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KINETICS OF THE OXYGEN-EVOLVING COMPLEX IN SALT-WASHED PHOTOSYSTEM II PREPARATIONS

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The kinetics of flash-induced electron transport were investigated in oxygen-evolving Photosystem II preparations, depleted of the 23 and 17 kDa polypeptides by washing with 2 M NaCl. After dark-adaptation and addition of the electron acceptor 2,5-dichloro-*p*-benzoquinone, in such preparations approx. 75% of the reaction centers still exhibited a period 4 oscillation in the absorbance changes of the oxygen-evolving complex at 350 nm. In comparison to the control preparations, three main effects of NaCl-washing could be observed: the half-time of the oxygen-evolving reaction was slowed down to about 5 ms, the misses and double hits parameters of the period 4 oscillation had changed, and the two-electron gating mechanism of the acceptor side could not be detected anymore. EPR-measurements on the oxidized secondary donor Z^+ confirmed the slower kinetics of the oxygen-releasing reaction. These phenomena could not be restored by readdition of the released polypeptides nor by the addition of $CaCl_2$, and are ascribed to deleterious action of the highly concentrated NaCl. Otherwise, the functional coupling of Photosystem II and the oxygen-evolving complex was intact in the majority of the reaction centers. Repetitive flash measurements, however, revealed P^+Q^- recombination and a slow Z^+ decay in a considerable fraction of the centers. The flash-number dependency of the recombination indicated that this reaction only appeared after prolonged illumination, and disappeared again after the addition of 20 mM $CaCl_2$. These results are interpreted as a light-induced release of strongly bound Ca^{2+} in the salt-washed preparations, resulting in uncoupling of the oxygen-evolving system and the Photosystem II reaction center, which can be reversed by the addition of a relatively high concentration of Ca^{2+}.

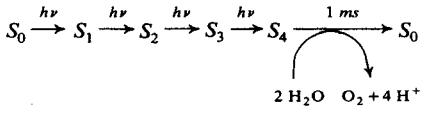
Introduction

Much recent research concerning photosynthetic oxygen evolution has been focussed on the function of three extrinsic polypeptides with molecular masses of about 33, 23 and 17 kDa [1–7]. The proteins are located at the inner side of the thylakoid membrane [1,5], where the process of oxygen evolution presumably takes place, and can be released from membrane preparations by a variety of treatments. Thus, treatment with concentrated NaCl released the 23 and 17 kDa proteins, while the 33 kDa protein and the strongly bound manganese were retained [4–7]. This manganese is assumed to play an essential role in the water-splitting process (see for a review Ref.

Abbreviations: Chl, chlorophyll; DCBQ, 2,5-dichloro-*p*-benzoquinone; Mes, 4-morpholineethanesulfonic acid; PS II, Photosystem II.

8). The salt-washed preparations exhibit strongly decreased rates of oxygen evolution in continuous light, which could be enhanced again by readdition of the 23 kDa polypeptide [3,9], or by addition of (at least) 5 mM Ca^{2+} [10,11]. The isolated 23 kDa protein appeared to lack redox centers or metal groups [12], and apart from increasing the affinity for the Ca^{2+} [9] or Cl^- [13] binding site, no specific function has been demonstrated so far.

It has been proposed that the inhibition by salt-washing only affects the higher oxidation states of the oxygen-evolving complex [6,7]. These oxidation states are generally denoted as 'S-states'. According to the Kok scheme (cf. Ref. 14), the system can be in five different redox states, since four photoreactions of PS II are needed to obtain oxygen evolution:



After dark-adaptation, the system is largely in S_1, and a series of saturating flashes produces a period 4 oscillation with release of oxygen mainly after the third flash. The oscillation is damped due to misses and double turnovers.

A powerful tool to study the S-state turnover is the ultraviolet absorbance change which has been found to oscillate with a periodicity of four [15]. Recently, this change was analyzed in detail, and it was concluded that it is caused by 3 Mn(III) ions which are oxidized to Mn(IV) in the first three S-states transitions successively, and which are all reduced again during the 1-ms phase of the $S_3 \rightarrow (S_4) \rightarrow S_0$ transition [16,17]. This phase was also reported to be accompanied by the EPR signal known as 'II_{vf}' which is caused by the oxidized form of the secondary donor Z [18,19].

