Synthesis of carbonic anhydrase with messenger RNA isolated from the cells of *Chlamydomonas reinhardtii* Dangeard C-9 grown in high and low CO_2

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Poly(A)$^+$RNA was isolated from *Chlamydomonas reinhardtii* cells grown autotrophically in ordinary air (low-CO_2 cells) and in air enriched with 4% CO_2 (high-CO_2 cells). In vitro translation products with the messenger RNA showed that the synthesis of the 37-kDa peptide was greater in low-CO_2 than in high-CO_2 cells. The immunoprecipitate produced by treating the translation products with antiserum against carbonic anhydrase (CA) from this alga was also 37 kDa. Comparison of radioactivities incorporated into these 37 kDa peptides suggested that CA induction by lowering atmospheric CO_2 concentration is regulated at the transcriptional level.

Carbonic anhydrase *Cell-free translation* *Immunoprecipitation*
Chlamydomonas reinhardtii *mRNA*

1. INTRODUCTION

Carbonic anhydrase (CA, EC 4.2.1.1), which catalyzes the reversible reaction between HCO_3^- and dissolved CO_2, is the only enzyme of photosynthetic carbon metabolism to fluctuate in activity in a number of algal species with changes in environmental CO_2 concentration (for literature, see [1]). High CO_2 concentration allowed the cells to express only trace CA activity with great contrast to low CO_2 concentration (normal air condition) under which high CA activity was induced. Recently it was shown that the increase of CA activity in green alga induced by shifting aeration conditions from high to low CO_2 concentration was regulated at the level of de novo protein synthesis [2,3].

Here we report the further study of the regulation mechanism of CA synthesis in green alga *C. reinhardtii* investigated by mRNA-dependent cell-free translation system and antiserum raised against purified *Chlamydomonas* CA.

2. MATERIALS AND METHODS

High-CO_2 cells of *C. reinhardtii* Dangeard C-9 (Algal Collection of Institute of Applied Microbiology, University of Tokyo) was obtained by growing them photoautotrophically under continuous illumination at 28°C in Sueoka's medium with constant bubbling of air enriched with 4% CO_2 [4]. Low-CO_2 cells possessing high CA activity were obtained by shifting the CO_2 concentration in air from 4% to the normal level (0.04%). The cells were harvested after 5 h. The cell density at this stage was about 1 ml pcv/l.

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Abbreviations: CA, carbonic anhydrase; high-CO_2 cells, algal cells grown in air containing 4% CO_2; low-CO_2 cells, algal cells grown in ordinary air (containing 0.04% CO_2); PAGE, polyacrylamide gel electrophoresis; pcv, packed cell volume

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CA from low-CO_2 cells of *C. reinhardtii* was purified by affinity chromatography and anti-CA antiserum was raised (in preparation). CA activity was measured as in [4].

Total RNA was extracted from both types of cells as in [5] with small modifications. After the first ethanol precipitation step, the pellet was dissolved in 25 mM Tris–HCl (pH 8.0) and 25 mM NaCl, then extracted with 2-methoxyethanol [6]. RNA was precipitated with cetyltrimethylammonium bromide and then dissolved in 10 mM Tris–HCl (pH 8.0). RNA was further purified by 2 M LiCl precipitation. (In one experiment shown in table 1, hydroxyapatite chromatography was carried out with potassium phosphate buffer (0.1–0.4 M) instead of 2-methoxyethanol precipitation.) Poly(A)$^+$ RNA was then separated by chromatography on oligo(dT)–cellulose (Sigma, St.Louis, MO) as in [7] and translated in a rabbit reticulocyte lysate cell-free translation system (Amersham, England) using [^{3}H]leucine (187 Ci/mmol, Amersham) or [^{35}S]methionine (1000–1200 Ci/mmol, Amersham) for 1 h at 30°C. The saturating level of poly(A)$^+$ RNA (40 μg/ml) was used for the reaction. Portions were taken for determination of isotope incorporation into the acid insoluble fraction and the rests were analyzed by 12.5% SDS-polyacrylamide gel electrophoresis (PAGE) and fluorography [8,9] as described below.

