Recovery of Photosystem II Activity in Photoinhibited *Synechocystis* Cells: Light-Dependent Translation Activity Is Required besides Light-Independent Synthesis of the D1 Protein†

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ABSTRACT: Irreversible photoinactivation of photosystem II (PSII) results in the degradation of the reaction center II D1 protein. In *Synechocystis* PCC 6714 cells, recovery of PSII activity requires illumination. The rates of photoinactivation and recovery of PSII activity in the light are similar in cells grown in minimal (MM) or glucose-containing medium (GM). Reassembly of PSII with newly synthesized proteins requires degradation of the D1 protein of the photoinactivated PSII. This process may occur in darkness in both types of cells. The degraded D1 protein is, however, only partially replaced by newly synthesized protein in MM cells in darkness while a high level of D1 protein synthesis occurs in darkness in the GM cells. The newly synthesized D1 protein in darkness appears to be assembled with other PSII proteins. However, PSII activity is not recovered in such cells. Illumination of the cells in absence but not in the presence of protein synthesis inhibitors allows recovery of PSII activity.

The exposure of photosynthetic oxygen-evolving organisms to high light intensities results in the decrease of CO_2 fixation and oxygen evolution activities [photoinhibition (PI)][1] (for reviews, see refs *1*−*3*). This phenomenon is paralleled by the degradation of the D1 protein (*4*), an essential constituent of the photosystem II (PSII). Together with the D2 protein, D1 forms the core of PSII and binds all the cofactors involved in the primary charge separation and stabilization leading to water oxidation and reduction of plastoquinone (for a review on PSII, see refs *5*−*7*). As a consequence of PSII photochemistry, photoinactivation of PSII and degradation of the D1 protein occur not only at high light intensities but also at light intensities far below those required to saturate electron flow (*8*, *9*). To maintain the PSII activity, the degraded protein has to be replaced by a new protein copy (*4*, *10*, *11*). Therefore, the level of PSII activity, at any light intensity, is the result of the balance between the rate of photoinactivation and protein degradation and the rate of de novo PSII core protein synthesis and assembly with the remaining PSII components. When the rate of photoinactivation and protein degradation exceeds that of the repair process, the photosynthetic activity progressively decreases. Lowering the light intensity slows down the rate of photodamage, and photosynthetic activity is progressively restored (e.g., see refs *4*, *12*, *13*). The optimal conditions for the recovery from photoinhibition vary for different organisms depending on their ability to degrade the damaged D1 and to replace it by a newly synthesized protein, thus allowing the assembly and activation of new PSII complexes.

The D1 protein is encoded by the *psbA* plastid gene, highly conserved in all higher plants, algae, and cyanobacteria. In photosynthetic eukaryotes, *psbA* is usually present as a single-copy gene in the chloroplast genome (*14*). In mature chloroplast, *psbA* expression is regulated by light mostly at the posttranscriptional level (for reviews, see refs *15*, *16*). Only minor changes in *psbA* transcription have been observed following illumination of dark-adapted plants (e.g., see refs *17*, *18*). However, transfer from low to high light intensities increases not only translational initiation and elongation but also the transcription of the *psbA* gene (*18*).

In higher plants, by regulating chlorophyll synthesis, light also controls the synthesis and accumulation of the D1 protein as well as other chlorophyll binding proteins (*19*−*23*). Complete translational elongation and accumulation of the D1 protein depend on chlorophyll binding, stabilizing the D1 synthesis intermediates (*20*−*24*). The synthesis of chlorophyll is tightly regulated by light in angiosperms. In contrast, gymnosperms and green algae are capable of synthesizing chlorophyll in darkness (for reviews, see refs *25*, *26*). Thus, algae such as *Chlamydomonas* and *Scenedesmus* sp. accumulate D1 protein and contain significant

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[1] Abbreviations: Chl, chlorophyll; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCPIP, 2,6-dichlorophenolindophenol; DPC, 1,5-diphenylcarbazide; F_o and F_m, initial and maximum fluorescence level; GM, glucose-enriched medium; MM, minimal medium; PI, photoinhibition; PQ, plastoquinone; PSI and PSII, photosystem I and II, respectively; Q_A and Q_B, primary and secondary quinone electron acceptors in PSII; RCIII, reaction center II.

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amounts of assembled PSII complexes capable of light-driven charge separation activity (*27*). However, even in organisms that can synthesize and assemble PSII in darkness, illumination enhances translation initiation by regulating binding of nuclear-encoded proteins to the 5′ untranslated region of *psbA* mRNA (*28*−*30*). This binding is controlled via the ATP level and the redox state of the chloroplast (*28, 31*−*34*). Recently, Edhofer et al. (*35*) reported that light also regulates the enzymatic activity of the translation and elongation process.

In cyanobacteria, *psbA* constitutes a gene family. Its expression is essentially regulated at the transcriptional level (*36*). It has been suggested that a blue receptor (*37*) or the redox state of the photosynthetic electron transport chain is involved in the light regulation of *psbA* expression (*38*−*40*). Most of these studies have been conducted in *Synechococcus* PCC 7942 and to a lesser extent in *Synechocystis* PCC 6803 and 6714. Each of these strains has three *psbA* genes: *psbA* I, *psbA* II, and *psbA* III. In *Synechococcus* PCC 7942, these genes encode two different forms of D1. The D1 protein encoded by *psbA* I is mainly present in low light conditions, while that encoded by *psbA* II and *psbA* III is mainly present under high light intensities (*36, 39, 41*−*44*). In *Synechocystis* PCC 6803 and 6714, the *psbA* I gene is not expressed (*45*), and only one form of D1 encoded by the *psbA* II and *psbA* III genes is present. Transferring cells grown at low light to higher irradiances increases the steady-state level of both *psbA* II and *psbA* III transcripts (*46*). In darkness, due to the stabilization of the transcript, the high level of these *psbA* mRNAs is maintained for several hours (*38*, *47*). Recently, He and Vermaas (*48*) have shown that in the cyanobacterium *Synechocystis* PCC 6803, *psbA* translation is also regulated by light. As in mature chloroplast, translation initiation of *psbA* mRNA depends on chlorophyll binding to D1 synthesis intermediates. However, in *Synechocystis* PCC 6803, the determinant element for D1 synthesis seems to be the chlorophyll availability rather than chlorophyll synthesis per se.

