Roles of Carbonic Anhydrase in Photosynthesis of *Skeletonema costatum*

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Abstract: The role of carbonic anhydrase (CA) in photosynthesis of the marine diatom *Skeletonema costatum* grown at ambient level of CO_2 was investigated. Extracellular CA activity was very low. In comparison, intracellular CA activity was great part of total CA activity. The inhibition of external CA by acetazolamide (AZ) caused little change in net photosynthetic rate (P_n), but the inhibition of intracellular CA by ethoxyzolamide (EZ) resulted in the strong reduction of P_n. EZ reduced the light-saturated photosynthesis, the saturation radiance and the affinity of inorganic carbon for photosynthesis, raised inorganic carbon compensation point and enhanced the inhibition of photosynthesis by high O_2 and light. It is concluded that extracellular CA exerted a minor role in the photosynthesis, but intracellular CA enhanced the efficiency of photosynthetic carbon fixation and the capacity of acclimation to stress conditions (high light, O_2 and low CO_2) by catalytically converting HCO_3^- to CO_2 and facilitating CO_2 supply to the cell.

Key words: *Skeletonema costatum*; acetazolamide; ethoxyzolamide; carbonic anhydrase; photosynthesis; photorespiration; photosynthetic affinity to inorganic carbon

Algae in both marine and freshwater environments like terrestrial higher plants use gaseous CO_2 as the substrate of photosynthetic carbon fixation. However, aquatic environments are quite different from the terrestrial ones. CO2 in water diffuses about 10^4 times slower than in air, though its partial pressure in water is almost equal to that in the air when air-water equilibrium is reached (Raven 1999). Therefore, photosynthesis of marine phytoplankton can be more CO_2-limited. Bicarbonate ions (HCO_3^-) accounts for more than 95% of the total dissolved inorganic carbon in natural seawater, while CO_2 accounts for in less than 1% (Raven and Falkowski 1999). Although HCO_3^- has been proved not to be used in carboxylation by algae, it can indirectly contribute to marine photosynthesis (Kaplan and Reinhold 1999).

In marine diatoms, the K_m (CO_2) of Rubisco is about 30–60 μmol/L (Badger *et al.* 1998), but CO_2 concentration is about 10 μmol/L in natural seawater. To compensate for the catalytic limitation of Rubisco, many taxa of phytoplankton employ inorganic carbon-concentrating mechanisms (CCM) to increase their efficiency of carbon fixation (Raven and Johnston 1991, Kaplan and Reinhold 1999). Carbonic anhydrase (CA, EC 4.2.1.1), a zinc metallaenzyme catalyzing the interconversion between CO_2 and HCO_3^-, has been taken as a component of CCM and plays a role in photosynthetic CO_2 fixation (Badger and Price 1994). Functional roles of CA vary much among photosynthetic organisms (Stemler 1997, Karlsson *et al.* 1998, Kaplan and Reinhold 1999). In some organisms, e.g. *Chlorella*, periplasmic CA is apparently absent, but in other organisms, e.g. *Chlamydomonas reinhardtii*, periplasmic CA is clearly present. It has shown that internal CA is essential to photosynthesis in both *C. reinhardtii* and *Dunaliella tertiolecta* but not in *Coccomyxa* (Badger and Price 1994).

In marine diatoms, little has been documented on the function of CA in photosynthesis. *Skeletonema costatum,* a unicellular marine diatom, is widely distributed in coastal waters all over the world and is a major component of most marine phytoplankton blooms (Eppley *et al.* 1971, Falciatore and Bowler 2002). The relationship between its CA and photosyn-

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thesis is still unclear. The present study was intended to examine the characteristics of its CA activity and its functional role in the photosynthesis of the marine diatom *S. costatum*.

