

Effects of Copper and Zinc Ions on Photosystem II Studied by EPR Spectroscopy<sup>†</sup>C. Jegerschöld,<sup>‡,§</sup> F. MacMillan,<sup>‡,||</sup> W. Lubitz,<sup>⊥</sup> and A. W. Rutherford<sup>\*,‡</sup>

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**ABSTRACT:** The effect of Zn<sup>2+</sup> or Cu<sup>2+</sup> ions on Mn-depleted photosystem II (PS II) has been investigated using EPR spectroscopy. In Zn<sup>2+</sup>-treated and Cu<sup>2+</sup>-treated PS II, chemical reduction with sodium dithionite gives rise to a signal attributed to the plastosemiquinone, Q<sub>A</sub><sup>•-</sup>, the usual interaction with the non-heme iron being lost. The signal was identified by Q-band EPR spectroscopy which partially resolves the typical *g*-anisotropy of the semiquinone anion radical. Illumination at 200 K of the unreduced samples gives rise to a single organic free radical in Cu<sup>2+</sup>-treated PS II, and this is assigned to a monomeric chlorophyll cation radical, Chl *a*<sup>•+</sup>, based on its <sup>1</sup>H-ENDOR spectrum. The Zn<sup>2+</sup>-treated PS II under the same conditions gives rise to two radical signals present in equal amounts and attributed to the Chl *a*<sup>•+</sup> and the Q<sub>A</sub><sup>•-</sup> formed by light-induced charge separation. When the Cu<sup>2+</sup>-treated PS II is reduced by sodium ascorbate, at ≥77 K electron donation eliminates the donor-side radical leaving the Q<sub>A</sub><sup>•-</sup> EPR signal. The data are explained as follows: (1) Cu<sup>2+</sup> and Zn<sup>2+</sup> have similar effects on PS II (although higher concentrations of Zn<sup>2+</sup> are required) causing the displacement of the non-heme Fe<sup>2+</sup>. (2) In both cases chlorophyll is the electron donor at 200 K. It is proposed that the lack of a light-induced Q<sub>A</sub><sup>•-</sup> signal in the unreduced Cu<sup>2+</sup>-treated sample is due to Cu<sup>2+</sup> acting as an electron acceptor from Q<sub>A</sub><sup>•-</sup> at low temperature, forming the Cu<sup>+</sup> state and leaving the electron donor radical Chl *a*<sup>•+</sup> detectable by EPR. (3) The Cu<sup>2+</sup> in PS II is chemically reducible by ascorbate prior to illumination, and the metal can therefore no longer act as an electron acceptor; thus Q<sub>A</sub><sup>•-</sup> is generated by illumination in such samples. (4) With dithionite, both the Cu<sup>2+</sup> and the quinone are reduced resulting in the presence of Q<sub>A</sub><sup>•-</sup> in the dark. The suggested high redox potential of Cu<sup>2+</sup> when in the Fe<sup>2+</sup> site in PS II is in contrast to the situation in the bacterial reaction center where it has been shown in earlier work that the Cu<sup>2+</sup> is unreduced by dithionite. It cannot be ruled out however that Q<sub>A</sub>-Cu<sup>2+</sup> is formed and a magnetic interaction is responsible for the lack of the Q<sub>A</sub><sup>•-</sup> signal when no exogenous reductant is present. With this alternative possibility, the effects of reductants would be explained as the loss of Cu<sup>2+</sup> (due to formation of Cu<sup>+</sup>) leading to loss of the Cu<sup>2+</sup> from the Fe<sup>2+</sup> site due to the binding equilibrium. The quite different binding and redox behavior of the metal in the iron site in PS II compared to that of the bacterial reaction center is presumably a further reflection of the differences in the coordination of the iron in the two systems.

Cu<sup>2+</sup> ions are known to inhibit electron transfer in photosystem II (PS II)<sup>1</sup> (for a review of Cu<sup>2+</sup> effects, see ref 1; for a review of PS II electron-transfer reactions see ref 2). The incubation of PS II-enriched membranes with high concentrations of Cu<sup>2+</sup> was shown to affect both the electron donor and electron acceptor sides of PS II (3) whereas the primary charge separation remained functional

(3–5). On the donor side of PS II, Cu<sup>2+</sup> had no effect on the EPR signal from the dark stable tyrosine radical (Tyr<sub>D</sub><sup>•</sup>), and despite the inhibition of oxygen evolution, the rapidly decaying tyrosine radical EPR signal (Tyr<sub>Z</sub><sup>•</sup>) could not be detected, indicating that the oxidation of Tyr<sub>Z</sub><sup>•</sup> by the photooxidized chlorophyll (P<sub>680</sub><sup>+</sup>) was inhibited (3). Loss of

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<sup>1</sup> Abbreviations: Chl *a*, chlorophyll *a*; cw, continuous wave; DCPIP, dichlorophenolindophenol; DPPH, α,α'-diphenyl-β-picrylhydroxyl; E<sub>m7</sub>, midpoint potential at pH 7; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance; ENDOR, electron nuclear double resonance; HEPES, N-(2-hydroxyethyl)piperazine-N'-ethanesulfonic acid; hfc, hyperfine coupling constant; Δ*H*<sub>pp</sub>, the line width of an EPR signal defined as the peak to peak width of the first derivative signal; MES, 2-morpholinoethanesulfonic acid; o.d./i.d., outer diameter, inner diameter; PS II, photosystem II; Q<sub>A</sub>, the first protein-bound plastoquinone acting as an electron acceptor; Q<sub>B</sub>, the second protein-bound plastoquinone acting as an electron acceptor; Q<sub>A</sub><sup>•-</sup>-Fe<sup>2+</sup>, the Q<sub>A</sub><sup>•-</sup> state magnetically coupled with the high-spin, non-heme ferrous ion; Tris, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; Tyr<sub>Z</sub><sup>•</sup>, redox-active tyrosine 161 of D1 protein which acts as a kinetically competent electron carrier; Tyr<sub>D</sub><sup>•</sup>, redox-active tyrosine 160 of D2 protein which forms a stable tyrosyl radical.

tyrosine oxidation was also indicated by the measurements of  $P_{680}^+$  reduction (4).

