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Irreversible photoinhibition of photosystem II is caused by exposure of *Synechocystis* cells to strong light for a prolonged period

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Abstract

Irreversible photoinhibition of photosystem II (PSII) occurred when *Synechocystis* sp. PCC 6803 cells were exposed to very strong light for a prolonged period. When wild-type cells were illuminated at 20 °C for 2 h with light at an intensity of 2,500 μmol photons m^{-2} s^{-1}, the oxygen-evolving activity of PSII was almost entirely and irreversibly lost, whereas the photochemical reaction center in PSII was inactivated only reversibly. The extent of irreversible photoinhibition was enhanced at lower temperatures and by the genetically engineered rigidification of membrane lipids. Western and Northern blotting demonstrated that, after cells had undergone irreversible photoinhibition, the precursor to D1 protein in PSII was synthesized but not processed properly. These observations may suggest that exposure of *Synechocystis* cells to strong light results in the irreversible photoinhibition of the oxygen-evolving activity of PSII via impairment of the processing of pre-D1 and that this effect of strong light is enhanced by the rigidification of membrane lipids.

Keywords: Photodamage; Photosystem II; D1 protein; S*ynechocystis*

1. Introduction

Photosynthetic organisms are often exposed to high irradiance and, under such conditions, the photochemical reaction center of photosystem II (PSII) is inactivated. This process is defined as photoinhibition or photodamage [1]. It is likely that, during photoinhibition, the Dl protein, one of the heterodimeric proteins in the photochemical reaction center of PSII, is modified. However, photoinhibition is generally reversible under normal conditions [1–6], and PSII is repaired by replacement of the D1 protein by newly synthesized D1 protein [2,7,8].

The first step in the repair of PSII is the degradation of the D1 protein in the photoinhibited PSII complex. Such degradation involves proteolysis by the DegP2 and FtsH proteases [5,6,9–11]. Subsequently or simultaneously, the precursor to Dl protein (pre-D1) is synthesized de novo and is integrated into the PSII complex via a multistep process [3–5]. First, *psbA* mRNA forms a complex with ribosomes in the cytosol and this complex is targeted to the thylakoid membrane. Then the elongating pre-D1 protein is inserted cotranslationally into the thylakoid membrane and forms a complex with the D2 protein. This complex combines with

Abbreviations: BQ, 1,4-benzoquinone; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; Chl, chlorophyll; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; PSII, photosystem II

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other components to form PSII monomers, which are photochemically active [5,12]. The pre-D1 protein in the PSII monomers is processed to yield the mature D1 protein, with subsequent formation of the dimeric form of PSII [5,12–14]. Finally, the oxygen-evolving machinery is integrated into PSII for full restoration of PSII activity [4–6]. The PSII complex undergoes this cycle of photodamage and repair upon exposure to light under normal environmental conditions.

Photoinhibition appears to be affected both by low temperature [15 16] and by the extent of the unsaturation of the fatty acids in membrane lipids [17 18]. The extent of photoinhibition in vivo depends on the balance between the photodamage to PSII and the repair of PSII [16,19,20]. Decreases in temperature markedly suppress the repair of PSII [21,22]. This effect of temperature is enhanced by a decrease in the extent of unsaturation of fatty acids in *Synechocystis* sp. PCC 6803 (hereafter, *Synechocystis*), which can be achieved by genetic engineering that leads to the replacement of polyunsaturated fatty acids by monounsaturated fatty acids [23,24].

We demonstrated previously that the PSII complex is irreversibly photoinhibited when leaves of *Vicia faba* are exposed to very strong light for a prolonged period (for example, 2500 μmol photons m^{-2} s^{-1} for 6 h; [25]). An attempt was made to explain this irreversible photoinhibition by a model in which the photochemical reaction center of the PSII complex loses its capacity for repair upon exposure to high-intensity light after reversible photoinhibition has been induced. However, the molecular mechanism of this irreversible photoinhibition remains to be characterized.

The present study was designed to examine the effects of temperature and the rigidification of membrane lipids on irreversible photoinhibition and to analyze the mechanism of irreversible photoinhibition in *Synechocystis*, in which ambient temperature and the rigidification of membrane lipids can be controlled with relative ease. Our results demonstrate that the target of irreversible photoinhibition is the oxygen-evolving complex of PSII and that impaired processing of pre-D1 prevents the assembly of a repaired oxygen-evolving complex. The fluidity of membrane lipids plays a key role in protecting cells from irreversible photoinhibition.

2. Materials and methods

2.1. Cells, culture conditions and photoinhibition

The original sample of *Synechocystis* sp. PCC 6803 was kindly donated by Dr. John G. K. Williams (DuPont de Nemours and Co., Wilmington, DE; [26]). The *desA*$^-$/*desD*$^-$ strain of *Synechocystis* sp. PCC 6803 was obtained as described previously [23]. Wild-type and *desA*$^-$/*desD*$^-$ cells were grown photoautotrophically at 30 °C under illumination from incandescent lamps at an intensity of 50 μmol photons m^{-2} s^{-1} in buffered BG-11 medium supplemented with 20 mM HEPES–NaOH (pH 7.5), with continuous aeration with sterile air that contained l% CO_2 [27]. Cultures of wild-type and mutant cells were adjusted to approximately the same optical density in buffered BG-11 medium and then exposed to strong light (2500 μmol photons m^{-2} s^{-1}) for photoinhibition and to low-intensity light (50 μmol photons m^{-2} s^{-1}) at 30 °C for recovery. Air that contained 1% CO_2 was bubbled through cultures during all experiments. Small aliquots of cells were withdrawn at intervals for measurements of PSII activity.

