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Recent chlorophyll-*a* fluorescence yield measurements, using single-turnover saturating flashes (STSFs), have revealed the involvement of a rate-limiting step in the reactions following the charge separation induced by the first flash (Magyar et al. 2018). As also shown here, in diuron-inhibited PSII core complexes isolated from *Thermosynechococcus vulcanus* the fluorescence maximum could only be reached by a train of STSFs. In order to elucidate the origin of the fluorescence yield increments in STSF series, we performed transient absorption measurements at 819 nm, reflecting the photooxidation and re-reduction kinetics of the primary electron donor P680. Upon single flash excitation of the dark-adapted sample, the decay kinetics could be described with lifetimes of 17 ns (~50%) and 167 ns (~30%), and a longer-lived component (~20%). This kinetics are attributed to re-reduction of $P680^{\bullet+}$ by the donor side of PSII. In contrast, upon second-flash (with Δt between 5 μs and 100 ms) or repetitive excitation, the 819 nm absorption changes decayed with lifetimes of about 2 ns (~60%) and 10 ns (~30%), attributed to recombination of the primary radical pair $P680^{\bullet+}Pheo^{\bullet-}$, and a small longer-lived component (~10%). These data confirm that only the first STSF is capable of generating stable charge separation - leading to the reduction of Q_A; and thus, the fluorescence yield increments elicited by the consecutive flashes must have a different physical origin. Our double-flash experiments indicate that the rate-limiting steps, detected by chlorophyll-*a* fluorescence, are not correlated with the turnover of P680.

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Abbreviations – bRC, purple bacterial reaction center; MES, 2-(*N*-morpholino) ethanesulfonic acid; OEC, oxygen-evolving complex; P680, primary electron donor of photosystem II; Pheo, pheophytin; PSII, photosystem II; PSII CC, PSII core complex; Q_A, quinone-type electron acceptor; RC, reaction center; STSF, single-turnover saturating flash; MTSF, multiple-turnover saturating flash; Y_Z, tyrosine residue.

Introduction

Photosystem II (PSII) is the redox-active pigment–protein complex embedded in the thylakoid membrane that catalyzes the oxidation of water and the reduction of plastoquinone. PSII is probably the most-studied light-inducible enzyme, not only for its relevance to biochemistry (it is the O_2-producing enzyme) but also because it serves as basis of inspiration for artificial photocatalyzers for water oxidation. The PSII reaction center complex (RC) is surrounded by CP47 and CP43 core antenna proteins. This PSII core complex (PSII CC) may be regarded as the minimal fully functional PSII complex capable of charge separation and stabilization, plastoquinone reduction and oxygen evolution (Ghanotakis and Yocum 1990, van Grondelle et al. 1994). The structure of PSII CC from *Thermosynechococcus vulcanus* has been determined at 1.9 Å resolution by synchrotron radiation X-ray crystallography (Umena et al. 2011); femtosecond X-ray free electron laser (XFEL) has provided a radiation-damage-free structure at a resolution of 1.95 Å (Suga et al. 2015). Each PSII CC contains the reaction center incorporated in the D1/D2 proteins, the α and β subunits of cytochrome b559 and two integral antenna proteins, CP43 and CP47, which carry 14 and 17 chlorophyll-*a* molecules (Chl-*a*), respectively. The RC D1/D2 proteins are located approximately symmetrically with respect to the transmembrane region, which is very similar to the arrangement of the L/M subunits in bacterial RC (bRC). Four Chls (two accessory chlorophylls Chl_{D1} and Chl_{D2} and a special pair of the Chl-*a* molecules P_{D1} and P_{D2}, conventionally denoted as P680), two pheophytins ($Pheo_{D1}$ and $Pheo_{D2}$), and two plastoquinones (Q_A and Q_B) are arranged in two symmetrical branches. (It is to be noted that the nature of the primary donor is still under debate, and either a monomer, dimer or multimer of Chl-*a* molecules have been proposed (Durrant et al. 1995, Boussaad et al. 1997, Barber and Archer 2001, Romero et al. 2017a).) The absorption of a photon or transfer of an exciton to the primary electron donor P680 forms an electronically excited state. As in the simpler purple bRC (Michel and Deisenhofer 1988, Rutherford 1989, Kálmán et al. 2008), the subsequent charge separation in PSII is known to proceed asymmetrically, only along the D1 branch with the formation of $P680^{\bullet+}Pheo^{\bullet-}$ and this primary charge separation is stabilized by a re-oxidation of $Pheo^{\bullet-}$ by Q_A, forming $P680^{\bullet+}Q_A^{\bullet-}$. The primary electron donor is re-reduced by electron donation from the nearby redox active tyrosine (Tyr161 of D1), forming a neutral tyrosyl radical $Y_Z^{\bullet}(H^+)$, which is then able to oxidize the Mn_4CaO_5 cluster, leading to the $S_2^{(+)}$ state, which denotes the state of the oxygen-evolving complex (OEC) after a single-turnover saturating flash (STSF) excitation (Fig. 1; Shen 2015).

