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Action of mercury on the photosynthetic apparatus of spinach chloroplasts

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Abstract

In chloroplasts of *Spinacea oleracea L.*, Hg^{2+} ions interact with some sites in the photosynthetic electron transport chain: (*1*) with the intermediates Z^+/D^+ situated in the D1 and D2 proteins and with the manganese cluster in the oxygen evolving complex which are located on the donor side of photosystem (PS) 2, (*2*) with the chlorophyll *a* dimer in the core of PS1 (P700). P700 is oxidized in the dark by $HgCl_2$. The Hg^{2+} ions form organometallic complexes with amino acids contained in chloroplast proteins.

Additional key words: EPR spectroscopy; fluorescence spectroscopy; $HgCl_2$; photosynthesis; radionuclide X-ray fluorescence analyses.

Introduction

Mercury is a potential environmental contaminant which strongly inhibits photosynthetic processes in algae and higher plants. Some authors situate the site of action of Hg^{2+} ions at the donor side of PS1: (*1*) without precise specification (Singh *et al.* 1989), (*2*) at the site of plastocyanin (Kimimura and Katoh 1972, Radmer and Kok 1974, Rai *et al.* 1991), or (*3*) at the acceptor side of PS1 either at the ferredoxin action site (Honeycutt and Krogmann 1972, De Filippis *et al.* 1981) or in the F_B iron-sulphur cluster (Jung *et al.* 1995). Another authors localize the site of Hg^{2+} action in PS2: (*a*) however, without exact determination of the site of action (Honeycutt and Krogmann 1972, Kimimura and Katoh 1972, Rai *et al.* 1991), (*b*) on the donor side

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Abbreviations: Chl - chlorophyll; DCMU - 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCPIP - 2,6-dichlorophenol-indophenol; $d\chi''/dB$ - the first derivative of the imaginary part of magnetic susceptibility χ with respect to magnetic induction B; EPR - electron paramagnetic resonance; IC_{50} - concentration of the inhibitor causing 50 % decrease of DCPIP photoreduction against the control sample; OEC - oxygen evolving complex; PS - photosystem; Q_A, Q_B - the first and the second quinone acceptors of PS2; RFA - radionuclide X-ray fluorescence analysis.
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of PS2, namely in the oxygen evolving complex (OEC) (De Filippis *et al.* 1981, Singh and Singh 1987, Samson *et al.* 1990, Bernier *et al.* 1993, Bernier and Carpentier 1995), (*c*) directly in the core of PS2 (Murthy *et al.* 1995), or (*d*) on the acceptor side of PS2 between the Q_A and Q_B quinones (Miles *et al.* 1973, Prokowski 1993). In *Spirulina platensis*, Murthy *et al.* (1989, 1995) and Murthy and Mohanty (1991, 1995) found the interaction of Hg^{2+} with phycobilisomes that form a part of the light-harvesting complex. Nahar and Tajmir-Riahi (1994, 1995) report that Hg^{2+} ions interact with the light-harvesting complex of PS2 in spinach chloroplasts. The Hg^{2+} ions inhibit also the formation of ATP (Honeycutt and Krogmann 1972, De Filippis *et al.* 1981, Singh and Singh 1987). Several authors explain the mechanisms of Hg^{2+} action by the formation of organometallic complexes of mercury with amino acids in the proteins of photosynthetic centres due to a strong affinity of Hg^{2+} ions to C=O, C-N, C-S, and C-SH groups (Nahar and Tajmir-Riahi 1994, 1995, Bernier and Carpentier 1995).

We studied the effect of a wide concentration range of $HgCl_2$ upon photosynthetic apparatus of spinach chloroplasts using several methods: radionuclide X-ray fluorescence analyses (RFA), electron paramagnetic resonance (EPR), and emission fluorescence spectroscopy. The goal of this work was to find new information about inhibitory action of mercury on photosynthetic apparatus, namely the direct oxidation of the core of PS1 by $HgCl_2$.

