Manganese oxidation states in photosystem II

J Messinger1, JH Robblee2,3, U Bergmann2, C Fernandez4, P Glatzel2,5, S Isgandarova1, B Hanssum1, G Renger1, SP Cramer2,5, K Sauer2,3 and VK Yachandra2

1Max Volmer Laboratorium der TU Berlin, PC 14, Strasse des 17. Juni 135, D-10623 Berlin, Germany, email: johannes@struktur.chem.tu-berlin.de
2Melvin Calvin Laboratory, Physical Biosciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA 94720, email: VKYachandra@lbl.gov
3Dept. of Chemistry, Univ. of California, Berkeley, CA 94720
4Faculdade de Medicina - Fundação do ABC, Santo André, SP 09060-650, Brazil
5Dept. of Applied Science, Univ. of California, Davis, CA 95616

Keywords: water oxidation, S states, oxygen evolution pattern, XANES, Kβ XES

Introduction

Photosynthetic water oxidation is catalyzed by the oxygen evolving complex (OEC), which is a functional part of photosystem II. The OEC interfaces the light-induced one-electron oxidations of the PS II reaction center with the four-electron chemistry of water oxidation. The OEC has therefore two functions: a) storage of oxidizing equivalents and b) positioning and activation of the substrate water molecules. The oxidation states of the OEC are termed the S0, S1, S2, S3 and S4 states according to the number of stored oxidizing equivalents relative to the lowest state of the redox cycle. A main constituent of the OEC is a tetranuclear Mn cluster. It is well accepted that Mn oxidations take place during the S0 → S1 and S1 → S2 transitions. In contrast, the nature of the S2 → S3 transition is highly disputed. We present new data obtained by EPR, XANES and Kβ XES measurements that strongly support the idea of a ligand-centered oxidation during the S2 → S3 transition. Another controversial point is the absolute calibration of the Mn redox states. Mn4(III2,IV2) and Mn4(III4) are discussed as oxidation states for the dark-stable S1 state. In the case of Mn4(III4), the S3 state would be the lowest possible oxidation state of the OEC, assuming that only Mn(IV) and Mn(III) can be reduced in a reversible fashion by exogenous reductants like N2H4. First results are shown which indicate the existence of the S4 and S5 states and thus support Mn4(III2, IV2) as Mn oxidation states in the S1 state.

Materials and methods

PS II membranes were prepared using the BBY protocol. Flash samples (0F,…, 6F) were obtained by double sided laser flash illumination of PS II membranes (9.5 mg Chl/ml) containing 1 mM PPBQ. Great care was taken to minimize and monitor radiation damage during X-ray experiments. Further details are described in (Messinger et al., 2001). Oxygen flash yield measurements were obtained with a frequency doubled Nd/YAG-laser (9 ns pulse width) and a home-built Joliot-type electrode. The flash frequency was 1 Hz. Polarographic signals were stored on a computer and flash artifacts and O2 uptake signals were subtracted from the original traces within Excel.
Results and Discussion

**EPR**
The S\(_2\) EPR multiline signals (S\(_2\) MLS) from all flash samples (109) were measured to obtain information on their S state distribution. In Figure 1A the normalized S\(_2\) MLS amplitudes, determined from the four largest low field peaks, are plotted as a function of flash number (diamonds). Each point is the average of about 20 (0F to 3F) or 8 (4F to 6F) essentially identical individual samples. The S state composition of the samples was extracted from this S\(_2\) MLS oscillation pattern by systematic fits with an extended Kok model. The best fit was obtained with 10.8 % miss probability, 0 % double hit probability and 5.7 % initial S\(_2\) state population. The latter is due to the applied preflash protocol. This fit is shown in Figure 1A as solid line. It also contains about 5 % of centers which are blocked after an initial S\(_1\) → S\(_2\) transition.

**XANES**
In Figure 1B, the Mn K-edge position is plotted as a function of flash number. The edge energy is determined from the first zero crossing of the second derivatives (i.e. the inflection point energy, IPE) as described by (Roelofs et al., 1996). A clear upshift in energy is observed between the 0F and 1F samples, indicating a Mn oxidation on the S\(_1\) → S\(_2\) transition. In contrast, a much smaller shift in edge position is seen between the 1F and 2F samples. This suggests a ligand-centered oxidation during the S\(_2\) → S\(_3\) transition. The same pattern is seen (Table 1) when the flash spectra are deconvoluted into pure S-state spectra using the EPR data discussed above. In line with a Mn oxidation on the S\(_0\) → S\(_1\) transition, a clear edge shift is seen for this turnover.

**K\(\beta\) XES**
It has been demonstrated that the first moments of the K\(\beta\)\(_{1,3}\) peaks in Mn K\(\beta\) fluorescence emission spectra are less sensitive to structural changes than XANES edge energies (Visser et al., 2001) and that they shift to lower energies in response to Mn oxidation (Bergmann et al., 1998). The results of Figure 1C show a large downshift between the 0F and 1F samples and only a minor change between the 1F and 2F samples. This reinforces the conclusion that a ligand

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![Fig. 1](image)

**Table 1:** Mn K-edge energies of PS II membrane fragments in various S-states of the OEC.
centered oxidation takes place during the $S_2 \rightarrow S_3$ transition (Messinger et al., 2001).

