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Control of the maximal chlorophyll fluorescence yield by the Q_B binding site

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Abstract

Differences in maximal yields of chlorophyll variable fluorescence (F_m) induced by single turnover (ST) and multiple turnover (MT) excitation are as great as 40%. Using mutants of *Chlamydomonas reinhardtii* we investigated potential mechanisms controlling F_m above and beyond the Q_A redox level. F_m was low when the Q_B binding site was occupied by PQ and high when the Q_B binding site was empty or occupied by a PSII herbicide. Furthermore, in mutants with impaired rates of plastoquinol reoxidation, F_m was reached rapidly during MT excitation. In PSII particles with no mobile PQ pool, F_m was virtually identical to that obtained in the presence of PSII herbicides. We have developed a model to account for the variations in maximal fluorescence yields based on the occupancy of the Q_B binding site. The model predicts that the variations in maximal fluorescence yields are caused by the capacity of secondary electron acceptors to reoxidize Q_A^-.

Additional key words: conformational change; electron transport; photosystem II; thylakoid membrane.

Introduction

Progress in commercial instrumentation development and methodology has widely increased the use of chlorophyll (Chl) variable fluorescence as a biophysical tool to probe photosynthetic processes (*e.g.*, Govindjee 1995, Kolber *et al.* 1998, Suggett *et al.* 2010, Kalaji *et al.* 2014, Lin *et al.* 2016). Although several biophysical models of varying complexity exist that describe the origin of variable Chl fluorescence within PSII and the relation between variable fluorescence and photochemical yields (*e.g.*, Butler and Strasser 1977, Dau 1994, Lavergne and Trissl 1995, Lazar and Schansker 2009, Vredenberg and Prasil 2009), the interpretation of variable fluorescence is still a subject of debate. The consensus is that the redox state of the primary quinone acceptor Q_A is the major factor responsible for the variability of the Chl fluorescence yield in photosynthesis (Duysens and Sweers 1963): when Q_A is oxidized, the yield is low (F_0), when Q_A is reduced, the yield is high (F_m). The controversy rises from the observations that Q_A is nearly fully reduced following short ST flash of μs duration (Joliot and Joliot 1981), but the fluorescence yield after ST flash reaches only approx. 60% of the maximal value. The maximal value of F_m is obtained only after much longer MT excitation (tens or hundreds of ms). Therefore several other processes, on both the donor and acceptor sides of PSII, have been suggested to result in up to *ca*. 40% of the variability in yields (reviewed *e.g.*, Dau 1994, Samson *et al.* 1999, Schansker *et al.* 2014). This issue is not trivial, as the interpretation of biophysical processes related to photosynthetic energy conversion is increasingly based on measurements of variable fluorescence, inherently dependent on selection of experimental protocols and data interpretation (Kolber *et al.* 1998, Kromkamp and Forster 2003, Suggett *et al.* 2003, Suggett *et al.* 2010).

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Abbreviations: 2,5 DMBQ – 2,5-dimethyl-*p*-benzoquinone; 2,6 DCBQ – 2,6-dichloro-*p*-benzoquinone; Chl – chlorophyll; DBMIB – 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCMU – 3-(3,4,-dichlorophenyl)-1,1-dimethylurea; FRR – fast repetition rate; F_0 – initial fluorescence yield in dark-adapted sample; F_i – initial fluorescence yield in light or following preillumination; F_m – maximal fluorescence yield; HF1 – F_m induced by first ST excitation in dark-adapted cells; HFD – F_m in the presence of saturating amounts of PSII herbicides; HFM – F_m induced by MT excitation; LF – F_m induced by the second ST excitation; MT – multiple turnover; PQ – plastoquinone-9; RCII – reaction center of PSII; ST – single turnover; TL – thermoluminescence; σ_{PSII} – functional cross-section of PSII.
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Several hypotheses have been proposed to explain the difference between maximal fluorescence yields observed after ST and MT excitations (reviewed in Samson *et al.* 1999, Schansker *et al.* 2014, Stirbet and Govindjee 2012). A number of experiments indicate that differences in F_m are related to nonphotochemical phenomena on the acceptor side of PSII (*i.e.*, the variations in F_m are not related to changes in the quantum yield of photochemical charge stabilization within PSII). Delosme (1967) proposed that, in addition to the reduction of Q_A resulting in a rapid increase in fluorescence yield (the photochemical phase of fluorescence induction), MT excitation also results in a slower reduction of a nonphotochemical quencher, R (the "thermal" phase). It was later suggested that R is at least partly related to the removal of static fluorescence quenching by oxidized plastoquinone molecules (Vernotte *et al.* 1979, Yaakoubd *et al.* 2002). Other authors have related the slowly reducible quencher to the structural or functional heterogeneity of the acceptor side of PSII (Diner and Mauzerall 1973, Joliot and Joliot 1977, 1979, 1981). Joliot and Joliot (1977, 1979) proposed the existence of an alternative quencher, Q_2, and Valkunas *et al*. (1991) proposed multiple quenching states of PSII (France *et al.* 1992). The concept of several, structurally independent quenchers located on the acceptor side of PSII seems difficult to reconcile with observations that F_m, induced by a ST flash, increases in the presence of DCMU and is comparable to F_m obtained by MT excitation (*e.g.*, Neubauer and Schreiber 1987). Samson and Ruce (1996) concluded that R is located beyond Q_A and interacts closely with the Q_B binding site. Using the series of ST flashes, Vredenberg *et al*. (2006) in a series of papers (Vredenberg *et al.* 2007, 2009, 2012; Vredenberg and Prasil 2013) extensively studied the modulation of the fluorescence yield related to the thermal phase. Although these experiments did not prove the role of Q_B site occupancy directly, they clearly demonstrated that the fluorescence yield changes during the thermal phase are caused by the photo-electrochemical processes on the acceptor side of PSII following multiple excitations and secondary electron transfers. Recently, Schansker *et al.* (2011) conducted a detailed analysis of the possible origin of the thermal phase and concluded that it reflects a fluorescence yield change caused by light-induced conformational change in the reaction center of PSII that occurs after the primary reduction of Q_A.

Alternatively, Schreiber and coworkers proposed that multiple turnovers are required to remove the nonphotochemical fluorescence quenching induced by ST flashes which results from kinetic limitation originating on the PSII donor side (Schreiber and Neubauer 1987, Schreiber *et al.* 1995a,b). The long pulses of actinic light used during the saturation pulse method (Schreiber *et al.* 1986) are thus deemed essential to observe the full extent of photochemical quenching of variable fluorescence. This proposal forms the basis of the widely used saturating pulse method for assessing photosynthetic yields (Schreiber 1984). Schreiber and Krieger (1996) have further suggested that differences in F_m induced by ST or MT excitations are due to variations in yields of recombination fluorescence in closed PSII reaction centers.

