Inhibition of the Water Oxidizing Complex of Photosystem II and the Reoxidation of the Quinone Acceptor $Q_A^-$ by $\text{Pb}^{2+}$

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Abstract

The action of the environmental toxic $\text{Pb}^{2+}$ on photosynthetic electron transport was studied in thylakoid membranes isolated from spinach leaves. Fluorescence and thermoluminescence techniques were performed in order to determine the mode of $\text{Pb}^{2+}$ action in photosystem II (PSII). The invariance of fluorescence characteristics of chlorophyll a (Chl a) and magnesium tetraphenylporphyrin (MgTPP), a molecule structurally analogous to Chl a, in the presence of $\text{Pb}^{2+}$ confirms that Pb cation does not interact directly with chlorophyll molecules in PSII. The results show that Pb interacts with the water oxidation complex thus perturbing charge recombination between the quinone acceptors of PSII and the S$_2$ state of the Mn$_4$Ca cluster. Electron transfer between the quinone acceptors $Q_A$ and $Q_B$ is also greatly retarded in the presence of $\text{Pb}^{2+}$. This is proposed to be owing to a transmembrane modification of the acceptor side of the photosystem.

Introduction

Heavy metals play essential cofactor roles as structural and catalytic components of enzymes in many physiological processes required for the normal development of plants. Over the course of evolution, plants have developed different mechanisms that control and respond to the intake and accumulation of both essential and nonessential heavy metals. However, some heavy metals such as lead can be highly toxic to cells and cell organelle functions even at very low concentrations. Although the influence of excessive dose of heavy metals on the photosynthetic activity of plants has been studied in many cultivated species (1–3), the mechanism of heavy metal toxicity on photosynthesis is still a matter of great debate. Some evidence points to their involvement as inhibitors of electron transport in light reactions (4–5) and in the inhibition of enzyme activity in dark reactions by the direct blocking of protein functions or displacement of endogenous metals (6–7).

Lead, found in the environment, comes from both natural and anthropogenic sources. The metal is present in the soil, but also in all other environmental compartments: water, air and even living beings (8). The toxicity of a metal depends on its chemical state as well as on environmental factors (9–10). In soil, Pb can be found in ionic form, or bound to the soil particles (11). It has two oxidation states, namely 2$^+$ and 4$^+$. The tetravalent state is a strong oxidant but is not abundant in the environment. The divalent state, on the other hand, is more stable and prominent in the environment (12). The accumulation of Pb from atmospheric deposition or contaminated waste is largely stored in the soil, mainly in the surface layers and, more specifically, in the organic-rich layers (13).

However, a small fraction of the metal is also absorbed by living organisms (micro-and meso-organisms, plants ...etc.). The photosystem II (PSII) complex is one of the two membrane-bound large multisubunit chlorophyll–protein complexes (PSII and PSI) of plants, algae and cyanobacteria embedded in the thylakoid membranes. PSI collects light energy, converts it into electro-chemical energy and drives electron transfer from water to PSI. On its acceptor side, PS II electron transport involves two acceptor quinones, $Q_A$ and $Q_B$ that are arranged around a non-heme iron. This non-heme iron is hexacoordinated by four histidines and two remaining ligand positions are taken by the oxygen atoms of bicarbonate as bidentate ligand (14–15). Further, the study of the effect of bicarbonate has suggested that the non-heme iron plays a role of an electron- transport regulator on the acceptor side of PSI. Although the precise mechanism of this process needs more study, the depletion of bicarbonate results in a decelerating of the electron transfer rate between $Q_A$ and $Q_B$ (16–20). The water oxidation complex (WOC) is located on the donor side of PSII. It is composed of a Mn$_4$Ca cluster where the successive absorption of four quanta by PSII results in the advancement of the S-states cycle from S$_0$ $\rightarrow$ S$_1$ $\rightarrow$ S$_2$ $\rightarrow$ S$_3$ $\rightarrow$ (S$_4$) $\rightarrow$ S$_0$. The S$_1$-state decays to the S$_0$-state after the 4$^th$ flash with the concurrent oxygen evolution. The electrons are passed from the WOC to the reaction center P680$^+$ through the secondary electron donor, TyrZ (Tyrosine 161 of D1 subunit) (21).

