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# The charge accumulation mechanism in NaCl-washed and in $Ca^{2+}$-reactivated Photosystem-II particles

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**In this report we show that, under flashing light, NaCl-washed Photosystem-II particles which do not evolve oxygen, are unable to go beyond the $S_3Z^+$ state. Addition of $Ca^{2+}$ alone restores the $S_3Z^+ \rightarrow S_0$ transition and oxygen evolution. These conclusions were reached by analysis of the oxygen and luminescence oscillations.**

## Introduction

Isolated oxygen-evolving Photosystem-II particles form a useful material for studying the mechanism of photosynthetic oxygen evolution. Washing these particles with 1 or 2 M NaCl results in partial inactivation of oxygen evolution and in the release of two of the three extrinsic polypeptides located on the inner face of the membrane where the water-splitting process takes place [1–7]. The 17 and 24 kDa proteins are released [4–7], whereas the 33 kDa protein remains attached to the membrane, and no manganese is removed.

The discovery by Joliot et al. [8] of the characteristic oscillatory pattern of oxygen evolution obtained under flashing light (oxygen sequence) led to Kok's interpretation [9] for the observed periodicity of 4. Namely, that the water-splitting complex of each PS-II center is able to store four oxidizing equivalents in order to oxidize a pair of water molecules. Therefore, there exist five different redox states of the donor side of PS II. The corresponding redox states of the water-splitting system are denoted $S_n$, $n$ varying between 0 and 4. In dark-adapted samples only the lower states $S_0$ and $S_1$ are stable, and with flashing light it is possible to go through the S cycle step by step, and to study the properties of the individual S states (see Ref. 10 for a review).

In PS-II particles four Mn atoms are essential for oxygen evolution, and in a recent model a change in their redox state was correlated to the $S_n \rightarrow S_{n+1}$ transition [11]. One intermediary donor, Z, has been clearly characterised by its visible, ultra-violet and electron paramagnetic resonance spectra [12–15].

After a photochemical charge separation the PS-II centers are in an inactive state $S_n^*$ unable to trap another exciton before further stabilization of the separated charges ($S_n^* \rightarrow S_{n+1}$) or disappearence of S* by back reaction with luminescence emission ($S_n^* \rightarrow S_n + h\nu$). On the donor side, the $S_n^*$ state is now thought to be the $S_nZ^+$ state as $P^+$ is rapidly reduced by Z [16]. On the acceptor side, $Q^-$ is reoxidised by the secondary electron acceptor B. In chloroplasts the half-time for the reoxidation of $Q^-$ is several hundred microseconds [17]. Joliot et al. [18] showed that the intensity of delayed

Abbreviations: PS-II, Photosystem II; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid; Mes, 4-morpholineethanesulphonic acid; DCBQ, 2,5-dichloro-*p*-benzoquinone.

luminescence oscillated in synchrony with the concentration of the $S_2$ and $S_3$ states formed per flash during a train of flashes. They measured the luminescence 30 ms after each flash, a time sufficiently long for the transition $S_n^* \rightarrow S_{n+1}$ to have taken place. For shorter times, as Zankel [19] demonstrated, the oscillations of delayed luminescence after each flash are in step with those of oxygen evolution (i.e., with the $S_3^*$ state). The $S_3^*$ state is a much better substrate for luminescence than the other S states [20].

During a train of saturating flashes each center has a small probability of not stabilising a positive charge (misses). This results in damping of the oxygen and luminescence oscillations. The degree of damping depends on the efficiency of the charge separation and stabilisation steps and can be calculated. The miss parameter $\Sigma_{i=0}^{3}\alpha$, representing the amount of damping, and the initial dark distribution between the $S_0$ and $S_1$ states can be computed using a fitting procedure described by Lavorel [21].

After NaCl-washing the photochemical charge separation is still operative, and it is important to determine the stage at which the step-wise mechanism of charge accumulation is blocked, in order to understand the role of the 17 and 24 kDa proteins and that of $Ca^{2+}$ on the positive charge storage mechanism. After Tris-washing which removes all three polypeptides and three of the four Mn atoms, it has been shown that two charges can still be stored on the donor side, but $Z^+$ is no longer able to transfer its charge to the oxygen-evolving complex. This results in a fast charge recombination as seen when the kinetics of $Z^+$ reduction or $Q^-$ oxidation are followed by fluorescence, luminescence, visible spectrophotometry or EPR spectrometry [10,22].

