

Photosynthesis of *Euglena gracilis* under Cobalamin-Sufficient and -Limited Growing Conditions¹

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ABSTRACT

Cobalamin is essential for growth of *Euglena gracilis* and photosynthesis. Methylcobalamin in *Euglena* chloroplasts (Y Isegawa, Y Nakano, S Kitaoka, 1984 Plant Physiol 76: 814-818) functions as a coenzyme of methionine synthetase. The requirement of cobalamin for photosynthesis appeared remarkably high in *Euglena* grown under the dark-precultured condition. The required amount of cobalamin for normal photosynthetic activity was 7.4×10^{-11} molar, while 7.4×10^{-10} molar cobalamin was required for normal growth. The lowered photosynthetic activity in cobalamin-limited cells was restored 20 hours after feeding cyanocobalamin or methionine to cobalamin-limited cells. Lowering of photosynthetic activity was due to loss of photosystem I activity. This photosynthetic activity was recovered after supplementation by methionine or cobalamin. The results suggest that methionine serves for the stabilization of photosystem I. This paper is the first report of the physiological function of cobalamin in chloroplasts of photosynthetic eukaryotes.

In *Euglena gracilis* the physiological function of Cbl² has not been well understood, although Cbl is essential for growth, is rapidly accumulated in the cells (2, 18) and is converted to coenzyme forms in this organism (8). *Euglena* cells, when grown on a CN-Cbl-limited medium (below 50 ng CN-Cbl·L⁻¹), show increases in chloroplast number and concomitant increases in cell components such as Chl, protein, RNA and DNA (3, 9); CN-Cbl-limited cells return to the normal state within 24 h after raising the CN-Cbl level to normal (2).

Although plants and yeasts had not been thought to require Cbl for growth, *Phaseolus vulgaris* has an Ado-Cbl-dependent enzyme (leucine 2,3-aminomutase [14]), extracts of *Solanum tuberosum* contain endogenous activities of two coenzyme Cbl-dependent enzymes (leucine 2,3-aminomutase and methylmalonyl-CoA mutase [15]) and two cobalamin-dependent enzyme systems have been found in *Candida utilis* (Me-Cbl dependent methionine biosynthesis and leucine 2,3-aminomutase [16]). The subcellular locations and physiological functions of these enzymes are not known. Nobody has defined a function of Cbl related to photosynthesis in photosynthetic organisms. In the

present paper we show that photosynthetic activity is affected by Cbl in *E. gracilis* Z, and discuss the physiological functions of Cbl and Cbl-dependent metabolic pathways.

MATERIALS AND METHODS

Organism and Culture. *Euglena gracilis* Klebs strain Z Pringsheim, cultured organotrophically in the dark under CN-Cbl-sufficient conditions ($5 \mu\text{g CN-Cbl}\cdot\text{L}^{-1}$), was then cultured photoorganotrophically at 27°C under illumination (Homolux, Panasonic) (2500 lux) in the Oda medium (13) under CN-Cbl-limited and -sufficient conditions with 0.05 and $5 \mu\text{g}$ of CN-Cbl·L⁻¹, respectively. Cells were harvested by centrifugation at 1000g for 5 min at 4°C. Cell number was calculated with hemacytometer.

Cell Extract. *E. gracilis* cells were disrupted by sonication (10 kHz) for a total of 1 min with 3 rest intervals of 15 s each at 4°C, and the sonicate was centrifuged at 100,000g at 2°C for 1 h. The supernatant fraction was used as the cell extract.

Subcellular Fractionation and Purification of Chloroplasts. Partial trypsin digestion of the pellicle followed by mild mechanical disruption of *Euglena* cells and subcellular fractionation by differential centrifugation were conducted according to Shigeoka *et al.* (19).

A suspension of chloroplasts was layered on the top of continuous Percoll (Lot No. 4634) gradients and centrifuged in a swing-out rotor (Hitachi RPS-25) for 30 min at 2300g at 4°C. The linear gradient with the Percoll solution from 10 to 80% (v/v) was prepared with a gradient former (model 570 Instrumentation Specialties Co.) into centrifuge tubes in the volume of 24 ml. The Percoll solution contains 25 mM glycylglycine-KOH buffer (pH 7.4), 3% (w/v) Ficoll, and 0.33 M sorbitol. The isopycnicly banded chloroplasts were then recovered from the gradients, washed, and resuspended in 0.33 M sorbitol glycylglycine-KOH buffer (pH 7.4).