During continuous illumination at room temperature of manganese-depleted PS II membranes in the presence of  $Cu^{2+}$ , an EPR-signal different from the well-known tyrosine signals was induced. This radical had a  $g$ -value of 2.0028, an unresolved line width ( $\Delta H_{pp}$ ) of  $\sim 9$  G, amounting to approximately 1 spin per PS II reaction center, and a half-time in the hundreds of milliseconds range (3). A similar EPR signal was formed by illumination at 200 K, and this was stable in the dark at low temperature. Since an earlier optical study on similar samples had reported that the donor to  $P_{680}^+$  at higher temperatures was neither a tyrosine nor a chlorophyll (4), this donor radical thus remained unidentified (3).

On the acceptor side of PS II, the site of inhibition seems to be after the primary quinone electron acceptor,  $Q_A$  (3–8). Flash-induced absorption spectroscopy indicated that  $Q_A^{\bullet-}$  was formed in most centers (4), while sodium dithionite reduction yielded only a fraction ( $\sim 30\%$  of a control) of the EPR signal arising from  $Q_A^{\bullet-}$  interacting magnetically with the non-heme iron,  $Fe^{2+}$  (3). However, in the same samples a free radical signal was observed with  $g = 2.0044$ ,  $\Delta H_{pp} \approx 9$  G, and constituting  $\sim 0.7$  spins. This EPR signal was assigned to the semiquinone anion radical  $Q_A^{\bullet-}$  magnetically uncoupled from  $Fe^{2+}$ . It was concluded then that the  $Cu^{2+}$  seemed to have uncoupled the semiquinone from the iron, possibly by displacing the iron from its site. It remained unclear why the  $Q_A^{\bullet-}$  signal was not observed in the presence of the light induced-electron donor radical in unreduced  $Cu^{2+}$ -treated PS II as described above.

In the present work we extend the earlier EPR study (3) of  $Cu^{2+}$  effects on PS II. We identify the radicals formed in PS II after treatment with  $Cu^{2+}$  ions using EPR spectroscopy at 34 GHz and  $^1H$ -electron nuclear double resonance (ENDOR) spectroscopy. The effect of  $Cu^{2+}$  ions is compared to the effects of the nonparamagnetic, nonredox ion,  $Zn^{2+}$ , by performing temperature and redox studies. The results of these experiments lead to an understanding of the effects of  $Cu^{2+}$  on the acceptor side of PS II.

## MATERIALS AND METHODS

PS II-enriched membrane fragments were prepared from spinach as in ref 9 with the modifications as in ref 10 and stored at  $-80$  °C prior to use. The storage buffer contained sucrose (0.4 M), NaCl (10 mM), and Mes–NaOH (25 mM, pH 6.5). The PS II membrane fragments were diluted to 0.15 mg of Chl  $mL^{-1}$  in Tris–HCl (0.8 M, pH 8.5) and incubated (4 °C) in weak light for 30 min to remove the extrinsic polypeptides and most of the Mn (Mn-depleted). The Tris buffer was removed by two washing steps in the storage buffer.  $Cu^{2+}$  or  $Zn^{2+}$  treatments were done by diluting Mn-depleted PS II-enriched membrane fragments to 0.53 mg of Chl  $mL^{-1}$  with  $CuCl_2$  (2.4 mM) or  $ZnCl_2$  (24 mM) which correspond to  $\sim 1000$   $Cu^{2+}/PS$  II and 10 000  $Zn^{2+}/PS$  II, respectively. After incubation (20 min) at room temperature in the dark the sample was centrifuged and resuspended in storage buffer to a final concentration of 2–3 mg of Chl  $mL^{-1}$  for EPR measurements or to 5 mg of Chl  $mL^{-1}$  for ENDOR samples. EDTA washing was performed using storage buffer containing EDTA (1 mM). Any further

treatments and washing steps are indicated in the text and in the table/figure legends.

For X-band EPR, samples were put inside standard (o.d. 4 mm) quartz EPR tubes while, for Q-band EPR, quartz tubes with 3 mm o.d. were used. Illuminations performed at 200 K (in solid  $CO_2$ /ethanol) for 10 min were done using an 800 W tungsten lamp filtered by a water bath and a  $>720$  nm cutoff filter to avoid infrared radiation. The sample was then dark-adapted at 200 K for 2 min before cooling to 77 K and transferred in the dark to the spectrometer. Illumination at 77 K was performed in the same way but in liquid nitrogen. Illuminations at 5 and 15 K were performed directly in the EPR cavity using the same lamp as above.

Reduced samples were generated by the addition under argon of sodium dithionite (40 mM) in the storage buffer (but with 200 mM Mes–NaOH, pH 6.5), incubation for 2–5 min, and then frozen in the dark. Reduction by sodium ascorbate (50 mM) was performed in the presence of dichlorophenolindophenol (DCPIP, 1 mM) in the same buffer and incubation time as for dithionite reduction. Illumination conditions are as indicated in the figure caption. The quantification of the EPR signals was determined by double integration and by comparison to  $Tyr_D^{\bullet}$ . The maximum yield of  $Tyr_D^{\bullet}$  was obtained in a sample frozen rapidly after a short illumination at room temperature. This was taken as being 1 spin/PS II.

