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# INTRACELLULAR INORGANIC CARBON EXISTS AS PROTEIN CARBAMATE IN PHOTOSYNTHESIZING EUGLENA GRACILIS Z*

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SUMMARY. The form of inorganic carbon accumulated in Euglena gracilis cells was determined. Euglena cell protein bound 0.96 nmol $CO_2$/mg protein. The binding of $CO_2$ was by the formation of protein carbamate as indicated by acid lability of the protein-$CO_2$ complex, stimulation of complex formation by high ionic strength and the carbamate resonance in $^{13}C$-NMR spectrum. The protein carbamate could also be isolated from photosynthesizing Euglena. The formation of the carbamate required light energy fixed photosynthetically.

There are two differences in photosynthetic carbon metabolism between higher $C_3$ plants and such photosynthetic unicellular organisms as algae and Euglena, a photosynthetic protozoon. One is the mechanism of glycolate metabolism (1) and the other is concentration of inorganic carbon (Ci) in the cells (2-5). When these photosynthetic microorganisms are grown under low $CO_2$ concentrations as in air, the Ci-concentrating system is operative and the intracellular Ci level is raised so as to maintain high photosynthetic activity (2, 3, 6). The system can accumulate extracellular Ci in the cells up to 0.9 to 6.0 mM (2, 3, 7). Free $CO_2$ in the cells could be expected to be 0.2 to 3.0 mM from the above Ci concentrations and intracellular pH. However, because this free $CO_2$ in the cells would be 100 to 1,000 times as high as the extracellular concentration of free $CO_2$ in these experiments and because $CO_2$ diffuses very rapidly through the cell membrane and into water (8), the occurrence of such high concentrations of free $CO_2$ in the unicellular organisms requires explanation. In the present communication, we provide evidences showing that a con-

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siderable part of intracellular Ci exists as protein carbamate in photosynthesizing, air-grown Euglena.

## MATERIALS AND METHODS

Organism, Culturing and Preparing Cell Extract. E. gracilis z was cultured photoautotrophically on air or 5% $CO_2$ in air in Cramer and Myer's medium (9) with illumination at 2,000 lux and 27 °C, or photoheterotrophically (10). Cell collection and preparation of cell extract followed the methods reported previously (10). Protein was determined according to Lowry et al. (11) using bovine serum albumin as a standard.

Determination of Protein Carbamate. Determination of protein carbamate followed the method established by Perrella and Rossi-Bernardi (12) with some modifications. The reaction mixture (1.0 ml) for the formation of protein carbamate in vitro contained 50 mM potassium phosphate buffer (pH 8.0), an appropriate concentration of $NaH^{14}CO_3$ and Euglena cell protein. The reaction was stopped after 10 min by adding 0.1 ml of 2 M glycine-NaOH (pH 13.5) with the effects of stabilizing protein carbamate and converting unreacted $CO_2$ and $HCO_3^-$ to $CO_3^{2-}$, and the reaction mixture cooled promptly in ice. The mixture was applied onto a column (1.5 x 30 cm) of Sephadex G-25 which had been equilibrated with 10 mM NaOH containing 0.2 M NaCl. The elution was performed with 10 mM NaOH-0.2 M NaCl at 4 °C at an elution rate of 1.0 ml/min.

$^{13}C$-NMR Spectrum of Protein Carbamate. Euglena was grown photoheterotrophically on a vitamin $B_{12}$-limited medium (13), which yields cells having very fragile cell membranes easily disrupted in a dense suspension. The cell extract obtained as described above was dialyzed against 50 mM potassium phosphate buffer (pH 8.0) over night, and to the dialyzed extract was added $NaH^{13}CO_3$ prepared from $Ba^{13}CO_3$ (90 atom % $^{13}C$, MSD ISOTOPES, Montreal, Canada) according to O'Learly et al. (14), to a concentration of 50 mM. $^{13}C$-NMR spectrum was obtained with a Varian spectrometer operated at 50.3 MHz and in the Fourier transform with a deuterium lock. Experimental temperature was 23 °C. The assignments of resonance peaks in the spectrum followed the method of O'Learly et al. (14).

