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Origins of the low yield of chlorophyll a fluorescence induced by single turnover flash in spinach thylakoids

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Abstract

In whole algae and isolated thylakoids, the maximum yield of Chl*a* fluorescence induced by a saturating single turnover flash is about half of the maximum yield observed under continuous illumination. The origins of this low fluorescence yield were investigated by measuring in fresh spinach thylakoids incubated under different conditions the fluorescence yield induced by a weak non-actinic flash fired alone (F_o) or 50 μs after a saturating laser flash (F_m) and also the PSII effective absorption cross-section (σ_{PSII}) derived from the flash energy saturation curves of F_v ($F_v = F_m - F_o$). We observed that: (1) In the presence of a background blue light or after the chemical reduction of the primary quinone electron acceptor Q_A, a saturating single turnover flash induced high fluorescence yields comparable to the maximum yield observed under continuous illumination. (2) Addition of carbonylcyanide-*m*-chlorophenylhydrazone (CCCP) in the assay medium increased the variable fluorescence ($F_v = F_m - F_o$) by 24% relative to the control and abolished its period-four oscillation under repetitive flashes. (3) After dark incubation of thylakoids under reducing conditions where most of the plastoquinone pool was chemically reduced, large increases of both F_v and σ_{PSII} were observed. (4) In thylakoids treated with the inhibitor DCMU, both F_o and F_m were increased by 30% relative to the control but no significant change of σ_{PSII} was observed. In contrast to intact thylakoids, F_m increased significantly under repetitive flashes in DCMU-treated thylakoids. Moreover, the enhancements of σ_{PSII} following the plastoquinone chemical reduction were largely abolished in DCMU-treated thylakoids. From these observations, we conclude that although some limitations of the PSII donor side contribute to the low fluorescence yield after a single turnover flash, most of the fluorescence quenching present after a single turnover flash originates from the oxidized plastoquinone pool and/or from a unidentified component, possibly the putative quencher Q_2.

Keywords: Absorption cross-section of photosystem II; Chl*a* fluorescence quenching; Plastoquinone pool; Pump-probe fluorescence method

1. Introduction

Chl*a* fluorescence is commonly considered as an intrinsic probe of the reactions affecting the PSII complexes in chloroplasts [1–3]. Illumination of dark-adapted photosynthetic material by a continuous saturating light may increase the yield of Chl*a* fluorescence up to five times from a minimum level (F_o) to a maximum unquenched level (F_m). The kinetic of Chl*a* fluorescence induction is related, although non-linearly, to the closure of the PSII centres, i.e., to the reduction of the primary quinone electron acceptor, Q_A [4]. Several photochemical (qP) and non-photochemical (qN) quenching mechanisms determine the yield of Chl*a* fluorescence [1]. Analysis of the different fluorescence quenching components by the saturation pulse method can provide meaningful physiological parameters such as the photochemical quantum yield and the efficiency of excitation energy capture by open PSII centres [5,6]. Equivalent parameters can also be determined using the pump-probe fluorescence method introduced by Mauzerall [7]. In this method, the Chl*a* fluorescence yield is measured with a weak and non-actinic probe flash fired

Abbreviations: ADRY, acceleration of the deactivation reactions of the oxygen-evolving complex Y; CCCP, carbonylcyanide-*m*-chlorophenylhydrazone; DQH_2, duroquinol; FeCN, ferricyanide; F_m, maximum yield of Chl-*a* fluorescence when the PSII reaction centers are closed; F_o, minimum yield of Chl-*a* fluorescence when the PSII reaction centers are open; F_v, F_m-F_o; PQ, plastoquinone; PSII, photosystem II; Q_A, primary quinone electron acceptor of PSII; σ_{PSII}, effective absorption cross-section of PSII.

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tens of μs after an actinic single turnover pump flash of variable intensity [7–9]. The light flashes used are short enough to elicit only single turnovers of the reaction centers.

