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Production of Phytochelatins in the Marine Diatom *Phaeodactylum tricornutum* in Response to Copper and Cadmium Exposure

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Plants, algae and some fungi are capable of synthesizing, on exposure to heavy metals, thiol-rich peptides $(\gamma\text{-glutamylcysteinyl})_n$-glycine with n=2 to 11, also known as phytochelatins (Grill et al. 1985). Phytochelatins (PCs) seem to be involved in detoxification and homeostasis of trace metals in plants and thus serve functions analogous to metallothioneins in animals. Phytochelatins are enzymatically synthesized by a specific γ-glutamylcysteine -dipeptidyl transpeptidase (phytochelatin synthase) which is activated by the presence of metal ions and uses glutathione as a substrate (Grill et al. 1989). Phytochelatins bind metal ions by thiolate coordination yielding intracellular metal complexes.

In the literature it has been reported that many trace metals can induce phytochelatin production in plants, although the cellular response depends on the particular metal (Grill et al. 1987; Steffens 1990).

Most of the studies concerning phytochelatin production in phytoplankton have generally been limited to their induction by Cd. To our knowledge, only two studies of phytoplankton, one in two species of chlorophytes (Gekeler et al. 1988) and the other in three marine species (Ahner and Morel 1995) have reported stimulation of phytochelatin production by other metals, including copper. The latter authors, by using a chemically defined, metal-buffered medium, report that Cd is the most effective inducer of PCs for most algae at metal concentrations naturally occurring in seawater.

The aim of this study was to investigate the induction of PCs in the marine phytoplanktonic alga *Phaeodactylum tricornutum* on copper exposure. The PC production in response to Cu exposure was evaluated as a function of the intracellular metal concentration, and compared to that obtained in response to Cd, which has previously been shown to be a good inducer in this alga species (Kawaguchi and Maita 1990; Morelli and Scarano 1995).

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MATERIALS AND METHODS

Phaeodactylum tricornutum Bohlin (Bacillariophyceae), was maintained axenically in a thermostatic room at 25°C under continuous illumination at 3000±500 lux using, as culture medium, natural seawater enriched with f/2 (Guillard 1975) without Cu and Zn. Seawater was collected in the Tyrrhenian Sea (close to the Tuscan Archipelago), then filtered through 0.45 µm membrane filters (Sartorius) and stored in the dark at +5°C. A stock culture was maintained in the exponential growth phase by inoculating every 7 days into a fresh sterilized medium. Suitable volumes of this stock solution were used to inoculate cultures at an initial cell concentration of $5 \cdot 10^5$ cells ml^{-1}. Five days after inoculation, near the end of the log-phase, 200 ml of the culture was centrifuged at 4000 rpm for 15 minutes and then rinsed three times with seawater. The washed algae were added to one litre of seawater to obtain an initial cell concentration of $3 \cdot 10^5$ cells ml^{-1}. The seawater was previously charcoal-treated in order to remove dissolved organic matter, sterilized by filtration through 0.22 µm sterile filters (Sartorius), and spiked with the appropriate amounts of $Cu(NO_3)_2$ or $CdCl_2$. Cell density was determined at the beginning and after 24 h exposure, by counting cells in a Thoma counting chamber under a microscope.

After a 24 h exposure, the cells to be assayed for cellular metal-binding material were harvested by gentle suction filtration through 0.45 pm membrane filters and rinsed with seawater containing $2 \cdot 10^{-3}$ M ethylenediamine (EN) and then with metal-free seawater, to remove metals adsorbed to the cell surface. The cells were resuspended in 6 ml 25 mM TRIS (hydroxymethyl) amino methane, 0.15 M NaCl pH 8.3 buffer, containing, as antioxidant, 1 mM 3-Mercapto-1,2-propanediol (monothioglycerol) and, as protease inhibitor, 1mM phenylmethylsulfonyl fluoride (PMSF). An aliquot of 100 µL of this suspension was appropriately diluted and measured by atomic absorption spectrometry (AAS) for the metal content. This value was used to calculate the total amount of metal in a single cell, and was referred to as the intracellular metal concentration. An untreated culture was submitted to the same experimental protocol and the intracellular metal concentration was found to be negligible. The cells were disrupted by sonication in an ice bath, filtered through a 0.2 µm membrane filter and immediately subjected to the gel-filtration procedure.

