finding reported here, that is, dissociation of LHCI from the PSI core, shows that PSI also needs some protection against over-illumination. In fact, the decrease in its antenna size may well represent a protective mechanism for the PSI reaction center. In this context, PSI does not differ from PSII, for which phosphorylation-independent dissociation of LHCII under photoinhibitory conditions has been reported (49) and is confirmed here. Further investigations on the effects of photoinhibition on PS I are now in progress.

Most of the data presented here refer to photosystem II and may conveniently be discussed in terms of Scheme I, which tentatively depicts the succession of events involved in PSII repair after photoinhibition, as far as "donor side" photodamage is concerned.

Step 1: Monomerization of Dimeric PSII Core

Recent data from EM of crystallized PSII membranes (11), fractionation studies of thylakoids, PSII membranes and other PSII subparticles by different methods (15, 42, 43) indicate a dimeric organization for the PSII core complex which, besides the reaction center components (9, 39), also contains inner antennae CP47 and CP43. Our method, based on sucrose gradient fractionation of dodecyl maltoside–solubilized thylakoids and immunoblotting making use of a set of polyclonal antibodies raised against individual PSII proteins, allowed two distinct PSII populations with the same polypeptide composition to be resolved. We tentatively assign the slower migrating band to the monomeric form and the faster band to the dimeric form of PSII. One reason why the dimeric form of PSII has not been detected before in single-step fractionation of thylakoids may be that the faster migrating band comigrates with the more abundant PSI-LHCI complex and that its presence can only be detected with specific antibodies. Moreover, the integrity of the dimeric PSII core strongly depends on solubilization conditions, which must be kept as mild as possible, and on light exposure, whose effect is quick monomerization of the complex, at least when donor side inactivated thylakoids are used. Multiple sucrose gradient bands of PSII, where no PSI was present, have been obtained previously by detergent solubilization of PSII complex (4) and PSII membranes (15) where no PSI was present. In the latter case, up to four bands for which the polypeptide composition was not strictly the same were observed, suggesting that they represent different solubilization products rather than different oligomerization forms. Protein phosphorylation has also been reported to affect the sucrose gradient fractionation of PSII membranes (21). However, in the present study, any heterogeneity due to different phosphorylation levels can be excluded since the thylakoids were extensively dark-adapted before use and no ATP was added. Therefore, the finding that illumination as short as 2.5 min brings about disappearance of the dimeric form of PSII is clear evidence that monomerization of its core is the very first effect of over-illumination. It is worth noticing that this first step is shared by the donor side and