Materials and methods

Chloroplasts were prepared from market spinach by the procedure of Walker (1980) partly modified by Šeršeň *et al.* (1990) using TRIS buffer (20 mol m^{-3}, pH = 7.0) containing 400 mol m^{-3} saccharose and 20 mol m^{-3} $MgCl_2$. The chlorophyll (Chl) content was determined according to Lichtenthaler and Wellburn (1983).

The rate of photosynthetic electron transport in spinach chloroplasts was monitored spectrophotometrically (*Specord UV-VIS, Zeiss*, Jena, Germany) as a reduction of 2,6-dichlorophenol-indophenol (DCPIP) in phosphate buffer containing 20 mol m^{-3} of phosphates (pH = 7.2), 400 mol m^{-3} of saccharose, 5 mol m^{-3} $MgCl_2$, and 15 mol m^{-3} NaCl. The Chl content in these experiments was 27 g m^{-3}. The samples were irradiated by a halogen lamp (250 W) from 0.1 m distance through a 4 cm water filter to prevent heating of the samples. This photochemical assay was carried out under saturating irradiance of "white light" (~ 900 μmol m^{-2} s^{-1} PAR) at 25 °C.

The Chl fluorescence emission spectra were recorded by a fluorescence spectrophotometer *F-2000* (*Hitachi*, Tokyo, Japan) at 25 °C (excitation slit: 10 nm, emission slit: 20 nm). The samples of spinach chloroplasts [9 g(Chl) m^{-3}] were excited with the radiation of λ_{ex} = 436 nm for monitoring fluorescence of Chl *a*, or at λ_{ex} = 275 nm for monitoring fluorescence of aromatic amino acids in photosynthetic proteins. The samples were kept in the dark for 10 min prior to the measurements.

EPR measurements were made 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 modulation amplitude at 25 °C. The samples contained 2.8 kg(Chl) m^{-3}, and they were irradiated by ~400 μmol m^{-2} s^{-1} PAR directly in the resonance cavity using a 250 W halogen lamp from 0.5 m distance through a 5 cm thick water filter.

Samples for RFA were prepared as follows: The chloroplasts suspended in the phosphate buffer [0.9 kg(Chl) m^{-3}] were treated with 1.0 mol m^{-3} $HgCl_2$, stored for 15 min in the dark at 4 °C, and then centrifuged (10 min at 15 000×*g*, 4 °C). The sediment was resuspended in the original phosphate buffer and centrifuged again (10 min, 15000×*g*, 4 °C). The last procedure was repeated three times, and finally the sediment was resuspended in a small amount of original buffer, collected on filtration paper (10 mm of diameter), and then dried. In thus prepared samples the Mn, Cu, Fe, and Hg contents were determined by RFA spectroscopy (Tölgyessy *et al.* 1990). This method is based on the interaction of a low-energetic γ- and X-radiation with the analyzed sample. The fluorescence of the elements contained in the samples was excited by a radionuclide source ^{238}Pu (half-life: 86.4 years; activity: 740 MBq; energy: 12-22 keV). The energy of ^{238}Pu is higher than the absorption edges of the studied elements, and it ensures efficient excitation of the fluorescence radiation. The energy of fluorescence radiation formed is characteristic for the emitting element (Kα Cu 8.047 keV; Kβ Cu 8.904 keV; Kα Mn 5.898 keV; Kβ Mn 6.490 keV; Kα Fe 6.403 keV; Kβ Fe 7.057 keV; Kα : Kβ 6:1, Kα Ca 3.691 keV, Kβ Ca 4.012 keV, Lα Hg 9.987 keV), and the magnitude of the analytic signal is proportional to the amount of the determined element in the analyzed sample. The maxima corresponding to fluorescence radiation of Mn, Fe, Ca, Cu, and Hg in the spectrum were identified using the corresponding standards [Cu—channel numbers 146(Kα) and 168 (Kβ), Mn—channel numbers 91 (Kα) and 106 (Kβ), Fe—channel numbers 105 (Kα) and 120 (Kβ), Ca—channel numbers 39 (Kα), and 46 (Kβ), Hg—channel number 197 (L α)]. At polycomponent RFA, lines of fluorescence radiation of the present elements can be separated by a detection technique with a good energetic resolution. For this reason a semiconductor Si(Li) detector with Be-inlet window with 12 mm diameter (*UJV* Řež, Czech Republic) linked with a *1024* channel analysator (*ORTEC*, USA) was used.

