The Light Sensitivity of ATP Synthase Mutants of *Chlamydomonas reinhardtii*[1]

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Chlamydomonas reinhardtii mutants defective in the chloroplast ATP synthase are highly sensitive to light. The ac46 mutant is affected in the *MDH1* gene, required for production or stability of the monocistronic *atpH* mRNA encoding CF$_O$-III. In this and other ATP synthase mutants, we show that short-term exposure to moderate light intensities—a few minutes—induces an inhibition of electron transfer after the primary quinone acceptor of photosystem II (PSII), whereas longer exposure—several hours—leads to a progressive loss of PSII cores. An extensive swelling of thylakoids accompanies the initial inhibition of electron flow. Thylakoids deflate as PSII cores are lost. The slow process of PSII degradation involves the participation of ClpP, a chloroplast-encoded peptidase that is part of a major stromal protease Clp. In the light of the above findings, we discuss the photosensitivity of ATP synthase mutants with respect to the regular photoinhibition process that affects photosynthetic competent strains at much higher light intensities.

The unicellular alga *Chlamydomonas reinhardtii* has long been used for the isolation of photosynthesis mutants because it can grow in heterotrophic conditions using acetate as an exogenous source of reduced carbon (Levine and Goodenough, 1970). In addition, strains showing various photosynthesis defects can be distinguished based on their fluorescence yield at room temperature (Bennoun and Delepelaire, 1982). This screening criterium is based on the early discovery (Duysens and Sweers, 1963) that the chlorophyll fluorescence yield at room temperature is primarily determined by the redox state of the primary quinone acceptor of photosystem II (Q$_A$) that loses its fluorescence quenching properties when reduced. Thus, the identification of photosystem II (PSII)-minus, cytochrome b_6f-minus, or photosystem I (PSI)-minus mutants as high fluorescence colonies is easily understood in terms of a block in electron transfer at various steps of the photosynthetic reaction chain. The fluorescence screen used to identify ATP synthase-minus mutants (Bennoun et al., 1978), although very powerful, has not yet been interpreted in molecular terms. Such mutants have a wild-type fluorescence phenotype when analyzed in dim light (i.e. below 6 μE m^{-2} s^{-1}) but display a fluorescence induction curve similar to that of PSII mutants when transferred for several hours to moderate light (i.e. between 40 and 140 μE m^{-2} s^{-1}).

Here we report a detailed study of the structural and functional modifications that accompany the changes in fluorescence properties of ATP synthase mutants when transferred from dim light to moderate light (70 μE m^{-2} s^{-1}). We show that, subsequent to an early and reversible block in photosynthetic electron transfer, the mutants undergo a considerable thylakoid swelling before flattening upon selective photodestruction of PSII. The critical role of the chloroplast protease Clp in PSII degradation in these experimental conditions is demonstrated.

RESULTS

The ac46 Mutant Lacks CF$_O$-III Due to the Absence of the *atpH* Transcript

The ac46 mutant lacking chloroplast ATP synthase was chosen for this study because its primary defect is the absence of CF$_O$-III, a major chloroplast-encoded CF$_O$ subunit (Lemaire and Wollman, 1989a). The absence of CF$_O$-III in the ac46 mutant is documented on Figure 1A by an immunoblotting experiment using whole-cell protein extracts. Sizeable amounts of CF$_1$ may still assemble in this strain since the α, β, and γ subunits can still be detected by immunoblotting, but they are not associated with the thylakoid membrane (Lemaire and Wollman, 1989a). Thus, this mutant cannot be suspected to undergo any proton leakage accompanying an assembly defect in ATP synthase since it lacks the major subunit of the proton channel.

Since ac46 is a nuclear mutant, the lack of synthesis of CF$_O$-III, a chloroplast-encoded subunit, points to a mutation in a nuclear gene that could encode a factor specifically controlling the expression of the chloroplast *atpH* gene at the posttranscriptional level. As previously described (Drapier et al., 1998), the *atpH*
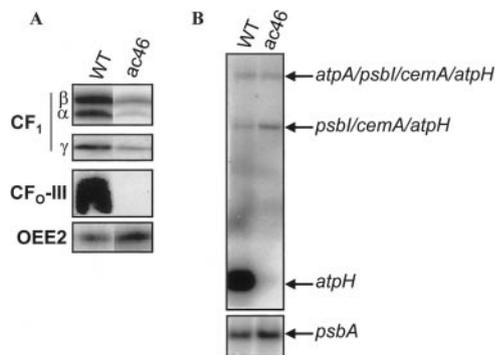
Figure 1. Characterization of the ac46 mutant. A, Immunoblots of whole cell protein extracts of the ac46 and WT strains, probed with specific antibodies for ATP synthase subunits. OEE2 was included as a loading control. B, Accumulation of the *atpH* transcripts in the ac46 and WT strains. The *psbA* probe was included as a loading control.

probe reveals three transcripts in the WT strain (Fig. 1B), *atpH* being the fourth gene in a polycistronic transcription unit containing *atpA*, *psbI*, *cemA*, and *atpH*. The monocistronic *atpH* transcript is specifically missing in ac46, whereas the di- and tricistronic transcripts are still present in similar amounts as in the wild type (Fig. 1B). Thus, the mutation defines a novel gene *MDH1*, whose mutated allele is *mdh1-ac46*, which is required for production or stability of monocistronic *atpH* mRNA.

Early Changes in Fluorescence of ATP Synthase Mutants Placed under Moderate Light

When kept in darkness or under low light, ATP synthase mutants of *C. reinhardtii* display fluorescence induction kinetics at room temperature that are similar to that of a wild-type strain (Joliot et al., 1998). This is shown on Figure 2A for ac46 grown under 6 μE m^{-2} s^{-1} and dark-adapted for 5 s before recording its fluorescence induction. The steady-state level of fluorescence (F_s) attained after a 1.4-s illumination in the apparatus (at 60 μE m^{-2} s^{-1}) remained well below the maximal fluorescence level (F_m) which can be measured by performing the same experiment in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), a PSII inhibitor that prevents reoxidation of the semiquinone Q_A^- by the secondary quinone acceptor of PSII (Q_B). This is indicative of an efficient electron transfer along the photosynthetic electron transfer chain insuring the rapid reoxidation of Q_A^- produced by PSII charge separation.

After as little as 4 min of pre-illumination at moderate light intensities (70 μE m^{-2} s^{-1}) followed by a 5-s dark adaptation period, ac46 displayed a dramatic change in its fluorescence induction kinetics (Fig. 2A). The fluorescence induction curve measured in the absence of DCMU was now very similar to that obtained in the presence of the inhibitor, with a rapid