2.2. Quantitation of photosynthetic activity

The activity of PSII was measured by monitoring the photosynthetic evolution of oxygen at 30 °C in the presence of l.0 mM *p*-benzoquinone as the electron acceptor with a Clark-type oxygen electrode. Actinic light was provided by an incandescent lamp in combination with a red optical filter (R-62; Hoya Glass, Tokyo, Japan) at a saturating intensity of 1200 μmol photons m^{-2} s^{-1}.

The Chl concentration of the suspension of thylakoid membranes was determined as described by Arnon et al. [28].

2.3. Fourier transform infrared (FTIR) spectroscopy

For FTIR spectroscopy, suspensions of thylakoid membranes, prepared as described below, were centrifuged in a model TL100 centrifuge (Beckman, Fullerton, CA) at 75,000 rpm for 20 min at 4 °C. Then they were resuspended in the identical but D_2O-based buffer. FTIR spectra were recorded with an FTIR spectrometer (model PU9800; Philips, Cambridge, UK) at a spectral resolution of 2 cm^{-1}. A total of 128 interferograms was accumulated for each background and sample spectrum. Experiments were performed by repeating the following cycle of measurements: sample spectrum; background spectrum; new temperature setting; a 10-min pause to allow the new thermal equilibrium to be reached; and reading the actual temperature. The temperature was increased in steps of 2 to 3 °C in a water-thermostatted sample holder. The variability of the adjusted temperature was about 0.1 °C. The entire procedure was computer-controlled.

Data were analyzed with SPSERV software (Bagyinka, Cs., Szeged, Hungary). The spectra were not manipulated prior to the analysis of data. Spectral regions of interest were fitted with Lorentzian components. All component parameters (frequency, bandwidth, and intensity) were freely optimized by the program. The error of determinations of component band frequencies in the C-H stretching region was smaller than 0.1 cm^{-1}. All other details have been described elsewhere [24].

2.4. Light-induced quenching of chlorophyll fluorescence

Light-induced quenching of Chl fluorescence, due to the reduction of pheophytin [29–31] in intact cells, was monitored 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 30 °C under anaerobic conditions (10 mM glucose, 50 units ml^{-1} glucose oxidase and 1000 units ml^{-1} catalase) in the presence of 20 μM 3-(3′, 4′-dichlorophenyl)-1,1-dimethylurea (DCMU) and 2 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) or in the presence of 20 μM 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}.

2.5. Isolation of thylakoid membranes and immunoblotting analysis of D1, pre-D1 and D2 proteins

Thylakoid membranes were isolated, as described previously [32,33], from wild-type and *desA*$^{-}$/*desD*$^{-}$ cells of *Synechocystis* that had been incubated in light as described above. Thylakoid membranes were solubilized by incubation for 5 min at 65°C in 60 mM Tris (pH adjusted to 6.8 with HCl) 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 polyacrylamide gel electrophoresis [12.5% (w/v) polyacrylamide] in the presence of 0.08% (w/v) SDS and 6 M urea, as described previously [26,34]. Molecular markers (Kaleidoscope prestained standards; Bio-Rad Laboratories, Hercules, CA) were used for estimations of the molecular masses of immunoreactive proteins. 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, D1, pre-D1 and D2 proteins were detected immunologically with an ECL Western blotting kit according to the protocol supplied with the kit (Amersham International, Buckinghamshire, UK).

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; [35]). 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. The D2 protein was detected with specific antibodies that had been raised in rabbits against amino acids 320–345 of the D2 protein of *Synechocystis* [36]. The pre-D1 protein was detected with antibodies raised in rabbits against a synthetic oligopeptide of 16 amino acid residues (SGEGAPVALTAPAVNG) that corresponded to the carboxyl terminus of pre-D1 (the products of the *psbAII* and *psbAIII* genes) from *Synechocystis*. As second antibodies, we used horseradish peroxidase-linked antibodies raised in donkeys against rabbit immunoglobulin G (Amersham International).

The antibodies raised in rabbit against D1 and D2 were kindly provided by Prof. Kimiyuki Satoh (Department of Biology, Okayama University, Okayama, Japan), and Prof. Eva-Mari Aro (Department of Plant Physiology and Molecular Biology, University of Turku, Finland), respectively, and the antibodies against pre-D1 were generated in our laboratory and purified by affinity chromatography with the 16 amino acid sequence of the carboxy-terminus of pre-D1 (ProtOn™-Kit 1; Funakoshi, Tokyo, Japan). A digital camera system (LAS-1000; Fuji Photo Film Co., Tokyo, Japan) was used to monitor signals from blotted membranes and to quantify the D1 and pre-D1 proteins.

2.6. Northern blotting analysis

Total RNA was extracted from cells and Northern blotting analysis was performed as described previously [37]. Equal amounts of RNA (4 μg) from each sample were loaded in individual wells of the gel and rRNA was visualized by staining with ethidium bromide. A 1.0-kb fragment of DNA that included the coding region of the *psbA* gene was amplified by the polymerase chain reaction with primers 5′-AACGACTCTCCAACAGCGCGAAA-3′ and 5′-CGTTCGTGCATTACTTCAAAACCG-3′ and genomic DNA from *Synechocystis* as the template. The amplified fragment of DNA was ligated into the TA cloning vector pT7Blue-T (Novagen, Darmstadt, Germany). The plasmid was digested at the *Hin*cII and *Nco*I sites within the insert. The resultant 700-bp fragment of DNA was conjugated with alkaline phosphatase using an Alkphos Direct kit (Amersham Pharmacia Biotech, Piscataway, NJ) and the conjugate was used as the probe. After hybridization, blots were soaked in CDP-star solution (Amersham Pharmacia Biotech) and signals from hybridized mRNAs were detected with the digital camera system.