In this contribution, we analyzed the period 4 oscillation of the oxygen-evolving mechanism in salt-washed, dark-adapted PS II preparations by the ultraviolet absorbance changes and, under steady-state conditions, by the EPR signal of Z^+. It will be shown that, despite of an about 4-times retardation of the oxygen-releasing reaction, the preparations are still able to perform a quite normal S-states turnover in the majority of the reaction centers. We conclude that in the absence of the 23 and 17 kDa proteins (and without externally added Ca^{2+}) the functional coupling between Z and the oxygen-evolving complex is gradually being lost upon illumination. Subsequent Ca^{2+} addition restores the coupling, although the retardation of the $S_3 \rightarrow (S_4) \rightarrow S_0$ reaction is still observed.

Materials and Methods

PS II particles were prepared from spinach chloroplasts with Triton X-100 as described by Ghanotakis et al. [7]. The final preparations were stored in 400 mM sucrose/50 mM Mes/15 mM NaCl/5 mM $MgCl_2$ (pH 6.0) at −80°C. Salt-washing was performed by incubation of the preparations at about 1 mg Chl/ml for 1 h in 2 M NaCl/50 mM Mes/5 mM $MgCl_2$ (pH 6.0). This treatment allowed for a complete removal of the 23 and 17 kDa polypeptides from the membranes [6,7,9,10]. The 23 and 17 kDa polypeptides were desalted and concentrated from the supernatant, obtained after the salt-washing by repeated dilutions and filtrations by Ultrafiltration (Amicon PM 10 Diaflo membrane).

The optical experiments were performed with a chlorophyll concentration of 200 μg/ml in 20 mM Mes-NaOH/5 mM $MgCl_2$/15 mM NaCl (pH 6.0) in an apparatus described in more detail in Ref. 16. The optical pathlength was 1.2 mm. The saturating 10 μs xenon flashes were spaced at 300 ms (unless stated otherwise), and the time constant of the apparatus was 0.3 ms for the period 4 measurements, and 30 μs for the measurements on the P^+Q^- back reaction.

The EPR experiments were carried out in East Lansing, MI (U.S.A.), using market spinach and the EPR spectrometer described previously [10]. Oxygen-evolving PS II particle preparation and salt-washing were carried out as described in Ref. 7. The salt-washed PS II preparations were washed once with 0.4 M sucrose/50 mM Mes/10 mM NaCl (pH 6.0), and stored frozen at −40°C prior to use. The chlorophyll concentration in recording EPR decays was 2.5 mg/ml. The 14-μs xenon flashes were of saturating intensity.

Results

Absorbance changes with periodicity four

PS II preparations were isolated from spinach chloroplasts with a high concentration of Triton X-100. It was shown before that these preparations exhibit high rates of oxygen evolution in continuous light and that a complete removal of the 23 and 17 kDa polypeptides by washing with 2 M NaCl decreases these rates considerably [7]. Addition of Ca^{2+}, or readdition of the 23 kDa polypeptide, however, restored these rates almost to control levels [9,10].

In this study, we examined the effects of these treatments on the flash-induced kinetics of PS II electron transfer. First, we analyzed the period 4 oscillation of the absorbance changes in the ultraviolet part of the spectrum. The preparations were dark-adapted for at least 15 min after which the lipophilic electron acceptor DCBQ (100 μM) was added, and a series of saturating flashes, spaced at 300 ms, was fired. This flash frequency allowed for an almost complete reoxidation of the acceptor side between the flashes, both in the nontreated [17], and in the salt-washed preparations (not shown).

Fig. 1A shows a typical trace of an absorbance difference measurement in the nontreated preparations at 350 nm, a wavelength where the changes are dominated by those of the oxygen-evolving complex [17]. The traces shown are 50 ms sweeps, the off-set being adjusted before each flash. Superimposed on a relatively slow decay of the acceptor side, a damped period 4 oscillation can be seen both in a fast transient, observed most easily after the third flash, presumably reflecting the $S_3 \rightarrow (S_4) \rightarrow S_0$ transition (the oxygen-releasing reaction), and in the changes remaining after the decay of the transient, reflecting the different S-states S_0–S_3. The sequence of the latter absorbance changes is $+1, +1, +1, -3$ for the $S_0 \rightarrow S_1$, $S_1 \rightarrow S_2$, $S_2 \rightarrow S_3$ and $S_3 \rightarrow S_0$ transitions, respectively [17]. The half-time of the fast transient on the third flash was calculated to be 1.3 ± 0.1 ms [20], indicating that the oxygen-releasing reaction proceeds with about the same rate in the PS II preparations and in chloroplasts (cf. Ref. 14).