All experiments were repeated at least three times, the reproducibility of the measurements was below experimental error (10 %).

Results

$HgCl_2$ inhibits the rate of electron transport through the photosynthetic chain of spinach chloroplasts. The intensity of this inhibition was monitored by the decrease of DCPIP photoreduction with respect to the untreated control. The concentration of $HgCl_2$ causing 50 % decrease of this photoreduction at the molar ratio of Chl : $HgCl_2$ ~ 1:1 (IC_{50} value) was 28 mmol m^{-3}.

As concerns the pigment-protein complexes in spinach chloroplasts (Fig. 1*A*), the presence of $HgCl_2$ caused quenching of Chl *a* fluorescence at 685 nm. The interaction of $HgCl_2$ with aromatic amino acids, which are present in the proteins of

spinach chloroplasts, was documented by the quenching of their fluorescence at 334 nm (Fig. 1*B*).

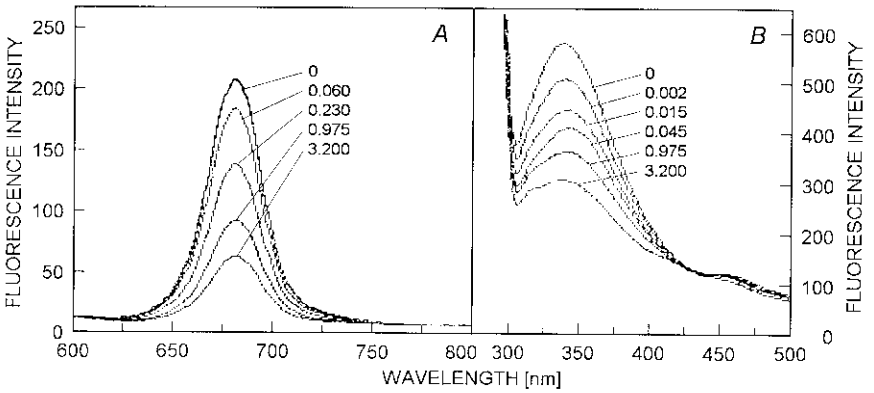

Fig. 1. The chlorophyll fluorescence emission spectra (*A*) and fluorescence emission spectra of aromatic amino acids (*B*) of untreated spinach chloroplasts (0), and of chloroplasts treated with the given concentrations [mol m^{-3}] of $HgCl_2$. Excitation wavelength was 436 (*A*) or 275 (*B*) nm, chlorophyll content was 9 g m^{-3}.

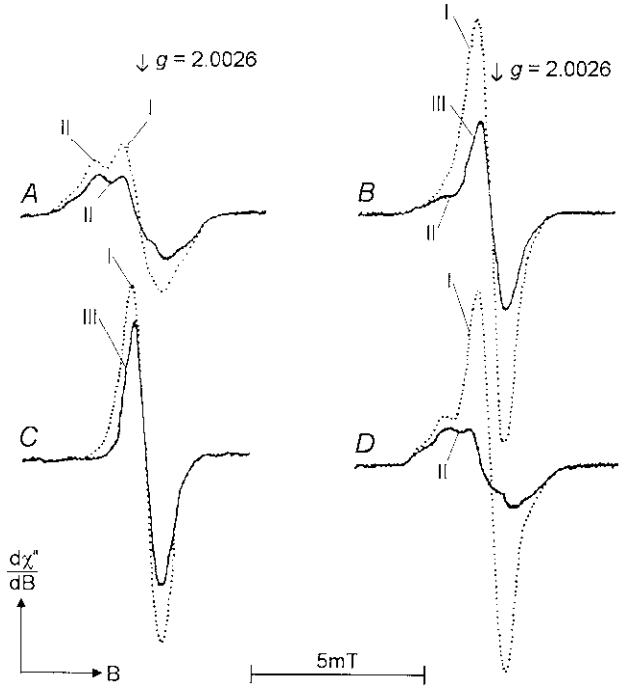

Fig. 2. EPR spectra of untreated spinach chloroplasts (*A*) and chloroplasts treated with 8 (B) or 40 (*C*) mol m^{-3} of $HgCl_2$ or with 5 mol m^{-3} DCMU (*D*). The full line spectra were recorded in the dark and the dotted ones were recorded in the light. B is magnetic induction given in milliTesla (mT), and $d\chi''/dB$ is the first derivative of the imaginary part of magnetic susceptibility χ with respect to B. Chl content was 2.8 kg m^{-3}.

