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Mössbauer Studies of Cu(II) Ions Interaction with the Non-Heme Iron and Cytochrome b_{559} in a *Chlamydomonas reinhardtii* PSI Minus Mutant

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Mössbauer spectroscopy was applied, for the first time, to study the interaction of copper ions with the non-heme iron and the heme iron of cytochrome b_{559} in photosystem II thylakoids isolated from a *Chlamydomonas reinhardtii* photosystem I minus mutant. We showed that copper ions oxidize the heme iron and change its low spin state into a high spin state. This is probably due to deprotonation of the histidine coordinating the heme. We also found that copper preserves the non-heme iron in a low spin ferrous state, enhancing the covalence of iron bonds as compared to the untreated sample. We suggest that a disruption of hydrogen bonds stabilizing the quinone–iron complex by Cu^{2+} is the mechanism responsible for a new arrangement of the binding site of the non-heme iron leading to its more "tense" structure. Such a diamagnetic state of the non-heme iron induced by copper results in a magnetic decoupling of iron from the primary quinone acceptor. These results indicate that Cu does not cause removal of the non-heme iron from its binding site. The observed Cu^{2+} action on the non-heme iron and cytochrome b_{559} is similar to that previously observed for α-tocopherol quinone.

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1. Introduction

Physiological function of some metals in photosystem II (PSII) and their action at toxic concentrations are still not fully understood. Copper, being a micronutrient extensively applied as a fungicide in agricultural practice, is one of the examples. In the photosynthetic electron transport chain, a copper ion is a component of plastocyanin, which is involved in the electron transfer between the PSII and PSI [1]. Recently it has been found that at very low, equimolar Cu^{2+}/PSII reaction center proportions copper stimulates oxygen evolution [2]. However, at elevated concentrations Cu(II) inhibits the photosynthetic electron transport [3–5]. Photosystem II is more sensitive to Cu^{2+} action than photosystem I [3–5]. In the literature, various sites of copper action have been suggested. Different action sites of copper ions on PSII are certainly dependent on the relative Cu(II) to reaction center proportions. The obtained results suggest that copper inhibits either the donor- or the acceptor side in PSII. The earliest report stated the proximity of a Cu binding site to the oxygen evolving complex (OEC) [6]. Further studies confirmed the existence of a highly sensitive site for Cu-inhibition on the oxidizing side of PSII [7–9]. Copper ions have been even suggested to substitute calcium and manganese ions in the OEC [10]. A reversible influence of Cu^{2+} on the redox states of tyrosine Z (Tyr Z) and tyrosine D (Tyr D) on the donor side of photosystem II has been reported [11, 12]. Simultaneously, the irreversible Cu(II) induced inactivation of the acceptor side has been observed [12]. The primary quinone acceptor Q_A [12], the pheophytin(Pheo)–Q_A–Fe region [13], the non-heme iron [14, 15] and a secondary quinone acceptor Q_B [16] have been identified as targets of Cu^{2+} action. Copper ions have also been found to influence redox properties of cytochrome b_{559}, one of the most intriguing components of PSII. It has been shown that Cu(II) decreased the level of photoreduced cytochrome b_{559} and slowed down its rate of photoreduction [17]. A conversion of the high potential (HP) form of cytochrome b_{559} to the low potential (LP) form has been reported in photosystem II treated with high concentrations of copper ions [12]. However, recent studies have demonstrated that Cu^{2+} does not influence the level of the HP and LP forms of cytochrome b_{559} [18]. Moreover, it has been shown that copper ions may oxidize the LP form of cytochrome b_{559} at low concentrations (several Cu^{2+} ions per reaction center) and the HP form of cytochrome b_{559} at higher concentrations [18]. A mechanism has been proposed responsible for Cu(II)-induced fluorescence quenching and inhibition of oxygen evolution in photosystem II based on the interactions of copper ions with Tyr Z, both forms of cytochrome b_{559} and chlorophyll Z [18].

