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Activation of a Reserve Pool of Photosystem II in *Chlamydomonas reinhardtii* Counteracts Photoinhibition[1]

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ABSTRACT

The effect of strong irradiance (2000 micromole photons per square meter per second) on PSII heterogeneity in intact cells of *Chlamydomonas reinhardtii* was investigated. Low light (LL, 15 micromole photons per square meter per second) grown *C. reinhardtii* are photoinhibited upon exposure to strong irradiance, and the loss of photosynthetic functioning is due to damage to PSII. Under physiological growth conditions, PSII is distributed into two pools. The large antenna size ($PSII_\alpha$) centers account for about 70% of all PSII in the thylakoid membrane and are responsible for plastoquinone reduction (Q_B-reducing centers). The smaller antenna ($PSII_\beta$) account for the remainder of PSII and exist in a state not yet able to photoreduce plastoquinone (Q_B-nonreducing centers). The exposure of *C. reinhardtii* cells to 60 minutes of strong irradiance disabled about half of the primary charge separation between P680 and pheophytin. The $PSII_\beta$ content remained the same or slightly increased during strong-irradiance treatment, whereas the photochemical activity of $PSII_\alpha$ decreased by 80%. Analysis of fluorescence induction transients displayed by intact cells indicated that strong irradiance led to a conversion of $PSII_\beta$ from a Q_B-nonreducing to a Q_B-reducing state. Parallel measurements of the rate of oxygen evolution revealed that photosynthetic electron transport was maintained at high rates, despite the loss of activity by a majority of $PSII_\alpha$. The results suggest that $PSII_\beta$ in *C. reinhardtii* may serve as a reserve pool of PSII that augments photosynthetic electron-transport rates during exposure to strong irradiance and partially compensates for the adverse effect of photoinhibition on $PSII_\alpha$.

Light energy drives photosynthesis but excess light is potentially damaging to the photosynthetic apparatus. The latter phenomenon is called photoinhibition and is manifested as a loss in chloroplast electron-transport capacity (for recent reviews see 38, 39). Though plants differ greatly in their sensitivity to photoinhibition, current evidence indicates widespread occurrence of photoinhibition in both terrestrial and aquatic environments (2, 33). In most cases, the loss of PSII function is responsible for decreased electron-transport activity, though damage to the PSI complex has also been reported (39).

Recent investigations from several laboratories have sought to establish the single or several sites of light-dependent damage within the PSII complex. One group of studies has focused on the primary electron-transport events within the PSII reaction center complex, *i.e.*

$$P680^* \text{ Pheo } Q_A \rightarrow P680^+\text{Pheo}^- Q_A \rightarrow P680^+\text{Pheo } Q_A^- \quad (1)$$

When isolated spinach thylakoids were illuminated with strong-irradiance (2500 $\mu mol \cdot m^{-2} \cdot s^{-1}$) at 0°C, both the Q_A[2] (320 nm absorbance change) and Pheo (685 nm absorbance change) signal were lowered in parallel (8, 9, 12), suggesting inhibition of primary photochemistry. The PSII primary charge separation was also inhibited when intact cells of the green alga, *Chlamydomonas reinhardtii*, were exposed to strong irradiance (12). The rapid loss of the pheophytin photoreduction during photoinhibition has also been detected using EPR difference spectroscopy (46). These results indicated that photoinhibitory damage affected the ability of PSII to form the $P680^+$ $Pheo^-$ charge separation.

An alternative proposal (22, 37) is that photoinhibition results from light-dependent damage to the plastoquinone binding site, which is located on the 32 kD 'D1' polypeptide of PSII. According to the latter model, only electron transfer from Q_A to the bound plastoquinone, Q_B, is disrupted, whereas no damage occurs to the water splitting enzyme or the reaction center. Support for this proposal was found in the rapid *in vivo* turnover of D1 in *C. reinhardtii* (22, 37). Subsequently, it has been accepted that the functional components of the PSII reaction center (Mn, Z, P_{680}, Pheo, Q_A and Q_B) are all bound to the D1/D2 heterodimer in analogy with the structure of the crystallized bacterial reaction center (32, 44). In the context of the latter model of PSII, enhanced turnover of D1 would be a likely consequence of processes that repair a damaged PSII reaction center (3, 28). The precise events leading to the loss of PSII primary charge separation remain to be resolved (36, 43).

There is considerable experimental support for the concept of heterogeneity among PSII reaction centers both in the functional antenna size and electron-transport properties on the reducing side of Q_A (5). The concept of PSII heterogeneity was originally introduced to explain the biphasic nature of PSII activity, measured either by fluorescence induction ki-

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[2] Abbreviations: Q_A, primary quinone electron acceptor of PSII; F_o, nonvariable fluorescence yield; F_{pl}, initial plateau of fluorescence yield in the fluorescence induction curve; F_p, peak fluorescence in the fluorescence induction curve; F_m, maximum fluorescence; F_v, variable fluorescence = $F_m - F_o$; P680, reaction center of PSII; Pheo, pheophytin; Z, secondary PSII donor; Q_B secondary quinone electron acceptor of PSII; LHC, light-harvesting complex; PS II-Q_B-nonreducing, PSII center with impaired Q_A-Q_B interaction.

netics or by the reduction of the primary quinone, Q_A (31). The biphasic nature was suggested to be due to the presence of two distinct populations of PSII, namely $PSII_\alpha$ and $PSII_\beta$. Their different kinetics were explained by different sizes of the light harvesting antenna (31). In higher plant chloroplasts, $PSII_\alpha$ accounts for 75 to 80% of PSII centers, contains about 250 Chl (*a* and *b*) molecules in its antenna, and is located in the grana region of the thylakoid membrane, while $PSII_\beta$ accounts for the remaining 20 to 25%, has a smaller antenna size of only about 120 Chl molecules, and is located in the stroma-exposed regions of the thylakoid membrane (1, 30). In *C. reinhardtii* grown at moderate irradiances, the $PSII_\beta$ comprises about 45% of total PSII content (34).

