Adenosylcobalamin-dependent methylmalonyl-CoA mutase isozymes in the photosynthetic protozoon *Euglena gracilis* Z

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The photosynthetic protozoon *Euglena gracilis* Z contains adenosylcobalamin-dependent methylmalonyl-CoA mutase (MCM) involved in propionate metabolism. The specific activity of the *Euglena* mutase was about 6.5-fold greater in propionate-adapted *Euglena* cells than in photoautotrophic cells (control). Although the control cells contained only one mutase (apparent M_r 72 000), the propionate-adapted cells contained two mutases with M_r values of 72 000 and 17 000; both enzymes were located in the mitochondria. These results provide evidence that propionate-adapted *Euglena* contains two MCM isozymes. The induced mutase (M_r 17 000) permits photoassimilation of propionate.

Keywords: propionate metabolism, adenosylcobalamin, methylmalonyl-CoA mutase, mitochondria, *Euglena gracilis* Z

INTRODUCTION

Euglena gracilis Z, a photosynthetic flagellate, combines characteristics of both plant and animal cells (Kitaoka *et al.*, 1989). *E. gracilis* Z requires cobalamin (Cbl) for growth (Watanabe *et al.*, 1992). It can take up and accumulate Cbl, which is converted into coenzymes 5'-deoxyadenosylcobalamin (AdoCbl) and methylcobalamin (Isegawa *et al.*, 1984). Cbl uptake by *Euglena* mitochondria is shown to be a biphasic process, consisting of energy-independent Cbl-binding to the mitochondrial membrane and energy-dependent active transport (Watanabe *et al.*, 1993a). The enzymes involved in the synthesis of AdoCbl are found only in the mitochondria (Watanabe *et al.*, 1987a). AdoCbl synthesized in the mitochondria is the cofactor of ribonucleotide reductase (EC 1.17.4.2), which functions in DNA synthesis (Hamilton, 1974). *Euglena* cells also contain methylcobalamin-dependent methionine synthase (EC 2.1.1.13) isozymes, which are located in the cytosol, chloroplasts and mitochondria (Isegawa *et al.*, 1994). The isozymes supply methionine for protein synthesis in the individual organelles.

Numerous non-enzymic Cbl-binding proteins are distributed in the mitochondria, microsomes, chloroplasts,

cytosol, pellicle and culture broth of *E. gracilis* Z. Some of them have been purified and characterized (Watanabe *et al.*, 1987b, c, 1988a, b). The Cbl-binding protein has an absolute requirement for the complete Cbl molecule with an α -axial ligand (the cobalt-coordinated nucleotide) and an intact *b*-propionamide side-chain (Watanabe *et al.*, 1993a, b, c). Thus, *E. gracilis* Z is suitable for elucidating physiological roles of Cbl in photosynthetic microorganisms.

E. gracilis Z can utilize propionate for growth as sole carbon source only under illumination, being converted into cell components through the methylmalonyl-CoA mutase (MCM; EC 5.4.99.2) pathway (Yokota *et al.*, 1982). This pathway metabolizes odd-chain fatty acids and branched-chain amino acids to methylmalonyl-CoA, which is converted to the Krebs cycle intermediate succinyl-CoA by MCM (Rosenberg & Fenton, 1989). There is little information available on the enzymological properties of the AdoCbl-dependent MCM in *E. gracilis* Z. Here we describe the occurrence and subcellular location of MCM isozymes and attempt to clarify propionate metabolism in this protozoon.

METHODS

Culture and organism. *E. gracilis* Z was photoautotrophically cultured for 12 d at 27 °C with illumination (8000 lx) in Cramer–Myers medium (Cramer & Myers, 1952) containing

Abbreviations: AdoCbl, 5'-deoxyadenosylcobalamin; Cbl, cobalamin; MCM, methylmalonyl-CoA mutase.

cyanocobalamin ($5 \mu\text{g l}^{-1}$). This autotrophic medium was supplemented with $6.6 \text{ g propionate l}^{-1}$ and the cells were subcultured several times (> 5) under the same conditions to adapt them to propionate.

