

Photoinhibition of *Chlamydomonas reinhardtii* in State 1 and State 2

DAMAGES TO THE PHOTOSYNTHETIC APPARATUS UNDER LINEAR AND CYCLIC ELECTRON FLOW*

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The relationship between state transitions and photoinhibition has been studied in *Chlamydomonas reinhardtii* cells. In State 2, photosystem II activity was more inhibited by light than in State 1. In State 2, however, the D1 subunit was not degraded, whereas a substantial degradation was observed in State 1. These results suggest that photoinhibition occurs via the generation of an intermediate state in which photosystem II is inactive but the D1 protein is still intact. The accumulation of this state is enhanced in State 2, because in this State only cyclic photosynthetic electron transport is active, whereas there is no electron flow between photosystem II and the cytochrome *b₆f* complex (Finazzi, G., Furia, A., Barbagallo, R. P., and Forti, G. (1999) *Biochim. Biophys. Acta* 1413, 117–129). The activity of photosystem I and of cytochrome *b₆f* as well as the coupling of thylakoid membranes was not affected by illumination under the same conditions. This allows repairing the damages to photosystem II thanks to cell capacity to maintain a high rate of ATP synthesis (via photosystem I-driven cyclic electron flow). This capacity might represent an important physiological tool in protecting the photosynthetic apparatus from excess of light as well as from other a-biotic stress conditions.

The photochemical utilization of absorbed light is a critical step in the photosynthetic process. Because harvesting of light, photochemistry, and electron transfer occur on widely different scales of time, a correct balance among these different processes is required to optimize the efficiency of CO₂ fixation.

When light is absorbed in excess of what can actually be utilized by photochemistry, damage to the photosynthetic apparatus may be induced. Impairment of both photosystem I (PSI)¹

(1) and photosystem II (PSII) (2) has been described, and this loss of activity has been termed photoinhibition (3). It has been also shown that the degradation of the PSII reaction center D1 subunit is a major consequence of photoinhibition (2).

Some mechanisms contribute to protecting the photosynthetic apparatus from an excess of light (4, 5). The first is the so-called energy-dependent quenching, qE, *i.e.* the increased thermal dissipation in the PSII antennae that follows the generation of the electrochemical proton gradient across the thylakoid membranes. It is supposed to protect the reaction center from the consequences of a strong illumination by reducing the amount of energy present in the antenna protein complexes (6).

The second one (6) is state transitions, a phenomenon that has been discovered in *Chlorella pyrenoidosa* (7) and in *Porphyridium cruentum* (8). It is a mechanism to balance light utilization between the two photosystems that is based on the reversible transfer of a fraction of the light-harvesting complex II (LHCII) from PSII to PSI (reviewed in Refs 9–11). It is also supposed to protect PSII from photoinhibition inasmuch as it can decrease the size of its antenna.

The migration of LHCII to PSI (State 1-State 2 transition) results from the phosphorylation of the former by a membrane-bound protein kinase, which is activated under reducing conditions (reviewed in Refs. 9 and 12). Under oxidizing conditions, the kinase is deactivated, and LHCII is dephosphorylated by a thylakoid-bound phosphatase, which is possibly regulated by the recently discovered immunophilin-like 40-kDa luminal TLP protein (13). After dephosphorylation, LHCII rebinds to PSII (State 2-State 1 transition).

In higher plants, only a small fraction of the LHCII (15–20%, reviewed in Ref. 10) migrates reversibly from PSII to PSI. In the green alga *Chlamydomonas reinhardtii*, on the contrary, a much larger fraction of the PSII antenna is transferred during State 1-State 2 transition (14), and a much larger decrease of PSII energy capture is accordingly observed (15). In addition, cytochrome *b₆f* complexes accumulate in the unstacked lamellae in State 2 (16). Therefore, it is unlikely that state transitions serve the purpose of balancing the absorption of PSII and PSI in *Chlamydomonas*. Instead, State 2 would represent a structural condition where most of the excitation energy is utilized by PSI photochemistry so that cyclic electron transport around PSI is likely to prevail over linear electron flow that involves both PSI and PSII.

In agreement with this idea, Finazzi *et al.* (17) show that

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¹ The abbreviations used are: PS, photosystem; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCMU, 3-(3',4'-dichlorophenyl)-1,1-dimethylurea; Fo, minimum value of fluorescence emission measured at open reaction centers; Fm, maximal value of fluorescence emission measured at closed reaction centers; Fv, variable fluorescence

(Fm – Fo); ¹O₂, singlet oxygen species; PC, plastocyanin; PQ, plastoquinone; PQH₂, plastoquinol; Qa, primary quinone acceptor of photosystem II; Qb, secondary quinone acceptor of photosystem II; LHCII, light-harvesting complex II.

although the cytochrome *b₆f* turnover was the same in State 1 and State 2, it was completely inhibited by the addition of the PSII inhibitor DCMU in State 1, whereas no effect of this inhibitor was observed in State 2. This result led Finazzi *et al.* (17) to propose that in State 2 the reducing equivalents involved in the reduction of the cytochrome *b₆f* are not produced at the level of PSII but rather at the level of PSI. Under these conditions PSII is not connected to the intersystem electron carriers but is still photochemically active (17).

To investigate whether this lack of functional connection between PSII and cytochrome *b₆f* complex might affect the sensitivity of the former to photoinhibition, we have measured the effects of strong illumination on fluorescence emission, O₂ evolution, and cytochrome *f* reduction in algae under State 1 or State 2 conditions. We have found that PSII is more prone to photoinhibition in State 2. However, in this state the loss of activity is not accompanied by a degradation of the D1 protein. The effect on PSII seems to be rather specific as neither PSI nor cytochrome *b₆f* activities nor the coupling of thylakoid membranes were affected by the treatment. Thus, we suggest that state transitions in *C. reinhardtii* represent a means to maintain a high ATP synthesis capacity, even when damages to PSII are induced by illumination with extremely intense light.

