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Biochimica et Biophysica Acta 1757 (2006) 173 – 181
BIOCHIMICA ET BIOPHYSICA ACTA
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The chlorophyll *a* fluorescence induction pattern in chloroplasts upon repetitive single turnover excitations: Accumulation and function of Q_B-nonreducing centers

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Received 20 June 2005; received in revised form 23 January 2006; accepted 9 February 2006
Available online 10 March 2006

Abstract

The increase of chlorophyll fluorescence yield in chloroplasts in a 12.5 Hz train of saturating single turnover flashes and the kinetics of fluorescence yield decay after the last flash have been analyzed. The approximate twofold increase in F_m relative to F_o, reached after 30–40 flashes, is associated with a proportional change in the slow (1–20 s) component of the multiphasic decay. This component reflects the accumulation of a sizeable fraction of Q_B-nonreducing centers. It is hypothesized that the generation of these centers occurs in association with proton transport across the thylakoid membrane. The data are quantitatively consistent with a model in which the fluorescence quenching of Q_B-nonreducing centers is reversibly released after second excitation and electron trapping on the acceptor side of Photosystem II.

Keywords: Chlorophyll *a*; Fluorescence quenching; Photosystem 2; Three state trapping mechanism (TSTM); Non-QB-reducing center; Single turnover excitation

Abbreviations: β, fraction of PSUs with Q_B-nonreducing RCs; DCMU, 3(3,4-dichlorophenyl)-1,1-dimethylurea; FCCP, carbonyl cyanide p-trofluoromethoxyphenylhydrazone; $F(t)$, fluorescence level at time t; F_m, maximum fluorescence in each STF in flash train; F_o, fluorescence level of system with 100% open PSUs in dark-adapted state; F_v, variable fluorescence at each STF in a 12.5 Hz flash train with $F_v = F_m - F_o$; ΔF, fluorescence increase in STF in 12.5 Hz flash train; ΔF_o, increase in fluorescence level at onset of STF in 12.5 Hz flash train with $\Delta F_o = F_m - \Delta F$; ΔF^{Qa}, fluorescence increase in STF associated with release of Q_A-quenching; ΔF^{Phe}, fluorescence increase in STF associated with release of Phe-quenching; ΔF^{nQb}, fluorescence increase in STF associated with release of quenching in Q_B-nonreducing RCs; F^{sc}, fluorescence of semi-closed (-open) Q_B-reducing RCs; F^{sc}_{nQb}, fluorescence of semi-closed (-open) Q_B-nonreducing RCs; F^{c}_{nQb}, fluorescence of closed Q_B-nonreducing RCs; k_{-1}, rate constant of radical pair recombination; $k_{AB1,2}$, rate constant of Q_A^- oxidation by Q_B and Q_B^-, respectively; $k_{1,2}$, rate constant of reoxidation of $[PheQ_A]^{2-}$ to $[PheQ_A]^-$ in Q_B-nonreducing RCs; $k_{nB1,2}$, rate constant of reoxidation $[PheQ_A]^-$ in Q_B-nonreducing RCs; k_e, rate constant of Q_A photoreduction; N, number of STFs in variable flash train; OEC, oxygen evolving complex; P_{680}, primary electron donor of PSII; Phe(or Ph), pheophytin, primary electron acceptor of PSII; $[PheQ_A]^{2-,-}$, double and single reduced acceptor pair of PSII, respectively; PQ, plastoquinone; PSII, photosystem II; PSU, photosynthetic unit; Q_A, primary quinone acceptor of PSII; Q_B, secondary quinone acceptor of PSII; RC, reaction center of PSII; TSTM, three-state trapping model; STF, single turnover flash; VMC, valinomyin; Y_Z, secondary electron donor of PSII

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doi:10.1016/j.bbabio.2006.02.006

1. Introduction

Variable chlorophyll *a* fluorescence in green plant cells and chloroplasts is a powerful non-invasive method for evaluating mechanisms of photosynthetic energy trapping, in relation to photosynthetic parameters associated with Photosystem II (PSII) [1]. Sensitivity and time resolution of fluorescence instruments have highly profited from skilful application of dedicated new photometric technologies [2,3] and of appropriate powerful routines in mathematical software (like Mathcad or Mathlab) to resolve fluorescence responses in single turnover excitations [4,5].

The light-dependent chlorophyll *a* fluorescence yield in chloroplasts and intact leaves is variable between a lowest level F_o at full photochemical quenching and a maximal level F_m at saturating light intensities at which quenching is released. Variable fluorescence is defined as $F_v=F_m-F_o$. The variable fluorescence induced by a saturating single turnover flash (STF) has been reported to be 40 to 60% of F_v in saturating multiturnover pulses (MTF) [6–9]. The primary quinone acceptor Q_A of PSII has since long been known as the major and principal quencher; the quenching is released upon its photoreduction [10]. Other electron transport intermediates at the acceptor- and donorside of PSII have been proposed as additional functional quenchers like pheophytin (Phe) [11], reduced forms of the secondary quinone acceptor Q_B^- [12] and Q_B^{2-} [13], plastoquinone (PQ) [14], oxidixed primary (P_{680}^+) [15], and secondary donor (Y_z^+) [16] or side products like triplet carotenoids (car^T) [17]. Their quenching has been discussed in relation to the increase in the fluorescence yield in an STF being substantially below F_v.

The decay of the chlorophyll fluorescence yield after saturating single turnover excitations is multi-phasic with, except for a slow component in the tens of seconds time range, two major kinetic components differing in their 2.5–5 and 1.2–1.4 ms^{-1} rate constants. These have been attributed to those of the dark reversion of light-driven Q_A-quenching release caused by reoxidation of Q_A^- by Q_B and Q_B^-, respectively in the dark [18,19]. The residual fluorescence signal after STF excitation recovering in the tens of seconds time range has been reported to originate for a major part from Q_B-nonreducing centers [3,20]. It is probably associated with the increased F_o level observed after a low intensity far red light pulse [21].

