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EPR studies of the oxygen-evolving complex reveal a light-adaptation process in Photosystem II

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Introduction

Photosystem II (PSII) catalyzes the light-driven oxidation of water, evolving one molecule of O_2 and four protons per two H_2O molecules oxidized. The site of water oxidation is the oxygen-evolving complex (OEC), situated on the lumenal side of the membrane-spanning protein complex, containing four Mn ions and a Ca and a Cl ion. Although the structure of PSII was recently determined by X-ray crystallography (Zouni *et al.*, 2001), the resolution is not yet high enough to unambiguously determine the detailed structure of the OEC.

Four oxidations of the OEC are required to build up enough oxidizing power to split water. The four quasi-stable oxidation states of the OEC are termed S_0, S_1, S_2 and S_3, with the index signifying the number of electrons that have been removed from the cluster in each state. Upon oxidation of the OEC poised in the S_3 state, it transiently passes through the S_4 state, is rereduced by H_2O to the S_0 state, and releases one molecule of O_2. When left in the dark, the OEC equilibrates to the S_1 state. The S_2, S_3, and S_0 states are semi-transient, and can be trapped by various methods. Particularly, the S_2 state has been studied extensively in samples that have first been dark-adapted then illuminated continuously at 200K. As described below, we trap the S-states by exposing the PSII samples to short, strong flashes of light, and subsequently freezing them. This method lets us study PSII samples given up to 10 flashes of light; that is, we can study the OEC step by step as it passes through more than two full enzymatic cycles.

Materials and methods

PSII membrane fragments were prepared as described by Pace (1991), and stored at –80ºC in a buffer containing 400 mM sucrose, 20 mM MES pH 6.0, 10 mM NaCl, 10 mM $MgCl_2$, and 5 mM $CaCl_2$. Prior to preparing EPR samples, methanol was added to a final concentration of 5 % (v/v). The samples were subjected to a preflash protocol which renders full oxidation of Y_D and close to 100% of the OEC centers poised in the S_1 state (Styring and Rutherford, 1988). After adding the electron acceptor phenyl-*p*-benzoquinone (PPBQ), dissolved in DMSO, to the samples, the appropriate number of laser flashes were given, upon which the samples were rapidly frozen. A single flash moves the preflashed PSII centers to the S_2 state, two flashes moves them to the S_3 state, etc. There is a miss parameter associated with the flash protocol; each flash brings 85-90% of the Mn centers forward in the S-cycle. This results in a dephasing of the centers. By monitoring the electron paramagnetic resonance

(EPR) S_2 multiline signal in each sample, we can estimate the miss factor and assure high S-state specificity in our samples.

The flashes were delivered by a 5 Hz Nd:YAG laser (532 nm, 7 ns, 300 mJ). Continuous wave (cw) EPR was performed on a Bruker E500 spectrometer, and pulsed EPR experiments on a Bruker 580 spectrometer fitted with a dielectric resonance cavity. Oxford Instruments liquid He cryostats were used.

Results

Microwave power saturation differences

By using the laser flash procedure described above, we obtained two samples dominated by the S_2 state: one which was frozen after one laser flash, and one that was frozen after five laser flashes. The S_2 multiline amplitude was twice as intense in the one-flash sample as in the five-flash sample (85% and 45% S_2 content, respectively), due to the increased mixing of the S-state populations with flash number. The EPR multiline spectrum from each sample is shown in Figure 1A. A spectrum taken with the same settings in a sample given four laser flashes is also shown. This spectrum shows very little S_2 multiline features. The weak S_2 multiline signal intensity after four flashes demonstrates that the multiline signal observed after five flashes has indeed appeared after a full turnover of PSII.

