Modulation of Flash-Induced Photosystem II Fluorescence by Events Occurring at the Water Oxidizing Complex†

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ABSTRACT: The mechanism of flash-induced changes with a periodicity of four in photosystem II (PSII) fluorescence was investigated with the aim of further using fluorescence measurements as an approach to studying the structural and functional organization of the water-oxidizing complex (WOC). The decay of the flash-induced high fluorescence state of PSII was measured with pulse amplitude modulated fluorometry in thylakoids and PSII enriched membrane fragments. Calculated Q_A^- decay was well described by three exponential decay components, reflecting Q_A^- reoxidation with halftimes of 450 and 860 μs, 2 and 7.6 ms, and 111 and 135 ms in thylakoids and PSII membranes, respectively. The effect of modification of the PSII donor side by changing pH or by removal of the extrinsic 17 and 24 kDa proteins on period four oscillations in both maximum fluorescence yield and the relative contribution of Q_A^- reoxidation reactions was compared to flash-induced oxygen yield. The four-step oxidation of the manganese cluster of the WOC was found to be necessary but not sufficient to produce modulation of PSII fluorescence. The capacity of the WOC to generate molecular oxygen was also required to observe a period four in the fluorescence; however, direct quenching by oxygen was not responsible for the modulation. Potential mechanisms responsible for the periodicity of four in both maximum fluorescence yield pattern and flash-dependent changes in proportion of centers with different Q_A^- reoxidation rates are discussed with respect to intrinsic deprotonation events occurring at the WOC.

In green plants, variable fluorescence under physiological conditions arises from photosystem II (PSII)[1] antenna (for a review, see ref *1*) and provides information on such processes as energy conversion in PSII (*2*), electron transport on the acceptor side of PSII (*3*, *4*), and capacity of the donor side of PSII (*5*). In dark-adapted chloroplasts, a damped binary oscillation as a function of flash number has been observed in the relaxation kinetics of chlorophyll (Chl) *a* fluorescence on a time scale of hundreds of microseconds (*3*). The binary oscillation pattern is suggested to originate from the difference in the rates of Q_A^- reoxidation by the second plastoquinone acceptor (Q_B or Q_B^-). The reaction of Q_A^- reoxidation by Q_B has a halftime of 150−250 μs, while Q_A^- reoxidation by semiquinone Q_B^- proceeds with a halftime of 400−600 μs (*3*, *6*). Joliot and Joliot (*7*) showed that the Chl *a* fluorescence yield measured at 74 ms and 2 s after excitation flashes had a periodicity of four, with minima after the fourth and the eighth flashes. They suggested increased quenching of fluorescence by the water-oxidizing complex (WOC) in the S_0 and S_1 states compared to the S_2 and S_3 states to explain this oscillatory pattern. Delosme (*8*) and Zankel (*9*) also observed a period of four oscillation in the fluorescence yield measured in chloroplasts on a microsecond time scale, with minima after the third and the seventh flashes. The pattern was shown to correspond well to the change of a sum of the S_2 and S_3 populations just before the flashes. However, as opposed to the quenching properties at long times ($t > 70$ ms) (*7*), the capacity of the WOC in the S_2 and S_3 states to quench fluorescence at short times ($t < 80$ μs) (*8*, *9*) is higher than that of the WOC in the S_0 and S_1 states. The period four modulation is eliminated by 100 mM methylamine, which does not alter flash-induced oxygen production (*9*).

A similarity between oscillation patterns of flash-induced absorption changes at 515 nm (electrochromic band shifts of carotenoids) and flash-induced Chl *a* fluorescence yield measured in thylakoid membranes at 80 and 800 ms after excitation flashes has been found (*10*). The absorption changes as a function of flash number have been suggested to reflect an appearance of a surplus of charge in the S_2 state (*11*). To explain the presence of uncompensated positive charge in the S_2 and S_3 states, it has been assumed that an intrinsic stoichiometry of protons released from the WOC

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[1] Abbreviations: CCCP, carbonyl cyanide-*m*-chlorophenylhydrazone; Chl, chlorophyll; cyt b_{559}, cytochrome b_{559}; DCBQ, 2,6-dichloro-*p*-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; F_{max}, maximal fluorescence; F_0, minimal fluorescence with open reaction centers; $F(t)$, fluorescence at time t after excitation flashes; fwhm, full width at half-maximum; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; PAM fluorometer, pulse amplitude modulated fluorometer; PSII, Photosystem II.; P_{680}, redox active specially bound chlorophyll *a* within the PSII reaction center; Q_A and Q_B, primary and secondary plastoquinone electron acceptors of photosystem II; S_i, redox state *i* of the water oxidase; WOC, water-oxidizing complex; Y_z, redox-active tyrosine of polypeptide D1 that serves as electron donor to $P680^+$.

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per P_{680} during S-state turnover is 1:0:1:2 for the S_0-S_1, S_1-S_2, S_2-S_3, and $S_3-(S_4)-S_0$ transitions, respectively (*11*, *12*). Brettel at al. (*13*) observed a retardation of electron transfer to P_{680}^+ in the S_2 and S_3 states compared to the S_0 and S_1 states. This effect was also explained in terms of the appearance of uncompensated positive charge in the S_2 state due to the above-mentioned intrinsic proton release pattern. However, extrinsic proton release to a solution, determined on the basis of measurements with sensitive glass electrodes, deviates from the intrinsic proton release and depends on pH (*14*, *15*). For example at pH 6.5 the stoichiometry of the extrinsic proton release was found to be 1.3:0.1:0.95:1.65. Such a noninteger pattern has been interpreted in terms of a combination of specific deprotonations and electrostatically induced pK_a shifts of protonatable amino acid residues. The debate over whether the extrinsic proton release directly reflects deprotonation events in the immediate vicinity of the WOC or is related to pK_a changes of amino acid residues located far from the Mn cluster of the WOC remains unresolved (for a review, see ref *16*).

A thorough analysis of the fluorescence yield oscillation pattern and the flash-induced absorption changes at 515 nm led Delrieu and Rosengard (*10*) to the conclusion that the physical processes underlying these two data sets are different. Electric field localized in the vicinity of the Mn cluster of the WOC and Y_z, was suggested to influence the fluorescence yield by a change in the free energy of the PSII charge separation reactions (*17*, *18*). In contrast, the absorption changes at 515 nm were suggested to be affected by electric fields far from the Mn cluster. Delrieu and Rosengard (*10*) concluded that S_2 associated charge surplus stably accumulated near the Mn cluster in a population of centers was responsible for the modulation of the flash-induced Chl *a* fluorescence yield measured on a long time scale ($t > 80$ ms).