Here we report on the kinetics of rise and decay of variable fluorescence in chloroplasts excited by variable number (N) of saturating single turnover flashes (STFs) given at 12.5 Hz frequency. The data show (i) a gradual increase in the maximal fluorescence level F_m with flash number reaching a nearly twofold higher steady state value for $N\geq 40$, (ii) a substantial change with progressing N in the fractional size of at least 6 kinetically distinguishable but invariable decay components, and (iii) a seeming Q_A-quenching release paradox. Data give support for the hypothesis that progressive single turnover excitation causes accumulation of trapping competent Q_B-nonreducing RCs. Specific sensitivity of the fluorescence induction pattern to membrane-modifying agents suggests that accumulation of these centers occurs in association with proton transport across the thylakoid membrane.

2. Material and methods

Plant growth (Pea), chloroplast isolations and suspending medium were as described elsewhere [22,23]. In the experiment with valinomycin (VMC) (see Fig. 6) the KCl concentration in the suspending medium was increased to 100 mM. Room temperature chlorophyll fluorescence yields were measured in dark-adapted chloroplast preparations (1 μg/ml) with the Dual-Modulation Kinetic Fluorometer (Photon Systems Instruments, Brno, Czech Republic), as described in detail in (3). The set-up was routinely used in a mode in which the fluorescence yield after the last of N ($50\geq N\geq 1$) single turnover excitations (STFs) in a 12.5 Hz flash train was probed by weak 2.5 μs measuring flashes, fired at progressing dark intervals in a time domain between 50 μs and 18 s with, on a log time scale 4 equidistant excitations per decade. STFs were found to be saturating as concluded from the constancy of the relative fluorescence signal F_m/F_o upon 50% decrease in flash intensity, or alternatively in chloroplast density. Further details about the use of this equipment can be found elsewhere (3, and see also http://www.psi.cz). Exponential decomposition and quadratic least square fitting of the fluorescence decay were done with standard routines provided by appropriate software (MathCad 11, MathSoft Inc. Cambridge, Mass.).

3. Results and interpretation

Fig. 1 shows the release and dark recovery of chlorophyll fluorescence quenching during and after a 12.5 Hz STF train, plotted as the fluorescence signal at time t, $F(t)$, relative to the fluorescence yield F_o at the onset of the 1st flash. The flash frequency of 12.5 Hz was chosen to guarantee a complete dark relaxation, except for the slow component in the 0.1 to 20 s time domain, of the STF-induced variable fluorescence ΔF during the 80 ms dark period between STFs. The $F(t)$ maximum reached in each STF is defined as F_m. Fig. 2 illustrates, for the same experiment, the amplitude ΔF and the rise ΔF_o ($=F_m-\Delta F$) in the level at each onset F_o^*($=F_o+\Delta F_o$) as a function of STF number N. The fluorescence levels F_o^* and F_m increase with flash number, after a distinct delay during the first flashes, and reach a steady level at $F/F_o\sim 2.8$ and ~ 4.5, respectively around the 40th flash. This is due to the nearly constancy, except for the 4-periodic modulation in the first 10 STFs, of the STF-induced response (ΔF). It is clear from Fig. 2 that, at the frequency used, i) the steady state level of ΔF_o in the flash train approaches the level of the invariable ΔF and ii) a pronounced change $\Delta F_o\sim 0.4$ occurs induced by the first STF, which is seen as the jump in F_o^* and F_m at the 2nd flash.

The dark decay of variable fluorescence, as illustrated in Fig. 1 for flashes with number $N=6$, 16, 26, 40 and 50, shows a fast ($t<0.1$ s) and a slow phase ($t<20$ s), associated in rough approximation with ΔF and ΔF_o, respectively. The kinetic pattern of $F(t)$ in response to the 12.5 Hz flash train with the nearly parallel rise of F_m and F_o^* suggests that the rise of F_m at the frequency used is mainly, if not exclusively due to increase in the fractional component(s) associated with the slow decay phase ΔF_o. The insert in Fig. 1 shows that the multi-phasic decay pattern of the slow phase ΔF_o is invariable with flash number, except for a slight enhancement in the 1–5 s time domain after the 6th flash. The somewhat deviant kinetic pattern for flashes with $N<6$, and the delay in the rise during the first flashes are presumed to be associated with the transfer of S states and the establishment of a homogeneous distribution of these states. It has been shown that these phenomena are at the

