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# The inhibitory mechanism of Cu(II) on the Photosystem II electron transport from higher plants

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**Abstract**

In a previous paper, we reported that Cu(II) inhibited the photosynthetic electron transfer at the level of the pheophytin-$Q_A$-Fe domain of the Photosystem II reaction center. In this paper we characterize the underlying mechanism of Cu(II) inhibition. Cu(II)-inhibition effect was more sensitive with high pH values. Double-reciprocal plot of the inhibition of oxygen evolution by Cu(II) is shown and its corresponding inhibition constant, $K_i$, was calculated. Inhibition by Cu(II) was non-competitive with respect to 2,6-dichlorobenzoquinone and 3-(3,4-dichlorophenyl)-1,1-dimethylurea and competitive with respect to protons. The non-competitive inhibition indicates that the Cu(II)-binding site is different from that of the 2,6-dichlorobenzoquinone electron acceptor and 3-(3,4-dichlorophenyl)-1,1-dimethylurea sites, the $Q_B$ niche. On the other hand, the competitive inhibition with respect to protons may indicate that Cu(II) interacts with an essential amino acid group(s) that can be protonated or deprotonated in the inhibitory-binding site.

*Abbreviations:* BSA – bovine seroalbumin; Chl – chlorophyll; DCBQ – 2,6-dichlorobenzoquinone; DCMU – 3-(3,4-dichlorophenyl)-1,1-dimethylurea; MES – 2-(N-morpholino)-ethanesulphonic acid; Pheo – pheophytin; $Q_A$ – primary quinone acceptor; $Q_B$ – secondary quinone acceptor; PS – Photosystem; RC – reaction center; Tricine – N-[Tris(hydroxymethyl)-methyl]-glycine

## Introduction

Certain herbicides are known to inhibit the photosynthetic electron transport. Among those, the 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) has widely been used in photosynthesis studies. A precise location of the inhibitory-binding site has been established for DCMU in plants (Lavergne 1982, Trebst 1987). This herbicide inhibits the electron transport on the reducing side of the Photosystem (PS) II at the level of $Q_B$ site (Pfister et al. 1981, Pfister and Schreiber 1983, Diner and Petrouleas 1987). A precise determination of the number of specific DCMU-binding sites was done by Tischer and Strotmann (1977) and Kaplanová and Szigeti (1985). They proposed that chloroplasts bound DCMU at sites which were approximately equimolar with the Photosystem II reaction center (PS II RC).

Photosynthetic organisms are also very sensitive to heavy metals. This effect is at present very important for the ecosystems, considering the increasing problem of ambiental pollution. Among heavy metals, copper inhibits the photosynthetic electron transport and its effect has been studied in higher plants (Mohanty et al. 1989, Hsu and Lee 1988), green algae (Samson et al. 1988) and cyanobacteria (Singh and Singh 1987). Despite these studies the location of

Cu(II)-binding site in the photosynthetic electron transport chain and the underlying inhibitory mechanism are as yet unclear. In a previous paper (Yruela et al. 1991) we have shown that oxygen evolution by Photosystem II membranes was inhibited by Cu(II) when DCBQ or ferricyanide, but not silicomolibdate, was used as electron acceptor. This indicated that Cu(II) affected the reducing side of Photosystem II. The paper also indicated, by using trypsin-treated thylakoids that Cu(II)-inhibitory site is located before $Q_B$-binding site and close to the pheophytin (Pheo)-$Q_A$-Fe domain of the PS II RC.

In the present paper we characterize the Cu(II)-inhibitory mechanism based on measurements of oxygen evolution by PS II membranes from sugar beet using DCBQ as electron acceptor. Data suggest that Cu(II) has a specific inhibitory-binding site and that its inhibitory mechanism is non-competitive with respect to DCBQ and DCMU, and competitive with respect to $H^+$.

## Materials and methods

### *Biological material*

Sugar beet (*Beta vulgaris* cv. Monohill) was grown hydroponically in a growth chamber in half-Hoagland nutrient solution, under 400 $\mu$E $m^2 s^{-1}$ from fluorescent lamps at 25 °C, 80% humidity and a 16 h photoperiod.

