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# Very strong UV-A light temporally separates the photoinhibition of photosystem II into light-induced inactivation and repair

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**Abstract**

When organisms that perform oxygenic photosynthesis are exposed to strong visible or UV light, inactivation of photosystem II (PSII) occurs. However, such organisms are able rapidly to repair the photoinactivated PSII. The phenomenon of photoinactivation and repair is known as photoinhibition. Under normal laboratory conditions, the rate of repair is similar to or faster than the rate of photoinactivation, preventing the detailed analysis of photoinactivation and repair as separate processes. We report here that, using strong UV-A light from a laser, we were able to analyze separately the photoinactivation and repair of photosystem II in the cyanobacterium *Synechocystis* sp. PCC 6803. Very strong UV-A light at 364 nm and a photon flux density of 2600 μmol photons $m^{-2}$ $s^{-1}$ inactivated the oxygen-evolving machinery and the photochemical reaction center of PSII within 1 or 2 min before the first step in the repair process, namely, the degradation of the D1 protein, occurred. During subsequent incubation of cells in weak visible light, the activity of PSII recovered fully within 30 min and this process depended on protein synthesis. During subsequent incubation of cells in darkness for 60 min, the D1 protein of the photoinactivated PSII was degraded. Further incubation in weak visible light resulted in the rapid restoration of the activity of PSII. These observations suggest that very strong UV-A light is a useful tool for the analysis of the repair of PSII after photoinactivation.


*Keywords:* D1 protein; Photoinhibition; Photosystem II; *Synechocystis*; UV-A light

## 1. Introduction

Light is essential for photosynthesis but is also harmful to the photosynthetic machinery. Exposure of photosynthetic organisms to strong light results in severe inhibition of the activity of photosystem II (PSII). This phenomenon is known as photoinhibition or light-induced inactivation of photosystem II. Photosynthetic organisms have the ability to repair inactivated PSII. Recent studies have demonstrated that the light-induced inactivation of PSII occurs in two steps: step 1 occurs at the oxygen-evolving site of PSII and step 2 occurs at the photochemical reaction center of PSII [1,2]. The multi-step repair of PSII includes the degradation of the D1 protein, synthesis of the precursor to the D1 protein (pre-D1), assembly of the PSII reaction center complex, and processing of pre-D1.

The mechanisms proposed for the light-induced inactivation and repair of PSII remain controversial [3–12] because it is difficult to separate photoinactivation from repair under normal laboratory conditions since the rate of inactivation is similar to the rate of repair. For example, under strong visible light, for example, 2000 μmol photons $m^{-2}$ $s^{-1}$, the maximum rate of light-induced inactivation corresponds to inactivation of

*Abbreviations:* Chl, chlorophyll; DCIP, dichlorophenolindophenol; DPC, diphenylcarbazide; EPR, electron paramagnetic resonance; PSII, photosystem II; Pheo, pheophytin

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approximately 2% of the total reaction center of PSII in 1 min [10]. By contrast, the maximum rate of repair corresponds to repair of 1% of the reaction center in 1 min [12]. Thus, inactivation and repair of PSII are basically simultaneous and, under the experimental conditions used to date, the inactivation and repair of PSII cannot be investigated separately. Therefore, new techniques are required for rapid inactivation of PSII, for example, within 1 min, during which no repair occurs.

UV-B light (280–320 nm) and UV-A light (320–400 nm) inactivate PSII much more efficiently than visible light [13–16]. In intact photosynthetic organisms, the UV light-induced inactivation of PSII can be efficiently reversed under visible light [4,9]. UV light can also induce the repair of PSII, although its efficiency is not as great as that of visible light. These observations suggest that UV light might allow the rapid inactivation of PSII in the absence of equally rapid repair.

In this study, we used very strong UV-A light at 364 nm from a UV laser to inactivate PSII almost entirely in 1 min, during which time the D1 protein was not degraded. During subsequent incubation in visible light, full activity of PSII was restored. Thus, strong UV-A light provides a novel tool for separate examinations of the light-induced inactivation and subsequent repair of PSII.

## 2. Materials and methods

### 2.1. Strain and culture conditions

*Synechocystis* sp. PCC 6803 was kindly donated by Dr. J.G.K. Williams of DuPont de Nemours (Wilmington, DE). Cells were grown photoautotrophically in glass tubes (2.5 cm i.d. × 20 cm; 120 ml) at 34 °C under constant illumination from incandescent lamps at 70 μE $m^{-2}$ $s^{-1}$ in BG-11 medium [17] supplemented with 20 mM HEPES–NaOH (pH 7.5). Cultures were aerated with sterile air that contained 1% (v/v) $CO_2$ [18]. Cultures of cells with an absorbance at 730 nm ($OD_{730}$) of 0.5 were used for experiments. The concentration of chlorophyll *a* was determined as described by Arnon et al. [19].

