Nonphotochemical Quenching of Chlorophyll Fluorescence in *Chlamydomonas reinhardtii*

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ABSTRACT: Unlike plants, *Chlamydomonas reinhardtii* shows a restricted ability to develop nonphotochemical quenching upon illumination. Most of this limited quenching is due to state transitions instead of ΔpH-driven high-energy state quenching, qE. The latter could only be observed when the ability of the cells to perform photosynthesis was impaired, either by lowering temperature to ∼0 °C or in mutants lacking RubisCO activity. Two main features were identified that account for the low level of qE in *Chlamydomonas*. On one hand, the electrochemical proton gradient generated upon illumination is apparently not sufficient to promote fluorescence quenching. On the other hand, the capacity to transduce the presence of a ΔpH into a quenching response is also intrinsically decreased in this alga, when compared to plants. The possible mechanism leading to these differences is discussed.

The absorption of light in excess of the capacity for photosynthetic electron transport is damaging to photosynthetic organisms (*1*). That capacity is, however, variable, depending mainly on the efficiency of CO_2 assimilation. For example, decreases in temperature lower the rate with which electrons can be transported within the electron transport chain as well as the capacity of metabolic processes to assimilate the reductant that has been photoproduced. In higher plants, the availability of CO_2 is liable to be limiting under conditions of water stress, due to the closure of stomata. This will again inhibit photosynthetic assimilation. Under such conditions, cells are likely to experience oxidative stress, due to the uncontrolled formation of reactive oxygen species associated with the absorption of light by chlorophyll.

Under conditions of excess light, a variety of mechanisms are initiated which protect chloroplasts from damage. In higher plants, a number of processes have been identified, primarily through application of measurements of chlorophyll fluorescence yield. These are all associated with photosystem (PS)[1] II and are collectively referred to as nonphotochemical quenching mechanisms (NPQ; *1*). However, this term comprises at least three processes: (i) qI, mainly related to photoinhibition, a slowly reversible damage to PSII reaction centers (*2*, *3*), although data suggest that a zeaxanthin-dependent quenching might contribute substantially to this process (*4*, *5*), (ii) qT, state transitions, a change in the relative antenna sizes of PSII and PSI, due to the reversible phosphorylation and migration of antenna proteins (LHCII) (*6*), and (iii) qE, also termed "high-energy state quenching", a form of quenching associated with the development of a low pH in the thylakoid lumen (e.g., ref *7*).

High-energy state quenching is largely thought to be associated with an increase in thermal dissipation within the light-harvesting apparatus (*1*, *8*, *9*), associated with the generation of a ΔpH (*7*, *10*) and with the formation of zeaxanthin via deepoxidation of violaxanthin (*11*). However, in vitro at least, this term also encompasses acidic pH-induced processes in the PSII reaction center, specifically following the release of a Ca^{2+} ion associated with the water-splitting complex (e.g., ref *12*). We recently presented evidence suggesting a mechanistic link between these two forms of quenching in vivo, with a reaction center process occurring at the onset of quenching and then "migrating" to the antenna as the process develops (*13*).

Despite our growing understanding of the mechanism of thermal dissipation of excess energy (see, e.g., refs *14* and *15*), the knowledge of the regulatory mechanisms that modulate the nonphotochemical quenching response remains at a basic level. It is known that the capacity for nonphotochemical quenching of fluorescence varies extensively between photosynthetic organisms, but the factors that determine that variation are poorly understood.

Recent work has provided valuable information about the nature of NPQ, thanks to the isolation and characterization of mutants, first obtained in the green alga *Chlamydomonas reinhardtii* (*16*, *17*) and then in the C3 plant *Arabidopsis thaliana* (*18*, *19*). In the latter, these mutants have shown that the nonphotochemical quenching is an essential part of

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[1] Abbreviations: DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; $\Delta\mu_{H^+}$, electrochemical proton gradient; ΔΨ, electric component of the electrochemical proton gradient; HA, hydroxylamine; NPQ, nonphotochemical quenching of fluorescence; PS, photosystem.

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the plant response to stress, as indicated by their slower growth and reduced fertility when exposed to naturally varying conditions (*20*). On the other hand, a wide variability of the capacity for nonphotochemical quenching is commonly seen in photosynthetic organisms. When calculated according to Bilger and Björkman [NPQ = $(F_m - F_m')/F_m'$, where F_m represents the maximum fluorescence emission recorded in dark-adapted material while F_m' represents the maximum fluorescence value recorded at discrete time intervals during illumination; *21*], a very low level of NPQ, around 0.2−0.3, can be estimated in the marine picoeukaryote *Ostreococcus tauri* (G. Finazzi and H. Moreau, unpublished results). This value increases to 0.5 in cyanobacteria (*22*) and up to 12 in diatoms (*23*). In addition, it appears that not only the amplitude but also *the nature* of the NPQ response can vary between organisms, even within the two model organisms mentioned above. Indeed, while it is acknowledged that *Chlamydomonas* can undergo very large state transitions, a more limited capacity for qT has been reported in plants (see, e.g., ref *24*).

To further explore the cause of such variability, we have reanalyzed the nonphotochemical quenching response in the two model photosynthetic organisms employed for the study of NPQ, *Chlamydomonas* and *Arabidopsis*. We observe that, under optimal physiological conditions, the dominant route for dissipating energy in *Chlamydomonas* is through photochemistry, with a minor nonphotochemical quenching that involves state transitions. High-energy quenching in this alga appears only when the capacity to perform photosynthesis is inhibited. It has specific traits that are not observed in higher plants: a reaction center-based quenching, particularly long-lived, which poorly converts into an antenna-based quenching.

