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# Role of two forms of the D1 protein in the recovery from photoinhibition of photosystem II in the cyanobacterium *Synechococcus* PCC 7942

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**Abstract**

The study of turnover of two distinct forms of the photosystem II (PSII) D1 protein in cells of the cyanobacterium *Synechococcus* PCC 7942 showed that the 'high-light' form D1:2 is degraded significantly faster at 500 μE $m^{-2}$ $s^{-1}$ as compared with 50 μE $m^{-2}$ $s^{-1}$ while the degradation rates of the 'low-light' form D1:1 under low and high irradiance are not substantially different. Consequently, the D1:1 turnover does not match photoinactivation of PSII under increased irradiance and therefore the cells containing this D1 form exhibit a decrease in the PSII activity. Monitoring of the content of each D1 form during a recovery from growth–temperature photoinhibition showed a good correlation between the synthesis of D1:2 and restoration of the PSII activity. In contrast, when photoinhibitory treatment was conducted at low temperature, a fast recovery was not accompanied by the D1:2 accumulation. The data suggest that photoinactivation at growth temperature results in a modification of PSII that inhibits insertion of D1:1 and, therefore, for restoration of the photochemical activity in the photoinactivated PSII complexes the D1:2 synthesis is needed. This may represent the primary reason for the requirement of *psbAII*/*psbAIII* expression under increased irradiance. 

*Keywords:* Cyanobacterium; D1 protein; Photoinhibition; Photosynthesis; Photosystem II; *Synechococcus* PCC 7942

## 1. Introduction

The D1 protein represents a key subunit of photosystem II (PSII), a multicomponent pigment–protein complex of oxygenic photosynthetic organisms (for recent review on PSII see [1]). The protein is encoded by the *psbA* gene that is usually present as a single copy in the chloroplast genome of algae and higher plants [2]. In contrast, the genome of cyanobacteria, the oxygenic photosynthetic prokaryotes, usually contains a *psbA* multigene family (for review see [3]). This is also the case of a cyanobacterium *Synechococcus* PCC 7942 that contains three genes encoding two different forms of the D1 protein [4]. As a response to increased irradiance, the amount of the *psbAI* transcript encoding the 'low light' form D1:1 is diminished while the level of the *psbAII* and *psbAIII* transcripts encoding the 'high light' form D1:2 is increased [5,6]. It has been shown that under high light conditions the *psbAI* transcription is

Abbreviations: CAP, chloramphenicol; Chl, chlorophyll; DM, dodecylmaltoside; HRA, Hill reaction activity; PS, Photosystem; PSIIPI, photoinactivation of photosystem II; RIF, rifampicin

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stopped and the breakdown of the *psbAI* transcript is accelerated by a newly synthesized protein factor [7]. On the other hand, a transcriptional induction but not an mRNA stabilization is responsible for the increased level of *psbAII* and *psbAIII* transcripts in high light [7]. Similar replacement of mRNA is detected also after the transfer of cells from growth to low temperature [8]. In parallel with the changes in *psbA* transcription, there is an appearance of the D1:2 form in the membrane while D1:1 is degraded [9,10]. The opposite process (i.e. removal of D1:2 from the membrane and appearance of D1:1 related to the induction of the *psbAI* and suppression of the *psbAII* and *psbAIII* transcription) occurs when cells are shifted from high to low irradiance [10,11].

It is usually assumed that the fast, light-dependent turnover of the D1 protein is triggered by the PSII photoinactivation (PSIIPI) (for reviews see [12,13]) and therefore, its main purpose consists in the repair of photoinactivated PSII complexes [14]. Both D1 forms in *Synechococcus* PCC 7942 exhibit a similar rate of turnover under low light conditions as judged from the radioactive pulse-chase labeling [15]. Clarke et al. [10] also observed similar degradation rates for both forms under high light conditions in the presence of streptomycin. However, the results of Krupa et al. [16] and Clarke et al. [10] pointed to a significantly lower rate of PSII repair in the mutant containing only D1:1 as compared with wild type or mutant with D1:2 only. On the other hand, Kulkarni and Golden [11] detected much faster degradation of D1:1 as compared with D1:2 in the presence of CAP. Although a clear conclusion cannot be drawn from these results they seem to indicate that the turnover of the two D1 forms exhibits significant differences under high light conditions.

The aim of the present work was to characterize the degradation of each D1 form under different light conditions and to establish their role in the process of recovery from photoinhibition in the wild type of *Synechococcus* PCC 7942. The results showed that the turnover of D1:1 efficiently ensures stability of the PSII activity in cells under low light conditions. However, when the irradiance is increased the rate of D1:1 turnover is not able to match the rate of PSIIPI possibly due to a temperature-dependent modification of photoinactivated PSII complexes that inhibits D1:1 insertion. The resulting loss of PSII activity can be efficiently compensated by the D1:2 synthesis.