Gel filtration was performed by using a Hi-Load Superdex 30 column (60 x 1.6 cm; Pharmacia) previously calibrated at 0.15 M ionic strength with molecular weight standards as cytochrome C (M_r 12000) aprotinine (M,

6500), vitamin B12 (M_r 1355) and lumichrome (M_r 242). Haemoglobin (M_r 64000) was used to determine the column exclusion volume. The chromatographic system consisted of a LKB 2150 pump, a Rheodyne model 7125 injection valve, an UV detector (Uvicord SD, Pharmacia, LKB) set at 254 nm and a Hewlett Packard model 3396 computing integrator as recorder. The injection volume was 4 ml. The elution buffer was 25 mM TRIS, 0.15 M NaCl pH 8.3, at a flow rate of lml min^{-1}. The eluate was collected in 2 ml fractions.

Cu and Cd concentrations were determined by AAS using a Perkin Elmer Spectrophotometer (Model 1100 B) equipped with a graphite furnace (Model HGA 700).

The concentration of the sulfhydryl groups (SH) in the fractions was determined using an adsorptive cathodic stripping voltammetic method described elsewhere (Scarano and Morelli 1996). Voltammetric measurements were carried out in stirred solutions, consisting of 0.5 ml of the chromatographic fraction and 19.5 ml of 25 mM TRIS, 0.1 M NaCl pH 8.3 buffer made $2.5 \cdot 10^{-6}$ M Cu. After 60 s of preconcentration time at -0.4 V, the potential was scanned in the cathodic direction from -0.6 to -1.4 V, in the differential pulse mode. The instrument was a Metrohm 646 VA processor connected to a 647 VA stand equipped with a hanging mercury drop electrode (HMDE) and the instrumental settings were: scan rate, 12 mV s^{-1}; pulse duration, 40 ms; pulse repetition time, 0.3 s; modulation amplitude, 50 mV.

All reagents were of analytical grade: PMSF and monothioglycerol were from Sigma; $Cu(NO_3)_2$, and $CdC1_2$ from BDH, ethylenediamine, TRIS and NaCl from Carlo Erba. HNO_3 was Suprapur grade (Merck). All solutions were prepared with pure water obtained by a Milli-Q purification system (Millipore).

RESULTS AND DISCUSSION

In order to investigate the presence of metal binding peptides in Cu-treated algae, *Phaeodactylum tricornutum* cells were exposed to a copper concentration of 2 µM in organic-free seawater, at a cell concentration of 3.10^5 cells ml^{-1}, for 24 h. The crude extracts of Cu-treated and untreated cells were subjected to gel filtration chromatography and the eluates assayed for copper. A copper-inducible UV-absorbing peak was eluted at a volume of 73±0.5 ml, corresponding to an apparent molecular weight of 4000-5000, and coinciding with the elution of Cu (Fig. 1). The Cu-binding material eluted from gel filtration column as a symmetrical peak, with a peak width at half-maximal height corresponding to 5-7% of the column volume.