1 Materials and Methods

1.1 Algal cells and growth conditions

Skeletonema costatum (Greville) Cleve strains No. 2042 was given by the Institute of Oceanography, Chinese Academy of Sciences. Culture was carried out at 20℃ and 180 μmol m^{-2} s^{-1} (12:12 LD cycle) in filtered seawater enriched with 3.0 mmol/L KNO_3, 0.1 mmol/L Na_2HPO_4, 70 μmol/L $NaSiO_3$, 1.0 μmol/L $FeSO_4$ and 25 μmol/L EDTANa. The water was aerated with ambient air of 360 μmol/mol CO_2. Cells were collected during the mid-exponential phase.

1.2 Measurement of CA activity

CA activity was measured by an electrometric method as described by Wilber and Anderson (1948). Cells harvested were washed and re-suspended in 20 mmol/L buffered veronal seawater (pH8.2). 0.5 mL of cell sample was added to 4 mL of buffer and mixed. After the addition of 2 mL CO_2-saturated icy pure water, the time required for a pH drift from pH 8.2 to 7.2 was recorded at 4℃. Extracellular CA activity was measured using intact cells. Then the total CA activity was assayed with homogenized crude extracts and intracellular CA activity was taken as the difference between the extracellular and total CA activities. Enzyme units are calculated from the equation $U=10\times(T_0/T-1)$, where T_0 and T represent the times required for the reaction in the absence and presence of the sample, respectively.

1.3 Measurements of photosynthetic oxygen evolution

Photosynthetic oxygen evolution was measured with a Clark-type oxygen electrode (YSI -5300, USA) in the presence and absence of acetazolamide (AZ) or ethoxyzolamide (EZ) (100 μmol/L). AZ and EZ are impermeable and permeable CA inhibitors, and inhibit extracellular and intracellular CA, respectively (Moroney *et al.* 1985). Cells harvested were washed and re-suspended in filtered seawater buffered with 20 mmol/L Tris-HCl at pH 8.2 and incubated in 5 mL buffer. Photosynthetic parameters associated with light curve (P-I) were analyzed according to Jassby and Platt (1976). Before determining the dissolved inorganic carbon (DIC)-dependent oxygen evolution, cells were allowed to photosynthesize to deplete possible intracellular pool of "CO_2" until no net O_2 evolution was observed. Following the addition of $NaHCO_3$ solutions, oxygen evolution rate was measured at 20℃ and 400 μmol m^{-2} s^{-1}. The K_m of DIC and CO_2 values (the DIC and CO_2 concentration required to give half-maximal photosynthetic rate) for photosynthesis were determined by fitting the Michaelis–Menten formula. The inorganic carbon compensation points (CCP) were determined by interpolation to the point of zero net photosynthesis (Lloyd *et al.* 1977).

The assessment of photorespiration was performed according to Reiskind and Bowes (1991). Net photosynthetic rates in the presence and absence of EZ were measured under 20% or 2% O_2 (200 and 20 μmol/L dissolved O_2, respectively) at 20℃ and 400 μmol m^{-2} s^{-1}. Cells were incubated in 5 mL buffered (20 mmol/L Tris-HCl, pH 8.2) "CO_2"-free seawater to which the 2.0 or 0.2 mmol/L $NaHCO_3$ was added. Photorespiratory rate was calculated as: $100\times(1-P_n^h / P_n^l)$, where P_n^h and P_n^l are P_n at 20% and 2% O_2, respectively.

1.4 Measurements of PS II photochemical efficiency

For the determination of photoinhibition, photochemical efficiency of PSII (F_v/F_m) was measured by using a Plant Efficiency Analyzer (PEA, Hansatech Instruments Ltd. UK) as described by Chen and Gao (2004). Cells were collected and re-suspended in a fresh medium. After being exposed to a high irradiance of 1 200 μmol m^{-2} s^{-1} at 20℃, cells were sampled at certain time intervals and were acclimated to dark for 10 min, and then tested for PSII photochemical efficiency (F_v/F_m).

1.5 Measurements of cell number and chlorophyll a concentration

The number of cells was determined microscopically by using a haemocytometer. Chlorophyll a concentration was determined spectrophotometrically as described by Jeffrey and Humphrey (1975).