rise from the initial fluorescence level (F_0) to an F_s level very close to F_m. This is reflected in a drop of the parameter ($F_m - F_s$)/F_v (Table I) and indicates an inhibition of electron transfer beyond Q_A. However, the persistence of a large variable fluorescence $F_v = F_m - F_0$ demonstrated that PSII charge separation and stable Q_A reduction still occurred. Similar changes in the fluorescence induction curves were observed with three other ATP synthase mutants: *atpC1* (Smart and Selman, 1991), *atpA-Fud16;ncc1* (Ketchner et al., 1995), and *tbc1-F54* (Drapier et al., 1992) (Table I). In contrast, the wild-type strain retained efficient electron transfer beyond Q_A after 4 min preillumination at 70 μE m^{-2} s^{-1}; F_s remained well below F_m (Fig. 2B and Table I). The kinetics of these early changes in fluorescence induction parameters of ATP synthase mutants upon a preillumination at 70 μE m^{-2} s^{-1} are shown for ac46 in Figure
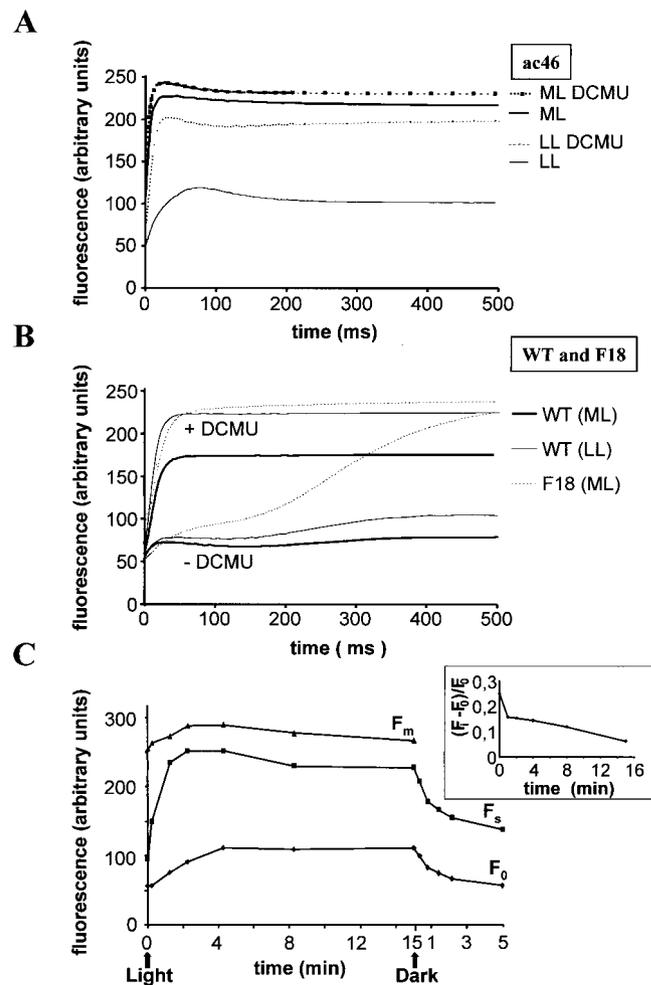
Figure 2. Early changes in room temperature fluorescence. Fluorescence induction kinetics were recorded with or without DCMU either after incubation in low light (LL = 6 μE m^{-2} s^{-1}) or after 4-min exposure to moderate light (ML = 70 μE m^{-2} s^{-1}). A, ac46; B, wild type and cytochrome b_6f mutant F18; C, evolution of F_0, F_s, and F_m during exposure of ac46 to moderate light. Inset, Evolution of ($F_i - F_0$)/F_0.

Table I. *Evolution of the fluorescence parameters in wild type and ATP synthase mutants after various times of illumination at 70 $\mu E\ m^{-2}\ s^{-1}$*

After 5 s dark adaptation, actinic light (60 $\mu E\ m^{-2}\ s^{-1}$) is turned on and the F_0 and F_s parameters are recorded at time 0 and 1.4 s, respectively. F_i and F_m are measured in the same manner except that DCMU is added during the dark adaptation period. The variable fluorescence F_v is $F_m - F_0$. The normalized parameters F_v/F_m and $(F_m - F_s)/F_v$ reflect the activity of stable PSII charge separation and the activity of electron transfer beyond Q_A, respectively. F_m at time t is indicated relative to time 0 of illumination at 70 $\mu E\ m^{-2}\ s^{-1}$.

	Time in Medium Light									
Strains	0			5 min			24 h		48 h	
	F_v/F_m	$(F_m - F_s)/F_v$	F_m	F_v/F_m	$(F_m - F_s)/F_v$	F_m	F_v/F_m	F_m	F_v/F_m	F_m
			%			%		%		%
ac46	0.77	0.61	100	0.52	0.13	115	0.28	68	0.24	25
F54	0.68	0.70	100	0.50	0.19	125	0.45	63	0.22	21
atpC1	0.67	0.70	100	0.53	0.19	127	0.37	63	0.30	25
atpA-Fud16;ncc1	0.64	0.74	100	0.44	0.10	126	0.35	100	0.19	52
Wild type	0.70	0.57	100	0.72	0.75	96	0.69	94	0.65	102

2C. The increase in F_s was completed within the first 2 min of preillumination reaching about 85% of the F_m value. The rise at F_s was indicative of a rapid and drastic decrease in the efficiency of Q_A^- reoxidation. In addition to the rise of F_s, we observed a limited but significant increase at F_m (Fig. 2B) that is due in part to an increased state I configuration (see "Discussion"). The inhibition of electron transfer in ac46 was reversible, as shown by the rapid decrease ($t_{1/2} = 1.5$ min) of the F_s level back to its initial level when ac46 was placed back into darkness after a 15-min illumination under moderate light.

The decrease in the rate of Q_A^- reoxidation in ATP synthase mutants is likely to result from the light-induced acidification of the lumen. This acidification should be caused by the release of protons, coupled to photosynthetic electron transfer, that cannot be translocated back to the stroma in the absence of an ATP synthase. We first considered a possible block in the reoxidation of plastoquinol (PQH_2) by the cytochrome b_6f complex. The latter reaction is coupled to a release of protons in the lumen and is therefore predicted to be considerably slowed down when lumenal pH becomes acidic (Hope, 1993; Finazzi and Rappaport, 1998). We compared the fluorescence induction behavior of ac46 exposed to moderate light with that of the cytochrome b_6f mutant F18 (Lemaire et al., 1986) blocked in PQH_2 reoxidation. As shown in Figure 2B, this mutant still displays a large difference in the rate of fluorescence rise with and without DCMU. This difference reflects the larger number of electron acceptors after PSII that are available without DCMU, i.e. the plastoquinone pool, whereas only the primary acceptor Q_A is available when the inhibitor is present. Thus, the similar fluorescence rises with and without DCMU that we observed with ac46 exposed to moderate light point to an impairment of electron transfer to the PQ pool. In order to rule out the possibility that the PQ pool had remained fully reduced in the 5 s of dark adaptation after preillumination of ac46, we added benzoquinone 5 s prior to the measurement of the fluorescence induction. This

treatement known to oxidize at least part of the PSII electron acceptors (Lavergne, 1984; Joliot and Joliot, 1985) did not restore electron transfer beyond Q_A in light-treated ATP synthase mutants (data not shown).

To directly test the hypothesis that electron transfer would be blocked between Q_A and Q_B, we compared the initial fluorescence levels observed in the absence (F_o) and presence (F_i) of DCMU. In vivo, F_i is usually higher than the genuine F_0 because binding of the inhibitor to the Q_B pocket requires that it is not occupied by a semiquinone (Wollman, 1978). In the fraction of centers with a semireduced Q_B^- acceptor in the dark, DCMU binding occurs only when the electron is transferred back to Q_A. Consequently, an interruption of electron transfer between Q_A and Q_B should prevent a DCMU-induced rise of the initial fluorescence level. Indeed, when the difference ($F_i - F_0$)/F_0 was plotted as a function of time of illumination under 70 $\mu E\ m^{-2}\ s^{-1}$ (Fig. 2C, inset), a rapid decrease was observed. The loss of the DCMU-induced rise of the initial level of fluorescence developed together with the inhibition of electron flow. Taken together, our results strongly suggest that electron transfer is blocked between Q_A and Q_B in light-treated ATP synthase mutants.