## RESULTS

Fig. 1 shows the separation of protein-bound $^{14}CO_2$ on Sephadex G-25 after incubation of the cell extract from air-grown Euglena with 6 mM $NaH^{14}CO_3$ for 10 min. The separated protein-$^{14}CO_2$ complex was completely acid-labile. The ratio of the radioactivity trapped by protein to the amount of protein was nearly constant in the protein fractions (4.5 to 6.5 nmol of $^{14}C$ trapped/mg protein). The same ratio was obtained with cell extracts of Euglena grown on 5% $CO_2$ and photoheterotrophically. The formation of protein-bound $^{14}CO_2$ was stimulated 50% by the addition of 0.15 M NaCl to the reaction mixture, as reported for the formation of glycyl-glycine carbamate (15). The amount of the protein-$^{14}CO_2$ complex separated per mg of protein was dependent on the concentration of $NaHCO_3$ added to the reaction mixture to at least 6 mM (Fig. 2) and was inde-

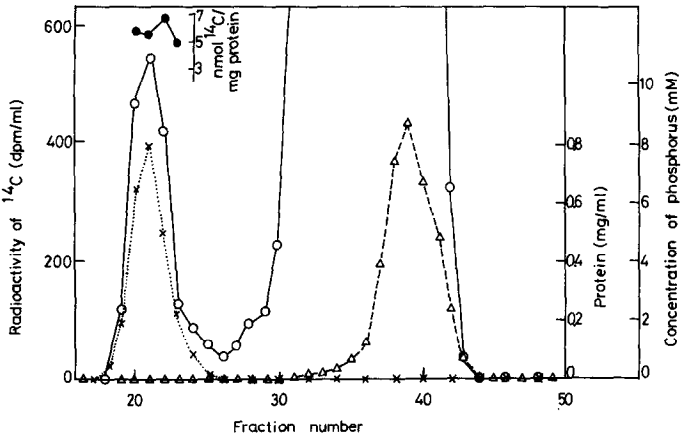


Fig. 1. Separation of air-grown Euglena cell protein-$^{14}CO_2$ complex formed in vitro on Sephadex G-25 at alkaline pH. The protein and $NaH^{14}CO_3$ concentrations in the reaction mixture (1.0 ml) were 7.4 mg and 6 mM (129 dpm/nmol), respectively. Radioactivity, O—O; protein, x·····x; concentration of phosphorus, Δ--Δ. Note that phosphorus used to buffer the reaction mixture did not contaminate the protein fractions at all.

pendent of the protein concentration. The slope of Fig. 2 shows that 0.96 nmol of the complex per mg of protein was formed when Euglena cell protein reacted with 1 mM $NaHCO_3$.

In Fig. 3 is shown $^{13}C$-NMR spectrum of Euglena cell protein incubated with 50 mM $NaH^{13}CO_3$. The broad resonance peak around 161 ppm was due to $H^{13}CO_3^-$ and $^{13}CO_3^{2-}$. Free $^{13}CO_2$ gave a sharp resonance peak at 125 ppm. The small, but significant peak of resonance at 167 ppm was assigned to protein $^{13}C$-carbamate. These peaks were not seen in the control spectrum.

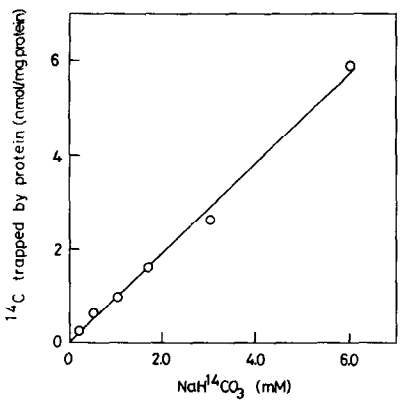


Fig. 2. Efficiency of cell protein-$CO_2$ complex formation by air-grown Euglena. The protein concentration in the reaction mixture was 8.8 mg.