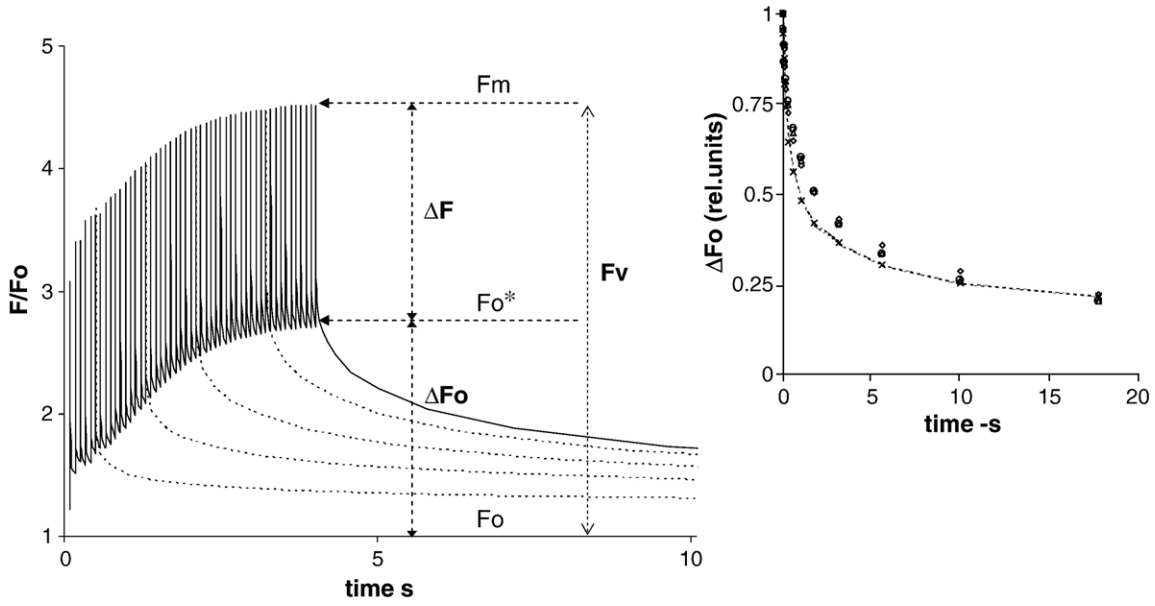

Fig. 1. Fluorescence response $F(t)$ to a 12.5 Hz flash train of 50 STFs plotted relative to the fluorescence F_o (=1) at the onset of the first flash. F_m is the maximum F-level reached in each STF; ΔF is the amplitude of the F-response induced by each STF; $F_o^*=F_m-\Delta F$ is the F-level at the onset of each STF; $\Delta F_o=F_o^*-F_o$, the rise in the F-level at the onset of an STF in the 12.5 Hz train, and $F_v=F_m-F_o$ is the variable fluorescence. Dotted curves are of the dark decay in the 10 s time range after a train with 6, 16, 26 and 40 STFs. Note (i) the 4-periodic modulation of F_o^* (ΔF_o) and F_m in the first flashes, (ii) the delay in the rise pattern of F_o^* (ΔF_o) which reaches a steady state, in this case after about 40 STFs, and (iii) two distinct decay phases: a fast component ΔF of about constant size and recovery within 1 s and a slow one ΔF_o into the tens of seconds time domain increasing in size with flash number. Inset shows the decay of slow phase, normalized to amplitude of 50th STF for $N=6$, 16, 26, 40 and 50. It shows, except for $N=6$ (curve), no difference in decay pattern of the slow phase.

basis of the 4-periodic fluorescence responses in the first flashes of a train [18,24–26].

The invariability of the decay pattern of ΔF_o with progressing flash number (insert Fig. 1) suggests that this fluorescence phase is associated with a single component that accumulates in a flash train and is active in releasing fluorescence quenching. We propose that the accumulating component is identical with a fraction of RCs in which Q_A is reduced. The recovery of this slow fluorescence phase, i.e., the return to the open (-quenched) state with $F_o^*=F_o$, then would mean a slow reoxidation of Q_A^- and rules out an oxidation by Q_B or Q_B^- which is known to occur within a fraction of ms [18,19]. This suggests that the increasing slow recovery phase in a STF train reflects the accumulation of antenna systems with Q_B-nonreducing centers. This is in line with interpretations of the slow recovery phase measured in isolated ($N=1$) [3,20] or in low frequency (~1 Hz) STF trains [5], or after low intensity far red pre illumination [21]. The multiphasic composition of ΔF_o dark recovery which can be fitted with a bi-exponential function with time (rate) constants $k_{nB1}=0.5\pm0.1$ and $k_{nB2}=0.03\pm0.003$ s^{-1} could be related with the S-state dependent rate constants of Q_A^- oxidation by the donor side components [26–28] and possibly modified by pH-dependent changes in the $Q_A^-Q_B \leftrightarrow Q_AQ_B^-$ equilibrium [29,21].

The nearly invariability of ΔF with progressing flash number at the frequency used, even at ΔF_o levels comparable to ΔF (Fig. 2), leads to a seeming paradox. How can STF excitation of photosystems with a high fraction of Q_A^- containing but Q_B-nonreducing RCs cause a nearly unaltered extent of quenching release as compared to that in a system of RCs with Q_A fully oxidized? Or, in other words why, when and how becomes the release of photochemical quenching in STF excitation independent of the redox state of the quencher Q_A? We propose, in agreement with postulates and predictions of the Three State Trapping Model (TSTM) of PSII [4,11,30], that charge separation in singly reduced Q_B-nonreducing RCs causes double reduction of the PSII acceptor pair [PheQ_A]. Only the trapping of a second electron at the acceptor (and donor) side causes the full closure of the RC and is associated with a concomitant release of fluorescence quenching of approximate equal size as that associated with single reduction. The excitability and electron trapping competence of singly reduced Q_B-nonreducing RCs, which in the TSTM concept are categorized as semi-open (-closed), is confirmed by the $F(t)$ response in a 12.5 Hz STF train in the presence of DCMU, as shown in Fig. 3. The figure shows for the response in the presence of the herbicide: (i) the sub-maximal fluorescence yield in a single turnover excitation and F_m being reached only after 5–6 STFs, (ii) the amply documented increase of the initial dark fluorescence level, ascribed to the dark conversion of RCs in the $S_0Q_B^-$ state into their Q_B-nonreducing form after DCMU addition [4,13,31–33], and (iii) the unaltered or even slightly higher STF response (ΔF) in the 1st STF, irrespective the presence of an altered fraction of Q_B-nonreducing RCs.

It is then of interest to study the dark decay of ΔF (recovery of photochemical quenching) in dependence of the flash number. In dark adapted samples with open and a relatively small fraction of Q_B-nonreducing RCs ($\Delta F_o<0.2$) the decay is expected to be governed for the major part by the rate of reoxidation of Q_A^-. If the reoxidation rate of the proposed double reduced acceptor side (e.g., in Q_B-nonreducing RCs) is different from that of the single reduced acceptor in 'normal' RCs, then one might expect a change

