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Evidence for a fluorescence yield change driven by a light-induced conformational change within photosystem II during the fast chlorophyll *a* fluorescence rise

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ABSTRACT

Experiments were carried out to identify a process co-determining with Q_A the fluorescence rise between F_0 and F_M. With 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea (DCMU), the fluorescence rise is sigmoidal, in its absence it is not. Lowering the temperature to −10 °C the sigmoidicity is lost. It is shown that the sigmoidicity is due to the kinetic overlap between the reduction kinetics of Q_A and a second process; an overlap that disappears at low temperature because the temperature dependences of the two processes differ. This second process can still relax at −60 °C where recombination between Q_A^- and the donor side of photosystem (PS) II is blocked. This suggests that it is not a redox reaction but a conformational change can explain the data. Without DCMU, a reduced photosynthetic electron transport chain (ETC) is a pre-condition for reaching the F_M. About 40% of the variable fluorescence relaxes in 100 ms. Re-induction while the ETC is still reduced takes a few ms and this is a photochemical process. The fact that the process can relax and be re-induced in the absence of changes in the redox state of the plastoquinone (PQ) pool implies that it is unrelated to the Q_B-occupancy state and PQ-pool quenching. In both +/−DCMU the process studied represents ~30% of the fluorescence rise. The presented observations are best described within a conformational protein relaxation concept. In untreated leaves we assume that conformational changes are only induced when Q_A is reduced and relax rapidly on re-oxidation. This would explain the relationship between the fluorescence rise and the ETC-reduction.

1. Introduction

Chlorophyll *a* fluorescence measurements are applied widely in the study of the photosynthetic apparatus, the effects of external factors affecting the photosynthetic apparatus, as well as for the study of the productivity of photosynthetic organisms (reviewed e.g. in [1]). One of the processes that has been extensively studied is the induction of photosynthesis by continuous light on a dark-to-light transition (reviewed among others in [2,3]). Kautsky and Hirsch [4] in 1931 were the first to observe that on a dark-to-light transition the fluorescence intensity initially increases after which it decreases again. The technological advances over the last 80 years allow us, today, to measure this phenomenon in much more detail and with a much higher time resolution than the eyes of Kautsky and Hirsch allowed in 1931. As the measuring equipment improved, more and more features of this fluorescence behavior were revealed. In the sixties of the last century, it was discovered that the fluorescence rise exhibited an intermediate step and under some conditions a dip [5–7]. In the late eighties/early nineties a second intermediate step was identified [8–11]. A repetition of the experiment of Kautsky and Hirsch with modern equipment, using a high light intensity of e.g. 3000 μmol photons m^{-2} s^{-1} would yield a fluorescence rise from a low value (F_0 or O) measured after 10–20 μs, via an intermediate step J (2–3 ms) and a second intermediate step I (~30 ms) to a maximum fluorescence intensity (F_M or P) that is reached after ~200 ms. The difference between the fluorescence intensity at F_M or P and the fluorescence intensity at F_0 or O is called variable fluorescence and the fluorescence rise can also be referred to as an OJIP-transient [10–12]. With respect to the interpretation of the kinetics of the fluorescence rise, a milestone was the formulation of the idea that the variable fluorescence reflects the reduction of Q_A or Q as it was known at the

Abbreviations: Chl, chlorophyll; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; DF, delayed fluorescence; ETC, electron transport chain; F_0 and F_M, fluorescence intensity measured when all photosystem II reaction centers are open or closed respectively; OJIP-transient, fluorescence induction transient defined by the names of its intermediate steps: O = 20 μs, J = 3 ms, I = 30 ms and P = the maximum fluorescence intensity; P680, reaction center pigments of photosystem II; PQ, plastoquinone; PSII and PSI, photosystem II and I, respectively; Q_A and Q_B, primary and secondary quinone electron acceptors of photosystem II respectively; S-states S_0, S_1, S_2, S_3 and S_4, different redox states of the oxygen-evolving complex

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time, the primary quinone acceptor of photosystem II, by Duysens and Sweers [13] in 1963. In mainstream publications on kinetic Chl *a* fluorescence measurements it is often implicitly assumed that variable fluorescence is a reflection of the redox state of Q_A (see e.g. [2,14,15]). In this concept the fluorescence intensity will increase if the population of Q_A molecules becomes more reduced. This idea can also be called a single-'quencher'-concept.

Over the last 50 years a number of observations have been made that are difficult to reconcile with a single-'quencher'-concept. They can be better explained if it is assumed that the fluorescence rise is determined by two major processes of which the reduction of Q_A is one. The first of these two-'quencher'-concepts was formulated by Delosme [16] in 1967. Morin [6] and Delosme [16] observed two fluorescence rise phases with a separating step at 2 ms (=J). Delosme [16] concluded that only the first rise phase (OJ) reflected the reduction of Q_A and that the second rise phase (JIP) represented another process. The fast rise phase was called the photochemical phase (strong dependence on the light intensity) and the slower (J-to-I-to-P) rise the thermal phase (less light intensity dependent and more sensitive to the temperature) [16]. With respect to the thermal phase, Delosme observed that the step J did not shift in response to changes in the light intensity. He assumed that the thermal phase was suppressed by inhibitors like 3-(p-chlorophenyl)-1,1-dimethylurea (CMU) and phenanthroline. Simulating his data, he further concluded that the other quencher had properties similar to R (Q_B). Delosme [17] also observed that the characteristics of the second quencher resembled the properties of the PQ-pool.

Joliot and Joliot [18,19] made another relevant observation. They simultaneously measured Chl *a* fluorescence and C550—an electric-field-induced spectral shift related to the reduction of Q_A- in DCMU-poisoned samples and observed a non-linearity between these two signals. This was interpreted in terms of two electron acceptors: Q_1 (Q_A) and Q_2 (an unknown quencher) of which Q_1 was efficiently reduced in the light and its reduction induced a C550 signal, whereas the reduction of Q_2 was far less efficient and its reduction did not induce a C550 signal. These authors further observed that Q_2 was more quickly re-oxidized than Q_1.

In the nineties, the problems associated with the single-' quencher'-concept gained renewed attention. In 1996, Samson and Bruce [20] considered the question why a single turnover flash induces considerably less fluorescence than a multiple turnover pulse. These authors explained the difference in terms of the quencher Q_2 mentioned above and quenching by oxidized PQ-molecules. Delosme [16] assumed that inhibitors would suppress the thermal phase; Samson and Bruce [20], on the other hand, showed that in the presence of DCMU the difference between a single turnover flash and a multiple turnover is considerably smaller than in its absence, but the difference is still there. This could mean that a single turnover flash in the presence of DCMU not only reduces nearly all Q_A, but also induces part of the thermal phase.

According to Vasil'ev and Bruce [21], Kolber, Prášil and Falkowski proposed that it was the occupancy state of the Q_B-site that determined the thermal phase, a point of view supported also by Vasil'ev and Bruce. Yaacoubd et al. [22] tried to prove this hypothesis using isolated thylakoid membranes, artificial quinones and DCMU. The authors noted that the thermal phase seemed to be more sensitive to quenching by non-natural quinones than the photochemical phase. Another observation that can be explained on the basis of the occupancy state hypothesis is the kinetic effect of electron flow through PSI on the fluorescence rise [23–25].

In purple bacterial reaction centers light-induced conformational changes have been described [26–29] and a link between light-induced conformational changes and changes in the fluorescence yield has been proposed [30]. The goal of this paper is to provide new experimental observations on the properties of the 'thermal phase' and to show that the essence of this phase is not so much its temperature dependence but the fact that it represents a fluorescence yield change. The argument will be put forward that this yield change is driven by light-induced conformational changes inside PSII reaction centers in which Q_A remains reduced for at least a few milliseconds.

2. Materials and methods

2.1. Plant material

Most measurements were carried out on mature leaves of 3–4 weeks old pea plants (*Pisum sativum* L.). Plants were grown in commercial soil in a greenhouse where the temperature was 20–25 °C during the day and 18–20 °C at night.