Studying transcription and translation activities during light stress and recovery, we have previously determined the characteristics of the irreversible step of photoinhibition in *Synechocystis* PCC 6714 cells (*49*). We have observed that light stress induces a large increase of the *psbA* mRNA level due to an increase of *psbA* transcription. A rapid turnover of *psbA* transcripts is maintained for a long time even after the transcription of other genes has already stopped. When the light stress is prolonged, *psbA* transcription and translation also decrease, and photoinhibition becomes irreversible. This suggests that photodamage of the transcription and translation machinery could be the cause of irreversible light stress. Recently, it was proposed that photosynthetic electron transport has an important role in *psbA* gene expression (*40*). Our data point out a control mechanism involving two different signals: (1) increase in the lifetime of Q_A^- specifically activating *psbA* transcription (see also ref *50*); (2) oxidation of the electron transport chain downstream of photosystem II decreases expression of the *psbA* gene as well as that of many other genes (*40, 50*).

In the present work, we have further studied the role of light in the recovery of photosynthetic activity of photoinhibited *Synechocystis* PCC 6714 cells able to grow under darkness in a glucose-containing medium. These cells possess a light-dependent as well as a light-independent pathway for chlorophyll synthesis (*51*). The results of this work demonstrate that cells grown in glucose-containing medium are able to synthesize the D1 protein in darkness. However recovery of PSII activity does not occur in darkness, and PSII cannot be photoactivated in the absence of de novo light-dependent protein synthesis.

MATERIALS AND METHODS

Strain Culture Conditions and Chlorophyll Measurements. Synechocystis PCC 6714 cells were grown on a rotary shaker (120 rpm) at 32 °C under a 5% CO_2-enriched atmosphere in the mineral medium described by Herdman et al. (*52*) with twice the concentration of nitrate in the absence or in the presence of 0.1% glucose. Illumination was provided by fluorescent white lamps, giving a total intensity of 70 μmol of photons·m^{-2}·s^{-1}.

Chl *a* concentration was spectrophotometrically determined in methanol extracts using the extinction coefficient at 665 nm of 74.5 mL mg^{-1} cm^{-1} (*53*).

Light Treatment and Radioactive Labeling. Cells in the exponential phase of growth were harvested by centrifugation and resuspended in fresh culture medium containing 25 mM HEPES (pH 6.8) at a final concentration of 30 μg of Chl·mL^{-1} (ca. 3×10^8 cells mL^{-1}). Cells were preincubated under culture conditions for 30 min before being used for experiments.

For photoinhibition experiments, cells (30 μg of Chl·mL^{-1}) were incubated at 22 °C in a refrigerated glass tube (3 cm diameter) under gentle stirring and illuminated with four Atralux spots of 150 W. Each lamp gave an intensity of about 1000 μmol of photons·m^{-2}·s^{-1}. The cells were preincubated for 5 min in low light at 22 °C to adapt them to this temperature. Samples were collected for various analyses at different times as indicated under Results. For recovery experiments, cells were transferred to the rotary shaker and incubated at 32 °C in growth light or in darkness. When indicated, lincomycin (100 μg·mL^{-1}) was added as an inhibitor of protein synthesis. For radioactive labeling, [^{35}S]-Met (10 μCi·mL^{-1}) was added at different times of recovery, and incubation was continued 45 min. Then, cells were pelleted (8−10 mL per sample), and thylakoid membranes were obtained as described below.

Fluorescence Measurements and PSII Activity Measurements. Photoinhibition and recovery were followed by measurements of Chl fluorescence induction. Fluorescence was monitored during the first second of induction using a home-built fluorometer. Excitation was provided by a tungsten lamp equipped with 5-59 and 4-96 Corning filters (shutter opened in 2 ms). Fluorescence was detected in the red region through a 2-64 Corning filter and a Wratten 90 filter. The recording was made with a multichannel analyzer connected to a personal computer. Cell suspension was normalized to 1 μg of Chl·mL^{-1}. DCMU [3-(3,4-dichlorophenyl)-1,1-dimethylurea] (2×10^{-5} M) was added in order to measure the Fm level (the fluorescence level reached when Q_A is reduced). Fo (the initial fluorescence at the onset of illumination) was determined in the absence of DCMU.

Oxygen evolution of intact cells (10 μg of Chl·mL^{-1}) was measured polarographically using a Clark-type oxygen electrode with addition of 1 mM 2,6-dichlorobenzoquinone (DCBQ) as an artificial PSII electron acceptor. The samples

were illuminated with continuous saturating white light. The amount of oxygen produced per flash during a sequence of saturating flashes was measured at 25 °C with a rate electrode as described by Joliot and Joliot (*54*). The short (5 μs) saturating flashes were produced by a Strobotac (General Radio Co.). The spacing between flashes was 0.6 s. *Synechocystis* PCC 6714 cells (100 mg of Chl·mL^{-1}) were resuspended in a medium containing 20 mM Hepes (pH 6.5), 100 mM KCl, and 5 mM $MgCl_2$. The cells were dark-adapted for 5 min prior to each flash sequence.

PSII activity of isolated thylakoid membranes (10 μg of Chl·mL^{-1}) was measured spectrophotometrically using diphenylcarbazide (DPC) (10^{-4} M) as an electron donor and 2,6-dichlorophenolindophenol (DCIP) (10^{-4} M) as an electron acceptor.

Preparation of Thylakoid Membranes. Cells were washed in a buffer containing 50 mM MES, 0.1 M sucrose, 5 mM $CaCl_2$, pH 6.5, 1 mM PMSF, and 1 mM benzamidine (MES buffer) and then resuspended in 500 μL of the same buffer. Cells were broken by 8 cycles of shaking with a bead-beater (Roucaire, Germany) in the presence of 450 μL of glass beads and in darkness. A cycle consisted of 15 s "on phase" and 15 s "off phase". The bead-beater chamber was cooled by CO_2 in the outer jacket. Glass beads and unbroken cells were eliminated by centrifugation at 1440*g* for 2 min. Thylakoids were sedimented by centrifugation at 19370*g* for 30 min. The pellet was washed in MES buffer and centrifuged again. Finally, the pellet was resuspended in 200 μL of MES buffer.

