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Interruption of the Calvin cycle inhibits the repair of Photosystem II from photodamage

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Abstract

In photosynthetic organisms, impairment of the activities of enzymes in the Calvin cycle enhances the extent of photoinactivation of Photosystem II (PSII). We investigated the molecular mechanism responsible for this phenomenon in the unicellular green alga *Chlamydomonas reinhardtii.* When the Calvin cycle was interrupted by glycolaldehyde, which is known to inhibit phosphoribulokinase, the extent of photoinactivation of PSII was enhanced. The effect of glycolaldehyde was very similar to that of chloramphenicol, which inhibits protein synthesis de novo in chloroplasts. The interruption of the Calvin cycle by the introduction of a missense mutation into the gene for the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) also enhanced the extent of photoinactivation of PSII. In such mutant 10-6C cells, neither glycolaldehyde nor chloramphenicol has any additional effect on photoinactivation. When wild-type cells were incubated under weak light after photodamage to PSII, the activity of PSII recovered gradually and reached a level close to the initial level. However, recovery was inhibited in wild-type cells by glycolaldehyde and was also inhibited in 10-6C cells. Radioactive labelling and Northern blotting demonstrated that the interruption of the Calvin cycle suppressed the synthesis de novo of chloroplast proteins, such as the D1 and D2 proteins, but did not affect the levels of *psbA* and *psbD* mRNAs. Our results suggest that the photoinactivation of PSII that is associated with the interruption of the Calvin cycle is attributable primarily to the inhibition of the protein synthesis-dependent repair of PSII at the level of translation in chloroplasts.
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Keywords: Calvin cycle; Photoinactivation; Photoinhibition; Photosystem II; Repair cycle

1. Introduction

Light is necessary for photosynthesis, but it also inactivates the photosynthetic machinery [1–6]. This phenomenon is referred to as "photoinactivation" or "photoinhibition". The extent of photoinactivation is enhanced when light stress is combined with other types of environmental stress that limit the activity of the Calvin cycle [7].

It is likely that the major site of photodamage in the photosynthetic machinery is Photosystem II (PSII), the complex of proteins and pigments that oxidizes water and reduces plastoquinone during photosynthesis. In one current model of the photoinactivation of PSII, it is suggested that strong light generates highly toxic singlet oxygen via the interaction of oxygen with triplet-excited P680 [8,9]. The resultant singlet oxygen itself or the radical produced from singlet oxygen damages the D1 protein in PSII in the model that is referred to as "acceptor-side photoinhibition" [10,11]. It has been also proposed that, when the transfer of electrons from water to P680 is impaired, strong light forms the harmful oxidant $P680^{+}$, which then damages the D1 protein [12,13]. This latter model is referred to as "donor-side photoinhibition" [10,11].

To prevent the accumulation of photodamaged PSII, photosynthetic organisms have developed a repair process, which consists of several steps, namely, degradation of the

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylure; PSII, Photosystem II; pre-D1, precursor to D1 protein; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; TAP, Tris–acetate phosphate

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D1 protein, synthesis de novo of the precursor to D1 protein (pre-D1), insertion of the newly synthesized precursor into the thylakoid membrane concomitantly with the assembly of other PSII proteins, maturation of the D1 protein via carboxy-terminal processing of pre-D1, and the assembly of the oxygen-evolving machinery [11,14]. The extent of photoinactivation of PSII is therefore the result of the balance between the rate of photodamage and the rate of repair [15,16].

The photoinactivation of PSII is accelerated and enhanced by glyceraldehyde [17] or glycolaldehyde [18], disrupters of the Calvin cycle that inhibit phosphoribulokinase [19,20]. Therefore, it is likely that the activity of the Calvin cycle is involved in the susceptibility of PSII to photoinactivation. It has been assumed that when the Calvin cycle is interrupted, excess of absorbed light energy accelerates damage to PSII. However, the molecular mechanism responsible for the acceleration of photoinactivation by factors that have a negative effect on the Calvin cycle has not yet been clarified.

In this study, we examined the effects on the photoinactivation of PSII of impairment of activities of enzymes in the Calvin cycle in *Chlamydomonas reinhardtii* cells, using glycolaldehyde and a strain (10-6C) of *C. reinhardtii* with a missense mutation in the gene for the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). We found that impairment of the activities of enzymes in the Calvin cycle did not directly accelerate the damage to PSII but appeared to suppress the repair of PSII by inhibiting the light-dependent synthesis of PSII proteins de novo, which is essential for the repair of PSII.

2. Materials and methods

2.1. Strains and culture conditions

A mutant strain of *C. reinhardtii*, 10-6C mt$^+$ (10-6C) [21], with a missense mutation in the gene for the large subunit of Rubisco and the parental wild-type strain, 2137 mt$^+$, were obtained from the Chlamydomonas Genetics Center (Duke University, Durham, NC, USA). Cells were grown at 25 °C in a Tris–acetate phosphate (TAP) medium [22] under dim light at 5 μmol photons m^{-2} s^{-1} or in minimum medium (TAP medium minus acetate) in light at 100 μmol photons m^{-2} s^{-1}. Cells that had reached the mid-logarithmic phase of growth (5 μg of Chl ml^{-1}, 4×10^6 cells ml^{-1}) were collected by centrifugation at 500×*g* for 3 min and resuspended in fresh growth medium at 5 μg of Chl ml^{-1}.

2.2. Photoinactivation treatment

Cells were incubated in darkness for 30 min in the presence or absence of glycolaldehyde, and then they were exposed to light from metal halide arc lamps, as indicated in the text. During experiments, cultures were bubbled continuously with air and the temperature of the medium was kept at 25 °C.