Tobacco plants (*Nicotiana tabacum L.*) were grown under similar conditions and were used when they were 7–8 weeks old.

2.2. DCMU treatment

Whole leaves were incubated in 0.2 mM DCMU (3-(3′,4′-dichlorophenyl)-1,1-dimethylurea) solution for at least 10 h in complete darkness before the fluorescence measurements. The solution contained 0.2% dimethylsulfoxide (DMSO) to dissolve the DCMU.

2.3. PSII-membranes

PSII-membranes were isolated according to the method of Bertholt et al. [31] with slight modifications. The PSII-membranes were stored at −80 °C in a medium consisting of 0.33 M sorbitol, 15 mM NaCl and 40 mM MES (pH 6.5). This medium was also used for the measurements described here. DCMU (100 μM) was added immediately before the fluorescence measurements.

2.4. Fast Chl a fluorescence (OJIP) measurements

Fluorescence measurements were carried out at room temperature or at low temperature with a Handy-PEA instrument (Hansatech Instruments Ltd., UK). Leaf samples were illuminated with continuous red light (650 nm peak wavelength; the spectral half-width was 22 nm; the light emitted by the LEDs is cut off at 700 nm by a NIR short-pass filter). The light intensity was 3500 μmol photons $m^{-2}\,s^{-1}$, if not stated otherwise. The light was provided by an array of three light-emitting diodes focused on a circle of 5 mm diameter of the sample surface. The first reliably measured point of the fluorescence transient is at 20 μs, which can be taken as F_0.

For the measurements of Fig. 1, a Handy-PEA instrument with a custom made illumination unit, allowing light intensities of up to 15,000 μmol photons $m^{-2}\,s^{-1}$, was used. For Figs. 5–7 an M-PEA instrument (Hansatech Instruments Ltd., UK, see below) was used allowing a time interval between pulses down to 1 ms. For further technical details of the M-PEA instrument see [32].

For the fluorescence measurements at low temperature the sample holder of a thermoluminescence-instrument (described in [33]) was used to control the temperature of DCMU-inhibited leaves and PSII-membranes. For the double pulse experiments at low temperature the pulse length was increased as the temperature was lowered in order to induce the maximum fluorescence intensity in all cases (−20 °C: 80 ms, −30 °C: 120 ms, −40 °C: 200 ms and −60 °C: 300 ms).

2.5. Delayed fluorescence

Delayed fluorescence (DF) measurements were carried out with an M-PEA instrument (Hansatech Instruments Ltd., UK). Pea leaves were illuminated with pulses of red light (either 1150 or 5000 μmol photons $m^{-2}\,s^{-1}$) of variable length (1–200 ms). On turning off the

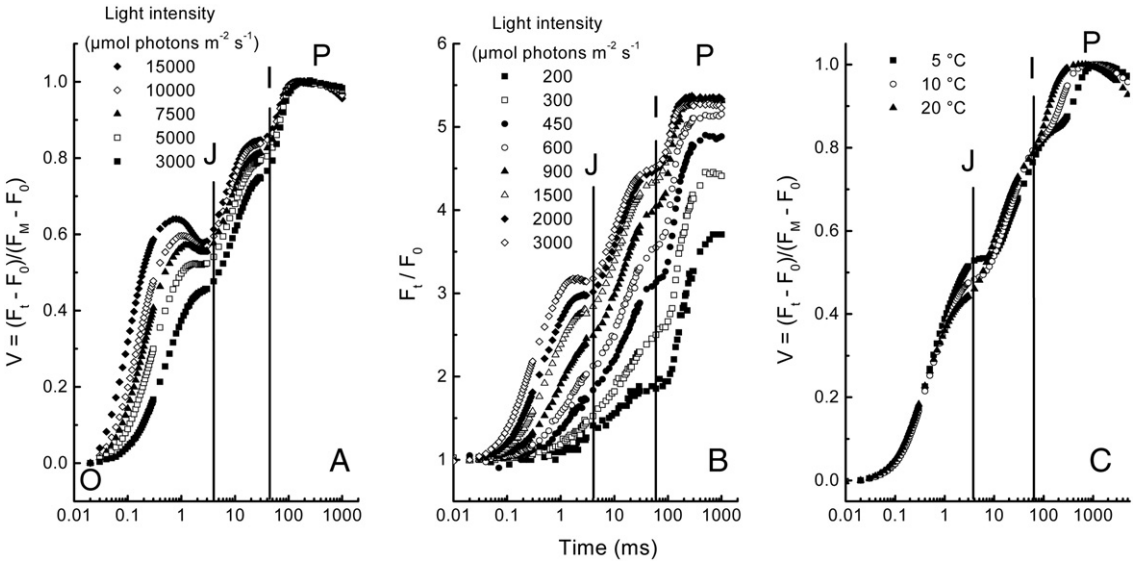

Fig. 1. Light intensity and temperature dependences of the form of the OJIP-transients measured on pea leaves (panels A and C) or tobacco leaves (panel B). In panel A, fluorescence transients induced by 3000–15,000 μmol photons m^{-2} s^{-1} of light are shown, whereas in panel B transients induced by more moderate light intensities of 200–3000 μmol photons m^{-2} s^{-1} are shown. In panel C, fluorescence transients induced by 3500 μmol photons m^{-2} s^{-1} were measured on pea leaves at three different temperatures. The lines in the panels indicate that the position of the I and J-steps does not depend on the light intensity. The fluorescence transients in panel A were normalized to F_M, in panel B to F_0 and the transients in panel C were double normalized between F_0 and F_M.

light the DF-decay was measured in darkness. The DF-decay kinetics were fitted with a sum of 3 exponentials.

The experimental approach used here, differs from that in [32] where the light pulse was interrupted at various times and where, during each interruption, the decay of the DF-signal was measured. The reduction of the photosynthetic electron transport chain and the accompanying fluorescence rise are determined to a considerable extent by dark reactions. During the repetitive dark intervals the dark reactions continue. This means that the time-dependence of the reduction of the electron transport chain using such a protocol will differ from the time dependence of the reduction of the electron transport chain using continuous light, complicating comparisons between both types of measurements.

3. Results

3.1. Light and temperature dependences of the kinetics of the OJIP-transients

In the Introduction, we noted that the fluorescence rise between 2 and 3 ms and 200 ms (the J–I–P-rise) is also called the '*thermal phase*'. In Fig. 1 three typical characteristics of the thermal phase are illustrated. Fig. 1A demonstrates that the photochemical phase (OJ) is strongly dependent on the light intensity even at very high light intensities (3000–15,000 μmol photons m^{-2} s^{-1}), but the JI and IP-rise phases, that together represent the thermal phase, show a limited response to an increase of the light intensity. The data on the light intensity dependence also show that it is impossible to make a sharp separation between the photochemical and the thermal phase since the J-step saturates only at high light intensities. It can also not be excluded that there is some kinetic overlap between the photochemical and the thermal phases. In Fig. 1B, the light intensity dependence of the OJIP-transients is shown in the 300 to 3000 μmol photons m^{-2} s^{-1} range. The figure illustrates that changing the light intensity does not shift the steps J and I to longer or shorter times, confirming the observation of Delosme [16] for the J-step. Instead, the J-step loses amplitude as the light intensity is decreased whereas the IP-rise gains amplitude. The shift of the steps to either shorter or longer times would have been typical for a photochemical rise phase, whereas the fixed position of the steps in time accompanied by changes in the amplitude is typical for rate limiting steps. For example, the exchange rate of PQH_2 for PQ at the Q_B-site does not depend on the light intensity. On the basis of kinetic considerations and measurements carried out on leaves treated with either dibromothymoquinone (DBMIB) or methylviologen it was proposed that the J-step is a reflection of the exchange of a reduced PQ-molecule for an oxidized one at the Q_B-site and that the I-step reflects the rate limitation imposed by the re-oxidation of plastoquinol molecules at the cyt b_6/f-complex [23]. In Fig. 1C OJIP-transients (double normalized between O and P) measured at 5, 10 and 20 °C are shown. Lowering the temperature increases the amplitude of the OJ phase (probably due to a slowdown of the reactions at the acceptor side of PSII), and slows the JI-rise down to some extent, whereas the IP-rise is strongly decelerated. The initial part of the OJ-rise remained completely unaffected by the temperature between 5 and 20 °C, confirming its photochemical nature.

