

New facts about CdCl_2 action on the photosynthetic apparatus of spinach chloroplasts and its comparison with HgCl_2 action

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Abstract

Using EPR spectroscopy it was found that CdCl_2 and HgCl_2 interact (1) with the intermediates Z^*/D^* , *i.e.* with the tyrosine radicals on the donor side of photosystem (PS) 2 situated in the 161st position in D_1 and D_2 proteins; (2) with the primary donor of PS1 (P700) whereby the oxidation of chlorophyll (Chl) α dimer in the reaction centre of PS1 occurs yet in the dark; (3) with the manganese cluster which is situated in the oxygen evolving complex. Due to these interactions of investigated metal chlorides with the photosynthetic apparatus, the interruption of the photosynthetic electron transport through photosynthetic centres occurs. Monitoring of time dependence of EPR signal I of chloroplasts treated with CdCl_2 or HgCl_2 after switching off the light suggests that all mechanisms, *i.e.* direct, cyclic, and non-cyclic reductions of $\text{P}700^+$ are damaged. The formation of complexes between mercury or cadmium ions and amino acid residues constituting photosynthetic peptides was suggested as possible mechanism of their inhibitory action. The higher HgCl_2 efficiency in comparison with that of CdCl_2 was explained by higher ability of mercury ions to form complexes with amino acids, what was demonstrated by their apparent binding constants: $K = 10\ 200\ \text{M}^{-1}$ for Hg^{2+} ions, and $K = 3\ 700\ \text{M}^{-1}$ for Cd^{2+} ions.

Additional key words: cadmium; EPR spectroscopy; fluorescence spectroscopy; mercury; photosynthetic electron transport; *Spinacia oleracea*.

Introduction

Cadmium and mercury belong to the major heavy metal pollutants that have toxic effects on living organisms (Siedlecka and Krupa 1999). They affect various plant processes, such as the efficiency of water use, transpiration, photosynthesis, and plant growth (Clijsters and van Assche 1985, El-Shintinawy 1999, Ghorbanli *et al.* 1999). Cadmium exhibits several toxic effects on higher plants, which are caused by direct and indirect mechanisms of its action on their photosynthetic apparatus (Krupa 1999). In our previous papers (Šeršeň *et al.* 1998a,b) we dealt with inhibitory effects of Hg on photosynthetic apparatus. In this paper we focus our attention on the Cd action on photosynthesis. The site of action of Cd^{2+} ions was situated into the photosystem (PS) 2 either without precise localisation (Hampp *et al.* 1976, Nedunchezhian and

Kulandaivelu 1995, Siedlecka and Krupa 1996, Purohit and Singh 1999) or on the donor side, particularly in the oxygen evolving complex (OEC) or in its vicinity (Bazzaz and Govindjee 1974, Van Duijvendijk-Matteoli and Desmet 1975, Baszyński *et al.* 1980, De Filippis *et al.* 1981, Atal *et al.* 1991). Other possibility was to situate it in the site of Q_A or Q_B on the acceptor side of PS2 (Singh and Singh 1987, Fodor *et al.* 1996). Paddock *et al.* (1999) found that Cd^{2+} reduced protonation of Q_B in the reaction centre of *Rhodobacter sphaeroides*. Several authors reported that PS1 activity is little sensitive or inactive to Cd^{2+} (Bazzaz and Govindjee 1974, Baszyński *et al.* 1980, Atal *et al.* 1991, Krupa *et al.* 1987, Siedlecka and Krupa 1996). Besides the above-mentioned effects Cd^{2+} ions cause: (1) inhibition of ATP (De Filippis *et al.* 1981)

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Abbreviations: Chl – chlorophyll; cw – continual wave; DCMU – 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCPIP – 2,6-dichlorophenolindophenol; $d\chi''/dB$ – the first derivative of the imaginary part of magnetic susceptibility χ with respect to magnetic induction B ; EPR – electron paramagnetic resonance; IC_{50} -value – concentration of CdCl_2 or HgCl_2 causing the 50 % inhibition of DCPIP photoreduction; OEC – oxygen evolving complex; PAR – photosynthetically active radiation, PET – photosynthetic electron transport, PS – photosystem; P700 – primary donor of PS1; Q_A , Q_B – the first and the second quinone acceptors of PS 2.