One notable difference between the two fluorescence methods is that the Chl*a* fluorescence yield induced by a saturating single-turnover flash (sub-microsecond duration) is about half the yield observed from a longer multi-turnover flash [10–12]. It has been demonstrated that the use of actinic pulses longer than ≈ 50 μs was necessary to induce high Chl*a* fluorescence yields similar to those observed under continuous illumination [12,13]. From these observations, a model was proposed where a first photoreaction converts an open PSII reaction center (Q_0) to a low fluorescence state (Q_I), this state being transformed within 50 μs in a dark reaction to a second state (Q_{II}). A second photoreaction then converts the state Q_{II} into a high fluorescence state (Q_{III}). It appears that the fluorescence properties of the PSII centres in the Q_I state (after a single-turnover flash) are related to the fast I_1/J transient [6,14] of the polyphasic rise of Chl*a* fluorescence induced by an intense illumination. However, the nature of the so-called Q_i states and the dark reaction has not yet been identified.

In the pump probe technique two potential quenching effects, electron transfer from Q_A^- to Q_B and the persistence of the primary electron donor P_{680}^+ can be considered insignificant at the end of an optimized time interval (≈ 50 μs) between the pump and the probe flashes. This is because the rate of Q_A^- oxidation is much slower and the rate of P_{680}^+ reduction much faster than the delay time between pump and probe flashes [15,16]. The low Chl*a* fluorescence yield observed with a single turnover flash may have at least five plausible origins: the PSII donor side, the non-heme iron on the PSII acceptor-side, the PSII antenna, the PQ pool and the presence of a putative quencher Q_2:

(1) Small oscillations (about 10% of F_m) with a period-four of the μs components of Chl*a* fluorescence yield have been observed following repetitive single turnover flashes [17,18]. These oscillations indicate a susceptibility of the fluorescence yield in the μs time domain to the presence of oxidizing equivalents, other than P_{680}^+, stored on the PSII donor side.

(2) Photochemical quenching by the PSII acceptor-side non-heme iron, previously identified as Q_{400}, could also affect the Chl*a* fluorescence yield after a single-turnover flash [19]. If the non-heme iron is oxidized prior to the flash, rapid oxidation of Q_A^- occurs with half-time of 25 μs or less at pH 7.5. This electron transfer is insensitive to the presence of the herbicide DCMU, an inhibitor which blocks Q_A^- re-oxidation by the secondary quinone acceptor Q_B [19]. Although exogenous oxidants such as FeCN are generally required to oxidize the non-heme iron, it was found that 30–40% of the non-heme iron was oxidized presumably by O_2 in a thylakoid suspension which was dark incubated for 15 min under aerobic conditions [20].

(3) Quenching of excitation energy in the PSII antenna owing to the presence of carotenoid triplets or singlet-singlet annihilation processes could result from an overexcitation of the PSII pigments during a flash [17,21].

(4) Non-photochemical quenching can be caused by the presence of oxidized plastoquinone pool [22]. This quenching is usually considered as minor [1,2] but Van Gorkom and collaborators [23] have observed a large variation (nearly zero up to 30%) in the extent of Chl*a* fluorescence susceptible to the quenching effect of the plastoquinone pool, depending on the origin and the integrity of the thylakoid membrane. Its importance on the Chl*a* fluorescence yield resulting from a single-turnover flash in dark-adapted thylakoids is unknown.

(5) The low fluorescence yield observed after a single turnover flash has been previously attributed to an unidentified putative quencher, the so-called Q_2 quencher [10,24]. Although its exact nature is far from clear, the Q_2 quencher has been proposed as a low quantum yield, side-path electron acceptor [26].

In this report, we used the pump-probe fluorescence method to investigate the origin of the low fluorescent state Q_I of PSII complexes. The yields of Chl*a* fluorescence induced by a single turnover flash and the effective absorption cross-sections of PSII were determined in fresh spinach thylakoids which were dark-adapted in the presence of different additives. Our results indicate that the redox state of the PQ molecules and/or of an unidentified component, possibly the putative quencher Q_2, have an important effect on these parameters, whereas minor effects could be attributed to the PSII donor side and the non-heme iron in our samples. The relation between these results and the photochemical and non-photochemical phases of Chl*a* fluorescence induction under continuous illumination is discussed.

