Copper(II) Inhibition of Electron Transfer through Photosystem II Studied by EPR Spectroscopy†

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ABSTRACT: EPR spectroscopy was applied to investigate the inhibition of electron transport in photosystem II by Cu^{2+} ions. Our results show that Cu^{2+} has inhibitory effects on both the donor and the acceptor side of photosystem II. In the presence of Cu^{2+}, neither EPR signal $II_{very\ fast}$ nor signal II_{fast}, which both reflect oxidation of tyrosine$_Z$, could be induced by illumination. This shows that Cu^{2+} inhibits electron transfer from tyrosine$_Z$ to the oxidized primary donor $P680^+$. Instead of tyrosine$_Z$ oxidation, illumination results in the formation of a new radical with $g = 2.0028 \pm 0.0002$ and a spectral width of 9.5 ± 0.3 G. At room temperature, this radical amounts to one spin per PS II reaction center. Incubation of photosystem II membranes with cupric ions also results in release of the 16 kDa extrinsic subunit and conversion of cytochrome b_{559} to the low-potential form. On the acceptor side, Q_A can still be reduced by illumination or chemical reduction with dithionite. However, incubation with Cu^{2+} results in loss of the normal EPR signal from Q_A^- which is coupled to the non-heme Fe^{2+} on the acceptor side (the Q_A^-−Fe^{2+} EPR signal). Instead, reduction of Q_A results in the formation of a free radical spectrum which is 9.5 G wide and centered at $g = 2.0044$. This signal is attributed to Q_A^- which is magnetically decoupled from the non-heme iron. This suggests that Cu^{2+} displaces the Fe^{2+} or severly alters its binding properties. The inhibition of tyrosine$_Z$ is reversible upon removal of the copper ions with EDTA while the modification of Q_A was found to be irreversible.

Copper is an essential micronutrient for higher plants. Despite this, it is also a very potent inhibitor of photosynthetic activity (Droppa & Horváth, 1990; Barón et al., 1995). Since the first studies on inhibition of photosynthesis by cupric ions (Macdowall, 1949), it has become clear that photosystem II (PS II)[1] is more sensitive to Cu^{2+} inhibition than photosystem I (Cedéño-Maldonado & Swader, 1972). PS II is a large protein complex in the thylakoid membrane with about 20 protein subunits [for reviews, see Andersson & Styring (1991) and Vermaas et al. (1994a)] that catalyzes the light-driven reduction of plastoquinone by electrons from water that is oxidized to molecular oxygen (Debus, 1992). A heterodimer of two homologous proteins, the D1 and D2 proteins, constitutes the PS II reaction center that binds all the redox components involved in the photochemistry. This includes the redox-active tyrosine residues (Tyr$_Z$ and Tyr$_D$), the primary electron donor (P680), the primary pheophytin acceptor, the secondary plastoquinone acceptors (Q_A and Q_B), the Mn-cluster involved in the oxidation of water, and the non-heme Fe^{2+} situated on the acceptor side between Q_A and Q_B. Also present in the PS II complex is Cyt b_{559} which might alternate between several forms with different oxidation potentials. Cyt b_{559} participates in slow electron transfer reactions in PS II and may function as an electron donor to P680 when the water-oxidizing complex is inactivated (Thompson & Brudvig, 1988; Buser et al., 1992).

The target for Cu^{2+} inhibition within the PS II complex is not clear. Several investigations indicate that the site for copper ions is on the donor side of PS II. From spectroscopic measurements, it was suggested that Cu^{2+} blocked the donor side of PS II close to the water-oxidizing system (Vierke & Struckmeier, 1977). On the basis of DCIP reduction measurements, it was proposed that the target was between P680 and a donor site localized after the water-splitting system (Shioi et al., 1978; Samson et al., 1988). Similar conclusions have been drawn on the basis of fluorescence measurements (Hsu & Lee, 1988; Samson & Popovic, 1988; Renganathan & Bose, 1989, 1990), where a drastic decrease of maximal fluorescence without changes in the initial (F_0) fluorescence was found in the presence of Cu^{2+}. There are also suggestions that cupric ions interact with the

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[1] Abbreviations: BBY, PS II membranes prepared by the method of Berthold et al. (1981); Chl, chlorophyll; Cyt, cytochrome; DCIP, 2,6-dichlorophenolindophenol; EDTA, ethylenediaminetetraacetate; EPR, electron paramagnetic resonance; Mes, 4-morpholineethanesulfonic acid; Pheo, pheophytin; PS II, photosystem II; P680, primary electron donor chlorophyll(s) of PS II; Q_A, Q_B, primary and secondary quinone acceptors; S-states (S_n, where $n = 0, 1, 2, 3,$ or 4), redox states of the oxygen-evolving complex; Tris, tris(hydroxymethyl)aminomethane; Tyr$_D$, redox-active tyrosine 161 of the D2 protein; Tyr$_Z$, redox-active tyrosine 161 of the D1 protein.

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acceptor side in PS II. From thermoluminescence and delayed luminescence studies, Mohanty et al. (1989) proposed that the inhibition site is at the level of the secondary quinone acceptor (Q_B). Recently, based on spectrophotometric measurements of Q_A and pheophytin reduction, Yruela et al. (1991, 1992, 1993) challenged the earlier studies and suggested an inhibition site on the acceptor side of PS II in the pheophytin−Q_A−non-heme Fe^{2+} region. However, these conclusions were disputed in recent optical studies (Schröder et al., 1994). It was shown by flash-induced absorption change measurements that Cu^{2+} did not lower the yield of the primary charge separation between P680 and Q_A. Instead, the electron transfer to $P680^+$ was slowed down, indicating that Tyr_Z was inactivated or inhibited.