When Q_A is pre-reduced, only the primary radical pair $P680^{\bullet+}Pheo^{\bullet-}$ can be formed. This pair then decays by charge recombination within a few nanoseconds. In this case, the lifetime and yield of fluorescence (emitted mostly by the antenna chlorophylls) are substantially increased because the energy transfer from the antennae to P680 becomes slower and less efficient and because delayed luminescence occurs during the (prolonged) lifetime of the $P680^{\bullet+}Pheo^{\bullet-}$ pair (Shuvalov et al. 1980, Nuijs et al. 1986, Schatz et al. 1987, Schlodder and Brettel 1988, van Mieghem et al. 1995).

Despite the detailed structural information and the wealth of functional data (Cardona et al. 2012, Nelson and Junge 2015, Duan et al. 2017, Romero et al. 2017b), our understanding of the processes associated with or accompanying the functioning of RC is incomplete: the photochemical activity of PSII is routinely monitored by Chl-*a* fluorescence induction but the presently available models and observations are still controversial (Stirbet and Govindjee 2012, Schansker et al. 2014) and the origin of variable fluorescence ($F_v = F_m - F_o$) remains to be elucidated. F_o and F_m are the minimum and maximum fluorescence levels in dark-adapted samples, obtained before and after a sufficiently long and strong excitation, respectively. The canonical view that the rise of fluorescence yield from F_o to F_m depends solely on the redox state of the primary quinone electron acceptor, Q_A (Duysens and Sweers 1963, Strasser et al. 2004) has been challenged by several authors (Joliot and Joliot 1979, Schansker et al. 2011, Vredenberg 2011). Recently, it has been shown that a substantial part of the gradual fluorescence rise of PSII elicited by multiple µs-STSFs in the presence of the PSII electron transport inhibitor diuron (DCMU, 3-(3′,4′ dichlorophenyl)-1,1′ dimethylurea) that permits only one stable charge separation, originates from light-induced processes that occur after the stabilization of the charge separated state, i.e. in already closed RC where Q_A is reduced (Magyar et al. 2018, Joliot and Joliot 1979). This phenomenon has been observed in a variety of samples, from PSII CC to whole cells and from intact to TRIS-washed thylakoid membranes and at temperatures between about 170 and 280 K. Further, experiments using double flashes with variable delays (Δt) between them, have revealed the involvement of a previously unknown rate-limiting step in the light-induced reactions that lead to the gradual increase of the fluorescence level from F_1 (after one STSF) to F_m (reached after several flashes). These observations cannot be reconciled with the mainstream model according to which "in order to reach [F_m], it is necessary, and sufficient, to have Q_A completely reduced in all the active PSII centers" (Stirbet and Govindjee 2012). However, the underlying physical mechanism (or mechanisms) of the fluorescence yield increments and of the rate-limiting step has (have) not been elucidated.

An analysis of the temperature dependence of the fluorescence transients strongly suggested the involvement of conformational changes/dielectric relaxation in the F_1-to-F_m fluorescence increments. With respect to the origin of the rate-limiting step, two hypotheses have been put forward (Magyar et al. 2018): (1) It might originate from a rate limitation at the level of primary photochemistry (e.g. the turnover of $P680^{\bullet+}Pheo^{\bullet-}$). Although in closed RC the generation and fast recombination of $P680^{\bullet+}Pheo^{\bullet-}$ have been thoroughly demonstrated (van Mieghem et al. 1995, Nadtochenko et al. 2014),

a gating mechanism operating in the time interval shortly after the first flash cannot be ruled out a priori - given the fact that the increments between F_1 and F_2, F_2 and F_3 etc. are light-induced events, which require waiting time. (2) Alternatively, the light-induced reactions might change the fluorescence yield of the PSII CC without gating the primary photochemistry. The fluorescence of PSII CC has been proposed to originate from the two antenna-proteins, and to depend on the excitation energy transfer reactions between $P680^*$ and the domains of CP47 and CP43 (Shibata et al. 2013).

In this work, in an attempt to elucidate the origin of Chl-*a* fluorescence yield increments and the physical mechanism underlying the rate-limiting step in PSII, we measured, at room temperature, Chl-*a* fluorescence kinetics induced by trains of µs-STSFs, and saturating laser-flash induced nanosecond 819 nm transient absorption changes (originating mainly from the oxidation and re-reduction of P680) in DCMU-treated PSII CC isolated from *T. vulcanus*. Our data confirm that the F_o-to-F_1 fluorescence rise is associated with stable charge separation (down to $S_2^{(+)}Q_A^{\bullet-}$; Fig. 1).