2. Materials and methods

Thylakoids were isolated from fresh market spinach as described earlier [26] except that the resuspension medium consisted of 330 mM sorbitol, 30 mM tricine/NaOH (pH 8.2), 10 mM KCl and 5 mM $MgCl_2$. Chl-*a* + *b* concentration was estimated according to [27]. Thylakoids kept in darkness at 0°C were used within 6–8 h after their isolation. For each measurement, a new sample was resuspended at a Chl $a+b$ concentration of 10 μM and dark-adapted for three min at room temperature in the absence or the presence of appropriate additives. Duroquinol (DQH_2) was prepared following the procedure of [28] and sodium dithionite (0.6 M) was dissolved in a buffer solution of Tricine 1 M (pH 9.0) and used within one hour.

The yield of Chl-*a* fluorescence induced by a non-actinic probe flash lamp was measured after a dark interval of 50

μs following a single turnover pump laser flash (250 ns duration). The probe flash was provided by a xenon flashlamp (Brock electronic shop), whereas the saturating actinic flash (650 nm) was generated by a Phase-R (New Durham, NH) DL-32 flash lamp pumped dye laser. Saturating flashes had a photon density (0.3 photons/Å^2) approx. 10 times the half saturation value. The xenon flashlamp was covered with a short pass blue filter and fluorescence was detected with a Hamamatsu RG967 photomultiplier tube protected with a 690 nm long pass filter. The flashes were both supplied to the bottom of a standard 10 × 10 mm sample cuvette via a randomized fiber optic bundle (Oriel) held 10 mm beneath the cuvette. Chl *a* fluorescence was collected from one side of the cuvette through a small 10 mm wide by 2 mm high window centred 10 mm above the bottom of the cuvette. The combination of randomized fiber optic and optically thin (2 mm) measurement region ensured homogeneous illumination of the thylakoid suspension contributing to the fluorescence emission.

The PSII effective absorption cross-section σ_{PSII} was determined from the flash intensity saturation curve of Chl *a* fluorescence yield. Data were fit with the cumulative single hit Poisson probability distribution:

$$F(I) = F_m(1 - e^{-I \cdot \sigma_{PSII}}) \quad (1)$$

where I is the pulse energy (photons/Å^2), $F(I)$ is the Chl *a* fluorescence yield, F_m is the maximal F yield measured at saturating flash energies and σ_{PSII} is the PSII effective optical absorption cross-section (Å^2) [7]. The actinic flash energy was adjusted with neutral density filters. A small fraction of the actinic flash was directed toward a light pulse pyranometer (Molectron) so that the energy of each laser pulse could be monitored. The ratio between the flash energy reflected toward the pyranometer and the flash energy at the sample was determined at the end of the experiment by measuring the flash energy with a 10 × 10 mm cuvette containing a sample placed in front of the pyranometer with the fiber optic held at 10 mm from the cuvette.

3. Results and discussion

3.1. F_m induced by a single turnover saturating flash

Table 1 presents the Chl *a* fluorescence yields measured from untreated and treated thylakoids illuminated with a non-actinic probe flash fired alone (F_o) or 50 μs after a saturating laser flash (F_m). On this time scale Q_A is still expected to be reduced in all photoactive PSII centres [16]. We have verified this expectation in our experiments by varying the delay time between pump and probe flash over a range from 20 μs to 10 ms (data not shown). For convenience the fluorescence yields have been expressed relative to a normalized value of $F_o = 1.0$ in untreated control thylakoids. In untreated thylakoids, the ratio of variable fluorescence $(F_m\text{-}F_o)/F_o$ is 2.52, in agreement with other measurements made elsewhere under similar

