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

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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 β-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., 1989). This pathway metabolizes odd-chain fatty acids and branched-chain amino acids to methylmalonyl-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...
cyanocobalamin (5 μg L⁻¹). This autotrophic medium was supplemented with 6.6 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 ml 10 mM potassium phosphate buffer, pH 7.0, containing 1 mM EDTA, disrupted by sonication (10 kHz, 20 s x 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 μM AdoCbl, 0.5 mM N₅-methylmalonyl-CoA, 5 mM lithium acetocetate, 0.15 mM NADH, 0.1 unit succinyl-CoA transferase, 1.5 unit β-hydroxyacyl-CoA dehydrogenase and crude enzyme. The components, except for N₅-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 N₅-methylmalonyl-CoA. MCM activity was calculated from the decrease in NADH concentration, measured by the change in A₄₅₀. 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 nmol succinate formed min⁻¹ (mg protein)⁻¹ ± SD of triplicate experiments.

**Subcellular fractionation.** Propionate-adapted cells were centrifuged at 20000 g for 10 min and washed twice with distilled water. The cells (31 g wet weight) were suspended in 50 ml 25 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 20000 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 ml 10 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 s x 5) and used as samples for Toyopearl HW55S gel-filtration experiments.

**Gel-filtration experiments.** The M₅₀ of the *Euglena* MCM was determined by HPLC (Shimadzu LC-7A pump and SPD-7A V spectrophotometer, and a Hitachi Chromato-data processor D-2500) using a Toyopearl HW55S column (10 x 28.5 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 Mₕ 200000), yeast alcohol dehydrogenase (Mₕ 150000), bovine serum albumin (Mₕ 66000), ovalbumin (Mₕ 45000) and soybean trypsin inhibitor (Mₕ 20000). Blue dextran was assayed by measuring the A₄₅₀. The enzymes and proteins were monitored by measuring the A₂₅₀.

**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 ± 0.13 nmol min⁻¹ (mg protein)⁻¹, respectively], and was similar to that of human liver MCM [24 nmol min⁻¹ (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 ± 7.35 nmol min⁻¹ (mg protein)⁻¹] and the control only changed slightly [2.42 ± 0.11 nmol min⁻¹ (mg protein)⁻¹], indicating that half of the activity of the MCM detected had bound AdoCbl as the cofactor. Only residual activity [<0.1 nmol min⁻¹ (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 [172.6 nmol min⁻¹ (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 nmol min⁻¹ (mg protein)⁻¹], while the chloroplast MCM activity disappeared. The MCM activity in the microsomal fraction was due to contamination by the
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![Graph showing subcellular localization of MCM in propionate-adapted Euglena cells.](image)

**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.

![Graph showing elution patterns of Euglena MCM on Toyopearl HW55S column chromatography.](image)

**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.

Cytoplasmic 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*ₙ of 72000, while that of the propionate-adapted cells eluted as two peaks with apparent *M*ₙ 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*ₙ 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*ₙ values of 72000–77500), while the bacterial enzyme consists of two non-identical subunits with *M*ₙ 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 Mr to the mammalian and bacterial subunits. The *Euglena* small MCM possibly has the smallest Mr 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.

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**REFERENCES**


