ENHANCEMENT OF RIBULOSE 1,5-BISPHOSPHATE CARBOXYLATION REACTION BY CARBONIC ANHYDRASE

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1. Introduction

It has been established that algal cells grown with ordinary air (low-CO_2 cells) have a much lower K_m value for CO_2 in photosynthesis than those grown with CO_2-enriched air (high-CO_2 cells) [1–4]. Carbonic anhydrase (EC 4.2.1.1) is almost exclusively located in low-CO_2 cells of *Chlorella* and its activity in high-CO_2 cells is marginal, if any [5,6]. CO_2 concentration during growth does not affect ribulose 1,5-bisphosphate (Ru-P_2) carboxylase (EC 4.1.1.39) activity [3,6,7]. The app. K_m (CO_2) in low-CO_2 cells is greatly increased by Diamox, an inhibitor of CA [3,6]. These results indicate the possibility that the transport of CO_2 from outside the algal cells to the site of Ru-P_2 carboxylase may be accelerated by CA and that the observed difference in K_m (CO_2) may be due to the difference in CA activity.

We have found [8] that, irrespective of CO_2 concentration during growth, the rate of photosynthetic CO_2 fixation in *Chlorella vulgaris* 11 h cells in the presence of low $NaHCO_3$ concentrations was greatly enhanced by the addition of CA, while the initial rate of CO_2 fixation immediately after bubbling of ordinary air was suppressed by this enzyme. Since CA catalyzes the dehydration of bicarbonate and the hydration of CO_2 in the suspending medium, the above evidence was taken as an indication that these algal cells absorb only free CO_2.

The only difference observed in the kinetics of CO_2 fixation between the above two types of *Chlorella* cells was that when CA was added to low-CO_2 cells under bubbling of air, the rate of CO_2 fixation, which was initially lowered, soon started to increase and then exceeded the rate in the absence of CA, while such a CA-induced increment in the rate of CO_2 fixation was not observed in high-CO_2 cells. Tsuzuki and Miyachi [9] derived the equations which express the changes in the concentrations of CO_2 and HCO_3^- in the medium during the experiment as well as the time course of CO_2 fixation. Simulation of CO_2 fixation with such an equation supported the conclusion that *Chlorella* cells fix only free CO_2, with the exception that both CO_2 and HCO_3^- contributed to photosynthesis when low concentrations of CO_2 were bubbled through a suspension of low-CO_2 cells in the presence of CA. It is highly unlikely that HCO_3^- is absorbed only when low-CO_2 cells are given low concentrations of CO_2 in the presence of CA. The equation also indicated that the concentrations of dissolved CO_2 under these conditions are far lower than those under other conditions. Since the concentration of CO_2 near the cell surface of *Chlorella* would be still smaller than that in the suspending medium, it is possible that HCO_3^- formed from CO_2 is converted again into CO_2 via CA around the cell surface, and then incorporated into the algal cells under such conditions (indirect use of CO_2). We therefore assumed that the contribution of HCO_3^- deduced by simulation is equated with the indirect use of CO_2.

Recently, we also found that only CO_2 can cross

Abbreviations: CA, carbonic anhydrase; Diamox, 5-acetamide-1,3,4-thiadiazole-2-sulfonamide; ethoxyzolamide, 6-ethoxy-2-benzolesulfonamide; high-CO_2 cells, algal cells which were grown with CO_2-enriched air; low-CO_2 cells, algal cells which were grown with ordinary air; Ru-P_2, ribulose 1,5-bisphosphate

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the chloroplast envelope [10]. The substrate for Ru-P_2 carboxylase is known to be free CO_2 [11]. In high-CO_2 cells which show very small CA activity, therefore, CO_2 which crossed the chloroplast envelope would be used directly as the substrate for this enzyme. On the other hand, in the stroma of low-CO_2 cells where most of CA is located, the 'indirect use of CO_2' by Ru-P_2 carboxylase can occur in addition to the direct use of CO_2, and this enhances CO_2 fixation under very low CO_2 concentrations [6]. If this inference is the case, it can be expected that CO_2 fixation by Ru-P_2 carboxylase under bubbling of low concentrations of CO_2 should be enhanced by the addition of CA. The positive results obtained are described here.

