On the origin of the '35-μs kinetics' of $P680^{+\bullet}$ reduction in photosystem II with an intact water oxidising complex

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Abstract The origin of the '35-μs kinetics' of $P680^{+\bullet}$ reduction in photosystem II (PS II) with an intact water oxidising complex has been analysed by comparative measurements of laser flash induced changes of the 830-nm absorption and the relative quantum yield of chlorophyll (Chl) fluorescence. The latter parameter was monitored at a time resolution of 500 ns by using newly developed home built equipment [Reifarth, F., Christen, G. and Renger, G. (1997) Photosynth. Res. 51, 231–242]. It was found that: (i) the amplitudes of the unresolved ns-kinetics of both 830-nm absorption changes and the rise of fluorescence yield exhibit virtually the same period four oscillation pattern when dark adapted samples are excited with a train of saturating laser flashes; (ii) the corresponding oscillation patterns of the normalised extent of the 35-μs kinetics under identical excitation conditions are strikingly different with maxima after the 3rd and 5th flash for the 830-nm absorption changes vs. pronounced maxima after the 4th and 8th flash for the rise of the fluorescence yield. The period four oscillations unambiguously show that the '35-μs kinetics' of $P680^{+\bullet}$ reduction are characteristic for reactions in PS II entities with an intact water oxidising complex. However, the disparity of the oscillation patterns of (ii) indicates that in contrast to the ns components of $P680^{+\bullet}$ reduction the 35-μs kinetics do not reflect exclusively an electron transfer from Y_Z to $P680^{+\bullet}$. It is inferred that a more complex reaction takes place which comprises at least two processes: (a) $P680^{+\bullet}$ reduction by Y_Z and (b) coupled and/or competing reaction(s) which give rise to additional changes of the chlorophyll fluorescence yield.

Key words: $P680^{+\bullet}$ reduction; Fluorescence rise; Photosystem II; 35-μs kinetics

1. Introduction

Photosynthetic water oxidation to molecular oxygen and four protons takes place via a sequence of redox steps at a manganese-containing operational unit referred to as water oxidising complex (WOC) (for reviews, see [1–3]). This sequence is energetically driven by the cation radical $P680^{+\bullet}$ that is formed as a result of the primary charge separation in photosystem II (PS II) (for review, see [4]). $P680^{+\bullet}$ extracts electrons from the WOC with tyrosine residue 161 (Y_Z) of polypeptide D1 [5,6] acting as intermediary redox carrier either in a conventional manner of electron transfer or as specific hydrogen abstractor from substrate (H_2O^- or OH^-) coordinated to manganese (for further discussion, see [7,8]). The kinetics of both $P680^{+\bullet}$ reduction by Y_Z and stepwise WOC oxidation by Y_Z^{OX} depend on the redox state S_i of the WOC (for a compilation of data, see [9]). The former reaction is dominated by ns kinetics [10–13]. In addition a smaller fraction (about 15–20%) exhibits a $P680^{+\bullet}$ reduction with about 35 μs. The extent of these '35-μs kinetics' was shown to exhibit a characteristic period four oscillation [14–16] that is typical for a dependence on the redox state S_i of the WOC. The origin of the '35-μs kinetics' is not yet clarified. It was proposed that it involves 'dead' centres [17], a recombination reaction [18], the reduction by carotenoids [19] or the reduction of a $Chl^{+\bullet}$ molecule different from P680 [20]. Recently it was ascribed to a stabilising relaxation process [16]. In this case the period four oscillation of the amplitudes was explained by an S_i-state dependent redox equilibrium between $P680^{+\bullet}/Y_Z$ and $P680/Y_Z^{ox}$, but the seemingly independent 20–40-μs kinetics is difficult to reconcile with this idea [16].

One possible approach for obtaining further information on the origin of the '35-μs kinetics' is to perform a comparative study where the $P680^{+\bullet}$ reduction in the microsecond time domain is monitored via 830-nm absorption changes and by flash induced changes of the fluorescence quantum yield as outlined recently [21].

2. Materials and methods

Thylakoid membranes were isolated from spinach as described by Winget et al. [22]. PS II membrane fragments were prepared from spinach as described in [23] with slight modifications [24]. LHC II particles were prepared according to a procedure described by Irrgang et al. [25].

Flash induced changes of the fluorescence quantum yield with a time resolution of about 500 ns were monitored with a home-built equipment [21]. The fluorescence was probed by short light emitting diode (LED) pulses. The pulse trains for the LEDs were generated by a programmable function generator (Stanford Research Systems, DS 345) in combination with a home-built current driver. Fluorescence measurements were performed with thylakoids ([Chl]=20 μg/ml) or LHC II particles ([Chl] = 10 μg Chl/ml) in the absence of artificial acceptors.