A reasonable solution to these seemingly conflicting results is that Cu^{2+} interferes with components both on the oxidizing and on the reducing side of PS II. In line with this, it was recently shown that Cu^{2+} has at least two effects on PS II: it modifies the atrazine binding affinity to the Q_B site, and it interferes with Tyr_Z oxidation (Renger et al., 1993).

Another controversial issue is the reversibility of inhibition with copper ions. The inhibition was found to be reversed by the addition of manganese (Haberman, 1969; Gupta, 1986) or by the removal of excess Cu^{2+} (Hsu & Lee, 1988). However, the reversibility was associated with binding of copper ions by buffers and reagents used to test copper toxicity, which prevented Cu^{2+} to reach its inhibition site (Renganathan & Bose, 1989). This opinion is supported by reports indicating that Cu^{2+}-induced inhibition of electron transport is irreversible (Cedéño-Maldonado & Swader, 1972; Samuelson & Öquist, 1980; Arellano et al., 1995).

In an attempt to resolve some of these questions, we have used EPR spectroscopy to study inhibition of the electron transfer components in PSII by cupric ions. It is found that Cu^{2+} reversibly inhibits the electron donation from Tyr_Z to P680. Illumination in the presence of Cu^{2+} resulted in the induction of a new as yet unidentified radical instead of Tyr_Z. Incubation with Cu^{2+} also resulted in rapid loss of the 16 kDa extrinsic subunit in PS II and conversion of Cyt b_{559} to its low-potential form. In addition, we observed irreversible changes on the acceptor side of PS II leading to uncoupling of Q_A^- from the acceptor side non-heme ferrous ion.

MATERIALS AND METHODS

PS II Preparations. PS II enriched membranes (BBY membranes) were isolated from spinach leaves according to Berthold et al. (1981) with slight modifications to remove starch and adventitious metal ions (Arellano et al., 1994). After the detergent treatment, the membranes were washed and stored at high concentration (5−6 mg of Chl mL^{-1}) at −80 °C. The washing and storage buffer consisted of 50 mM Mes−NaOH, pH 6.0, 15 mM NaCl, 5 mM $MgCl_2$, and 400 mM sucrose. Tris-washed PS II membranes, lacking the 33, 23, and 16 kDa polypeptides of the water-splitting complex and the Mn-cluster, were prepared by washing the BBY membranes at 4 °C in dim light for 20 min with 0.8 M Tris at pH 8.4. The Tris buffer was removed by washing the membranes twice in the storage buffer (Ljungberg et al., 1986).

Preparation of EPR Samples. EPR samples of BBY membranes or Tris-washed PS II membranes were prepared at a concentration corresponding to 2−3 mg of Chl/mL. In the inhibition experiments, $CuSO_4$ was added to the EPR samples (corresponding to a Cu^{2+}/PS II reaction center ratio of 1000−1500) and allowed to react for 10−20 min in the dark at room temperature. Thereafter, excess copper was removed by washing the EPR sample with 4 volumes of storage buffer. After being washed, the sample was resuspended to 2−3 mg of Chl mL^{-1} in the storage buffer and either used directly (in room-temperature EPR experiments) or stored in the dark at liquid nitrogen temperature. In experiments aimed at testing the reversibility of the inhibition, $CuSO_4$ was added as described above. After the defined incubation time, the copper ions were removed by washing the samples 3 times in 4 volumes of storage buffer containing 5 mM EDTA; finally, the sample was washed once with the storage buffer without EDTA.

EPR Spectroscopy. X-band room-temperature and low-temperature EPR spectra were recorded with a Bruker ESP 300 spectrometer equipped with an Oxford Instruments cryostat and temperature controller. Data acquisition and data handling, were performed with the ESP300 program. Room-temperature measurements were carried out in a flat cell while measurements at liquid helium temperature were performed in calibrated quartz EPR tubes. For kinetic measurements, the samples were given saturating flashes in the EPR cavity from a Nd-YAG laser (Spectra Physics DCR-3G, 250 mJ per flash at 532 nm; flash duration 8 ns). Integration of the radical spectra and the EPR spectrum from oxidized Cyt b_{559} was done with the ESP300 software after correcting the spectral size for chlorophyll concentration and EPR tube size. The size of signal II_{slow} (originating from the Tyr_D^{ox}) in PS II enriched membranes was used as an internal spin-standard for radical spectra since it corresponds to one oxidized Tyr_D per PS II reaction center (Miller & Brudvig, 1991). The size of oxidized Cyt b_{559} was estimated using the amount of Cyt b_{559} that was photooxidized by illumination at 77 K (see below) in intact PS II membranes as a standard. Illumination at 77 K induces one stable charge separation in PS II. At 77 K, the electron comes either from a chlorophyll radical or from Cyt b_{559} (de Paula et al., 1986; Thompson & Brudvig, 1988; Miller & Brudvig, 1991). The fraction of chlorophyll that was oxidized could be estimated from the size of the chlorophyll radical which allowed estimation of the amount of Cyt b_{559} signal. Ascorbate treatment to reduce Cyt b_{559} was carried out according to Thompson and Brudvig (1988).

EPR spectra from Tyr_D^{ox} and Tyr_Z^{ox} (signal II_{slow} and signal II_{fast}), respectively, were measured at 20 °C at nonsaturating microwave power. Signal II_{slow} was measured immediately after illumination in the EPR cavity to oxidize any Tyr_D that was reduced during the sample preparation (Vass & Styring, 1991). Signal II_{fast} was measured during continuous illumination in the EPR spectrometer using saturating light provided from a 1000 W projector filtered through a $CuSO_4$ heat filter. Kinetic EPR measurements of photoinduced radicals (signal II_{fast} or a radical that was induced after Cu^{2+} incubation; see below) were done using single flashes from the Nd-YAG laser spaced long enough to allow complete decay of the measured radical between the flashes.