Resolution of Chl−Protein Complexes and Thylakoid Polypeptides. For the separation of Chl−protein complexes by nondenaturing Deriphat-PAGE, thylakoids were isolated as above and resuspended at a final Chl concentration of 0.6 mg of Chl/mL in buffer A (20 mM MES, pH 6.35, 5 mM $MgCl_2$, 5 mM $CaCl_2$, 25% glycerol), as described by Barbato et al. (*55*). The membranes were solubilized by incubation of 4 volumes of thylakoid suspension with 1 volume of the solubilization medium (9% dodecyl maltoside, 1% SDS) for 15 min on ice. Unsolubilized material was removed by centrifugation at 19370*g* and 4 °C for 2 min. A volume of 12.5 μg of Chl was loaded per slot on the Deriphat-PAGE (5−12% gradient) slab as described by Barbato et al. (*55*). Migration was carried out at 100 V and 4 °C for 60 min. The resolved green bands were excised and stored in the cold and darkness until further use.

Thylakoid proteins were resolved by denaturing electrophoresis according to Laemmli (*56*). Thylakoid samples were denatured in the presence of 2% SDS (1 h on ice followed by heating for 90 s at 80 °C), and samples containing 4.5 μg of chlorophyll were loaded and resolved by SDS−PAGE (9−15% acrylamide gradient) containing 4 M urea. For separation of protein constituents of the Chl−protein complexes, the bands excised from the Deriphat gels were incubated in the presence of sample buffer (2% SDS) at 37 °C for 2 h. The solubilized protein extract of each band was further resolved by SDS−PAGE. Migration was performed overnight at constant amperage (5 mA) and 4 °C. The resolved proteins were detected by staining with Coomasie Brilliant Blue R and autoradiographed using X-ray film (KODAK).

Immunodetection of D1 Protein. Thylakoid proteins separated on SDS−PAGE (14% acrylamide) were transferred to a nitrocellulose membrane (1 mA/1 cm^{-2} for 1 h). The membrane was saturated by PBS−Tween solution with 10% skimmed milk for 30 min and then washed in PBS−Tween (5 min × 3) before being hybridized with a monospecific D1 antibody (*57*) (1/250^e in PBS−Tween) overnight at 4 °C. The membrane was then washed (10 min × 3) in PBS−Tween and incubated in the presence of peroxidase coupled to secondary antibodies (1/200^e in PBS−Tween with 2.5% skimmed milk) at room temperature for 2 h. The membrane was washed, and D1 was revealed by the ECL method (Amersham).

Scanning of the Autoradiogram. The analysis of the autoradiograms was performed by combining a scanner (studioscan *II*si, AGFA) and a Macintosh Power 7100/80 computer using the public domain NIH Image program (developed at the National Institutes of Health and available from the Internet by anonymous FTP from zippy.nimh.nih.gov or on diskette from the National Technical Information Service, Springfield, VA, part number PB-500195GEI).

RESULTS

Photoinhibition and Recovery of PSII Activity. In *Synechocystis* cells, high irradiance leads to a large decrease of the PSII variable fluorescence (Fv) due to the formation of a quencher which specifically quenches Fm. Fo remains almost constant. Under the same conditions, oxygen evolution is also inhibited (photoinhibition); however, this process occurs at a somewhat slower rate (*13, 58, 59*). Thus, the quencher can be present in centers which are still active for oxygen evolution but "marked" for D1 degradation (*60*). After 30 min of photoinhibition, cells have lost 90−95% of their variable fluorescence and 75−85% of their O_2-evolving activity (Figure 1A). This was observed in cells in the absence as well as in the presence of glucose in the medium (data not shown). After 30 min light-stress, the cells were transferred and incubated in low light intensity or in darkness for 4 h. Photosynthetic activity was recovered in the cells incubated in low light. However, only 10−20% of the initial activity was recovered in the photoinhibited cells incubated in darkness, and this process occurred during the first 2 h of the dark incubation (Figure 1B). Measurements of the oxygen flash-yield activity showed a marked decrease in the amount of active PSII centers in the cells exposed to the photoinhibitory light (Figure 2). Almost complete recovery of the oxygen flash-yield activity occurred in the low light incubated cells. In contrast, only a limited recovery of this activity was observed in the cells incubated in darkness (15−20%) (Figure 2). When the sequences were normalized to the yield of the third flash, the oscillation pattern of the oxygen evolution with the flash number was identical in all cases (*61* and data not shown). This indicates that the residual oxygen-evolving PSII centers present in the photoinactivated cells as well as after recovery in the light or darkness had a normal charge separation activity and S-state cycle. The presence of glucose slightly increases the extent of PSII recovery (20−30%) (Figure 2).

D1 Protein Degradation and Synthesis during Recovery from Photoinhibition in the Light or Darkness. In *Chlamydomonas* cells, the rate of accumulation of stable newly synthesized D1 protein is regulated by the rate of D1 degradation (*62*−*64*). A similar relationship between D1

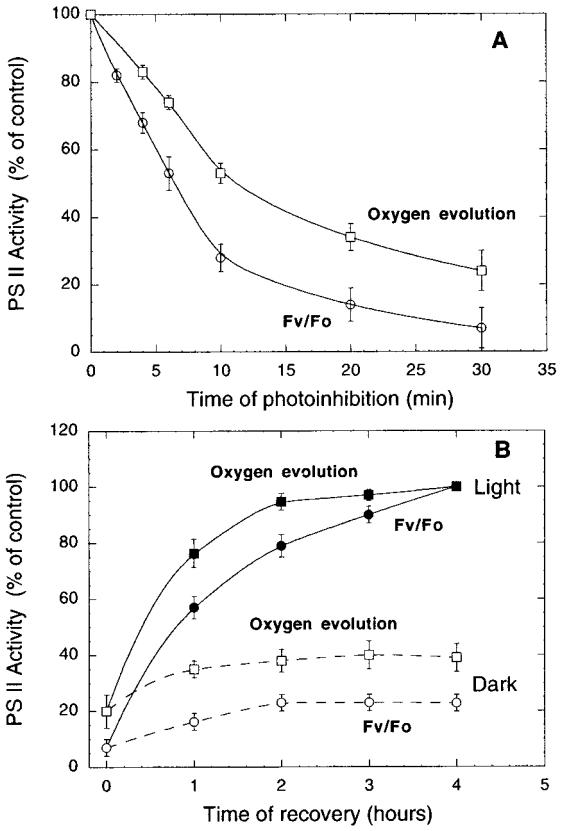

FIGURE 1: Kinetics of photoinactivation and recovery of PSII activity. (A) Decrease of oxygen evolution (open squares) and of variable fluorescence (Fv/Fo; Fv = Fm − Fo) (open circles) as a function of time in *Synechocystis* PCC 6714 cells (30 μg of Chl·mL^{-1}) exposed to 4000 μmol of photons·m^{-2}·s^{-1}, 22 °C. (B) Recovery of oxygen-evolving activity (squares) and of variable fluorescence (circles) in the photoinactivated cells exposed to 70 μmol of photons·m^{-2}·s^{-1}, 32 °C (closed symbols), or in darkness (32 °C) (open symbols). The 100% oxygen evolution measured using DCBQ (1 mM) as an external electron acceptor of PSII was 150 μM O_2 (mg of Chl)$^{-1}$ h^{-1}. Fm was measured in the presence of DCMU (2×10^{-5} M). The 100% value of Fv/Fo was 1.8.