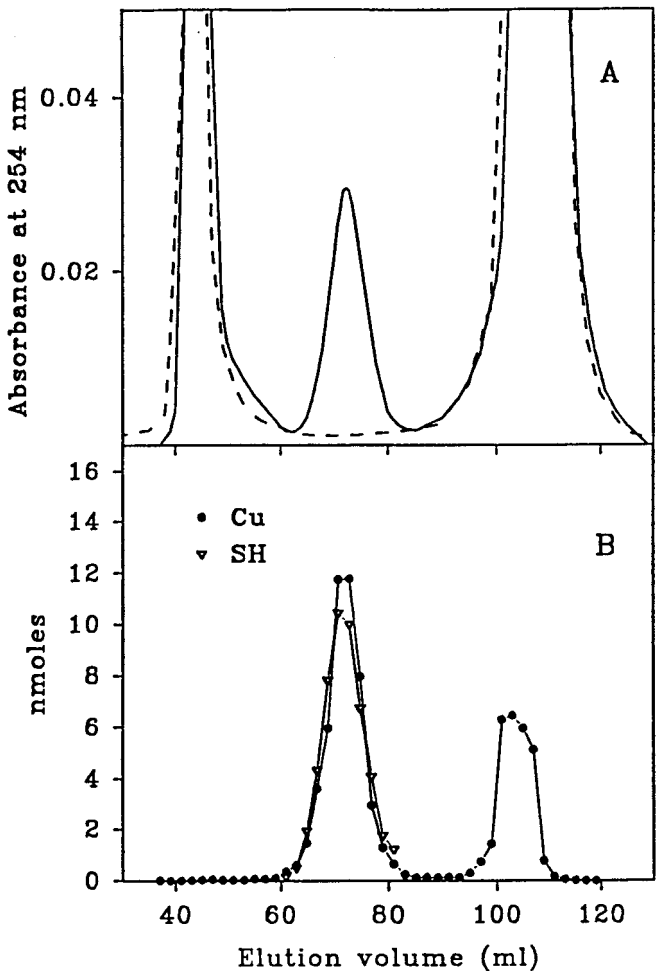

Figure 1. A) Gel filtration profiles of crude extracts of $2.3 \cdot 10^8$ cells of *Phaeodactylum tricornutum* grown for 24 h in the absence (- - - -) and in presence of 2 µM Cu (——). B) Cu and SH distribution in the gel filtration eluates of copper-treated cells.

The Cu-containing fractions were assayed for the SH content and a clear association of the induced material with Cu and sulfhydryl groups was found. This result indicates the presence of a copper-phytochelatins complex (Cu-PCs) in this alga. *Phaeodactylum tricornutum* cells, grown in the absence of Cu, did not exhibit any detectable metal binding peptide. For Cu-treated and untreated cells, two other UV-absorbing peaks appeared, which can be attributed to high molecular weight material and to a thioglycerol-copper complex, respectively. In the extract of untreated cells, in which an amount of copper similar to that of the Cu-exposed cells was added before the fractionation, neither Cu or SH could be detected at the Cu-induced peak position, and about 90% of the Cu was recovered at the

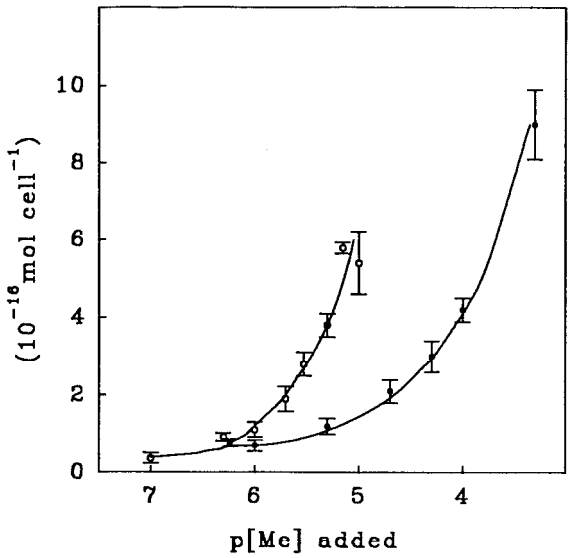

Figure 2. Intracellular Cu (o) and Cd (•) concentrations as a function of the external metal concentration. Error bars refer to the standard deviations for three replicate experiments.

thioglycerol peak. In the extract of the Cu-exposed cells about 70% of the total amount of Cu injected into the column was bound to the Cu-induced peak, 1-2% was recovered in the high molecular weight fractions, and 25-30% eluted with the thioglycerol peak. The SH:Cu molar ratio in the Cu-PCs complex was calculated in each fraction collected from the Cu-induced peak obtained from three separate experiments, and a mean value of 0.97±0.21 (n=24) was found. This value suggests a binding stoichiometry SH:Cu in the Cu-PCs complex close to 1. This result agrees with the value suggested by Grill et al. (1991) for the copper-phytochelatin complexes.

