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# Effect of Heavy Metals on the Activity of External Carbonic Anhydrase of Microalga *Chlamydomonas reinhardtii* and Microalgae from Karst Lakes

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Carbonic anhydrase (CA, EC4.2.1.1) is a zinc metalloenzyme that catalyzes the reversible hydration of carbon dioxide: $CO_2 + H_2O \longleftrightarrow HCO_3^- + H^+$. External CA ($CA_e$) enables algal cells to utilize $HCO_3^-$ as inorganic carbon source in case that $CO_2$ is limited externally to the plasma membrane (Tsuzuki and Miyachi, 1989). $HCO_3^-$, when it is charged, cannot freely cross the non-polar lipid cell membrane whereas $CO_2$ can. $CA_e$ is a major component of the $CO_2$-concentrating mechanism in some algal species (Aizawa and Miyachi, 1986). Not all autotrophic species of aquatic unicellular protists express external carbonic anhydrase. Algal $CA_e$ activity is species-specific (Colman and rotatore, 1995; Nimer et al., 1997) and also varies with season and phase of growth (Hobson et al., 2001).

Heavy metals are of environmental interest both as trace nutrients and as toxicants at elevated concentrations. It is well understood that the aqueous chemistry of metals such as metal speciation and metal-metal interaction is crucial for controlling metal uptake and toxicity to aquatic organisms (Sunda and Huntsman, 1998). Numerous studies have been conducted with respect to interactions between heavy metals and microalgae in molecular and/or cell levels, such as algal bioaccumulation of heavy metals and the effect of heavy metals on algal photosynthesis and main nutrients assimilation (Haritonidis and Malea, 1999; Danilov and Ekelund, 2001; Devriese et al., 2001). However, there are few studies that have considered the influence of heavy metals on external carbonic anhydrase of microalgae, specifically dealing with microalgae from karst lakes.

In the present study we have investigated the influences of different heavy metals (Cu, Zn, Cd, Pb) on the activity of $CA_e$ for microalgae collected from karst lakes and microalga *Chlamydomonas reinhardtii* in laboratory culture. The latter provides an excellent model for this purpose (Devriese et al., 2001). We also identified microalgal species in these karst lakes. The aim of this study was to understand: (i) the effect of heavy metals on external CA from various microalgae; and (ii) the relationship of this effect and microalgal species; and (iii) the relationship of $CA_e$ activities and microalgal species. The possible mechanism in which heavy metals affect external carbonic anhydrase is also discussed.

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## MATERIALS AND METHODS

All chemicals used in this study were A.R. grade. Heavy metal stock solution consists of distilled water and $CdCl_2 \cdot 2.5H_2O$, $ZnSO_4 \cdot 7H_2O$, $CuSO_4$, $Pb(NO_3)_2$, respectively. The concentration of heavy metal stock solution is $10^{-2}$ M.

The microalgal samples were collected from two karst lakes (106°26'E, 26°31'N): Aha Lake and Hongfeng Lake, which are located in the central part of the Yunnan-Guizhou Plateau. Both lakes are important sources of drinking water for Guiyang, the capital of Guizhou province. Lake water is $CO_2$ - $HCO_3^-$ buffered. $HCO_3^-$ is the main anion in both lakes (2.23mmol/L in Aha Lake and 2.17mmol/L in Hongfeng Lake, respectively). Due to a high pH, the concentration of dissolved trace metals is very low (for Cu, 3.40ng/mL in Aha Lake and 2.64ng/mL in Hongfeng Lake, respectively; Wang et al., 2003).

Microalgae were collected from surface waters at Aha and Hongfeng lakes. 55L surface water from each lake was immediately filtered by 270 meshes fabric in order to remove impurities. Subsequent filtration with cellulose acetate membrane filters (1.2 μm pore size, Millipore) yielded the microalgae that were used in the following experiments. Microalgae were scraped off the cellulose acetate membrane and diluted to 400mL by final filtrated surface water. This provided our microalgal stock solution. After being fixed with a 2% formaline solution, 5mL microalgal stock solution was used to identify microalgal species and analyze microalgal quantity by light microscope. By centrifugation at 1600g for 10min, 10mL microalgal stock solution was utilized to determine chlorophyll *a* (chl *a*). The centrifuged microalgae were soaked in 90% ethanol at 4℃ in the dark for 12 hrs, then centrifuged (1600g for 10 minutes). The extract was analyzed at 665 and 750 nm wavelength against a 90% ethanol blank. The same extract was acidified with a drop of 1 N HCl and the steady absorbance at 665 and 750 nm was subsequently detected within 10min. The concentration of chl *a* was calculated according to the following equation (Lorenzen, 1967):

chlorophyll*a*(mg/L)=$11.49 \cdot R \cdot ((A_{665}-A_{750})_{na}-(A_{665}-A_{750})_a) \cdot V_e/(V_c \cdot L \cdot (R-1))$.

where $(A_x)_{na}$ and $(A_x)_a$ are absorbances at x nm under non-acidified and acidified conditions, respectively. R is maximun absorbance ratio of $(A_{665})_{na}$ / $(A_{665})_a$, that is 1.7 here. $V_e$ is extraction volume in milliliter. L is cuvette light-path in centimeter. $V_c$ is centrifuged volume in litre.