X-band EPR spectra were measured on a Bruker ESP-300E spectrometer using a standard rectangular Bruker EPR cavity equipped with an Oxford helium flow cryostat (ESR 900). The microwave frequency was measured using a Hewlett-Packard (HP5350B) frequency counter. The magnetic field was measured using a Bruker gaussmeter (ER035M). The measured  $g$ -values were corrected for an offset against a known  $g$ -standard (DPPH). Q-band EPR spectra were measured on a Bruker ER 200D EPR spectrometer equipped with a Bruker Q-band microwave bridge, Bruker ER 5103 QTH resonator, and an Oxford CF 935 cryostat.  $^1H$ -cw-ENDOR spectra were recorded using a lab-built ENDOR extension consisting of a Rhode & Schwarz radio frequency (rf) synthesizer (SMG), an ENI solid-state rf amplifier (A200L), and a self-built  $TM_{110}$  ENDOR cavity adapted to an Oxford helium flow cryostat (ESR 910) which has been described previously (11). The Q-band EPR powder spectra were analyzed using a simulation and fitting program described previously (12).

## RESULTS

Figure 1A,C shows the effects of 200 K illumination and Figure 1B,D of chemical reduction in the dark on Mn-depleted PS II-enriched membranes incubated at room temperature in the presence of  $Cu^{2+}$  (Figure 1A,B) and  $Zn^{2+}$  (Figure 1C,D), respectively. The spectra in Figure 1A,C were obtained by subtracting the spectrum of the dark stable tyrosyl radical,  $Tyr_D^{\bullet}$ , which was present in the dark. In  $Cu^{2+}$ -treated PS II the light-induced radical is centered at  $g = 2.0028$ , is 9.5G wide, and amounted to approximately 1 spin/PS II. From  $^1H$ -ENDOR spectra (see below) this signal was identified as a monomeric chlorophyll cation radical. A similar EPR signal is observed in control, Mn-depleted PS II (not shown, but see e.g. refs 13 and 14).

In  $Zn^{2+}$ -treated PS II (Figure 1C), the signal consists of approximately 2 spins/PS II with an apparent  $g$ -value of

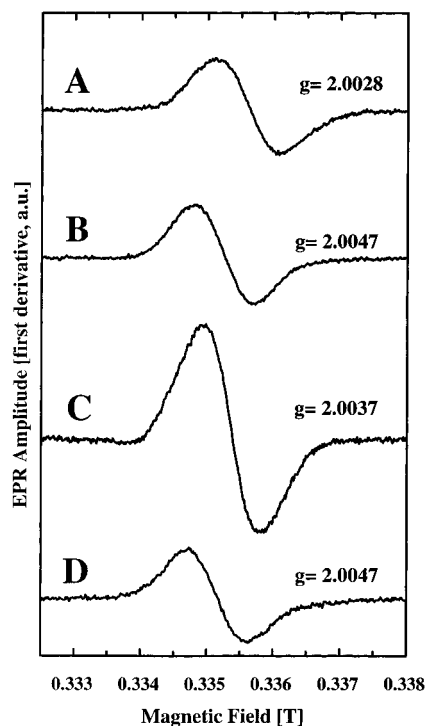


FIGURE 1: EPR spectra of spinach membrane fragments treated with either Cu<sup>2+</sup> (A and B) or Zn<sup>2+</sup> (C and D). Spectra A and C are recorded after illumination at 200 K for 10 min. Spectra B and D are recorded under reducing conditions (in the presence of dithionite) in the dark (see Materials and Methods). Experimental conditions: microwave power, 0.1 mW; field modulation depth, 0.2 mT; accumulation time, 160 s; temperature, 20 K.

2.0037 and is approximately 10 G wide. This spectrum is attributed to the overlap of the EPR signal from the electron donor, Chl *a*<sup>+</sup>, and the EPR signal arising from Q<sub>A</sub><sup>•-</sup> (see below) generated in stoichiometric amounts by illumination.

In control, Mn-depleted PS II, low-temperature illumination gives rise to an EPR signal at  $g = 1.9$  from Q<sub>A</sub><sup>•-</sup> interacting with Fe<sup>2+</sup> (Q<sub>A</sub><sup>•-</sup>Fe<sup>2+</sup>) (15). No such signals attributable to Q<sub>A</sub><sup>•-</sup>–Fe<sup>2+</sup> was detectable in the Cu<sup>2+</sup>- and Zn<sup>2+</sup>-treated PS II measured here.

The traces in Figure 1B,D show the spectra obtained when Cu<sup>2+</sup>- and Zn<sup>2+</sup>-treated PS II are reduced with sodium dithionite. In both samples, a radical is observed with a  $g$ -value of  $g = 2.0047$  and a width of 9.5 G; it constitutes approximately 1 spin/PS II. These signals have a  $g$ -value typical of a semiquinone anion radical (16) and are attributed to the EPR signal from Q<sub>A</sub><sup>•-</sup> decoupled from the non-heme iron. This assignment is in accordance with earlier work on Cu<sup>2+</sup>-treated PS II (3) and is confirmed by EPR spectroscopy at Q-band frequencies (see below).

In control, Mn-depleted PS II, virtually no free radical signal is present upon reduction with dithionite. Instead, the EPR signal at  $g = 1.9$  arising from Q<sub>A</sub><sup>•-</sup>–Fe<sup>2+</sup> is present. No such signal is observed under these conditions in Cu<sup>2+</sup>- or Zn<sup>2+</sup>-treated PS II confirming the absence of the magnetically coupled iron (not shown).