The consecutive F_1-to-F_2 (and to F_m) increment(s) cannot be quantitatively explained by stable charge separation. Instead, rapid recombination of $P680^{\bullet+}Pheo^{\bullet-}$ prevails upon second and repetitive flashes. We also conclude that the rate limitation in PSII does not mainly originate from gating of the primary charge separation and must involve other light-dependent processes affecting the Chl-*a* fluorescence yield.

Materials and methods

Photosystem II core complexes and treatments

PSII core complex of *T. vulcanus* was isolated as described earlier (Shen and Inoue 1993, Shen and Kamiya 2000). For Chl-*a* fluorescence measurements, the Chl content of the PSII CC was adjusted to ~25 µg ml^{-1} in 20 mM MES (pH 6.0), 20 mM NaCl, 3 mM $CaCl_2$ and 5% glycerol. DCMU was dissolved in dimethyl sulfoxide (DMSO) and added to all samples immediately before the fluorescence measurements at a final concentration of 40 µM (the final concentration of DMSO did not exceed 1%). Before the measurements, the samples were dark adapted for 5 min at room temperature. For transient absorption spectroscopy, the same protocol was used for the dilution of PSII CC to a Chl concentration of ~250 µM and 40 µM DCMU was added to the samples immediately before the measurements.

Chlorophyll *a* fluorescence

Relative fluorescence yields were measured using a customized PAM 101 fluorometer (Walz), as described earlier (Magyar et al. 2018) - with minor modifications. Variable fluorescence was induced by single-turnover saturating Xe flashes (Excelitas LS-1130-3 Flashpac with FX-1163 Flashtube with reflector) of 1.5 µs duration at half-peak intensity. The frequency of the modulated measuring light (low intensity, virtually non-actinic) was 1.6 kHz. In order to determine F_m a 100 ms long multiple-turnover saturating flash (MTSF) was used after the first pair of STSFs. MTSFs were delivered from a

700 mW blue laser diode (ULV-445-700, Roithner Laser Technik GmbH). The sample was placed at the sample holder of a thermoluminescence (TL) apparatus in order to control the temperature. The timing of the flashes was controlled by a programmable digital pulse generator (BNC 577, Berkeley Nucleonics Corp) via custom-designed LabVIEW software. The kinetics of each measurement was recorded using a USB Multifunction I/O Device (NI DAQ 6001) via custom-designed LabVIEW software, with 20 ms^{-1} sampling rates. However, the time resolution of the measurements was determined by the 1.6 kHz modulation frequency of the measuring beam of the PAM 101 fluorometer.

Transient absorption spectroscopy

The transient absorption spectroscopy system is optimized for fast response, high fidelity, and low noise; the setup has been described in detail in (Byrdin et al. 2009, Müller et al. 2014, Müller et al. 2018). Two pulsed lasers were used in single- and double-flash experiments. In double flash excitation experiments, the first excitation flash (~5 ns, 532 nm) was provided by a Nd:YAG pumped optical parametric oscillator (OPO; Brilliant B/Rainbow) and the second flash by a Continuum Leopard SS-10 Nd:YAG laser (~100 ps, 532 nm). The delay between the laser flashes was controlled by a high precision digital pulse and delay generator (DG535 from Stanford Research Systems, Inc.). The kinetic traces were recorded upon the second flashes. For single- and multiple-flash excitation experiments, the 100 ps laser was used. The laser energies were set to ~5 mJ cm^{-2} for the 5 ns pulse and ~1 mJ cm^{-2} for the 100 ps pulse, as estimated from energy measurements behind a cell filled with H_2O using an energy meter (Gentec QE25SP-H-MB-D0) and from transient absorption signals using $[Ru(bpy)_3]^{2+}$ as an actinometer (Müller and Brettel 2012). A small fraction of the 100 ps pulse was used to trigger the detection system via a fast photodiode. Continuous measuring light was provided by a laser diode emitting at 819 nm (SDL-5411-G1, Spectra Diode Labs), passed through a cut-off filter RG780 (to suppress weak contaminations below 750 nm in the emission of the laser diode). The measuring light was perpendicular to the excitation laser beams and passed through the sample along the 10 mm path of a 2 × 8 × 10 mm (width×height×length) quartz cell with self-masking solid black walls (from Starna). Flash-induced changes of the transmission of the sample were monitored behind the sample and an 820 nm interference filter (to suppress stray light and the fluorescence induced by the excitation flash) by a Si photodiode (Alphalas UPD-200-UP; rise time, 200 ps; sensitive area, 0.1 mm^2) coupled to a digital oscilloscope (Agilent Infinium 81004B; sampling rate, 40 Gsamples s^{-1}; rms noise at 40 mV full scale, 340 µV; bandwidth, dc–10 GHz). To estimate the decay parameters, Levenberg-Marquardt least-squares optimization algorithm was used in OriginLab2018 (OriginLab Corporation,) to fit trend lines according to the equation: $y(t) = A_1 \cdot e^{-t/\tau_1} + A_2 \cdot e^{-t/\tau_2} + y_0$.

