Stimulation of Oxygen Evolution in Photosystem II by Copper(II) Ions

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Z. Naturforsch. **57c**, 853–857 (2002); received June 3/June 20, 2002

Copper(II) Ions, Stimulation of Oxygen Evolution, Photosystem II

We have found that Copper(II) ions at about equimolar Cu^{2+}/photosystem II (PS II) reaction center proportions stimulate oxygen evolution nearly twofold. This high affinity Cu-binding site is different from the binding sites of Mn and Ca ions. The analysis of the Cu^{2+} content in PS II preparations isolated from wild-type tobacco and a tobacco mutant deficient in light-harvesting complex suggests that Cu^{2+} may be a native component of PS II and may take part in the oxygen evolution process. At higher concentrations, Cu^{2+} ions inhibit oxygen evolution and quench fluorescence.

Introduction

Copper is an essential microelement for plants, however, at higher concentrations it shows toxic effects (Droppa and Horváth, 1990; Barón *et al.*, 1995; Prasad and Strzałka, 1999). In the photosynthetic apparatus, photosystem II (PSII) is the most sensitive site to Cu^{2+} ions. In the experiments reported in the literature, the amount of Cu^{2+} ions present in the investigated systems exceeded by far the amount of photosynthetic reaction centers (RC). Such high copper concentrations resulted in the inhibition of oxygen evolution accompanied by quenching of variable fluorescence (Hsu and Lee, 1988; Samson *et al.*, 1988; Arellano *et al.*, 1995). It was found that Cu^{2+} inhibits both the donor and the acceptor side of PS II but the most sensitive site of Cu-inhibition was located on the oxidizing side of PS II (Haberman, 1969; Cedeno-Maldonado and Swader, 1972; Vierke and Stuckmeier, 1977). The primary quinone acceptor QA (Jegerschöld *et al.*, 1995), the pheophytin -QA-Fe region (Yruela *et al.* 1996), the non-heme iron (Singh and Singh, 1987; Jegerschöld *et al.*, 1999) and the secondary quinone acceptor QB (Mohanty *et al.*, 1989) are among the suggested inhibition sites of Cu^{2+} on the acceptor side of PS II. On the donor side of PSII a reversible inhibition of TyrZ oxidation by Cu^{2+} has been observed (Schröder *et al.*, 1994; Jegerschöld *et al.*, 1995).

On the other side, involvement of Cu^{2+} in photosynthetic reactions of PSII as its native component has already been suggested in several reports. Lightbody and Krogmann (1967) suggested that there was a site close to the OEC which was sensitive to inhibition by Cu^{2+} chelators. This site was not dependent on plastocyanin. Other experiments with lipophilic chelators indicated the existence of a copper-protein within PSII (Bar and Crane, 1976). Copper deficiency experiments showed that PSII activity decreased in plants grown under Cu-deficiency (Barón *et al.*, 1990). Analyses of PS II preparations obtained by many authors showed that Cu^{2+} is often found in these particles (Droppa and Horváth, 1990; Barón *et al.*, 1995) but this was usually attributed to the contamination of PS II preparations with starch and nuclear fractions (Arellano *et al.*, 1994; Barón *et al.*, 1993).

The present study is mainly aimed at the investigation of the mechanism of Cu^{2+} action on oxygen evolution. In our experiments, we used equimolar

Abbreviations: Chl, chlorophyll; conc., concentration; cyt b559, cytochrome *b*559; LHC, light-harvesting complex; OEC, oxygen evolving complex; Pheo, pheophytin; PS II, photosystem II; PS II BBY particles, membranes enriched in photosystem II; RC, reaction center; Trp, tryptophan; Tyr, tyrosine.

0939–5075/2002/0900–0853 $ 06.00

Cu^{2+}/PSII RC proportions. The native content of Cu^{2+} in PS II particles isolated from tobacco and from a light-harvesting complex (LHC)-deficient tobacco mutant (Specht *et al.*, 1987) was measured.

