Photoinactivation of Photosystem II and Degradation of the D 1 Protein are Reduced in a Cytochrome b_6/f-Less Mutant of *Chlamydomonas reinhardtii*

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The effect of unoccupancy of the Q_B site by plastoquinone on the photoinactivation of reaction center II in a Cyt b_6/f-less mutant of *Chlamydomonas reinhardtii*, B_6, was investigated. In these cells the oxidation of plastoquinol generated by electron flow *via* RC II to plastoquinone and thus the turnover of PQH_2/PQ *via* the Q_B site are drastically reduced. Reaction center II of the mutant cells was resistant to photoinactivation relative to the control cells as demonstrated by measurements of light-induced destabilization of S_2-Q_B^- charge recombination, rise in intrinsic fluorescence and loss of variable fluorescence. These parameters relate to functions involving the reaction center II D 1 protein. The light-induced degradation of D 1 in the mutant cells was also considerably reduced, with a $t1/2$ value of 7 h as compared, under similar conditions, to about 1.5 h for the control cells. These results indicate that the photoinactivation of RC II and turnover of the D 1 protein are related and require occupancy of the Q_B site by PQ and its light-driven reduction.

Introduction

It is now well established that the D 1 protein, one of the major components of the photochemical reaction center II [1], is rapidly degraded and resynthesized (turnover), when oxygen evolving photosynthetic membranes of both eukariotic and prokariotic organisms are exposed to light *in vivo* (reviewed in [2] and [3]). Light fluency exceeding that required to saturate photosynthesis, causes a gradual inactivation of photosystem II (reviewed in [2] and [4]), (photoinhibition). Recovery from extensive photoinhibition, requires *de novo* synthesis of chloroplast translated proteins, specifically that of the reaction center II protein D 1 [5]. We have proposed previously that the process of photosystem II photoinactivation and turnover of the D 1 protein are related phenomena [6, 7]. Recently we have obtained experimental evidence for the involvement of the Q_B binding site located within the D 1 protein [8, 9], in this process [6, 7]. Using the thermoluminescence technique for characterization of the charge recombination of the S states and Q_B^- [10, 11], it was demonstrated that exposure of intact *Chlamydomonas* cells to light intensities saturating photosynthesis induces a destabilization of the reduced semiquinone Q_B^- compatible with a change in its redox potential and shortening of its life time, [6]. These changes correlated with a reduction in the oxygen evolution capacity and coincided with the loss of variable fluorescence characteristic of photoinhibition and with an increase in the turnover of the D 1 protein which persisted even when the cells were transferred to lower light intensities [6, 7].

Participation of the Q_B^- semiquinone in these processes requires occupancy of the Q_B site of RC II by plastoquinone. Arguments in favor of this hypothesis could be found in the observation that herbicides binding at the same site such as Diuron prevents the D 1 protein light-dependent degradation *in vivo* [12] and the light-induced destabilization of Q_B^- [7]. As opposed to that, *in vitro* experiments in which thylakoids were depleted of plastoquinone showed an increased sensitivity of the D 1 protein to proteolysis by trypsin. These results were considered as an indication for an enhanced susceptibility of the D 1 toward degradation when the Q_B site is unoccupied by plastoquinone [13]. In the present work we have used a

Abbreviations: Chl, chlorophyll; Cyt b_6/f, Cytochrome b_6/f complex; F_0, F_s, F_{max}, intrinsic, steady-state and maximal fluorescence, respectively; PQ, plastoquinone; PQH_2, plastoquinol; PS II, photosystem II; RC II, reaction center II; TL, thermoluminescence.

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cytochrome b_6/f-less mutant of *Chlamydomonas* to measure the light-dependent turnover of the D1 protein and stability of the RC II toward photoinactivation. In such a mutant the plastoquinone pool should be mostly reduced in the light to plastoquinol, a condition which favors unoccupancy of the Q_B binding site. The results clearly demonstrate that in this mutant RC II is protected against the light-induced D1 turnover and photoinhibition.

Materials and Methods

Chlamydomonas reinhardtii y-1 and the B_6 mutant cells were grown in a liquid medium containing acetate as a carbon source as described before [14]. The *y-1* mutant does not synthesize chlorophyll in the dark but is indistinguishable from the wild type cells when grown in the light. Light-grown cells are photosynthetically competent and exhibit normal photosynthetic electron flow [14]. B_6 is a chloroplast mutant lacking Cyt b_6/f activity [15]. As reported before for other cytochrome b_6/f-less mutants [16–18], this mutant does not exhibit a light-dependent or redox-dependent light harvesting II complex protein phosphorylation [19].