Another type of PSII heterogeneity has been reported, *i.e.* the PSII reducing side heterogeneity (24, 25, 29, 45). This type of heterogeneity was identified by the inability of a small fraction of PSII centers to transfer electrons from Q_A to Q_B. These PSII centers have been termed Q_B-nonreducing and are normally inactive in the process of plastoquinone reduction (7, 15, 24, 25, 29). One convenient method to quantify these Q_B-nonreducing centers is from the PSII fluorescence induction kinetics measured with thylakoid membranes in the absence of the PSII inhibitor DCMU (7, 29). Because Q_B-nonreducing centers are unable to transfer electrons from Q_A^- to Q_B, electrons accumulate on Q_A promptly upon illumination and this is reflected in the initial fluorescence yield increase from F_o to $F_{p'}$ (13). In mature chloroplasts, the kinetics of the Q_B-nonreducing centers are identical to those of $PSII_\beta$ (7, 29), suggesting that there is a strong overlap between Q_B-nonreducing centers and $PSII_\beta$.

In the present study, the dynamics of PSII heterogeneity of low-light (LL) grown *C. reinhardtii* were examined during exposure of cells to strong irradiance. The loss of PSII reaction center activity was monitored using absorbance change measurements at 685 nm (Pheo photoreduction). In addition, we investigated the effect of strong-irradiance exposure on the pool size of Q_B-nonreducing the Q_B-reducing forms estimated from fluorescence kinetics. These changes were correlated with the properties of light-saturated oxygen-evolution in *C. reinhardtii*. The results suggest that under certain stress conditions, Q_B-nonreducing centers may be converted to a Q_B-reducing form and, thus, become active in the process of plastoquinone reduction. At the same time, strong-irradiance exposure leads to a loss of $PSII_\alpha$ primary charge separation activity. The 'activation' of $PSII_\beta$ appears to counter the loss of a major fraction of PSII primary charge separation due to photoinhibition.

MATERIALS AND METHODS

Chlamydomonas reinhardtii strain CC-124 (mt-) (Duke University culture collection) was grown in an ammonium/phosphate/trace-metal media which was continuously stirred and bubbled with 3% CO_2. Experimental cultures were grown in 1 L culture flasks which had 5 cm pathlength in the direction of illumination from a warm white fluorescent light source. Neutral density screens were used to obtain a low-light growth intensity (LL) of 15 μmol m^{-2} s^{-1}. Cultures were used in midlog phase (0.5 -1.0×10^6 cells mL^{-1}). Growth rate was ca. 0.5 d^{-1} under these conditions (25°C).

Strong-irradiance treatments were administered using a halogen light source with a 15 cm thick water heat filter at an intensity of 2000 μmol m^{-2} s^{-1}. Before exposure, cells were concentrated by settling for 30 min, after which supernatant was removed in sufficient quantity to obtain an approximate 10-fold higher cell concentration. An aliquot of 10 mL from the concentrated cell suspension was then exposed to strong irradiance, during which cells were vigorously stirred with a continuous stream of 3% CO_2 in air. In other treatments, a larger light source and incubation chamber was used so that preconcentration of cells was not necessary. Similar results were obtained with either set up. Temperature was maintained at 25°C using a water bath.

A polarographic, Clark-type electrode was used to measure O_2 evolution. Illumination was provided by a halogen projector lamp. The incident intensity to the sample was varied by neutral density filters. After the treatment period, cells were either used directly for measurements of oxygen evolution or prepared for other assays as described below. Before transfer to the chamber of the oxygen electrode, cells were resuspended in fresh growth media (kept under 3% CO_2 in air) to the original culture density. A circulating water bath (Lauda LM6) was used to maintain both media and electrode chamber at the culture temperature (25°C). Initial O_2 concentration was lowered to 20% saturation by bubbling with N_2 for approximately 20 s. The rate of O_2 evolution was then measured under an irradiance of 1500 μmol m^{-2} s^{-1} (HL) for a period of 3 min, followed by a transition to 230 μmol m^{-2} s^{-1} (ML) in which the rate of O_2 evolution was again monitored for a period of 3 min. Gross O_2 evolution rates were computed after correction for the subsequently determined rate of dark O_2 uptake. The rationale for the O_2 evolution measurement is discussed further below.

The *in vivo* fluorescence of intact *C. reinhardtii* cells was induced with green light defined by a Corning CS 4-96 and CS 3-69 filter. Control cells maintained in LL growth conditions were dark adapted 20 s before measurement. Additional dark adaptation (5 min) was used after cells received strong irradiance exposure prior to fluorescence measurements. The fluorescence probing illumination had a flux of 24 to 100 μmol m^{-2} s^{-1} as indicated.

Material for the determination of PSII content was rapidly cooled to 0°C in an ice-brine mixture, pelleted at 5000*g* for 5 min and the pellets were kept frozen at −80°C until analysis. Frozen cells were resuspended in a hypotonic buffer containing 20 mM Tris-HCl (pH 7.8), 35 mM NaCl, and 2 mM $MgCl_2$. Broken thylakoids were obtained by sonicating the cells in ice for 1 min pulsed mode (50% duty cycle, power = 5, Branson sonifier model 200). The concentration of the primary electron acceptor, Pheo, of PSII was determined from the light-minus-dark absorbance difference change at 685 nm (ΔA_{685}) as described (12, 21). Thylakoids were diluted with additional hypotonic buffer to a Chl $a + b$ concentration of about 10 μM and 2 μM methyl viologen, 2 μM indigodisulphonate, 2 mM $MnCl_2$, 0.05% (v/v) Triton X-100. Sufficient dithionite to lower the redox potential to −490 mV was added to the reaction mixture just before measurement. The Pheo photoreduction was induced by blue (Corning CS 4-96) excitation light at an intensity of 600 μmol m^{-2} s^{-1}. The measuring

beam had a half-bandwidth of 1 nm. The absorbance difference measurements were corrected for the effect of particle flattening (40), and an extinction coefficient of 65 $mM^{-1} \cdot cm^{-1}$ was applied (12). Under these conditions, the concentration of PSII is equal to that measured by the quantitation of Q_A (light-minus-dark absorbance change at 320 nm) (12, 18).