3. Results

3.1. Irreversible photoinhibition depends on temperature and the duration of irradiation

Fig. 1 shows the extent of the photoinhibition of the oxygen-evolving activity of PSII in wild-type cells of *Synechocystis* during exposure to light at 2500 μmol photons m^{-2} s^{-1} and the subsequent recovery of cells from the photoinhibited state. The numbers in circles in Figs. 1A and 2A correspond to samples analyzed in experiments for which results are shown in Figs. 4–7. At 25 °C, wild-type cells lost approximately 70% and 80% of their oxygen-evolving activity upon exposure to light at 2500 μmol photons m^{-2} s^{-1} for 1 and 2 h, respectively. This activity of PSII in these photoinhibited cells recovered rapidly after they were transferred to light at 50 μmol

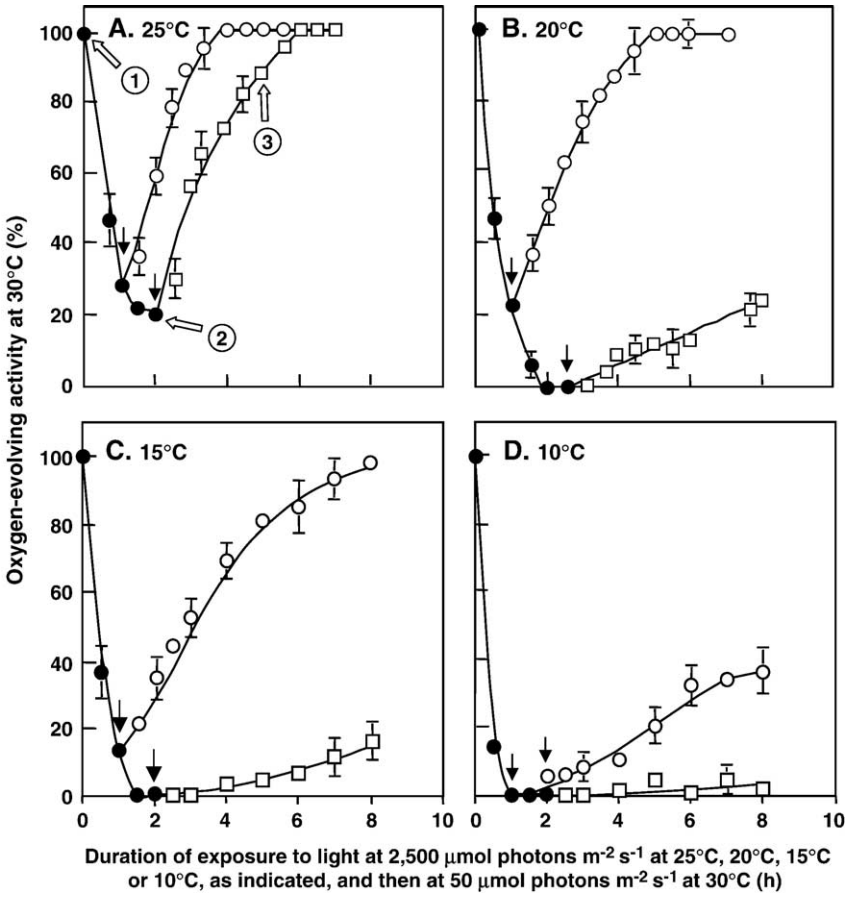

Fig. 1. Effects of temperature on the photoinhibition and subsequent recovery of PSII in wild-type cells of *Synechocystis*. Cells that had been grown at 30 °C were exposed to light at 2500 μmol photons m^{-2} s^{-1} for 1 or 2 h at 25 °C (A), 20 °C (B), 15 °C (C) or 10 °C (D). After exposure to strong light, cells were exposed to light at 50 μmol photons m^{-2} s^{-1} at 30 °C. The PSII activity was determined by monitoring the oxygen-evolving activity at 30 °C in light at 1200 μmol photons m^{-2} s^{-1} in the presence of 1.0 mM *p*-benzoquinone. The activity corresponding to 100% was 590±30 μmoles O_2 (mg Chl)$^{-1}$ h^{-1}. Thin arrows indicate the time at which cells were transferred from light at 2,500 μmol photons m^{-2} s^{-1} (closed symbols) to light at 50 μmol photons m^{-2} s^{-1} (open symbols). Open circles and open squares indicate recovery after exposure to strong light for 1 and 2 h, respectively. The open arrows indicate the times at which aliquots were removed for Western and Northern blotting and for analysis of the light-induced quenching of Chl fluorescence for which results are shown (with the corresponding encircled numbers) in Figs. 4 through 7. Values are the means±S.D. of results from three independent experiments. Where error bars are not shown, they fall within symbols.

photons m^{-2} s^{-1} (Fig. 1A). When wild-type cells were exposed for 1 h to light at 2500 μmol photons m^{-2} s^{-1} at 20 °C, the extent of photoinhibition was greater than that at 25 °C (Fig. 1B). However, the PSII activity again recovered completely after exposure of the cells to light at 50 μmol photons m^{-2} s^{-1}. Recovery after exposure to strong light for 2 h was incomplete.

