Note

Induction of Glyoxylate Cycle–Key Enzymes, Malate Synthase, and Isocitrate Lyase in Ethanol-grown *Euglena gracilis*

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Received August 26, 1993

The effects of ethanol on the glyoxylate cycle–key enzymes of *Euglena*, malate synthase (EC 4.1.3.2) and isocitrate lyase (EC 4.1.3.1), were investigated. The addition of ethanol to *Euglena* cells increased malate synthase and isocitrate lyase activities. Immunoblot analysis with antibody raised against malate synthase showed that the increase in malate synthase activity was due to newly synthesized protein. The experimental results reported here demonstrate that ethanol is assimilated by the glyoxylate cycle including the alcohol metabolizing enzymes in *Euglena*.

In a previous paper, we reported that *E. gracilis* grew on either myristic acid or myristyl alcohol as a sole carbon source and proposed that the β-oxidation pathway and glyoxylate cycle contributed to the metabolism of the fatty acid and alcohol. Hosotani et al. have reported that *E. gracilis* grows on ethanol with or without illumination. However, information concerning glyoxylate cycle enzymes and their compartments are not yet available. To elucidate the regulation mechanism of malate synthase and isocitrate lyase in the glyoxylate cycle, we investigated the effects of ethanol on two glyoxylate cycle–key enzymes. In this paper, we report the induction of malate synthase and isocitrate lyase caused by ethanol and the physiological participation of these two enzymes in the assimilation of ethanol in *Euglena*.

*E. gracilis* SM-ZK, a streptomycin-bleached mutant of *Euglena graciila* Z, was cultured heterotrophically at 27°C for 6 days under illumination (2000 lux) in Cramer and Myers medium5 containing D-glucose (1 g/liter) or ethanol (0.5% v/v) and (NH₄)₂SO₄ as the sole carbon and nitrogen sources. Growth was measured by counting the cells with a hemocytometer. The harvested cells were washed twice, resuspended in a 100 mM HEPES-KOH buffer containing 10 mM MgCl₂, pH 7.5, and disrupted by ultrasonic treatment (10 kHz) for 1 min (15 s x 4). The resulting lysate was centrifuged at 14,000 x g for 15 min and the supernatant was used as a crude homogenate. Malate synthase from ethanol-grown cells was purified at 0–4°C by polyethylene glycol-6000 fractionation followed by Phenyl-Sepharose and Superose-6-column chromatographies on a FPLC (Pharmacia FPLC system). The enzyme was purified about 20-fold over the crude extract of *E. gracilis* with a yield of 6%. Polyacrylamide disk-gel electrophoresis showed only one detectable protein band and SDS-polyacrylamide gel electrophoresis of the purified enzyme gave a single protein band (116 kDa). Isocitrate lyase was assayed by following the increase in absorbance of glyoxylate-phenylhydrazone at 334 nm. Malate synthase activity was measured by following the decrease in absorbance of acetyl-CoA at 322 nm. Western blotting was done by a modification of the method of Burnette with skim milk to cover the background. Protein was measured by the method of Bradford with bovine serum albumin as a standard. All other chemicals were of reagent grade.

The effects of carbon sources on the growth rate and the activities of malate synthase and isocitrate lyase were investigated. Figure 1 shows that the cell number of *Euglena* grown on ethanol is equal to that of glucose-grown cells (A and B). At this time glucose or ethanol as a carbon source was completely consumed in 4 days of culture. The levels of malate synthase and isocitrate lyase were compared with extracts from cells harvested during cultivation on two different carbon sources. The levels of the two glyoxylate cycle–key enzyme activities in ethanol-grown cells were about 2.5 to 6 times higher than those in glucose-grown cells throughout the cell growth (C and D). The immunochromatography and malate synthase was examined by using an antiserum against malate synthase from ethanol-grown cells. The result of immunochromatography showed that the enzyme from both carbon sources was completely precipitated by the anti-malate synthase antiserum (data not shown). This result indicates that the malate synthase from cells grown on different carbon sources is immunochemically indistinguishable and that only one kind of the active enzyme is present in the cells, regardless of the growth substrates.