In this report, we present studies on the interaction of copper ions with the non-heme iron and cytochrome b_{559} using the Mössbauer spectroscopy. This experimental method is unique for investigations of local electronic and structural properties of the probe, in our case ^{57}Fe. It gives also direct information on diamagnetic states of the iron and in this respect it can be used as a method

complementary to EPR. The experiments have been performed on intact thylakoid membranes isolated from a mutant of *Chlamydomonas reinhardtii* deficient in photosystem I (PSI minus) to avoid in the Mössbauer spectra contribution of other Fe components than those of photosystem II [19]. The results indicate that copper oxidizes the heme iron changing its low spin state into a high spin state whereas it keeps the non-heme iron in a low spin reduced state. The possible role of the non-heme iron and cytochrome b_{559} in cyclic electron flow around PSII and their role in the protection against photoinhibition based on their interactions with copper ions is discussed.

2. Materials and methods

A PSI-deficient mutant of *Chlamydomonas reinhardtii* was cultivated in tris-acetate-phosphate (TAP) buffer medium [20] containing Hunter's trace solution enriched in the iron isotope ^{57}Fe. $FeSO_4 \cdot H_2O$ in the trace metal solution was replaced by $^{57}FeCl_3$. The content of ^{57}Fe in the growth medium was about 4.5 times higher than that reported by other groups [21]. Thylakoid membranes were isolated according to Diner and Wollman [22] by sucrose cushion centrifugation. The PSI deficiency of strain Xba9 was caused by the disruption of a psaA exon downstream of the psbD gene. The mutation was achieved by an in vitro mutagenesis approach, using the plasmid vector pCA1 and the aadA-selectable marker [23] for chloroplast transformation with a gun-powder driven DNA particle-delivery instrument (Shearline–MK2, UK). Copper ions were added to the sample in a form of $CuCl_2$ solution at a Cu^{2+}/Chl molar ratio of 1:1. The sample was incubated in the presence of copper ions in darkness at room temperature.

We applied a gas-flow cryostat to record the Mössbauer spectra. Measurements were performed in the temperature range of 78–200 K. Temperature stabilization was within 0.1 K. The source of γ-ray, 40 mCi Co/Cr was kept at room temperature. Isomer shifts are given vs. metallic Fe at room temperature.

Our control sample contained PSII thylakoid membranes with a total chlorophyll content of 50 mg. These PSII thylakoids contain 2.0–3.2 iron atoms per reaction center as calculated from the Mössbauer effect (assuming 160–250 chlorophyll molecules per reaction center). This sample was later divided into several subsamples, each of which was used in different measurements in order to make sure that each subsample originates from the same reference sample material. The iron content of each subsample was large enough to ensure the total absorption effect of 0.5% for doublets. The Mössbauer effects of this magnitude are well detectable and can be used in the quantitative analysis.

3. Results and discussion

The high quality of the spectrum shown in Fig. 1A for a control sample allowed us to exclude a single doublet fit with broadened lines. We fitted the most general form, which could be fitted to the data, namely the data was evaluated

assuming a Gaussian distribution of the quadrupole splitting as shown in the inset of Fig. 1. In this case an isomer shift was a fitted parameter but a line width was a fixed parameter and set to be 0.23 mm/s as has been obtained from the fit of the subspectra shown in Fig. 1B. Such line widths of about 0.23 ± 0.02 mm/s suggest homogeneous iron states. From the experimental spectra obtained for the samples treated with α-tocopherol quinone (α-TQ) [19] or Cu(II) we could learn that they are a superposition of two components of comparable fraction of the total signal. Since they contained the same material as the reference sample, we could

