Effect of chromium on photosystem 2 in the unicellular green alga, *Chlorella pyrenoidosa*

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Abstract

We investigated the effect of chromium (20–40 g m^{-3}, 8–72 h) on the photosystem 2 (PS2) activities of *Chlorella pyrenoidosa* cells. By using chlorophyll fluorescence transients, thermoluminescence, oxygen polarography, and Western blot analysis for D1 protein we found that inhibition of PS2 can be accounted for by the enhanced photodestruction of the reaction centres in the cells cultivated in the presence of Cr(VI) at 25 °C in "white light" (18 W m^{-2}). Hence photodestruction of D1 is caused by an enhanced oxidative stress and lipid peroxidation, as indicated by the appearance of a high-temperature thermoluminescence band.

Additional key words: chlorophyll fluorescence; D1 protein; Western blot analysis; thermoluminescence.

Introduction

Chromium is a highly toxic non-essential metal for plants that inhibits a variety of metabolic activities, including photosynthesis (Clijsters and van Assche 1985). Interest in chromium originates from its widespread industrial use and consequent pollution in diverse environmental settings. Large quantities of Cr compounds are discharged in liquid, solid, and gaseous wastes into the environment, resulting in significant adverse biological and ecological effects (Kabata-Pendias and Pendias 2001). Chromium can exist in several chemical forms, displaying oxidation numbers from 0 to VI. Only trivalent and hexavalent chromium are stable enough to occur in the environment. Chromium (IV) and (V) forms are only unstable intermediates in reactions of trivalent and hexavalent oxidation states as oxidizing and reducing agents, respectively (Shriver *et al*. 1990, Ball and Nordstrom 1998). Cr(VI) is considered the most toxic form of Cr, which usually occurs associated with oxygen as chromate (CrO_4^{2-}) or dichromate ($Cr_2O_7^{2-}$) oxy-anions. The toxic effects of Cr in plants are primarily dependent on the metal speciation, which determines its uptake, translocation, and accumulation (Shanker *et al*. 2005), but photosynthesis is clearly an important target of its toxicity (Krupa and Baszyński 1995, Küpper *et al*. 2002, Appenroth *et al*. 2003).

Chlorella pyrenoidosa is a unicellular green alga, which is found both in fresh and marine waters. Its physiological and biochemical properties and photosynthetic apparatus are similar to higher plants, but its growth is very fast. For these reasons, *Chlorella* is used in various metabolic and stress investigations (Rachlin and Grosso 1993, Lustigman *et al*. 1995). The quantum yield of photosystem 2 (PS2) of microalgae is often used in phytotoxicity assays (Nash *et al*. 2005). This is based on the high sensitivity of PS2 toward abiotic stresses and takes advantages on the use of rapid, non-invasive, sensitive chlorophyll (Chl) fluorescence kinetic measurement. In the present work we investigated by using Chl fluorescence, thermoluminescence, O_2-polarography, and D1-Western blot analysis the toxic effect of Cr(VI) on PS2 of *Chlorella* cells at different concentrations and lengths of treatment in the dark and in the light.

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Abbreviations: Chl – chlorophyll; F_v, F_m – variable and maximum chlorophyll fluorescence, respectively; PpBQ – phenyl-*p*-benzoquinone; PS – photosystem; TL – thermoluminescence.
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Materials and methods

Organism and growth: *Ch. pyrenoidosa* (strain *IAM-C128*) cells were grown in sterile tubes containing a synthetic modified C-30 medium. Cultures were aerated by filtered air bubbled with 4 % CO_2, which promoted algal suspension and stabilized the algal suspension homogeneity and pH at 7.2. Cells were permanently irradiated with "white fluorescent light" (18 W m^{-2}) and were kept at 25 °C during the growth. The cultures were started from agar plates and were grown in liquid medium for 3 d. When the cultures reached approximately 1×10^5 cells per cm^3 in the mineral medium, algae were treated with 0.1–50.0 g m^{-3} of chromium (VI) as $K_2Cr_2O_7$.