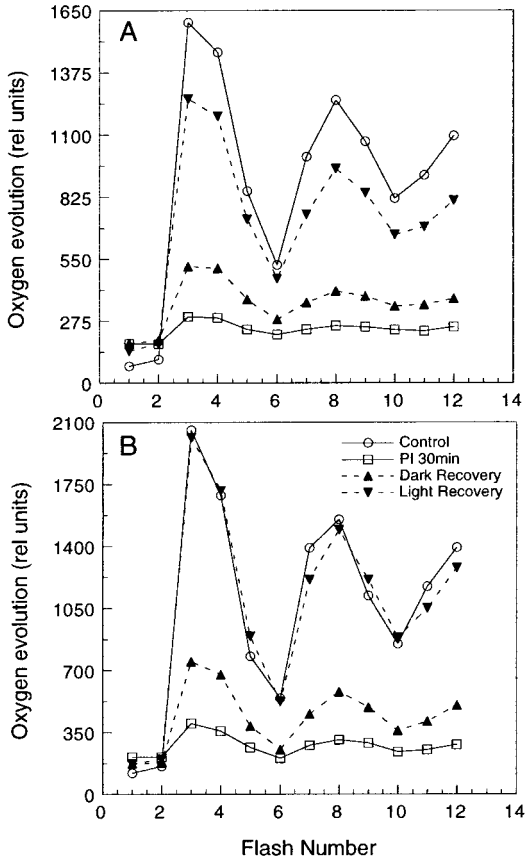

FIGURE 2: Oxygen flash-yield activity of control, photoinhibited cells and cells after recovery in the light or darkness. Cells were photoinhibited for 30 min (PI) and allowed to recover in the light or darkness for 2 h. Oxygen flash yield was measured as described under Materials and Methods. Panels A and B, cells grown and maintained in minimum or in glucose-enriched medium, respectively.

degradation and *psbA* mRNA translation has been reported in mutants of *Synechocystis* 6803 (*65*). We therefore determined whether the limited recovery of PSII activity in the *Synechocystis* PCC 6714 cells incubated in darkness could be due to their inability to degrade the D1 protein of the photoinactivated PSII. The level of the D1 protein was quantified by immunodecoration in thylakoids of cells, grown in the MM medium, following various treatments. Cells were harvested before photoinhibition, after 30 min of light stress, and after 2 h of recovery in low light or darkness in the absence of glucose and in the presence or in the absence of lincomycin. The SDS−PAGE-resolved D1 protein band appeared as a doublet (Figure 3A) in the absence of urea or as a single band in the presence of 4 M urea (Figure 3B). The appearance of the D1 doublet using monospecific polyclonal antibodies was observed also by other authors (Vermaas, personal communication). Phosphorylation of the protein that could result in formation of a D1 protein conformer with different electrophoretic mobility cannot explain this phenomenon since this protein is not phosphorylated in *Synechocystis* 6803 (*66*). Nevertheless, under our conditions, the level of the D1 protein band decreases by 30% in the photoinactivated cells as compared to in control cells (Figure 3A). After 2 h of incubation in low light or in darkness in the presence of lincomycin, both bands have almost completely disappeared. In the absence of lincomycin, a significant amount of the D1 protein doublet could be seen in the cells incubated in the light which have recovered PSII activity while only traces of the D1 doublet were detected in the dark-incubated cells. Similar kinetics of D1 degradation were observed under light and dark conditions (Figure 3B). Other PSII proteins such as D2, CP43, and CP47 decreased only by about 10% (data not shown). These results indicate that the D1 protein present in photoinactivated cells can be degraded in the light as well as in darkness even in the absence of glucose. Thus, the degradation of the D1 protein cannot be the limiting factor in the recovery of PSII activity in the dark-incubated cells.

Synthesis of the D1 Protein during Recovery from Photoinhibition in the Light or in Darkness. The results presented in Figure 3 suggest that in the absence of glucose, the inability of the photoinhibited cells to recover PSII activity in the darkness could be due to lack of de novo synthesis of D1 protein. To confirm this hypothesis, radioactive labeling experiments were carried out to assay the de novo protein synthesis and incorporation into the thylakoid membranes during the dark recovery process. [^{35}S]Met was added at the beginning of the recovery period or after 90 min of incubation in low light or in darkness of cells grown and incubated during the recovery process in low (MM) or rich (GM) glucose media. Radiolabeling was performed in each

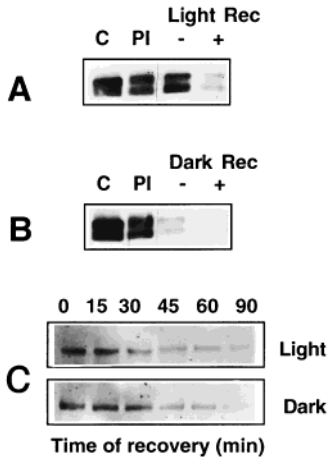

FIGURE 3: Loss and de novo synthesis of the D1 protein during the photoinhibiton and recovey process. (A and B) The D1 protein was detected by immunodecoration in thylakoid membranes isolated from nonphotoinhibited cells (control, C), from cells photoinhibited for 30 min (PI), and from photoinhibited cells after recovery for 2 h in the light (A, Light Rec) or in darkness (B, Dark Rec) in the presence (+) or in the absence (−) of lincomycin. The proteins were separated by SDS−PAGE (14% acrylamide). (C) The D1 protein was detected by immunodecoration in thylakoid membranes isolated from photoinhibited cells during different times of light and dark recovery in the presence of lincomycin. The thylakoid proteins were separated by SDS−PAGE (14% acrylamide, 4 M urea). The autoradiograms were scanned to obtain the values mentioned in the text. The photoinhibited samples presented 3% and 10% of their initial variable fluorescence and oxygen-evolving activity, respectively.