In all previous works fluorescence yields have been studied at certain fixed time delays after excitation flashes. Recently Shinkarev et al. (*19*) attempted to extract more information about the nature of a period four modulation of the Chl *a* fluorescence by analyzing its decay in a time interval from 70 μs to 50 ms. Their study revealed the presence of a quencher of the Chl *a* fluorescence arising with a delay of approximately 0.5 ms after excitation. Flash-number dependent changes in the amount of this quencher were characterized by a periodicity of four, with maxima after the third and the seventh flashes. It was proposed that the quencher is a product forming during the $S_3-(S_4)-S_0$ transition. The concentration of the quencher did not decrease significantly at least until 50 ms after the excitation. Accordingly, photosynthetic oxygen could only partly be responsible for this quenching.

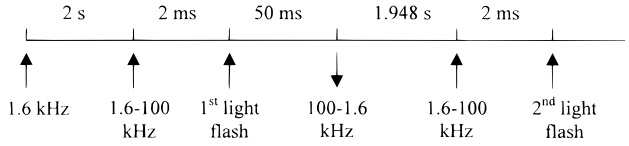
The mechanisms underlying period four oscillations in the Chl *a* fluorescence yield are complex, and no agreement has been achieved in interpretation of the experimental data. Clarification of the given question would make fluorescence measurements a powerful tool to study photosynthetic water oxidation. It is possible to selectively alter individual processes occurring at the WOC in PSII-enriched membrane fragments and thereby to examine the effects of these processes on the fluorescence decay. In this work we have approached the problem by measuring the decay kinetics of the flash-induced high fluorescence state of PSII along with flash-induced oxygen yield and photoinduced changes in the PSII fluorescence yield in thylakoids and PSII-enriched membrane fragments with modified WOC. Calculated Q_A^- decay was fit with a sum of three exponential decays. The decay components reflecting reoxidation of Q_A^- with different rates and maximum fluorescence yield show different oscillation patterns and different responses to modifications of the WOC. Our data do not support the idea that photosynthetic oxygen or a product forming during the $S_3-(S_4)-S_0$ transition is responsible for the period four modulations of fluorescence. Instead, such modulations reflect a control over the PSII charge separation and Q_B site properties by uncompensated positive charge in the S_2 and S_3 states of the WOC.

MATERIALS AND METHODS

Thylakoids were isolated from freshly harvested spinach as described in Whitmarsh and Ort (*20*) and stored at −80 °C at a concentration of about 2 mg of Chl/mL. For measurements, the thylakoids were diluted in buffer 1 (0.3 M sucrose, 40 mM Mes−NaOH, pH 6.5, and 35 mM NaCl) to a final concentration. PSII-enriched membrane fragments (PSII membranes) were prepared from the spinach using Triton X-100 (*21*). After thawing at 4 °C, thylakoids and PSII membranes were washed and resuspended in buffer 1. Thawing and washing of the preparations were carried out under dim green light (20−25 min). Before measurements, the preparations were dark-adapted for an additional 20 min at 4 °C. The rate of oxygen evolution under continuous illumination of PSII membranes with 0.6 mM $K_3Fe(CN)_6$ and 0.6 mM DCBQ as electron acceptors was 300−350 μmol of O_2 (mg of Chl)$^{-1}$ h^{-1}.

To remove the extrinsic 24 and 17 kDa polypeptides, PSII membranes were diluted in a medium containing 1 M NaCl, 30 mM Mes−NaOH, pH 6.5, and 0.3 M sucrose to a concentration of 2 mg of Chl/mL (*22*). After 30 min incubation at 4 °C under room light the PSII membranes were pelleted. The pellet was washed twice and resuspended in buffer 1.

Decay kinetics of the high fluorescence state of PSII following single-turnover saturating light flashes and photoinduced changes in fluorescence yield were measured with a pulse amplitude modulated fluorometer (PAM 101, Walz, Germany). A light-emitting diode (type NSPB, Nichia, emission maximum at 465 nm, 30 nm half-width) produced measuring light pulses of 1 μs duration at frequencies of 1.6 or 100 kHz. A short-pass filter (SP 695, Schott) removed spurious longer wavelength emission of the LED. A PIN-photodiode (type S 3590−01, Hamamatsu, 10 × 10 mm active area, 35 MHz bandwidth, 5 nA dark current) was protected by a long-pass filter (KC-19, Mashpriborintorg, Russia) against reflected and scattered measuring light. Low-frequency (1.6 kHz) measuring light switched to high-frequency (100 kHz) measuring light 2 ms before excitation flashes and then back after 50 ms according to the following scheme:



Signals from the PAM were digitized by a home-built ADC with data acquisition program (Brock electronics shop). Data points were sampled every 50 μs for the fluorescence decay measurements and 10 decays were averaged in each experiment.

Flash-induced O_2 evolution was measured with a Clark-type horizontal electrode covered by a 5 μm Teflon membrane in a home-built microcell with 5 μL volume and a 0.3 mm sample thickness (*23*). A high-accuracy oxygen polarograph was built according to a scheme described previously by Meunier and Popovic (*24*). The difference current required to keep polarization between working (Pt) and reference (Ag) electrodes at 700 mV was amplified and digitized by the above-mentioned ADC with the data acquisition program.

Flash-induced O_2 yield and fluorescence in PSII membranes were measured in the presence of 1 mM $K_3Fe(CN)_6$, 1.2 mM DCBQ, or 0.7−2 μM DCBQ, respectively, added to the assay medium 2 min before light flashes. Concentration of the preparations was 8−10 μg of Chl/mL for fluorescence measurements and 300−350 μg of Chl/mL for O_2 yield measurements. All measurements were done at room temperature.

A xenon flash lamp (FX-224, EG&G, Princeton) with a 9 μs fwhm was used in fluorescence and O_2 yield measurements. The flash frequency was 0.5 Hz. An incandescent lamp (KL 1500 Electronic, Walz, Germany) was used in measurements of photoinduced changes in fluorescence yield. The sample was illuminated for 1 s with 400 $\mu M/m^2s$ white light.

Normalized concentration of Q_A^- was calculated, assuming a nonlinear relationship between the fluorescence yield $F(t)$ and $[Q_A^-(t)]$ (*25*):

$$\frac{F_{(t)} - F_0}{F_{max} - F_0} = b[Q_A^-(t)] + (1 - b)\frac{(1 - p)[Q_A^-(t)]}{1 - p[Q_A^-(t)]}$$

Here, F_{max} is the maximum fluorescence yield, F_0 is the fluorescence yield before flashes (when all Q_A is in the oxidized state), b is the fraction of PSII units which are not connected via interunit excitation energy transfer, and p is the interunit excitation energy transfer probability. Values of b and p were 0.3 and 0.5, respectively, as described previously (*26*, *27*).