Figs. 1C and D show the effects at 15 °C and at 10 °C of high-intensity light at 2500 μmol photons m^{-2} s^{-1} on the photoinhibition of PSII in wild-type cells of *Synechocystis* and the subsequent recovery from photoinhibition. Exposure of cells to high-intensity light at 15 °C for 1 and 2 h resulted in loss of 85% and 100% of the original activity of PSII, respectively. After exposure of such photoinhibited cells to light at 50 μmol photons m^{-2} s^{-1} for 5 h, the oxygen-evolving activity recovered completely (Fig. 1C). Exposure for 2 h resulted in incomplete recovery. When wild-type cells were exposed to high-intensity light for 2 h at 10 °C, rather than at 15 °C, PSII activity disappeared entirely. During subsequent incubation of these cells in light at 50 μmol photons m^{-2} s^{-1}, the recovery of PSII was extremely slow and minimal (Fig. 1D). These observations indicate that exposure of wild-type cells to high-intensity light for 2 h at 10 °C resulted in the irreversible photoinhibition of PSII (Fig. 1D).

3.2. Rigidification of membrane lipids enhances irreversible photoinhibition

In a previous study, we demonstrated that the extent of (reversible) photoinhibition of PSII in *Synechocystis* is enhanced by decreases in temperature [22]. Rigidification of membrane lipids by targeted mutagenesis of both the *desA* and *desD* genes for Δ12 and ω3 fatty acid desaturases allows the replacement of polyunsaturated fatty acids by monounsaturated fatty acids [23] and also enhances the extent of reversible photoinhibition [19,23]. Therefore, in the present study, we examined whether the replacement of

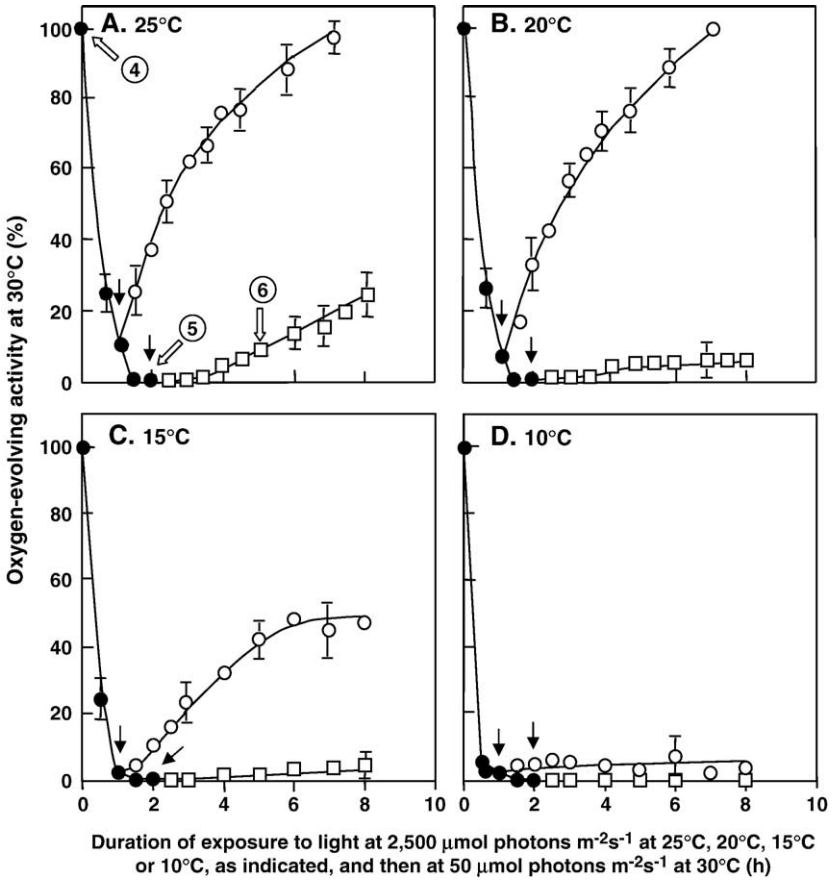

Fig. 2. Effects of temperature on the photoinhibition and subsequent recovery of PSII in *desA*$^-$/*desD*$^-$ cells of *Synechocystis.* For experimental conditions and definitions of symbols, see the legend to Fig. 1. The absolute activity corresponding to 100% was 620±35 μmol O_2 (mg chl)$^{-1}$ h^{-1}. The values are the means ± S.D. of results of three independent experiments. Where error bars are not shown, they fall within symbols.

polyunsaturated by monounsaturated fatty acids would mimic the effects of a decrease in temperature. We compared the photoinhibition in *desA*$^-$/*desD*$^-$ cells, which contained monounsaturated but no polyunsaturated fatty acids, to that in wild-type cells, which contained polyunsaturated fatty acids and only very low levels of monounsaturated fatty acids.

Fig. 2 shows the photoinhibition of PSII in *desA*$^-$/*desD*$^-$ mutant cells of *Synechocystis* during their exposure to light at 2500 μmol photons m^{-2} s^{-1} and the subsequent recovery of PSII from the photoinhibited state in light at 50 μmol photons m^{-2} s^{-1}. At 25 °C (Fig. 2A), PSII activity fell to 90% and 100% of the original activity upon exposure of cells to high-intensity light for 1 and 2 h, respectively. Thus, the extent of photoinhibition in mutant cells was higher than that in wild-type cells. However, after exposure of the mutant cells to light at 50 μmol photons m^{-2} s^{-1} for 1 h, PSII activity recovered completely.