MATERIALS AND METHODS

Strains and Culture Conditions—*C. reinhardtii* wild type (from strain 137C) was kindly provided by the Laboratoire de Physiologie Membranaire du Chloroplaste at the Institut de Biologie Physico-Chimique de Paris (France). Cells were grown at 24 °C in acetate-supplemented medium (18) under 60 μE m⁻² s⁻¹ of continuous white light. They were harvested during exponential growth and resuspended at the required chlorophyll concentration in an high salt minimal medium (19). The use of this medium prevented the spontaneous transition to State 2 otherwise observed in the presence of acetate (see Ref. 20).² Chlorophyll concentration was measured as the absorbance at 680 nm of the cell cultures in a spectrophotometer equipped with a scatter attachment on the basis of a calibration curve constructed after extraction of the chlorophyll with 80% acetone.

State Transitions and Photoinhibitory Treatments—State 1 was obtained through incubation of the cells in the dark under strong agitation, whereas State 2 was obtained through dark incubation in anaerobic conditions obtained by argon bubbling. Photoinhibition was performed by illuminating the sample with white light on a thin layer (~1 mm) of cells in a Petri dish ([chlorophyll] = 500 μg ml⁻¹) at room temperature. The light was screened with a layer of water and infrared- and UV-absorbing filters. The intensity of the light reaching the sample was 2300 μE m⁻² s⁻¹. We ensured that the layer of cells was sufficiently thin to minimize mutual shadowing. When indicated, plastidial protein synthesis was inhibited by adding lincomycin at the final concentration of 1 mM. Samples were collected at the indicated times and used in the different experiments at the required chlorophyll concentration.

Oxygen Evolution and Fluorescence Emission Measurements—Photosynthesis and respiration were measured as the O₂ exchange with a Clark-type electrode (Radiometer, Denmark) at 24 °C. The actinic light was filtered through a heat filter, and its intensity was 850 μE m⁻² s⁻¹. Fluorescence was measured in the same chamber used for O₂ recordings using a PAM fluorometer (Walz, Germany).

Spectroscopic Measurements—Spectroscopic measurements were performed on whole cells at room temperature using a homemade spectrophotometer as described by Joliot *et al.* (21).

In continuous light experiments, actinic light was provided by a light-emitting diode array, placed on both sides of the cuvette. Its intensity was 1500 μE m⁻² s⁻¹. Measurements were performed on algae kept under State 1 conditions obtained through a strong agitation in the dark in air. Estimation of the rates of cytochrome *f* turnover was done using a procedure previously employed (17). Briefly, starting from the consideration that the rate of cytochrome *f* oxidation and reduction is the same at steady state, the latter can be expressed as $df/dt = [f^-] \times k_{ox} \times [PC^+]$, where $[f^-]$ is the fraction of reduced cytochrome *f*, and $k_{ox} \times [PC^+]$ represents the product of the second order rate constant for cytochrome *f* oxidation times the concentration of oxidized

plastoquinin. Both parameters can be easily calculated experimentally from the traces of Fig. 3; $[f^-]$ is estimated comparing the plateau absorption level measured in the absence and presence of DBMIB, whereas $k_{ox} \times [PC^+]$ is given by the initial rate of cytochrome *f* oxidation provided that it is measured when its reduction is inhibited, *i.e.* in the presence of DBMIB.

In single turnover flash experiments, excitation was provided by a xenon lamp (EG&G). Light was filtered through a Schott filter (RG 695) and was of saturating intensity. Measurements were performed on algae kept in State 2 to ensure dark reduction of the plastoquinone pool. Repetitive (usually 10) illuminations were performed at the frequency of 0.15 Hz. The transmembrane potential was estimated from the amplitude of the electrochromic shift at 515 nm, which is known to give a linear response with respect to the electric component of the transmembrane potential (22). Under the conditions employed here, the kinetics of the electrochromic signal exhibited two phases previously characterized in Joliot and Delosme (23): a fast phase (phase a), associated with PSI and PSII charge separation, and a slow phase, which develops in the millisecond time scale and is associated with the turnover of the cytochrome *b₆f* complex (phase b).

The kinetics of phase b was deconvoluted from membrane potential decay assuming that the latter process exhibited first-order kinetics. Phase b was then computed considering that the rate of membrane potential decay between two consecutive acquisitions was linearly related to its mean value in the same interval. Cytochrome *f* redox changes were evaluated as the difference between the absorption at 554 nm and a base line drawn between 545 and 573 nm. We have checked that this procedure for deconvolution of cytochrome *f* signals was reliable also in the case of continuous illumination (17).

Protein Analysis—For protein analysis, algae were collected at the indicated times, washed in 20 mM HEPES containing protease inhibitors (200 μM phenylmethylsulfonyl fluoride, 5 mM amino-ε-caproic acid, and 1 mM benzamidin) and 1 mM lincomycin, and resuspended in 100 mM dithiothreitol, 100 mM Na₂CO₃. The algae were then solubilized in the presence of 2% SDS and 20% (w/v) sucrose at 100 °C for 1 min. Polypeptides were separated by denaturing SDS-polyacrylamide gel electrophoresis in the presence of 6 M urea. Immunoblotting was performed with monospecific polyclonal antibodies against D1, as described in Barbato *et al.* (24).

RESULTS

To investigate the influence of state transitions on the sensitivity of *C. reinhardtii* to photoinhibition, we have performed experiments on cells placed either in State 1 or in State 2. We have measured fluorescence emission, photosynthetic activity (as O₂ evolution), and the rate of cytochrome *f* reduction after exposure to strong illumination. The measurements were performed on the same batch of algae, collected either before starting the light treatment or after different irradiation times.