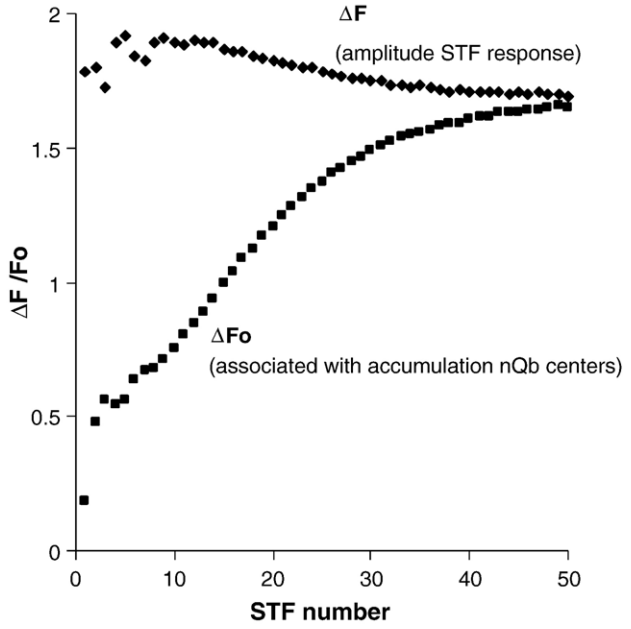

Fig. 2. Amplitude (ΔF) of STF-induced fluorescence response in a 12.5 Hz train of 50 STFs (upper data, diamonds) and the rise (ΔF_o) in the quasi-steady state fluorescence level at the onset of STFs in the train plotted as function of STF number (same data as in Fig. 1). The *x*-axis corresponds with a linear time range of 4 s. The fluorescence level (F_o) at the start of the 1st flash has been corrected for the presence of an approximately 15% fraction of semi-open Q_B-nonreducing (nQb) RCs (with Q_A^-). Figure illustrates that ΔF_o in STF train rises to level close to that of ΔF_o associated with 100% Q_A^-, and a nearly unaltered STF response (ΔF). This suggests, conclusive with kinetics (see Fig. 4), (i) substantial accumulation and conversion of nQb RCs, and (ii) occurrence of charge separation and electron trapping in semi-open Q_B-nonreducing RCs.

in the dark recovery kinetics with flash number. This is because the fractional distribution of 'normal' and Q_B-nonreducing RCs alters with flash number as reflected by changes in ΔF_o (see Fig. 2). Fig. 4 shows the kinetics of the recovery of fluorescence quenching release after the 6th and 40th STF after correction for (subtraction of) the slow recovery of quenching associated with ΔF_o. The kinetics are distinctly different. It is clear that after the 6th STF at which the fraction β of Q_B-nonreducing RCs, according to the relative size of ΔF_o, is approximately 25% (see Fig. 2), 75% of the quenching has recovered within 3 ms, whereas after the 40th flash with ~60% Q_B-nonreducing RCs, much less recovery is observed within this time. This leads to the conclusion that the recovery of photochemical quenching in the 80 ms dark period after a STF in a 12.5 Hz flash train is likely to originate from at least two fractions of RCs of which the distribution changes with flash number. The data suggest that one fraction (1-β) is identical with 'normal' Q_B-reducing RCs and the other β-fraction consists of Q_B-nonreducing RCs.

These qualitative conclusions with respect to extent and kinetics of distinguishable components in the fluorescence response support the hypothesis on the functional role of an accumulating fraction of Q_B-nonreducing RCs in the release and recovery of quenching during and after a train of STF excitations. Thus, a global target analysis [34] has been applied on the fluorescence decay after STF excitations. This allows the fluorescence kinetics to be quantified in terms of quenching properties and activities of 'normal' (Q_B-reducing) and Q_B-nonreducing RCs.

At the firing of the *N*th flash with excitations every 80 ms, the major part of the system is assumed to consist of two RC-fractions; one (1-β) in which Q_A is oxidized (open RCs), and the other (β) in which Q_A is reduced and Phe oxidized (semi-open

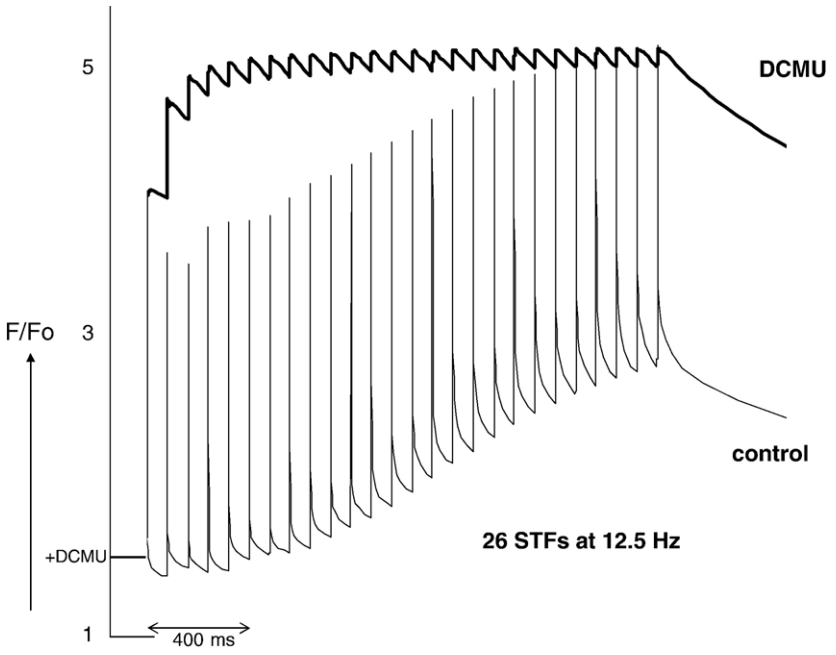

Fig. 3. Fluorescence response to a 12.5 Hz flash train of 26 STFs plotted relative to the fluorescence F_o (=1) at the onset of the first flash in the absence (control) and presence of 10 μM DCMU. The response of the control shows the same pattern as illustrated in Fig. 1 with the 4-periodic modulation of F_m and F_o^* and a pronounced delay in the rise pattern of F_o^* (ΔF_o) in the first 5 to 10 STFs. The response in the presence of DCMU shows a higher F-level at the onset of the 1st STF with $F(0)/F_o$ ~1.3, a sub-maximal F_m level in the 1st flash and a F_m level that is reached after approximately 5 to 7 STFs.