Flash induced absorption changes at 830 nm with microsecond time resolution (maximum electrical bandwidth: 300 kHz) were measured with a single beam flash photometer as outlined previously [26,27]. A compensation technique involving a second photo diode was used to enhance base line stability. The absorption measurements were performed with PS II membrane fragments at a concentration of 70 μg Chl/ml in the presence of 200 μM DCBQ. PS II membrane fragments were used instead of the more intact thylakoids in order to remove contributions by P700 turnover and because of the markedly reduced scattering of this sample type.

All experiments were performed at 4°C in a pH 6.5 low salt buffer medium (50 mM MES, 10 mM NaCl). The samples were excited by 10-ns (FWHM) laser flashes at 532 nm from a frequency-doubled Nd/

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Abbreviations: ^{3}Car, carotenoid triplet; Chl, chlorophyll; DCBQ, 2,6-dichloro-*p*-benzoquinone; FWHM, full width at half maximum; LHC II, light harvesting complex II; MES, morpholinoethane sulfonic acid; P680, photoactive chlorophyll of PS II; PS II, photosystem II; WOC, water oxidising complex; Y_Z, redox active tyrosine between WOC and P680

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YAG laser at a repetition rate of 1 Hz. Absorption changes were fitted with three exponentials and a constant using a Levenberg-Marquart fitting procedure.

3. Results and discussion

The top traces in Fig. 1 show absorption changes at 830 nm induced in dark adapted PS II membrane fragments by a series of 8 saturating laser flashes. The detectable initial amplitudes oscillate because most of the nanosecond kinetics are not observed owing to limited time resolution. Similar period four oscillation patterns were observed previously [15,16]. A data analysis reveals that the microsecond decay comprises at least three different kinetics with half times of 3–5 μs, 20–40 μs and 130–200 μs. In a target fit using fixed half times of 3 μs, 35 μs and 130 μs the normalised amplitudes of the 3-μs and the 35-μs component are strongly oscillating with period four whereas those of the 130-μs phase are virtually independent of the flash number. The oscillation of the 3-μs component is in line with recent findings [16,28] but in contrast to a previous report [15]. As the period four oscillation is a characteristic fingerprint of the reactions in the water oxidising complex (WOC) the 3-μs and 35-μs kinetics reflect $P680^{+\bullet}$ reduction steps in PS II with an intact WOC while the 130-μs kinetics are attributed to centers lacking a competent WOC. The assignment of the 3-μs kinetics is in perfect agreement with the original conclusion on the nature of this reaction [29].

$P680^{+\bullet}$ is known to be a strong quencher [30]. Accordingly the kinetics of $P680^{+\bullet}$ reduction should also be observed in the corresponding flash-induced fluorescence rise. The fluorescence yield changes induced in thylakoids by the same train of saturating laser flashes were monitored with a time resolution of 500 ns and sweep time of about 100 μs (in order to verify that the rise kinetics of the fluorescence yield changes are not obscured by decay kinetics due to $Q_A^- \rightarrow Q_B$ electron transfer, check experiments were performed with longer sweeps of about 400 μs at 4°C). The traces obtained are depicted in the bottom part of Fig. 1. They reveal three characteristic features: (i) a fast rise that is not resolved at 1 μs; (ii) a multiphasic rise in the μs range; and (iii) the maximum level achieved 100 μs after the flash. All three parameters exhibit a pronounced period four oscillation. In samples with destroyed

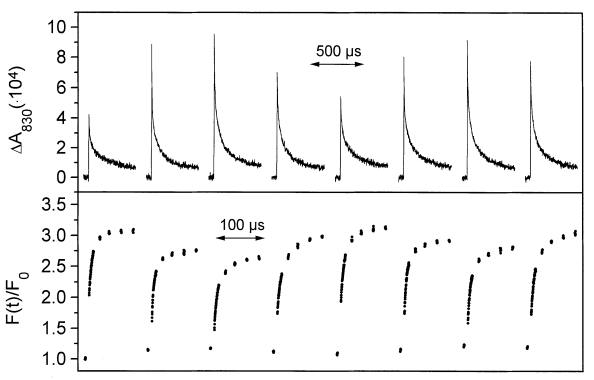

Fig. 1. Changes of absorption at 830 nm (top) and fluorescence (bottom) induced by a train of 8 saturating laser flashes in dark adapted PS II membrane fragments (top) and thylakoids (bottom) from spinach. For further experimental details see Section 2. 30 and 16 signals were averaged for the traces on top and bottom, respectively.