Table 1
Chl *a* fluorescence yields measured from untreated and treated spinach thylakoids illuminated with a non-actinic probe flash fired alone (F_o) or 50 μs after a saturating laser flash (F_m)

Treatments	F_o	F_m	$F_v = F_m - F_o$
Control	1.00 ± 0.04	3.52 ± 0.19	2.52
Background blue light [1]	n.d.	6.25 ± 0.44	n.d.
Dithionite 2 mM (15 min)	5.70 ± 0.57	6.44 ± 0.19	0.74
CCCP 2 μM	0.98 ± 0.02	4.11 ± 0.09	3.13
Nigericin + Valinomycin [2]	0.97 ± 0. 03	3.38 ± 0.16	2.40
FeCN + DCMU [3]	0.97 ± 0.13	2.33 ± 0.19	1.36
PreFlash [4]	1.10 ± 0.09	3.60 ± 0.37	2.50
Repetitive flashes [5]	1.00 ± 0.04	3.47 ± 0.07	2.47
DQH_2 50 μM	1.36 ± 0.07	4.77 ± 0.16	3.41
DQH_2 250 μM	1.58 ± 0.11	4.77 ± 0.31	3.19
Dithionite 2 mM (15 s)	1.97 ± 0.10	5.63 ± 0.32	3.66
DCMU 10 μM + DQH_2 50 μM	1.35 ± 0.12	5.06 ± 0.40	3.71
DCMU + Dithionite 2mM 15 s	1.37 ± 0.07	5.63 ± 0.22	4.26
DCMU 10 μM	1.34 ± 0.12	4.59 ± 0.44	3.25
DCMU 10 μM Repetitive flashes [5]	n.d.	5.80 ± 0.13	n.d.

Only one pair of flashes was given per sample, unless indicated otherwise. The data are the mean values (± standard deviation) of 3–4 independent measurements. n.d.: not determined.

[1] Background blue light intensity ≈ 100 μE · m^{-2} · s^{-1}

[2] Both nigericin and valinomycin at 0.5 μM

[3] Thylakoids incubated in presence of 400 μM FeCN for 2 min, then addition of 10 μM DCMU 1 min prior the pump flash.

[4] Saturating preflash given 15 s prior the pump flash.

[5] Fluorescence yields averaged from the 2nd to the 17th flashes given at a frequency of 0.17 Hz.

conditions with intact green algae or isolated thylakoids [11–13]. When Q_A was chemically reduced after 15 min of dark incubation in the presence of 2 mM sodium dithionite, the Chl*a* fluorescence yields at F_o and F_m were 5.70 and 6.44 respectively. The persistence of a small variable fluorescence ($F_v = F_m$-F_o) indicates an incomplete chemical reduction of Q_A under these conditions probably due to some inaccessibility of the reductant in the absence of a redox mediator. A high F_m yield (6.25) was also observed when the sample was illuminated with a background blue light ($\approx 100\ \mu E \cdot m^{-2} \cdot s^{-1}$). As these high F_m yields, observed when Q_A was reduced either chemically or photochemically, were obtained in the presence of the saturating flash, then the relatively low F_m yield observed in the control cannot be attributed to the presence of any long lived (ca. 50 μs) fluorescence quenchers produced by the saturating flash itself [17,21].