At 20 °C (Fig. 2B), after photoinhibition by exposure to light at 2500 μmol photons m^{-2} s^{-1} for 1 h, these mutant cells were able to regain normal PSII activity. However, when the duration of photoinhibitory illumination was extended to 2 h, the subsequent recovery of PSII at 50 μmol photons m^{-2} s^{-1} was minimal. These observations suggest that the saturation of fatty acids in membrane lipids might have a significant effect on the development of irreversible photoinhibition.

Figs. 2C and D show the extent of photoinhibition of PSII in *desA*$^-$/*desD*$^-$ cells upon exposure to light at 2500 μmol photons m^{-2} s^{-1} at lower temperatures and the subsequent recovery from photoinhibition. At 15 °C (Fig. 2C), exposure to strong light for 1 h completely eliminated the activity of PSII. However, the subsequent transfer of cells to light at 50 μmol photons m^{-2} s^{-1} allowed the partial restoration of PSII activity. When the duration of exposure to high-intensity light was extended to 2 h, we detected only minimal recovery from the photoinhibited state (Fig. 2C). At 10 °C (Fig. 2D), exposure to high-intensity light for 1 or 2 h resulted in almost complete loss of the reversibility of photoinhibition. These results suggested that such conditions had caused the irreversible photoinhibition of PSII.

The results obtained in *desA*$^-$/*desD*$^-$ cells at 15 °C, 20 °C and 25 °C, which are shown in Fig. 2, were very similar to those for wild-type cells at 10 °C, 15 °C and 20 °C, respectively, in Fig. 1. This similarity indicates that the effects of the rigidification of membrane lipids by the genetic modification of fatty acid desaturases resemble those of a decrease in ambient temperature.

3.3. Quantitation of changes in membrane fluidity caused by low temperatures and the genetic manipulation of the unsaturation of fatty acids

The frequencies of $\nu_{sym}CH_2$ bands in FTIR spectra are indicators of the fluidity of the fatty acyl chains in membrane lipids. The method used for the determination of these frequencies from the C-H stretching region of the infrared spectrum is explained elsewhere [24]. However, the general rule is that the higher the frequency of the $\nu_{sym}CH_2$ band, the greater is the disorder (fluidity) of the fatty acyl chains of the membrane lipids; in other words, greater disorder of fatty acyl chains corresponds to lower microviscosity of membrane lipids.

Fig. 3 shows that the membrane lipids in *desA*$^-$/*desD*$^-$ cells were less fluid than those in wild-type cells over a range of temperatures from 10 to 25 °C. The results in Fig. 3 also demonstrate that the difference in fluidity between the two types of membrane lipid was larger with decreases in temperature. The difference in temperature for the same fluidity was 7–10 °C. These results might provide some clues to explain the results of our photoinhibition experiments (Figs. 1 and 2). In these experiments, the time-dependent development of irreversible photoinhibition was observed to occur in a similar manner in membranes from *desA*$^-$/*desD*$^-$ mutant cells (Fig. 2) and from wild-type cells (Fig. 1), when the temperature was lowered by 5–7 °C in the latter case.

3.4. Irreversible photoinhibition does not occur in the reaction center of PSII

In cells that had suffered irreversible photoinhibition, the light-dependent transport of electrons from H_2O to *p*-benzoquinone was inhibited. These observations led us to ask whether the photochemically active reaction center of PSII could be repaired even after irreversible photoinhibition. To investigate the activity of the reaction center of PSII, we measured the light-dependent reduction of pheophytin by monitoring the light-induced quenching of Chl fluorescence under anaerobic conditions [29–31].

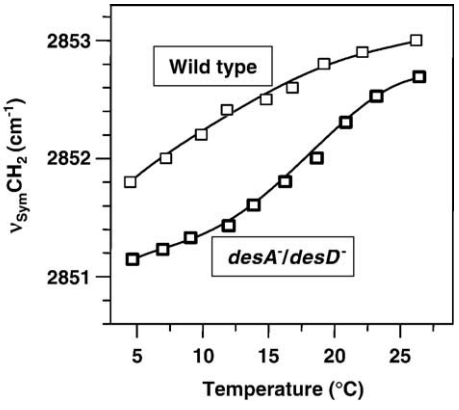

Fig. 3. Thermotropic response curves for $\nu_{sym}CH_2$ frequencies, as determined by FTIR spectroscopy in thylakoid membranes from wild-type cells and *desA*$^-$/*desD*$^-$ cells of *Synechocystis* that had been grown at 30 °C. The disorder of fatty acyl chains (fluidity) in thylakoid membranes from *desA*$^-$/*desD*$^-$ cells was similar to that in thylakoid membranes from wild-type cells when the temperature in the former case was 7–10 °C higher than in the latter. The range of standard errors in the determination of the $\nu_{Sym}CH_2$ frequencies was smaller than 0.1 cm^{-1}.

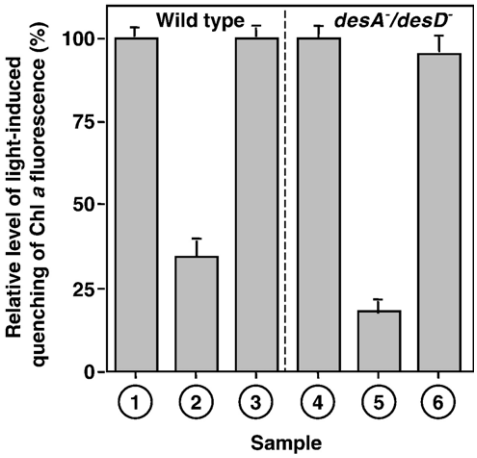

Fig. 4. Effects of temperature and strong light on the light-induced quenching of Chl fluorescence due to the photoreduction of pheophytin during exposure of wild-type and *desA*$^-$/*desD*$^-$ cells of *Synechocystis* to strong light and during subsequent recovery of PSII in weak light. The timing of sampling is indicated by the corresponding encircled numbers in Figs. 1A and 2A. The light-induced quenching of Chl fluorescence was measured at 30 °C under anaerobic conditions, as described in Materials and methods. The light-induced quenching of Chl fluorescence due to the photoreduction of pheophytin before exposure to strong light was taken as 100%. Experiments were repeated four times and means+S.D. of results are shown.

