FORM OF INORGANIC CARBON UTILIZED FOR PHOTOSYNTHESIS ACROSS THE CHLOROPLAST MEMBRANE

Yoshihiro SHIRAIWA and Shigetoh MIYACHI*

Institute of Applied Microbiology and **Radioisotope Centre, University of Tokyo, Bunkyo-ku, Tokyo 113, Japan*

Received 5 September 1978

1. Introduction

Carbon dioxide, and not bicarbonate, was reported to be taken up by the chloroplasts via simple diffusion [1,2]. In contrast, it was concluded [3] that bicarbonate, and not CO_2, can cross the chloroplast envelope. With envelope membranes isolated from different species, it was shown [4] that bicarbonate transport capabilities paralleled their ATPase activities. Inorganic carbon was assumed [5] to be transported into the chloroplasts through the simple diffusion of CO_2 across the envelope as well as through an ATP-dependent process. Thus, a unified conclusion has not been drawn as to the form of inorganic carbon supplied through the chloroplast envelope for CO_2 fixation.

We have studied [6] the form of inorganic carbon utilized for photosynthesis in *Chlorella vulgaris* cells with carbonic anhydrase (EC 4.2.1.1) as a tool. The rate of photosynthetic $^{14}CO_2$ fixation in the presence of $NaH^{14}CO_3$ at concentrations which limit photosynthesis was greatly enhanced by the addition of carbonic anhydrase to the suspending medium. Since the conversion of HCO_3^- to CO_2 in the suspending medium is accelerated by adding this enzyme, the above result indicates that the active species of inorganic carbon absorbed by *Chlorella* cells is free CO_2 and that bicarbonate cannot be utilized by these cells. Using the same technique, we studied the form of inorganic carbon which can cross the chloroplast envelope. The results obtained with the chloroplasts isolated from spinach and the green alga *Bryopsis maxima* lead to the conclusion that the species of inorganic carbon which crosses the chloroplast envelope is also free CO_2.

* To whom correspondence should be addressed

2. Materials and methods

2.1. *Plant materials*

Spinach (*Spinacia oleracea*) was bought from a local market. The green alga, *Bryopsis maxima*, was collected at Choshi on the Pacific coast of the Boso penninsula and stored at 4°C in a water bath containing sea water.

2.2. *Isolation of intact chloroplasts*

The intact chloroplasts from spinach leaves were isolated as in [7] with the following modifications. Before homogenization, the spinach leaves were placed on ice and illuminated with a 200 W incandescent lamp placed 30–40 cm above them for about 1 h in the cold room (4°C). The homogenate was then passed successively through 82 μm and 20 μm nylon nets. After centrifuging the filtrate, the surface of the pellet was rinsed with a small amount of the isolation medium.

Intact chloroplasts of *Bryopsis maxima* were isolated according to Muto et al. (unpublished): The alga was illuminated with a bank of fluorescent lamps ($3.7–4.0 \times 10^4$ lux) at 25°C for 1 h and then washed with deionized water. The long cells of the alga were cut with scissors and their contents squeezed out through 82 μm and 40 μm nylon nets. Of 150 mM Hepes–KOH buffer (pH 6.8) containing 3 M sorbitol

2 ml was added to 4 ml of the juice thus obtained. Suspension (2 ml) was placed in the centrifuge tube on 4 ml 50 mM Hepes–KOH buffer (pH 6.8) containing 1.5 M sorbitol and centrifuged at 170 × *g* for 5 min. The pellet was suspended in 2 ml 50 mM Hepes–KOH buffer (pH 6.8) containing 1 M sorbitol (suspending medium) and then placed in a centrifuge tube on 4 ml same buffer but containing 1.5 M sorbitol. After centrifugation at 170 × *g* for 10 min, the pellet was suspended in 2 ml suspending medium. The suspension was centrifuged at 650 × *g* for 90 s and the pellet was kept in ~0.5 ml suspending medium.

2.3. *Determination of photosynthetic $^{14}CO_2$ fixation and oxygen evolution*

The composition of the reaction media used for spinach and *Bryopsis* chloroplasts were 330 mM sorbitol, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA in 50 mM Hepes–NaOH buffer (pH 7.8) and 1 M sorbitol, 2 mM KH_2PO_4 in 50 mM Tricine–KOH buffer (pH 8.5), respectively. To prepare each medium, all of the above chemicals, except NaOH (or KOH), were dissolved in CO_2-free water. CO_2-free air was then bubbled through the solution. After about 30 min, the pH value of each suspension was adjusted by adding CO_2-free NaOH (or KOH) under continued bubbling of CO_2-free air.