Reactions of Photosynthesis. Photosynthesis resulted in net O₂ evolution from cells, which were cultured in the dark in the presence of Cbl and then in the light with limiting or supplementing Cbl, was measured with a Kalbani-type oxygen electrode (Kyusui Kagaku Co.) in 2 ml of a reaction mixture containing an aliquot of the cell culture and 25 mM NaHCO₃, 1 mM MgCl₂, and 50 mM potassium phosphate buffer (pH 7.8). The mixture which contained 60 to 100 $\mu\text{g Chl}\cdot\text{ml}^{-1}$, was incubated at 25°C and 10,000 lux with wolfram lamp. ¹⁴CO₂ fixation was carried out under the same conditions as described above except that 10 mM (0.4 μCi) (final concentration) NaH¹⁴CO₃ was added instead of 25 mM NaHCO₃. A sample of 0.2 ml was withdrawn after a definite period through Eppendorf pipette and injected into a vial containing 0.3 ml of 0.3 N HCl. The vial was shaken for 30 min to remove free ¹⁴CO₂ and the fixed radioactivity in the sample was counted with Aloka LSC 903 scintillation counter.

¹ This paper is the second in a series on the physiological function of cobalamin in *Euglena gracilis* z.

² Abbreviations: Cbl, cobalamin; CN-Cbl, cyanocobalamin; Me-Cbl, methylcobalamin; Ado-Cbl, 5'-deoxyadenosylcobalamin; DCIPH₂, 2,6-dichlorophenolindophenol reduced form; MV, methyl viologen; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; RuBPC, ribulose 1,5-bisphosphate carboxylase.

Ferricyanide-dependent O_2 evolution by isolated chloroplasts was estimated according to Lilley *et al.* (10). The MV-Hill and DCIPH₂-MV photoreduction reactions were measured basically according to Epel and Neumann (6).

Enzyme Assays. The activity of RuBPC (EC 4.1.1.39) was measured according to Rabinowitz *et al.* (17). GAPDH was assayed according to Velick (20).

Chl and protein were determined by the methods of Mackinney (12) and Lowry *et al.* (11), respectively.

RESULTS

Effects of Growth, Cbl, and Light on Photosynthetic Activity.

Figure 1 shows variation of the photosynthetic activity in *E. gracilis* during growth under Cbl-sufficient and -limited conditions. The photosynthetic activity was affected by the amount of Cbl in the growth medium and the period of growth. The activity of Cbl-sufficient cells was about twice as high as Cbl-limited cells in the period of 4 to 6 d. The highest activity was found in the 5 d light-exposed cultures of both types of cells, and only Cbl-sufficient cells decreased substantially in activity by 7 d of growth. Both cultures reached the stationary phase of growth in 4 d, and the cell numbers under Cbl-sufficient and -limited conditions were 16 and 3.5×10^6 cells \cdot ml⁻¹, respectively. Figure 2 shows that the activity of photosynthesis increases in parallel with the increase of Cbl in the growth medium. The activity

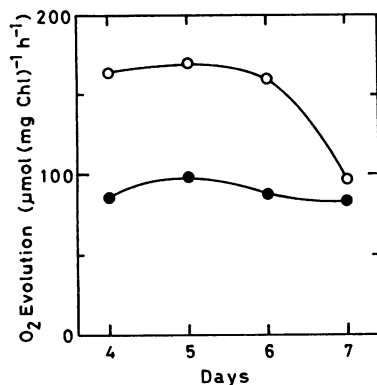


FIG. 1. Photosynthetic activity of CN-Cbl-sufficient and -limited cells during greening. O_2 evolution was assayed along with growth in the light of dark-grown precultured cells under the Cbl-sufficient and -limited conditions. Greening was carried out under illumination at 2500 lux. CN-Cbl-sufficient cells, \circ ; CN-Cbl-limited cells, \bullet .

