Carbonic anhydrase located on cell surface increases the affinity for inorganic carbon in photosynthesis of *Dunaliella tertiolecta*

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Low-CO_2 cells of *Dunaliella tertiolecta* had higher carbonic anhydrase (CA) activity on their surfaces than inside. The treatment with subtilisin completely abolished CA activity on the cell surface, while the activity inside the cells was not affected by this treatment. Correspondingly, the apparent $K_m(NaHCO_3)$ for photosynthesis was increased two-fold, while V_{max} was not affected by this treatment. The apparent $K_m(NaHCO_3)$ was lowered to the original level when CA was added to the suspension of subtilisin-treated cells. These results indicate that CA on the cell surface enhances the affinity for inorganic carbon in photosynthesis of *D. tertiolecta*.

Carbonic anhydrase *Photosynthetic O_2 evolution* *Subtilisin treatment* *Cell surface* Dunaliella *Marine alga*

1. INTRODUCTION

It has been established with a number of microalgae that the apparent $K_m(CO_2)$ for photosynthesis in cells grown in air-levels of CO_2 (low-CO_2 cells) is as low as in terrestrial C_4 plants, although these algae fix CO_2 via the C_3 pathway. In contrast, the apparent $K_m(CO_2)$ in algal cells grown under 1–5% CO_2 is as high as that observed in terrestrial C_3 plants [2,3].

Studies with *Chlorella vulgaris* 11h gave evidence indicating that CA which is located inside the cells (possibly within chloroplasts) plays an important role in enhancing the transport of CO_2 from outside the cells to the site of carboxylation reaction, and hence lowers the apparent $K_m(CO_2)$ in photosynthesis of low-CO_2 cells [3–5].

Recently, it was shown that there are 3 groups in microalgae with respect to the form of inorganic carbon utilized for photosynthesis. The first group which includes *C. vulgaris* 11h and *C. miniata* utilized CO_2 irrespective of the CO_2 concentration during growth, while the second group which includes *Chlorella* sp. K and *C. vulgaris* C-3 took up HCO_3^- in addition to CO_2. On the other hand, high-CO_2 cells of *C. pyrenoidosa* and *Chlamydomonas reinhardtii* mainly utilized CO_2, whereas low-CO_2 cells of these species utilized HCO_3^- in addition to CO_2 [6,7]. It was further shown that the algae which utilized CO_2 had CA only inside the cells, while those which utilized HCO_3^- had this enzyme on their cell surfaces. The effects of Diamox, an inhibitor of CA, on HCO_3^- uptake and other results indicated that HCO_3^- in the reaction medium was first converted to CO_2 by CA located outside the plasmalemma, and then the CO_2 molecule thus formed crossed the plasmalemma into the algal cells [1,6,8,9]. However, there is no direct evidence indicating whether CA on the cell surface can lower $K_m(CO_2)$ for photosynthesis.

Abbreviations: CA, carbonic anhydrase; Diamox, 2-acetylamino-1,3,4-thiadiazole-5-sulfonamide; high-CO_2 cells, algal cells grown in air enriched with 1.5–4% CO_2; low-CO_2 cells, algal cells grown in ordinary air (containing 0.04% CO_2)

Part of this investigation was reported at the International Symposium on Kinetics of photosynthetic carbon metabolism in C_3-plants, Tallin, 1983 [1]

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The present experiments were carried out to investigate this possibility. *Dunaliella tertiolecta* was chosen as experimental material. This alga lacks rigid cell walls [10]. It is therefore reasonable to assume that when CA is located on the cell surface, it will be susceptible to some of the proteases. We found that CA was actually located on the cell surface and that its activity was abolished by subtilisin. The effects of subtilisin on the photosynthetic characteristics were also studied here.

2. MATERIALS AND METHODS

2.1. *Algal culture*

The cells of *D. tertiolecta* Butcher were grown axenically, in an inorganic medium. This medium, which was modified from [11], contained 20 mM Tris–HCl (pH 8.0) and micronutrients, as in [12] but no $NaHCO_3$ was added. The algal suspension was bubbled with air under constant illumination with a bank of fluorescent lamps (10 klx) at 25°C. Three-day-old cultures were always used for the experiment.