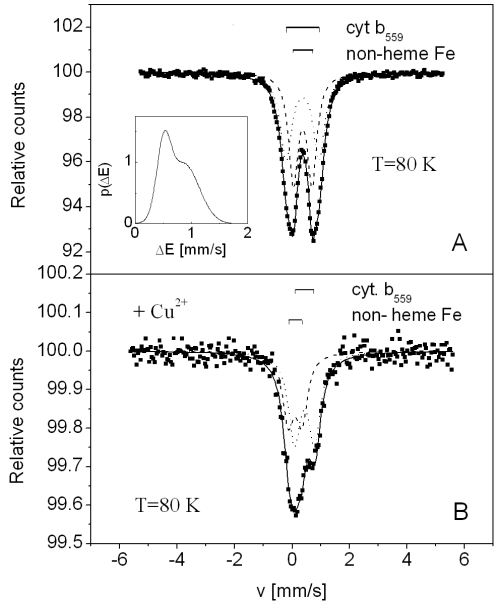

Fig. 1. ^{57}Fe Mössbauer absorption spectra of PSII thylakoid membranes isolated from the *Chlamydomonas reinhardtii* Xba9 PSI$^-$ mutant: (A) untreated sample at $T = 80$ K, (B) after dark incubation with Cu^{2+} at 80 K. Subspectra correspond to the heme iron in cytochrome b_{559} and the non-heme iron. In the inset in (A) the quadrupole splitting distribution in the case of the untreated sample is presented.

immediately conclude that the spectrum shown in Fig. 1A also contains basically two fractions. For further discussion, based also on the fact that the obtained distribution of quadrupole splitting shows clearly two maxima (inset in Fig. 1A), the spectra of thylakoid membranes recorded at temperatures below 269 K were fitted assuming two independent doublets. The fitted quadrupole splittings of the subspectra correspond to values estimated from the Gaussian distribution. The doublets have different splittings and similar isomer shifts within a wide range of temperatures (Fig. 1A, Fig. 2). The slight asymmetry of the spectrum shown in Fig. 1A comes from the small difference in isomer shifts (see Fig. 2A) and contri-

butions of the two components at 80 K. The ratio of the fraction of the component, later assigned to the non-heme iron, and of the one ascribed to the heme iron is about 1.2 : 1 (at 80 K). This ratio results from the different Debye temperatures of those two iron binding sites [19]. The fitted hyperfine parameters are presented

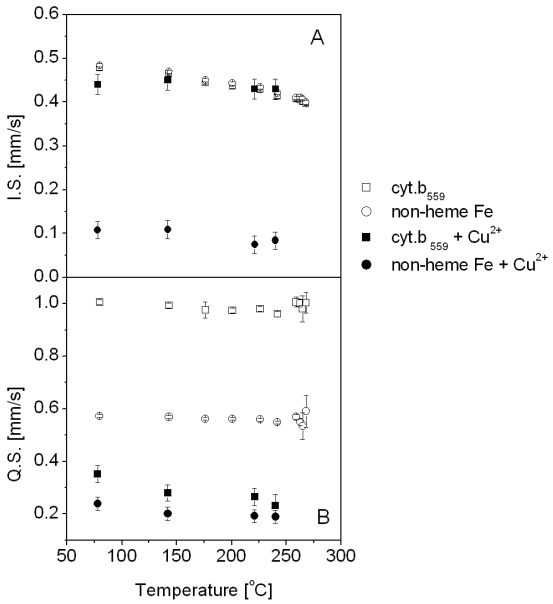

Fig. 2. Temperature dependence of (A) isomer shifts and (B) quadrupole splittings of the heme iron in cytochrome b_{559} (squares) and the non-heme iron (circles) in untreated PSII thylakoids isolated from the *Chlamydomonas reinhardtii* Xba9 PSI^- mutant (open symbols) and incubated with Cu^{2+} (closed symbols). Let us note that some error bars are of the size of symbols.