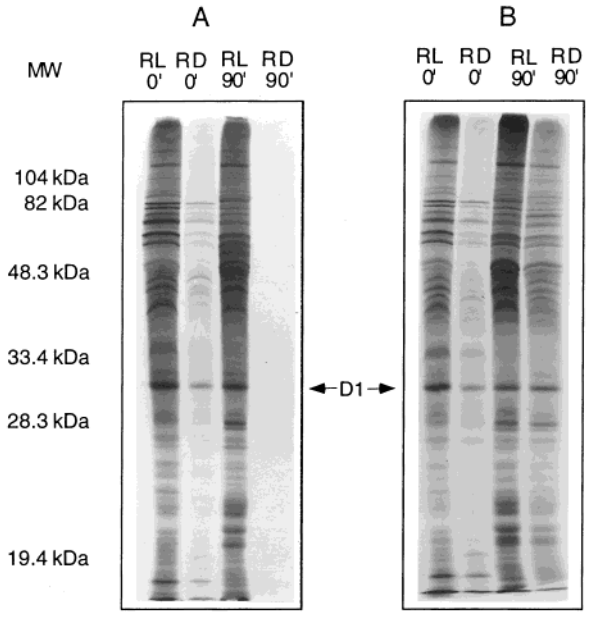

FIGURE 4: Protein synthesis during light and dark recovery in cells grown in minimum medium (A) and in a glucose-enriched medium (B). Cells were photoinhibited for 30 min and then transferred for recovery of PSII activity to low light (RL) or darkness (RD). [^{35}S]-Met was added at the onset of the recovery (0′) or after 90 min of incubation under recovery conditions. Radioactive labeling and autoradiograms of thylakoid proteins were carried out as described under Materials and Methods.

case for 45 min. The results of such experiments are shown in Figure 4.

During recovery in the light, a large number of thylakoid polypeptides were synthesized in both types of cells. Among these, the D1 protein was one of the most radiolabeled proteins. Following radioactive labeling started from the beginning of the light recovery (L0), about 5% of the total label present in the thylakoid membranes appeared in this protein in the MM and GM cells. Synthesis of the D1 protein continued, and the protein was highly labeled also if the radioactive methionine was added after 90 min of recovery (L90). In this case, the radioactivity incorporated in the D1 protein band amounted only to about 2% of the total thylakoid radioactivity. These results suggest that the rate of D1 protein synthesis decreases during the recovery process.

The D1 protein was heavily labeled in the thylakoids of the cells incubated in darkness during the first 30 min of the recovery process and amounted to about 4% of the total radioactivity of the thylakoids obtained from both the MM and GM cells. Synthesis of thylakoid proteins ceased progressively in the MM cells, and no radioactive labeling of proteins occurred after 90 min of incubation in darkness while significant de novo protein synthesis continued during this period in the GM cells (Figure 4). In this case, the D1 protein was highly labeled relative to the other thylakoid proteins (about 3% of the total radioactivity), i.e., almost the same relative labeling as that in the thylakoids of MM cells after 90 min recovery in the light. However, after 90 min recovery in darkness of cells grown in glucose-enriched medium, the total radioactive labeling of thylakoid proteins was still weaker than that incorporated in the light-exposed cells. These results could indicate that the energy source required for protein synthesis may become limiting in darkness and in the absence of glucose in the photoinactivated cells. Considering that the photosynthetic electron-transfer chain in cyanobacteria shares the plastoquinone pool with the respiratory chain, it is conceivable that the pool may become oxidized in the absence of photosynthetic electron flow and glucose and contribute to the down-regulation of D1 protein synthesis in these cells.

Effect of Darkness on D1 Protein Synthesis in Nonphotoinhibited Cells. To test whether the regulation of D1 protein synthesis by light is expressed only in the photoinhibited cells, we tested the radiolabeling of de novo synthesized proteins in control, MM, and GM nonphotoinhibited cells exposed to 70 μmol of photons·m^{-2}·s^{-1} or in darkness. [^{35}S]-Met was added at the start of light (L0) or dark (D0) incubation and after 30 (D30) or 90 min (D90) of dark incubation, and labeling was performed for 45 min (Figure 5). In the light, the labeling of the D1 protein relative to the total radioactivity of the thylakoid proteins was lower as compared to that of the photoinhibited cells (about 1% as compared to about 5% in the thylakoids of photoinhibited cells), indicating that the synthesis of the D1 protein is stimulated in cells that have been preexposed to light stress. In darkness, thylakoid protein synthesis progressively decreased in the cells incubated in the absence of added glucose (Figure 5). Furthermore, the synthesis of the D1 protein was more rapidly affected than the synthesis of other thylakoid proteins. In contrast, in the presence of glucose, synthesis of the D1 protein as well as other thylakoid proteins was maintained as active as in the light-exposed cells, even after a long period of dark incubation (Figure 5).

The results presented in Figures 4 and 5 showed that synthesis of thylakoid proteins and particularly that of the D1 protein ceases gradually in photoinhibited and nonpho-

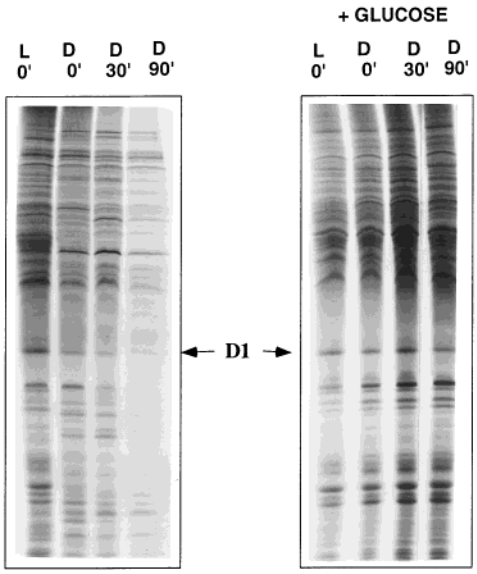

FIGURE 5: Thylakoid protein synthesis in the light or darkness in cells grown in minimal (MM) or glucose-enriched (GM) medium. Cells grown in MM or GM medium were preincubated for 30 min under low light conditions and then transferred to darkness or further incubated in the light. [^{35}S]Met was added at the onset of the transfer time to darkness or light incubation (D0 and L0, respectively) and after 30 and 90 min of incubation. Radioactive labeling was continued for 45 min. The figures are autoradiograms showing the pattern of protein labeling following SDS−PAGE of thylakoid proteins.

toinhibited cells incubated for prolonged times in darkness. The presence of an exterior source of energy and reducing power totally prevented this situation in nonphotoinhibited cells but only partially in photoinhibited cells. We have already reported that during photoinhibition the levels of several mRNAs decrease and that light stress also damages the transcriptional and translational machinery (*49*). This could explain the above differences between photoinhibited and nonphotoinhibited cells.