Materials and Methods

PS II BBY particles were isolated from tobacco (*Nicotiana tabacum*, cv. John Williams Broadleaf or from the Su/su var. Aurea mutant) according to the method of Berthold *et al.*, (1981) with the modifications of Arellano *et al.*, (1994). The amperometric oxygen evolution measurements were performed on PS II particles at a chlorophyll (Chl) concentration (conc.) of 42 μg/ml in 50 mM Hepes (N-[2-hydroxyethyl]-piperazine-N′–2-ethane sulfonic acid) buffer, pH 7.0 containing 10 mM KCl, 5 mM $MgCl_2$ and 2.5 mM $CaCl_2$ using a three-electrode-system (Schmid and Thibault 1979). Saturating light flashes of 5 μs duration at half intensity were provided by a xenon lamp (Stroboscope 1539A from General Radio, Concord, Massachusets USA). The samples were illuminated by 15 flashes spaced 300 ms apart. Measurements of fluorescence induction kinetics were performed on a home-built fluorimeter using excitation with blue light (BG12 filters) and detection at 685 nm through a monochromator. Fluorescence was measured at Chl conc. of 50 μg/ml in the same medium as that for the oxygen evolution measurements. The content of Mn and Cu in the PS II preparations was determined using atomic absorption spectroscopy.

Results

Measurements of oxygen evolution under short saturating flashes show that 0.25 and 0.5 μM $CuCl_2$, corresponding to a Cu^{2+}/PS II ratio of 1.5 and 3.0, respectively, stimulated oxygen evolution almost two-fold (Fig. 1). These $CuCl_2$ concentrations had no effect on the fluorescence kinetics. With increasing $CuCl_2$ concentrations, oxygen evolution decreased, which was accompanied by fluorescence quenching (data not shown). In the case of $CuSO_4$, stimulation of oxygen evolution was observed already at 50 nM $CuSO_4$ (Fig. 2).

In order to get better insights into the molecular mechanism of the observed effect of Cu^{2+} on oxygen evolution, we analyzed the results using the

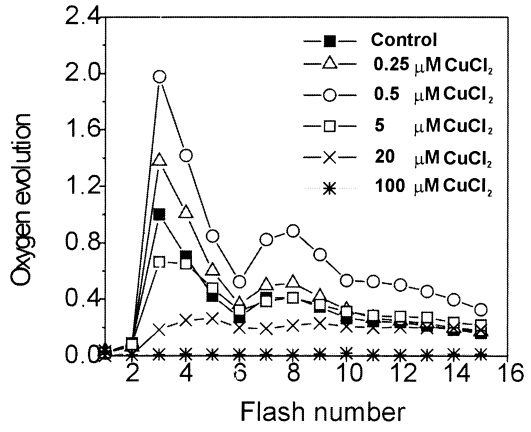

Fig. 1. Effect of $CuCl_2$ on flash-induced oxygen evolution in PS II BBY particles isolated from tobacco. Chl conc. – 42 μg/ml assay in 50 mM Hepes pH 7.0 containing 10 mM KCl, 5 mM $MgCl_2$ and 2.5 mM $CaCl_2$. All amplitudes have been normalized to the amplitude under the 3rd flash in the control sample.

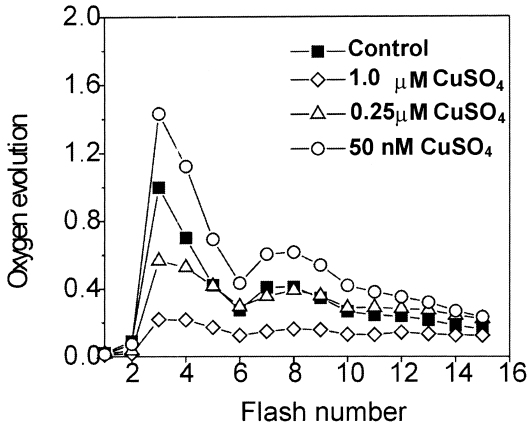

Fig. 2. Effect of $CuSO_4$ on flash-induced oxygen evolution in PS II BBY particles isolated from tobacco. Other conditions as in Fig. 1.