In previous works, Wensink et al. [6] have also studied the reconstitution of photosynthetic water splitting after salt-washing of PS-II particles. They concluded that the NaCl-washed particles were only able to perform the $S_0 \rightarrow S_1 \rightarrow S_2$ transitions, but their medium was depleted in $Cl^-$ which is also known to affect the donor side of PS II [23].

In PS-II particles washed in the dark with NaCl, and dark-adapted, Dekker et al. [24] found that period 4 oscillations in luminescence and absorbance changes due to the donor side, were still occurring in 75% of the PS-II centers. However, upon repetitive flash illumination, the $Z^+$ decay is slowed down in salt-washed particles. These results are explained by assuming that in dark-adapted NaCl-washed particles, $Ca^{2+}$ is still at its original binding site and that light induces the release of $Ca^{2+}$ from this site. We carried out the NaCl washing in room light which resulted in a greater inhibition of oxygen evolution than that observed by Dekker et al. [24]. In another paper [25] we present additional evidence for the release of $Ca^{2+}$ by illumination.

In the present report we used EGTA (ethylene glycol bis($\beta$-aminoethyl ether)-*N*,*N'*-tetraacetic acid) to complex the $Ca^{2+}$ remaining in dark-adapted NaCl-washed PS-II particles. By luminescence studies we show that in salt-washed particles with no $Ca^{2+}$ present, the step-wise mechanism of charge accumulation is blocked at the $S_3Z^+ \rightarrow S_0$ transition. The study of oxygen evolution and luminescence emission after reactivation by $Ca^{2+}$ enabled us to conclude that $Ca^{2+}$ was sufficient to restore a fully operative water-splitting system.

## Materials and Methods

PS-II particles of the Kuwabara and Murata type (type 1) [26] or the Berthold, Babcock and Yocum type (type 2) [27], slightly modified as in Ref. 28, were prepared from spinach or pea chloroplasts, respectively. The oxygen measurements were done with type-1 particles washed with 1 M NaCl, luminescence measurements were done with type-2 particles washed with 2 M NaCl. We checked that the variations between these two types of preparations were not larger than the variations between different batches of the same type of preparation. The residual activity after NaCl washing in the light, done according to Ref. 5, varied from 10% to 40% of the total restorable activity which was equal to 70–80% of the activity of native PS-II particles (approx. 250–300 $\mu$M $O_2$/mg total chlorophyll/h).

For measurements with the rate electrode, the PS-II particles were used at a concentration of 1 mg total chlorophyll/ml. The flow medium was 0.3 M sorbitol/80 mM KCl/10 mM $CaCl_2$/25 mM Mes (pH 6.5). Flash sequences were programmed using a Goupil 3. Data acquisition was

carried out via a Didac (Intertechnique) multichannel analyser after differentiation and amplification of the signals. Because of the bare platinum electrode, no electron acceptor was added. The low size of the plastoquinone pool associated with the PS-II particles [29,30] resulted in a rapid decrease in the oxygen yield during a flash series. 10 min darkness between two sequences were required for full reoxidation of the plastoquinone pool by the oxygen dissolved in the medium.

The kinetics of deactivation were measured as described in Ref. 31. Dark-adapted particles are in state $S_1$ or $S_0$. Preillumination with two saturating flashes transforms the centers in state $S_1$ to state $S_3$. A detecting flash of saturating intensity is given at a time $t$ after the second preilluminating flash. The amount of oxygen evolved after the detecting flash is proportional to the number of centers in state $S_3$ at time $t$. After only one preilluminating flash, centers in state $S_1$ are transformed to state $S_2$. Two detecting flashes separated by 0.5 s are given at time $t$ after the preillumination. The amount of oxygen evolved after the last flash is proportional to the number of centers in state $S_2$ at time $t$.