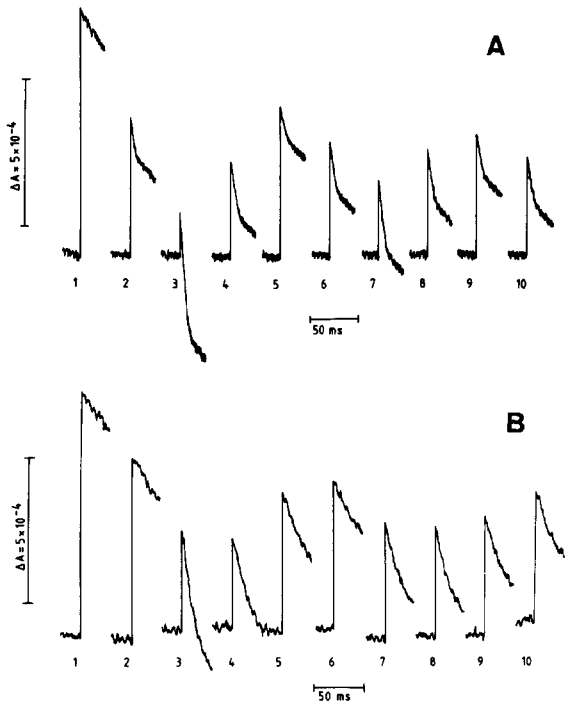


Fig. 1. Absorbance changes at 350 nm in dark-adapted, nontreated (A) or salt-washed (B) preparations, suspended at a chlorophyll concentration of 200 μg Chl/ml in 20 mM Mes-NaOH (pH 6.0) with 5 mM $MgCl_2$, 15 mM NaCl and 100 μM DCBQ. The flashes were spaced at 300 ms. The recordings are the average of 25 measurements.

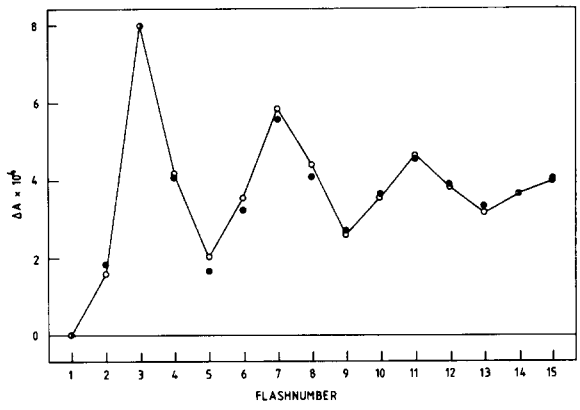


Fig. 2. Amplitudes of the 1.3 ms phase of the $S_3 \rightarrow (S_4) \rightarrow S_0$ transitions after the first 15 flashes in dark-adapted, nontreated PS II preparations as in Fig. 1, recorded at 300 nm (open circles). These amplitudes are compared to those of the $S_3 \rightarrow (S_4) \rightarrow S_0$ transition calculated for a dark S-states distribution of 75% S_1 and 25% S_0, and 9% misses and 9% double hits on all transitions (closed circles). The values at 300 nm were the average of 75 measurements.

Fig. 1B shows a trace of a 350 nm absorbance difference measurement in a salt-washed preparation. It appears that these preparations still perform a period 4 oscillation in a considerable fraction of the centers. We noticed that the presence of a lipophilic acceptor like DCBQ was a prerequisite for the appearance of this oscillation. Addition of an acceptor system containing ferricyanide and ferrocyanide, as used in Refs. 7 and 10, prevented the oscillations, even in the presence of DCBQ. In the absence of artificial acceptors, period 4 oscillations could not be observed either [6]. Remarkably, the presence of the ferricyanide acceptor system, or the absence of artificial acceptors, did not prevent the period 4 oscillations in the nontreated preparations. When comparing the trace of the salt-washed preparations (Fig. 1B) to that of the nontreated preparations (Fig. 1A), two main differences can be observed: the decay of the fast phase on the third flash, reflecting the oxygen-releasing reaction, is slowed down considerably in the salt-washed preparations and the damping of the oscillation seems to be somewhat more pronounced. The half-time of the slowed-down decay was calculated to be 5 ± 2 ms, the variation being dependent on the salt-washed preparation used.