The fluorescence decay kinetics were fit using the MICROCAL ORIGIN 4.1 program. Analysis of oscillation patterns based on the Kok model (*28*) was performed using a genetic algorithm (*29*). The fit program was based on the formula

$$S_i^{n+1} = (1 - \alpha - \beta)S_{i-1}{}^n + \alpha S_i^n + \beta S_{i-2}{}^n$$

where n is a flash number and α and β are probabilities of misses and double hits, correspondingly. Simulation of the $Q_B(Q_B^-)$ binary oscillation pattern was performed using the formula

$$Q_B(Q_B^-)^{n+1} = (1 - \alpha - \beta)Q_B^-(Q_B)^n + \alpha Q_B(Q_B^-)^n + \beta Q_B(Q_B^-)^n$$

Parameter z, which represents a number of closed centers

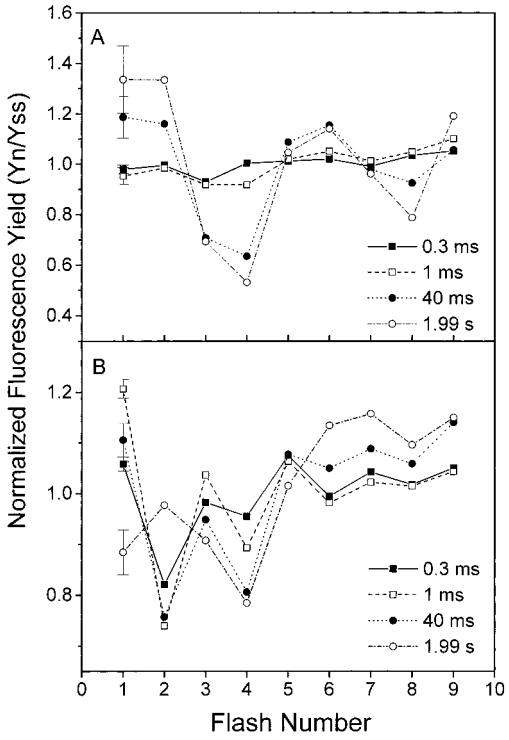


FIGURE 1: Normalized Chl *a* fluorescence yield (Y_n/Y_{ss}) measured as a function of flash number in thylakoids (10 μg of Chl/mL) (A) and PSII membranes (8 μg of Chl/mL) (B) at time t after excitation flashes. t = 300 μs, 1 ms, 40 ms, and 1.99 s. Dark time between the flashes was 2 s. The assay medium contained 40 mM Mes−NaOH, pH 6.5, 35 mM NaCl, and 300 mM sucrose. 0.7 μM DCBQ (final concentration) was added to the assay medium for PSII membranes.

with reduced Q_A forming after each flash (*30*, *31*), was used in the analysis of fluorescence oscillation patterns.

RESULTS

Flash-Number Dependent Changes in the Fluorescence Yield in Thylakoids and PSII Membranes. The fluorescence yield, measured in thylakoids at pH 6.5 and plotted as a function of flash number at various times after the excitation flashes (Figure 1A), is characterized by a periodicity of four that reflects the four-step process of water oxidation (*7*, *9*). The period four oscillation pattern has minima after the third and the seventh flashes at short times (t < 700 μs) and after the fourth and the eighth flashes at longer times (t > 700 μs). By using pump and probe fluorometry (*19*, *32*) and fast repetition rate fluorometry (*33*), earlier investigators showed the same modulation pattern of the fluorescence yield in thylakoids. For measurements of the fluorescence relaxation kinetics as a function of flash number in PSII membranes, 0.7 μM DCBQ was added to the assay medium. At this concentration DCBQ acts effectively as an electron acceptor and does not quench fluorescence directly. In contrast to thylakoids, the fluorescence yield measured in PSII membranes after the first flash was significantly higher than that of the second flash (Figure 1B). This difference was also observed in the absence of DCBQ when the fluorescence yield was measured only on the first two flashes (data not shown) and thus not caused by the acceptor. An irregular period two oscillation in the fluorescence yield was seen in PSII membranes at all time intervals except the yield measured 1.99 s after the flashes which had minima after

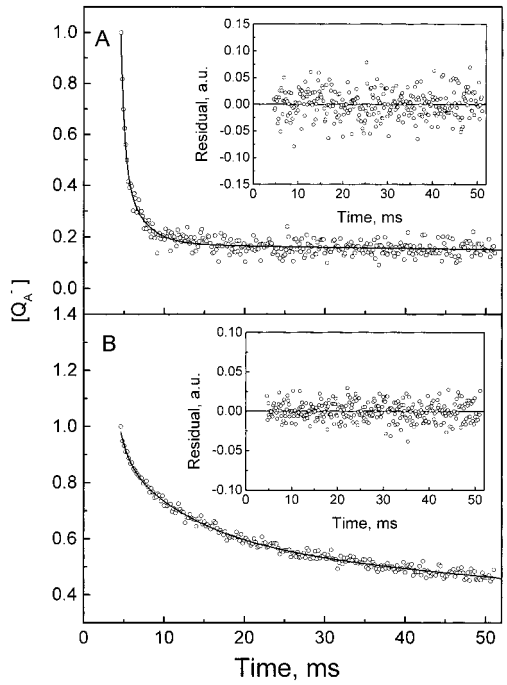


FIGURE 2: Kinetics of Q_A^- reoxidation, which were calculated from the decay of PSII high fluorescence state following the first single-turnover light flash (open circles), and the theoretical fit assuming three exponential decay components (solid line) in thylakoids (A) and PSII membranes (B). The half-times were kept constant at 450 μs, 2 ms, and 111 ms and 860 μs, 7.6 ms, and 135 ms for thylakoids and PSII membranes, respectively. The small panels show the weighted difference between the experimental data and the theoretical fit curve. Reduced χ^2 values were 1.09 and 1.16 for thylakoids and PSII membranes, respectively.

the fourth and the eighth light flashes. The binary oscillation reflects the two-step process of Q_B reduction (*3*).

Analysis of the Q_A^- Decay Kinetics in Thylakoids and PSII Membranes. Q_A^- reoxidation involves reactions with different rates (*32*, *34*). These reactions may be modulated by processes occurring at the WOC in different ways. To separate these reactions and to obtain their modulation patterns, we analyzed the Q_A^- decay in thylakoids as well as PSII membranes with a model function, a sum of exponential decay components with offset:

$$[Q_A^-(t)] = \sum_{i=1}^{n} A_i \exp(-t/\tau_i) + [Q_A^-(t = 2s)]$$

Here A_i are amplitudes of decay components, τ_i are lifetimes of these components, and $[Q_A^-(t = 2\ \mathrm{s})]$ is concentration of Q_A^-, which remains reduced 2 s after the excitation flashes. In this way, flash-induced changes in amplitudes of the decay components reflect flash-number dependent difference in a relative contribution of reactions with different rates to Q_A^- reoxidation. It was found that three components were required for satisfactory description of the data (Figure 2 and Table 1). A cross-correlation between the model parameters was observed when the decay kinetics from different flash numbers were fit independently, assuming free running half-times. Therefore, in our work we fit the decay kinetics for different flash numbers simultaneously, assuming the lifetimes to be independent of flash number. In this case, reduced χ^2 increased insignificantly in comparison to that generated for the model with free running halftimes (Table 1).

