Photoinhibition of Photosystem II from Higher Plants

EFFECT OF COPPER INHIBITION*

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Strong illumination of Cu(II)-inhibited photosystem II membranes resulted in a faster loss of oxygen evolution activity compared with that of the intact samples. The phenomenon was oxygen- and temperature-dependent. However, D1 protein degradation rate was similar in both preparations and slower than that found in non-oxygen evolving PSII particles (i.e. Mn-depleted photosystem II). These results seem to indicate that during illumination Cu(II)-inhibited samples do not behave as a typical non-oxygen evolving photosystem II. Cytochrome b$_{559}$ was functional in the presence of Cu(II). The effect of Cu(II) inhibition decreased the amount of photoreduced cytochrome b$_{559}$ and slowed down the rate of its photoreduction. The presence of Cu(II) during illumination seems to protect P680 against photodamage as occurs in photosystem II reaction centers when the acceptor side is protected. The data were consistent with the finding that production of singlet oxygen was highly reduced in the preparations treated with Cu(II). EPR spin trapping experiments showed that inactivation of Cu(II)-treated samples was dominated by hydroxyl radical, and the loss of oxygen evolution activity was diminished by the presence of superoxide dismutase and catalase. These results indicate that the rapid loss of oxygen evolution activity in the presence of Cu(II) is mainly due to the formation of ‘OH radicals from superoxide ion via a Cu(II)-catalyzed Haber-Weiss mechanism. Considering that this inactivation process was oxygen-dependent, we propose that the formation of superoxide occurs in the acceptor side of photosystem II by interaction of molecular oxygen with reduced electron acceptor species, and thus, the primarily Cu(II)-inhibitory site in photosystem II is on the acceptor side.

The photosynthetic activity decreases when oxygenic organisms are exposed to prolonged illumination with high light intensities. This process, which includes the functional impairment of photosystem II (PSII) electron transport and the structural damage of the PSII reaction center (RC) D1 protein, is known as photoinhibition (for reviews see Refs. 1–3). The molecular mechanism of photoinhibition remains to be elucidated. However, it seems clear that the primary target of photoinhibition is the PSII RC. There has been a great deal of discussion on the processes involved in photoinhibition, which has focused on differentiating between whether the continuous strong illumination damages electron transport at the acceptor or the donor side of the initial charge transfer reaction between P680 and pheophytin (Pheo). It is possible that both of these mechanisms operate in vivo with a relative balance being dependent on other factors besides light intensity (2, 4). In the donor side-mediated photoinhibition, the electron donation to P680$^-$ is inhibited and the functioning of Tyr$Z$ and/or P680 is affected. Electron transfer from Tyr$Z$ to P680$^-$ is slowed down leading to an accumulation of long-lived highly oxidizing radicals at the donor side (P680$^-$ and Tyr$Z^+$), which in turn would have a high probability of inducing rapid inactivation of PSII electron transport activity and oxidation of nearby pigments, redox components, or amino acids (3, 5). In contrast, acceptor side-mediated photoinhibition seems to be due to an overaccumulation of reduced QA. Indeed, the presence of nonfunctional primary quinone electron acceptor QA, which in an intact system stabilizes charge separation (6, 7), induces the primary radical pair (P680$^-$Pheo$^+$) recombination either to the excited singlet state (P680$^1$Pheo$^+$) or via a radical pair mechanism to the triplet state (P680$^3$Pheo$^+$). A direct recombination to the ground state (P680Pheo$^-$) is also possible (8). Oxygen has been implicated in photoinhibition processes (2, 9), and the production of damaging oxygen species may be a mechanism that activates D1 protein degradation. The involvement of such species in photoinhibition has been concluded on the basis of detection of photoinduced $^1$O$_2$ formation using both steady-state and time-resolved measurements of its emission at 1270 nm (10). Under aerobic conditions light treatment led to an irreversible loss of electron transport and no triplet state of P680 was detected (11). It was suggested that P680 triplet was quenched by molecular oxygen leading to the production of its first excited state O$_2$(^1Ag) which is highly reactive. When this reaction occurs, it has been shown that there is an irreversible bleaching of chlorophores within the PSII RC (12–14). The primary damage is to the chlorophylls (Chls) of P680, but further exposure to illumination results in the bleaching of other bound chlorophores and in the D1 protein degradation (15, 16). However, the mechanism of this process has not been fully characterized yet.