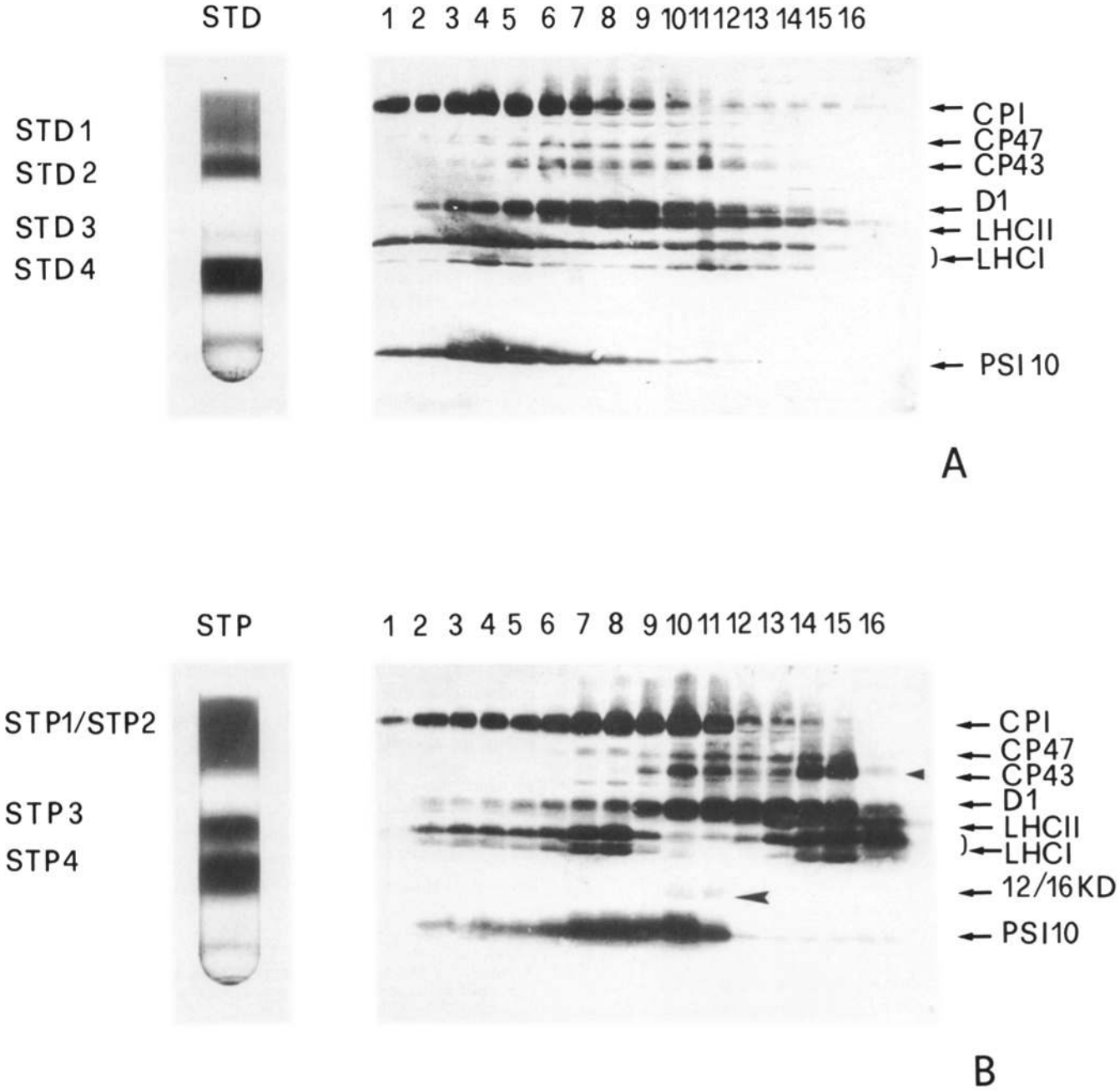


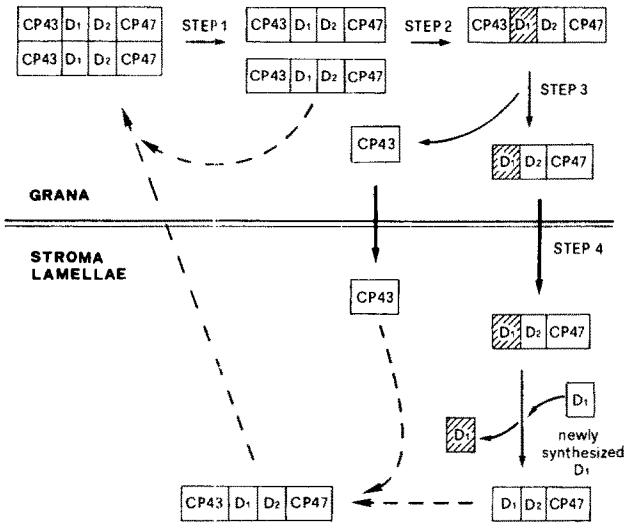
Figure 8. Sucrose gradient sedimentation of stroma lamellae solubilized in 1% dodecyl maltoside and immunoblot analysis of gradient (see Fig. 2 for details) with a mixture of polyclonals directed to PSI 200, CP47, CP43, and D1. (*A*) Dark control sample; (*B*) light-treated (30 min) sample. Attribution of fragment in lanes 10 and 11 to COOH-terminal 16-kD fragment is uncertain, since 12-kD is present in other analyses (e.g., see Fig. 4 *D*) may be masked by strong reaction of anti-PSI 200 polyclonal with PSI 10 protein.

acceptor side photoinhibition pathway, since it is observed independently of Tris-washing.