EXPERIMENTAL PROCEDURES

Growth Conditions. Chlamydomonas WT cells from strain 137C, RubisCO mutants 18.5 and 31.4 (*25*), and the NPQ-deficient mutant *npq1 lor1* (*16*) were grown in Tris−acetate−phosphate medium (TAP; *26*) under moderate illumination (6 μmol of photons m^{-2} s^{-1}), unless otherwise indicated. Cells were collected during the exponential phase and resuspended in a 20 mM HEPES buffer, pH 7.2, with 20% Ficoll to prevent sedimentation. All batches examined were dark adapted, with continuous shaking, for at least 4 h before use. *Arabidopsis* plants were grown on soil at an energy flux of 150 μmol of photons m^{-2} s^{-1} of white light under a 12 h dark−12 h light regime at a temperature of 25 °C.

Fluorescence Measurements. Fluorescence emission was measured using a laboratory-built instrument, as described elsewhere (*27*). This apparatus provides continuous illumination and saturating short light pulses (200 μs), provided by a green LED array (emission peak 520 nm, 30 nm full width at half-maximum). Fluorescence is measured in the near-infrared. For fluorescence measurements, *Chlamydomonas* cells were placed in a laboratory-built metal cuvette, containing a small glass chamber (∼20 μL), and used at the concentration of ∼5 × 10^7 cells mL^{-1}.

Spectroscopic Measurements. Spectroscopic measurements were performed using a laboratory-built spectrophotometer (*27*). Actinic light was provided by the same LED source employed in the case of fluorescence. Light-induced absorption changes were measured as absorption of flashed monochromatic light at discrete times. PSII charge separation was measured as the extent of the electrochromic signal at 515−545 nm, 100 μs after excitation with a single turnover laser pulse at 695 nm. This signal is linearly proportional to reaction center photochemistry (*28*). In our conditions, this deconvolution was sufficient to discriminate the eletrochromic shift from other superimposed signals (see also refs *27* and *29*). This was checked by measuring time-resolved spectra of the light-induced signals in the 500−600 nm region (not shown). PSII contribution was deduced as the difference between the signals measured in the absence and presence of the PSII inhibitors DCMU and hydroxylamine (HA; *30*). The latter compound was added to destroy the manganese cluster responsible for oxygen evolution and to slow recombination between the donor and acceptor side of PSII, which would preclude correct estimation of the PSI/PSII ratio. Measurements of continuous light-induced electrochromic signals were performed as previously described (*27*, *29*). Cytochrome *f* redox changes were calculated as the difference between the absorption at 554 nm and a baseline drawn between 545 and 573 nm (*31*) and corrected for the contribution of the electrochromic signal (5% of the signal observed at 515 nm; *31*).

In Vivo Phosphorylation of Antenna Proteins. Chlamydomonas cells grown at 3 × 10^6 cells mL^{-1} were harvested and resuspended in a phosphate-depleted medium containing 1 μCi mL^{-1} $^{33}P_i$. Then, they were treated as described in Wollman and Delepelaire (*32*).

Pigment Analysis. The pigments were extracted with 80% acetone and then separated and quantified by HPLC (*33*) and by fitting of the acetone extract with the spectra of individual pigments (*34*).

RESULTS

Light-Induced Photochemical and Nonphotochemical Quenching of Fluorescence in Chlamydomonas and Arabidopsis. Illumination of *Chlamydomonas* with saturating light intensity (1000 μmol of photons m^{-2} s^{-1}) resulted in the limited development of nonphotochemical quenching (Figure 1A,C), at variance with *Arabidopsis*, where a sustained NPQ was observed (Figure 1B,D). Previous work has underlined that the NPQ response can vary in plants grown at different light intensities (e.g., ref *35*). This prompted us to check the light dependence of the NPQ response in *Chlamydomonas*. No substantial differences were observed in *Chlamydomonas* cells grown heterotrophically at 6 μE m^{-2} s^{-1} (open triangles) or autotrophically at 100 μE m^{-1} s^{-2} (closed triangles) as in previous work (*16*, *17*). The same limited NPQ response was observed within a wide range of actinic light intensities. At 80 μmol m^{-2} s^{-1} (Figure 1F), a transient fluorescence quenching was seen in *Arabidopsis* (thick arrow), which was previously interpreted as the signature of reaction center-driven qE (e.g., ref *13*). This quenching was also absent in *Chlamydomonas* (Figure 1E). In addition, all wild-type strains of *Chlamydomonas* behaved similarly whether they derived from strain 137C (as presented here) or CC125, which has been previously employed to study nonphotochemical quenching in *Chlamydomonas* (*16*, *17*, *36*).