Photosynthetic organisms are normally protected from damage by $^1$O$_2$ by carotenoids (17–19). This fact has been demon-
CuCl2 were done at 4°C for 10 min with occasional shaking by hand.

...donor side of PSII was impaired by treatment with some re-...catalase, and 50 ml of Chl were incubated with 250 ml of catalase prior to light treatment and maintaining N2 bubbling during illumination. Control samples were kept in the dark under identical conditions to the illuminated samples in order to monitor dark reactions. The samples were exposed to heat-filtered white light (5,500 μEm−2s−1) for 10 min while this time was sufficient to abolish the activity of Cu(II)-inhibited preparations. However, removal of oxygen from the medium abolished the inactivation independently of the presence of Cu(II). At 4 °C these differences were smaller, and the loss of activity was slower than that at 25 °C (Fig. 1B). It is known that photoinhibition is a temperature-dependent phenomenon, and it increases at higher temperatures (42). A comparison of Fig. 1, A and B, indicates that the Cu(II) effect on photoinhibition is also temperature-dependent. How-
and 4°C (samples had no effect on the DCIP reduction suggesting that
1. The addition of DPC to the photoinhibited Cu(II)-treated
min illumination, respectively, in agreement with data of Fig.
photoreduction which decreased up to 8 and 4% after 15 and 30
side in these experimental conditions. Cu(II)-treated samples
exhibited much stronger loss of activity in terms of DCIP-
activity was 40 and 92%, respectively. In Mn-depleted prepa-
rations, D1 degradation went up to 40% after the same illumi-
and the presence of Cu(II) showed no effects on D1 protein
degradation at 25°C (Fig. 2). In contrast, a faster D1 degrada-
tion was observed in a non-oxygenic PSII (i.e. manganese-
depleted PSII membranes). These results indicate that the dif-
ficencies in the inactivation curves (Fig. 1) are not due to the
loss of the protein itself. D1 protein was degraded around 20%
after 10 min illumination (Fig. 2B) in both nontreated and
Cu(II)-inhibited samples, whereas the loss of oxygen evolu-
tion activity was 40 and 92%, respectively. In Mn-depleted prepa-
rations, D1 degradation went up to 40% after the same illumi-
nation time. It seems, therefore, that Cu(II) inhibitory and
TEMED treatment effects are the consequence of different
molecular mechanisms. Since TEMED treatment removes the
extrinsic proteins and the manganese cluster of the PSII donor
side (35), it can be suggested that the Cu(II)-inhibitory site, in
these experimental conditions, is not located on the donor side
of PSII. In addition, the presence of Cu(II) did not modify the
D1 protein degradation in the non-oxygenic (i.e. manganese-
depleted) samples (data not shown). The more pronounced im-
pairment of the electron transport compared with D1 protein
degradation in the intact samples has also been described by
others (3).

Table I summarizes the DCIP reduction measurements in
nontreated and Cu(II)-treated photoinhibited samples. The rate
of DCIP reduction in nontreated PSII membranes decreased 50 and 68% after 15 and 30 min of illumination, re-
spectively. After that, addition of 300 μM DPC almost totally
restored the rate of DCIP reduction. A noncomplete restoration of activity in the presence of DPC after illumination indicates that a partial damage occurs at a place other than the donor side in these experimental conditions. Cu(II)-treated samples exhibited much stronger loss of activity in terms of DCIP-
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samples had no effect on the DCIP reduction suggesting that

FIG. 1. Inhibition of oxygen evolution activity during aerobic
(A, □) and anaerobic (C, ▨) photoinhibition in the presence (C, ■)
and absence (□) of 250 μM (Cu(II)/RC = 275) CuCl₂ at 25 (A)
and 4 °C (B). Samples were illuminated with
5,500 μEm⁻²s⁻¹ and washed twice before measurements. The 100%
oxygen evolution activity without addition was about 560 pmol of O₂
mg of Chl⁻¹h⁻¹. Values represent means ± S.E. (n = 4).