When the Q_A^-−Fe^{2+} EPR signal was measured, the samples were incubated with 50 mM sodium formate for 10 min to enhance the size of the signal (Vermaas & Rutherford, 1984). Q_A was then reduced either chemically or photochemically. The full size of the Q_A^-−Fe^{2+} signal in control

Table 1: Structural Effects of Copper Treatment of BBY Membranes

samples/treatment	Cu ions/PS II[a]	protein content	oxygen evolution [μmol O_2 (mg of Chl)$^{-1}$ h^{-1}]	Mn ions/PS II[a]
BBY membranes; control	0	normal	530	4.2
BBY + copper; buffer-washed[b]	250	90% loss of 16 kDa	0	2
BBY + copper; EDTA-washed[c]	3−4	100% loss of 33, 23, and 16 kDa	0	2

[a] With the assumption that each PS II center contains 220 chlorophylls. Metals were determined by atomic absorption. [b] Cupric ions were added to BBY membranes in the dark (1000−1500 Cu^{2+}/PS II). The BBY membranes were then diluted 3 times with Mes buffer, centrifuged, and resuspended again to a Chl concentration of 2−3 mg/mL. [c] Copper-incubated BBY membranes were diluted 3 times with 5 mM EDTA in Mes buffer and shortly centrifuged. After repeating this 2 times, the BBY membranes were washed once with Mes buffer and resuspended to a Chl concentration of 2−3 mg/mL.

or Cu^{2+}-treated samples was measured after incubating the samples with 40 mM fresh dithionite for 10 min in the dark at room temperature. The photochemical ability of PS II was also tested by illumination at low temperatures (Styring et al., 1990; Miller & Brudvig, 1991). In order to produce one single stable charge separation in PS II, the samples were illuminated in an unsilvered dewar at 77 K in a liquid nitrogen bath during 20 min or at 200 K in a dry ice−ethanol bath during 5 min using white, heat-filtered light from two 1000 W projector lamps. Illumination at 200 K was applied in experiments where the efficiency of the transition between the S_1- and S_2-states was investigated (Brudvig et al., 1983) while illumination at 77 K was applied when the efficiency of electron donation from an accessory chlorophyll electron donor and/or Cyt b_{559} was tested (de Paula et al., 1986; Miller & Brudvig, 1991).

Other Measurements. The chlorophyll concentration was determined according to Porra et al. (1989). SDS−PAGE electrophoresis with 4 M urea and 17.5% polyacrylamide was carried out according to Laemmli (1970). The gels were stained with Coomassie brilliant blue R-250. The proteins were quantified using a Molecular Dynamics densitometer and the Image Quant software. The metal content was determined by atomic absorption spectrometry using a hollow cathode lamp and a graphite furnace (Perkin-Elmer HGA-400, Überlingen, Germany) (Arellano et al., 1993).

RESULTS

Cu(II) Binding to PS II. It has been shown with a variety of methods that Cu^{2+} ions at a concentration of about 100 μM efficiently inhibit electron transfer through PS II when the experiments are performed in dilute solutions of 10−50 μg of Chl mL^{-1} (e.g., Arellano et al., 1995; Renger et al., 1993; Schröder et al., 1994). Here, however, we have studied the mechanism for copper inhibition by EPR spectroscopy which is carried out at 100−200 times higher concentrations of PS II. To obtain equivalent concentration ratios (1000−1500 Cu^{2+} ions/PS II reaction center), we used 6−7 mM Cu^{2+} in the EPR samples. These concentrations interfered negatively with the base line in EPR spectroscopy. To avoid this, the samples were washed with storage buffer after 10 min incubation, thereby removing about 75% of the excess copper but leaving the inhibitory pattern unchanged (see below).

Effects of Cu^{2+} on the Structure of PS II. There are several effects of this concentration of copper on the structure of PS II (Table 1). Interestingly, the 16 kDa extrinsic subunit on the donor side was almost quantitatively released from the BBY membranes while the 23 and 33 kDa subunits remained bound. In addition, 50% of the Mn ions were lost from the Mn-cluster (Table 1). Consequently, the oxygen evolution and the ability to form the S_2-state multiline EPR signal (Miller & Brudvig, 1991) were irreversibly inhibited (not shown).

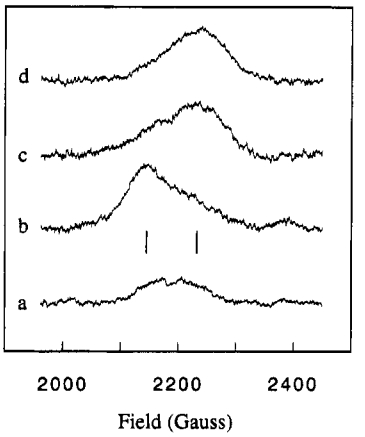

FIGURE 1: Effect of Cu^{2+} on the oxidation state of Cyt b_{559}. EPR spectra were recorded around the g_z-turning point region of the oxidized Cyt b_{559} signal in intact PS II enriched membranes (a) in the dark in the absence of Cu^{2+}. (b) After illumination at 77 K for 20 min in the absence of Cu^{2+}. (c) In the dark in the presence of 250 equiv of Cu^{2+} and (d) after reduction of the sample in (c) with 15 mM ascorbate (10 min) after removal of the copper with 5 mM EDTA. The EPR conditions were as follows: temperature, 15 K; microwave power, 6.32 mW; modulation amplitude, 15.7 G; microwave frequency, 9.237 GHz. The bars indicate the positions for the high-potential form (at g = 3.08, left) and low-potential form (at g = 2.96, right) of Cyt b_{559}.