At present, there are only a few reports regarding the adverse action of $\text{Pb}^{2+}$ on the photosynthetic apparatus (22 and references therein). A decline of the photochemical quantum yield of PSII was observed in isolated thylakoid membranes from spinach (23). It was proposed that $\text{Pb}^{2+}$ affects oxygen evolution by removing...
recently shown to affect PSI electron transport presumably due to binding near or at plastocyanin (25).

In this study, we have further investigated the mechanism of the action of Pb$^{2+}$ in thylakoid membranes. The effects of the metal ion on the electron transport were studied using thermoluminescence and fluorescence spectroscopic techniques. Functional assays were used to determine the site of action and consequences of metal ion interaction in the thylakoid membranes and to explore the mode of action of the metal that causes the loss of photosystem II functions.

**Materials and Methods**

**Thylakoid Membranes Isolation**

Thylakoid membranes were prepared from fresh market spinach leaves (*Spinacia oleracea* L.) as described elsewhere (26), and were stored in the dark in 50 mM Hepes NaOH (pH 7.6), 0.33 M sorbitol, 2 mM EDTA, 1 mM MgCl$_2$, 1 mM NaCl, and 10 mM KCl.

**Chlorophyll Fluorescence Induction**

Chlorophyll a fluorescence induction (FI) measurements were performed at room temperature using the Plant Efficiency Analyser (Hansatech, King' Lynn, Norfolk, UK). The assay medium consisted of 50 mM Hepes-NaOH (pH 7.6), 0.33 M sorbitol, 2 mM EDTA, 1 mM MgCl$_2$, 1 mM MnCl$_2$, 10 mM KCl, and 10 mM NaCl with a final Chl concentration of 25 $\mu$g mL$^{-1}$ for thylakoid membranes. Red excitation light peaking at 433 nm was used.

**Figure 1**. Typical traces of Chl a fluorescence rise in isolated thylakoid membranes in the absence (Ctrl) or in the presence of PbCl$_2$ added in various concentrations ($\mu$M, unless specified in mM) as indicated by numbers adjacent to traces. See Materials and methods for details. doi:10.1371/journal.pone.0068142.g001

**Figure 2**. Fluorescence emission spectra of: (A) ethanolic solution of Chl a (2.5 $\mu$M) alone (–) and in presence of 1 mM PbCl$_2$ (–); (B) ethanolic solution of MgTPP (2.5 $\mu$M) alone (–) and in presence of 1 mM PbCl$_2$ (–). In both cases, the excitation wavelength was 433 nm. doi:10.1371/journal.pone.0068142.g002

**Figure 3**. Effect of various concentration of PbCl$_2$ on Chl fluorescence induction parameters in thylakoids membranes. (A) $F_{m}$, $F_{v}$ and $F_{o}$ vs PbCl$_2$ (B) Fv/Fm vs PbCl$_2$. Each point is the average of nine experiments. doi:10.1371/journal.pone.0068142.g003
655 nm with an intensity of 1800 μmol m$^{-2}$ s$^{-1}$ was obtained from six light emitting diodes. As the fluorescence signal during the first 40 μs is ascribed to artifacts due to a delay in response time of the instrument, these data were not included in the analysis of FI traces.

**Thermoluminescence**

Measurements of thermoluminescence were performed using home-built equipment. The complete description of the design and functional aspects are described elsewhere (27–28). Thylakoid membranes were diluted to a final Chl concentration of 200 μg mL$^{-1}$ in a medium containing 50 mM Hepes-NaOH (pH 7.6), 0.33 M sorbitol, 2 mM EDTA, 1 mM MgCl$_2$, 1 mM MnCl$_2$, 10 mM KCl, and 10 mM NaCl. About 300 μL of the suspension was added to the sample compartment (15 mm diameter) positioned just above Peltier plate and covered with a Hellma 202-OS disc window. The sample chamber was closed with a holder bearing the light guide connected to the photomultiplier. The sequence of incubation periods and flash illumination was as follows. The samples were pre-incubated for 120 s at 20°C, then the temperature was brought down to 2°C within 36 s and kept for 60 s. Two actinic single turn-over saturating white flashes of about 25 μs red actinic pulse width (setting 10, XE-STC, Walz, Germany) were then applied to initiate charge separation in PSII. Finally, a linear warming (0.5°C s$^{-1}$) of the samples in total darkness activated the recombination of PSII charge pairs that can be detected by the appearance of emission bands with characteristic temperature optima (27–28).