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1. The addition of DPC to the photoinhibited Cu(II)-treated
samples had no effect on the DCIP reduction suggesting that

FIG. 2. Immunoblot with rabbit antiserum anti-D1 (A) and
quantitative plot of the effect of strong white light illumination
(5,500 μEm⁻²s⁻¹) on D1 protein degradation (B) in photoinhib-
ted PSII membranes without any addition (a, ○), in the presence
of 250 μM (Cu(II)/RC = 275) CuCl₂ (b, □), and Mn-depleted
c, ▽). Samples were illuminated with 5,500 μEm⁻²s⁻¹ and
washed twice before measurements. The 100%
percentage of DCIP-photoreduction activity compared with the con-

TABLE I

| Sample          | DCIP-reduced | Illumination time (min) |
|-----------------|--------------|------------------------|
| Non-treated     |              |                        |
| -DPC            | 74.5 ± 3.1   | 15                     |
| +DPC            | 95.7 ± 2.8   | 30                     |
| Cu(II)-treated  |              |                        |
| -DPC            | 60.5 ± 2.3   | 15                     |
| +DPC            | 61.2 ± 3.3   | 30                     |

* Values represent means ± S.E. (n = 4).
† Illumination time in min.
‡ Percentage of DCIP-photoreduction activity compared with the control for each substrate.

the irreversible damage occurred beyond the DPC donation
side, i.e. the P680 and/or the acceptor side, but not the donor
side.

Effect of Cu(II) Inhibition on the Cyt b₅₅₉ Photoreduction—
The effect of Cu(II) on the photoreduction of Cyt b₅₅₉ is shown
in Fig. 3A. The measurements were performed at pH 7.8; at
this pH the percentages of oxidized and reduced Cyt b₅₅₉ in
the dark were 53 and 47%, respectively. These values correspond
well with those published by Buser et al. (24). The positive band
centered at 560 nm, which corresponds to a fraction of 40% of
total Cyt b₅₅₉ decreased in Cu(II)-inhibited samples. The pres-
ence of Cu(II) did not modify the ratio Cyt red/Cyt ox in the dark.
This result is not in agreement with those reported recently by
Jegerschöld et al. (32). These authors showed that Cu(II) dis-
turbed the luminal side of PSII affecting the potential of Cyt
b₅₅₉ which is transformed to the low potential form. This dis-
crepancy may be due to the different concentrations of CuCl₂
used in the experiments that were much lower in our case. The
light-induced kinetics of Cyt b<sub>559</sub> in the 0–250-s range were also measured in the absence and in the presence of Cu(II) (Fig. 3B). The PSII intact samples depicted positive kinetics (Fig. 3B, a); however, in the presence of increasing amounts of CuCl<sub>2</sub> (≥50 μM, Cu(II)/RC = 185) negative kinetics were measured (Fig. 3B, b and c). The Cu(II)/RC ratio used in these measurements provides 75–80% inhibition of oxygen evolution activity (28). A possible direct chemical interaction between Cyt b<sub>559</sub> and Cu(II) was discarded since it has no effect on the dithionite-reduced minus ferricyanide-oxidized spectrum (data not shown). These results indicated that in addition to the low Cu(II) concentration effect on the photoreduction of Cyt b<sub>559</sub> (Fig. 3A), another effect at higher Cu(II) concentrations (≥50 μM) occurs that induces Cyt b<sub>559</sub> oxidation.