Cyt b_{559} was also modified by copper treatment. In control BBY membranes, Cyt b_{559} is mainly in the high-potential form which remains reduced in the dark. The EPR spectrum shows a small absorption from some low-potential Cyt b_{559} (Figure 1a). Illumination at 77 K results in the oxidation of about one copy of high-potential Cyt b_{559} per PS II center, which is shown by the increase of the peak at g = 3.08 (Figure 1b; Miller & Brudvig, 1991). When the EPR spectrum from Cyt b_{559} was measured in the copper-treated samples where more than 90% of the 16 kDa subunit was lost but the 23 and 33 kDa proteins remained, it showed a large peak at g = 2.96 (Figure 1c). Quantification revealed that the treatment resulted in quantitative oxidation of Cyt b_{559}. This most likely reflects a shift of Cyt b_{559} to its low-potential form. This was further substantiated by the concomitant shift in the g-value to g = 2.96, which is normal for low-potential forms of Cyt b_{559} (Miller & Brudvig, 1991), and the fact that ascorbate was unable to reduce Cyt b_{559} after incubation with copper ions (Figure 1d). Furthermore, almost complete removal of Cu^{2+} (<4 Cu^{2+} ions remaining; Table 1) did not alter the spectral shape of Cyt b_{559}, indicating that the modification was not reversible simply by removing the Cu^{2+} ions (not shown).

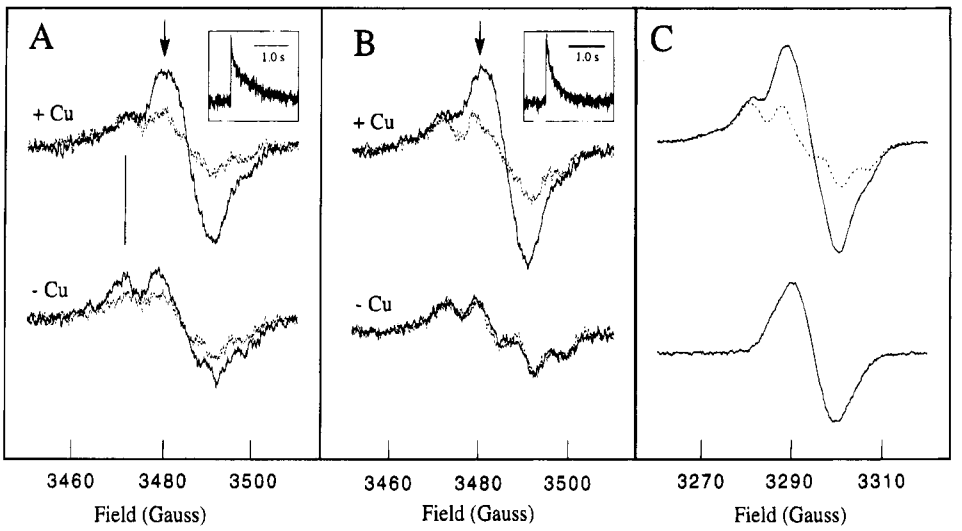

FIGURE 2: Effect of Cu^{2+} ions on the formation of radical EPR spectra in (A) Tris-washed PS II membranes and (B) intact BBY membranes. The spectra are recorded in the absence (lower spectra) and presence (upper spectra) of Cu^{2+}. The spectra were recorded in the dark (dotted spectra; representing Tyr_D^{ox}) and during continuous illumination (solid curve spectra; representing light-induced radicals). The insets show the decay kinetics of the light-induced radical species measured at the field position indicated by an arrow in the upper spectrum. (C) Induction of radicals by illumination at 200 K for 5 min of intact PS II enriched membranes in the presence of Cu^{2+} (upper spectra, outer trace). The spectrum from Tyr_D^{ox} (upper spectra, inner trace) was recorded in the dark prior to illumination at 200 K. The subtraction of the inner from the outer spectrum provides the spectrum (lower spectrum) of a light-induced radical species with $g \approx 2.0028$ and line width 9.5 ± 0.3 G. EPR conditions in panels A and B: temperature, 295 K; microwave power, 2 mW; modulation amplitude, 2.92 G; microwave frequency, 9.773 GHz. In panel C: temperature, 15 K; microwave power, 0.5 μW; modulation amplitude, 2.49 G; microwave frequency, 9.237 GHz. The kinetic traces are the sum of 100 individual flash-induced kinetic transients.

Table 2: Photochemical Properties of Copper- and EDTA-Treated Tris-Washed PS II Membranes

sample/treatment	Cu ions/PS II[a]	signal II_{fast} from Tyr_Z^{ox} (%)[b]	radical at $g = 2.0028$ (%)[b]	radical(s) induced at 200 K (%)[c]	Q_A^-–Fe^{2+} (%)[d]	Q_A^-–Fe^{2+} (%)[e]	Q_A^- at $g = 2.0044$ (%)[f]
control Tris-washed PS II	0	90	0	50 ($g = 2.0026$)	50	100	0
control Tris-washed PS II + EDTA wash[g]	0	70	0	50 ($g = 2.0026$)	50	100	0
Tris-washed PS II + Cu[h]; buffer wash[i]	≈250	0	100	100 ($g = 2.0028$)	nd	30	70
Tris-washed PS II + Cu[h]; EDTA wash[g]	2–3	70	40	80 ($g = 2.0031$)	30	30	70

[a] Determined by EPR spectroscopy assuming that each PS II reaction center contains 220 chlorophylls. [b] Compared to signal II_{slow}. The EPR signals were induced by illumination at 295 K. [c] The sample was illuminated at 200 K. The EPR signal may be a mix of radicals; see text for explanation. [d] Induced by illumination at 200 K. Percent of maximal amplitude when Q_A was reduced by dithionite. [e] Q_A^- was reduced chemically with with 40 mM dithionite. Amplitude in percent of maximal signal. [f] Q_A^- was reduced chemically with 40 mM dithionite. Radical size in percent of signal II_{slow}. [g] The membranes were washed 3 times with EDTA and then resuspended in buffer without EDTA. [h] The membranes were incubated with copper (1000–1500 Cu^{2+}/PS II) for 20 min. [i] After the copper incubation, a part of the copper was removed by one wash in copper-free medium.

