Biochemistry **1996,** *35,* 9469−9474

Cu(II)-Inhibitory Effect on Photosystem II from Higher Plants. A Picosecond Time-Resolved Fluorescence Study†

Inmaculada Yruela,‡,§ Guido Gatzen,‡ Rafael Picorel,|| and Alfred R. Holzwarth*,‡

Max-Planck-Institut für Strahlenchemie, Stiftstrasse, 34−36, D-45470 Mülheim an der Ruhr, Germany, and Estación Experimental de Aula Dei (C.S.I.C.), Apdo. 202, E-50080 Zaragoza, Spain

Received July 20, 1995; Revised Manuscript Received May 6, 1996⊗

ABSTRACT: The influence of Cu(II) inhibition on the primary reactions of photosystem II (PSII) electron transport was studied by picosecond time-resolved fluorescence on isolated PSII membranes. The fluorescence decay from Cu(II)-inhibited PSII centers showed a dominant amplitude of a fast phase (100−300 ps) similar to PSII centers in the uninhibited "open state" and minor contributions of components around 600 ps and 2.6 ns. These data indicate efficient primary charge separation in PSII membranes incubated with Cu(II). The quantum yield of primary reactions in the inhibited PSII centers was similar to that of "open" PSII centers. Kinetic analysis of the decay curves in the framework of the exciton/radical pair equilibrium model showed no significant changes in the rate constants associated with the charge separation/recombination equilibrium. However, in closed centers (Q_A reduced), a decrease in the rate constant k_{23}, associated with the back-reaction of a relaxed radical pair, by a factor of 4 was calculated. The free energy losses upon primary charge separation (ΔG_1) and during subsequent radical pair relaxation (ΔG_2) were also determined in Cu(II)-inhibited centers and were compared with uninhibited centers. No changes in the ΔG_1 values and a significant decrease in the ΔG_2 values were found as compared with those of control PSII centers in the "closed" state. These data indicate that Cu(II) does not affect primary radical pair formation, but strongly affects the formation of a relaxed radical pair, by neutralizing the negative charge on Q_A^- and eliminating the repulsive interaction between $Pheo^-$ and Q_A^- and/or by modifying the general dielectric properties of the protein region, surrounding these cofactors. Moreover, a close attractive interaction between $Pheo^-$, Q_A^-, and Cu^{2+} can be proposed. Our results are in good agreement with very recent EPR results indicating an additional effect of Cu^{2+} on the acceptor side [Jegerschöld et al. (1995) *Biochemistry 34*, 12747−12758].

Photosynthetic organisms perform the conversion of light into chemical energy in their reaction centers. In photosystem II (PSII),[1] this process leads to the light-driven oxidation of water and transfer of the liberated electrons to the membrane-soluble plastoquinone pool. Light absorbed by antenna complexes excites the primary electron donor, P680, whose subsequent photooxidation leads to the formation of the primary radical pair $P680^+Pheo^-$. In PSII membranes with typically 200−250 Chl/P680 (Bowlby et al., 1988), this process occurs in several hundreds of picoseconds (Holzwarth et al., 1985; Schatz et al., 1987). In "open" PSII centers, the primary radical pair is stabilized by transferring the electron from the primary electron acceptor, $Pheo^-$, to the primary quinone acceptor, Q_A, within about 300−500 ps (Nuijs et al., 1986; Schatz et al.,1988; Trissl & Leibl, 1989). However, if Q_A is already reduced ("closed" PSII centers), the charge-separated state $P680^+Pheo^-Q_A^-$ cannot transfer its electron from $Pheo^-$ to Q_A^-, and the decay of the photoinduced radical pair $P680^+Pheo^-$ occurs *via* several channels, including charge recombination to the ground state and/or to singlet excited states as well as triplet radical pair formation (Roelofs & Holzwarth, 1990).

It is known that some heavy metals, among which Cu(II) is the most effective, inhibit the photosynthetic electron transport in higher plants (Clijsters & Van Asche, 1985). In earlier reports, the donor side of PSII (Cedeno-Maldonado et al., 1972; Shioi et al., 1978a,b; Bohner et al., 1980; Samuelson & Öquist, 1980; Vierke & Struckmeier, 1977; Renger et al., 1993), the cytochrome b_6/f complex (Singh & Singh, 1987), or charge separation (Hsu & Lee, 1988) have been reported as possible Cu(II)-inhibitory sites [for a review, see Baron et al. (1995)]. The paper of Renger et al. (1993) also reported an additional effect of Cu(II) on atrazine binding, *i.e.,* an acceptor side effect.

Recently, we have made an extensive study on this subject and particularly on the Cu(II)-inhibitory effect (Yruela et al., 1991), its inhibitory mechanism (Yruela et al., 1992), and the location of the Cu(II)-binding site (Yruela et al., 1993). Our work suggested that Cu(II) impairs the photosynthetic electron transport on the acceptor side between Pheo and Q_A and that Cu(II) would bind to an amino acid(s) which is (are) necessary for the electron transport between

† I.Y. was supported by a postdoctoral fellowship from the European Union (Human Capital and Mobility Program). This project has also been supported in part by the Sonderforschungsbereich 189 (Heinrich-Heine-Universität Düsseldorf and Max-Planck-Institut für Strahlenchemie, Mülheim a.d. Ruhr).