Measurement of photosynthetic activities: Whole chain electron transport rate (from H_2O to CO_2) was measured by using a Clark-type oxygen electrode (*Hansatech*, Kings Lynn, UK) in a temperature controlled cell at 25 °C under saturating "white light" in the culture medium. PS2 electron transport rate was measured in the presence of 250 μM phenyl-*p*-benzoquinone (PpBQ). The absolute rates of O_2 evolution of untreated samples in the absence and presence of PpBQ were *ca*. 36 and 44 mmol(O_2) kg^{-1}(Chl) s^{-1}, respectively.

Chl fluorescence induction transients were measured at room temperature with a pulse-modulated *PAM-101* Chl fluorometer (*H. Walz*, Germany). The samples [10 g m^{-3}(Chl)] after 5 min dark adaptation were irradiated with saturating actinic "white light" (3 000 μmol m^{-2} s^{-1}) provided by *KL 1500* (*Schott*) halogen lamp for 5 s.

Thermoluminescence was measured in a home-built apparatus as described by Wiessner and Demeter (1988). 0.4 cm^3 aliquots of algal sample [10 g(Chl) m^{-3}] taken directly from the culture vessel were excited by continuous "white light" (50 μmol m^{-2} s^{-1}) at –80 °C. The emitted thermoluminescence was measured during heating the sample in the dark at a heating rate of 20 ºC per min by a *Hamamatsu* end-window photomultiplier.

Western blot was performed according to Towbin *et al.* (1979). The proteins were probed with mono-specific polyclonal antibodies raised against *Chlamydomonas reinhardtii* D1 protein. Blots were developed by using goat anti-rabbit secondary antibodies conjugated with alkaline phosphatase using standard nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate staining protocol. The amount of proteins was quantified by scanning the nitrocellulose membranes with a *Bio-Rad* densitometer.

Results and discussion

As shown in Fig. 1, upon exposure of *Ch. pyrenoidosa* cells to chromium, F_v/F_m, which is proportional to the quantum yield of PS2 reaction centre (Björkman and Demmig 1987, Genty *et al*. 1990), is reduced substantially, and this inhibitory effect increases with increasing the concentration of Cr(VI) (Fig. 1*A*). Inhibition of PS2, as reflected by O_2-polarography in the presence of PpBQ, develops gradually (Fig. 1*B*). These data are in reasonable agreement with literature on the inhibitory effect of Cr and other heavy metal ions on the PS2 activity of thylakoid membranes (Clijsters and van Assche 1985, Krupa and Baszyński 1995, Zeid 2001, Küpper *et al.* 2002). The lag phase and the relatively slow onset of the inhibition, however, suggest that the inhibition of PS2 activity might depend on a slow uptake of Cr, or it might be brought about by other effects on the photosynthetic machinery and/or on the chloroplast ultrastructure (cf. Appenroth *et al*. 2003, Shanker *et al*. 2005).