Step 2: Cleavage of D1 Protein

Light-induced degradation of D1 in thylakoids or PSII particles with inactivated donor side is oxygen-independent (5, 26, 51) and significantly faster than that observed in the presence of an active donor side (27). Accordingly, D1 fragments are easily observed after a few minutes (~5) of light exposure. However, sucrose gradient sedimentation shows that breakdown products are only found in the green band containing the other proteins of the PSII core, while they are completely absent in the more slowly migrating bands. We interpret this finding as evidence for cleavage of the D1-protein while still assembled in the PSII core, where it also remains after cleavage. This interpretation is in accordance with the finding that no stable PSII units are observed in mutants lacking the D1-protein (12). The presence of D1-protein, although damaged and cleaved, within the PSII structure is sufficient to prevent its dismounting.

This conclusion was also reached by Adir et al. (1) through



Scheme 1. Proposal for succession of events involved in repair cycle during photoinhibition of photosystem II. Steps 1–4 are based on experimental results reported here; dashed arrows represent speculative guesses.

photoinhibition experiments in the presence of chloramphenicol which prevents resynthesis of plastidial proteins. Other lines of evidence suggest the stability of a PSII core deprived of the CP43 subunit. Such a CP43-less core is able to perform photochemistry in mutants in which CP43 has been genetically removed (47) and has also been identified as an intermediate in the biogenetic assembly of PSII (17).

Step 3: Dissociation of the CP43 Inner Antenna

The presence of CP43 as a free chlorophyll-protein in the sucrose gradient sedimentation pattern of photoinhibited thylakoids indicates that dissociation of CP43 from the PSII core also takes place as a consequence of exposure to excessive light. In view of the recent finding that CP43 plays a role in defining the cleavage site(s) for D1 (7), we propose that dissociation of CP43 follows the lytic event on D1. In fact, if cleavage occurred after CP43 dissociation, then a 24-kD COOH-terminal fragment would appear, as occurs for the CP43-less PSII core and for the D1-D2-Cyt b 559 reaction center (6, 7). No dissociation of CP43 from the core was observed in dark control thylakoids, irrespectively of whether they had or not been subjected to Tris-washing.

Step 4: Migration to the Stroma Lamellae

The distribution of the apoproteins of CP47, CP43, and D1 in the stroma lamellae of a photoinhibited sample among the sucrose gradients of Fig. 8 indicates that, after CP43 dissociation in the grana region, this complex, together with the remaining CP43-less core units carrying a cleaved D1-protein, migrate to the stroma lamellae. In fact, while CP43 is found in stroma lamellae mainly as a free protein, CP47 at least in part is found associated to a core unit. Two circumstances contribute to make this result only partially clear: the first is the presence of the PSII units which normally reside in the stroma lamellae (2); the second is that some of the damaged cores are probably dismantled when they are in the stroma fraction, if D1 is not promptly resynthesized.

Migration of PSII subunits from grana membrane to stroma lamellae after photoinhibition has been reported previously (25). The nonstoichiometric relative amounts of the various PSII proteins in stroma lamellae after photoinhibition have been taken as an indication that disassembly of damaged PSII cores takes place in the grana and is followed by independent migration of the dissociated subunits, including COOH- and NH_2-terminal fragments of D1 (5, 20). In our case of donor side photoinhibition, some of the PSII components, and in particular CP43, are also found to migrate independently to the stroma lamellae. However, the results presented here support the view of migration of damaged PSII cores retaining their structure and topological organization. The repair cycle continues in the stroma lamellae with the substitution of the cleaved D1-protein by a newly synthesized one. This is in agreement with the report of Adir et al. (1), who have shown that, during recovery from photoinhibition, the newly synthesized D1 protein is found in stroma-exposed membranes assembled with the other proteins of the PSII core except CP43. It remains to be clarified whether a different mechanism of protein migration is associated with the different pathways for degradation for the D1 protein, occurring during acceptor and donor side photoinhibition.

The mechanism by which damaged cores and dissociated CP43 migrate from grana to stroma lamellae is also still unclear. Since no ATP was present in our assay conditions, phosphorylation of polypeptides should not be involved. One possibility is that dissociation of CP43 after cleavage of D1 and its reassociation after D1 resynthesis both contribute to create the driving force for migration.