Assembly of Active PSII Complexes during Recovery from Photoinhibition. The fact that GM cells do not restore their initial photosynthetic activity during dark recovery whereas they are able to synthesize D1 and other thylakoid proteins led us to consider that the D1 protein synthesized in darkness may not be assembled in PSII complexes. To test this possibility, we compared the distribution of radioactively labeled D1 protein in the thylakoid membrane complexes of GM cells incubated for 90 min in light or in darkness after light stress. Pigment−protein complexes of membranes from cells treated as in Figure 4 were separated by nondenaturing Deriphat-PAGE as described under Materials and Methods. Three green bands (1−3), among them band 1 being the major one, and a yellow band (4) were resolved (Figure 6). Between bands 1 and 2, a series of very weak green bands were also observed (bands a−e, Figure 6). The pigmented bands were excised, and their proteins were resolved by denaturing SDS−PAGE. The gel was stained, dried, and exposed to X-ray film. The autoradiograms obtained in these experiments are shown in Figure 7. The green band 1 corresponds to PSI-enriched membranes (equivalent to the B1 band reported by *55*). Band 3 corresponds to PSII (equivalent to band B4 of 54). The green bands between bands 1 and 3 contained similar proportions of PSI and PSII complexes. The autoradiograms showed a

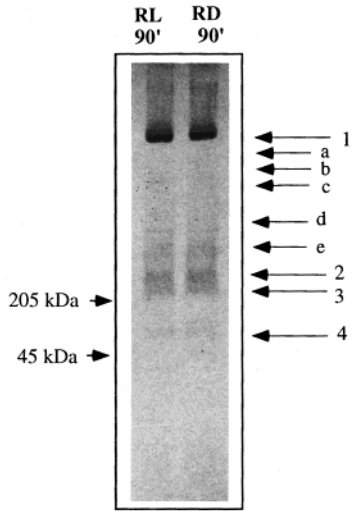

FIGURE 6: Pattern of protein−chlorophyll complexes of thylakoid membranes isolated from photoinhibited cells after recovery in the light or darkness. Thylakoid membranes were isolated from cells photoinhibited for 30 min and then allowed to recover PSII activity in the light or darkness. Radioactivity was added after 60 min of recovery. Radioactive labeling was continued for 45 min. The thylakoid protein−chlorophyll complexes were resolved by nondenaturing electrophoresis as described under Materials and Methods. All the bands were green except band 4 that was yellow. The mobilities of myosin (205 kDa) and ovalbumin (45 kDa) are indicated by arrows.

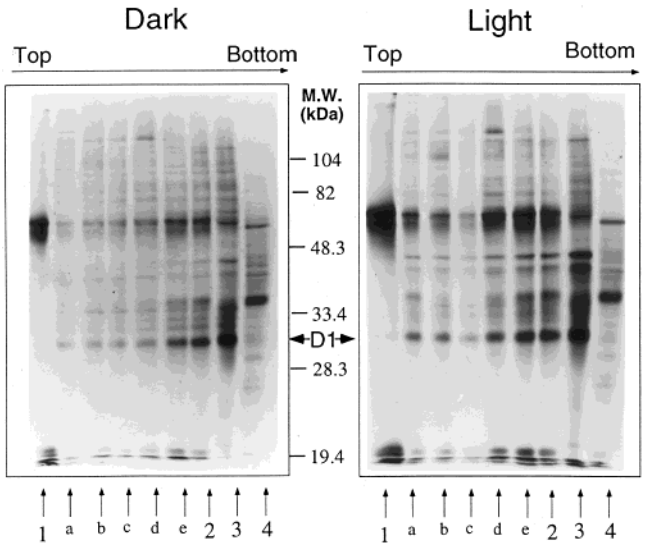

FIGURE 7: Distribution of the newly synthesized proteins in protein−chlorophyll complexes. Same experimental conditions as in Figure 6. The pigment-containing bands were excised from the gels shown in Figure 6, and their protein content was resolved by denaturing SDS−PAGE followed by autoradiography. Apparent molecular masses are indicated in the center of the figure.

similar profile for the thylakoid proteins synthesized during recovery in the light or in darkness (Figure 7). The distribution of the newly synthesized D1 protein in the different bands was also similar in both recovery conditions. The D1 protein was principally present in bands 2 and 3, containing the PSII complex. The D1 protein was found only in pigment−protein-containing regions of the nondenaturating gel (data not shown). These results suggest that the D1 protein synthesized during the recovery process in the light or darkness may be present in PSII Chl-containing complex.

Photoactivation of Dark-Assembled PSII. Since the D1 protein is synthesized in the light-stressed cells during the recovery period of incubation in darkness and seems to be integrated in complexes identified as PSII, we considered the possibility that the light-limiting step for recovery of PSII activity is the photoactivation of dark-assembled complexes. According to the current models, complete assembly and activation of the Mn cluster are light-driven processes. We have therefore measured PSII activity in thylakoids isolated from cells treated as described in the first section but bypassing the Mn cluster by using DPC as an electron donor to Yz and DCIP as an electron acceptor. The reduction of DCIP using DPC was compared to that using water oxidation as an electron donor. After 30 min of photoinhibition, PSII activity was lowered by 70% of its initial value. During the following low-light incubation, most (~70%) of the PSII electron-transport activity from DPC to DCIP was recovered. However, no recovery of electron-transfer activity was detectable in the thylakoids obtained from the cells incubated in darkness (not shown). The limited PSII activity measured in the cells that have been exposed to recovery in darkness (Figure 2) seemed to be unstable and was lost following the breaking of the cells and isolation of the thylakoid membranes. Thus, light is necessary to recover both electron-transfer activities, from water to DCIP as well as from DPC to DCIP. These results suggested that the loss of oxygen evolution was due not only to lack of assembly of the Mn cluster in the PSII donor side but also to lack of recovery of the acceptor side activity or stable charge separation. Another possibility is that the Mn cluster is misassembled and cannot be photoactivated. A series of 50 saturating white flashes (data not shown) or continuous illumination (see below) were unable to photoactivate the dark-assembled PSII complexes. Under these conditions, DPC is unable to act as an electron donor. In contrast, photoactivation of dark-grown *Chlamydomonas rehinhardtii* (*67*) or *Synechocystis* (A.-L. Etienne, personal communication) cells is very efficient and rapid. Most of the photoactivation occurs in less than 1 min of continuous illumination or after several white saturanting flashes.