## 2. Material and methods

A cyanobacterium *Synechococcus* PCC 7942 was grown at 35°C in medium A [17] and bubbled with air containing 2% $CO_2$. The cylindrical cuvette with the culture (40 mm internal diameter) was irradiated by tungsten filament bulbs to give 100 μE $m^{-2}$ $s^{-1}$ at the cylinder surface. The culture was diluted every day to maintain the chlorophyll concentration at about 25 μg $ml^{-1}$. Before experiments the culture was diluted to 7 μg (Chl) $ml^{-1}$ and placed into 18-mm thick planparallel cuvettes in a temperature controlled bath and bubbled with air containing 2% $CO_2$. The irradiance was provided by tungsten filament bulbs. When inhibitors chloramphenicol (CAP; 50 μg $ml^{-1}$ final concentration), rifampicin (RIF, 100 μg $ml^{-1}$ final concentration) or diuron ($10^{-5}$ M final concentration) were used, cells were incubated for 5 min before the start of the light treatment. Each experiment was repeated 2–4 times to confirm the trend and one typical example is shown in the figures.

A light-saturated (3500 μE $m^{-2}$ $s^{-1}$) steady-state rate of oxygen evolution (Hill reaction activity, HRA) in cell suspensions was measured at 35°C using a temperature controlled chamber [18] equipped with a Clark-type electrode (YSI, USA). The artificial electron acceptors *p*-benzoquinone (0.5 mM final concentration) and ferricyanide (1 mM final concentration) were added just before measurement. The HRA of cells was 900–1200 μmol $O_2$ mg $(Chl)^{-1}$ $h^{-1}$). For the measurement of HRA in diuron-poisoned cells, these were washed twice with the cultivation medium before the measurement. The HRA of the diuron-treated and subsequently washed cells was lower by 10–20% as compared with the untreated cells.

The set-up for the radioactive labeling was identical as for the photoinhibitory treatment. Radiolabeled L-[$^{35}$S]-methionine (>1000 Ci $mmol^{-1}$, Amersham) was added (final activity 1 mCi $ml^{-1}$) to the diluted cell suspension which was exposed to either 50 μE $m^{-2}$ $s^{-1}$ for the cells containing D1:1 or 500 μE $m^{-2}$ $s^{-1}$ for the cells pre-exposed to this light

intensity for a previous 3 h and containing mainly D1:2. The pulse proceeded for 20 min and then cold methionine was added to a final concentration of 2 mM. For characterization of light induced D1 degradation (chase), cells were subjected to 50 or 500 μE $m^{-2}$ $s^{-1}$ and 10 ml aliquots were withdrawn at appropriate time intervals and used for the preparation of thylakoid membranes (see below).

Thylakoid membranes were prepared by vortexing the cells with glass beads at 4°C as described in Komenda et al. [19]. Thylakoids for trypsinization were isolated using the same procedure but in all buffers 0.5 M mannitol was present.

Digestion of thylakoids by Lys-C protease was conducted at 25°C for 60 min and then the thylakoids were immediately solubilized with SDS for electrophoresis. Chl concentration of the thylakoid suspension during digestion was 450 μg $ml^{-1}$ and the protease was used at concentration 20 μg $ml^{-1}$. Trypsinization of thylakoids was performed at Chl concentration 200 μg $ml^{-1}$ and trypsin concentration 50 μg $ml^{-1}$. After 5, 15, 30 and 60 min incubation at 25°C, aliquots were withdrawn and proteolysis was stopped by transfer to ice and addition of 2 mM Pefabloc SC (Merck).

The protein composition of thylakoids was assessed by electrophoresis in a denaturing 12–20% linear gradient polyacrylamide gel containing 7 M urea [20]. The thylakoids were solubilized in 60 mM Tris/HCl, pH 9, containing 2% SDS (w/v) and 2% dithiothreitol (m/v) at 20°C for 60 min. Samples were loaded with an equal amount of chlorophyll (0.5 μg for immunoblotting and 2.5 μg for autoradiography) and proteins in the gel were transferred onto nitrocellulose membrane (0.2 μm, Schleicher-Schuel, Germany) by semi-dry blotting. Membrane was incubated with specific antibodies and then with a secondary antibody–alkaline phosphatase conjugate. Proteins were visualized by colorimetric reaction using the BCPIP-NBT system. The following specific antibodies were used: (i) form-specific antibodies raised against the first 25 amino acid residues of D1:1 and D1:2 from *Synechococcus* PCC 7942 [21]; (ii) antibody raised against residues 58–86 of the spinach D1 (D1 Nt-antibody); (iii) antibody raised against the last 29 residues of the pea D1 precursor (D1 Ct-antibody) and (iv) antibody raised against the last 12 residues of the pea D2 (D2 Ct-antibody). For autoradiography, the membrane was exposed to the sensitive film for 48–96 h. The autoradiograms were scanned using the Genius GS-FC60 scanner and the band intensities were estimated from the digitized image using the SigmaGel software (Jandel, USA).

The thylakoids for separation of chlorophyll-proteins were solubilized with dodecylmaltoside (DM/Chl = 20, w/w) and quickly spun down to remove insoluble material. The extracts were analyzed by non-denaturing electrophoresis in a 5–10% linear gradient polyacrylamide gel according to Laemmli [20] except that SDS was replaced by 0.2% Deriphat 160 in the electrophoretic buffer and the gel contained no detergent. Samples were loaded with an equal amount of chlorophyll (10 μg).