The results shown here for the Cu<sup>2+</sup>-treated sample are comparable to those reported earlier (3) except that the Cu<sup>2+</sup> treatment used here seems to affect the acceptor side in all centers while, in the earlier work, ~30% of the centers still showed the Q<sub>A</sub><sup>•-</sup>–Fe<sup>2+</sup> EPR signal and accordingly the

radical at  $g = 2.0047$  accounted for only ~70% of the centers (3). The more complete effect in the present work is presumably due to the lower concentration of membranes now used during incubation (0.53 mg of Chl mL<sup>-1</sup> vs 2–3 mg of Chl mL<sup>-1</sup>; see ref 3). Indeed, we expressly used these conditions in order to obtain a more complete effect at the level of the electron acceptors.

For the Zn<sup>2+</sup> treatment, the effects described required concentrations which were 10 times higher (i.e. 24 mM) than those required for the Cu<sup>2+</sup> treatment. When 2.4 mM Zn<sup>2+</sup> was used, i.e., the concentration which gave a complete effect when Cu<sup>2+</sup> ions were used, the uncoupled Q<sub>A</sub><sup>•-</sup> was present in only 10–20% of the centers (not shown).

When Cu<sup>2+</sup>-treated PS II was washed in EDTA-containing buffer (see Materials and Methods), a single wash eliminated more than 90% of the remaining copper from solution as monitored by the Cu<sup>2+</sup> EPR signal. A 200 K illumination of such a sample revealed a slightly altered EPR spectrum. The yield of signals observed increased to ~1.2 spins/PS II, and there was a very slight shift in the  $g$ -value to a higher value. After a second EDTA wash, 200 K illumination induced 2 radicals/PS II which gave an EPR signal approximately 10 G wide with a  $g$ -value of 2.0037. Thus EDTA washing renders the Cu<sup>2+</sup>-treated PS II comparable to Zn<sup>2+</sup>-treated PS II. EDTA washing did not, however, influence the EPR signals observed under reducing conditions in either Cu<sup>2+</sup>- or Zn<sup>2+</sup>-treated PS II. These observations indicate that, (1) both the Cu<sup>2+</sup> and the Zn<sup>2+</sup> treatment resulted in the displacement of Fe<sup>2+</sup> from its site, (2) the presence of the Cu<sup>2+</sup> in the site resulted in the absence of the uncoupled Q<sub>A</sub><sup>•-</sup> signal, and (3) removal of the Cu<sup>2+</sup> by washings with EDTA resulted in the appearance of the uncoupled Q<sub>A</sub><sup>•-</sup> signal from PS II with an unoccupied iron site.

Figure 2 shows the Q-band ( $\nu \sim 34$  GHz) EPR spectra of the radicals formed after treatment with Cu<sup>2+</sup> and Zn<sup>2+</sup> under reducing conditions. A simulation (dotted line) is also shown using the  $g$ -tensor principal values (from Table 1). The observed  $g$ -anisotropy, typical of semiquinones (16, 17), indicates that the radical observed at X-band frequencies with an average  $g$ -value of 2.0047 originates from a semiquinone radical anion. The comparison of the  $g$ -tensor principal values in Table 1 shows  $g_{zz}$  and  $g_{yy}$  values almost identical to the values of the plastoquinone-9 anion radical (PQ-9<sup>•-</sup>) in frozen basic 2-propanol solution, as well as for Q<sub>A</sub><sup>•-</sup> observed in “Fe-depleted” and “cyanide-treated” PS II membrane fragments (18, 19). The  $g_{xx}$  value is, however, different from the  $g_{xx}$  value obtained for the model PQ-9<sup>•-</sup> system (2.0063). The value obtained here for Cu<sup>2+</sup>-treated PS II ( $g_{xx} = 2.0068$ ) lies between that from cyanide-treated (2.0065) and Fe-depleted (2.0070) PS II preparations. This  $g_{xx}$  value has been shown to be very sensitive to the electrostatic environment and in particular the presence of hydrogen bonding to the carbonyl groups of semiquinone radical anions (17, 20) and tyrosine radicals (21).

The Q-band EPR spectrum (not shown) of the radical generated by 200 K illumination in the absence of a chemical reductant also contains the underlying Tyr<sub>D</sub><sup>•</sup> EPR signal, and therefore, an exact determination of the  $g$ -tensor is difficult. As compared to X-band, no discernible broadening of the EPR line width is observed. The lack of resolved  $g$ -anisotropy at Q-band would indicate that the signal does not

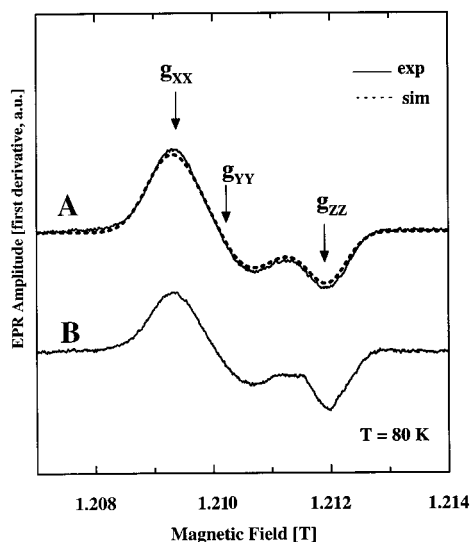


FIGURE 2: (A) 34 GHz EPR spectrum of the radical generated under reducing conditions from  $\text{Cu}^{2+}$ -treated samples. Dark-adapted samples were treated with sodium dithionite (for details, see Materials and Methods). The simulated spectrum (dotted) is shown using the  $g$ -tensor (Table 1) and an isotropic 4 G line width. Experimental conditions: microwave power, 0.006 mW; field modulation depth, 0.5 mT; accumulation time, 20 min; temperature, 80 K. (B) 34 GHz EPR spectrum of the radical generated under reducing conditions from  $\text{Zn}^{2+}$ -treated samples. Dark-adapted samples were treated with dithionite (for details, see Materials and Methods). Experimental conditions: as in (A) except accumulation time 35 min.

