Binding of Copper(II) to Proteins of the Photosynthetic Membrane and its Correlation with Inhibition of Electron Transport in Class II Chloroplasts of Spinach

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EPR of Copper(II) Complexes with Membrane Proteins, Inhibition of Photosynthetic Electron Transport

Incubation of class II chloroplasts of spinach with copper in the light at pH = 8 in concentrations that inhibit oxygen evolution results in the formation of a copper(II) protein complex with the photosynthetic membrane. The EPR spectra indicate that the four nearest ligands to Cu(II) consist of three oxygen atoms and one nitrogen atom. The copper(II) protein appears to be predominantly associated with photosystem II. The formation of this protein as measured by the EPR signal amplitude of its room temperature spectrum correlates with the inhibition of oxygen evolution and of electron transport within photosystem I. This result indicates that the inhibition of photosynthetic electron transport by copper may be due to the formation of a copper(II) chelate with a membrane protein.

Introduction

Copper is known to be an effective inhibitor of photosynthesis in algae [1–4] and in chloroplasts of higher plants [5–9]. It has been demonstrated that copper intoxication of chloroplasts leads to inhibition of photosynthetic electron transport [5–7]. Both photosystems are affected but oxygen evolution turned out to be most sensitive to copper poisening [7]. Very little is known, however, on the mechanism through which copper exerts its toxic action on the photosynthetic membrane. The work of Cedeno-Maldonado, Swader, Heath and Chan [7–9] suggests that inhibition of photosynthetic electron transport may be due to the reaction of cupric ions wih sulfhydryl groups. However, though copper is most likely to inhibit the activity of enzymes in many cases through the formation of copper mercaptides with the sulfhydryl group of cysteinyl residues [10], inhibition of enzymes through the formation of copper(II) chelates with the protein has also been reported [10, 11]. We investigated, therefore, the interaction of copper(II) with the photosynthetic membrane in class II-chloroplasts by EPR spectroscopy. Our results show that the formation of a copper(II) membrane protein may be responsible for the inhibition of photosynthetic electron transport.

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Experimental

Class II-chloroplasts were prepared from market spinach according to the method of Sane *et al.* [12]. The incubation buffer contained 67 mM phosphate (pH = 8.0) and 5 mM $MgCl_2$. These hypotonically suspended chloroplasts still exhibit all activities of intact electron transport chains [13, 14]. Sonicated chloroplasts have been obtained from class II chloroplasts by sonication at 40 kc (250 W) for 2 min (Schoeller & Co., type WP 10/40-6, Germany) followed by centrifugation of the thylakoid fragments at $4700 \times g$ for 15 min.

PSI- and PSII-particles * were prepared as described by Anderson and Boardman [15].

Chlorophyll was determined according to the method of Arnon [16].

Tris inactivation of chloroplasts was performed as described by Yamashita and Butler [17].

DCIP photoreduction with water as electron donor was measured at 586 nm in stirred chloroplast suspensions with the apparatus described by Schmidt and Rosenkranz [18]. To ascertain that the recordings are not due to light scattering changes the spectrum of the rate of DCIP photoreduction between 540 nm and 670 nm was compared with its absorption spectrum – a method first suggested by Izawa [19]. The transmission changes corresponded to the absorption spectrum within 10%. The rate of dye reduction was obtained from the initial slopes of the recorded transmission curves.

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

As revealed by EPR spectroscopy the amount of free copper(II) present in phosphate buffer solutions strongly depends on the preparation procedure and the age of the buffer. Therefore, copper containing buffer solutions were prepared always in the same way (dissolution of $CuSO_4$ in 2 ml distilled water and dilution with phosphate buffer to 100 ml) and were used immediately to avoid aging processes of the $Cu(OH)_2$-sol.

Inhibition of photosynthesis by copper is stimulated in the light [2, 7]. 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.

EPR measurements were carried out with the Varian E-12 spectrometer. The microwave frequency was measured with a Systron Donner counter 1037 and the magnetic field with a proton resonance gaussmeter connected to a digital frequency meter (Racal 806 R).