### 2.2. Preparation of thylakoid membranes

Thylakoid membranes were isolated from cells as described previously [8] with minor modification. Cells were harvested by centrifugation at 3000×*g* for 5 min at room temperature. The pelleted cells were resuspended in a solution that contained 50 mM HEPES–NaOH (pH 7.5) and 20 mM $CaCl_2$ and the suspension was centrifuged at 6000×*g* for 5 min. Finally, cells were suspended in 50 mM HEPES–NaOH (pH 7.5), that contained 1.0 M glycinebetaine, 0.6 M sorbitol, 15 mM $CaCl_2$, 15 mM $MgCl_2$, and 1 mM 6-amino-*n*-caproic acid. The above procedures were performed at room temperature but all subsequent steps were performed at 4 °C. The suspension of cells at a concentration of 1.0 mg Chl $ml^{-1}$ was passed through a French pressure cell (SLM Instruments, Urbana, IL, USA) at 160 Mpa. The homogenate was centrifuged at 6000×*g* for 10 min to remove unbroken cells and cellular debris. The supernatant was centrifuged at 20,000×*g* for 45 min. Sedimented thylakoid membranes were suspended in 50 mM MES–NaOH (pH 6.5) supplemented with 1.0 M glycinebetaine, 10 mM $CaCl_2$, 15 mM $MgCl_2$ and 10 mM NaCl and the suspension was centrifuged again at 20,000×*g* for 45 min. Pelleted thylakoid membranes were resuspended in the same buffer as above at 1 mg Chl $ml^{-1}$, frozen in liquid nitrogen, and stored at −80 °C.

### 2.3. Inactivation of PSII by UV-A light

Strong UV-A light was provided by a laser (model 2085-25; Spectra-Physics Lasers, Inc., Mountain View, CA) that produced UV light at 363.8 nm±0.5 nm. The intensity of light was equalized over the sample with a Homogeneous Irradiation System box (Hitachi, Tokyo, Japan). The intensity of UV-A light at the surface of the sample was 2400–2600 μmol photons $m^{-2}$ $s^{-1}$, as measured with a radiometer (model RM S-101; Rayon, Tsukuba, Japan). A suspension of cells at 2 μg Chl $ml^{-1}$ was irradiated with UV-A light in a Petri dish (i.d., 2.8 cm), which contained the suspension of cells to a height of 1.0 cm with continuous stirring at room temperature. The total volume of the exposed suspension of cells was 6.5 ml in each independent experiment.

### 2.4. Effects of irradiation with strong UV-A light on the growth rate

A suspension of cells ($OD_{730}$=0.5) was exposed to strong UV-A light at 2600 μmol photons $m^{-2}$ $s^{-1}$ for 1 min. Then the suspension was diluted 5-fold and incubated in a test tube under normal growth conditions. The growth rate of cells was monitored in terms of absorbance at 730 nm with a spectrophotometer (model 200-20; Hitachi, Tokyo, Japan).

### 2.5. Measurement of the oxygen-evolving activity of PSII

We examined the activity of PSII in *Synechocystis* cells by monitoring electron transport from $H_2O$ to 1.4-benzoquinone (BQ) at 1.0 mM with a Clark-type oxygen electrode (Hansatech Instruments, King's Lynn, UK), as described previously [11]. Actinic light at 2000 μmol photons $m^{-2}$ $s^{-1}$ was provided by the lamp of a slide projector with a red optical filter (R-60; Toshiba, Tokyo, Japan) and an infrared-absorbing filter (HA-50; Hoya Glass, Tokyo, Japan). The activity of PSII in thylakoid membranes was examined by monitoring the light-induced transport of electrons from $H_2O$ to 2,6-dichlorophenolindophenol (DCIP) or from diphenylcarbazide (DPC) to DCIP, as described previously [1]. The reduction of DCIP was monitored in terms of decreases in absorption at 580 nm with a spectrophotometer (model 557; Hitachi), which was equipped with a 4-96 filter (Corning, New York, NY) and a red cutoff filter (VR-67; Toshiba) for the measuring beam and actinic light, respectively. Actinic light at 1000 μmol photons $m^{-2}$ $s^{-1}$ was provided by the lamp of a slide projector. The reaction mixture contained thylakoid membranes that corresponded to 5 μg of chlorophyll, 0.1 mM DCIP, and 0.5 mM DPC when necessary.