The rates of the transitions $S_n^* \rightarrow S_{n+1}$ can be estimated using a similar method [32]. One, two or three preilluminating flashes are given, followed by a short, variable dark time and a train of detecting flashes spaced by 0.5 s. The oxygen yield is measured on the third flash, i.e., on the first and second detecting flashes for $S_2^* \rightarrow S_3$ and $S_1^* \rightarrow S_2$ respectively. For the transition $S_3^* \rightarrow S_0$ the oxygen yield is measured on the seventh flash, i.e., the fourth detecting flash.

Luminescence measurements were made with a stopped-flow fluorimeter described in Ref. 33. Luminescence was excited with a short electronic flash through a Corning C-S 4-96 filter, and detected by a photomultiplier tube through a Wratten (92) and Corning C-S 2-64 filter. The photomultiplier tube was gated off during the flash. The anode output was terminated to ground with a 100 kΩ resistor, and measured with a signal averager SEIN (Interzoom). The flash artefact did not exceed 5% of the signal, and was subtracted from the measurements in all experiments. The luminescence intensity was detected either at 200 μs or 2 ms after each flash of a series (flash spacing, 0.5 s.). The medium used was 0.3 M sucrose/10 mM NaCl/25 mM Mes (pH 6.5)/50 μM EGTA. For luminescence, except for the deactivation experiments, the particles were incubated at a concentration of 10 μg total chlorophyll/ml for 10 min in the dark before the addition of the electron acceptor DCBQ (2,5-dichloro-p-benzoquinone) (50 μM). For the reactivation experiments 10 mM $CaCl_2$ was added to the medium after dark adaptation of the sample.

## Results

*Luminescence during a flash sequence*

For NaCl-treated particles, Dekker et al. [24] showed that the luminescence detected in the ms range after each flash of a sequence oscillated in synchrony with oxygen evolution. We repeated

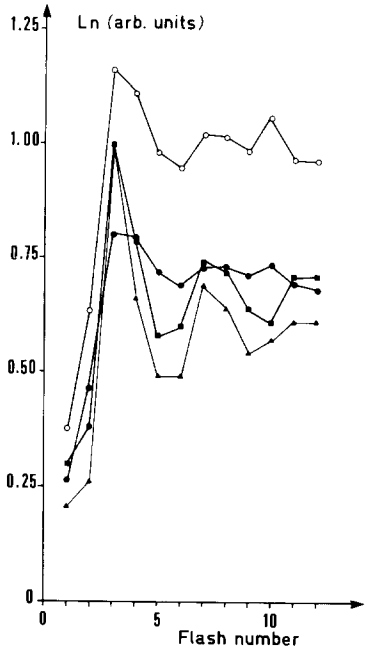


Fig. 1. Amplitudes of the luminescence intensity ($L_n$) detected 2 ms after the first 12 flashes in dark-adapted native PS-II particles (▲) or $Ca^{2+}$-reactivated NaCl-washed PS-II centers (■) suspended at a chlorophyll (Chl) concentration of 10 μg Chl/ml. Trace (○) corresponds to $L_t$. Trace (●) corresponds to PS-II centers which did not evolve oxygen, and it was obtained using the equation $L_i = L_t - L_r$ x% activity (see the text). For native and $Ca^{2+}$-reactivated particles the sequences were normalized to $L_3$. Temperature was 20°C. See Materials and Methods for the other conditions.

that experiment and found that in native oxygen-evolving particles and $Ca^{2+}$-reactivated NaCl-washed PS-II particles the best oscillating pattern was detected for the luminescence emitted 2 ms after each flash. These oscillations concerned 75% of the total luminescence intensity (Fig. 1). The decay kinetics of the luminescence in the ms range were similar for native and $Ca^{2+}$-reactivated particles (not shown). For untreated and $Ca^{2+}$-reactivated particles it was striking to see that the luminescence intensity oscillated in synchrony with oxygen evolution. Moreover the $S_0$, $S_1$ and $\Sigma_{i=0}^{3}\alpha$ parameters computed from luminescence or oxygen sequences are in good agreement (Table I). This means that the oxygen precursor $S_3^*$ lasts longer in PS II particles than in chloroplasts, and that it is the main substrate 2 ms after the flash.