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and Chl (Stobart *et al.* 1985) synthesis; (2) disorganisation of thylakoid membranes (Fernandez-Pinas *et al.* 1995, Ozounidou *et al.* 1997, Stoyanova and Tchakalova 1999); (3) changes in the lipid composition of thylakoid membranes (Skórzyńska-Polit and Baszyński 1997).

This study was focused on the more precise specifica-

tion of the site and mechanism of action of Cd^{2+} ions upon photosynthetic electron transport chain using emission fluorescence and EPR spectroscopy and on the comparison of $CdCl_2$ and $HgCl_2$ effects on the photosynthetic apparatus of spinach chloroplasts.

Materials and methods

Cadmium chloride, mercury chloride, and calcium chloride were purchased from *Lachema* (Brno, Czech Republic). All chemicals were of analytical grade purity and they have been applied in all experiments without further purification.

Chloroplasts were prepared from market spinach by the procedure of Walker (1980) partly modified by Šeršeň *et al.* (1990) using TRIS buffer (20 mM, pH = 7.0) containing 0.4 M saccharose, 15 mM NaCl, and 5 mM $MgCl_2$. Chl content was determined according to Lichtenthaler and Wellburn (1983).

The photosynthetic electron transport (PET) through PS2 and PS1 in the suspension of spinach chloroplast [30 g(Chl) m^{-3}] was measured in phosphate buffer (pH = 7.2) containing 5 mM $MgCl_2$, 15 mM NaCl, and 0.4 M saccharose according to Xiao *et al.* (1997) by monitoring the rates of photoreduction of 2,6-dichlorophenolindophenol (DCPIP) or photooxidation of DCPIPH₂ using methyl viologen as a final acceptor. These photoprocesses were recorded as changes in the absorbance of DCPIP at 595 nm by a spectrophotometer *Specord UV-VIS* (Zeiss, Jena, Germany). We irradiated the chloroplast suspen-

sions by the 250-W halogen lamp through the 5-cm water filter. The irradiance was 900 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PAR, temperature was 25 °C.

EPR was measured with an instrument *ERS 230* (ZWG, AdW, Berlin, Germany) which operates in the X-band. The EPR spectra of spinach chloroplasts were recorded at 5 mW of microwave power with 0.5 mT amplitude modulation at 25 °C. The samples containing 3.4 kg of Chl in m^3 were measured in flat quartz cell and their irradiance (~400 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PAR) was carried out directly in the resonance cavity with a 250-W halogen lamp from 0.5 m distance through 5 cm water filter.

The fluorescence emission spectra were recorded by a fluorescence spectrophotometer *F-2000* (Hitachi, Tokyo, Japan) at 25 °C (excitation slit of 10 nm, emission slit of 20 nm). The samples of spinach chloroplasts [9 g(Chl) m^{-3}] were excited at $\lambda_{ex} = 436$ nm for monitoring fluorescence of Chl *a* (in 1 min time intervals). For monitoring fluorescence from aromatic amino acid residues contained in photosynthetic proteins the excitation UV radiation ($\lambda_{ex} = 275$ nm) was used. The samples were kept in the dark for 10 min before the measurements.

Results and discussion

Previously (Šeršeň *et al.* 1998a,b) we found that the spinach chloroplasts treated with $HgCl_2$ exhibited a decrease of the rate of DCPIP photoreduction with the $IC_{50} = 28 \mu\text{M}$. In the present experiments $CdCl_2$ inhibited the DCPIP photoreduction as well, however, with pronouncedly lower effectiveness. The determined value of $IC_{50} = 1 \text{ mM}$ was in good agreement with that found by Hampp *et al.* (1976). On the other hand, we observed that 1 mM of $CdCl_2$ or 28 μM $HgCl_2$ completely inhibits the photo-oxidation of DCPIPH₂. Hence $CdCl_2$ and $HgCl_2$ interact with both photosynthetic centres.