Light-Induced Thylakoid Swelling in ATP Synthase Mutants

Ultra-thin sections of ac46 were prepared at various time points of illumination at 70 $\mu E\ m^{-2}\ s^{-1}$. Prior to exposure to moderate light, low-light-grown ac46 displayed a chloroplast ultrastructural organization very similar to that in a wild-type strain: a dense array of thylakoid membranes extended along a large cup-shaped chloroplast (Fig. 3A). The most conspicuous change that developed upon 70 $\mu E\ m^{-2}\ s^{-1}$ illumination was an extensive swelling of the thylakoids, which allowed clear observation of the lumenal space. This change was already apparent after 30 min (Fig. 3B) and increased during the first
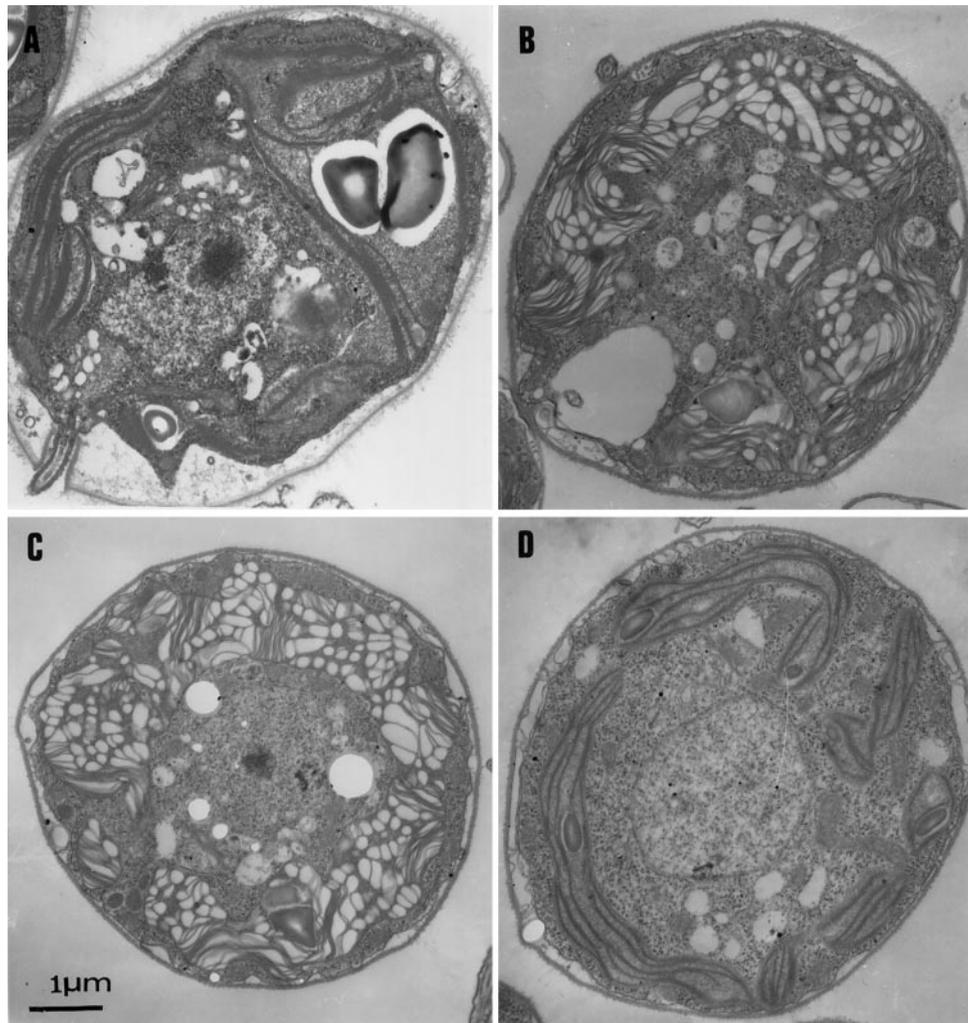
Figure 3. Light-induced thylakoid swelling in ac46. Cells were fixed in low light (A), or after exposure to moderate light for 30 min (B) or 3 h (C). D, Control experiment, wild-type cells exposed for 3 h to moderate light.

3 h of illumination (Fig. 3C). A similar swelling was observed with F54 and other ATP synthase mutants (data not shown), but not with a wild-type strain (Fig. 3D).

The above observations suggested that swelling resulted from the light-induced electro-chemical proton gradient ($\Delta\tilde{\mu}_{H^+}$) that accumulated in the absence of ATP synthase activity. Therefore, we preincubated ac46 with uncouplers [200 μM Crown, 5 μM carbonylcyanide-4-tri(fluoromethoxy)phenylhydrazone]. These high concentrations of uncouplers were necessary to shorten the half time of the light-induced transmembrane electric field to less than 20 ms (F. Rappaport and F.-A. Wollman, unpublished data). Upon illumination, the uncoupler-treated ac46 cells no longer displayed any light-induced swelling (Fig. 4A).

Since the light-induced $\Delta\tilde{\mu}_{H^+}$ results from proton translocation across the membrane upon photosynthetic electron flow, we also assessed the behavior of ATP synthase mutants that were altered in their electron transfer capacity. The double mutant F54-F18,

which lacks the ATP synthase and displays neither linear nor cyclic electron transfer due to the absence of the cytochrome b_6f complex, showed no light-induced thylakoid swelling (Fig. 4B). Strangely enough, the double mutant F54-F34, devoid of ATP synthase and PSII, did not undergo thylakoid swelling either (Fig. 5A), despite the fact that it should still be capable of cyclic electron flow between cytochrome b_6f and PSI. This unexpected finding was confirmed with ac46 cells when illuminated in the presence of DCMU. No swelling was observed in these conditions (Fig. 5B). Here again, the inhibitor should selectively inhibit linear electron flow from PSII to PSI but not PSI-driven cyclic electron flow. We conclude that the ATP synthase mutants were not able to perform PSI-driven cyclic electron flow at a rate high enough to establish the $\Delta\tilde{\mu}_{H^+}$ required for swelling. It has been suggested (Vallon et al., 1991; Finazzi et al., 1999) that cyclic electron flow is active mostly in state II, i.e. when cytochrome b_6f moves to unappressed membranes in the vicinity of PSI. Our
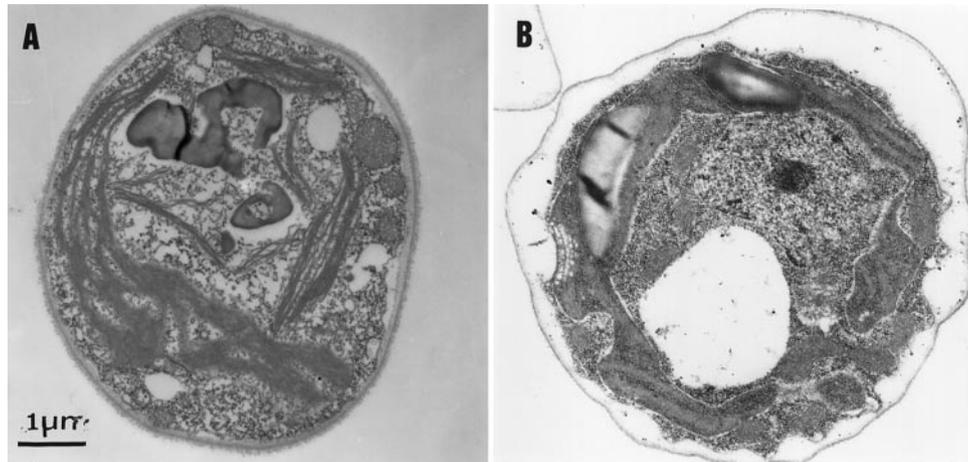
Figure 4. Light-induced swelling is prevented by addition of uncouplers or the absence of cytochrome b_6f. A, ac46 treated with uncouplers [200 μM Crown, 5 μM carbonylcyanide-4-tri(fluoromethoxy)phenylhydrazone], then exposed to moderate light for 3 h. B, F54-F18 exposed to moderate light for 3 h.