In 1998, we introduced the fast repetition rate (FRR) method for measurements of variable fluorescence (Kolber *et al.* 1998). The FRR permits both ST and MT excitations of PSII and substantially increases the amount of information that can be obtained from measurements of variable fluorescence. The FRR fluorescence technique applies a sequence of subsaturating excitation pulses ('flashlets') at microsecond intervals to induce fluorescence transients. This approach allows the generation of both single-turnover (ST) and multiple-turnover (MT) flashes and allows to measure a suite of photosynthetic parameters: fluorescence quantum yields, photochemical and nonphotochemical quenching, functional absorption cross-section of PSII, energy transfer between PSII units, and the kinetics of electron transfer on the acceptor side of PSII. The interpretation of the ST-derived data is based on the assumption that the time constant of Q_A^- reoxidation remains constant during the excitation protocol. Using this technique, in conjunction with thermoluminescence and molecular genetics, we present experimental data that support our earlier hypothesis that differences in maximal fluorescence yields are controlled by the occupancy of the Q_B site (Samson *et al.* 1999). We discuss these results in relation to the interpretation of variable fluorescence measurements.

Materials and methods

Experimental organisms: For most experiments, we used the unicellular chlorophytes *Chlorella pyrenoidosa* and *Chlamydomonas reinhardtii*. Cells were grown in batch cultures in mineral medium at 25°C in 500-ml bottles under continuous light at an irradiance of 50 μmol (quantum) m^{-2} s^{-1} supplied by cool white fluorescent tubes (LL - low light conditions). High light (HL) cells were grown at 400 μmol(quantum) m^{-2} s^{-1}. Cultures were maintained in logarithmic growth phase. The DCMU-4 (Erickson *et al.* 1984) (cc 2473), cc 2964 [pet A-A15E mutation (Smith and Kohorn 1994), resulting in a reduced accumulation of cytochrome *f*] and a series of photosynthetically competent mutants with substitution of D1 residue Ala251 (Lardans *et al.* 1997) (cc3388 A251I, cc3389 A251L, cc3390 A251C, cc3391 A251P, cc3392 A251S, cc3394 A251A) of *Chlamydomonas reinhardtii* were obtained from the Chlamydomonas Genetics Center, Duke University, Durham, NC, USA. All *Chlamydomonas* strains were grown at LL conditions in HS mineral medium (Sueoka 1960). The marine unicellular algae *Phaeodactylum tricornutum* and *Thalassiosira weissflogii* (diatoms), *Nannochloropsis sp.* (Eustigmatophyceae),

and *Aureococcus anophagefferens* (Pelagophyceae) were grown in ASW medium enriched with f/2 nutrients (Guillard and Ryther 1962) at 20°C, under LL growth conditions.

Thylakoid membranes: Thylakoids were prepared from freshly harvested spinach leaves according to Whitmarsh and Ort (1984). After the final centrifugation step, the membranes were resuspended in the measuring medium (0.2 M sorbitol, 10 mM $MgCl_2$, 10 mM NaCl, and 50 mM Hepes, pH 6.8/NaOH) with a concentration of 3 mg(Chl) ml^{-1} and kept on ice. For fluorescence measurements, thylakoids were resuspended in the measuring medium to less than 1 μg(Chl) ml^{-1}. Thylakoids were used within 8 h after preparation.

Photosystem II particles: PSII particles (BBY) were isolated from spinach as described by Berthold *et al.* (1981). Freshly prepared, nonfrozen particles were diluted in measuring medium (0.3 M sucrose, 10 mM NaCl, 5 mM $MgCl_2$, and 30 mM MES, pH 6.2/NaOH) and were used within 10 h after isolation to examine fluorescence and thermoluminescence characteristics.

Thermoluminescence was measured with a computerized laboratory-built apparatus, as described previously (Prasil *et al.* 1996). Five ml of algal suspension containing 5 μg(Chl) were gently filtered on *Millipore HA 25* filters (25 mm diameter, 0.8 μm pore size) and placed on a temperature-regulated sample holder. Care was taken to keep the algae covered at all times by a layer of medium. Algae were kept for 180 s in the dark at 25°C. The sample holder was then cooled to +2°C and after 60-s temperature equilibration, the sample was excited by 2 single turnover saturating flashes provided by a xenon flash lamp at 1 Hz. The TL emission signal was recorded from +2 to +70°C with linear heating at a rate of 0.5°C s^{-1}.

Fluorescence measurements: Fluorescence transients were acquired in unicellular algae, suspensions of thylakoid membranes, and BBY particles using custom-built FRR fluorometer (Kolber *et al.* 1998).

Results

A wide range of variable fluorescence responses to ST and MT FRR excitation protocols can be elicited, depending upon the sequence and interval between flash sets (Fig. 1). The magnitude of variation can be significant. In *Chlorella*, for example, F_m can vary by up to 40%, depending on the excitation protocol and sample conditions used (Kolber *et al.* 1998). The variations in maximal fluorescence yield can be characterized by four general levels: LF, HF1, HFM, and HFD (Fig. 1). During the first ST excitation, the fluorescence yield rises to a level (HF1) that is, on average, ~15–20% higher than maximal fluorescence yield, LF, observed during the second, and subsequent ST flashes. Long (>50 ms) MT excitation results in an increase in fluorescence yield to the HFM level which is significantly higher in intact algal cells and in thylakoid membranes than both the HF1 and LF. In the presence of PSII herbicides, such as DCMU, which blocks electron transfer from Q_A to Q_B, the maximal fluorescence yield reaches an HFD level that is 5–10% lower than HFM level (Fig. 1B). The HFD level is independent of the ST or MT excitation protocol. This general pattern was observed in a wide number of cyanobacterial and algal taxa and in isolated thylakoid membranes, but not in PSII particles (Table 1S, *supplement available online*), with the maximal yields almost identical. The variability in F_m is not correlated with variations in PSII/PSI ratios and can significantly influence calculations of photochemical yields [F_v/F_m, F_v/F_0 (Table 1S)]. As the results in Fig. 1 cannot be readily explained by the level of Q_A^- reduction and its influence on variable fluorescence yields alone, we examined alternative hypotheses to account for the observed patterns. In the following section, we described results of experiments directed at unraveling some of the mechanisms that modify fluorescence yields of closed PSII reaction centers.

