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Effect of acetazolamide on stable carbon isotope fractionation in *Chlamydomonas reinhardtii* and *Chlorella vulgaris*

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The effect of extracellular carbonic anhydrase (CAex) on stable carbon isotope fractionation in algae is still unclear. The stable carbon isotope composition and algal growth in the presence and absence of the membrane-impermeable CA inhibitor acetazolamide were compared in *Chlamydomonas reinhardtii* and *Chlorella vulgaris*. The CAex of both algal species contributed about 9‰ of the stable carbon isotope fractionation and exhibited a dosage effect. Therefore, evidence *in vivo* that CAex leads to a larger carbon isotope fractionation of algae is presented.

acetazolamide, algae, bicarbonate, carbonic anhydrase, stable carbon isotope

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The isotopic composition of sedimented algal material is an indicator of paleoenvironmental conditions because of the well-documented effect of CO_2 concentration on marine algal carbon fractionation [1–3]. However, use of existing models for prediction of algal carbon isotope fractionation revealed a large deviation in some aquatic ecosystems [4]. This deviation, if not considered in prediction models, would affect the precision of predictions of paleoenvironmental CO_2 concentrations based on the carbon isotope composition.

The deviation from the model-predicted isotope fractionation results not only from environmental factors, but also from some important physiological factors. Extracellular carbonic anhydrase action might be one of the main physiological processes that lead to the deviation. Carbonic anhydrase (CA; EC 4.2.1.1), a zinc-containing metalloenzyme, catalyzes the reversible interconversion between bicarbonate (HCO_3^-) and CO_2. The uncatalyzed, slow interconversion between CO_2 and HCO_3^- produced about 10‰ of the stable carbon isotope fractionation, whereas the interconversion *in vitro* catalyzed by CA had only 1.1‰ fractionation [5,6].

The present study examines the effects of extracellular CA (CAex) on carbon isotope fractionation by comparison of the stable carbon isotope composition and algal growth with and without the membrane-impermeable CA inhibitor acetazolamide (AZ) in *Chlamydomonas reinhardtii* and *Chlorella vulgaris*. Evidence *in vivo* showed that extracellular CA leads to higher algal carbon isotope fractionation.

1 Materials and methods

Chlamydomonas reinhardtii and *Chlorella vulgaris* samples were obtained from the Institute of Hydrobiology, Chinese Academy of Sciences. Both species were grown axenically in artificial freshwater soil extract (SE) medium. Cultures were incubated at 25.0 ± 1.0°C under 150 μmol m^{-2} s^{-1} light intensity and a 16/8 h day/night cycle. Experiments were conducted with the following treatments.

Treatment 1: *C. reinhardtii* and *C. vulgaris* were grown in SE media that contained different $NaHCO_3$ concentra-

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tions (0, 0.5, 2, 8, 16, or 20 mmol L^{-1}) with or without AZ (10 mmol L^{-1}). The cultures were treated for 12 d, with the first 8 d for expanding the culture, and the last 4 d for strengthening the culture. The added $NaHCO_3$, which has a $\delta^{13}C$ value of −17.4‰ in solid state, and −16.6‰ in solution, was a tracer for the dissolved inorganic carbon (DIC) sources used by algae.

Treatment 2: *C. reinhardtii* and *C. vulgaris* were grown in SE media with different AZ concentrations (0, 0.01, 0.10, 0.50, or 1.00 mmol L^{-1}) and 0.5 mmol L^{-1} added $NaHCO_3$. The cultures were treated for 14 d, with the first 10 d for expanding the culture, and the last 4 d for strengthening the culture. The added $NaHCO_3$ was the same $\delta^{13}C$ value as that of Treatment 1.

All experimental treatments consisted of five replicates. Algal proteins were assayed using Coomassie brilliant blue. Aquamerck was used in the titration of bicarbonate concentrations in the media.

The algal cultures were dried prior to analysis and were converted to CO_2 at 800°C in a quartz tube over copper oxide in an oxygen atmosphere. Water and oxygen were removed from the gas stream in a liquid N_2 trap, and CO_2 was double distilled and collected into a sample tube. The CO_2 sample was analyzed with an isotope ratio mass spectrometer (Finnigan MAT 252, Bremen, Germany). All isotopic compositions ($\delta^{13}C$) are expressed as per mille (‰) and compared with a standard (Pee Dee Belemnite) (see Formula (1)). The analytical precision was ±0.1‰.

$$\delta^{13}C\ (‰) = [(R_{sample}/R_{standard})-1]\times 1000, \quad (1)$$

where R_{sample} and $R_{standard}$ are the ratio of heavy to light isotopes ($^{13}C/^{12}C$) of the sample and the standard, respectively.