3.2. The fluorescence rise in the presence of DCMU

For a further characterization of the thermal phase, first a simpler system will be treated. In samples inhibited with DCMU, the same or a similar process that is responsible for the fluorescence rise of the thermal phase has been suggested to occur [18,19,34].

Strasser and coworkers [35,36] have suggested that the fluorescence rise kinetics in the presence of DCMU are similar to the rise kinetics of the OJ-rise. In Fig. 2 a detailed comparison is made of the fluorescence rise of untreated and DCMU-treated leaves. Fig. 2A shows that in the presence of DCMU more time is needed to reach the F_M-level than the J-step in the absence of DCMU. This difference in the rise time is already observed after 1–2 ms of illumination. In Fig. 2B a comparison is made of the initial kinetics of the fluorescence rise of untreated and DCMU-treated leaves at three different light intensities. To allow a meaningful comparison of the kinetics a linear time scale was used. The initial rise kinetics during the first 100–150 μs of illumination are the same in both cases. Then, the fluorescence rise in the presence of DCMU accelerates (the curve becomes sigmoidal), whereas this acceleration is absent without DCMU. This pattern is observed for all three light intensities shown. Fig. 2C shows that the

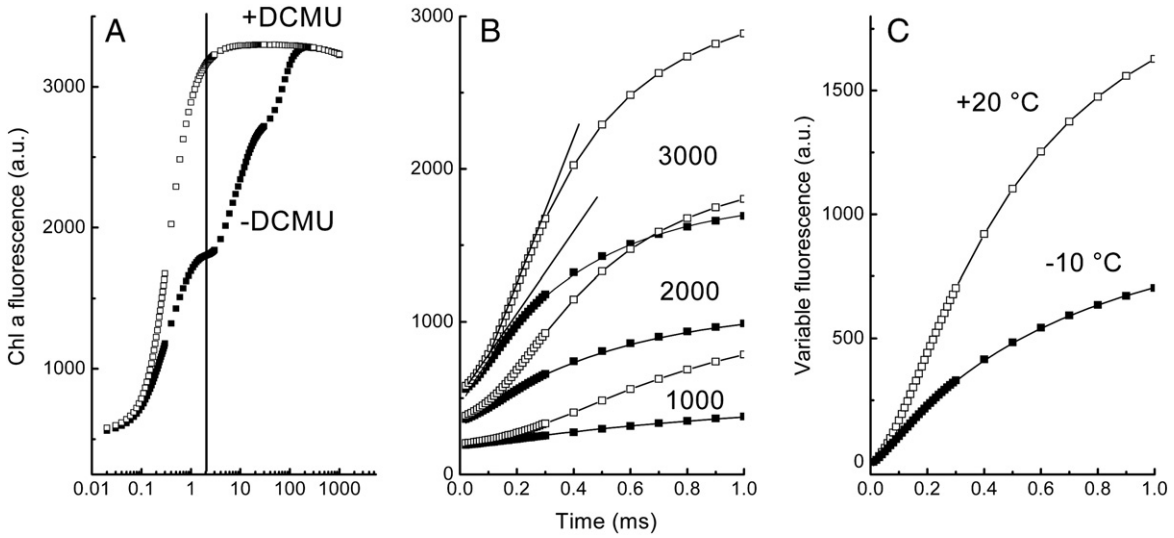

Fig. 2. Comparison of fluorescence induction curves measured at room temperature on control or DCMU-inhibited pea leaves. In panel A, the entire transients are compared (on a logarithmic time scale) and in panel B, the initial fluorescence rise (1 ms) on a linear time scale at three different light intensities (in μmol photons m^{-2} s^{-1}). In panel C, a comparison is made between the initial fluorescence rise at 20 and −10 °C induced by 3500 μmol photons m^{-2} s^{-1}; in both cases DCMU-inhibited pea leaves were measured. The untreated leaves (closed symbols) in panel B were fitted with a single exponential and in panel C, the low temperature measurement was fitted with two exponentials. In panel B, two tangent lines to the transients measured at 3000 μmol photons m^{-2} s^{-1} were drawn to illustrate the acceleration of the fluorescence transients measured on DCMU-inhibited leaves.

sigmoidicity of the fluorescence in the presence of DCMU also disappears if the temperature is lowered, for example to −10 °C. To confirm the claim that the OJ-phase of the fluorescence transients in panel 2B were approximately exponential, the transients measured at the three light intensities were fitted with a single exponential function. The transient measured at −10 °C in panel 2C was fitted with two exponentials. The obtained fits are shown in Fig. 2B and C as well. In both cases the exponential fits approximated the measured transients quite well.

3.3. Temperature dependence of the fluorescence rise of DCMU-inhibited pea leaves and PSII-membranes

The temperature dependence of the fluorescence kinetics of DCMU-inhibited pea leaves and PSII-membranes was studied in Figs. 3 (induction) and 4 (relaxation). If the process associated with the thermal phase would represent a process other than a redox reaction, the temperature dependence of the induction and relaxation kinetics of the photochemical phase could differ quite strongly from the induction and relaxation of the thermal phase. The presence of DCMU allowed us to avoid complications due to the inhibition by low temperature of e.g. Q_A to Q_B electron transport below −30 °C [9]. Fig. 3 shows that below −20 °C a strong slowdown of the fluorescence rise as a function of the temperature is occurring. This is associated with the appearance of a slow induction phase at temperatures below −20 °C (Fig. 3B). In Fig. 3C the effect of a saturating single turnover pre-flash on the fluorescence rise induced by a strong pulse of light of a leaf frozen to −80 °C is shown. The three curves were fitted with the sum of 3 exponentials. Without a pre-flash the amplitudes of the three phases were: A1 = 19.6% (τ = 0.2 ms),

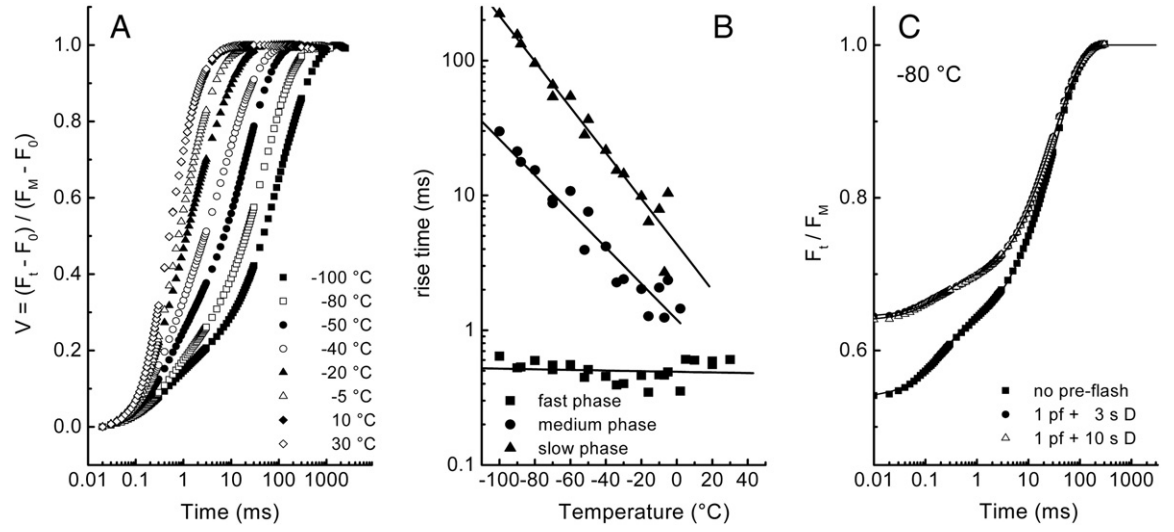

Fig. 3. Temperature dependence of fluorescence induction transients measured on DCMU-poisoned pea leaves. In panel A, examples of fluorescence transients measured at different temperatures and double normalized between O and P are shown and in panel B, the apparent rise times were determined for the 3 rise components of fluorescence induction transients like those in panel A. In panel C, the effect of a pre-flash on the fluorescence rise measured at −80 °C, normalized to the maximum fluorescence intensity, after a 3 or 10 s dark interval is shown. The light intensity was in all cases 3500 μmol photons m^{-2} s^{-1}.