2.3. Measurements of the light-dependent evolution of oxygen

The light-dependent activity of oxygen production and PSII activity in intact cells were measured at 25 °C by monitoring the light-dependent evolution of oxygen with a Clark-type oxygen electrode. Light-dependent activity of oxygen production was measured in the presence of 5 mM $NaHCO_3$ in light at 1000 μmol photons m^{-2} s^{-1}. The activity of PSII was measured in the presence of 0.2 mM phenyl-*p*-benzoquinone, as an artificial electron acceptor in light, at 800 μmol photons m^{-2} s^{-1} that had been passed through a red optical filter (R-60; Hoya, Tokyo, Japan) and an infrared-absorbing filter (HA-50; Hoya, Tokyo, Japan).

2.4. Measurement of chlorophyll fluorescence

The fluorescence of chlorophyll *a* was measured with a chlorophyll fluorometer (PAM-2000; Heinz Walz, Effeltrich, Germany). After the incubation of cells in darkness for 30 min, the minimum fluorescence yield (Fo) was monitored in weak red light at 0.3 μmol photons m^{-2} s^{-1} and then the chlorophyll fluorescence yield (Fs) was measured after the addition of actinic light at 250 μmol photons m^{-2} s^{-1} in the presence of 5 mM $NaHCO_3$. During the illumination with actinic light, the maximum fluorescence yield in the light-adapted state (Fm′) was monitored during repeated pulses of saturating white light at 6000 μmol photons m^{-2} s^{-1} for 1.2 s at intervals of 20 s. The fluorescence yield that was measured after the exposure of cells to light at 250 μmol photons m^{-2} s^{-1} for 5 min in the presence of 20 μM DCMU was used as the maximum fluorescence yield (Fm). The effective quantum yield (Yield), non-photochemical quenching (NPQ), and photochemical quenching (qP) were calculated as $[(Fm' - Fs)/Fm']$, $[(Fm - Fm')/Fm']$, and $[(Fm' - Fs)/(Fm' - Fo)]$, respectively [23,24].

2.5. Western blotting analysis

Cells that had been grown in the TAP medium in light at 5 μmol photons m^{-2} s^{-1} were incubated in darkness for 30 min in the presence or absence of 200 μg ml^{-1} chloramphenicol and in the presence or absence of 5 mM glycolaldehyde. Then cells were incubated in light at 100 μmol photons m^{-2} s^{-1}. The cells were collected by centrifugation at 2000×*g* for 3 min, and total cell proteins corresponding to 0.5 μg of Chl were solubilized by heating in 2% SDS plus 0.1 M dithiothreitol at 60 °C for 3 min and fractionated on an SDS-polyacrylamide gel (15% acrylamide) that contained 6 M urea and 0.08% SDS [25]. Separated proteins were blotted onto a nitrocellulose

membrane and allowed to react with antibodies raised in rabbit against the purified D1 protein from spinach. Each band of immunologically reactive protein was detected with peroxidase-linked second antibodies and enhanced chemiluminescence Western blot detection reagents (RPN 2106; Amersham Biosciences, Tokyo, Japan). Chemiluminescence was detected by exposure of the membrane to X-ray film. Levels of the D1 protein were determined densitometrically.

2.6. Pulse labeling of proteins

Cells that had been grown to the mid-logarithmic phase (4×10^6 cells ml^{-1}) in sulfate-reduced TAP medium (0.1 mM sulfate; 8% of the concentration of sulfate in TAP medium) were collected by centrifugation at 500×*g* for 3 min, resuspended at 5 μg of Chl ml^{-1} in TAP medium that did not contain sulfur (sulfur-depleted TAP medium), and incubated in light at 5 μmol photons m^{-2} s^{-1} for 2 h. Then cells were incubated in darkness for 30 min in the presence or absence of 10 μg ml^{-1} cycloheximide and in the presence or absence of 5 mM glycolaldehyde. The uptake of sulfate was initiated by the addition of 0.1 mM $[^{35}S]$sulfate as Na_2SO_4 (10 μCi ml^{-1}; Amersham Biosciences) in light at 100 μmol photons m^{-2} s^{-1}. The uptake of sulfate was terminated by the addition of 200 μg ml^{-1} chloramphenicol and 10 μg ml^{-1} cycloheximide. Total cell protein from *Chlamydomonas* cells, corresponding to 1 μg of Chl, was solubilized in 2% SDS and 0.1 M dithiothreitol by heating at 60 °C for 3 min and was fractionated on an SDS-polyacrylamide gel (15% acrylamide) that contained 6 M urea and 0.08% SDS [25]. The labeled proteins on the dried and fixed gel were exposed to an imaging plate (BAS-III; Fuji-photo Film, Tokyo, Japan) and visualized with a Bio-image Analyzer (BAS 2000; Fuji-photo Film).