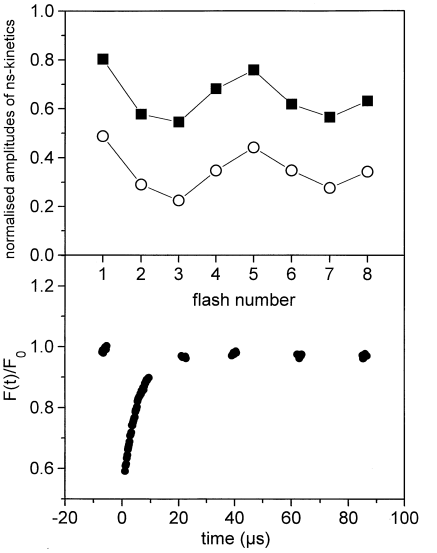

Fig. 2. Normalised amplitudes of the ns kinetics of 830-nm absorption changes (filled squares) and the rise of fluorescence (open circles) as a function of flash number (top) and flash-induced changes of the normalised fluorescence in solubilised LHCII (bottom trace). The data of the top figure were gathered from evaluation of the experimental results presented in Fig. 1 (for further details, see text). The bottom trace is a single flash experiment. For other experimental details see text.

WOC the kinetics are changed and no oscillation is observed [21].

The extent of the normalised value $[F_n(1\ \mu s)-F_0]/[F_{max}-F_0]$ is ascribed to the ns kinetics of $P680^{+\bullet}$ reduction by Y_Z (see [21] and references therein). Therefore its oscillation pattern is expected to be congruent with that of the normalised ns kinetics of 830-nm absorption changes, $\Delta A_n^{830}(ns)/\Delta A_{max}^{830}$, where ΔA_{max}^{830} is the initial amplitude measured in samples completely deprived of a functional WOC (treatment with NH_2OH at high concentrations) because in this case ΔA_{max}^{830} reflects the total amount of photoactive P680 [26]. The data gathered from the experimental results are depicted in the top panel of Fig. 2. A comparison of both patterns readily shows that the extent of the unresolved fast fluorescence rise exhibits virtually the same period four oscillation as $\Delta A_n^{830}(ns)/\Delta A_{max}^{830}$ but the levels of the fluorescence yield are shifted by about 0.25 units towards lower values. The latter phenomenon is due to fluorescence quenching by carotenoid triplets (^{3}Car). This effect cannot be eliminated because excitation with saturating flashes is indispensable to avoid additional misses in the S_i-state turnover that give rise to a blurring of the oscillation (see [21] and references therein). To illustrate the effect of ^{3}Car formation on the flash induced fluorescence transients, experiments were performed with isolated LHC II complexes. The bottom trace in Fig. 2 shows a kinetically unresolved fluorescence decay that is followed by a recovery with a half time of 3 μs which perfectly fits with recent data on the ^{3}Car decay in the same type of LHC II preparation [31]. Accordingly, the measured values of $[F_n(1\ \mu s)-F_o]/[F_{max}-F_o]$ have to be corrected for the fluorescence decrease caused by carotenoid triplet formation. This contribution was recently found to be 0.2–0.3 units [21] which exactly corresponds with the difference between the patterns in Fig. 2 (top). Thus, the findings of Fig. 2 (top) unambiguously

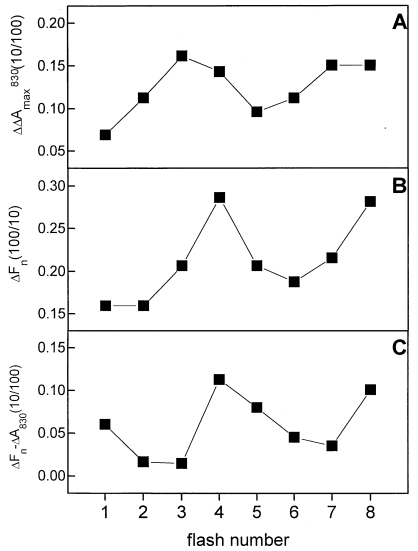

Fig. 3. Normalised extent of the '35-μs kinetics' of the decay of 830-nm absorption change (A), the rise of fluorescence yield (B) and the difference between both parameters (C) as a function of flash number in dark adapted samples. The data were gathered from evaluation of the experimental results presented in Fig. 1 (for further details, see text). The values in pattern C were corrected for ^{3}Car contribution (3%).

show that the corrected $[F_n(1\ \mu s)-F_o]/[F_{max}-F_o]$ values provide a direct measure for $P680^{+\bullet}$ reduction in the ns time domain.