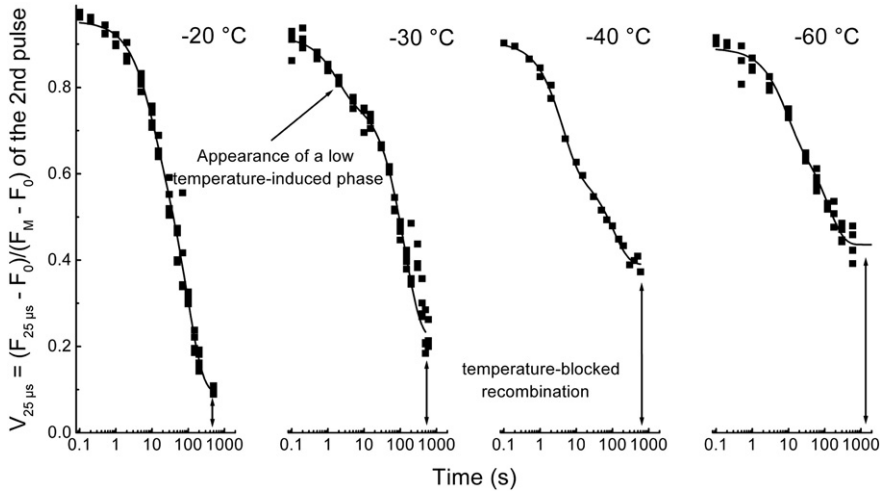

Fig. 4. Dark-relaxation kinetics of the initial fluorescence intensity ($V_{25\ \mu s}$) of PSII-membranes following a saturating pulse at low temperatures. The vertical arrows indicate the part of the initial fluorescence intensity that did not relax within 15 min. The light intensity was in all cases 3500 μmol photons $m^{-2}\ s^{-1}$ and the pulse lengths were 80 ms (−20 °C), 120 ms (−30°), 200 ms (−40 °C), and 300 ms (−60 °C).

A2 = 17. 2% (τ = 7 ms) and A3 = 63.2% (τ = 41 ms). If the fluorescence transient was measured 3 and 10 s after the pre-flash, respectively, the amplitudes were A1 = 9.4/10.2% (τ = 0.2/0.2 ms), A2 = 17.3/17.4% (τ = 9/9 ms) and A3 = 50.7/49.3% (τ = 44/45 ms). Thus, the pre-flash, reduced the amplitude of the fast and the slow phases. The amplitude of the medium phase remained nearly unchanged. In Fig. 3B, the rise times of the three fluorescence induction phases are shown as a function of the temperature. The rise time of the fast phase was nearly temperature independent, which suggests that it represents the photochemical phase. For this phase an apparent activation energy of 2 kJ/mol was calculated. The other two phases were strongly temperature dependent (apparent activation energies of 13 and 16 kJ/mol). The rise time of the intermediate phase was slowed down from ~2.4 to ~15 ms as the temperature was decreased from −30 to −80 °C. In the same temperature range the slowest rise phase slowed down from ~14 s to ~95 ms. It is expected that the process associated with the thermal phase will be the least affected by a single pre-flash and for that reason the medium phase, that remained unaffected by a pre-flash, was ascribed to the thermal phase.

The fluorescence relaxation kinetics of DCMU-poisoned PSII-membranes following a saturating pulse were determined at low temperature as well (Fig. 4). The initial fluorescence value of the second pulse ($F_{25\ \mu s}$) of a double pulse experiment was determined as a function of the dark-interval at four different temperatures. On lowering the temperature from −20 °C to −30 °C a new faster relaxation phase became visible. The amplitude of this phase increased as the temperature was lowered further and, in addition, the relaxation kinetics of this phase slowed down as the temperature was lowered from −30 to −60 °C. In the second place, the slow phase that was still observed at −20 °C gradually disappeared and instead the relative fluorescence level towards which the fluorescence declined, increased. This increase leveled off around a value of 0.44. Compared with [37] where a 40 min halftime for the decay of the S_2-multiline was observed at −20 °C and a nearly stable S_2-multiline at −30 °C, this stabilization was shifted by approximately 10 °C towards lower temperatures in Fig. 4. This difference could be due to presence of 50% glycerol in the samples of [37], which was absent in our samples. Finally, the fast phase at −20 °C which turned into the intermediate phase at temperatures ≤ −30 °C slowed down as well as the temperature was lowered. The appearance of a new kinetic phase at −30 °C can be explained by the assumption that charge stabilization occurs no longer in all reaction centers due to temperature induced changes on the donor side of PSII [37,38]. This would also explain the observation that a preflash reduces the amplitude of the slowest fluorescence rise phase at −80 °C. On each flash, charge stabilization will occur in a fraction of the PSII reaction centers and as a consequence the fraction of charge unstabilized reaction centers will decline. The amplitudes of the three phases of the fluorescence rise (data not shown) show that the amplitude of the medium phase increases as the temperature is lowered from +20 to −10 °C. This agrees with the idea of a kinetic overlap between the photochemical and thermal phases in the presence of DCMU at room temperature; an overlap that disappears as the temperature is lowered. Below −20 °C the amplitudes of the photochemical and thermal phases decrease as the fraction of centers in which charge stabilization did not occur increased reaching a value of more than 50% of the total amplitude below −40 °C.

3.4. Dark-recovery kinetics of the OJIP-transient in the presence and absence of DCMU following a saturating pulse

In Fig. 5A some examples of induction curves measured on DCMU-inhibited pea leaves at various times after a short saturating pulse (15 ms 3500 μmol photons $m^{-2}\ s^{-1}$) are presented. In Fig. 5B, the dark-adaptation kinetics of the initial fluorescence intensity (O) are shown (see arrow in Fig. 5A for the corresponding time points). Fitting the fluorescence relaxation kinetics with the sum of two exponentials yielded one fast phase of 72 ms representing 29% of the amplitude and a slower phase with a τ-value of 740 ms representing 71% of the amplitude. The slower phase most probably represented a charge recombination between Q_A^- and the S_2 state of the oxygen-evolving complex. Literature values for the decay time (τ) of the recombination between reaction Q_A^- and the S_2/S_3 states depend on the sample type and measuring conditions with published values of ~260 ms in untreated PSI-less *Synechocystis* PCC 6803 cells [39], ~500 ms for non-Q_B-reducing PSII reaction centers in PSII-membranes [40], ~600 ms in untreated pea leaves [23] and 1.44 s in DCMU-inhibited *Synechocystis* PCC 6803 cells [41]. The value of 72 ms is quite close to the 120 ms obtained by Dekker et al. [42] for the recombination reaction between Q_A^- and Tyr_Z^+, however, Tyr_Z^+ is quickly re-reduced by the manganese cluster and as a consequence its concentration will be very low at the end of the saturating pulse where the electron transport chain has become reduced and Q_A^- has become stably reduced as well. Other charge recombination pairs that could explain the fast phase are unknown to us.