experimental conditions (illumination of cells with impaired PSII activity) should instead cause a transition to state I, due to a light-induced oxidation of the PQ pool (Keren and Ohad, 1998). Therefore, we repeated our experiments with strains that were preadapted to state II before illumination. This was achieved by adding Glc/Glc oxidase to the resuspension buffer prior to illumination, in order to establish anaerobic conditions (Wollman and Delepelaire, 1984). Remarkably, pretreatment in state II restored a light-induced thylakoid swelling both in the F34-F54 double mutant and in the ac46 mutant treated with DCMU (Fig. 5, C and D). Thus, the $\Delta\tilde{\mu}_{H^+}$ responsible for swelling can be generated by linear as well as cyclic electron transfer, the latter only if the cells are placed in State II.

Effects of Long-Term Exposure to Moderate Light

Much to our surprise, ac46 cells showed flattened thylakoids after 48 h at 70 μE m^{-2} s^{-1} (Fig. 6A). They appeared only slightly swollen and more densely stacked than in cells that were continuously kept under dim light. Thus, subsequent to their swelling, ac46 thylakoids undergo a deflation process. This deflation starts between 3 and 6 h of exposure to moderate light, as shown on Figure 6B where we have quantified the average width of the thylakoids at various time points of illumination.

We first considered that prolonged swelling could have caused mechanical damage to the thylakoids, so that membrane leakiness would increase sufficiently to allow flattening. The stability of the light-induced electrochromic shift at 515 nm is a well-established probe of the ionic conductance of the thylakoid membrane in vivo (Junge and Witt, 1968). The 515-nm signal rises with the transmembrane electric field in two steps: in the sub-microsecond time range upon charge separation within the reac-

tion centers and then more slowly, in the millisecond time range, with the electron transfer across the cytochrome b_6f complex. In a wild-type strain, the latter phase of rise is most often truncated by a rapid decay phase due to proton extrusion through the ATP synthase that collapses the light-induced electric field while generating ATP (Junge et al., 1970). Typical kinetics of a 515-nm absorbance change after one flash in the wild-type strain are presented on Figure 7 (double crosses), showing a 15-ms half-time of decay of the electric field. As we reported previously (Lemaire and Wollman, 1989a), ATP synthase mutants display a long-lived, light-induced 515-nm absorbance change since the transmembrane electric field is no longer consumed by the ATP synthase. Thus, the slow phase of the electrochromic shift in ac46 adapted to dim light is now well resolved and the half-time of the decay phase is longer than 1 s (Fig. 7, crosses). It is interesting that the decay of the light-induced 515-nm absorbance change was similarly slow after 6 and 48 h of illumination, indicating that the thylakoid membranes retained their impermeability throughout the light treatment. Therefore, restoration of membrane flattening could not be attributed to an increased leakiness of the thylakoids.

A complete inhibition of photosynthetic electron transport could also end up in membrane flattening since it would extinguish the source of proton accumulation in the thylakoid lumen. Figure 8 shows the changes at F_m and F_0 over a 48-h time period of exposure to moderate light. Besides the initial rise at F_0 and F_m that was better resolved in Figure 2B, two subsequent phases were observed. A further rise of F_m up to 6 h is probably correlated with changes in thylakoid swelling, since the fluorescence yield in *C. reinhardtii* varies with the osmotic pressure applied to the cells (F.-A. Wollman and R. Delosme, unpublished data). It is followed by an extensive fluores-
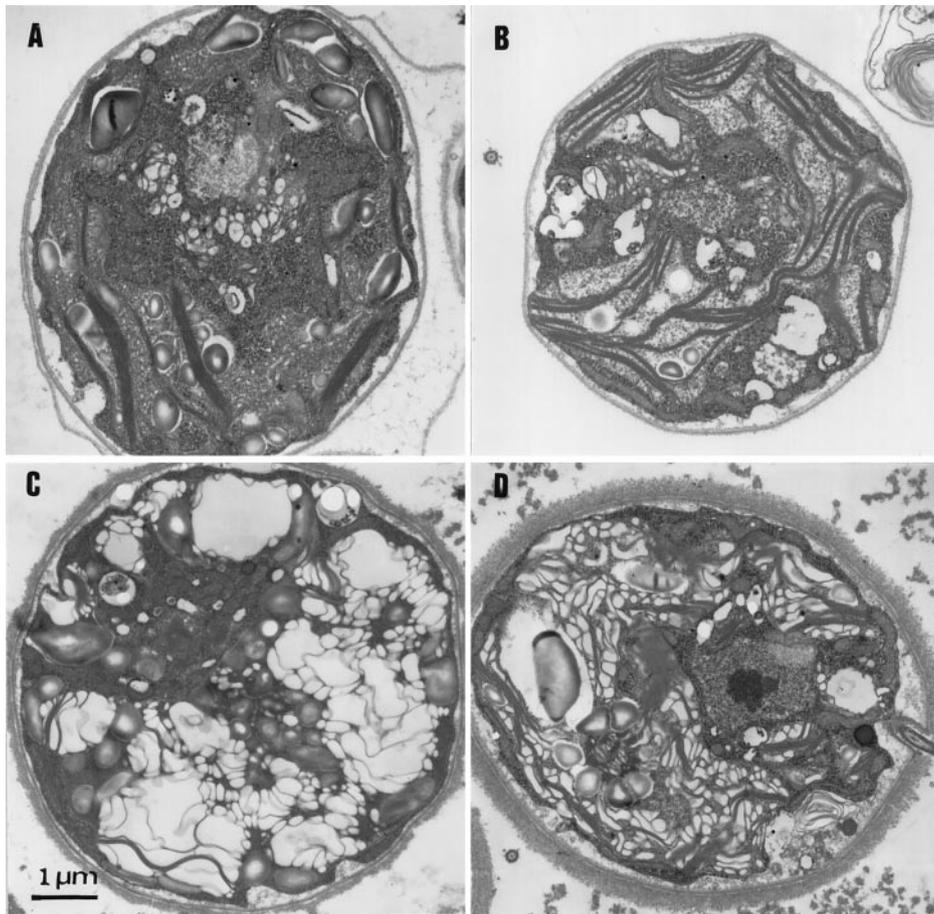
Figure 5. Effect of state transitions on thylakoid swelling in the absence of PSII-driven electron transfer; F54-F34 double mutant lacking ATP synthase and PSII (A and C) and ac46 incubated with DCMU (B and D). Cells were exposed to moderate light for 30 min, either without Glc/Glc oxidase, i.e. in state I conditions (A and B), or with Glc/Glc oxidase, i.e. in state II conditions (C and D).

cence quenching, which is much more pronounced at F_m than at F_0. Thus, after 24-h illumination at moderate light, ac46 cells had lost most of their variable fluorescence, which is indicative of a loss of PSII charge separation. A similar evolution of the parameters of fluorescence induction was recorded during light exposure of the *atpC1*, *atpA-Fud16;ncc1*, and F54 strains (Table I).

