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The state of manganese in the photosynthetic apparatus. 5. The chloride effect in photosynthetic oxygen evolution. Is halide coordinated to the EPR-active manganese in the O_2-evolving complex? Studies of the substructure of the low-temperature multiline EPR signal

Vittal K. Yachandra, R.D. Guiles, Kenneth Sauer and Melvin P. Klein *

Laboratory of Chemical Biodynamics, Lawrence Berkeley Laboratory, University of California, Berkeley, CA 94720 (U.S.A.)

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The role of chloride in the manganese-containing oxygen-evolving complex of Photosystem II has been studied by observing the amplitude of the multiline EPR signal as a function of Cl^- concentration or when Cl^- is replaced by Br^- or F^-. The correlation of the multiline EPR signal intensity and O_2 activity with the concentration of Cl^- shows that chloride is involved in oxygen evolution at the S_2 or earlier S states, and that it is necessary for the production of an EPR-detectable S_2 state. We have developed a new method for the preparation of subchloroplast PS II particles containing Br^- and F^- and have used these particles for studying the EPR fine structure at high resolution. The fine structure shows a multiplet of 4–6 lines with 10–15 G spacing; at the resolution of our experiment there are no significant differences between the Cl^-- and Br^--containing samples, suggesting that the halide is not a ligand of the EPR-active Mn. Various structural possibilities for the Mn complex, which would account for the observed fine structure of the multiline EPR spectrum are discussed.

Introduction

It has been known since the early work of Warburg and Lüttgens [1] that chloride is an essential cofactor in photosynthetic oxygen evolution. Recent studies have confirmed that Cl^- is closely related to Photosystem II [2,3], and it has been suggested that the Mn-containing oxygen-evolving complex is the site of Cl^- action [4–7]. Models have been presented suggesting that the chloride ion could be ligated to Mn [3,8] or may be required to stabilize a positive charge on the Mn-containing oxygen evolving enzyme [9,10]. Recent kinetics studies have concluded that Cl^- depletion inhibits the advancement of the Mn-oxygen-evolving complex beyond the S_2 state [11,12], while other studies have implicated a role for Cl^- in the S_3^*-to-S_0 transition [13]. It has also been postulated that Cl^- interacts with cytochrome *b*-559 [14,15].

Although the requirement for Cl^- on the donor side of PS II and its involvement with the Mn-oxygen-evolving complex has been established,

* To whom correspondence should be addressed.

Abbreviations: Chl, chlorophyll; DMBQ, 2,6-dimethyl-*p*-benzoquinone; EXAFS, extended X-ray absorption fine structure; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PS, Photosystem; Mes, 4-morpholineethanesulfonic acid; ETW and TWE, exchanged and Triton-washed, and Triton-washed and exchanged, respectively.

both its mechanism and site of action are still matters of controversy.

The light-induced multiline EPR signal containing 19 or more lines extending over more than 1000 G in width observed at low temperature in spinach chloroplasts has been assigned to a manganese species on the basis of its hyperfine splittings [16–18]. The flash dependence and the temperature dependence of its formation and decay indicate that it is correlated with the presence of the S_2 state in the O_2-evolving complex [16,19]. Using EDTA-washed, Cl^--depleted chloroplasts, we have investigated in detail the Cl^- requirements for generating the multiline EPR signal. We present evidence to suggest that Cl^- is required for the generation of the S_2 state as defined by EPR criteria and also that the fine structure of the multiline signal is invariant (at a modulation amplitude of 4 G) when Cl^- is replaced with Br^-, indicating that the site of the halide ion is not as a ligand to the EPR active Mn at the S_2 state. However, it cannot be ruled out that the transferred hyperfine coupling with the halogen nuclei is too small to be evident at this resolution.