Flash-number dependent changes in amplitudes of all components of the Q_A^- decay in thylakoids were characterized by a periodicity of four (Figure 3A). It is difficult to assign the oscillation pattern of the fast ($t_{1/2} = 450$ μs) and the middle ($t_{1/2} = 2$ ms) decay component to flash-dependent changes in individual S population(s) due to the rates of S-state transitions ($t_{1/2} = 30-1300$ μs (*35*)). Most of the decay of the slow component (half-time of 111 ms) occurs on a time scale after all S-state transitions have occurred. Thus, the Kok model can be applied for the analysis of its oscillation pattern. Assuming the WOC in the S_0 and S_1 states to be a more efficient fluorescence quencher than in the S_2 and S_3 states, we were able to achieve a good fit to the data (Table 2, Figure 4A).

In comparison to thylakoids, Q_A^- reoxidation in PSII membranes proceeds more slowly, and the contribution of the slow reactions to the reoxidation increases (Figure 3B). The middle component ($t_{1/2} = 7.6$ ms) was modulated with a clear period two. The amplitudes of the fast ($t_{1/2} = 860$ μs) and the slow ($t_{1/2} = 135$ ms) decay components were characterized by complex oscillation patterns that were the sum of a binary oscillation and a period four oscillation. Taking into account the effect of the Q_B redox states on the fluorescence yield and using the same values of parameters α and β for both periods, we simulated well the oscillation pattern of the slow component (Figure 4A). Period two and period four oscillations obtained by decomposition of the pattern are shown in Figure 4B. The periodicity of four in the slow component is similar to the slow component pattern in thylakoids. This result suggests that the nature of period four modulation of the fluorescence in thylakoids and PSII membranes is the same.

Despite some differences in the experimental conditions (see Materials and Methods), parameters of the Kok model used for the simulation of the slow component pattern match well those of the O_2 yield pattern in both thylakoids and PSII membranes (Table 2). This implies that PAM fluorometry is suitable for detecting S-state dependent changes in the fluorescence, and weak high frequency (100 kHz) measuring light does not randomize the S populations. It should also be noted that due to the 2 s dark interval between flashes, Y_D can reduce the Mn cluster in the S_2 and S_3 states in some centers. Therefore the S_0/S_1 ratio obtained from the simulation of the slow decay component and O_2 yield patterns may not correspond to a true S_0/S_1 ratio calculated in experiments with closely spaced flashes.

Effect of CCCP on the Oscillation Pattern of the Decay Components. To prove the effect of S-state turnover on Q_A^-, we used CCCP, a lipophilic uncoupler of photophosphorylation in chloroplasts (*36*), known to accelerate the decay of the S_2 and S_3 states (*37*, *38*). The presence of 5 μM CCCP completely inhibited flash-induced O_2 production by thylakoids and eliminated period four modulations of the decay components (Figure 3C). Appearance of a period two behavior in oscillation patterns of all components and an increase in the contribution of the slow decay component ($t_{1/2} = 59$ ms) to Q_A^- reoxidation compared to the control also occurred. The enhanced binary oscillation may be explained by an additional effect of CCCP on Q_B/Q_B^- distribution in darkness. Addition of 5 μM CCCP to PSII membranes tended to simplify the binary oscillation pattern (Figure 3D). No significant changes were seen in the half-

Table 1: Reduced χ^2 Values Generated for Exponential Decay Models of the Q_A^- Decay in Thylakoids and PSII Membranes

	two exponential decay model, free running half-times	three exponential decay model, free running half-times	three exponential decay model, fixed half-times
thylakoids	1.26	1.07	1.09
PSII membranes	1.47	1.14	1.16

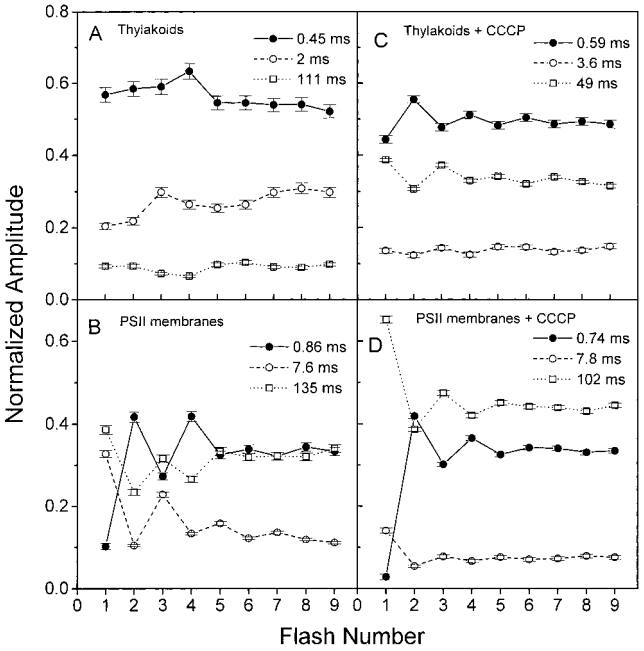


FIGURE 3: Components of the Q_A^- decay as a function of flash number in thylakoids (A, C) and PSII membranes (B, D) in the absence (A, B) and in the presence of 5 μM CCCP (C, D). $t_{1/2}$ = halftimes of the components ($t_{1/2} = \ln 2(\tau)$).The O_2 yield on the third light flash in thylakoids and PSII membranes in the presence of CCCP was inhibited by 100% and 95%, respectively, compared to the control.

Table 2: Parameters of the Kok Model, the Number of Closed Reaction Centers (z), and Q_B/Q_B^- Populations in the Dark Used for Simulation of the O_2 Yield and the 111 (135) ms Decay Component Patterns in Thylakoids and PSII Membranes

oscillation pattern	S_0	S_1	α	β	z	Q_B	Q_B^-
O_2 yield in thylakoids	12%	88%	0.11	0.04	*a*		
O_2 yield in PSII membranes	17%	83%	0.16	0.06			
111 ms component[b] (thylakoids)	13%	87%	0.1	0.05	0.3%		
135 ms component (PSII membranes)	16%	84%	0.12	0.07	0.8%	85%	15%

[a] Parameters were not required for satisfactory description of the oscillation patterns. [b] The simulation of the decay components patterns assumed the WOC in the S_0 and S_1 states to be a more efficient fluorescence quencher than in the S_2 and S_3 states. The S_2 population was taken into account in all calculations, but was negligible.

times of the decay components in thylakoids or PSII membranes in response to CCCP. It is interesting to note that there was a big difference between the relative contribution of the fast and the slow decay components observed on the first and following flashes in PSII membranes in the presence of CCCP. This indicates a smaller population of centers, in which Q_A^- reoxidation proceeds quickly, in dark-adapted PSII membranes in comparison to PSII membranes illuminated with one or more light flashes. Such a difference could be explained by photoinduced binding of a plastoquinone molecule at the Q_B site in a population of centers in which presence of CCCP in the dark caused a release of plastoquinone. These results clearly show that CCCP affects the Q_B site prior to illumination. This effect, which is more pronounced in PSII membranes, is probably due to an increase in the microviscosity of the thylakoid membrane in the presence of CCCP (*39*, *40*).