Similarly to the Cu-exposed cells, cultures of *Phaeodactylum tricornutum* exposed to cadmium excess showed the appearance of an inducible, UV-absorbing, Cd- and SH- containing chromatographic peak, with a SH:Cd molar ratio of 1.9 ± 0.4, as described in details in a previous paper (Morelli and Scarano 1995) thus indicating the presence of cadmium-phytochelatins complexes (Cd-PCs). Here, carrying out exposure tests with Cu or Cd additions, the response of the algal cells to excess Cu has been compared with that of Cd.

Since the PC production should be regulated by the intracellular level of the metal, the Cu and Cd intakes were evaluated and compared. The concentration of intracellular Cu and Cd as a function of the respective metal added in the culture medium is reported in Fig. 2. The figure shows

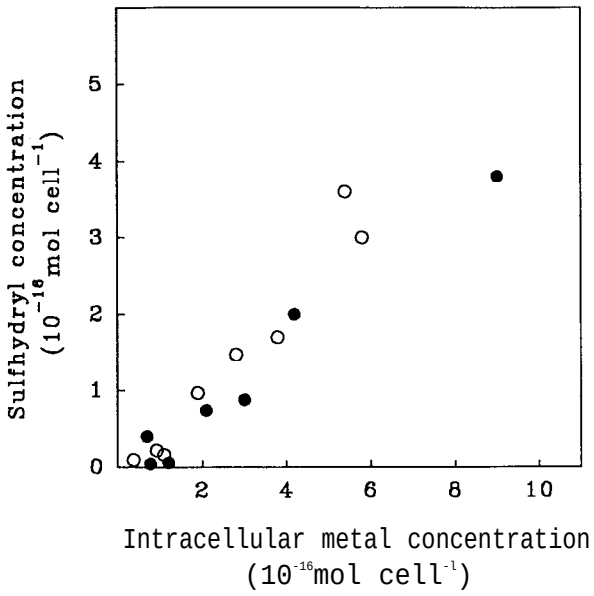

Figure 3. SH concentration iu the inducible material as a function of the Cu (o) or Cd (•) intracellular concentration.

that the exposure to either metal produces an increase in the intracellular metal concentration. At concentration lower than 10^{-6} M, the metal intake is low and comparable for both Cu and Cd. Above this level the intracellular amount of Cu increases, while the intracellular Cd increases only at concentrations at least one order of magnitude higher. In particular,at 10^{-5} M extracellularly, the intracellular Cu was close to $6 \cdot 10^{-16}$ mol cell^{-1} and the intracellular Cd was as low as $1 \cdot 10^{-16}$ mol cell^{-1}. From these data it can be inferred that to obtain $6 \cdot 10^{-16}$ mol cell^{-1} of Cd, concentrations higher than 10^{-4} M Cd must be added to the culture media. Hence, in order to compare the PC production induced by similar level of Cu and Cd inside the cell, cultures of *Phaeodactylum tricornutum* were exposed to Cd or Cu at external concentration to give approximately the same amount of intracellular metal.

The production of PCs induced by Cu or Cd, taken as the sum of the sulfhydryl groups in the chromatographic fractions corresponding to the metal-induced peak, is reported in Figure 3 as a function of the intracellular metal. The figure shows that the patterns of the SH contents induced by Cu and Cd are superimposable over the entire experimental range and both increase almost linearly with the intracellular metal concentration. This result indicates that Cu induces as much phytochelatin as Cd, when the same intracellular metal concentration is reached. In addition, the relationship between metal ion concentration inside the cell and PCs

suggests that the mechanism of PC synthesis is controlled by the metal entered inside the cell. The metal influx, in its turn, is dependent from the external metal concentration, as shown in Fig. 2, and from the capability of the membrane to regulate the passage of a specific metal.

It has been reported that at environmentally relevant metal concentrations, algae are capable to synthesize the exact concentration of PCs necessary to bind intracellular metal (Ahner et al., 1995). This should be the necessary requirement of an effective detoxification mechanism. However, our data, obtained using high metal concentrations and short exposure time, show that the amount of PCs synthesized is inadequate to sequester all the intracellular metal. In fact, taking into account that the SH:Me molar ratio was found to be 1:1 for Cu and 2:1 for Cd, it can be calculated that less than 50% of intracellular metal is sequestered by PCs. Further investigation at lower metal concentrations are in progress in order to clarify this point.

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