* Author to whom correspondence should be addressed.

‡ Max-Planck-Institut für Strahlenchemie.

§ Present address: Estación Experimental de Aula Dei (C.S.I.C.), Apdo. 202, E-50080 Zaragoza, Spain.

|| Estación Experimental de Aula Dei.

⊗ Abstract published in *Advance ACS Abstracts*, June 15, 1996.

[1] Abbreviations: Chl, chlorophyll; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; MES, 4-morpholineethanesulfonic acid; Pheo, pheophytin; PSII, photosystem II; P680, primary electron donor of PSII; Q_A, first quinone acceptor of PSII; Q_B, second quinone acceptor of PSII; RC, reaction center.

S0006-2960(95)01667-9 CCC: $12.00

Pheo and Q_A (Yruela et al., 1993). However, despite all of these studies, the Cu(II)-inhibitory mechanism remains unclear, and the precise localization of the primary target of the Cu(II)-binding site is still a matter of debate. Based on the study of P680$^+$ reduction in the microsecond time range, it has been proposed that Cu(II) modifies Tyr_z and thus the electron transport between Tyr_z and P680 is blocked (Schröder et al., 1994). However, these measurements give only a partial view of the dynamics of the photosystem II reaction center because they do not provide information on the primary processes on the reducing side of PSII. More recently, EPR studies have shown indeed that copper in addition has inhibitory effects also on the acceptor side (Jegerschöld et al., 1995).

The processes of charge separation, charge stabilization, and charge recombination in PSII have been studied by several groups using laser spectroscopy in the nanosecond and picosecond time ranges (Klimov & Krasnovskii, 1982; Karukstis & Sauer, 1983; Holzwarth, 1986, 1987), and time-resolved Chl fluorescence has been proven to be a powerful tool for the determination of the rate constants of these early electron transfer processes. In order to better understand the influence of Cu(II) inhibition on the electron transfer kinetics in PSII, we report here picosecond time-resolved fluorescence measurements in PSII preparations treated with $CuCl_2$ in both "open" and "closed" states.

MATERIALS AND METHODS

PSII Membrane Isolation. PSII membranes from spinach thylakoids were prepared according to Berthold et al. (1981) with the modifications described by Van Leeuwen et al. (1991) and were stored at −80 °C in 0.4 mM sucrose, 15 mM NaCl, 5 mM $MgCl_2$, and 20 mM Mes−NaOH (pH 6.5). The Chl concentration was determined as described by Arnon (1949).

Inhibition with Cu(II). PSII membranes (10 μg of Chl/mL) in 15 mM NaCl, 5 mM $MgCl_2$, and 20 mM Mes−NaOH (pH 6.5) were preincubated with various concentrations of $CuCl_2$ for 10 min at 4 °C (Yruela et al., 1991).

Time-Resolved Fluorescence Measurements. Fluorescence decay kinetics were measured with the single photon timing technique, using the apparatus described previously (Wendler & Holzwarth, 1987). The sample was excited at 651 nm by laser pulses with full width at half-maximum <15 ps at a repetition rate of 800 kHz. The fluorescence was selected by a double monochromator (spectral bandwidth 4 nm) and detected by a R2809U-07 MCP-photomultiplier (Hamamatsu, Iwata-gun, Japan). The resolution of the time-to-amplitude converter was 10 ps/channel. After deconvolution of the decay curves with the system response function, a time resolution of better than 20 ps could be achieved.

Samples for the fluorescence decay measurements were diluted to 10 μg of Chl/mL at 4 °C. The "open" state of control samples was maintained by dark adaptation combined with sufficiently low excitation density (E < 0.2 μJ/cm^2 per pulse) and a high sample pumping rate of 500 mL/min through a flow cuvette (cross section of 1.5 × 1.5 mm). For measurements in the "closed" state, samples were preincubated with 5 μM DCMU before the treatment with $CuCl_2$ and were then preilluminated with weak white light before entering the measuring cuvette. The fluorescence decay curves were measured at 680 and 685 nm with 10 ps/channel time.