EPR spectra in the region of free radicals at $g \sim 2$ showed that with the increase in $HgCl_2$ concentration the intensities of EPR signals II_{slow} and $II_{very\ fast}$ (Fig. 2*B,C*) decreased. Moreover, at high $HgCl_2$ concentrations (molar ratio Chl : $HgCl_2 \leq 1 : 1$), a new EPR signal with $g = 2.0024 \pm 0.0002$ and line width $\Delta B_{pp} = 0.8$ mT occurred (with regard to better preliminary identification denoted further as signal III in Fig. 2*B,C, full lines*). On the other hand, the intensity of the EPR signal I pronouncedly increased in the light with increasing $HgCl_2$ concentration (Fig. 2*B,C, dotted lines*). Besides these effects, in the EPR spectra of spinach chloroplasts treated with high concentrations of $HgCl_2$ (30 mol m^{-3}; Chl : $HgCl_2 \leq 1 : 10$) the occurrence of 6 lines of the Mn^{2+} free ions was observed (Fig. 3*B*).

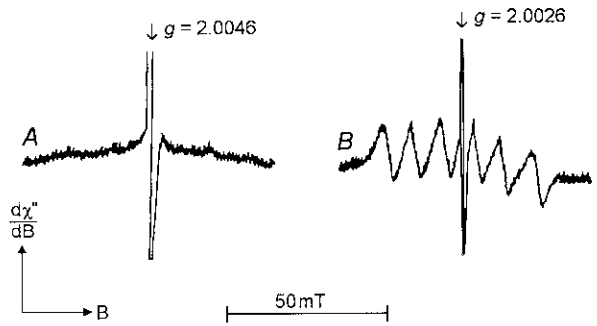

Fig. 3. The EPR spectrum of Mn^{2+} ions in untreated spinach chloroplasts (*A*) and in chloroplasts treated with 50 mol m^{-3} of $HgCl_2$ (*B*). For explanation of B and dχ"/dB see Fig. 2. Chl content was 2.8 kg m^{-3}.

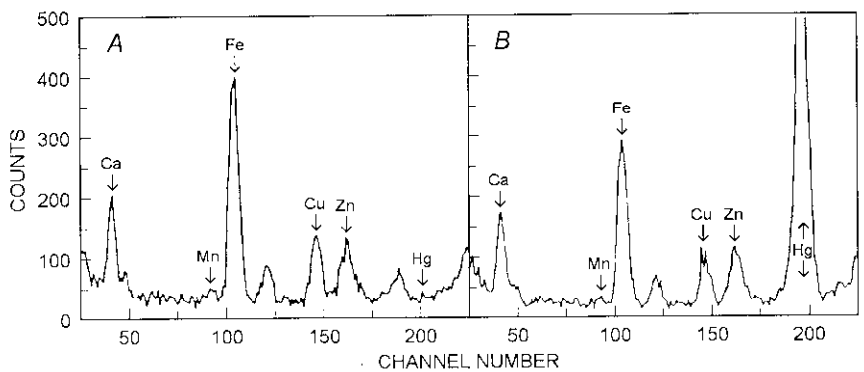

Fig. 4. The RFA spectrum of untreated spinach chloroplasts (*A*) and of chloroplasts treated with 1 mol m^{-3} $HgCl_2$ (*B*). The fluorescence bands belonging to individual elements contained in spinach chloroplasts are denoted by arrows.