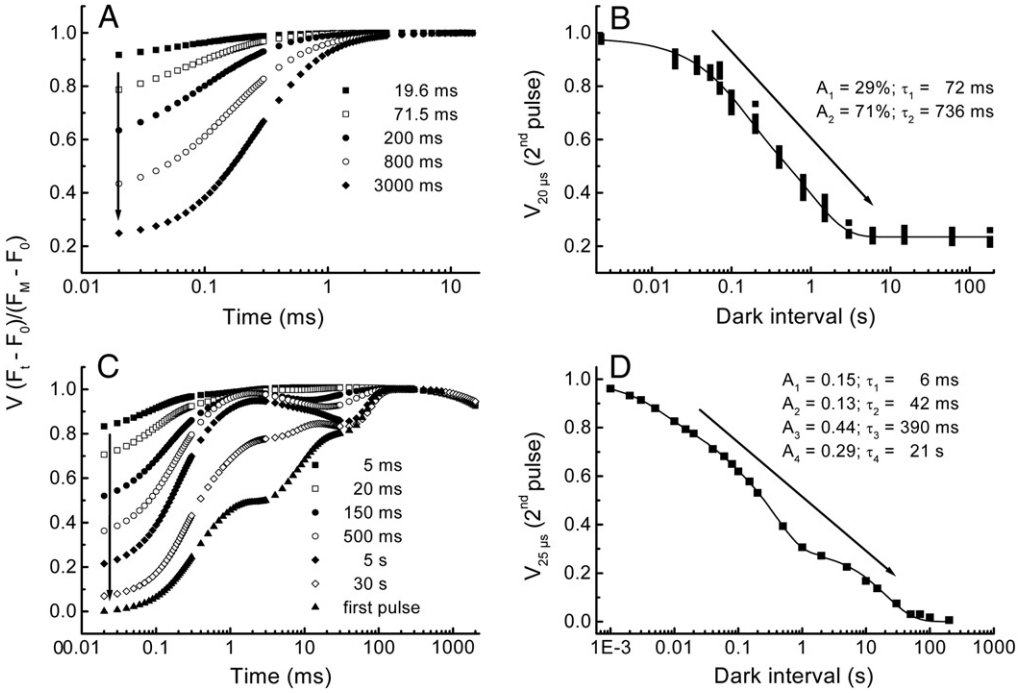

Fig. 5. Dark-recovery of the O-step measured at room temperature on pea leaves following a saturating pulse in the presence (panels A and B) and absence (panels C and D) of DCMU. In panels A and C, examples of the fluorescence transients measured at various times after a first saturating pulse of light are shown. The arrows in panels A and C indicate the measuring points used in panels B and D. The dark-recovery kinetics in panel B were fitted with the sum of two exponentials and the kinetics in panel D with the sum of four exponentials. The intensity of the pulses was 3500 µmol photons m^{-2} s^{-1} for panels A and B and 5000 µmol photons m^{-2} s^{-1} for panels C and D. Each point in panel D is an average of three independent measurements.

The dark-relaxation kinetics in the absence of DCMU are considerably more complex. In Fig. 5C examples are given of OJIP-transients measured at various times after a saturating pulse (700 ms 5000 µmol photons m^{-2} s^{-1}). The dark-relaxation kinetics of the O-level following a saturating pulse (dark intervals of 100 ms to 200 s) were studied in an earlier paper [23]. In the 100 ms to 200 s range, two relaxation phases were observed of which the fastest was assigned to the recombination of Q_A^- with the S_2 and S_3 states of the oxygen-evolving complex, whereas the slower relaxation phase was ascribed to forward electron transfer towards a re-oxidizing PQ-pool in those centers that were in the S_0 and S_1 states at the end of the saturating pulse. Due to technical limitations at that time, it was not possible to study the relaxation kinetics in the 0–100 ms range, during which ~40% of the fluorescence relaxed [23]. This amplitude corresponded quite closely to the amplitude of the JIP (or thermal) phase (Fig. 5C). In Fig. 5D the relaxation kinetics of the O-level (see arrow in Fig. 5C for the corresponding time points) between 1 ms and 200 s of darkness were studied and fitted with four exponentials. In addition to the two phases described above, two new, rapidly relaxing phases were observed with τ-values of 6 ms (15%) and 42 ms (13%). These two phases relaxed too rapidly to be ascribed to either Q_A^- reoxidation by charge recombination within PSII, or forward electron transport (in the presence of a reduced PQ pool). The two other phases had τ-values of 390 ms and 21 s, which were in agreement with our earlier results [23]. Based on the similar relaxation times and amplitudes (cf. fast phase of Fig. 5B and the two fast phases of Fig. 5D), we assume that the rapidly relaxing phase in the presence of DCMU corresponds to the relaxation of the process associated with the thermal phase detected in the absence of DCMU.

3.5. Delayed fluorescence

To discriminate between the concepts of Duysens and Sweers [13] on the one hand and Delosme [16] on the other, it is critical to understand at what time Q_A is reduced. Schreiber [43] and Strasser and Strasser [35] have claimed that the OJ-rise represents a single charge separation. Delayed fluorescence (DF) can be used to test this. DF is a reflection of charge recombinations between the acceptor and the donor side of PSII (see e.g. [44])—i.e. it can only occur after a stable charge separation has taken place. The DF measured following a pulse of light of a certain length (1–200 ms) is dominated by a decay component with a lifetime of ~40 µs (data not shown). This component is attributed to a recombination between Q_A^- and $P680^+$ [45–48]. In [48] it was shown that the DF-intensity due to the recombination between Q_A^- and $P680^+$ is S-state dependent and is maximal during the S_3-to-S_4-to-S_0 transition. In Fig. 6A OJIP-transients representative for the measurements in Fig. 6B are shown. In Fig. 6B the amplitude of the fast decay phase of the DF is shown as a function of the pulse length. The DF-intensity depends on the light intensity of the pulse. A 4.3-fold decrease of the pulse light intensity yields a ~2.6-fold decrease of the maximum DF-intensity measured. At 5000 µmol photons m^{-2} s^{-1}, the maximum amplitude of the fast decay phase of the DF-signal was observed following a 4 ms light pulse. This peak was shifted to 10 ms when the light intensity was decreased to 1150 µmol photons m^{-2} s^{-1}. The fact that the $P680^+$ concentration is at its maximum during the S_3-to-S_4-to-S_0 transition of the oxygen evolving complex [48] suggests that for the majority of PSII reaction centers 4 ms of illumination with 5000 µmol photons m^{-2} s^{-1} equated 3 charge separations. Fig. 6B demonstrates that the peak value of the amplitude of the fast DF-decay component is followed by a strong decline. The recombination rate during the fluorescence rise depends on the presence of $P680^+$. Due to its short lifetime, the $P680^+$-concentration depends strongly on a continuous generation of $P680^+$ which in turn depends on a turnover of PSII. The decrease of the DF-signal suggests that the turnover of PSII decreases strongly during the J-to-I-to-P fluorescence rise and as demonstrated in Fig. 6C the decrease of the DF-intensity is approximately inversely proportional to the fluorescence rise during the thermal phase. The observed relationship implies that the number of charge separations is inversely proportional to JIP-fluorescence rise and, therefore, to the reduction/oxidation rate of Q_A as well.