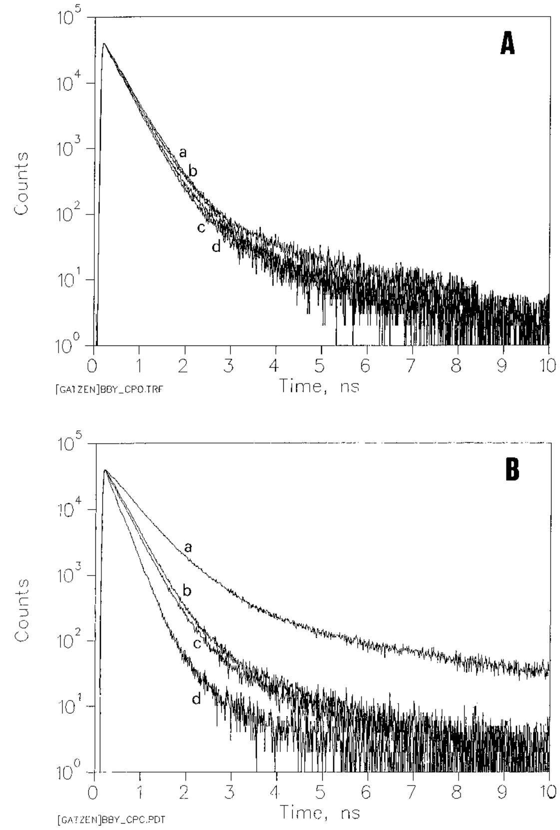

FIGURE 1: Effect of Cu(II) treatment on the fluorescence decay kinetics of PSII membranes. The fluorescence decays were measured in the open (A) and the closed (B) states after incubation with (a) 0 μM, (b) 5 μM, (c) 20 μM, and (d) 100 μM $CuCl_2$. All decay curves were accumulated up to 50 000 counts at the peak channel.

Data Analysis. The fluorescence decay data were analyzed by global lifetime and global target analysis as described by Roelofs et al. (1992). This included data sets measured at two different wavelengths and time resolution of 10 ps/channel. The data were fitted over a window of 10 ns for the control and Cu(II)-treated samples. The relative yield was calculated by $\langle \tau_{av} \rangle = \sum a_i \tau_i / \sum a_i$ (a_i is the relative amplitude of component i and τ_i is its lifetime in nanoseconds). Due to normalization to the total amplitude ($\sum a_i$), this expression gives the average lifetime (in nanoseconds) of the decay, which is proportional to the fluorescence yield (Roelofs et al., 1992).

RESULTS

The influence of Cu(II) inhibition on the fluorescence decay kinetics in the picosecond time range was studied in isolated PSII membranes. Fluorescence decays in "open" (F_o) and "closed" (F_{max}) PSII centers were recorded at 680 and 685 nm with excitation at 651 nm. In the "open" state of the control PSII centers, *i.e.,* when Q_A is oxidized, the electron transport rapidly quenches fluorescence, and F_o fluorescence was dominated by fast decaying components and disappeared within 3−5 ns (Figure 1A). However, a long fluorescence lifetime component of 3−4 ns was observed in closed PSII centers (Figure 1B) due to the fact that excitation energy cannot be used to drive photosynthetic electron transport via the reduction of Q_B. The fluorescence decay curves in Cu(II)-inhibited centers were similar to those of "open" control PSII centers (Figure 1A,B).

Biochemistry, Vol. 35, No. 29, 1996 9471

Table 1: Influence of Cu(II) Inhibition on the Fluorescence Decay Lifetimes (τ_i) and the Relative Amplitudes (a_i) in Isolated PSII Membranes[a]

sample	τ_1, ns (a_1, %)	τ_2, ns (a_2, %)	τ_3, ns (a_3, %)	τ_4, ns (a_4, %)	average lifetime, $\langle\tau_{av}\rangle$ (ns)
control					
open	0.07 (30)	0.28 (59)	0.52 (11)	2.7 (0.1)	0.24
closed	0.10 (22)	0.40 (47)	0.80 (30)	3.9 (0.5)	0.50
Cu(II)-treated (open)					
5 μM	0.06 (27)	0.25 (60)	0.46 (12)	2.4 (0.1)	0.22
20 μM	0.06 (30)	0.24 (58)	0.45 (11)	2.2 (0.09)	0.21
100 μM	0.07 (21)	0.23 (57)	0.46 (21)	2.2 (0.08)	0.24
Cu(II)-treated (closed)					
5 μM	0.10 (35)	0.30 (58)	0.52 (7)	2.1 (0.1)	0.24
20 μM	0.10 (40)	0.30 (57)	0.66 (2)	2.6 (0.06)	0.22
100 μM	0.08 (45)	0.21 (54)	0.65 (0.6)	4.2 (0.01)	0.15

[a] The fluorescence decay curves were measured at 680 and 685 nm with 10 ps/channel time resolution. The lifetimes (ns) and the relative amplitudes (%) were obtained with a global lifetime fit at the two wavelengths, by using a 10 ns window.

In order to characterize more quantitatively the fluorescence decay kinetics, a global kinetic analysis procedure was applied. First the decay curves were analyzed in terms of a sum of exponential components. To achieve good fits, at least four components were necessary to describe the fluorescence kinetics adequately, as judged from the weighted residual plots and the χ^2 values (not shown). Deconvolution with only three components resulted in much less satisfactory fits in both open and closed states. The calculated lifetimes and relative amplitudes (Table 1) confirmed the qualitative observations from Figure 1. In the "open" state of PSII, the decay of the F_o fluorescence was dominated by two fast components of about 70 and 280 ps. A smaller contribution from a 520 ps component and an almost negligible amount of a 2−3 ns component were also presented. After closing the centers by a single reduction of Q_A, the decay was dominated by the 400 and 800 ps components. The relative fluorescence yield for "closed" PSII centers was 2 times higher than those in the "open" state, which is quite typical for isolated PSII particles. In the Cu(II)-inhibited samples, the most prominent component of the decay had a lifetime of about 210−300 ps, similar to that of "open" centers. In addition, smaller contributions from 650 ps and 2.6 ns components were calculated.

