The redox state of the plastoquinone pool directly modulates minimum chlorophyll fluorescence yield in *Chlamydomonas reinhardtii*

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A B S T R A C T

The effect of the plastoquinone (PQ) pool oxidation state on minimum chlorophyll fluorescence was studied in the green alga *Chlamydomonas reinhardtii*. In wild type and a mutant strain that lacks both photosystems but retains light harvesting complexes, oxygen depletion induced a rise in minimum chlorophyll fluorescence. An increase in minimum fluorescence yield is also observed when the PQ pool becomes reduced in the presence of oxygen and after application of an ionophore that collapses the transmembrane proton gradient. Together these results indicate that minimum chlorophyll fluorescence is modulated by the PQ oxidation state.

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1. Introduction

Chlorophyll (Chl) fluorescence is an intrinsic feature of all Chl-based photosynthetic organisms [1–6]. This signature of photosynthesis [7] is a non-invasive reporter of the state of the photosynthetic machinery. In cyanobacteria, green algae, and higher plants the understanding of the mechanisms that modulate Chl fluorescence has reached a sophistication that allows the assessment of excitation transfer, electron transfer, biochemical reactions and physiology [3].

There remain substantial gaps in our understanding of Chl fluorescence. Several lines of evidence suggest that Chl fluorescence is directly modulated by the plastoquinone (PQ) oxidation state [reviewed by [8–10]]. However, there has not been a clear demonstration that PQ operates as a Chl fluorescence modulator in an in vivo system. This is due to the complex interactions with other potential Chl fluorescence modulators, in particular the modulation by the status of the electron acceptors of photosystem II (PSII).

In PSII, as well as photosystem I (PSI), excitation energy is utilized to generate reducing equivalents through a charge separation event. The cofactors involved in charge separation and stabilization are housed in the reaction center core. The increase of the absorption cross-section of each photosystem is accomplished through monomeric and trimeric antenna protein complexes, termed light harvesting complexes (LHCS).

Interaction of light with a Chl molecule can result in an excited-state Chl, which can recover to the ground state by converting most of the excited-state energy into fluorescence. In photosynthetic organisms the likelihood of Chl fluorescence occurring is determined by competing deexcitation pathways. Excitation can be transferred to another molecule, be used to accomplish charge separation, or be emitted as fluorescence or another form of radiation. Because Chls are bound to several different protein complexes (i.e. PSs and LHCS) and interact with different classes of molecules (e.g. other Chls, carotenoids, quinones, oxygens), Chl fluorescence is a very complex and information-rich reporter.

When plants and green algae are illuminated, a complex modulation of Chl fluorescence yield is observed [11]. While Chls of both photosystems contribute to fluorescence emission, the fluorescence emitted by Chls energetically-coupled to PSII exhibits a large dynamic modulation at room temperature. In contrast, the contribution of those associated with PSI to overall Chl fluorescence is rather small, with estimates ranging from 5% to 20% [10] and appears largely static and not modulated by the status of the electron transport chain of PSI.

Abbreviations: AA, antimycin A; Chl, chlorophyll; F₀, minimum fluorescence yield obtained by a non-actinic measuring light; Fₚₚ, maximal fluorescence yield obtained by a saturating light pulse; LHC, light harvesting complex; PS, photosystem; OG, octyl gallate; TAP, Tris–acetate–phosphate; WT, wild type

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Several mechanisms have been proposed and identified to modulate Chl fluorescence in plants and green algae. (1) The main modulator of Chl fluorescence is the oxidation state of the first electron acceptor of PSII, QA [12], a tightly-bound plastoquinone (PQ) [13]. While it was first assumed that QA is the only modulator of chlorophyll fluorescence, additional fluorescence-modulating mechanisms have been identified: (2) the reduction state of the primary electron donor (P680) [14–16], (3) the electrochemical potential across the thylakoid membrane [17,18], and (4) the association of LHCs with either PSI or PSII [19–21].