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References

1. Adir, N., S. Shochat, and I. Ohad. 1990. Light-dependent D1 protein synthesis and translocation is regulated by reaction center II. *J. Biol. Chem.* 265:12563–12568.
2. Anderson, J. M., and A. Melis. 1983. Localization of different photosystems in separate regions of chloroplast membranes. *Proc. Natl. Acad. Sci. USA.* 80:745–749.
3. Arnon, D. I. 1949. Copper enzymes in isolated chloroplasts. Polyphenol oxidase in *Beta vulgaris*. *Plant Physiol.* 24:1–15.
4. Barbato, R., F. Rigoni, M. T. Giardi, and G. M. Giacometti. 1989. The minor antenna complexes of an oxygen evolving photosystem II preparation: purification and stoichiometry. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 251:147–154.
5. Barbato, R., G. Friso, M. T. Giardi, F. Rigoni, and G. M. Giacometti. 1991. Breakdown of the photosystem II reaction center D1 protein under photoinhibitory conditions: identification and localization of the C-terminal degradation products. *Biochemistry.* 30:10220–10226.
6. Barbato, R., C. A. Shipton, G. M. Giacometti, and J. Barber. 1991. New evidence suggests that initial photoinduced cleavage of the D1-protein may not occur near the PEST sequence. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 290:162–166.
7. Barbato, R., A. Frizzo, G. Friso, F. Rigoni, and G. M. Giacometti. 1992. Photoinduced degradation of the D1 protein in isolated thylakoids and various photosystem II particles after donor side inactivation: detection of a C-terminal 16 kDa fragment. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 304:136–140.
8. Barber, J., and B. Andersson. 1992. Too much of a good thing: light can be bad for photosynthesis. *Trends Biochem. Sci.* 17:61–66.
9. Barber, J., D. J. Chapman, and A. Telfer. 1987. Characterization of a PSII reaction center isolated from the chloroplasts of *Pisum sativum*. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 220:67–73.
10. Bassi, R., and D. J. Simpson. 1987. Chlorophyll-protein complexes of bar-