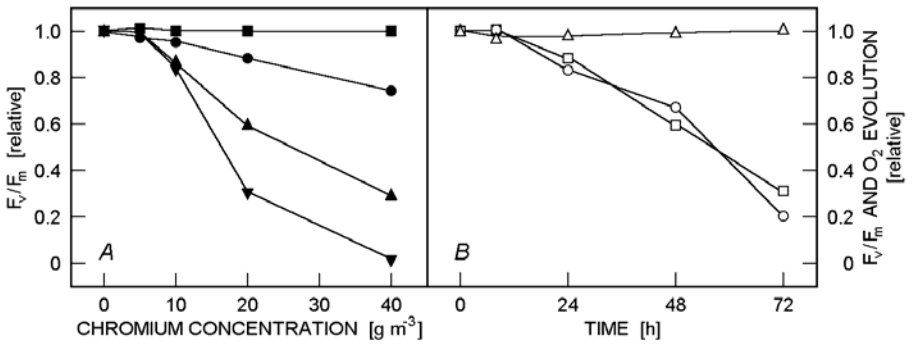

Fig. 1. Effect of chromium on PS2 activities in *Chlorella* cells at different Cr(VI) concentrations (*A*) and different lengths of treatment (*B*), characterized by chlorophyll fluorescence (F_v/F_m) and oxygen evolution in the presence of 250 μM PpBQ. The concentration dependence (*A*) is shown for 8 (■), 24 (●), 48 (▲), and 72 (▼) h of treatment. The time course of the inhibition at 20 g m^{-3} Cr(VI) (*B*) is shown for F_v/F_m (□) and O_2-evolution (○). *B* also shows that Cr(VI) treatment in the dark has virtually no effect on the O_2-evolution (△). The values were normalized to the untreated control at t = 0 h.

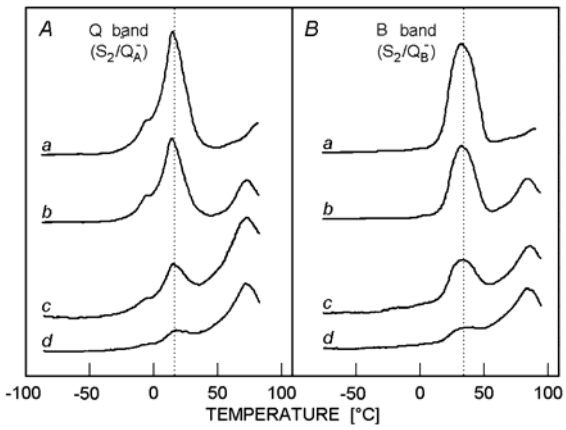

Fig. 2. Effect of 20 g m^{-3} Cr(VI) on the thermoluminescence of *Chlorella* cells after 0 (*a*), 24 (*b*), 48 (*c*), and 72 (*d*) h-treatment. Thermoluminescence was excited by continuous "white light" of 50 μmol m^{-2} s^{-1} for 30 s at –80 ºC in the presence (*A*) or absence (*B*) of 10 μM DCMU.

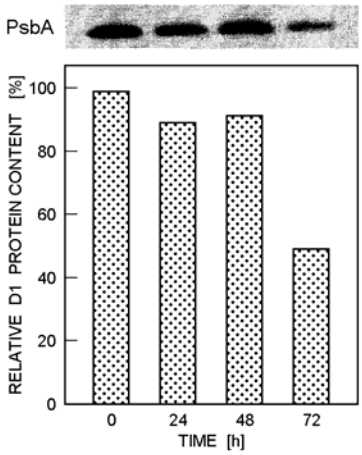

Fig. 3. Immunoblot SDS PAGE and densitometric analysis of D1 protein of *Chlorella pyrenoidosa* cultivated for different time periods in the presence of 20 g m^{-3} Cr(VI). For further details see Materials and methods.

The fact that this inhibitory effect is virtually absent in algal cells exposed to Cr in the dark (Fig. 1*B*) shows that either the uptake in algal cells is light-dependent or, which is more likely, the inhibitory effect on PS2 originates from other deleterious effect(s) on the photosynthetic system.