Effect of Cu^{2+} on the Induction of Tyr_Z^{ox} and Tyr_D^{ox}. The function of the two tyrosine electron donors, Tyr_Z and Tyr_D, was investigated by EPR spectroscopy in the presence and absence of Cu^{2+}. Tyr_D^{ox} gives rise to the EPR signal II_{slow} (Figure 2, dotted lines), which is stable for hours in intact PS II and for about an hour in Tris-washed PS II (Vass & Styring, 1991; Miller & Brudvig, 1991). Incubation of PS II with cupric ions had no effect on the amplitude of signal II_{slow} (Figure 2A,B).

The function of Tyr_Z was investigated in Tris-washed PS II membranes. In the absence of the water-splitting complex, the reduction of Tyr_Z^{ox} is slowed down, allowing Tyr_Z^{ox} (signal II_{fast}) to be observed during continuous illumination of the sample, leading to a doubling of the amplitude of signal II (figure 2A, lower spectra; Table 2) indicative of complete oxidation of Tyr_Z (Babcock et al., 1989).

In contrast, in a Tris-washed sample incubated with Cu^{2+} ions for 10 min, signal II_{fast} could not be induced by the continuous illumination, indicating that the function of Tyr_Z had been impaired. In Figure 2A (upper spectra), the trace recorded during illumination shows no increase in the amplitude at 3471.5 G, a field position characteristic for the low-field shoulder of signal II_{fast} (marked with a bar in Figure 2A, lower spectra). Instead, the continuous illumination induced a new featureless radical signal on top of signal II_{slow} (Figure 2A, upper spectra) in the presence of Cu^{2+}. Quantification of the new radical showed that it corresponded to one radical per PS II reaction center (Table 2). Furthermore, the radical was quantitatively induced by illumination in presence of 5 mM ferricyanide (not shown), indicating that it has a cationic origin and does not represent a reduced component on the acceptor side. The decay kinetics of the radical in the dark could be determined in a repetitive flash experiment. The kinetic trace showed an essentially monophasic decay with $t_{1/2} = 700 \pm 25$ ms (Figure 2A, upper inset). The size of the flash-induced radical corresponded to approximately 60% of the radical induced by continuous illumination. The lower yield of the radical by flash induction probably reflects difficulties for the laser flash to saturate the sample. Resolution of the kinetic spectrum

demonstrated that the same radical was induced by both flashes and continuous illumination (not shown).

Figure 2B shows the effects of Cu^{2+} ions on intact, oxygen-evolving BBY membranes. In intact PS II, Tyr_Z^{ox} is reduced very rapidly by the water-splitting complex, and, consequently, the EPR spectrum of the light-induced Tyr_Z^{ox} (denoted signal $II_{very\ fast}$) decays too fast to be resolved. Therefore, no induction of Tyr_Z^{ox} could be observed during continuous illumination (Figure 2B, lower spectra). Note that the illumination did not induce any other observable radical. However, when the membranes were treated with Cu^{2+} ions for 10 min, the effect was similar to that in the Tris-washed membranes, and the same transient, narrow, and structureless radical was formed during illumination in the presence of copper (Figure 2B; upper spectra). Comparison with the size of signal II_{slow} showed that the radical was induced in more than 90% of the PS II centers. The decay kinetics of the new radical after a flash were monophasic with a $t_{1/2}$ for the decay of 325 ± 25 ms (Figure 2B, inset). Furthermore, we observed no induction of Tyr_Z^{ox} in the copper-treated sample.

Our interpretation of these results is that Tyr_Z oxidation is inhibited by the addition of cupric ions. Instead, another radical with a totally different EPR spectrum appeared during flash or continuous illumination. Thus, the primary charge separation reaction must still be operational. The question then arises, which of the other redox components are still functional in the presence of Cu^{2+}. In the room-temperature experiments described above, each sample underwent several turnovers during the measurement. Therefore, we investigated the effect of Cu^{2+} ions in samples where only one charge separation was allowed to take place. This was achieved by illumination at 200 K and then recording the EPR spectrum in the frozen state (at 77 K). Figure 2C shows the EPR spectra in the radical region in the dark (inner spectrum) and after illumination at 200 K (outer spectrum) of intact PS II membranes in the presence of 250 Cu^{2+} ions per PS II. The lower spectrum in Figure 2C is the difference spectrum and represents a radical EPR spectrum with g = 2.0028 ± 0.0002 and a line width of 9.5 ± 0.3 G. The size of the signal corresponds to one radical per PS II reaction center. This radical is similar to that induced at room temperature which implies that the same electron transfer pathway dominates at both high and low temperatures. Furthermore, the same amount of the same radical was induced by illumination at 200 K of Tris-washed PS II membranes incubated with copper ions (Table 2).

Reversibility of the Copper Effects. Table 1 shows the results after removal of copper from BBY membranes by repeated EDTA washes. The EDTA washes removed the remaining 16 kDa and 100% of the extrinsic 23 and 33 kDa subunits. When BBY membranes underwent the same washing procedure without preincubation with Cu^{2+}, no loss of subunits or manganese was observed (not shown). Thus, it seems likely that the Cu^{2+}-induced loss of manganese and the 16 kDa subunit renders the rest of the oxygen-evolving complex more susceptible to treatment with EDTA.