Materials and Methods

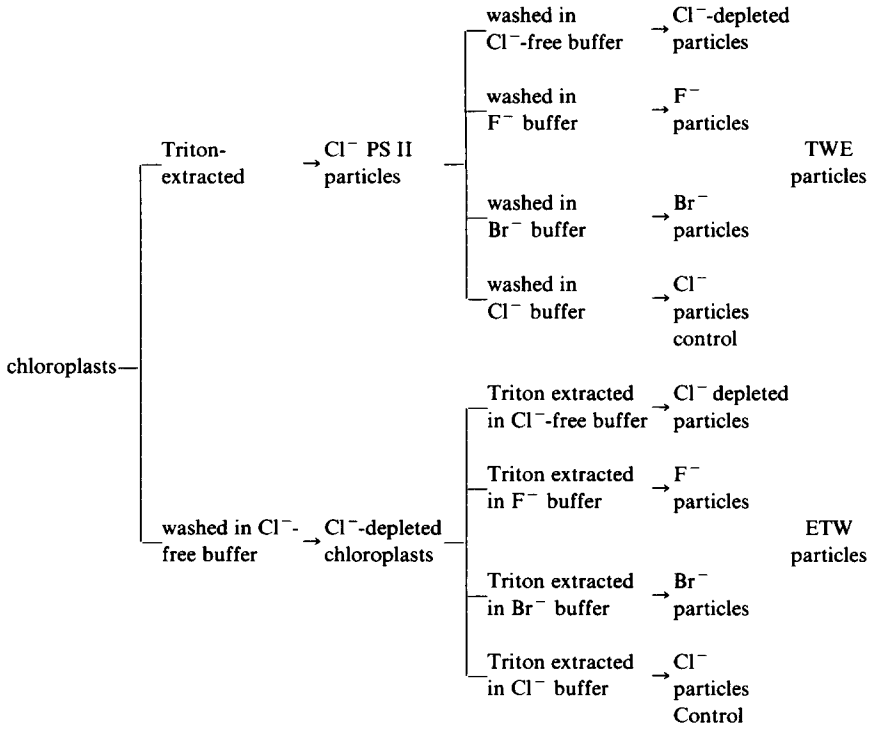
Broken chloroplasts were prepared from destemmed market spinach by a procedure described elsewhere [20]. Chloride-depleted chloroplasts were prepared by washing the chloroplasts 3–4 times in a chloride-free buffer containing 50 mM Hepes (pH 8.0)/10 mM Na_2SO_4/5 mM $MgSO_4$/0.5 mM EDTA. Fewer washes are required when higher pH (8.3–8.5) buffers are used [6,21]. Two more such washes were carried out in an EDTA-free buffer at pH 7.5. The suspensions were incubated for 5 min each time in the dark before centrifugation. O_2 evolution activity of the chloroplasts was monitored in a Cl^--free buffer after each wash; after 3–4 washes the chloroplasts were totally inactivated.

The Cl^- samples were prepared by suspending the Cl^- depleted chloroplasts in buffers containing 50 mM Hepes (pH 7.5), 5 mM $MgSO_4$, 10 mM Na_2SO_4 and various concentrations of NaCl ranging from 0.1 to 15 mM. The Br^- samples were prepared by suspending Cl^--depleted chloroplasts in 50 mM Hepes buffer (pH 7.5)/5 mM $MgSO_4$/15 mM NaBr. The F^- sample was prepared in a similar manner, except that $MgSO_4$ was not added because of the limited solubility of MgF_2. The O_2 activity and the amplitude of the multiline EPR signal are reported as a fraction of the control sample, which contained 15 mM NaCl.

The preparation of Cl^--free or Br^-- or F^--substituted subchloroplast membranes was accomplished by a modification of the method of Kuwabara and Murata [22]. The method consisted of initially preparing Cl^--depleted chloroplasts followed by suspension in Cl^--free (50 mM Hepes (pH 7.5)/10 mM Na_2SO_4/5 mM $MgSO_4$) or Cl^- (50 mM Hepes (pH 7.5)/15 mM NaCl/5 mM $MgCl_2$) or Br^- (50 mM Hepes (pH 7.5)/15 mM NaBr/5 mM $MgSO_4$) or F^- buffers (50 mM Hepes (pH 7.5)/10 mM Na_2SO_4/15 mM NaF). PS II particles were then prepared by Triton X-100 treatment of the chloroplasts in the respective 50 mM Mes buffers at pH 6.0 [22] (Exchanged and Triton-Washed: the ETW method). This was by far the most efficient method for preparing functionally Cl^--depleted or Br^-- or F^--containing PS II particles, as will be shown in the results section.

We also prepared Cl^--depleted PS II particles by washing PS II particles prepared by the method of Kuwabara and Murata in a Cl^--free buffer containing 50 mM Mes (pH 6.3)/5 mM $MgSO_4$/10 mM Na_2SO_4. Dialysis of the PS II particles overnight in a Cl^--free buffer with three buffer changes yielded similar results. The PS II samples were made by suspending the Cl^--depleted PS II particles in 50 mM Mes at pH 6.0, 6 mM $MgSO_4$, 10 mM Na_2SO_4 and various concentrations of NaCl ranging from 0.1 to 15 mM. Bromide or fluoride samples were prepared by suspension in buffers containing 15 mM NaBr or 15 mM NaF. The F^- sample did not contain any $MgSO_4$. We also prepared the F^- and Br^- samples by directly exchanging the Cl^- PS II particles in F^- and Br^- buffers (Triton-Washed and Exchanged: the TWE method) instead of initially preparing the Cl^- depleted thylakoids. The protocol for the preparation of ETW and TWE particles is summarized in Scheme I.