In order to obtain more information about the changes in the damping, caused by the changes in the parameters of the period 4 oscillation (i.e., the dark S-states distribution, and the amounts of misses and double hits), we analyzed the oscillation patterns of the $S_3 \rightarrow (S_4) \rightarrow S_0$ transitions in both types of preparations. Fig. 2 (open circles) shows this pattern in the nontreated preparations, measured as described in Ref. 17 by the absorbance changes at 300 nm, together with a Kok model fit assuming a dark S-states distribution of 25% S_0 and 75% S_1, and 9% misses and 9% double hits on each turnover (closed circles). The relatively high amount of double hits is due to the tail of the 10-μs xenon flash. Similar patterns were reported for the sequence of oxygen release in chloroplasts [14].

The pattern of the 5-ms phase, presumably reflecting the $S_3 \rightarrow (S_4) \rightarrow S_0$ transition in the salt-washed preparations, is shown in Fig. 3. We measured the relative amplitudes of this phase by its luminescence signals. Measurement of the ab-

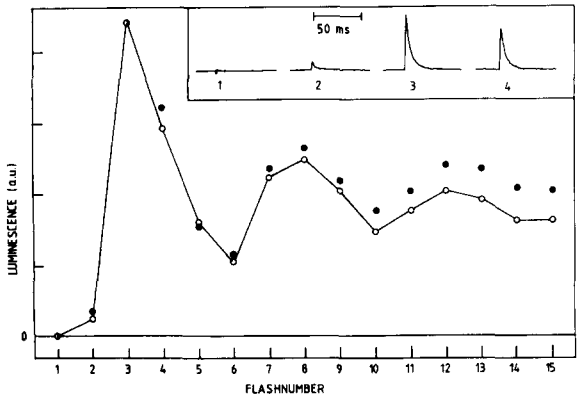


Fig. 3. Amplitudes of the 5 ms phase of the $S_3 \rightarrow (S_4) \rightarrow S_0$ transition after the first 15 flashes in dark-adapted salt-washed PS II preparations as in Fig. 1, recorded in luminescence (open circles). The amplitudes are compared to those of the $S_3 \rightarrow (S_4) \rightarrow S_0$ transition calculated for a dark S-state distribution of 75% S_1 and 25% S_0, and 18% misses and 4% double hits on all transitions (closed circles). Inset: trace of the luminiscence changes due to the first four flashes. The measurements were averaged 25 times.

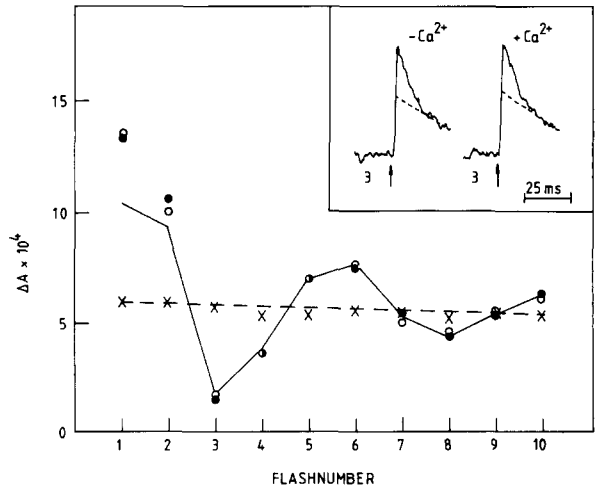


Fig. 4. Absorbance changes at 310 nm due to the first 10 flashes in dark-adapted salt-washed PS II preparations as in Fig. 1 (open circles), or as in Fig. 1 but with addition of 20 mM $CaCl_2$ (closed circles), or 250 μM hydroxylamine (crosses). The oscillating changes are compared to a Kok model fit using the same parameters for the oscillation as in Fig. 3, the turnover of absorbance changes of +1, +1, +1, −3 for the $S_0 \rightarrow S_1$, $S_1 \rightarrow S_2$, $S_2 \rightarrow S_3$ and $S_3 \rightarrow S_0$ transitions respectively [17], and the dashed line as base-line. All measured values are recorded 30 ms after the flashes and are the average of 25 experiments. Inset: traces of the absorbance changes due to the 3rd flash in the absence or presence of 20 mM $CaCl_2$.

sorbance changes of this phase was less convenient, since they were accompanied by a 20 ms decay phase, caused by reoxidation of the reduced primary acceptor Q^- by DCBQ (see also Ref. 17). The inset in Fig. 3 shows that in the luminescence traces no significant contamination by longer decay phases was detected. The measured amplitudes (open circles) can apparently be fitted with the Kok model using a dark S-states distribution of 25% S_0 and 75% S_1, with 18% misses and 4% double hits on each turnover (closed circles). So, the period 4 oscillation of photosynthetic oxygen evolution changes upon salt-washing due to an increase of the number of misses (from 9 to 18%) and a decrease of the number of double hits (from 9 to 4%). It was checked that the observed differences were not due to a diminished light-saturation in the salt-washed preparations: with 2-times more intense xenon flashes the same parameters of the oscillation were obtained.