In addition to these established mechanisms, it has been suggested that molecular oxygen [22] and oxidized PQ molecules [23–25] can also act as quenchers, and thus modulate in vivo Chl fluorescence. This study investigates the possibility that oxidized PQ can directly quench Chl fluorescence by isolating and distinguishing this mode from all other known modes of Chl fluorescence modulation.

2. Materials and methods

2.1. Algal strains and growth conditions

Chlamydomonas reinhardtii wild type (WT) (2137c mt−), cytochrome oxidase-deficient mutant (M90.23), and strains unable to accumulate PSI and PSII (ΔPSII/II) were utilized in this study. A ΔPSII/II strain has been generated biologically with tungsten particles coated with pAA400 plasmid [26] in which the psaA gene was deleted, and a host ΔPSII mutant FuD7 [27] that we obtained from the Chlamydomonas Center (http://www.chlamy.org/). An independently derived ΔPSII/II strain, C2 [28], was also used. This strain carries two chloroplast DNA deletions, one encompassing the tscA, chlN and one copy of psbA genes, and the other deleting the other copy of the psbA gene.

All strains were maintained under phototrophic conditions on Tris–acetate–phosphate (TAP) plates under incandescent illumination of 100–120 μmol photons m−2 s−1 for WT and M90.23 mutant, and 5–20 μmol photons m−2 s−1 for the ΔPSII/II mutants. Cells were grown in liquid TAP media until mid-logarithmic growth phase and concentrated to ~40 μg Chl/ml (WT and M90.23) or ~20 μg Chl/ml (ΔPSII/II) in fresh TAP medium.

2.2. Induction of anaerobic and respiration inhibited conditions

Anaerobic conditions were achieved by cellular respiration in a sealed electrode chamber or cuvette. Inhibitors were used to specifically terminate respiration. Octyl gallate (OG) and antimycin A (AA) were dissolved in ethanol (stock solution 10 mM and 5 mM, respectively). For experiments a final concentration of 10 μM OG and 5 μM AA was added to cells. Nigericin (stock 1 mM in water) was applied at a final concentration of 1 μM.

2.3. Data acquisition

Simultaneous oxygen and Chl fluorescence measurements were recorded using two different systems. In one setup a Clark-type oxygen electrode (Rank Brother, United Kingdom) was interfaced with a PAM fluorometer (Walz, Germany). An alternative configuration utilized an inhouse-built fluorometer [29] that interfaces a custom-made cuvette with an oxygen-modulated luminescence lifetime sensor (Presens, Germany) [30]. The presented figures show the results recorded with the former system, which were further confirmed by the latter system. The oxygen signal was calibrated using aerated water and electrode readings in cell suspensions in which oxygen was depleted by cellular respiration. Low light intensities were chosen to avoid actinic effects of the measuring light by the PAM fluorometer (1.6 kHz setting). Short (20 μs) infrequent (1 pulse every 5 s), low-intensity probing pulses were applied with the in-house-built fluorometer to avoid the accumulation of reduced QA between pulses. To ensure the non-actinic nature of the probing light we used intensities and frequencies that did not induce an increase in F0 when the QA site was completely blocked by a bound herbicide (10 μM DCMU). To obtain the maximum fluorescence yield (Fm'), saturating light pulses >2000 μmol photons m−2 s−1 were applied for 1 s.