For measurements of D1 protein degradation, cells were incubated at the light intensities and times as indicated in the presence of chloramphenicol (200 μg/ml) added to prevent synthesis of the chloroplast translated D1. Thylakoid membranes were prepared as described before [20], and protein immunodetection using various antibodies was performed after resolving the thylakoid proteins by SDS-PAGE as described by Laemmli [21] followed by transfer to nitrocellulose paper and detection of the immunodecorated polypeptides by alkaline phosphatase or ^{125}iodinated protein A.

Thermoluminescence measurements were performed on intact cells or isolated thylakoids obtained from cells exposed to various light treatments as previously described [6].

Fluorescence rise kinetics was recorded using a home-made apparatus consisting of a cell holder, excited by a DC light source equipped with an electronic shutter (opening time, 2 msec) and filtered through a blue Corning 4-96 filter. The signal was detected at a right angle by a photodiode protected by a 685 nm cut-off red filter (Schott). The signal was digitized, stored and analyzed by an IBM-PC computer programmed to operate the shutter and record the voltage rise at different rates (up to 30000 points/sec) so as to cover efficiently both the fast and slow phase of the fluorescence rise.

Photoinhibitory light was provided by a projector equipped with a heat and UV filter (Pro Cabin, 680 W Tungsten-halogen lamp) supplying about 80% of the light energy in the visible region. The incubation vessel was temperature controlled by a surrounding water jacket (25 °C) as described before [6] and Chl concentration was 30–35 μg/ml.

Results

Effect of high light treatment on the fluorescence kinetics of the B_6 mutant cells

The absence of the cytochrome b_6/f polypeptides in the mutant as demonstrated by immunoblotting with antibodies against cytochrome b_6, cytochrome f and the Rieske polypeptide is shown in Fig. 1. Comparison of the thylakoid polypeptide pattern of the mutant to that of the *y-1* cells did not disclose other significant differences. To assay the sensitivity of the b_6 mutant relative to that of the *y-1* cells to high light intensities, cell suspensions were incubated in growth light or exposed to 250 Wm^{-2} for 30 min and the fluorescence rise kinetics of both types of cells was recorded after preadapting them to the dark for 1 min [6, 7]. The *y-1* cells in which electron flow is uninhibited, showed a low steady state fluorescence (F_s) as compared to the maximal fluorescence exhibited in the presence of the photosystem II inhibitor, diuron (F_{max}, Fig. 2). As expected, the steady-state

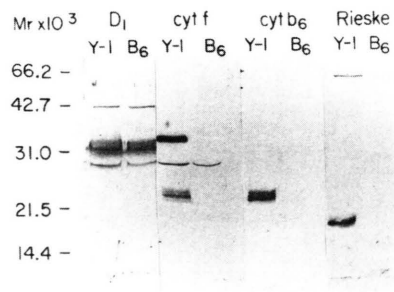

Fig. 1. Absence of the Cyt b_6/f polypeptides in thylakoids of the B_6 mutant. Thylakoids from *y-1* and B_6 mutant cells were resolved by SDS-PAGE and transferred to nitrocellulose paper. Strips of the blot were incubated with *anti* D1 antibodies [20] and antibodies against Cyt *f*, Cyt b_6 and Rieske protein subunit respectively. Detection was by alkaline phosphatase.

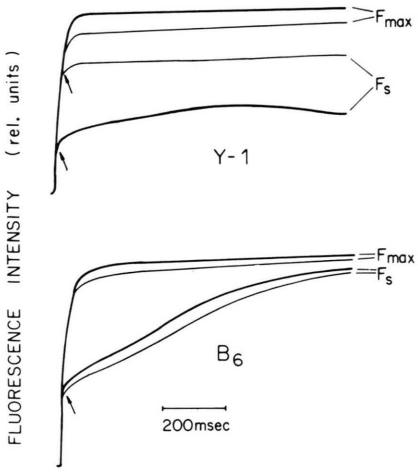

Fig. 2. Kinetics of fluorescence rise in intact *y-1* and B_6 mutant cells. Cells suspended in growth medium (35 μg chlorophyll/ml) were exposed to growth light (25 Wm^{-2}) or photoinhibitory light (125 Wm^{-2}) for 20 min. The cells were diluted to 4 μg chlorophyll/ml and fluorescence kinetics was measured as described in the Methods; F_{max} traces were recorded in presence of diuron (5 μM) and steady-state (F_s) traces, in absence of the inhibitor; heavy lines, control, light lines, cells exposed to high light; F_v/F_0 values were 5.7 and 0.3 for the *y-1* cells and 1.9 and 2.0 for the B_6 mutant, for the control and high light exposed cells respectively.