For the determination of the level of *psbA* transcript, total RNA was isolated from the cells according to the phenol-extraction procedure described in [22]. RNA was then fractionated on 1% agarose gels containing 20 mM sodium phosphate buffer, pH 7.0 and 17% formaldehyde. Equal amounts of RNA (10 μg) were loaded for each sample. Fractionated RNA was transferred to Hybond N+-membrane and fixed by UV cross-linking. Prehybridization (30 min) and hybridization (18 h) were performed at 55°C in AlkPhos Direct hybridization buffer (Amersham). As the *psbA* gene-specific probe, a PCR-generated DNA fragment containing codons 60–304 of the *psbA2*/*psbA3* genes from *Synechocystis* 6803 was used. The probe was labeled with thermostable alkaline phosphatase using an AlkPhos Direct kit. After hybridization, the membrane was washed in AlkPhos Direct primary washing buffer at 55°C for 10 min and twice in AlkPhos Direct secondary washing buffer at room temperature for 5 min. Finally, the membrane was immersed in the chemiluminescence substrate CDP-Star, wrapped in Saran wrap and exposed for 1–3 h to X-ray film.

The concentration of chlorophyll was assayed in 100% methanol [23].

## 3. Results

In the initial experiments we characterized the rate of D1:1 and D1:2 degradation in the *Synechococcus* cells by radioactive pulse-chase protocol (without use

of protein synthesis inhibitors) at 50 and 500 μE $m^{-2}$ $s^{-1}$ and compared them with the time course of PSII activity measured under identical light conditions in the absence and presence of CAP (Fig. 1). At 50 μE $m^{-2}$ $s^{-1}$ the rate of D1 degradation was similar for cells containing D1:1 and D1:2 (Fig. 1C,D) and it approached (for the D1:2-containing cells) or exceeded (for the D1:1-containing cells) the rate of PSIIPI in the presence of CAP (Fig. 1A,B). There was no decrease of PSII activity in the absence of CAP documenting the ability of the D1:1 and D1:2 turnover to counteract PSIIPI. When irradiance was increased ten times, the rate of degradation was accelerated proportionally to the acceleration of CAP-induced PSIIPI only in the D1:2-containing cells while it did not substantially change in the D1:1-containing cells. Consequently, the cells containing D1:1 exhibited a significant decrease of PSII activity even in the absence of CAP during the first 90 min of the high light treatment (Fig. 1A). The following increase of the activity observed during the next 90 min was related to the synthesis of

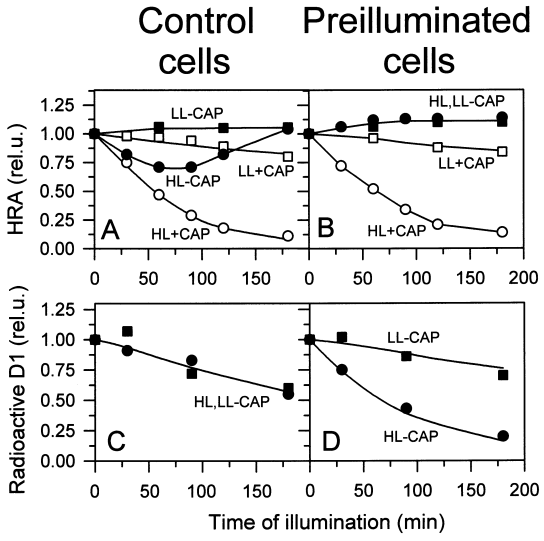


Fig. 1. Time course of HRA and degradation of the radiolabelled D1 protein during low and high light treatment at 35°C in the *Synechococcus* cells containing D1:1 or D1:2. The control cells (containing D1:1; A, C) or the cells pre-illuminated for 180 min at 500 μE $m^{-2}$ $s^{-1}$ (containing D1:2; B, D) were illuminated at 50 (LL, squares) or 500 μE $m^{-2}$ $s^{-1}$ (HL, circles) in the absence (closed symbols) or presence (empty symbols) of CAP. Samples were taken at indicated time intervals for measurements of HRA or for isolation of thylakoids, electrophoresis of thylakoid proteins and autoradiography as described in Section 2.