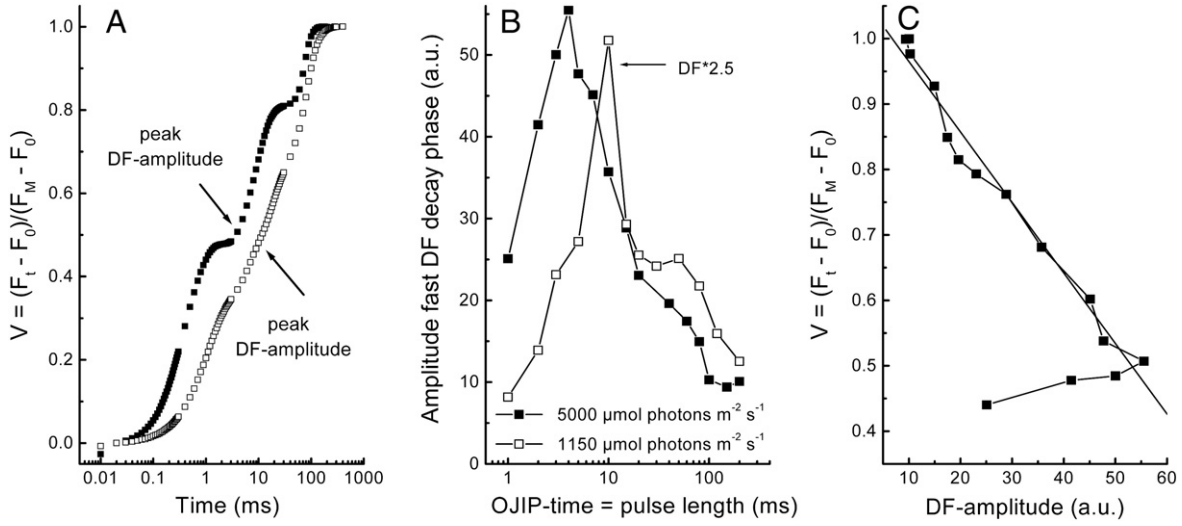

Fig. 6. Dependence of the amplitude of the fast (µs) decay phase of the delayed fluorescence (DF) on the length of a light pulse (1–200 ms) of 1150 and 5000 µmol photons $m^{-2}\,s^{-1}$, respectively. In panel A, representative OJIP-transients for the DF-measurements in panel B are shown; in panel B, the dependence of the amplitude of the fast DF decay phase on the length of the excitation pulse; and in panel C, the relationship between the DF and the (prompt) fluorescence intensity elicited in both cases by 5000 µmol photons $m^{-2}\,s^{-1}$ pulses. Every point in panel B is an average of 4 independent measurements.

3.6. The properties of the 'thermal phase'

In Fig. 7 some properties of the generation and relaxation of the thermal phase are summarized. On illuminating a dark-adapted leaf with a strong pulse of red light, approx. 200 ms were needed to reach the maximum fluorescence intensity (Fig. 7A) and this fluorescence rise followed the reduction of the electron transport chain closely [23]. During a subsequent dark interval of 80–100 ms the process associated with the thermal phase relaxed, whereas the re-oxidation of Q_A^- was still very limited (see Fig. 5D). The process associated with the thermal phase could then be regenerated within 2–3 ms by a second strong pulse of light (Fig. 7B). The experiment shown in Fig. 7 provides us with a method to study the properties of the thermal phase. Varying the light intensity of the second pulse of light, the light intensity dependence of the process associated with the thermal phase can be studied (Fig. 8). Fig. 8 demonstrates that the induction of this process is indeed light intensity dependent. It was further observed that the light intensity of the first pulse did not have an effect on the rise time of the fluorescence induced by the second pulse of light as long as the light intensity of this pulse was saturating (data not shown). The fluorescence rise induced by the second pulse of light could be described by the sum of two exponentials whose τ-values exponentially decreased as the light intensity of the pulses was increased (Fig. 8B). The amplitude of the slow phase decreased and the amplitude of the fast phase increased as the light intensity was increased (data not shown).

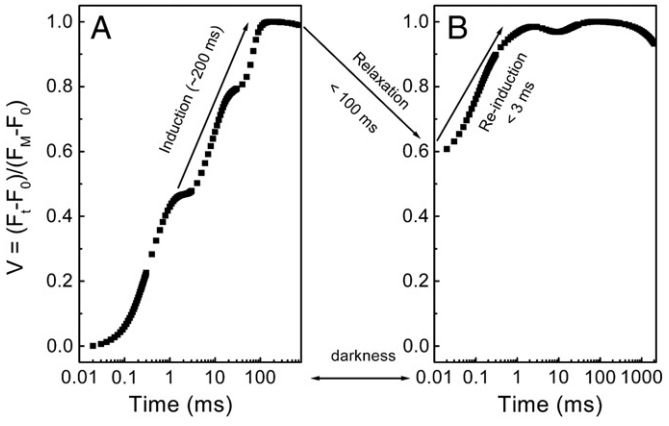

Fig. 7. Summary of the induction and relaxation kinetics of the process associated with the thermal phase. The light intensity of the two light pulses was 5000 µmol photons $m^{-2}\,s^{-1}$.

4. Discussion

4.1. Arguments against a purely Q_A-based interpretation of the fluorescence rise

Looking at the data of the present study and the literature a number of problems with the single-'quencher'-concept can be listed:

1. A saturating single turnover flash cannot generate more than 60–65% of the maximum fluorescence intensity [8,20]. This observation applies as well to continuous light where 15,000 µmol photons $m^{-2}\,s^{-1}$ induces a J-step that is only 60–65% of the F_M-value (Fig. 1).
2. In untreated leaves electron flow through PSI has a kinetic effect on the Chl *a* fluorescence rise although there are two rate limiting steps between Q_A and PSI. Or to formulate it differently, a reduced electron transport chain is a pre-condition for reaching the F_M [23].
3. Although high light intensities reduce the contribution of the IP-phase (electron transport through PSI) to the fluorescence rise, 15,000 µmol photons $m^{-2}\,s^{-1}$ is not sufficient to eliminate the IP-phase (Fig. 1, [24]).
4. The J-step does not represent a single charge separation, but 2–3 charge separations (Fig. 6, and compare the simulations of the OJIP transients in [36,47,49]) and the J and I steps behave as expected for a rate limitations in the sense that they do not change position in response to changes in the light intensity (Figs. 1A and B).
5. The relaxation kinetics of the thermal phase, occurring within 100 ms, cannot be explained by any known redox reaction involving the re-oxidation of Q_A^- (Fig. 5).
6. In samples pre-treated with DCMU and hydroxylamine the halftime for the re-oxidation of Q_A^- is greater than 20 min at room temperature [19]. Under these conditions there is a partial relaxation of the fluorescence intensity unaccompanied by changes

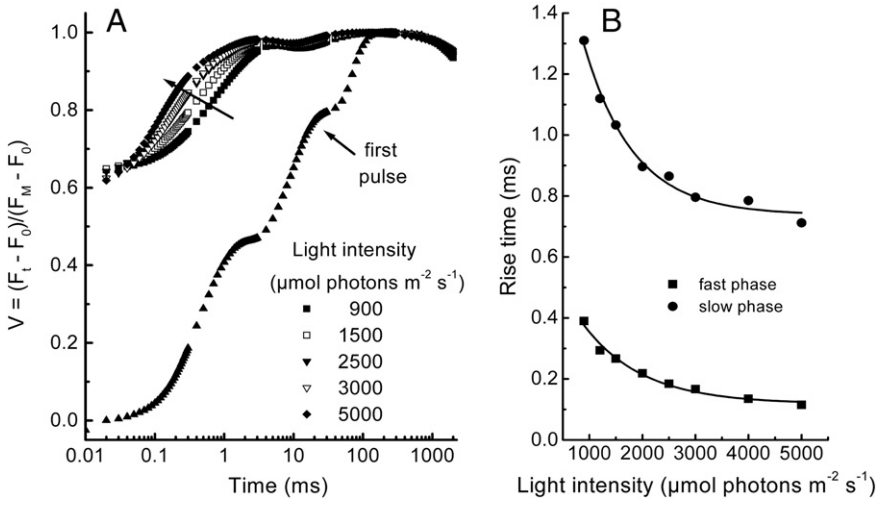

Fig. 8. Regeneration of the thermal phase as studied by a double pulse experiments in which a first pulse of 700 ms duration and 5000 μmol photons m^{-2} s^{-1} was followed by a second pulse of 2 s duration, 80 ms later. The intensity of the second pulse was varied between 900 and 5000 μmol photons m^{-2} s^{-1} (panel A) and the first 3 ms of the fluorescence rise induced by the second pulse was fitted with the sum of two exponentials of which the light intensity dependence of the τ-values is shown in panel B. Each transient in panel A represents the average of four independent measurements.

in the Q_A-redox state [18,19] implying as well that Q_A^- is not easily oxidized by oxygen.

7. There is relaxation of the fluorescence intensity (Fig. 4) at temperatures where the recombination between Q_A^- and the S_2 state is known to be completely inhibited [37].
8. The kinetics of the fluorescence rise at low temperatures do not agree with a photochemical reaction leading to the reduction of Q_A, even if there would be PSII heterogeneity (Fig. 3).