The multiline signals obtained after one and five flashes arise from the same S-state and appear similar in shape, but they have different microwave power saturation behavior. Figure 1B shows the microwave power saturation of the S_2 multiline signal in the two samples. The S_0-content of the five-flash sample is no more than 14%, and we carefully avoid regions where the S_0 signal is intense when quantifying the spectra. Consequently, the S_0 signal (which is difficult to saturate (Peterson *et al.* 1999)) does not contribute significantly to the S_2 multiline saturation behavior in the five-flash sample. Comparing these data with our earlier
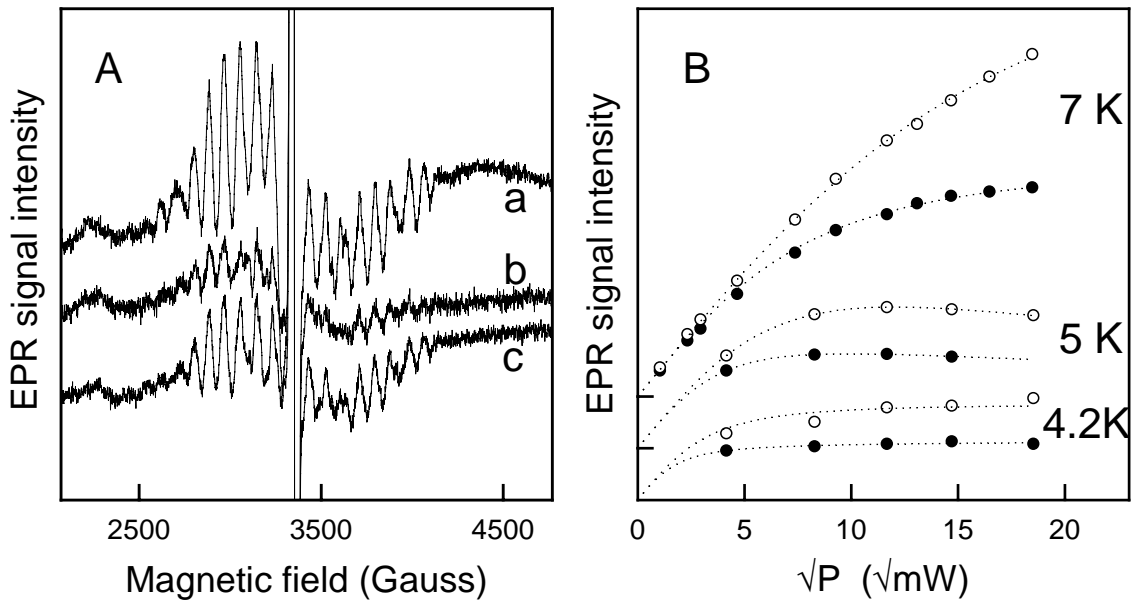

Fig. 1. A: The S_2 multiline signal recorded in samples given (a) 1 flash, (b) 4 flashes, and (c) 5 flashes. EPR settings: 7K, microwave power 5.9 mW, microwave frequency 9.47 GHz, mod. amplitude 20 G, mod. frequency 100 kHz.

B: Microwave power saturation at different temperatures of the S_2 multiline signal after one flash (filled circles) and five flashes (open circles).

published study (Peterson *et al.*, 1999), it is also evident that the five-flash S_2 multiline signal relaxes faster than the S_0 signal throughout the temperature region studied. Thus, the S_2 multiline signal produced by five flashes saturates differently than the S_2 multiline signal produced by 1 flash.

Changes at room temperature

   To investigate the stability of the fast-relaxing form of the S_2 multiline signal obtained by five flashes, we designed the following experiment. First, five-flash samples were prepared as usual. After the excitation flashes, the sample was not frozen immediately, but kept at room temperature in absolute darkness for a period of time, Δt, before freezing. The value of $P_{1/2}$ (at 5 K) was measured for each sample. The same procedure was used for one-flash S_2 samples, and also for the S_0 state signal in three-flash samples. This experiment is limited in time by the room temperature decay of the S_2 and S_0 states: the decay of the S_2 state has a half-time of 1.5 minutes, and the decay of S_0 has a half-time of 12 minutes (Åhrling *et al.*, 1997). As long as the signal is intense enough to be detected at several powers, it is possible to establish a $P_{1/2}$-value for the signal.