Effect of Modification of the WOC in PSII Membranes on the Oscillation Patterns of the Decay Components. To determine which events occurring during the process of water oxidation modulate the fluorescence yield, we modified the WOC in PSII membranes in different ways. Changing the pH of the assay medium allowed us to alter the stoichiometry of proton release during S-state transitions with a minimal modification of the acceptor side of PSII. This allowed us to examine the effects of deprotonation events on the relative contribution of the Q_A^- reoxidation reactions. The oscillation patterns of the decay components were not altered remarkably at pH 5.5 in comparison to the control (Figure 5A). However, at pH 7.6 the contribution of the periodicity of four to the oscillation pattern of the fast component diminished by about 60%, and the slow component was modulated with a clear period two (Figure 5B). The lifetimes of the decay components also changed slightly at pH 7.6 compared to the control. Two exponential decay components were required to model the Q_A^- decay in NaCl-treated PSII membranes (Figure 5C). Q_A^- reoxidation in such PSII membranes proceeded mostly through the slow reaction ($t_{1/2}$ = 235 ms), and period four and period two oscillations in both decay components were absent.

Modulation of the F_{max} Yield. To reveal the effect of events occurring at the WOC on charge separation in the PSII reaction center, we measured flash-number dependent changes in the F_{max} yield (Figure 6). The F_{max} yield measured in thylakoids oscillated with a periodicity of four, with minima after the third and the seventh flashes. In PSII membranes the F_{max} yield showed a complex oscillation pattern including period two and period four oscillations. The presence of CCCP eliminated a period four oscillation in the F_{max} yield in thylakoids and PSII membranes and caused appearance of a weak binary oscillation. No remarkable changes were observed in the F_{max} yield oscillation pattern in PSII membranes at pH 5.5. At the same time, at pH 7.6 a periodicity of two dominated the pattern. NaCl-treated PSII membranes showed neither period four nor period two oscillations in the F_{max} yield. These results show that the oscillatory behavior of both F_{max} and relative contribution of Q_A^- reoxidation reactions are similar in response to the modifications of the WOC properties.

Influence of Modification of the WOC on the Activity of PSII Membranes. Effects of the applied modification procedures on electron transport from the donor side of PSII were determined from measurements of the rise time and magnitude of variable fluorescence in PSII membranes (Table 3). The magnitude of variable fluorescence was markedly decreased only in NaCl-treated membranes. This could result from a strong donor side inhibition, changes in excitation

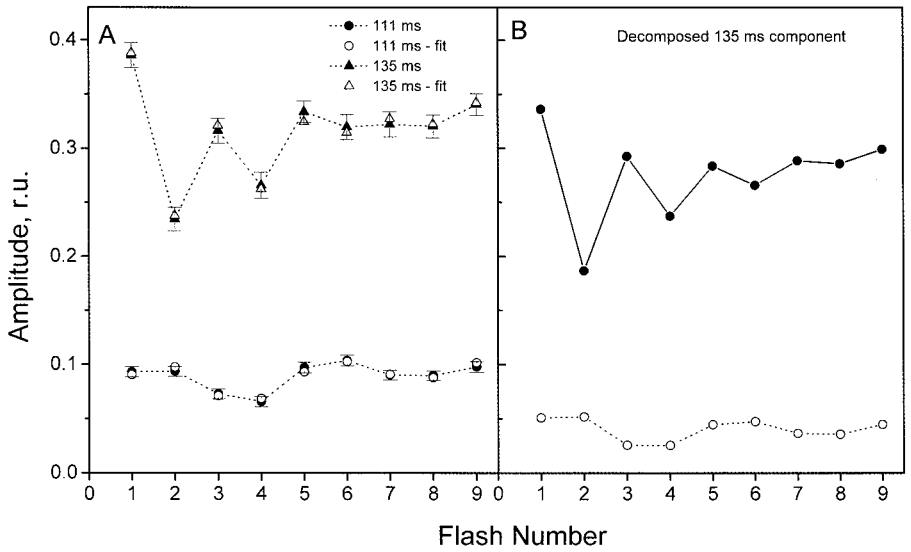


FIGURE 4: Comparison of the oscillation patterns of the 111 and 135 ms decay components in thylakoids and PSII membranes, respectively, with fit (A), using the Kok model and assuming the WOC in S_0 and S_1 states to be a more efficient quencher than in the S_2 and S_3 states. Conditions of the fit procedure are indicated in Table 2. Right panel (B) shows oscillation patterns obtained by decomposition of the 135 ms component pattern on period two and period four behaviors.

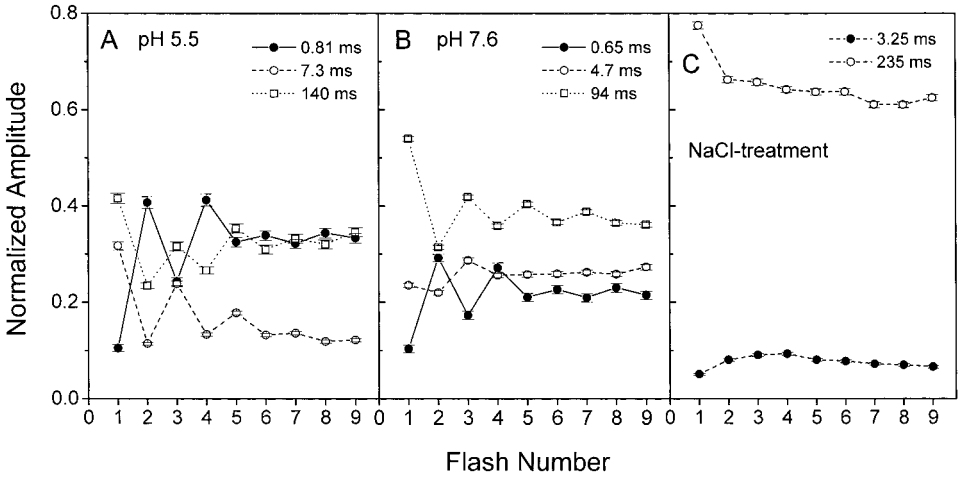


FIGURE 5: Components of the Q_A^- decay as a function of flash number in PSII membranes at pH 5.5 (A), at pH 7.6 (B), and in NaCl-treated PSII membranes (C). The assay medium with pH 5.5 and 7.6 contained 40 mM MES−NaOH and 10 mM Hepes−NaOH, respectively.

energy transfer and/or charge separation efficiency, or formation of a small population of quenching centers. The rise time of variable fluorescence was increased in the presence of 5 μM CCCP and in NaCl-treated PSII membranes indicating a significant decrease in donor side electron transport. The decay of variable fluorescence associated with reoxidation of the plastoquinone pool was slowed the most in the presence of CCCP.

No significant inhibition of flash-induced O_2 yield was seen in PSII membranes at pH 5.5 (Figure 7). A decrease in the yield by 80% was observed in PSII membranes at pH 7.6. Such a decrease correlates with a diminution of the contribution of the periodicity of four to the oscillation pattern of the fast component at this pH (compare Figure 5 with Figure 7). However, the flash-induced O_2 yield decreased to only 60% of its original magnitude after NaCl-treatment while flash-dependent changes in the amplitudes of the decay components with periods two and four were completely eliminated.

