Cadmium inhibits epoxidation of diatoxanthin to diadinoxanthin in the xanthophyll cycle of the marine diatom *Phaeodactylum tricornutum*

Martine Bertrand[a,b,*], Benoît Schoefs[b,1], Pavel Siffel[c], Karel Rohacek[c], Istvan Molnar[c]

[a] *Institut National des Sciences et Techniques de la Mer, Conservatoire National des Arts et Métiers, BP 324, F-50103 Cherbourg Cedex, France*
[b] *Laboratory of Biomembranes, University of South Bohemia, Branisovska 31, CZ-370 05 Ceske Budejovice, Czech Republic*
[c] *Laboratory of Photosynthesis, Institute of Plant Molecular Biology, Academy of Sciences of the Czech Republic, Branisovska 31, CZ-370 05 Ceske Budejovice, Czech Republic*

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Abstract Cd has pleiotropic effects on plant physiology and in particular on photosynthesis. It has not been established yet if Cd alters the functioning of the xanthophyll cycle. To answer this question, an exponentially growing culture of the marine diatom *Phaeodactylum tricornutum* was incubated with Cd (20 mg/l) for 24 h and irradiated with a light activating the xanthophyll cycle, which in diatoms, consists of the reversible deepoxidation of diadinoxanthin to diatoxanthin. The measurements show that the deepoxidation step is not influenced by Cd. In contrast, the Cd concentration used sharply inhibits the epoxidation of diatoxanthin to diadinoxanthin.

Key words: Algae; Microalgae; Cd; Heavy metal stress; Photoprotection mechanism; Pollution

1. Introduction

There is considerable concern about the increasing levels of heavy metals in the environment and the potential harmful effects of these elements on living organisms. This is especially important in the case of heavy metals like Cd, which have no biological functions (but see [1]).

The photosynthetic process is very sensitive to Cd: it reduces growth, inhibits chlorophyll (Chl) biosynthesis, depresses the photosynthetic carbon assimilation, etc. ([2–5], reviewed in [6]). To our knowledge, only scant information is available about the influence of Cd on the xanthophyll cycle, an important photoprotection process of photosynthesis triggered under high-light conditions ([7], reviewed in [8]). In higher plants and green algae, the xanthophyll cycle consists in the reversible conversion of violaxanthin to zeaxanthin via antheraxanthin, whereas in chromophytes, it consists in the interconversion of diadinoxanthin (DD) and diatoxanthin (DT) ([9], reviewed in [8]). The activity of this cycle has been correlated with the thermal dissipation of the excess energy at the antenna side of the photosynthetic apparatus [10,11].

In this contribution we report evidence, based on absorbance and fluorescence measurements and pigment quantifications, for the partial inhibition by Cd of the epoxidation step of the xanthophyll cycle in the marine diatom *Phaeodactylum tricornutum*.

2. Materials and methods

2.1. Cultivation of algae

The experiments were performed with the marine diatom *P. tricornutum* BOHLIN COUGHLAN/-632 axenic strain from the collection at the Institute of Botany at Trebon, Czech Republic. The cells were grown in a medium (pH 8.3) containing filtered reconstituted sea water (33 g/l of commercial salts from the Red Sea), Na_2HPO_4 (140 μM), $NaNO_3$ (1560 μM), Na_2SiO_3 (130 μM), $CuSO_4$ (1.4 μM), $Co(NO_3)_2{\cdot}6H_2O$ (1.6 μM), $MnCl_2$ (3.2 μM), Mo (0.7 μM), $ZnCl_2$ (0.3 μM), Fe–EDTA (100 μM), vitamin B1 (54 μM), vitamin B12 (0.7 μM) and vitamin H (700 μM). The chemicals (P.A. quality) were purchased by Fluka, and the water was of Milli-Q quality (Millipore).

Erlenmeyer flasks containing 100 ml of culture medium were inoculated with 25 ml of a mother diatom culture. The microalgae were grown under a light–dark cycle (16 h/8 h, illumination: fluorescent tubes, 40 μmol photons/m^2/s PAR) in a phytotron thermostated at 20 ± 2°C.

Different concentrations of $CdCl_2$ (0–25 mg/l of Cd) were applied the 4th day after inoculation (exponential phase). Measurements were performed 24 h after Cd addition.

2.2. Induction of the xanthophyll cycle and fluorescence measurements

Chl fluorescence induction kinetics were measured using a PAM 101-103 fluorimeter (Walz, Germany). The weak modulated measuring light (ML; 1.6 kHz) was delivered by a weak blue LED ($\lambda = 450$ nm, Nichia, Japan). The light was filtrated through a Corning 4-96 filter (380 nm $< \lambda <$ 600 nm). The detector was protected by a red filter (RG645, Schott, $\lambda > 650$ nm).