The change in the activity of PS2 induced by chromium exposure was also assessed by thermoluminescence (TL) measurements *in vivo*, *i.e.* on intact algal cells (Fig. 2). TL is a powerful tool to examine, *via* thermally induced charge re-combinations, the charge separation and the consecutive charge stabilisation steps in PS2. Irradiation of the algal cell suspension at –80 °C induces the B band originating from the charge recombination between the S_2 state of the water splitting complex and the reduced secondary quinone acceptor (Q_B) of PS2, with a maximum at around 32 °C. In the presence of DCMU, which inhibits the electron transport between Q_A and Q_B, the charge recombination occurs between the S_2 state and Q_A^-. According to the lower activation energy of that recombination reaction, the maximum of the corresponding TL band (Q band) appears at lower temperatures. Under our experimental conditions in *Ch. pyrenoidosa* the maximum of Q band was found at 15 ºC. Addition of 20 g m^{-3} Cr(VI) into the cultivation medium resulted in a gradual suppression of both the Q and B bands (Fig. 2). The fact that the B band is not replaced by Q band upon Cr(VI) treatment proves that the electron transfer between Q_A and Q_B is not hampered. Also, the decrease of the amplitudes of Q and B bands without shifting their maximum position indicates that the inactivation of PS2 during Cr(VI) treatment is due to the decreased number of the generated charges by the reaction centres in the primary charge separation or their subsequent stabilisation by formation of the S states and Q_A^- rather than to the alteration of redox properties of the S_2 state or the quinone acceptor. In other terms, these data suggest that the non-damaged reaction centres are not affected by chromium but Cr(VI) accelerates

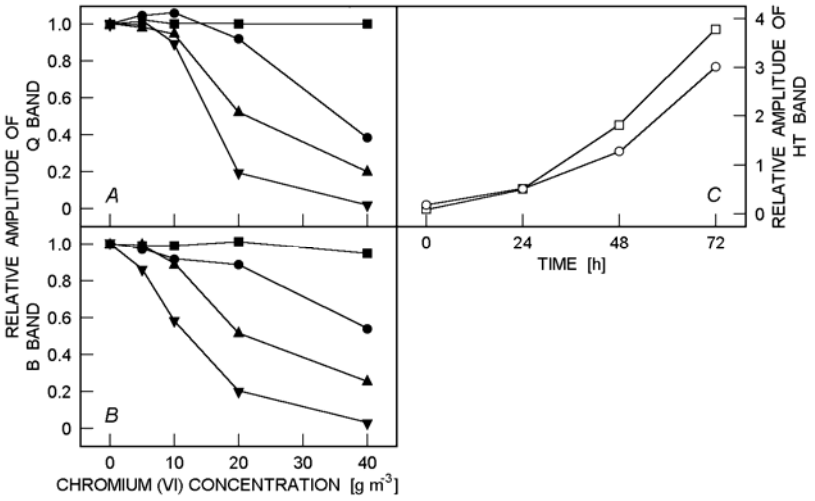

Fig. 4. Effect of chromium on the amplitudes of thermoluminescence bands. *A* and *B*: Q and B bands, respectively, relative to the untreated control, as a function of Cr(VI) concentration after 8 (■), 24 (●), 48 (▲), and 72 (▼) h of cultivation of the cells in the presence of chromium. *C*: Enhancement of the HT band [HT/Q (□) and HT/B (○)] during the cultivation of the cells in the presence of 20 g m^{-3} Cr(VI).

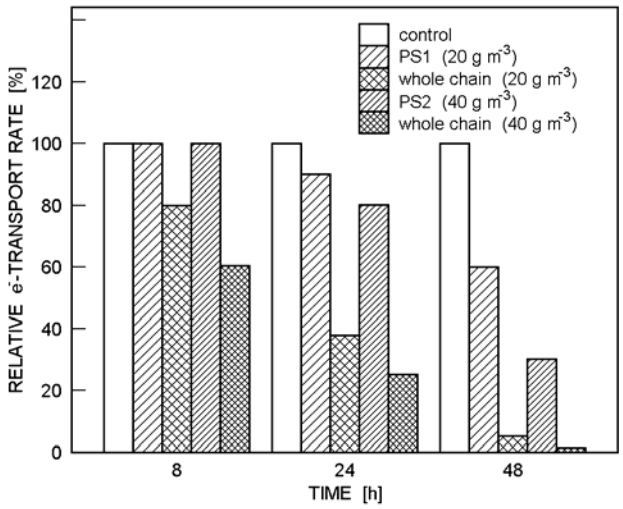

Fig. 5. Effect of chromium on photosystem 2 (PS2) and whole chain electron transport after different periods of cultivation times in the presence of 20 and 40 g m^{-3} $K_2Cr_2O_7$. The rates in the Cr(VI)-treated cells are compared to the corresponding rates in the untreated cells.