### 2.6. Measurement of light-induced quenching of chlorophyll fluorescence

Reduction of pheophytin was monitored by means of the light-induced quenching of Chl fluorescence as described in Refs. [20,21] with a fluorometer (PAM-101; Heinz Walz, Effeltrich, Germany) in the pulse-amplitude modulation mode. The light-induced quenching of Chl fluorescence was measured at 34 °C in the presence of 20 μM 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea (DCMU) and 1 mg $ml^{-1}$ sodium dithionite after continuous exposure of the sample to actinic light ($\lambda$>520 nm) from an incandescent lamp (KL-1500 Electronic; Schott Glasswerke, Wiesbaden, Germany) at 2700 μmol photons $m^{-2}$ $s^{-1}$. The presence of dithionite is important to measure the reduction of pheophytin, because it establishes conditions under which all the electron carriers, except pheophytin, are reduced.

### 2.7. Measurement of the release of Mn from PSII by electron paramagnetic resonance

EPR signals due to Mn were measured with an EPR spectrometer (model ER 200-D; Brucker, Karlsruhe, Germany). *Synechocystis* cells were washed with 0.1 mM EDTA after irradiation with UV-A light. Then thylakoid membranes were isolated as described above. The thylakoid membranes were suspended in 50 mM MES–NaOH (pH 6.5) supplemented with 1.0 M glycinebetaine [22], 10 mM $CaCl_2$, 15 mM $MgCl_2$ and 10 mM NaCl at 1.2 mg Chl $ml^{-1}$, and enclosed in a sealed glass capillary tube (i.d., 0.02 cm) in a final volume of 40 μl. EPR spectra were recorded at room temperature in darkness under the following conditions: microwave power, 65 mW; modulation amplitude, 20 G; time constant, 1.0 s; and scan rate, 200 G $min^{-1}$.

### 2.8. Immunoblotting analysis of the Dl protein

Isolated thylakoid membranes were solubilized by incubation for 5 min at 65 °C in 60 mM Tris–HCl (pH 6.8) that contained 2% (w/v) sodium dodecyl sulfate (SDS), 5% (v/v) 2-mercaptoethanol and 10% (v/v) glycerol, and then proteins were separated by gel electrophoresis on a 12.5% polyacrylamide gel that contained 0.08% (w/v) SDS and 6 M urea, as described previously [8]. After electrophoresis, the separated proteins were blotted onto a nitrocellulose membrane (Schleicher and Schuell Inc., Keene, NH) in a semi-dry transfer apparatus (Atto, Tokyo, Japan). Then the D1 protein was detected immunologically with an ECL Western blotting kit according to the protocol supplied with the kit (Amersham Biosciences, Piscataway, NJ, USA).

The D1 protein was detected with antibodies raised in rabbits against a synthetic oligopeptide that corresponded to the AB loop of the D1 protein from spinach (amino acids 55–78, counted from the amino terminus of spinach D1 protein [23]). These antibodies recognize the products (D1) of the *psbAI*, *psbAII* and *psbAIII* genes because the amino acid sequence of the AB loop is exactly the same in the product of each of these genes. As second antibodies, we used horseradish peroxidase-linked antibodies raised in donkeys against rabbit immunoglobulin G (Amersham Biosciences). The antibodies raised in rabbit against D1 were kindly provided by Prof. Kimiyuki Satoh (Department of Biology, Okayama University, Okayama, Japan). A digital camera system (LAS-1000; Fuji Film, Tokyo, Japan) was used to monitor signals from blotted membranes and to quantify the D1 protein.

## 3. Results and discussion

### 3.1. The oxygen-evolving activity of PSII was depressed within 1 min by very strong UV-A light

Very strong UV-A light at 264 nm and 2600 μmol photons $m^{-2}$ $s^{-1}$ from a UV laser rapidly inactivated PSII in *Synechocystis* cells (Fig. 1). The oxygen-evolving activity fell to approximately 50% and 10% of the original level during such illumination for 0.3 min and 1 min, respectively. Similarly rapid inactivation of PSII has not been previously reported. Lincomycin, an inhibitor of protein synthesis, did not affect the rate of inactivation.

