Inhibition of Millisecond Luminescence by Copper(II) in Spinach Chloroplasts

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Millisecond Luminescence of Chloroplasts, Effect of Copper (II)

Intoxication of class II chloroplasts of spinach with Cu (II) leads to inhibition of millisecond luminescence. The degree of inhibition depends on Cu (II) -concentration. The investgation of the pH dpendence of the inhibition curve of luminescence revealed that (1) there is an inhibition site of copper on the donor side of photosystem II, (2) copper (II) does not act as an uncoupler of photophosphorylation, (3) a protonation equilibrium is involved in the inhibition mechanism, and (4) copper (II) binds to a dissociated residue of a membrane protein.

Introduction

In isolated chloroplasts copper inhibits photosynthetic electron transport in both photosystems [1] but photosystem II was found to be much more sensitive to copper intoxication than photosystem I [1, 2]. Total inhibition of oxygen evolution was observed to occur at total free copper concentrations in the suspension as low as 1 – 2 Cu/Chl, whereas total inhibition of photosystem I requires approximately the threefold amount of copper [1, 2].

Inhibition of oxygen evolution may be brought about by inhibition of any component of photosystem II. The investigation of the influence of copper on several Hill reactions has revealed that the most sensitive inhibition site of copper must be on the oxidizing side of photosystem II. The copper inhibition curve of the DPC-DCIP-Hill reaction * in Tris-washed chloroplasts was found to be very similar to that of photosystem I [2]. This has been interpreted to mean that copper blocks electron transport *before* the DPC entry site of photosystem II. Since DPC is known to donate electrons to some unknown component located between the oxygen evolving system and the photoact [3 – 5], the inhibition site of copper must indeed be very close to the water oxidation site. However, the Hill reaction in heat-treated chloroplasts with Mn (II) as electron donor was observed to be even more sensitive to copper than in untreated chloroplasts with water as electron donor [1]. It was concluded, therefore, that the inhibition site of copper must be located *after* the water oxidation site. So the experimental results obtained thus far seem to indicate that copper inhibits a component of photosynthetic electron transport that is very close to the oxygen evolving system.

No clear-cut picture as by what mechanism copper (II) exerts its toxic action has emerged as yet. Since the Cu (II) ion does not penetrate into lipophilic regions, it is most likely a membrane protein that is modified by interaction with Cu (II). This has been shown to be true [2]. Cedeno-Maldonado, Swader, Heath and Chan [6, 7] observed SH-group oxidation upon interaction of the thylakoid membrane with copper and attributed its inhibitory action to this effect. An alternative suggestion is that the formation of an artificial Cu (II) -membrane protein may be responsible for the inhibition of photosynthetic electron transport through photosystem II because both phenomena appeared to be correlated to each other [2].

In this communication we present experimental results which provide further evidence for the latter hypothesis. We studied the effect of Cu (II) on the properties of the millisecond luminescence intensity in class II chloroplasts of spinach. Millisecond delayed light intensity is sensitive to uncouplers of photophosphorylation [8 – 12] and is controlled by the rate of linear electron transport [13 – 16]. It has been demonstrated by Sorokin [13] that luminescence intensity strongly depends on whether the oxidizing or reducing side of photosystem II is blocked. This provides an independent approach to the problem where to locate the inhibition site of copper.

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* The following abbreviations have been used in the text: DCIP, 2,6-dichloroindophenol; DPC, 1,5-diphenyl carbazide.

It is shown in this paper that the luminescence measurements corroborate the notion that the most sensitive inhibition site of copper must be on the oxidizing side of photosystem II.

Furthermore, the investigation of the pH-dependence of the inhibition curve of luminescence revealed that (1) Cu(II) does not act as an uncoupler of photophosphorylation, (2) that a protonation equilibrium is involved in the inhibition mechanism, and (3) that Cu(II) binds to a dissociated residue of a membrane protein.

Experimental

Class II chloroplasts were prepared from market spinach according to the method of Sane *et al.* [17], and were incubated in phosphate buffer (67 mM) containing 5 mM $MgCl_2$. Inhibition of photosynthesis by copper is stimulated in the light [1]. Therefore, before measurement the chloroplast suspension was irradiated in the presence of copper with saturating red light ($\lambda \geqq 640$ nm) for 5 min at 4 °C. Luminescence measurements were carried out at 4 °C as described before [18, 19]. Copper (II) concentrations were measured by EPR-spectroscopy (E 12, Varian).