The fluorescence induction curve of PSII was measured using thylakoids freshly isolated from *C. reinhardtii* as previously described (34). Fluorescence was induced using broad band green light transmitted by a combination of Corning CS 4-96 and CS 3-69 filters at an intensity of 25 μmol m^{-2} s^{-1}. The proportion of $PSII_\beta$ was determined from kinetic analysis of the area growth over the fluorescence curve measured in the presence of DCMU (20 μM) as previously described (29, 30).

Thylakoid membrane proteins were resolved by SDS-PAGE using the discontinuous buffer system of Laemmli (23) modified as follows. The reservoir buffer contained 0.025 M Tris and 0.25 M glycine (pH 8.3), the pH of the stacking buffer and resolving buffer was lowered to 6.7 and 8.7, respectively. Polyacrylamide slabs (1.5 mm × 16 cm) were prepared using a 5% stacking gel and a 12.5 to 22% linear gradient resolving gel. The samples were solubilized in 100 mM Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 3% β-mercaptoethanol, and incubated at 60°C for 15 min. The gels were loaded on an equal Chl basis (Chl *a*+*b* of 12 nmol) and electrophoresis was performed with a constant current of 16 mA for 16 h at 20°C.

The LHC-II and D1 proteins of the *C. reinhardtii* thylakoids resolved by SDS-PAGE were identified by immunoblotting (Western blot analysis). Electrophoretic transfer to nitrocellulose and subsequent incubations with rabbit antibodies to maize LHC-II (gift of Dr. R. Malkin) and D1 (gift of Dr. G. Schuster) and with alkaline-phosphate conjugated goat-anti-rabbit antibody was performed as in (19). Color development of the blots was performed as described previously (11).

RESULTS

Loss of Primary Charge Separation Activity during Strong-Irradiance Treatment

The absorbance change at 685 nm due to Pheo photoreduction was used to quantitate the PSII reaction centers active in primary charge separation in *Chlamydomonas reinhardtii* thylakoid membranes. Control LL-grown cells contained an average of 2.0 mmol of photochemically competent PSII per mol Chl *a*+*b* (Chl:Pheo = 500). The LL-grown cells were sensitive to photoinhibition. After 60 min exposure to strong irradiance less than half of the PSII reaction centers were able to form a stable primary charge separation (Fig. 1). The decrease in photoreducible Pheo content occurred continuously during strong-irradiance treatment (Fig. 2).

The Pheo measurements defined the decrease in total capacity for PSII primary photochemistry; however, additional information was sought on the relative sensitivity of $PSII_\alpha$ and $PSII_\beta$ to strong-irradiance treatment. The kinetics of PSII photochemistry were defined from the fluorescence induction curve of isolated thylakoid membranes in the presence of DCMU. A comparison of the curves observed for control and photoinhibited thylakoids (30 min treatment) is given in Figure 3A. After strong-irradiance treatment, F_m of thylakoid membranes was dramatically lowered, but F_o was only slightly lower (cf. 8–10). The kinetics of the area increase over the fluorescence induction curve of thylakoid membranes from control and strong-irradiance treated cells were analyzed on semi-logarithmic plots (Fig. 3B). The proportion of $PSII_\beta$ in LL-grown control *C. reinhardtii* averaged 30% of the total PSII in the thylakoid membrane (intercept of the slow phase with the ordinate at zero time in Fig. 3B [31]). The strong-

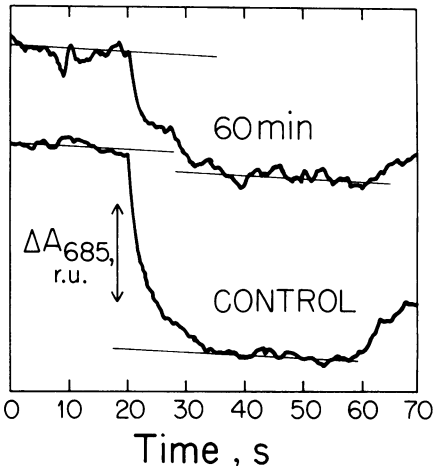

Figure 1. Light-induced absorbance change at 685 nm in isolated *C. reinhardtii* thylakoids attributed to the photoreduction of the primary PSII acceptor, Pheo. Thylakoids (10 μM Chl *a* + *b*) were suspended in the presence of 2 mM $MnCl_2$, 2 μM methyl viologen, 2 μM indigodisulfonate, and 0.05% (v/v) Triton X-100. The redox potential of the degassed suspension was adjusted to −490 mV with sodium dithionite. Blue actinic illumination of 600 μmol $m^{-2}s^{-1}$ defined by a Corning CS 4-96 was turned 'on' at time = 20 s, and turned 'off' at time = 60 s. The upper trace is from thylakoids isolated from cells of *C. reinhardtii* after 60 min exposure to strong irradiance. The lower trace is from thylakoids isolated from control cells of *C. reinhardtii*.

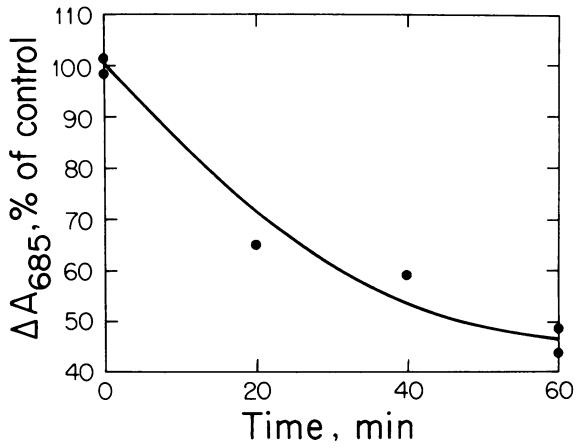

Figure 2. Amplitude of the absorbance change at 685 nm (ΔA_{685}) as a function of strong-irradiance exposure of *C. reinhardtii* cells. The amplitude of the absorbance change of control cells (see Fig. 1) is taken as 100%. Treated cells were incubated for up to 60 min at 2000 μmol m^{-2} s^{-1}. Each point represents the average of 4 to 6 exposures (*e.g.* Fig. 1) of one preparation.