2 Results and discussion

2.1 Stable carbon isotope composition with and without acetazolamide

The CO_2 and generated HCO_3^- concentrations varied with the amount of added HCO_3^-. The total DIC content increased with increasing added HCO_3^- concentration (Table 1). The $\delta^{13}C_{DIC}$ and the proportion of HCO_3^- obtained from conversion of CO_2 to total DIC were high at low added HCO_3^- concentrations (0–2.00 mmol L^{-1}), and low at high added HCO_3^- concentrations (8.00–20.00 mmol L^{-1}) regardless of the presence or absence of AZ (Table 1). The pH in the presence of AZ was lower than that without AZ. Therefore, the CO_2 concentrations in the culture media that contained

Table 1 Concentration of dissolved inorganic carbon and $\delta^{13}C_{DIC}$ in original culture media of *C. reinhardtii* and *C. vulgaris*

Treatment	pH	$[HCO_3^-]^{a)}$ (mmol L^{-1})	$[HCO_3^-]^{b)}$ (mmol L^{-1})	$[HCO_3^-]^{c)}$ (mmol L^{-1})	$[CO_2]^{d)}$ (mmol L^{-1})	Total DIC (mmol L^{-1})	$\delta^{13}C_{DIC}$ (‰, PDB)
Chlamydomonas reinhardtii −AZ	6.50	0	0.80	0.80	0.61	1.41	−11.8
	6.87	0.50	1.60	1.10	0.52	2.12	−13.7
	7.75	2.00	2.90	0.90	0.12	3.02	−14.8
	8.54	8.00	8.20	0.20	0.06	8.26	−15.9
	8.82	16.00	15.70	−0.30	0.06	15.76	−16.5
	8.88	20.00	19.40	−0.60	0.06	19.46	−16.6
Chlorella vulgaris −AZ	6.50	0	0.80	0.80	0.61	1.41	−11.8
	6.87	0.50	1.45	0.95	0.47	1.92	−13.5
	7.75	2.00	2.70	0.70	0.11	2.81	−15.6
	8.54	8.00	8.20	0.20	0.06	8.26	−16.5
	8.82	16.00	15.45	−0.55	0.06	15.51	−16.6
	8.88	20.00	19.5	−0.50	0.06	19.56	−16.6
Chlamydomonas reinhardtii +AZ	6.00	0	1.00	1.00	2.40	3.40	−10.5
	6.40	0.50	1.80	1.30	1.72	3.52	−11.9
	7.00	2.00	2.90	0.90	0.70	3.60	−14.5
	7.62	8.00	8.40	0.40	0.48	8.88	−16.1
	7.90	16.00	15.90	−0.10	0.48	16.38	−16.4
	8.00	20.00	19.50	−0.50	0.47	19.97	−16.5
Chlorella vulgaris +AZ	6.00	0	0.80	0.80	1.92	2.72	−10.5
	6.40	0.50	1.60	1.10	1.53	3.13	−12.0
	7.00	2.00	2.80	0.80	0.67	3.47	−14.5
	7.62	8.00	8.25	0.25	0.47	8.72	−16.1
	7.90	16.00	15.70	−0.30	0.47	16.17	−16.4
	8.00	20.00	19.50	−0.50	0.47	19.97	−16.5

a) $NaHCO_3$ concentration added to the culture medium; b) $NaHCO_3$ concentration in the culture medium; c) HCO_3^- concentration from the conversion of CO_2; d) concentration of CO_2 in the medium.

AZ were higher than in the media lacking AZ.

The stable carbon isotope composition and growth of *C. reinhardtii* and *C. vulgaris* varied with the total DIC with and without AZ (Figure 1). Protein content increased at low added HCO_3^- concentrations and decreased at high added HCO_3^- concentrations without AZ. However, in the presence of AZ, the protein content increased independent of HCO_3^- concentration.

The stable carbon isotope composition without AZ was significantly different from that with AZ. The mean $\delta^{13}C$ values of *C. reinhardtii* and *C. vulgaris* without AZ were about 9.1‰ and 11.4‰ more positively skewed than that with AZ at low added HCO_3^- concentrations (0–2.00 mmol L^{-1}), respectively. The $\delta^{13}C$ values of the two algal species were similar (about −25.5‰) at 8.00 mmol L^{-1} added HCO_3^- regardless of the presence or absence of AZ. The $\delta^{13}C$ values of *C. reinhardtii* and *C. vulgaris* without AZ were more negatively skewed than that with AZ (16.00 and 20.00 mmol L^{-1} added HCO_3^-, respectively).