3. Results

3.1. F0 increase and PQ pool reduction in dark anaerobic conditions

When WT C. reinhardtii cells were placed in an airtight compartment in the dark, oxygen was consumed by cellular respiration and the cells encountered anaerobic conditions. This caused a biphasic increase in F0 (Fig. 1a) and a decrease in Fm' (Fig. 1b). During anaerobic conditions mitochondrial respiration cannot dispose of metabolically-generated electrons, leading to the accumulation of reducing equivalents throughout the cell [19,31]. Electrons generated in mitochondria, cytoplasm, or within the chloroplast may enter the photosynthetic electron transport chain by the same complexes that are involved in cyclic photosynthetic electron...
transport (cytochrome b6f complex and a chloroplastic NAD(P)H oxidoreductase) [32]. The chloroplastic NAD(P)H plastoquinone oxidoreductase may also be a portal for electrons for chlororespiration (reviewed by [33,34]). The reduction of the PQ pool triggers a relocation of LHC from PSII to PSI, which subsequently lowers FM (state 2 transition). While the mechanism responsible for the decrease in FM is well established [35,36], that for the increase in F0 remains unclear.

3.2. Qa-dependent quenching

The reduction of the PQ pool coincided with the F0 increase under dark anaerobic conditions. Since QA, QA, and the PQ pool are in a redox equilibrium, the observed F0 increase could be caused by the reduction of QA under these conditions. To test if the observed F0 increase was due to reduced QA, the effect of anaerobic conditions on fluorescence was checked in a mutant strain that is unable to assemble PSI and PSII core complexes ([33,34]). The reduction of the PQ pool triggers a relocation of LHC from PSII to PSI, which subsequently lowers FM (state 2 transition). While the mechanism responsible for the decrease in FM is well established [35,36], that for the increase in F0 remains unclear.

3.3. Energy-dependent quenching

To test the possibility that the observed fast F0 increase was due to the anaerobic-induced relaxation of the transmembrane proton gradient, effects of the ionophore nigericin, which disrupts the proton gradient, were examined. The results show no effects of nigericin on F0 increase in WT and fluorescence in ΔPSI/II (Fig. 2a and b), suggesting that the F0 increase induced by anaerobic conditions was not due to the relaxation of energy-dependent quenching.

3.4. O2-dependent quenching

Since molecular oxygen is a potential Chl fluorescence quencher [37], oxygen depletion could lead to the fast F0 rise observed above. To distinguish oxygen-mediated quenching from that dependent on oxidized PQ molecules, the PQ pool was reduced through the application of respiratory inhibitors in the presence of oxygen [30,38]. Since C. reinhardtii possesses two oxygen-dependent mitochondrial oxidases, the cytochrome oxidase and alternative oxidase (AOX), both activities must be inhibited to completely abolish mitochondrial oxygen consumption. This can be accomplished by applying antimycin A (AA) and octyl gallate (OG). AA inhibits the cytochrome bc1 complex, terminating electron transport to cytochrome oxidase. OG inhibits AOX in mitochondria and is also an efficient inhibitor of a chloroplastic oxidase (PTOX) [39]. Application of AA alone or OG alone to WT or the ΔPSI/PSII strain did not induce an increase in fluorescence yield (data not shown). When we added both AA and OG to WT cells to test the effects of complete termination of cytochrome oxidase, AOX and PTOX activity, an F0 increase was observed (Fig. 3a). We also tested the effect of OG addition to cytochrome oxidase-deficient mutant (M90.23), which also led to an increase in F0 (Fig. 3c).

It is of note that the F0 increase induced by the application of AA and OG in WT (Fig. 3a) and by the application of OG in the cytochrome oxidase mutant M90.23 (Fig. 3c) is much more pronounced than the F0 increase caused by oxygen depletion (Fig. 1a). These extra F0 increases were reset by the application of saturating light pulses to WT in the presence of AA and OG and to M90.23 cells in the presence of OG (Fig. 3b and d, respectively). The F0 level achieved by these pulses is similar to the F0 level induced at the onset of anaerobic conditions (Fig. 1a and b). Inhibition of cellular respiration in ΔPSI/II cells induces an increase in fluorescence (Fig. 3e) similar to that observed under anaerobic conditions (Fig. 1c).

4. Discussion

4.1. F0 increase in anaerobic conditions

The accumulation of electrons in the electron transport chain induced by oxygen depletion proceeds in two phases indicative of (1) reduction of the PQ pool, followed by (2) reduction of QA. This interpretation is in agreement with previous studies using different terminology [40,41].