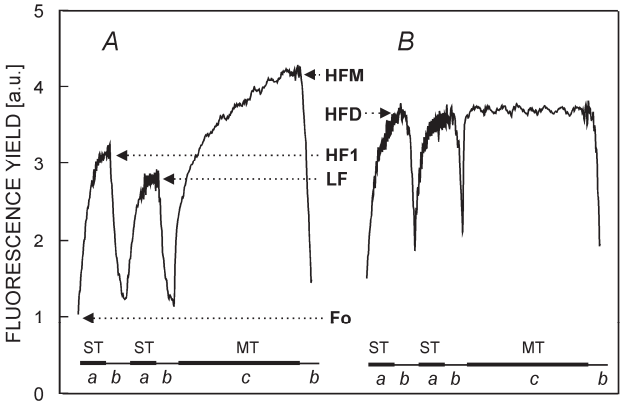

Fig. 1. Changes in fluorescence yield induced by series of two single turnover (*a*) and multiple turnover (*c*) FRR excitation protocols. Low light [50 μmol(quantum) m^{-2} s^{-1}) grown *Chlorella* cells [0.5 μg(Chl) ml^{-1}] were kept in the dark for 120 s before the first ST flash. The measurements were performed on control cells (panel *A*) or after addition of 1 μM DCMU (panel *B*). The x-axis is linear in number of excitation flash lets, but not in time. Although the flashlets were always of 1-μs duration, they were spaced by dark intervals of different duration: the single-turnover excitation protocols consisted of 80 flashlets spaced by 0.6-μs interval, *i.e.*, total duration of 120 μs (*a*). Then the relaxation of fluorescence yields was followed for 1.5 s (*b*). Finally, the multiple turnover excitation protocol consisted of 3000 flashlets spaced by 50 μs, with total duration of 150 ms (*c*). In the DCMU measurements, the herbicide was added after 60 s in the dark.

If algal cells or spinach thylakoids are kept in the dark for more than 120 s and subjected to a sequence of ST

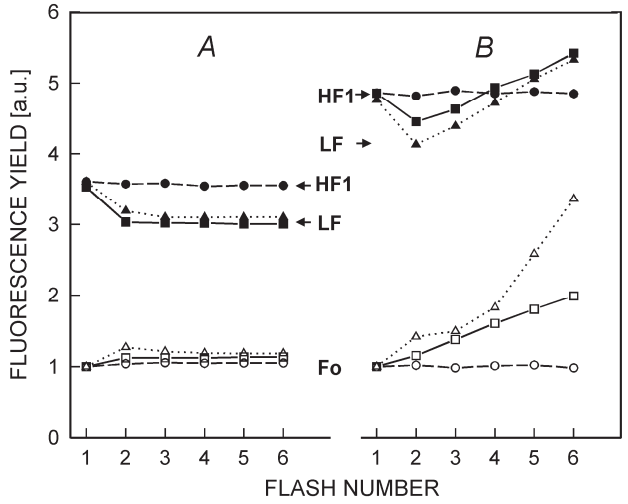

Fig. 2. Dependence of the initial (*open markers*) and maximal (*closed markers*) variable fluorescence yields on the number of single turnover excitations and on the time interval between ST flashes for *Chlorella* cells (*A*) and spinach thylakoid membranes (*B*). Chlorophyll concentration in samples and parameters of FRR single turnover excitation protocols were the same as in Fig. 1. The time interval between individual ST excitations was 0.125 s (*triangles*), 1.5 s (*squares*), and 60 s (*circles*). Samples were kept for 120 s in the dark before the first flash.

flashes, the fluorescence yield induced by the first flash rises to the HF1 level, while on the second ST flash it rises to the lower, LF level. If ST flashes are delivered at a frequency that permits reoxidation of the acceptor side of PSII [typically 1–10 Hz in whole cells (Fig. 2*A*) or less in isolated spinach thylakoids (Fig. 2*B*)], F_m remains at the LF level. If, however, ST flashes are repeated frequently enough to gradually reduce the acceptor side, F_m increases (Fig. 2*B*). Under such conditions, the initial fluorescence yield (F_i) also rises.

In intact algae, flash period oscillations of F_m are very small (Fig. 2*A*, *see* also Fig. 1S in Kolber *et al.* 1998) with the exception of the initial transition from the HF1 state in dark-adapted samples to the LF state by the first ST flash. Despite numerous experimental observations of the effect of donor side on fluorescence (Delosme 1971a, Delosme 1971b, Joliot and Joliot 1971, Zankel 1973, Bowes and Crofts 1980, Robinson and Crofts 1983, Kramer *et al.* 1990, Shinkarev *et al.* 1997, Koblizek *et al.* 2001), the lack of significant F_m oscillations under our experimental conditions suggests that the differences between HF1 and LF cannot be quantitatively accounted for by changes in the redox state on the donor side of PSII.

To test the hypothesis that the transition from HF1 to LF is primarily due to processes on the acceptor side of PSII, we analyzed the fluorescence characteristics of photoautotrophic *Chlamydomonas* wild type and mutants with a modified single amino acid in the Q_B-binding pocket of the D1 protein. In wild type (WT) cells (Fig. 3*A*), reoxidation of Q_A^- proceeds with τ of 300–600 μs when Q_B is bound to the reaction center, but this time constant increases to 1.5–3 ms in centers with an empty pocket at the time of the flash (Crofts *et al.* 1993, Crofts and Wraight 1983, Taoka 1990). Q_A^- reoxidation kinetics in the HF1 state can be inferred from the decay of variable fluorescence yield (F) measured just prior to the second ST flash (open symbols, Fig. 3*A*). The transition from HF1 to LF follows the overall rate of Q_A^- reoxidation, with time constant of about 2 ms. Thus, the LF state is formed by the second ST flash only when Q_A^-, formed by the first ST flash, becomes reoxidized.

In the DCMU-4 mutant, Ser264 in the herbicide-binding pocket of the D1 protein is replaced by Ala. Binding of PQ molecules is impaired and the rate of electron transfer from Q_A^- to Q_B is significantly reduced (Crofts *et al.* 1993), as is the sensitivity to PSII herbicides (Erickson *et al.* 1984). In this mutant, differences in maximal fluorescence yields between the first and subsequent ST flashes occur, but the transition from HF1 to LF is significantly slower (with time constant of about

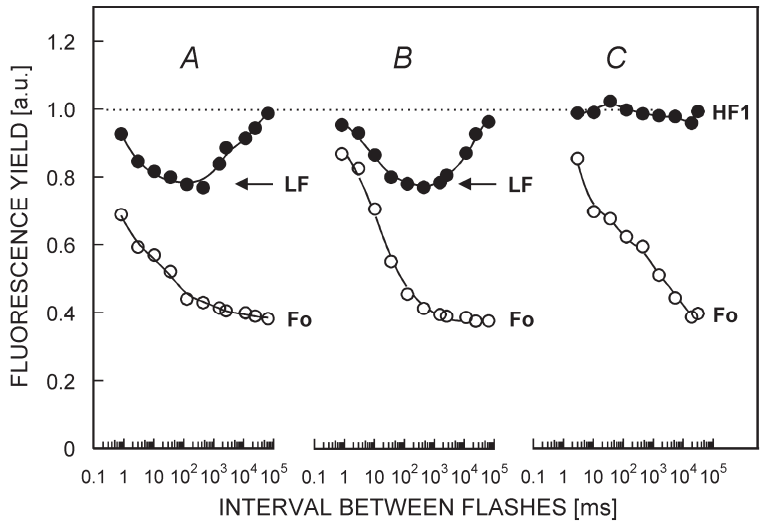

Fig. 3. Initial (F', *open markers*) and maximal (F_m, *closed markers*) fluorescence yields induced by the second ST FRR excitation in dependence on the time interval between the first and the second ST excitation in *Chlamydomonas* wild type (*A*), mutant DCMU-4 (*B*), and mutant A251L (*C*). Cell concentration and conditions for variable fluorescence measurements the same as in Fig. 1.