2.7. Northern blotting analysis

Cells (5 μg of Chl ml^{-1}) grown in TAP medium in light at 5 μmol photons m^{-2} s^{-1} were incubated in darkness for 30 min in the presence or absence of 5 mM glycolaldehyde. Then cells were incubated in light at 100 μmol photons m^{-2} s^{-1} for indicated times and total RNA was isolated from 15 ml of the suspension of cells and purified with an RNeasy Plant Mini Kit (Qiagen, Tokyo, Japan). Approximately 1 μg of the purified RNA was denatured by heating for 10 min at 65 °C in a solution that contained 50% (v/v) formamide, 5% formaldehyde (v/v), and 20 mM MOPS and was fractionated by electrophoresis on a 1.0% agarose gel that contained 3.7% formaldehyde. Bands of RNA on the gel were blotted onto a nylon hybridization transfer membrane (Hybond-N+; Amersham Biosciences) and then cross-linked to the membrane with a cross-linker (FS-800; Funakoshi Co., Ltd., Tokyo, Japan) under UV light. A 1047-bp fragment of DNA that contained the coding region of the *psbA* gene (nucleotides +3 to +1049; counted from the site of initiation of translation) was amplified by PCR with pBA157 [26], which contains an intron-free *psbA* gene, as template and primers 5′-GACAGCAATTTTAGAACGTCGTG-3′ and 5′-GAGCTAGAGTTAGTTGAAGCTAAG-3′. A 1235-bp fragment of DNA that contained the coding region of the *psbD* gene (nucleotides + 481 to + 1716) was amplified by PCR with total DNA, isolated with a DNeasy Plant Mini Kit (Qiagen), as template and primers 5′-GCTGATGACTGGCTTCGT-3′ and 5′-GGACCTTTTGACAGAGTAC-3′. A 493-bp fragment of DNA that contained the coding region of 16S ribosomal RNA (nucleotides + 1 to + 493) was amplified by PCR with total DNA, prepared as described above, as template and primers 5′-ATCCATGGAGAGTTTGATC-3′ and 5′-CCTCTGTATTACCGCG-3′. Each amplified fragment was purified with the QIAquick PCR purification Kit (Qiagen) and labeled with the AlkPhos Direct Labelling Reagents (Amersham Biosciences). Protocols and reagents for the chemiluminescent visualization of RNA were supplied as components of the Gene Images AlkPhos Direct Labeling and Detection System with CDP-Star (Amersham Biosciences). Signals from hybridized mRNA were detected with a luminescence image analyzer (Las-1000; Fuji-photo Film).

3. Results

3.1. Inhibition of phosphoribulokinase by glycolaldehyde enhanced the extent of photoinactivation of PSII

When wild-type cells of *C. reinhardtii* were grown photoautotrophically in the minimum medium in continuous light at 100 μmol photons m^{-2} s^{-1}, the light-dependent activity of oxygen production, which reflects the rate of electron flow from H_2O to CO_2, was 210 μmol O_2 mg Chl^{-1} h^{-1}. Glycolaldehyde, which inhibits the activity of phosphoribulokinase, a component of the Calvin cycle, decreased the light-dependent activity of oxygen production to 67%, 41%, and 14% of the original level when it was present in the medium at 2.5 mM, 5 mM, and 10 mM, respectively (Table 1).

In darkness, glycolaldehyde at 2.5 mM, 5 mM, and 10 mM had no direct effect on the activity of PSII, as measured in terms of the evolution of oxygen in the presence of artificial electron acceptor phenyl-*p*-benzoquinone (Fig. 1A). When cells were incubated in light at 250 μmol photons m^{-2} s^{-1} for 240 min in the absence of glycolaldehyde, the PSII activity remained unchanged (Fig. 1A). However, the activity decreased gradually in the presence of glycolaldehyde, and the effect of glycolaldehyde was concentration dependent. Thus, glycolaldehyde at 2.5 mM, 5 mM, and 10 mM decreased the activity to 86%, 21%, and 9% of the initial level, respectively, during incubation for 240 min in light (Fig. 1A). In the absence of glycolaldehyde, chloramphenicol, which inhibits protein

Table 1
Effects of glycolaldehyde on light-dependent activity of oxygen production

Glycolaldehyde (mM)	Light-dependent activity of oxygen production [μmol O_2 mg Chl^{-1} h^{-1} (%)]		
	Photoautotrophic growth at 100 μmol photons m^{-2} s^{-1}	Photoheterotrophic growth at 5 μmol photons m^{-2} s^{-1}	
	Wild type	Wild type	10-6C
0	209.7 ± 13.7 (100)	169.2 ± 4.0 (100)	26.3 ± 8.6 (100)
2.5	139.9 ± 6.0 (67)	118.6 ± 9.2 (70)	26.0 ± 6.2 (99)
5	85.8 ± 13.4 (41)	69.8 ± 7.1 (41)	28.6 ± 8.6 (109)
10	30.2 ± 11.8 (14)	14.5 ± 8.5 (9)	25.8 ± 7.5 (98)

Chlamydomonas cells were grown photoautotrophically in light at 100 μmol photons m^{-2} s^{-1} in minimum medium or photoheterotrophically in light at 5 μmol photons m^{-2} s^{-1} in the TAP medium. Cells were incubated in darkness for 30 min in the presence or absence of glycolaldehyde (GA) at 2.5, 5, or 10 mM. The light-dependent activity of oxygen production was measured in the presence of 5 mM $NaHCO_3$. Values are means±S.D. of results from three independent experiments. Activities in the absence of glycolaldehyde were taken as 100%.

synthesis in chloroplasts, accelerated the decrease in PSII activity during incubation in light [27]. Moreover, chloramphenicol completely abolished the effects of glycolaldehyde on photoinactivation (Fig. 1B). When cells were incubated in light at 0, 100, 250, and 500 μmol photons m^{-2} s^{-1} for 30 min in the presence of chloramphenicol, the extent of photoinactivation was related to light intensity (Table 2). Moreover, in the presence of chloramphenicol, glycolaldehyde had no additional effect on the extent of photoinactivation at any light intensity tested (Table 2). These results suggest that glycolaldehyde enhanced the extent of photoinactivation of PSII without directly accelerating the photodamage.