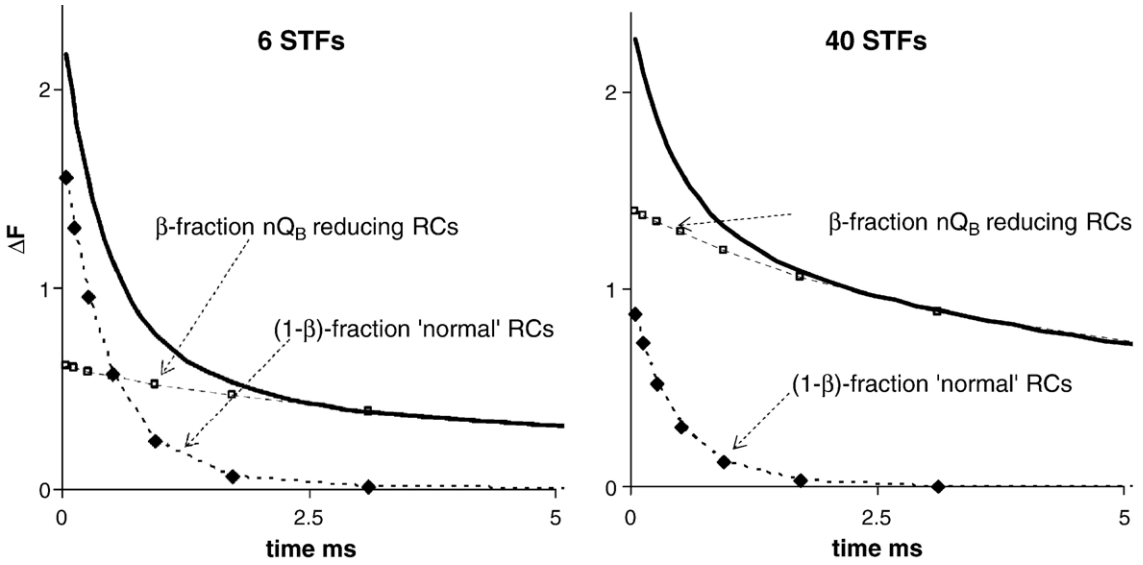

Fig. 4. Fluorescence response in the linear 0–5 ms time range (bold solid curve) in dark adapted pea chloroplasts after the last excitation in a 12.5-Hz flash train with 6 and 40 STFs (left and right hand panel, respectively). Response curves are corrected for slow ΔF_o decay (tens of seconds, see Fig. 1) associated with recovery of the β-fraction with semi-open (Q_A^- containing) Q_B-nonreducing RCs. The dashed curves with closed and open symbols are simulated curves calculated with Eqs. (1)–(4) for nearest fit of the experimental curve. Fit parameters are given in the legend of Fig. 5. Note the pronounced decrease (from 74 to 41%) in the (1-β)-fraction of 'normal' Q_B-reducing RCs with flash number.

RCs). The β-fraction appears to be particular in a sense that reoxidation of Q_A^- by Q_B or Q_B^- is hindered in these (Q_B-nonreducing) centers. This β-fraction of the single reduced (semi-open) Q_B-nonreducing RCs might contain a small sub-fraction of closed RCs in which the acceptor pair [Phe Q_A] is double reduced. Excitation of the (1-β)-fraction of open centers causes their semi-closure and gives rise to a fluorescence response $F^{sc}(t)$ that is described by

$$F^{sc}(t) = \Delta F^{Qa}(e^{-k_{AB1}t} + e^{-k_{AB2}t}) \quad (1)$$

in which k_{AB1} and k_{AB2} are rate constants of Q_A^- reoxidation by Q_B or Q_B^-, respectively and ΔF^{Qa} is the fluorescence increase associated with release of Q_A-quenching in a single turnover excitation. Excitation of the β-fraction of single reduced Q_B-nonreducing centers causes reduction of the acceptor pair $PheQ_A^-$ and full closure of the RC. This will give rise to a fluorescence response

$$F^{c}_{nQb}(t) = \Delta F^{Phe}(e^{-k_1 t} + e^{-k_2 t}) \quad (2)$$

due to release of Phe-quenching with amplitude ΔF^{Phe} and subsequent reoxidation of the double reduced acceptor pair $[PheQ_A]^{2-}$ towards the Q_B-nonreducing form $[PheQ_A^-]$ with rate constants k_1 and k_2. Finally, the decay (full opening) of the fraction of semi-open Q_B-nonreducing centers is represented by the slow response

$$F^{sc}_{nQb}(t) = \Delta F^{nQb}(e^{-k_{nB1}t} + e^{-k_{nB2}t}) \quad (3)$$

and attributed to disappearance of Q_A^- with rate constants k_{nB1} and k_{nB2} and amplitude ΔF^{nQb}, caused by interaction with PSII donor side components and modified by changes in the $Q_A^-Q_B \leftrightarrow Q_AQ_B^-$ equilibrium [35]. The experimental data for a 12.5-Hz STF train, as illustrated with ΔF and ΔF_o in Fig. 1, indicate that the amplitudes of the STF responses in open and semi-open RCs are equal. This means, in confirmation with postulates of TSTM, that $\Delta F^{Qa} = \Delta F^{Phe} = \Delta F^{nQb}$. The decay of $F(t)$, starting at $t=0$ from F_m for each N, assuming photoelectric effects on $F(t)$ [30] to be invariable with N for $N>6$, has been fitted and solved for β, F_v^{QA} and the 6 rate constants with a least square difference routine, applied to

$$F(t) = (1-\beta)*F^{sc}(t) + \beta*[F^{c}_{nQb}(t) + F^{sc}_{nQb}(t)] \quad (4)$$