5S-state model developed by Burda and Schmid (1996). It is well known that the oxygen evolving complex (OEC) accumulates successively four positive charges in the Mn-active site before oxygen is evolved. The redox states of the Mn complex are assigned by S_i, where the subscript denotes the accumulated charges (i changes from 0 to 4). The probability of the non-successful transition between the $S_i \rightarrow S_{i+1}$ states is given by α_i (the miss parameter). The probability of the O_2 yield, accompanied by a fast $S_3 \rightarrow (S_4) \rightarrow S_0$ transition is described by parameter *d* (Burda and Schmid,

Table I. Parameters of the 5S-state model fitted to the experimental data of $CuCl_2$ – and $CuSO_4$ – treated PSII BBY particles and total oxygen evolution of these samples; S-state distribution S_i (i = 0, 1, 2); α_t – total miss parameter (the sum of probabilities of unsuccessful transitions between $S_i \rightarrow S_{i+1}$); *d* – probability of the fast $S_3 \rightarrow (S_4) \rightarrow S_0$ transition associated with oxygen evolution; rate of total O_2 evolution is the sum of O_2 evolution signals of all 15 flashes normalized to the control samples. The rate is given in arbitrary units (a.u.). The values are averages of 20 experiments with maximal deviation of ± 3%.

[μM]	S_0 (%)	S_1 (%)	S_2 (%)	α_t	*d*	Total O_2 evolution (a.u.)
$CuCl_2$						
0.00	19.1	74.5	6.9	1.46	0.38	1.00
0.25	13.7	83.6	3.4	1.69	0.33	1.26
0.50	17.2	79.8	2.7	1.69	0.18	1.93
1.00	15.2	79.9	5.6	1.58	0.30	0.93
5.00	32.7	60.9	7.1	1.03	0.30	1.00
10.00	33.3	63.4	2.3	1.06	0.35	0.97
20.00	–	–	–	–	–	0.54
100.00	–	–	–	–	–	0.00
$CuSO_4$						
0.00	19.1	74.5	6.9	1.46	0.38	1.00
0.05	20.7	75.9	3.6	1.57	0.28	1.51
0.25	25.0	68.8	5.4	0.96	0.33	0.92
1.00	33.9	61.8	5.3	1.08	0.33	0.40

1996; Burda and Schmid, 2001). The above parameters, fitted to the observed patterns of oxygen evolution, are given in Table I. It can be seen that higher Cu^{2+} concentrations, which alter either the initial S-state distribution or the transition probabilities between S-states, lowered the O_2 yield. Cu^{2+} cations at concentrations above 5 μM $CuCl_2$ or 1 μM $CuSO_4$ caused an increase of the S_0 state population and at the same time a decrease of the S_1-state population. The lowering of the total miss parameter, α_t, is accompanied by changes of the initial S-state distribution. The non-successful transitions between the $S_0 \rightarrow S_1$ states and the $S_2 \rightarrow S_3$ states give the main contribution to the α_t parameter. The decrease of α_t originates from more efficient transitions between the $S_0 \rightarrow S_1$ states. The parameter *d* was significantly influenced only by such Cu^{2+} concentrations which stimulated oxygen evolution.

The question to be asked is whether the stimulation of oxygen evolution at low Cu^{2+} concentrations is due to natively PSII-bound Cu^{2+} which during preparation was released from its binding site in PSII but can be restituted by the addition of external Cu^{2+} ions. Therefore, we analyzed the native Cu and Mn content of PS II preparations used for oxygen evolution measurements. Since it was suggested that Cu^{2+} can bind non-specifically to the LHC of PS II (Droppa and Horváth, 1990; Barón *et al.*, 1995), we also measured the Cu content in PS II BBY particles isolated from a chlorophyll deficient tobacco mutant (Okabe *et al.*, 1977). In the isolation procedure of PS II from the mutant, different Triton X-100/Chl ratios were applied to investigate whether the possible Cu-binding sites are sensitive to the detergent treatment. The results shown in Table II, normalized to 4 Mn atoms, indicate that there was 1 Cu/PS II in PS II isolated from the wild-type of tobacco and about 2 Cu/PS II in the LHC-mutant, independently on the detergent concentration used. The Chl/PS II ratios indicate that the LHC antenna size in the Su/su aurea mutant was 2–2.5 times reduced in comparison to that of the wild-type. This data suggests that Cu was not bound to the LHC in our PSII preparations.

Table II. Cu and Mn content in PS II particles isolated from wild-type tobacco *N. tabacum* JWB and from the LHC-mutant Su/su var. Aurea. The values in brackets denote the Chl/Triton X-100 ratio (w/w) used for the isolation of PS II particles. The values came from 3 different particle preparations which deviated by less than 5%.