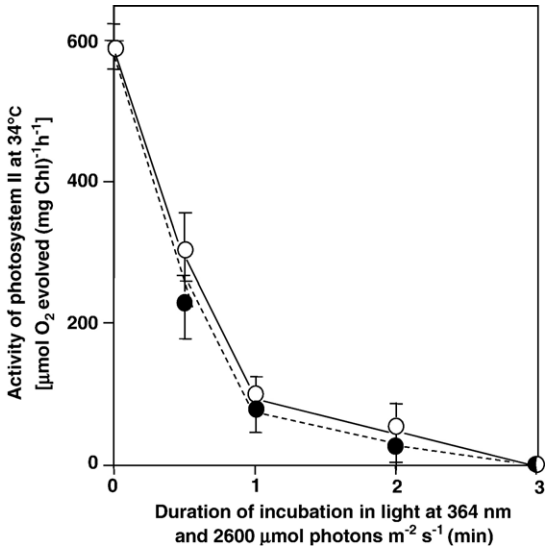


Fig. 1. Inactivation of PSII induced by very strong UV-A light. *Synechocystis* cells were incubated in light at 364-nm and 2600 μmol photons $m^{-2}$ $s^{-1}$ at 25 °C in the presence of 250 μg $ml^{-1}$ lincomycin or in its absence. At designated times, aliquots of the suspension of cells were withdrawn and PSII activity was monitored in terms of the photosynthetic evolution of oxygen in the presence of 1.0 mM BQ as the electron acceptor. Each point and bar represents the average±S.E. of results from four independent experiments. ○–○, No addition (control); ●–●, 250 μg/ml lincomycin was added just before the start of UV-A irradiation.

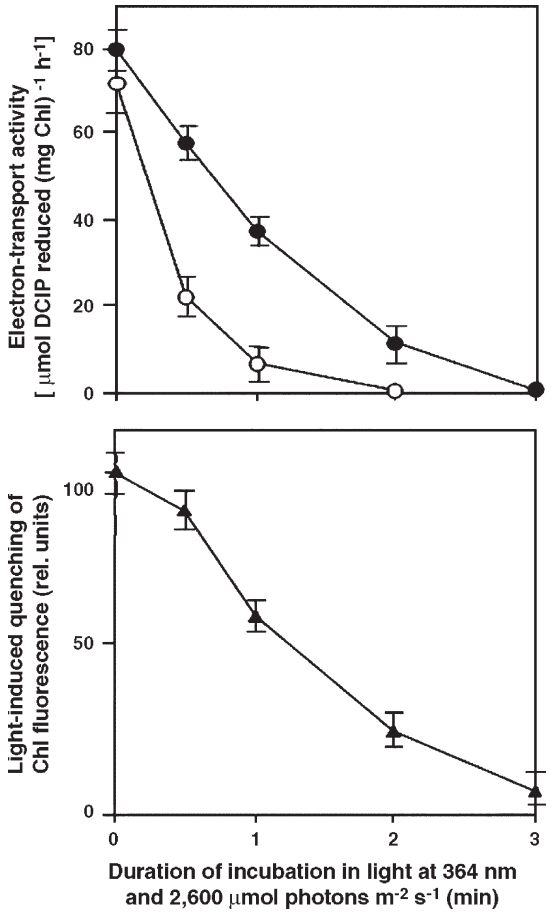


Fig. 2. Very strong UV-A light induced the inactivation of PSII, as monitored in isolated thylakoid membranes. *Synechocystis* cells were incubated in light at 364 nm and 2600 μmol photons $m^{-2}$ $s^{-1}$ at 25 °C. At designated times, an aliquot of the suspension of cells was withdrawn and thylakoid membranes were isolated as described in Materials and methods. The PSII activity of thylakoid membranes was measured by monitoring electron transport from $H_2O$ to DCIP (○–○), electron transport from DPC to DCIP (●–●), and the light-induced quenching of Chl fluorescence in the presence of 1 mg $ml^{-1}$ sodium dithionite (▲–▲). Each point and bar represents the average±S.E. of results from three independent experiments.

In a previous study, we demonstrated that UV-A light inactivated the oxygen-evolving machinery of PSII more rapidly than it inactivated the photochemical reaction center of PSII in thylakoid membranes from *Thermosynechococcus elongatus* [1]. In the present study, we compared the rate of UV-A light-induced inactivation of the oxygen-evolving machinery with that of the photochemical reaction center of PSII in *Synechocystis* cells. We first irradiated *Synechocystis* cells with strong UV-A light for designated periods of time. Then, we isolated thylakoid membranes from these cells and measured the activities of the oxygen-evolving machinery and the photochemical reaction center. We monitored the former in terms of electron transport from $H_2O$ to DCIP and the

latter in terms of electron transport from DPC to DCIP and, also, in terms of the light-induced quenching of chlorophyll fluorescence that results from the photochemical reduction of pheophytin.

