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Alterations in Photosystem II electron transport as revealed by thermoluminescence of Cu-poisoned chloroplasts

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Abstract

The thermoluminescence characteristics of functionally intact thylakoids and TRIS-washed BBY particles were studied under Cu(II) poisoned conditions. In thylakoids, both the A and B thermoluminescence bands corresponding to S_3QA^- and $S_2S_3QB^-$ charge recombinations, respectively showed specific responses to Cu(II) treatment. The amplitude of the B band was gradually decreased, which corresponds to the Cu(II) induced inactivation of TyrZ. The simultaneous stepwise shift in the peak position of the B band indicated, however, that S_3QB^- charge combination is more resistant to Cu(II) poisoned conditions. The shifted peak position of the A band toward the higher temperature in Cu(II) treated thylakoids also showed a change in the redox span between the recombination partners generating the A band of the glow curve. The A_T band due to the $HisCQA^-$ recombination in TRIS-washed BBY particles was insensitive to Cu(II) addition indicating that Cu(II) did not affect either HisC or QA^- . The unaffected intensity of the A and A_T bands when Cu(II) inhibits TyrZ function favours the assumption of an alternative pathway in which functional TyrZ is not required. In addition, Cu-induced changes of the TL bands were compared to those produced by the Tyr and His modifiers NBD and DEPC, respectively. We obtained very similar results regarding TL bands by either adding NBD or Cu-poisoning in functional thylakoids. Regarding DEPC, the A and A_T bands were abolished by increasing concentrations of the His modifier. This effect was associated with the decrease of the B band and its replacement by the Q band at around 0 °C. Comparing our data obtained by Cu, NBD and DEPC treatments, we have found a strong interrelation between HisC and S_3 state. We assume that in some inhibitory conditions in the S_3 state His is oxidized in place of Mn and this alternative pathway does not require functional TyrZ.

Abbreviations: DCMU– 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DEPC– diethyl pirocarbonate; NBD– 7-chloro-4-nitrobenz-2-oxa-1,3-diazole; PS II – Photosystem II; QA and QB – the primary and secondary quinone acceptors of PS II; TL – thermoluminescence; TyrZ – tyrosine species acting as a donor to P680.

Introduction

A large number of publications have demonstrated that the main action site of Cu(II) inhibition is the PS II mediated electron transport (for review see Droppa and Horváth 1990; Barón et al. 1995). On the one hand, the acceptor side of PS II was found to be affected by Cu. Different reports have proposed the acceptor side of PS II (Yruela et al. 1992, 1993, 1996; Mohanty et al. 1989; Renger et al. 1993), the reaction center (Hsu and Lee 1988; Renganathan and Bose 1989) or the donor side (Cedeño-Moldonado and Swader 1972; Schioi et al. 1978a,b; Samson et al. 1988) as putative Cu-inhibitory sites. Most recent publications have indicated that the different theories concerning the action sites of Cu(II) toxicity has to be reevaluated (Renger et al. 1993; Králová et al. 1994; Schröder et al. 1994; Jegerschöld et al. 1995; Arellano et al. 1995). Evidence was presented that the main donor side inhibition of Cu(II) was the deactivation of TyrZ with a concomitant appearance of a new, unidentified radical at $g=2.0028$ with a spectral shift of 9.5 G. Additional effects under Cu(II) toxicity conditions were also observed in the oxygen evolving complex and in the acceptor side with uncoupling of QA⁻ from the non-heme ferrous ion (Jegerschöld et al. 1995).

Although the Cu-sensitivity of PS II electron transport is well documented (Droppa and Horváth 1990; Barón et al. 1995; Böhner et al. 1980; Singh and Singh 1987; Samuelson and Öquist 1980; Renganathan and Bose 1990), the site(s) and the mechanism of Cu(II) inhibition are still a matter of debate. For obtaining further information about the inhibitory mechanism of Cu(II) within PS II, thermoluminescence (TL) seems to be a useful technique, since it originates from the charge recombination between positively charged donors and negatively charged acceptors of PS II and even small changes in the redox properties of PS II electron transport are reflected in TL characteristics (Horváth 1986; Demeter and Govindjee 1989; Vass and Govindjee 1996).