   Figure 2 shows the change of $P_{1/2}$ of the S_0 and S_2 multiline EPR signals during room temperature dark adaptation, reflecting changes (note: *not* the decay) of the S_0 and S_2 states during this time. The S_2 multiline signal in the one- and five-flash samples frozen immediately after the excitation flashes show the discrepancy in $P_{1/2}$ discussed earlier. During dark-adaptation at room temperature, the one-flash multiline signal remains at the same low saturation level. In contrast, the five-flash multiline shows a decrease of $P_{1/2}$ from the initial fast-relaxing level down to a level similar to that of the one-flash multiline signal. The half-time of this transition is approximately 30 seconds.
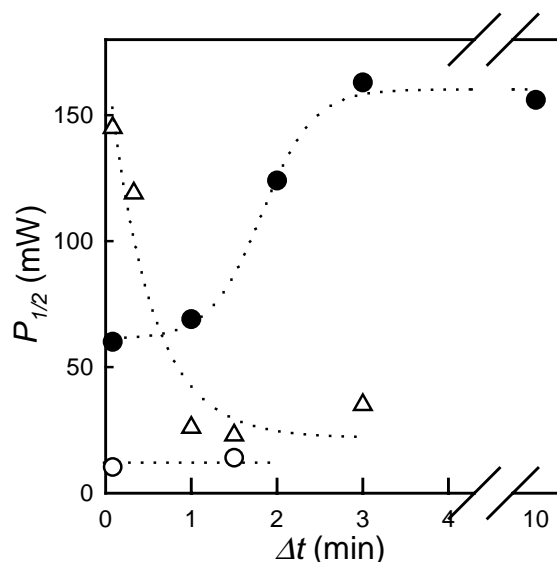

Fig. 2: Microwave power of half-saturation ($P_{1/2}$) at 5 K as a function of dark adaptation at 295 K (Δt). Open circles: the S_2 multiline signal after one flash, open triangles: the S_2 multiline signal after five flashes, filled circles: the S_0 signal.
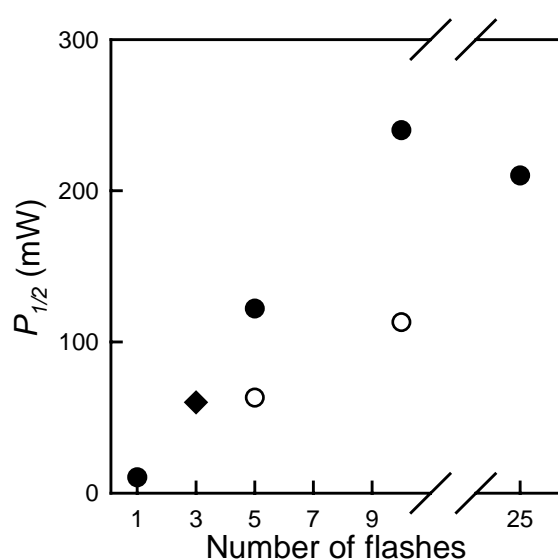
Fig. 3: $P_{1/2}$ at 5 K as a function of the number of flashes given to the sample before immediately freezing it. Filled circles: the S_2 multiline signal, open circles: the S_2 multiline signal after 3 weeks at 77 K, diamond: the S_0 EPR signal.

Surprisingly, the microwave power saturation of the S_0 signal also changes during room temperature dark adaptation, but in a very different manner. The relaxation rate does not change much the first minute, but then grows dramatically to a maximum level, reached after three minutes. The change is of a roughly sigmoidal shape, and in the opposite direction than that of the five-flash S_2 multiline.

The S_2 state after several turnovers

It is apparent from our data that the Mn cluster undergoes some sort of change during the first turn-over after dark adaptation. This may be part of a process that takes several cycles to complete. In order to investigate this possibility, we prepared PSII samples given 10 and 25 flashes, respectively. After 10 flashes, the S-state population is rather scrambled, but the S_2 state still dominates, with a sample composition of 30-40% S_2 centers and only ~10% S_0 centers. Thus, it is possible to study the S_2 state separately from the S_0 state in the 10-flash sample. Most of these S_2 centers are on the third turn-over after dark adaptation. The 25-flash sample is completely scrambled, with 25% of the centers in each S-state. What we can study in such a sample is 25% S_2 and 25% S_0, both several cycles away from dark adaptation. The values obtained from the S_2 multiline signal in this sample probably contains contributions from the S_0 signal, but the S_2 multiline behavior clearly dominates the strong central peaks used for our amplitude analysis.