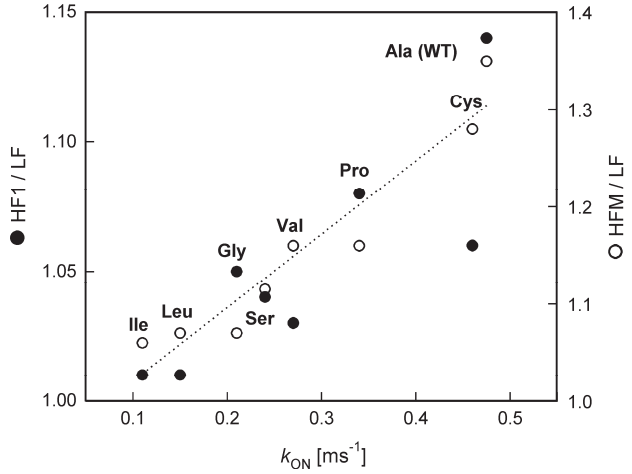

Fig. 4. Differences in F_m induced by ST (● HF1/ LF) and MT excitation (❍HFM/LF) in dependence on k_{ON}, the rate constant for plastoquinone binding to the Q_B pocket in several site-directed mutants of *Chlamydomonas* with single amino acid substitution of Ala251 of the D1 protein.

10 ms) than in the WT (Fig. 3*B*). This lag in the progression to the LF state corresponds with the decrease in the reoxidation rate of Q_A^- ($\tau \sim 10$ ms, Fig. 3*B*).

Another amino acid important for proper function of the Q_B-binding site is Ala251 of the D1 protein (Lardans *et al.* 1997, 1998). We analyzed the fluorescence characteristics of several photosynthetically competent site-directed mutants of this particular amino acid, which is on the small stromal helix (IV-V loop) of the D1 protein (Trebst 1986). The kinetics of electron transport between Q_A and Q_B, and the stability and exchange of the PQ in the Q_B pocket, depend strongly on the length and hydrophobicity of the amino acid chain in the D1-251 position (Lardans *et al.* 1998). For example, no differences between the first and subsequent ST flashes were observed in the A251I mutant (Fig. 3*C*, with Ala251 of the D1 protein is replaced by isoleucine, strain cc3388). Like DCMU-4, the A251I mutant exhibits a decrease in the initial rate of Q_A^- reoxidation and a much slower rate of PQ exchange in the Q_B pocket, to the extent that Q_B binding site is mostly unoccupied. In this mutant, Q_A^- reoxidation is limited by the slow rate of binding of PQ molecules to the pocket, decreasing the overall rates of electron transport far below that observed in the DCMU-4 strain. We calculated the kinetic parameters characterizing the binding and exchange of PQ molecules in the Q_B pocket for several of the site-directed mutants of D1-251, using the approach proposed by Crofts *et al*. (1993) (*see* Appendix and Lardans *et al.* 1998). In all the mutant strains studied, the ratio between the HF1 and LF yields was linearly correlated with k_{ON}, the rate constant for PQ binding to the Q_B pocket (Fig. 4).

Thermoluminescence: Differences between mutant strains in their binding affinity and stabilization of PQ molecules in the Q_B pocket can be inferred from TL glow curves (Fig. 1S, *supplement available online*, panel *A*). TL curves of the wild type and the DCMU-4 strains were very similar, with two main peaks, one centered at *ca*. 20°C and the other at *ca*. 30°C. That these bands originate from recombination between $S_{2/3}$ states and Q_B^- is supported by period four oscillations of their intensities (Fig. 1S, panel *A*, insert). In contrast, in A251I, most of the charge recombination proceeds at lower temperatures (<10°C) and lacks the oscillation pattern. These features are characteristic of $S_2Q_A^-$ recombination (Rutherford *et al.* 1982). Only a minor peak is observed around 35–40°C. This peak shows limited oscillations (Fig. 1S, panel *A*, insert) and might originate from the fraction of centers with bound PQ.

A similar TL analysis with BBY PSII particles reveals a strong Q band ($S_2Q_A^-$ recombination) and a lack of a B band (Fig. 1S, panel *B*), indicating that a Q_B was not occupied by a bound plastoquinone. In such particles, the ratio between HF1 and LF is approximately 1, similar to that following addition of DCMU in the native sample (Table 1S). In contrast, isolated spinach thylakoids show characteristic TL B band and high ratio HF1/LF and HFD/LF.

Taken together, our results suggest that variations in maximal fluorescence yields are related to the occupancy of the Q_B site by PQ and by the resulting kinetics of Q_A^- reoxidation. The LF state is formed only after the Q_B-bound state is stabilized by a presence of single electron supplied by the previous charge-separation event. All the *Chlamydomonas* mutants studied evolve oxygen and are grown under conditions where inorganic carbon was the sole carbon source. The photochemical efficiency of PSII, determined as F_v/F_m measured with a single turnover flash, was virtually identical in all the mutants, averaging 0.65 (Lardans *et al.* 1998). These results suggest that the primary charge separation and the function of the donor side of PSII are not affected by mutations either in amino acids A251 or S264. Since the rates of $S_n \rightarrow S_{n+1}$ transitions of the water splitting complex do not depend on the rates of electron transfer from Q_A to Q_B, our results clearly demonstrate that the variations in ST fluorescence yield cannot be explained by processes on the donor side of PSII.

