EPR, MAGNETIC SUSCEPTIBILITY AND ENDOR STUDIES OF THE WATER OXIDIZING COMPLEX AND DIMANGANESE MODELS: CONSEQUENCES OF CL- EXCHANGE AND CA2+ DEPLETION.

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1. INTRODUCTION

Calcium, chloride and manganese are inorganic cofactors that are required for photosynthetic water oxidation. While manganese serves as the redox agent which catalyzes water oxidation, the biological roles of calcium and chloride have not been determined.

Here we report evidence from EPR spectroscopy and magnetic susceptibility for the occurrence of a major structural change in the manganese cluster upon extraction of one Ca from spinach PSII membranes. We also show that replacement of Cl- by F- produces no detectable change in the magnetic susceptibility for the S1 -> S2 transition while the EPR data yield the g=4.1 signal instead of the normally occurring multiline g=1.98 signal.

Promising results from synthetic dimanganese(III,IV) complexes show that proton and nitrogen ENDOR resonances can serve to identify the coordinating ligand groups for first and second shell magnetic atoms. Direct detection of 55Mn ENDOR signals holds promise for the assignment of the coordination symmetry and oxidation state of Mn in PSII.

2. MATERIALS and METHODS

Highly active Triton-solubilized spinach PSII membranes1 (BBY type, 400μM O2/h mgChl) and reaction center core complexes extracted using β-octylglycopyranoside-2 (OGP, 600-900μM O2/h/mgChl) were prepared as previously described.3 F-substitution was done by washing twice with Cl- free buffer at pH 7.5 followed by incubation in the F-containing buffer at pH 6.4. Extraction of a single Ca2+ involved in O2 evolution was done using citrate buffer (pH=3) as outlined by Ono and Inoue.5 Cyanobacteria Synechococcus sp., a gift of Prof. S. Katoh, were cultured at 55°C for three days and crude oxygen evolving core complexes (1500-2000μM O2/h/mgChl at 40°C) were prepared with OGP according to Katoh.6 Laser flash induced changes in magnetic susceptibility were measured using the superconducting SQUID magnetometer described by J. Philo,7 with sample preparation conditions as previously described.8 Samples were given a single preflash followed by a 20 min dark period prior to the train of flashes. DCBQ (1 mM) was used as the electron acceptor.

X-band EPR and ENDOR measurements were performed using Bruker spectrometers (ESP300 and ER250SH) with broadband rf excitation (1-150MHz) and a gasflow helium cryostat for low temperature measurements (T=4-80K, Oxford ESR 900).

3. RESULTS and DISCUSSION

3.1. Chloride extraction. Prior studies have shown that Cl- depletion modifies the S2 state (spin S = 1/2) by reversible loss of the normal S2 multiline EPR signal4 while the
g = 4.1 signal (spin S=3/2 ground state) is still present.\(^9\) Both F\(^-\) substitution and 140 K illumination lead to an increase of this signal.\(^{10}\) This change in EPR has been attributed to either an increase in the electronic ground state spin from S = 1/2 to 3/2 for a tetramanganese cluster\(^{11}\) or to oxidation of an isolated ion Mn(III) → Mn(IV).\(^{12}\) These proposals can be readily distinguished by magnetic susceptibility. Fig. 1 gives the changes in magnetic susceptibility (Δχ) produced by 3 single turnover laser flashes in untreated spinach core complexes (g), F\(^-\) substituted core complexes (i) and Cl\(^-\) reconstituted core complexes (h). For the first flash, the S\(_1\) → S\(_2\) reaction, Δχ = +6μB\(^2\)/PSII remains large and unaltered upon F\(^-\) substitution compared to the reconstituted level. All the following flashes produce no change, indicating either a blockage of S\(_2\) photooxidation or that subsequent S states have the same χ as the modified S\(_2\) state. The retention of the large increase in Δχ for S\(_1\)→S\(_2\) indicates that photooxidation of a magnetically isolated high spin Mn(III) → Mn(IV) does not occur, since this is predicted to produce a decrease in Δχ by -9μB\(^2\)/center (8).

**Figure 1.** Light induced changes in magnetic susceptibility (a, b, c) for different O\(_2\)-evolving PSII preparations: a) OGP spinach core complexes; b) BBY spinach membranes; c) Synechococcus sp. OGP core complexes; d,e,f) Effects of calcium depletion; d) BBY spinach membranes; e) Ca\(^{2+}\) reconstituted; f) Ca\(^{2+}\) depleted. (g,h,i) Effects of replacing Cl\(^-\) with F\(^-\): g) spinach OGP core complexes; h) Cl\(^-\)-reconstituted; i) F\(^-\)-substituted. All data at 0°C except c)20°C; flash interval 2 secs; flash duration 300 ns.