2. Materials and methods

To make a buffer solution in which dissolved $^{14}CO_2$ and $H^{14}CO_3^-$ are in equilibrium, air containing 400 or 4000 ppm $^{14}CO_2$ was bubbled through 4–5 ml of 100 mM Tris–HCl buffer solution (pH 7.5) containing 20 mM $MgCl_2$ and 4 mM dithiothreitol which was placed in a test tube kept at 20°C. The flow rate of the bubbling gas was ~40 ml/min. At intervals, portions of the buffer solution (10–30 μl) were taken out and quickly transferred into a vial containing 3.5 ml of a mixture of dioxane-scintillator, methanol and monoethanolamine (8:3:1, v/v/v) and radioactivity dissolved in the buffer solution was determined with a liquid scintillation spectrometer (Packard 3380). The dioxane-scintillator contained 10 g PPO, 25 mg POPOP and 100 g napthalene in 1 liter of dioxane. Radioactivity in the buffer solution increased with time and the increase stopped 2–2.5 h after start of the bubbling when $^{14}CO_2$ in the bubbling gas, that dissolved in the buffer solution, and $H^{14}CO_3^-$ in the same solution reached equilibrium. Then 500 μl each of the buffer solution was transferred into small test tubes. Bubbling of $^{14}CO_2$ through the respective buffer solutions continued throughout the experimental period and the temperature was kept at 20°C. Five minutes after the transferral, 10 μl 50 mM Ru-P_2 (final conc. 0.94 mM) and 10 μl CA solution (210 Wilbur-Anderson units; 100 μg protein) were added. In some experiments, CA was substituted for with bovine serum albumin solution (100 μg) or deionized water. The reaction was started by adding 10 μl Ru-P_2 carboxylase solution (containing 15 μg protein) which had been dissolved in the same buffer solution equilibrated with the same concentration of $^{14}CO_2$ as in the reaction medium and then kept at 0°C at least for 30 min. To study the effect of CA inhibitor, 50 μl 50 mM Diamox or ethoxyzolamide was dissolved in 50 μl CA solution. After the preincubation period, which is indicated in section 3, 20 μl of the mixture was added instead of CA solution (final inhibitor conc. 0.94 mM).

At intervals after the start of the reaction, 50 μl of the reaction mixture was taken out with a microsyringe and quickly transferred into a vial containing 200 μl 6 N HCl. The vial was vigorously shaken by hand and 200 μl deionized water was added. Unfixed $^{14}CO_2$ was purged with N_2, then 4 ml liquid scintillator solution (Aquazol 2, Packard) was added. Radioactivity was determined as above. The pH value (pH 7.5) selected in this experiment is ideal neither for activator nor reaction of Ru-P_2 carboxylase [12]. The reason for adopting this pH value is that an impractically long period of gas-bubbling was necessary to establish the equilibrium between gaseous and dissolved $^{14}CO_2$ at higher pH values.

Ru-P_2 carboxylase (partially purified from spinach leaves), CA (from bovine erythrocytes), Ru-P_2 (tetrasodium salt) and ethoxyzolamide were products from Sigma, St Louis. Diamox was purchased from Japan Lederle, Tokyo.

3. Results and discussion

Figure 1 (left) shows that the rate of $^{14}CO_2$ fixation in the reaction medium in which the dissolved $^{14}CO_2$ had been in equilibrium with 400 ppm $^{14}CO_2$ (low-CO_2 medium) was greatly enhanced by the addition of CA. The same enhancing effect was observed even when the added CA was diluted 10 000-fold. The molecular weights of Ru-P_2 carboxylase and CA have been reported as 557 000 [13] and 31 000 [14], respectively. Therefore, CA given at the molar ratio of ~1/100 that of Ru-P_2 carboxylase could enhance the carboxylation reaction. In the reaction medium in which the dissolved $^{14}CO_2$ was in equilibrium with 4000 ppm $^{14}CO_2$ gas, the rate of $^{14}CO_2$ fixation by Ru-P_2 carboxylase was >10-times higher than that in the low-$^{14}CO_2$ medium (fig.1,