Electron flow through photosystem I was measured by the steady state amplitude of EPR signal I which was generated using monochromatic saturating far-red light (715 ± 10 nm). The signal amplitude was corrected for the contribution of EPR signal II. The rate of electron transport is then given by [20]

$$R = k\left(1 - \frac{[\mathrm{P}\,700^+]}{[\mathrm{P}\,700]_{\mathrm{tot}}}\right).$$

k is the number of photochemical acts within PS I per sec.

$[\mathrm{P}\,700]_{\mathrm{tot}}$ was determined by EPR spectroscopy after oxidation of P 700 with $K_3Fe(CN)_6$ and subsequent illumination with saturating far-red light (715 nm) – a procedure that creates the maximal amplitude of EPR signal I. Excitation of EPR signal I by far-red light assures that the contribution of noncyclic electron flow from photosystem II to electron transport in photosystem I is negligible. Hence R represents the rate of cyclic electron transport. In untreated class II chloroplasts usually no EPR signal I is observable because $\mathrm{P}\,700^+$ is kept reduced by cyclic electron flow. But in chloroplasts treated with inhibitors of electron transport through photosystem I the amplitude of signal I increases.

Results and Discussion

I. EPR spectra of copper(II) compounds present in the incubation buffer at pH = 8.0

By means of EPR spectroscopy two different copper(II) compounds were shown to be present in freshly prepared incubation buffer solutions (Table I)) the well-known polycrystalline $Cu(OH)_2$ [21] and $Cu(H_2O)_6^{2+}$. In aged buffer solutions, however, no hydrated copper(II) was present. The EPR signal of an as yet unidentified polycrystalline copper(II) species with axial microsymmetry appeared instead in addition to the spectrum of polycrystalline $Cu(OH)_2$ (Table I) *. Contrary to the action of freshly prepared buffer incubation of class II chloroplasts in *aged* buffer solutions in the light did not lead to inhibition of photosynthetic electron transport. This indicates that $Cu(H_2O)_6^{2+}$ is the species that is responsible for the toxic action of copper.

II. EPR spectra of copper(II) complexes with proteins of the photosynthetic membrane

Total inhibition of oxygen evolution in class II chloroplasts was observed to occur below 5 μM Cu_{tot}/mg Chl at pH = 8 (Fig. 5). As revealed by EPR spectroscopy incubation of chloroplasts with copper in this concentration range leads to the for-

* The unknown Cu(II) species might be identical with the sparingly soluble $Cu_4H(PO_4)_3 \cdot 3\,H_2O$ which is stable in buffer solutions up to pH = 8 [22].

Compound	Solvent	T[°K]	g_0	$g_{\|\|}$	$g_\perp$	$A_{\|\|}$[cm^{-1}]
Polycryst.	Buffer	90	0.83			No hyperfine structure
$Cu(OH)_2$		293	No signal observable			
$Cu(H_2O)_6^{2+}$	water	90	—	2.400	2.084	0.0144
		293	2.187 *	—	—	—
	buffer	90		2.400	2.076	0.0140
		293	2.193	—	—	—
Unknown Cu(II) species in aged buffer solution	buffer	90		2.182 +	2.050 +	—

Table I. EPR parameters of copper(II) compounds present in the incubation buffer at pH = 8 and, for comparison, those of $Cu(H_2O)_6^{2+}$ in water. Experimental accuracy for g-factor measurements: ± 0.005.

* From Samaraev and Tikhomirova [22].

\+ g values were assigned according to the theory of Kneubühl [23].

Table II. EPR parameters of the two copper(II) chelates with membrane proteins formed upon interaction of copper(II) with class II chloroplasts at pH=8. g_0, g-factor at room temperature. a, hyperfine splitting at room temperature.