Effect of Cu(II) Treatment on the Photobleaching of Pigments During Photoinhibition—The changes in the absorption spectra of PSII membranes as a function of pre-illumination with strong white light were also measured (data not shown). A gradual loss of the absolute Q<sub>y</sub> absorption band in both nontreated and Cu(II)-treated PSII membranes was observed. Illumination of PSII membranes with strong light resulted in a photobleaching of Chls and carotenoids. It is known that in the presence of molecular oxygen, the illumination of the PSII RC causes a selective photobleaching of Chl absorbing at about 680 nm, which almost certainly constitutes the primary electron donor P<sub>680</sub> (13). To better identify the Chl species that are affected during illumination in the presence of Cu(II), we recorded the absorption changes in photoinhibited OECC samples which are more purified preparations, deprived of most peripheral antenna complexes. The light minus dark difference absorption spectra of 0.25–1.5-min illuminated OECC samples (Fig. 4) presented a main peak at around 681–682 nm and a shoulder at around 670 nm in both nontreated and Cu(II)-treated samples. Photobleaching in the red spectral region was also accompanied by a loss of the absorbance at around 435 nm, which is attributed to the loss of Chl a. In addition, a large photobleaching in the carotenoid region (450–500 nm) was observed in the Cu(II)-treated samples (Fig. 4B). Fig. 5 presents in more detail the behavior of the different groups of pigments contained in the RC of PSII with illumination time. It has been described that two Chl species absorbing at 670 and 680 nm are involved in the irreversible bleaching brought about by strong white illumination (13, 43). The relative extent

Fig. 3. Light-induced absorbance change in the spectral range 540–585 nm (A) after 25 s of continuous illumination of dark-adapted PSII membranes and time course of the light-induced absorbance change at 560 nm in dark-adapted PSII membranes (B). Measurements were done at pH 7.8 without any addition (●, a) and preincubated with 50 μM CuCl<sub>2</sub> (Cu(II)/RC = 185) (■, b) and 125 μM (Cu(II)/RC = 462) CuCl<sub>2</sub> (c).

Fig. 4. Effect of strong illumination at 25°C under aerobic conditions on the absorption spectrum of nontreated (A) and incubated with 250 μM CuCl<sub>2</sub> (B) (Cu(II)/RC = 275) OECC samples. Spectra 1–3 are the differences between the spectrum of OECC samples illuminated for 0.25, 0.75, and 1.25 min, respectively, and the initial spectrum before light treatment.
of the decrease of the absorbance at 680, 670, and 495 nm depended on the presence of copper. When copper was absent the illumination of OEC samples caused a preferential irreversible bleaching at 680 nm compared with 670 nm (Fig. 5A). The addition of CuCl₂ reduced the rate of bleaching of both groups of pigments, but the effect was more pronounced on the 680-nm pigments. The rate of bleaching at 495 nm was also measured; the decrease of the absorbance at this wavelength was faster than that at 680 nm in the samples treated with CuCl₂ (Fig. 5B). A comparison between Fig. 1, Fig. 5, and Table I supports the view that the presence of Cu(II) stimulates the damage of a site beyond P₆₈₀ in the acceptor side.