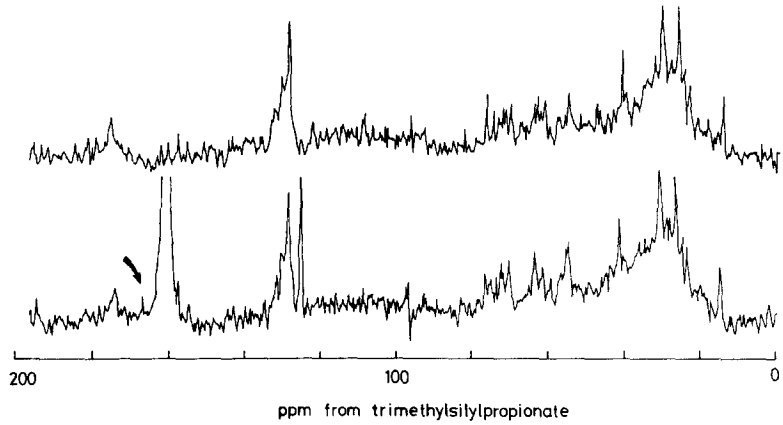


Fig. 3. $^{13}C$-NMR spectrum of Euglena cell protein-$^{13}CO_2$ complex. Upper spectrum is a control without added $NaH^{13}CO_2$ in the reaction mixture. The protein concentration was 62.5 mg/ml. The spectrometer was run with a tube of 5 mm in diameter, $^{13}C$ excitation pulse width of 6 μsec, spectral widths of 10 kHz, and an acquisition time of 0.7 sec. The tube was spun at the rate of 25 rps. The spectra represent data accumulations in excess of 70,000 transients. The arrow indicates the resonance peak of protein $^{13}C$-carbamate.

Euglena is coated with a proteinous pellicle easily solubilized by alkali above pH 12 containing 0.1% sodium dodecylsulfate (SDS). To air-grown Euglena ($5.9 \times 10^6$ cells) photosynthesizing in the presence of 1 mM $NaH^{14}CO_3$ (3550 dpm/nmol) under illumination at 10,000 lux was added 2 M glycine-NaOH (pH 13.5) containing 1% SDS to give 0.2 M glycine-NaOH containing 0.1% SDS, and the suspension cooled promptly. After centrifugation of the alkaline solution to remove cell debris, it was applied onto a column (1 x 100 cm) of Sephadex G-25 which had been equilibrated as in Fig. 1. As shown in Fig. 4, the protein-$^{14}CO_2$ complex was extracted from photosynthesizing, air-grown Euglena. The complex was estimated to be about 1 nmol/mg protein (Table I). The formation of the protein-$CO_2$ complex in the photosynthesizing cells was suppressed by darkening the cells or adding 50 μM 3-(3',4'-dichlorophenyl)-1,1'-dimethylurea (DCMU), an inhibitor of photosynthetic electron transport chain (16) (Table I).

## DISCUSSION

It is known that protein carbamate formed by the reaction of non-protonated protein amino groups and free $CO_2$ is relatively stable in alkali at low temperature (17, 18). In the present experiments, the complex formed from Euglena cell protein and $CO_2$ was separated under thus conditions (Fig. 1). The

Table I. Requirements of light and photosynthetic electron transport for the formation of protein-$^{14}CO_2$ complex in photosynthesizing, air-grown Euglena

| Experimental conditions | Amount of protein-$^{14}CO_2$ complex (nmol of $^{14}CO_2$/mg protein) |
|---|---|
| Light | 0.943 |
| Dark | 0.334 |
| Light + 50 μM DCMU | 0.310 |

Cell number in the reaction mixture was $10^7$. The other conditions were the same as in Fig. 4.

identification of the separated protein-$CO_2$ complex as protein carbamate was based on the acid lability of the protein-bound $^{14}C$, the stimulation of the formation of the complex by high ionic strength (15), and the carbamate resonance in $^{13}C$-NMR spectrum (Fig. 3). The protein carbamate was also isolated from photosynthesizing Euglena (Fig. 4).