We further analyzed the results in the framework of a model for the excited state dynamics in photosystem II, called the exciton/radical pair equilibrium model (Schatz et al., 1988; Schatz & Holzwarth, 1986) which has been proven to properly describe the primary processes in PSII. This model assumes that P680 constitutes a shallow trap for the excitation energy which equilibrates over the antenna system and P680 much faster than primary charge separation occurs. Thus, the excitation decay is limited by the rate of the primary charge separation (trap-limited quenching). The primary radical pair is in equilibrium with P680* and the excited antenna. In its simplest form the model predicts biexponential fluorescence decay for the equilibrated (Chl antenna/P680)* state. The faster decaying fluorescence component reflects mainly the process of primary charge separation and its reversal back to the excited state, whereas the slower component reports primarily on the charge stabilization process (in "open" centers), or the relaxation of the radical pair (in "closed" centers) (see model in Figure 2).

The results of this analysis are summarized in Tables 2 and 3 which include also the free energy changes, ΔG, between the states involved as calculated from the obtained rate constants according to Schatz et al. (1988) by assuming equilibrium kinetics. Due to the problem in determining the rate constants k_{11} and k_{21} simultaneously, we did two different analyses by fixing either k_{11} or k_{21} and calculating k_{21} or k_{11}, respectively. These calculations were done for both the "open" (Table 2) and the "closed" (Table 3) states of PSII with and without added Cu(II). The data showed no major changes in the rate constant values for the "open" state of Cu(II)-inhibited PSII centers as compared with those of the control (Table 2) except for small changes in rates k_{32} and k_{23} related to P680$^+$Pheo$^-$ radical pair relaxation and a ca. 20% increase in the rate of Pheo$^-$ to Q_A transfer (rate k_{33}). For the "closed" state, the rate constant values associated with the charge separation equilibrium (k_{12}, k_{21}) were practically constant when k_{21} was fixed. However, significant changes in k_{32} and k_{23} values, associated with the formation and back-reaction of the relaxed radical pair, were found (Table 3). Particularly, the rate constant k_{23} (see model Figure 2) decreased by a factor of 4. It is interesting to note that a different result was obtained when the rate constant k_{11} was fixed. In that case, smaller values for k_{12} in the Cu(II)-inhibited centers were obtained. This dependence could in principle be explained by a model where Cu(II) affects the primary charge separation equilibrium. This latter interpretation can be ruled out, however, if we take into account the data obtained for the Q_A oxidized (open) state, where no changes in the rate constants associated with this equilibrium were found.

The data also showed an about 14 meV and 84 meV free energy loss upon charge separation (ΔG_1) and during the subsequent radical pair relaxation (ΔG_2), respectively, in control "open" PSII membranes. These values of ΔG_1 and ΔG_2 are in good agreement with recent data from PSII membranes (Schatz et al., 1988) and thylakoids (Roelofs et al., 1992). In the "open" state, Cu(II)-inhibited PSII centers were characterized by similar ΔG_1 and ΔG_2 values as the control samples (Table 2, note that the small changes in actual numbers are considered to be within the error limits). In Cu(II)-inhibited PSII centers with Q_A reduced, no significant changes in the ΔG_1 values were observed either. However, the free energy difference values associated with radical pair relaxation (ΔG_2) decreased substantially when PSII particles were incubated with increasing Cu(II) concentrations (Table 3).

In addition to the Cu(II) effect on the rate constants of radical pair relaxation and the associated ΔG_2, the data showed a second and independent significant Cu(II) effect