Q_B site occupancy as inferred from recombination: If the time interval between repetitive ST flashes is longer than a few seconds, maximal fluorescence yields induced by the second and subsequent flashes are higher than LF and begin to approach the HF1 level (Fig. 3*A*,*B*). In *Chlamydomonas*, the interval between flashes necessary to increase F_m from LF to HF1 is the same in both the wild type and the DCMU-4 mutant (half of the increase from LF to HF1 occurs within ~15 s). The transition from LF to HF1 closely follows the kinetics of the $S_{2/3}Q_B^-$ charge recombination (τ ~15 s, data not shown). This correlation suggests that the LF state develops when the reduced

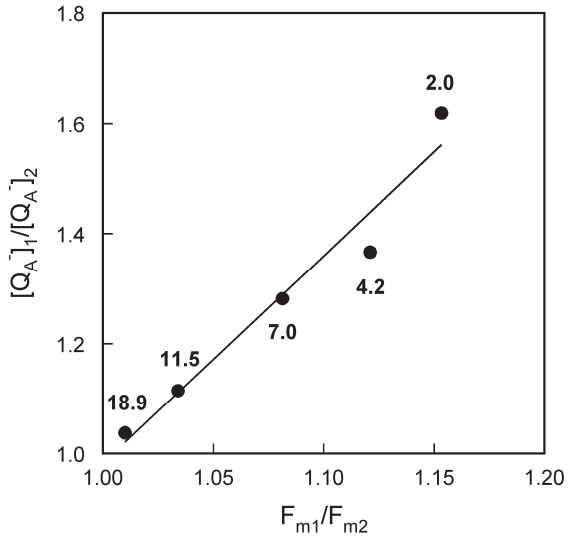

Fig. 5. Relation between occupancy of the Q_B site and differences in maximal fluorescence yields F_m induced by the first (F_{m1}) and second (F_{m2}) ST FRR excitation. Wild type *Chlamydomonas* cells were kept for >60 s in the dark and then excited by sequence of two ST FRR excitations that were spaced by time interval varying from 2.0 to 18.9 s. The relative changes in the occupancy of the Q_B site was then inferred from the kinetics of Q_A^- reoxidation. The occupancy of the Q_B site is inversely proportional to the concentration of Q_A^- at times >2 ms. In the case shown, $[Q_A^-]$ was estimated 5 ms following the first ($[Q_A^-]_1$) and the second ($[Q_A^-]_1$) ST excitation.

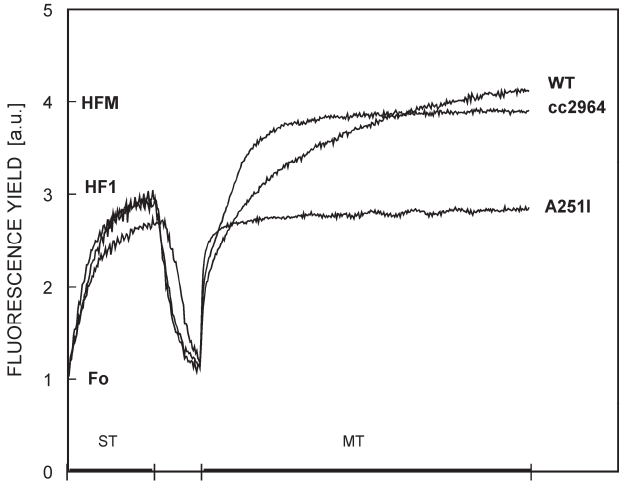

Fig. 6. The dependence of the slow rise of fluorescence yield during MT excitation protocol on the ability to reduce and oxidise the PQ pool. Raw data are shown for *Chlamydomonas* WT, for a mutant strain with reduced activity of the cyt b_6/f complex (cc2964), and for a mutant strain with impaired binding of PQ molecules to the Q_B pocket (A251I). For comparison, the MT excitation protocol (3000 flashlets delivered during 150 ms) was preceded by ST excitation of 80 flashlets (120-μs duration). The decay of fluorescence after the ST excitation was monitored for 1.5 s. Like in Fig. 1, the x-axis is linear in number of flashlets, not in time.

semiquinone molecule, Q_B^-, is bound to the Q_B site. Increasing the interval between flashes up to 30 min does not result in an increase in maximal fluorescence yields above the HF1 level. The changes in occupancy of the Q_B pocket during LF → HF1 transition can be inferred from the analysis of Q_A^- reoxidation following ST flash. The relative amplitude of the slow (~ ms) component of Q_A^- reoxidation reflects the fraction of centers with an empty Q_B pocket at the time of the flash (Crofts *et al.* 1993). The relative number of centers with a vacant Q_B pocket increases in parallel with the increase of F_m from LF to HF1 (Fig. 5).

Yields under multiple turnover excitation: The variability of fluorescence induction curves during MT excitation protocol in *Chlamydomonas* wild type and mutant strains impaired in either the reduction (A251I) or oxidation (cc2964) of the PQ pool is shown in Fig. 6. While the fluorescence yields induced by a ST excitation in each of the dark-adapted samples are comparable (*i.e.*, the primary photochemical energy conversion efficiency in these strains was not modified), the rise in fluorescence yield during the subsequent MT excitation differed significantly. In the wild type, MT excitation induced a relatively slow fluorescence rise (>120 ms) that reached an asymptotic HFM value which was 40–50% higher than LF. In the mutant with an impaired cyt b_6/f complex (cc 2964), the HFM level was comparable to the WT, but the rise in fluorescence yield was significantly steeper and the maximal level was reached more rapidly due to decreased rates of PQ pool reoxidation relative to rates of electron delivery to the PQ pool. Similar effect was observed upon addition of 0.1–0.5 μM DBMIB to the WT cells. On the other hand, MT excitation resulted in only a slight increase in maximal fluorescence yield above HF1 in mutants with decreased affinity of PQ molecules for the Q_B binding site (*e.g.*, mutant A251I). The increase in fluorescence yields above LF that occurred during the MT protocol are linearly correlated with the kinetics of binding (exchange) of the plastoquinone to the Q_B binding site in the same way as the difference between HF1 and LF (Fig. 4).

Relaxation of fluorescence yield following MT flash excitation and reduction of the PQ pool is slower than that following ST excitation. The relaxation kinetics can be manipulated by factors that influence PQ pool reoxidation rates, both *in vitro* and *in vivo*. Such factors include the addition of weak far-red light or addition of artificial PSII acceptors (Table 2S, *supplement available online*). Both ST- and MT-induced variable fluorescence are quenched following the addition of artificial PSII electron acceptors 2,6-DCBQ (10 μM) and 2,5-DMBQ (50 μM). However, quenching of HFM is higher than that of LF and the difference between HFM and LF decreases after addition of benzoquinones. The response to ST flash given during the relaxation from HFM to F_0 was similar to high fluorescence state formed after dark adaptation, such that the first ST flash given after the reduction of the PQ pool

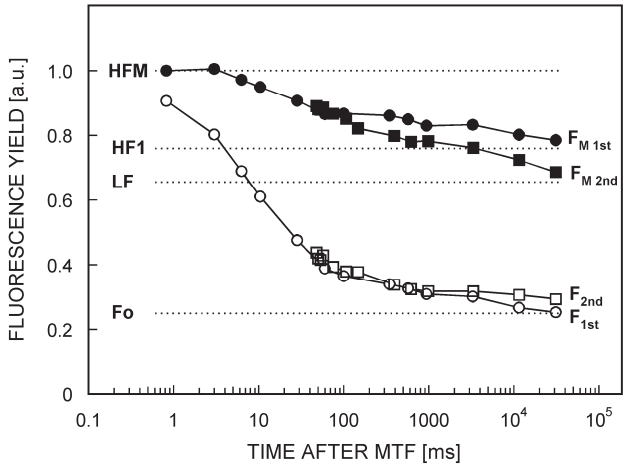

Fig. 7. The dependence of the initial (*open markers*) and maximal (*filled markers*) fluorescence yields measured by ST FRR protocol on the time delay following the MT excitation in *Chlorella.* The cells were excited by 3000 subsaturating flashlets given within 30 ms that caused increase of fluorescence yield to the HFM level. ST excitations spaced by 50 ms were provided at different time intervals following the end of MT excitation.

induced a maximal fluorescence yield higher than or equal to HF1, while the LF state could be induced only by a subsequent flash (Fig. 7).