Thermoluminescence (TL)

TL was measured using a custom-made apparatus similar to the one described by Ducruet and Vass (2009). Samples of PSII core complexes, equivalent to 12 µg Chl, on a 13-mm-diameter glass-fiber filter paper (GF-2, Macherey-Nagel GmbH) were placed on a copper sample holder, which was connected to a cold finger immersed in liquid nitrogen. A heater coil, placed under the sample holder, ensured the desired temperature and heating rate of the sample during the measurements. Dark-adapted samples were excited by one STSF at 10°C, or by 1 and 20 STSFs at -60°C. The glow curves were measured while heating the sample to 70°C with a constant heating rate of 0.5°C s^{-1}. The TL emission was measured with a Hamamatsu end-window photomultiplier (Hamamatsu Photosensor Modules H1071-20). The detector current was amplified using a DC transimpedance amplifier with a 10 V/µA conversion ratio (Hamamatsu C7319).

Results

Upon the excitation of DCMU-treated dark-adapted PSII CC of *T. vulcanus* by double STSFs with variable waiting times (Δt) between the two flashes, the Chl-*a* fluorescence yield at room temperature (23°C) displayed a stepwise rise (Fig. 2A). A similar pattern was observed earlier using a train of STSFs at 5°C (Magyar et al. 2018). STSF excitation of PSII CC in the presence of PSII inhibitor DCMU leads to the formation of $S_2^{(+)}Q_A^{\bullet-}$ state via three intermediate states (Fig. 1**)**. As shown in Figs 2A and S1, the first STSF raised the fluorescence yield from F_o to F_1, with F_1 being significantly smaller than F_m, 59 ± 3% of F_m at room temperature. This value at room temperature and at 5°C varied from batch to batch between about 45 and 60% and about 35 and 45 %, respectively; the F_v/F_m parameter under the same conditions was 0.845 ± 0.035 (n = 9, obtained from 5 different batches; data not shown). These observations are in good agreement with the data reported earlier on PSII CC by Magyar et al. (2018) and in reasonable accordance with observations on thylakoid membranes (Joliot and Joliot 1979, Prášil et al. 2018). By using a second STSF, the fluorescence level could be further raised, by about another 15% – but only after a sufficiently long Δt waiting time between the two flashes, and further excitations were required to reach the maximum level F_m; here MTSF excitation was used (the second MTSF laser pulse was applied to confirm that F_m was reached).

As reported earlier (Magyar et al. 2018), the fluorescence level at the second and later flashes depended on the dark waiting time Δt between the flashes (Fig. 2B). Compared to F_1, no fluorescence increment could be induced with the second flash if two STSFs were fired simultaneously, or with a short waiting time (Δt < 100 µs); the F_1-to-F_2 increment was maximal at waiting times longer than 10 ms. The half-rise time ($\Delta t_{1/2}$) was about 3 ms. The effect of waiting time is also demonstrated in Fig. S1: the F_1-to-F_2 fluorescence yield increment, i.e. the increase between the fluorescence levels after the first and second STSF, was negligible with $\Delta t \leq$ 100 µs, and reached its maximum with $\Delta t \geq$ 10 ms.

In order to elucidate the physical mechanism underlying the waiting-time dependent stepwise fluorescence yield rise in DCMU-treated PSII CC, we performed laser-flash-induced transient

absorption measurements at 819 nm to monitor the photooxidation and re-reduction kinetics of P680, and the fate of $P680^{\bullet+}Pheo^{\bullet-}$ primary radical pair. Note that both $P680^{\bullet+}$ and $Pheo^{\bullet-}$ absorb at this wavelength with extinction coefficients, respectively, $\varepsilon(P680^{\bullet+})_{819\ nm} \approx 7\ mM^{-1}$ (Mathis and Setif 1981) and $\varepsilon(Pheo^{\bullet-})_{819\ nm} \approx 6\ mM^{-1}$ (Fujita et al. 1978). We recorded single, double and repetitive flash-induced transient absorption changes in the DCMU-treated dark-adapted PSII CC, using variable waiting times (Δt) between consecutive laser flashes.