### 3.2. Comparison of PSII in spinach vs. a thermophilic algae

Fig. 1 shows that the pattern of flash induced changes in magnetic susceptibility for different PSII preparations isolated from spinach as OGP core complexes (a) and BBY membranes (b) are very similar to those seen in OGP complexes from the thermophilic algae *Synechococcus sp.* (c). The data are given in terms of Δμ\(_{eff}\)/PSII where the number of PSII centers is based on a chlorophyll determination of 225 Chl/PSII (BBY, Fig 1b,d,e,f) or 70 Chl/PSII (OGP, Fig 1a, g,h,i) or the measured Mn content and an assumed ratio of 4Mn/PSII yielding 120 Chl/PSII (*Synechococcus*, Fig 1c). The Chl normalization includes all PSII centers (active + dead) as an upper limit, while the normalization to Mn content ignores dead centers which are free of Mn. The pattern of changes consists of positive increases on flashes 1,3 and 5 and zero or small increases on flashes 2, 4 and 6. The amplitude differences between spinach and *Synechococcus* may not be real owing to
the different normalizations and temperatures used (273K vs. 298 K). The data indicate the presence of a similar pattern of magnetic coupling between Mn ions in the WOC and hence similar bridging ligand structures. This similarity in magnetic properties of the WOC exists despite the different conditions for an EPR detectable S2 multiline signal (Synechococcus: absent in sucrose buffer, present in glycerol) and a 3 fold higher O2 evolution rate (at 40-45°C) compared to spinach (at 20°C).

![Diagram of Mn23/4(Pz3BH)2OOAc(Ph)3](image)

**Fig.2a: Structure elements of Mn23/4(Pz3BH)2OOAc(Ph)3:**

- **b:** EPR spectrum 10^-2M in CDCl3, T=5K, Mod.: 2Gpp, 9.4 GHz;
- **c:** ENDOR spectra 3-23 MHz pos 2 and 3, mw=65mW, rfp=100W, fmod.: 80kHz, T=5-10K, 20 scans a 40s;
- **d:** ENDOR spectra 80-160MHz, fmod=250kHz, scan time 10 min.

3.3. Ca2+ depletion. Ca2+ depletion has been shown to abolish O2 evolution while restricting photooxidation of the WOC to a single turnover.5ab Photooxidation produces an unusually stable S2QB state that is resistant to recombination. In Fig. 1f it is seen that spinach PSII membranes depleted of a single Ca2+ ion exhibit no Δχ on any flash compared to the undepleted control (d) and the Ca2+ reconstituted sample (e). The reconstituted level of Δχ matched the 70% reconstitution of O2 evolution rate. The absence of a change in Δχ observed on the first measuring flash indicates that the modified S2 state possibly already produced by the preflash (see 2. Mat. & Met.), is either very
stable (hence no change in \( \chi \)), or that Ca depletion abolishes any difference in \( \chi \) for the \( S_1 \leftrightarrow S_2 \) reaction. Evidence for a major structural change is also seen by EPR which reveals two new EPR signals in the \( g=2 \) region (data not shown). One reached by illumination at 273K has an unresolved 200G wide signal, and a second is maximized by further dark adaptation at 273 K. The latter signal shows a similarity with the \( S_2 \) multiline signal, but has reduced Mn hyperfine splittings and contains more hyperfine lines. Ca\(^{2+}\) depletion was also found to increase the accessibility of the Mn site to \( \text{NH}_2\text{OH} \).

3.4. Mn-dimer studies. As a basis for beginning to identify the possible ligands which coordinate Mn in the WOC, we have undertaken a systematic study of the ENDOR spectra of synthetic Mn\(_{2}\)(III,IV) complexes of the di-\( \mu\)-oxo-\( \mu\)-carboxylato type shown in Fig. 1a. In Fig. 2b the well-resolved 16-line EPR spectrum typical of this family [RCO\(_2\)=tripheny lacetate, P\(_2\)3BH=hydrotri(pyrazolyl)borate] at 5 K. Good spectral resolution of an axial tensor component on the high field EPR transition made it possible to select the spin centers according to their orientation in the magnetic field for ENDOR study. The very large Mn hyperfine field associated with the EPR signal also made it possible to exploit the difference in the Zeeman effects for \( ^1\text{H} \), \(^{14}\text{N} \), \(^{11}\text{B} \) and even \(^{55}\text{Mn} \) to assign resonances, as shown in Fig. 2c,d. ENDOR recorded at the two resolved orientations noted in the EPR spectrum as positions 2(II) and 3( ) showed clear separation into two tensor components due either to g or hyperfine anisotropy. ENDOR couplings recorded at all other EPR field positions could be deconvoluted into a sum of these two spectra. Detailed assignments of these resonances are in progress using isotopically labelled derivatives.

Direct detection of \(^{55}\text{Mn} \) ENDOR couplings can be used to provide a very precise determination of the hyperfine and quadrupole couplings of this nucleus and hence gives information about the strength and symmetry of the Mn ligand field (Fig. 2d). The spectrum shown covers the frequency range 80-160 MHz for a single Mn nucleus possessing the smaller of the two hyperfine couplings predicted for these dimers. This can be assigned to the Mn(IV) oxidation state.

Ligand ENDOR measurements on the \( S_2 \) multiline state of PSI are also in progress. Preliminary results show evidence for \( \text{D}_2\text{O} \) exchangeable matrix proton couplings to Mn and only weak or no low frequency couplings which could be assigned to nitrogen ligands.

Acknowledgements.
This work was supported by NIH (GM 39932 and HL-24644). M.B. thanks the DFG for a generous grant.

4. REFERENCES
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