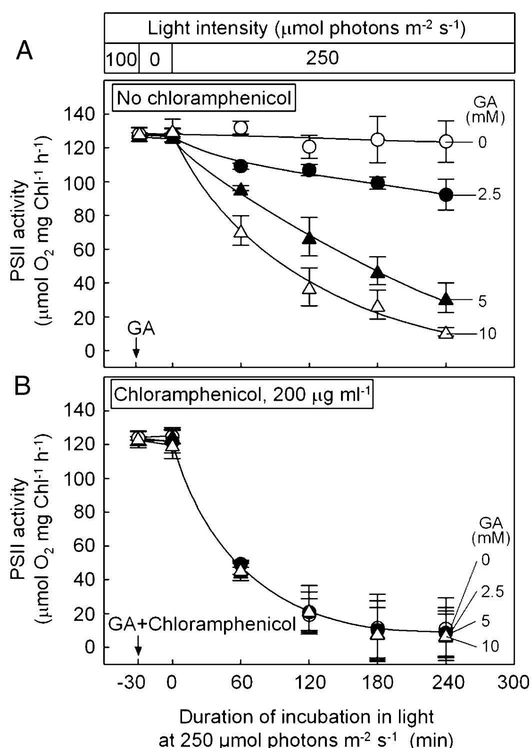

Fig. 1. Effects of glycolaldehyde and chloramphenicol on the photoinactivation of PSII in photoautotrophically grown wild-type cells of *C. reinhardtii* during incubation in light. Cells were grown in the minimum medium in continuous light at 100 μmol photons m^{-2} s^{-1}. After incubation in darkness for 30 min with glycolaldehyde (GA), as indicated, cells were exposed to light at 250 μmol photons m^{-2} s^{-1}. PSII activity was measured at intervals by monitoring oxygen evolution in the presence of 200 μM phenyl-*p*-benzoquinone. (A) No chloramphenicol added. (B) Chloramphenicol added at 200 μg ml^{-1} 30 min before the exposure of light. Values are means±S.D. (bars) of results from three independent experiments.

To demonstrate the effect of interruption of the Calvin cycle on electron transport in PSII, we investigated, using a chlorophyll fluorometer in the pulse amplitude modulation mode, the effects of glycolaldehyde on the redox state of Q_A, the primary acceptor of electrons in PSII, in wild-type cells that had been grown photoautotrophically (Fig. 2A). When these cells were exposed to light at 250 μmol photons m^{-2} s^{-1}, glycolaldehyde decreased the effective quantum yield of PSII, and the effect of glycolaldehyde was dose dependent (Fig. 2B). However, glycolaldehyde did not significantly affect the value of 1 − qP (where qP represents photochemical quenching; Fig. 2C), suggesting that the interruption of the Calvin cycle by glycolaldehyde did not increase the proportion of Q_A in the reduced state. When 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) was added

Table 2
Effects of glycolaldehyde on the photoinactivation of PSII in the presence of chloramphenicol at various light intensities

Light intensity (μmol photons m^{-2} s^{-1})	PSII activity after photoinactivation [μmol O_2 mg Chl^{-1} h^{-1} (%)]	
	No glycolaldehyde	Glycolaldehyde (10 mM)
Darkness	125.0 ± 2.8 (100)	127.0 ± 4.4 (100)
100	118.8 ± 6.3 (95)	119.1 ± 2.0 (94)
250	82.2 ± 10.0 (66)	83.2 ± 11.7 (67)
500	24.6 ± 4.0 (20)	26.1 ± 1.6 (21)

Wild-type cells, which had been grown photoautotrophically in the minimum medium in light at 100 μmol photons m^{-2} s^{-1}, were incubated with 200 μg ml^{-1} chloramphenicol in the presence or absence of 10 mM glycolaldehyde in darkness for 30 min. Cells were then incubated in light at 100, 250, or 500 μmol photons m^{-2} s^{-1} or in darkness for 30 min. PSII activity was measured in the presence of 200 μM phenyl-*p*-benzoquinone. Values are means±S.D. of results from three independent experiments. Initial PSII activities, measured before incubation in light or darkness, were taken as 100%.