**Cu(II)-mediated Free Oxygen Radical Formation During Photoinhibition**—The experiments of Fig. 1 gave evidence that oxygen-reactive species are the main cause to the loss of photosynthetic activity during photoinhibition. The presence of Cu(II) clearly stimulated this phenomenon. However, these data give no information on the identity of the presumed damaging oxygen species or on the reaction mechanism leading to their formation. In order to investigate these issues the chemical trapping technique was used. Particularly, the spin trapping is a powerful tool to detect oxygen free radicals (44). The spin traps most commonly used are nitroso and nitrone compounds that react with free radicals yielding a more stable adduct (45) that are detected by electron paramagnetic resonance (EPR) spectroscopy. With both of these traps the adduct is a nitroxide that gives well-resolved splitting constants. Singlet oxygen, which is a strong electrophile but not a radical, can oxidize sterically hindered amines to a stable N-oxyl radicals (39). This technique allows a specific study of singlet oxygen formation without interference from other possible side products (39, 41). The appearance of the EPR signal in photoinhibited PSII membranes in the presence of TEMP (Fig. 6b) showed that stable paramagnetic radicals are produced in the reaction between a product of photoinhibition and the diamagnetic amine. The symmetrical triplet is characteristic of the EPR spectrum of the paramagnetic N-oxyl radical (TEMPO), which is formed selectively when singlet oxygen (¹O₂) is trapped by TEMP. This is evidenced in Fig. 6a which shows the EPR spectrum of TEMPO. No EPR signal appeared after illuminating TEMP in a buffer only (data not shown). Comparing the EPR signal of the nitrooxide radical TEMPO in photoinhibited PSII membranes (Fig. 6b) with that from a known concentration of TEMPO (Fig. 6a), approximately 10⁻⁶ mol of ¹O₂ were produced. Cu(II)-treated samples (Fig. 6c) depicted an EPR spectrum of similar shape to that of non-treated PSII membranes (Fig. 6b), but the amplitude of the signal was much smaller. The relative intensity of this EPR signal was 2.36 times smaller than that of the nontreated preparations (Table II). The relative intensities were proportional to the area under the EPR signal. Anaerobic photoinhibition did not result in a significant ¹O₂ production (Fig. 6d), demonstrating that the N-oxyl radical resulted from a reaction requiring molecular oxygen. When nontreated and Cu(II)-treated PSII membranes were photo-inhibited in the presence of DMPO, which is the most useful trap for the oxygen-centered free radicals (44), different EPR signals appeared as shown in Fig. 7. Based on the coupling constants (Table II) two different DMPO radical adducts were identified in the absence of Cu(II). The more intense signal (Fig. 7a, not marked) corresponded to the DMPO carbon-centered radical and the smaller one (Fig. 7a, marked) to hydroxyl radical adduct. The carbon-centered radical had hyperfine coupling constant of 15.6 G and 15.0 G, and the hydroxyl radical of aN = αH = 15.0 G which corresponds well with those published by Chamulitrat et al. (40). Similar acyl radical adducts from the decomposition of lipid hydroperoxides have been reported (46, 47). In the presence of Cu(II) the hydroxyl radical adduct was the only paramagnetic signal to show up (Fig. 7b). This signal disappeared in the absence of oxygen (Fig. 7c). These results indicated that in the presence of Cu(II) hydroxyl radicals are preferentially produced compared with that of intact preparations. Since the relative intensity values calculated under the DMPO-radical adduct EPR signals are in the same range in both nontreated and Cu(II)-treated samples, it can be suggested that oxygen species formed under photoinhibitory conditions are converted to 'OH in the presence of Cu(II). ¹O₂ can be transformed into 'OH (48, 49) which can also be generated from hydrogen peroxide (H₂O₂) and superoxide radical (O₂⁻) via Cu(II)-catalyzed reactions (47, 50, 51).

In order to investigate which is the mechanism of Cu(II)-mediated oxygen-reactive species formation, we studied the influence of several oxygen-radical scavengers on the oxygen evolution activity of photoinhibited PSII membranes in the absence and in the presence of Cu(II) (Fig. 8). Compared with
5,000 mM Mes-NaOH (pH 6.5). As shown in Fig. 7, EPR spectra were recorded in ethyl acetate solutions. Values represent means ± S.E. (n = 2).

FIG. 7. EPR spectra of trapping free oxygen radicals by DMPO in isolated PSII membranes. Samples (200 μg of Chl ml⁻¹) containing 100 μM DMPO were photoinhibited with continuous white light of 5,000 μE m⁻² s⁻¹ for 6 min at 25°C with no addition (a), in the presence of 250 μM CuCl₂ (Cu(II)/RC = 275) (b), and in the absence of oxygen (c). The control, where the inactivation was 40% after 10 min of illumination, the loss of activity was reduced to ~20% with the addition of SOD and azide, respectively, in the nontreated samples (Fig. 8A). In the Cu(II)-treated samples (Fig. 8B), SOD showed the maximal protection (less than 10% inactivation) after 10 min illumination, and azide also reduced the inactivation. The strong protective effects observed by SOD in both experimental conditions indicate a direct contribution to the photoinactivation process. The finding that SOD only recovered 50% of the lost activity in the nontreated samples (Fig. 8A) and gave the maximal protection in the presence of Cu(II) (Fig. 8B) suggests that damage in nontreated samples occurs via two different mechanisms (i.e. OH radical) seems to be responsible for the loss of the oxygen evolution activity.