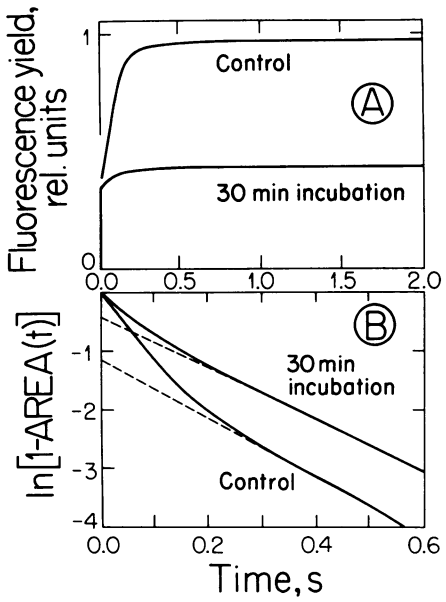

Figure 3. Fluorescence induction kinetics of *C. reinhardtii*. A, Time course of fluorescence induction by weak green actinic light (25 μmol m^{-2} s^{-1}) in the presence of DCMU (20 μM), for thylakoid membranes isolated from either control cells of *C. reinhardtii*, or from cells after 30 min incubation at 2000 μmol m^{-2} s^{-1} strong irradiance; B, semi-logarithmic kinetic analysis of the area growth over the fluorescence induction curve [area (t)] for thylakoids isolated from control cells or from cells incubated for 30 min under strong irradiance. The slope of the slow (exponential) component associated with $PSII_\beta$ is indicated by a dashed line. The extrapolation of this line to the zero time intercept indicates the relative (ln of the) proportion of $PSII_\beta$. The intercept for the 30 min incubated cells is closer to origin, indicating a significantly larger relative proportion of $PSII_\beta$ after strong-irradiance incubation.

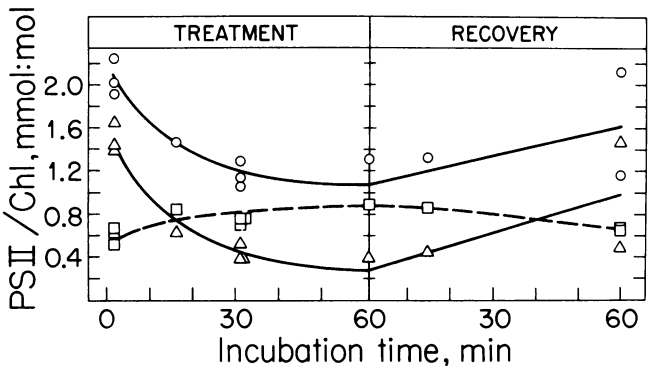

Figure 4. Strong-irradiance effects on absolute pool sizes of total PSII (○), $PSII_\alpha$ (△), and $PSII_\beta$ (□) in the thylakoid membrane of *C. reinhardtii*. Cells were incubated for up to 60 min under strong-irradiance and then returned to growth irradiance for recovery. The total PSII active in primary charge separation was estimated from light-induced absorbance change measurements at 685 nm (*e.g.* Fig. 1). The $PSII_\alpha$ and $PSII_\beta$ components were estimated using the kinetic analysis of the fluorescence induction curve (*e.g.* Fig. 3). Each point represents a separate thylakoid preparation with independent absorbance change and fluorescence induction measurements.

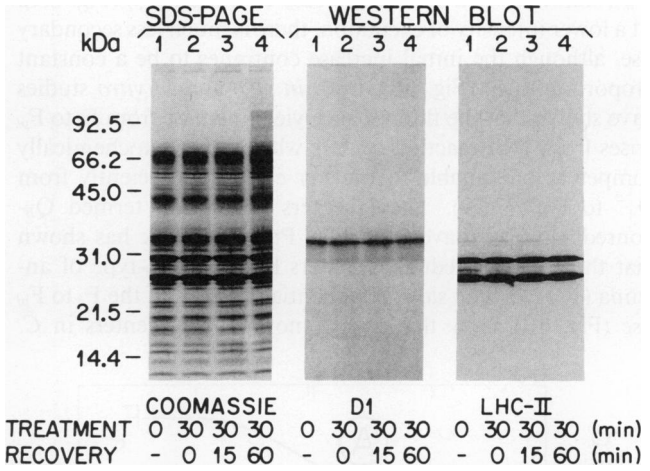

Figure 5. Polyacrylamide gel electrophoresis and immunoblot analysis of thylakoid membrane proteins from *C. reinhardtii*. Left panel, thylakoid membrane proteins were resolved by electrophoresis (SDS-PAGE) and stained with Coomassie brilliant blue. Thylakoid membranes were loaded on an equal Chl basis, with a total of 12 nmol of Chl *a* + *b* per lane. Lane 1, control cells; lane 2, cells exposed to 30 min strong irradiance; lane 3, cells exposed to 30 min strong irradiance followed by 15 min recovery at growth irradiance; lane 4, as for lane 3 but 60 min recovery. Center panel, SDS-PAGE prepared and samples loaded as for left panel, followed by electrophoretic transfer to nitrocellulose and immunological detection of the D1 polypeptide (Western blot). Right panel, SDS-PAGE prepared and samples loaded as for left panel, followed by Western Blot for the PSII light harvesting Chl binding polypeptides (LHC-II).

irradiance treatment resulted in a more than doubling in the relative proportion of $PSII_\beta$, which then accounted for the majority of the fluorescence emitting PSII (Fig. 3B). To quantitate the effect of strong-irradiance treatment on the pool sizes of $PSII_\alpha$ and $PSII_\beta$, the relative proportion of each PSII was applied to the total functional PSII measured by Pheo photoreduction in each membrane preparation (Fig. 4). This analysis showed that $PSII_\alpha$ was rapidly photoinhibited by strong irradiance, whereas the $PSII_\beta$ content remained more or less constant. Such results are consistent with previous measurements of strong irradiance treated spinach thylakoids (9, 27), and show that photoinhibition damage is directed mainly at $PSII_\alpha$.