The loss of PSII activity correlated with a major change in PSII content in ac46. Freeze-fracture analysis of *C. reinhardtii* thylakoid membranes allows visualization of the various transmembrane particles that correspond to the major photosynthetic proteins (for review, see Staehelin, 1986). Figure 9 shows the exoplasmic fracture face (EF) of ac46 grown in dim light and after 48-h illumination at 70 μE m^{-2} s^{-1}. Most of the intramembrane particles of the EF face correspond to PSII cores (Olive et al., 1979). While ac46 grown under dim light displayed EF faces covered with large particles at a density close to that in the wild type (Table II), their number dropped drastically after 48-h exposure to moderate light. Quan-

tification of the EF particle density at various time points (Table II) showed that the loss started at 6 h and was completed at 24 h, in parallel with the decline of thylakoid swelling and variable florescence. More than 50% of the EF particles were lost during this time period, which suggests that some disassembly or degradation of the PSII core complexes had occurred upon prolonged illumination. The actual loss of PSII subunits upon illumination of ac46 was demonstrated in western blot analysis. A marked decrease in the content of CP43, CP47, and D2 was observed between 6 and 24 h (Fig. 10A). This loss was specific of the PSII complex since neither the cytochrome b_6f nor the PSI content varied in the same conditions (Fig. 10A). Taken together, the above data provide the molecular basis for membrane flattening upon prolonged illumination of ac46. Because of the degradation of PSII proteins, ac46 becomes similar to a PSII-ATP synthase double mutant that is unable to perform cyclic electron flow in these state I conditions. Thus, $\Delta\tilde{\mu}_{H^+}$ collapses and membranes resume a flat configuration.
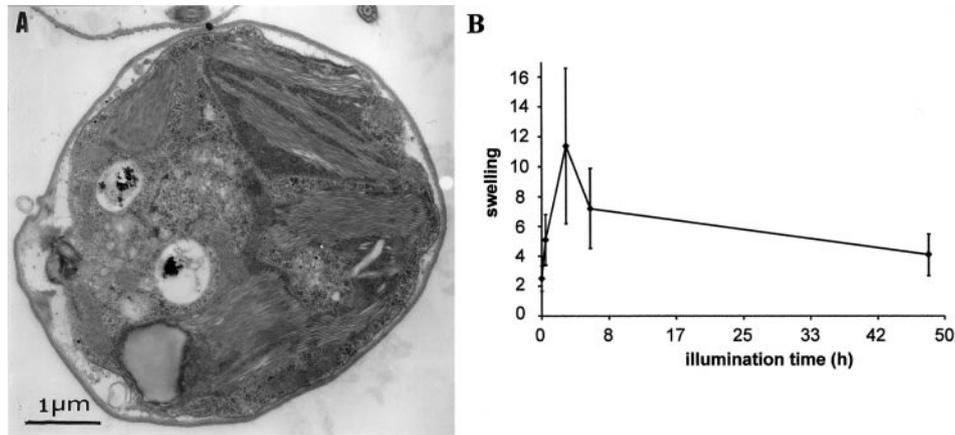
Figure 6. Loss of thylakoid swelling upon long-term exposure to light. A, ac46 cells exposed to 70 μE m^{-2} s^{-1} light for 48 h. B, Evolution of the average thylakoid width (arbitrary units) during light treatment.

## PSII Degradation Is Controlled by the Amount of Clp Protease

In a previous study (Majeran et al., 2000), we have shown that ClpP, the chloroplast-encoded peptidase subunit of the stromal protease Clp, contributes to the degradation of a thylakoid membrane protein, the cytochrome b_6f complex. In order to examine whether the Clp protease is also involved in the light-induced degradation of PSII in ATP synthase mutants, we constructed double mutants combining a nuclear mutation preventing ATP synthase accumulation (*mdh1-ac46*) with the chloroplast *clpP-AUU* mutation that leads to an attenuation of translation initiation for ClpP. In the double mutant ac46-AUU (*mdh1-ac46;clpP-AUU*), the accumulation of ClpP was reduced, as expected, to about 20% of that in ac46 (data not shown). The double mutant showed the same fluorescence behavior as ac46, which indicated that PSII inactivation still occurred in this strain (data not shown). However, there was only a limited de-

crease in the content in EF particles during exposure to 70 μE m^{-2} s^{-1} (Table I). Accordingly, immunoblotting experiments indicated a moderate loss of integral PSII subunits between 6 and 24 h of illumination (Fig. 10B). Similar results (data not shown) were obtained when comparing the F54 (*tda1-F54*) ATP synthase mutant to a double mutant F54-AUU (*tda1-F54;clpP-AUU*).