The stable carbon isotope composition in algae reflects the utilization of DIC [7]. Without AZ, the algal cells mainly utilized the HCO_3^- generated from the rapid interconversion of CO_2 catalyzed by CAex at low added HCO_3^- concentrations (0–2.00 mmol L^{-1}). The carbon isotope fractionation was very low (about 1.1‰) [6]. However, in the presence of AZ, the algal cells mainly used the HCO_3^- generated from the slow (uncatalyzed) interconversion of CO_2 at low added HCO_3^- concentrations. The slow interconversion between CO_2 and HCO_3^- produced about 10‰ of the stable carbon isotope fractionation [5]. Therefore, $\delta^{13}C$ values in the presence of AZ at low added HCO_3^- concentrations were about 9‰ less than those in the absence of AZ, regardless of algal growth rate or cell size [8].

Algal growth rate and cell size may affect isotope fractionation [8]. The $\delta^{13}C$ value is inversely correlated with algal growth rate or cell size [8]. The difference in the $\delta^{13}C$ values of *C. reinhardtii* with and without AZ was approximately 9‰. This value could be regarded the absolute contribution of CAex. However, greater differences in the $\delta^{13}C$ values than 9‰ (mean 11.4‰) were observed in *C. vulgaris*. A small, additional difference (mean 2.4‰) in the $\delta^{13}C$ values was recorded in *C. vulgaris* above that produced by CAex. The additional difference might reflect the lower algal growth rate and smaller cell size when *C. vulgaris* was cultured in media that contained AZ.

A linear or stoichiometric relationship existed between the $\delta^{13}C$ values in the algal cells and the total DIC in culture media lacking AZ at low added HCO_3^- concentrations. In natural water bodies, the concentration of HCO_3^- is much lower than 2.0 mmol L^{-1} [9]. Thus, we also deduced that the difference in carbon isotopic fractionation is large between algae with high CAex activity and that without CAex activity.

The $\delta^{13}C$ value of added HCO_3^- was more negative than that of the control (0 mmol L^{-1} added HCO_3^-) (Table 1). At high concentrations of added HCO_3^- (8.00–20.00 mmol L^{-1}), the algal cells mainly used the added HCO_3^- in the presence or absence of AZ. Therefore, the more negative the algal $\delta^{13}C$ values, the more the algal cells used the added HCO_3^- regardless of the growth restrictions. In medium lacking AZ,

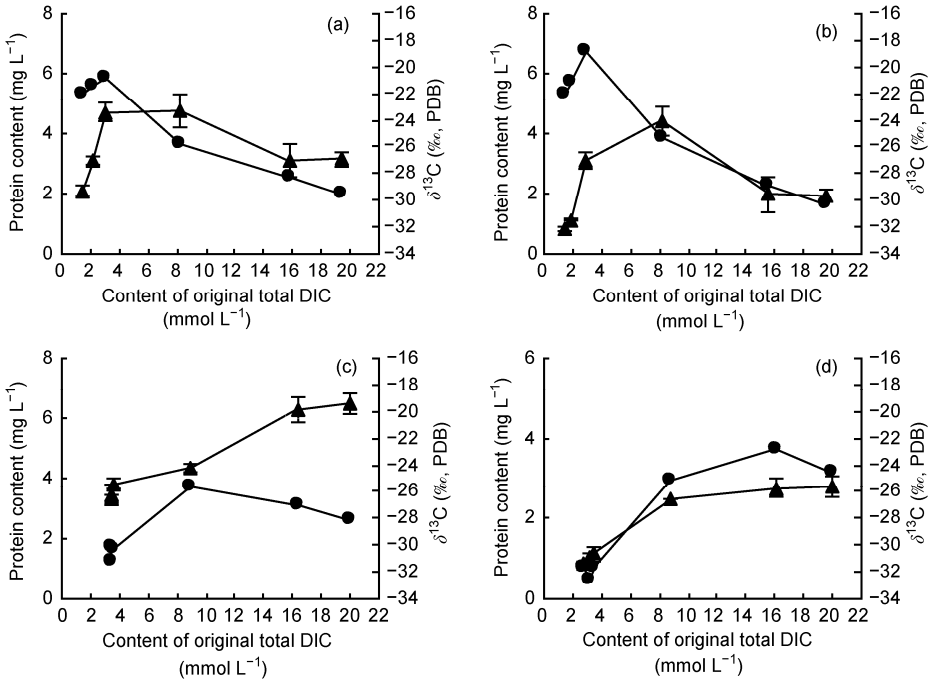

Figure 1 Stable carbon isotope composition (●) and growth (▲) of *Chlamydomonas reinhardtii* and *Chlorella vulgaris* with total dissolved inorganic carbon. (a) *C. reinhardtii* without AZ; (b) *C. vulgaris* without AZ; (c) *C. reinhardtii* + AZ; (d) *C. vulgaris* + AZ.