Thylakoid membrane proteins of control and strong-irradiance treated *C. reinhardtii* were analyzed using SDS-PAGE and immunoblot techniques in order to test whether any significant amount of protein degradation occurred during strong-irradiance exposure. Of particular interest was the thylakoid membrane content of the D1 protein, the degradation of which has been correlated with the loss of PSII activity during strong-irradiance exposure (37). In the analysis of the thylakoid membranes of the *C. reinhardtii* used in this study, the proteins displayed the same electrophoretic pattern and the same staining intensity (loaded on equal Chl basis) independent of treatment (Fig. 5, left panel). In particular, there was no observable change in the protein bands in the 25 to 34 kD region where the D1 and PSII light harvesting Chl-binding (LHC-II) polypeptides are expected to be found (Fig. 5, left panel). The content of D1 and LHC-II polypeptides

was detected by probing with specific antibodies. Again, the amounts of the D1 polypeptide in the thylakoid membrane was independent of strong-irradiance treatment as evidenced by the equal amounts of antibody binding to D1 (Fig. 5, center panel). Thylakoids that were isolated from strong-irradiance treated cells had about half the primary charge separation activity of control cells (*cf.* Fig. 4). Moreover, no evidence of a net change in D1 was found over the recovery period (Fig. 5). The content of the PSII light harvesting polypeptides in the thylakoid membrane also did not change during strong-irradiance treatment and subsequent recovery (Fig. 5, right panel). Thus, photoinhibition occurs without any net loss in antigenically competent D1 or LHC-II polypeptides. Similar conclusions have been drawn by other investigators (42, 46).

Strong-Irradiance Effects on PSII Reducing Side Heterogeneity

The fluorescence induction curve of intact cells of *C. reinhardtii* in the absence of PSII herbicides (Fig. 6) resembles that of other green algae and higher plants. Fluorescence increases from a nonvariable yield (F_o) to an initial plateau (F_{pl}), followed by a second increase to a peak (F_p) (Fig. 6A). At a lower intensity of excitation, there is much less secondary rise, although the initial increase continues to be a constant proportion of F_o (Fig. 6B). Both *in vivo* and *in vitro* studies have shown that the fluorescence yield increase from F_o to F_{pl} arises from PSII reaction centers which are photochemically competent but unable to transfer electrons efficiently from Q_A^- to Q_B (7, 29). These centers have been termed Q_B-nonreducing (*cf.* Lavergne [24]). Previous work has shown that these Q_B-nonreducing centers have a $PSII_\beta$-type of antenna (18, 29). The slow, exponential kinetics of the F_o to F_{pl} rise (Fig. 6B) show that the Q_B-nonreducing centers in *C. reinhardtii* also have the characteristics of $PSII_\beta$. The increase of fluorescence emission from the PQ-reducing PSII centers (Q_B-reducing) is delayed until reduction of the plastoquinone pool, upon which electrons accumulate on Q_A and fluorescence undergoes a further increase from F_{pl} to F_p. Total variable fluorescence, $F_v = F_m - F_o$ was calculated using F_m measured in the presence of 10 μM DCMU (Fig. 3). The fluorescence emitted from the Q_B-nonreducing centers was thus calculated to be about 15% of total variable fluorescence.

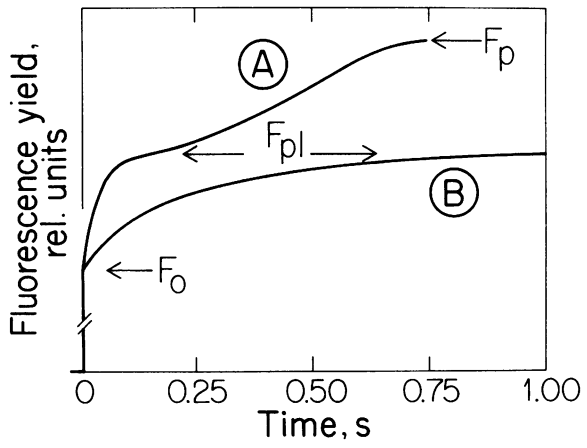

Figure 6. Fluorescence induction kinetics of intact *C. reinhardtii* cells suspended in the absence of PSII herbicides. The actinic light came on at zero time. A, Actinic irradiance was 96 μmol m^{-2} s^{-1} green light which induced the F_o, F_{pl}, to F_p sequence; (B) actinic irradiance was 24 μmol m^{-2} s^{-1} so that only F_o to F_{pl} amplitude is induced. Curve (B) is plotted with an ordinate scale factor of 4 to compensate for the lower actinic excitation. Note that the same F_o to F_{pl} fluorescence yield increase occurs in both (A) and (B).

The amplitude of the exponential fluorescence increase from F_o to F_{pl} was lowered upon strong-irradiance treatment, suggesting that the relative concentration of Q_B-nonreducing PSII centers was lowered (Fig. 7). The lowering occurred rapidly, only 20% of the exponential amplitude remained after 15 min of strong-irradiance exposure (Fig. 7). The drop in the F_o to F_{pl} yield was confirmed by independent measurements of the F_o to F_{pl} amplitude with thylakoid membranes isolated from control and strong-irradiance treated cells (data not shown). As was noted above (Fig. 4), such lowering in the amplitude of the initial fluorescence yield increase should not be attributed to photoinhibition of $PSII_\beta$, which is resistant to damage (9, 27). The photoinhibition rate of $PSII_\beta$ has been reported to be several times slower than the rate at which $PSII_\alpha$ is damaged (8), and much slower than the rate of lowering of the F_{pl} amplitude. Instead, the lowering may be a manifestation of the conversion of Q_B-nonreducing centers into a Q_B-reducing state (see also below). The F_o to F_{pl} amplitude recovered very slowly when strong-irradiance treated cells were returned to normal growth irradiance (Fig. 7), suggesting that $PSII_\beta$ did not revert to a Q_B-nonreducing status. Similar lowering in F_o to F_{pl} amplitude has been observed in other species of algae and higher plants upon strong-irradiance exposure, but has not been attributed to changes in the functional status of PSII Q_B-nonreducing centers (4, 10, 47).