Compound	T[°K]	g_0	$g_\perp$	$g_\parallel$	$A_\parallel$[cm^{-1}]	a[G]
A	90		2.068	2.297	0.0174	
	277	2.142				70
B	90		2.076	2.367	0.0151	
	277	No signal observable				

mation of an apparently single Cu(II) complex compound. Its EPR spectra at room and low temperature are shown in Fig. 1 and Fig. 2. The experimentally determined g-values are listed in Table II. The line at highest field in Fig. 1 originates from the "overshoot" of one of the copper hyperfine lines [25]. An alternative explanation – the assignment to a second copper(II) compound with different $g_\perp$ but the same $g_\parallel$ and $A_\parallel$ – is highly unlikely in view of the small line width (20 G) and because of the fact that the EPR parameters of copper(II) compounds differ much more in $g_\parallel$ and $A_\parallel$ than in $g_\perp$ [26]. It cannot be ruled out at present, however, that several binding sites of Cu(II) which give rise to identical or nearly identical EPR spectra might contribute to the observed spectrum. Three poorly resolved superhyperfine lines with a splitting of approximately 10 G indicate superhyperfine interaction of Cu(II) with one nitrogen atom. The evaluation of the EPR parameters $g_\parallel$ and $A_\parallel$ (complex A in Table II) according to the method of Peisach and Blumberg [26] reveals that the following configurations for the four in-plane ligands of Cu(II) are possible: 4O, 2N2O, and 1N3O. On the basis that three nitrogen superhyperfine lines can be seen in the region of $g_\perp$ we conclude that the four nearest ligands to Cu(II) most likely consist of three oxygen atoms and one nitrogen atom.

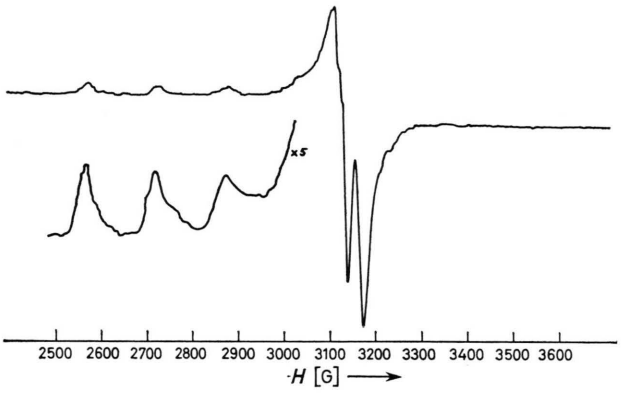

Fig. 1. Low temperature EPR spectrum of the copper(II) membrane protein A. T=90 °K; pH=8.0; chlorophyll concentration, 3.0 mg/ml; copper concentration, 15 mM/L ($CuSO_4$); modulation amplitude, 5 G; time constant, 0.1 sec; scan rate, 250 G/min; microwave frequency, 9.0388 Gc; microwave power, 50 mW; receiver gain, 500.

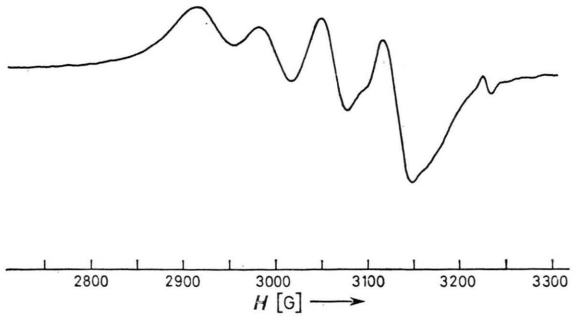

Fig. 2. EPR spectrum of the copper(II) membrane protein A measured at 4 °C. Modulation amplitude, 10 G. Time constant, 1 sec; scan rate, 125 G/min; microwave frequency, 9.071834 Gc; microwave power, 5 mW; receiver gain, 5000; other data as in Fig. 1.

The same copper(II) compound was found to be present in the sediment of sonicated chloroplasts which consists of fragments of the thylakoid membrane and in the protein fraction of class II chloroplasts after extraction of the lipids with an acetone-water mixture (80% acetone). No copper(II) was detected by EPR spectroscopy within the lipid fraction. These results indicate that copper(II) is bound to a membrane protein of the photosynthetic membrane. Attempts to isolate this protein by means of SDS-acrylamide gel electrophoresis [27] have not been successful as yet since the copper(II) protein complex was destroyed upon solubilization of the acetone-extracted protein fraction with 0.2% SDS.