In order to confirm the calculated parameters of the oscillation in the salt-washed preparations, we analyzed the absorbance changes at 310 nm, remaining at 30 ms after the flashes. The measured values are indicated by the open circles of Fig. 4. Addition of hydroxylamine (250 μM) resulted in a complete disappearance of the oscillations (Fig. 4, crosses). The hydroxylamine concentration used was low enough to prevent the inhibitory action between Z and the primary donor P-680 [21]. When the changes in the presence of hydroxylamine are taken as 'baseline' then the measured values (open circles) can be fitted with the Kok model using the parameters of Fig. 3, and the sequence of absorbance changes of $+1$, $+1$, $+1$, -3 for the $S_0 \rightarrow S_1$, $S_1 \rightarrow S_2$, $S_2 \rightarrow S_3$ and $S_3 \rightarrow S_0$ transitions, respectively (solid line). Only the first and, to a lesser degree, the second flash did not conform to this pattern, as was observed earlier for the nontreated preparations [17]. So, the sequence of donor-side absorbance changes is not influenced by the salt-treatment. However, the additional period 2 oscillation in the nontreated preparations [17] cannot be detected in the salt-washed preparations.

We used the absorbance changes at 310 nm for a calculation of the fraction of centers that contribute to the period 4 oscillation. The kinetics observed on the third flash were analyzed and after proper subtraction of the 20 ms phase of the acceptor side change we calculated a differential extinction coefficient of about 5.5 $mM^{-1} \cdot cm^{-1}$ for the 5 ms phase on the third flash. This yields, after correction for the contributing centers according to the fit of Fig. 3, a value of about 13.5 $mM^{-1} \cdot cm^{-1}$ for the 100% change. In nontreated preparations, this extinction coefficient was found to be about 18 $mM^{-1} \cdot cm^{-1}$ [17]. So we conclude that after salt-washing the period 4 oscillation still occurred in approximately 75% of the centers.

The closed circles in Fig. 4 show the 310 nm absorbance changes after the addition of 20 mM $CaCl_2$ to dark-adapted, salt-washed preparations. It appears that Ca^{2+} does not affect the parameters of the period 4 oscillation, does not affect the amplitude of the oscillation and does not accelerate the retarded oxygen-releasing reaction, as can be seen from the changes after the third flash, shown in the inset of Fig. 4 (the dashed lines indicate the contribution of the acceptor side). The same was found after addition of various amounts of the 23 and 17 kDa polypeptides (not shown). So, despite a more than 2-fold enhancement of the rates of oxygen evolution in continuous light

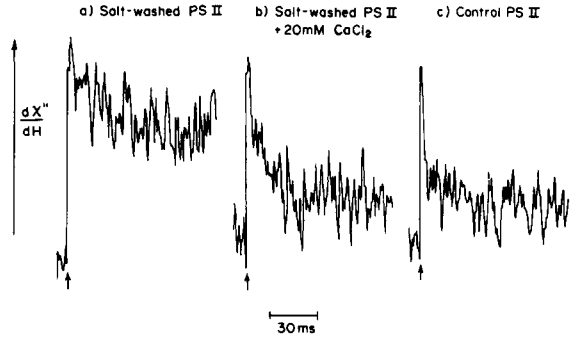


Fig. 5. EPR detection of the decay of Z^+ under steady-state flashing light conditions in (a) salt-washed PS II preparations, (b) salt-washed PS II preparations plus 20 mM $CaCl_2$ and (c) control, oxygen-evolving PS II preparations. For each trace, the decay transients for six identical samples, each of which received 80 flashes, were averaged. In each experiment, there was a dark time of 2 s between flashes. The instrument time constant was 1 ms, the microwave power was 50 mW and the spectrometer was set to the low-field peak of the Z^+ EPR spectrum. The buffer consisted of 0.4 M sucrose, 50 mM Mes buffer and 10 mM NaCl; 600 μM DCBQ was added as the electron acceptor. For trace b, the sample was incubated in the dark for 10 min following Ca^{2+} addition.

[7,9,10], these additions do not affect the flash-induced kinetics of the period 4 oscillation of oxygen evolution.