Table 1: Principal Values of the  $g$ -Tensors of the Electron Acceptor Radical Anion  $\text{Q}_A^{\bullet-}$  in  $\text{Cu}^{2+}$ - and  $\text{Zn}^{2+}$ -Treated PS II under Reducing Conditions at  $T = 80$  K, Compared to  $\text{Q}_A^{\bullet-}$  Observed in Other PS II Preparations and Model Systems

sample	$g_{xx}$	$g_{yy}$	$g_{zz}$	$1/3\text{Tr } g$	note
	PS II $\text{Q}_A^{\bullet-}$				
$\text{Cu}^{2+}$ -treated	2.0068	2.0052	2.0022	2.0047	<i>a</i>
$\text{Zn}^{2+}$ -treated	2.0070	2.0054	2.0023	2.0049	<i>a</i>
$\text{Fe}^{2+}$ -depleted	2.0070	2.0053	2.0023	2.0048	<i>b</i>
$\text{CN}^-$ -treated	2.0065	2.0052	2.0022	2.0046	<i>c</i>
	Models				
PQ-9 $^-$	2.0063	2.0052	2.0022	2.0046	<i>d</i>
PQ-1 $^-$	2.0062	2.0052	2.0023	2.0046	<i>e</i>

<sup>a</sup> All values obtained by simulation of the powder Q-band (34 GHz) EPR spectra. The error is  $\pm 0.0001$ . <sup>b,c</sup> Iron-depleted PS II and cyanide-treated PS II, respectively, both from Q-band (34 GHz) EPR measurements (18, 19). The error is  $\pm 0.0001$ . <sup>d,e</sup> References 17 and 22, respectively, in frozen basic 2-propanol; W-band (95 GHz) EPR measurements. The error is  $\pm 0.0001$ .

arise from a semiquinone or a modified tyrosine; however, it is consistent with it being a Chl  $a^{\bullet+}$  radical (23, 24).

Figure 3 shows the  $^1\text{H}$ -ENDOR spectrum of the same sample. As mentioned above, this sample also contains  $\text{Tyr}_D^{\bullet}$ . Hyperfine interactions from  $\text{Tyr}_D^{\bullet}$  have previously been observed at cryogenic temperatures using ENDOR spectroscopy (2). It is possible to suppress these contributions from the spectra by performing the ENDOR measurements at slightly higher temperatures (130 K instead of 5–20 K). In frozen solutions protons from methyl groups give rise to narrow and intense ENDOR lines (25). Lines from  $\alpha$ -protons and from fixed  $\beta$ -protons ( $\text{CH}_2$  groups) are broadened and not easily detected. From Figure 3 several line pairs spaced symmetrically around the free proton Larmor frequency are clearly resolved. These observed hyperfine couplings (hfc's)

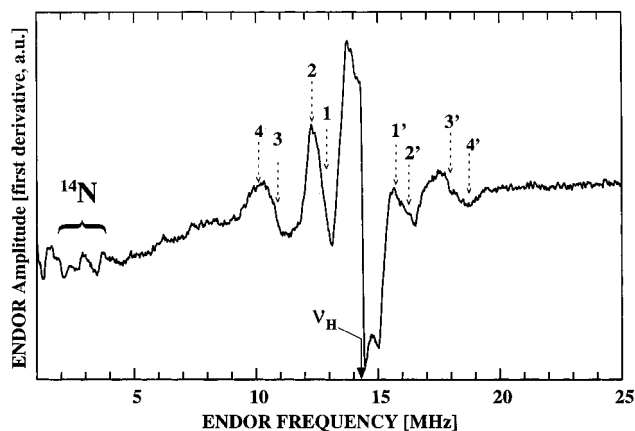


FIGURE 3: CW-ENDOR spectrum of the sample treated with  $\text{Cu}^{2+}$  after illumination at 200 K. The line pairs belonging to certain proton hyperfine couplings (hfc's) are labeled (1,1', etc.) and are listed in Table 2. The ranges in which lines associated with  $^{14}\text{N}$  nuclei occur are indicated in the spectrum but are not analyzed here. Experimental conditions: microwave power, 10 mW; radio frequency power, 200 W; frequency modulation, 12.5 kHz; frequency modulation depth,  $\pm 100$  kHz; accumulation time, 60 min; temperature, 123 K.

Table 2: Principal Values of the Methyl Proton Hyperfine Tensors (in MHz) and Assignments for the Observed Chlorophyll  $a$  Radical Cations in PS II and in Organic Solvents

feature <sup>a</sup>		hyperfine coupling (MHz)				assign <sup>t</sup>
		$\text{Cu}^{2+}$ (PS II) <sup>b</sup>	Chl $a^{\bullet+}$ (PS II) <sup>c</sup>	Chl $a^{\bullet+}$ (PS II) <sup>d</sup>	Chl $a^{\bullet+}$ in $\text{CH}_2\text{Cl}_2/\text{THF}$ <sup>e</sup>	
<b>1,1'</b>	$A_{\perp}$	3.2	3.4	3.0	2.75	<b>2-,7-methyl</b>
<b>2,2'</b>	$A_{\parallel}$	3.8	4.1	3.6	3.90	
	$A_{\text{iso}}^f$	3.4	3.6	3.2	3.15	
<b>3,3'</b>	$A_{\perp}$	7.0	7.3	7.2	7.15	<b>12-methyl</b>
<b>4,4'</b>	$A_{\parallel}$	8.6	8.2	8.5	8.30	
	$A_{\text{iso}}$	7.5	7.6	7.6	7.55	

<sup>a</sup> Taken from Figure 3. <sup>b</sup>  $\text{Cu}^{2+}$ -treated PS II, this work; error is  $\pm 0.1$  MHz. <sup>c</sup> Reference 28. <sup>d</sup> Reference 18. <sup>e</sup> In  $\text{CH}_2\text{Cl}_2/\text{THF}$  (10:1 v/v) (26, 27). <sup>f</sup>  $A_{\text{iso}} = 1/3(A_{\parallel} + 2A_{\perp})$ .

and their assignments are very typical of a monomeric Chl  $a$  cation radical in organic solvents (Table 2; see also refs 23, 26, and 27). Previously monomeric Chl  $a$  cations have been observed in PS II and investigated with ENDOR spectroscopy (18, 28), and the hfc's observed here and their interpretation are very similar to those seen previously (see Table 2).