Functionally active O₂ evolving thylakoids or PS II enriched preparations, depending on the measuring conditions, exhibit characteristic bands of TL between -30 and -10 °C and +17 and +35 °C designated as A and B bands, respectively (Inoue and Shibata 1982). The A band was found to be due to S₃QA⁻ charge recombination (Koike et al. 1986; Demeter and Govindjee 1989; Vass and Govindjee 1996). The B band is an integration of two overlapping bands, namely B₁ (+17 °C) and B₂ (+37 °C) bands corresponding to S₃QB⁻ and S₂QB⁻ recombination, respectively (Inoue and Shibata 1982). Deactivation of the water splitting complex with either NH₂OH or TRIS completely abolished the B band due to the loss of the S

states. In contrast, the A band (very probably corresponding to the A_T band) seems to be resistant to such a treatment (Demeter et al. 1985). In PS II samples, where the electron donation from the Mn-cluster is interrupted by different treatments as mentioned above a new TL band appears. The so-called A_T band was peaked at similar temperature as the A band in functionally intact membranes. Earlier studies about the origin of this band led to the conclusion that the recombination partner on the donor side is different from that of the A band and, therefore, it was designated as A_T band (Koike et al. 1986).

Comparative studies of the EPR signal II_{fast} and TL characteristics as well as treatment of thylakoids with the histidine modifier diethyl pyrocarbonate (DEPC) led to the conclusion that the A_T band originated from HisCQA- recombination (Ono and Inoue 1991; Allakhverdiev et al. 1992). Site directed mutagenesis of *Chlamydomonas reinhardtii* showed that the replacement of His195 in the vicinity of Tyr_z did not influence the peak position of the A_T band, while the conversion of His190 residue to phenylalanine completely abolished this band (Kramer et al. 1994). This is in good agreement with the earlier observation that the tyrosine modifier NBD (7-chloro-4-nitrobenz-2-oxa-1,3-diazole) had no influence on the A_T band (Allakhverdiev et al. 1992).

In the present study therefore, we have used TL to investigate Cu(II) induced changes within PS II electron transport. We have found that the increasing concentration of Cu(II) added to functionally intact thylakoids resulted in a gradual decrease of intensity of the B band with a stepwise shift in its peak position toward the lower temperature. In contrast, an upward shift was observed in the peak position of the A band without any changes in its intensity indicating different action mechanism of Cu(II) for the A and B band. The A_T band of TRIS-washed BBY particles was completely resistant to Cu(II) treatment up to 500 μM. In addition we have compared the Cu effect on the TL bands with those produced by both the NBD and DEPC, a Tyr and a His modifier, respectively.

Materials and methods

Spinach thylakoids were prepared as described by Ford and Evans (1983). Oxygen evolving PS II (BBY) particles were isolated according to the method of Berthold et al. (1981), with modifications of Arellano et al.

(1994), in which the Triton X-100 treated thylakoids were centrifuged for 4 min at $10\,000 \times g$ in order to remove contaminating starch, DNA and metal ions.

Both thylakoids and BBY particles were stored at high concentration (5–6 mg chl/ml) at $-80\text{ }^{\circ}\text{C}$ in a buffer containing 50 mM MES (pH 6.5) 15 mM NaCl, 5 mM MgCl_2 and 400 mM sucrose. The oxygen evolving capacity of the samples was checked in a Clark type O_2 electrode in an assay medium containing 20 mM MES (pH 6.5), 10 mM NaCl, thylakoid membranes equivalent to $17\text{ }\mu\text{g chl/ml}$ and 0.4 mM 2,6-dimethyl-*p*-benzoquinone as electron acceptor. The values varied between 157–198 $\mu\text{mol O}_2/\text{mg chl}\cdot\text{h}$ depending on the preparation used.