fluorescence level, F_s, of the B_6 mutant was similar in the absence or presence of diuron, since the mutant can not oxidize plastoquinol. However the rise time of the F_s level in the B_6 mutant is considerably slower than that of the F_{max} level due to the time required for the reduction of the plastoquinone pool in the light (Fig. 2). The variable fluorescence, (Fv), calculated as $(F_{max}-F_0)/F_0$, indicative of the relative content of active RCIII, was higher in the *y-1* cells as compared to the B_6 mutant due to a higher level of intrinsic fluorescence (F_0) in the mutant cells (Fig. 2). Following exposure to high light, the variable fluorescence in the *y-1* cells decreased significantly, due to a rise in the F_0 level and reduction of the F_{max}, as previously reported for photoinhibited cells [4, 6, 7]. No such reduction in the F_v level was observed however in the B_6 mutant cells, indicating that these cells were not photoinhibited by this treatment (Fig. 2).

It was previously reported that in *Chlamydomonas* cells chlororespiration activity can account for the oxidation of the plastoquinol pool [22, 23]. A high rate of oxygen-dependent oxidation of plastoquinol could permit continuous reduction of plastoquinone in the light-exposed cells and thus increase at least to some extent the occupancy of the Q_B site by plastoquinone even in absence of an active cytochrome b_6/f complex. To estimate the rate of plastoquinol reoxidation the effect of the dark adaptation time following light exposure of cells on the fluorescence rise kinetics in the B_6 mutant cells was assayed. The results of such an experiment are shown in Fig. 3. Mutant cells were incubated in low or high light and then the fluorescence rise kinetics was measured repeatedly after short dark adaptation time intervals. In low light exposed cells, the fluorescence rise kinetics after 15 sec of dark adaptation was comparable but not identical to that obtained following dark adaptation for 1 min indicating that part of the plastoquinone pool was still reduced. In cells exposed to high light the plastoquinone pool remained partially reduced even after 1 min of dark adaptation of the preilluminated cells (Fig. 3). These results

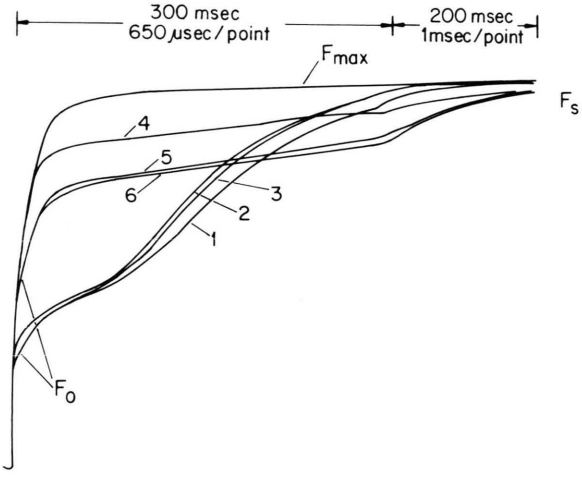

Fig. 3. Reoxidation in the dark of the plastoquinol pool in B_6 cells following exposure to low and high light. Mutant cells were incubated in low or high light (as in Fig. 2). The cells were dark-adapted for 1 min and the fluorescence rise was traced as in Fig. 2; 1–3, control; 4–6, cells exposed for 5 min to high-light intensity; 1, trace taken after 2 min dark adaptation; F_{max}, trace obtained in presence of 5 μM diuron; 2, 3, traces taken consecutively after 5 and 15 sec dark intervals following exposure of the cells to the exciting beam for 30 sec; 4, 5, and 6, traces taken consecutively after exposure to the exciting beam for 30 sec at 5, 15, and 60 sec intervals; F_s, steady-state fluorescence, equals the F_{max} in the mutant.

indicate that the B_6 mutant can oxidize plastoquinol *in vivo*, however the rate of this reaction is slow and further reduced in cells which have been exposed to high light intensity.