Fig. 4 shows that, during incubation of cells under anaerobic conditions in light at 2,500 μmol photons m^{-2} s^{-1} at 25 °C for 2 h, the extent of light-induced quenching decreased to 34% and 18% of the original level in wild-type and *desA*$^-$/*desD*$^-$ cells, respectively. When these cells were transferred to light at 50 μmol photons m^{-2} s^{-1} at 30 °C for 3 h, the level of light-induced quenching of Chl fluorescence returned to 100% and 94% of the original level in wild-type and *desA*$^-$/*desD*$^-$ cells, respectively. Similar results were observed in the presence of 1 mg^{-1} ml^{-1} sodium dithionite. These results suggest that irreversible photoinhibition was an event that occurred in the oxygen-evolving complex but not in the photochemical reaction center.

3.5. Irreversible photoinhibition is related to the impaired processing of pre-D1

To examine whether the irreversibility of photoinhibition might be related to loss of the ability to synthesize pre-D1 or to process pre-D1, we performed Western blotting analysis to monitor changes in levels of the Dl and pre-D1 proteins in thylakoid membranes from wild-

type and *desA*$^{-}$/*desD*$^{-}$ cells. In the analysis of some samples, we observed two bands of immunoreactive protein above the one that was due to D1 protein. We postulated that these bands might correspond to the precursor to the D1 protein (pre-D1-1) and to partially processed pre-D1 (pre-D1-2), respectively [26,38]. A clear difference with respect to irreversible photoinhibition was evident when wild-type cells and *desA*$^{-}$/*desD*$^{-}$ cells were exposed to photoinhibiting conditions at 25 °C for 2 h, as shown in Figs. 1A and 2A. Figs. 5A and C show that the level of the Dl protein fell considerably after exposure of both wild-type and mutant cells to light at 2500 μmol photons m^{-2} s^{-1}. However, this loss was compensated by increases in levels of pre-D1-1 and pre-D1-2 in both wild-type and *desA*$^{-}$/*desD*$^{-}$ cells. After subsequent exposure for 3 h to light at 50 μmol photons m^{-2} s^{-1}, the level of the Dl protein in wild-type cells clearly increased in the expense of pre-D1-1 and pre-D1-2, suggesting that pre-D1-1 and pre-D1-2 were processed to the D1 protein.

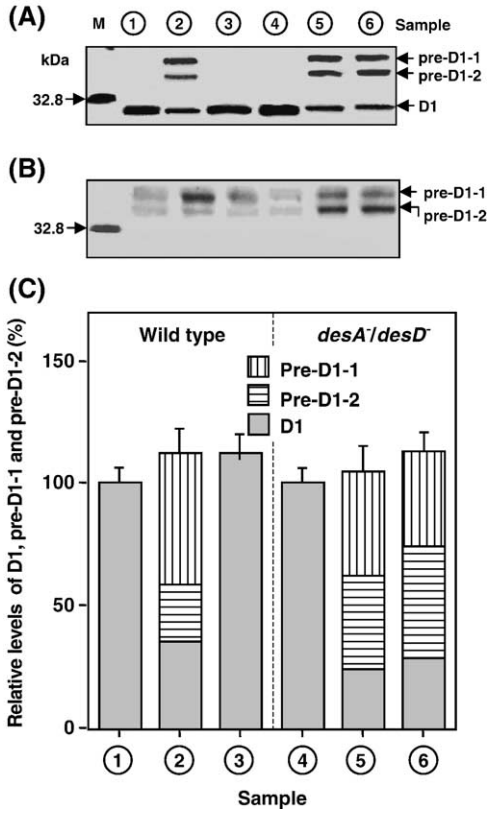

Fig. 5. Changes in levels of the D1, pre-D1-1 and pre-D1-2 proteins during exposure of wild-type and *desA*$^{-}$/*desD*$^{-}$ cells to strong light and during subsequent recovery in weak light. The timing of sampling is indicated by the corresponding encircled numbers in Figs. 1A and 2A. At designated times, aliquots of cell suspensions were withdrawn for preparation of thylakoid membranes, which were subjected to Western blotting analysis, as described in Materials and methods, with antibodies against the D1 protein (A) and with antibodies against the carboxy-terminal extension of the pre-D1 protein (B). Quantitation of the data in (A) is presented in (C). Experiments were repeated four times [values in (C) are means+S.D.], and essentially the same results were obtained in each experiment.

This observation appeared to be correlated with the recovery of PSII activity (Fig. 1A). These results indicated that, in wild-type cells, the Dl protein was degraded and pre-D1 was synthesized de novo during photoinhibition under strong light and that pre-D1 was processed to yield D1 during the repair of PSII under weak light.