### *PS II membrane isolation*

PS II membranes with high rate of oxygen evolution were prepared by the method of Berthold et al. (1981), with some modifications. The chloroplast pellet was centrifuged at 13 350 × *g* for 10 min and the precipitate washed once with buffer containing 150 mM NaCl/5 mM $MgCl_2$/0.2% BSA (w/v)/20 mM Tricine (pH 8.0). The resultant pellet (thylakoid fraction) was resuspended in 10 mM NaCl/5 mM $MgCl_2$/50 mM MES–NaOH (pH 6.0) at a chlorophyll (Chl) concentration of 3 mg $ml^{-1}$. The thylakoid suspension was incubated with Triton X-100 (Boehringer, for membrane research) at a Triton:Chl ratio of 25:1 (w/w). The 25 min incubation with gentle stirring was followed by a centrifugation of 3500 × *g* for 5 min. The resultant pellet was discarded and the supernatant centrifuged at 40 000 × *g* for 30 min. The pellet (PS II membranes) was resuspended at a Chl concentration of 10 mg $ml^{-1}$ in a solution containing 15 mM NaCl/5 mM $MgCl_2$/400 mM sucrose/50 mM MES–NaOH (pH 6.0), and rapidly frozen and stored in liquid nitrogen until used. All purification steps were done at 4 °C under dim light. Chl concentration was determined as described by Arnon (1949).

### *Inhibition with Cu(II) and DCMU*

PS II membranes (10–60 $\mu$g Chl) in 3 ml of buffer containing 300 mM sucrose/10 mM NaCl/25 mM MES–NaOH (pH 6.5) were pre-incubated with various concentrations of $CuCl_2$ and DCMU for 10 min at 4 °C with occasional shaking by hand. Then, the reaction mixture was heated at 25 °C in a water bath and the oxygen evolution measured. In the case of competition experiments between $CuCl_2$ and DCMU, the later inhibitor was added after PS II membranes were pre-incubated with $CuCl_2$ for 10 min at 4 °C. The free Cu(II) in the assay medium was measured at 325.7 nm in a Jobin Yvon 1500 inductively-coupled plasma atomic emission spectrophotometer (ICP–AES). To do this, PS II membranes were incubated with $CuCl_2$, centrifuged, and the concentration of Cu(II) was determined in the membrane-free supernatant.

### *Oxygen evolution activity*

Oxygen evolution was measured with a Clark-type oxygen electrode fitted with a circulating water jacket at 25 °C. The standard assay medium consisted of 300 mM sucrose/10 mM NaCl/25 mM MES–NaOH (pH 6.5). DCBQ at a saturating concentration of 0.5 mM was used (unless it is stated) as an artificial electron acceptor. Actinic light from two projector lamps placed on both sides of the electrode cuvette was filtered through 9.5 cm of water. The light intensity on the surface of each side of the sample cuvette was 2200 $\mu$E $m^{-2} s^{-1}$, which was saturating in all the assays. DCBQ was dissolved in ethanol and added just before measuring Hill

activity (the DCBQ final dilution was 100 folds). The Chl concentration was kept in the range of 3.3–20 $\mu g\ ml^{-1}$. The oxygen evolution activity of controls PS II membranes was about 500 $\mu mol\ O_2\ mg\ Chl^{-1}\ h^{-1}$.

## Results

Double-reciprocal plot of the inhibition of photosynthetic electron transport by Cu(II) is shown in Fig. 1. Considering that the specific inhibitor binding is directly related to the inhibition, I, of the electron transport, as shown by Tischer and Strotmann (1977), the inhibition curve can be described by

$$1/I = K_i/a + 1 . \quad (1)$$

Figure 1 was obtained from the corresponding inhibition curve, plotting 1/% Inhibition vs. $1/a_t$ ($a_t$, concentration of total inhibitor). From these data we determined the inhibition constant, $K_i$, of Cu(II), obtaining the value of 5.88 $\mu$M. Note that to calculate the $K_i$, equilibrium concentrations of free inhibitor rather than total inhibitor concentrations are required (Eq. (1)). However, considering that the concentrations of inhibitor used in our experiments are far in excess than that of the RC (ca. between 680 and 500-folds before and after PS II membranes incubation with $CuCl_2$), the concentration of the inhibitor-binding site complex is negligible compared to the total concentration of inhibitor. In Fig. 1, a linear regression curve with a single slope is obtained, indicating that the inhibition curve is monophasic and Cu(II) inhibits at a single specific site of the electron transport chain.

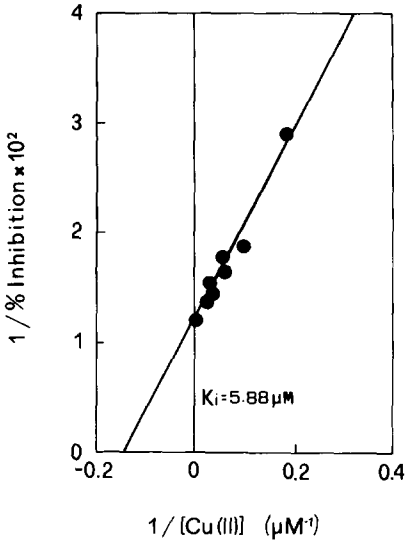


*Fig. 1.* Double-reciprocal plot of inhibition of oxygen evolution activity by $CuCl_2$. The activity was measured in the presence of 0.5 mM DCBQ as an electron acceptor.