In order to directly measure the degradation rate of PSII subunits, chase experiments were performed in which lincomycin was added at the beginning of the illumination period. This antibiotic blocks translation of chloroplast proteins shortly after initiation so that the fate of preexisting proteins can be analyzed by immunoblotting without interference from de novo protein synthesis. In these conditions, we observed an increase in the half-life of CP47 and CP43 in the F54-AUU double mutant compared to the F54 parent (Fig. 10C). Similar results were obtained with strains ac46 and ac46-AUU (data not shown). We conclude that the proteolytic disposal of PSII cores that occurred in ATP synthase mutants after PSII inactivation involves, directly or indirectly, the Clp protease.
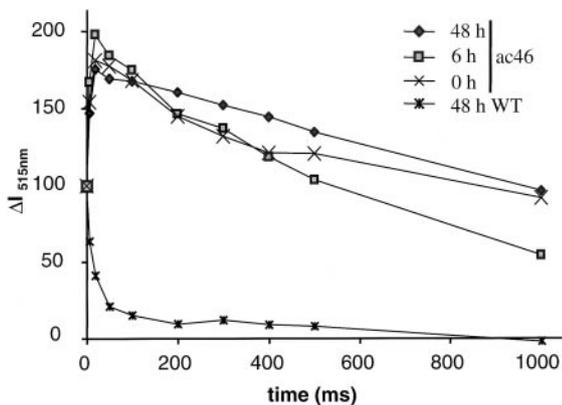
Figure 7. Flash-induced electrochromic signal recorded at 515 nm. PSII activity is inhibited by DCMU + hydroxylamine prior to the measurements. Curves are normalized to the electrochromic shift recorded 100 μs after the actinic flash (phase a, representing PSI charge separation). Times of exposition to moderate light are indicated in hours.
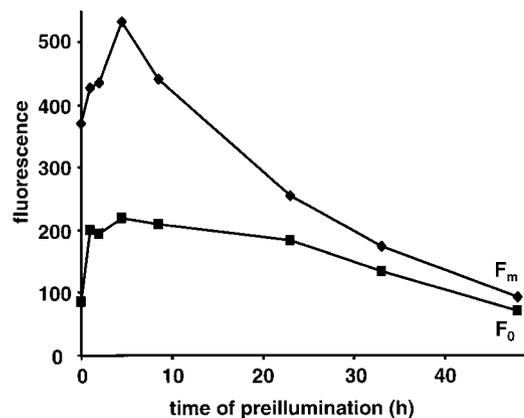
Figure 8. Changes at F_m and F_O upon prolonged exposure of ac46 to moderate light.
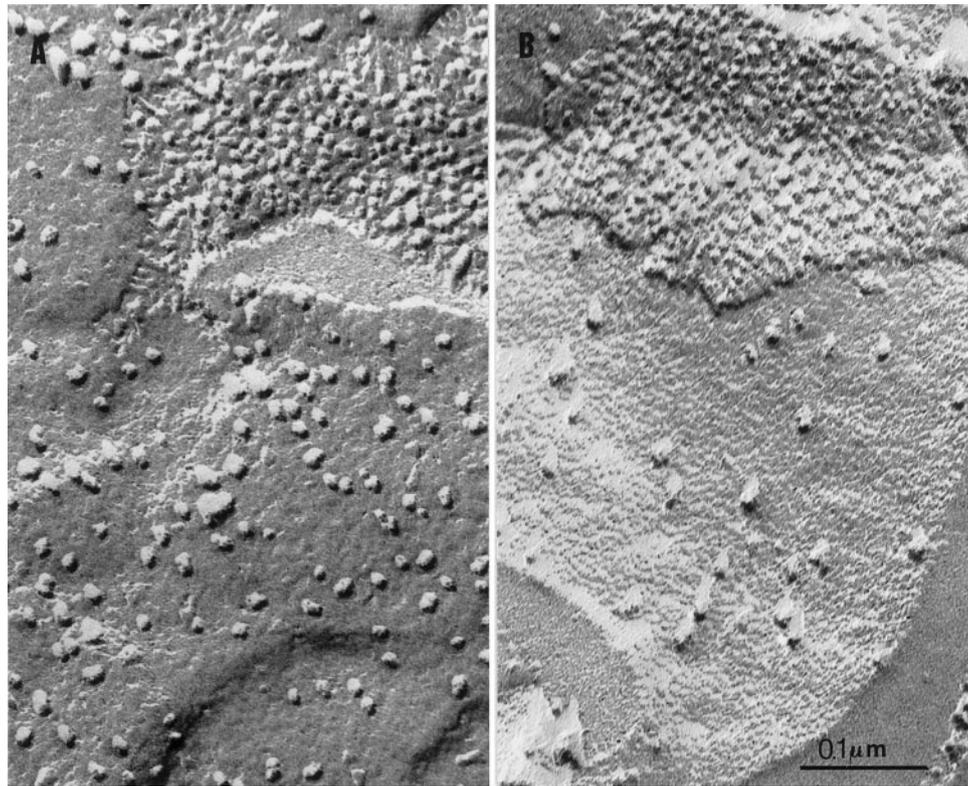
Figure 9. Freeze fracture analysis of ac46 grown in low light (A) or after 48-h exposure to 70 μE m^{-2} s^{-1} light (B). Note the loss in most of the large EF particles, whereas protoplasmic faces (top right) remain unaltered.
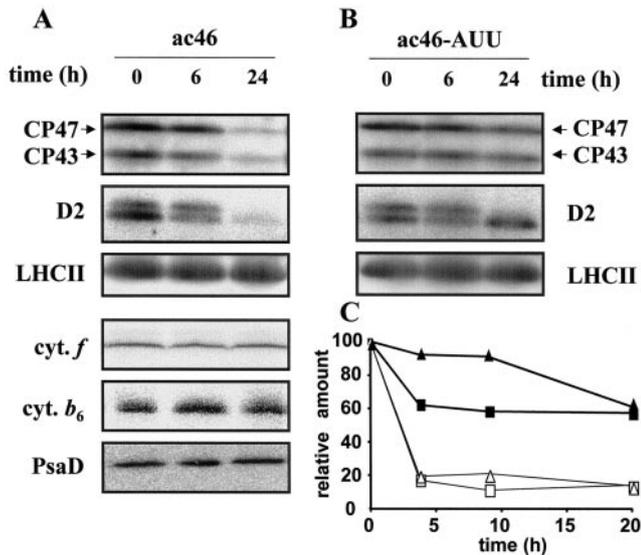
DISCUSSION

The present analysis of the physiological changes induced by exposure to moderate light in *C. reinhardtii* mutants lacking the ATP synthase provides the basis for their genetic screening as high fluorescence colonies (Bennoun et al., 1978). The ac46 mutant has been chosen for this analysis because it lacks the CF_O ATP synthase subcomplex. This mutant has been described as lacking synthesis of CF_O-III, the proteolipid subunit that forms the bulk of the CF_O transmembrane channel (Lemaire and Wollman, 1989a). Here we confirm the absence of CF_O-III accumulation and present a molecular characterization of

the primary defect: the absence of the monocistronic *atpH* mRNA. This mutation defines a novel gene *MDH1* that is required for the production or stability of the monocistronic *atpH* mRNA. It is interesting that the di- and tricistronic mRNAs were still present in normal amounts. Thus, polycistronic messengers in which *atpH* is the downstream cistron appear incompetent for translation of subunit III.

When kept under dim light, ATP synthase mutants behave as wild type, showing a low steady-state level of fluorescence, owing to an efficient electron flow between PSII and PSI. However, after illumination at moderate light intensities (40–140 μE m^{-2} s^{-1}), they behave as high fluorescent strains. The various

Table II. *EF particle content in wild type, ac46, and ac46-AUU membranes grown in dim light and after 6, 24, and 48 h illumination at 70 μE m^{-2} s^{-1}*

Strain	Illumination Time at 70 μE m^{-2} s^{-1}			
	0	6	24	48
			h	
Wild type	1,248	1,368	1,208	1,280
	942–1,547	896–1,633	1,025–1,284	1,111–1,711
ac46	1,039	948	434	463
	867–1,584	806–1,250	290–685	260–672
Ac46-AUU	1,311	1,021	1,185	1,188
	981–1,710	846–1,314	883–1,536	1,006–1,815

Figure 10. Accumulation of photosynthetic complexes in ATP synthase mutants after exposure to moderate light. A, ac46 was exposed to moderate light for 0, 6, and 24 h. PSII, PSI subunits, and cytochrome b_6 were detected by immunoblotting, cytochrome f by TMBZ staining. B, Strain ac46-AUU treated as in A. Only PSII subunits are shown. C, Strains F54 (white symbols) and F54-AUU (black symbols) were exposed to moderate light in the presence of lincomycin, an inhibitor of chloroplast protein synthesis. The levels of CP47 (squares) and CP43 (triangles) were measured by immunoblotting and phosphoimager quantification (ImageQuant software, Molecular Dynamics, Sunnyvale, CA).

changes in functional and supramolecular organization of the photosynthetic apparatus in an ATP synthase mutant over a 48-h illumination period at 70 μE m^{-2} s^{-1} are summarized on the scheme of Figure 11.