The binding of Hg^{2+} ions to spinach chloroplasts is documented in RFA spectra (Fig. 4): after treatment of chloroplasts with $HgCl_2$ (Chl : $HgCl_2 \sim 1 : 1$) a part of mercury was irreversibly bound to chloroplasts what was reflected by the occurrence

of a band at channel number 197 belonging to Hg (Lα) (Fig. 4*B*). On the other hand, the disappearance of manganese band at channel numbers 91 (Kα) and 106 (Kβ) was observed. In the presence of $HgCl_2$ the decrease of intensities of the bands belonging to Fe, Cu, and Ca was not significant (Fig. 4*B*).

Discussion

The value of IC_{50} = 0.028 mol m^{-3} found for the inhibition of DCPIP photoreduction rate by $HgCl_2$ is in good accordance with the findings of Kimimura and Katoh (1972), Bernier *et al.* (1993), *etc.* This inhibition of photosynthetic electron transport is caused by interactions of $HgCl_2$ with some constituents of the photosynthetic apparatus which results in its subsequent damage.

The interaction of $HgCl_2$ with photosynthetic centres indicates the decrease of intensity of the Chl fluorescence emission band at 685 nm (λ_{ex} = 436 nm) with increasing $HgCl_2$ concentration (Fig. 1*A*). The band belongs to Chl *a* which is present mainly in the pigment-protein complexes of PS2 (Govindjee 1995). This effect, *i.e.*, the quenching of Chl fluorescence at high $HgCl_2$ concentrations is in accordance with the findings of other authors, and it can be explained by the oxidation of quinones on the acceptor side of PS2 (Miles *et al.* 1973), by binding of mercury on the proteins in the water-splitting complex (Samson *et al.* 1990), or by unspecified injury of PS2 (Kimimura and Katoh 1972).

In an effort to determine the site of $HgCl_2$ action, studies of EPR spectra of spinach chloroplasts showed that low concentrations of $HgCl_2$ caused a decrease of both signal parts, II_{slow} and $II_{very\ fast}$ (Fig. 2*B*). These signals belong to the intermediates Z^+/D^+, corresponding to the tyrosine radicals situated in position 161 in D1 (Tyr_Z) and D2 (Tyr_D) proteins on the donor side of PS2 (Svensson *et al.* 1991, Noren and Barry 1992). Due to the interaction of $HgCl_2$ with Tyr_Z and Tyr_D the electron transport between photosynthetic centres PS2 and PS1 will be disrupted. This disruption is accompanied with a great increase of the signal I intensity in the light (Fig. 2*B,C, dotted line*). As the EPR signal I belongs to the Chl *a* dimer in P700 (Hoff 1979), it could be assumed that $HgCl_2$ did not damage P700 and a part of the acceptor side of PS1.

Besides the above mentioned $HgCl_2$ effects, in the EPR spectra of spinach chloroplasts recorded in the dark, a new signal III with g = 2.0024±0.0002 occurred, with ΔB_{pp} = 0.8 mT (Fig. 2*B,C, full line*). The shape of this new signal was similar to that of signal I (Fig. 2*C, full line*), however, the corresponding g values differed from each other (2.0026 for signal I and 2.0024 for signal III). From the position and the shape of signal III we suppose that it belongs to some form of the cation radical of Chl, either to the cation radical in the core of PS2 (spectroscopic parameters: g = 2.002 and ΔB_{pp} = 0.7 mT) (Van Gorkom *et al.* 1974, 1975) or to the cation radical of the Chl *a* dimer in the core of PS1 (spectroscopic parameters are the same as for the above mentioned signal I) (Hoff 1979). In order to solve this problem, we made an experiment with chloroplasts treated with 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) (Fig. 2*D*). DCMU is an electron acceptor that inhibits photosynthetic