TRIS-washed PS II particles were prepared by washing the BBY membranes at $4\text{ }^{\circ}\text{C}$ in the dark for 30 min with 0.8 M TRIS at pH 8.4. The TRIS buffer was removed by washing the membranes twice in storage buffer (Ljungberg et al. 1986). Before TL measurements, samples were diluted to chlorophyll equivalent of $250\text{ }\mu\text{g/ml}$ containing 60% of glycerol to avoid the distortion of TL curves during the ice to water transition (Vass et al. 1981).

TL of 0.5 ml suspension containing $125\text{ }\mu\text{g chl}$ was measured using an apparatus similar to that described earlier (Demeter et al. 1979). The light source was a 650 W tungsten lamp provided 10 W/m^2 intensity at the surface of the sample. Samples were illuminated at the specified temperature and rapidly cooled down to $-80\text{ }^{\circ}\text{C}$. TL was measured during heating the sample in darkness with a constant heating rate of $20\text{ }^{\circ}\text{C/min}$.

Results and discussion

Control thylakoids isolated from spinach exhibited the A band at $-17\text{ }^{\circ}\text{C}$ and the B band at $+30\text{ }^{\circ}\text{C}$ and both bands were sensitive to PS II herbicides (Figure 1A). As has been shown previously, the A and B bands corresponded to S_3QA^- and $\text{S}_2\text{S}_3\text{QB}^-$ charge recombinations, respectively, and were strongly influenced by inhibitors interrupting electron transport between QA and QB (for review see Horváth 1986; Demeter and Govindjee 1989; Vass and Govindjee 1996). The elimination of both bands with PS II herbicides can be explained by the fact that neither S_3 state is formed (due to one turnover) nor QB reduction can occur (Demeter et al. 1979; Droppa et al. 1981). Treatment of thylakoids with increasing concentrations of Cu(II)

showed a drastic decrease in the intensity of the B band, while that of the A band was not significantly decreased (Figures 1B, C and 2). The decrease of the amplitude of the B band is in agreement with the earlier observation of Mohanty et al. (1989), who found a gradual decrease in the B band intensity by increasing Cu(II) concentration. Since they used one single turnover flash excitation which allowed $S_1 \rightarrow S_2$ transition only, the A band was not generated in their experiments. In our experiments, however, continuous illumination generated the A band as well. Characteristic bandshifts were also observed after Cu(II) addition (Figure 3). Increasing the Cu(II) concentration resulted in a stepwise shift toward the lower temperature in the position of the B band. Up to 20 μM of Cu(II) the B band peaked at around +30 °C, which corresponds to $S_2(S_3)Q_B^-$ recombinations (Horváth 1986; Demeter and Govindjee 1989). Between 50–100 μM of Cu, the B band was positioned at around +24 °C indicating that $(S_2)S_3Q_B^-$ recombination became dominant. At higher concentrations than 500 μM Cu, only $S_3Q_B^-$ recombination was observed indicated by the shift of the B band to +15 °C. Mohanty et al (1989) also found a slight (3–4 °C) downshift of the peak position. Their conclusion that Cu(II) interacts with the Q_B binding site hardly explains our finding since they demonstrated that Cu(II) had only a minor effect on the redox potential of the Q_B . An alternative explanation for Mohanty's observation is that the slight bandshift may be due to a small proportion of S_3 state generated by double hits (Joliot and Joliot 1981). The large (15 °C) temperature shift in the peak position of the B band under Cu(II) poisoned condition observed in our experiments, therefore, seems to be more likely a donor side effect of Cu(II). These results seem to be in agreement with the Cu(II) induced inactivation of TyrZ as found by Schröder et al. (1994) and Jegerschöld et al. (1995).

The A band also showed an upward shift in the peak position without any decrease in the intensity of the band (cf. Figures 2 and 3). The temperature shift of the A band indicated that the increasing Cu(II) concentration led to changes in the redox span between the positive and negative charge located on redox partners generating the A band (Vass et al. 1981).