2 Results

CA activities were detected in the intact cells and crude extracts of *S. costatum*, which correspond to the extracellular and total CA. The extracellular CA activity accounted for only 6% of the total CA and the intracellular CA accounted for 94% of the total CA (Fig.1a). Photosynthetic rate decreased by 36% by the intracellular CA inhibitor EZ, but changed little by the extracellular CA inhibitor AZ (Fig.1b).

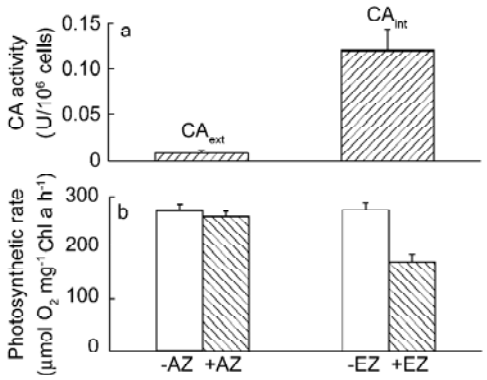

Fig.1 Extracellular CA (CA_{ext}) and intracellular CA (CA_{int}) activities (a) and effects of CA inhibitors AZ and EZ on photosynthetic oxygen evolution (b) in *Skeletonema costatum*
Values are means ±SE, n=3 (a) and n=4 (b).

The parameters of the light-dependent photosynthetic oxygen evolution (P-I) of *S. costatum* were shown in Table 1. Photosynthetic efficiency (α), the light compensation point for photosynthesis (I_c) and dark respiration rate (R_d) were not affected by the EZ (P>0.5). Only the light-saturated photosynthetic rate (P_m) and the light saturation point for photosynthesis (I_k) were significantly affected, being reduced by 40% with EZ (Table 1).

The dependence of photosynthetic oxygen evolution of the alga on DIC was investigated in the presence of EZ (Fig.2). The K_m values (μmol/L)for DIC (or CO_2) were 340±20 (or 2.0±0.1) and 710±70 (or 4.2 ±0.4) in the absence and in the presence of EZ, respectively, and increased 109% by EZ, which indicated that the photosynthetic affinity to inorganic carbon ($1/K_m$) markedly decreased when cells were exposed to intracellular CA inhibitor EZ. The compensation points for DIC (or CO_2) were 13.4 (or 0.08) and 5.8 (or 0.035) μmol/L with and without EZ, respectively, increasing 131% with EZ (Fig.3).

Table 1 The parameters of P_n-light (P-I) curve for *Skeletonema costatum* in the absence and presence of 100 μmol/L EZ

	P_m	α	I_k	R_d	I_c
−EZ	952±113	2.1±0.3	452±51	100±19	49±9
+EZ	572± 66	2.1±0.2	272±31	106± 5	50±3

Values are mean±SE, n=3. P_m & R_d: μmol O_2 mg^{-1} Chl a h^{-1}; α: (μmol O_2 mg^{-1} Chl a h^{-1})/(μmol m^{-2} s^{-1}); I_k & I_c: μmol m^{-2} s^{-1}.

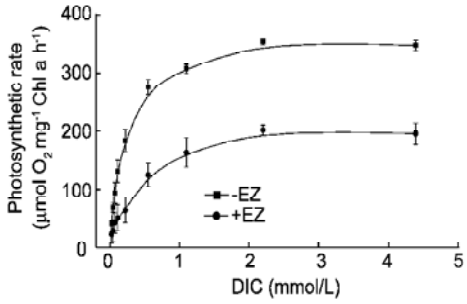

Fig.2 Photosynthetic oxygen evolution of *Skeletonema costatum* as a function of DIC concentrations in the absence and presence of 100 μmol/L EZ
Values are means ±SE, n=3.