The current results indicate that the initial rise of F0 upon encountering anaerobic conditions is due to the loss of oxidized PQ quenching as the PQ pool becomes reduced. This initial increase is followed by a slower rise in F0 due to the accumulation of reduced QA in WT. The assignment of these two phases to the loss of oxidized PQ quenching, followed by the reduction of QA, is in agreement with the observations made in the ΔPSI/II strain, where in the absence of QA, only the first fast increase in fluorescence was observed.

The secondary slow increase in F0 in the WT strain that occurs after prolonged anaerobic adaptation is reset by saturating light pulses to the fluorescence level reflecting a reduced PQ pool. These pulses oxidize the PQ pool through the action of PSI. The oxidized PQ pool can in turn oxidize QA that has been generated in the preceding dark period. The action of saturating light pulses in resetting F0 to a level reflecting the reduced PQ pool is especially well observed in cells where respiration has been chemically inhibited. In these cells the initial increase in F0 reflects not only the loss of oxidized PQ-mediated quenching, but also accumulation of QA that occurs more rapidly compared to anaerobically-adapted cells.
A likely reason for the slower accumulation of reduced QA in anaerobically incubated cells compared to chemically-inhibited cells is that low oxygen concentrations may trigger a physiological response preceding anaerobic conditions. In contrast, the termination of respiration by inhibitors does not permit cells to limit electron mobilization in anticipation of a switch to anaerobic metabolism.

4.2. Exclusion of other known Chl fluorescence modulation mechanisms

Our study demonstrates that in vivo modulation of F₀ by the oxidized PQ molecules in the PQ pool is distinct from other known modes of fluorescence modulation: (1) the use of a mutant strain that lacks the charge separating PSI and PSII complexes excludes Chl fluorescence modulation by QA; (2) since all experiments were conducted essentially under dark conditions, P₆₈₀-dependent quenching can also be excluded; (3) application of nigericin indicates that loss of energy-dependent quenching is not the reason for the observed increase in F₀ under anaerobic conditions; (4) the state transition induced by anaerobic conditions decreased Fₘ and occurs on a slower time scale in WT and the M90.23 strain and is absent in the ΔPSI/II strain. Furthermore, the presence of oxygen in cells with chemically-inhibited respiration does not prevent the increase in F₀ while the PQ pool becomes reduced.

4.3. Oxidized PQ as a quencher of Chl fluorescence in WT and ΔPSI/II

The fluorescence increase caused by anaerobic conditions in the ΔPSI/II strains indicates that fluorescence modulation occurs in LHCs, with relaxation of direct quenching of Chl fluorescence within the LHC system by oxidized PQ molecules as a likely mechanism. Recent in vivo measurements show similarly short fluorescence lifetimes in the ΔPSI/II and WT cells [26]. This surprising finding is consistent with a similar degree of fluorescence modulation that occurs in the ΔPSI/II strain and WT when quenching through oxidized PQ is abolished by anaerobic transitions.

The ability of oxidized quinones to quench Chl fluorescence has been suggested in studies using chemically treated membrane fragments [23,24] and thylakoids and reconstituted systems with added non-native quinones [42,43] and native PQ [8,44,45]. Chl fluorescence quenching by oxidized quinones has been suggested to occur via a transient charge transfer and recombination between Chl and quinone [46,47].

4.4. Magnitude of Chl fluorescence modulation

More than 90% of fluorescence in intact cells is emitted by chlorophyll molecules in the LHC complexes [1]. WT cells and the mutants lacking the charge separation machinery show a similar extent of fluorescence modulation dependent on the reduction state of the PQ pool. Fluorescence yield under dark aerobic conditions is 84% (ΔPSII/I) and 75% (WT) of the fluorescence yield under dark anaerobic conditions. The difference in fluorescence modulation in WT and the ΔPSII/I strain may be due to differences in the PQ reduction state in the dark and due to additional quenching by oxidized PQ acting on the chlorophylls associated with the photosystems.