DISCUSSION

Origin of the Decay Components. Our studies confirm the complex kinetics of reoxidation of Q_A^- in thylakoids and in PSII membranes. The fastest component of this process, with half-times of 450 and 860 μs in thylakoids and PSII membranes, respectively, reflects electron transfer from Q_A^- to Q_B in active reaction centers, with plastoquinone bound to the Q_B site before excitation (*41*). These centers are identified as type A centers in Figure 8. The middle component ($t_{1/2}$ = 2 (7.6) ms) is likely associated with reoxidation of Q_A^- in centers with an empty Q_B site in the dark (*41*). In such reaction centers (type B) Q_A^- reoxidation would be limited by diffusion of a plastoquinone molecule to the empty Q_B site (Figure 8). The half-times of the fast and the middle decay components presented in this report do not quite correspond to those previously reported by Renger et al. (*34*) for thylakoids and PSII membranes (300 (670) μs and 3.3 (10.5) ms). This could result from differences in PSII preparation, growth conditions, or spinach type; however, it may also reflect the assumption in our

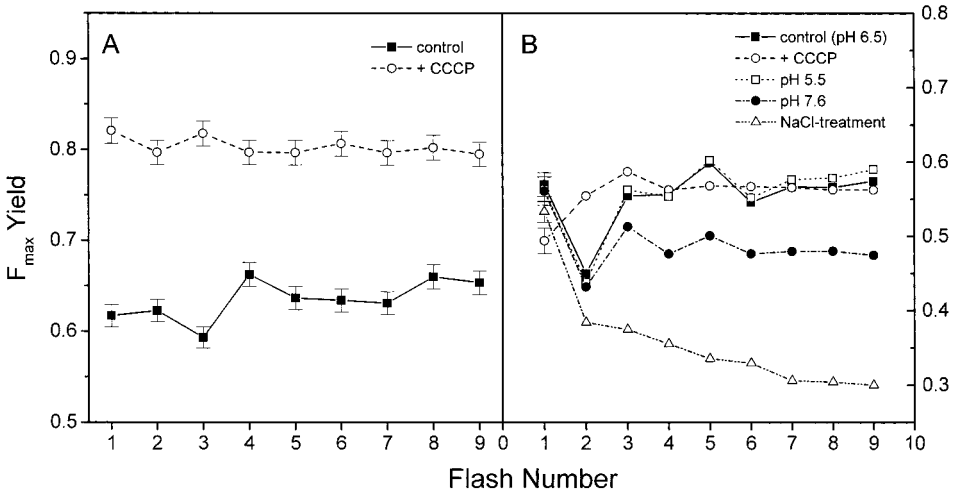


FIGURE 6: F_{max} yield as a function of flash number in thylakoids (A) and PSII membranes (B) in the absence (control) and in the presence of 5 μM CCCP, at pH 5.5 and 7.6 (B), and in NaCl-treated PSII membranes. The assay medium contained 40 mM Mes−NaOH, pH 6.5 (5.5), or 10 mM Hepes−NaOH, pH 7.6, 35 mM NaCl, and 300 mM sucrose. 0.7−2 μM DCBQ (final concentration) was added to the assay medium for PSII membranes.

Table 3: Rise Time, Decay Rate, and Magnitude of Variable Fluorescence (F_v/F_0) in PSII Membranes at Different pH of the Assay Medium, in the Presence of 5 μM CCCP or in NaCl-Treated PSII Membranes

modification	rise time ($t_{1/2}$, ms)	decay rate ($t_{1/2}$, s)	F_v/F_0[a]
pH 6.5 (control)	27	0.6	1
pH 5.5	27	0.6	1
pH 7.6	28	0.8	0.94
5 μM CCCP	39	1	0.97
NaCl treatment	46	0.7	0.78

[a] Magnitude of variable fluorescence was set as 1 for the control.

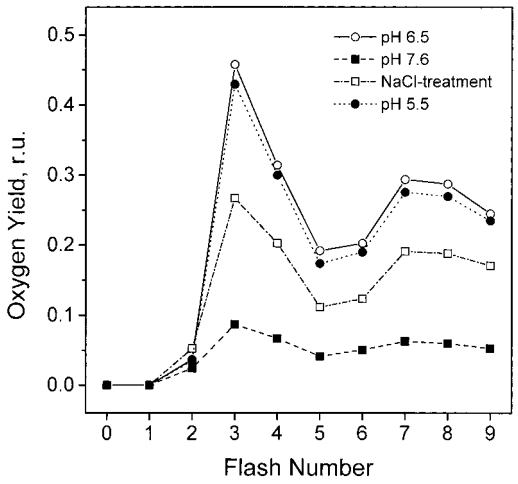


FIGURE 7: Flash-induced O_2 yield measured in PSII membranes (350 μg of Chl/mL) at different values of pH of the assay medium (5.5, 6.5 (control), and 7.6) and in NaCl-treated PSII membranes. Dark time between flashes was 2 s. The assay medium contained 40 mM Mes−NaOH, pH 6.5 (5.5), or 10 mM Hepes−NaOH, pH 7.6, 35 mM NaCl, 300 mM sucrose, 1 mM $K_3Fe(CN)_6$, and 1.2 mM DCBQ.

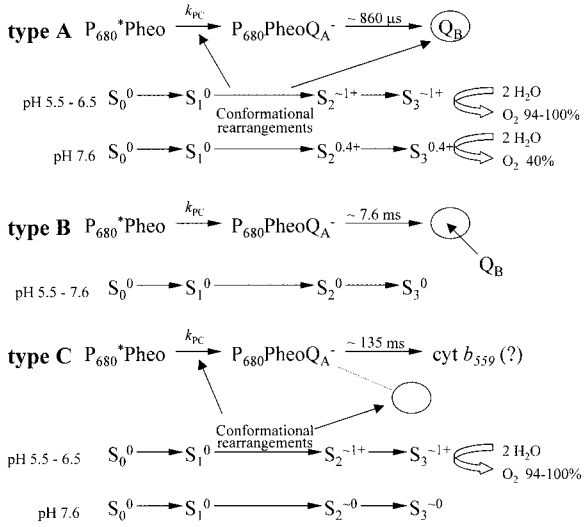


FIGURE 8: Proposed mechanism for period four modulation of the Chl *a* fluorescence in PSII membranes at different pH. Centers in which Q_A^- reoxidation proceeds with half-times of 860 μs, 7.6 ms, and 135 ms are identified as type A, B, and C centers, respectively. In type A centers the Q_B site is occupied by plastoquinone before excitation. In type B centers this site is empty in the dark, and Q_A^- reoxidation is limited by diffusion of plastoquinone to the Q_B site. Type C centers are considered as inactive centers with an empty Q_B site, and therefore, Q_A^- is reoxidized by cyt b_{559}. K_{PC} represents the rate constant of primary charge separation. Numbers with the S states signify values of uncompensated positive charge in these states. According to the proposed mechanism, appearance of the uncompensated positive charge in the S_2 and S_3 states (except type B centers) results in conformational rearrangements in the PSII reaction center which affect both charge separation reactions and plastoquinone-binding properties of the Q_B site (see text). Type A and C centers also differ in values of the uncompensated positive charge in the S_2 and S_3 states at pH 5.5−6.5 and 7.6.

model that half-times of the decay components were independent of the flash number and correspond to an averaged magnitude calculated for all kinetics.