Yields in the presence of PSII herbicides: Our basic hypothesis that maximal fluorescence yields are dependent upon the occupancy of the Q_B binding site is also supported by measurements of fluorescence properties in the presence of herbicides that interact with the Q_B pocket. We measured fluorescence yield changes during titrations of DCMU and other PSII herbicides (*Atrazine*, *Dinoseb*, *Ioxynil* - data not shown) from 1 nM to 10 μM. The titration curves showed a significant degree of variability, specifically in whole algal cells. An example of a DCMU titration profile for intact *Chlorella* cells grown in low light is shown in Fig. 2S (*supplement available online*). The LF level began to increase towards HF1 as PSII centers were blocked by the herbicide and the maximal fluorescence yield induced by MT excitation showed an initial decrease as the pool of secondary PQ acceptors could not be fully reduced (Fig. 2S). Fifty percent of Q_A^- reoxidation was

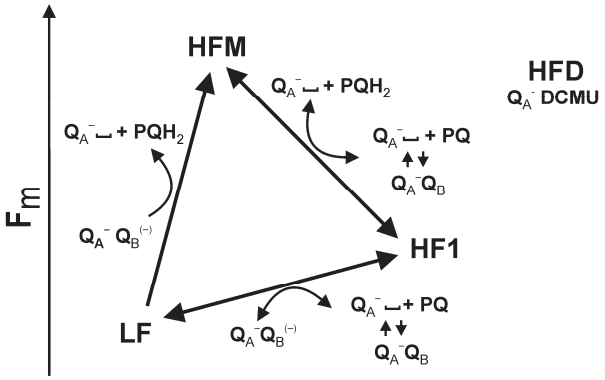

Fig. 8. The proposed model of modulation of the maximal yield F_m of Chl *a* fluorescence by the occupancy of the Q_B binding site of PSII. F_m, the fluorescence yield of closed PSII, with reduced primary quinone acceptor (Q_A^-) can vary depending on the presence of the secondary plastoquinone acceptor in the Q_B binding pocket. F_m is low (LF) when Q_B is mostly occupied by PQ molecules (either in reduced semiquinone or oxidised quinone form) and the highest one when the Q_B pocket is empty (HFM) or when PQ is substituted by herbicides interacting with the Q_B pocket (HFD). After relaxation in the dark, which results in recombination of stored charges, the fraction of centers with PQ bound in the pocket decreases. This results in the increased fluorescence yield HF1 observed after the first ST flash. Beside the modulation by the occupancy of the Q_B binding site, part of the increase of fluorescence observed during the MT protocol can be ascribed to the removal of static quenching by the oxidised PQ molecules. This accounts for the difference between HFM and HFD.

inhibited by 30–60 nM DCMU. At such DCMU concentrations, the transition from HF1 → LF did not occur. Further increases in herbicide concentration resulted in complete substitution of PQ in the Q_B binding pockets and an increase of maximal fluorescence yield to the HFD level which was independent of ST or MT excitation protocols. Titration with DCMU measured with ST flash resulted in parallel increases in both F_0 and F_m; F_v remained virtually constant. Since addition of herbicides does not affect the distribution of the S states in the dark, the differences between HF1 and HFD cannot be due to the redox status on the donor side of PSII, but rather due to the occupancy of the Q_B.

Discussion

The results presented indicate that variations in maximal fluorescence yields are related to the nonphotochemical quenching effects by electron carriers on the acceptor side of PSII. While the effects of plastoquinone pool on F_m were described 50 years ago, our results indicate that this is not due to the direct effect of the PQ pool, but rather to the occupancy of the Q_B site that modifies the fluorescence yields. This modulation accounts for up to 40% of F_m and does not reflect changes in primary photochemical energy conversion efficiency in PSII. Consequently, the calculations of the photochemical quantum yield of PSII from variable fluorescence yields induced by MT flashes, using formulae developed by Butler (1972) and Lavergne (1974) must be corrected for this nonphotochemical quenching phenomena.

What does cause the variations in the maximal fluorescence yields? Oscillations in F_m induced by ST flashes were first observed in *Chlorella* by Delosme (1971a), who

concluded that fluorescence yield is modulated by the oxidation state of the donor side and that the fluorescence yield is higher in S_0 and S_1 states and lower in S_2 and S_3. While the maximal extent of variation in F_m induced by ST illumination (HF1/LF) reported here is comparable to that reported by Delosme, there are several reasons why the F_m variations cannot be fully explained by the influence on the donor side. First, except for the initial HF1 → LF transition, we do not observe any significant (>5%) oscillations in F_m that would indicate modulation by the redox state of the donor side using an ST protocol (Bowes and Crofts 1980, Delosme 1971a, Delosme 1971b, Joliot and Joliot 1971, Zankel 1973, Robinson and Crofts 1983, Kramer *et al.* 1990, Kolber *et al.* 1998, Koblizek *et al.* 2001). FRR ST flashes produce classical period of four oscillations in the oxygen flash yield (Y_{O2}, *see* Fig. 5 in Kolber *et al.* 1998). The discrepancy between the observed oscillations in Y_{O2} and weak oscillations in F_m might be partly due to the increased number of double-hits inherent to the FRR protocol. To test this, we used the observed pattern of Y_{O2} oscillations to calculate the fraction of PSII reaction centers in S_2 and S_3. This model predicts that even if the number of double-hits and misses is high (≥ 15%), the oscillation of F_m should be observable for several periods (Fig. 3S, *supplement available online*). For example, the predicted difference in F_m between the 3rd and 5th ST flashes should be > 10% of LF.

There is also evidence of transient quenching of variable fluorescence occurring during the ST flash that can be ascribed either to the accumulation of the oxidized intermediates on the donor side or the presence of triplet states (Mathis and Galmiche 1967, Zankel 1973). This transient quenching accounts for only ~5% of variable fluorescence yield in the HF1 or LF states, has a lifetime of 5–30 μs, and is observed only if double hits occur during the ST protocol (Kolber *et al.* 1998). These calculations and observations indicate that while nonphotochemical quenching on the donor side of PSII occurs, the effect does not quantitatively account for the variations in fluorescence yields induced by ST and MT flashes. Moreover, the correlation between the kinetics of the HF1 → LF transient observed in the WT and DCMU-4 mutant and the kinetics of electron transfer from Q_A^- to Q_B indicate that the donor side (which is not influenced by the site-directed mutation in DCMU-4) does not limit fluorescence yields during ST excitation. We can also rule out that differences in maximal fluorescence yields induced by ST and MT protocols are due to the presence of an alternative fast ($\tau < 100$ μs) reoxidation pathway for Q_A^-. If such a pathway were operating in all PSII reaction centers, a decrease in fluorescence yield would be observed when the rate of FRR excitation is decreased. This is not the case (Kolber *et al.* 1998).