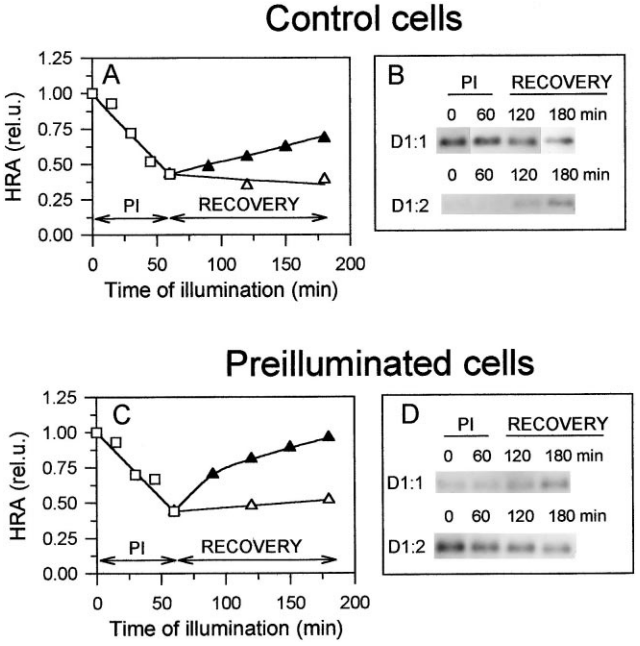


Fig. 2. Time course of HRA and level of two D1 forms during photoinhibition at 35°C and the subsequent recovery from photoinhibition in the *Synechococcus* cells containing D1:1 or D1:2. The control cells (containing D1:1; A, B) or the cells pre-illuminated for 180 min at 500 μE $m^{-2}$ $s^{-1}$ (containing D1:2; C, D) were photoinhibited at 500 μE $m^{-2}$ $s^{-1}$ for 60 min in the presence of CAP (PI, open squares), then the cells were washed and exposed to 50 μE $m^{-2}$ $s^{-1}$ (RECOVERY) in the absence (closed triangles) or presence of CAP (empty triangles). Samples were taken at indicated time intervals for measurements of HRA or for isolation of thylakoids, electrophoresis of thylakoid proteins and immunoblotting as described in Section 2.

the D1:2 form [19]. The inability of the cells containing D1:1 to cope with the fast PSII photodamage under increased irradiance is in line with the results obtained with a mutant containing just the D1:1 form [16].

Our previous studies of the thermophilic *Synechococcus* [24], *Synechococcus* PCC 7942 [25] and especially *Synechocystis* PCC 6803 [26], showed that the rate of the D1 degradation in cyanobacteria is dependent on the functional protein synthesis and most probably on the synthesis of the new D1 protein [26]. Therefore, our finding that the accelerated PSIIPI under 500 μE $m^{-2}$ $s^{-1}$ did not result in the proportionally increased rate of D1:1 degradation led us to a hypothesis that the D1:1 insertion is inhibited in the photoinactivated PSII complexes and as a consequence, also the degradation of D1:1 protein to be

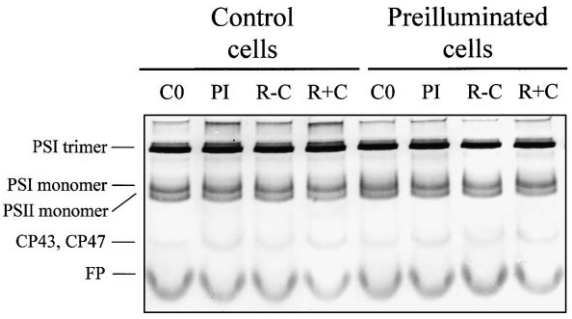


Fig. 3. Analysis of chlorophyll-proteins in the thylakoids isolated from D1:1- and D1:2-containing cells before photoinhibition, after photoinhibition and after subsequent recovery from photoinhibition in the absence and presence of CAP at 35°C. The control cells (D1:1-containing cells) or the cells pre-illuminated for 180 min at 500 μE $m^{-2}$ $s^{-1}$ (D1:2-containing cells) were photoinhibited at 500 μE $m^{-2}$ $s^{-1}$ for 60 min in the presence of CAP (PI), then the cells were washed and exposed to 50 μE $m^{-2}$ $s^{-1}$ for 120 min in the absence (R−C) or presence of CAP (R+C). Electrophoresis of chlorophyll-proteins was performed in the presence of Deriphat 160 as described in Section 2. FP: free pigments.

replaced is slowed down. This hypothesis was tested by further experiments. We monitored the D1 content during recovery from photoinhibition. The cyanobacterial cells were illuminated for 60 min with 500 μE $m^{-2}$ $s^{-1}$ at 35°C in the presence of CAP and then the cells were washed and subjected to 50 μE $m^{-2}$ $s^{-1}$ for recovery from photoinhibition. We found that during the high light treatment PSII activity decreased to about 50% of the initial value and subsequent recovery was rather slow: HRA increased only by about 25% in 2 h (Fig. 2A). The corresponding appearance of D1:2 in the membranes and slight decrease of the D1:1 content (Fig. 2B) indicated that the synthesis of D1:2 was responsible for the partial restoration of the PSII function.

The same experiment was conducted with the cells preilluminated with 500 μE $m^{-2}$ $s^{-1}$ for 3 h containing the D1:2 form [19]. The rate of PSIIPI in the presence of CAP was similar to the cells with the D1:1 form. However, restoration of HRA after washing the cells and their exposure to low light was faster as compared with the cells containing D1:1 and reached nearly 100% of the initial activity after 2 h (Fig. 2C). Monitoring of the D1 replacement showed that the amount of D1:2 decreased while the content of D1:1 increased during the recovery process (Fig. 2D). However, as HRA of the cells reached nearly 80% of the initial activity in the first hour of the recovery process and only small amount of D1:1 appeared in the membranes during this period, we concluded that the photoinactivation of PSII complexes with D1:2 was also counterbalanced by the D1:2 synthesis. Similar to the previous experiment, we found the constant steady-state level of the total D1 and D2 proteins accompanied by no increase in the chlorophyll content and cell number during the recovery phase of the experiment (data not shown) indicating that the restoration of the PSII activity proceeds by the insertion of D1:2 into preexisting complexes rather than by the assembly of new PSII complexes. In summary, these results supported our hypothesis since they showed that insertion of D1:2 but not D1:1 is responsible for the repair of photoinactivated PSII.