At higher copper concentrations ($\approx$ 10 μM Cu_{tot}/mg Chl) the formation of a second copper(II) protein complex (B) has been observed in addition to the compound A described above. The low tempera-

ture EPR spectrum of B obtained with excess amounts of copper is shown in Fig. 3. The EPR signal of A is not seen because of the low concentration of A as compared to B. In contrast to A compound B can easily be destroyed by washing the chloroplasts with buffer. The EPR parameters of B (Table II) indicate[26] that copper(II) coordinates to four oxygen atoms.

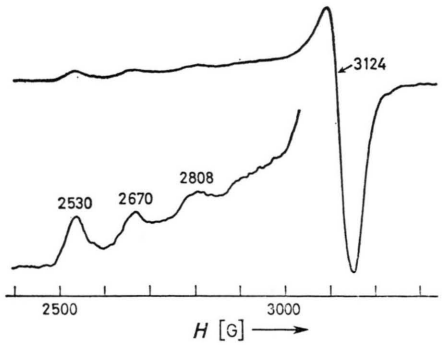

Fig. 3. Low temperature EPR spectrum of the copper(II) membrane protein B. $T=90$ °C; pH=6.0; chlorophyll concentration, 3.0 mg/ml; copper concentration, >30 mM/L; modulation amplitude, 10 G; time constant, 0.3 sec; scan rate, 500 G/min; microwave frequency, 9.0762 Gc; microwave power, 200 mW; receiver gain, 63.

The formation of compound B is certainly not related to the toxic effects of copper on photosynthetic electron transport because B is only formed at comparatively high copper concentrations. We have concentrated, therefore, on the study of the properties of compound A.

III. Saturation effects of the copper(II) membrane protein A in photosystem I and photosystem II particles

The degree of complex formation of copper(II) with the thylakoid membrane with increasing copper concentration was followed by measuring the signal amplitude of the fourth line of the EPR spectrum of compound A obtained at 4 °C (Fig. 2). Comparison of the saturation curves for PS I and PS II particle (Fig. 4) shows that the number of copper binding sites per Chl associated with the PS I fraction is only half that of PS II. Since the PS II fraction usually exhibits considerable PS I activity, the ratio of P 700/Chl was measured for both preparations. For PS I we obtained 1.9 (relative units) and for PS II 0.8 at the same Chl-concentration. Hence the number of binding sites within PS II is 2.7 fold that within PS I. This

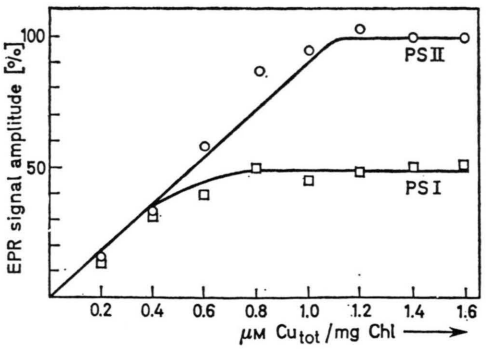

Fig. 4. Saturation effect upon formation of the copper(II) membrane protein A in PS I and PS II particles. □, PS I particles; ○, PS II particles; $T=20$ °C; pH=8.0; chlorophyll concentration, 3.0 mg/ml.

result demonstrates that the artificial copper(II) membrane protein A is predominantly associated with PS II.

IV. Correlation between inhibition of photosynthetic electron transport and binding of copper(II) to the membrane protein A

Copper inhibits photosynthetic electron transport through both photosystems but PS II is much more sensitive to copper poisening than PS I[7]. This has been confirmed by our measurements. Electron flow from PS II as measured by the rate of DCIP photoreduction is abolished at a copper concentration of 4 μM Cu_{tot}/mg Chl whereas electron transport through PS I is inhibited to 30% only (Fig. 5) *.