The results for STFs 6, 16, 26 and 50, plotted on a logarithmic time scale, are shown in Fig. 5. The calculated curves are given with the indicated symbols. The parameters corresponding with the fits for the respective STFs (N) are listed in the legend of Fig. 4. Rate constants of Q_A^- reoxidation by Q_B and Q_B^- (k_{AB1} and k_{AB2}) and of Q_A^- reoxidation in Q_B-nonreducing centers (k_{nB1} and k_{nB2}) are in the range reported by others [18–21]; those of Phe^- reoxidation in closed Q_B-nonreducing centers (with $k_1 \sim 0.3$ ms^{-1} and $k_2 \sim 7$ s^{-1}) are reported for the first time and are distinctly smaller than of Q_A^- reoxidation in semi-closed 'normal' (Q_B-reducing) centers. The resolved responses in the 0–5 ms linear time range associated with the (1-β)- and β-fraction of 'normal' ($F^{sc}(t)$) and Q_B-nonreducing RCs ($F^{c}_{nQb}(t)$), respectively are shown for the 6th and 40th STF in Fig. 4. They confirmingly show a change in the fractional composition of the pattern with increase in $F^{c}_{nQb}(t)$ which is in harmony with an increase in the fraction of antennas with single reduced Q_B-nonreducing centers with flash number and quenching properties similar to that of Q_A. It should be stressed that the global target analysis of the $F(t)$ curves in Fig. 4 has been done with the minimal number of parameters (rate constants) and with approximately equal weight factors for the components. A deconvolution with less rate constants gave a

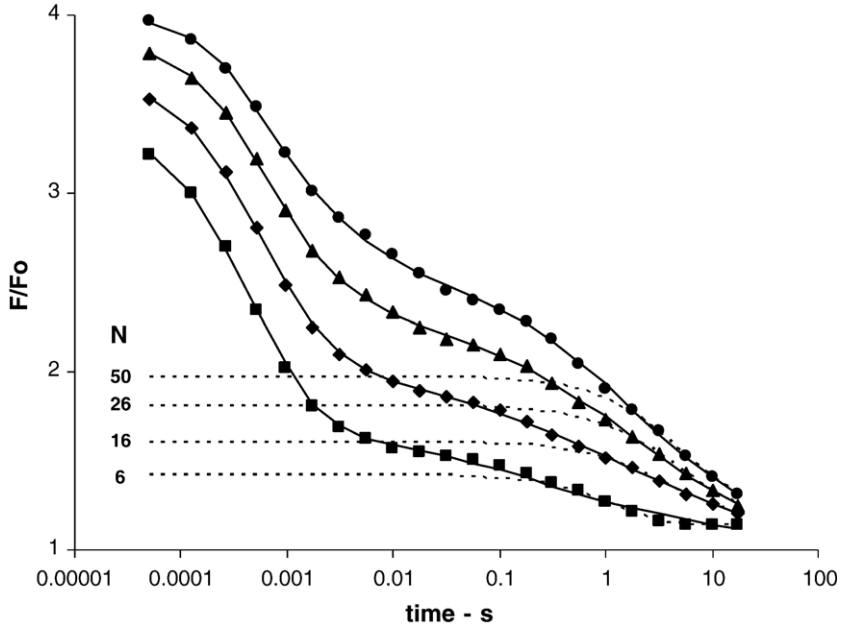

Fig. 5. Experimental (solid curves) and calculated (symbols) fluorescence decay in the dark after a Nth STF in a 12.5 Hz flash train for $N=6$ (boxes), 16 (diamonds), 26 (triangles) and 40 (circles) in the 50 μs to 20 s time range plotted on a log time scale. Calculated curves (symbols) were derived using a quadratic least square fitting routine and Eqs. (1–4) (see text and Fig. 1). Dotted curves with N—numbers are of the slow phase $F^{sc}_{nQb}(t)$ expressed by Eq. (3) (see text). Parameter values corresponding for each N with the calculated curve are given in the table below. The mean deviation for the individual data points in each of the curves is found to be less than 1%.

N	ΔF^{Qa}	β	k_{AB1} (ms^{-1})	k_{AB2}	k_1	k_2 (10^2)	k_{nB1} (10^3)	k_{nB1} (10^4)
6	1.9	0.26	2.6	1.3	0.3	0.7	0.7	0.1
16	2.0	0.38	2.3	1.2	0.2	0.5	0.3	0.2
26	2.1	0.52	2.1	1.1	0.1	0.3	0.3	0.3
40	2.1	0.59	1.9	1.0	0.1	0.2	0.3	0.3

worse fit with higher deviation factor. It cannot be excluded that the reaction pattern is more complex than targeted here.

4. Discussion

The extent and recovery kinetics of the fluorescence quenching release in a 12.5 Hz train of single turnover flashes STFs suggests that electron-trapping-competent Q_B-nonreducing reaction centers have a functional role in the primary process of photosynthetic energy conversion in PS II. The presence of a small fraction of these RCs in dark adapted chloroplasts has been concluded from the fluorescence response upon DCMU addition [36], in low intensity light [21,24,36–38] and from the recovery kinetics after single [3,20,23] or multiple excitations [5] with STF(s). DCMU addition causes, as compared to F_o in a 10 to 20 min dark adapted control preparation, a light-independent increase ΔF_o in the initial fluorescence level with $\Delta F_o/F_o$ in the range between 0.3 and 0.5 [31–33]. In the experiment with DCMU, illustrated in Fig. 3, $\Delta F_o/F_o=0.49$. Values of $\Delta F_o/F_o$ in the range between 0.3 and 0.5 after DCMU addition indicate the presence of a β-fraction of 17 to 25% semi-open Q_B-nonreducing RCs (assuming [7,4,30] that the average fluorescence increase associated with release of Q_A-quenching in a single turnover excitation is $\Delta F^{Qa}/F_o \sim 2$). This β-fraction has been argued to be identical to the fraction of centers in S_0 state which is reported [28,33,36] to be commonly present in 10 to 20 min dark adapted preparations with S_0/S_1 ratio equal to 25/75. The mere fact that the STF-induced quenching release upon the 1st STF after DCMU addition is not reduced in the presence of a substantial β-fraction of Q_B-nonreducing RCs, and in some experiments is even higher, supports the hypothesis that these RCs are excitable, competent of charge separation and functional in release of quenching concomitant with the double reduction of the PSII acceptor side. The same applies to the single reduced Q_B-nonreducing RCs (because of the presence of DCMU) that are formed in the 1st STF. These become fully closed in the subsequent STFs. The number of STFs required for full closure, approximately 5–10 in the experiment of Fig. 3, is determined by the electron trapping efficiency at the donor side [4,11]. The failure of a single saturating excitation to cause maximal fluorescence quenching release in the presence of DCMU and full closure of open Q_B-nonreducing RCs has been reported before [9,23] and is in confirmation with the basic postulates of TSTM. This phenomenon was alternatively interpreted in terms of a single hit trapping mechanism with, under the experimental conditions used, a reduced electron trapping efficiency of PSII RCs caused by an enhanced radical pair recombination [39].