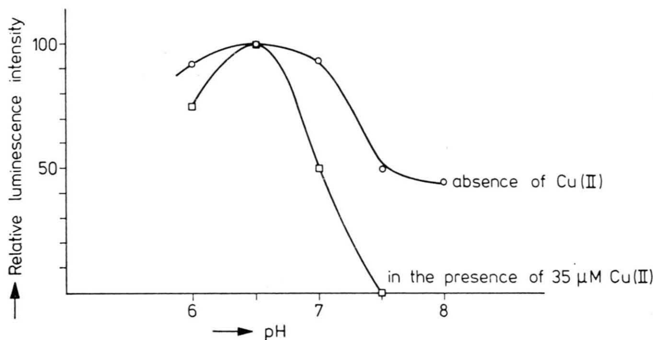


Fig. 1. pH dependence of 10 ms luminescence intensity in class II chloroplasts of spinach in the presence and absence of copper. $T=4$ °C, 0.3 mg/ml Chlorophyll, 67 mM phosphate buffer containing 5 mM $MgCl_2$. Luminescence intensity was normalized to 100 in both cases. ○ In the absence of Cu(II); □ in the presence of 35 µM Cu(II).

Results and Discussion

In the absence of factors affecting the membrane potential (electric fields, addition of salts, artificially created pH-gradients) the quenching of luminescence in chloroplasts by copper can be attributed either to inhibition of photophosphorylation [9] or to inhibition of photosynthetic electron transport through photosystem II [13]. Inhibition of photophosphorylation by uncoupling agents eliminates the dependence of luminescence intensity from the external pH in the range 6 – 8 [20]. The pH dependence of luminescence intensity is not eliminated, however, by copper (Fig. 1). This reveals that copper does not act as an uncoupler of photophosphorylation.

Millisecond delayed light emission in chloroplasts is not inhibited when electron transport is blocked after the plastoquinone pool by electron transport inhibitors [21]. The decrease of millisecond luminescence intensity in the presence of copper (Fig. 2) must be solely due, therefore, to inhibition of electron transport through photosystem II.

Delayed light intensity is strongly correlated with the rate of electron transport through photosystem II [13]. The theory of Sorokin [13] predicts in accordance with experimental results that, at low excitation intensity (<1 mW/cm^2), the luminescence intensity increases when electron transport is blocked on the reducing side of photosystem II and decreases after blocking the oxidizing

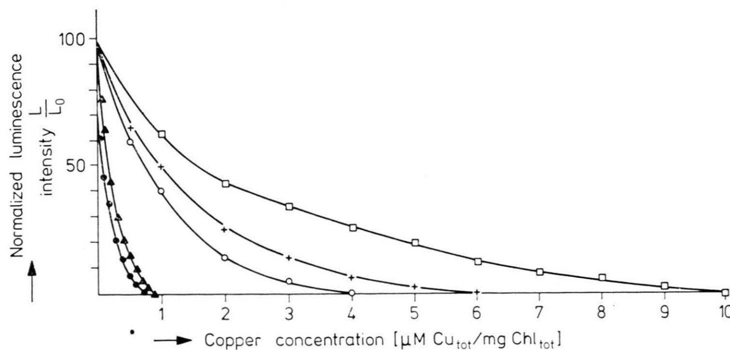


Fig. 2. Concentration dependence of luminescence quenching by copper at different pH values. Same data as in Fig. 1. Luminescence intensity was normalized to 100 in each case. □ pH=8, + pH=7.5, ○ pH=7.0, ▲ pH=6.5, ● pH=6.0.

side. Hence according to the results shown in Fig. 2 the site of copper inhibition must be on the donor side of photosystem II.

The interpretation of the pH dependence of luminescence intensity (Fig. 1) leads to the same conclusion. According to Sorokin [13] the rate of photosynthetic electron transport and the intensity of delayed light emission change mutually in a supplementary way at low excitation intensities when the rate constant of electron transport k_1 on the reducing side of photosystem II is smaller than that of the oxidizing side ($k_1 < k_{H_2O}$). Hence luminescence intensity should decrease at higher pH values because the rate of electron transport increases [22]. This is actually observed (Fig. 1). In the presence of copper, however, the intensity of delayed light is further suppressed accompanied by a decrease of the electron transport rate [1, 2]. This behavior is predicted by the theory of Sorokin when $k_{H_2O} < k_1$. This clearly shows that copper intoxication leads to an inhibition of the water oxidizing side of photosystem II thus confirming the result obtained earlier [1, 2].