Portions of the translation products were diluted with 3 vols of 1 M NaCl containing 1% Triton X-100 and 5 μl null serum, then 25 μl of 10% formalin-fixed *Staphylococcus aureus* Cowan I cell suspension was added. After these treatments, the translation products were kept at 25°C for 30 min. Non-specific binding portion to serum immunoglobulin was pelleted by centrifugation. To this supernatant, 5 μl specific anti-CA antiserum was added and incubated at 25°C for 30 min, then at 4°C overnight. After precipitation of the immunocomplex by treatment with the cells of *S. aureus* as mentioned above, the pellet was thoroughly washed using sucrose pad centrifugation (0.5 ml of 1 M sucrose solution containing 0.75 M NaCl and 1% Triton X-100). The precipitation with anti-CA antiserum was repeated twice. The washed pellet was resuspended in SDS electrophoresis buffer and boiled for 5 min to release the immunoprecipitated CA peptide. After centrifugation, the supernatant was applied to 12.5% SDS–PAGE [8], and the gel was processed for fluorography [9]. The fluorogram was scanned with gel scanner (ISCO, Model 1310). In one experiment, the gel containing the 37-kDa peptide (1 mm thick) from each sample lane was cut out and solubilized, then the radioactivity was determined with a scintillation spectrophotometer [10].

3. RESULTS AND DISCUSSION

Five hours after lowering the CO_2 concentration, CA activity attained a 4–12 times higher level than in the high-CO_2 cells. The magnitude of the

Table 1

Improvement of translational activity of poly(A)$^+$ RNA by treatments with 2-methoxyethanol and LiCl

Additional procedures to the standard method[a]	Stimulation of translational activity[b]	
	High-CO_2 cells	Low-CO_2 cells
None	1–5	1–7
Hydroxyapatite chromatography and 2 M LiCl precipitations	[c]	18
Extraction with 2-methoxyethanol and precipitation with 2 M LiCl	40–80	30–55

[a] Dobberstein et al. [5]

[b] Stimulation of translational activity = The radioactivity of translation products with poly(A)$^+$ RNA/The radioactivity of translation products without RNA

[c] Data not obtained

increase in CA activity was greatly affected by culture conditions, such as cell concentration and the rate of aeration. It has been revealed that the level of the CA peptide immunoprecipitated with anti-CA antiserum increased in parallel to its activity (in preparation). In order to find out which regulation point of CA synthesis is affected by changes in CO_2 concentration, we extracted RNA and compared the nature of poly(A)$^+$ RNA obtained from both types of algal cells with respect to the level and translation products as described below. During this study, special precautions were taken on the extractability of poly(A)$^+$ RNA from the cells. Table 1 shows that translatability of poly(A)$^+$ RNA was greatly improved by the addition of two steps, extraction with 2-methoxyethanol and precipitation with 2 M LiCl, as in [5]. This improvement in translation activity of poly(A)$^+$ RNA might be related to the high contamination of polysaccharides in the original RNA fractions from the algal cells. The level of poly(A)$^+$ RNA purified as above was about 0.16 and 0.75% of total RNA in low- and high-CO_2 cells, respectively (table 2). This difference may reflect that in growth rates.