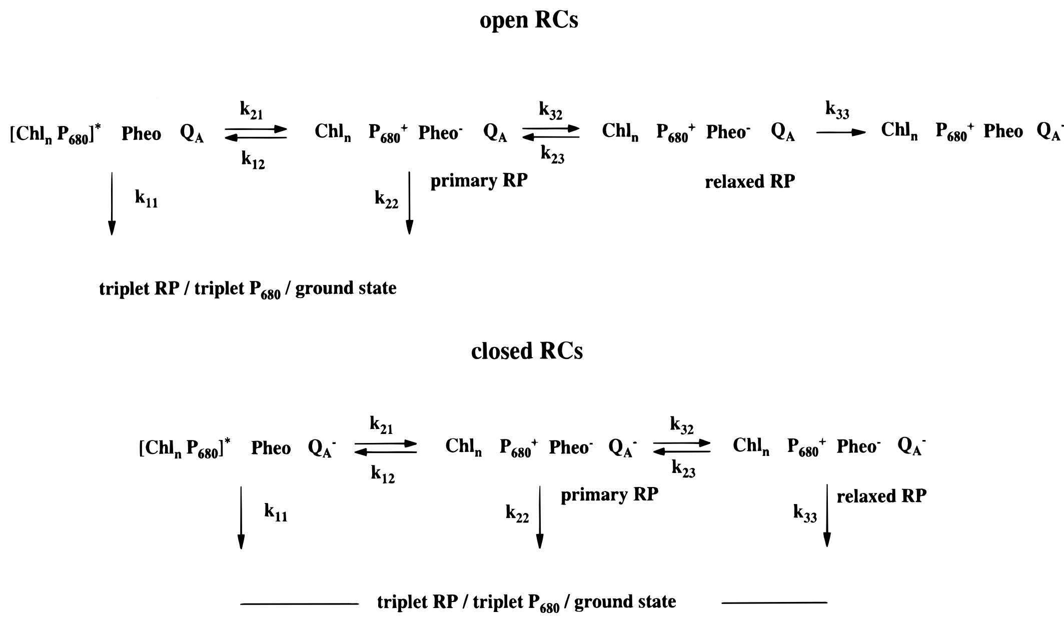

FIGURE 2: Extended exciton/radical pair equilibrium model for the primary processes in the "open" (top) and "closed" (bottom) states of PSII (Roelofs & Holzwarth, 1990). The model is characterized by seven rate constants: k_{21} is the apparent rate constant for the primary charge separation; k_{12} describes the primary radical pair (PRP) recombination back to the excited state of P*; k_{32} and k_{23} are the rate constants for the formation and back-reaction of the relaxed radical pair (RRP), respectively, in the closed state; k_{22} and k_{33} are the rate constants for the deactivation of the relaxed and primary radical pair, respectively, to the ground state and/or triplet states; k_{11} describes the radiative and nonradiative decay of excited states in the Chl anntena complex. In the open state, k_{33} stands for the electron transfer rate from Pheo$^-$ to Q_A.

Table 2: Kinetic and Thermodynamic Analysis of Fluorescence Decays from "Open" PSII Membranes Incubated with Cu(II)[a]

sample	k_{11} (ns^{-1})	k_{21} (ns^{-1})	k_{12} (ns^{-1})	k_{22} (ns^{-1})	k_{32} (ns^{-1})	k_{23} (ns^{-1})	k_{33} (ns^{-1})	ΔG_1 (meV)	ΔG_2 (meV)
control	0.30	6.11	3.35	0.00	6.41	0.19	2.09	−14.1	−83.5
$CuCl_2$[b]									
5 μM	0.30	6.54	4.69	0.00	8.31	0.29	2.47	−7.9	−79.5
20 μM	0.30	6.86	4.28	0.00	7.71	0.27	2.52	−11.3	−80.0
100 μM	0.30	5.59	3.63	0.00	8.06	0.58	2.77	−10.3	−62.7
$CuCl_2$[c]									
5 μM	0.72	6.11	5.01	0.00	8.00	0.31	2.46	−4.7	−79.5
20 μM	1.03	6.11	4.79	0.00	7.21	0.29	2.50	−5.8	−76.7
100 μM	3.00	6.11	3.53	0.00	8.01	0.54	2.76	−13.1	−64.3

[a] The kinetic analyses have been performed for two methods differing in the rate constants that were kept fixed in the analysis (see text and below). The rate constants were calculated on the basis of the kinetic model in Figure 2. ΔG_1 and ΔG_2 are the changes in free energy due to charge separation and subsequent relaxation of the radical pair, respectively, as calculated according to ref 5. [b] k_{11} was fixed in the analysis at 0.3 ns^{-1} (sum of radiative and nonradiative decay rate constants for the antenna excited states). [c] k_{21} in the control was calculated fixing k_{11} to 0.3 ns^{-1}, and then the resulting value of 6.1 ns^{-1} obtained for k_{21} was kept constant for Cu(II) inhibited samples.

Table 3: Kinetic and Thermodynamic Analysis of Fluorescence Decay in "Closed" PSII Membranes Incubated with Cu(II)[a]

sample	k_{11} (ns^{-1})	k_{21} (ns^{-1})	k_{12} (ns^{-1})	k_{22} (ns^{-1})	k_{32} (ns^{-1})	k_{23} (ns^{-1})	k_{33} (ns^{-1})	ΔG_1 (meV)	ΔG_2 (meV)
control	0.30	4.32	3.54	0.00	4.59	0.23	1.67	−4.8	−71.1
$CuCl_2$[b]									
5 μM	0.30	5.38	2.08	0.00	5.39	0.10	2.02	−22.7	−93.9
20 μM	0.30	5.76	1.94	0.00	5.18	0.08	1.57	−26.0	−100.2
100 μM	0.30	7.54	1.88	0.00	7.02	0.06	1.56	−33.2	−112.2
$CuCl_2$[c]									
5 μM	1.36	4.32	2.59	0.00	4.88	0.12	2.01	−12.2	−88.9
20 μM	1.74	4.32	2.58	0.00	4.54	0.09	1.56	−12.3	−93.6
100 μM	3.54	4.32	3.28	0.00	5.60	0.08	1.54	−6.6	−101.5

[a] The kinetic analyses have been performed for two methods differing in the rate constants that were kept fixed in the analysis (see text and below). The rate constants were calculated on the basis of the kinetic model in Figure 2. ΔG_1 and ΔG_2 are the changes in free energy due to charge separation and subsequent relaxation of the radical pair, respectively, as calculated according to ref 5. [b] k_{11} was fixed in the analysis at 0.3 ns^{-1} (sum of radiative and nonradiative decay rate constants for the antenna excited states). [c] k_{21} was calculated fixing k_{11} to 0.3 ns^{-1} in the control and then the resulting value of 4.32 ns^{-1} obtained for k_{21} was kept constant for Cu(II) inhibited samples.

at higher Cu(II) concentrations [$\geq$20 μM Cu(II)]. The rate constant ascribed to nonradiative relaxation of Chl excited states in the antenna complex (k_{11}) increased under these conditions (see Tables 2 and 3). Performing the kinetic analysis on both open and closed centers simultaneously allowed us to separate the two effects.