Fluorescence Emission and Oxygen Evolution—Illumination of the algae with high light intensity largely modified their fluorescence emission parameters; a large decrease of the maximal fluorescence emission (F_m) was observed in State 1 cells (Fig. 1A), whereas an increase of the minimal one (F_o) occurred in State 2 (Fig. 1B). This suggests that the consequences of illumination on the photosynthetic apparatus of *Chlamydomonas* were not identical in the two conditions. In both cases, however, the effect of illumination was to reduce the F_v/F_m ratio (Fig. 1C), a parameter related to the photochemical efficiency of PSII (25). This indicates that PSII was the major target of photoinhibition in both State 1 and State 2. The F_v/F_m decline was reversible in the dark, unless an inhibitor of protein synthesis, lincomycin, was present in the medium (not shown). The addition of this compound during illumination enhanced the photoinhibition, and its effect was larger in State 2 (Fig. 1D).

Samples were also collected to measure the effects of illumination on the photosynthetic O₂ evolution. To this aim, State 1 was re-established (by oxygenation in the dark) in algae preilluminated in State 2 before O₂ evolution was recorded. We have already shown indeed that no oxygen is evolved by the algae in State 2 (17). During the State 2 to State 1 transition,

² F.-A. Wollman, personal communication.

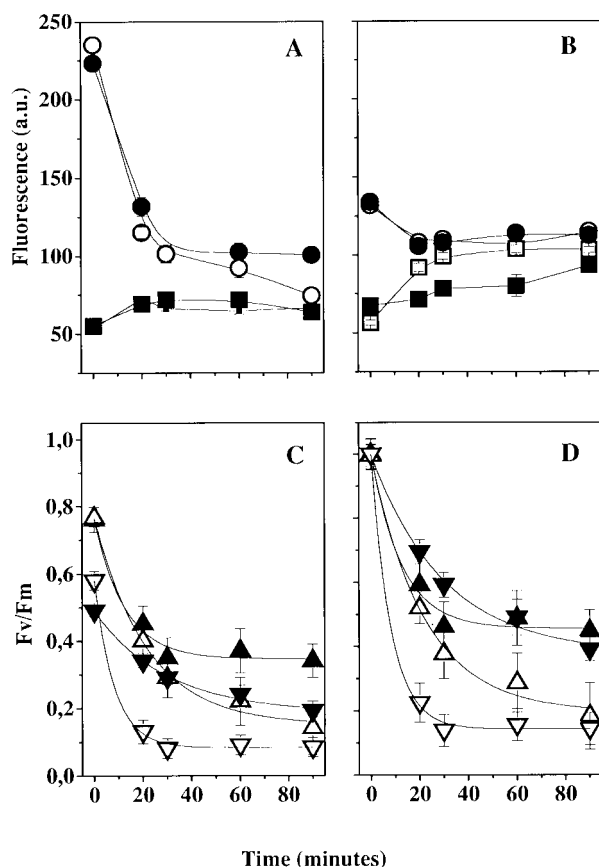


FIG. 1. Photoinhibition of *C. reinhardtii* cells in State 1 and State 2. Algae were collected during the exponential growth phase and resuspended in the minimal high salt medium (19). State 1 was obtained by incubating the algae in the dark under vigorous agitation to prevent reduction of the PQ pool. State 2 was obtained through dark incubation of the algae under an argon atmosphere. Photoinhibitory treatments were performed illuminating a thin layer of cells (~1 mm) with white light of $2300 \mu\text{E m}^{-2} \text{s}^{-1}$ at a chlorophyll concentration of $500 \mu\text{g ml}^{-1}$. **Panel A**, changes in the F_0 (squares) and F_m (circles) parameters in State 1-treated cells. **Closed symbols**, control; **open symbols**, 1 mM lincomycin. **Panel B**, State 2-treated cells. **Panel C**, decrease of the F_v/F_m parameter upon photoinhibition in State 1 (upward triangles) and State 2 (downward triangles). In **panel D**, the same traces as in **panel C** are presented after normalization of the initial value. The data represent the result of five independent experiments. *a.u.*, absorbance units.

no recovery of inhibition occurred; after the transition to State 1, the F_v/F_m of State 2-treated algae was still largely inhibited if compared with an untreated (State 1) sample (not shown).

The oxygen evolution rates (measured before and after the photoinhibitory treatment) are shown in Table I. No decrease of oxygen evolution was observed in both State 1- and State 2-illuminated samples in the absence of lincomycin. In its presence, a loss of activity was observed, which was again larger in State 2- than in State 1-treated cells.

Electron Transport from PSII to Cytochrome b_6/f Complex—A more direct way to characterize the effects of irradiation on PSII photochemical activity would be to measure directly the rate of plastoquinone reduction. It is very difficult to measure this parameter *in vivo*, where the redox changes associated to PQH_2 formation (observed around 260 nm) are largely masked by other absorption signals. However, it is possible to obtain this information indirectly by measuring the rate of cytochrome f reduction.

This rate can be expressed indeed as $k_{\text{red}} \times [f\text{Fe}^{3+}\text{S}\cdot b_1^+ \cdot b_h] \times [\text{PQH}_2]$, where k_{red} is the second order rate constant for plastoquinol oxidation, $[f\text{Fe}^{3+}\text{S}\cdot b_1^+ \cdot b_h]$ represents the concentra-

TABLE I
Effects of photoinhibition on photosynthetic oxygen evolution in Chlamydomonas cells

Oxygen evolution and consumption were measured by a Clark-type electrode at a chlorophyll concentration of $20 \mu\text{g ml}^{-1}$ in the presence of 5 mM NaHCO_3 . Photosynthetic oxygen evolution rate ($\mu\text{mol mg}^{-1}$ chlorophyll h^{-1}) is expressed as the sum of O_2 evolution (in the light) and consumption (in the dark). The latter was not affected by the treatment. Other conditions are as in Fig. 1. Light intensity was $850 \mu\text{E m}^{-2} \text{s}^{-1}$. Data represent the result of five different experiments.

