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Carbonic anhydrase, one of major soluble proteins in the marine diatom *Phaeodactylum tricornutum* grown in air-level CO_2.

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Introduction

The aquatic environment is known to have a limited capacity to hold gaseous CO_2 and limits the diffusion rate of $CO_{2(aq)}$ which is about 10^{-4} times that in the atmosphere. In order to overcome such disadvantageous factors, a number of aquatic photoautotrophs such as algae and cyanobacteria are thought to have evolved an ability to take up and accumulate dissolved inorganic carbon (DIC) intracellularly which allows algal cells to photosynthesize efficiently even under CO_2 limitation. This DIC acquisition mechanism is termed a carbon concentrating mechanism (CCM) and a number of workers have suggested that carbonic anhydrase (CA) plays a key role in the CCM.

The role of CA in CCM has been studied extensively in freshwater species of cyanobacteria and green algae and has strongly suggested that the intracellular location of CA seems to be a crucial factor for the operation of the CCM. One form of cyanobacterial CAs, which has been shown to be critical component of CCM (Fukuzawaet al., 1992), is known to be localized in the carboxysome. The occurrence of CA in the cytoplasm resulted in a high CO_2-requiring phenotype presumably due to stimulation of HCO_3^- dehydration in the cytoplasm and therefore an efflux of CO_2 from the cells (Price and Badger, 1989). As a mechanism to enhance the rate of CO_2 supply to Rubisco in *Chlamydomonas reinhardtii*, it has been suggested that a lumenal form CA catalyzes the dehydration of HCO_3^- to give an ample efflux of CO_2 from the lumen to the stroma or the pyrenoid (Park et al., 1999).

The role of CA in marine microalgae is however largely unknown and remain to be studied. Diatoms are widespread in aquatic environments, and marine species are considered to be some of the most important CO_2 fixers in the hydrosphere. John-McKay and Colman (1997) reported that all *Phaeodactylum tricornutum* strains they studied possessed internal CA but that some of them also possessed external CA, which was also suggested to be essential for carbon acquisition under carbon-limited conditions by maintaining CO_2 equilibrium in the periplasmic layer (Iglesias-Rodriguez and Merrett, 1997). In contrast, the function of the internal form of CA in marine algae has not been studied extensively. To date, only two studies of diatom CAs have been reported at the molecular level, namely those of the marine diatom *Thalassiosira weissflogii* (TWCA1) (Cox et al., 2000) and *P. tricornutum* (Satoh et al., 2001) from which the enzymes were isolated and the genes cloned. TWCA1 was shown to share no homology with other known CAs. The structure of Zn coordination site of TWCA1 was determined by X-ray absorption spectrometry (Cox et al., 2000), which was shown to be very similar to that of mammalian α-CAs (Cox et al., 2000). In contrast, *P. tricornutum* CA was shown to be β-type with a completely conserved Zn coordination site (Satoh et al., 2001) and found to be induced at the transcriptional level in response to CO_2 limitation (Satoh et al., 2001). The function of these CAs are still largely unknown and this study describes

physiological status of β-CA of *P. tricornutum* and its possible function in the CCM is discussed.

Materials and Methods

2.1 Cells and culture conditions

The marine diatom, *P. tricornutum* (UTEX 642) was obtained from University of Texas Culture Collection at Austin and was cultured axenically in Harrison's f/2-enriched artificial seawater (F2AW) under continuous illumination at a photon flux density of 100 μmol m^{-2} s^{-1} at 20°C. The culture was aerated with 5% CO_2 (high CO_2-grown cells) or air (air-grown cells).

2.2 Measurement of CA activity

CA activity was measured by the potentiometric method described by Wilbur and Anderson (1948).

Results and Discussion

Identification of β-type CA in P. tricornutum

Internal CA was recently purified from the marine diatom *P. tricornutum* UTEX642 by ammonium sulfate precipitation, and two step column chromatography on DEAE-Sephacel and *p*-aminomethylbenzene sulfonamide (*p*-AMBS) agarose (Satoh et al., 2001). The purified CA was shown to comprise an electrophoretically single polypeptide of 28 kD. The entire sequence of the cDNA of this CA was obtained by the RACE method and indicated that the cDNA encodes 282 amino acids (Satoh et al., 2001). The mature CA was found to consist of 236 amino acids and the sequence was homologous to β-type CAs (Satoh et al., 2001). Even though the zinc-ligand amino acid residues were shown to be completely conserved, the amino acid residues which may constitute a CO_2-binding site appeared to be unique among the β-CAs so far reported (Satoh et al., 2001).

3.2 Clues for intracellular status of P. tricornutum CA

Comparison of above-described putative precursor sequence of CA with the N-terminal amino acid sequence of the purified CA, indicated that it included a possible ER signal sequence of up to 46 amino acids at the N-terminus (Satoh et al., 2001). This sequence is similar to the fucoxanthin-chlorophyll *a/c* proteins (FCPs) which are associated with photosystem II in *P. tricornutum* (Grossman et al., 1990) and are required for some of the cytoplasmically-synthesized proteins of *P. tricornutum* to be directed to the plastids (Bhaya and Grossman, 1991). This suggests that *P. tricornutum* CA could be located in organelles. An interesting and reproducible result is that the native form of *P. tricornutum* CA was not separated into any distinct sharp peak after gel filtration but was distributed in a broad range of fractions which corresponded to molecular masses of 800 kD to 29 kD (Fig. 1), suggesting that the quaternary structure of this enzyme may be very large and be disassembled during purification. *P. tricornutum* is known to possess a pyrenoid and it is possible that the large subunit structure may be related to the architecture of pyrenoid of this alga.