Sample	Cu : Mn : Chl molar ratio
JWB (1:25)	1.0 : 4 : 244
Su/su var. aurea (1:5)	2.7 : 4 : 120
Su/su var. aurea (1:10)	2.1 : 4 :100
Su/su var. aurea (1:20)	2.5 : 4 : 93
Su/su var. aurea (1:25)	2.3 : 4 :108

We have not observed any release of the extrinsic PS II proteins (17 kDa, 23 kDa and 33 kDa) throughout the whole range of $CuCl_2$ and $CuSO_4$ concentrations.

Discussion

The strong stimulation of oxygen evolution by Cu^{2+} at low concentrations raises the question as to whether this effect is caused by a specific, so far not recognized, native Cu-binding site within PSII or by another metal-binding site where Cu^{2+} only substitutes for this metal. The potential candidates

could be Mn^{2+} or Ca^{2+}. Manganese substitution by Cu^{2+} seems rather improbable at low Cu^{2+} concentrations and would certainly give an inhibitory effect on oxygen evolution. Calcium substitution by Cu^{2+} is more probable and could take place at a non-specific binding site of Ca^{2+}, for example in the region of the extrinsic proteins of the OEC. A stimulatory effect on oxygen evolution at this site was already observed for europium and dysprosium ions (Burda *et al.*, 1995). However, in those experiments the lanthanides stimulated oxygen evolution in the absence of external Ca^{2+} ions, while the stimulation by Cu^{2+} in the present experiments was observed in the presence of an excess of Ca^{2+} ions ($5 \cdot 10^3$ Ca:1 Cu). This excludes the possibility that Cu^{2+} stimulates oxygen evolution at the Ca-binding site(s) in PS II. These observations might indeed suggest that there exists a specific Cu-binding site within PS II. The copper detected in our PS II particles was certainly not a contamination from starch and nuclear fractions since we used an isolation procedure which removes these fractions from PSII preparations (Barón *et al.*, 1993; Arellano *et al.*, 1994). Moreover, the higher Cu^{2+} content in PS II particles isolated from the LHC-deficient tobacco mutant (compared to the Cu^{2+} content in particles isolated from the wild-type) indicates that Cu^{2+} detected in our PS II preparations is not a component of the LHC.

Our results point to a high-affinity Cu-binding site within PSII since Cu^{2+} ions very efficiently stimulated oxygen evolution at concentrations as low as 1.5–3 Cu^{2+}/PS II for $CuCl_2$. The stimulatory effect of Cu^{2+} on oxygen evolution was observed only with fresh PS II preparations, suggesting that the Cu-binding site is labile and easily inactivated. It should be emphasized that the stimulatory action of Cu^{2+} does neither change significantly the initial S-states distribution nor the transition probabilities between the S-states. Copper ions only enhance the efficiency of oxygen evolution and this can indicate structural changes within the OEC caused by Cu^{2+}. It is known that copper ions, in a highly specific way, induce and stabilize α-helix and β-sheet conformations of peptides acting on the Trp-His interaction (Zou and Sugimoto, 2000). However, it cannot be excluded that Cu^{2+} interacts with Trp, His or Tyr residues in the vicinity of the Mn-complex. Manganese substitution by Cu^{2+} is improbable at such low copper concentrations and could result in the inhibition of oxygen evolution. It is clear that this binding site of copper(II) is sensitive to SO_4^{2-} ions, which do not compete at all with Cl^- (Critchley *et al.*, 1982; Lindberg and Andreasson, 1992). Such a specific amplification of interaction within PSII by SO_4^{2-} ions has been observed only for NH_4^+ (Schiller *et al.*, 1995), which has been shown to bind to the Mn site in the OEC (Beck and Brudvig, 1986).

Summarizing, we have shown a direct involvement of Cu^{2+} ions in the stimulation of oxygen evolution in PS II for an equimolar Cu^{+2}/PS II RC concentration in the presence of Ca^{2+}. This gives the evidence that the binding site of copper ions is different from that of lanthanides, which have been found to stimulate O_2 evolution at equimolar Eu^{3+} (Dy^{3+})/PS II RC concentrations but only in the absence of calcium ions.The Cu^{2+} binding site is sensitive to SO_4^{2-} ions.

Acknowledgements

This work was supported by grant No. 6 P04A 03817 from the Committee for Scientific Research (KBN) of Poland. K. B. thanks the European Commission for a IHP Marie Curie Fellowship.

The authors would like to thank Dr A. Radunz for his help by AAS measurements.

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