Another consequence of the transient ^{3}Car formation is a marked increase of the 3-μs fluorescence rise coinciding with ^{3}Car decay. This interference prevents a comparative study with the corresponding 3-μs kinetics of 830-nm absorption changes. On the other hand, the analysis of the 35-μs kinetics is only marginally affected. In order to minimise this effect and furthermore to avoid complications by a possible flash dependent variation of the half time, the amplitude of the '35-μs kinetics' was determined in a first order approximation by using the difference of the values measured at 10 μs and 100 μs, respectively, of both the flash induced changes of absorption at 830 nm and the fluorescence yield. Taking into account the different directions, i.e. fluorescence rise and absorption decay caused by $P680^{+\bullet}$ reduction, the following normalised quantities were used: $\Delta\Delta A_n^{830}(10/100) = [\Delta A_n^{830}(10\ \mu s)-\Delta A_n^{830}(100\ \mu s)]/\Delta A_{max}^{830}$, and $\Delta F_n(100/10) = [F_n(100\ \mu s)-F_n(10\ \mu s)]/[F_{max}-F_0]$, where F_{max} is the maximum level of $F_n(100\ \mu s)$ measured in the flash sequence. At this stage of approximation it is not necessary to take into account the non-linear relationship between quencher concentration and fluorescence [32]. The results obtained are depicted in Fig. 3A,B. A comparison of the oscillation patterns readily reveals that in contrast to the ns components (see Fig. 2, top) there exist drastic differences between both parameters $\Delta\Delta A_n^{830}(10/100)$ and $\Delta F_n(100/10)$. The data of the 830-nm absorption changes exhibit the expected features with minima after the 1st and 5th flash and maxima after the 3rd and 7th flash [14–16]. A strikingly different pattern, however, emerges for the normalised fluorescence data that are also characterised by a period four oscillation but exhibit pronounced maxima after the 4th and 8th flash and comparatively small values after the other flashes. The most obvious explanation for this disparity is the conclusion that the 35-μs reduction of $P680^{+\bullet}$ is not simply an electron transfer from Y_Z as in the case of the ns kinetics but comprises an additional coupling and/or competition with other processes. Therefore, questions arise on the nature of these other reactions. As an attempt to obtain further information the differences between both patterns were determined. The results are depicted in Fig. 3C. An inspection of this trace reveals that there is a marked surplus in the extent of $\Delta F_n(100/10)$ in the 1st, 4th, 5th and 8th flash, while the differences are rather small after the 2nd, 3rd, 6th and 7th flash. This pattern is indicative for a dependence on S_0/S_1 and S_2/S_3, respectively. Basically, two alternative explanations can be offered: (i) $\Delta A_n^{830}(10/100)$ reflects $P680^{+\bullet}$ reduction by Y_Z and the surplus of $\Delta F_n(100/10)$ is due to an additional fluorescence rise in S_0 and S_1 which could originate for instance from protolytic reactions at amino acid residues which affect the Chl fluorescence [33]; or (ii) there exists a relaxation process which leads to an almost constant fluorescence rise in each flash. In this case the oscillation pattern of $\Delta F_n(100/10)$ is the result of $P680^{+\bullet}$ reduction by competing electron donors with different quenching properties (e.g. Y_Z, $Q_A^{-\bullet}$). The extent of these reactions oscillates for kinetic reasons as a function of the redox state S_i. The latter effect could also readily explain the oscillation pattern of the 35-μs component of delayed fluorescence emission [34,35].

At present, a distinction between both models cannot be achieved, but it is clear that a transfer of proton and/or hydrogen is involved as detected by marked kinetic H/D isotope exchange effects (Christen and Renger, unpublished results). These details are beyond the scope of this study and will be outlined in a forthcoming paper.

3.1. Concluding remarks

The present study has unambiguously shown that the '35-μs kinetics' of $P680^{+\bullet}$ reduction takes place in PS II with an intact WOC. However, this reaction is not simply an electron transfer from Y_Z to $P680^{+\bullet}$ but is coupled with additional processes (most likely proton and/or hydrogen transfer) which give rise to changes of the fluorescence quantum yield. The underlying mechanism of this interesting effect and its functional relevance remain to be clarified in further studies.

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