A 15 to 25% residual quenching release is reproducibly observed 80 ms after a first single turnover flash in dark adapted

samples [3,23,40]. It shows up as the jump in ΔF_o at the 2nd STF (Fig. 2). It is tempting to assume that this quenching originates from the single reduced β-fraction with Q_B-nonreducing RCs in state S_0. This fraction, as discussed above, is of a comparable size and contains Q_B^- which shifts its electron to Q_A in the presence of DCMU causing ΔF_o in the dark upon addition of the inhibitor [41]. Excitation of the β-fraction will–of course–generate Q_A^- and cause, if we deal with Q_B-nonreducing RCs, a comparable ΔF_o as DCMU addition. The absence of a significant response associated with ΔF_o in dark adapted chloroplasts after the 2nd, 3rd and 4th flash (Figs. 1 and 2) suggests that singly reduced RCs in states S_1, S_2 and S_3, in contrast to those in S_0 state, hardly contain, if at all, Q_B-nonreducing RCs. The small ΔF_o response after the 2nd flash might result from S_0 RCs that were not hit in the 1st STF. The pattern of Fig. 2 for the STFs 1–4 is found to be in agreement with a STF mishit parameter $\alpha \sim 0.8$ (not shown). In confirmation with our hypothesis are the small but distinct ΔF_o responses after the 5th and 6th STF in which, for $\alpha \sim 0.8$ and a S_0/S_1 ratio of 0.25/0.75 at the first flash, a fraction of 0.4 and 0.3 of S_0 RCs are excited.

The fluorescence decay kinetics from F_m after the first flash in 10 to 20 min dark adapted chloroplasts (not shown, but see Fig. 4 for similar although higher response in the 6th flash) indicate the presence of a small decay component associated with the quenching release of doubly reduced (fully closed) Q_B-nonreducing RCs related with $F^c_{nQb}(t)$ (Eq. (2)). As an average this sub-β-fraction was found to be 10% of the fraction of 'normal' open RCs. This means, because of the presence of non-quenching Q_A^- in the sub-β-fraction, that F_o estimated at the onset of the 1st flash is about 10% above the correct dark level. This correction was done in Fig. 2 and explains $\Delta F_o/F_o \sim 0.2$ at the 1st STF.

Figs. 1–3 show that after the sizeable jump caused by the 1st STF and a subsequent delay during the next 4 to 6 STFs, a sigmoidal rise in ΔF_o occurs towards a steady state level that is reached after 40 to 50 STFs. The analyses of the recovery kinetics of ΔF_o and ΔF (Figs. 4, 5) provide evidence that this rise is associated with accumulation of singly reduced Q_B-nonreducing RCs. Q_B-nonreducing RCs have been defined as RCs that either are blocked in $Q_A^- \rightarrow Q_B$ electron transfer related to altered occupancy properties of the Q_B-binding site [42], or have a low equilibrium constant of the $Q_A^-Q_B \leftrightarrow Q_AQ_B^-$ equilibrium [43]. It has been shown [19,44] that the acceptor side equilibrium is pH-dependent: it shifts to the left (increase in Q_A^-) at alkaline pH. If the accumulation of Q_B-nonreducing RCs with progressing STFs in a flash train were caused by an alkaline shift of the $Q_A^-Q_B \leftrightarrow Q_AQ_B^-$ equilibrium, then one might