Table II

| Samples       | Hyperfine coupling constants | Relative intensity* |
|---------------|-----------------------------|---------------------|
|               | a¹       | a²       | G       |
| TEMPO⁻        |          |          |         |
| Nontreated    | 16.0     | 15.0     | 9.1 ± 0.2 |
| Cu(II)-treated| 15.0     | 15.0     | 3.8 ± 0.1 |
| DMPO⁺ nontreated | 15.0 | 15.0 | 18.1 ± 4.2 |
| OH            | 15.0     | 16.0     | 15.3 ± 0.5 |
| ClO₂⁻         | 15.6     | 23.5     | 119.8 ± 5.1 |
| Cu(II)-treated| 15.0     | 15.0     | 181.9 ± 4.2 |

* Values represent means ± S.E. (n = 2).

DISCUSSION

Strong illumination of PSII membranes preincubated with copper resulted in a faster inactivation of the oxygen evolution activity. This phenomenon was dependent on the temperature and the presence of oxygen. It has been reported that acceptor side-induced photoinactivation of oxygen evolving PSII is oxygen-dependent. Reactive oxygen species generated at the acceptor side of PSII contribute to the irreversible inactivation of oxygen evolving PSII during illumination (1, 11). However, photoinhibition of non-oxygen evolving PSII is independent of molecular oxygen (3). According to that, our results seem to indicate that photoinhibition of Cu(II)-inhibited PSII occurs as in PSII preparations that evolve oxygen, at least under the experimental conditions used in this work. In addition, no differences in the D1 protein degradation were observed between both Cu(II)-treated and nontreated PSII samples, which in turn was slower than that in manganese-depleted PSII preparations. It has been described that using similar illumination conditions to those in our experiments, photoinhibition results in a greater inactivation of PSII preparations that evolve oxygen, at least under the experimental conditions used in this work. An additional inactivation process occurs when the donor side of PSII is unable to supply electrons to P680 leading to an accumulation of photoinhibition of O₂-evolving thylakoids (52). As shown in Fig. 8A, catalase addition did not significantly modify the time course of photoinhibition in control PSII membranes; however, it did protect in the Cu(II)-treated samples (~30% inactivation compared with ~100% inactivation with no catalase). Thus, these results further implicate superoxide in the photoinactivation mechanism and give evidence that hydroxyl radicals are formed via Cu(II)-catalyzed Haber-Weiss reaction and contribute to the photoinhibitory mechanism. As a control we measured the oxygen evolution activity of PSII membranes in the presence of a hydroxyl radical generating system (Fig. 8B). Hydroxyl radicals were produced in vitro from Fenton’s reaction (48) using 200 μM FeSO₄ and 600 μM H₂O₂. The similarities between the inactivation rates in the presence of Cu(II) and the hydroxyl radical generating system (Fig. 8B) further support this conclusion. The finding that SOD only recovered 50% of the lost activity in the non-oxygen evolving PSII preparations (Fig. 8A) and gave the maximal protection in the presence of Cu(II) (Fig. 8B) further supports this conclusion. The finding that SOD only recovered 50% of the lost activity in the nontreated samples (Fig. 8A) and gave the maximal protection in the presence of Cu(II) (Fig. 8B) further supports this conclusion. The finding that SOD only recovered 50% of the lost activity in the nontreated samples (Fig. 8A) and gave the maximal protection in the presence of Cu(II) (Fig. 8B) further supports this conclusion. The finding that SOD only recovered 50% of the lost activity in the nontreated samples (Fig. 8A) and gave the maximal protection in the presence of Cu(II) (Fig. 8B) further supports this conclusion.
of abnormally long-lived oxidizing radicals (i.e. P680^+, TyrZ^−) on the donor side of PSII (3). Recently, a preferential Cu(II) inhibition of the donor side compared with the acceptor side was reported (32, 33). These authors proposed that Cu(II) binds to TyrZ, so the electron transfer to P680 becomes blocked as is the case for manganese-depleted PSII membranes. However, in these conditions we observed a much faster D1 degradation compared with what happened in Cu(II)-treated samples (Fig. 2). The findings indicated that at least Cu(II)-inhibited PSII membranes did not behave as a typically donor side-damaged PSII. In such case, a protective secondary electron donor that could reduce the photoinduced positive charges on the donor side, which inhibited the additional D1 degradation, should be postulated. Jegerschöld et al. (32) have in fact reported that a new electron donor instead of TyrZ\(^-\) appears in the Cu(II)-inhibited samples using EPR spectroscopy. However, no Chl or carotenoid radicals with a lifetime longer than 1 ms could be detected by flash absorption measurements (33). If we assume a model for Cu(II)-inhibited RCs where Cu(II) blocks TyrZ oxidation and an alternative electron donor reducing P680 appears, then D1 degradation differences between a non-treated and Cu(II)-treated non-oxygen evolving PSII (i.e. manganese-depleted PSII membranes) should be observed. However, no such differences were found. Besides no restoration by DPC of the activity in the Cu(II)-treated samples was measured. Considering that DPC is an artificial electron donor that restores the PSII activity in donor side photoinhibited samples (26), our data suggested that the site affected during illumination must be located after the DPC donation site. On the other hand, it is noteworthy that the experimental conditions used in other recent works (32, 33, 53) are quite different to those used in this report and in our previous works (28–31). Particularly, the Cu(II) concentrations used in our measurements (Cu(II)/RC = 250) were much lower than those used by these authors (Cu(II)/RC = 1,000–1,500).