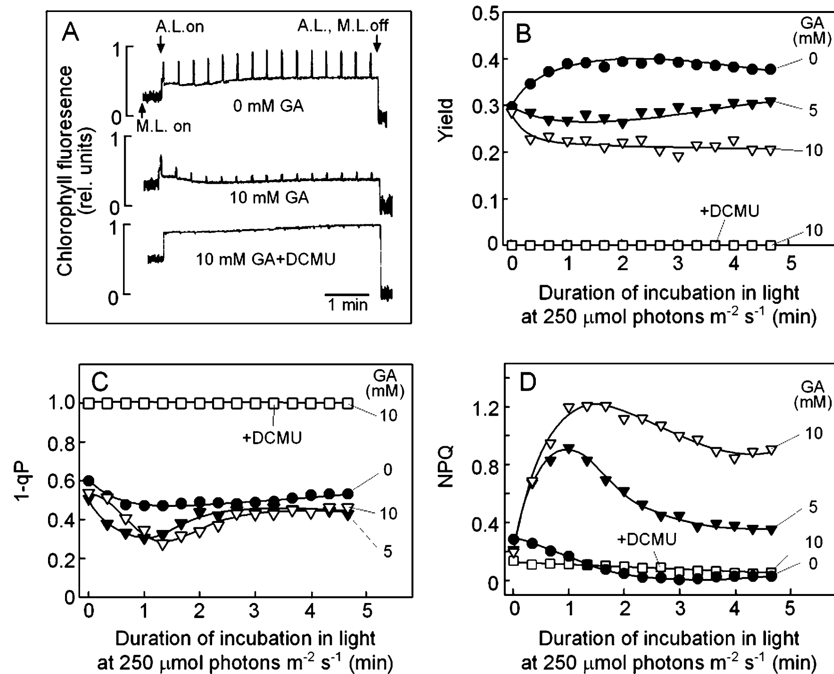

Fig. 2. Effects of glycolaldehyde on the kinetics of chlorophyll fluorescence in photoautotrophically grown wild-type cells of *C. reinhardtii*. Cells were grown in the minimum medium in light at 100 μmol photons m^{-2} s^{-1}. After the incubation of cells in darkness for 30 min with glycolaldehyde (GA), as indicated, the fluorescence of chlorophyll *a* was measured with a chlorophyll fluorometer in the pulse amplitude modulation mode in light at 250 μmol photons m^{-2} s^{-1} in the presence and absence of 20 μM DCMU. (A) Kinetics of chlorophyll fluorescence. Cells were exposed to a saturating pulse of light at 20-s intervals. A.L., Actinic light; M.L., measuring light. (B) Effective quantum yield of PSII (Yield). (C) Determination of $1 - qP$ (i.e., 1 minus photochemical quenching), which corresponds to the relative level of the reduced form of Q_A. (D) Non-photochemical quenching (NPQ), which corresponds to the nonradiative dissipation of excitation energy as heat. In panels B, C, and D, values are the averages of the results of three independent experiments.

to inhibit the transport of electrons from Q_A to Q_B, the value of $1 - qP$ was equal to one, indicating that Q_A was fully reduced under such conditions (Fig. 2C). By contrast, the presence of glycolaldehyde enhanced the extent of NPQ (non-photochemical quenching), which corresponds to the nonradiative dissipation of excitation energy as heat, and the effect of glycolaldehyde was dose dependent (Fig. 2D).

3.2. Effects of a missense mutation in the gene for the large subunit of Rubisco on photoinactivation of PSII

To confirm the contribution of interruption of the Calvin cycle to the photoinactivation of PSII, we examined the effect of a missense mutation in the gene for the large subunit of Rubisco in 10-6C cells, which reduces the CO_2-assimilating activity to 2% of the original level [21], on the photoinactivation of PSII. To circumvent the effects of the mutation on PSII activity during growth, we grew both wild-type and 10-6C cells photoheterotrophically in an acetate-containing TAP medium in dim light. The light-dependent activity of oxygen production of wild-type cells was 170 μmol O_2 mg Chl^{-1} h^{-1}, while that of 10-6C cells was 26 μmol O_2 mg Chl^{-1} h^{-1}. Glycolaldehyde at 5 mM decreased the light-dependent activity of oxygen production to 41% of the initial activity in wild-type cells, whereas glycolaldehyde had no significant effect on the activity in 10-6C cells (Table 1).

The PSII activity in 10-6C cells was similar to that in wild-type cells when the cells were grown photoheterotrophically (Fig. 3), in agreement with a previous report by Spreitzer and Mets [21]. Glycolaldehyde had no effect on PSII activity in both wild-type cells (Fig. 3A) and 10-6C cells (Fig. 3D) during incubation in darkness for 60 min. In light at 100 μmol photons m^{-2} s^{-1}, glycolaldehyde enhanced the extent of the photoinactivation of PSII in wild-type cells (Fig. 3B) but no further effect of glycolaldehyde was observed in the presence of chloramphenicol (Fig. 3C). This result was consistent with the results obtained with photoautotrophically grown wild-type cells (as shown in Fig. 1). PSII in photoheterotrophically grown cells was photoinactivated in light at 100 μmol photons m^{-2} s^{-1} (Fig. 3B), whereas that in photoautotrophically grown cells was not in light at 250 μmol photons m^{-2} s^{-1} (as shown in Fig. 1). We presume that this result was due to a difference between two kinds of cell in susceptibility to light. This is validated by the observation of Aro et al. [28] that low-light grown leaves were more sensitive to strong light than are high-light grown leaves.

In 10-6C cells, PSII was much more susceptible to photoinactivation than it was in wild-type cells, and neither glycolaldehyde nor chloramphenicol had any effect on the extent of photoinactivation of PSII (Figs. 3E and 3F). These results indicated that the mutation in the large subunit of Rubisco enhanced the extent of photoinactivation of PSII

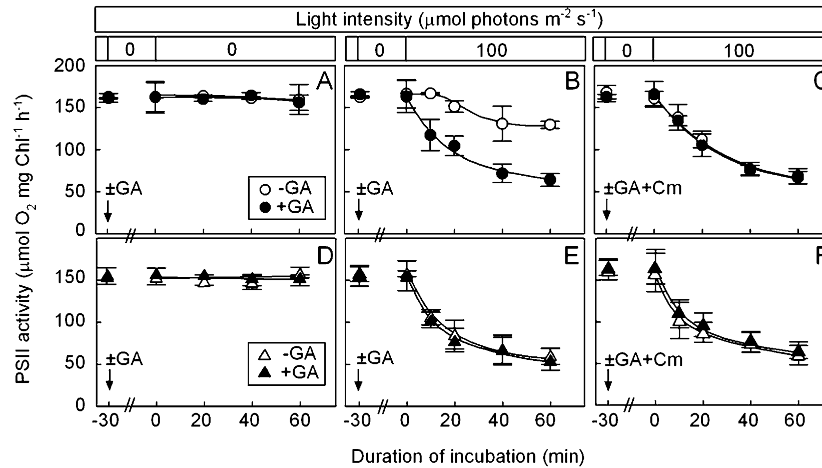

Fig. 3. Effects of a missense mutation in the gene for the large subunit of Rubisco on the photoinactivation of PSII in photoheterotrophically grown cells. Wild-type cells and 10-6C cells were grown in the acetate-containing TAP medium in light at 5 μmol photons m^{-2} s^{-1}. Wild-type cells (circles; A, B, and C) and 10-6C cells (triangles; D, E, and F) were incubated in darkness for 30 min in the presence or absence of 5 mM glycolaldehyde (GA), in the presence (C and F) or absence (A, B, D, and E) of 200 μg ml^{-1} chloramphenicol (Cm), and then they were incubated in darkness (A and D) or light (B, C, E, and F) at 100 μmol m^{-2} s^{-1} for designated periods of time. PSII activity was measured by monitoring the photosynthetic evolution of oxygen in the presence of 200 μM phenyl-*p*-benzoquinone. Values are means±S.D. (bars) of results from four independent experiments.

and the effect of the mutation was very similar to that of glycolaldehyde or chloramphenicol. Moreover, the results suggest that the interruption of the Calvin cycle might inhibit the protein synthesis-dependent repair of photodamaged PSII.