Rates of O_2 evolution were measured using a Clark-type oxygen electrode. Illumination was with a 200 W quartz lamp. Chloroplast samples were suspended at 20–30 μg Chl/ml in 50 mM Hepes



Scheme I. Schematic presentation of the preparation of TWE and ETW PS II particles.

(pH 7.5)/5 mM $MgSO_4$/3 mM $K_3Fe(CN)_6$/500 μM DMBQ and contained the same concentration of the halide ion as the samples. In the case of PS II particles 50 mM Mes at pH 6.0 was used. O_2-evolution rates were typically 250–300 μmol O_2 per mg Chl per h for PS II particles and 150–200 μmol O_2 per mg Chl per h for the chloroplasts.

For EPR measurements samples were placed in quartz tubes and dark adapted for 1 h at 4°C. After equilibration at 190 K in a dry ice/methanol bath, the samples were illuminated with a tungsten lamp for 1 min and then were immediately frozen in liquid N_2.

EPR spectra were recorded with a Varian E109 spectrometer equipped with a model E102 microwave bridge and an Air Products Helitran cryostat. EPR spectra were recorded at 8–10 K using 50 mW microwave power at 9.19 GHz, 100 kHz field modulation of 32 G amplitude, scan time 4 min and time constant 0.25 s. EPR spectra at high resolution (4 G modulation amplitude) were collected using PS II particles with 6–8 mg Chl/ml. To achieve a signal-to-noise ratio at which the substructure became evident, we collected multiple scans (approx. 60, 4 min scans at a time constant of 0.128 s) using a signal averager built in our laboratory, and the averaged data was then transferred to a VAX 11/780 computer for analysis.

Results

Fig. 1a shows the amplitude of the multiline EPR signal generated by 1 min of continuous illumination at 190 K plotted as a function of the Cl^- concentration. The curve drawn in Fig. 1a is a hyperbolic plot derived from a linear least squares fit to a Lineweaver-Burk plot of the data. The profile of the curve is similar to that observed in earlier studies [2,3] of O_2-evolution activity. The EPR signal amplitude reaches half maximum at about 0.5 mM Cl^- and a maximum at 5–10 mM Cl^- ion concentration. Fig. 1b is a plot of the oxygen evolution activity vs. the multiline EPR signal amplitude at each Cl^- concentration

studied. The line drawn is a linear least squares fit to the data. Both response inhibitions were reversible and are strongly correlated.

The results with Br^- or F^- substituted chloroplasts show that Br^- is an effective replacement for Cl^- in terms of O_2 activity (95%), but F^- does not restore O_2 activity to more than 30% of the control sample. The amplitude of the multiline EPR signal correlates with O_2 activity in both cases, 83% for Br^- and 30% for F^- (Table I).

Similar studies were carried out using TWE PS II particles. Repeated washing or dialysing against Cl^--free buffers decreased the O_2-evolution activity by about 40%, with a corresponding decrease in multiline EPR signal amplitude, but we were unable to prepare PS II particles with total functional impairment by this method. The results are shown in Fig. 2a and b. Both the O_2-evolution activity and the amplitude of the multiline EPR signal amplitude reach a maximum at 5–10 mM Cl^- concentration.

The Br^- and F^- substituted particles prepared by the TWE method follow patterns observed with chloroplasts. Br^- is an effective replacement for Cl^-, with O_2-evolution activity of 100% and a multiline amplitude of 84%. The F^--substituted particles exhibit an O_2-evolution activity of about 23% and a multiline amplitude of about 46%. It is interesting that the F^--substituted particles exhibit a smaller multiline amplitude and O_2 activity than the Cl^--depleted particles.

The behavior of PS II particles prepared by the ETW method was very different. By this method we were able to prepare Cl^- depleted PS II particles with considerably less O_2 evolution activity and multiline EPR signal (10–20%) than the particles prepared using the TWE method (approx. 60%). On readdition of Cl^- to Cl^--depleted ETW particles only 40–50% of control activity was restored (control was the Cl^- ETW particles, see Scheme I for details). It appears that the O_2 activity and multiline EPR signal amplitude of the PS II particles can be reduced to only about 60% by washing in a Cl^--free medium but, once completely Cl^--depleted PS II particles are prepared (by utilizing completely Cl^--depleted chloroplasts), only about 50% of the O_2 activity and multiline EPR signal can be restored on readdition of Cl^-.