Effect of high-light exposure on the thermoluminescence glow curves of the y-1 and B_6 mutant cells

Charge recombination between the $S_{2,3}$ states and the primary or secondary stable reduced acceptors, Q_A^- or Q_B^-, can be elicited by heating in the dark, isolated chloroplasts or whole cells, after preillumination by a saturating single turnover light flash in the presence or absence of diuron respectively [10, 11]. The recombination results in light emission and a glow curve (thermoluminescence), can be obtained in which the intensity of the light emitted as a function of the sample temperature is recorded. Under specific experimental conditions, the temperature at which the emission is maximal is characteristic of the recombining species, and related to the redox potential or their stability [10, 11]. Charge recombination between the S states and Q_B^- (B band [10, 11]), in intact *Chlamydomonas* cells is characterized by a maximal light emission at about 37–39 °C while that resulting from the recombination of Q_A^- (D band [10, 11]), peaks at about 7–10 °C (Fig. 4, [6, 7]). Similar results were obtained with isolated thylakoids (data not shown). It has been shown before that the B band emission peak is shifted to a lower temperature, comparable to that of the D band in cells [6, 7], or thylakoids [7] isolated from cells which have been exposed to photoinhibitory light. Recent experimental results demonstrate that this shift in the B band temperature peak is indicative of changes in the D1 protein properties, and correlated with the loss of reaction center II activity and the light-dependent D1 protein synthesis [6] and modification [7]. Thus thermoluminescence measurements could provide information on the light-induced changes in the reaction center II of the B_6 mutant as compared to the *y-1* cells. Results of such experiments are shown in Fig. 5. The peak temperature of the B band emission is reduced in *y-1* cells as a function of exposure to high-light intensity. The temperature shift correlates with loss of variable fluorescence due to rise in the F_0 and decrease in the F_{max} values (Table I, see also Fig. 5) as reported before [6, 7]. In contradistinction, the glow curve of the B_6 mutant cells shows a broad emission temperature including a shoulder at about 16 °C which is shifted to a higher temperature following high-light treatment. This indicate that prior to the high light exposure part of the reaction centers of the mutant cells are already altered and the glow curve shape is due to the presence of a mixed population of intact and altered reaction centers which revert to the unaltered form during the high-light exposure.

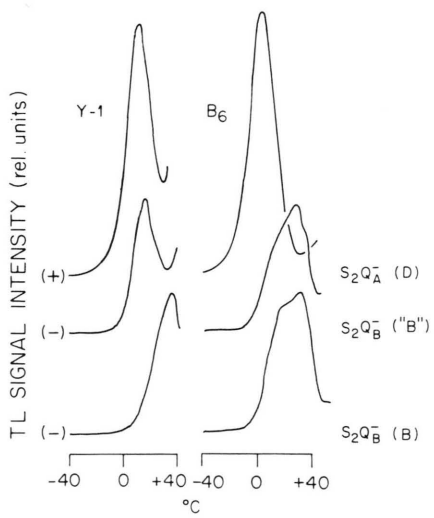

Fig. 4. Thermoluminescence glow curves of *y-1* and the B_6 mutant cells exposed to high light intensity. Cells were incubated as in Fig. 2; the thermoluminescence emission resulting from S_2-Q_B^- or S_2-Q_A^- was recorded after 2 min of dark adaptation and excitation by a single turnover flash in absence (−) or presence of 5 μM diuron respectively; note the downshift of the temperature emission peak in the *y-1* cells as compared to the small upshift in the emission temperature of the mutant cells.

Table I. Resistance *in vivo* to light-induced damage of PS II in the B_6 mutant.

Treatment	Cells	F_0 (% of control)	F_v/F_0 (% of control)	Δ °C
125 Wm^{-2}; 50 min	*y-1*	240	12	−21
	B_6	65	157	−4
250 Wm^{-2}; 30 min	*y-1*	190	24	−24
	B_6	76	118	+10

Δ °C, shift in the emission temperature of S_2-Q_B^- charge recombination; for experimental details, see Methods.