growth of the two algal species was inhibited by the DIC sources because of the high pH of the culture media. The HCO_3^- concentration was too high for algal growth in these conditions. The algal cells prioritized uptake of the light ^{12}C isotope, which resulted in a negatively skewed $\delta^{13}C$ value and higher carbon isotope fractionation. Furthermore, carbon isotope fractionation increased with increasing pH and HCO_3^- concentration in the medium. Thus, a linear relationship was observed between the $\delta^{13}C$ values in algal cells and the total DIC in culture medium lacking AZ at high added HCO_3^- concentrations (8.00–20.00 mmol L^{-1}).

Growth of the two algal species was not inhibited by the DIC sources at high added HCO_3^- concentrations (8.00–20.00 mmol L^{-1}) in the presence of AZ because of the moderate pH of the culture medium. The algal cells produced little carbon isotope fractionation during the growth period because of the unrestricted DIC. Therefore, the $\delta^{13}C$ values of the algal cells with AZ were higher than those in medium lacking AZ.

The algal growth rate and cell size in *C. reinhardtii* were higher than those of *C. vulgaris* at 16.00 and 20.00 mmol L^{-1} added HCO_3^-. Therefore, the $\delta^{13}C$ values of *C. reinhardtii* were lower than those of *C. vulgaris* at 16.00 and 20.00 mmol L^{-1} added HCO_3^-.

2.2 Dosage effect of acetazolamide on the stable carbon isotope signature

Low AZ concentrations (0–0.1 mmol L^{-1}) promoted growth of *C. reinhardtii*, whereas high AZ concentrations (0.5–1 mmol L^{-1}) slightly inhibited growth. In the concentration range tested, AZ exhibited no significant effect on the growth of *C. vulgaris*. The effect of AZ on the stable carbon isotope fractionation of *C. reinhardtii* was similar to that of *C vulgaris* (Figure 2). The two algal species showed positively skewed $\delta^{13}C$ values because of the slight inhibition of CAex activity by AZ at low concentrations, and negatively skewed $\delta^{13}C$ values because of the high inhibition of CAex activity by high AZ concentrations. These results indicated AZ has a dose-dependent effect on algal stable carbon isotope fractionation.

3 Conclusions

Extracellular carbonic anhydrase can significantly influence stable carbon isotope fractionation of *C. reinhardtii* and *C. vulgaris* under normal growth conditions. The CAex of *C. reinhardtii* and *C. vulgaris* contributes approximately 9‰ of the stable carbon isotope fractionation, which was derived from the difference between the smaller fractionation

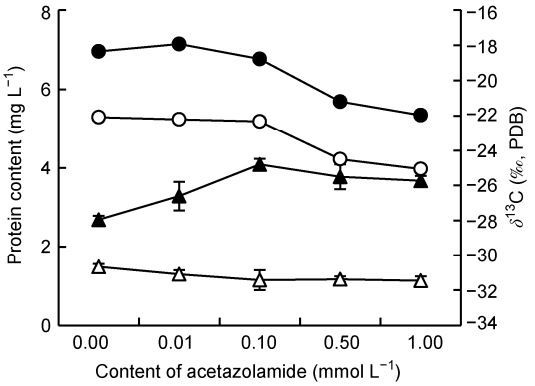

Figure 2 $\delta^{13}C$ values and growth of *Chlamydomonas reinhardtii* and *Chlorella vulgaris* at different AZ concentrations. ●, $\delta^{13}C$ of *C. reinhardtii*; ○, $\delta^{13}C$ of *C. vulgaris*; ▲, protein content of *C. reinhardtii*; △, protein content of *C. vulgaris*.

from the catalyzed conversion of CO_2 to HCO_3^- and the larger fractionation from the uncatalyzed, slow interconversion. Moreover, CAex had a dose-dependent effect on algal stable carbon isotope fractionation, which could cause a large deviation in the predicted paleoenvironmental CO_2 concentration based on the algal carbon isotope composition.

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