The data above show that both  $\text{Cu}^{2+}$  and  $\text{Zn}^{2+}$  treatments lead to a displacement of  $\text{Fe}^{2+}$  from its site. The only evident spectroscopic difference between the  $\text{Cu}^{2+}$ - and  $\text{Zn}^{2+}$ -treated PS II is that the  $\text{Q}_A^{\bullet-}$  radical is absent upon 200 K illumination of the unreduced sample in  $\text{Cu}^{2+}$ -treated material, while in  $\text{Zn}^{2+}$ -treated PS II this signal is present. This is presumably due to the magnetic or redox properties of  $\text{Cu}^{2+}$ . Thus the  $\text{Q}_A^{\bullet-}$  radical may be missing either because it is magnetically interacting with  $\text{Cu}^{2+}$ , as occurs in the bacterial reaction center when  $\text{Cu}^{2+}$  is substituted for  $\text{Fe}^{2+}$  (29, 30), or because the  $\text{Cu}^{2+}$  acts as an electron acceptor from the semiquinone.

As mentioned above, it seems clear that the  $\text{Cu}^{2+}$  is indeed in the site since its removal by EDTA washing leads to the appearance of the uncoupled  $\text{Q}_A^{\bullet-}$  signal upon 200 K illumination. Since the  $\text{Cu}^{2+}$ -treated sample does show the uncoupled  $\text{Q}_A^{\bullet-}$  signal upon chemical reduction with dithio-

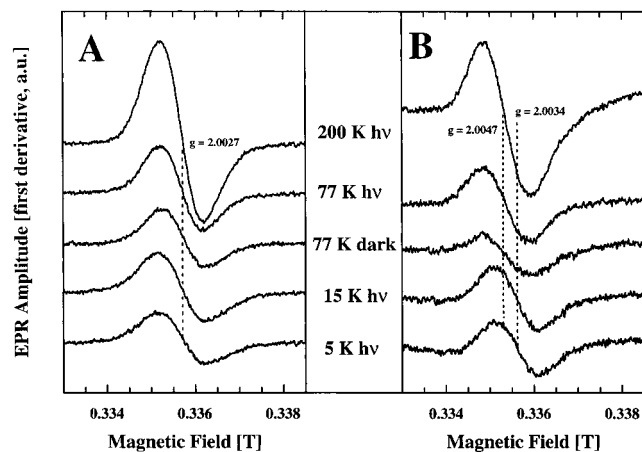


FIGURE 4: (A) EPR spectra of PS II membrane fragments after treatment with Cu<sup>2+</sup>. Samples were illuminated with no additions. Key: from the bottom up at 5 K, 15 K (both illuminated in the cavity), and left in the dark at 77 K, and then illuminated at 77 K and then finally (top spectrum) at 200 K (in ethanol/dry ice bath). For light sources and illumination times, see Materials and Methods. Experimental conditions: microwave power, 0.1 mW; field modulation depth, 0.2 mT; accumulation time, 160 s; temperature, 20 K. (B) EPR spectra of PS II membrane fragments after treatment with Cu<sup>2+</sup>. After being frozen in the dark, samples were illuminated at different temperatures in the presence of sodium ascorbate and DCPIP (see Materials and Methods). Key: from the bottom up at 5 K, 15 K, then left in the dark for 5 min at 77 K, illuminated at 77 K and then finally (top spectrum) at 200 K. Illumination and experimental conditions were as in (A).

nite, it also seems clear that the Cu<sup>2+</sup> in the Fe<sup>2+</sup> site is chemically reduced, forming the Cu<sup>+</sup> ion. This, however, does not help us distinguish whether the Cu<sup>2+</sup> is interacting magnetically or acting as an electron acceptor, since chemical reduction would eliminate both effects. It may be possible to distinguish between these two possible roles from experiments using sodium ascorbate as a reductant.

When Cu<sup>2+</sup>-treated PS II is reduced with sodium ascorbate in the presence of DCPIP, the sample shows no free radical signals in the dark; upon illumination at 200 K, however, a free radical signal equivalent to ~1 spin/reaction center is observed (Figure 4B top spectrum). On the basis of its *g*-value, this signal is attributed to Q<sub>A</sub><sup>•-</sup>. The reactions occurring in this experiment can be understood on the basis of the following studies done with illumination given at lower temperatures.

Figure 4B shows a series of illuminations of Cu<sup>2+</sup>-treated PS II in the presence of ascorbate and DCPIP. In Figure 4B, illumination at 5 K induces a radical with a relatively low *g*-value and a line width of ~9.8 G. After illumination at 15 K, the light-induced radical is about 11 G with *g* = 2.0034. This signal is attributed to a low yield of both the electron donor radical and the uncoupled Q<sub>A</sub><sup>•-</sup> signals indicating that the Cu<sup>2+</sup> is chemically reduced by the ascorbate/DCPIP prior to illumination. When this sample is warmed in the dark to 77 K the signal decreases in size and the remaining signal is about ~9 G wide and has a *g*-value ~2.0047. It seems clear that upon warming to 77 K, the radical from the oxidized electron donor is chemically reduced by the ascorbate/DCPIP (or by an intrinsic component reduced by this electron donor system) leaving the uncoupled Q<sub>A</sub><sup>•-</sup> signal. Further illumination at 77 K increases the size of this Q<sub>A</sub><sup>•-</sup> signal, and illumination at 200 K results

in formation of ~1 spin of Q<sub>A</sub><sup>•-</sup> per PS II, presumably with the Chl *a*<sup>•+</sup> being reduced by the exogenous electron donors.