2.2. *Measurement of O_2 exchange*

Oxygen exchange was followed with a Clark type oxygen probe (Yellow Spring, OH). The cells were collected by centrifugation at 500 × *g* for 10 min and were suspended in fresh culture medium which had been bubbled with N_2 gas for 3 h to minimize the dissolved CO_2. The algal suspension was pre-illuminated with a projector lamp until endogenous O_2 evolution ceased. Photosynthesis was then started by adding known amounts of $NaHCO_3$ solutions. The light intensity was 20 klx. Assay of dark respiration was carried out in the culture medium which had been bubbled with air. Concentrations of cells were 0.1 and 0.2% packed cell volume in the assay of photosynthesis and respiration, respectively. The temperature was kept at 25°C during measurement of O_2 exchange.

2.3. *Assay of carbonic anhydrase*

CA activity was measured by the electrometric method in [13], with slight modifications. The cell suspension or sonicate of algal cells (0.1 ml) was mixed with 2.9 ml of 17.2 mM Veronal buffer containing 0.5 M NaCl (pH 8.2) and kept at 3°C. Enzyme reaction was initiated by adding 2 ml of 0.5 M NaCl solution saturated with CO_2 at 0°C, and the time required for the pH to change from 8.0 to 7.0 was determined. Enzyme unit was calculated as in [6].

2.4. *Treatment with protease*

The algal cells were suspended in fresh culture medium containing 2% (w/v) of various proteases at a density of 100 μl packed cell volume per 5 ml. The suspensions which were kept in an L-shaped glass vessel were gently shaken at 28°C under illumination at 3 klx. After 1 h, the cells were collected by centrifugation and resuspended in fresh culture medium.

3. RESULTS AND DISCUSSION

Similar to *Ch. reinhardtii* [7] and *C. pyrenoidosa, C. vulgaris* var. vulgaris, *C. vulgaris* var. vulgaris C-1 and *C. ellipsoidea* [6], the suspensions of intact low-CO_2 cells of *D. tertiolecta*, showed CA activity (43.6 ± 8.1 units/mg chlorophyll, $n = 5$). The activity was almost completely (99%) inhibited by 10 μM Diamox. The shape, number and halo of the cells observed under a phase contrast microscope after the CA assay indicated that the cells were not broken and kept an intact cell surface. The supernatant obtained after centrifuging the cell suspensions at 500 × *g* for 10 min did not show any CA activity. These results indicate that the low-CO_2 cells of *D. tertiolecta* have CA on their surfaces.

The CA activity in the suspension of intact low-CO_2 cells of *D. tertiolecta* decreased in parallel with the concentration of as well as the time of incubation with subtilisin (Boehringer, Mannheim), a protease. The activity was completely abolished by treatment with 2% subtilisin for 60 min (fig.1). When these cells were broken with a sonicator, the resulting homogenates showed CA activity which was almost the same as the difference in CA activities between the sonicate and cell suspension (fig.1a, ●). We assumed that these activities represent those of CA located inside the algal cells. These results indicate that CA was located inside, as well as on the surface of low-CO_2 cells of *D. tertiolecta*. The supernatant obtained after centrifugation of subtilisin-treated cell suspensions did not show CA activity (fig.1a; △) indicating that the catalytic activity of this enzyme was destroyed

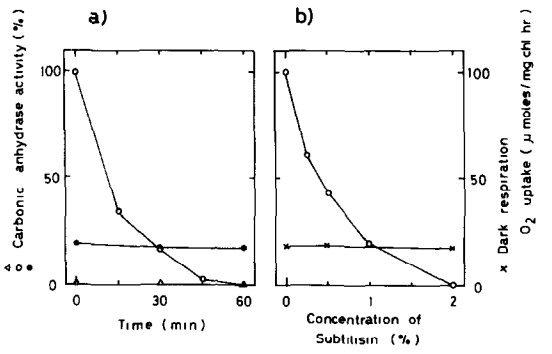

Fig.1. Effect of subtilisin treatment on CA activity and dark respiration in low-CO_2 cells of *D. tertiolecta*. (a) Time course of the effects of subtilisin treatment. (○) CA activity measured with the suspension of unbroken cells, (●) difference in CA activity between the sonicate and unbroken cells, (△) CA activity in the supernatant obtained after centrifuging the subtilisin-treated cell suspension. (b) (○) CA activity in the suspension of unbroken cells, (×) dark respiration activity. Subtilisin concentration was fixed at 2% (w/v) in (a), and time of subtilisin treatment at 60 min in (b); 100% of the CA activity corresponds to 37.6 units/mg chlorophyll.