3.2. Quenching of F_m by oxidized donor side intermediates

As mentioned earlier, oxidizing equivalents present on the PSII donor side contribute to some extent to the low Chl*a* fluorescence yield after a single turnover flash [17,18]. One class of compounds, the ADRY reagents, is recognized to decrease the lifetimes of those oxidizing equivalents, namely the tyrosine Y_Z^+ and the S_i states of the oxygen-evolving complex [29,30]. We observed that the addition of the ADRY reagent CCCP (2 μM) increased the extent of variable fluorescence by 24% compared to the control (Table 1). This effect was not related to the uncoupling effect of CCCP as a control level Chl*a* fluorescence yield was observed in thylakoids treated with 0.5 μM nigericin and 0.5 μM valinomycin (Table 1). A period-four oscillation of the μs component of Chl*a* fluorescence yield is observed when intact thylakoids are exposed to repetitive flashes [17,18]. This oscillation depends on the four oxidizing equivalents S_i of the oxygen-evolving complex that are formed following each photochemical reaction in the reaction centre. As previously observed [17,18], the Chl*a* fluorescence yields of untreated thylakoids were maximum at the first and fifth flashes and minimum after the third and seventh flashes (flash frequency of 1 Hz) (Table 2). In contrast, the enhanced yield of Chl*a* fluorescence measured in the presence of 2 μM CCCP was found to be independent of the flash number (Table 2). This indicates that the enhancement of the flash-induced Chl*a* fluorescence yield by CCCP is related to its accelerating effects on the reduction of some oxidizing equivalents on the PSII donor side.

Table 2
Yields of normalized variable Chl*a* fluorescence ($F_m - F_o / F_o$) measured after different numbers of actinic flashes from spinach thylakoids resuspended in the absence and presence of CCCP 2 μM

	1st flash	3rd flash	5th flash	7th flash
Control	2.46±0.10	2.25±0.18	2.52±0.15	2.43±0.17
CCCP 2 μM	2.98±0.16	2.99±0.14	2.98±0.18	2.96±0.14

The data are the mean values (±standard deviation) of 4–5 independent measurements.

Schreiber and Neubauer [31] previously reported a preflash number dependency and a stimulatory effect of CCCP on the I_1 transient of the polyphasic rise of fluorescence emission induced by an intense illumination. The similar effects of preflashes and CCCP on the fluorescence yields observed at both the I_1 transient and after a single turnover flash (Q_1 state) support the idea of a common fluorescence quenching which limits the fluorescence yield under these conditions, as previously suggested by Schreiber and Neubauer [31]. These authors observed that the removal of the fluorescence quenching at the I_1 transient during the non-photochemical subphase D-I_2 was insensitive to the presence of the electron acceptor methyl viologen and thereby concluded that the low fluorescence yield at I_1 was dependent on the PSII donor side rather than its acceptor side [31,32]. However, under the intense illumination used in their experiments, no quenching effect of methyl viologen would be expected as the limiting step of Q_A and PQH_2 re-oxidation is at the Cyt b_6/f complex rather than at the reducing side of PSI. [33,34].

Our results with CCCP suggest that only a minor part of the quenching observed with single turnover flashes arises from the PSII donor side.

3.3. Quenching of F_m by the oxidized non-heme iron of PSII

In the pump-probe fluorescence method, the yield of Chl*a* fluorescence can also be susceptible to photochemical quenching caused by the PSII acceptor-side non-heme iron. Indeed, fast re-oxidation of flash-induced Q_A^- can occur with a half-time of 25 μs or less by electron transfer to the non-heme iron if it has been previously oxidized by exogenous oxidants such as FeCN [19,20]. The quenching effect of this DCMU-independent electron transfer is shown in Table 1 with thylakoids dark-incubated in the presence of 400 μM FeCN prior the addition of DCMU. Even if some attenuation of the probe flash via absorption by FeCN has contributed to this decreased Chl*a* fluorescence yield, a significant quenching effect is clearly demonstrated by the low (F_m-F_o)/F_o ratio of 1.36, which is about half of the control. On the other hand, we observed that F_m measured in untreated thylakoids 15 s after a saturating preflash is not significantly different from the control (Table 1). Similar results were obtained when the fluorescence yield was averaged from the second to the 17th flash (one flash every 6 s). Considering the slow oxidation rate of the non-heme iron [19], these results indicate that in our experiments, the non-heme iron does not significantly contribute to the low yield of Chl*a* fluorescence induced by a single turnover flash.