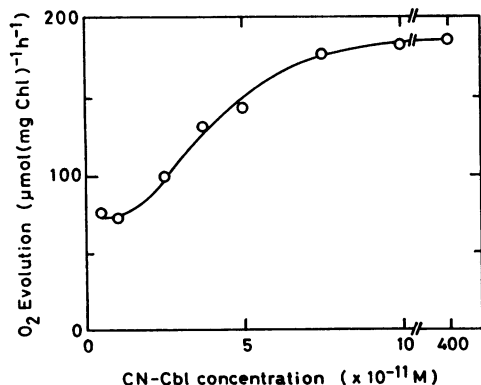


FIG. 2. Effect of CN-Cbl concentration on the photosynthetic activity. Cells, which were precultured in the dark under Cbl-limited condition, were grown for 5 d under illumination (2500 lux) and with various concentration of CN-Cbl.

reached the maximum at 7.4×10^{-11} M ($0.1 \mu\text{g} \cdot \text{L}^{-1}$) Cbl in the medium, while the cell growth was the best at 7.4×10^{-10} M ($1.0 \mu\text{g} \cdot \text{L}^{-1}$) Cbl, indicating that the amount of Cbl required for normal growth was ten times as much as that required for normal photosynthetic activity.

Figure 3 shows effect of light intensity on the photosynthetic activity under Cbl-sufficient and -limited conditions in the dark- and light-precultured cells. The light saturation point for the activity was 10,000 lux both in Cbl-sufficient and -limited cells. The activities were 120 and $60 \mu\text{mol} (\text{mg Chl})^{-1} \text{h}^{-1}$ in Cbl-sufficient and -limited cells precultured in the dark in the presence of Cbl, and 100 and $80 \mu\text{mol} (\text{mg Chl})^{-1} \text{h}^{-1}$ in Cbl-sufficient and -limited cells precultured in the light with Cbl, respectively. Since the effect of Cbl is more pronounced in the cells precultured in the dark, thereafter the photosynthetic activities are determined under such conditions.

Effects of Feeding Cbl or Methionine on Photosynthesis. Figure 4 shows variation of the photosynthetic activity due to adding Cbl to *Euglena* cells which had been grown for 4 d in the Cbl-limited medium. An increase in the activity was observed 12 h after Cbl addition and the level of Cbl-sufficient cells was reached by 20 h; the cell number (14×10^6 cells \cdot ml⁻¹) was restored to that of Cbl-sufficient cells within 24 h after Cbl feeding. However, during active cell division (12 h after Cbl feeding) the photosyn-

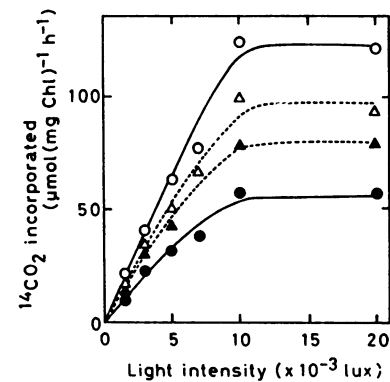


FIG. 3. Effect of light intensity on the CO_2 fixation. Photosynthetic activities are assayed by the use of Cbl-sufficient and -limited 5 d grown cells whose seed cells are precultured in the presence of CN-Cbl in the dark or light. See "Materials and Methods" for the estimation of CO_2 fixation activity. Dark-precultured Cbl-sufficient cells, \circ ; dark-precultured Cbl-limited cells, \bullet ; light-precultured Cbl-sufficient cells, Δ ; light-precultured Cbl-limited cells, \blacktriangle .

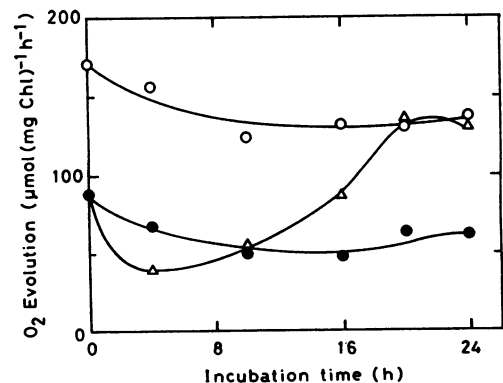


FIG. 4. Effect of feeding CN-Cbl on photosynthetic activity. Growth conditions are presented in Figure 1. Cbl-supplemented cells received 10^{-8} M CN-Cbl at 0 h in figure to Cbl-limited cells grown for 4 d. O_2 evolution was assayed along with growth for 24 h. Cbl-sufficient cells, \circ ; Cbl-limited cells, \bullet ; Cbl-supplemented cells, Δ .

thetic activity was not restored and after cell division ceased the activity was restored, indicating that *E. gracilis* requires Cbl for normal photosynthesis independently of cell division.