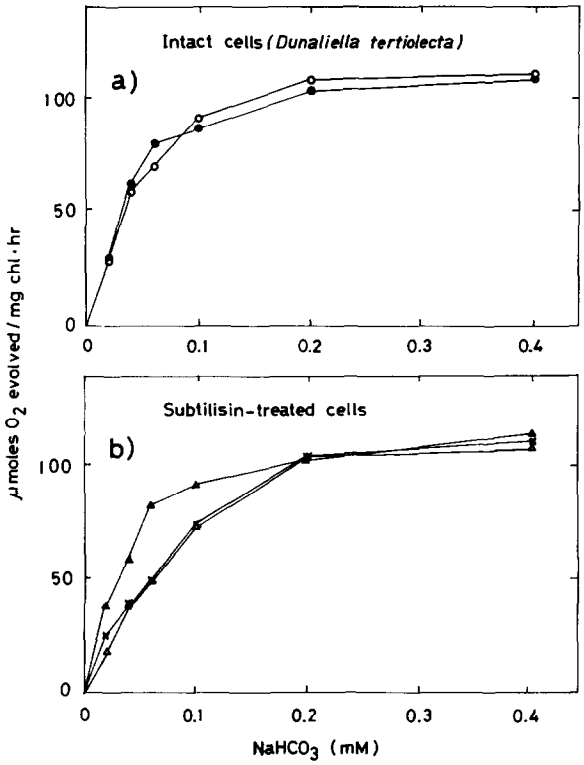

Fig.2. Effect of subtilisin treatment on photosynthetic characteristics in low-CO_2 cells of *D. tertiolecta*. (a) Rate of photosynthetic O_2 evolution determined in the absence (○) and presence (●) of bovine CA (Sigma). (b) (△) Subtilisin-treated cells, (×) boiled bovine CA was added to the subtilisin-treated cells, (▲) CA was added to the subtilisin-treated cells. Bovine CA added to the 5-ml assay mixture was 2250 units.

by this treatment. The figure further shows that the dark respiratory activity was not affected by subtilisin. Likewise, subtilisin treatment did not affect the shape and kinesis of the cells. On the other hand, treatment with other proteases (2%, w/v), for 1 h such as type III, IV, IX, XIII, XIV, XVIII, XIX (Sigma), α-chymotrypsin, trypsin and thermolysin (Boehringer, Mannheim) did not affect CA activity in the intact cell suspensions.

Fig.2a shows that the addition of CA obtained from bovine erythrocytes (Sigma, no.C-7500) to the cell suspension did not affect the rates of photosynthetic O_2 evolution at all $NaHCO_3$ concentrations tested. Subtilisin treatment lowered the rates of photosynthesis under low $NaHCO_3$ concentrations, while it did not affect the rates under saturating $NaHCO_3$ concentrations (fig.2b). As a consequence, the apparent $K_m(NaHCO_3)$ at pH 8.0 estimated from Hanes plots [14] of the data shown in the figure was increased from 0.051 to 0.112 mM by subtilisin treatment. The addition of CA to the suspension of subtilisin-treated cells lowered the apparent K_m to the original level (0.041 mM). On the other hand, the addition of boiled CA which lost catalytic activity did not show any effect. These results indicated that CA located on the cell surface increased the affinity for CO_2 in photosynthesis of *D. tertiolecta*. The fact that the maximum rate of photosynthesis was not decreased by subtilisin also indicates that other photosynthetic reactions such as the photoelectron transport system were not inhibited by this treatment.

The present results seem to support the assumption that CA on the cell surface accelerates the conversion of HCO_3^- to CO_2, and that CO_2 is the active species taken up by *D. tertiolecta*. The form of inorganic carbon utilized for photosynthesis in the low-CO_2 cells of this alga is currently under investigation.

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