Strong-Irradiance Effects on Photosynthetic Rates

Only Q_B-reducing centers participate in steady-state photosynthetic electron-transport (oxygen evolution) *in vivo*,

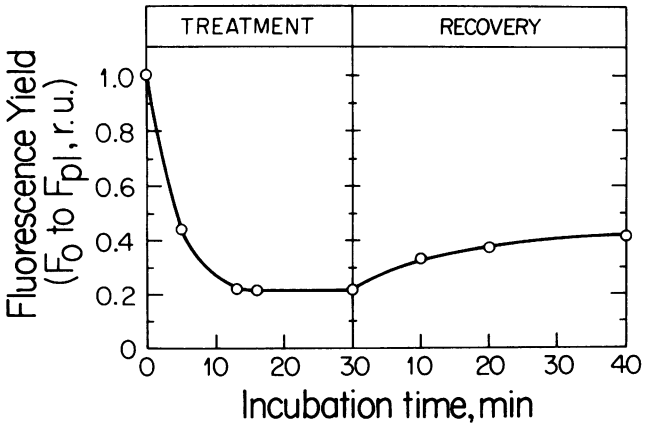

Figure 7. Amplitude of the fluorescence yield increase from F_o to F_{pl} (pool size of PSII Q_B-nonreducing) measured on samples taken at various times during a 30 min strong-irradiance incubation and subsequent 'recovery' at normal growth irradiance. The lowered F_o to F_{pl} amplitude after strong-irradiance exposure suggests an activation of $PSII_\beta$ (Q_B-nonreducing) in electron transfer from Q_A to Q_B.

while Q_B-nonreducing centers are not involved in oxygen evolution (15, 24). Hence, upon photoinhibition, it is expected that rates of oxygen evolution should decrease in proportion with the loss of $PSII_\alpha$ photochemical activity. We tested whether such a relationship was observed during photoinhibition of intact cells. This was accomplished by comparing the extent of photoinhibition of $PSII_\alpha$ with the rates of oxygen evolution measured under both medium and high actinic excitation. Photosynthetic O_2 evolution was measured for 2 to 3 min under intermediate-light (ML; 230 μmol m^{-2} s^{-1}) which saturates electron-transport through $PSII_\alpha$ in LL grown cells (34). Short-term measurements at a much higher light intensity (HL; 1500 μmol m^{-2} s^{-1}) were used to gauge electron flow through both $PSII_\alpha$ and $PSII_\beta$. This HL intensity is more than six times greater than the ML to ensure that any electron transport by the small antenna $PSII_\beta$ would be saturated.

It was established that prior to strong-irradiance treatment, the rate of photosynthesis (oxygen evolution) under HL exceeded that under ML by less than 10% (see legend, Fig. 8). This observation confirmed that, in control cells, $PSII_\beta$ contributed little to the process of H_2O oxidation and plastoquinone photoreduction. Figure 8 shows that, following a strong irradiance treatment, there was a loss in oxygen evolution capacity (photoinhibition). After 60 min of strong irradiance only 40% of the initial activity, as measured by ML, had remained (Fig. 8, open circles). However, the extent of photoinhibition of $PSII_\alpha$ was consistently greater than that of ML oxygen evolution (Fig. 8, triangles). After 60 min of strong-irradiance exposure, only 20% of active $PSII_\alpha$ had remained. In contrast, the inhibition of photosynthesis was much less when measured by HL oxygen evolution than when measured by ML oxygen evolution (Fig. 8, solid circles). After 60 min

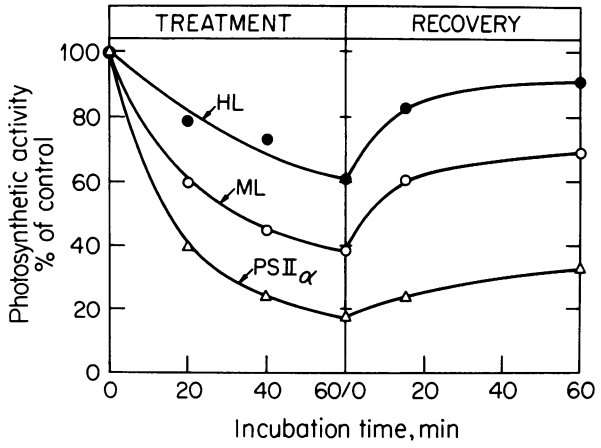

Figure 8. Rates of photosynthetic activity (O_2 evolution) and concentration of photochemically active $PSII_\alpha$ centers as a function of strong-irradiance treatment and subsequent recovery. All quantities are plotted relative to control values = 100% (△), ($PSII_\alpha$) the $PSII_\alpha$ activity as estimated from photoreducible Pheo and proportion of $PSII_\alpha$ in the fluorescence induction curve (*cf.* Fig. 2 and Fig. 4). (○), (ML) rate of oxygen evolution at 230 μmol m^{-2} s^{-1}, control cells had an average rate of 675 fmol O_2 $cell^{-1}$ h^{-1}. (●), (HL) rate of oxygen evolution at 1500 μmol m^{-2} s^{-1}, control cells had an average rate at HL of 714 fmol O_2 $cell^{-1}$ h^{-1}.

in strong irradiance, HL activity decreased only to 63% of the initial activity. No change occurred in the Chl content per cell (about 5 fmol Chl $a+b$ $cell^{-1}$) during the strong-irradiance incubation. These results suggest that the net loss of $PSII_\alpha$ activity due to photoinhibition was partially compensated through conversion of a portion of the $PSII_\beta$ pool from a Q_B-nonreducing to Q_B-reducing status. Because of such conversion, the decrease of oxygen evolution rate in HL was limited to about half of the loss of $PSII_\alpha$ activity.