In Figure 4A the effects of low-temperature illumination of a Cu<sup>2+</sup>-treated sample in the absence of ascorbate and DCPIP are shown. After illumination at 5 K a small radical signal ~10 G wide and with *g* = 2.0025 is present. This is attributed to the donor side radical alone. There is a slight increase in this signal after an additional illumination at 15 K. After incubation in the dark at 77 K the signal narrows to 9.5 G and during subsequent illuminations at 77 and 200 K the line width and *g*-value do not noticeably change. This signal is attributed to a donor-side radical being photogenerated in increasing amounts as the temperature is raised until the Chl *a*<sup>•+</sup> radical, at approximately 1 spin/PS II, is generated at 200 K, as seen previously in Figure 1. The slight changes in width in the radical generated at different temperatures probably reflect carotenoid oxidation in place of the chlorophyll at lower temperatures as reported recently (31) (see also ref 32). From the experiments in Figure 4B, Cu<sup>2+</sup> seems to be chemically reducible by ascorbate while Q<sub>A</sub><sup>•-</sup> requires reduction by dithionite (Figure 1); thus, the absence of a photoinduced Q<sub>A</sub><sup>•-</sup> EPR signal in the experiments of Figures 1 and 4 is attributed to Cu<sup>2+</sup> acting as an electron acceptor from Q<sub>A</sub><sup>•-</sup>.

There is however an alternative explanation for these results. We have seen above that two washings with chelator are sufficient for the Cu<sup>2+</sup> to be lost from the site. If the Cu is weakly bound and in equilibrium with the Cu<sup>2+</sup> ions in solution, their removal from solution by their reduction to Cu<sup>+</sup> could also result in the loss of Cu<sup>2+</sup> from the site. This explanation is less attractive since we would have to assume that the simple reduction of the Cu by 5 min of incubation in the EPR tube would be as effective as diluting and washing with chelator. Nevertheless, this alternative cannot be ruled out, and this then leaves open the possibility that the absence of the semiquinone radical signal in the absence of exogenous reductant could be due a magnetic interaction with the Cu<sup>2+</sup>.

As expected Zn<sup>2+</sup>-treated Mn-depleted samples reduced with ascorbate and DCPIP showed the Q<sub>A</sub><sup>•-</sup> signal after the illumination at 200 K. The absence of a light-induced Chl *a*<sup>•+</sup> radical is again attributed to electron donation at this temperature from DCPIP/ascorbate as seen with the Cu<sup>2+</sup>-treated sample (data not shown).

## DISCUSSION

The present data with Cu<sup>2+</sup>-treated PS II agree with and extend the earlier EPR study (3). The biochemical treatments used here provide more complete effects and thus more homogeneous material. We now have shown that Zn<sup>2+</sup> treatment of PS II produces a state which is comparable to that induced by Cu<sup>2+</sup> but is simpler to understand since Zn<sup>2+</sup> is neither paramagnetic nor redox active. The influence of Cu<sup>2+</sup> when it occupies the Fe<sup>2+</sup> site is rationalized on the basis of studies with dithionite, ascorbate, and illumination at a range of temperatures. The identities of the radicals are clarified through the use of Q-band EPR and <sup>1</sup>H-ENDOR spectroscopy. From these results a better understanding of the effect of Cu<sup>2+</sup> ions on PS II is obtained.

The results clarify the origin of the radicals formed in Cu<sup>2+</sup>-treated PS II. The identity of the signal seen under reducing conditions as a semiquinone already seemed likely

from the  $g$ -value of the signal measured at X-band (3); however, the Q-band EPR spectra reported here are clear evidence in favor of this assignment. This corroborates the identification of this radical as uncoupled  $Q_A^{\bullet-}$ .

For the other radical ( $g = 2.0028$ ), the Q-band EPR showed no detectable  $g$ -tensor anisotropy which is consistent with it arising from a Chl  $a^{\bullet+}$  molecule. The  $^1\text{H}$ -ENDOR spectra are similar to those arising from monomeric Chl  $a^{\bullet+}$ . It seems likely then that this is indeed the origin of the radical. This conclusion disagrees with the earlier report in which it was concluded that the electron donor in Cu-treated PS II was a species other than chlorophyll. This was based on kinetic optical studies done on similar samples at room temperature. The most likely explanation for this discrepancy is the difference in conditions used: Here we have shown that the stable state formed after donation of an electron at 200 K is likely to be a chlorophyll radical; in the work of Schröder et al. (4) it was shown that the state responsible for the rapid reduction of  $P_{680}^+$  at room temperature is unlikely to be chlorophyll. It seems quite possible that a different species is being looked at under these two different sets of conditions.

The results also clarify what occurs upon incubation with  $\text{Cu}^{2+}$  and what reactions take place in the  $\text{Cu}^{2+}$ -treated PS II. Incubation of Mn-depleted PS II with  $\text{Cu}^{2+}$  or  $\text{Zn}^{2+}$  leads to a displacement of the non-heme iron atom (see below). Given that  $\text{Cu}^{2+}$  can be depleted from the site by two washes with EDTA, it seems clear that the binding site is not as tight as that in the bacterial system. The higher concentration required to obtain the  $\text{Zn}^{2+}$  effect might indicate that  $\text{Zn}^{2+}$  is even more weakly bound. Incubations with divalent cations followed by chelator washes may then prove useful as an additional method for removing the iron from PS II for spectroscopic studies. Given this observation, it seems that when PS II preparations are used in which it is thought that  $\text{Zn}^{2+}$  occupies the iron site, it is likely that the iron site is unoccupied unless high concentrations of  $\text{Zn}^{2+}$  are present in the medium (for example ref 33).