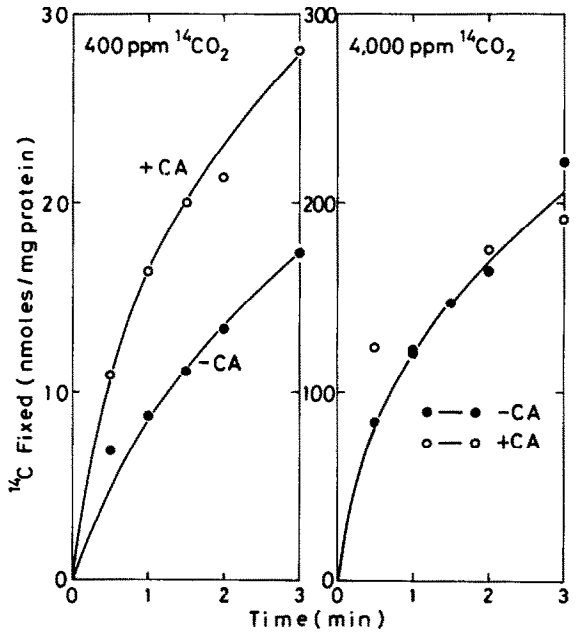

Fig.1. Effects of $^{14}CO_2$ concentrations on enhancement of Ru-P_2 carboxylation reaction by CA. $^{14}CO_2$ fixation was started after equilibrium between $^{14}CO_2$ in the bubbling gas and that dissolved in the reaction medium had been established.

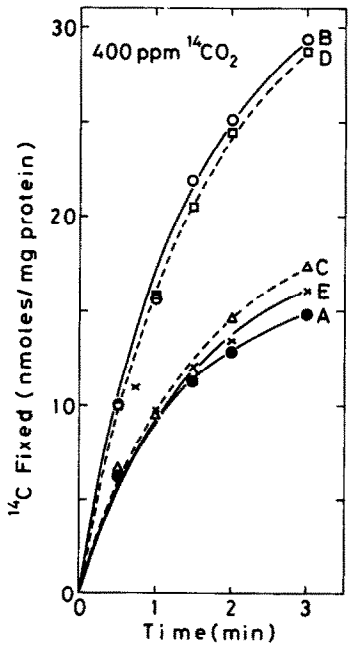

Fig.2. Effects of various reagents on CA-induced enhancement of Ru-P_2 carboxylation reaction. (A) No addition (+H_2O); (B) +CA; (C) +Diamox (final conc. 0.94 mM) which had been preincubated with CA for 30 min; (D) +Diamox and CA without preincubation; (E) +bovine serum albumin.

right). However the addition of CA did not cause any enhancement in $^{14}CO_2$ fixation, indicating that CA enhances CO_2 fixation by Ru-P_2 carboxylase only under very low CO_2 concentrations.

Figure 2 shows that the enhancing effect of CA was abolished when this enzyme had been preincubated with Diamox for 30 min (compare A and C). The enhancing effect was also abolished when CA had been pretreated with ethoxyzolamide for 40 min (data not shown). Diamox did not exert any inhibitory effect when this inhibitor and CA were added to the reaction medium without preincubation (compare C and D). Figure 2 further shows that CA could not be substituted for with bovine serum albumin (compare B and E), indicating that the enhancement of CO_2 fixation by CA was not induced by its protective action against oxidation of Ru-P_2 carboxylase.

The above results clearly indicate that CO_2 fixation by Ru-P_2 carboxylase under low CO_2 concentrations (i.e., in a reaction medium in which the dissolved CO_2 was equilibrated with CO_2 in air) is greatly enhanced by the catalytic action of CA. It has been shown that K_m (CO_2) in low K_m form of Ru-P_2 carboxylase is 20 μM, while that in high K_m form is >200 μM [15]. The CO_2 concentration in the medium in equilibrium with 400 ppm CO_2 gas at 20°C and pH 7.5 is 15.7 μM [16]. According to the data in [12], Ru-P_2 carboxylase would have been only partially activated under the CO_2 concentration and pH value adopted during the preincubation in the present experiment. Therefore, the indirect supply of CO_2 by CA will not only increase the supply of substrate (CO_2) but also activate Ru-P_2 carboxylase.

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