Time	State 1		State 2	
	Control	Lincomycin, 1 mM	Control	Lincomycin, 1 mM
<i>min</i>				
0	105 ± 10	104 ± 5	98 ± 10	112 ± 10
20	110 ± 8	106 ± 7	93 ± 11	25 ± 11
30	109 ± 11	100 ± 10	97 ± 11	13.5 ± 8
60	111 ± 9	83 ± 8	100 ± 9	9 ± 6
90	108 ± 10	54 ± 6	99 ± 7	9 ± 9

tion of active cytochrome b_6/f complexes, and $[\text{PQH}_2]$ expresses the concentration of plastoquinol. Although $[\text{PQH}_2]$ is proportional to the fraction of active PSII, at least in State 1 conditions (Ref. 17, see also below), the product $k_{\text{red}} \times [f\text{Fe}^{3+}\text{S}\cdot b_1^+ \cdot b_h]$ depends on the catalytic efficiency of the cytochrome complex. Therefore, it is important first to check that the turnover rate of cytochrome b_6/f complex *per se* is not affected by the photoinhibitory treatment. Only in this case, the product $k_{\text{red}} \times [f\text{Fe}^{3+}\text{S}\cdot b_1^+ \cdot b_h]$ can be taken as a constant, and the cytochrome f reduction rate can be used to obtain information on PSII activity.

The intrinsic cytochrome b_6/f activity can be easily measured in State 2 conditions (*i.e.* anaerobiosis) under a single turnover flash illumination regime of low actinic light frequency. In these conditions indeed the PQ pool is fully rereduced in the dark time between two consecutive illuminations (26), and the catalytic properties of the complex can be studied independently of the rate of PQ photoreduction. Thus, the kinetics of cytochrome b_6/f under single flash illumination was always measured in State 2: State 1 preilluminated cells were dark-adapted to anaerobiosis before their cytochrome b_6/f kinetics were measured.

Fig. 2 shows the kinetics of the electrochromic shift (**panels A and B**) and of cytochrome f redox changes (**panel C and D**) measured before and after a photoinhibitory treatment of *C. reinhardtii*. The slow phase of the electrochromic shift (phase b, see "Materials and Methods") and cytochrome f redox changes are representative of electron injection into the low and high potential electron transfer chains of the cytochrome b_6/f complex, respectively (23, 26). Fig. 2 shows that all the electron transfer steps that follow plastoquinol oxidation were not affected by the photoinhibitory illumination. No differences were observed between cells treated in State 1 and State 2 (not shown).

During the measurements, PSII activity was inhibited with DCMU and hydroxylamine (27). Their addition did not affect cytochrome b_6/f kinetics (as expected, since it does not depend on PSII activity in State 2, see above) but reduced the amplitude of the fast phase of the electrochromic signal (phase a) because of the loss of PSII photochemistry. In the presence of DCMU and hydroxylamine, phase a only depends on PSI-driven charge separation. Its constancy before and after the photoinhibitory treatment (Fig. 2, **A and B**) suggests that PSI was not affected by photoinhibition in our conditions.

The treatment did not affect the permeability of the thylakoid membrane either, as indicated by the finding that the addition of an ionophore induced the same acceleration of cytochrome b_6/f turnover in both untreated and treated cells. The kinetic effect of ionophores is quantitatively related to the magnitude of the electrochemical proton gradient (reviewed in

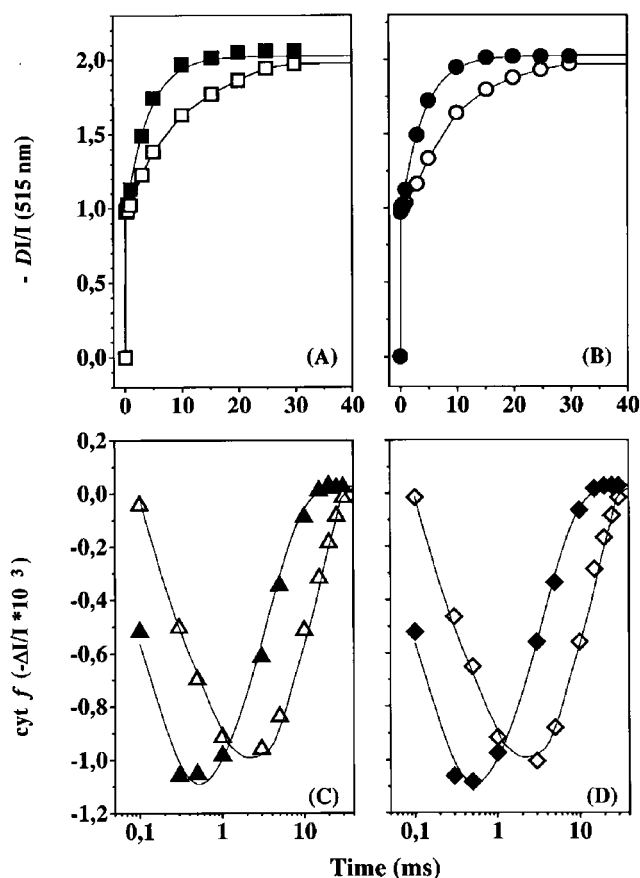


FIG. 2. Effects of preillumination on the kinetics of the electrochromism signal (A and B) and cytochrome f ($cyt f$; C and D) redox changes. Panels A and C, untreated algae; panels B and D, preilluminated algae. Open symbols, control samples; closed symbols, 1 μ M carbonyl cyanide p -trifluoromethoxyphenylhydrazone. The cells (50μ g of chlorophyll ml^{-1}) were illuminated with red flashes at the frequency of 0.15 Hz. DCMU and hydroxylamine were added at the concentrations of 10 μ M and 1 mM, respectively, to block PSII activity. Treated algae were illuminated for 90 min under the same conditions as in Fig. 1.