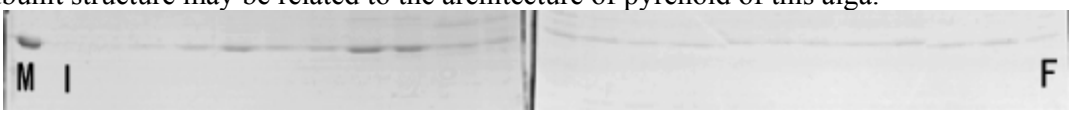

Fig. 1. SDS-PAGE of fractions after gel filtration of partially purified *P. tricornutum* β-CA on a Superdex 200 column. M, purified CA; I, the initial fraction. F, the final fraction. CA is eluted into a broad range of fractions.

CA activities in cells under various different growth conditions

Internal CA seems to be one of major soluble proteins in *P. tricornutum* and can be detected easily in crude extracts by SDS-PAGE followed by CBB staining (Fig.2). Cells grown in 5% CO_2 contained little CA (Fig. 2) even though constitutive levels of mRNA have been detected in 5% CO_2-grown cells (Satoh et al., 2001). In contrast, a considerable amount of CA was found to be accumulated in air-grown cells independent of growth pH (Fig. 2). There was significant difference in levels of CA activity among cells grown at three different pHs from 7 to 9 (Table 1). Namely, at pH 8, CA activity was shown to be 1.5 and 3.6 times those of cells grown under pHs 7 and 9, respectively (Table 1).

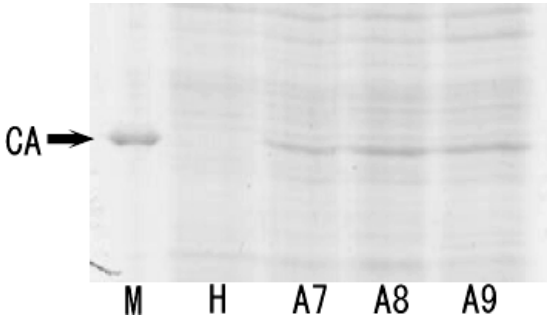

Fig. 2. SDS-PAGE of purified CA and cell lysates of *P. tricornutum* grown under various different conditions. M, purified CA; H, lysate of 5% CO_2-grown cells; A7, A8, and A9, lysates of cells grown in air at pHs 7, 8 and 9, respectively.

Table 1. CA activities[a] in cell suspensions and cell lysates of *P. tricornutum* grown in air at various pHs.

Growth pH	Cell suspension	Cell lysate
	WAU[b] mg Chl*a*$^{-1}$	
7	N.D.[c]	137 ± 19
8	N.D.[c]	211 ± 64
9	N.D.[c]	58 ± 23

[a] Values are the mean ± SD of five separate experiments. [b]Wilbur-Anderson Unit. [c]Not detected.

The use of CO_2 increases when the pH of the growth medium is lowered which would account for the decrease in internal CA activity at pH 7, i.e., CCM would be less crucial than in the case under high pH around 8. It is interesting that CA activity is reduced drastically by growth under pH 9 (Table 1), a pH at which cells should require an efficient CCM to function. It is not likely that high pH around 9 is toxic for *P. tricornutum* because cells grew as fast as at the other two pHs (data not shown). Given the data, it seems that the function of internal CA in CCM might change as the pH of bulk medium increases and certainly becomes less important at around pH 9 as compared to pH 8. It has been shown recently in *P. tricornutum* UTEX640 that the expression of the CCM is regulated by CO_2 in the bulk medium rather than other DIC species and severe CO_2 starvation below atmospheric levels confers on cells an extremely efficient HCO_3^- uptake system (Matsuda et al., 2001). The reduction of CA activity at pH 9 might relate to this extremely efficient HCO_3^- utilization which would be due to severe CO_2 starvation at this high growth pH.

Concluding remarks

β-CA found in the marine diatom *P. tricornutum* seems to be one of the major soluble proteins. Although, this protein possesses a putative chloroplast- and/or thylakoid- targeting signal, the intracellular location is not known. Given the results of gel filtration, this enzyme is likely to form hetero-sized structures from polymers of about 30 subunits to monomers *in vitro* suggesting that it occurs intracellularly as part of a large protein complex.

The expression of this CA is shown to be regulated by [CO_2] and pH in the bulk medium. The intriguing aspect is that the expression revealed an apparent peak of activity at around pH 8 and was reduced drastically toward pH 9 even though the cells were probably under optimal growth state over wide range of alkaline pHs from 7 to 9. These results show that the requirement for internal CA is not relative to the increment of pH, but appears to be reduced above pH 8. This suggests that cells might undergo an alteration of mechanisms to acquire DIC at around pH 8.

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