In wild-type plants and algae, a well-documented effect of a large $\Delta\tilde{\mu}_{H^+}$ is to induce qE, the "energetic" component of non-photochemical quenching (for review, see Lavergne and Briantais, 1995; Yamamoto and Bassi, 1996). In this respect, it is paradoxical that ATP synthase mutants that should accumulate a large electrochemical gradient upon illumination do not show rapid fluorescence quenching. qE could be ineffective in our experimental conditions. The sequence of events that transduce the $\Delta\tilde{\mu}_{H^+}$ signal into a quenching state for excitons encompasses deepoxidation of violaxanthin to antheraxanthin and zeaxanthin, followed by nonradiative dissipation of light energy through a mechanism that remains poorly understood. It has been reported that the capacity for antheraxanthin and zeaxanthin formation depends on the light intensity used for growing plants (Demmig-Adams et al., 1995) or algae (Niyogi et al., 1997). In particular, the wild-type strain of *C. reinhardtii* that contains exclusively violaxanthin when grown at 100 μE m^{-2} s^{-1} requires exposure to 800 μE m^{-2} s^{-1} to display conversion to antheraxanthin and zeaxanthin (Niyogi et al., 1997). Thus, there is a threshold of light intensity, independent of $\Delta\tilde{\mu}_{H^+}$, under which nonphotochemical quenching does not

develop in *C. reinhardtii*. The activity of the xanthophyll cycle of the ATP synthase mutants that are grown in dim light and exposed to only 70 μE m^{-2} s^{-1} may be too low to elicit transduction of the $\Delta\tilde{\mu}_{H^+}$ effect.

The high fluorescence behavior of ATP synthase mutants can be separated kinetically into two phases that develop on widely different time scales. Within the first 5 min of illumination at 70 μE m^{-2} s^{-1}, most of the ability to reoxidize photoreduced Q_A is lost. Therefore, the mutant shows a large variable fluorescence nearly reaching F_m even in the absence of DCMU. Then, between 6 and 48 h illumination, the number of active PSII centers decreases steadily and the PSII proteins are degraded. The mutant is initially blocked in a high fluorescence state. After 6 h, a fluorescence quenching develops, leading the mutant cells to lose their high fluorescence properties together with their PSII activity.

The primary defect of a chloroplast ATP synthase mutant resides in its inability to use the proton motive force that develops across the thylakoid membranes during photosynthetic electron transport. During illumination, the lumenal space is then expected to undergo a progressive acidification, limited only by proton leakage and conversion to transmembrane electric potential. Therefore, we suspected that inactivation of photosynthetic electron transport could result from an alteration of a pH-dependent reaction occurring at the inner side of the thylakoid membrane (for review, see Kramer et al., 1999). In particular, lumen acidification can be expected to impair the oxidation of PQH_2 at the Q_o site of the cytochrome $b_6 f$ complex, a reaction that requires the release of two protons in the lumenal space (for review, see Hope, 1993). One of the predicted consequences is an increase of F_s, indicative of a more reduced state of the PQ pool under illumination. In this case, however, a substantial difference should remain between the fluorescence induction curves with and without DCMU, reflecting the need to reduce the PQ pool before reaching a high level of fluorescence in the latter case. In our experimental setup, the 5 s of dark adaptation are sufficient to ensure reoxidation of most of PQH_2 accumulated during illumination, as documented in Figure 2B
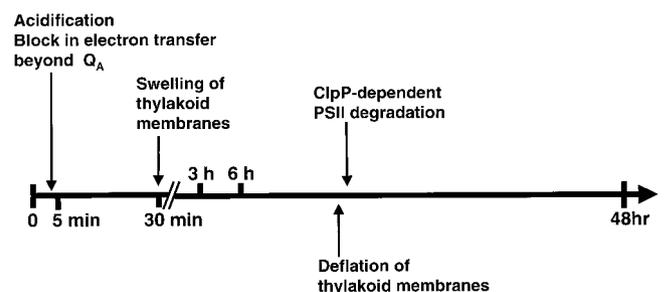
Figure 11. Summary of the major events occurring upon illumination of an ATP synthase mutant at 70 μE m^{-2} s^{-1}.

with a cytochrome b_6f mutant. Furthermore, the rapid fluorescence rise observed in illuminated ATP synthase mutants was also observed in the presence of benzoquinone, an oxidant of the PQ pool (Bulte and Wollman, 1990). Hence, inactivation of the Q_o site, even though it may well occur, is not the cause of the fluorescence rise observed in light-treated ATP synthase mutants.

Our results rather suggest a rapid block between Q_A and Q_B. The induction curves with and without DCMU become similar. This inhibitor binds to the Q_B pocket by displacing Q_B but not Q_B^- (Velthuys, 1981). Because in darkness a fraction of PSII centers are found in the Q_B^- state (Wollman, 1978), these centers will bind the inhibitor only when the electron from the charged semiquinone is visiting Q_A, an event with low but significant probability, with the equilibrium constant between Q_A/Q_B^- and Q_A^-/Q_B being about 20 (Diner, 1977). Thus, addition of DCMU will normally increase the initial fluorescence, by blocking these centers in the Q_A^- state. Our finding (Fig. 2C) that light treatment of ATP synthase mutants decreased the effect of DCMU on the initial fluorescence level is therefore in favor of a block of electron transfer between Q_A and Q_B. In essence, PSII centers become similar to the "non Q_B-reducing" centers (Lavergne and Briantais, 1995), closed after a single turnover, which have been described in plants and algae and can make up between 10% and 35% of PSII centers under certain growth conditions.

The mechanism by which proton accumulation in the lumen causes such dramatic changes at the stromal acceptor side of PSII is still unclear. However, studies by Krieger and coworkers provide interesting clues. They have shown that inactivation of the donor side affects the mid-point redox potential (E_m) of Q_A, raising it from -80 mV to $+65$ mV (Krieger and Weis, 1992). This probably reflects a physiologically significant conformational change of the PSII center, especially since photoactivation of PSII in dark-grown cells induces a change in the reverse direction, from $+110$ mV to -80 mV (Johnson et al., 1995). These changes appear to correlate with the Ca^{2+} binding capacity of the donor side. Interestingly, incubation of PSII membranes at low pH has been shown to cause Ca^{2+} release, thus inactivating oxygen evolution (Ono and Inoue, 1988). We propose that the rapid acidification of the lumen upon illumination of an ATP synthase mutant could raise the E_m of Q_A to a value too positive to allow downstream electron transfer to Q_B and the plastoquinone pool, whose $E_{m(pH8)}$ is about $+30$ mV (Thielen and van Gorkom, 1981). Such a change in the mid-point potential of Q_A would also explain both the rise of the initial fluorescence level (since Q_A would now be reducible by PQH_2) and its relative insensitivity to DCMU (since single-reduced centers would now have the charge mostly located on Q_A already before addition of the inhibitor).

Following the rapid block in PSII reoxidation, we observed a loss in variable fluorescence together with a quenching at F_m that slowly developed over the next 48 h of illumination. This behavior is highly reminiscent of the photoinhibition process observed in wild-type algae or in plants, by which excess illumination leads to loss of PSII activity (for review, see Keren and Ohad, 1998). Photoinhibition has been described as a two-step process: an initial reversible stage characterized by a block in electron transfer between Q_A and Q_B, and an irreversible stage characterized by a strong fluorescence quenching and a degradation of PSII proteins. Thus, the behavior of ATP synthase mutants in moderate light is strikingly similar to a regular photoinhibition process, the second step of which would be very much delayed. However, the presently described photoinhibition of ATP synthase mutants occurs at light intensities about $30\times$ lower than that required for wild-type cells.