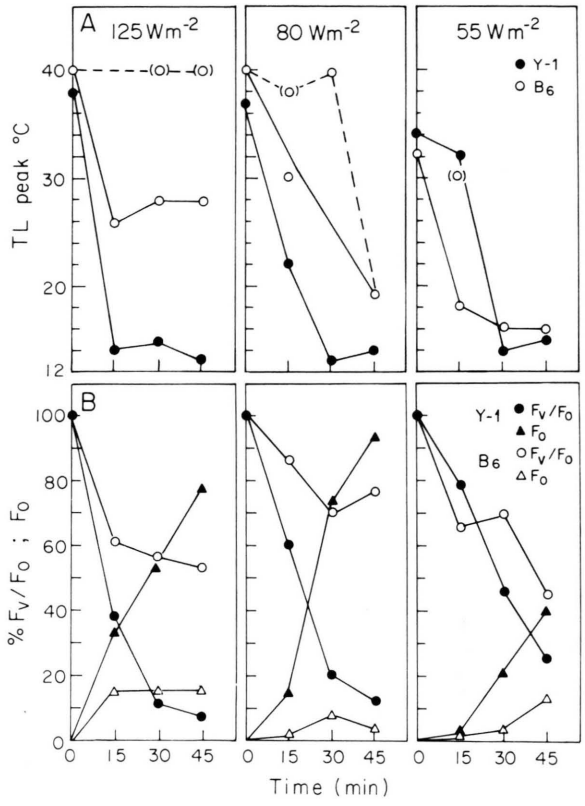

Fig. 5. Effect of light exposure on the thermoluminescence emission temperature and fluorescence rise. Cell suspensions were incubated at low growth light intensity (25 Wm^{-2}) for 30–60 min and the time 0 sample were taken; the cells were then transferred to light intensities as indicated and samples were taken at various times for measurements of thermoluminescence and fluorescence parameters as described in the Methods; the glow curves of the B_6 mutant cells were asymmetric (*cf.* Fig. 4) indicating the presence of a population of unaltered reaction centers displaying an emission temperature as in control samples (values in parenthesis); note the gradual reduction in the effect of time exposure on the TL temperature peak and loss of variable fluorescence in the B_6 mutant cells exposed to higher light intensity as compared to the *y-1* control cells.

Since the B_6 mutant cells exhibited an anomalous glow curve showing a lower emission temperature peak of the B band in low light exposed cells which shifted to a higher temperature in high light exposed cells, it was of interest to test whether this phenomenon is correlated with the total amount of absorbed energy. Thus the effect of light intensity and time of exposure on the kinetics of the changes in the thermoluminescence and fluorescence parameters was assayed. Results of such an experiment are shown in Fig. 5. A clear tendency is demonstrated toward an improved response to higher light intensities in terms of the stability of the thermoluminescence and fluorescence parameters in the b_6/f-less mutant cells as compared to the *y-1* control cells.

Light-induced turnover of the D 1 protein

The results presented so far demonstrate that photosystem II of the cytochrome b_6/f-less mutant is resistant to the light-induced loss of reaction center II activity relative to that of the *y-1* cells as demonstrated by the reduced temperature peak shift of the B band thermoluminescence signal and the persistence of the variable fluorescence in the high light-treated cells. If indeed the light-induced turnover of the D 1 protein is related to the above light-induced changes in reaction center II, one would predict a significant reduction in the light-dependent degradation of the D 1 protein in the B_6 mutant cells. To test this possibility, *y-1* and B_6 mutant cells were exposed to high light intensity for various times in the presence of chloramphenicol to prevent the *de novo* synthesis of the D 1 protein and thus allow the detection of the light-dependent loss of the D 1 as assayed by protein immunoblotting. Results of such experiments are shown in Fig. 6 and demonstrate unequivocally the resistance of the D 1 protein in the B_6 mutant to the light-dependent degradation *in vivo* as com-

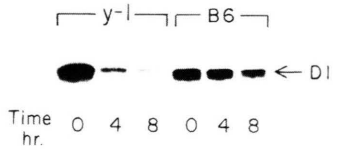

Fig. 6. Light-dependent degradation of the D 1 protein is significantly reduced in the B_6 mutant cells. Cell suspension were exposed to 250 Wm^{-2}. At times as indicated samples were taken and the residual amount of D 1 protein in isolated thylakoids was detected by use of ^{125}iodinated protein A. The estimated t 1/2 value of D 1 was about 1.5 and 7 h in the *y-1* and the B_6 mutant cells respectively.

pared to that of the *y-1* cells. The relative amounts of the D 1 protein on a chlorophyll basis indicates that the amount of this protein (and thus of RC II) is somewhat lower in the mutant as compared to the *y-1* cells. This could imply that the ratio antennae/RC II is higher and thus more energy is absorbed by RC II in these cells.