The particles prepared using the ETW method

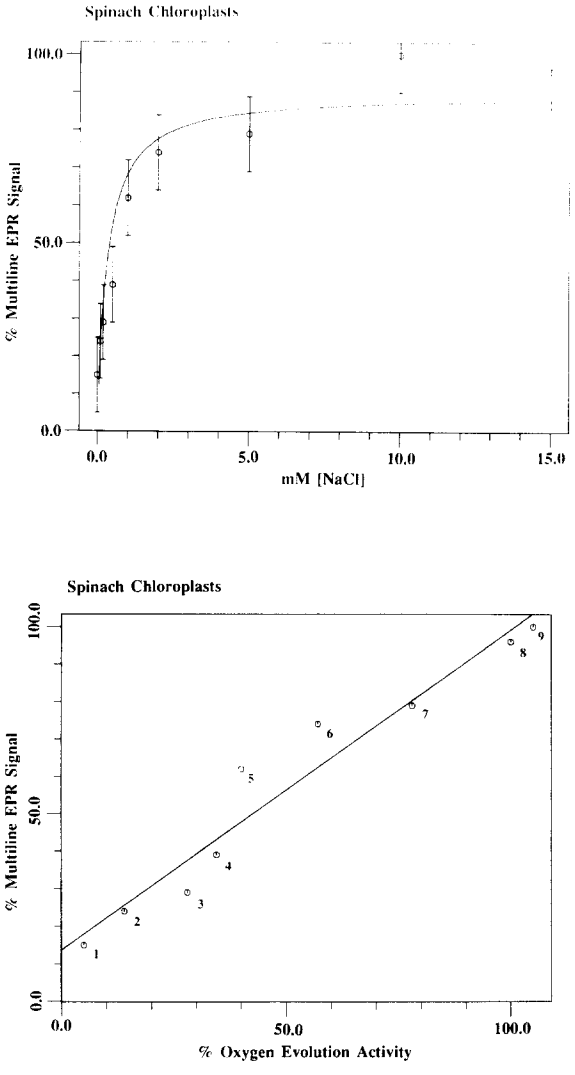


Fig. 1. (a) The effect of Cl^- concentration on the amplitude of the low-temperature multiline EPR signal of spinach chloroplasts. Illumination and EPR protocols are described in the text. The amplitudes of the multiline signal were taken as the average peak-to-peak height of 4 lines downfield and 4 lines upfield from $g = 2.0$ and normalized with respect to the Chl content of each sample. Sample buffers contained 50 mM Hepes at pH 7.5, 5 mM $MgSO_4$, 10 mM Na_2SO_4 and a variable concentration of Cl^-. The curve drawn is a hyperbolic fit derived from a linear least squares fit to a Lineweaver-Burk plot of the data. (b) The relation between multiline EPR signal amplitude and O_2-evolution activity of spinach chloroplasts at various Cl^- concentrations. Illumination, EPR and oxygen-activity assay protocols are described in the text. The line drawn is a linear least-squares fit to the data. Cl^- concentrations corresponding to each point plotted are: (1) 0.0; (2) 0.1; (3) 0.2; (4) 0.5; (5) 1.0; (6) 2.0; (7) 5.0 (8) 10.0 and (9) 15 mM.