The PQ pool of C. reinhardtii is partly reduced in dark aerobic conditions [48]. In darkadapted Arabidopsis thaliana leaves, direct determination of oxidized and reduced PQ indicates that 40% of PQ is reduced [49]. Assuming similar dark aerobic PQ reduction states in A. thaliana and C. reinhardtii, as well as a linear relationship between the amount of oxidized PQ and decrease in fluorescence [44], a total decrease of about 40% in F₀ in the presence of an oxidized PQ pool compared to a reduced PQ pool may be extrapolated.

4.5. Minimum Chl fluorescence yield

F₀ is a parameter that is used to calculate many photosynthetic performance indicators [3,7,17]. Usually F₀ is defined as the fluo-

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**Fig. 3.** Fluorescence (black, solid) and oxygen (red, dotted) traces in WT (a and b), cytochrome oxidase-deficient strain M90.23 (c and d), and ΔPSII/I mutant (e). AA and OG were added to a final concentration of 5 μM (AA) and 10 μM (OG) to inhibit cytochrome bc₁ and AOX/PTOX activity, respectively. F₀ was assessed in (a, c and e) and FM was probed by saturating light pulses (b and d).
rescence yield in a system with oxidized QA in all PSII centers. Our results indicate, however, that 0 is also significantly modulated independently of the reduction state of QA. Experimental protocols such as several minutes of dark adaptation or the use of a pulse of far red light, have been developed with the intention of oxidizing QA and thereby achieving the ‘true F’ or F. Our data suggest that these procedures achieve this lowest fluorescence level by inducing additional quenching through oxidizing the PQ pool. The amount of modulation by the PQ pool reduction state determined in this study justifies the care taken to obtain a ‘true F’.

4.6. Steady state and maximum Chl fluorescence

Under steady state conditions the redox states of QA, QB and PQ are in equilibrium. Therefore under a range of light intensities the reduction state of QA and the reduction state of the PQ pool are related. The correlation between PQ pool and QA reduction status breaks down, however, during short saturating light pulses that are used to determine 0. The multi-photon requirement and temperature dependency for obtaining 0 [50,51] may indicate a link to the light-dependent loss of quenching provided by oxidized PQ [10,37,52–54].

4.7. Physiological function of PQ-mediated excitation modulation

The ability of oxidized PQ to quench fluorescence appears to be counterproductive to physiological requirements. Under low light conditions PQ remains oxidized, and precious excitation energy is lost. Under high light conditions a reduced PQ pool would ensure that excitation is efficiently coupled to the reaction centers. However, a reduced electron transport chain has been shown to cause damage under illumination, especially to PSII.

This seemingly illogical interaction of physiology with excitation modulation may be comprehensible in an evolutionary context. Quinones were likely recruited as mobile electron carriers before substantial amounts of oxygen were present in the atmosphere. In oxygen-free conditions, modulating excitation transfer before substantial amounts of oxygen were present in the atmosphere may have provided an efficient mechanism for adjusting a photosynthetic system without incurring photodamage. There has been a proposal that excitation modulation through the reduction state of PQ enables energy dissipation in green algae and plants [54]. To what extent this ‘legacy modulation’ has been adapted to ‘tune’ energy transfer within the complex oxygenic photosynthetic system remains to be further clarified in the future.

5. Conclusions

Anaerobic transitions allow a physiology-based manipulation of the reduction state of the PQ pool in an in vivo system without the application of light or chemicals. The responses of WT and mutants to anaerobic conditions were utilized to identify the effect of the PQ pool oxidation state on Chl fluorescence. Our study indicates that the quenching ability of oxidized PQ contributes substantially to modulating of chlorophyll fluorescence.

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