in Fig. 2 ((A) isomer shifts, (B) quadrupole splittings). It can be concluded that there is no reduced high spin state of iron. The treatment of thylakoids with hydroquinone and ascorbate did not influence the hyperfine parameters of the two states of iron. This confirms that both components originate from low spin Fe^{2+}. Differences in quadrupole splittings indicate two different environments of the iron atoms. The component characterized by a quadrupole splitting of about 1 mm/s can be ascribed to cytochrome b_{559} [19, 24–26] and that one characterized by a quadrupole splitting of about 0.55 mm/s to the non-heme iron [19]. The more detailed description of these data is given in [19]. Figure 1B shows a Mössbauer spectrum of PSII thylakoid membranes treated with Cu^{2+} at 80 K. The spectra of Cu^{2+} treated samples consist of two symmetric quadrupole doublets with apparently different hyperfine parameters compared to those obtained in the case of untreated thylakoids. The fitted parameters are presented in Fig. 2. The com-

ponent characterized by an isomer shift of about 0.44 mm/s and a quadrupole splitting of about 0.3 mm/s is assigned to a high spin state of Fe^{3+}, whereas the component with an isomer shift of about 0.1 mm/s and a quadrupole splitting of about 0.2 mm/s to a low spin state of Fe^{2+}.

3.1. Cytochrome b_{559}

Copper ions may oxidize both potential forms (HP and LP) of cytochrome b_{559} [18] but not the non-heme iron [11, 15]. Consequently, the high spin oxidized state of iron originates from the heme iron of cytochrome b_{559}. Having in mind that the midpoint redox potential of Cu^{2+} ($E_{m,7} = +0.167$ V) is too low to oxidize directly the HP form of cytochrome b_{559}, one may expect that the change of the heme-iron valence state is rather due to a deprotonation process [18, 27]. The quadrupole splitting of high spin Fe^{3+} assigned to the heme iron results from anisotropic covalence of iron bonds and their tetragonal distortion that lowers the symmetry of the iron complex to D_{4h}. Therefore, the most probable target of Cu^{2+} is one of the histidine residues coordinating the heme of cytochrome b_{559} [18]. A role of the hydrogen bond between one of the imidazole nitrogen and an amide carbonyl group of the polypeptide chain has been suggested to cause the conversion between different potential forms of cytochrome b_{559} [27]. It seems that the geometry of the axial histidines of the heme-iron [28] as well as its exposure to a more hydrophilic environment are responsible for the variable character of cytochrome b_{559} potential forms [29].

The oxidation of cytochrome b_{559} by copper ions has been already reported [12]. Contrary to a high spin state of heme Fe^{3+} reported here, the authors of Ref. [12] speak of a low spin ferric state, which they assigned to the low potential form of cytochrome b_{559}. This is in contradiction to our finding, which has been confirmed by others [30], that Cu^{2+} does not convert the HP form of cytochrome b_{559} to its LP form [18]. It is evident that the Mössbauer spectra presented here do not contain any contribution of a low spin ferric state. To our current knowledge, only the considerably higher copper concentrations used in the EPR measurements could be a possible source of these discrepancies. This issue should be further investigated. High spin oxidized iron states of cytochrome b_{559} have been observed occasionally by different groups and some of these states have been ascribed to native forms of cytochrome b_{559} [31–33]. Usually, in samples treated with high potential quinones, particularly 2,3-dichloro-5,6-dicyano--*p*-benzoquinone (DDQ), a high spin Fe^{3+} state was detected. The appearance of a high spin ferric ion was explained by OH^- ligation of the heme iron [32, 33] or by a direct interaction of a high potential quinone with the iron of cytochrome b_{559} via substitution of one of the imidazole groups from the axial position [34]. However, of particular interest in the context with our copper action on photosystem II is the conversion of the low spin ferrous heme iron into a high spin ferric one in PSII thylakoid membranes from the *Chlamydomonas reinhardtii* PSI minus mutant after treatment with α-TQ. Apart from the similar influence of α-TQ and Cu^{2+} on

the Mössbauer spectra of Xba9 thylakoids [19], it is known that α-TQ acts on photosystem II in a way similar to copper ions: (a) α-TQ ($E_{m,7} = 0$ mV) has a redox potential even lower than the Cu^{2+}/Cu^{+} couple [35], (b) α-TQ is not an electron acceptor of PSII [36], (c) it inhibits electron transport slowing down the mode of oxygen evolution and affects the S-states distribution of the oxygen evolving complex [18, 36], (d) it oxidizes both potential forms of cytochrome b_{559} in darkness [18, 37, 38] and (e) quenches effectively PSII fluorescence probably by a mechanism similar to that of carbonylcyanide-*p*-trifluoromethoxy-phenyl-hydrazone (FCCP) [18, 38, 39]. Therefore, the model of electron transfer pathways within PSII show-