The $P_{1/2}$-values obtained for the S_2 multiline signal in the multiple flash samples at 5 K were 240 mW for the 10-flash sample and 210 mW for the 25-flash sample (Figure 3, filled circles). Given the relative uncertainty of $P_{1/2}$ determination at such high powers of half-saturation, along with the possibility of S_0 interference in a scrambled sample, these values are rather similar. They are both significantly higher than the $P_{1/2}$ of the S_2 multiline in the five-flash sample, which is 150 mW at the same temperature. These data indicate that the form of the S_2 state that is present during continuous illumination of PSII centers is a fast-relaxing form, with $P_{1/2}=(220\pm30)$ mW at 5 K, which is reached after two complete turn-overs of a dark-adapted center.

When we remeasured the five- and ten-flash samples after three weeks of storage on liquid nitrogen, the $P_{1/2}$ values had receded (Figure 3, open circles), although the non-saturated intensity of the multiline signal had not changed.

Pulsed EPR investigations

The parameter of half-saturation, $P_{1/2}$, is proportional to both the transverse relaxation rate, T_2^{-1}, and the spin-lattice relaxation rate, T_1^{-1}. Pulsed EPR can be used to distinguish between these two parameters. We have commenced such studies on our samples, measuring T_2 with two-pulse ESEEM and T_1 with ESE-detected saturation recovery. The measurements are conducted on both the Mn-derived multiline signal and on Y_D^{ox}, since the radical is likely to be affected by the phenomenon as well. Our preliminary results (not shown) show that the T_2-values for the multiline signal and Y_D^{ox} are the same in both types of S_2 state samples, while the T_1-values from the two samples differ. The T_1 traces show more than one exponent in some cases, and we are not ready to present a full analysis at this stage. We have found that the S-states that show no EPR signal in modulated, perpendicular mode cw EPR still show absorption in the pulsed field swept mode (Peterson, 2000). Therefore, the pulsed data from a five-flash S_2 state sample must be treated with care, to avoid contributions from other S-states present after five flashes.

Discussion

There are some reports in the literature of phenomena reminiscent of that described here. Beck *et al.* (1985) discovered that a period of continuous illumination induces changes of the OEC such that the S_1 state obtained after a short period of dark-adaptation (6 minutes) is different from that obtained from a long-term dark-adapted sample (4 hours). The two forms of the S_1 state were termed "resting" and "active" forms. Both forms yielded full oxygen evolution on the third flash, but they were different in the form of the S_2 multiline signal that resulted from low-temperature illumination of the S_1 samples. They reported differences in spectral shape and in relaxation rate: the multiline signals derived from the active S_1 form were found to relax faster than the ordinary multiline signal (de Paula and Brudvig, 1985). The S_2 multiline spectra we obtain from the one- and five-flash samples both resemble the "resting state multiline" in shape, although they relax differently. When Y_D^{ox} relaxation was measured in the different S_1 samples (Koulougliotis *et al.*, 1992), it was found that in samples with the OEC in the resting S_1 state, Y_D^{ox} relaxed slowly – as slowly as in Mn depleted samples. With the OEC in the active S_1 state, Y_D^{ox} relaxation was faster. The transformation of the S_1 state in the dark from the active to the resting form had a half-time of 30 minutes at room temperature, as measured by the rate of O_2 consumption (Beck *et al.*, 1985), and 3.5 hours at 0°C, as measured by the relaxation rate of Y_D^{ox} (Koulougliotis *et al.*, 1992).