substantially the photo-destruction of the reaction centres and/or decelerates their repair (Mizusawa *et al*. 2003, Ohira *et al*. 2005, Zsiros *et al.* 2006). Indeed, as shown in Fig. 3, cultivation of the cells in the presence of Cr(VI) lead to a gradual destruction of the D1 protein of PS2.TL measurements also revealed that exposure of the cells to Cr(VI) led to the emergence of a high temperature (HT) band around 75 ºC, in parallel with the diminishment of the Q and B bands (Fig. 4). This high temperature band has been related to the peroxidation of the lipid components of thylakoid membranes initiated by oxidative stress (Ducruet and Vavilin 1999, Havaux 2003). Increased lipid peroxidation was also indicated by a 3–4 times increase in the malondialdehyde production of Cr(VI)-treated cells (20 g m^{-3}, 24 h) (Hörcsik *et al*. in preparation). The origin of this lipid peroxidation and the sequence of events are unclear. It might arise from reactions on the acceptor side of photosystem 1 rather than on PS2. This notion is supported by the data that the inhibition of whole chain electron transport is more significant and progresses more rapidly than the inhibition of PS2 reactions (Fig. 5). The enhancement of lipid peroxidation, coupled to the inhibition of electron transport, has been reported in different systems (Somashekaraiah *et al*. 1992, Sandalio *et al.* 2001, Dixit *et al.* 2002, Rai *et al.* 2004), and the absorption of excess light, in the absence of partially inactive electron transport system, can result in the formation of highly reactive oxygen species leading to further oxidative stress (Niyogi 1999). Such a mechanism might be held responsible for the enhanced net photo-destruction of PS2.

References

Appenroth, K.J., Keresztes, A., Sárvári, É., Jaglarz, A., Fischer, W.: Multiple effects of chromate on *Spirodela polyrhiza*: Electron microscopy and biochemical investigations. – Plant Biol. **5**: 315-323, 2003.

Ball, J.W., Nordstrom, D.K.: Critical evaluation and selection of standard state thermodynamic properties for chromium metal and its aqueous ions, hydrolysis species, oxides, and hydroxides. – J. chem. Eng. Data **43**: 895-918, 1998.

Björkman, O., Demmig, B.: Photon yield of O_2 evolution and chlorophyll fluorescence characteristics at 77 K among vascular plants of diverse origins. – Planta **170**: 489-504, 1987.

Clijsters, H., van Assche, F.: Inhibition of photosynthesis by heavy metals. – Photosynth. Res. **7**: 31-40, 1985.

Dixit, V., Pandey, V., Shyam, R.: Chromium ions inactivate electron transport and enhance superoxide generation *in vivo* in pea (*Pisum sativum* L. cv. Azad) root mitochondria. – Plant Cell Environ. **25**: 687-693, 2002.

Ducruet, J.M., Vavilin, D.: Chlorophyll high-temperature thermoluminescence emission as an indicator of oxidative stress: Perturbating effects of oxygen and leaf water content. – Free Radical Res. **31**: S187-S192, 1999.

Genty, B., Harbinson, J., Briantais, J.-M., Baker, N.R.: The relationship between nonphotochemical quenching of chlorophyll fluorescence and the rate of photosystem 2 photochemistry in leaves. – Photosynth. Res. **25**: 249-257, 1990.

Havaux, M.: Spontaneous and thermoinduced photon emission: new methods to detect and quantify oxidative stress in plants. – Trends Plant Sci. **8**: 409-413, 2003.

Kabata-Pendias, A., Pendias, H. Trace Elements in Soils and Plants. 3rd Ed. – CRC Press, Boca Raton – London – New York – Washington 2001.