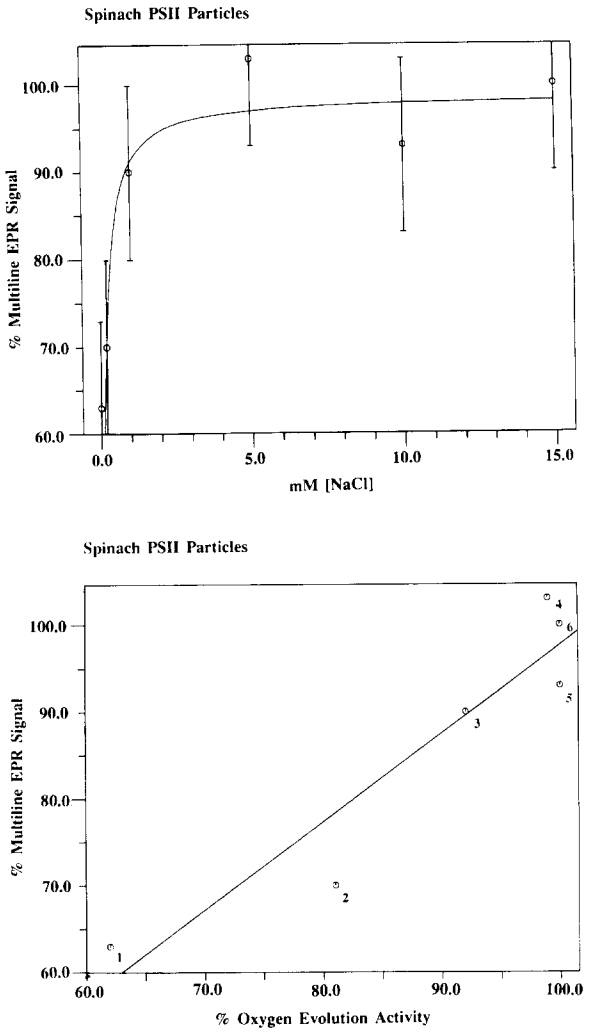


Fig. 2. (a) The effect of Cl^- concentration on the amplitude of low temperature multiline EPR signal of TWE PS II particles. Conditions are similar to those described in Fig. 1a. (b) The relation between multiline EPR signal amplitude and O_2-evolution activity of TWE PS II particles at various Cl^- concentrations. The line drawn is a linear least-squares fit to the data. Cl^- concentrations corresponding to each point plotted are: (1) 0.0; (2) 0.2; (3) 1.0; (4) 5.0; (5) 10.0; and (6) 15.0 mM. Illumination, EPR and oxygen-activity assay protocols are described in the text.

resulted in Cl^- and Br^- particles which were indistinguishable from the particles prepared using the TWE procedure in terms of activity and multiline EPR signal amplitude. The F^- samples prepared by the two protocols exhibited distinctly different properties. The O_2 activity was less than 5% and we were unable to generate any multiline EPR signal using particles prepared by the ETW method, compared to about 30% activity and multiline EPR signal that we could generate with the TWE method. This raises the possibility that the O_2 activity and multiline amplitude in F^- samples prepared by washing or exchanging is actually due to the presence of residual amounts of Cl^- in the preparations, which is then completely removed by our newer procedure of preparing F^- particles giving rise to negligible activity and multiline signal. All of these results are summarized in Table I.

Other studies [2–7] have implicated Cl^- as a ligand to Mn or in the electrostatic stabilization of the complex. These studies and our results presented above suggest that the Cl^- requirement for O_2 activity is due to the involvement of Cl^- in the Mn-containing oxygen-evolving complex. To test for the possibility that the halide is in the first coordination sphere of the metal atom, where it could give rise to superhyperfine splittings from the halogen nuclei, we have recorded the multiline

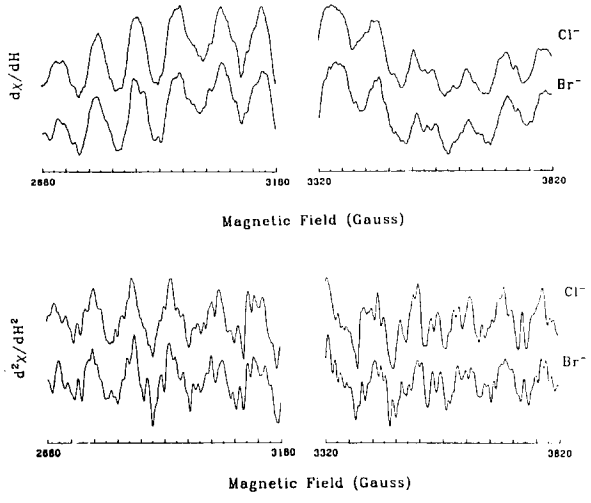


Fig. 3. (a) Multiline EPR spectra observed for spinach ETW PS II particles in Cl^- and Br^- buffers. The EPR spectra were recorded at 7 K using 50 mW microwave power at 9.19 GHz; 100 kHz field modulation at 4 G amplitude; scan time, 4 min; time constant, 0.128 s. The spectra shown are the sum of 60 individual scans. (b) The second derivative $d^2\chi/dH^2$ of the multiline EPR spectra for spinach ETW PS II particles in Cl^- and Br^- buffers. The second derivatives were obtained numerically from a 10 point (5 G) zero-order sliding fit to the data.