**Fluorescence Measurements**

Fluorometric experiments were carried out at room temperature 24°C with a Perkin Elmer LS35 Spectrometer equipped with a red-sensitive photomultiplier R928. Samples were excited at 434 nm and fluorescence emission spectra were measured from 600 to 800 nm as described by Rajagopal et al (2003) (29). The excitation and emission spectral widths were fixed at 5 and 2.5 nm, respectively, and emission spectra were corrected according to the photomultiplier sensitivity using the correction factor spectrum provided by Perkin-Elmer.

**Flash-induced Fluorescence Decay Kinetics**

In order to examine the reduction and oxidation kinetics of QA, Chl fluorescence rise and its relaxation in the dark were measured with FL3500 Fluorometer (Photon Systems Instruments, Brno, Czech Republic) as described previously (30–31). Thylakoid membranes (Chl concentration of 25 μg mL$^{-1}$) were incubated for 3 min at room temperature in complete darkness without or with 50 μM of DCMU before initiating the fluorescence measurements. Samples were excited with a 20 μs red actinic flash from a LED peaking at 625 nm and prompt fluorescence was measured for 1 min. The first measurement was taken 20 μs after the flash was given. The traces were averaged to estimate the half-life times and amplitudes of the fluorescence decay components using the following three exponential functions:

\[
F(t) = F' + A_1 e^{-K_1 t} + A_2 e^{-K_2 t} + A_3 e^{-K_3 t}
\]

where $F(t)$ is the fluorescence value at time $t$, $K_n$ is the rate constant, $A_n$ is the amplitude of the fluorescence relaxation phase, and $F'$ is the stable minimal fluorescence at the end of the decay.

**Results**

**Chlorophyll Fluorescence Induction**

The kinetic curves of the fast Chl fluorescence rise were measured in isolated thylakoid membranes both untreated and those treated with various concentrations of PbCl$_2$ as shown in Fig. 1. The FI traces, normalized at minimal values ($F_0$), are characterized by a series of inflections in the rate of rise in the fluorescence intensity termed as OJIP transient (32–33). In isolated thylakoid membranes, the I step of the OJIP fluorescence, as observed by Bukhov et al 2003 (34), cannot be resolved visually.

| Without DCMU | Fast Phase | Middle Phase | Slow Phase |
|--------------|------------|--------------|------------|
| PbCl$_2$ (μM) | $t_{1/2}$ (±0.1 s) | A (±5%) | $t_{1/2}$ (±0.3 ms) | A (±3%) | $t_{1/2}$ (±0.4 s) | A (±2%) |
| 0 | 430 | 69 | 6.1 | 22 | 5.9 | 9 |
| 10 | 690 | 61 | 7.5 | 26 | 7.1 | 13 |
| 100 | 987 | 45 | 11.3 | 35 | 12.1 | 20 |
| 1000 | 1440 | 28 | 16.8 | 43 | 15.6 | 29 |
| 2000 | 1590 | 19 | 18.2 | 46 | 17.2 | 35 |

| With DCMU | Fast Phase | Middle Phase | Slow Phase |
|------------|------------|--------------|------------|
| PbCl$_2$ (μM) | $t_{1/2}$ (±0.5 ms) | A (±4%) | $t_{1/2}$ (±0.03 s) | A (±3%) | $t_{1/2}$ (±0.3 s) | A (±2%) |
| 0 | 298 | 41 | 1.79 | 47 | 18.5 | 12 |
| 10 | 1130 | 26 | 1.92 | 53 | 22.1 | 21 |
| 100 | 1560 | 19 | 2.25 | 58 | 23.6 | 23 |
| 1000 | – | – | 4.35 | 68 | 31.2 | 32 |
| 2000 | – | – | 4.86 | 69 | 31.9 | 31 |

Table 1. Effect of PbCl$_2$ on the relative amplitude (A) and half-life time ($t_{1/2}$) of the exponential decay components of Chl a fluorescence yield after a single turnover flash measured in thylakoid membranes in the absence and in the presence of 50 μM DCMU.