Z^+ decay kinetics

A kinetic study on the EPR signal of Z^+ was performed to find out whether the slowed-down kinetics of the oxygen-releasing reaction, as detected by the absorbance changes of the manganese complex, is also reflected by those of the secondary donor. It was reported before that the decay of Z^+ is strongly biphasic under repetitive flash conditions with half-times of approx. 25 and 800 ms when using an equimolar mixture of ferricyanide and ferrocyanide at pH 7.5 [7]. Here, we analyzed the Z^+ decay at pH 6.0 in the presence of DCBQ.

Fig. 5c shows a trace of its decay in the non-treated preparations. A fast phase with 1–2 ms half-time can be detected, reflecting the involvement of Z^+ in the oxygen-releasing reaction. The same measurement in the salt-washed preparations with 20 mM $CaCl_2$ is shown in Fig. 5b. The fast phase seems to be slowed down to 5–10 ms, indicating that the kinetics of Z^+ and manganese still coincide after salt-washing. In the absence of Ca^{2+}, however, the 5–10 ms phase is strongly reduced and a much slower phase appears (Fig. 5c), demonstrating a clear effect of Ca^{2+} on the kinetics of Z under repetitive flash conditions. This seems inconsistent with the period 4 oscillation observed after dark-adaptation of the preparations (which is not influenced by Ca^{2+}), but is in agreement with the partial inactivation of the oxygen-evolving complex, observed in continuous light [3,4,7] and in repetitive flash excitation [22] of salt-washed PS II. These observations are discussed in more detail below.

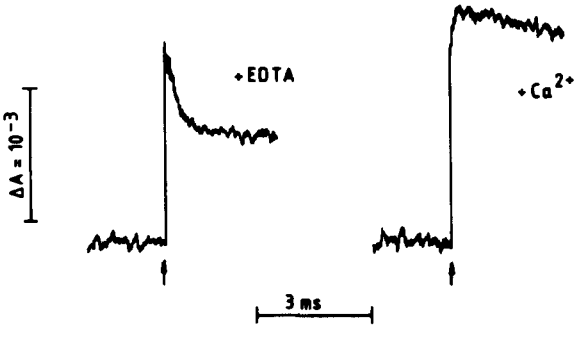


Fig. 6. Absorbance changes at 325 nm due to the first 150 flashes, spaced 500 ms, in dark-adapted, salt-washed PS II preparations as in Fig. 1, but with 200 μM DCBQ, and with 2 mM EDTA (left) or 20 mM $CaCl_2$ (right). The shown traces are 5 ms sweeps, measured with a time resolution of 30 μs.

P^+Q^- recombination

In order to check whether the different behavior of the system is due to the different illumination conditions, we have studied the P^+Q^- recombination, which should appear when the reduction of Z^+ becomes slower than the flash-repetition rate. At room temperature, the back reaction of P^+Q^- proceeds with about 200 μs half-time [23]. Fig. 6 shows the averaged absorption changes at 325 nm induced by 150 flashes, spaced at 500 ms, in salt-washed preparations with 200 μM DCBQ and either 2 mM EDTA (left) or 20 mM $CaCl_2$ (right). The rapid decay phase, $t_{1/2} = 220 \pm 20$ μs,

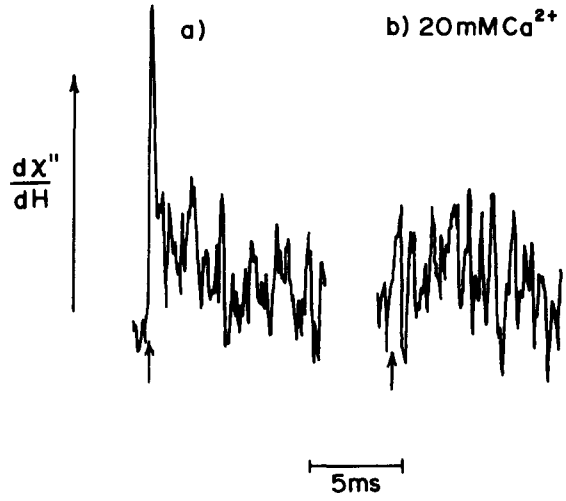


Fig. 7. EPR traces of the behavior of P-680$^+$ in (a) salt-washed PS II preparations and (b) salt-washed PS II preparations plus 20 mM $CaCl_2$. For each trace, the decay transients for two identical sampels, each of which received 80 flashes, were averaged. In each experiment, there was a dark time of 1 s between flashes. The instrument time constant was 60 μs, the microwave power was 50 mW and the spectrometer magnetic field was set to the zero crossing of Z^+. At this field, the P-680$^+$ signal intensity is near maximal [24]; traces recorded at other field positions showed that the rapid transient in (a) has the same lineshape as P-680$^+$ (not shown). The buffer/acceptor system were the same as Fig. 5; the dark-incubation following Ca^{2+} addition for trace (b) was 10 min.

observed with EDTA, but not with $CaCl_2$, may be largely due to the reoxidation of Q^-. A 3-times larger, negative transient with the same decay time was observed at 435 nm (not shown), suggesting that the transient is indeed due to P^+Q^-. Its amplitude indicated that, dependent on the preparation used, 30–50% of the centers were involved.