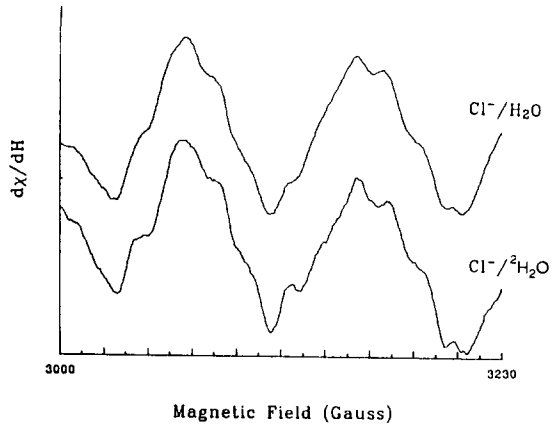


Fig. 4. The multiline EPR spectra of spinach TWE PS II particles in Cl^-/H_2O or in $Cl^-/^2H_2O$ buffers on the low field side of $g = 2$. The PS II particles were washed in ^2H_2O buffer 3 times and cycled through the S states by illumination and then again washed in ^2H_2O buffer before dark adaptation and preparation of the S_2 state by continuous illumination at 190 K. The instrument settings were identical to those decribed for Fig. 3a.

EPR signal at high resolution. Fig. 3a shows the EPR spectra of PS II particles in Cl^- and Br^- buffers at 4 G modulation amplitude. Distinct fine structure is seen in both spectra and is even more evident in the second derivative presentation shown in Fig. 3b. Each major line is split into a multiplet of 4–6 lines with splittings of the order of 10–15 G and there are no statistically significant differences between the spectra of the Cl^- and Br^- samples.

Fig. 4 shows a portion of the multiline spectrum on the low field side of $g = 2$, on an expanded scale. The peaks are clearly resolved into a multiplet and appear to be more complex than was previously assumed [23]. The multiplet spectrum of a suspension of PS II particles in ^2H_2O buffer is also shown in Fig. 4, but no clear differences are seen in the spectra of the Cl^-/H_2O or $Cl^-/^2H_2O$ samples.

Discussion

Correlation of Cl^- concentration with multiline EPR signal amplitude

The correlation of the multiline amplitude with the concentration of Cl^- in chloroplasts is a direct indication that Cl^- is required for the generation of the S_2 state as defined by EPR criteria, and that its involvement in the Mn-containing oxygen-evolving complex occurs prior to the S_2 state However, it is possible that Cl^- is also required for the generation of the higher S states, as suggested in other studies [11–13]. Earlier studies with Cl^--depleted chloroplasts indicate that two oxidizing equivalents can accumulate on the oxidizing side of PS II, forming the S_2 state in the dark [14]. The more recent luminescence studies by Itoh et al. [11] and Theg et al. [12] have concluded that Cl^- depletion inhibits the advancement beyong the S_2 state, but does not inhibit the earlier transitions. This discrepancy between the luminescence and EPR studies may be due to either (1) the presence of intermediates between the Mn-containing oxygen-evolving complex and Z on the donor side of PS II or (2) an alteration in the structure of the Mn-containing oxygen-evolving complex sufficient to change its EPR properties, without preventing S_2 or even S_3 formation from a functional point of view. Work is in progress in our laboratory to resolve this question by monitoring the oxidation state of the Mn-containing oxygen-evolving complex in Cl^--depleted particles by studying the Mn K-edge X-ray spectra, which provides an independent measure of the number of oxidizing equivalents stored in the Mn-containing oxygen-evolving complex [24].

The results with TWE PS II particles (Table I) also indicate that Cl^- or Br^- is required for O_2 activity and that the site of action is prior to the S_2 state. However, the O_2 activity decreases to only 60% upon washing in Cl^--free buffer, and subsequent washes in Cl^--free buffer are not effective in reducing the activity or the multiline amplitude. One explanation for this behaviour is that to maintain the functional integrity of the PS II particles it is necessary to work at a pH of 6, and that at such a low pH it is not possible to deplete the particles of Cl^-. It has been reported [6,21] that high pH facilitates the removal of Cl^- in chloroplasts. Similar results have been observed in PS II particles [25], but are complicated by the fact that at high pH peptides are also released from the PS II particles [22]. Another possibility is that there are two forms of the Mn complex, only one of which is susceptible to Cl^- depletion in PS II particles. A proposal for two forms of the

TABLE I

THE CORRELATION OF OXYGEN ACTIVITY AND EPR MULTILINE SIGNAL AMPLITUDE IN CHLOROPLASTS, AND TWE AND ETW PS II PARTICLES

In each case the sample with 15 mM NaCl concentrations is the control sample and is explained in Scheme I. MLS, EPR multiline signal amplitude. All figures are percentages.