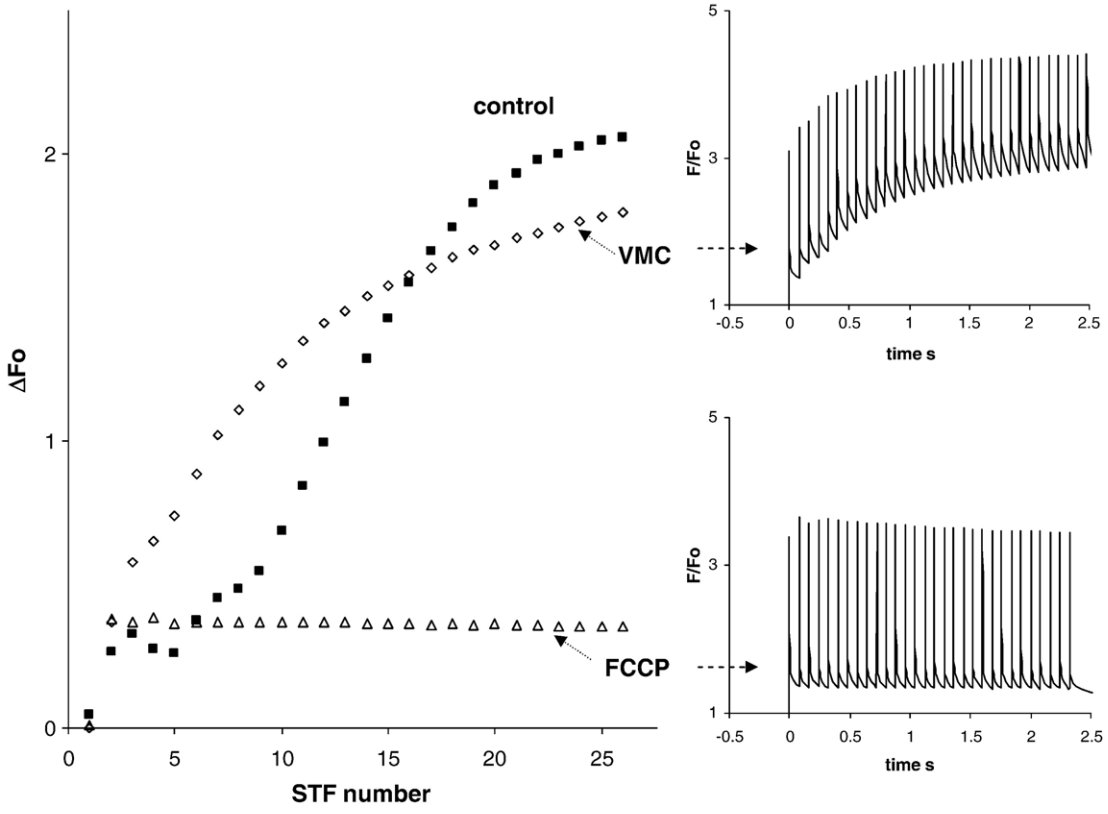

Fig. 6. (Left hand panel) The rise (ΔF_o) in the quasi-steady state fluorescence level at the onset of STFs in a 12.5 Hz train of 26 STFs plotted as function of STF number in absence (control, closed boxes) and presence of 6 μM VMC +100 mM KCl (open diamonds) or 0.2 μM FCCP (open triangles), respectively. The *x*-axis for 25 STFs corresponds with a linear time range of 2 s. (Right hand panel) The original recordings of the F-response in the 30STF 12.5 Hz flash train in the presence of VMC (top) or FCCP (bottom). The original response of the control is similar as the one reproduced in Fig. 1. Figure illustrates an inhibition of the delay in the accumulation of Q_B-nonreducing RCs (ΔF_o) and decrease in its steady state by VMC and full inhibition of the accumulation by FCCP without, for both membrane-modifying agents, an effect of the 1st flash on ΔF_o.

expect that agents interfering with photo-electro-chemical events at the photosynthetic membrane, in particular those which modify proton movements and pH changes, will affect the ΔF_o and ΔF kinetics. This appears to be the case as illustrated in Fig. 6 where ΔF_o kinetics in a 12.5 Hz flash train in absence and presence of uncouplers valinomycine (VMC) or FCCP, are plotted as function of STF number. The original recordings of the F-response in the flash train in the presence of VMC or FCCP are shown at the right hand side of Fig. 6; the response of the control is similar as the one reproduced in Fig. 1. Figure illustrates (i) no effect on ΔF_o of the 1st flash for both membrane-modifying agents, (ii) an inhibition of the delay in the accumulation of Q_B-nonreducing RCs (ΔF_o) in the first 6 to 8 STFs with a decrease in its steady state by VMC and (iii) full inhibition of the accumulation by FCCP. The absence of an effect on the 1st flash is consistent with the earlier conclusion that this response is caused by $S_0[Q_B^-]$ RCs that are present, apparently with a low $Q_A^-Q_B \leftrightarrow Q_AQ_B^-$ equilibrium, in the dark adapted state. The ionophores were found no to change the decay kinetics of the STF-induced changes in the 0–5 ms time range (see Figs. 4 and 5) markedly. The effect of the ionophores on the ΔF_o response pattern in subsequent excitations is in confirmation with their amply documented interaction with the rate of proton uptake which is enhanced by VMC and inhibited by FCCP [45,46]. An enhanced proton uptake in the thylakoid lumen at each excitation (STF) caused by VMC will lead to an enhanced alkalization at the stroma side, and consequently result in an faster alkaline shift of the $Q_A^-Q_B \leftrightarrow Q_AQ_B^-$ equilibrium with an associated enhanced rise in ΔF_o. Conversely the inhibition of proton uptake by FCCP will inhibit the ΔF_o response in progressing STFs. The lower ΔF response upon STF excitations in the presence of VMC has been reported before and indicates the attenuation of the photo-electric effect on the release of photochemical fluorescence quenching [23]. The data of Figs. 1 and 6 do not allow conclusions about differences in pH sensitivity of the $Q_A^-Q_B \leftrightarrow Q_AQ_B^-$ equilibrium in S_1-, S_2-, and S_3-RCs. Validation of the correctness of our interpretation on the coupling between accumulation of Q_B-nonreducing RCs and lumenal acidification requires additional experiments. Experiments with STF trains of variable frequency combined with titration of ionophores that modify the proton conductance of the thylakoid are a promising means in this respect. These should be critical with respect to the frequency range. On one side the frequency limit is determined by the allowance of a complete turnover of the trans-thylakoid proton pump (~10 ms). This would give a limit of the order of 100 Hz. Variation in the low frequency range would yield information on the dark recovery of the STF-induced pH gradient across the membrane in connection with an altered time pattern of the ΔF_o rise in the flash train. In this connection, it is of interest to refer to results of similar experiments [5] which show that the ΔF_o rise in a 1 Hz flash train with 20 STFs is largely reduced as compared to the rise found upon repetitive STF excitation at 10 Hz (Figs. 1 and 2).

We might conclude, in confirmation with a double hit three state trapping model (TSTM), that the increase of the slow recovery phase after an increasing number of repetitive STFs is the reflection of that of an accumulating fraction of antenna systems with Q_B-nonreducing singly reduced RCs with approximately half of the maximal fluorescence yield. This accumulation is postulated to be related with photo-electrochemical membrane responses of the RCs in one or more of the 4 donor S-states. The electrochemical response, notably the proton uptake associated with excitations, is presumed to cause amongst other an alkaline shift of the $Q_A^-Q_B \leftrightarrow Q_AQ_B^-$ equilibrium in the RCs and will promote the fraction of non Q_B-reducing centers therein. The relatively high accumulation of these centers in the first flash (Fig. 1) suggests a particular property in this respect of the donor state S_0.

The progressive accumulation of non Q_B-reducing centers in flash trains would on first sight result in loss of electron trapping efficiency. However, the non-zero trapping efficiency in second hits causing double reduction of the acceptor side and the subsequent reoxidation to the single reduced Q_A^-, probably by Q_B or Q_B^- will circumvent this inefficiency. In other words, Q_B-nonreducing RCs are photo-converted in a double hit photo-process into Q_B-reducing centers.

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