Tischer and Strotmann (1977), using radioactively labelled herbicides proved that the relationship between $I_{50}$ (defined as the concentration of herbicide which produces 50% inhibition) and Chl concentration was linear as described by

$$I_{50} = K_i + x_t/2 . \quad (2)$$

Taking these observations into account, they concluded that $K_i$ can also be determined by measuring the $I_{50}$ at different Chl concentrations and extrapolating to zero Chl. Figure 2 shows

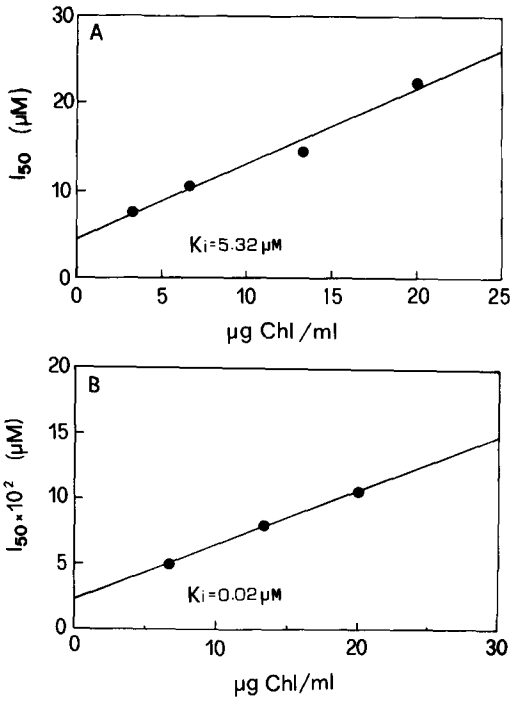


*Fig. 2.* Dependence of $I_{50}$ values of oxygen evolution inhibition by $CuCl_2$ (A) and DCMU (B) on the chlorophyll concentration. Each point was obtained from an inhibition curve using 0, 5, 10, 20 and 30 $\mu$M $CuCl_2$ or 0.01, 0.02, 0.05, 0.1 and 0.2 $\mu$M DCMU. DCBQ was used as artificial electron acceptor at a concentration of 0.5 mM.

the $I_{50}$ values of oxygen evolution inhibition by Cu(II) and DCMU at various Chl concentrations. The inhibition constants, $K_i$, for Cu(II) and DCMU were calculated from these plots (Fig. 2) by extrapolating the linear regression curves to zero Chl concentration. The resultant $K_i$ were 5.32 $\mu$M for Cu(II) and 0.023 $\mu$M for DCMU. The values corresponded well to the $K_i$ calculated for Cu(II) from Fig. 1 and for DCMU by others (Tischer and Strotmann 1977, Kaplanová and Szigeti 1985). Furthermore, the linear dependency between $I_{50}$ and Chl concentration observed for both Cu(II) (Fig. 2A) and DCMU (Fig. 2B) implies that, as in the case of the DCMU (Tischer and Strotmann, 1977), the non-specific sites are negligible respect to inhibition.

To further characterize the Cu(II)-inhibitory mechanism, we studied the influence of pH on the Cu(II) inhibition and determined the enzymatic mechanism respect to DCBQ, DCMU and to $H^+$ using the Lineweaver–Burk plot. Figure 3 presents 1/% Activity vs. 1/[S]; [S] being the concentration of DCBQ, in the presence of different concentrations of the inhibitor Cu(II). We obtained linear regression curves with different slope for each concentration of inhibitor, showing the same intercept with the

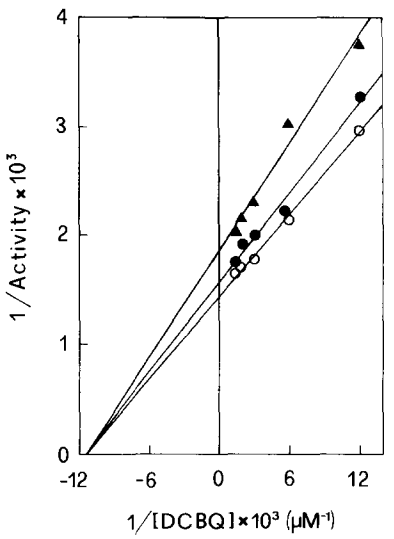


*Fig. 3.* Reciprocal plot of the effect of DCBQ on the oxygen evolution activity. The activity ($\mu$moles $O_2$ mg $Chl^{-1}$ $h^{-1}$) was measured in the absence of $CuCl_2$ (○), and in the presence of 5 $\mu$M (●) and 10 $\mu$M $CuCl_2$ (▲).