One can assume that recovery of PSII activity requires an additional light-dependent protein synthesis. To test this possibility, we measured the oxygen-evolving activity in vivo during recovery of photoinhibited GM cells in the light, or in darkness in the absence or presence of lincomycin for 2 h. The dark-incubated cells were then transferred to the light, and incubation was continued in the presence or absence of this protein synthesis inhibitor. The sustained oxygen-evolving activity in continuous light was measured using DCBQ as an electron acceptor (Figure 8A) or by a Joliot-type, rate oxygen electrode (Figure 8B). A limited recovery of PSII activity was observed in the cells incubated in darkness (see also Figure 2). Lincomycin completely inhibited recovery of PSII activity in darkness. When the cells incubated in darkness in the absence of lincomycin were transferred to the light, the initial level of PSII activity was recovered. The recovery of activity was considerably lower (10%) if lincomycin was present during the incubation in the light (Figure 8). These results indicate that most of the PSII activity recovered under these conditions was not related to the D1 protein synthesized in darkness but rather to the light-induced synthesis of D1 and/or other proteins. We

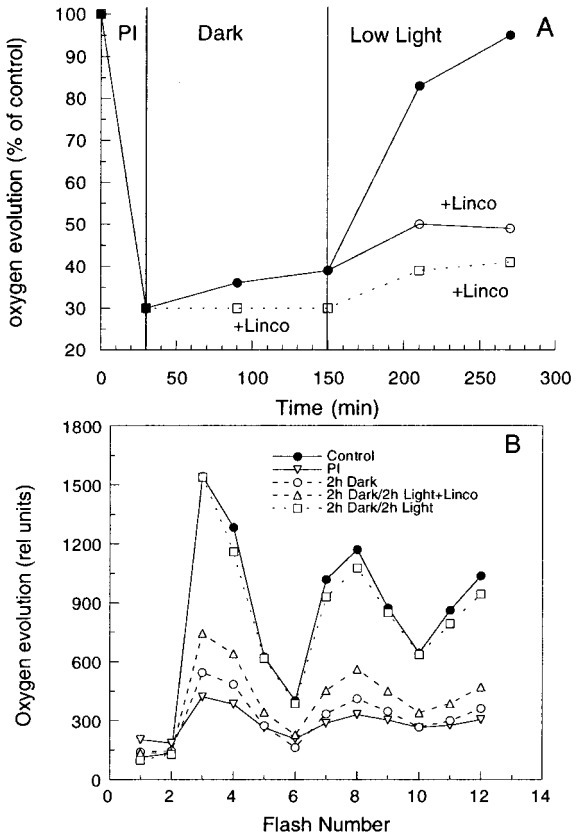

FIGURE 8: Photoactivation of PSII complexes formed during recovery in the light or darkness in photoinhibited cells. (A) Time course of oxygen evolution (measured using DCBQ as an electron acceptor) during photoinhibition (PI) and recovery in darkness (Dark) in the absence (closed circles) or in the presence (open squares) of lincomycin followed by further incubation in light in the absence (closed circles) or presence of lincomycin (open circles and squares). (B) Oxygen flash-yield activity of the same samples as in (A).

conclude that the D1 protein synthesized in darkness cannot be photoactivated in the absence of light-dependent synthesis of proteins that so far are not identified.

DISCUSSION

The aim of this work was to determine the role of light in the recovery of PSII activity of photoinhibited cyanobacterial cells. Until recently, the inability of these cells to recover the activity of PSII in darkness was largely ascribed to the accepted idea that the D1 protein is not synthesized in the dark. However, we have reported before that D1 synthesis occurs in darkness after photoinhibition of *Chlamydomonas reinhardtii* y-1 and *Scenedesmus* cells (*64*, *68*). The photodamaged D1 protein could be degraded and replaced by a new copy, even in *C. reinhardtii* y-1 cells which are unable to synthesize chlorophyll in darkness. In a more recent study, we have demonstrated that the OEC33 protein, stabilizing the Mn cluster of the oxygen-evolving complex, dissociates from PSII following degradation of the D1 protein during light stress. The OEC33 protein reassociates with PSII under recovery conditions in light as well as in darkness (*69*). Despite this, only a very limited extent of the initial photosynthetic activity has been recovered in the dark-incubated cells. Thus, in eukaryotic photosynthetic organisms, D1 protein synthesis and PSII reassembly are not the only determinant elements for the recovery of photosynthetic

activity in darkness. Other light-dependent reactions are required for activation of dark-assembled complexes.

In this work we show that in the prokaryote *Synechocystis* PCC 6714 cells light is essential for synthesis of the D1 protein and of other thylakoid proteins in the absence of an external source of carbon. In the presence of glucose, synthesis of these proteins may occur even in darkness. However, synthesis of these proteins is not sufficient for recovery of PSII activity in photoinhibited cells.

D1 Synthesis during Recovery of PSII Activity in the Light or Darkness in the Absence of Glucose. Under light-recovery conditions, active protein synthesis and incorporation of the newly synthesized proteins into the thylakoid membranes occurred from the onset of the recovery process and continued for at least 2 h. This suggests that transcription and translation are activated during light recovery. During photoinhibition, the transcription activity of several genes encoding photosynthetic proteins decreases quickly, and the corresponding transcripts disappear (*49*). After 30 min of photoinhibition, the level of these transcripts is very low or undetectable. The initial levels are restored only after 2 h of light recovery (*49*). However, *psbA* mRNA presents the particularity of a higher turnover during light stress than under growth light conditions, and a high level of *psbA* transcripts is maintained during the photoinhibition process. The high level of *psbA* mRNA may allow rapid translation and replacement of the degraded D1 protein (*49*). Indeed, in this paper we show that newly synthesized D1 protein is incorporated into the thylakoid membranes at higher rates than other proteins, especially at the initial period of recovery. This has been observed in the cells incubated in the dark as well as in the light during the recovery process.