Crude enzyme preparation. Propionate-adapted and photoautotrophic *Euglena* cells were grown for 12 d at 27°C with illumination in the Cramer-Myers medium with and without propionate (6.6 g l^{-1}), respectively. The cells were washed twice with distilled water, suspended in $20 \text{ ml } 10 \text{ mM}$ potassium phosphate buffer, pH 7.0, containing 1 mM EDTA, disrupted by sonication (10 kHz , $20 \text{ s} \times 4$), and centrifuged at 20000 g for 30 min. The supernatant fraction was used as a crude enzyme.

Enzyme assay. MCM was spectrophotometrically assayed (Hitachi 200-10 spectrophotometer) by the succinyl-CoA transferase/ β -hydroxyacyl-CoA-dehydrogenase-coupled method (Watanabe *et al.*, 1993d). Briefly, the assay mixture (0.5 ml) contained 10 mM potassium phosphate buffer, pH 7.0, $10 \mu\text{M}$ AdoCbl, 0.5 mM DL-methylmalonyl-CoA, 5 mM lithium acetoacetate, 0.15 mM NADH, 0.1 unit succinyl-CoA transferase, 1.5 unit β -hydroxyacyl-CoA dehydrogenase and crude enzyme. The components, except for DL-methylmalonyl-CoA, were mixed in a 0.7 ml cuvette, and the temperature was equilibrated by incubation in a water-jacketed cuvette holder maintained at 30°C . The reaction was started by addition of DL-methylmalonyl-CoA. MCM activity was calculated from the decrease in NADH concentration, measured by the change in A_{340} . The activity of apo-MCM was calculated by subtracting activity in the absence of AdoCbl from that in the presence of AdoCbl. Results are expressed as $\text{nmol succinate formed min}^{-1} (\text{mg protein})^{-1} \pm \text{SD}$ of triplicate experiments.

Subcellular fractionation. Propionate-adapted cells were centrifuged at 2000 g for 10 min and washed twice with distilled water. The cells (31 g wet weight) were suspended in $50 \text{ ml } 25 \text{ mM}$ HEPES/KOH buffer, pH 7.0, containing 0.33 M mannitol and then disrupted by gently grinding with 30–40-mesh sea sand (5 g) for 10 min using a mortar and pestle. The suspension was filtered through a double layer of gauze to remove the sand and then centrifuged at 2000 g for 10 min to remove unbroken cells. The supernatant was subjected to differential centrifugation according to Tokunaga *et al.* (1979).

The following marker enzyme activities were assayed by the methods described in the cited references: glutamate dehydrogenase (EC 1.4.1.2; Tokunaga *et al.*, 1979), a marker enzyme of *Euglena* cytosol; succinate-semialdehyde dehydrogenase (EC 1.2.1.16; Tokunaga *et al.*, 1976), a marker enzyme of the mitochondrial matrix; ribulose-bisphosphate carboxylase/oxygenase (EC 4.1.1.39; Rabinowitz *et al.*, 1975), a marker enzyme of chloroplasts; and glucose-6-phosphatase (EC 3.1.3.9; de Duve *et al.*, 1955), a marker enzyme of microsomes.

The mitochondria and chloroplasts were further purified by a Percoll density gradient centrifugation (Watanabe *et al.*, 1987b; Isegawa *et al.*, 1984), and suspended in $2.0 \text{ ml } 10 \text{ mM}$ potassium phosphate buffer, pH 7.0, containing 100 mM potassium chloride and 1 mM EDTA. These organelles were disrupted by sonication (10 kHz , $10 \text{ s} \times 5$) and used as samples for Toyopearl HW55S gel-filtration experiments.

Gel-filtration experiments. The M_r of the *Euglena* MCM was determined by HPLC (Shimadzu LC-7A pump and SPD-7AV spectrophotometer, and a Hitachi Chromato-data processor D-2500) using a Toyopearl HW55S column ($1.0 \times 28.5 \text{ cm}$). The column was equilibrated with 10 mM potassium phosphate buffer, pH 7.0, containing 100 mM potassium chloride and

1 mM EDTA and eluted with the same buffer at a flow rate of 0.5 ml min^{-1} . The column was calibrated with blue dextran (mean M_r 2000000), yeast alcohol dehydrogenase (M_r 150000), bovine serum albumin (M_r 66000), ovalbumin (M_r 45000) and soybean trypsin inhibitor (M_r 20000). Blue dextran was assayed by measuring the A_{650} . The enzymes and proteins were monitored by measuring the A_{280} .

Protein assay. Protein was assayed by the method of Bradford (1976), with ovalbumin as standard.