DISCUSSION

In the following discussion, we will focus first on the main effect of Cu(II) inhibition, *i.e.*, on the modification of the electron transfer rates in the RC. Finally, we will comment on the additional Cu(II) effect of modification of the antenna deactivation rate k_{11}.

Our previous investigations on the Cu(II)-inhibitory effect on the photosynthetic electron transport have been concentrated mainly on the characterization of the Cu(II)-binding site and the mechanism that regulates the Cu(II) inhibition in PSII (Yruela et al., 1991, 1992, 1993). We have suggested that Cu(II) affects the electron transport on the reducing side of PSII, close to the Pheo$-$$Q_A$$-$Fe domain, the cofactors which are involved in the primary photochemical reactions of PSII. In the present work, we studied in detail the influence of Cu(II) on the rate constants of electron transfer processes using picosecond time-resolved measurements. The fluorescence decay curves and the quantum yield given by Cu(II)-inhibited PSII centers were comparable with PSII centers in the "open" state, indicating that PSII particles treated with Cu(II) do still have an efficient charge separation. Our result is consistent with the work of Renger et al. (1993) and Schröder et al. (1994) but is not in agreement with our earlier interpretation of an impaired charge separation up to Q_A (Yruela et al., 1991, 1992, 1993) although it is still in agreement with our previous suggestion of an acceptor side effect of Cu(II) (Yruela et al., 1993).

To further investigate in detail which are the effects of Cu(II) on the primary electron transport reactions in the PSII, we have analyzed the picosecond fluorescence decay in the framework of the exciton/radical pair equilibrium model described by Schatz et al. (1988). This analysis provides the complete set of rate constants which describe the steps of exciton trapping from the excited antenna as well as the charge separation, charge stabilization, and charge recombination processes in the RCs. In addition, the effects of the reduction of Q_A on the primary processes can be evaluated (Schatz et al., 1988; Roelofs & Holzwarth, 1990). The rate of P680$^+$Pheo$^-$ formation is controlled by the redox state of Q_A, and this is considered to be a consequence of the electrical field created by the negative charge on Q_A and the smaller distance between Q_A^- and Pheo than Q_A^- and P680. This electrical field increases the energy content of the electrical dipole in the radical pair P680$^+$Pheo$^-$. In PSII core particles with only 80 Chl/P680, it was found that (a) charge separation in "open" RCs is exergonic and associated with a decrease in the standard free energy of 38 meV and (b) in "closed" RCs charge separation is endergonic, giving a standard free energy increase of 12 meV. Our previous work (Yruela et al., 1991, 1992, 1993, 1994) indicated that the Cu(II)-binding site is quite close to Q_A. For this reason, Cu(II) could substantially influence one or more of the rate constants of the electron transfer processes. Thus, the determination of these rate constants within the framework of the excited state/radical pair equilibrium model should be useful for a better understanding of the Cu(II)-inhibited PSII system. If a significant interaction between the electrical fields created by the negative charges on Q_A^- or Pheo$^-$ and the positive ones on Cu(II) occurs, this effect should show up in modified electron transfer rates and/or in modified rates of radical pair relaxation and would provide information on the localization and mechanistic effect of Cu inhibition.

In our experiments, Cu(II) inhibition did not affect strongly the rate constants calculated for PSII centers with oxidized Q_A except for some 20% increase in the rate for Pheo$^-$ to Q_A transfer. This means that up to Q_A the electron transfer is quite normal in Cu-treated centers. This does not support our previous suggestion that Cu(II) inhibition impairs Q_A reduction (Yruela et al., 1993). This conclusion was based on steady-state light-induced absorption measurements in the time range where several transitions such as Q_A, Q_B, and Z reduction as well as S0$-$S4 transitions contributed to the absorption change signal. Thus, this discrepancy can be resolved by the determination of detailed rate constants of the experiments described in this work.

Significant changes were found for the rate constants in the "closed" state of PSII. The presence of the fast fluorescence component (60$-$70 ps) under these conditions is again consistent with an efficient primary charge separation similar to that in "open" control centers (Table 1). The results are summarized in Tables 2 and 3 and include also the free energy changes, ΔG, determined from the rate constants according to Schatz et al. (1988). The data show about 7$-$12 meV and 89$-$102 meV free energy losses upon charge separation and during the subsequent radical pair relaxation, respectively, in "closed" PSII centers inhibited with Cu(II). The calculated values of ΔG_1, *i.e.*, the free energy difference for primary charge separation, are in good agreement with recent data from uninhibited PSII particles (Schatz et al., 1988) and thylakoids (Schatz & Holzwarth, 1986). However, the values of ΔG_2, *i.e.*, the free energy difference for radical pair relaxation, are substantially smaller than reported for control "closed" PSII centers (Roelofs et al., 1992; see also references in Clijsters & Van Asche, 1985). Overall Cu(II) increases the free energy difference as compared to the control.