The conclusion about insertion of D1:2 into the assembled PSII complexes was supported by monitoring PSII integrity in *Synechococcus* cells during photoinhibition and the recovery phase by the electrophoresis of pigment–protein complexes in the presence of Deriphat. Similar to the situation in the thermophilic *Synechococcus* [24] electrophoresis separated chlorophyll-proteins of the control cells into three bands representing trimeric and monomeric PSI complex and monomeric PSII complex (Fig. 3) as determined by absorption spectroscopy and polypeptide composition (not shown, see [24]). After 1 h of strong illumination there was maximally a 15% decrease in the intensity of the PSII core band and corresponding appearance of free CP47 and CP43 reflecting very limited disassembly of PSII complexes despite the 50% loss of PSII activity. Significant disassembly of PSII cores did not occur even during the subsequent low light treatment of the photoinhibited cells in the presence of CAP. As under the same conditions in the absence of CAP the activity of D1:2-containing PSII was fully restored, it implies that the PSII disassembly was not a prerequisite for PSII repair and that during the recovery from photoinhibition mostly assembled PSII core complexes were targets for the insertion of the newly synthesized D1:2 molecule. Moreover, the same electrophoretic pattern showed that the rate of PSII disassembly under photoinhibitory conditions is very similar for PSII containing D1:1 and D1:2. Thus, the different rate of PSII disassembly could not explain the

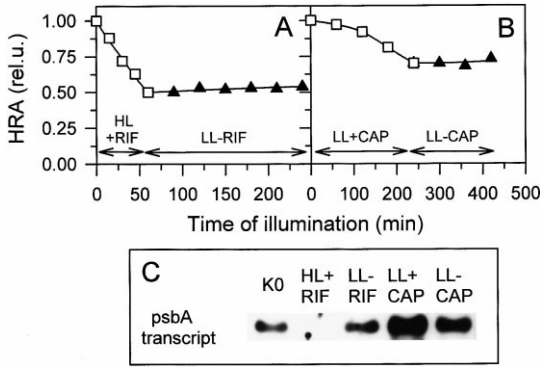


Fig. 4. Time course of HRA and level of *psbA* transcript during high light treatment in the presence of RIF or during low light treatment in the presence of CAP and during the subsequent recovery phase after washing out the inhibitors in the cells containing D1:1 at 35°C. (A) Cells were photoinhibited at 500 μE $m^{-2}$ $s^{-1}$ for 60 min in the presence of RIF (HL+RIF, open squares), then washed and further incubated for another 180 min at 50 μE $m^{-2}$ $s^{-1}$ (LL−RIF, closed triangles). (B) Cells were photoinhibited at 50 μE $m^{-2}$ $s^{-1}$ for 240 min in the presence of CAP (LL+CAP, open squares), then washed and further incubated for another 180 min at 50 μE $m^{-2}$ $s^{-1}$ (LL−CAP, closed triangles). (C) Levels of the *psbA* transcript at the beginning of the light treatment (K0), after photoinhibition at 500 μE $m^{-2}$ $s^{-1}$ for 60 min in the presence of RIF (HL+RIF) and subsequent washing out of the inhibitor and incubation at 50 μE $m^{-2}$ $s^{-1}$ for another 180 min at 50 μE $m^{-2}$ $s^{-1}$ (LL−RIF); or after illumination at 50 μE $m^{-2}$ $s^{-1}$ for 240 min in the presence of CAP (LL+CAP) and subsequent washing out of the inhibitor and incubation at 50 μE $m^{-2}$ $s^{-1}$ for another 180 min at 50 μE $m^{-2}$ $s^{-1}$ (LL−CAP).

different rates of PSII repair observed in the cells containing D1:1 and D1:2.

When transcription of *psbAII*, *psbAIII* and other genes was inhibited by rifampicin (RIF) during the photoinhibitory treatment at 500 μE $m^{-2}$ $s^{-1}$, after 1 h of illumination the PSII activity decreased to 50% of its initial value (Fig. 4A). After washing out the inhibitor, no restoration of the activity was found during subsequent incubation of the cells at 50 μE $m^{-2}$ $s^{-1}$ for 3 h and there was no appearance of D1:2 in the cells during the experiment (data not shown). Using Northern blot we found that no *psbA* transcript was present after 1 h of the high light treatment while after washing the cells and subsequent incubation at 50 μE $m^{-2}$ $s^{-1}$ the amount of *psbA* transcript achieved a similar level as the cells before the high light treatment (Fig. 4C). This result confirmed the inhibitory effect of RIF on the cyanobacterial gene transcription and also documented reversibility of this inhibition after removal of the inhibitor. Thus, the lack of *psbA* transcript could not be the reason for no observed recovery of PSII activity. Similarly, no restoration of PSII activity and no D1:2 accumulation were found after illumination of the CAP-treated *Synechococcus* cells for 4 h at 50 μE $m^{-2}$ $s^{-1}$ and subsequent washing of the cells and incubation at 50 μE $m^{-2}$ $s^{-1}$ (Fig. 4B). Illumination of the cells at 50 μE $m^{-2}$ $s^{-1}$ in the absence of CAP does not lead to appearance of D1:2 in the membrane (data not shown) indicating no significant activating effect of this illumination on the *psbAII* and *psbAIII* transcription. During the low light treatment of the cells in the presence of CAP the amount of *psbA* transcript significantly increased and after washing the cells and their incubation in low light its level partially decreased (Fig. 4C). Again, the non-availability of *psbA* transcript could not be the reason for no recovery of PSII activity observed in this experiment.