The minor quenching developed in *Chlamydomonas* during illumination was not sensitive to the dissipation of the

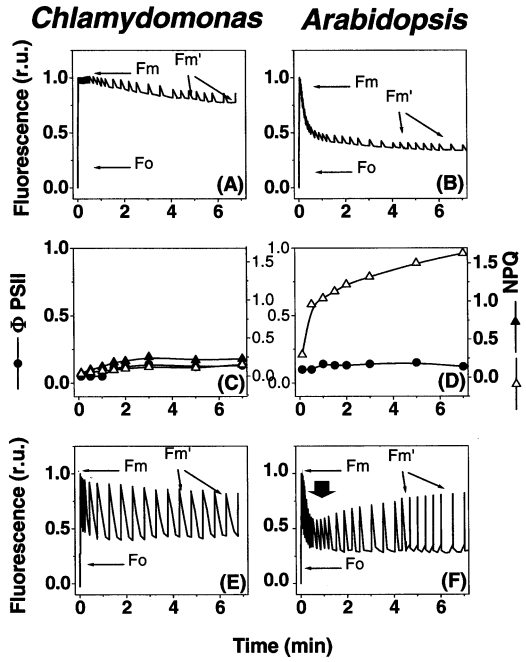

FIGURE 1: Light-induced nonphotochemical quenching generation in *Chlamydomonas* cells (A) and *Arabidopsis* leaves (B) upon illumination with high photon flux. Light intensity was ~1000 μmol of photons m^{-2} s^{-1} of green light. (C, D) Calculation of NPQ and ΦPSII. (E, F) Nonphotochemical quenching measured at a light density of 80 μmol of photons m^{-2} s^{-1}. NPQ was calculated as $(F_m - F_m')/F_m'$, where F_m represents the maximum fluorescence emission recorded in dark-adapted material, while F_m' represents the maximum fluorescence value recorded at discrete time intervals during illumination (*21*). F_m and F_m' values were obtained by superimposing a saturating flash (200 ms of duration) to the continuous actinic light. ΦPSII is calculated as $(F_m' - F_s)/F_m'$ ($\Delta F/F_m'$; *70*), where F_s represents the steady-state level of fluorescence emission upon illumination. This parameter represents the quantum yield of linear electron flow (*70*). Key: circles, ΦPSII; open triangles, NPQ; solid triangles, NPQ measured in cells grown autotrophically at 100 μE m^{-2} s^{-1} white light.

trans-thylakoid ΔpH by addition of the H^+/K^+ exchanger nigericin (not shown). This suggests that state transitions and/or photoinhibition represent(s) the prominent form of nonphotochemical quenching in *Chlamydomonas* upon illumination with saturating light. Examination of the extent of light-induced phosphorylation of LHCII subunits confirmed the occurrence of a limited transition to state 2 under these conditions (see below). In contrast, the extensive nonphotochemical quenching measured in *Arabidopsis* was sensitive to addition of nigericin (not shown), indicating that this quenching reflects the occurrence of high-energy state quenching (*1*, *8*, *9*, *37*). The absence of high-energy state quenching in *Chlamydomonas* was confirmed by the lack of absorbance changes at 535 nm during illumination (data not shown), a linear indicator for the generation of this type of quenching in plants (*38*).

The fluorescence of dark-adapted cells of *Chlamydomonas* was slightly quenched as compared to that in *Arabidopsis* (compare their respective Fv in Figure 1). This quenching, which was not sensitive to nigericin (not shown), reflected a small transition to state 2 in darkness, owing to the higher level of reduction of the plastoquinone pool by stromal reductants in this alga (reviewed in ref *24*). This suggests that no high-energy state quenching could be induced in the dark-adapted *Chlamydomonas* cells, although a substantial

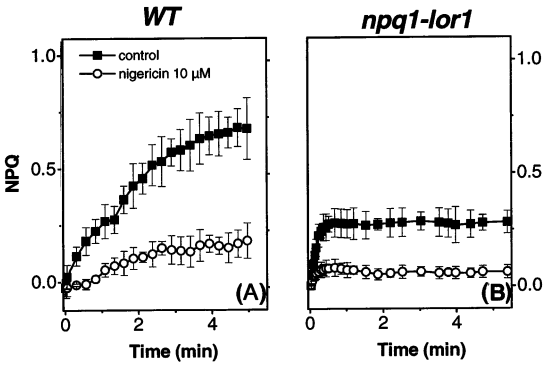

FIGURE 2: Fluorescence behavior of *Chlamydomonas* cells at ~0 °C. (A) Estimation of NPQ in WT cells. (B) Estimation NPQ in *npq1 lor1* cells. Key: squares, control; circles, 10 μM nigericin. This compound was added to cells 5 min prior to illumination. In low-temperature experiments, cells were dark adapted at room temperature and then loaded into a cuvette that was prerefrigerated in an ice/water bath for 20 min. After a few seconds of further cold incubation, fluorescence parameters were measured. Light was as in Figure 1A. Panels refer to three independent experiments.

ΔpH is generated in these algae, through ATP hydrolysis by the CF_0/F_1 complex (*39*). Again, a different behavior has been reported in plants, where no ΔpH is present in the dark (*40*). However, a large high-energy state quenching could be established in the absence of illumination, upon generation of the ΔpH by ATP hydrolysis (*41*) in isolated thylakoid.

High-Energy State Quenching Can Be Generated in Chlamydomonas When Photosynthetic Performance Is Impaired. The above results indicate that even under conditions of saturating light, where photochemistry is largely or completely saturated, no high-energy state quenching is induced in *Chlamydomonas*. We next considered whether this applies under conditions where the overall photosynthetic capacity is limited. In higher plants, it has been shown that high-energy state quenching is enhanced with decreasing temperatures (e.g., ref *42*). To test whether the same treatment promoted the generation of high-energy state quenching in *Chlamydomonas*, we performed experiments on cells that were incubated at ~0 °C. Under these conditions, the induction of nonphotochemical quenching was clearly visible when illumination was sustained over 5 min (Figure 2A), though it still remained much lower than in higher plants. This quenching was reversible in the dark, in both the wild-type and the mutant strain. Addition of nigericin demonstrated that the bulk, but not all, of this quenching was pH-dependent. The induction of quenching in these conditions was found to be strongly sensitive to temperature, the high-energy state quenching relaxing rapidly upon warming the cuvette (not shown). This quenching was severely impaired in the *npq1 lor1* mutant (Figure 2B) that has been previously described as defective in nonphotochemical quenching due to altered xanthophyll cycle turnover and lutein synthesis (*16*). The mutant displayed a limited induction of high-energy state quenching at ~0 °C that was kinetically distinct from that in the wild type. While the wild type displayed multiphasic kinetics, with slow components developing in the time range of minutes, the onset of NPQ was essentially monophasic in the *npq1 lor1* mutant. This behavior is consistent with our previous observations in plants (*13*) that the onset of reaction center-based quenching precedes generation of antenna-based quenching, owing to