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due to the significant overlap between JI and IP phases (Fig. 1, Ctrl). However, Pospisil and Dau 2002 (35) and Boisvert et al 2006 (36) have decomposed the OJIP traces by fitting the experimental curves with a sum of three mono-exponential components. This method showed a good fit of the OJIP traces with the three components OJ, JI and IP, even though the inflection step I was absent (36). The I step can be restored by the addition of some different exogenous electron acceptors at the Q_B site of PSII (37). The treatment with different concentrations (10–400 μM) of lead increased the relative fluorescence intensity at J step while the rise towards the P step was retarded and the fluorescence intensity at P declined (Fig. 1). This suggests that the electron transfer between Q_A^- and Q_B was slowed down with the increasing Pb^{2+} concentration. However, at Pb^{2+} concentrations greater than 400 μM, the OJ phase also began to decrease and the overall fluorescence induction was strongly damped (Fig. 1). Furthermore, the intensity of JIP phase also diminished with increased concentration of Pb cation. In other words, one observed a quenching of Chl fluorescence as the concentration of PbCl_2 increased.

Fluorescence of Chl a and MgTPP

The observed Chl fluorescence quenching (Fig. 1) may be the result of the direct interaction of PbCl_2 with the excited states of Chl of PSII thereby altering its radiative characteristics. Therefore, in order to verify this possibility, the fluorescence of Chl a in ethanolic solution was studied in absence and presence of PbCl_2 (Fig. 2A). The fluorescence of Chl a in this solution exhibits a maximum at 675 nm, which is characteristic of monomeric Chl a (38). As seen from the figure, the fluorescence properties of Chl a practically remained unchanged upon addition of PbCl_2. This suggests that PbCl_2 has no direct effect on the excited singlet states of Chl a, and thus on its radiative properties. To further confirm this observation, fluorescence studies of MgTPP were performed in the presence of Pb^{2+} (Fig. 2B). The use of MgTPP is due to its structural analogy with Chl a as it is composed of the same porphyrin macrocycle with central Mg. In addition, the added...
advantage of using MgTPP is that it does not contain the phytol chain as is present in Chl a, and, as a result, can be more easily accessible to the additives. It is shown in Fig. 2B that PbCl₂ had no effect on the fluorescence properties of MgTPP thus confirming the results obtained with Chl a.

Chlorophyll Fluorescence Induction Parameters

The Chl fluorescence properties of thylakoid membranes in the presence of Pb²⁺ were further subjected to the comprehensive analysis of fluorescence induction kinetics (Fig. 3). The initial fluorescence F₀ (O-level), which describes the functional state of PSII reaction centers in terms of its openness in the dark-adapted state (39), remained virtually unchanged by the addition of Pb cations (Fig. 3A). However, in order to assess the effect of PbCl₂ on the maximum quantum yield of the primary photochemistry of PSII in thylakoid membranes, the changes in the maximal fluorescence observed in dark-adapted samples, Fm, when the excitons have been trapped and all the reaction centers of PSII are in closed state (40), were also examined. As seen from Fig. 3A, Fm greatly diminished as Pb²⁺ concentration increased. This decline in Fm leads to a decrease in the variable fluorescence Fv (Fv = Fm - F₀) and, consequently, Fv/Fm, the maximal PSII photochemical quantum yield, also decreased (Fig. 3B). The fluorescence rise greatly decreased when the concentration of PbCl₂ increased, especially at concentrations above 100 μM PbCl₂. This decline of the band was accompanied by an upward shift of the maximum temperature (Tm) from 33°C to 41°C.

