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Molecular characterization of a bifunctional glyoxylate cycle enzyme, malate synthase/isocitrate lyase, in *Euglena gracilis*

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Abstract

Euglena gracilis induced glyoxylate cycle enzymes when ethanol was fed as a sole carbon source. We purified, cloned and characterized a bifunctional glyoxylate cycle enzyme from *E. gracilis* (EgGCE). This enzyme consists of an N-terminal malate synthase (MS) domain fused to a C-terminal isocitrate lyase (ICL) domain in a single polypeptide chain. This domain order is inverted compared to the bifunctional glyoxylate cycle enzyme in *Caenorhabditis elegans*, an N-terminal ICL domain fused to a C-terminal MS domain. Purified EgGCE catalyzed the sequential ICL and MS reactions. ICL activity of purified EgGCE increased in the existence of acetyl-CoA at a concentration of micromolar order. We discussed the physiological roles of the bifunctional glyoxylate cycle enzyme in these organisms as well as its molecular evolution.

Keywords: Bifunctional enzyme; Glyoxylate cycle; *Euglena gracilis*; Inverted order; Malate synthase; Isocitrate lyase; Ace operon; Gene fusion; Acetyl-CoA; Mitochondrial localization

1. Introduction

The glyoxylate cycle is a metabolic pathway which is very important for organisms to synthesize carbohydrates from C2 compounds, as first proposed by Kornberg and Krebs (1957). This cycle has two key specific enzymes; isocitrate lyase (ICL; EC 4.1.3.1) and malate synthase (MS; EC 2.3.3.9). ICL catalyzes the cleavage of D-isocitrate to glyoxylate and succinate, and glyoxylate formed by the ICL reaction is condensed with acetyl-CoA to produce L-malate by the action of MS. These two enzymes ensure the bypass of two of the decarboxylation steps of the tricarboxylic acid (TCA) cycle in the synthesis of succinate. Thus, the glyoxylate cycle is very important especially under carbon limiting conditions. In certain higher plants, the glyoxylate cycle has been reported to play a pivotal role in the synthesis of carbohydrates from storage lipids during seedling (Eastmond and Graham, 2001).

Isocitrate lyases have been found in a wide range of species including bacteria (Kornberg, 1966), archaea (Serrano et al., 1998), yeast (Taylor et al., 1996), fungi (Lorenz and Fink, 2001) and higher plants (Eastmond and Graham, 2001). ICLs commonly consist of four identical subunits in either prokaryotes or eukaryotes. However, the subunit molecular mass is significantly different between bacterial (approximately 47 kDa) and eukaryotic (60–64 kDa) ICLs.

On the basis of the amino acid sequence features, MSs have been divided into two major families, isoforms A (MSA) and G (MSG). MSA with a molecular mass of about 65 kDa occurs in bacteria (Kornberg, 1966), yeast (Hartig et al., 1992), fungi (Lorenz and Fink, 2001) and higher plants (Eastmond and Graham, 2001), whereas MSG with around

Abbreviations: MS, Malate synthase; ICL, Isocitrate lyase; EgGCE, *Euglena gracilis* bifunctional glyoxylate cycle enzyme; CeGCP, *Caenorhabditis elegans* bifunctional glyoxylate cycle protein.

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80 kDa has been found only in bacteria. Certain bacteria, such as *Escherichia coli*, express both the two types of MSs (Molina et al., 1994).

Liu et al. (1997, 1995) found that ICL and MS are encoded by a single gene and expressed as a single bifunctional polypeptide in nematode *Caenorhabditis elegans*. The polypeptide, called bifunctional glyoxylate cycle protein (GCP), consists of two separate domains for ICL and MS, and the ICL domain locates in the amino-terminal side of the polypeptide. The physiological functions of the GCP during development in nematode have been well studied; however, the catalytic properties of the unique enzyme have not been reported.

It has been reported that *Euglena gracilis*, a unicellular protist containing chloroplasts, has both ICL and MS activities, and these enzyme activities are greatly enhanced when ethanol is fed as a sole carbon source (Inui et al., 1992). In the present paper, we have purified a bifunctional enzyme having the ICL and MS activities from *E. gracilis* grown on ethanol, and cloned cDNA encoding this bifunctional enzyme. We reported that the bifunctional glyoxylate enzyme found in *E. gracilis* (EgGCE), as well as the GCP in *C. elegans*, consists of two functional components, the ICL and MS domains, but that the ICL domain is found in the carboxy-terminal side in the EgGCE, in contrast to the nematode enzyme. In addition, it is also shown that the ICL reaction is activated in the presence of acetyl-CoA, a substrate of the MS reaction, in EgGCE; *V*max increases whereas *K*m for Mg^{2+}–isocitrate complex decreases in the presence of acetyl-CoA.