Similar results were obtained by EPR. Fig. 7 shows that the P^+Q^- recombination reaction can be detected as a transient P-680$^+$ EPR signal which decays rapidly ($t_{1/2} < 400$ μs) in salt-washed PS II preparations. This experiment was done under essentially identical conditions to that for the Z^+ experiment in Fig. 5a and demonstrates that P^+Q^- recombination occurs when Z^+ decays slowly. Addition of Ca^{2+} results in the disappearance of the P-680$^+$ decay transient indicating, in agreement with Fig. 5b that rapid P-680$^+$ reduction occurs when Z^+ is reduced in the dark time between flashes.

In our optical set-up, the first 150 flashes on preparations without Ca^{2+} or EDTA yielded a 200 μs phase with only small amplitude. Fig. 8 shows the averaged amplitudes of this phase due to the first 30, the second 30 flashes, etc. After the first 60 flashes, the P^+Q^- recombination hardly takes place, but after 150 flashes the amplitude is almost the same as with EDTA. This result suggests a gradual inactivation of the oxygen-evolving system upon illumination. However, when Ca^{2+} was added after the 150 flashes the 200 μs phase disappeared again, indicating a reactivation of the oxygen-evolving complex.

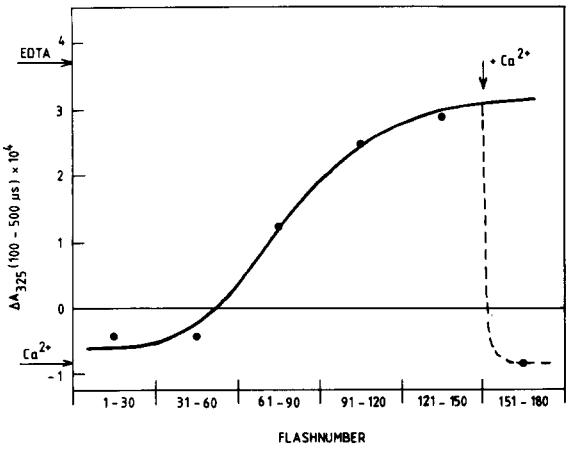


Fig. 8. Amplitudes of the 200 μs decay phase of P^+Q^- recombination due to the first 30, the second 30, etc., flashes in salt-washed, dark-adapted PS II preparations as in Fig. 1, but with 200 μM DCBQ. The indicated values are the differences between 100 and 500 μs after the flashes, measured at 325 nm as in Fig. 6. The dashed line indicates the expected amplitude of the 200 μs phase after addition of 20 mM $CaCl_2$. The horizontal arrows indicate the amplitude of the first 150 flashes in the presence of 2 mM EDTA or 10 mM $CaCl_2$.

Discussion

In this contribution, we analyzed characteristics of electron transport in PS II preparations depleted of the 23 and 17 kDa polypeptides by washing with 2 M NaCl and reported that these preparations were, under certain conditions, able to perform a period 4 oscillation of oxygen evolution in about 75% of the reaction centers. The results show unambiguously that the functional coupling between the secondary donor Z and the manganese complex, which is responsible for the oscillations [17], remains intact in the majority of the centers upon salt-washing (provided that the preparations were not exposed to too much light). This is in line with the suggestions of Ghanotakis et al. [7], made on the basis of the saturation behavior of the EPR signal of Z^+ in the salt-washed preparations. Our results only partially confirm the conclusions of Wensink et al. [6], which imply that the 23 and/or the 17 kDa polypeptide is required for an efficient turnover of higher S-states. Apparently, this requirement is not observed when DCBQ is used as the electron acceptor. In salt-washed inside-out vesicles the period 4 oscillation may behave differently [22].