An unexpected result was obtained when the photoinhibitory treatment in the presence of CAP was done at 20°C instead of 35°C. In this experiment the restoration of HRA at low light and growth temperature after removal of CAP was completed in 1 h (Fig. 5A) and monitoring of the D1 content revealed

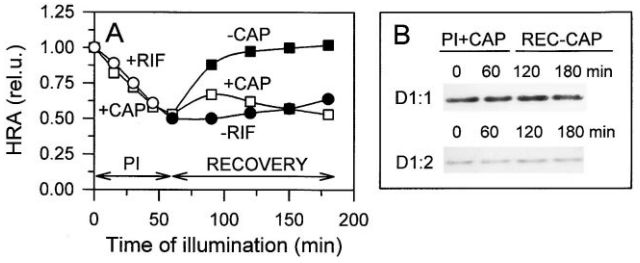


Fig. 5. Time course of HRA and level of two D1 forms during photoinhibition at 20°C and the subsequent recovery from photoinhibition at 35°C in the cells containing D1:1. The control cells containing D1:1 were photoinhibited (PI) at 500 μE $m^{-2}$ $s^{-1}$ for 60 min at 20°C in the presence of CAP (open squares, PI+CAP) and then were washed and exposed to 50 μE $m^{-2}$ $s^{-1}$ (RECOVERY) in the absence (closed squares, REC−CAP) or presence of CAP (empty squares) at 35°C; or the cells were photoinhibited at 500 μE $m^{-2}$ $s^{-1}$ for 60 min at 20°C in the presence of RIF (PI, open circles) and then were washed and exposed to 50 μE $m^{-2}$ $s^{-1}$ at 35°C (RECOVERY, closed circles). Samples were taken at indicated time intervals for measurements of HRA or for isolation of thylakoids, electrophoresis of thylakoid proteins and immunoblotting as described in Section 2.