Recovery from Photoinhibition

Recovery from photoinhibition of photosynthesis in *C. reinhardtii* (26, 37) and other plants (16) has been reported to be completely dependent on chloroplast protein synthesis. This protein synthesis is presumably directed toward the apoproteins of the PSII reaction center (37, 42). However, the possibility remained that conversion of $PSII_\beta$ to a Q_B-reducing state could enhance recovery rates. In this regard, short-term O_2 evolution measurements were made immediately following 60 min strong-irradiance treatment, or after 60 min strong irradiance followed by 10 to 60 min incubation in low growth irradiance (15 μmol m^{-2} s^{-1}). The change in rates was compared to relative changes in concentration of $PSII_\alpha$ (Fig. 8, right panel). The recovery of $PSII_\alpha$ activity was slow during incubation of photoinhibited cells in the LL growth irradiance (*cf.* 41). However, we observed a fast phase of recovery during which the increase in the rates of ML and HL oxygen evolution exceeded the increase in total $PSII_\alpha$ primary charge separation activity (Fig. 8). The short-term (0–10 min) increase in O_2 evolution rate was not inhibited by addition of the chloroplast protein translation inhibitors chloramphenicol (200 μg mL^{-1}) or lincomycin (20 μg mL^{-1}) (data not shown). Both ML and HL O_2 evolution rates increased, and a large difference remained between photosynthetic rates at these two irradiances. Such an increase in electron-transport rates without a corresponding increase in number of $PSII_\alpha$ able to carry out a primary charge separation could be accounted for by continuing conversion of $PSII_\beta$ to a Q_B-reducing status, at least in the initial period following strong-irradiance exposure. This fast phase of recovery may have restored some photosynthetic capacity. However, full restoration of PSII charge separation (which probably involves turnover of component PSII polypeptides is required before photosynthetic rates recover completely [28]).

DISCUSSION

The quantitation of PSII primary charge separation, as measured by pheophytin photoreduction, is a specific assay for the amount of damage that occurs during photoinhibition. Measurements of pheophytin photoreduction in strong-irradiance treated *Chlamydomonas reinhardtii* are consistent with previous findings of loss of PSII primary photochemistry upon photoinhibition (6, 9, 12, 35, 46). Also in agreement with earlier studies (9, 27), we found that photoinhibition adversely affected $PSII_\alpha$ whereas $PSII_\beta$ appeared resistant to damage (Fig. 4). The maintenance of $PSII_\beta$ primary charge separation activity during strong-irradiance treatment combined with the simultaneous decrease in the amplitude of the

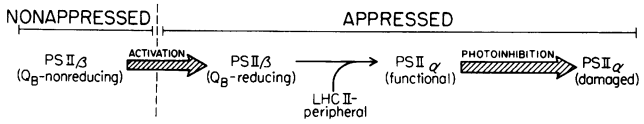

Figure 9. A schematic of part of a proposed PSII repair cycle, adapted from Guenther and Melis (17). The wide, hatched arrows indicate those steps emphasized during strong-irradiance exposure.

F_o to $F_{p/}$ amplitude suggests that after strong-irradiance treatment a large proportion of $PSII_\beta$ are beginning to contribute to the electron-transport process.

The quantitative contribution of $PSII_\beta$ can be estimated by comparing the size of each oxygen-evolving PSII pool before and after strong-irradiance treatment. The variation in oxygen evolution rates, relative to the activity of $PSII_\alpha$ centers, provides one way of estimating the changes in PSII pool sizes. Strong-irradiance exposure of 60 min lead to a decline in Q_B-reducing centers to 63% of the initial activity. However, $PSII_\alpha$ declined to about 20% of the activity in the control. The remaining Q_B-reducing activity is attributed to centers with the smaller $PSII_\beta$ configuration. We suggest that these $PSII_\beta$ Q_B-reducing centers originated from Q_B-nonreducing centers upon conversion to a Q_B-reducing form. We estimate that after exposure to strong-irradiance up to 77% of $PSII_\beta$-Q_B-nonreducing was converted to $PSII_\beta$-Q_B-reducing. This estimate is based on the calculation of how many centers would have been activated to a Q_B-reducing state in order to maintain the observed rates of light-saturated oxygen evolution (Appendix).

Thus, it appears that as a result of the strong-irradiance treatment, and in spite of substantial activity loss by $PSII_\alpha$ (photoinhibition), a new set of PSII centers is now engaged in O_2 evolution. The exact nature of the mechanism and regulation of the conversion of $PSII_\beta$-Q_B-nonreducing to $PSII_\beta$-Q_B-reducing is unknown and more work is required. Depending on the photosynthetic organism, $PSII_\beta$ may comprise between 20 to 45% of the total number of PSII reaction center complexes (7, 30, 34). The results from this work suggest that $PSII_\beta$ centers are, in part, a reserve pool of PSII, readily available to the chloroplast in case of catastrophic photoinhibition.

This activation of $PSII_\beta$ appears to be a special aspect of the general role of Q_B-nonreducing centers in a proposed PSII repair cycle (17, 18). A schematic depicting the role of this activation in the general PSII repair cycle is given in Figure 9. Operation of the cycle can lead to the accumulation of $PSII_\beta$-Q_B-reducing centers during strong-irradiance exposure. Activation may include movement of the complex from the stroma-exposed region of the thylakoid membrane to the grana partition region (18, 28). The process could occur rapidly during strong irradiance exposure because no additional protein complexes need be synthesized. Furthermore, the peripheral LHC-II and the Q_B-reducing $PSII_\beta$ centers remain separate, perhaps by a mechanism similar to the reversible separation between LHC-II and PSII known to occur in strong-irradiance (*i.e.* state transitions, reviewed in [2]). Indeed, state-transitions probably occurred during our strong-irradiance treatments of *C. reinhardtii* and may have reduced the functional antenna size of some $PSII_\alpha$ centers. According to our estimates, this process made only minor contributions to the increase of Q_B-reducing $PSII_\beta$ centers during strong-irradiance exposure (Appendix).