To obtain further information concerning the induction of the two glyoxylate cycle–key enzymes in *Euglena*, the effects of ethanol on the enzyme activity were investigated. When 0.5% (v/v) ethanol was added to the glucose-grown *E. gracilis* cells at the stationary

Fig. 1. Levels of Malate Synthase and Isocitrate Lyase Activities in *Euglena* Cells Grown on Different Carbon Sources.

Cells were cultivated aerobically in glucose or ethanol medium: A, cell growth on glucose; B, cell growth on ethanol; C, effects of ethanol addition after 4 days cultured on glucose and activities of malate synthase (○, glucose; ●, after ethanol addition) and isocitrate lyase (△, glucose; ▲, after ethanol addition); D, activities of malate synthase and isocitrate lyase from ethanol-grown cells. All enzyme units are expressed as nmol min⁻¹ per mg protein.

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phase, the activities of malate synthase and isocitrate lyase were observed as shown in Fig. 1C. The addition of ethanol increased linearly the activity of both enzyme to reach about 2 to 3-fold in enzyme activity over a period of 48 h, however the addition of cycloheximide (0.1 mM) together with ethanol stopped the increase in enzyme activity (data not shown). To find whether the increases in malate synthase and isocitrate lyase were due to de novo synthesis of enzyme proteins or the activation of pre-existing proteins, we measured the increases in the enzyme levels in the glucose-grown cells after addition of ethanol by Western blot analysis. Proteins from crude homogenates at some cultivation stages after ethanol addition were electrophoresed to nitrocellulose after SDS-polyacrylamide gel electrophoresis, and then probed with anti-malate synthase serum followed by anti-mouse IgG conjugated with peroxidase. Each lane contained the same amount of protein. In all cases, only a single band in the 116 kDa region of the subunit molecular mass of malate synthase appeared on the blots, as shown in Fig. 2. The increases in the enzyme levels in the glucose-grown cells after addition of ethanol were immunochemically shown to be an increase in the amount of enzyme proteins, and the enzyme activity was inhibited by cycloheximide. These results indicate that the increase in malate synthase level in *Euglena* at the late-stationary growth phase is attributable to the induction of the enzyme synthesis by the addition of ethanol. As shown above, ethanol was found to be a factor regulating the malate synthase formation in *E. gracilis*.

The glyoxylate cycle, a metabolic bypass of the tricarboxylic acid cycle, is an anaplerotic pathway providing 4-carbon dicarboxylic acids. Definitive studies by Kornberg have shown that the growth of many microorganisms on C₂ compounds, including algae, is dependent on anaplerotic reactions due to the glyoxylate cycle. We have reported recently that the glyoxylate cycle participates in the conversion from wax esters to paramylon in *Euglena* cells fed with [1-¹⁴C]-acetate and [U-¹⁴C]-acetate both of which are the oxidized form of ethanol. Furthermore, we have found that malate synthase and isocitrate lyase are not induced by acetyl-CoA produced endogenously by decomposition of wax esters. In the above case, the *Euglena* cells were grown on glucose and glutamate, hence the constitutive malate synthase and isocitrate lyase appear to be involved in the conversion from wax esters to paramylon, indicating that activities of malate synthase and isocitrate lyase are enough to sustain its conversion. In ethanol-grown cells, we conclude that both enzymes are induced and that the induced enzymes contribute to sustain the cell growth. However, malate synthase from glucose- and ethanol-grown cells was immunochemically indistinguishable, showing that a single enzyme participates in ethanol and wax ester metabolisms.

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**Fig. 2.** Western Blot Analysis of Anti-malate Synthase Antibody.

Proteins were electrophoresed on a SDS polyacrylamide slab gel and transferred to a nitrocellulose sheet by Western blotting. The sheet was incubated with anti-malate synthase antibody, and the antibody-antigen reaction was detected by using alkaline phosphatase. Lane 1, malate synthase of stationary phase cells grown on glucose; lane 2, malate synthase of one-day cultured cells after addition of ethanol; lane 3, malate synthase of two-day cultured cells after addition of ethanol; lane 4, malate synthase from ethanol-grown cells at stationary phase.