Krupa, Z., Baszyński, T.: Some aspects of heavy metals toxicity towards photosynthetic apparatus – direct and indirect effects on light and dark reactions. – Acta Physiol. Plant. **17**: 177-190, 1995.

Küpper, H., Šetlík, I., Spiller, M., Küpper, F.C., Prášil, O.: Heavy metal-induced inhibition of photosynthesis: Targets of in vivo heavy metal chlorophyll formation. – J. Phycol. **38**: 429-441, 2002.

Lustigman, B., Lee, L.H., Khalil, A.: Effects of nickel and pH on the growth of *Chlorella vulgaris*. – Bull. Environ. Contamin. Toxicol. **55**: 73-80, 1995.

Mizusawa, N., Tomo, T., Satoh, K., Miyao, M.: Degradation of the D1 protein of photosystem II under illumination *in vivo*: Two different pathways involving cleavage or intermolecular cross-linking. – Biochemistry **42**: 10034-10044, 2003.

Nash, S.M.B., Quayle, P.A., Schreiber, U., Muller, J.F.: The selection of a model microalgal species as biomaterial for a novel aquatic phytotoxicity assay. – Aquat. Toxicol. **72**: 315-326, 2005.

Niyogi, K.K.: Photoprotection revisited: Genetic and molecular approaches. – Annu. Rev. Plant Physiol. Plant mol. Biol. **50**: 333-359, 1999.

Ohira, S., Morita, N., Suh, H.J., Jung, J., Yamamoto, Y.: Quality control of photosystem II under light stress – turnover of aggregates of the D1 protein in vivo. – Photosynth. Res. **84**: 29-33, 2005.

Rachlin, J.W., Grosso, A.: The growth response of the green alga *Chlorella vulgaris* to combined cation exposure. – Arch.

Environ. Contamin. Toxicol. **24**: 16-20, 1993.

Rai, V., Vajpayee, P., Singh, S.N., Mehrotra, S.: Effect of chromium accumulation on photosynthetic pigments, oxidative stress defense system, nitrate reduction, proline level and eugenol content of *Ocimum tenuiflorum* L. – Plant Sci. **167**: 1159-1169, 2004.

Sandalio, L.M., Dalurzo, H.C., Gomez, M., Romero-Puertas, M.C., del Rio, L.A.: Cadmium-induced changes in the growth and oxidative metabolism of pea plants. – J. exp. Bot. **52**: 2115-2126, 2001.

Shanker, K.A., Cervantes, C., Loza-Tavera, H., Avudainayagam, S.: Chromium toxicity in plants. – Environ. int. **31**: 739-753, 2005.

Shriver, D.F., Atkins, P.W., Langford, C.H.: Inorganic Chemistry. 2nd Ed. – Oxford University Press, Oxford 1990.

Somashekaraiah, B.V., Padmaja, K., Prasad, A.R.K.: Phytotoxicity of cadmium ions on germinating seedlings of mung bean (*Phaseolus vulgaris*): Involvement of lipid peroxides in chlorophyll degradation. – Physiol. Plant. **85**: 85-89, 1992.

Towbin, H., Stachelin, T., Gordon, J.: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. – Proc. nat. Acad. Sci. USA **76**: 4350-4354, 1979.

Wiessner, W., Demeter, S.: Comparative thermoluminescence study of autotrophically and photoheterotrophically cultivated *Chlamydobotrys stellata*. – Photosynth. Res. **18**: 345-356, 1988.

Zeid, I.M.: Responses of *Phaseolus vulgaris* to chromium and cobalt treatments. – Biol. Plant. **44**: 111-115, 2001.

Zsiros, O., Allakhverdiev, S.I., Hihashi, S., Watanabe, M., Nishiyama, Y., Murata, N.: Very strong UV-A light temporally separates the photoinhibition of photosystem II into light-induced inactivation and repair. – Biochim. biophys. Acta **1757**: 123-129, 2006.