The importance of Cu(II) concentration to evaluate the effects of Cu(II) inhibition is also shown in Cyt b\(559\) photoreduction experiments. In our experimental conditions, Cyt b\(559\) was functional, and the ratio Cyt\(\text{red}/\text{Cyt}^{2+}\) was not modified in the presence of CuCl\(_2\) in contrast to the results of Jegerschöld et al. (32). Light-induced difference spectra in the 540–585-nm range and kinetics of Cyt b\(559\) at 560 nm were drastically modified in Cu(II)-treated samples compared with those of intact preparations. Copper treatment inhibited the photoreduction of Cyt b\(559\). Buser et al. (24) proposed that the reducing equivalent for Cyt b\(559\) reduction must come from the electron acceptor side of PSII. They suggested reduced Qh as the direct source of reductant to oxidize Cyt b\(559\). The fact that in the presence of Cu(II) no reduction of Cyt b\(559\) is observed after 20 s illumination indicates that this ion may modify the reducing side of PSII. We also measured a slower rate of Cyt b\(559\) reduction in Cu(II)-inhibited samples as reported by these authors in PSII membranes inhibited by 3-(3,4-dichlorophenyl)-1,1-dimethylurea (2). In addition to Cu(II) effect on the photoreduction of Cyt b\(559\), the data showed a second Cu(II) effect at higher concentrations (>50 μM, Cu(II)/RC = 185) which provides the photooxidation of Cyt b\(559\). In non-treated Q\(_{\text{ECC}}\)-evolving PSII membranes, the rate of electron transfer from Y\(_{\text{a}}\) to P680\(^-\) is several orders of magnitude faster than the rate of electron donation from Cyt b\(559\) to P680\(^-\), and the quantum yield of Cyt b\(559\) oxidation by P680\(^-\) is very low. However, in inactivated Q\(_{\text{ECC}}\)-evolving complexes the Y\(_{\text{a}}\) to P680\(^-\) electron donation kinetics is significantly slowed down and the oxidation of Cyt b\(559\) accelerated (24). Assuming that P680\(^-\) is the oxidant to Cyt b\(559\), our results are consistent with the suggestion that in the conditions of excess of Cu(II) the P680\(^-\) formation is stabilized and a faster rate of Cyt b\(559\) photooxidation should occur as is the case in non-oxygenic (i.e. NH\(_2\)OH-treated) PSII membranes (24). This finding corresponds well with data published by Schröder et al. (33) where higher concentrations of Cu(II) (Cu(II)/RC = 400–2300) were used and they would correspond with a donor side Cu(II) inhibition. On the other hand, the absorption spectral changes during illumination suggest that a significant protection against pigment photobleaching was achieved in the presence of Cu(II) as occurs when the acceptor side is protected by some electron acceptors (13). Addition of the artificial acceptor silicomolybdate, which takes electrons close to the Pheo-Q\(_{\text{A}}\) domain (7), delays P680 photodamage at expense of 670 nm Chls and inhibits P680 triplet formation (13). Recently, we have measured the influence of Cu(II) inhibition on the primary reactions of PSII electron transport (31). The results indicated that Cu(II) strongly impaired the formation of a relaxed radical pair probably by neutralizing the negative charge on QA and eliminating the repulsive interaction between Pheo\(^+\) and QA and/or by modifying the general dielectric properties of the protein region surrounding these cofactors. A similar charge compensating effect has been observed upon double reduction of QA and subsequent protonation (54). However, in our system the decrease of the free energy difference for the radical pair relaxation was even stronger than that reported by Vass et al. (54). Based on these findings we postulated that a close attractive interaction between Pheo\(^-\) and Cu\(^{2+}\) can occur. In addition, the fluorescence decay from Cu(II)-inhibited PSI centers did not show a long-lived component in the range of about 10 ns which is characteristic of a double reduction of QA (31, 54), a phenomenon that facilitates the light-induced formation of a \(^5\)P680 due to charge recombination P680\(^-\)/Pheo\(^-\) (11). According to these results no higher production of \(^3\)P680 should be expected in the presence of Cu(II). This prediction is consistent with our singlet oxygen measurements where a lower yield of this oxygen species was detected during illumination in the presence of Cu(II) compared with the control. However, the findings of a lower photobleaching at 680 nm (Fig. 4) and a lower yield of singlet oxygen production (Fig. 6) are in agreement with the fact that Cu(II) may act in the acceptor side. Schröder et al. (33) reported that Cu(II) inhibition induced a shift of the P680\(^+\) reduction from ns to μs time domain, much slower than in the case of NH\(_2\)OH-treated PSII. This phenomenon would lead to the accumulation of long-lived oxidizing radicals on the donor side and irreversible photobleaching of the accessory Chls absorbing at 670 nm during illumination, finally promoting a faster final P680 damage. However, the presence of Cu(II) during illumination prevented the blue shift of the long wavelength absorption band in both PSII membranes and OECC compared with that of NH\(_2\)OH-treated PSII samples (data not shown). In Cu(II)-treated samples the carotenoid bleaching was much more rapid than the Chl loss. This phenomenon can be achieved by (a) a direct interaction with Chl triplets or (b) a direct interaction with a donor side Cu(II) where the triplet yield would be lower than that in intact samples, the second possibility is in accordance to our results.