The observations listed above show that there is a growing number of problems with an interpretation of the fluorescence kinetics based on a pure Q_A-model. In the rest of the Discussion section an alternative interpretation that is more consistent with the observations is proposed.

4.2. DCMU and the 'thermal phase'

In [35,43] it has been argued that the J-step represents a single charge separation based on the observation that the fluorescence rise in the presence of DCMU and the OJ-rise in the absence of DCMU have similar rise kinetics. As demonstrated in Fig. 2, the fluorescence rise kinetics of control and DCMU-inhibited leaves differ considerably. In the presence of DCMU an acceleration of the fluorescence rise is observed after 150 μs of illumination that is missing in untreated leaves. This sigmoidicity in the fluorescence rise has traditionally been ascribed to connectivity between PSII antennae [12,50]. Vredenberg [51] showed that it is also possible to get a sigmoidal fluorescence rise with two kinetically overlapping exponential functions. That this may be an explanation for the fluorescence rise in the presence of DCMU is also suggested by Fig. 2C where it is shown that the sigmoidicity is lost on lowering the temperature to −10 °C indicating that the lowering of the temperature causes a kinetic separation of the two phases.

A feature observed in Fig. 2A is the relative slowness of the fluorescence rise in the presence of DCMU. The F_M is reached at times longer than the J-step, even though the J-step represents 2–3 charge separations (Fig. 6) and the F_M in the presence of DCMU only 1. This surprising observation can be explained if we assume that the fluorescence rise in the presence of DCMU equates one charge separation plus the induction of the process associated with the thermal phase (Fig. 5), whereas in untreated leaves the process associated with the thermal phase is mainly induced during the JIP-rise. The data presented in Fig. 2 suggest that upon reduction of Q_A the process associated with the thermal phase can be induced quickly (see also Figs. 7 and 8). The fact that this does not lead to a rapid rise of the fluorescence intensity to F_M in the absence of DCMU suggests that the process associated with the thermal phase may be induced continuously, but probably relaxes immediately on re-oxidation of Q_A (some support for this is found in [52], which deals with twin-flash excitation of thylakoid membranes). The turnover of Q_A will only stop once the electron transport chain is reduced and, therefore, a full induction of the process associated with the thermal phase will only occur once the electron transport chain becomes fully reduced. In the presence of DCMU one charge separation is enough to stably reduce Q_A and the process associated with the thermal phase can be induced immediately. Therefore, in the presence of DCMU the induction of the state associated with the thermal phase can occur in PSII reaction centers with a reduced Q_A in parallel with the reduction of Q_A in the reaction centers that had not yet been excited by a photon. This overlap explains the observation that the fluorescence rise in the presence of DCMU has a thermal phase of only 10–15% [20], whereas the relaxation kinetics indicate that it represents 30% of the fluorescence rise (Fig. 5B). Fig. 2 demonstrates that the acceleration point shifts to shorter times as the light intensity is increased. This light intensity dependence implies that it cannot be due to the release of donor side quenching as suggested in [51] because the electron transport reactions on the donor side do not depend on the light intensity. The fact that the acceleration/sigmoidicity is no longer observed at −10 °C is then due to a temperature dependent kinetic separation between the reduction of Q_A and the subsequent generation of the thermal phase. This idea finds support in the observation that the apparent activation energies of the photochemical and thermal phases are quite different (2 versus 13 kJ/mol) (Fig. 4).

In other words, to explain the observations presented here, the introduction of a second process that depends for its induction on the reduction of Q_A is needed.

4.3. The JIP-rise, the 'thermal phase' and the underlying process

On introducing the concept of the photochemical and the thermal phases, Delosme [16] assumed that at high light intensities both phases could be separated completely. However, as shown here, this is a simplification. At light intensities of 3000–5000 μmol photons m^{-2} s^{-1} the J-step is not yet saturated which means that there is an overlap between the photochemical phase and the thermal phase

under many conditions, nor can it be excluded that already some thermal phase is induced in parallel to the photochemical phase. This is something that occurs in DCMU-inhibited samples (Fig. 2). $P680^+$ is a known fluorescence quencher [53,54]. There is little $P680^+$ at F_0 or F_M, but around the J-step, there is a relatively high concentration of the S_4-state of the oxygen-evolving complex with concomitant high levels of $P680^+$ (Fig. 6 and [47]). High light intensities keep the reaction centers synchronized during the first few charge separations and as a consequence the S_4-state is generated in most PSII reaction centers within a narrow time window and it is likely that the dip around the J-step observed at high light intensities (Fig. 1) is due to quenching by $P680^+$. On the basis of Fig. 5 (70% photochemical phase and 30% thermal phase) one would expect the J-step to occur around 70% of the F_M-value. A combination of non-saturation at lower light intensities and $P680^+$-quenching at high light intensities can explain the discrepancy between the expected and the measured fluorescence intensity at the J-step. It is also important to point out that the process associated with the thermal phase is photochemical in nature. The induction of the process associated with the thermal phase depends on the reduction of the electron transport chain, which is determined by dark reactions that are temperature dependent. However, once the electron transport chain is in the reduced state the process associated with the thermal phase can relax and be re-induced quickly in a light-dependent way (Figs. 7 and 8).

4.4. Low temperature kinetics

A complication with respect to the study of the process associated with the thermal phase at low temperature was that between 0 and −20 °C a new fluorescence rise phase showed up that also appeared around −30 °C in the fluorescence relaxation kinetics. This phase we ascribe to the occurrence at low temperatures of unstabilized charge separations in agreement with [37,38]. As described in the Results section we assigned the medium fluorescence rise phase to the process associated with the thermal phase and the slow rise phase to centers in which charge stabilization was slow, based on the analysis of the effect of a pre-flash on the fluorescence rise at −80 °C. On lowering the temperature from +20 to −10 °C the amplitude of the fluorescence rise phase determined by the process associated with the thermal phase increased from 10 to 15% of the fluorescence amplitude at +20 °C to ~40% around −10 °C with a concomitant decrease of the amplitude of the phase ascribed to the photochemical phase (data not shown). This observation confirmed the idea that there is kinetic overlap between the photochemical and thermal phase in the presence of DCMU at room temperature. The lack of charge stabilization in an increasing number of reaction centers at temperatures below −20 °C introduces a practical problem. At −20 °C the relaxation of the thermal phase is 100-fold slower than at room temperature (τ ~ 9.3 s), but this is still relatively fast. Lowering the temperature further leads to a further slowdown of the relaxation of the thermal phase, but at the same time its generation becomes more and more incomplete.

Below −20 to −30 °C the charge stabilization process becomes less efficient [37,38]. This means that in a fraction of reaction centers one excitation is still enough to induce a stable charge separation whereas in others several excitations are needed. It adds not only a new kinetic phase (Figs. 3 and 4), it also causes kinetic overlap between the induction of the different processes. This made it more difficult to judge if the ratio between the fraction of the fluorescence due to the reduction of Q_A (photochemical phase) and fraction of the fluorescence rise due to the process associated with the thermal phase changed on lowering the temperature. The experiments in Fig. 4 are best suited to address this question since the relaxation kinetics of the three phases are different allowing a kinetic separation and, in addition, at the end of the saturating first pulse the PSII reaction centers have been excited many times reducing the contribution of the unstabilized charge separations to the fluorescence rise (25% instead of more than 50%). The fraction of the fluorescence intensity that does not relax within 15 min seems to saturate at ~0.44 around −50/−60 °C. However, this fraction is an underestimation of the potential contribution of the photochemical phase to the fluorescence rise due to the fact that there is still the fraction of centers with an unstabilized charge separation to consider. If we would distribute the ~25% of this fraction 70:30 between the fluorescence rise due to the reduction of Q_A and the fluorescence rise due to the process associated with the thermal phase, the contribution of the photochemical phase to the fluorescence rise would be ~62% which is not far from the 70% we observed at room temperature in Fig. 5B and D. This result contrasts strongly with the conclusion of Moise and Moya [55] that the thermal phase was completely suppressed below −50 °C. Neubauer and Schreiber [9] also concluded that the thermal rise phase was suppressed at low temperatures (below −35 °C), however in their case the authors observed that the JIP-rise disappeared below −35 °C. This is due to the inhibition of the electron transfer from Q_A to Q_B and as we have shown here, this is unrelated to the process associated with the thermal phase.