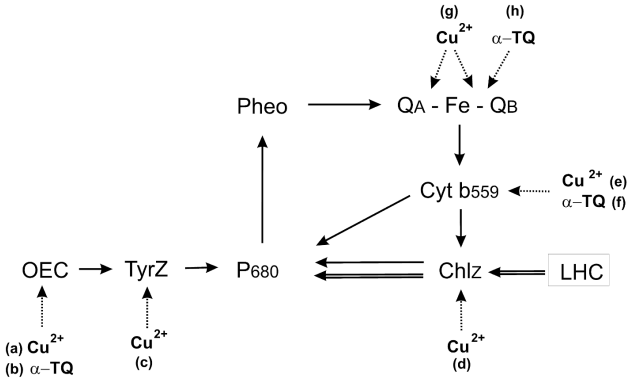

Fig. 3. Scheme showing the predicted sites of Cu^{2+} ((a) [2, 7, 10], (c) [2, 11, 12, 18], (d) [12, 18], (e) [12, 17, 18, 30, this article], (g) [12–16, 40, 41, this article]) and α-TQ ((b) [36], (f) [19, 38], (h) [19]) interaction with PSII components. Their influence on electron ($\rightarrow$) and energy ($\Longrightarrow$) transfer routes causing inhibition of oxygen evolution and fluorescence quenching are indicated.

ing possible interaction sites of Cu(II) responsible for the inhibition of oxygen evolution and fluorescence quenching [18] can be directly adapted also for α-TQ (Fig. 3).

3.2. Non-heme iron

The action of copper ions on the acceptor side of photosystem II ($Q_A - Fe - Q_B$) is still an intriguing and unresolved issue. Depending on the material used and experimental methods applied, various binding sites of Cu^{2+} within the quinone–Fe complex have been suggested. Copper has been found to affect the binding affinity of atrazine to PSII [40] and to modify the Q_B pocket [16]. Flash-induced absorption spectroscopy studies have shown that the primary charge separation ($P680^{+}Q_A^{-}$) is not modified by copper ions [11]. Nevertheless, EPR measurements provide evidence that Cu(II) leads to a magnetic decoupling of $Q_A^{\cdot -}$ from the non-heme iron [11, 12, 41]. The appearance of an EPR signal at $g = 2.0044$, attributed to a free radical originating from the semiquinone form of the primary

quinone electron acceptor [42], was interpreted as the non-heme Fe^{2+} displacement or at least an alteration of the iron binding site by copper ions. It has been shown that other metal ions (Zn^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, and Mn^{2+}) may substitute the non-heme iron reversibly, but only moderately are able to slow down the electron transfer in bacterial reaction centers [43]. In PSII reaction centers, depletion of the iron is much more difficult and attempts of its substitution usually failed [44, 15] although there is a close similarity of the binding site of $Q_A^{\cdot -}$ in photosystem II and in bacterial reaction centers [45].

The detection of the semiquinone radical of the primary electron acceptor allows for another possible interpretation than removal of the non-heme iron from its binding site, namely that a high spin state of the ferrous ion is converted into a low spin ferrous state due to the binding of Cu(II) in the vicinity of the quinone–iron complex. The results presented here indicate that the low spin state of the reduced iron is strongly influenced by Cu^{2+} causing more covalent binding of the Fe^{2+} in the quinone–iron complex. This means that the interaction of Fe^{2+} with the ligands is stronger and the length of the Fe bonds decreases upon sample treatment with Cu(II). For example, ligation of Fe^{2+} by cyanide also leads to the formation of low spin Fe^{2+} [46]. Recently, a low spin state of non-heme Fe^{2+} has been observed in PSII treated with α-TQ [19]. The hyperfine parameters of the ferrous ions with a spin state $S = 0$ are similar in these cases. The much lower isomer shift observed here, than in the case of CN^- or α-TQ treatments, results from higher ligand–metal σ-donation and metal–ligand π-donation (π — back-donation) due to the higher overlapping of iron and ligand orbitals. The difference in the strength of non-heme iron bonds in its diamagnetic state is even larger in comparison to the high spin state of Fe^{2+} iron usually observed in PSII and bacterial reaction centers [24, 47–49].