Another possible explanation for the observed variations in F_m is heterogeneity of PSII complexes. PSII has been proposed to be composed of a mixture of centers with different antenna size, electron transfer kinetics, and localization within thylakoid membranes (for review, *see* Govindjee 1990). We suggest that while heterogeneity in the antenna size of PSII may well exist, it cannot play an important role in the mechanism responsible for the observed differences in fluorescence yields. First, if present, the variations in the antenna size of PSII should (by definition) be revealed from analyses of the functional absorption cross sections based on ST excitation. Specifically, in the fast FRR induction curve, multiple cross sections would be manifested by a deviation from a cumulative one-hit Poisson function with a lag in the flash saturation profile with increased excitation. The lag would reflect a second smaller cross section independent of the turnover time for electron transfer within a reaction center. Such flash saturation profiles are not observed with ST flashes. Second, we have shown that the transitions between states with different fluorescence yields (LF, HF1, HFM) are time-dependent processes on the order of >1 ms, that require a secondary electron transfer following the reduction of Q_A. If the observed differences were due to different antenna sizes, formation of the LF, HF1, and HFM states would be dependent solely on the absorbed energy delivered during the excitation protocol. Finally, PSII antenna heterogeneity cannot explain the ST-induced differences in fluorescence yields in the presence of DCMU (HFD *vs.* LF). Lower HFM/LF ratios were observed in the presence of benzoquinone acceptors 2,6-DCBQ and 2,5-DMBQ (Table 2S) which prevent reduction of the PQ pool by accepting electrons directly from the Q_B binding site or from PQH_2, respectively (Satoh *et al.* 1995).

Based on the results from kinetic analyses, pharmacological studies and mutant characterization, we present a conceptual, working model to account for the variations in maximal fluorescence yields between ST and MT (Fig. 8). If Q_A is reduced and the Q_B site is occupied by a plastoquinone molecule (*i.e.*, $Q_A.Q_B$), the observed fluorescence yield is low (LF state). If the Q_B site is empty ($Q_A._$) or if plastoquinone is substituted by an herbicide (Q_A.DCMU), maximal fluorescence increases by up to 40% (HFM and HFD, resp.). In the presence of weak background light, or following a ST flash, most of the Q_B sites are occupied by bound plastoquinone, in both the oxidized and the semiquinone form (LF state). In PSII centers that have a Q_B site occupied by the semiquinone, the electron resides primarily on Q_B [the apparent equilibrium constant, K_{app} is 5 to 15 (Robinson and Crofts 1984)]. During relaxation in the dark, the centers in the $S_{2/3}Q_B^-$ configuration recombine, leading to the oxidation of Q_B^-. Consequently, the dark-adapted state is characterized by an increased fraction of centers with oxidized $Q_A.Q_B$ complexes. Since the dissociation constant for oxidized plastoquinone in the Q_B pocket is higher than for semiquinone (Robinson and Crofts 1983), PQ tends to dissociate from the pocket. Thus, the fraction of PSII centers with a vacant Q_B site ($Q_A._$) is higher during the first ST flash than after ”activation“ of the acceptor side

and rebinding of the PQ molecule to the Q_B site, [*e.g.*, following the first flash or in the weak background light (LF)]. The difference in the occupancy is reflected in fluorescence yield that is higher in the HF1 state than in the LF state. The highest fluorescence yields are observed when the Q_B site is vacant, either following addition of PSII herbicides (HFD) or after long MT excitation, leading to the reduction of the plastoquinone pool (HFM). In some cases, we observed that the maximal fluorescence yield after complete reduction of the PQ pool was higher than after inhibition by PSII herbicides (one such example is shown in Fig. 1). This result indicates that the oxidized PQ pool might induce some form of static quenching of variable fluorescence, as it has been previously proposed (Amesz and Fork 1967, Vernotte *et al.* 1979).

Since the maximal fluorescence yield induced by MT flashes is attained when the PQ pool is reduced, the variations in F_m measured by MT protocol (HFM) does not necessarily correlate with changes in photochemical yields of PSII, calculated as F_v/F_m. Full reduction of the PQ pool can only occur when the rate of PQ reduction exceeds the rate of PQH_2 reoxidation. The fastest turnover time for the PQ molecule at the Q_B site is 2–3 ms, while the PQH_2 reoxidation in steady state is limited by the dark reactions, with $\tau \sim 10$ ms for low light-grown cells and much slower in isolated thylakoids in the absence of terminal electron acceptors (Sukenik *et al.* 1987). However, there are several physiologically relevant cases when the complete reduction of the PQ pool by MT excitation becomes much more difficult, or even impossible, due either to an increase in the reoxidation rate of PQH_2 or the effective slowing down of PQ pool reduction by PSII. For example, the overall electron transfer time from water to the terminal acceptor decreases < 5 ms in algal cells grown at high light intensities (Myers and Graham 1971, Sukenik *et al.* 1987). This prevents full reduction of the PQ pool and results in a decreased maximal fluorescence yield (HFM state), even if the single turnover-induced fluorescence yields remain unaffected. Many environmentally relevant stresses (*e.g.*, diurnal mid-day photoinhibition) affect PSII preferentially and decrease photochemical activity or $Q_A \rightarrow Q_B$ electron transport rates, and thereby, the rate of PQ reduction as well. Finally, we observed that longer-term (>15 min) dark adaptation leads to up to three times slowdown of PQ pool reoxidation rates, presumably due to downregulation of PSI activity. As a result, MT flash induced fluorescence yields (HFM) display much higher level of short-term variability compared to that induced by ST flashes (LF). If the MT method were used to monitor the effects of the stress, a decline in photochemical yields and increase of nonphotochemical quenching might be erroneously interpreted from the change in HFM.