2. Materials and methods

2.1. Organism and culture

Euglena gracilis SM-ZK (Oda et al., 1982), a non-photosynthetic mutant derived from strain *Z* by treatment with streptomycin, was cultured in Cramer–Myers medium (Cramer and Myers, 1952), supplemented with ethanol at 85 mM as a sole carbon source, with aeration at 27 °C for 4 days.

2.2. Enzyme purification

All operations during the enzyme purification were conducted at 4 °C. *E. gracilis* cells grown on ethanol (about 10 g wet basis) were harvested, washed and suspended in buffer A (10 mM potassium phosphate buffer, pH 7.0, containing 1 mM dithiothreitol, 1 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride and 10 μM leupeptin). The cells were disrupted by sonication (10 kHz, 1 min × 8) and centrifuged at 20,000 ×*g* for 30 min to obtain a crude enzyme solution. The solution was further centrifuged at 100,000 ×*g* for 1 h. Ammonium sulfate was added to the supernatant to the 40% saturation and the solution was centrifuged at 18,000 ×*g* for 20 min. The concentrations of ammonium sulfate in the supernatant increased to 45% saturation after centrifugation, and the solution was centrifuged at 18,000 ×*g* for 20 min to collect the precipitate. The precipitate was dissolved in buffer A and dialyzed to remove ammonium sulfate.

The enzyme solution was applied onto a DEAE–Sepharose FF column (1.5 × 11 cm), which had been equilibrated with the buffer A. The column was washed with 75 mL of the same buffer and the enzyme was eluted with 300 mL of a linear concentration gradient (10 to 400 mM) of potassium phosphate in the buffer. Active fractions were collected and applied onto a Phenyl–Sepharose column (1.3 × 4.5 cm), which had been equilibrated with buffer B (50 mM potassium phosphate buffer, pH 7.0, containing 1 mM dithiothreitol, 1 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride and 10 μM leupeptin). The column was washed with 30 mL of buffer B containing 40% ethyleneglycol, and the enzyme was eluted by increasing the concentration of ethyleneglycol to 55% in the buffer. Active fractions were collected and dialyzed against the buffer A, and applied onto a hydroxyapatite column (1.7 × 5 cm) pre-equilibrated with buffer A. The column was washed with 50 mL of buffer A, and the enzyme was eluted with 200 mL of a linear concentration gradient (10 to 500 mM) of potassium phosphate in the buffer. Active fractions were combined and concentrated by membrane filtration with a Centricon 30 filter (Millipore, MA, USA). The enzyme solution (2 mL) was chromatographed on a Hi-Load Superdex column (Amersham Biosciences, NJ, USA) pre-equilibrated with buffer B containing 0.1 M KCl, using a fast protein liquid chromatography system (Amersham Biosciences). The active fractions obtained were combined and used as the purified preparation of EgGCE. The protein content was determined according to Bradford (1976) with bovine serum albumin as a standard.

2.3. Enzyme assays

ICL reaction, the cleavage reaction of isocitrate to succinate and glyoxylate, was assayed by measuring the formation of glyoxylate–phenylhydrazone at 334 nm (Malhotra and Srivastava, 1982) at 30 °C. The reaction mixture (1 mL) contained 100 mM potassium phosphate buffer, pH 6.5, 30 mM $MgCl_2$, 2 mM dithiothreitol, 4 mM phenylhydrazine–HCl, 30 mM sodium isocitrate, and the enzyme.

MS activity was assayed by measuring the acetyl-CoA degradation (Cook, 1970) at 30 °C. The reaction mixture contained 100 mM Tris–HCl buffer, pH 8.0, 150 μM acetyl-CoA, 10 mM $MgCl_2$, 1 mM glyoxylate, and the enzyme in a total volume of 1 mL. The activity was determined by following the decrease in absorbance at 232 nm owing to the degradation of acetyl-CoA.