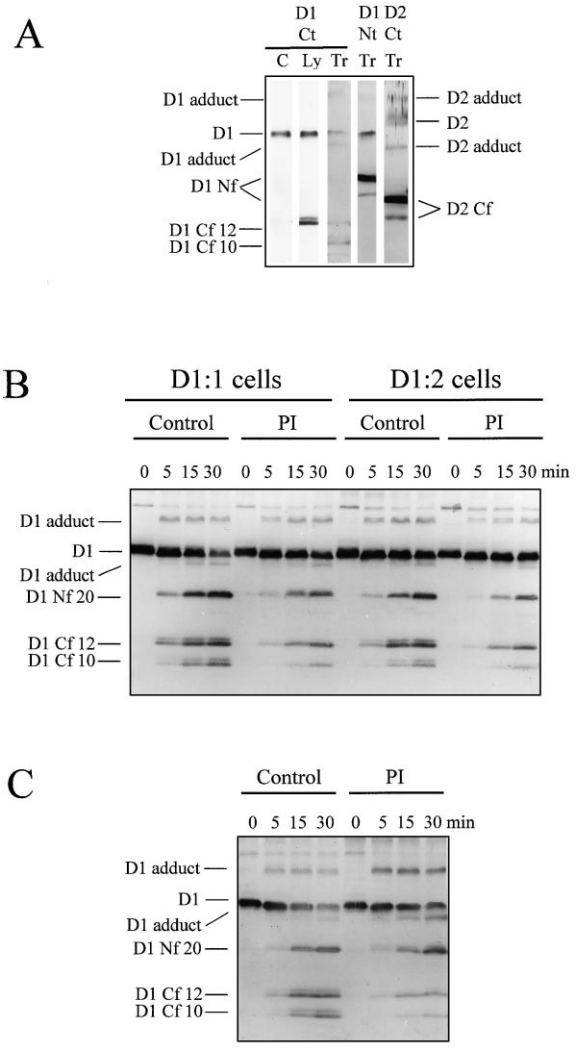


Fig. 6. Effect of trypsin and Lys-C protease on the D1 protein in thylakoids isolated from control cells and time course of the D1 proteolysis by trypsin in thylakoids isolated from control cells and cells photoinhibited at 35 or 20°C. (A) The thylakoids from control cells (containing D1:1) were digested with Lys-C protease (Ly) or trypsin (Tr) for 60 min as described in Section 2. Nitrocellulose membranes with separated thylakoid proteins were probed with N- and C-specific anti-D1 antibody (D1 Nt and D1 Ct) and the C-specific anti-D2 antibody (D2 Ct). C, undigested control thylakoids; D1 Nf, N-terminal D1 fragment; D1 Cf, C-terminal D1 fragment; D2 Cf, C-terminal D1 fragment. (B) The control cells (D1:1 cells) or the cells pre-illuminated for 180 min at 500 μE $m^{-2}$ $s^{-1}$ (D1:2 cells) were photoinhibited under 1000 μE $m^{-2}$ $s^{-1}$ for 120 min at 35°C in the presence of CAP and diuron ($10^{-5}$ M). Thylakoids isolated from non-photoinhibited (CONTROL) and photoinhibited (PI) cells were then incubated with trypsin and samples were taken at indicated time intervals for electrophoresis of proteins and immunoblotting as described in Section 2. Designation of fragments as in (A). (C) The control cells containing D1:1 were photoinhibited under 750 μE $m^{-2}$ $s^{-1}$ for 120 min at 20°C in the presence of CAP. Thylakoids isolated from non-photoinhibited (CONTROL) and photoinhibited (PI) cells were then incubated with trypsin and samples were taken at indicated time intervals for electrophoresis of proteins and immunoblotting as described in Section 2. Designation of fragments as in (A). ←

that no D1:2 accumulated in the membrane (Fig. 5B). Thus, the D1:1 containing PSII complexes photoinactivated at low temperature could be easily repaired by the D1:1 synthesis while such repair did not occur after PSIIPI at growth temperature. The result of the experiment was similar when CAP was absent during the photoinhibitory treatment (not shown). However, similar to the photoinhibition at growth temperature the presence of rifampicin during the high light treatment strongly inhibited the following recovery phase (after removal of the inhibitor) (Fig. 5A) suggesting that functional transcription during photoinhibition is a prerequisite for the efficient repair of PSII complexes.

In agreement with our hypothesis, previous results (namely slow D1:1 turnover in high light and the photoinhibitory temperature-dependent involvement of D1:1 in the PSII repair) could be explained by the PSIIPI-induced and temperature dependent structural changes in PSII affecting the D1:1 insertion. Therefore we attempted to visualize such changes by monitoring the accessibility of the D1 protein to trypsin [27] in thylakoids isolated from control and photoinhibited cells. The cells with each D1 form were illuminated at 1000 μE $m^{-2}$ $s^{-1}$ in the presence of CAP and diuron, the latter being added to the suspension in order to limit the D1 degradation during the light treatment [19]. After 120 min of illumination when about 30% of the initial HRA in the cells left, thylakoid membranes were isolated from both the control and photoinhibited cells and they were subjected to trypsinization. Analysis of the D1 breakdown in thylakoids from the control cells by Western blotting using both Nt- and Ct-antibody showed formation of one 20 kDa N-terminal fragment (plus another very tiny 17 kDa fragment) and two C-terminal double-bands of about 12 and 10 kDa (Fig. 6A). Formation of the same 12 kDa double-band using Lys-C protease confirmed that the primary cut occurred at lysine 239 residue

of D1 (Fig. 6A) and that the two bands in the 12 kDa doublet reflect two different structural states of a single fragment (there is just one lysine residue on the stromal side of both D1 forms). The 10 kDa doublet originated from the secondary cleavage of the 12 kDa doublet as documented by its slower appearance in comparison with 20 kDa and 12 kDa fragments (Fig. 6B). An interesting phenomenon was a fast trypsin-induced generation of two D1- and D2-reactive bands with an Mr of about 28 and 40 kDa, respectively. As the 40 kDa band was reactive with the D1-specific Nt- and Ct-antibody as well as with the D2-C-terminus-specific antibody (Fig. 6A) we assume that it is an adduct of the entire D1 protein and C-terminal fragment of D2 formed by trypsin cut at arginine 234 [23]. In contrast, the 28 kDa band was only reactive with C-terminus-specific D1 and D2 antibody suggesting that it originates from cross-linking of C-terminal trypsin-induced fragments of each protein (Fig. 6A). Comparison of the D1 proteolysis in thylakoids from control and illuminated cells showed that fragmentation of each form was slower in the light-treated cells as compared with the control cells (Fig. 6B). This is evident from both the slower disappearance of the original D1 band and from the slower appearance of the D1 fragments, especially the 10 kDa one, in thylakoids from illuminated cells. In addition, both 12 and 10 kDa doublets were converted during illumination into the single electrophoretic bands. Nevertheless, both D1 forms were fragmented similarly in thylakoids from control and high light-exposed cells suggesting no difference between the modification of PSII containing D1:1 and D1:2. We also subjected to trypsinization thylakoids isolated from the D1:1-containing cells exposed to 750 $\mu E\ m^{-2}\ s^{-1}$ for 2 h at 20°C (Fig. 6C). This illumination led to 70% loss of PSII activity in the cells and electrophoresis of digested thylakoids again showed a slower proteolysis of the D1 protein as compared with thylakoids from the control cells. However, in contrast to the control cells and the cells photoinhibited at 35°C, there was a markedly increased level of the 40 and 28 kDa adducts. In summary, the results of trypsinization supported our hypothesis that photoinactivation at growth temperature induced a structural modification. This modification was similar in PSII complexes containing D1:1 and D1:2 and could be related to the inhibition of the D1:1 insertion into the PSII complexes photoinactivated at 35°C (Fig. 2). In contrast, PSII modification induced by low-temperature photoinhibitory treatment exhibited certain specific features that could enable the efficient repair of the low-temperature inactivated PSII complexes by the D1:1 insertion.