Messenger RNA purified as above was translated with the in vitro system and analyzed by SDS–PAGE (fig.1, lanes a–d). High-CO_2 cells translated peptides of rather lower molecular mass, lower than 45 kDa, while low-CO_2 cells translated peptides of much higher molecular masses in addition to those of lower molecular masses. Qualitatively, both showed almost identical patterns. Labelling with [^{3}H]leucine and [^{35}S]methionine showed preferential synthesis of 22- and 37-kDa peptides, and suppressed synthesis

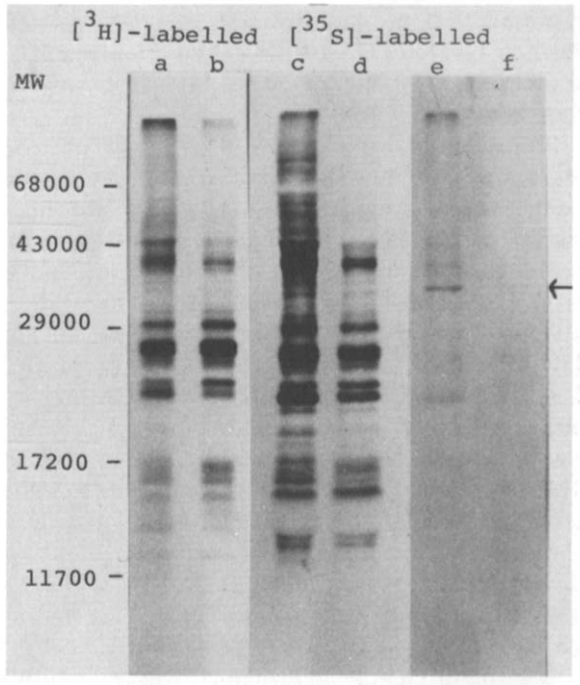

Fig.1. Effect of CO_2 concentration during growth of *C. reinhardtii* cells on cell-free translation products. Forty μg each of poly(A)$^+$ RNA and [^{3}H]leucine or [^{35}S]methionine was used for the translation, and the products were separated by SDS–PAGE (12.5%). Lanes a–d are fluorograms of total translation products and lanes e and f are those of immunoprecipitates with anti-CA antiserum. Lanes a,c and e are from low-CO_2 cells and lanes b,d and f are from high-CO_2 cells. Arrow indicates the position of purified CA peptide detected by Coomassie brilliant blue R-250 staining on the same gel.

Table 2

Levels of poly(A)$^+$ RNA extracted from high- and low-CO_2 cells

Steps	High-CO_2 cells	Low-CO_2 cells
Total RNA	7.6 mg/ml pcv	12.7 mg/ml pcv
Poly(A)$^+$ RNA	45 μg/ml pcv	21 μg/ml pcv
(% of total RNA)	(0.75%)	(0.16%)

The total RNA fraction in this table was obtained by adopting the treatments with 2-methoxyethanol and 2 M LiCl

of 23- and 26-kDa peptides in low-CO_2 cells as compared to high-CO_2 cells. Addition of antiserum against CA from the same algal cells to the translation products resulted in the major immunoprecipitate of the 37-kDa peptide (fig.1, lanes e,f). This peptide showed the same mobility with the purified CA peptide (37 kDa) which had been run on the same polyacrylamide gel plate and detected by Coomassie brilliant blue R-250 staining (arrow in fig.1).

Yang et al. (in preparation) have shown that the molecular size of CA in the cells of *C. reinhardtii* is also 37 kDa. The identity of sizes between the translated and native CA molecules indicates that, with respect to the molecular size, this enzyme is not processed after the translation. Authors in [11]

reported that the molecular size of CA isolated from *Chlamydomonas* cells was 26 kDa. The reason for this discrepancy is not clear at this moment.

The density of the 37-kDa band on the fluorogram of the ^{35}S-labeled immunoprecipitate in the presence of saturating RNA was 3–4 times higher in low-CO_2 cells than in high-CO_2 cells. Likewise, the radioactivity of the 37-kDa peptide from low-CO_2 cells, which was determined with a scintillation spectrophotometer, was 3 times higher (300 cpm) than that of high-CO_2 cells (100 cpm). It has been shown that the induction of CA activity caused by the CO_2 reduction during algal growth was in parallel with the increase in the amount of CA (in preparation). These results indicate that the induction of CA in *C. reinhardtii* is regulated by the transcriptional level.

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