The intact chloroplasts suspended in 3 ml reaction medium were placed in a water-jacketed transparent cylinder equipped with a Clark type oxygen probe (Rank Brothers, London) and illuminated from both sides with projector lamps (2 × 24 000 lux at the surface of the reaction vessel). The temperature of the suspension was kept at 20°C by water running through the water-jacket and a thermostat. Reaction was started by adding $NaH^{14}CO_3$ and the change in O_2 concentration in the suspension was recorded. At intervals, 50 μl suspension was quickly transferred into a vial containing 200 μl methanol. The vial was kept in the hood with ventilator and a drop of 20% acetic acid was added to the methanol suspension. After about 2 h, 10 ml scintillation solution (Aquasol 2, Packard) was added and the radioactivity of fixed ^{14}C was determined with a liquid scintillation spectrophotometer (Packard 3380).

Solution (50 μl) containing 105 Wilbur-Anderson units of carbonic anhydrase (from bovine erythrocytes, Sigma) was added at the time indicated in the figures.

3. Results

The rate of photosynthetic $^{14}CO_2$ fixation (μmol $^{14}CO_2$ fixed/mg chl·h) by the intact chloroplasts isolated from spinach leaves in the presence of low concentration of $NaH^{14}CO_3$ (0.058 mM, pH 7.8, 20°C) was enhanced from 6.6–7.7 (17% increase, fig.1a) or from 3.4–5.0 (47% increase, fig.1b) by adding carbonic anhydrase. The rate of photosynthesis (μmol O_2 evolved/mg chl·h) in the presence of a high concentration of $NaHCO_3$ (2 mM) was 49.3 and 35.2 in the chloroplasts used for the experiments shown in fig.1a,b, respectively. In the presence of high concentration (3.5 mM) of $NaH^{14}CO_3$, the addition of carbonic anhydrase did not show any enhancement in photosynthesis.

The rate of photosynthetic $^{14}CO_2$ fixation of intact chloroplasts isolated from *Bryopsis maxima* in the presence of 0.23 mM $NaH^{14}CO_3$ (pH 8.5, 20°C) was enhanced from 3.6–8.0 (120% increase) when carbonic anhydrase was added just before the start of the reaction, or to 8.8 (140% increase) when the

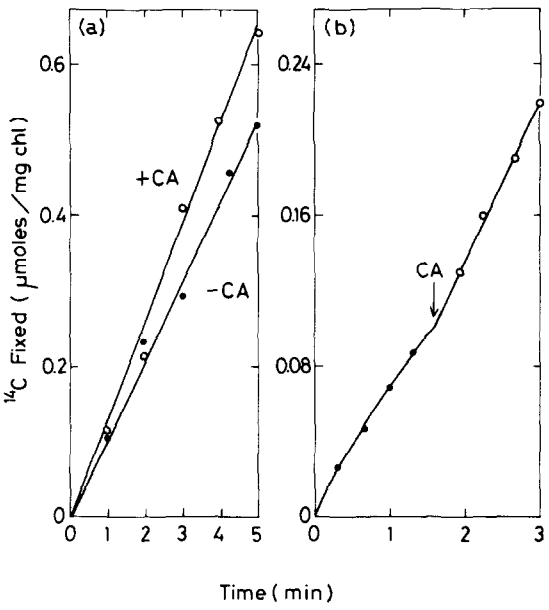

Fig.1. Effect of carbonic anhydrase on photosynthetic $^{14}CO_2$ fixation by intact chloroplasts isolated from spinach. (a) Carbonic anhydrase was added just before the start of photosynthetic $^{14}CO_2$ fixation; (b) Carbonic anhydrase was added 1 min 35 s after the start of the reaction.

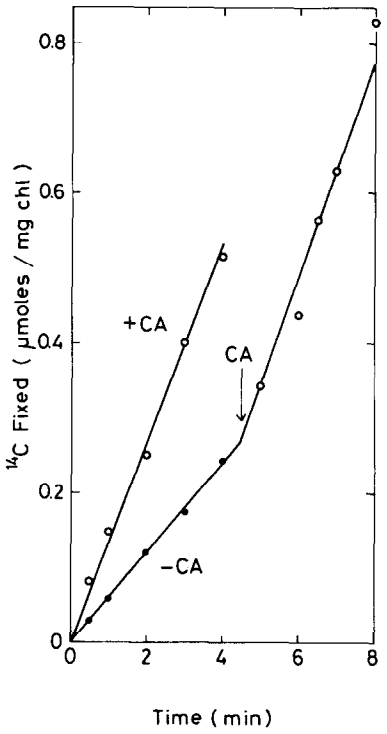

Fig.2. Effect of carbonic anhydrase on photosynthetic $^{14}CO_2$ fixation by intact chloroplasts isolated from *Bryopsis maxima*. Carbonic anhydrase was added 30 s before or 4.5 min after the start of photosynthetic $^{14}CO_2$ fixation.

enzyme was added 4.5 min after the start of photosynthetic $^{14}CO_2$ fixation (fig.2). The rate of O_2 evolution in the presence of 6.8 mM $NaHCO_3$ was 77.7. Under this condition, the addition of carbonic anhydrase did not show any enhancement in photosynthetic $^{14}CO_2$ fixation.