Our finding that $CdCl_2$ inhibits the PET through PS1 differs from the findings of other authors who suggested that $CdCl_2$ does not interact with PS1. This contradiction can be caused by lower applied $CdCl_2$ concentrations in the experiments of Baszyński *et al.* (1980) and Krupa *et al.* (1987) or by other sort of plant chloroplasts (maize) used by Bazzaz and Govindjee (1974).

For more precise recognition of the site and mechanism of $CdCl_2$ inhibitory action on the photosynthetic apparatus of spinach chloroplasts the emission fluorescence and EPR spectroscopy were used. When chloroplasts were irradiated with $\lambda = 436$ nm, an emission band with the maximum at $\lambda = 686$ nm was observed. This band belongs to the pigment-protein complexes present mainly in PS2 (Atal *et al.* 1991, Govindjee 1995). Chloroplasts treated with $CdCl_2$ exhibited in our experiments a quenching of emission of Chl *a*-molecules. The quenching of Chl *a*-fluorescence increased with increasing $CdCl_2$ concentration (not documented here). This finding is in good agreement with that of Atal *et al.* (1991). The quenching of the emission of Chl *a* was observed also in chloroplasts treated with $HgCl_2$ (Šeršeň *et al.* 1998a,b). When chloroplast suspension was excited by radiation of $\lambda = 275$ nm an emission band at 334 nm was observed. This emission band belongs to fluorescence of aromatic

amino acid residues, mainly to tryp-tophan (Chen 1986). Fig. 1 demonstrates interaction of CdCl₂ with the aromatic amino acid residues in photosynthetic proteins: with increasing CdCl₂ concentration the intensity of fluorescence emission band at 334 nm decreased. The decay of this emission band is caused by the formation of complexes between Cd ions and aromatic amino acid residues contained in the peptides of the photosynthetic apparatus. Quenching of emission of aromatic amino acid residues was observed also in chloroplasts treated with HgCl₂ (Šeršeň *et al.* 1998a).

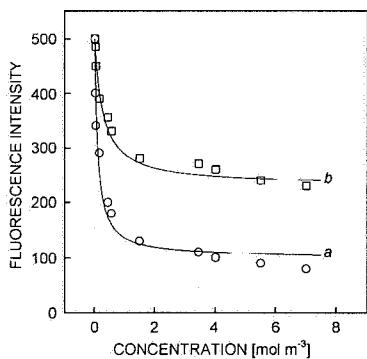


Fig. 1. The dependencies of fluorescence intensity of aromatic amino acid residues in chloroplast peptides on concentrations of HgCl₂ (a) and CdCl₂ (b).

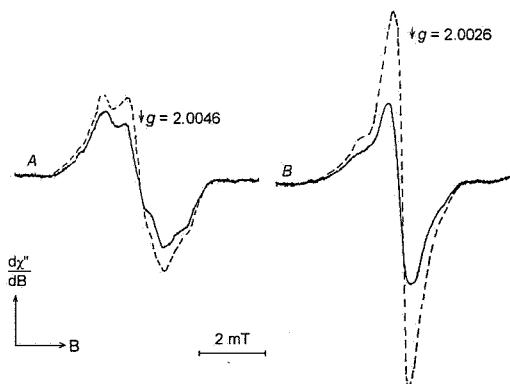


Fig. 2. The EPR spectra of untreated spinach chloroplasts (A) and chloroplasts treated with 0.033 M CdCl₂ (B). The *full lines* were recorded in the dark and the *dashed ones* under continuous irradiation.

The EPR spectroscopy is appropriate to determine the site of action of photosynthesis inhibitors because all green algae and higher plants exhibit EPR signals in the region of free radicals ($g \approx 2.00$) yet at room temperature. These signals are: (1) The EPR signal I ($g = 2.0026$, $\Delta B_{pp} = 0.8$ mT) belonging to the cation radical of Chl α dimer in the reaction centre of PS1 (P700) (Hoff 1979). This signal is observable in Fig. 2B as narrower part of the dashed line. (2) The EPR signal II composed from two parts, namely from signal II_{slow} and signal II_{very fast}. The