Ref. 28), *i.e.* to the permeability of membrane to ions. This conclusion is in agreement with previous results with isolated thylakoid membranes (29, 30).

Having ascertained that the intrinsic activity of PSI and cytochrome b_6f complex was not affected by photoinhibition, we have estimated the consequences of illumination on PSII by measuring the turnover rate of cytochrome f under steady state illumination conditions, as stated above.

Fig. 3 shows the results of such measurements in the case of one representative experiment. Panels A and B refer to measurements performed on dark-adapted algae under both State 1 (A) and State 2 (B). Similar cytochrome f kinetics was observed in both states; switching the light on generated an oxidation signal (absorption decrease) that rapidly attained a plateau level. After the light was switched off, a reduction was observed that brought the signal to its initial level. In State 1 and State 2 conditions, the extent of the oxidation signal was equally sensitive to the addition of DBMIB, an inhibitor of cytochrome f reduction by plastoquinol (31) (Figs. 3, A and B, compare squares and triangles). DCMU, which blocks plastoquinone reduction by PSII (27), inhibited electron flow only in State 1 cells (circles). This result confirms previous findings from Finazzi *et al.* (17) that the transition from State 1 to State 2 corresponds to a shift from a linear (involving PSII and PSI) to a cyclic (involving only PSI) electron transport system.

Therefore, in State 2 it is not possible to measure PSII

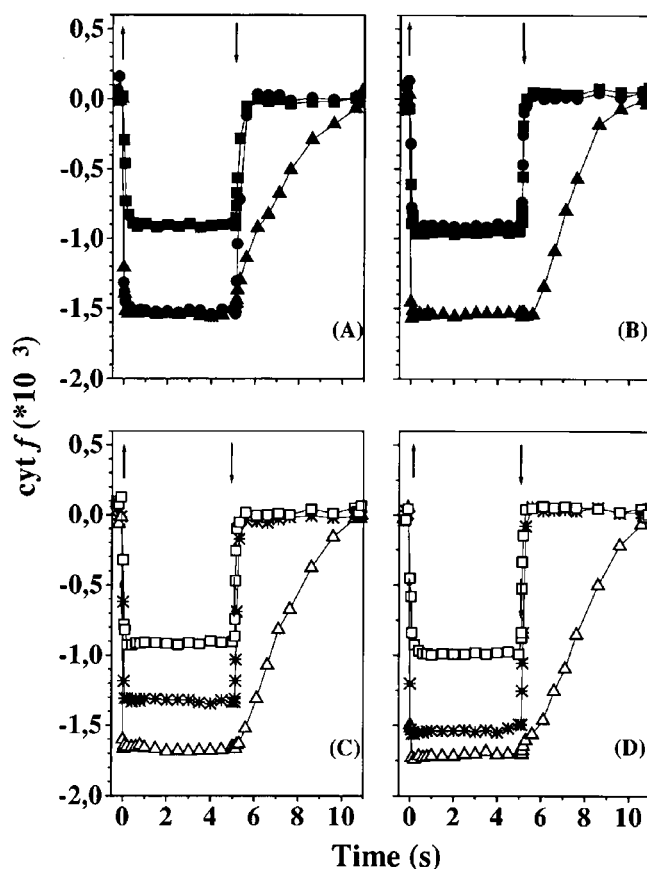


FIG. 3. Effects of photoinhibition on redox changes of cytochrome b_6f ($cyt f$) complex observed under continuous illumination. Panel A, State 1, untreated. Panel B, State 2, untreated. Panel C, State 1, 90 min of illumination. Panel D, State 2, 90 min of illumination. Squares, no additions; circles, 10 μ M DCMU; triangles, 2 μ M DBMIB; asterisks, 1 mM lincomycin (added during preillumination). Note that in panel D, State 1 was re-established before measuring cytochrome f kinetics. Other conditions are as in Fig. 1. Upward arrow, actinic light on. Downward arrow, actinic light off. Light intensity was $1500 \mu E m^{-2} s^{-1}$. [Chlorophyll] = 50μ g of chlorophyll ml^{-1} .

activity on the basis of cytochrome f turnover. For this reason, the consequences of preillumination on cytochrome f kinetics in the case of continuous illumination regime were measured in State 1, at variance with single flash measurements. In State 2-preilluminated cells, State 1 was re-established by dark oxygenation of the cells. The same treatment did not affect the rate of electron transfer in dark-adapted cells (not shown). Panels C and D of Fig. 3 present the results of such measurements. In the absence of lincomycin, no differences were observed between preilluminated and dark-adapted cells (squares). The steady state redox level of cytochrome f was more oxidized, however, in lincomycin-treated samples (asterisks). This suggests that photoinhibition reduced the rate of plastoquinol generation by PSII in the presence of the antibiotic, in agreement with the finding that O_2 evolution was inhibited (Table I). This conclusion is also in agreement with previous results obtained under similar experimental conditions in higher plant leaves (32). Again, the consequences were more severe in the case of State 2- than State 1-treated cells (compare C and D, asterisks). In untreated cells, lincomycin did not affect cytochrome f turnover (not shown). The time courses of the decrease in PSII-driven cytochrome f electron flow are shown in Table II.

Stability of the PSII Reaction Center—All the measurements performed so far indicate that the activity of PSII is decreased by photoinhibition both in State 1 and State 2 provided that linco-

TABLE II

Effect of photoinhibition on the electron flow through cytochrome *b₆f*

The rates of cytochrome *f* reduction were calculated from traces as in Fig. 3 as explained under "Materials and Methods". Rates are expressed as mol e⁻ s⁻¹. Data represent the result of five independent experiments.