We examined that the inhibition of the repair of PSII upon the interruption of the Calvin cycle by monitoring the recovery of the PSII activity during the activity of PSII fell to approximately 20% of the initial activity by the incubation of wild-type and 10-6C cells for 30 min in light at 1000 and 750 μmol photons m^{-2} s^{-1}, respectively (Fig. 4). After the incubation of cells in darkness in the presence or absence of chloramphenicol, cells were exposed to weak light at 40 μmol photons m^{-2} s^{-1} to allow the recovery of PSII activity. In wild-type cells, in the absence of chloramphenicol, PSII activity returned to close to the initial level (Fig. 4A), whereas recovery was negligible and only transient in the presence of chloramphenicol (Fig. 4B). These results indicate that the recovery of PSII activity is attributable primarily to protein synthesis and only partially to protein synthesis-independent factors [29]. When wild-type cells were incubated in the presence of glycolaldehyde after photoinactivation, recovery was negligible, and only a transient increase in PSII activity was detected both in the presence (Fig. 4B) and absence (Fig. 4A) of chloramphenicol. Furthermore, the recovery of PSII activity was also only negligible and transient in 10-6C cells in the presence and absence of chloramphenicol (Fig. 4). The addition of glycolaldehyde to 10-6C cells did not produce any further effect on the recovery of PSII activity (Fig. 4). These results suggest that interruption of the Calvin cycle by glycolaldehyde or by a missense mutation in the large subunit of Rubisco inhibits the protein synthesis-dependent recovery of photoinactivated PSII as does chloramphenicol.

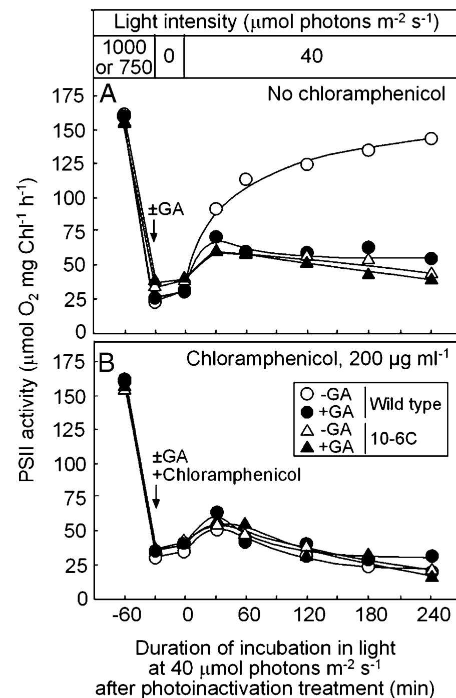

Fig. 4. Effect of glycolaldehyde and the missense mutation in the gene for the large subunit of Rubisco on the recovery of PSII activity after photoinactivation. Wild-type cells (circles) and 10-6C cells (triangles), which had been grown photoheterotrophically in the TAP medium in light at 5 μmol photons m^{-2} s^{-1}, were incubated for 30 min in light at 1000 μmol photons m^{-2} s^{-1} and 750 μmol photons m^{-2} s^{-1}, respectively, to photoinactivate PSII. Cells were then incubated in darkness for 30 min in the presence (closed symbols) or absence (open symbols) of 5 mM glycolaldehyde (GA), with further incubation in light at 40 μmol photons m^{-2} s^{-1}. (A) In the absence of chloramphenicol. (B) In the presence of 200 μg ml^{-1} chloramphenicol. Values are the average of results from two or three independent experiments.

3.3. Interruption of the Calvin cycle did not influence degradation of the D1 protein directly

To determine whether interruption of the Calvin cycle might influence the degradation of the D1 protein, which is the first step in the protein synthesis-dependent repair of photodamaged PSII, we monitored decreases in the level of D1 protein in the presence of chloramphenicol during incubation in light by Western blotting (Fig. 5). When wild-type cells were incubated in light at 100 μmol photons m^{-2} s^{-1}, the level of the D1 protein decreased gradually to less than 25% of the original level after incubation for 180 min. In 10-6C cells, the light-dependent decrease in the level of D1 protein was similar to that in wild-type cells. The presence of glycolaldehyde had no effect on changes in the level of D1 protein in wild-type cells and 10-6C cells. These results suggest that the degradation of photodamaged D1 protein in photodamaged PSII was unaffected by the interruption of the Calvin cycle.

3.4. Interruption of the Calvin cycle inhibited protein synthesis de novo

To investigate whether the inhibition of the synthesis of the D1 protein de novo occurs upon the interruption of the Calvin cycle, we monitored newly synthesized proteins by radioactive labeling. When wild-type cells were incubated in light with [^{35}S]sulfate in the presence of cycloheximide, which inhibits the expression of nuclear genes, the D1 protein was radiolabeled and the extent of labeling increased with the duration of incubation (Fig. 6A). Although we also observed labeling of D1 in the presence of glycolaldehyde, the extent of labeling was much lower than that in the absence of this inhibitor (Fig. 6A). In the absence of glycolaldehyde, the labeling of other plastid genome-encoded proteins that are found in PSII, such as CP47, CP43, and D2, was apparent after incubation for 60 min. However, these proteins were not labeled in the presence of glycolaldehyde even after incubation for 90 min (Fig. 6A). Fig. 6A also shows that, in 10-6C cells, the extent of labeling of the plastid genome-encoded proteins of PSII was similar to that in wild-type cells in the presence of glycolaldehyde.