In *desA*$^{-}$/*desD*$^{-}$ cells, the Dl protein was degraded and pre-D1-1 and pre-D1-2 were synthesized de novo during photoinhibition under strong light, as occurred also in wild-type cells. However, during the subsequent exposure of *desA*$^{-}$/*desD*$^{-}$ cells to light at 50 μmol photons m^{-2} s^{-1}, levels of D1 protein, pre-D1-1 and pre-D1-2 did not change, suggesting that processing of pre-D1 was inhibited in *desA*$^{-}$/*desD*$^{-}$ cells. This phenomenon was correlated with the inability of these cells to regain the oxygen-evolving activity of PSII (Fig. 2A) and it suggests that irreversible photoinhibition might have been due to loss of the ability to process pre-Dl.

When we performed Western blotting analysis with antibodies against the SGEGAPVALTAPAVNG polypeptide, which corresponds to the carboxy-terminal extension of pre-D1 [39,40] and is not part of the mature D1 protein, we observed bands of both pre-D1-1 and pre-D1-2. These results confirmed that pre-D1-1 and pre-D1-2 were synthesized in a similar manner in both types of cell and that pre-D1-1 and pre-D1-2 were subsequently processed to D1 in wild-type cells but were not converted to D1 in *desA*$^{-}$/*desD*$^{-}$ cells (Fig. 5B).

3.6. Irreversible photoinhibition has no effect on the level of D2 protein

To examine whether another important component of the PSII reaction center, namely, the D2 protein, is modified during reversible photoinhibition and subsequent repair in wild-type cells and during irreversible photoinhibition in *desA*$^{-}$/*desD*$^{-}$ cells, we investigated, by Western blotting, changes in the level of the D2 protein. Fig. 6 shows that the level of D2 was not significantly affected by exposure of either wild-type or *desA*$^{-}$/*desD*$^{-}$ cells to strong light at 2500 μmol photons m^{-2} s^{-1} at 25 °C for 2 h. These results suggest that the level of D2 protein is unaffected when *Synechocystis* cells undergo irreversible photoinhibition.

3.7. Irreversible photoinhibition has no effect on the level of psbA transcripts

In *Synechocystis*, pre-D1 is encoded by a small multigene family that consists of the *psbAI*, *psbAII* and *psbAIII* genes [41–43]. The *psbAII* and *psbAIII* genes are expressed in response to light and the *psbAI* gene is nonfunctional [42–44]. The *psbAII* and *psbAIII* genes encode identical pre-D1 proteins.

We monitored changes in the level of a mixture of *psbAII* and *psbAIII* transcripts by Northern blotting (Fig. 7).

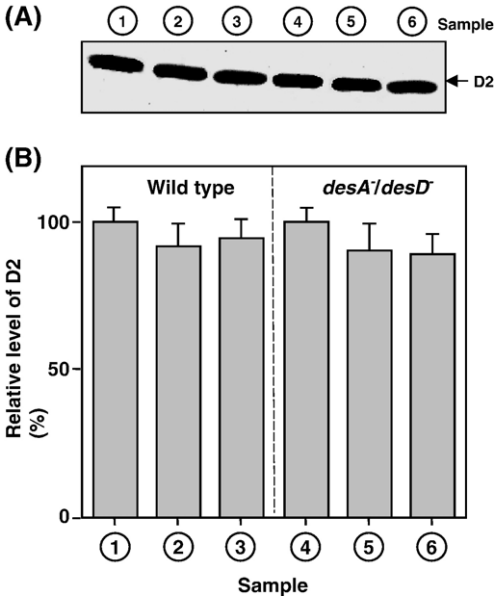

Fig. 6. Changes in levels of the D2 protein during exposure of wild-type and *desA*$^{-}$/*desD*$^{-}$ cells to strong light and during subsequent recovery in weak light. The timing of sampling is indicated by the corresponding encircled numbers in Figs. 1A and 2A. (A) Results of Western blotting. (B) Quantitation of the results shown in (A), namely, levels of the D2 protein. Signals due to D2 before exposure to strong light were taken as 100%. Experiments were repeated three times [(B) shows means+S.D.], and essentially the same results were obtained in each experiment.

Transfer of wild-type and *desA*$^{-}$/*desD*$^{-}$ cells from light at 50 μmol photons m^{-2} s^{-1} to light at 2,500 μmol photons m^{-2} s^{-1} caused the rapid accumulation of *psbA* transcripts of 1.2 kb in both strains. The level of *psbA* transcripts increased about four-fold during the exposure to strong light for 2 h in both wild- type and mutant cells. During subsequent exposure of cells to light at 50 μmol photons m^{-2} s^{-1}, the level of *psbA* transcripts decreased similarly. These results suggest that changes in the abundance of these transcripts did not differ between wild-type and *desA*$^{-}$/*desD*$^{-}$ cells.

4. Discussion

In the present study, we demonstrated that the oxygen-evolving activity of the PSII complex was irreversibly lost when cyanobacterial cells were exposed, for a prolonged period, to strong light at an intensity that exceeded intensities that cause reversible photoinhibition. In addition to light intensity, lower temperatures (Fig. 1) and the lack of polyunsaturated fatty acids in membrane lipids (Fig. 2), both of which decreased the microviscosity (fluidity) of membrane lipids (Fig. 3), enhanced the extent of irreversible photoinhibition.

We demonstrated previously that low temperatures inhibit or delay the repair of the photoinhibited PSII complex after mild photoinhibition (reversible photoinhibition) [19]. We also demonstrated that genetic modification of the extent of saturation of the fatty acids in membrane lipids delayed the repair of the PSII complex [21,22]. A careful examination of the results in Figs. 1 and 2 suggests that both low temperature and the enhanced saturation of fatty acids reduced the time required for the total elimination of the oxygen-evolving activity of PSII.