Time	State 1		State 2	
	Control	Lincomycin, 1 mM	Control	Lincomycin, 1 mM
<i>min</i>				
0	70 ± 5	65 ± 7	68 ± 5	74 ± 5
20	65 ± 6	56 ± 7	67 ± 6	18 ± 3
30	79 ± 7	48 ± 3	62 ± 4	9 ± 4
60	71 ± 7	35 ± 4	65 ± 5	6 ± 4
90	66 ± 8	26 ± 5	70 ± 6	3 ± 3

mycin is added to the cell suspension. This loss of activity is generally associated to a damage of the D1 subunit of PSII, which is subsequently rapidly degraded (see *e.g.* Refs. 2 and 33). To verify if this was the case in our conditions, we have measured the amount of D1 in both State 1- and State 2-treated samples using an immunoblotting assay (Fig. 4). We found remarkable differences between State 1 and State 2 cells; although the amount of D1 was reduced upon photoinhibition in State 1-treated cells, no substantial degradation was observed in State 2 despite a massive loss of PSII activity (Fig. 4A).

It has previously been demonstrated that DCMU protects the D1 protein from degradation (34–35), possibly by reducing the accessibility to the protease(s) to the damaged PSII centers (34). Therefore, we have repeated the photoinhibitory treatments in the presence of this inhibitor. As shown in Fig. 4 (panel B), DCMU protected against D1 degradation in State 1-treated cells. At the same time, it deeply affected the fluorescence parameters of State 1 cells, strongly enhancing the Fv/Fm decrease (Fig. 5, compare *open* and *closed squares*). In State 2, no substantial effects of DCMU were observed (Fig. 5, *circles*). This effect was not due to any overestimation of the F_o parameter due to incomplete reoxidation of Qa⁻. We checked indeed that it was rapidly oxidized in the dark (not shown), in agreement with previous work (36). Very similar kinetics of Qa⁻ relaxation was also observed in State 1 and State 2 cells (not shown). This suggests that the Qa-Qb equilibration rate (37) is not affected by the state transitions, in agreement with our previous findings that indicate a full photochemical competence of PSII in State 2 (17).

DISCUSSION

Relationship between Fluorescence Emission, Oxygen Evolution, Cytochrome f Reduction, and D1 Protein Levels during Photoinhibition of C. reinhardtii Cells under State 1 and State 2 Conditions—We report here on the sensitivity of *C. reinhardtii* to photoinhibition in State 1 and State 2 conditions. In both States, we have observed that the PSI and cytochrome *b₆f* complex intrinsic activities were not affected by a preillumination with very intense light (Fig. 2), whereas the photochemical efficiency of PSII was reduced. This results in a modification of several parameters, all related to PSII; the fluorescence emission (Fig. 1), the rate of O₂ evolution (Table I) and of electron transport to cytochrome *f* (Fig. 3, Table II), and the level of the D1 protein (Fig. 4) are reduced during photoinhibitory treatments. A comparison of light on the different parameters reveals that the Fv/Fm is decreasing in the absence as well as in the presence of lincomycin (Fig. 1), whereas the O₂ evolution (Table I) and the cytochrome *f* reduction rates (Fig. 3, Table II) are affected by the treatments only in the presence of the inhibitor. Their sensitivity is lower than that of the Fv/Fm, the cytochrome *f* reduction rate parameter being nevertheless more affected. In addition, the loss of photosynthetic activity

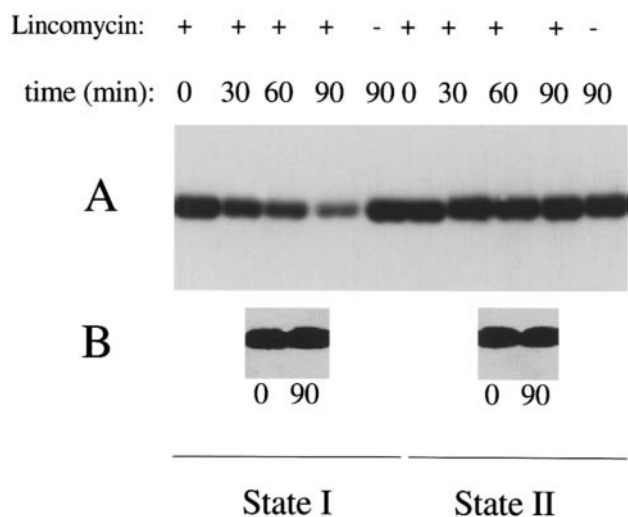


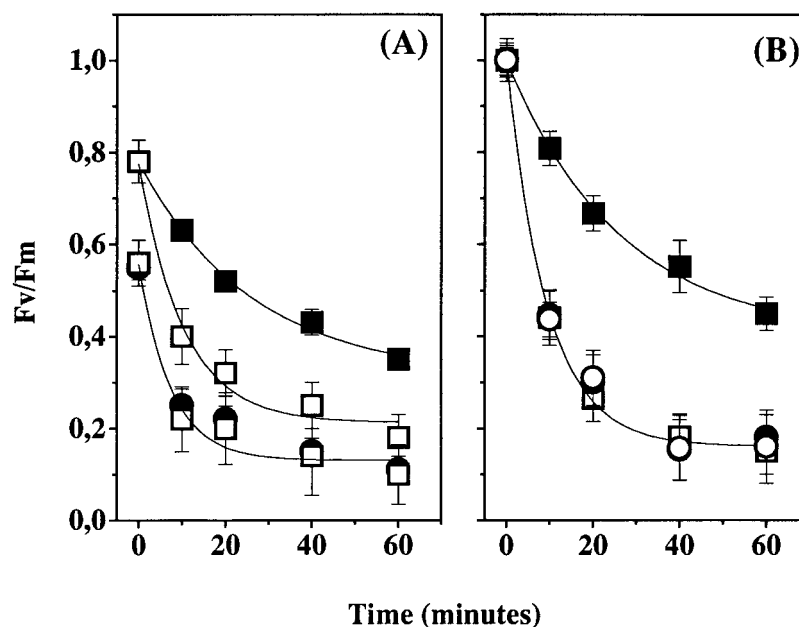
FIG. 4. Light-induced degradation of the D1 protein in State 1 and State 2 photoinhibited *C. reinhardtii* cells. State 1 and State 2 cells were incubated in light as in Fig. 1. Samples were collected after the indicated minutes, thylakoid membranes were isolated, and their content of D1 subunit was assayed by Western blotting. Panel A, control; panel B, DCMU-treated samples.