Before irradiation of a cell suspension by actinic light (AL), each sample was dark-adapted for 15 min and irradiated by a weak far-red (FR) light ($\lambda = 735$ nm, ~10 μmol/m^2/s) for 5 s to re-oxidize the electron transport carriers. Then, the minimum Chl fluorescence level (F_0) corresponding to open photosystem II (PSII) reaction centers was recorded using ML. Next, one polychromatic saturation pulse (SP; 1000 μmol/m^2/s, 1 s, light source: KL 1500 lamp, Walz, Germany) was applied to determine the maximum fluorescence level (F_M). After three min of relaxation in the dark, the suspension was illumi-

*Corresponding author. Fax: (33)-2-33 88 73 39.
E-mail address: mbertran@intechmer.cnam.fr (M. Bertrand).

[1] Present address: Plasticité et Expression des Génomes Microbiens, Université Joseph Fourier, CNRS-FRE 2383, BP 53, F-38041 Grenoble Cedex 9, France.

Abbreviations: AL, actinic light; Chl, chlorophyll; DD, diadinoxanthin; DT, diatoxanthin; DTT, dithiothreitol; F_0, minimum chlorophyll fluorescence level; F_0', minimum chlorophyll fluorescence level of the light-adapted state; F_M, maximum chlorophyll fluorescence level; F_M', maximum chlorophyll fluorescence level of the light-adapted state; F_v, variable fluorescence; FR, far-red; ML, modulated measuring light; NPQ, non-photochemical quenching; PSII, photosystem II; SP, saturation pulse

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nated with AL (1000 μmol/m^2/s, light source: KL 1500 lamp, Walz, Germany) for 7 min to induce fluorescence quenching. The intensity of AL was high enough to keep the PSII closed throughout the exposure and thus the observed fluorescence decline only represents non-photochemical quenching (NPQ).

When AL was switched off and FR was applied, the minimum Chl fluorescence level corresponding to the light adapted state (F_0') was determined. Then, dark relaxation of both F_0' and maximum Chl fluorescence level of the light-adapted state (F_M') were measured. During this period, light pulses were given at a regular interval of 5 min. The first one was triggered 1 min after switching off the AL (see Fig. 1). Variable fluorescence (F_v) was calculated as ($F_M - F_0$).

2.3. Pigment extraction and separation by HPLC

One milliliter of cells was taken at selected moments of the light–dark treatment described above (see Fig. 2). The cells suspension was spun down at 4000×*g*. The supernatant was discarded and the pigments were extracted with 800 μl tetrahydrofuran. The extract was centrifuged at 15 000×*g* and the pellet was extracted again with 400 μl acetone. The operation was repeated once, but using 800 μl methanol. After the last centrifugation, the pellet was colorless. The colored supernatants were pooled, filtered on a PTFE filter membrane (pore diameter: 0.45 μm), and dried under a nitrogen stream. The dried pigments were either directly used for HPLC analysis or stored at −80°C. All the operations were performed in a cold room and under a dim green light to avoid pigment alterations [12].

The dried samples were dissolved in 250 μl of methanol and analyzed according to [13]. The diode-array detector, the pump system and the column were described elsewhere [14]. All solvents were of HPLC quality. DD and DT were detected on the basis of the chromatograms recorded at 445 and 451 nm, respectively.

2.4. In vivo absorbance measurements

Light-induced absorbance difference spectra were measured on cell suspensions with the laboratory-built flash spectrophotometer composed of a microsecond Xe flash lamp (FX-1160, EG$_\Sigma$G, USA, flash duration of 1 μs), an imaging monochromator (MS257, Oriel, USA) and an acquisition and triggering unit FL100 (Photon System Instruments, Czech Republic). The detector was composed of two large area photodiode arrays (S4111, Hamamatsu) for sample and reference. Measuring light pulse was filtered using a combination of GG475 filter (Schott, $\lambda > 480$ nm) and a Corning 4-96 filter (380 nm $< \lambda <$ 600 nm), divided to sample and reference channels by a broadband beam splitter and focused to sample and reference cells. After passage of light through a sample and a reference, both light beams were focused to two bundles of optical fibers and directed to the monochromator input. This experimental setup gave a noise as low as 2×10^{-4} per one flash with a spectral resolution of 2.1 nm per one spectral point. Each absorbance measurement represents an accumulation of 10 spectra measured within 1 s. The Chl concentration was approximately 20 mg/l. To reach this concentration, the cells were sedimented without centrifugation (15 min in darkness). For the experiments using dithiothreitol (DTT), the algae were incubated in the presence of the compound (1 mM) in the dark for 20 min.