The results also show that irreversible photoinhibition started to occur when cyanobacterial cells that had already suffered the reversible loss of PSII activity were exposed to high-intensity light. This might explain how conditions for the initiation of irreversible photoinhibition of the PSII complex are established. In a recent study [45], we have demonstrated that the limited supply of electrons from PSII, which is achieved by the presence of DCMU, decreases the intracellular level of ATP and delays the repair of PSII. The complete elimination of PSII activity by exposure of *Synechocystis* cells to high-intensity light for a prolonged period is similar to that by the presence of DCMU with respect to the supply of electrons from PSII. Under such conditions, all the electron-transport components are oxidized and, therefore, ATP is not synthesized. This mechanism may explain why the repair does not occur after the PSII activity is totally lost.

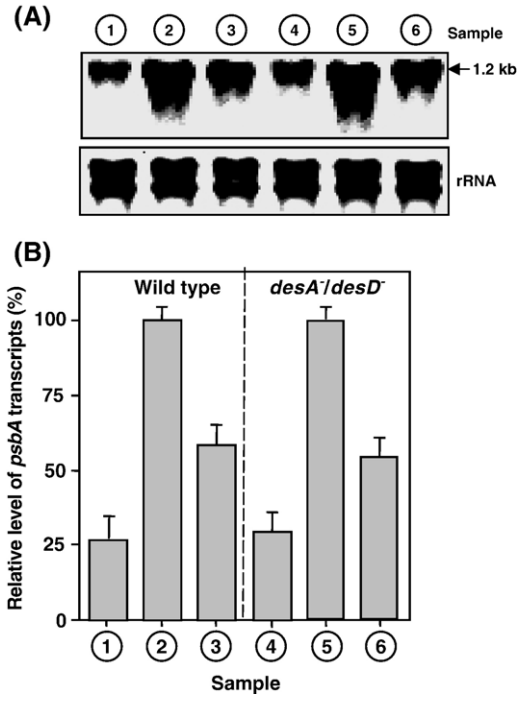

Fig. 7. Changes in levels of *psbA* transcripts during exposure of wild-type and *desA*$^{-}$/*desD*$^{-}$ cells to strong light and during subsequent recovery in weak light. The timing of sampling is indicated by the corresponding encircled numbers in Figs. 1A and 2A. At designated times, aliquots of cell suspensions were withdrawn for extraction of RNA, which was subjected to Northern blotting analysis as described in Materials and methods. (A) Gel-electrophoretic patterns. The levels of transcripts were normalized by reference to levels of rRNA and the results are shown quantitatively in (B). Experiments were repeated three times and similar results were obtained in each case. Values in (B) are means+S.D.

Our results in (Figs. 1, 2, and 4) suggest that, even after the oxygen-evolving activity is irreversibly inhibited, the activity of the photochemical reaction center of PSII was recovered with pre-D1. The photochemical reaction center of PSII that is active with pre-D1 has been observed in the LF-1 mutant of *Scenedesmus obliquus* [46] and the CtpA-deficient mutant of *Synechocystis* [47]. However, the PSII complex in these mutants is unable to incorporate Mn into the oxygen-evolving center and, consequently, it is inactive in the photosynthetic evolution of oxygen. Therefore, our observations corroborate that the active PSII complex was reassembled with pre-D1.

Our findings might explain both the reversible and the irreversible photoinhibition of the PSII complex. It appears that repair involves a series of steps (reactions), as follows: degradation of the D1 protein in photoinhibited PSII; transcription of the *psbA* genes to yield *psbA* mRNAs; translation of *psbA* mRNAs to yield pre-D1; reassembly of the PSII monomeric complex by insertion of pre-D1; proteolytic cleavage of the carboxy-terminal extension of pre-D1 to produce the D1 protein in the PSII monomer; dimerization of PSII monomers; and assembly of the oxygen-evolving complex in PSII. The results in the present study indicate that the steps that are unaffected in *Synechocystis* cells that have been subject to irreversible photoinhibition are the degradation of D1 protein (Figs. 5A and C), and the transcription (Fig. 7) and translation (Fig. 5A) of *psbA* transcripts. The impediment that prevents the repair of PSII after it has been irreversibly photoinhibited is a defect in the processing of pre-D1 (Fig. 5). All our observations together suggest that processing of pre-D1 is the principal target for the irreversible photoinhibition of PSII.

In the repair process that is an integral part of the normal photodamage-repair cycle, pre-D1 is incorporated into the monomeric PSII complex as it is being translated [38,48]. Then, it is processed by the lumenal protease CtpA. After the proteolytic cleavage that yields mature D1, PSII monomers form dimers and become fully active [5,6,12]. There are several possible mechanisms that might explain how the proteolytic activity of CtpA in irreversibly photoinhibited cells is regulated: (1) the expression of the *ctpA* gene might be inhibited; (2) the processing of the precursor to CtpA might be inhibited; (3) CtpA might be inactivated; or (4) some specific conformation of the processing site of pre-D1 might be resistant to CtpA. Since PSII appeared to become irreversibly photoinhibited after it had been reversibly photoinhibited, the defect in the processing of pre-D1 must have occurred when PSII that had been reversibly photoinhibited was exposed to strong light. It is unlikely that the fluidity of membrane lipids is involved directly in the defect in the function of CtpA. It is more likely that the rigidification of membrane lipids acts primarily to prevent PSII from undergoing repair of reversible photodamage [19,20], with resultant high levels of reversibly photoinhibited PSII (Figs. 1A, B, 2A and B), which is, in turn, the target of irreversible photoinhibition.

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