Based on Fig. 5 and the argument developed above we can conclude that neither the presence of DCMU nor freezing of the sample to e.g. −60 °C affect the relative contribution of the thermal phase to the fluorescence rise.

4.5. An alternative model: Q_A-reduction + a fluorescence yield change caused by a light-induced conformational change

Based on the eight observations made above it can be argued—we think convincingly—that the concept of Duysens and Sweers [13]—i.e. the single-'quencher'-concept—is insufficient to explain the fluorescence rise. But what is then the process associated with the thermal phase? In general, changes in the fluorescence intensity can be due to either of two processes: photochemical quenching (associated with the redox chemistry of the photosynthetic electron transport chain (mainly Q_A), which changes the rate constant k_P) and non-photochemical quenching that is related to fluorescence yield changes and changes the rate constant k_F. Although oxidized PQ-molecules lead to a strong non-photochemical quenching of Chl *a* fluorescence in PSII-membranes [56], we will not consider it here, because the measurements on PSII-membranes were all carried out in the presence of DCMU, minimizing changes in the PQ-redox state; and in leaves PQ-pool quenching does not occur [57]. This was confirmed by Figs. 7 and 8 where it is shown that the fluorescence relaxation and regeneration associated with the process studied here take place on a time-scale where no changes in the PQ-redox state occur (τ-value of re-oxidation of the PQ-pool is 60–70 s in pea leaves [23,58]). All other forms of non-photochemical quenching like energy-dependent quenching (qE) are related to structural changes of PSII: low lumenal pH induced changes (e.g. [59]) and conformational changes in the light harvesting complexes (e.g. [60–62]). We have shown here that the thermal phase has approximately the same amplitude in the absence as well as presence of DCMU. As argued in the previous paragraph, the data further indicate that the ratio between the photochemical and thermal phase does not change significantly as the temperature is lowered. It is still possible to induce stable charge separations at −80 °C, but below approx. −40 °C the recombination reaction is blocked [37], see also Fig. 5). However, as shown by Fig. 5 the thermal phase can still relax at −60 °C although the relaxation kinetics are more than 1000-fold slower than at room temperature. The block of the recombination reaction between Q_A^- and the S_2-state can be explained if we assume that it is due to a complete shift of the equilibrium between the S_2-state and P680 towards P680 (i.e. the electron is 100% of the time localized on P680). In the absence of $P680^+$ no re-combination reaction can take place. This also implies that the thermal phase cannot be due to a second unknown redox

factor on the acceptor side of PSII (its recombination with the acceptor side would also be blocked). At the same time a redox factor on the donor side transferring an electron to the S_2 state would not lead to the emission of fluorescence. On this basis we can exclude a redox reaction as an explanation for the generation and relaxation of the process associated with the thermal phase. As we argued above if the thermal phase is not a form of photochemical quenching then it would probably be due to a fluorescence yield change induced by a conformational change. Charge separations induce strong electric fields and these fields can be seen as a potential driving force of such a conformational change [63]. Studying the relationship between the fluorescence yield Φ and the fluorescence lifetime Moise and Moya [55] also concluded that the thermal phase represented a conformational change. They localized this conformational change tentatively in the PSII core antennae. However, to what extent the phenomena described in their paper are identical to the ones studied here is difficult to judge.

Light-induced conformational changes have also been described in bacteriorhodopsin (e.g. [64,65]) and bacterial reaction centers (e.g. [26–29]). A link between such conformational changes and the fluorescence yield has been made for bacterial reaction centers of *Rhodobacter sphaeroides* [30].

At this point it is still necessary to explain the link between a conformational change and a fluorescence yield change. Obvious targets would in this respect be the couple P680/Pheo and their environments. Other studies have shown that changes in the environment of either of these two redox factors (e.g. mutations of amino acids) can affect thermoluminescence yield [66,67]. A study on the reaction centers of *R. sphaeroides* suggests that in these organisms changes in the environment of P680 can affect the fluorescence yield [68].

4.6. The conformational change and room temperature measurements

At room temperature, in the absence of DCMU, the situation is somewhat more complicated because under such conditions the reduction of the electron transport chain is a precondition for the generation of the thermal phase. In [24] it was observed that there is a kinetic effect of electron flow through PSI on the fluorescence rise kinetics (the IP-phase) despite the fact that there are two rate limitations between Q_A and PSI. Vasil'ev and Bruce ([21], and references therein) have suggested that the thermal phase could be explained if it was assumed that the occupancy state of the Q_B-site would modulate the fluorescence yield. If this were the case, it could explain the kinetic effect of electron transport through PSI on the fluorescence rise, since the occupancy state of the Q_B-site depends on the redox state of the PQ-pool which in turn is modulated by electron flow towards PSI. There are two problems with this idea though: (1) it is not clear how the occupancy state of the Q_B-site could affect the fluorescence yield, but more importantly (2), as we have shown here (Figs. 7 and 8), the process associated with the thermal phase can relax and be regenerated in the absence of changes of the redox state of the PQ-pool (which also excludes PQ-pool quenching as an explanation). However, there is another aspect that was not considered before. The occupancy state of the Q_B-site has also an effect on Q_A. The re-oxidation kinetics of Q_A^- depend on the occupancy state of the Q_B-site and once the electron transport chain becomes stably reduced, Q_A becomes completely and stably reduced as well. In other words, the occupancy state of the Q_B-site modulates the stability of the reduction of Q_A. Fig. 1A shows that even at 15,000 μmol photons (~1 excitation every 40 μs) 2 ms of illumination is not enough to reach F_M, although this illumination time should be more than enough to reduce nearly all Q_A. The reduction of the electron transport chain that remains a pre-condition for reaching the F_M will mean that initially the Q_A molecules in all reaction centers will become oxidized and reduced again every few ms. In other words, even when nearly all Q_A is reduced, this is a dynamic state with a turnover of Q_A every few ms. Above we argued that the process associated with the thermal phase could very well represent a fluorescence yield change due to a light-driven conformational change. To explain the room temperature data on this basis we have to assume that a precondition for the generation of the conformational change is a reduced Q_A and, in addition, we have to assume as well that the conformational change will relax immediately on re-oxidation of Q_A. This way we can understand that the induction of the conformational change in all reaction centers depends on the flow of electrons through the whole electron transport chain of which the DF-intensity is an indicator (Fig. 6C). The data in Fig. 5 further suggest that light is needed to maintain the conformational change. In darkness, in the presence of Q_A^-, the conformational change will relax within 100 ms. But the data in Fig. 7 suggest as well that on re-illuminating the leaf the conformational change can be regenerated within a few ms if the electron transport chain is still reduced. An important point to make here is that the process associated with the thermal phase has a photochemical nature, but since its generation depends on the reduction of the photosynthetic electron transport chain, which is determined by several rate limiting dark reactions that are sensitive to temperature, it has been thought of as a 'thermal' process in the literature. Although fluorescence measurements of untreated and DCMU-poisoned leaves have often been treated separately, our data suggest that the effect of the conformational change on the fluorescence yield is very similar in both cases. There is a kinetic difference, though: in the absence of DCMU the generation/relaxation of the conformational change is a biphasic process, whereas in the presence of DCMU only one phase is detected and in addition, the conformational change relaxes with slower kinetics in the presence of DCMU (Fig. 5).

As we have shown here, the key to understanding the induction of the conformational change is the stability of the reduction of Q_A. In the presence of DCMU a single charge separation is enough to induce a stable reduction of Q_A and therefore the F_M can be reached within a few ms. In the absence of DCMU the reduction of the whole electron transport chain is a pre-condition for a stable reduction of Q_A and therefore the F_M is reached only after 200 ms or more, even at very high light intensities.

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