In accordance with previous studies on this topic (7, 18, 29), we have attributed the variable fluorescence yield from F_o to $F_{p/}$ to a significant fraction of PSII centers that are Q_B-nonreducing. However, alternative explanations for the origin of the F_o to $F_{p/}$ transition have appeared in the literature. One hypothesis is that Q_A reverts to an 'inactive' state, Q_i, with a half time of 30 s in the dark. In the state Q_i, Chl *a* fluorescence is quenched more efficiently than in the state Q_A. Therefore, F_o corresponds to the Q_i state, and $F_{p/}$ corresponds to the state Q_A (20). A second hypothesis is that the F_o to $F_{p/}$ transition represents the activation of the oxygen evolution system. When the oxygen evolving system is dark-adapted, S_0 and S_1 are in high concentration and quench fluorescence more than the S_2 and S_3 states, which are generated upon illumination (13). We favor the interpretation that F_o to $F_{p/}$ reflects electron accumulation on Q_A in reaction centers impaired in the Q_A to Q_B electron transport (Q_B-nonreducing centers). A number of observations support this interpretation. The amplitude of the F_o to $F_{p/}$ component is similar to the proportion of F_v accounted for by $PSII_\beta$ and the kinetics of the F_o to $F_{p/}$ increase resemble the exponential kinetics of $PSII_\beta$ (Fig. 6), (14, 18, 29). The amplitude of the fluorescence yield increase from F_o to $F_{p/}$ in thylakoid membranes is unaffected by the presence of the artificial electron acceptor ferricyanide or the PSII electron donor hydroxylamine (29). These 'DCMU-like' Q_B-nonreducing centers have been isolated in stroma-exposed thylakoid membranes from *C. reinhardtii* (PJ Neale, A Melis, unpublished data), indicating that they are localized in a thylakoid membrane domain which is spatially separate from that of the Q_B-reducing centers. Further evidence that the F_o to $F_{p/}$ transition corresponds to the Q_B-nonreducing centers has been provided by observing the recovery time of $F_{p/}$ which has a half-time on the order of 2 s (7, 29). This reoxidation rate is too slow for these centers to make any significant contribution to steady-state electron transport.

The availability of a reserve pool of PSII offers a number of advantages to algal cells in minimizing the damage due to strong-irradiance exposure. Since the potential drop in electron-transport rates is partially offset by $PSII_\beta$, the cell can maintain a supply of reducing equivalents needed to synthesize new reaction center proteins. Also, the small antenna size of $PSII_\beta$ is an advantage in protecting against further photoinhibition of the newly integrated centers. In summary, these results suggest that $PSII_\beta$ is a light-activated 'reserve' pool of PSII which may play an important role in sustaining plant growth and productivity under adverse light conditions in the natural environment.

APPENDIX

The following procedure was used to estimate the change in the pool size of the $PSII_\beta$-Q_B-reducing centers due to strong-irradiance treatment. For the purpose of calculation, the total PSII content of control cells is set to 1.0. Since the $PSII_\alpha$ are

the main contributors to electron transport in the control cells, we set

$$PSII_{Q_B\text{-}reducing}(control) = PSII_{\alpha}(control) \quad (A1)$$

The number of Q_B-reducing centers after strong-irradiance treatment is estimated from the change in PSII electron transport

$$PSII_{Q_B\text{-}reducing}(treatment) = PSII_{Q_B\text{-}reducing}(control) \times PSII_{electron\ transport}(treatment) \quad (A2)$$

where, again, $PSII_{electron\ transport}$ is expressed as a proportion of the rate in control cells. It was assumed that only $PSII_{\alpha}$ centers are damaged during strong-irradiance exposure, so that the number of $PSII_{\alpha}$ remaining after strong-irradiance treatment is

$$PSII_{\alpha}(treatment) = PSII_{\alpha}(control) - PSII_{photoinhibited} \quad (A3)$$

where $PSII_{photoinhibited}$ is the decrease in number of PSIIs active in primary charge separation. The number of $PSII_{\beta}$-Q_B-reducing after strong-irradiance treatment is therefore

$$PSII_{\beta} - Q_B - reducing(treatment) = PSII_{Q_B\text{-}reducing}(treatment) - PSII_{\alpha}(treatment) \quad (A4)$$

Now consider a specific estimate based on our PSII quantitations for *C. reinhardtii*. The relative $PSII_{\alpha}$ content of control cells was 0.70 (Fig. 4). Electron transport estimated from HL O_2 evolution, after exposure to strong irradiance, was 0.63 relative to control (Fig. 8). The strong-irradiance treatment resulted (on average) in a lowering to 52% of PSII able to perform a primary charge separation (Figs. 2, 4, 8), so that $PSII_{photoinhibited} = 0.48$. Therefore

$$PSII_{Q_B\text{-}reducing}(treatment) = 0.70 \times 0.63 = 0.45$$

$$PSII_{\alpha}(treatment) = 0.70 - 0.48 = 0.22$$

Note that the $PSII_{\alpha}$ content estimated this way results in a somewhat larger pool size after strong-irradiance treatment than that indicated in Figure 4. This is attributed to the fact that some (probably less than 10%) of the $PSII_{\alpha}$ centers have been converted to $PSII_{\beta}$ by the process of a strong-irradiance induced state-transition (2). Using equation A4 corrects for the minor contribution to the $PSII_{\beta}$-Q_B-reducing pool made by this process. Finally,

$$PSII_{\beta} - Q_B - reducing(treatment) = 0.45 - 0.22 = 0.23$$

Since the $PSII_{\beta}$ in the control cells was 0.30 of PSII, we derived 0.23/0.30 = 0.77. Thus, we conclude that up to 77% of $PSII_{\beta}$ is in the Q_B-reducing state after strong-irradiance exposure.

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