Since the shift in the peak positions of both A and B bands showed similar concentration dependence of Cu(II) poisoning, we might assume that the observed bandshifts belongs to the donor rather than the acceptor side of PS II since the S states are the common pool for positive charges of both bands. This assumption also seems to be supported by the work of Jegerschöld et al. (1995) who demonstrated that Cu treatment resulted in: (a) at least 50% lost of Mn ions; (b) complete release of the 16 kD extrinsic subunit;

(c) irreversible conversion of the high potential cyt *b559* to a low potential form; (d) inhibition of the electron donation between Tyr_z and P₆₈₀ (Krállová et al. 1994; Jegerschöld et al. 1995). The Tyr_z deactivation by Cu(II) and, as a consequence, the inhibition of electron donation to P₆₈₀ well explains the complete elimination of the B band by high Cu(II) concentrations. The unaffected intensity of the A band otherwise indicates that the formation of S₃ states as well as the charge recombination generating this band does not require functional Tyr_z in such conditions.

If the above mentioned effects of Cu-poisoning are really the consequence of Tyr_z inhibition, we must get very similar results by adding the Tyr_z modifier NBD (Deters et al. 1975; Allakhverdiev et al. 1992) to functional thylakoids. As shown in Figure 4, the increasing concentration of NBD gradually decreases the intensity of the B band with a concomitant shift of the peak position from +32 °C to +19 °C corresponding to S₂(S₃)Q_B⁻ and (S₂)S₃Q_B⁻ charge recombination, respectively (Horváth 1986; Inoue and Shibata 1982; Demeter and Govindjee 1989). These data also demonstrate that the S₂Q_B⁻ recombination (B₂ band) very quickly disappears after the Tyr_z inhibition while the S₃Q_B⁻ recombination pathway (B₁ band) is more resistant against the Tyr_z desactivation. Simultaneously, the shift of the A band also could be observed similarly to that of Cu-poisoned thylakoids (cf. Figures 1 and 4). Comparing our results obtained by either Cu(II) or NBD treatments, we can conclude that in our experimental conditions Cu(II) addition primarily inhibits Tyr_z on the donor side of PS II.

In TRIS-washed thylakoids or TRIS-treated BBY particles, the A band corresponding to S₃Q_A⁻ is replaced by the A_T band (Koike et al. 1986) which originates from HisCQ_A⁻ charge recombination (Ono and Inoue 1991; Allakhverdiev et al. 1992). In our further experiments the relationship between the A and A_T bands were tested by applying the histidine modifier DEPC (Allakhverdiev et al. 1992; Ehrenberg et al. 1976; Hedge et al. 1993). As shown in Figure 5, both the A and A_T band could be abolished by the increasing concentrations of DEPC. In thylakoid membranes the disappearance of the A band was associated with an upward shift of the peak position of the B band and at higher DEPC concentrations a drastic reduction of the amplitude with a concomitant appearance of a new band at around 0 °C (Figure 5A). The shift in the peak position of the B band indicates that the S₂(S₃)Q_B⁻ is substituted with S₂Q_B⁻ charge recombination (Inoue and Shibata 1982; Horváth 1986; Demeter and Govindjee 1989). The decrease in the intensity of the B band and its replacement with the band at around 0 °C (Q band) is in a good agreement with the earlier finding

demonstrated that the higher concentrations of DEPC inhibited electron transfer between QA and QB and, therefore, only S₂QA⁻ charge recombination (Q band) could be observed (Hedge et al. 1993). The fact that the histidine modifier DEPC abolishes the A band generated by S₃QA⁻ recombination indicates that histidine is in a strong connection with the S₃ state (Boussac et al. 1990; Berthomieu and Boussac 1995; Haumann et al. 1997).

As shown in Figure 6 the A_T band which was also abolished by DEPC can be generated with higher temperature excitation but it cannot be charged with low temperature (-80 °C) illumination because S₃ states are not formed (Demeter et al. 1985). In contrast to the A band (Figures 3 and 4), both the amplitude and peak position of the A_T band were absolutely unaffected by Cu(II) treatment (Figure 6). The insensitivity of the A_T band to Cu(II) poisoning indicates that in TRISwashed BBY particles, Cu(II) does not interfere with either QA⁻ or HisC required to generate the A_T band (Ono and Inoue 1991; Allakhverdiev et al. 1992). The recent finding that at higher concentrations of Cu(II) or NBD, Tyr_z is completely inhibited suggests that HisCQA⁻ recombination does not require active Tyr_z and verifies the earlier assumption that the generation of the A and A_T bands might have alternative recombination pathways (Kramer et al. 1994). This is in accordance with the earlier observation that in the presence of the tyrosine modifier NBD the A_T band was absolutely unaffected (Allakhverdiev et al. 1992).