Materials. DL-Methylmalonyl-CoA, succinyl-CoA transferase, β -hydroxyacyl-CoA dehydrogenase and AdoCbl were obtained from Sigma. Toyopearl HW55S was obtained from Tosoh.

RESULTS AND DISCUSSION

Occurrence of MCM in *E. gracilis* Z

MCM activity was found in homogenates of *Euglena* cells grown photoautotrophically (control) or adapted to the propionate-supplemented medium. The specific activity of total MCM (both holo- and apoenzymes) was about 6.5-fold higher in the propionate-adapted cells than in the control cells [17.41 ± 2.87 and $2.64 \pm 0.13 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$, respectively], and was similar to that of human liver MCM [$24 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$; Fenton *et al.*, 1982]. In the absence of AdoCbl, the MCM activity of the propionate-adapted cells decreased to about 50% of the activity [$7.97 \pm 7.35 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$] and the control only changed slightly [$2.42 \pm 0.11 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$], indicating that half of the activity of the MCM detected had bound AdoCbl as the cofactor. Only residual activity [$< 0.1 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$] of MCM was found in a cell homogenate of *E. gracilis* Z grown heterotrophically in Koren-Hutner medium (Koren & Hutner, 1967). Increased MCM activity in propionate-adapted cells is therefore due to induction of the MCM enzyme by propionate photoassimilation.

Subcellular distribution of MCM

Fig. 1 shows activities of MCM and marker enzymes in the subcellular fractions from differential centrifugation of an homogenate of the propionate-adapted *E. gracilis* Z. The enzyme activities were plotted as relative specific activity in each fraction versus percentage protein (Beaufay *et al.*, 1964). Based upon the distribution of the marker enzymes succinate-semialdehyde dehydrogenase (mitochondria), ribulose-bisphosphate carboxylase/oxygenase (chloroplasts), glucose-6-phosphatase (microsomes) and glutamate dehydrogenase (cytosol), these organelles were separated satisfactorily. MCM activity was predominantly located in the mitochondrial fraction (Fig. 1), which also had the highest specific activity [$172.8 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$]. When the mitochondrial and chloroplast fractions were purified by Percoll density gradient centrifugation, the specific activity of MCM increased about twofold in the Percoll-purified mitochondrial fraction [$354.6 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$], while the chloroplast MCM activity disappeared. The MCM activity in the microsomal fraction was due to contamination by the

Fig. 1. Subcellular localization of MCM in propionate-adapted *Euglena* cells. The procedures for subcellular fractionation and assay of MCM and marker enzymes are described in Methods. The distribution pattern is typical of that obtained in three separate experiments. Homogenates of *Euglena* cells were subjected to differential centrifugation, with increasing *g* force from left to right. □, Chloroplast fraction; ■, mitochondrial fraction; ▨, microsomal fraction; ▩, cytosolic fraction. MCM and marker enzymes are plotted as relative specific activity in each fraction versus percentage protein. Recoveries were 103% for MCM (a), 101% for glutamate dehydrogenase (b; cytosolic marker enzyme), 101% for succinate-semialdehyde dehydrogenase (c; mitochondrial marker enzyme), 103% for ribulose-bisphosphate carboxylase/oxygenase (d; chloroplastic marker enzyme), 103% for glucose-6-phosphatase (e; microsomal marker enzyme) and 104% for protein.

Fig. 2. Elution patterns of *Euglena* MCM on Toyopearl HW55S column chromatography. MCM activity was separated and assayed as described in Methods. The data are from a run of four separate experiments. (a) Crude homogenate from photoautotrophic *Euglena* cells (control); (b) crude homogenate from propionate-adapted cells; (c) mitochondrial fraction from the propionate-adapted cells. Solid line, protein; ●, MCM activity.

cytosolic fraction since washing the fraction with 10 mM HEPES/KOH buffer, pH 7.0, containing 0.25 M sucrose removed the activity. The cytosolic MCM activity was

also due to contamination of the soluble fraction by damaged mitochondria since a minor amount of succinate-semialdehyde dehydrogenase, a mitochondrial marker enzyme, was also recovered in the cytosolic fraction. These results indicate that *Euglena* MCM is enriched in the mitochondrial fraction; it is identical in subcellular location to the mammalian MCM (Rosenberg & Fenton, 1989).