3.4. Quenching of F_m by oxidized PQ

The presence of Chl *a* fluorescence quenching associated with an oxidized PQ pool has been demonstrated in the past but its relative importance remains a subject of debate [22,23,35,36]. The effect of chemical reduction of the PQ pool on the Chl *a* fluorescence yield was therefore assessed under our experimental conditions. Dark incubation of thylakoids in the presence of DQH_2 ($E_{m,7.0} = +5$ mV) for 3 min or in the presence of sodium dithionite for 15 s resulted in large increases of the F_m yield (Table 1). In the later case, F_m is increased by 60% relative to the control and accounts for 90% of the F_m yield observed in the presence of background light. The effectiveness of DQH_2 and sodium dithionite to reduce the PQ pool in our experiments was confirmed by an independent measure with a pulse amplitude modulated (PAM) fluorimeter of fluorescence decay kinetics following a single turnover saturating flash. The millisecond time scale decrease in fluorescence yield back towards the F_o level is much slower when the PQ pool is reduced [37]. Under our experimental conditions, both DQH_2 and sodium dithionite treatments caused a large increase in the half time of recovery by 100% or more relative to the control (results not shown).

The increases in F_m resulting from the addition of DQH_2 or sodium dithionite were similar in the absence or presence of DCMU (Table 1). However, the increase in F_o induced by sodium dithionite was significantly inhibited in DCMU treated thylakoids which resulted in a maximal value for F_v under these conditions. This is consistent with previous reports [22,38] that the chemical reduction of Q_A by sodium dithionite is retarded in the presence of DCMU.

3.5. Effect of DCMU on F_m quenching

DCMU alone had a notable stimulatory effect (about 30%) on both F_o and F_m yields. The effect of DCMU on F_o has previously been reported and related to a back flow of the electron from Q_B^- to Q_A in some PSII complexes [37]. However, the effect of DCMU on F_m induced by a single turnover flash was somewhat unexpected. It is unlikely that this increase of F_m is related to the inhibition of a fast Q_A^- re-oxidation. It is important to note that contrary to untreated thylakoids, the enhancement of the Chl *a* fluorescence yield in DCMU-treated thylakoids increases with the number of saturating flashes (Table 1; see Ref. [10]. This effect, clearly not due to photoreduction of the PQ pool, was already reported and attributed to the putative fluorescence quencher Q_2 [10]. Under repetitive single turnover flashes (< 1 Hz) in the absence of DCMU, electrons are transferred from Q_A^- to Q_B and the fluorescence yield remains low. In the presence of DCMU, inhibition of electron transfer from Q_A^- to Q_B would allow the reduction of the Q_2 quencher by Q_A^- or by $Pheo^-$, resulting in the observed increase of the fluorescence yield under these conditions (Table 1).

The stimulatory effect of DCMU on flash-induced F_m is analogous to the effects of DCMU and related inhibitors which raise the I_1 fast transient and eliminate the non-photochemical phase of Chl *a* fluorescence induction observed at the onset of a strong continuous illumination [32,39]. This again suggests a similarity between the flash induced F_m and the I_1 fast transient observed after the onset of intense illumination [6,31].

3.6. The mechanism of F_m quenching. Are there changes in the absorption cross-section?

The increase of F_m induced by CCCP observed in this work indicates that some of the quenching observed with a single turnover flash is related to oxidized intermediates on the donor side. However, our observations with sodium dithionite, DQH_2 and to a lower degree DCMU, indicate that much of the quenching after a single turnover flash is dependent on the presence of some oxidized PQ molecules or other oxidants such as the Q_2 quencher.

Previous observations have suggested the presence of a non-photochemical quenching resulting from a direct interaction between oxidized PQ and Chl *a* causing the dissipation of excitation energy in the pigment bed [22,36]. This can be verified with the pump-probe fluorescence method by the determination of the effective absorption cross-section of PSII 'σ_{PSII}' from the flash intensity saturation curves of variable Chl *a* fluorescence [7,40]. Chl *a* fluorescence quenching due to processes occurring during the actinic laser flash, such as non-photochemical deactivation of exciton energy in the pigment bed, will decrease the value of σ_{PSII}. In contrast, fluorescence quenching due to inactivated reaction centres or due to an electron transfer within the PSII reaction centre and occurring after the actinic flash will not affect σ_{PSII} [7,40].