ley photosystem I. *Eur. J. Biochem.* 163:2218–2230.
11. Bassi, R., A. Ghiretti Magaldi, G. Tognon, G. M. Giacometti, and K. R. Miller. 1989. Two-dimensional crystals of the photosystem II reaction center complex from higher plants. *Eur. J. Cell. Biol.* 50:84–93.
12. Bennoun, P., M. Spierre-Herz, J. Erickson, J. Girard-Bascou, Y. Pierre, M. Delosme, and J. D. Rochaix. 1986. Characterization of photosystem II mutants of *Chlamydomonas reinhardtii* lacking the psbA gene. *Plant Mol. Biol.* 6:151–160.
13. Chua, N.-H., and N. Gillham. 1977. The sites of synthesis of the principal thylakoid membrane polypeptides in *Chlamydomonas reinhardtii. J. Cell Biol.* 74:441–452.
14. Cleland, R. E. 1988. Molecular events of photoinhibitory inactivation in the reaction centre of photosystem II. *In* Ecology of Photosystems in sun and shade. J. R. Evans, S. von Caemmerer, and W. W. Adams III, editors. CSIRO, Melbourne, Australia. 135–150.
15. Dainese, P., and R. Bassi. 1991. Subunit stoichiometry of the chloroplast photosystem II antenna system and aggregation state of the component chlorophyll-a/b binding proteins. *J. Biol. Chem.* 266:8136–8142.
16. Dekker, J. P., N. R. Bowlby, and C. F. Yocum. 1989. Chlorophyll and cytochrome b-559 content of the photochemical reaction center of photosystem II. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 254:150–154.
17. de Vitry, C., J. Olive, D. Drapier, M. Recouvreur, and F.-A. Wollman. 1989. Posttranslational events leading to the assembly of photosystem II protein complex: a study using photosynthetic mutants from *Chlamydomonas reinhardtii. J. Cell Biol.* 109:991–1006.
18. Diner, B. A., D. F. Ries, B. N. Cohen, and J. G. Metz. 1988. COOH-terminal processing of polypeptide D1 of the photosystem II reaction center of *Scenedesmus obliquus* is necessary for the assembly of the oxygen-evolving complex. *J. Biol. Chem.* 263:8972–8980.
19. Di Paolo, M. L., A. Peruffo dal Belin, and R. Bassi. 1990. Immunological studies on chlorophyll-a/b proteins and their distribution in thylakoid membrane domains. *Planta (Berl.).* 181:275–286.
20. Ghirardi, M. L., F. E. Callahan, S. K. Sopory, T. D. Elich, M. Edelman, and A.-K. Matoo. 1990. Cycling of the photosystem II reaction center core between grana and stroma lamellae. *In* Current Research in Photosynthesis. Vol. II. M. Baltscheffsky, editor. Kluwer Academic Publishers, The Netherlands. 733–738.
21. Giardi, M. T., F. Rigoni, R. Barbato, and G. M. Giacometti. 1991. Relationships between heterogeneity of the PSII core complex from grana particles and phosphorylation. *Biochem. Biophys. Res. Commun.* 176: 1298–1305.
22. Gounaris, K., and J. Barber. 1985. Isolation and characterization of a photosystem II reaction centre lipoprotein complex. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 188:68–72.
23. Grebanier, A. E., D. M. Coen, A. Rich, and L. Bogorad. 1978. Membrane proteins synthesized but not processed by isolated maize chloroplasts. *J. Cell Biol.* 78:734–745.
24. Greenberg, B. M., V. Gaba, A.-K. Mattoo, and M. Edelman. 1987. Identification of a primary in vivo degradation product of the rapidly-turning-over 32 kd protein of photosystem II. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:2865–2869.
25. Hundal, T., I. Virgin, S. Styring, and B. Andersson. 1990. Changes in the organization of photosystem II following light-induced D1-protein degradation. *Biochim. Biophys. Acta.* 1017:235–241.
26. Jegerschöld, C., and S. Styring. 1991. Fast oxygen-independent degradation of the D1 reaction center protein in Photosystem II. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 280:87–90.
27. Jegerschöld, C., I. Virgin, and S. Styring. 1990. Light-dependent degradation of the D1 protein in Photosystem II is accelerated after inhibition of the water splitting reaction. *Biochemistry.* 29:6179–6186.
28. Johnstone, A., and R. Thoorpe. 1982. Immunochemistry in Practice, Blackwell, London. 1–298.
29. Kuhn, M., A. Thiel, and P. Boger. 1988. The 9-kDa phosphoprotein involved in photoinhibition. *Z. Naturforsch. Sect. C. Biosci.* 43*c*:413–417.
30. Kyle, D. J., and I. Ohad. 1990. The mechanism of photoinhibition in higher plants and green algae. *In* Encyclopedia of Plant Physiology (New Series). L. A. Staehelin and C. J. Arntzen, editors. Springer, Heidelberg. 19:468–476.
31. Kyle, D. J., T. Y. Kuang, J. L. Watson, and C. J. Arntzen. 1984. Movement of a sub-population of the light harvesting complex (LHCII) from grana to stroma lamellae as a consequence of its phosphorylation. *Biochem. Biophys. Acta.* 765:89–96.
32. Kyle, D. J., I. Ohad, and C. J. Arntzen. 1984. Membrane protein damage and repair: selective loss of a quinone-protein function in chloroplast membranes. *Proc. Natl. Acad. Sci. USA.* 81:4070–4074.