Thermoluminescence

Thermoluminescence was used to further explore the effects of PbCl₂ on charge recombination between donor and acceptor sides of PSII. The TL glow curves for untreated (Ctrl) and Pb²⁺-treated thylakoid membranes following two single turn-over white flashes are displayed in Fig. 5A. The TL signal (Fig. 5A, Ctrl) attained its maximal intensity at the temperature of 38°C, characteristic of the temperature optimum for the B band appearing in the range between 30 and 40°C, as previously reported for this type of material (46–47). The B band is attributed to the charge recombination of S₂/Qₐ– pairs produced by linear electron transport in PSII (48–51). The intensity of the B band progressively diminished as the concentration of PbCl₂ increased (Fig. 5A). The addition of 20 μM PbCl₂ produced 13% decrease in TL intensity, and in the presence of 2 mM PbCl₂, the TL intensity was suppressed completely. Also, the decline of the band was accompanied by an upshift of the maximum temperature (Tm) from 38°C to 41°C.

The changes in the amplitude and Tm of the B band could be related to changes in the properties of the Sₐ states of the Mn₄Ca cluster and/or to modification of the Qₐ– binding site in the presence of PbCl₂. In order to elucidate the site of action of PbCl₂ in the electron transport chain, the TL glow curves were recorded following two single turn-over white flashes in the presence of 50 μM of PSII inhibitor DCMU, known to block the electron flow beyond Qₐ. DCMU eliminated the B band with a simultaneous appearance of Q band with a maximum at 17°C, attributed to the back-flow of electrons from Qₐ– to the S₂ state (48–51) (Fig. 5B). The reason for the absence of B band is that since DCMU stops the electron flow past Qₐ, the formation of Qₐ– and state S₂ is not realized (49). The addition of 20 μM PbCl₂ already suppressed 12% of the Q band intensity. A progressive decrease of the Q band was observed when the concentration of PbCl₂ was further increased. The addition of 2 mM PbCl₂ caused the loss of more than 90% of Q band intensity. This loss was accompanied by a strong upshift of Tm from 17°C to 27°C.

Discussion

The negative action of Pb²⁺ on PSII photochemistry and electron transport, uninfluenced by PSI activity, was studied in thylakoid membranes using various approaches specific for PSII. Chlorophyll fluorescence induction kinetics measurements (Fig. 1) have shown that the fluorescence was greatly quenched when PbCl₂ was added. Several authors postulated that damage caused by heavy metal ions (such as Zn²⁺, Cu²⁺, and Pb²⁺) to plants was due to the substitution of the central Mg from the Chl a molecules thus causing fluorescence quenching (32–54, 22). However, measurements of pure Chl a or MgTPP fluorescence in ethanolic solution (Fig. 2) have demonstrated that the addition of PbCl₂ has no effect on the excited states of Chl a and the structure of the pigment remains intact. The fluorescence quenching observed during Chl fluorescence induction is, therefore, related to the modifications in the photochemical activity of PSI.

The OJIP traces constitute an essential tool to study the activity and integrity of the photosynthetic apparatus under different stress conditions, providing the information on PSII photochemistry such as the electron transport on both donor and acceptor sides of PSII.
the photosystem (32–33, 55). The IP step of the Chl fluorescence induction has been correlated with the photoreduction of the PQ pool (36–57). Thus, the observed decline in IP phase indicates a strong inhibition of the accumulation of reduced PQ especially at Pb2+ concentrations above 400 μM (Fig. 1). This coincided with a decrease in the P/Fm values due to a decrease in Fm (Fig. 3) (23). This part of the induction is known to be more sensitive to the unfavourable treatments in comparison with the photochemical phase (QII) (58). Indeed, the perturbation in the structure-function relations of the WOC has been shown to correlate with the quenching of the IP fluorescence rise that results in a decline of Fm (36, 58). The above is in line with the previous reports showing that Pb2+ causes the release of extrinsic polypeptides associated with the WOC together with the Ca2+ and Cl– required as cofactors (5, 25). Therefore, the inhibition of JIP rise and the more significant damping of the whole fluorescence induction kinetics above 400 μM Pb2+ are the result of the disorganization of the WOC causing the lack of electron flow towards the acceptor side of PSII. The damage of the Mn4Ca cluster is also supported by the decline of both Q and B thermoluminescence bands. Such inhibition of both S2QA and S2QB charge recombination (Q and B band, respectively) shows that the S2 state of the WOC becomes unavailable as the common recombination partner with increasing concentrations of PbCl2 and indicates a dysfunction of the WOC.