Fig. 2 shows that the rate of electron transport from $H_2O$ to DCIP decreased rapidly, with the activity disappearing almost completely within 1 min and with a half-inactivation time of approximately 0.3 min. This profile was similar to that observed in the case of electron transport from $H_2O$ to 1,4-benzoquinone in intact cells. By contrast, complete inactivation of the electron transport from DPC to DCIP required 2 min, and the half-inactivation time was 1 min. The kinetics of inactivation of the light-induced reduction of pheophytin was very similar to those of the electron transport from DPC to DCIP. These observations confirmed our previous observation that the inactivation of the oxygen-evolving machinery preceded that of the photochemical reaction center when isolated thylakoid membranes were irradiated by UV-A light, for example, at 350 nm [1].

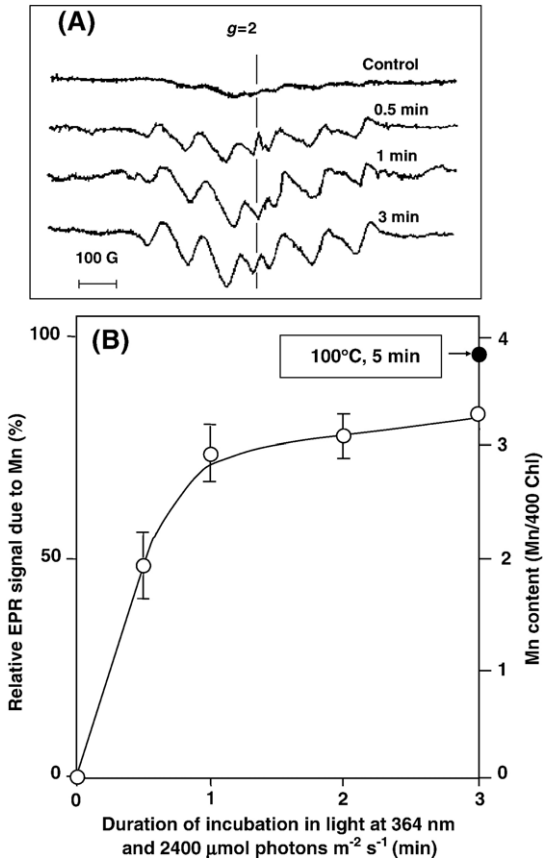


Fig. 3. The release of Mn from PSII during incubation of thylakoid membranes in very strong UV-A light. Thylakoid membranes from *Synechocystis* cells were incubated in light at 364 nm and 2400 µmol photons $m^{-2}$ $s^{-1}$ and, at designated times, aliquots were withdrawn from the suspension of thylakoid membranes. (A) EPR spectra of thylakoid membranes that had been exposed to UV-A light for the indicated times. (B) Increases in the level of free Mn during incubation in UV-A light. Free Mn was quantified by reference to EPR spectra. The filled circle indicates the amount of Mn released by heating thylakoid membranes at 100 °C for 5 min. Each point and bar represents the average ± S.E. of results from three independent experiments.