4. Conclusion and discussion

The present results showed that the addition of carbonic anhydrase enhanced photosynthetic $^{14}CO_2$ fixation in spinach and *Bryopsis maxima* chloroplasts in the presence of $NaH^{14}CO_3$ at 0.058 mM (pH 7.8, 20°C) and 0.23 mM (pH 8.5, 20°C), respectively. The concentrations of $^{14}CO_2$ in equilibrium with $NaH^{14}CO_3$ at the above concentrations are 2.3 μM and 1.9 μM. The K_m values for CO_2 in photosynthesis in spinach and *Bryopsis maxima* chloroplasts are 30–50 μM [8] and 10 μM (Muto et al., unpublished), respectively. Therefore, the concentrations of $^{14}CO_2$ in equilibrium with the added $NaH^{14}CO_3$ are far lower than the respective K_m values for CO_2. Under such low CO_2 conditions, carbonic anhydrase, which accelerates the conversion of HCO_3^- to CO_2, enhanced photosynthetic CO_2 fixation in the isolated chloroplasts. No effect of carbonic anhydrase was observed in the presence of higher concentrations of bicarbonate (3.5 mM for spinach and 6.8 mM for *Bryopsis maxima* chloroplasts). The $^{14}CO_2$ concentrations in equilibrium with $NaH^{14}CO_3$ at the respective concentrations are 140 μM and 55 μM, which are significantly higher than K_m values for CO_2. It is reasonable to assume that CO_2 dehydrated from $NaHCO_3$ at such high concentrations is sufficient to maintain the maximum rate of photosynthetic CO_2 fixation even without the addition of carbonic anhydrase. Based on these results we conclude that the active species of 'CO_2' absorbed by chloroplasts is CO_2.

The procedure adopted [3] to determine the active species of 'CO_2' is based on the fact that the attainment of equilibrium between HCO_3^- and CO_2 requires more than 1 min at 10°C in the absence of carbonic anhydrase [9]. Thus, the initial uptake of the active ^{14}C-labeled species should be more rapid than that of the non-active ^{14}C-labeled one. The time course was followed [3] of $^{14}CO_2$ uptake by the chloroplasts which were placed in the medium containing $H^{14}CO_3^-$ and CO_2 or HCO_3^- and $^{14}CO_2$ at 10°C. Since the dark ^{14}C-incorporation from the former medium was much faster than that from the latter, it was concluded that active species involved in the uptake is HCO_3^-. When $^{14}CO_2$ was the desired species, the $H^{14}CO_3^-$ solution was reacted with an equivalent amount of HCl and then the resulting solution was added to the chloroplast suspension. We showed that $^{14}CO_2$ produced by neutralization of $H^{14}CO_3^-$ easily escaped to the gas phase. It is therefore likely that the concentration of $^{14}CO_2$ added to the chloroplast suspension was smaller than that expected. Thus, the amount of $^{14}CO_2$ given to the chloroplast suspension would have been smaller than that of $H^{14}CO_3^-$, and this difference might have brought about the difference in ^{14}C-uptake observed [3].

Acknowledgements

This work carried out at Radioisotope Centre of University of Tokyo was supported by grants from Japanese Ministry of Education, Science and Culture and Ministry of Agriculture, Forestry and Fishery.

References

[1] Werdan, K., Heldt, H. W. and Geller, G. (1972) Biochim. Biophys. Acta 283, 430–441.
[2] Heber, U. and Purczeld, P. (1977) Proc. 4th Int. Congr. Photosynthesis (Hall, D. O. et al. eds) pp. 107–118, The Biochemical Society, London.
[3] Poincelot, R. P. (1974) Plant Physiol. 54, 520–526.
[4] Poincelot, R. P. and Day, P. R. (1976) Plant Physiol 57, 334–338.
[5] Champigny, M.-L. and Bismuth, A. (1977) Photosynthetic organelles Structure and Function; spec. iss. Plant Cell Physiol. 3, 365–375.
[6] Miyachi, S. and Shiraiwa, Y. (1978) submitted.
[7] Miyachi, S. and Hogetsu, D. (1970) Plant Cell Physiol. 11, 927–936.
[8] Lilley, R. McC. and Walker, D. A. (1975) Plant Physiol. 55, 1087–1092.
[9] Cooper, T. G., Tchen, T. T., Wood, H. G. and Benedict, C. R. (1968) J. Biol. Chem. 243, 3857–3863.