Comparing our data obtained by Cu-, NBD- and DEPC treatment of thylakoids and TRIS-washed BBY particles we can speculate about the arrangement of the HisC and Tyr_zC in the recombination pathway generating the A and A_T bands. The fact that histidine modifier DEPC abolished both A and A_T band indicates a strong interrelation between HisC and S₃ state. We might assume that in such inhibitory conditions S₃ state is substituted by HisC in charge recombinations resulted in a modified A band. If S₃ or HisC and Tyr_zC are arranged in series, the inhibition of Tyr_z with either NBD or Cu(II) should block the recombination of HisC or S₃ states and in turn quench the A_T and A bands. The resistance of both modified A and A_T bands against NBD or Cu(II) treatments indicates that in such conditions their recombination pathway differs from that of Tyr_z.

In the present paper we have demonstrated that Cu(II) strongly influenced the TL characteristics of isolated thylakoids by affecting the donor side of PS II. On the one hand, Cu(II) shifted the S₂QB⁻ to S₃QB⁻ charge recombination responsible for the B band generation. On the other hand, Cu(II) influenced the redox

span of the recombination partners generating the A band. The Tyr modifier NBD treatment resulted in similar changes in TL characteristics as Cu(II) poisoning conditions. The His modifier DEPC, however, abolished both the A and A_T bands. This effect was associated with the decrease of the B band and its replacement by the Q band at around 0 °C. Comparing our data obtained by Cu, NBD and DEPC treatments, we have found a strong interrelation between Hisc and S₃ state. We assume that in some inhibitory conditions S₃ state is substituted by Hisc and this alternative pathway does not require functional TyrZ.

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Figure Legends

Figure 1. Effect of Cu(II) on the glow curve of isolated spinach thylakoid suspension excited by continuous white light at -20°C for 1 min. A: control (grey line shows the inhibitory effect of $10\ \mu\text{M}$ DCMU); B: $50\ \mu\text{M}$ Cu; C: $500\ \mu\text{M}$ Cu.

Figure 2. Influence of various Cu(II) concentrations on the TL intensity measured at the actual peak positions of the A ($\square$); AT (Δ) and B ($\bullet$) bands. Samples were illuminated at -20°C for 1 min.

Figure 3. Effect of the increasing Cu(II) concentration on the peak positions of the A and B bands of isolated thylakoids and that of the AT band measured in TRIS-washed BBY particles. All samples were excited at -20°C for 1 min.

Figure 4. Effect of increasing concentrations of the tyrosine modifier NBD on the glow curve of isolated thylakoids. A: control; B: $5\ \mu\text{M}$ NBD; C: $10\ \mu\text{M}$ NBD; D: $20\ \mu\text{M}$ NBD. All samples were excited with continuous white light at -20°C for 1 min.

Figure 5. Effect of the histidine modifier DEPC on the glow curves of isolated thylakoids (a) and TRIS-washed BBY particles (b). Samples were measured after 45 min of DEPC incubation. (A) no addition; (B) $1\ \text{mM}$; (C) $10\ \text{mM}$; (D) $20\ \text{mM}$; (E) no addition; (F) $1\ \text{mM}$; (G) $2.5\ \text{mM}$; (H) $5\ \text{mM}$; (I) $20\ \text{mM}$. All samples were illuminated at -20°C for 1 min with continuous white light.

Figure 6. Glow curves of TRIS-washed BBY particles measured at various Cu(II) concentrations. (A) control; (B) 20 μ M Cu; (C) 500 μ M Cu. (A–C) samples were illumination at -20°C for 1 min. (D) control excited at -80°C for 1min.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6