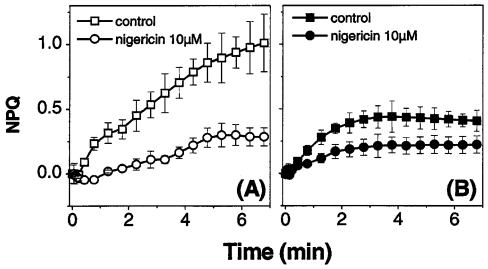

FIGURE 3: Relative contribution of nonphotochemical quenching in 18.5 cells of *Chlamydomonas* under illumination with high (A) and low (B) light. NPQ was calculated as in Figure 1. Key: squares, control; circles, 10 μM nigericin. This compound was added to cells 5 min prior to illumination. Very similar results were obtained in another RubisCO mutant, 31.4 (*25*) (not shown). Same light intensity as in Figure 1. Panels refer to five independent experiments.

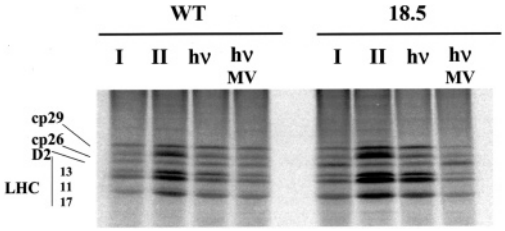

FIGURE 4: Autoradiogram of ^{33}P-radiolabeled antenna polypeptides in the 15−35 kDa region in the wild type and the 18.5 strain. I: State 1 (antenna proteins dephosphorylated), induced through vigorous agitation in the dark in air. II: State 2 (antenna proteins phosphorylated), obtained through addition of glucose and glucose oxidase (*32*). *hν*: Cells were illuminated for 7 min with 100 μmol of photons m^{-2} s^{-1}. *hν* MV: Cells were illuminated in the presence of 1 mM methyl viologen.

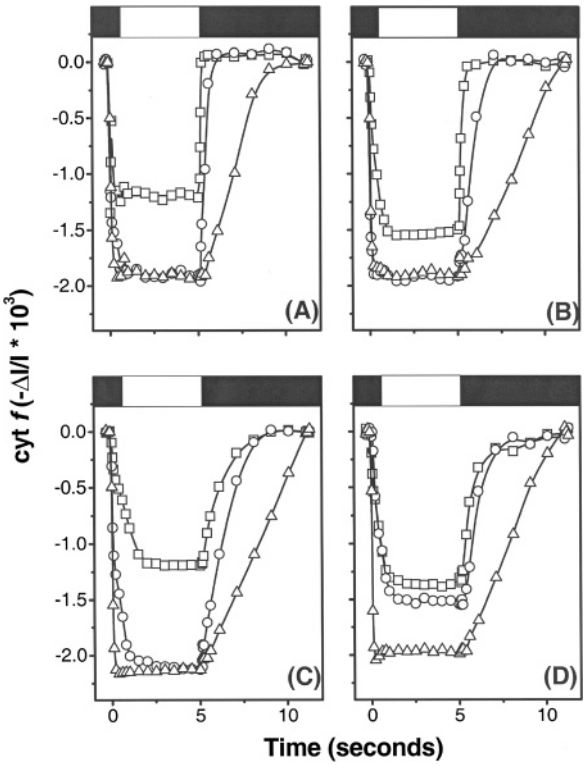

FIGURE 5: Cytochrome *f* redox changes in continuous light as measured by absorbance changes. (A, C) WT cells. (B, D) 18.5 cells. Panels: A and B, dark-adapted cells; C and D, illuminated cells, as in Figure 3. Symbols: squares, no inhibitors; circles, 10 μM DCMU; triangles, 2 μM DBMIB. Other conditions are described in Experimental Procedures. Key: black bar, actinic light off; white bar, actinic light on. A decrease of absorbance corresponds to oxidation of cytochrome *f*. Traces were normalized to the amplitude of a PSI-driven charge separation signal measured at 515 nm.

the longer induction time of zeaxanthin synthesis. Our observations suggest that, at ~0 °C, the *npq1 lor1* mutant develops only a reaction center-based quenching whereas the wild type also develops a slower zeaxanthin-mediated and antenna-based form of quenching.