Relative changes in σ_{PSII} are often inferred from relative changes in F_m and F_o via a simple 'ratio of rate constants' analysis based on Butler's bipartite model [41]. Nonphotochemical quenching mechanisms that would result in decreases in σ_{PSII} (antenna based mechanisms) would also result in quenching of F_o while reaction center based mechanisms would not. The bipartite model is, however, limited by its incorrect assumption of irreversible trapping and direct formation of Q_A^- [2]. The currently accepted origin of variable fluorescence as recombination of the primary radical pair ($P680^+/Phe^-$) in the presence of Q_A^- is not addressed by the bipartite model [2]. Due to these limitations we have not completed the classical quenching analysis of our fluorescence parameters (F_m,F_o) to determine changes in σ_{PSII} in terms of the bipartite model. We have rather measured σ_{PSII} directly, a powerful tool which frees us from the limiting assumptions of the bipartite model concerning the origins of variable fluorescence.

Curves of the flash intensity dependency of the variable Chl *a* fluorescence were measured from spinach thylakoids

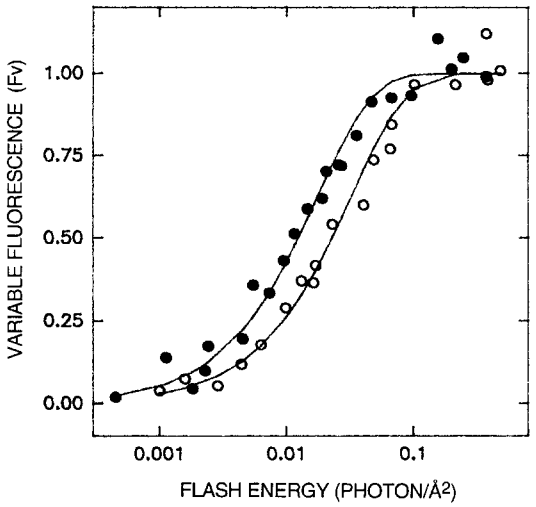

Fig. 1. Flash saturation curves of variable Chl-*a* fluorescence F_v measured 50 μs after a single-turnover flash from spinach thylakoids incubated in the absence (O) or the presence of 5 mM of sodium dithionite added 15 s prior the flash (●). The maximum F_v values from both treatments were normalized to unity and the curves represent the fit of experimental data to Eq. (1) in the text.

incubated under different conditions. Representative curves measured from thylakoids incubated in the absence and presence of sodium dithionite for 15 s are presented in Fig. 1. The two curves clearly show that lower flash energies were required to induce variable Chl*a* fluorescence in thylakoids incubated under reducing conditions compared to untreated thylakoids. The data could be satisfactorily fit to the cumulative single-hit Poisson distribution from which the values of the PSII effective absorption cross-sections σ_{PSII} were derived [7,40]. The σ_{PSII} values for the different treatments are summarized in Table 3. Firstly, dark incubation of thylakoids in the presence of DCMU or CCCP resulted in a small increase of σ relative to the control that can be considered insignificant. France et al. [13] have previously observed no effect of DCMU on the σ_{PSII} value estimated from the flash energy saturation curves of F_v generated by a 300 ps pump laser flash. On the other hand, large increases of σ_{PSII} relative to the control resulted from the incubation of thylakoids in the presence of sodium dithionite (+93%) or DQH_2 (+62%) (Table 3).