The  $\text{Zn}^{2+}$ -treated system when illuminated at 200 K gives rise to two radicals arising from the electron donor and electron acceptor radical pair, Chl  $a^{\bullet+}Q_A^{\bullet-}$ , while in the presence of the reductant dithionite only the  $Q_A^{\bullet-}$  signal is present in the dark. This is easy to understand since  $\text{Zn}^{2+}$ , when present in the  $\text{Fe}^{2+}$  site, can have no magnetic or redox effects. What is observed in  $\text{Cu}^{2+}$ -treated PS II is more complicated. Illumination of an unreduced  $\text{Cu}^{2+}$ -treated sample results in formation of only a donor side radical, Chl  $a^{\bullet+}$ , while the acceptor side radical,  $Q_A^{\bullet-}$ , is not detected. When the sample is treated with chelator, the missing  $Q_A^{\bullet-}$  signal appears indicating that the  $\text{Cu}^{2+}$  is removed by this treatment. This also confirms that when the  $\text{Cu}^{2+}$  is present, it is responsible for the absence of the uncoupled  $Q_A^{\bullet-}$  signal.

In the presence of dithionite, the uncoupled  $Q_A^{\bullet-}$  signal appears stoichiometrically just as it does as in  $\text{Zn}^{2+}$ -treated PS II, while, in the presence of a weaker reductant, ascorbate,  $Q_A^{\bullet-}$  is photoinducible at low temperature. These results indicate that treatment with reductants removes the paramagnetic  $\text{Cu}^{2+}$  by reducing it to the  $\text{Cu}^+$  state. It appears then the  $E_{m7}$  of  $\text{Cu}^{2+}/\text{Cu}^+$  is higher than that of ascorbate ( $E_{m7}$  of ascorbate is 60 mV). With such a high  $E_{m7}$ , it seems likely that  $\text{Cu}^{2+}$  can act as an electron acceptor from  $Q_A^{\bullet-}$ , explaining the absence of a light-induced  $Q_A^{\bullet-}$  signal in

unreduced  $\text{Cu}^{2+}$ -treated PS II. The alternative explanation that reduction of the  $\text{Cu}^{2+}$  in the medium upon addition of reductant leads to loss of  $\text{Cu}^{2+}$  in the site cannot be ruled out. Thus, we must leave open the possibility that the absence of the semiquinone signal after illumination without a reductant could be due to a magnetically coupled  $Q_A-\text{Cu}^{2+}$  state.

We looked for the light-induced disappearance of a  $\text{Cu}^{2+}$  EPR signal associated with its role as an electron acceptor (or alternatively the appearance of a  $Q_A-\text{Cu}^{2+}$  signal) but were unable to get reliable results, mainly due to the presence of relatively large underlying  $\text{Cu}^{2+}$  signals arising from  $\text{Cu}^{2+}$  in solution and  $\text{Cu}^{2+}$  nonspecifically bound to the membranes. Washing of the samples to remove nonspecific  $\text{Cu}^{2+}$  also lead to loss of the  $\text{Cu}^{2+}$  from the site as shown by the appearance of a light-induced  $Q_A^{\bullet-}$  signal.

The suggestion that  $\text{Cu}^{2+}$  displaces the  $\text{Fe}^{2+}$  and can act as an electron acceptor is in apparent contradiction with the report of Schroder et al. (4), who observed absorption kinetics indicating that  $P^+Q_A^-$  charge recombination occurs in Cu-treated PS II. In the present work we based conditions of our Cu treatment on the loss of the magnetically coupled  $\text{Fe}^{2+}$  (as monitored by the  $Q_A-\text{Fe}^{2+}$  signal). This required relatively long incubations with high concentrations of  $\text{Cu}^{2+}$  ions. In the work of Schroder et al. (4), their interest was focused on the inhibition of electron donation from Tyr<sub>Z</sub>, and for this it was sufficient to add the  $\text{Cu}^{2+}$  ions immediately prior to measuring the kinetic traces. It seems likely then that under these conditions  $\text{Cu}^{2+}$  ions would have little effect on the iron site.

The weak binding of  $\text{Cu}^{2+}$ , its proposed redox behavior, and its apparently high potential when in the  $\text{Fe}^{2+}$  site is very different from the behavior of  $\text{Cu}^{2+}$  in the bacterial reaction center where it remains  $\text{Cu}^{2+}$  even in the presence of sodium dithionite (29, 30). The ligand environment of the metal site in the bacterial reaction center is well established from X-ray crystallography; the metal is six coordinate with four imidazole ligands, and the fifth and sixth ligand both come from the carboxylic acid group of a glutamate (34). PS II has the 4 histidines conserved, but the glutamate is missing. It has been suggested that exchangeable carboxylic acid ligands replace the glutamate (34–36). The presence of exchangeable Fe ligands in PS II has been taken as the origin of several of the differences occurring between PS II and the bacterial reaction center including the fact that the  $\text{Fe}^{2+}$  is redox active (35–37). The relatively trivial exchangeability of the  $\text{Fe}^{2+}$  for other divalent cations, the weak binding of these ions, and the proposed redox activity of the  $\text{Cu}^{2+}$  are taken as further evidence for the more flexible ligand environment around the metal in PS II. It is still unclear what is the functional significance of such flexibility; however, roles in the regulation of electron transfer can be imagined.

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