The half-times of the slow component determined in our work as 111 ms for thylakoids and 135 ms for PSII membranes significantly vary from those reported by Renger et al. (*34*) (590 and 290 ms). It has been proposed that the slow component reflects the reoxidation of Q_A^- by the Mn cluster of the WOC in the S_2 (S_3) state in inactive centers, lacking the capability of Q_A^- reoxidation via Q_B (*32*, *34*). This conclusion is based on the fact that in the presence of DCMU a redox reaction between the Mn cluster and Q_A^- occurs ($t_{1/2} = 1.5$ s) (*42*). It was also found that in a fraction of PSII reaction centers reoxidation of Q_A^- was slow, with a half-time of 1.5 ± 0.3 s (*43*), and the WOC in these centers

functioned at physiological rates in the presence of exogenous quinone acceptors (*44*). However, the electron acceptor for Q_A^- reoxidation in such centers was not identified. Our data do not support the conclusion stated above for the following reasons. First, the halftimes of the Q_A^- reoxidation in the inactive centers and back-reaction between Q_A^- and the Mn cluster are significantly slower than those found in the present study. Second, if the slow decay component reflected a redox reaction with a participation of the Mn cluster of the WOC in the S_2 or S_3 states, addition of CCCP should eliminate the contribution of this component. Instead of this, addition of CCCP caused an increase in the contribution of the slow component. Meanwhile, the half-time of the slow component in thylakoids (111 ms) is close to that of the reduction of cyt b_{559}, measured in thylakoids at pH 7.8 ($t_{1/2}$ = 100 ms) (*45*). On the basis of comparisons of the half-time of cyt b_{559} reduction and maximum halftime for the reduction of the plastoquinone pool (6−10 ms), Whitmarsh and Cramer (*45*) concluded that cyt b_{559} is an acceptor for no more than 1 of every 10 electrons accepted by the pool. There are controversies concerning the source of the electrons for oxidized cyt b_{559}. Since DCMU concentrations which appeared to block electron transport from water to methyl viologen were found to only partially inhibit the rate of cyt b_{559} photoreduction, Samson and Fork (*46*) suggested reduced Q_A^- as a reductant to cyt b_{559}. At the same time, Buser et al. (*47*) found that DCMU inhibited the reduction of cyt b_{559} under conditions where the plastoquinone pool and Q_A were reduced. Thus, they concluded that $Q_B^{\bullet -}(H^+)$ or Q_BH_2 was the most likely source of the reduction of the oxidized cyt b_{559}. In contrast to thylakoids, the halftime of the reduction of cyt b_{559} in PSII membranes was reported to be drastically slower (70 s at pH 8.0) (*47*). However, the alkaline pH of the assay medium in that study, although not effecting flash-induced oxygen yield in thylakoids (*15*), would strongly inhibit the oxygen flash yield in PSII membranes (pH 7.6) (Figure 7). Therefore, one can expect the rate of the reoxidation of cyt b_{559} to be different in PSII membranes at pH 6.5 and 8.0 due to damage of the donor side. Physical differences between active and inactive reaction centers could be related to heterogeneity exhibited by cyt b_{559} (*48*). We speculate that the slow decay component reflects the reoxidation of Q_A^- by cyt b_{559} in inactive centers with modified Q_B site. This assumption can be supported by the fact that the population of PSII centers with the slow Q_A^- reoxidation rate (type C centers in Figure 8) is smaller in thylakoids than in PSII membranes and increases in the modified PSII membranes. However, further studies are required to confirm this suggestion.

Analysis of the Q_A^- decay in thylakoids and PSII membranes shows that type B centers in these preparations differ in properties of the WOC. Thus the amount of such centers in PSII membranes as opposed to thylakoids was not S-state dependent and they seemed to be incapable of O_2 production. Such a difference is likely due to damage of these centers during preparation of PSII membranes. There is also variance in the WOC properties between type A and type C centers in PSII membranes at different pH. On the basis of these results, one can consider Q_B site properties to be related somehow to capacity of the WOC.

Interestingly, the period two changes in the amount of the type A centers and the type B and type C centers are opposite in phase. It is not possible to observe this in control thylakoids due to the absence of a binary oscillation in the fluorescence; however, the same phase shift was also seen in thylakoids in the presence of CCCP. These results imply that the proportion of active and inactive PSII reaction centers is dependent on the redox state of Q_B.

Which Process Is Responsible for Period Four Oscillation in PSII Fluorescence? The rate of reduction of P_{680}^+ is dependent on S state ($t_{1/2}$ = 23 ns in the S_0 and S_1 states and a biphasic reduction with $t_{1/2}$ = 50 and 260 ns in the S_2 and S_3 states (*13*)) which results in S-state dependent changes in the concentration of P_{680}^+ during a microsecond flash. As P_{680}^+ is a known quencher of fluorescence, this could result in period four oscillations in fluorescence yield. However, this possibility can be ruled out as the period four oscillation is still present at times longer than 5 ms after excitation even though P_{680}^+ is fully reduced to P_{680} on this time scale (*49*).

Oxygen, another known quencher of fluorescence, has also been proposed to contribute to period four oscillations in fluorescence yield (*19*). However, oxygen does not yet appear at 100 μs after excitation, even though period four oscillations do, and oxygen reaches equilibration between its concentration in local and bulk phases in a few milliseconds (*50*) so could not explain the persistence of period four oscillations at longer times. In addition, the removal of the extrinsic 17 and 24 kDa proteins, which inhibits the flash-induced O_2 yield by only 40%, resulted in complete elimination of period four oscillations in fluorescence.

Even though oxygen is not likely the direct quencher of fluorescence, in our studies using various procedures to alter donor side capacity, we have found that a decrease in the oxygen yield does correlate with a diminution of the period four modulation. For example the period four changes in the amount of type A centers is significantly decreased at pH 7.6 as is the oxygen yield. Plijter et al. (*51*) reported that PSII membranes at pH 8.3 still exhibited a period four oscillation in absorption changes at 345 nm, which are attributed to the oxidation of the Mn cluster, even though oxygen evolution was completely inhibited at this pH. A 1.5 ms phase of the 345 nm absorption changes related to the reduction of the Mn cluster during the $S_3-(S_4)-S_0$ transition slowed to 3.2 ms at pH 8.3. Putting together our results (Table 3 and Figure 5) and results presented above, one can conclude that the four-step oxidation of the Mn cluster alone is not sufficient to produce modulation of the PSII fluorescence. Normal chemistry, proceeding at the WOC and resulting in oxidation of water and formation of O_2, appears to be required to observe a period four in the fluorescence. If oxygen itself is not responsible, it is reasonable to suggest that deprotonation events occurring during water oxidation are responsible for the period four modulation of both PSII charge separation and Q_B site properties.