The characteristics of the period 4 oscillations described in this paper indicate three different changes in PS II electron transfer upon washing with 2 M NaCl, none of which could be reversed by addition of the 23 and 17 kDa polypeptides or $CaCl_2$. The $S_3 \rightarrow (S_4) \rightarrow S_0$ transition slows down to about 5 ms, the misses and double hits parameters of the period 4 oscillation change, and the period 2 oscillation of the two-electron gating mechanism disappears. This last change, reported earlier [6] on the basis of period 2 measurements as described by Pulles et al. [15], clearly indicates an effect on the acceptor side. In addition, we

found that in the presence of DCBQ the period 2 oscillation at the acceptor side, which accompanied the period 4 oscillation in untreated preparations [17], is also removed by salt-washing. The changed acceptor side behavior may be the cause of some other effects, such as the decreased number of double hits. The 1 ms phase in the reoxidation of Q^-, which allowed a second turnover of some centers during the tail of the xenon flash in untreated preparations [17], is absent after salt-washing and the 20 ms phase is correspondingly larger (Dekker, J.P., unpublished data.)

In addition to the effects on the acceptor side, an effect on the donor side by salt-washing was evident: the $S_3 \rightarrow (S_4) \rightarrow S_0$ transition was slowed down to about 5 ms, as indicated both by the manganese absorbance changes and by the EPR signal of Z^+. Sinclair [25] recently reported that the addition of nitrate has a similar effect. This retardation by a factor of about 4 has no effect on the rates of oxygen evolution in continuous light. Presumably, the rate-limiting step is still at the acceptor side: the 5 ms half-time of the formation of oxygen would yield a maximum rate in continuous light of about 2000 μmol O_2/mg Chl per h (assuming 280 Chl/PS II [16]), which is considerably faster than the rates reported in Ref. 10. The effects of salt-washing on the donor side and the acceptor side may be mutually independent, and may not be related to the removal of the 23 and 17 kDa polypeptides either. The fact that the effects were not reversed by addition of the polypeptides or $CaCl_2$ may indicate that they are due to the high concentration of NaCl only.

Our results suggest an explanation for the apparent contradictions concerning the function of the 23 kDa protein as described in the literature. Åkerlund et al. [22,26] postulated a direct involvement on the oxidizing site of PS II, Wensink et al. [6] assumed involvement in the higher S-states only, while the EPR power saturation of Z^+ suggested a normal coupling between the manganese complex and Z in the absence of the 23 kDa polypeptide [7]. Besides, measurements on the oxygen-evolving capacity in continuous light suggested a role as Ca^{2+}- or Cl^--binding protein [9,13,27]. The latter measurements, however, were rather indirect, since the rate-limiting step of electron transfer in continuous light presumably is at the acceptor side.

The results in Ref. 9 indicate that bound Ca^{2+} can be released (in the dark) using extreme conditions only. Probably, Ca^{2+} was still at its original binding site during the period 4 measurements in dark-adapted preparations. The appearance of the P^+Q^- recombination reaction after prolonged illumination and the observation of a very long lived Z^+ in such conditions clearly indicate an inhibition of electron transfer from the oxygen-evolving complex to Z^+. Since these phenomena disappeared upon Ca^{2+} addition, the inhibition is ascribed to a light-induced release of Ca^{2+} from its binding site, which apparently can be reversed by increasing the Ca^{2+} concentration. The fact that not all centers show the back reaction, or the slow phase in Z^+ reduction (cf. Ref. 7), is possibly related to the fact that the oxygen-evolving capacity is not inhibited completely after salt-washing. The fraction of system II involved, and the amount of light needed for Ca^{2+} release may be expected to vary between different types of PS II preparations.

In conclusion, the results from this paper and that of Ref. 9 may be explained by the following scheme:

$$\mathrm{MZPQ} \xrightarrow[\text{23, 17 kDa}]{\text{2 M NaCl}} (\mathrm{MZPQ}) \xrightarrow[\mathrm{Ca^{2+}}]{h\nu} (\mathrm{M/ZPQ}) \xrightarrow[\text{(23, 17 kDa)}]{\mathrm{Ca^{2+}}} (\mathrm{MZPQ})$$

(M represents the manganese cluster of the oxygen-evolving complex; the slash indicates its uncoupling from the donor Z). The washing with 2 M NaCl removes the 23 and 17 kDa polypeptides and creates a modified PS II, represented here by parentheses, with a damaged secondary acceptor mechanism and a slower $S_3 \rightarrow (S_4) \rightarrow S_0$ transition. Subsequent illumination causes release of strongly bound Ca^{2+} and disconnection of the oxygen-evolving complex from the PS II reaction center, which can be reversed by addition of a high concentration of Ca^{2+}, or by a low concentration if the released polypeptides are added again.

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