*Chlamydomonas reinhardtii* was obtained from the Institute of Hydrobiology, Chinese Academy of Sciences and cultured sterile in the artificial freshwater medium SE, which is provided by the Freshwater Algae Culture Collection (http://www.ctcccas.ac.cn). Concentrations of Cd, Pb, Cu, Zn in SE medium are 0, 0, 0.02, 0.05mg/L, respectively. The exposure concentrations were nominal. *Chlamydomonas reinhardtii* was cultured in 150mL flasks inside an incubation chamber at 25.0℃±1.0℃, under a 16:8 h light:dark regime, in 4000LX illumination, with reciprocative shaking (90times/min).

By adding heavy metal stock solution to SE medium and microalgal stock

solution, *Chlamydomonas reinhartii* and microalgae collected from karst lakes were treated under Cu, Zn, Cd, Pb-excessive conditions, at 0.5, 1.0, 1.5, 2.0 mg/L concentrations, respectively. After 24 hours, these microalgae were immediately harvested by centrifugation at 1600g for 10min. Harvested microalgae were suspended in 15mL ice-cold barbital buffer (PH 8.30) for one hour in order to adequately extract $CA_e$. 10mL ice-cold $CO_2$-saturated water was subsequently added to this buffer and recorded the time (T) in which pH decreased from 8.20 to 7.20. The activity of $CA_e$ was computed with the equation: $CA = (T_{nonmicroalgae}/T_{microalgae}-1) / \text{chl } a$, where CA is the activity (Wilbur-Anderson units $\cdot$ (ug chl $a$)$^{-1}$) (Wilbur and Anderson, 1948). The relative activity of $CA_e$ (RCA) was computed according to: $RCA = CA_{metal}/CA_{nonmetal}$. Each treatment consisted of three replicates. The mean and standard deviation are calculated for each treatment. One-way ANOVA and pairwise comparison test (Tukey) are conducted for each group.

## RESULTS AND DISCUSSION

In order to assess the contribution of a changing algal biomass on our experiments, we have measured biomass change of *Chlamydomonas reinhartii* over time. Within 24 hours, biomass of *Chlamydomonas reinhartii* hardly increases at Cu, Pb, Cd, Zn-excessive conditions, even at normal culture conditions; but after 24 hours biomass of *Chlamydomonas reinhartii* increases over time (Table 1). From this result, we concluded that 24 hours is the optimum time for heavy metal exposure.

**Table 1.** Biomass change of *Chlamydomonas reinhartii* over time under Cu, Zn, Cd, Pb-excessive conditions (2.0mg/L for each heavy metal) and normal culture condition.

| Time | Biomass of *Chlamydomonas reinhartii* (Chl *a*, mg/L) | | | | |
|---|---|---|---|---|---|
| (hr) | Cu | Zn | Cd | Pb | Normal |
| 0 | 1.33 ± 0.10 | 1.33 ± 0.10 | 1.33 ± 0.10 | 1.33 ± 0.10 | 1.33 ± 0.10 |
| 24 | 1.31 ± 0.04 | 1.42 ± 0.01 | 1.31 ± 0.06 | 1.35 ± 0.07 | 1.51 ± 0.02 |
| 48 | 1.37 ± 0.07 | 1.86 ± 0.03 | 1.63 ± 0.05 | 1.86 ± 0.02 | 2.15 ± 0.02 |
| 72 | 1.40 ± 0.02 | 1.84 ± 0.16 | 1.62 ± 0.16 | 1.81 ± 0.16 | 2.34 ± 0.04 |
| 96 | 1.51 ± 0.05 | 1.99 ± 0.06 | 1.76 ± 0.17 | 2.12 ± 0.06 | 2.43 ± 0.12 |

Values are means ± SD of three replicates.