MCM isozymes

Fig. 2(a, b) shows the elution profile of MCM activity in control and propionate-adapted *Euglena* cells during Toyopearl HW55S gel filtration. The MCM activity of control cells eluted as a peak with an apparent M_r of 72000, while that of the propionate-adapted cells eluted as two peaks with apparent M_r values of 72000 and 17000; an identical elution profile for the MCM activity of the mitochondria fractionated from the propionate-adapted cells was obtained (Fig. 2c). These results indicate that the mitochondria of the propionate-adapted *Euglena* cells contain the two MCM isozymes with apparent M_r values of 72000 (large MCM) and 17000 (small MCM), and that the large and small MCMs are constitutive and inducible enzymes, respectively.

The enzyme has been isolated from human (Fenton *et al.*, 1982) and sheep (Cannata *et al.*, 1965) livers and from *Propionibacterium shermanii* (Francalanci *et al.*, 1986). The human enzyme consists of a homodimer (identical subunits with M_r values of 72000–77500), while the bacterial enzyme consists of two non-identical subunits with M_r values of 79000 and 67000. The human MCM contains 2 mol AdoCbl (mol enzyme)⁻¹. The AdoCbl content of

the bacterial enzyme has not been reported because the enzyme is purified as an apoenzyme. Sheep-liver MCM is sensitive to thiol reagents such as *N*-ethylmaleimide, which has no effect on the activity of the bacterial enzyme. Kinetic properties of the mammalian and bacterial enzymes also differ.

The *Euglena* large MCM had a similar M_r to the mammalian and bacterial subunits. The *Euglena* small MCM possibly has the smallest M_r among the Cbl-dependent enzymes which have been reported. In this paper, we have demonstrated the first occurrence of the propionate-induced small MCM in organisms.

The results presented here provide evidence that the propionate-adapted *Euglena* cells contain the two MCM isozymes. The small MCM is induced significantly, presumably to enable photoassimilation of propionate.

ACKNOWLEDGEMENTS

This study was supported in part by a research fund of the Japanese Private School foundation (F.W. and Y.T.).