The labeling of proteins in the absence of cycloheximide (i.e., both plastid- and nuclear-encoded proteins) was also suppressed by glycolaldehyde or the missense mutation in Rubisco (data not shown). These results suggest that the inhibitory effect of the interruption of the Calvin cycle on the synthesis of proteins is universal in plastid or nuclear genome. The prominent effect of glycolaldehyde on the synthesis of PSII proteins demonstrated in Fig. 6A is supposed to be a result of the rapid turnover of PSII proteins during incubation in light.

In order to examine whether glycolaldehyde directly inhibits the protein synthesis, we performed experiments of [^{35}S]sulfate labeling in 10-6C cells in the presence or absence of cycloheximide in light at 100 μmol photons m^{-2} s^{-1} (Fig. 6B). The result shows that glycolaldehyde did not affect the extents of the labeling of plastid and nuclear genome-encoded proteins after incubation in light for 60 min (Fig. 6B). These observations suggest that glycolaldehyde has no direct effect on protein synthesis.

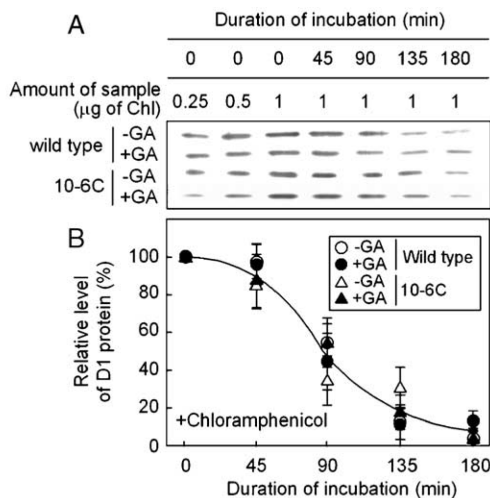

Fig. 5. Western blotting analysis of the effects of glycolaldehyde and the missense mutation in the gene for the large subunit of Rubisco on levels of the D1 protein. (A) Wild-type and 10-6C cells, which had been grown photoheterotrophically in TAP medium in light at 5 μmol photons m^{-2} s^{-1}, were incubated in darkness for 30 min in the presence of 200 μg ml^{-1} chloramphenicol in the presence (+GA) or absence (−GA) of 5 mM glycolaldehyde. Cells were exposed to light at 100 μmol photons m^{-2} s^{-1} for 0, 45, 90, 135, or 180 min. Total cell protein was fractionated by SDS-polyacrylamide gel electrophoresis. Immunoblotting analysis was performed with antibodies against the D1 protein from spinach leaves. To estimate changes in the level of proteins during incubation, the results of a dilution series (25% and 50%) of total cell proteins before incubation were also shown. (B) Quantified results. Values are means ± S.D. (bars) of results from three independent experiments.

3.5. Interruption of the Calvin cycle had no effects on levels of psbA and psbD transcripts

To determine whether the interruption of the Calvin cycle might inhibit the synthesis of chloroplast proteins at the transcriptional level, we examined the levels of transcripts of the *psbA* gene, which encodes the pre-D1, and the *psbD* gene, which encodes the D2 protein, by Northern blotting (Supplementary Fig. 1 in Appendix A). During incubation in light at 100 μmol photons m^{-2} s^{-1}, there were no changes in the level of 16S ribosomal RNA in the presence and absence of glycolaldehyde in wild-type cells and in 10-6C cells. When wild-type cells were incubated in light in the absence of glycolaldehyde, levels of *psbA* and *psbD* transcripts increased gradually with time. The presence of glycolaldehyde did not affect the levels of any of these transcripts. The levels of *psbA* and *psbD* transcripts in 10-6C cells were slightly higher than those in wild-type cells throughout the experiment. These results suggest that the inhibition of the light-dependent synthesis of the D1 and D2