In higher plants, high-energy state quenching also increases when the supply of CO_2 to the leaf is reduced (e.g., ref *43* and references cited therein). Controlling the CO_2 concentration inside *Chlamydomonas* cells is less straightforward than in leaves, due to the presence of a CO_2 concentrating mechanism (*44*, *45*). Instead, we investigated the development of nonphotochemical quenching in mutants unable to assimilate CO_2. We examined the 18.5 strain, which lacks the RubisCO small subunit (*25*). A significant level of nonphotochemical quenching was observed in this RubisCO mutant upon illumination with either saturating light (Figure 3A) or limiting light (Figure 3B) at room temperature. Addition of nigericin resulted in the relaxation of some, but not all, of the quenching, which we thus attribute to high-energy state quenching. Based on its kinetics, the nigericin-insensitive quenching could be attributed to the occurrence of state transitions, an assignment that is supported by the extent of light-induced phosphorylation of LHCII subunits at low light (Figure 4). This phosphorylation was sensitive to the addition of the electron acceptor methyl viologen, which oxidizes the electron transport chain, confirming that this light-induced phosphorylation is indeed mediated by the redox state of plastoquinones. The same pattern of LHCII phosphorylation was observed in the wild type, but to a smaller extent. Still, even in the mutant, the extent of light-induced LHCII phosphorylation remained lower than upon a transition to state 2 due to an anaerobic incubation in the dark. The same behavior was observed in the mutant strain 31.4 (not shown), an independent mutant devoid of RubisCO (*25*).

In *Chlamydomonas*, transition to state 2 activates cyclic electron flow around PSI (*31*, *46*, *47*). This has been demonstrated using the PSII inhibitor DCMU: a DCMU-insensitive electron flow through the cytochrome b_6f complex and PSI develops in state 2 but not in state 1 (*31*). To assess whether the same phenomenon was induced by illumination of the RubisCO mutant, we measured the kinetics of cytochrome *f* redox changes in dark-adapted and preilluminated cells of both the WT and the 18.5 strain (Figure 5). In both strains, a similar behavior was observed in dark-adapted cells (Figure 5A,B): switching the actinic light on resulted in the oxidation of cytochrome *f* (squares), which rapidly reached a plateau. After the light was switched off, cytochrome *f* reduction was observed, and the absorption signal returned to its initial value. In both the WT and the 18.5 strain, cytochrome *f* oxidation yield was increased by addition of DCMU (circles) or of DBMIB (triangles), an inhibitor of cytochrome *f* reduction by plastoquinol (reviewed in ref *48*). This indicates that cytochrome *f* reduction can be inhibited with a similar efficiency by blocking either PQ reduction by PSII or PQH_2 oxidation by the b_6f complex, as expected from the operation of a linear electron flow between the two

Table 1: Carotenoid Composition of Thylakoid Membranes from Dark-Adapted and Illuminated Cells of the WT and the 18.5 Mutant of *Chlamydomonas*[a]

	neoxanthin	violaxanthin	antheraxanthin	zeaxanthin	β-carotene	lutein
WT dark	6.33 ± 0.25	8.38 ± 0.45	0.28 ± 0.09	0.74 ± 0.11	12.13 ± 1.80	6.49 ± 0.15
WT light	6.46 ± 0.17	9.74 ± 1.17	0.48 ± 0.02	0.84 ± 0.12	15.65 ± 0.58	5.64 ± 0.51
18.5 dark	6.17 ± 0.07	8.96 ± 0.03	0.52 ± 0.24	0.76 ± 0.02	10.26 ± 0.13	5.99 ± 0.06
18.5 light	6.19 ± 0.12	7.01 ± 0.08	0.80 ± 0.03	1.19 ± 0.13	10.80 ± 0.06	6.17 ± 0.36

[a] Cells were illuminated as in Figure 1A for 7 min and then frozen in liquid nitrogen. Samples were analyzed for carotenoid composition as indicated in Experimental Procedures. Numbers refer to moles of pigment per 100 mol of chlorophyll *a* + *b*. Values refer to two independent measurements, repeated three times.

photosystems. While the same pattern of inhibition was conserved in the preilluminated wild type (Figure 5C), the oxidation yield of cytochrome *f* became largely insensitive to the addition of DCMU in the preilluminated mutant (Figure 5D, circles) while still remaining sensitive to DBMIB (Figure 5D, triangles). This is indicative of a genuine light-induced transition to state 2 (Figure 4) since it was indeed able to promote cyclic electron flow (*31*). This light-induced cyclic electron flow is likely to provide the basis for the ΔpH responsible for the qE part of nonphotochemical quenching in the RubisCO mutant.

High-Energy State Quenching in the RubisCO Mutant Is Largely Comprised of Reaction Center Quenching. A major difference between reaction center quenching and antenna quenching is the involvement of xanthophyll deepoxidation in the latter only (*1*, *8*, *9*). When the amount of antheraxanthin plus zeaxanthin synthesized during illumination was tested in the RubisCO mutant, it turned out to be well below that observed in illuminated plants. While only 0.7 mol per 100 mol of chlorophyll was synthesized in the case of *Chlamydomonas* (Table 1), more than 5 mol per 100 mol of chlorophyll was produced in plants in the same conditions (*13*).

To test whether a long-lived reaction center quenching was induced in the RubisCO mutant during illumination, we measured the extent of PSII charge separation on dark-adapted samples at the onset of and after prolonged illumination. To this aim, we used the generation of a field-indicating electrochromic shift around 515 nm as a probe (see Experimental Procedures and ref *13* for details). A single turnover laser pulse, the intensity of which was saturating, was used to detect PSII activity. Indeed, quenching in the antenna should only promote loss of activity upon illumination with subsaturating light intensity, because of the reduced light-harvesting capacity. In contrast, inhibition of charge separation upon illumination with saturating light is only expected in the case of reaction center quenching, which affects the intrinsic photochemical capacity of the reaction centers, independently of the light intensity used (*13*).