REFERENCES

- Beaufay, H., Jacques, P., Baudhuin, P., Sellinger, O. Z., Berthet, J. & de Duve, C. (1964). Resolution of mitochondrial fractions from rat liver into three distinct populations of cytoplasmic particles by means of density equilibration in various gradients. *Biochem J* **92**, 184–205.
- Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. *Anal Biochem* **72**, 248–254.
- Cannata, J. J. B., Focesi, A., Jr, Mazumder, R., Warner, R. C. & Ochoa, S. (1965). Metabolism of propionic acid in animal tissues. XII. Properties of mammalian methylmalonyl coenzyme A mutase. *J Biol Chem* **240**, 3249–3257.
- Cramer, M. C. & Myers, J. (1952). Growth and photosynthetic characteristics of *Euglena gracilis*. *Arch Mikrobiol* **17**, 384–402.
- de Duve, D., Pressman, B. C., Gianetto, R., Wattiaux, T. G. & Appelmans, F. (1955). Tissue fractionation studies. 6. Intracellular distribution patterns of enzymes in rat liver. *Biochem J* **60**, 604–617.
- Fenton, W. A., Hack, A. M., Willard, M. F., Gertler, A. & Rosenberg, L. E. (1982). Purification and properties of methylmalonyl coenzyme A mutase from human liver. *Arch Biochem Biophys* **214**, 815–823.
- Francalanci, F., Davis, N. K., Fuller, J. Q., Murfitt, D. & Leadlay, P. F. (1986). The subunit structure of methylmalonyl-CoA mutase from *Propionibacterium shermanii*. *Biochem J* **236**, 489–494.
- Hamilton, F. D. (1974). Ribonucleotide reductase from *Euglena gracilis*: a 5'-deoxyadenosylcobalamin-dependent enzyme. *J Biol Chem* **249**, 4428–4434.
- Isegawa, Y., Nakano, Y. & Kitaoka, S. (1984). Conversion and distribution of cobalamin in *Euglena gracilis* z, with special reference to its location and probable function within chloroplasts. *Plant Physiol* **76**, 814–818.
- Isegawa, Y., Watanabe, F., Kitaoka, S. & Nakano, Y. (1994). Subcellular distribution of cobalamin-dependent methionine synthase in *Euglena gracilis* z. *Phytochemistry* **35**, 59–61.
- Kitaoka, S., Nakano, Y., Miyatake, K. & Yokota, A. (1989). Enzymes and their functional location. In *The Biology of Euglena*, vol. 4, pp. 1–135. Edited by D. E. Buetow. San Diego: Academic Press.
- Koren, L. E. & Hutner, S. H. (1967). High-yield media for photosynthesizing *Euglena gracilis* z. *J Protozool* **14S**, 17.
- Rabinowitz, H., Reisfeld, A., Sagher, D. & Edelman, M. (1975). Ribulose diphosphate carboxylase from autotrophic *Euglena gracilis*. *Plant Physiol* **56**, 345–350.
- Rosenberg, L. E. & Fenton, W. A. (1989). Disorders of propionate and methylmalonate metabolism. In *The Metabolic Basis of Inherited Disease*, pp. 822–844. Edited by C. R. Scriver, A. L. Beaudet, W. S. Sly & D. Valle. New York: McGraw Hill.
- Tokunaga, M., Nakano, Y. & Kitaoka, S. (1976). Separation and properties of the NAD-linked and NADP-linked isozymes of succinic semialdehyde dehydrogenase in *Euglena gracilis* z. *Biochim Biophys Acta* **429**, 55–62.
- Tokunaga, M., Nakano, Y. & Kitaoka, S. (1979). Subcellular location of the GABA-shunt enzymes in *Euglena gracilis* strain Z. *J Protozool* **26**, 471–473.
- Watanabe, F., Nakano, Y. & Kitaoka, S. (1987a). Purification and characterization of aquacobalamin reductase (NADPH) from *Euglena gracilis*. *J Biol Chem* **262**, 11514–11518.
- Watanabe, F., Nakano, Y. & Kitaoka, S. (1987b). Isolation and some properties of soluble and membrane-bound cobalamin binding proteins of *Euglena* mitochondria. *Arch Microbiol* **149**, 30–35.
- Watanabe, F., Nakano, Y. & Kitaoka, S. (1987c). Purification and some properties of cytosolic cobalamin-binding protein in *Euglena gracilis*. *Biochem J* **247**, 679–685.
- Watanabe, F., Ito, T., Tabuchi, T., Nakano, Y. & Kitaoka, S. (1988a). Isolation of pellicular cobalamin-binding proteins of the cobalamin uptake system of *Euglena gracilis*. *J Gen Microbiol* **134**, 67–74.
- Watanabe, F., Nakano, Y., Ochi, H. & Kitaoka, S. (1988b). Purification, some properties and possible physiological role of an extracellular cobalamin binding protein from *Euglena gracilis*. *J Gen Microbiol* **134**, 1385–1389.
- Watanabe, F., Nakano, Y. & Stupperich, E. (1992). Different corrinoid specificities for cell growth and the cobalamin uptake system in *Euglena gracilis* z. *J Gen Microbiol* **138**, 1807–1813.
- Watanabe, F., Nakano, Y. & Stupperich, E. (1993a). Uptake of cobalamin by *Euglena* mitochondria. *J Biochem* **114**, 793–799.
- Watanabe, F., Nakano, Y., Tamura, T. & Stupperich, E. (1993b). Corrinoid specificity of cytosolic cobalamin-binding protein of *Euglena gracilis* z. *J Biochem* **113**, 97–100.
- Watanabe, F., Nakano, Y., Stupperich, E., Ushikoshi, K., Ushikoshi, S., Ushikoshi, I. & Kitaoka, S. (1993c). A radioisotope dilution method for quantitation of total vitamin B₁₂ in biological sample using isolated *Euglena* pellicle fragments as a solid-phase vitamin B₁₂-binding material. *Anal Chem* **65**, 657–659.
- Watanabe, F., Tamura, Y., Saido, H. & Nakano, Y. (1993d). Enzymatic assay for adenosylcobalamin-dependent methylmalonyl coenzyme A mutase. *Biosci Biotechnol Biochem* **57**, 1593–1594.
- Yokota, A., Hosotani, K. & Kitaoka, S. (1982). Mechanism of metabolic regulation in photoassimilation of propionate in *Euglena gracilis* z. *Arch Biochem Biophys* **249**, 530–537.

Received 17 November 1995; revised 1 March 1996; accepted 26 April 1996.