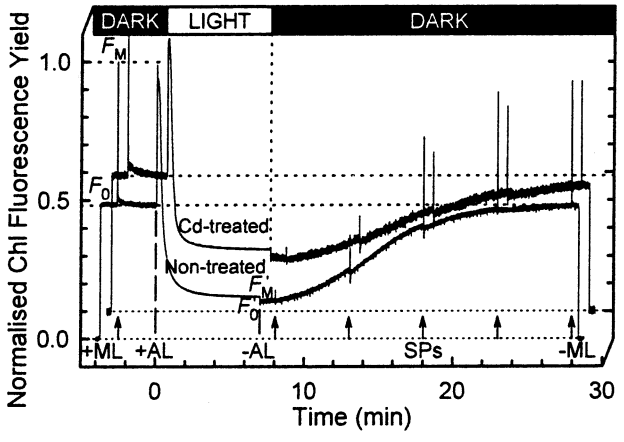

Fig. 1. Typical Chl fluorescence induction kinetics recorded with living cells of *P. tricornutum* in the absence or presence of Cd (20 mg/l). Each arrow designates one SP.

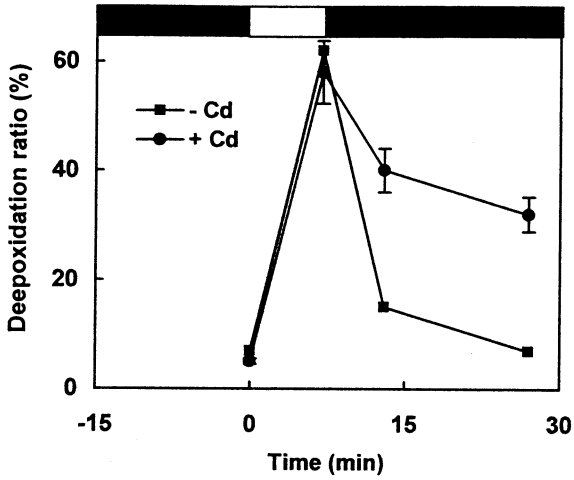

Fig. 2. Variations of the relative amount of DT during the light–dark sequence applied on *P. tricornutum* grown in the absence or presence of 20 mg Cd/l ($n = 6$).

3. Results and discussion

3.1. Influence of Cd concentration on the cell growth

The increase of Cd concentration in the culture medium reduced the growth rate: the higher the concentration in Cd, the lower the rate (data not shown). The growth rate was reduced by 50% using a concentration of 20 mg Cd/l. A similar value was reported in [15]. Therefore, this concentration was used for all the experiments.

3.2. Cd slows down the fluorescence yield relaxation

In photosynthetic organisms, the relative decrease of the fluorescence yield is correlated with the activity of the xanthophyll cycle (higher plants: [16], diatoms: [17,18]) although it cannot be fully explained by the xanthophyll conversion [10,17,18]. Fig. 1 presents typical Chl fluorescence induction kinetics of non-treated and Cd-treated cells. The comparison of the figures presented in Table 1 clearly indicated that Cd has no effect on the maximum yield of PSII photochemistry (F_v/F_M) or the NPQ mechanism (($F_M - F_M'$)/F_M'), which did not differ significantly in Cd-treated algae. However, the rate of the F_0' relaxation in the dark is reduced by 58% in the Cd-treated cells.

It has been shown that the F_0 quenching is of non-photochemical nature. It could be induced by the formation of high-energy state of thylakoid membranes [19,20], photoinhibition of PSII reaction centers and/or by the xanthophyll cycle activity [21,22]. Depression of fluorescence intensity by photoinhibition is accompanied by a pronounced decrease of the F_v/F_M ratio, which is not observed in the presence of Cd (Table 1). High-energy state of thylakoid membranes of high-

Table 1
Effects of the Cd addition on the Chl fluorescence induction parameters ($n = 6$)

	F_v/F_M	($F_M - F_M'$)/F_M'	F_0 Relaxation rate (/min)
−Cd	0.58 ± 0.02	2.21 ± 0.20	0.12 ± 0.02
+Cd	0.58 ± 0.03	2.31 ± 0.28	0.05 ± 0.02

er plants, and related fluorescence decrease, usually relax within 1 min after switching off AL. For *Phaeodactylum* cells, the relaxation lasted 10–30 min (Fig. 1). This duration corresponds to the rate of DT epoxidation [23]. Hence, the decrease in the rate of F_0 relaxation, observed in the presence of Cd, may reflect an inhibition of the xanthophyll epoxidation reaction.