How Proton Release Could Affect the PSII Fluorescence. It is widely accepted that the absence of proton release during the S_1-S_2 transition results in the appearance of uncompensated positive charge in the S_2 and S_3 states (*11, 13*). Several studies indicate the influence of an external electric field on fluorescence (*17, 18*). The oscillation patterns of the slow decay component observed in thylakoids and PSII membranes are explained well with respect to the presence of charge surplus in the S_2 and S_3 states. However, it is most

unlikely that this charge surplus directly affects the Q_B site. We suggest that conformational rearrangements of protein(s) in the reaction center induced by the appearance of the uncompensated positive charge influence Q_B site properties. This suggestion is supported by recent work of Christen and Renger (*52*). They concluded that S*i* state dependence of proton/deuterium exchange effect on the fast P_{680}^+ reduction kinetics is not easily reconcilable with a simple electrostatic effect caused by a single localized charge and may rather reflect structural differences of the WOC in redox states S_0, S_1 versus S_2, S_3. However, the F_{max} yield may be directly influenced by the uncompensated charge.

In accordance with pH-dependent pattern of the extrinsic proton release accompanying the S-state transitions of the WOC (*14*), the predicted stoichiometry of uncompensated charge is 0:−0.75:+0.25:+0.25 at pH 5.5, 0:−0.3:+0.6:+0.65 at pH 6.5, and 0:−0.05:+0.5:+0.5 at pH 7.6 for the S_0, S_1, S_2, and S_3 states, correspondingly. However, no correlation is observed between the pH-dependent changes in the given stoichiometry and changes in the period four modulation of the fluorescence at the same pH (Figures 3B and 5A,B). This implies that the extrinsic proton release does not affect the given processes. It has been suggested that the extrinsic proton release is related to amino acid pK_a shifts indirectly caused by events at the WOC (*53*, *54*). Our data are consistent with this idea if the fluorescence modulation is proposed to reflect intrinsic deprotonation events. It is reasonable to expect that deprotonation of a protonatable group, which is not occurring or negligible at pH 5.5−6.5 during the S_1-S_2 transition, could rise significantly at pH 7.6. This suggestion can be supported by the fact that the extrinsic proton release during the S_1-S_2 transition and absorption changes at 435 nm, associated with this release, are small at pH 5.5−6.5 (0−0.1) but become maximal near pH 7.65 (approximately 0.5) (*14*). pH-dependent changes in the period four modulation of the population of type A centers suggest that the p*K* of the proposed protonatable group in such centers is in the 7.3−7.5 range. Histidine residues that can titrate in this range have been suggested to be ligands to the Mn cluster (*55*, *56*). In type C centers the p*K* of this protonatable group shifts to approximately 6.9−7.1. The proposed mechanism for the period four modulation of the fluorescence at different pH in PSII membranes is shown in Figure 8. Such a mechanism suggests that deprotonation in the vicinity of the Mn cluster at pH 7.6 in the type A and type C centers, resulting in a decrease in local uncompensated charge in the higher S_2 and S_3 states, appears to disturb the normal chemistry of water oxidation.

Formation of H_2O_2 attributed to the donor side of PSII in PSII core complexes at pH 7.6 was reported by Fine and Frasch (*57*). However, it is unclear whether uncompensated positive charge itself and/or conformational rearrangements of the WOC induced by the charge are sufficient for water oxidation. Alternatively, disturbance of the normal chemistry of water oxidation at pH 7.6 may be explained by pH-induced change in the Mn cluster properties. However this mechanism would imply that, in contrast to fluorescence, the water oxidation process is not effected by intrinsic deprotonation events.

The mechanism proposed in this work to account for a period four oscillation in the fluorescence is in contradiction with the study of Delrieu and Rosengard (*10*). They ascribed the oscillation pattern of the fluorescence yield measured 80 ms after exitation flashes in thylakoids to flash-dependent changes in the S_2 population. The simulation of the oscillation pattern with respect to S_2-state considered the dark distribution of S_0, S_1, S_2, and S_3 populations and parameters α, β, and *z* to be 18, 63, 17, and 2 and 0.025, 0, and 10 (*30*). The dark distribution of S populations and the values of the parameters varied from those generated for O_2 yield pattern (12.5, 79, 8.5, 0; 0.06, 0, 9) within the same preparation. They also differed considerably from generally accepted ones (*31*, *58*). These discrepancies do not support simulations of the fluorescence yield pattern, which assume period four oscillations arise from the S_2 state.

Effect of NaCl Treatment. Interestingly, disappearance of period four as well as period two modulation of the fluorescence was observed in NaCl-treated PSII membranes while the flash-induced O_2 yield was inhibited by only 40%. Elimination of a binary oscillation in the absorption changes at 350 nm was previously detected in NaCl-washed PSII membranes in the presence of hydroxylamine as an electron donor for PSII (*59*). This effect was explained in terms of an alteration of the acceptor side of PSII due to removal of the 17 and 24 kDa proteins. The redox potential of Q_A/Q_A^- shifts from −80 to +65 mV in Ca-depleted PSII membranes (*60*). Both of these results imply long-range allosteric coupling of the WOC to the Q_A site across the thylakoid membrane. Recently, using fluorometric and Mössbauer spectroscopy, Garbers et al. (*61*) found a correlation between electron transfer from Q_A^- to Q_B and protein flexibility in PSII membranes. Thus, elimination of a binary oscillation in the fluorescence as a result of the removal of the extrinsic proteins is more likely related to conformation changes in the Q_A and/or Q_B site(s) than to changes in the Mn cluster properties. The elimination of the period four oscillation by NaCl treatment supports a mechanism of indirect modulation of the fluorescence by uncompensated positive charge through conformational rearrangements of protein(s) in the PSII reaction center.

CONCLUSIONS

This work shows that the Q_A^- decay in thylakoids and PSII membranes is well described by three exponential decay components which reflect Q_A^- reoxidation in three distinct types of PSII centers with differing Q_B site properties. The F_{max} yield pattern and the flash-dependent changes in the proportion of the PSII centers are characterized by a periodicity of four. Neither the four-step oxidation of the Mn cluster, the flash-induced production of photosynthetic oxygen, nor the extrinsic proton release is found to be responsible for the period four modulation of the fluorescence. We suggest that both PSII charge separation reactions and properties of the Q_B site are sensitive to intrinsic deprotonation events in the immediate vicinity of the Mn cluster. The mechanism of such a modulation suggests that appearance of uncompensated positive charge due to the absence of a deprotonation of a histidine residue during the S_1-S_2 transition results in conformational rearrangements in the PSII reaction center.

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