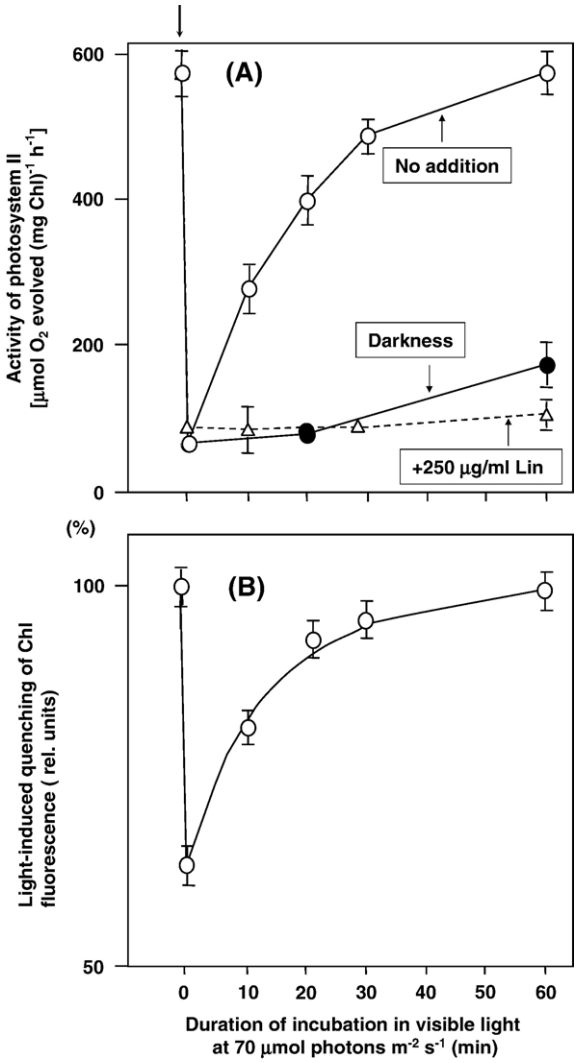


Fig. 4. Recovery of PSII activity after inactivation by very strong UV-A light. *Synechocystis* cells in suspension ($OD_{730}$=0.5) were exposed for 1 min to light at 364 nm and 2600 µmol photons $m^{-2}$ $s^{-1}$ (indicated by the large arrow) and then incubated in weak visible light at 70 µmol photons $m^{-2}$ $s^{-1}$ or in darkness. Aliquots were withdrawn at designated times. (A) Recovery of the oxygen-evolving activity. ○–○, Incubation in weak visible light in the absence of lincomycin; △–△, incubation in weak visible light in the presence of 250 µg/ml lincomycin, which was added at zero time; ●–●, incubation in darkness in the absence of lincomycin. (B) Increase in Chl fluorescence due to the reduction of pheophytin. Each point and bar represents the average ± S.E. of results from three independent experiments.

The catalytic center of the oxygen-evolving machinery includes four Mn atoms [24]. Renger et al. [13] demonstrated that the UV light-induced inactivation of the oxygen-evolving machinery is associated with the release of Mn atoms from the catalytic center. To investigate whether Mn was released upon inactivation of the oxygen-evolving machinery by very strong UV-A light, we exposed thylakoid membranes to UV-A. Fig. 3 shows that close to the maximum amount of Mn was released in response to UV-A irradiation for 1 min, during which time the oxygen-evolving machinery was almost completely inactivated. The number of released Mn atoms was approximately three per PSII complex.

### 3.2. Repair of PSII after UV-A irradiation

The multi-step repair of PSII includes the degradation of the D1 protein, the synthesis of pre-D1, the assembly of the PSII complex, and the processing of pre-D1. To examine whether such repair occurs after photoinactivation by UV-A light, we irradiated *Synechocystis* cells for 1 min to decrease the oxygen-evolving activity to 10% of the original level and then we incubated the cells in visible light at 70 μmol photons $m^{-2}$ $s^{-1}$, namely, under conditions that allow the repair of PSII after photoinactivation by strong visible light [10–12].

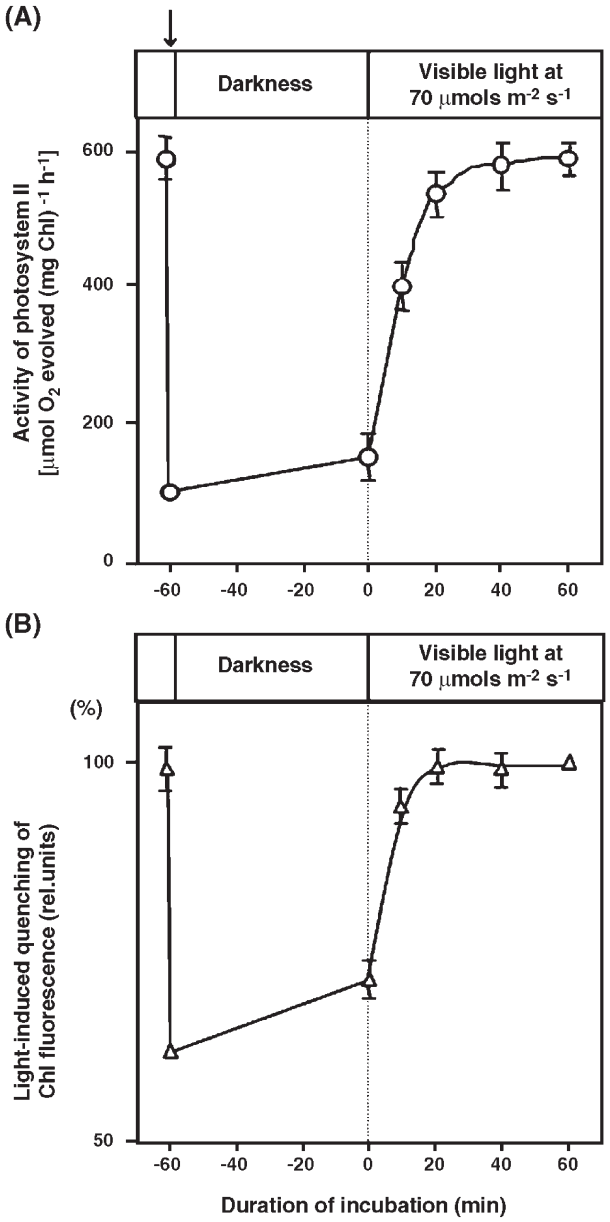


Fig. 5. Incubation in darkness after UV-A irradiation accelerated the subsequent recovery of PSII activity. *Synechocystis* cells in suspension ($OD_{730}$=0.5) were exposed for 1 min to light at 364 nm and 2400 μmol photons $m^{-2}$ $s^{-1}$ and then transferred to darkness. After 60 min, cells were exposed to visible light at 70 μmol photons $m^{-2}$ $s^{-1}$. The recovery was monitored by means of the oxygen-evolving activity (A) and the light-induced quenching of chl fluorescence (B).