largely precedes the degradation of the D1 protein (Fig. 4).

The differences observed in the Fv/Fm decrease between cells treated or not with the antibiotic are consistent with the occurrence of a protein synthesis-dependent recovery in both State 1- and State 2-treated cells (reviewed in Ref. 38). On the other hand, the comparison between the fluorescence parameter on one side and the electron transport measurements on the other suggests that this protein synthesis-dependent recovery is able to minimize the consequences of photoinhibition on electron transport by keeping the loss of PSII activity within a level compatible with the functioning of the overall photosynthetic process. This value can be estimated from the traces of Fig. 1 and corresponds to a Fv/Fm decline of ~50% (*i.e.* of the maximal decrease measured in the absence of the antibiotic, Fig. 1D). Consistently with this idea, also in the presence of lincomycin a substantial inhibition of cytochrome *f* reduction rate and of O₂ evolution can be observed only between 30 and 60 min of treatment in State 1 (Fv/Fm equal to 40% of the initial value) and after 20 min in State 2 (Fv/Fm = 25%). We believe that this apparent insensitivity of electron transport parameters to photoinhibition is a consequence of the use of high light intensities to induce photosynthesis. Under these conditions, the kinetic performance of the photosynthetic apparatus is saturated, and the rate of oxygen evolution is limited by the rates of the reactions occurring in the dark, most probably those of CO₂ assimilation by the Calvin Benson cycle, and not by the light-driven electron flow. The latter has to be reduced beyond a certain level (~50%) before becoming rate-limiting.

As stated before, the degradation and re-synthesis of the D1 subunit are deeply involved in the loss of photosynthetic activity observed here. This confirms previous findings suggesting that light affects the stability of the D1 subunit (reviewed in Ref. 38). However, our data indicate that the damage (and the subsequent repair) occurs in a time scale of minutes, which is apparently considerably faster than the rate of degradation of the D1 protein. This inconsistency is probably due (at least to some extent, see below) to a misestimation of the D1 turnover rate, due to the presence of the protein synthesis inhibitor in the reaction medium. It has been already reported that protein synthesis inhibitors reduce the rate of D1 degradation (39), which is otherwise very rapid (2, 34–35, 40–41). Their effect

FIG. 5. Effect of DCMU on the fluorescence parameters of *C. reinhardtii* cells in State 1 and State 2 conditions. Same conditions as in Fig. 1. Squares, State 1 cells. Circles, State 2 cells. Closed symbols, control. Open symbols, 10 μ M DCMU. In panel B the same traces as in panel A are presented after normalization of the initial value. The data represent the result of five independent experiments.



has been interpreted as the consequence of a synchronization existing *in vivo* between the synthesis and the degradation of this PSII subunit, which is expected to reduce the rate of the synthesis when the degradation is prevented. Alternatively, the involvement of non-nuclear factors in the replacement of the newly synthesized proteins in the membranes has also been proposed as an explanation for this phenomenon (42–43). At present, our data do not allow discrimination between the two possibilities.

The data presented here suggest that the effect of light on PSII efficiency is likely due to a damage to its reaction center. This is confirmed by the measurements of the amount of the protein D1, at least in State 1 conditions (Fig. 4A, see under “Results”), and by the effect of DCMU (Fig. 4B). It is therefore a “classical” acceptor side photoinhibition, *i.e.* it is due to the accumulation of reduced PSII acceptors that induce the formation of P680 triplets. They may react with O_2 , generating the oxidant species 1O_2 , which is responsible for the impairment of PSII activity (44). In principle, the oxygen requirement of this reaction could explain the differences observed between State 1- and State 2-treated cells. However, we consider this possibility rather unlikely, because oxygen entered the Petri dish during the treatment in State 2 despite the fact that argon was bubbled to maintain anaerobiosis, and its concentration at the end of the treatment was 50–100 μ M. This amount is largely sufficient to react with 3P680 .

Mechanism of Photoinhibition under State 1 and State 2 Conditions—It was previously reported that the damage to the PSII reaction center proceeds via the generation of an intermediate state (45–49), where charge separation in PSII is already perturbed (low Fv/Fm), but the D1 protein is still present in the reaction center. After its formation, degradation of the D1 protein occurs, giving rise to an irreversible loss of activity (38, 45–49) that can be repaired only by *de novo* protein synthesis (reviewed *e.g.* in Ref. 38).

The data reported here are in agreement with this idea, since the decrease of the activity of PSII precedes the degradation of the protein in State 1. The phenomenon is even more evident in State 2, where no degradation of the protein is observed, despite the fact that PSII activity is more severely inhibited by the illumination (Figs. 1 and 5). Therefore, our results suggest that photoinhibition in State 2 induces a higher accumulation of the intermediate state. The large increase of the Fo during

photoinhibition in State 2 (Fig. 1) is also consistent with this conclusion, as an increased Fo is a typical signature of the formation of the intermediate state (49). The similar consequences of photoinhibition in State 2 and in State 1 in the presence of DCMU (enhanced Fv/Fm decrease and no D1 degradation) are also consistent with this hypothesis, as this compound has been shown to enhance the generation of the PSII intermediate state during photoinhibition (49).