As shown in Fig. 3A (black trace), upon single-flash excitation, the transient absorption signal at 819 nm exhibits a fast rise and an initial sub-ns decay phase (which is not well-resolved in our experiment and which we tentatively attribute to the re-oxidation of $Pheo^{\bullet-}$ by Q_A in a few hundred picoseconds; Fig. 1). This ultrafast phase is followed by an essentially biphasic decay, which can be well fitted in the time window from 1 to 800 ns by a bi-exponential decay with time constants of 16.6 ns (~50%) and 167 ns (~30%) and a constant (~20%) that accounts for decay components on the microsecond time scale (Table 1), in good agreement with literature data for the reduction of $P680^{\bullet+}$ by the donor side of PSII (van Best and Mathis 1978, Brettel et al. 1984, Schlodder et al. 2015). The fastest decay component (~20 ns) has been clearly attributed to proton-coupled electron transport from TyrZ in the dark-adapted state (S_1) of the OEC (Tommos and Babcock 2000, Schlodder et al. 2015). Slower decay components are thought to indicate relaxation processes involving hydrogen bond networks that shift the equilibrium $P680^{\bullet+}TyrZ \leftrightarrow P680TyrZ^{\bullet}(H^+)$ to the right and reduction of $TyrZ^{\bullet}(H^+)$ by the OEC (Schilstra et al. 1998, Christen and Renger 1999, Schlodder et al. 2015). Reduction of $P680^{\bullet+}$ is also known to be slowed down in the presence of higher oxidation states (S_2 and S_3) of the OEC (Brettel et al. 1984, Schlodder et al. 1985) and in PSII with inactive OEC (Conjeaud et al. 1979, Conjeaud and Mathis 1980).

In contrast to the transient absorption changes upon a single laser-flash excitation, following one or several pre-flashes, the kinetics was dominated by a rapid decay in a few nanoseconds. This was most easily detected in an experiment with repetitive ($2\ s^{-1}$) excitation by 100 ps pulses. The red trace in Fig. 3A represents an average of 128 signals (which substantially improved the signal-to-noise ratio) following 10 pre-flashes given to bring PSII to the state where F_m is observed in the fluorescence yield measurements (Fig. 2A).

Even though the observed initial amplitudes of the signals of the single-flash and repetitive-flashes experiments were similar, note that this does not imply a similar yield of $P680^{\bullet+}$ formation – the signal of the repetitive flash experiment contains a considerable contribution of $Pheo^{\bullet-}$, whereas $Pheo^{\bullet-}$ is essentially absent at the beginning of our fit (1 ns) in the single-flash experiment. Considering the molar absorption coefficients given above, the $P680^{\bullet+}$ $Pheo^{\bullet-}$ pair yield in the repetitive flashes experiments amounts only to ~65% of the $P680^{\bullet+}$ yield obtained in the single-flash experiment, close to the value (60±15%) previously reported for PSII complexes from *Synechococcus* (Schlodder and Brettel 1988).

A bi-exponential fit of the repetitive flashes experiment in the interval between 360 ps (where the signal rise was completed) and 50 ns yielded τ_1 of 2 ns (~55%), τ_2 of 9 ns (~35%) and a minor longer-lived component (10%). In line with previous works (Shuvalov et al. 1980, Nuijs et al. 1986, Schlodder and Brettel 1988, van Mieghem et al. 1995), we attribute the decay phases on the ns timescale to recombination of the primary radical pair $P680^{\bullet+}Pheo^{\bullet-}$ in closed RC (containing $Q_A^{\bullet-}$). The time constants previously attributed to $P680^{\bullet+}Pheo^{\bullet-}$ recombination vary considerably (between 1.6 ns (Schatz et al. 1987) and ~25 ns (slower component of a biphasic decay; van Mieghem et al. 1995), which may reflect differences in origins and types of the PSII preparations (with (photo)chemically semi- or fully (pre-)reduced Q_A) and in the time window and resolution of the used techniques. It has been suggested that a biphasic nature of the decay is due to relaxation of the $P680^{\bullet+}Pheo^{\bullet-}$ pair (Schlodder and Brettel 1988). The small absorption change remaining after the ns decay may represent triplet states of Chl-*a* in the antenna and/or in the RC (van Mieghem et al. 1995).

We next studied the yield and kinetics of the $P680^{\bullet+}Pheo^{\bullet-}$ pair upon the second flash in double-flash experiments as a function of the delay between the two flashes (between 5 μs and 100 ms). Dependence of the initial amplitude on the delay would be a testament to gating of the primary charge separation, which could, in principle, be related to the lack of F_1-to-F_2 fluorescence increment. In other terms, we wanted to clarify if the lack of F_1-to-F_2 fluorescence increment upon the second STSF excitation with Δt < 100 μs, is caused, or not, by the lack (gating) of primary photochemistry.