Previous reports are in favor of the importance of oxygen radicals in photoinhibition, and it is known that superoxide radical and hydrogen peroxide can be generated by reactions with molecular oxygen in the acceptor side of PSII from oxidation of the quinones electron acceptors (52, 55, 56). Our experiments using EPR spin traps and oxygen radical scavengers suggest that photodamage in Cu(II)-treated PSII membranes is dominated by \(\cdot\)OH which is produced from superoxide via
Cu(II)-catalyzed Haber-Weiss reaction. According to that Cu(II) reduces the P680 triplet formation in our experimental conditions, it is possible that the accumulation of reducing components at the acceptor side promotes the formation of superoxide species that yields hydroxyl radicals in Cu(II)-treated samples. Recently, in non-oxygen-evolving PSII has been proposed that superoxide (57) and hydroxyl (58) radicals contribute to photodamage; however, these processes were non-oxygen-dependent in contrast to our data. Cu(II) could partially affect the donor side of PSII, and a potential H2O oxidation via H2O oxidation should also be taken into account (59) as a source of ‘OH production causing PSII inactivation. However, this mechanism is also non-oxygen-dependent. Considering that ‘OH is an even more reactive species than O2 (48) and the production of active oxygen species during a donor side-induced photoinhibition does not require the presence of oxygen (58, 60, 61), we postulate that the faster inactivation of PSII in the presence of Cu(II) compared with that of nontreated samples is due to damage by hydroxyl radicals generated at the acceptor side of PSII.

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