Thus, we suggest that the condition required to enhance photoinhibition is the lack of electron transfer from Qa^- to the intersystem chain, a condition achieved in State 2 (17) or in State 1 in the presence of DCMU. This induces over-reduction of the Qa quinone acceptor, promoting acceptor side photoinhibition (33–35, 38, 41, 50).

Using *Chlamydomonas* mutants devoid of the cytochrome b_6f complex, it has been previously reported that the presence of a plastoquinol molecule in the Qb site of PSII exerts a protective role against the degradation of the D1 protein by proteases by a mechanism resembling that of DCMU (48). The finding that no degradation of D1 occurs in State 2 (Fig. 4) suggests that the Qb site of PSII complexes is occupied by a PQH_2 during the photoinhibitory treatment under these conditions. This would stem from the fact that PSII and the cytochrome b_6f complex are not connected functionally (Ref. 17; see also Fig. 3, A and B).

It has also been suggested that the PQ pool is not homogeneously distributed in thylakoid membranes (51). The existence of PQ-diffusing domains, the equilibration of which is very slow, have been already reported (52–53). Thus, it is possible to think that the structural rearrangements of the photosynthetic apparatus that follow State 2 transition (14–16) cause a physical separation between PSII and the b_6f complex by placing them in different PQ domains. The cytochrome would be still connected to PSI (explaining the shift to cyclic electron flow; see Ref. 17), whereas photosystems II would be in separate domains, where plastoquinone would be reduced very rapidly without being oxidized (at least, not in a fast time scale). This would give rise to over-reduction of the acceptor of PSII and to the loss of its photochemical capacity. At the same time, the D1 protein would not be degraded because of the protecting role played by the PQH_2 present in the Qb site.

It is important to note, however, that the properties of PSII centers in State 2 are different from those of the Qb nonreducing centers (54), *i.e.* PSII, where PQ reduction in the Qb site is

impaired. In these centers, indeed, Q_a^- is oxidized at a rate that is ~ 1000 times slower, whereas we have observed that the reoxidation of Q_a^- (measured as the fluorescence decline after an illumination) is rapid in both in State 1 and State 2 cells (see "Results").

To summarize, we believe that the following events occur during photoinhibition of State 2 cells. Transition to State 2 isolates PSII from the b_6f complex because of physical constraints to PQH₂ diffusion. Thus, PSII does not participate to electron flow but is nevertheless able to reduce Q_a and the plastoquinone molecules that are still connected to it. During a prolonged illumination, Q_a is over-reduced, and all the PQ pool connected to PSII is in the PQH₂ form. This promotes loss of photochemical activity while protecting the D1 subunit from degradation.

Physiological Consequences of Photoinhibition in State 1 and State 2 Conditions—We have previously demonstrated that cyclic electron flow around PSI is induced by transition to State 2 in *C. reinhardtii* cells (17). Here, we have demonstrated that PSII is the major target of photoinhibition (Figs. 1, 3, and 4), in agreement with previous results (e.g. Refs. 2 and 3), whereas PSI activity is not affected by our illumination protocol (Fig. 2). For these reasons, we think that the consequences of photoinhibition should be more serious on the overall photosynthetic activity of cells placed in State 1, where PSII photochemistry contributes to the electron flow, than in State 2, where it does not (17).

In particular, in State 2 the capacity of light-induced ATP synthesis should not be affected by photoinhibition despite the fact that PSII is degraded. Indeed, in this condition ATP synthesis depends exclusively on PSI-driven cyclic electron flow (Ref. 17; see also Refs. 55–57). The ability to maintain a high level of ATP synthesis in photoinhibited cells might have important consequences on several cellular processes. In particular, the ability to recover from the photoinhibition itself depends on the ATP-requiring protein synthesis (2, 38, 58). In addition, the ability to re-establish linear electron flow (via State 2 to State 1 transition) is also influenced by the availability of ATP (41).

Important consequences may then be envisaged in the case of environmental stress. For example, a shortage of oxygen in the external milieu would induce the reduction of the PQ pool and the transition to State 2 (see e.g. Ref. 10). Illumination under these conditions would promote acceptor side inhibition, reducing or suppressing linear electron flow and lowering the ability of the cells to generate the electrochemical proton gradient and, consequently, to synthesize ATP. The activity of cyclic electron flow and the dependent ATP synthesis would however protect the cells from severe energy crisis and provide the conditions for recovery of photosynthetic activity. The stronger inhibition of PSII taking place under these conditions, therefore, would be largely compensated by the higher capacity to recover from the damage.

Interestingly, the phenotype reported here is similar to that already reported in the case of other typical stress conditions as the nutrient deficiency (see e.g. Refs. 59–61). It has been shown that under phosphorous and sulfur deficiencies a decrease in the rate of oxygen evolution is observed that correlates with a systematic transition to State 2 and a loss of the ability to reduce the PQ pool (60). Nitrogen starvation also induces a systematic transition to State 2 (61) due to over-reduction of the PQ pool and a loss of photosynthetic activity (59). It seems therefore that the transition from State 1 and State 2 is a common response to a-biotic stress, which might protect the photosynthetic ability to perform ATP synthesis (see also Ref. 60 for a further discussion), thus allowing the maintenance of vital processes.

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Photoinhibition of *Chlamydomonas reinhardtii* in State 1 and State 2: DAMAGES TO THE PHOTOSYNTHETIC APPARATUS UNDER LINEAR AND CYCLIC ELECTRON FLOW

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