**$S_{-4}$ and $S_{-5}$ states**

In spinach thylakoids, it was shown that N$_2$H$_4$ reacts predominantly as a two-electron reductant with the OEC, i.e. $S_1 \rightarrow S_{-1} \rightarrow S_{-3}$ ($\rightarrow S_{-5}$) or $S_0 \rightarrow S_{-2} \rightarrow S_{-4}$ (Messinger et al., 1997). In this study we use *Synechococcus elongatus* thylakoids because they retain higher O$_2$ evolution activity during incubation with high (30-100 mM) hydrazine concentrations (data not shown). Flash patterns of dark-adapted thylakoids (mostly $S_1$ state) were best fit with a miss parameter of 9.5 % and a double hit parameter of 0.0 % (Fig. 2A). These parameters are almost unaffected by hydrazine incubation (data not shown). The fit results in Table 2 show that the $S_{-4}$ and $S_{-5}$ states are required for acceptable fits of the O$_2$ flash patterns obtained after hydrazine incubation. It should be noted that so far we have observed the $S_{-5}$ state only in samples that were not washed to remove N$_2$H$_4$ after incubation (Fig. 2B and fits 2 and 4, Table 2). In contrast, the $S_{-4}$ state can still be observed (to a lesser extent) after one washing step, which decreases the N$_2$H$_4$ concentration from 50 mM to approx. 0.5 mM (Fig. 2C and fits 4 and 6, Table 2). After extensive washing (3x) $S_{-4}$ is also lost, but $S_3$ and higher states (if present) are not affected (not shown). This could be a consequence of the expected instability of the $S_{-4}$ and $S_{-5}$ states. Other explanations for the high O$_2$ yields in the 8th and 9th flashes involving for example back reactions of the $S_2$ and $S_3$ states with N$_2$H$_4$ or $Y_D$ within the flash train cannot be fully ruled out at the moment. However, if back reactions with N$_2$H$_4$ were involved, then the difference in $S_{-4}$ state population would be expected to be larger between patterns C and D. In contrast, back reactions with $Y_D$ should not be affected at all by washing steps; thus $S_{-4}$ would not be expected to disappear after 3 washing steps.

<table>
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<th>Sample</th>
<th>Fit</th>
<th>$S_{-1}$</th>
<th>$S_{-2}$</th>
<th>$S_{-3}$</th>
<th>$S_{-4}$</th>
<th>$S_{-5}$</th>
<th>$f_{q} \times 10^5$</th>
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<td>-</td>
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<td>39.9</td>
<td>43.4</td>
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</table>

**Table 2:** Fit of O$_2$ oscillation patterns of *Synechococcus elongatus* thylakoids incubated with N$_2$H$_4$ (Fig. 2). Sample B: 10 min incubation of dark-adapted thylakoids with 30 mM N$_2$H$_4$ on ice (+ 5 min on electrode). Sample C was enriched in $S_0$ by 3 flashes and then incubated 10 min with 50 mM N$_2$H$_4$ on ice. Sample D: same as B, but washed once in N$_2$H$_4$ free buffer. In Fits 1, 3 and 5 the $S_{-4}$ and $S_{-5}$ states were excluded. The fit quality ($f_{q}$) was calculated as sum(dy$_i^2$)/(data points - free parameters). The O$_2$ yields of the first 16 flashes were included in the fits. Fixed parameters: misses 10.0 %, double hits 0.0 %, other $S$-states 0.0 %
The results of this study are consistent with the following oxidation states of the OEC: $S_0$, $\text{Mn}_4(\text{II,III,IV}_2)$ or $\text{Mn}_4(\text{III},\text{IV})$; $S_1$, $\text{Mn}_4(\text{III}_2,\text{IV}_2)$; $S_2$, $\text{Mn}_4(\text{III},\text{IV}_3)$; $S_3$, $\text{Mn}_4(\text{III},\text{IV}_3)^*$. The dot represents a ligand centered oxidation. Based on the observed lengthening of all Mn-Mn distances during the $S_2 \rightarrow S_3$ transition (Liang et al., 2000), a $\mu$-oxo-bridge radical most likely exists in the $S_3$ state.

**Acknowledgments**

This research was supported by the National Institutes of Health Grant (GM-55302), and the Director, Office of Science, Office of Basic Energy Sciences, Division of Energy Biosciences, U. S. Department of Energy under contract DE-AC03-76SF00098. Synchrotron radiation facilities were provided by SSRL. GR gratefully acknowledges the financial support by the DFG (Sfb 498, TP C4). JM thanks the DFG for support through grants Me 1629/1-1, 2-1 and 2-2. JM and SI thank P. Fromme, A. Zouni, J. Kern and H.T. Witt for the kind gift of *Synechococcus elongatus* thylakoids.

**References**


**Fig. 2** Normalized oxygen flash yield patterns (symbols) and fits (lines) from control (A) and hydrazine reduced (B, fit 2; C, fit 4) from *Synechococcus elongatus* thylakoids. For details see Table 2 and text.