In NaCl-washed particles the luminescence sequence corresponding to PS-II centers which did not evolve oxygen could be computed by subtracting from the total luminescence emission that corresponding to the oxygen-evolving centers. The estimation of the luminescence emitted by PS II centers which did not evolve oxygen in salt-washed particles is dependent on the percentage of activity, used in the formula:

$$L_i = L_t - L_r \times \% \text{ activity}$$

where $L_i$ is the luminescence from centers unable to evolve oxygen; $L_t$ is the total luminescence from salt-washed particles; $L_r$ is the luminescence of $Ca^{2+}$-reactivated particles; % activity was obtained from oxygen evolution measured under continuous illumination.

The luminescence corresponding to the inactive PS II centers is shown in Fig. 1. The luminescence intensity reached a maximum on the third flash only and remained high for the following flashes. If the charge accumulation could only go to $S_2^*$ then a large luminescence emission should be obtained on the second flash, with a small increase between the second and third flash. As there was a large increase between the second and third flash, the most likely explanation is that the centers inhibited by salt washing reached the $S_3^*$ state, but were unable to be reduced back to $S_0$ by oxidising water. The luminescence intensity of salt-washed

TABLE I

PARAMETERS COMPUTED FROM LUMINESCENCE OR OXYGEN SEQUENCES

The values shown were calculated from oxygen sequences ($O_2$) or luminescence intensity oscillations, detected 2 ms after each flash of a sequence (Lum.) and are the average of 3–5 experiments. $\Sigma_{i=0}^{3}\alpha$ is the miss parameter. Temperature was 20°C, except otherwise indicated. The deactivation of the $S_n$ states were obtained using the $Y_3(t)/Y_3$ (0.5 s) ratio for $O_2$ measurements and the $(L_3 - L_2)(t)/(L_3 - L_2)$ (0.5 s) ratio for luminescence measurements. The turnover of $S_1^*$ and $S_2^*$ was calculated as for the deactivation. The turnover of $S_3^*$ was calculated as $(Y_7(t) - Y_6(0.5\text{ s}))/(Y_7(0.5\text{ s}) - Y_6(0.5\text{ s}))$ [32].

| Particles Experience | Native | | $Ca^{2+}$-reactivated NaCl-washed | | NaCl-washed |
|---|---|---|---|---|---|
| | $O_2$ | Lum. | $O_2$ | Lum. | Lum. |
| $\Sigma_{i=0}^{3}\alpha$ | 0.45 | 0.44 | 0.52 | 0.54 | 2 ms × 1.5<br>200 µs × 2.5 |
| $S_1$ (%) | 70 | 63 | 61 | 58 | – |
| $S_0$ (%) | 30 | 37 | 39 | 42 | – |
| Deactivation | | | | | |
| $S_3$ (s) | 40 | – | 28 | 15–20 | 4 |
| Deactivation | | | | | |
| $S_2$ (s) | 35 | – | 34 | 10 | 10 |
| $S_1^* \rightarrow S_2$ (ms) | 23°C: 2.5<br>18°C: 6.0 | – | 4.2 | – | – |
| $S_2^* \rightarrow S_3$ (ms) | 1.5 | – | 1.5 | – | – |
| $S_3^* \rightarrow S_0$ (ms) | 12 | – | 13 | – | – |

particles, measured 200 μs after the flashes, was 2.5-times larger than that of untreated particles. This can be attributed to some $P^+Q^-$ being formed by the flashes in centers where Z is still oxidised when the flash is fired, $P^+Q^-$ is a good luminescence substrate in the microsecond region [34].

In the absence of the 17 and 24 kDa proteins and without $Ca^{2+}$ added the separated charges were unstable. Indeed the deactivation of $S_3$ was faster (4s) (Table I) in the absence than in the presence of $Ca^{2+}$ (15–20 s). The life-time of $S_2$ (10 s) was not sensitive to the presence of $Ca^{2+}$.