To test the reversibility of the copper-mediated inhibition of Tyr_Z, we turned to Tris-washed PS II membranes (Table 2). The Tris-washed membranes were incubated with copper (≈1000 Cu^{2+}/PS II reaction center) for 20 min in the dark. This resulted in complete inhibition of Tyr_Z and quantitative oxidation of the radical at g = 2.0028 (Figure 2A; Table 2). Thereafter, three EDTA washes were applied before the sample was finally resuspended in EDTA-free buffer (in the dark). After this washing procedure, only about 2−3 Cu^{2+} ions per PS II remained in the sample. The results show that 70% of signal II_{fast} from Tyr_Z^{ox} now could be induced by continuous illumination. The radical signal was induced in a smaller fraction (30−40%), approximately corresponding to the fraction of centers where Tyr_Z was not functional. In Tris-washed PS II membranes, that were not treated with copper ions, a corresponding EDTA treatment resulted in approximately 30% loss of signal II_{fast}, indicating that the EDTA wash itself was slightly inhibitory. These results indicate that the Cu^{2+}-induced inhibition of Tyr_Z electron transfer to P680 is reversible. In addition, our results clearly show that Tyr_Z might be functional although several Cu^{2+} ions are bound to the membranes. This indicates that several high-affinity Cu^{2+} sites exist in PS II membranes. These might well be on other proteins than the PS II reaction center (Takahashi & Asada, 1986; Tajmir-Riahi & Ahmed, 1993).

Interestingly, we observed that illumination at 200 K of Cu^{2+}-incubated samples that had been washed with EDTA induced a new, nonstructured radical signal with g = 2.0031. This radical signal amounted to 0.8 spin per PS II reaction center (Table 2). In part, this signal corresponds to the well-known chlorophyll donor (g = 2.0026; Miller & Brudvig, 1991) that normally is induced by illumination of Tris-washed PS II membranes (Table 2, radical induced by illumination at 200 K). In part, this signal originates from Q_A^- which has been decoupled from the iron (see below).

Effects of Copper on the Reduction of Q_A. Q_A^- can be studied and quantified by EPR spectroscopy. Normally, Q_A^- interacts with the non-heme iron, giving rise to the Q_A^-−Fe^{2+} EPR signal that depends on the presence and correct coupling of both the reduced quinone and the ferrous iron (Butler et al., 1984) and is characterized by two g-values of g = 1.82 and 1.7 (Rutherford & Zimmermann, 1984). However, in cases when Q_A^- does not interact magnetically with the iron (for example, after extraction of the ferrous ion or after CN^- treatment), it has a simple radical EPR spectrum with a high g-value, g = 2.0044, which is about 9.5 G wide (Klimov et al., 1980; Sanakis et al., 1994).

In a sample containing cupric ions, the Q_A^-−Fe^{2+} EPR spectrum could not be detected due to the very large EPR signal from the Cu^{2+} ions (Table 2). Therefore, the Q_A^-−Fe^{2+} signal had to be measured in EDTA-washed samples. The amplitude of the Q_A^-−Fe^{2+} EPR spectrum induced by chemical reduction with dithionite was about 30% of its maximal size (Figure 3; Table 2) in Tris-washed PS II membranes incubated with cupric ions and washed with EDTA. Thus, the Q_A−Fe^{2+} complex had not been modified in these centers. Furthermore, dithionite reduction also resulted in the formation of a featureless radical with EPR properties similar to those described for Q_A^- in the absence of magnetic interaction with the acceptor side iron (Figure 3, inset; Klimov et al., 1980; Sanakis et al., 1994). This radical was present in 70% of the PS II centers (as compared to signal II_{slow} in the same sample prior to chemical reduction) (Table 2). Together, these results indicate that Q_A could be reduced chemically in all PS II centers but that the Cu^{2+} incubation had modified the Q_A−Fe^{2+} complex. The modification seemingly was irreversible as it remained although Cu^{2+} was removed by EDTA or reduced by dithionite (Table 2). The fraction of uncoupled Q_A^- was

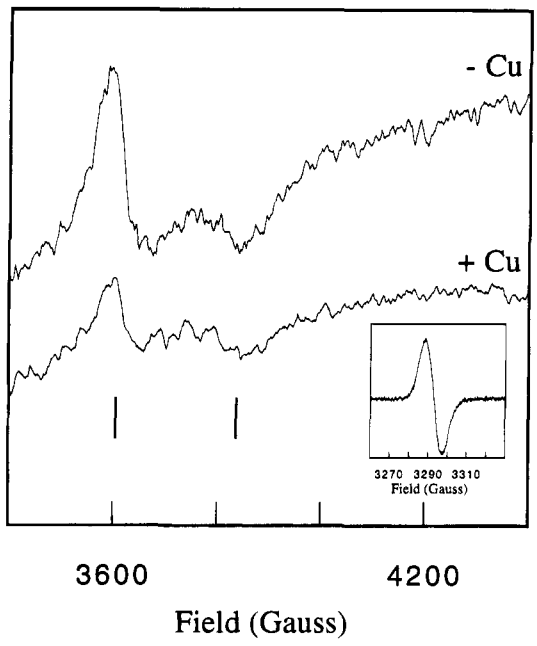

FIGURE 3: Effect of Cu^{2+} ions on the induction of the EPR spectra from Q_A reduced chemically in the dark by 50 mM dithionite in Tris-washed membranes. The bars indicate the spectral features at $g \approx 1.82$ (to the left) and $g \approx 1.7$ (to the right) that belong to the Q_A^-–Fe^{2+} EPR signal. The inset shows the size of the uncoupled Q_A^- radical EPR signal in the Cu^{2+}-treated sample after reduction with dithionite. All spectra are recorded in the presence of 100 mM formate to enhance the size of the Q_A^-–Fe^{2+} EPR signal (see Materials and Methods). Most of the Cu^{2+} ions were removed prior to measurements with three EDTA washes. EPR conditions: Q_A^-–Fe^{2+} EPR spectra; temperature, 4 K; microwave power, 32 mW; modulation amplitude, 32 G; microwave frequency, 9.236 GHz. The conditions for the free radical spectrum of uncoupled Q_A^- (inset): temperature, 15 K; microwave power, 0.5 μW; modulation amplitude, 3.2 G; microwave frequency, 9.236 GHz.