It is known that the Coulombic interaction energy between Pheo$^-$ and Q_A^- in the radical pair state in PSII centers with Q_A^- reduced, which is not present when Q_A is oxidized, increases the energy content of the radical pair state P680$^+$Pheo$^-$$Q_A^-$ (Schatz et al., 1988). The fact that the presence of Cu(II) increases the value of $|\Delta G_2|$, *i.e.*, the free energy difference for radical pair relaxation of P$^+$Pheo$^-$, can be explained in such a way that Cu^{2+} neutralizes the negative charge on Q_A^- thus eliminating the repulsive interaction between the negative charges on Q_A^- and Pheo$^-$ which is responsible for the large increase in fluorescence yield upon closing PSII centers. In order to exert this effect, Cu must be bound at a site close to Q_A. A similar charge-compensating effect was observed recently upon double reduction of Q_A and subsequent double protonation (Vass et al., 1994). However, in our system, the decrease of ΔG_2 was even stronger than that reported by Vass et al. (1994) where Q_A was doubly reduced and neutral. This fact could be interpreted as an additional attractive interaction between Pheo$^-$ and the bivalent Cu^{2+} which could easily produce a very pronounced decrease of the relaxed radical pair energy. Thus, these data are consistent with our previous work that locates the Cu(II)-binding site close to the Pheo$-$$Q_A$$-$Fe domain (Yruela et al., 1993). Quite recently, flash-induced absorption spectroscopy has been used to study the effect of copper on the oxidation and rereduction of P680 (Renger et al., 1993; Schröder et al., 1994). Furthermore, the copper effect on atrazine binding to PSII has been studied (Renger et al., 1993). The localization of Cu(II) binding on the acceptor side of PSII and the effects on electron transfer and free energy differences of radical pairs as proposed here are in principal agreement with the finding of a reduced atrazine-binding constant upon Cu(II) treatment. There is also agreement between our data and the work of Schröder et al. (1994) in the finding that the primary charge separation is not affected by Cu(II) binding. On the one hand, the flash photolysis method as used in those works does not give any information on primary radical pair relaxation. For this reason, the Cu(II) effects on the acceptor side were not revealed so clearly in those studies. On the other hand, time-resolved fluorescence as applied here does not provide any information on the rate of $P680^+$ reduction. Thus, the further finding of Schröder et al. (1994) of a reduced $P680^+$ reduction rate upon Cu(II) treatment which points to an additional Cu(II)-binding site near the secondary electron donor tyrosine Z is not incompatible with our data.

More recently, EPR spectroscopy studies on Cu(II) inhibition have shown for the first time that Cu(II) has inhibitory effects on the acceptor side of PSII (Jegerschöld et al., 1995). They observed that Q_A can be reduced by illumination or chemical reduction; however, treatment with copper results in the loss of the normal EPR signal from Q_A^- which is coupled to the non-heme Fe(II), and the formation of a free radical signal which is attributed to Q_A^- decoupled from the non-heme iron. These results of Jegerschöld et al. (1995) are in good agreement with our data. However, we measure the Cu^{2+} effects on the energy of radical pairs, while the EPR study provides direct information on the modified coupling of Q_A^- to its environment upon Cu^{2+} binding. In summary, we thus propose that all effects, *i.e.*, (i) decreased atrazine binding (Renger et al., 1993), (ii) suppression of magnetic coupling between high-spin Fe^{2+} and Q_A^-, and (iii) the effect on radical pair relaxation (present study), might originate from a particular modification of the PSII acceptor side by Cu(II). Probably all three studies describe complementary facets of the same underlying effect.

The pronounced changes in k_{11} or alternatively k_{12} values (see the two alternative analyses in Table 3), as observed only in "closed" PSII centers inhibited with Cu(II), indicate an additional Cu(II) effect on the antenna system or on the primary charge separation. The second possibility, *i.e.*, modification of k_{12}, can be ruled out due to the fact that no such pronounced effect on that rate constant (k_{12}) was found in the "open" PSII centers inhibited with Cu(II) (Table 2). Thus, we discard the analysis with fixed k_{11} values. In order to explain the drastic k_{11} changes at high copper concentrations, we thus have to conclude that there must exist a further, perhaps unspecific, Cu(II)-binding site in the antenna complex of PSII. The binding constant of this site must be substantially smaller than the one(s) for binding in the reaction center, as can be deduced from the fact that only at concentrations $\geq 20\ \mu$M does this effect start to become significant and it still increases at 100 μM Cu(II). This quenching effect of Cu on Chl excited states can be explained easily in terms of the heavy-atom effect of Cu which increases strongly the intersystem crossing rate from the singlet to the triplet state when it is located very close to an antenna Chl. We finally note that most previous studies of Cu(II) inhibition applied Cu(II) concentrations chosen to our maximal value or higher. Thus, the antenna quenching effect should have been quite pronounced in most previous studies and should be taken into accout when interpreting the data.