Electron transport in Tris-washed chloroplasts with DPC as electron donor and DCIP as acceptor is much less sensitive to copper intoxication than the $H_2O \rightarrow$ DCIP-reaction (Fig. 5). Since DPC donates electrons within PS II to a component located between the oxygen evolving system and the photoact[28–30], the site most sensitive to copper must be located before the DPC entry site, that is close to the oxygen evolving system. In chloroplasts with intact thylakoids DCIP is preferentially reduced by PS I[13]. The inhibition curve of the DPC – DCIP reaction may reflect, therefore, the influence of several inhibition sites between the DPC donor site within PS II and the DCIP reduction site within PS I. The shape of the curve and its

* This result is in remarkably good agreement with that reported by Cedeno-Maldonado *et al.*[7] (25%) though different chloroplast preparations and buffer solutions for the measurement of photosynthetic electron transport were used.

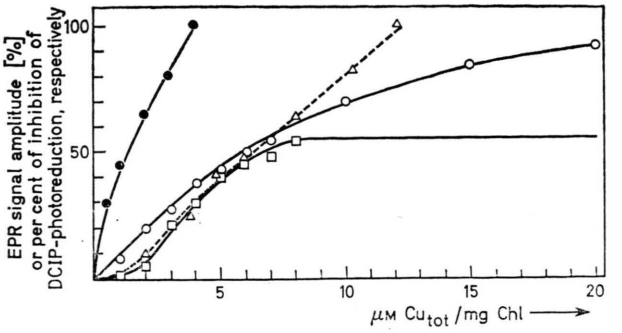

Fig. 5. Inhibition of photosynthetic electron transport by copper and its correlation with the formation of the copper(II) membrane protein A. ○, EPR signal amplitude of copper(II) membrane protein A; □, EPR signal amplitude of $P\,700^+$ generated at 715 nm; ●, inhibition of DCIP photoreduction $1-R/R_0$ (R_0-control rate); △, inhibition of the DPC–DCIP photoreaction; $T=4$ °C; pH=8.0; chlorophyll concentration, 3.0 mg/ml (EPR measurements), 45 μg/ml otherwise.

close correspondence to the inhibition curve of cyclic electron transport in its copper sensitive part, however, indicates that the inhibition of the DPC – DCIP photoreaction predominantly reflects the inhibitory influence of copper on PS I.

Though *linear* electron flow through PS I can be totally inhibited [7], Fig. 5 shows that cyclic electron transport is inhibited maximally up to approximately 50%. This was observed even at copper concentrations as high as 40 μM C_{tot}/mg Chl. This discrepancy is not understood at present. Excessive binding of copper to the thylakoid membrane might either create an unphysiological pathway for cyclic electron transport thus circumventing the inhibition site or favour the direct recombination of $P\,700^+$ and the primary electron acceptor $P\,430^-$ – a reaction that has recently been demonstrated to occur in chloroplasts when no secondary acceptor is present [31].

Copper(II) is known to interact with proteins in two principal ways: (1) formation of artificial Cu(II) protein compounds [*e. g.* [26]]; (2) catalysis of the oxidation of SH-groups to disulfides under formation of Cu^- [*e. g.* [32]]. Both phenomena might be involved in the mechanism of inhibition of photosynthetic electron transport. A clear-cut decision whether reaction (1) or reaction (2) or both are responsible for the toxic effects of copper cannot be reached at present. However, the fact that the inhibition curves for PS I and PS II activity closely parallel the formation of the copper protein compound A (Fig. 5) clearly indicates that inhibition of photosynthetic electron transport might be related to the formation of a Cu(II) membrane protein.

Oxygen evolution is totally inhibited long before the concentration of the copper(II) protein complex A has reached its maximal value (Fig. 5). Furthermore, A is also found in PS I particles. As the possibility that several Cu(II) protein complexes might contribute to the room temperature EPR signal (see Sect. II) appears to be less probable, these results are interpreted to indicate that the EPR detectable Cu(II) does not bind to the water splitting enzyme itself.

The number of bound Cu(II) ions that is necessary for total inhibition of electron transport is always lower than the total number of binding sites (Fig. 5). Thus, if only one binding site for Cu(II) is assumed to be responsible for the EPR signal shown in Fig. 2, copper does not inhibit electron transport by way of binding to any other component of the electron transport system either.

We prefer, therefore, the explanation that binding of Cu(II) to a membrane protein that is not involved in photosynthetic electron transport leads to structural changes of the photosynthetic membrane with subsequent inhibition of electron transport.

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