## 4. Discussion

Monitoring of the D1 degradation using the radioactive pulse-chase labeling in the absence of protein synthesis inhibitors disclosed an important difference between the two forms of the D1 protein present in *Synechococcus* PCC 7942. The degradation rate of the high-light form D1:2 was proportional to the light intensity and in this respect D1:2 behaved as the D1 protein in other cyanobacterial strains, for instance *Synechocystis* PCC 6803 [26,28]. It is evident that the turnover of this form efficiently copes with PSIIPI even under high irradiance. In contrast, the degradation of the low-light form D1:1 was not significantly accelerated when irradiance was ten times increased and it became apparently slower than the rate of PSIIPI at the same light intensity. There was no decrease of PSII activity in the cells containing D1:1 under low light conditions in the absence of CAP but in its presence the activity was slowly decreasing (Fig. 1A). It shows that the rates of the D1:1 degradation and synthesis under low light conditions are sufficient to counteract PSIIPI. Furthermore, similar to the *Synechocystis* cells [26], the rate of D1:1 turnover in low light seems to exceed that of PSIIPI indicating that the turnover is not just a simple mean for replacement of the D1 protein in the photoinactivated PSII. In contrast, under high light conditions the D1:1 turnover is not fast enough to completely match the rate of PSIIPI. We assume that this inability of PSII repair to compensate PSIIPI and the subsequent decrease of the PSII activity can explain the transient period of inhibited growth observed in the cells of the *Synechococcus psbAII/psbAIII* deletion mutant transferred to higher irradiance [29].

Our results also showed that the increase of HRA during the recovery from photoinhibition in low light was accompanied by the incorporation of D1:2. The

faster recovery from photoinhibition in the cells containing D1:2 as compared with the cells containing D1:1 is in agreement with results of Krupa et al. [16]. This can be explained by the higher probability of D1:2 synthesis in the preilluminated cells since the D1:1-containing cells were subjected to high light only for 1 h and moreover in the presence of CAP that was shown to significantly retard the degradation of *psbAI* transcript in high light [7]. In line with this explanation, the restoration of activity was inhibited when the photoinhibitory treatment in the presence of CAP was conducted in low light that does not induce the D1:2 synthesis.

Also the complete absence of recovery from RIF-induced photoinhibition conducted at 35°C suggests that transcription of *psbAII* and *psbAIII* genes during high irradiance is needed for the following PSII repair. However, a similar absence of recovery was also observed after the RIF-induced photoinhibition at 20°C. When RIF is absent during this type of photoinhibitory treatment, the following PSII repair does not require D1:2 synthesis. This finding shows that functional transcription of genes that encode other protein factors than D1:2 is a prerequisite for the efficient PSII repair. For instance, heat shock proteins are good candidates for such factors: Schroda et al. have recently shown that synthesis of the heat shock protein HSP 70 very significantly affects the repair of PSII in the green alga *Chlamydomonas* [30].

A higher rate of the D1:1 degradation in comparison with the rate of CAP-induced PSIIPI at 50 μE $m^{-2}$ $s^{-1}$ indicates that at this irradiance D1:1 replacement may occur in still functional PSII complexes (although they may be already tagged for replacement) with a rate that ensures D1 replacement before PSIIPI occurs. Non-denaturing electrophoresis confirmed that PSII complexes that are subject for the D1 replacement are recognized still in the assembled form containing all main protein subunits (at least CP47, CP43, D2, D1 and cytochrome *b*-559). The insertion of the D1:1 into functional assembled PSII is in line with the fast and PSIIPI-independent replacement of D1:2 for D1:1 after the transfer of the D1:2-containing cells from high to low irradiance [25].

We assume that when irradiance is increased, there is an accumulation of inactive and structurally modified PSII complexes that have a low affinity for the newly synthesized D1:1 but increased affinity for D1:2. Consequently, there is a fast insertion of D1:2 into photoinactivated PSII. This hypothesis is in line with our previous results showing that the replacement of D1:1 by D1:2 is triggered by PSIIPI at 35°C [25]. The different efficiency of the D1:1 insertion into PSII complexes photoinhibited at 20 and 35°C and trypsinization experiment suggest that PSIIPI at 35°C is accompanied by a PSII structural modification that inhibits the D1:1 insertion. We could not accelerate repair of the photoinactivated complexes by oxygen removal (data not shown) excluding the possibility that this modification is related to the attack by reactive species as observed in the thermophilic *Synechococcus* [31]. In contrast, PSIIPI at 20°C modifies PSII in the way that D1:1 insertion is enabled. It is of interest that this modification was characteristic by the increased formation of the adducts of the entire D1 or its C-terminal fragment and the trypsin-induced C-terminal D2 fragment. This may indicate that the mutual position of the D2 amino acid chain near the trypsin cutting site and the D1 protein can play an important role during the recognition process between the newly synthesized D1 protein and the PSII complex.

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