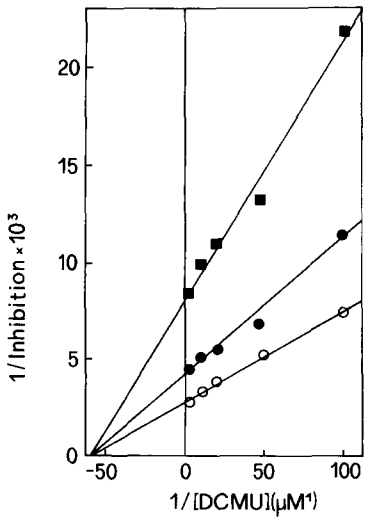


*Fig. 4.* Reciprocal plot of the inhibitory effect of DCMU on the inhibition of oxygen evolution activity by $CuCl_2$. The DCMU-inhibition was measured in the absence of $CuCl_2$ (○), and in the presence of 2.5 $\mu$M $CuCl_2$ (●) and 10 $\mu$M $CuCl_2$ (■), and is given as $\mu$mol $O_2$ mg $Chl^{-1}$ $h^{-1}$. DCBQ was used as electron acceptor at a concentration of 0.5 mM.

x-axis. This indicates that Cu(II) shows a non-competitive inhibition with respect to DCBQ. In Fig. 4 we plotted 1/Inhibition vs. 1/[DCMU] in the presence of different Cu(II) concentrations and saturating amounts of DCBQ. We obtained linear regression curves with the same intersect on the x-axis, which implies that Cu(II) is also a non-competitive inhibitor with respect to DCMU.

Figure 5 displays the influence of pH on the Cu(II) inhibition effect. PS II membranes treated with Cu(II) were more sensitive with pH rise using DCBQ as electron acceptor. Note that the pH effect on other segments of the PS II electron transport pathway, such as the donor side, was eliminated in this graphic since each value was referred to the corresponding non-treated control. Thus, the pH influence observed is related exclusively to the inhibition on the acceptor side. The pH dependency of Cu(II) inhibition may indicate that this inhibitor competes with $H^+$ for the same binding site. This indication is corroborated in Fig. 6, which shows the reciprocal plot 1/Activity vs. $1/[H^+]$. All linear regression curves showed the same intercept on the y-axis, which suggests that Cu(II) is a

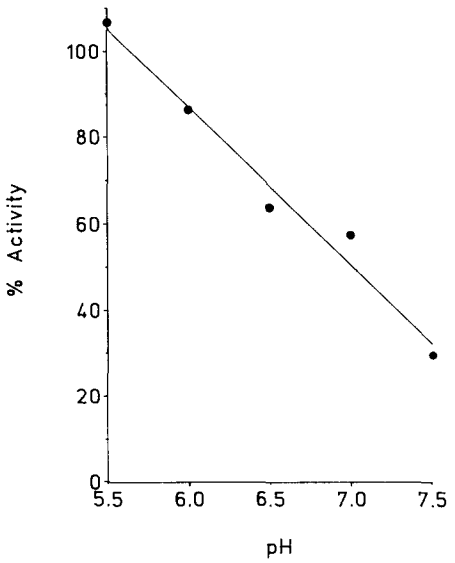


*Fig. 5.* Influence of pH on the inhibitory action of 5 $\mu$M $CuCl_2$ on the oxygen evolution activity. DCBQ as a concentration 0.5 mM was used as artificial electron acceptor.

competitive inhibitor with respect to $H^+$. From this plot the $K_M$ for proton binding (Ka) and the $K_i$ for Cu(II) inhibition can be calculated, obtaining a value of 0.025 $\mu$M (pKa = 7.60) and 5.48 $\mu$M, respectively. The value of 5.48 $\mu$M corresponds well to the $K_i$ determined from Figs. 1 and 2. Note that saturating amounts of DCBQ were used in this assay.