Figure 5 shows the effect on the photosynthetic activity after supplementing 4 d grown cells in the Cbl-limited medium with methionine. The photosynthetic activity in methionine-fed cells was considerably restored by 20 h; 89% of the activity was found compared with the activity of the Cbl-sufficient cells. The cell number (4×10^6 cells·ml⁻¹) was not completely restored to that of the Cbl-sufficient cells (16×10^6 cells·ml⁻¹) by the methionine supplement, but the results indicate a similar pattern to Cbl feeding, indicating that Cbl required for normal photosynthesis is at least partially replaced by methionine.

Effect of Cbl on Calvin Cycle and Photosystems. Table I shows that the activities of the Calvin cycle enzymes, GAPDH, and RuBPC in Cbl-limited cells are not different from those in Cbl-sufficient cells. These enzyme activities were higher required for net photosynthetic activity.

The partial activities of the photosystems are shown in Table II. In the PSII reaction the activity of ferricyanide reduction was the same in Cbl-sufficient and -limited cells. In the PSI reaction the activity of DCIPH₂-MV reduction was 665.0 μmol (mg Chl)⁻¹h⁻¹ in Cbl-sufficient cells and 387.2 in Cbl-limited cells, and the values for O₂ evolution calculated from the above activities were 166.3 and 96.8 μmol (mg Chl)⁻¹h⁻¹, respectively. The activity of the MV-Hill reaction, PSI and PSII, was 166.0 and 97.7 μmol (mg Chl)⁻¹h⁻¹, in Cbl-sufficient and -limited cells, respectively. The DCIPH₂-MV reaction and MV-Hill reaction of Cbl-sufficient cells was about 1.7 times as high as in Cbl-limited cells. These values corresponded to the net photosynthetic activities, 164.5 μmol (mg Chl)⁻¹h⁻¹ in the Cbl-sufficient cells and

Table II. Photochemical Activities of Chloroplasts of CN-Cbl-Sufficient and -Limited Cells during Greening

Growth conditions were presented in Figure 3. See "Materials and Methods" for details of the estimation of photosystem activities.

	CN-Cbl-Sufficient Cells	CN-Cbl-Limited Cells
	μmol (mg Chl) ⁻¹ h ⁻¹	
Ferricyanide reduction	148.7	151.2
DCIPH ₂ -MV reduction	665.0	387.2
MV-Hill reaction	166.0	97.7
Whole cells (O ₂ evolution)	164.5	89.8

89.8 in the Cbl-limited ones. These results suggest that Cbl-deficiency causes a lowering of the PSI activity.

DISCUSSION

Photosynthetic activity of cells grown in Cbl-limited medium increased with the concentration of added Cbl. The maximum activity was obtained at 7.4×10^{-11} M Cbl, while the amount of Cbl required for normal photosynthesis was 1/10 of that required for normal growth. These results suggest that a Cbl-dependent metabolism occurs in the photosynthetic system and a smaller amount of Cbl is effective for photosynthesis compared with growth.

The light dependency in Cbl-sufficient and -limited cells was not different, but the specific activity of photosynthesis in Cbl-limited cells, when precultured in the dark, was half, compared with that in Cbl-sufficient cells. The activity of Cbl-limited cells precultured in the light was 20% as low as that of Cbl-sufficient cells. These seed cells were precultured in the presence of CN-Cbl in the dark or light. The photosynthetic activity of Cbl-sufficient cells precultured in the dark was higher than those precultured in the light, while the activity of Cbl-limited ones was the reverse, indicating a high photosynthetic activity in the light-precultured cells. These results suggest that the high activity in dark-precultured and Cbl-sufficient cells is due to a synchronous development of proplastids through the transfer to the light of dark-grown cells. The reverse phenomenon in Cbl-limited cells appears to be observed by the expression of chloroplast development due to depletion of Cbl in the dark-grown cells. Accordingly Cbl is suggested to function for chloroplast development. However, the proteins of light harvest pigments were not damaged under Cbl-limited condition (Y Isegawa, Y Nakano, S Kitaoka, unpublished data). The photosynthetic activity in Cbl-limited cells was restored within 20 h after Cbl feeding to the same activity as Cbl-sufficient cells. The lag time for the recovery of photosynthesis was more than 12 h, corresponding to the period of cell division. The factor necessary for maximum photosynthesis is probably synthesized after the halt of cell division. The restoration of the photosynthetic activity by addition of methionine to the Cbl-limited cells was partial with kinetics similar to Cbl-supplemented cells. The results indicate that Cbl can be partly replaced by methionine and suggest that this amino acid is synthesized by a Cbl-dependent enzyme in *Euglena* cells. We have shown that Me-Cbl functions as a cofactor in methionine synthesis in *Euglena* chloroplasts (8). The lag observed in the recovery of photosynthetic activity after addition of methionine might be due to a number of factors. One is that methionine may be taken up poorly by chloroplasts. Another is that the amino acid is preferentially utilized in protein synthesis and methylation unrelated to photosynthesis. Still another possibility is that a long time is necessary for the synthesis and turnover of components related to photosynthetic activity.