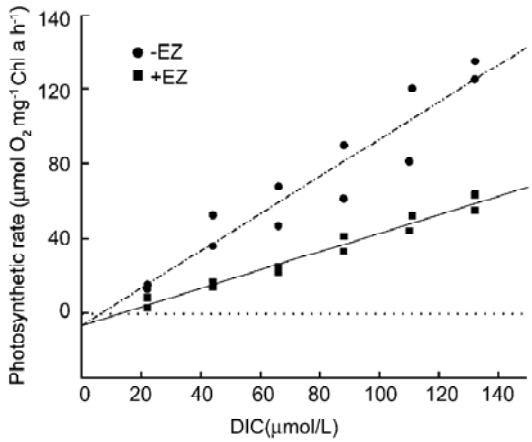

Fig.3 Rate of photosynthetic oxygen evolution of *Skeletonema costatum* at low concentrations of DIC in the absence and presence of 100 μmol/L EZ
Inorganic carbon compensation points were determined from such plots.

The effects of inhibitor EZ on photorespiration were shown in Fig.4. In the absence of EZ, photosyn-

thetic rates for *S. costatum* grown at 0.2 or 2.0 mmol/L DIC exhibited no significant difference between 20% and 2% O_2 (P>0.1) (Fig.4), indicating the absence of photorespiration. In the presence of EZ, however, the photosynthetic rates at 20% O_2 decreased to 75% and 88% of those at 2% O_2 for cells grown at 0.2 and 2.0 mmol/L DIC, respectively (Fig.4), which indicated that there was considerable photorespiration in the presence of EZ (P<0.01), and the photorespiratory rates were 26 and 13 for cells grown at 0.2 and 2.0 mmol/L DIC, respectively.

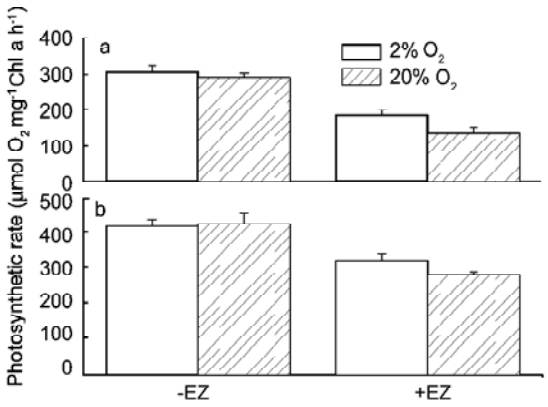

Fig.4 Photosynthetic oxygen evolution at 20% or 2% O_2 in the absence and presence of 100 μmol/L EZ for *Skeletonema costatum* grown at DIC 0.2(a)and 2.0(b) mmol/L
Values are means ±SE, n=3.

The photochemical efficiency of PS Ⅱ (F_v/F_m) decreased during the exposure of cells to high irradiance (Fig.5), the F_v/F_m values reduced by 43%–54% in 60 min. The fall was faster in the presence of EZ (Fig.5).

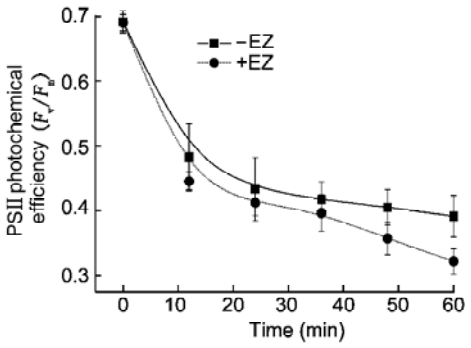

Fig.5 The PS Ⅱ photochemical efficiency (F_v/F_m) of *Skeletonema costatum* in the absence and presence of 100 μmol/L EZ as a function of exposure time to high irradiance (1 200 μmol m^{-2} s^{-1})
Values are means ±SE, n=9.