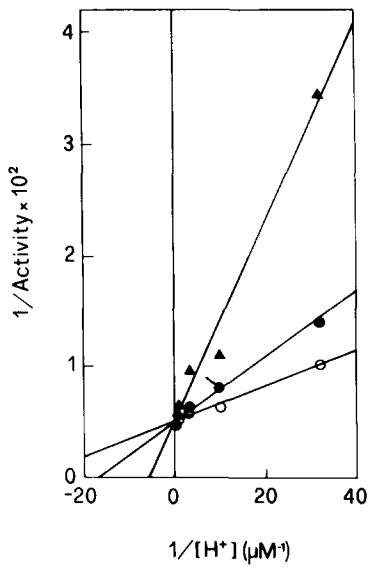


*Fig. 6.* Reciprocal plots of the influence of pH on the inhibitory action of $CuCl_2$. The concentrations of the inhibitor were: no inhibitor (○), 5 $\mu$M (●), and 10 $\mu$M (▲) $CuCl_2$. DCBQ was used as electron acceptor at a concentration of 0.5 mM. The activity is given as $\mu$mol $O_2$ mg $Chl^{-1}$ $h^{-1}$.

## Discussion

Cu(II) impairment of the photosynthetic electron transfer has recently been suggested to take place on the reducing side of PS II by Cu(II) binding to the Pheo-$Q_A$-Fe domain of the reaction center (Yruela et al. 1991). In the present paper, we characterize the Cu(II)-inhibitory mechanism on the PS II electron transport using DCBQ as an artificial electron acceptor. The inhibition constant, $K_i$, for Cu(II) was determined from the corresponding double-reciprocal plot of the oxygen evolution inhibition curves in the presence of DCBQ as a Hill reactant. The linear regression obtained in this plot indicated that the inhibition curve is monophasic and Cu(II) inhibited at a single specific site of the electron transport chain. Furthermore, comparing the linear dependency between $I_{50}$ and Chl concentration for Cu(II) and DCMU, and considering the theoretical conditions that support these plots (Tischer and Strotmann 1977), the presence of a specific Cu(II)-inhibitory binding site which impairs the electron transfer can also be concluded.

The reciprocal plot treatment of the oxygen evolution with DCBQ as electron acceptor in the presence of different concentrations of Cu(II) resulted in a non-competitive inhibition. A similar result was obtained in the reciprocal plot of the DCMU-inhibition in presence of different concentrations of Cu(II). These results indicate that Cu(II)-binding site does not overlap with that of the DCBQ electron acceptor site and that of the DCMU-binding site. These results are consistent with our previous location of the Cu(II)-binding site at the level of the Pheo-$Q_A$-Fe domain (Yruela et al. 1991), separated from the $Q_B$ niche (the DCBQ electron acceptor site). On the other hand, the herbicide DCMU was shown to be a competitive inhibitor with respect to $Q_B$ (Bowlby et al. 1988), in agreement with the location of this herbicide-binding site at the

level of the $Q_B$ niche (Pfister et al. 1981, Diner et al. 1991). However, the reciprocal plots of the influence of $H^+$ on the inhibitory action of Cu(II) showed a competitive inhibitory mechanism. This indicates that Cu(II) and $H^+$ compete for the same binding site, which is consistent with the results of Fig. 5. This suggests that Cu(II) may interact with amino acid residues that can be protonated and deprotonated. The straight line of the control in Fig. 6 may indicate that a single pKa on the acceptor side of PS II reaction center is responsible for the rate of the electron transfer. This does not necessarily mean, however, that this pKa corresponds to a single amino acid that controls the PS II electron transfer. It could well be due to several amino acid groups with similar pKa (close to 7.6), which constitute a niche where a single Cu(II) coordinates. Thus, the Cu(II)-inhibitory mechanism may be to prevent $H^+$ binding to an essential amino acid(s) involved in the photosynthetic electron transfer.

The fact that Cu(II) has a high affinity to amine, triazole or imidazole nitrogen atoms (Yoe 1983–1984, Renganathan and Bose 1990), point to a His or Trp as the Cu(II)-inhibitory target. Indeed, copper is used in immobilized metal-affinity chromatography (IMAC) (Sulkowski 1985) for protein purification. In this chromatography, metal ions are attached to a column in such a way that they can form coordination bonds with unprotonated His and Trp residues exposed on the surface of proteins. Proteins are then eluted with a decreasing pH gradient. The presence of these amino acid residues in the $Q_A$ and $Q_B$ sites has been established in spinach PS II (Diner et al. 1991) and bacterial reaction center (Coleman and Youvan 1990). More work has to be done to identify the essential amino acid group(s) where the inhibitory Cu(II) binds at the Pheo-$Q_A$-Fe domain of the Photosystem II.

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