Van Vliet and Rutherford (1996) showed that the S_1 state produced by giving the sample four excitation flashes shows fast-relaxing Y_D^{ox}, irrespective of the initial, zero-flash form of the S_1 state. It is a significant finding, since it shows that a change similar to the activation described by Beck *et al.* (1985) is obtained after one full turn-over of the OEC. They also showed that a single flash of light followed by 10 minutes of dark adaptation leaves the sample in the resting S_1 state. During the preflash treatment, our samples are subjected to continuous illumination at room temperature followed by 15+15 minutes of dark-adaptation in the absence of PPBQ. The two periods of dark-adaptation are separated by a single preflash, but this should not change the status of the sample according to van Vliet, above. Since the half-time of deactivation of illuminated PSII samples is 30 minutes at room temperature (Beck *et al.*, 1985), our preflash treatment probably results in ~50% active and ~50% resting S_1 centers, in the terms of Beck *et al.*

Styring and Rutherford also did an experiment analogous to that of Fig. 2: they produced three-flash samples, waited different periods of time, and then froze them. After freezing, they measured the $P_{1/2}$ of Y_D^{ox}. They found that the saturation behavior of Y_D^{ox} changes with time in the S_0 state: the initially high value of $P_{1/2}$ recedes to a lower value with a half-time of 30 seconds. While some of this reduction can be ascribed to the decay of the S_2 and S_3 centers in the three-flash sample, this only accounts for approximately half of the observed change. Their results show, like ours, a change at room temperature in the S_0 state. In a later study (Evelo *et al.*, 1989), it was

Table 1. See text.

S_0	S_1	S_2
S_0 signal $P_{1/2}$ increase: $t_{1/2}^{(293\ K)} \approx 2$ min *(this work)* $Y_D^{ox} P_{1/2}$ decrease: $t_{1/2}^{(RT)} \approx 1$ min *(Styring, 1988)* NMR rel. rate decrease: $t_{1/2}^{(RT)} \approx 50$ s *(Srinivasan, 1986)*	$Y_D^{ox} T_1^{-1}$ decrease: $t_{1/2}^{(RT)} \approx 30$ min $t_{1/2}^{(273\ K)} \approx 3.5$ h *(Beck, 1985;* *Koulougliotis, 1992)*	Five-flash multiline signal $P_{1/2}$ decrease: $t_{1/2}^{(RT)} \approx 30$ s $t_{1/2}^{(77\ K)} \approx 3$ weeks *(this work)*

shown that the high $P_{1/2}$ first observed for the S_0 state was due to a temperature resonance phenomenon, indicative of a very strong relaxation enhancer, much stronger than the OEC poised in the S_2 state.

Srinivasan and Sharp made a similar discovery, when measuring proton NMR relaxation rates in PSII as a function of S-state (Srinivasan and Sharp, 1986). After one and two excitation flashes, the relaxation rate was enhanced by the increased oxidation state of the Mn. The observed enhancement decayed on the time-scale of S_2 and S_3 state decay to the S_1 state. However, the enhancement induced by three excitation flashes decayed with a half-time of 50 s, although the S_0 state is stable for over 10 minutes at room temperature. Thus, the observed decay of the relaxation rate was not due to the decay of the S_0 state; rather, a change on the order of 1 min was taking place in the three-flash sample.

Table 1 summarizes the different findings. Taken together, it seems that the S_0, S_1 and S_2 states of the OEC all come in different forms, as judged by the relaxation properties of the Mn cluster and Y_D^{ox}. For each S-state, one form is related to PSII under illumination, and the other one to PSII in the dark. Dark-adaptation lets the former relax to the latter form in each S-state, at different rates.

These data imply a single mechanism of change for all the S-states, that occurs during the first few turn-overs after dark-adaptation. The change does not increase the rate of O_2 evolution – it is maximal from the beginning – but it may well be a necessary preparation for continuous water-splitting. We introduce the term *light-adaptation* to describe the observed phenomenon.

Our present observations may be caused by the appearance of a relaxation enhancer on the donor side of PSII, or by a change of the magnetic couplings within the Mn cluster. We favor the latter alternative, and speculate that such a change of couplings may be driven by proton release from the water binding sites of the OEC, as the first few molecules of H_2O are oxidized. It may also be driven by Tyrosine$_Z$, which accepts an electron and a proton from the Mn cluster.

By continuing our pulsed EPR investigations, we hope to elucidate the exact mechanism of light-adaptation.

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