How does Cu^{2+} influence the chemical bonds within the $Q_A{-}Fe{-}Q_B$ complex? The postulated impairment of the electron transfer between the primary and secondary quinone acceptors in PSII by copper ions can be explained taking into account the architecture of the quinone–iron complex and its location in the protein matrix. It should be realized that there are some differences in the properties of the non-heme iron between bacterial reaction centers and PSII. In both cases, the iron has four conserved histidine ligands but the fifth ligand is glutamate in purple bacteria, whereas in PSII it is most probably replaced by bicarbonate [50]. It is obvious from the proposed models of the $Q_A{-}Fe{-}Q_B$ complex [51, 52] that hydrogen bonds are important in the stabilization of the quinone–iron complex. Thus, Cu^{2+} competing for hydrogen may significantly disrupt the structure of the complex and modify the interaction between its components. It has been reported that cyanide binding at the non-heme Fe^{2+} causes the loss of magnetic interaction of $Q_A^{\cdot -}$ with the iron due to conversion of the high spin ferrous state into a low spin ferrous state [46]. The diamagnetic state of the non-heme iron observed in these studies due to the action of Cu^{2+} gives evidence that the free radical signal of

uncoupled semiquinone $Q_A^{\cdot -}$, observed in EPR experiments may also result from the conversion of a high to low spin Fe^{2+} state. The g-tensors of the electron acceptor radical anion $Q_A^{\cdot -}$ in untreated PSII or PSII treated with Cu^{2+}, Zn^{2+}, CN^- [15, 44, 53] support independently this conclusion. Such a modification of the quinone–iron complex induced by Cu(II) may lead to a more tense structure of the iron ligands with a simultaneous increase in the Q_A–Fe distance but without influencing the Pheo–Q_A interaction. According to the models proposed for the quinone–iron complex [51, 52], analogous modification may cause disconnection between Fe–Q_B and finally lead to the breaking of the linkage between the primary and secondary quinone acceptors. As a result of this, the electron transfer within the quinone–iron complex could be inhibited. A histidine residue has low selectivity towards transition metals and therefore all of them may give similar effects as copper ions, for example Zn^{2+} ions. On the other hand, one should also consider other possible interactions as for example deprotonation of peptide nitrogen in the vicinity of the non-heme iron. In theory, such an action is predicted exclusively for copper ions [54]. Indeed, hydrogen binding to the peptide amide nitrogen, which stabilizes the quinone–iron complex, is predicted: such as between Q_A and Phe261 of protein D2 and between Q_B and Phe265 of protein D1 [52].

Taking into account other copper ligands than only imidazole nitrogen, as for example tyrosine Tyr-, one can expect more than one Cu(II) binding site within the quinone–iron complex, as has been suggested in [41]. It is clear that more complex studies are necessary to give an unambiguous answer on the copper interaction with the Q_A-Fe-Q_B complex. However, from the data presented here it is obvious that Cu^{2+} may convert the non-heme iron into a diamagnetic state via structural modifications of the quinone–iron complex.

These Mössbauer measurements together with the data reported in [18, 19, 36] indicate common action sites of copper and α-TQ within PSII as shown in Fig. 3. These interactions can be responsible for similar modifications of the electron and energy transfer within photosystem II. Some of these actions could be explained by deprotonation processes in the vicinity of the heme- and non-heme iron.

Acknowledgments

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