slow component of signal II belongs to the intermediate D[•], *i.e.* the tyrosine radical situated in the 161st position in D₂ protein present on the donor side of PS2 (Debus *et al.* 1988). The signal II_{slow} is visible in Fig. 2A as a full line ($g = 2.0046$, $\Delta B_{pp} = 1.9$ mT). The fast component of signal II (signal II_{very fast}) is observable in light and belongs to the intermediate Z[•] (Blankenship *et al.* 1975), *i.e.* the tyrosine radical in the 161st position in D₁ protein present on the donor side of PS2 (Barry and Babcock 1987, Svensson *et al.* 1991). Because the intermediate Z[•] is reduced by Mn cluster in the microsecond time regime (Blankenship *et al.* 1975), only a certain part (*ca.* 29 %) of signal II_{very fast} is observable by cw EPR spectroscopy. This is shown in Fig. 2A as an increase of signal II in the light. The spectroscopic parameters of signal II_{very fast} are the same as those of signal II_{slow}, *i.e.* $g = 2.0046$, $\Delta B_{pp} = 1.9$ mT. Fig. 2B shows that CdCl₂ interacts with the intermediates Z[•]/D[•] what results in the intensity decrease of both components of signal II (Fig. 2B, *both lines*). The interaction of CdCl₂ with the intermediate Z[•] was better manifested at higher metal chloride concentrations ($c > 0.05$ M; not shown here). Besides these interactions with Z[•]/D[•] intermediates, CdCl₂ interacts also with the primary donor of PS1 (P700). Due to this interaction, oxidation of Chl α dimer occurs yet in the dark what is reflected in EPR spectrum by the increase of signal I intensity (Fig. 2B, *full line, its narrower part*). The evidence that the narrower part of the spectra in Fig. 2B belongs to oxidised form of P700⁺ was presented in Šeršeň *et al.* (1998a).

Van Duijvendijk-Matteoli and Desmet (1975), Baszyński *et al.* (1980), De Filippis *et al.* (1981), and Atal *et al.* (1991) suggested the OEC as possible site of CdCl₂ action on the base of indirect evidences. We present here the direct proof of CdCl₂ interaction with the Mn cluster, which is situated in the OEC. This is manifested by six lines belonging to free Mn²⁺ ions, which appear in EPR spectrum of chloroplasts treated with higher concentration of CdCl₂ (Fig. 3B). About 80 or 40 % of manganese was released from the OEC by HgCl₂ and CdCl₂, respectively. Thus, the efficiency of HgCl₂ was 1.7 fold higher than that of CdCl₂ used at the same molar concentration (0.07 M).

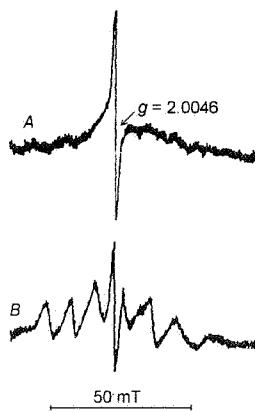
All the above-mentioned interactions of CdCl₂ with the photosynthetic apparatus result in the interruption of PET through photosynthetic centres. This interruption of electron transport is reflected in EPR spectra of spinach chloroplasts treated with CdCl₂ by a great increase of signal I intensity in the light (Fig. 2B, *dashed line*). This increase is caused by inability of the reduction of P700, which was oxidised by light.

By using the EPR method we found previously (Šeršeň *et al.* 1998a,b) that the sites of HgCl₂ inhibitory action on PET in spinach chloroplasts were the same as the sites of CdCl₂ action, but the effectiveness of HgCl₂

Table 1. The intensities of EPR signal I [relative] registered in EPR spectra of chloroplasts treated with HgCl_2 or CdCl_2 .

Concentration [M]	Signal I intensity in the light	in the dark	
	HgCl_2	CdCl_2	HgCl_2
0.01	17.1	13.9	11.7
0.03	18.3	14.2	14.1
0.05	19.7	18.1	15.6
			2.2
			5.5
			9.0

effects on the photosynthetic apparatus was more intensive. The measure of PET damage by CdCl_2 or HgCl_2 can be expressed as an increase in intensity of the EPR signal I. From EPR spectra of chloroplasts treated with CdCl_2 and HgCl_2 we evaluated the signal I intensities (Table 1). They showed that HgCl_2 was more deleterious for photosynthetic apparatus than CdCl_2 .