3.3. Inhibition of the epoxidation step of the xanthophyll cycle by Cd

In order to determine whether the slowing down of the F_0 relaxation rate in Cd-treated cells is correlated with a change in the xanthophyll cycle activity, the previous experiment was repeated and the variations of the DD and DT amounts were measured after HPLC separation. Fig. 2 presents the variations of the relative amount of DT during the light–dark sequence in the absence or in the presence of Cd. Regardless of the presence of Cd, the same level of deepoxidation was reached at the end of the high-light illumination. In contrast, the reversion of the xanthophyll cycle was strongly slowed down in the presence of Cd. Remarkably, the inhibition of epoxidation rate was very similar in magnitude (approximately 60%) to that of the relaxation rate (Table 1). This definitively confirms that both phenomenons are tightly linked.

3.4. Measurements of the xanthophyll cycle in vivo

It has been shown with higher plants that the deepoxidation of violaxanthin to zeaxanthin is reflected by an increase of the absorbance at approximately 505 nm [24–26]. In order to verify whether the activity of the xanthophyll cycle in diatoms is also correlated to similar absorbance changes, we recorded absorbance spectra at room temperature during the light–dark sequence (see Figs. 1 and 2). The spectra were subtracted from the spectrum recorded before illumination. The 7 min AL, which triggers the conversion of DD to DT (Fig. 2), induced a large increase of the absorbance at approximately 465 and 500 nm (Fig. 3A, circles). The absorbance changes were fully reversible in the dark (Fig. 3A, squares). This result clearly establishes that the activity of the xanthophyll cycle in diatoms can be followed by measuring absorbance modifications at 500 nm. To obtain an additional evidence for this conclusion, we incubated the algae with DTT, a specific inhibitor of violaxanthin deepoxidase in higher plants [24] and DD deepoxidase in diatoms [18]. In this condition, the absorbance changes at approximately 500 nm were totally inhibited (Fig. 3B, triangles). This result suggests that the two deepoxidases have a similar mode of functioning. This conclusion is in line with the recent finding of Jakob and collaborators [27] who showed that DD deepoxidase can also use violaxanthin as a substrate.

Fig. 3B presents the absorbance changes measured with algae grown in the absence or presence of Cd. As expected, regardless of the presence or absence of Cd, the absorbance changes at 500 nm were equivalent after the light treatment (Fig. 3B, circles). In contrast, the decrease of the absorbance at 500 nm, which reflects the epoxidation of DT to DD, was slower in the presence of Cd (Fig. 3B, compare squares (+Cd) with triangles (−Cd)).

3.4.1. To summarize. Cd does not influence the deepoxidation of DD to DT, but partially inhibits the rate of the epoxidation reaction. The comparison of fluorescence and pigment quantification data reveals that the rate of F_0 relaxation reflects the rate of DT epoxidation. Similarly to higher plants, the xanthophyll cycle in diatoms can be followed by absorbance variations at approximately 500 nm.

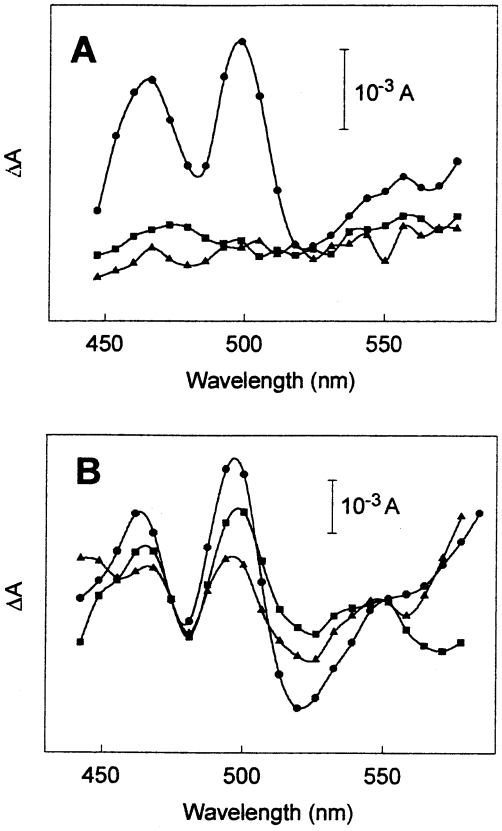

Fig. 3. Effect of Cd on the absorbance difference spectra of *P. tricornutum* (the control stayed in darkness). A: (●) −Cd+7 min AL; (■) −Cd+7 min AL+40 min darkness; (▲) −Cd+DTT+7 min AL. B: (●) +Cd+7 min AL; (■) +Cd+7 min AL+7 min darkness; (▲) −Cd+7 min AL+7 min of darkness.

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