In the mutant, PSII charge separation was substantially inhibited at the onset of illumination. This could be detected at both saturating (Figure 6) and subsaturating (not shown) flash intensities, in agreement with our previous observations in higher plants (*13*). However, in contrast to higher plants, the inhibition of PSII photochemistry did not relax with longer periods of exposure. It remained of similar amplitude when detected after 2 or 10 min of illumination, indicating that reaction center quenching was preserved. By contrast, a very limited decline in PSII charge separation was observed in the wild type, which tended to accumulate slightly over time. This is consistent with the finding that no significant high-energy state quenching was generated in the wild type at room temperature.

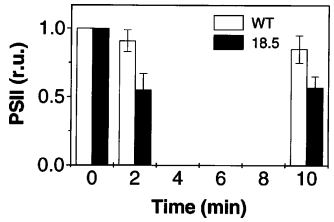

FIGURE 6: Measurements of PSII photochemical efficiency in dark-adapted and illuminated cells of the WT and the 18.5 mutant of *Chlamydomonas*. PSII activity was estimated as the extent of the electrochromic signal, as described in Experimental Procedures. Dark-adapted cells were measured as a reference for 100% activity of PSII. Activity was then measured after 2 and 10 min of illumination and the relative variation in activity calculated. Experimental conditions were as in Figure 1. At time = 0, the ratio between the DCMU/HA-sensitive (PSII) and -insensitive (PSI) component was set to 1. [DCMU] = 20 μM; [HA] = 1 mM. No changes in the fraction of the signal insensitive to DCMU and HA addition were observed, suggesting no changes in PSI photochemical activity. Panels refer to four independent measurements, repeated three times.

Storage of the $\Delta\mu_{H^+}$ in the Light Is Different in Chlamydomonas WT and in the RubisCO-Deficient Mutant. To confirm whether the extent of ΔpH maintained in the RubisCO mutant was indeed higher than in the wild type at high light, we measured the inversion of the electrochromic shift seen upon its relaxation from steady state upon a light to dark transition (*49*). When the light is switched off, relaxation of the electric component ($\Delta\Psi$) of the $\Delta\mu_{H^+}$ occurs before the complete dissipation of the ΔpH, owing to the low dielectric constant of the thylakoid membranes (*50*) and to the high H^+ buffering capacity of the lumen (see, e.g., ref *51*). This gives rise to an excess of positive charges in the stroma, which transiently inverts the membrane potential. Then, the slower flux of counterions promotes the relaxation of the membrane potential to the dark level. While it is not fully quantitative, the inversion of the membrane potential can be taken as a measurement of the relative amplitude of the ΔpH, with respect to the $\Delta\Psi$, under appropriate conditions (see refs *49* and *52* for a further discussion).

In our conditions, this measurement clearly supports the notion that the RubisCO mutant maintains a higher ΔpH than the wild type at steady state. Strikingly, most of the trans-thylakoid electrochemical gradient in the mutant could be assigned to ΔpH, in contrast to both the wild type of *Chlamydomonas* and *Arabidopsis*, where a substantial proportion was stored as a $\Delta\Psi$ (compare panel C with panels A and B in Figure 7; see also refs *52* and *49* for previous studies in *Chlamydomonas* and plants, respectively).

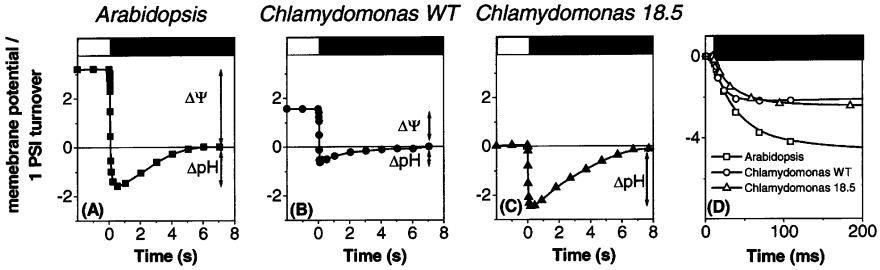

FIGURE 7: Estimation of the electrochemical proton gradient that is generated upon steady-state illumination in *Arabidopsis* leaves (A) and *Chlamydomonas* WT (B) and 18.5 (C) cells. Samples were illuminated for 5 min with saturating light (as in Figure 1). At time 0, the light was switched off, and the membrane potential decay was followed by measuring absorption changes at 515−545 nm. The amplitude of the membrane potential was normalized to 1 membrane charge separation, generated by a saturating laser flash to cells where PSII charge separation was inhibited by additions of DCMU and HA (*27*). Inversion of the membrane potential upon switching the light off is assumed to stem from fast H^+ flux from the CF_0/F_1 ATPase complex. Thus the amplitude of the inverted membrane potential can be taken as a growing function of the size of the light-induced ΔpH (*52*, *59*). (D) Transmembrane potential decay in *Arabidopsis* leaves (squares) and *Chlamydomonas* WT (circles) and 18.5 (triangles) cells upon switching the light off. The same traces as in panels A−C are presented on an expanded scale, upon normalization of the initial level of the membrane potential to 0. Key: white bar, light on; black bar, light off.

At the same time, we measured the initial rate of decay of the transmembrane potential upon switching the light off. We found that the decay of the potential, which gives an indication of the rate of proton flux through the CF_0/F_1 ATPase, was still significant in the RubisCO mutant, although its half-time (∼25 ms) was larger than in the wild type ($t_{1/2}$ ∼15 ms). Therefore, although the rate of ATP turnover is reduced at steady state in the mutant, other metabolic processes drive ATP turnover even in the absence of CO_2 assimilation. In our experimental conditions, the H^+ efflux measured in *Arabidopsis* leaves was slower than that observed in *Chlamydomonas* WT cells.