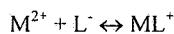
Fig. 3. The EPR spectra of Mn^{2+} ions in intact chloroplasts (A) and in chloroplasts treated with 0.05 M CdCl_2 (B).

Figs. 2 and 3 show that the CdCl_2 effects on photosynthetic apparatus are observed only at very high CdCl_2 concentrations (0.033 and 0.050 M, respectively). However, when we compared the ratios of molar concentrations $\text{Chl} : \text{CdCl}_2$ used in our EPR experiments (1 : 7 and 1 : 11, respectively) with that used at determination of IC_{50} for inhibition of electron transport through PS2 (1 : 30), it was evident that in EPR experiments the $\text{CdCl}_2 : \text{Chl}$ ratio was approximately 3-4 fold lower than that applied for Hill reaction measurement. To exclude possible interference of metal with the used buffer (effect of the ionic strength of solution) at higher CdCl_2 or HgCl_2

concentrations we carried out an EPR experiment with the same concentration of CaCl_2 . 0.05 M CaCl_2 caused no observable changes in EPR spectra of spinach chloroplasts. It denotes that CdCl_2 or HgCl_2 interact directly with photosynthetic apparatus and not through interaction of buffer with studied metals.

The time behaviour of EPR signal I of spinach chloroplasts after switching-off the light also supported the interaction of CdCl_2 and HgCl_2 with PS1. Fig. 4 presents the decrease of signal I intensity after switching-off the light. In the control sample, where all mechanisms (direct, cyclic, and non-cyclic) of reduction of $\text{P}700^+$ were not damaged, the decrease of signal I intensity to the original value after switching-off the light was very fast, in the range of 5 s (Fig. 4, line a). As the control we used chloroplasts treated with 5 mM DCMU, which caused complete inhibition of the electron flow from PS2 to PS1 without damage of PS1. After adding CdCl_2 or HgCl_2 to chloroplast suspension the rate of the decrease of signal I intensity was restricted (Fig. 4, lines b and c) what indicates that the applied metal chlorides damaged all reduction mechanisms of $\text{P}700^+$. This finding is in accordance with that of Sakurai *et al.* (1991) who found that HgCl_2 destroys Fe-S centre (F_B) on the acceptor side of PS1. Due to this interaction cyclic and non-cyclic electron transport through PS1 can be damaged. Fig. 4b,c also shows that the HgCl_2 action on PS1 is more effective than that of CdCl_2 .

Both CdCl_2 and HgCl_2 similarly affected the photosynthetic apparatus. However, the inhibitory effect of HgCl_2 was larger than that of CdCl_2 (cf. the IC_{50} values of 1 mM CdCl_2 and 28 μM HgCl_2). Similarly, larger efficiency of HgCl_2 on the fluorescence quenching of emission bands of aromatic amino acid residues ($\lambda = 334 \text{ nm}$) and chlorophyll ($\lambda = 686 \text{ nm}$) was observed. Using EPR spectroscopy we documented also higher effects of HgCl_2 on the photosynthetic apparatus with respect to those of CdCl_2 (different intensities of EPR signals I and II at the same applied metal concentration). To determine the reason of greater inhibitory efficiency of HgCl_2 with respect to that of CdCl_2 we evaluated the apparent equilibrium constants K of complex formation between metal ions and aromatic amino acid residues according to Tominaga *et al.* (1995). We calculated K values from the concentration dependence of the fluorescence band at 334 nm according to formulae:



[1]

$$K = \frac{[\text{ML}^+]}{[\text{M}^{2+}] [\text{L}^-]} = \frac{\alpha C_L}{(C_M - \alpha C_L) C_L (1 - \alpha)}$$