Abundances of different principal microalgae species from the investigated karst lakes are given in table 2. We had extracted $CA_e$ from *Chlamydomonas reinhartii* and microalgae collected from Hongfeng Lake and Aha Lake. Inhibition of different heavy metals on $CA_e$ increased with increasing heavy metal concentrations (Table 3). However, cadmium increases the activity of $CA_e$ at low concentrations. It is well known that the addition of cadmium increases the activity of CA in culture experiments (Morel et al., 1994; Lee et al., 1995). Obviously, cadmium is special to carbonic anhydrase, normally a zinc-based metalloenzyme, possibly due to the fact that Zn and Cd share a common group in the periodic table. But with increasing concentration, Cd also inhibits the activity of $CA_e$, just like Zn, Cu, Pb. The inhibitory effect of Cd, Cu, Zn on carbonic

anhydrase activity of estuarine crabs had also been reported (Vitale et al., 1999; Skaggs and Henry, 2002). Our data indicate that the magnitude in which Cu, Zn, Cd, Pb affect $CA_e$ depends on the concentration (Table 3). Furthermore, it appears that the inhibitory effect of Cu, Zn, Cd, Pb on $CA_e$ is species-specific. Not all algae possess external CA, and different algal species have different tolerance thresholds to heavy metals toxicity.

**Table 2.** Species number and abundance of main microalgae from Hongfeng Lake and Aha Lake in March, 2003.

| | Hongfeng Lake | | Aha Lake | |
|---|---|---|---|---|
| | Numbers | Quantity($\times10^5$cells/L) | Numbers | Quantity($\times10^5$cells/L) |
| Cyanophyta | 5 | 6.40 | 3 | 7.69 |
| Pyrrophyta | 0 | 0.00 | 2 | 0.25 |
| Chrysophyta | 1 | 0.11 | 2 | 0.05 |
| Chlorophyta | 21 | 3.53 | 11 | 0.97 |
| Bacillariophyta | 6 | 2.14 | 4 | 1.81 |

**Table 3a.** The relative activity of $CA_e$ from *Chlamydomonas reinhartii* under Cu, Zn, Cd, Pb-excessive conditions.

| Treated concentration | The relative activity of $CA_e$ | | | |
|---|---|---|---|---|
| (mg/L) | Cu | Zn | Cd | Pb |
| | $1.00 \pm 0.13^n$ | $1.00 \pm 0.13$ | $1.00 \pm 0.13$ | $1.00 \pm 0.13$ |
| 0.5 | $1.17 \pm 0.11$ | $0.88 \pm 0.17$ | $1.16 \pm 0.42$ | $0.61 \pm 0.15^*$ |
| 1.0 | $0.92 \pm 0.06$ | $0.82 \pm 0.09$ | $1.03 \pm 0.18$ | $0.73 \pm 0.02^*$ |
| 1.5 | $0.77 \pm 0.11$ | $0.54 \pm 0.21^*$ | $1.10 \pm 0.11$ | $0.84 \pm 0.07$ |
| 2.0 | $0.49 \pm 0.08^*$ | $0.31 \pm 0.09^*$ | $1.05 \pm 0.09$ | $0.77 \pm 0.11$ |

$^n$ normal culture condition; $^*$ The mean difference is significant between metal-excessive condition and normal culture condition (Tukey, $P<0.05$). Values are means ± SD of three replicates.

**Table 3b.** The relative activity of $CA_e$ from microalgae collected from Aha Lake under Cu, Zn, Cd, Pb-excessive conditions.

| Treated concentration | The relative activity of $CA_e$ | | | |
|---|---|---|---|---|
| (mg/L) | Cu | Zn | Cd | Pb |
| | $1.00 \pm 0.08^n$ | $1.00 \pm 0.08$ | $1.00 \pm 0.08$ | $1.00 \pm 0.08$ |
| 0.5 | $0.18 \pm 0.06^*$ | $0.73 \pm 0.08^*$ | $1.25 \pm 0.10$ | $0.78 \pm 0.05$ |
| 1.0 | $0.15 \pm 0.05^*$ | $0.65 \pm 0.09^*$ | $0.66 \pm 0.14^*$ | $0.57 \pm 0.09^*$ |
| 1.5 | $0.11 \pm 0.03^*$ | $0.40 \pm 0.09^*$ | $0.34 \pm 0.13^*$ | $0.23 \pm 0.05^*$ |
| 2.0 | $0.03 \pm 0.04^*$ | $0.06 \pm 0.02^*$ | $0.23 \pm 0.07^*$ | $0.23 \pm 0.14^*$ |

$^n$ normal culture condition; $^*$ The mean difference is significant between metal-excessive condition and normal culture condition (Tukey, $P<0.05$). Values are means ± SD of three replicates.