DISCUSSION

Characteristics of Nonphotochemical Quenching in Chlamydomonas. The results presented here demonstrate that *Chlamydomonas* does not develop an extensive quenching upon illumination, under conditions that favor high-energy state quenching in higher plants. This poor ability to develop high-energy state quenching in *Chlamydomonas* is observed *irrespective* of the light intensity used to grow the cells (i.e., at a light intensity similar to that used for growing *Arabidopsis*, as well as in low light conditions that are required to sustain growth of photosynthetic mutants) and to promote the NPQ response. Moreover, this different capacity to generate a sustained nonphotochemical quenching in the light cannot be ascribed to the presence of a quenched state generated before illumination, which would originate from a trans-thylakoid ΔpH established by ATP hydrolysis in darkness. Indeed, uncoupling of dark-adapted *Chlamydomonas* cells by ionophores had no effect on the variable fluorescence, in contrast to plants placed in conditions where high-energy state quenching is induced in the dark (*41*).

On the basis of measurements of phosphorylation of thylakoid membrane proteins, we showed that the slightly smaller variable fluorescence of well-aerated cells of *Chlamydomonas* is due to the establishment of a limited transition to state 2 in darkness. Transition to state 2, which accounts for the quenching of ∼20% of the variable fluorescence, is also induced by light, even when its intensity is saturating saturation for photosynthesis. Thus, in contrast to higher plants, state transitions rather than high-energy state quenching constitute the major form of nonphotochemical quenching in *Chlamydomonas*.

High-energy state quenching can nevertheless be induced in *Chlamydomonas* under conditions that prevent CO_2 fixation, i.e., by lowering the temperature or in mutants that lack RubisCO. However, its extent remains far below that in higher plants. In these circumstances, the mechanism generating high-energy state quenching shares similarities to that which develops in higher plants. It involves zeaxanthin synthesis, as suggested by the lower high-energy state quenching observed with the *npq1 lor1* mutant, as well as by the zeaxanthin plus antheraxanthin synthesis observed in the RubisCO mutant upon saturating illumination (Table 1). However, there are also striking differences. While the transient inactivation of PSII centers disappears upon prolonged illumination in plants (*13*), this quenching is maintained at steady state in *Chlamydomonas*. This indicates that both reaction center and antenna nonphotochemical quenching processes coexist in the alga under steady-state conditions and, thus, that the transition from reaction center to antenna quenching is less efficient in *Chlamydomonas* than in plants (see below).

A Reduced Capacity To Maintain a ΔpH at Steady State under Illumination Precludes Development of High-Energy State Quenching in Chlamydomonas. The present study, as well as earlier reports from Niyogi and co-workers (*16*, *17*, *36*), demonstrates, however, that *Chlamydomonas* possesses the ability to quench excitation energy via a qE-type mechanism in some particular conditions (see also ref *53*). Unlike higher plants, however, this organism does not show a sustained high-energy state quenching under physiological conditions when exposed to irradiance that is saturating for photosynthesis (present work; see also ref *52*). An additional stress, either low temperature or limitation of CO_2 fixation, is needed to induce the qE-type quenching. It is therefore likely that differences in the physiological conditions in which nonphotochemical quenching was measured in *Chlamydomonas* cells might account for the rather high variability in the extent of nonphotochemical quenching reported up to now (e.g., from ∼0.5 in ref *36* to ∼2 in refs *16* and *17*).

When detectable, high-energy state quenching develops together with a transition to state 2 and, therefore, with the onset of cyclic electron flow around PSI (Figure 5). Cyclic electron flow has been implicated in the generation of a sustained ΔpH across the thylakoid membranes to regulate light harvesting under conditions of reduced photosynthetic

performances (*54*). Thus, an increased generation of ΔpH by cyclic electron flow might account for the appearance of high-energy state quenching in the RubisCO mutant. This hypothesis is supported by the estimation of the size of the electrochemical proton gradient based on measurements of the relaxation of the light-induced membrane potential performed in Figure 7. The size of ΔpH is larger in the RubisCO mutant than in the WT. However, other regulatory mechanisms have been proposed in plants, which may also contribute to the increased capacity to build up a ΔpH upon illumination and, thus, to the establishment of nonphotochemical quenching under conditions of restricted electron transport rate.

Kramer and co-workers have proposed that a downregulation of the ATPase activity might account for the maintenance of a sustained ΔpH under conditions where the overall rate of electron flow to CO_2 is restricted (*55, 56*). Under the conditions explored here, this process seems to be operating since the rate of H^+ flow observed in the RubisCO mutant is smaller than in the WT. This behavior can be easily explained in terms of the very large increase in ATP/ADP ratio (up to ∼∞; see, e.g., ref *57*) in the mutant, which cannot utilize photoproduced ATP in the Benson−Calvin cycle. Owing to this change in the ΔG_p, an increase in the inward proton flux at steady state is expected, leading to a decrease in the net outward proton flow, as observed in Figure 7.

Finally, in plants it has been shown that the parsing between the ΔpH and the ΔΨ components of the electrochemical proton gradient can be changed in favor of the former under conditions of very low concentration of electron acceptors (i.e., low CO_2 and O_2 concentration; *56*). These conditions further enhance the ability of plants to establish nonphotochemical quenching, although the overall rate of electron flow is drastically reduced (*56*). The mechanism leading to this different parsing under conditions of inhibited electron flow is not fully understood. It has been argued, however, that changes in the ionic stromal balance, or in the lumenal buffer capacity, are likely to be the major effectors of this phenomenon in plants (*49*, *56*, *58*, *59*).