Further information on the mechanism of copper inhibition comes from the analysis of the concentration dependence of inhibition of luminescence at different pH values. It is seen from Fig. 3 that the logarithmus of the total Cu(II) concentration in the chloroplast suspension that is necessary for total inhibition of luminescence is a reciprocal linear function of pH. Therefore, a protonation equilibrium must be involved in the inhibition process.

The result shown in Fig. 3 can be explained by assuming that copper inhibits luminescence through

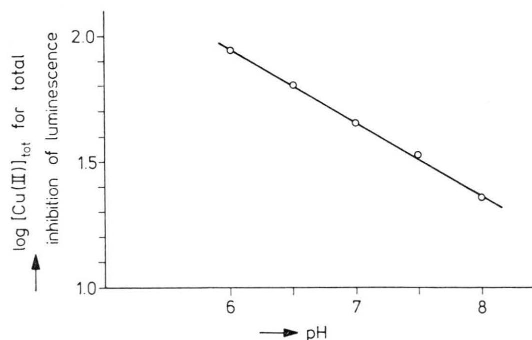
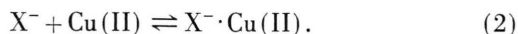


Fig. 3. pH dependence of the logarithmus of the total Cu(II) concentration (μM/L) in the buffer that is necessary for total inhibition of luminescence.

interaction with the anion of a protein residue that contains a dissociable proton. The dissociated residue X^-

$$XH \rightleftharpoons X^- + H^+ \tag{1}$$

is assumed to bind one Cu(II) ion.



$$X^- + Cu(II) \rightleftharpoons X^- \cdot Cu(II) . \tag{2}$$

The dissociation constant of the residue is given by

$$K_1 = \frac{[X^-][H^+]}{[XH]} \tag{3}$$

and the dissociation constant of the copper(II) complex by

$$K_2 = \frac{[Cu(II)]_{free} \cdot [X^-]}{[X^- \cdot Cu(II)]} . \tag{4}$$

Hence by combining both expressions

$$\frac{[Cu(II)]_{free}[XH]}{[X^- \cdot Cu(II)][H^+]} = \frac{K_2}{K_1} . \tag{5}$$

In Fig. 3 the total concentration of Cu(II) before equilibration with chloroplasts was measured and not $[Cu(II)]_{free}$. Both concentrations are related by

$$[Cu(II)]_{tot} = [Cu(II)]_{free} + [X^- \cdot Cu(II)] . \tag{6}$$

Measuring $[Cu(II)]_{free}$ (after equilibration of the chloroplasts with Cu(II) and separation of the chloroplasts from the supernatant by ultrafiltration) revealed that $[X^- \cdot Cu(II)]$ is equal to or below 5% of $[Cu(II)]_{tot}$ so that $[Cu(II)]_{tot} \gg [X^- \cdot Cu(II)]$.

Then Eqn (5) may be written in the form

$$\log [Cu(II)]_{tot} + \log \frac{[XH]}{[X^- \cdot Cu(II)]} = \log \frac{K_2}{K_1} - pH . \tag{7}$$

Assuming that the ratio $[XH]/[X^- \cdot Cu(II)]$ does not strongly depend on pH this equation may be simplified into

$$\log [Cu(II)]_{tot} = \log A - pH \tag{8}$$

where

$$A = \frac{K_2}{K_1} \frac{[X^- \cdot Cu(II)]}{[XH]}$$

is assumed to be approximately constant as long as the pH range is sufficiently small.

Eqn (8) shows that the reciprocal linear relationship between $\log [Cu(II)]_{tot}$ and pH depicted