Synthesis of the D1 protein occurs initially also in darkness albeit at a lower rate as compared to the light-incubated cells. Nevertheless, the D1 protein was still specifically synthesized and incorporated into the thylakoid membranes. After longer incubation in darkness, the de novo synthesis of the protein was drastically reduced. Since the degradation of the D1 protein following photoinactivation of PSII occurs to the same extent during the recovery period in light or darkness, the decrease of the de novo D1 protein present in the thylakoid membrane results more likely from cessation of its synthesis rather than a decrease in the degradation of the photoinactivated PSII D1 protein required for its replacement.

The incubation of *Synechocystis* cells in darkness causes a rapid decrease of mRNAs including those encoding photosynthetic proteins. However, the level of the *psbA* mRNA remains high for a long time due to the stabilization of the transcript (*45, 65*). This balances the decreasing transcriptional activity of *psbA* in darkness (*48*). Despite the relatively high level of *psbA* mRNA, the D1 protein synthesis and/or incorporation into the membrane is gradually inhibited in darkness. This could indicate that light regulates *psbA* mRNA translation in *Synechocystis* PCC 6714. It has already been suggested that in *Synechocystis* cells, transcript levels do not constitute the sole mechanism regulating D1 synthesis (*47, 49, 50*). The translation of *psbA* mRNA can occur in the absence of active transcription (*49*), or, alternatively, the accumulation of the D1 protein is not always directly proportional to the level of the transcript (*47, 49, 50*). In addition, there is a relation between the accumulation of the PSII D1 protein and the availability of chlorophyll (*47*). In conclusion, light seems to act at the transcriptional and posttranscriptional levels in *Synechocystis* PCC 6714 cells.

Glucose Metabolism Can Substitute for the Light Requirement of D1 Protein Synthesis but Not for the Formation of Active PSII. The presence of glucose had little effect on photoinhibition and PSII activity recovery processes. The *Synechocystis* PCC 6714 cells did not recover their initial photosynthetic activity in darkness irrespective of the presence or absence of glucose in the medium. However, the presence of glucose allowed synthesis of thylakoid proteins including the D1 protein during dark incubation. This was observed in both photoinhibited and control cells. We have already demonstrated that transcription of *psbA* and of other photosynthetic genes is maintained in the presence of glucose (*48*). The results of that work suggested that the effect of glucose may be related to the redox state of the photosynthetic electron-transport chain. The electron-transport chain including the plastoquinone pool become more oxidized during dark incubation due to a decrease of the $NADPH+H^+$ concentration (*70, 71, 48*). The glucose metabolism via the pentose phosphate pathway leads to the production of $NADPH+H^+$, and a large level of reducing power is conserved even in darkness. This reducing power can be used for oxidative phosphorylation as well as for reduction of the thioredoxin, allowing the maintenance of the intersystem chain as more reduced. However, the involvement of the redox state of the electron-transport chain in the regulation of D1 synthesis and the nature of other signal(s) involved in D1 translation remain to be elucidated.

In pea chloroplasts, dark-synthesized D1 protein is very poorly incorporated into PSII complexes (*24*). In this work, we showed that in *Synechocystis* cells grown in a glucose-containing medium, the D1 protein is synthesized in darkness and possibly incorporated in PSII complexes. A similar distribution of the D1 protein synthesized during light and dark recovery was observed. The light- and dark-synthesized protein was mostly incorporated in the green band 3 as detected by nondenaturing electrophoresis. This band contains essentially the proteins of the PSII complex (*55*). Based on the apparent molecular weight of band 3, one can assume that this band probably contains monomers of PSII complexes (see also *55*). The molecular mass of monomers is around 240−250 kDa and that of dimers 400−500 kDa (*72*). The D1 protein was the most radioactively labeled polypeptide of this band, suggesting that de novo synthesized D1 could be assembled, at least partially, with preexisting proteins of PSII under both light and dark conditions. In light, PSII activity was recovered, indicating that the newly synthesized D1 protein not only comigrates with PSII but also incorporates in a functional PSII form. In darkness, although the D1 protein is synthesized and incorporated into band 3, PSII activity is not recovered. We cannot totally discard the possibility that unassembled D1 protein comigrates with PSII in the electrophoretic system used in this work. However, in vivo the D1 protein is not stable and does not accumulate in thylakoids when not assembled into Chl−protein complexes (see ref *1* for recovery, refs *73*−*75* for *Synechocystis* mutants, and ref *76* for *Chlamydomonas* mutants).

The dark-assembled PSII complexes were not photoactivatable in the absence of additional light-dependent protein synthesis. These results suggest that binding of manganese

ions to the donor side of PSII may not be the only limiting step for the recovery in darkness of photosynthetic activity in photoinhibited cells.

Interestingly, light is not required for the biogenesis of active RCII during growth of *Synechocystis*, *Scenedesmus*, and *Chlamydomonas* wild-type cells in the dark in the presence of a carbon source. Moreover, when dark-grown *C. reinhardtii* y-1 cells exposed to the light in the presence of chloramphenicol (lacking all plastid-encoded thylakoid proteins and photosynthetic activity but containing significant amounts of chlorophyll and assembled LHCII) are then transferred to darkness after chloramphenicol washing, oxygen evolution activity is partially recovered (*77*). Basically a similar effect was obtained in greening *Euglena gracilis* cells (*78*). These results suggest that the assembly of D1 protein synthesized in darkness concomitantly with all other plastid-encoded components of PSII allows the activation of PSII without additional light-dependent protein synthesis. However, in *Scenedesmus* (*67*) and *Chlamydomonas* (*64*) as well as in cyanobacteria cells, light is necessary to recover PSII activity after photoinhibition (this work and see also *79*). Gong and Ohad (*67*) have suggested that the process of activation of RCII assembled from preexisting components and newly synthesized ones may involve light-regulated steps. The results presented in this paper indicate that this light-regulated step needs protein synthesis (probably other than D1 synthesis). Further studies will be necessary to elucidate which protein(s) is (are) involved in this process. This (these) protein(s) may be a component of the PSII (also degraded during photoinhibition) or a chaperone-type protein needed for the attachment of the cofactors or for the correct assembly of PSII in a dimer, that is the active form of these complexes. Another possibility is that in the case of recovery from photoinhibition, light is required for the binding of cofactors (and/or correct PSII assembly) that should occur simultaneously to the synthesis of the D1 protein to obtain active PSII complexes.

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