Our data clearly indicate that the same phenomenon is also observed in *Chlamydomonas* under conditions where high-energy state quenching is induced. While the overall amplitude of the $\Delta\mu_{H^+}$ is the same, the relative contribution of ΔpH is larger in the RubisCO mutant than in the WT. An additional ATPase activity has been identified in the thylakoid membranes, the "X" pump, which shows a selectivity for ions other than *protons* (*60*). Its contribution to the generation and dissipation of the $\Delta\mu_{H^+}$ is almost negligible in physiological conditions (see ref *60* for a further discussion). This would imply that either this pump requires a higher $\Delta\mu_{H^+}$ than the CF_0/F_1 enzyme to become active (*61*) or that it has an unfavorable ATP/ion ratio if compared to the CF_0/F_1 enzyme. Therefore, the activity of this pump would be negligible in the WT, but it would contribute significantly to the $\Delta\mu_{H^+}$ turnover in the RubisCO mutant upon illumination. In this strain, sustained generation of the electrochemical proton gradient, coupled to reduced ATP consumption, might transiently bring the $\Delta\mu_{H^+}$ to the threshold value required for the activation of the X pump. Because its ion specificity is different from that of the CF_0/F_1 enzyme, activation of the X pump should lead to a specific dissipation of ΔΨ. While this hypothesis fits with the data reported here in *Chlamydomonas*, at present no reports for the existence of such activity have been published with plants. Therefore, its possible involvement in the modulation of the parsing of the $\Delta\mu_{H^+}$, which has been reported in tobacco leaves (*56*), remains to be investigated.

Chlamydomonas Offers a Poor NPQ Response to an Increased ΔpH. Whatever the mechanism responsible for the ΔpH increase in the RubisCO mutant, our data suggest that the size of the ΔpH generated in the wild type is not sufficient to reach the threshold for the activation of the NPQ response (see, e.g., ref *62*), even at saturating light intensities (see also ref *52*). Even in the case of the RubisCO mutant, which develops a large ΔpH at steady state, the high-energy state quenching remained lower that in plants, with a prevalence of reaction center quenching over antenna-driven quenching. *This indicates that Chlamydomonas has a low ability to transduce the ΔpH signal into an NPQ response*.

What is the mechanism for the downregulation of the NPQ response observed in *Chlamydomonas*? In plants, the small PSII subunit PsbS has been shown to play a major role in the modulation of antenna-driven high-energy state quenching (*19*), probably by sensing the lumenal pH via the protonation of two lumen-exposed glutamate residues (*38*). In addition, our recent results suggest that this protein is also required for the transition from a reaction center- to an antenna-driven mode of nonphotochemical quenching (*13*). An orthologue gene for PsbS has only recently been detected in *Chlamydomonas* (*63*−*65*). However, our preliminary observations of the level of the PsbS protein in *Chlamydomonas* membranes indicate that it would be significantly lower than in plants (Bonente et al., unpublished result). It is tempting therefore to suggest that *Chlamydomonas* has never acquired the PsbS-mediated response to increasing ΔpH, because of a lack of selective pressure. Therefore, the nonphotochemical quenching response observed in *Chlamydomonas* under conditions of restricted electron flow capacity (i.e., in the RubisCo mutant) would represent a ΔpH-dependent, but PsbS-independent quenching mechanism, yet to be mechanistically defined. The same mechanism is likely at the origin of the very large qE observed in diatoms (*23*), which lack PsbS (*66*). Clearly, *Chlamydomonas* still benefits from the synthesis of zeaxanthin, e.g., through its role as an antioxidant (*67*) or through its ability to alter membrane fluidity. However, it seems that this process is not integrated within a process of dynamic regulation of light harvesting. This difference likely reflects the specific constraints of its natural environment that has not provided the conditions for selecting an optimization of this photoprotective process. For instance, *Chlamydomonas* can escape from high light by negative phototaxis. Although appealing, this hypothesis per se cannot account for not having selected a high-energy state quenching strategy since negative phototaxis exists both in *Chlamydomonas* (see, e.g., ref *68*) and in diatoms (*69*), which clearly have a high capacity to develop a ΔpH-mediated quenching response (see above). We favor, however, a different view, where *Chlamydomonas* seems to have optimized its capacity to modulate the ratio of ATP to NADPH synthesized in the light, to optimize light excitation and utilization. In contrast to plants, *Chlamydomonas* is able to enhance the synthesis of ATP in the light by modulating cyclic electron flow via state transitions (*57*). This is expected

to result in a better coupling of ATP synthesis to the generation of reducing power in order to achieve the correct stoichiometry, as required for the assimilation of CO_2 by the Benson and Calvin cycle (*57*). By doing so, *Chlamydomonas* might be able to prevent ATP shortage during fluctuating conditions in its natural environment conditions and to avoid overreduction of its photosynthetic electron transport chain. This is clearly a prerequisite to prevent the onset of a high-energy quenching response, as explained in the introduction. In light of this hypothesis, it is clear that the only form of NPQ response required for efficient photosynthesis is the ability to adjust the relative photochemical efficiency of the two photosystems. This probably explains why *Chlamydomonas* uses state transitions, i.e., redox-mediated changes in the light capture ability of PSII over PSI, as the prevailing form of NPQ.

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