Remarkably, already for the delay (Δt) of 5 μs, the 'typical' $P680^{\bullet+}Pheo^{\bullet-}$ recombination signal was observed with a relative amplitude of as much as ~65% of the signal in the repetitive-flash experiment (with 500 ms intervals). This relative amplitude increased with delay length to ~90% at 100 ms (Fig. 3B). The signals at all delays (averages of 5-8 experiments each) could be well fitted with bi-exponential decay functions with shared time constants τ_1 of 2 ns and τ_2 of 10 ns (i.e. very similar to the repetitive flashes experiment). The amplitudes of the two phases, of the longer-lived component, and their sum (corresponding to the initial amplitude) are plotted against the delay time (Δt) in the inset of Fig. 3B. Note that between Δt of 5 μs and 5 ms, the growth of the initial amplitudes ($A_1 + A_2 + y_0$ in the inset of Fig. 3B) is essentially due to the growth of the amplitude of the 2 ns phase. Between Δt of 5 and 100 ms, there seems to be a significant increase in the longer-lived component y_0.

Discussion

The goal of this work was to elucidate a potential connection between nanosecond redox transients of P680 and the stepwise, waiting-time-dependent rise of the Chl-*a* fluorescence yield in dark-adapted, DCMU-treated PSII CCs excited with a series of STSFs. Earlier, Magyar et al. (2018) have shown that in these samples, which are capable of only a single stable charge separation, the fluorescence yield maximum (F_m), cannot be reached by exciting the sample with only one STSF. One flash has been shown to lead to a fluorescence level, termed F_1, significantly lower than F_m. The maximal

fluorescence yield F_m could only be reached by providing additional excitations to the sample, e.g. with a train of several flashes – which then gradually raised the fluorescence yield, from F_1 to F_2 and so forth (Fig. 2). It has also been shown, in accordance with earlier literature data obtained on isolated thylakoid membranes (Joliot and Joliot 1979), that only the first STSF leads to stable charge separation (Q_A reduction) and the F_1-to-F_2 and the further fluorescence yield increments are given rise to by different physical mechanisms. Recently, Prášil et al. (2018) have shown that variations in the fluorescence yield depend on the occupancy of the Q_B binding site and on the capacity of the secondary electron acceptors to re-oxidize the reduced Q_A. However, under our experimental conditions, significant participation of Q_B could be ruled out (Fig. S2), and thus also the dependence of the fluorescence yield on the Q_B site reactions.

Magyar et al. (2018) suggested the involvement of light-induced conformational changes and/or dielectric relaxation processes in the gradual STSF-induced rise in PSII fluorescence yield. It has been hypothesized, but not substantiated, that the fluorescence yield increments, induced by STSFs, were caused by the formation and rapid recombination of the $P680^{\bullet+}Pheo^{\bullet-}$ primary radical pair – via its intense transient electric field, superimposed on that generated by $Q_A^{\bullet-}$, and/or via local thermal jumps due to dissipation associated with the recombination of charges. Here, by measuring the transient absorption changes at 819 nm on DCMU-treated PSII CC, we monitored the laser-flash-induced photooxidation of P680 and its decay kinetics on the nanosecond timescale. Single, double, and multiple laser-flash experiments were performed.

Analysis of the decay kinetics of $P680^{\bullet+}$ (Fig. 3, Table 1) confirmed that, as expected, the photochemical events elicited by the first and second flash excitations differed substantially from each other. The kinetics of ΔA_{819} following a single-flash excitation of the dark-adapted DCMU-treated PSII CC could be assigned to the formation of the radical pair $P680^{\bullet+}Q_A^{\bullet-}$ and re-reduction of $P680^{\bullet+}$ by the donor side of PSII – as reflected by the multiphasic decay kinetics containing an ~20 and an ~200 ns component and a slow component on the µs timescale, with ~50, ~30 and ~20% contributions, respectively (see Results for more details and for references). In contrast, in the presence of a pre-flash or under repetitive flash excitations, ΔA_{819} decayed much faster, dominated by components of ~2 ns (~60%) and ~10 ns (~30%), – that can be assigned to charge recombination of $P680^{\bullet+}Pheo^{\bullet-}$; small longer-lived absorption changes (~10%) are essentially attributed to Chl-*a* triplet states (see Results for more details and for references). In good accordance with the conclusion that after the first STSF the additional flashes do not lead to stable charge separation, no additional TL band was present following multiple-STSF excitation of the sample (Fig. S3).

Our data confirm that, in the presence of DCMU, stable charge separation in PSII can only be induced by the first saturating flash. Hence the gradual fluorescence yield increments from F_1 to F_m upon additional flashes are not caused by stable charge separation and must be triggered by other events (Joliot and Joliot 1979, Magyar et al. 2018).