On the other hand, the OJ phase is related to the reduction state of Qs (36, 59). The relative increase of OJ in the presence of low concentrations of PbCl2 (Fig. 1) is strongly indicative of a delayed electron transfer from QA to Qb. This was indeed verified using the measurements of Chl fluorescence decay kinetics following a single-turn-off flash (Fig. 4). The fluorescence decay was greatly retarded with the life-time of all three components being significantly increased even at concentrations below 400 μM PbCl2 (Table 1). The amplitude of the fast component, attributed to electron transfer from QA to Qb, diminished with a concurrent increase of the other components. Also, the decreased rate of QA reoxidation resulted in an increased amplitude of the slow component attributed to the back reactions with the S2 state of the Mn4Ca cluster (42–43). This corresponds with the increased amplitude of the middle component of the decay measured in the presence of DCMU (Table 1), a component also attributed to S2/QA recombination (44–45). Therefore, the population of PSII centers with a reduced QA that is reoxidized through S2/QA recombination is increased but the rate of this reoxidation is strongly declined most likely due to the stabilization of the S2 state of the WOC (see below).

The delayed reoxidation of QA+ may be interpreted in terms of an active site of Pb2+ near QA or Qb. Indeed, similar data were previously used to conclude that an inhibitory site of various metal cations was located between QA and Qb (Fig. 6) (60–62). However, the destabilization of the WOC discussed above may also cause the delayed QA– reoxidation. It was indeed shown that the removal of the extrinsic polypeptides or Ca2+ from the WOC can cause the diminished rate of QA– reoxidation through a transmembrane conformational effect (42). Removal of Ca2+ from the WOC also produces a modification in the mid-point potential of QA, thus altering the electron transfer process between QA and Qb (63, 43). It can be postulated that this conformational change modifies the bicarbonate binding that is required for proper electron transfer from QA to Qb (17, 10). Therefore, it is plausible that the action of Pb2+ at the WOC would cause this same transmembrane effect as was also proposed for the inhibitory action of Ni2+ and polyamines (64–65). This view is supported by the strong progressive upshift of the Tm of Q and B thermoluminescence bands with increasing concentrations of Pb2+ (Fig. 5).

Such large increase in thermoluminescence temperature was previously associated with the stabilization of the S2 state of the WOC due to the modification in the ligand environment of the Mn4Ca complex following the depletion in Cl– or in 33 kDa extrinsic polypeptide (66–67). Therefore, the shift of Tm towards higher temperatures may be due to a change in the population of PSII centers with a stabilized S2 state owing to the action of Pb2+ causing a retarded QA– reoxidation at low Pb2+ concentrations. This may represent an intermediate step in the inhibition of the WOC that precedes the serious damping of the fluorescence induction observed at high Pb2+ concentrations (Fig. 1).

Although an active site of Pb2+ at or near Qb cannot be fully excluded, the negative action of Pb2+ is postulated to proceed in two steps. During the intermediate step, the environment of the Mn4Ca complex is disorganized and the S2 state of the WOC is stabilized which consequently affects QA– reoxidation and increases S2/QA– charge recombination (though the recombination proceeds at a slower rate compared to the control). During the final phase, the WOC is damaged more seriously leading to a loss of charge recombination and of PQ reduction.

**Author Contributions**

Conceived and designed the experiments: AB RC. Performed the experiments: AB. Analyzed the data: AB SH RC. Contributed reagents/materials/analysis tools: SH RC. Wrote the paper: AB SH RC.

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