Table 3
Values of the effective absorption cross-sections of PSII (σ_{PSII}) determined from the flash intensity saturation curves of variable Chl*a* fluorescence from untreated and treated spinach thylakoids

Treatments	σ (Å²)
Control	29.2±4.0
CCCP 2 μM	34.7±2.2
DCMU 10 μM	33.6±2.0
DQH_2 50 μM	47.3±4.3
Dithionite 2 mM (15 s)	56.3±8.5
DQH_2 50 μM+DCMU 10 μM	32.2 (n = 2)
Dithionite 2 mM (15 s) +DCMU 10 μM	38.3±3.5

The data are the mean values (±standard deviation) of 3 independent measurements (unless indicated otherwise).

The closure of a significant number of reaction centres can lead to an increase in the apparent cross-section of those remaining open if the reaction centers share a common antenna (connectivity) [12,24]. However, connectivity cannot be responsible for the increases in σ_{PSII} we observed upon reduction of PQ as thylakoids dark incubated in the presence of DCMU displayed a similar increase in F_o level (due to closed centres) to those incubated with DQH_2 (Table 1) but did not show the large increase in σ_{PSII}.

It is well established that exogenous quinones in their oxidized form can efficiently quench the excited state of Chl in solution and in thylakoid membranes and thereby decrease the fluorescence yield [42]. The dramatic increases in σ_{PSII} we observed in the thylakoids treated with DQH_2 or sodium dithionite would therefore be consistent with the idea that the oxidized PQ pool causes a quenching of Chl*a* fluorescence localized in the antenna [22,36]. However, the effects of DQH_2 and sodium dithinonite on σ_{PSII} are completely or mostly abolished in DCMU-treated thylakoids (Table 3). These observations indicate that the mechanism by which the oxidized PQ pool quenches F_m is more complex than a direct quenching in the bulk antenna chlorophyll. It is possible that the interaction between oxidized PQ and the antenna pigment bed is somehow localized in the vicinity of the DCMU binding site.

An alternative explanation for the smaller effects of DQH_2 and sodium dithionite on σ_{PSII} in the presence of DCMU would be an inhibition by DCMU of the reduction of a redox component which affects the estimation of σ_{PSII}. It is already known that DCMU retards the chemical reduction of Q_A by sodium dithionite ([22,38]; see Table 1) as well as the oxidation of the non-heme iron by FeCN [19]. Also, Joliot and Joliot [24] observed that the chemical reduction of the Q_2 quencher was inhibited in the presence of DCMU. Based on these previous observations and on our results, we can suggest that whereas the reduction of either the Q_2 quencher or the PQ pool increases F_m yield, only the reduction of Q_2 induces an increase of σ_{PSII}. Mathis and Rutherford [25] suggested that the Q_2 quencher phenomenon was a manifestation of a low quantum yield reduction of a second Pheo in the PSII reaction centre. We may further suggest that the reduction of 'Q_2' would result in an increase of the quantum yield of PSII primary photochemistry. Such increase would consequently lead to an increase of the estimation of σ_{PSII} [7,40]. Although the nature of the putative quencher Q_2 is still enigmatic, its influence on F_m and σ_{PSII} cannot be disregarded. Further studies are required to reconcile the presence of this quencher with current perceptions of Chl*a* fluorescence and the PSII complex.

4. Conclusions

Our results indicate that the maximum yield of Chl*a* fluorescence after a single turnover flash (1) is somewhat quenched by the presence of oxidizing equivalents on the donor side of PSII, (2) is more dramatically affected by the redox state of the plastoquinone pool and/or the putative fluorescence quencher Q_2 and (3) is equivalent to the fast fluorescence transient (I_1) observed at the onset of very intense illumination [6,31]. Incubation of thylakoids under reducing conditions in the presence of DQH_2 (3 min) or sodium dithionite (15 s) results in an increase of σ_{PSII}. As DCMU significantly inhibits these increases either the interaction between oxidized PQ and the antenna pigment bed is somehow localized in the vicinity of the DCMU binding site and/or only the putative quencher Q_2, whose reduction by sodium dithionite and DQH_2 is inhibited by DCMU, is responsible for the observed increase of σ_{PSII}.

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