3 Discussion

Nimer *et al.* (1997) reported that extracellular CA activities in marine diatoms grown in natural environment were absent or very low. Matsuda *et al.* (2001) reported that there was little effect of inhibitor AZ on the photosynthesis of *Phaeodactylum tricornutum* grown at ambient level of CO_2. Our results showed that the extracellular CA activity of *S. costatum* was also very low (Fig.1a). Experiments with extracellular and intracellular CA inhibitors proved that intracellular CA exhibited an important role in maintaining the normal photosynthetic activity, but extracellular CA had little effect on photosynthesis (Fig.1b).

In the presence of EZ, P_m and I_k decreased, but α and I_c did not change, indicating that the intracellular CA of *S. costatum* could enhance the photosynthetic capacity in high light region (Table 1). High rate of photosynthetic carbon fixation under high light requires rapid supply of CO_2, but the uncatalyzed rate for conversion of HCO_3^- to CO_2 is far lower than the rate of CO_2 fixation by Rubisco in marine microalgae grown under optimal conditions (Badger and Price 1994). The intracellular CA must be high to keep a high rate of conversion of HCO_3^- to CO_2 within the cell via catalyzing.

The increase in K_m and CCP values by EZ showed that inhibition of intracellular CA led to decrease in photosynthetic inorganic carbon affinity (Figs.2 and 3). The photorespiration was active in the presence of EZ, the rate was higher at 0.2 mmol/L than at 2.0 mmol/L DIC, implying that intracellular CA could, in particular under carbon-limited condition, alleviate O_2 inhibition to photosynthesis (Fig.4). The intracellular CA as a component of CCM could catalyze conversion of HCO_3^- to CO_2 and then elevate CO_2 concentration and the ratio of CO_2/O_2 around the site of Rubisco, which favoring photosynthesis against photorespiration.

In green algae, pyrenoids play an important role in the CCM, where the intracellular CA and Rubisco are concentrated (Kaplan and Reinhold 1999). The advantage of the dense package within pyrenoids may lie in the substantial barrier to diffusion via the pyreniod sheath, which enhances CO_2 levels in the close vicin-

ity of Rubisco and closes the functional linkage between CA and Rubisco (Reinhold *et al.* 1991). But little is known on the relationship between intracellular CA of marine diatoms and their pyrenoids. So it is interesting to examine the location of intracellular CA of *S. costatum* within the pyrenoids.

The CA inhibitor has been used to examine the role of CA in photosynthesis (Moroney *et al.* 2001). In this paper, experiments with CA inhibitors showed that extracellular CA had little effect on photosynthesis, but intracellular CA exerted an important role in photosynthesis. The most direct approach to establish the functional role of CA in photosynthesis is via the isolation and analysis of genetic mutants, but unfortunately up to now, it has been proved to be difficult in photosynthetic organisms (Kaplan and Reinhold 1999).

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碳酸酐酶在中肋骨条藻光合作用中的作用

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摘要：探讨了在正常空气条件下生长的中肋骨条藻(*Skeletonema costatum*)的碳酸酐酶(CA)在其光合固碳中的作用。在中肋骨条藻的胞内和胞外均有CA活性，但胞外CA活性很低。CA抑制剂AZ(乙酰唑磺胺)对中肋骨条藻的光合放氧速率没有明显影响，而CA抑制剂EZ(乙氧苯唑胺)对其光合放氧速率有强烈的抑制作用。EZ的抑制作用使细胞最大光合速率、饱和光强和无机碳亲和力下降，无机碳的补偿点和光呼吸提高，使强光下光抑制作用增强。这些结果表明：中肋骨条藻的胞外CA在其光合作用中所起的作用较小，而其胞内CA通过催化胞内碳库中的HCO_3^-快速转化成CO_2，提高胞内CO_2的有效供给，从而提高细胞光合固碳能力和对逆境(高O_2、强光和低CO_2)的适应能力。

关键词：中肋骨条藻；乙酰唑磺胺(AZ)；乙氧苯唑胺(EZ)；碳酸酐酶；光合作用；光呼吸作用；光合无机碳的亲和力

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