electron transport between PS2 and PS1 in the site of Q_B (Izawa 1980, Trebst 1980). Fig. 2*D* shows that the increase in intensity of signal I in the light was the same for chloroplasts treated with DCMU (Fig. 2*D*, *dotted line*) and for chloroplasts treated with $HgCl_2$ (Fig. 2*C*, *dotted line*). The difference between EPR spectra of the above mentioned treated chloroplasts was only in the shape of the signals in the dark. EPR spectra of chloroplasts treated with DCMU contained undamaged signals II_{slow} and $II_{very\ fast}$, indicating that DCMU did not damage the donor side of PS2 (Fig. 2*D*). On the other hand, in the EPR spectra of chloroplasts treated with $HgCl_2$ a decrease of intensities of EPR signals II_{slow} and $II_{very\ fast}$ and the appearance of signal III was found. If the signal III did not belong to the Chl *a* dimer in PS1 of irradiated chloroplasts treated with $HgCl_2$, an increase of signal I by the value of EPR signal recorded in the dark would be observed. However, Fig. 2*B,C,D* (*dotted lines*) showed that the size of signal I in all three cases was approximately the same. Consequently, in dependence on $HgCl_2$ concentration, P700 is in the dark partially or almost fully oxidized.

At higher $HgCl_2$ concentrations, in the EPR spectra of spinach chloroplasts a further new signal consisting of 6 lines was observed (Fig. 3*B*). This signal belongs to free Mn^{2+} ions (Blankenship and Sauer 1974). Therefore, it can be assumed that $HgCl_2$ interacts with the manganese cluster which is present in the oxygen evolving complex on the donor side of PS2. Due to this interaction, the Mn^{2+} ions are released from the manganese cluster into the interior of thylakoid membranes. Due to mutual interactions of the manganese ions occurring in the cluster in different oxidized states, in the EPR spectra of untreated chloroplasts the signal of manganese can not be detected at room temperature. However, after $HgCl_2$ treatment of chloroplasts, the released manganese ions are only in the form of Mn^{2+} (the spin moment of their electrons is 5/2) and, consequently, they can be detected by EPR also at room temperature.

In the RFA spectra of $HgCl_2$-treated spinach chloroplasts, an intensive signal at channel number 197 belonging to Hg (Lα) occurred (Fig. 4*B*). Thus besides the above mentioned sites of action, mercury may be bound also to further sites of photosynthetic apparatus, probably to the proteins which are able to associate with C=O, C-N, C-S, or C-SH groups. In RFA spectra of $HgCl_2$-treated chloroplasts the disappearance of Mn bands at channel numbers 91 (Kα) and 106 (Kβ) was observed [the intensity of these bands in untreated spinach chloroplasts was fairly low - at the threshold of the noise of the instrument - and so the observed intensity decrease by $HgCl_2$ was not significant (Fig. 4*B*)]. This is in accordance with the results obtained by EPR spectroscopy proving that $HgCl_2$ releases Mn^{2+} ions from the manganese cluster (Fig. 3*B*).

The binding of Hg^{2+} ions to the chloroplast proteins and the formation of organometallic complexes was also documented by the quenching of fluorescence emission of aromatic amino acids. The fluorescence spectra of aromatic amino acids excited with the radiation of 275 nm show two emission bands at 334 and 305 nm belonging to tryptophan and tyrosine, respectively (Lakovich 1986). In the fluorescence spectra of $HgCl_2$-treated chloroplasts the decrease in intensity of the above mentioned emission bands was observed (Fig. 1*B*). This is the evidence for the

binding of mercury ions to amino acids which are present in chloroplasts.

Conclusion: At higher concentrations (molar ratio Chl : $HgCl_2 \leq 1 : 2.5$), $HgCl_2$ interacts with the intermediates Z^+/D^+, *i.e.*, with Tyr_Z and Tyr_D located in D1 and D2 proteins on the donor side of PS2. EPR spectroscopy showed that at high concentrations (Chl : $HgCl_2 \leq 1 : 10$), $HgCl_2$ releases Mn^{2+} ions from the manganese cluster situated in the water-splitting complex on the donor side of PS2. These findings were previously assumed by some researchers (Honeycutt and Krogman 1972, Singh and Singh 1987, Samson *et al.* 1990), however, without direct proofs. We demonstrated for the first time, using EPR spectroscopy, that $HgCl_2$ oxidizes the core of PS1 (P700) in the dark if the molar ratio Chl : $HgCl_2 \leq 1 : 1$. The findings obtained by IR spectroscopy by Nahar and Tajmir-Riahi (1994, 1995), namely the formation of organometallic complexes of mercury with amino acids contained in chloroplasts, were confirmed by fluorescence spectroscopy.

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