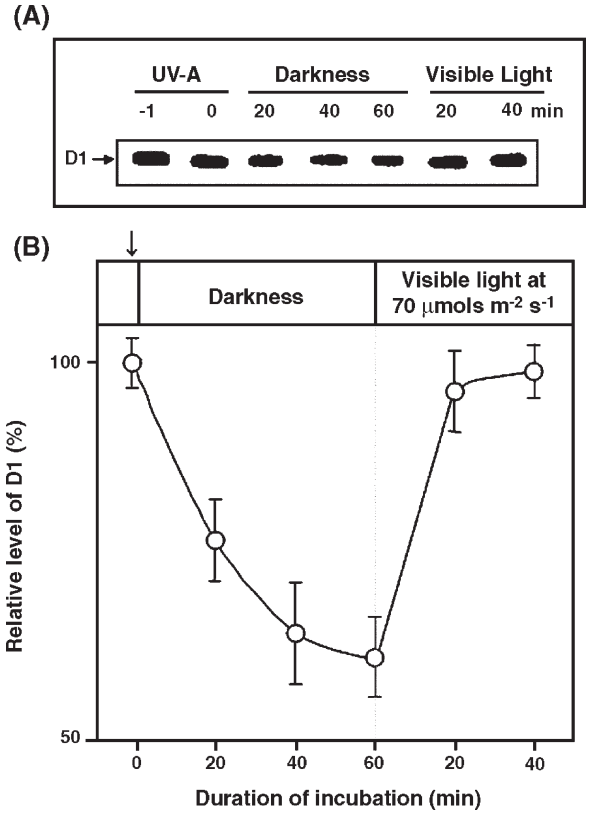


Fig. 6. Changes in the level of the D1 protein upon UV-A irradiation at 364 nm and 2400 μmol photons $m^{-2}$ $s^{-1}$, during incubation in darkness, and during subsequent incubation in visible light at 70 μmol photons $m^{-2}$ $s^{-1}$. At designated times aliquots of the suspension of cells were withdrawn for preparation of thylakoid membranes. Thylakoid membranes were subjected to Western blotting analysis with antibodies against the D1 protein as described in Materials and methods. (A) Results of Western blotting. Experiments were performed three times, and essentially the same results were obtained in each experiment. (B) Quantification of results in (A). Each range of deviation represents results from three independent experiments.

Fig. 4 shows that the oxygen-evolving activity was efficiently restored under normal growth conditions, for example, at 34 °C under continuous visible light at 70 μmol photons $m^{-2}$ $s^{-1}$. The original activity was restored within 60 min, with a half-recovery time of 15 min. In the presence of lincomycin, cells were unable to recover their oxygen-evolving activity (Fig. 4A). In darkness, the oxygen-evolving activity was only partially restored. These findings indicate that photoinactivation of PSII by very strong UV-A light is reversible and that the repair of UV-A light-inactivated PSII requires protein synthesis that might be accelerated by weak visible light. These conditions are the same as those required for repair after PSII has been photoinactivated by strong visible light [11,12].

Irradiation with UV-A for 1 min reduced by 50% the activity of the photochemical reaction center. We monitored the repair of the photochemical reaction center during incubation in light at 70 μmol photons $m^{-2}$ $s^{-1}$. Fig. 4B shows that the activity of the photochemical reaction center returned to its original level in 30 min, with a half-recovery time of 12 min, when monitored in terms of the light-induced reduction of pheophytin. These