*Oxygen evolved during a flash sequence*

The oxygen sequences of native and $Ca^{2+}$-reactivated NaCl-washed PS-II particles are shown in Fig. 2. They are very similar. The values for the turnover of the PS-II centers, the deactivation half-times of the $S_2$ and $S_3$ states, and the misses and the $S_0$ and $S_1$ dark distribution computed from luminescence and oxygen sequences are given in Table I. The differences between native and $Ca^{2+}$-reactivated PS-II centers were: (i) a smaller $S_1$ concentration and a slight increase in misses for the latter and (ii) a less stable $S_3$ state in re-

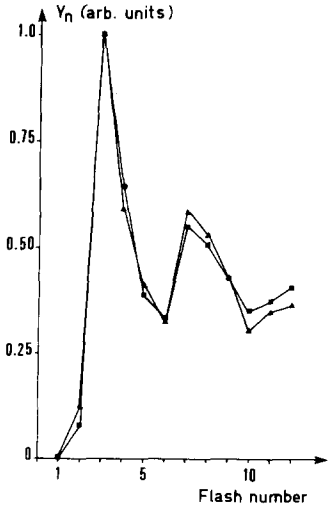


Fig. 2. Oxygen yield sequence (normalized to $Y_3$) as a function of flash number ($Y_n$) for native particles (▲) and $Ca^{2+}$-reactivated NaCl-washed PS-II particles (■). The flow medium was: 0.3 M sorbitol/25 mM Mes (pH 6.5)/80 mM KCl/10 mM $CaCl_2$. Chlorophyll total was 1 mg/ml. Temperature, 20°C. See Materials and Methods for other conditions.

activated than in native particles. The life-time of $S_2$ was equivalent in both preparations. The turnover times were equivalent, but the transition $S_1^* \rightarrow S_2$ was very sensitive to temperature, possibly because the binding of the secondary acceptor B to its apoprotein [35,36] was dependent on a diffusion-limited step. The transition $S_2^* \rightarrow S_3$ coupled with the reduction of the bound $B^-$ is less temperature-sensitive. The life-times of the $S_2$ and $S_3$ states obtained from the luminescence experiments were systematically smaller than those estimated from oxygen sequences. For luminescence both oxidizing and reducing equivalents are necessary and most of the deactivation reaction can proceed through a route different from the back reaction. Thus the luminescence intensity drops faster than the concentration of the $S_2$ and $S_3$ states.

## Discussion

The isolation of PS-II particles from chloroplasts of higher plants results in a decrease of the turnover rates of the S states and in less efficient charge stabilization. There is a slight increase in the 'miss' coefficient as compared to chloroplasts [37]. The limiting step in the $S_3^* \rightarrow S_0$ transition is considerably slower in PS II particles (12 ms) than in chloroplasts (2 ms). The increased life-time of $S_3^*$ must result from less efficient water oxidation by the oxygen-evolving complex. After NaCl-washing, 60–90% of the PS-II centers were unable to evolve oxygen. In these centers the 17 and 24 kDa proteins are lost, but the 4 Mn atoms and the 33 kDa protein are still present [4–7]. In the absence of $Ca^{2+}$ it is possible to form the $S_3Z^+$ state, but not to evolve oxygen. Rutherford et al. (personal communication) reached the same conclusion from thermoluminescence experiments. In $Cl^-$-depleted chloroplasts Theg et al. [23] concluded from equivalent luminescence experiments that only the $S_2Z^+$ state could be reached. When NaCl-washed PS-II particles are reactivated by the addition of $Ca^{2+}$ the full S-cycle is restored, oxygen is evolved and the kinetics of the turnover reactions are hardly affected by the absence of the 17 and 24 kDa proteins. We therefore conclude that the absence of the 17 and 24 kDa proteins with no $Ca^{2+}$ present inhibits the last step of the charge storage device, but that $Ca^{2+}$ is sufficient to re-

store it. Thus the inhibition of water oxidation by salt-washing is due to the loss of $Ca^{2+}$ rather than to the loss of the 17 and 24 kDa proteins. The previous works of Dekker et al. [24] and our other report [25] concerning the reactivation of NaCl-washed PS-II particles also support a hypothesis in which the role of the 24 kDa protein is to stabilize $Ca^{2+}$ on its site.

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