The 819 nm transient absorption experiments using pre-flashes (Fig. 3) also answer the question whether the rate-limiting reaction in PSII CC, reported by Chl-*a* fluorescence (Fig. 2), can be assigned to gating of the primary photochemical event (formation of $P680^{\bullet+}Pheo^{\bullet-}$). If this were the case, the dependence of the initial amplitude of the ΔA_{819} on the delay Δt between the first (pre-)flash and second flash (inducing the observed absorption changes), should directly correlate with the appearance of the F_1-to-F_2 fluorescence yield increment. Inspection of our experimental data (compare Fig. 3B, Table 1 and Fig. 2B) reveals, however, that both the extents of the effects and the delay times of their appearance are clearly different: While the fluorescence yield increment was virtually zero for Δt up to 10 µs, the initial ΔA_{819} at Δt = 5 µs was already ~65% of the value obtained with repetitive flashes at 500 ms interval. Furthermore, most of the (moderate) increase of the initial ΔA_{819} occurred between Δt = 5 µs and 100 µs, while most of the fluorescence yield increment occurred between Δt = 1 ms and 10 ms. We hence conclude that the rate limitation observed in fluorescence experiments does not originate mainly from gating of the primary charge separation.

We would like to mention that an increase of the initial ΔA_{819} with increasing Δt was expected because charge separation is only possible in PSII in which $P680^{\bullet+}$ formed by the pre-flash is already re-reduced. The extent of the increase (from ~65% (of the value under repetitive excitation) at Δt = 5 µs to ~90% at Δt = 100 ms) is, however, somewhat more pronounced than expected from the re-reduction kinetics of $P680^{\bullet+}$ formed by the pre-flash: according to the first-flash signal in Fig. 3A, at least 80% of $P680^{\bullet+}$ should already be re-reduced at Δt = 5 µs. As a possible explanation, we suggest that the yield of primary charge separation may be diminished during the lifetime (~50 µs; Fig. 1) of $Y_Z^{\bullet}(H^+)$ due to electrostatic interaction with $P680^{\bullet+}$. It is also of note that the amplitude y_0 of the long-lived component of ΔA_{819} increased slightly (from 12 to 17% of the initial amplitude), but significantly, between Δt = 5 ms and 100 ms. This may indicate that, in part of PSII, $Q_A^{\bullet-}$ formed by the pre-flash became re-oxidized in this time window so that a stable charge separation (forming long-lived $P680^{\bullet+}$) was possible upon the second flash. Re-oxidation of $Q_A^{\bullet-}$ may occur by charge recombination with the oxidizing equivalent on the donor side of PSII or by electron transfer to the secondary quinone Q_B in a fraction of PSII in which this electron transfer was not completely blocked by DCMU.

Summarizing the direct conclusions from our experimental data, the waiting-time related fluorescence yield increment cannot be explained at the level of primary photochemistry of P680. Instead, we must assume that the waiting time is needed for allowing the relaxation (or completion) of a presently unidentified process that affects the fluorescence yield of Chl-*a*, which has been proposed to be determined by an interplay between the RC and the two antenna proteins of PSII CC (Shibata et al. 2013). It must be clearly seen that this process is induced by light and it does not occur without additional excitation of the sample. Thus, simple mechanisms, such as allowing a quencher (e.g. $P680^{\bullet+}$) or triplet states to relax, do not explain our observations. On the other hand, conformational changes and/or dielectric relaxation might have cumulative nature, and the occurrence of consecutive

events may depend on the completion of the previous steps. In this context it is interesting to note that using a pump-probe technique, Schreiber and Krieger (1996) have shown that in isolated DCMU-treated intact chloroplasts the initial fluorescence yield rise (measured at 20 µs, limited by the time resolution of the instrument) upon a STSF excitation was followed by a slower rise in the dark, which was completed in ~1 ms, with $t_{1/2}$ ~ 50 µs (Note that our Chl-*a* fluorescence measurements, such as shown in Fig. 2, do not resolve this fast rise). These data, on the one hand, confirm that the reduction of Q_A alone, occurring on the (sub-)ns timescale (Fig. 1), cannot be held responsible for F_v. On the other hand, given the comparable delay times, there is a possibility that the rise time in the fluorescence, observed by Schreiber and Krieger (1996), and Δt, the (dark) waiting time needed for the F_1-to-F_2 and F_2-to-F_3 etc. fluorescence yield increments (Magyar et al. 2018; Fig. 2), share the same physical mechanism. This hypothesis should be tested experimentally – by comparing the outcomes of both techniques applied to the same sample under comparable conditions. As to the putative common underlying physical mechanism, while we cannot rule out other explanation, the slow rise after a STSF excitation may also originate from conformational changes/dielectric relaxation due to the generation of the local electric field around $Q_A^{\bullet-}$. Further experiments are needed to clarify the nature of such putative, probably subtle, conformational changes and their possible role in adjusting the kinetic constants affecting the recombination and energy transfer pathways in PSII CC during the dark-to-light transition of PSII. It is also to be noted that in high light, in the absence of DCMU, excitations arrive to the RC before the electron transfer from $Q_A^{\bullet-}$ to Q_B is completed; and thus, the rate limitation (Δt) must affect the fluorescence induction kinetics even under natural conditions – experiments are underway to test this assumption and to construct a mathematical model taking into account the observations pointing to the role of light-induced processes in the presence of $Q_A^{\bullet-}$.