[2]

$$\alpha = \frac{(KC_M C_L + KC_L^2 + C_L) \pm [(KC_M C_L + KC_L^2 + C_L) - 4 K^2 C_M C_L^3]^{1/2}}{2 KC_L^2} \quad [3]$$

$$F = F_i - \alpha (F_i - F_s) \quad [4]$$

where M^{2+} is metal ion (Cd^{2+} or Hg^{2+}), L is ligand (aromatic amino acid residues present in the peptides of chloroplast), C_M is the initial metal concentration, C_L is the initial ligand concentration, α is the fraction of bounded metal, F_i is the fluorescence intensity in the absence of metal, and F_s is the fluorescence intensity in the presence of saturating amount of metal ions. Fig. 1 shows dependencies of fluorescence quenching on the metal concen-

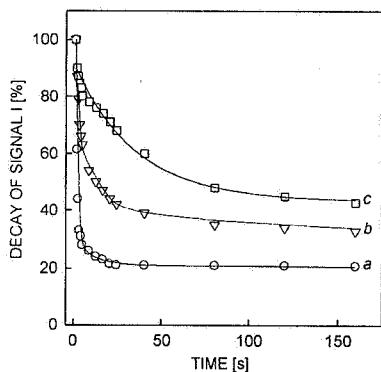


Fig. 4. The time dependencies of EPR signal I intensity after switching off light in chloroplasts treated with 5 mM DCMU (a), 0.05 M CdCl₂ (b), and 0.05 M HgCl₂ (c).

tration. From these dependencies we calculated equilibrium constants K using Eqs. (3) and (4). The K values obtained by computer fitting of the quenching curves α and b in Fig. 1 at the proposed concentration $C_L = 5 \mu M$ were $K = 10\ 200 M^{-1}$ ($r^2 = 0.97$) for HgCl₂ and $K = 3\ 700 M^{-1}$ ($r^2 = 0.97$) for CdCl₂. The values of these calculated binding constants are only apparent because the final quenching can be affected by several factors, e.g. formation of complexes between cadmium (mercury) chloride and cysteins, histidines, glutamates, etc. We assume that interactions of the studied metal ions with aromatic amino acids are important in their fluorescence quenching. The

absorption spectra of chloroplasts treated with the studied metal chlorides exhibited only a small absorption increase at 334 nm with respect to the absorption of control chloroplasts. Therefore the decrease of the emission band due to this absorption can be considered as negligible. Moreover, we determined the stability constants of the complexes between tryptophan and CdCl₂ or HgCl₂ in distilled water by the same method. The values of these constants are $K = 182\ 100 M^{-1}$ ($r^2 = 0.99$) for HgCl₂ or $K = 17\ 130 M^{-1}$ ($r^2 = 0.94$) for CdCl₂. Hence the affinity of CdCl₂ to tryptophan is lower than that of HgCl₂. On the base of this finding we conclude that the larger inhibitory efficiency of HgCl₂ than CdCl₂ is caused by the higher ability of mercury ions to form complexes with aromatic amino acids.

Thus we found new sites of CdCl₂ action on photosynthetic apparatus which were not published up to now, namely Z[•]/D[•] intermediates and the primary donor of PS1 (P700). The interaction of CdCl₂ with P700 resulted in a damage of direct, cyclic, and non-cyclic reduction of P700⁺. By registration of released Mn²⁺ ions in EPR spectra of chloroplasts treated with CdCl₂ we confirmed the Mn cluster in the water splitting complex as a further site of CdCl₂ action. Some authors (Van Duijvendijk-Matteoli and Desmet 1975, Baszyński *et al.* 1980, De Filippis *et al.* 1981, Atal *et al.* 1991) proposed this site of CdCl₂ action on the base of indirect proofs. We found that CdCl₂ has the same sites of action on the photosynthetic apparatus of spinach chloroplasts as HgCl₂, but the efficiency of HgCl₂ is much greater. As possible mechanism of CdCl₂ action the formation of complexes between CdCl₂ and amino acid residues in photosynthetic proteins is suggested. The quenching of fluorescence of chloroplast aromatic amino acid residues manifested this. The higher efficiency of HgCl₂ than CdCl₂ is caused by higher ability of Hg²⁺ ions to form these complexes.

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