dependent on the incubation time with copper. After 10 min incubation, it was detected in about 20% of the PS II centers (not shown) while it was present in 70% of the centers after 20 min incubation (Figure 3, inset). Furthermore, control experiments where Q_A and the Cu^{2+} that was present were reduced by dithionite prior to the EDTA washes revealed that the fraction of uncoupled Q_A^- did not increase during the EDTA washes. Thus, the decoupling was not induced by the EDTA treatment but by the incubation with Cu^{2+}.

We also tested whether both types of Q_A signals could be photochemically induced. Tris-washed PS II membranes, incubated with or without Cu^{2+} and then EDTA-washed, were illuminated at 200 K. In both controls (with or without EDTA wash), the Q_A–Fe^{2+} EPR signal was formed in about 50% of the centers. In addition, a chlorophyll radical donor ($g = 2.0026$) was induced in a corresponding fraction of the centers. This seemingly low value of stable charge separation probably reflects the instability of the Chl^+–Q_A^- charge pair at 200 K (Thompson & Brudvig, 1988). In the copper-treated samples, after removing the Cu^{2+} ions with EDTA, 30% of the maximal Q_A^-–Fe^{2+} EPR signal was induced (Table 2). In addition, a featureless radical with $g = 2.0031$ and about 9–10 G wide corresponding to 0.8 radical per PS II center was formed (Table 2). Most likely, this radical is of a mixed origin. We hypothesize that stable charge separation (at 200 K) in the copper-treated samples occurred in about 50% of the PS II centers, similar to the control. If so, a fraction of the radical signal (≈20–25% on a radical basis) corresponds to Q_A^- that was uncoupled from the iron, having a free radical EPR spectrum with $g \approx 2.0044$ (compare Figure 3). The remaining part of the radical spectrum (≈0.5 spin per PS II reaction center) probably reflects oxidation of the chlorophyll electron donor, with $g = 2.0026$. This would result in a g-value between $g = 2.0026$ and 2.0044 in agreement with the measured $g = 2.0031$.

It should be noted that neither EPR signal (at $g = 2.0044$) from uncoupled Q_A^- or from Q_A^-–Fe^{2+} could be observed in the presence of high amounts of Cu^{2+}. However, the signals became observable when copper either was removed from PS II by repeated EDTA washes or was quantitatively reduced to monovalent Cu^+ by dithionite. Under these circumstances, both of the EPR signals from Q_A^- could be induced by illumination at 200 K or chemical reduction with dithionite (Figure 3; Table 2). The ratio between the two forms of the Q_A^- signals depended on the amount of Cu^{2+} and the incubation time (see above). Interestingly, illumination at room temperature (after EDTA washes) did not induce the free radical EPR spectrum of Q_A^- (Table 2). Either the electron was transferred to Q_B or the plastoquinone pool or was Q_A doubly reduced.

These observations also explain the seemingly contradictory results in Figure 2C. In this experiment, only the donor side radical (1 spin per PS II center) was observable following illumination at 200 K although a large fraction of uncoupled Q_A^- should have been formed during the illumination. However, this is explainable since the experiment was performed in the presence of 250 Cu^{2+} per PS II which prevents observation of signals from Q_A^-.

DISCUSSION

Our results reveal that there are two major inhibitory effects of Cu^{2+} ions on electron transfer through PS II. First, on the donor side, EPR measurements have directly demonstrated that oxidation of Tyr_Z is reversibly inactivated by Cu^{2+} (Figure 2A,B). In addition, we observed that a new radical, most probably on the donor side of PS II, was induced by illumination. These results are in agreement with conclusions from earlier flash-induced absorption change measurements where Cu^{2+} ions were found to inhibit fast reduction of $P680^+$ from Tyr_Z (Schröder et al., 1994).

Second, on the acceptor side, our results show that Cu^{2+} ions irreversibly perturb the interaction between Q_A^- and Fe^{2+} in the acceptor complex. After incubation with Cu^{2+}, Q_A can still be quantitatively reduced, in accordance with earlier optical measurements (Schröder et al., 1994), but the structure around Q_A is modified, leading to magnetic decoupling of Q_A from the non-heme iron. These results lend further strength to conclusions that cupric ions exert effects on both the donor and the acceptor side of PS II and should relieve some of the controversy concerning the mechanism for inhibition of PS II with copper.

Our results also show that Cu^{2+} disturbs the lumenal side of PS II, provoking loss of the 16 kDa protein and manganese and altering the potential of Cyt b_{559} (Table 1). It is likely that Cu^{2+} in this respect acts similarly to other divalent ions like Pb^{2+} and Zn^{2+} that are thought to displace inorganic factors (Cl^-, Ca^{2+}, Mn^{2+}) and remove the extrinsic subunits from the water-oxidizing complex (Rashid et al., 1994). Copper treatment induced loss of only the 16 kDa extrinsic

protein (Table 1), yet Cyt b_{559} is transformed to the low-potential form. This transformation is often brought about by treatments known to remove both the 16 and the 23 kDa extrinsic subunits. Thus, our results indicate that not only the 23 kDa extrinsic protein regulates the redox potential of Cyt b_{559}. In this respect, it is interesting that treatments known to release only Ca^{2+} from PS II membranes might transform a substantial amount of high-potential Cyt b_{559} to a low-potential form (McNamara & Gounaris, 1994). It has also been shown that the presence of $CaCl_2$ increased the amount of Cyt b_{559} that could be reconstituted to its high-potential form upon readdition of both the 16 and 23 kDa proteins (Bergström, 1986).