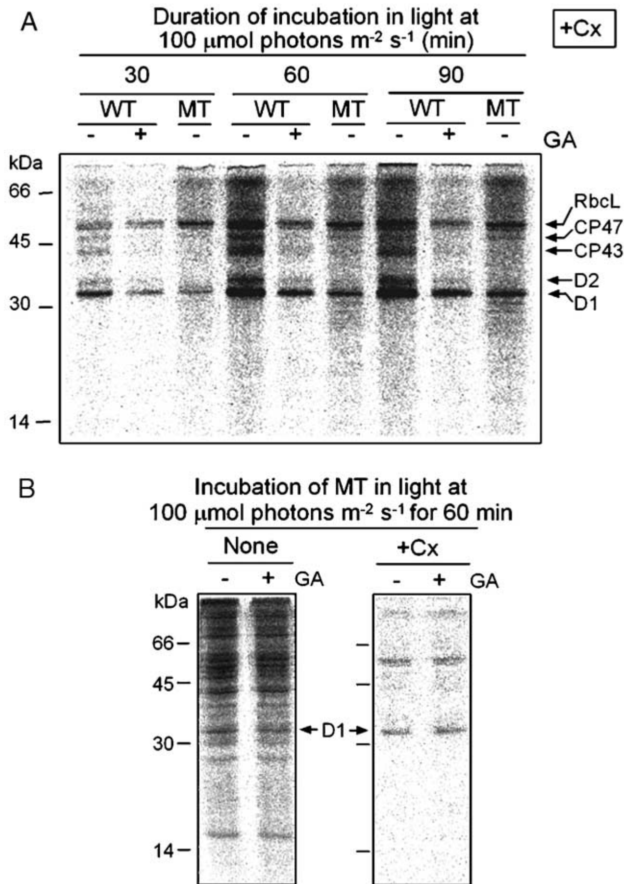

Fig. 6. Radioactive labeling analysis of the effects of glycolaldehyde and the missense mutation in the gene for the large subunit of Rubisco on the synthesis de novo of plastid-encoded proteins. Wild-type cells (WT) and 10-6C cells (MT), which had been grown photoheterotrophically in sulfate-reduced TAP medium in light at 5 μmol photons m^{-2} s^{-1}, were incubated in sulfate-depleted TAP medium in light at 5 μmol photons m^{-2} s^{-1} for 2 h. Newly synthesized proteins were labeled with [^{35}S]sulfate (10 μCi ml^{-1} as Na_2SO_4) in the presence (+) or absence (−) of 5 mM glycolaldehyde (GA). (A) Sulfate uptake was allowed to proceed in the presence of 10 μg ml^{-1} cycloheximide (Cx) in light at 100 μmol photons m^{-2} s^{-1} for the indicated times. (B) Sulfate uptake was allowed to proceed in the presence of 10 μg ml^{-1} cycloheximide (Cx) or in its absence (None) in light at 100 μmol photons m^{-2} s^{-1} for 60 min in 10-6C cells. Three independent experiments were performed, and essentially the same result was obtained in each case.

proteins by the interruption of the Calvin cycle (Fig. 6) is not attributable to decreases in the levels of the *psbA* and *psbD* transcripts.

4. Discussion

4.1. Interruption of the Calvin cycle does not directly accelerate photodamage to PSII but inhibits the repair of PSII

The present study demonstrated that interruption of the Calvin cycle neither enhanced the level of reduced Q_A (Fig. 2C) nor accelerated the photodamage to PSII directly, even though the extent of photoinactivation of PSII was enhanced (Figs. 1 and 3). These results suggest that a decrease in energy utilization caused by the interruption of the Calvin cycle does not induce the oxidative damage to D1 protein that was assumed to occur after the over-reduction of Q_A [8,9]. It should be noted that the photodamage to PSII is unaffected by oxidative stress in cyanobacteria [30,31]. The dissipation of absorbed light energy through NPQ [32] and the Mehler reaction [33,34], both of which are enhanced by the interruption of the Calvin cycle, might contribute to the predominance of Q_A in its oxidized form.

Fig. 4 demonstrates that the presence of glycolaldehyde and the missense mutation in Rubisco acted separately on the inhibition of the protein synthesis-dependent repair of PSII. The interruption of the Calvin cycle inhibited the synthesis of the D1 protein de novo during incubation in light (Fig. 6A), even though the level of the *psbA* transcript was unaffected (Supplementary Fig. 1 in Appendix A). These observations suggest that the interruption of the Calvin cycle inhibited the synthesis of the D1 protein at a post-transcriptional level and, thus, presumably, at the translational level in chloroplasts. The interruption of the Calvin cycle also inhibited the synthesis of other proteins that make up PSII, such as CP43, CP47, and D2 (Fig. 6A). These proteins are essential for the reassembly of D1 proteins during the repair of PSII, and the results suggest that not only the inhibition of the synthesis of D1 but also that of other proteins in PSII might be involved in the inhibition of the repair of PSII. It is unclear whether defects in the synthesis of nuclear genome-encoded proteins upon the interruption of the Calvin cycle might be involved in the disruption of the repair of PSII. However, it is likely that PSII activity can be, at least temperately, restored even when nuclear genome-encoded proteins are not synthesized [15].

4.2. How does the interruption of the Calvin cycle inhibit the synthesis of proteins in chloroplasts?

Wiese et al. [18] observed that the glycolaldehyde-dependent photoinactivation of PSII depends on the presence of molecular oxygen. The interruption of the Calvin cycle decreases the utilization of NADPH, with subsequent acceleration of the reduction of O_2 to H_2O_2 via O_2^- in PSI [33–35]. We showed previously that H_2O_2 inhibits the synthesis of proteins, in particular D1, during translational elongation in the repair cycle of PSII in the cyanobacterium *Synechocystis* sp. PCC 6803 [30]. A similar inhibitory effect of H_2O_2 on protein synthesis at the level of translation has been also demonstrated in rat liver [36] and in *Escherichia coli* [37]. It is likely that the inhibition of protein synthesis by H_2O_2 is universal in many organisms. Although H_2O_2 can be dissipated in chloroplasts by the water–water cycle or by direct reaction with antioxidants so that it remains at a low level, H_2O_2 might efficiently inhibit

the synthesis of chloroplast proteins de novo. Furthermore, the production of H_2O_2 in chloroplasts may potentially inhibit the synthesis of nuclear-encoded proteins because of its ready permeation of membranes [38].

There have been a number of evidences that environmental stress, such as high-temperature stress [39,40], low-temperature stress [16,39], and salt stress [41], inhibits the repair of photodamaged PSII. Environmental factors, such as temperature, water supply, salt stress, gas exchange (CO_2) stress, and pollutants, may potentially decrease the activity of the Calvin cycle directly in higher plants, algae, and cyanobacteria or indirectly via stomatal closure in higher plants [7,42]. Because the interruption of the Calvin cycle inhibited the repair of photodamaged PSII (Fig. 4), the susceptibility of the Calvin cycle to environmental stress might determine the tolerance of PSII to stress.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbabio.2005.04.003.

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