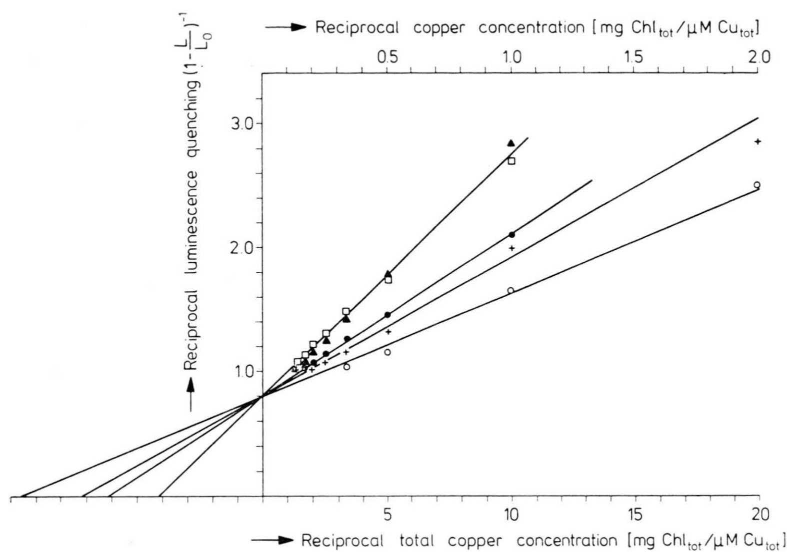


Fig. 4. Hughes-Klotz plot of the concentrations of free and bound copper (II). The concentration of bound Cu (II) is monitored by the relative luminescence quenching $1\text{-}L/L_0$. The total copper concentration $[Cu]_{tot}$ is approximately proportional to the free copper concentration (see text). Data are taken from Fig. 4. The upper scale for the reciprocal copper concentration is valid for pH=7, 7.5, and 8 and the lower one for pH=6 and 6.5. The straight line for pH=6.5 has not been given, since it is almost identical to that of pH=8 in this presentation. □ pH 8; + pH 7.5; ○ pH=7.0; ▲ pH=6.5; ● pH=6.0.

in Fig. 3 is consistent with the theory denoted above *.

The notion that inhibition of luminescence must be attributed to binding of Cu (II) to a dissociable protein residue is further confirmed by analysing the concentration dependence of luminescence quenching (Fig. 2). It does not obey the Stern-Volmer law thus indicating that Cu (II) does not quench luminescence by binding to chlorophyll a molecules.

Luminescence quenching by copper may be represented instead in the form of a Hughes-Klotz plot [23] (Fig. 4), if it is assumed that luminescence quenching $1-L/L_0$ is proportional to the concentration of bound Cu (II) **. It has been established by EPR measurements of the concentration of the free Cu (II) ion in the buffer solution that in the concentration range used in these experiments the concentration of free copper (II) in the suspension is approximately proportional to the total copper concentration $[Cu]_{tot}$. So the plot $(1-L/L_0)^{-1}$ *vs* $1/[Cu]_{tot}$ may be interpreted as representing a Hughes-Klotz plot of the concentrations of bound and free copper (II).

In this presentation the intercepts of the straight line with the ordinate and the abscissa are proportional to the number of binding sites and to the reciprocals of the apparent dissociation constants, respectively. Fig. 4 shows that the total number

* It is seen, however, that the slope of the straight line in Fig. 3 is only −0.3 and not −1 as predicted by Eqn (8). This is due to the fact that there is some pH dependence of $[XH]/[X^- \cdot Cu(II)]$. At pH=8 this ratio is expected to be smaller than at pH=6 so that, as is seen from Fig. 3, representing the data according to Eqn (7) would yield a more negative slope.

** The concentration of bound Cu (II) cannot be determined directly at present because it has not been excluded as yet that there might be additional binding sites of Cu (II) in the membrane which are not related to luminescence quenching. So determination of bound Cu (II) by standard methods would not yield the real amount of Cu (II) bound to the luminescence inhibiting site in this case.

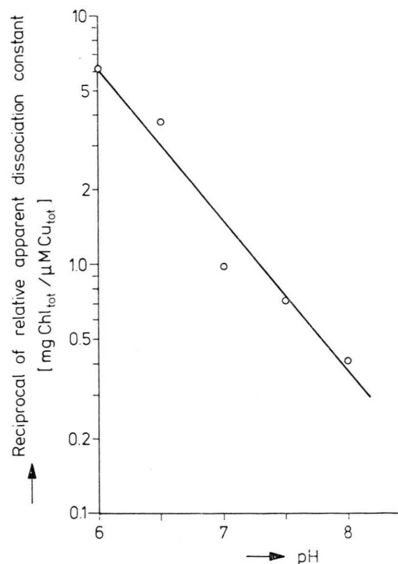


Fig. 5. pH dependence of the reciprocals of the relative dissociation constant of the copper (II) binding site. (Semilogarithmic plot.) Data are taken from Fig. 4.

of inhibiting binding sites available for Cu(II) is the same for all pH values investigated and Fig. 5 indicates that the relative apparent dissociation constants taken from Fig. 4 depend linearly on pH. Both results are consistent with Eqn (5) thus indicating that the interpretation of Fig. 4 as Hughes-Klotz plot of the binding of Cu(II) to a membrane protein is in accordance with the theory described before.

These results, therefore, provide evidence for the view that copper(II) inhibits oxygen evolution by way of binding to an unprotonated protein residue of a component which is very close to the water splitting system.

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