Author contributions

G.G. conceived the study together with G.S., P.M. and K.B. Transient absorption spectroscopy measurements were designed, performed and analyzed by K.B. and P.M. together with G.S. Flash-induced fluorescence measurements were designed and performed by G.S. and M.M. Thermoluminescence measurements were designed, performed and analyzed by L.K. together with G.S. and M.M. PSII CC were provided by J.R.S., Q.Z., Y.X. and G.H. The paper was written by G.S., G.G., P.H.L, P.M., K.B. and J.R.S.

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Supporting information

Fig S1. Kinetic traces of chlorophyll-*a* fluorescence yield upon double-STSF and a MTSF excitations of PSII core complex from *T. vulcanus* at room temperature in the presence of 40 μM DCMU.

Fig. S2. Thermoluminescence of PSII core complexes of *T. vulcanus* to demonstrate the inhibitory efficiency of DCMU on the electron transfer between Q_A and Q_B.

Fig. S3. Thermoluminescence curves of DCMU-treated PSII core complexes following their excitation at -60°C with 1 and 20 STSFs – showing that no additional TL band is produced upon multiple-flash excitation while the fluorescence yield increases.

Table 1. Amplitude (A_1 and A_2) and lifetime (τ_1 and τ_2) parameters of the transient absorption kinetics fitted with two exponentials and the amplitude of the residual long component (y_0) (see Methods). Numbers marked with a * are results of a global fit with shared time constants.

observed flash	τ_1 (ns)	τ_2 (ns)	A_1 (mOD)	A_2 (mOD)	y_0 (mOD)	$A_1 + A_2 + y_0$
1st flash	16.6	167	8.8	5.5	4.2	(18.5)
repetitive flashes	1.96	8.59	12.7	7.6	2.1	22.4
2nd flash, Δt 5 µs	2.05*	10.1*	9.5	3.8	1.2	14.5
2nd flash, Δt 100 µs	2.05*	10.1*	11.9	4.6	1.6	18.1
2nd flash, Δt 5 ms	2.05*	10.1*	13.1	4.5	2.3	19.9
2nd flash, Δt 100 ms	2.05*	10.1*	12.1	4.7	3.6	20.4

Figure legends

Fig. 1. Energy scheme of the electron transfer steps (until Q_A reduction) in PSII occurring upon excitation of P680 and assuming that all centers are in the dark-adapted stable state (S_1). Based on Cser and Vass (2007).

Fig. 2. (A) Two kinetic traces of chlorophyll-*a* fluorescence yield changes induced by trains of double-STSFs and two MTSFs in PSII core complexes from *T. vulcanus* at room temperature in the presence of 40 µM DCMU. Traces in black and blue, 1 µs and 100 ms waiting time (Δt) between the first and second STSF, as indicated; the MTSFs were 100 ms long. The traces are normalized to $F_m - F_o$; the F_v/F_m was 0.84 ± 0.02. (B) Dependence of the F_1-to-F_2 increment on the delay (Δt) between the first and second STSFs. The dashed line represents a single-exponential fit of the data points, with a half-rise time ($\Delta t_{1/2}$) of 2.9 ± 0.4 ms. Data points, mean values ± SD (n = 4); the curve is normalized to the maximal variable fluorescence ($F_m - F_o$).

Fig. 3. (A) Transient absorption kinetics in DCMU-treated, dark adapted PSII core complex from *T. vulcanus* at 819 nm using single flash excitation (black trace) and multiple flash excitations at 2 s^{-1} (red trace). The traces are averages of 4 and 128 signals, respectively. To reach the stationary state with multiple flash excitations, 10 pre-flashes were applied at the same repetition rate. The inset shows the kinetics up to 800 ns upon a single-flash excitation. The smooth lines represent bi-exponential fits of the curves (from 1 to 800 ns for the black curve and from 360 ps to 50 ns for the red one). (B) Traces in green, black, blue and red were recorded upon a second flash excitation, which followed a saturating (pre-)flash after Δt = 5 µs, 100 µs, 5 ms and 100 ms, respectively. The plotted signals are averages of 5 to 8 experiments on fresh samples from the same stock; the smooth lines represent global biexponential fits of the curves (fitting interval: 360 ps – 50 ns, shared time constants); inset: dependence of the fitted amplitudes on the delay between the first (pre-) flash and the actinic flash (Table 1).