The connection between Cu^{2+} incubation, release of the 16 kDa protein, and modification of Cyt b_{559} makes it tempting to speculate if Cu^{2+} ions might interfere with the Ca^{2+} binding site in the PS II reaction center presumably by replacing Ca^{2+}. In this respect, it is interesting to compare Cu^{2+} inhibition to the effects of lanthanoide ions on PS II electron transfer. Lanthanoides have been suggested to replace Ca^{2+} in many proteins [see Bakou et al. (1992) and references cited therein]. In PS II, the presence of lanthanoides results in removal of extrinsic subunits, loss of part of the Mn-cluster (Ghanotakis et al., 1985), conversion of Cyt b_{559} to the low-potential form (Bakou et al., 1992), and incomplete oxidation of Tyr_Z (Bakou & Ghanotakis, 1993). In addition, illumination at 200 K of lanthanoide-inhibited PS II results in oxidation of a component tentatively identified as a chlorophyll donor (Bakou et al., 1992). This has EPR characteristics quite similar (but seemingly not identical) to those of the Cu^{2+}-induced radical described here.

The chemistry behind the inhibition of Tyr_Z by Cu^{2+} and the damage to the oxygen-evolving complex probably involves binding in their vicinity. In proteins, Cu^{2+} often binds to histidyl or carboxylic side chains. There are several candidates close to Tyr_Z on the D1 protein (Svensson et al., 1990; Debus, 1992), and an interesting possibility is that Cu^{2+} coordinates to His190 on the D1 protein. From modeling work and site-directed mutagenesis studies, this residue is known to be close to Tyr_Z and the manganese cluster (Svensson et al., 1990; Roffey et al., 1993; Kramer et al., 1994) and has recently been proposed to be involved in proton transfer from Tyr_Z during oxygen evolution (Hoganson et al., 1995). It is highly likely that binding of a Cu^{2+} ion in this position would interfere severely with Tyr_Z oxidation as well as the binding of the manganese cluster.

The copper-induced radical that is formed instead of Tyr_Z has EPR parameters that resemble those of an accessory donor that might reduce $P680^+$ during illumination below 230 K. This donor, which has $g = 2.0026$ and is 10−11 G wide, has been assigned to a chlorophyll or a carotenoid molecule (Vermeglio & Mathis, 1973; de Paula et al., 1986; Miller & Brudvig, 1991). Initially, it was our hypothesis that the copper-induced radical was of the same origin. However, the EPR parameters are not identical. Furthermore, in flash absorption measurements in Cu^{2+}-inhibited PS II membranes, no chlorophyll radical with a longer lifetime than 1 ms (Schröder et al., 1994) nor any long-lived carotenoid radical (measured at >950 nm) could be detected (Schröder, unpublished results). The half-time for the decay of the Cu^{2+}-induced radical in the EPR measurements was about 300 ms. Therefore, we conclude that it has a different origin and an alternative candidate could be an oxidized amino acid side chain.

An unexpected effect was that Cu^{2+} modified the Q_A−non-heme iron complex such that Q_A was magnetically decoupled from Fe^{2+}. The modification was irreversible, and the coupled Q_A^-−Fe^{2+} EPR signal did not reappear after EDTA washes. It is thus tempting to speculate that one or more Cu^{2+} ion(s), which have a preference for nitrogen-containing ligands, is/are coordinated to one or several of the histidine ligands to the non-heme Fe^{2+} ion which might thereby leave its site. An indication that Cu^{2+} might actually replace the iron is that the noncoupled EPR signal from Q_A^- could only be observed when the Cu^{2+} ions had been removed or reduced to Cu^+ which is diamagnetic. This could indicate that a Cu^{2+} ion (which is paramagnetic) binds very close to Q_A^-, thereby broadening its EPR signal beyond detection by magnetic interaction. However, at present we cannot substantiate this suggestion. Instead, it is possible that copper binding in the acceptor complex induces secondary changes that alter the magnetic interaction between Q_A^- and the iron or leads to the formation of low-spin Fe^{2+} similar to the changes induced by CN^- (Sanakis et al., 1994).

Despite the modification, Q_A could still be almost quantitatively reduced by dithionite or illumination after copper treatment. In addition, the same amount of Q_A^- was induced by low-temperature illumination in both copper-treated and control Tris-washed membranes, indicating that the lifetime of Q_A^- is not modified by the copper incubation (Table 2). These results corroborate earlier conclusions from flash-induced absorption changes at 820 and 325 nm that the primary charge separation was unaffected by the presence of Cu^{2+} ions (Schröder et al., 1994). At the same time, our results lend support to studies indicating that Cu^{2+} modifies the electron transfer in the acceptor side complex of PS II [for example, see Mohanty et al. (1989) and Renger et al. (1993)] as this often is an effect of structural modifications in this region. A relevant example is that exchange of one of the presumed ligands to the non-heme Fe^{2+}, His268(D2) to Gln, resulted in inefficient electron transfer to Q_B and altered herbicide binding in the Q_B site although Q_A could still be photochemically reduced with low efficiency (Vermaas et al., 1994b).

In conclusion, our results show that Cu^{2+} ions have multiple effects on both the donor and the acceptor side of PS II which probably explains the conflicting reports on the inhibition site in the literature. One major consequence is the damage of the oxygen-evolving complex that is irreversibly inhibited since the 16 kDa subunit and part of the manganese cluster are lost. Second, Tyr_Z oxidation is reversibly inhibited. Finally, Cu^{2+} modifies the acceptor side irreversibly. Q_A can still be reduced, but the properties of the acceptor complex are severly altered.

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