33. Marder, J. B., P. Golubinoff, and M. Edelman. 1984. Molecular architecture of the rapidly metabolized 32-kilodalton protein of photosystem II. *J. Biol. Chem.* 259:3900–3908.
34. Mattoo, A.-K., and M. Edelman. 1987. Intramembrane translocation and posttranslational palmitoylation of the chloroplast 32-kDa herbicide-binding protein. *Proc. Natl. Acad. Sci. USA.* 84:1497–1501.
35. Mattoo, A.-K., U. Pick, H. Hoffman-Falk, and M. Edelman. 1981. The rapidly metabolized 32,000-dalton polypeptide of the chloroplast is the "proteinaceous shield" regulating photosystem II electron transport and mediating diuron herbicide sensitivity. *Proc. Natl. Acad. Sci. USA.* 78:1572–1576.
36. Melis, A. 1991. Dynamics of photosynthetic membrane composition and function. *Biochim. Biophys. Acta.* 1058:87–106.
37. Michel, H., D. F. Hunt, J. Shabanowitz, and J. Bennet. 1988. Tandem mass spectrometry reveals that three photosystem II proteins of spinach chloroplasts contain N-acetyl-O-phosphothreonine at their N-termini. *J. Biol. Chem.* 263:1123–1139.
38. Mullet, J. E., J. J. Burke, and C. J. Arntzen. 1980. Chlorophyll proteins of photosystem I. *Plant Physiol.* 65:814–822.
39. Nanba, O., and K. Satoh. 1987. Isolation of a photosystem II reaction center consisting of D-1 and D-2 polypeptides and cytochrome b-559. *Proc. Natl. Acad. Sci. USA.* 84:109–112.
40. Ohad, I., D. J. Kyle, and J. Hirschberg. 1985. Light-dependent degradation of the Q_B-protein in isolated pea thylakoids. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:1655–1659.
41. Ohad, I., N. Adir, H. Koike, D. J. Kyle, and Y. Inoue. 1990. Mechanism of photoinhibition in vivo. A reversible light-induced conformational change of reaction center II is related to an irreversible modification of the D1 protein. *J. Biol. Chem.* 265:1972–1979.
42. Peter, G. F., and J. P. Thornber. 1991. Biochemical composition and organization of higher plant Photosystem II light-harvesting pigment-proteins. *J. Biol. Chem.* 266:16745–16754.
43. Peter, G. F., and J. P. Thornber. 1991. Biochemical evidence that the higher plant photosystem II core complex is organized as a dimer. *Plant Cell Physiol.* 32:1237–1250.
44. Powles, S. B. 1984. Photoinhibition of photosynthesis induced by visible light. *Annu. Rev. Plant Physiol.* 35:15–44.
45. Prasil, O., N. Adir, and I. Ohad. 1992. Dynamics of photosystem II: mechanism of photoinhibition and recovery process. *In* The Photosystems. J. Barber, editor. Elsevier Biomedical, Amsterdam. 11:220–250.
46. Reisfeld, A., A.-K. Mattoo, and M. Edelman. 1982. Processing of a chloroplast-translated membrane protein *in vivo*. Analysis of the rapidly synthesized 32000-dalton shield protein and its precursor in *Spirodela oligorrhiza. Eur. J. Biochem.* 124:125–129.
47. Rögner, M., D. A. Chisholm, and B. A. Diner. 1991. Site-directed mutagenesis of psbC gene of photosystem II: isolation and functional characterization of CP43-less photosystem II core complexes. *Biochemistry.* 30:5387–5395.
48. Satoh, K., H. J. Nakatani, K. E. Steinback, J. Watson, and C. J. Arntzen. 1983. Polypeptide composition of a photosystem II core complex. Presence of a herbicide-binding protein. *Biochim. Biophys. Acta.* 724:142–150.
49. Schuster, G., M. Dewit, L. A. Staehelin, and I. Ohad. 1986. Transient inactivation of the thylakoid photosystem II light-harvesting protein kinase system and concomitant changes in intramembrane particle size during photoinhibition of *Chlamydomonas reinhardtii. J. Cell Biol.* 103:71–80.
50. Setlik, I., S. I. Allakhverdiev, L. Nedbal, E. Setlikova, and V. V. Klimov. 1990. Three types of photosystem II photoinactivation. I. Damaging processes on the acceptor side. *Photosynth. Res.* 23:39–48.
51. Shipton, C. A., and J. Barber. 1991. Photoinduced degradation of the D1 polypeptide in isolated reaction centers of photosystem II: evidence for an autoproteolytic process triggered by the oxidizing side of the photosystem. *Proc. Natl. Acad. Sci. USA.* 88:6691–6695.
52. Styring, S., I. Virgin, A. Ehrenberg, and B. Andersson. 1990. Strong light photoinhibition of electron transport in photosystem II. Impairment of the function of the first quinone acceptor, Q_A. *Biochim. Biophys. Acta.* 1015:269–278.
53. Telfer, A., J. B. Marder, and J. Barber. 1987. Photosystem II reaction centers isolated from phosphorylated pea thylakoids carry phosphate on the D1 and D2 polypeptide subunits. *Biochim. Biophys. Acta.* 893: 557–563.
54. Theg, S. M., L. J. Filar, and R. A. Dilley. 1986. Photoinactivation of chloroplasts already inhibited on the oxidising side of Photosystem II. *Biochim. Biophys. Acta.* 894:104–111.
55. Topf, J., H. Gong, L. Mets, and I. Ohad. 1992. Thylakoid membrane energization and swelling in photoinhibited *Chlamydomonas* cells is prevented in mutants unable to perform cyclic electron flow. *Photosynth. Res.* 32:59–69.