The activities of external CA ($\times10^{-3}$) of *Chlamydomonas reinhardtii*, microalgae collected from Aha Lake and Hongfeng Lake were 5.27 ± 0.66, 10.39 ± 0.17, 10.15±0.23, respectively (values are means ± SD from three replicates). The

activity of $CA_e$ from karst lakes is obviously higher than that from *Chlamydomonas reinhardtii*, whereas that from Hongfeng Lake and Aha Lake is nearly identical. The function of external CA is to accelerate the equilibration of $CO_2$ and $HCO_3^-$ in alkaline medium so that $CO_2$ is formed at a sufficient rate to support photosynthesis. $HCO_3^-$ is the main anion in karst lakes, whereas $HCO_3^-$ in SE culture medium was nearly zero. Obviously the activity of external carbonic anhydrase, which is located outside of cell membrane and surface active, is correlated with ambient $HCO_3^-$ in our study. This has also been found in some algae (Nara et al., 1990; Nimer et al., 1999).

**Table 3c.** The relative activity of $CA_e$ from microalgae collected from Hongfeng Lake under Cu, Zn, Cd, Pb-excessive conditions.

| Treated concentration | | The relative activity of $CA_e$ | | |
|---|---|---|---|---|
| (mg/L) Cu | Zn | Cd | Pb |
| | $1.00 \pm 0.09^n$ | $1.00 \pm 0.09$ | $1.00 \pm 0.09$ | $1.00 \pm 0.09$ |
| 0.5 | $0.87 \pm 0.13$ | $0.87 \pm 0.11$ | $1.37 \pm 0.12^*$ | $1.07 \pm 0.07$ |
| 1.0 | $0.90 \pm 0.12$ | $0.74 \pm 0.06^*$ | $1.18 \pm 0.09$ | $0.94 \pm 0.11$ |
| 1.5 | $0.83 \pm 0.11$ | $0.69 \pm 0.05^*$ | $1.17 \pm 0.07$ | $0.87 \pm 0.06$ |
| 2.0 | $0.56 \pm 0.08^*$ | $0.46 \pm 0.13^*$ | $0.88 \pm 0.11$ | $0.82 \pm 0.06$ |

$^n$ normal culture condition; $^*$ The mean difference is significant between metal-excessive condition and normal culture condition (Tukey, $P<0.05$). Values are means ± SD of three replicates.

**Table 4**. The relative activity of $CA_e$ from *Chlamydomonas reinhardtii.*

| | Cu | Zn* | Cd | Pb |
|---|---|---|---|---|
| After | $0.57 \pm 0.06$ | $0.93 \pm 0.19$ | $1.02 \pm 0.15$ | $0.68 \pm 0.10$ |
| Before | $0.47 \pm 0.08$ | $0.31 \pm 0.09$ | $1.05 \pm 0.09$ | $0.78 \pm 0.12$ |

$^*$ The mean difference is significant between "Before" and "After" (Tukey, $P<0.05$). Values are means ± SD for three replicates. "Before" refers to the relative activity of $CA_e$ from *Chlamydomonas reinhardtii* that was exposed to 2.0 mg/L heavy metals for 24 hours before extracting. "After" refers to the relative activity of $CA_e$ that was exposed to 2.0 mg/L heavy metals after being extracted from *Chlamydomonas reinhardtii.*

In order to understand the mechanism of interaction between heavy metals and external CA, we have measured the activity of external CA during exposure to 2.0mg/L heavy metals after being extracted from *Chlamydomonas reinhardtii* growing under normal culture condition. This was compared to the activity of $CA_e$ from *Chlamydomonas reinhardtii* exposed in 2.0 mg/L heavy metals for 24 hours before extracting. The effect of Cd, Cu, Pb on $CA_e$ is nearly identical following both kinds of treatment. Solely Zn is different (Table 4). This indicates that Cd, Cu, Pb probably affect $CA_e$ by directly interacting with $CA_e$, whereas Zn probably interacts indirectly with $CA_e$, such as influencing the metabolism of $CA_e$ in *Chlamydomonas reinhardtii* cell. In algal cells, metal binding sites are never entirely specific for a single nutrient metal. They also bind competing metals with similar ionic radii and coordination geometry. This could be the reason of inhibition on $CA_e$ by Cu and Cd. In addition to competition of metals for

biological ligands, some heavy metals, such as Pb, can directly change the bioactivity of proteins.

In conclusion, algal $CA_e$ activity is species-specific and correlated with ambient $HCO_3^-$. The effects of Cu, Zn, Cd, Pb on $CA_e$ from the same algal species are also different. Cadmium increased the activity of $CA_e$ at low concentrations and inhibited the activity of $CA_e$ with increasing concentrations. The extent of Cu, Zn, Cd, Pb influencing $CA_e$ is variable and correlated with the algal species. The effect of heavy metals on $CA_e$ appears to be threefold: ( i ) competing with $CA_e$ cofactor-Zinc; ( ii ) indirectly affecting the metabolism of $CA_e$ in algal cell; and (iii) directly changing bioactivity of $CA_e$.

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