ACKNOWLEDGMENT

We thank Michael Reus for able technical assistance.

REFERENCES

Arnon, D. I. (1949) *Plant Physiol. 24*, 1−15.
Baron, M., Arellano, J. B., & Lopez-Gorge, J. (1995) *Physiol. Plant. 94*, 174−180.
Berthold, D. A., Babcock, G. T., & Yocum, C. F. (1981) *FEBS Lett. 134*, 231−234.
Bohner, H., Böhme, H., & Bögner, P. (1980) *Biochim. Biophys. Acta 592*, 103−112.
Bowlby, N. R., Ghanotakis, D. F., Yocum, C. F., Petersen, J., & Babcock, G. T. (1988) in *Light Transduction in Photosynthesis: Higher Plant and Bacterial Models* (Stevens, S. E., Jr., & Bryant, D. A., Eds.) pp 215−226, The American Society of Plant Physiologists, Rockville, MD.
Cedeno-Maldonado, A., & Swader, J. A. (1972) *Plant Physiol. 50*, 698−701.
Clijsters, H., & Van Asche, F. (1985) *Photosynth. Res. 7*, 731−740.
Holzwarth, A. R. (1986) *Photochem. Photobiol. 43*, 707−725.
Holzwarth, A. R. (1987) *Top. Photosynth. 8*, 95−157.
Holzwarth, A. R., Wendler, J., & Haehnel, W. (1985) *Biochim. Biophys. Acta 807*, 155−167.
Hsu, B.-D., & Lee, J.-Y. (1988) *Plant Physiol. 87*, 116−119.
Jegerschöld, C., Arellano, J. B., Schröder, W. P., Van Kan, P. J. M., Baron, M., & Styring, S. (1995) *Biochemistry 34*, 12747−12758.
Karukstis, K. K., & Sauer, K. (1983) *J. Cell. Biochem. 23*, 131−158.
Klimov, V. V., & Krasnovskii, A. A. (1982) *Biophysics 27*, 186−198.
Nuijs, A. M., van Gorkom, H. J., Plijter, J. J., & Duysens, L. N. M. (1986) *Biochim. Biophys. Acta 848*, 167−175.
Renger, G., Gleiter, H. M., Haag, E., & Reifarth, F. (1993) *Z. Naturforsch. 48C*, 234−240.
Roelofs, T. A., & Holzwarth, A. R. (1990) *Biophys. J. 57*, 1141−1153.
Roelofs, T. A., Lee, C. H., & Holzwarth, A. R. (1992) *Biophys. J. 61*, 1147−1163.
Samson, G., Morissette, J.-C., & Popovic, R. (1988) *Photochem. Photobiol. 48*, 329−332.
Samuelson, G., and Öquist, G. (1980) *Plant Cell Physiol. 21*, 445−454.
Schatz, G. H., & Holzwarth, A. R. (1986) *Photosynth. Res. 10*, 309−318.
Schatz, G. H., & Holzwarth, A. R. (1987) in *Progress in Photosynthesis Research* (Biggins, J., Ed.) Vol. 1, pp 67−69, Martinus Nijhoff Publishers, Dordrecht, The Netherlands.
Schatz, G. H., Brock, H., & Holzwarth, A. R. (1988) *Biophys. J. 54*, 397−405.
Schröder, W. P., Arellano, J. B., Bittner, T., Baron, M., Eckert, H. J., & Renger, G. (1994) *J. Biol. Chem. 269*, 32865−32870.
Shioi, Y., Tamai, H., & Sasa, T. (1978a) *Physiol. Plant. 44*, 434−438.
Shioi, Y., Tamai, H., & Sasa, T. (1978b) *Plant Cell Physiol. 19*, 203−209.
Singh, D., & Singh, S. (1987) *Plant Physiol. 83*, 12−14.
Trissl, H. W., & Leibl, W. (1989) *FEBS Lett. 244*, 85−88.
Van Leeuwen, P. J., Nieveen, M. C., Van de Meent, E. J., Dekker, J. P., & Van Gorkom, H. J. (1991) *Photosynth. Res. 28*, 149−153.
Vass, I., Gatzen, G., & Holzwarth, A. R. (1994) *Biochim. Biophys. Acta 1183*, 388−396.
Vierke, G., & Struckmeier, P. (1977) *Z. Naturforsch., C: Biosci. 32*, 605−610.
Wendler, J., & Holzwarth A. R. (1987) *Biophys. J. 52*, 717−728.
Yruela, I., Montoya, G., Alonso, P. J., & Picorel, R. (1991) *J. Biol. Chem. 266*, 22847−22850.
Yruela, I., Montoya, G., & Picorel, R. (1992) *Photosynth. Res. 33*, 227−233.
Yruela, I., Alfonso, M., Ortiz de Zarate, I., Montoya, G., & Picorel, R. (1993) *J. Biol. Chem. 268*, 1684−1689.

BI951667E