	Chloride-depleted		Bromide		Fluoride	
	activity	MLS	activity	MLS	activity	MLS
Chloroplasts	5	15	95	83	30	30
TWE particles	62	63	100	84	23	46
ETW particles	8	18	92	95	5	5

Mn-containing oxygen-evolving complex has recently been presented by Brudvig et al. [26]. This proposal is supported by our observation that Cl^--depleted PS II particles prepared using the ETW procedure showed only 10–20% O_2 activity and multiline amplitude and only about 40–50% activity could be restored on readdition of Cl^-. However, further experiments are necessary before the observed data can be fully explained. The lack of good quantitative estimates of the halide requirements has been a major obstacle to a better understanding of the role of Cl^- in photosynthetic oxygen evolution.

Addition of F^- to Cl^--depleted PS II particles decreases the O_2 activity and the multiline amplitude to about 30% in exchanged PS II particles and to less than 5% in PS II particles made using F^--substituted chloroplasts. This reduction in activity and in multiline amplitude from that of Cl^--depleted PS II particles indicates a clear inhibitory role for F^- in O_2 evolution, possibly at the point of advancement to the S_2 state. This observation is in accord with our earlier studies of the EPR signal at $g = 4.1$ which pointed to a site of action of F^- between the S_1 and S_2 states [20].

Fine structure of the multiline EPR signal

A close examination of each of the hyperfine lines (Fig. 3) indicates a multiplicity of 4–6 lines separated by 10–15 gauss. Based on models that have been proposed for the Mn site [16–18] in PS II it seems probable that most, if not all, of this finer structure can be attributed to a non-degenerate superposition of the hyperfine lines due to Mn alone; this becomes evident when simulations (not shown) using an antiferromagnetically coupled Mn(III)Mn(IV) binuclear model have been carried out to second order (similar to those performed by Dismukes [17]). The fine structure can also be accounted for by including hyperfine or g-tensor anisotropy or by including nuclear quadrupole interactions. In addition, it can be argued that the EPR substructure is due to ligand superhyperfine interactions. The most likely ligand atoms capable of such magnetic interactions in the context of what is known about the manganese-containing oxygen-evolving complex are H, N and Cl.

In this report we address the question of whether the EPR substructure is due to an interaction of the halide ion with the Mn complex. Our approach to this problem is to look for differences in the high resolution EPR spectrum of Cl^-- and Br^--containing ETW particles. The rationale is that Br^- is an effective replacement for Cl^- functionally and in its ability to generate the multiline signal, so it is reasonable to assume that structurally it plays a role similar to that of Cl^-. All stable isotopes of Cl and Br have nuclear spins of $\frac{3}{2}$ and, the Cl^- and Br^- nuclei would be expected to split each of the major lines in the EPR spectrum into quartets. However, there are some significant differences in the magnetic properties of these two elements. The magnetic moments of the Br^- isotopes (μ of $^{79}Br = 2.09\ \mu_N$ and of $^{81}Br = 2.26\ \mu_N$) are about 2.5 times larger than the magnetic moments of the isotopes of Cl (μ of $^{35}Cl = 0.82\ \mu_N$ and of $^{37}Cl = 0.68\ \mu_N$). The difference in the magnitude of the magnetic moments and the natural abundance of the nuclei involved ($^{35}Cl/^{37}Cl = 3:1$, $^{79}Br/^{81}Br = 1:1$) would lead to very different ligand superhyperfine splitting patterns, when Cl^- is replaced by Br^- as a ligand.

Some examples of Cl^- and Br^- ligand superhyperfine coupling parameters (A(Cl) and A(Br)) in transition metal complexes are repesented in Table II. In all of these reports the Br^- splittings are larger than the Cl^- splittings. Unfortunately, we could not find any reports in the literature for A(Br) in Mn complexes; however, there is a report of a A(Cl) for Mn^{2+} in NaCl which is about 2 G [33]. So it is very likely that, if Cl is being replaced by Br as a ligand to Mn, a large change in the superhyperfine structure would be evident.