The activities of the enzymes related to the Calvin cycle were more than sufficient to support net photosynthetic activity in

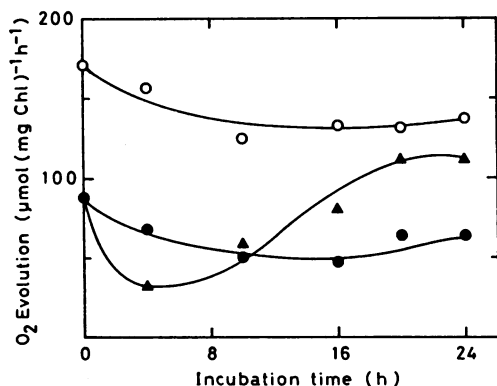


FIG. 5. Effect of feeding methionine on the photosynthetic activity. Methionine-supplemented cells received 1 mM methionine at 0 h in figure to Cbl-limited cells grown for 4 d. See Figure 4 for details. Cbl-sufficient cells, O; Cbl-limited cells, ●; methionine-supplemented cells, ▲.

Table I. Specific Activity of Some Enzymes in Chloroplasts of CN-Cbl-Sufficient and -Limited Cells during Greening

GAPDH and RuBPC were assayed along with greening of dark-grown cells under the Cbl-sufficient and Cbl-limited conditions. Greening condition was presented in Figure 1.

Enzyme	CN-Cbl	Activity after			
		4 d growth		6 d growth	
GAPDH	+	513.0 ^a	1.50 ^b	411.0 ^a	2.58 ^b
	-	505.8	2.10	397.2	2.28
RuBPC	+	258.0	0.72	197.4	1.26
	-	253.2	1.20	209.4	1.20

^a μmol (mg Chl)⁻¹h⁻¹.

^b μmol (mg protein)⁻¹h⁻¹.

both Cbl-sufficient and -limited *Euglena* cells. However, the activities of the photosystems in Cbl-limited cells were different from those of Cbl-sufficient cells. PSII activity in Cbl-limited cells was equivalent to that of Cbl-sufficient cells, while the PSI activity in Cbl-limited cells showed only 59% of the activity of Cbl-sufficient cells. These PSI activities corresponded to the net photosynthetic activities in the Cbl-sufficient and -limited cells. Thus, *Euglena* PSI appears to act as a limiting step in photosynthesis during Cbl deficiency. In normal higher plants, the rate-limiting step of photosynthesis is not RuBPC, under the best conditions at least, while in green algae, one of the rate-limiting steps is this enzyme (21). Since the effect of Cbl supplement on the photosynthetic activity of the Cbl-limited cells is similar to the methionine supplementation, reduction of PSI activity may be due to a deficiency of methionine in the chloroplasts, which is synthesized by a Cbl-dependent system.

Quinones related to the electron transport system of chloroplasts consist of plastoquinone, phyloquinone, and tocopherol quinone (7). These compounds contain some methyl groups which are transferred from *S*-adenosylmethionine. Baszynski (1) has reported that PSI activity lost by heptane treatment of spinach chloroplasts is completely restored by the addition of α -tocopherol, suggesting that α -tocopherol functions as an essential component in electron transport of PSI or as a stabilizer of PSI. In Cbl-limited *Euglena* cells, the contents of α -tocopherol and α -tocopherol quinone are reduced, compared with those in Cbl-sufficient cells (Y Isegawa, Y Nakano, S Kitaoka unpublished data). So it is suggested that the deficiency of methionine causes the a lowered synthesis of these quinones resulting in the decrease of PSI activity.

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