There appears to be a significant degree of variability in photochemical yields of PSII calculated as F_v/F_m among different photosynthestic organisms (Table 1S). This difference is most pronounced when MT excitation protocols are used, which might be ascribed to the different efficiencies of PQ pool reduction or PQ pool size. Nevertheless, it continues to be a variability in photochemical yields induced by ST excitation, namely between algae and higher plants. This variability may be attributable to differences in membrane architecture. Extensive stacking occurs in the spinach thylakoids, whereas photosynthetic membranes of green algae and diatoms are much more loosely organized (Staehelin 1986, Falkowski and Raven 1997). When spinach thylakoids are incubated in the absence of divalent cations, variable fluorescence decreases (while F_0 remains almost unchanged) due to the spillover of energy to PSI (reviewed *e.g.*, in Stys 1995). While the absolute values of variable fluorescence yields decrease as a result of unstacking, the relative differences between LF, HF1, HFD and HFM states can still be observed (data not shown). The differences in membrane architecture can qualitatively explain the generally lower variable fluorescence yields in algae, but not the variations in maximal fluorescence yields induced by different excitation protocols. In support of this, we have not observed any changes in the differences between maximal fluorescence yields induced by ST and MT excitation protocols in several mutants of *Chlamydomonas* that lack Chl *b* (*e.g.*, cc1355, cc2419, cc2449 – data not shown) and have altered or no thylakoid membrane stacking (Michel *et al.* 1983). Additionally, using a custom designed system for measurements of 90° light scattering at 780 nm, we failed to observe any changes in the light scattering in spinach thylakoids measured during the MT protocol that would indicate dynamical reorganization of the thylakoid membrane occurring on the time scales of ~100 ms (Gorbunov and Prášil, unpublished results).

We can only speculate about the possible physical process responsible for the variations in fluorescence yields. It does not seem likely that the presence or absence of plastoquinone in the Q_B pocket alone would modulate the exciton trapping rate in the reaction center of PSII. If that hypothesis were true, the intrinsic fluorescence yield (F_0) should be high following dark relaxation, before the first ST flash that induces the HF1 state, when we expect the increased fraction of Q_B sites to be vacant. However, F_0 is usually low after dark adaptation. Instead, the increase in fluorescence yield occurs during the fluorescence rise induced by the first ST excitation, suggesting that the occupancy state of the Q_B pocket does not have a direct influence on fluorescence yields, but instead influences the transition to high fluorescence state(s), manifesting itself in an elevated F_0 level observed in the subsequent ST flash applied two seconds later (Kolber *et al.* 1998). Whether this ability reflects an increased yield of the change in the redox state of some auxiliary quencher present on the acceptor side (*e.g.*, Q_{400}, non-heme Fe or the second "inactive" branch of redox components in PSII), a conformational change of the RCII

occurring during the reduction of Q_A^- that would modulate the redox potentials of cofactors inside PSII (Schansker *et al.* 2014) or the interaction between RCII and the surrounding PSII antenna, a change in the yields of the recombination luminescence (Schreiber and Krieger 1996) or a local electrostatic effect that modifies the rate of excitation trapping within PSII (Leibl *et al.* 1989) remains to be elucidated. Nevertheless, the results presented here are consistent with the hypothesis that nonphotochemical effects on fluorescence yields are most strongly modified by a presence of the secondary plastoquinone acceptor in the Q_B binding pocket of the D1 protein. The variability in yields does not reflect changes in the true photochemical yields of PSII, but significantly affects determinations of photosynthetic yields and rates that are based solely on fluorescence parameters. Care must be taken to measure all fluorescence parameters under the conditions that do not change the occupancy of the Q_B pocket, and, by the same token, under conditions that do not modify the level of PQ pool reduction. We suggest the use of a combination of ST and MT flash protocols can provide a significant amount of information on PSII that would not otherwise be accessible from either protocol alone. Saturating ST excitation is exceptionally useful in quantifying photochemical yields, the functional absorption cross section of PSII, and oxidation kinetics between Q_A and Q_B. MT flash protocols can provide insight into the redox state of the plastoquinone pool and its oxidation/reduction kinetics. The interpretation of MT yields in the context of photochemical efficiency is problematic, however, as a consequence of the inevitable modulation of fluorescence yield above and beyond the effects of Q_A reduction. The extent of this quenching is strongly affected by both the rates of electron flow into the PQ pool, and by the rates of PQ pool reoxidation.

Appendix

The kinetic and thermodynamic characteristics of the acceptor side of PSII in the *Chlamydomonas* site-directed mutants (Lardans *et al.* 1998) were determined using the two electron gate model as described by Crofts and Wraight (1983). For each mutant we obtained the following set of parameters:

K_{APP} apparent equilibrium constant for sharing of an electron between Q_A and Q_B

K_E semiquinone equilibrium constant for describing the electron distribution between Q_A and Q_B [$Q_A^-Q_B \rightarrow Q_A\,Q_B^-$]

K_O dissociation constant for plastoquinone in the Q_B pocket [$Q_A^-Q_B \rightarrow Q_A$- (+PQ_{POOL})]

r_1, r_2 reoxidation rates of Q_A^- (slow and fast components)

k_{AB} the rate constant $Q_A^-Q_B \rightarrow Q_A\,Q_B^-$

k_{BA} the rate constant $Q_A\,Q_B^- \rightarrow Q_A^-Q_B$

k_{ON} the rate of PQ binding to the Q_B pocket $Q_A^- + PQ_{POOL} \rightarrow Q_A^-Q_B$

k_{OFF} the rate for PQ dissociation from the Q_B pocket $Q_A^-Q_B \rightarrow Q_A^- + PQ_{POOL}$

K_O was experimentally determined as ratio of amplitudes (α) of the first two monoexponential components ($\tau_1 \sim 300$ μs and $\tau_2 \sim 2$ ms in wild type) of Q_A^- reoxidation in dark-adapted samples, following ST flash (*see* Eq. 6 in Kolber *et al.* 1998).

$$K_O = \alpha_2/\alpha_1 \qquad \text{(A1)}$$

The kinetics of Q_A^- reoxidation, determined from measurements of the fluorescence decay, provided values of apparent rate constants r_1 and r_2. The semiquinone equilibrium constant K_E was determined from fluorescence decay kinetics by calculating the fraction of reduced Q_A^- 25 ms following a ST flash (Kless and Vermaas 1995). Thus:

$$K_E = 1/[Q_A^-]_{25ms} - 1 \qquad \text{(A2)}$$

and K_{APP} was calculated as:

$$K_{APP} = K_E/(1 + K_O) \qquad \text{(A3)}$$

Alternatively, K_{APP} can be determined experimentally from analysis of the decay of delayed luminescence in the 0.1–100 s time interval as ratio of number of centers recombining from Q_B^- (slow component, $\tau_1 \sim 6$ s) to number of centers recombining from Q_A^- (fast component, $\tau_1 \sim 0.7$ s) or, as a ratio of the half-times of these two decay components (Crofts *et al.* 1993). Though each of these methods provided slightly different absolute values of K_{APP} (K_{APP} was the highest when determined from $[Q_A^-]_{25ms}$), the relative changes of K_{APP} between wild type and each of the mutant strain were independent on the method used. The remaining kinetic parameters of the two electron gate model (k_{ON}, k_{OFF}, k_{BA}, k_{AB}) were calculated solving the set of equations previously described in Crofts *et al.* (1993).

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