At 4 G modulation no significant differences are seen in the substructure of the multiline EPR signal for Cl^- or Br^- exchanged samples (Fig. 3). The criterion we used to arrive at this conclusion is as follows. We compared the difference spectrum of two independent Cl^- samples with the difference spectrum between a Cl^-- and a Br^--exchanged sample. The autocorrelation of the residuals obtained from the difference between two chloride spectra (each the sum of 60 individual scans) was a spectrum of points satisfying the 95% confidence interval criterion for white noise [34]. This analysis also indicated that no artifacts were introduced in taking the difference between two independently collected spectra. The autocorrelation of the residuals obtained from the difference between the chloride and bromide spectra also yielded a distribution of points satisfying the 95% confidence interval criteria for white noise. Thus we have rigorously demonstrated that there are no statistically significant differences between the chloride and bromide spectra. This result demonstrates that the substructure of the multiline EPR signal is not due to the exchanged halide ion, and we conclude that the halide ion is not coordinated to the EPR-active Mn. We qualify our conclusions by noting that there are two implicit but reasonable assumptions in our rationale, (1) that Cl^- is exchangeable and (2) if the halide is bound to the EPR-active Mn, some unpaired electron density is delocalized on to the ligands.

Recently, we have analysed the EXAFS spectrum of Mn in PS II particles poised in both the S_1 and S_2 states: the evidence does not support first shell coordination of Cl to Mn either in the S_1 or S_2 state [35,36].

Alternatively, the superhyperfine lines might be explained by isotropic superhyperfine coupling to other ligand nuclei. Nitrogen ligands are ubiquitous in metallo-proteins, and the substructure in the multiline EPR signal might be explained by isotropic coupling to nitrogen nuclei. The magnitude of the observed splittings, which are 10–15 G, are in the range observed for ligand superhyperfine coupling to ^{14}N [37]. Couplings to protons could also give rise to splittings of this magnitude. For example, superhyperfine coupling to ^1H of 9.35 G for Mn in $AlCl_3 \cdot 6H_2O$ [38] and Mn in $Ca_2Mg_3(NO_3)_{12} \cdot 24H_2O$ [39] have been attributed to Mn-OH_2 moieties.

TABLE II

SUPERHYPERFINE SPLITTINGS A(Cl) AND A(Br) IN TRANSITION METAL COMPLEXES [34]

Cp, cyclopentadiene.

	A(Cl) (gauss)	A(Br) (gauss)	Refs.
Fe^{3+} in AgCl	2.4		27, 28
Fe^{3+} in AgBr		10.6	27, 28
$Co(NCCH_3)_4Cl_2$	13.2		29, 30
$Co(NCCH_3)_4Br_2$		73.7	29, 30
Cu^{2+} in $CdCl_2$	9.5		31
Cu^{2+} in NH_4Br		24.9	31
$[Cp_2MoCl_2]^+$	2.3		32
$[Cp_2MoBr_2]^+$		15.8	32

If the substructure is due to coupling to exchangeable protons then deuterium substitution should significantly alter the appearance of the fine structure. In general, deuteron exchange for protons will result in a collapse of doublets into unresolved triplets because deuterons have $I = 1$ and the hyperfine splittings by deuterons are 7 times smaller than those due to protons. An example of this approach involves the rapid Mo(V) signal of xanthine oxidase, which exhibits proton doublets with 14 G splittings. These splittings collapse to single lines upon exchange in ^2H_2O, and it was concluded from these studies that the protons or deuterons were not directly coordinated to the Mo atom [40,41]. Similar effects are seen for Mo(V) complexes with coordinated –NH groups, which display proton superhyperfine coupligns of 7.4 G that are reduced to 2.0 G on deuteration [42].

Fig. 4 shows a portion of the multiline spectrum for a sample which was prepared using ^2H_2O

buffers. It is clear from a comparison of this spectrum with the multiline spectrum of the sample prepared in H_2O buffers that the substructure is not due to coupling to exchangeable protons; however, it is possible that the splittings are due to non-exchangeable protons or that they are not directly observable. We are presently addressing the question of ligation and structure of the Mn complex by ENDOR and Electron Spin Echo studies.

Conclusions

(1) Cl^- is required for the production of the multiline EPR signal, which indicates that it is required for the generation of an S_2 state as defined by EPR criteria and that its involvement in the Mn containing O_2 complex is prior to the S_2 state.

(2) The similarity of the high resolution EPR spectra with Cl^- and Br^- suggests that the halide ions are not exchangeable ligands of the paramagnetic center of S_2. However, it is possible that the differences are too small to be evident under the conditions of our experiment.

(3) The observed fine structure is not due to exchangeable protons.

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