Discussion

The mechanism whereby light causes the degradation of the reaction center II D 1 protein is not yet understood. Furthermore the relation of this phenomenon to that of the light-dependent inactivation of photosystem II *in vivo* (photoinhibition), is a controversial issue [4, 24]. In our working hypothesis the two phenomena are considered as related [7]. It has been proposed before [2, 4, 5] that electron flow *via* the Q_B secondary acceptor quinone of reaction center II may induce reversible changes in the conformation of the reaction center complex which could facilitate formation of harming free radical species. These could be oxygen or hydroxyl radicals as well as the semiquinone anion [2, 4, 5, 25] or the primary donor, Z [7]. These could cause further specific irreversible covalent changes in the D 1 protein resulting in its exposure to proteolytic degradation. Reversible light-induced conformational change in reaction center II affecting primarily the redox/stability of the Q_B^- have been recently demonstrated to occur *in vivo* [7], and found to correlate with the initial loss of variable fluorescence and partial loss of reaction center II activity. Furthermore under these conditions we could also demonstrate that a fraction of the D 1 protein undergoes a covalent modification [7], which correlates with an increase in the turnover of this protein [26]. The above hypothesis is supported by the findings that reversible conformational changes affecting the redox potential of the acceptor quinone are induced by light in isolated bacterial reaction centers in which the D-Q_B^- species have been generated [27]. The presence of a protease sensitive "PEST" sequence [25], specific to the D 1 protein which could be the primary site of degradation of this protein *in vivo* supports the concept that in the irreversibly modified protein this sequence became accessible to proteolysis. However the validity of our hypothesis rest on the demonstration that indeed occupancy of the Q_B site by plastoquinone and thus the ensuing light-dependent electron flow *via* this site is required for these phenomena to occur. The data presented here strongly support the contention that indeed continuous PQ/PQH_2 turnover within the Q_B site is essential for the light-induced loss of reaction center II activity and degradation of D 1 protein. In the mutant lacking the cytochrome b_6/f complex, light-dependent electron flow *via* reaction center II is rate-limited by reoxidation of the plastoquinol due to chlororespiration [22, 23]. This process is apparently slow as indicated by the data presented here and may account for the observed partial sensitivity to the light of the B_6 mutant reaction center II. In cells exposed to higher light fluency the mechanism(s) responsible for the plastoquinone oxidation, for reasons not yet understood, became less efficient and thus the occupancy of the Q_B site is further reduced. In these cells, the light-induced loss of reaction center II activity and the downshift in the TL emission temperature are less affected than in cells exposed to intermediate light intensity and may account for the observed "recovery" of TL signal temperature in B_6 cells transferred from low to high light (Fig. 3, 5).

It has been reported previously that in *Spirodella* ultra-violet (UV B) light causes enhanced degradation of the D 1 protein as compared to that induced by similar fluency of photosynthetic light [28]. This may be explained if one considers that in cells exposed to UV light direct excitation of the bound quinone could induce the specific modification of the D 1 protein only when the site is occupied. Degradation of the D 1 protein was elicited also by far red light which supported photosystem I cyclic electron flow but only a limited photosystem II activity [29]. In this case, generation of plastoquinol will be rate limiting relative to its oxidation by photosystem I and occupancy of the Q_B site by plastoquinone will be favored. The light-induced modification of reaction center II which appears to affect specifically the D 1 protein as detected by the thermoluminescence measurements and loss of variable fluorescence, is also prevented partially in cells exposed to the light in the presence of DBMIB [7], an inhibitor which at low concentrations blocks electron flow *via* cytochrome b_6/f [30] and thus favors accumulation of plastoquinol as in the case of the B_6 mutant. Based on the data presented here and elsewhere [7, 26] we

thus conclude that the light-dependent degradation of the D 1 protein is related to the loss of reaction center II activity. The mechanism of this phenomenon involves sustained turnover of the reduced/oxidized plastoquinone *via* the Q_B site.

Additional *Chlamydomonas* mutants lacking the cytochrome b_6/f complex, plastocyanin or photosystem I (manuscript in preparation) show a similar reduction in the light-dependent turnover of D 1 protein. Experiments devised to further elucidate the role of plastoquinol oxidation rate in the turnover of D 1 protein are now in progress.

Acknowledgements

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