Both α- and ε-amino groups of protein should participate in the carbamate formation with free $CO_2$ (12, 15). However, the protein carbamate separated in the present experiments is considered to involve α-amino group mainly, since

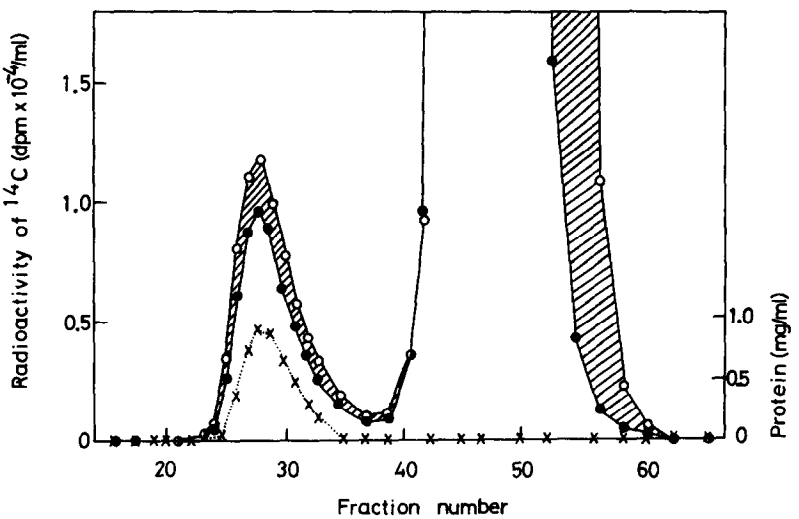


Fig. 4. Separation of protein-$^{14}CO_2$ complex formed in vivo in photosynthesizing Euglena on Sephadex G-25 at alkaline pH. Open and closed circles represent radioactivity of $^{14}C$ before and after, respectively, addition of 50 μl of 2 N $H_2SO_4$ to 0.2 ml of eluted fractions. Accordingly, the shaded area represents acid-labile $^{14}C$. X's show protein concentration.

the pK of the α-amino groups in protein is near 8, 2 pK units lower than that of ε-amino group (15). Stabilization of $CO_2$ on ε-amino groups may require the presence of divalent metal cations, as shown by O'Learly et al. (14).

The formation of protein carbamate in the photosynthesizing cells of Euglena was dependent on operation of the photosynthetic electron transport (Table I). Light energy, such as ATP, may be required for the active transport of Ci from the outside of the cells and alkalinization of chloroplast stroma to facilitate the carbamate formation. The dependency of the carbamate formation upon light energy is consistent with the reported fact that light energy is required for accumulation of Ci in algal cells (2, 3, 7). However, the amount of the protein carbamate (per mg protein) formed in vitro was the same in the both air- and 5% $CO_2$-grown cells of E. gracilis, and this contradicts the report that the capacity of the former cells to concentrate Ci in the cells is superior to that of the latter (2-4, 6, 7). This discrepancy may be overcome by the postulated presence of a Ci transporter in cell membrane or chloroplast envelope of the air-grown cells, as proposed by Kaplan et al. (19).

It is of interest that the protein carbamate was estimated to be 1 nmol/mg protein when the Euglena cell protein was incubated with 1 mM $NaHCO_3$ (Fig. 2). This means that when E. gracilis accumulates 1 mM Ci in the cells the protein carbamate amounts to 1 nmol/mg protein, corresponding to about 0.5 mM in concentration in the cells, since $10^6$ cells of Euglena contain about 300 μg of protein and are about 1 μl in volume (20). Accordingly, protein carbamate constitutes about 50% of the intracellular Ci in E. gracilis. The remainder may exist as free $CO_2$ and $HCO_3^-$, the concentrations of which depend on intracellular pH. Thus, protein carbamate decreases the intracellular concentration of free $CO_2$ and prevents the loss of $CO_2$ from the cells. The rates of association and dissociation of the protein carbamate are generally high (15, 21), so that the protein carbamate can contribute to temporal accumulation of Ci in the cells. The functional significance of this protein carbamate in the glycolate metabolism and photorespiration in E. gracilis is under study in this laboratory.

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