Thylakoid swelling is another feature common to both processes. It has been observed by Topf et al. (1992) using wild-type cells of *C. reinhardtii* photoinhibited at 625 μE m^{-2} s^{-1}. Swelling appears as a result of an osmoregulation process driven by lumen acidification and has been suggested to result from amonium uptake into the lumen. In that study, mutants lacking either PSI or plastocyanin or cytochrome b_6f failed to show swelling upon photoinhibition, indicating that it is driven by photosynthetic electron flow. Our observation that a double mutant lacking the ATP synthase and cytochrome b_6f does not show swelling leads us to the same conclusion. In addition, our experiments with uncoupler-treated ATP synthase mutants clearly point to $\Delta\tilde{\mu}_{H^+}$ as the driving force for thylakoid swelling. The exact mechanism for swelling remains unknown, but it is unlikely that protons themselves act as the osmotic species because of the unacceptably low pH that this would entail in the lumen. Linear as well as cyclic electron flow should be capable of generating $\Delta\tilde{\mu}_{H^+}$. Our initial observation that the absence of PSII activity prevented swelling was therefore rather intriguing. However, we observed that swelling resumed when PSII-inactive cells were placed in state II prior to illumination. This finding supports the view that, in *C. reinhardtii*, cyclic electron flow is prevented in state I but is activated in state II, most likely due to the redistribution of cytochrome b_6f complexes along the thylakoid membranes (Vallon et al., 1991). It nicely corroborates the recent study by (Finazzi et al., 1999) that shows an interruption of linear electron flow when wild-type cells of *C. reinhardtii* are placed in state II.

It sounds paradoxical that one of the first effects of light in ATP synthase mutants is an impairment of linear electron transport, because this should in turn counteract the effect of illumination. As outlined by Ohad et al. (1994), the same paradox holds for photoinhibition in the wild type, where both donor and

acceptor side models predict that inactivation of PSII will protect the remaining centers. Here, in view of the extreme impermeability of the thylakoid membrane to H$^+$ in the absence of ATP synthase (Lemaire and Wollman, 1989b), we may postulate that even a limited electron transfer activity can maintain a high $\Delta\tilde{\mu}_{H^+}$ once the thylakoid ability to extrude H$^+$ is overwhelmed.

The loss of variable fluorescence in ATP synthase mutants was accompanied by a degradation of PSII centers, documented by the loss in the major PSII subunits and in the corresponding EF particles observed on freeze-fractured thylakoid membranes. Here again, the ATP synthase mutant behaved as a wild type upon photoinhibition, but at much lower light intensities and according to a much slower process. We show here that disposal of inactivated PSII centers in ATP synthase mutants depends largely on the action of the Clp protease. This view is supported by our finding that ATP synthase mutants where accumulation of ClpP was reduced by the *clpP-AUU* mutation display a reduced degradation rate of PSII proteins. This observation is strikingly similar to our previous finding that ClpP controls the rate of degradation of another multisubunit photosynthetic protein, cytochrome b_6f (Majeran et al., 2000). Thus, this soluble stromal protease participates in the degradation of large transmembrane proteins. Of possible significance in this regard is the observation that a small fraction of ClpP is consistently found associated with the membrane (W. Majeran and O. Vallon, unpublished observation). Similarly, the cytosolic proteasome is responsible for the degradation of membrane proteins of the endoplasmic reticulum (Wiertz et al., 1996).

However, our results do not imply that Clp is the sole protease carrying out PSII degradation. In fact, PSII degradation in photoinhibitory conditions can readily be observed in vitro, in conditions where ClpP is certainly absent from the preparation (Shipton and Barber, 1992). During photoinhibition, the D1 subunit is the first target of proteolysis, and its degradation in vitro occurs in two steps (De Las Rivas et al., 1992). First, a GTP-stimulated endoproteolytic cleavage generates a 10-kD and a 23-kD fragment. The latter is then degraded by an ATP- and Zn^{2+}-dependant protease, which may be the membrane-bound FtsH protease (Spetea et al., 1999). Hence, photoinactivation may direct the PSII center into two divergent routes of degradation, depending upon the experimental conditions. Indeed, we have found that attenuation of ClpP in a photosynthesis-competent strain does not retard degradation of D1 or the other PSII subunits during photoinhibition (W. Majeran and O. Vallon, unpublished observation). During high light photoinhibition, a conformational change has been implicated in targeting D1 for proteolysis (Zer et al., 1994). The signal that triggers degradation of PSII in ATP synthase mutants is generated under a much lower photon flux and may be of a different nature.

MATERIALS AND METHODS

Strains

All strains were grown under dim light (cool fluorescent white light, 6 μE m^{-2} s^{-1}) in Tris-acetate medium (Harris, 1989) at 25°C with gentle shaking. The *mdh1-ac46 atpC1*, *atpA-fud16;ncc1*, and *tda1-F54* nuclear and chloroplastic mutations that prevent formation of the chloroplast ATP synthase have been described before (Lemaire and Wollman, 1989a; Smart and Selman, 1991; Drapier et al., 1992; Ketchner et al., 1995). The *tbc1-F34* and *ccs3-F18* nuclear mutations preventing the assembly of PSII and cytochrome b_6f, respectively, have been previously described (Chua and Bennoun, 1975; Lemaire et al., 1986). They were combined in genetic crosses (Harris, 1989) with the chloroplast *clpP-AUU* mutation (Majeran et al., 2000), which decreases the accumulation level of ClpP protease. Double mutant segregants were analyzed for the presence of the mutated *clpP* gene by polymerase chain reaction, and the reduced ClpP protein level was checked by immunoblotting using a homemade anti-ClpP serum (Majeran et al., 2000).

Functional Measurements

Exponentially growing cells were concentrated by centrifugation to a final density of 2 × 10^7 cells mL^{-1}. After resuspension under dim light for 30 min, cells were placed under moderate light (70 μE m^{-2} s^{-1}), provided by a tungsten light source. For measurements of fluorescence induction at room temperature, cells were rapidly transferred to a 1-mL cuvette placed in a home-built fluorimeter (Joliot et al., 1998), and fluorescence induction was recorded after 5 s of dark adaptation. When necessary, 10 μM DCMU was added before measurement. The half-life of the light-induced transmembrane electric field was measured by that of the induced electrochromic shift at 515 nm as previously described (Joliot et al., 1998).

Electron Microscopy

For thin-section electron microscopy, cells were fixed by addition of 1% (w/v) glutaraldehyde under stirring in the light, pelleted by centrifugation and further fixed for 1 h on ice, after which they were treated as by Baldan et al. (1991). Freeze fracturing was performed as described (Baldan et al., 1991). Estimation of thylakoid swelling was achieved by measuring the average width of cross-sectioned thylakoids on micrographs of thin sections.

RNA and Protein Analysis

Total RNA was extracted and analyzed as by Drapier et al. (1998). An *atpH* probe corresponding to the entire reading frame was prepared by photosynthetic carbon reduction using oligonucleotides ATPH1 (5'-ACCCTA-TCGTAGCTGCAACTTCTGTT-3') and ATPH2 (5'-AAC-

CAGCGAATGGGTTAGCGAATAG-3′). Plasmid pcp60 (containing the R8 fragment of the chloroplast genome (Rochaix, 1978) was used as a template. The *Eco*RI fragment R14 of the chloroplast genome was used as a *psbA* probe.

Whole cell protein extracts were separated in urea/SDS polyacrylamide gels and then transferred to nitrocellulose as by de Vitry et al. (1989). Binding of polyclonal antibodies was detected with ^{125}I-protein A and phosphor-imaging. The monoclonal antibody to CF_O subunit III was detected with anti-mouse peroxidase-labeled antibody and enhanced chemiluminescence detection (Amersham, Orsay, France). Heme staining was detected using 3,3′,5,5′-tetramethylbenzidine (Thomas et al., 1976).

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