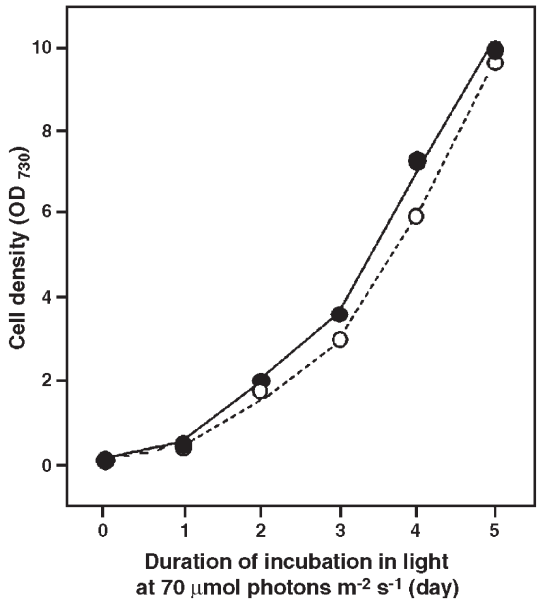


Fig. 7. Effects of the UV-A irradiation on the subsequent growth of cyanobacterial cells. *Synechocystis* cells in suspension ($OD_{730}$=0.5) were exposed for 1 min to light at 364 nm and 2600 μmol photons $m^{-2}$ $s^{-1}$ (at zero time) and then incubated for 5 days in weak visible light at 70 μmol photons $m^{-2}$ $s^{-1}$. Aliquots were withdrawn at designated times and the growth of cells was monitored by means of turbidity at 730 nm. Control cells (no UV-A irradiation), ●–●; UV-A-irradiated cells, ○–○.

results indicate that the rate of repair of the photochemical reaction center was shorter than that of the oxygen-evolving machinery in PSII.

### *3.3. Incubation in darkness after UV-A irradiation accelerated subsequent repair*

To dissect the repair process, we examined the effects of incubation in darkness after UV-A irradiation on the repair of PSII. In darkness, we observed the slight recovery of the oxygen-evolving activity and the capacity for the light-induced reduction of pheophytin (Fig. 5), confirming previous observations that the activity of PSII is partially restored in darkness [25,26]. After incubation in darkness for 60 min, cells were transferred to recovery conditions, namely, light at 70 μmol photons $m^{-2}$ $s^{-1}$. The oxygen-evolving activity returned to its original level in 30 min, with a half-recovery time of 10 min, whereas the activity of the photochemical reaction center recovered in 10 min, with a half-recovery time of approximately 5 min. These observations indicate that the hour-long incubation in darkness accelerated the subsequent repair of PSII and suggest that some step(s) in the repair process occurred in the dark after inactivation by very strong UV-A light.

The first step in the repair process after the inactivation of the photochemical reaction center is the degradation of the D1 protein [4]. Therefore, we performed Western blotting analysis to determine whether the D1 protein was degraded during the incubation in darkness. We withdrew aliquots of the cell suspension at designated times and isolated thylakoid membranes from the cells as described in Materials and methods. As shown in Fig. 6, Western blotting with antibodies that had been raised against amino acids 55–78 in the AB loop of the D1 protein revealed that UV-A irradiation for 1 min did not affect the level of the D1 protein. This result confirmed that the inactivation of PSII and the degradation of the D1 protein were separate processes [10]. During the 1-h incubation in darkness, the level of the D1 protein fell to about half of the original level. This decrease might correspond to the observed decrease in the activity of the photochemical reaction center (Figs. 4A and 5). During the subsequent incubation in weak visible light, the D1 protein returned to its original level. This pattern resembled the recovery of activity of the photochemical reaction center of PSII. In a previous study, we demonstrated that the synthesis of pre-D1 occurs in light [8,12]. Therefore, it is likely that, of many steps in the repair process, only degradation of the D1 protein occurs in darkness. Subsequent steps might require light [12].

### *3.4. Effects of strong UV-A light on the growth of Synechocystis cells*

To examine the long-term effects of short irradiation with very strong UV-A light on the growth of cells, we illuminated *Synechocystis* cells for 1 min with strong UV-A light. After irradiation, the culture was diluted 5-fold and transferred to normal growth conditions and then growth was monitored for 5 days. There were no significant differences between the growth profiles of control and UV-irradiated cells (Fig. 7). These results suggest that all physiological activities in *Synechocystis* cells recover rapidly after very strong but brief irradiation with UV-A light, such that cell viability is not significantly affected.

## 4. Conclusions

The present study indicates that very strong UV-A light from a laser inactivates PSII within a short time, during which no steps in the repair process occur. Therefore, very strong UV-A light allows us to investigate photoinactivation and repair separately. It also allows us to investigate directly the repair of photoinactivated PSII in which the D1 protein has not been degraded or in which the D1 protein has been degraded but the synthesis of the D1 protein de novo has not occurred. It is likely that the UV-A irradiation, which fully inactivates PSII in a minute, does not induce significant damage to DNA and other cellular components, since it did not affect the subsequent growth of cyanobacterial cells.

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